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 Sphe3^T, 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 Sphe3^T consisted of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The dominant fatty acids were iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, C_{16:0} and anteiso-C_{17:0}, representing >86 % of the total fatty acids. The predominant isoprenoid quinone of strain Sphe3^T was menaquinone-8 (MK-8). Based on 16S rRNA gene sequence analysis, strain Sphe3^T showed 99 and 98.9 % similarity to the type strains of *Arthrobacter oxydans* and *Arthrobacter polychromogenes*, respectively. Strain Sphe3^T 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 Sphe3^T represents a novel species of the genus *Arthrobacter*, for which the name *Arthrobacter phenanthrenivorans* sp. nov. is proposed. The type strain is Sphe3^T (=DSM 18606^T =LMG 23796^T).

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 Sphe3^T 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

Abbreviation: PAHs, polycyclic aromatic hydrocarbons.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *recA* gene sequences of strain Sphe3^T are AM176541 and AM931439, respectively.

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'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (5'-AAGGAGGTGATCCAGCC-3') corresponding to *Escherichia coli* positions 9–27 and 1525–1542, respectively (Stackebrandt & Liesack, 1993). Hot-start PCR at 95 °C for 5 min was followed by 30 cycles of 94 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min, and a final elongation step at 72 °C for 10 min. Following electrophoresis on a 0.7 % agarose gel, the amplified DNA fragment of 1.5 kb was purified by using a Nucleospin Extract II kit (Macherey-Nagel) and sequenced at Macrogen (Korea) by using primers 16SF and 16SR.

The *recA* gene sequence of strain Sphe3^T was provided by N. Kyrpides (personal communication); at the time of writing, sequencing of the genome of strain Sphe3^T at the JGI had almost been completed.

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 Cashion *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 Sphe3^T (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 Sphe3^T 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 Sphe3^T was found to be sensitive to various antibiotics, the minimal inhibitory concentrations of which were estimated as follows: ampicillin, 20 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 Sphe3^T 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.

Fig. 2. Neighbour-joining phylogenetic tree showing the position of strain Sphe3^T among closely related species of the genus *Arthrobacter* based on *recA* gene sequences. Numbers at nodes are bootstrap percentages based on 1000 resamplings. Bar, 0.02 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 Sphe3^T 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 Sphe3^T contained menaquinones MK-8 and MK-9(H₂) at a ratio of 3.6:1. Major fatty acids were anteiso-C_{15:0} (36.2%), iso-C_{16:0} (15.7%), iso-C_{15:0} (14.3%), anteiso-C_{17:0} (12.0%), C_{16:0} (8.3%), iso-C_{17:0} (4.0%), C_{16:1}ω7c (2.5%) and C_{14:0} (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 Sphe3^T was 65.7±0.2 mol% (mean±SD of four determinations). This value is char-

acteristic 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 Sphe3^T was aligned with several homologues from other *Arthrobacter* species. Phylogenetic analysis was conducted by comparing the 16S rRNA and *recA* genes from strain Sphe3^T 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 Sphe3^T 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 Sphe3^T with homologues of the above two species revealed 91% similarity. Table 1 details several phenotypic traits of strain Sphe3^T that could be used to differentiate it from phylogenetically related *Arthrobacter* species. Strain Sphe3^T 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 Sphe3^T exhibited no blue pigmentation on carbohydrate peptone medium. In addition, Sphe3^T 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 Sphe3^T and its closest phylogenetic neighbours were 19.0%

Table 1. Differential characteristics between strain Sphe3^T and its closest relatives in the genus *Arthrobacter*

Data for reference species were taken from Sgueros (1955) (*A. oxydans*), Schippers-Lammertse *et al.* (1963) (*A. polychromogenes*) and Westerberg *et al.* (2000) (*A. chlorophenolicus*). NA, Not applicable; ND, no data available.

Characteristic	Strain Sphe3 ^T	<i>A. oxydans</i>	<i>A. polychromogenes</i>	<i>A. chlorophenolicus</i>
Motility	–	–	–	+
Growth with nicotine	+	+	–	+
Xenobiotic compounds allowing growth	Phenanthrene, anthracene	–	–	Phenols
Colony pigmentation on:				
Nicotine agar	Cream to yellow	Blue	NA	Pearl grey
Peptone medium	Cream to yellow	Pearl grey to yellow	Blue	Pearl grey
Biotin requirement	–	+	+	–
Temperature range (°C)	4–37	ND	10–37	3–37
Gelatinase	–	+ (slow)	+ (rapid)	+ (slow)
Isolation source	Soil	Tobacco leaves and air	Airborne infection	Soil

(reciprocal value 28.2 %) with *A. oxydans* DSM 20119^T and 42.3 % (40.7 %) with *A. polychromogenes* DSM 20136^T, 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 Sphe3^T represents a novel species of the genus *Arthrobacter*, for which the name *Arthrobacter phenanthrenivorans* sp. nov. is proposed.

Description of *Arthrobacter phenanthrenivorans* sp. nov.

Arthrobacter phenanthrenivorans (phen.an'thre.ni.vo'rans. N.L. n. *phenanthrenum* phenanthrene; L. v. *vorare* to devour; L. part. adj. *vorans* devouring, digesting; N.L. part. adj. *phenanthrenivorans* digesting phenanthrene).

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 gelatinase. Does not produce H₂S. Acid is not produced from glucose, lactose or sucrose. Utilizes phenanthrene and anthracene as sole carbon sources. The major menaquinone is MK-8 and the major fatty acids are anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{15:0} and anteiso-C_{17:0}.

The type strain, Sphe3^T (=DSM 18606^T =LMG 23796^T), was isolated from creosote-contaminated soil in Greece.

Acknowledgements

We wish to thank Dr T. Sakellaridis and Dr A. Badeka for mass spectra of quinones obtained on an Agilent 1100 series LC-MSD-Trap-SL spectrometer funded by the horizontal laboratory and Unit Centres of the University of Ioannina. This work was co-funded by the European Union within the framework of the program 'Pythagoras II' and by the Greek Secretariat for Research and Technology (Programme PENED 1999).

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