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EXPERIMENTAL ARTICLES

Methylophaga murata sp. nov.: a Haloalkaliphilic Aerobic Methylotroph from Deteriorating Marble

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Abstract—The haloalkaliphilic methylotrophic bacterium (strain Kr3) isolated from material scraped off the deteriorating marble of the Moscow Kremlin masonry has been found to be able to utilize methanol, methylamine, trimethylamine, and fructose as carbon and energy sources. Its cells are gram-negative motile rods multiplying by binary fission. Spores are not produced. The isolate is strictly aerobic and requires vitamin B_{12} and Na^+ ions for growth. It is oxidase- and catalase-positive and reduces nitrates to nitrites. Growth occurs at temperatures between 0 and 42°C (with the optimum temperatures being 20–32°C), pH values between 6 and 11 (with the optimum at 8–9), and NaCl concentrations between 0.05 and 3 M (with the optimum at 0.5–1.5 M). The dominant cellular phospholipids are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. The major cellular fatty acids are palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), and octadecenoic ($C_{18:1}$) acids. The major ubiquinone is Q_8. It accumulates ectoine and glutamate, as well as a certain amount of sucrose, to function as osmoprotectants and synthesizes an exopolysaccharide composed of carbohydrate and protein components. It is resistant to heating at 70°C, freezing, and drying; utilizes methanol, with the resulting production of formic acid, which is responsible for the marble-degrading activity of the isolate; and implements the 2-keto-3-deoxy-6-phosphogluconate variant of the ribulose monophosphate pathway. The G+C content of its DNA is 44.6 mol %. Based on 16S rRNA gene sequencing and DNA–DNA homology levels (23–41%) with neutrophilic and alkaliphilic methylobacteria from the genus *Methylophaga*, the isolate has been identified as a new species, *Methylophaga murata* (VKM B-2303^T = NCIMB 13993^T).

Key words: *Methylophaga murata*, marble, restricted facultative methylotroph, haloalkaliphile, ribulose monophosphate pathway, osmoprotectants, ectoine.

Stone monuments are subject to active degradation by microbial communities including bacteria, fungi, and algae [1, 2]. The microorganisms that occur in biofilms on the stone surface are related by complex trophic interactions. The surface of deteriorating marble is particularly rich in microflora. Some representatives of this microbiota are resistant to unfavorable environmental conditions and show an ability to penetrate into the marble subsurface layer and develop there, causing gradual destruction of the marble [1, 2].

Since the species composition of marble-degrading microorganisms is as yet poorly understood, the aim of this study was to characterize taxonomically, physiologically, and biochemically a new strain of methylobacteria that has been isolated from the deteriorating marble of a Moscow Kremlin crypt.

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MATERIALS AND METHODS

Strain and cultivation conditions. Strain Kr3 was isolated from material scraped off the eroded part of a marble crypt in the Moscow Kremlin. The pH of the marble at the scraping site was 9.1. DNA–DNA hybridization studies were performed with the type cultures of methylobacteria *Methylophaga marina* ATCC 35842^T, *Methylophaga thalassica* ATCC 33146^T [3], *Methylophaga sulfidovorans* RB1 LMD 95.210^T [4], *Methylophaga alcalica* ATCC BAA-297^T [5], and *Methylophaga natronica* VKM B-2288^T [6] as the reference strains.

Alkaliphilic methylobacteria were isolated and cultivated using the following basal mineral (M) medium (g/l): KH_2PO_4, 1.0; KNO_3, 1.0; and $MgSO_4 \cdot 7H_2O$, 0.2. After sterilization, the medium was supplemented with a trace element solution (5 ml/l) containing (g/l) $FeNH_4$ citrate, 3; $CaCl_2 \cdot 2H_2O$, 3; $MnCl_2 \cdot 4H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.5; and $CuSO_4 \cdot 5H_2O$, 0.05. The medium was

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prepared using distilled water. The pH of the medium was adjusted to 9.0 by adding 5 ml of 2 M $NaHCO_3$ and 1 ml of 1 M Na_2CO_3 per 200 ml of the medium.

