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BACTERIAL COMMUNITY PROFILES IN RHIZOSPHERE OF PADDY RICE BASED ON 16S RRNA SEQUENCE ANALYSIS

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ABSTRACT

Rice is a staple food to almost one-third population of the world. The crop sustainability must therefore be considered to keep pace with the growing world population. Microorganims are one of the key factors affecting crop productivity and quality. Of particular interest is the plant rhiszosphere microbiome which has gained enormous attention in recent years due to their role both as pathogen and plant growth promoting agents. Accordingly, the present study is aimed at identification of bacteria isolated from soil samples collected from rice fields of district Jaffarabad, which belongs to the major rice growing area of Pakistan. Six strains B9, B15, B34, B40, B52 and B53 were identified as genus *Bacillus* whereas isolate B12 as genus *Staphylococcus* based on morphological and biochemical properties and 16S rRNA gene sequence analysis. Isolates B9, B40 and B53 showed 99% similarity to *Bacillus thuringiensis*, *Bacillus anthracis* and *Bacillus endophyticus* respectively. Conflicting results were observed for B15, B34 and B52 for sequence analysis based on BLAST and SeqMatch alignment. The isolate B12 showed 99% similarity to *Staphylococcus aureus*. Our findings indicate that instead of resorting rice bacterial populations through biochemical tests, the validation through 16S rRNA technique provides a more objective view.

Key words: Rice Rhizosphere, 16S rRNA, Bacilli, *Staphylococcus*

INTRODUCTION

Rice is a staple food to more than 3 billion population of the world. Rice contributes to 30-75% of the daily calorie intake of people living mostly in Asia, Africa and Latin America (Prasanna *et al.*, 2012). Therefore, the rice crop yield is required to be increased to keep pace between the rapidly escalating population and food demand of these areas. There are various challenges to achieve this target including insects, pests and pathogens which are a serious threat to crop production. The uncontrolled application of pesticides and fertilizer is posing severe health hazards and disrupting the environmental balance. The agriculture industry is therefore demanding an alternative which is equally effective and less hazardous. In view of this fact, soil microbial community has gained much attention in recent years due to their contribution to nutrient acquisition, growth and productivity of plant.

Bacteria are the most abundant and diverse constituent of the soil microbial community. A single gram of soil has been reported to contain 4000-7000 different bacterial genomes (Torsvik *et al.*, 1990). These bacteria perform a variety of metabolic activities. Five most crucial of which are: (i) turnover and formation of organic matter (ii) nutrient cycling (iii) prevention and transmission of disease (iv) degradation of pollutants and (v) soil structure improvement (Nannipieri *et al.*, 2003). Thus an understanding of diversity and function of the bacterial community would allow to produce crops in a more balanced and sustainable manner.

Most groups of beneficial bacteria are located in the rhizosphere termed as plant growth promoting rhizobecateria (PGPR). PGPR activity has been extensively reported for strains belonging to several genera such as

Arthrobacter, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Burholderia*, *Cellulomonas*, *Enterobacter*, *Pseudomonas*, *Rhizobium*, and *Serratia* (Elango and Rajasekar, 2011). There is still a long and growing list of PGPR which are capable of promoting plant growth by enhancing tolerance to abiotic stress (Berendsen *et al.*, 2012; Yang *et al.*, 2009) and hampering the growth of soil-borne pathogens (Bakker *et al.*, 2007; De Bruijn *et al.*, 2007; Kamilova *et al.*, 2008). In one study by Sokolova *et* al., (2011) growth promoting effects of PGPR strains *Azotobacter chroococcum* Az d10, *Bacillus megaterium* PI-04, and *Bacillus mucilaginosus* B-1574 with cucumber (*Cucumis sativus* L.) was reported. PGPR strain inoculation in this study stimulated seed germination and increase in the growth rate, the biomass of shoots, the number of lateral roots, and the root hair area by producing cytokinins (CKs) and indol-acetic acid (IAA) phytohormones. In another study (Ali *et al.*, 2009), a thermotolerant *Pseudomonas* spp. strain AMK-P6 induced thermotolerance in sorghum seedlings, reduced membrane injury, and improved the levels of cellular metabolites like proline, chlorophyll, sugars, amino acids, and proteins due to synthesis of high molecular weight protein in leaves at elevated temperatures.

