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Research Paper

Identification and characterization of rhizospheric microbial diversity by 16S ribosomal RNA gene sequencing

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Abstract

In the present study, samples of rhizosphere and root nodules were collected from different areas of Pakistan to isolate plant growth promoting rhizobacteria. Identification of bacterial isolates was made by 16S rRNA gene sequence analysis and taxonomical confirmation on EzTaxon Server. The identified bacterial strains were belonged to 5 genera *i.e. Ensifer*, *Bacillus*, *Pseudomona*, *Leclercia* and *Rhizobium*. Phylogenetic analysis inferred from 16S rRNA gene sequences showed the evolutionary relationship of bacterial strains with the respective genera. Based on phylogenetic analysis, some candidate novel species were also identified. The bacterial strains were also characterized for morphological, physiological, biochemical tests and glucose dehydrogenase (*gdh*) gene that involved in the phosphate solublization using cofactor pyrroloquinolone quinone (PQQ). Seven rhizoshperic and 3 root nodulating stains are positive *for gdh* gene. Furthermore, this study confirms a novel association between microbes and their hosts like field grown crops, leguminous and non-leguminous plants. It was concluded that a diverse group of bacterial population exist in the rhizosphere and root nodules that might be useful in evaluating the mechanisms behind plant microbial interactions and strains QAU-63 and QAU-68 have sequence similarity of 97 and 95% which might be declared as novel after further taxonomic characterization.

Key words: rhizosphere, root nodules, 16S rRNA gene sequence, glucose dehydrogenase, pyrroloquinolone quinone.

Introduction

Microbial diversity plays a vital role for maintaining the ecosystem functions which support life on earth. There are over 1.7 million strains registered and stored in World Data Centre for Microorganisms. Microbial interactions with plants together with cell signaling are known as plant microbial interaction (Hooper and Gordon, 2001). This interaction results in revealing important information and application in the field of biofertilizer, biofilming, bioinoculant and bioprocessing. In recent period, their importance in different capacities has been highlighted such as phosphate solubilization, nitrogen fixation, induced systematic resistance and plant growth improvements (Hayat *et al.*, 2010; Berg, 2009; Choi *et al.*, 2008; Rodriguez *et al.*, 2004; Bloemberg and Lugtenberg, 2001). Still a lot more to be discovered which may be linked to unearthing novel discoveries, identification, studying their potential role in biodegradation, reclamation of polluted soils and industrial waste managements.

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In soil, the major microbial activity is restrained to organic matter decomposition in the rhizosphere (Lynch, 1990). Plant and soil type, both have influence on the microbial diversity and community structure in the rhizosphere (Liu and Sinclair, 1993). Rhizobacteria colonize and proliferate on all ecological niches of plant roots at all stages of plant growth, in the presence of a competing microflora (Antoun and Kloepper, 2001). Soil contains nitrogen-fixing bacteria to fix atmospheric nitrogen to supply the partial needs of growing plants. In the association of rhizobia and its host plant, the bacteria enter into the cortex and induced nodule formation, reproduce and eventually differentiate into bacteriods, which further produce nitrogenase enzyme complex and convert atmospheric nitrogen into ammonia in presence of low oxygen concentration created by nodule. Plants provide carbon source to bacteria in return of nitrogen fixation (Berg, 2009).

Limited studies are available on the identified bacterial natural resources of Pakistan. The present study was undertaken to explore the potential of rhizobacteria and nodulating bacteria collected and isolated from field grown crops, leguminous and non-leguminous plants. This study is also an attempt to identify and characterize the bacterial strains by morphological, biochemical, physiological and molecular methods that play an important role in plant growth promotion.

