Arthrobacter nitroguajacolicus sp. nov., a novel 4-nitroguaiacol-degrading actinobacterium

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Three bacterial isolates from soil, capable of degradation or transformation of nitroaromatic compounds and displaying a rod–cocci growth cycle, were studied by a polyphasic approach. On the basis of 16S rRNA sequence analysis and of chemotaxonomic characteristics, such as type A3 α peptidoglycan with an interpeptide bridge Ala–Thr–Ala, the major menaquinone MK-9(H₂) and fatty acid composition, the isolates were assigned to the genus *Arthrobacter*. DNA–DNA hybridization, ribotyping and phenotypic studies revealed that the three strains constitute a single species, distinct from phylogenetically neighbouring *Arthrobacter aurescens* and *Arthrobacter ilicis*. A novel species, *Arthrobacter nitroguajacolicus* sp. nov., with the type strain G2-1^T (= CCM 4924^T = DSM 15232^T) is proposed.

The genus *Arthrobacter* (Conn & Dimmick, 1947; Koch *et al.*, 1995) embraces more than 30 species of bacteria isolated mostly from soil. Arthrobacters are metabolically versatile, and some species of this genus are characterized by the ability to metabolize xenobiotics (Westerberg *et al.*, 2000). Strain 8/3 (= CCM 4925 = DSM 15233), identified at first as *Corynebacterium* sp., was isolated from contaminated soil (Kotoučková *et al.*, 1997) and showed the ability to degrade nitroaromatic compounds. Two additional strains, G2-1^T and P1P (= CCM 7049 = DSM 15234), capable of degrading 4-nitroguaiacol (4-NG), were later isolated from forest soil.

For isolation, approximately 1 g soil was transferred into 100 ml mineral medium (MM) supplemented with 0.1 mM 4-nitrophenol (4-NP), and incubated at 28 °C on a rotary

shaker. The composition of MM (g l⁻¹) was as follows: CaCl₂, 0.0275; MgSO₄·7H₂O, 0.0225; FeCl₃·6H₂O, 0.00025; (NH₄)₂SO₄, 0.05; KH₂PO₄, 0.17; K₂HPO₄, 0.42; Na₂HPO₄·12H₂O, 0.88. The enrichment products (10 ml) were transferred to 100 ml fresh MM supplemented with 4-NP (strain P1P), or to MM supplemented with 4-NG (strain G2-1^T). After degradation of the nitroaromatic compounds, indicated by decolorization of the medium, the procedure was repeated three times. Subsequent streaking on solid MM supplemented with 4-NP or 4-NG resulted in the formation of single colonies. Degradation of nitroaromatic compounds was confirmed by cultivation of the strains in liquid MM supplemented with 4-NP (0.05 g l⁻¹) or 4-NG (0.004 g l⁻¹), and by HPLC analysis. A Waters HPLC system, with a reverse-phase Nova-Pak C18 column and acetonitrile/0.1% acetic acid (15:85) as the mobile phase, was used for HPLC analysis. Strain 8/3 was cultivated in MM with 0.2% (w/v) peptone and 0.05 g 4-NP l⁻¹. Identification of 4-NG as a product of 4-NP transformation by strain 8/3 was performed with a liquid HP 1100 chromatograph (Hewlett-Packard) coupled with an ion trap mass spectrometer (Bruker Daltonik).

Nutrient agar (CM2, Oxoid) was used for the characterization of colonies and cell morphology. Cell size was

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Abbreviations: 4-NG, 4-nitroguaiacol; 4-NP, 4-nitrophenol.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain G2-1^T is AJ512504.

