Marinobacter excellens sp. nov., isolated from sediments of the Sea of Japan

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Five strains of halophilic, Gram-negative marine bacteria (KMM 3809^T, KMM 3814, KMM 3815, KMM 3817 and KMM 3818) were isolated from sediments collected from Chazhma Bay, Sea of Japan. Phylogenetic 16S rRNA gene sequence-based analysis placed these bacteria in a clade within the genus *Marinobacter* in the γ -*Proteobacteria*. KMM 3809^T showed highest 16S rRNA gene sequence similarity of 97.3% to *Marinobacter litoralis* and 96.9% to *Marinobacter hydrocarbonoclasticus* and *Marinobacter aquaeolei*. DNA–DNA hybridization between the five isolates was at the conspecific level (94–96%) and that among the closest phylogenetic neighbours ranged from 45.0 to 62.5%. The new organisms were susceptible to polymyxin. Predominant fatty acids were C_{16:0}, C_{16:1 ω 9c}, C_{16:1 ω 7c} and C_{18:1 ω 9c}. Phylogenetic evidence, along with phenotypic and genotypic characteristics, showed that the bacteria constituted a novel species of the genus *Marinobacter*. The name *Marinobacter excellens* sp. nov. is proposed for this species, with the type strain KMM 3809^T (=CIP 107686^T).

The genus *Marinobacter*, with the type species *Marinobacter hydrocarbonoclasticus*, was created in 1992 to accommodate Gram-negative, moderately halophilic, aerobic γ -*Proteobacteria* that used a variety of hydrocarbons as the sole source of carbon and energy (Gauthier *et al.*, 1992). One more species, *Marinobacter aquaeolei*, was described recently (Nguyen *et al.*, 1999) and the misclassified species [*Pseudomonas*] *nautica* was assigned to *M. hydrocarbonoclasticus* (Spröer *et al.*, 1998). This group of marine *Proteobacteria* comprises efficient biodegraders of hydrocarbons, polycyclic aromatic carbons and acyclic isoprenoid

compounds (Rontani *et al.*, 1999; Hedlund *et al.*, 2001; Melcher *et al.*, 2002). Intensive studies of *Marinobacter* spp. metabolites of sufficient structural specificity suggest that they may have an important role as biological markers for microbial degradation in the aquatic environment.

In this study, we present the characterization of novel bacteria of the genus *Marinobacter* that were isolated from sediments collected in Chazhma Bay, Sea of Japan. This work was part of the taxonomic investigation of free-living marine bacteria dwelling in the Bay, sediments of which were contaminated by radionuclides (Ivanova *et al.*, 2002). Sediment samples were collected in 2001 from a depth of 0.5 m (salinity, 32‰; temperature, 12 °C) at Chazhma Bay, Sea of Japan. Bacteria were isolated by plating 0.1 ml of a suspension of 1 g sediment in 10 ml sterilized natural sea water onto marine 2216 agar plates (Difco) or plates with medium B that contained 0.2% (w/v) Bacto peptone (Difco), 0.2% (w/v) casein hydrolysate (Merck), 0.2% (w/v) Bacto yeast extract (Difco), 0.1% (w/v) glucose,

Abbreviations: AFM, atomic force microscopy; DPG, diphosphatidylglycerol; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KMM 3809^T is AY180101.

A table showing cellular fatty acid composition of *Marinobacter* species and further AFM images of cells of *M. excellens* are available as supplementary material in IJSEM Online.

0.02 % (w/v) KH_2PO_4 , 0.005 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 % (w/v) Bacto agar (Difco), 50 % (v/v) natural sea water and 50 % (v/v) distilled water at pH 7.8. Plates were incubated aerobically at room temperature (approx. 22–25 °C) for 5, 7 or 10 days. Strains were stored at –80 °C in marine 2216 broth (Difco) supplemented with 20 % (v/v) glycerol. In total, 145 viable bacterial strains have been recovered from sea water and sediment samples. During isolation studies, bacteria of different taxonomic groups, including *Shewanella*, *Halomonas*, *Pseudoalteromonas* and *Kocuria*, have been isolated (Ivanova *et al.*, 2002). From this collection, several bacterial strains with *Marinobacter*-like phenotypes were identified initially and studied further in detail.