Materials and Methods

Soil samples collection

The rhizosphere and the root nodule samples of different field grown crops and wild leguminous and non-leguminous plants were collected from different regions of Pakistan (Figure 1). Washing-off soil particles adhering to roots is considered as the best method to separate rhizospheric bacteria. Firstly, the root system together with adhering soil is carefully removed from the soil by shaking

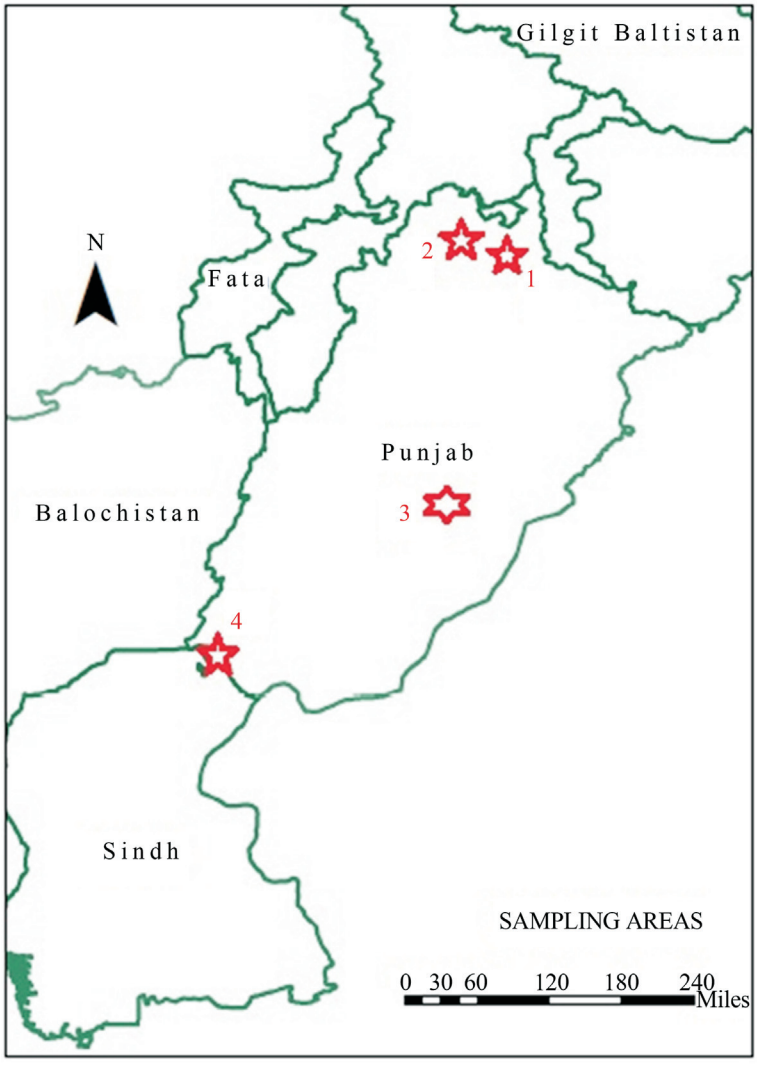

Figure 1 - Map of Pakistan, rectangular areas showing the areas where samples were collected: 1) Islamabad, 2) Rawalpindi, 3) Multan and 4) Jacobabad.

the root system for 5 min in Milli-Q water followed by isolation.

Bacterial isolation and phenotypic characterization

The soil samples were homogenized in Milli-Q water containing 0.89% NaCl (w/v) and serially diluted according to serial dilution method by using phosphate buffered saline (PBS, 1x) as the saline solution. Aliquots of each dilution were spread on Luria Bertani (LB) medium (pH-7.5) and incubated at 30 °C for 18-24 h. Selected colonies of bacteria were sub-cultured repeatedly on LB medium to obtain pure cultures. These cultures were preserved in 20% glycerol at -80 °C for further experimentation.

Leguminous plant root nodules were used to isolate nodulating strains by Vincent (1970) method. The nodules extract were streaked on yeast extract mannitol (YEM) agar which contained manitol 10 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ 0.2 g/L, NaCl 0.1 g/L, yeast extract 0.6 g/L, congored (0.25%) 10 mL/L, distilled water 1 L and 2% agar. The pH of the medium was maintained at 6.8-7.0 and allowed to grow at 36 ± 1 °C. Single rhizobial colonies that appeared on YEM agar plates within 48 to 72 h after incubation were picked and sub-cultured repeatedly on fresh YEM media to obtained purified cultures

Phenotypic characterization of rhizobacterial and nodulating isolates was carried out through morphological and microscopic observations.

