

Hoeflea olei sp. nov., a diesel-oil-degrading, anoxygenic, phototrophic bacterium isolated from backwaters and emended description of the genus *Hoeflea*

K. Rahul,¹ M. Azmatunnisa,¹ Ch. Sasikala¹ and Ch.V. Ramana²

Correspondence

Ch. Sasikala

sasi449@yahoo.ie

Ch.V. Ramana

cvr449@gmail.com

¹Bacterial Discovery Laboratory, Centre for Environment, Institute of Science and Technology, JNT University Hyderabad, Kukatpally, Hyderabad 500 085, India

²Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, India

A Gram-stain-negative, diesel-oil-degrading, rod-shaped bacterium (designated JC234^T) was isolated from a water sample collected from diesel-oil-contaminated backwaters in Kerala, India. Strain JC234^T was oxidase- and catalase-positive, and grew at 20–35 °C and at pH 7–9. Cells contained bacteriochlorophyll-*a*, hydroxydemethylspheroidene and three unidentified carotenoids. Growth occurred under aerobic, microaerobic and phototrophic anaerobic conditions. Strain JC234^T could utilize diesel-oil as a sole source of carbon and energy. Based on the 16S rRNA gene sequence analysis, strain JC234^T belonged to the genus *Hoeflea* within the family *Phyllobacteriaceae*, and was closely related to *Hoeflea alexandrii* AM1V30^T (98.1 % 16S rRNA gene sequence similarity), *Hoeflea halophila* JG120-1^T (97.6 %) and other members of the genus *Hoeflea* (<96.4 %). Strain JC234^T showed 22 ± 2 % and 28 ± 1.5 % DNA–DNA hybridization with *Hoeflea alexandrii* KCTC 22096^T and *Hoeflea halophila* KCTC 23107^T, respectively. The DNA G + C content of strain JC234^T was 54.3 mol %. The major cellular fatty acids were C₁₈:1ω7c/C₁₈:1ω6c, C₁₆:0 and C₁₆:1ω7c/C₁₆:1ω6c. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and phosphatidylglycerol were the major polar lipids. Strain JC234^T contained Q10 as the predominant ubiquinone. On the basis of morphological, physiological, genetic, phylogenetic and chemotaxonomical analyses, we conclude that strain JC234^T represents a novel species of the genus *Hoeflea*, for which the name *Hoeflea olei* sp. nov. is proposed. The type strain is JC234^T (=KCTC 42071^T=LMG 28200^T). An emended description of the genus *Hoeflea* is also provided.

Reclassification of *Agrobacterium ferrugineum* LMG 128 (Ahrens, 1968; Rüger & Höfle, 1992) resulted in the creation of the genus *Hoeflea* (Peix *et al.*, 2005) with *Hoeflea marina* as the type species. At the time of writing, the genus *Hoeflea* comprises six species with validly published names (<http://www.bacterio.net/hoeflea.html>): *Hoeflea marina* (Peix *et al.*, 2005), *Hoeflea phototrophica*

(Biebl *et al.*, 2006), *Hoeflea alexandrii* (Palacios *et al.*, 2006), *Hoeflea anabaenae* (Stevenson *et al.*, 2011), *Hoeflea halophila* (Jung *et al.*, 2013) and *Hoeflea suaedae* (Chung *et al.*, 2013), all isolated from marine habitats. Members of the genus *Hoeflea* are Gram-stain-negative, aerobic or microaerobic, non-endospore-forming and halotolerant. The DNA G + C content of members of the genus ranges from 53 to 60 mol %. Diphosphatidylglycerol, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), phosphatidylglycerol (PG), sulfoquinovosyldiacylglycerol (SQDG), a few unidentified glycolipids, unidentified aminolipids and unidentified lipids are the major polar lipids of the genus *Hoeflea*. *Hoeflea phototrophica* is the only species of this genus reported to have bacteriochlorophyll-*a* (Bchl-*a*) and *puf* genes; however it lacks anaerobic growth in the dark or light (Biebl *et al.*, 2006). In this study, we characterize a newly isolated strain (JC234^T) as a novel species

Abbreviations: Bchl-*a*, bacteriochlorophyll-*a*; DCM, dichloromethane; DDH, DNA–DNA hybridization; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmonomethylethanolamine; SQDG, sulfoquinovosyldiacylglycerol

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *puf* gene sequences of strain JC234^T are HG965759 and LN811447, respectively.

