JOURNAL OF BACTERIOLOGY, Aug. 1971, p. 563-569

Vol. 107, No. 2
Printed in U.S.A.

Comparison of the Morphology and Deoxyribonucleic Acid Composition of 27 Strains of Nitrifying Bacteria[1]

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Received for publication 3 May 1971

The gross morphology, fine structure, and per cent guanine plus cytosine (GC) composition of deoxyribonucleic acid of 27 strains of nitrifying bacteria were compared. Based on morphological differences, the ammonia-oxidizing bacteria were separated into four genera. *Nitrosomonas* species and *Nitrosocystis* species formed one homogenous group, and *Nitrosolobus* species and *Nitrosospira* species formed a second homogenous group in respect to their deoxyribonucleic acid GC compositions. Similarly, the nitrite-oxidizing bacteria were separated into three genera based on their morphology. The members of two of these nitrite-oxidizing genera, *Nitrobacter* and *Nitrococcus*, had similar GC compositions, but *Nitrospina gracilis* had a significantly lower GC composition than the members of the other two genera.

Nitrifying bacteria are chemoautotrophs which oxidize ammonia to nitrite and nitrite to nitrate and are categorized in the family *Nitrobacteraceae*. *Bergey's Manual* (2) lists the genera of ammonia-oxidizing bacteria as *Nitrosomonas*, *Nitrosocystis*, *Nitrosogloea*, *Nitrosospira*, and *Nitrosococcus* and the genera of nitrite-oxidizing bacteria as *Nitrobacter* and *Nitrocystis*. All of the genera listed above were originally described by Helene or Sergei Winogradsky (14-18).

The taxons of this family are poorly defined at present. The original strains of nitrifying bacteria described by the Winogradskys were not preserved in laboratory culture. Until recently, subsequent investigators succeeded in isolating only *Nitrosomonas* and *Nitrobacter* species. Since investigators following the Winogradskys worked only with the above two genera, doubts have been expressed as to the validity of the other genera (3, 4).

Recent investigations have established that nitrifying bacteria exist which are morphologically different from the rod-shaped *Nitrosomonas* or *Nitrobacter* species; for example, Watson (10) has isolated a large coccus *Nitrosocystis oceanus*. Recently this same investigator (11) isolated a spiral-shaped organism which oxidized ammonia and resembled *Nitrosospira briensis* described by the Winogradskys (19). Watson et al. (12) isolated and described a lobular-shaped ammonia-oxidizing bacterium and proposed that it should be categorized in a new genus called *Nitrosolobus*.

Watson and Waterbury (13) recently isolated and described two new nitrite-oxidizing bacteria which were morphologically different from any member of the genus *Nitrobacter* or *Nitrocystis*. One of these organisms, *Nitrospina gracilis*, was a long slender rod and the other, *Nitrococcus mobilis*, was a large coccus.

Several morphological types of nitrifying bacteria exist, but it is questionable whether all of the genera in *Bergey's Manual* (2) are valid. For example, the genera *Nitrosogloea*, *Nitrosocystis*, and *Nitrocystis* are reserved for the nitrifying bacteria which grow in aggregates forming either zoogloea or cysts. In our own experience (Watson, *unpublished data*), all nitrifying bacteria can form aggregates under certain cultural conditions, and those that form cysts do so only in mixed cultures. Aggregation and cyst formation are variable rather than constant characteristics and we question their use as taxonomic criteria.

Revision of the taxonomy of the nitrifying bacteria is needed. These bacteria can be separated into two groups based upon their ability to oxidize either ammonia or nitrite, but other biochemical characteristics cannot be used since

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these organisms are chemoautotrophs. Thus the separation of nitrifying bacteria into genera is dependent primarily upon their cellular morphology. If observations are limited to the light microscope, then shape and size are the only two morphological characteristics which can be used. Electron micrographs have shown that each nitrifying bacterium has a unique fine structure which distinguishes one genus from another (8, 11–13).

The value of using deoxyribonucleic acid (DNA) base compositions as an adjunct for classifying bacteria has been well established, but this method has not been used in categorizing nitrifying bacteria because so few strains have been analyzed (7). Previously, the DNA base composition of only two nitrifying bacteria had been examined (1, 5, 9). The aim of the present investigation was to compare the gross morphology, fine structure, and DNA base compositions of 27 strains of nitrifying bacteria to determine whether the taxonomy of these bacteria could be based on GC composition as well as morphology.

