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ORIGINAL PAPER

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Molecular characterization and PCR detection of a nitrogen-fixing *Pseudomonas* strain promoting rice growth

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Abstract Nitrogen-fixing plant growth-promoting rhizobacteria (PGPR) from the genus *Pseudomonas* have received little attention so far. In the present study, a nitrogen-fixing phytohormone-producing bacterial isolate from kallar grass (strain K1) was identified as *Pseudomonas* sp. by *rrs* (16S ribosomal RNA gene) sequence analysis. *rrs* identity level was high with an uncharacterized marine bacterium (99%), *Pseudomonas* sp. PCP2 (98%), uncultured bacteria (98%), and *Pseudomonas alcaligenes* (97%). Partial *nifH* gene amplified from strain K1 showed 93% and 91% sequence similarities to those of *Azotobacter chroococcum* and *Pseudomonas stutzeri*, respectively. The effect of *Pseudomonas* strain K1 on rice varieties Super Basmati and Basmati 385 was compared with those of three non-*Pseudomonas* nitrogen-fixing PGPR (*Azospirillum brasilense* strain Wb3, *Azospirillum lipoferum* strain N4 and *Zoogloea* strain Ky1) used as single-strain inoculants. *Pseudomonas* sp. K1 was detected in the rhizosphere of inoculated plants by enrichment culture in nitrogen-free growth medium, which was followed by observation under the microscope as well as by PCR using a *rrs*-specific primer. For both rice varieties, an increase in shoot biomass and/or grain yield over that of noninoculated control plants was recorded in each inoculated treatment. The effect of *Pseudomonas* strain K1 on grain yield was comparable to those of *A. brasilense* Wb3 and *Zoogloea* sp. Ky1 for both rice varieties. These results show that nitrogen-fixing pseudomonads deserve attention as potential PGPR inoculants for rice.

Keywords Plant growth-promoting rhizobacteria (PGPR) · *Azospirillum* · *Pseudomonas* · *nifH* · PCR detection

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Introduction

Plant growth-promoting rhizobacteria (PGPR) associate with major agricultural crops like rice, wheat, maize, and sugarcane, and stimulate plant growth through various mechanisms. Direct mechanisms that result in plant-growth promotion include biological nitrogen fixation, the solubilization of phosphorus, and the synthesis of phytohormones (Costacurta and Vanderleyden 1995; Bashan and Holguin 1997; Mirza et al. 2000; Penrose and Glick 2003; Lucy et al. 2004), while secretion of biocontrol compounds like siderophores, antibiotics, and lytic enzymes improve plant growth indirectly by inhibiting phytopathogens (Dunne et al. 1997; Bally and Elmerich 2005; Moënne-Loccoz and Défago 2004). Some plant-beneficial bacterial species are considered general root colonizers while others live endophytically in plant tissues and show some degree of specificity towards the host (Bashan and Levanony 1990; Fages 1994; James and Olivares 1997). Significant genotypic differences also exist among crop varieties in their ability to support plant-beneficial activities of microbes (Shrestha and Ladha 1996).

A number of PGPR strains belonging to diverse genera have been isolated from the plant family Gramineae (Poaceae) (Baldani et al. 1997; Bally et al. 1983; Döbereiner 1992; James and Olivares 1997; Reinhold-Hurek et al. 1993; Mirza et al. 2001). In the case of kallar grass [*Leptochloafusca* (L.) Kunth], a gramineous plant that colonizes salt-affected soils in Pakistan (Malik et al.

1986), nitrogen-fixing bacterial isolates have been obtained in several studies. They have been identified as belonging to the genera *Azospirillum* (Reinhold-Hurek et al. 1987; Bilal et al. 1990b), *Azoarcus* (Reinhold-Hurek et al. 1993), *Enterobacter* (Bilal et al. 1990a), and *Zoogloea* (Bilal and Malik 1987), while other kallar grass isolates could not be properly identified as they showed morphological/physiological characters different from those of known diazotrophs (Bilal et al. 1990b). As DNA-based techniques, like ribosomal RNA (*rrs*) sequence analysis, were not employed, definitive identification of these isolates from kallar grass could not be achieved at the time of their isolation.

