Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice

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Abstract: The present study deals with the isolation of plant growth promoting rhizobacteria (PGPR) from rice (variety NIAB IRRI-9) and the beneficial effects of these inoculants on two Basmati rice varieties. Nitrogen-fixing activity (acetylene-reduction activity) was detected in the roots and submerged shoots of field-grown rice variety NIAB IRRI-9. Estimation of the population size of diazotrophic bacteria by ARA-based MPN (acetylene reduction assay-based most probable number) in roots and shoots indicated about 10^5–10^6 counts/g dry weight at panicle initiation and grain filling stages. Four bacterial isolates from rice roots and shoots were obtained in pure culture which produced phytohormone indoleacetic acid (IAA) in the growth medium. Among these, three isolates S1, S4, and R3 reduced acetylene to ethylene in nitrogen-free semi-solid medium. Morphological and physiological characteristics of the isolates indicated that three nitrogen-fixing isolates S1, S4, and R3 belonged to the genus *Enterobacter*, while the non-fixing isolate R8 belonged to the genus *Aeromonas*. 16S rRNA sequence of one isolate from root (R8) and one isolate from shoot (S1) was obtained which confirmed identification of the isolates as *Aeromonas veronii* and *Enterobacter cloacae*, respectively. The 1517-nucleotide-long sequence of the isolate R8 showed 99% similarity with *Aeromonas veronii* (accession No. AF099023) while partial 16S rRNA sequence (two stretches of total 1271 nucleotide length) of S1 showed 97% similarity with the sequence of *Enterobacter cloacae* (accession No. AJ251469). The seedlings of two rice varieties Basmati 385 and Super Basmati were inoculated with the four bacterial isolates from rice and one *Azospirillum brasilense* strain Wb3, which was isolated from wheat. In the rice variety Basmati 385, maximum increase in root area and plant biomass was obtained in plants inoculated with *Enterobacter* S1 and *Azospirillum* Wb3, whereas in the rice variety Super Basmati, inoculation with *Enterobacter* R3 resulted in maximum increase of root area and plant biomass. Nitrogen fixation was quantified by using ^{15}N isotopic dilution method. Maximum fixation was observed in Basmati 385 with the inoculants *Azospirillum* Wb3 and *Enterobacter* S1 where nearly 46% and 41% of the nitrogen was derived from atmosphere (%Ndfa), respectively. In general, higher nitrogen fixation was observed in variety Basmati 385 than in Super Basmati, and different bacterial strains were found more effective as inoculants for the rice varieties Basmati 385 and Super Basmati.

Key words: phytohormones, nitrogen fixation, *Enterobacter, Aeromonas*.

Résumé : La présente étude s'intéresse à l'isolation de rhizobactéries favorisant la croissance des plantes (PGPR) de riz (variété NIAB IRRI-9) et des effets bénéfiques qu'auraient ces inoculants sur deux variétés de riz Basmati. L'activité fixatrice d'azote (activité réductrice d'acétylène) fut détectée dans les racines et les pousses submergées de la variété de riz cultivé NIAB IRRI-9. La taille des populations de bactéries diazotrophiques telle qu'estimée par MPN (nombre le plus probable) basé sur l'ARA (activité réductrice d'acétylène) dans les racines et les pousses était d'environ 10^5–10^6 comptes/g de poids sec, lors des stades d'établissement des panicules et de remplissage des grains. Quatre isolats bactériens provenant de racines et de pousses de riz et produisant la phytohormone acide indoleacétique (AIA) dans le milieu de croissance furent obtenues en culture pure. Parmi ceux-ci, trois isolats S1, S4 et R3 ont réduit l'acétylène en éthylène dans un milieu semi-solide sans azote. Les caractéristiques morphologiques et physiologiques des isolats ont indiqué que les trois isolats fixant l'azote S1, S4 et R3 appartenaient au genre *Enterobacter* alors que l'isolat non fixateur R8 appartenait au genre *Aeromonas*. La séquence des ARNs 16S d'un isolat des racines (R8) et d'un isolat de la pousse (S1) fut obtenue et confirma l'identité des isolats comme étant *Aeromonas veronii* et *Enterobacter cloacae*, respectivement. La séquence de 1517 nucléotides de l'isolat R8 afficha une similitude de 99% avec

