*J. Microbiol. Biotechnol.* (2010), **20**(12), 1614–1623
doi: 10.4014/jmb.1005.05014
First published online 30 September 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# Genetic and Phenotypic Diversity of Plant Growth Promoting Rhizobacteria Isolated from Sugarcane Plants Growing in Pakistan

**Mehnaz, Samina[1,3]*, Deeba N. Baig[2], and George Lazarovits[3]**

[1]*Department of Microbiology and Molecular Genetics, Quaid-e-Azam Campus, Punjab University, Lahore 54590, Pakistan*
[2]*School of Biological Science, Quaid-e-Azam Campus, Punjab University, Lahore 54590, Pakistan*
[3]*Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario, N5V4T3, Canada*

Received: May 10, 2010 / Revised: September 9, 2010 / Accepted: September 13, 2010

**Bacteria were isolated from roots of sugarcane varieties grown in the fields of Punjab. They were identified by using API20E/NE bacterial identification kits and from sequences of 16S rRNA and amplicons of the *cpn60* gene. The majority of bacteria were found to belong to the genera of *Enterobacter*, *Pseudomonas*, and *Klebsiella*, but members of genera *Azospirillum*, *Rhizobium*, *Rahnella*, *Delftia*, *Caulobacter*, *Pannonibacter*, *Xanthomonas*, and *Stenotrophomonas* were also found. The community, however, was dominated by members of the Pseudomonadaceae and Enterobacteriaceae, as representatives of these genera were found in samples from every variety and location examined. All isolates were tested for the presence of five enzymes and seven factors known to be associated with plant growth promotion. Ten isolates showed lipase activity and eight were positive for protease activity. Cellulase, chitinase, and pectinase were not detected in any strain. Nine strains showed nitrogen fixing ability (acetylene reduction assay) and 26 were capable of solubilizing phosphate. In the presence of 100 mg/l tryptophan, all strains except one produced indole acetic acid in the growth medium. All isolates were positive for ACC deaminase activity. Six strains produced homoserine lactones and three produced HCN and hexamate type siderophores. One isolate was capable of inhibiting the growth of 24 pathogenic fungal strains of *Colletotrichum*, *Fusarium*, *Pythium*, and *Rhizoctonia* spp. In tests of their abilities to grow under a range of temperature, pH, and NaCl concentrations, all isolates grew well on plates with 3% NaCl and most of them grew well at 4 to 41°C and at pH 11.**

**Keywords:** *Klebsiella*, Pseudomonads, *Enterobacter*, *Rhizobium*, *Azospirillum*, sugarcane

*Corresponding author
Phone: +92-423-99238531; Fax: +92-423-99230481;
E-mail: saminamehnaz@yahoo.com

The potential use of biofertilizers is now being seriously considered as a means to reduce the quantity of fertilizers required for crop production. This would help to minimize pollution and soil infertility, and above all reduce grower's costs. Plant growth promoting rhizobacteria (PGPR) have been reported to be present in high populations, in the rhizosphere and as endophytes of many crops. They include species of *Enterobacter*, *Bacillus*, *Klebsiella*, *Herbaspirillum*, *Burkholderia*, *Azospirillum*, and *Gluconacetobacter* [12]. The most common bacteria isolated from sugarcane tissues have been *Gluconacetobacter diazotrophicus*, *Herbaspirillum rubrisubalbicans*, and *H. seropedicae* [1], whereas *Enterobacter cloacae*, *Erwinia herbicolla*, *K. pneumoniae*, *K. oxytoca*, *Azotobacter vinelandii*, *Paenibacillus polymyxa*, and *Azospirillum* were found less often [19, 22].

It has been a general practice to apply 250 kg of N $ha^{-1}$ $y^{-1}$ or more in most sugarcane cultivating countries. Applying such high rates of fertilizer, however, could inhibit the interactions and associations between microorganisms and their host plant [21]. In Brazil, farmers use 50 kg N $ha^{-1}$ $y^{-1}$ and work is under way to reduce even this amount by one half through improvements in biological nitrogen fixation (BNF). In Pakistan, 150–240 kg N $ha^{-1}$ is recommended for sugarcane, grown in an average fertile soil. Currently, this crop is grown on an area of about one million hectares of which 62% is in the Punjab. There is little information as to what microorganisms are associated with sugarcane growing in the fields of Punjab, and therefore in this study, we undertook to carry out isolations and characterizations of potential beneficial bacteria that may be associated with various sugarcane varieties. Bacteria were isolated from the rhizosphere, the root, and the stems of sugarcane plants. The identity of isolates of interest was determined by 16S rRNA and *cpn60* genes sequence analyses and biochemical tests. The growth promoting potential, antifungal activity, detection of different enzymes, and production of metabolites were determined by the various bioassays described herein.

## Materials and Methods

### Isolation

Sugarcane plants of different varieties (*i.e*., LHF240, SHF240, CPF240, and CP77400) were collected from a selection of fields in Punjab. Bacteria were isolated from washed and surface-sterilized roots, shoots, and the rhizosphere soil using three culture media; namely, combined carbon medium (CCM) [29], M medium [40], and LGI medium [2]. For each sugarcane sample, 1 g of rhizosphere soil (brushed off of roots) was suspended in 9 ml of sterilized saline solution, and 100 µl of the suspension was then inoculated to CCM, LGI, and M media. Root and shoot pieces were thoroughly washed with sterilized water. One gram of each sample was separately macerated in 9 ml of saline solution, and 100 µl of this suspension was inoculated in the above-mentioned media. Root and shoot pieces were surface sterilized by using bleach and ethanol, as described by Coombs and Franco [3]. The tissues were macerated and suspensions inoculated into media as described earlier. Isolation was done in semisolid media and bacterial cultures were streaked on CCM, LGI, and M plates. Purification of isolated colonies was done on Luria–Bertani (LB) plates.

### Characterization of Isolated Bacteria

To identify the isolates to the species level, we used characteristics related to colony morphology, results from biochemical tests, and results of analyses of the gene sequences encoding 16S rRNA and *cpn60*.

**Colony morphology and biochemical tests.** Colony morphology was observed on LB medium. Bacterial growth was measured at different temperatures (4–41°C), pH ranges of 5–12, and various NaCl concentrations (0.5–5%) using LB medium as substrate. Biochemical tests were performed by using API20E and API20NE kits (bioMeriux, France). Catalase was identified by the method of MacFadden [14], using $H_2O_2$ and pure culture colonies from agar plates. Cellulase and chitinase activities were tested on LB plates, with the respective substrate added (carboxymethyl cellulose 1%, chitin 0.5%). Pectinase and protease activities were tested on the media described by Kumar *et al*. [12]. Lipase production was identified on the LB medium containing 1% butyrin [28] and by the Tween 80 hydrolysis assay, described by Sierra [36]. The presence of clear zones around the colonies after 2–8 days of incubation at 28°C were considered as a positive result for cellulase, chitinase, lipase, pectinase, and protease activities.