This study focuses on K1, a nitrogen-fixing bacterium isolated from kallar grass, which was initially and mistakenly identified as *Azospirillum brasilense* on the basis of a similar C-source utilization pattern in spite of the fact that it showed cell morphology and motility clearly different from those of azospirilla (Bilal et al. 1990b). Indeed, the relatedness of strain K1 to *Pseudomonas* rather than *Azospirillum* was reported in a later study (Rasul, personal communication), based on the catabolism of 96 carbon compounds in BIOLOG plates (BIOLOG Inc., Hayward, CA). The majority of gram-negative PGPRs studied so far belong either to the *Pseudomonas* genus or to nitrogen-fixing non-*Pseudomonas* taxa, and reports on nitrogen-fixing *Pseudomonas* PGPR are rare. Therefore, in the present work, the *rrs* gene of the strain was amplified by PCR and sequenced for taxonomic identification. Its partial *nifH* sequence was also determined. Strain K1 promotes the growth of kallar grass, a plant used as green manure in particular on saline soils (Malik et al. 1986), but this strain's usefulness on crop plants requires investigation. On this basis, strain K1 was compared with three other PGPR strains for the stimulation of the growth of two rice varieties. All four bacteria had been shown to fix nitrogen by acetylene reduction assay (ARA) and produce the phytohormone indoleacetic acid in pure culture (Bilal and Malik 1987; Bilal et al. 1990b; Rasul et al. 1998). Finally, a PCR method was developed to detect the strain in the roots of inoculated rice.

Materials and methods

PCR amplification and *rrs* sequence analysis

Strain K1 was isolated from kallar grass roots in Pakistan (Bilal et al. 1990b). Taxonomic characterization of strain K1 was done by sequence analysis of the 16S rRNA gene *rrs*, as follows: For the extraction of DNA, the strain was grown in Luria-Bertani (LB; Sambrook et al. 1989) broth for 12 h and cell pellets from 1.5-ml volumes were sedimented by centrifugation at 13,000×*g* for 5 min. The cell pellets were washed with TE buffer (10 mM Tris–Cl, 1 mM EDTA; pH 8) and then suspended in 200 µl of TE buffer. The cells were lysed by placing them in boiling water for 5 min in the presence of 1% sodium dodecyl sulfate. The lysate was extracted twice with phenol/chloroform (in a 1:1 ratio), followed by two extractions with chloroform/isoamyl alcohol (24:1). After adding 0.1 vol of sodium acetate (3 M; pH 5.2) and 0.5 vol of isopropanol, the supernatant was incubated for 30 min at −20°C. The nucleic acids were then sedimented by centrifugation at 13,000×*g* for 20 min and the resulting pellet was washed with 70% vol/vol ethanol before drying under vacuum. The nucleic acid pellet was then dissolved in 100 µl TE buffer and used as template for PCR amplification of the *rrs* gene. Each reaction mixture (50 µl) contained 0.5 µl Taq DNA polymerase (5 U μl^{-1}; Gibco/BRL, Cergy Pontoise, France); 5 µl Taq buffer; 5 µl of deoxyribonucleotide triphosphates (dNTPs) (200 µM of each); 5 µl (100 ng μl^{-1}) of each primer, i.e., primers FGPS4-281 bis (AGA GTT TGA TCC TGG CTC AG) and FGPS1509′-153 (AAG GAG GTG ATC CAG CCG CA) (Normand 1995); 24.5 µl sterile water; and 1 µl of template DNA. After denaturation of the template at 94°C for 4 min, 30 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 product was gel purified (NuSieve 1.2% w/vol) using QIAquick spin kits (QIAGEN, Courtaboeuf, France) and sequenced by Genome Express (Grenoble, France) on an automated sequencer (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 sequence has been deposited in the European Molecular Biology Laboratory (EMBL) databank (accession number AJ278107). An unrooted phylogenetic tree was inferred using the neighbor joining method (Saitou and Nei 1987), using Kimura's 2-parameter method (Kimura 1980) for distance calculation. Nodal robustness was assessed using 1,000 bootstrap replicates (Felsenstein 1985).

