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EXPERIMENTAL ARTICLES

Xanthobacter xylophilus sp. nov., a Member of the Xylotrophic Mycobacterial Community of Low-Mineral Oligotrophic Waters

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Abstract—A novel obligately aerobic heterotrophic bacterium belonging to the genus *Xanthobacter*, strain Z-0055, was isolated from the bacterial community of dystrophic waters formed by xylotrophic fungi grown on decaying spruce wood. The cells are small (0.4 ± 0.7 μm), ovoid, gram-negative, and nonmotile. The strain reproduces by nonuniform division. Strain Z-0055 is a moderately acidophilic microorganism and grows in a pH range of 4.8–6.8, with an optimum at pH 5.5. The temperature range for growth is 10–28°C, with an optimum at 20°C. The bacterium utilizes salts of organic acids (citrate, oxalate, succinate, and gluconate), as well as xylose and xylan as carbon and energy sources. No growth was detected at NaCl concentrations higher than 1.5 g/l. The DNA G+C base content is 63.6 mol %. According to phylogenetic analysis of the 16S rRNA gene sequences, strain Z-0055 belongs to the cluster containing *X. flavus, X. aminoxidans, X. autotrophicus*, and *X. viscosus*. The levels of similarity between the studied strain and these species are almost the same (96.0–97.0%). The genetic and ecophysiological properties of strain Z-0055 support classification of this acid-tolerant inhabitant of oligotrophic waters within the genus *Xanthobacter* as a new species *X. xylophilus* sp. nov.

Key words: oligotrophic bacteria, dissipotrophs, wood decomposition, myco–bacterial community, acidotrophic microorganisms, oligotrophic water.

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Bacteria of the genus *Xanthobacter* are a widespread group of microorganisms. They can be found in different habitats, including water and bottom sediments of lakes and seas, rice paddy soil and rice rhizosphere, wet meadow soils, oil-polluted soils, and precipitation tanks [1–4]. Wide distribution of bacteria of the genus *Xanthobacter* may be attributed to their physiology. Xanthobacters are aerobic bacteria capable of dinitrogen fixation. They grow both under chemoheterotrophic and lithoautotrophic conditions [1]. Their capacity for autotrophic growth is achieved via the energy-releasing reaction $2H_2 + O_2 = 2H_2O$; therefore, these microorganisms may be considered as typical hydrogen-oxidizing bacteria. Most species of the genus *Xanthobacter* utilize organic acids, alcohols (ethanol, butanol, and propanol) and C_1 compounds (methanol and methylated amines), as well as a limited number of carbohydrates, as carbon and energy sources [5, 6]. Some species are able to utilize complex organic compounds as growth substrates. For instance, *X. tagetidis* utilizes heterocyclic compounds [7], while "*X. polyaromaticivorans*'' utilizes polycyclic and aromatic compounds under microaerophilic conditions [8]. Morphologically, most representatives of the genus *Xanthobacter* are pleomorphic rods; they produce a yellow carotenoid pigment, zeaxanthin dirhamnoside, with the exception of *"X. polyaromaticivorans"*, which produces zeaxanthin, a salmon pink pigment. All members of the genus *Xanthobacter* are mesophiles and neutrophiles and grow at a pH optimum of 6.8–7.8 [1].

The aim of the present work was to study strain Z-0055, an acid-tolerant bacterium inhabiting dystrophic waters formed by xylotrophic fungi in the course of spruce wood degradation, as well as to determine its phylogenetic position.

MATERIALS AND METHODS

Object of study and source of isolation. Strain Z-0055 was isolated from low-humified acidic (pH 4.3) low-mineral oligotrophic (conductivity 140 μS) dystrophic water of a microlysimeter in which spruce wood was degraded by a xylotrophic fungal community [9].

