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ENVIRONMENTAL BIOTECHNOLOGY

Analysis of ammonia-oxidizing bacteria dominating in lab-scale bioreactors with high ammonium bicarbonate loading

Dana Vejmelkova · Dimitry Y. Sorokin · Ben Abbas · Olga L. Kovaleva · Robbert Kleerebezem · Marlies J. Kampschreur · Gerard Muyzer · Mark C. M. van Loosdrecht

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Abstract The ammonia-oxidizing bacterial community (AOB) was investigated in two types of laboratory-scale bioreactors performing partial oxidation of ammonia to nitrite or nitrate at high (80 mM) to extremely high (428 mM) concentrations of ammonium bicarbonate. At all conditions, the dominant AOB was affiliated to the *Nitrosomonas europaea* lineage as was determined by fluorescence in situ hybridization and polymerase chain reaction in combination with denaturing gradient gel electrophoresis. Molecular analysis of the mixed populations, based on the 16S rRNA and *cbb*L genes, demonstrated the presence of two different phylotypes of *Nitrosomonas*, while microbiological analysis produced a single phylotype, represented by three different morphotypes. One of the most striking features of the AOB populations encountered in the bioreactors was the domination of highly aggregated obligate microaerophilic *Nitrosomonas*, with unusual cellular and colony morphology, commonly observed in nitrifying bioreactors but rarely investigated by cultural methods. The latter is probably not an adaptation to stressful conditions created by high ammonia or nitrite concentrations, but oxygen seems to be a stressful factor in these bioreactors.

Keywords Ammonia-oxidizing bacteria (AOB) · DGGE · FISH · *Nitrosomonas* · Partial nitrification

D. Vejmelkova (✉)
Department of Water Technology and Environmental Engineering,
Institute of Chemical Technology,
Technicka 5, Prague 6,
166 28 Prague, Czech Republic
e-mail: dana.vejmelkova@vscht.cz

D. Y. Sorokin · O. L. Kovaleva
Winogradsky Institute of Microbiology,
Russian Academy of Sciences,
Prospect 60-let Octyabrya 7/2,
117811 Moscow, Russia

D. Vejmelkova · D. Y. Sorokin · B. Abbas · R. Kleerebezem · M. J. Kampschreur · G. Muyzer · M. C. M. van Loosdrecht
Department of Biotechnology, Delft University of Technology,
Julianalaan 67,
2628 BC Delft, The Netherlands

Introduction

In recent years the production of biogas from activated sludge, manure and agricultural residues is strongly increasing (Appels et al. 2008; Deublein and Steinhauser 2010). The effluent of these processes still contains high amounts of ammonium nitrogen with bicarbonate as counter ion and several other minerals. For these specific wastewaters, specialised processes have been developed like the Sharon process (Hellinga et al. 1998) in which the specific process conditions are used to select for desired nitrifying populations. These processes allow efficient ammonium conversion in nitrogen gas based on nitrification–denitrification with nitrite as intermediate (partial nitrification). For nitrogen recovery, it is possible to convert ammonium bicarbonate to ammonium nitrate. The latter solution is chemically stable and can be concentrated by for instance vapour compression techniques.

Partial nitrification at high substrate loading has been the subject for many studies (Hellinga et al. 1998; van Dongen et al. 2001; Sinha and Annachhatre 2004; Ganigué et al. 2007; van Hulle et al. 2007), but so far, only a few attempted to look behind the kinetic parameters and the general characterization of the ammonium-oxidizing biomass with molecular or microbiological methods (Logemann et al. 1998; Tan

et al. 2008). This is remarkable since the microscopic observation of the *Nitrosomonas* population in such systems looks clearly unusual, i.e. different from what is common in pure cultures. The known pure cultures of *Nitrosomonas* all grow as free, regular-shaped suspended cells, while the commonly present ammonia-oxidizing bacteria (AOB) biomass from the bioreactors consists of highly aggregated clusters with irregularly shaped cells surrounded by multilayered exopolymers (Arp and Bottomley 2006; Bock and Wagner 2006). The latter is a common phenomenon for biofilm organisms, but such a feature has never been demonstrated for pure cultures of *Nitrosomonas*. This warrants the necessity to look more closely to the aggregate-forming *Nitrosomonas* developing in high loaded ammonium systems.

The formation of aggregates or biofilms is common for microorganisms living in natural environments and man-made systems (Aoi 2002). Its creation depends on environmental conditions and could be a response to stress. In the case of AOB, the stress conditions, which might lead to aggregation are e.g., high concentration of ammonia, nitrite, salt or low pH (De Boer et al. 1991). Growth in a biofilm or aggregate brings to microorganisms many advantages. Next to the protection from inhibitors or predators, it will also enhance cooperation among members of the community (Marvasi et al. 2010). For example, AOB provide substrate for nitrite-oxidizing bacteria (NOB), and immediate oxidation of nitrite by NOB prevents its accumulation.

This paper describes results of molecular, microscopic and microbiological characterization of AOB populations in several partial nitrification lab-scale bioreactors operated at high to extremely high ammonium bicarbonate loading. The results indicated the domination of several phylo- and morphotypes of AOB related to the *Nitrosomonas europaea* lineage.