Seven supplementary figures and a supplementary table are available with the online Supplementary Material.

of the genus *Hoeflea* which can utilize diesel-oil for growth, contains *Bchl-a* and *puf* genes and can grow anaerobically in the light.

Strain JC234^T was isolated from an oil-contaminated backwater sample collected from Vembanad Lake, Kerala, India (GPS coordinates 9° 35' N 76° 23' E) during December 2013. The water sample that yielded strain JC234^T had a pH of 7, a temperature of 29 °C and a salinity of 1.8 % (w/v). The water sample with traces of oil was inoculated into 250 ml conical flasks containing 100 ml mineral salts medium [comprising (g l⁻¹): KH₂PO₄ (0.5), MgSO₄·7H₂O (0.2), NH₄Cl (0.6), CaCl₂·2H₂O (0.05) and 5 ml ferric citrate solution (0.1 %, w/v)] with 2 % (v/v) diesel-oil as a carbon source, and was incubated at 30 °C with shaking at 100 r.p.m. for 3 days. Purification of bacteria was achieved by repeated streaking on nutrient agar. Strain JC234^T was preserved as glycerol stocks and by lyophilization.

Genomic DNA was extracted and purified from strain JC234^T according to the method of Marmur (1961) and the DNA G+C content was determined by HPLC (Mesbah *et al.*, 1989). 16S rRNA gene amplification and sequencing was done as described previously (Subhash *et al.*, 2013). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (Kim *et al.*, 2012). The CLUSTAL W algorithm of the MEGA 5.2 software package was used for sequence alignments and MEGA 5.2 software (Tamura *et al.*, 2011) was used for phylogenetic analysis of the individual sequences. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Kimura, 1980). Neighbour-joining, maximum-likelihood and minimum-evolution methods in the MEGA 5.2 software were used to reconstruct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure based on 1000 replications.

The taxonomic relationship between strain JC234^T and its closest phylogenetic neighbours was examined using DNA–DNA hybridization (DDH) studies. Genomic relatedness was determined by the membrane-filter technique as described previously (Seldin & Dubnau, 1985; Tourova & Antonov, 1987; Subhash *et al.*, 2014). Hybridization was performed with three replications for each sample (control: reversal of strains was used for binding and labelling).

Morphological properties (cell shape, cell size, motility) of strain JC234^T grown on nutrient broth was observed directly or after Gram-staining using Olympus BH-2 phase-contrast microscope. The hanging-drop method was performed as outlined in Cappuccino & Sherman (1998) to test motility. Malachite green was used for spore staining as described by Schaeffer & Fulton (1933). Internal membrane structures were viewed with a transmission electron microscope (H-7500; Hitachi), after the cells had been processed as described by Hanada *et al.* (2002). *In vivo* absorption spectra were measured with a Spectronic Genesys 2 spectrophotometer using sucrose

solution for cell suspension (Trüper & Pfennig, 1981). Carotenoid composition was determined by C₁₈-HPLC (Subhash *et al.*, 2014). Amplification of the *puf* gene fragment was performed as described by Nagashima *et al.* (1997).

The pH range for growth was tested using nutrient broth, adjusted to different pH values (pH 4.0–11.0, intervals of 0.5 pH units) by using the appropriate biological buffers as described previously (Subhash *et al.*, 2014). NaCl [0–10 % (w/v) at 0.5 % intervals] and temperature (4, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C) ranges for growth were examined in nutrient broth and growth was measured turbidometrically at 540 nm in a colorimeter (Systronics).