**16S rRNA and *cpn60* gene sequence analyses.** Pure colonies of bacterial isolates were inoculated in LB broth. After overnight growth at 30°C, DNA was isolated from the cultures using the QIAGEN blood and cell culture DNA Midi kit. The DNA was dissolved in 100 µl of TE buffer and used as a template for PCR amplification of 16S rDNA. The amplification primers and PCR conditions were the same as previously described by Mehnaz *et al*. [18]. Each reaction mixture (50 µl) contained 0.5 µl *Taq* polymerase (5 U/µl), 5 µl PCR buffer, 2.5 µl $MgCl_2$ (50 mM), 1 µl dNTPs (10 mM), 1 µl (10 µM) of each primer (FGPS4-281 and FGPS1509-153), 37 µl filter-sterilized milli Q water, and 2 µl of template DNA. The PCR products were purified by using a QIAquick PCR purification kit (QIAGEN), cloned in Promega PGEM T-easy vector, and sequenced on the Applied Biosystems 3730 Analyzer. The sequences were deposited in the GenBank database (Accession Nos. GU459201 to GU459220; EU439419 to EU439425; EU761590, GU737688, GU737689, GU938486, GU938487).

The chaperonin gene (*cpn60*) was amplified from bacterial genomic DNA, by using universal *cpn60* degenerate primers, H729 and H730 [9]. The PCR conditions were 5 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 50°C, 45 s at 72°C, and 10 min at 72°C. The sequences were deposited in the GenBank database (Accession Nos. GU459180 to GU459200, GU737690, GU737691).

An alignment of nucleotide sequences of the 16S rDNA and *cpn60* genes of these isolates was carried out by using ClustalW, version 1.83. The evolutionary history was inferred using the neighbor-joining method [31]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches [7]. The evolutionary distances were computed using the Maximum Composite Likelihood method [37] and are in the units of number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 502 and 640 positions in the final dataset of *cpn60* and 16SrRNA, respectively. Phylogenetic analyses were conducted in MEGA4 [38]. For the 16S rRNA phylogenetic tree, only those strains for which more than 1 kb sequence was available were included.

### Assays for Growth Promoting Abilities of Isolates

All bacterial isolates were screened for nitrogen fixation, indole acetic acid production, phosphate solubilization, antifungal activity, siderophore production, HCN production, ACC deaminase activity, and quorum sensing by using following assays.

**Acetylene reduction assay.** Nitrogenase activity was detected by an acetylene reduction/ethylene production assay. Single bacterial colonies of each isolate were inoculated in vials containing CCM, LGI, or M semisolid media (5 ml/vial). After 24 h growth at 30°C, acetylene [10% (v/v)] was injected into all vials, which were then re-incubated at 30°C for 20 h. Ethylene production was measured as described by Mehnaz and Lazarovits [15].

**Indole acetic acid (IAA) production.** Bacterial cultures were grown for 7 days in 50 ml of CCM (ammonium chloride was added at 1.0 g/l) or King's B medium [10] (for fluorescent pseudomonads). L-Tryptophan (100 mg/l) was added to both media as a precursor of IAA biosynthesis. Cells were harvested at 10,000 rpm for 15 min. The pH of the supernatant was adjusted to 2.8 with hydrochloric acid and then extracted three times with equal volumes of ethyl acetate [39]. The extract was evaporated to dryness and resuspended in 1 ml of ethanol. The samples were analyzed by high-performance liquid chromatography as described by Mehnaz and Lazarovits [15].

**ACC deaminase activity.** The ACC deaminase activity was determined on solid DF salts minimal medium [6]. Filter-sterilized ACC solution (3 mM) was spread over the agar plates, allowed to dry for 10 min, and inoculated with bacterial strains. These plates were incubated for three days at 28°C, and growth on the plates was checked daily. For the colorimetric assay, bacterial cells were grown to the mid up to the late-log phase in 15 ml of tryptic soy broth. Bacterial cell pellets were processed for ACC deaminase activity as described by Penrose and Glick [25].

**Solubilization of organic phosphate.** Aliquots (10 µl) of bacterial cultures, after overnight growth in LB medium, were spot inoculated onto NBRIP [21] and calcium phytate agar medium [30]. Plates were incubated for 14 days at 28°C. Formation of a clear zone

around the bacterial growth spot was considered as positive for phosphatase and phytase activities.

**Antifungal activity.** The antifungal activity of all bacterial isolates was checked against four strains of a fungal pathogen *Colletotrichum falcatum*, isolated from sugarcane varieties (*i.e*., BF166, C01148, CP77400, and SPF234). These strains were kindly provided by Shukur Gunj Research Institute. Potato dextrose agar (PDA; Difco, MI, USA) was used for this assay. A small plug cut from a fresh agar culture of the target fungus was seeded at the center of the medium in a Petri plate. Aliquots (10 µl) of bacterial cultures grown in LB medium were spotted 2 cm away from the center (one isolate per plate) and air dried in a laminar flow cabinet prior to incubation. Plates were incubated for 10–15 days at 24°C. Antifungal activity was observed by the formation of an inhibition zone of mycelial growth, based on agar diffusion of extracellular bacterial metabolites. Isolate PB-St2 was screened against 24 fungal pathogens in addition to the above-mentioned fungi. These fungal isolates were provided by George Lazarovits, Agriculture and Agri-Food Canada.

**Hydrocyanic acid production.** To qualitatively determine the hydrocyanic acid (HCN) production, bacterial strains were streaked on LB medium. Filter paper saturated with an alkaline picrate solution (picric acid, 2.5 (g/l); $Na_2CO_3$, 12.5 (g/l); pH 13) was placed in the lid of a Petri plate containing bacterial culture [19]. The Petri plate was sealed with parafilm and incubated for four days. HCN production was assessed by the color change of yellow filter paper to brown/reddish brown.

**Siderophore production.** Siderophore production was detected by using the O-CAS method [27]. The pellets of overnight-grown bacterial cultures were suspended in sterilized water, and 10 µl of each culture was spotted on LB plates. The plates were incubated at 30°C for 48 h. CAS medium was prepared according to Schwyn and Neilands [35]. Ten ml of this medium was used as overlay and applied on LB agar plate containing 48-h grown bacterial culture. A change in color from blue to orange (hydroxamate-type siderophore) or purple (catechol-type siderophore) was considered as a positive reaction. Sterilized LB and water were used as the negative control.

