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EXPERIMENTAL ARTICLES

A Novel Bacterium Carrying out Anaerobic Ammonium Oxidation in a Reactor for Biological Treatment of the Filtrate of Wastewater Fermented Sludge

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Abstract—A new genus and species of bacteria capable of ammonium oxidation under anaerobic conditions in the presence of nitrite is described. The enrichment culture was obtained from the Moscow River silt by sequential cultivation in reactors with selective conditions for anaerobic ammonium oxidation. Bacterial cells were coccoid, ~0.4 × 0.7 μm, with the intracellular membrane structures typical of bacteria capable of anaerobic ammonium oxidation (anammoxosome and paryphoplasm). The cells formed aggregates 5–25 μm in diameter (10 μm on average). They were readily adhered to solid surfaces. The cells were morphologically labile: they easily lost their content and changed their morphology during fixation for electron microscopy. The organism was capable of ammonium oxidation with nitrite. The semisaturation constants Ks for nitrite and ammonium were 0.38 mg $N–NO_2$/L and 0.41 mg $N–NH_4$/L, respectively. The maximal nitrite concentrations for growth were 90 and 75 mg $N–NO_2$/L for single and continuous application, respectively. The doubling time was 32 days, $\mu_{max} = 0.022$ day^{-1}, the optimal temperature and pH were 20°C and 7.8–8.3, respectively. According to the results of 16S rRNA gene sequencing, the bacterium was assigned to a new genus and species within the phylum *Planctomycetes*. The proposed name for the new bacterium is *Candidatus* Anammoximicrobium moscowii gen. nov., sp. nov. (a microorganism carrying out anaerobic ammonium oxidation, isolated in the Moscow region).

Keywords: anaerobic ammonium oxidation, new bacteria, description, wastewater treatment

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Anammox bacteria capable of anaerobic ammonium oxidation with nitrite were discovered relatively recently, in 1999 [1]. Their existence had been predicted in 1977 based on analysis of the possible energy-gaining biochemical reactions [2]. Eight presently known species of anammox bacteria belong to five genera. They were retrieved from both natural environments (in marine and freshwater bottom sediments) and from waste treatment facilities [3]. These bacteria are unique in their ability to oxidize ammonium with nitrite with formation of a specific intermediate (hydrazine), as well as in the presence of specific lipids (ladderanes) [4] and of the intracellular membrane structure (anammoxosome) [5]. These bacteria exhibit more pronounced evolutionary divergence within the group than other bacterial groups [3]. The group was recently discovered, and new species are still emerging. As a rule, every new habitat is considered to harbor a single species [6]. This group is of importance due to its significant role in the nitrogen cycle in aquatic ecosystems (up to 35% in the oceans) [3, 7]. They are also found in wastewater treatment facilities (over a half of the known species have been isolated from the sludge of wastewater treatment facilities) [3], and have been used in technologies for ammonium removal from wastewater. The anammox technology was industrially implemented in about a dozen cases. Mosvodokanal Co. (Moscow, Russia) introduced this technology at the pilot (semiindustrial) level at Kur'yanovo waste water treatment plant (KWWTP, Moscow) [8, 9].

The goal of the present work was to investigate the bacteria responsible for anaerobic ammonium oxidation with nitrite in a bioreactor treating wastewaters with high ammonium concentrations (filtrate of the KWWTP wastewater fermented sludge).

MATERIALS AND METHODS

Enrichment culture of anammox bacteria was obtained in two stages. First, the sludge collected from the Moskva River bottom below the KWWTP outlet (500 g) was inoculated in a 50-L anaerobic reactor containing the filtrate of the KWWTP water fermented

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sludge (pH 7–8) supplemented with $N–NO_2$ (70 mg/L) and $N–NH_4$ (280 mg/L) [8]. Consumed nitrite was replenished to its original concentration. Intense consumption of equimolar amounts of nitrite and ammonium commenced after 254 days. The biomass was grown for the following 100 days. Afterwards, the cultivation was carried out in a flow mode. At this second stage, the sludge biomass obtained during the first stage (120 g) was used to inoculate a 61-L laboratory sequenced-batch reactor (SBR reactor) with complete biomass retention [8]. The reactor was fed with the filtrate of the KWWTP water fermented sludge (after methane fermentation at 55°C) (1–25 L/day) treated in a partial nitrification reactor. The filtrate contained the following: $N–NH_4$, 105 mg/L; $N–NO_2$, 135 mg/L; $N–NO_3$; 2 mg/L. The biological and chemical oxygen demand (BOD and COD were 18 and 65 mg/L, respectively, the molar ration of nitrite to ammonium was ~1.3, and the concentration of suspended matter did not exceed 10 mg/L. In the course of reactor operation, nitrogen load increased 13 times during 200 days, from 0.01 to 0.13 g $N–NH_4$ and $N–NO_2$/g volatile suspended solids, VSS) of the biomass per day. After 500 days of operation, the biomass was collected and transferred into a 3 m^3 semiindustrial installation [9]. The reactor was fed with partially nitrified filtrate of fermented sludge similar in composition to the one described above, but with up to 50 mg/L suspended matter. For phylogenetic, microscopic, and physiological investigation, as well as for analysis of the lipid composition, free-floating unattached activated sludge was collected directly from the laboratory flow reactor after 350–400 days of operation. The biomass of attached activated sludge was collected by scraping the biofilm with a rubber scraper, transferred into a flask, and homogenized by vigorous shaking.

