Pseudoalteromonas arctica sp. nov., an aerobic, psychrotolerant, marine bacterium isolated from Spitzbergen

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A novel aerobic, psychrotolerant marine bacterium was isolated at 4 °C from seawater samples collected from Spitzbergen in the Arctic. The strain was a polar-flagellated, Gram-negative bacterium that grew optimally at 10–15 °C and pH 7–8 in media containing 2–3 % NaCl (w/v), using various carbohydrates and organic acids as substrates. The main fatty acid components included 16 : 0 (12.7 % of total fatty acids), straight-chain saturated fatty acid methyl ester (FAME) and 16 : 1 ω 7c (40.2 %) monounsaturated FAME. Phylogenetic analysis revealed a close relationship (99 % 16S rRNA gene sequence similarity) between the novel isolate and *Pseudoalteromonas elyakovii* KMM 162^T and some other species of the genus *Pseudoalteromonas*. The DNA G + C content of the novel strain was 39 mol%. DNA–DNA hybridization showed only 47.6 % DNA–DNA relatedness with *P. elyakovii* KMM 162^T, 44.2 % with *Pseudoalteromonas distincta* KMM 638^T and 22.6 % with *Pseudoalteromonas nigrifaciens* NCIMB 8614^T. Based on phylogenetic and phenotypic characteristics, this isolate represents a novel species of the genus *Pseudoalteromonas* for which the name *Pseudoalteromonas arctica* is proposed; the type strain is A 37-1-2^T (=LMG 23753^T=DSM 18437^T).

Marine *Pseudomonas*-like bacteria comprise several related genera, including *Alteromonas*, *Marinomonas*, *Pseudoalteromonas*, *Moritella*, *Marinobacter*, *Psychrobacter*, *Colwellia*, *Shewanella*, *Halomonas* and several others. Classification of these aerobic heterotrophic micro-organisms from the marine environment remains difficult and time-consuming due to their phenotypic diversity and the absence of robust chemotaxonomic markers. Fortunately, phylogenetic information based on 16S rRNA gene sequences is useful in classifying and identifying micro-organisms belonging to previously poorly defined taxa (Ivanova *et al.*, 2000).

The genus *Pseudoalteromonas*, originally called *Alteromonas*, includes non-pigmented, Gram-negative, heterotrophic, aerobic, polar-flagellated species of marine bacteria that have DNA G + C contents ranging from 38 to 50 mol%. These characteristics differentiate this bacterial group from the previously described genus *Pseudomonas*. Since Baumann *et al.* (1972) proposed the genus *Alteromonas* in 1972, several species have been assigned to this genus. A recent revision of the genera based on phylogenetic analysis (Gauthier *et al.*, 1995) divided the

genus *Alteromonas* into two genera, *Pseudoalteromonas* and *Alteromonas*.

Pseudoalteromonas is one of the largest genera within the class *Gammaproteobacteria* and currently comprises more than 30 species (Ivanova *et al.*, 2004). These bacteria play an important role in marine environments owing to their abundance and high metabolic activities. *Pseudoalteromonas* are highly capable of surviving in nutrient-poor marine environment by adjustment of their biochemical pathways and production of a wide variety of metabolites, including biologically active compounds and enzymes (Ivanova *et al.*, 2003).

In this study, a novel aerobic bacterium was isolated from ice and seawater samples obtained from the area of Spitzbergen in the Arctic. This novel isolate was found to belong to the genus *Pseudoalteromonas*. Taxonomic and physiological analysis of this isolate demonstrated that it represents a novel species of the genus *Pseudoalteromonas*.