**Table 1.** Identification of sugarcane isolates on the basis of API20E/NE bacterial identification kits, and *cpn60* and 16SrRNA gene sequence analyses.

| Isolates | Identification methods | | | | |
|---|---|---|---|---|---|
| | API20E/NE | *cpn60* | (% identity; Accession No.) | 16S rRNA | (sequence length; % identity; Accession No.) |
| BN-St1 | *Pseudomonas putida* | - | | *P. putida** | (1,553 bp; 99.0; CP000712) |
| BN-St2 | *Enterobacter cloacae* | *E. cloacae* | (99.8; EU790580) | *E. cloacae* | (1,119 bp; 99.7; AY787819) |
| BN-St3 | *E. cloacae* | *E. cloacae* | (99.6; EU 790580) | *E. cloacae* | (1,118 bp; 99.7; AY787819) |
| BN-St4 | *E. cloacae* | *C. freundii* | (93.6; AY301234) | *E. oryzae/ K. oxytoca* | (1,467 bp; 99.7/99.6; EF488857/EU931550) |
| LH-S1 | *P. putida* | - | | *P. putida** | (1,530 bp; 99.5; AE015451) |
| LH-S2 | *Citrobacter freundii* | *C. freundii* | (93.4; AY301234) | *K. oxytoca* | (1,431bp; 99.1; EU931550) |
| LH-S3 | *S. maltophilia* | *E. cloacae* | (97.2; AB375470) | *S. maltophilia* | (1,416 bp; 99.7; AM743169) |
| LH-S4 | Unknown | *R. aquatilis* | (92.6; AY922367) | *Azospirillum brasilense* | (1,396 bp; 99.5; DQ288687) |
| LH-R1 | *P. putida* | - | | *P. putida** | (1,529 bp; 99.5; AE015451) |
| LH-R2 | *E. cloacae* | *E. cloacae* | (100; EU790580) | *E. cloacae* | (290 bp; 99.3; GQ995670) |
| LH-R3 | Unknown | *R. aquatilis* | (93.0; AY922367) | *Rhizobium* sp. | (1,375 bp; 99.6; GQ355317) |
| LH-R4 | *C. freundii* | *C. freundii* | (93.8; AY301234) | *K. oxytoca* | (950 bp; 99.5; EU931550) |
| LH-St1 | *E. cloacae* | *E. cloacae* | (100; EU790580) | *E. cloacae* | (1,120 bp; 99.7; AY787819) |
| PB-S1 | *E. cloacae* | *E. cloacae* | (97.7; AB375470) | *E. cloacae* | (1,439 bp; 98.8; AM778415) |
| PB-S2 | *E. cloacae* | *E. cloacae* | (97.4; AB375470) | *E. cloacae* | (1,437 bp; 98.5; AM778415) |
| PB-Rt1 | Unknown | *E. cloacae* | (97.7; AB375470) | *Pannonibacter phragmitetus* | (1,361 bp; 99.8; AJ314748) |
| PB-Rt2 | *Rahnella aquatilis* | *R. aquatilis* | (93.0; AY922367) | *R. aquatilis* | (744 bp; 98.9; AU90757) |
| PB-SRSt | *C. freundii* | *C. freundii* | (93.8; AY301234) | *K. oxytoca* | (1,435 bp; 99.7; EU931550) |
| PB-St1 | *P. fluorescens* | - | | *P. fluorescens** | (1,530 bp; 99.5; CP000094) |
| PB-St2 | *P. fluorescens* | *P. fluorescens* | (97.0; CP000076) | *P. aurantiaca*** | (1,529 bp; 99.8; DQ682655) |
| PB-St3 | *P. fluorescens* | *P. fluorescens* | (94.1; CP000072) | *P. reactans* | (1,423 bp; 99.9; DQ257418) |
| PB-St4 | *E. cloacae* | *E. cloacae* | (97.9; AB375470) | *E. aerogenes* | (1,119 bp; 99.3; EU855221) |
| PB-St5 | *C. freundii* | *C. freundii* | (93.7; AY301234) | *K. oxytoca* | (283 bp; 98.2; EU931550) |
| QS1 | Unknown | - | | *Delftia acidovorans* | (1,526 bp; 99.5; CP000884) |
| QS2 | Un-known | - | | *Caulobacter crescentus* | (1,475 bp; 98.4; CP001340) |
| QS3 | *S. maltophilia* | - | | *S. maltophilia* | (1,482 bp; 98.1; AB294556) |
| QR1 | Unknown | *Rhizobium etli* | (87.6; CP001074) | *Rhizobium* sp. | (1,378 bp; 99.5; EU841541) |
| QR2 | *P. putida* | *P. putida* | (91.3; CP000712) | *P. putida** | (1,530 bp; 99.9; AM411059) |
| QSt1 | *E. cloacae* | *E. cloacae* | (97.8; AB375470) | *E. cloacae* | (1,103 bp; 98.6; FJ796202) |
| QSt2 | *S. maltophilia* | *E. cloacae* | (97.6; AB375470) | *Xanthomonas* sp. | (1,442 bp; 99.6; EF522125) |
| QSt3 | *P. putida* | - | | *P. putida** | (1,529 bp; 99.9; AE015451) |
| OK-St | *P. putida* | - | | *P. putida** | (1,553 bp; 98.8; CP000712) |

*, **; 16S rRNA sequences of these strains are taken from Mehnaz *et al*. [16] and [17] respectively.

**Quorum sensing assay.** This assay was carried out as described by Mehnaz *et al*. [16]. A single colony of each culture was inoculated in 5 ml of LB medium and grown for 24 h. Aliquots (10 µl) of each bacterial culture and *R. leguminosarum* pRL1j1 were spotted on agar plates containing *C. violaceum* CV026 and air dried. Plates were incubated for 2–3 days at 28°C. Quorum sensing was detected by the appearance of a violet halo around the spot caused by violacein production resulting from activation of the reporter gene in *C. violaceum* CV026. *R. leguminosarum* pRL1j1 was used as the positive control. TY and LB media were used as negative controls. *C. violaceum* CV026 and *R. leguminosarum* pRL1j1 were kindly provided by Allen Downie, John Innes Centre, Norich, U.K.

## RESULTS

### Characterization of Bacterial Isolates

**Physiological and biochemical tests.** On the basis of API 20E and 20NE bacterial identification kits, bacteria were identified as members of the Enterobacteriaceae and Pseudomonadaceae (Table 1). Nine strains were identified as *Enterobacter cloacae*, six as *Pseudomonas putida*, three as *Pseudomonas fluorescens*, four as *Citrobacter freundii*, three as *Stenotrophomonas maltophilia*, and one as *Rahnella aquatilis.* Six isolates were not identified as their data profile did not fit the description of any bacterial species of the databank of the API20E or NE kits. A complete list of bacterial strains and results concluded from the bacterial identification kits is provided in Table 1.

Detailed data of the phenotypic characterization of these isolates are shown in Table 2. All strains grew well in the presence of 3% NaCl, and 16 isolates tolerated up to 5% NaCl. Twenty-six strains showed good growth at 4°C, four strains showed weak growth, and two strains did not grow at this temperature. Twenty-five strains grew well at 41°C. Twenty-six strains grew well at pH 11, and 17 strains showed good growth at pH 12. Eight isolates showed protease activity and 10 were positive for lipase activity. None of the isolates showed pectinase, cellulase, or chitinase activities.

