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EXPERIMENTAL ARTICLES

Phylogenetic Analysis of a Microbial Community Involved in Anaerobic Oxidation of Ammonium Nitrogen

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Abstract—The phylogenetic diversity of a microbial community involved in anaerobic oxidation of ammonium nitrogen in the DEAMOX process was studied. Analysis of clone libraries containing 16S rRNA gene inserts of *Bacteria*, (including *Planctomycetes*) and *Archaea* revealed the presence of nucleotide sequences of the microorganisms involved in the main reactions of the carbon, nitrogen, and sulfur cycles, including nitrifying, denitrifying, and ANAMMOX bacteria. In the bacterial clone library, 16S rRNA gene sequences of representatives of the phyla *Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Verrucomicrobia, Lentisphaerae, Spirochaetales,* and *Planctomycetes,* as well as of some new groups, were detected. In the archaeal clone library, nucleotide sequences of methanogens belonging to the orders *Methanomicrobiales, Methanobacteriales*, and *Methanosarcinales* were found. It is possible that both ANAMMOX bacteria and bacteria of the genus *Nitrosomonas* are involved in anaerobic ammonium oxidation in the DEAMOX reactor. Many sequences were similar to those from the clone libraries obtained previously from the ANAMMOX community of marine sediments. It is also probable that the DEAMOX reactions occur in natural ecosystems (in marine and freshwater sediments and the oceanic water column), thereby providing for the coupling of the nitrogen and sulfur cycles.

Key words: ANAMMOX, DEAMOX, *Planctomycetes*, phylogeny, 16S rRNA genes, sequence analysis.
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It was long believed that microbial oxidation of ammonium nitrogen occurs only under aerobic conditions. In the 1970s, a thermodynamic calculation revealed that ammonium oxidation to dinitrogen under anaerobic conditions was possible with nitrite as an electron acceptor [1]. In the 1980–1990s, the possibility of anaerobic microbial ammonium oxidation, termed the "ANAMMOX process" (ANAMMOX, ANaerobic AMMonium OXidation), was demonstrated [2].

Later, a number of publications were produced reporting losses of ammonium nitrogen in the course of anaerobic wastewater treatment that could be attributed to the ANAMMOX reaction. Such losses were observed at industrial wastewater treatment plants in Germany, Belgium, Japan, and Australia and in the filtrate treatment units of solid waste landfills (SWF) in Switzerland and the United Kingdom, as well as in natural ecosystems (marine and freshwater sediments and the oceanic water column) [3, 4]. Molecular biological studies of the relevant microbial communities revealed the presence of ANAMMOX bacteria of the phylum *Planctomycetes*.

All the ANAMMOX bacteria described so far form a monophyletic branch within the phylum *Planctomycetes* and belong to the genera *Candidatus* 'Brocadia', *Candidatus* 'Kuenenia', *Candidatus* 'Anammoxoglobus', *Candidatus* 'Scalindua', and *Candidatus* 'Jettenia' that exhibit low 16S rRNA gene sequence similarity to each other (about 90%), as well as to other planctomycetes of the genera *Gemmata, Isosphaera, Planctomyces*, and *Pirellula* [5–8].

The ANAMMOX process occurs at high ammonium concentrations and depends on the availability of an electron acceptor (nitrite). In reactors with a limited oxygen supply, ANAMMOX microorganisms are usually accompanied by aerobic ammonium-oxidizing bacteria that oxidize ammonium to nitrite and, at the same time, shield ANAMMOX bacteria from oxygen. Nitrite can also be produced in the process of denitrification (nitrate reduction to nitrite) [2, 9].

To solve the problem of nitrite supply, as well as of nitrogen removal from wastewater, a new purification process, DEAMOX (DEnitrifying AMmonium OXidation), was recently developed [9]. In this process, traditional nitrification, denitrification (reduction of nitrate to nitrite by autotrophic denitrifying bacteria),

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Table 1. Main biochemical transformations occurring in the course of the DEAMOX process

Reactor	Reaction
UASB reactor	N_{org} compounds + $SO_4^{2-} \longrightarrow NH_4^+ + HCO_3^- + CH_4 + HS^-$ (1)
Nitrifying reactor	$NH_4^+ + O_2 \longrightarrow NO_3^- + NO_2^-$ (2)
DEAMOX reactor	$4NO_3^- + HS^- \longrightarrow 4NO_2^- + SO_4^{2-}$ (3)
	$NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O$ (4)

and the ANAMMOX process (anaerobic oxidation of ammonium nitrogen) (reactions 1–4, Table 1) are combined.

The feasibility of the DEAMOX process was demonstrated under laboratory conditions in a series of experiments with nitrogen-rich yeast plant wastewater [9]. The technological scheme for nitrogen removal included several stages. At the first stage, an anaerobic UASB-type reactor was used to remove most organic matter, as well as for mineralization of nitrogen-containing compounds and conversion of sulfates into sulfide at 35°C (reaction 1). At the second stage of the process, half of the anaerobically treated wastewater was fed to the aerobic reactor to convert ammonium nitrogen into nitrate nitrogen via nitrification at 22°C (reaction 2). At the third stage, the wastewaters from the first and second reactors were combined; ammonium and nitrate nitrogen were converted into molecular nitrogen at 35°C in the anaerobic reactor using the DEAMOX process (reactions 3 and 4). Importantly, sulfide oxidized to sulfate served as an electron donor for denitrification. As a result, 85% of nitrogen was removed after 410 days of treatment in the DEAMOX reactor at a nitrogen loading rate of about 1000 mg N/l/day [9].

The species composition of the microbial community involved in the DEAMOX process should be known for understanding and control of the processes occurring in the reactor. The goal of the present work was to assess the phylogenetic diversity of the microbial community from a DEAMOX reactor by the 16S rRNA gene analysis.

MATERIALS AND METHODS

DNA extraction and amplification of the 16S rRNA gene fragments. In our study, we investigated sludge samples collected from the third reactor in which the DEAMOX process had been carried out by microorganisms for 1.5 years [9]. A sludge sample (1 ml) was centrifuged; DNA was extracted from the pellet using the Diatom^{tm}DNAprep kit (Biokom Co., Russia) according to the manufacturer's recommendations. The pellet was resuspended in double-distilled water, and a cycle of freezing in liquid nitrogen and thawing at 65°C was repeated twice. The sediment was then resuspended in a guanidine chloride solution and incubated at 65°C for 1 h. The resulting lysate was supplemented with a carrier (Diatomid/silica); the supernatant was removed, and the carrier was washed with a buffer solution supplemented with 70% ethanol. Purified DNA was dissolved in 100 μl of double-distilled water and used as a template for PCR.

For amplification of the 16S rRNA gene fragments, the universal primers targeting the representatives of the domains *Bacteria* and *Archaea* were used, as well as specific primers targeting the *Planctomycetes* and ANAMOX bacteria. Amplification of the 16S rRNA gene fragments of the representatives of the domain *Bacteria* was performed using the direct primer 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primers 519r (5'-G(T/A)ATTACCGCGGC(T/G)GCTG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'), corresponding to the *Escherichia coli* positions 8–27, 536–519, and 1510–1492, respectively [10].

