NOTE

Pseudoalteromonas ruthenica sp. nov., isolated from marine invertebrates

Elena P. Ivanova,[1]† Tomoo Sawabe,[2] Anatolii M. Lysenko,[3] Nataliya M. Gorshkova,[1] Vassilii I. Svetashev,[4] Dan V. Nicolau,[5] Noboru Yumoto,[6] Takahisa Taguchi,[6] Susumu Yoshikawa,[6] Richard Christen[7] and Valery V. Mikhailov[1]

Author for correspondence: Elena P. Ivanova. Tel: +61 3 9214 5137. Fax: +61 3 9214 5050. e-mail: eivanova@groupwise.swin.edu.au

[1] Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences, 690022 Vladivostok, Pr. 100 Let Vladivostoku 159, Russia

[2] Laboratory of Microbiology, Graduate School of Fisheries Sciences, Faculty of Fisheries, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan

[3] Institute of Microbiology of the Russian Academy of Sciences, 117811 Moscow, Russia

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

[5] Industrial Research Institute Swinburne, Swinburne University of Technology, PO Box 218, Hawthorn, Vic 3122, Australia

[6] National Institute of Advanced Industrial Science and Technology, AIST, Ikeda, Osaka 563, Japan

[7] Centre National de la Recherche Scientifique et Université, Pierre et Marie Curie Station Zoologique, Villefranche-sur-Mer 06230, France

On the basis of phenotypic and genotypic characteristics and analysis of 16S rRNA sequences, a novel species belonging to the genus *Pseudoalteromonas* is described. Two pale-orange-pigmented strains, KMM 300^T and KMM 290, isolated respectively from a mussel, *Crenomytilus grayanus*, and a scallop, *Patinopecten yessoensis*, are marine, Gram-negative, aerobic, rod-shaped bacteria that produce a number of antimicrobial compounds. The strains are able to degrade gelatin, elastin, starch, DNA and Tween 80. Chitin and agar are not degraded. The isolates from marine invertebrates grew at NaCl concentrations of 1–9% and a temperature range of 10–35 °C and did not utilize most of the wide range of carbohydrates tested, with the exception of D-glucose, cellobiose and sucrose. The DNA G+C content was 48·4–48·9 mol%. The level of DNA homology of the two strains was 98%. DNA from the strains isolated from marine invertebrates showed 5–15% genetic relatedness to the DNA of other type strains of the genus *Pseudoalteromonas*. 16S rRNA analysis indicated a clear affiliation of the novel bacteria to other species of the genus. The strains are assigned to a novel species, *Pseudomonas ruthenica* sp. nov., with the type strain KMM 300^T (= LMG 19699^T = CIP 106857^T).

Keywords: marine *Proteobacteria*, *Pseudoalteromonas ruthenica*

Marine aerobic heterotrophic bacteria of the genus *Pseudoalteromonas* (Gauthier *et al.*, 1995) comprise one of the most abundant taxonomic groups of the proteobacteria, widely distributed in the marine environment (Baumann & Baumann, 1981; Baumann *et al.*, 1984; Gauthier & Breittmayer, 1992). A study conducted along the Russian Far-Eastern coast of the Sea of Japan to analyse the diversity of marine bacteria has led to the isolation of a collection of strains from seawater and invertebrates (Ivanova *et al.*, 1996, 1998a, 2000a; Sawabe *et al.*, 2000). In this paper, we present the results of a phenotypic, genetic and phylogenetic examination of two strains isolated from a mussel, *Crenomytilus grayanus*, and a scallop, *Patinopecten yessoensis*. The data obtained suggest that the bacteria represent a novel species and the name *Pseudoalteromonas ruthenica* sp. nov. is proposed.

Mussels and scallops were collected in July 1990 at a depth of 5–8 m (salinity 33‰, temperature 12 °C) at the Pacific Institute Bio-organic Chemistry Marine Experimental Station, Troitza Bay, Gulf of Peter the Great, Sea of Japan. Invertebrates were prepared aseptically. The strains were isolated from tissue homogenates by plating 0·1 ml aliquots on agar plates of marine agar 2216 (Difco) and on plates with medium B. Medium B contained 0·2% (w/v) Bacto peptone (Difco), 0·2% (w/v) casein hydrolysate (Merck),

†**Present address:** Industrial Research Institute Swinburne, Swinburne University of Technology, PO Box 218, Hawthorn, Vic 3122, Australia.

