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EXPERIMENTAL ARTICLES

A Novel Filamentous Planctomycete of the *Isosphaera–Singulisphaera* Group Isolated from a *Sphagnum* Peat Bog

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Abstract—Planctomycetes are common inhabitants of northern wetlands. A significant proportion of these bacteria revealed in peat with the *Planctomycetes*-specific probes PLA46 and PLA886 is represented by filamentous forms which have not been cultured under laboratory conditions. In the present work, one of such organisms was isolated from a *Sphagnum* peat bog in a monoculture. The organism had large spherical cells assembled in long filaments. It could grow only in associations with bacterial satellites; attempts to isolate it in pure culture were unsuccessful. The organism was identified by PCR amplification, cloning, and subsequent analysis of its 16S rRNA gene fragment. Comparative sequence analysis revealed its affiliation with the *Isosphaera–Singulisphaera* group within the order *Planctomycetales*. The nucleotide sequence of the 16S rRNA gene of the new organism exhibited 94–96% similarity to those of the unicellular peat-inhabiting planctomycete *Singulisphaera acidiphila* and uncharacterized filamentous planctomycete "*Nostocoida limicola* III" from activated sludge. The new planctomycete utilized heteropolysaccharides of microbial origin as growth substrates and could grow at the low pH and temperatures typical of the northern wetlands.

Keywords: planctomycetes, filamentous forms, wetlands, *Singulisphaera, "Nostocoida"*

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Planctomycetes are a cosmopolitan but poorly studied phylogenetic group within the domain *Bacteria*, which has a number of unique morphological and ultrastructural characteristics [1–3]. These bacteria inhabit a broad range of natural environments, including aquatic and terrestrial ecosystems with diverse physicochemical conditions [2].

Recently, planctomycetes were detected in acidic northern wetlands [4–6]. The abundance of these organisms determined by in situ hybridization using the equimolar mixture of *Planctomycetes*-specific fluorescent probes PLA46 and PLA886 was up to 10^7 cells per gram wet peat, i.e., 2 to 14% of the total bacterial number [7]. The cells were of spherical or ellipsoid shape, in aggregates or multicellular filaments (chains). Further studies resulted in the isolation of pure cultures of a number of moderately acidophilic peat-inhabiting planctomycetes, which were described as new genera and species *Schlesneria paludicola, Singulisphaera acidiphila, Singulisphaera rosea*, and *Zavarzinella formosa* [8–11]. While the cells of these planctomycetes could form aggregates or, in some cases, rosettes, none of them were capable of forming long filaments. Among the known members of the *Planctomycetes*, only two have this kind of morphology: the thermophilic *Isosphaera pallida* from hot springs [12] and the taxonomically uncharacterized *"Nostocoida limicola"* from activated sludge [13]. Since both of these organisms were isolated from neutral environments, it was unlikely that they could occur in acidic wetlands. Therefore, the nature of the filamentous planctomycetes revealed in peat by oligonucleotide probes remained unclear.

This article presents characterization of the first filamentous planctomycete isolated from a *Sphagnum* peat bog. Its growth under laboratory conditions was possible only in an association with bacterial satellites. The goal of the present work was, therefore, to identify this organism within the community and to investigate its ecophysiological characteristics.

MATERIALS AND METHODS

Isolation and cultivation conditions. The planctomycete was isolated from acidic (pH 4.2) *Sphagnum* peat collected at a depth of 5–10-cm of the ombrotrophic peat bog Obukhovskoe (Yaroslavl oblast, Russia, 58°14′N, 38°12′E). The culture of the filamentous organism was obtained by plating the peat suspension on a mineral medium containing the following (g/L distilled water): KH_2PO_4, 0.1; $MgSO_4 \times 7H_2O$, 0.05; $(NH_4)_2SO_4$, 0.1; $CaCl_2 \times 2H_2O$, 0.01; trace element solution, 1 mL; vitamin solution [14], 1 mL; pH 4.8. As a solidifying agent, 1% solution of a microbial polysaccharide phytagel was used (Phyta-Gel, Fluka). The plates were incubated at 22°C for 6 weeks in a desiccator with 5% CO_2 generated by

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GENbox CO_2 system envelops (BioMerieux, France). The capacity of the filamentous planctomycete for anaerobic growth was determined by incubation in desiccators with AnaeroGen system envelopes (Oxoid). Growth characteristics of the culture were determined in liquid medium of the same composition under continuous microscopic control. The substrates tested as carbon and energy sources were added in the concentration of 0.5 g/L. Purification from bacterial satellites by plating on solid medium and by terminal dilutions in liquid medium was attempted.

