International Journal of Systematic and Evolutionary Microbiology (2002), **52**, 229–234 Printed in Great Britain

NOTE

Pseudoalteromonas issachenkonii sp. nov., a bacterium that degrades the thallus of the brown alga *Fucus evanescens*

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Eleven non-pigmented strains of Gram-negative, aerobic, marine bacteria with polar flagella were isolated from the thallus of the brown alga *Fucus evanescens* collected in the Kraternaya Bight of the Kurile Islands in the Pacific Ocean. These organisms were conspecific and exhibited high levels of genetic relatedness (up to 91%). The G+C contents of the DNAs of these strains were 42·9–43·3 mol%. These halophilic bacteria had bacteriolytic, proteolytic and haemolytic activities and degraded algal polysaccharides, synthesizing a number of glycoside hydrolases (fucoidanases, laminaranases, alginases, agarases, pullulanases, β-glucosidases, β-galactosidases, β-*N*-acetylglucosaminidases and β-xylosidases). By 16S rDNA analysis, the bacteria were shown to belong to the genus *Pseudoalteromonas*, a member of the γ-subclass of the *Proteobacteria*. DNA from the strains isolated from the brown alga showed 27–54% genetic relatedness with respect to DNAs of other type strains of the genus *Pseudoalteromonas*. The phenotypic characteristics, together with the genetic evidence, indicate that this group of epiphytic bacteria represents a distinct species, *Pseudoalteromonas issachenkonii* sp. nov., for which the type strain is KMM 3549^T (= LMG 19697^T = CIP 106858^T).

Keywords: marine, *Proteobacteria*, *Pseudoalteromonas issachenkonii*

Marine aerobic heterotrophic bacteria of the genus *Pseudoalteromonas* (Gauthier *et al.*, 1995) comprise one of the most abundant groups of proteobacteria, widely distributed in the marine environment (Baumann & Baumann, 1981; Baumann *et al.*, 1984; Gauthier & Breittmayer, 1992). We previously isolated a collection of marine aerobic *Alteromonas*-like proteobacteria from both seawater and an array of hydrocoles, namely mussels, ascidians, echinoderms, sea grass and algae, that are common inhabitants of the seas of Japan and Okhotsk, the Coral Sea and the South China Sea (Ivanova *et al.*, 1996, 1998, 2000a; Sawabe *et al.*, 2000). In this paper, we present the results of a phenotypic, genetic and phylogenetic examination of 11 strains isolated from the thallus of the brown alga *Fucus evanescens*. The data obtained suggest that the bacteria represent a novel species, for which we propose the name *Pseudoalteromonas issachenkonii* sp. nov.

Algae were collected by scuba-divers in mid-summer (July 1999) at the Kraternaya Bight of the Kurile Islands in the Pacific Ocean during the 23rd scientific expedition of the RV '*Akademician Oparin*'. The algae were transferred into sterile plastic bags and transported to the laboratory on board the vessel on the same day. A piece of algal thallus (5 g) was placed in a flask with 200 ml sterilized natural seawater and left for 2 months at room temperature (approx. 22 °C) to observe the degradation of the algal thallus. The strains were isolated from the resulting suspension by plating (0·1 ml) on agar plates of marine agar 2216 (Difco) and on plates with medium B [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, 0·002% (w/v) KH_2PO_4, 0·005% (w/v) $MgSO_4 .7H_2O$,

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The GenBank accession number for the 16S rRNA gene sequence of *Pseudoalteromonas issachenkonii* KMM 3549^T is AF316144.

1·5% (w/v) Bacto agar (Difco), 50% (v/v) natural seawater and 50% (v/v) distilled water, pH 7·5–7·8] as described elsewhere (Ivanova *et al.*, 1996). After the initial isolation, strains were purified on medium B and were 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, the phenotypic properties used for the characterization of the *Alteromonas*-like species were determined by using standard procedures (Baumann *et al.*, 1972; Baumann & Baumann, 1981; Smibert & Krieg, 1994) and as described elsewhere (Ivanova *et al.*, 1996, 1998). Growth at different temperatures, NaCl concentrations or pH values was measured using optical density at 660 nm after 24 h incubation in medium B. The incubation temperatures employed ranged from 4 to 42 °C. The NaCl concentrations used were in the range 0–20% (w/v). The pH was adjusted by using HCl and NaOH, and was in the range 4·5–12·0. Cultures were incubated on a rotary shaker at 160 r.p.m. for 24–36 h at 28 °C. The utilization of various organic substrates (0·1%, w/v; listed in Table 3) as sole carbon sources was determined using BM broth medium (Baumann *et al.*, 1972).

