Psychrobium conchae gen. nov., sp. nov., a psychrophilic marine bacterium isolated from the Iheya North hydrothermal field

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A novel psychrophilic, marine, bacterial strain designated BJ-1T was isolated from the Iheya North hydrothermal field in the Okinawa Trough off Japan. Cells were Gram-negative, rod-shaped, non-spore-forming, aerobic chemo-organotrophs and motile by means of a single polar flagellum. Growth occurred at temperatures below 16 °C, with the optimum between 9 and 12 °C. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the closest relatives of strain BJ-1T were Shewanella denitrificans OS-21T (93.5% similarity), Shewanella profunda DSM 15900T (92.9%), Shewanella gaetbuli TF-27T (92.9%), Paraferrimonas sedimentiola Mok-106T (92.1%) and Ferrimonas kyonanensis Asr22-7T (91.7%). The major respiratory quinone was Q-8. The predominant fatty acids were C16:1ω7c and C16:0. The G+C content of the novel strain was 40.5 mol%. Based on phylogenetic, phenotypic and chemotaxonomic evidence, it is proposed that strain BJ-1T represents a novel species in a new genus, for which the name Psychrobium conchae gen. nov., sp. nov. is proposed. The type strain of Psychrobium conchae is BJ-1T (=JCM 30103T=DSM 28701T).

The genus Shewanella, currently the only member of the family Shewanellaceae (Ivanova et al., 2004b), belongs to the order Alteromonadales. The genus was defined by the following features: aerobic or facultatively anaerobic,Gram-negative, motile, rod-shaped bacteria, nitrate-reducing, with C14:0, C16:1ω7c, C16:0 and C17:1ω6c as the major fatty acids. In this study, we used a polyphasic taxonomic approach to investigate a psychrophilic strain that was found to represent a novel genus and species belonging to the family Shewanellaceae.

Strain BJ-1T was isolated from gill tissue of the deep-sea hydrothermal vent mussel Bathymodiolus japonicus at the Ihey North hydrothermal field in the Okinawa Trough, off Japan (27° 47.438′ N 126° 53.736′ E; depth 990 m), collected by the remotely operated vehicle Hyper-Dolphin during the JAMSTEC NT12-06 cruise in March 2012 (Kawagucci et al., 2013). Fresh gill tissue was crushed to a fine paste with a small amount of sterilized artificial seawater (ASW; RohtoMarine, Rei-Sea Co.) with ice cooling by using a mortar and pestle. The tissue paste suspended in ASW was spread on marine agar 2216 (MA; Difco) plates and incubated at 10 °C for isolation. After isolation of strain BJ-1T, the strain, maintained on MA plates or in marine broth 2216 (MB; Difco), was incubated aerobically for 2 or 3 days at 12 °C and stored at −80 °C. Unless indicated otherwise, physiological tests were performed with a slight modification (use of ASW) of the general procedures described by Barrow & Feltham (1993) and Baumann et al. (1972). The effects of temperature, NaCl concentration and pH on growth were determined by examining the time-course of optical density (temperature gradient incubator with a bio-photorecorder, model TVS1). Growth at 4–20 °C was tested in MB. Cell growth was observed at 16 °C or below (optimum 12 °C), but not above 18 °C. Growth at 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, 4 and 5 % (w/v) NaCl was examined in Luria–Bertani (LB) medium [1.0 % (w/v) tryptone (Difco), 0.5 % (w/v) yeast extract (Difco)] and incubated at 12 °C. Cell growth was observed at NaCl concentrations of 2–4 % (optimum 3 %), but not at less than 1 % or more than 5 % NaCl. Growth at pH 5.0–10.0 (in increments of 0.5 pH units) was measured in 3 % NaCl LB medium with incubation at 12 °C. Growth was observed at pH 5.5–8.5 (optimum pH 6.0–6.5), but not at pH 5.0 or at pH 9.0 or above.

Species of the genera Shewanella, Paramoritella and Ferrimonas that do not grow at 5 % NaCl can be grown at 1 % NaCl, and species that cannot be grown at 1 % NaCl

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BJ-1T is AB930131.
can be grown at 5%. Strain BJ-1T is therefore unique among this group of organisms in being limited to growth at NaCl concentrations both greater than 1% and less than 5% (Table 1).

The morphology of living and non-living stained cells was determined by light microscopy and transmission electron microscopy, respectively. For negative staining, a drop of the culture was placed on a copper grid coated with Pioloform and carbon and stained with 1% potassium phosphotungstic acid adjusted to pH 6.5 with potassium hydroxide. Negatively stained cells were observed with a model Tecnai 20 transmission electron microscope (FEI) at an accelerating voltage of 200 kV.