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Aeromonas veronii (n° de référence AF099023) alors que la séquence partielle de l'ARNr 16S (deux segments d'une longueur totale de 1271 nucléotides) de S1 afficha une similitude de 97% avec la séquence de *Enterobacter cloacae* (n° de référence AJ251469). Les semis de deux variétés de riz, Basmati 385 et Super Basmati, furent inoculés avec les quatre isolats bactériens du riz et avec une souche de *Azospirillum brasilense* Wb3 qui a été isolée du blé. Dans le riz de variété Basmati 385, une augmentation maximale de la superficie des racines et de la biomasse végétale fut obtenue pour des plantes inoculées avec *Enterobacter* S1 et *Azospirillum* Wb3, alors que chez la variété de riz Super Basmati l'inoculation avec *Enterobacter* R3 a entraîné une augmentation maximale de la superficie des racines et de la biomasse. La fixation de l'azote a été mesurée en utilisant la méthode de dilution isotopique du ^{15}N. Une fixation maximale fut observée dans le Basmati 385 en association avec les inoculants *Azospirillum* Wb3 et *Enterobacter* S1, chez qui près de 46 % et 41 % de l'azote était dérivé de l'atmosphère (%Ndfa), respectivement. En général, une meilleure fixation d'azote était observée chez la variété Basmati 385 comparativement au Super Basmati. Des souches bactériennes différentes se sont avérées être d'efficaces inoculants pour les variétés de riz Basmati 385 et Super Basmati.

Mots clés : phytohormones, fixation de l'azote, *Enterobacter*, *Aeromonas*.

[Traduit par la Rédaction]

Introduction

A variety of beneficial bacteria have been found to colonize the roots and aerial parts of rice, wheat, maize, sugarcane, and other graminaceous plants (Bilal and Malik 1987; Diem et al. 1978; Hassan et al. 1998; Hurek et al. 1994; James et al. 1994; Magalhaes et al. 1979; Patriquin and Dobereiner 1978). Interest in the beneficial rhizobacteria associated with cereals has increased recently due to their potential use as biofertilizers (Ali et al. 1995; Bashan and Levanony 1990; Dobereiner and Baldani 1998; Hegazi et al. 1998; Okon and Labandera-Gonzalez 1994; Omar et al. 1994). Application of bacterial inoculants as biofertilizers has resulted in improved growth and increased yield of cereal crops (Boddey et al. 1986; Fages 1994; Kapulnik et al. 1981; Kennedy and Tchan 1992; Pereira et al. 1988). Beneficial effects of these plant growth promoting rhizobacteria (PGPR) have been attributed to biological nitrogen fixation (Boddey et al. 1995; Lima et al. 1987; Malik et al. 1988, 1991; Urquiaga et al. 1992) and production of phytohormones that promote root development and proliferation resulting in more efficient uptake of water and nutrients (Haahtela et al. 1990; Jacoud et al. 1999; Sarig et al. 1992; Tien et al. 1979). Nitrogen-fixing bacteria belonging to the genera *Azospirillum*, *Acetobacter*, *Azoarcus*, *Enterobacter*, and *Herbaspirillum* appear to be frequent colonizers of important cereal crops and grasses (Baldani et al. 1986; Bally et al. 1983; Bilal et al. 1990*b*; Dobereiner and Day 1976; Gillis et al. 1989; Reinhold-Hurek et al. 1993).

Ribosomal RNA sequence analysis has been extensively used to study phylogenetic relationships between microorganisms as well as for taxon identification (Woese 1987; Woese et al. 1985, 1990). Sequence information from the conserved regions of the 16S rRNA gene (*rrs*) is useful for studying phylogenetic relationships as well as for the design of specific or generic oligonucleotide probes and primers used for identification by hybridizations and discriminant PCR-amplifications, respectively (Givannoni 1991). Variable regions of 16S rRNA provide sequence data to develop specific probes and primers for detection of bacteria by hybridization or with polymerase chain reaction (Stahl and Amann 1991; Ward et al. 1992). The availability and use of PCR-based amplification methods and sequencing of the PCR products on automated sequencers has dramatically expanded RNA databases during the past few years. Now sequences of over 16 000 rRNA molecules from different organisms have been catalogued (Ludwig and Schleifer 1999; Normand 1999). This wealth of sequence information is now readily available in public databases for ever finer identification of new bacterial isolates by sequence comparisons.

