Arthrobacter phenanthrenivorans sp. nov., to accommodate the phenanthrene-degrading bacterium Arthrobacter sp. strain Sphe3

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A novel phenanthrene-degrading bacterium, designated strain Sphe3T, was isolated from a creosote-contaminated soil in Greece. Cells were non-motile, Gram-positive, aerobic, and rod- to coccus-shaped. The strain was isolated on the basis of formation of a clear zone on agar plates sprayed with phenanthrene. Optimal growth occurred at 30°C. The G+C content of the DNA was 65.7 mol%. The polar lipid pattern of strain Sphe3T consisted of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The dominant fatty acids were iso-C15:0, anteiso-C15:0, iso-C16:0, C16:0 and anteiso-C17:0, representing >86% of the total fatty acids. The predominant isoprenoid quinone of strain Sphe3T was menaquinone-8 (MK-8). Based on 16S rRNA gene sequence analysis, strain Sphe3T showed 99 and 98.9% similarity to the type strains of Arthrobacter oxydans and Arthrobacter polychromogenes, respectively. Strain Sphe3T showed 91% similarity to homologues of A. oxydans and A. polychromogenes based on recA gene sequence analysis. Based on 16S rRNA and recA gene sequence analysis and DNA–DNA hybridization analysis, as well as physiological and chemotaxonomic characteristics, it is concluded that strain Sphe3T represents a novel species of the genus Arthrobacter, for which the name Arthrobacter phenanthrenivorans sp. nov. is proposed. The type strain is Sphe3T (=DSM 18606T =LMG 23796T).

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that are found in many polluted soils as a result of natural or industrial activities, including those of creosote wood-treatment facilities (Mueller et al., 1989). PAHs have attracted considerable attention because of their potential toxicity for higher organisms and resistance to microbial degradation (Kanaly & Harayama, 2000). A wide range of micro-organisms have been discovered that are able to degrade highly stable, toxic organic compounds such as polycyclic and aliphatic hydrocarbons (Habe & Omori, 2003; Kanaly & Harayama, 2000; Van Hamme et al., 2003). Among these micro-organisms, several Arthrobacter species are able to degrade PAHs (Grifoll et al., 1992; Seo et al., 2006). We have previously reported the phenanthrene uptake activity and membrane lipid alterations of a PAH-degrading Arthrobacter strain, Sphe3, isolated from a creosote-contaminated soil in Greece (Kallimanis et al., 2007). In the present study, on the basis of phylogenetic analysis of 16S rRNA and recA gene sequences, together with physiological and chemotaxonomic characteristics and DNA–DNA hybridization analysis, we demonstrate that strain Sphe3 represents a novel species of the genus Arthrobacter.

Strain Sphe3T was isolated from Perivleptos, a creosote-polluted site in Epirus, Greece (12 km north of the city of Ioannina), in which a wood-preserving industry had operated for over 30 years (Kallimanis et al., 2007). Soil samples (10 g) were mixed with 100 ml of minimal medium M9 (Sambrook et al., 1989) supplemented with 0.01% (w/v) phenanthrene (in crystal form) as the sole carbon and energy source for growth. Following incubation at 30°C for 1 week, the soil samples were serially diluted and screened for the presence of phenanthrene-degrading micro-organisms (Kiyohara et al., 1982; Zhang et al., 2004). Five colonies, able to grow in the presence of phenanthrene as the sole carbon source, were isolated and designated as Sphe3–Sphe8. As these organisms were all identified as belonging to the genus Arthrobacter based on preliminary
16S rRNA gene sequence analysis, we selected the first isolate, strain Sphe3, for further characterization.

Cell morphology and motility were examined by using a microscope equipped with phase-contrast optics. Biochemical tests were performed as outlined by Smibert & Krieg (1994) and Cappuccino & Sherman (1996) at 37 °C in the appropriate medium. Appropriate positive and negative controls were included in all experiments. Formation of pigments characteristic of Arthrobacter polychromogenes or Arthrobacter oxydans was investigated on peptone-yeast agar plates or on nicotine agar (0.4 % nicotine) as described by Loveland-Curtze et al. (1999). The minimal inhibitory concentration of various antibiotics such as ampicillin, chloramphenicol, erythromycin, neomycin, rifampicin and tetracycline was estimated by growth tests on Luria agar medium.