Physiology of anammox bacteria. Standard techniques were used to determine the physiological parameters of bacteria responsible for anaerobic ammonium oxidation (growth rate, kinetic parameters, and temperature and pH optima). The growth rate was considered proportional to the rates of ammonium and nitrite consumption at their excess in the medium and was calculated for bacteria grown in the laboratory flow reactor with increasing substrate load. Specific rates of ammonium and nitrite consumption, as well as the semisaturation constants Ks, were calculated from the rates of decrease of their concentrations. The experiments were carried out in 100-mL glass Falcon vials under standard conditions (the biomass concentration was 0.9–1.1 g VSS/L). To prevent oxygen access into the reaction mixture, the vials were filled to capacity, bubbled with nitrogen, and sealed with rubber stoppers under nitrogen flow. All the content of a vial was used for each experimental point. The study was carried out at pH 7.8 and 19–22°C. The optima of temperature and pH were determined in a similar way. Specific activity of the activated sludge biomass was expressed in the amount of $N–NH_4$ or $N–NO_2$ metabolized by 1 g VSS per hour [mgN/(g activated sludge VSS h)].

Concentrations of components of the medium were determined by the standard techniques. NH_4 and NO_2 were determined photometrically with the Nessler Griess reagent, respectively, on a DR2010 spectrophotometer (Hach, United States). Suspended matter was determined gravimetrically (AC211S balance, Sartorius, Germany). Chemical oxygen demand (COD) was determined by the bichromate method. Biological oxygen demand was determined by the manometry (in Oxitop vials, WTW, Germany) according to [10].

Cell morphology was studied by phase and epifluorescence microscopy (Zeiss Axioplan 2 epifluorescence microscope, Germany) and by electron microscopy (JEM-100C transmission electron microscope, Jeol, Japan at 80 kV and ×20000 magnification). For electron microscopy, the activated sludge suspension and the preparations were treated as described previously [11]. Anammox bacteria were visualized and identified using the FISH technique (fluorescent in situ hybridization) with Cy3-labeled oligonucleotide probes Pla46 (specific for the whole group of *Planctomycetes*) and Amx368 (specific for the known anammox bacteria of the family *Brocadiaceae*) [12, 13]. The probe Amx148, which was developed for the novel bacteria described in the present work, was also used.

Lipid composition was determined by gas and liquid chromatography–mass spectrometry (GC–MS and HPLC–MS) on a 6890/5975N gas chromatograph–mass spectrometer (Agilent Technologies, United States) and an Acella/LTQ Velos liquid chromatograph–mass spectrometer, Thermo Fisher Scientific, United States). Identification of the compounds was carried out using the NIST08 and Wiley8n mass spectral libraries. The lipids were extracted from the cells of organisms of the activated sludge with dichloromethane–methanol mixture (1 : 1) or with methyl-*tret*-butyl ether after washing with methanol. The solvents were removed by evaporation, and fatty acid methyl and silyl ethers were prepared [14].

Molecular biological and phylogenetic investigation. Total DNA of the bacterial community of the activated sludge was isolated as was described previously [15]. Amplification of the 16S rRNA genes was carried out using the Univ11F–Univ1385R universal primer system [16]. Cloning of the amplified 16S rRNA gene fragments was carried out using the pGEM-T Easy system reagent kit (Promega, United States) according to the manufacturer's recommendations. Sequencing was carried out according to Sanger et al. [17] using the Big Dye Terminator v.3.1 (Applied Biosystems, Inc., United States) on an ABI PRIZM 3730 automatic sequencer (Applied Biosystems, Inc., United States). For cluster analysis, the sequences were aligned with the relevant sequences of

the most closely related bacteria using the CLUSTAL W software package [18]. The TREECONW software package [19] was used to construct phylogenetic trees.