Neutrophilic bacteria of the genus *Methylophaga* were grown in a K medium [7] containing 3% NaCl, 20 μg/l vitamin B_{12}, and 0.5% CH_3OH (pH 7.2–7.4). Enrichment cultures were obtained by placing a sample aliquot (0.5 g) into a 700-ml flask containing 200 ml of the M medium supplemented with 3% NaCl, 0.5 vol % methanol, and 0.02 vol % yeast extract. The flask was incubated at 29°C on a shaker (180 rpm) for 2 days. After 3–4 successive transfers of the flask contents to fresh K medium, the enrichment was exhaustively plated onto K medium solidified with agar. The isolated colonies of methylobacteria were transferred to agar slants, and the whole procedure (cultivation in liquid K medium and the obtaining of individual colonies of methylobacteria on K agar) was repeated. The isolated culture was tested for microbiological purity by light and electron microscopy of cells and colonies grown on agar media containing methanol and methylamine.

Study of the cultural, physiological, and biochemical properties of the isolate. In order to investigate the morphology of the cells and colonies, as well as to evaluate the cell motility, strain Kr3 was grown on M agar. The ability of the isolate to reduce nitrates was studied using cells grown in liquid M medium for 1, 2, and 3 days. The formation of indole from L-tryptophan was assayed with the Salkowski reagent [8]. The hydrolysis of starch was assayed with the Lugol solution using bacterial cells grown on M agar containing 0.2 wt % of soluble starch. Oxidase was assayed with a 1 wt % solution of tetramethyl-*p*-phenylenediamine dihydrochloride. Catalase activity was determined by placing 3% hydrogen peroxide solution onto the plate culture grown on M agar. The requirement for Na^+ ions was investigated by replacing the sodium salts in the medium with respective K, Mg, or Li salts. The halotolerance of the isolate was studied by growing it in liquid K and M media with different concentrations of NaCl (0–20 wt %). The growth temperature range of the isolate was determined by cultivating it on M agar containing 3% NaCl at temperatures ranging from 0 to 50°C. The pH range suitable for growth was determined by cultivating the isolate on carbonate-free K and M media containing 3% NaCl. The pH of the media was adjusted to 6.0–12.0 by adding the necessary amount of 1 M NaOH. The ability of the isolate to utilize various organic compounds as sources of carbon and energy was studied at pH 8.0 after replacing methanol in the liquid M medium with methylamine, trimethylamine, fructose, formate, amino acids, alcohols, etc., taken at a concentration of 0.3 wt %. The flasks were incubated on a shaker at an optimum growth temperature for 14 days. Volatile compounds were added at a concentration of 0.5 vol %. The ability of the isolate to utilize methane was studied using 700-ml flasks containing 100 ml of the M medium. The flasks were sealed with rubber stoppers furnished with two cotton-wool traps. The headspace of the flasks was filled with a mixture of methane and air in a volume proportion of 1 : 1. The ability of the isolate to grow autotrophically was tested by incubating it in an atmosphere containing H_2, O_2, and CO_2 gases in a proportion of 7 : 2 : 1. The ability of the isolate to utilize various nitrogen sources was studied by replacing KNO_3 in the M medium with equivalent amounts of nitrogen sources. When $(NH_4)_2SO_4$ and methylamine were tested, the medium did not contain carbonates, and its pH was adjusted to 8.0 by adding 1 M NaOH. Vitamin requirements were studied using M medium containing thiamine (50 μg/l), biotin (50 μg/l), vitamin B_{12} (20 μg/l), or yeast extract (0.01 vol %). The control medium contained no vitamins.

The concentration of formate in the culture liquid was determined by ^{1}H-NMR and by an enzymatic method with a formate dehydrogenase isolated from *Candida boidinii* (Boehringer, Germany).

Electron microscopy. Whole cells and their thin sections were examined by routine electron microscopic methods [5].

Chemotaxonomic analysis. The cellular fatty acid and phospholipid profiles of strain Kr3 were studied according to the method described in [5]. Ubiquinones were extracted from the cells by the Collins method [9] and analyzed with a Finnigan MAT-8430 mass spectrometer (Germany).