Various biochemical tests such as hydrolysis of starch, casein, gelatin and Tweens 20 and 80, oxidase, catalase, nitrate reduction, nitrite reduction, H₂S production, methyl red and Voges–Proskauer tests were performed by the procedures as outlined in Cappuccino & Sherman (1998). Arginine dihydrolase, phenylalanine deaminase, ornithine decarboxylase and lysine decarboxylase activities were determined as described by Smibert & Krieg (1981). Utilization of organic carbon compounds as carbon and energy sources for organo-heterotrophic growth was tested in a mineral medium as previously described (Lakshmi *et al.*, 2011) replacing sodium pyruvate with specific organic compounds (0.35 % w/v or v/v); growth was measured turbidometrically at 540 nm after 48 h. Nitrogen source utilization was tested by replacing ammonium chloride with 7 mM (final concentration) different nitrogen sources (NaNO₃, NaNO₂, glutamate, aspartate, glutamine and urea). Utilization of glutamate, glutamine, methionine, aspartate, peptone, Casamino acids and urea as sole source of carbon, nitrogen and energy was also determined. Phototrophic growth (light anaerobic) was tested in nutrient broth in fully filled screw-cap test tubes incubated at 2400 lx at 28–30 °C and chemotrophic growth was determined in nutrient broth incubated in aerobic dark at 100 r.p.m. at 28–30 °C.

Cellular fatty acids, polar lipids and quinone composition were analysed from cultures that attained 70 % maximal optical density (at their late exponential growth phase). Fatty acid analysis was done as described previously (Sasser, 1990; Subhash *et al.*, 2014). Polar lipids analysis was performed as described previously (Tindall, 1990; Tindall *et al.*, 1987; Oren *et al.*, 1996; Subhash *et al.*, 2014). Respiratory quinones were analysed as described by Xie & Yokota (2003).

To determine the diesel-oil degradation capability, strain JC234^T was grown in 250 ml conical flasks containing 100 ml mineral salts medium with 5 % (v/v) diesel-oil as a carbon source and was incubated at 30 °C under shaking at 100 r.p.m. for 5 days. Cells were harvested by centrifugation (10 000 g for 15 min at 4 °C). The diesel-oil in the supernatant was extracted with 50 ml dichloromethane (DCM) and collected by centrifugation at 10 000 g, 4 °C for 10 min. The DCM fraction containing diesel-oil was concentrated and the extracted hydrocarbons were analysed

by GC-MS. A control containing diesel-oil and no cells was used to determine background baseline of growth and degradation.

GC-MS analysis was done on a Pegasus HT TOF (high-throughput time-of-flight)-MS system (Leco) equipped with an Agilent series (7890) gas chromatograph. One micro-litre of sample was injected into a HP-5 column (30 m, internal diameter 0.32 mm, thickness 0.25 μ m), with helium as the carrier gas at a constant flow of 1.2 ml min⁻¹. The initial oven temperature of 60 °C was ramped to 300 °C at 3 °C min⁻¹ and held for 5 min. Inlet temperature was 250 °C, ion source temperature 250 °C and ionization energy -70 eV. Mass spectra were recorded at 50–1000 *m/z*. LecoChromaTOF software (version 4.21) was used to process the chromatograms and the metabolites were identified based on mass spectral comparison to a standard NIST (National Institutes of Standards and Technology) 98 library. Mass spectral peaks showing similarity above 700 were accepted, with maximum match equal to 1000 for statistical analysis.

The EzTaxon-e server search analysis revealed that strain JC234^T was most closely related to members of genus *Hoeflea*, and the highest sequence similarity was observed with *Hoeflea alexandrii* AM1V30^T (98.1 %), *Hoeflea halophila* JG120-1^T (97.6 %) and other members of the genus *Hoeflea* (<96.4 %). The results of phylogenetic analysis of the 16S rRNA gene sequences suggested that strain JC234^T clustered with the members of the genus *Hoeflea* and formed a separate clade along with the type strains of *Hoeflea alexandrii* and *Hoeflea halophila* (Fig. 1).

