Note

Pseudoalteromonas agarivorans sp. nov., a novel marine agarolytic bacterium

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The phenotypic, genomic and phylogenetic characteristics of four aerobic, Gram-negative, non-fermentative, motile, non-pigmented, agarolytic *Pseudoalteromonas*-like bacteria, isolated from marine environments, have been investigated. These bacteria share DNA–DNA similarities above 86 %. Comparative 16S rDNA sequence analysis of strain KMM 255^T revealed its membership of the genus *Pseudoalteromonas*; it shares 99.9 % sequence similarity with *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas atlantica* and *Pseudoalteromonas espejiana*. DNA–DNA reassociation levels obtained for strain KMM 255^T and type strains of these four species and other *Pseudoalteromonas* species were below 45 %. The marine isolates differed from known species of the genus by the fact that the cells are motile by means of a single flagellum or two to four polar unsheathed flagella and by an inability to utilize most organic compounds. On the basis of phenotypic, DNA–DNA hybridization and phylogenetic data, it is concluded that the isolates represent a novel species within the genus *Pseudoalteromonas*, for which the name *Pseudoalteromonas agarivorans* sp. nov. is proposed. The type strain is strain KMM 255^T (= DSM 14585^T).

Aerobic, Gram-negative, non-fermentative, heterotrophic, *Pseudomonas*-like bacteria able to decompose algal polysaccharides such as agar, alginate and carrageenan have been isolated from marine environments in earlier studies (ZoBell & Upham, 1944; Humm, 1946; Yaphe & Baxter, 1955; Yaphe, 1957, 1962; Akagawa-Matsushita *et al.*, 1992). The genus *Alteromonas*, originally described by Baumann *et al.* (1972) for marine aerobic, Gram-negative, non-fermentative, polarly flagellated bacteria with a DNA G+C content of 37–50 mol%, was later divided into two genera on the basis of phylogenetic analysis: *Alteromonas*, comprising the single species *Alteromonas macleodii*, and *Pseudoalteromonas*, now including 22 species either previously belonging to *Alteromonas* (Gauthier *et al.*, 1995) or described subsequently (Bozal *et al.*, 1997; Bowman, 1998; Holmström *et al.*,

1998; Ivanova *et al.*, 2000a, 2001; Romanenko *et al.*, 1995; Sawabe *et al.*, 1998, 2000; Venkateswaran & Dohmoto, 2000). The present study was performed to clarify the taxonomic status of four *Pseudoalteromonas*-like, agarolytic, marine isolates belonging to the genus *Pseudoalteromonas*.

Four strains were isolated from sea-water samples and ascidian specimens collected from coastal and open oceanic waters during 1985–1992. Sampling, strain isolation and cultivation procedures have been described previously (Romanenko *et al.*, 1994a, b). The strains, designated KMM 255^T, KMM 232, KMM 254 and KMM 644, have been deposited in Collection of Marine Micro-organisms (KMM), Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia. The bacteria were maintained on Marine 2216 agar (MA; Difco) plates at 15 °C and stored at –80 °C in 30 % (v/v) glycerol. For reference type strains and their origins, see Table 3. The novel strains were grown routinely at 28 °C on MA or Marine 2216 broth (MB; Difco) and nutrient agar medium, containing natural sea water (SWM).

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The GenBank accession number for the 16S rDNA sequence of strain KMM 255^T is AJ417594.

For negative-staining, samples were fixed in 3 % glutaraldehyde/5 % formaldehyde in PBS (100 mM phosphate, 150 mM NaCl, pH 6.9) for 1 h on ice. After being washed in TE buffer (20 mM Tris/HCl, 1 mM EDTA, pH 7.0), samples were adsorbed onto a thin carbon film, washed in TE buffer and negatively stained with 4 % uranyl acetate. After air-drying, samples were examined in a Zeiss EM910 transmission electron microscope at an acceleration voltage of 80 kV. Standard phenotypic characterization of the strains was performed using the methods described by Baumann *et al.* (1984), Gauthier & Breittmayer (1992) and Smibert & Krieg (1994). Hydrolysis of κ -carrageenan was determined as described by Yaphe & Baxter (1955). Growth at different temperatures (4–40 °C) and pH values (5.0–10.0) was tested by using MB. The sodium-ion requirement and tolerance of NaCl were determined in SWM medium, prepared on the artificial sea-water base supplemented with the appropriate amount of NaCl, ranging from 0 to 15 % (w/v). Acid production from sugars (with 1 %, w/v, of the test sugar) was determined using the method of Leifson (1963). Additional biochemical tests were carried out using API 20NE test kits (bioMérieux) as described by the manufacturer, with the exception that strains were suspended in 3 % (w/v) NaCl solution. Isolates were characterized physiologically by the Biolog GN MicroPlate method. The strains were grown for 24 h at 28 °C on MA 2216 medium and the microtitre plates were inoculated with cells suspended in 2.5 % (w/v) NaCl. Results were read automatically with a spectrophotometer after 24 and 48 h incubation at 28 °C. Antibiotic sensitivity was tested on MA plates by using the agar diffusion method involving discs impregnated with the following 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;

