Nitrogen-fixers—*Pseudomonads* and other Aerobic Bacteria—from Rhodesian Soils

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SUMMARY

Very numerous small transparent colonies appeared on plates of nitrogen-poor agar inoculated with soils from various places in Rhodesia. Eighty-three cultures of bacteria were isolated from these colonies, all but two of them small Gram-negative rods. Two cultures contained Gram-positive cocci. Thirty-nine of the isolates fixed nitrogen, ten of them fixing as much as or more than a *Beijerinckia* culture grown under the same conditions. The Gram-negative isolates are not all alike; their identity is uncertain, but some might be *Pseudomonas* species, some *Achromobacter* (or *Acinetobacter*), and one good fixer might be a *Flavobacterium*. There are also a few isolates which attack glucose fermentatively. Nitrogen-fixers of these types—aerobic bacteria with very small colonies—appear to be numerous and widely distributed in Rhodesian soils.

INTRODUCTION

In the course of ecological studies on bacteria of various physiological groups in soils in the neighbourhood of Salisbury, Rhodesia (Meiklejohn, 1965, 1967), plates were prepared for counting *Azotobacter* and *Beijerinckia* species by Jensen's method (1940). These plates, of nitrogen-poor sucrose agar, were examined after two or three days of incubation, to see whether *Azotobacter* colonies had developed. On many of the plates, prepared from soils of varying properties and from many different sites, numerous very small transparent colonies appeared during the first 2 or 3 days. As these small colonies grew abundantly on nitrogen-poor agar, it was thought possible that some of them might be nitrogen-fixers.

In some preliminary experiments, an isolate from such a small transparent colony (culture B), derived from soil sample 10 (see Table 3), was compared for nitrogen-fixing ability with a *Beijerinckia* culture isolated from the same soil sample. The results of two such experiments, testing different concentrations of molybdenum and of calcium, are given in Table 1. From this table it is apparent that culture B fixed about as much nitrogen as the *Beijerinckia* culture, and that, like *Beijerinckia*, culture B seemed to require molybdenum, but not calcium, when grown in a nitrogen-poor medium.

Culture B was found not to be a pure culture, so it was purified, and cultures 1, 7 and 10 were derived from it. These were all found to be nitrogen-fixers (see Table 3), and were used as the first cultures of a series systematically isolated from soil samples and tested for their ability to fix nitrogen (or rather, for ability to fix enough nitrogen to be detectable by a Kjeldahl determination).

METHODS

Isolation of cultures

Forty-nine samples of surface soil (down to 4 in.) were collected from selected sites in the following seven places: Atlantica Ecological Research Station, 16 miles W. of Salisbury; College Farm, in the Mazoe valley, 24 miles N.; Glen Rosa farm, near Raffingora, 90 miles N.; Grasslands Research Station, Marandellas, 50 miles S.E.; Henderson Research Station, Mazoe valley, 20 miles N.; Mount Pleasant vlei, and the University College site (U.C.R.N.), both in the northern outskirts of Salisbury.

Table 1. *Effect of molybdenum, and of calcium, on nitrogen fixation by culture B and a Beijerinckia culture*

Medium	Total nitrogen (mg./25 ml.)					Nitrogen fixed	
	Control (not inoculated)	Beijerinckia		Culture B		(mg./25 ml.) Beijerinckia	(mean) Culture B
		(1)	(2)	(1)	(2)		
+ 2 p.p.m. Mo	1.75	11.06	—	12.25	11.73	9.31	10.24
+ 0.01 p.p.m. Mo	1.68	8.16	10.50	7.88	7.00	7.65	5.76
No Mo	1.68	4.38	3.94	5.61	3.05	2.48	2.65
Sucrose medium without yeast extract. Cultures incubated 25 days at 30°.							
+ 100 p.p.m. Ca	1.75	6.13	7.28	4.94	4.46	4.95	2.95
+ 20 p.p.m. Ca	1.60	6.70	6.55	4.81	4.38	5.03	2.99
No Ca	1.81	4.08	6.34	3.94	5.83	3.40	3.07

Sucrose medium without yeast extract. Cultures incubated 28 days at 30°.

