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Characterization of *Azospirillum* and related diazotrophs associated with roots of plants growing in saline soils

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Diazotrophs, especially of genus *Azospirillum* were readily isolated from roots of many plants using semi-solid nitrogen free malate medium (NFM). These isolates formed fine, white sub-surface pellicle in nitrogen-free malate medium within 24 h, which gradually moved to the surface, and exhibited high acetylene reduction rates. Using selected cultural and biochemical tests, most of the isolates were identified as *Azospirillum brasilense*. Four isolates from Kallar grass root surface and one isolate from *Atriplex* root interior formed phenotypically a homogenous group. It shared many characteristics with the species of genus *Azospirillum* except shape. All the biochemical tests performed, categorized them with *A. brasilense*. However, the shape and the protein profile on SDS polyacrylamide gel electrophoretograms suggested that the group of these five isolates is clearly distinct and differs widely from all the type strains, belonging to various genera.

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Nitrogen fixation associated with the roots of grasses has over recent years been recognized as an important factor in the maintenance of soil fertility (Dart 1986). Such studies received an impetus with the report of the occurrence of *Azospirillum* in the rhizosphere of various grasses and its close association with the roots (Dobereiner *et al.* 1976; Weier *et al.* 1981; Reingold *et al.* 1986). Selective media for isolating diazotrophs belonging to this genus have been described (Dobereiner *et al.* 1976; Cacares 1982) and used by many workers (Baldani *et al.* 1986; Sundaram *et al.* 1988). The ease of recognition of *Azospirillum* by its characteristic sub-surface pellicle in the semi-solid agar medium has led many workers to conclude its preponderance in the rhizosphere and on root surfaces (Hegazi *et al.* 1979; Rao & Venkateswarlu 1985). However close investigation of such pellicles may reveal presence of microorganisms other than *Azospirillum* (R. Bilal, unpublished data).

Associative nitrogen fixation has previously been reported in *Leptochloa fusca*, locally known as Kallar grass (Malik *et al.* 1981; 1982). It grows luxuriantly in highly saline low-fertility soils and possesses the C-4 photosynthetic pathway (Zafar & Malik 1984). Detailed investigation into the rhizosphere of this grass has previously been carried out (Zafar *et al.* 1986), which resulted in the elucidation of seasonal variation in the nitrogenase activity of excised roots as determined by acetylene reduction assay and enumeration of diazotrophs in the rhizoplane and histoplane fractions of the root. A number of diazotrophic bacteria have been isolated from the roots of this grass and some of them identified (Reinhold *et al.* 1986; Bilal & Malik, 1987; Zafar *et al.* 1987).

In this paper, we report the isolation and identification of a group of organisms which resemble *A. brasilense* in many characteristics but differ in shape and protein electrophoretic behaviour.

Materials and Methods

Reference Strains

The bacterial cultures used as reference were: *Azospirillum brasilense* JM82; *A.*

Il est apparu facile d'isoler des diazotrophes, principalement du genre *Azospirillum* à partir de racines de nombreuses plantes et utilisant un milieu malate semi-solide exempt d'azote (NFM). Ces isolats forment une pellicule fine et blanche sous la surface dans le milieu malate exempt d'azote, endéans les 24 h. Ces pellicules se déplacent progressivement vers la surface et exhibent des vitesses élevées de production d'acétylène. L'emploi de tests de culture sélective ou biochimique, a permis d'identifier la plupart des isolats comme *Azospirillum brasilense*. Quatre isolats à partir de la surface des racines d'herbe Kallar et un isolat à partir de l'intérieur d'une racine d'*Atriplex* forment un groupe homogène sur le plan phénotypique. Celui-ci partage de nombreuses caractéristiques avec les espèces du genre *Azospirillum* excepté la forme. Tous les tests biochimiques exécutés les rangent avec *A. brasilense*. Toutefois, leur forme ainsi que le profil protéique sur un électrophérogramme sur gel de polyacrylamide-SDS suggèrent que le groupe de cinq isolats est nettement distinct et diffère largement de toutes les souches types, appartenant à de nombreux genres.

brasilense CdJa, ATCC 29710 (from D.H. Hubbell & S. Albrecht, Gainesville, Fla.); *A. brasilense* sp7 ATCC 29145; *Herbaspirillum seropedicae* ZM-176; *A lipoferum* R-1; *A. amazonense* Y-1; *A. amazonense* A-14; *Acetobacter nitrocaptans* PAL5 (from J. Doberiener, Brazil); *Azotobacter vinelandii* (DSM 2289); *Enterobacter cloacae* DSM 30075; *Pseudomonas* sp. DS8 and DK3 (local) *Azotobacter* spp. K5, K9 and K12 (isolated locally from rhizosphere of Kallar grass).

