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Klebsiella sp. *NIAB-I:* A new diazotroph, associated with roots of kallar grass from saline sodic soils

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Abstract

A nitrogen fixing organism containing a plasmid has been isolated from the rhizosphere fraction of *Leptochloa fusca* (L) Kunth (kallar grass) growing on saline soils in the Punjab area. This bacterium can grow aerobically in a medium containing 1 *M* NaCl and can fix nitrogen efficiently under microaerobic conditions on semi-solid medium with glucose or sucrose as a carbon source. Maximum N_2-fixation in batch cultures occurred with 100 m*M* NaCl at pH 8.0 and 35°C. DNA hybridization and analysis of the protein pattern were carried out to establish its taxonomic position. On the basis of protein electrophoretic pattern, physiological characteristics, DNA relatedness, and better growth in the presence of high NaCl concentration, we regard this strain as a new species of *Klebsiella*.

Introduction

Diazotrophs are ubiquitous. Several genera of dinitrogen fixing bacteria are reported to be associated with the roots, stems and leaves of various plants (Ladha *et al.*, 1983; Dart, 1986; Bilal and Malik, 1987). Kallar grass (*Leptochloa fusca* L. Kunth) a salt-tolerant grass, is a primary colonizer of salt-affected lands in Pakistan (Sandu and Malik, 1975). Nitrogenase activity (acetylene reduction) associated with the roots of this grass has been reported (Malik *et al.*, 1980, 1982; Zafar 1985) and the grass roots are infected by nitrogen fixing bacteria (Zafar *et al.*, 1986, Reinhold *et al.*, 1987). One of these isolates, NIAB-I, resembled *Klebsiella* in morphology, biochemical characteristics and DNA base composition (Zafar *et al.*, 1987). This paper describes further characteristics of this isolate (NIAB-I) and its comparison with the known N_2-fixing wild type *Klebsiella pneumoniae* strain M5A1. Furthermore, on the basis of physiological behaviour, protein electrophoretic pattern and DNA—DNA hybridization we propose this strain as a new species of *Klebsiella*.

Materials and methods

Bacterial strains

Two wild type *Klebsiella* strains M5A1 and NIAB-I, were used as experimental organisms. M5A1, known as *Klebsiella pneumoniae* NCIB 12204 (identified in European Collections as *Klebsiella oxytoca*) was supplied by Dr. David Lowe, AFRC Unit of N_2 – Fixation, University of Sussex, England. NIAB-I strain was isolated form the roots of kallar grass (Zafar, 1985). Both organisms were kept on nutrient agar slants and subcultured monthly.

Media and growth conditions

The minimal medium (MM) described by Yoch and Pengra (1966) was used with slight modification, containing ($g.l^{-1}$) Na_2HPO_4, 6.25; KH_2PO_4, 0.75; $MgSO_4.7H_2O$, 0.2; Na_2MoO_4, 0.01; $FeSO_4.7H_2O$, 0.01, glucose, 20.0. pH and sodium chloride concentration were adjusted as required. This

medium was routinely prepared by autoclaving phosphates and the remaining ingredients separately in distilled water. $FeSO_4.7H_2O$ was added after filter-sterilization.

Organisms were grown at 35°C under an atmosphere of either N_2 or N_2 + air in a 6 l fermenter (Eyla laboratory fermenter. Model No. M-160 Tokyo, Japan). The pH of the growth medium was stabilized with 2*N* NaOH added by an automatic bench pH controller (Model No. FC-1 Eyla, Tokyo, Japan). The 6 l fermenter was inoculated with 300 ml of a culture grown aerobically for 24 h on minimal medium with 300 mg ammonium sulphate per litre.

Preparation of cell extracts for protein analysis

Whole cells protein samples, each from a 5 ml overnight culture of a bacterial strain, were prepared by SDS, protease inhibitor (phenylmethylsulphonyl fluoride) and lysozyme treatment as described earlier (Shavikumar *et al.*, 1986). SDS polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970).

Molecular studies on bacterial strains M5A1 and NIAB-I

Labelled DNA was extracted from cells which had been grown in a medium containing 1mCi of [^{3}H] thymidine per 300 ml of Luria broth. The extraction and purification of labelled DNA from M5A1 strain and unlabelled DNA from NIAB-I was performed according to the method of Marmour (1961). The concentration and purity of DNA was determined by measuring the absorbance at 260 nm and 280 nm.

