***Leisingera methylohalidivorans* gen. nov., sp. nov., a marine methylotroph that grows on methyl bromide**

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A marine methylotroph, designated strain MB2^T, was isolated for its ability to grow on methyl bromide as a sole carbon and energy source. Methyl chloride and methyl iodide also supported growth, as did methionine and glycine betaine. A limited amount of growth was observed with dimethyl sulfide. Growth was also noted with unidentified components of the complex media marine broth 2216, yeast extract and Casamino acids. No growth was observed on methylated amines, methanol, formate, acetate, glucose or a variety of other substrates. Growth on methyl bromide and methyl iodide resulted in their oxidation to CO₂ with stoichiometric release of bromide and iodide, respectively. Strain MB2^T exhibited growth optima at NaCl and Mg²⁺ concentrations similar to that of seawater. Phylogenetic analysis of the 16S rDNA sequence placed this strain in the α -Proteobacteria in proximity to the genera *Ruegeria* and *Roseobacter*. It is proposed that strain MB2^T (= ATCC BAA-92^T = DSM 14336^T) be designated *Leisingera methylohalidivorans* gen. nov., sp. nov.

Keywords: methyl halides, methylotrophic bacterium, marine, *Ruegeria*, *Roseobacter*

INTRODUCTION

Release of methyl bromide (MeBr) and methyl chloride (MeCl) into the atmosphere leads to transport of bromine and chlorine atoms to the stratosphere, which contributes to the catalytic destruction of ozone (Mellouki *et al.*, 1992). There have been several reports of microbiological degradation of methyl halides in recent years, which suggest the importance of bacteria in the global biogeochemical cycle of methyl halides. MeBr can be degraded by natural populations of bacteria in agricultural and forest soils (Hines *et al.*, 1998; Miller *et al.*, 1997; Varner *et al.*, 1999), in seawater (Goodwin *et al.*, 1998; King & Saltzman, 1997; Tokarczyk & Saltzman, 2001), and in freshwater, estuarine and hypersaline/alkaline aquatic

systems (Connell *et al.*, 1997; Goodwin *et al.*, 1998). Methanotrophs and ammonia oxidizers co-oxidize methyl halides via their respective mono-oxygenases (Duddleston *et al.*, 2000; Keener & Arp, 1993; Oremland *et al.*, 1994). However, neither methanotrophs nor ammonia oxidizers play a significant role in the oxidation of MeBr in coastal waters (Goodwin *et al.*, 1998).

Marine isolates able to grow on methyl halides have not previously been described in detail, but several terrestrial bacteria are now known. These include several facultative methylotrophs in the genera *Hyphomicrobium* and *Methylobacterium* (Doronina & Trotsenko, 1997; McDonald *et al.*, 2001). Strains CC495 (Coulter *et al.*, 1999) and IMB-1 (Connell Hancock *et al.*, 1998) can also grow on methyl halides. Of all of the above listed bacteria, only strain IMB-1 was isolated using MeBr as the sole carbon source; the others were isolated with MeCl. Hoeft *et al.* (2000) reported isolation of several marine methylotrophs capable of co-oxidizing MeBr during growth on dimethyl sulfide (DMS), and one strain was reported able to grow on MeBr.

Abbreviations: AODC, acridine orange direct counts; DMS, dimethyl sulfide; MeBr, methyl bromide; MeCl, methyl chloride; MeI, methyl iodide.

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The GenBank accession number for the 16S rRNA sequence of strain MB2^T is AY005463.

The oceans act as a net biological and chemical sink for MeBr (Yvon & Butler, 1996; Yvon-Lewis & Butler, 1997). However, relatively little is known about the actual organisms and the enzymes involved in the degradation of methyl halides in the oceans. In this paper, the isolation and characterization of a marine methylotroph able to grow on methyl halides as a sole carbon and energy source are reported. This organism, strain MB2^T, is the first species isolated from seawater using MeBr as its sole growth substrate. Based on its 16S rDNA gene sequence alignment and DNA–DNA hybridization studies, this strain is proposed as a new member of a new genus of the α -*Proteobacteria*, *Leisingera methylolalidivorans* gen. nov., sp. nov.