Biochemical and physiological characterization

Biochemical tests includes Indole acetic acid (IAA) production (Joseph *et al.*, 2007), catalase production (MacFadden, 1976) and physiological tests includes N-acylhomoserine lectone (McClean *et al.*, 1997), nitrogenase activity (Dobereiner and Day, 1976). The ability of bacteria for phosphate solubilization was determined by Pikovskaya medium culture plates contained bromo phenol blue. The phosphate solubilization efficiency of these isolated strains was determined by ratio of colony diameter with the halo zone diameter (Edi-Premoto *et al.*, 1996). The change in pH by bacterial strains in Pikovskaya broth medium was determined as described previously by Islam *et al.* (2005) which reported that the growth of bacteria is inversely related to change in pH of the medium.

PCR amplification, phylogenetic analysis and sequencing of 16S rRNA gene

Genomic DNA of bacterial strains was extracted by the CTAB method. The most promising eight rhizobacteria and five nodulating strains were identified using 16S rRNA gene sequence. Universal primers P1 (5' - PAGAGTTTGATCCTGGTCAGAACGAACGCT - 3') and P6 (5' -TACGGCTACCTTGTTACGACTTCACCCC - 3') were used corresponding to *E.coli* positions 8-37 for forward primer and 1479-1506 for reverse primer, respectively, to amplify about 1500 bp fragment of 16S rRNA gene according to the procedure described previously (Ahmed *et al.*, 2007). Amplified PCR products of the selected strains were sequenced using commercial service of MACROGEN Seoul, Korea (http://macrogen.com/eng/). The gene sequences were assembled using BioEdit software ver 7.1 (Hall, 1999). The sequence of all the stains were submitted to National center for biotechnology information (NCBI) Data Bank under the accession numbers as mentioned in Table 1.

The strains were identified using nearly complete sequence of 16S rRNA gene on Ez-Taxon Server (http://eztaxon-e.ezbiocloud.net) and BLAST search on DDBJ / NCBI servers. Sequences of closely related validly published type strains used for constructing the phylogenetic tree of *Bacillus* strains were selected and retrieved from the EzTaxon Server (http://eztaxon-e.ezbiocloud.net) database. The phylogenetic and molecular analyses were performed with all the closely related taxa according to procedure as described previously (Roohi *et al.*, 2012) using MEGA version 5.1 (Tamura *et al.*, 2011). The stability of the relationship was assessed by bootstrap analysis by performing 1000 re-samplings for the tree topology of the neighbour-joining method.

Amplification of glucose dehydrogenase gene by PCR amplification

A primer set *gdh* Fp (5'-CCCGAATTCGGCGTGATCCGTGGTT - 3') and *gdh* Rp (5'- ATGCGTCGACTAGTCGCCCATCTT - 3') was used to amplify the region of 1.4 kb encoding membrane glucose dehydrogenase (*gdh*) gene. The product of *gdh* gene works with cofactor pyrroloquinolone quinone (PQQ). The 25 µL reaction mixture was prepared for *gdh* gene amplification. The amplification reaction was performed with initial temperature of 94 °C for 2 min followed by 35 cycles consisting of 94 °C for 1 min; primer annealing at 55 °C for 1 min and primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min in a thermal cycler.

