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ORIGINAL PAPER

Dimitry Sorokin · Tat'jana Tourova
Markus C. Schmid · Michael Wagner
Hans-Peter Koops · J. Gijs Kuenen · Mike Jetten

Isolation and properties of obligately chemolithoautotrophic and extremely alkali-tolerant ammonia-oxidizing bacteria from Mongolian soda lakes

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Abstract Five mixed samples prepared from the surface sediments of 20 north-east Mongolian soda lakes with total salt contents from 5 to 360 g/l and pH values from 9.7 to 10.5 were used to enrich for alkaliphilic ammonia-oxidizing bacteria. Successful enrichments at pH 10 were achieved on carbonate mineral medium containing 0.6 M total Na^+ and ≤4 mM NH_4Cl. Five isolates (ANs1–ANs5) of ammonia-oxidizing bacteria capable of growth at pH 10 were obtained from the colonies developed on bilayered gradient plates. The cells were motile and coccoid, with well-developed intracytoplasmic membranes (ICPM) and carboxysomes. At pH 10.0, ammonia was toxic for growth at concentrations higher than 5 mM NH_4Cl. The bacteria were able to grow within the salinity range of 0.1–1.0 M of total Na^+ (optimum 0.3 M). In media containing 0.3–0.6 M total Na^+, optimal growth in batch cultures occurred in the presence of a bicarbonate/carbonate buffer system within the pH range 8.5–9.5, with the highest pH limit at pH 10.5. At pH lower than 8.0, growth was slower, most probably due to decreasing free ammonia. The pH profile of the respiratory activity was broader, with limits at 6.5–7.0 and 11.0 and an optimum at 9.5–10.0. In pH-controlled, NH_3-limited continuous culture, isolate ANs5 grew up to pH 11.3, which is the highest pH limit known for ammonia-oxidizing bacteria so far. This showed the existence of extremely alkali-tolerant ammonia-oxidizing bacteria in the soda lakes. Comparative 16S rDNA sequence analysis of the five isolates demonstrated that they possess identical 16S rDNA genes and that they are closely related to *Nitrosomonas halophila* (sequence similarity 99.3%), a member of the β-subclass of the Proteobacteria. This affiliation was confirmed by comparative sequence analysis of the *amoA* gene, encoding the active-site subunit of the ammonia-monoxygenase, of one of the isolates. DNA-DNA hybridization data further supported that the soda lake isolates are very similar to each other and represent an alkali-tolerant subpopulation of *N. halophila* whose species description is herewith amended.

Keywords Alkalitolerant · Ammonia-oxidizing bacteria · Nitrification · Soda lakes · *Nitrosomonas*

J.G. Kuenen · M. Jetten
Department of Biotechnology, Kluyver Laboratory,
Delft University of Technology,
Julianalaan 67, 2628 BC Delft, The Netherlands

D. Sorokin · T. Tourova
Institute of Microbiology, Russian Academy of Sciences,
Moscow, Russia

H.-P. Koops
Abteilung für Mikrobiologie, Institut für Allgemeine Botanik,
Universität Hamburg, 22609 Hamburg, Germany

M.C. Schmid · M. Wagner
Lehrstuhl für Mikrobiologie, Technische Universität München,
85350 Freising, Germany

M. Jetten (✉)
Department of Microbiology, University of Nijmegen,
Toernooiveld 1, 6525 ED Nijmegen
e-mail: M.Jetten@sci.kun.nl,
Tel.: +31-24-3652940, Fax: +31-24-3652830

Introduction

Autotrophic nitrifying bacteria that are able to obtain energy from the oxidation of ammonia and nitrite have been known for more than a century. They were one of the first discovered chemolithoautotrophs (Winogradsky 1890) and have been subject of detailed studies (Laanbroek and Woldendorp 1995). Because of the relatively high specific substrate-conversion rates, they play a key role in nitrogen cycling in many different environments, mostly in aquatic environments, (especially in sediments) and soils (Abeliovich 1987; Laanbroek and Woldendorp 1995). They are also an extremely important component of wastewater treatment systems (Jetten et al. 1997; Juretschko et al. 1998; Purkhold et al. 2000; Wagner et al. 1995, 1996).

Most of the ammonia oxidizers isolated in pure culture grow well at moderate temperature, within a relatively

narrow pH range from neutral to slightly alkaline, and at low to moderate salt concentrations (Koops and Möller 1992). One of the reasons for such restrictions may be the relatively low energy efficiency of nitrification reactions which might not allow these bacteria to survive energetically expensive extreme conditions (Oren 1999). Therefore the ability of nitrifying bacteria to grow under extreme conditions should be limited. In particular, almost nothing is known about ammonia-oxidizing bacteria able to grow under extremely alkaline conditions.

Soda lakes represent a rare example of a natural environment where two extreme factors are combined (high salinity and high pH) due to the presence of alkaline salts at high concentrations, in particular sodium carbonates. Surprisingly, such lakes may be very productive because of the bloom of alkaliphilic nitrogen-fixing cyanobacteria (Melack 1981; Cloern et al. 1983) that facilitate an active nitrogen cycle. Substantial evidence for the presence of an autotrophic ammonia-oxidation process in one specific layer, containing ammonia and small amounts of oxygen, of the stratified alkaline and saline Mono Lake in California has recently been obtained by using specific inhibitors (Joye et al. 1999) and by molecular probing (Ward et al. 2000). The latter investigations indicated members of the *Nitrosomonas europaea* lineage to be present in this environment; however, these results might have been biased due to the limited coverage of the specific primers used for PCR amplification (Purkhold et al. 2000). Furthermore, these studies were not substantiated by the isolation of pure cultures of the responsible bacteria.