METHODS

Isolation. An MeBr-degrading enrichment culture was established from seawater collected from a tide pool off the coast of central California as described by Goodwin *et al.* (1998). The enrichment (and the subsequent pure culture) was maintained in liquid culture for > 3 years with MeBr as its sole source of carbon and energy. Isolation of a pure culture was achieved by plating on a mineral salts medium containing 0.5% (w/v) Phytigel (Sigma). Plates were incubated in a gas-tight container containing 100 µmol MeBr l⁻¹ in the gas phase (0.34%, v/v). The medium (MAMS) was adapted from Thompson *et al.* (1995) and contained (g l⁻¹): NaCl (16), (NH₄)₂SO₄ (1.0), MgSO₄·7H₂O (1.0), CaCl₂·2H₂O (0.2), FeSO₄·7H₂O (0.002), Na₂MoO₄·2H₂O (0.002), Na₂WO₄ (0.003), KH₂PO₄ (0.36), K₂HPO₄ (2.34) and 1.0 ml SL-10 trace metals (Widdel *et al.*, 1983). The phosphates were added after autoclaving from sterile stock solutions. The final pH of the medium was 6.9–7.1.

The culture was streaked for purity four times prior to transferring back into liquid mineral salts medium having the same composition as given above. Liquid cultures were maintained in Balch tubes, crimp-sealed with an air gas phase (16 ml) and 10 ml MAMS medium. Liquid cultures typically received three additions of MeBr, each about ~ 0.63% (v/v) gas phase, which is equivalent to a dissolved concentration of ~ 300 µM per addition (see below) prior to transfer to fresh medium. This course of pulsed MeBr injection was followed to prevent incubation of cultures with toxic levels of MeBr, which would preclude growth (Miller *et al.*, 1997; Connell Hancock *et al.*, 1998). Following isolation, strain MB2^T could be successfully grown on marine agar 2216 (Difco) or MAMS containing 1.5% agar and yeast extract (0.5%, w/v).

Growth experiments. Potential growth substrates were added to strain MB2^T in liquid MAMS medium either with or without a vitamin mix that included B₁₂ (1 ml l⁻¹) (Pfennig, 1978). Cultures were grown in Balch tubes (see above) or in 159 ml serum vials containing 50 ml media, each of which was sealed with butyl rubber stoppers and incubated at 21 °C on a rotary shaker at 200 r.p.m. Cultures were grown with an air:liquid ratio of ~ 2:1 to reduce oxygen limitation. This was effective because no oxygen limitation was noted when comparing growth on yeast extract in sealed Balch tubes with growth obtained in cotton-stoppered conical flasks (data not shown). MeBr and MeCl were added to sealed cultures as a gas, whereas methyl iodide (MeI) was added to cultures from aqueous solutions in closed vials lacking any headspace.

Growth was monitored by measuring changes in OD₆₈₀ on a Spectronic 20 spectrophotometer or by acridine orange direct counts (AODC) (Hobbie *et al.*, 1977). Specific growth rates (μ) were determined by a least-squares linear fit to the following formula: $\mu = \ln(N/N_0)/t$ where N is the number of cells at time t (in h) and N_0 is the number of cells at $t = 0$. Growth on the following compounds was measured by AODC: acetate, cysteine, DMS, dimethylamine, methane-sulfonic acid, methionine, methyl halides and trimethylamine. Growth on all other substrates was tested by measuring OD₆₈₀. Due to the pleomorphic nature of the cells when grown on yeast extract and glycine betaine, accurate measurements of direct cell counts were not possible for these substrates.

Optimal growth conditions (salinity, pH and temperature) were determined in Balch tubes containing MAMS media supplemented with 0.2% (w/v) yeast extract. The magnesium requirements of the culture were tested by varying the MgSO₄ concentration (0.01–15 g l⁻¹) and compared with growth in which Na₂SO₄ was added in lieu of MgSO₄. Variations in pH were achieved by adjusting the medium with acid or base prior to autoclaving and by verifying the pH after autoclaving. Magnesium and calcium salts were added as sterile solutions after cooling. Some precipitation was noticeable in media with pH ≥ 8. For the purpose of making physiological comparisons, strain IMB-1 (= ATCC 202197), an MeBr-oxidizing terrestrial bacterium (Connell Hancock *et al.*, 1998; Miller *et al.*, 1997; Schaefer & Oremland, 1999), was subjected to the same growth conditions of temperature, pH and salinity.