Materials and methods

Bioreactors

fThree types of lab-scale nitrifying bioreactors were investigated. The 2.5-L completely stirred tank reactor (CSTR) AOB1 was operated in continuous mode with ammonium bicarbonate as substrate at concentration up to 80 mM. The medium contained NH_4HCO_3 6.77 g/L, KH_2PO_4 100 mg/L, $MgSO_4{\cdot}7H_2O$ 100 mg/L, $CaCl_2{\cdot}H_2O$ 150 mg/L, KCl 0.835 g/L, yeast extract 1 mg/L, $FeSO_4.7\ H_2O$ 12.5 mg/L, trace elements solution (0.15 g H_3BO_3, 0.25 g $CuSO_4{\cdot}5H_2O$, 0.18 g of KI, 1 g $MnCl_2{\cdot}4H_2O$, 0.2 g $NiCl_2{\cdot}6H_2O$, 0.1 g $NaSeO_4$, 0.22 $(NH_4)Mo_7O_{24}{\cdot}4H_2O$, 0.1 g $AlNH_4(SO_4)_2$ 0.43 g $ZnSO_4{\cdot}7H_2O$, 0.24 g $CoCl_2{\cdot}6H_2O$, 0.05 g $NaWO_4{\cdot}2H_20$ and 15 g EDTA in 1 L of demineralized water) 1.25 mL/L. Proton formation and the availability of inorganic carbon limited ammonium oxidation to the level of 45% conversion of the ammonium to nitrite. Nitrite oxidation was prevented by application of a solid retention time of 1.5 days. The pH was not controlled and fluctuated within a range from 6.5 to 7.0, depending on the ammonia-oxidizing activity in the reactor. The 10-L CSTR reactor (AOB2**)** was operated in continuous mode with ammonium bicarbonate as a substrate at concentrations from 71 to 428 mM, aiming at partial oxidation of ammonium to nitrate with ammonium nitrate as the final product. Without pH control, a pH of 6.3–6.6 was established. This bioreactor was operated at a 5-day hydraulic retention time (HRT) which was equal to the solid retention time (SRT) in order to investigate the possibility of partial oxidation of ammonium bicarbonate to ammonium nitrate. The third 2.5-L CSTR reactor (AOB3) was a variant of the AOB1 operated with a longer solid retention time (2 days) allowing for the development of NOB. The operational temperature in all reactors was maintained at 35±1 °C.

Inoculum for the bioreactors

The initial material for AOB1 was taken from the sludge of a full-scale nitritation bioreactor operating in Rotterdam-Sluisjesdijk WWTP (Mulder et al. 2001). After 8 months of operation, the SRT of AOB1 was prolonged to 2 days (this reactor operation mode is referred here as AOB3), allowing NOB to grow in the reactor. The inoculum for AOB2 consisted mainly of the same sludge which was used as inoculum for AOB1 (80%) and was mixed with the sludge from a lab-scale reactor where both AOB and NOB were active (Kampschreur et al. 2008).

Enrichment of AOB from the bioreactor AOB1

Effluent aggregated biomass was collected from the reactor and allowed to settle by gravity. The concentrated cells were homogenized in a sterile glass potter and immediately subjected to serial dilutions into the mineral medium containing 80 mM ammonium bicarbonate. The medium was supplemented with 0.7 mM KH_2PO_4, 1 mM MgSO4 and 1 mL/L of trace metal solution (Pfennig and Lippert 1966) and dispensed in cotton-plugged 20-mL serum bottles in 5 mL portions. The bottles were placed into a closed jar and incubated at 30 °C under the gas mixture containing 2% O_2 and 10% CO_2 balanced by argon. CO_2 was necessary to keep the pH below 8. Incubations that showed growth and nitrite formation were plated on solid medium of the same composition after pottering and incubated in the same way as the liquid serial dilutions. Separate colonies were placed into 5 mL portion of the

liquid medium, and their growth was checked by nitrite formation with the test strips (Merck). The purity of the isolates was checked by microscopy, absence of growth in rich organic media and by 16S rRNA gene sequencing.

Analytical procedures

In the bioreactors, mixed liquor suspended solids, ammonium, nitrite and nitrate were off-line, by respectively standard filtration methods and standard spectrophotometric methods (Dr. Lange spectrophotometry kit). Nitrite formation in batch AOB cultures was detected colorimetrically according to Gries-Romijn-van Eck (1966). Cell protein was measured by the Lowry method (Lowry et al. 1951). Phase contrast microphotographs were obtained using microscope model Zeiss Axioplan Imaging 2 (Göttingen, Germany). Samples for electron microscopy were prepared as was described by Dawes (1971). The cells were washed and prefixed in glutaraldehyde (final concentration 3% *v*/*v*) at pH 7.0 in 0.05 KP buffer, then postfixed in 1% (*w*/*v*) OsO_4 for 12 h at room temperature, washed and stained overnight with 1% (*w*/*v*) uranyl acetate, dehydrated in an ethanol series and embedded in Epon resin. Thin sections were contrasted with 1% (*w*/*v*) lead citrate. The preparations were observed in JEOL 100 (Japan) transmission electron microscope.

Fluorescence in situ hybridization

Samples for fluorescence in situ hybridization (FISH) analysis were fixed in 4% (*w*/*v*) paraformaldehyde and stored in 80% (*v*/*v*) ethanol at −20 °C. The hybridization of fixed samples was performed according to Pernthaler et al. (2001). Seven probes were used to classify the AOB population in the reactors. In addition, the oligonucleotides specific for the *Betaproteobacteri*a, BET42a and for the domain *Bacteria*, EUB338mix were used (Loy et al. 2007). The probes (except for the competitors) were 5′-labelled with the Cy3, Cy5 or FLUOS dye. All probes used in this study are listed in Table 1. Hybridized samples were examined with Zeiss Axioplan-2 Optical Microscope.