The DNA–DNA reassociation value between strain JC234^T and *Hoeflea alexandrii* KCTC 22096^T was 22 \pm 2 %, and between strain JC234^T and *Hoeflea halophila* KCTC 23107^T was 28 \pm 1.5 %; these hybridization values are well within the recommended standards to delineate a bacterial species based on DDH (Stackebrandt & Goebel, 1994).

The DNA G + C content of strain JC234^T was 54.3 mol % (by HPLC).

On nutrient agar, colonies of JC234^T were round, flat to raised, initially colourless or light reddish-brown but later

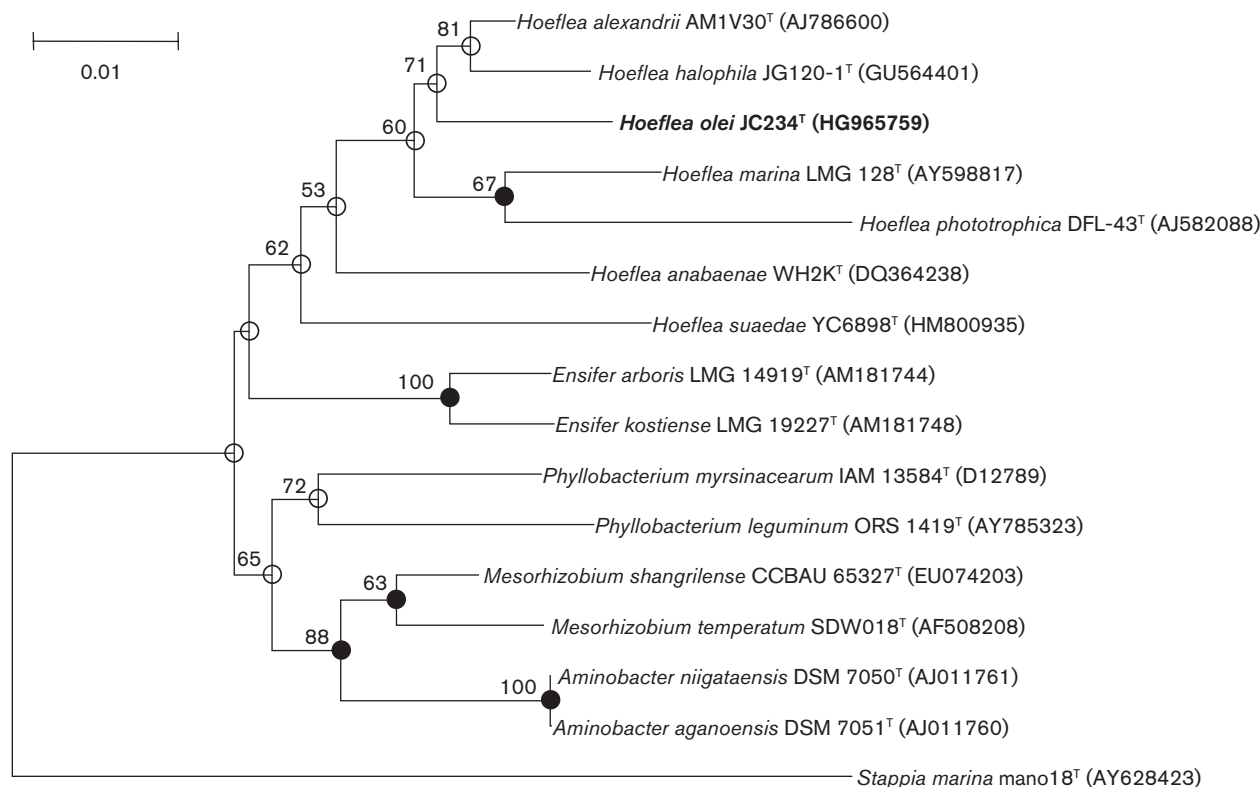


Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between strain JC234^T and closely related taxa. The tree was reconstructed by the neighbour-joining method using MEGA 5.2 software and rooted by using *Stappia marina* mano18^T as an outgroup. Numbers at nodes are bootstrap percentages based on 1000 resamplings. Filled circles indicate nodes that were obtained by all treeing methods; empty circles represent nodes that were recovered by neighbour-joining and minimum-evolution methods. GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 1 nt substitution per 100 nt.