**16S rRNA and *cpn60* gene sequence analyses.** The results of 16S rRNA and *cpn60* analyses partially

**Table 2.** Phenotypic characterization of sugarcane isolates.

| Strains | Protease | Lipase | Growth at | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 4°C | 41°C | 3% NaCl | 5% NaCl | pH 11 | pH 12 |
| BN-St1* | - | - | + | - | + | + | - | - |
| BN-St2 | - | - | + | + | + | + | + | + |
| BN-St3 | + | + | + | + | + | - | + | + |
| BN-St4 | - | - | w | + | + | + | + | + |
| LH-S1* | - | - | + | + | + | + | + | + |
| LH-S2 | - | - | + | + | + | + | + | + |
| LH-S3 | + | + | + | + | + | - | + | + |
| LH-S4 | - | + | - | + | + | - | + | - |
| LH-R1* | - | - | + | + | + | + | + | + |
| LH-R2 | - | - | w | + | + | - | + | - |
| LH-R3 | - | - | w | + | + | - | + | - |
| LH-R4 | - | - | + | + | + | + | + | + |
| LH-St1 | - | - | + | + | + | - | + | - |
| PB-S1 | - | - | + | + | + | + | + | + |
| PB-S2 | - | - | + | + | + | + | + | + |
| PB-SRST | - | - | + | + | + | + | + | - |
| PB-RT1 | - | + | + | + | + | - | + | + |
| PB-RT2 | + | + | + | + | + | - | - | - |
| PB-St1* | + | + | + | - | + | - | + | - |
| PB-St2** | + | + | + | - | + | - | - | - |
| PB-St3 | + | + | + | - | + | - | + | - |
| PB-St4 | - | - | + | + | + | + | + | + |
| PB-St5 | - | - | + | - | + | + | + | - |
| Q-S1 | - | - | + | + | + | - | + | + |
| Q-S2 | - | - | + | + | + | + | + | + |
| Q-S3 | + | + | w | + | + | - | + | + |
| Q-R1 | - | - | + | + | + | - | + | - |
| Q-R2* | - | - | + | - | + | + | - | - |
| Q-St1 | - | - | + | + | + | + | + | + |
| Q-St2 | + | + | - | + | + | - | - | - |
| Q-St3* | - | - | + | + | + | + | + | + |
| OK-St* | - | - | + | - | + | - | - | - |

w=weak growth

*, **=data is taken from Mehnaz *et al* [16] and [17] respectively.

supported the results of the API20E and NE identification kits, as most of the strains were identified as members of Pseudomonadaceae and Enterobacteriacae, but owing to the small database of identification kits, variances were encountered at the genus and species levels (Table 1). On the basis of the 16S rRNA gene sequence analysis, seven isolates were identified as *Enterobacter cloacae*, one as *Enterobacter aerogenes*, one as *Enterobacter oryzae*, four as *Klebsiella oxytoca*, six as *Pseudomonas putida*, two as *Stenotrophomonas maltophilia*, and two as *Rhizobium* sp. The remainder of the isolates were identified as *Pseudomonas fluorescens*, *P. aurantiaca*, *P. reactans*, *Xanthomonas* sp., *Rahnella aquatilis*, *Delftia acidovorans*, *Caulobacter crescentus*, *Pannonibacter phragmitetus*, and *Azospirillum brasilense*. In excess of 1 kb fragments were sequenced for most of the isolates, and between 98–99% similarity with identified bacterial genera/species was observed.

For the *cpn60*, at least 550 bp sequences were characterized for each isolate. On the basis of *cpn60*, 11 isolates were identified as *E. cloacae*, five as *Citrobacter freundii*, three as *Rahnella aquatilis*, six as *Pseudomonas putida*, three as *Pseudomonas fluorescens*, and one as *Rhizobium etli*. The detailed list is provided in Table 1. As identification of *P. putida* strains, on the basis of 16S rRNA, has already been published [17], only one *P. putida* strain (QR2) was amplified and sequenced for the *cpn60* gene. Unfortunately, we were unable to sequence the *cpn60* genes of QS1, QS2, and QS3.

Phylogenetic trees for *cpn60* and 16S rRNA genes, showing homology of these strains with one another, are shown in Fig. 1 and 2, respectively. Each tree is divided in two groups, one big and one small. The upper part of the *cpn60* tree included 13 members of Enterobacteriaceae, one member of Pseudomonadaceae, and one member of Rhizobiacaea, keeping the last two separate from the rest of the members and from each other as well. The lower part included six members of Enterobacteriaceae and two of Pseudomonadaceae. Five members of Enterobacteriacea grouped together at the extreme end of the tree, indicating that although they are members of Enterobacteriacea, they are very different from the group positioned at the top of the branch. A sixth member (LH-S4) identified as *R. aquatilis* is separated out from both groups of Enterobacteriacea.

The lower branch of the 16S rRNA phylogenetic tree was dominated by Enterobacteriacaea and Pseudomonadacea,

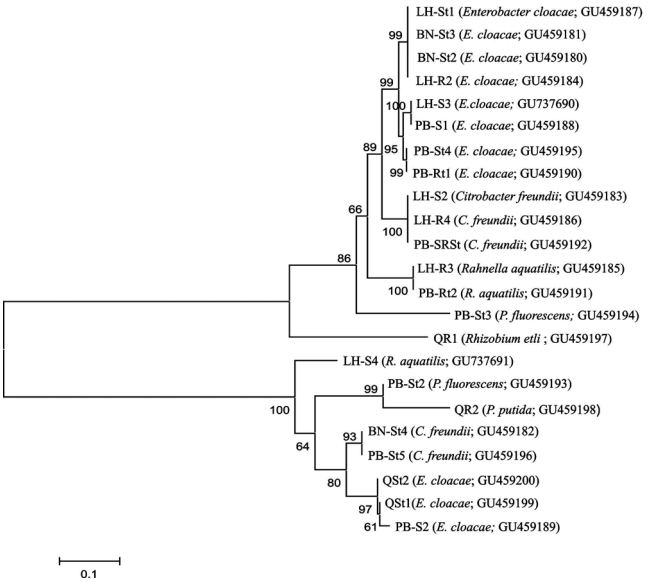


**Fig. 1.** Neighbor-joining tree of *cpn60* gene sequences of bacterial isolates from sugarcane.
Identification on the basis of the *cpn60* database and accession numbers are in parentheses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of number of base substitutions per site.

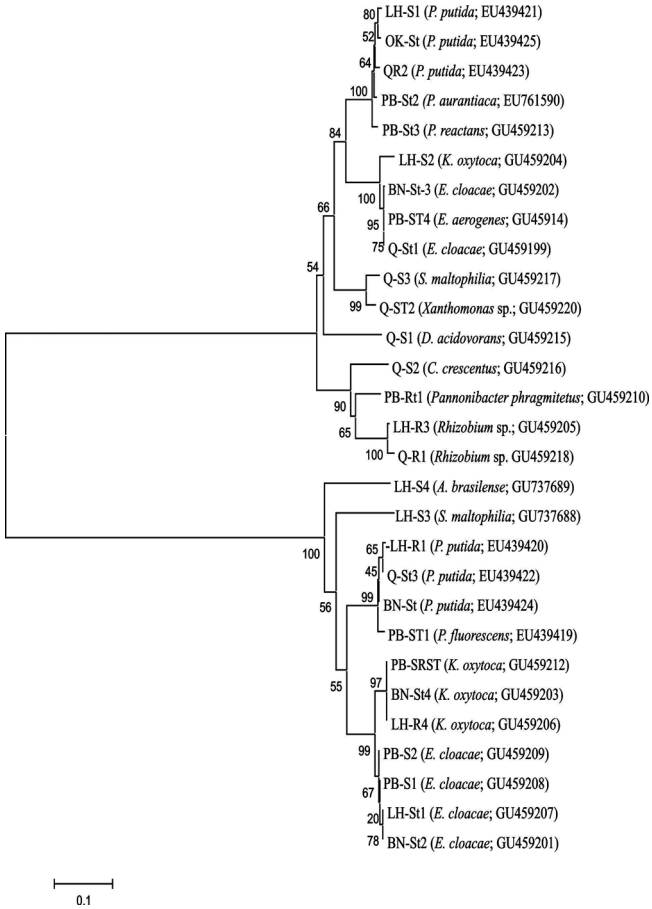


**Fig. 2.** Neighbor-joining tree of 16S rRNA gene sequences of bacterial isolates from sugarcane.
Identification on the basis of the 16S rRNA database and accession numbers are in parentheses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of number of base substitutions per site.