Pigment composition. For analysis of the pigment composition, the culture was grown under light in liquid medium with 0.1% phytagel as a carbon and energy source. The suspension was concentrated by filtration through a 3-μm-pore-size membrane filter and the biomass was washed with sterile water to remove some of the bacterial satellites. Absorption spectra of the pigments extracted with the acetone–methanol mixture (7 : 2 vol/vol) within the 250–1000 nm wave range were obtained on an Sph-56 spectrophotometer.

Fluorescent in situ hybridization (FISH). The consortium composed of the filamentous organism and bacterial satellites was grown in liquid medium with phytagel and fixed for 1.5 h with 4% formaldehyde in phosphate buffer containing the following (g/L): NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.44; NaH_2PO_4, 0.2; pH 7.0. The cells were then collected by centrifugation and washed with the same buffer. Fixed cells were resuspended in a mixture of the phosphate buffer and 100% ethanol (1 : 1 vol/vol) and stored at –20°C. The fixed samples were spread on slides with wells and hybridized with the probes according to Shahl and Amann [15] at 46°C. Primary identification of the filamentous organism was carried out by hybridization with Eub338-mix, the equimolar mixture of Cy3-labeled probes specific to all members of the *Bacteria* domain [16]. For subsequent hybridization, the Cy3-labeled probes PLA46 + PLA886 specific to the phylum *Planctomycetes* [17] were used. The Cy3-labeled oligonucleotide probes were synthesized by Syntol (Moscow, Russia). The samples were analyzed using an Axioplan 2 epifluorescence microscope (Zeiss, Germany) with the Zeiss 20 and Zeiss 02 filters for CY3-labeled probes and detection of the cell autofluorescence, respectively.

Identification by polymerase chain reaction (PCR). Total DNA from the cells of a submerged culture of the planctomycete was extracted using the FastDNA SPIN kit for soil (Biol 101, United States) according to the manufacturer's recommendations. It was used as a template for polymerase chain reaction. PCR amplification of the fragment (~1350 bp) of the 16S rRNA gene of the planctomycete was carried out using the forward primer Pla46F (5'-GGATTAGGCATGCAAGTC-3'), which is highly specific to planctomycetes and the reverse universal bacterial primer Univ1390R (5'-CGGGCGGTGTCTACAA-3') [18]. The DNA of the planctomycete *Singulisphaera acidiphila* MOB10^T was used as a positive control. PCR was carried out on a PE GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied Biosystems, United States). The reaction mixture contained 1 μL DNA, 1 μL of the primers Pla46F and Univ1390R (at 30 pmol/μL), 50 μL MasterMix (Promega), and sterile water to 100 μL. The temperature profile was as follows: initial denaturing (1 min at 94°C); 35 cycles of denaturing (1 min at 94°C), primer annealing (1 min at 59°C), and elongation (1.5 min at 72°C); and final elongation (7 min at 72°C). PCR products were checked by electrophoresis on a 1.2% agarose gel and visualized under a UV transilluminator after being stained with ethidium bromide. The amplicons were cloned using the Gem-T Easy Vector System II kit (Promega) according to the manufacturer's recommendations. Recombinant clones were selected by amplification of the cloned fragments with the vector-specific primers T7 and SP6. Isolation and purification of the plasmid DNA was carried out using the Wizard® Plus Minipreps DNA Purification System (Promega). The nucleotide sequences were determined using the ABI 377A sequencer (Perkin-Elmer Applied Biosystems, United States).

The sequences were edited using the SeqMan software package (Laser Gene 7.0; DNA Star Package). Comparison of the sequences with those of the GenBank database was carried out using the Blast software package (http://blast.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using the ARB software package (http://www.arb-home.de). Statistical reliability of the branching order was determined by bootstrap analysis of 1000 alternative trees using the Phylip software package.

The 16S rRNA gene sequence of the filamentous peat-inhabiting planctomycete was deposited in GenBank, accession no. JQ067914.

