

Full Length Research Paper

DNA fingerprinting of spore-forming bacterial isolates, using *Bacillus cereus* repetitive polymerase chain reaction analysis (Bc-Rep-PCR)

N. Du Rand and M. D. Laing

Discipline of Plant Pathology, School of Agriculture, Earth and Environmental Sciences, College of Agriculture and Environmental Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3201, Pietermaritzburg, South Africa.

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Bc-repetitive extragenic palindromic polymerase chain reaction (Bc-Rep PCR) analysis was conducted on seven *Bacillus thuringiensis* isolates accessed from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection and on five local isolates of entomopathogenic spore-forming bacteria. The five isolates were three strains of *B. thuringiensis*, one strain of *B. cereus* and one strain of *Brevibacillus laterosporus*. All five isolates were distinguished from each other using Bc-Rep PCR analysis. The three *B. thuringiensis* isolates were closely related. The isolate of *B. laterosporus* was not related to any of the *B. cereus* group isolates. Serotyping was also conducted on the five local isolates. However, only one of these isolates could be identified with serotyping and was identified as *B. thuringiensis* subsp. *kenyae*.

Key words: Bc-repetitive extragenic palindromic polymerase chain reaction, *Bacillus* sp., serotyping.

INTRODUCTION

The most commonly used technique for the sub-specification of varieties of *Bacillus thuringiensis* has been H-serotyping (Lecadet et al., 1999). This also has been the most common technique to date used for identifying and characterizing novel *Bacillus* strains (Lecadet et al., 1999). Since the classification by De Barjac and Frachon (1990), the numbers of serotypes has increased from 27 to 69 and is still increasing. Biochemical tests on their own have proven ineffective in classifying *B. thuringiensis* into serotypes (Keshvarshi, 2008). However, in situations of conflicting serotype results, they may be used for clarification (Lecadet et al., 1999).

H-serotyping has several major limitations, which have resulted in a search for better techniques for classifying *B. thuringiensis*. Firstly, H-serotyping is unable to differentiate between *B. cereus* and *B. thuringiensis*.

Secondly, phylogenetic relationships between serotypes cannot be drawn (Lecadet et al., 1999). Thirdly, self-agglutinated and non-motile strains cannot be distinguished by H-serotyping (Lecadet et al., 1999). Lastly, only a few laboratories worldwide are able to perform a full H-serotyping assay (Lecadet et al., 1999).

Molecular techniques have been used as alternatives for typing *B. thuringiensis*. The most common techniques include ribosomal DNA restriction fragment polymorphism (RFLP) (Joung and Côte, 2001), Arbitrary Primer PCR technology (Brousseau et al., 1993) and amplified fragment length polymorphism (AFLP) (Pattanayak et al., 2000). Relatively few strains of *B. thuringiensis* have been analyzed using these techniques so far. Early results using these techniques suggest that the members of the *B. cereus* group and the *B. thuringiensis* group could be considered as a single species, and that *B. anthracis* is closely related to these two groups. Techniques such as the Reverse Transcriptase Sequencing of 16S rRNA have demonstrated that there are high levels of sequence similarity (>90%) amongst the members of the *B. cereus* group

*Corresponding author. E-mail: 962082021@stu.ukzn.ac.za.
Tel: 0027332605828. Fax: 027332606495.

(Ash et al., 1991). The only attribute that distinguishes these genera from each other is the functional genes that are carried on plasmids (Carlson et al., 1994; Helgason et al., 2000). These pathological and phenotypic attributes may easily be transferred between "species" through the horizontal transfer of plasmids (Helgason et al., 2000).