Suspensions of 1/10, 1/40 and 1/160 in sterile water were made from each soil sample, and 1 ml. portions spread over the surface of pre-dried agar plates (Jensen, 1940). The plates were incubated at 30° for 2 days, and then colonies were picked to agar slopes, and afterwards purified by making poured plates and picking colonies again. The medium used for the isolation and purification of the cultures was the 'sucrose agar' of Brown, Burlingham & Jackson (1962) (g./l.): sucrose, 5; K₂HPO₄, 0.8; MgSO₄·7H₂O, 0.2; FeSO₄·7H₂O, 0.04; CaCl₂·2H₂O, 0.5; Na₂MoO₄·2H₂O 0.005; agar 15; deionized water, 1000 ml.

Stock cultures were maintained on the same medium, but with the addition of 10 ml 0.1% yeast extract (Difco) per litre.

Testing for nitrogen fixation

Culture conditions: The isolates were tested in batches, by growing them on a nitrogen-poor liquid sucrose medium, of the same composition as the medium used for plates, without agar but with yeast extract. Forty ml. of this medium in a 250 ml. conical flask was inoculated with 1 ml. of a suspension made by emulsifying the growth on a 7-day-old agar slope culture with 10 ml. sterile liquid medium. The flasks were stoppered with cotton wool, which was covered with metal foil, and were incubated at 30° for 5 weeks. With each batch of cultures, a Beijerinckia culture prepared in the same way and a flask of uninoculated sterile medium were also incubated.

Estimates of total nitrogen were made by Kjeldahl digestion, followed by determination of the ammonia formed by the micro-diffusion method of Conway (1950).

Ten ml. samples of the cultures, and of the uninoculated control, were digested with sulphuric acid, with selenium dioxide as a catalyst. After cooling, the contents of each Kjeldahl flask were diluted to 50 ml., and 1 ml. samples of this taken for ammonia determinations. Three Conway units were prepared for each culture, and for the control, and 2 blank units, with reagents but no test solution, were also prepared. All the units were incubated overnight at 25° and the ammonia estimated by titration with 0.01 M-hydrochloric acid.

Because the uninoculated controls always contained a small amount of nitrogen, the estimates from the inoculated flasks were corrected for this amount, to give an estimate of nitrogen fixed (in 5 weeks at 30°). In the tables this is given as mg. nitrogen/25 ml. culture.

Observations and tests on nutrient agar slope cultures

The isolates were subcultured to nutrient agar (Oxoid) slopes for observations and biochemical tests. The following observations were made: *colour* of growth on slope; *motility*, on hanging drop from a 24 hr culture; *Gram stain*, *cell length* and *shape*, on Gram-stained films.

Biochemical tests made by methods described by Cowan & Steel (1965) were:

Oxidation-fermentation (O/F) test—Hugh & Leifson's medium with 1% glucose, incubated at 28° for up to 14 days, *Escherichia coli* as fermentative control.

Catalase activity—a few drops of 3% hydrogen peroxide run down a 24–72 hr slope, *Staphylococcus albus* as positive control.

Oxidase activity by Kovacs method; *Pseudomonas aeruginosa* as positive control, *E. coli* as negative control.

Gelatin liquefaction by Frazier method: *P. aeruginosa* as positive control, *E. coli* and an uninoculated plate as negative controls.

Nitrate reduction was tested on tubes of Meiklejohn medium (1940) containing Durham tubes, which were incubated at 30° for 7 days, and then examined for gas formation and tested for nitrate with Griess-Ilosvay reagent. The medium contained (g./l.): KNO₃, 1; glycerol, 3.6; KH₂PO₄, 0.5; K₂HPO₄, 0.5; CaCl₂·2H₂O, 0.1; NaCl, 0.1; MgSO₄·7H₂O, 0.3; FeCl₃, 0.01; deionized water, 1000 ml.

