Nocardioides oleivorans sp. nov., a novel crude-oil-degrading bacterium

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The crude-oil-degrading strain BAS3^T represents a novel *Nocardioides* species, according to a taxonomic study. The 16S rRNA gene sequence of strain BAS3^T was most similar to that of *Nocardioides ganghwensis* (IMSNU 14028^T; 99 % similarity), but the DNA–DNA relatedness to this type strain was only 32 %. The physiological properties of strain BAS3^T differ from those of *N. ganghwensis* (IMSNU 14028^T) and other species of *Nocardioides*. The diamino acid in the cell-wall peptidoglycan of strain BAS3^T is LL-diaminopimelic acid and the major menaquinone is MK-8(H₄). The name *Nocardioides oleivorans* sp. nov. is proposed for the novel *Nocardioides* species, since its type strain, BAS3^T (= DSM 16090^T = NCIMB 14004^T), is able to degrade crude oil.

Crude oil consists of various hydrocarbons that can be degraded by micro-organisms (Jobson *et al.*, 1972; Bosecker *et al.*, 1991; Zengler *et al.*, 1999). Several genera of hydrocarbon-oxidizing bacteria are known (Rosenberg, 2000; Van Hamme *et al.*, 2003). The aromatic-compound-degrading species of the genus *Nocardioides* comprise the pyridine-degrading species *Nocardioides pyridinolyticus* (Yoon *et al.*, 1997), the *p*-nitrophenol-degrading species *Nocardioides nitrophenolicus* (Yoon *et al.*, 1999) and the pyric acid (2,4,6-trinitrophenol)-degrading *Nocardioides* species strain CB 22-2 (Rajan *et al.*, 1996; Behrendt & Heesche-Wagner, 1999).

Here we describe the classification of the crude-oil-degrading strain BAS3^T as a member of the genus *Nocardioides*. The Gram-positive strain was isolated from crude oil sample 19 from the oilfield Oerrel of the Gifhorn Trough, North-West Germany (Bosecker *et al.*, 1991). For enrichment, artificial sea-water medium, supplemented with 1–5 % (w/v) crude oil as the carbon source, in Erlenmeyer flasks was inoculated and incubated on a rotary shaker at 30 °C in the dark for several weeks. The medium (pH 7.3) consisted of the following (l⁻¹): 23.4 g NaCl, 0.75 g KCl, 7.0 g MgSO₄·7H₂O, 1.0 g NH₄NO₃, 0.7 g K₂HPO₄ and 0.3 g KH₂PO₄ (Fedorak & Westlake, 1981).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BAS3^T is AJ698724.

A phylogenetic tree for strain BAS3^T along with a table of its whole-cell fatty acid composition are available as supplementary material in IJSEM Online.

For isolation via subculturing on agar plates, a basal medium (pH 7.3), without oil, was used, consisting of the following (l⁻¹): 23.4 g NaCl, 0.75 g KCl, 7.0 g MgSO₄·7H₂O, 0.5 g peptone from meat, 0.5 g peptone from casein, 1.0 g yeast extract and 18 g agar.

The morphology of the cells, their motility and the occurrence of spores were investigated by using phase-contrast light microscopy (Axioskop microscope; Zeiss). A Gram-stain and a test for catalase were performed according to Burghardt (1992) and Gerhardt *et al.* (1994). Anaerobic growth was checked by means of incubation in the presence and absence of oxygen, using the Anaerocult system (Merck). Further physiological tests were carried out as described by Kämpfer *et al.* (1991). Briefly, the utilization of various carbon sources as sole substrate and the hydrolysis of various compounds were studied using a complex medium containing trace elements and vitamins in microplates. To confirm oil degradation, 40 ml medium (Kämpfer *et al.*, 1991; modified) in 100 ml Erlenmeyer flasks was supplemented with 1 ml crude oil as the sole carbon source, inoculated and then incubated on a rotary shaker (120 r.p.m.) at 25 °C. Cell growth was checked after 3 weeks. The modified medium had a pH of 7.0 and the following composition (l⁻¹): 1.0 g NaCl, 0.1 g MgSO₄·7H₂O, 1.0 g (NH₄)₂SO₄, 3.2 g K₂HPO₄, 6.18 g Na₂HPO₄·2H₂O, 0.17 g CaSO₄·2H₂O, 0.001 g H₃BO₃, 0.002 g CuSO₄·5H₂O, 0.003 g ZnI₂·8H₂O, 0.2 g FeSO₄·7H₂O, 0.002 g NiCl₂·6H₂O, 0.004 g CoCl₂·6H₂O, 0.01 g MnCl₂·5H₂O, 0.003 g Na₂MoO₄·2H₂O, 0.5 g EDTA·2H₂O and vitamins according to Kämpfer *et al.* (1991).

