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Discrimination between *B. cereus* and *B. thurengenesis* using 16S rDNA sequencing

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ABSTRACT

This paper mainly emphasizes on the methods for the identification and discrimination of closely related species. Two isolates resembling Bacillus thuringenesis were isolated from soil and dead insects and analyzed by traditional biochemical methods. Biochemical results for this isolate were similar to that of Bacillus thuringenesis. For further confirmation a set of synthetic oligonucleotide homologous to broadly conserved sequence was used for in-vitro amplification via polymerase chain reaction followed by direct sequencing. The 16S rDNA sequencing after Blast analysis confirmed the bacterial isolate as Bacillus cereus, which causes food born disease syndrome associated with enterotoxin, whereas Bacillus thuringenesis is an insect pathogen. The 16S and 23S rRNA currently considered as the most useful molecules for the determination of prokaryotic phylogeny. Analysis of these rRNA sequences has resulted in tremendous expansion of our knowledge of prokaryotic diversity and has demonstrated the limitations of the existing prokaryotic taxonomy which is based primarily on the analysis of phenotypic traits. Discrimination between B. cereus and Bacillus thuringenesis is a challenging debate to which this paper makes contribution. Thus it is difficult task to discriminate between closely related species like Bacillus thuringenesis from Bacillus cereus by any traditional methods and the fact that they have grouped together in our analysis and other recent studies is not surprising.

Key words : *Bacillus cereus, B. thuringenesis,* 16S rDNA sequencing and bacterial identification

INTRODUCTION

Spore forming micro-organisms are widely distributed in nature due to resistance in their endo-spores to various stresses and their long term survival under unfavorable conditions. Therefore, most aerobe spore-formers can be isolated form a wide variety of sources including foods. Bacillus species are important, as food spoilage organism and can be isolated from fruits, vegetable products, nuts, cereals, milk and dairy products, meat, dried foods and spices. The current classification of Bacillus isolated from dead insects is based on endosperm and sporangium morphology and further subdivision to species level on the basis of biochemical tests. Differentiation between *Bacillus* can be difficult and a large number of phenotypic tests are used to distinguish between them, although sometimes only a single feature separates species. Several *B. cereus* isolates are present in food and involved in food poisoning so these isolates needs to be properly identified by using specific and sensitive methods. As the traditional methods are insufficient to discriminate *Bacillus thuringenesis* and *B. cereus* like microbes many recent studies grouped them together (Carlson *et al.,* 1994 and Daffonchio *et al.,* 2000).

MATERIALS AND METHODS

Bacterial Isolates: Bacillus isolate PDKV-II included in this study was collected from soil and dead insects from fields of PDKV campus. Isolated bacteria were grown on nutrient broth and used for analysis.

Morphological cultural and biochemical characterization of isolates or conventional identification of isolates:

For identification of *Bacillus* spp. in our lab morphological characteristics such as shape, size, endspore production etc. were observed. Cultural characteristics were also studied along with growth in broth. Slides were observed under phase contrast microscope. Bacterial isolates and were also identified by conventional biochemical methods comprising of IMVIC test (Indole, Methyl Red, Voges Proskauer and Citrate Test), sugar fermentation eg. Glucose, lactose, maintol, arabinose and sucrose with acid and gas production and enzymes like catalase, oxidases, urease, gelatinase, amylase, caseinase, lecthinase, deaminase, cellulase, lipse, β-galactosidase, nitrate-reductase were studied (Deshpande *et al.,*).

Genotypic identification:

Pure cultures of Bacilli were used for DNA extraction using the Prep-Man[Tm]. DNA extraction protocol was provided in the Microseq Manual. A 500bp 16S ribosomal DNA (rDNA) fragment was amplified from 5'

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end of the gene in a reaction volume of 50 µl (25 µl PCR master mix + 25 µl genomic DNA). Positive control (*E. Coli* Genomic DNA; Sigma corporation) and a negative control (sterile deionized water) were also included in experiment. Tubes were capped and placed in Gene Amp PCR System 9600 (PE-ABD). Thermal cycling parameters included an initial of 10 min at 95^{0}C; 30 cycles of 30sec at 95^{0}C, 30sec at 60^{0}C and 45sec at 70^{0}C and final extension at 72^{0}C for 10 min.

