Continuous culture enrichments of ammonia-oxidizing bacteria at low ammonium concentrations

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Abstract

Until now enrichments of ammonia-oxidizing bacteria from natural ammonium-limited environments have been performed mainly in the presence of much higher ammonia concentrations than those present in the natural environment and many have resulted in the enrichment and isolation of environmentally less important bacteria. Therefore, we used continuous cultures to enrich ammonia-oxidizing bacteria at growth-limiting ammonium concentrations of around 5 µM from the root zone of the macrophyte *Glyceria maxima* from the lake Drontermeer (The Netherlands). Molecular analysis at the end of the enrichment experiments showed that all continuous cultures consisted of *Nitrosomonas* cluster 6a, which comprises also *Nitrosomonas ureae* and *Nitrosomonas oligotropha*. This was independent of whether *Nitrosomonas*- or *Nitrosospira*-like bacteria were dominant in the inoculum. Thus all known ammonia-oxidizing bacteria belonging to *Nitrosomonas* cluster 6a are able to grow at very low ammonium concentrations. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

Ammonia oxidation, the chemolithoautotrophic oxidation of ammonium to nitrite via hydroxylamine [1] is in many ecosystems limited by the availability of ammonium [2,3]. Additionally the ammonia-oxidizing bacteria have to compete with heterotrophic bacteria and plants for the available ammonium in natural environments. Laboratory experiments are showing that ammonia-oxidizing bacteria, like *Nitrosomonas europaea*, are very bad competitors for ammonium compared to heterotrophic bacteria [4,5] and plants [6,7]. These results indicate that there is low ammonium availability for the ammonia-oxidizing bacteria in the oxygenated parts around roots of submerged plants. For example, the ammonium concentration in pore water samples from the root zone of the macrophyte *Glyceria maxima* varied between 0.3 and 0.8 mM over one year [8] and in a fertilized paddy soil close to the rice root it was 0.15 mM [9].

Enrichments and isolations of ammonia-oxidizing bacteria have been done up to now at a wide range of initial ammonium concentrations: 0.67 mM [10], 0.75 mM [11], 1.4 mM [12], 3.78 mM [13], 10 mM [14,15], up to more than 100 mM ammonium [11,12,16,17]. The pH value of the medium was in the most cases between 7 and 8. But unfortunately present culture methods resulted only in the isolation of a fraction of the total diversity of the ammonia-oxidizing bacteria [18].

Therefore we chose a different approach to enrich ammonia-oxidizing bacteria at low ammonium concentrations. The goal of this study was to investigate whether there were ammonia-oxidizing bacteria which are specifically adapted to grow at low ammonium concentrations and whether other factors like low oxygen or the addition of carbon had also an influence on the outcome of the enrichment experiments. We present continuous culture experiments in which ammonia-oxidizing bacteria were enriched at low ammonium concentrations. As an inoculum we used sediment from the root zone of the macrophyte *G. maxima* from the lake Drontermeer (The Netherlands). The community development in the continuous cultures was followed with denaturing gradient gel electrophoresis (DGGE). The bands belonging to the ammonia-oxidizing bacteria were identified by sequencing the excised bands from the DGGE.

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Table 1
Experimental set up of the different continuous culture experiments to enrich ammonia-oxidizing bacteria

Continuous culture experiments	Initial NH_4^+ concentration in the reservoir (mM)	pO_2 (%)	Casamino acids concentration in the reservoir (mg l^{-1})	Inoculum	Sampling date	Start of the chemostat
Ch1	0.5	16		sediment	Nov. 1997	Nov. 1997
Ch2	0.5	16		extracted bacteria	Nov. 1997	Nov. 1997
Ch3	0.05	16		extracted bacteria	Nov. 1997	Jan. 1998
Ch4	0.05	20	4	extracted bacteria	Nov. 1997	Jan. 1998
Ch5	0.05	1		extracted bacteria	Nov. 1997	Jan. 1998
Ch6	0.1	16		sediment	Apr. 1998	Jan. 1999

2. Materials and methods

2.1. Site description and sampling

Sediment samples were taken inside the root zone of the macrophyte *G. maxima* (reed sweet grass) at the lake Drontermeer, near the township of Elburg (52°58′N, 5°50′E), transported to the lab, and stored in PVC cylinders at 4°C. For a more detailed site description we refer to Bodelier et al. [8].

2.2. Extraction of bacteria

Bacteria were extracted according to the method described by Jacobsen and Rasmussen [19]. Sediment samples (85 g each) were shaken at 150 rpm for 30 min in 0.85% NaCl solution and spun down for 5 min at 3000 rpm. The pellet was extracted twice with 8.5 g Chelex 100, 100 glass beads ($\emptyset = 3$ mm) and 170 ml NDP solution (0.1% sodium deoxycholate and 2.5% polyethylene glycol 6000) at 150 rpm for 1 h and spun down again at 3000 rpm for 5 min. All supernatants were pooled and spun down for 20 min at 12 000 rpm. The pellets were washed twice with 0.85% NaCl. Afterwards the cells were diluted in mineral salt medium without ammonium to get an aliquot of cells present in 10 g sediment, which was used as inoculum for the chemostats. This method of extraction based on Chelex 100 was used for the continuous culture experiments Ch2–Ch5. Chelex 100 produces hydrogen peroxide, which inhibits the hydroxylamine oxidoreductase [20]. Therefore we inoculated Ch1 and Ch6 directly with 10 g sediment and observed no differences between the two methods.

2.3. Medium

Mineral salt medium containing different amounts of ammonium, 10 mM NaCl, 1 mM KCl, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$ and 1 ml l^{-1} trace elements [5] was used to enrich the ammonia-oxidizing bacteria. For the batch experiments we added two and a half times more HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) than ammonium calculated on the molar basis to keep the pH value around 7.0. The pH of the media used for the batch experiments was adjusted to 7.8 with sodium hydroxide before autoclaving. After autoclaving a KH_2PO_4 solution was added to get a final phosphate concentration of 1 mM.

2.4. Continuous culture experiments

The continuous culture enrichments were carried out in Biostat M fermenters (B. Braun, Melsungen, Germany) [5]. All cultures were kept at a temperature of 25°C and a stirrer speed of 300 rpm. The pH was adjusted to 7.5 ± 0.1 by adding Na_2CO_3 solution.