The GenBank accession numbers for the 16S rDNA sequences of *Pseudoalteromonas ruthenica* KMM 300^T and KMM 290 are AF316891 and AF316890.

0·2 % (w/v) Bacto yeast extract (Difco), 0·1 % (w/v) glucose, 0·002 % (w/v) KH_2PO_4, 0·005 % (w/v) $MgSO_4 . 7H_2O$, 1·5 % (w/v) Bacto agar (Difco), 50 % (v/v) natural seawater and 50 % (v/v) distilled water at pH 7·5–7·8, as described elsewhere (Ivanova *et al.*, 1996). After the initial isolation, the strains were purified on medium B and maintained on the same semi-solid medium B in tubes under mineral oil at 4 °C and stored at −80 °C in marine broth (Difco) supplemented with 30 % (v/v) glycerol. All isolates were streaked on agar plates from broth cultures every 6 months to ensure purity and viability.

Unless indicated otherwise, phenotypic properties used for the characterization of *Alteromonas*-like species were investigated by using standard procedures (Baumann *et al.*, 1972; Baumann & Baumann, 1981; Smibert & Krieg, 1994) and as described elsewhere (Ivanova *et al.*, 1996, 1998a). The temperature tolerance for growth was examined on medium B incubated at 4, 10, 30, 35, 37 and 42 °C. The use of various organic substrates [0·1 % (w/v), listed in Table 2] as sole sources of carbon was tested using BM broth medium (Baumann *et al.*, 1972). The bacteria were grown at 24–26 °C. The ability to use organic substrates was investigated using API 50 CH strips (bioMérieux).

Two strains, KMM 300^T isolated from a mussel, *Crenomytilus grayanus*, and KMM 290 isolated from a scallop, *Patinopecten yessoensis*, had all of the phenotypic characteristics of the genus *Pseudoalteromonas*. The cells were Gram-negative, strictly aerobic, oxidase- and catalase-positive, rod-shaped, 0·7–0·9 μm in diameter and 1·0–1·2 μm long with a single polar flagellum. A previous report described the production of a number of antimicrobial compounds by these strains (Ivanova *et al.*, 1998b). Colonies were uniformly round, 2–3 mm in diameter, circular, regular, convex, translucent, smooth and slightly orange-pigmented after incubation for 48 h on marine agar. No diffusible pigment was produced in the medium. The bacteria did not form endospores, did not accumulated poly-β-hydroxybutyrate as an intracellular reserve product, did not have an arginine dihydrolase and required sea water or 1–9 % NaCl for growth, with optimum growth at 1–3 % NaCl. The temperature range for growth was 10–35 °C, with optimum growth at 25–30 °C. No growth was detected at 42 °C. The pH range for growth was 6·0–10·0, with optimum growth at pH 7·5–8·0. The strains decomposed gelatin, elastin, Tween 80, DNA and starch. Agar and chitin were not hydrolysed. Of the 49 carbohydrates tested, the two strains assimilated the carbohydrates D-glucose, cellobiose and sucrose. Cells of strain KMM 300^T also assimilated salicin and arbutin. Neither strain was susceptible to benzylpenicillin, ampicillin, oleandomycin or streptomycin.

Analysis of fatty acid methyl ethers (FAME) was performed by GLC as described by Svetashev *et al.* (1995). The fatty acid patterns of the two strains studied were very similar to those found for other *Pseudoalteromonas* species (Ivanova *et al.*, 2000b). The level of major fatty acids were 2–11 % for 14:0, 13·8–18·7 % for 16:0, 31–42 % for 16:1ω7, 4–8 % for 17:1ω8 and 4–16 % for 18:1ω7.

