# *Thalassolituus oleivorans* gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons

Michail M. Yakimov,[1] Laura Giuliano,[1] Renata Denaro,[1] Ermanno Crisafi,[1] Tatiana N. Chernikova,[2] Wolf-Rainer Abraham,[2] Heinrich Luensdorf,[2] Kenneth N. Timmis[2] and Peter N. Golyshin[2]

Correspondence
Michail M. Yakimov
iakimov@ist.me.cnr.it

[1]Istituto per l'Ambiente Marino Costiero, CNR Consiglio Nazionale Ricerche, Spianata San Raineri 86, 98122 Messina, Italy

[2]Division of Microbiology, GBF National Research Centre for Biotechnology, Mascheroder Weg 1, 38124 Braunschweig, Germany

An aerobic, heterotrophic, Gram-negative, curved bacterial strain, designated MIL-1$^T$, was isolated by extinction dilution from an n-tetradecane enrichment culture that was established from sea water/sediment samples collected in the harbour of Milazzo, Italy. In the primary enrichment, the isolate formed creamy-white, medium-sized colonies on the surface of the agar. The isolate did not grow in the absence of NaCl; growth was optimal at 2·7 % NaCl. Only a narrow spectrum of organic compounds, including aliphatic hydrocarbons ($C_7$–$C_{20}$), their oxidized derivatives and acetate, were used as growth substrates. The isolate was not able to grow under denitrifying conditions. The DNA G+C content and genome size of strain MIL-1$^T$ were estimated to be 53·2 mol% and 2·2 Mbp, respectively. The major cellular and phospholipid fatty acids were palmitoleic, palmitic and oleic acids (33·5, 29·5 and 11·0 % and 18, 32 and 31 %, respectively). 3-Hydroxy lauric acid was the only hydroxy fatty acid detected. Thirteen different compounds that belonged to two types of phospholipid (phosphatidylethylamine and phosphatidylglycerol) were identified. 16S rRNA gene sequence analysis revealed that this isolate represents a distinct phyletic lineage within the $\gamma$-*Proteobacteria* and has about 94·4 % sequence similarity to *Oceanobacter kriegii* (the closest bacterial species with a validly published name). The deduced protein sequence of the putative alkane hydrolase, AlkB, of strain MIL-1$^T$ is related to the corresponding enzymes of *Alcanivorax borkumensis* and *Pseudomonas oleovorans* (81 and 80 % similarity, respectively). On the basis of the analyses performed, *Thalassolituus oleivorans* gen. nov., sp. nov. is described. Strain MIL-1$^T$ (=DSM 14913$^T$=LMG 21420$^T$) is the type and only strain of *T. oleivorans*.

## INTRODUCTION

Many marine bacteria that are capable of degrading petroleum hydrocarbons have recently been isolated from sites all over the world (Dyksterhouse *et al.*, 1995; Button *et al.*, 1998; Yakimov *et al.*, 1998; Hedlund *et al.*, 1999; Syutsubo *et al.*, 2001; Golyshin *et al.*, 2002). Analysis of 16S rRNA gene sequences of these marine hydrocarbonoclastic bacteria revealed that they all belong to the $\gamma$-subclass of the *Proteobacteria*; however, they are separate and distinct from other bacteria of this group and represent the genera *Alcanivorax* (Yakimov *et al.*, 1998), *Cycloclasticus* (Dyksterhouse *et al.*, 1995), *Marinobacter* (Gauthier *et al.*, 1992), *Neptunomonas* (Hedlund *et al.*, 1999), *Oleiphilus* (Golyshin *et al.*, 2002) and *Oleispira* (Yakimov *et al.*, 2003). The genera *Marinobacter* and, especially, *Alcanivorax*, seem to play a major role in the first step of crude oil biodegradation in marine environments (Harayama *et al.*, 1999; Kasai *et al.*, 2001). These marine hydrocarbonoclastic bacteria appear to be novel in a number of respects. They are obligate for hydrocarbon substrates and additionally use only a small number of low-molecular-mass organic acids, such as acetate and pyruvate. Only a few rRNA operons (one to three) and cytoplasmic proteins (not more than 300) and small genome sizes (2·0–3·0 Mbp) are characteristic for these micro-organisms

Published online ahead of print on 11 July 2003 as DOI 10.1099/ijs.0.02424-0.

Abbreviations: CID, collision-induced dissociations; GLFA, glycolipid fatty acids; PE, phosphatidylethylamine; PG, phosphatidylglycerol; PLFA, phospholipid fatty acids; TLFA, major cellular fatty acids.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and putative alkane hydroxylase gene sequences of strain MIL-1$^T$ are AJ431699 and AJ431700, respectively.

(Button *et al.*, 1998; Golyshin *et al.*, 2002; Yakimov *et al.*, 2002). However, information about the genetics and biochemistry of hydrocarbonoclastic bacteria is still very limited (Wang *et al.*, 1996; Hedlund *et al.*, 1999; Dutta & Harayama, 2001). Further studies on the nutrient requirements of hydrocarbonoclastic bacteria, coupled with investigations into the degradation pathways and genomic analysis, need to be performed to achieve a better understanding of the superior metabolic capacities of these bacteria, which would contribute significantly to the management of oil pollution in marine environments.

