Leisingera aquimarina sp. nov., isolated from a marine electroactive biofilm, and emended descriptions of *Leisingera methylohalidivorans* Schaefer *et al.* 2002, *Phaeobacter daeponensis* Yoon *et al.* 2007 and *Phaeobacter inhibens* Martens *et al.* 2006

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Strain LMG 24366^T was isolated from a marine electroactive biofilm grown on a stainless steel cathode (Genova, Italy) and was investigated by using a polyphasic taxonomic approach. This study demonstrated that strain LMG 24366^T represents a novel species within the genus *Leisingera*, which shared 98.9 % 16S rRNA gene similarity with its nearest phylogenetic neighbour, *Leisingera methylohalidivorans*. Strain LMG 24366^T grew on betaine (1 mM) as a sole carbon source, whereas no growth was observed on L-methionine (10 mM). The phenotypic and genotypic analyses showed that strain LMG 24366^T could be differentiated from established *Leisingera* species and that it represented a novel species, for which the name *Leisingera aquimarina* sp. nov. is proposed. The type strain is LMG 24366^T (=CCUG 55860^T) and has a DNA G+C content of 61.4 mol%.

The genus *Leisingera* was created by Schaefer *et al.* (2002) to accommodate *Leisingera methylohalidivorans*. The only species of this genus was isolated from a tide pool off the coast of California and was able to grow on methyl bromide as a sole energy and carbon source (Schaefer *et al.*, 2002). Although oceans act as a sink for methyl bromide (Yvon & Butler, 1996), it was the first species shown to be able to grow on methyl bromide. Growth of *L. methylohalidivorans* has also been observed on methyl chloride, methyl iodide and methionine. These data indicate a role for *L. methylohalidivorans* in the degradation of methyl halides and thus in the release of bromide and chlorine atoms in the stratosphere, which contributes to the catalytic destruction of ozone (Butler, 1999).

The genus *Leisingera* is a member of the *Roseobacter* lineage of the *Alphaproteobacteria*. This lineage is one of the most abundant marine groups (González *et al.*, 2000), as *Roseobacter* species can constitute up to 20 % of coastal bacterioplankton communities and are found in almost all marine environments (Buchan *et al.*, 2005).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LMG 24366^T is AM900415.

The present study formed part of an analysis of the microbial diversity of a marine electroactive biofilm grown on a stainless steel cathode [EA BIOFILMS-508866 (NEST)], exposed to natural seawater at the ISMAR-CNR Marine Station, located in the port of Genoa, Italy (Faimali *et al.*, 2008; Vandecandelaere *et al.*, 2008). The biofilm was removed from the stainless steel cathode by sonication (Branson 3200) (90 s) in a sterile plastic tube containing 30 ml 0.85 % NaCl solution. Diluted cell suspensions (10^{-1} to 10^{-6}) were inoculated on marine agar 2216 (MA; Difco) and incubated aerobically at 20 °C for several days. Pure cultures were obtained and isolates were stored at –20 or –80 °C using MicroBank vials.

Whole-cell fatty acid methyl ester analysis, performed as described by Mergaert *et al.* (2001), indicated that strain LMG 24366^T belonged to the *Alphaproteobacteria*. The predominant fatty acids were $C_{10:0}$ 3-OH (2.0 %), $C_{12:0}$ 3-OH (2.1 %), $C_{16:0}$ (3.5 %), $C_{16:0}$ 2-OH (4.2 %), $C_{14:1}$ iso E (11.6 %), $C_{18:1}\omega7c$ (71.6 %) and an unknown fatty acid of equivalent chain-length 11.799 (2.7 %). The remaining fatty acids constituted minor fractions only (<1.0 %). The predominant fatty acids observed were very similar to those of *Phaeobacter daeponensis*, *Phaeobacter gallaeciensis*,

Phaeobacter inhibens and *L. methylohalidivorans* (Yoon *et al.*, 2007), indicating that strain LMG 24366^T was closely related to *Phaeobacter* species and to *L. methylohalidivorans*.

DNA was extracted according to Pitcher *et al.* (1989) and an almost-complete 16S rRNA gene sequence was obtained (1396 bp) using the universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards *et al.*, 1989), as described by Mergaert *et al.* (2001). The FASTA program was used to find the most similar sequences. These almost-complete 16S rRNA gene sequences (1248–1465 bp) were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and a neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) using BioNumerics 4.61 software (Applied Maths) (Fig. 1).