neomycin, 15 µg; oxacillin, 20 µg; and O/129, 150 µg. Cell morphology and motility were examined by transmission electron and phase-contrast microscopy on bacterial cells grown for 24 h in MB. Analysis of methylated fatty acids and lipids was performed as described by Svetashev *et al.* (1995) and Ivanova *et al.* (2000b). Isolation of DNA and determination of the base composition were performed according to Marmur (1961), Marmur & Doty (1962) and Owen *et al.* (1969). DNA–DNA relatedness was measured spectrophotometrically (De Ley *et al.*, 1970) under optimal reassociation conditions in $2 \times$ SSC at 64 °C. 16S rRNA gene sequences were determined and compared as described by Rainey *et al.* (1996). Previously published 16S rRNA gene sequences were obtained from the EMBL/GenBank databases. The analysis of sequences used to generate the dendrogram in Fig. 2 was based on 1391 bases, containing 826 polymorphic sites. Accession numbers are indicated on the dendrogram.

The four isolates, strains KMM 255^T, KMM 232, KMM 644 and KMM 254, were aerobic, Gram-negative, non-fermentative, oxidase- and catalase-positive, rod-shaped bacteria, motile by means of unsheathed, single, polar flagella. In addition, cells with a tuft of two to four polar flagella were observed (Fig. 1). The strains required Na⁺ ions for growth and grew in 1–9 % NaCl. They did not grow at 4–5 or 40 °C; they grew slowly at 6–7 °C and had a temperature optimum ranging between 25 and 28 °C. On MA medium, the bacteria formed whitish or yellowish S- and R-colonies, depressed into the agar. The phenotypic characteristics of the isolates and the phylogenetically closest relatives are summarized in Table 1. The novel isolates were characterized by the hydrolysis of agar, alginate, carrageenan and other polymeric molecules and by the inability to produce acid from D-glucose according to Leifson's method. Weak D-glucose utilization was observed in the API 20NE test. The strains

Fig. 1. Transmission electron micrographs showing general morphology of negatively stained cells of strains KMM 232 (a, b) and KMM 255^T (c–e), displaying cells with unsheathed, single, polar flagella and cells bearing a tuft of two to four polar flagella. Bars, 0.5 µm.

Table 1. Phenotypic characteristics of *Pseudoalteromonas agarivorans* sp. nov. and phylogenetically related *Pseudoalteromonas* species

Strains: 1, *P. agarivorans* sp. nov. KMM 255^T, KMM 232, KMM 254 and KMM 644 (reactions in parentheses refer to strain KMM 255^T); 2, *P. distincta* KMM 638^T; 3, *P. elyakovii* KMM 162^T (data from Sawabe *et al.*, 2000); 4, *P. atlantica* IAM 12376^T (Akagawa-Matsushita *et al.*, 1992); 5, *P. carrageenovora* IAM 12622^T (Akagawa-Matsushita *et al.*, 1992); 6, *P. espejiana* IAM 12640^T (Chan *et al.*, 1978). +, Positive; –, negative; v, variable between strains; w, weak; ND, not determined. Acid production was determined according to Leifson (1963). All strains were positive for the following tests: sodium-ion requirement for growth, growth at 25–28 °C, motility by a single polar flagellum, oxidase, catalase, production of lipase, caseinase, DNase, gelatin liquefaction and sensitivity to streptomycin (30 µg) and polymyxin (300 U); all strains were negative for growth at 40 °C, indole production, nitrate reduction, denitrification, arginine dihydrolase, chitin hydrolysis, L-arabinose utilization and sensitivity to lincomycin, benzylpenicillin (10 U) and O/129 (150 µg).