For amplification of the 16S rRNA gene fragments of planctomycetes, the Pla46f (5'-GACTTGCATGCCTAATCC-3') primer [5] combined with the universal 519r, 1390r, and 1492r primers, as well as with the AMX820R primer (5'-AAAACCCCTCTACTTAGTGCCC-3') targeting ANAMMOX bacteria [5], and a combination of the Pla58f (5'-GGCATGGATTAGGCATGC-3') and Pla926r (5'-CCACCGCTTGTGTGAGCCCC-3') primers [11] were used.

The 16S rRNA gene fragments of archaea were amplified using the direct A109f (5'-ACG/TGCTCAGTAACACGT-3') and reverse A1041r (5'-GGCCATGCACCWCCTCTC-3') primers corresponding to *E. coli* positions 109–125 and 1058–1041, respectively [12].

The PCR cycle parameters were as follows: 94°C for 3 min, 30–35 cycles including DNA denaturation (94°C for 0.5 min), annealing at 50°C for 0.5 min, elongation at 72°C for 0.5 and 1.5 min for bacterial 16S rRNA gene fragments or at 72°C for 1 min for the 16S rRNA gene fragments of archaea and planctomycetes, and final elongation at 72°C for 7 min. The annealing temperature of the Pla58f-Pla926r primers was 58°C. The PCR products obtained were then analyzed by electrophoresis in 1% agarose gel and precipitated with 70% ethanol containing 0.75 M ammonium acetate (pH 5.0).

Cloning and sequencing of the PCR products. Cloning of the PCR products of the 16S rRNA gene fragments was performed using the pGEM-T plasmid vector system (Promega, United States). A total of 165 unique clones containing 16S rRNA gene inserts of the required size were selected. The four clone libraries consisted of 18, 48, 48, and 51 clones containing inserts of 16S rRNA gene fragments of bacteria,

archaea, and planctomycetes and were obtained using the 8f-1492r (clone library B), 8f-519r (clone library MB), Pla58f-Pla926r (clone library Pla), and A109f-1041r (clone library MA) primers, respectively.

Sequencing of the amplification products was performed on an ABI 3100 Avant Genetic Analyser sequencer (Applied Biosystems Inc., United States), using a Dyenamic Terminator Cycle Sequencing Ready Reaction kit (Amersham, United Kingdom) according to the manufacturer's protocol.

The 16S rDNA sequences of bacteria were obtained using the T7 plasmid primer (for MB clones) and the 8-27f primer (for B clones). The 16S rDNA sequences of archaea were obtained using the A109f primer (for MA clones).

Phylogenetic analysis. Preliminary analysis of the obtained nucleotide sequences was performed using the NCBI BLAST software package. The sequences were edited with the BioEdit software package (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). The obtained nucleotide sequences were aligned with the corresponding sequences of the most closely related microorganisms using the CLUSTAL W v. 75 software package [13]. The online CHECK_CHIMERA system and the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) were used to detect chimerical sequences. The phylogenetic trees were constructed using the neighbor-joining method [14] implemented in the TREECONW software package (http://bioinformatics.psb.ugent.be/psb/Userman/treeconw.html) [15].

The 16S rRNA gene fragments obtained in this work were deposited in the GenBank under accession numbers DQ507137–DQ507199 and FJ788643–FJ788663.

RESULTS AND DISCUSSION

The 16S rRNA genes of representatives of the domain *Bacteria*. The B and MB clone libraries consisted of 66 bacterial clones obtained and analyzed in this study. All the 16S rRNA gene sequences were grouped into 50 phylotypes belonging to 11 known phyla and 11 unclassified bacterial groups. The predominance of the nucleotide sequences of representatives of the phyla *Proteobacteria* and *Bacteroidetes* was detected (Figs. 1 and 2).

Most of the proteobacterial 16S rDNA sequences (18 out of 24 clones) belonged to *Betaproteobacteria* and were grouped into 15 phylotypes (Fig. 1). The similarity of the obtained sequences to those from various gene banks was low, which prevented us from affiliating them with any previously described species.

The nucleotide sequences of the order *Rhodocyclales* were most closely related to those of *Thauera* (three phylotypes, representative clones B-20, MB-23, and MB-33) and *Azoarcus* (one phylotype, clone B-16); one phylotype (clone MB-59) formed an isolated phylogenetic branch. The microorganisms of the order *Rhodocyclales*, including representatives of the genera *Thauera* and *Azoarcus*, are known to decompose various organic compounds, both under aerobic and anaerobic conditions (coupled to denitrification) [16, 17]. Thus, they may be involved in the degradation of residual organic matter in the nitrifying reactor and the DEAMOX reactor during denitrification. In addition, microorganisms closely related to *Thauera aromatica* were described (96% similarity of 16S rDNA sequences), which oxidized sulfide under anaerobic conditions [18].

The sequences of the representatives of the order *Nitrosomonadales* isolated from the reactor were similar to those of ammonium-oxidizing bacteria of the genus *Nitrosomonas* (clone MB-48). Two phylotypes (clones MB-30 and B-9) formed a separate cluster within this order. These bacteria probably arrived in the DEAMOX reactor from the nitrifying reactor. The role of nitrifying bacteria in the studied microbial community is probably not limited to nitrification. Bacteria *Nitrosomonas europaea* and *Nitrosomonas eutropha* are known to carry out complete conversion of ammonium to N_2 under anaerobic conditions, with nitrite as an electron acceptor [19].

A number of unknown representatives of the order *Burkholderiales* were detected among *Betaproteobacteria*. One phylotype (clone MB-22) was found to be most closely related the genera *Aquaspirillum* and *Acidovorax* of the family *Comamonadaceae*; five phylotypes (clones B-7, B-10, MB-9, MB-18, and MB-21) formed an isolated cluster. A group of closely related nucleotide sequences had previously been discovered when analyzing clone libraries obtained from wastewater [17] and the ANAMMOX community (clone MB-21; AB194898).

Among the sequences of *Betaproteobacteria*, the 16S rRNA gene (clone B-3) was detected, belonging to the cluster formed by the representatives of the genus *Thiobacillus* (family *Hydrogenophilaceae*). This group of microorganisms oxidizes sulfide under both aerobic (nitrifying reactor) and anaerobic conditions in the course of denitrification (DEAMOX reactor).

The remaining six proteobacterial clones formed five phylotypes belonging to the classes *Gamma-*, *Delta-*, and *Epsilonproteobacteria* (Fig. 1). One sequence of the class *Gammaproteobacteria* was most closely related to that of the genus *Nevskia* of the family *Xanthomonadaceae* (clone MB-3); the position of another one (clone MB-51) within this class was uncertain. Two phylotypes belonged to the class *Epsilonproteobacteria* (clones B-1 and MB-8); one phylotype (clone MB-57) was distantly related to the species *Desulfuromonas acetexigens* of the class *Deltaproteobacteria* (95% similarity).

