



## AMMONIUM-OXIDATION AT LOW pH BY A CHEMOLITHOTROPHIC BACTERIUM BELONGING TO THE GENUS *NITROSOSPIRA*

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**Summary**—Ammonium-oxidizing bacteria of the genus *Nitrosospira* have often been isolated from acid soils. However, the lower pH-limit for nitrifying activity of these bacteria in pure cultures or in mixed cultures with *Nitrobacter* sp. is much higher than ambient soil-pH. This study shows that the acid-sensitive, ammonium-oxidizing bacterium *Nitrosospira* strain AHB1, which has been isolated from an acid heathland soil, could be adapted to oxidize ammonium at pH 4 in the presence of the acid-tolerant nitrite-oxidizing bacterium *Nitrobacter* strain NHB1. Adaptation could be achieved in two different ways: (1) by immobilizing the bacteria in alginate beads; and (2) by exposing them to pH-fluctuations. Thus, *Nitrosospira* spp may be involved in the oxidation of ammonium at an ambient low soil-pH, even though they appear to be acid-sensitive after isolation.

### INTRODUCTION

Acid forest soils with a high availability of ammonium are located in areas of northwestern Europe where the atmospheric input of  $\text{NH}_x$  has increased due to the intensification of animal husbandry. It has been well-established that nitrification proceeds in these soils (Van Breemen *et al.*, 1987; Tietema *et al.*, 1992). In general, the formation of nitrate in acid forest soils has adverse effects on the environment, as it may result in further acidification of the soils and pollution of ground- and surface-waters (Van Breemen and Van Dijk, 1988).

In order to estimate reliably the amount of nitrate that ultimately may be produced and leached in N-saturated acid forest soils, the microbiology of the nitrification process has to be known. The use of inhibitors, such as acetylene and nitrapyrin, indicates that chemolithotrophic bacteria are the most important nitrifying microorganisms, despite the acidity of the soils (Stams *et al.*, 1990; De Boer *et al.*, 1991, 1992). Microscopic examinations of acid enrichment cultures of a Dutch forest soil have revealed the occurrence of aggregates of bacteria morphologically similar to strains of the known chemolithotrophic genera *Nitrosospira* (ammonium-oxidizer) and *Nitrobacter* (nitrite-oxidizer) (De Boer *et al.*, 1991). It has been shown that the aggregates were active at pH 4, whereas single cells were not. It has been suggested that only the ammonium-oxidizing bacteria might need to be aggregated for activity at low pH, since the existence of acid-tolerant nitrite-oxidizing

bacteria, growing as single cells, had already been reported (Hankinson and Schmidt, 1988).

Yet, direct proof for ammonium-oxidation at low pH by aggregated *Nitrosospira* cells did not exist because attempts to obtain the aggregated, acid-tolerant cells in pure culture were not successful. On the contrary, acid-sensitive strains of the genus *Nitrosospira* have been isolated from acid soils quite often (Walker and Wickramasinghe, 1979; Hankinson and Schmidt, 1984; Martikainen and Nurmiaho-Lassila, 1985; De Boer *et al.*, 1989a, b; Allison and Prosser, 1991). Therefore, we examined the possibility of adapting an acid-sensitive *Nitrosospira* strain to low pH. This was done by growing cultures under two different conditions that were expected to result in formation of bacterial aggregates. These were: (1) Immobilization in alginate beads; and (2) Exposure to pH-fluctuations. The formation of microcolonies of nitrifiers after immobilization in gels is well-known (Van Neerven *et al.*, 1990; Hunik *et al.*, 1993). Formation of nitrifying aggregates during a period of pH-fluctuation was reported by De Boer *et al.* (1991). The effects of both culturing conditions on the ammonium-oxidizing activity of the *Nitrosospira* bacteria at low pH are reported here.