Characteristic	1	2	3	4	5	6
Pigmentation	–	+	–	–	–	–
Cells with > 1 polar flagellum	+	–	–	–	–	–
Lateral flagella	–	+	–	–	–	–
Growth at 4–5 °C	–	+	+	+	+	–
Hydrolysis of:						
Starch	+	–	+	+	–	+
Alginate	+	–	+	+	+	+
Agar	+	–	–	+	–	–
Carrageenan	+	–	–	v	+	–
Acid production from:						
D-Glucose	–	–	+	+	+	+
Maltose	v (+)	–	+	+	+	+
D-Mannitol	–	–	+	+	+	+
Oxidation of (API 20NE):						
D-Glucose	w	–	+	+	+	+
Mannitol	+	–	+	+	+	+
Maltose	v (+)	–	+	+	+	+
Caprate	–	ND	ND	+	+	+
Adipate	–	ND	ND	–	–	–
L-Malate	–	–	–	–	+	–
Phenylacetate	–	–	ND	v	–	–
Oxidation of (Biolog):						
D-Fructose	–	–	+	+	+	v
Mannose	–	–	+	+	–	v
N-Acetylglucosamine	–	–	v	–	–	–
Sucrose	–	–	+	+	+	+
Glycerol	–	–	v	+	+	–
Citrate	–	–	v	+	+	+
Propionate	–	ND	+	+	+	+
Butyrate	–	ND	+	+	–	+
Glycogen	+	ND	ND	+	–	ND
Sensitivity to (µg per disc):*						
Ampicillin (10)	+	+	+	+	+	–
Gentamicin (10)	+	+	+	–	+	+
Kanamycin (30)	+	+	–	–	–	+
Carbenicillin (25)	v (–)	–	+	–	+	–
Oleandomycin (15)	v (+)	+	+	+	–	+
Tetracycline (30)	–	–	+	+	–	+
Neomycin (15)	v (–)	–	–	–	–	ND
Oxacillin (20)	–	–	–	ND	–	ND
DNA G + C content (mol%)	42.2–(43.8)	43.8	38.5–38.9	40.6–41.7	39.5	43–44

*Data for type strains of *P. atlantica*, *P. carrageenovora* and *P. espejiana* were determined in this study.

utilized a small number of substrates and did not oxidize D-glucose or other carbohydrates, carboxylic acids, amino acids or other compounds when tested with the Biolog identification system. Table 1 includes the differentiating phenotypic properties of those type strains with which strain KMM255^T shares the closest phylogenetic relationships. Comparison with type strains of other non-pigmented, phylogenetically less closely related species, i.e. *Pseudoalteromonas antarctica* CECT 4664^T, *Pseudoalteromonas haloplanktis* IAM 12915^T, *Pseudoalteromonas nigrificiens* IAM 13010^T, *Pseudoalteromonas prydzensis* ACAM 620^T, *Pseudoalteromonas tetraodonis* IAM 14160^T and *Pseudoalteromonas undina* NCIMB 2128^T (Ivanova *et al.*, 2002), clearly indicated significant phenotypic differences in the formation of melanin-like pigments, the production of hydrolytic enzymes and the utilization of sugars and acids (not shown).

All strains exhibited similar whole-cell phospholipid and fatty acid profiles (Table 2). The predominant fatty acids were 16:0 (20.9–30.4%) and 16:1 ω 7c (38.9–44.0%). The main phospholipids were phosphatidylethanolamine and phosphatidylglycerol (respectively 66–73 and 23–30%); these results are in accordance with those found for other *Pseudoalteromonas* species (Svetashev *et al.*, 1995; Bozal *et al.*, 1997; Ivanova *et al.*, 2000b). Diphosphatidylglycerol (0.9–1.3%) and bis-phosphatidic acid (2.0–2.7%) were minor components. It has been noted previously that, except in the case of *P. nigrificiens* (1.4%), diphosphatidylglycerol is not detected in *Pseudoalteromonas* type strains (Frolova *et al.*, 2000).