In our preliminary investigation on the presence of nitrifying bacteria in soda lakes and soda soils in the southeast Siberian dry steppe and in the Kenyan Rift Valley, some evidence was obtained that both ammonia- and nitrite-oxidizing bacteria are active in these environments (Sorokin 1998). However, only nitrite-oxidizing enrichments from several samples at pH 10 were successful, resulting in the isolation and description of a new alkaliphilic *Nitrobacter* species (Sorokin et al. 1998). The possibility of ammonia oxidation to nitrite at pH ≥10 has been demonstrated so far only in pure culture of *Methylomicrobium* sp., an alkaliphilic methanotrophic bacterium isolated from Kenyan soda lake sediments (Sorokin et al. 2000a).

Here we describe the isolation and properties of extremely alkali-tolerant, lithoautotrophic, ammonia-oxidizing bacteria from Mongolian soda lake sediments that are capable of growth at extremely high pH values.

Materials and methods

Samples

Surface sediments from 20 soda lakes in the north-east dry steppe of Mongolia (Choibolsan Province), obtained in September 1999, were used to enrich for alkaliphilic ammonia-oxidizing bacteria. Samples were combined into five groups according to the salt content (Table 1) of the lakes from which the samples were obtained.

Table 1 Characteristics of the composite samples (sediments/water 1:1) from Mongolian soda lakes (September, 1999) used for the enrichment of alkaliphilic ammonia-oxidizing bacteria

Isolated bacterium	Number of subsamples	Composite soda lake sediment samples	
		pH	Total g salts l^{-1}
ANs5	5	9.5–10.5	5– 10
ANs1, ANs2	7	9.4–10.0	15– 30
ANs4	6	9.5–10.2	40– 65
None	4	10 –10.2	200–220
ANs3	2	9.8–10.0	360–390

Bacterial cultures

Pure cultures of ammonia-oxidizing bacteria of the genus *Nitrosomonas* were used as a reference to carry out comparative studies with the new isolates from the soda lakes. These strains were obtained from the culture collection of the University of Hamburg.

Culture conditions and media composition

Ammonia-oxidizing bacteria from soda lakes were enriched and routinely cultivated in basal alkaline salt medium containing 0.6 M Na^+ [$NaHCO_3$-Na_2CO_3 buffer (0.5 M Na^+), NaCl (0.1 M) and K_2HPO_4 (5 mM)]. After sterilization the pH of this mineral medium was 10.05–10.1 and sufficiently stable during aerobic cultivation: in sterile controls the pH did not change, and in cultures the maximal pH drop did not exceed 0.2 pH units. After sterilization 1 ml l^{-1} of trace element solution (Pfennig and Lippert 1966) and 1 mM $MgSO_4 \cdot 7H_2O$ were added. NH_4Cl was supplied in a fed-batch manner from the 4 M stock solution so that its concentration did not exceed 4 mM. Batch cultivation was performed in serum flasks of 30–1000 ml or in 20-l bottles closed with rubber septa (to prevent ammonia loss) with 1:10 liquid to air ratio. Bottles were incubated at 30 °C with gentle agitation (50–100 rpm). To investigate the influence of pH on growth and respiration activity, 0.1 M HEPES-NaCl buffer for a pH range of 7.0–8.0 and $NaHCO_3$-Na_2CO_3 buffer for a higher pH range were used with the same total Na^+ content. These allowed prolonged aerobic cultivation at stable pH conditions up to pH 10.3. Short-term activity tests with washed cells were possible up to the highest pH created with sodium carbonate system (11.5).

To study the possibility of growth at pH>10.3, a pH-controlled continuous cultivation was carried out using a fermentor (Applikon, Schiedam, The Netherlands) with a 2 l working volume. The set-point pH was maintained within ±0.05 units by automatic titration with 2 M NaOH. The 2-component medium (acidic and basic components separately) contained 0.25 M total Na_2CO_3 and 0.05 M NaCl. Influent NH_4Cl concentration was 22 mM. Dissolved oxygen concentration and temperature were controlled at 50% air saturation and at 30 °C.

Colonies of ammonia-oxidizing bacteria from soda lakes were obtained on solid medium with an ammonia gradient. The vertical gradient was created as follows: the bottom layer contained 4% agar and 10 mM NH_4Cl. This layer was covered with a mineral alkaline agar (2% final agar concentration) of pH 10, prepared by 1:1 mixing of the carbonate mineral base medium and 4% agar at 50 °C. The plates were allowed to equilibrate for 24 h before use.

The potential for heterotrophic growth of the isolates was tested in 0.3 M Na^+ mineral base medium at pH 10 using nitrate as nitrogen source. Carbon was supplied as 0.5 g yeast extract l^{-1} or 5 mM pyruvate or 5 mM acetate.

