Rheinheimera pacifica sp. nov., a novel halotolerant bacterium isolated from deep sea water of the Pacific

Lyudmila A. Romanenko,¹ Masataka Uchino,² Enevold Falsen,³
Natalia V. Zhukova,⁴ Valery V. Mikhailov¹ and Tai Uchimura²

Correspondence

Lyudmila A. Romanenko
piboc@stl.ru

¹Pacific Institute of Bioorganic Chemistry, Far-Eastern Branch, Russian Academy of Sciences, 690022 Vladivostok, Prospect 100 Let Vladivostoku, 159, Russia

²Laboratory of Food Science and Technology, Department of Applied Biology and Chemistry, Faculty of Applied Bioscience, Tokyo University of Agriculture, Sakuragaoka 1-1-1, Setagaya-ku, Tokyo 156-8502, Japan

³Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden

⁴Institute of Marine Biology, Far-Eastern Branch, Russian Academy of Sciences, 690041, Vladivostok, Russia

An aerobic, Gram-negative, non-fermentative, rod-shaped, motile, non-pigmented bacterium, KMM 1406^T, was isolated from a sample of Pacific deep sea water and investigated for phenotypic characteristics, chemotaxonomic features and phylogenetic relationships. The deep-sea isolate exhibited growth in 0–8 % (w/v) NaCl and at 4–37 °C, hydrolytic activity on gelatin, Tween 80 and starch and lack of D-glucose utilization. The major fatty acids were C_{16:0}, C_{16:1}ω9c, C_{17:1}ω8c and C_{18:1}ω7c. The DNA G + C content was 49.6 mol%. 16S rRNA gene sequence analysis revealed that strain KMM 1406^T was related closely to *Rheinheimera baltica* DSM 14885^T within the γ -*Proteobacteria*, with 96.8 % sequence similarity. On the basis of phenotypic and molecular data, a novel species, *Rheinheimera pacifica* sp. nov., is proposed. The type strain is KMM 1406^T (=IAM 15043^T=JCM 12090^T=NRIC 0539^T=CCUG 46544^T).

Gram-negative, aerobic, heterotrophic, non-fermentative bacteria that are associated with marine habitats belong to different genera within the γ -*Proteobacteria*. Among them, the recently described genus *Rheinheimera*, which currently comprises a single species, *Rheinheimera baltica* DSM 14885^T, was proposed for motile, blue-coloured, non-halotolerant bacteria isolated from Baltic sea-water samples (Brettar *et al.*, 2002). Phylogenetic relatives of *R. baltica* DSM 14885^T were a clinical isolate of *Alishewanella fetalis* (Fonnesbech Vogel *et al.*, 2000) with sequence similarity of 94.8 % and, more closely related (96.5–96.8 % sequence similarity), some deep-sea bacteria that were reported by Takami *et al.* (1999) (Brettar *et al.*, 2002). During the course of studying biodiversity of deep-sea micro-organisms of the Pacific, we isolated the motile, non-pigmented, halotolerant bacterium KMM 1406^T and characterized it by using polyphasic taxonomy. Several phenotypic and physiological properties of the new deep-sea isolate were consistent with those of *R. baltica*. 16S rRNA gene sequence analysis

confirmed the affiliation of strain KMM 1406^T to the genus *Rheinheimera*. Sequence similarity with its close phylogenetic relative, *R. baltica* DSM 14885^T, was 96.8 %. Based on phenotypic features and genetic analysis, we concluded that strain KMM 1406^T represents a novel species within the genus *Rheinheimera*, for which the name *Rheinheimera pacifica* sp. nov. is proposed.

Strain KMM 1406^T was isolated from a sea-water sample that was obtained from a depth of 5000 m in the north-western part of the Pacific Ocean in July 1985. The sea-water sample was taken by a plastic hydrological bathometer. Aliquots (100 µl) of sea water were spread onto agar plates of sea-water medium (SWM) that contained (l⁻¹): 5.0 g peptone, 2.5 g yeast extract, 1.0 g glucose, 0.2 g K₂HPO₄, 0.05 g MgSO₄, 750 ml sea water, 250 ml distilled water and 15.0 g agar. Inoculated plates were incubated for 10 days at 28 °C. The bacterium was cultivated aerobically on marine agar 2216 (MA), marine broth (MB; Difco) or tryptic soy agar (TSA) at 25–28 °C and stored at –80 °C in liquid medium supplemented with 30 % (v/v) glycerol. Strain KMM 1406^T has been deposited in the Collection of Marine Microorganisms (KMM) of the Pacific Institute of

Abbreviation: PNPG, *p*-nitrophenyl α -D-glucopyranoside.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of KMM 1406^T is AB073132.