Methods such as the use of 16S to 23S ribosomal intergenic spacer Sequences (ISR) have failed to aid in the design of species-specific oligonucleotide probes to differentiate between members of this group (Bourque et al., 1995). Ash et al. (1991) stated that techniques using 16S rRNA to attempt to group members of the *B. cereus* group into one species should be supported with other techniques because similar results have been obtained in other genospecies. With more advanced techniques that use very specific primers, differences between these species have been highlighted. Strains of *B. thuringiensis* have a high degree of relatedness but are genetically distinct (Joung and Côte, 2001; Nakamura, 1998). The relatedness between these members is between 85 to 100%. In contrast to earlier research using 16S rRNA, the genetic relatedness between *B. thuringiensis* and *B. cereus* has been demonstrated to be between 60 to 70%, by using sensitive DNA techniques. In order for these two species to be seen as a single species, the relatedness needs to be greater than 70% (Joung and Côte, 2001; Nakamura, 1998). Research by Nakamura (1998) and Joung and Côte (2001) suggest that some serotypes of the *B. thuringiensis* groups should be reassigned. It has also been suggested that some members of *B. thuringiensis* are in fact members of *B. cereus*, but that, due to horizontal plasmid migration, these *B. thuringiensis* species have lost the ability to produce crystal proteins (Hill et al., 2004; Nakamura, 1998; Peruca et al., 2008). For this reason they may be identified as *B. cereus* due to the absence of the crystal protein.

A new technique for fingerprinting and potentially classifying novel strains of *B. thuringiensis* was developed by Reyes-Ramirez and Ibarra (2005). The technique is Repetitive Extragenic Palindrome Sequence-Based PCR analysis (rep-PCR). The technique originated from the designing of PCR primers from Rep sequences found in *Escherichia coli* Migula and *Salmonella typhimurium* genomes. This technique is based on the fact that Rep sequences have been shown to be common in prokaryote genomes and have been used in the design of primers specific to certain species or groups.

Reyes-Ramirez and Ibarra (2005) found a 26-base Rep that is common in the six members of the *B. cereus* group: CCCACTGATTAAGTTTCACTTTAT (accordingly named Bc-Rep). This was used to design two specific primers for the *B. cereus* group: 18-mer primer Bc-Rep-1 (5'-ATTAAGTTTCACTTTAT-3') and 14-mer primer Bc-Rep-2 (5'-TTTAATCAGTGGG-3'). These primers have been shown to be applicable to *B. thuringiensis*, *B. cereus*, *B. anthracis* and *B. mycoides*.

Analysis using this technique results in discrete and reproducible patterns. Almost all the serotypes of *B. thu-*

ringiensis have been shown to have distinct patterns (Reyes-Ramirez and Ibarra, 2005). The aim of this study is thus, to determine how closely related the isolates were to each other, and how they compare from known serotypes.

MATERIALS AND METHODS

Bacterial samples and sample collection

Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* spp. collected in sugarcane producing areas in KwaZulu-Natal, (KZN), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures. Mushroom compost was sampled from a mushroom farm (Karkloof, KwaZulu-Natal), compost from an urban garden (Hillcrest, KwaZulu-Natal) and grain dust was sampled from a chicken grain storage facility (Pietermaritzburg, KwaZulu-Natal). Diseased *T. molitor* larvae were collected from the insect rearing facility (University of KwaZulu-Natal). Adult beetles and white grubs were collected from light traps and soil, respectively, from the sugarcane areas in the KwaZulu-Natal midlands, KZN. These were provided by the South African Sugar Research Institute (SASRI), Mount Edgecombe, KZN. All the samples were stored at 4°C in a refrigerator.

Isolation of endospore-forming bacteria

Isolation of *Bacillus* sp. was conducted using a similar pasteurization method as described by Ohba and Aizawa (1978). Approximately 0.5 g of each sample was suspended in 10 ml sterile distilled water. Suspensions were shaken vigorously for 30 s at full speed on a vortex shaker. Mixed suspensions were then left to stand for 1 h at room temperature. Suspensions were vortexed a second time at full speed for 30 s and then subjected to a pasteurization process in a preheated water bath for 10 min at 80°C. After pasteurization, 0.1 ml aliquots of the suspensions were plated out onto nutrient agar (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (Thiery and Frachon, 1997).