Washed cell experiments. Cells were harvested in late exponential phase, washed twice with MAMS medium lacking nitrogen, trace metals and carbon, and then resuspended in this adapted medium (Connell Hancock *et al.*, 1998). To test if MeBr oxidation was constitutive or inducible, strain MB2^T was subcultured five times on MAMS medium with hydrolysed casein as the carbon and energy source. Washed cell suspensions (20 ml) were dispensed into 57 ml serum bottles, stoppered and crimp-sealed. Chloramphenicol (20 µg ml⁻¹) was added to half the bottles just prior to the addition of MeBr.

Incubation experiments with ¹⁴C-MeBr were conducted using washed suspensions of methionine-grown cells. Cells (3 ml) were dispensed into 13 ml serum bottles, stoppered and crimp-sealed with an air atmosphere and injected with 0.55 µCi (2.04 × 10⁴ Bq) ¹⁴C-MeBr (specific activity, 52.6 mCi mmol⁻¹ or 195 × 10⁷ Bq mmol⁻¹; purity = 99.9%; NEN). The reaction was stopped by the injection of 0.25 ml 6 M HCl, also liberating ¹⁴CO₂ into the gas phase.

Analytical techniques. Headspace methyl halide concentrations were determined by GC (Miller *et al.*, 1997). Unless otherwise noted, methyl halide concentrations in the aqueous phase are reported. Aqueous concentrations were determined using the following dimensionless Henry's coefficients: MeBr, 0.24 (DeBruyn & Saltzman, 1997); MeCl, 0.45 (Schaufler *et al.*, 1998); and MeI, 0.23 (Hunter-Smith *et al.*, 1983). Bromide and iodide in solution were determined by ion chromatography (Connell Hancock *et al.*, 1998). The concentration of ¹⁴CO₂ in acidified bottles was measured by GC in series with gas proportional counting (Culbertson *et al.*, 1981).

Morphological and phenotypic analyses. Samples for scanning electron microscopy (SEM) (Hitachi S-4700) were prepared according to the procedure employed by Smith *et al.* (1985). Cells were tested for catalase via the SpotTest kit (Difco). Sensitivity to penicillin G (50 µg ml⁻¹) was

assessed by following MeBr ($\sim 250 \mu\text{M}$) degradation and visual turbidity in stoppered Balch tubes containing 10 ml fresh MAMS medium and a 0.5 ml inoculum. This culture was monitored for 1 month and compared to an otherwise identical inoculated control tube that lacked penicillin G.

Phylogenetic analysis. DNA of strain MB2^T was extracted (Marmur, 1961) and the 16S rRNA genes were PCR-amplified with primers f27 and r1492 (Lane, 1991). PCR products were checked on 1% (w/v) agarose gels and fragments of the correct size were cloned using the TOPO TA cloning kit (Invitrogen). Small-scale preparations of plasmid DNA (Saunders & Burke, 1990) from 20 clones were screened by randomly amplified polymorphic DNA analysis to ensure the purity of the DNA prior to sequencing. DNA sequencing reactions (Lane, 1991) were carried out from DNA of two clones by cycle sequencing using the ABI PRISM Dye Terminator kit (PE Applied Biosystems). The resulting sequences from the two clones were identical. The complete 16S rRNA sequence (1431 bp) was aligned, using the ARB program for sequence alignment (Strunk *et al.*, 2000), to representative organisms from the same and related genera of bacteria. Phylogenetic position was determined using the DNADIST, DNAML, DNAPARS and SEQBOOT programs of the PHYLIP package and supported by bootstrap values (Felsenstein, 1993). Phylogenetic dendrograms were constructed from distance data using the Fitch–Margoliash method and the dendrograms were drawn using the TREEVIEW program V1.5 (Page, 1996).