Sequence analysis

Genomic DNA was extracted from the cell pellet using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, USA), following the manufacturer's instructions. For the pure cultures, the nearly complete 16S rRNA gene was obtained using general bacterial primers GM3f (5′-AGAGTTTGATCCTGGCTCAG-3′) and GM4r (5′TACGGTTAC-CTTGTTACGACTT-3′). For the denaturing gradient gel electrophoresis (DGGE) analysis, partial amplification with a primer pair 341F+GC/907R was employed (Schäfer and Muyzer 2001). For amplification of the *cbb* genes (coding for RuBisCO large subunit) primers and protocols described by Tourova et al. (2010) were used. DGGE of small ribosomal subunit coding genes was performed as described by Muyzer et al. (1993) using a denaturing gradient of 20–35% to 60–70% denaturants in 6% polyacrylamide gel. Individual bands were excised, reamplified and run again on a denaturing gradient gel to check their purity. A 16S rRNA gene-based and a *cbb* gene clone libraries from the reactor AOB2 were constructed using pCR4–TOPO vector (Invitrogen Corp., Carlsbad, CA) according to manufacturer's protocol. The PCR products for cloning and sequencing were purified using the QIAquick PCR purification kit (QIAGEN, The Netherlands) and sequenced. The obtained sequences were first compared with sequences stored in GenBank using the BLAST algorithm

Table 1 List of the 16S rRNA-targeted oligonucleotide probes used in this study (Loy et al. 2007)

Probe name	Sequence 5′-3′	Specificity
Nso190	CGATCCCCTGCTTTTCTCC	Betaproteobacterial ammonia-oxidizing bacteria
Nso1225	CGCCATTGTATTACGTGTGA	Betaproteobacterial ammonia-oxidizing bacteria
NEU	CCCCTCTGCTGCACTCTA	Most halophilic and halotolerant *Nitrosomonas* spp.
cNEU	TTCCATCCCCCTCTGCCG	NEU competitor
Nse1472	ACCCCAGTCATGACCCCC	*Nitrosomonas europaea*, *N. halophila*, *N. eutropha*, Kraftisried-Isolat Nm103
Cluster6a192	CTTTCGATCCCCTACTTTCC	*Nitrosomonas oligotropha* lineage
cCluster6a192	CTTTCGATCCCCTGCTTTCC	Cluster6a192 competitor
Nsv443	CCGTGACCGTTTCGTTCCG	*Nitrosospira* spp.
NmV	TCCTCAGAGACTACGCGG	*Nitrosococcus mobilis* ("*Nitrosomonas*") lineage
BET42a	GCCTTCCCACTTCGTTT	Betaproteobacteria
GAM42a	GCCTTCCCACATCGTTT	BET42a competitor
EUB338 I	GCTGCCTCCCGTAGGAGT	most *Bacteria*
EUB338 II	GCAGCCACCCGTAGGTGT	*Planctomycetales*
EUB338 III	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*

EUB338mix is a mixture of EUB338 I, II and III

(http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, the 16S rRNA gene sequences were imported into the ARB, automatically aligned, and added to a phylogenetic tree using the Quick-add tool. The tree was constructed using maximum likelihood algorithm, and 1,000 rounds of bootstrap were performed. The species used for the 'base' tree were long quality checked sequences >1,200 bp and were taken from the 'All species Living tree project' (SILVA; Ref). A filter, pos_var_ssuref:bacteria, was applied to remove part of the sequence before basepair with *Escherichia coli* pos. 127 and after 1333 in order to use sequences of the same length. Small sequences <600 bp were calculated into the tree using the build-in ARB parsimony function (the same filter as above was used). Gene sequences showing more than 97% similarity were considered to belong to the same operational taxonomic unit (OTU; Elsaied and Naganuma 2001). A neighbour-joining tree of translated amino acid *cbb* sequences was constructed using the TreeConW program package (Van de Peer and De Wachter 1994).

Nucleotide sequence accession numbers

The GenBank/EMBL accession numbers of the 16S rRNA and the *cbb*L gene sequences determined in this study are JF508893-JF508900 and GQ345128-GQ345131, respectively.

Results

Reactor performance

Operational conditions of AOB1 allowed growing only microorganisms capable of nitritation. The alkalinity in the reactor enabled approximately 45% of ammonia to be oxidized to nitrite. A larger conversion leads to pH decrease and thereby inhibition of the nitrification. In AOB3, due to prolonged HRT/SRT, NOB were also growing resulting in complete oxidation of nitrite to nitrate. AOB2 reactor was steadily transforming half of incoming ammonia to nitrate so both, AOB and NOB, were present as in AOB3. Table 2 summarizes the most important parameters of all three reactors. Concentrations of free ammonia (FA) and free nitrous acid (FNA) were calculated according to Anthonisen et al. (1976). The difference between $N–NH_4^+$ in the influent and $N–NH_4^+$ plus $N–NO_2^-$ in the effluent is present in the form of $N–NO_3^-$.