### MATERIALS AND METHODS

#### *Microorganisms and preculture medium*

All experiments were performed using mixed cultures of the acid-sensitive ammonium-oxidizing bacterium *Nitrosospira* strain AHB1, and the acid-tolerant nitrite-oxidizing bacterium *Nitrobacter*

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strain NHB1 (De Boer and Laanbroek, 1989; De Boer *et al.*, 1989a). Mixed cultures were used to prevent the toxicity of nitrite at low pH (De Boer and Laanbroek, 1989). Both strains had been isolated from an acid (pH 3.8) heathland soil (De Boer *et al.*, 1988, 1989a). Mixed batch cultures of these strains were grown in liquid medium of the following composition (per litre of deionized water):  $(\text{NH}_4)_2\text{SO}_4$ , 330 mg;  $\text{KH}_2\text{PO}_4$ , 100 mg;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 40 mg;  $\text{CaCl}_2$ , 15 mg and 1 ml of a trace-element solution (De Boer and Laanbroek, 1989). Bromothymol blue ( $5 \text{ ml l}^{-1}$  of 0.04% solution in water) was added as a pH-indicator. The pH was adjusted using NaOH; after autoclaving (20 min,  $120^\circ\text{C}$ ) it was about 7.5. The medium was inoculated (1%, v/v) with an older, mixed culture. Incubation was at  $20^\circ\text{C}$  without shaking. During incubation without pH-adjustment, nitrification stopped after the pH had decreased to about 4.5. By then, 0.3–0.4 mmol  $\text{l}^{-1}$  of ammonium had been oxidized and the cultures contained  $1\text{--}2 \times 10^6$  cells  $\text{ml}^{-1}$  of both strains. Within 1 wk, these cultures were used as inocula for pH-stat and immobilization experiments. In cases, where it was desirable to have a higher cell density in the inoculum, the pH of the mixed cultures was adjusted with sterile NaOH solution during the incubation to 7.0–7.5, as indicated by the pH-indicator. The purity of the mixed batch cultures was checked by inoculating 5 ml of liquid peptone(0.1%)–glucose(0.1%)–yeast extract (0.01%) medium with a few droplets of the cultures. If no growth was observed after 3 wk at  $20^\circ\text{C}$ , the cultures were considered free of heterotrophic contaminants.

#### Immobilization experiments

A 4% (w/v) solution of sodium-alginate (Sigma) in deionized water was mixed 1 : 1 (v/v) with a culture of *Nitrosospira* AHB1 and *Nitrobacter* NHB1. The mixture was transferred dropwise into a stirring solution of  $0.2 \text{ mol l}^{-1}$   $\text{CaCl}_2$  using a syringe that was kept 20 cm above the  $\text{CaCl}_2$  solution. This procedure resulted in the formation of calcium-alginate beads with an average diameter of 2 mm. These beads were allowed to harden for 20 min in the  $\text{CaCl}_2$  solution. They were collected on a sieve, and washed with deionized water containing  $5 \text{ mmol l}^{-1}$   $\text{CaCl}_2$ . Portions of alginate (10 g) were transferred to Erlenmeyer flasks containing 100 ml of liquid growth medium with the same composition as the preculture medium, except that it contained  $5 \text{ mmol l}^{-1}$   $\text{CaCl}_2$  and no bromothymol blue. The initial pH of the medium was 6 or 4. The flasks were kept at  $20^\circ\text{C}$  on a rotary shaker operating at 150 rev  $\text{min}^{-1}$ . The pH was measured daily using a pH-electrode. As soon as the pH dropped below 4, it was adjusted daily to 4 with  $0.1 \text{ N Na}_2\text{CO}_3$ . Samples were taken weekly for analysis of nitrite and nitrate. The rate of nitrate formation by the immobilized bacteria was compared with that of non-immobilized bacteria that were simultaneously incubated under comparable conditions, i.e. in the

presence of alginate beads. Each treatment was done 3 times.