Plates were incubated for 24 h at 30°C in an incubator and examined for colonies with typical *B. cereus* morphology (fast growing, rhizoid, irregular, raised, smooth, and opaque white) or colonies with an 'ice crystal' appearance with a colony diameter larger than 2 mm (Damgaard et al., 1997; Prescott et al., 1999; Selvakumar et al., 2007). Various other white coloured bacterial colonies that predominated and were larger than 2 mm in diameter were also selected for microscopic evaluation. The number of colonies isolated from each isolate was estimated and counted using a colony counter. Selected colonies were then purified by sub-culture onto nutrient agar and incubated for 3 d at 30°C in an incubator. Colonies were stored on nutrient agar slants.

Selected isolates were subjected to Gram staining, as well as, Coomassie Blue staining [Coomassie Blue stain 0.133% (w/v) and methanol 50% (v/v)] for 1 min. Slides were then rinsed gently for 30 s with distilled water and blotted dry with tissue paper (Kimberly-Clark, SA) (Ammons et al., 2002). Isolates were screened for the presence of crystal proteins using light microscopy with a 100 × oil immersion objective lens (Bernhard et al., 1997; Young et al., 1998). Crystal proteins stained dark blue (Ammons et al., 2002). Isolates were screened for size of cells, spore morphology and the presence of crystal proteins. Isolates without crystal proteins, but with cells broader than 0.9 µm, were also selected, in order to include *B. cereus* which does not produce crystal proteins. Rod-shaped bacterial cells containing oval spores were selected because this fits the criteria of *B. cereus*, *B. thuringiensis* and *B. laterosporus* cells (Thiery and Frachon, 1997). Selected cultures were purified by sub-culturing and were assigned numbers NDR1-

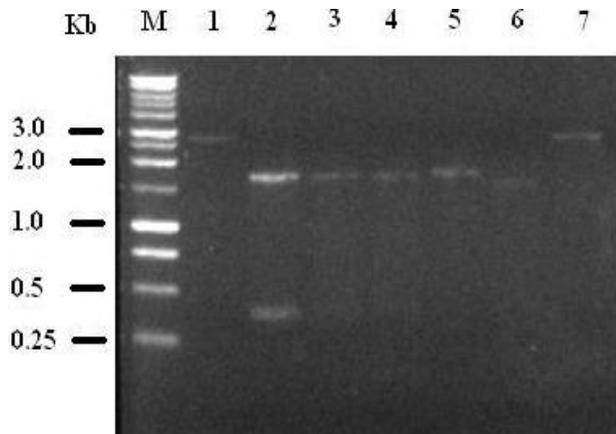


Figure 1. Bc-Rep-PCR fingerprint profiles of the DSMZ *Bacillus thuringiensis* strains. Lane 1, *B. thuringiensis* subsp. *thuringiensis*; Lane 2, subsp. *aizawai*; lane 3, subsp. *kurstaki*; lane 4, subsp. *kenya*; lane 5, subsp. *nigeriae*; lane 6, subsp. *tochiensis*; lane 7, subsp. *morrisoni*; lane M, 1 kb molecular weight markers (Promega).

NDR14.

***Bacillus thuringiensis* samples**

Eight known subspecies of *B. thuringiensis* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection (www.dsmz.de). The subspecies were: *B. thuringiensis* subsp. *nigeriae*, *B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* subsp. *morrisoni*, *B. thuringiensis* subsp. *israelensis*, *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *thuringiensis*, *B. thuringiensis* subsp. *kenya* and *B. thuringiensis* subsp. *tochiensis*.

Inoculum preparation

The isolates were used to inoculate 150 ml of sterile tryptone soy broth (TSB) (Biolab) (Merck, SA) and incubated in a shaker water bath (GFL 1083) at 250 rpm for 24 h at 30°C.