In order to prepare exopolysaccharide (EPS), the cells from a late exponential culture were removed by centrifugation. The culture liquid was diluted twofold with water and again centrifuged. The supernatant was mixed with two volumes of cold acetone. The precipitate was washed with acetone and dried in vacuum. The amount of isolated EPS was determined gravimetrically. In order to determine the carbohydrate composition of the EPS, it was hydrolyzed in 1 M trifluoroacetic acid at 120°C for 6 h. The hydrolyzate was analyzed with a Biotronic C2000 sugar analyzer (Germany). To determine the amino acid composition of the EPS, it was hydrolyzed in 4 N HCl at 100°C for 12 h. The hydrolyzate was analyzed with a Microtechna T339 amino acid analyzer (Czech Republic).

In order to identify and quantify intracellular osmoprotectants, 100 mg of exponential-phase cells were suspended in 5 ml of methanol and the suspension was centrifuged at 4500 *g* for 40 min. The supernatant was evaporated using a rotary vacuum pump. The residue was analyzed with a high-resolution WP 80 SY NMR spectrometer (Bruker, Germany) [7, 10].

The enzymes were assayed by conventional methods [11, 12].

DNA isolation and analysis. DNA was isolated by the Marmur method [13]. The G+C content of the DNA was determined with a Beckman DU-8B spectrophotometer (United States) by the heat denaturation method at a heating rate of 0.6°C/min. The DNA of *Escherichia coli* K-12 was used as the standard. The DNA–DNA homology levels of strain Kr3 and the type

Table 1. The effect of the NaCl concentration in the medium on the level of various phospholipids in the cells of strain Kr3

Phospholipid	NaCl concentration, %			
	0	3	6	9
Phosphatidylethanolamine	51.4	48.0	47.0	37.3
Phosphatidylglycerol	37.0	40.7	34.8	44.0
Cardiolipin	11.6	11.3	18.2	18.7

Note: The cultivation temperature was 29°C. The content of particular phospholipids is expressed as a % of the total.

Table 2. The fatty acid composition of Kr3 cells grown at different temperatures

Fatty acids	Cultivation temperature, °C	
	4	29
Conventional		
$C_{12:0}$	2.9	3.6
$C_{14:0}$	0.1	1.3
$C_{15:0}$	0.6	0.7
$C_{16:0}$	15.0	31.0
$C_{16:1}$	50.6	36.0
$C_{17:0}$	0.4	0.4
$C_{17:1}$	0.5	0.2
$C_{17:2}$	0	0
$C_{18:0}$	0.5	0.8
$C_{18:1}$	29.1	15.5
Cyclic		
$C_{17:0}$	0	0.6
$C_{19:0}$	0	0
Hydroxy acids		
3-OH $C_{10:0}$	0.3	4.2
3-OH $C_{14:0}$	0	5.5
3-OH $C_{16:0}$	0	0.2

Note: The content of particular fatty acids is expressed as a % of the total.

cultures of the genus *Methylophaga* were determined from DNA–DNA reassociation values [14].

The 16S rRNA gene was amplified by the PCR method using the universal prokaryotic primers 27f and 1492r: 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAAGGTGATCCAGCTCGT-3', respectively. The PCR amplifications were carried out in a Hybaid thermal cycler (United Kingdom), with the initial DNA denaturation step at 95°C for 2 min, followed by 25 cycles of DNA denaturation at 94°C for 40 s, primer annealing at 60°C for 40 s, and primer extension at 72°C for 40 s, with the final extension step at 72°C for 4 min. The reaction mixture (30 μl) contained 1 μl DNA preparation and 5 pmol of the respective primer in 10 mM Tris–HCl buffer containing 68 mM $(NH_4)_2SO_4$, 1 mg/ml BSA, and 2.5 mM $MgCl_2$. The mixture was supplemented with dNTP (0.2 μM each) and 1 U *Taq* DNA polymerase. The reaction products were separated by electrophoresis in 1% agarose gel. The PCR product of the 16S rRNA gene was purified by electrophoresis in low-melting-point agarose with agarase in accordance with the manufacturer's protocol (Fermentas, Lithuania). The PCR fragment was sequenced with an automatic CEQ2000 XL sequenator (Beckman Coulter, United States) using a CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter).

Phylogenetic analysis. A preliminary phylogenetic screening of the nucleotide sequences of the 16S rRNA gene of strain Kr3 over the GenBank (NCBI) database was carried out with the aid of the BLAST program package (http://ncbi.nlm.nih.gov). In order to specify the phylogenetic position of strain Kr3, the nucleotide sequences of the 16S rRNA gene were manually aligned with the aid of the CLUSTAL W program (http://www.genebee.msu.su/clustal) using the latest relevant sequences available from the NCBI Database Project.

