Note

Psychromonas arctica sp. nov., a novel psychrotolerant, biofilm-forming bacterium isolated from Spitzbergen

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Using starch as a carbon source at a cultivation temperature of 4°C, a number of Gram-negative, aerobic strains was isolated from sea-ice and sea-water samples collected at Spitzbergen in the Arctic. Analysis of the genetic diversity of the novel isolates by random amplification of polymorphic DNA (RAPD) and ERIC fingerprinting revealed a homogenic group of biofilm-forming bacteria that contained small extrachromosomal elements. As a representative of the group, strain Pull 5.3T, isolated from a sea-water sample, was used for detailed characterization. The results of phylogenetic analysis indicated that the newly isolated strain is a member of the γ-subclass of the Proteobacteria and belongs to the genus Psychromonas. On the basis of DNA–DNA hybridization experiments, chemotaxonomic studies and phenotypic characterization, strain Pull 5.3T (= CECT 5674T = DSM 14288T) clearly represents a novel species, for which the name Psychromonas arctica sp. nov. is proposed.

Cold-adapted micro-organisms are found in both permanently and temporarily cold habitats, which comprise more than 80 % of the Earth’s biosphere. Oceans, covering three-quarters of the Earth, polar regions (14 % of the Earth’s surface), high mountains and deep lakes provide various aquatic and terrestrial cold environments where the temperature seldom or never reaches 5°C (Gounot, 1999). The ability of micro-organisms to grow at low temperatures is not restricted to prokaryotes. A wide variety of micro-organisms, including bacteria, archaea, yeast, fungi and algae, is found in cold environments. These micro-organisms are free-living in soil and fresh and saline waters or are associated with plants and cold-blooded animals such as fish or crustaceans. Among the bacteria, almost all types have been identified either after isolation (Ravenschlag et al., 1999; Bowman et al., 1997) or by detection in their natural habitats using a 16S rRNA approach (Fuhrman et al., 1993; DeLong et al., 1994; Vetriani et al., 1998). Unlike hyperthermophiles, they do not belong to new phyla. The majority of psychrophiles studied to date belong to the Gram-negative Proteobacteria. This is not surprising, since Gram-negatives are pre-dominant in marine waters, where most investigations have been performed.

Psychrophiles and psychrotolerant micro-organisms have a wide range of adaptations, including alterations in the protein and lipids of their membrane, energy-generation systems, protein synthesis and hydrolytic enzymes (Russell, 1998). Higher specific activity at low temperatures and thermostensitivity of cold-active enzymes provide a valuable source for exploration of novel biotechnological processes (Feller & Gerday, 1997). Since it has become clear that psychrophiles also represent a naturally occurring model for investigating protein adaptation to cold (Aghajari et al., 1998), an increasing number of novel bacterial strains from different Arctic and Antarctic environments have been isolated.

In this study, employing a broad screening programme, a number of heterotrophic bacteria was isolated from sea-ice and sea-water samples obtained from the area of Spitzbergen in the Arctic. Among the novel isolates, a group of strains was found to be similar to, but distinct from in a number of characteristics, a previously described psychrophilic species,
*Psychromonas antarctica*, isolated from sediment of a high-salinity pond on the McMurdo Ice Shelf, Antarctica (Mountfort *et al.*, 1998). Taxonomic and physiological analysis of the newly isolated strains demonstrated that they represent a novel species of the genus *Psychromonas*, and we propose the name *Psychromonas arctica* sp. nov. for the species represented by the type strain Pull 5.3T.

