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SHORT COMMUNICATIONS

Identification of *Methylobacterium* Strains Using Sequence Analysis of 16S rRNA Genes

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Lack of phenotypic characteristics that differentiate *Methylobacterium* species hinders species identification of the bacteria of this genus. The aim of the present work was to identify new isolates of methylotrophic bacteria using the analysis of the nucleotide sequences of their 16S rRNA genes. While doing this, we adhered the recommendations of the International Committee, according to which the number of unidentified nucleotides should not exceed 0.5% of their total number [1].

The subjects of study were methylotrophic bacteria that we isolated from the soil of the Chernobyl Nuclear Power Plant (UCM B-3362 and UCM B-3389) or from plant phyllosphere: UCM B-3368 and UCM B-3383 (*Vitia* sp. leaves) and UCM B-3360 (*Trifolium pratense* leaves) [2, 3]. Preliminary identification of bacteria was performed based on the study of their morphological–cultural and physiological–biochemical properties by commonly accepted methods described in the manual by Gerhardt *et al.* [4] and our earlier paper [2].

Isolation of cellular DNA was performed as described in [5], and amplification of 16S rDNA according to Lane, using oligonucleotide primers (37f and 1492r) universal for most eubacteria [6]. 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 reverse and forward primers (27f and 1492r) on the CEQ 2000XL sequencer (Beckman Coulter), using the accompanying CEQ DTCS Kit according to the manufacturer's recommendations. For comparative phylogenetic analysis, we retrieved from GenBank the 16S rRNA gene sequences of 14 species of *Methylobacterium: M. organophilum* JCM 2833^T (D32226), *M. extorquens* JCM 2802^T (D32224), *M. rhodesianum* JCM 2810^T (D32228), *M. zatmanii* JCM 2819 (D32230), *M. rhodinum* JCM 2811^T (D32229), *M. mesophilicum* JCM 2829^T (AJ400919), *M. radiotolerans* JCM 2831^T (D32227), *M. fujiawaense* DSM 5686 (AJ250801), *M. suomiense* NCIMB13778^T (AY009404), *M. lusitanum* NCIMB13779^T (AY009403), *M. dichloromethanicum* DSM6343^T (AF227128), *M. nodulans* ORS1924 (AF220762), *M. chloromethanicum* CM4 (AF198624), and *M. podarium* (AF514774).

Based on the main diagnostic features, the pink facultative methanol-utilizing bacteria that we isolated from soil and plant phyllosphere were assigned to the genus *Methylobacterium.* Investigation of their morphological–cultural and physiological–biochemical properties allowed strains UCM B-3360 and UCM B-3368 to be identified as *M. extorquens.* The species affiliation of the other strains was not established. From the bacterial biomass, DNA samples were isolated, which were sufficiently pure for further work as determined by horizontal electrophoresis [7] and spectrophotometry [8]. PCR allowed us to obtain amplificates of 16S rRNA genes, which were further sequenced. The nucleotide sequences determined were deposited with GenBank under the following accession numbers: strain UCM B-3360, accession number AY460189; UCM B-3362, AY365229; UCM B-3368, AY366072; UCM B-3383, AY366073; and UCM B-3389, AY460187.

Comparison of the nucleotide sequences of 16S rRNA genes of the bacteria studied with sequences available from GenBank, performed with the use of the BLASTN 2.2.4 software, showed that all of them belonged to the class *Proteobacteria* and, particularly, to the genus *Methylobacterium.* The similarity levels were 99.4% between strain UCM B-3383 and *M. mesophilicum*; 98.5% between strain UCM B-3389 and *M. organophilum*; and 97.7–99.4% between *M. extorquens* and UCM B-3360, UCM B-3362, and UCM B-3368. These values allowed us to assign our isolates to the corresponding species. With other *Methylobacterium* species, the isolates exhibited a similarity of 93–96%.

Construction of a phylogenetic tree based on comparative analysis of the 16S rRNA genes was performed with the use of various algorithms implemented in the TreeView 1.5.2 and ClustalX 1.81 software pack-