colour intensified and become dark reddish-brown with a diameter of 1–3 mm. Cells were Gram-stain-negative, motile, straight rods measuring 2–5 µm long and 1–2 µm wide (Fig. S1 available in the online Supplementary Material). The colour of the phototrophically (anaerobic light, 2400 lx) grown cell suspension of strain JC234^T was reddish-brown. *In vivo* absorption spectra exhibited maxima at 475, 512, 589, 802, 846 and 877 nm (Fig. 2) indicating the presence of bacteriochlorophyll-*a* in phototrophically grown cells of strain JC234^T. Transmission electron micrographs revealed that cells of strain JC234^T grown phototrophically had no definite internal membrane structures (Fig. S2). Carotenoid composition from phototrophically grown cells indicated the presence of hydroxydemethylspheroidene and three unidentified carotenoids (Fig. S3). Phototrophic growth, Bchl-*a* and *puf* genes were identified in strain JC234^T but not in *Hoeflea alexandrii* KCTC 22096^T or *Hoeflea halophila* KCTC 23107^T. The highest *puf* gene sequence similarity (94 %) of strain JC234^T was observed with an uncultured bacterial clone based on comparison with sequences in the GenBank database by using the program BLASTN (Altschul *et al.*, 1997). Among other members of the genus *Hoeflea*, phototrophic growth was not reported but the presence of *puf* genes was reported in *Hoeflea phototrophica* DFL-43^T (Biebl *et al.*, 2006). Although strain JC234^T and *Hoeflea phototrophica* share the presence of Bchl-*a* and carotenoids, they differ importantly in phototrophic growth (anaerobic light). Bchl-*a* and carotenoids of strain JC234^T were observed only from phototrophically grown cells, while the culture grows equally well under both phototrophic and chemotrophic conditions (Fig. S4).

Strain JC234^T grew at pH 7–9 with optimum growth at pH 7, and differs from *Hoeflea alexandrii* KCTC 22096^T and *Hoeflea halophila* KCTC 23107^T which have a pH range of 6–9. NaCl was not required for growth of strain JC234^T but could be tolerated up to 8 % (w/v), while the type strains of *Hoeflea alexandrii* and *Hoeflea halophila* are more and less tolerant, respectively (Table 1). The temperature range for growth further differentiates strain

JC234^T from its closest phylogenetic neighbours. Other physiological and biochemical results are presented in the species description and Table 1.

The major fatty acids of strain JC234^T were C_{18:1}ω7c/C_{18:1}ω6c, C_{16:0} and C_{16:1}ω7c/C_{16:1}ω6c, with minor amounts of C_{18:1}ω7c 11-methyl and C_{18:0}. Strain JC234^T shared the presence of major fatty acids with *Hoeflea alexandrii* KCTC 22096^T and *Hoeflea halophila* KCTC 23107^T. However, significant differences in the relative amounts of C_{16:1}ω7c/C_{16:1}ω6c and C_{18:1}ω7c 11-methyl were found between strain JC234^T and the type strains of *Hoeflea alexandrii* and *Hoeflea halophila* (Table S1).

The polar lipid profile of strain JC234^T contained PC, PME, PG, PE, SQDG and unidentified lipids L1, L2 and L3 (Fig. S5). Identification of SQDG in strain JC234^T was based on the motility of the spot as demonstrated by Chung *et al.* (2013) in *Hoeflea suaedae* YC6898^T, *Hoeflea marina* KACC 12993^T and *Hoeflea alexandrii* KACC 12994^T. The spot corresponding to SQDG was eluted from the TLC plate, dissolved in chloroform/methanol (2 : 1, v/v) and the Fourier transform infrared (FTIR) spectrum of the chloroform/methanolic extract was recorded on a Bruker FTIR spectrometer in the wave number range of 3500–500 cm⁻¹. The infrared spectrum of the chloroform/methanolic extract showed characteristic absorption bands for sulfur-containing compounds at 959 cm⁻¹ and 792 cm⁻¹ indicating the presence of strong dehydration of SO₃ and symmetrical C-O-S association with a C-O-SO₃ group. The above two characteristic bands and the other absorption bands indicating the -CH₃ bending and C-H stretching confirmed the presence of SQDG (Fig. S6). The polar lipid profile of strain JC234^T was well in consonance with the type species of the genus *Hoeflea* (*Hoeflea marina*) which contained PC, PME, PG and PE as major lipids as reported by Chung *et al.* (2013) and data from this study (data not shown). The major quinone of strain JC234^T was ubiquinone Q10, which is in line with members of the genus *Hoeflea*.