Arctic sea-ice and sea-water samples were collected in 1998 during an expedition to Svalbard, Spitzbergen. Samples were collected and transported to the laboratory at ambient temperature, 2–10 °C. An aliquot (0.5 ml) of liquid samples or a piece of membrane filter (cellulose acetate, pore size 0.2 μm) through which sea water was filtered were used for inoculation of 10 ml complex marine medium containing starch as a carbon source in 20 ml tubes. The complex medium contained (l)g starch as a carbon source in 20 ml tubes. The complex medium contained (l)g NaCl, 28.13 g; KCl, 0.77 g; CaCl₂.H₂O, 0.02 g; MgSO₄.7H₂O, 0.5 g; NH₄Cl, 1.0 g; iron ammonium citrate, 0.02 g; yeast extract, 0.5 g; 10-fold-concentrated trace element solution (DSM 141), 1 ml; 10-fold-concentrated vitamin solution (DSM 141), 1 ml; KH₂PO₄, 2.3 g; Na₂HPO₄.2H₂O, 2.9 g; starch, 5 g. The pH was adjusted with NaOH to 7.2. In order to identify and isolate the producers of two main starch-degrading enzymes, α-amylase (hydrolyses α-1,4-glycosidic bonds) and pullulanase (specifically cleaves α-1,6-glycosidic bonds), enrichment cultures were screened on agar plates containing complex medium and 0.5 % dyed starch-degrading enzymes, α-amylase (hydrolyses α-1,4-glycosidic bonds) and pullulanase (specifically cleaves α-1,6-glycosidic bonds), enrichment cultures were screened on agar plates containing complex medium and 0.5 % dyed amylopectin (Jørgensen *et al.*, 1997) or dyed pullulan (Megazyme). The degradation of dyed amylpectin indicates the presence of α-amylase activity, whereas halo formation on dyed pullulan is due to pullulanase (hydrolyses α-1,6-linkage in pullulan) or pullulan hydrolase (hydrolyses α-1,4-linkage in pullulan). Colonies that formed clearing zones on any of the dyed substrates were selected and transferred to liquid cultures. For the isolation of pure cultures, serial dilution and plating techniques were applied.

Enrichment cultures were obtained by inoculation of the complex medium containing starch as a carbon source with sea-ice and sea-water samples from Spitzbergen. After incubation for 6 weeks at 4 °C, stable enrichment cultures were obtained. Isolation was achieved by serial dilution and subsequent cultivation on agar plates containing dyed substrate (amylopectin or pullulan) at 4 °C. A total of 12 pure cultures were obtained, including seven strains isolated on dyed pullulan (Pull 1.5; Pull 5.3T; Pull 6.3; Pull 6.5; Pull 7.2; Pull 15.3; Pull 16.2) and five strains isolated on dyed amylopectin (Amyl 8.2; Amyl 18.6; Amyl 18.7; Amyl 20.2; Amyl 20.3). Interestingly, all strains isolated on dyed pullulan were also able to hydrolyse dyed amylopectin. The strains isolated on dyed amylpectin were not able to degrade dyed pullulan, however.

Gram staining and catalase tests were performed as described by Smibert & Krieg (1994). Cytochrome oxidase activity was determined with the Bacident Oxidase assay (Merck). Growth was measured by determining the optical density at 600 nm (1 cm path length) using a Shimadzu UV 1602 spectrophotometer.

To determine the salt requirement for growth, media were prepared with ten different NaCl concentrations between 0 and 6 % (w/v). The concentrations of other salts were kept constant. The vitamin requirement was tested after at least ten transfers on medium without vitamins. The pH optimum for growth was tested between pH 4 and 11. Growth with different electron donors (yeast extract, Casamino acids, starch, xylan, chitin, cellulose, gelatin, glycogen, acetate, ethylene glycol, betaine, mannitol, sorbitol, valine, isoleucine, L-histidine, L-arginine, L-serine, alanine, D-fructose, glucose, lactose, maltose, mannose, sucrose, xylose, glycerol, citrate, fumarate, propionate, lactate, pyruvate, malate and succinate) was tested using oxygen as electron acceptor on complex media lacking starch and yeast extract. Tubes without electron donors were inoculated and served as negative controls. Growth tests on different electron acceptors were made under anaerobic conditions in sulfate- and nitrate-free medium that was supplemented with glucose as the carbon source. The following electron acceptors were tested: thiosulfate (10 mM), elemental sulfur, nitrate (5 mM), nitrite (2 mM) or iron(III) citrate (30 mM). All test tubes were inoculated with sulfate- and nitrate-free pre-culture. The same pre-culture was used as the inoculum for growth experiments without electron acceptors using glucose, starch, lactate, pyruvate, fumarate, malate or propionate as a carbon source at a final concentration of 10 mM. Growth was measured by direct cell counting under the phase-contrast microscope. All tests were incubated at least in triplicate at the optimal temperature for growth of 20 °C.