RESULTS AND DISCUSSION

Enrichment culture of anammox bacteria. The sampling site for the inoculum for the enrichment was chosen based on the presence of both ammonium and nitrites (formed from ammonium in the course of denitrification) in the river water. Prolonged cultivation yielded the activated sludge with high specific anammox activity [9], confirming the choice of the sampling site.

Enrichment of the anammox bacteria in the sequence "enrichment reactor → laboratory flow reactor → semiindustrial flow reactor" resulted in increasing specific anammox activity of the sludge, which indicated an increase in the share of anammox bacteria. The rate of this process increased from the values below the sensitivity of the method [0.1 mg $N-NO_2 + N-NH_4$/(g dry bacterial biomass day)] in the first enrichment reactor to 1 g $N-NO_2 + N-NH_4$/(g dry bacterial biomass day) in the semiindustrial reactor.

FISH investigation of the activated sludge from the enrichment reactor and from both flow reactors revealed efficient staining only with the Pla46 probe for the *Planctomycetes,* while Amx368, the universal probe for all known anammox bacteria, yielded only weak staining [8]. This finding indicated that anammox bacteria different from the previously known ones were responsible for anaerobic ammonium oxidation in the reactors.

Low specificity of the Amx368 in the case of the anammox bacteria in the sludge under study made it necessary to develop a new probe.

Development of the FISH probe for detection of new anammox bacteria. The universal primer pair Univ11F–Univ1385R was used to obtain PCR fragments of the 16S rRNA genes. The primer pair Pla46F–Univ1390R yielded no PCR fragments suitable for cloning. The inserts of 150 clones from the library thus obtained were sequenced and compared to the known sequences from the GenBank database. According to BLAST analysis, two sequences, ANAMMOX Clone 20 and ANAMMOX Clone 26, belonged to the *Planctomycetes*. They were not related to any known species of anammox bacteria. Full-sized PCR fragments of these clones were sequenced. Testing with the Find Chimeras v. 1 software package (http://decipher.cee.wisc.edu/FindChimeras.html) revealed that the sequence ANAMMOX Clone 20 was a chimerical one; it was therefore excluded from further analysis. A dendrogram was constructed for the full-sized ANAMMOX Clone 26 sequence and the 16S rRNA genes of the known planctomycetes (Fig. 1). ANAMMOX Clone 26 formed a separate cluster with the sequence of the type species *Pirellula staleyi* ATCC27377 (M34126) located in the cluster of the 16S rRNA sequences of the family *Planctomycetaceae*. Homology with the *P. staleyi* sequence was 87.2%, while within the *Blastopirellula/Pirellula/Rhodopirellula* cluster the similarity was 84.5–92.5% (table). This value probably indicated that the sequence ANAMMOX Clone 26 belonged to a new species of planctomycetes. The sequence was deposited to GenBank under accession no. KC467065.

Based on the ANAMMOX Clone 26 nucleotide sequence, Cy-3-labeled probes were constructed. Their specificity was determined using the ProbeCheck database [20]. The probe for the clone 26 Amx148-24 (a 24-nucleotide probe for anammox bacteria, starting at no. 148) had the following structure: (Cy3)GGACCAAAGGTGTGATTCCGCCGG. This probe made it possible to discriminate between the new anammox bacteria and other clones obtained in the present study, as well as other known *Planctomycetes*. FISH investigation with the probe revealed groups of cells forming dense aggregates (microcolonies), which are typical of anammox bacteria (Fig. 2). The microcolonies of anammox bacteria were 5 to 25 μm in size, probably according to different stages of microcolony development (from young to older ones). Phase microphotographs revealed that larger microcolonies consisted of several small ones.

FISH analysis suggested that clone 26 contained the 16S rRNA fragment of the new anammox bacteria from the pilot installation. The nucleotide sequence of the 16S rRNA gene of clone 26 as a representative of a new species and genus of anammox bacteria was deposited to GenBank (KC467065).

Electron microscopy of the free-floating activated sludge from the semiindustrial reactor and of the biofilm from the feed and walls of this reactor revealed characteristic cell aggregates (microcolonies, Fig. 3a) similar to those of the previously described anammox bacteria [3, 6, 21]. Cell package in the microcolonies was moderately loose, so that they touched each other at 1–3 points, rather than adjoining with their whole surface. The intercellular matrix occupied about half of the photographed area.