DNA was isolated following the method of Marmur (1961). The G+C content of the DNA of strains KMM 300^T and KMM 290 was 48·4 and 48·9 mol %, respectively, determined using the thermal denaturation method (Marmur & Doty, 1962). For DNA–DNA hybridization experiments, reference strains were obtained from the ATCC (Manassas, VA, USA), the National Collection of Industrial and Marine Bacteria (NCIMB, UK) and the Marine Biotechnology Institute Collection (MBIC, Japan) or were kindly provided by U. Simidu, J. Guinea and C. Holmström. All reference strains were cultured routinely on marine agar 2216 plates (Difco). DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described elsewhere (Marmur & Doty, 1962; De Ley *et al.*, 1970). The level of DNA homology of the two strains isolated from marine invertebrates was 98 % and therefore they were assigned genotypically to a single species. The genetic similarity of KMM 300^T and KMM 290 to the type strains of the genus *Pseudoalteromonas* listed in Table 1 was rather low and ranged from 5 to 16 %. Based on generally accepted criteria of the definition of genomic species (Wayne *et al.*, 1987), strains KMM 300^T and KMM 290 are assigned to the separate species.

DNA for PCR was prepared using the Promega Wizard genomic DNA extraction kit according to the instruction manual. Aliquots (100 ng) of DNA templates were used for PCR amplification of small-subunit rRNA genes as described previously (Sawabe *et al.*, 1998a, b). The PCR conditions were as follows: initial denaturation step at 94 °C for 180 s, an annealing step at 55 °C for 60 s and an extension step at 72 °C for 90 s. The thermal profile consisted of 30 cycles. The amplification primers used in this study gave a 1·5 kb PCR product and corresponded to positions 25–1521 of the *Escherichia coli* sequence. The PCR products were purified using a Promega Wizard PCR Preps DNA purification kit and sequenced directly by using a *Taq* FS dye terminator sequencing kit (ABI) and the protocol recommended by the manufacturer. DNA sequencing was performed with an Applied Biosystems model 373S automated sequencer. Nine sequencing primers were used for sequencing (Sawabe *et al.*, 1998a).

The 16S rDNA sequences were aligned automatically and then manually by reference to a database of 20000 already-aligned bacterial 16S rDNA sequences. Phylogenetic trees were constructed according to three different methods (bioNJ, maximum-likelihood and maximum-parsimony). For the neighbour-joining (NJ) analysis, a matrix distance was calculated according to Kimura's two-parameter correction. Boot-

Table 1. DNA relatedness among tested *Pseudoalteromonas* strains

Organism	G+C content (mol%)	Hybridization with DNA from KMM 300^T (%)
P. ruthenica KMM 300^T	48·4	100
P. ruthenica KMM 290	48·9	98
P. antarctica CECT 4664^T	42·3	14
P. atlantica IAM 12927^T	42·1	15
P. aurantia DSM 6057^T	44·1	10
P. carrageenovora IAM 12662^T	38·9	10
P. citrea ATCC 29719^T	42·1	15
P. distincta KMM 638^T	43·6	15
P. elyakovii KMM 162^T	40·1	10
P. espejiana NCIMB 2127^T	42·7	14
P. haloplanktis ATCC 14393^T	40·2	14
P. luteoviolacea NCIMB 1893^T	42·0	10
P. maricaloris KMM 636^T	39·1	16
P. nigrifaciens IAM 13010^T	41·7	8
P. peptidolytica MBIC 1416^T	37·9	5
P. piscicida ATCC 15057^T	42·7	15
P. rubra ATCC 29570^T	39·0	11
P. tetraodonis IAM 14160^T	42·1	15
P. tunicata CCUG 26757^T	42·3	10

