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EXPERIMENTAL ARTICLES

Isolation of Bacteria of the Genus *Variovorax* from the *Thioploca* Mats of Lake Baikal

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Abstract—Three strains of gram-negative bacteria were isolated from the mats of colorless sulfur bacteria *Thioploca* (Lake Baikal). The cells of new strains are motile with peritrichous flagella. Bacteria are aerobic, obligate chemoorganoheterotrophs growing within the pH range of 3.0–8.8 with the optimum at 8.3 and within the temperature range of 5–42°C with the optimum at 28°C. The cells contained menaquinones MK-8 H2 as the major component, as well as MK-7 H2 (less than 15%), while the content of ubiquinone Q8 was at least an order of magnitude lower. The G+C content of DNA in the new strains varied from 67.4 to 69.9 mol %. The level of DNA–DNA hybridization between the strains ranged from 80 to 94%, indicating that all the isolates belonged to one species. Analysis of the 16S rRNA gene nucleotide sequences of the type strain (GenBank HQ400611) revealed close homologues among the known species of the genus *Variovorax*: 98% resemblance with the type strains of the species *V. paradoxus, V. soli, V. ginsengisoli,* and *V. boronicumulans* and 96% similarity with the type strain of *V. dokdonensis*. However, since the isolates differed significantly in the composition of fatty acids and isoprenoid quinones from the nearest neighbors in the phylogenetic tree, they cannot be related implicitly to the known species.

Keywords: Frolikha Bay, Lake Baikal, mats of colorless sulfur bacteria of the genus *Thioploca*, bacteria of the genius *Variovorax*, phylogeny.

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Colorless sulfur bacteria of the genus *Thioploca* inhabiting both marine and freshwater ecosystems are characterized by a unique metabolism. Bacteria migrating actively between sulfide-enriched deep sediment and nitrate-enriched surface layer couple sulfide oxidation to nitrate reduction [1, 2].

Bacteria of the genus *Thioploca*, in spite of their large size, remain a poorly studied group of microorganisms, mainly because of the absence of pure cultures, which can be isolated only on the basis of the knowledge of the *Thioploca* habitats in natural ecosystems and peculiar metabolic properties of the associated bacteria.

Colorless sulfur bacteria of the genus *Thioploca* were revealed in Lake Baikal [3]; the studies of their species diversity and ecological physiology showed that the habitats of *Thioploca* mats were characterized by an abundance of plant debris and continuous inflow of hydrothermal subterranean fluids of unusual composition. *Thioploca* mats were found to contain thiobacilli, methane-oxidizing, and sulfate-reducing bacteria [4].

The common sheath is an important structure of the representatives of the genus *Thioploca* bacteria which is of great ecological importance. It is assumed that organic material forming the sheath represents a reserve substrate [5].

A number of publications is known concerning the associations of *Thioploca* spp. with other bacteria [1, 5–10]; most of them deal with bacterial associations with sheaths of marine *Thioploca* species [1, 6–8]; however, there are few reports about species inhabiting freshwater or brackish waters [5]. For example, the symbiosis of *Thioploca* sp. bacteria with sulfate-reducing bacteria *Desulfonema* spp. and lithotrophic bacteria similar to Anammox was reported [6]. Filamentous sulfate-reducing bacteria (SRB) of the genus *Desulfonema* were often found attached to the sheath of *Thioploca* [5, 7]. *Thioploca* sheaths are a reserve substrate for SRB and promote their migration together with *Thioploca* in order to find the optimal conditions for their development in the bottom sediments, enriched with available sulfates and intermediate reduced sulfur compounds [1]. Bacteria of the genus *Thioploca*, in addition, obtain organic substances as carbon sources and sulfide as an energetic substrate, which is extremely important for deep layers where sulfide concentration is low [1].

Lithotrophic bacteria of the Anammox group are often associated with *Thioploca* spp. Symbiotic relations between *Thioploca* and anaerobic methane-oxidizing (anammox) bacteria have been confirmed by

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both geochemical data and results of analytical modeling [6, 9, 10]. The chemolithoautotrophic sulfur-oxidizing bacterium *Thiomicrospira chilensis* sp. nov. has been also isolated from a *Thioploca* spp. mat (sediment of the continental shelf, Chile) [10]. Organotrophic bacteria of the phylum *Bacteroidetes* and the *Desulforhopalus/Desulfobulbus* cluster were revealed in the bacterial community associated with the sheaths of *Thioploca* spp. from freshwater Lake Biwa (Japan) and Lake Constance (Germany) and from the brackish Lake Ogawara (Japan). It was assumed that these bacteria used the sheath substances as a growth substrate [5].

The aforementioned information was concerned to the organisms associated with an outer surface of the *Thioploca* sheaths, whereas no data is available for bacteria occurring inside the sheaths.

The goal of the present work was to isolate and characterize the bacteria occurring inside the sheaths of freshwater bacterium *Thioploca* spp. from Lake Baikal.

