

STUDIES OF AEROBIC NON-SYMBIOTIC NITROGEN-FIXING BACTERIA¹

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Abstract

The occurrence of aerobic, non-symbiotic, nitrogen-fixing bacteria was determined in samples of soil collected in the various soil zones of Alberta and Saskatchewan. Mannitol and sodium benzoate dust-plates, and mannitol solution cultures with subsequent inoculation onto mannitol agar demonstrated that *Azotobacter* were not widespread in the Canadian prairie province soils. These procedures also led to the isolation of smaller, aerobic, non-symbiotic, nitrogen-fixing organisms from all the Alberta and Saskatchewan soils studied. These smaller, nitrogen-fixing bacteria which developed as 1- to 3-mm circular, convex, unpigmented colonies on mannitol and glucose agar were classified as *Pseudomonas*. Flagellation of the 0.75 to 1 μ by 1.5- to 2- μ Gram-negative, coccoid rods was polar. Starch was hydrolyzed; gelatin was not liquified. Indol, acid, and gas were not produced; litmus milk was not reduced, but hydrogen sulphide was formed. The pseudomonads, capable of initiating growth at a pH of 4.9, could also grow at 8° C, whereas the *Azotobacter chroococcum* required higher temperatures and reactions above pH 6.2. *Azotobacter chroococcum* fixed up to 12 mg nitrogen per gram of carbohydrate. The smaller bacteria, classified as *Pseudomonas azotogensis*, fixed from 0.1 to 3.9 mg N per gram of mannitol.

Introduction

The agricultural soils of the Canadian prairies, developed primarily under grassland condition, have relatively high contents of organic matter and nitrogen. Because of the scarcity of legumes in the native prairie vegetation, the role of symbiotic nitrogen fixation in the accumulation of this nitrogenous organic matter is of doubtful significance, especially in the semiarid regions (9). The dry-land agriculture practised in this area does not include extensive nitrogen additions by either fertilization or legumes; maintenance of the nitrogen content therefore appears to depend largely on non-symbiotic nitrogen fixation.

The fact that agricultural soils of this region contain organisms capable of fixing atmospheric nitrogen has been previously demonstrated (8, 9, 10). Soils of the brown, black, and grey-wooded soil zones were found to fix significant quantities of nitrogen when incubated with plant residues.

Azotobacter were isolated from the irrigated soils of the brown soil zone (3). Their occurrence under conditions of dry-land cultivation was, however, found to be spasmodic. The black and grey-wooded soils, which Newton found to fix atmospheric nitrogen, did not contain *Azotobacter* (3, 9). Therefore, although it was known that these soils contained non-symbiotic nitrogen-fixing organisms, their characteristics had not been studied.

Methods

Organisms capable of developing on media without added nitrogen were

¹Manuscript received July 7, 1960.

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isolated from 44 cropped or virgin sites in Alberta and Saskatchewan. The soils studied ranged from the irrigated and non-irrigated semiarid soils of the brown soil zone to the subhumid grey-wooded zone.

The isolation media consisted of mannitol and sodium benzoate agar without added nitrogen (K_2HPO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $Na_2MoO_4 \cdot 7H_2O$, 0.05 g; agar 15 g; distilled water 1000 ml). Two per cent mannitol or 0.1% sodium benzoate served as the energy source. Approximately one-tenth gram of pulverized soil was shaken from a spatula onto the agar and incubated. A solution of mannitol with the necessary mineral salts was also inoculated with soil and subsequently subcultured onto mannitol and sodium benzoate agar.

The macroscopic growth characteristics and the microscopic morphological features were determined for organisms capable of fixing significant quantities of nitrogen as determined by a micro-Kjeldahl procedure. Unless otherwise specified, the techniques were those suggested by the Manual of Microbiological Methods (15).

Microscopic examination and growth on sugar-free peptone media were used for purity controls of the cultures. Unless otherwise specified, the incubation period was 7 days at 28° C.