Eleven strains isolated from the degraded thallus of the brown alga *Fucus evanescens* had all of the phenotypic characteristics of the genus *Pseudoalteromonas*. They were Gram-negative, aerobic, oxidase- and catalase-positive, rod-shaped bacteria, 0·7–0·9 μm in diameter and 1·0–1·2 μm long with single polar flagella. Colonies were uniformly round, 2–3 mm in diameter, circular, regular, convex, translucent and smooth after incubation for 48 h on medium B. No diffusible pigment was produced in the medium. The bacteria did not form endospores, did not accumulate poly-*β*-hydroxybutyrate as an intracellular reserve product, did not have an arginine dihydrolase, were oxidase- and catalase-positive and required seawater or 0·5–15% NaCl for growth (the optimum being 1–3% NaCl). The temperature range for growth was 4–37 °C, optimum growth being at 28–30 °C. No growth was detected at 42 °C. The pH range for growth was 6·0–10·0, optimum growth being at pH 7·5–8·0. The bacteria were haemolytic and decomposed gelatin, casein, alginate, Tween 80, DNA and chitin. Agar, carrageenan and starch were not hydrolysed. We report elsewhere that the novel species synthesizes a number of glycoside hydrolases (fucoidanases, laminaranases, alginases, agarases, pullulanases, *β*-glucosidases, *β*-galactosidases, *β*-*N*-acetylglucosaminidases and *β*-xylosidases) (Ivanova *et al.*, 2002b).

Analysis of the fatty acid methyl ethers was performed by GLC as described by Svetashev *et al.* (1995). The cellular fatty acid composition of the strains isolated from the algae is shown in Table 1. The fatty acid patterns were very similar to those found for other *Pseudoalteromonas* species (Ivanova *et al.*, 2000b). The levels of the main fatty acid methyl ethers were 12·3–15·5% for 16:0, 38–46% for 16:1ω7, 8·2–13·6% for 17:1ω8 and 4–7·5% for 18:1ω7 and were very similar to those found for other *Pseudoalteromonas* species (Ivanova *et al.*, 2000b).

Table 1. Cellular fatty acid composition of *P. issachenkonii* sp. nov.

Values are percentages of the total fatty acids.

Fatty acid	KMM 3549^{T}	Ten other strains (range)
11:0	0	0·1–0·2
12:0	1·8	1·3–1·9
12:1	0·5	0·3–0·5
13:0	0·2	0·3–0·4
13:1	0·2	0·2–0·6
14:0	1·4	1·3–1·5
14:1ω7	1·3	0·9–1·2
a15:0	0·3	0·1–0·2
15:0	3·8	3·6–6·4
15:1ω8	5·0	4·9–7·9
15:1ω6	0	0·4–0·6
i16:0	0·8	0·8–1·0
16:0	15·1	12·3–15·5
16:1ω7	45·6	38·0–46·0
16:1ω5	0·1	0·2
i17:0	0·6	0·6–0·9
a17:0	0	0·19
12:0-3OH	0·4	0·2–0·4
17:0	2·4	2·0–4·0
17:1ω8	8·2	8·9–13·6
17:1ω6	0·8	0·8–1·0
18:0	0·4	0·3–0·6
18:1ω9	0·1	0·1
18:1ω7	6·6	4·1–7·5
19:1	0·2	0·2–0·3

DNA was isolated according to the method of Marmur (1961) and the G+C content of DNA of strains KMM 3549^{T} and KMM 3558 (determined using the thermal denaturation method of Marmur & Doty, 1962) was 48·4–48·9 mol%. For DNA–DNA hybridization experiments, *Pseudoalteromonas antarctica* CECT 4664^{T}, *Pseudoalteromonas atlantica* IAM 12927^{T}, *Pseudoalteromonas aurantia* DSM 6057^{T}, *Pseudoalteromonas carrageenovora* IAM 12662^{T}, *Pseudoalteromonas citrea* ATCC 29719^{T}, *Pseudoalteromonas distincta* KMM 638^{T}, *Pseudoalteromonas elyakovii* KMM 162^{T}, *Pseudoalteromonas espejiana* IAM 12640^{T}, *Pseudoalteromonas haloplanktis* ATCC 14393^{T}, *Pseudoalteromonas luteoviolacea* NCIMB 1893^{T}, *Pseudoalteromonas peptidolytica* MBIC 1416^{T}, *Pseudoalteromonas piscicida* ATCC 15057^{T}, *Pseudoalteromonas prydzensis* ACAM 620^{T}, *Pseudoalteromonas nigrifaciens* IAM 13010^{T}, *Pseudoalteromonas rubra* ATCC 29570^{T}, *Pseudoalteromonas tetraodonis* IAM 14160^{T} and *Pseudoalteromonas tunicata* CCUG 26757^{T} were obtained from the ATCC (Manassas, VA,