Hybridization procedure

The denaturation of unlabelled DNA was carried out by boiling the DNA (10 μg ml^{-1} of 0.1 × Sodium Saline Citrate Buffer; SSC) three times at 95°C in closed pyrex tubes in a water bath for 2 min. SSC contains 0.15*M* NaCl and 0.15*M* sodium citrate (pH 7.0). The buffers 0.1 × SSC and 2 × SSC (see below) contain one tenth of, and twice, these concentrations, respectively. After denaturation, the tubes were placed in an ethanol ice bath for 10–15 min. The procedure for DNA–DNA hybridization followed that of McConaughy *et al.*, (1969) with slight modifications. The denatured suspensions were poured on pre-soaked nitro-cellulose filters and allowed to flow under gravity. After 24 to 32 h the filters were removed, placed in sterile Petri-dishes (glass) and baked for 2 h at 80°C in a vacuum oven (this denaturation enables the DNA to stick to the filters). The immobilized DNA filters were then placed in vials with different concentrations of labelled DNA (in 2 × SSC) and incubated for 18–24 h at 67°C. The hybridization reactions were terminated by washing the filters with 2 × SSC solution (thrice) and then incubated in another vial containing DNAase for 1 h at 37°C.

DNA filters were again washed three times with cold (2 × SSC), dried in a vacuum oven at 70°C and placed in 10 ml of scintillation fluid. The amount of input DNA bound to the immobilized DNA was estimated by measuring the radioactivity of the nitrocellulose filter in a liquid scintillation counter (Packed Tricarb 4388). The percent hybrids was determined for each sample relative to the control.

Nitrogenase assay and protein estimation

The nitrogenase activity (acetylene reduction) of 5 ml samples from batch cultures was measured in

Table 1. Effect of NaCl and pH on the N_2ase activity of *Klebsiella* strains M5A1 and NIAB-I in batch cultures at 35°C

Klebsiella strain	pH	Conc. of NaCl (m*M*)	Max. N_2ase activity at (h)[b]	Max. N_2ase activity (nmoles $C_2H_4^{-1}$ min^{-1} mg protein^{-1} ml^{-1})
M5A1	6	100	32	180
	7[a]	100	24	290
	7	34.2	26	278
	7	100	24	316
	7	250	38	140
	8	100	44	270
NIAB-I	7	100	30	233
	8	34.2	26	320
	8	100	28	418
	8	250	38	270
	8[a]	100	24	350
9	10	34	250	
	0			

[a] Strains were grown under anaerobic N_2-fixing conditions.
[b] Maximum N_2-ase activity observed after inoculation.