as it included four members of Pseudomonadaceae and seven members of Enterobacteriacea. Two members, one of Rhodospirillaceae and one of Xanthomonadaceae, are also included. The upper branch showed more diversity and included members of Pseudomonadaceae, Enterobacteriaceae, Rhizobiaceae, Rhodobacteriaceae, Xanthomonadacea, Caulobacteriaceae, and Comamonadaceae.

**Assays for Characteristics Associated with Plant Growth Promotion Potentials**

**Acetylene reduction assay.** Nine out of 32 isolates were positive for acetylene reduction and thus are considered as having nitrogen fixation activity (Table 3). *K. oxytoca* isolates LH-S2, LH-R4, and PBSRSt, *E. oryzae* BN-St4, and *S. maltophilia* isolate LH-S3 showed activity in NFM, LGI, and CCM media. *E. cloacae* LH-St1 showed activity in NFM and CCM, and *Klebsiella oxytoca* sp. PB-St5 was positive in LGI and CCM. *Azospirillum brasilense* LH-S4 showed activity in NFM, and *Rhizobium* sp. QR1 was positive in CCM. The isolates that were positive for acetylene reduction assay in more than one medium showed higher activity in CCM medium as compared with LGI and NFM. The highest activity was shown by *Rhizobium* sp. QR1 (468–640 μmol/h/vial) in CCM, and the lowest activity was shown by *K. oxytoca* LH-S2 (27–33 μmol/h/vial) in CCM.

**Indole acetic acid production.** In the presence of tryptophan, all isolates except *Pannonibacter phragmitetus* PB-Rt1 produced IAA (Table 3). *E. cloacae* BN-St2 was the highest and *P. aurantiaca* PB-St2 was the lowest IAA producer, at 174 μg/ml and 0.15 μg/ml, respectively. Eleven isolates, including six strains of *E. cloacae* (BN-St2, BN-St3, LH-R2, LH-St1, PB-S1, PB-S2) *E. aerogenes* PB-St4, *E. cloacae* Q-St1, *A. brasilense* LH-S4, *K. oxytoca* PB-SRSt, and *Rhizobium* sp. Q-R1 showed higher IAA production as the concentration for these strains ranged from 121–174 μg/ml. Three *Pseudomonas* strains (PB-St2, QSt3, LH-S1) and *S. maltophilia* QS3 showed low

**Table 3.** Determination of characteristics associated with plant growth promotion of bacterial strains isolated from sugarcane.

| Strains | ARA[a] (μmol/h/vial) | Phosphate solubilization | IAA production (μg/ml medium) | ACC deaminase (nmol/h/mg protein) | Quorum sensing | Siderophore production | HCN production |
|---|---|---|---|---|---|---|---|
| BN-St1 | 0 | + | 51.38±4.35 | 147±13 | - | - | - |
| BN-St2 | 0 | + | 174.04±11.26 | 138±27 | - | - | - |
| BN-St3 | 0 | + | 152.02±16.55 | 130±14 | - | - | - |
| BN-St4 | 233–302 | + | 14.14±1.39 | 251±20 | - | - | - |
| LH-S1 | 0 | + | 0.90±0.08 | 88±11 | - | - | - |
| LH-S2 | 27–33 | + | 15.64±1.45 | 296±42 | - | - | - |
| LH-S3 | 281–372 | - | 5.71±0.16 | 75±17 | - | - | - |
| LH-S4 | 30–35 | - | 151.56±14.40 | 109±12 | - | - | - |
| LH-R1 | 0 | + | 3.31±0.05 | 87±6 | - | - | - |
| LH-R2 | 0 | + | 169.84±27.41 | 842±61 | - | - | - |
| LH-R3 | 0 | + | 5.36±1.25 | 140±29 | - | - | - |
| LH-R4 | 62–79 | + | 12.37±2.72 | 261±58 | - | - | - |
| LH-St1 | 216–219 | + | 155.70±7.18 | 75±19 | - | - | - |
| PB-S1 | 0 | + | 172.00±37.13 | 65±8 | + | - | - |
| PB-S2 | 0 | + | 132.50±13.55 | 65±12 | + | - | - |
| PB-SRST | 262–347 | + | 121.80±15.64 | 146±34 | - | - | - |
| PB-RT1 | 0 | + | 0.0 | 1,012±109 | - | - | - |
| PB-RT2 | 0 | + | 13.22±1.93 | 108±27 | - | - | - |
| PB-St1 | 0 | + | 12.48±1.57 | 88±16 | - | + | + |
| PB-St2 | 0 | - | 0.15±0.02 | 232±58 | + | + | + |
| PB-St3 | 0 | + | 1.45±0.03 | 77±10 | - | - | - |
| PB-St4 | 0 | + | 145.08±22.70 | 110±34 | + | - | - |
| PB-St5 | 179–228 | + | 12.08±2.99 | 123±17 | - | - | - |
| Q-S1 | 0 | - | 3.53±0.16 | 336±89 | - | - | - |
| Q-S2 | 0 | + | 7.17±1.51 | 658±91 | + | - | - |
| Q-S3 | 0 | + | 0.28±0.01 | 85±13 | - | - | - |
| Q-R1 | 468–640 | + | 160.00±18.39 | 785±103 | - | - | - |
| Q-R2 | 0 | + | 5.19±0.11 | 106±25 | - | + | + |
| Q-St1 | 0 | + | 160.94± 8.17 | 182±31 | + | - | - |
| Q-St2 | 0 | - | 6.10±1.63 | 185±9 | - | - | - |
| Q-St3 | 0 | + | 0.48±0.03 | 90±15 | - | - | - |
| OK-St | 0 | + | 8.56±1.93 | 105±7 | - | - | - |

[a]ARA for each strain was checked in NFM, LGI, and CCM media, and values given show the range of maximum activity of the strain in one of these media.

production of IAA, as values ranged between 0.15 and 0.90 µg/ml. Seventeen isolates produced IAA in the range of 1–51 µg/ml.