Activity measurements

The oxidation kinetics of NH_3 and NH_2OH was assayed either directly with a chemostat culture or with washed cells using the

buffer systems mentioned above. Oxygen consumption rates were monitored with a Clark dissolved-oxygen electrode (Yellow Spring Instruments, Ohio, USA) in a thermostated chamber with a magnetic stirrer (total volume of 5 ml) at 30 °C. Cells were collected by centrifugation, washed with sodium carbonate buffer and resuspended in appropriate buffers at a protein concentration of 10 mg ml^{-1}. For measurements the cells were diluted 100-fold in buffer.

Electron microscopy

Cells were collected from batch or chemostat cultures by centrifugation, washed and resuspended in 0.5 M NaCl, and fixed with glutaraldehyde (3% final). After removal of glutaraldehyde, preparations were post-fixed with OsO_4 (1% final) containing 0.5 M NaCl, dehydrated and embedded into resin. Thin sections were double stained with lead citrate and uranyl acetate. Cells for total preparations were stained with 1% uranyl acetate after fixation with glutaraldehyde.

Biochemical analysis

Total cell proteins of soda lake isolates and neutrophilic *Nitrosomonas* species were compared by SDS-PAGE under denaturing conditions. The cells were sonicated, and the resulting total protein extracts were denatured in sample buffer by boiling for 5 min. Acrylamide (10%) gels were used to separate the proteins. Nitrite, ammonium, and nitrate in the supernatants were measured colorimetrically (Sorokin et al. 1998). The biomass protein was determined by the method of Lowry (1951).

DNA extraction

Cell pellets of the isolates ANs1–ANs5 were suspended in 280 µl TE-buffer [10 mM Tris-HCl (pH 7.4), 10 mM sodium EDTA (pH 7.4)], 40 µl SDS and 80 µl 5 M NaCl. For cell lysis, the mixture was incubated for 1 h at 65 °C. After addition of 400 µl phenol-chloroform-isoamyl alcohol (25:24:1) and 10 s vortexing the mixture was centrifuged for 10 min at 10,000×*g*. The aqueous phase was carefully transferred to a fresh tube, mixed with one volume of chloroform-isoamyl alcohol by vortexing and again centrifuged for 10 min at 10,000×*g*. Subsequently, the aqueous phase was transferred to a fresh tube, and the DNA was allowed to precipitate by incubation with 0.6 volumes of isopropanol for 1 h at room temperature. After centrifugation for 20 min at 10,000×*g* the pellet was washed with 1 ml of 70% ethanol, dried and finally resuspended in 50 µl double-distilled H_2O.

PCR amplification, sequencing and phylogeny of 16S rDNA and *amo*A

To obtain almost full-length 16S rDNA PCR products, the DNA of the isolates ANs1–ANs5 was amplified with the forward primer 616V (*Escherichia coli* positions 8–27; 5′-AGAGTTTGATYMTGGCTCAG-3′) and the reverse primer 630R (*E. coli* positions 1529–1545: 5′-CAKAAAGGAGGTGATCC-3′). PCR was performed in a 96-microwell plate with a gradient cycler (Eppendorf, Hamburg, Germany). Reaction mixtures were prepared in a total volume of 50 µl containing 2 mM $MgCl_2$, 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega, Madison). Thermal cycling was carried out with an initial denaturation of 3 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 50 s, and elongation at 72 °C for 3 min. Cycling was completed by a final elongation step at 72 °C for 10 min. Negative controls (no DNA added) were included in all sets of amplifications. The presence and size of the amplification products were determined by agarose (0.8%) gel electrophoresis of 5-µl aliquots of the PCR products. The pure culture-derived 16S rDNA PCR products were sequenced directly using a previously published procedure (Purkhold et al. 2000).

The *amoA* gene fragment of isolate ANs1 was amplified, cloned and sequenced as described in Purkhold et al. (2000).

Phylogenetic analyses of the retrieved 16S rDNA and *amoA* sequences were performed by use of the ARB program package (Felsenstein 1993; Maidak et al. 1996; Strunk and Ludwig 1997) as described in Purkhold et al. (2000).

The isolation of complete genomic DNA and subsequent DNA-DNA hybridization were carried out according to Marmur (1961) and De Ley et al. (1970), respectively.

Nucleotide sequence accession numbers

The 16S rDNA sequences of isolates ANs1–5 obtained in this study are available in GenBank under accession no. AY026313-AY026317. The *amoA* sequence of isolate ANs1 received the GenBank accession no. AY026907

Results

Enrichment and isolation of pure cultures

Alkaliphilic NH_3-oxidizing bacteria were enriched with soda-based mineral medium at pH 10.05 containing 0.6–2 M total Na^+ using five combined sediment samples from Mongolian soda lakes (see Table 1). Positive results (nitrite production concomitant with ammonia consumption) were obtained in 2–4 weeks with all five samples on medium containing 0.6 M total Na^+ and with a NH_4Cl concentration less than 4 mM. The most rapid nitrite formation was observed with sample group 2 from the lakes containing 15–30 g salts l^{-1}. Stable development of the nitrifying population was achieved using a fed-batch mode of NH_4Cl supply (2 mM doses). But even after three to four repeated 100-fold dilutions of the enrichments, heterotrophic bacteria outnumbered the nitrifiers in subsequent dilution series. Pure cultures were successfully purified and isolated by using double-layered solid medium with an ammonia gradient. The plates were incubated in closed jars under an atmosphere containing 99% argon and 1% O_2. A total of five isolates (ANs1–5) of obligately autotrophic ammonia-oxidizing bacteria were obtained (see Table 1). The isolates did not show any potential for heterotrophic growth. Addition of a small amount of organic compounds even inhibited ammonia oxidation.