Determination of Nitrogen Fixation

The nitrogen-fixing ability of the organisms was determined in a solution buffered at pH 7.2 by 1.8 g K_2HPO_4 and 0.7 g KH_2PO_4 per liter of 2% mannitol solution plus mineral salts. Two milliliters of a washed resting suspension of cells, adjusted to a concentration which gave a reading of 15% transmittance at 585 m μ on a "Spectronic 20" colorimeter, were added to 5 ml of mannitol solution. The controls immediately received 1.5 ml of concentrated H_2SO_4 . The cultures were incubated at 25° C for 48 hours in a constant temperature room equipped with a reciprocating shaker.

The determination of total nitrogen employed the principles of a method developed by Polly (11). The cultures after incubation were digested with 1.5 ml concentrated H_2SO_4 , 0.05 ml of 10% $HgSO_4$, and 0.5 g K_2SO_4 . After 30 minutes' digestion, the solution was cooled and five drops of 30% hydrogen peroxide were added. Then digestion was continued for another 10 minutes. The flasks were allowed to cool, the mercury precipitated with sodium sulphide and aliquots of the supernatant diluted for Nesslerization.

Results

Azotobacter in Alberta Soils

Dusting mannitol and sodium benzoate agar plates with soil demonstrated, on incubation, that *Azotobacter* were present in the three irrigated soils collected from the brown soil zone, and in one adjacent cultivated non-irrigated soil. The other dry-land cultivated and the virgin soil sites were devoid of these nitrogen-fixing organisms.

Inoculation of mannitol solution with subsequent transfer onto mannitol and sodium benzoate agar verified that *Azotobacter* were absent from most of the Alberta soils tested. Characterization of the isolates obtained from the various soils showed that the *Azotobacter* produced large, mucous, coalescent

TABLE I

Atmospheric nitrogen fixed by *Azotobacter chroococcum* during 48 hours' incubation in 5 ml of 2% mannitol solution

Location of soils from which organisms were isolated	Nitrogen content		Nitrogen fixed	
	Initial	After incubation	$\mu\text{g/ml}$	mg/g of mannitol
Taber	0.18 (0.13-0.25)*	1.24 (1.19-1.35)*	212	10.6
Vauxhall	0.17 (0.17-0.18)	1.45 (1.19-1.58)	256	12.8
Brooks	0.19 (0.17-0.18)	0.35 (0.27-0.41)	35	1.65

*Range of four determinations.

colonies having a greyish-brown water-insoluble pigment. On sodium benzoate agar, 3- to 4-mm, flat, circular colonies with a black, water-soluble pigment were formed. The growth on mannitol agar slants was brown filiform showing evidence of slime production. On sodium benzoate slants filiform to beaded growth produced a black, soluble pigment.

Growth of the 2- to 3- μ , yeastlike *Azotobacter* cells was very limited in nutrient broth, in peptone water, and on nutrient agar. Potassium nitrate slightly inhibited growth on mannitol agar. Starch was utilized. Gelatin was not liquified, nor was indol produced from tryptophan. Hydrogen sulphide was not produced nor was litmus milk reduced after 14 days.

The *Azotobacter* were capable of initiating growth between pH values of 6.2 and 7.9 with an optimum growth rate at pH 7.0. The organisms developed more profusely at 28° C than at either 25° C or 37° C. Growth did not occur at 8° C. The thermal death temperature for a 10-minute exposure was 55° C.

Table I shows that approximately 12 mg nitrogen was fixed per gram of mannitol when a heavy, washed resting suspension of cells was inoculated into the energy source and incubated for 48 hours. One of the cultures tested, however, fixed only 1.65 mg of nitrogen under these conditions.

Other Nitrogen-fixing Organisms

Every one of the 72 individual samples of soil from the brown, dark brown, black, and grey-wooded soils of Alberta contained organisms, other than *Azotobacter*, capable of developing on media with no nitrogen added. The colonial characteristics, microscopic appearance and biochemical properties of the organisms isolated from the 0- to 6-in., and 6- to 12-in. cultivated and virgin sites from each of the areas sampled did not differ significantly, and the organisms were all classified as *Pseudomonas*.

Figure 1 shows the microscopic morphology of the Gram-negative rods which were found to be 0.75 by 1.5 μ in size when stained with methylene blue. Copious amounts of slime were produced. Part of this appeared to be stained with carbol fuchsin for the cells appeared larger in size when this stain was employed.

