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Isolation and identification of a N_2-fixing zoogloea-forming bacterium from kallar grass histoplane

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Semi-solid medium was used to isolate an aerobic, N_2-fixing (C_2H_2-reducing), H_2-utilizing bacterium from the roots of kallar grass (*Leptochloa fusca*). The organism was identified by morphological, cultural and biochemical characteristics. The N_2-fixing, zoogloeal floc-forming isolate described here is a new species.

Dinitrogen fixation associated with the roots of kallar grass (*Leptochloa fusca*) has been reported by Malik *et al.* (1980). Aerobic N_2-fixing bacteria have also been reported in the rhizosphere, rhizoplane and histoplane of this salt-tolerant grass (Zafar *et al.* 1986) and several bacterial genera have been identified in the N_2-fixing population. Because of their diversity, various isolation media and conditions are required to obtain these bacteria from the same source.

This paper presents the results of identification studies of a dinitrogen-fixing organism isolated from the histoplane fraction of kallar grass roots. Preliminary characterization, based on growth in liquid medium, suggests that it resembles members of the genus *Zoogloea*, family Pseudomonadaceae. Its generic standing as well as characterization is discussed.

Materials and Methods

ISOLATION PROCEDURE

Excised roots of kallar grass were washed free of soil with sterile distilled water, soaked in 5% NaOCl (w/v) for 15 min and rinsed with sterile distilled water. The roots were then macerated in a blender with 0·85% NaCl solution for 15 min. The turbid supernatant fluid, called the histoplane (HP) fraction, was used for the enrichment of diazotrophs in nitrogen-free semi-solid combined carbon medium (CCM) of Rennie (1981) which contained (g/l in distilled water): K_2HPO_4, 0·8; KH_2PO_4, 0·2; NaCl, 0·1; mannitol, 5·0; sucrose, 5·0; sodium lactate, 0·5 ml (60% v/v); $MgSO_4.7H_2O$, 0·2; $CaCl_2.2H_2O$, 0·06; yeast extract, 0·1; Na_2MoO_4, 0·025; $Na_2FeEDTA$, 0·028; biotin, 5 μg; *p*-aminobenzoic acid, 10 μg; pH adjusted to 7·0. Volumes of 0·1 ml from the HP fraction were added to 5 ml of CCM medium contained in cotton-plugged bottles. Incubation was for 48 h at 30°C. Cultures showing growth were used for streaking on CCM agar plates.

After 24–48 h of incubation at 30°C different types of colonies appeared on agar plates and were subjected to acetylene reduction assay (ARA). Individual colonies were transferred to CCM (5 ml medium per 17 ml bottle) and incubated at 30°C for 24–48 h. Cotton wool plugs were replaced by Suba seals (William Freeman, Barnsley, Yorkshire, UK) and a 10% v/v C_2H_2 atmosphere provided. Gas samples (100 μl) were analysed for ethylene on a gas chromatograph (Carlo Erba Model Fractovap 2150) fitted with a 1 m × 2 mm steel column filled with Porapak N (80–100 mesh, Waters Associates Inc., MA, USA) and a flame ionization detector. Nitrogen was used as a carrier gas at a flow rate of 32 ml/

min. The detector and injector temperature was 125°C and the oven temperature was 75°C.

CHARACTERIZATION

Morphological, cultural and biochemical characteristics of the strain were examined by conventional procedures including the commercial API 20E (API System S.A., La Balme Les Grottes, 38390 Montalieu Vercieu, France) test kits. The results of API kits were evaluated by comparison with standard biochemical tests as described by MacFaddin (1985). The ability to grow under chemolithoautotrophic conditions was determined by the method of Wiegel & Schlegel (1976).

The effect of various concentrations of NaCl, pH and different N sources on growth and nitrogenase activity was studied using media of the following composition.

Malate yeast medium (MYM) contained (g/l): K_2HPO_4, 0·1; KH_2PO_4, 0·4; sodium malate, 5·0; $Na_2MoO_4.2H_2O$, 0·002; NaCl, 0·1; $CaCl_2.2H_2O$, 0·02; $MgSO_4.7H_2O$, 0·2; yeast extract, 0·1; $NaFe_2EDTA$, 0·028. The pH was adjusted to $6·8 \pm 0·2$ with NaOH. Agar at 15 g/l for solid and 2 g/l for semi-solid medium was added.

