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Isolation and identification of diazotrophic bacteria from rice, wheat and kallar grass

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Abstract

Three bacterial isolates were obtained from the roots of rice, wheat and kallar grass. These isolates fix nitrogen in pure culture as confirmed by acetylene reduction assay in semi-solid nitrogen-free growth media. Production of growth hormones by these isolates was confirmed by colorimetric method. The isolates from wheat (strain Wb-3) and rice (strain N-4) were identified as *Azospirillum brasilense* and *A. lipoferum*, respectively, on the basis of their morphology, carbon utilization pattern and biochemical tests. The isolate K-1 from kallar grass, previously identified as *Azospirillum brasilense*, was confirmed to belong to a newly named genus *Azoarcus* (Reinhold-Hurek *et al.*, 1993) by using 16S rRNA-targeted oligonucleotide probes and some morphological and physiological characteristics.

Introduction

After the discovery of a very active association between *Azospirillum* species and the root system of various graminaceous plants (Dobereiner and Day, 1976), the use of these bacteria as biofertilizers fixing atmospheric nitrogen and producing phytohormones has attracted the attention of agronomists. Bacteria of the genus *Azospirillum* and other nitrogen-fixing soil bacteria associate with the roots of many plants including important crops such as wheat, rice, maize and sugarcane (Tarrand *et al.*, 1978; Dobereiner *et al.*, 1976; Elmerich *et al.*, 1992; Malik *et al.*, 1994). The organisms now classified in the genus *Azospirillum* were originally described in 1925 as *Spirillum lipoferum* (Beijerinck, 1925). At present five species of the genus *Azospirullum*, namely *A. lipoferum, A. brasilense, A. irakense, A. halopraeferens* and *A. amazonense*, have been recognized (Tarrand *et al.*, 1978;

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Khammas *et al.*, 1989; Reinhold *et al.*, 1987; Dobereiner, 1992). In the rhizosphere of grasses diazotrophic bacteria belonging to at least 11 genera have been found (Elmerich *et al.*, 1992). These include *Azoarcus*, an isolate from kallar grass which grows on saline sodic, alkaline soils having low fertility (Sandhu and Malik, 1975). These diazotrophic Gram-negative rods could not be assigned to any previously described taxon on the basis of phenotypic characteristics (Reinhold *et al.*, 1986). As a result of a polyphasic study, a new genus *Azoarcus* was proposed to accommodate these organisms (Reinhold-Hurek *et al.*, 1993). These bacteria constituted a separate rRNA branch in rRNA superfamily III, which corresponds to the beta subclass of the Proteobacteria. This affiliation of *Azoarcus* to the beta subdivision of the Proteobacteria was further confirmed by reverse transcriptase sequencing of 16S rRNA (Hurek *et al.*, 1993).

In the present study isolation and identification of three diazotrophs from wheat, rice and kallar grass on the basis of morphological and physiological characteristics and 16S rRNA-targeted oligonucleotide probe hybridizations was carried out.

Materials and methods

Isolation of diazotrophs

The roots of wheat were thoroughly washed with sterile water to remove adhering soil. One gram root pieces were homogenized in 10 ml sterile water and serial dilutions were prepared. These dilutions were used to inoculate combined carbon medium (CCM; Rennie, 1981) and nitrogen free malate medium (NFM; Okon *et al.*, 1977) and incubated at 30°C. The vials showing bacterial growth and acetylene reduction activity were used to inoculate plates of the same media to obtain pure colonies. Isolation of the strain N-4 from rice and strain K-1 from kallar grass has been previously described (Bilal *et al.*, 1990; Malik *et al.*, 1994).