All other 16S rDNA sequences of the bacterial species not belonging to the phylum *Proteobacteria* were grouped into 30 phylotypes of the phyla *Bacteroidetes, Chloroflexi, Firmicutes, Verrucomicrobia, Len-*

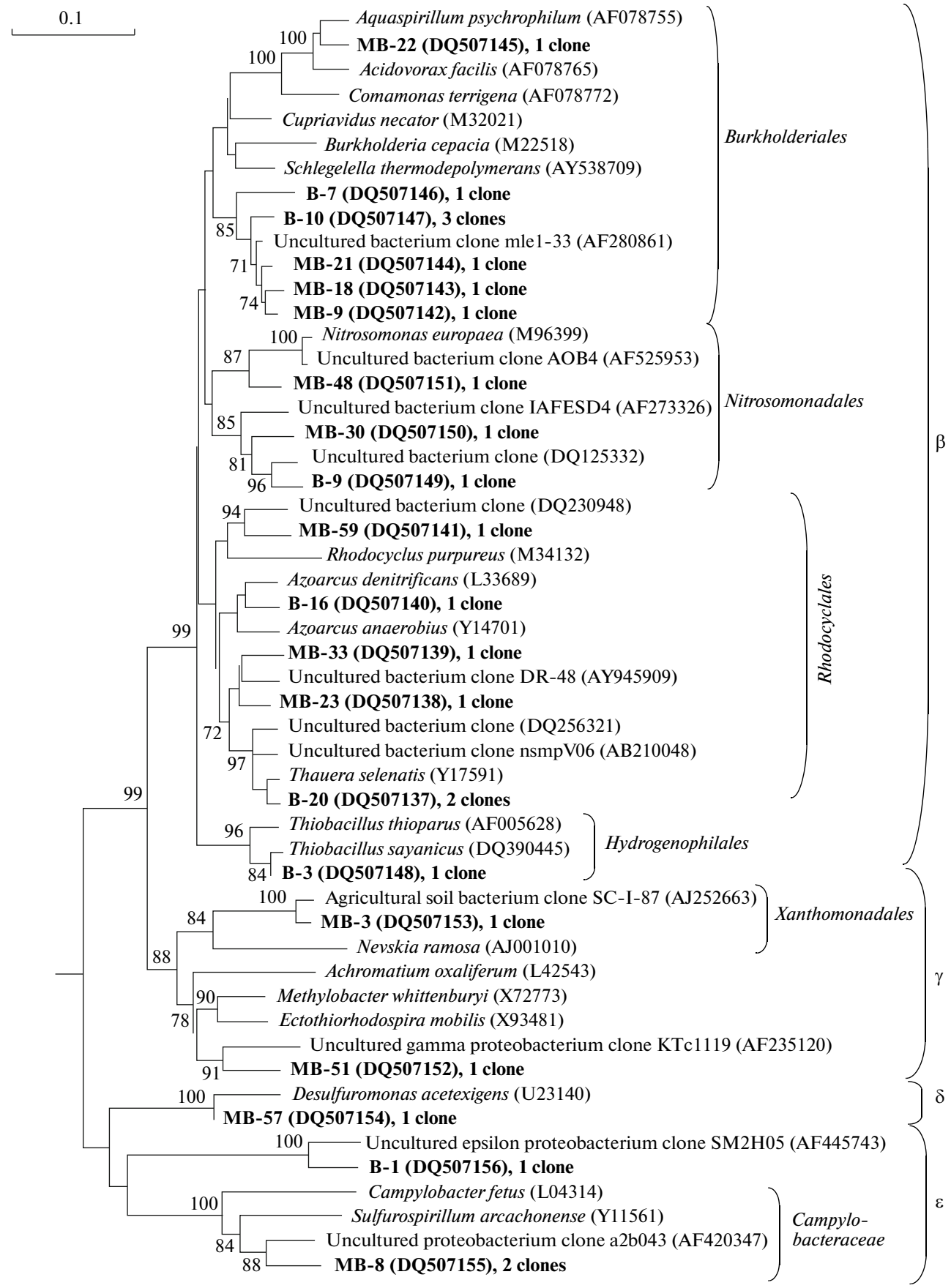
0.1
Aquaspirillum psychrophilum (AF078755)
MB-22 (DQ507145), 1 clone
Acidovorax facilis (AF078765)
Comamonas terrigena (AF078772)
Cupriavidus necator (M32021)
Burkholderia cepacia (M22518)
Schlegelella thermodepolymerans (AY538709)
B-7 (DQ507146), 1 clone
B-10 (DQ507147), 3 clones
Uncultured bacterium clone mle1-33 (AF280861)
MB-21 (DQ507144), 1 clone
MB-18 (DQ507143), 1 clone
MB-9 (DQ507142), 1 clone
Burkholderiales
Nitrosomonas europaea (M96399)
Uncultured bacterium clone AOB4 (AF525953)
MB-48 (DQ507151), 1 clone
Uncultured bacterium clone IAFESD4 (AF273326)
MB-30 (DQ507150), 1 clone
Uncultured bacterium clone (DQ125332)
B-9 (DQ507149), 1 clone
Nitrosomonadales
Uncultured bacterium clone (DQ230948)
MB-59 (DQ507141), 1 clone
Rhodocyclus purpureus (M34132)
Azoarcus denitrificans (L33689)
B-16 (DQ507140), 1 clone
Azoarcus anaerobius (Y14701)
MB-33 (DQ507139), 1 clone
Uncultured bacterium clone DR-48 (AY945909)
MB-23 (DQ507138), 1 clone
Uncultured bacterium clone (DQ256321)
Uncultured bacterium clone nsmpV06 (AB210048)
Thauera selenatis (Y17591)
B-20 (DQ507137), 2 clones
Rhodocyclales
Thiobacillus thioparus (AF005628)
Thiobacillus sayanicus (DQ390445)
B-3 (DQ507148), 1 clone
Hydrogenophilales
β
Agricultural soil bacterium clone SC-I-87 (AJ252663)
MB-3 (DQ507153), 1 clone
Nevskia ramosa (AJ001010)
Xanthomonadales
Achromatium oxaliferum (L42543)
Methylobacter whittenburyi (X72773)
Ectothiorhodospira mobilis (X93481)
Uncultured gamma proteobacterium clone KTc1119 (AF235120)
MB-51 (DQ507152), 1 clone
γ
Desulfuromonas acetexigens (U23140)
MB-57 (DQ507154), 1 clone
δ
Uncultured epsilon proteobacterium clone SM2H05 (AF445743)
B-1 (DQ507156), 1 clone
Campylobacter fetus (L04314)
Sulfurospirillum arcachonense (Y11561)
Uncultured proteobacterium clone a2b043 (AF420347)
MB-8 (DQ507155), 2 clones
Campylo-bacteraceae
ε

Fig. 1. Phylogenetic tree constructed on the basis of analysis of the 16S rRNA gene sequences of *Proteobacteria* revealed in the clone library of the microbial community carrying out anaerobic oxidation of ammonium nitrogen. Scale bar, ten nucleotide substitution per 100 nucleotide base pairs. The numerals at the branching points show the significance of the branching order as determined by bootstrap analysis of 100 alternative trees (only bootstrap values above 90% were considered significant).

tisphaerae, Spirochaetales, and *Planctomycetes* (Figs. 2 and 3) or were representatives of 11 unclassified bacterial groups.