Malate KNO_3 medium (MKM) was obtained by replacing yeast extract with 1·0 g/l KNO_3 in MYM to get a C : N ratio of 1 : 10, to promote zoogloeal growth. Sodium malate was 2·6 g/l in MKM to give a C : N ratio of 1 : 5, which produces dispersed growth. For studying the utilization of various C sources for growth and nitrogenase activity, 5 g/l of each C source (Table 2) was used in MKM.

For the formation of flocs in the presence of different N sources the yeast extract was replaced by 40 mg/l N in the form of NH_4Cl, $(NH_4)_2SO_4$ or KNO_3.

Results

The highest dilution enrichment of HP fraction which exhibited ARA activity was 10^{-5}. Three main types of colonies appeared on CCM agar plates when streaked from the 10^{-5} enrichment bottle. Only one strain, designated as Y-1, displayed appreciable nitrogenase activity (ARA) (500–600 nmol C_2H_4/h/culture bottle) in pure culture, and was used in this study.

Strain Y-1 grew rather slowly on N-free as well as nutrient agar medium. It formed a tough and unbreakable surface pellicle. Young cultures on semi-solid CCM or MYM were not distinctly yellow, but as growth continued they became ochre yellow and the pellicle thick and mat-like. Maximum acetylene reduction rates of up to 2 μmol C_2H_4/h/culture were observed on semi-solid MYM in 17 ml screw-capped bottles.

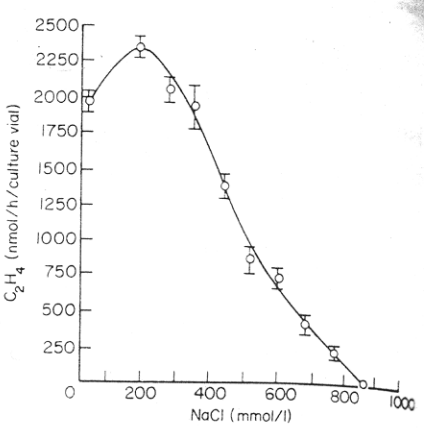

Fig. 1. Effect of varying concentration of NaCl on nitrogenase activity of zoogloea-forming isolate (Y-1) from kallar grass histoplane. Vertical bars represent standard error.

On nutrient agar the colonies are tenacious, cohesive and yellow with a crenate edge. A whole colony could be lifted easily from the agar with a wire loop. Under the stereomicroscope a raised undulating surface and a spreading margin was observed in older plates. Some characteristics of the organisms are presented in Tables 1 and 2.

Under the microscope the flocs showed finger-like dendritic out-growths, each originating from a cluster of cells. The cells in the floc seemed to be embedded in a matrix and were static, but some showed rhythmic movements along the margins, whereas free cells in the suspension were actively motile.

Because this bacterium was isolated from kallar grass growing in highly saline soil, the effects of varying concentrations of NaCl on growth and nitrogenase activity were studied. Maximum nitrogenase activity was observed at 170 mmol/l NaCl and declined steadily with increase in salt concentration; it was negligible at 800 mmol/l NaCl (Fig. 1).

Table 1. Comparison of characteristics of strain Y-1 with Zoogloea and Xanthobacter

Character	Strain Y-1	Zoogloea		Xanthobacter	
		Genus	*Zoogloea ramigera*	Genus	*Xanthobacter autotrophicus*
Cell shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	v	–
Flagellation	Monopolar	Monopolar	Monopolar	Peritrichous	None
Gram reaction	–	–	–	v	v
Colony	Cohesive, irregular, small	Punctiform, cohesive, circular	Tenacious, cohesive	Opaque, slimy	v
Colony edge	Irregular, lobate	Entire or lobate	Erose	Entire	Entire
Colony on 10^{-5} M crystal violet agar	Yellow	*	*	*	Red
Pigmentation	Yellow	None or straw	–	Yellow	Yellow
Slime	Water-insoluble	Water-insoluble	Water-insoluble	Water-insoluble	Water-insoluble
Pleomorphic	+	With age	+	+	On succinate
Zoogloeal growth†	+	+	+	–	–
Fixation of atmospheric N	+	–	–	+	+
O_2 requirement	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
NO_3 respiration	–	+	+	–	–
NO_3 reduced	+	+	+	+	+
Acid from carbohydrates	–	–	–	–	–
Gelatinase	+	+	+	–	–
pH range	6–8	5–10	*	5·8–9	*
Oxidase, catalase	+	+	*	+	+
Urease	+	+	+	*	d