The numerical analysis indicated that the nearest phylogenetic neighbour of strain LMG 24366^T was *L. methylohalidivorans* LMG 23656^T with 98.9 % 16S rRNA gene sequence similarity. Members of the genus *Phaeobacter* (*P. daeponensis* LMG 24139^T, *P. inhibens* LMG 22475^T and *P. gallaeciensis* LMG 23163^T) (Martens *et al.*, 2006; Yoon *et al.*, 2007) were also closely related to strain LMG 24366^T, with 16S rRNA gene sequence similarities of 96.9–97.2 %.

Strains LMG 24366^T, *L. methylohalidivorans* LMG 23656^T, *P. gallaeciensis* LMG 23163^T and *P. inhibens* LMG 22475^T were investigated by using repetitive-PCR fingerprinting using the BOX-A1R-primer 5′-CTACGGCAAGGCGACGCTGACG-3′ (Rademaker *et al.*, 2000; Versalovic *et al.*, 1994). Numerical analysis, using BioNumerics 4.61 software showed that strain LMG 24366^T could be distinguished from its nearest phylogenetic neighbours (Fig. 2).

The DNA G+C content of strain LMG 24366^T was determined. DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was separated using a Water Breeze HPLC system and Xbridge Shield RP18 column thermostabilized at 37 °C. The solvent used was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) and *Escherichia coli* LMG 2093 DNA were used as calibration reference and control, respectively. The DNA G+C content of LMG 24366^T was 61.4 mol%, which correlates with that of the genus *Leisingera* as described by Schaefer *et al.* (2002).

DNA–DNA hybridization experiments were carried out with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature used was 45 °C and reciprocal reactions were performed for each pair of strains. The mean DNA–DNA hybridization value between strain LMG 24366^T and its nearest phylogenetic neighbour, *L. methylohalidivorans* LMG 23656^T, was 56±2 %; the mean DNA–DNA hybridization value between LMG 24366^T and *P. gallaeciensis* LMG 23163^T was very low (2±0 %). These data indicated that LMG 24366^T represents a novel species within the genus *Leisingera* (Wayne *et al.*, 1987).

The following morphological, physiological and biochemical characteristics were evaluated for strain LMG 24366^T. Colony morphology was determined after 4 days incubation at 20 °C on MA. Cells were tested for their Gram reaction and the presence of catalase and oxidase activity.

Growth was examined on nutrient agar (NA), trypticase soy agar (TSA), R2A and peptone/yeast extract/glucose (PYG) agar (Tan & Rüger, 1999). Growth on L-methionine (10 mM) and betaine (1 mM) was tested as described by Martens *et al.* (2006). The optimal salinity and the optimal growth temperature were determined using R2A supplemented with 1–20 % NaCl, incubated for 2 weeks at 20 °C, and MA incubated at 4–45 °C for 2 weeks, respectively. The effect of pH on growth was analysed using marine broth 2216 (Difco) with a pH that ranged from 5.0 to 10.0 (at intervals of 0.5 pH units), with incubation at 20 °C for 7 days.

Degradation of casein and chitin (Reichenbach & Dworkin, 1981), DNA [using DNA agar (Difco) containing 0.01 % toluidine blue (Merck)], starch and L-tyrosine (Barrow & Feltham, 1993) was tested and the reactions were read after 5 days incubation at 20 °C. Strain LMG 24366^T was inoculated on Sierra's medium to determine its lipolytic activity and incubated for 10 days at 20 °C (Sierra, 1957).

The susceptibility to the following antibiotics (Oxoid) was examined on MA plates using the disc diffusion method: cefoxitin (30 μg), gentamicin (30 μg), erythromycin (15 μg), tetracycline (30 μg), streptomycin (25 μg), vancomycin (30 μg), trimethoprim (1.25 μg) and clindamycin (2 μg). Results were read after 5 days incubation at 20 °C.

Biochemical characteristics from commercial microtest galleries (API ZYM and API 20NE; bioMérieux) were studied according to the manufacturer's instructions. API ZYM was read after 4 h incubation at 20 °C, whereas API 20NE was read after 48 h incubation at 20 °C.

The cell morphology of strain LMG 24366^T was determined by using transmission electron microscopy. Cells were negatively stained with 2 % uranyl acetate. Ultrathin sections were prepared and analysed as described by Mast *et al.* (2005) (Fig. 3).

The results of the phenotypic tests are given in Table 1. Strain LMG 24366^T could be differentiated from *L. methylohalidivorans* and *Phaeobacter* species. On the basis of the phylogenetic, genomic and phenotypic data, LMG 24366^T represents a novel species within the genus *Leisingera*, for which the name *Leisingera aquimarina* sp. nov. is proposed.