RESULTS

The nitrogen-poor agar plates made from all the 49 soil samples were covered with very numerous tiny colonies after two days' incubation at 30°. The colonies were raised and transparent, looking like little drops of clear syrup on the agar surface. Plates inoculated with 1/10 and 1/40 suspensions were too crowded to count, but an approximate count could usually be done on plates made from 1/160 suspensions. Table 2 gives approximate counts from 14 soil samples, which give rough estimates of 32,000 to 128,000 organisms per gram soil. All the samples but one also contained *Beijerinckia* sp., which were very much fewer than the small-colony organisms.

Nitrogen-fixing ability of isolates

Eighty-three cultures were isolated from the small transparent colonies, and all of them were bacteria. Most of them grew very slowly in the liquid medium used to test for nitrogen fixation, and it was therefore assumed that they were slow fixers, and the

incubation period was prolonged to 5 weeks. Nitrogen determinations at the end of 5 weeks showed that 39 of the 83 isolates could fix nitrogen; there were 10 good fixers (as good as *Beijerinckia* or better), 19 moderately good, and 10 poor fixers.

Soils yielding good nitrogen-fixers. There were 7 soil samples which yielded good nitrogen-fixers (Table 3). They had three things in common: none of them was very

Table 2. *Numbers of small transparent colonies and of Beijerinckia*

Soil sample	Place	Colonies per plate 1/160 dilution (approx.)	Estimated numbers per gram soil	<i>Beijerinckia</i> per gram soil
416	Henderson	500	80,000	29
420	Henderson	400	64,000	0
433	College Farm	800	128,000	1,905
434	College Farm	400	64,000	13,000
556	College Farm	200	32,000	288
557	College Farm	300	48,000	465
558	College Farm	200	32,000	645
559	College Farm	300	48,000	542
560	College Farm	300	48,000	606
562	College Farm	200	32,000	5,143
573	Glen Rosa	200	32,000	384
574	Glen Rosa	200	32,000	5,362
575	Glen Rosa	500	80,000	2,055
577	Glen Rosa	300	48,000	438

Table 3. *Soil samples yielding good nitrogen-fixers*

Sample	pH	Place: type of soil	Cultures isolated	N fixed (mg./25 ml.)
456	7.6	Atlantica, termite md. nr. stream, brown clay loam	456 B	5.60
425	6.8	Henderson, Rhodes grass pasture, yellow-brown clay loam	425	3.62
430	6.4	Henderson, Rhodes grass pasture, red clay loam	430	3.56
10	6.4	Atlantica, grass near stream, dark brown loam	10 1 52 A 7	3.50 2.45 2.03 1.35
420	6.0	Henderson, Panicum pasture, brown clay loam	420	3.40
537	6.2	Atlantica, under <i>Brachystegia spiciformis</i> , brown sandy loam	537 B 537 A	2.75 2.70
536	7.2	Atlantica, under <i>Combretum molle</i> , brown loam	536 B 536 A	2.22 1.75
423	6.2	Henderson, Panicum pasture, yellow-brown clay loam	Beijerinckia	2.80*
428	6.4	Henderson, <i>Desmodium discolor</i> , light brown clay loam	Beijerinckia	2.24†

* Mean of 7 determinations; † Mean of 8 determinations.

acid, all but one (537) were heavy soils, and all were covered with permanent vegetation. Four of the 7 were under grass, 2 were from among the roots of trees, and one (456) was from a termite mound which had bushes and trees, including a large *Acacia galpinii*, growing on it.

The 10 good nitrogen-fixers fixed from 2.03 to 5.60 mg. N/25 ml. culture in 5 weeks at 30°; average nitrogen fixation by the two *Beijerinckia* cultures grown under the same conditions was 2.80 mg. N/25 ml., and 2.24 mg. N/25 ml.