Culture Media

The nitrogen-free malate medium as described by Dobereiner *et al.* (1976) was used for enrichment and for nitrogenase activity by acetylene reduction assay. Potato/dextrose/agar (PDA) and nutrient agar (Difco) were used for purity check (Baldani & Dobereiner 1980). For semi-solid medium 2.0 g Bacto-agar/l and for solid medium 20 g agar/l was added. Five ml of autoclaved semi-solid medium was dispensed into 17 ml glass vials and were inoculated with the root fractions.

Isolation Procedure

The plant rhizospheres were collected from Bio-Saline Research Sub Station of Nuclear Institute for Agriculture and Biology (NIAB) near Lahore. The soil of the area is highly saline-sodic (pH 8 to 9.5; electrical conductivity 10 to 40 mS/cm; total organic N is>0.02% and organic C is 0.2%). Excess soil was removed by placing the rhizosphere under a gentle stream of water. When the roots were free of adhering soil, these were thoroughly washed in several changes of sterile distilled water. For the isolation of bacteria from root interior, the roots were immersed in 5% NaOCl for 30 min, followed by washing in several changes of sterile distilled water.

The roots were excised to 2 cm small pieces and were inoculated in sterile semi-solid nitrogen-free malate medium. After 3 to 4 days of incubation at 30°C, a loopful of bacterial growth was transferred into a second vial of nitrogen-free malate medium and was incubated further. The vials were observed daily for alkali production and the formation of fine sub-surface pellicle. All the vials showing signs of growth with visible blue colour in nitrogen-free malate medium were assayed for the presence of nitrogenase activity.

The screw caps were replaced by serum stoppers and 10% (v/v) acetylene was added. The cultures were incubated at 30°C for 1 h, 100 µl of the gas sample was removed and analysed for ethylene by gas chromatography (Bilal & Malik 1987). For isolation of diazotrophs, the cultures yielding more than 100 nmol ethylene/h/vial were streaked on nitrogen-free malate medium plates supplemented with 0.01% yeast extract. Individual colonies were picked and reinoculated on semi-solid nitrogen-free medium for determining the nitrogenase activity. Various purified cultures giving positive acetylene reduction were retained, after being checked for purity on potato-dextrose or nutrient agar plates.

Characterization and Identification of N_2-fixing Isolates

Bacteria were characterized by the formation of a sub-surface pellicle on nitrogen-free malate medium and production of alkali which changed the colour of the medium from green to deep blue. Isolates were streaked on nutrient agar and potato-dextrose agar plates to check the purity.

Morphological observations were made by phase contrast as well as by electron microscopy. Electron microscopy preparations were negatively stained by 2% (w/v) phosphotungstic acid before observing in a transmission electron microscope. Sole carbon source utilization media, semi-solid glucose

medium, peptone based glucose broth as well as biotin requirement medium was prepared as described by Tarrand *et al.* (1978).

Maintenance of Cultures

All the isolates were maintained on nutrient agar slants under paraffin oil. Each culture was re-streaked on nutrient agar or nutrient agar plates and tested for nitrogenase activity on semi-solid nitrogen-free malate medium before each study.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Reference strains and the isolates were grown to stationary phase in 5 ml of nutrient broth at 30°C with shaking. Cultures were centrifuged at 1000 *g* for 10 to 15 min. The cell pellet was processed for total soluble protein profile analyses by employing the method described by Shivakumar *et al.* (1986). Samples were subjected to one dimensional SDS-PAGE using 10% (w/v) resolving and 4.5% (w/v) stacking gels. A 10 µl sample containing 50 µg of protein was loaded per well on the gels. After electrophoresis the gels were stained overnight in 25% (v/v) ethanol and 5% (v/v) glacial acetic acid containing 0.025% (w/v) Coomassie Brilliant Blue R-250, destained for 2 to 3 h in 40% (v/v) absolute ethanol and then 5% (v/v) glacial acetic acid. The gels were left in methanol-glycerol-water (55:2:43 by volume) for 12 to 24 h. Coomassie Brilliant Blue stained protein patterns in the gels were photographed using a Fortepan 200 black and white film.

