Microbacterium oleivorans sp. nov. and *Microbacterium hydrocarbonoxydans* sp. nov., novel crude-oil-degrading Gram-positive bacteria

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A taxonomic study of two crude-oil-degrading, Gram-positive bacterial strains, designated BAS69^T and BNP48^T, revealed that they represent two novel *Microbacterium* species. 16S rRNA gene sequence similarity to their closest phylogenetic neighbours was 98.5 % for BAS69^T (*Microbacterium paraoxydans* DSM 15019^T and *Microbacterium saperdae* DSM 20169^T) and 99 % for BNP48^T (*Microbacterium luteolum* DSM 20143^T). Levels of DNA–DNA relatedness to the closest phylogenetic neighbours of both strains were between 11 and 38 %. According to phylogenetic analysis, the two strains are distinguishable from all recognized species of *Microbacterium*. Morphological and physiological characteristics of strains BAS69^T and BNP48^T were different from those of phylogenetically closely related *Microbacterium* species. The diamino acid in the cell-wall peptidoglycan of BAS69^T is lysine and of BNP48^T is ornithine. The major menaquinones are MK-11 and MK-12 for both strains. Based on their ability to degrade crude oil, the name *Microbacterium oleivorans* sp. nov. is proposed for strain BAS69^T (= DSM 16091^T = NCIMB 14003^T) and *Microbacterium hydrocarbonoxydans* is proposed for strain BNP48^T (= DSM 16089^T = NCIMB 14002^T).

Crude oil consists of various hydrocarbons, which can be degraded by micro-organisms, and several hydrocarbon-oxidizing bacterial genera have been described (Jobson *et al.*, 1972; Bosecker *et al.*, 1991; Rueter *et al.*, 1994; Zengler *et al.*, 1999; Rosenberg, 2000; Van Hamme *et al.*, 2003). Here we describe the classification of two crude-oil-degrading, Gram-positive bacteria that showed characteristics of members of the genus *Microbacterium*. The genus *Microbacterium* comprises more than 30 physiologically versatile species isolated from various environments (Yokota *et al.*, 1993; Takeuchi & Hatano, 1998a, b; Collins & Bradbury, 1999; Schumann *et al.*, 1999; Behrendt *et al.*, 2001; Zlamala *et al.*, 2002; Laffineur *et al.*, 2003).

The two strains were isolated from different oil-containing environments. Strain BAS69^T was isolated from oil storage cavern 126 near Etzel, Germany (Bock *et al.*, 1994). Strain BNP48^T was isolated from an oil-contaminated soil in Germany. For enrichment, medium supplemented with 1–5 % crude oil as carbon source in Erlenmeyer flasks was inoculated and incubated on a rotary shaker at 30 °C in the dark for several weeks. For growth of strain BAS69^T, we

prepared an artificial sea water medium (Fedorak & Westlake, 1981) consisting of (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 (pH 7.3), and for strain BNP48^T we used a salt-poor medium (BNP; modified from Jobson *et al.*, 1972) consisting of (l⁻¹) 2.0 g Na₂SO₄, 0.2 g MgSO₄·7H₂O, 2.0 g KNO₃, 1.0 g NH₄Cl, 0.5 g K₂HPO₄ and traces of FeSO₄ (pH 7.3). For isolation via subculturing on agar plates different media without oil were used. Strain BAS69^T was isolated on a basal medium (BAS) consisting of (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 (pH 7.3). Strain BNP48^T was isolated on a modified BNP medium consisting of (l⁻¹) 2.0 g Na₂SO₄, 0.2 g MgSO₄·7H₂O, 2.0 g KNO₃, 1.0 g NH₄Cl, 0.5 g K₂HPO₄, traces of FeSO₄, 1.5 g sodium lactate, 1.0 g yeast extract and 18 g agar (pH 7.3).

Cell morphology, cell motility and the occurrence of spores were examined by phase-contrast light microscopy. Gram-staining and catalase testing was performed according to Burghardt (1992) and Gerhardt *et al.* (1994). Anaerobic growth was investigated by 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, use of various carbon sources

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *M. oleivorans* sp. nov. BAS69^T and *M. hydrocarbonoxydans* sp. nov. BNP48^T are AJ698725 and AJ698726.

as the only substrate and hydrolysis of various compounds were studied using a complex medium containing trace elements and vitamins in microplates. To confirm oil degradation, 40 ml medium (modified from Kämpfer *et al.*, 1991) in 100 ml Erlenmeyer flasks was supplemented with 1 ml crude oil as the only carbon source, inoculated and incubated on a rotary shaker (120 r.p.m.) at 25 °C. Cell growth was checked after 3 weeks. The modified medium (pH 7.0) comprised (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 dihydrate and vitamins according to Kämpfer *et al.* (1991).

