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The Properties of Novel Mesophilic Denitrifying *Bacillus* Cultures Found in Tropical Soils

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Fifteen endospore-forming, mesophilic, denitrifying cultures were isolated from tropical soil samples by anaerobic enrichment culture in peptone medium containing nitrite. The cultures are of the second morphological group of the genus *Bacillus* and contain 39·1 to 41·5 mol % GC in their DNA. They are, however, phenotypically quite heterogeneous and differ from all previously described *Bacillus* species. Seven of the new isolates can grow anaerobically with nitric oxide as the terminal electron acceptor, a character not previously observed in any bacterium.

INTRODUCTION

Endospore-forming denitrifying bacteria can be isolated from soils by anaerobic enrichment in peptone media fortified with NO_3^-, NO_2^- or N_2O. The species selected depends on the nature of the terminal electron acceptor supplied. Media of high salinity with KNO_3 favour the isolation of *Bacillus licheniformis* because of its high osmotic tolerance (Pichinoty, 1973; Verhoeven, 1952). However, this species, which belongs to morphological group I (Gordon *et al.*, 1973), cannot use NO_2^- or N_2O as electron acceptor under anaerobic conditions; moreover, it generally produces only small quantities of N_2 in peptone medium containing NO_3^-, and some strains do not produce gas after 8 d incubation at 32 or 40 °C (Pichinoty *et al.*, 1978*b*).

We have introduced new selective methods to determine whether there are *Bacillus* species having high denitrifying activity. By using N_2O instead of NO_3^-, we have already isolated a new species, *Bacillus azotoformans*, which belongs to morphological group II (Gordon *et al.*, 1973) and which vigorously reduces NO_3^-, NO_2^- and N_2O (Pichinoty *et al.*, 1978*a*). We now describe bacteria isolated in the presence of KNO_2, that also belong to group II but are clearly different from *B. azotoformans*; some preliminary results have already been published (Pichinoty *et al.*, 1978*c*) describing the utilization of NO as terminal electron acceptor by some bacteria and this character has been looked for in numerous denitrifying bacteria.

This work is of some ecological interest as present knowledge of the denitrifying flora of tropical regions is meagre. All the soils used came from rice fields in Senegal which are particularly favourable environments for denitrification (Broadbent & Clark, 1965).

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METHODS

Reference bacterial strains. The following denitrifying bacteria were used: 2 *Pseudomonas aeruginosa* strains from the Collection of the Pasteur Institute; *Pseudomonas fluorescens* strain 108 belonging to biotype B (Stanier *et al.*, 1966); strain 229 (Stanier *et al.*, 1966) and 12 other strains of *Pseudomonas stutzeri* (Pichinoty *et al.*, 1977*c*); strain 291-75 related to *Pseudomonas pickettii* (Garcia *et al.*, 1977); strain 301-75 related to *Pseudomonas lemoignei* (Pichinoty *et al.*, 1977*d*); 7 *Alcaligenes denitrificans* strains and 4 *Alcaligenes odorans* strains (Pichinoty *et al.*, 1978*d*); 6 *Paracoccus denitrificans* strains (Pichinoty *et al.*, 1977*b*); 2 *Agrobacterium* strains (Pichinoty *et al.*, 1977*a*); *Flavobacterium* strain 12-75 (Pichinoty *et al.*, 1976*a*); 15 *B. licheniformis* strains (Pichinoty *et al.*, 1978*b*); 17 *B. azotoformans* strains (Pichinoty *et al.*, 1978*a*).

Soils. Soil samples came from Senegalese rice fields located mainly in two regions, the Casamance in the south and the river region in the north. Samples were air-dried then sifted before use, some having been stored for several years in the laboratory. Table 1 indicates the origin of each sample.

Isolation. Flasks (250 ml) containing 50 ml medium I (see below) were inoculated with 1 g soil and incubated *in vacuo* at 32 °C; after 3 to 5 d, bubbles of N_2 appeared in the liquid and the NO_2^- disappeared. Flasks that did not show abundant gas production were discarded. Plates of nutrient agar were then streaked and incubated aerobically at 32 °C for 48 h. Colonies were transferred to tubes of peptone broth (Difco) containing 0·5 % (w/v) KNO_3. Gas production was noted using inverted vials within the tubes and successive passages were made to select individual isolates with active denitrification capacity. Cultural purity was verified by colonial uniformity and by microscopic examination.