A rooted phylogenetic tree was constructed by the neighbor-joining method (NEIGHBOR) with the TREECON algorithm [15]. Evolutionary distances were calculated as the number of substitutions per 100 nucleotides. In order to assess the reliability of the branching points on the tree, the sequence data were subjected to bootstrap analysis with the aid of the TREECON program.

The nucleotide sequence of the 16S rDNA of strain Kr3 has been deposited in GenBank under accession number AY694421.

RESULTS

Cell morphology. The cells of strain Kr3 were found to be motile, gram-negative, non-spore-forming rods, 0.7×1.7–2.0 μm in size, with one polar flagellum. The cells multiplied by binary fission and had no complex system of intracytoplasmic membranes.

The physiological, biochemical, and chemotaxonomic properties of strain Kr3. The strain grew in liquid media without cell aggregation. Pigments were not produced. The strain required oxygen and vitamin B_{12} for growth. The colonies produced on agar medium with 0.5% methanol after 3 days of incubation at 29°C were round, smooth, even-edged, convex, homogeneous, nonpigmented, translucent, and 1 mm in diameter.

Strain Kr3 was found to be able to utilize methanol, methylamine, trimethylamine, and fructose as sources of carbon and energy and unable to utilize formaldehyde, formate, dimethyl sulfide, organic acids, amino acids, and C_2–C_6 alcohols. The strain could not grow on nutrient or malt extract agars, or in atmospheres of

($CH_4 + O_2$) and ($H_2 + O_2 + CO_2$). Gelatin and starch were not hydrolyzed. Ammonia and hydrogen sulfide were not produced on the test media. Indole was produced from L-tryptophan. The cells were oxidase- and catalase-positive. Nitrates were reduced to nitrites. The cells were able to utilize nitrates, glutamate, urea, methylamine, and ammonium ions in media containing 3% NaCl or 3% Na_2SO_4 at a pH equal to or less than 8.0. The strain was resistant to ampicillin, gentamicin, kanamycin, lincomycin, nalidixic acid, and novobiocin (10–50 μg per disc) but sensitive to streptomycin (10 μg per disc), neomycin (30 μg per disc), and erythromycin (15 μg per disc). The isolate could grow in liquid media at temperatures between 4 and 42°C (with the optimum temperatures being 20–32°C) and pH values between 6 and 11 (with optimum at 8–9) and obligately required Na^+ ions for growth, as is evident from the fact that growth was not observed in media in which the sodium salts were replaced with potassium, magnesium, or lithium salts. At the same time, the isolate did not require Cl^- ions for growth, as is evident from the fact that growth was observed in neutral media in which NaCl was replaced with Na_2SO_4 and in alkaline media in which NaCl was replaced with sodium carbonates. The strain was found to be able to grow at NaCl concentrations of up to 20% (with the optimum at 3–9% NaCl), suggesting that it is a moderate halophile.

The strain grew rather well at low temperatures. For comparison, in the medium containing 0.5–1.5 M NaCl (pH 8–9), $\mu = 0.3$ and 0.04 h^{-1} at 29 and 4°C, respectively. On the agar media, the strain even grew at 0°C. The methanol concentrations suitable for growth were found to be 0.02–7 vol % (the optimal concentration of methanol is 0.5 vol %). The strain produced EPS composed of carbohydrate and protein moieties. The carbohydrate moiety was primarily found to contain arabinose and glucose, as well as some galactose, mannose, rhamnose, and ribose. Analysis showed that the main components of the protein moiety are glutamate and asparagine, with the minor components being lysine, histidine, arginine, threonine, serine, glycine, alanine, valine, isoleucine, leucine, tyrosine, and phenylalanine. When growth was limited by oxygen or sources of nitrogen, phosphorus, or iron, the EPS yield increased from 0.1 to 2.3 g/g biomass. The strain produced formic acid from methanol in amounts of 4–10 mM, acidifying the cultivation medium.