Two phylotypes of the genus *Dysgonomonas* (clones B-28 and MB-38), as well as one phylotype of each of the genera *Chryseobacterium* (clone MB-43), *Sphingobacterium* (clone MB-26), *Chitinophaga* (clone MB-14), and *Flexibacter* (clones MB-11 and MB-47), were members of the phylum *Bacteroidetes*. Clone B-5 formed a separate branch with an uncertain position within this phylum. According to the published data, the presence of phylotypes belonging to the phylum

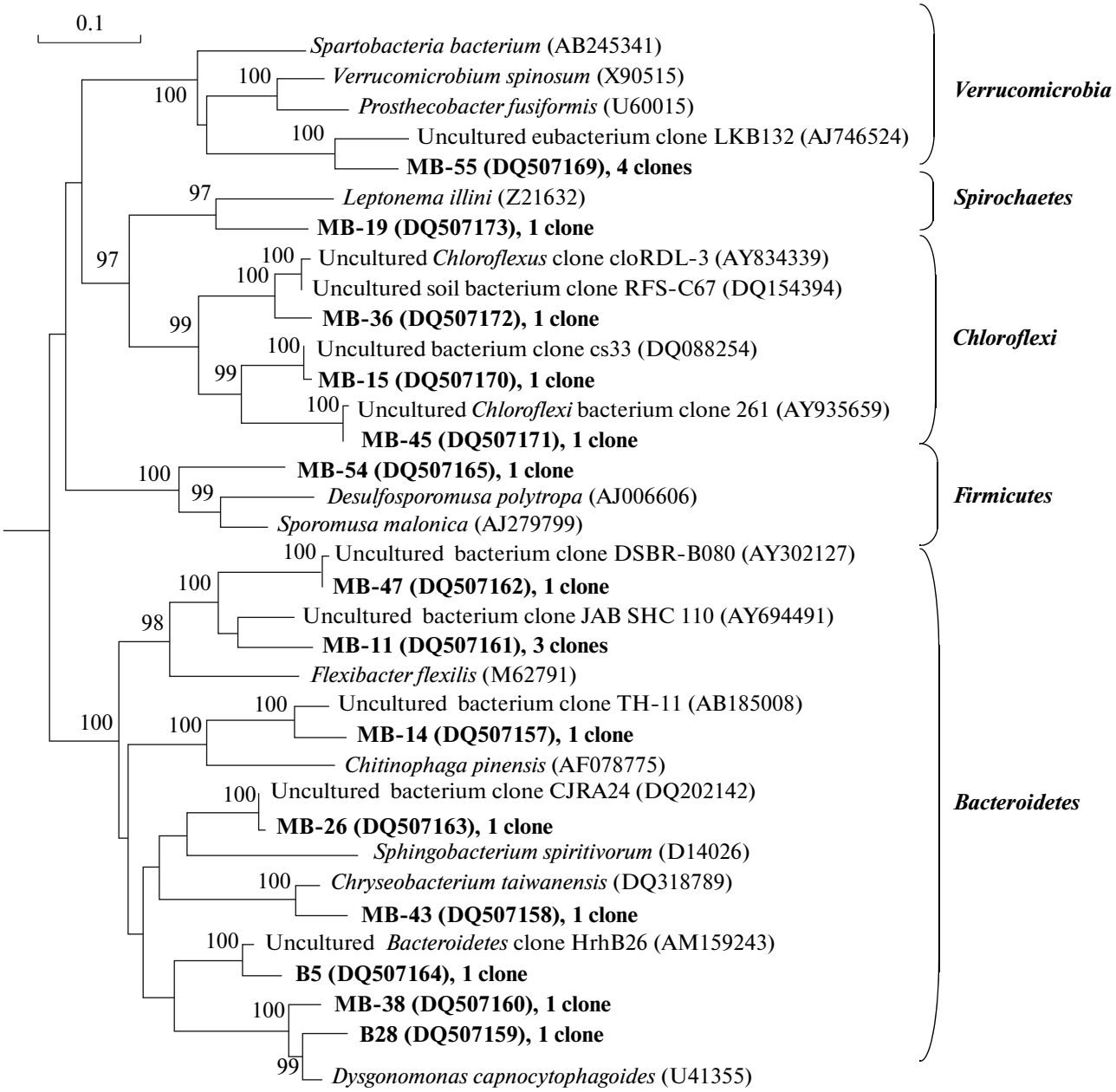

Fig. 2. Phylogenetic tree constructed on the basis of analysis of the 16S rRNA gene sequences of other members of the domain *Bacteria* revealed in the clone library of the microbial community carrying out anaerobic oxidation of ammonium nitrogen. Scale bar, ten nucleotide substitutions for each 100 nucleotide base pairs.