Media. The basal mineral medium contained (per litre): $Na_2HPO_4.12H_2O$, 3·575 g; KH_2PO_4, 0·98 g; $MgSO_4.7H_2O$, 0·03 g; NH_4Cl, 0·5 g; trace metal solution (Pichinoty *et al.*, 1977*d*), 0·2 ml; pH 7·0. To the above basal medium the following additions were made (per litre). Medium I: nutrient broth (Difco), 8 g; yeast extract (Difco), 5 g; KNO_2, 5 g. Medium II: Bacto-peptone (Difco), 1 g; yeast extract (Difco), 0·5 g; glucose (sterilized separately at 110 °C), 5 g. Medium III: nutrient broth (Difco), 8 g; yeast extract (Difco), 5 g; Bacto-tryptone (Difco), 5 g; L-asparagine, 2 g (or sodium acetate, 4 g); pH 8·0, adjusted with 1 M-NaOH. Medium IV: Casamino acids (Difco), 10 g; Bacto-tryptone (Difco), 10 g; sodium acetate, 5 g; biotin, 50 μg; folic acid, 0·1 mg; riboflavin, 5 mg; thiamin, 25 mg; nicotinic acid, 25 mg; pyridoxine, 25 mg; calcium pantothenate, 25 mg; L-tryptophan, 100 mg; DL-threonine, 100 mg; cystine, 10 mg; pH 8·0, adjusted with 1 M-NaOH. Medium V: yeast extract (Difco), 5 g; Biotrypcase (Bio-Mérieux), 10 g; KNO_3 or KNO_2, 5 g. Medium VI: nutrient broth (Difco), 8 g; KNO_3, 5 g. Medium VII: yeast extract (Difco), 4 g.

Gases. N_2, N_2O and NO were of the purest grade from 'L'Air Liquide'.

Morphology. For measuring cells and spores, wet mounts were prepared from 5 d cultures grown on nutrient agar at 32 °C. Flagella were stained by the technique of Rhodes (1958). Capsules were observed in India ink preparations. Cells cultured in medium VII containing 2% (w/v) sodium DL-3-hydroxybutyrate were examined after staining with Sudan Black for the presence of poly-β-hydroxybutyrate. Confirmation of the absence of the latter was obtained by the method of Law & Slepecky (1961).

Extraction of an intracellular pigment. A 7 l fermenter culture was grown aerobically at 32 °C in 0·4% (w/v) yeast extract (Difco) plus 0·2% (w/v) Proteose-peptone (Difco) for 7 h. Exponential phase cells were harvested by centrifuging, washed and lyophilized. The yellow pigment was extractable with cold methanol but not with ethanol, acetone, benzene, petroleum ether or carbon tetrachloride. Methanol was removed *in vacuo*.

Diagnostic tests. All tests were carried out at 32 °C. The following tests were performed as described by Skerman (1967): gelatin liquefaction, hydrolysis of Tween 80, Voges–Proskauer reaction and urease production. The following tests were done as described by Gordon *et al.* (1973): tyrosine decomposition, deamination of phenylalanine, production of indole and of dihydroxyacetone, hydrolysis of hippurate and of starch, pigmentation from glucose and tyrosine, NO_3^- reduction to NO_2^-, growth in the presence of azide, growth at pH 5·6, lysozyme resistance, NaCl tolerance, egg-yolk reaction and catalase production. The oxidase test (Kovacs, 1956) was applied to colonies as well as to cell-free extracts. Nitrite tolerance was determined in peptone broth at pH 7·0 containing 0·25 to 4% (w/v) KNO_2.

Glucose fermentation and oxidation of carbohydrates. For the anaerobic fermentation of glucose, 250 ml flasks containing 50 ml medium II were inoculated from 48 h nutrient agar cultures and then evacuated. Growth was observed after 6 d. Parallel observations were made in tubes of the semi-solid medium of Hugh & Leifson (1953) which, after inoculation, were covered with a 3 cm layer of agar to ensure anaerobiosis. Both procedures yielded the same results. Production of acid from carbohydrates under aerobic conditions was determined by the method of Hugh & Leifson (1953).

Utilization of organic acids. The medium used contained (g l^{-1}): NaCl, 1; $MgSO_4.7H_2O$, 0·2; KH_2PO_4, 0·5; NH_4Cl, 0·5; yeast extract (Difco), 1; agar, 15; organic acid, 2; phenol red, 0·008; pH 6·8, adjusted with

1 M-KOH. The medium was dispensed into tubes, autoclaved and used as slopes which were heavily inoculated from 48 h nutrient agar cultures. Blanks of uninoculated slopes were incubated as well as inoculated controls lacking any organic acid additions. After incubation for 4 to 6 d, positive reactions were indicated by a distinct redness compared with the controls.

Enzyme activities. Anaerobic cultures were grown in 3 l flasks containing 1 l medium devoid of air. Aerobic cultures were agitated in Fernbach or Erlenmeyer flasks. Cells were harvested after 24 to 48 h at 32 °C. Nitrate reductases A and B (Pichinoty & Piéchaud, 1968) and respiratory nitrite reductase (Miyata & Mori, 1968) were assayed by manometric techniques in extracts of cells grown anaerobically in medium VI. Dehydrogenases for L-glutamate (EC 1.4.1.4), L-alanine (EC 1.4.1.1), and L-leucine (EC 1.4.1.9) were determined spectrophotometrically (Hong *et al.*, 1959; Sanwal & Zink, 1961) with extracts of cells grown aerobically in medium VII. L-Arginine iminohydrolase (EC 3.5.3.6) activity was measured in these cells (Stanier *et al.*, 1966). β-Galactosidase (EC 3.2.1.23) was measured (Hestrin *et al.*, 1955) in extracts of cells grown aerobically in medium VII containing 0·4% (w/v) lactose.

