Immunofluorescent Assay for the Marine Ammonium-
Oxidizing Bacterium *Nitrosococcus oceanus*†

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Nitrification is one of the important microbial transformations of nitrogen in the ocean. Traditional enrichment-culture methods for enumerating the autotrophic bacteria which oxidize ammonium to nitrite are very time consuming (months) and are believed to seriously underestimate natural abundances. A fluorescent-antibody assay for a marine ammonium-oxidizing bacterium was developed to provide a rapid and direct means of identifying these microorganisms. Antibodies to *Nitrosococcus oceanus* were prepared and tested against pure cultures of marine, freshwater, and soil ammonium oxidizers and against bacteria from natural seawater samples. Cell counts of culture samples determined by the fluorescent-antibody assay agreed with hemacytometer and acridine orange counts. Our results demonstrated that the immunofluorescent assay is a powerful tool for the detection of *Nitrosococcus* in the marine environment.

Nitrite occupies a key position in the nitrogen cycle in the ocean as an intermediate in nitrification, denitrification, and nitrate respiration. The rates of these processes are important in determining the distribution and abundance of inorganic nitrogen in the ocean. Microbial production of nitrite is mediated by two very different biochemical transformations: oxidation of ammonium to nitrite by autotrophic nitrifying bacteria (7, 29) and reduction of nitrate to nitrite by both heterotrophic denitrifying bacteria (13) and nitrate-respiring facultative anaerobes (9). Phytoplankton release nitrite under conditions of light limitation (6, 15, 27). The relative importance of the oxidative and reductive transformations is a function of environmental parameters in a specific oceanic regime (6, 16, 21). Denitrification, for example, is believed to be the prime mechanism of nitrite production in waters with low dissolved-oxygen concentrations (13); nitrate respiration also occurs under these conditions (9, 11). The occurrence of autotrophic nitrification is not as well documented, nor are the specific environmental conditions favoring nitrification well defined (7).

Ammonium-oxidizing bacteria have been isolated from the Atlantic (S. W. Watson, Abstract, Int. Congr. Microbiol. 8th, Montreal, B,6:12, 1962) and Pacific (10) oceans. In culture media with environmentally realistic concentrations of ammonium, rates of both growth and nitrite production are very low. Watson (29) and Carlucci and Strickland (10) have estimated from ammonium enrichment studies that in situ abundances of nitrifying bacteria in the ocean may be as low as one bacterium per liter, and certainly less than $10^5$ cells per liter. Such low abundances, coupled with low estimated rates of nitrification, lead these investigators to conclude that in situ nitrification is insufficient to account for the observed nitrite distribution in the sea.

The ammonium enrichment techniques used for enumerating nitrifying bacteria, however, require long incubations and may seriously underestimate in situ abundances. We developed an immunofluorescent-antibody assay as an alternative to the enrichment methods and as a more direct approach for determining in situ concentrations of nitrifying bacteria in the ocean. The fluorescent-antibody (FA) technique is successfully used by Schmidt and co-workers to study *Rhizobium* (25) and nitrifying bacteria (2, 12) in freshwater and soils; Apel and co-workers (1) and Reed and Dugan (24) use a similar approach for enumerating *Thiobacillus* and methylo- trophs, respectively, in their natural environments. In this paper we describe the preparation and testing of an immunofluorescent assay of sufficient sensitivity and specificity for the detection and enumeration of low concentrations of nitrifiers in the marine environment.