Table 2. DNA relatedness among *Pseudoalteromonas* strains tested

Organism	G + C content (mol %)	Hybridization with DNA from *P. issachenkonii* KMM 3549^T (%)
P. issachenkonii KMM 3558	42·9	91
P. antarctica CECT 4664^T	42·3	43
P. atlantica IAM 12927^T	42·1	54
P. aurantia DSM 6057^T	44·1	48
P. carrageenovora IAM 12662^T	38·9	48
P. citrea ATCC 29719^T	42·1	32
P. distincta KMM 638^T	43·6	30
P. elyakovii KMM 162^T	40·1	33
P. espejiana IAM 12640^T	42·7	38
P. maricaloris KMM 636^T	39·0	20
P. haloplanktis IAM 12915^T	40·2	42
P. luteoviolacea NCIMB 1893^T	42·0	30
P. peptidolytica MBIC 1416^T	37·9	21
P. piscicida IAM 12932^T	42·7	40
P. prydzensis ACAM 620^T	39·2	36
P. nigrifaciens IAM 13010^T	41·7	28
P. rubra ATCC 29570^T	39·0	30
P. ruthenica KMM 300^T	48·4	25
P. tetraodonis IAM 14160^T	42·1	50
P. tunicata CCUG 26757^T	42·3	31
P. undina NCIMB 2128^T	42·2	43

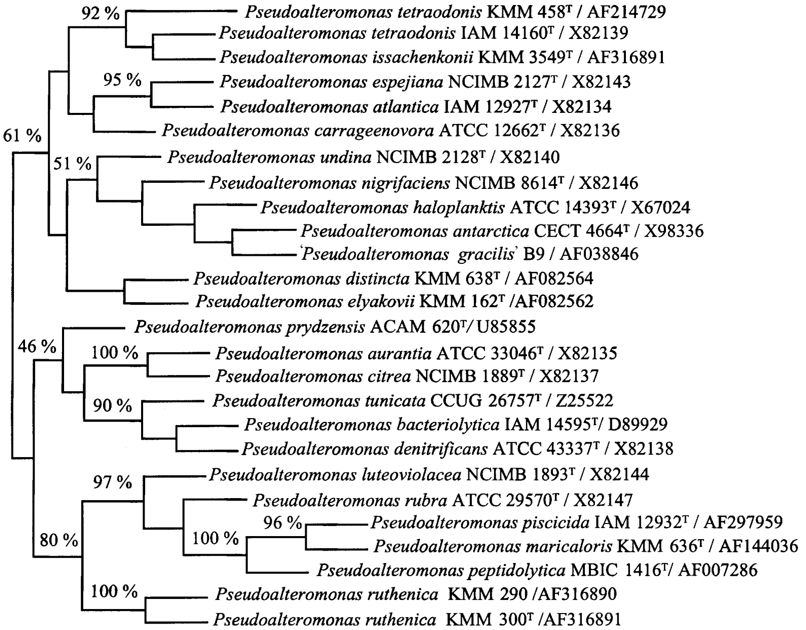

Fig. 1. Phylogenetic position of isolates of *Pseudoalteromonas issachenkonii* KMM 3549^T within the genus *Pseudoalteromonas*. The unrooted tree was obtained using a bioNJ algorithm, Kimura's two-parameter correction for distance calculations and 500 replications in a bootstrap analysis. Bootstrap percentages are indicated only for those branches that were also found in maximum-likelihood ($P < 0·01$) and parsimony (most parsimonious tree) analyses.