During studies on the diversity of hydrocarbonoclastic marine bacteria that occur naturally in coastal ecosystems that have been chronically exposed to oil hydrocarbon pollution, a heterotrophic $\gamma$-proteobacterium that was obligate for hydrocarbon utilization was isolated. In this work, the phenotypic characterization of strain MIL-1$^T$, its phylogenetic assignment and DNA base and lipid compositions are presented. The isolate constitutes a species within a novel genus for which, by considering its origin, morphology and metabolism, the name *Thalassolituus oleivorans* gen. nov., sp. nov. is proposed. Strain MIL-1$^T$ (=DSM 14913$^T$=LMG 21420$^T$) is designated as the type strain.

## METHODS

**Bacterial strain isolation.** Strain MIL-1$^T$ was isolated from sea water/sediment samples that were collected in the harbour of Milazzo, Sicily, Italy, from a depth of about 5 m, by using an enrichment culture with 0·2 % (v/v) n-tetradecane as the sole carbon source in ONR7a mineral medium (Dyksterhouse *et al.*, 1995). Replicate tenfold dilutions of the primary enrichment were made in 10 ml ONR7a mineral medium that was supplemented with sterile n-tetradecane (0·2 %, v/v). Tubes were incubated in the dark at 20 °C until turbidity changes due to bacterial growth ceased (approx. 2 weeks). Positive tubes that represented the highest dilution ($10^{-5}$) were plated further onto solid ONR7a mineral medium that was supplemented with n-tetradecane; single colonies were observed after 7 days incubation.

**Growth conditions and phenotypic analysis.** The isolate was cultivated aerobically in ONR7a medium that was supplemented with 0·2 % (v/v) n-tetradecane. Bacto agar (Difco) (15 g l$^{-1}$) was added for the preparation of solid medium. For all phenotypic tests, cultures were pre-grown in ONR7a medium that was supplemented with n-hexadecane. Growth under anaerobic conditions and utilization of carbon sources were determined and routine tests were carried out as described previously (Golyshin *et al.*, 2002).

The effects of hydrocarbons, salinity and temperature on growth were also examined. The capacity of various aliphatic hydrocarbons to serve as the sole source of carbon and energy was determined at 20 °C in liquid ONR7a medium. Substrates were sterilized separately and added aseptically at 0·2 % (v/v). To determine the salinity range for growth, ONR7a medium that contained no sodium ions and was supplemented with n-tetradecane was prepared by adjustment with appropriate concentrations of NaCl [0·01–2·0 M, i.e. 0·06–12 % (w/v)]. Temperature range for growth was determined by incubation of cultures in the same medium at 4, 10, 15, 20, 25 and 30 °C. In all experiments, growth was scored by measuring $OD_{600}$. Five replicates of test cultures of each strain were analysed after three serial transfers under identical conditions.

**Electron microscopy.** The isolate was cultivated aerobically in ONR7a medium that was supplemented with 0·2 % (v/v) n-tetradecane; cells in mid-exponential growth phase were sedimented and fixed in 5 % glutaraldehyde that was buffered with 50 mM PBS, pH 7·1. Negative-staining, shadow-casting, embedding and ultra-thin sectioning were done according to methods described previously (Yakimov *et al.*, 1998; Golyshina *et al.*, 2000).

**Cellular fatty acid analysis.** Lipids were extracted from mid-exponential cells that had been grown in ONR7a/tetradecane medium by using a modified Bligh–Dyer procedure (Bligh & Dyer, 1959). Later on, fatty acid methyl esters were generated and analysed by GC as described previously (Vancanneyt *et al.*, 1996).

**Phospholipid analysis.** Lipids of cells that had been harvested in the mid-exponential phase were extracted and polar lipids were separated by flash chromatography, as described previously (Abraham *et al.*, 1997). The polar lipid fraction was analysed by using electrospray ionization in the negative mode in a quadrupol-time-of-flight mass spectrometer. Abundant molecular ions were separated and the parent ions then underwent collision-induced dissociations (CID); resulting fragments were finally detected in the time-of-flight part of the instrument.

**G+C content and genome format.** The DNA G+C content of strain MIL-1$^T$ was determined by using an HPLC method that was described previously (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984). Purified non-methylated $\lambda$-phage DNA (Sigma) was used as a control. PFGE separation of the DNA digests by endonucleases *Asc*I, *Pac*I, *Pme*I, *Swa*I and *Sfi*I (New England Biolabs) was performed by using a Gene Navigator Electrophoresis device (Pharmacia) with switch times that ramped between 2 and 64 s at 6 V cm$^{-1}$. In order to obtain plasmids, cells of MIL-1$^T$ were extracted with a Large Construct kit (Qiagen). The extracted DNA was later analysed by gel electrophoresis.

**16S rRNA gene sequence analysis.** To investigate the phylogenetic relationships of strain MIL-1$^T$, isolation of genomic DNA, PCR amplification, determination of the sequence of the 16S rRNA gene and its subsequent phylogenetic affiliation were performed according to previously described protocols (Golyshin *et al.*, 2002).

**Cloning of the putative *alkB* gene.** Chromosomal DNA of strain MIL-1$^T$ was amplified by using oligonucleotides and conditions described by Smits *et al.* (1999) and the deduced putative AlkB protein sequence from MIL-1$^T$ was aligned manually by using the Se-Al sequence alignment editor, version 1.0 $\alpha$1 (Rambaut, 1996). Maximum-likelihood evolutionary distances of the proteins were calculated by using the PROTDIST program and a dendrogram depicting phylogenetic relationships was derived by using the Fitch–Margoliash method (FITCH version 3.572c) with random-order input of sequences and using the global rearrangement option (Felsenstein, 1993).