Isolation of phytohormone-producing and diazotrophic bacteria from rice and their characterization on the basis of morphological and physiological studies as well as by 16S rRNA sequence analysis is presented. Effects of these bacteria on inoculated rice seedlings and nitrogen fixation are also discussed.

Materials and methods

Detection of nitrogen-fixing activity in roots and submerged shoots of rice

Acetylene reduction assay (ARA) was used to detect the presence of nitrogen-fixing bacteria associated with rice. Roots and shoots of rice submerged under water (5 cm pieces of shoot near the base) were collected from a farmer's field in Gakhar (Gujranwala District), which belongs to the major rice growing area of Pakistan situated between Ravi and Chenab river. The roots and shoot samples (approximately 5 g fresh weight) were washed with sterile water and transferred to 16 mL capacity glass tubes with rubber stoppers. Acetylene (10%) was injected and the tubes were incubated at 30°C for 16 h. Triplicate samples of roots and shoots, collected from 3 different plants growing in the same field, were used for ARA. The tubes with plant material (roots and shoots) but without C_2H_2 were used as control. Another set of tubes containing only 10% C_2H_2 and no plant material was also used as control. Ethylene production was measured on a gas chromatograph (Gasukuro kogyo model 370, Tokyo, Japan) using Porapak N column (Supelco Inc., Bellefonte, Pennsylvania). Quantitative estimations of ethylene gas produced in the samples were made by measuring peak height relative to the standard (1% C_2H_4). Root and shoot samples were dried in an oven at 70°C to a constant weight.

Detection of bacterial populations and isolation of bacteria from rice

The roots and shoots of rice submerged under water were collected from the field and thoroughly washed with sterile water to remove adhering soil. One gram of roots or shoots were homogenized with a pestle and mortar in sterile water, and serial dilutions (10×) were prepared. Aliquots (100 μL) from these dilutions were used to inoculate semi-solid combined carbon medium (CCM; Rennie 1981) and incubated at 30°C for 48 h. Acetylene (10% v/v)

was injected to vials showing bacterial growth, and acetylene reduction activity was measured for determining MPN counts on a gas chromatograph (Gasukuro kogyo model 370), using a Porapak N column. One hundred microlitres of the serial dilutions prepared from roots and shoots of rice were also used to inoculate LB agar plates (Maniatis et al. 1982). For isolation of diazotrophic bacteria, serial dilutions from ARA-positive vials were prepared and used to inoculate LB and CCM agar plates to get single (pure) colonies.

Nitrogen fixation by bacterial isolates

Nitrogen fixation by the bacterial isolates was determined in semisolid combined carbon medium (CCM; Rennie 1981) and nitrogen free medium (NFM; Okon et al. 1977) by the acetylene reduction method. The vials containing 5 mL of the CCM and NFM semisolid media were inoculated with single bacterial colonies and incubated at 30°C for 16 h. Acetylene (10%) was injected into the inoculated vials and again they were incubated at 30°C for 16 h. Acetylene reduction activity was measured on a gas chromatograph (Gasukuro kogyo model 370) using Porapak N column. To measure specific activity of the cultures protein estimation was carried out by the method described by Lowry et al. (1951).

Indoleacetic acid (IAA) production

For detection and quantification of IAA production by the bacterial isolates, cultures were grown in CCM enriched with sucrose (5 g/L), mannitol (5 g/L) and ammonium chloride (1 g/L). Tryptophan (100 mg/L) was added as precursor of IAA biosynthesis. Bacterial cultures were grown for one week and the cells were pelleted by centrifugation at 10 000 rpm for 15 min. The pH of the supernatant was adjusted to 2.8 with HCl and then extracted three times with equal volumes of ethyl acetate (Tien et al. 1979). The extract was evaporated to dryness and resuspended in 1 mL of ethanol. The samples were analysed on HPLC (Perkin-Elmer, USA; Series 200) using UV detector and Techsphere 5-ODS C-18 column. Indoleacetic acid was quantified with Turbochrom software using an interface (Perkin-Elmer, U.S.A.) with attached computer. Pure indole-3-acetic acid (Sigma, U.S.A.) was used as standard for identification and quantification of IAA produced by the bacterial strains. Methanol: acetic acid: water (30:1:70 v/v/v) was used as a mobile phase at the rate of 1.2 mL/min (Rasul et al. 1998).