Ubiquinone Q_8 was found to be the major quinone. Cellular phospholipids were dominated by phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. Experiments with ^{14}C-methanol showed that the level of phosphatidylglycerol and cardiolipin rises, whereas that of phosphatidylethanolamine declines as the medium salinity increases (Table 1). The major fatty acids were palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), and octadecenoic ($C_{18:1}$) acids. The cells grown at 29°C were dominated by saturated fatty acids, whereas the cells grown at 4°C were dominated by the unsaturated fatty acids $C_{16:1}$ and $C_{18:1}$ (Table 2).

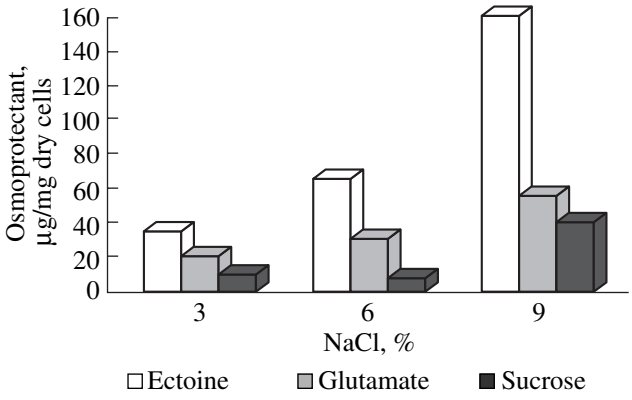

Fig. 1. The effect of the NaCl concentration in the medium on the accumulation of osmoprotectants in the cells of strain Kr3.

The osmolarity of the medium and the cultivation temperature influenced the intracellular content of some of the low-molecular-weight organic metabolites. Ectoine (2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) and glutamate were found to be the major, and sucrose the minor, osmoprotectants. The concentration of these compounds in the cells rose as the osmolarity of the medium increased (Fig. 1) and the cultivation temperature decreased. For instance, the intracellular content of ectoine at 4°C was two times higher than at 29°C. The cells grown at high NaCl concentrations (6–9%) were found to withstand heating at 70°C for 15–20 min, repeated freezing–thawing cycles, and lyophilization without cryoprotectants, which was probably due to the high content of ectoine in such cells.

The assay of enzymes in the methanol-grown cells of strain Kr3 (Table 3) showed the presence of methanol dehydrogenase, formaldehyde dehydrogenase, formate dehydrogenase, and 3-hexulose-6-phosphate synthase (the key enzyme of the ribulose monophosphate RuMP pathway), as well as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, transaldolase, transketolase, and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase. Enzymes specific to the serine pathway (hydroxypyruvate reductase and serine–glyoxylate aminotransferase) and the Calvin cycle (ribulose bisphosphate carboxylase) were not detected. Consequently, strain Kr3 implements the KDPG variant of the RuMP pathway. Pyruvate kinase, 2-oxoglutarate dehydrogenase, 6-phosphofructokinase, fructose-bisphosphate aldolase, isocitrate lyase, and malate synthase were not detected either. Ammonium ions were assimilated through the reductive amination of 2-oxoglutarate and via the glutamate cycle with the involvement of glutamate synthase/glutamine synthetase.

The extracts of methylamine- or trimethylamine-grown cells showed the presence of inducible dehydrogenases of methylamine and trimethylamine, which exhibited activity of 449 and 327 nmol/(min mg protein),

Table 3. Enzyme activities in the extracts of Kr3 cells grown on methanol

Enzyme	Cofactor	Activity, nmol/(min mg protein)
Methanol dehydrogenase	PMS	110
Formaldehyde dehydrogenase	PMS	34
	NAD^+	0
	NAD^+, GSH	0
Formate dehydrogenase	PMS	20
	NAD^+	0
Hydroxypyruvate reductase	NAD(P)H	0
Serine–glyoxylate aminotransferase	NAD(P)H	0
Ribulose bisphosphate carboxylase		0
3-Hexulose-6-phosphate synthase		346
Glucose-6-phosphate dehydrogenase	NAD^+	460
	$NADP^+$	2076
6-Phosphogluconate dehydrogenase	NAD^+	0
	$NADP^+$	119
2-Keto-3-deoxy-6-phosphogluconate aldolase		64
Fructose-bisphosphate aldolase	ATP, PP_i	0
6-Phosphofructokinase		0
Transaldolase		73
Transketolase		252
Citrate synthase		45
α-Oxoglutarate dehydrogenase	NAD^+	0
Isocitrate dehydrogenase	NAD^+	182
Isocitrate lyase		0
Malate synthase		0
Glutamate dehydrogenase	NADH	61
	NADPH	12
Glutamine synthetase	ATP, Mn^{2+}	1069
Glutamate synthase	NADH	44
	NADPH	0

Note: PMS and GSH stand for phenazine methosulfate and reduced glutathione, respectively.

respectively. The enzymes of the *N*-methylglutamate pathway (*N*-methylglutamate dehydrogenase and γ-glutamylmethylamide lyase) were not detected.