Most of the cells were characterized by amorphous angular shape, which has been attributed to plasmolysis developing in the course of their fixation for electron microscopy [6]. Some of the cells were found outside the microcolonies (Fig. 3b). A small fraction of the cells retained the "normal" rounded-cell morphology (Fig. 3d). The cells contained extensive subcellular membranous structures dividing them into compartments and characteristic of the anammox bacteria (anammoxosome and paryphoplasm). Anammoxosome is an intracellular membrane structure occupying 30 to 60% of the cell volume. Paryphoplasm is extensive space between the outer and inner membranes, discernable as a ring on the photographs.

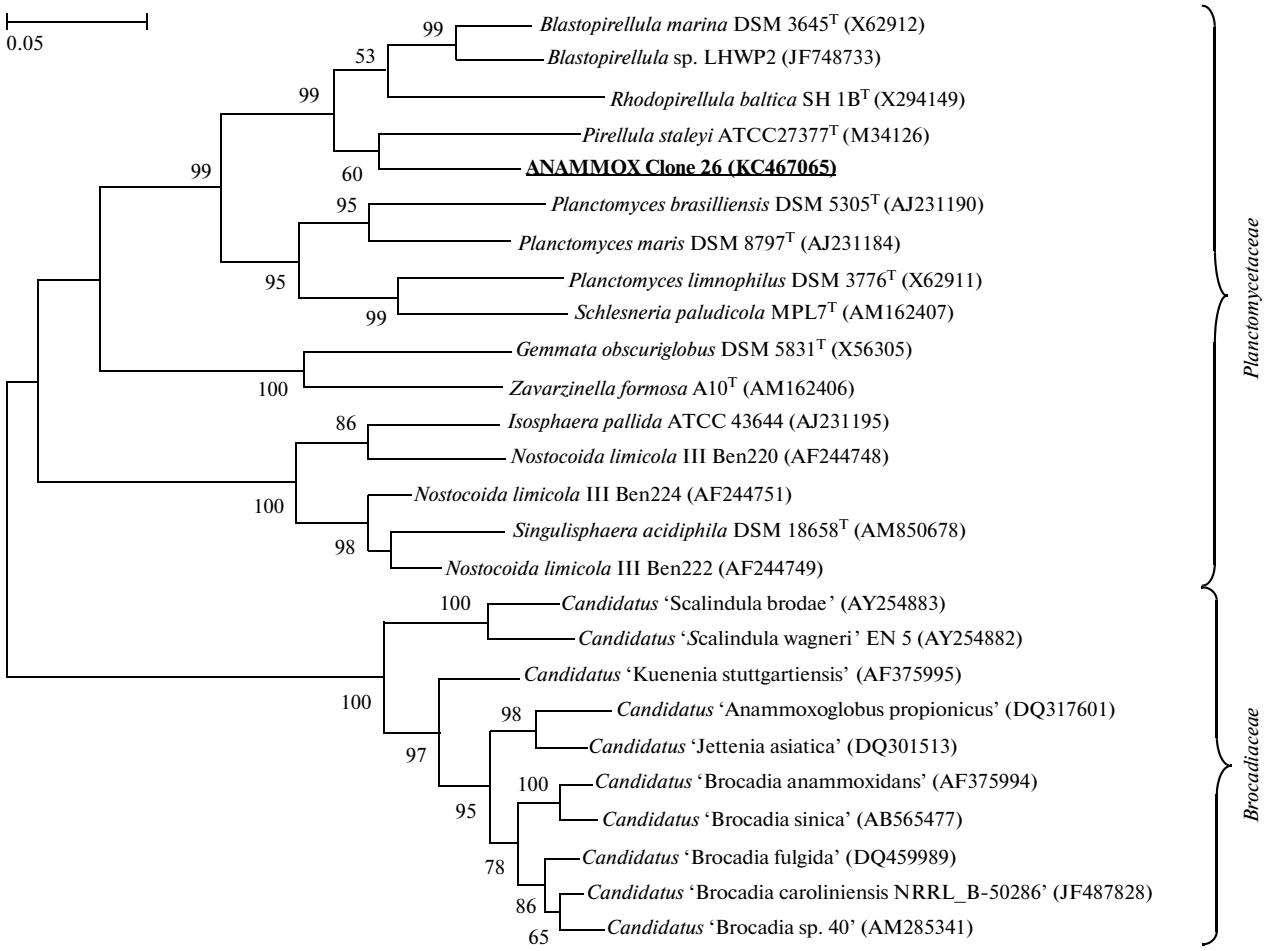

Fig. 1. Unrooted phylogenetic tree of the 16S rRNA gene sequences for the clone ANAMMOX Clone 26 and bacteria of the order *Planctomycetales*. The tree was constructed based on 1321 nucleotides of the16S rRNA gene and chosen out of 500 alternative trees. The numerals indicate the branching order. The name of the studied clone is in underlined boldface. The evolutionary distance scale is shown at the top. The maximum likelihood algorithm was used. Type strains are designated by a superscript T.