Arctic sea ice and seawater samples were collected from Spitzbergen, Norway, and transported to the laboratory at an ambient temperature of 10 °C. A 1 ml aliquot of each of the 50 liquid samples was incubated at 4 °C in a complex marine aerobic liquid medium named EMP-2 (enrichment medium for psychrophiles 2). This medium consisted of a basal medium supplemented with a mixture of different

Abbreviations: FAME, fatty acid methyl ester; AZCL, azurine-crosslinked.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A 37-1-2^T is DQ787199.

carbon sources. The basal medium contained (g l^{-1}): 28.13 g NaCl, 0.77 g KCl, 0.02 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g NH_4Cl , 0.02 g iron ammonium citrate, 0.5 g yeast extract, 1 ml 10-fold concentration trace element solution (from DSMZ medium 141), 1 ml 10-fold concentration vitamin solution (from DSMZ medium 141), 2.3 g KH_2PO_4 and 2.9 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$. Carbon sources were added at the following concentrations (g l^{-1}): 0.5 g sodium acetate, 0.5 g disodium succinate, 0.5 g sodium pyruvate, 0.5 g DL-malate, 0.5 g D-mannitol and 2 g glucose. The final pH of the medium was 7.0. Incubation was carried out at 4 °C for about 4–7 days before growth and colonies were observed on agar plates. Colonies were selected on the basis of morphological differences. For the isolation of a pure culture, serial dilution and plating techniques were applied. The pure isolates were routinely cultivated on EMP-2 agar plates containing 2 % (w/v) agar at 15 °C for 4 days.

The Gram staining test was performed using the Hucker method (Gerhardt *et al.*, 1994). For the sporulation test, cells were grown for up to 6 days in basal medium. The presence of spores and the morphology of the cells were determined by phase-contrast microscopy (Zeiss/Axioskop).

The maximal growth temperature was tested in the complex medium between 4 and 35 °C at pH 7.0. The pH range for growth was determined by testing between pH 2 and 10 at 15 °C. The salt requirement was determined at different NaCl concentrations ranging from 0 to 10 % (w/v), with no change in the other salt component concentrations at pH 7.0 and 15 °C. Growth was measured by determining the OD_{600} .

Substrate utilization was tested on API 20NE strips (bioMérieux), API 20E strips (bioMérieux) and Biolog GN2 microplates. API 20NE and API 20E strips were also used for testing the ability of the novel strain to produce indole (from tryptophan), acetoin and H_2S (from sodium thiosulfate) and to reduce nitrate to nitrite and nitrite to nitrogen. Carbohydrate metabolism was also tested using API 50CH strips (bioMérieux).

The strips and microplates were inoculated with a cell suspension that was grown on complex medium [cells were resuspended in 0.85 % (w/v) NaCl to a final OD_{600} of 1.0] and incubated at 15 °C overnight.

Enzyme formation by the isolated aerobic strain was tested on diffusion agar plates containing the basal medium and 0.1 % (w/v) of one of the following substrates: red pullulan (pullulanase), azo-casein (protease), azurine-crosslinked (AZCL)-pullulan (pullulanase), AZCL-HE-cellulose (cellulase), AZCL-arabinan (arabinase), AZCL-arabinoxylan (arabinoxylanase), AZCL-curdlan (curdalanase), AZCL-amylose (amylase), AZCL-dextran (dextranase), AZCL-galactan (galactanase), AZCL-galactomannan (galactomannanase), AZCL- β -glucan (β -glucanase), AZCL-xylan (xylanase) and AZCL-xyloglucan (xyloglucanase).

Substrate degradation was detected by a clearing zone/colour diffusion halo around the colonies after growth on agar plates at 10 °C for 2–4 days. AZCL polymers were purchased from Megazyme.

Cells of the isolated strain were harvested by centrifugation after growth in 2 l complex medium at 15 °C and pH 7.0 and were used for fatty acid analysis. Fatty acid methyl ester (FAME) analysis was performed according to the method of Kämpfer & Kroppenstedt (1996) using minor modifications of the methods of Miller (1982) and Kuykendall *et al.* (1988). The FAME mixtures were separated using the Sherlock Microbial Identification system (MIS; MIDI), which consisted of an Agilent model 6890N GC fitted with a 5 % phenyl-methyl polysiloxane capillary column (0.2 mm $\times$ 25 m), a flame-ionization detector, an Agilent model 7683A automatic sampler and a Hewlett-Packard computer with the MIDI database. Peaks were automatically integrated and fatty acid names and percentages were calculated using the MIS Standard Software (Microbial ID).