MATERIALS AND METHODS

Source of isolation and methods of cultivation. The samples of bottom sediments from Lake Baikal (Frolikha Bay) were collected in late August–early September 2003 using sample collectors (Okean grab, benthic tube) during the R/V *Vereshchagin* expeditions from a depth of 430 m, from the areas where in this period mass development of colorless sulfur bacteria of the genus *Thioploca* was observed. The *Thioploca* mats were easily visually identified, as large accumulations of bacteria were formed in the surface sediment layers; the sheaths with snow-white trichomes were strongly encrusted with iron salts and covered with numerous bacterial overgrowth. The filaments were up to 2 cm long.

The concentrations of sulfide, oxygen, and bicarbonate were determined by the standard methods [11]. The temperature, pH, and Eh were measured with portable devices: a Prima sensory electric thermometer (Singapore), a PRO pH meter (Singapore), and an ORP redox potential meter (Portugal).

Before inoculation, the overgrowths were removed from the sheath surface with a sealed end of a Pasteur pipette; the sheaths were washed in the drops of sterile Lake Baikal water in petri dishes. The filaments were carefully disengaged from the sheaths with a sharp end of a Pasteur pipette and washed quickly in sterile Lake Baikal water to prevent their contact with air. Since the filaments in sheaths were interwoven, bundles of them were used. Test tubes with the medium were inoculated with 10 trichomes of *Thioploca*.

To obtain enrichment cultures, bacteria were cultivated in a medium based on the *Macromonas* medium [12], containing the following (g/L): $NaNO_3$, 0.1; $CaCl_2$, 0.1; sodium acetate, 1.0; acidic casein hydrolysate, 0.1; yeast extract (Difco, United States), 0.1; fermented silt, 1 mL; agar (Difco, Unites States), 1.0; the solution of vitamins and trace elements according to Pfennig [13], 1 mL; distilled water, 1 L. Initial pH was adjusted to 8.1 with a 10% solution of $NaHCO_3$. The medium was dispensed into the tubes containing 0.2 g of FeS. The same medium with agar (14 g/L) was used for the isolation of the strains on petri dishes.

Morphological and physiological characteristics. Morphology of the cultures was studied under a phase contrast microscope (Carl Zeiss, Jena, GDR). The size of living cells was measured with an ocular micrometer; the cell structure was examined under a JEM-100 electron microscope (JEOL, Japan) at accelerating voltage of 80 kV [14, 15].

The ability of bacteria to utilize various sulfur compounds as electron donors was determined in the liquid medium containing the following (g/L): NH_4Cl, 0.1; $CaCl_2$, 0.1; K_2HPO_4, 0.022; KH_2PO_4, 0.017; sodium acetate and lactate, 0.1; D-glucose, 0.2; the solution of vitamins and trace elements according to Pfennig [13], 1 mL; distilled water, 1 L. Initial pH was adjusted to 8.1 with a 10% solution of $NaHCO_3$. The medium was supplemented with 0.03% of one of the sulfur compounds tested: thiosulfate, sulfide, sulfite, and sulfate (as control).

Utilization of reduced inorganic sulfur compounds was determined by iodometric titration [16].

The ability of bacteria to utilize various nitrogen sources was determined using the aforementioned liquid medium supplemented with $Na_2S_2O_3 \cdot 5\ H_2O$, 1 g/L; the concentration of nitrogen sources was 0.01%.

Utilization of various carbon sources was tested by the cultivation of bacteria on mineral medium with 0.05% of a carbon source; since the growth of bacteria in liquid media was unstable, the medium in petri dishes was supplemented with 14 g/L of washed agar (Difco, United States). The growth of bacteria was determined by enumerating the colonies grown on petri dishes at tenfold dilutions.

The results were analyzed after three successive platings on the medium with the same source. The number of bacterial cells was determined under a phase contrast microscope. The growth of bacteria was assayed as protein content by staining with Coomassie Blue [17].

The capacity for hydrogen oxidation was studied using 30-mL vials filled with a gas mixture containing 80% H_2, 10% CO_2, and 10% O_2. The vials were inoculated with a 2-day culture (10% of the volume of liquid media) and then turned upside-down. The uninoculated vials containing the gas mixture were used as controls.

Resistance to antibiotics was tested using the aforementioned agar medium. The antibiotic-containing test discs were placed onto the bacterial lawn on the agar surface. The following concentrations of antibiotics were used (μg): novobiocin and rifampicin, 5; ampicillin, benzylpenicillin, gentamycin, doxycy-

cline, methicillin, oxacillin, and streptomycin, 10; lincomycin, oleandomycin, and erythromycin, 15; carbenicillin, 25; amicyclin, vancomycin, ristomycin, nalidixic acid, neomycin, polymyxin, levomycetin, tetracycline, and kanamycin, 30.

Analysis of carotenoids. The pigments were extracted from the cells grown on petri dishes with a mixture of acetone–methanol (7 : 2) and analyzed on an SF-56 spectrophotometer (LOMO, Russia).