Physiological and biochemical tests used for the identification of isolates

The bacterial isolates were first segregated into two groups, enterobacters (facultatively anaerobic, Gram-negative, and with the ability to produce acid and gas from glucose) and the non-enterobacters. Physiological and biochemical tests were performed using the QTS-20 miniaturized identification system (DESTO Laboratories, Karachi, Pakistan). The bacterial cultures grown on LB plates were suspended in saline solution and used to inoculate QTS cupules. The oxidation fermentation test was performed as described by Hugh and Leifson (1953). Catalase was identified by the MacFadin (1980) method, using H_2O_2 and pure culture colonies from agar plates.

PCR-amplification and 16S rRNA sequence analysis

Cells of the isolate R8 from rice root and the isolate S1 from shoot were grown in LB broth for 24 h at 30°C. The cell pellets from 1.5 mL cultures were obtained by centrifugation at 13 000 rpm for five minutes and washed with TE buffer (10 mM Tris·Cl; 1 mM EDTA, pH 8). The cell pellets were then dissolved in 200 µL of TE. Cell lysis was obtained at 37°C for 30 min with lysozyme (2 mg/mL; final concentration) and by using SDS (1%). The lysate was extracted twice with phenol/chloroform followed by two extractions with chloroform/isoamyl alcohol (24:1). After adding 1/10 volume of sodium acetate (3 M, pH 5.2) and 0.5 volume of isopropanol, the supernatant was incubated at –20°C for 30 min. The precipitated nucleic acids were then sedimented by centrifugation at 13 000 rpm for 20 min and the resulting pellet was washed with 70% ethanol before drying under vacuum. The nucleic acid pellets were then dissolved in 100 µL TE and used as template for PCR amplification of 16S rRNA gene. Each reaction mixture (50 µL) contained 0.5 µL Taq DNA polymerase (5 U/µL; Gibco/BRL), 5 µL Taq buffer, 5 µL dNTPs (200 µM), 5 µL (100 ng/µL) of each primer (Primer FGPS4-281 bis: AGA GTT TGA TCC TGG CTC AG; Primer FGPS1509′-153: AAG GAG GTG ATC CAG CCG CA; Normand 1995), 24.5 µL sterile water and 1 µL of template. After denaturation of the template at 94°C for 4 min, thirty rounds of temperature cycling (94°C for 1 min, 55°C for 1 min and 72°C for 1 min) were followed by incubation at 72°C for 7 min. The PCR products were gel purified (NuSieve, 1.2%) by using QIAquick spin (QIAGEN) kits and sequenced on Perkin-Elmer ABI PRISM Model 373. Amplification primers as well as internal primers (Normand 1995) were used for sequencing both strands of the PCR products. The sequences were deposited in the EMBL databank (*Aeromonas veronii* R8, accession No. AJ278105; *Enterobacter cloacae* S1, accession No. AJ278106).

Inoculation of rice with bacterial isolates

Rice seeds were surface sterilized with 0.1% mercuric chloride for five minutes and then washed three times with sterilized water. The seeds were germinated on water-agar (1.5% agar) plates and one-week-old contamination-free rice seedlings of variety Basmati-385 and Super Basmati were transplanted in Pyrex tubes (19.5 × 2.2 cm) containing sterilized vermiculite. Half strength N-free Hoagland solution was used as nutrient source. ^{15}N labelled ammonium sulfate (0.75 mg N/tube) of 10% atom excess was added as a tracer to quantify nitrogen fixation. For inoculation of the plants, bacterial cultures were grown in LB broth for 16 h at 30°C. The cell pellets were obtained by centrifugation at 10 000 rpm for 5 min, washed and resuspeded in sterile water. The plants were inoculated with 1 mL bacterial suspension (10^8–10^9 cells/mL) of individual strains. For each treatment 8 plants were used. Heat-killed cells were used to inoculate the plants used as non fixing control. Plants were kept at 30 ± 2°C during the day, and 25 ± 2°C at night for 8 weeks. At the time of harvesting, root area of the plants was measured with the Root Image Analysis Program (Washington State University Research Foundation, Washington State University, U.S.A.). The plants were dried in an oven at 70°C until no change in weight was noted. The dried plant samples were ground to a fine powder and total N in these samples was determined by using a semi micro-Kjeldahl method based on wet combustion in a Rapid Kjeldahl System (Labconco, Kansas city, Missouri). The analysis for ^{15}N excess was carried out on a double inlet mass spectrometer (Varian MAT GD 150). Quantification of nitrogen fixation based on isotope dilution was calculated by the formula of Fried and Middleboe (1977) which is:

$$\%\ \mathrm{N\ fixed} = 1 - \frac{(^{15}\mathrm{N\,atom\%\,excess})\mathrm{fs}}{(^{15}\mathrm{N\,atom\%\,excess})\mathrm{nfs}} \cdot 100$$

Where fs is fixing system and nfs is non fixing system.