The DNA G + C content was 42.2–43.8 mol%. DNA–DNA binding between the four isolates ranged between 88 and 97% (Table 3). Comparison of the almost complete 16S rDNA sequence of strain KMM 255^T with the corresponding sequences of type strains of *Pseudoalteromonas* species revealed 99.9% sequence similarity to *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas atlantica* and *Pseudoalteromonas espejiana*. Slightly lower values (98.5–99.8%) were found with the other species indicated in Fig. 2, including the recently described species *Pseudoalteromonas issachenkonii* (99.6% similarity) (Ivanova *et al.*, 2002). DNA–DNA similarities determined for strain KMM 255^T and a range of closely and more distantly related type strains of species of *Pseudoalteromonas* were significantly below 60% (19–45%), indicating that the four isolates belong to a separate genospecies (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). The type strains of *P. prydzensis* and *P. antarctica* were not included in the DNA binding studies because the 16S rRNA gene sequence similarities were distinctly lower (respectively 97.2 and 98.5% similarity) than the gene sequence similarities determined for the other non-pigmented *Pseudoalteromonas* species. *P. issachenkonii* was not included because of its recent description. However, as DNA–DNA similarities determined for the type strain KMM 3549^T and the type strains of non-pigmented species, including strains that are highly

Table 2. Cellular fatty acid and phospholipid composition of strains of *P. agarivorans* sp. nov.

Values are percentages of total fatty acids/phospholipids. Major fatty acids (>10%) are shown in bold. Phospholipids are abbreviated as: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; BA, bis-phosphatidic acid.

Component	KMM 255 ^T	KMM 232	KMM 644	KMM 254	Mean
Fatty acid					
11:0	0	0	0	0.2	0.1
12:0	1.8	2.1	1.9	1.1	1.7
12:1	0.4	0.6	0.5	0.2	0.4
13:0i	0	0	0.1	0	0.1
13:0	0.1	0	0.1	0.2	0.1
13:1	0	0.1	0.2	0.4	0.3
14:0	1.1	1.0	0.8	0.8	0.9
14:1 ω 7c	0.4	0.3	0.5	0.3	0.4
14:1 ω 5c	0	0	0.4	0	0.1
15:0i	0.3	0	0	0	0.1
15:0a	0	0.2	0.7	0.2	0.3
15:0	2.7	1.3	2.7	2.6	2.3
15:1 ω 8c	1.2	0.4	1.5	0.7	0.9
15:1 ω 6c	0.2	0	0.2	0.2	0.2
16:0i	1.3	0.9	2.2	0.7	1.3
16:0	27.3	30.4	20.9	23.1	25.4
16:1ω7c	42.4	42.3	44.0	39.2	42.0
16:1 ω 5c	0	0.2	0.2	0.1	0.1
17:0i	0.4	0.4	1.3	0.3	0.6
17:0a	1.9	1.3	3.2	1.0	1.9
17:0	3.4	2.9	2.7	4.6	3.4
17:1 ω 8c	5.9	4.5	6.3	10.6	6.8
17:1 ω 6c	0.5	0.4	0.5	0.7	0.5
18:0i	0.2	0.2	0.2	0.2	0.2
18:0	0.6	0.7	0.6	0.7	0.7
18:1 ω 11c	0.3	0.4	0.3	0.2	0.3
18:1 ω 9c	0.2	0.2	0.2	0.3	0.2
18:1 ω 8c	7.1	8.6	7.4	10.2	8.3
19:1	0.2	0.3	0.1	0.6	0.4
Phospholipid					
PE	72.2	73.1	69.0	66.4	70.2
PG	24.5	23.1	28.0	30.1	26.4
DPG	0.9	1.1	1.0	1.3	1.1
BA	2.3	2.7	2.0	2.2	2.3

related to the novel genospecies in terms of 16S rRNA gene sequence similarity, were well below 54%, we are confident that the type strains of the novel genospecies and *P. issachenkonii* are not members of the same species. Morphological, phenotypic and genomic characteristics suggested that the novel isolates should be assigned to a novel species of *Pseudoalteromonas*. The moderate DNA–DNA similarities (Table 3) between species correlated with the presence of a combination of distinct metabolic differences determined for the phylogenetically closest relatives (Table 1).