The physiological characteristics of the filamentous isolate were determined by measuring CO_2 accumulation in the gas phase of the cultures growing in hermetically sealed 500-mL serum bottles with 100 mL of liquid medium. Dense suspensions of the filamentous microorganism were used as inocula. The suspension was obtained by filtration through 3-μm pore membranes, washing the filters with sterile medium, and resuspending the biomass in sterile water. Growth characteristics of the filamentous microorganism were determined within the temperature range from 4 to 37°C and pH range from 3.6 to 8.1. The suspension of bacterial satellites obtained by filtration of the culture was used as the control inoculum for analysis of the spectrum of utilized substrates (determined from the dynamics of CO_2 accumulation in the gas phase). The initial optical density of the culture in the control and experimental variants was 0.2 U. CO_2 content in the gas phase was determined on an INFRALYT 4 infra-

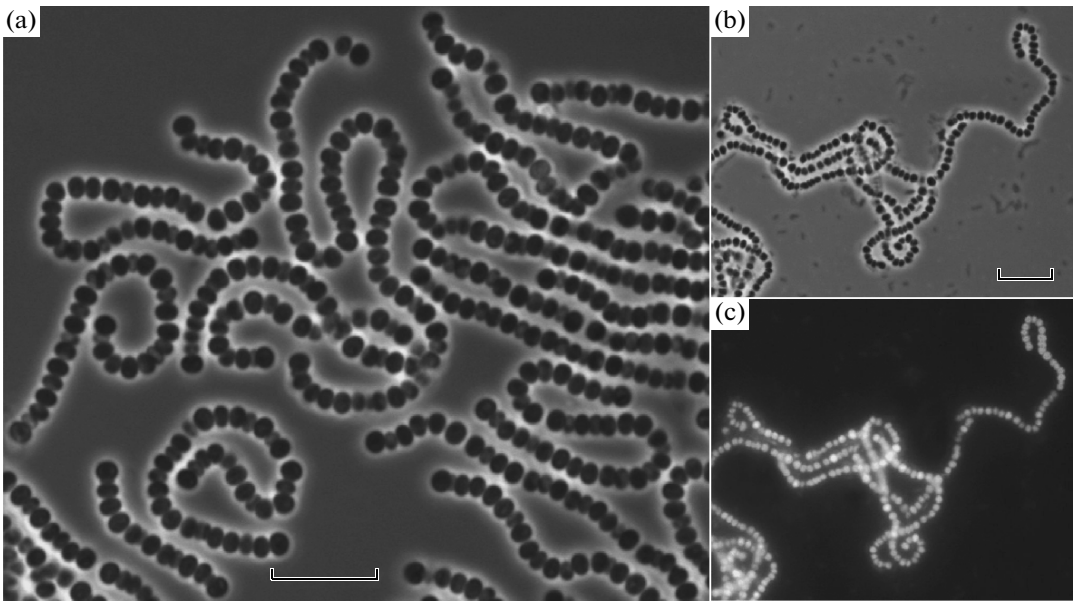

Fig. 1. Cell morphology of the filamentous planctomycete isolated from a *Sphagnum* peat bog, phase contrast (a); FISH identification: cell chain, phase-contrast image (b) and epifluorescent micrograph of whole-cell hybridization with the probes PLA46 + PLA886 specific to members of the phylum *Planctomycetes* (c). Scale bars, 10 μm.

red gas analyzer (Dessau, Germany). Sugars (glucose, cellobiose, and arabinose), salts of organic acids (pyruvate, succinate, and acetate), and polymeric compounds (phytagel, starch, pectin, and xylan) were tested as the growth substrates.

RESULTS AND DISCUSSION

Morphological and cultural characteristics of the filamentous microorganism. After 6 weeks of incubation of the mineral medium with phytagel inoculated with peat suspension, a lawn of nonpigmented bacteria developed, which contained pink, slightly convex colonies ~2 mm in diameter. Microscopic examination revealed that these colonies contained large (2–3 μm in diameter) spherical cells assembled in stable long filaments (Fig. 1a). Transfer of the colonies into liquid medium with phytagel as the carbon and energy source resulted in growth of the filamentous microorganism as light-pink aggregates composed of long chains (100 μm and more) of spherical cells. Growth of the filament occurred by budding of the terminal cells. The filamentous microorganism was nonmotile and had no gas vacuoles. Apart from this organism, small (0.5 to 1 μm long) bacterial satellites, both motile and nonmotile, developed in the culture; their number varied depending on the culture age and the growth substrate. In order to obtain pure cultures of the filamentous microorganism, three approaches were used, namely, plating on the surface of a solid medium, terminal dilutions in liquid medium with 0.1% phytagel, and cell collection on filters (3 μm pore diameter) with subsequent washing with sterile water to remove the small satellites and inoculating of the biomass to solid and liquid media. None of these attempts was successful. Growth of the filamentous microorganism occurred only in the presence of bacterial satellites, which had significantly higher growth rates. Thus, direct identification of this morphologically conspicuous peat-inhabiting microorganism was impossible.