Table 4. *Soil samples yielding moderate nitrogen-fixers*

Sample	pH	Place: type of soil	Cultures isolated	N fixed (mg./25 ml.)
569	6.2	UCRN, sprouting grass, brown organic	569B	1.98
451	6.0	Grasslands, nr. river, dark brown clay	451	1.94
559	6.1	College farm, Rhodes grass, brownish yellow loam	{ 559A 559B	1.93 1.40
574	6.6	Glen Rosa, burnt bush, brown organic	574B	1.93
454	7.6	Atlantica, Termite md. in wood, light brown clay	454	1.89
560	6.4	College farm, Rhodes grass, yellow-brown loam	560A	1.80
562	6.1	College farm, Rhodes grass, red-brown clay loam	562A	1.80
571	6.2	UCRN, mown grass, brown loam	571B	1.75
557	6.2	College farm, Rhodes grass, brownish yellow loam	557B	1.68
556	6.1	College farm, Rhodes grass, brownish yellow loam	556B	1.63
577	6.4	Glen Rosa, grass fallow, light brown sandy loam	577A	1.58
539	6.4	Atlantica, under <i>Terminalia sericea</i> , brown organic	539A	1.52
433	6.0	College farm, burnt grass, brown sandy loam	433	1.23
570	6.5	UCRN: Termite md., brown clay loam	{ 570B 570A	1.17 0.96
573	6.2	Glen Rosa, grass after maize, light brown loam	{ 573B 573A	1.05 0.88
575	7.2	Glen Rosa, bush, dark brown organic	575A	1.00

Table 5. *Soil samples yielding poor nitrogen-fixers, or none*

Sample	pH	Place: type of soil	Cultures isolated	N fixed (mg./25 ml.)
434	6.0	College farm, wood, brown sandy loam	434B	0.81
416	5.8	Henderson, pasture, dark brown organic	416	0.63
566	6.0	UCRN, top end, mown grass, brown organic	566A	0.58
314	c. 5.6	Grasslands, maize, brown sandy	34A	0.53
558	6.2	College farm, Rhodes grass, brownish yellow loam	{ 558A 558B	0.53 0.53
450	6.1	Grasslands, Hyparrhenia, brown sandy	450B	0.40
457	6.6	Atlantica, Hyparrhenia, brown loam	457B	0.35

Soil samples yielding only isolates that did not fix nitrogen: 2 from Atlantica, 4 from College Farm, 2 from Glen Rosa, 2 from Grasslands, 5 from Henderson, 2 from Mount Pleasant vlel, 2 from University College site (UCRN). Total 19.

Table 6. *Characteristics of the nitrogen-fixing isolates*

Group	Culture no.	Gram reaction	Shape	Cell length (μ , approx)	Motility	Colour (nutrient agar)	Reactions in tests*					
							O/F test	Catalase	Oxidase	Nitrate to nitrite	Gelatin liquefaction	N-fixing ability†
A 1	‡425	—	Oval rod	1.0	+	Yellow	F	+	—	+	—	Good
	539A	—	Short rod	1.2	+	Orange	F	+	—	+	—	Moderate
	573A	—	Short rod	1.3	—	Orange	F	+	—	—	—	Poor
	573B	—	Short rod	1.3	—	Orange	F	+	—	+	—	Moderate
A 2	571B	—	Short rod	1.3	—	Pink	(F)	+	+	+	+	Moderate
B 1	10	—	Short rod	1.0	+	Buff	(O)	+	—	+	—	Good
	430	—	Short rod	1.0	+	Buff	(O)	+	+	+	—	Good
	433	—	Short rod	1.0	+	Buff	O	+	(+)	—	—	Moderate
B 2	559A	—	Short rod	1.1	+	Green	O	+	+	—	+	Moderate
	559B	—	Short rod	1.1	+	Green	O	+	+	—	+	Moderate
	560A	—	Short rod	1.0	+	Green	O	+	+	—	+	Moderate
B 3	34A	—	Short rod	1.0	+	Yellow	O	?	?	+	+	Poor
	434B	—	Short rod	1.0	+	Yellow	O	+	+	—	+	Poor
	457B	—	Oval rod	1.0	+	Yellow	O	+	—	—	+	Poor
B 4	52A	—	Short rod	1.0	+	White	O	+	+	—	—	Good
	416	—	Short rod	1.0	+	Grey	(O)	—	+	—	—	Poor
	556B	—	Short rod	1.4	+	Grey	O	+	+	—	—	Moderate
	558B	—	Short rod	1.1	+	Grey	(O)	+	+	—	—	Poor
	562A	—	Oval rod	0.8	+	Grey	(O)	+	+	—	—	Moderate
	566A	—	Short rod	1.1	+	Grey	O	(+)	—	—	+	Poor
B 5	450B	—	Short rod	1.0	+	Grey	O	?	?	+	+	Poor
	451	—	Short rod	1.0	+	Grey	O	?	?	+	+	Moderate
	454	—	Rod	1.0	+	Grey	N	(+)	—	+	+	Moderate
	537A	—	Oval rod	0.5	+	Grey	(O)	+	+	+	+	Good
	557B	—	Short rod	1.1	+	Grey	(O)	+	+	+	—	Moderate
C 1	456B	—	Short rod	1.0	—	Yellow	(O)	+	+	—	—	Good
C 2	1	—	Short rod	1.0	—	Buff	(O)	+	(+)	—	—	Good
	7	—	Short rod	1.0	—	Buff	(O)	+	(+)	—	+	Moderate
	536B	—	Short rod	1.6	—	Buff	N	(+)	+	—	—	Good
	558A	—	Short rod	0.7	—	Grey	N	+	+	—	—	Poor
	569B	—	Short rod	1.1	—	Grey	(O)	+	(+)	—	—	Moderate
	575A	—	Short rod	1.0	—	Grey	N	+	+	+	—	Moderate
	577A	—	Short rod	1.7	—	Grey	O	+	+	—	+	Moderate
C 3	420	—	Short rod	1.0	—	Yellow	N	(+)	—	—	—	Good
	536A	—	Short rod	0.8	—	Green	N	+	—	—	+	Moderate
	537B	—	Rod	1.5	—	White	N	+	—	+	—	Good
	574B	—	Oval rod	0.7	—	Grey	N	—	—	—	—	Moderate
D	§570A	+	Coccus	2.8	—	Buff	(F)	+	—	—	—	Poor
	§570B	+	Coccus	2.8	—	Buff	(F)	+	—	+	—	Moderate