Tables showing the differentiating physiological reactions and cellular fatty acid composition of *Arthrobacter nitroguajacolicus* sp. nov. strains are available as supplementary material in IJSEM Online.

determined by light microscopy with image analysis software (Laboratory Imaging). To observe cell shape during growth phases, scanning electron microscopy was employed: cells grown in nutrient broth (Oxoid) were fixed with 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and dehydrated samples were coated with gold in a sputter-coater (Polaron) and examined with a Philips CM12/STEM electron microscope (Pospíšil *et al.*, 1998). Images were stored digitally (AnalySis 3.2 Pro, Soft Imaging System). Gram staining was examined as described previously (Smibert & Krieg, 1994). Motility was determined by the hanging drop method (Smibert & Krieg, 1994). The physiological and biochemical characteristics of the strains grown on nutrient agar were studied as described previously (Smibert & Krieg, 1994). The API CORYNE system was used for examination of isolates as a routine system, and API ZYM was chosen for additional enzymic characterization. The ability of strains to utilize a broad range of carbon sources was determined by using Biolog GP2 microplates (Biolog, Inc.). All commercial kits were used according to the manufacturers' instructions.

Cells for fatty acid analysis were harvested from 24 h cultures grown at 28 °C on Trypticase soy broth agar. Fatty acids were extracted and analysed following the instructions of the Microbial Identification System operating manual (MIDI, Inc., 1999). Peptidoglycan structure was determined using purified cell-wall hydrolysates, according to the method of Schleifer (1985). Amino acids and peptides were separated by two-dimensional ascending TLC on cellulose plates with the solvent systems of Schleifer & Kandler (1972). The N-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer (1985). Menaquinones were extracted according to Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1996).

Genomic-DNA extraction, PCR-mediated amplification of the 16S rRNA and purification of PCR products were carried out as described previously (Rainey *et al.*, 1996).

The ae2 editor (Maidak *et al.*, 1999) was used to align the 16S rRNA sequences obtained in this study with the 16S rRNA sequences of representatives of the principal bacterial lineages available from public databases. Evolutionary distances were calculated by the method of Jukes & Cantor (1969). Phylogenetic dendrograms were constructed using neighbour-joining algorithms (De Soete, 1983).

For determination of G+C content and for DNA–DNA hybridization, DNA was isolated by breaking cells in a French press, followed by purification by hydroxyapatite chromatography (Cashion *et al.*, 1977). G+C content was determined by reverse-phase HPLC of nucleosides (Mesbah *et al.*, 1989). DNA–DNA reassociation was performed under optimal conditions (2 × SSC at 67 °C) and recorded using a Gilford 2600 spectrophotometer (Huß *et al.*, 1983; Jahnke, 1992). Automated ribotyping was carried out using the RiboPrinter microbial characterization system (Qualicon,

DuPont) and *EcoRI* and *PvuII* to generate restriction fragments. Band patterns were compared by BioNumerics software (Applied Maths). Clustering was performed by the unweighted pair group method with arithmetic means (UPGMA) based on Pearson's correlation coefficient, using an optimization coefficient of 1.2%.

An almost complete 16S rRNA gene sequence of 1488 nt was determined for strain G2-1^T, ranging from position 18 (5') to 1526 (3'), following the *Escherichia coli* numbering of Brosius *et al.* (1978). Phylogenetic analyses, based on a dataset consisting of 1150 unambiguous nucleotides between positions 41 and 1158, showed that strain G2-1^T belongs to the radiation of the genus *Arthrobacter* (Fig. 1). The highest binary 16S rRNA similarity value was found with *Arthrobacter aureescens* DSM 20116^T (99.7%) and *Arthrobacter ilicis* DSM 10138^T (99.1%). The DNA–DNA reassociation values between strain G2-1^T and its closest phylogenetic neighbours were 38.4% (*A. ilicis* DSM 10138^T) and 44.8% (*A. aureescens* DSM 20116^T), clearly below the 70% considered to be the threshold value for the delineation of genomic species (Wayne *et al.*, 1987). The DNA–DNA similarity of strains G2-1^T and 8/3 was 78.2% (repetition 81.5%). The RiboPrint patterns of strains G2-1^T, 8/3 and P1P were nearly identical and differed from those of the type strains of *A. ilicis* and *A. aureescens* (Fig. 2). The results of DNA–DNA hybridization and RiboPrint analyses indicated that strains G2-1^T, 8/3 and P1P constituted a homogeneous genomic species.

