Marinomonas pontica sp. nov., isolated from the Black Sea

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A Gram-negative, polarly flagellated bacterium was isolated from a sea-water sample collected from the Karadag Natural Reserve of the Eastern Crimea and characterized to clarify its taxonomic position. 16S rRNA gene sequence-based phylogenetic analysis of this novel organism revealed *Marinomonas vaga*, *Marinomonas communis*, *Marinomonas mediterranea*, *Marinomonas primoryensis* and '*Marinomonas protea*' as its closest relatives (similarity 95–97%). The G + C content of the DNA was 46.5 mol%. The organism grew between 4 and 33 °C, tolerated 10% NaCl, was slightly alkaliphilic and was not able to degrade starch, gelatin, agar or Tween 80. Phosphatidylethanolamine (53.4%) and phosphatidylglycerol (46.6%) were the predominant phospholipids. The major fatty acids were 16:0 (15.5%), 16:1 ω 7 (26.7%) and 18:1 ω 7 (47.1%). The phylogenetic, genetic and physiological properties of the organism placed it within a novel species, proposed as *Marinomonas pontica* sp. nov., the type strain of which is 46-16^T (=LMG 22531^T=KMM 3492^T=UCM 11075^T).

The genus *Marinomonas* van Landschoot and De Ley 1984 was created to accommodate two misclassified *Alteromonas* species, *Marinomonas communis* and *Marinomonas vaga* (van Landschoot & De Ley, 1983; Baumann *et al.*, 1984; Gauthier & Breittmayer, 1992). More recently, two additional species, *Marinomonas mediterranea* and *Marinomonas primoryensis*, have been described (Solano & Sanchez-Amat, 1999; Romanenko *et al.*, 2003).

In this study, we report on the characterization of a novel mesophilic bacterium of the genus *Marinomonas* isolated from sea-water samples collected in the Karadag Natural Reserve of the Eastern Crimea. This work was part of a

taxonomic survey of free-living microbial populations of the Black Sea. During the course of this work, 51 *Alteromonas*-like strains of different phenotypes were isolated. The phenotype of the majority of the strains closely resembled *Pseudoalteromonas*, while a few had distinct phenotypes. Further detailed taxonomic investigation of one such strain, 46-16^T, revealed a number of particular phenotypic traits, e.g. lack of amylase, gelatinase, lipase and chitinase, and utilization of glycerol, lactate and some other carbon sources, which led us to assume that this strain belonged to the genus *Marinomonas*. Genetic and phylogenetic analyses confirmed this conclusion and allowed us to conclude that this organism constitutes a novel species, for which we propose the name *Marinomonas pontica* sp. nov.

Water samples were collected in July 2000 from a depth of 1–3 m (salinity 17‰, temperature 16 °C) using a standard hydrological plastic bathometer in the Karadag Natural Reserve (a central part of the Black Sea coast line). Samples

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were kept at 4 °C and processed within 4–8 h. A portion of sea water (0.1 ml) was plated on to marine agar 2216 (Difco) or medium B containing 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, 0.02 % (w/v) KH₂PO₄, 0.005 % (w/v) MgSO₄·7H₂O, 0.5 % (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 (~22–25 °C) for 5, 7 or 10 days. The isolation and purification of bacterial strains was done as described elsewhere (Ivanova *et al.*, 1996). Strains were stored at –80 °C in marine 2216 broth (Difco) supplemented with 20 % (v/v) glycerol.