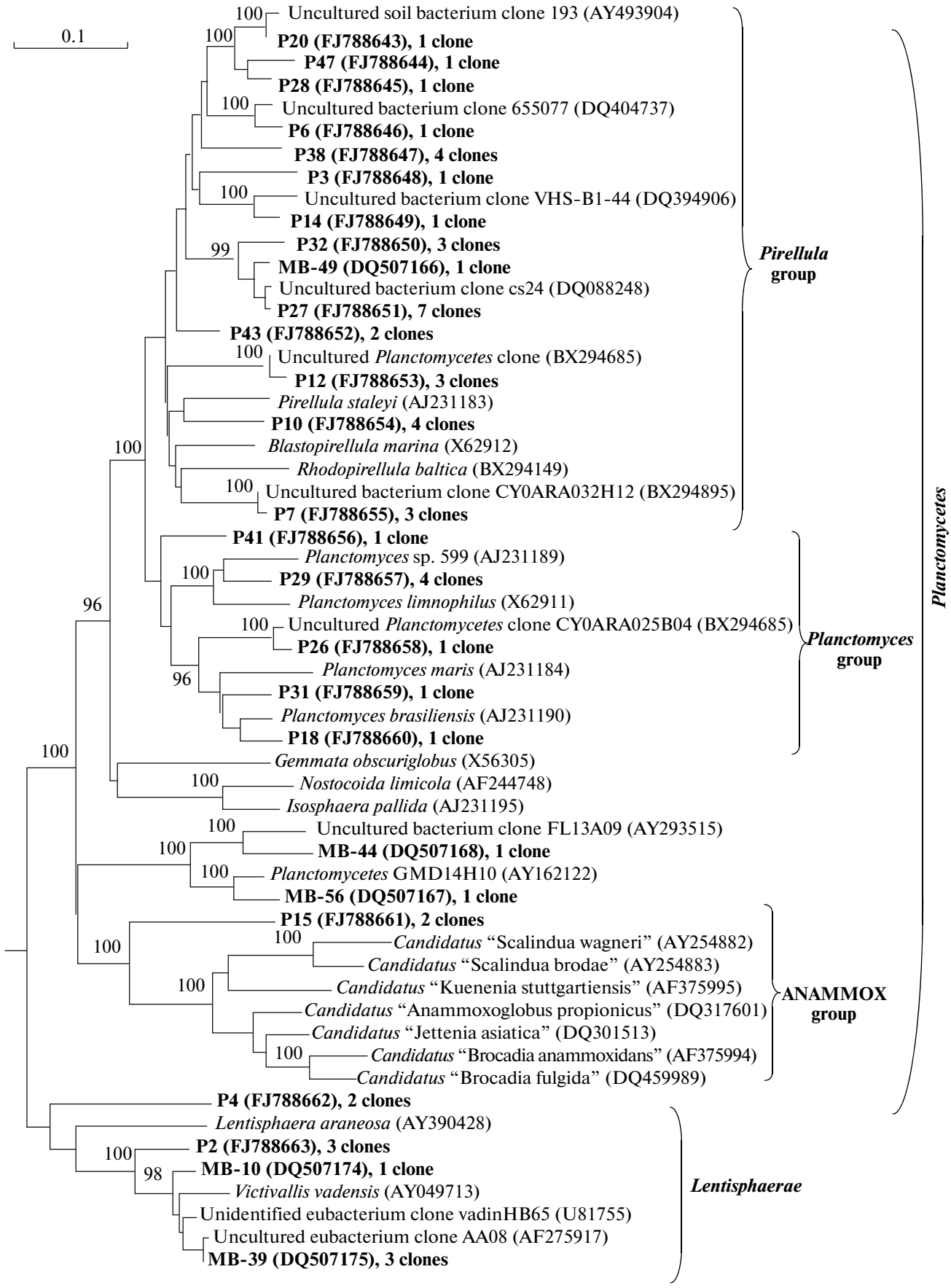
0.1
Uncultured soil bacterium clone 193 (AY493904)
P20 (FJ788643), 1 clone
P47 (FJ788644), 1 clone
P28 (FJ788645), 1 clone
Uncultured bacterium clone 655077 (DQ404737)
P6 (FJ788646), 1 clone
P38 (FJ788647), 4 clones
P3 (FJ788648), 1 clone
Uncultured bacterium clone VHS-B1-44 (DQ394906)
P14 (FJ788649), 1 clone
P32 (FJ788650), 3 clones
MB-49 (DQ507166), 1 clone
Uncultured bacterium clone cs24 (DQ088248)
P27 (FJ788651), 7 clones
P43 (FJ788652), 2 clones
Uncultured Planctomycetes clone (BX294685)
P12 (FJ788653), 3 clones
Pirellula staleyi (AJ231183)
P10 (FJ788654), 4 clones
Blastopirellula marina (X62912)
Rhodopirellula baltica (BX294149)
Uncultured bacterium clone CY0ARA032H12 (BX294895)
P7 (FJ788655), 3 clones
Pirellula group
P41 (FJ788656), 1 clone
Planctomyces sp. 599 (AJ231189)
P29 (FJ788657), 4 clones
Planctomyces limnophilus (X62911)
Uncultured Planctomycetes clone CY0ARA025B04 (BX294685)
P26 (FJ788658), 1 clone
Planctomyces maris (AJ231184)
P31 (FJ788659), 1 clone
Planctomyces brasiliensis (AJ231190)
P18 (FJ788660), 1 clone
Planctomyces group
Gemmata obscuriglobus (X56305)
Nostocoida limicola (AF244748)
Isosphaera pallida (AJ231195)
Uncultured bacterium clone FL13A09 (AY293515)
MB-44 (DQ507168), 1 clone
Planctomycetes GMD14H10 (AY162122)
MB-56 (DQ507167), 1 clone
P15 (FJ788661), 2 clones
Candidatus "Scalindua wagneri" (AY254882)
Candidatus "Scalindua brodae" (AY254883)
Candidatus "Kuenenia stuttgartiensis" (AF375995)
Candidatus "Anammoxoglobus propionicus" (DQ317601)
Candidatus "Jettenia asiatica" (DQ301513)
Candidatus "Brocadia anammoxidans" (AF375994)
Candidatus "Brocadia fulgida" (DQ459989)
ANAMMOX group
P4 (FJ788662), 2 clones
Lentisphaera araneosa (AY390428)
P2 (FJ788663), 3 clones
MB-10 (DQ507174), 1 clone
Victivallis vadensis (AY049713)
Unidentified eubacterium clone vadinHB65 (U81755)
Uncultured eubacterium clone AA08 (AF275917)
MB-39 (DQ507175), 3 clones
Lentisphaerae
Planctomycetes

Fig. 3. Phylogenetic tree constructed on the basis of analysis of the 16S rRNA gene sequences of other members of the phylum *Planctomycetes* detected in the clone library of the microbial community carrying out anaerobic oxidation of ammonium nitrogen. Scale bar, ten nucleotide substitution for each 100 nucleotide base pairs.

Bacteroidetes in a clone library is associated with denitrification [20].

Members of the phylum *Chloroflexi* utilize organic compounds during phototrophic growth and are capable of chemoorganotrophic growth; they are common inhabitants of activated sludge and various bioreactors. The clone library contained the sequences of representatives of the phylum *Chloroflexi* (3 phylotypes, clones MB-15, MB-45, and MB-36). Due to translucence of the material of laboratory reactors, the presence of phototrophs in the studied community cannot be ruled out. Phylotypes of phototrophic bacteria of the genus *Rhodocyclus*, which are capable of utilizing organic compounds in the course of photosynthesis, were retrieved, as well as those of bacteria of the genus *Ectothiorhodospira*, which utilize sulfide as the sole electron donor for photosynthesis, oxidizing it to sulfate via elemental sulfur that is then removed from the cells [clones MB-51 (*Gammaproteobacteria*) and MB-59 (order *Rhodocyclales*), Fig. 1]. The detection of these phylotypes provides an explanation for accumulation of sulfur on the walls of the UASB reactor, in addition to the chemical or bacterial (by members of the genus *Thiobacillus*) sulfide oxidation, which we have previously observed [9]. Detection of the 16S rRNA genes of anaerobic organotrophic sulfur-reducing bacteria of the genus *Desulfuromonas* (clone MB-57) and the family *Campylobacteraceae* (clone MB-8) is therefore also accounted for.

