Olivibacter oleidegradans sp. nov., a hydrocarbon-degrading bacterium isolated from a biofilter clean-up facility on a hydrocarbon-contaminated site

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A novel hydrocarbon-degrading, Gram-negative, obligately aerobic, non-motile, non-sporulating, rod-shaped bacterium, designated strain TBF2/20.2^T, was isolated from a biofilter clean-up facility set up on a hydrocarbon-contaminated site in Hungary. It was characterized by using a polyphasic approach to determine its taxonomic position. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate is affiliated with the genus *Olivibacter* in the family *Sphingobacteriaceae*. It was found to be related most closely to *Olivibacter ginsengisoli* Gsoil 060^T (93.3% 16S rRNA gene sequence similarity). Strain TBF2/20.2^T grew at pH 6–9 (optimally at pH 6.5–7.0) and at 15–42 °C (optimally at 30–37 °C). The major fatty acids were iso-C_{15:0} (39.4%), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 26.0%), iso-C_{17:0} 3-OH (14.5%) and C_{16:0} (4.5%). The major menaquinone was MK-7 and the predominant polar lipid was phosphatidylethanolamine. The DNA G+C content of strain TBF2/20.2^T was 41.2 mol%. Physiological and chemotaxonomic data further confirmed the distinctiveness of strain TBF2/20.2^T from recognized members of the genus *Olivibacter*. Thus, strain TBF2/20.2^T is considered to represent a novel species of the genus *Olivibacter*, for which the name *Olivibacter oleidegradans* sp. nov. is proposed. The type strain is TBF2/20.2^T (=NCAIM B 02393^T =CCM 7765^T).

The genus *Olivibacter*, family *Sphingobacteriaceae* (Euzéby, 1997), was proposed by Ntougias *et al.* (2007) and, at the time of writing, comprised four recognized species: *Olivibacter sitiensis*, isolated from alkaline olive-oil mill wastes (Ntougias *et al.*, 2007), and *Olivibacter ginsengisoli*, *Olivibacter terrae* and *Olivibacter soli* (Wang *et al.*, 2008), isolated from soil of a ginseng field and compost in South Korea. The present study describes the taxonomic characterization of a novel *Olivibacter*-like bacterial strain, designated TBF2/20.2^T.

Strain TBF2/20.2^T was isolated from a clean-up facility at a volatile hydrocarbon-contaminated site in Hungary. A biofilter was used for bioremediation of contaminated groundwater by using *ex situ*, on-site treatment. A sample was taken from the biofilter and 10 g of the sample was

stirred thoroughly for 20 min in 90 ml physiological saline (0.9%, w/v, NaCl) solution and glass beads. Serial dilutions were made and plated on TGY5 agar plates [per litre distilled water: 5 g tryptone, 2.5 g yeast extract, 5 g glucose (all from BioLab Inc.) and 15 g agar agar (Merck)] and incubated at 30 °C for 72 h. Colonies were selected randomly and subsequently purified twice on TGY5 agar medium at 30 °C.

Hydrocarbon degradation was tested on prediluted and sterilized diesel oil solution prepared by a Hungarian accredited analytical laboratory (Wessling Hungary Ltd). To confirm oil degradation, 450 ml of this solution was inoculated with strain TBF2/20.2^T growing in 50 ml TGY5 broth (final volume 500 ml) and incubated for 5 days in a rotary shaker (120 r.p.m.) at 25 °C. Uninoculated solution with the addition of 50 ml sterile distilled water was used as a control and incubated under the same conditions. After incubation, oil degradation rates were determined by the same analytical laboratory. Strain TBF2/20^T degraded

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TBF2/20.2^T is HM021726.

A supplementary table is available with the online version of this paper.

more than 70 % of the diesel oil during incubation compared with the control.