MATERIALS AND METHODS

All nitrifying bacteria used in this investigation (Table 1), unless otherwise indicated, were isolated at the Woods Hole Oceanographic Institution by using enrichment cultures and serial dilution techniques as described previously (10–13).

The growth medium for the fresh water and soil ammonia-oxidizing bacteria had the following composition: distilled water, 1 liter; $(NH_4)_2SO_4$, 1.7 g; $CaCl_2 \cdot 2H_2O$, 20 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; chelated iron (Atlas Powder), 1.0 mg; $Na_2MoO_4 \cdot 2H_2O$, 100 μg; $MnCl_2 \cdot 4H_2O$, 200 μg; $CoCl_2 \cdot 6H_2O$, 2.0 μg; $ZnSO_4 \cdot 7H_2O$, 100 μg; K_2HPO_4, 15.0 mg; $CuSO_4 \cdot 5H_2O$, 20 μg. The *p*H of this medium after autoclaving was 7.5. The growth medium used for seawater ammonia-oxidizing bacteria was identical to that listed above except that seawater replaced the distilled water and the K_2HPO_4 concentration was lowered 10-fold. The growth medium for the soil and fresh water nitrite-oxidizing bacteria had the following composition: distilled water, 1 liter; $MgSO_4 \cdot 7H_2O$, 100 mg; $CaCl_2 \cdot 2H_2O$, 5 mg; chelated iron (Atlas Powder), 1.0 mg; $Na_2MoO_4 \cdot 2H_2O$, 25 μg; $MnCl_2 \cdot 4H_2O$, 50 μg; $ZnSO_4 \cdot 7H_2O$, 25 μg; $CoCl_2 \cdot 6H_2O$, 0.6 μg; $CuSO_4 \cdot 5H_2O$, 5 μg; K_2HPO_4, 3.4 mg; and $NaNO_2$, 690 mg. The *p*H of this medium was adjusted to 7.5 after autoclaving. The medium used for the seawater nitrite-oxidizing bacteria was the same except that seawater rather than distilled water was used and the K_2HPO_4 concentration was halved.

To collect sufficient cellular material for DNA analysis, cultures were grown at room temperature in 20-liter carboys which were agitated with magnetic stirrers and sparged with air sterilized through a 0.22-μm membrane filter (Millipore Corp.). All carboys were stoppered with cotton plugs or silicon stoppers and fitted with glass tubes for the aseptic addition of nutrients. As the *p*H dropped in the ammonia-oxidizing cultures, they were readjusted to *p*H 7.5 by the addition of 0.3 M K_2CO_3. As the nitrite concentration was depleted during growth, increments of nitrite were added to maintain the original concentration.

All cells were harvested by means of a continuous-flow centrifuge (Sorvall model RC2-B) operated at 17,000 × *g* and at a temperature of 5 C. After harvesting, the organisms were washed in 10 ml of 0.15 M NaCl containing 0.1 M Na_2 ethylenediaminetetraacetate (EDTA) at *p*H 8.0 (saline-EDTA). The cells were then removed by centrifugation, suspended in 3 ml of the same medium, and lysed by the addition of sodium dodecyl (lauryl) sulfate to a final concentration of 3% (w/v). The above procedure was carried out at the Woods Hole Oceanographic Institution. The lysates were then shipped to Houston, Tex. There they were deproteinized with fresh phenol saturated with saline-EDTA at neutral *p*H, and the aqueous phase was separated by centrifugation. The DNA was precipitated by the addition of two volumes of 95% (v/v) ethanol, and the strands were "spooled" on a clean glass rod. The spool was freed of phenol by successive immersions in two changes of 70% (v/v) ethanol in water, drained, and finally dissolved in 0.15 M NaCl plus 0.01 M Na_3 citrate at *p*H 7.0. The DNA was analyzed by CsCl density-gradient centrifugation as previously described (6). Each sample was analyzed twice by using DNA of bacteriophage 2C (P_{CsCl} = 1.742 g/cm^3) as the reference. The host for the bacteriophage was *Bacillus subtilis*.

