Autecological Study of the Chemoautotroph *Nitrobacter* by
Immunofluorescence

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Fluorescent antibodies (FA) prepared for *Nitrobacter agilis* and *N. winogradskyi* were highly reactive in homologous staining. Low-level cross-reactions between the two species were removed by adsorption. All 15 pure-culture isolates of *Nitrobacter* tested reacted strongly with either *N. agilis* FA or *N. winogradskyi* FA. All pure-culture isolates from soils were determined to be *N. winogradskyi*. Those from Mammoth Cave sediments and a cattle waste oxidation ditch were *N. agilis*. No cross-reaction was found in extensive tests that included five isolates of *Nitrosomonas europaea* and 668 heterotrophic aerobic and anaerobic bacteria isolated from soil, sewage, and cave sites. The FA preparations were used to detect *Nitrobacter* species in Mammoth Cave sediments, in a cattle waste oxidation ditch, and in surface waters and sediments of a river and to observe that *N. winogradskyi* can outgrow *N. agilis* in enrichment culture.

Ecological studies of autotrophic nitrifying bacteria have been limited by a lack of suitable techniques. Although the process of nitrification can be followed easily by chemical means, microbial agents responsible for the nitrification process cannot be studied directly. Direct plating, even with strictly inorganic media, is useless because the organic materials introduced with the inoculum permit growth of nonnitrifying heterotrophs, and nitrifying chemoautotrophs, if present, are unrecognizable. Also, because of these faster-growing heterotrophic contaminants, isolation of autotrophic nitrifiers is a tedious process requiring careful and extensive serial enrichment cultures (9).

The usual approach to the microbiology of the nitrification process is indirect. Some modification of the most probable number (MPN) procedure (1, for example) is used for statistical estimation of the numbers of chemoautotrophic nitrifiers present in the nitrifying environment. The accuracy and sensitivity of the MPN for quantification of these bacteria is unknown, because no standard exists. Qualitatively, the MPN gives no information on the nature of the chemoautotrophs present nor on the species diversity of the nitrifying habitat.

We have explored the use of immunofluorescence for the direct detection of nitrifying bacteria in natural environments. Efforts were concentrated on the genus *Nitrobacter*, because it is an excellent indicator organism in the two-step ammonia-to-nitrate reaction carried out by the autotrophic nitrifying bacteria. The purpose of this paper is to demonstrate that fluorescent antibodies (FA) can be prepared for *Nitrobacter*, that these are specific, and that they may be used to detect and study *Nitrobacter* in various ecosystems.

**MATERIALS AND METHODS**

* Cultures. The stock culture of *Nitrobacter winogradskyi* was obtained originally from H. Laudefout, University of Louvain, Belgium; that of *Nitrobacter agilis* was obtained from the American Type Culture Collection. Both were grown routinely in shaken cultures at room temperatures. *Nitrobacter* medium was prepared as follows. Solution I was composed of (per liter): NaH2PO4, 5.0 g; KH2PO4, 0.5 g; NaNO2, 1.4 g; FeSO4·7H2O, 1.0 mg/ml; and ethylenediaminetetraacetic acid, 1.3 mg/ml; and trace solution (ZnSO4·7H2O, 0.02 mg/ml; CuSO4·5H2O, 0.02 mg/ml; NaMoO4·2H2O, 0.02 mg/ml), 1.0 ml. Solution II was composed of (per 100 ml) MgSO4·7H2O, 2.0 g. To 100 ml of autoclaved and cooled solution I, 1.0 ml of autoclaved solution II was added aseptically. Both *Nitrobacter* species were cloned on standard medium with Noble agar (Difco) prior to growth for use as antigens. Pure-culture isolates of *Nitrobacter* used in specificity tests were obtained from each of the following habitats: agricultural soils, cattle feed lot oxidation ditch, lenitic waters, and cave sediments. Procedures used to obtain the pure cultures have been reported (9).

A large number of unidentified heterotrophic bacteria were isolated from the study habitats to determine if bacteria other than *Nitrobacter* reacted with...
the FA. Two basic media were employed: soil extract agar (6) and peptonized milk agar (4), both with and without sodium nitrite at 500 μg/ml. Media were amended with antibiotic, cycloheximide at 100 μg/ml. Serial 10-fold dilutions were made from each habitat sample, and 0.1 ml of each dilution was inoculated on the four types of media. Anaerobic and aerobic incubations were carried out at room temperature. Anaerobic colonies were isolated in oxygen-free roll tubes prepared under continuous gassing with nitrogen. Morphologically distinct colonies from each dilution of each plate or roll tube were picked and transferred to microscope slides. The bacteria were heat fixed and stained with N. agilis and N. winogradskyi FA.