GC parameters were as follows: carrier gas, ultra-high-purity hydrogen; column head pressure, 60 kPa; injection volume, 2 μl ; column split ratio, 100:1; septum purge, 5 ml min^{-1} ; column temperature, 170–270 °C at 5 °C min^{-1} ; injection port temperature, 240 °C; and detector temperature, 300 °C.

For 16S rRNA gene amplification and sequencing, cells were harvested from a 500 μl culture sample by centrifugation and resuspended in 100 μl water. A sample of 1 μl was used as a template for amplification of the 16S rRNA gene. PCR-mediated amplification of the 16S rRNA gene was carried out according to Rainey & Stackebrandt (1993). PCR products were purified using the QIAquick PCR purification kit (Qiagen). Purified PCR products were sequenced directly using the Tag Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems). PCR products were electrophoresed using an Applied Biosystems model 373S DNA sequencer. Both strands of amplification products were sequenced using primers 8F, 518F and 1504R (Buchholz-Cleven *et al.*, 1997). The complete 16S rRNA gene sequence of the isolated strains was determined by the assembly of all sequence products using Vector NTI Advance 9.1 (Invitrogen) software.

BLAST analysis of the 16S rRNA gene sequence was carried out using the NCBI online database to determine the phylogenetic grouping to which the isolated strains were most closely related. Reference sequences utilized in phylogenetic analysis were retrieved from the NCBI database and aligned with the newly determined sequences of the isolated strains by using CLUSTAL W (1.83) software. Phylogenetic and molecular evolutionary analysis was performed by the neighbour-joining method by using the software PHYLIP version 3.65 (Felsenstein, 1995). The DNADIST program with the Kimura-2 factor was used to compute the pairwise evolutionary distances for the above aligned sequences (Kimura, 1980). Topology of the phylogenetic trees was evaluated by performing a bootstrap

(algorithm version 3.6b) with 1000 bootstrapped trials. Phylogenetic trees were drawn using TREEVIEW 32 software (Fig. 1).

The 16S rRNA gene sequence data of the isolated aerobic strain were compared to the available sequences of micro-

organisms belonging to the genus *Pseudoalteromonas* and related micro-organisms.

Cells of the isolated strains were harvested by centrifugation after growth at 15 °C on 2 l complex medium and used for determination of the genomic DNA G+C

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequence (1.499 kb) comparisons indicating the position of strain A 37-1-2^T within the genus *Pseudoalteromonas*. Analysis was performed by the neighbour-joining method using software from PHYLIP version 3.65 (see text for further details). *Bacillus halodurans* LB 35 was used as the outgroup. Bar, 2 nt substitutions per 100 nt. Significant bootstrap values (%) are given at the nodes.

content. Cells were disrupted with a French press and purified by chromatography on a hydroxyapatite column (Cashion *et al.*, 1977). The DNA G+C content was determined by HPLC (Shimadzu) (Mesbah & Whitman, 1989). The analytical column used was a Vydac 201 SP 54, C18 5 μ m (250 $\times$ 4.6 mm) equipped with a guard column 201 GD 54H (Vydac).

DNA–DNA hybridization of the novel isolate with the reference strains *Pseudoalteromonas elyakovii* LMG 14908^T, *Pseudoalteromonas distincta* KMM 638^T and *Pseudoalteromonas nigrifaciens* DSM 8810^T was carried out using 3 g cell mass of each strain. To isolate the DNA, cells were French-pressed (Thermo Spectronic) and DNA was purified by chromatography on a hydroxyapatite column as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modification described by Huß *et al.* (1983). In the case of *P. elyakovii*, a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories) was used. A Cary 100 Bio UV/VIS-spectrometer equipped with a Peltier thermostatted 6 $\times$ 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian) was used in the case of *P. distincta* and *P. nigrifaciens*. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992).