Pigment composition. The presence of a pink pigment was one of the features differentiating the filamentous microorganism from bacterial satellites. Unlike chlorophyll-containing photosynthetic organisms, the filamentous isolate did not exhibit autofluorescence under UV illumination. Analysis of the absorption spectrum of the pigments extracted from the cells by acetone–methanol did not reveal chlorophylls or bacteriochlorophylls. Absorption maxima at 380, 460, 495, and 527 nm indicated the presence of carotenoids. Thus, although some cyanobacteria have similar cell morphology, they were excluded from consideration. Apart from cyanobacteria, some planctomycetes are the only known microorganisms forming chains of spherical cells [12, 13]. Molecular approaches were used to confirm the classification of the filamentous microorganism as a member of the phylum *Planctomycetes*.

FISH identification. Hybridization of the cells of the filamentous isolate with the Cy3-labeled probes Eub338-mix gave a positive result, confirming that this organism belonged to the *Bacteria* domain. At the next stage, Cy3-labeled probes PLA46 + PLA886 specific

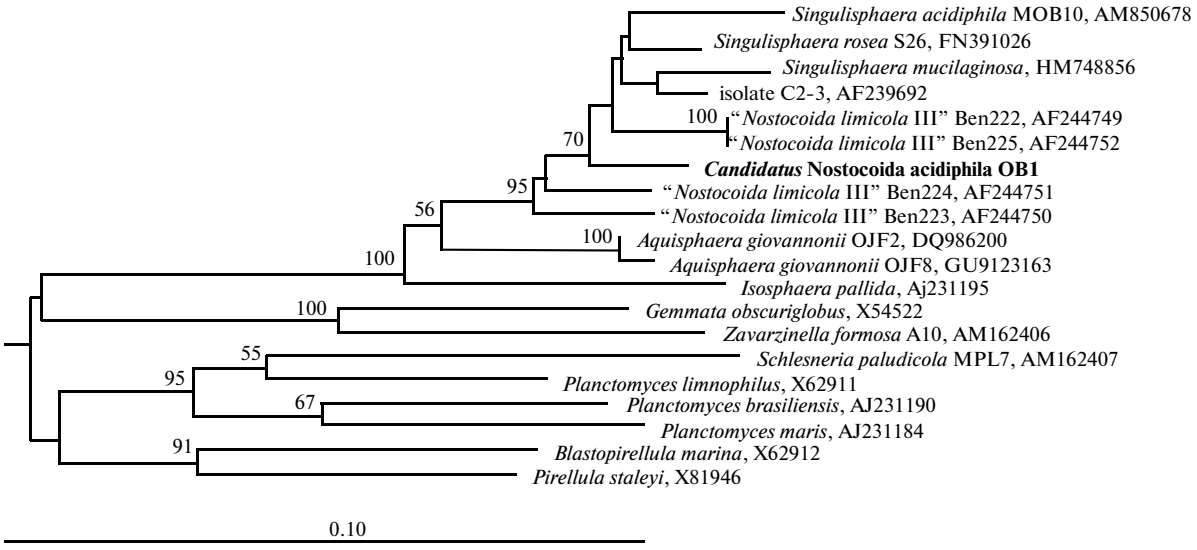

Fig. 2. Phylogenetic tree constructed based on comparative analysis of the 16S rRNA gene sequences of the filamentous peat-inhabiting planctomycete and other members of the *Planctomycetes*. The 16S rRNA gene sequences of five anammox planctomycetes (AF375994, AF375995, AY254883, AY254882, and AY257181) were used as an outgroup. The marker indicates 0.1 substitutions per nucleotide position.

to members of the phylum *Planctomycetes* were used [17]. The preparations hybridized with these probes displayed brightly fluorescent filaments of spherical cells (Figs 1b, 1c). Thus, preliminary identification of the filamentous isolate by means of FISH suggested that it belonged to planctomycetes.