Unless otherwise indicated, phenotypic characteristics were studied using standard procedures (Baumann *et al.*, 1972; Smibert & Krieg, 1994) as described previously (Ivanova *et al.*, 1996, 1998). The following physiological and biochemical properties were examined: oxidation/fermentation of glucose, denitrification, catalase and oxidase activities, gelatin liquefaction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, and the ability to hydrolyse starch, gelatin, chitin and Tween 80. Growth rate was studied under optimal physiological conditions and the requirement for Na⁺ ions was studied on medium containing (w/v) 0.25 % yeast extract, 0.1 % glucose, 0.02 % K₂HPO₄ and 0.005 % MgSO₄·7H₂O (pH 7.8). Salt-tolerance tests were performed on trypticase soy agar (TSA; Difco) with NaCl concentrations of 0.6–20.0 % (w/v). Cellular morphology was examined by phase-contrast light microscopy of 24 h cultures grown on agar plates. Electron micrographs of negatively stained cells were prepared using a Zeiss EM 10 CA electron microscope (80 kV). A drop of particle-free (autoclaved and ultracentrifuged) distilled water was placed on the bacterial growth (a few colonies) of a 24 h culture grown on agar plates. The sample (30 µl) of resulting bacterial suspension was applied to carbon- and Formvar-coated 400-mesh copper grids, a drop of 1.25 % uranyl acetate was added and the bacteria were allowed to adhere for 1 min at room temperature. Superfluous liquid was gently removed using a piece of filter paper.

Susceptibility to antibiotics was tested by the routine diffusion-plate method, employing medium B agar and discs impregnated with the following antibiotics: benzylpenicillin (10 µg), lincomycin (15 µg), oleandomycin (10 µg), polymyxin (25 µg), streptomycin (30 µg), erythromycin (20 µg), tetracycline (10 µg), cephalosporin (30 µg), furadonin (10 µg), nalidixic acid (10 µg) and ciprofloxacin (15 µg). Antibacterial activity was determined by the agar-diffusion assay, based on the method described by Barry (1980). Cultures (0.1 ml) of indicator test strains were spread on TSA plates in which circular wells (8 mm diameter) had been cut. Samples (0.1 ml) of the supernatant were tested and 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. Antimicrobial activities were tested

against *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 885-653, the phytopathogenic fungi *Penicillium chrysogenum* UCM F-57672 and *Cochliobolus sativus* UCM F-11224 and the cyanobacteria *Synechocystis minuscula* UCM A-14 and *Synechococcus cedrorum* UCM A-15. The culture fluid of strain 46-16^T grown in marine broth 2216 for 24 h inhibited the growth of *C. sativus*, while its cellular extract was active against *Synechocystis minuscula*. The results of the examination of the other morphological and physiological properties are shown in Table 1 and given in the species description.

For analysis of phospholipids and fatty acids, strains were grown at 28 °C on marine agar 2216. After 48 h growth, cells were harvested. Lipids were extracted by a method modified from Bligh & Dyer (1959). Polar lipids were separated by two-dimensional micro-TLC in solvent systems described by Vaskovsky & Terekhova (1979). Lipids were detected by TLC using 10 % H₂SO₄ in methanol with subsequent heating to 180 °C and using specific reagents for phospholipids (Vaskovsky *et al.*, 1975) and amino-containing lipids (2 % ninhydrin in acetone) and Dragendorff's reagent for choline lipids. Phospholipids were quantified by the method of Vaskovsky *et al.* (1975). Lipids were treated with 5 % HCl in methanol at 80 °C for 180 min to produce fatty acid methyl esters (FAMES) (Christie, 1982). FAMES were analysed by flame ionization detection GC (Shimadzu GC-17) with a fused silica capillary column (30 m × 0.25 mm) coated with Supelcowax 10 at 210 °C. Helium was used as a carrier gas. FAMES were identified by comparing the retention times with those of authentic standards and using equivalent chain length (ECL) measurements. To ensure correct identification, FAMES were analysed by GC-MS using a model GCMS-QP5050A (Shimadzu) fitted with an MDN-5S capillary column (30 m × 0.25 mm). The column temperature was programmed as follows: 1 min at 170 °C, followed by an increase to 240 °C at a rate of 2 °C min⁻¹, and held at 240 °C for 20 min. The temperature of the injector and detector was 250 °C. Phosphatidylethanolamine and phosphatidylglycerol were the major constituents of the phospholipids, accounting for 53.4 ± 0.7 and 46.6 ± 0.7 % of the total phospholipids, respectively. The cellular fatty acids comprised 14:0 (1.6 %), 15:0-ai (2.0 %), 16:0 (15.5 %), 16:1ω7 (26.7 %), 17:0-ai (0.6 %), 18:0 (5.7 %), 18:1ω7 (47.1 %) and 19:0-ai (0.8 %), with the most diagnostic *cis*-hexadecenoic (16:1ω7) and *cis*-octadecenoic (18:1ω7) and hexadecanoic (16:0) fatty acids. Overall, the phospholipid and fatty acid patterns of the novel isolate possessed a profile characteristic of the genus *Marinomonas* (Ivanova *et al.*, 2000).