Statistical analyses

Results of the measurements were subjected to analysis of variance (ANOVA) and significance at the 5% level was tested by Duncan's multiple range test (DMRT) using a computer software program. Mean values and the standard error were calculated, and the data were analysed by randomised complete block design (RCBD).

Table 1. Detection of acetylene reduction activity (ARA) and bacterial populations associated with roots and submerged shoots of rice variety NIAB IRRI-9.

	Root		Shoot	
	Panicle initiation	Grain filling	Panicle initiation	Grain filling
ARA*	10±3.6	39±14	10±2.8	120±43
MPN counts ($\times 10^6$)**	1.2±0.2	3.5±1.0	0.1±0.01	10.3±2.1
CFU ($\times 10^6$)***	8.2±2.7	8.1±3.4	0.5±0.05	3.9±1.4

*nmol C_2H_4/g dry weight/day.
**ARA-based MPN counts.
***Colony forming units on LB plates.

Table 2. Production of indoleacetic acid and acetylene reduction activity of the bacterial isolates from rice and wheat. The results are average of three replicates ± standard deviation.

	IAA Production (IAA mg/L of growth medium)		ARA (nmol C_2H_4/h/mg protein)	
Bacterial isolates	Without tryptophan	With tryptophan	NFM[a]	CCM[b]
Enterobacter S1	2.6±0.4	77±13	20±3	626±51
Enterobacter S4	2.4±0.2	167±21	5±1	94±15
Enterobacter R3	2.5±0.2	750±35	51±12	259±43
Aeromonas R8	0.9±0.1	276±17	ND	ND
[c]*Azospirillum brasilense* Wb 3	0.5±0.2	253±19	1080±175	2021±284

[a]NFM, nitrogen-free malate medium (Okon et al. 1977).
[b]CCM, combined carbon medium (Rennie 1981).
[c]An isolate from wheat (Hassan et al. 1998).
ND, not detected (no ethylene peak was detected).

Results and discussion

By using acetylene reduction assay (ARA) technique, nitrogen-fixing activity was detected in roots as well as in submerged shoots of rice variety IRRI NIAB-9 (Table 1). This indicates colonization of rice roots and shoots by diazotrophic bacteria. In aerial parts of rice plants above the level of floodwater, no ARA activity was found. Higher ARA was determined at grain filling stage than panicle initiation stage. In submerged shoots, relatively higher ARA was detected as compared to roots, where about 3 times more activity (120 nmol C_2H_4 per g dry weight per day) was recorded at grain filling stage. Similar variation in ARA with the plant growth stage has been reported by Watanabe et al. (1979) in two rice varieties, IR36 and IR26, where maximum ARA was detected at heading stage. Rao and Rao (1984) determined maximum activity 60 days after transplantation, while Barraquio et al. (1986) also detected maximum activity at heading stage. Higher ARA detected during a particular growth stage may be due to reduction in the inhibitory nitrogen concentrations in the soil or overproduction of root exudates creating conducive conditions for growth and activity of diazotrophs (Dobereiner and De-Polli 1980; Jagnow 1983). Population of diazotrophs as estimated by ARA-based MPN counts was also higher at grain filling stage in both roots and submerged shoots (Table 1). Acetylene reduction activity detected in shoots and the presence of diazotrophs in high numbers may be of practical significance, as the isolation and use of these bacteria as biofertilizers along with root colonizing bacteria may enhance efficiency of such inocula. In wetland rice, contribution of the basal portion of shoot to nitrogen fixation has been reported by Watanabe et al. (1981).