Morphology

Cells of the isolates grown at pH 10 were mostly coccoid and 1–2.5 µm in diameter. Some of the cells were motile by a tuft of flagella (Fig. 1a). In old cultures most of the cells became refractile under the light microscope. The cell ultrastructure of isolates ANs1 and ANs5 was typical for ammonia-oxidizing bacteria of the genus *Nitrosomonas.* The cells contained extensive multiple layers of intracytoplasmic membranes and carboxysome-like bodies (Fig. 1b). Electron-transparent granules of storage material could be seen in some of the cells. It was not clear whether or not the cells of the new isolates contained an

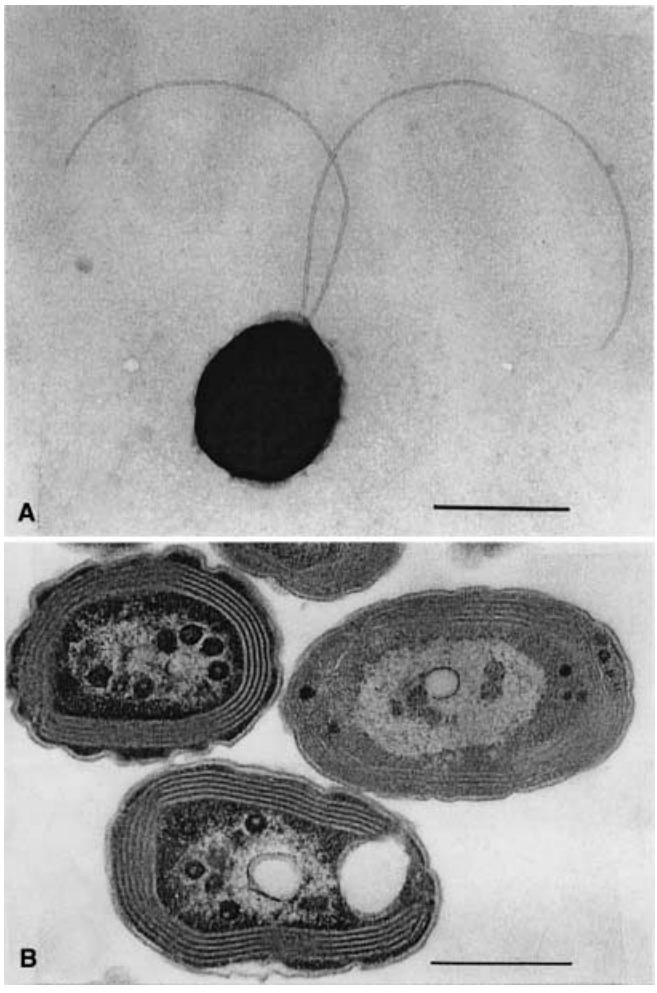

Fig. 1a, b Morphology of the soda lake ammonia-oxidizing bacterium isolate ANs1 grown at pH 10 and salt content 0.6 M Na^+. **a** Negatively stained whole cell preparation; *bar* 1 μm. **b** Thin-section; *bar* 1 μm

S-layer. Fragments of lysed cells that looked similar to subunit layers were observed, but it was not possible to see them in cell sections. Isolates ANs1, ANs2 and ANs4 formed tiny (1 mm) reddish colonies covered with a skin-like surface layer. In liquid cultures they formed aggregates. Isolates ANs3 and ANs5 formed much larger (up to 5 mm) reddish colonies with a refractile center. Compared to isolates ANs1, ANS2, and ANs4, they grew homogeneously and notably faster in liquid cultures.

Ammonia toxicity for growth at pH 10

At pH 10 more than 90% of the added NH_4Cl was present as NH_3, which is known to be a very toxic compound for ammonia-oxidizers. The importance of this fact for growth of soda lake ammonia-oxidizing bacteria was already observed during isolation of pure cultures. Isolate ANs1 was tested for the ability to start growth at pH 10 with 2–10 mM NH_4Cl. Growth started without a lag phase only at ≤4 mM NH_4Cl. At 5–8 mM, a 2- to 5-day lag phase preceded growth. At concentrations above 9 mM NH_4Cl the isolate failed to grow. Therefore all subsequent batch-cultivation experiments were performed using a fed-batch ammonia supply of 4 mM NH_4Cl doses after at least 80% oxidation of the previous dose. By using this strategy, high-density cultures were obtained that consumed up to 80 mM NH_3.

