Studies on the Nitrogen-Fixing Bacteria of the Genus *Azotobacter* in Libyan Soils

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ABSTRACT

The numbers of *Azotobacter* in some Libyan soils, determined by the most probable number technique, varied from few cells (less than 10) to as many as 30 thousands per gram of soil. The abundance was favourably affected by cultivation of the soil and the amount of organic materials, and deterimentally affected by the high salt concentration in soil.

Studies on the morphological, cultural and nutritional characteristics of 12 isolates revealed that one isolate resembled *A. vinelandii* (Lipman) and the rest of the isolates resembled *A. chroococcum* (Beijerinck). The differential use of the pentoses: xylose, arabinose and ribose by *A. vinelandii* and not by *A. chroococcum* was used as a taxonomic criterion for differentiating between these two species.

INTRODUCTION

The nonsymbiotic nitrogen-fixing bacteria of the genus *Azotobacter* are widely distributed in nature. Reports on their incidence in soils all over the world, as well as in aquatic environments were reviewed by several investigators (1,11,14,17). The abundance of *Azotobacter* in soils, however, is subject to great variation depending upon the environmental conditions prevailing. The fact that the abundance of *Azotobacter* in soil is correlated to the amount of carbonaceous materials was reported by many investigators (6,8,15,21). Other factors, especially soil pH and salinity, are also limiting for numbers of azotobacter in soils. Elwan and El-Sayed (6) made a survey for azotobacter population in 10 uncultivated and 22 cultivated localities in various types of Egyptian soil. Azotobacter was not recorded in saline localities; and water soluble salts above 1 % was considered the limiting factor for the absence of azotobacter in Egyptian soils. Also, the increase in azotobacter counts on leaching saline soils has been shown experimentally by Taha *et al.* (20). In culture media, Fustec-Mathen *et al.* (9) reported that most of the isolated strains of *A. chroococcum* and *A. agilis* were able to grow in media containing up to 2–2.5 % NaCl. Few strains isolated from soil closely influenced by sea water, however, were able to grow on media with up to 8 % NaCl.

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The purpose of the present investigation was to study the abundance of these nitrogen-fixing bacteria in different Libyan soils and to isolate representative strains and study their characteristics.

MATERIAL AND METHODS

The abundance of *Azotobacter* was determined in five composite soil samples collected from: (1) the farm of the Faculty of Agriculture, Tripoli (cultivated area); (2) Virgin soil near the farm of the Faculty of Agriculture; (3) Wady-El-Mait Project, 70 km east of Tripoli, (4) Taworga Project, 300 km east of Tripoli, an area of saline sandy soil under reclamation in which the land was leveled and leached 7 times; (5) same as (4) but before leaching.

The soil samples were air dried for 2 days, sieved through a 2 mm sieve and used for determining the number of azotobacter cells by the dilution to extinction technique using tables of the Most Probable Number (22). The nitrogen-free medium used for enumeration, cultivation, and isolation of *Azotobacter* spp. had the following composition; K_2HPO_4 0.5 gm, $MgSO_4$ $7H_2O$ 0.2 gm, NaCl 0.2 gm, CaCl $2H_2O$ 0.1 gm, $NaMoO_4$ $2H_2O$ 0.01 gm, $MnSO_4$ $4H_2O$ trace, $FeCl_3$ trace, sourose 10 gm, distilled water 1,000 ml. Twenty grams of Bacto-agar (Difco) was added per litre of medium when solid medium was desired.

The ability of the isolated *Azotobacter* strains to use different organic compounds as carbon and energy sources was tested by replacing sucrose with the organic compound at the same concentration.

The cultures were incubated at 28°C for 10–15 days for detecting pellicle formation.