Genotypic characterization. The 16S rRNA gene sequencing showed that strain Kr3 belongs to the class *Gammaproteobacteria* (87–93% homology level) and has a 94.3–96.2% level of homology to the known species of the genus *Methylophaga.* The G+C content of the DNA was found to be 44.6 mol %. The DNA–DNA homology levels of strain Kr3 to the type representatives of the genus *Methylophaga* (*M. marina* ATCC 35842^T, *M. thalassica* ATCC 33146^T, *M. sulfidovorans* RB1 LMD 95.210^T, *M. alcalica* ATCC BAA-297^T, and *M. natronica* VKM B-2288^T) ranged from 23 to 41%, suggesting that strain Kr3 belongs to this genus.

DISCUSSION

Although the interaction of the microorganisms occurring in biofilms on the surface of stone has been extensively studied, many aspects of this interaction remain unknown. The surface of stones have been found to contain methanotrophs from the genera *Methylocystis* and *Methylosinus.* The concentration of methane in the atmosphere (1.7 ppm) is insufficient to maintain the growth of these bacteria. However, the local concentration of methane in masonry may be higher due to the metabolism of methanogenic microorganisms [16]. Methanotrophs can excrete methanol (an intermediate of methane oxidation) into the environment [17]. Plants are also a global source of atmospheric methanol, which comprises 40–46% (or more than 100 million tons per year) of the total emission of

Table 4. General and differentiating characteristics of bacteria from the genus *Methylophaga*

Characteristic	Strain Kr3	*M. marina* ATCC 35842^T	*M. thalassica* ATCC 33146^T	*M. sulfidovorans* LMD 95.210^T	*M. alcalica* VKM B-2251^T	*M. natronica* VKM B-2288^T
Cell size, μm	0.7 × 1.7–2.0	0.2 × 1	0.2 × 1	0.2 × 0.9	0.4 × 1.2–2.8	0.6 × 2.8
Type of methylotrophy	Restricted facultative	Restricted facultative	Restricted facultative	Obligate	Obligate	Restricted facultative
Utilization of						
Fructose	+	+	+	–	–	+
Methanol	+	+	+	+	+	+
Methylamine	+	+	+	+	+	+
Dimethylamine	–	–	–	+	–	–
Trimethylamine	+	–	–	–	–	–
Dimethyl sulfide	–	–	–	+	–	–
C_1 assimilation pathway	RuMP	RuMP	RuMP	RuMP	RuMP	RuMP
Krebs cycle	Incomplete	ND	ND	ND	Incomplete	Incomplete
Vitamin requirement	B_{12}	B_{12}	B_{12}	B_{12}	B_{12}	B_{12}
Reduction of NO_3^- to NO_2^-	+	–	–	ND	+	+
Temperature range (optimum), °C	0–42 (20–32)	10–40 (30–37)	8–42 (30–37)	17–35 (22)	4–35 (25–29)	4–37 (25–29)
pH range (optimum)	6.0–11.0 (8.0–9.0)	5.0–9.0 (7.0–7.5)	5.0–9.0 (7.0–7.5)	6.0–9.0 (7.4–7.8)	7.0–11.0 (9.0–9.5)	7.0–11.0 (8.5–9.0)
Maximum NaCl concentration (optimum), %	20 (3–9)	12 (1–4)	12 (1–4)	12 (1.5–2.5)	10 (3–4)	10 (2–3)
Major ubiquinone	Q_8	Q_8	Q_8	Q_8	Q_8	Q_8
Major fatty acids	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$	$C_{16:0}$, $C_{16:1}$	$C_{16:0}$, $C_{16:1}$	ND	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$
G + C content, mol %	44.6	43.0	44.0	42.4	48.3	45.0

Note: ND stands for "not determined". The symbols "+" and "–" indicate, respectively, the presence and the absence of a particular property.

organic carbon into the atmosphere [18]. This methanol may provide for the growth of the methylobacteria occurring in biofilms on the surface of stone.