## RESULTS AND DISCUSSION

### Phenotypic and ultrastructural characteristics

Strain MIL-1$^T$ was isolated after serial dilutions from an enrichment culture that was established from sea water/sediment samples collected in the harbour of Milazzo, Italy, by addition of n-tetradecane as the sole carbon source. Exponentially growing cells were subjected to ultra-thin sectioning after embedding in epoxy resin and were analysed with an energy-filtered transmission electron microscope. Characteristically, the bacteria showed a curved, vibrioid, occasionally screw-like morphology (Fig. 1a) and distinctly

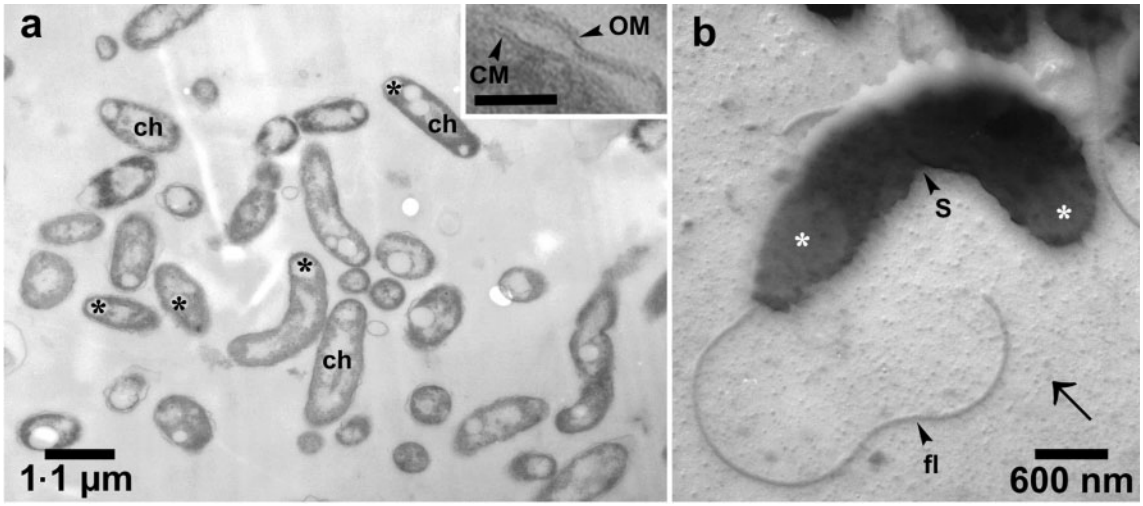


**Fig. 1.** Electron micrograph of (a) ultrathin-sectioned and (b) shadow-casted exponentially growing cells of *Thalassolituus oleivorans*. The nucleoplasm (ch) occupies most of the cell lumen and electron-translucent inclusions (asterisks) are found mainly at the cell poles. Inset shows the cytoplasmic (CM) and outer (OM) membranes. A single flagellum (fl) is inserted at one cell pole of a dividing cell, which shows the start of septation (S). Direction of shadow-casting is marked by an arrow. Bars, 1·1 µm (a); 100 nm (inset); 600 nm (b).

presented a Gram-negative cell wall architecture with an outer membrane. However, under the fixation protocol used, the murein sacculus could be not be recognized as a typical central periplasmic layer (Fig. 1a, inset). Cells were of various lengths in the range 1·2–3·1 µm and measured 0·32–0·77 µm in diameter (mean value: $0{\cdot}566 \pm 0{\cdot}108$ µm; $n = 37$) and, under the growth conditions used, the cytoplasm contained electron-translucent inclusions, possibly of hydrocarbon polymers (Fig. 1a, asterisk). From shadow-casted samples, inclusions were located mainly at cell poles (Fig. 1b, asterisk). The bacteria characteristically showed monopolar, monotrichous flagellation (Fig. 1b, fl), whereas a monopolar tuft of four flagella was also detected.

The new isolate required NaCl for growth; growth was observed at NaCl concentrations of 0·5–5·7 % (w/v). Optimum growth occurred at 2·7 % NaCl. The isolate grew at 4–30 °C, with an optimum growth temperature of 20–25 °C. The pH range for growth was 7·5–9·0, with optimum growth at pH 8·0.

## Physiology and biochemical characteristics

Consistent with its phylogenetic placement, strain MIL-1$^T$ shares many phenotypic properties with *Oceanospirillum* and related genera. However, there are some crucial phenotypic differences that suggest that the new strain does not belong to any previously described genus. Isolate MIL-1$^T$ was oxidase-positive and did not catabolize any substrate tested except for acetate, aliphatic hydrocarbons with a carbon chain length between $C_7$ and $C_{20}$ and their oxidized derivatives. Poor growth was observed in ONR7a medium that was supplemented with L-arabinose and psicose. During growth on Tweens 20, 40 and 80, production of extracellular lipase was detected. Neither nitrate reduction nor denitrifying activity was detected. The reaction for catalase was positive. Biochemical and physiological characteristics that differentiate isolate MIL-1$^T$ from related genera are summarized in Table 1. In contrast with the genera *Marinobacter*, *Marinomonas* and *Oceanobacter*, which are characterized by nutritional versatility, uptake by isolate MIL-1$^T$ is almost restricted to aliphatic hydrocarbons. Such a narrow spectrum of substrates that support growth of MIL-1$^T$ is a typical physiological feature for marine, obligately alkane-degrading $\gamma$-proteobacteria that belong to the recently described genera *Alcanivorax*, *Oleiphilus* and *Oleispira* (Yakimov *et al.*, 1998, 2002; Golyshin *et al.*, 2002).