DNA–DNA hybridization. Genomic DNA from strain MB2^T was compared to reference strains, *Ruegeria algicola* strain FF3^T (= ATCC 51440^T) and *Roseobacter gallaeciensis* strain BS107^T (= ATCC 700871^T) by DNA–DNA hybridization assays following Johnson (1994). DNA was extracted as mentioned previously (Marmur, 1961) and sonicated to give fragments < 1 kb. DNA from strain MB2^T was labelled with [methyl-³H]-dTTP (NEN DuPont) using the Nick Translation kit (Gibco-BRL) and separated from unreacted [methyl-³H]-dTTP by centrifugation using Nu-Clean D50 spin columns (Shelton Scientific). Hybridization was carried out at 62 °C for 24 h and the S1 nuclease method was used to digest unhybridized DNA. Reassociated DNA was collected on GF/F filters following acid precipitation with HCl-PPi (1 M HCl; 1%, w/v, Na₄P₂O₇ · 7H₂O; 1%, w/v, NaH₂PO₄). Filters were counted using a liquid scintillation spectrophotometer. Each hybridization reaction was done in triplicate. The percentage similarity between strain MB2^T and the two reference strains was based on a relative scale. Salmon sperm DNA was used to define 0% similarity and unlabelled DNA from strain MB2^T was used to define 100% similarity. The G+C content was determined at Deutsche Sammlung von Mikroorganismen und Zellkulturen.

RESULTS AND DISCUSSION

Morphology

Strain MB2^T exhibited pleomorphism, depending on the growth substrate. The strain formed single or paired rods (1.1–1.4 × 0.4–0.5 μm) (Fig. 1, top) when grown with methyl halides, methionine or DMS on mineral medium. When cultured with yeast extract or glycine betaine, the rods became enlarged and elongated (2.4–8.2 × 0.7–0.8 μm) (Fig. 1, bottom). Yeast-grown cell lines returned to mineral salts medium with

Fig. 1. SEMs of strain MB2^T grown on MeBr (top; bar, 1 μm) and yeast extract (bottom; bar, 4 μm).

MeBr as the substrate re-established their original form. In contrast, cells grown on marine broth showed the standard rod morphology.

Colonies were non-pigmented, smooth, with an entire edge when grown on solid media regardless of carbon source. When grown on MeBr on solid media, colony diameter was < 1 mm within 2–4 days; however, when grown on solid media with yeast extract or marine agar, the diameter was ~ 2 –3 mm within 2–3 days. Cells stained Gram-negative and they displayed obvious motility when viewed by phase-contrast microscopy. Additional phenotypic characteristics of this strain are given in Table 1.

Growth substrates

Strain MB2^T had a limited substrate range, being able to grow only on the following defined substrates out of the variety tested (μ , substrate concentration): glycine betaine (0.24 h⁻¹, 1 mM), methionine (0.09 h⁻¹, 10 mM), MeBr (0.07 h⁻¹, 0.24 mM), MeCl (0.05 h⁻¹,

Table 1. Comparison of *Leisingera methylohalidivorans* MB2^T to its nearest relatives within the α -*Proteobacteria*

All three type strains are positive for catalase and oxidase. +, Positive reaction; –, negative reaction. For growth substrates: –, OD₆₈₀ < 0.01; +, OD₆₈₀ ≥ 0.02; ++, OD₆₈₀ ≥ 0.1; +++, OD₆₈₀ > 0.5; ND, not determined.

Characteristic	<i>Leisingera methylohalidivorans</i> MB2 ^T	<i>Ruegeria algicola</i> FF3 ^{T*}	<i>Roseobacter gallaeciensis</i> BS107 ^{T†}
Morphology	Rods or ovoid rods	Ovoid rods	Ovoid rods
Starch hydrolysis	+	+	–
Salinity range (‰)	15–65	6–120	6–120
Temperature optimum (°C)	27	25–30	23–27
pH optimum	7.7	7.5	7.0
Vitamin requirement:			
Thiamin	–	+	+
Biotin	–	+	–
Penicillin G resistance	–	–	+
Pigment	None	Beige	Brownish, diffusible
G + C content (mol %)	60.5	60	57.6–58.0
Substrates for growth (5 mM):			
Acetate	–	+	+
Lactate	–	ND	ND
Methanol (1–5 mM)	–	–	–
Malate	–	+	ND
Pyruvate	–	+	+
Citrate	–	+	+
Glucose	–	+	+
Fructose	–	+	+
Galactose	–	–	+
Glycerol	–	–	+
Glycine	–	ND	+
Glutamic acid	–	+	+
Casamino acids (0.2 %)	+++	+	+
Yeast extract (0.5 %)	+++	+	+
Marine broth (Difco 2217)	+++	+++	+++

* Except for marine broth, data as reported by Lafay *et al.* (1995); data reported only as + or –.