Amplification and sequencing of partial *nifH* from strain K1

DNA from strain K1 was obtained using a DNA purification kit (QIAGEN). For PCR amplification of partial *nifH*, 1 µl of template DNA was added to the reaction mixture (50 µl) described above, which contained 1 µl of each *nifH* primer, i.e., PolF (TGCGAYCCSAARGCB GACTC) and PolR (ATSGCCATCATYTCRCCGGA) (Poly et al. 2001). These primers amplify a 360-bp region between sequence positions 115 and 476 [referring to *Azotobacter vinelandii nifH* coding sequence (M20568)]. PCR conditions, purification, and sequencing of the PCR product were the same as mentioned above. The EMBL accession number for the *nifH* sequence is AY660961.

Inoculation of rice seedlings grown in nonsterilized soil

During the first week of July, 5-week-old seedlings of the rice varieties Super Basmati and Basmati 385 (provided by the Rice Research Institute, Kala Shah Kaku, Pakistan) were transplanted into 25-cm-diameter fiberglass pots (five

seedlings per pot). Each pot contained 10 kg of air-dried soil (about 25 cm deep). The soil used in this experiment belonged to the Hafizabad series (coarse loamy, mixed, hyperthermic, Ustalfic, Haplargid, Aridisol). It was collected from the top 30 cm of an experimental field of the NIBGE Institute in Faisalabad (Pakistan), and had an electrical conductivity of saturation extract 2.5 dS m^{-1}, pH 8.2, organic matter 0.60%, available P 7.5 mg kg^{-1}, and total N 0.059%. For inoculation, cells from 24-h-old LB cultures were washed twice with saline solution (0.85% NaCl). In addition to *Pseudomonas* sp. K1, inoculants also included *Azospirillum lipoferum* N4 and *A. brasilense* Wb3 (Hassan et al. 1998), as well as *Zoogloea* sp. Ky1 (Bilal and Malik 1987). The seedlings were inoculated at the time of transplantation by dipping the root systems in the cell suspensions [approximately 10^8 colony forming units (CFU) ml^{-1}, as determined on LB plates] for 30 min. Heat-killed cells of strain K1 were used as noninoculated control. Each pot was kept flooded with water until 2 weeks before rice harvest.

Three pots were used for each treatment. All data were obtained from six plants per treatment (n=6; two plants per pot). For calculation of plant biomass, two plants from each pot were harvested at maturity and dried to a constant weight in an oven at 70°C. The root systems from two other plants were used for bacterial enumerations (see below).

ARA-based most probable number (MPN) of nitrogen-fixing bacteria associated with roots

Root samples were collected from plants under each treatment 4 weeks after transplantation. Some of the soil was carefully removed from the pots with a spoon, partially exposing the root system (for two plants per pot). Root segments were taken (a few grams from each of two plants), and the soil was then placed back and the plants were allowed to grow till harvest. These sampled plants grew as well as the others based on visual comparisons of shoots, but they were excluded from plant biomass calculations. One gram of each sample (root and tightly adhering soil) was homogenized with a pestle and mortar in 9 ml sterile water, and serial dilutions (10×) were prepared from this suspension. Of each dilution, 100 µl was used to inoculate 5 ml of semisolid (0.2% agar) nitrogen-free combined carbon medium (CCM; Rennie 1981) in glass vials. Five vials were used for each dilution. After 24 h of bacterial growth at 30°C, vials were incubated with C_2H_2 (10% v/v) at 30°C. ARA was measured on a gas chromatograph (Model 370; Gasokuro Kogyo, Japan) after 48 h, and ARA-positive vials were used for the estimation of the most probable number (MPN) with the help of a probability table (Cochran 1950). Agar plates (in triplicate) of LB medium were also inoculated with 100 µl of each dilution of root macerate suspension. For microscopic examination of the cells from (1) the different types of bacterial colonies appearing on these plates and (2) CCM enrichment cultures, the cells were suspended in sterilized saline solution and observed without staining for comparison with cell morphology of strain K1. Due to their characteristic motility pattern, the cells of strain K1 are easily distinguishable from other bacteria in unstained preparations.