## Lipid analysis

After a whole-cell methanolysis procedure and saponification of phospho- and glycolipids, three different fatty acid profiles were detected in strain MIL-1$^T$ (Table 2). The fraction of the saturated fatty acids $C_{12}$–$C_{18}$ represented $>92$ % of total extracted glycolipid fatty acids (GLFA), with lauric acid as a major component. The major cellular and phospholipid fatty acid (TLFA and PLFA, respectively) profiles were characterized by an almost equal presence of saturated and monounsaturated fatty acids, with a strong predominance of $C_{14:0}$, $C_{16:1}$, $C_{16:0}$ and $C_{18:1}$. These profiles were different from that of *Oceanobacter kriegii*, which is characterized by the strong abundance of monounsaturated fatty acids (63 %) (Gonzalez & Whitman, 2001). Analysis of hydroxy fatty acids in strain MIL-1$^T$ TLFA revealed the presence of a single hydroxy fatty acid, $C_{12:0}$ 3-OH, whereas three different 3-hydroxy fatty acids are

**Table 1.** Selected phenotypic properties that distinguish the genus *Thalassolituus* from other related genera of marine *γ-Proteobacteria*

Genera: 1, *Marinobacterium*; 2, *Marinomonas*; 3, *Oceanobacter*; 4, *Oleispira*; 5, *Thalassolituus*. Data from Bowditch *et al.* (1984), Gonzalez & Whitman (2001), Satomi *et al.* (2002), Yakimov *et al.* (2002) and this study.

| Trait | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Cell shape* | St | St | St | Cr | Cr, Cb |
| Flagellation† | 1 | 1–2 | 1 | 1 | 1–4 |
| Accumulation of PHB‡ | + | − | + | − | − |
| Lipase | − | − | + | + | + |
| Reduction of nitrate | + | + | + | + | − |
| Utilization of: | | | | | |
| D-Glucose | + | + | − | − | − |
| D-Fructose | V§ | + | + | − | − |
| Ethanol | + | + | + | − | − |
| n-Propanol | + | + | + | − | − |
| n-Butanol | + | + | + | − | − |
| Mannitol | ND‖ | + | + | − | − |
| Sorbitol | + | + | + | − | − |
| Citrate | + | + | + | − | − |
| *p*-Hydroxybenzoate | + | + | + | − | − |
| DL-*β*-Hydroxybutyrate | + | − | + | − | − |
| 2-Oxoglutarate | + | V | + | − | − |
| DL-Lactate | + | + | + | − | − |
| DL-Malate | V | + | + | − | − |
| Quinate | + | + | + | − | − |
| Propionate | + | + | + | − | − |
| DL-Alanine | + | + | + | − | − |
| Aliphatic hydrocarbons | − | − | ND | + | + |
| Major 3-OH fatty acid (%) | $C_{10:0}$ (100) | $C_{10:0}$ (60) | $C_{12:0}$ (54) | None | $C_{12:0}$ (100) |
| DNA G+C content (mol%) | 55 | 44–48 | 55 | 41–42 | 53·2 |

*Cb, Coccoid bodies; Cr, curved rods; St, straight rods.
†Number of flagella at one pole.
‡PHB, Poly-*β*-hydroxybutyrate.
§V, Variable among strains
‖ND, No data available.

present in *Oceanobacter kriegii*: $C_{10:0}$ (19 %), $C_{12:0}$ (54 %) and $C_{16:0}$ (27 %).

## Analysis of intact phospholipids

Analysis of CID-MS spectra revealed the presence of two different types of phospholipid: the phosphatidylethylamine (PE) and phosphatidylglycerol (PG) types. Thirteen different compounds could be identified and are listed in Table 3. The position of the two fatty acids at the glycerol moiety could be deduced because for the fatty acid positioned at *sn*-2, the neutral loss as free fatty acid, as well as substituted ketene, is more frequent than for that positioned at *sn*-1 (Murphy & Harrison, 1994). From the structure of the lipids, it was evident that all lipids possessed an unsaturated fatty acid at *sn*-2 of the glycerol moiety, whereas the *sn*-1 position was mainly occupied by saturated fatty acids. Such a preference for having longer and saturated fatty acids at *sn*-1 was described previously as a general feature of bacterial phospholipids (Lechevalier, 1977), with only a few exceptions (Fang *et al.*, 2000). As the distribution of fatty acids in the molecule has some influence on the rigidity of the cell wall, the finding that the proportion of saturated fatty acids at the *sn*-1 position is higher for PG than for PE may have consequences for the stability of cell-wall contact with hydrocarbons.