FA techniques. Cloned isolates of the stock cultures of N. winogradskyi and N. agilis were grown in pure culture to a density of about 10^6 cells/ml (95 Klett units) for the preparation of individual antisera. Cells were harvested by centrifugation, washed, suspended in phosphate buffer (pH 7.2), and injected intravenously into rabbits. After injections were completed, test bleedings were made, and antibody levels were determined by tube agglutination; these were shown to be greater than 1:2560 for each homologous system. Antiserum was harvested by cardiac puncture, fractionated, and conjugated to a fluorochrome dye. All procedures were as described previously (8).

Cross-reactions between the two species were removed by adsorption of the FA with the heterologous antigen. Each Nitrobacter culture was grown to a density of 10^8 cells/ml in shaken flasks. The entire culture of 100 ml was collected on a sterile 0.4 μm membrane filter ("Nucleopore," General Electric, Pleasanton, Calif.) and transferred aseptically to sterile screw-cap test tubes containing heterologous FA. Adsorption was carried out with constant agitation for 24 h at 4 C. The adsorbed FA was removed by filtration, checked for heterologous cross-reactions, and stored at ~20 C until used. Only one reciprocal adsorption procedure was necessary to obtain species-specific FA.

Methods for preparation and staining of contact slides and for all fluorescence microscopy and photomicrography are detailed elsewhere (8). Gelatin-rhodamine isothiocyanate (RhITC) conjugate (8 μg of RhITC per mg of gelatin) was used to suppress nonspecific adsorption of FA (2). The techniques for preparation of terrestrial and aquatic samples for immunofluorescence examination on membrane filters were as reported previously (3). All FA were diluted 1:4 for staining and, for purposes of detecting Nitrobacter in natural habitats, the two FA were combined for use as a single reagent.

RESULTS

As seen in Table 1, initial tests with the unconjugated sera gave good agglutination titers, and those with each conjugated serum (FA) demonstrated excellent immunofluorescence staining reactions with its homologous antigen. Cross-reactions between the two species of Nitrobacter were minimal with respect to the staining of N. winogradskyi by N. agilis FA, but were of moderate (2+) intensity in the reverse system. Adsorption of N. agilis and N. winogradskyi FA with their heterologous antigens removed all interfering cross-reactions. The slight loss in fluorescence staining intensity sustained by N. agilis FA as a result of the adsorption procedure did not affect its usefulness. All subsequent studies were carried out with adsorbed FA. Immunofluorescence typical of each Nitrobacter species when reacted with its homologous FA is shown in Fig. 1A. The two Nitrobacter species were identical in size and shape.

Behavior of the Nitrobacter FA relative to known bacteria related to the antigen organisms is summarized in Table 2. Isolates of the chemosynthetic autotrophic nitrifiers are not readily available in culture collections because of the well-known difficulties in the isolation and the maintenance of pure cultures. Most of those listed in Table 2 represent successful isolations made over the past several years by means of an enrichment isolation protocol (9).

All tested isolates of Nitrobacter reacted strongly with one of the two Nitrobacter FA, and none of the isolated stained with more than one FA. Thus, every Nitrobacter obtained from widely different habitats was clearly characterized by FA reaction as being either N. agilis or N. winogradskyi. Clearly, too, the majority of these Nitrobacter isolates, as judged by FA reactivity, proved to be N. winogradskyi. Although Nitrosomonas is related to Nitrobacter both in terms of taxonomic position and ecosystem distribution, none of the Nitrosomonas cultures listed in Table 2 cross-reacted with either Nitrobacter FA.

Specificity of the FA was checked further in tests with unrelated bacteria. These data are given in Table 3 and summarize experience with heterotrophic bacteria isolated from soil, sewage, and cave sites of interest. None of the 336 aerobic and 332 anaerobic bacteria evidenced more than trace cross-reactions in these tests. In the light of all of the specificity test data, the two Nitrobacter FA preparations were considered to be highly specific at the species level and excellent reagents for the detection of Nitrobacter in natural environments.

Detection of Nitrobacter species in distinctly different habitats by FA techniques is shown in Fig. 1B to F. The ability to observe Nitrobacter is of interest in relation to the special features of each ecosystem. Photomicrographs 1C to F show typical cells that reacted to the combined
Fig. 1. Photomicrographs of immunofluorescing Nitrobacter cells from various habitats. (A) Pure culture smear of *N. winogradskyi* isolated from soil. (B) *N. agilis* (125-8) membrane filter preparation of Mammoth Cave National Park cave sediments. (C) Nitrobacter species membrane filter preparation of cattle waste oxidation ditch stained with combined *N. agilis*-*N. winogradskyi* FA. (D) Single cell of Nitrobacter species on a contact slide incubated for 3 weeks near the surface of the St. Louis River in Minnesota and stained with combined FA. Note unstained, sheathed bacteria above and below the Nitrobacter cell. (E, F) Microcolonies of Nitrobacter species on contact slides incubated for 3 weeks in the upper 5 cm of St. Louis River sediment and stained with combined FA. Irregular, white areas (F) represent organic matter that was orange-yellow as viewed by microscope. Scale insert represents 10 μm; all preparations were photographed at ×1,000.