† Except for marine broth, as reported by Ruiz-Ponte *et al.* (1998); data reported only as + or –.

0.37 mM), MeI (0.04 h⁻¹, 0.13 mM) and DMS (0.01 h⁻¹, 0.05 mM). Glycine betaine supported growth at 1 mM, but not at 5 mM. Growth was also observed on the following complex substrates: Casamino acids, yeast extract and marine broth (Table 1). These complex substrates each contained one or more of the defined substrates shown to support growth, although additional growth substrates may have been present. For example, Casamino acids and yeast extract both contain methionine, and yeast extract contains 1–3 % (w/w) glycine betaine (Farrell *et al.*, 1993; Oren, 1999; Jansen & Hansen, 2001). Growth on 1 mM glycine betaine could be monitored by OD₆₈₀ (versus by AODC), although readings were low (0.1) compared to growth on yeast extract (0.9).

Strain MB2^T was an obligate aerobe, being unable to grow in the absence of oxygen on yeast extract with or without nitrate (data not shown). In addition to the compounds given in Table 1, no growth was observed on the following substrates: no added carbon, as-

paragine (5 mM), cysteine (4 mM), dimethylamine (5 or 0.05 mM), formate (5 mM), glutamine (2.5 mM), homocysteine (5 mM), methanesulfonic acid (5 or 0.05 mM), monomethylamine (5 mM), serine (5 mM), threonine (5 mM), trimethylamine (5 or 0.05 mM) or urea (5 mM). Furthermore, no growth was observed on a mixture of L-amino acids that included (0.1 %, w/v, each) arginine, cysteine, glutamate, glycine, phenylalanine, serine and tyrosine.

Strain MB2^T demonstrated exponential growth on MeBr (Fig. 2), MeCl, MeI and methionine. Lag periods were not observed for methionine or MeBr consumption, but did occur with MeCl and MeI (data not shown). Experiments with radiolabelled MeBr demonstrated that strain MB2^T oxidized ¹⁴C-MeBr to ¹⁴CO₂ (18 % in 2 h), whereas no ¹⁴CO₂ was detected in sterile controls. When strain MB2^T was grown with MeBr and MeI, it was necessary to add these substrates as discrete pulses over the course of the incubation to prevent toxicity (Connell Hancock *et al.*, 1998; Miller

Fig. 2. Growth of strain MB2^T on MeBr. ○, MeBr consumption; ■, bromide release; ▲, cells per ml. No growth was observed without MeBr (not shown). Arrows indicate pulsed additions of MeBr. Symbols represent the mean of three cultures; bars, ± 1 SD.

et al., 1997). Pulsed additions were not required for methionine or MeCl, presumably because of the lower reactivity of these compounds.

In contrast to other substrates, growth on DMS (0.5 or 0.05 mM) ceased after about ~ 50 h whether or not pulsed additions were used (data not shown), raising the possibility that strain MB2^T could not be maintained on DMS. Although growth was limited, a cell-line was maintained on 0.05 mM DMS through three sequential transfers. After each transfer, cell densities increased ~ 10-fold in 72 h, typically reaching a maximum of ~ 2×10^5 cells ml⁻¹. For comparison, cells grown on MeBr or methionine increased > 100-fold in 48 h, reaching densities of 10^7 – 10^8 cells ml⁻¹. No growth was observed on 1.4 or 5 mM DMS.