PCR detection of the inoculated strain K1 in the roots of rice variety Super Basmati

A specific primer (P-K1, i.e., GAA CTG CAT CCA AAA CTA CT) based on the *rrs* sequence was developed for PCR detection of inoculated strain K1 in combination with primer FGPS1509′-153 mentioned above. The specificity of the primer set was tested using non-nitrogen-fixing *Pseudomonas* sp. 96-51 (Rasul et al. 1998) and nitrogen-fixing *Zoogloea* Ky1 (Bilal and Malik 1987), *Enterobacter cloacae* S1 (Mehnaz et al. 2001) and SC20 (Mirza et al. 2000), *Aeromonas veronii* R8 (Mehnaz et al. 2001), *A. lipoferum* N4 (Hassan et al. 1998) and Sp59b (Tarrand et al. 1978), *A. brasilense* Wb3 (Hassan et al. 1998) and Sp7 (Tarrand et al. 1978), and *Herbaspirillum seropedicae* Z176 (Fu and Burris 1989). The strains were grown in LB broth, and cell pellets from 1.5 ml LB culture were sedimented by centrifugation at 1,000×g for 5 min. The cells were resuspended in 100 µl of TE buffer and placed in boiling water for 10 min for cell lysis. The lysate was centrifuged for 10 min at 1,000×g and the supernatant was used as template for PCR.

To study inoculant survival in the rhizosphere, template DNA for PCR was also obtained from CCM enrichment cultures originating from root macerates of plants inoculated with strain K1, as well as control plants (at 4 weeks after inoculation). Aliquots (100 µl) from ARA-positive CCM vials were diluted with equal volumes of sterile distilled water and placed in boiling water for 10 min for cell lysis. The supernatant obtained by centrifugation at 1,000×g for 10 min was used as template for PCR. Each reaction mixture (50 µl) contained 0.5 µl Taq DNA polymerase (5 U μl^{-1}; Gibco/BRL), 5 µl Taq buffer, 5 µl dNTPs (200 µM of each), 5 µl (100 ng μl^{-1}) of each primer, 24.5 µl sterile water, and 1 µl of template DNA. After denaturation of the template at 94°C for 3 min, 35 rounds of temperature cycling (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min) were followed by 72°C for 5 min. After PCR, 10 µl from the reaction mixture was used for agarose gel (1%) electrophoresis. The number of CCM vials giving specific PCR products was used for MPN estimation of the population size of inoculated strain K1 with the help of the probability table of Cochran (1950).

Statistical analyses

The pot experiment followed a randomized complete block design. Means and standard errors (n=6) were calculated. Quantitative results were subjected to ANOVA, and significance at the 5% level was tested by Duncan's Multiple Range Test.

Results and discussion

Identification of strain K1 as *Pseudomonas* by *rrs* and *nifH* sequence analysis

The 1,475-bp *rrs* sequence obtained from the nitrogen-fixing kallar grass isolate K1 showed highest similarity to those of the putative marine diazotroph BAL281 (99%; AY972868), *Pseudomonas* sp. PCP2 (98%; AF326380), and uncultured bacteria obtained by direct amplification from the environment (98%; AJ306778 and AY693817). Among the recognized species of the genus *Pseudomonas*, the sequence from K1 showed highest similarity with those of *P. alcaligenes* (97%; AF390747.1), *P. pseudoalcaligenes* (96%; Z76666.1), and *Pseudomonas stutzeri* (96%; U26262.1). In the phylogenetic tree (Fig. 1) based on *rrs* sequences, in which sequences representing the main *Pseudomonas* groups recognized by Anzai et al. (2000) were included, strain K1 formed a large cluster with species from the "*P. aeruginosa*" group (*P. aeruginosa*, *P. resinovorans*, and *P. alcaligenes*).

The amplification of partial *nifH* (360 bp) from strain K1 further confirmed (in addition to acetylene reduction activity) the presence of a nitrogen fixation ability in this strain. The *nifH* sequence showed highest similarity to those of *Azotobacter chroococcum* (93% in DNA sequence; X03916) and *P. stutzeri* (91%; AJ297529). Anzai et al. (2000) have noted a close relatedness of the *rrs* sequence of *Azotobacter* to those of the *Pseudomonas* spp., noticeably *P. aeruginosa* (with which strain K1 shares several phenotypic properties).