PCR was performed on Perkin-Elmer Gene Amp PCR System 9600. Amplified products were characterized by electrophoresis on 1% agarose gel. Later, amplified products were purified with EXO-SAP according to the manufacture's instructions.

Prior to sequencing, cycle sequencing reactions of 16S rDNA was performed by using the Microseq 16S rDNA bacterial sequencing kit and recommended protocol. Forward and Reverse sequencing reactions were performed for each amplified product. The sequencing reaction consisted of 13µl of Microseq sequencing mix 4µl of sterile distilled water, and 3ìl of purified amplified product. Sequencing reactions were purified by using master mix, containing 3M sodium acetate (10µl), ethanol (250µl), according to manufactures instructions, and all sequence analysis was performed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer/ Applied Biosystems, Foster city, Calif) (Patel *et al.,* 2000).

Sequence data analysis:

All the sequencing data was analyzed with the Microseq software version 1.36. The consensus sequence was used for database comparison. A dendogram consisting of close matches with the distance score was generated with Alignment Tool of Microseq software.

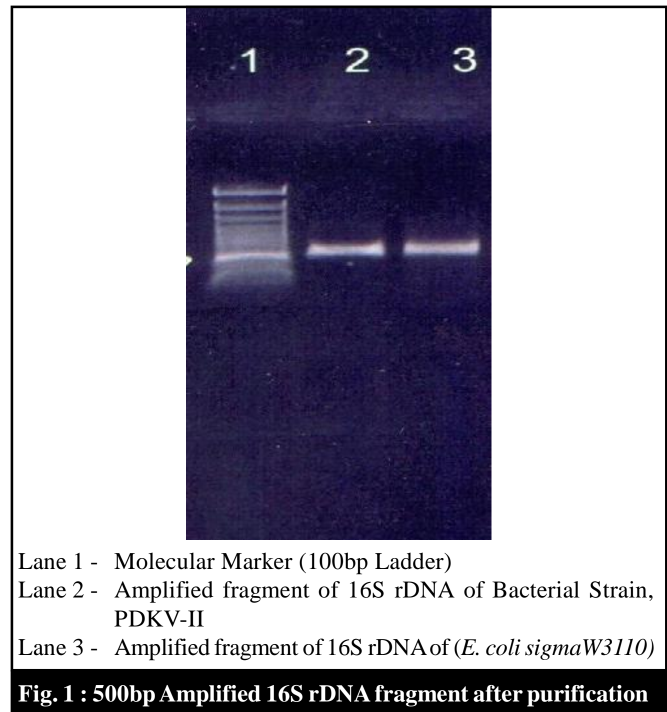
This distance score indicated the per cent difference between unknown sequence and the database sequence. For the purpose of comparing an isolate's original identification to its Microseq identification, the Microseq identity was considered to be the closest match in the Microseq database.

RESULTS AND DISCUSSION

Isolates included in our study were identified to genus level by conventional morphological, cultural and biochemical methods, and for species level identification of isolate was subjected to 16SrDNA sequence analysis. Various morphological, cultural characteristics were also studied and it was found that growth on nutrient agar medium was rapid and abundant. The isolates formed rough, off-white, opaque flat colonies on nutrient agar with undulated edge. When the isolates were grown in broth, the medium became turbid with the formation of a pellicle. The isolate was found to be gram positive. Biochemical test comprising of IMVIC test, Sugar fermentation and enzyme production was studied (Catalase, lecitihinase, deaminase, cellulase, lipase, b-galactosidase, nitrate-reductase. Results of all biochemical tests are presented in (Table 1).