## DNA G+C content and genome format

The G+C content of the genomic DNA of strain MIL-1$^T$ is 53·2 mol%, which is comparable with the DNA G+C contents of *Marinobacterium* and *Oceanobacter* (Table 1). The G+C content of the amplified 16S rRNA gene sequence of strain MIL-1$^T$ is 53·37 mol%. As revealed by PFGE

**Table 2.** Fatty acid profiles of *T. oleivorans* MIL-1$^T$ and another marine hydrocarbonoclastic $\gamma$-proteobacterium, *Oleispira antarctica* RB-8$^T$

Abbreviations: GL, glycolipids; PL, phospholipids; TL, total lipids. Values given are percentages of the total for each type of lipid.

| Fatty acid | *T. oleivorans* | | | *O. antarctica** | |
|---|---|---|---|---|---|
| | PL | GL | TL | PL | TL |
| $C_{12:0}$ | 0·0 | 37·2 | 2·0 | 1·4 | 1·2 |
| $C_{12:0}$ 3-OH | 0·0 | 0·0 | 1·4 | 0·0 | 0·0 |
| $C_{14:1}\omega9$ | 0·0 | 0·0 | 1·2 | 2·4 | 0·2 |
| $C_{14:0}$ | 8·1 | 28·7 | 10·2 | 5·3 | 1·2 |
| $C_{16:1}\omega7$ | 17·6 | 0·0 | 33·5 | 49·9 | 29·9 |
| $C_{16:0}$ | 31·6 | 20·6 | 29·5 | 32·5 | 23·9 |
| $C_{18:1}\omega6/\omega9$ | 30·7 | 7·9 | 10·9 | 2·4 | 32·0 |
| $C_{18:0}$ | 12·0 | 5·6 | 7·7 | 0·0 | 1·2 |
| Other† | 0·0 | 0·0 | 3·6 | 6·1 | 10·4 |
| Total | 100·0 | 100·0 | 100·0 | 100·0 | 100·0 |

*Grown at 20 °C.

†Other mean content of unidentified fatty acids in TL of *T. oleivorans* and different fatty acids detected only in *O. antarctica*.

analysis of endonuclease digests of the genomic DNA of isolate MIL-1$^T$, the genome size was about 2·2 Mbp. No plasmids were observed.

## Molecular phylogenetic analysis

An almost-complete 16S rDNA sequence (1366 bp) was determined for isolate MIL-1$^T$. Preliminary sequence comparison against the 16S rRNA sequences held in GenBank and the Ribosomal Database Project database (Altschul *et al.*, 1997; Maidak *et al.*, 1997) indicated that the organism belongs to the $\gamma$-subclass of the *Proteobacteria*. The sequence was aligned manually against representatives of the $\gamma$-*Proteobacteria* by using the secondary structure model of bacterial 16S rRNA (Gutell, 1994). On the basis of 16S rDNA similarity, strain MIL-1$^T$ showed an apparent relationship with bacteria that belonged to the *Marinomonas* assemblage, within a heterogeneous group that also contained the genus *Oceanospirillum*. The closest relatives are *Oceanobacter kriegii* ATCC 27133$^T$ (94·4 % 16S rDNA sequence similarity), *Oleispira antarctica* LMG 21398$^T$ (92·5 %), *Marinobacterium georgiense* IAM 1419 (91·6 %), *Oceanospirillum multiglobuliferum* NBRC 13614$^T$ (91·5 %), *Marinomonas mediterranea* ATCC 700492$^T$ (91·2 %) and *Oceanospirillum linum* ATCC 11336$^T$ (90·9 %). According to the method of analysis (Satomi *et al.*, 2002), strain MIL-1$^T$ formed a stable phyletic group with *Oceanobacter kriegii* and *Oleispira antarctica* and was evidently placed in the *Marinomonas* assemblage. The branching point of MIL-1$^T$ was stable, as the corresponding bootstrap values were very high (100, 71 and 78 %, respectively; Fig. 2). A very similar tree topology was reconstructed by using the Jukes–Cantor treeing algorithm (data not shown).

**Table 3.** Polar lipids identified by CID-MS in lipid extract of *T. oleivorans* MIL-1$^T$

| No. | Mass | Type | *sn*-1 | *sn*-2 |
|---|---|---|---|---|
| 1 | 687 | PE | $C_{16:1}$ | $C_{16:1}$ |
| 2 | 687 | PE | $C_{14:1}$ | $C_{18:1}$ |
| 3 | 687 | PE | $C_{14:0}$ | $C_{18:2}$ |
| 4 | 689 | PE | $C_{16:0}$ | $C_{16:1}$ |
| 5 | 689 | PE | $C_{14:0}$ | $C_{18:1}$ |
| 6 | 715 | PE | $C_{16:1}$ | $C_{18:1}$ |
| 7 | 717 | PE | $C_{16:0}$ | $C_{18:1}$ |
| 8 | 717 | PE | $C_{18:0}$ | $C_{16:1}$ |
| 9 | 720 | PG | $C_{16:0}$ | $C_{16:1}$ |
| 10 | 720 | PG | $C_{14:0}$ | $C_{18:1}$ |
| 11 | 746 | PG | $C_{16:1}$ | $C_{18:1}$ |
| 12 | 748 | PG | $C_{16:0}$ | $C_{18:1}$ |
| 13 | 776 | PG | $C_{18:0}$ | $C_{18:1}$ |

## Alkane hydroxylase gene (*alkB*)

The putative gene for alkane hydroxylase, the key enzyme of alkane catabolism, was cloned by using the approach of Smits *et al.* (1999). Searches for coding areas revealed that the sequenced DNA represented part of a larger ORF that encoded a protein of 185 aa. Phylogenetic analysis of this deduced polypeptide is shown in Fig. 3. The protein sequence exhibited 80 % similarity to the corresponding part of the 404 aa alkane hydroxylase of *Alcanivorax borkumensis* and clustered distinctly with the branch of pseudomonad alkane hydroxylases. Interestingly, we failed to amplify the putative *alkB* gene from the most closely related micro-organisms, *Oceanobacter kriegii* ATCC 27133$^T$ and *Oleispira antarctica* DSM 14852$^T$.