Influence of salts on growth in batch culture at pH 10

Isolate ANs1 was capable of growth at pH 10 in sodium-carbonate-based medium within the range of 0.1–0.8 M total Na^+. If sodium-carbonate base was completely replaced by NaCl, adjusted up to pH 10 by NaOH, no growth was observed. The minimal carbonate concentration supporting growth on 0.3 M NaCl-based medium had to be above 0.05 M Na^+ (Fig. 2a). Maximum salt tolerance was observed on medium containing 0.2 M Na^+ carbonates and NaCl (Fig. 2b) with a growth optimum around 0.3–0.5 M Na^+. Overall, the results demonstrated relatively low salt tolerance of the ANs isolates (even those

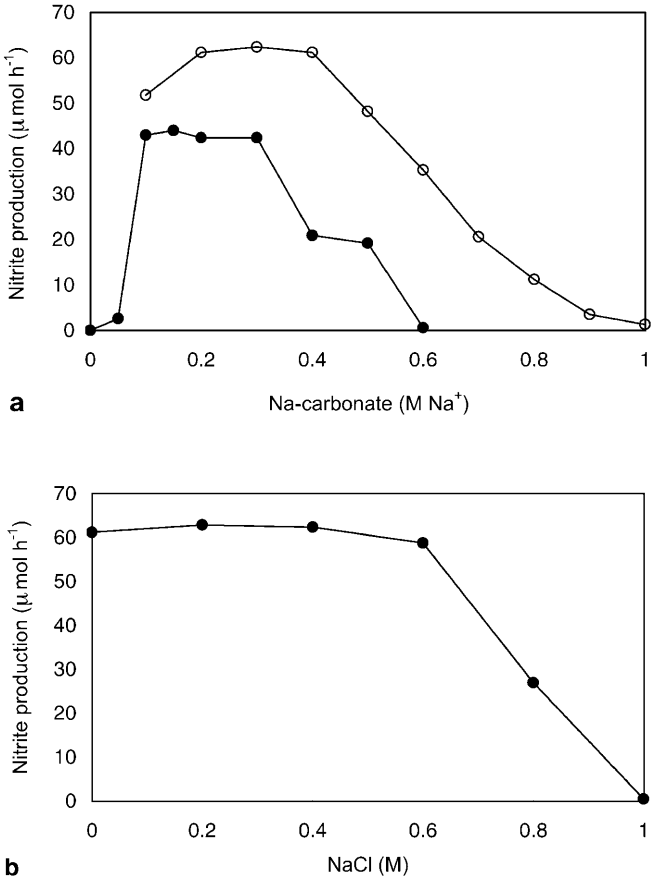

Fig. 2 The influence of sodium carbonate (**a**) and sodium chloride (**b**) on growth of ammonia-oxidizing isolate ANs1 in batch culture at pH 10. The basic medium contained no NaCl (○) or 0.3 M NaCl (●) in variant (**a**) and 0.2 M Na^+ sodium carbonates in variant (**b**)

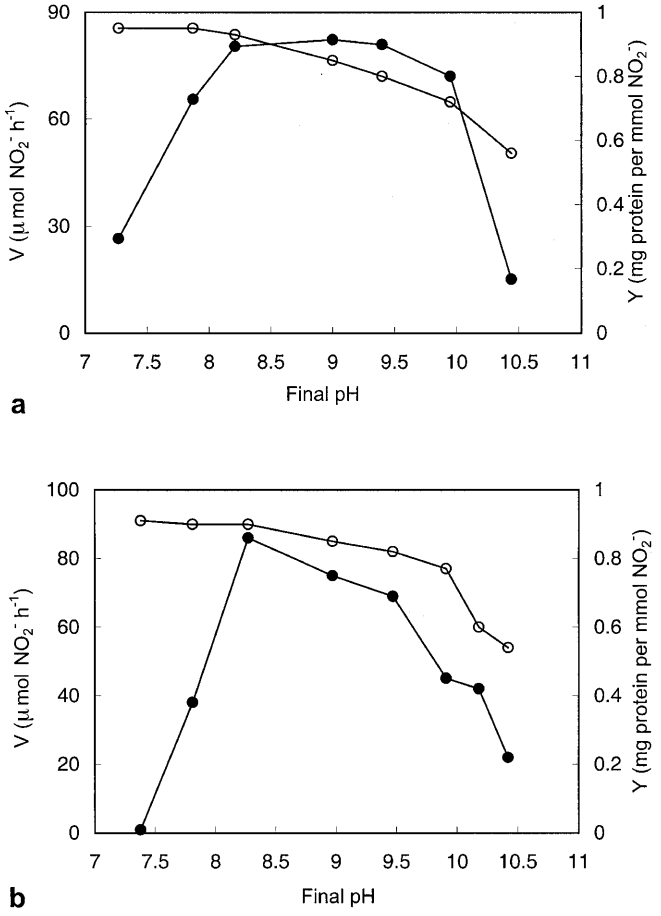

Fig. 3a The pH profiles for growth of ammonia-oxidizing isolates ANs1 (**a**) and ANs5 (**b**) in batch culture at 0.6 M Na^+. ❍ Molar growth yield, ● average rate of nitrite production (*V*). Buffering system for pH range 7–8 was 0.1 M HEPES+0.6 M NaCl and for higher pH values sodium bicarbonate/carbonate buffer, 0.6 M total Na^+

isolated from lakes containing more than 50 g salt l^{-1}) with an optimum corresponding to salt concentrations of diluted soda lakes.

Influence of pH on growth in batch culture at 0.6 M Na^+

All five ANs isolates grew best on sodium bicarbonate-/carbonate-buffered medium within the pH range 8.5–9.5 (Fig. 3). Isolates ANs1 and ANs2 had a lower pH limit at pH 7.2, while the other three isolates started to grow at pH≥7.5. The maximum pH limit achieved in batch cultures was 10.5, but the buffering capacity of carbonate base was not sufficient to keep the pH constant: it already started to drop after cultures had oxidized only 3–4 mM NH_3. The growth yield was within the range of 0.6–1 mg protein per mmol NO_2^- produced with a clear tendency to decrease at pH>10.