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, J. Bowman and C. Holmström. Reference strains were routinely cultured on marine agar 2216 plates (Difco). DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970). The level of DNA hybridization between two representative strains isolated from the algae was 91 %. Therefore, these bacteria were assigned genotypically to a single species. The genetic similarity of strains KMM 3549^T and KMM 3558 to the type strains listed in Table 2 ranged from 27 to 54 %. On the basis of generally accepted criteria for the definition of genomic species

Table 3. Phenotypic characteristics that distinguish *P. issachenkonii* sp. nov. from other non-pigmented *Pseudoalteromonas* species

Strains: 1, *P. antarctica* CECT 4664^{T}; 2, *P. atlantica* IAM 12927^{T}; 3, *P. carrageenovora* IAM 12662^{T}; 4, *P. distincta* KMM 638^{T}; 5, *P. elyakovii* KMM 162^{T}; 6, *P. espejiana* IAM 12640^{T}; 7, *P. haloplanktis* IAM 12915^{T}; 8, *P. nigrifaciens* IAM 13010^{T}; 9, *P. prydzensis* ACAM 620^{T}; 10, *P. tetraodonis* IAM 14160^{T}; 11, *P. undina* NCIMB 2128^{T}. All of the strains studied were polarly flagellated bacteria, required sodium ions for growth, were positive for oxidase and catalase and for the hydrolysis of gelatin and Tween 80, grew at 1–6% NaCl, utilized D-glucose, did not utilize D-sorbitol, γ-aminobutyrate, α-ketoglutarate, D-glucosamine or fucoidan, were susceptible to gentamicin, polymyxin and tetracycline and were not susceptible to benzyl-penicillin. +, Positive; −, negative; V, strain variation; ND, no data available.

Characteristic	*P. issachenkonii* sp. nov.		1	2	3	4	5	6	7	8	9	10	11
	KMM 3549^{T}	Ten other strains*											
Melanin-like pigments	−	0	−	−	−	+	−	−	−	+	−	−	−
Growth at:													
4 °C	+	10	+	−	+	−	+	+	−	+	+	+	+
37 °C	+	10	−	−	+	−	+	+	V	−	−	−	−
Growth in NaCl at:													
8–10%	+	10	+	−	+	+	+	+	+	+	+	+	+
12%	+	10	−	−	+	ND	−	+	+	−	+	+	+
15%	+	10	−	−	−	−	−	−	−	−	+	−	−
Production of:													
Amylase	−	0	−	+	−	−	+	+	V	+	+	−	+
Alginase	+	10	ND	+	+	+	+	+	−	+	−	ND	−
Agarase	−	0	−	+	−	−	−	−	−	−	−	−	−
κ-Carrageenase	−	0	−	−	+	−	−	−	−	−	ND	−	−
Chitinase	+	8	−	−	−	−	−	−	V	−	+	−	+
DNase	+	10	+	+	+	+	+	+	+	+	−	+	+
Utilization of:													
D-Mannose	−	0	+	+	−	+	+	−	+	+	+	−	−
D-Galactose	+	10	−	+	−	−	+	+	V	+	V	+	−
D-Fructose	+	10	+	+	+	+	+	V	+	+	−	−	−
Sucrose, maltose	+	10	+	+	+	−	+	+	+	+	+	+	+
Melibiose, lactose	+	10	+	+	+	+	−	+	−	+	−	−	−
D-Gluconate	−	0	−	−	−	−	−	−	−	+	+	−	−
N-Acetylglucosamine	−	0	−	−	−	−	−	−	ND	ND	+	ND	+
Succinate, D-mannitol	+	10	+	+	+	−	+	−/+	V	+/−	+	−	+/−
Fumarate	+	10	+	+	+	−	+	−	ND	−	+	ND	+
Citrate	+	10	+	−	+	+	−	+	+	+	+	+	−
m-Erythritol	ND	ND	ND	−	ND	−	−	−	+	ND	ND	−	−
Glycerol	−	0	ND	+	+	+	−	+	−	+	+	−	−
L-Tyrosine	ND	ND	ND	−	+	−	−	+	+	+	V	+	+
Xylose	−	0	ND	−	−	−	+	+	−	+	−	−	−
Trehalose	−	0	ND	+	−	−	−	+	−	+	+	−	+
Acetate	+	10	+	+	+	+	−	+	+	+	+	+	+
Pyruvate	+	10	+	+	+	+	+	+	+	+	+	+	−
Susceptibility to:													
Kanamycin (30 μg)	−	2	+	+	−	ND	−	−	+	−	ND	−	+
Oleandomycin (15 μg)	+	10	−	+	+	ND	+	+	+	−	ND	−	+
Ampicillin (10 μg)	+	10	−	−	−	ND	+	−	+	+	ND	+	+
Carbenicillin (15 μg)	+	10	+	−	+	ND	+	+	+	+	ND	+	+
Streptomycin (10 μg)	+	9	−	+	+	ND	−	−	+	+	ND	+	+
G+C content (mol%)	43	42–43	42·3	42·1	42·0	43·6	40	42·7	43·3	41·7	39	42·1	42·2

* Number of strains positive (or range, for G+C content).