Microscopic analysis and identification of AOB in the reactors

Phase contrast microscopy of the aggregated biomass fraction from the AOB reactors showed a dominance of large, coccoid, irregularly shaped cells arranged in tight clusters (Fig. 1a, d). FISH revealed that most of the aggregated coccoid cells react with the probes specific for the betaproteobacterial AOB, belonging to the genus *Nitrosomonas* (Fig. 1b, c ,e, f). The probes NSV443, NmV and Cluster6a192, which are specific for the *Nitrosospira* spp, *Nitrosococcus mobilis* and *Nitrosomonas oligotropha* lineage, respectively, did not give any signal. The signal of the probes NSO190, NSO1225, Nse1472 and NEU653 was mostly overlapping, indicating that AOB present in the reactors are belonging to the group of halophilic and halotolerant *Nitrosomonas*.

Isolation of the dominant AOB from the bioreactors

We obtained an active enrichment culture in a serial dilution up to 10^{-8} from the aggregated biomass of reactor AOB1. Growth was only observed at microaerophilic conditions and in the presence of buffering CO_2 in the gas phase. The resulting culture consisted of two phases; the main biomass fraction was represented by highly aggregated large coccoid cells, similar to what was observed in the bioreactors, while the suspended biomass was dominated by large, motile, lemon-shaped cells. The latter (*type 1*) could be easily isolated in pure culture after plating and it grew in liquid medium with ammonium bicarbonate as the only substrate at fully aerobic conditions (Fig. 2a). Further efforts in isolating AOB from the aggregated biomass proved to be very difficult since nearly every colony formed even after thorough mechanical homogenization of the starting material, which was a mixture of AOB and heterotrophic 'satellite' bacteria. These colonies were very unusual for AOB, i.e. they grew as a single strong aggregate. Only few of these colonies with more regular circular shape and semisolid consistence turned out to consist of pure AOB cells and

Table 2 The most important parameters of the reactors investigated in this study

Reactor	pH	*T* (°C)	SRT [days]	c $(N–NH_4^+)_{in}$ [mg/L]	c $(N–NH_4^+)_{ef}$ [mg/L]	c $(FA)_{ef}$ [mg/L]	c $(N\text{-}NO_2^-)_{ef}$ [mg/l]	c $(FNA)_{ef}$ [mg/L]
AOB1	6.5–7	35±1	1.5	1,100	600	2.1–6.7	500	0.1–0.3
AOB2	6.3–6.6	35±1	5	6,000	3,000	6.8–13.5	n.s.	n.s.
AOB3	6.5–7	35±1	2	1,100	600	2.1–6.7	5	n.s.