RESULTS AND DISCUSSION

The diverse morphological types of nitrifying bacteria studied in this investigation are illustrated in Fig. 1–16. Four basic morphological types of ammonia-oxidizing bacteria and three types of nitrite oxidizers can be seen. All of the ammonia oxidizers which were rod shaped were categorized, for the purpose of this investigation, in the genus *Nitrosomonas*. Their fine structure was characterized by peripherally located cytomembranes which formed flattened lamellae (Fig. 2, 4). Although all of the strains of *Nitrosomonas* were rod shaped and all had peripheral cytomembranes, the cells varied in shape and size. Three basic morphological types were found. The cells in the first group (consisting of strains C-31, C-49, and C-51) were short rods, 0.8 by 1 to 2.0 μm, with pointed ends (Fig. 1). The guanine plus cytosine (GC) compositions of these strains were 50.5, 50.5, and 51.0%, respectively, as deduced from the buoyant density in CsCl (Table 2).

The marine strains of *Nitrosomonas* (C-13, C-15, C-21, C-25, C-56, and C-139) were rods, 1.0 to 1.3 μm wide and 2.0 to 2.5 μm long, with rounded ends (Fig. 2). The GC compositions of these strains ranged from 47.4 to 48.5% (Table 2).

The third morphological type of *Nitrosomonas*

TABLE 1. *Organisms utilized in this study*

Organism	Strain no.	ATCC no.	Source
Nitrosomonas species	C-31	25978	Soil[a]
	C-49	25979	Paramaribo River, Surinam, South America
	C-51	25980	Soil[b]
	C-13		Seawater, South Pacific
	C-15	25981	Seawater, South Pacific
	C-21		Seawater, South Pacific
	C-25	25982	Seawater, South Atlantic
	C-56	25983	Seawater, North Atlantic
	C-139		Seawater, South Atlantic
	C-91	25984	Chicago sewage disposal plant
	C-93	25985	Chicago sewage disposal plant
	C-97		Chicago sewage disposal plant
	C-99		Chicago sewage disposal plant
Nitrosocystis oceanus	C-27		Seawater, Barbados Harbor
	C-29		Seawater, Pacific Ocean[c]
	C-107	19707	Seawater, North Atlantic
Nitrosolobus multiformis	C-22	25198	Soil, Windhoek, Southwest Africa
	C-57	25917	Soil, Santa Cruz Island, Galapagos Archipelago
	C-71	25196	Soil, Paramaribo, South America
Nitrosospira briensis	C-76	25961	Soil, Island of Crete
Nitrobacter species	NB-253	25390	—[d]
	NB-255	25391	—[e]
	NB-215	25385	Soil, Santa Cruz Island, Galapagos Archipelago
	NB-107	28383	—[g]
	NB-213	25384	—[f]
	NB-106	14123	—[g]
Nitrococcus mobilis	NB-231	25380	Seawater, South Pacific Ocean
Nitrospina gracilis	NB-211	25379	Seawater, South Atlantic Ocean

[a] Received from David Pramer.
[b] Received from Michael Rees who obtained it from E. L. Schmidt.
[c] Received from A. F. Carlucci.
[d] Received from A. P. Van Gool; this culture was designated as Engel No. 1 strain of *Nitrobacter winogradskyi*.
[e] Received from A. P. Van Gool; this culture was designated as Lees No. 1 strain of *Nitrobacter winogradskyi*.
[f] Received from Alvin Nason; designated as *Nitrobacter agilis*.
[g] Received from Helen Funk; designated as *Nitrobacter agilis*.

species was represented by strains C-91, C-93, C-97, and C-99. The cells of these strains were 1.2 μm wide and 2.0 to 3.5 μm long and had pointed ends (Fig. 3 and 4). In dividing cells, a prominent plasmodesmid (Fig. 3) appeared between daughter cells. Electron microscopic observations showed that two or more cells were often interconnected, forming a chainlike structure, but there was no cross wall separating the cells. The GC compositions of these strains varied from 48 to 49% (Table 2).

The GC compositions of all strains of *Nitrosomonas* species ranged from 47.4 to 51.0%. Although three morphological types of *Nitrosomonas* species exist, it is possible to distinguish only the shorter rods of the first group from the remainder on the basis of their slightly higher GC contents.