Strain Kr3, which was isolated from deteriorating marble, is a new haloalkaliphilic restricted facultative methylotroph implementing the RuMP pathway of C_1 metabolism. *Methylophaga* is the only known genus of moderately halophilic, aerobic, gram-negative methylobacteria with the RuMP pathway. Neutrophilic representatives of this genus (*M. marina, M. thalassica* [3], and *M. sulfidovorans* [4]) have been isolated from seawater, whereas the alkaliphilic species *M. alcalica* [5] and *M. natronica* [6] have been isolated from soda lakes.

Bacteria of the genus *Methylophaga*, which belongs to *Gammaproteobacteria*, are obligate or restricted facultative methylotrophs. They have no 2-oxoglutarate dehydrogenase, depend on vitamin B_{12}, possess ubiquinone Q_8 and fatty acids $C_{16:0}$ and $C_{16:1}$, and are characterized by a low G+C content of DNA (43–49 mol %) (Table 4). According to all these characteristics, strain Kr3 is close to the methylobacteria of the genus *Methylophaga.* Strain Kr3 is also close to the type cultures of the genus *Methylophaga* according to DNA–DNA homology levels (23–41%) and 16S rDNA sequencing data (94–96% homology). However, unlike the known *Methylophaga* species, the new isolate grows in wider ranges of pH, NaCl concentration, and temperature (Table 4); i.e., it is more adaptable to variations in the environment. Like the alkaliphilic species *M. alcalica* and *M. natronica* [5, 6], strain Kr3 synthesizes the bioprotectants ectoine, glutamate, and sucrose. The content of these compounds in the Kr3 cells depends not only on the osmolarity of the medium but also on the cultivation temperature.

The discovery that Kr3 cells with a high content of ectoine withstand heating at 70°C, repeated freezing–thawing cycles, and lyophilization suggests that ectoine serves as a thermo- and cryoprotectant. It is known that the heterocyclic amino acid ectoine exerts a profound stabilizing effect on biopolymers (proteins and DNA) and whole cells and possesses a high water-bearing capacity. When cells of strain Kr3 are grown at low temperatures, they are distinguished by a relatively

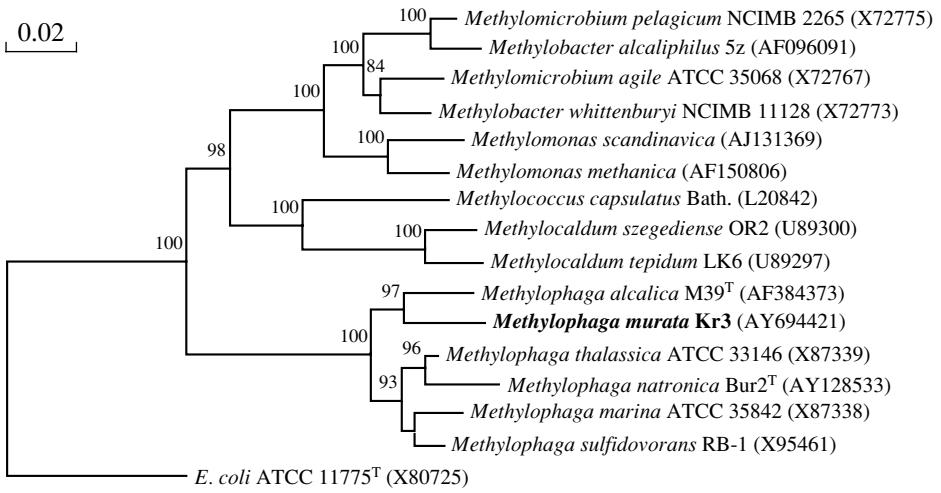

Fig. 2. The phylogenetic position of *Methylophaga murata* Kr3 among the *Gammaproteobacteria* methylotrophs. The scale bar corresponds to two nucleotide substitutions per 100 nucleotides. The statistical significance of the branching points on the tree was assessed by bootstrap analysis.

high content of unsaturated fatty acids. In bacteria, fatty acids mainly occur in membrane phospholipids. The membranes that contain unsaturated phospholipids in greater amounts pass from a liquid to crystalline state at lower temperatures than membranes that contain unsaturated phospholipids in smaller amounts. Since the fluidity of the membranes determines their functional activity, the changes observed in the fatty acid composition of strain Kr3 may represent a homeostatic mechanism.

