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-235 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 100-120ppm, 7.5-8.0 and 4-5mg/liter 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 5,000rpm for 10 min and DNA was extracted by the method as described [14]. DNA was precipitated by the addition of one volumes of isopropanol. The DNA was then dried and dissolved into 50µl 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′AGT TTG ATC CTG GCT CAG 3′ and the reverse primer was 5′GTT AGT TTG ATC CTG GCT CAG 3′.

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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 2 hr in 1X TBE (Tris-Boric acid-EDTA) buffer with 5 ml aliquots of PCR products. The gel images were digitized through UVP GelDoc-IT 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-IT 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-rihamnose, D-melitotose 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 expect DCC genotype. Three bands of 632, 430, 242bp molecular size were exclusively present in DCC genotype. A unique band of 85bp
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 between AAA and BAB genotypes, whereas DCC genotype could digest pattern there was no difference in the banding pattern molecular size was present only in BAB genotypes. In AAA genotype and DCC genotype molecular size was absent in BAB genotype. In AAA genotype and DCC genotype molecular size was absent in BAB genotype. In AAA genotype and DCC genotype molecular size was absent in BAB genotype.

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 TOLERANT |
|---------------|--------------------------------|----------|-----------|---------|--------------------------|-------------|--------------|
|               |                                |          |           |         |                          |             | 5%           | 10%          |
| C7A           | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C8K1          | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C3E           | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C3F           | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C14J          | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C1G           | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C2B           | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C2F           | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C11A          | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C11B1         | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C11B2         | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C8K3          | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C11D          | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C13A          | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | Negative    | Positive     | Positive     |
| C14E          | Bacillus subtilis              | AAA      | High      | Positive| Positive                 | 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 TOLERANT |
|---------------|--------------------------------|----------|-----------|---------|--------------------------|-------------|--------------|
|               |                                |          |           |         |                          |             | 5%           | 10%          |
| C14D          | Bacillus pumilus               | BAB      | Medium    | Negative| Negative                 | Negative    | Positive     | Positive     |
| C14K          | Bacillus pumilus               | BAB      | Medium    | Positive| Positive                 | Negative    | Positive     | Positive     |
| C10F          | Bacillus pumilus               | BAB      | Medium    | Negative| Negative                 | Negative    | Positive     | Positive     |
| C14A          | Bacillus pumilus               | BAB      | Medium    | Negative| Negative                 | Negative    | Positive     | Positive     |
| C8N           | Bacillus pumilus               | BAB      | Medium    | Negative| Negative                 | Negative    | Positive     | Positive     |
| C8P           | Bacillus pumilus               | BAB      | Medium    | Negative| Negative                 | Negative    | Positive     | Positive     |
| C5K           | Bacillus pumilus               | BAB      | Medium    | Positive| Positive                 | Negative    | Positive     | Positive     |
| C5M           | Bacillus pumilus               | BAB      | Medium    | Negative| Negative                 | Negative    | Positive     | Positive     |
| C14N          | Bacillus pumilus               | BAB      | Medium    | Negative| Positive                 | Negative    | Positive     | Positive     |
| C5R           | Bacillus pumilus               | BAB      | Medium    | Negative| Positive                 | Negative    | Positive     | Positive     |
| C6C           | Bacillus pumilus               | BAB      | Medium    | Negative| Positive                 | Negative    | Positive     | Positive     |
| C7E           | Bacillus pumilus               | BAB      | Medium    | Negative| Negative                 | Negative    | Positive     | Positive     |
| C6M           | Bacillus pumilus               | BAB      | Medium    | Negative| Positive                 | Negative    | Positive     | Positive     |
| C14L          | Bacillus pumilus               | BAB      | Medium    | Negative| Negative                 | Negative    | Positive     | Positive     |

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

| SAMPLE NUMBER | SPECIES IDENTIFIED BY PCR-RFLP | GENOTYPE | CELLULASE | AMYLASE | PHOSPHATE SOLUBILISATION | LECITHINASE | SALT TOLERANT |
|---------------|--------------------------------|----------|-----------|---------|--------------------------|-------------|--------------|
|               |                                |          |           |         |                          |             | 5%           | 10%          |
| C5F           | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C9E           | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C1H           | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C5A           | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C11C          | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C11E          | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C9EP          | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C12C          | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C12I          | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C121P         | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
| C12L          | Bacillus cereus                | DCC      | Low       | Negative| Negative                 | Positive    | Positive     | Negative     |
frequent occurrence of interoperon variability of the 16S rRNA gene in Bacillus and Paenibacillus. Because PCR-RFLP detects interspecies and interstrain as well as interoperon 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 ≤ 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 (amylose 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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