The organisms (C-27, C-29, and C-107) in the genus *Nitrosocystis* (10) all belonged to one species. The cells were large cocci ranging in size from 1.8 to 2.2 μm (Fig. 5). The ultrastructure of this organism was characterized by cytomembranes (Fig. 6) which formed flattened lamellae in the central region of the cell (8). The GC compositions of these three strains were 50.5, 51.0, and 51.0% (Table 2). The gross morphology and fine structure of *N. oceanus* were strikingly different from those of *Nitrosomonas* strains, the GC compositions of the organisms in

these two genera were too similar to be used as a basis for generic separation.

The third ammonia-oxidizing bacterium studied was a lobular organism, 1.0 to 1.5 μm wide and 1.0 to 2.5 μm long, called *Nitrosolobus multiformis* (reference 12; *see* Fig. 7). The fine structure of this cell was characterized by the presence of cytomembranes which tended to compartmentalize the cells (Fig. 8). Three strains of *N. multiformis* (C-22, C-57, and C-71) had DNA GC base compositions of 53.6, 55.1, and 54.6% (Table 2). This organism was clearly distinguishable from the other ammonia-oxidizing bacteria by its gross morphology, fine structure, and DNA base composition.

TABLE 2. *Buoyant densities in CsCl and guanine plus cytosine contents (GC) in the DNA of nitrifying bacteria*

Organism	Strain	*CsCl* density[a] (g/cm³)	*GC content* (moles %)	Group means ± σ
Nitrosomonas species Group 1	C-31	1.7095	50.5	
	C-49	1.7095	50.5	1.7097 ± 0.0005
	C-51	1.710	51.0	50.7 ± 0.5%
Group 2	C-13	1.7075	48.5	
	C-15	1.707	48.0	1.7069 ± 0.0007
	C-21	1.707	48.0	47.9 ± 0.7%
	C-25	1.707	48.0	
	C-56	1.7065	47.4	
	C-139	1.7065	47.4	
Group 3	C-91	1.707	48.0	1.7075 ± 0.0008
	C-93	1.708	49.0	48.5 ± 0.8%
	C-97	1.7075	48.5	
	C-99	1.708	49.0	
Nitrosocystis oceanus	C-27	1.7095	50.5	
	C-29	1.710	51.0	1.7098 ± 0.0004
	C-107	1.710	51.0	50.8 ± 0.4%
Nitrosolobus multiformis	C-22	1.7125	53.6	
	C-57	1.714	55.1	1.7133 ± 0.0008
	C-71	1.7135	54.6	54.4 ± 0.8%
Nitrosospira briensis	C-76	1.713	54.1	
Nitrobacter species	NB-253	1.720	61.2	
	NB-255	1.7205	61.7	
	NB-215	1.719	60.2	1.7198 ± 0.0009
	NB-107	1.720	61.2	61.0 ± 0.9
	NB-213	1.720	61.2	
	NB-106	1.7195	60.7	
Nitrococcus mobilis	NB-231	1.720	61.3	
Nitrospina gracilis	NB-211	1.7165	57.7	