At the start continuous cultures containing mineral salt medium without ammonium were inoculated with 10 g sediment or bacteria extracted from 10 g sediment. Then the pumps were started to add new mineral salt medium with ammonium in different concentrations (Table 1) at a dilution rate of 0.014 h^{-1}. Different conditions like reduced oxygen partial pressure and addition of casamino acids were applied to the continuous cultures (Table 1). When the ammonium was consumed completely, we increased the ammonium concentration in the reservoir to get more biomass in the cultures.

The chemostats were sampled daily to determine the ammonium, nitrite and nitrate concentrations and every third day (i.e. after every volume change) for molecular analysis.

2.5. Batch culture experiments

Batch culture experiments were done in 100 ml Erlenmeyer flasks with 50 ml HEPES-buffered medium. In some experiments the ammonium was measured from time to time and if it was consumed, new ammonium and sodium hydroxide were added to restore the ammonium concentration and pH value. The growth rate was determined by measuring the nitrite and nitrate concentrations and calculated via ln transformation of the nitrite plus nitrate contents [21].

2.6. Chemical analysis

The samples were analyzed immediately or stored at −20°C. Ammonium was determined colorimetrically [22]. Nitrite and nitrate were measured with high-pressure

liquid chromatography (HPLC). We used a HPLC consisting of a LC-XPD pump (Pye Unicam), a guard column Ionpac AG9-SC and a separator column Ionpac AS9-SC (Dionex, Breda, The Netherlands). The columns were flushed with a helium degassed carbonate buffer (2 mM Na_2CO_3, 0.75 mM $NaHCO_3$, pH 9.3) with a flow rate of 0.8 ml min^{-1}. Nitrite and nitrate were detected with an UV detector (Milton Roy, Riviera Beach, FL, USA) at a wavelength of 214 nm. Sampling was done with a Marathon autosampler (Spark Holland, Emmen, The Netherlands).

2.7. Molecular analysis

For the molecular analysis after every volume change 50 ml of chemostat culture liquid or 10 ml of the batch cultures were filtered through a 0.2-μm membrane filter NC20 (Schleicher and Schuell, Dassel, Germany). These filters were cut into two pieces, put into 2-ml screw-cap tubes and stored until further analysis at −80°C. Sediment samples (0.5 g) from the beginning of the experiment were also stored in 2-ml screw-cap tubes at −80°C.

2.8. DNA extraction

We added 0.7 ml high salt $MgCl_2$ lysis buffer (HSB) [23] and 0.5 g 0.1 mm diameter acid-washed zirconium beads to the filters or to the sediment in the 2-ml screw-cap tubes. The tubes were shaken twice on a Mini Bead beater for 30 s at 5000 rpm and stored in-between on ice. Afterwards the samples were stored for at least 30 min on ice. Then 0.07 ml HSB with CTAB (hexadecyltrimethylammoniumbromide) was added to get a final CTAB concentration of 1% and the samples were again shaken on the Mini Bead beater. After centrifugation for 5 min at 10 000 rpm, 0.5 ml of the aqueous phase was transferred to a new screw-cap tube and extracted once with buffer saturated phenol (pH 8) and twice with phenol/chloroform/isoamylalcohol (25:24:1; pH 8). The DNA in 0.4 ml of the aqueous phase was precipitated with 0.04 ml 3 M sodium acetate (pH 5.2), 0.8 ml 96% ethanol and 2 μl glycogen overnight at −20°C. After centrifugation at 13 000 rpm for 30 min the DNA was washed once with 70% ice-cold ethanol and spun down again (13 000 rpm, 5 min). The pellet was vacuum-dried for 2 min, resuspended in 100 μl TE buffer (1×TE = 10 mM Tris, 1 mM EDTA, pH 8), dissolved for 30 min at 60°C, and stored at 4°C (short term) or −80°C (long term).

2.9. PCR, DGGE and phylogenetic analysis with ammonia oxidizer-specific primers

The DNA was directly amplified with the CTO189F-GC/CTO654-R primer set [24,25] with the following modifications: we used 1.75 mM Mg^{2+} and 40 cycles of PCR.

We were not able to amplify the DNA from all starting cultures with this direct PCR approach, so we used a nested approach for the comparison of the different continuous cultures with their starting cultures. First the DNA was amplified with the βAMOf/βAMOr primer set [17]. The PCR products were diluted 1:100 or 1:1000 and used as template for a PCR with the CTO primers [24,25].

DGGE (denaturing gradient gel electrophoresis) was done as described by Kowalchuk et al. [24] with modifications from De Bie et al. [25] and the sequencing of the DGGE bands and the analysis of the data as described by De Bie et al. [25]. For sequencing the center of the band was cut out and incubated overnight in 50 μl TE at 4°C. The supernatant was used for a PCR with the CTO primer set. Afterwards the PCR products were reanalyzed on DGGE to confirm the recovery of the excised bands and used without further purification for sequencing. Sequencing was done with internal Cy5-labeled primers 357F and 518R [26] and a cycle sequence kit (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. Sequence reactions were analyzed on an ALF express II (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions.

Sequences were determined with ALF express Sequence Analysis Software (Amersham Pharmacia Biotech, Little Chalfont, UK). Alignment of the sequences was done with the Dedicated Comparative Sequence Editor program (DCSE) [27] including sequences from the ssu rRNA database (Antwerp, Belgium) [28]. Distance analysis of 402 nucleotide positions (*Escherichia coli* numbering: 206–608) of the alignment was performed with Treecon version 1.3b software [29] using Jukes-Cantor (1969) [55] correction. Gaps were not taken into account in the analysis. The bootstrap analysis was based on 100 replicates. Additionally maximum parsimony and distance analyses of the sequences were done with the PHYLIP version 3.5c [30]. All methods showed the same grouping in the phylogenetic tree. The overall clustering of the different clusters of ammonia-oxidizing bacteria in the tree remained the same, when using 400 or 1100 bp for tree building (results not shown). The sequences determined in this study have been deposited in the EMBL database under the accession numbers: AJ299043–AJ299057.

2.10. PCR with amoA-specific primers and restriction fragment analysis of the products

The DNA from the end cultures of the continuous culture enrichments was also amplified with the *amo*A1F*/*amo*A2R primer set specific for the *amo*A gene of the β-subgroup ammonia-oxidizing bacteria [31,32]. The PCR products were digested with the restriction enzyme *Msp*I and analyzed on a 2.5% agarose gel [32,33].

3. Results

Ammonium limitation in the littoral zone of macrophytes in the lake Drontermeer is assumed to be the controlling factor of the activity of the ammonia-oxidizing bacteria [8]. We used continuous cultures to enrich these bacteria under more natural conditions, i.e. low ammonium availability.