**MATERIALS AND METHODS**

**Cultures.** Isolates of marine nitrifying bacteria were kindly supplied by A. F. Carlucci, Scripps Institution of Oceanography (*Nitrosococcus [Nitrosocystis] oceanus* and an unidentified marine ammonium-oxidizing isolate, no. 5), and by S. W. Watson, Woods Hole Oceanographic Institution (*N. oceanus*). Soil and freshwater isolates were obtained from E. L. Schmidt, Department of Microbiology and Soil Science, University of Minnesota (*Nitrosomonas Tara, Nitrosomonas europaea, Nitrosomonas E-K, and Nitrosomona-
nas WH-2). The marine isolates were maintained in two seawater culture media: (i) C medium which was modified from Carlucci and Strickland (10) to contain 0.3 g of CaCO₃, 0.05 g of K₂HPO₄, 0.16 g of NH₄Cl, and 1.0 ml of chelated metals solution (3) per liter of filtered seawater; and (ii) W medium (29), containing 0.635 g of NH₄Cl, 0.02 g of CaCl₂·2H₂O, 0.357 g of MgSO₄·7H₂O, 0.043 g of K₂HPO₄, 0.005 g of phenol red, and 1.0 ml of chelated metals solution per liter of filtered seawater. W medium required periodic adjustment to pH 7.5 with sterile 0.1 M K₂CO₃. Nitrosomonas isolates were grown in a medium containing 0.5 g of (NH₄)₂SO₄, 0.04 g of MgSO₄·7H₂O, 0.2 g of KH₂PO₄, 0.04 g of CaCl₂·2H₂O, 1.0 ml of basic phenol red solution (0.05% phenol red in 0.0014 N NaOH), and 1.0 ml of chelated metals solution per liter of distilled water (26); pH was maintained by periodic addition of 0.47 M Na₂CO₃ when indicated by color change of the phenol red indicator. All the above isolates were maintained in liquid culture (50- or 100-ml volumes in 125- or 250-ml Erlenmeyer flasks) at 20°C in the dark and were transferred to fresh media every 3 weeks. Freedom of the cultures from heterotrophic bacterial or fungal contaminants was monitored by inoculating a sample of the culture into sterility-test medium (after Carlucci and Pramer [8]; modified CP medium: 2.5 g of bacto-tryptone, 0.1 g of yeast extract, 0.1 g of K₂HPO₄, and 1.0 ml of chelated metals solution per liter of distilled water or 90% seawater).

The bacterial isolates used in heterologous testing of the assay specificity were maintained in appropriate media. Escherichia coli (supplied by D. Cramer, Department of Microbiology, University of Washington) was added to plates or slants on a basal salts solution with 1.0 g of glucose per liter (20). Benekeia gazogenes, BV1-SJG (another marine pigmented vibrio, supplied by S. J. Giovannoni, Department of Biology, Boston University), and unidentified marine heterotrophs (isolated from waters off the Washington coast by the senior author) were all maintained on plates or in liquid culture of modified CP medium. Thiosulfate-oxidizing isolate M1 was isolated from a Massachusetts salt marsh by the senior author and maintained on the Thiobacillus thioparus medium of Vinblad and Santer (28). Nitrobacter agilis (supplied by E. L. Schmidt) was grown in the Nitrobacter medium of Fliermans et al. (12).

Field samples. Seawater samples were collected in 40-liter polyvinyl chloride water bottles during several cruises off the Washington coast. Seawater was prefiltered through 10-µm Nitex netting to remove zooplankton and large phytoplankton and then concentrated by continuous-flow centrifugation at 27,000 × g at 4°C on board ship using a gimballed-mounted, highspeed centrifuge. Pellets were suspended in 200 to 400 ml of filtered seawater and preserved in 2% Formalin (sodium acetate buffered) for subsequent fluorescent-antibody staining in the laboratory.

Immunization procedure. A total of 137 liters of N. oceanus (Carlucci isolate) culture were grown in several 12- and 20-liter glass carboys, filled to 75% capacity, and were harvested by continuous-flow centrifugation at 27,000 × g. The pellets were washed in saline solution (0.15 M NaCl), resuspended at 39,000 × g for 30 min, covered with saline, and frozen at -40°C until use. A 3-ml sample of this saline cell suspension, containing 10 mg of protein per ml (assayed according to Lowry et al. [19]), was used for the immunization of each of two female New Zealand white rabbits. A modification of the immunization schedule of Kenny (18) was followed (Table 1). A sample of blood was drawn before immunization to test for reactions between N. oceanus and this pre-immunization serum. At 7 days after the last injection, antisera were collected (by cardiac puncture) directly into sterile vacuum bottles. The whole blood was allowed to coagulate for 24 h at room temperature, after which the supernatant serum was collected and stored at 0°C for 10 min at 3,000 × g. The clear serum was frozen at -40°C and used without further purification in all subsequent antibody-antigen reactions.