**ACC deaminase activity.** All isolates were positive for the presence of ACC deaminase enzyme. All isolates showed growth within 24 h on the plates containing 1-amino cyclopropane 1-carboxylic acid (ACC) as nitrogen source. The results of the colorimetric assay are described in Table 3. The highest activity was observed for *Pannonibacter phragmatetus* PB-Rt1. *E. cloacae* LH-R2 and *Rhizobium* sp. QR1 also showed higher activities as compared with the rest of the strains. The lowest activity was observed for *E. cloacae* PB-S1 and PB-S2.

**Phosphate solubilization, antifungal activity, quorum sensing, and HCN and siderophore production.** Results of these assays are given in Table 3. Twenty-seven isolates were positive for phosphate solubilization. *P. aurantiaca* PB-St2 was the only isolate that showed antifungal activity against *Colletotrichum falcatum* isolates. This isolate was further screened for antifungal activity against several pathogenic fungal isolates. It inhibited growth of 20 isolates of *Fusarium*, *Colletotrichum*, and *Pythium* spp. A complete list of the fungal strains tested is provided in Table 4.

Six isolates (*E. cloacae* PB-S1 and PB-S2, *P. aurantiaca* PB-St2, *E. aeoregenes* PB-St4, *Caulobacter crescentus* QS2, and *E. cloacae* QSt1) were positive for quorum sensing. Three isolates (*P. fluorescens* PB-St1, *P. aurantiaca* PB-St2, and *P. putida* QR2) produced HCN and hexamate-type siderophores.

## DISCUSSION

In this study, we characterized 32 bacterial strains, isolated from sugarcane varieties growing in Punjab. Most of the bacteria were members of the Enterobacteriacae and Pseudomonadacea families. *Gluconacetobacter* and *Herbaspirillum* spp., which are well known for their association with sugarcane, were not isolated from these plants, suggesting they may be missing from Pakistani cultivars.

The bacteria were characterized using biochemical tests, and micro- and molecular biology techniques. We also

**Table 4.** Antifungal activity of *P. aurantiaca* PB-St2 against pathogenic fungal isolates.

| Fungal isolates | Disease/plant | Antifungal activity |
|---|---|---|
| *Colletotrichum falcatum* BF166 | Red rot/sugarcane | ++ |
| *C. falcatum* C01148 | Red rot/sugarcane | +++ |
| *C. falcatum* CP77400 | Red rot/sugarcane | ++++ |
| *C. falcatum* SPF 234 | Red rot/sugarcane | +++ |
| *C. acutatum* | Anthracnose/blue berries | +++ |
| *C. coccodes* JAT2241 | Anthracnose/tomato | ++ |
| *C. lindemuthanium* 2221 | Anthracnose/common bean | ++ |
| *C. orbiculare* 2195 | Anthracnose/melons & cucumbers | ++++ |
| *Cylindrocarpan destructans* 1378 | Anthracnose/ | ++ |
| *Fusarium lateritium* 543 | Shisham root | ++ |
| *F. graminearum* V20251 | Headblight/corn | ++ |
| *F. graminearum* V14435 | Headblight/corn | ++ |
| *F. graminearum* RS29B01 | Headblight/corn | ++ |
| *F. graminearum 212698* | Headblight/corn | ++ |
| *F. oxysporum* | Wilt | ++++ |
| *F. oxysporum 540* | Shisham root | ++ |
| *F. oxysporum. lycopersicum. FOL 1835* | Wilt/tomato | ++++ |
| *F. oxysporum. radicis lycopersici 1833* | Wilt/tomato | +++ |
| *F. solani 120* | Rose root | - |
| *F. solani* 1888 | Wilt/cucumber | ++ |
| *F. solani* 1891 | Wilt/pepper | ++ |
| *F. solani* 1892 | Canker/pepper root | ++++ |
| *Pythium aphanidermatum* 2102 | Damping off, rot & blights/cucumber | ++ |
| *P. aphanidermatum* 2190 | Damping off, rot & blights | ++ |
| *P. capsici* | Fruit rot/peppers | ++ |
| *P. ultimum* | Damping off | - |
| *Rhizoctonia solani* | Damping off | + |
| *Phytophthora cactum* | Root rot/Ginseng | - |

-, no activity; + , weak activity; ++, moderate; +++, strong; ++++, very strong.

compared results derived from three commonly used methods for bacterial identification namely, API20E/NE kits, and *cpn60* and 16Sr RNA gene sequence analyses. The results from all three methods were identical for 15 isolates (most of the *Enterobacter* and *Pseudomonas* strains). For 22 isolates, results as to the identities that were derived from the bacterial identification kits and *cpn60* gene sequences were identical, but for the remaining strains the various methods provided conflicting identities, likely due to the small databanks available for both the API kits and *cpn60* genes as compared with 16S rRNA gene databank. Those strains that were identified as *Citrobacter freundii,* by bacterial identification kits and *cpn60* gene analyses were identified as *Klebsiella oxytoca* by 16S rRNA gene sequence analyses. Although *K. oxytoca* is present in the databank of API20E, our isolates showed a higher similarity to *C. freundii* than *K. oxytoca*. *K. oxytoca* has also been entered into the *cpn60* databank, but our isolates showed higher similarity with *C. freundii* (94%) than *K. oxytoca* (91%). This variance may be due to the only one sequence that has been deposited for *K. oxytoca* in the *cpn60* gene databank. So far, the 16S rRNA gene sequence analyses is the most reliable technique for bacterial identification, and therefore we rely on these data and consider it as a final identification rather than the biochemical and *cpn60* gene sequence results.

It was observed that the bacterial community associated with Pakistani sugarcane cultivars was dominated by *Enterobacter* (28%) and *Pseudomonas* spp. (28%), as 56% of strains were members of these two genera. These bacteria reside into the rhizosphere, the root, and/ or the shoot of all sugarcane varieties. *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp., although common isolates of sugarcane reported from different parts of the world [23], were not found here. However, the isolations of *E. cloacae*, *Pseudmonas*, *Bacillus*, *Burkholderia cepacia*, and *Klebsiella pneumoniae* from a sugarcane agroecosystems of Brazil, and *Pantoea* spp. from sugarcane cultivars of Cuba were recently reported [4, 13]. Interestingly, despite quite extensive work on the microorganism residents of sugarcane, *Rhizobium, P. aurantiaca*, *P. reactans*, *Rahnella aquatillis*, *Delftia*, *Caulobacter*, *S. maltophilia*, *E. aerogenes*, *E. oryzae* and *Pannonibacter* have not been previously reported. *K. oxytoca* was isolated only by Mirza *et al*. [20] and they presented partial sequence of one isolate. In this study, we isolated five strains of *K. oxytoca* from three different locations of Punjab.

Pakistani soils are known for their calcarious and sodic nature as well as high pH. Soil pH is mostly around 8.0 to 8.4, and for sodic soils it is around 10 [34]. These factors are reducing the yield of major crops like sugarcane, wheat, rice, cotton, *etc*. Any bacterial strain or community with the ability to survive at high pH and show salt tolerance will be ideal for these soils. Considering this fact, the growth of all isolates at high pH and salt concentration was checked and most of the organisms showed good growth with both parameters. Similarly, as the sugarcane is an annual crop and the temperature of Pakistan is mostly above 40°C in summer and below 10°C in winter, it is therefore important that bacterial inoculums (biofertilizer) should be able to survive at this temperature range. Twenty-five isolates showed the ability to grow at 4 and 41°C, providing the reason to be used as a biofertilzer at these temperatures. The PGPR are not very host specific like *Rhizobium* and can be use as a biofertilizer for its non-host plant, and these isolates can also be use as inoculum for the crops that grow at extreme environmental conditions.