n.s. not significant

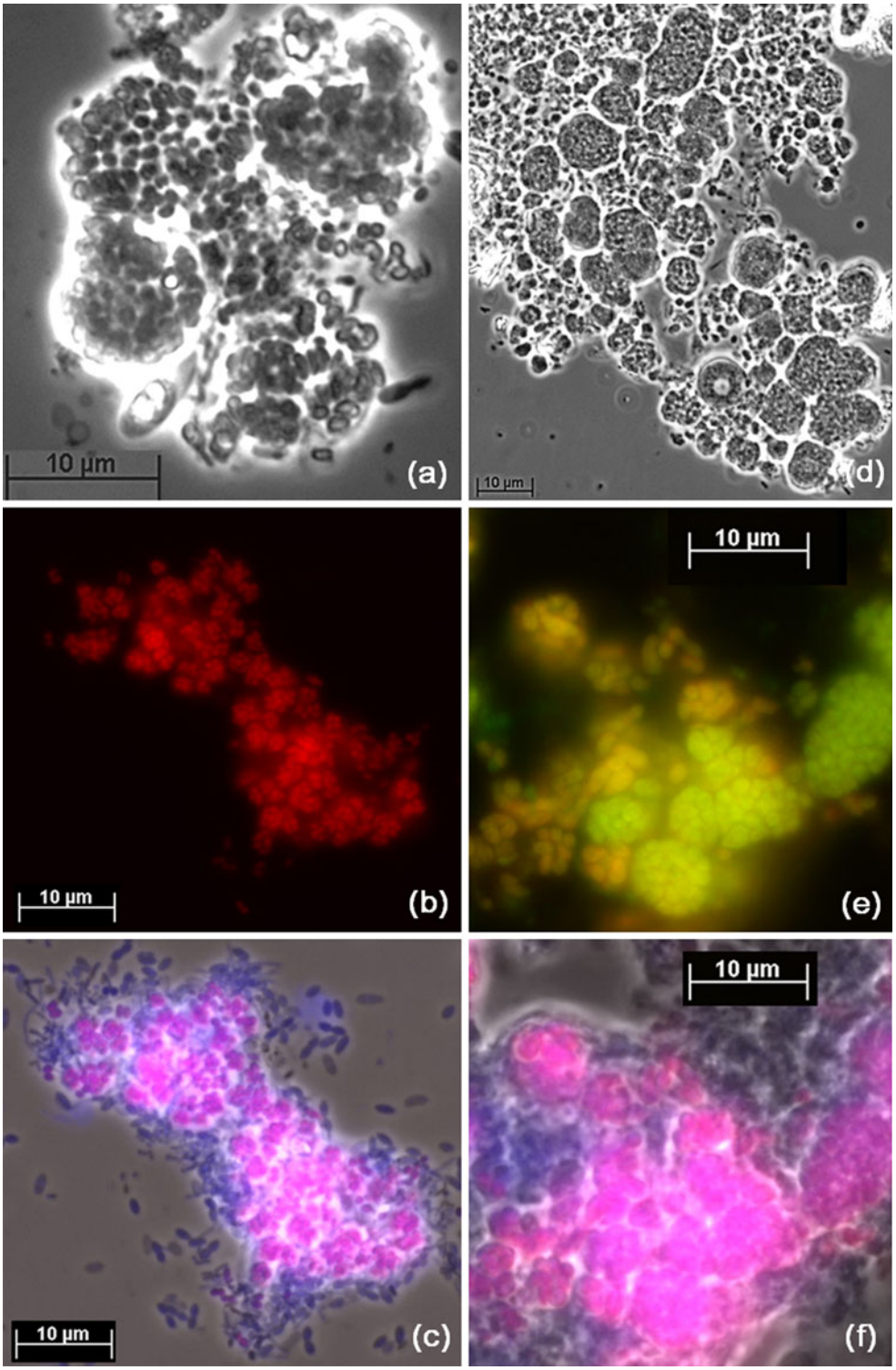

Fig. 1 Aggregated AOB in the reactor biomass. **a, d** Phase contrast microphotographs; **b**, **c, e, f** FISH results. *Red* NSO190+ NEU653, *green* BET42a, *blue* EUBmix. **a–c** cells from the reactor AOB2; **d–f** cells from the reactor AOB3

resulted in several isolates (*type 2*). This AOB morphotype was represented by elongated cells growing mostly in tight clusters, although free-living cells were also observed (Fig. 2b). In contrast to the free-living type 1 with regular soft colonies, type 2 only grew at microaerophilic conditions. So, one of the reasons for the aggregated growth in this type of AOB might be the necessity to protect the cells from high oxygen concentrations. Most of the solid AOB colonies had irregular shape and contained a mixture of large coccoid AOB cells and small rod-shaped heterotrophic contaminants, which, after transfer to the liquid culture medium, grew solely as a firm aggregate without any suspended cells

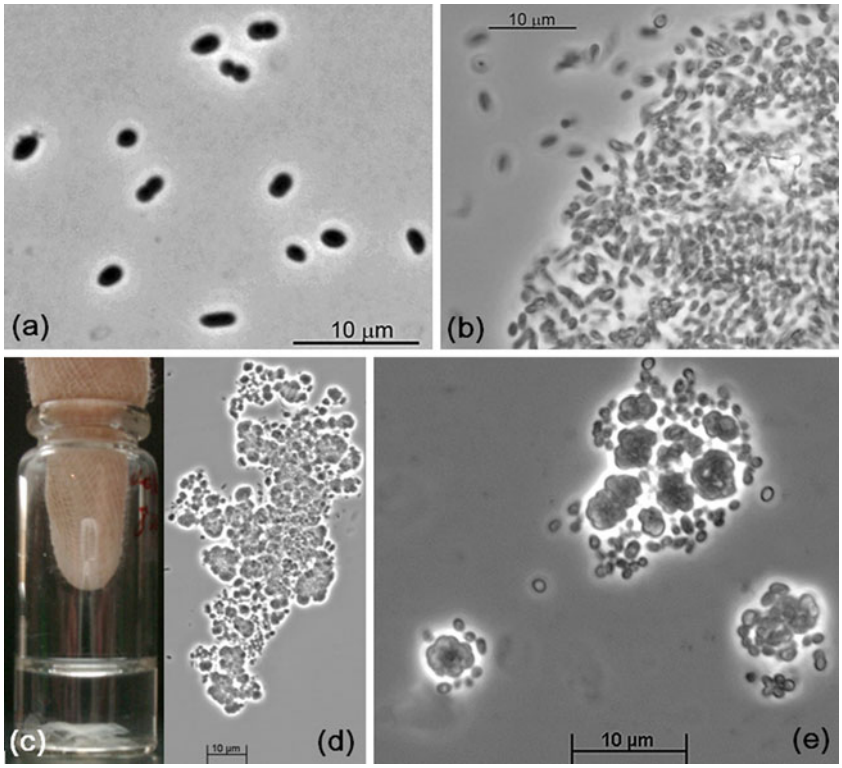

Fig. 2 Cell morphology (phase contrast) of AOB cultures enriched and isolated from reactor AOB1. **a** *Nitrosomonas* strain a5 with free-living cells (type 1), **b** *Nitrosomonas* strain a15 (type 2), **c–d** highly enriched culture of *Nitrosomonas* type 3 (**c** liquid culture showing skin-like aggregated growth, **e** phase contrast microphotograph)

(Fig. 2c–e). This AOB (*type 3*), although highly enriched, resisted further purification. Similar to the type 2, it grew only at low oxygen concentration.

Electron microscopy (Fig. 3) of the aggregated biomass from the AOB reactors and the type 3 AOB batch cultures revealed a dominance of cell organization usual for aggregates, but very uncharacteristic of the AOB normally found in pure cultures. The cells were embedded in structured exopolymer matrix, sometimes doubled (individual cells and microcolonies). The cell content was very dense, and only in some cases was the intracytoplasmic membranes' characteristic for *Nitrosomonas* could be distinguished. Most striking, the cell shape was far from regular. Harsh mechanical disintegration resulted only in fragmentation of the aggregates, but failed to produce individual cells, which rapidly reintegrated. Incubation at low oxygen and ammonia concentrations (2% O_2 and 10 mM ammonium bicarbonate) did not result in the appearance of any suspended cells. All three types of isolated AOB were able to grow in the presence of up to 100 mM NH_4HCO_3, and the washed cells respired ammonium in the presence of up to 200 mM nitrite.