Double immunodiffusion procedure. Antigen-antiserum reactions were tested by a thin-layer gel diffusion technique (23). Melted agarose (1.2 ml of 0.5% [wt/vol], Sigma Chemical Co., St. Louis, Mo.) in Veralon buffer (0.0167 M barbital, 0.0834 M sodium barbital, pH 8.6) with 0.5% Triton X-100 was plated on a 25- by 75-mm glass slide. Once the agarose had solidified, Plexiglas templates were placed on the gel surface. Wells in the templates were filled with 10 to 12 µl of the appropriate test isolate or antiserum preparation. The slides were placed in a humidified chamber for 48 h and then in a TES-saline solution [5 mM Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid in 0.15 M NaCl, pH 7.3] for 24 h, washed in distilled water for 4 h, and dried overnight at 37°C. Dried slides were stained with Coomassie brilliant blue (1.0 g of Coomassie brilliant blue in 200 ml of destain solution on a glass slide). Dried slides were washed with distilled water, or distilled water-chlороform solution (9:2) for 7 min and then destained with 5 to 15% isopropanol in destain solution. Antigens used in the immunodiffusion procedure were obtained from large-volume cultures, which were harvested, washed, and stored as described for N. oceanus.

FA staining procedure. An indirect FA staining method (employing a fluorescent-labeled nonspecific sheep anti-rabbit antibody) was used (Fig. 1). The

<table>
<thead>
<tr>
<th>Day</th>
<th>Protocol</th>
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<tbody>
<tr>
<td>0</td>
<td>Draw 15 ml of preblood from marginal ear vein</td>
</tr>
<tr>
<td>1</td>
<td>Inject each flank intramuscularly with 1:1 emulsion of antigen: Freund incomplete adjuvant (Difco Laboratories)</td>
</tr>
<tr>
<td>21</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>24</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>27</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>30</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>37</td>
<td>Collect antisera by cardiac puncture</td>
</tr>
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</table>
protocol was derived, in part, from Reed and Dugan's (24) indirect FA method and, in part, from the direct FA method (employing a fluorescent-labeled specific antibody) of Bohlool and Schmidt (5). Filters (0.40-μm nominal pore size, Nuclepore Corp., Pleasanton, Calif.) were dyed by immersion in Irgalan black (0.2% in 2.0% acetic acid, Union Color and Chemical Co., Boston, Mass.) for a minimum of 0.5 h, rinsed in distilled water, placed on filter holders, and washed with phosphate-buffered saline (PBS: 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of NaH2PO4, and 0.2 g of KH2PO4 per liter of distilled water; pH adjusted to 7.4 with 1.0 N NaOH). Samples of cultures or natural seawater containing material to be FA stained were filtered and washed with PBS. For field samples, filters were removed from holders and placed on glass slides; a gelatin solution (0.2 ml of alkaline hydrolyzed gelatin, pH 10.5 [4]) was placed on the filters, and the slides were incubated at 50 to 60°C until nearly dry. When pure cultures were FA stained, the filters were kept on the filter holders, covered with 0.2 ml of 2% bovine serum albumin (Sigma Chemical Co.) in PBS for 30 min, and then rinsed with PBS. At this point in the procedure, filters of field samples were returned to holders and washed with PBS. Slight positive pressure was then established by reversing vacuum pump connections; sufficient PBS was added to just submerge the filter, and then 10 μl of the specific antiserum was added. The filters were incubated for 30 min and washed with 30 ml of PBS. Positive pressure was reestablished, and 10 to 15 μl of fluorescein isothiocyanate-conjugated sheep anti-rabbit immunoglobulin G (IgG; Miles Yeda Ltd., Elkhart, Ind.) was added. The filters were incubated for 30 min and washed with 30 ml of PBS, followed by 20 ml of 0.5 M carbonate buffer (pH 9.6). Filters were then placed on clean slides, topped with 2 drops of mounting solution (glycerol-PBS, 9:1 [vol/vol]), and mounted under a cover slip. FA-stained slides were viewed with a Zeiss Universal research microscope using an epifluorescence illuminator with a 100-W tungsten halogen lamp (10× ocular, 40× objective). A blue excitation filter (450 to 500 nm) and a yellow-green fluorescence barrier filter (528 nm) were used.

Enumeration. Counting efficiency and reliability of the FA assay were tested by comparing it with two independent methods of enumeration. Concentrated pure culture samples were counted using a hemacytometer with Nomarsky optics at 400× magnification. Dilutions of these samples were counted by the FA assay described above and by acridine orange direct counting (17).