It has been known for more than a decade that PGPR enhance the plant growth by direct and indirect mechanisms. These mechanisms include nitrogen fixation, phosphate solubilization, phytohormones, siderophore, ACC deaminase, homoserine lactone, antibiotics production (secondary metabolites), *etc*. By using these mechanisms, bacteria provide the plant with nitrogen, phosphorus, and iron, lower down the ethylene concentration, and give defence against pathogens. Some of these mechanisms are interrelated; that is, phytohormone and ACC deaminase synthesis, homoserine lactone and secondary metabolites production. IAA produced by bacteria is taken up by plants and together with endogenous plant IAA, it can stimulate plant cell proliferation and or elongation as well as induce the synthesis of ACC synthetase. The ACC is exuded out by the plants and taken up by the ACC-deaminase-containing bacteria, which act as a sink for ACC; they cleave it into ammonia and alpha ketobutyrate, and readily metabolize them. The direct consequences of this interaction are to increase plant biomass and protect the plant from the inhibitory effect of ethylene (synthesized from biotic and abiotic factors) [8]. Quorum sensing has been shown to regulate a number of diverse phenotypes including secondary metabolites production (siderophores, HCN, exopolysacchrides, biosurfactant. exoenzymes, phenazines, *etc*.) and playing a key role in biocontrol activity. The mechanism is explained by Diggle *et al*. [5] with an example, which states 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.

To assess the growth promoting potential, all strains were subjected to the above-mentioned assays. Each bacterial isolate possessed more than one trait associated with PGPR activity. Seventeen isolates were positive for ACC deaminase activity, IAA production, and phosphate solubilization, and six were positive for nitrogen fixation in addition to these three traits. Six strains were positive for homoserine lactone production. Most strains with ACC deaminase activity showed an inverse capacity to produce IAA. ACC deaminase activity has been reported previously to be

present in *Rhizobium*, *P. putida*, *P. fluorescens*, *Enterobacter cloacae*, *E. aerogenes*, *Rahnella aquatilis*, and *A. lipoferum* [11, 26, 32, 33]. We were unable to find reports of ACC deaminase activity in *P. aurantiaca*, *Klebsiella*, *Delftia*, *Caulobacter*, *P. reactans*, *Xanthomonas*, *Stenotrophomonas maltophilia*, and *Pannonibacter phragmatetus.* Nine strains showed nitrogen fixing activity, which included four strains of *K. oxytoca* and one strain each of *E. cloacae*, *E. oryzae*, *A. brasilense*, *Rhizobium*, and *S. maltophilia*. In a previous study, we reported the presence of the *nifH* gene in *P. putida* strains [17], but in the present study, the nitrogen fixing activity by these strains was not observed. Nitrogen fixing isolates of *S. maltophilia* were recently reported from wheat and the members of family Compositae [24], but its isolation from sugarcane has not been reported.

Three isolates, *i.e.*, Q-R2, PB-St1, and PB-St2, showed the presence of five beneficial traits. *P. putida* Q-R2 and *P. fluorescens* PB-St1 were positive for ACC, IAA, phosphate solubilization, and HCN and siderophore production. *P. aurantiaca* PB-St2 showed potential for inhibiting the fungal growth in addition to ACC, IAA, HCN, siderophore, and homoserine lactone production, and such traits were characterized in more detail in a separate study [16]. In those studies, we could not detect IAA production by PB-St2, as a high quantity of pigment produced by this organism interfered with the colorimetric assay used. In this study, IAA production was measured using HPLC and we were able to detect IAA production by this organism. Considering the affiliation of PB-St2 with *P. chloraphis* (very well known for antifungal activity), it was expected that it might show antipathogenic behavior for several other organisms. To confirm that, it was screened against pathogens of other crops. As expected, the strain inhibited the growth of 25 fungal pathogens of important crops, which suggests that it may be an excellent candidate as a biocontrol agent for several crops including sugarcane.

Two media were used to measure for phosphate solubilization and lipase production to ensure that we do not underestimate the potential capacity of any strain for such activities. Although most strains solubilized both sources of phosphate, some strains could solubilize only calcium triple phosphate or phytic acid, but not both. Similarly, when evaluating lipase activity, we found that some strains hydrolyzed either Tween 80 or butyrin, but rarely both. Using more than one medium for such tests greatly enhanced the identification of such traits, as they may be important in PGPR activities.

Looking at the data, it can be suggested that strains of *Klebsiella*, *Rhizobium*, and *Azospirillum* are the best choices for a biofertilizer, owing to their abilities of nitrogen fixation, and indole acetic acid and ACC deaminase production. Strains of *Enterobacter* and *Pseudomonas* can also be used as inoculums for their IAA production abilities, and *P. aurantiaca* in particular as a biocontrol agent for sugarcane and other crops as well. Since many PGPR posses several of these traits, a bacterium may utilize different traits at various times during the life cycle of a plant.

In conclusion, this study provides a picture of the bacterial community associated with sugarcane growing in Pakistan. It includes multiple genera of alpha, beta, and gamma proteobacteria. Most of the isolates belong to the families Enterobacteriaceae and Pseudomonadaceae. At least 10 of these species have not been previously reported from sugarcane. Similarly, the presence of ACC deaminase activity in many of these isolates was not previously recognized. The complete information about the basic characteristics of these organisms including growth at extreme conditions and potential for plant growth promotion provides the researchers an opportunity to choose the organism according to the requirement. All of the strains showed potential growth promoting activities and this makes them good candidates for including into a biofertilizer formulation, not only for sugarcane but for other crops as well. Future studies will examine the effects of these bacteria on sugarcane plants under different growth conditions.

## Acknowledgments

We are thankful to Dr. Keri Wang, A & L Canada Laboratories Inc., London, Ontario, Canada, for his help in sequencing of strains. This work was partially supported by a grant from the Agriculture and Agri-Food Canada MII Initiative.

## References

1. Boddey, R. M., S. Urquiaga, B. J. R. Alves, and V. Reis. 2003. Endophytic nitrogen fixation in sugarcane: Present knowledge and future application. *Plant Soil* **252:** 139–149.
2. Cavalcante, V. A. and J. Dobereiner. 1988. A new acid tolerant nitrogen fixing bacterium associated with sugarcane. *Plant Soil* **108:** 23–31.
3. Coombs, J. T. and C. M. M. Franco. 2003. Isolation and identification of actinobacteria from surface sterilized wheat roots. *Appl. Environ. Microbiol.* **69:** 5603–5608.
4. De Lima, T. C. S., B. M. Grisi, and M. C. M. Bonato. 1999. Bacteria isolated from a sugarcane agroecosystem: Their potential production of polyhydroxyalcanoates and resistance to antibiotics. *Rev. Microbiol.* **30:** 214–224.
5. Diggle, S. P., S. A. Crusz, and M. Camara. 2007. Quorum sensing. *Curr. Microbiol.* **17:** 907–910.
6. Dworkin, M. and J. Foster. 1958. Experiments with some micro-organisms which utilize ethane and hydrogen. *J. Bacteriol.* **75:** 592–601.
7. Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783–791.