Enzyme extracts. Cells, grown as described above, were harvested and washed by centrifugation, resuspended in water and disrupted ultrasonically for 2 min at 0 °C at 5 A using an MSE ultrasonic disintegrator. Cell debris was removed by centrifuging at 25000 *g* for 10 min at 4 °C.

Cytochrome spectra. Organisms were cultivated in medium VII aerobically, or anaerobically in the presence of 0·5% (w/v) KNO_3 for 24 to 48 h. Reduced–oxidized spectra of the cytochromes in the cell suspensions were obtained in a Cary 15 spectrophotometer at the temperature of liquid nitrogen using Plexiglass cuvettes of 3 mm light path. Sodium dithionite was added to one cuvette and H_2O_2 to the other. Spectra were also obtained at room temperature in the presence of CO with the addition of dithionite using 10 mm path length cuvettes.

Denitrification studies. Cultures were grown anaerobically in medium V for 48 h. Reactions were carried out in 125 ml serum bottles containing: cell suspension, 1 ml; yeast extract (Difco), 500 mg; potassium phosphate buffer pH 7·0, 450 μmol; chloramphenicol, 2·5 mg; KNO_3 or KNO_2, 50 mg; water, to 25 ml; the gaseous phase consisted of 98 ml helium and 2 ml krypton. In some experiments, the NO_3^- or NO_2^- was omitted and 10 ml NO or N_2O was added to the gaseous phase. The bottles were agitated in a water bath at 37 °C. Samples of gas (1 ml) were removed at 40 min intervals over 6 h and analysed by gas chromatography (Garcia, 1974). Specific activities were calculated from the rates of production or consumption of each gas. These were expressed in μl (22 °C, normal atmospheric pressure) per mg cellular nitrogen per hour. Nitrogen contents of cells were measured by the micro-Kjeldahl method (Bailey, 1967).

Growth studies. Growth was observed for up to 13 h in conical 1 l flasks containing 450 ml medium IV. For anaerobic cultivation, an over-pressure of about 250 g was maintained in the stoppered flasks during the experiment and samples were taken through capillary tubes equipped with stopcocks. Cultures were agitated in a water bath. When added, KNO_3 or KNO_2 were at 0·2% (w/v) using N_2 as gas. Nitrite production was measured by the assay of Rider & Mellon (1946) using 1 ml culture added to 3 ml acetone. Other anaerobic cultures were effected under N_2O or 10% (v/v) NO in N_2. Growth was estimated turbidimetrically at 600 nm (1 cm light path): an absorbance of 0·5 was equivalent to 220 mg dry wt cells l^{-1}. Aerobic cultures and cultures with NO were inoculated from 24 h aerobic starter cultures in medium IV; cultures containing NO_3^-, NO_2^- or N_2O were inoculated from anaerobic starters grown with the same electron acceptor. Cell counts were made with a Coulter counter and as colony-forming units on nutrient agar plates.

Base composition of DNA. DNA was extracted and purified by the method of Marmur (1961) from cells grown on medium VII for 12 h at 32 °C. GC contents were calculated from the buoyant densities in CsCl by analytical ultracentrifugation relative to an internal reference standard of DNA from bacteriophage 2C (ρ_{CsCl} = 1·742 g cm^{-3}) (Mandel *et al.*, 1968; Schildkraut *et al.*, 1962).

RESULTS

Isolation

Although pasteurization was not initially practised, inoculated vessels in later experiments were held at 80 °C for 10 min to eliminate non-spore-forming bacteria. Fifteen cultures designated RS1 to RS15 were isolated from different soil sources (Table 1). They have been maintained on nutrient agar slopes and lyophilized samples of each are conserved at the Collection of the Pasteur Institute (Table 1).

Morphology

Isolated bacteria were all rods (1·5 to 7·0 × 0·4 to 0·5 μm) with rounded ends giving variable Gram-staining. Motility was difficult to observe in wet mounts. Those cultures which

Table 1. *Origin and some properties of the strains isolated from Senegalese soils*

Strain	Origin of the soil (locality; region)	Maximum growth temp. (°C)	Tolerance to NO_3^- (mM)	GC (mol %)	CIP* accession no.
RS1	Loudia; Casamance	41	117	40·8	R902
RS2	Boundoum; river region	41	117	40·3	R903
RS3	Bambato; Casamance	42	117	39·8	R904
RS4	Tanaff; Casamance	41	117	39·8	R905
RS5	Oussouye; Casamance	41	117	41·3	R906
RS6	Djibelor; Casamance	41	117	40·8	R907
RS7	Djibelor; Casamance	42	117	40·8	R908
RS8	Sébikotane; Cap-Vert	41	117	41·5	R909
RS9	Keur-Diallo; river region	42	59	40·3	R899
RS10	Diango; Casamance	43	411	39·5	R895
RS11	Bounkilinn; Casamance	41	294	40·8	R896
RS12	Ntiagar; river region	45	411	39·1	R897
RS13	River region	44	411	39·1	R898
RS14	Boundoum Dam; river region	42	117	41·3	R900
RS15	M'Bang; river region	45	353	41·3	R901

* CIP, Collection de l'Institut Pasteur.