Description of *Leisingera aquimarina* sp. nov.

Leisingera aquimarina (a.qui′ma.ri′na. L. fem. n. *aqua* water; L. adj. *marinus* from the sea; N.L. fem. adj. *aquimarina* from seawater).

Cells are ovoid (1 × 1.4 μm), Gram-negative and motile by means of a single polar flagellum. Poly-β-hydroxybutyrate inclusion bodies are present. Colonies are dark beige–pink,

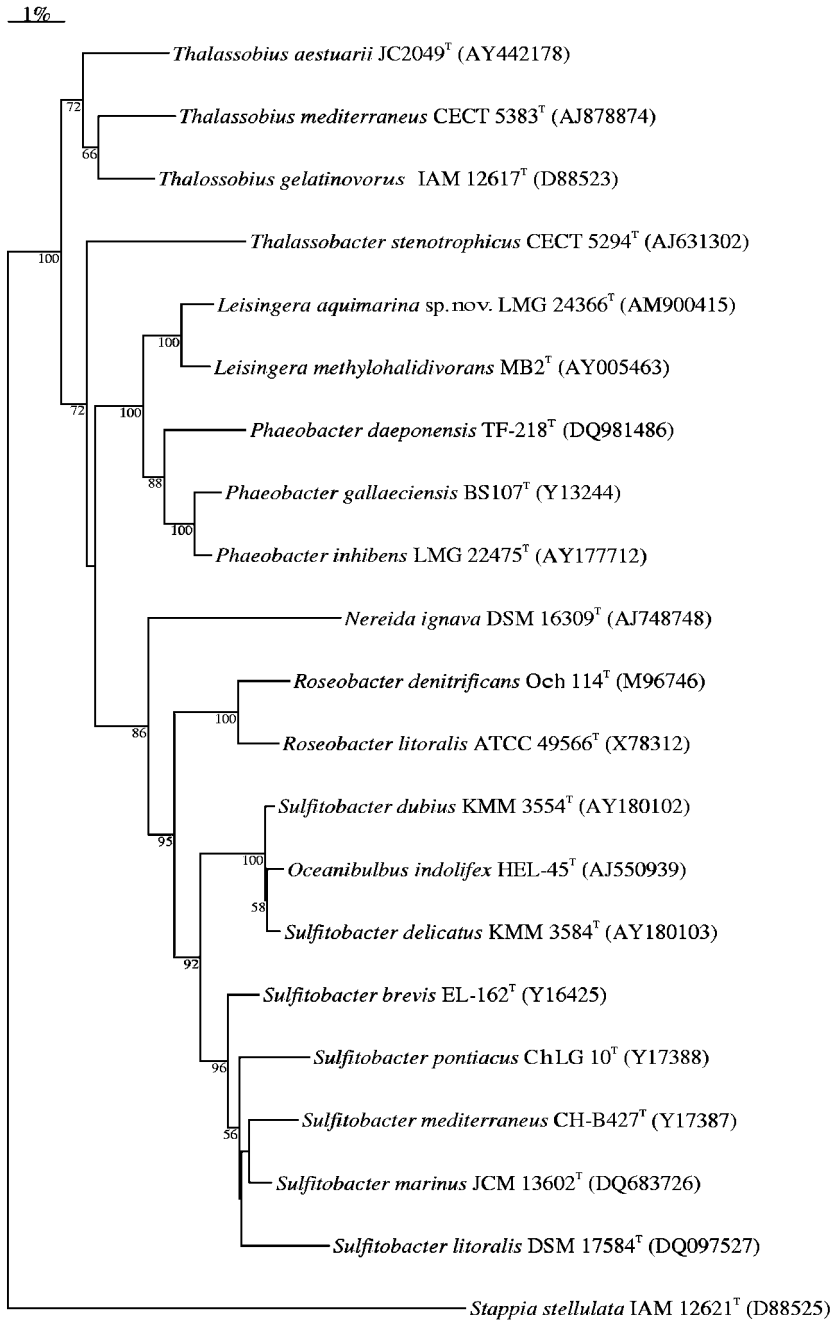

Fig. 1. Neighbour-joining dendrogram depicting the 16S rRNA gene sequences of strain LMG 24366^T (*Leisingera aquimarina* sp. nov.) and related species. Bar, 1 % 16S rRNA gene sequence diversity. Bootstrap percentages (1000 replicates) above 50 % are shown.