Thus, the high sequence similarities of *rrs* and (partial) *nifH* genes of strain K1 to those of *Pseudomonas* provide strong evidence that the strain belongs to this genus rather

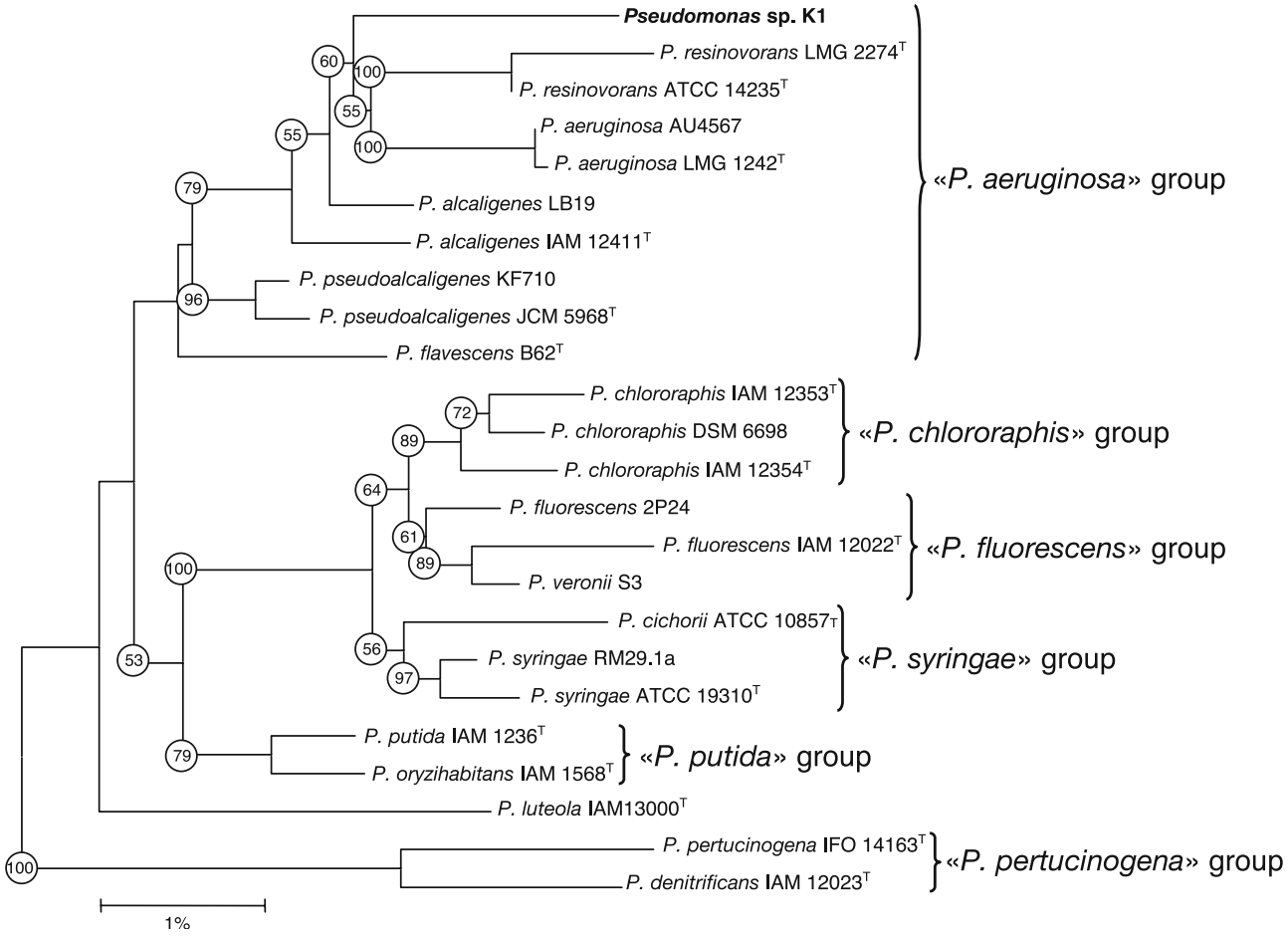

Fig. 1 Phylogenetic relationship based on *rrs* sequences between *Pseudomonas* strain K1 and the main *Pseudomonas* groups defined by Anzai et al. (2000). The unrooted neighbor joining phylogenetic tree was inferred using Kimura's 2-parameter distance. Bootstrap values (1,000 replicates) are shown. The *scale bar* represents the percentage of substitutions per site. Accession numbers of *Pseudomonas* strains were AJ278107 (*Pseudomonas* sp. K1), AY509898 (*P. chlororaphis* DSM 6698), D84011 (*P. chlororaphis* IAM 12354), D84008 (*P. chlororaphis* IAM 12353), AY574914 (*P. syringae* RM29.1a), D84026 (*P. syringae* ATCC 19310), AB021398 (*P. cichorii* ATCC 10857), AY447045 (*P. fluorescens* 2P24), D84013 (*P. fluorescens* IAM 12022), AY179328 (*P. veronii* S3), D84020 (*P. putida* IAM 1236), D84004 (*P. oryzihabitans* IAM 1568), AB109888 (*P. pseudoalcaligenes* KF710), AB021379 (*P. pseudoalcaligenes* JCM 5968), D84006 (*P. alcaligenes* IAM 12411), AF390747 (*P. alcaligenes* LB19), U01916 (*P. flavescens* B62), Z76668 (*P. resinovorans* LMG 2274), AB021373 (*P. resinovorans* ATCC 14235), AY486366 (*P. aeruginosa* AU4567), Z76651 (*P. aeruginosa* LMG 1242), AB021380 (*P. pertucinogena* IFO 14163), AB021419 (*P. denitrificans* IAM 12023), and D84002 (*P. luteola* IAM 13000). *T* Type strain

than to *Azospirillum* as previously reported (Bilal et al. 1990b). The isolation of nitrogen-fixing *Pseudomonas* strains from rhizospheres has been reported (Barraquio et al. 1983; Watanabe et al. 1987), though some controversy existed over the nitrogen-fixing ability in some members of this genus. The ability to fix nitrogen claimed earlier for *Pseudomonas glathei* was later shown to be due to nitrogen scavenging (Zolg and Ottow 1975), but this species has been reclassified as belonging to the *Burkholderia* genus. While the taxonomic position of some of these species has been revised (Willems et al. 1989; Anzai et al. 2000), Vermeiren et al. (1999) have shown that strain A15 was a nitrogen-fixing *P. stutzeri* strain. Overall, at least five species of *Pseudomonas*, i.e., *P. stutzeri*, *P. diazotrophicus*, *P. saccharophila*, *P. paucimobilis*, and *P. azotocolligans*, have been included in the list of diazotrophs (Eady 1992). In this context, the possibility that nitrogen-fixing pseudomonads could display plant-growth promotion abilities is interesting because the majority of gram-negative PGPR studied so far correspond to either nondiazotrophic pseudomonads or nitrogen-fixing non-*Pseudomonas* strains (Lucy et al. 2004).

Effect of *Pseudomonas* sp. K1 on rice

Beneficial effects of bacterial inoculation were observed on both rice varieties grown in nonsterilized soil, regardless of the inoculant used (Table 1). Plant-growth-promotion traits found in these strains include nitrogen fixation and phytohormone production (Rasul et al. 1998), but the experimental design did not enable us to determine the contribution of each mode of action.

Differences were found between bacterial treatments, as (1) *A. brasilense* Wb3 was more effective than the other treatments when considering specifically plant shoot biomass (straw+grain weight) of Basmati 385, and (2) *A. lipoferum* N4 was less effective than the three other treatments (Table 1). In this study, *A. lipoferum* N4, *A. brasilense* Wb3, and *Zoogloea* sp. Ky1 were selected as efficient nitrogen-fixing PGPR, and it is interesting to find that strain K1 was as effective as these reference strains when considering the effect on yield.

Differences between both rice varieties were also observed when comparing the effect of inoculation. Indeed, *A. lipoferum* N4 had a significant effect on grain yield for Super Basmati, but not for Basmati 385, whereas the effect of *A. brasilense* Wb3 on plant shoot biomass was higher than those of *Zoogloea* sp. Ky1 and strain K1 with Basmati 385, but not with Super Basmati. These results indicate that it may be important to take into consideration the genotypes of both partners when studying the interaction between plant and PGPR, as previously observed in experiments with other types of PGPR, pedoclimatic conditions, and/or crops (Charyulu et al. 1985; Sharifi-Tehrani et al. 1998; Gyaneshwar et al. 2001; Simon et al. 2001). In the case of *Pseudomonas* sp. K1, initially studied for its beneficial effect on kallar grass growth, yield rice increased by 55% for Super Basmati and 93% for Basmati 385 (Table 1), vs only 12% in a preliminary experiment with rice variety NIAB-6 grown in saline soil.