Bioorganic Chemistry, Vladivostok, Russia. The type strains of *Rheinheimera baltica* (DSM 14885^T) and *Alishewanella fetalis* (CCUG 30811^T) were used in this study for comparison. Cell morphology was examined with a phase-contrast microscope. Motility was observed by the hanging-drop method. Cell morphology was examined by transmission electron microscopy on exponential-phase cells that were grown in TSB. Cells were negatively stained with potassium phosphotungstate (1%, w/v; pH 7.0). Gram-reaction, oxidase and catalase activity, nitrate reduction and production of caseinase, deoxyribonuclease, gelatinase and lipase (Tween 80) were tested according to standard methods described by Smibert & Krieg (1994). Hydrolysis of starch was determined after 2 days incubation on nutrient agar medium that contained 10% (w/v) soluble starch by flooding plates with 1% (w/v) iodine solution. Formation of H₂S from thiosulfate was detected with lead acetate paper. Ability to grow at different temperatures was tested on MA and TSA at 4, 10, 15, 25, 30, 35, 37 and 42 °C. The pH range for growth (5.0–10.0) was tested by using MB with pH values adjusted by addition of 5 M NaOH or HCl. Sodium ion requirement and tolerance of various NaCl concentrations were examined by using SWM prepared with artificial sea water with NaCl concentrations of 0, 1, 3, 5, 8, 10, 12, 15 and 20% (w/v). The strain was tested for its ability to ferment glucose and produce acid from carbohydrates with a supplement of 1% (w/v) of each compound by the use of marine oxidation–fermentation medium (Leifson, 1963). The ability to utilize various compounds as sole carbon and energy sources was examined in a mineral liquid medium that contained (g l⁻¹): NH₄Cl, 1.0; K₂HPO₄, 0.075; CaCl₂, 1.45; NaCl, 5.0; MgCl₂, 6.15; KCl, 0.75; and FeSO₄, 0.028, supplemented with 0.2% (w/v) of one of the test substrates. Bacterial growth was determined spectrophotometrically after 2 days cultivation. Strain KMM 1406^T was characterized additionally by using API 20NE and API ZYM identification systems (bioMérieux) at 28 °C. Antibiotic sensitivity was tested by the agar-diffusion method on TSA plates by using discs impregnated with antibiotics (content per disc): ampicillin, 10 µg; benzylpenicillin, 10 U; gentamicin, 10 µg; kanamycin, 30 µg; carbenicillin, 25 µg; lincomycin, 15 µg; oleandomycin, 15 µg; polymyxin, 300 U; streptomycin, 30 µg; tetracycline, 30 µg; and neomycin, 15 µg. DNA base composition was determined as described by Marmur & Doty (1962) and Owen *et al.* (1969). Whole-cell fatty acids were determined as described by Svetashev *et al.* (1995). 16S rRNA gene sequences were determined and compared as described by Shida *et al.* (1997). Previously published 16S rRNA gene sequences were obtained from GenBank/EMBL/DBJ.

Deep-sea isolate KMM 1406^T was an aerobic, Gram-negative, oxidase- and catalase-positive, chemoheterotrophic, non-pigmented, rod-shaped bacterium that measured 0.6–0.8 µm in diameter and 1.8–2.0 µm in length. Cells were motile by means of four to seven polar or bipolar and lateral flagella (Fig. 1). Strain KMM 1406^T did not require

Fig. 1. Electron micrograph of a negatively stained cell of KMM 1406^T. Bar, 1 µm.

sodium ions for growth and grew in 0–8% (w/v) NaCl. Growth temperature was 4–37 °C. No growth was observed in 10% NaCl or at 40 or 41 °C. The new bacterium formed non-pigmented, smooth, transparent, shining, convex and circular colonies with entire margins that were 3–5 mm in diameter on TSA and MA after 24 h incubation and released a slightly brown diffusible pigment into the medium. Strain KMM 1406^T was unable to produce acid from carbohydrates aerobically or to ferment glucose under anaerobic conditions. Hydrolytic reactions on starch, gelatin and casein were observed after 24 h, but Tween 80 and DNA were hydrolysed after 3 days incubation. Other phenotypic characteristics of strain KMM 1406^T are shown in Table 1.