Results

Isolation of bacteria

The rhizosphere and root nodules of different non-leguminous and leguminous plants were used for bacterial isolation. Roots of ten different plant species were collected for rhizospheric samples and nine plant species for nodule. A total of eighty one strains were obtained, out of which fifty eight isolates were rhizosphere and twenty three strain isolated from root nodules. In rhizosphere samples, 11 strains were obtained from *Oryza sativa*, 3 from *Zea mays*, 10 from *Lycopersicon esculentum*, 5 from *Gossypium hirsutum*, 2 from *Artemisia* sp., 4 from *Rhyncosia minima*, 6 from *Alysicarpus bupleurifolius*, 6 from *Cassia occidentalis*, 8 from *Vigna mungo* and 3 from *Pisum sativum*. Whereas in nodule of plants, 8 strains were ob-

Table 1 - The source and location of isolated strains, identification based on 16S rRNA gene sequence and their accession numbers.

Strain ID	Strain name / Genus	Source of isolation	Location of isolation	Number of nucleotides of 16S rRNA gene	Accession number of 16S rRNA gene	Closely related taxa identified by using the EzTaxaon Server Database[a]	Sequence similarity (%) of 16S rRNA gene with closely related taxa	Sequence query coverage (%)
QAU53	*Ensifer* sp.	Nodules of *Melilotus indicus*	Islamabad	1383	KC679988	*Ensifer arboris* LMG 14919^T (AM181744)	98.76	99.6
QAU54	*Bacillus* sp.	Nodules of *Indigofera linifolia*	Islamabad	1543	KC679987	*Bacillus drentensis* LMG 21831^T (AJ542506)	99.22	100
QAU56	*Ensifer* sp.	Nodules of *Crotalaria medicaginea* Islamabad	1398	KC679989	*Ensifer kostiensis* LMG 19227^T (AM181748)	99.64	100	
QAU62	*Bacillus* sp.	Rhizosphere of *Gossypium hirsutum*	Jacobabad	1077	KC679986	*Bacillus anthracis* ATCC 14578^T (AB190217)	99.71	73.5
QAU63	*Bacillus* sp.	Rhizosphere of *Lycopersicon esculentum*	Jacobabad	1483	KC679985	*Bacillus subtilis* subsp. spizizenii NRRL B-23049^T (CP002905)	97.01	100
QAU64	*Leclercia* sp.	Rhizosphere of *Vigna mungo*	Islamabad	1501	KC886280	*Leclercia adecarboxylata* GTC 1267 ^{T} (AB273740)	99.46	100
QAU65	*Pseudomonas* sp.	Rhizosphere of *Pisum sativum* Islamabad	1431	KC679990	*Pseudomonas moorei* RW10 ^{T} (AM293566)	99.79	98.7	
QAU66	*Leclercia* sp.	Rhizosphere of *Vigna mungo*	Islamabad	1500	KC679993	*Leclercia adecarboxylata* GTC 1267 ^{T} (AB273740)	99.39	100
QAU67	*Pseudomonas* sp.	Rhizosphere of *Gossypium hirsutum*	Multan	1431	KC679991	*Pseudomonas moorei* RW10 ^{T} (AM293566)	99.93	98.0
QAU68	*Bacillus* sp.	Rhizosphere of *Zea mays*	Multan	1470	KC679984	*Bacillus anthracis* ATCC 14578^T (AB190217)	95.75	100
QAU69	*Pseudomonas* sp.	Rhizosphere of *Zea mays*	Multan	1492	KC679992	*Pseudomonas vancouverensis* ATCC 700688^T (AJ011507)	99.52	100

[a]http://eztaxon-e.ezbiocloud.net.

tained from *Vigna mungo*, 1 from *Pisum sativum*, 5 from *Cassia occidentalis*, 1 from *Alysicarpus bupleurifolius*, 2 from *Crotolaria medicaginea*, 2 from *Indigofera linifolia*, 2 from *Melilotus indicus*, 1 from *Melilotus polymorpha* and 1 from *Medicago polymorpha.*

Phenotypic, biochemical and physiological characterization of bacteria

Eight rihzospheric strains showed good results for phosphate solubilization and positive for either *gdh* gene and nitrogenase activity or indole acetic acid (IAA) production. All these strains were found Gram negative else than *Bacillus* strains (QAU-62, QAU-63 and QAU-68) which were Gram positive. Among these strains, the dominant character was *coccus* (Diplo, strepto or in cluster) except for QAU-68 which was *bacillus* (Table 2). In these strains, QAU-67 was the only which showed positive results for *N*-acyl-homoserine lactone (AHL) production and all the strains collected from rhizospere were negative for nitrogenase activity. All rihzospheric strains were positive for catalase and IAA production except QAU-62 (Table 2). Out of thirty one, five nodulating strains were positive for phosphate activity.