16S rRNA gene analysis of the reactor biomass and enrichment cultures

16S rRNA gene DGGE gels from the three bioreactors contained two distinct bands belonging to two different *Nitrosomonas* phylotypes (Fig. 4a, b). Additional sequences were generated by DGGE from enrichment and pure AOB cultures from AOB1 reactor (Fig. 4c). In total, eight environmental *Nitrosomonas* sequences were retrieved from this analysis and deposited in GenBank as JF508893-

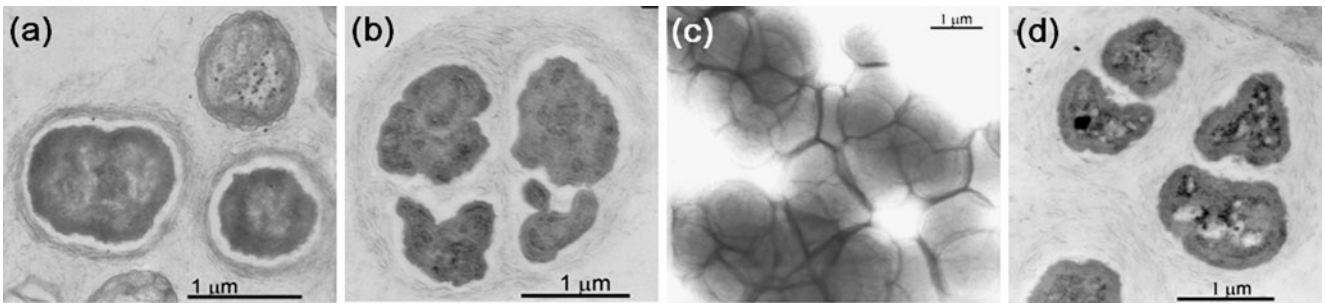

Fig. 3 Cell ultrastructure of aggregated AOB in the reactor AOB1 (**a**) and AOB2 (**b**) and in highly enriched mixed culture *Nitrosomonas* type 3 (**c–d**). **a**, **b**, **d** thin sections; **c** total preparation