Growth on nitrogen-free mannitol agar as demonstrated in Fig. 2, produced small, colorless, circular, convex colonies with entire edges and butyrous centers. Colony growth on sodium benzoate agar was similar to that obtained with mannitol or glucose agar. Samples from the grey-wooded areas, however, produced a dark brown, water-soluble pigment on sodium benzoate agar.

The small coccoid rods grew profusely on nutrient agar producing 2- to 4-mm, entire, circular, white colonies, which tended to turn a light yellow with age. Growth on nutrient agar slants was white and filiform. Inoculation of nutrient broth produced a flocculent growth, whereas peptone water became cloudy and had a slight ring on the surface.

Growth was enhanced by 0.1% nitrate producing 2- to 4-mm colonies with a light yellow tinge. Growth in nitrate glucose broth resulted in both nitrite and gas being formed. The addition of 0.2% glycine to the sugar plus salt media, however, suppressed development of the organisms. Eosin methylene blue agar produced raised, glistening, pink colonies that darkened in the center after 7 to 10 days' incubation.

The isolates were motile under conditions of the hanging drop motility test. Flagellation, as determined by Liefson's flagella stain (6), was polar.

Gelatin was not liquified, nor was indol produced. Litmus milk was unchanged after 14 days. Brilliant green bile broth showed growth but no gas. Neither acid nor gas was produced from a number of carbohydrates and the Voges-Proskauer test for acetyl methyl carbinol was negative.

The lead acetate strip test for H₂S production showed erratic results but Kligler's iron agar demonstrated H₂S production.

The pseudomonads have a greater ability than *Azotobacter* to withstand

TABLE II

Atmospheric nitrogen fixed by *Pseudomonas azotogensis* after 48 hours' incubation in 5 ml of 2% mannitol solution

Location of soils for which organisms were isolated	Nitrogen content		Nitrogen fixed	
	Initial	After incubation	µg/ml	mg/g of mannitol
Youngstown	.220 (.19-.26)*	.307 (.27-.34)*	17.4	0.87
Gleichen	.087 (.08-.10)	.478 (.47-.50)	78	3.91
Halkirk	.028 (.020-.030)	.048 (.030-.070)	4	0.20
Strome	.132 (.11-.16)	.237 (.15-.32)	21	1.05
Airdrie	.158 (.14-.17)	.212 (.18-.25)	11	0.55
Edmonton	.152 (.10-.25)	.465 (.43-.50)	63	3.13
Waskatenau	.113 (.10-.13)	.143 (.11-.17)	6	0.30
Lac La Biche	.218 (.21-.23)	.270 (.19-.32)	10	0.52
Breton	.168 (.13-.18)	.228 (.16-.29)	12	0.60

*Range of four determinations.