For the isolation of beneficial bacteria from rice roots and shoots, nitrogen-free semisolid media were inoculated with serial dilutions prepared from homogenized plant materials. Bacterial growth became visible within 48 h as a veil-like pellicle just below the surface of the medium. This pellicle formation was initially considered a characteristic of *Azospirillum*, which finds suitable oxygen concentration just below the surface due to its microaerophilic nature (Tarrand et al. 1978). However, later studies (Gillis et al. 1989; Haahtela et al. 1981, 1983; Seldin et al. 1984; Watanabe and Barraquio 1979) showed that other microaerophilic bacteria including diazotrophs can also be isolated in semisolid media. Serial dilutions of the bacterial growth were used to inoculate NFM, CCM, and LB agar plates to get single cell colonies. Two isolates (R3 and R8) were obtained from rice roots and two (S1 and S4) from the submerged shoots of rice in pure culture. All these isolates produced the phytohormone IAA in growth medium while only three isolates S1, S4, and R3 showed acetylene reduction activity in nitrogen-free media (Table 2). The amount of IAA produced in the presence of tryptophan added as a precursor of IAA biosynthesis was considerably higher as compared to the medium from which tryptophan was omitted (Table 2). Among the strains tested, strain R3 produced the highest amount (750 mg/L of the medium) of IAA. Production of phytohormones by different PGPR strains and their beneficial effects on plants have been reported (Haahtela et al. 1990; Hartmann et al. 1983; Tien et al. 1979). However, detrimental effects of the phytohormones have also been observed as reported by Zelena et al. (1988) in corn where strong inhibition of root elongation was noted.

Colony and cell morphology characteristics and physiological/biochemical data of the isolates are given in Table 3. The cells of all isolates were Gram negative, short or me-

Table 3. Physiological and biochemical tests* used for the identification of PGPR isolated from rice variety NIAB-6.

Test	Isolate S1	Isolate S4	Isolate R3	Isolate R8
ARA	+	+	+	–
[a]Resistance to antibiotics				
Ampicillin	+	+	+	+
Gentamycin	–	+	–	–
Kanamycin	–	+	–	–
Rifampicin	–	–	–	–
Spectinomycin	–	+	–	–
Streptomycin	–	–	–	–
Tetracyclin	–	–	–	–
Oxidase	–	–	–	+
[b]ONPG	+	–	+	+
Ornithine decarboxylase	–	+	+	–
Lysine decarboxylase	–	+	–	+
Organism identified	*Enterobacter*	*Enterobacter*	*Enterobacter*	*Aeromonas*

*All the isolates formed off-white round colonies on LB agar plates and the cells were Gram-negative, short motile rods. All the isolates were positive for the following tests: Acid and gas production from D-glucose, catalase, nitrate reduction, Voges Proskauer, utilization of sodium malonate and sodium citrate, acid production from sugars (glucose, sucrose, mannitol, maltose, rhamnose, sorbitol). All the isolates were negative for the following tests: H_2S production, indole, urea hydrolysis, arginine dihydrolase and acid production from of inositol.

[a]50 μg/mL concentration of the antibiotics was used.

[b]Ortho nitro phenyl β-D-galactopyranoside.

Table 4. Effect of PGPR on root area, plant biomass, and nitrogen fixation of rice varieties Basmati 385 and Super Basmati. Plants were grown under microbiologically controlled conditions in sterile vermiculite for 8 weeks. Eight plants were used for each treatment.

	Basmati 385			Super Basmati		
Treatments	Root area (cm^2)	Plant biomass (mg/plant)	*%Ndfa	Root area (cm^2)	Plant biomass (mg/plant)	*%Ndfa
[a]Control	32.2 C	293 C	—	31.7 C	302 D	—
Enterobacter S1	60.0 A	522 A	41.1 AB	52.6 A	457 B	28.7 B
Enterobacter S4	44.3 B	397 ABC	7.5 C	52.9 A	501 B	34.3 A
Enterobacter R3	44.4 B	369 BC	40.3 B	54.4 A	553 A	8.8 C
Aeromonas R8	59.6 A	477 AB	ND	52.0 A	466 B	ND
[b]*Azospirillum* Wb3	60.8 A	516 A	45.7 A	43.9 B	419 C	31.5 AB

*Percentage of nitrogen derived from air; ND, not determined. Means followed by the same letter are not statistically different at 5% level according to Duncan's Multiple Range Test (DMRT).

[a]Plants inoculated with heat-killed cells.