After adapting the conventional methods we have identified the bacterial isolates by using highly advanced genotypic identification system the Microseq 500 16S bacterial sequencing kit (Perkin-Elmer (PE) Biosystems, Foster City, Calif.)

16S ribosomal DNA from bacterial strains PDKV-II was isolated using prep Man^{Tm} DNA extraction protocol. Amplification was done using specific primers which resulted in expected 500bp PCR product. The weakly non-specifically amplified PCR products were completely removed by the gel electrophoresis and DNA clean-up procedure (Exo-I SAP). DNA recovery results after purification is shown in (Fig. 1).



Lane 1 - Molecular Marker (100bp Ladder)
Lane 2 - Amplified fragment of 16S rDNA of Bacterial Strain, PDKV-II
Lane 3 - Amplified fragment of 16S rDNA of (*E. coli sigmaW3110)*

Fig. 1 : 500bp Amplified 16S rDNA fragment after purification

Partial gene sequencing of 500bp 16S rDNA was performed for bacterial isolate PDKV-II. The 500bp 16S rDNA sequence was amplified with Microseq 500 16S rDNA PCR module. Forward and reverse sequencing reactions resulted in 500bp 16S rDNA sequences. By assembling these forward and reverse sequences consensus sequences were derived. Identification of bacteria was done by comparing the sequences of

unknown bacterial strain with the Microseq-database.

Sequence analysis:

The DNA Sequence obtained was subjected to 'BLAST' search with micro sequence Database for identification and analysis. 'BLAST' search generated a list of closest matches with distance score. The distance score indicated differences between the unknown sequence and database sequence. The Microseq identity was considered to be the closest match in Microseq database. Prior to adoption of sequencing system the isolate was identified by biochemical characterization but, biochemical characteristics of PDKV-II was so, similar to both the species *i.e. B. cereus* and *B. thurengenesis* that we can't correctly identify the isolate. Microseq software generated a distance matrix for PDKV-II, which was showing *B. Cereus* as the closet match for PDKV-II with 10.54 as distance score. Distance matrix was also showing the other matches but *B. mycoides* and *B. thuringiensis* were the closer next to *B. Cereus.*

Dendograms were constructed based upon the set distances between all pairs of sequences of the input files which include unknown sequences, any of the sequences from the Microseq database and sequences from other sources or database. A scale is included describing a percentage difference that the segment length represents and dendograms were showing that *B. cereus*, *B. thurengenesis* fall in same cluster in the neighbor Joining tree along with isolate PDKV - II. The positive control was also correctly identified as *E. coli* sigma W3110 (Fig. 2).

In this study, bacterial isolate from soil was identified and characterized by biochemical tests and later confirmed by ribosomal sequence analysis. The results of biochemical tests were not confirmatory but sequencing correctly identified the isolate as *B. cereus.*

Table 1 : Biochemical characters of *Bacillus* isolates

Characteristics of *Bacilli*	*Bacillus* isolates	
	II	IV
IMViC		
Indole production, Methyl Red	+	+
Voges-proskauer, Citrate utilization	-	-
Sugar fermentation		
Glucose, Sucrose	A/-	A/-
Lactose , Mannitol, Arabinose	-/-	-/-
Enzyme study		
Catalase, Oxidase, Urease, Gelatinase	+	+
Amylase, Caesinase	-	-
Lecithinase, Deaminase	+	+
Cellulase, Lipase, B-Galactosidase, Nitrate reductase	-	-

A/-: Acid / No gas, -/-: No Acid / No gas.