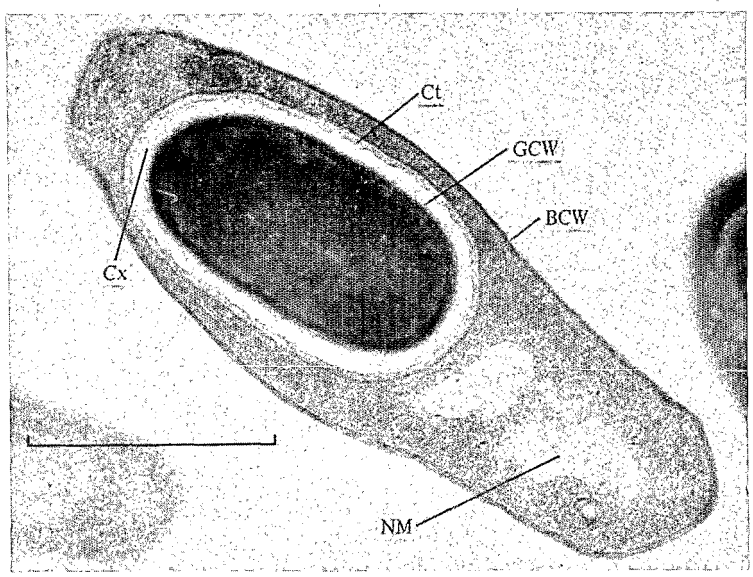

Fig. 1. Ultrathin section of a strain RS5 cell with an endospore: BCW, bacterial cell wall; NM, nuclear material; Ct, spore coat; Cx, cortex; GCW, germ cell wall. Bar marker represents 1 μm.

were motile had only a few flagella arranged peritrichously. Ultrathin sections of cells of strain RS5 showed a somewhat thin cell wall structure characteristic of Gram-positive bacteria (Fig. 1). Endospores (1·1 × 0·6 to 0·8 μm) were oval, distinctly bulged the sporangium and had the typical structure of endospores of *Bacillus*, though with a thinner spore coat. Capsules were never observed.

Growth of isolates in liquid medium was without pellicle formation. Colonies on solid medium varied in appearance according to age. On nutrient agar, colonies of RS10, RS12, RS13, RS14 and RS15 were convex and translucent (Fig. 2*a*); colonies of the other strains were circular and convex with opaque centres and translucent margins (Fig. 2*b*) except RS11 which was pale yellow and entirely opaque.

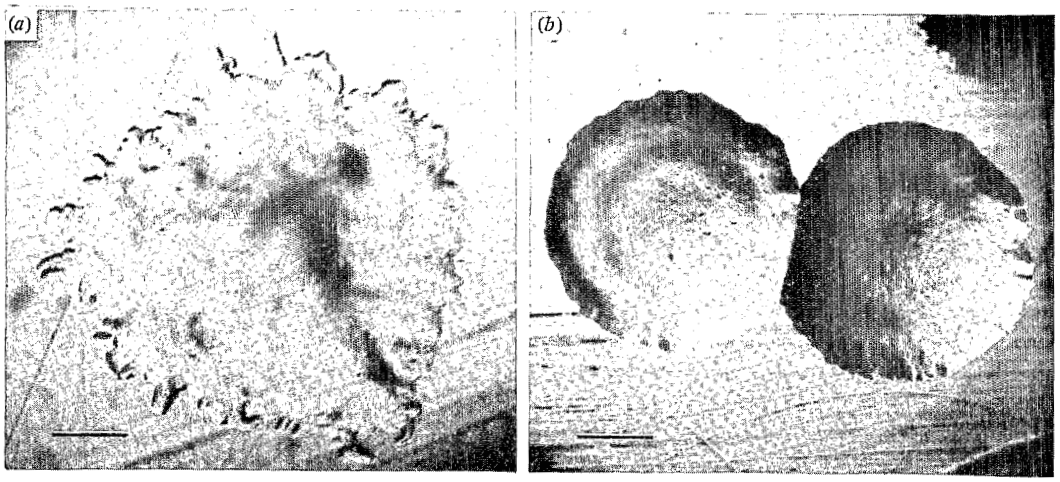

Fig. 2. Colonies of strains RS13 (*a*) and RS8 (*b*) on nutrient agar. Bar markers represent 1 mm.