One clone, MB-54 (Fig. 2), belonged to the phylum *Firmicutes* and the genera *Sporomusa* and *Desulfosporomusa*. These bacteria probably arrived into the DEAMOX reactor from the UASB reactor. The representatives of both genera utilize organic acids, including formate and butyrate, alcohols, and some amino acids and carbohydrates. Bacteria of the genus *Desulfosporomusa* grow on these substrates, both with and without sulfate; they are capable of fermentation and autotrophic sulfate-reducing growth with H_2/CO_2 [21].

The clone library included the nucleotide sequences of members of the phyla *Spirochaetes* (one phylotype, clone MB-19, 90% similarity) and *Verrucomicrobia* (four clones, representative clone MB-55, 90–95% similarity); the latter are often retrieved in microbial communities of soils, freshwater and marine ecosystems, and hot springs. The detected *Lentisphaerae* phylotypes (representative clones MB-10 and MB-39, Fig. 3) were closely related to the phylotypes detected previously in the communities of anaerobic reactors [22]. The representatives of the orders *Victivallales* (phylum *Lentisphaerae*) include anaerobic organotrophic bacteria with fermentative metabolism.

Using the 8f-519r bacterial primers, we retrieved three types of 16S rRNA gene sequences of representatives of the phylum *Planctomycetes* belonging to the family *Planctomycetaceae* (clone MB-49) and to a new cluster (clones MB-44 and MB-56), the position within this phylum of which remains uncertain (Fig. 3).

A number of nucleotide sequences (16 clones) belonged to 11 groups that form new branches within the domain *Bacteria*. The sequences assigned to group 1 (six clones, representative clone B-8) and group 2 (clone B-17), were almost identical (99% similarity) to the 16S rRNA gene fragments revealed by analysis of the clone libraries of microbial communities from ANAMMOX wastewater reactors.

16S rRNA genes of representatives of the domain *Archaea*. Although the application of molecular techniques based on determination of nucleotide sequences of 16S rRNA genes makes it possible to determine the phylogenetic position of the constituents of a microbial community, it is seldom sufficient to determine their biological functions. Methanogenic archaea are among the rare microbial groups, for which their phylogenetic position indicates their pathways of energy production.

A total of 51 archaeal clones from the MA genome library were grouped into 13 phylotypes belonging to the phylum *Euryarchaeota*. Most of the phylotypes belonged to the orders *Methanomicrobiales, Methanosarcinales*, and *Methanobacteriales* (Table 2); in addition, two new putative *Euryarchaeota* groups were retrieved (Table 2, groups 1 and 2). The nucleotide sequences of hydrogen-utilizing methanogens of the orders *Methanomicrobiales* (16 clones) and *Methanobacteriales* (13 clones), as well as of acetate-utilizing methanogens of the order *Methanosarcinales* (15 clones, Table 2), were detected. Most sequences from the archaeal clone library were close to the sequences of the 16S rRNA gene fragments of the known *Archaea*.

The nucleotide sequences of hydrogen-utilizing methanogens of the order *Methanomicrobiales* belonged to six phylotypes (16 clones), some of which were closely related to the 16S rRNA gene sequence of *Methanospirillum hungatei* (three clones, representative clone MA-15, 99% similarity). The nucleotide sequences of the order *Methanobacteriales* (three phylotypes, 12 clones) exhibited 99% similarity to those of the 16S rRNA gene fragments of *Methanobacterium beijingense* (11 clones, representative clone MA-72) and *Methanobacterium subterraneum* (one clone, MA-20).

The 16S rRNA genes of organotrophic methanogens *Methanosaeta concilii* (13 clones, 99% similarity)

Table 2. Phylogenetic diversity of archaeal 16S rRNA gene fragments detected in the clone library of the microbial community carrying out anaerobic oxidation of ammonium nitrogen

Phylogenetic group	Representative clone of the phylotype	Phylogenetically most closely related sequences in the GenBank	Similarity, %	Accession number	Number of related clones
Methanomicrobiales	MA-15	*Methanospirillum hungatei* JF-1	99	CP000254	3
	MA-61	Uncultured *Methanospirillaceae* archaeon, clone LF-Eth-B	95	AB236101	1
	MA-74	Uncultured *Methanomicrobiaceae* archaeon, clone MP-H2-B	97	AB236085	1
	MA-8	Uncultured *Methanomicrobiaceae* archaeon, clone X4Ar27	98	AY607252	1
	MA-26	Uncultured archaeon, clone CG-6	99	AB233296	3
	MA-9	Uncultured *Methanomicrobiales* archaeon, clone SL-Pro-A	98	AB236081	7
Methanobacteriales	MA-72	*Methanobacterium beijingense* 4-1	99	AY552778	11
	MA-20	*Methanobacterium subterraneum* DSM 11075	99	X99045	1
	MA-4	Uncultured archaeon, clone OuI-11	99	AJ556498	1
Methanosarcinales	MA-43	*Methanosaeta concilii* Opfikon	99	X51423	13
	MA-75	*Methanomethylovorans* archaeon, clone 2H1	99	AY426485	2
Unclassified *Euryarchaeota*					
group 1	MA-5	Uncultured archaeon, clone 69-1	99	AF424763	4
group 2	MA-37	Uncultured archaeon, clone CG-8	99	AB233298	3

and *Methanomethylovorans* sp. (2 clones) were detected as well. In addition to $H_2 + CO_2$, members of the order *Methanosarcinales* are able to grow on acetate, methanol, and methylated amines. Cells of filamentous microorganisms morphologically similar to *Methanosaeta* were often present on the photographs of granules from the DEAMOX reactor [9]. According to the published data, aceticlastic methanogens *Methanosaeta* spp. and hydrogen-utilizing methanogens of the genera *Methanospirillum, Methanobacterium*, and *Methanobrevibacter* contribute to formation of the granules of anaerobic biomass [3, 23].

The metabolic properties of unclassified archaea (two phylotypes, seven clones) detected in the microbial community are as yet unidentified. Similar nucleotide sequences are often found during molecular biological studies of microbial communities from various natural and anthropogenic habitats.

16S rRNA genes of representatives of the phylum *Planctomycetes*. Representatives of the phylum *Planctomycetes* are presently believed to play an important role in both the ANAMMOX and DEAMOX processes. Using universal bacterial primers, we detected only three *Planctomycetes* phylotypes. It is well known that bacteria belonging to this phylum possess a number of distinctive features in their 16S rRNA gene structures [3–8]; therefore, even some known members of this phylum are not detected with universal bacterial primers [5].

To assess the diversity of members of the phylum *Planctomycetes* in the microbial community under study, analysis of the efficiency and specificity of a number of primers described in the literature [5, 8, 11, 24] was carried out; the optimal amplification conditions were determined. We failed to determine the *Planctomycetes* diversity in the microbial community using the bacterial primers 519r, 1390r, and 1492r. In this connection, amplification of the 16S rRNA gene fragments of planctomycetes was performed with the primers targeting this group of bacteria, Pla58f, Pla926r, Pla46f, and AMX820R. The synthesis of a specific PCR product was observed only when the Pla58f–Pla926r primer combination was used [11]. A total of 48 clones belonging to 21 phylotypes and representing two different phyla were obtained.