DNA was extracted from cells grown overnight on medium B following the method of Marmur (1961). The G+C content of the DNA was determined as described by Marmur & Doty (1962) and was 46.5 ± 0.4 mol%.

The 16S rRNA gene for strain 46-16^T was amplified and

Table 1. Characteristics that differentiate *M. pontica* sp. nov. from phylogenetically related species

Taxa: 1, *M. pontica* sp. nov.; 2, *M. vaga*; 3, *M. communis*; 4, *M. mediterranea*; 5, *M. primoryensis*. Data from this study, Baumann *et al.* (1972), Solano *et al.* (1997), Solano & Sanchez-Amat (1999) and Romanenko *et al.* (2003). All strains were Gram-negative, motile, rod-shaped organisms, negative for amylase production, arginine dihydrolase and L-tyrosine utilization. All utilized citrate and acetate. V, Variable reaction depending on the strain; ND, data not available.

Characteristic	1	2	3	4	5
G + C content (mol%)	46	47–49	46–48	46	45
Flagellation	Subpolar/polar	Polar	Polar	Polar	Polar
Pigment	None	None	None	Melanin-like	Yellowish
Oxidase	+	–	+	–	+
Growth at:					
4 °C	+	–	–	–	+
35 °C	–	+	+	–	–
40 °C	–	–	+	–	–
Growth on 8 % NaCl	+	+	+	–	–
Production of:					
Lipase	–	–	–	+	–
Gelatinase	–	–	–	+	–
Utilization of:					
D-Glucose	+	+	+	+	V
Maltose	+	V	V	–	+
<i>m</i> -Hydroxybenzoate	+	+	+	–	+
Malate	+	+	+	+	V
L-Arginine	+	V	–	ND	V
L-Lysine	+	V	–	ND	+

sequenced as described elsewhere (Ivanova *et al.*, 2001, 2003a, b). Phylogenetic analyses were done as described previously (Ivanova *et al.*, 2004) and as described in detail at <http://bioinfo.unice.fr>. Briefly, a BLAST query allowed us to retrieve the 100 most similar sequences from the EMBL public database. These sequences were aligned and alignments were checked manually. Phylogenetic trees were constructed using three different methods: bio-neighbour joining, maximum likelihood and maximum parsimony. For the neighbour-joining analysis, distance matrices were calculated using the Kimura two-parameter correction and the analysis was performed according to Gascuel (1997). Maximum likelihood (using the global option) and maximum parsimony were from PHYLIP (Felsenstein, 1993). Phylogenetic trees were drawn using NJPLOT (Berry & Gascuel, 1996). For the final tree, only closely related sequences (as deduced from the global phylogenetic analyses) from cultured strains and, where possible, from type strains were retained.

According to the phylogenetic analyses, strain 46-16^T formed a cluster with unidentified marine bacterium Tw-9 but not with other species with validly published names of the genus *Marinomonas*. The 16S rRNA gene sequences shared 95–97 % identity with genes from *M. primoryensis*, *M. vaga*, *M. communis*, *M. mediterranea*

and two unpublished species, '*Marinomonas protea*' and '*Marinomonas alkaliphila*' (Fig. 1). Since bacteria that differ by more than 2.5 % at the 16S rRNA gene sequence level are unlikely to exhibit more than 60–70 % DNA–DNA hybridization values (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001; Keswani & Whitman, 2001), it could be inferred that the new strain from sea water represented a novel species.