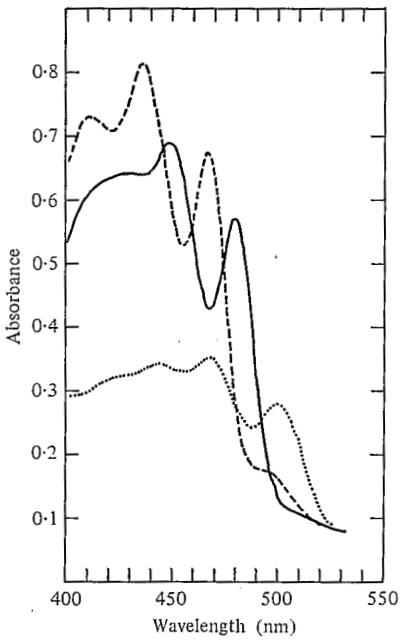

Fig. 3. Absorption spectra of the carotenoid pigment of strain RS9 in benzene (——), petroleum ether (– – –) and carbon disulphide (· · ·).

Properties of the intracellular pigment of strain RS9

Centrifuged pellets of cells of strain RS9 were yellow, though the colour was not seen in colonies. Portions of the cell mass turned blue when suspended in 50% (v/v) H_2SO_4 suggesting the presence of a carotenoid (Skerman, 1967). Spectra are shown in Fig. 3. The pigment exhibited major absorption peaks at 407, 436 and 464 nm in methanol, 427, 448 and 479 nm in benzene, 411, 437 and 467 nm in petroleum ether and 420, 443, 468 and 501 nm in carbon disulphide; these are characteristic of carotenoids (Liaaen-Jensen & Jensen, 1971; Weedon, 1965).

Table 2. *Variable characters*

Character	Total no. of positive strains	Negative strains
Motility and presence of flagella	13	RS3, RS14
Growth on N_2O	12	RS7, RS9, RS14
Growth on NO	7	RS7, RS9, RS10, RS11, RS12, RS13, RS14, RS15
Fermentation of glucose	9	RS10, RS11, RS12, RS13, RS14, RS15
Growth in the presence of 5% (w/v) NaCl	6	All except RS7, RS10, RS11, RS12, RS13, RS15
Growth in the presence of 8% (w/v) KNO_3	12	RS7, RS8, RS9
Growth at pH 5·6	1	All except RS14
Nitrate reductase A	1	All except RS15
Unidentified nitrate reductase	14	RS15
Production of a brown pigment in media containing tyrosine	3	All except RS10, RS12, RS13
Hydrolysis of:		
Hippurate	5	All except RS7, RS11, RS12, RS14, RS15
Gelatin	3	All except RS9, RS11, RS14
Starch	2	All except RS9, RS11
Tween 80	11	RS10, RS11, RS12, RS13
Urea	4	All except RS7, RS11, RS13, RS14
β-Galactosidase	10	RS10, RS12, RS13, RS14, RS15
Arginine iminohydrolase	3	All except RS6, RS8, RS11
L-Glutamate dehydrogenase	14	RS9
Egg-yolk reaction	1	All except RS5
Production of a yellow pigment	1	All except RS9

Physiological characters

All 15 cultures were facultative anaerobes, grew anaerobically in medium VII containing 0·5% (w/v) KNO_3 or 0·2% (w/v) KNO_2 and produced N_2. Anaerobic growth did not occur in this medium if 0·2% (w/v) $K_2S_4O_6$, $Na_2S_2O_3$ or sodium fumarate replaced the NO_3^- or NO_2^-.

Growth at the expense of NO was studied in 250 ml flasks containing 50 ml medium III or IV with an atmosphere of 10% NO in N_2 and sealed with silicone rubber stoppers. The results are presented in Table 2. In medium III, limited anaerobic growth under 100% N_2 was obtained by the fermentation of the carbohydrates present in the yeast extract and consequently medium IV was preferred for this evaluation. The eight cultures listed in Table 2 as not growing in the presence of NO did not do so even if the medium was supplemented with 0·5% (w/v) KNO_3.

All cultures were oxidase positive, possessed cytochrome *c* and catalase (EC 1.11.1.6). All required accessory growth factors (which have not been identified). None grew in peptone media containing 0·02% (w/v) NaN_3 or 0·001% (w/v) lysozyme (EC 3.2.1.17), nor did any synthesize poly-β-hydroxybutyrate. None decomposed tyrosine, produced indole or acetylmethylcarbinol. Dihydroxyacetone was not produced from glycerol. Brown or black pigments were never observed on glucose-containing media. L-Phenylalanine deaminase was absent but L-alanine dehydrogenase, L-leucine dehydrogenase and respiratory nitrite reductase were present. Cultures able to ferment glucose did not produce H_2.