Assay of fatty acid composition. Fatty acids were analyzed on an HP-5973D chromatograph–mass spectrometer (Agillent Technologies, previously Hewlett-Packard, United States). The samples were treated as described earlier [18].

Determination of the isoprenoid quinone composition. Wet biomass of strain Fr1a was broken up in a porcelain mortar under liquid nitrogen and extracted with cold acetone. The extract was evaporated. The residue was separated by chromatography on Armsorb TSKh KSKG-UV 254 plates in hexane–diethyl ether (85 : 15). Mass spectra of the quinones were registered on a Finnigan-MAT 8430 mass spectrometer (Bremen, Germany) under standard electron impact (70 eV) [19].

Analysis of the DNA nucleotide composition was performed by the method of thermal denaturation; DNA–DNA hybridization was carried out by the method of optical reassociation as described earlier [20]. DNA was isolated by the Marmur method [21].

Phylogenetic analysis. PCR amplification of the 16S rRNA genes was carried out with the universal eubacterial primers [22, 23].

The nearest homologues were found using the BLAST software package, the Ribosomal Data Base Project (http://rdp.cme; http://www.ncbi.nih.gov/BLAST), and a msu.edu database. The nucleotide sequences were analyzed with the programs BioEdit and CLUSTALW; the phylogenetic tree was constructed by the neighbor-joining method using the MEGA software package [24]. The nucleotide sequence with a length of 1415 bp for strain Fr1a was deposited in the GenBank database under the accession number HQ400611.

Amplification of the RuBisCO (ribulose bisphosphate carboxylase/oxygenase) genes from the "green" variant of forms I and II was performed using the primers and protocol of Tourova et al. [25]. Genes of the "red" type (form I) RuBisCO were amplified with the aid of primers RubIrF/RubIrR [26].

RESULTS AND DISCUSSION

Isolation of bacteria. No growth of *Thioploca* was observed in the medium used for the *Macromonas* cultivation, whereas the cell number of concomitant microorganisms increased. Bacteria with microcapsules, which formed yellowish colonies on solid media, were prevalent among the latter. The isolated three bacterial strains (Fr1a, Fr1v, and Fr1g) were similar in their phenotypic characteristics (Table 1). Strain Fr1a was studied in detail.

Cell morphology and ultrastructure. All the studied isolates were gram-negative bacteria with oval or rod-shaped cells, motile with peritrichous flagella, and capable of forming cell conglomerates (microcolonies) and cell chains (Figs. 1a and 1b). The cells were 0.6–0.7 μm in diameter and 0.7–1.4 μm in length (Figs. 1c–1e). The cell reproduction was realized as uniform division by constriction (Figs. 1f–1i).

As seen in ultrathin sections (Fig. 1j), the cell wall was multilayered, typical of gram-negative bacteria; the outer membrane had a wavy surface; the cells were covered with slimy microcapsules (Figs. 1c–1f).

In the nucleus zone of cytoplasm, large round electron-dense inclusions were found out which are usually identified as polyphosphates (Fig. 1j).

Cultural, physiological and biochemical characteristics. Strains Fr1a, Fr1v, and Fr1g grew within a temperature range from 5 to 42°C with an optimum at 28°C. Bacteria preferred the media with low mineralization; 30 g/L of NaCl suppressed cell growth; the maximum NaCl concentration supporting slow growth was 45 g/L. The isolates grew in the media with initial pH from 3.0 to 8.8 with an optimum of 8.3.

Colonies formed on solid media were round, point-like, glossy, and slightly yellowish in color. The spectra of acetone–methanol (7 : 2) extracts showed absorption peaks at (425), 444, and (477) nm that is typical of carotenoid pigments. The colonies had a smooth surface, droplet-shaped profile and even edges; their structure was coarse-grained with oily or viscous consistency.

In liquid medium, the growth was moderate with homogeneous turbidity; a dense precipitate was formed at the end of cultivation.

All the strains were obligate organoheterotrophs (Table 1). No cell growth was observed either in mineral medium with thiosulfate without organic substances or on hydrogen independently of the presence of organic compounds. All the isolates were able to utilize the following carbon sources: L(+)-arabinose, D-glucose, D(+)-maltose, D(–)-mannitol, sucrose, D(+)-sorbitol, fructose, acetate, butyrate, valerate, L-glutamate, caproate, α-ketoglutarate, lactate, D,L-malate, maleate, pyruvate, propionate, succinate, fumarate, and citrate. No growth was observed on D(+)-cellobiose, pantothenate, L-α-alanine, L- arginine, D,L-valine, L-histidine, D,L-lysine, D,L-methionine, L-proline, ethanol, starch, creatine, phenol, or L-cysteine. The type strain Fr1a was able to grow on galactose, D(+)-lactose, D(+)-raffinose, D(+)-trehalose, benzoate, D,L-tryptophan, D,L-leucine, glycerol, and urea. Growth was inhibited by dulcitol, D(+)-xylose, D-rhamnose, malonate, D(+)-glucosamine, and D,L-ornithine.