Results and Discussion

Nitrogen-fixing bacteria capable of making fine sub-surface pellicles in semi-solid nitrogen-free malate medium were readily isolated from the rhizoplane and histoplane of some plants growing in saline sodic soils of Lahore, Pakistan (Table 1). The pure cultures exhibiting higher rates of N_2-fixation (more than 100 nmol ethylene/h/culture vial) were identified using morphological and biochemical characteristics. We report here the characterization of *Azospirillum* and a new organism that resembles *Azospirillum* closely from the Kallar grass root surface, and *Atriplex* root interior.

The putative *Azospirillum* populations were regularly detected as thin sub-

Table 1. List of diazotrophs isolated from different root fractions using Malate (NFM) medium.

Plant Origin	Root Fraction	Isolate Code	
Leptochloa fusca (Kallar grass)	RP*	KR5†, KR6, KR7, KR8, KR9, KR10, KR11, KR12, KR13, KR14, KR1, KR2, KR3, KR4,	17
	HP**	KH1, KY1	
Atriplex amnicola	RP	AR6, AR7, AR8, AR9, AR10, AR11, AR12, AR13, AR15	11
	HP	AH1, AH2	
Cynodon dactylon	RP	CR1, CR2, CR3, CR4, CR5, CR6	13
	HP	CH1, CH2, CH3, CH4, CH5, CH6, CH7	
Triticum aestivum	RP	TR1†, TR2†	2
TOTAL			43

* = RP rhizoplane or root surface. Roots were washed excessively with tap water and then with sterile distilled water. 1cm long root pieces were inoculated in semi solid nitrogen-free malate medium and incubated at 30°C.

** = HP Histoplane or root interior. The washed roots were immersed in NaOCl for 30 min and rinsed repeatedly in sterile distilled water before inoculating as above.

† = Isolates obtained on nitrogen-free malate medium supplemented with NaCl 2.5g/l.

surface pellicles when washed or surface sterilized root pieces were placed in vials containing semi-solid nitrogen-free malate medium. Such pellicles were streaked on nitrogen-free malate medium agar supplemented with yeast extract. All the individual colony types appearing on agar plates were tested for acetylene reducing activity. A total of 43 bacterial strains which exhibited more than 100 nmol ethylene/h/culture vial were stored on nutrient agar slants. Later, 13 isolates were selected for further study based on their ability to form fine sub-surface pellicles, higher rates of nitrogenase activity and spinning motility. These strains were designated as AH1, KH1, CH1-7 and KR1-4. [The first letter of the strain code refers to plant origin (A = *Atriplex*; K = Kallar grass and C = *Cynodon*; T = *Triticum aestivum*) where as the second letter refers to the rhizosphere fraction from where it was isolated (R = Rhizoplane and H = Histoplane)].

All 13 strains were compared with the standard *Azospirillum* strains for the growth characteristic in nitrogen-free malate medium, where they form fine sub-surface pellicles, and as growth continues it turns white like paper. This growth behaviour is well defined for *Azospirillum* (personal communication, Johanna Dobereiner). The growth in nitrogen-free malate medium was always accompanied by alkali production and high rates of nitrogenase activity (more than 100 nmol ethylene/h/culture vial). Phase contrast microscopic examination of wet mount of the actively growing cultures showed helical cells resembling *Azospirillum* or straight rods, but all were actively motile. Almost always some cells in the field exhibited typical spinning motility. Morphologically, *Azospirillum* can be differentiated from all other organisms because of their helical shape from which it derives it name. All the isolated diazotrophs included in the present study were helical in shape except isolate KR1-4 and AH1. The cells of these strains were actively motile and showed a variety of motions, but in fixed preparation these were found to be straight rods. The cells from semi-solid nitrogen-free malate medium varied in length from few µm to 30 µm in length and 2 to 3 µm in width. The cells were always longer when organic acids were used as substrate.