In morphological characterization, all nodulating bacterial isolates were *streptococci* except QAU-54 which was strepto*bacilii*. In nodulating strains, QAU-60 was the only strain, which showed positive results for AHL production. QAU-53 and QAU-54 were positive for catalase test, whereas the remaining 3 showed negative results. For nitrogenase activity, all isolates were positive except QAU-54. No IAA production was seen in these isolates except QAU-56 (Table 2).

In Pikovskaya broth, all the strains drastically decreased the pH of medium after 4 days of incubation. The pH change was dropped from an initial value 7.0 to 4.0 pH units. The highest drop in pH was observed by QAU-69 (4.0) followed by QAU-65 (4.4) and QAU-64 (4.4). (Table 1).

16S rRNA identification and phylogenetic analysis

Eight rhizobacterial (QAU-62, QAU-63 QAU-64, QAU-65, QAU-66, QAU-67, QAU-68 and QAU-69) and five nodulating strains (QAU-51, QAU-53, QAU-54, QAU-54 and QAU-60) were identified by 16S rRNA gene sequence on Ez-Taxon Server. Based on the sequences of strains QAU-63 and QAU-68, BLAST search results showed that the both strains are more closely related to the species of genus *Bacillus* (Figure 2) with 95.75% and 97.01% sequence similarity, respectively. The 16S rRNA gene sequence similarity of the strains with other validly published species is presented in Table 1.

The sequence analysis showed that six strains were homologous with previously characterized bacterial species however two strains (QAU-63 and QAU-68) showed less similarity values (97.01% and 95.75%) with previously

Table 2 - Phenotypic biochemical and physiological characterization of isolated strains.

Strain ID	Colony morphology [a]	Gram's staining [b]	IAA production [b]	Catalase production [b]	N-acyl homoserine lectone [b]	Nitrogenase activity	Phosphate solubilization [c]	pH change [d]	GDH [e]
QAU51	T, R, S, C	+	–	–	–	+	2.6	6.7	+
QAU53	T, P, S, M, C	-	–	+	–	+	3.8	6.1	+
QAU54	T, P, S, C	-	–	+	–	–	3.8	6.2	–
QAU56	T, R, S, C, M	-	+	–	–	+	3.2	6.5	+
QAU60	T, R, S, C	-	–	–	+	+	2.6	4.0	–
QAU62	W, R, D, Cn	+	–	–	–	–	3.5	5.8	–
QAU63	W, R, D, C	-	+	+	–	–	2.7	5.0	+
QAU64	S, T, C, R	-	+	+	–	–	3.0	4.4	+
QAU65	S, T, C, R	-	+	+	–	–	3.3	4.4	+
QAU66	S, T, C, R	-	+	+	–	–	3.3	6.1	+
QAU67	M, S, O, C, R	-	+	+	+	–	2.9	4.6	+
QAU68	O, R, Cn, D	+	+	+	–	–	3.3	5.3	+
QAU69	M, S, O, C, R	-	+	+	–	–	3.3	4.0	+

[a] S (shiny), M (Mucoid), T/W/O (Transparent/ White/ Off-White), C/Cn (Convex/ Concave), R (Rounded).
[b] + Tested positive, – Tested negative.
[c] Tri calcium phosphate ($Ca_3(PO_4)_2$)solubilization efficiency calculated according to Edi-Premoto *et al.* (1996) method on Pikovskaya medium plat.
[d] Change in pH calculated by subtracting final value from initial value.
[e] GDH (Glucose dehydrogenase presence tested by PCR).