The maximum growth temperature in peptone broth was between 41 and 45 °C. All the cultures tolerated at least 59 mM-NO_2^- and some tolerated almost sevenfold higher concentrations (Table 1). Additional features which varied amongst the 15 cultures are presented in Table 2.

The nine cultures having fermentative activity (Table 2) grew slowly on glucose anaerobically and slightly acidified the medium. Seven carbohydrates (L-rhamnose, L-sorbose, D-melibiose, D-dulcitol, D-arabitol, aesculin and *meso*-erythritol) were not oxidized by any culture. Twenty-five other carbohydrates were oxidized by some, but not all, of the 15

Table 3. *Acid production from carbohydrates*

Substrate	Total no. of positive strains	Negative strains
D-Glucose	14	RS15
D-Mannose	8	RS7, RS10, RS11, RS12, RS13, RS14, RS15
D-Fructose	12	RS12, RS13, RS15
D-Galactose	9	RS9, RS10, RS12, RS13, RS14, RS15
L-Fucose	1	All except RS11
D-Fucose	1	All except RS14
Cellobiose	8	RS9, RS10, RS11, RS12, RS13, RS14, RS15
Maltose	14	RS15
Lactose	3	All except RS7, RS9, RS11
D-Trehalose	12	RS12, RS13, RS15
Sucrose	7	RS3, RS7, RS8, RS10, RS12, RS13, RS14, RS15
Salicin	8	RS7, RS10, RS11, RS12, RS13, RS14, RS15
Raffinose	5	All except RS1, RS2, RS3, RS5, RS6
D-Melezitose	8	RS9, RS10, RS11, RS12, RS13, RS14, RS15
D-Xylose	8	RS7, RS9, RS10, RS12, RS13, RS14, RS15
D-Ribose	14	RS15
L-Arabinose	6	All except RS1, RS2, RS3, RS4, RS5, RS8
D-Arabinose	1	All except RS11
α-Methyl-D-glucoside	6	All except RS1, RS2, RS4, RS5, RS6, RS9
D-Gluconate	6	All except RS2, RS3, RS5, RS6, RS9, RS10
D-Sorbitol	5	All except RS1, RS3, RS5, RS6, RS8
D-Mannitol	10	RS9, RS12, RS13, RS14, RS15
L-Arabitol	1	All except RS14
Arbutin	1	All except RS9
Glycerol	14	RS9

cultures (Table 3). Culture RS15 was readily distinguished from all others by its inability to oxidize any carbohydrate except glycerol.

Of 24 organic acids tested, only pyruvate and L-asparagine were oxidized by all isolates; propionate was oxidized by none. The reactions with respect to the remaining 21 compounds are given in Table 4.

Two bacterial nitrate reductases A and B are known and can be distinguished by their activity with chlorate which serves as substrate for nitrate reductase A but inhibits enzyme B (Pichinoty & Piéchaud, 1968). For 14 isolates, chlorate served neither as substrate nor inhibitor; hence an unidentified nitrate reductase activity is presumed. In cell-free extracts of strain RS5, 96% of this nitrate reductase activity was recovered in the pellet after centrifuging at 140000 *g* for 2 h.

DNA base compositions

The GC content of the DNA of each isolate is given in Table 1. The mean of all the analyses was $40{\cdot}4 \pm 0{\cdot}8$ mol % GC.

Cytochrome spectra

Components of the respiratory chain were identified in cells grown aerobically. The absorption peaks for cytochromes *b*, *c* and *a* at liquid nitrogen temperature were not significantly different in the various cultures though the relative intensities of the peaks characteristic of each protein did vary. Two cytochrome oxidases were observed: $a+a_3$, with an absorption maximum at 600 to 602 nm, and the CO-binding pigment (cytochrome *o*), whose Soret band was at 415 to 418 nm at room temperature. Some doubt nevertheless must

Table 4. *Organic acids utilized for growth*

Substrate	Total no. of positive strains	Negative strains
Acetate	14	RS8
Butyrate	8	RS1, RS4, RS6, RS10, RS11, RS12, RS14
Isobutyrate	9	RS1, RS3, RS4, RS5, RS8, RS13
Valerate	1	All except RS10
Isovalerate	5	All except RS7, RS10, RS11, RS14, RS15
DL-Lactate	11	RS9, RS10, RS12, RS13
DL-β-Hydroxybutyrate	9	RS1, RS3, RS11, RS12, RS13, RS14
Glycollate	3	All except RS10, RS11, RS14
Succinate	4	All except RS2, RS7, RS9, RS15
L-Malate	9	RS1, RS4, RS5, RS6, RS10, RS13
Fumarate	6	All except RS1, RS2, RS7, RS9, RS14, RS15
Citrate	3	All except RS7, RS11, RS14
α-Ketoglutarate	11	RS1, RS2, RS3, RS5
Malonate	3	All except RS5, RS7, RS8
Glutarate	2	All except RS11, RS15
Tartrate	1	All except RS15
Glycine	10	RS3, RS4, RS5, RS6, RS14
L-α-Alanine	14	RS14
DL-Aspartate	11	RS1, RS4, RS5, RS12
L-Glutamate	11	RS3, RS4, RS6, RS14
L-Glutamine	3	All except RS11, RS12, RS15

Table 5. *Rates of reduction of nitrogen oxides by cell suspensions of strains* RS3 *and* RS14

Evolution and consumption of gas are expressed as μl (mg N)$^{-1}$ h^{-1}.