Polyphasic taxonomic treatment of strain MIL-1$^T$ unequivocally indicates that the phylogenetic and phenotypic differences between strain MIL-1$^T$ and its closest relatives justify the description of a novel genus and species, *Thalassolituus oleivorans* gen. nov., sp. nov.

## Description of *Thalassolituus* gen. nov.

*Thalassolituus* (Tha.las.so.li.tu′us. Gr. fem. n. *thalassa* the sea; L. masc. n. *lituus* a curved rod, crook; N.L. masc. n. *Thalassolituus* a marine, curve-shaped organism).

Gram-negative, vibrioid to spiral, motile cells, 1·2–3·5 μm long by 0·6 μm wide. Strictly halophilic: $Na^+$ ions are required for growth. Chemoorganoheterotrophic; strictly aerobic; unable to grow under anaerobic conditions by fermentation, nitrate reduction or phototrophically. Oxidase-positive. Ammonia and nitrate may serve as nitrogen sources. Indole-, arginine dihydrolase- and gelatinase-negative. Acetate, $C_7$–$C_{20}$ aliphatic hydrocarbons and their oxidized derivatives are the only carbon sources that are

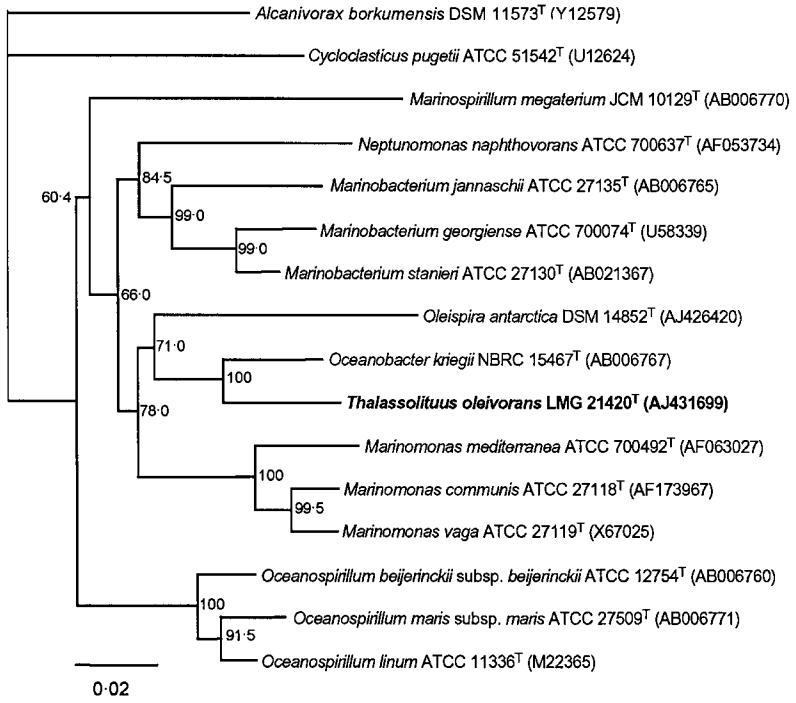


**Fig. 2.** Position of *T. oleivorans* MIL-1$^T$ among related representatives of the *Oceanospirillum* assemblage with validly published names, based on 16S rRNA phylogenetic analysis. The tree, based on 1360 nucleotide positions, was constructed by using the neighbour-joining method and nucleotide substitution rates were computed by using Kimura's two-parameter model, as described previously (Satomi *et al.*, 2002; Yakimov *et al.*, 2002). Numbers at nodes are bootstrap values (percentage of 500 trees analysed; only values >60 % are shown). Bar, 0·02 substitution per sequence position.

used for growth. Principal cellular fatty acids are laurate, palmitate and octadecenoate. According to 16S rRNA gene sequence analysis, the genus belongs to the $\gamma$-subgroup of the *Proteobacteria*, namely to the *Oceanospirillum*/*Marinomonas*/*Marinobacterium* assemblage. The type and only species (to date) of the genus is *Thalassolituus oleivorans.*

## Description of *Thalassolituus oleivorans* sp. nov.

*Thalassolituus oleivorans* (o.le.i.vo′rans. L. n. *oleum* oil; L. part. adj. *vorans* devouring; N.L. adj. *oleivorans* oil-devouring).

Polymorphic bacteria that are motile by means of one to four polar flagella. Genome size is about 2·2 Mbp. Marine; requires at least 25 % sea water salinity for growth. $Na^+$ ions are required; growth occurs at NaCl concentrations of 0·5–5·7 % (w/v), with optimum growth at 2·3 % NaCl. Growth occurs at 4–30 °C, with optimum growth at 20–25 °C. pH range for growth is 7·5–9·0, with optimum growth at pH 8·0. Tweens 20, 40 and 80 are degraded, whereas agarase, amylase, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, gelatinase and aesculinase activities are not detected. Nitrate is not reduced to nitrite. Acetate, aliphatic hydrocarbons with a chain-length between $C_7$ and $C_{20}$ and their oxidized derivatives are the only substrates that support growth. The principal fatty