8. Glick, B. R., B. Todorovic, J. Czarny, Z. Cheng, J. Duan, and B. McConkey. 2007. Promotion of plant growth by bacterial ACC deaminase. *Crit. Rev. Plant Sci.* **26:** 227–242.
9. Hill, J. E., A. Paccagnella, K. Law, P. L. Melito, D. L. Woodward, L. Price, *et al*. 2006. Identification of *Campylobacter* spp. and discrimination from *Helicobacter* and *Arcobacter* spp. by direct sequencing of PCR-amplified *cpn60* sequences and comparison to *cpn*DB, a chaperonin reference sequence database. *J. Med. Microbiol.* **55:** 393–399.
10. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* **44:** 301–307.
11. Kumar, K. V., S. Srivastava, N. Singh, and H. M. Behl. 2009. Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of *Brassica juncea. J. Haz. Mat.* **170:** 51–57.
12. Kumar, R. S., N. Ayyadurai, P. Pandiaraja, A. V. Reddy, Y. Venkatesvarlu, O. Prsakash, and N. Sakthivel. 2005. Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad spectrum antifungal activity and biofertilizing traits. *J. Appl. Microbiol.* **98:** 145–154.
13. Loiret, F. G., E. Ortega, D. Kleiner, P. Ortega-Rodes, R. Rodes, and Z. Dong. 2004. A putative new endophytic nitrogen-fixing bacterium *Pantoea* sp. from sugarcane. *J. Appl. Microbiol.* **97:** 504–511.
14. MacFadden, J. F. 1980. *Biochemical Tests for Identification of Medical Bacteria*. Williams and Wilkins, Baltimore.
15. Mehnaz, S. and G. Lazarovits. 2006. Inoculation effects of *Pseudomonas putida*, *Gluconaacetobacter azotocaptans* and *Azospirillum lipoferum* on corn plant growth under green house conditions. *Microb. Ecol.* **51:** 326–335.
16. Mehnaz, S., D. N. Baig, F. Jamil, B. Weselowski, and G. Lazarovits. 2009. Characterization of a phenazine and hexanoyl homoserine lactone producing *Pseudomonas aurantiaca* strain PB-St2, isolated from sugarcane stem. *J. Microbiol. Biotechnol.* **19:** 1688–1694.
17. Mehnaz, S., B. Weselowski, F. A. Mufti, S. Zahid, G. Lazarovits, and J. Iqbal. 2009. Isolation, characterization and effect of fluorescent pseudomonads on micropropagated sugarcane. *Can. J. Microbiol.* **55:** 1007–1011.
18. Mehnaz, S., M. S. Mirza, J. Haurat, R. Bally, P. Normand, A. Bano, and K. A. Malik. 2001. Isolation and 16S rRNA sequence analysis of beneficial bacteria from the rhizosphere of rice. *Can. J. Microbiol.* **47:** 110–117.
19. Miller, R. L. and V. J. Higgins. 1970. Association of cyanide with infection of birdsfoot trefoil by *Stemphylium loti*. *Phytopathology* **60:** 104–110.
20. Mirza, M. S., W. Ahmed, F. Latif, J. Haurat, R. Bally, P. Normand, and K. A. Malik. 2001. Isolation, partial characterization and the effect of plant growth promoting bacteria on micropropagated sugarcane *in vitro*. *Plant Soil* **237:** 47–54.
21. Nautiyal, C. S. 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* **170:** 265–270.
22. Oak, A. 1992. A re-evaluation of nitrogen assimilation in roots. *Bioscience* **42:** 103–111.
23. Olivares, F. L., V. L. D. Baldani, V. M. Reis, J. I. Baldani, and J. Dobereiner. 1996. Occurrence of the endophytic diazaotrophs *Herbaspirillum* spp. in roots, stems and leaves predominantly of Gramineae. *Biol. Fertil. Soils* **2:** 197–200.
24. Park, M., C. Kim, J. Yang, H. Lee, W. Shin, S. Kim, and T. Sa. 2005. Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microb. Res.* **160:** 127–133.
25. Penrose, D. M. and B. R. Glick. 2003. Methods for isolating and characterizing ACC deaminase containing plant growth promoting rhizobacteria. *Physiol. Plant* **118:** 10–15.
26. Penrose, D. M., B. A. Moffat, and B. R. Glick. 2001. Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) to assess the effects of ACC deaminase-containing bacteria on roots of canola seedlings. *Can. J. Microbiol.* **47:** 77–80.
27. Perez-Miranda, S., N. Cabirol, R. George-Tellez, L. S. Zamudio-Rivera, and F. J. Fernandez. 2007. O-CAS, a fast and universal method for siderophore detection. *J. Microbiol. Methods* **70:** 127–131.
28. Rashid, N., Y. Shimada, S. Ezaki, H. A. Tomi, and T. Y. Imanaka. 2001. Low temperature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A. *Appl. Environ. Microbiol.* **67:** 4064–4069.
29. Renni, R J. 1981. A single medium for the isolation of nitrogen fixing bacteria. *Can. J. Microbiol.* **27:** 8–14.
30. Rosado, A. S., F. S. de Azevedo, D. W. da Cruz, J. D. Van Elsa, and L. Seldin. 1998. Phenotypic and genetic diversity of *Penibacillus azotofixans* strains isolated from the rhizoplane soil of different grasses. *J. Appl. Microbiol.* **84:** 216–226.
31. Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.
32. Saleem, M., M. Arshad, S. Hussain, and A. S. Bhatti. 2007. Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. *J. Ind. Microbiol. Biotechnol.* **34:** 635–648.
33. Saravanakumar, D. and R. Samiyappan. 2007. ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. *J. Appl. Microbiol.* **102:** 1283–1292.
34. Sarwar, G., H. Schmeisky, N. Hussain, S. Muhammad, M. Ibrahim, and E. Safdar. 2008. Improvement of soil physical and chemical properties with compost application in rice-wheat cropping system. *Pak. J. Bot.* **40:** 275–282.
35. Schwyn, B. and J. B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Anals Biochem.* **160:** 46–56.
36. Sierra, G. 1957. A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty acid substrates. *Antonie van Leeuvenhoek* **23:** 15–22.
37. Tamura, K., M. Nei, and S. Kumar. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA.* **101:** 11030–11035.
38. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*. **24:** 1596–1599.
39. Tien, T. M., M. H. Gaskins, and D. H. Hubbel. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of Pearl Millet (*Pennisetum americanum* L.). *Appl. Environ. Microbiol.* **37:** 1016–1027.
40. Xie, C. H. and A. Yokota. 2005. *Azospirillum oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the roots of the rice plant *Oryza sativa*. *Int. J. Syst. Evol. Bacteriol.* **55:** 1435–1438.