* Reactions in tests: F, fermentative attack; (F) slow fermentative attack; O, oxidative attack; (O), slow oxidative attack; N, no attack; +, positive; (+), weakly positive; —, negative.

† Nitrogen-fixing ability: for details see Tables 3–5.

‡ Culture 425 gives acid on glucose and sucrose, not on lactose (1% in peptone water).

§ Cultures 570A and 570B contained Gram-negative cells as well as Gram-positive.

Soils yielding moderate nitrogen-fixers. Two moderate nitrogen-fixers (7 and 536A) were obtained from soils that also yielded good fixers. The other 17 isolates in this group came from 16 soil samples of differing properties, with nothing in common except that none was very acid (Table 4). The 19 isolates classed as moderate nitrogen-fixers fixed from 1 to 1.98 mg. N/25 ml.; that is, their performance ranged from strains fixing nearly as much nitrogen as *Beijerinckia* to strains fixing only half as much.

Soils yielding poor nitrogen-fixers, or none. Seven soil samples, of differing properties, yielded isolates of poor nitrogen-fixing ability (less than 1 mg. N/25 ml.). There were also 19 soil samples from which no nitrogen-fixers of the small-colony type were isolated, though they all contained *Beijerinckia* species.

Characteristics of the isolates

On the nitrogen-poor sucrose agar all the isolates looked much alike, with tiny glassy colonies on plates, and colourless gummy streaks on slopes. But on nutrient agar, on which most of them grew quite well, differences in colour appeared.

The isolates also differed in other respects, and were not a uniform group, though nearly all of them were small Gram-negative rod-shaped bacteria. Two cultures only, from soil sample 570, contained large Gram-positive cocci (group D). The Gram-negative isolates can be divided into three groups, A, B and C, which can be further subdivided (see Table 6):

Group A: attacking glucose fermentatively in the O/F test.

- A 1 Fast fermenters
- A 2 Slow fermenters

Group B: attacking glucose oxidatively or not at all: motile.

- B 1 Buff on nutrient agar
- B 2 Green on nutrient agar
- B 3 Yellow on nutrient agar
- B 4 White or grey: nitrate not reduced
- B 5 Grey: nitrate reduced to nitrite

Group C: attacking glucose oxidatively or not at all: non-motile.