On the photographs, the cytoplasm proper, in anammox bacteria termed riboplasm due to the presence of ribosomes, was darker and occupied up to 50% of the cell area. A still darker nucleoid was discernable.

The microcolonies were surrounded with a single-layer envelope, which was of higher contrast than the matrix (Fig. 3a). The extracellular exopolymer matrix of the colonies was homogeneous, with fibrillar–vesicular structure (Fig. 3b).

About 70% cells in the preparations remained morphologically intact, while the remaining cells were partially or completely disturbed and looked like "sheaths" of the lysed cells (Fig. 3d). Lysed cells were more numerous in free-floating sludge, while the morphologically intact cells prevailed in the biofilm, in agreement with higher activity of the biofilm cells and with the reports on attachment as a requirement for successful growth of anammox bacteria [3].

Different morphologies of anammox bacteria from the same samples have been reported previously, specifically, characteristic shape of plasmolysed cells with a rigid outer envelope and compressed intracellular content and cells with soft envelopes and "crumpled" angular appearance [6]. This is in agreement with our findings.

Physiology of anammox bacteria. The main physiological characteristics of the new anammox bacteria were determined in a laboratory flow reactor.

Accumulation of anammox bacteria in the reactor resulted in the granules of activated sludge changing their color from black-brown to beige with a pink shade; some granules were red-orange. Red or orange color of the cytoplasm is typical of anammox bacteria, which contain high amounts of cytochromes [3]. In our experiments, granules of intense red or orange color occurred infrequently, since the incoming water always contained suspended matter, which masked the color of anammox bacteria.

The doubling time of anammox bacteria determined from the rates of ammonium oxidation and equimolar nitrite reduction (which are proportional to the biomass of the bacteria) was 32 days, which corre-

Similarity of the 16S rRNA gene sequences for bacteria of the *Pirellula* cluster and ANAMMOX Clone 26

	Blastopirellula marina DSM 3645T (X62912)	*Pirellula staleyi* ATCC27377T (M34126)	*Rhodopirellula baltica* SH 1BT (X294149)	*Blastopirellula* sp. LHWP2 (JF748733)	**Clone 26-2011**
Blastopirellula marina DSM 3645T (X62912)	1	0.845	0.847	0.925	0.856
Pirellula staleyi ATCC27377T (M34126)	0.845	1	0.827	0.855	0.872
Rhodopirellula baltica SH 1BT (X294149)	0.847	0.827	1	0.864	0.857
Blastopirellula sp. LHWP2 (JF748733)	0.925	0.855	0.864	1	0.853
Clone 26-2011	0.856	0.872	0.857	0.853	1

sponds to the growth rate of 0.02 day^{-1}. The semisaturation constants for $N–NH_4$ and $N–NO_2$ calculated using the Michaelis–Menten kinetics for conditions of an excess of one of the substrates were 0.41 and 0.38 mg/L, respectively. The limiting nitrite concentrations were 90 and 75 mg $N–NO_2$/L for one-time nitrite introduction and for a constant nitrite concentration, respectively. Irreversible inhibition of the process and cell death occurred at nitrite concentrations of 120 mg/L or higher. Anammox bacteria with the same [22], higher [23], and lower sensitivity to nitrite [24] have been described in the literature. In the presence of oxygen, the organism did not grow, oxidize ammonium, or reduce nitrite. Our results differ from those reported previously for anammox bacteria, which had three times higher growth rates, 400 times lower affinity constant, and 40% lower specific rate of the energy substrate transformation [24].

Importantly, bacteria from the activated sludge were capable of retained activity during prolonged survival in the absence of nutrient sources. For example, after 90 days without nutrients (substrate feed) to the