All six continuous culture enrichments were following a similar pattern, resulting in a growth-limiting substrate concentration of around 5 μM, and the enrichment of a special group of *Nitrosomonas*-like bacteria.

3.1. Continuous culture enrichment Ch6

First we show the continuous culture enrichment Ch6 in detail (Fig. 1A,B): The ammonium concentration was increasing in the beginning (Fig. 1A). Nitrite and nitrate production started after one and two volume changes, respectively. Already after 4.5 volume changes the ammonium concentration in the continuous culture was lower than 10 μM and reached a steady-state value of around 5 μM. After 10 volume changes the ammonium concentration in the chemostat increased a little, because the ammonium concentration in the reservoir was increased from 0.1 to 0.2 mM ammonium. At the same time the nitrite concentration in the culture reached a value below the detection limit indicating that the complete nitrification sequence took place.

After every volume change a sample was taken for molecular analysis, comprising DNA isolation, PCR, DGGE and sequencing. DGGE of PCR products obtained with the ammonia oxidizer-specific primers resulted partly in multiple bands due to the use of degenerated primers [24] (Figs. 1, 2, and 5). All bands indicated in the figures by circles were excised, reamplified, and separated again
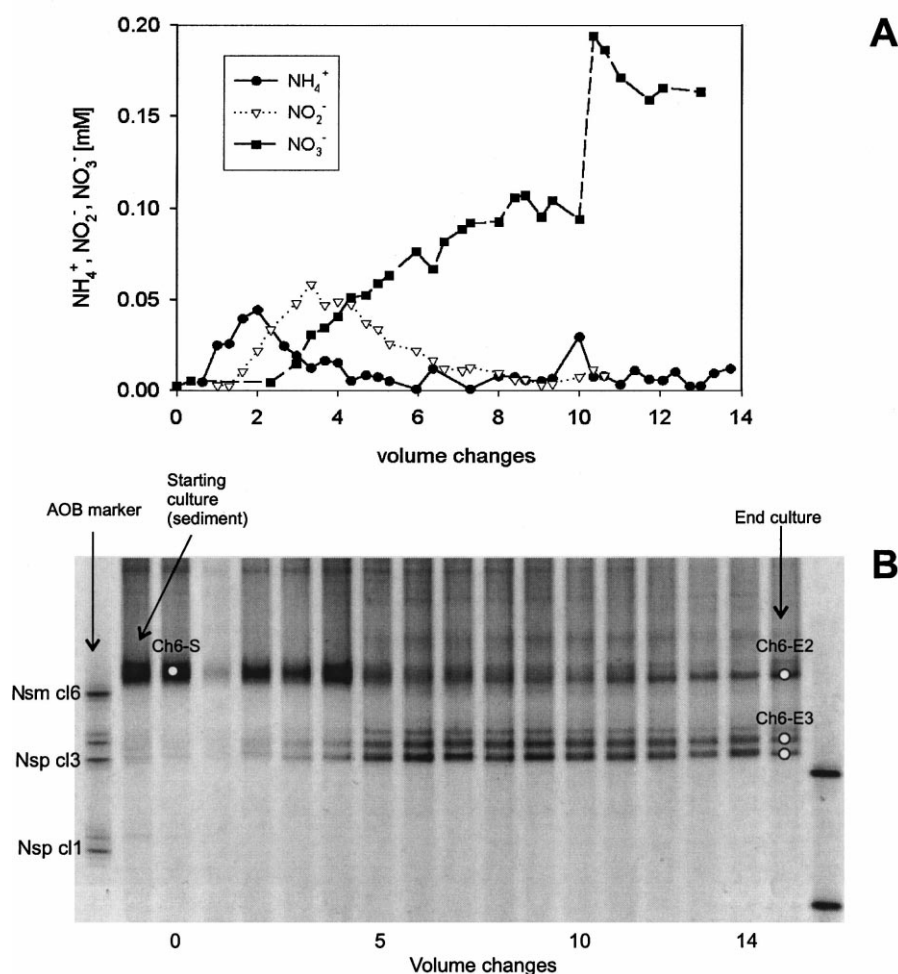

Fig. 1. Inorganic nitrogen dynamics and molecular analysis of the continuous culture experiment Ch6 during 14 volume changes (= 42 days). A: Ammonium, nitrite and nitrate concentrations. B: Denaturing gradient gel electrophoresis (DGGE) of PCR products obtained with direct PCR with ammonia oxidizer-specific primers. Codes indicate excised bands from which sequences have been determined (see Fig. 3). (AOB marker, ammonia oxidizer marker; Nsm cl6, *Nitrosomonas* cluster 6; Nsp cl1, *Nitrosospira* cluster 1; Nsp cl3, *Nitrosospira* cluster 3.)
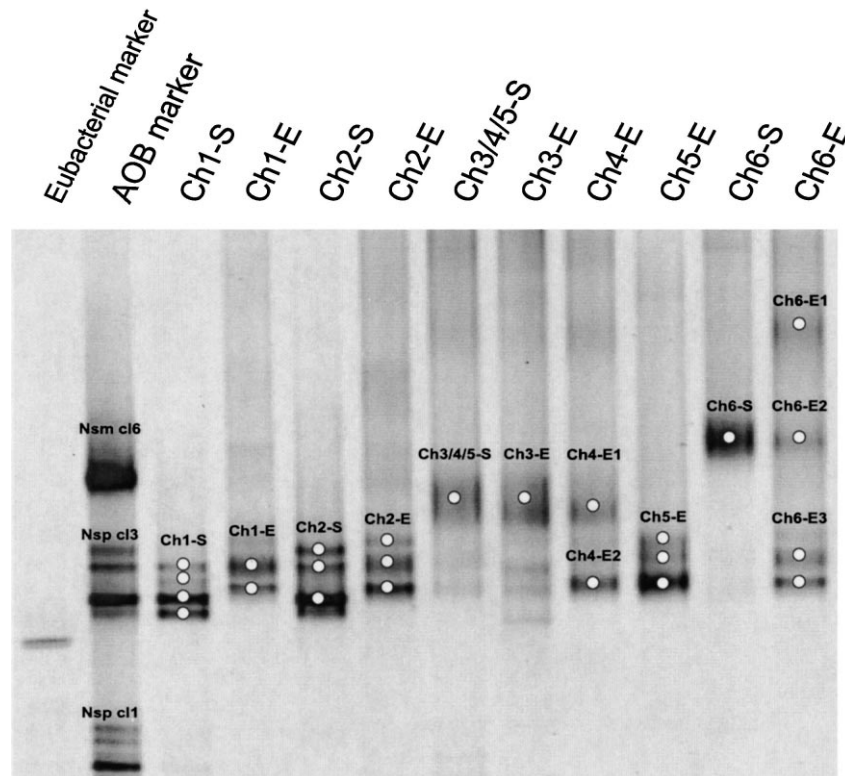
Fig. 2. DGGE of the nested PCR products from the starting and end cultures of the different continuous culture enrichments. Codes indicate excised bands from which sequences have been determined (see Fig. 3) (S, starting culture; E, end culture). (AOB marker, ammonia oxidizer marker; Nsm cl6, *Nitrosomonas* cluster 6; Nsp cl1, *Nitrosospira* cluster 1; Nsp cl3, *Nitrosospira* cluster 3.)