Table 3. DNA–DNA binding of the novel isolates, *Pseudoalteromonas* species and *A. macleodii*

Strain	G + C content (mol%)	DNA–DNA binding (%) to:		
		1	7	12
<i>P. agarivorans</i> sp. nov.				
1. KMM 255 ^T	43.8	100	45	31
2. KMM 232	42.2	95	51	NT
3. KMM 254	42.7	86	48	29
4. KMM 644	42.5	88	48	29
5. <i>P. elyakovii</i> KMM 162 ^T	39.5	29	NT	54
6. <i>P. espejiana</i> IAM 12640 ^T	43.2	41	54	33
7. <i>P. atlantica</i> IAM 12376 ^T	40.5	45	100	NT
8. <i>P. carrageenovora</i> IAM 12622 ^T	41.3	26	31	NT
9. <i>P. haloplanktis</i> ATCC 14393 ^T	42.7	29	32	30
10. <i>P. undina</i> IAM 12922 ^T	41.1	34	NT	14
11. <i>P. tetraodonis</i> IAM 14160 ^T	42.1	33	NT	17
12. <i>P. distincta</i> KMM 638 ^T	43.8	31	NT	100
13. <i>P. nigrifaciens</i> IAM 13010 ^T	41.7	19	31	31
14. <i>A. macleodii</i> IAM 12920 ^T	45.3	8	9	12

NT, Not tested.

Description of *Pseudoalteromonas agarivorans* sp. nov.

Pseudoalteromonas agarivorans (a.gar.i.vor'ans. N.L. n. *agarum* agar-agar, algal polysaccharide; L. v. *vorare* to devour, to digest; N.L. adj. *agarivorans* agar-devouring).

Gram-negative, strictly aerobic, rod-shaped cells, 0.8–0.9 µm in diameter and 2.5–3.8 µm long, motile by a single, polar, unsheathed flagellum. Cells with two to four polar flagella are observed. Does not form endospores. Oxidase- and catalase-positive. Sodium ions are essential for growth. Mesophilic and neutrophilic chemo-organotroph.

Grows at 7–35 °C, with optimal growth at 20–28 °C; no growth at 4 or 40 °C. Non-pigmented, whitish or pale yellowish S- and R-colonies, depressed into the agar. Positive for lipase, caseinase, DNase, gelatinase and β-galactosidase. Some strains produce acid from sucrose, maltose and melibiose. ONPG test is positive. As determined by using the Biolog identification system, Tweens 80 and 40, cyclo-dextran, dextran and glycogen are utilized; acetic and succinic acids are weakly utilized. In addition to metabolic properties used in the differentiation of the species from other *Pseudoalteromonas* strains indicated in Table 1, negative for the utilization of L-arabinose and gluconate (according to API 20NE), negative for urease, indole production and aesculin hydrolysis and shows no acid formation from L-arabinose, lactose, D-mannose, D-galactose, D-xylose, rhamnose or glycerol. D-Glucose utilization is weak or slow in the API test and negative in the Biolog GN identification system. As determined by the Biolog GN panel, does not utilize cellobiose, L-fucose, D-galactose, D-lactose, D-melibiose, D-raffinose, L-rhamnose, gluconate, *m*-inositol, erythritol, adonitol, methylpyruvate, mono-methylsuccinate, β-hydroxybutyric acid, acetic acid, *cis*-aconitic acid, citric acid, formic acid, L-lactic acid, propionic acid, succinic acid, bromosuccinic acid, L-aspartic acid, L-glutamic acid, L-serine, L-alanine, L-proline, L-threonine, D-glucuronic acid, α-ketoglutaric acid, α-ketovaleric acid, malonic acid, L-alanylglycine, L-asparagine, hydroxy-L-proline, acetate, DL-lactate, L-leucine, L-histidine, L-ornithine, L-phenylalanine, D-serine, DL-carnitine, γ-aminobutyric acid, uridine, thymidine, putrescine, glucose 1-phosphate or glucose 6-phosphate. Resistant to lincomycin (15 µg), benzylpenicillin (10 U), oxacillin (20 µg), tetracycline (30 µg) and O/129 (150 µg). Major fatty acids are hexadecanoic acid (16:0) and hexadecenoic acid (16:1ω7c).

Fig. 2. 16S rDNA dendrogram showing the position of strain KMM 255^T among some phylogenetically closely related *Pseudoalteromonas* species. Bar, 2 inferred substitutions per 100 nucleotides. Numbers at branching points refer to bootstrap values (500 resamplings).

Dominant phospholipids are phosphatidylethanolamine and phosphatidylglycerol; bis-phosphatidic acid and diphosphatidylglycerol are minor components. DNA G+C content is 42.2–43.8 mol% (thermal denaturation). The strains were isolated from sea water deep in the Pacific Ocean and from the marine ascidians *Halocynthia aurantium*, *Polysyncraton* sp. and *Clarelina molucensis*. The type strain is strain KMM 255^T (=DSM 14585^T).

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