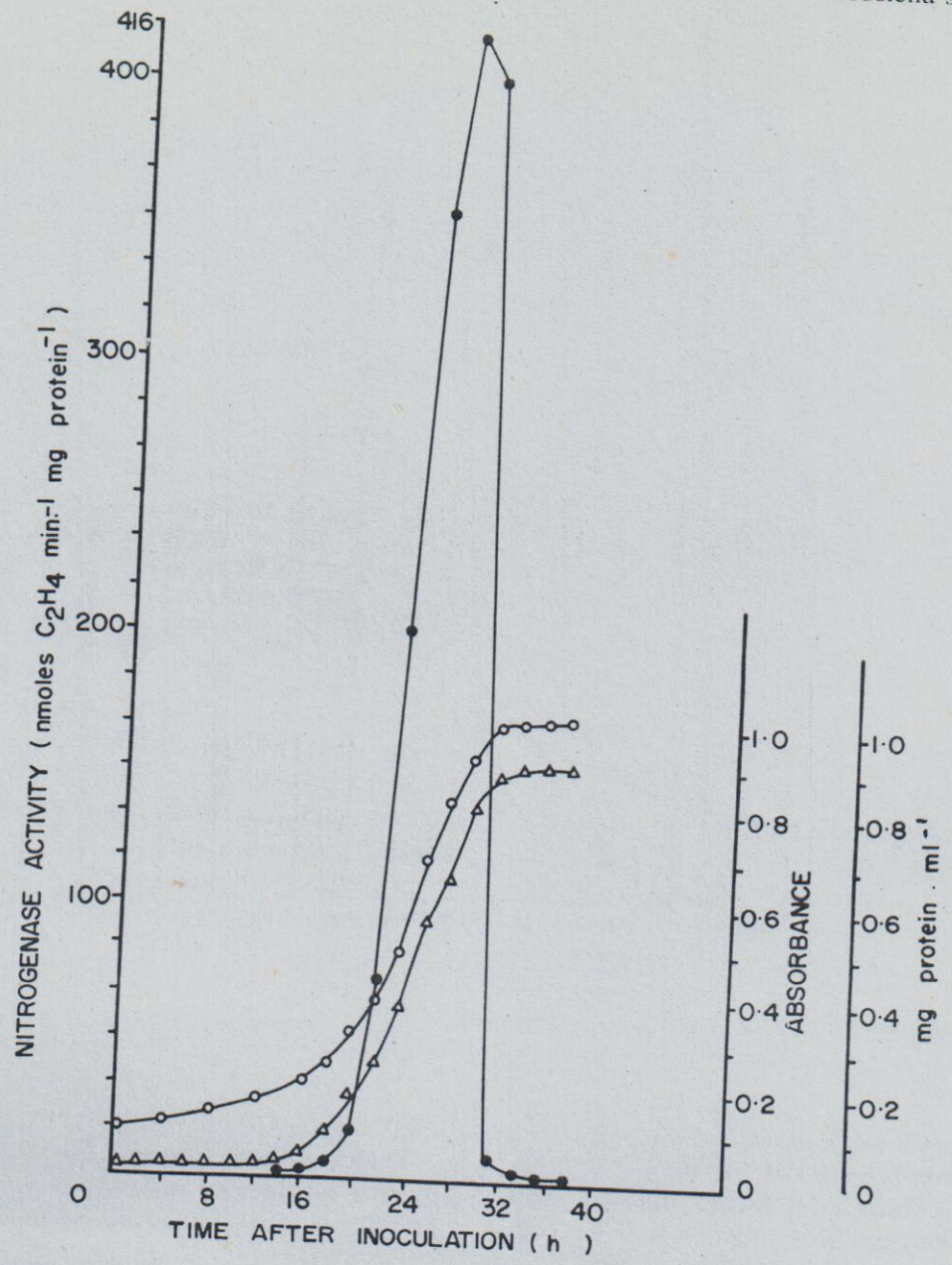

Fig. 1. Nitrogenase activity by *Klebsiella* sp. NIAB-I during growth on glucose at pH 8.0, with 100 m*M* NaCl and temperature 35°C.

13 ml serum vials as described by Hill (1976). Total protein of whole cells was determined by the method of Gowa (1953).

Results and discussion

The comparison of the nitrogenase activities of two wild type *Klebsiella* strains M5A1 and NIAB-I in batch cultures at various pH values and NaCl concentrations is summarized in Table 1. *Klebsiella* strains are facultative anaerobes and N_2-fixation in these organisms is mainly associated with anaerobic metabolic processes but our results provide evidence that a gas mixture (3% air and 97% N_2) and 100 m*M* NaCl in the growth medium improves the physiological state of the cells and nitrogen fixing ability of the two experimental organisms. This is an indication that the two organisms (M5A1 and NIAB-I) grow and fix N_2 actively in the