on DGGE. If they resulted in the same multiple banding pattern (i.e. Fig. 2, Ch1S) only one of these bands was used for sequencing. The DGGE of the PCR products amplified with ammonia oxidizer-specific primers (Fig. 1B) showed in the starting culture one dominant band. After two volume changes, when ammonium oxidation had started, a second band came up. The two bands coexisted in the chemostat over 10 volume changes until the end of the experiment, indicating that bacteria corresponding to these bands remained present in the culture. Sequencing of the bands showed that both sequences are belonging to different groups of *Nitrosomonas* cluster 6a (Fig. 3).

Parallel to the continuous culture enrichment experiment Ch6 we inoculated batch cultures containing 0.5 mM ammonium with the sediment (1% w/v) to investigate a possible difference in the outcome of the enrichment related to different initial ammonium concentrations. In these batch culture enrichments ammonium was oxidized within a few days and DGGE showed the enrichment of the ammonia-oxidizing bacterium, which was already dominant in the starting culture (Ch6S; results not shown).

3.2. Comparison of the different continuous culture enrichments

Ammonium and nitrite consumption in the other continuous culture enrichments showed almost the same picture as continuous culture experiment Ch6. In the continuous culture enrichments Ch1–Ch4 nitrite production started later after 3–5 volume changes and nitrate production after 8–14 volume changes. The continuous culture experiment Ch5 running under oxygen-limited conditions with an oxygen partial pressure of 1% O_2 produced no nitrite for eight volume changes (24 days) and two subsequent weeks running without further medium addition. After this time, the continuous culture was completely switched off. However, testing for nitrite 3 days later showed low amounts of nitrite. At that moment the oxygen partial pressure in the culture had increased to 5% O_2. Therefore everything was switched on again to reestablish the previous conditions. Three days later 0.05 mM ammonium was completely oxidized to nitrite and the pumps were started to add new medium. Nitrate was never detected in this continuous culture indicating that no nitrite oxidation took place.

The growth-limiting substrate concentrations presented in Table 2 were determined in all continuous culture enrichments as the mean of the ammonium concentration over the last 3–5 volume changes assuming the cultures to be at the steady state. They were lower than 10 μM ammonium in all cultures.

To compare all starting cultures with the end cultures of the different continuous culture enrichments we performed

Fig. 3. Neighbor-joining phylogenetic tree of the 16S rRNA sequences from the ammonia-oxidizing bacteria enriched in continuous and batch cultures. Codes (in bold) are corresponding with the names in Figs. 1, 2 and 5.

DGGE analysis of PCR products obtained with a nested PCR approach (Fig. 2). The additional PCR step was necessary to get PCR products of all starting cultures. In contrast the DNA of the end cultures of all continuous culture enrichments could be amplified also without the nested approach. A comparison by DGGE of the PCR products from the nested and the direct approach showed only one additional DGGE band in the nested PCR products from the continuous culture enrichment Ch6 (Figs. 1 and 2). Sequencing the excised bands obtained by the di-

Table 2
Growth-limiting ammonium concentrations in the different continuous culture experiments

Continuous culture experiment	Growth-limiting ammonium concentration (μM)[a]
Ch1	7.0 ± 1.1
Ch2	7.5 ± 1.9
Ch3	6.1 ± 2.4
Ch4	4.1 ± 2.5
Ch5	4.5 ± 1.9
Ch6	6.8 ± 3.6

Continuous cultures were running under comparable conditions: 25°C, 300 rpm stirrer speed, $D = 0.014$ h^{-1}, pH $= 7.5 \pm 0.1$.

[a]Values are calculated as mean ($\pm$ S.D.) from the ammonium concentrations at the last 3–5 volume changes ($n = 9$–15).

rect and the nested PCR approach showed almost no differences ($< 1\%$) between the sequences (results not shown). For the cultures Ch1 and Ch2, DGGE analysis showed that the location of the bands representing the starting culture was different from those representing the end culture (Fig. 2). Sequencing the bands resulted in sequences closely related to *Nitrosospira* sp. Np2221 and *Nitrosospira* sp. C128 in the starting culture and to *Nitrosomonas* cluster 6a-A in the end culture (Fig. 3). The continuous cultures Ch3, Ch4 and Ch5 were inoculated with a starting culture, which was closely related to the end culture, and all were belonging to *Nitrosomonas* cluster 6a-A (Fig. 3). The culture Ch6 started with an ammonia-oxidizing bacterium belonging to *Nitrosomonas* cluster 6a-B and resulted in a mixture of the starting culture (*Nitrosomonas* cluster 6a-B) and two other ammonia-oxidizing bacteria belonging to the *Nitrosomonas* clusters 6a-A and 6a-C, respectively. In comparison we enriched members of *Nitrosomonas* cluster 6a-A in all continuous cultures. At the end of the different enrichments wall growth was observed in all continuous cultures. DGGE analysis indicated that there were no differences in the ammonia-oxidizing com-

munities present in the culture liquid and in the biofilm on the wall of the vessel (results not shown).

In addition to the 16S rRNA-based PCR approach, we amplified the DNA of the end cultures of all continuous culture experiments with *amo*A-specific primers and digested the PCR products (Fig. 4). The restriction pattern obtained from the different continuous cultures was similar to the pattern of newly isolated (Bollmann, A., unpublished results) and already cultured members of *Nitrosomonas* cluster 6a.