- C 1 Yellow on nutrient agar: oxidase-positive
- C 2 Buff or grey: oxidase-positive
- C 3 Oxidase-negative

Nitrogen-fixing ability does not seem to be connected with any other of the properties studied; 13 isolates, 5 of which fix nitrogen, are in group A; group B contains 20 fixers and 20 non-fixers, and group C 12 fixers and 15 non-fixers. Both of the Gram-positive cultures (D) fixed nitrogen, and there was one non-fixer which grew so badly on nutrient agar that it could not be grouped. The data for the 39 nitrogen-fixers are given in Table 6, and it can be seen that good, moderate and poor fixers are found in all of the groups A, B and C.

DISCUSSION

The bacteria which form small transparent colonies on nitrogen-poor sucrose agar were found in many soil samples collected from various places in eastern Rhodesia, including all of the 49 samples described in this paper. The numbers of small colonies

(Table 2) indicate a large population, of tens of thousands per gram of soil. Since nearly half of the eighty-three colonies picked at random contained nitrogen-fixers, it is probable that aerobic nitrogen-fixers of this type are both widespread and abundant in eastern Rhodesian soils, and account for more nitrogen fixation than organisms from either of the genera *Azotobacter* or *Beijerinckia*.

Species of the genus *Azotobacter* are rare in eastern Rhodesian soils, probably because most soils are too acid. We have found *A. chroococcum* at Henderson, but consistently in one soil only, a red clay from the bottom of the Mazoe valley. Species of the genus *Beijerinckia* are widespread in Rhodesian soils, but usually in small numbers (Table 2), and seem to be more numerous in poor soils (Meiklejohn, 1965). They are slow fixers of nitrogen, and no more efficient than the best of the new isolates. The average nitrogen fixed by the two *Beijerinckia* cultures used as controls in this work was 2.80 mg. and 2.24 mg. N/25 ml. Ten of the small-colony isolates fixed more than 2 mg. N/25 ml., the best of all, culture 456B, fixing 5.6 mg. N/25 ml.

The identity of the new isolates is doubtful. They are not all alike (Table 6), but none of them can be identified as a species of the genera *Azotobacter* or *Beijerinckia*, or as *Derxia gummosa* (Jensen, Petersen, De & Bhattacharya, 1960), from all of which they differ in colony type, in cell size, and in other characters.

The new isolates are smaller in cell size than any species of *Azotobacter*, even *A. vinelandii* (Jensen, 1954), except for the two in group D, which are Gram-positive. The isolates in groups A, B and C are all small rods, the largest being only $1.7\ \mu$ long. *Azotobacter chroococcum* forms colonies on nitrogen-poor sucrose agar in 2 to 3 days (Brown *et al.* 1962), but the colonies are large and opaque, and turn brown after some time, unlike the small glassy colourless colonies of the new isolates.

Beijerinckia colonies, unlike those of the new isolates, are very slow to appear on sucrose agar plates. They are usually not visible until after 10–14 days' incubation at 30°. They are white, opaque, and very sticky, even rubbery (Jensen, 1954). *Beijerinckia* organisms have a very characteristic appearance, with an oil drop at each end, which was never shown by any of the new isolates.

Derxia gummosa (Jensen *et al.* 1960) is a slow nitrogen-fixer isolated from an Indian soil. It forms yellow colonies, which become large and dark brown later, on nitrogen-poor mannitol agar. The cells are rod-shaped and large, 3–6 by $1-1.2\ \mu$ in young cultures, bigger than any of the new isolates. Also *D. gummosa* is catalase-negative and fermentative, a combination of characters not found in any of the new isolates.

The new isolates, however, resemble the small-celled aerobic nitrogen-fixers described by Skinner (1928), Stapp (1940), Anderson (1955), Voets & Debacker (1956) and V. Jensen (1956). Stapp (1940) isolated a small Gram-negative nitrogen-fixing rod from vegetable material sent from Egypt, and called it *Azotomonas insolita*. It was motile, colourless on nutrient agar, and fermented sugars with acid and gas. Nitrogen-fixing strains of the fermenting species *Aerobacter aerogenes* were isolated by Skinner (1928) in U.S.A., and V. Jensen (1956) in Denmark. (Mahl, Wilson, Fife & Ewing (1965), however, found that several small-celled nitrogen-fixers originally classified as strains of *A. aerogenes* could be identified serologically as types of *Klebsiella pneumoniae*.)

