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Nitrification at Low pH by Aggregated Chemolithotrophic Bacteria

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A study was performed to gain insight into the mechanism of acid-tolerant, chemolithotrophic nitrification. Microorganisms that nitrified at pH 4 were enriched from two Dutch acid soils. Nitrate production in the enrichment cultures was indicated to be of a chemolithoautotrophic nature as it was (i) completely inhibited by acetylene at a concentration as low as 1 μmol/liter and (ii) strongly retarded under conditions of carbon dioxide limitation. Electron microscopy of the enrichment cultures showed the presence of bacteria that were morphologically similar to strains of known chemolithotrophic nitrifying genera. Many of the enriched bacteria, in particular those that were identified as ammonium oxidizers, were aggregated. Filtration experiments indicated that aggregated cells were able to nitrify at low pH, whereas single cells were not. It is hypothesized that cells inside the aggregates are protected against the toxicity of nitrous acid. Nitrification by aggregated chemolithoautotrophic bacteria may be the dominating process of nitrate formation in many acid soils as it does not appear to depend on the existence of microsites of high pH (acid-sensitive autotrophic nitrification) or on the availability of organic carbon (heterotrophic nitrification).

It is established that nitrification in acid forest soils increases after disturbance of the forest ecosystem, e.g., by clear cutting or a high deposition of ammonium (14, 20). This may result in undesirable leaching of nitrate and aluminum into the groundwater (16, 21).

Depending on the type of acid soil studied, nitrate production is believed to be mainly caused by fungi (13, 19) or chemolithoautotrophic bacteria (5, 25). Autotrophic nitrification in acid soils is thought to depend on the presence of microsites of relatively high pH because all chemolithotrophic, ammonium-oxidizing strains that have been isolated from such soils appear to be acid sensitive (10, 17). Recently, evidence has been presented (6) for the existence of chemolithotrophic bacteria that are able to oxidize ammonium at pH 4. Acetylene-sensitive, acid-tolerant nitrate production was generally found to occur in Dutch heathland and forest soils with a high availability of ammonium, indicating that acid-tolerant ammonium-oxidizing chemolithotrophs may commonly be present in such soils (5, 8). Nevertheless, chemolithotrophic oxidation of ammonium at low pH remains a rather obscure process as, thus far, the responsible bacteria could not be isolated. To obtain more information on the morphology and growth characteristics of acid-tolerant ammonium-oxidizing strains, we did electron microscopic investigations of enrichment cultures nitrifying at low pH.

MATERIALS AND METHODS

Enrichment procedures. Acid-tolerant nitrifying bacteria were enriched at pH 4 in a liquid mineral medium [2.5 mM $(NH_4)_2SO_4$, 0.2 mM KH_2PO_4, 0.2 mM $MgSO_4$, 0.2 mM $CaCl_2$, trace elements (2)] that was inoculated (0.1%, wt/wt) with organic material (FH layer) of either a heathland soil (pH 3.6) or a Douglas fir forest soil (pH 3.7). Characteristics of both soils are presented elsewhere (6, 8). Previously, it had been shown that nitrate accumulation in both soils was completely inhibited by 60-Pa acetylene (6, 8), indicating that nitrification may be of a chemolithotrophic nature (12). The pH of the 0.1% suspensions was measured daily and adjusted to 4 with Na_2CO_3. Incubations were done at 20°C on a rotary shaker (100 rpm). After an incubation of 5 to 10 weeks, more than 90% of the ammonium was converted to nitrate. Subsequently, fresh mineral medium containing 1 mM $(NH_4)_2SO_4$ was inoculated (1:10) with nonfiltered or 5-μm-filtered (nylon) 0.1% suspension. The 5-μm filtration treatment was performed to remove most of the particulate organic matter. The pH of the subcultures was measured daily and adjusted to 4 or 6 with Na_2CO_3. Incubation conditions were the same as described above. Samples for determination of ammonium and nitrate were taken weekly. In a similar way, the 5-μm-filtered subcultures that had been adapted to nitrify at pH 4 were subcultured.