3.3. Influence of different initial ammonium concentrations on the enrichment culture Ch6

The results of the molecular analyses indicate that members of *Nitrosomonas* cluster 6a were dominant among the ammonia-oxidizing bacteria in the continuous culture enrichments.

The continuous culture enrichment Ch6 ended up with a coculture of two (direct PCR approach) or three (nested PCR approach) different ammonia-oxidizing bacteria. We did a batch experiment with this coculture to investigate if the initial ammonium concentration had an influence on the final community structure of the ammonia-oxidizing bacteria. Therefore we inoculated 10% of the culture liquid from the continuous culture experiment Ch6 into batch cultures with different initial ammonium concentrations (0.1, 0.25, 1.0, 2.5, 10 mM). When these cultures had consumed all ammonium, they were supplied with ammonium and sodium hydroxide to restore the initial ammonium concentration and a pH value of 7.5–7.8. Molecular analysis showed that the two major bands Ch6E2 (*Nitrosomonas* cluster 6a-B) and Ch6E3 (*Nitrosomonas* cluster 6a-A) of the continuous culture enrichment experiment had disappeared in all batch enrichments (Fig. 5). Only the ammonia-oxidizing bacteria corresponding to the third band (Ch6E1, *Nitrosomonas* cluster 6a-C, Fig. 3) could be recovered in the batch cultures. With the exception of one of
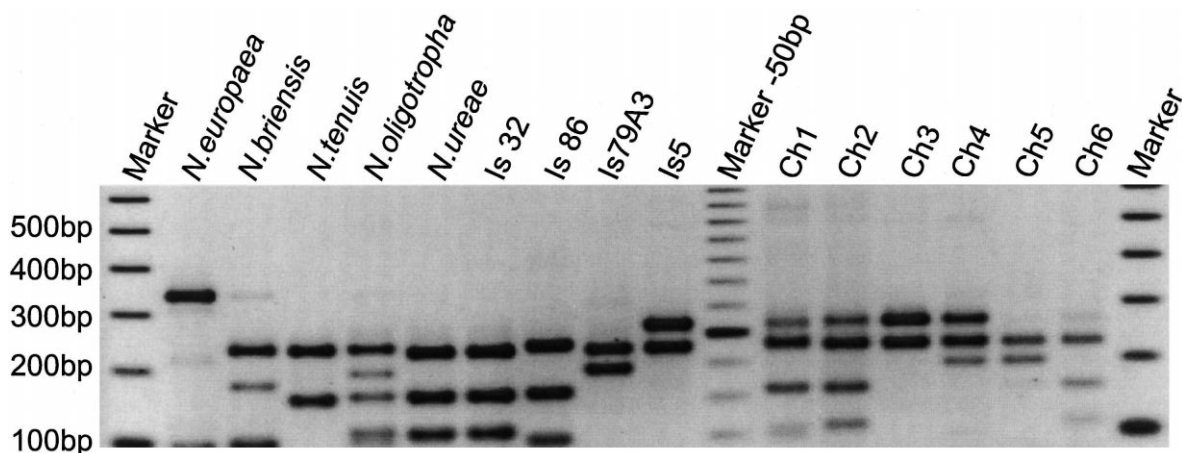

Fig. 4. Restriction patterns of *amo*A PCR products after digestion with *Msp*I from the different continuous culture enrichments in comparison with isolates. Isolates Is32 and Is86 are belonging to *Nitrosomonas* cluster 6a and Is79A3 and Is5 to *Nitrosomonas* cluster 6a-A based on 16S rRNA sequence analysis (Bollmann, A., unpublished results).

the two ammonia-oxidizing bacteria appearing in the cultures incubated at an initial ammonium concentration of 10 mM (Fig. 5, Ba 10-1) we observed a clear shift of the dominant ammonia-oxidizing bacteria with increasing initial ammonium concentration. At initial ammonium concentrations of 0.1 mM and 0.25 mM, members of *Nitrosomonas* cluster 6a-C, at 0.25 mM, 1 mM, and 2.5 mM, members of *Nitrosomonas* cluster 6a-D and at 10 mM, a member of *Nitrosomonas* cluster 6, related to strains from marine and estuarine environments arose (Figs. 3 and 5). The additional band in the cultures incubated at 10 mM ammonium (Ba 10-1) could originate from cells that started to grow when the ammonium concentration again reached lower values.

3.4. Growth behavior of the enrichment culture Ch5

The ammonia-oxidizing bacteria in the continuous culture enrichments were growing at ammonium concentrations around 5 µM. DGGE analysis of three continuous cultures showed only one band, indicating the dominance of only one ammonia-oxidizing strain. In the three other cultures more than one band appeared, indicating cocultures of more than one ammonia-oxidizing bacterium. We enriched the culture from the continuous culture Ch5 further, to make sure that only one ammonia-oxidizing bacterium was present. This culture was incubated at different ammonium concentrations to investigate whether these bacteria are also able to grow at higher ammonium concentrations (0.5–10 mM, Fig. 6). The ammonia-oxidizing bacteria started to grow exponentially after 28–40 days, depending on the initial ammonium concentration. The
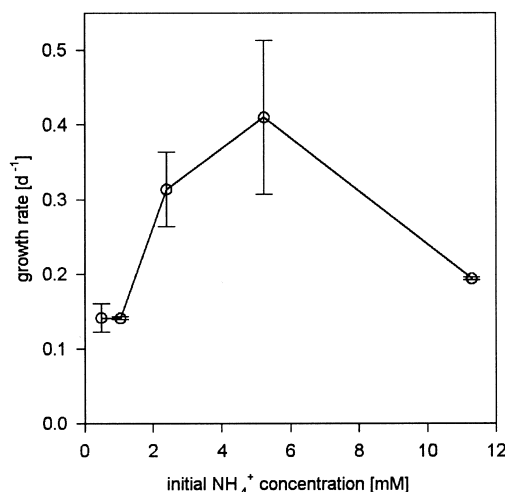

Fig. 6. Growth rates (day^{-1}) (mean ± range, $n = 2$) of the community of continuous culture Ch5 observed at different initial ammonium concentrations.

calculated growth rates showed an optimum curve with the highest value at an initial ammonium concentration of 5 mM ammonium. We monitored the ammonia-oxidizing bacteria with DGGE. No new bands appeared, indicating that no other ammonia-oxidizing bacteria, which can be detected with the primer system, grew up (results not shown). Additionally at higher ammonium concentrations members of *Nitrosomonas* cluster 6a-A will be out competed by other ammonia-oxidizing bacteria if present (Fig. 5).