Fatty acid methyl ester (FAME) analysis was performed according to the modified method of LePage & Roy (1984). Total lipids were extracted according to Bligh & Dyer (1959). For derivatization, an aliquot of 3 mg total lipid extract was resolved in 2 ml methanol/hexane (4:1, v/v) plus pyrogallol and was methylated with 200 μl acetylchloride at 100 °C for 1 h; 5 ml 6 % K₂CO₃ was added and the mixture was centrifuged for 10 min at 2200 g. The upper, hexane phase containing the FAMEs was removed and dried with Na₂SO₄. FAMEs were analysed by capillary GC performed on an LS 32 GC (Chrompack; Kohn, 1996). For separation of fatty acid species, a fused silica capillary column (D23, 40 m; Fisons) was used. The chromatographic conditions were as follows: injector temperature (PTV), 65–270 °C; split ratio, 15:1; carrier gas, helium at a flow rate of 40 cm s⁻¹. The column oven temperature profile was: initial temperature, 60 °C for 0-1 min; from 60 to 180 °C at 40 °C min⁻¹; 180 °C for 2 min; from 180 to 210 °C at 2 °C min⁻¹; 210 °C for 3 min; from 210 to 240 °C at 3 °C min⁻¹; 240 °C for 10 min. Spectra were recorded by a flame-ionization detector at 280°C.

Chromosomal DNA of the newly isolated strains was prepared according to the method described by Ausubel *et al.* (1992) and used as template DNA (5–100 ng) in a
Cells of strain Pull 5.3T were harvested from 2 ml culture samples by centrifugation and resuspended in 100 μl water. A subsample of 1 μl was used as a template for the amplification of 16S rDNA with primers 9–27f and 1492–1515r (Buchholz-Cleven et al., 1997). The PCR incorporated a hot start at 94 °C for 5 min and at 80 °C for 1 min before the addition of HiFi DNA polymerase mixture, followed by 30 cycles of 94 °C for 1·5 min, 46 °C for 1·5 min and 68 °C for 1·5 min. The amplification was performed with reagents from the Expand High Fidelity PCR System kit (Roche Diagnostics) following the recommendations of the manufacturer and using a Gene Amp PCR System 2400 thermal cycler. Negative controls with water instead of DNA showed no amplification. Amplicons were separated on a 1·0% agarose gel stained with ethidium bromide and purified by the Qiagen PCR purification kit. Both strands of the amplification product were sequenced using primers 7F, 787F, 787R, 1175R, 1099F and 1492R (Buchholz-Cleven et al., 1997) (primer nomenclature refers to the 5’ end of the respective target on the 16S rDNA according to Escherichia coli numbering).

Almost the complete sequence of the 16S rRNA gene of strain Pull 5.3T was determined. To determine the closest relatives of the novel isolate, preliminary searches in the EMBL database were performed with the program FASTA. Reference sequences utilized in phylogenetic analysis were retrieved from the EMBL database and aligned with the newly determined sequence of the novel isolate by using
CLUSTAL W. Software from PHYLIP version 3.57c (Felsenstein, 1993) and MEGA version 2.0 (Kumar et al., 2001) was used for phylogenetic and molecular evolutionary analyses. DNADIST, with the maximum-likelihood option, was employed to analyse sequence similarities and NEIGHBOR (Kimura’s two-parameter correction) was used to create a phylogenetic tree. The 16S rRNA sequence of Bacillus subtilis TB11 (AF058766) was used as the outgroup.