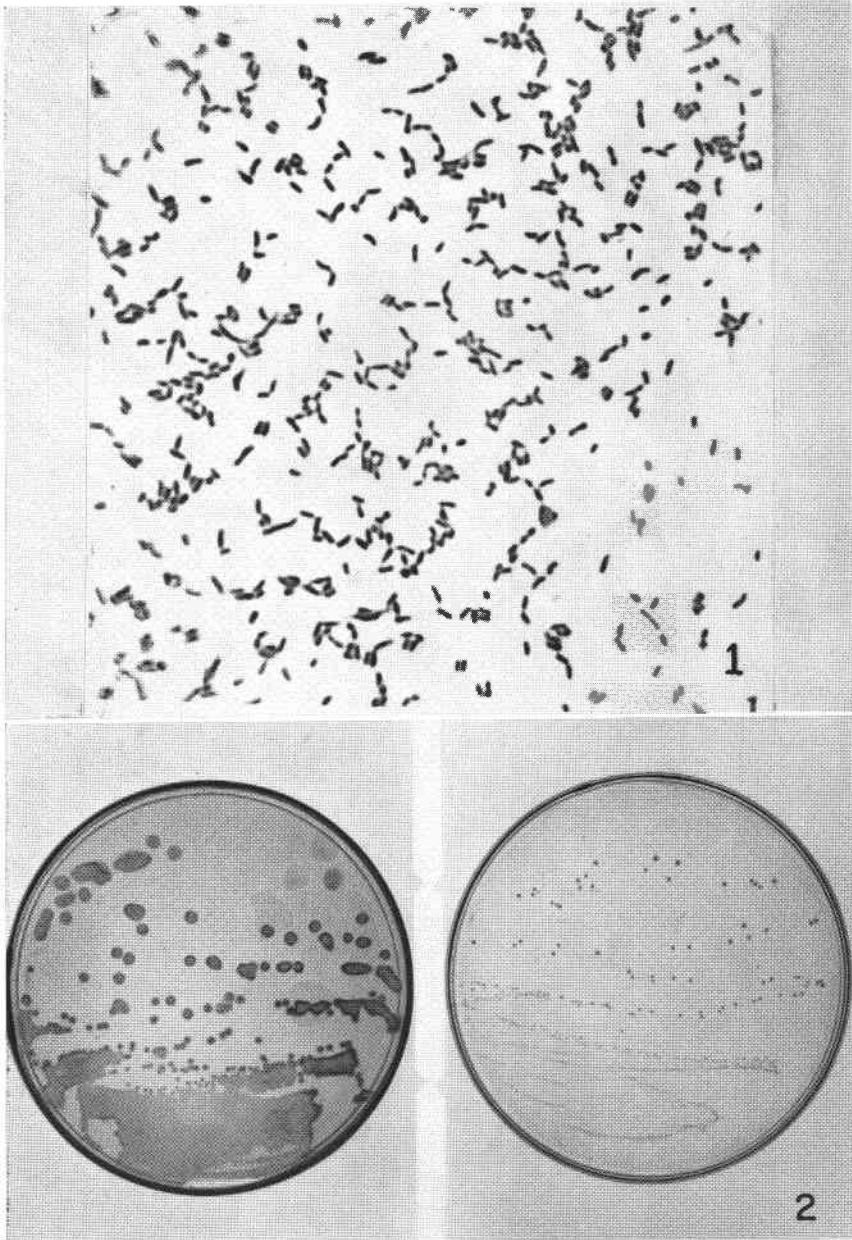


FIG. 1. Microscopic morphology of nitrogen-fixing pseudomonads.

FIG. 2. Growth of nitrogen-fixing organisms on nitrogen-free media; (A) *Azotobacter*, (B) *Pseudomonas*.

acidic pH values. *Azotobacter* did not develop below a pH of 6.2 whereas the growth of the smaller nitrogen-fixing organisms, although scant, extended from pH 4.9 to 7.9.

The optimum growth temperature for *Pseudomonas* was 25° to 28° C and growth could be initiated at temperatures as low as 8° C. The thermal death temperature for a 10-minute exposure was found to lie between 50° and 55° C.

The data obtained from the micro-Kjeldahl determination of the amount of atmospheric nitrogen fixed by isolates from various soils of Alberta are summarized in Table II. Non-symbiotic nitrogen fixation is usually considered to be growth bound. Therefore, visual observations of growth should be a fairly safe criterion for determining the ability of an organism to fix nitrogen. Ascertaining the extent of growth with organisms such as these is, however, difficult because of the extensive amounts of slime produced on nitrogen-free media. This is demonstrated by the variation in the nitrogen content of the different isolates shown in the first column of Table II. The inocula for all the isolates had been adjusted to 15% transmittance at the maximum adsorption wave length of 585 μ . The results in Table II show that the nitrogen fixed in 5 ml of 2% mannitol varied from 4 to 78 μ g/ml. The fixation per gram of mannitol therefore ranged from 0.2 to 3.9 mg N per gram of mannitol. Fixation was statistically significant at or above the 5% level of significance for all the cultures tested.

Nitrogen-fixing Organisms in Saskatchewan Soils

The studies of the non-symbiotic nitrogen-fixing organisms in Alberta soils had been conducted with soils collected in 1954 and 1955. In the spring of 1959 the stored soil samples were again tested for the occurrence of organisms capable of developing on a nitrogen-free medium. *Azotobacter* had not survived storage, but the small Gram-negative pseudomonads were still abundant.