The novel isolate 46-16^T could be clearly distinguished from other species of the genus *Marinomonas* by the combination of the following features: salinity tolerance and temperature for growth; ability to hydrolyse starch, gelatin and casein; susceptibility to certain antibiotics; and carbon source utilization. In particular, strain 46-16^T differed from *M. primoryensis* by the ability to grow above 30 °C, tolerance to concentrations of NaCl greater than 6 % and the ability to utilize glycerol; from *M. mediterranea* by the lack of pigments, the positive oxidase reaction, the inability to reduce nitrate and produce lipase and gelatinase, and the ability to utilize glycerol; and from *M. vaga* and *M. communis* by the growth temperature range, oxidase reaction, non-susceptibility to benzylpenicillin and some carbon compounds utilization (Table 1). Consequently, we propose that strain 46-16^T isolated from the Black Sea be classified as a novel species, *Marinomonas pontica* sp. nov.

Fig. 1. Phylogenetic position of *M. pontica* sp. nov. according to 16S rRNA gene sequence analysis. An unrooted tree is shown resulting from neighbour-joining analysis. Percentages of bootstrap analysis (1000 replications) are indicated only for branches also retrieved by maximum parsimony and maximum likelihood.

Description of *Marinomonas pontica* sp. nov.

Marinomonas pontica (pon.ti'ca. L. fem. adj. *pontica* related to the Black Sea, of the Black Sea).

Cells are rod shaped, 0.8–1.6 µm in length and 0.4–0.6 µm in diameter (by electron microscopy), motile with a single subpolar flagellum and Gram-negative. Aerobic and chemo-heterotrophic. They do not form endospores. Colonies on marine agar 2216 are slightly creamy, circular, smooth and convex with an entire edge. Organic growth factors are not required. The organism requires Na⁺ ions and grows on 0.5–10% NaCl. No growth is detected on 15% NaCl. The temperature growth range is 4–33 °C, with optimum growth occurring at 20–25 °C and no growth detected at 40 °C. The pH for growth ranges from 6.0 to 10.0, with the optimum at pH 7.5–8.5. Oxidase- and catalase-positive. Does not reduce nitrate to nitrite. Arginine dihydrolase and lysine decarboxylase are not exhibited. Does not produce amylase, esterase (Tween 80), proteinase (gelatinase) or agarase. Chitin is not hydrolysed. D-Glucose is utilized as a sole source of carbon. The following substrates are utilized: α-D-glucose, sucrose, D-trehalose, cellobiose, α-D-lactose, maltose, D-ribose, L-rhamnose, D-fructose, glycogen, dextrin, acetate, formate, butyrate, propionate, malate, lactate, citrate, pyruvate, ethanol, fumarate, D-mannitol, *m*-hydroxybenzoate, betaine, D-alanine, L-alanine, L-glycine, sarcosine, sodium succinate, L-proline, L-glutamate, L-asparagine, L-serine, L-ornithine, L-arginine, L-lysine, L-histidine and L-phenylalanine. Does not utilize D-xylose, L-arabinose, D-arabinose, D-mannose, D-sorbitol,

D-galactose, D-raffinose, valerate, glycerol, adonitol, *m*-inositol, benzoate, *o*-hydroxybenzoate, phenylacetate, fumarate, Tween 80, leucine, L-threonine L-cysteine, methionine, L-phenylalanine or L-tyrosine. Non-susceptible to benzylpenicillin; susceptible to lincomycin, oleandomycin, polymyxin, streptomycin, erythromycin, tetracycline, cephalosporin, furadonin, nalidixic acid and ciprofloxacin. Phosphatidylethanolamine and phosphatidylglycerol are the predominant phospholipids. Major cellular fatty acids are *cis*-hexadecenoic (16:1ω7), *cis*-octadecenoic (18:1ω7) and hexadecanoic (16:0) (~89%).

The type strain is 46-16^T (=LMG 22531^T=KMM 3492^T=UCM 11075^T). The G+C content of its DNA is 46.5 mol%. Isolated from a sea-water sample collected in the Karadag Natural Reserve of the Eastern Crimea, the Black Sea.

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