Enrichment cultures (pH 7) inoculated with seawater samples from Spitzbergen showed bacterial growth after 1 week of aerobic incubation at 4 °C. Microscopy revealed the presence of short-rod-shaped cells. After a number of serial dilutions, one strain (A 37-1-2^T) was investigated further.

As observed for members of the genus *Pseudoalteromonas*, cells of the newly isolated strain were Gram-negative, motile, polar-flagellated, short rods (0.15–0.2 μ m wide and 0.4–0.75 μ m long) (Bozal *et al.*, 1997; Ivanova *et al.*, 2000, 2004; Romanenko *et al.*, 2003; Sawabe *et al.*, 2000). They occurred as single cells or in pairs. Spores were not detected. A comparison of the morphological characteristics of the isolated strain and related strains is shown in Table 1.

No growth was observed under anaerobic conditions with glucose. Growth occurred only under aerobic conditions between 4 and 25 °C. The optimal growth temperature and pH of the novel strain (10–15 °C and pH 7–8) were similar to those of *Pseudoalteromonas antarctica* (10–15 °C and pH 7–8). Only a few *Pseudoalteromonas* strains show growth at 4 °C; these include *Pseudoalteromonas espejiana*, *Pseudoalteromonas carrageenovora* and *Pseudoalteromonas haloplanktis*. The newly isolated strain grew in the presence of a wide range of NaCl concentrations, from 0 to 9% (w/v) [optimum 2–3% (w/v)], which is broader than the range for *P. distincta* (0.5–8%) and *Pseudoalteromonas aliena* (0.5–6%), (Bozal *et al.*, 1997; Ivanova *et al.*, 2004).

Strain A 37-1-2^T was able to utilize a large number of substrates, including α -cyclodextrin, dextrin, cellobiose, D-galactose, α -D-glucose, maltose, D-mannitol, melibiose, sucrose, pyruvic acid methyl ester, D-gluconic acid, L-alanine, L-alanylglycine, glycyl-L-glutamic acid, L-serine, aesculin, ferric citrate and capric acid. *P. elyakovii*, *P. espejiana*, *Pseudoalteromonas citrea* and the novel isolate shared the ability to utilize D-galactose, sucrose, maltose, melibiose, D-mannitol and pyruvate. Only *P. haloplanktis*, *P. nigrifaciens* and the novel isolate were able to utilize D-gluconate.

The isolated strain produced several enzymes: pullulanase, caseinase, β -glucanase, esterase, pectinase, β -glucosidase and β -galactosidase. *P. aliena*, *P. antarctica* and *Pseudoalteromonas agarivorans* are known to produce caseinase (Bozal *et al.*, 1997; Ivanova *et al.*, 2004; Romanenko *et al.*, 2003). *P. agarivorans* produces an esterase and a β -galactosidase (Bozal *et al.*, 1997).

The FAME composition of the novel strain after growth at the optimal temperature of 15 °C is shown in Table 2. The FAME profiles display only those fatty acids comprising $\geq 0.05\%$ of the total. Straight-chain saturated FAMES comprised 20.3%, terminally branched saturated FAMES comprised 20.2% and monounsaturated FAMES comprised 56.1%. The monounsaturated FAME 16:1 ω 7c (40.2%) was the most abundant FAME found. Interestingly, the high content of monounsaturated fatty acids in the isolate is a characteristic of psychrophilic micro-organisms where the lipid bilayers of psychrophilic cells are composed mostly of *cis*-unsaturated double bond chains, which create a 30° bend resulting in formation of cavities in the lipid layer, thus reducing packing density and maintaining fluidity in near- or subfreezing conditions (Deming, 2002; Feller & Gerday, 2003). The FAME composition of the isolate and related strains belonging to the genus *Pseudoalteromonas* is listed in Table 2.