Tests for chemolithoautotrophy. Nitrate production in subcultures was tested for its sensitivity to (i) acetylene and (ii) limitation of carbon dioxide. For these experiments, subcultures were used that had been inoculated with nonfiltered 0.1% suspensions and adjusted to pH 4. In the acetylene inhibition experiment, 25-ml portions of these subcultures (0.01% suspensions) were transferred into screw-cap bottles (315 ml). Subsequently, the bottles were closed with a rubber septum. Acetylene, which was made free of acetone and CO by passing the gas through a solution of $CuCl_2$ in concentrated HCl and then water (22), was added through the septum to give equilibrium concentrations of 0, 1, 10, and 100 μM, respectively. Since no pH adjustments were possible in the screw-cap bottles, incubations were started at pH 4.5 instead of pH 4.0. The bottles were kept closed for 10 days while being incubated on a rotary shaker. At the end of the incubation period, the gas phase was analyzed for the presence of oxygen by using a gas chromatograph equipped with an electron capture detector. Oxygen concentrations were always above 15% (vol/vol). The pH of the incubated subcultures was determined. Samples for nitrate measurement were taken before and after the incubation. Each treatment was done three times.

To study the effect of carbon dioxide limitation on nitrate

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TABLE 1. Effect of acetylene on nitrate accumulation at pH 4.5 in nonfiltered subcultures

Acetylene concn (μM)	Nitrate accumulation (μmol/ml in 10 days)[a]	Final pH[a]
0	0.25 ± 0.01	3.4 ± 0.1
1	0.00 ± 0.00	4.5 ± 0.1
10	0.00 ± 0.00	4.5 ± 0.1
100	0.00 ± 0.00	4.5 ± 0.1

[a] Mean and standard deviation of three replicates.

production, portions of 50 ml of the 0.01% suspensions (pH 4) were transferred into Erlenmeyer flasks (300 ml). Subsequently, the flasks were closed with a bulb-tube that contained a separate reservoir. Liquid in the reservoir is in open connection with the suspension but cannot reach it. Carbon dioxide was withdrawn by filling the reservoir with 15 ml of 1 N NaOH, whereas bulb-tubes without NaOH served as a control. Incubation conditions were the same as described above. The bulb-tubes were removed daily to allow pH adjustment (pH 4) and to prevent oxygen limitation. Samples for nitrate measurements were taken weekly. Each treatment was done twice.

Transmission electron microscopy. Subcultures that nitrified at pH 4 were examined for the presence of chemolithotrophic nitrifying bacteria by transmission electron microscopy. Cells with or without (i.e., 5-μm inoculation) organic particles were concentrated by centrifugation (18,000 × *g*). Cells were fixed for 1 h in a solution of 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 0°C, postfixed for 60 min in a solution of 1% (wt/vol) OsO_4 and 2.5% (wt/vol) K_2CrO_7 in the same buffer at room temperature, and subsequently poststained overnight in 1% (wt/vol) uranyl acetate. After dehydration in a graded ethanol series, the cells were embedded in Epon 812, sectioned with a diamond knife on an LKB microtome, and examined in a Philips EM 300 electron microscope. In a similar way, thin sections were made of *Nitrosospira* strain AHB1, an acid-sensitive ammonium-oxidizing strain isolated from an acid heathland soil (3), to compare its morphology with that of the bacteria in the enrichment cultures. Cells of *Nitrosospira* strain AHB1 that were examined as such had been cultured in a pH-stat (pH 6) in the presence of nitrite-oxidizing cells (*Nitrobacter* strain NHB1) in the same mineral medium as was used for the enrichment cultures.

Analytical procedures. The concentrations of ammonium, nitrite, and nitrate were determined by continuous flow analysis (methods 824-87T and 795-86T; Bran and Luebbe Analyzing Technologies, Elmsford, N.Y.).