Isolation of Azotobacter Strains

Five grams soil sample from the Faculty of Agriculture farm was mixed with 100 ml nitrogen-free liquid medium in 250 ml capacity Erlenmeyer flask. After incubation for 4 days at 28°C a loopfull of the pellicle formed was streaked on the surface of the solid medium, described above, in Petri plates. After 10 days incubation, 12 of the typical *Azotobacter* colonies developed on the agar medium were isolated and purified by repeated streaking on the same medium. Finally, the isolated cultures were streaked on the nitrogen-free medium containing 0.5% sucrose and 1.0% sodium benzoate as carbon and energy sources. This medium proved excellent for purification since the sodium benzoate at the concentration used was found to inhibit most of the contaminating organisms, and *Azotobacter* spp. produced on this medium, within 4–7 days, characteristic brown colonies with blackish secretion around them. The purity of the isolated cultures was then tested microscopically before studying their characteristics. In studying the characteristics of these isolates, comparisons were made with *A. chroococcum* strain 25, and *A. vinelandii* strain 4 obtained from the Ministry of Agriculture, Cairo, Egypt.

Cyst formation was tested by preparing wet mounts from 3 week old cultures using 0.25% aqueous safranin solution as a mounting fluid. The diluted solution of this dye was found to be absorbed differentially by the different parts of azotobacter cysts as well as the vegetative cells.

The efficiency of the strains to fix atmospheric nitrogen in culture medium was tested by growing the isolates in 50 ml aliquots of the nitrogen-free sucrose medium in duplicate. After 10 days incubation at 28°C the total nitrogen content of the cultures as well

as the uninoculated medium was determined by the Kjeldahl procedure followed by Nesslerization (2).

RESULTS

The Abundance of Azotobacter

The characteristics of the composite soil samples used and the average number of *Azotobacter* per gram of dry soil are shown in Table 1. Highest counts obtained were from soil samples of the farm of Faculty of Agriculture. Average numbers of azotobacter in the virgin soil sample was 1320 cells per gram of dry soil. The cultivated soil in the same area contained 20 times as much azotobacter as the virgin soil.

Counts of azotobacter in the soil from Wadi-El-Mait were relatively low in comparison with those from Tripoli area. However, lowest counts were found in soil samples from Taworga. The numbers ranged from none to very few cells (less than 10 per gram of soil). The leached soil contained slightly higher counts than the unleached soil.

The type of azotobacter colonies developing on sodium benzoate medium is shown in Fig. 1. The colonies were smooth, glistening and dark brown surrounded with blackish diffusable pigment.

Morphology and Motility of the Isolated Strains

Wet preparations and Gram stained smears from 24 and 72-hour old cultures grown on nitrogen-free sucrose medium were examined. Young cultures of all of the isolated strains grown on liquid medium showed active motility.

Cells from 24 hour-old cultures were rods 2–4 μ in length and 0.5–1 μ in diameter, uniformly stained, Gram negative, and usually found in pairs. At 72 hours most of the cells were rods in pairs, but some tended to be spherical, highly vaculated and showed great variation in size. These spherical cells were either singular, in pairs, or in groups.

In wet mount preparation with safranin solution the cytoplasmic materials of the vegetative cells intensily absorbed the dye and stained red, whereas the granules of B-hydroxy butyrate did not absorb the dye and remained unstained. Photographs of the vegetative cells of some of the isolates in wet mount preparation are shown in Fig. 2.

All of the isolated strains were found to produce cysts characterized by their spherical shape and the thick refractile cell wall. Variations in cyst size among the different strains as well as within cysts of the same strain were observed. In the course of encystment the vegetative cells become round in shape, yet their cell walls remain thin and the internal structure of the rounded cell still undifferentiated. This is followed by a stage of accumulation of capsular refractile material around the immature cyst and the central body becomes differentiated. The walls of cyst get thicker and three distinct layers become differentiated. Probably the difference in chemical composition of these layers and accordingly the difference in the rate of safranin absorption in wet mount preparations made it possible to distinguish their boundaries under the light microscope (Fig. 3). Probably, because the conversion of vegetative cells to cysts was not synchronized, it was possible to find at any one time cells in different stages of encystment.

Cultural Characteristics

Young cultures of all of the isolated strains, except one, formed white to cream glistening butyrous to mucous colonies on sucrose nitrogen-free agar medium. Two to three

Table 1 Characteristics of the soil samples and average counts of total microflora and *Azotobacter* per gram of dry soil.