Based on the GC-MS analysis of diesel layer, various hydrocarbons were seen to decrease in abundance sequentially based on their carbon chain length and branching. Strain JC234^T degraded almost all the hydrocarbon present in diesel (C10–C28) which suggests that this strain could be efficiently used for the treatment processes of various hydrocarbon-contaminated sites (Fig. S7).

Distinct phylogenetic, genotypic, chemotaxonomic and phenotypic properties thus justify the description of strain JC234^T as representative of a novel species of the genus *Hoeflea*, for which the name *Hoeflea olei* sp. nov. is proposed. An emended description of the genus *Hoeflea* is also provided. Phototrophy is a recognized genus-specific character (Imhoff & Caumette, 2004) and hence it is debatable if a new genus needs to be described in future to accommodate this anoxygenic phototrophic bacterium based on multiple strains. Such description of a new genus based on phototrophy was recently proposed for

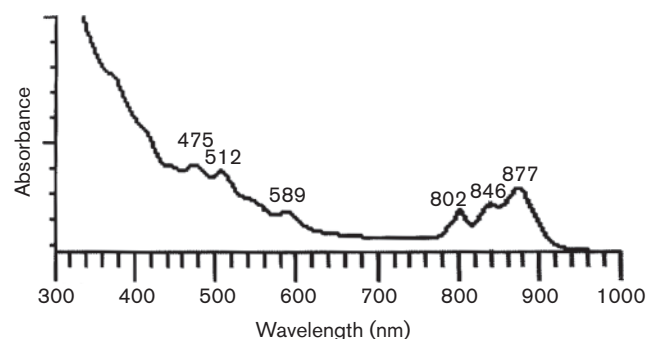


Fig. 2. Whole-cell absorption spectrum of strain JC234^T

Table 1. Differentiating characteristics between strain JC234^T and the type strains of phylogenetically related species of the genus *Hoeflea*

Taxa: 1, JC234^T; 2, *Hoeflea alexandrii* KCTC 22096^T; 3, *Hoeflea halophila* KCTC 23107^T. All data from this study. All strains are Gram-stain-negative, motile, non-spore-forming rods, positive for catalase activity and show optimal growth at pH 7.0. All strains are negative for nitrate and nitrite reduction, H₂S production, methyl red, Voges–Proskauer, hydrolysis of starch, gelatin and casein, and activity of arginine dihydrolase, phenylalanine deaminase, ornithine decarboxylase and lysine decarboxylase. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3
Isolation source	Hydrocarbon-contaminated backwaters	Marine sediment	Dinoflagellate
NaCl tolerance range (optima) for growth (% w/v)	0–8 (0–1)	0–10 (0–2)	0–5 (1)
Temperature range (optima) for growth (°C)	20–35 (30)	10–40 (30)	15–30 (25)
pH range for growth	7–9	6–9	6–9
Oxidase activity	+	w	+
Anaerobic phototrophic growth	+	–	–
Hydrolysis of:			
Tween 20	–	+	–
Tween 80	–	+	–
Degradation of diesel-oil	+	–	–
Organic substrates utilized for growth			
Acetate	+	–	–
Arabinose	–	–	+
Butyrate	+	w	–
Citrate	+	–	+
Fumarate	–	+	+
Galactose	–	+	–
Glucose	w	+	–
Glutamate	–	+	–
Glycerol	–	–	w
Pyruvate	+	+	–
Sucrose	–	+	+
Bacteriochlorophyll- <i>a</i>	+	–	–
DNA G + C content (mol%)	54.3	58.2	57.1

Gemmobacter changlensis reclassifying into *Cereibacter changlensis* (Suresh *et al.*, 2015). Similarly a chemotrophic bacterium, *Rhodobacter massiliensis* (Greub & Raoult, 2003) was reclassified as *Haematobacter massiliensis* (Helsel *et al.*, 2007).