Phospholipid and fatty acid methyl ester analyses were performed as described previously (Kallimanis et al., 2007, and references therein). Isoprenoid quinone extraction was performed as described by Rosa-Putra et al. (2001) and isoprenoid quinones were characterized by electrospray ionization mass spectrometry (ESI-MS).

DNA was isolated according to the standard Joint Genome Institute (JGI) protocol for bacterial genomic DNA isolation by using CTAB. The 16S rRNA gene (1.5 kb) was amplified from the genomic DNA by using primers 16SF (5'-AGAGTTTGATCCTGCTCAG-3') and 16SR (5'-AAGGAGGTGATCCAGCC-3') corresponding to Escherichia coli positions 9–27 and 1525–1542, respectively. 16S rRNA gene sequence analysis, we selected the first isolate, strain Sphe3, for further characterization.

For DNA–DNA hybridization, DNA was isolated by using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashon et al. (1977).

DNA–DNA hybridization was carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huß et al. (1983) by using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in-situ temperature probe (Varian). Hybridization reactions were carried out in 2 × SSC plus 10 % formamide at 69 °C.

The DNA G+C content was determined by the fluorimetric dye-binding method (Johnson, 1994) based on the differential binding of Hoechst 33258 (benzidine) and ethidium bromide to A + T and G + C regions of DNA, respectively. E. coli DH5α (52 mol%), Agrobacterium tumefaciens UBAPF2 (60 mol%) and Pseudomonas aeruginosa PAO (67 mol%) were used as controls.

Taxonomic analysis was conducted by using the GenBank BLAST program. For phylogenetic and molecular evolutionary analysis, MEGA version 3.1 (Kumar et al., 2004) was used. The 16S rRNA gene sequence of strain Sphe3T (1500 bp) was aligned with sequences from related taxa by using the CLUSTAL W program (Thompson et al., 1994). The resultant tree topology was evaluated by bootstrap analysis by using the neighbour-joining method based on 1000 resamplings.

Strain Sphe3T was found to be a Gram-positive, aerobic, non-motile bacterium exhibiting a rod–coccus cell cycle, with a cell size of approximately 1.0–1.5 × 2.5–4.0 μm. Colonies were slightly yellowish on Luria agar. The temperature range for growth was 4–37 °C with optimum growth at 30–37 °C. The pH range for growth was 6.5–8.5 with optimal growth at pH 7.0–7.5. Strain Sphe3T was found to be sensitive to various antibiotics, the minimal inhibitory concentrations of which were estimated as follows: ampicillin, 0.01 mg l⁻¹; chloramphenicol, 10 mg l⁻¹; erythromycin, 10 mg l⁻¹; neomycin, 20 mg l⁻¹; rifampicin, 10 mg l⁻¹; tetracycline, 10 mg l⁻¹.

Fig. 1. Neighbour-joining phylogenetic tree showing the position of strain Sphe3T among closely related species of the genus Arthrobacter based on 16S rRNA gene sequences. Numbers at nodes are bootstrap percentages based on 1000 resamplings. Bar, 0.01 substitutions per nucleotide position.
Amylase, catalase and nitrate reductase tests were positive, whereas those for arginine dihydrolase, gelatinase, lipase, lysine and ornithine decarboxylases, oxidase, urease, citrate assimilation and H₂S production were negative. No acid was produced in the presence of glucose, lactose or sucrose. Strain Sphe3T could also grow by utilizing anthracene (a three-ring PAH) as carbon source, but not naphthalene (a two-ring aromatic hydrocarbon). Growth was also observed in the presence of 1-hydroxy-2-naphthoate, 2-carboxybenzaldehyde, phthalate or protocatechuate, which constitute intermediate metabolites of the phenanthrene degradation phthalate pathway (Kiyohara & Nagao, 1978; Barnsley, 1983). In addition, enzyme assays performed as described by Iwabuchi & Harayama (1997, 1998) revealed activity of 1-hydroxy-2-naphthoate dioxygenase and 2-carboxybenzaldehyde dehydrogenase (data not shown).