Analysis of the *Planctomycetes* clone library indicated considerable diversity of members of this phylum within the studied microbial community (19 phylotypes, Fig. 3), which is hardly accidental. Within this phylum, five phylotypes (clones P-18, P-26, and P-31) can be assigned to the genus *Planctomyces*. Within the *Pirellula–Blastopirellula–Rhodopirellula* group, 13 phylotypes formed separate branches. One phylotype (clone P15) belonged to the phylogenetic cluster of candidate species capable of anaerobic ammonium oxidation (ANAMMOX group) [8].

The 16S rRNA gene sequences retrieved in this analysis did not display any relatedness to the plancto-

mycete sequences that were previously discovered using universal bacterial primers (clones MB-49, MB-44, and MB-56), which confirms that the structure of the planctomycete 16S rRNA genes possesses some unique features.

Intriguingly, synthesis of the PCR product of about 500 bp was observed when the Pla46f–519r primer was used (data not presented). Analysis of the obtained sequences revealed their similarity to the various functional genes of the bacterium *Nitrosomonas europaea*. The 16S rRNA genes of the *N. europaea* cluster, were also detected with the universal bacterial primers. Nonspecific annealing of the primers for the ribosomal gene with the sequences of the other genes of *N. europaea* may indicate the predominance of this microorganism in the microbial community.

Thus, the obtained results of molecular biological investigations of the sludge samples collected from the third reactor in which microorganisms were involved in the DEAMOX process demonstrated the presence of microorganisms typical of the communities inhabiting the anaerobic (UASB) and nitrifying reactors (Table 1) that arrived to the DEAMOX reactor with the total runoff. The nucleotide sequences of 16S rDNA gene fragments of the main constituents of the microbial trophic chain in the UASB reactor, in which the bulk of organic matter from the yeast plant wastewater was destroyed, were detected both among the bacterial and archaeal clones.

This study demonstrated the complex composition of the microbial communities inhabiting the DEAMOX reactor. The 16S rRNA genes of the microorganisms carrying out the main reactions of the carbon, nitrogen, and sulfur cycles in the DEAMOX reactor (Table 1) were detected, which enabled us to formulate a general concept of the microbial trophic chain in the reactor. The majority of the *Planctomycetes* and *Verrucomicrobia* phylotypes detected in the clone libraries from the DEAMOX reactor were similar to those detected in the course of the analysis of the ANAMMOX communities isolated from wastewater reactors and anaerobic marine sediments [25]. The simultaneous presence of *Verrucomicrobia, Lentisphaera*, and *Planctomycetes* phylotypes was also observed in the clone libraries from the marine water samples collected in the suboxygenic zone of the Black Sea [11]. We believe that the DEAMOX reactions may also occur in natural ecosystems (in marine and freshwater sediments and the oceanic water column), thereby providing coupling of the nitrogen and sulfur cycles. The results of the recent studies of the genome of *Candidatus* "Kuenenia stuttgartiensis" isolated from the microbial community of a laboratory bioreactor suggest that ANAMMOX bacteria play an important role in the biological cycles of nitrogen, carbon, and metals [26].

As was noted above, almost no nitrite is found in the emitted flux of the DEAMOX reactor. This is possibly due to the syntrophic interspecific transfer of nitrite from sulfide-oxidizing denitrifiers to ANAMMOX bacteria. Such syntrophy was demonstrated for *Thioploca* and ANAMMOX-like bacteria from marine sediments [27]. In our case, such microorganisms as *Thauera* sp. (clones B-20, MB-23, MB-33) or *Thiobacillus* (clone B-3), as well as the putative ANAMMOX bacteria (clone P15) or new representatives of this group, may be involved in syntrophic growth. It is likely that both ANAMMOX bacteria and members of the genus *Nitrosomonas* are involved in anaerobic ammonium oxidation in the DEAMOX reactor [19]. This work is the first stage in a study of the composition of the microbial community inhabiting a DEAMOX reactor. To elucidate the key agent of the DEAMOX process, further microbiological and genetic studies are required (for example, FISH analysis).

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REFERENCES

1. Broda, E., Two Kinds of Lithotrophs Missing in Nature, *Z. Allgem. Mikrobiol.*, 1977, vol. 17, pp. 491–493.
2. Jetten, M., Schmid, M., van de Pas-Schoonen, K., Sinninghe-Damsté, J., and Strous, M., Anammox Organisms: Enrichment, Cultivation, and Environmental Analysis, *Methods Enzymol.*, 2005, vol. 397, pp. 34–57.
3. Anyusheva, M.G. and Kalyuzhnyi, S.V., Anaerobic Oxidation of Ammonium: Microbiological, Biochemical, and Biotechnological Aspects, *Usp. Sovrem. Biol.*, 2007, vol. 127, no. 1, pp. 34–43.
4. Kuypers, M.M.M., Sliekers, A.O., Lavik, G., Schmid, M., Jørgensen, B.B., Kuenen, J.G., Sinninghe, Damsté, J.S., Strous, M., and Jetten, M.S.M., Anaerobic Ammonium Oxidation by Anammox Bacteria in the Black Sea, *Nature*, 2003, vol. 422, pp. 608–611.
5. Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J.W., Schleifer, K.H., and Wagner, M., Molecular Evidence for Genus Level Diversity of Bacteria Capable of Catalyzing Anaerobic Ammonium Oxidation, *Syst. Appl. Microbiol.*, 2000, vol. 23, pp. 93–106.
6. Schmid, M., Walsh, K., Webb, R.I., Rijpstra, W.I., van de Pas-Schoonen, K., Verbruggen, M.J., Hill, T., Moffett, B., Fuerst, J., Schouten, S., Damsté, J.S., Harris, J., Shaw, P., Jetten, M., and Strous, M. *Candidatus* "Scalindua brodae", sp. nov., *Candidatus* "Scalindua wagneri", sp. nov., Two New Species of Anaerobic Ammonium Oxidizing Bacteria, *Syst. Appl. Microbiol.*, 2003, vol. 26, pp. 529–538.
7. Kartal, B., Rattray, J., van Niftrik, L., van de Vossenberg, J., Schmid, M.C., Webb, R.I., Schouten, S., Fuerst, J.A., Sinninghe Damsté, J., Jetten, M.S.M.,

and Strous, M., *Candidatus* "Anammoxoglobus propionicus", a New Propionate Oxidizing Species of Anaerobic Ammonium Oxidizing Bacteria*, Syst. Appl. Microbiol.,* 2007, vol. 30, pp. 39–49.