#### pH-stat experiments

The pH-stat experiments were conducted using Biostat M fermenters (B. Braun, Melsungen, Germany) with culture volumes of 1250 ml, and Applikon ADI 1030 fermenters (Applikon, Delft, The Netherlands) with culture volumes of 750 ml. All fermenters were equipped with automatic regulation of pH and temperature. In addition, the Biostat M fermenters were equipped with continuous recording of the oxygen-tension. Growth medium (4/5 of the final volume) was autoclaved in the culture vessels. After cooling, mixed batch precultures of *Nitrosospira* AHB1 and *Nitrobacter* NHB1 were added to give the final volumes mentioned. After inoculation, the composition of the medium was the same as that of the preculture medium except that it contained more  $(\text{NH}_4)_2\text{SO}_4$  ( $5\text{--}7 \text{ mmol l}^{-1}$ ) and some nitrate ( $0.5\text{--}1 \text{ mmol l}^{-1}$ ). The bacteria in the pH-stat were exposed to three successive pH-conditions:

- (I) Initial period (5–10 days), in which the pH was constantly maintained at 6.
- (II) Intermediate period (20–30 days), in which the pH fluctuated as it was adjusted to 6 manually only once a day.
- (III) Final period (30–40 days), in which the pH was constantly maintained at 4.

The bacteria were grown at a constant temperature of  $20^\circ\text{C}$ . The cultures were aerated by stirring (150 rev  $\text{min}^{-1}$ ). Samples were taken regularly for determination of ammonium, nitrite and nitrate, and for enumeration of bacteria. Whenever the concentration of ammonium dropped to less than  $1 \text{ mmol l}^{-1}$ , a sterile solution of  $100 \text{ mmol l}^{-1}$   $(\text{NH}_4)_2\text{SO}_4$  was added to give a concentration of  $5\text{--}6 \text{ mmol l}^{-1}$   $\text{NH}_4^+$ . In total, four of these pH-stat experiments were completed. Two extra pH-stat experiments were conducted in which the intermediate period of pH-fluctuations was omitted.

An additional experiment was done to investigate the possibility that the ammonium-oxidation at pH 4 in pH-stat cultures was restricted to cells that were attached to the glass walls of the vessels. Fresh inorganic liquid medium (same composition as the preculture medium but with bromocresol green as pH-indicator) was inoculated with different amounts of the liquid phase of a non-aggregated pH-stat culture that was producing nitrate at pH 4. The final volume (medium + inoculum) was 100 ml. Incubation was at  $20^\circ\text{C}$  in Erlenmeyer flasks that were placed on a rotary shaker operating at 150 rev  $\text{min}^{-1}$ . If necessary the pH was kept at 4 using  $0.1 \text{ N Na}_2\text{CO}_3$ . Samples were taken weekly for nitrate measurements. These experiments were performed in triplicate.

### Enumeration techniques and chemical analyses

Bacteria were enumerated microscopically (magnification 400 $\times$ ) using a counting chamber. The percentage of aggregated cells was determined by comparing the numbers in samples before (A) and after (B) an ultrasonic treatment (10 s, 50 kHz). The difference, (B) – (A), was considered to be the amount of aggregated cells. In addition, bacterial aggregates were counted directly.

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, NY). Nitrite was also measured using the more sensitive chemiluminescent technique (Garside, 1982). This was done by reducing nitrite to NO in culture samples (5 ml) according to Goretski *et al.* (1990). Subsequently, NO was measured using a chemiluminescence detector (NO-NO<sub>2</sub>-NO<sub>x</sub> analyser Model 42S, Thermal Environmental Instruments Inc., Franklin, MA) equipped with a sample mixing unit. Nitrogen gas (purity > 99.999%, Hoekloos, Schiedam, The Netherlands) was used as a carrier. Using the chemiluminescent technique, NO<sub>2</sub><sup>-</sup> could be measured to concentrations as low as 50 nmol l<sup>-1</sup>.

## RESULTS

### Immobilization experiments

Mixed cultures of immobilized nitrifying bacteria started to produce nitrate at pH 6, and the pH dropped to 4 within 2 wk (Fig. 1). Thereafter, while the pH was adjusted daily to 4, nitrate production continued during the whole experimental period. The nitrifying activity of non-immobilized cells resulted in a drop of the pH to 4.2. Despite the presence of alginate beads, these cells did not show further nitrate production. Neither immobilized nor non-immobilized cells produced nitrate when the pH was low (4) at the start of the incubation (not shown).