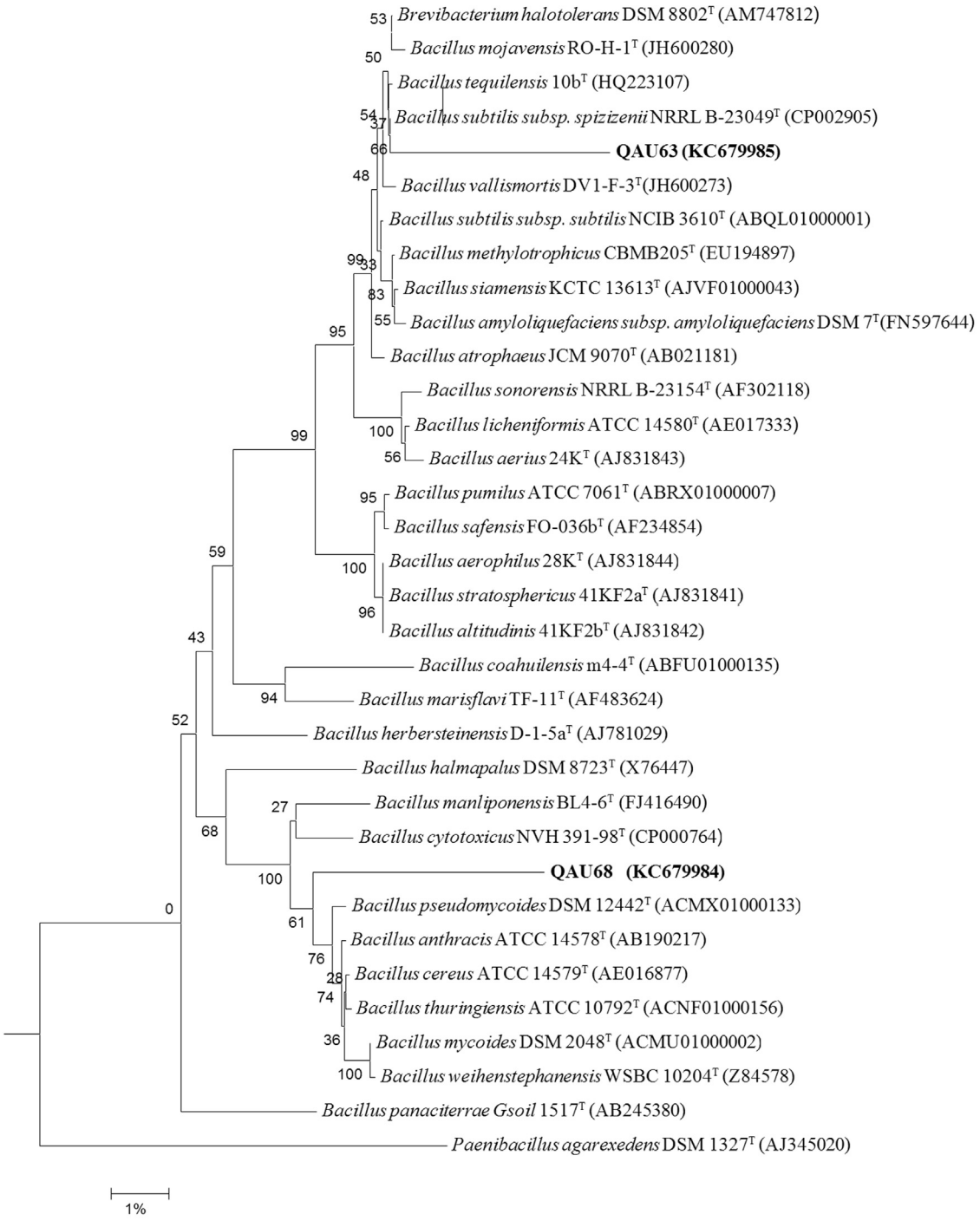

Figure 2 - Phylogenetic tree showing inter-relationship of Strain QAU63 and QAU68 with closely related species of the genus *Bacillus* inferred from aligned unambiguous sequences (1259 ntd) of 16S rRNA gene. Tree was generated using the neighbour-joining method and was rooted by *Paenibacillus agarexedens* (AJ345020) as an out group. Bootstrap values (more than 50%), expressed as percentage of 1000 replications, are indicated at the nodes. Accession number of each type strain is shown in parantheses.