To verify and extend the findings obtained from Alberta soils, samples from virgin and cultivated sites of the brown, dark brown, and black soil zones of Saskatchewan were collected and studied. Soils from 16 sites varied in texture from a fine sandy loam to a clay and had pH values of 5.5 to 7.6.

Azotobacter were found in three of the soils studied. A brown solodized-solonetz clay loam having a pH value of 7.6 mapped as a member of the Echo Association, a silty clay loam (Elstow pH 6.4) from the dark brown soil zone, and a similar orthic member (Blaine Lake pH 6.6) from the black soil zone were the only soils from which it was possible to isolate these nitrogen-fixing organisms.

The study of Alberta soils indicated that *Azotobacter* were associated with irrigated soils mainly. Only one irrigated soil was sampled in Saskatchewan. Plots at the South Saskatchewan predevelopment station at Outlook had been irrigated for 10 years but *Azotobacter* were not present in these soils.

Liquid cultures and dust-plate techniques indicated that the soils of Saskatchewan all contained other organisms capable of growing on media which did not contain added nitrogen. The colonies on nitrogen-free glucose agar, some of which reached a size of 5 to 6 mm, were opalescent, raised, viscous with smooth edges. Microscopic observations showed a variety of organisms growing in conjunction with small, hard to stain, Gram-negative rods similar

to those isolated from Alberta soils. Further isolation procedures separated the small rods which were probably the source of nitrogen for the other contaminating organisms. These contaminants were often yeasts and spore-forming rods having a decided tendency to turn Gram-negative with age.

Discussion

The characteristics of the *Azotobacter* found in Alberta and Saskatchewan soils agree with those described in Bergey's Manual (2) by Jensen (4) as those belonging to *A. chroococcum*. The part played by these organisms in the nitrogen economy of the prairie soils is, however, questionable. In Alberta, *Azotobacter* were found primarily in irrigated soils and in Saskatchewan only 3 of 16 dry-land sites sampled contained these nitrogen-fixing organisms. Jensen (4) questioned the ability of *Azotobacter* to add substantial amounts of nitrogen to the soil under field conditions. Their importance even when present in the soils is probably negligible.

Aerobic organisms other than *Azotobacter* have been often described as capable of growing on nitrogen-free media (16, 7, 5). The Kjeldahl method of determining nitrogen in media inoculated with small numbers of the organisms has, however, failed to show conclusive evidence of fixation. Ross (14) in a series of biological studies of some Tussock grassland soils in New Zealand isolated 11 strains of *Bacterium radiobacter* and three strains of organisms described as *Pseudomonas* capable of growth on media containing no added nitrogen. Kjeldahl determination of nitrogen content of the cultures after 28 days' incubation demonstrated that nitrogen fixation was insignificant under the conditions utilized.

More refined techniques have, however, demonstrated that the ability to fix nitrogen is fairly widespread. Proctor and Wilson (12, 13) found that six randomly selected strains of *Pseudomonas* and eight of *Achromobacter* fixed nitrogen. An inducible enzyme system appeared to be involved in the fixation of 1.1 to 4.3 mg N per gram of carbohydrate.

The Gram-negative, nitrogen-fixing rods isolated in this study are similar to those described by Voets and Debacher (17) for a nitrogen-fixing *Pseudomonas* which they called *Pseudomonas azotogensis*. The size, slime production, staining reactions, biochemical properties, and growth on various media were all similar. Anderson (1) has also isolated a similar nitrogen-fixing *Pseudomonas* to which he assigned the species name *azotocolligans*. The two species designations may be reconcilable. They appear to be similar in that they fix approximately 1 to 4 mg of atmospheric nitrogen per gram of mannitol, and have identical characteristics except for variations in H₂S formation and pigment production.

Acknowledgments

Grateful acknowledgment is accorded to Dr. G. R. Anderson, Bacteriology Department, University of Idaho, for supplying cultures of *P. azotocolligans*, and to J. P. Voets, Rijklandbouwhogeschool, Ghent, Belgium, for the culture of *P. azotogensis*. This work was supported in part by the National Research Council, Ottawa, Canada.

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