### pH-stat experiments

All pH-stat cultures were able to continue nitrification at pH 4 after a period of daily pH-fluctuations, but not without such a period (Fig. 2). The cultures that nitrified at pH 4 consisted largely (>99%) of non-aggregated cells [Fig. 2(B)]. More than 90% of the ammonium added was oxidized to nitrate at all pH-conditions. Nitrite concentrations were generally low (10–50  $\mu$ mol l<sup>-1</sup>) during the initial period at pH 6 and became lower than the detection level of 50 nmol l<sup>-1</sup> as soon as the pH decreased. Within 24 h after the automatic titration was stopped, the pH dropped below 4 (Fig. 3). This strong drop in pH was observed during the whole period of daily titration to pH 6. Cell numbers increased strongly during the initial period of pH 6 to about 10<sup>8</sup> cells ml<sup>-1</sup>. A further increase in cell numbers was observed during the period of constantly low pH (Fig. 3).

The oxygen concentration (percentage of air saturation) was measured in two pH-stat cultures. In these cultures, it decreased to about 10% during the initial period of pH 6. Subsequently, during the period of pH-fluctuations, it increased again. During the period at pH 4, the oxygen concentration fluctuated between 50–70% of air saturation.

The nitrifying activity at pH 4 of non-aggregated bacteria that were cultured in the pH-stat could be transferred to a sterile fresh mineral medium at pH 4, but high inoculum densities were required to maintain activity throughout the whole incubation period (Fig. 4).

## DISCUSSION

The ammonium-oxidizing bacterium *Nitrosospira* strain AHB1, which was isolated from an acid heathland soil, was reported to be acid-sensitive (De Boer and Laanbroek, 1989). However, it appeared that the *Nitrosospira* cells could oxidize ammonium at pH 4 after immobilization in alginate beads, or after exposure to pH-fluctuations. Growth of immobilized nitrifying bacteria has been shown to result in the formation of microcolonies (Hunik *et al.*, 1993). The immobilized *Nitrosospira* cells were active at low pH only if they started to nitrify initially at a higher pH. This indicates that growth and, consequently, formation of microcolonies was essential for their

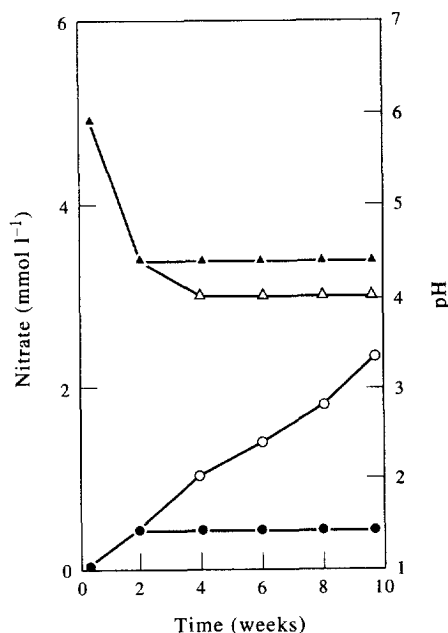


Fig. 1. Cumulative nitrate accumulation (○, ●) and pH-changes (△, ▲) in mixed batch cultures of *Nitrosospira* AHB1 and *Nitrobacter* NHB1 that were either immobilized in alginate (open symbols) or non-immobilized (closed symbols). The non-immobilized cultures contained the same amount of alginate beads as the immobilized ones. The pH was adjusted daily to 4 as soon as it dropped below this value. Symbols represent the mean of three experiments (CV < 5%).