FA and hence do not distinguish between *N. agilis* and *N. winogradskyi*. Fig. 1B shows cells released from a Mammoth Cave sediment, concentrated onto a membrane filter, and stained with *N. agilis* FA only. The habitats represented by Fig. 1B are terrestrial, but much different from humid agricultural soils in that they are very dry, of constant temperature, free of higher plants, low in organic matter, slightly alkaline, and high in nitrates and carbonates. The nitrifying system represented in Fig. 1C is an oxidation ditch containing impounded cattle
wastes. The habitat is basically aquatic and is characterized by a progressive accumulation of nitrogenous and food-waste solids, which are continuously circulated in a closed, aerated ditch below confined, feeding cattle. Despite a high percentage of organic solids (4 to 6%) in the oxidation ditch, nitrification may proceed vigorously. The organic matter does not interfere with detection of *Nitrobacter* cells on the membrane filter.

Figures 1D to F represent stream environments as sampled from the St. Louis River in Carlton County, Minnesota, and show *Nitrobacter* cells that developed on contact slides. Of particular interest is the capability to detect *Nitrobacter* in sediments (Fig. 1E and F), because more attention should be given to nitrification transformations in aquatic sediments.

One question raised by the FA specificity tests with related bacteria (Table 2) concerns the high frequency with which *Nitrobacter*
isolates derived from various habitats, especially agricultural soils, proved to be *N. winogradskyi*. These data suggest that *N. winogradskyi* predominates in most of the soil environments studied or that the lengthy enrichment procedures preliminary to isolation favor *N. winogradskyi* over *N. agilis*. The behavior of the two species when grown together in the enrichment culture medium was studied by direct microscopy count of each species after specific FA staining on membrane filters.

Data for the mixed-culture growth show that *N. winogradskyi* can outgrow *N. agilis* rapidly under the cultural conditions used in enrichment (Fig. 2). The occurrence of *N. agilis* in agricultural soils apparently must be studied by direct examination of those soils by using the specific FA; such studies are under way. The fact that the Mammoth Cave isolates obtained by the same enrichment and isolation procedures proved to be *N. agilis* strongly suggests that these cave habitats favor the dominance of *N. agilis*. Again, more detailed studies based on direct FA examination of these sites are under way.

**Fig. 2.** Response of *N. agilis* and *N. winogradskyi* to mixed culture conditions. Direct counts were made on membrane filters by using specific fluorescent antibodies. One cell/field = 1.2 × 10^6 Nitrobacter/ml. Data plotted are the mean of duplicate experiments with an average of 500 fields counted for each time period. Initial populations were: *N. agilis*, 4.3 × 10^6/ml, and *N. winogradskyi*, 3.7 × 10^6/ml.

**DISCUSSION**

In recent years development of the FA technique for effective use in microbial ecology has greatly expanded the potential for autecological study of microorganisms (7). The inherent advantages that this technique uniquely provides are particularly appropriate for study of the nitrifying bacteria. Refractory to ready culture, difficult to isolate, and of nondescript morphology, the nitrifying bacteria function in complex habitats characterized by great species diversity. The species-specific FA reported in this study made it possible for the first time to observe and recognize members of one nitrifying genus, *Nitrobacter*, by direct microscopy examination of nitrifying habitats.

According to classical concepts of nitrification, the capability to observe *Nitrobacter* species directly should provide an insight as well into the ecology of the ammonia-oxidizing bacteria on which the *Nitrobacter* depend for their specific energy source. This makes it possible to better assess the role of the chemoautotrophic bacteria in a given nitrifying situation and to evaluate, indirectly at least, the possible contribution of heterotrophs to that situation. The FA technique also suggests an approach to long-standing questions concerning the microbiology of nitrification in acid soil environments. These and other questions are likely to be addressed most effectively by quantifying the technique, according to protocols developed for the enumeration of *Rhizobium japonicum* (3), used in this study to obtain the data for Fig. 2.

Each nitrifying habitat examined by immunofluorescence was found to contain *Nitrobacter* cells. Whether those seen were representative of all autotrophic nitrite oxidizing genera present is not known with certainty, but this seems likely in view of the limited genus diversity reported by Watson and Waterbury (11). They note that the little-known genus *Nitrocystis* probably is *Nitrobacter* with zoogloeae and that the two new genera, *Nitrooccus* and *Nitrospina*, apparently are restricted to marine environments. All 15 of the nitrite-oxidizing isolates that we had isolated in pure culture from a number of diverse habitats were typical of *Nitrobacter* in size and shape and could be clearly characterized by immunofluorescence as either *N. agilis* or *N. winogradskyi* (Table 2). These data suggest that it would be premature to accept the recommendation of Watson and Mandel (10) that *N. agilis* be dropped as a species epithet and that only *N. winogradskyi* be recognized. Similarly, it appears that the conclusion of Pan (5) that there are no adequate
criteria to distinguish between *N. agilis* and *N. winogradskii* is unwarranted. Our data indicate that simple agglutination or immunofluorescence does indeed provide a useful and definitive criterion.

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