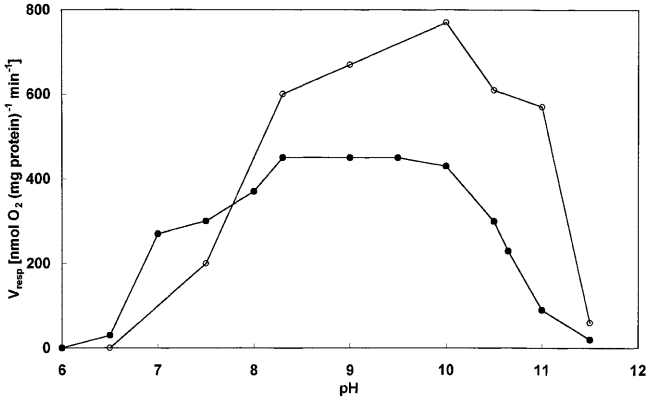

Fig. 4 The pH profiles for respiratory activity (V_{resp}) of ammonia-oxidizing isolate ANs5 grown in batch culture at pH 10 and 0.6 M Na^+ (●) and in NH_3-limited and pH-controlled continuous culture at pH 11.0 and 0.3 M Na^+ (❍). The buffering system was as described in Fig. 3, except that for cells from the chemostat buffers contained 0.3 M Na^+

Influence of pH on respiratory activity of washed cells

In general, the pH profiles for NH_3-dependent oxygen consumption by washed cells of ANs isolates grown at pH 10 were similar to their growth pH profiles with somewhat higher tolerance at pH>10.5 (Fig. 4). Some isolates were even active up to pH 11.0. In sodium bicarbonate/carbonate buffers, the optimum pH was between 8.2 and 10. If the biomass was obtained from cultures in which most of the cells entered refractile phase, a very long lag phase (up to 20 min) was observed before oxygen consumption started. Ammonia toxicity for respiration increased with increasing pH. K_i 50 values of 8, 4.5 and 0.5 mM were measured at pH 9.0, 10.0, and 11.0, respectively. K_m values for ammonia estimated from the V (rate of substrate oxidation) vs S (substrate concentration) plot at pH 10 were in the range of 40–50 µM for all ANs isolates. With hydroxylamine, the pH dependence of the oxidation was difficult to measure because of its high spontaneous auto-oxidation at high pH. Measured for cells of isolate ANs4, NH_2OH rates of oxidation were much lower than rates of ammonia oxidation and had an pH optimum of 9.0.

Growth of isolate ANs5 in ammonia-limited continuous culture at pH>10

As was mentioned above, it was not possible to keep the pH constant during batch cultivation of ANs isolates at a pH higher than 10.3–10.4. Moreover ammonia toxicity increased dramatically at these pH values. To avoid these problems and to reveal the true pH limits for growth of the soda lake isolates, strain ANs5 was grown in ammonia-limited and pH-controlled continuous culture in medium containing 0.3 M total Na^+. Under these conditions the bacteria exhibited a remarkable potential to grow at extremely high pH values (Table 2). The cultures grew very

Table 2 Parameters of growth of alkaliphilic ammonia-oxidizing bacterium isolate ANs5 in ammonia-limited continuous culture at pH>10 containing ammonia, 22.5 mM; temperature 30 °C; salts: Na_2CO_3, 0.25 M Na^++0.05 M NaCl). *Q* Ammonia-dependent respiration activity measured directly in the culture taken from the fermentor (culture) or after centrifugation and resuspension of the cells in buffers containing 0.3 M total Na^+, μ specific growth rate, *Y* molar growth yield

Culture pH	μ (h^{-1})	Y (mg protein $mmol^{-1}$)	Q [nmol O_2 (mg protein)$^{-1}$ min^{-1}]		
			Culture	Washed cells	
				Culture pH	pH 10
10.05	0.110	1.23	1970	300	300
10.52	0.092	1.12	1670	620	610
10.70	0.075	0.95	1290	480	620
10.90	0.052	0.68	1180	410	640
11.02	0.035	0.64	1080	480	770
11.18	0.028	0.57	1050	150	220
11.28	0.012	0.35	820	Nd	Nd

fast at pH 10, with a μ_{max} 0.11 h^{-1}, which is within the highest rate known for autotrophic ammonia-oxidizing bacteria, and were still able to grow actively up to pH 11.2. The other interesting fact revealed in chemostat culture was the much higher activity of ammonia-dependent respiration in the cell suspensions taken directly from the chemostat than from washed cells (Table 2). It is clear that the washing step partially inactivated the respiratory activity of ANs isolates. The cells grown at pH 11.0 in a chemostat showed a definite alkaline shift in their respiratory pH profile as compared to cells grown at pH 10, which was especially pronounced at pH 11.0 (Fig. 4).