(Wayne *et al.*, 1987), the strains isolated from the brown alga *Fucus evanescens* can be assigned to a separate species.

Bacterial DNA for PCR was prepared using the Promega Wizard genomic DNA extraction kit according to the instruction manual. DNA templates

(100 ng) 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, annealing step at 55 °C for 60 s and 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. PCR products were purified using the Promega Wizard PCR preps DNA-purification kit and were sequenced directly by using a *Taq* FS dye terminator sequencing kit (ABI) according to the protocol recommended by the manufacturer. DNA sequencing was performed with an Applied Biosystems model 373S automated sequencer. Nine 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 previously 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 distance matrix was calculated according to Kimura's two-parameter correction. Bootstraps were done using 500 replications, bioNJ and Kimura's two-parameter corrections. BioNJ was performed according to Gascuel (1997), and the 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). Phylogenetic trees were drawn using NJPLOT (Perrière & Gouy, 1996) and CLARIS DRAW software for Apple Macintosh computers.

The domains used to construct the phylogenetic trees were regions of the small-subunit rDNA sequences that were available for all sequences and excluding positions likely to show homoplasy. For Fig. 1, only those sequences of the genus *Pseudoalteromonas* for which almost the entire 16S rDNA sequence, corresponding to positions 45–1351 of the KMM 3549^T sequence, was included in the analysis were retained. See Ivanova *et al*. (2002a) for further consideration of the treeing analysis.

Analysis of the 16S rDNA gene sequence revealed that the novel bacteria are members of the γ-subclass of the *Proteobacteria*. These data indicate clearly that KMM 3549^T represents a lineage closely related to other species of *Pseudoalteromonas*; this is consistent with the phenotypic characteristics. Strain KMM 3549^T forms a robust clade with *P. tetraodonis* (Fig. 1). However, a definitive allocation of this strain to a separate species is supported by the data obtained using DNA–DNA hybridization experiments.

Bacteria of the novel species can be easily distinguished from other non-pigmented species by virtue of a number of phenotypic traits (Table 3). For example, bacteria of three species of the genus *Pseudoalteromonas* (including *Pseudoalteromonas undina*, *P. prydzensis* and some strains of *P. haloplanktis*) are able to produce chitinase. In contrast to these bacteria, strains of the novel species are halophilic (they grow in NaCl concentrations up to 15%), bacteriolytic, haemolytic, produce alginase and utilize lactose. On the basis of the data obtained, we propose that the strains isolated from the degraded thallus of the brown algae *Fucus evanescens* should be named *Pseudoalteromonas issachenkonii* sp. nov.

Description of *Pseudoalteromonas issachenkonii* sp. nov.

Pseudoalteromonas issachenkonii (i.ssa′chen.ko.ni.i. N.L. gen. n. *issachenkonii* in honour of Boris Issachenko, the Russian pioneer of marine microbiology).

The cells are rod-shaped, single, approximately 0·7–0·9 μm in diameter and motile by means of single polar flagella. Gram-negative. Aerobic. Chemorganotrophic with respiratory metabolism. Cells do not form endospores. They do not accumulate poly-β-hydroxybutyrate as an intracellular reserve product and they do not have an arginine dihydrolase system. Oxidase- and catalase-positive. Requires NaCl or seawater for growth. Growth occurs in media with 0·5–15% NaCl. The temperature for growth ranges from 4 to 37 °C with optimum growth at 28–30 °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. Bacteriolytic and haemolytic. Gelatin, casein, chitin, alginate, DNA and Tween 80 are decomposed. Agar-agar, carrageenan and starch are not hydrolysed. D-Galactose, D-fructose, D-mannitol, sucrose, maltose, melibiose, lactose, fumarate, citrate and pyruvate are utilized. Susceptible to gentamicin, polymyxin and tetracycline but not benzyl-penicillin. The main cellular fatty acids are $16:1\omega7$, $16:0$, $17:1\omega8$ and $18:1\omega7$ (approx. 80% of the total).

Isolated from the brown alga *Fucus evanescens* collected from the Kraternaya Bight of the Kurile Islands in the Pacific Ocean. The G+C content of the DNA is 42·9–43·3 mol%. The type strain is KMM 3549^T (= LMG 19697^T = CIP 106858^T).

Acknowledgements

This study was supported by funds from the Russian Foundation for Basic Research (grant no. 99-04-48017) and by a grant from the State Committee for Science and Technologies of the Russian Federation (no. 00-03-19).

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