The best growth was observed on three substrates: acetate, lactate, and glucose. The highest biomass

Table 1. Variability of the physiological and biochemical characteristics of three strains of the new bacteria

Characteristics	Strains		
	Fr1a^T	Fr1v	Fr1g
Utilization of carbon sources:			
Sugars:			
L(+)-arabinose	+	+	+
Galactose	+	–	+
D-glucose	+	+	+
Dulcitol	–	+	–
D(+)-xylose	–	+	–
D(+)-lactose	+	+	–
D(+)-maltose	+	+	+
D(–)-mannitol	+	+	+
D-rhamnose	–	+	–
D(+)-raffinose	+	–	+
Sucrose	+	+	+
L(–)-sorbose	–	–	–
D(+)-sorbitol	+	+	+
D(+)-trehalose	+	–	+
Fructose	+	+	+
D(+)-cellobiose	–	–	–
Acids:			
Acetate	+	+	+
Benzoate	+	–	+
Butyrate	+	+	+
Valerate	+	+	+
L-glutamate	+	+	+
Caproate	+	+	+
α-ketoglutarate	+	+	+
Lactate	+	+	+
D,L-malate	+	+	+
Maleate	+	+	+
Malonate	–	+	+
Pantothenate	–	–	–
Pyruvate	+	+	+
Propionate	+	+	+
Succinate	+	+	+
Fumarate	+	+	+
Citrate	+	+	+
Amino acids:			
L-α-alanine	–	–	–
L-arginine	–	–	–
D,L-valine	–	–	–
L-histidine	–	–	–
D,L-leucine	+	–	–
D,L-lysine	–	–	–
D,L-methionine	–	–	–
L-proline	–	–	–
D,L-tryptophan	+	–	–
D,L-β-phenylalanine	+	–	+
Alcohols:			
Ethanol	–	–	–
Glycerol			
Other compounds:	+	–	+
D(+)-glucosamine	–	+	+

Table 1. (Contd.)

Characteristics	Strains		
	Fr1a^T	Fr1v	Fr1g
Starch	–	–	–
Kreatine	–	–	–
Urea	+	–	+
D,L-ornithine	–	+	+
Phenol	–	–	–
L-Cysteine	–	–	–
Utilization of nitrogen sources:			
L-α-alanine	+	+	+
Asparagine	+	+	+
Aspartate	+	+	+
D,L-valine	–	+	–
Casein hydrolysate	+	+	+
Glycine	+	+	+
Glutamine	+	+	+
L-glutamate	+	+	+
D(+)-glycosamine	–	–	–
Yeast extract	+	+	+
D,L-leucine	+	+	+
D,L-lysine	–	+	–
D,L-methionine	+	+	–
Urea	+	+	+
Peptone	+	+	+
L-proline	+	+	+
D,L-β-phenylalanine	–	+	–
L-cysteine	–	+	–
N_2	–	–	–
$NaNO_3$	–	–	–
$NaNO_2$	–	–	–
NH_4Cl	+	+	+
NH_4NO_3	+	+	+
Effect of antibiotics:			
Amicyclin	–	–	–
Ampicillin	+	+	+
Benzylpenicillin	+	+	+
Vancomycin	+	+	+
Gentamycin	–	–	–
Doxycycline	–	–	–
Kanamycin	–	–	–
Carbenicillin	–	–	–
Levomycetin	–	–	–
Lincomycin	+	+	+
Methicillin	+	+	+
Nalidixic acid	–	–	–
Neomycin	–	–	–
Novobiocin	+	+	+
Oxacillin	+	+	+
Oleandomycin	–	–	–
Polymyxin	–	–	–
Ristomycin	+	+	+
Rifampicin	+	+	+
Streptomycin	–	–	–
Tetracycline	–	–	–
Erythromycin	–	–	–

Note: + and – stand for growth and no growth, respectively. Medium composition for the analyses is given in the Materials and Methods.