Colonies of strains G2-1^T, P1P and 8/3 grown aerobically on nutrient agar were yellow-coloured, circular, convex and opaque. Cells were Gram-positive, irregular, non-spore-forming rods and displayed a rod–coccus life cycle (Fig. 3). Growth and physiological characteristics of the three strains are given in the species description and are also shown in Supplementary Table A in IJSEM Online. In addition, strains G2-1^T and P1P use 4-NG as a growth substrate, and strain 8/3 produces 4-NG in the course of 4-NP transformation. Characteristics that differentiate strain G2-1^T from the type strains of the phylogenetically related species *A. aureescens* and *A. ilicis* are summarized in Table 1.

The cell wall peptidoglycan of strains G2-1^T, 8/3 and P1P contained lysine in position 3 of the peptide subunit and the interpeptide bridge, Ala–Thr–Ala. This polymer type is consistent with the types A3α (Schleifer & Kandler, 1972) and A11.17, according to the DSMZ catalogue of strains (DSMZ, 2001). Labelling by 1-fluoro-2,4-dinitrobenzene confirmed that alanine represents the N terminus of the interpeptide bridge.

The major menaquinone of strains G2-1^T, 8/3 and P1P was MK-9(H₂). MK-8(H₂) and MK-10(H₂) occurred as minor components. The peptidoglycan type and menaquinone pattern place strain G2-1^T in group II of *Arthrobacter* species along with *Arthrobacter aureescens*, *Arthrobacter ilicis*, *Arthrobacter ureafaciens*, *Arthrobacter histidinovorans* and

Fig. 1. Neighbour-joining tree for 16S rRNA, showing the phylogenetic position of strain G2-1^T compared to other species of the genus *Arthrobacter*. Members of the *Actinobacteria* were used to root the dendrogram. The bar represents 5 substitutions per 100 nt.

Arthrobacter nicotinovorans (Stackebrandt & Schumann, 2000) which also form a common phylogenetic group (Fig. 1).

Anteiso-C_{15:0} was found to be the major fatty acid (65–70%). Iso-C_{14:0}, iso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0} and some straight-chain saturated fatty acids (C_{14:0}, C_{16:0}) were also present. The DNA base composition of strain G2-1^T was 61.9 mol% G+C. The fatty acid profile and DNA base composition are in agreement with data reported

for other *Arthrobacter* species (Stackebrandt & Schumann, 2000). The presence of isoH-C_{16:1} (0.8–0.9%), anteiso-C_{17:1}ω9c (0.6–0.8%) and C_{16:1}ω7c (1.2–1.3%) differentiates the isolates from the type strains of *A. ilicis* and *A. aurescens*. The fatty acid profiles are shown in Supplementary Table B in IJSEM Online.

Based on the results of phylogenetic analysis, riboprinting, DNA–DNA hybridization, and chemotaxonomic and physiological studies, it is proposed to classify the isolates

Fig. 2. Riboprint patterns generated by *EcoRI* and *PvuII* for strains G2-1^T, P1P, 8/3 and the type strains of *A. aurescens* and *A. ilicis*. VCA, Patterns obtained by *EcoRI*; VCC, patterns obtained by *PvuII*. The relationship dendrogram was calculated by BioNumerics software (Applied Maths).

Fig. 3. Scanning electron micrographs of strains G2-1^T and 8/3 grown in nutrient broth at 28 °C. (a) G2-1^T long rods after 8 h cultivation. Bar, 2 µm. (b) G2-1^T short rods, coccoid and club-shaped forms after 24 h cultivation. Bar, 1 µm. (c) Strain 8/3, coccoid and club-shaped forms after 24 h cultivation. Bar, 1 µm.

as representing a novel species, *Arthrobacter nitroguajacolicus* sp. nov., with the type strain G2-1^T (=CCM 4924^T = DSM 15232^T).

Description of *Arthrobacter nitroguajacolicus* sp. nov.

Arthrobacter nitroguajacolicus (ni.tro.gu.a.ja.co'li.cus. N.L. masc. adj. *nitroguajacolicus* pertaining to the chemical nitroguaiacol, whose name is based on N.L. *guaiacum* – from Spanish *guayaco*, a tropical American tree and the resin derived thereof).

Table 1. Differentiating characteristics of strain G2-1^T and type strains of *A. aurescens* and *A. ilicis*

Strains P1P and 8/3 correspond to G2-1^T in all characteristics except positive reaction for arbutin. W, weakly positive reaction.