Composition of the media and cultivation conditions. Strain Z-0055 was isolated on low-mineral PC medium containing the following: Hutner's basal salt

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solution, 5 ml/l [10]; fungal culture liquid as a carbon and energy source, 10 ml/l; and yeast extract as a growth factor, 0.05 g/l. The culture liquid was obtained as follows. Xylotrophic fungi (*Aspergillus ustus, Trichoderma harzianum, Cladosporium* sp., *Penicillium* sp., and *Paecilomyces* sp.) grown on spruce wood in a microlysimeter were isolated and cultivated on the mineral base of PC medium with xylose (2 g/l) as a carbon and energy source. Then, the culture liquid was filtered and used for the preparation of PC medium (pH 4.3). The medium conductivity was 140 μS, which corresponded to the water conductivity in the microlysimeter at the moment of sampling. Strain Z-0055 was isolated by inoculating aliquots of low-humified water from the microlysimeter onto agar medium. To inhibit fungal growth during the isolation of strain Z-0055, nystatin was used.

The colonies grown on agarized PC medium were transferred to liquid medium of the same composition and then plated onto the agar medium to obtain the pure culture. The pure culture was maintained in liquid PC medium with succinate (0.2 g/l) as a substrate and yeast extract (0.05 g/l).

Microscopic investigations. Cell morphology was studied under a light microscope with a phase-contrast device (Amplival, Germany), as well as by electron microscopy (JEM 100C, Japan) of negatively stained preparations and ultrathin sections. The preparations were stained with 1% uranyl acetate. To obtain ultrathin sections, the cell preparations were fixed with glutaraldehyde with subsequent additional fixation with osmic acid in cacodylate buffer and then embedded in Epon. Ultrathin sections were obtained with an LKB ultramicrotome, stained with lead citrate, and then additionally stained with a 3% aqueous solution of uranyl acetate.

Physiological properties and growth characteristics. The range of substrates utilized by strain Z-0055 was determined on PC medium (pH 5.5) with yeast extract (0.05 g/l) at 20°C. The tested substrates were added to a concentration of 0.5 g/l. Sugars (arabinose, xylose, glucose, fructose, galactose, mannose, lactose, maltose, sucrose, raffinose, starch, and xylan), alcohols (glycerol, sorbitol, and mannitol), salts of organic acids (acetate, butyrate, propionate, pyruvate, fumarate, succinate, oxalate, oxaloacetate, citrate, and benzoate), primary alcohols (methanol and ethanol), amino acids (methylalanine, glutamate, leucine, cysteine, and aspartate), and methylamines were tested as carbon and energy sources. The biomass yield was assessed by the optical density (OD) of the cell suspension measured on a UNICO 2100 spectrophotometer at 600 nm.

The growth rate of strain Z-0055 at a pH range of pH 4.8–8.0 was determined by the addition of 0.05 M solutions of Na_2HPO_4 and KH_2PO_4 to the medium. Growth in a pH range of 3.0–4.8 was determined in the medium acidified with 0.1 N HCl to the required pH level. The medium pH was determined potentiometrically using an Expert 001 pH/ion meter (Russia).

Growth of Z-0055 was investigated within a temperature range of 4–37°C.

The dependence of growth on NaCl concentrations was determined in a liquid medium supplemented with NaCl (0.5–3.0 g/l) at 20°C and pH 5.5.

The temperature and pH optima for growth of strain Z-0055, as well as the dependence of growth on NaCl concentrations in the media were determined with succinate as a substrate.

The capacity for lithoautotrophic growth was assessed by measuring optical density (OD_{600}) of the cell suspension and by monitoring hydrogen utilization during cultivation of strain Z-0055 in liquid medium with the gas phase $H_2 : O_2 : CO_2$ (7 : 2 : 1). The hydrogen concentration was measured on an LKhM-80 gas chromatograph (Russia) with a katharometer detector. The separation was carried out on a column packed with a 5A molecular sieve.

The ability of the culture to grow on various nitrogen sources was tested using inorganic salts (ammonium sulfate and potassium nitrate), as well as amino acids (phenylalanine, tryptophan, leucine, aspartic, arginine, and glutamate).

The nitrogen-fixing activity of the strain was determined by the acetylene method.

Two primer systems, F1/R6 [11] and PolF/PolR [12], designed for the *nifH* gene, were used to detect the presence of the *nifH* gene in the DNA.