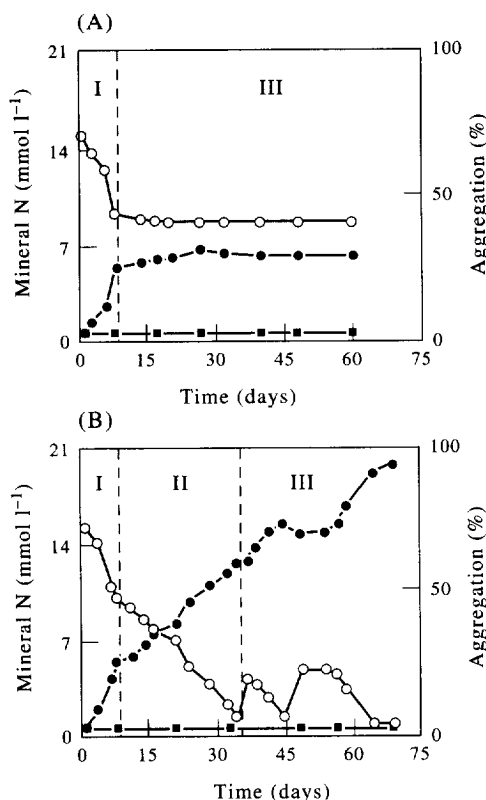


Fig. 2. Changes in the concentrations of ammonium (○) and nitrate (●) and in the percentage of aggregated cells (■) in a mixed pH-stat culture of *Nitrosospira* AHB1 and *Nitrobacter* NHB1. (A) Two cultures that were exposed to two different pH-conditions: I (pH 6, autotitration); III (pH 4, autotitration). (B) Four cultures that were exposed to three different pH-conditions: I (pH 6, autotitration); II (pH 6±4, titration to pH 6 once a day); III (pH 4, autotitration). Increases in the concentrations of ammonium are due to additions of sterile solutions containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.

nitrifying activity at low pH. In other studies evidence has been provided for ammonium-oxidation at low pH by chemolithotrophic bacteria that were present in cel-aggregates or in biofilms (De Boer et al., 1991; Allison and Prosser, 1993).

Yet, the pH-stat experiments showed that, after a period of pH-fluctuation, *Nitrosospira* cells adapted to acid conditions without aggregation. It is unlikely that this adaptation was restricted to cells that were attached to the glass wall since samples of the liquid phase, which contained only non-aggregated cells, continued to oxidize ammonium at pH 4 after being transferred to fresh media. Hence, neither aggregation nor biofilm formation appeared to be an absolute prerequisite for acid-tolerance. This implies that the cells had really adapted to low pH because they were not dependent on special pH-conditions in aggregates or biofilms.

As yet, it remains unclear which factors resulted in acid-tolerance. It has been shown that *Nitrosospira* strain AHB1 was not active below pH 5.5 if NO<sub>2</sub><sup>-</sup> was accumulating in the medium (De Boer and Laanbroek,

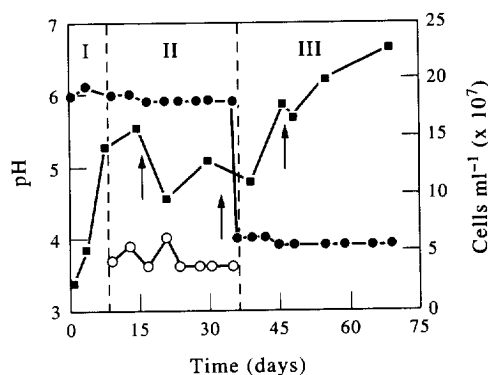


Fig. 3. Changes in pH (○, ●) and total cell numbers (■) in a mixed pH-stat culture of *Nitrosospira* AHB1 and *Nitrobacter* NHB1 that was exposed to three different pH-conditions [see legend to Fig. 2(B)]. During period II, the pH fluctuated daily between the upper (●) and lower (○) values indicated. Arrows indicate dilutions of the culture due to the addition of a sterile solution containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.

1989). However, it is unlikely that acid-sensitive cells had to adapt to deal with toxic levels of HNO<sub>2</sub> in the pH-stat cultures, as the concentration of NO<sub>2</sub><sup>-</sup> was always kept low (< 50 nmol l<sup>-1</sup>) by *Nitrobacter* strain NHB1. It may be that acid-sensitive *Nitrosospira* cells had to adapt to deal with the rather low concentration of NH<sub>3</sub> (NH<sub>4</sub><sup>+</sup> ↔ NH<sub>3</sub> + H<sup>+</sup>, pK = 9.25 at 25°C) at pH 4, since it is believed that NH<sub>3</sub> is the actual substrate of the ammonia monooxygenase (Prosser, 1989). Similarly, acid-sensitive cells may have had problems with the fixation of C at pH 4, because at this pH the