Unless indicated otherwise, phenotypic characteristics were studied by using standard procedures (Baumann *et al.*, 1972; Smibert & Krieg, 1994) as described previously (Ivanova *et al.*, 1996, 1998). Tests for utilization of various organic substrates as sole carbon sources at a concentration of 0.1 % (w/v) were performed in 10 ml liquid BM medium (Baumann *et al.*, 1972). Ability to oxidize organic substrates was investigated by using Biolog GN plates as described previously (Ivanova *et al.*, 1998). The following physiological and biochemical properties were examined: oxidation/fermentation of glucose, denitrification, catalase and oxidase activities, gelatin liquefaction, sodium requirement [at 0, 1, 3, 6, 8, 10, 12, 15 and 20 % (w/v) NaCl], production of indole and H_2S and ability to hydrolyse starch, Tween-85 and casein. Haemolytic activity of the strains was detected on blood agar (1^{-1} : trypticase soy agar, 40 g; mouse blood, 50 ml; water, 950 ml). Cytotoxic and antibacterial activities were assessed by the agar-diffusion assay, based on methods described elsewhere (Barry, 1980; Sasaki *et al.*, 1985). Cultures (0.1 ml) of indicator test strains were spread on tryptic soy agar plates in which circular wells (diameter, 10 mm) had been cut. Areas of inhibited bacterial growth were measured after incubation for 48 h at 28 °C. Zones of inhibited growth of the indicator strains

surrounding the wells were observed, mean diameters were measured and 10 mm was subtracted to represent the diameter of the well. Antimicrobial activities were tested against *Staphylococcus aureus* CIP 103594, *Escherichia coli* ATCC 25290, *Proteus vulgaris* NBRC 3851^T, *Enterococcus faecium* CIP 104105, *Bacillus subtilis* ATCC 6051^T and yeast *Candida albicans* KMM 455. Atomic force microscopy (AFM) was employed to characterize the morphology of the cells, by using a TopoMetrix Explorer (model no. 4400-11; ThermoMicroscopes) in the non-contact mode, with either a 2 µm liquid scanner (0.8 µm z-range; model no. 5270-00) or a 100 µm liquid scanner (10 µm z-range; model no. 5180-00). Silicon cantilevers with a spring constant of 42 N m⁻¹ and resonant frequency of 320 kHz (model no. 1650.00) were used; all imaging was performed in ethanol. All samples were prepared on freshly cleaved mica. Morphological and physiological properties are shown in Fig. 1, Table 1 and Supplementary Table A (available in IJSEM Online) and given in the species description.

The 16S rRNA gene was amplified and sequenced by MIDI Laboratories, Newark, USA. Briefly, primers used for amplification corresponded to *E. coli* positions 5 and 1540. Amplification products were purified by using Microcon 100 (Millipore) molecular mass cut-off membranes and checked for quality and quantity on an agarose gel. Cycle sequencing of 16S rRNA gene amplification products was carried out by using AmpliTaq ES DNA polymerase and rhodamine dye terminators (Applied Biosystems). Samples were electrophoresed on an ABI Prism 377 DNA sequencer. The new 16S rRNA gene sequence was added and aligned by reference to a database of more than 60 000 already aligned sequences. Automatic alignments were first obtained by using PCaligne, a program designed specifically for that purpose (Abdeddaïm, 1997). Related sequences were selected according to BLAST queries against the latest release of the 'Bacteria' division of GenBank. The 100 most closely related sequences were selected and alignments were refined manually by using SeaView (Galtier *et al.*, 1996).

Fig. 1. Dimensional AFM deflection image showing (a) a cell of *M. excellens* KMM 3809^T grown for 24 h; (b) line profile of the cell, showing length (vertical line) and width (horizontal line).