Genomic DNA was extracted by using the UltraClean Microbial DNA Isolation kit (MoBio Laboratories). The 16S rRNA gene was amplified from the chromosomal DNA by using primers 27F and 1492R (Lane, 1991). Amplification was performed by using an Eppendorf Mastercycler. PCR products were purified with a PCR Advanced PCR Clean Up kit (Viogene). The almost-complete 16S rRNA gene sequence of strain TBF2/20.2^T was determined by using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequencing products were separated on a model 310 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequence of strain TBF2/20.2^T was compared with those of the type strains of recognized species of the genus *Olivibacter* and of representative members of related genera obtained from GenBank (Chun *et al.*, 2007). Multiple alignments of 16S rRNA gene sequences were made with the CLUSTAL X program (Thompson *et al.*, 1997). The MEGA4 software (Tamura *et al.*, 2007) was used to construct phylogenetic trees with the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Swofford, 1993) algorithms. Bootstrap values were determined based on 5000 replications. Evolutionary distances were calculated with Kimura's two-parameter model (Kimura, 1983).

Analysis of the almost-complete 16S rRNA gene sequence (1406 bp) of strain TBF2/20.2^T revealed that it is a member of the genus *Olivibacter*, being related most closely to *O. ginsengisoli* Gsoil 060^T (93.3 % similarity). As the type strains of all recognized *Olivibacter* species showed less than 97 % 16S rRNA gene sequence similarity to strain TBF2/20.2^T, it

can be concluded that they differ at the species level (Stackebrandt & Goebel, 1994; Tindall *et al.*, 2010). Interestingly, we observed 99.2 % 16S rRNA gene sequence similarity between strain TBF2/20.2^T and the uncharacterized bacterial strain BPC8, a member of a benzo[a]pyrene-degrading bacterial consortium (Kanaly *et al.*, 2002). The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) shows clearly that strain TBF2/20.2^T belongs to the lineage of the family *Sphingobacteriaceae*. The topologies of the neighbour-joining, minimum-evolution and maximum-parsimony phylogenetic trees were very similar, and strain TBF2/20.2^T clustered together with recognized members of the genus *Olivibacter* at a bootstrap level of 99 %. On the basis of 16S rRNA gene sequence analysis, the phylogenetic position of strain TBF2/20.2^T among recognized members of genus *Olivibacter* is unique and distinct.

Samples for electron microscopy were taken from 48-h cultures grown in R2A broth at 30 °C. Cell suspension was dropped onto a carbon-coated grid and allowed to be absorbed for 5 min at room temperature. A drop of 1 % uranyl acetate (negative stain) was placed on the grid for 1 min, excess liquid was wicked off and the grids were allowed to air-dry. Cells were then critical-point dried by using liquid CO₂. Samples were examined with a Zeiss EM910 electron microscope at 40 kV. An electron micrograph of cells of strain TBF2/20.2^T is presented in Fig. 2. The cells were rod-shaped, about 1.8 µm long and 0.6 µm in diameter. The surface of the cells was totally smooth without flagella, fimbriae or capsules. Cells of strain TBF2/20.2^T as observed by electron microscopy closely resemble those of *O. sitiensis* (see Supplementary Fig. S1 of Ntougias *et al.*, 2007). From these results it can be speculated that the lack of flagella is a common

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relative position of strain TBF2/20.2^T among various representative members of the family *Sphingobacteriaceae*. Bootstrap values are based on 5000 repetitions. Bar, 0.02 substitutions per nucleotide position.

Fig. 2. Electron micrographs of cells of strain TBF2/20.2^T. The unflagellated rod-shaped cells are about 1.8 μm long and 0.6 μm in diameter. Bars, 1 μm (a) and 2 μm (b).

characteristic of members of the genus *Olivibacter*. Cell morphology was also observed at ×1000 magnification with a light microscope (Zeiss Docuval) by using cells grown for 72 h at 30 °C on TGY5 and R2A agar plates. Colonies of strain TBF2/20.2^T grown on R2A agar were smooth, circular, convex, 1.0–2.0 mm in diameter, opaque, yellowish white or yellow depending on length of incubation. After a longer incubation period, the colonies became light brown. On TGY5 agar, strain TBF2/20.2^T formed regular and rounded colonies.

