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EXPERIMENTAL ARTICLES

Emended Description of *Methylomonas rubra* sp. nov.

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Abstract—Strain *Methylomonas rubra* 15sh^T, deposited in several collections of microorganisms (NCIMB 11913^T, = UCM B-3075^T = ACM 3303^T), is the subject of numerous studies. However, the name of this strain is not valid, primarily due to the phenotypic similarity of t– species *M. rubra* to the species *M. methanica.* The results of the present study and data available in the literature indicate that *M. rubra* deserves the status of a separate species. Strains of *M. rubra* differ from strains of *M. methanica* in a number of properties, such as the ability to reduce nitrates to nitrites, the structure of intracytoplasmic membranes, and the presence of a new coenzyme Q. The distinctions between the species *M. rubra* and *M. methanica* were confirmed by comparison of electrophoretic patterns of their cellular proteins, by results of DNA–DNA hybridization, and by the data from 16S rRNA gene sequencing (the level of phylogenetic homology between these two species was below 95%). Phylogenetic and phenotypic analyses showed that strains of *M. rubra* cannot be assigned to any species of the genus *Methylomonas.* Results of polyphasic analysis suggest an independent taxonomic status of strain *Methylomonas rubra* 15sh^T. This paper contains description of *Methylomonas rubra* sp. nov. with the type strain 15sh^T = NCIMB 11913^T = UCM B-3075^T = ACM 3303^T. The nucleotide sequence of the 16S rRNA gene of strain 15sh^T has been deposited in the GenBank database under the accession number AY995198.

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Key words: Methylomonas rubra sp. nov., species description, obligate methane-oxidizing bacteria.

The first strain of *Methylomonas rubra* was isolated in 1970 [1]; however, the description of this species was incomplete, and no attempt to validate it was undertaken. Later, we isolated strains that corresponded to the description of this species; a formalized description of the species *Methylomonas rubra* was provided, and strain 15sh^T was proposed as the type strain [2]. Strain *Methylomonas rubra* 15sh^T has been deposited in several collections of microorganisms under designations NCIMB 11913^T, UCM B-3075^T, and ACM 3303^T and still remains the object of studies [3–7]. Since the name *Methylomonas rubra* is not valid, we carried out phylogenetic analysis and provide an amended description of this species with the aim to validate it.

MATERIALS AND METHODS

The objects of study were strains of obligate methane-oxidizing bacteria isolated from drainage waters of a coal-mine in Donets Basin and deposited in the Ukrainian Collection of Microorganisms (UCM) under the designations UCM B-3075^T (= 15sh^T) and UCM B-3005 [8].

Isolation of DNA. Bacteria were cultivated as described earlier [9]. Cellular DNA was isolated according to the method described in the manual by Gerhardt et al. [10]. The purity of the DNA preparation was tested spectrophotometrically at 260, 230, and 280 nm [11] and by horizontal electrophoresis in agarose gel [12].

PCR amplification and 16S rRNA gene sequencing. The 16S rRNA genes were amplified using oligonucleotide primers (27f and 1492r) universal for most eubacteria [13]. PCR was run on a Gene Amp PCR System 2400 thermal cycler (Perkin Elmer) using a DTCS Master Mix standard kit supplied together with a CEQ 2000XL sequencer (Beckman Coulter). The temperature program of the amplification involved 30 cycles of DNA denaturation at 96°C for 20 s, primer annealing at 50°C for 20 s, and primer extension at 60°C for 4 min. Sequencing of the amplified 16S rRNA genes was performed in both directions with the help of forward and reverse primers (27f and 1492r) on a CEQ 2000XL sequencer (Beckman Coulter) using the accompanying CEQ DTCS Kit according to the manufacturer's recommendations.