The occurrence of diaminopimelic acid in the cell wall and

also the peptidoglycan type were determined as described by Schleifer (1985) and Schleifer & Kandler (1972), using TLC with cellulose plates (Merck). Menaquinones were extracted as described by Collins *et al.* (1977) and were analysed by HPLC according to Groth *et al.* (1996). Analysis of the whole-cell fatty acid pattern of cells grown on the basal medium was performed with the MIDI system (Microbial ID), using previously described methods (Kroppenstedt, 1985; Meier *et al.*, 1993).

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were carried out as described previously (Rainey *et al.*, 1996). Purified PCR products were sequenced with *Taq* DyeDeoxy terminator cycle sequencing kits (Applied Biosystems) according to the manufacturer's protocol. An Applied Biosystems 373A DNA sequencer was used for electrophoresis of the sequence reaction products. The ae2 editor (Maidak *et al.*, 1999) was used to align the 16S rRNA gene sequence determined in this study against the 16S rRNA gene sequences (available from the public databases) of representatives of the main bacterial lineages. Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). The least-squares distance method of De Soete (1983) contained in the PHYLIP package (Felsenstein, 1993) was used in the construction of the phylogenetic dendrogram from distance matrices. Bootstrap analyses were done as described by Felsenstein (1993).

For DNA–DNA reassociation experiments, DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite, as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out under optimal conditions for DNA–DNA reassociation as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

The almost-complete 16S rRNA gene sequence of strain BAS3^T was compared with those of members of closely related genera. Members of the genus *Nocardioides* were the closest phylogenetic neighbours. A maximum pairwise similarity value of 99% was found for *Nocardioides ganghwensis* IMSNU 14028^T. Pairwise similarity values higher than 95% were also found for *Nocardioides aquiterrae* KCCM 41647^T (95.4%), *N. pyridinolyticus* KCTC 0074BP^T (95.2%), *Nocardioides simplex* KCTC 9106^T (95.2%) and *N. nitrophenolicus* KCTC 0457BP^T (95.1%); a phylogenetic tree of the *Nocardioides* species and species of closely related genera is available as supplementary material in IJSEM Online.

Because of the high pairwise similarity value (99%) between strain BAS3^T and *N. ganghwensis*, the DNA–DNA relatedness of these two strains was determined. The value obtained was 32%, which is well below the threshold value

of 70% recommended for the definition of bacterial species (Wayne *et al.*, 1987).

The morphological, physiological and chemotaxonomic characteristics of strain BAS3^T were analysed. The properties are given in the description section. Several physiological properties of strain BAS3^T and the closest phylogenetic neighbour, *N. ganghwensis* IMSNU 14028^T (Yi & Chun, 2004), were compared. For the latter strain, oil degradation could not be observed, and the following carbon sources were utilized by this strain only: citrate, salicin and L-arabinose. L-Rhamnose, however, was utilized only by strain BAS3^T. Both strains utilized D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, L-ornithine and N-acetylglucosamine.

