Genotypic and phenotypic diversity of *Bacillus* spp. isolated from Freshwater Ecosystems

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Abstract

The efficacy of the restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplified rrs (16S ribosomal ribonucleic acid [rRNA] gene) for the identification of *Bacillus* spp. isolated from freshwater pond ecosystems was evaluated. Results showed that no restriction site differences occur between rrs operons within the bacteria belonging to the same species and as a result members of the same species yield very similar RFLP patterns at 100% similarity level. Cluster analysis of PCR-RFLP patterns grouped all the isolates into three clusters; each cluster represented a single species except one for which no placement was obtained. Phenotypic characteristics of the isolates were found to be well concordance with the results of PCR-RFLP. Our findings suggested that PCR- RFLP is suitable tool for discrimination and identification of endoglucanase positive *Bacillus* spp. isolated from freshwater ecosystems.

Keywords: Cellulolytic *Bacillus* spp.; 16SrDNA PCR- RFLP; 16SrDNA gene Sequencing

Introduction

Cellulose is a prominent carbonaceous constituent of plants and the most abundant organic compound in nature. In freshwater ecosystems it is a major constituent of aquatic plants [9] and major source of nutrients for other organisms [13]. The degradation of cellulose involves three enzymes β -glucosidase, endo- β -(1-4)-D-glucanase and exo- β -(1-4)-D-glucanase, where these enzymes interact synergistically for the hydrolysis of cellulose [20]. The genus *Bacillus* is characterized by Gram-positive, aerobic or facultative anaerobic, rod-shaped bacteria that form spores, and contains more than 60 species that have quite different phenotypes and produces cellulase. These spore-forming bacteria represent a major microflora in many natural biotopes, where they play an important role in ecosystem development; they are able to transform many chemical compounds. DNA typing techniques using various methods for molecular characterization of *Bacillus* spp. have been developed, including the 16S-23S rDNA Intergenic Transcribed Spacer PCR (ITS-PCR) amplification [6,8], Restriction Fragment Length Polymorphism of the ITS-PCR (ITS-PCR RFLP) for differentiation of species and strains [6], Randomly Amplified Polymorphic DNA (RAPD) [6] and Single Strand Conformation Polymorphism (SSCP) [4]. The composition of benthic microbial communities can be readily studied by whole-cell fluorescence in situ hybridization [10]. Although FISH with fluorescently monolabeled oligonucleotide probes is an effective tool for studying microbial community structuring in various aquatic environments [2], its sensitivity is thought to depend on the growth state (i.e., ribosome content) of the target cells [1]. The objective of this study was to discriminate endoglucanase positive *Bacillus* spp. based on 16SrDNA PCR-RFLP.

Materials and Methods

Isolation of cellulolytic bacillus spp

Seventy sediment samples from carp rearing ponds were collected from uppermost layer at a depth of 4-5 cm. The sizes of the rearing ponds were 0.1-0.4 hectares. Alkalinity, pH and DO were 100-120 ppm, 7.5-8.0 and 4-5 mg/lit respectively. The samples were pre-enriched in carboxymethyl cellulose (CMC) broth for 48 hrs. Screening was carried out, by spreading the serially diluted pre-enriched samples in CMC agar

plates and well separated colony was screened for cellulolytic activity by congo red binding assay [18]. All the strains were preserved in 40% glycerol at -20°C for further use.

Morphological, physiological, biochemical characterization

The morphological characteristics of different isolates were studied by observing colony characters in CMC agar plates, Gram staining, endospore staining and subjected to further characterization by different biochemical tests. Physiological parameters included growth at different ranges of temperature (25°C to 65°C) and sodium chloride concentration (2% to 10%).

DNA extraction

The isolates were grown in cellulose enrichment broth for overnight at 37°C. Cultures (10ml) were pelleted by centrifugation at 5,000 rpm for 10 min and DNA was extracted by the method as described [14]. DNA was precipitated by the addition of one volume of isopropanol. The DNA was then dried and dissolved into 50 μ l TE buffer. DNA quality was checked by 0.8% agarose gel electrophoresis and quantified spectrophotometrically (6505 UV/VIS Spectrophotometer, Jenway).