Acetylene reduction assay

Acetylene reduction activity of the vials inoculated with bacterial isolates was measured by injecting 10% acetylene after 48 h of growth 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 Okon's malate medium (Okon *et al.*, 1977) enriched with

sucrose 5 g, mannitol 5 g and ammonium chloride 1 g per litre of the medium. Tryptophan 100 mg/L was added as precursor of IAA. After 4 weeks of growth, qualitative estimation of indoleacetic acid was performed by Fe-$HClO_4$ and Fe-H_2SO_4 reagents (Gordon and Weber, 1951). For quantitative estimation of IAA by HPLC, the ethyl acetate extraction method was used (Tien *et al.*, 1979).

Antibiotic resistance pattern

Resistance of the isolates to antibiotics was tested on LB plates.

Physiological and biochemical tests

The 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 oxidation fermentation test was performed as described by Hugh and Leifson (1953). Catalase was identified by the MacFaddin method (1980), using H_2O_2 and pure culture colonies from nutrient agar plates. The isolates found to be non-enterobacters (cytochrome-oxidase positive, oxidation-fermentation negative or positive) and morphologically similar to *Azospirillum* or *Azoarcus* were further tested for carbon source utilization for comparison with these genera.

Dot-blot hybridization

Bacterial cells of the two isolates from rice, i.e. K-1 and *Zoogloea* Ky-1 (Bilal and Malik, 1987) were grown in LB for 24 h at 30°C and centrifuged at 13 000 rpm for 5 min. The cell pellets from 1.5 ml cultures were washed with TE buffer (10 mM Tris. Cl; 1 mM EDTA, pH 8) and 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 kept at –20°C for 30 min. The nucleic acids were then precipitated by centrifugation at 13 000 rpm for 20 min and the pellet was washed with 70% ethanol before drying under vacuum. The nucleic acid pellets were then dissolved in 100 µl TE. The nucleic acids (100 ng) were applied on GeneScreen filters (Dupont) using Hybri.Dot manifold (BRL), immobilized by UV light and hybridized according to Church and Gilbert

(TH15: GACATCGGCCGCTCCAATCGCG) developed against *Azoarcus* sequence (Hurek *et al.*, 1993) was 5'-labelled using phage T4 polynucleotide kinase (BRL) and 10–20 μCi of [γ-^{32}P] adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) according to Maniatis *et al.* (1982).

Results and discussion

Bacterial growth became visible within 48 h in the nitrogen-free semi-solid media inoculated with homogenized roots of wheat. Initially growth was visible as a veil-like pellicle just below the surface of the media. This pellicle formation is considered a characteristic of *Azospirillum*, which finds suitable oxygen concentration just below the surface due to its microaerophilic nature (Tarrand *et al.*, 1978). The vials showing pellicle formation and high ARA activity were used to inoculate NFM, CCM and LB plates to obtain single cell colonies. Pure cultures were again subjected to ARA. All cultures showed nitrogen-fixing ability and produced IAA in liquid media (Table 1). Production of phytohormones by plant-associated bacteria and their beneficial effects on plant growth have been described (Costacurta and Vanderleyden, 1995).

Isolation and identification of Azospirillum brasilense *Wb-3 from wheat*

The isolate Wb-3 obtained from wheat formed subsurface white pellicle in semi-solid NFM and CCM media which spread to the surface of the medium as the culture grew older. The cells were short Gram-negative rods (2–3 μm long) and showed helical shape and motility characteristic of genus *Azospirillum*. The ARA detected in semi-solid NFM and CCM was 1.1 and 1.6 μmol C_2H_4/mg protein per hour, respectively. Among the antibiotics tested, the isolate was found resistant only to ampicillin (Table 2). The isolate could grow on the organic acids malate and lactate, but failed to utilize glucose, sucrose and mannitol as carbon source

Table 1. Acetylene reduction activity (ARA) and indoleacetic acid (IAA) production by the isolates from rice, wheat and kallar grass

Bacterial strains	NFM (μmol C_2H_4/mg proteins per hour)	CCM (μmol C_2H_4/ mg proteins per hour)	IAA (μg/ml)
Azospirillum brasilense (WB-3)	1.1	1.64	16.1
Azospirillum lipoferum (N-4)	0.81	1.89	6.3
Azoarcus sp. K-1	1.6	1.96	10.5