MB2^T is a methylotroph because it grows on methyl halides (MeBr, MeCl, MeI) and to a limited extent on DMS. Curiously, no growth was noted with any other C₁ substrate, including methylated amines, methanol and formate. The original enrichment culture from which strain MB2^T was isolated did not grow on methane (Goodwin *et al.*, 1998) and it was unable to degrade methyl fluoride or dibromomethane (K. Goodwin and J. K. Schaefer, unpublished results). Although the mixed culture could utilize methylated amines (Goodwin *et al.*, 1998), strain MB2^T could not. In this respect, strain MB2^T differed markedly from the facultative methylotrophs, strains IMB-1 and CC495, which grow on methylated amines in addition to methyl halides (Connell Hancock *et al.*, 1998; Coulter *et al.*, 1999).

Strain MB2^T grew on glycine betaine and methionine, which would seem to suggest that it is a facultative rather than an obligate methylotroph. However, the

inability of strain MB2^T to grow on demethylated analogues such as homocysteine, glycine and cysteine suggests that growth on glycine betaine and methionine occurred at the expense of their methyl groups. Therefore, strain MB2^T appears to be an obligate methylotroph, capable of growth on a limited suite of substrates that include methyl halides, DMS and some compounds having C₁ units attached as methyl groups to S or N atoms.

Methyl halide degradation

A 1:1 stoichiometry between MeBr oxidation and the accumulation of Br⁻ in the medium was observed (Fig. 2). There was negligible MeBr loss (~ 2.6%) in sterile controls (data not shown) attributable to chemical hydrolysis and halide exchange (Elliott & Rowland, 1993). A similar relationship was observed between MeI oxidation and the accumulation of I⁻ in the medium. After 3 days incubation, cells had consumed 10 μmol MeI, whereas 12 μmol I⁻ was detected in the medium. After 7 days incubation, the amount of I⁻ recovered increased to 22 μmol, which agreed with the 19 μmol MeI consumed. The abiotic loss of MeI that occurred in controls (~ 16% of initial level) was insignificant compared to the consumption in live samples. Accumulations of Cl⁻ during MeCl oxidation could not be followed because of the high background levels of this ion in the marine medium. No significant loss of MeCl (< 1%) occurred in sterile controls (data not shown).

After ~ 19 h incubation in the presence of 80 or 420 μM MeBr, suspensions of Casamino acid-grown cells demonstrated MeBr consumption, whereas suspensions incubated with chloramphenicol were unable to oxidize MeBr (data not shown), indicating that the enzyme(s) for MeBr degradation required induction. Methyl halide degradation was also found to require induction in *Methylobacterium* CM4^T (Vannelli *et al.*, 1998) and strain CC495 (Coulter *et al.*, 1999). Oxidation of high levels (> 100 μM) of MeBr required induction in strain IMB-1, although there appeared to be a constitutive ability to consume low levels of MeBr (18 nM) (Schaefer & Oremland, 1999). Therefore, the pathway of methyl halide degradation does not appear to be constitutive amongst methyl halide-degrading bacteria, at least at high concentrations.

Physiological characteristics

Strain MB2^T was isolated from seawater and is clearly a marine bacterium. Growth occurred over a salinity range of 10–60 g NaCl l⁻¹, with an optimum at the salinity of seawater (Fig. 3a). In contrast, strain IMB-1, a terrestrial organism, had an optimum at ~ 2 g l⁻¹ and did not grow at > 10 g l⁻¹. The optimum Mg²⁺ concentration for strain MB2^T was 40–80 mM, which overlaps with the 54 mM concentration found in seawater (data not shown). Strain MB2^T had a lower

Fig. 3. Effect of salinity (a), temperature (b) and pH (c) on growth of strain MB2^T (●) and strain IMB-1 (○), grown on 0.2% yeast extract and 10 mM glucose, respectively. OD₆₈₀ measurements were taken at same time for all conditions. Symbols represent the mean of three cultures; bars, ± 1 SD.

optimum temperature for growth than strain IMB-1 (Fig. 3b). The lack of growth above pH 8 (Fig. 3c) was most likely due to precipitation of the magnesium and calcium salts, rather than a response to a physiological constraint of pH. Other characteristics of strain MB2^T are given in Table 1.