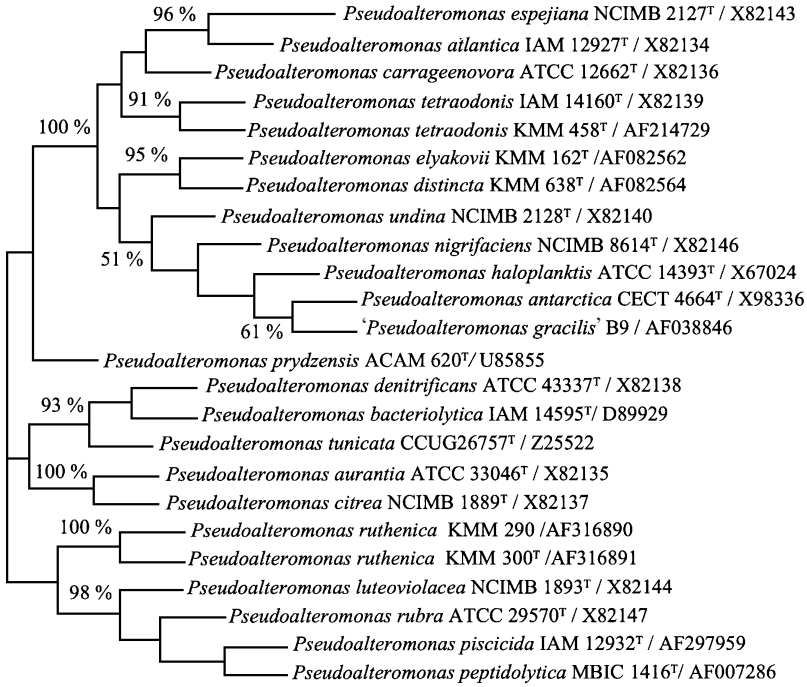

Fig. 1. Phylogenetic position of *Pseudoalteromonas ruthenica* isolates KMM 300^T and KMM 290 within the genus *Pseudoalteromonas*. Unrooted tree obtained using a bioNJ algorithm, Kimura's two-parameter correction for distance calculations and 500 replications in a bootstrap analysis. Bootstrap percentages are only indicated for branches also found in maximum-likelihood ($P < 0·01$) and parsimony (most-parsimonious tree) analyses.

straps were done using 500 replications, bioNJ and Kimura's two-parameter corrections. BioNJ analysis was done according to Gascuel (1997), maximum-likelihood and maximum-parsimony data were from PHYLIP (Phylogeny Inference Package, version 3.573c, distributed by J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA, USA). The phylogenetic trees were drawn using NJPLOT (Perrière & Gouy, 1996) and Claris DRAW software for Apple Macintosh.

Domains used to construct phylogenetic trees were regions of the small-subunit rDNA sequences available for all sequences and excluding positions likely to show homoplasy. When bacterial sequences from different phyla or different genera were used to determine that the novel species indeed belonged to the genus *Pseudoalteromonas*, these domains were more (genus determination) or less (phylum determination) extended, since the number of positions that can be aligned properly decreases and homoplasy increases

Table 2. Phenotypic features that distinguish *P. ruthenica* from other related *Pseudoalteromonas* species

Strains are indicated as: 1, *P. ruthenica* KMM 300^T and KMM 290; 2, *P. maricaloris* KMM 636^T; 3, *P. piscicida* ATCC 15057^T; 4, *P. citrea* ATCC 29719^T; 5, *P. aurantia* DSM 6057^T; 6, *P. peptidolytica* MBIC 1416^T; 7, *P. rubra* ATCC 29570^T; 8, *P. luteoviolacea* NCIMB 1893^T. Data were taken from this study, Gauthier & Breittmayer (1992) and Ivanova *et al.* (1998a). All strains exhibit polar flagella, require sodium ions for growth, are positive for oxidase and catalase and negative for denitrification and arginine dihydrolase activity, do not produce chitinase, produce gelatinase, lipase, amylase and DNase, grow at 10–35 °C and 3–6% NaCl, utilize D-glucose, are susceptible to gentamicin and polymyxin and are not susceptible to benzylpenicillin or ampicillin. +, Positive; −, negative; V, strain variation; ND, no data available. Features that differ for KMM 290 are given in parentheses.

Characteristic	1	2	3	4	5	6	7	8
Requirement for organic growth factors	−	−	+	+	+	−	+	+
Growth at:								
4 °C	−	−	−	−	+	−	−	−
37 °C	+(−)	+	+	−	−	+	+	+
Growth with:								
8% NaCl	+	+	+	+	−	+	−	−
10% NaCl	−	+	+	+	−	−	−	−
Production of agarase	−	−	−	V	−	−	−	−
Utilization of:								
Maltose	−	+	−	−	−	+	+	−
D-Arabinose	−	+	−	−	−	−	−	−
Sucrose	−	+	−	−	−	−	−	−
Melibiose	−	+	−	−	−	−	−	−
Mannitol	−	+	−	−	−	−	−	−
Sorbitol	−	+	−	−	−	−	−	−
Citrate	−	+	+	−	−	−	−	−
Glycerol	−	+	−	−	−	−	−	−
L-Arginine	−	+	ND	−	+	−	−	+
Susceptibility to:								
Kanamycin (30 μg)	−(+)	−	+	−	+	ND	−	−
Oleandomycin (15 μg)	−	+	+	+	+	+	+	+
Streptomycin (10 μg)	−	+	+	+	+	ND	−	+