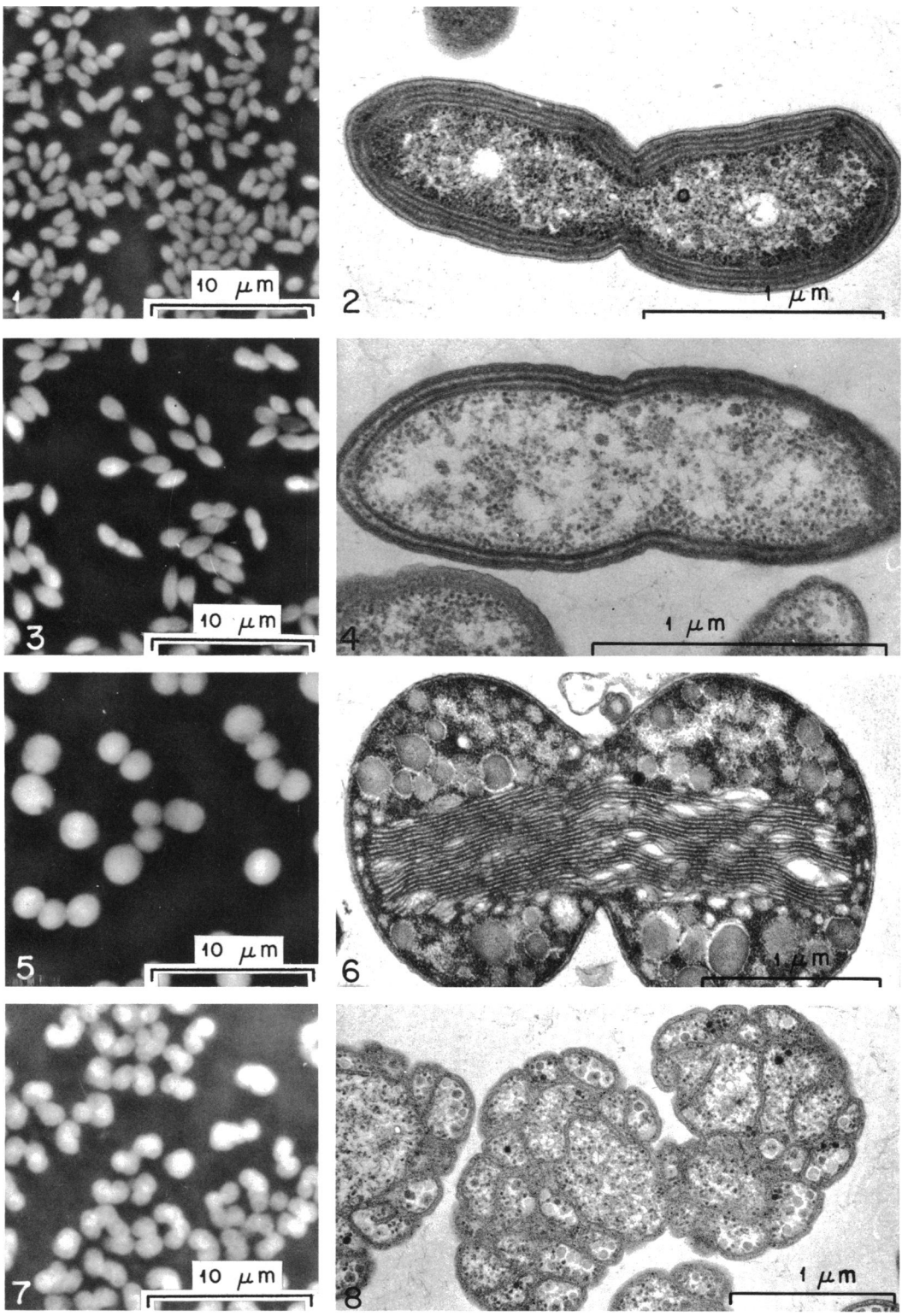
[a] Mean of two determinations.

The fourth morphological type of ammonia-oxidizing bacteria studied was a spiral-shaped organism called *Nitrosospira briensis* (reference 19; *see* Fig. 9 and 10); the fine structure of this organism has been described by Watson (11). In contrast to all other ammonia-oxidizing bacteria, it was void of cytomembranes. The DNA base composition of the one strain analyzed was 54.1%, indistinguishable from the base composition of *Nitrosolobus multiformis*.