In the present study, a direct correlation between the total number of nitrogen-fixing bacteria and plant data was not observed, but the number of nitrogen-fixing bacteria was high and fluctuated little ($3–8\times10^8$ cells g^{-1} dry root). Nitrogen fixers were also detected in the roots of control plants, indicating the colonization of roots by indigenous populations of diazotrophs. Therefore, if nitrogen fixation by root-associated bacteria is important for rice growth, then both the indigenous microbiota and the PGPR inoculants could have contributed to this microbial function.

Survival of *Pseudomonas* sp. K1 in rice rhizosphere

Survival of *Pseudomonas* strain K1 was studied in the rhizosphere of rice variety Super Basmati, which is the most extensively cultivated aromatic fine grain variety in Pakistan. In the present study, bacterial colonies with morphological characteristics of *Pseudomonas* strain K1

Table 1 Effect of inoculation with *Pseudomonas* sp. K1 or other nitrogen-fixing PGPR on rice varieties Super Basmati and Basmati 385 grown in nonsterile soil

Treatments	Super Basmati			Basmati 385		
	Diazotrophs[a] (log MPN g^{-1} dry root)	Shoot dry weight[b] (g $plant^{-1}$)	Grain yield (g $plant^{-1}$)	Diazotrophs (log MPN g^{-1} dry root)	Shoot dry weight (g $plant^{-1}$)	Grain yield (g $plant^{-1}$)
Control[c]	8.67±0.03 b	14.8±0.8 c	5.1±0.3 c	8.50±0.03 c	13.2±0.6 d	2.7±0.4 b
A. brasilense Wb3	8.86±0.02 a	24.2±2.2 a	7.8±0.7 a	8.58±0.03 b	28.3±1.1 a	5.4±0.5 a
A. lipoferum N4	8.86±0.02 a	19.6±1.6 b	6.7±0.8 b	8.66±0.05 a	16.2±0.7 c	3.2±0.3 b
Pseudomonas sp. K1	8.62±0.05 c	23.6±1.2 a	7.9±0.6 a	8.54±0.11 bc	21.1±1.2 b	5.2±0.4 a
Zoogloea sp. Ky1	8.68±0.03 b	24.8±1.3 a	8.4±0.7 a	8.64±0.05 ab	20.5±0.9 b	5.1±0.5 a

Data are shown as mean ± standard deviation (n=6). In each column, means followed by the same letter are not statistically different at the 5% level (ANOVA followed with Duncan's Multiple Range Test)

[a]Population level of root-associated nitrogen-fixing bacteria, determined by ARA-based MPN

[b]Shoot means straw + grain

[c]Rice was treated with heat-killed cells of *Pseudomonas* sp. K1

were not observed among colonies (9.6×10^8 CFU g^{-1} dry root) on LB plates inoculated with serial dilutions of root macerates.

The specificity of the primer set developed for PCR detection of *Pseudomonas* sp. K1 was verified against strain K1 and ten other bacterial strains in pure culture. The expected 0.9-kb PCR product was obtained only with the template DNA of *Pseudomonas* strain K1 (Fig. 2).

In CCM enrichment cultures originating from root macerates of plants inoculated with *Pseudomonas* sp. K1, cells resembling strain K1, along with *Azospirillum*-like cells, were clearly distinguishable under the microscope on the basis of peculiar cell morphology and motility, but K1-like cells were not seen when control plants were used. PCR products of the expected size (0.9 kb) were obtained from enrichment cultures of up to the 10^{-5} dilution (Fig. 3), whereas no PCR product was found when studying control plants. This indicated the absence of any cross-reacting bacterial diazotrophs having target *rrs* sequences similar to *Pseudomonas* strain K1 in the enrichment cultures.

Using PCR-based MPN, the population of strain K1 was estimated to be 1.7×10^4 cells g^{-1} dry root at 4 weeks after inoculation. Several studies have shown that *Pseudomonas* strains decreased in number in the rhizosphere within weeks or months of inoculation (Mirleau et al. 2000; Moënne-Loccoz and Défago 2004), even when plant-beneficial effects took place (Moënne-Loccoz et al. 1999). In the roots of plants inoculated with strain K1, total population of the diazotrophs, as estimated by ARA-based MPN, was 4.2×10^8 cells g^{-1} dry root at 4 weeks after transplantation (Table 1).