8. Schmid, M.C., Maas, B., Dapena, A., van de Pas-Schoonen, K., van de Vossenberg, J., Kartal, B., van Niftrik, L., Schmidt, I., Cirpus, I., Kuenen, J.G., Wagner, M., Sinninghe Damsté, J.S., Kuypers, M., Revsbech, N.P., Mendez, R., Jetten, M.S., and Strous, M., Biomarkers for in situ Detection of Anaerobic Ammonium-Oxidizing (Anammox) Bacteria, *Appl. Environ. Microbiol.*, 2005, vol. 71, pp. 1677–1684.
9. Kalyuzhnyi, S., Gladchenko, M., Mulder, A., and Versprille, B., DEAMOX—New Biological Nitrogen Removal Process Based on Anaerobic Ammonia Oxidation Coupled to Sulphide Driven Conversion of Nitrate Into Nitrite, *Water Res.*, 2006, vol. 40, pp. 3637–3645.
10. Weisburg, W., Barns, S.M., Pelletier, D.A., and Lane, D.J., 16S Ribosomal DNA Amplification for Phylogenetic Study, *J. Bacteriol.*, 1991, vol. 173, pp. 697–703.
11. Kirkpatrick, J., Oakley, B., Fuchsman, C., Srinivasan, S.T., Staley, J.T., and Murray, J.W., Diversity and Distribution of *Planctomycetes* and Related Bacteria in the Suboxic Zone of the Black Sea, *Appl. Environ. Microbiol.*, 2006, vol. 72, pp. 3079–3083.
12. Kolganova, T.V., Kuznetsov, B.B., and Tourova, T.P., Designing and Testing Oligonucleotide Primers for Amplification and Sequencing of Archaeal 16S rRNA Genes, *Mikrobiologiya*, 2002, vol. 71, pp. 283–285 [*Microbiology* (Engl. Transl.), vol. 71, no. 2, pp. 243–246].
13. Thompson, J.D., Higgins, D.G., and Gibson, T.J., CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Positions-Specific Gap Penalties and Weight Matrix Choice, *Nucleic Acids Res.*, 1994, vol. 9, pp. 3251–3270.
14. Saitou, N. and Nei, M., The Neighbour-Joining Method: A New Method for Reconstructing Phylogenetic Trees, *Mol. Biol. Evol.*, 1987, vol. 4, pp. 406–425.
15. Van de Peer, Y. and De Wachter, R., TREECON for Windows: A Software Package for the Construction and Drawing of Evolutionary Trees for the Microsoft Windows Environment, *Comput. Appl. Biosci.*, 1994, vol. 10, pp. 569–570.
16. Juretschko, S., Loy, A., Lehner, A., and Wagner, M., The Microbial Community Composition of a Nitrifying-Denitrifying Activated Sludge from an Industrial Sewage Treatment Plant Analyzed by the Full-Cycle rRNA Approach, *Syst. Appl. Microbiol.*, 2002, vol. 25, pp. 84–99.
17. Liu, B., Zhang, F., Feng, X., Liu, Y., Yan, X., Zhang, X., Wang, L., and Zhao, L., *Thauera* and *Azoarcus* as Functionally Important Genera in a Denitrifying Quinoline-Removal Bioreactors Revealed by Microbial Community Structure Comparison, *FEMS Microbiol. Ecol.*, 2006, vol. 55, pp. 274–286.
18. Cytryn, E.D., van Rijn, J., Schramm, A., Gieseke, A., de Beer, D., and Minz, D., Identification of Bacteria Potentially Responsible for Oxic and Anoxic Sulfide Oxidation in Biofilters of a Recirculating Mariculture System, *Appl. Environ. Microbiol.*, 2005, vol. 71, pp. 6134–6141.
19. Bock, E., Schmidt, I., Stuven, R., and Zart, D., Nitrogen Loss Caused by Denitrifying *Nitrosomonas* Cells Using Ammonium or Hydrogen as Electron Donors and Nitrite as Electron Acceptor, *Arch. Microbiol.*, 1995, vol. 163, pp. 16–20.
20. Hoshino, T., Terahara, T., Tsuneda, S., Hirata, A., and Inamori, Y., Molecular Analysis of Microbial Population Transition Associated with the Start of Denitrification in a Wastewater Treatment Process, *J. Appl. Microbiol.*, 2005, vol. 99, pp. 1165–1175.
21. Sass, H., Overmann, J., Rutters, H., Babenzien, H.-D., and Cypionka, H., *Desulfosporomusa polytropa* gen. nov., sp. nov., a Novel Sulfate-Reducing Bacterium from Sediments of an Oligotrophic Lake, *Arch. Microbiol.*, 2004, vol. 182, pp. 204–211.
22. Delbes, C., Moletta, R., and Godon, J.J., Monitoring of Activity Dynamics of an Anaerobic Digester Bacterial Community Using 16S rRNA Polymerase Chain Reaction-Single-Strand Conformation Polymorphism Analysis, *J. Environ. Microbiol.*, 2000, vol. 2, pp. 506–515.
23. Grotenhuis, J.T.C., Smit, M., Plugge, C.M., Yuansheng, X.U., van Lammeren, A.A.M., Stams, A.J.M., and Zehnder, A.J.B., Bacteriological Composition and Structure of Granular Sludge Adapted to Different Substrates, *Appl. Environ. Microbiol.*, 1991, vol. 57, pp. 1942–1949.
24. Neef, A., Amann, R.I., Schlesner, H., and Schleifer, K.-H., Monitoring a Widespread Bacterial Group: In situ Detection of Planctomycetes with 16S rRNA-Targeted Probes, *Microbiology* (UK), 1998, vol. 144, pp. 3257–3266.
25. Freitag, T.E. and Prosser, J.I., Community Structure of Ammonia-Oxidizing Bacteria within Anoxic Marine Sediments, *Appl. Environ. Microbiol.*, 2003, vol. 69, pp. 1359–1371.
26. Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W., Horn, M., Daims, H., Bartol-Mavel, D., Wincker, P., Barbe, V., Fonknechten, N., Vallenet, D., Segurens, B., Schenowitz-Truong, C., Médigue, C., Collingro, A., Snel, B., and Dutilh, B.E., Op den Camp, H.J., van der Drift, C., Cirpus, I., van de Pas-Schoonen, K.T., Harhangi, H.R., van Niftrik, L., Schmid, M., Keltjens, J., van de Vossenberg, J., Kartal, B., Meier, H., Frishman, D., Huynen, M.A., Mewes, H.W., Weissenbach, J., Jetten, M.S., Wagner, M., and Le Paslier, D., Deciphering the Evolution and Metabolism of an Anammox Bacterium from a Community Genome, *Nature*, 2006, vol. 440, pp. 790–794.
27. Prokopenko, M.G., Hammond, D.E., Berelson, W.M., Bernhard, J.M., Stott, L., and Douglas, R., Nitrogen Isotopes, Diagenesis and Possible Chemosymbiosis between Two Lithotrophs (*Thioploca* and *Anammox*)—"Riding on a Glider", *Earth Planet Sci. Lett*, 2006, vol. 242, pp. 186–204.