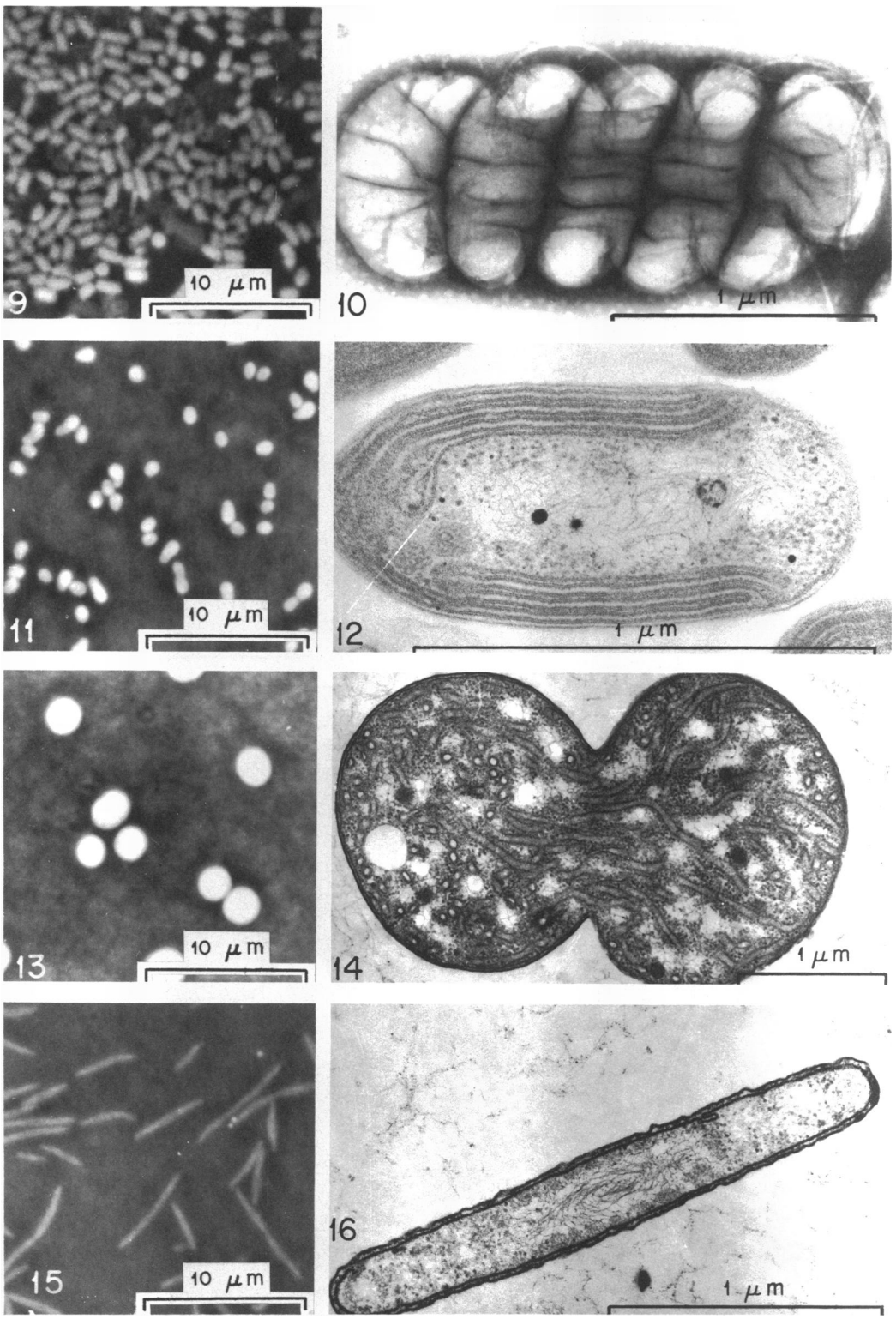
The nitrite-oxidizing bacteria studied included *Nitrobacter winogradskyi*, *Nitrobacter agilis*, *Nitrococcus mobilis*, and *Nitrospina gracilis*. Six strains of *Nitrobacter* were studied. All six of these strains were short rods, often pear shaped, and were 0.8 μm wide and 1.0 to 2.0 μm long (Fig. 11). The fine structure also was similar and was characterized by a polar cap of cytomembranes as described by Murray and Watson (reference 8; *see* Fig. 12). Three strains designated as *Nitrobacter agilis* (NB-213, NB-106, and NB-107) by other investigators had GC compositions of 61.2, 61.2, and 60.7%, and two strains designated as *Nitrobacter winogradskyi* (NB-253 and NB-255) had GC compositions of 61.2 and 61.7% (Table 2). An unidentified strain (NB-215) had a GC composition of 60.2% (Table 2). The gross morphology, fine structure, and GC compositions of all strains were so similar that it appeared all should be considered to be the same species and designated *Nitrobacter winogradskyi*.

The second nitrite-oxidizing bacterium studied, *Nitrococcus mobilis*, was a large coccus, 1.5 to 1.8 μm in diameter just after division, and elongate, 1.8 by 3.5 μm just prior to division (Fig. 13; see reference 13). *N. mobilis* had tubular cytomembranes which were scattered at random throughout the cytoplasm (Fig. 14). The GC composition of the one strain of *Nitrococcus mobilis* (NB-231) analyzed was 61.3% (Table 2) and thus similar to the *Nitrobacter* species.