DNA extraction

The DNA was extracted from an overnight culture with Mobio UltraClean™ microbial DNA isolation kit (Mobio, USA).

DNA quantification

A test on the quality and quantity of DNA extracted was conducted electrophoretically as follows: 5 µL of DNA extract aliquots were loaded on a 1.2% horizontal agarose gel slab (Bio-Rad, SA). Samples were run in TAE at pH 8.0 (40 mM Tris-acetate and 1 mM EDTA). Ethidium bromide (Bio-Rad, SA) is a DNA stain that fluoresces under UV light. This was added to the tank of TAE buffer at pH 8.0 (40 mM Tris-acetate and 1 mM EDTA) in order to visualize the DNA bands. Gels were run at 90 V for 55 min.

Primers

The Bc-Rep sequence primers designed by Reyes-Ramirez and

Ibarra (2005) were used. The primers were manufactured by Integrated DNA Technologies (IDT, SA) and were as follows: Direct 18 mer primer, 5'-ATT AAA GTT TCA CTT TAT-3' and a 14 mer reverse primer, 5'-TTT AAT CAG TGG GG-3'.

Conditions for Bc-Rep-PCR amplification

The PCR mixtures were prepared as follows: 1 µg of template DNA, 15 pmol of each primer, 5 mM MgCl₂, 5 µL thermophilic DNA polymerase 10x reaction buffer, 1 µL 10 mM PCR nucleotide mix, 0.25 µL of 5 u/µL *Taq* DNA polymerase and nuclease free water to make the volume up to 50 µL (PCR Core Systems, Promega) (Whitehead Scientific, SA). The conditions of PCR amplification were as follows: 5 min initial denaturation at 94°C, followed by 34 cycles of 1 min denaturation at 94°C, 1 min annealing at 42°C and 1.5 min polymerization at 72°C. The amplification was completed with an extension step of 7 min at 72°C (Reyes-Ramirez and Ibarra, 2005). Amplifications were conducted in an Applied Biosystems Gene Amp PCR System 2400. Amplified samples were stored at -20°C (Conquest).

Electrophoresis

The Bc-Rep-PCR fragments were analyzed as follows: 5 µL aliquots of each of the amplified products were loaded on to a 1.2% agarose slab (110 x 140 mm). The gel was run in TAE buffer pH 8.0 (40 mM Tris-acetate and 1 mM EDTA) at 90V for 55 min. A 1 kb DNA ladder (Promega) (Whitehead Scientific, SA) was used as a molecular weight marker. A gel documentation system, Versa Doc 2000 (Bio-Rad), was used to photograph the gels. Quantity One Version 1.1 computer software was used to analyze the molecular weight patterns (Figures 1 and 2).

Bc-Rep-PCR analysis

The individual patterns of the polymorphic bands of each of the isolates were identified according to the migration rates. An estimation of the banding sizes was used to determine relatedness.

***B. thuringiensis* serotyping**

B. thuringiensis isolates were sent to Dr M. Ohba at the Graduate School of Agriculture, Kyushu University Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka, B12-8581 in Japan for serotyping. A standard method was used, similar to that developed by Thiery and Frachon (1997).

RESULTS

The Bc-Rep-PCR analysis of the seven DSMZ isolates did not produce many distinguishing bands. The bands from lanes 3 to 6 were very similar to each other (Figure 1) and were estimated at approximately 1.8 kb. Lanes 1 and 7 had similar bands to each other and were distinct from the bands in Lanes 3 to 6 (Figure 1). These bands were estimated at approximately 2.9 kb. Lane 2 had two bands, estimated to be approximately 1.8 and 0.35 kb (Figure 1). This demonstrated that *B. thuringiensis* subsp. *thuringiensis* and *B. thuringiensis* subsp. *morrisoni* are closely related to each. Similarly the *B. thuringiensis* subsp.