		Strain RS3					Strain RS14				
		Evolution			Consumption		Evolution			Consumption	
Culture conditions	Electron acceptor	NO	N_2O	N_2	NO	N_2O	NO	N_2O	N_2	NO	N_2O
Anaerobic, containing NO_3^-	NO_3^-	0	0	146	—	—	0	0	70	—	—
	NO_2^-	0	0	291	—	—	0	0	92	—	—
	NO	—	0	174	295	—	—	0	144	304	—
	N_2O	—	—	2287	—	2349	—	—	1198	—	1168
Anaerobic, containing NO_2^-	NO_3^-	0	242	342	—	—	0	0	493	—	—
	NO_2^-	0	251	519	—	—	0	0	1030	—	—
	NO	—	0	445	604	—	—	0	230	443	—
	N_2O	—	—	2626	—	2380	—	—	2351	—	2541

remain as to the true identity of this cytochrome *o* as the photochemical action spectrum (Lemberg & Barrett, 1973) has not been obtained. Also observed were cytochrome *c*, whose α peak was at 547 to 549 nm, and possibly two or even three cytochromes *b*, with α peaks between 551 and 564 nm.

Strains RS3, RS7, RS12 and RS15, when cultivated anaerobically in the presence of NO_3^-, all possessed cytochromes *c* and *o*. In contrast, the cytochrome oxidase $a+a_3$ was absent from all and only a single cytochrome *b* was visible in each. There was no peak of absorption between 625 and 630 nm which would be characteristic of cytochrome d_1c which catalyses the reduction of NO_2^- to NO and thus functions as a respiratory nitrite reductase in many different non-sporulating denitrifying bacteria (Miyata & Mori, 1968).

Denitrification by cell suspensions

Culture RS3 was selected as representative of those strains which grew with NO or N_2O as terminal electron acceptors and culture RS14 as representative of those which responded

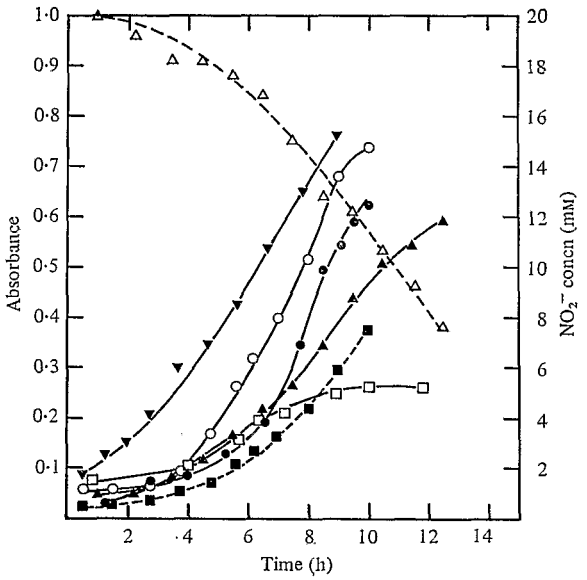

Fig. 4. Growth of strain RS1 at 37 °C and strain RS8 at 32 °C in medium IV in the presence of different electron acceptors. Strain RS1: ▼, growth on air; ○, growth on NO_3^- under a N_2 atmosphere; ■, production of NO_2^- during growth on NO_3^-; ▲, growth on NO_2^- under a N_2 atmosphere; △, reduction of NO_2^- during growth on NO_2^-; ●, growth on N_2O. Strain RS8: □, growth on 10% NO in N_2.

to neither of these gases (Table 2). Activities of the cell suspensions cultivated anaerobically on NO_2^- or NO_3^- are shown in Table 5. N_2O was more rapidly reduced than NO by both cultures, irrespective of whether NO_3^- or NO_2^- served as electron acceptor during growth. The stoicheiometries were as expected, 1 mol N_2O yielding 1 mol N_2, and 2 mol NO yielding 1 mol N_2. Cells grown in the presence of NO_2^- denitrified NO_3^-.

Growth in the presence of various electron acceptors

Growth curves are shown in Fig. 4. When NO served as the electron acceptor, a growth lag of from 40 to 60 h was always observed (not shown). Though this lag might be due to the selection of mutants resistant to the toxic action of NO, this was not so as cultures grown in the presence of NO did not show a shorter lag when inoculated into fresh medium; independent clones of RS8 on nutrient agar from a culture grown in 10% NO displayed the same lag as the parent culture.