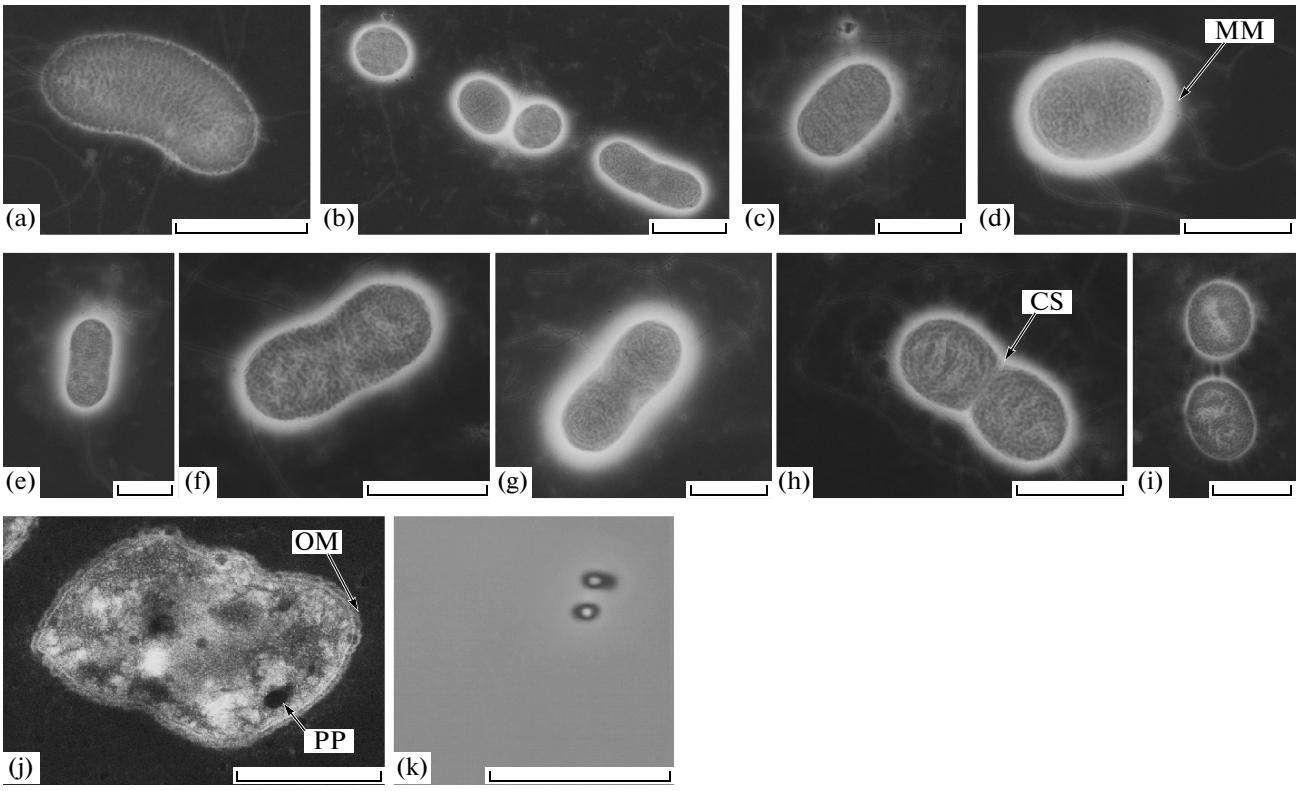

Fig. 1. Morphology and ultrastructure of the cells of strain Fr1a^T: whole cells stained with ammonium molybdate (a)–(i) and ultrathin structure of the cells (j). Electron microscopy: (a) and (c)–(i), scale bar of 0.7 μm; (b) and (j), scale bars of 1.0 and 0.4 μm, respectively. Phase contrast microscopy: (k), scale bar of 10 μm. Arrows indicate polyphosphates (PP); outer membrane (OM); cell septum (CS); and mucous microcapsule (MM).

(15 mg protein/L) was accumulated during cultivation of strain Fr1a for 6 days.

No lithoautotrophic growth on H_2 or reduced sulfur compounds was revealed, that was confirmed by the absence of the RuBisCO genes. When grown on media with acetate and lactate in the presence of various sulfur compounds ($S_2O_3^{2-}$, SO_3^{2-}, SO_4^{2-}), the cell yield (protein yield) was similar in all the variants.

The isolated bacteria were aerobes, oxidase- and catalase-positive.

The strains utilized the following nitrogen sources: alanine, asparagine, aspartate, casein hydrolysate, glycine, glutamine, L-glutamate, yeast extract, D,L-leucine, urea, peptone, L-proline, NH_4Cl, and NH_4NO_3. No growth was observed with such nitrogen sources as atmospheric N_2, $NaNO_3$, $NaNO_2$, and D(+)-glucosamine. Strain Fr1a was able to utilize D,L-methionine, whereas D,L-valine, D,L-lysine, D,L-β-phenylalanine, and L-cysteine did not support growth.

It was shown that ampicillin, benzylpenicillin, vancomycin, lincomycin, methicillin, novobiocin, oxacillin, ristomycin, and rifampicin had no effect on the growth of all strains. The growth of bacteria was inhibited by amicyclin, gentamycin, doxycycline, kanamycin, carbenicillin, levomycetin, nalidixic acid, neomycin, oleandomycin, polymyxin, streptomycin, tetracycline, and erythromycin.

Chemotaxonomic characteristics. Analysis of the fatty acid composition revealed the predominance of straight-chain $C_{16:0}$ and unsaturated $C_{18:1}$ ω7c acids (Table 2). Chromatograms of isoprenoid quinones had the bands typical of menaquinones. Mass spectrometric analysis showed the presence of menaquinone MK-8 H2 with molecular mass of 718 Da as the major quinone, MK-7 H2 with molecular mass of 650 Da as the minor one (less than 15%), and ubiquinone Q8, although the content of the latter was lower by an order of magnitude.