Characteristic	G2-1 ^T	<i>A. aurescens</i> CCM 1649 ^T	<i>A. ilicis</i> CCM 4967 ^T
Oxidase	+	+	–
Hydrolysis of:			
Aesculin	+	+	–
Starch	+	+	–
Elastase	+	+	–
Pyrrolidonyl arylamidase	–	–	+
Motility	+	–	+
Utilization of gluconate	–	+	–
Used for respiration:			
D-Xylose	–	W	+
Uridine	–	–	+
Arbutin	W	W	–
Sucrose	+	–	+
Propionic acid	+	–	W
α-Cyclodextrin	–	+	–
D-Melibiose	–	W	W
3-Methylglucose	–	W	W
D-Raffinose	+	W	W
Salicin	–	–	W

Cells are Gram-positive irregular rods, club-shaped with typical V-forms, motile and non-acid-fast. They display a rod–coccus life cycle. Cocci are 0.7–1 µm in diameter; rods are 0.6–1.0 µm wide and 1.0–4 µm long. Spores are not formed. Colonies are yellow, circular, convex and opaque. Growth occurs with a suitable carbon source in mineral salts medium; no additional growth factors are required. Obligately aerobic. Growth at 4–37 °C, with optimum at 25–30 °C. Growth occurs in the pH range 6.0–8.0 and in the presence of up to 6 % (w/v) NaCl. Catalase and oxidase positive. Nitrate not reduced. Methyl red test, urease and haemolysis negative. Gelatin, starch, casein, aesculin, ONPG and tyrosine are hydrolysed. Tween 80, DNA and lecithin are not hydrolysed. Elastase is produced. Pyrrolidonyl arylamidase and arginine dihydrolase are not produced. Production of alkaline phosphatase, acid phosphatase, α-galactosidase and α-fucosidase is variable between strains: the type strain shows positive reactions. Simmon's citrate is utilized, but not gluconate. Acid is not produced from ribose, mannitol, sorbitol, lactose, trehalose, raffinose, sucrose, L-arabinose, D-arabitol, cyclodextrin, glycogen, pullulan, maltose, melibiose, melezitose, methyl β-D-glucopyranoside or tagatose. In the Biolog test system, the following compounds are utilized for respiration: dextrin, glycogen, arbutin, D-cellobiose, D-fructose, D-galactose, α-D-glucose, maltose, maltotriose, D-mannitol, D-mannose, palatinose, D-psicose, D-raffinose, D-ribose, D-sorbitol, sucrose, turanose, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, p-hydroxyphenylacetic acid, L-malic

acid, methyl pyruvate, propionic acid, pyruvic acid, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyrroglutamic acid, L-serine, putrescine and glycerol. Utilization of gentiobiose, α -ketoglutaric acid and β -ketoglutaric acid is strain dependent: the type strain is positive. A negative reaction is seen with α -cyclodextrin, β -cyclodextrin, inulin, mannan, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, amygdalin, L-arabinose, D-arabitol, L-fucose, D-galacturonic acid, α -D-lactose, lactulose, D-melezitose, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, 3-methylglucose, methyl α -D-glucoside, methyl β -D-glucoside, methyl α -D-mannoside, L-rhamnose, salicin, sedoheptulosan, D-tagatose, D-trehalose, xylitol, D-xylose, γ -hydroxybutyric acid, lactamide, D-lactic acid methyl ester, D-malic acid, monomethyl succinate, succinamic acid, succinic acid, *N*-acetyl glutamic acid, 2,3-butanediol, adenosine, 2-deoxyadenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate and DL- α -glycerol phosphate. The cell wall diamino acid is lysine. The peptidoglycan type is A3 α , with an Ala-Thr-Ala interpeptide bridge. The major menaquinone is MK-9(H₂). The cellular fatty acid pattern is dominated by anteiso-C_{15:0}. The G + C content is 61.9 mol%.

The type strain of *Arthrobacter nitroguajacolicus* is G2-1^T (= CCM 4924^T = DSM 15232^T), isolated from forest soil.

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