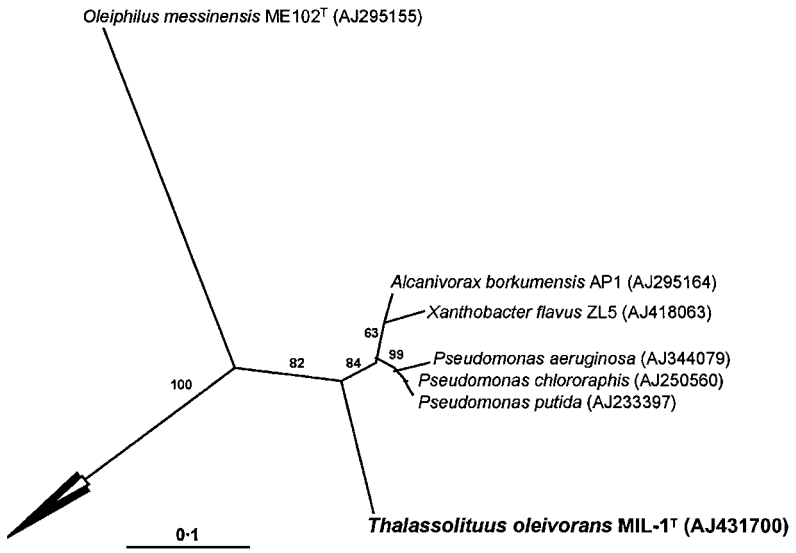


**Fig. 3.** Phylogenetic position of the deduced protein sequence for the *T. oleivorans* MIL-1$^T$ cloned putative alkane hydroxylase AlkB, among relevant enzymes of the $\gamma$-*Proteobacteria*. Numbers at nodes are bootstrap confidence values (percentage of 100 bootstrap replications). Tree was rooted with the sequence of the alkane-1-monooxygenase of *Rhodococcus erythropolis* (GenBank accession no. AJ301871). Bar, 0·1 substitution per sequence position.

acids in total TLFA, PLFA and GLFA profiles are $C_{12:0}$, $C_{16:0}$ and $C_{18:1}$. The TLFA and PLFA profiles are characterized by an almost equal presence of saturated and monounsaturated fatty acids, with a strong predominance of $C_{14:0}$, $C_{16:1}$, $C_{16:0}$ and $C_{18:1}$. Phospholipids are represented by the PE and PG types. DNA G+C content is 53·2 mol%. According to analysis of the 16S rRNA gene sequence, this bacterium belongs to the $\gamma$-subclass of the *Proteobacteria* and forms a stable phyletic group with *Oceanobacter kriegii*.

The type and only strain to date, MIL-1$^T$ (=DSM 14913$^T$=LMG 21420$^T$), was isolated after serial dilutions from an enrichment culture that was established from sea water/sediment samples collected in the harbour of Milazzo, Sicily, Italy, by addition of n-tetradecane as the sole carbon source.

## ACKNOWLEDGEMENTS

We are indebted to Rene Huppmann for assistance in fatty acid analysis. This work was supported by grants from the Italian Ministry for Education, University and Research (PEA 1999–2000, Research Project 1.4 and CLUSTER 10, Project 'SAM'). K. N. T. gratefully acknowledges the generous support of the Fonds der Chemischen Industrie. We thank Hans Trüper (University of Bonn) for advice and corrections of the bacterial name.

## REFERENCES

**Abraham, W.-R., Meyer, H., Lindholst, S., Vancanneyt, M. & Smit, J. (1997).** Phospho- and sulfolipids as biomarkers of *Caulobacter sensu lato*, *Brevundimonas* and *Hyphomonas*. *Syst Appl Microbiol* **20**, 522–539.

**Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997).** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

**Bligh, E. G. & Dyer, W. J. (1959).** A rapid method for total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.

**Bowditch, R. D., Baumann, L. & Baumann, P. (1984).** Description of *Oceanospirillum kriegii* sp. nov. and *O. jannaschii* sp. nov. and assignment of two species of *Alteromonas* to this genus as *O. commune* comb. nov. and *O. vagum* comb. nov. *Curr Microbiol* **10**, 221–230.

**Button, D. K., Robertson, B. R., Lepp, P. W. & Schmidt, T. M. (1998).** A small, dilute-cytoplasm, high-affinity, novel bacterium isolated by extinction culture and having kinetic constants compatible with growth at ambient concentrations of dissolved nutrients in seawater. *Appl Environ Microbiol* **64**, 4467–4476.

**Dutta, T. K. & Harayama, S. (2001).** Biodegradation of *n*-alkylcycloalkanes and *n*-alkylbenzenes via new pathways in *Alcanivorax* sp. strain MBIC 4326. *Appl Environ Microbiol* **67**, 1970–1974.

**Dyksterhouse, S. E., Gray, J. P., Herwig, R. P., Lara, J. C. & Staley, J. T. (1995).** *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. *Int J Syst Bacteriol* **45**, 116–123.

**Fang, J., Barcelona, M. J., Nogi, Y. & Kato, C. (2000).** Biochemical implications and geochemical significance of novel phospholipids of the extremely barophilic bacteria from the Mariana Trench at 11,000 m. *Deep-Sea Res Part I Oceanogr Res Pap* **47**, 1173–1182.

**Felsenstein, J. (1993).** PHYLIP (phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle, USA.

**Gauthier, M. J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P. & Bertrand, J.-C. (1992).** *Marinobacter hydrocarbonoclasticus* gen nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int J Syst Bacteriol* **42**, 568–576.