+, Positive or presence of character; –, negative or absence of character; v, variable; d, 11–89% strains give positive result.
* Information not available.
† Pertains to macroscopic star-like flocs, with dendritic out-growth.

Table 2. Other characteristics of zoogloeal strain Y-1 from kallar grass histoplane

Characteristic	Y-1	Characteristic	Y-1
Amino acid decarboxylase		Utilization as sole C source in broth	
lysine decarboxylase,	+	methanol, ethanol, *n*-propanol, *n*-butanol	+
ornithine decarboxylase		gluconic acid	−
Arginine dehydrolase	+	lactic acid, malic acid	+
β-Galactosidase (ONPG)	+	sucrose, glucose, raffinose, D-fructose,	−
Tryptophane deaminase	−	lactose, arabinose, mannose	
Sodium citrate, sodium malonate	−	Acid from sugars†	−
Voges-Proskauer	−	Utilization of N source	
H_2S production	−	$KNO_3(NH_4)_2$, NH_4Cl yeast extract	+
Indole	−	Nitrogenase activity on‡	
Denitrification (to N_2)	−	malate, ethanol	+
Vitamin requirement	−	*n*-propanol, *n*-butanol, methanol	−
Utilization of Krebs cycle intermediates			
pyruvate, citrate, malate, succinate,	+		
oxaloacetate			
Oxidation–fermentation of carbohydrates*	o		

o, Oxidative utilization.
* Carbohydrates tested: sucrose, glucose, mannitol, xylose, maltose, lactose, sorbitol, raffinose, inositol.
† Carbohydrates tested: glucose, maltose, sucrose, mannitol, arabinose, rhamnose, sorbitol, inositol.
‡ In semi-solid malate yeast medium malate was replaced by respective source.

Discussion

The organism designated as Y-1 is not one of those commonly occurring on the kallar grass histoplane. Generally there are not many yellow-coloured N_2-fixing organisms. The only nitrogen-fixing organism known to produce yellow, water-insoluble pigment is *Xanthobacter autotrophicus* (Wiegel & Schlegel 1976). Strain Y-1 was not thought to be *Xanthobacter*, although it was similar in colour, because of its peculiar growth in shaken broth culture, where it aggregates to form macroscopic star-like flocs (Fig. 2) similar to those of *Zoogloea ramigera* (Krieg & Holt 1984). It appears that identification of species of genus *Zoogloea* is based primarily on the presence of characteristic extracellular capsular or zoogloeal matrix. Under the microscope the flocs of Y-1 show an arrangement of cells similar to that of bacteria in columns each of which protrudes from an aggregate of cells, which has been reported for the historically recognized form of *Z. ramigera* (Unz & Dondero 1967).

The extracellular slime produced by strain Y-1 was water-insoluble and visually similar to that reported for *Zoogloea*, as the culture does not show any turbidity during growth and floc formation. At a later stage of the growth, free motile cells were seen in hanging drop preparation. The matrix or capsular exopolymer of *Z. ramigera* is reported to be water-insoluble and does not thicken the medium visibly. Therefore, preliminary observations regarding the capsular polymer of strain Y-1 suggest that it is similar to that reported for the type strain of the genus *Zoogloea*, family Pseudomonadaceae.

There are reports of *Zoogloea* strains that resemble a number of species belonging to various genera; for example, *Pseudomonas denitrificans*, some species of *Gluconobacter* and *Acetobacter* (Dugan 1981), but the unique trait that places such strains in the genus *Zoogloea* is the presence of finger-like projections and a zoogloeal matrix (Dugan 1981). The extent of zoogloea production, however, varies among strains and with the culture conditions and may diminish or be lost during frequent transfers on enriched culture media.