Description of *Hoeflea olei* sp. nov.

Hoeflea olei (o'le.i. L. gen. n. olei of oil)

Colonies are circular with entire margin, and texture is moist with flat to raised elevation. On nutrient agar, colonies are reddish-brown and measure 1–3 mm in diameter. Cells are straight rods (1–2 µm × 2–5 µm), Gram-stain-negative, motile and non-endospore-forming. Aerobic, microaerobic and phototrophic anaerobic growth is possible. Whole-cell absorption spectra of phototrophically grown cells exhibit maxima at 475, 512, 589, 802, 846 and 877 nm. Phototrophically grown cells have Bchl-*a*. Hydroxydemethylspheroidene and three unidentified carotenoids are present in phototrophically grown cells. Growth occurs on diesel-oil. Growth temperature range

is 20–35 °C (optimum 30 °C). Growth pH range is pH 7–9 (optimum pH 7). NaCl is not required for growth but can be tolerated up to 8 % (w/v). Catalase- and oxidase-positive. Negative for hydrolysis of casein, gelatin, starch and Tweens 20 and 80. Tests for arginine dihydrolase, phenylalanine deaminase, lysine decarboxylase, ornithine decarboxylase, methyl red, Voges–Proskauer and production of H₂S are negative. Indole is not produced from tryptophan. Negative for nitrate and nitrite reduction. Good growth occurs with acetate, butyrate, citrate and pyruvate as sole carbon and energy sources. Arabinose, capric acid, cellobiose, fumarate, galactose, glycerol, lactate, lactose, ribose, salicin, sucrose, sorbitol, tagatose and xylose are not used as sole sources of carbon and energy. Ammonium chloride is used as sole nitrogen source; cannot use glutamine or glutamate as sole source of carbon/nitrogen and energy. PC, PME, PG, PE, SQDG and three unidentified lipids (L1, L2 and L3) are the polar lipids. The major quinone is ubiquinone Q10. Major fatty acids are C_{18:1}ω7c/C_{18:1}ω6c, C_{16:0} and C_{16:1}ω7c/C_{16:1}ω6c along with C_{18:1}ω7c 11-methyl and C_{18:0} in minor quantities.

The type strain JC234^T (=KCTC 42071^T=LMG 28200^T) was isolated from a diesel-oil-contaminated backwaters of Vembanad Lake, Kerala, India. The DNA base composition of the type strain is 54.3 mol %.

Emended description of the genus *Hoeflea* Peix *et al.* 2005

The description of the genus is as given by Peix *et al.* (2005) with the following modifications. Some species show microaerobic growth and lack motility. Oxidase activity is negative in some species. Some members of the genus contain photosynthetic reaction-centre genes *puf* and form photosynthetic pigments such as bacteriochlorophyll-*a* and carotenoids. Chemo-organotrophic with or without photosynthetic pigments, but some species are phototrophic. Phosphatidylcholine, unidentified glycolipids, unidentified aminolipid and unidentified lipids are also present in some species. C_{16:1}ω7c/C_{16:1}ω6c can also be present in major amounts (>3 %). C_{18:1}ω7c 11-methyl and C_{16:0} can be present in minor amounts (<3 %). C_{19:0}cycloω8c is absent in some species. Some species require NaCl for growth and can tolerate concentrations up to 10–11 % (w/v) and pH of 5–10. Temperature range for growth is 4–42 °C. Some species can degrade diesel-oil. The DNA G+C content is 53.1–59.7 mol %.

The type species is *Hoeflea marina*.

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