NFM = Nitrogen free malate medium (Okon *et al.*, 1977); CCM = Combined carbon medium (Rennie, 1981)

Table 2. Antibiotic resistance pattern of isolates from rice, wheat and kallar grass

Antibiotics (100 µg/ml)	K-1	N-4	WB-3
Ampicilin	–	+	+
Gentamycin	–	+	–
Kanamycin	–	–	–
Rifampicin	+	+	–
Spectinomycin	–	–	–
Streptomycin	–	+	–
Tetracyclin	–	–	–

(Table 3). Based on the morphological and physiological characteristics (Tables 3 and 4) the strain Wb-3 was identified as *Azospirillum brasilense*, to which it shows maximum resemblance (Tarrand *et al.*, 1978). The isolate accumulated polyhydroxy butryate (PHB) granules which were also identified in azospirilla by other workers (Reinhold *et al.*, 1986).

Isolation and identification of Azospirillum lipoferum *N-4 from rice*

The isolate N-4 obtained from histoplane fraction of roots formed off-white colonies on solid LB and CCM media. In semi-solid NFM medium it formed fine subsurface white pellicle within 24 h which gradually spread to the surface. The colour of the semi-solid NFM medium turned blue due to the rise in pH of the medium. Examination of the wet mounts of the culture in NFM and CCM semi-solid media under a phase contrast microscope showed plump, highly motile

Table 3. Physiological characterization of the isolates from rice, wheat and kallar grass

Characteristics	*A. halopraeferens*[1]	*A. irakense*[2]	*A. lipoferum*[3]	*A. brasilense*[3]	WB-3	N-4	K-1
Catalase	+	+	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+	+	+
Biotin requirement	+		+	–	–	+	–
Acidification of peptone–based glucose medium	–	–	+	–	–	+	–
Sole carbon source for growth in N-free semi-solid medium							
Glucose	–	+	+	–	–	+	–
Mannitol	+	+	+	–	–	+	–
Sucrose	–	+	–	–	–	–	–
Maltose	–	+	–	–	–	–	–
Malate	+	+	+	+	+	+	+

[1] Reinhold *et al.*, 1987; [2] Khammas *et al.*, 1989; [3] Tarrand *et al.*, 1978.

Table 4. Biochemical characterization of the isolates from rice, wheat and kallar grass

Biochemical tests	*A. irakense*[1]	*A. brasilense*[2]	*A. lipoferum*[2]	*A. halo-praeferens*[3]	WB-3	N-4	K-1
ONPG	–	+	+	+	+	–	+
Sodium citrate	–	+	+	–	+	+	–
Sodium malonate	+	+	+	+	–	+	–
Lysine decarboxylase	+	+	+	+	+	+	–
Arginine dihydrolase	–	+	+	–	+	+	–
Ornithine decarboxylase	+	+	+	–	+	+	+
H_2S production	–	–	–	–	–	–	–
Urea hydrolysis	+	+	+	–	+	+	–
Tryptophan deaminase	–	–	–	+	–	–	–
Indole	–	–	–	–	–	–	–
Acetoin (VP)	–	–	–	–	–	–	–
Gelatine hydrolysis	–	–	–	–	–	–	+
Acid from glucose	–	–	+	–	–	+	–
Nitrate reduction	+	+	+	+	+	+	+
Acid from maltose	–	–	–	–	–	–	–
Acid from sucrose	–	–	–	–	–	–	–
Acid from mannitol	–	–	+	–	–	+	–
Acid from arabinose	–	–	–	–	–	–	–
Acid from rhamnose	–	–	–	–	–	–	–
Acid from sorbitol	–	–	–	–	–	–	–
Acid from inositol	–	–	–	–	–	–	–

[1] Khammas *et al.*, 1989; [2] Tarrand *et al.*, 1978; [3] Reinhold *et al.*, 1987.