**Golyshin, P. N., Chernikova, T. N., Abraham, W.-R., Lünsdorf, H., Timmis, K. N. & Yakimov, M. M. (2002).** *Oleiphilaceae* fam. nov., to include *Oleiphilus messinensis* gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons. *Int J Syst Evol Microbiol* **52**, 901–911.

**Golyshina, O. V., Pivovarova, T. A., Karavaiko, G. I. & 7 other authors (2000).** *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. nov., comprising a distinct lineage of the *Archaea*. *Int J Syst Evol Microbiol* **50**, 997–1006.

**González, J. M. & Whitman, W. B. (2001).** *Oceanospirillum* and related genera. In *The Prokaryotes* (available at http://link.springer-ny.com/link/service/books/10125/tocs.htm).

**Gutell, R. R. (1994).** Collection of small subunit (16S- and 16S-like) ribosomal RNA structures: 1994. *Nucleic Acids Res* **22**, 3502–3507.

**Harayama, S., Kishira, H., Kasai, Y. & Shutsubo, K. (1999).** Petroleum biodegradation in marine environments. *J Mol Microbiol Biotechnol* **1**, 63–70.

**Hedlund, B. P., Geiselbrecht, A. D., Bair, T. J. & Staley, J. T. (1999).** Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov. *Appl Environ Microbiol* **65**, 251–259.

**Kasai, Y., Kishira, H., Syutsubo, K. & Harayama, S. (2001).** Molecular detection of marine bacterial populations on beaches contaminated by the Nakhodka tanker oil-spill accident. *Environ Microbiol* **3**, 246–255.

**Krieg, N. R. (1984).** Genus *Oceanospirillum* Hylemon, Wells, Krieg and Jannasch 1973, 361$^{AL}$. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 104–110. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

**Lechevalier, M. P. (1977).** Lipids in bacterial taxonomy – a taxonomist's view. *Crit Rev Microbiol* **5**, 109–210.

**Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1997).** The RDP (Ribosomal Database Project). *Nucleic Acids Res* **25**, 109–111.

**Mesbah, M., Premachandran, U. & Whitman, W. B. (1989).** Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

**Murphy, R. C. & Harrison, K. A. (1994).** Fast atom bombardment mass spectrometry of phospholipids. *Mass Spectrom Rev* **13**, 57–75.

**Pot, B., Gillis, M. & De Ley, J. (1992).** The genus *Oceanospirillum*. In *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, vol. 4., pp. 3230–3236. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

**Rambaut, A. (1996).** Se-Al (Sequence Alignment Editor), version 1.0 $\alpha$1. Distributed by the author and available via http://evolve.zoo.ox.ac.uk/software.html?id=seal. Department of Zoology, University of Oxford, UK.

**Satomi, M., Kimura, B., Hayashi, M., Shouzen, Y., Okuzumi, M. & Fujii, T. (1998).** *Marinospirillum* gen. nov., with descriptions of *Marinospirillum megaterium* sp. nov., isolated from kusaya gravy, and transfer of *Oceanospirillum minutulum* to *Marinospirillum minutulum* comb. nov. *Int J Syst Bacteriol* **48**, 1341–1348.

**Satomi, M., Kimura, B., Hamada, T., Harayama, S. & Fujii, T. (2002).** Phylogenetic study of the genus *Oceanospirillum* based on 16S rRNA and *gyrB* genes: emended description of the genus *Oceanospirillum*, description of *Pseudospirillum* gen. nov., *Oceanobacter* gen. nov. and *Terasakiella* gen. nov. and transfer of *Oceanospirillum jannaschii* and *Pseudomonas stanieri* to *Marinobacterium* as *Marinobacterium jannaschii* comb. nov. and *Marinobacterium stanieri* comb. nov. *Int J Syst Evol Microbiol* **52**, 739–747.

**Smits, T. H. M., Röthlisberger, M., Witholt, B. & van Beilen, J. B. (1999).** Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environ Microbiol* **1**, 307–317.

**Syutsubo, K., Kishira, H. & Harayama, S. (2001).** Development of specific oligonucleotide probes for the identification and *in situ* detection of hydrocarbon-degrading *Alcanivorax* strains. *Environ Microbiol* **3**, 371–379.

**Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

**Vancanneyt, M., Witt, S., Abraham, W.-R., Kersters, K. & Fredrickson, H. L. (1996).** Fatty acid content in whole-cell hydrolysates and phospholipid fractions of pseudomonads: a taxonomic evaluation. *Syst Appl Microbiol* **19**, 528–540.

**Wang, Y., Lau, P. C. K. & Button, D. K. (1996).** A marine oligobacterium harboring genes known to be part of aromatic hydrocarbon degradation pathways of soil pseudomonads. *Appl Environ Microbiol* **62**, 2169–2173.

**Yakimov, M. M., Golyshin, P. N., Lang, S., Moore, E. R. B., Abraham, W.-R., Lünsdorf, H. & Timmis, K. N. (1998).** *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *Int J Syst Bacteriol* **48**, 339–348.

**Yakimov, M. M., Giuliano, L., Gentile, G., Crisafi, E., Chernikova, T. N., Abraham, W.-R., Lünsdorf, H., Timmis, K. N. & Golyshin, P. N. (2003).** *Oleispira antarctica* gen. nov., sp. nov., a novel hydrocarbonoclastic marine bacterium isolated from Antarctic coastal sea water. *Int J Syst Evol Microbiol* **53**, 779–785.