Non-fermenting small-celled aerobic nitrogen-fixers have also been described. In 1955, Anderson found a nitrogen-fixing *Pseudomonas* in soils from Idaho, in which

it was much more abundant than *Azotobacter*. The growth on Martin's nitrogen-poor agar was colourless and transparent at first, becoming yellow or orange after 2 weeks. Anderson called it *Pseudomonas azotocolligans*, n.sp. (Anderson, 1955). In the following year, Voets & Debacker isolated, from a greenhouse soil in Belgium, a non-pigmented *Pseudomonas* which fixed about 1 mg. N/100 ml. medium in 7 days; they called it *P. azotogensis* n.sp. (Voets & Debacker, 1956).

Voets' & Debacker's culture, and 5 other *Pseudomonas* cultures, were examined by Proctor & Wilson (1958), who found that all of them, and also all of eight *Achromobacter* cultures (one of which had been isolated in Denmark by V. Jensen), fixed nitrogen. The aerobic fixation by the *Pseudomonas* cultures was from 3 to 50 μ g./ml. culture in 4 days at 30°. The *Achromobacter* cultures fixed from 3 to 17 μ g. N/ml. under the same conditions.

But, though the new isolates resemble those previously described, they cannot be definitely identified as any of them. The cultures in group A 1, which ferment glucose rapidly in the O/F test, could belong to some genus in the Enterobacteriaceae, as they are fermenting, catalase-positive and oxidase-negative; but not to the genus *Aerobacter* or *Klebsiella*. Nor can they be *Azotomonas insolita*, because they are pigmented on nutrient agar and give no gas on sugars.

The motile, oxidative cultures in group B are nearly all catalase- and oxidase-positive, and could be *Pseudomonas* species, the non-pigmented forms in groups B 4 and B 5 resembling the *P. azotogensis* of Voets & Debacker (1956).

Of the non-motile oxidative forms in group C, culture 456B, which is put by itself in group C 1, forms yellow pigment on nutrient agar and might be a *Flavobacterium* (Table 6). This culture was the best nitrogen-fixer of the new isolates, (5.6 mg. N/25 ml.). The cultures in group C 2, which are oxidase-positive, could belong to the genus *Achromobacter*, and those in group C 3 to the genus *Acinetobacter* (Cowan & Steel, 1965).

The identity of the Gram-positive cocci in the two cultures from sample 570 is a complete puzzle (group D). The cultures also contained Gram-negative cells, and it is possible that they, not the cocci, are the real nitrogen-fixers.

It may seem rather extraordinary that we should have isolated 39 cultures of nitrogen-fixers, and also 44 cultures which resembled one or other of the 39 in every respect save that they could not fix nitrogen. This, however, has been observed by Skinner (1928), who isolated 23 strains of *Aerobacter aerogenes*, only 3 of which fixed nitrogen, and by V. Jensen (1956). He had two *A. aerogenes* strains which were identical, except that one fixed nitrogen and the other did not. Mahl *et al.* (1965), in addition to their nitrogen-fixing strains of *Klebsiella pneumoniae*, tested 19 strains which were found not to fix nitrogen.

It might be asked why, since these small-celled aerobic nitrogen-fixers seem to be abundant and widespread in Rhodesian soils, nothing of the kind has been observed elsewhere, except by Anderson (1955) in Idaho. The reason may well be that this type of nitrogen-fixer either does not occur, or is very rare, in soils in Europe, and perhaps in northern North America too. Brown *et al.* (1962) found that nitrogen-poor agar plates inoculated on the surface with a variety of soils from Britain yielded, after 2 or 3 days of incubation, either *Azotobacter* colonies or nothing. There was never any abundant development of small colonies, such as we observed in Rhodesia. Colonies of other organisms than *Azotobacter* sometimes developed on plates inoculated with

soils from Britain, but these did not appear till the plates had been incubated for a week or longer.

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