Altogether, PGPR offer an environmentally sustainable approach for biocontrol and improvement of plant and soil health. The application of molecular tools is enhancing our ability to understand the rhizosphere which will lead to new products with improved effectiveness. Several techniques have been devised for accessing the soil microorganisms and various signature sequences have been identified for characterization. However, 16S rRNA sequence has been by far the most commonly used housekeeping genetic marker.

There are several intrinsic characteristic of 16S rRNA sequence making it suitable for phylogenetic analysis. Most notable of which are (i) its presence in almost all bacteria (ii) the function of the 16S rRNA gene over time has not changed (iii) the 16S rRNA gene is large enough for informatics purposes (1,500 bp). Sequencing of 16S rRNA is the most common approach due to both its phylogenetic properties and large amount of 16S rRNA sequence data available for analysis. 16S rRNA gene amplicon pyrosequencing root microbiome of *Arabidopsis thaliana* has recently provided a comprehensive picture of the *Arabidopsis thaliana* root microbiome (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012).

Agriculture has an important direct and indirect role in generating economic growth in Pakistan. Therefore, soil and improving crop management practices receive considerable importance. Accordingly, this study is aimed at the isolation and identification of bacteria isolated from rhizosphere of rice. Although, the diversity and function of microbes in rice rhizosphere have been studied in different areas of Pakistan (Malik *et al.*, 2002; Mehnaz *et al.*, 2001; Mirza *et al.*, 2006; Naureen *et al.*, 2009), this study is however, the first attempt to document the soil microbial flora of an agriculturally important area, Jaffarabad; situated in Balochistan.

MATERIALS AND METHODS

Soil Sampling and Bacterial Isolation

Ten soil samples from the rhizospheric region of rice (variety IRRI-6) were collected from paddy fields of Jaffarabad district, which belongs to the major rice growing area of Pakistan. Seven morphologically distinct bacterial isolates B9, B12, B18, B34, B40, B52 and B53 were isolated from rice rhizospheric soil samples. Isolation of these organism involved dilution (10^{-2}) of the soil samples and plating by spread plate method on nutrient agar media. Pure isolates were obtained and subjected to identification based on morphological and biochemical characteristics, and 16S rRNA gene sequence analysis (Gerhardt *et al.*,1994).

Morphological and Biochemical Characteristics

Gram staining and biochemical tests were performed for the identification of bacterial isolates following the method described by Cappuccino and Sherman, (2002). Cell morphology was studied by light microscope (Olympus microscope CX 21, Japan) and colony morphology was determined by using a stereo microscope (Olympus model SZ 11, Japan).

16S rRNA Sequence Determination

Genomic DNA was extracted from overnight-grown bacterial cultures incubated at 37°C in Luria-Bertani broth medium at 120 rev min^{-1}(Oxoid). DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega®, England) following the manufacturer's instructions. Amplification of 16S rDNA was performed according to the method described by Hasnain and Thomas, (1996) by using forward primer 920(5'-AAACTCAAATGAATTGACGG-3') and reverse primer 1392 (5'-ACGGGCGGTGTGTAC-3'). The PCR product was excised from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN® Inc., Valencia, CA). The purified PCR product was sent to Centre of Applied Molecular Biology (CAMB), Lahore for sequencing.

Sequence and Phylogenetic Analysis

The 16S rRNA sequence of the isolates was compared to those generated by BLAST of GenBank (www.ncbi.nlm.nih.gov/BLAST/) and SeqMatch of RDP (http://rdp.cme.msu.edu/seq-match/seqmatch_intro.jsp) for assignment to closet match. The sequences were aligned with 16S rRNA reference sequences retrieved from NCBI. The phylogenetic tree was constructed in MEGA version 5 (Tamura *et al.*, 2011), based on the Tamura-Nei model (Tamura and Nei, 1993).The evolutionary history was inferred from 1000 replicates by using the Maximum Likelihood method (Felsenstein, 1985).