characterized validly published species. QAU-62, QAU-63 and QAU-68 clustered together and belonged to the genus *Bacillus*, QAU-64 and QAU-66 were identified as *Leclercia* species and QAU-65, QAU-67, and QAU-69 found as Pseudomonas (Table 1). Among the nodulating strains, QAU-53 and QAU-56 clustered together and belonged to genus *Ensifer*, QAU-54 showed homology with Bacillus, whereas QAU-51 and QAU-60 strains in nodulating bacteria did not show enough level of homology due to insufficient sequence data on full length 16S rRNA gene (Table 1).

Identification of *gdh* gene

The PCR amplification with *gdh* primer of glucose dehydrogenase gave good amplification at annealing Temperature 54 °C. An amplicon of about 1400 bp was obtained in strains QAU-63, QAU-64, QAU-65, QAU-66, QAU-67, and QAU-69 whereas it could not amplify in other strains. The nodulating bacterial strains QAU-51, QAU-53 and QA56 also gave good amplification at 54 °C (Figure 3).

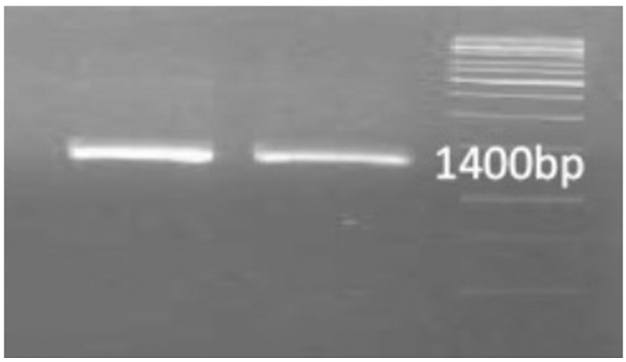

Figure 3 - PCR Amplification of glucose dehydrogenase (*gdh* gene).

Discussion

Bacteria perform different functions in many capacities and under different situations. Nature has placed them in the subsurface and is largely untapped in certain soils where specific conditions prevail. Considering this and many open ended questions, we collected bacteria present in several ecological niches (soil and nodules). Since Pakistani soil are either calcareous or sodic in nature, the pH found around 8.0 or 10 respectively. These conditions provide one of unique ecological conditions to study the bacterial communities, most of the bacterial strains were similar to the members of *Bacillaceae, Enterobacteriacae, Rhizobiaceae* and *Pseudomonadaceae* families. This was further tested through their phosphate solubility which is largely dependent on PQQ and *gdh* genes (Rodriguez *et al.*, 2004; Gyaneshwar *et al.*, 1998). Such a capability has been reported in *Pseudomonas aerginosa* (Midgley and Dawes, 1973) and *Enterobacter asturiae* (Tripura *et al.*, 2007). In our studies, ten strains out of 13 showed the presence of *gdh* gene thus indicate the potential to solubilize organic phosphate in soil. On the contrary, few strains did not show the presence of *gdh* gene, however these also demonstrated the capability to solubilize phosphate.

The absence of a PCR product, when trying to amplify *gdh* gene from phosphate solubilizing strains does not necessarily mean that it is absent from their genomes. Somewhat, this result may be endorsed to an inefficient amplification reaction or mismatched primer region. Nevertheless, *gdh* gene was not detected when testing the rest of the strains, even though these significantly acidify culture supernatants after four days of the growth. The absence of *gdh* gene might be due to production and excretion of other organic acids, which may also act as chemical agents for mobilizing insoluble phosphates. The reported *gdh* sequence of *Enterobacteriaceae* members such as *Escherichia coli*, *Serratia marcescens*, *Salmonella* sp. and *Shigella* sp. is highly conserved (Tripura and Podile, 2007).