The G+C content of DNA of new isolates was 67.4–69.9 mol %. The results of DNA–DNA hybridization (80–94%) indicate a close resemblance between these strains (Table 3).

Phylogenetic analysis of the 16S rRNA genes. The results of the 16S rRNA gene sequence analysis showed that the strains belonged to the class *Betaproteobacteria* and were most closely related to the genus *Variovorax* (Fig. 2). The isolates had 16S rRNA similarity of 98% with the type strains of *V. paradoxus, V. soli, V. ginsengisoli*, and *V. boronicumulans* and of 96% similarity with the type strain of *V. dokdonensis*.

Table 2. Fatty acid composition of the new isolates and *Variovorax* strains

The acid with the number of carbon atoms	Fatty acid composition (% of the total)							
	Fr1a^T	Fr1v	Fr1g	*Variovorax boronicumulans* sp. nov., BAM–48^T	*Variovorax dokdonensis* sp. nov., DS-43^T	*Variovorax paradoxus* comb. nov., KCTC 12459, DSM 30034^T	*Variovorax soli* sp. nov., GH9–3^T	*Variovorax ginsengisoli* sp. nov.
Straight-chain fatty acids								
10:0	–	–	–	–	0.56–0.7	–	0.57	0.53
12:0	–	–	–	3.5	3.1–6.29	3.1–3.92	3.2–3.29	3.65
*i*14:0	–	0.34	–	–	–	–	–	–
14:0	–	0.55	0.17	1.1	1.0–1.25	0.9–1.37	0.77	4.91
*i*15:0	1.40	4.21	–	–	–	–	–	–
*a*15:0	1.57	4.67	–	–	–	–	–	–
15:0	–	0.47	–	–	1.8	6.7–7.09	1.17	0.49
*i*16:0	6.27	7.00	–	–	–	–	–	–
16:0	**18.22**	**19.54**	**18.51**	**36.1**	**25.2–34.95**	**26.8–31.6**	**33.2–38.11**	**39.07**
*a*17:0	–	1.08	–	–	–	–	–	–
17:0	–	–	–	–	1.6–2.1	1.2–2.74	1.0–1.22	–
18:0	3.52	–	–	–	–	–	–	–
Unsaturated fatty acids								
15:1ω6c	–	–	–	–	1.1	0.9	–	–
16:1ω5c	–	–	–	–	0.3	2.2	–	–
16:1ω7c	**24.54**	**26.59**	**26.78**	**–**	**–**	n.d.	**–**	**–**
16:1ω7t	–	1.29	–	–	–	n.d.	–	–
18:1ω7c	**41.52**	**31.66**	**51.55**	**11.3**	**13.1–16.1**	**12.6**	**9.0**	**–**
16:1ω7c/15:0 *i*2OH*	**–**	**–**	**–**	**21.40**	**23.45**	**16.98**	**21.48**	**19.72**
18:1ω7c/wgt/w12t*	**–**	**–**	**–**	**11.30**	**7.10**	**11.93**	**5.09**	**9.28**
Fatty hydroxyacids hydroxy acids								
10:0 3OH	–	–	–	2.9	2.0–3.27	2.1–3.7	4.77–5.1	3.54
12:0 3OH	–	–	–	–	2.4–3.3	–	–	–
14:0 2OH	2.96	–	–	2.2	–	2.2–2.7	–	–
16:1 2OH	–	–	–	–	–	0.6	n.d.	–
*i*16:0 3OH	–	0.32	–	–	–	–	n.d.	–
16:0 3OH	–	1.71	–	–	–	–	n.d.	–
*i*17:0 3OH	–	–	–	–	–	1.4	n.d.	–
Fatty cyclo acids								
17:0 *cyclo*	**–**	**–**	**–**	**19.0**	**15.1–23.6**	**17.1–26.3**	**15.9–22.53**	**18.82**
17 cyc	–	–	0.54	n.d.	–	–	–	–
19:0 *cyclo* ω8c	–	–	–	–	–	0.7	–	–
19 cyc	–	–	0.88	–	–	–	–	–
11-Me18:1	–	–	0.26	–	–	–	–	–

* The summarized amount of two or three fatty acids which could not be separated; – and n.d. stand for no acid and no data, respectively. If the amount of acids in two strains (*V. dokdonensis* DS-43^T and *V. paradoxus* KCTC 12459) was less than 0.5%, they were not taken into account. The predominant components are shown in bold.