RESULTS AND DISCUSSION

To study the bacteria inhabiting rice rhizosphere, strains isolated by culturing method were identified, based on biochemical and phenotypic characteristics followed by PCR amplification of 16S rRNA gene and sequencing of the amplicons extracted from the gel. 16S rRNA gene sequences were aligned using BLAST of GenBank and SeqMatch of RDP to identify the isolates. Sequence analysis of isolates B9, B12, B40 and B53 showed 99% sequence similarity to *Bacillus thuringiensis, Staphylococcus aureus, Bacillus anthracis* and *Bacillus endophyticus*. For specie delineation an extensive survey of literature reveals that there are no accepted guidelines in the scientific community regarding the threshold values for 16S rDNA based identification. There are several reasons for this fact. A commonly used cut-off value is 97% similarity. However, this recommendation has been questioned in several studies. Even a difference rate of >0.5% is also suggested to be considered as an indicative of a new species (Palys *et al.*, 1997). As the bacterial genera do not evolve at the same rate (Drancourt *et al.*, 2000), it may be necessary to use different cutoff values depending on the bacterial genus under investigation. For *Bacillus* species even a sequence pairwise similarity <98.5% are considered as different species. For example, the 16S rRNA gene sequences of the type strains of *Bacillus bataviensis, Bacillus soli, Bacillus dretensis, Bacillus novalis* and *Bacillus vireti* are distinct species, show 98.7–99.6% pairwise similarity (Ko *et al.*, 2006). This paradox has led to the adoption of a polyphasic approach pyramiding morphological, biochemical and molecular data for the characterization of bacteria.

Based on microscopic analysis of the stained bacterial cells, six strains were spore forming rod shaped; Bacilli, whereas one strain was non-spore forming, round shaped in a cluster type arrangement; *Staphylocoocus* (Table 1). The biochemical tests results of the isolates were compared to *Bergey's* Manual of systematic bacteriology (Bergey, 2001). Two isolates B9 and B40 presented similar biochemical profile (Table 1). However, subsequent sequence analysis of 16S rRNA gene resolved these isolates as closest match (99% similarity) to the members of *Bacillus cereus* group; *Bacillus thuringiensis* (B9) and *Bacillus anthracis* (B40). Despite of having considerably distinct virulence characteristics, *Bacillus anthracis* and *B. thuringiensis* are so closely related species (Daffonchio *et al.*, 2000; Helgason *et al.*, 2000; Vilas-Boas *et al.*, 2002) to each other that they are treated as one species by some authors (Helgason *et al.*, 2000; Bavykin *et al.*, 2004).

However, other studies have obtained sufficient genetic discrimination between two species (Chang *et al.*, 2003; Keim *et al.*, 1997; Radnedge *et al.*, 2003; Harrell *et al.*, 1995; Ash *et al.*, 1991). The biochemical reaction results of isolate B9 are in agreement with previously reported biochemical characteristic of *Bacillus thuringiensis* (Kati *et al.*, 2007; Chatterjee *et al.*, 2007). Also, the biochemical results of B40 (Table 1) were in accordance to the traditional identification results of *Bacillus anthracis* (Bergey, 2001), except for citrate utilization which was demonstrated as variable characteristic of the bacteria by Gordon *et al.*, (1973). Thus the two isolates B9 and B40 are identified as the closest match to *Bacillus thuringiensis* and *Bacillus anthracis*

The 16S rRNA gene sequence alignment of isolate B34 showed 95% similarity to *Bacillus flexus* and *Bacillus megaterium*. However, the alignment by SeqMatch tool of RDP (Cole *et al.*, 2009) returned *Bacillus megaterium* as closest match to the sequence. The dissimilarity of 5% in this case is greater than the commonly accepted threshold of 3% dissimilarity for species delineation (Stackebrandt and Goebel, 1994; Wayne *et al.*, 1987). Also results of biochemical test of the isolate B34 were similar to its close relatives *Bacillus flexus* and *Bacillus megaterium* (Bergey, 2001; Priest *et al.*, 1988). Further investigation including complete 16S rRNA gene sequencing of the isolate is required to come up to a final conclusion.