Location	Depth of sample cm	pH	Organic matter content %	E. C. mmhos/cm at 25°C	Average[1] number of Total microflora × 1,000	Azotobacter
1 Faculty farm (Cultivated soil)	0–20	8.1	0.58	0.56	1960	24,800
2 Faculty farm (virgin soil)	0–20	8.1	0.25	0.62	146	1,320
3 Wadi-El-Mait (orchard)	0–30	7.4	0.51	—	313	19
4 Taworga (leached soil)	0–16	7.4	0.33	4.1	23.3	10
5 Taworga (unleached soil)	0–17	6.9	0.23	42.7	1.6	3

[1]Average of 3 replicates.

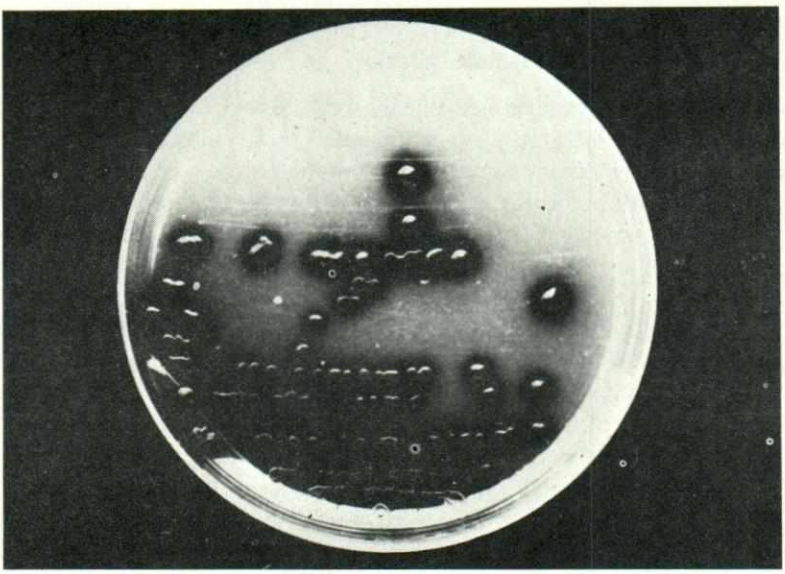

Fig. 1. Typical colonies of *Azotobacter* grown on nitrogen-free benzoate medium.

weeks old cultures on the same medium showed brown non-diffusable pigmentation; the intensity of color in the different strains varied from light brown to very dark brown. Isolate no. (3) formed light creamy colonies and was the only isolated strain that produced water soluble yellowish green pigment diffused in the surrounding medium. Young culture of this strain (72 hr) grown on glucose nitrogen-free medium was tested under ultraviolet lamp (360 mμ) and was found to produce greenish fluorescence.

Nutritional Characteristics

The ability of the strains to utilize different sugars and organic compounds was tested by inoculating plates of the nitrogen-free sugar medium by a single streak with a