[b]An isolate from wheat (Hassan et al. 1998).

dium sized (0.5–1 μm in diameter and 1–4 μm in length), motile rods. All the isolates were facultatively anaerobic, having both a respiratory and a fermentative type of metabolism. The isolates were able to catabolize D-glucose and other carbohydrates with the production of acid. The isolate R8 was oxidase positive and catalase positive, whereas the isolates R3, S1, and S4 were oxidase negative and catalase positive. Following morphological characterization and QTS-20 biochemical tests, the isolate data were compared with those of standard species using *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994). Based on these morphological/physiological characteristics (Table 3), the isolate R8 was identified as *Aeromonas veronii* and the remaining three strains (R3, S1, and S4) as *Enterobacter cloacae* because these isolates shared maximum common characters with these two bacterial genera (Farmer et al. 1992; Grimont and Grimont 1992; Holt et al. 1994).

One isolate from shoot (S1) and one from root (R8) were further identified by 16S rRNA sequence analysis. Use of conserved primers allowed amplification of the nearly complete *rrs* gene. PCR products were obtained from both isolates which were sequenced directly. For the isolate R8 the sequence information for 1517 nucleotides was obtained. The comparison of the almost complete sequence with the databank showed highest similarity (1503 identities /1517 positions or 99% similarity) with the sequence of *Aeromonas veronii* (accession No. AF099023). In addition to this high sequence similarity, this isolate showed maximum similarity with the phenotypic profile of this species (Table 3). Strains of *Aeromonas* occupy a wide variety of ecological niches, causing human extraintestinal infections and also diseases in both cold-blooded and warm-blooded animals. However, members of this genus have been considered primarily aquatic organisms due to their isolation from a wide variety of water samples (Farmer et al. 1992). The isolation of *Aeromonas* strain R8 from rice reflects the presence of these bacteria in the rice rhizosphere, which remains submerged under water during most part of the growing season. The se-

quence of the two stretches of 16S rRNA PCR product of the isolate S1 from shoot was also determined. This strain showed highest 16S rRNA sequence similarity (1234 identities /1271 positions 97%) with *Enterobacter cloacae* (accession No. AJ251469). Nitrogen fixation by *Enterobacter* isolated from plant roots including rice as well as from paper-mill-process water has been reported (Bally et al. 1983; Barraquio and Watanabe 1981; Haahtela et al. 1983; Ladha et al. 1983). Bilal et al. (1990*a*) identified most of the diazotrophs as *Enterobacter agglomerans* and *Enterobacter cloacae* from *Atriplex* growing in low-fertility saline sodic soils of Pakistan. The isolation of *E. cloacae* was recently reported by Mukhopadhay et al. (1996) from rice variety IR42 from surface-sterilized seeds.

The isolates from rice were used to inoculate seedlings of rice varieties Basmati 385 and Super Basmati. All isolates showed beneficial effects on root area of both rice varieties (Table 4). *Aeromonas* R8 and *Enterobacter* S1 from rice and *Azospirillum brasilense* Wb3 showed maximum increase in root area of Basmati 385 where almost a doubling in root area over uninoculated plants was observed. In Super Basmati, all *Enterobacter* and *Aeromonas* strains used in the present study proved to be more effective in increasing root area compared to *Azospirillum brasilense* strain. Estimation of root area is considered the most reliable characteristic for evaluation and measurement of the plant growth response to bacterial inoculations (Fallik et al. 1988). A positive effect of bacterial inoculations on roots of maize and wheat has been reported (Arsac et al. 1990; Creus et al. 1996; Jacoud et al. 1999). Similar beneficial effects of bacterial inoculations were also observed on plant biomass of both rice varieties. In Basmati 385, *Enterobacter* strain S1 and *Azospirillum brasilense* Wb3 showed maximum increase in plant biomass where 78% and 76% increase over control was noted, respectively. In Super Basmati maximum increase (83%) in plant biomass was shown by *Enterobacter* strain R3. Quantification of nitrogen fixation by ^{15}N isotopic dilution method showed that the effect of bacterial inoculation was more prominent in variety Basmati 385 as compared to Super Basmati (Table 4). In this rice variety *Azospirillum brasilense* Wb3 and *Enterobacter* S1 showed maximum fixation with 46% and 41% Ndfa recorded, respectively. In Super Basmati *Enterobacter* strain S4 was found to be the most efficient with 34% Ndfa observed. However, the same strain showed poor performance with Basmati 385.

In the present study four plant growth promoting rhizobacteria belonging to genera *Enterobacter* and *Aeromonas* were obtained, which showed beneficial effects on rice seedlings. These isolates, after further testing under field conditions, could prove useful for developing a biofertilizer for rice.

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