A high percentage of the isolates accumulated poly-β-hydrooxybutyrate (PHB), as observed for *Azospirillum* strains by other workers (Becking 1981; Reinhold *et al.* 1986). However, the strains KR1-4 and AH1 did not accumulate PHB.

The majority of the isolates when grown on nutrient agar medium formed light pink colonies, however the pigment was more characteristic and dark pink when the nutrient broth grown cells were pelleted by centrifugation. Some *Azospirillum* strains formed pink colonies, e.g., *A. brasilense* Sp7 ATCC 29145 and one strain, Cd from California, produced bright red colonies, although some strains isolated from Israel have been reported to be yellow (Nur *et al.* 1980).

The morphological and physiological characterization of these isolates is summarized in Table 2. None of the isolates used glucose, sucrose and mannitol as sole carbon source, but grew on lactate, malate and fructose. The biochemical characteristics of our isolates show that they resemble closely *A. brasilense* which differs from *A. lipoferum* in its biotin requirement and ability to utilize glucose as a carbon source (Tarrand *et al.* 1978). However, our isolates produce acid in peptone-based glucose medium unlike *A. brasilense*, *A. amazonense* and like *A. lipoferum*. All the isolates, with the exception of AH1, exhibited weak reaction in oxidation fermentation tubes with fructose as C source. However, no gas was produced even after 10 days incubation. Most of the cultural, physiological and morphological characteristics of the KR group isolate and AH1 matched those of reference strains of *A. brasilense* but were straight rods.

Table 2. Morphological and physiological characterization of diazotrophs isolated from the roots of plants growing in saline soils.

Characteristics	Test Group no. 1	2	3	4	5
Cell shape	r	h	h	r	r
Acid from peptone in glucose based medium	+	+	+	+	–
Acid from glucose	Alk	Alk	Alk	–	–
Oxidation Fermentation	A	–	A	w^+	w^+

Test Group no. 1 = strain KH1; no. 2 = AH1; no. 3 = CH1, CH2, CH3, CH4, CH5, CH6, CH7; no. 4 = KR1; no. 5 = KR2, KR3, KR4.
All the strains were positive for motility, pigment, and utilization of lactate, malate and fructose as sole carbon source while negative for biotin requirement succinic acid, mannitol and sucrose utilization as sole carbon source.
A = acid; Alk = alkaline; h = helical; r = rod; + = positive; – = negative; w^+ = weak positive.

Whole cell protein pattern comparison by appropriate choice of separation method can discriminate microorganisms at the genus, species or strain level as required (Jackman 1988). Sundaram *et al.* (1988) have characterized various *Azospirillum* isolates using a one-dimensional SDS-PAGE method.

We used a one-dimensional SDS-PAGE for total soluble bacterial proteins to compare our isolates with various *Azospirillum* species. Comparison with *Azospirillum lipoferum*, *A. brasilense* and *A. amazonense* strains showed that all our strains except KR group resembled closely *A. brasilense* strain CdJA whereas these differed slightly from the *A. brasilense* strain Sp-7 in their low molecular weight proteins, and from *A. amazonense* in several low and high molecular weight proteins. The protein profile pattern of KR group was homogenous and entirely different from rest of the local isolates as well as three standard *Azospirillum* strains that were used (Fig. 1).

In order to ascertain any taxonomic affiliation of KR group, their protein profiles were compared with various diazotrophic type strains belonging to seven different genera (Fig. 2). The electrophoretic pattern of total soluble proteins of one-dimensional SDS-PAGE demonstrated that the KR group diazotrophs are entirely different from all the strains belonging to various genera that were included in the study.

Our studies provide evidence that in addition to the widespread occurrence of *Azospirillum* (Dobereiner *et al.* 1976) there are many new diazotrophic organisms that are present in association with the grass roots. The occurrence of *Azospirillum* is much more confined to niches closer to the root surface, as is evidenced by the high number of isolates from surface, sterilized roots (Tables 1 & 2). In addition, our studies further suggest that there might be many more organisms which are sufficiently similar to *Azospirillum* in some aspects yet very different in other respects to be categorized with *Azospirillum*. This emphasizes the need to devise media for isolation of other unexplored organisms and methods for their characterization, in order to understand the true diversity that exists among the diazotrophs.