Table 3. Genotypic characteristics of the new isolates

Strains	DNA G+C content, mol %	DNA–DNA homology, %		
		Fr1a^T	Fr1v	Fr1g
Fr1a^T	69.9	100		
Fr1v	67.4	80	100	
Fr1g	68.7	93	94	100

Bacteria of the genus *Variovorax* belong to the family *Comamonadaceae*, subclass Betaproteobacteria. In the last years, bacteria of the genus *Variovorax* attracted great interest of researchers [27–29]; they were found both in soils and freshwater environments. The domination of these bacteria among rhizobacteria capable of utilization of sulfonates as the sulfur source made it possible to assume their involvement in the biogeochemical sulfur cycle [28]. It was shown that some strains of *V. paradoxus* were capable of hydrogen oxidation in soils, which is useful for the plant growth, particularly for leguminous plants, resulting in the yield increasing by 15–30% in the soils inoculated with *V. paradoxus* strains [29].

The temperature maximum for the growth of the isolates (42°C) was higher than that for other species (no more than 37°C). Unlike some strains of *V. paradoxus* most closely related to our strains in their 16S rRNA sequences, the isolates were unable to grow on hydrogen but utilized maltose, valerate, and propionate as carbon sources. The isolates differed from the known *Variovorax* species in the composition of fatty acids and quinones (Tables 2 and 4). New strains did not contain unsaturated fatty acids $C_{16:1}\omega7c/C_{15:0}$ *iso*2OH, and $C_{18:1}\omega7c$/wgt/w12t, and the cyclo acid $C_{17:0}$ *cyclo*. Lipids of strain Fr1a contained no straight-chain fatty acids ($C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{17:0}$), unsaturated fatty acids ($C_{15:1}\omega6c$, $C_{16:1}\omega5c$), fatty hydroxy acids ($C_{10:0}$ 3OH, $C_{12:0}$ 3OH, $C_{16:1}$ 2OH, and *iso*$C_{17:0}$ 3OH), and cyclo acids ($C_{19:0}$ *cyclo* ω8c. Type strains of the species of the genus *Variovorax*: *V. boronicumulans, V. dokdonensis, V. paradoxus,* and *V. ginsengisoli*, contained ubiquinone Q8 and no menaquinones, whereas the type strain Fr1a contained mainly menaquinones MK-8 H2 and MK-7 H2, and slight amounts of ubiquinone Q8.

Thus, along with numerous microorganisms inhabiting the surface of the *Thioploca* sheaths, organotrophic microorganisms located inside the sheaths were isolated and assigned to the genus *Variovorax*. Comparative studies of the morphological, physiological, biochemical, and genetic characteristics of new strains and the known type strains of *Variovorax* species revealed a number of essential differences between them. Later on, this will allow us to designate the isolates as a new species of the genus *Variovorax* and to change the description of this genus (composition of quinones and fatty acids). It can be assumed that

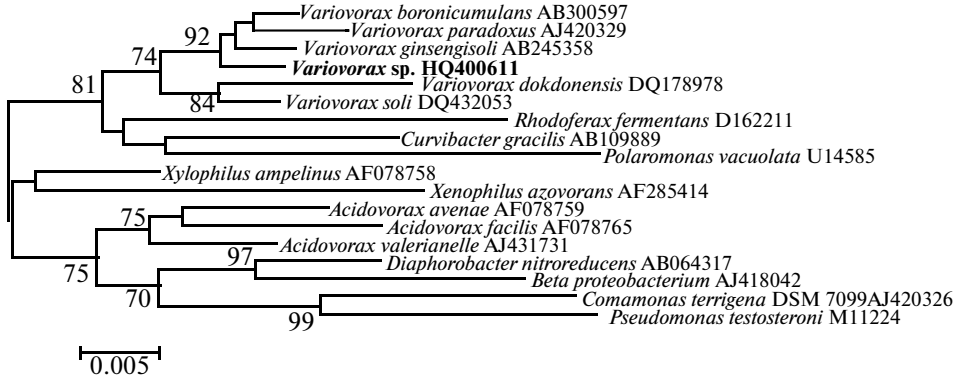

Fig. 2. Position of the type strain *Variovorax* sp. Fr1a^T in the unrooted phylogenetic tree constructed by the neighbor-joining method. Nucleotide sequences determined in this study are shown in bold. The numerals indicate the reliability of the branching points on the tree determined by bootstrap analysis of 1000 alternative trees (values of over 90% were considered as significant). The tree was constructed on the basis of the analysis of the 16S rRNA gene nucleotide sequences. The scale bar represents the evolutionary distance corresponding to five nucleotide substitutions per 1000 nucleotides.