Carbon-source utilization and enzyme activities were tested by using API 20NE, API 20E, API ID 32GN, API 50CH and API ZYM test kits (bioMérieux) according to the manufacturer's instructions. Comparisons were made between strain TBF2/20.2^T and *O. soli* LMG 23492^T, *O. ginsengisoli* LMG 23491^T, *O. terrae* LMG 23494^T and *O. sitiensis* DSM 17696^T (see Supplementary Table S1, available in IJSEM Online). Examination of the OF test, citrate utilization, catalase activity, aesculin hydrolysis, nitrate reduction, Tween 80 hydrolysis, gelatin hydrolysis, growth in 5% NaCl, malonate utilization, phenylalanine deamination and hydrogen sulphide production was made

according to the methods described by Barrow & Feltham (1993), verifying the API test results. The Gram-reaction was performed by using the non-staining method, as described by Buck (1982). Growth at 3, 4, 5, 10, 15, 20, 25, 28, 30, 32, 37, 40, 42 and 45 °C and at pH 2.0–10.0 (in increments of 1 pH unit) was assessed after 5 days of incubation on TGE (Merck) agar (for temperature) or in TGE broth (for pH). Salt tolerance was tested after 5 days of incubation on R2A agar supplemented with 1–10% (w/v) NaCl. Growth on nutrient agar, trypticase soy agar (TSA) and MacConkey agar (Merck) was also evaluated at 30 °C. Strain TBF2/20.2^T grew on nutrient agar and TSA but not on MacConkey agar. It reduced nitrate to nitrite. Other physiological characteristics of strain TBF2/20.2^T are summarized in the species description. Phenotypic characteristics that serve to differentiate this strain from its closest phylogenetic relatives are listed in Table 1.

Chemotaxonomic analyses (quinone and fatty acid methyl esters) and determination of the DNA G+C content were carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Strain TBF2/20.2^T, *O. soli* LMG 23492^T, *O. ginsengisoli* LMG 23491^T, *O. terrae* LMG 23494^T and *O. sitiensis* DSM 17696^T were grown under identical conditions before the fatty acid compositions were estimated with the TSBA40 method (Miller, 1982; Kuykendall *et al.*, 1988; Kämpfer & Kroppenstedt, 1996). DNA for determination of the genomic G+C content was extracted based on the method of Cashion *et al.* (1977). G+C ratio determination was carried out according to Tamaoka & Komagata (1984) and Mesbah *et al.* (1989). Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material by using the two-stage method described by Tindall (1990a, b) and Tindall *et al.* (2007). Chemotaxonomic analysis of strain TBF2/20.2^T showed some similarities to its closest relatives. The DNA G+C content of strain TBF2/20.2^T was 41.2 mol%. The major respiratory quinone was MK-7 (98%); a trace amount of MK-6 (2%) was also observed. However, the fatty acid profile of strain TBF2/20.2^T [iso-C_{15:0} (39.4%), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 26.0%), iso-C_{17:0} 3-OH (14.5%) and C_{16:0} (4.5%)] showed characteristic differences from those of its closest relatives (Table 2). Although similar fatty acid profiles were observed for strains of species belonging to the genus *Olivibacter*, in which iso-C_{15:0} and summed feature 3 are the major components, significant differences in the proportions of these latter fatty acids were observed. This also confirms that strain TBF2/20.2^T differs at the species level from recognized species of the genus *Olivibacter*.

Therefore, on the basis of the low levels of 16S rRNA gene sequence similarity between strain TBF2/20.2^T and its closest relatives within the genus *Olivibacter*, and the results of chemotaxonomic, biochemical and physiological analysis, strain TBF2/20.2^T is considered to represent a novel species of the genus *Olivibacter*, for which the name *Olivibacter oleidegradans* sp. nov. is proposed.