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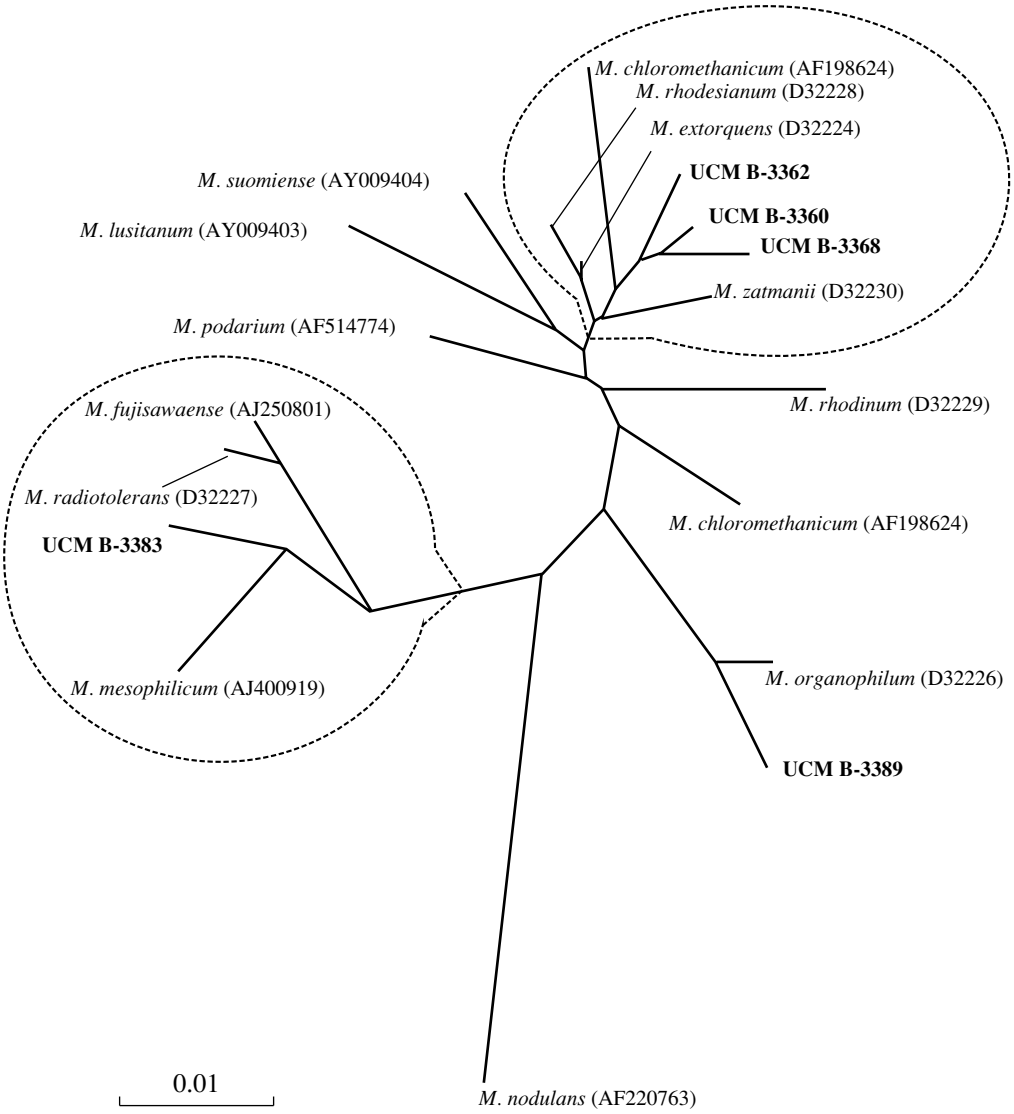

Unrooted phylogenetic tree showing clusters of *Methylobacterium* species revealed by the analysis of the nucleotide sequences of the 16S rRNA genes. The 16S rRNA gene sequences of 14 species of *Methylobacterium* were retrieved from GenBank; the 16S rRNA gene sequences of strains UCM B-3360, UCM B-3362, UCM B-3368, UCM B-3383, and UCM B-3389 were determined in this work. Dashed lines outline clusters within which strains exhibit high similarity levels. The scale bar corresponds to 1 substitution per 100 nucleotides. UCM is Ukrainian Collection of Microorganisms, Zabolotnyi Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kiev.

ages. In the tree constructed (see figure), strains UCM B-3360, UCM B-3362, and UCM B-3368 formed a monophyletic cluster with *M. extorquens, M. zatmanii*, and *M. rhodesianum.* The similarity values between these three species ranged from 98.4 to 99.4%. The species *M. chloromethanicum* also fell within this cluster, although its similarity values were lower (97.6–98.3%) with strains of *M. extorquens.* A second coherent cluster was formed by the species *M. mesophilicum, M. fujiawaense*, and *M. radiotolerans* (97.8–98.9% similarity within the cluster). The species within the clusters were close both by their 16S rRNA gene sequences (more than 97.5% similarity) and by phenotypic properties. They also exhibited a rather high level of total DNA hybridization (33–43%) [9]. Such a close phylogenetic relatedness of species within clusters seems to mean strain rather than species distinctions and raises the question of whether the existence of some of these *Methylobacterium* species as independent taxonomic units is justified.

Based on the sequence analysis of 16S rRNA genes and study of the phenotypic properties, our isolates of methylotrophic bacteria were assigned to the species *M. mesophilicum* (strain UCM B-3383), *M. extorquens* (strains UCM B-3360, UCM B-3362, and UCM B-3368), and *M. organophilum* (strain UCM B-3389).

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