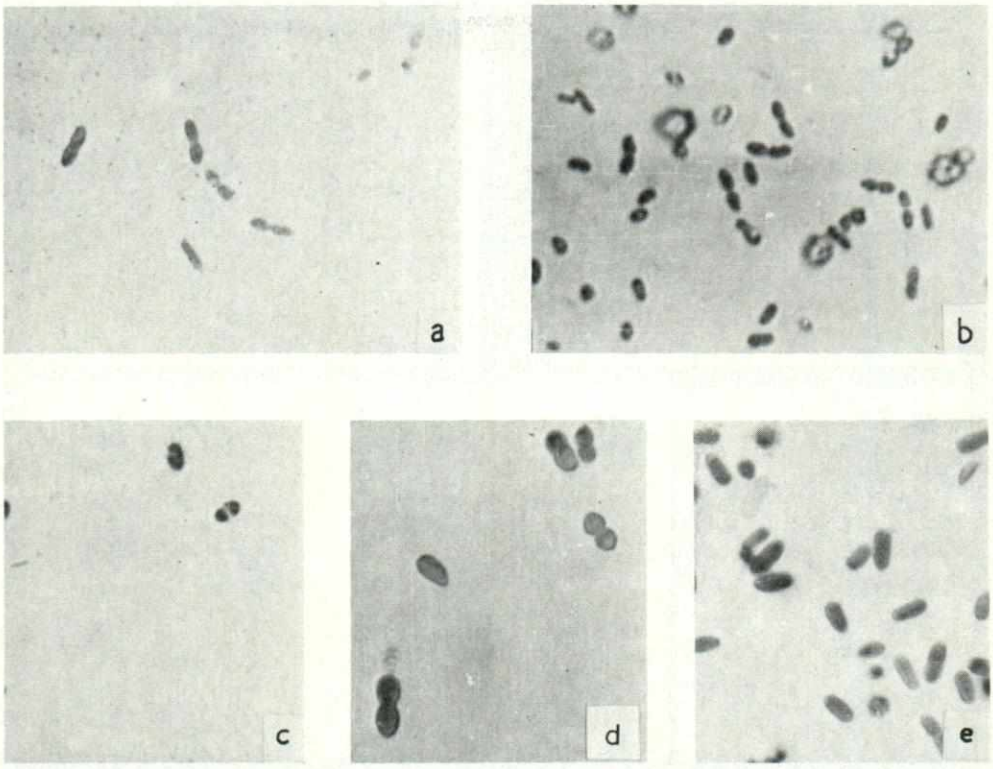

Fig. 2. Photomicrographs of wet preparations of *Azotobacter* cells (× 6000); (a) isolate 2, 24-hr old culture; (b) isolate 2, 72-hr old culture; (c) isolate 3, 48-hr old culture; (d) isolate 5, 24-hr old culture; (e) isolate 8, 48-hr old culture.

loopful from a 48-hour old culture. Growth on the inoculated plates was checked after 7 days incubation at 28°C. The compounds tested and the results obtained are shown in Table 2.

All isolates, as well as the reference strains *A. chroococcum* and *A. vinelandii*, utilized glucose, maltose, raffinose, mannitol, starch, acetate, and benzoate as the sole carbon/energy source in the medium. But *A. vinelandii* and the isolated strain no. (3) were the only strains able to use the sugar rhamnose, ribose, xylose or arabinose as sole carbon/energy source. Also, strain no. (3) and *A. vinelandii* were the only two strains that gave abundant growth on mannose medium, while *A. chroococcum* and all the other isolates either did not grow or gave very poor growth on this medium.

Efficiency of the isolates to fix nitrogen

Amounts of atmospheric nitrogen fixed in culture medium by the six isolates tested are shown graphically in Fig. 4. The range of fixation was from 8.8 to 12.4 mg nitrogen per 100 ml of the culture. Assuming that all the amount of sucrose in the medium was used by the growing cultures, fixation of 9–12 mg nitrogen per gram of sugar indicates intermediate fixing efficiency of the isolates.