The occurrence of diaminopimelic acid in the cell wall and the peptidoglycan type were determined as described by Schleifer (1985) and Schleifer & Kandler (1972), using plates of cellulose for TLC. Menaquinones were extracted as described by Collins *et al.* (1977) and were analysed by HPLC according to the method of Groth *et al.* (1996). The acyl type of the peptidoglycan was determined as described by Uchida *et al.* (1999).

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced with *Taq* Dyedexy 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 sequences determined here against those of representatives of the main bacterial lineages available from the public databases. 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.

For DNA–DNA reassociation experiments, DNA was isolated using a French pressure cell (Thermo Spectronic) followed by purification by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out by the method described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

The almost complete 16S rRNA gene sequences of strains BAS69^T and BNP48^T, consisting of 1490 and 1495 nt, respectively, were compared to sequences of members of the genus *Microbacterium*, which were their closest phylogenetic neighbours (Fig. 1).

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequence analysis showing the phylogenetic position of *Microbacterium oleivorans* sp. nov. BAS69^T and *Microbacterium hydrocarbonoxydans* sp. nov. BNP48^T compared to closely related recognized species of the genus *Microbacterium*. The sequences of *Curtobacterium luteum* DSM 20542^T and *Clavibacter michiganensis* subsp. *michiganensis* DSM 46364^T served as external references. Bar, 3 nucleotide substitutions per 100 nucleotides.

For strain BAS69^T, a maximum pairwise similarity value of 98.5% was obtained for *Microbacterium paraoxydans* DSM 15019^T and *Microbacterium saperdae* DSM 20169^T. Pairwise similarity values above 98% were also found for *Microbacterium maritipicum* ATCC 19260^T, *Microbacterium luteolum* DSM 20143^T, *Microbacterium testaceum* DSM 20166^T and *Microbacterium schleiferi* DSM 20489^T (98.4% each), *Microbacterium oxydans* DSM 20578^T (98.3%), *Microbacterium foliorum* DSM 12966^T, *Microbacterium phyllosphaerae* DSM 13468^T and strain BNP48^T (98.2% each), *Microbacterium liquefaciens* DSM 20638^T and *Microbacterium keratanolyticum* DSM 8606^T (98.0% each).

For strain BNP48^T, maximum pairwise similarity values of 99.0 % were obtained for *M. luteolum* DSM 20143^T, 98.9 % for *M. foliorum* DSM 12966^T and 98.8 % for *M. oxydans* DSM 20578^T, *M. maritipicum* ATCC 19260^T, *M. phyllosphaerae* DSM 13468^T and *M. saperdae* DSM 20169^T. Pairwise similarity values above 98 % were also found for *M. liquefaciens* DSM 20638^T and *M. paraoxydans* DSM 15019^T (98.6 % each), strain BAS69^T (98.2 %), *M. schleiferi* DSM 20489^T (98.2 %), *M. testaceum* DSM 20166^T and *M. keratanolyticum* DSM 8606^T (98.1 % each).

Based on these high pairwise 16S rRNA gene sequence similarity values, DNA–DNA relatedness of strains BAS69^T and BNP48^T to several closest phylogenetic neighbours was determined. Levels of DNA–DNA relatedness of strain BAS69^T to *M. saperdae*, *M. foliorum* and *M. phyllosphaerae* were 18, 21 and 19 %, respectively. Those of strain BNP48^T to *M. maritipicum*, *M. oxydans*, *M. luteolum*, *M. foliorum* and *M. phyllosphaerae* were 23, 16, 38, 36 and 11 %, respectively. DNA–DNA relatedness between the two novel strains was 41 %. These low values demonstrate that strains BAS69^T and BNP48^T each represent members of single novel species of the genus *Microbacterium*.

Morphological, physiological and chemotaxonomic characteristics of the two strains are different from those of phylogenetically closely related *Microbacterium* species (Table 1). The two strains were obligately aerobic, Gram-positive, irregular rods without spores. Cells of strain BAS69^T were immotile, whereas those of strain BNP48^T were motile. Cells were small, varying in size from 0.3 to 1.1 µm for strain BAS69^T and from 0.3 to 1.5 µm for strain BNP48^T. Colonies of both strains were circular, smooth, translucent and pigmented. Maximum colony diameters were 3 and 5 mm after 2 weeks of growth for strains BAS69^T and BNP48^T, respectively. Neither strain contained meso-diaminopimelic acid in the cell wall. The peptidoglycan type of strain BAS69^T was B1γ, [L-Glu] D-Glu(Hyg)–Gly_{1–2}–L-Lys and that of strain BNP48^T was B2β, [L-Hsr] D-Glu(Hyg)–Gly–D-Orn (Schleifer & Kandler, 1972). The major menaquinones were MK-11 and MK-12, with minor amounts of MK-9, MK-10 and MK-13 for both strains.