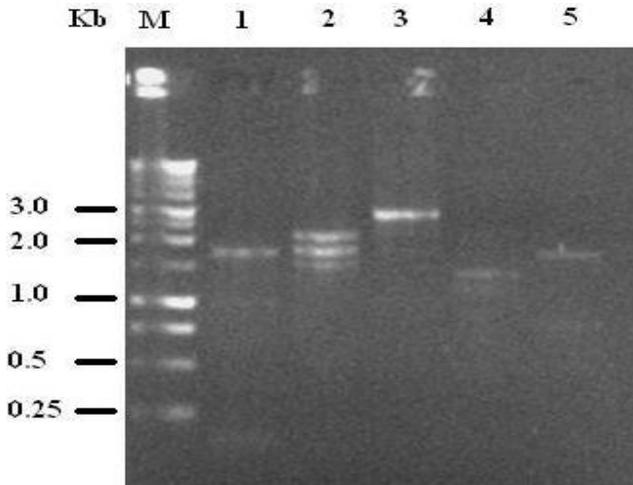


Figure 2. Bc-Rep-PCR fingerprint profiles of the five local, entomopathogenic bacterial isolates. Lane M, 1 kb molecular weight markers (Promega); lane 1, NDR 1 (*Bt*); lane 2, NDR 3 (*Bt*); lane 3, NDR 5 (*B. cereus*); lane 4, NDR 2 (*B. laterosporus*); lane 5, NDR 11 (*Bt*).

from Lanes 2 to 6 could be regarded as being related to each other, with one common band, although the second band of *B. thuringiensis* subsp. *aizawai* separated this subspecies from the other three (Figure 1).

The bands obtained from Bc-Rep-PCR of the five isolates (NDR1, NDR2, NDR3, NDR5, and NDR11) were found to be distinct from each other (Figure 2). This demonstrated that these isolates were not closely related to each other. NDR1 shared a common band with NDR3 and NDR11, at approximately 1.8 kb (Figure 2). Isolate NDR1 and NDR11 were also not distinguishable from each other. NDR3 had three bands at 2.0 kb, 1.8 kb and 1.6 kb respectively (Figure 2). NDR5 produced one clear band at approximately 2.9 kb (Figure 2). NDR4 also produced only one band at approximately 1.5 kb (Figure 2).

Bc-Rep PCR analysis was used to determine the relatedness of the five local entomopathogenic isolates, and to relate them to known serotype of *B. thuringiensis*. Overall, the DNA bands were not as clear and distinct as the bands obtained by Reyes-Ramirez and Ibarra (2005). The bands of the local isolates were markedly more distinct than the bands of the DMSZ isolates, which were not distinct. The bands of the DMSZ isolates were very similar to each other (Figures 1 and 2). The serotyping was done to identify the *B. thuringiensis* strains and demonstrated that this method is limited because only one strain could be identified (Table 1).

DISCUSSION

Serotyping has several limitations, as discussed earlier, which were apparent when only one of three local *B. thuringiensis* strains could be serotyped (Table 1). Such a limited technique cannot be used as the basis for classi-

fying *B. thuringiensis* strains because not all strains can be identified using this technique. The technique of Bc-Rep-PCR analysis is a powerful alternative to serotyping that is applicable to all strains of *B. thuringiensis*. However, the technique is difficult to standardize due to the number of variables that require optimization for each isolate. Laboratory conditions may differ from each other and the sources of known isolates may differ. As a result, it will be difficult to produce the same banding patterns at different laboratories (Figures 1 and 2). Equipment, chemicals, conditions and isolates vary from laboratory to laboratory. Resources differ from country to country. It is essential that inter-laboratory calibrations using identical *B. thuringiensis* cultures should be undertaken to ensure that different laboratories generate similar results. Further research in terms of standardization of current laboratory protocols is required in order to determine whether certain variables may be eliminated to create a more streamline process. Problems arose when following the Bc-Rep-PCR protocol of Reyes-Ramirez and Ibarra (2005). They claimed that their technique is quick and easy to conduct. In practice, it has proven time consuming and laborious, and some runs were not successful. The magnesium chloride optimization for the DMSZ group was at 5 mM of $MgCl_2$, similar to that of Reyes-Ramirez and Ibarra (2005). However, the $MgCl_2$ optimization varied for each of the local isolates, ranging from 2 mM to 5 mM. Therefore, the quantity of 5 mM of $MgCl_2$ is not a universally applicable quantity. This is in spite of Reyes-Ramirez and Ibarra (2005) having stated that this fixed quantity is all that is required for optimization.