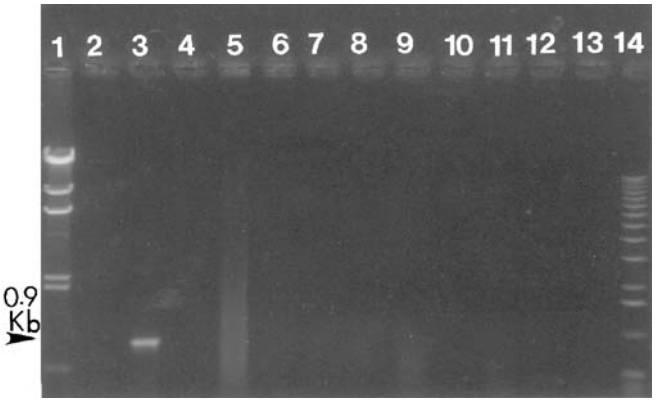

Fig. 2 Amplification of a *rrs* fragment using a primer pair specific for *Pseudomonas* sp. K1. *Lane 1* corresponds to a DNA size marker (Lambda DNA/*Hin*dIII, Promega) and *lane 2* to a blank (control). Bacteria studied included *Pseudomonas* sp. K1 (*lane 3*), *Zoogloea* sp. Ky1 (*lane 4*), *Pseudomonas* sp. 96-51 (*lane 5*), *Enterobacter cloacae* S1 (*lane 6*), *Aeromonas veronii* R8 (*lane 7*), *E. cloacae* SC20 (*lane 8*), *Azospirillum lipoferum* N4 (*lane 9*), *Azospirillum brasilense* Wb3 (*lane 10*), *A. lipoferum* Sp59b (*lane 11*), *A. brasilense* Sp7 (*lane 12*), and *Herbaspirillum seropedicae* Z176 (*lane 13*). *Lane 14* corresponds to another DNA size marker (1 kb DNA ladder, Promega). A PCR product (0.9 kb) was detected only with template DNA from strain K1 (*lane 3*)

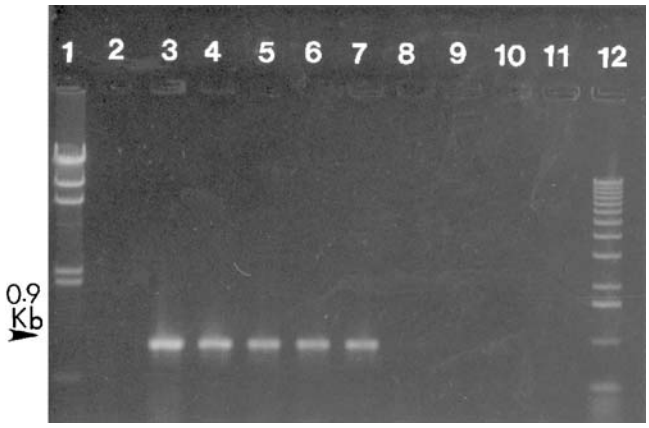

Fig. 3 PCR detection of *Pseudomonas* strain K1 in the inoculated roots of rice variety Super Basmati 4 weeks after transplantation. Template DNA was prepared by cell lysis of CCM enrichment cultures obtained by inoculation with serial dilutions of root macerates. *Lane 1* corresponds to DNA size marker Lambda DNA/*Hin*dIII (Promega), *lane 2* to the control (10^{-1} dilution from rice treated with heat-killed cells), *lanes 3–11* to serial dilutions 10^{-1} to 10^{-9} from rice inoculated with strain K1, and *lane 12* to DNA size marker 1 kb DNA ladder (Promega)

Conclusion

In the present study, strain K1 was reidentified as *Pseudomonas* on the basis of *rrs* (and partial *nifH*) sequence analysis. The beneficial effects of diazotrophic strain K1 on the growth and yield of two rice varieties were comparable to those of established PGPR corresponding to nitrogen-fixing non-*Pseudomonas* bacteria, and strain K1 could be detected by PCR from roots of inoculated rice using a primer designed to specifically target this pseudomonad. These results indicate that nitrogen-fixing *Pseudomonas* strain K1 could be useful as rice inoculant.

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