16S rDNA amplification and restriction digestion

The 16S rDNA gene from individual bacterial isolates was amplified by the polymerase chain reaction (PCR) with the 16S primers [18]. The PCR reaction mixtures (50ml) contained, dNTPs each 2.5mM; 10X PCR buffer 5ml; each primer 20 pmol; Taq DNA polymerase (Genetix, India) 0.75U and bacterial DNA 100 ng. The forward primer was 5'AAG AGT TTG ATC CTG GCT CAG 3' and the reverse primer was 5' GGT

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TAC ATT GTT ACG ACT T 3'. The PCR was carried out in a thermal cycler (M. J. Research, Inc., Waltham, Massachusetts, USA) with initial denaturation of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 49°C for 2 min., extension at 72°C for 2 min and a final extension at 72°C for 8 min. Amplified DNA fragments were examined by horizontal electrophoresis in 2% agarose gel (Genei, India) containing ethidium bromide (0.5 mg/ml) at 100V for 1 hr in 1X TBE (Tris-Boric acid-EDTA) buffer with 5 ml aliquots of PCR products. The gel images were digitized through UVP GelDoc-itTM imaging system, USA.

Three restriction endonucleases used in this study were Alu I, Hae III and Msp I (Genetix, India). PCR product (1µg) was digested with 1.5U of each enzyme at 37°C overnight. Digested PCR products were electrophoresed through 3% agarose gels containing 0.5mg/ml ethidium bromide for approximately 2.5 hrs at 100V using 1X TBE buffer and photographed in UVP GelDoc-itTM imaging system, USA.

16s rDNA sequencing and identification

PCR products of 17 different isolates from each composite genotype were purified by QIAquick PCR purification kit according to the manufacturer's instructions (QIAGEN, Germany). The purified PCR products were sequenced by Chromous Biotech, Chennai, India. A database search was performed using BLAST programme (NCBI, Maryland, USA) to identify the microorganisms at species level.

Data analysis

For cluster analysis, data were transformed to estimate distances [12]. The Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) was used for cluster analysis for 16s PCR RFLP [16].

Results and Discussion

Forty one cellulolytic isolates from 70 pond sediment samples were obtained on the basis of CMC congo-red interaction assay, which showed halo zones, ranging from 14 to 44 mm diameter indicating quantitative variation in the enzyme production among the isolates. All the strains were found to be Gram positive, catalase and oxidase positive, spore-forming rods. Most of the strains utilized dextrose, D-fructose, gelatin, casein, urea, mannose, trehalose, sucrose whereas, all the isolates were negative for indole production, L-rhamnose, D-melzitose phenylalanine deamination, hydrogen sulphide production. The isolates revealed variation in citrate utilization, amylase production, nitrate reduction, acetyl methyl carbinol production and sugar utilisation like; mannitol, sorbitol, L-arabinose, D-xylose. Twenty isolates showed amylase activity, 24 nitrate reduction activity, 19 phosphate solubilization activity and 11 isolates lecithinase activity. The physiological studies showed the growth of 23 isolates at 60°C and only 8 isolates tolerate upto 10% salt concentration.

A specific product of 1.5 kb was obtained in 16S rDNA PCR in all the isolates. PCR product digested with AluI (Figure 1A) showed one common band of molecular size 184bp, Hae III (Figure 1B) digestion produced three common bands of molecular size 435, 294 and 123bp and banding pattern obtained with MspI (Figure 1C) showed three common bands of 422, 220 and 132bp molecular size in all the isolates. Four different profiles were obtained with PCR products digested with AluI and three with both HaeIII and MspI. Patterns of each enzyme were combined together and each strain was assigned a composite genotype [19]. Based on this, 4 genotypes were obtained. In *AluI* digestion a common band of 265bp molecular size was found in all the isolates except DCC genotype. Three bands of 632, 430, 242bp molecular size were exclusively present in DCC genotype. A unique band of 85bp

Figure 1: Agarose gel electrophoresis of *AluI*(A), *HaeIII*(B), *MspI*(C) digested PCR products of few representative samples. Lane M, size marker (100bp DNA ladder).

Figure 2: Dendrogram showing the genetic relationship of different isolates of cellulolytic *Bacillus* sp. constructed on the basis of 16S rDNA PCR-RFLP pattern.

SAMPLE NUMBER	SPECIES IDENTIFIED BY PCR-RFLP	GENOTYPE	CELLULASE	AMYLASE	PHOSPHATE SOLUBILISATION	LECITHINASE	SALT TOLRANT	
							5%	10%
C7A	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C8K1	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C3E	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C3F	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C14J	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C1G	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C2B	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C2F	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C11A	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C11B1	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C11B2	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C8K3	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C11D	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C13A	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive
C14E	<i>Bacillus subtilis</i>	AAA	High	Positive	Positive	Negative	Positive	Positive

Table 1: Detailed characteristics of the isolates grouped in cluster-I.