RESULTS AND DISCUSSION

Tests for chemolithoautotrophy. Even at the lowest concentration (1 μM), acetylene completely blocked the nitrate accumulation in 0.01% suspensions of both the forest soil (Table 1) and the heathland soil (data not shown). The control suspensions showed a considerable acidification during the incubation, whereas the suspensions containing acetylene did not acidify at all. Ammonium oxidation by *Nitrosomonas europaea* was inhibited by about 60% by 1 μM acetylene (12), which implies that the ammonium-oxidizing microorganisms in these suspensions were even more sensitive to acetylene. The depletion of carbon dioxide (absorption by NaOH) had a pronounced delaying effect on nitrate production in acid 0.01% suspensions of both the forest soil (Fig. 1) and the heathland soil (data not shown). Nitrite never accumulated in detectable (>5 μM) amounts. This implies that the effect of carbon dioxide depletion on nitrate production is not due to nitrous acid toxicity, which could occur if autotrophic nitrite oxidation was more repressed by carbon dioxide depletion than autotrophic ammonium oxidation. Therefore, the results show a direct dependency of the ammonium-oxidizing microorganisms on the availability of carbon dioxide, which is in agreement with autotrophic but not with heterotrophic nitrification. Thus, the effects both of acetylene and of carbon dioxide limitation on nitrate accumulation indicate that chemolithoautotrophic bacteria are the main nitrifying organisms in the enrichment cultures.

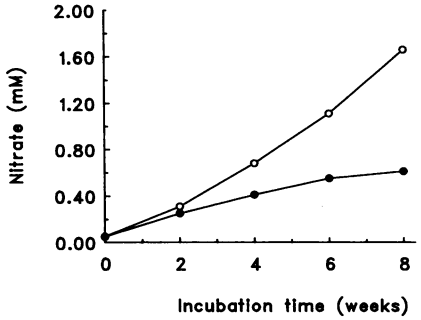

FIG. 1. Effect of carbon dioxide withdrawal (absorption by NaOH) on nitrate production at pH 4 in a mineral medium containing 1 mM $(NH_4)_2SO_4$ that was inoculated (1:10) with a nonfiltered nitrifying 0.1% suspension (pH 4) of the organic layer of a Douglas fir forest soil. ○, nitrate concentrations in the controls, i.e., Erlenmeyer flasks that were closed with bulb-tubes containing no NaOH; ●, nitrate concentrations in Erlenmeyer flasks that were closed with NaOH-containing bulb-tubes. Every day the bulb-tubes were removed to prevent oxygen limitation and to allow pH (4) adjustment.

Effect of filtration. Tenfold dilutions of the nonfiltered 0.1% suspensions immediately started to accumulate nitrate at pH 6 and 4 (Fig. 2). Nitrite was never present in detectable (>5 μM) amounts. The onset of nitrate accumulation in medium inoculated with 5-μm-filtered 0.1% suspension was much slower and occurred at pH 6 but not at pH 4. As the pH was adjusted only once a day, the daily drop in pH due to overnight nitrification could be observed. Interestingly, the overnight pH drop in medium of pH 6 that had been

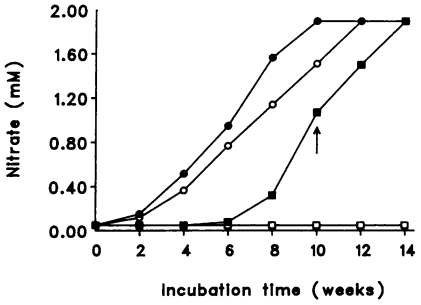

FIG. 2. Cumulative nitrate accumulation at pH 4 (○, □) and 6 (●, ■) in a mineral medium containing 1 mM $(NH_4)_2SO_4$ that was inoculated (1:10) with nonfiltered (○, ●) or 5-μm-filtered (□, ■) nitrifying 0.1% suspension (pH 4) of the organic layer of a Douglas fir forest soil. The pH was not kept constant but was adjusted daily. Arrow indicates a switch of the daily pH adjustment from 6 to 4 for the medium inoculated with 5-μm-filtered suspension.

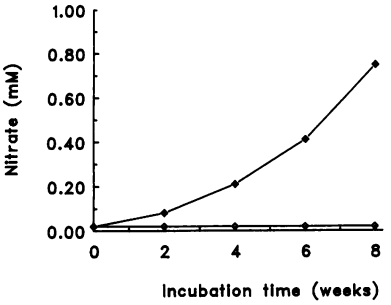

FIG. 3. Cumulative nitrate accumulation at pH 4 in a mineral medium containing 1 mM $(NH_4)_2SO_4$ that was inoculated with (1:10) nonfiltered (◆) and 5-μm-filtered (◇) portions of the enrichment culture (Fig. 2, ■) that had been nitrifying at pH 6 and subsequently at pH 4.

inoculated with 5-μm-filtrate became more than 2 pH units (<pH 4) during the incubation. At that time, a daily adjustment to pH 4 was sufficient to maintain nitrate production (Fig. 2). New 10-fold dilutions without 5-μm filtration of these acid-adapted enrichment cultures showed exponential nitrate production at pH 4 (Fig. 3), indicating that the nitrifying bacteria did proliferate despite the low pH. Filtration (5 μm) of the acid-adapted enrichment cultures completely removed the nitrate-producing activity (Fig. 3). The results indicate that aggregated nitrifying bacteria (>5 μm) can be active and grow at low pH, whereas single cells and small aggregates (<5 μm) cannot.