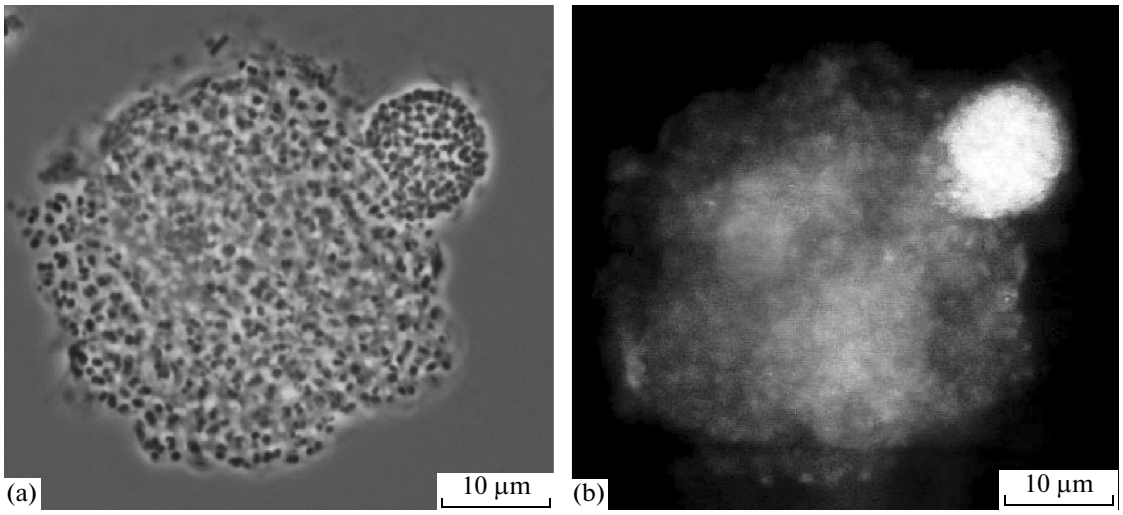

Fig. 2. Activated sludge from the laboratory flow reactor after 24 months of operation: phase contrast (a) and staining with the fluorochrome-labeled oligonucleotide probe Amx148-24 (b).