Phylogenetic analysis was carried out using the 16S rRNA gene sequences of the type strains of obligate methane-oxidizing bacteria of the family *Methylococcaceae* (figure). Comparative analysis of nucleotide sequences of the 16S rRNA genes of various strains belonging to the genus *Methylomonas* was performed

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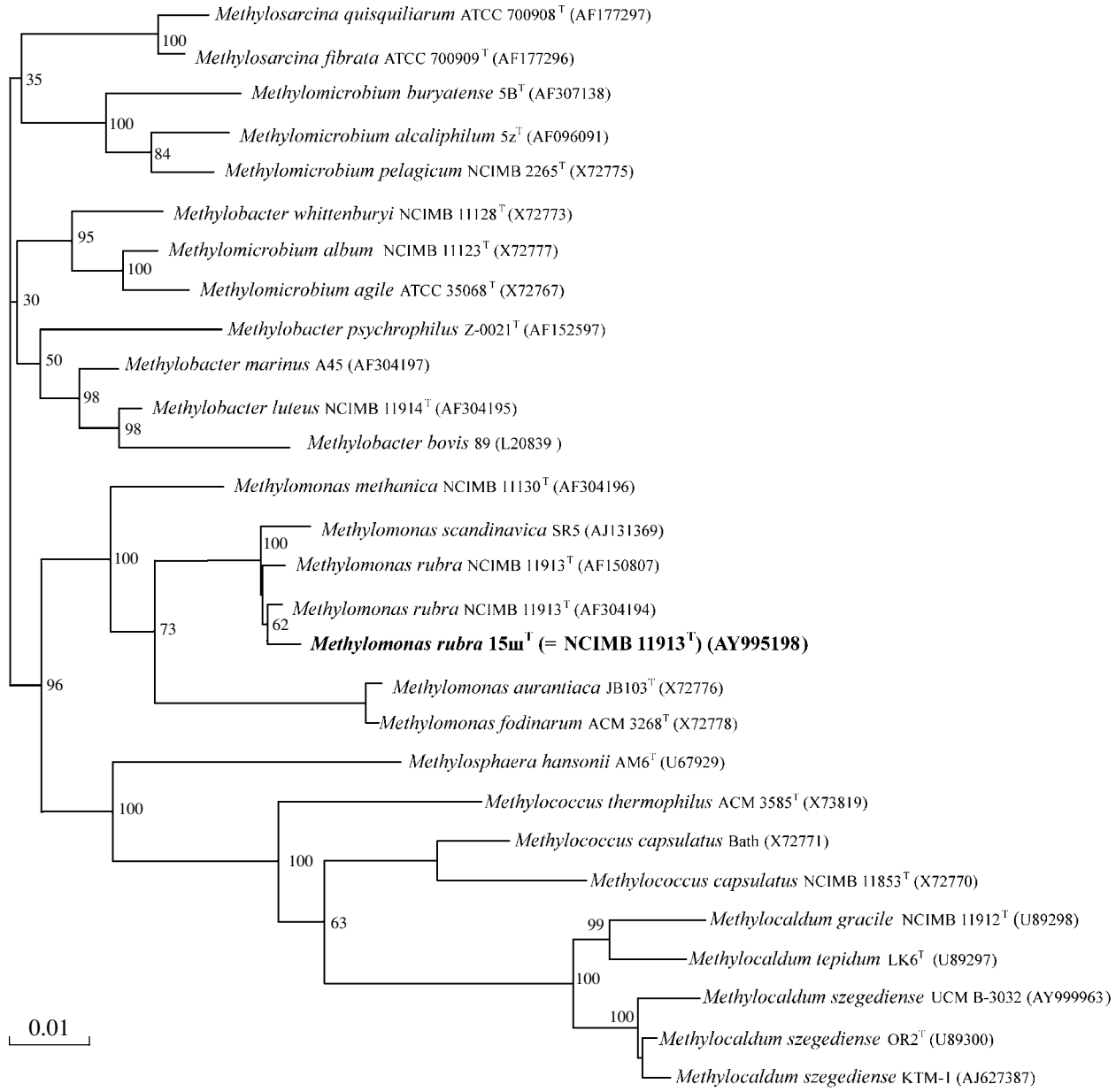

Position of methane-oxidizing bacteria of the genus *Methylomonas* in the phylogenetic tree of the family *Methylococcaceae* (class *Gammaproteobacteria*). The scale bar corresponds to 1 nucleotide substitution per 100 nucleotides.

with the use of the BLASTN (version 2.2.4) software package. The construction of a 16S rDNA-based phylogenetic tree was performed with the use of various algorithms implemented in the TreeView (version 1.5.2) and ClustalX (version 1.81) software packages. To assess the reliability of branching order in the tree, bootstrap values were derived by analyzing 1000 alternative trees.