with the depth of the phylogenetic tree. For that reason, a detailed phylogenetic tree that analyses the position of a species within a genus cannot usually be properly presented with outgroups (unless some outgroups are so closely related that the entire sequence is orthologous and with little suspected homoplasy). One should note that rooting a clade with outgroups is of importance only when one wants to derive theoretical considerations concerning some traits and to define, for example, which are ancestral and which are derived.

For the tree shown in Fig. 1, retaining only sequences of the genus *Pseudoalteromonas* allowed the inclusion in the analysis of almost the entire 16S rDNA sequences, corresponding to positions 71–1375 of the KMM 300^T sequence. The topology shown is that of the bootstrap analysis, as it as been demonstrated that this topology is often better than that of a simple NJ or maximum-parsimony analysis (Berry & Gascuel, 1996). As a result, there is no scale bar in this tree; note also that one should consider the scale bar with caution in a simple tree, as the scale bar represents distances calculated after correction (Kimura's two-parameter; Jukes & Cantor, 1969), and that the lengths of the branches do not represent simply the real number of differences between the sequences themselves (Gascuel, 1997).

16S rDNA sequence analysis revealed that the bacteria studied are the members of the *γ-Proteobacteria*. These data indicate clearly that the two strains KMM 300^T and KMM 290 form a clade that should be included in the genus *Pseudoalteromonas*. Because this clade did not cluster robustly with any recognized species of *Pseudoalteromonas*, phylogenetic analysis suggests that this clade represents a novel species within the genus *Pseudoalteromonas*, a result that is consistent with phenotypic characteristics and DNA–DNA hybridization experiments. Strain KMM 300^T should then be recognized as the type strain of a novel species, distinct from *Pseudoalteromonas luteoviolacea*, *Pseudoalteromonas rubra*, *Pseudoalteromonas piscicida* and *Pseudoalteromonas peptidolytica*, although these species clearly form a distinct subclade within *Pseudoalteromonas* (Fig. 1).

Bacteria of the novel species can be distinguished easily from phylogenetically related pigmented species by a number of phenotypic traits: from *P. rubra* and *P. luteoviolacea* by the ability to grow with 8 % NaCl and the inability to utilize maltose or L-arginine; from *P. peptidolytica* by the inability to utilize maltose; and from *P. piscicida* by the inability to grow with 10 % NaCl and resistance to oleandomycin (Table 2). Therefore, we propose that strains KMM 300^T and KMM 290 be placed in a novel species as *Pseudoalteromonas ruthenica* sp. nov.

Description of *Pseudoalteromonas ruthenica* sp. nov.

Pseudoalteromonas ruthenica (ru.the′ni.ca. M.L. fem. adj. *ruthenica* of Russia).

Cells are rod-shaped, single, about 0·7–0·9 μm in diameter, Gram-negative and motile, with a single polar flagellum. Strictly aerobic. Chemorganotroph with respiratory metabolism. Does not form endospores. Does not accumulate poly-β-hydroxybutyrate as an intracellular reserve product and has an arginine dihydrolase system. Oxidase- and catalase-positive. Requires sodium ions or seawater for growth. Growth occurs in media with 1–9 % NaCl. Temperature for growth ranges from 10 to 35 °C, with optimum growth at 25–30 °C. No growth is detected at 40 °C. The pH for growth ranges from 6·0 to 10·0, with optimum growth at pH 7·5–8·0. Decomposes gelatin, elastin, Tween 80, DNA and starch. Agar and chitin are not hydrolysed. Utilizes D-glucose, cellobiose and sucrose (according to bioMérieux API 50 CH strips). The main cellular fatty acids are 16:1ω7, 16:0, 17:1ω8 and 18:1ω7 (about 80 % of the total).

Isolated from a mussel, *Crenomytilus grayanus*, and a scallop, *Patinopecten yessoensis*, collected from the Sea of Japan. The G+C content of the DNA is 48·4–48·9 mol %. The type strain is KMM 300^T (= LMG 19699^T = CIP 106857^T).

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