Major fatty acids of strain KMM 1406^T were C_{16:1}ω9c (25.5%), C_{16:0} (19.1%), C_{18:1}ω7c (15.7%), C_{17:0} (8.1%) and C_{17:1}ω8c (11.7%); fatty acids C_{15:0} (2.4%), C_{15:1}ω8c (3.3%), i-C_{16:0} (3.7%) and i-C_{18:0} (1.3%) were detected as minor components. The DNA G + C content of strain KMM 1406^T was 49.6 mol%.

Phylogenetic relationships among strain KMM 1406^T and some related taxa are shown in Fig. 2. Comparative 16S rRNA gene sequence analysis showed that the new isolate was phylogenetically most closely related to *R. baltica* DSM 14885^T and deep-sea isolates HBT 019, HTB 010 and HTB 021 (Takami *et al.*, 1999), with 96.8–97.4% sequence similarity, and less closely related to *A. fetalis* CCUG 30811^T (Fonnesbech Vogel *et al.*, 2000), with 95.4% sequence similarity. This 16S rDNA sequence similarity value with *R. baltica* DSM 14885^T is appropriate for determining intrageneric relationships for species definition (Stackebrandt & Goebel, 1994), indicating that strain KMM 1406^T probably represents a novel species within the genus *Rheinheimera*. The novel deep-sea isolate shared main physiological characteristics, assimilation pattern and spectrum of enzyme activities with *R. baltica* DSM 14885^T. Its DNA G + C content was close to those of *R. baltica* strains (47.8–48.9 mol%). The fatty acid profiles of KMM 1406^T

Table 1. Phenotypic characteristics of strain KMM 1406^T and the related bacteria *Rheinheimera baltica* DSM 14885^T and *Alishewanella fetalis* CCUG 30811^T

Taxa: 1, KMM 1406^T; 2, *Rheinheimera baltica* DSM 14885^T; 3, *Alishewanella fetalis* CCUG 30811^T. +, Positive; –, negative; W, weak reaction; ND, not determined; NG, no growth. Data are from Brettar *et al.* (2002), Fønnesbech Vogel *et al.* (2000) and the present study. All strains are Gram-negative, positive for oxidase, catalase, gelatinase and growth in 3 % NaCl and negative for growth in 10 % NaCl, arginine dihydrolase and urease activities, acid production from D-glucose and mannitol and assimilation of D-mannose, caprate, adipate, L-malate, phenylacetate and gluconate.

Feature	1	2	3
Pigmentation	–	+	–
Motility	+	+	–
Flagella arrangement:			
Polar	+	+	–
Bipolar, lateral	+	–	–
Growth at (°C):			
4	+	+	–
25	+	+	W
37	+	–	+
40–42	–	–	+
Growth in NaCl (%):			
0	+	+	–
6	+	–	+
8	+	–	+
Hydrolysis of:			
Tween 80	+	+	+
Starch	+	+	–
DNA	+	ND	NG
API test:			
Nitrate reduction	–	–	+
Aesculin hydrolysis	+	–	+
Utilization of:			
Glucose	–	+	–
L-Arabinose	+	–	–
N-Acetylglucosamine	+	+	–
Maltose	+	+	W
Citrate	+	–	–
DNA G+C content (mol%)	49.6	48.9	50.6
Isolation source	Pacific deep sea water	Baltic surface sea water	Human foetus

*In the present study, *R. baltica* DSM 14885^T did not grow without NaCl.