For two isolates B12 and B53 BLAST and SeqMatch arrived at similar results with a closest match (99% similarity) to *Staphylococcus aureus* and *Bacillus endophyticus.* Evidence exists for the association of *Staphylocoous aureus* with soil (Krishnaveni, 2013; Hamann, 1986). In the case of isolates B15 and B52, conflicting results for identification by sequence analysis were observed. The BLAST search returned *Bacillus infantis* and *Bacillus firmus* as closest match (99% similarity) to B15 and *Bacillus safensis* and *Bacillus pumilus* as closest match (99% similarity) to B52 whereas SeqMatch search resulted in *Bacillus firmus* and *Bacillus pumilus* for B15 and B52, respectively. All four isolates (B12, B53, B15 and B52) were misidentified on the specie level by biochemical

identification. This discrepancy in identification may be attributed to misinterpretation of the results of the biochemical tests performed. Further discriminatory tests may be helpful to give a reasonably clear picture for the identification of these isolates on the specie level.

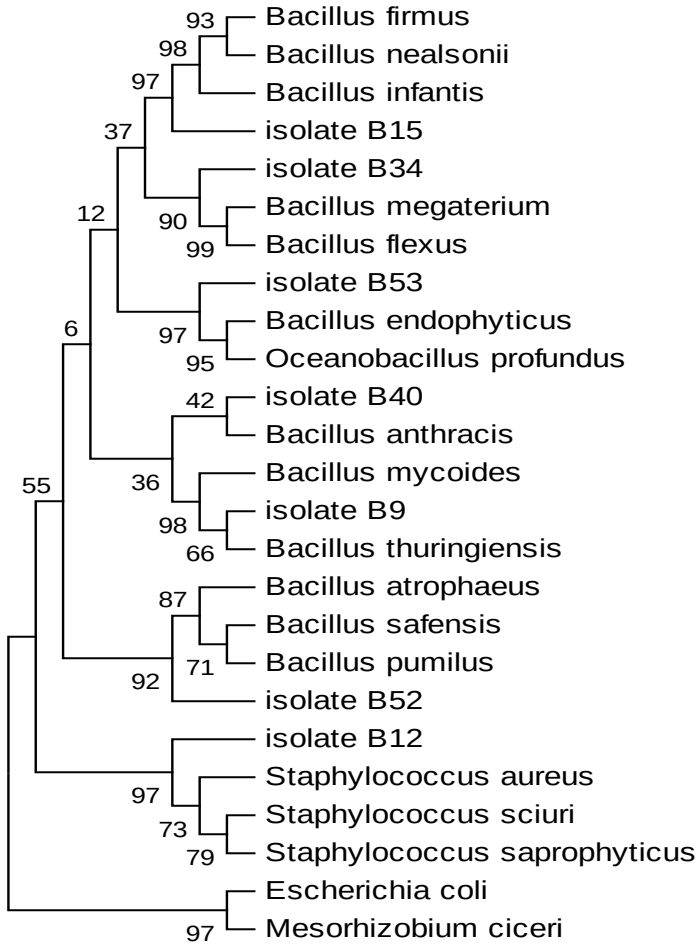

Fig. 1. Bacterial 16S rRNA sequence analyzed by Maximum Likelihood. Number above the nodes represent the boostrap replicate percentages using 1000 replicates by Maximum Likelihood.

In the phylogenetic tree (Figure 1) generated from the aligned sequence data, the isolate B9 and B40 clustered and grouped with the member of *Bacillus cereus,* positioned close to *Bacillus thuringiensis* and *Bacillus anthracis*, respectively. The position of the other isolates was found congruent to that of BLAST alignment results of these isolates. Poor bootstrap values are most likely due the short sequence of the 16S rRNA used to construct phylogenetic tree in this study since short sequence yield less accurate trees.

The majority of the isolates in this study belonged to the genus *Bacillus*, supported by the fact that genus *Bacillus* is the most frequent genus in soil and terrestrial environment. *Bacillus* is the genus of gram positive, aerobic, endospore forming bacteria. Soil is the main reservoir of genus *Bacillus* (Watanabe and Hayano, 1993). The predominance of the *Bacillus* in the soil is thought to be due to the endospore forming ability of the bacteria and production of substances that act as growth inhibitors for other microorganisms (Lunares *et al.*, 1993; Weller *et al.*, 2002). Therefore, this suggests that these species may be attractive biological control agents and good plant growth promoting bacteria (PGPB) (Landa *et al.*, 1997). The *Bacillus* species isolated in the present study have been reported extensively for their growth enhancement effect in rice as well as in other crops. Watanabe and Hayano (1993) identified *Bacillus thuringiensis*, *Bacillus megaterium*, *Bacillus firmus*, and *Bacillus pumilus* in rice paddy field and demonstrated the proteolytic activity of the isolates Xie *et al.*, (1998) reported the nitrogen fixing activity of *Bacillus firmus*, *Bacillus megaterium* and *Bacillus pumilus* isolated from the rice rhizosphere. *Bacillus thuringiensis* has been isolated and characterized from the rice ecologies in several studies (Das and Dangar, 2007;