G + C content and DNA–DNA hybridization

The G + C content of *Leisingera methylohalidivorans* MB2^T was determined to be 60.5 ± 0.2 mol %, which is similar to that reported for DNA from *Ruegeria algicola* and *Roseobacter gallaeciensis* (Table 1). Since the G + C contents of DNA from these strains were so similar, hybridization of DNA from strain MB2^T to DNA from *Ruegeria algicola* FF3^T and *Roseobacter gallaeciensis* BS107^T were performed. Unlabelled

DNA from salmon sperm, *Ruegeria algicola* or *Roseobacter gallaeciensis* was allowed to hybridize with labelled DNA from strain MB2^T resulting in duplex products with mean counts of 2893, 2721 and 2999 d.p.m. per filter, respectively. In contrast, DNA from strain MB2^T hybridized to itself resulted in a final duplex with counts of 50025 c.p.m. per filter. Therefore, DNA from strain MB2^T had very low (< 1%) hybridization to DNA from either *Ruegeria algicola* or *Roseobacter gallaeciensis*.

16S rRNA sequence analysis

Phylogenetic analysis of the 16S rDNA sequence of strain MB2^T grouped this bacterium with species in proximity to the *Ruegeria* and *Roseobacter* genera, being most closely related to *Roseobacter gallaeciensis*, *Ruegeria algicola* and strain GAI-5, a facultative methylotroph isolated from coastal seawater (González & Moran, 1997) (Fig. 4). The 16S rDNA sequences obtained from two separate clones of MB2^T were identical. Analysis with DNAML and DNAPARS produced identical phylogenies to those produced by DNADIST. The 16S rDNA sequence of MB2^T had closest identity to those from *Ruegeria algicola* (97.5%) and *Roseobacter gallaeciensis* (97.1%).

Strain MB2^T was distinct from *Roseobacter denitrificans* and *Roseobacter litoralis*, which are pink-pigmented bacteria that contain bacteriochlorophyll *a* (Shiba, 1991). *Roseobacter* was originally defined as the genus containing these aerobic photosynthetic species, and the lack of bacteriochlorophyll *a* was a primary motivation for reclassifying *Roseobacter algicola* as *Ruegeria algicola* (Uchino *et al.*, 1998; Trüper & Imhoff, 1999). Yurkov & Beatty (1998) also called for reclassification on these grounds. Moreover, work to differentiate *Roseobacter*-related bacteria using 16S–23S internal transcribed spacer sequences confirms a clear separation of *Ruegeria algicola* from *Roseobacter denitrificans* and *Roseobacter litoralis* (Söller *et al.*, 2000). Strain MB2^T is unpigmented and lacks bacteriochlorophyll *a*, and therefore lacks the main distinguishing feature of the genus *Roseobacter*. Furthermore, it is noted that *Roseobacter gallaeciensis* (Ruiz-Ponte *et al.*, 1998), *Roseobacter* sp. DSS8 and *Roseobacter* sp. GA15 also lack bacteriochlorophyll *a*, suggesting that the placement of those organisms in the *Roseobacter* genus should be re-examined. In addition, phylogenetic analysis (Fig. 4) indicates that there are at least two groups of *Ruegeria* species, the first comprising *Ruegeria atlantica*, *Ruegeria gelatinovorans* and *Roseobacter* sp. DSS8. The second group comprises *Ruegeria algicola*, which groups with *Roseobacter gallaeciensis*, and *Roseobacter* sp. GA15. In the future, the phylogeny of these bacteria should be clarified.

Strain MB2^T had a similar G + C content to *Roseobacter gallaeciensis* and *Ruegeria algicola* (Table 1) and was related to these bacteria as indicated by 16S rDNA sequences (Fig. 4). However, DNA–DNA hybrid-

Fig. 4. Phylogenetic analysis of the 16S rRNA sequence of strain MB2^T. The dendrogram shows the results of DNADIST analysis, based on the comparison of 1126 nt. Bootstrap values greater than 65% derived from 100 replicates are also shown (DNADIST values; DNAPARS values given in parentheses). Bar, 0.01 Jukes-Cantor substitutions per nucleotide.

ization results showed a very low level of hybridization to either *Ruegeria algalicola* or *Roseobacter gallaeciensis*. Based on this lack of hybridization and the lack of pigmentation, it is proposed that strain MB2^T be designated a new species in a new genus, *Leisingera methylohalidivorans* gen. nov., sp. nov.