RESULTS AND DISCUSSION

The properties of strains UCM B-3075^T and UCM B-3005, which were preliminarily assigned to the species *Methylomonas rubra*, have been described in our previous work [2]. These strains utilize only methane as the carbon substrate, which is assimilated via the ribulose monophosphate pathway; they are characterized by predominance of monounsaturated fatty acids with 16 carbon atoms and possess a system of intracytoplasmic membranes (ICMs) of type I. Based on these properties, the strains under study were assigned to the family *Methylococcaceae*. Reasoning from the morphological features of the cells, the presence of a pink pigment, and the fatty acid composition, these strains were assigned to the genus *Methylomonas*. However,

Table 1. Coefficients of similarity between the sequences of the 16S rRNA gene of *Methylococcus rubra* 15sh^T determined by different authors

no.	Strain 15sh^T clones	GenBank accession number	Coefficients of similarity,%
1	UCM B-3075^T	AY995198	99.1
	NCIMB 11913^T	AF304194	
2	UCM B-3075^T	AY995198	99.0
	clone pAMC265	AF150807	
3	NCIMB 11913^T	AF304194	99.5
	clone pAMC265	AF150807	

Note: The 16S rRNA gene sequences of NCIMB 11913^T [5] and pAMC265 [4] were taken from the GenBank database. The 16S rRNA gene sequence of strain 15sh^T (= UCM B-3075^T) was determined in the present work.

phenotypically, the species *Methylomonas rubra* and *M. methanica* are very similar.

Further studies revealed that the species *M. rubra* and *M. methanica* exhibit different electrophoretic patterns of cellular proteins [14]. These species are characterized by different composition of respiratory quinones; in particular, strain *M. rubra* NCIMB 11913^T (= UCM B-3075^T) contains 11-methylene-18-dimethyl-ubiquinone-6 (MQ-6), whereas *M. methanica* contains 18-methylene-Q-8 (MQ-8) [15]. Although the physiological role of the novel quinone that was revealed in *M. rubra* remains unknown, the authors suggested that it fulfills a respiratory function similar to that of ubiquinones. We believe that the distinctions between *M. rubra* and *M. methanica* in the composition of coenzymes Q are an additional reason to consider them to be independent species. Hybridization of DNA from *M. rubra* with DNA from several strains of *M. methanica* showed a low level of DNA–DNA homology (12–28%) [16], which prevents assignation of these strains to one species. The species *M. rubra* and *M. methanica* are also different in the structure of ICMs; in particular, *M. methanica* has usual type I ICMs, represented by bundles tightly packed along the longitudinal axis of the cell, whereas in *M. rubra*, the type I ICMs are oriented radially and have several initial points of formation [14].

To verify the taxonomic status of the species *M. rubra*, we performed 16S rRNA gene sequencing. The DNA preparation was isolated from biomass of strain UCM B-3075^T; by using horizontal electrophoresis, it was proved that the preparation contained no low-molecular-weight nucleotides and was suitable for further work. The 16S rRNA gene was amplified by PCR and then sequenced. The nucleotide sequence of the 16S rRNA gene of strain UCM B-3075^T was deposited in GenBank under the accession number AY995198. Comparative analysis of the data on 16S rRNA gene sequencing of strain *Methylomonas rubra* 15sh^T (Table 1) showed that the discrepancy between the results obtained by different authors was about 0.5%, which is within the accuracy of the analysis.