Description of *Microbacterium oleivorans* sp. nov.

Microbacterium 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-spore-forming, immotile, irregular rods 0.3–1.1 µm in size. Colonies are circular, smooth, translucent and orange pigmented with a maximum colony diameter of 3 mm after 2 weeks. Growth occurs at 30 and 37 °C and at 2 and 4 % NaCl. Catalase-positive and oxidase-negative. Crude oil is used as substrate. H₂S is not produced, arginine is not hydrolysed, urease is not present and the Voges–Proskauer reaction is negative. Acid is produced from sucrose and

xylose. Acid is not produced from D-glucose, rhamnose, adonitol, inositol or sorbitol. The following are utilized: L-arabinose, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-maltose, D-mannose, α-D-melibiose, L-rhamnose, D-ribose, D-sucrose, salicin, D-trehalose, L-xylose, D-mannitol, sorbitol, fumarate, DL-lactate, L-malate, pyruvate, L-aspartate, L-histidine, putrescine and 4-hydroxybenzoate. The following are not utilized: N-acetyl-D-glucosamine, α-D-galacturonate, glycogen, adonitol, i-inositol, acetate, propionate, *trans*-aconitate, adipate, citrate, DL-3-hydroxybutyrate, suberate, L-alanine, L-hydroxyproline, L-proline, L-serine, 3-hydroxybenzoate, phenylacetate, N-acetyl-D-galactosamine and L-ornithine. The following are hydrolysed: aesculin, *p*-nitrophenyl (pNP) N-acetyl-β-D-galactosaminide, pNP N-acetyl-β-D-glucosaminide, pNP α-L-arabinopyranoside, pNP β-D-cellobioside, pNP β-D-galactopyranoside, pNP α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP α-D-mannoside, pNP α-D-maltoside, pNP β-D-xyloside, bis-pNP phosphate, benzolphosphonacid-pNP ester, L-alanine *p*-nitroanilide (pNA), glycine pNA, L-leucine pNA, L-lysine pNA and L-proline pNA. The following are not hydrolysed: pNP β-D-glucuronide, pNP β-D-lactoside, pNP phosphocholine, 2-deoxythymidine-5'-pNP phosphate, γ-L-glutamate pNA, L-glutamate-γ-3-carboxy pNA and L-valine pNA. The type strain does not contain meso-diaminopimelic acid in its cell wall and the peptidoglycan-type is B1γ, [L-Glu] D-Glu(Hyg)–Gly_{1–2}–L-Lys. The major menaquinones are MK-11 and MK-12.

The type strain, BAS69^T (= DSM 16091^T = NCIM 14003^T), was isolated from oil storage cavern 126 near Etzel, Germany (Bock *et al.*, 1994).

Description of *Microbacterium hydrocarbonoxydans* sp. nov.

Microbacterium hydrocarbonoxydans (hy'dro.car.bon.ox'y.dans. N.L. part. adj. *hydrocarbonoxydans* oxidizing hydrocarbons).

Cells are obligate aerobic, Gram-positive, non-spore-forming, motile, irregular rods 0.3–1.5 µm in size. Colonies are circular, smooth, translucent and yellow pigmented with a maximum colony diameter of 5 mm after 2 weeks. Growth occurs at 30 and 37 °C and at 2 and 4 % NaCl. Catalase-positive and oxidase-negative. Crude oil is used as substrate. H₂S is not produced, arginine is not hydrolysed, urease is not present and the Voges–Proskauer reaction is negative. Acid is produced from rhamnose, sucrose and xylose. Acid is not produced from D-glucose, adonitol, inositol or sorbitol. The following are utilized: N-acetyl-D-glucosamine, L-arabinose, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, glycogen, D-maltose, D-mannose, L-rhamnose, D-ribose, D-sucrose, salicin, D-trehalose, L-xylose, D-mannitol, acetate, propionate, citrate, fumarate, DL-lactate, L-malate, pyruvate, L-alanine, L-aspartate, L-histidine, L-hydroxyproline, L-proline, L-serine, putrescine, phenylacetate and

Table 1. Differential morphological and physiological characteristics of *M. oleivorans* sp. nov. BAS69^T, *M. hydrocarbonoxydans* sp. nov. BNP48^T and their phylogenetically closest relatives