round and 1–2 mm in diameter after 3 days incubation on MA. Growth occurs after 2 days incubation at 20 °C on MA, but not on R2A, NA, TSA or PYG. Temperature range for growth is 4–37 °C; no growth occurs at 40 °C or higher. NaCl range for growth is 1–7 %. pH range for growth is 5.5–9.0; optimal pH for growth is 6.5–8. Growth occurs on betaine (1 mM) as a sole carbon source, but not on L-methionine (10 mM). Catalase- and oxidase-positive. Degradation of gelatin is weakly positive; does not degrade tyrosine, DNA, starch, casein, chitin, aesculin or Tween 80. Positive for leucine arylamidase activity; weak alkaline phosphatase, esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase activities. No activity is detected for esterase (C4), valine arylamidase, acid phosphatase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, arginine dihydrolase, urease or α-fucosidase. Nitrate is not reduced to nitrite or nitrogen. Indole is not produced and glucose is not fermented. Does not assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid. Susceptible to cefoxitin (30 μg), erythromycin (15 μg), tetracycline (30 μg) and

Fig. 2. BOX-PCR fingerprints of strain LMG 24366^T (*Leisingera aquimarina* sp. nov.) and related strains.

streptomycin (25 μg). Resistant to vancomycin (30 μg), trimethoprim (1.25 μg), clindamycin (2 μg) and gentamicin (30 μg). Dominant fatty acids are $C_{10:0}$ 3-OH, $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{16:0}$ 2-OH, $C_{14:1}$ iso E, $C_{18:1}\omega7c$ and an unknown fatty acid of equivalent chain-length of 11.799; other fatty acids constitute trace amounts only (<1.0 %). The DNA G+G content of the type strain is 61.4 mol%.

The type strain, LMG 24366^T (=CCUG 55860^T), was isolated from a marine electroactive biofilm grown on a stainless steel cathode (Genoa, Italy).

Emended description of the genus *Leisingera* Schaefer *et al.* 2002

The description is as given by Schaefer *et al.* (2002) and Martens *et al.* (2006) with the following additions. Do not degrade tyrosine, casein or DNA. Do not hydrolyse aesculin or gelatin. No indole production or fermentation of glucose. Do not grow on NA, R2A or PYG. Positive for leucine arylamidase activity. No activity is detected for esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase or urease. Do not assimilate D-mannose, D-mannitol, maltose, potassium gluconate, capric acid, adipic acid or phenylacetic acid. Susceptible to cefoxitin (30 μg), erythromycin (15 μg), streptomycin (25 μg) and tetracycline (30 μg). Resistant to vancomycin (30 μg), trimethoprim (1.25 μg) and clindamycin (2 μg). The type species is *Leisingera methylohalidivorans*.

Emended description of *Leisingera methylohalidivorans* Schaefer *et al.* 2002

The description is as given by Schaefer *et al.* (2002) and Martens *et al.* (2006) with the following additions. Does not degrade tyrosine, casein or DNA. Does not hydrolyse aesculin or gelatin. No indole production or fermentation of glucose. Grows weakly on TSA; does not grow on NA, R2A or PYG. Positive for leucine arylamidase activity; weak valine arylamidase and naphthol-AS-BI-phosphohydrolase activities. No activity is detected for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase or urease. Does not assimilate D-mannose, D-mannitol, maltose, potassium gluconate, capric acid, adipic acid or phenylacetic acid. Susceptible to cefoxitin (30 μg), erythromycin (15 μg), streptomycin (25 μg) and tetracycline (30 μg). Intermediately susceptible to gentamicin (10 μg). Resistant to vancomycin (30 μg), trimethoprim (1.25 μg) and clindamycin (2 μg).

Emended description of *Phaeobacter inhibens* Martens *et al.* 2006

The description is as given by Martens *et al.* (2006) with the following additions. Grows weakly on TSA; does not grow on NA, PYG or R2A. Does not hydrolyse DNA or aesculin. No fermentation of glucose. Positive for leucine arylamidase activity; weak acid phosphatase and α-glucosidase activities. No activity is detected for esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase or urease. Susceptible to cefoxitin (30 μg). Intermediately susceptible to erythromycin (15 μg) and streptomycin (25 μg). Resistant to tetracycline (30 μg), gentamicin (10 μg), vancomycin (30 μg), trimethoprim (1.25 μg) and clindamycin (2 μg).