**RESULTS**

The double immunodiffusion tests of the homologous system (N. oceanus, Carlucci isolate, antigen and its specific anti-N. oceanus antiserum) yielded 10 to 12 precipitin bands. The Watson isolate and the unidentified Carlucci isolate no. 5 gave the second and third strongest reactions for the double immunodiffusion test; the soil and freshwater ammonium oxidizers also showed positive reactions (Table 2). The non-ammonium-oxidizing autotrophs and the heterotrophs showed little or no response to N. oceanus antiserum. A positive FA reaction was observed only for the three marine ammonium-oxidizing isolates (Table 2). For controls, the reactivity of fluorescein isothiocyanate sheep anti-rabbit IgG was tested against saline solution, pre-immunization serum, and N. oceanus antiserum; only the specific antiserum gave a positive fluorescent result.

Auto- and nonspecific fluorescence did not interfere in FA-stained culture samples, but were adequately suppressed by bovine serum albumin. Although a minimal amount of intense localization of green or orange fluorescence was often present, the bacteria were easily distinguished by characteristic size and shape. Careful pretreatment of filters and pH control in washing the filtered samples resulted in a black background which contrasted well with the green.

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**FIG. 1. FA staining procedure.**
fluorescence of the FA-stained cells (Fig. 2). Auto- and nonspecific fluorescence was more severe in field samples; several methods of di-

Table 2. Immunodiffusion and FA reactions of bacterial isolates to N. oceanus antiserum. Immunodiffusion results were number of precipitin bands formed; FA results were either a strong fluorescence (+) or no detectable fluorescence (−)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Immunodiffusion</th>
<th>FA</th>
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<tbody>
<tr>
<td>1. <em>Nitrosococcus oceanus</em></td>
<td>10–12</td>
<td>+</td>
</tr>
<tr>
<td>(Carlucci)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. <em>N. oceanus</em> (Watson)</td>
<td>9–10</td>
<td>+</td>
</tr>
<tr>
<td>3. No. 5 (Carlucci)</td>
<td>5–6</td>
<td>+</td>
</tr>
<tr>
<td>4. <em>Nitrosomonas Tara</em></td>
<td>3</td>
<td>−</td>
</tr>
<tr>
<td>5. <em>Nitrosomonas europaea</em></td>
<td>3–4</td>
<td>−</td>
</tr>
<tr>
<td>7. <em>Nitrosomonas E-K</em></td>
<td>2–4</td>
<td>−</td>
</tr>
<tr>
<td>8. 14-1*</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>9. 14-2*</td>
<td>0–1</td>
<td>−</td>
</tr>
<tr>
<td>10. 14-3*</td>
<td>1–2</td>
<td>−</td>
</tr>
<tr>
<td>11. 16-2*</td>
<td>1</td>
<td>−</td>
</tr>
<tr>
<td>12. 18-2*</td>
<td>1</td>
<td>−</td>
</tr>
<tr>
<td>13. 18-3*</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>14. 20-1*</td>
<td>1</td>
<td>−</td>
</tr>
<tr>
<td>15. 20-3*</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>16. <em>E. coli</em></td>
<td>ND*</td>
<td>−</td>
</tr>
<tr>
<td>17. Thiosulfate oxidizer M1</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>18. <em>Nitrobacter agilis</em></td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>19. <em>B. gazogenes</em></td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>20. BV1-SJG</td>
<td>ND</td>
<td>−</td>
</tr>
</tbody>
</table>

* Unidentified marine heterotrophs isolated off the Washington coast.
ND, Not determined.

minishing this unwanted fluorescence were examined since incubation with bovine serum albumin proved ineffective. The procedure of Bohlool and Schmidt (4) was found to be a satisfactory method for attenuating auto- and nonspecific fluorescence; the gelatin coating gave a nearly black background which allowed easy detection of fluorescent cells in the presence of noncellular particulate fluorescence.

The precision of the FA assay as a method for enumerating ammonium-oxidizing bacteria was tested by comparing counts of FA-stained cells with both hemacytometer and acridine orange cell counts for cell concentrations spanning four orders of magnitude, and including concentrations likely to be encountered in field samples (Fig. 3). There was no significant difference (P > 0.95) from the predicted slope of 1 in the regression of count data; FA counts gave good replication (Fig. 3).