The Bc-Rep-PCR banding patterns of NDR1, *B. thuringiensis* subsp. *kenyae* (Figure 2), were compared to that of the DMSZ group *B. thuringiensis* subsp. *kenyae* (Figure 1). The banding patterns of these two subspecies of *B. thuringiensis* were found to be similar to each other (Figure 1 and 2). This demonstrates that these two isolates are the same organism but they produce different shaped crystal proteins (Table 1). The Bc-Rep-PCR banding pattern of the *B. thuringiensis* subsp. *morrisoni* isolate obtained from the DMSZ was not similar to that published by Reyes-Ramirez and Ibarra (2005). The banding patterns published by Reyes-Ramirez and Ibarra (2005) did not have a band at 3.0 Kb (Figure 1). This observation confirmed that the current protocol for Bc-Rep-PCR is not as reliable as claimed.

A further confounding factor is that various natural inhibitors may be present in the extracted DNA although, the manufacturer claims that their DNA isolation kit does eliminate inhibitors (Mobio) (Lamboy, 1994). The optimization with $MgCl_2$ does affect PCR reactions and this could be the main source of discrepancies (Lamboy, 1994). This could affect the binding of the primers to the template DNA hence resulting in relatively fewer bands being detected (Lamboy, 1994). Other unknown factors may affect the PCR reactions. These protocols are sensitive and the reproducibility of amplification patterns may be

Table 1. Serotypes and crystal protein shapes of the entomopathogenic bacterial isolates and the DSMZ *Bacillus thuringiensis* strains.

Bacterial sample	Crystal protein shape	Serotype
<i>B. thuringiensis</i> isolate NDR1	Bp ^α	4a, 4c
<i>B. laterosporus</i> isolate NDR2	Cs ^α	—
<i>B. thuringiensis</i> isolate NDR3	Tri ^α	Non-motile
<i>B. cereus</i> isolate NDR5	β	—
<i>B. thuringiensis</i> isolate NDR11	S ^α	Strong self agglutination
<i>B. thuringiensis</i> subsp. <i>thuringiensis</i>	S ^α	1
<i>B. thuringiensis</i> subsp. <i>azawai</i>	S ^α	7
<i>B. thuringiensis</i> subsp. <i>kurstaki</i>	Bp ^α	3a, 3b,3c
<i>B. thuringiensis</i> subsp. <i>kenyae</i>	S ^α	4a, 4c
<i>B. thuringiensis</i> subsp. <i>nigeriae</i>	U ^α	8a, 8d
<i>B. thuringiensis</i> subsp. <i>tochigiensis</i>	R ^α	19
<i>B. thuringiensis</i> subsp. <i>morrisoni</i>	S ^α	8a, 8b

^αBp, Bipyramidal; Cs, Canoe shaped; Tri, triangular; S, spherical, R, rhomboidal, U, undetermined; ^β Does not produce parasporal inclusion bodies.

affected by factors such as precise concentrations, cycling temperature and differences in parameters (Damiani et al., 1996).