Phylogenetic analyses

All five ANs isolates possessed a 16S rDNA gene with identical sequence. Comparative 16S rDNA sequence analysis demonstrated that the isolates are affiliated with the *Nitrosomonas europaea* lineage, which comprises besides *Nitrosomonas europaea* the species *Nitrosomonas eutropha* and the halophilic species *Nitrosomonas halophila* (Fig. 5). The low sequence divergence of the 16S rDNA (below 1%) with the latter species indicated that the new alkali-tolerant isolates might belong to the species *N. halophila.* Consistent with these results the *amoA* sequence of the isolate ANs1 showed the highest sequence similarity (95.6% on the nucleic acid level and

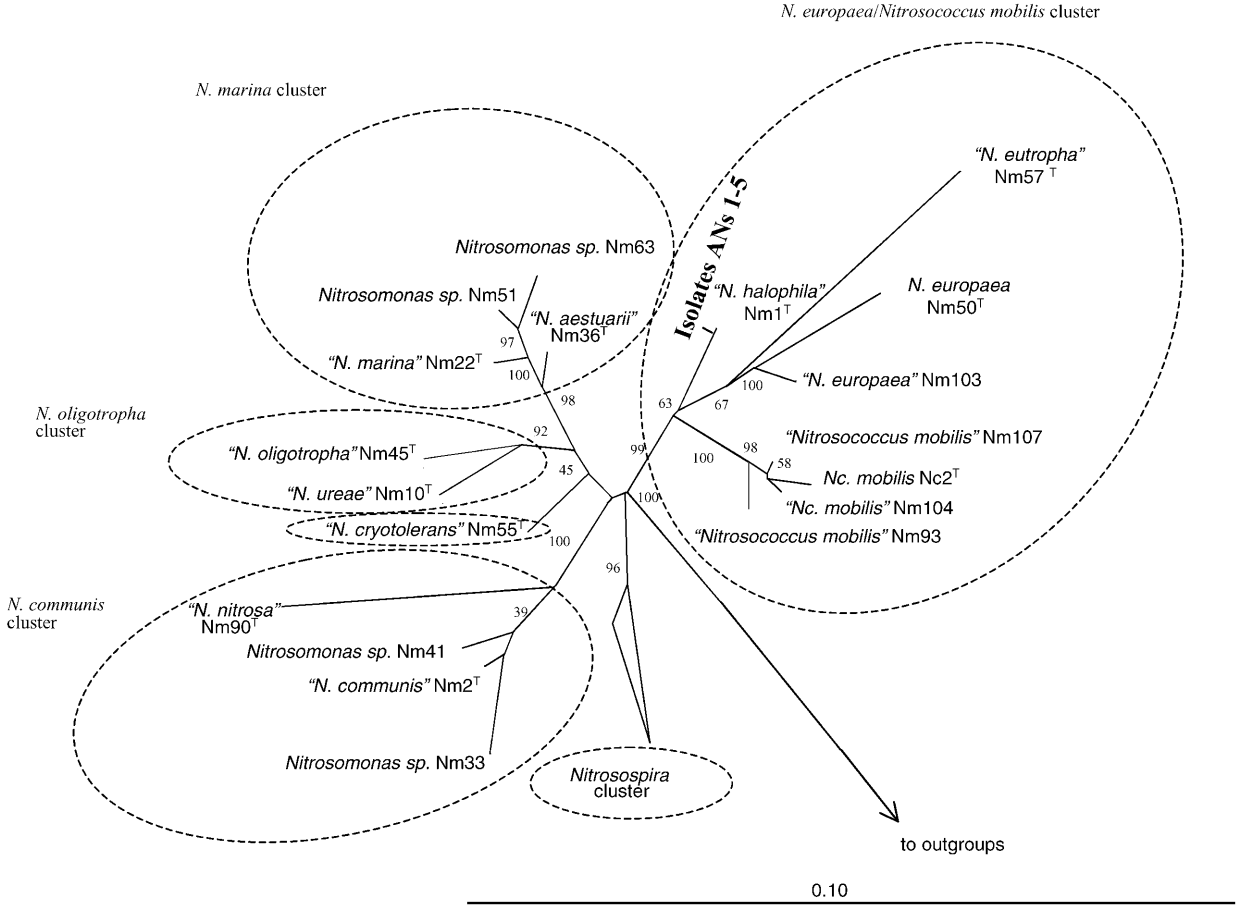

Fig. 5 Phylogenetic neighbor-joining 16S rRNA tree showing the affiliation of the ammonia-oxidizing isolates ANs1–5 from the Mongolian soda lakes to recognized ammonia-oxidizers of the β-subclass of *Proteobacteria*. *Bar* 10% sequence divergence

98.6% on the amino acid level) with the respective gene of *N. halophila*. DNA-DNA hybridization experiments finally proved that the isolates indeed belong to the species *N. halophila*, since the observed DNA-DNA similarity values of the isolates with the type strain of *N. halophila* were higher than 70% (73–87%).

Discussion

In a recent investigation of the total bacterial DNA collected from the water of alkaline and saline Mono Lake, a number of 16S rDNA clones were identified that belonged to the *Nitrosomonas europaea* cluster (Ward et al. 2000). Some of the clones were almost identical to the species *N. europea* and *N. eutropha*, respectively. These two species have no obligate salt requirement, but both reveal relatively strong salt tolerance (Koops and Harms 1985; Koops et al. 1991). Clearly, the molecular approach by itself was not sufficient for the characterization and understanding of specific adaptation of the ammonia-oxidizing population to specific conditions of the highly alkaline and saline environment.