It was previously reported that bacterial strains showing catalase activity must be highly resistant to environmental, mechanical, chemical stress, enhances the growth, seed emergence, crop yield, and contribute to the protection of plants against certain pathogens and pests (Dey *et al.*, 2004; Herman *et al.*, 2008; Kloepper *et al.*, 2004; Minorsky, 2008; Kokalis-Burelle *et al.*, 2006). We have tested all strains for catalase production in which 9 strains showed positive results both from rhizobacteria and root nodulating strains. Diggle *et al.* (2007) stated that sensing the "signal molecule" (homoserine lactone produced by bacteria) in tomato rhizosphere, the plant increases the salicylic acid production in leaves, which enhances the systemic resistance against fungal pathogen. Two of our strains QAU-60 and QAU-67 also produced AHL (signal molecule).

Molecular phylogeny extends our knowledge regarding organism relationships and provides the foundation for the conventional identification techniques (Singh *et al.*, 2007). Based upon 16S rRNA gene sequences analysis, strains QAU-65, QAU-67, QAU-69 were identified as *Pseudomonas*. Similarly, strains QAU-64 and QAU-66 appear in same cluster and revealed as close to the members of *Leclercia*. Comparative sequence analysis of 16S rRNA is currently the most widely used approach for the reconstruction of microbial phylogeny (Rasche *et al.*, 2006). In our study, we found that strains QAU-62, QAU-63 and QAU-68 belong to *Bacillus* whereas strains QAU-63 and QAU-68 showed sequence similarity of 97% or less (Table 2). This low sequence similarity of the strain QAU-63 and QAU-68 with the closely related members of *Bacillus* gives a further opportunity to investigate these strains taxonomically for delineation of possible novel species; however, the taxonomic studies are beyond the scope of this manuscript. 16S rRNA gene sequence of bacterial strains with similarity less than 97% can be declared as novel after complete taxonomic characterization as reported by Lim *et al.* (2006).

Our strains revealed diverse morphological, physiological and biochemical behavior. The idea here was not only to identify but also to find some promising strain with unique traits such as potential candidates to solubilize phosphate, induced systematic resistance, plant growth improvements and antioxidant activity. The novelty of *Ensifer* sp. and its symbiotic association with other plants were previously reported by Degefu *et al.* (2012). In this study, the symbiotic association of *Ensifer arboris* with legumes of *Melilotus indicus* and *Crotalaria medicaginea* and associa-

tion of *Leclercia* sp. with *Vigna mungo* has been reported for the first time in Islamabad region of Pakistan.

The significant positive association of *Bacillus* with *Gossypium hirsutum* was previously reported by Saharan and Nehra (2011). In our study, we reported the association of *Bacillus* sp. isolated from rhizosphere of *Gossypium hirsutum*, *Lycopersicon esculentum* and *Zea mays* of Jacobabad and Multan areas respectively. *Pseudomonas* sp. also showed good association with *Gossypium hirsutum, Zea mays* and *Pisum sativum* that were isolated from Multan and Islamabad areas respectively. The availability and association of these bacterial strains with plants is very useful for planning future studies by seeing the critical role of these rizhospheric and nodulating bacteria in crop improvement studies.

Conclusions

The present study deals with investigating the bacterial diversity in root nodules and rizosphere in highly diversified agricultural areas of Pakistan. We attempted to culture indigenous microbes collected from these areas. Their identification based on molecular analysis gives us an edge to have more cultured microorganisms with their taxonomy from indigenous environments. The microbial diversity can prove to be a valuable future resource in various industrial and biotechnological processes. Such microbes can also be used as a source of gene(s) that can increase phosphorus and nitrogen uptake in different crop species through genetic transformation.

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