PCR identification. PCR with the primers specific to members of the phylum *Planctomycetes* and the DNA of the culture yielded an amplicon of expected length (~1350 bp). An amplicon of the same length was obtained in the control PCR with *Singulisphaera acidiphila* MOB10^T DNA. The amplified fragment of the 16S rRNA gene of the culture was cloned and sequenced. Comparative analysis of the nucleotide sequence demonstrated that the filamentous bacterium belonged to the group of genera *Isosphaera–Singulisphaera* within the order *Planctomycetales* (Fig. 2). The 16S rDNA nucleotide sequence exhibited 95.0% similarity to that of the unicellular peat-inhabiting planctomycete *Singulisphaera acidiphila* and 94.8–96.3% similarity to the sequences of some taxonomically undescribed filamentous planctomycetes isolated from activated sludge and tentatively named "*Nostocoida limicola* III". The novel peat-inhabiting isolate exhibited 93.4–93.6% similarity to members of the genus *Aquisphaera* and only 89.3% similarity to the thermophilic filamentous planctomycete *Isosphaera pallida*.

Ecophysiological characteristics. Since all of our attempts to purify the culture of the filamentous peat-inhabiting planctomycete from bacterial satellites failed, we developed the cultivation conditions resulting in the highest possible proportion of the filamentous planctomycete within the association. Investigation of its growth within the community at different pH values showed that the filamentous planctomycete was acidophilic, growing well at pH 3.6–3.8 (Fig. 3a). Microscopic control confirmed its predominance in the community grown in acidic media, while neutral or alkaline conditions resulted in preferential development of the satellite bacteria. Further experiments on assessment of the physiological characteristics of the filamentous peat-inhabiting planctomycete were carried out in acidic media (pH 3.6–4.0) under continuous microscopic control of the development of the target organism.

Test on its response to oxygen and temperature showed that the filamentous planctomycete was a strictly aerobic mesophilic organism. Attempts to cultivate it under anaerobic or microaerobic conditions in a tube filled with phytagel medium or in a desiccator with AnaeroGen system envelopes were unsuccessful. The temperature range for growth was 10–30°C with the optimum at 25–28°C (Fig. 3b). Growth occurred in liquid media or on solid media with phytagel. No growth occurred on media with agar (or washed agar). Among the utilized substrates tested, most (yeast extract, sugars, organic acids, starch, pectin, and xylan) inhibited growth of the planctomycete even at low concentrations in liquid medium (0.05%). Heteropolysaccharides of microbial origin, phytagel (Fig. 4) and (to a lesser degree) xanthan, were the only substrates supporting growth of the peat-inhabiting planctomycete.

Thus, analysis of the characteristics of the new filamentous planctomycete revealed that it can not be