Transmission electron microscopy. Following the inoculation procedures described above, it was possible to prepare subcultures that nitrified at pH 4 in both 5-μm-filtered and nonfiltered 0.1% suspensions. Subcultures of nonfiltered suspensions contained organic particles, but those of 5-μm-filtered suspensions did not. Both types of subcultures were examined for the presence of chemolithotrophic nitrifying bacteria by transmission electron microscopy. In all filtered and nonfiltered subcultures nitrifying at pH 4, aggregated bacteria were present that appeared to be morphologically similar to the acid-sensitive, ammonium-oxidizing bacterium *Nitrosospira* strain AHB1 that has been isolated from an acid heathland soil (Fig. 4). The various shapes of *Nitrosospira* strains that were obtained when the cells were sectioned are characteristic and are due to the spiral structure of the cells, i.e., the configuration depends on the plane of sectioning of the cell (23). In most cases, a *Nitrosospira* bacterium cannot be seen as a complete cell but only as a few separated segments (coils) that are in close proximity (Fig. 4A). Many aggregates contained U-shaped cells (pseudococci) which closely resembled the morphology of aggregated cells (cysts) of *Nitrosospira briensis* (23). Both *Nitrosospira* strain AHB1 cells and aggregated *Nitrosospira*-like cells contained granules (Fig. 4C and D), which consist most probably of polyphosphate since accumulation of polyphosphate by nitrifying bacteria is commonly observed (24). The extensive cytomembrane system that is characteristic for *Nitrosomonas* sp. and *Nitrobacter* sp. (24) was lacking in both *Nitrosospira* strain AHB1 cells and aggregated *Nitrosospira*-like cells. The lack of an extensive cytomembrane system was also reported for *Nitrosospira briensis* (23, 24). The aggregates containing the *Nitrosospira*-like bacteria appeared to be more or less spherical with a diameter of 3 to 20 μm.

Bacteria that possessed an extensive peripheral cytomembrane system similar to that of the nitrite-oxidizing cells of the genus *Nitrobacter* (24) were also present in the nitrate-producing acid subcultures (Fig. 4E and F). These *Nitrobacter*-like cells occurred both singly (not shown) and aggregated (Fig. 4E and F). Aggregates (up to 50 μm) containing both *Nitrosospira*-like and *Nitrobacter*-like bacteria were also detected (Fig. 4E). In these mixed aggregates, the two cell types appeared to be located in separate groups, with the *Nitrobacter*-like cells on the outside of the aggregate (Fig. 4E). Since the aggregates remained intact after the electron microscopic pretreatments (dehydration), it is evident that the bacteria have to be embedded in a stabilizing polymer matrix. It is well known that aggregated nitrifying bacteria that occur in so-called zoogloeae or cysts are embedded in slime layers (24).