Table 4. Comparative characterization of the representatives of the genus *Variovorax* and the new isolates

Characteristics	The studied strains			*Variovorax boronicumulans* sp. nov. *	*Variovorax dokdonensis* sp. nov. **	*Variovorax paradoxus comb.* nov. ***	*Variovorax soli* sp. nov. ****	*Variovorax ginsengisoli* sp. nov. *****
Motile rods, μm	0.6–0.7 × 0.7–1.4			0.5–0.7 × 1.0–2.20	0.3–0.5 × 0.7–2.8	0.5–0.6 × 1.2–3.0	0.5–0.7 × 1.0–1.5	0.3–0.5 × 0.5–3.0
Peritrichs	+			1–2	+	+	n.d.	n.d.
Pigments	+			n.d.	n.d.	+	n.d.	n.d.
Nitrate reduction	–			–	–	+	–	–
Anaerobic growth on NO_3	–			–	–	+	–	–
Chemolithotrophic growth on hydrogen	–			n.d.	n.d.	+	n.d.	n.d.
Oxidase	+			n.d.	n.d.	+	n.d.	n.d.
Catalase	+			n.d.	n.d.	+	n.d.	n.d.
Starch hydrolysis	–			n.d.	+	–	–	n.d.
Growth on nutrient agar (Difco)	+			n.d.	n.d.	+	n.d.	n.d.
Tolerance to 1.5% NaCl	+			–	+	+	+	–
NaCl growth range, %	0–3			0–1	0–5		0–3	0–1
	Fr1a^T	Fr1v	Fr1g					
Carbon sources:								
D-rhamnose	–	+	–	n.d.	n.d.	n.d.	n.d.	n.d.
L-rhamnose	n.d.	n.d.	n.d.	–	–	–	–	–
D(+)-maltose	+	+	+	–	–	–	±	n.d.
D-glucose	+	+	+	–	+	+	+	–
L-arabinose	+	+	+	±	±	+	+	+
D(–)-mannitol	+	+	+	+	–	+	+	n.d.
Citrate	+	+	+	+	–	±	–	–
Butyrate	+	+	+	n.d.	+	+	+	+
Acetate	+	+	+	n.d.	–	+	–	+
Malonate	–	+	+	n.d.	–	+	–	+
Benzoate	+	–	+	n.d.	+	+	+	+
Valerate	+	+	+	n.d.	+	–	+	+
Propionate	+	+	+	n.d.	+	–	+	+
Lactate	+	+	+	n.d.	+	+	+	+
Caproate	+	+	+	n.d.	+	+	+	n.d.
L-histidine	–	–	–	n.d.	+	+	+	n.d.
L-α-alanine	–	–	–	n.d.	+	–	–	+
Urea	+	–	+	–	+	±	–	–
Resistance to antibiotics:								
Ampicillin	+	+	+	n.d.	n.d.	n.d.	n.d.	+
Kanamycin	–	–	–	n.d.	–	–	n.d.	–
Lincomycin	+	+	+	n.d.	n.d.	n.d.	n.d.	+
Tetracycline	–	–	–	n.d.	n.d.	–	–	–

Table 4. (Contd.)

Characteristics	The studied strains			*Variovorax boronicumulans* sp. nov. *	*Variovorax dokdonensis* sp. nov. **	*Variovorax paradoxus comb.* nov. ***	*Variovorax soli* sp. nov. ****	*Variovorax ginsengisoli* sp. nov. *****
Novobiocin	+	+	+	n.d.	–	n.d.	n.d.	+
Streptomycin	–	–	–	n.d.	–	–	n.d.	–
Erythromycin	–	–	–	n.d.	n.d.	n.d.	n.d.	–
Neomycin	–	–	–	n.d.	–	–	n.d.	–
Gentamycin	–	–	–	n.d.	n.d.	n.d.	n.d.	+
Oleandomycin	–	–	–	n.d.	n.d.	n.d.	n.d.	–
Carbenicillin	–	–	–	n.d.	–	–	n.d.	n.d.
Isolated from	S, FW			FW	FW	n.d.	S, FW	FW
Menaquinone[1]								
MK-8 H2	+			–	–	n.d.	–	n.d.
MK-7 H2	+			–	–	n.d.	–	n.d.
Ubiquinone Q-8	±			+	+	n.d.	+	n.d.
pH range for growth (pH_{opt})	3.0–9.0 (8.3)			5.0–9.0 (7.0)	5.0–9.5 (7.0–8.0)	(n.d.)	5.0–9.0 (n.d.)	5.0–8.5 (n.d.)
Temperature range for growth ($T°_{opt}$°C)	5–42 (28)			4–37 (30)	(30)	(n.d.)	10–35 (n.d.)	4–30 (n.d.)
The DNA G+C content, mol %	69.9			71.2	66.0	67.0	67.1	66.0

Note: N.d stands for no data; + and – designate the presence and the absence of a feature, respectively; [1] designates characteristics given for type strain Fr1a^T (the data beginning from menaquinone and further those given in the second column); pH_{opt} means the optimum initial pH of the medium; S and FW stand for soil and freshwater, respectively; *, **, ***, ****, and *****, stand for references [30], [31], [27], [32], and [33], respectively.

organisms of the genus *Variovorax* utilize organic compounds of the sheaths and ammonium produced by *Thioploca* sp. (as a nitrogen source). So far it remains unclear whether the presence of *Variovorax* is favorable for *Thioploca* and whether their relationship is mutually beneficial.

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