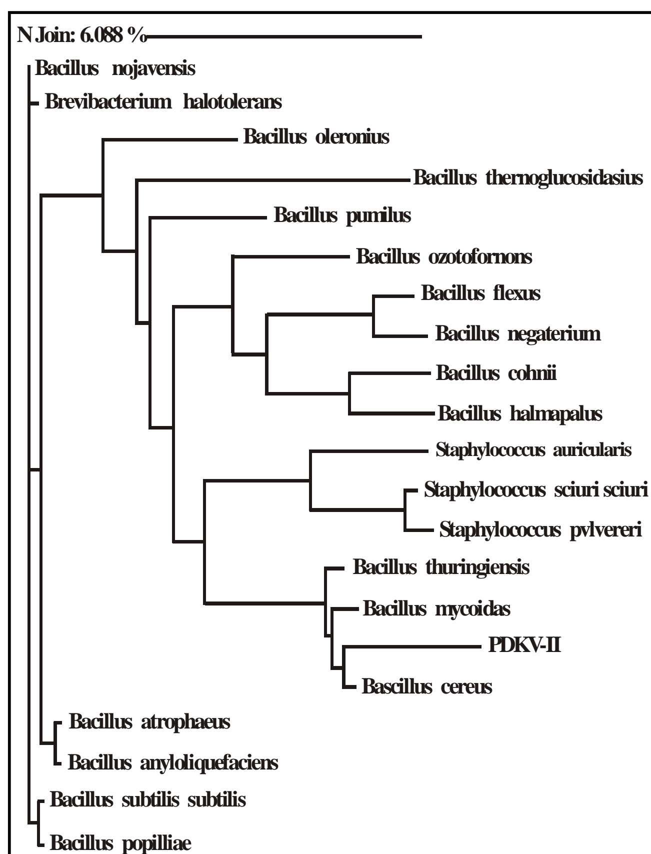


Fig. 2 : Phylogenetic tree for bacterisl isolate PDKV-II

Assessment of genetic relationship of *Bacillus cereus* and *B. thurengenesis* serovars from measurements of DNA reassociation DNA relatedness studies and 16S rDNA sequence data have indicated that *B. cereus* and *B. thurengenesis* are closely related (Nakamura *et al.*, 1994). Most of the biochemical characters of the *B. cereus* and *B. thurengenesis* are very much similar to each other. *B. thurengenesis* can only be distinguished from *B. cereus* by production of toxin crystals. All isolates identified as *B. thuringiensis* by sequencing reacted with the primers for the three known *B. thuringensis* delta -toxin gene sequences. However, this characteristic is plasmid encoded and transmissible to *B. census* by conjunction (Damgaard *et al.*, 1996 and Damgaard *et al.,* 1997). *B. thuringensis* preparation for use in controlling both agricultural pest and vectors of human and animal disease are commercially available. The results presented in this study emphasize the need to screen strains carefully for use as insecticides, as some of the strains identified as *B. thuringensis* were involved in food poisoning eg. Strain L3 and 1230. These strains produced enterotoxin determined by western

immunoblot technique, PCR and Vero cell assays. It has already been reported in previous studies that strains of *B. thuringensis*, including strains isolated from commercial insecticides, produced a *B. cereus* diarrheal type enterotoxin (Claus *et al.,* 1986) and some incidences linking *B. thuringenesis* to human infection have been described (Jackson *et al.,* 1995 and Kramer *et al.,* 1989). Cases of *B. Cereus* diarrheal outbreaks from ingestion of raw contaminated vegetable sprouts and from improperly cooked green bean have been reported should be intrigued to avoid misclassification of microclines result isolated. (Damgaard *et al.*, 1996 and Damgaard *et al.,* 1997) These cases may in-fact have been caused by *B. thuringenesis*, due to misclassification of isolates. This could suggest that the role of *B. thuringenesis* in food poisoning might be underestimated. Based on these observations and the results presented in this study, it seems necessary to reevaluate the taxonomy of this organism. As specific and sensitive identification method to discriminate between the species, like *B. thuringenesis* and *B. cereus* 16S rDNA sequence analysis is available and should be utilized to avoid misclassification microbial of isolates.

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