Table 1. Characteristics that differentiate *M. excellens* from phylogenetically related species

Taxa: 1, *M. excellens* KMM 3809^T; 2, *M. hydrocarbonoclasticus*; 3, *M. aquaeolei*. All strains are straight rod-shaped organisms, are oxidase-positive, exhibit lipase, grow in 15% NaCl, do not hydrolyse gelatin, casein or chitin, are negative for haemolysis and are susceptible to polymyxin. w, Weakly positive. Data are from this study, Gauthier *et al.* (1992) and Nguyen *et al.* (1999).

Characteristic	1	2	3
DNA G + C content (mol%)	56.0	52.7	55.7
Optimal growth temperature (°C)	28	32	30
Growth at/in:			
10 °C	+	+	–
45 °C	–	+	+
0% NaCl	–	–	+
Catalase	w	+	+
Denitrification	+	+	–
Production of:			
Amylase	+	–	–
Alginase	–	+	–
Utilization of:			
D-Glucose	–	–	–
D-Galactose	– (1*)	–	–
DL-Lactate	–	+	+
Succinate	–	+	+
Citrate	–	+	+
Susceptibility to:			
Gentamicin	–	–	+
Kanamycin	– (1)	+	+
Neomycin	–	–	+

*No. strains tested that are positive.

Phylogenetic trees were constructed according to three different methods: BIONJ as described by Gascuel (1997), maximum-likelihood with the 'global' option (ML) and maximum-parsimony (MP), both from PHYLIP (Phylogeny Inference Package, version 3.573c; distributed by J. Felsenstein, Department of Genetics, UW, Seattle, WA, USA). For the neighbour-joining (NJ) analysis, a distance matrix was calculated according to the Kimura two-parameter correction. Bootstraps were done by using 1000 replications and the BIONJ and Kimura two-parameter corrections. Phylogenetic trees were drawn by using NJPLOT (Perrière & Gouy, 1996). Preliminary phylogenetic analyses were done by using the most conserved parts of the sequence, which were not likely to show too much homoplasy. These analyses, as well as a table sorting available sequences according to decreasing similarity, allowed us to select 13 sequences for the final analysis shown in this paper. Domains used to construct the final phylogenetic trees (positions 88–1469 of KMM 3809^T) were regions of the small-subunit rRNA gene sequences that were available for all sequences and excluded positions

Fig. 2. Phylogenetic position of *M. excellens* according to 16S rRNA gene sequence analysis. The topology shown was obtained by using the BIONJ algorithm and 1000 bootstrap replications with the Kimura two-parameter distance correction. Bootstrap values are indicated only for branches that were also retrieved by MP and ML ($P < 0.01$); these branches should be considered as the only robust clusters identified by this analysis.

that were likely to show homoplasy or notoriously difficult to sequence, i.e. the 5' end of the sequences.

16S rRNA gene sequence analyses revealed that strain KMM 3809^T is a member of the γ -Proteobacteria and, more precisely, that it is included in the clade formed by the genus *Marinobacter* (Fig. 2). The topology of the phylogenetic tree shown in Fig. 2 is that of the bootstrap analysis, as it has been demonstrated that this topology is often better than that of a simple NJ analysis (Gascuel, 1997). As a result, there is no distance bar in this tree; note also that the distance bar should be considered with caution in a tree, as it represents distances calculated after corrections (transversions being accounted for more than transitions) and branch-lengths do not represent the real number of differences between the sequences themselves. Bootstrap numbers are indicated only for branches that were also retrieved in the ML and MP trees (consensus tree). 16S rRNA gene sequence similarities with other available sequences were calculated by parsing the result of a BLAST analysis of KMM 3809^T on the 'Bacteria' division of GenBank (at 25 November 2002), with the options 'no filter' and $W=7$. The sequence of strain KMM 3809^T had 97.3% or less similarity to its nearest phylogenetic relatives, i.e. *M. hydrocarbonoclasticus*, *M. aquaeolei* and *M. litoralis*.