FIG. 1–8. *Phase-contrast and electron micrographs of Nitrosomonas, Nitrosocystis, and Nitrosolobus species.* Fig. 1. *Phase-contrast photomicrograph of Nitrosomonas europaea (C-31).* ×*2,500.* Fig. 2. *Electron micrograph of a marine Nitrosomonas species (C-56) showing peripheral cytomembranes.* ×*37,600.* Fig. 3. *Phase-contrast photomicrograph of Nitrosomonas species (C-91) isolated from the Chicago sewage disposal plant; note connections between cells.* ×*2,500.* Fig. 4. *Electron micrograph of Nitrosomonas strain shown in Fig. 3; note peripheral membranes.* ×*45,000.* Fig. 5. *Phase-contrast photomicrograph of Nitrosocystis oceanus (C-107).* ×2,500. Fig. 6. *Electron micrograph of Nitrosocystis oceanus (C-107) showing flattened vesicles in the central region of the cell.* ×*27,100.* Fig. 7. *Phase-contrast photomicrograph of Nitrosolobus multiformis (C-71) showing lobular shape of cells.* ×*2,500.* Fig. 8. *Electron micrograph of Nitrosolobus multiformis (C-71) showing lobular shape of cells and partial compartmentalization of cytoplasm by cytomembranes.* ×*25,000.*



FIGS. 1-8.



FIGS. 9–16.

The third nitrite-oxidizing bacterium, *Nitrospina gracilis*, was a long slender rod, 0.3 to 0.4 μm wide and 2.7 to 6.5 μm long (Fig. 15), and lacked an extensive cytomembrane system (Fig. 16; see reference 13). The strain studied was NB-211 and it had a GC composition of 57.7% (Table 2).

Based on morphology and fine structure, the ammonia-oxidizing bacteria were separable into four genera and the nitrite-oxidizing bacteria into three. Comparison of the GC compositions of the ammonia-oxidizing bacteria permitted the separation of these organisms into two groups but not into specific genera. Similarly, by comparison of the GC compositions of the nitrite-oxidizing bacteria, it was possible to separate them into two groups but not into specific genera.

Knowledge of the GC compositions of various strains should be useful in categorizing the nitrifying bacteria in the future. For example, all the rod-shaped organisms studied in this investigation had similar but not identical GC compositions and were placed in the genus *Nitrosomonas*. The GC compositions of the first group of rods was significantly different from the other two groups at the 95% confidence level (23 degrees of freedom) and indicates that the three groups should be classified into more than one species. As diverse morphological strains of nitrifying bacteria are isolated in the future, their DNA base ratios should be determined to decide whether all strains of a morphological type should be included in the same genus or species.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of Janet Bergendahl, Linda Graham, and Frederica Valois.

This investigation was supported by contract AT(30-1)-4138 (Reference No. NYO-4138-11) and by Public Health Service grant GM-11214 from the National Institute of General Medical Sciences.

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FIG. 9–16. *Phase-contrast and electron micrographs of Nitrosospira, Nitrobacter, Nitrococcus, and Nitrospina species.* Fig. 9. *Phase-contrast photomicrograph showing Nitrosospira briensis (C-76); note that the spiral nature of the cells is not immediately apparent.* ×*2,500.* Fig. 10. *Electron micrograph showing negatively stained cell of Nitrosospira briensis (C-76); note spiral nature of cells.* ×*40,100.* Fig. 11. *Phase-contrast photomicrograph of Nitrobacter winogradskyi (NB-255) showing wedged- to pear-shaped cells.* ×*2,500.* Fig. 12. *Electron micrograph of Nitrobacter winogradskyi (NB-255) showing polar cap of cytomembranes.* ×*71,800.* Fig. 13. *Phase-contrast photomicrograph of Nitrococcus mobilis (NB-231).* ×*2,500.* Fig. 14. *Electron micrograph of Nitrococcus mobilis (NB-231) showing tubular cytomembranes.* ×*23,900.* Fig. 15. *Phase-contrast photomicrograph of Nitrospina gracilis (NB-211).* ×*2,500.* Fig. 16. *Electron micrograph of Nitrospina gracilis (NB-211) showing cells lacking cytomembranes.* ×*42,600.*