Growth under anaerobic conditions was tested with the AnaeroPak system (Mitsubishi Gas Chemical) on MA 1 week after the addition of 0.1% KNO₃ to the MA plates. Acid production from sugars was assessed using a modified oxidative-fermentative medium (Hugh & Leifson, 1953) containing ASW, 0.05% (NH₄)₂SO₄, 0.01% yeast extract (Difco), 0.05% Tris, 1% test sugar and 0.003% bromothymol blue (adjusted to pH 7.2 at 20°C) with incubation at the optimum temperature. Oxidase activity was determined by spreading cell pellets on oxidase test paper (Nissui Pharmaceutical). Hydrolysis of gelatin, casein, starch, Tween 20, 40 and 80, chitin and ascellin was detected on MA plates using substrate concentrations of 1% (w/v). DNase activity was assessed using DNase test agar (Difco). The susceptibility of the strain to antibiotics was tested using MA plates and 6 mm sensitivity discs (Becton Dickinson) according to the manufacturer's instructions. The following antibiotics were examined: ampicillin (10 µg), cefotaxime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 IU), polymyxin B (300 µg), rifampicin (5 µg), tetracycline (30 µg) and vancomycin (30 µg). The effects of antimicrobial compounds on cell growth were assessed after 2 days at 12°C. The diameter of the inhibition zone was used to judge susceptibility according to the manufacturer's manual.

Cells of strain BJ-1T were rod-shaped, Gram-negative, strictly aerobic, non-spore-forming and motile by means of a single polar flagellum. Colonies were whitish, smooth, circular and 1.0–2.0 mm in diameter after 3 days of incubation at 12°C on MA. Detailed results from phenotypic and biochemical tests are given in the species description and in Table 1. Among related genera, only strain BJ-1T and some species of the genus *Shewanella* cannot be grown under anaerobic conditions.

Chromosomal DNA was purified using the phenol extraction method (Saito & Miura, 1963). The DNA G+C content was determined using reversed-phase HPLC (Tamaoka & Komagata, 1984). The 16S rRNA gene was amplified using the PCR method with primers 27F and 1492R (Lane, 1991). The 16S rRNA gene sequence of strain BJ-1T was obtained by direct sequencing of PCR-amplified DNA as described previously (Uchida et al., 2012). The resulting 16S rRNA gene sequence (1482 nt) of strain BJ-1T was compared with available 16S rRNA gene sequences from the DDBJ using the BLAST program (http://blast.ddbj.nig.ac.jp/top-j.html) to determine an approximate phylogenetic affiliation, and gene sequences were aligned with those of closely related species using the CLUSTAL_X software (Thompson et al., 1997). In addition, sequence similarity values between the novel strain and related strains were calculated using the GENETYX-MAC program version 17.0.2 (SDC Software Development).

Phylogenetic analyses were conducted in MEGA 5.2 (Tamura et al., 2011) using the neighbour-joining (NJ) method (Saitou & Nei, 1987). Bootstrap analysis to evaluate the stability of phylogenetic trees was performed by obtaining a consensus tree based on 1000 randomly generated trees (Felsenstein, 1985). All major branching nodes were subjected to maximum-likelihood (ML) and unweighted pair group method with arithmetic means (UPGMA) analyses (data not shown), which confirmed the robust nature of the phylogeny of this group of organisms, the branching order and high bootstrap values. The results of the phylogenetic analyses indicated that the novel organism belonged to the class *Gammaproteobacteria*. The novel bacterium was most closely related to *Shewanella denitrificans* OS-217T, *Shewanella profunda* LT13aT, *Shewanella waksmanii* KMM 3823T and *Shewanella gaetbuli* TF-27T, with pairwise similarity of 93.5, 93.2, 92.9 and 92.9%, respectively (Fig. 1). More distantly related organisms included *Paraferrimonas sedimenticola* Mok-106T (92.1%), *Moritella marina* ATCC 15381T (91.3%), *Paramoritella alkaliphila* A3F-7T (91.1%) and *Ferrimonas baleareca* DSM 9799T (90.8%). This cluster was supported by a significant bootstrap value of 90%. There is no precise correlation between 16S rRNA gene sequence divergence and species delineation, but it is generally recognized that divergence values of 3% or more are significant (Stackebrandt & Goebel, 1994). The sequence divergence of 6% or more displayed between the novel isolate and members of the genus *Shewanella*, combined with physiological and chemotaxonomic criteria, suggest strongly that the new isolate represents a novel genus.