The sensitivity of strain Z-0055 to antibiotics (lincomycin, 10 mg; novobiocin, 30 mg; ampicillin, 10 mg; chloramphenicol, 30 mg; neomycin, 10 mg; gentamycin, 10 mg; kanamycin, 30 mg; and streptomycin, 10 mg) was determined by the diameter of growth inhibition zones surrounding antibiotic discs (Oxoid) on the agar surface.

Catalase activity was assayed by monitoring the formation of gas bubbles on addition of a 3% hydrogen peroxide solution to the cells; the presence of oxidase was detected by changes in the colony pigmentation when the reactant REF-55635 was applied.

Pigment. The affinity of the pigment to the group of carotenoids or xanthophylls was determined by phase separation of carotenoids [13].

Molecular genetic analysis. DNA isolation and purification, as well as determination of the DNA G+C content, were performed as described earlier [14].

Determination of the nucleotide sequence of the 16S rRNA gene of strain Z-0055 was performed as follows. DNA was extracted by the phenol method [15]. PCR amplification of the 16S rRNA gene was carried out with the universal eubacterial primers 27f and 1492r on a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, United States). Sequencing of amplification products was performed on a CEQ2000 XL automatic sequencer (Beckman Coulter, United

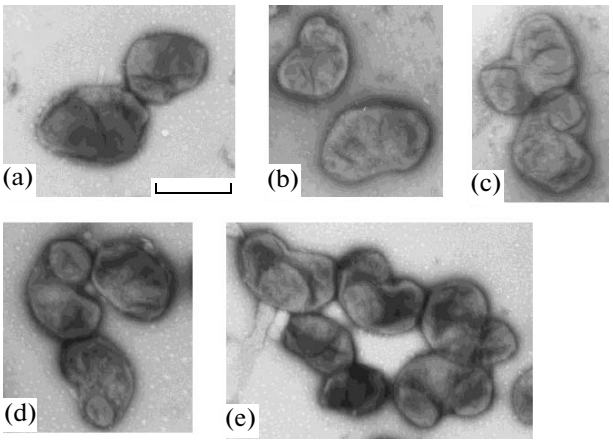

Fig. 1. Electron microphotographs of the cells of strain Z-0055 at various stages of reproduction (scale bar, 0.5 μm). The preparations were stained with 1% uranyl acetate.

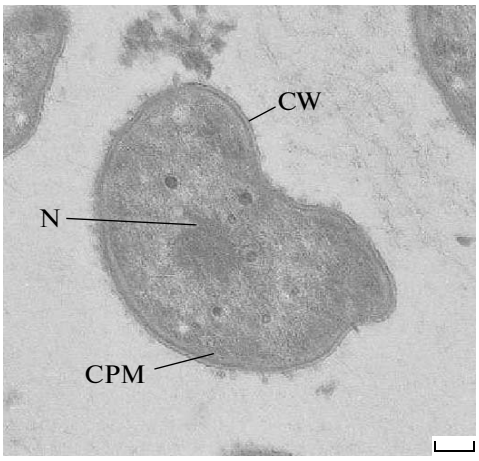

Fig. 2. Electron microphotograph of an ultrathin section of a cell of strain Z-0055. CW, cell wall; CPM, cytoplasmic membrane; N, nucleoid (scale bar, 0.1 μm).

States) according to the manufacturer's instructions. For the identification of strains closely related to strain Z-0055, the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) was used. The phylogenetic tree was constructed by the methods implemented in the TREECON software package [16]. The obtained 16S rRNA gene sequence of strain Z-0055 was deposited in the GenBank under the accession number FJ882073.

RESULTS

Isolation source. Strain Z-0055 was isolated from dystrophic low-humified, acidic (pH 4.3) low-mineral oligotrophic (conductivity 140 μS) water of a microlysimeter in which spruce wood was degraded by xylotrophic fungi.