Table 1. Differential characteristics between strain TBF2/20.2^T and the type strains of recognized *Olivibacter* species

Strains: 1, TBF2/20.2^T; 2, *O. soli* LMG 23492^T; 3, *O. ginsengisoli* LMG 23491^T; 4, *O. terrae* LMG 23494^T; 5, *O. sitiensis* DSM 17696^T. Data were obtained in this study unless indicated. All strains grew at 15 and 30 °C and were positive for activities of catalase, acid phosphatase, alkaline phosphatase, leucine arylamidase, valine arylamidase, α -glucosidase and *N*-acetyl- β -glucosaminidase and for assimilation of sucrose, D-mannose and D-glucose. All strains were negative for the Gram stain and sporulation. w, Weakly positive.

Characteristic	1	2	3	4	5
Growth at/with:					
5 °C	+	–	–	–	+
37 °C	+	+	–	–	+
42 °C	+	–	–	–	+
4 % NaCl	–	+	+	+	–
Gelatin hydrolysis	–	+	+	–	–
Enzyme activity					
Esterase (C4)	–	–	+	+	+
Esterase lipase (C8)	–	–	–	+	–
Naphthol-AS-BI-phosphohydrolase	+	–	–	–	+
β -Galactosidase	+	–	–	–	–
β -Glucuronidase	–	–	–	+	–
α -Mannosidase	+	–	–	–	–
α -Fucosidase	–	+	–	–	–
Assimilation of:					
D-Adonitol	+	+	–	–	–
D-Lyxose	+	+	–	–	–
D-Mannitol	–	+	–	–	–
Melezitose	+	w	–	–	–
Turanose	+	w	–	w	–
Inositol	–	–	+	–	–
L-Arabinose	+	+	–	w	–
L-Histidine	–	–	–	–	+
<i>N</i> -Acetylglucosamine	+	–	–	–	–
DNA G + C content (mol%)	41.2	39.2 ^a	43.6 ^a	40.6 ^a	45.6 ^b

*Data taken from: a, Wang *et al.* (2008); b, Ntougias *et al.* (2007).

Description of *Olivibacter oleidegradans* sp. nov.

Olivibacter oleidegradans [o.le.i.de.gra'dans. L. n. *oleum* oil; L. part. adj. *degradans* returning to the original order, referring to the ability of the type strain to degrade oil (hydrocarbons); N.L. part. adj. *oleidegradans* capable of degrading oil (hydrocarbons)].

Cells are Gram-negative, obligately aerobic, non-spore-forming, non-motile rods (0.6 × 1.8–2.0 µm) that lack flagella. Colonies grown on R2A agar plates are smooth, circular, convex, non-luminescent, opaque and yellowish after 5 days at 30 °C. Good growth is observed on TGE agar, TSA and nutrient agar but not on MacConkey agar. Growth is observed at 15–45 °C and at pH 6.0–9.0. Optimal growth occurs at 30–37 °C and pH 6.5–7.0. Unable to grow in the presence of 2 % NaCl. Oxidase- and catalase-positive.

Table 2. Cellular fatty acid profiles of strain TBF2/20.2^T and the type strains of recognized *Olivibacter* species