The G+C content of the DNA from the novel isolate is 39.0 mol%. The 16S rRNA gene sequence (1.499 kb) was analysed and compared to available 16S rRNA gene sequences of organisms belonging to the genus *Pseudoalteromonas*. The most closely related species, with 16S rRNA gene sequence similarities of 99%, were *P. elyakovii* (1484 out of 1489 nt; 99.7% sequence similarity), *P. citrea* (1469/1474 nt; 99.7%), *P. haloplanktis* (1484/1494 nt; 99.3%), *P. distincta* (1473/1481 nt; 99.5%), *P. agarivorans* (1475/1484 nt; 99.4%) and *Pseudoalteromonas gracilis* (1484/1494 nt; 99.3%). Although the level of 16S rRNA similarity with the closest relative (99%) is high for two species, the observed value is still low for the mean level of similarity within the genus (Gauthier *et al.*, 1995). Strain A 37-1-2^T formed a cluster with *P. elyakovii* and *P. distincta*. Hybridization of DNA of strain A 37-1-2^T with that of the most related strains, i.e. *P. elyakovii*, *P. distincta* and *P. nigrifaciens*, showed only 47.6, 44.2 and 22.6% DNA–DNA relatedness, respectively, despite the fact that the 16S rRNA gene sequence similarity between all of them

Table 1. Comparative characteristics of strain A 37-1-2^T and related members of the genus *Pseudoalteromonas*

Strains: 1, A 37-1-2^T; 2, *P. elyakovii* KMM 162^T; 3, *P. espejiana* IAM 12640^T; 4, *P. citrea* KMM 216; 5, *P. carrageenovora* NCIMB 302^T; 6, *P. atlantica* NCIMB 301^T; 7, *P. distincta* KMM 638^T; 8, *P. haloplanktis* IAM 12915^T (Sawabe *et al.*, 2000); 9, *P. tetradonis* IAM 14160^T; 10, *P. antarctica* CECT 4664^T; 11, *P. nigrifaciens* IAM 13010^T; 12, *P. undina* NCIMB 2128^T (Ivanova *et al.*, 2001, 2002). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Growth at:												
4 °C	+	–	+	–	+	–	–	+	+	+	+	+
37 °C	–	–	–	–	–	–	–	–	–	–	–	–
40 °C	–	–	–	–	–	–	–	–	–	–	–	–
Production of:												
Amylase	–	+	+	+	–	+	–	–	–	–	+	+
Alginase	–	+	+	+	+	+	+	–	ND	ND	+	–
Agarase	–	–	–	+	–	+	–	–	–	–	–	–
Utilization of:												
D-Mannose	–	+	–	+	–	+	+	–	–	+	+	–
D-Galactose	+	+	+	+	–	+	–	–	+	–	+	–
D-Fructose	–	+	+	+	+	+	+	–	–	+	+	–
Sucrose, maltose	+	+	+	+	+	–	+	–	+	+	+	+
Melibiose	+	+	+	+	+	+	–	–	–	+	+	–
D-Lactose	–	+	+	+	+	+	–	–	–	+	+	–
D-Gluconate	+	–	–	–	–	–	–	+	–	–	+	–
N-Acetylglucosamine	–	–	–	–	–	–	–	–	ND	–	ND	+
D-Mannitol	+	+	+	+	+	–	+	+	–	+	–	–
Succinate	–	+	+	+	+	–	+	+	–	+	+	+
Citrate	–	+	+	–	+	+	–	–	+	+	+	–
meso-Erythritol	–	–	–	–	–	–	–	+	–	ND	ND	–
Glycerol	–	–	+	–	+	+	+	+	–	ND	+	–
γ-Aminobutyrate	–	–	–	–	–	–	–	–	ND	ND	ND	ND
Xylose	–	+	+	+	–	–	–	–	–	ND	+	–
Trehalose	–	–	+	+	–	+	–	+	–	ND	+	+
Acetate	–	+	+	+	+	+	–	+	+	+	+	+
D-Glucosamine	–	–	–	–	–	–	–	+	ND	ND	ND	ND
Pyruvate	+	+	+	+	+	+	+	–	+	+	+	–
Alginate	–	+	+	+	+	+	+	–	ND	ND	ND	ND
Aconitate, gluconate	–	+	–	–	–	–	–	–	+	ND	ND	ND

was very high (99 %). This strongly indicates that the newly isolated strain is a novel species within the genus *Pseudoalteromonas*.