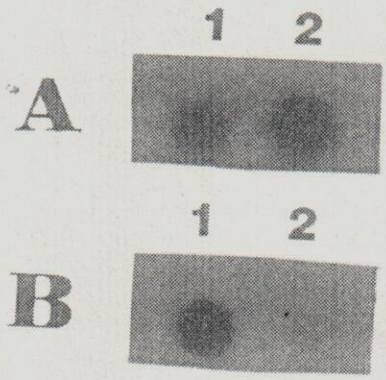

Figure 1. Dot–blot hybridization of 16S rRNA-targeted oligonucleotide probes. **A**: Hybridization with the universal eubacterial probe (p1115), indicating presence of rRNA target on the blot. **B**: Hybridization with oligonucleotide probe specific for *Azoarcus* (TH15; Hurek *et al.*, 1993). This probe strongly hybridized with rRNA of *Azoarcus* K-1 but did not hybridize with the rRNA of *Zoogloea* strain Ky-1. **1** = rRNA of *Azoarcus* K-1; **2** = rRNA of *Zoogloea* Ky-1.

helical cells resembling *Azospirillum*. The cells range in length from about 5 µm in CCM and NFM to 30 µm in LB liquid medium. This variability in cell size has also been noted in other strains of *A. lipoferum* (Krieg and Dobereiner, 1984).

Among the antibiotics tested in the present study, strain N-4 showed resistance to ampicillin, gentamycin, rifampicin and streptomycin, while it was sensitive to kanamycin, spectinomycin and tetracyclin (Table 2). The resistance of strain N-4 to antibiotics gentamycin, rifampicin and streptomycin can prove useful in differentiation of this *Azospirillum* strain from *A. brasilense* strain Wb-3 in ecological studies. The isolate could grow on glucose, mannitol, lactate and malate, while it could not utilize sucrose, maltose, etc. (Table 3) and required biotin for its growth. The morphological characteristics and physiological tests (Tables 3 and 4) showed that this strain belongs to *Azospirillum lipoferum* (Tarrand *et al.*, 1978). Isolation of *A. lipoferum* has often been reported from maize, while *A. brasilense* has been commonly obtained from wheat and rice (Baldani and Dobereiner, 1980).

Isolation and identification of Azoarcus *K-1 from kallar grass*

The isolate K-1 from kallar grass forms thin subsurface pellicle in semi-solid NFM and CCM media. On LB plates it formed off-white colonies. The organism is an aerobic, Gram-negative, highly motile straight rod (cell width 0.5–0.8 μm; length 2–4 μm). In NFM and CCM the strain was able to reduce acetylene at the rate of 1.6 and 1.96 μmol C_2H_4/mg protein per hour, respectively. The isolate could grow well on salts of organic acids such as lactate and malate, but could not utilize carbohydrates such as glucose, sucrose and mannitol, as carbon source. The morphological characteristics and carbon utilization pattern of the isolate K-1 resembled *Azoarcus* described by Reinhold-Hurek *et al.* (1993). Previously, due to the non-availability of the description of *Azoarcus* at that time, the strain K-1 was classified as *Azospirillum brasilense* mainly on the basis of its ability to fix nitrogen and carbon source utilization pattern (Bilal *et al.*, 1990). The genus *Azoarcus* was first isolated from kallar grass, and later its association with kallar grass was confirmed by fluorescent antibodies (Reinhold *et al.*, 1987) and immunogold electron microscopy (Hurek *et al.*, 1991). The isolate K-1 was further confirmed as *Azoarcus* by using a 16S rRNA-targeted oligonucleotide probe (Hurek *et al.*, 1993) which hybridized strongly with RNA of K-1 on a dot-blot but did not show signals with the RNA of *Zoogloea* strain Ky-1 (Figure 1). *Zoogloea* strain Ky-1 is a diazotrophic bacterium which was isolated from kallar grass by Bilal and Malik (1987).

Three isolates discussed here can fix nitrogen and produce phytohormones, and have great potential for use as biofertilizers for the important cereal crops like wheat and rice.

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