Strains: 1, TBF2/20.2^T; 2, *O. soli* LMG 23492^T; 3, *O. ginsengisoli* LMG 23491^T; 4, *O. terrae* LMG 23494^T; 5, *O. sitiensis* DSM 17696^T. Data were obtained in this study; values are percentages of total fatty acids. –, Not detected; ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5
Saturated					
C _{14:0}	1.57	1.12	1.59	2.10	0.34
C _{16:0}	4.54	4.88	5.30	4.22	4.54
Unsaturated					
C _{14:1} ω5 <i>c</i>	0.24	–	–	–	–
C _{16:1} ω5 <i>c</i>	0.54	1.53	0.58	–	1.17
C _{15:1} ω6 <i>c</i>	0.30	–	–	–	–
Branched chain					
iso-C _{15:0}	39.43	29.60	28.24	27.61	31.18
anteiso-C _{15:0}	0.30	0.66	–	0.32	–
iso-C _{15:0} 3-OH	1.94	2.51	2.51	2.49	1.87
iso-C _{15:1} F	0.13	–	–	–	–
iso-C _{16:0} 3-OH	0.24	0.34	0.39	–	–
iso-C _{17:0}	0.97	0.13	0.37	0.43	0.31
iso-C _{17:0} 3-OH	14.46	12.86	13.97	12.34	10.97
iso-C _{17:1} ω9 <i>c</i>	1.88	1.16	0.96	1.29	1.75
Hydroxy					
C _{14:0} 2-OH	0.68	0.27	0.31	0.34	–
C _{15:0} 2-OH	0.26	–	–	–	–
C _{16:0} 2-OH	1.68	2.23	2.62	2.87	0.59
C _{16:0} 3-OH	0.7	2.18	2.85	1.64	0.63
C _{16:1} 2-OH	0.14	–	0.33	0.48	–
C _{17:0} 2-OH	–	0.33	–	–	–
Cyclopropane acids					
C _{17:0} cyclo	0.86	–	–	–	–
Unknown					
ECL 13.565	0.71	0.64	0.80	0.80	1.21
ECL 16.582	1.18	0.96	0.91	0.92	0.81
Summed features*					
1	0.33	–	–	–	–
3	26.01	38.42	38.26	42.16	44.23
4	0.38	0.19	–	–	0.41

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the Microbial Identification System (MIDI). Summed feature 1 consisted of iso-C_{15:1} H and/or C_{13:0} 3-OH; summed feature 3 consisted of iso-C_{15:0} 2-OH and/or C_{16:1}ω7*c*; summed feature 4 consisted of iso-C_{17:1} I and/or anteiso-C_{17:1} B.

Assimilates amygdalin, arbutin, D-adonitol, D-arabinose, cellobiose, D-fructose, D-glucose, lactose, D-lyxose, maltose, D-mannose, melezitose, melibiose, raffinose, D-ribose, sucrose, trehalose, turanose, aesculin, L-arabinose, L-rhamnose, gluconate, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, *N*-acetylglucosamine, salicin and trypsin. Positive for acid phosphatase, alkaline phosphatase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase, β -glucosidase, α -mannosidase and *N*-acetyl- β -glucosaminidase. Negative

reactions are observed for gelatin hydrolysis, urease, Tween hydrolysis, leucine arylamidase, esterase (C4), esterase lipase (C8), β -glucuronidase and α -fucosidase and for assimilation of adipate, malonate, caprate, citrate, 3-hydroxybenzoate, 4-hydroxybenzoate, 3-hydroxybutyrate, D-arabitol, D-fucose, D-mannitol, D-sorbitol, D-tagatose, dulcitol, erythritol, phenylacetate, gentiobiose, glycerol, glycogen, inositol, itaconate, lactate, L-alanine, L-fucose, L-histidine, L-proline, L-serine, L-sorbose, L-xylose, 2-keto-D-gluconate, potassium 5-ketogluconate, L-proline, propionate, suberate, valerate and xylitol. The major fatty acids are iso-C_{15:0}, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), iso-C_{17:0} 3-OH and C_{16:0}. Menaquinone 7 (MK-7) is the predominant respiratory quinone; MK-6 is present as a minor component. Phosphatidylethanolamine, two aminophospholipids, three phospholipids, four lipids and an atypical glycolipid are present as polar lipids. The DNA G+C content of the type strain is 41.2 mol%.

The type strain, TBF2/20.2^T (=NCAIMB 02393^T =CCM 7765^T), was isolated from an on-site, *ex situ* groundwater-cleaning biofilter facility on a former airbase in Hungary.

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