The G+C content of the DNA from strain Pull 5.3T was 40.1 mol%. The complete 16S rRNA gene sequence of strain Pull 5.3T (1528 nt, E. coli positions 8–1534) showed that the novel isolate belongs to the γ-subclass of the Proteobacteria (Fig. 2) and is related to species of the genera Colwellia, Moritella, Vibrio and Shewanella with sequence similarities of 89–0–89.8%. The closest relative is P. antarctica, sharing 94.8% 16S rDNA similarity, which, at the time of writing, is the only species described in the genus Psychromonas (Mountfort et al., 1998). The relative binding ratio for DNA–DNA hybridization of strain Pull 5.3T and P. antarctica is 25.0%, well below the threshold value of 70% accepted for the distinction of different species (Wayne et al., 1987).

On a medium containing glucose or starch as the carbon source, strain Pull 5.3T formed white colonies up to 2 mm in diameter and cells stained Gram-negative. The motile, rod-shaped cells measured 1.2–2.6 μm in length (Fig. 3a). In old cultures (2–3 days), the cells became more pleomorphic and non-motile coccoid cells, 1.3–1.7 μm in diameter, were observed. Electron microscopic observations revealed the presence of blebs on the cell surface (Fig. 3b) when the cells were grown at the optimal growth temperature of 20°C. Similar membrane structures have been reported for both Gram-negative and Gram-positive bacteria (Antranikian et al., 1987). Although the function of the blebs of strain Pull 5.3T is still not clear, these structures could be connected with instability of membranes caused by the high incubation temperature. Cytoplasmic glycogen-like inclusions were found by microscopic investigation to be abundant in actively growing cells (Fig. 3a).

The cells grew either singly, in pairs or in dense aggregates enclosed by fibrous exopolysaccharides, forming a multilayered biofilm within a few days at any cultivation temperature from 4 to 20°C and independent of the carbon source used. The extracellular matrix may be an additional survival mechanism, protecting the cells from frost and/or regulating the nutrient supply within the multispecies biofilm community in the natural environment under starvation conditions (Costerton et al., 1995). Subsequent cultivation of the strain, independent of conditions used, resulted in complete loss of its ability to form biofilms within 2 months.

No spores were detected in either actively growing or old cultures, regardless of whether the newly isolated strain was cultivated on the regular complex medium or on the medium of Duncan & Strong (1968).

A culture grown at 4°C was used for the determination of total cellular fatty acid composition. The novel isolate contained 16:1ω7c as a major fatty acid (nearly 50%). Other dominant fatty acids included 16:0, 16:1ω7t and 18:1ω7 as determined by GC (7.0–16.2%). On the other hand, 12:0 and 14:1ω5t represented relatively minor...
components (2.74–5.22%). A fatty acid with an equivalent chain length of 14:33 could not be identified. The fatty acid profile of isolate Pull 5.3T resembles those of other marine bacteria characterized by the predominance of 16:1 and other monounsaturated species and short-chain fatty acids (Herbert, 1981). The conversion of cis- to trans-unsaturated fatty acids is believed to change the membrane fluidity in response to an environmental stimulus and was also reported for a heterogenic group of bacteria that included psychrophilic pollutant-degrading bacteria (Keweloh & Heipieper, 1996). The ability to synthesize trans-unsaturated fatty acid isomers could be an ecological advantage for Pull 5.3T, similar to a phenomenon described for the marine psychrophilic Vibrio strain ABE-1 (Okuyama et al., 1991). This allows the organism to react to changes in temperature and salinity much faster than by changing the degree of membrane saturation (Keweloh & Heipieper, 1996). Polyunsaturated fatty acids, which have been reported recently for a number of marine psychrophilic bacteria (Russell & Nichols 1999; Nichols et al., 1999), could not be detected.

Strain Pull 5.3T grew aerobically at temperatures between 0 and 25°C (data not shown). Maximum growth rate as well as an increasing cell size, decrease in membrane stability and lower final cell yield were observed at 20°C. No growth was