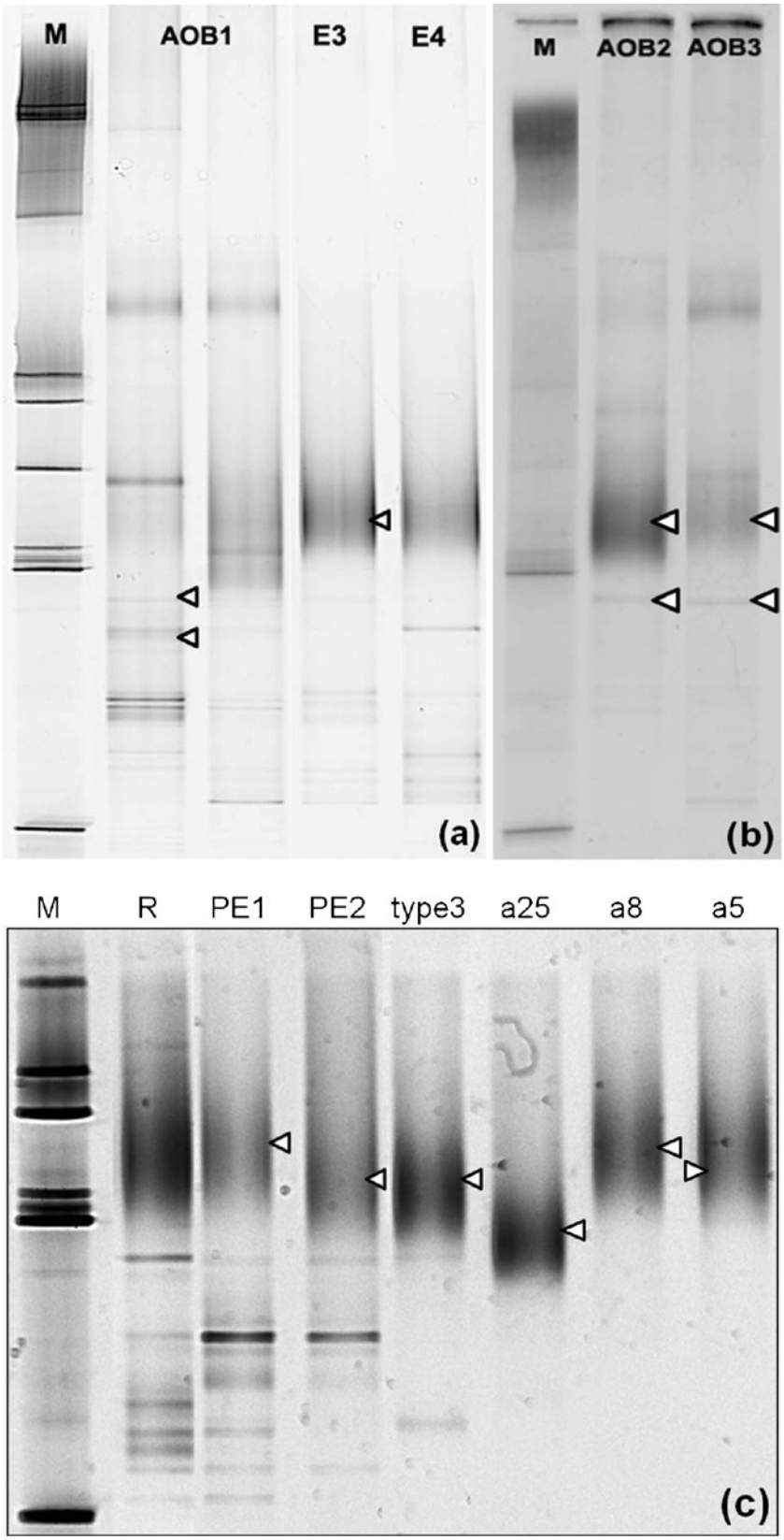

Fig. 4 Results of the DGGE analysis of the AOB reactor biomass (**a**–**b**), enrichment and pure AOB cultures from AOB1 (**c**). *M* marker, *E* AOB enrichment cultures from reactor AOB1, *R* biomass from the reactor AOB1, *PE1* and *PE2* primary AOB enrichments, *type 3* highly enriched aggregated *Nitrosomonas* culture, *a25* pure culture of loosely aggregated *Nitrosomonas* (*type 2*) from PE, *a5* and *a8* pure cultures of free-living *Nitrosomonas* from PE (*type 1*). Cut bands belonging to *Nitrosomonas* are indicated by *arrows*

JF508900. Phylogenetic analysis demonstrated that the obtained sequences cluster together with sequences from other members within the cluster *N. europaea–Nitrosomonas eutropha* (Fig. 5)

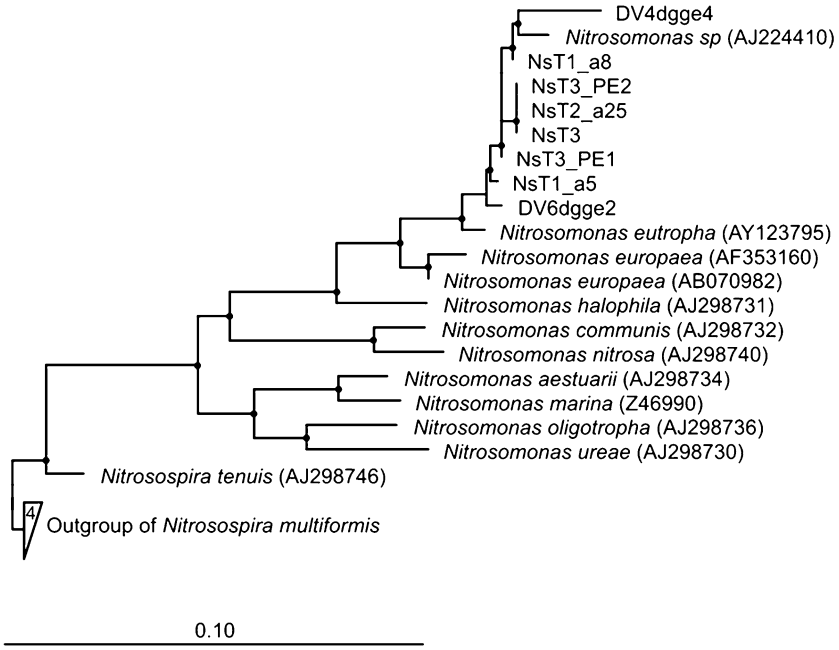

Fig. 5 Phylogenetic position of the AOB sequences obtained from the reactor biomass by DGGE (see Fig. 4) and cloning and from cultures based on 16S rRNA gene analysis. The sequences obtained in this study are in *bold type*. The sequences from enriched and pure AOB cultures are marked as *Ns*. The abbreviations *T1*, *T2* and *T3* indicate three types of enriched AOB and abbreviations *a5*, *a8* and *a25* mark isolated pure cultures of AOB. The sequence of *Nitrosospira multiformis* was used as an outgroup. The *bar* indicates 10% sequence difference. Bootstrap values greater than 90% are indicated by a *dot placed onto the node*

*cbb*L gene analysis

Amplicons of the form I RuBisCO genes (*cbb*L) were obtained from biomass AOB2 reactor. PCR with primers specific for form II RuBisCO (*cbb*M) did not give positive results. Phylogenetic analysis of the recovered *cbb*L sequences revealed a presence of two distinct OTU both belonging to *Nitrosomonas* cluster (Fig. 6), corresponding well to the results obtained by 16S rRNA gene-based analysis.

Despite the difference in macro- and micromorphology, the phylogenetic analysis of both the 16S rRNA and the