However, the problem of taxonomy of KR group organisms was confused because of their morphological and biochemical similarity and a wide variation in protein profile with *Azospirillum* and also from other respresentative genera. Further comparison with other diazotrophic and non-diazotrophic bacteria might reveal a possible affiliation with any genus.

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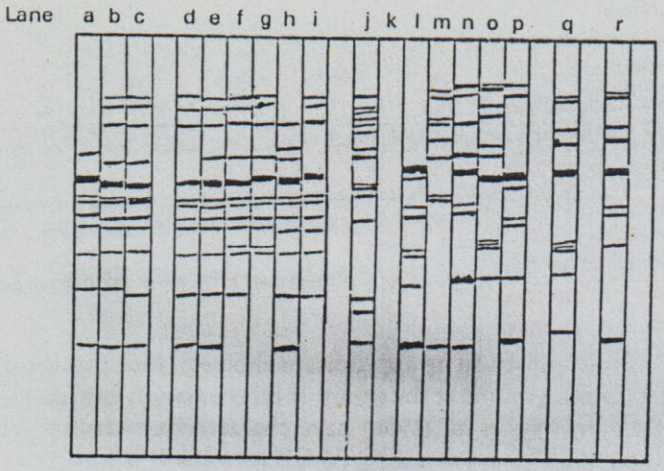

Figure 1. SDS-PAGE pattern of total soluble protein of different bacterial strains (a) KH1; (b) CH4; (c) CH7; (d) CH1; (e) CH2; (f) CH3; (g) CH5; (h) AH1; (i) KR1; (j) TR1; (k) TR2; (l) KR5; (m) *Herbaspirillum seropedica* ZM 176; (n) *A. brasilense* CdJA; (o) *A. lipoferum* R1; (p) *A. amazonense* A14; (q) *A. brasilense* Sp7; (r) *A. brasilense* JM82.

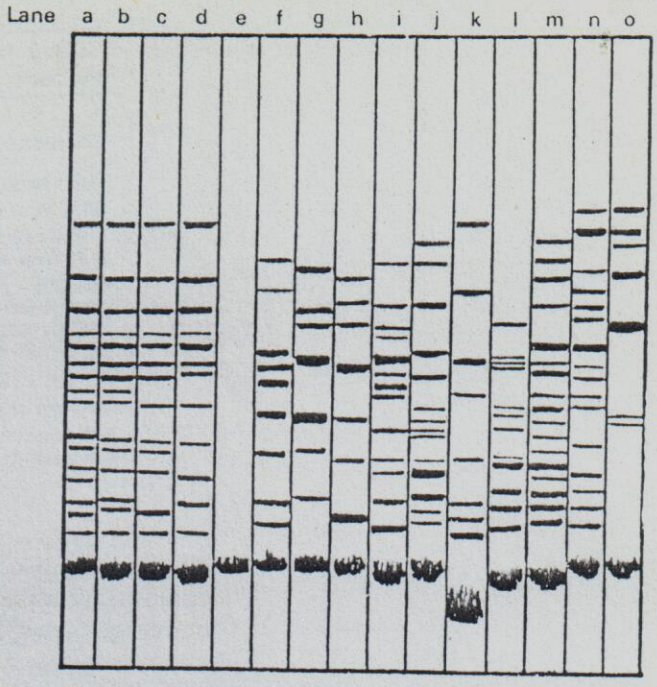

Figure 2. SDS-PAGE pattern of total soluble proteins of different bacterial strains. Lane (a) KR1; (b) KR2; (c) KR3; (d) KR4; (e) *Azotobacter vinelandii* DSM 2289; (f) *E. cloacae* DSM 30075 (g) *Pseudomonas* sp. DS8; (h) *Pseudomonas* sp. DK3; (i) *A. brasilense* JM 82; (j) *A. brasilense* Sp7; (k) *A. amazonense* A14; (l) *A. lipoferum* R1; (m) *A. brasilense* CdJA ATCC 29710; (n) KR5; (o) *Herbaspirillum seropedica* ZM176.

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