DSMZ *B. thuringiensis* subspecies only distinguished between three of the seven subspecies (Figure 1). Four of the *B. thuringiensis* subspecies could not be distinguished from each other using this method (Figure 1). When comparing the results of these two groups of entomopathogenic spore-formers, *B. thuringiensis* subsp. *kenyae* produced a similar sized band as Isolates NDR1 and NDR11 (Figures 1 and 2). The common band shared between NDR1, NDR3 and NDR11 was not surprising because all three isolates are subspecies *B. thuringiensis* (Figure 2). NDR5 and NDR2 did not share any common bands with any of the isolates in Figure 2. This was expected because these two isolates are not *B. thuringiensis* subspecies. However NDR5 did share a common band with *B. thuringiensis* subsp. *thuringiensis* at 2.9kb (Figure 1 and 2). NDR5 is an isolate of *B. cereus* and it has been suggested that *B. cereus* and *B. thuringiensis* may be the same species of *Bacillus*, with the only difference being that *B. thuringiensis* produces crystal proteins (Bernhard et al., 1997; Priest et al., 2004).

The primers used for the Bc-Rep-PCR were meant to be *B. cereus* group specific. However, isolate NDR2 was an isolate of *B. laterosporus* that did not belong to this group. Hence it was surprising to find that the Bc-REP PCR generated clear bands for Isolate NDR2. This result may have been due to random REP-PCR amplification. This demonstrates another flaw in the technique as an alternative to serotyping for *B. thuringiensis* and closely related entomopathogenic bacteria. Only one of the isolates was identified with serotyping (Table 1). No prior research was found that reported toxicity by isolates of *B. thuringiensis* subsp. *kenyae* towards Coleoptera. Hence this isolate is the first of *B. thuringiensis* subsp. *kenyae*

reported to show toxicity to this insect order. Serotypes such as *B. thuringiensis* subsp. *morrisoni* are toxic towards Lepidoptera, Diptera and Coleoptera.

Brevibacillus laterosporus was unfortunately not serotyped. Isolate NDR5 (*B. cereus*) could not be serotyped because the Graduate School of Agriculture, Kyushu University in Japan only serotypes *B. thuringiensis* isolates although *B. cereus* can be serotyped (Ohba, 2005). Serotyping is a technique for identifying strains of *B. thuringiensis*. However, its limitations have become apparent as more of *B. thuringiensis* and related bacteria are isolated (Reyes-Ramirez and Ibarra 2005). Some of the problems that have been highlighted include:

1. *B. cereus* and *B. thuringiensis* isolates sometimes have a strong cross reactivity (Ohba and Aizawa, 1986);
2. Serotyping is unable to discriminate between closely related intra-subspecies strains such as *B. thuringiensis* subsp. *entomocidus* and *B. thuringiensis* subsp. *subtoxicus* (Joung and Côté, 2001).
3. Different esterase patterns are present on different serotypes (Lysenko, 1983). Cross-reactions due to these patterns make it difficult to differentiate between the subspecies.
4. Serotyping is not a good indicator of pathogenic specificity (Kriege et al., 1987). *B. thuringiensis* subsp. *morrisoni* isolates have been found that are pathogenic to Lepidoptera, Diptera and Coleoptera (Thiery and Frachon, 1997).
5. Serotyping is a laborious and time-consuming technique that involves the production of flagellar antisera in rabbits (Thiery and Frachon, 1997).

Given these problems, many institutes have since abandoned serotyping. As such, serotyping is probably no longer valuable as a method of identifying *B. thuringiensis* strains. Reyes-Ramirez and Ibarra (2005) could

not distinguish between various subspecies such as *B. thuringiensis* subsp. *amagiensis* and *B. thuringiensis* subsp. *seoulensis*; subsp. *sotto* and subsp. *Dakota*. These subspecies can however be identified from each other through serotyping (Reyes-Ramirez and Ibarra, 2005). Databases based on these techniques may thus be of limited value. However, the replacement technology of Bc-Rep-PCR is also not selective enough because different strains cannot be distinguished from each other by this method. This technique needs to be conducted in conjunction with serotyping to identify subspecies. However, this is not always applicable as not all subspecies are serotypable. A more sensitive and more stable technique is required for the classification of *B. thuringiensis* and related bacteria.

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