Description of *Pseudoalteromonas arctica* sp. nov.

Pseudoalteromonas arctica (arc'ti.ca. L. fem. adj. *arctica* from the Arctic, referring to the site where the type strain was isolated).

Cells are straight, polar-flagellated, Gram-negative and rod-shaped, 0.15–0.2 µm wide and 0.4–0.7 µm long. They occur singly and are non-spore-forming and strictly aerobic. Colonies on agar medium are slightly orange, circular, smooth and convex. Temperature range for growth is 4–25 °C, with an optimum between 10 and 15 °C. No growth observed at 30 °C. pH range for growth is 6–8, with an optimum at pH 7–8. Growth is observed at 0–9 % NaCl (w/v), with an optimum between 2 and 3 %

(w/v). Growth is observed with α-cyclodextrin, dextrin, cellobiose, D-galactose, α-D-glucose, maltose, D-mannitol, melibiose, sucrose, pyruvic acid methyl ester, D-gluconic acid, L-alanine, L-alanylglycine, glycyl-L-glutamic acid, L-serine, aesculin, ferric citrate and capric acid. Positive for activities of the following enzymes: pullulanase, protease, β-glucanase, esterase, pectinase, β-glucosidase and β-galactosidase. FAMES comprise 20.3 % straight-chain saturated FAMES, 20.2 % terminally branched saturated FAMES and 56.1 % monounsaturated FAMES. Phylogenetic analysis reveals a close relationship to *P. elyakovii*, *P. distincta* and *P. nigrifaciens* with 99 % 16S rRNA gene sequence similarity and 47.6, 44.2 and 22.6 % DNA–DNA relatedness, respectively. Habitat: Arctic seawater.

The type strain is A 37-1-2^T (=LMG 23753^T=DSM 18437^T), isolated from seawater samples taken from Spitzbergen. The DNA G+C content of the type strain is 39 mol%.

Table 2. Comparative fatty acid content (%) of the novel strain and related strains belonging to the genus *Pseudoalteromonas*

Strains: 1, A 37-1-2^T; 2, *P. haloplanktis* CECT 4188^T; 3, *P. tetraodonis* IAM 14160^T (Ivanova *et al.*, 2001); 4, *P. agarivorans* KMM 255^T (Romanenko *et al.*, 2003); 5, *P. atlantica* IAM 14164 (Bozal *et al.*, 1997). ND, No data available.

Fatty acid	1	2	3	4	5
10:0 3-OH	1.2	ND	ND	ND	1.4
12:0	2.4	2.0	1.5	1.8	2.5
11:0 3-OH	1.1	0.3	0.4	ND	1.8
13:0	0.2	0.2	0.26	0.1	ND
12:0 3-OH	6.4	1.9	0.9	ND	3.8
14:0	1.1	2.0	2.0	1.1	0.7
15:1 ω 8c	1.8	2.3	6.3	1.2	ND
15:1 ω 6c	0.3	0.2	0.6	0.2	ND
15:0	2.2	3.3	8	2.7	2.7
16:1 ω 7c	40.2	40.5	35.0	42.4	22.0
16:1 ω 5c	0.2	0	0.2	0	ND
16:0	12.7	30.1	18.2	27.3	19.4
17:1 ω 8c	5.5	6.0	10.7	5.9	8.2
17:0	1.5	3.9	5.5	3.4	9.6
18:1 ω 9c	0.3	0.2	0.2	0.2	ND
18:1 ω 7c	7.8	2.4	2.5	7.1	ND
18:0	0.4	1.9	0.6	0.6	1.9

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