Strain Sphe3T contained menaquinones MK-8 and MK-9(H₂) at a ratio of 3.6:1. Major fatty acids were anteiso-C₁₅ : 0 (36.2 %), iso-C₁₆ : 0 (15.7 %), iso-C₁₅ : 0 (14.3 %), anteiso-C₁₇ : 0 (12.0 %), C₁₆ : 0 (8.3 %), iso-C₁₇ : 0 (4.0 %), C₁₆ : 1ω7c (2.5 %) and C₁₄ : 0 (1.4 %). The major phospholipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine (63.8, 27.5 and 4.0 % respectively; data not shown).

The G+C content of strain Sphe3T was 65.7 ± 0.2 mol% (mean ± SD of four determinations). This value is characteristic of recognized *Arthrobacter* species, which typically have a G+C content in the range 59–70 mol% (Keddie et al., 1986). The nucleotide sequence of 1520 bp of the 16S rRNA gene of strain Sphe3T was aligned with several homologues from other *Arthrobacter* species. Phylogenetic analysis was conducted by comparing the 16S rRNA and recA genes from strain Sphe3T with the corresponding genes from other representative taxa deposited in the GenBank database, and phylogenetic trees were constructed based on the neighbour-joining method (Figs 1 and 2). Comparative 16S rRNA gene sequence analysis showed that strain Sphe3T was most closely related to the type strains of *A. oxydans* and *A. polychromogenes* (99 and 98.9 % similarity, respectively). Alignment of the recA gene sequence of strain Sphe3T with homologues of the above two species revealed 91 % similarity. Table 1 details several phenotypic traits of strain Sphe3T that could be used to differentiate it from phylogenetically related *Arthrobacter* species. Strain Sphe3T was able to grow on nicotine but it differed from *A. oxydans* in that it did not form a blue pigment. Furthermore, unlike *A. polychromogenes*, strain Sphe3T exhibited no blue pigmentation on carbohydrate peptone medium. In addition, Sphe3T was able to grow in mineral salts medium, whereas *A. oxydans* and *A. polychromogenes* require vitamins such as biotin.

Levels of DNA–DNA relatedness between strain Sphe3T and its closest phylogenetic neighbours were 19.0 %

**Table 1.** Differential characteristics between strain Sphe3T and its closest relatives in the genus *Arthrobacter*

Data for reference species were taken from Sguros (1955) (*A. oxydans*), Schippers-Lammertse et al. (1963) (*A. polychromogenes*) and Westerberg et al. (2000) (*A. chlorophenolicus*). NA, Not applicable; ND, no data available.
(reciprocal value 28.2%) with *A. oxydans* DSM 20119T and 42.3% (40.7%) with *A. polychromogenes* DSM 20136T, clearly below the 70% considered to be the threshold value for the definition of bacterial species (Wayne et al., 1987).

On the basis of the data presented, we consider that strain Sphe3T represents a novel species of the genus *Arthrobacter*, for which the name *Arthrobacter phenanthrenivorans* sp. nov. is proposed.

**Description of Arthrobacter phenanthrenivorans sp. nov.**


Cells are aerobic and non-motile, stain Gram-positive and exhibit a rod–coccus growth cycle. Colonies are cream to yellow in colour. Grows at 4–37°C in mineral salts medium with a suitable carbon source; optimum growth occurs between 30 and 37°C. No additional growth factors are required. Catalase- and amylase-positive. Reduces nitrate to nitrite. Negative for oxidase, urease, lipase and lactic acid. Reduction of phenanthrene is catalytic, and membrane lipid alterations of the PAH degrading *Arthrobacter* sp. strain Sphe3. *Appl Microbiol Biotechnol* 76, 709–717.

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**References**