SAMPLE NUMBER	SPECIES IDENTIFIED BY PCR-RFLP	GENOTYPE	CELLULASE	AMYLASE	PHOSPHATE SOLUBILISATION	LECITHINASE	SALT TOLRANT	
							5%	10%
C14D	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive
C14K	<i>Bacillus pumilus</i>	BAB	Medium	Positive	Negative	Negative	Positive	Positive
C10F	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive
C14A	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive
C8N	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive
C8P	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive
C5K	<i>Bacillus pumilus</i>	BAB	Medium	positive	Positive	Negative	Positive	Positive
C5M	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive
C14N	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Positive	Negative	Positive	Positive
C5R	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive
C6C	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Positive	Negative	Positive	Positive
C7E	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive
C8M	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Positive	Negative	Positive	Positive
C14L	<i>Bacillus pumilus</i>	BAB	Medium	Negative	Negative	Negative	Positive	Positive

Table 2: Genotypic and phenotypic characteristics of the isolates in cluster-II.

SAMPLE NUMBER	SPECIES IDENTIFIED BY PCR-RFLP	GENOTYPE	CELLULASE	AMYLASE	PHOSPHATE SOLUBILISATION	LECITHINASE	SALT TOLRANT	
							5%	10%
C5F	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C9E	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C1H	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C5A	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C11C	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C11E	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C9EP	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C12C	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C12I	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C121P	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative
C12L	<i>Bacillus cereus</i>	DCC	Low	Negative	Negative	Positive	Positive	Negative

Table 3: Detailed characteristics of the isolates grouped in cluster-I.

molecular size was present only in BAB genotypes. In *HaeIII* restriction digestion pattern there was no difference in the banding pattern between AAA and BAB genotypes, where as DCC genotype could be differentiated from other two groups by a specific band of 565bp molecular size. In *MspI* restriction digestion a band of 154bp molecular size was absent in BAB genotype. In AAA genotype and DCC genotype all bands were same except a band of 581bp which were present in AAA

genotype. 16S rDNAs of 17 isolates, eight from AAA genotype, seven from DCC genotype and two from BAB genotype were sequenced. A database search was performed using BLAST programme (NCBI, Maryland, USA) to identify the microorganisms at species level. All the isolates from AAA, BAB and DCC genotypes were identified as *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus cereus* respectively. The alignment showed 99-98% sequence similarity. Heyndrickx et al. [7] observed the

frequent occurrence of interperon variability of the 16S rRNA gene in *Bacillus* and *Paenibacillus*. Because PCR-RFLP detects interspecies and interstrain as well as interperon variability and enables a relatively fast multiple strain analysis per taxon. This PCR-RFLP fingerprinting allows the construction of a database for identification purposes. Shangkuan et al. [15] reported that PCR-RFLP is simple to perform and has potential as a rapid method for typing and discriminating *B. anthracis* strains from other *B. cereus* group bacteria. On the contrary, PCR-RFLP fingerprinting failed to identify *Brevibacillus agri* and *Brevibacillus brevis* [11]. Dendrogram (Figure 2) constructed from 16s rDNA PCR-RFLP data showed three different clusters. Cluster-I comprised of all AAA genotypes, cluster-II BAB genotype and cluster-III DCC genotypes. In between cluster-I and cluster-II similarity of $\leq 90\%$ was observed where as cluster-III was distantly related to both cluster-I and cluster-II. However isolate C4B couldnot be clustered.

Analysis of phenotypic/functional characteristics of these clusters revealed that cellulase production in terms of zone size was highest in *Bacillus subtilis*, intermediate in *Bacillus pumilus* and lowest in *Bacillus cereus*. (Tables1,2,3) All the isolates of cluster-I two of cluster-II were positive for starch degradation (amylase production), and the property was absent in all the isolates of cluster-III. Phosphate solubilization was absent in all the isolates of cluster-III where as all the isolates of cluster-I and four isolates of cluster-II were positive for phosphate solubilization. Lecithinase activity was exclusively present in all isolates of cluster-III. Salt resistance of the isolates was less in cluster-III (5%) where as it was comparatively high (10%) in other two clusters. It indicates that genetic relatedness of three species of *bacillus* coincided well with the phenotypic relationship of the species.

The identification of microbial species by phenotypic methods can sometimes be uncertain, complicated and time-consuming. The use of molecular methods has revolutionised their identification, by improving the quality and effectiveness of this identification. Some of these methodologies use either the rDNA spacer region or its target. These techniques are useful for the identification and reliable detection of different bacterial species as well as the monitoring of the species [3]. Our approach using 16S rRNA gene has enabled comprehensive characterization of cellulolytic *Bacillus* spp. predominating in freshwater ecosystems. This study shows that PCR-RFLP as an alternative to more laborious techniques used for endoglucanase positive *Bacillus* spp. identification and characterization, such as the morphological and physiological analyses or the determination of the 16S rRNA gene sequence, can be used as an identification tool. The results were validated by 16S rDNA sequencing of isolates selected at random from each cluster.

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