The accumulation of negatively charged phosphatidylglycerol and cardiolipin in cells grown at high medium salinities against the background of a diminished level of phosphatidylethanolamine may further stabilize the membranes of strain Kr3.

Strain Kr3 responds to a deficiency of nitrogen, phosphorus, iron, and oxygen by enhancing the synthesis of EPS, which stabilizes the cells and prevents them from drying.

The formic acid that is produced by strain Kr3 in relatively large amounts (up to 10 mM) acidifies the medium and may cause the erosion of marble. It can be suggested that the new methylotroph is well-adapted to life on the surface of stone, which is exposed to considerable variations in ambient temperature, pH, and salinity over the course of time.

The pheno- and genotypic differences between strain Kr3 and the known representatives of the genus *Methylophaga* make it possible to consider this strain as a new species, which has been named *Methylophaga murata.* The phylogenetic position of *M. murata* Kr3 among the known *Methylophaga* species and other members of the class *Gammaproteobacteria* is shown in Fig. 2.

The description of the new species is as follows:

Methylophaga murata nov. sp. (mu'ra. ta. L. adj. *murata*, stone). Cells are motile, gram-negative, nonspore-forming rods, 0.7×1.7–2.0 μm in size, with one polar flagellum. They multiply by binary fission and do not have a complex system of intracytoplasmic membranes. Growth is strictly aerobic. No cell aggregation occurs in liquid media. Pigments are not produced. Vitamin B_{12} and Na^+ ions are required for growth. Colonies produced on agar medium with 0.5% methanol are round, smooth, even-edged, convex, homogeneous, nonpigmented, translucent, and 1 mm in diameter. Growth occurs at temperatures between 0 and 42°C (with optimal growth at 20–32°C), pH values between 6 and 11 (with optimal growth at 8–9), NaCl concentrations between 0.3 and 20% (with optimal growth at 3–9% NaCl), and methanol concentrations between 0.02 and 7 vol % (with optimal growth at 0.5 vol % methanol). Formic acid is produced from methanol. Oxidase and catalase tests are positive. Nitrates are reduced to nitrites. Methanol, methylamine, trimethylamine, and fructose are utilized as sources of carbon and energy. Formaldehyde, formate, dimethyl sulfide, organic acids, amino acids, and C_2–C_6 alcohols are not utilized. No growth occurs on nutrient or malt extract agars or in the atmospheres of $CH_4 + O_2$ or $H_2 + O_2 + CO_2$. Gelatin and starch are not hydrolyzed. Ammonia and hydrogen sulfide are not produced on test media. Indole is produced from L-tryptophan. Nitrates, glutamate, and urea (as well as methylamine and ammonium ions in NaCl-containing media at pH values not higher than 8.0) are utilized as nitrogen sources.

The 2-keto-3-deoxy-6-phosphogluconate variant of the ribulose monophosphate pathway is operative. The Krebs cycle is opened at the level of 2-oxoglutarate dehydrogenase. Pyruvate kinase and the glyoxylate cycle enzymes isocitrate lyase and malate synthase are absent. Ammonium ions are assimilated through the

reductive amination of 2-oxoglutarate and via the glutamate cycle. The major ubiquinone is Q_8. Cellular phospholipids are dominated by phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. The major fatty acids are palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), and octadecenoic ($C_{18:1}$) acids. Ectoine and glutamate are the major osmoprotectants, and sucrose is as a minor osmoprotectant. Cells grown in the presence of 6–9% NaCl are resistant to heating at 70°C, freezing, and drying. The G+C content of the DNA is 44.6 mol %. Type strain *M. murata* $Kr3^T$ has been isolated from material scraped off the deteriorating part of a marble crypt in the Moscow Kremlin. The type strain is deposited in the All-Russia Collection of Microorganisms as VKM B-2303 and the National Collection of Industrial and Marine Bacteria as NCIMB 13993.

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