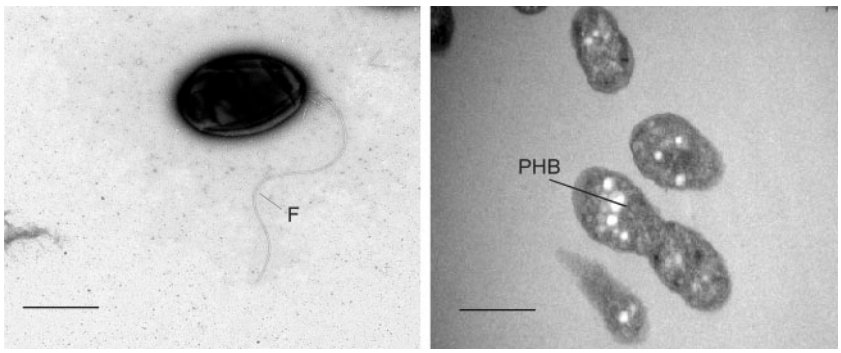

Fig. 3. Electron micrographs of cells of LMG 24366^T, showing a polar flagellum (F; left) and poly-β-hydroxybutyrate (PHB) inclusion bodies (right). Bars, 0.5 μm.

Table 1. Phenotypic characteristics of *Leisingera aquimarina* sp. nov., *Leisingera methylohalidivorans* and *Phaeobacter* species

Strains: 1, LMG 24366^T (*Leisingera aquimarina* sp. nov.); 2, *L. methylohalidivorans* LMG 23656^T (data from Schaefer *et al.*, 2002; Martens *et al.*, 2006); 3, *P. inhibens* LMG 22475^T (Martens *et al.*, 2006); 4, *P. gallaeciensis* LMG 23163^T (Ruiz-Ponte *et al.*, 1998; Martens *et al.*, 2006); 5, *P. daeponensis* LMG 24139^T (Yoon *et al.*, 2007). All strains are Gram-negative, grow in 2 % NaCl, and are oxidase- and catalase-positive and susceptible to streptomycin (25 μg). +, Positive; −, negative; W, weakly positive; I, intermediate susceptibility to an antibiotic; ND, no data available.

Characteristic	1	2	3	4	5
Origin	Marine EAB*, Italy	Tidal pool, USA	Tidal flat, Germany	Scallop, Spain	Tidal flat, Korea
Colony colour	Dark beige–pink	Non-pigmented	Dark brown	Brown	Yellowish white
Growth at:					
4 °C	+	+	+	−	+
40 °C	−	−	−	−	+
Growth in NaCl at:					
1 %	W	−	+	+	+
7 %	W	−	+	+	+
10 %	−	−	−	+	−
Growth on:					
NA	−	−†	−†	ND	W†
TSA	−	W†	W†	ND	W†
Growth on:					
Betaine	+	+	+	−	+†
Methionine	−	+	+	+	+†
Degradation of:					
Tyrosine	−	−†	+	−†	+
Starch	−	+	−	−	−
Gelatin	W	−†	−	−	−
Reduction of nitrate to nitrite	−	−	−	−	+
Susceptibility to:					
Erythromycin (15 μg)	+	+†	I†	+	I†
Tetracycline (30 μg)	+	+†	−†	ND	−
Gentamicin (30 μg)	−	I†	−†	+	+
Vancomycin (30 μg)	−	−†	−†	ND	+†
Enzymic activity†					
Alkaline phosphatase	W	−	−	ND	+
Esterase (C4)	−	−	−	ND	+
Esterase lipase (C8)	W	−	−	ND	+
Valine arylamidase	−	W	−	ND	−
Acid phosphatase	−	−	W	ND	+
Naphthol-AS-BI-phosphohydrolase	W	W	−	ND	−
α-Glucosidase	−	−	W	ND	−
DNA G+C content (mol%)	61.4	60.5	55.7	58	64.9

*Electroactive biofilm.
†Data from this study.

Emended description of *Phaeobacter daeponensis* Yoon *et al.* 2007

The description is as given by Yoon *et al.* (2007) with the following additions. Grows weakly on TSA and NA; does not grow on PYG or R2A. Does not hydrolyse DNA. No fermentation of glucose. No activity is detected for urease. Susceptible to cefoxitin (30 μg) and vancomycin (30 μg). Intermediately susceptible to erythromycin (15 μg). Resistant to trimethoprim (1.25 μg) and clindamycin (2 μg).

Acknowledgements

This work is funded by the European Commission [EA BIOFILMS-508866 (NEST)]. The Fund for Scientific Research – Flanders (FWO – Vlaanderen) is acknowledged for financial support of P. V.

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