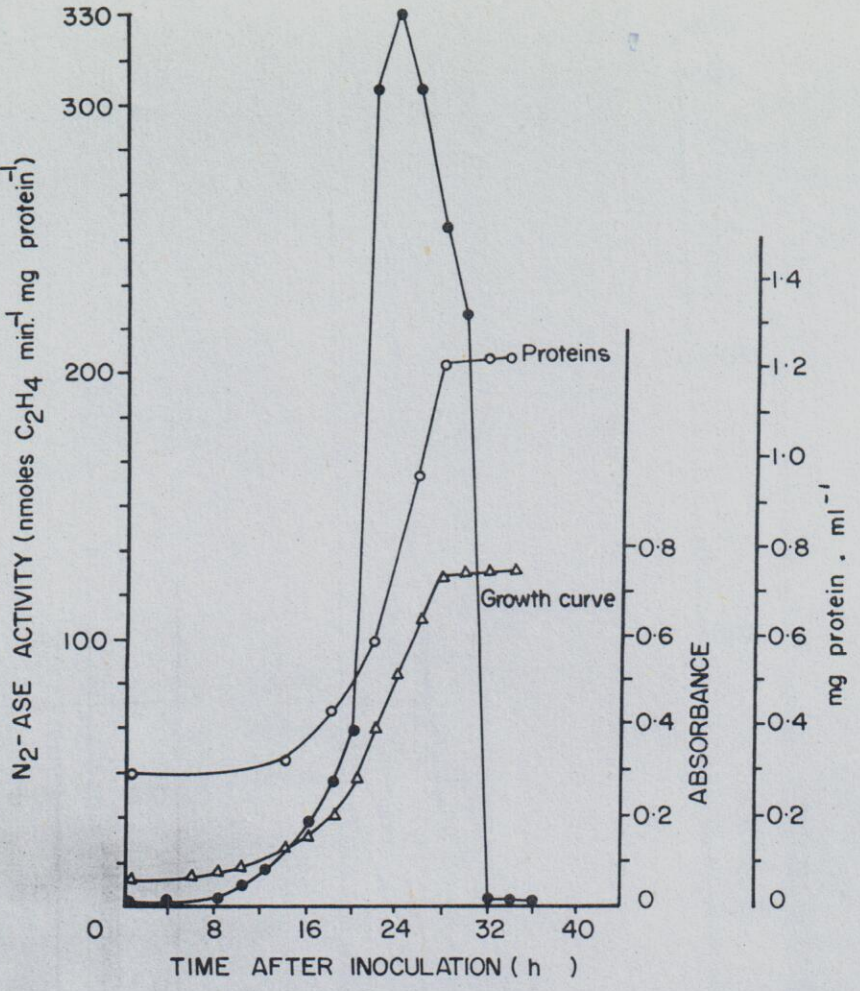

Fig. 2. Nitrogenase activity by *Klebsiella pneumoniae* M5A1 during growth on glucose at pH 7.0 with 100 m*M* NaCl and temperature 35°C.

presence of a small amount of air. Similar observations have been made by others working with *Klebsiella pneumoniae* M5A1 (Klucas, 1972; Hill, 1976; Bergersen *et al.*, 1981; Hill *et al.*, 1984.)

It is of interest that NaCl concentration and pH of the growth medium also has an important effect on the growth rate and on the nitrogenase activity of the intact organisms; 100 m*M* NaCl in the growth medium increases the N_2ase activity by 12% in M5A1 and about 24% in NIAB-I at the optimal pH value. The pH optimum for NIAB-I was 8.0 and for M5A1, 7.0. A higher concentration of salt (NaCl), however, decreases the nitrogen fixing ability of the cells. Thus, with 250 m*M* NaCl in the growth medium, M5A1 and NIAB-I showed a 55 and 35% decrease, respectivley, in N_2ase activity.

Typical growth curves of NIAB-I and M5A1 as regards nitrogenase activity, protein content and growth in batch cultures are presented in Figs 1 and 2.

The aerobic growth patterns of M5A1 and NIAB-I in Luria broth medium supplemented with different NaCl concentrations are presented in Fig. 3a,b. It can be seen that *Klebsiella* sp NIAB-I grows well even at 1*M* NaCl; M5A1 does not grow at this NaCl concentration.

Comparison of electrophoretic pattern of proteins

SDS–PAGE patterns of some diazotrophs are shown in Fig. 4. The three genera differ from each other in this respect. The two *Azospirillum*

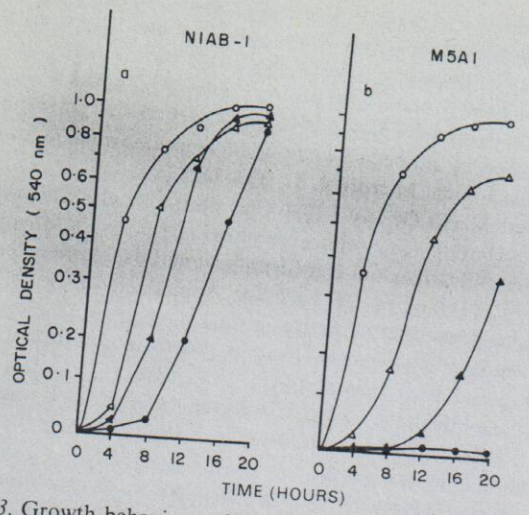

Fig. 3. Growth behaviour of (a) *Klebsiella* sp. NIAB-I and (b) *Klebsiella pneumoniae* M5A1 in Luria broth medium supplemented with different NaCl concentrations. The optical density is plotted as a function of incubation time. Open circle represents control; closed circle, 1*M* NaCl; closed triangle, 0.8*M* NaCl; open triangle, 0.6*M* NaCl.

brasilense strains 1690 and 2915 have identical protein patterns whereas the three *Klebsiella* strains M5A1, NIAB-I and 681 show differences in their electrophotograms. Bacteria with identical protein patterns have a high genomic similarity (Kersters and De Ley 1975, 1980). Izard *et al.*, (1981) identified four groups of *Klebsiella* spp. by the electrophoretic behaviour of proteins.

DNA–DNA hybridization

Because of the protein electrophoretic differences between the two *Klebsiella* spp. studied, it became important to investigate their DNA homology. Binding of 60–70% between the DNAs from M5A1 and NIAB-I was found (Fig. 5). At lower values of DNA (in μg) the binding is 100% but as the amount of DNA is increased the binding is decreased indicating that, genetically, the two species are different.