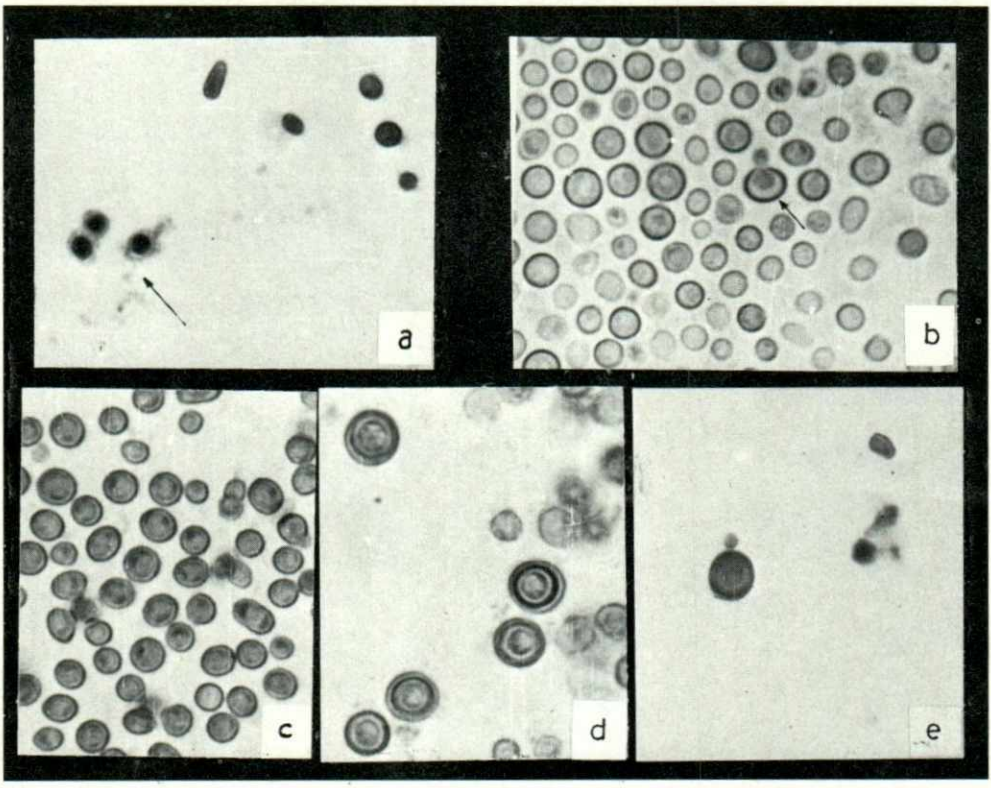

Fig. 3. Photomicrographs of wet preparations of *Azotobacter* cysts at different stages of maturity (× 6000); (a) rod and round vegetative cells started to encyst, with three rounded cells surrounded by capsular material; (b) round vegetative cells at first stage of encystment, arrow shows one mature cyst in a state of germination; (c) cysts with thick walls and undifferentiated central bodies; (d) mature cysts with differentiated central bodies and very thick walls of 3 distinct layers; (e) large mature cyst germinating.

DISCUSSION

In the virgin soil of Tripoli area the relatively low count of *Azotobacter* is due to the very low content of organic matter and to the dry conditions prevailing in this semi arid region. This is also reflected in the low numbers of total microflora in the soils of this region (Table 1). On the other hand, the absence or the presence of very few cells of azotobacter in Taworga is due to the high soluble salts in soil (above 4 mmhos/cm).

Species identification of the 12 isolates of *Azotobacter* obtained in this study was performed according to Bergey's manual (3) and Norris and Chapman classification (16). The results of morphological and cultural characteristics, and utilizable carbon/energy source indicate that eleven of the isolates belong to the species *A. chroococcum*, whereas, only one isolate (no. 3) belongs to *A. vinelandii*. This latter strain was the only isolated strain that produced water soluble diffusable pigment, which gave fluorescence under the ultraviolet light. The species of *Azotobacter* that are known to produce water soluble pigment that fluoreses are *A. vinelandii* and *A. agilis* (*Beijerinck*). Isolate no. (3), however, was found to form cysts, a characteristic that is not demonstrated in strains

Table 2 Utilization of organic compounds as carbon/energy source by the isolated and known strains of *Azotobacter*.

Isolate number	Glucose	Mannose	Rhamnose	Ribose	Xylose	Arabinose	Maltose	Raffinose	Mannitol	Starch	Acetate	sodium benzoate
1	+	±	−	−	−	−	+	+	+	+	+	+
2	+	±	−	−	−	−	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+
5	+	±	−	−	−	−	+	+	+	+	+	+
6	+	+	−	−	−	−	+	+	+	+	+	+
7	+	−	−	−	−	−	+	±	+	−	+	+
8	+	±	−	−	−	−	+	+	+	+	+	+
9	+	−	−	−	−	−	+	+	+	+	+	+
11	+	±	−	−	−	−	+	+	+	+	+	+
12	+	±	−	−	−	−	+	+	+	+	+	+
14	+	±	−	−	−	−	+	+	+	+	+	+
15	+	±	−	−	−	−	+	+	+	+	+	+
A. chroococcum	+	±	−	−	−	−	+	+	+	+	+	+
A. vinelandii	+	+	+	+	+	+	+	+	+	+	+	+

(+) good growth
(±) very slight growth
(−) no growth.