Results of the chemotaxonomic analysis are given in Supplementary Table A (available in IJSEM Online) and the species description. Isoprenoid quinones were extracted from lyophilized cells and analysed as described previously (Ivanova *et al.*, 2003) according to Moule & Wilkinson (1987). Lipids were extracted as described previously (Ivanova *et al.*, 2000). TLC revealed that phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were the major phospholipids; the amount of PE ranged from 48.1

to 54.8 %, whilst the amount of PG ranged from 29.0 to 38.4 %. Diphosphatidylglycerol (DPG) was also detected, at 9.9–19.4 %. Fatty acid methyl esters were prepared as described by Svetashev *et al.* (1995). The resulting fatty acid methyl esters were analysed on a Shimadzu GC-14A gas chromatograph with a flame-ionization detector by using both a non-polar SPB-5 fused-silica column (30 m × 0.25 mm, i.d.) at 210 °C and a polar Supelcowax-10 fused-silica column (30 m × 0.25 mm, i.d.) at 200 °C. The fatty acid composition of the five strains was similar (see Supplementary Table A in IJSEM Online). Three components, C_{16:0}, C_{16:1}ω9c and C_{18:1}ω9c, accounted for >70 % of total fatty acids. Minor fatty acids included C_{12:0}, C_{14:0}, C_{15:0}, C_{17:0}, C_{18:0} and C_{17:1}ω8c. In their main features, the fatty acid profiles were similar to those reported for *Marinobacter* species (Nguyen *et al.*, 1999; Yoon *et al.*, 2003). These authors found a relatively high proportion of hydroxy fatty acids (up to 10 %), whilst in our experiments, C_{12:0} 3-OH was detected in lower amounts (up to 0.7 %). Such variation in the proportion of fatty acids was observed previously for other *Proteobacteria* (Huys *et al.*, 1994; Ivanova *et al.*, 2000) and can be explained by differing experimental conditions employed in different laboratories. In addition, a greater proportion of C_{16:1}ω9c in the fatty acid profiles of the new isolates compared to those of other type strains and a number of differences in distribution of fatty acids present in minor amounts, i.e. accounting for <5–7 %, namely C_{15:0}, C_{17:0} and C_{17:1}ω8c, were also found. Notably, all bacteria of the genus *Marinobacter* exhibited an abundance of ω9c isomers of the fatty acids C_{16:1} and C_{18:1}, which is in agreement with results reported previously for type strains grown under different cultivation conditions (Nguyen *et al.*, 1999; Yoon *et al.*, 2003). We suggest that ω9c isomers of fatty acids C_{16:1} and C_{18:1} might be characteristic chemotaxonomic markers of the genus *Marinobacter*.

For genotypic characterization, DNA was isolated from the strains by following the method of Marmur (1961). The G+C content of the DNA was determined by using the thermal denaturation method (Marmur & Doty, 1962). The type strains of *M. hydrocarbonoclasticus* (ATCC 49840^T), *M. aquaeolei* (ATCC 700491^T) and *M. litoralis* (KCCM 41591^T) were used for comparison of phenotypic properties and DNA–DNA hybridization experiments. All reference strains were cultured routinely on marine agar 2216 plates (Difco). DNA–DNA hybridization experiments were performed by using covalent attachment of the DNA in micro-wells, according to the method described by Christensen *et al.* (2000). Briefly, 300 ng DNA (400–700 bp fragments) diluted in ice-cold 1-methylimidazole (Sigma), pH 7.0 and 25 µl 40 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma) dissolved in sterilized Nanopure H₂O (18.2 MΩ cm⁻¹) was added to each well of NucleoLink micro-well strips (Nalge Nunc International) to bind the DNA covalently to the NucleoLink surface. After incubation at 50 °C for 18 h (without shaking), unbound DNA was washed continuously.

DNA labelling with photoactivated biotin, hybridization, detection and quantification were performed as described by Christensen *et al.* (2000). DNA–DNA hybridization data revealed a high level of DNA relatedness among KMM 3809^T, KMM 3814, KMM 3817 and KMM 3818, ranging from 93 to 96 %, which indicated that the strains belonged to the same species (Wayne *et al.*, 1987). As the phenotypic and chemotaxonomic characteristics of KMM 3815 were identical to those of KMM 3814, the former was excluded from DNA–DNA hybridization experiments. Genetic similarity of KMM 3809^T with type strains of the genus *Marinobacter*, listed in Table 2, was 45–63 %. Based on the generally accepted criterion of the definition of genomic species (Wayne *et al.*, 1987), strains KMM 3809^T, KMM 3814, KMM 3817 and KMM 3818 are assigned to the novel species.