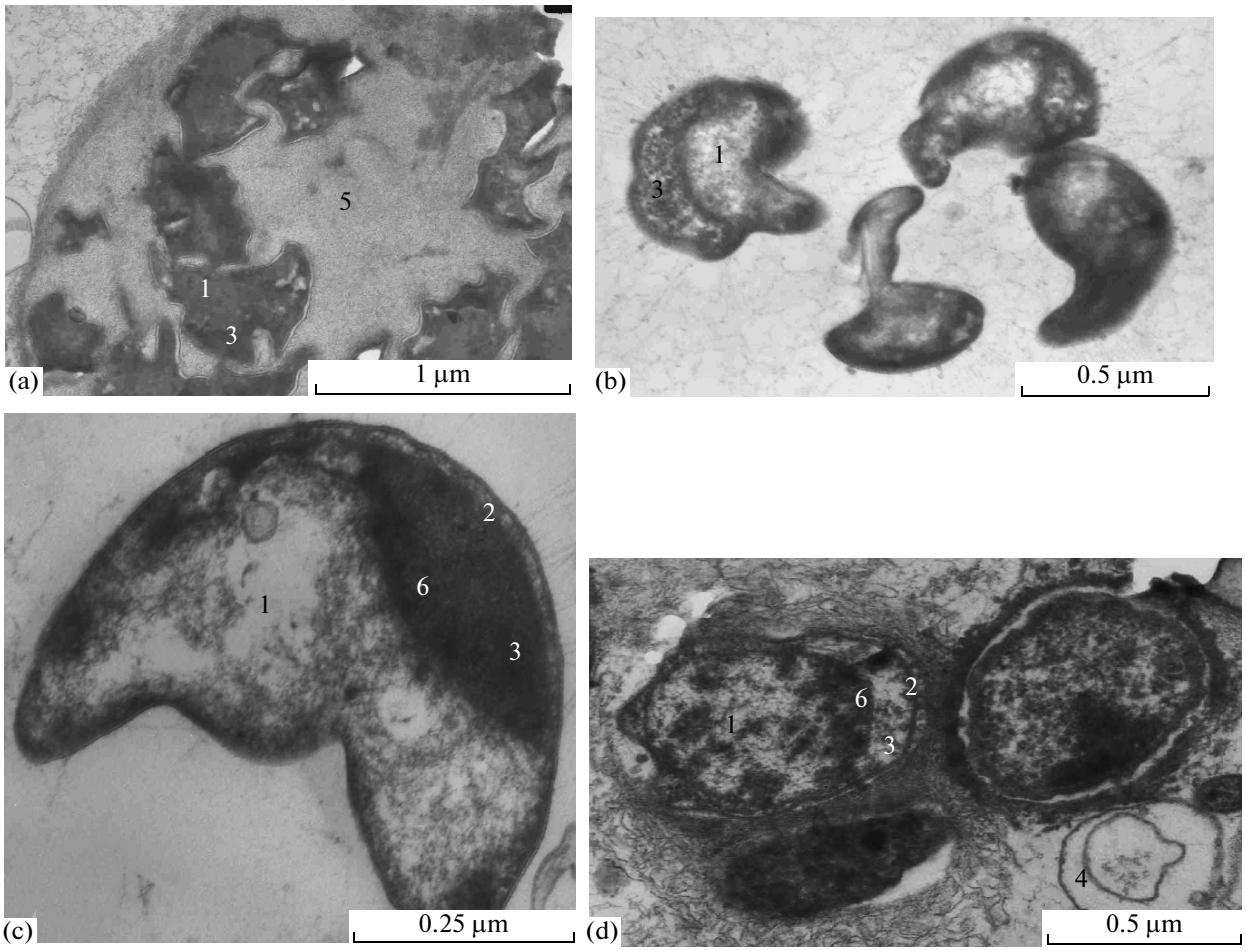

Fig. 3. Electron micrographs of the activated sludge (biofilm) from the semiindustrial anammox reactor: typical microcolony consisting of modified (plasmolysed) cells (a); individual cells outside the colonies (b); a plasmolysed cell (c); and cells of the regular shape from outside the colony, with well-pronounced structure (d). Numeric designations: anammoxosome (1), parypho-plasm (2), riboplasm (3), lysed cell with visible membrane structures (4), extracellular exopolymer matrix (5), and nucleoid (6).

laboratory flow reactor, the original rate of ammonium oxidation with nitrite was restored after two days.

Figure 4 shows that the temperature optimum for growth of anammox bacteria was 20°C, while optimal pH was 7.8–8.3.

In the course of cultivation of the activated sludge, the cells tended to adhere to the reactor walls, which is typical of anammox bacteria [3]. Specific activities of the biofilm and free-floating sludge were 36.5 and 1.4 mg $N–NH_4$/(g VSS h), respectively, indicating selective adhesion of anammox bacteria on solid surfaces.

Lipid composition. A chromatogram of fatty acid methyl ethers from the cells of the activated sludge of the laboratory flow reactor is presented on Fig. 5. The lipids were mostly represented by normal and *iso*-C_{14}–C_{18} fatty acids. Ladderane acids (C_7-[3]-ladderane and C_7-[5]-ladderane) constituted up to 5%.

GC–MS and HPLC–MS analyses of the activated sludge biomass revealed the presence of ladderane lipids, alcohols, and fatty acids characteristic for the anammox bacteria: C_{20}-[3]-ladderane alcohol, C_{20}-[5]-ladderane alcohol, C_{18}- and C_{20}-[3]-ladderane acid, C_{18}- and C_{20}-[5]-ladderane acid, as well as the ethers and esters of these compounds with phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol residues (identification will be presented in our forthcoming paper). The presence of these lipids was an indication of high abundance of anammox bacteria in the activated sludge. The properties of ladderane lipids and their ratio to regular lipids were similar to those described previously for anammox bacteria [4, 14].

Since the described microorganism capable of ammonium oxidation with nitrite was isolated from a new habitat, had a unique 16S rRNA gene sequence, and differed from the previously described members of this physiological group in a number of characteristics,

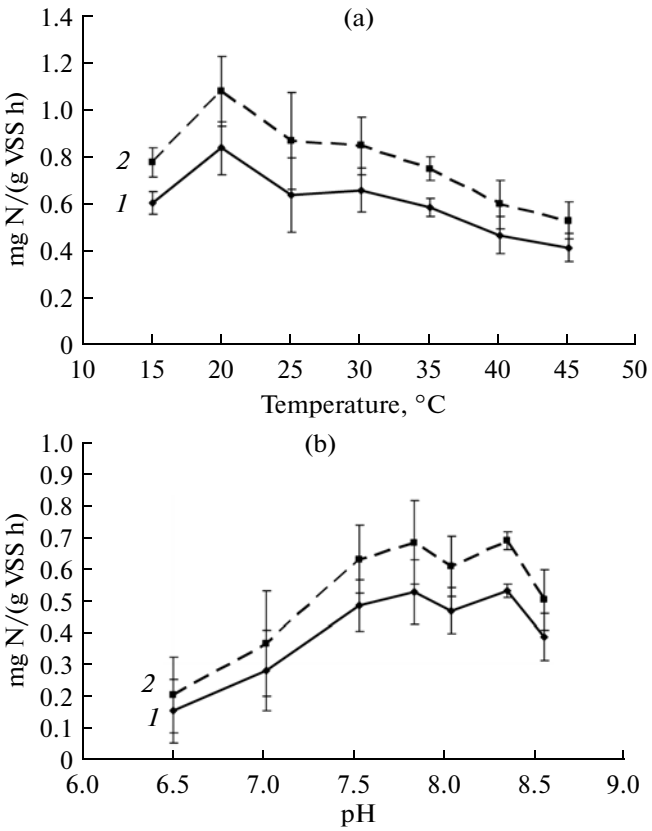

Fig. 4. Rates of ammonium (*1*) and nitrate (*2*) consumption by anammox bacteria of free-floating activated sludge at different temperatures (a) and pH (b).