The phylogenetic relatedness of strain 15sh^T to *Methylomonas* species and other taxa of the family *Methylomonas* is shown in the dendrogram (figure). Species of the genus *Methylomonas* formed a separate cluster; the similarity levels between species ranged from 94.6 to 99.4%. *M. rubra* and *M. methanica* formed separate branches in this cluster. As seen from Table 2, strains of *M. rubra* differed from strains of *M. methanica* in their ability to reduce nitrates to nitrites, a different structure of ICMs, and the presence

Table 2. Differentiating properties of the species of the genus *Methylomonas*

Species	Pigmentation			Growth at		Nitrate reduction	Phosphatase activity	ICM structure (type I)	The G+C of DNA, mol %	Isoprenoid quinones
	pink	red	orange	15°C	37°C					
M. rubra	+	+	–	–	+	+	ND	Radial orientation[a]	51–52	2MQ-6[b]
M. methanica	+	–	–	–	B	–	–	Bundles along the cell axis	51–53	3MQ-8[c]
M. fodinarum	–	–	+	–	–	+	–	Bundles along the cell axis	58–59	ND
M. aurantiaca	–	–	+	–	+	–	+	Bundles along the cell axis	55–56	ND
M. scandinavica	+	–	–	+	–	ND	ND	Packages of vesicles	53.3	MQ-8

Note: The properties of *M. methanica*, *M. fodinarum*, and *M. aurantiaca* are cited from [17], and those of *M. scandinavica* are cited from [18]. "ND" means not determined; "+," "–," and "V" stand for positive, negative, and variable, respectively.

[a] Several initial points of ICM formation occur [14].

[b] MQ-6 is 11-methylene-18-dimethyl-ubiquinone-6 [15].

[c] MQ-8 is 18-methylene-ubiquinone-8 [15].

of a specific coenzyme Q (11-methylene-18-dimethyl-ubiquinone-6). As mentioned above, the distinctions between the species *M. rubra* and *M. methanica* were confirmed by analyses of electrophoretic patterns of their cellular proteins and data from DNA–DNA hybridization and sequencing of 16S rRNA genes. The species *M. rubra* and *M. methanica* exhibited a low level of 16S rRNA gene homology (94.9%). All the above is indicative of the independent taxonomic status of strain *M. rubra* 15sh^T

The species most closely related to *M. rubra* proved to be *M. scandinavica* (with a similarity level of the 16S rRNA genes of 97.7%); however, this species differs from *M. rubra* in a number of phenotypic properties. Unlike *M. rubra*, *M. scandinavica* is a psychrotrophic bacterium with the temperature optimum of 15°C; no growth was observed at 37°C; its ICMs are represented by packages of vesicles; and its cells contain ubiquinone MQ-8 (Table 2). Strain *M. rubra* 15sh^T is phylogenetically distinct from strains *M. fodinarum* ACM 3268^T and *M. aurantiaca* JB103^T (94.9 and 94.5% similarity levels, respectively) and differs from them phenotypically (Table 2); therefore, strain 15sh^T cannot be assigned to these species. Thus, the results of polyphasic analysis provide strong evidence for the assignment of *M. rubra* to a separate species.

In the dendrogram (the figure), the genera *Methylobacter, Methylocaldum, Methylococcus, Methylomicrobium, Methylomonas, Methylosarcina*, and *Methylosphaera* formed separate clusters. However, some aspects of the tree topology were at odds with the currently accepted taxonomy. In particular, the type strains of the species *Methylomonas aurantiaca* and *M. fodinarum* showed a high similarity level of their 16S rRNA gene sequences (99.4%). These species are similar in their phenotypic properties and, according to Bowman et al. [17], they exhibit a rather high level of DNA–DNA hybridization (83–47%). All these data are indicative of strain-level rather than species-level distinctions between *M. aurantiaca* and *M. fodinarum*.