**Table 1. Comparative characteristics of *P. arctica* sp. nov. and *P. antarctica***

Data for *P. antarctica* were taken from Mountfort et al. (1998). Both taxa require NaCl for growth, showing optimum growth in 2% NaCl, are motile, ferment glucose and starch, are oxidase and catalase positive, hydrolyse starch and utilize fructose, glucose, maltose, mannitol and sucrose. Both taxa are negative for hydrolysis of cellulose, chitin and xylan and utilization of lactate, malate and xylose. ND, Not determined.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>P. arctica</em> Pull 5.3T</th>
<th><em>P. antarctica</em> DSM 10704T</th>
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<tbody>
<tr>
<td>Temperature for growth (°C):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>Maximum</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Optimal pH for growth</td>
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<td>6.5</td>
</tr>
<tr>
<td>NaCl concentration for growth</td>
<td>1–7</td>
<td>1–6</td>
</tr>
<tr>
<td>Oxygen relationship</td>
<td>Aerobe</td>
<td>Aerotolerant anaerobe</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
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<td>Hydrolysis of glycogen</td>
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</tr>
<tr>
<td>Utilization of:</td>
<td></td>
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<tr>
<td>Alanine</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acetate</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Propionate</td>
<td>–</td>
<td>ND</td>
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<tr>
<td>Pyruvate</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Succinate</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Citrate</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
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</tr>
<tr>
<td>Mannose</td>
<td>+</td>
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</tr>
<tr>
<td>Galactose</td>
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</tr>
<tr>
<td>Rhamnose</td>
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<tr>
<td>Glycerol</td>
<td>+</td>
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observed at 27 °C or above. The organism grew well at salt concentrations of 1–7 % (w/v) with an optimum at 2 % (data not shown). The pH range for growth was 6·5–9·8, with an optimum at pH 8·8 (data not shown). Under optimal conditions, using starch or glucose as a carbon source, the growth rate was 0·68 h⁻¹.

Of a number of polymeric substrates tested (starch, xylan, chitin, cellulose, gelatin, glycopycen), only starch was found to support growth. However, the monosaccharides fructose, glucose, mannose and mannitol as well as the disaccharides lactose, maltose and sucrose were utilized. Acetate, pyruvate, succinate, fumarate, glycerol and alanine supported growth, but propionate, malate, citrate, lactate and xylose did not. Isolate Pull 5.3T fermented glucose and starch, resulting in the formation of acetate, ethanol, formate, lactate and CO₂. No hydrogen, 1-propanol, 1-butanol, propionate or succinate could be detected as fermentation products. Nitrate, nitrite, sulfate, sulfite, iron(III) and elemental sulfur were tested as possible electron acceptors during growth of the strain on starch or glucose. Additional electron acceptors had little or no effect on growth or on the formation of fermentation products. At the end of growth, the concentration of electron acceptors had not decreased and no reduction products were found.

Although the fermentation pattern of Pull 5.3T and its ability to utilize carbohydrates are similar to those reported for P. antarctica, the novel isolate differs from its closest relative in a number of characteristics (Table 1). Differences in morphology, temperature and pH optima, the ability to form biofilms and the low DNA–DNA hybridization value indicate that the novel isolate Pull 5.3T represents a novel species of the genus Psychromonas, for which we propose the name Psychromonas arctica sp. nov.

**Description of Psychromonas arctica sp. nov.**

*Psychromonas arctica* (arc-ˈti.ca. L. fem. adj. *arctica* from the Arctic, referring to the site where the type strain was isolated).

Cells are 0·7–1·7 μm wide and 1·3–2·6 μm long. In old cultures, cells become more pleomorphic, with non-motile cocoid cells that are 1·3–1·7 μm in diameter. The pH optimum for growth is 8·5–8·8. Growth occurs in the presence of 1–7 % NaCl (w/v) with an optimum at 2 % NaCl. The temperature range for growth is 0–25 °C, with an optimum at 20 °C. Does not survive at temperatures above 27 °C for more than 4 h. The G + C content is 40·1 mol%. Sucrose, fructose, glucose, mannose, mannitol, lactose, maltose, acetate, pyruvate, succinate, fumarate, glycerol and alanine serve as carbon sources. Glucose and starch are fermented to acetate, ethanol, formate, lactate and CO₂. Xylan, chitin, gelatin, propionate, malate, citrate, lactate and xylose do not support growth. Vitamins are not required for growth. The type strain, strain Pull 5.3T (= CECT 5674T = DSM 14288T), was isolated from a sea-water sample taken near Svalbard, Spitzbergen.

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**References**