it may be concluded that it is a new member of the *Planctomycetes* and may be assigned to a new genus and species. Since the bacterium was not isolated in pure culture, it can not be described as a new taxon in accordance with the requirements of the International Code of Nomenclature of Bacteria. [25]. The *candidatus* status has been provided for such occasions [26]. In accordance with the practice of description of such microorganisms, description of the new bacterial genus and species *Candidatus* Anammoxomicrobium moscowii gen. nov., sp. nov. (a microorganism carrying out anaerobic ammonium oxidation, isolated in the Moscow region) is presented below.

Bacteria have coccoid cells 0.4 × 0.75 μm in size. The cells contain the membrane structures typical of the anammox bacteria (anammoxosome and paryphoplasm); they occur in aggregates of 5 to 25 μm (10 μm on average), easily adhere to solid surfaces, and are morphologically labile (change their morphology and are easily lysed). The organism grows only under anaerobic conditions and is capable of ammonium oxidation with nitrite. The semisaturation constants are Ks = 0.38 mg $N–NO_2$/L for nitrite and Ks = 0.41 mg $N–NH_4$/L for ammonium; the doubling time under optimal conditions is 32 days (μ_{max} = 0.02 day^{-1}). The temperature and pH optimal for growth are 20°C and 7.8–8.3, respectively. The new organism belongs to the phylum *Planctomycetes*.

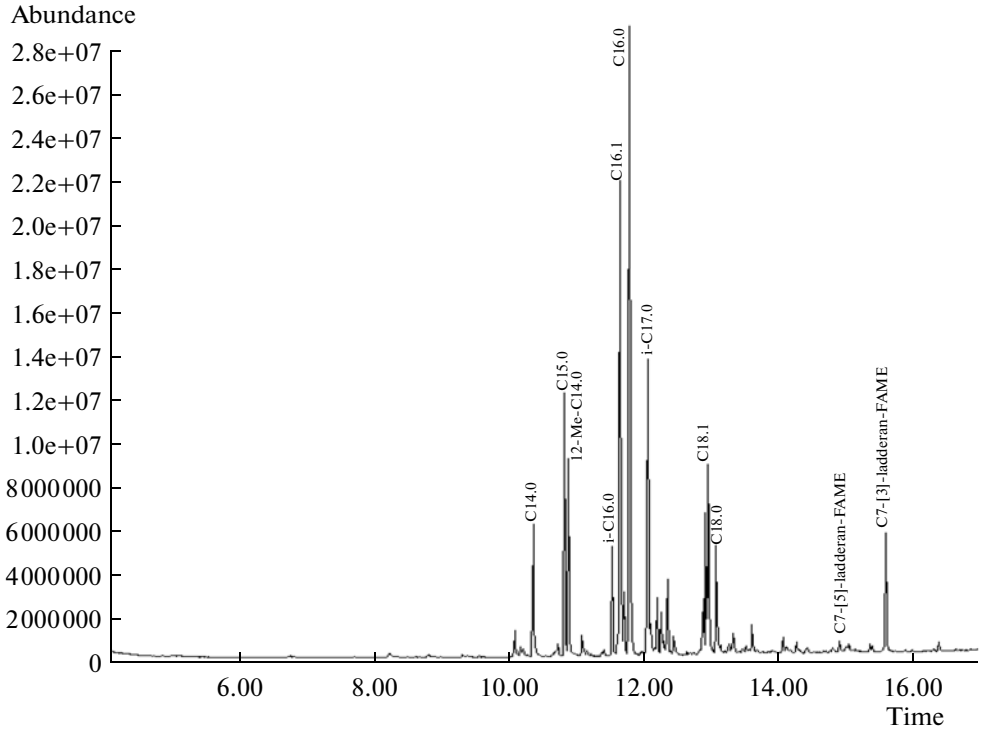

Fig. 5. Chromatogram of fatty acid methyl ethers (FAME) form the activated sludge recorded as full ionic current. The chromatogram reflects approximate ratios of the regular and ladderane fatty acids in the lipids.

The new bacterium is phylogenetically related to the *Pirelula* group, while physiologically it is similar to the family *Brocadiaceae*. Planctomycetes possessing anammox activity but not belonging to the family *Brocadiaceae* have not been described previously, although such possibility is in agreement with the data of [27], where in the anammox community of the sediment of a freshwater lake most of the clones characterized by their 16S rRNA gene sequences were different from the known *Brocadiaceae* species.

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