4. Discussion

Despite the different starting cultures and the different conditions applied (Table 1) we enriched bacteria from *Nitrosomonas* cluster 6a-A in all continuous cultures (Figs. 2 and 3), indicating that *Nitrosomonas* cluster 6a-A had excellent competitive abilities under the prevailing conditions. We assume that the low growth-limiting ammonium concentration (< 10 µM) is the factor, which makes the conditions favorable for the enrichment of this group.

4.1. Nitrosomonas cluster 6a

The good competitive abilities of the *Nitrosomonas* cluster 6a-A-like bacteria enriched at low ammonium concentrations could be due to the fact that the bacteria belong to a phylogenetic group that is adapted to grow at low ammonium concentrations. Cultured representatives like *Nitrosomonas* sp. JL21, *Nitrosomonas* sp. AL212, *Nitrosomonas ureae*, *Nitrosomonas oligotropha*, and two other isolates had lower K_s or K_m values for ammonia than *N. europaea*, *Nitrosomonas eutropha* and close relatives [12,35–37]. Thus all known isolated ammonia-oxidizing bacteria belonging to *Nitrosomonas* cluster 6a are able to
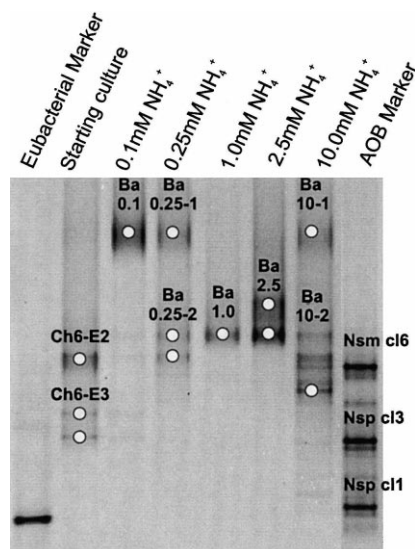

Fig. 5. DGGE of PCR products obtained with direct PCR with ammonia oxidizer-specific primers from the batch experiment inoculated with the end culture of the continuous culture enrichment experiment Ch6. Codes indicate excised bands from which sequences have been determined (see Fig. 3). (AOB marker, ammonia oxidizer marker; Nsm cl6, *Nitrosomonas* cluster 6; Nsp cl1, *Nitrosospira* cluster 1; Nsp cl3, *Nitrosospira* cluster 3.)

grow at low ammonium concentrations. But these bacteria are also able to grow at higher initial ammonium concentrations (Fig. 6), although they showed a long lag phase, indicating that the bacteria had to adapt to the higher ammonia availabilities. Three genes encode the ammonia monooxygenase, the key enzyme converting ammonia to hydroxylamine [1] and each gene is present in two or more copies [38,39]. *N. europaea* has two copies of the *amo*A gene [40]. Mutations in one of the copies of the gene showed that *N. europaea* is able to grow with only one active copy [41]. One of these mutants is growing slower than the wild-type, the other one at the same rate [41]. So one of the reasons that these bacteria are able to grow at very low ammonium concentrations could be that there is a differential expression or regulation of *amo*A.

In all continuous culture systems we observed wall growth. Molecular analysis showed no difference between the ammonia-oxidizing bacteria in the culture and on the wall of the vessel (results not shown). Attachment of ammonia-oxidizing bacteria can have several positive effects on the ammonia-oxidizing bacteria, like the increase of the ammonia-oxidizing activity [42,43], the reduction of the lag phase [44], and the increase of the activity at low pH values [45,46]. Hence attachment of the ammonia-oxidizing bacteria could decrease the growth-limiting substrate concentration in the continuous cultures.

The closest relatives of the enriched ammonia-oxidizing bacteria were clones obtained from lakes and lake sediments in the Netherlands and Belgium and from contaminated groundwater by growth-independent molecular methods [47,48], indicating that we enriched a widely distributed and probably environmental important group of ammonia-oxidizing bacteria. There are only a few other studies investigating ammonia-oxidizing bacteria from freshwater environments. *Amo*A sequences from clones from the Schöhsee in Germany clustered together with *N. ureae* [31,33]. In other oligotrophic and eutrophic lakes other *Nitrosomonas*-like ammonia-oxidizing bacteria have been found. Some belong to *Nitrosomonas* cluster 7, whereas the affiliation of others is not clear [10,49–51].

4.2. Nitrosomonas cluster 6a versus Nitrosospira

Nitrosospira-like bacteria, which were present in starting cultures of the continuous culture experiments Ch1 and Ch2 (Figs. 2 and 3) and also in sediment samples from the root zone of *G. maxima* from the same sampling location [48,52] were never enriched. This indicates that the conditions in the continuous cultures fitted better for *Nitrosomonas* cluster 6a than for the *Nitrosospira*-like bacteria.

The growth rate applied to the continuous cultures could have been too high for the *Nitrosospira*-like bacteria. Pure culture experiments with different *Nitrosospira*-like bacteria showed maximum growth rates between 0.006 h^{-1} and 0.035 h^{-1} [21,53]. In the continuous cultures we applied a growth rate of 0.014 h^{-1}. So the *Nitrosospira*-like bacteria present in the starting culture could have had lower maximum growth rates than the applied dilution rate and consequently could have been washed out from the continuous culture system.

Secondly the long- and the short-term storage at 4°C had a different effect on the *Nitrosospira*- and on the *Nitrosomonas*-like bacteria. In the samples taken in November 1997 both *Nitrosospira*-like bacteria and different members of *Nitrosomonas* cluster 6a were present. The dominance of both groups in the starting cultures depended on the storage time of the cores. If we used the sediment directly after taking it to the laboratory, we found *Nitrosospira*-like bacteria in the starting culture (Fig. 3, Table 1). When using sediment, which had been stored for 2–3 months at 4°C, *Nitrosomonas* cluster 6-like bacteria were dominant in the starting culture (Fig. 3, Table 1). Different activities and growth rates of ammonia-oxidizing bacteria at low temperatures have been shown for *Nitrosospira*-like bacteria [53]. *Nitrosomonas cryotolerans* is able to grow at 0°C [54]. These observations indicate that different ammonia-oxidizing bacteria have different sensitivities to low temperatures, which could be a reason for the shift in the dominant bacteria in the starting cultures during the storage at 4°C.