Combining the results of filtration experiments and electron microscopy, it appears that nitrification by chemolithotrophic bacteria can occur at low pH provided the cells are aggregated. This may apply particularly to the ammonium-oxidizing bacteria, since single cells of certain *Nitrobacter* strains have been shown to oxidize nitrite at low pH (11). The ability to be active at low pH in aggregates is not a general feature of known ammonium-oxidizing strains. Biofilms containing *Nitrosomonas* sp. as well as aggregated cells of *Nitrosospira* sp. were not able to oxidize ammonium below pH 5 (1, 15, 17, 23). Thus, ammonium oxidation in enrichment cultures at pH 4 is probably done by hitherto unidentified strains that resemble strains of known genera. It is possible that the acid-tolerant *Nitrosospira*-like bacteria are aggregated cells of *Nitrosospira* strains that have been isolated (10, 15) from acid soils. However, with respect to *Nitrosospira* strain AHB1 this seems to be unlikely since antibodies against this strain did not react with the aggregated *Nitrosospira*-like bacteria (4). Still, the possibility that acid-sensitive *Nitrosospira* strains isolated from acid soils adapt to acid conditions by aggregation should be taken into consideration. The results of the 5-μm filtration experiments indeed indicate that such an adaptation can take place during a period of fluctuating pH, although the adapted bacteria may already have occurred in small (<5-μm) aggregates. The isolation procedures that are commonly used, i.e., serial dilution of enrichment cultures (18), select for nitrifying bacteria that grow as single cells. If aggregation is necessary to allow activity at low pH, it is not surprising that the strains isolated in this way can no longer grow at low pH. The other possibility, namely, that acid-sensitive and acid-tolerant strains are different species, may of course be equally true. In that case, new methods have to be developed to isolate the acid-tolerant strains because, as mentioned above, the routine methods will select for the acid-sensitive ones. It will be very difficult to separate the aggregates from contaminating heterotrophs by using the serial dilution technique at low pH because the aggregates will provide the heterotrophs with an energy source (polymer matrix).

As yet, the mechanism of nitrification at low pH by chemolithotrophic bacteria is not understood. It has been argued that slime layers modify the microenvironment of nitrifying bacteria (1, 17). The inability of nitrifying bacteria to be active at low pH has been associated with either the dependency of ammonium-oxidizing bacteria on NH_3 (26) or the toxicity of nitrous acid (HNO_2) (9). Therefore, the most simple solution for explaining the ability to nitrify in aggregates is given by the assumption that the pH inside the aggregates is higher than that in the surrounding medium. However, this is rather unlikely, because the surrounding medium is continuously acidified, which implies that protons