Our previous physiological studies with methanotrophic (Sorokin et al. 2000a) and nitrite-oxidizing (Sorokin et al. 1998) bacteria isolated from soda lake sediments revealed that both stages of nitrification can be carried out at much higher pH values than was previously recognized. The isolation of specific autotrophic ammonia-oxidizing bacteria able to grow well at pH values above 10 confirmed that nitrifying bacteria can play an active role in the soda lake microbial system. Taking into account that at a high pH ammonia has an increasing potential to escape into the atmosphere and the toxicity of ammonia at high pH, the role of the alkali-tolerant ammonia-oxidizing bacterial population in nitrogen cycling of the soda lakes may be very important. The physiological properties of the ammonia-oxidizing strains isolated from the Mongolian soda lakes demonstrated that they indeed can be active under conditions common for diluted soda lakes.

Based on their tolerance to extremely high pH values, the new ammonia-oxidizing isolates may be considered as absolute leaders among alkaliphilic chemolithoautotrophic bacteria isolated so far from soda lakes (Sorokin 1998; Sorokin et al. 1998, 2000a, b, c). There is only one example described in the literature demonstrating the ability of a heterotrophic *Bacillus* species to grow up to pH 11.4 in chemostat under pH-controlled conditions (Sturr et al. 1994). This value seems to be a trustful alkaline pH limit for growth of bacteria. Above this limit, the maintenance of pH homeostasis became impossible even for highly effective heterotrophic energy metabolism. Therefore, the ammonia-oxidizing isolates from the soda lakes belong to the most alkali-tolerant groups of bacteria known yet. One of the reasons for the limitation of growth of alkaliphilic autotrophs at pH>10.5 might be carbon limitation due to the dominance of carbonate ion over bicarbonate. Assuming the much bigger cell volume of ammonia-oxidizing isolates from soda lakes than of other types of chemolithoautotrophic alkaliphiles, it might be speculated that the former can overcome the carbon limitation at pH>10.5 by increasing the specific number of carboxysomes. Comparison of the thin sections prepared from the cells of isolate ANs5 grown at pH 10 and 11 revealed a sharp increase in the number of carboxisomes per cell (from 2–4 to 10–15) with a concomitant decrease of their volume.

The salt concentration seems to be a much more crucial factor for ANs isolates than the high pH values. In this respect, the new ammonia-oxidizing alkali-tolerant isolates resemble the low-salt-tolerant nitrite-oxidizing *Nitrobacter alkalicus*, also obtained from the soda lake environments (Sorokin et al. 1998). In contrast, some of the sulfur-oxidizing alkaliphiles from soda lakes are able to grow up to saturating salt concentrations (Sorokin et al. 2001). One of the reasons for the low salt tolerance of the autotrophic nitrifying bacteria may be their relatively low efficiency of energy production, which might limit their ability to synthesize energetically expensive organic compatible solutes (Oren 1999).

Phylogenetic analysis clearly demonstrated that the soda lake isolates do not represent a new bacterial species but rather represent a specific alkali-tolerant subpopulation of *Nitrosomonas halophila*, which was originally isolated from the North Sea. It is very interesting to note that in environmental surveys, including marine ecosystems, *N. halophila* 16S rDNA sequences were never detected among the many sequences retrieved for other ammonia-oxidizing bacteria (Purkhold et al. 2000). This might indicate that the soda lakes are the actual habitat of this bacterium. The type strain *N. halophila* Nm1 was described as a neutrophilic bacterium but revealed a relatively strong salt tolerance (Koops et al. 1991). The genetic relation on the species level among isolates obtained from substantially different environments is surprising. Our careful reexamination of the influence of pH on growth and activity of *N. halophila* Nm 1, however, demonstrated a remarkable tolerance of this marine isolate to high pH. This tolerance was missing among another species of the *N. europea* lineage, *N. eutropha*. *N. halophila* Nm1 grew up to pH 9.5 with an optimum at pH 8.5–9 in bicarbonate-based medium with a total Na^+ concentration of 0.3 M. But, in contrast to the soda lake ANs isolates, *N. halophila* Nm1 was not able to grow at pH 10 and higher in carbonate-dominating medium. Accordingly, here we suggest a modification of the species description of *Nitrosomonas halophila* (based on the original description of Koops et al. 1991), including the properties of the new alkali-tolerant representatives from the soda lakes:

Nitrosomonas halophila (hal.o'phi.la. Gr. n. *hal*, salt; Gr. adj. *philos*, loving; L. fem. adj. *halophila*, salt-loving).

Cells are 1.1–1.8×1.5–2.5 μm in size. In some isolates cells are motile by a tuft of flagella. Carboxysomes and intracytoplasmic membranes are present. Have an obligate salt requirement with a growth optimum around 0.3 M Na^+ and upper limit of 0.8 M Na^+. The marine-type isolate can grow up to pH 9.5, and soda lake isolates are extremely alkali-tolerant, with a maximum pH limit for

growth up to 11.2. Utilization of urea not observed. The G+C content in DNA is 53.8 mol % (T_m). Habitat: the type strain Nm 1 was isolated from the North Sea; isolates ANs1–5 were obtained from the Mongolian soda lakes. Type strain: Nm 1 is deposited and maintained in the culture collection of the Institut fur Allgemeine Botanik der Universitaet Hamburg. Mikrobiologische Abteilung, Germany. ANs1–5 isolates are maintained in the same collection and in the culture collection of the Department of Biotechnology, Kluyver Laboratory, TU Delft, Delft, The Netherlands.

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