Taxa: 1, strain BAS69^T; 2, strain BNP48^T; 3, *M. maritypicum*; 4, *M. oxydans*; 5, *M. luteolum*; 6, *M. saperdae*; 7, *M. paraoxydans*; 8, *M. foliorum*; 9, *M. phyllosphaerae*. Data are from Takeuchi & Hatano (1998b), Yokota *et al.* (1993), Behrendt *et al.* (2001), Laffineur *et al.* (2003) and this study. Identical characteristics are not included. Properties were analysed as described by Kämpfer *et al.* (1991). +, Positive; –, negative; (+), only positive for certain strains; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9
Colour of colonies*	O	Y	LY	Y	YW	YW/Y	Y	Y	Y
Motility	–	+	+	+	–	+	+	+	+
Type of peptidoglycan	B1 γ	B2 β	B2 β	B2 β	B2 β	B2 β	B2 β	B2 β	B2 β
Cell wall diamino acid	Lys	Orn	Orn	Orn	Orn	Orn	Orn	Orn	Orn
Major menaquinones (MK)	11, 12	11, 12	12	11, 12	12	11, 12	ND	10, 11, 12	10, 11, 12
Growth at 37 °C	+	+	+	+	–	–	+	(+)	(+)
Growth in 2 % NaCl	+	+	+	+	–	–	ND	+	+
Growth in 6.5 % NaCl	–	–	+	ND	ND	ND	ND	ND	ND
H ₂ S production	–	–	–	+	+	+	ND	–	–
Arginine dihydrolase	–	–	–	–	–	+	ND	–	–
Urease	–	–	ND	ND	+	+	ND	–	ND
Acid production from:									
Glucose	–	–	+	+	+	+	+	+	(+)
Rhamnose	–	+	–	ND	ND	ND	ND	(+)	+
Inositol	–	–	ND	ND	ND	ND	ND	–	(+)
Xylose	+	+	–	ND	ND	ND	ND	+	(+)
Utilization of:									
N-Acetyl-D-glucosamine	–	+	+	+	+	+	ND	ND	ND
L-Arabinose	+	+	–	–	+	+	ND	+	+
Glycogen	–	+	ND	ND	ND	ND	ND	ND	ND
α -D-Melibiose	+	–	ND	ND	ND	ND	ND	ND	ND
Sorbitol	+	–	ND	ND	ND	ND	ND	ND	ND
Acetate	–	+	–	ND	+	+	ND	ND	ND
Propionate	–	+	–	ND	+	+	ND	ND	ND
Citrate	–	+	+	+	–	–	ND	(+)	–
Fumarate	+	+	–	ND	+	+	ND	ND	ND
DL-Lactate	+	+	–	ND	+	+	ND	ND	ND
L-Alanine	–	+	ND	ND	ND	ND	ND	ND	ND
L-Hydroxyproline	–	+	ND	ND	ND	ND	ND	ND	ND
L-Proline	–	+	ND	ND	ND	ND	ND	ND	ND
L-Serine	–	+	ND	ND	ND	ND	ND	ND	ND
4-Hydroxybenzoate	+	–	ND	ND	ND	ND	ND	ND	ND
Phenylacetate	–	+	ND	ND	ND	ND	ND	(+)	(+)
N-Acetyl-D-galactosamine	–	+	ND	ND	ND	ND	ND	ND	ND
Hydrolysis of:									
pNP β -D-lactoside	–	+	ND	ND	ND	ND	ND	ND	ND
pNP phosphocholine	–	+	ND	ND	ND	ND	ND	ND	ND
2-Deoxythymidine-5'-pNP phosphate	–	+	ND	ND	ND	ND	ND	ND	ND

*LY, Light yellow; O, orange; Y, yellow; YW, yellowish-white.

N-acetyl-D-galactosamine. The following are not utilized: α -D-galacturonate, α -D-melibiose, adonitol, i-inositol, sorbitol, *trans*-aconitate, adipate, DL-3-hydroxybutyrate, suberate, 3-hydroxybenzoate, 4-hydroxybenzoate and L-ornithine. The following are hydrolysed: pNP N-acetyl- β -D-galactosaminide, pNP N-acetyl- β -D-glucosaminide, pNP α -L-arabinopyranoside, pNP β -D-cellopyranoside,

pNP β -D-galactopyranoside, pNP α -D-glucopyranoside, pNP β -D-glucopyranoside, pNP β -D-lactoside, pNP α -D-mannoside, pNP α -D-maltoside, pNP β -D-xyloside, bis-pNP phosphate, benzolphosphonacid-pNP ester, pNP phosphocholine, 2-deoxythymidine-5'-pNP phosphate, L-alanine pNA, glycine pNA, L-leucine pNA, L-lysine pNA and L-proline pNA. The following are not hydrolysed: pNP

β -D-glucuronide, γ -L-glutamate pNA, L-glutamate- γ -3-carboxy pNA and L-valine pNA. The type strain does not contain *meso*-diaminopimelic acid in its cell wall and the peptidoglycan-type is B2 β , [L-Hsr] D-Glu(Hyg)-Gly-D-Orn. The major menaquinones are MK-11 and MK-12.

The type strain, BNP48^T (= DSM 16089^T = NCIMB 14002^T), was isolated from an oil-contaminated soil in Germany.

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