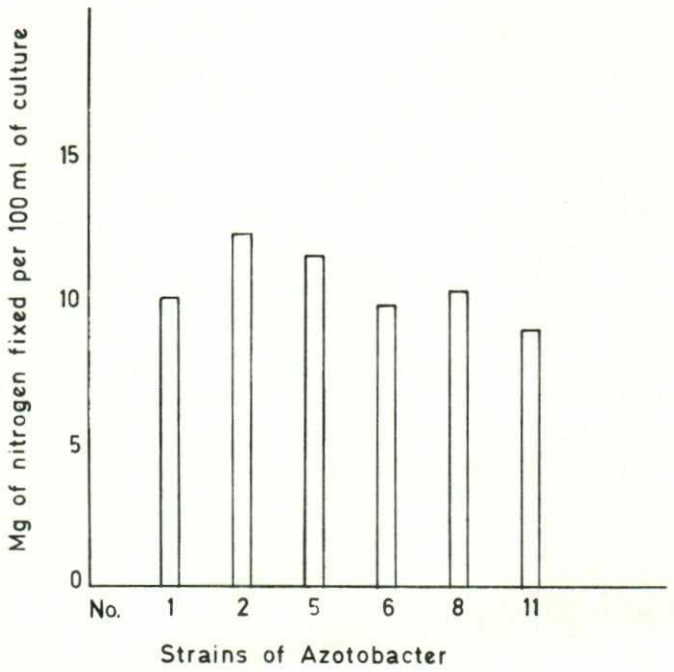

Fig. 4. Amounts of nitrogen fixed by some of the *Azotobacter* isolates.

of *A. agilis* (16). Furthermore, isolate no. (3) also utilized benzoate which is not used by *A. agilis* as a carbon source (18). Therefore, it can be concluded that isolate (3) belongs to *A. vinelandii* rather than *A. agilis*.

In agreement with previous report (18), strains of *A. vinelandii* were found able to use rhamnose as sole carbon source while *A. chroococcum* strains could not. Also, the finding that the pentose sugars: xylose, arabinose and ribose are used by *A. vinelandii* and not by *A. chroococcum* as carbon/energy source was used as a taxonomic criterion in the differentiation between these two species. Variations between species and strains of *Azotobacter* to utilize different organic compounds as carbon/energy source were reported by other investigators (5,18).

Cyst formation is also an important criterion in the identification of *Azotobacter* species. The different stages from vegetative cells to mature cysts observed for the isolated *Azotobacter* strains are in agreement with the reports of other investigators (13,23). Hitchins and Saddoff (10) reported that as encystment of *A. vinelandii* proceeds the vegetative cells convert to rounded nonmotile cells surrounded by non-structural capsular material and the exine coat develops by the continuous excretion of membraneous components into capsule surrounding the cell.

The internal structure of cysts was also studied by several investigators (7,10,12,19, 23). Structurally two distinct areas surrounding the compact central body were revealed in the electron micrographs; an electron transparent area or 'intine' and electron dense multilayered 'exine' (10). Frozen-etched preparations examined by Cagle *et al.* (4) using the electron microscope also revealed a matrix of extra exinic polymer consisting of polysaccharides extending some distance beyond the exine. The distribution of extracellular polysaccharides around cells and cysts was reported to be an important factor and prerequisite for mature cyst formation (4,7).

In the present investigation three outer layers surrounding the cyst central body were

observed in the wet mount preparations using the safranin dye (Fig. 3,d). It is probable that the outer (third) layer is extracapsular material that faintly absorbed the safranin dye. It is also possible that the difference in the degree of compactness in the multi-layered exine caused variation in the intensity of safranin absorption.

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