Bacteria of the novel species have a number of distinct phenotypic features that allow them to be distinguished from other *Marinobacter* species (Table 2). For example, the newly isolated bacteria were not able to grow at temperatures higher than 41 °C and were able to denitrify and produce amylase. Notably, bacteria isolated from Chazhma Bay were only susceptible to one antibiotic (polymyxin) and remarkably resistant to the other 10 listed above; strain KMM 3814 was weakly susceptible to kanamycin. In contrast, *M. aquaeolei* was susceptible to gentamicin, kanamycin and neomycin as well as polymyxin and *M. hydrocarbonoclasticus* was susceptible to kanamycin.

Description of *Marinobacter excellens* sp. nov.

Marinobacter excellens (ex'cell.ens. L. masc. adj. *excellens* remarkable, exceptional).

The majority of cells are rod-shaped, with lengths and widths that vary from 1 to 8 µm and from 0.6 to 1.4 µm, respectively (Fig. 1). They are motile, polarly flagellated, Gram-negative strains that are strictly aerobic heterotrophs. Anaerobic growth occurs by fermentation of D-glucose by

Table 2. DNA relatedness among tested strains

Organism	G+C content (mol%)	Hybridization (%)*
<i>M. excellens</i> :		
KMM 3809 ^T	56.0	100
KMM 3814	55.7	95.8
KMM 3817	55.0	93.8
KMM 3818	55.2	93.0
<i>M. hydrocarbonoclasticus</i> ATCC 49840 ^T	52.7	62.5
<i>M. aquaeolei</i> ATCC 700491 ^T	55.7	57.0
<i>M. litoralis</i> KCCM 41591 ^T	55.0	45.0

*Values are hybridization (%) with biotinylated DNA from *M. excellens* KMM 3809^T.

anaerobic respiration of nitrate. No endospores are formed. Colonies on marine 2216 agar are circular, smooth, convex with an entire edge, transparent and 1–3 mm in diameter after 2 days incubation at room temperature (approx. 22–24 °C). Organic growth factors are not required. Growth occurs at 1–15 % NaCl. No growth at 20 % NaCl. Growth temperature ranges from 10 to 41 °C, with optimum growth at 20–25 °C. No growth is detected at 45 °C. pH range for growth is 6.0–10.0, with optimum growth at pH 7.5. Oxidase-positive and weakly positive for catalase. Amylase and lipase are hydrolysed, whereas gelatin, casein, chitin, agar, alginate and laminaran are not. Bacteria are non-haemolytic on mouse blood agar, non-cytotoxic, do not exhibit antimicrobial activity, are susceptible to polymyxin and resistant to ampicillin, benzylpenicillin, gentamicin, kanamycin, carbenicillin, neomycin, tetracycline, lyncomycin, oleandomycin and streptomycin. Positive for lipase and amylase, but negative for agarase, chitinase, caseinase and gelatinase; able to utilize a limited range of carbohydrates. Of the 95 carbon sources in the Biolog system, strain KMM 3809^T utilized Tween-85, N-acetyl-D-glucosamine, D-fructose, maltose, D-mannitol, L-rhamnose, D-sorbitol, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, D-galactonic acid lactone, α -hydroxybutyric acid, γ -hydroxybutyric acid, succinic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, L-pyrogutamic acid, D-serine, DL-carnitine, urocanic acid and 2-aminoethanol. Major respiratory lipoquinone is Q9; PE, PG and DPG are major phospholipids; cellular fatty acids are C_{16:0}, C_{16:1} ω 9c, C_{16:1} ω 7c and C_{18:1} ω 9c. Organisms belong to the γ -Proteobacteria, based on 16S rRNA gene sequences. DNA G + C content is 55.0–56.0 mol%.

The type strain is KMM 3809^T (=CIP 107686^T). Isolated from sediments collected in Chazhma Bay, Sea of Japan.

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