Another problem is posed by the genera *Methylobacter* and *Methylomicrobium*, which, in addition to two separate clusters, also formed a cluster common for both genera (figure). This common cluster included the type strains *Methylobacter whittenburyi* NCIMB 11128^T, *Methylomicrobium agile* ATCC 35068^T, and *Methylomicrobium album* NCIMB 11123^T. The genera *Methylomicrobium* and *Methylobacter* exhibit certain distinctions in the morphology of vegetative cells, colony pigmentation, and content of the prevalent monounsaturated fatty acids with 16 carbon atoms [19]. Species of these genera have been repeatedly reclassified. In particular, strain *Methylomicrobium agile* ATCC 35068^T (A30) [19] was earlier described as *Methylomonas agile* A30 [1] and as *Methylobacter agilis* NCIMB 11124^T (= A30 = ACM 3308 = ATCC 35068^T) [17]; strain *Methylomicrobium album* NCIMB 11123^T (= ACM 3314 = IMET 10526 = BG8) [19] was designated as *Methylomonas albus* BG8 [1] and as *Methylobacter albus* BG8 (= ACM 3314) [17]. Apparently, the taxonomic status of some species assigned to the genera *Methylobacter* and *Methylomicrobium* needs further refinement.

As a rule, the genera of methylotrophs that have been classified on the basis of sequence analysis of 16S rRNA genes, e.g., *Methylocaldum* [20] and *Methylosarcina* [21], combine species with a high level of phylogenetic relatedness. At the same time, several genera of methylotrophs, such as *Methylococcus*, *Methylomonas*, and *Methylobacter*, were described on the basis of their phenotypic properties [1, 2, 14, 17], and phylogenetic analysis of their 16S rRNA genes allowed the taxonomic status of some of them to be refined.

Description of Methylomonas rubra sp. nov. (ex Romanovskaya, Malashenko, and Bogachenko 1978, 120)

rub'ra. F. L. adj. *rubra*, red. Synonym: *Methylomonas rubrum* (sic) Whittenbury et al 1970, 205.

Cells are gram-negative rods (0.6–0.8 × 1.2–1.8 μm), occurring singly or in pairs and motile by means of a single polar flagellum. No capsule or spores have been observed. Reproduction occurs by binary cell fission. Cells contain well-developed type I intracytoplasmic membranes, which are radially oriented and have several initial points of formation. Resting forms (cysts) occur only in the stationary growth phase; they are sensitive to heating and drying. Colonies are pink to red in color, round, slightly convex, with even or uneven edges, oily or slimy, from 2 to 6 mm in diameter. When grown in liquid medium, cells form homogeneous or sometimes flocculent suspension; no pigment is released into the medium. Methane carbon is assimilated via the ribulose monophosphate pathway; low activity of hydroxypyruvate reductase (the key enzyme of the serine pathway) is also detectable. NADP- or NAD-dependent isocitrate dehydrogenase is present. Only soluble methane monooxygenase is present [22]. Methanol (0.03–0.06%) can also be utilized. Cells contain 11-methylene-18-dimethyl-ubiquinone-6 (MQ-6) [15]. The predominant fatty acids of the cell envelope are $C_{14:0}$ and $C_{16:1}$. Methane-grown cells are able to reduce nitrates to nitrites. Growth occurs within the temperature range from 20 to 37°C and pH range from 6.2 to 8.0, with the optima at 28–30°C and pH 6.5–7.0. The G+C content of DNA is 51–52 mol%. Other characteristics are consistent with those given in the description of the genus M*ethylomonas* and the family *Methylococcaceae* [17].

The type strain *Methylomonas rubra* 15sh^T (= NCIMB 11913^T = UCM B-3075^T = ACM 3303^T) was isolated from drainage waters of a coal mine (Donets Basin, Ukraine) [2]. The nucleotide sequence of the 16S rRNA gene of strain 15sh^T has been deposited in GenBank under the accession number

AY995198. A reference strain is UCM B-3005 (= IMV B-3005 = 2t).

Thus, this paper presents a description of the species *Methylomonas rubra* sp. nov. (the type strain 15sh^T = NCIMB 11913^T = UCM B-3075^T), which belongs to the family *Methylococcaceae*, the order *Methylococcales*, class *Gammaproteobacteria*.

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