Cell morphology and ultrastructure. When grown under optimal cultivation conditions on the medium with succinate as a substrate, strain Z-0055 formed small (0.4 ± 0.7 μm) ovoid nonmotile cells (Fig. 1a). During reproduction, one of the cell ends elongated (Fig. 1b, 1c, 1d) and a curved U-shaped cell (0.4–0.7 ± 0.8–1.2 μm) was formed (Fig. 1e). This cell then divided nonuniformly into two ovoid cells. The cells of strain Z-0055 did not produce resting forms. No slimy capsules were detected. Ultrathin sections of the cells of strain Z-0055 gram-negative structure of their cell wall (Fig. 2).

Cultural properties. The strain produced small (up to 2 mm in diameter), slimy, dense, rounded, convex, orange-colored (due to the pigment) colonies with smooth edges.

Pigments. Investigation of the pigment in the three-phase system hexane : water : methanol (1 : 0.5 : 1) revealed that the pigment concentrated in the upper hexane fraction, which confirmed its affinity to carotenoids.

Physiological properties. Strain Z-0055 was an obligately aerobic bacterium. The bacterium grew within a temperature range of 10–28°C with an optimum at 20°C. The microorganism was moderately acidophilic and grew within a pH range of 4.8–6.8 with an optimum at 5.5. The strain was NaCl-sensitive: the NaCl content in the media above 1.5 g/l inhibited growth; this suggests that this microorganism is a typical inhabitant of oligotrophic waters.

Strain Z-0055 utilized a limited range of organic compounds, including salts of organic acids (succinate, citrate, oxalate, and gluconate) and carbohydrates (xylose), as well as a polysaccharide (xylan), as carbon and energy sources. The maximum growth rate was observed on succinate (μ_{max} 0.023 h^{-1}).

The bacterium was oligotrophic and grew at substrate concentrations ranging from 0.1 to 5 g/l. The optimal substrate concentration in the medium was 0.2 g/l.

Strain Z-0055 utilized ammonium sulfate, potassium nitrate, asparagine, arginine, and glutamate as nitrogen sources. The strain was not capable of nitrogen fixation.

The organism was catalase- and oxidase-positive.

Antibiotics. The bacterium was resistant to novobiocin, chloramphenicol, neomycin, and gentamycin, as well as to antibiotics of fungal origin (lincomycin and ampicillin). Strain Z-0055 was sensitive to kanamycin and streptomycin.

Molecular genetic analysis. The content of the G+C base pairs in the DNA of strain Z-0055 was 63.6 mol %.

The sequence of the 16S rRNA gene fragment (1310 bp) was determined for strain Z-0055. Phylogenetic analysis confirmed affiliation of strain Z-0055 within the genus *Xanthobacter*. The levels of 16S rRNA similarity between strain Z-0055 and each species of this genus were virtually the same (96.0–