There are reports (Steigerwalt *et al.*, 1976; Izard *et al.*, 1981; Reinhold *et al.*, 1987) that DNA–DNA homology values within the genus range between 20 and 100%.

Klebsiella pneumoniae M5A1 is indole positive whereas our isolate is indole negative and has a G + C content 56.9 (Zafar *et al.*, 1987). Bagley *et al.*, (1981) proposed a new species, *Klebsiella planticola*, on biochemical and physiological

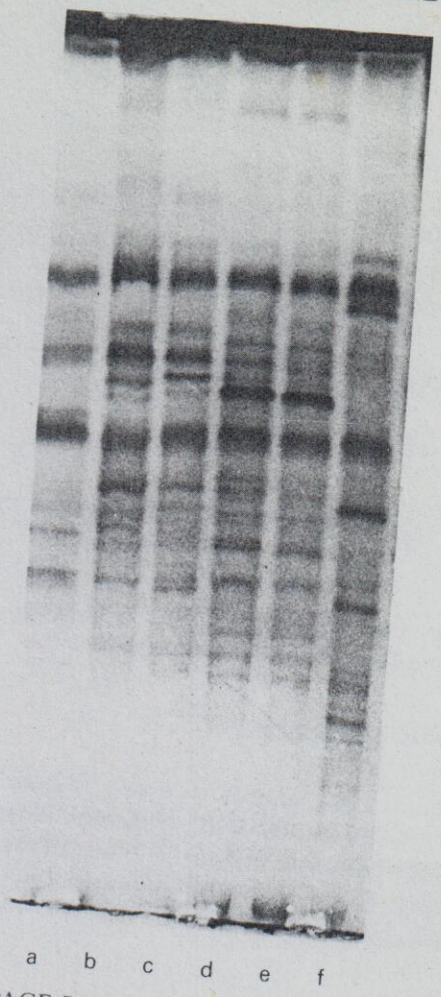

Fig. 4. SDS–PAGE Pattern of (a) *Bacillus polymyxa* 372, (b) *Azospirillum brasilense* 1690, (c) *Azospirillum brasilense* 2915, (d) *Klebsiella pneumoniae* 681, (e) *Klebsiella* sp. NIAB-I and (f) *Klebsiella pneumoniae* M5A1. Strain 681 is a non-N_2-fixer from the German Culture Collection whereas M5A1 strain is a N_2-fixer. M5A1 strain is now held with the National Collection of Industrial Bacteria as *Klebsiella pneumoniae (oxytoca)* NCIB 12204.

characteristics. Similarly Ladha *et al.* (1983) have identified new species on biochemical and immunological characteristics. *Klebsiella terrigena* was also identified as a new species using electrophoretic and DNA hybridization technique (Izard *et al.*, 1981). Recently, Reinhold *et al.* (1987) and Kersters and De Ley (1980) have used the SDS–PAGE method to classify bacteria. The present studies on the physiological behaviour, protein pattern and molecular studies on *Klebsiella* spp. NIAB-I provide evidence that our isolate is a new species of *Klebsiella*. Recently we have found that NIAB-I contains a plasmid which confers salt tolerance whereas M5A1 has no plasmid.

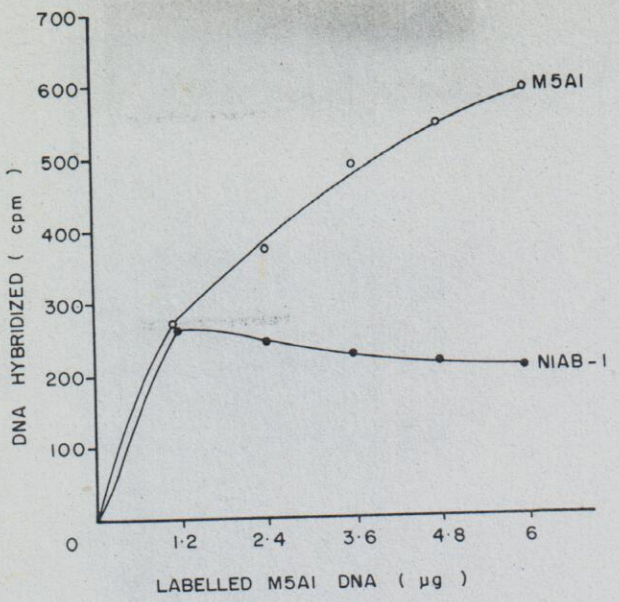

Fig. 5. DNA–DNA hybridization of *Klebsiella* sp. NIAB-I and *Klebsiella pneumoniae* M5A1.

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