2008; Das *et al.*, 2008; Schoenly *et al.*, 2010). *Bacillus megaterium* is a beneficial bacterium widely distributed and mainly being found in soil, rice paddy fields, embedded in plant ovule and seed tissue (Mundt and Hinkle, 1976; Sneath, 1986; Vary, 1994; Watanabe and Hayano, 1993). *Bacillus megaterium* isolated from rice roots in Taiwan, reduced attraction to and penetration of rice root and diminished the egg hatch rate (Padgham and Sikora, 2007).

Table 1. Biochemical and morphological characteristics of isolated strains.

Biochemical Tests	B9	B12	B15	B34	B40	B52	B53
Gram Staining	+	+	+	+	+	+	+
Cell Shape	Rod	Round	Rod	Rod	Rod	Rod	Rod
Spore Staining	+	-	+	+	+	+	+
Cytochrome Oxidase	-	-	-	+	-	-	+
Catalase Test	+	+	+	+	+	+	+
Methyl Red Test	+	+	-	-	+	-	-
Starch Hydrolysis Test	+	-	ND	+	+	-	+
Indole Test	-	-	+	-	-	-	-
Nitrate Reduction Test	+	+	+	-	+	-	+
Citrate Utilization Test	+	-	+	+	-	+	+
Lactose Fermentation	ND	+	+	+	ND	ND	+

+, tested positive/utilized as substrate; -, tested negative/not utilized as substrate; ND= Not Determined

Table 2. Closest match to the isolates inferred by SeqMatch and BLAST alignment.

Isolate	% identity	Closest phylogenetic neighbor
B9	99%	*Bacillus thuringiensis*
B12	99 %	*Staphylococcus aureus*
B15	99 %	*Bacillus firmus or Bacillus infantis*
B34	95 %	*Bacillus flexus or Bacillus megaterium*
B40	99 %	*Bacillus anthracis*
B52	99 %	*Bacillus pumilus* or *Bacillus safensis*
B53	99 %	*Bacillus endophyticus*

Bacillus thuringiensis is among the most widely studied PGPR *Bacillus* (Bai *et al.*, 2002a; Bai *et al.*, 2002b). *Bacillus thuringiensis* is one of the most potent and versatile pathogen capable of infecting protozoa, nematodes, flatworms, mites and insects that are either plant pests or human and animal health hazards (Joung and Horticultural, 2000; Feitelson, 1993). Theunis *et al.*, (1998) observed more than 50% insect infection in the rice environment are caused by *Bacillus thuringiensis*. *Bacillus megaterium* has been reported to improve the percentage of seed germination under saline condition (Kaymak and Yarali, 2009), reduce metal potentially increasing the bioavailability of iron (Chakraborty *et al.*, 2006), stimulation of growth due to cytokinin signaling (Ortíz-Castro *et al.*, 2008) and IAA production (Srinivasan *et al.*, 1996). Similarly, *Bacillus pumilus* strains are known to produce several biologically active compounds, antagonist to variety of plant pathogenic fungi (Yan *et al.*, 2002; Wei *et al.*,

1996; Raupach and Kloepper, 1998; Raj *et al.*, 2003). High amounts of gibberellins (GAs) have been detected in *Bacillus pumilus* by GC-MS analysis (Gutiérrez Mañero *et al.*, 2001). Thus these isolates have been proved to render significant contribution to enhancement of growth and biocontrol of pests and insect in rice rhizosphere as well as in other crops.

In conclusion, the results of the present study provide a basis for an interesting group of potent biocontrol and growth promoting Bacilli and suggest that this area is rich in important endospore forming Bacilli. Further investigation and isolation may reveal new species. Further investigation is needed for the characterization of the isolated bacteria with regard to their host.

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