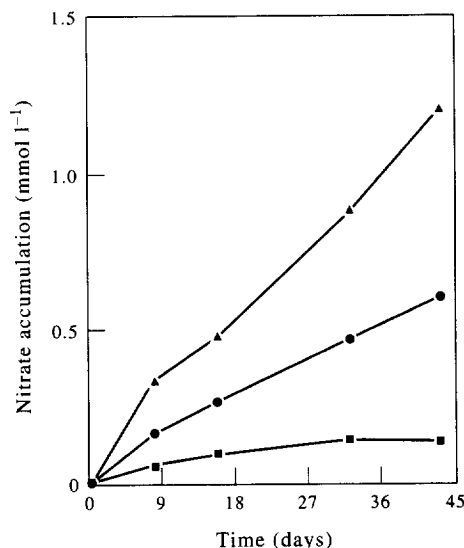


Fig. 4. Cumulative nitrate accumulation at pH 4 in mixed batch cultures of *Nitrosospira* AHB1 and *Nitrobacter* NHB1 that were inoculated with different amounts of a non-aggregated pH-stat culture that was nitrifying at pH 4. The inocula densities (v/v) were 10% (▲), 5% (●) and 1% (■), respectively. Sterile adjustments to pH 4 were done as indicated by the pH-indicator bromocresol green. Symbols represent the mean of three experiments (CV < 5%).

concentration of inorganic C is relatively low due to the instability of bicarbonate.

The inoculation of non-aggregated, acid-tolerant cells from pH-stat cultures into fresh mineral medium of pH 4 showed that they could continue their nitrifying activity, but a high cell density appeared to be required. Hence, it appears that an unknown factor which is linked to a high cell density is essential for the acid-tolerant ammonium-oxidizing activity. Interestingly, also the microcolonies inside alginate beads represent small volumes with a high bacterial density. A density-dependent factor that may be of particular interest is the formation of extracellular polymeric substances. It is known that chemolithotrophic nitrifying bacteria produce extracellular polymeric substances (Prosser, 1989). Acid-sensitive, nitrite-oxidizing bacteria that attached to glass slides became embedded in their own extracellular polymeric substances. The embedded cells could oxidize nitrite at lower pH than free-living cells (Keen and Prosser, 1987). Hence, the extracellular polymeric substances enabled the cells to be active at low pH, although the mechanistic aspects were not well-understood (Prosser, 1989). Since the pH-stat cultures represent batch cultures, extracellular polymeric substances may have accumulated. Therefore, the possibility that extracellular polymeric substances have been involved in the adaptation of *Nitrosospira* cells to low pH in pH-stat cultures cannot be excluded, even though free-living cells rather than attached ones appeared to be adapted.

The inability of adapted cells to remain active at pH 4 if the cell density is low, may explain why acid-tolerant *Nitrosospira* bacteria have never been isolated, since isolation of an acid-tolerant strain requires that one cell is able to proliferate under acid conditions. Recently, Hayatsu (1993) reported the first isolation of an acidophilic, ammonium-oxidizing bacterium (genus *Nitrosococcus*) from an acid tea soil. However, the descriptions of both the culturing methods (no culturing at constant low pH) and the results (cells could not be counted by dilution in the medium of pH 5 from which the strain was isolated) indicate that this strain is not acidophilic but rather able to adapt to become acid-tolerant as is the case for *Nitrosospira* strain AHB1.

The ability of adapted *Nitrosospira* strain AHB1 cells to nitrify and proliferate at pH 4 shows that nitrate production in acid soils can be the result of the activity of acid-tolerant, chemolithotrophic bacteria. This supports the results of earlier studies that provided evidence for such bacteria to be responsible for nitrate formation in N-saturated, acid forest and heathland soils (De Boer *et al.*, 1989b, 1991). The results of Hayatsu (1993) provided evidence for the occurrence of acid-tolerant, chemolithotrophic nitrification in N-fertilized, acid tea soils. Thus, acid-tolerant, chemolithotrophic nitrification appears to be a generally occurring process in many acid soils with a high availability of  $\text{NH}_4^+\text{-N}$ . However, the

mechanistic aspect of this process needs further examination.

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