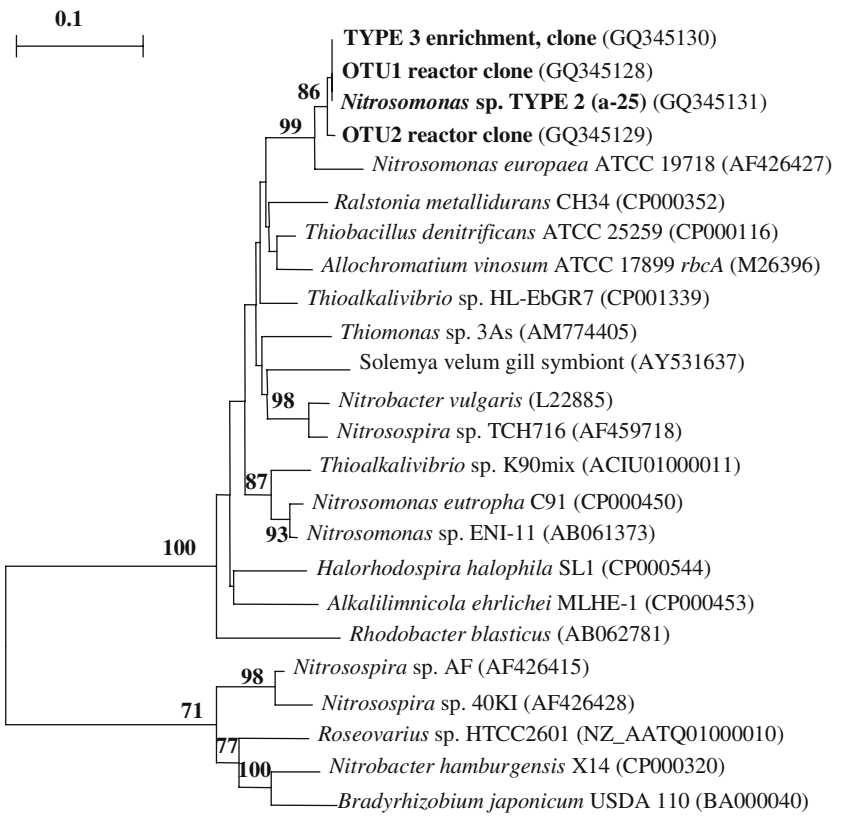

Fig. 6 Phylogenetic tree based on translated amino acid *cbb*L sequences. The tree was constructed using neighbour-joining algorithm. Bootstrap values were calculated from 1,000 replicates. The percentages of bootstrap values above 70% are indicated at the corresponding nodes. The *scale bar* represents 10% amino acid sequence divergence. Sequences obtained in this study are shown in *bold*

functional *cbb*L genes showed that all three cultured AOB types belonged to a single phylotype clustering within the *N. europaea* group (Fig. 5, 16S tree; Fig. 6, *cbb*L tree). Furthermore, all three were nearly identical to one of the two *Nitrosomonas* phylotypes in the three AOB bioreactors as identified by DGGE and cloning.

Discussion

It is well known that the concentration of ammonia influences the community composition of AOB. In general, members of the genus *Nitrosospira* and the *N. oligotropha* clusters prevail in environments with low ammonia concentrations (Bollmann and Laanbroek 2001; Limpiyakorn et al. 2005; Otawa et al. 2006; Wang et al. 2010), whereas environments rich in ammonia are dominated by members of the *N. europaea–N. eutropha* clusters (Logemann et al. 1998; Limpiyakorn et al. 2005; Tan et al. 2008). Hence, the finding that the dominant AOB in the investigated nitrifying reactors belonged to the *N. europaea* lineage is not surprising and corresponds to what was observed before (Logemann et al. 1998; Tan et al. 2008; Matsumoto et al. 2010). However, the peculiar morphology has no analogues among the so far described pure cultures of this AOB genus despite of being a well-documented phenomenon in the wastewater treatment plants and bioreactors. For example, a photograph presented by Bock and Wagner (2006) in their recent review chapter on nitrifying bacteria looks very similar to what we observed in highly enriched aggregated *Nitrosomonas* type 3. Such "biofilm"-like ecotype of *Nitrosomonas* is nearly impossible to cultivate separately from the heterotrophic 'satellite' bacteria, and it grows much slower than the free-living ecotype. These might be the reasons of its absence among the pure cultures studied so far. Despite the close phylogenetic affiliation to "normal" free-living ecotypes, the aggregated *Nitrosomonas* was clearly different in oxygen tolerance. Such sensitivity is difficult to explain for fully aerobic organisms like *Nitrosomonas*. Rather, we would expect that biofilm formation should be a reaction on the extremely high ammonium presence in the investigated reactors. However, just in AOB2 reactor were the calculated FA concentrations reached the values which were predicted as inhibitory for nitrification (Anthonisen et al. 1976). The nitrite sensitivity could play a role only in AOB1 where the calculated FNA concentrations varied between 0.1 and 0.3 mg/L. Furthermore, we could not find substantial differences in nitrite sensitivity between the three different morphotypes of isolated *Nitrosomonas*. So, there must be other significant reasons for the observed domination and stability during cultivation outside the reactor of the biofilm *Nitrosomonas* ecotype. It could be a sort of stress response, similar to, for example, what was observed for a nitrifying population at low pH (de Boer et al. 1991). Another feature, which might play a role, is the solid retention time in the system. If the aggregates are big enough, they may settle rapidly allowing some biomass retention, which should not be common for a CSTR. This phenomenon would give a competitive advantage to the slow-growing, aggregate-forming AOB ecotype. It would be interesting to measure the biomass concentration in the reactor and compare it with the effluent. The remarkable difference in oxygen requirements and morphology among the three AOB morphotypes observed in this study may reflect different conditions of substrate supply, metabolites or oxygen concentration, pH, etc. which could play a role in a distinct gene expression (Jefferson 2004). After a consideration of all mentioned possible stress factors, it is most probably that the oxygen sensitivity is the main causation of AOB aggregation. It is well known that AOB do not need such high oxygen concentration as NOB (Hanaki et al. 1990). However, to our knowledge, it has not been shown so far that oxygen could be inhibitive for these aerobic microorganisms. For better understanding of background and mechanism of such inhibition further experiments need to be carried out.

Our findings confirmed that a proper combination of phenotypic and genotypic methods brings more comprehensive information about investigated community in the environment (Nichols 2007). Since molecular biological tools started to be widely used in microbial ecology (Muyzer et al. 1993; Amann et al. 1995), cultivation-dependent techniques are being suppressed. However, these techniques have definitely still the place in this field. 16S-based typing of microbial communities might give very small variations between strains, whereas as observed in this study, still large phenotypic differences exist. This is logical since the phenotype is influenced by the full genome and not by the 16S RNA itself.

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