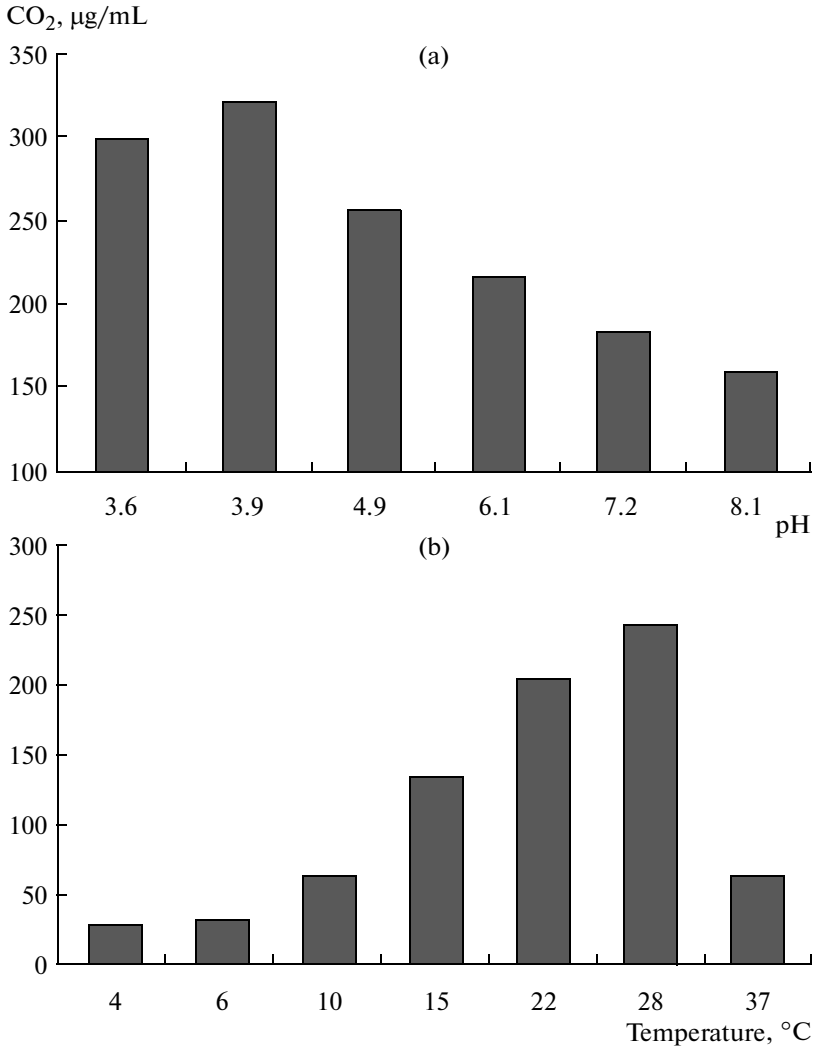

Fig. 3. Growth of the filamentous peat-inhabiting planctomycete depending on pH (a) and temperature (b).

assigned to the genus *Singulisphaera* due to significant differences in morphology and in the spectrum of utilized substrates. Phylogenetically and morphologically it is most similar to "*Nostocoida limicola* III", the taxonomically uncharacterized neutrophilic filamentous planctomycete isolated from activated sludge. Unlike the latter, however, it is acidophilic and does not use sugars, including arabinose, the preferred substrate of "*Nostocoida limicola* III" [13]. The ability of the studied filamentous planctomycete to grow on heteropolysaccharides of microbial origin suggests microbial biofilms and other structured microbial communities as some of its possible ecological niches.

Although we failed to obtain the new filamentous planctomycete in a pure culture, in accordance with the recommendations of the International Committee on Systematics of Prokaryotes [19], its morphological, physiological, and genetic characteristics make it possible to describe it as a candidate for a new species of planctomycetes, *Candidatus* "Nostocoida acidiphila".

Description of *Candidatus* "Nostocoida acidiphila" (a.ci.di'phila. N.L. n. *acidum* acid from L. adj. *acidus* acidic; Gr. adj. *philos* loving; N.L. fem. adj. *acidiphila* acid-loving). Pink-pigmented spherical cells 2–3 μm in diameter, which are assembled in long stable filaments (chains). Cells are nonmotile and contain no gas vacuoles. Carotenoids are present with absorption maxima at 380, 460, 495, and 527 nm. The organism is an aerobic mesophile and acidophile with growth optimum at 22–28°C and pH 3.6–4.0. Only heteropolysaccharides of microbial origin support growth: phytagel, Gellan-Gum (its commercial analogue), and xanthan. Acidic *Sphagnum* peat bogs are the main habitat.

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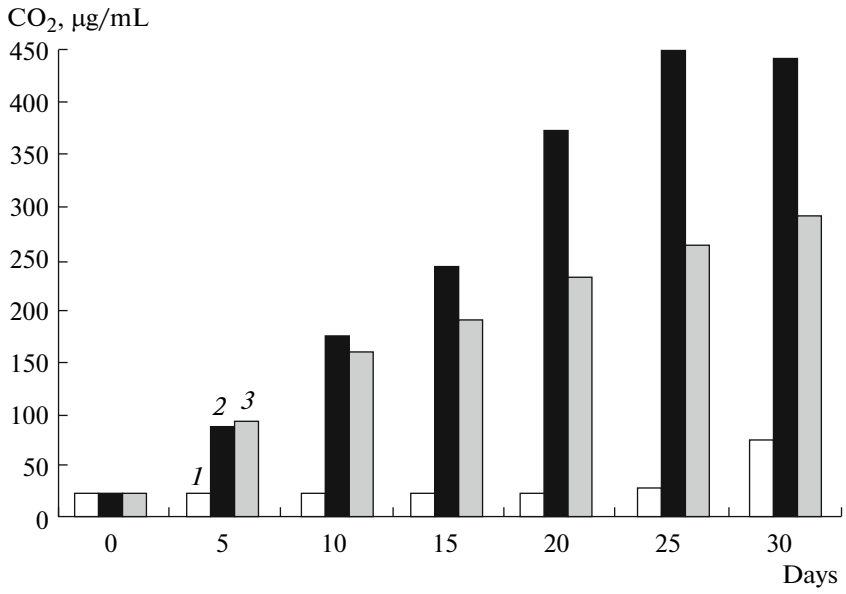

Fig. 4. Dynamics of CO_2 production by the consortium growing in liquid medium with phytagel: control (no phytagel) (*1*), growth of the planctomycete associated with bacterial satellites (*2*), and growth of bacterial satellites separated by filtration from the filamentous planctomycete (*3*).

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