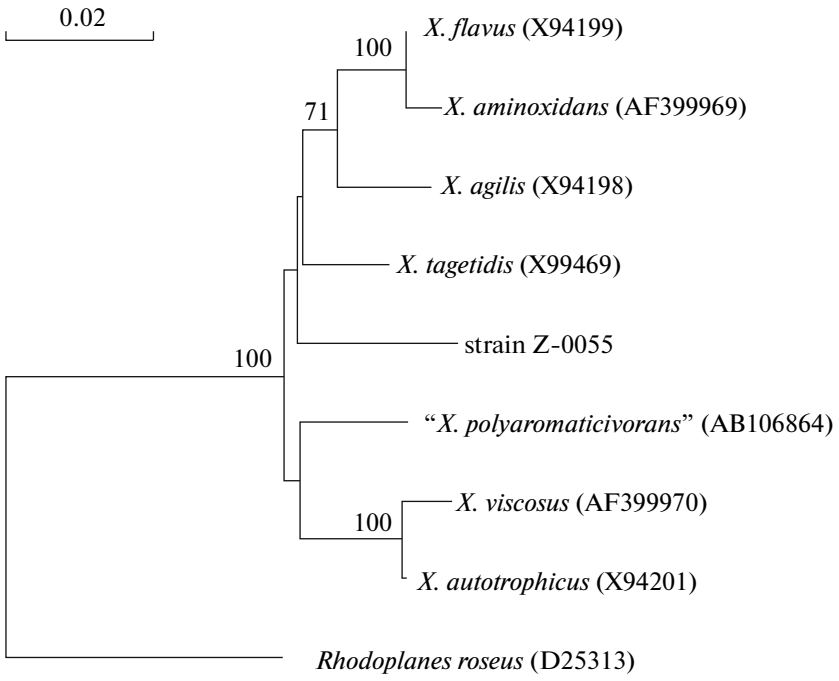

Fig. 3. Phylogenetic position of strain Z-0055 among members of the genus *Xanthobacter* according to the 16S rRNA sequence analysis. The bar shows evolutionary distance, corresponding to two replacements per 100 nucleotides. The numerals show the significance of the branching order as determined by bootstrap analysis.

97.0%); in the phylogenetic tree, this strain fell into a cluster with *X. flavus, X. aminoxidans, X. agilis*, and *X. tagetidis* (Fig. 3).

Despite the fact that all known representatives of the genus *Xanthobacter* are able to fix molecular nitrogen, we failed to detect the acetylene reduction activity of strain Z-0055. Thus, we tested the new isolate for the presence of the *nifH* gene. In the DNA of strain Z-0055, the *nifH* gene fragment was not detected.

DISCUSSION

Strain Z-0055 was isolated from dystrophic waters formed by a xylotrophic fungal community in the course of spruce wood degradation. Comparative phylogenetic analysis of the 16S rRNA gene sequence of strain Z-0055 demonstrated that the isolate belongs to the genus *Xanthobacter*. In accordance with *Bergey's Manual of Systematic Bacteriology* (2005), the genus *Xanthobacter* belongs to the class *Alphaproteobacteria*, order *Rhizobiales*, family *Hyphomicrobiaceae* [2]. An independent family, *Xanthobacteraceae*, was presently established within the class *Alphaproteobacteria* [17]. The family *Xanthobacteraceae* includes the genera *Azorhizobium, Ancylobacter, Labrys, Starkeya*, and *Xanthobacter*. The genus *Xanthobacter* is represented by the following species: *X. autotrophicus*^{T} [18], *X. flavus* [19], *X. agilis* [20], *X. tagetidis* [7], *X. aminoxidans* (*Blastobacter aminooxidans*), and *X. viscosus* (*Blastobacter viscosus*) [4]. The levels of 16S rRNA similarity between strain Z-0055 and all species of the genus *Xanthobacter* are almost the same (96.0–97.0%) (Fig. 3). The levels of similarity between the 16S rRNA gene sequences of all the species belonging to this genus are above 96.0%; the similarity levels between *X. flavus* and *X. aminoxidans* and between *X. autotrophicus* and *X. viscosus* are even higher, over 99%. Hence, according to the data obtained by the 16S rRNA gene analysis, strain Z-0055 is genetically distinct from the other species within the genus *Xanthobacter*.

The phenotypic, physiological, and biochemical properties of strain Z-0055 (table) indicate that this microorganism differs significantly from the known species of the genus *Xanthobacter*. The cells of strain Z-0055 are ovoid. Pleomorphism, a characteristic trait of most representatives of the genus *Xanthobacter*, was not observed. The cells contain an orange pigment, which was found to be a carotenoid rather than a xanthophyll, as in other species. Strain Z-0055 utilizes primarily organic acids as carbon and energy sources. The novel strain is not capable of lithoautotrophic growth on the gas mixture $H_2 : O_2 : CO_2$ (7 : 2 : 1) and is not able to utilize C_1 compounds, which distinguish strain Z-0055 from the validated representatives of the genus *Xanthobacter* (except for "*X. polyaromaticivorans*"). Unlike "*X. polyaromaticivorans*" and *X. flavus*, strain Z-0055 does not utilize aromatic and polycyclic compounds as carbon and energy sources. Unlike the known species of the genus *Xanthobacter*, which grow at a pH optimum of 6.8–7.8, this strain is a moderately acidophilic bacterium with a growth