4.3. Influence of the ammonium concentration on the enrichment of ammonia-oxidizing bacteria

Observations about the selectivity of the ammonium concentration have been made before in other studies. Batch enrichments from sediments and freshwater lakes resulted in the enrichment of *Nitrosomonas* cluster 6a at an initial ammonium concentration of 0.5/0.67 mM, and of *Nitrosospira*-like ammonia-oxidizing bacteria at 1/12.5 mM ammonium ([10], Bollmann, A. unpublished results). Suwa et al. [12,35] enriched *Nitrosomonas* cluster 7-like bacteria at 70 mM and *Nitrosomonas* cluster 6a-like bacteria at 1.4 mM ammonium. This selectivity of the ammonium concentration on the enrichment of ammonia-oxidizing bacteria could be caused by the better competitive abilities of certain bacteria under the prevailing conditions, but it could also be due to different sensitivities against the toxic effects of ammonium. The first hypothesis is favored by our observations concerning the competitive abilities of the different bacteria under different ammonium concentrations in continuous and batch cultures and the second hypothesis by the observation that the growth of the *Nitrosomonas* cluster 6a-A is inhibited at ammonium concentrations of 10 mM.

Finally we can conclude, that continuous cultures are a good additional tool to enrich specific groups of ammonia-oxidizing bacteria (for example *Nitrosomonas* cluster 6a-A), because members of this group have been enriched only in continuous cultures. However, other approaches seem to be required to enrich e.g. *Nitrosospira*-like bacte-

ria. The enrichment of new ammonia-oxidizing bacteria offers also the possibility to isolate these strains and to investigate their ecological niches.

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References

[1] Wood, P.M. (1987) Monooxygenase and free radical mechanisms for biological ammonia oxidation. In: The Nitrogen and Sulfur Cycles (Cole, J.A. and Ferguson, S.J., Eds.), pp. 219–243. Cambridge University Press, Cambridge.

[2] Belser, L.W. (1979) Population ecology of nitrifying bacteria. Annu. Rev. Microbiol. 33, 309–333.

[3] Laanbroek, H.J. and Woldendorp, J.W. (1995) Activity of chemolithotrophic nitrifying bacteria under stress in natural soils. In: Advances in Microbial Ecology, Vol. 14 (Jones, J.G., Ed.), pp. 275–304. Plenum Press, New York.

[4] Van Niel, E.W.J., Arts, P.A.M., Wesselink, B.J., Robertson, L.A. and Kuenen, J.G. (1993) Competition between heterotrophic and autotrophic nitrifiers for ammonia in chemostat cultures. FEMS Microbiol. Ecol. 102, 109–118.

[5] Verhagen, F.J.M. and Laanbroek, H.J. (1991) Competition for ammonium between nitrifying and heterotrophic bacteria in dual energy-limited chemostats. Appl. Environ. Microbiol. 57, 3255–3263.

[6] Bodelier, P.L.E., Duyts, H., Blom, C.W.P.M. and Laanbroek, H.J. (1998) Interactions between nitrifying and denitrifying bacteria in gnotobiotic microcosms planted with the emergent macrophyte *Glyceria maxima*. FEMS Microbiol. Ecol. 25, 63–78.

[7] Verhagen, F.J.M., Hageman, P.E.J., Woldendorp, J.W. and Laanbroek, H.J. (1994) Competition for ammonium between nitrifying bacteria and plant roots in soil in pots; effects of grazing by flagellates and fertilization. Soil Biol. Biochem. 26, 89–96.

[8] Bodelier, P.L.E., Libochant, J.A., Blom, C.W.P.M. and Laanbroek, H.J. (1996) Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptations of ammonia-oxidising bacteria to low oxygen or anoxic habitats. Appl. Environ. Microbiol. 62, 4100–4107.

[9] Arth, I. and Frenzel, P. (2000) Nitrification and denitrification in the rhizosphere of rice: The detection of processes by a new multi-channel electrode. Biol. Fertil. Soils 31, 427–435.

[10] Hastings, R.C., Saunders, J.R., Hall, G.H., Pickup, R.W. and McCarthy, A.J. (1998) Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Appl. Environ. Microbiol. 64, 3674–3682.

[11] Bruns, M.A., Stephen, J.R., Kowalchuk, G.A., Prosser, J.I. and Paul, E.A. (1999) Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled and successional soils. Appl. Environ. Microbiol. 65, 2994–3000.

[12] Suwa, Y., Imamura, Y., Suzuki, T., Tashiro, T. and Urushigawa, Y. (1994) Ammonia-oxidizing bacteria with different sensitivities to (NH₄)₂SO₄ in activated sludges. Water Res. 28, 1523–1532.

[13] Utåker, J.B., Bakken, L., Jiang, Q.Q. and Nes, I.F. (1995) Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA sequences. Syst. Appl. Microbiol. 18, 549–559.

[14] Koops, H.P., Harms, H. and Wehrmann, H. (1976) Isolation of moderate halophilic ammonia-oxidizing bacterium, *Nitrosococcus mobilis* nov. sp.. Arch. Microbiol. 107, 277–282.

[15] Soriano, S. and Walker, N. (1968) Isolation of ammonia-oxidizing autotrophic bacteria. J. Appl. Bacteriol. 31, 493–497.

[16] Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Röser, A., Koops, H.P. and Wagner, M. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64, 3042–3051.

[17] McCaig, A.E., Embley, T.M. and Prosser, J.I. (1994) Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol. Lett. 120, 363–368.

[18] Stephen, J.R., McCaig, A.E., Smith, Z., Prosser, J.I. and Embley, T.M. (1996) Molecular diversity of soil and marine 16S rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. 62, 4147–4154.

[19] Jacobsen, C.S. and Rasmussen, O.F. (1992) Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation-exchange resin. Appl. Environ. Microbiol. 58, 2458–2462.

[20] Hooper, A.B. and Terry, K.R. (1977) Hydroxylamine oxidoreductase from *Nitrosomonas*: Inactivation by hydrogen peroxide. Biochemistry 16, 455–459.

[21] Belser, L.W. and Schmidt, E.L. (1980) Growth and oxidation kinetics of three genera of ammonia oxidizing nitrifiers. FEMS Microbiol. Lett. 7, 213–216.

[22] Kandeler, E. and Gerber, H. (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertil. Soils 6, 68–72.

[23] Bateson, M.M. and Ward, D.M. (1995) Methods for extracting DNA from microbial mats and cultivated micro-organisms: high molecular weight DNA from french press lysis. Molecular Microbial Ecology Manual 1.1.4.

[24] Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M. and Woldendorp, J.W. (1997) Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. 63, 1489–1497.