and *R. baltica* (Brettar *et al.*, 2002) were found to be similar to each other, but strain KMM 1406^T contained a higher proportion of C_{17:0} and C_{17:1ω8c}. Together with the findings mentioned above, significant characteristics for clear differentiation of KMM 1406^T from *Rheinheimera baltica* include its lack of blue-coloured pigmentation, presence of bipolar and lateral flagella, growth at 37 °C and in 6–8 % NaCl, positive reactions for aesculin hydrolysis, PNPG (*p*-nitrophenyl α-D-glucopyranoside) test, arabinose and citrate utilization and inability to utilize D-glucose. It has been reported that *R. baltica* strains do not require NaCl for growth and that sodium ions stimulate their growth (Brettar *et al.*, 2002). In the present study, we failed

to grow *R. baltica* DSM 14885^T without NaCl, whilst strain KMM 1406^T gave good growth on the same media. In addition, NaCl was not needed to support growth of KMM 1406^T. Major phenotypic differences of the new strain from the phylogenetically less related species *A. fetalis* were as follows: motility, inability to reduce nitrate or thiosulfate, lack of growth at 40 or 42 °C or without NaCl, hydrolysis of some compounds and assimilation pattern (Table 1). On the basis of phenotypic characterization and phylogenetic analysis, we propose that strain KMM 1406^T should be classified as a novel species, *Rheinheimera pacifica* sp. nov., with the type strain KMM 1406^T (=JCM 12090^T = NRIC 0539^T = CCUG 46544^T).

Fig. 2. Phylogenetic relationships of KMM 1406^T and some related genera, based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Significances for particular nodes were obtained by bootstrap analysis. GenBank accession numbers are given in parentheses. Bar, 0.01 nucleotide substitution per site.

Description of *Rheinheimera pacifica* sp. nov.

Rheinheimera pacifica (pa.ci'fi.ca. N.L. fem. adj. *pacifica* from the Pacific Ocean, pertaining to the Pacific Ocean sea-water sample from which the type strain was isolated).

Strictly aerobic, Gram-negative, chemoheterotrophic, oxidase- and catalase-positive, rod-shaped, 1.8–2.0 µm long and 0.6–0.8 µm wide and motile with four to seven polar or bipolar and lateral flagella. Sodium ions are not required for growth. Growth is observed in 0–8% (w/v) NaCl, but not in 10% NaCl. Grows at 4–37 °C, but not at 40–41 °C. Non-pigmented, smooth, transparent and circular colonies with entire margins are formed on MA. Gelatin, starch, casein, Tween 80 and DNA are hydrolysed. Glucose fermentation and H₂S production are not detected. Glycerol, acetate, L-arginine, valine, asparagine and DL-lysine are utilized, but methionine and L-glutamic acid are not. According to the API 20NE test (bioMérieux), aesculin and gelatin hydrolysis, PNPG test, arabinose, N-acetylglucosamine, maltose and citrate assimilation are positive and the following tests are negative: nitrate reduction, indole production, arginine dihydrolase, urease and assimilation of glucose, mannose, mannitol, gluconate, L-malate, caprate, adipate and phenylacetate. Assimilation of trehalose and sucrose is positive. In API ZYM analysis, positive reactions are exhibited for alkaline phosphatase, esterase C4, esterase lipase C8, leucine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase; negative reactions are exhibited for lipase C14, valine arylamidase, cystine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase. Susceptible to gentamicin (10 µg); weakly susceptible to polymyxin (300 U), streptomycin (30 µg), kanamycin (30 µg) and neomycin (15 µg) and resistant to carbenicillin (25 µg), lincomycin (15 µg), oleandomycin (15 µg), tetracycline (30 µg), ampicillin (10 µg) and benzylpenicillin (10 U). DNA G+C content of the type strain is 49.6 mol% (determined by the thermal denaturation method). Predominant cellular fatty acids are C_{16:0}, C_{17:0}, C_{16:1}ω9c,

C_{17:1}ω8c and C_{18:1}ω7c. Fatty acids C_{15:0}, C_{15:1}ω8c, i-C_{16:0} and i-C_{18:0} are minor components.

The type strain, KMM 1406^T (=IAM 15043^T=JCM 12090^T=NRIC 0539^T=CCUG 46544^T), was isolated from deep sea water of the Pacific Ocean.

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