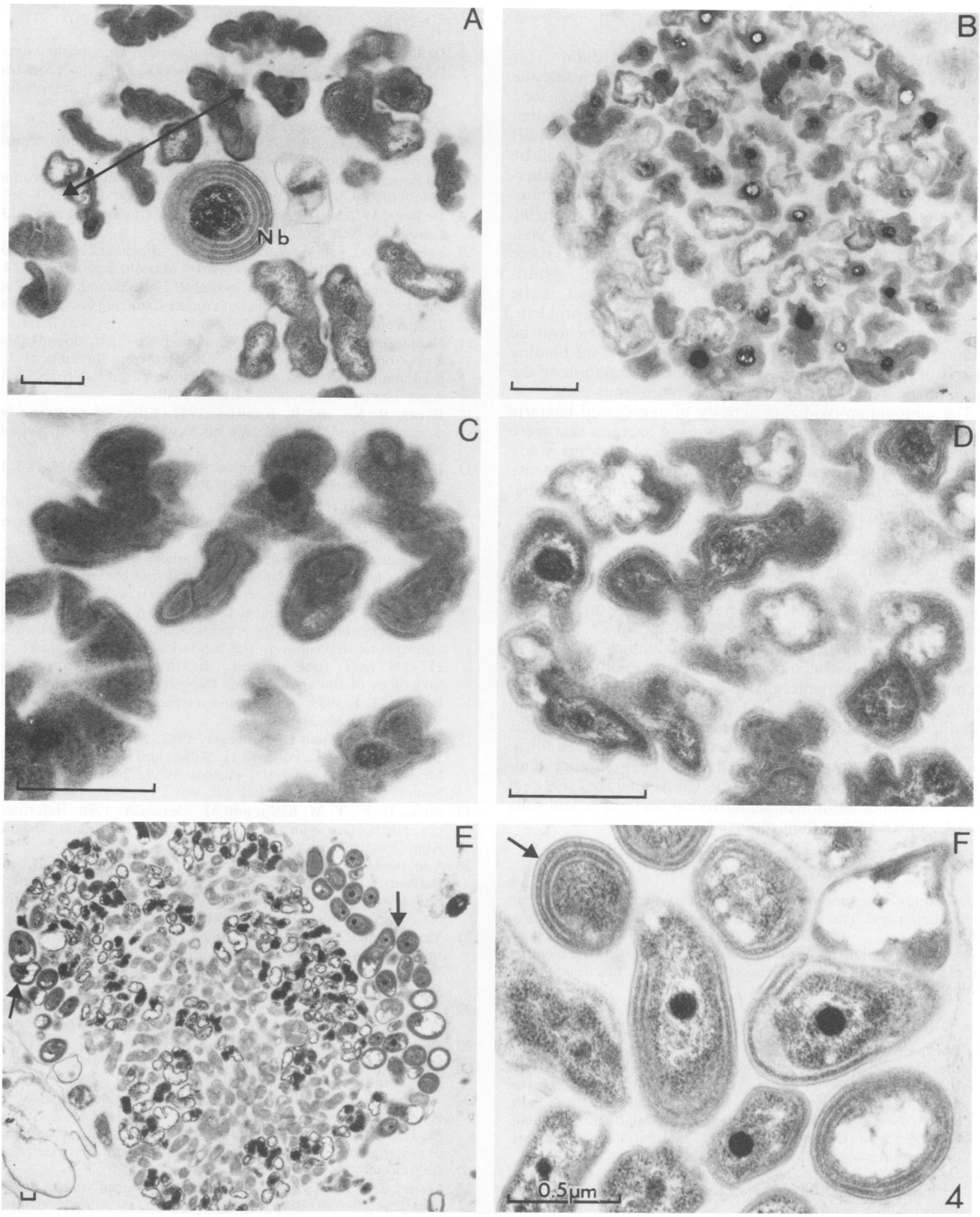

FIG. 4. Transmission electron micrographs of *Nitrosospira* strain AHB1 that was cocultured with *Nitrobacter* strain NHB1 at pH 6 in a pH-stat and of aggregated bacteria that were present in enrichment cultures nitrifying at pH 4. (A) Characteristic capricious forms of *Nitrosospira* strain AHB1 cells caused by its spiral morphology. Double arrow indicates one cell; Nb indicates a cell of *Nitrobacter* strain NHB1. (B) Aggregated *Nitrosospira*-like bacteria. (C) Segments (coils) of *Nitrosospira* strain AHB1 cells showing the lack of an extensive cytomembrane system. (D) Segments of aggregated *Nitrosospira*-like bacteria. (E) Aggregated *Nitrosospira*-like bacteria surrounded by *Nitrobacter*-like bacteria (arrow). (F) Aggregated *Nitrobacter*-like bacteria showing the characteristic peripheral cytomembrane system (arrow). Scale bars, 0.5 μm.

are excreted from the aggregates into the medium. This requires a proton gradient, indicating that the pH inside the aggregates is even lower than that outside the aggregates. Evidently, the aggregated, chemolithotrophic bacteria that can oxidize ammonium at low pH must possess a transport system for NH_4^+, because concentrations of NH_3 will be extremely low (pK NH_4^+/NH_3 = 9.25) at pH 4. Furthermore, if it is assumed that the ammonia monooxygenase does not differ from that of other ammonium-oxidizing bacteria, these bacteria must somehow be able to keep their internal pH high to allow NH_3 oxidation (7). Still, these proposed adaptations alone are not sufficient to allow nitrification at low pH because aggregation appeared to be required. Thus, it is believed that the second problem, namely, the toxicity of nitrous acid, may somehow be counteracted by aggregation. Characterization of the binding matrix of the aggregates may reveal valid information on the mechanistic aspects of protection against nitrous acid.

The ammonium-oxidizing activity of aggregated bacteria at low pH is not merely a rest activity of bacteria that were formerly growing at suitable pH conditions. In this study (Fig. 3) and in a previous study (6), it was shown that the rate of nitrate accumulation at pH 4 increased during the incubation period, indicating proliferation of nitrifying bacteria. Therefore, it is believed that the described type of nitrification may be the dominating process of nitrate formation in many acid soils, as it does not appear to depend on the existence of microsites of high pH (acid-sensitive autotrophic nitrification) or on the availability of organic carbon (heterotrophic nitrification). The importance of aggregated chemolithotrophic ammonium-oxidizing bacteria in acid soils may have been overlooked because of the dilution techniques that are generally used in enumerating and isolating nitrifying bacteria (18). Dilution methods assume that the nitrifying bacteria occur as single cells after the soil has been suspended. However, it was observed that even ultrasonic treatment was not very effective in disrupting acid-tolerant nitrifying aggregates (4). Consequently, the real numbers of chemolithotrophic nitrifying bacteria in acid soils may be much higher than reported.

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