[25] De Bie, M.J.M., Speksnijder, A.G.C.L., Kowalchuk, G.A., Schuurman, T., Zwart, G., Stephen, J.R., Diekmann, O.E. and Laanbroek, H.J. (2001) Shifts in the dominant populations of ammonia-oxidizing β-subclass Proteobacteria along the eutrophic Schelde estuary. Aquat. Microb. Ecol. 23, 225–236.

[26] Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

[27] De Rijk, P. and de Wachter, R. (1993) DCSE v2.54, an interactive tool for sequence alignment and secondary structure research. Comput. Appl. Biosci. 9, 735–740.

[28] Van de Peer, Y., Jansen, J., de Rijk, P. and de Wachter, R. (1997) Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res. 24, 111–116.

[29] Van de Peer, Y. and De Wachter, R. (1994) TREECON for windows: a software package for the construction and drawing of evolutionary trees for the Microsoft windows environment. Comput. Appl. Biosci. 10, 569–570.

[30] Felsenstein, J. (1993) PHYLIP (Phylogeny Inference Package) version 3.5c, Department of Genetics, University of Washington, Seattle, WA.

[31] Rotthauwe, J.H., Witzel, K.P. and Liesack, W. (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63, 4704–4712.

[32] Stephen, J.R., Chang, Y.J., Macnaughton, S.J., Kowalchuk, G.A., Leung, K.T., Flemming, C.A. and White, D.C. (1999) Effect of toxic metals on indigenous soil β-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. Appl. Environ. Microbiol. 65, 95–101.

[33] Speksnijder, A.G.C.L. (1999) Community analysis of β-subgroup ammonia-oxidizing bacteria in aquatic environments: a molecular approach. Ph.D. thesis. University of Nijmegen, Nijmegen.

[35] Suwa, Y., Sumino, T. and Noto, K. (1997) Phylogenetic relationships of activated sludge isolates of ammonia oxidizers with different sensitivities to ammonium sulfate. J. Gen. Appl. Microbiol. 43, 373–379.

[36] Koops, H.P. and Pommerening-Roser, A. (2001) Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol. 37, 1–9.

[37] Stehr, G., Böttcher, B., Dittberner, P., Rath, G. and Koops, H.P. (1995) The ammonia-oxidizing nitrifying population of the river Elbe estuary. FEMS Microbiol. Ecol. 17, 177–186.

[38] Norton, J.M., Low, J.M. and Klotz, M.G. (1996) The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in *Nitrosospira* sp. NpAV. FEMS Microbiol. Lett. 139, 181–188.

[39] Klotz, M.G. and Norton, J.M. (1995) Sequence of an ammonia monooxygenase subunit A-encoding gene from *Nitrosospira* sp. NpAV. Gene 163, 159–160.

[40] McTavish, H., Fuchs, J.A. and Hooper, A.B. (1993) Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. 175, 2436–2444.

[41] Hommes, N.G., Sayavedra-Soto, L.A. and Arp, D.J. (1998) Mutagenesis and expression of *amo*, which codes for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. 180, 3353–3359.

[42] Underhill, S.E. and Prosser, J.I. (1987) Surface attachment of nitrifying bacteria and their inhibition by potassium ethyl xanthate. Microb. Ecol. 14, 129–139.

[43] Diab, S. and Shilo, M. (1988) Effect of adhesion to particles on the survival and activity of *Nitrosomonas* sp. and *Nitrobacter* sp.. Arch. Microbiol. 150, 387–393.

[44] Powell, S.J. and Prosser, J.I. (1992) Inhibition of biofilm populations of *Nitrosomonas europaea*. Microb. Ecol. 24, 43–50.

[45] Allison, S.M. and Prosser, J.I. (1993) Ammonia oxidation at low pH by attached populations of nitrifying bacteria. Soil Biol. Biochem. 25, 935–941.

[46] De Boer, W., Klein Gunnewiek, P.J.A., Veenhuis, M., Bock, E. and Laanbroek, H.J. (1991) Nitrification at low pH by aggregated chemolithotrophic bacteria. Appl. Environ. Microbiol. 57, 3600–3604.

[47] Ivanova, I.A., Stephen, J.R., Chang, Y.J., Brugggemann, J., Long, P.E., McKinley, J.P., Kowalchuk, G.A., White, D.C. and Macnaughton, S.J. (2000) A survey of 16S rRNA and *amo*A genes related to autotrophic ammonia-oxidizing bacteria of the β-subdivision of the class proteobacteria in contaminated groundwater. Can. J. Microbiol. 46, 1012–1020.

[48] Speksnijder, A.G.C.L., Kowalchuk, G.A., Roest, K. and Laanbroek, H.J. (1998) Recovery of a *Nitrosomonas*-like 16S rDNA sequence group from freshwater habitats. Syst. Appl. Microbiol. 21, 321–330.

[49] Smorczewski, W.T. and Schmidt, E.L. (1991) Numbers, activities, and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, eutrophic lake sediment. Can. J. Microbiol. 37, 828–833.

[50] Ward, B.B., Voytek, M.A. and Witzel, K.P. (1997) Phylogenetic diversity of natural populations of ammonia oxidizers investigated by specific PCR amplification. Microb. Ecol. 33, 87–96.

[51] Whitby, C.B., Saunders, J.R., Rodriguez, J., Pickup, R.W. and McCarthy, A. (1999) Phylogenetic differentiation of two closely related *Nitrosomonas* spp. that inhabit different sediment environments in an oligotrophic freshwater lake. Appl. Environ. Microbiol. 65, 4855–4862.

[52] Kowalchuk, G.A., Bodelier, P.L.E., Heilig, G.H.J., Stephen, J.R. and Laanbroek, H.J. (1998) Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol. Ecol. 27, 339–350.

[53] Jiang, Q.Q. and Bakken, L.R. (1999) Comparison of *Nitrosospira* strains isolated from terrestrial environments. FEMS Microbiol. Ecol. 30, 171–186.

[54] Koops, H.P., Böttcher, B., Möller, U.C., Pommerening-Röser, A. and Stehr, G. (1991) Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov. and *Nitrosomonas halophila* sp. nov.. J. Gen. Microbiol. 137, 1689–1699.

[55] Jukes, T.H. and Cantor, C.R. (1969) Evolution of protein molecules. In: Mammalian Protein Metabolism, Vol. 3 (Munro, H.N., Ed.), pp. 21–132. Academic Press, New York.