Of all the oxidized forms of nitrogen tested as electron acceptors, growth on NO exhibited the longest generation times and lowest growth yield. Microscopically, cells so grown were quite normal – motile strains remained motile; cell viability was somewhat variable, but frequently attained 70%. The gaseous products obtained during growth with NO were examined by gas chromatography. During growth, N_2O and N_2 were produced followed by the disappearance of the NO and N_2O which were completely reduced to N_2 in a few days. Uninoculated media with NO in the atmosphere did not produce these gases, showing that reduction of NO was a biological process.

Cultivation of diverse denitrifying species in the presence of NO

The 70 denitrifying bacteria examined tolerated relatively high NO_2^- concentrations but none of them grew in medium III at 32 °C under 10% NO in N_2, even if the medium contained 0·5% (w/v) KNO_3.

Alteration of medium IV by NO

NO is an active oxidant and reacts slowly with components of medium IV to lessen its nutritive value: when 250 ml glass-stoppered flasks containing 50 ml medium and an atmosphere of 50% NO in N_2 were sealed for 1 week at 32 °C, the medium became acidic and turned pink, and when such flasks were seeded with garden soil and incubated aerobically at 32 °C, they showed no growth over several weeks. Thus NO cannot be used for direct enrichment of organisms capable of using it as terminal electron acceptor.

DISCUSSION

The bacteria described were an assemblage of heterogeneous phenotypes. It may be fortuitous that the GC contents of their DNAs showed little variation. All had common morphological features and had some characters of *Bacillus brevis*: oxidase and catalase positive and ability to reduce NO_3^- to NO_2^-; six of the cultures were also incapable of fermenting carbohydrates (De Barjac & Bonnefoi, 1972; Gibson & Gordon, 1974; Gordon *et al.*, 1973). Nevertheless, *B. brevis* cannot denitrify and has a higher GC content in its DNA (Gibson & Gordon, 1974; Pichinoty *et al.*, 1978*a*). Five cultures of *B. brevis* erroneously reported as denitrifiers by Gordon *et al.* (1973) are incapable of anaerobic growth in the presence of NO_3^-, NO_2^- or N_2O (Pichinoty *et al.*, 1978*a*).

Bacillus azotoformans, which also belongs to the same morphological group, is distinguished from the 15 cultures described here in many ways: colonial morphology, failure to retain the Gram-stain, very active motility with numerous flagella, poly-β-hydroxybutyrate synthesis, utilization of tetrathionate and fumarate as electron acceptors and failure to decompose H_2O_2 (Pichinoty *et al.*, 1976*b*, 1978*a*).

The new isolates could not be identified using existing schemes, but we make no taxonomic proposals until more strains have been isolated and examined.

The absence of a peak between 625 and 630 nm does not prove the absence of cytochrome d_1c. The method employed may not have been sufficiently sensitive to detect it.

The inability of culture RS14 to grow at the expense of N_2O or NO could not be explained by any enzymic deficiency since the reductases for both substrates were clearly evident. This bacterium was inhibited by NO gas. Inability to grow on N_2O must have some other explanation, because this gas is not toxic.

The seven cultures growing in the presence of NO fermented glucose, but one culture (RS9) which fermented glucose could not utilize NO.

It was possible that NO was not used directly as electron acceptor, but rather reacted with one or more organic medium components and was thus converted to a more tractable electron acceptor. This seems unlikely for the following reasons: all denitrifying bacteria appear to possess a nitric oxide reductase (Payne, 1973; Pichinoty, 1973); reduction of NO by cell suspensions was rapid with no indication of a latent phase. Resistance to high concentrations of NO_2^- and to NO suggests that these toxic and mutagenic compounds are reduced at the cell boundary and do not penetrate to the cytoplasm. Nitrate reductase of *Escherichia coli* has such a location (Haddock & Jones, 1977), but the locations of nitrite and nitric oxide reductases in denitrifying bacteria are not yet known.

Growth with NO as terminal electron acceptor is an exceptional property of 7 of the 15 cultures isolated from the Senegalese soils, suggesting that this oxide of nitrogen (which is found *in vitro* in the course of the reduction of nitrite by nitrite reductase) is an intermediate in denitrification (Miyata & Mori, 1968; Payne, 1973; Pichinoty, 1973). The 70 collection strains and 8 of the 15 strains described could not grow in the presence of NO.

Biological reduction of NO could occur *in situ*; the chemical decomposition of NO_2^- has been observed in various soils (acid and neutral as well as alkaline) with production of this gas (Bulla *et al.*, 1970). Knowledge of soil bacteria is limited and new isolation procedures

regularly reveal new isolates whose form and function often modify our understanding of the soil microflora. Enrichment cultures will be made using soils of temperate regions in order to determine whether the bacteria described here are specific to tropical soils.

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