Matrix formation and its morphological appearance are reported to be controlled nutritionally by C and N sources, C : N ratio and by turbulence of the growth medium (Dugan 1981). When the strain was grown on a lower C : N ratio of 1 : 5, the flocs did not appear and dispersed growth was observed.

Unz & Dondero (1967) reported the existence of other bacteria adhering to the zoogloeal exopolymer which can lead to erroneous results. In order to ascertain the purity of strain Y-1, dilution platings from dispersed growth were made on nutrient agar. They yielded only a single

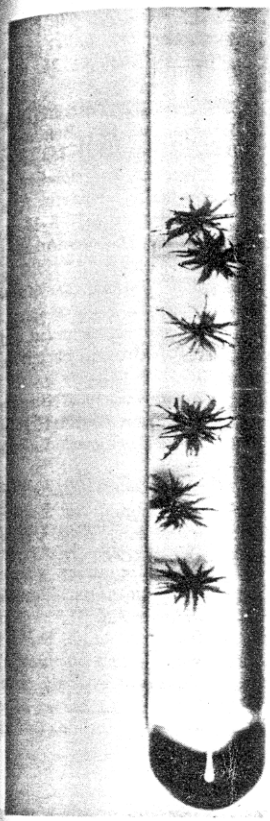

Fig. 2. Zoogloeal growth showing macroscopic star-like flocs in broth culture (malate yeast medium). The flocs were stained with crystal violet and placed in clean solution again to increase the contrast. × 1·5.

type of colony. These colonies, when re-inoculated on the high C : N ratio (1 : 10) MKM broth, produced typical star-like flocs. These observations indicate that isolated colonies on agar plates originated from zoogloeal growth of only one type of bacteria.

Under static conditions, strain Y-1 formed a pellicle but no lacy tape-like flocs extending from it were seen. Also it did not utilize glucose or sucrose even when supplemented with 0·1% yeast extract, NH_4Cl or KNO_3 in the broth medium. Itzigoshn (1867), however, who had originally given it its name observed lace-like floc formation and reported utilization of glucose in the presence of arginine.

Non-utilization of glucose as C source, the yellow pigment, the property of N_2 fixation and chemolithoautotrophic growth and lack of nitrate respiration and denitrification suggest that it is a *Xanthobacter* species. However, there are no reports so far of any *Xanthobacter* species forming macroscopic flocs in broth culture. Only one anaerobic species of the genus *Bacteroides*, namely *B. zoogleoformans*, has been reported to form zoogloea (Cato *et al.* 1982).

The property of denitrification in the genus *Zoogloea* was reported to be 90–100% for group I strains and absent for group II, although some 15 isolates (group II) were reported to produce yellow pigments but were not floc-forming or zoogloeal (Unz & Dondero 1967). Nitrogen fixation in *Pseudomonas* species has recently been reported by Barraquio *et al.* (1983). The capacity of N_2 fixation has been claimed earlier for *Ps. glathei* which later proved to be a N_2 scavenger rather than a N_2 fixer (Zolg & Ottow 1975), because it failed to perform the function under strictly controlled conditions, and also did not reduce acetylene.

From the comparison of various characteristics of *Zoogloea* and *Xanthobacter* strains studied so far (Table 1) it is difficult to assign any taxonomic position to strain Y-1, especially when the two genera themselves suffer some nomenclatural problems (genus *Zoogloea*) and uncertainty of the status of genus *Xanthobacter* on account of DNA-RNA homologies (DeSmedt *et al.* 1980).

The antigenic relatedness and G + C analysis results are considered better tools for ascertaining systematic relationship among organisms. Very little work has been done so far in this direction for these two genera. The G + C analysis data, although not conclusive, reinforce the view that the floc-forming isolates are similar organisms (Chorpenning *et al.* 1978), and are sufficiently distinctive to allow differentiation from other Gram-negative rods. At present, however, there is no definitive description of either the genus *Zoogloea* or its species other than the gelatinous matrix and finger-like projections when they are present.

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