DISCUSSION

Only the marine ammonium-oxidizing isolates gave a positive reaction with FA staining and were visually indistinguishable from the homologous Carlucci strain of *N. oceanus* used to produce the antibody. Both immunodiffusion and FA tests gave a positive reaction for the marine ammonium oxidizers but did not give identical results for *Nitrosomonas* isolates (Table 2). Although the FA test is considered generally more sensitive than gel diffusion methods

Fig. 2. FA-stained cells of *N. oceanus*. Cells appeared bright green, especially at periphery; background was black. Average cell diameter was 3.3 μm.
To be detectable by double immunodiffusion, the antigen must be capable of both diffusing through the agarose matrix and forming a precipitate with the antibody. Although whole-cell preparations which had been frozen and thawed were used in the diffusion tests, only the soluble or solubilized constituents can react. Thus, a positive reaction by immunodiffusion and a negative one by FA, such as for *Nitrosomonas* isolates, were likely due to these species having internal antigens in common with *Nitrosococcus* but immunologically very different cell wall antigens.

Nitrifying bacteria share a unique pathway of energy production: the oxidation of reduced nitrogen compounds. The results of our immunological studies also give support to the biochemical basis for the systematic classification of ammonium-oxidizing bacteria. The strains of marine, freshwater, and soil ammonium oxidizers we examined did exhibit antigenic similarity in immunodiffusion tests, although the nitrate oxidizer we examined did not (Table 2). Our results indicated that the nonspecific cross-reactions observed by immunodiffusion did not contribute to an FA-positive reaction and that the FA assay was specific for the detection of *Nitrosococcus*.

Using the direct FA method, Belser and Schmidt (2) compared cross-reactions of 16 soil ammonium-oxidizing strains from four genera. Of the antibodies prepared from each of the six strains of *Nitrosomonas* they tested, only two were essentially species specific, i.e., did not react with congeneric strains. The other four strains had significant cross-reactions with each other; i.e., heterologous tests gave positive reactions. However, cross-reactions with other related genera of ammonium-oxidizers were minimal. Because these authors did not perform gel diffusion tests, the specificity of their antisera cannot be directly compared with specificity results from immunodiffusion assays (Table 2).

The usefulness of the FA assay as a technique for enumerating concentrations of nitrifying bacteria in the ocean depends on the specificity of the antigenic reaction, the visual resolution of the stained cell from background nonspecific and autofluorescence, and precision of the actual counting procedure. The antigenic reaction was found to be specific for the marine ammonium oxidizers tested (Table 2). The inclusion of a gelatin or bovine serum albumin treatment in the FA-staining protocol was important in suppressing background fluorescence and in attaining sharp visual resolution of the FA-stained cell. The final carbonate buffer rinse increased absolute fluoroscein isothiocyanate fluorescence and enhanced contrast between fluorescent cells and background; in addition, the alkaline pH

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**Fig. 3.** Log-log transformed count data showed no difference between calculated regressions and predicted lines with slope 1 and intercept 0 (P ≥ 0.95). (A) FA versus predicted counts: cells/ml = 0.9761 FA + 0.0839, df = 11, r = 0.9733. (B) Acridine orange versus predicted counts: cells/ml = 1.003 acridine orange (AO) + 0.3874, df = 15, r = 0.9677.

(14), our results suggested that the two tests did not necessarily measure the same set of antigens. To detect an antigen by the FA method used here, the antigen-antibody complex formed by the specific reaction must be retained on a 0.40-μm Nuclepore filter. A few outer membrane components are important in FA staining of whole cells (22); soluble cytoplasmic components are not visible in FA-stained whole cells and may also be lost through the filter if the cells rupture during the FA staining procedure.
also aided in stabilizing fluorescence so that the FA-stained filters could be stored for a few weeks and recounted, if necessary. The results of the comparison of enumeration methods—of counts of FA-stained cells with hemacytometer and acridine orange counts—indicated that the FA technique was both accurate and precise (Fig. 3). Nitrifying bacteria in field samples from the primary nitrite maximum layer off the coast of Washington have been identified and enumerated by the FA assay; these results will be reported in a subsequent publication.

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LITERATURE CITED