Differentiating characteristics of representatives of the genus *Xanthobacter*

	Z-0055	*X. agilis*	*X. viscosus*	*X. tagetides*	"*X. polyaromaticivorans*"	*X. flavus*	*X. aminoxidans*	*X. autotrophicus*
Pleomorphic rods, mm	– 0.4 × 0.7	– 0.7 × 1.1–3.6	+ 0.5–0.9 × 1.0–3.2	+ 0.5 × 1.0	N/D	+ 0.5–0.7 × 1.0–2.5	+ 0.8–1.0 × 1.5–3.5	+ 0.4–0.8 × 0.8–4.0
Motility	–	+	–	+	–	+	–	+, –
Reproduction, nonuniform division	+	–	+	–	–	–	+	–
Pigment	Orange	Yellow	Yellow	Yellow	Salmon pink	Yellow	Yellow	Yellow
Autotrophic growth	–	+	+	+	–	+	+	+
Reaction to chloramphenicol	–	+	N/D	+	N/D	–	N/D	–
Utilization of organic acids	+	+	+	+	–	+	+	+
Utilization of hexoses	+	(+)	+	+	–	+	+	+
Utilization of aromatic compounds	–	–	–	–	+	+	–	–
Utilization of C_1 compounds	–	+	+	+	–	+	+	+
Dinitrogen fixation	–	+	+	+	+	+	+	+
G+C base content, mol %	63.6	N/D	66.3	68	N/D	N/D	69.1	N/D
Optimal growth temperature	20	20	28–30	28–31	N/D	N/D	29–32	30
Optimal pH	5.5	6.8–7.2	6.8–7.2	7.6–7.8	N/D	N/D	7.2–7.8	6.8–7.2

Note: N/D stands for "no data."

optimum at pH 5.5. Strain Z-0055 is not able to fix molecular nitrogen, unlike all the known representatives of this genus. Its DNA G+C base content is lower (63.6 mol %) than that of the other species of this genus (66.3–69.1 mol %).

Thus, the phenotypic properties of strain Z-0055 differ considerably from those of the other species of the genus *Xanthobacter*. The ecophysiological properties of strain Z-0055 (resistance to a number of antibiotics, including those of fungal origin; preferential utilization of organic acids produced by xylotrophic fungi; low concentrations of the utilized substrate; acid tolerance; and adaptation to low NaCl concentrations) allow us to consider this strain a dissipotroph that inhabits acidic dystrophic waters and belongs to the trophic myco–bacterial community. We propose that strain Z-0055 be classified into the genus *Xanthobacter* as a novel species, *X. xylophilus* sp. nov.

Description of *Xanthobacter xylophilus* sp. nov. *xy.lo'phi.lus.*, Gr. n. *xylon*, wood; Gr. n. *philos*, friend; M.L. masc. *xylophilus* friend of wood.

The cells are ovoid (0.4 × 0.7 μm). The bacterium reproduces by nonuniform division and does not form capsules. U-shaped cells, 0.4–0.7 × 0.8–1.2 μm, are formed before the division and split into two ovoid cells. The cell wall structure is of the gram-negative type.

The colonies are small (2 mm in diameter), slimy, dense, rounded, convex, orange-colored, with smooth edges. Carotene is the main pigment.

The organism is an obligate aerobe. Yeast extract is required for growth. Succinate, citrate, oxalate, gluconate, carbohydrates (xylan and xylose) are utilized as carbon and energy sources. Mono-, di-, and polysaccharides, sugar alcohols, and amino acids are not utilized as carbon sources.

Growth occurs in the presence of lincomycin, novobiocin, ampicillin, chloramphenicol, neomycin, and gentamycin. Kanamycin and streptomycin suppress growth.

The organism is catalase- and oxidase-positive and is not capable of lithoautotrophic growth. The ability to assimilate molecular nitrogen was not detected.

The pH range for growth is 4.8–6.8 with an optimum at 5.5. The bacterium is a mesophile growing in a temperature range from 10 to 28°C with a growth

optimum at 20°C. Active growth occurs at NaCl concentrations not exceeding 1.5 g/l.

The DNA G+C base content is 63.6 mol %.

The organism was isolated from acidic (pH 4.3), low-mineral oligotrophic water formed by the xylotrophic fungal community grown on decaying spruce wood.

The type strain is Z-0055^T (= VKM B-2535).

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