Comparison of *Leisingera methylohalidivorans* MB2^T to members of the genera *Roseobacter* and *Ruegeria*

Phylogenetic neighbours of *L. methylohalidivorans* MB2^T were tested for their ability to degrade MeBr in order to assess whether methyl halide degradation was also a general ability of the *Roseobacter*/*Ruegeria* genera. Neither *Roseobacter gallaeciensis* BS107^T nor *Ruegeria algalicola* FF3^T was able to consume MeBr, even at concentrations as low as 8.5 µM. *Ruegeria algalicola* was also tested using 200 µM MeBr, a concentration routinely used to grow strain MB2^T, but this amount of MeBr appeared to kill the culture. Furthermore, *Roseobacter* group strains GAI-37 and DSS-3 (González *et al.*, 1999) were unable to consume MeBr (data not shown).

Many bacteria in the genera *Roseobacter* and *Ruegeria* can utilize a wide variety of substrates, including glucose and acetate (González *et al.*, 1999; Lafay *et al.*, 1995; Ruiz-Ponte *et al.*, 1998), whereas strain MB2^T exhibited a narrower substrate range. Two other non-pigmented isolates, namely *Roseobacter* sp. GA15 (González & Moran, 1997) and *Roseobacter* sp. DSS-8 (González *et al.*, 1999), can oxidize DMS to DMSO, but it is not known if this reaction supports growth (J. M. Gonzalez, personal communication). Niches for MeBr-oxidizing bacteria could perhaps be found near

MeBr-producing phytoplankton (Saemundsdóttir & Matrai, 1998; Scarratt & Moore, 1998) or in shallow coastal waters that contain large macroalgal communities that produce methyl halides (Goodwin *et al.*, 1998; Manley & Dastoor, 1987).

Description of *Leisingera* gen. nov.

Leisingera (Lei.sin'ge.ra. N.L. fem. n. *Leisingera* in honour of Thomas Leisinger, on the occasion of his retirement and for his contributions to our understanding of the biochemistry of bacterial methyl halide metabolism).

Obligatory aerobic, moderately halophilic rods. Gram-negative reaction. Grows by oxidation of methyl halides, or select methylated substrates like methionine. The G+C content of the only strain isolated thus far is 60.5 mol%. Found in coastal marine tide pools. Type species is *Leisingera methylohalidivorans*.

Description of *Leisingera methylohalidivorans* sp. nov.

Leisingera methylohalidivorans (me.thy.lo.ha.li.di.vo'rans. N.L. n. *methylohalidium* methyl halide; L. part. pres. *vorans* devouring; *methylohalidivorans* degrading methyl halides).

Cells are motile, Gram-negative rods which occur singly or in pairs (1.1–1.4 × 0.4–0.5 µm) when grown on methyl halides, methionine or DMS. Cells demonstrate pleomorphism when grown on yeast extract or glycine betaine, becoming enlarged and elongated (2–10 µm in length). Colonies are non-pigmented,

smooth, with an entire edge when grown on solid media regardless of carbon source. When grown on methyl bromide on solid media, colony diameter is < 1 mm; however, when grown on solid media with yeast extract or marine agar, the diameter is ~ 2–3 mm. pH and temperature optima are 7.7 and 27 °C, respectively. Unable to grow at NaCl concentrations of < 10 g l⁻¹. Cells have a fairly narrow substrate range being restricted to a limited number of methylotrophic substrates such as methyl halides, DMS, and the methyl groups of methionine and glycine betaine. Growth also occurs on complex substrates such as yeast extract, Casamino acids and marine broth. Simple carbohydrates, amino acids (other than methionine) and small organic acids cannot serve as sole sources of carbon and energy. Catalase- and oxidase-positive. Does not hydrolyse starch. Unable to use nitrate as an electron acceptor. Does not require vitamins for growth. Inhibited by penicillin G. G+C content is 60.5 ± 0.2 mol %. Type strain is *Leisingera methylohalidivorans* MB2^T (= ATCC BAA-92^T = DSM 14336^T).

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