The fatty acid profile of strain BAS3^T was characteristic of members of the genus *Nocardioides* (iso-C_{16:0} as the branched fatty acid plus an abundance of 10-methyl fatty acids). The major fatty acids of strain BAS3^T were C_{18:1ω9c}, iso-C_{16:0}, C_{18:0} and 10-methyl fatty acids (the whole-cell fatty acid composition of strain BAS3^T and those of closely related *Nocardioides* type strains are available as a supplementary table in IJSEM Online). Strain BAS3^T contained LL-diaminopimelic acid in the cell wall and had A3γ-type peptidoglycan (LL-diaminopimelic acid–Gly). MK-8(H₄) was the major menaquinone and MK-8(H₂) was a minor menaquinone component.

On the basis of the polyphasic evidence, we suggest that strain BAS3^T represents a novel species, for which we propose the name *Nocardioides oleivorans* sp. nov.

Description of *Nocardioides oleivorans* sp. nov.

Nocardioides oleivorans [o.le.i.vor'ans. L. n. *oleum* oil; L. v. *vorare* to devour; N.L. part. adj. *oleivorans* capable of utilizing oil (hydrocarbons)].

Cells are obligate aerobic, Gram-positive, non-endospore-forming, non-motile, irregular rods about 0.3 µm wide and up to 1.1 µm long. Catalase-positive and oxidase-negative. Colonies are circular, smooth, translucent and orange-pigmented with a maximum colony diameter of 2 mm after 2 weeks. Growth occurs at 30 °C and with 2% (w/v) NaCl. Crude oil is used as substrate. Acid is not produced from D-glucose, rhamnose, sucrose, adonitol, inositol, xylose or sorbitol. Utilization of N-acetyl-D-glucosamine, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-maltose, D-mannose, α-D-melibiose, L-rhamnose, D-sucrose, D-trehalose, D-mannitol, acetate, propionate, fumarate, DL-3-hydroxybutyrate, DL-lactate, L-malate, pyruvate, L-aspartate, L-histidine, L-proline, putrescine, phenylacetate and L-ornithine is observed. L-Arabinose, α-D-galacturonate, glycogen, D-ribose, salicin, L-xylose, adonitol, i-inositol, sorbitol, *trans*-aconitate, adipate, citrate, suberate, L-alanine, L-hydroxyproline, L-serine, 3-hydroxybenzoate, 4-hydroxybenzoate and N-acetyl-D-galactosamine are not

utilized. Hydrolysis of *p*-nitrophenyl (pNP) *N*-acetyl- β -D-glucosaminide, pNP β -D-galactopyranoside, pNP α -D-glucopyranoside, pNP β -D-glucopyranoside, pNP α -D-maltoside, bis-pNP phosphate, benzolphosphonacid pNP-ester, pNP phosphocholine, 2-desoxythymidine 5'-pNP phosphate, L-alanine *p*-nitroanilide (pNA), γ -L-glutamate pNA, L-glutamate- γ -3-carboxy pNA, L-leucine pNA and L-lysine pNA is detected. pNP *N*-acetyl- β -D-galactosaminide, pNP α -L-arabinopyranoside, pNP β -D-cellobioside, pNP β -D-glucuronide, pNP β -D-lactoside, pNP α -D-mannoside, pNP β -D-xyloside, glycine pNA, L-proline pNA and L-valine pNA are not hydrolysed. The strain contains LL-diaminopimelic acid in its cell wall and possesses A3 γ -type peptidoglycan (LL-diaminopimelic acid-Gly). The cellular fatty acids comprise saturated, unsaturated, iso-, anteiso- and 10-methyl-branched types and hydroxy fatty acids. The major fatty acids are C_{18:1} ω 9c, iso-C_{16:0}, C_{18:0} and 10-methyl fatty acids. The major menaquinone is MK-8(H₄).

The type strain is strain BAS3^T (=DSM 16090^T=NCIMB 14004^T), which was isolated from a crude oil sample from the oilfield Oerrel of the Gifhorn Trough, North-West Germany (Bosecker *et al.*, 1991).

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