Cellular fatty acids, isoprenoid quinones and polar lipids were extracted from the isolated strain after culture in MB at 12°C for up to 2 days. Cells were washed twice with 0.7% NaCl at 4°C. The fatty acids were obtained from cells by saponification, methylation and extraction according to the Sherlock Microbial Identification System (MIDI, 1999). The fatty acid composition was determined using a Finnigan TRACE DSQ GC-MS system (Thermo Fisher Scientific) equipped with a DB-5 column (J&W Scientific) under a helium flow of 1.5 ml min⁻¹ and an oven temperature program increasing from 140°C (5 min) to 280°C (5 min) at 4°C min⁻¹. The fatty acids that made up more than 1% of the total in strain BJ-1T were C₁₆:₀ (67.0%), C₁₆:₁ (26.3%), C₁₄:₁ (2.2%), C₁₈:₁ω7c (1.9%), C₁₄:₀ 3-OH (1.5%) and C₁₄:₀ (1.2%).

Isoprenoid quinones were extracted from dried cells (200 mg) with chloroform/methanol (2:1), purified on
Table 1. Differential characteristics of strain BJ-1<sup>T</sup> (Psychrobium gen. nov.) and related genera


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Psychrobium (n=1)</th>
<th>Shewanella (n=61)</th>
<th>Paramoritella (n=2)</th>
<th>Moritella (n=7)</th>
<th>Ferrimonas (n=8)</th>
<th>Paraferrimonas (n=1)</th>
</tr>
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<tr>
<td>Metabolism*</td>
<td>A</td>
<td>F (93.3)</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
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<tr>
<td>4 °C</td>
<td>+</td>
<td>+ (80.3)</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>20 °C</td>
<td>−</td>
<td>+ (95.1)</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>37 °C</td>
<td>−</td>
<td>− (59.0)</td>
<td>+</td>
<td>−</td>
<td>+ (62.5)</td>
<td>+</td>
</tr>
<tr>
<td>42 °C</td>
<td>−</td>
<td>− (83.6)</td>
<td>−</td>
<td>−</td>
<td>− (62.5)</td>
<td>−</td>
</tr>
<tr>
<td>1% NaCl</td>
<td>−</td>
<td>+ (96.6)</td>
<td>+</td>
<td>ND</td>
<td>+ (62.5)</td>
<td>+</td>
</tr>
<tr>
<td>5% NaCl</td>
<td>−</td>
<td>+ (86.9)</td>
<td>+ (50)</td>
<td>ND</td>
<td>+ (87.5)</td>
<td>−</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>16:0 16:0 16:0 16:0</td>
<td>i-13:0 i-15:0 14:0 15:0 16:0</td>
<td>16:0 16:0 16:0 16:0</td>
<td>16:1 16:0 16:0 16:0</td>
<td>i-15:0 16:0 16:1 16:0</td>
<td>i-15:0 16:0 16:0 16:0</td>
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<tr>
<td>Major quinone(s)</td>
<td>Q-8</td>
<td>Q-8, Q-8, MK-7</td>
<td>Q-8</td>
<td>Q-8</td>
<td>Q-7, Q-8, MK-7</td>
<td>Q-7, MK-6, MK-7</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>40.5</td>
<td>39–54</td>
<td>56–57</td>
<td>41–47</td>
<td>54–60</td>
<td>50–51</td>
</tr>
</tbody>
</table>

*A, Aerobic; F, facultatively anaerobic.
TLC and analysed using reversed-phase HPLC according to methods described previously (Miyazaki et al., 2006). Standard quinones (Q-6, Q-7, Q-9 and Q-10) were obtained from Sigma. Ubiquinone 8 (Q-8) was the only quinone detected.

Polar lipids were extracted using the procedures described by Minnikin et al. (1984) and identified using two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). Polar lipid analysis of strain BJ-1T showed the presence of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidyglycerol and phosphatidylmethylethanolamine and small amounts of an unidentified phospholipid (Fig. 2).

Based on its distinctive genotypic, chemotaxonomic and other phenotypic characteristics, strain BJ-1T is considered to represent a new genus and species within the Gammaproteobacteria, for which the name Psychrobium conchae gen. nov., sp. nov. is proposed.

**Description of Psychrobium gen. nov.**

*Psychrobium* (Psy.chro’bi.um. Gr. adj. psychros cold; Gr. n. bios life; N.L. neut. n. *Psychrobium* a living entity coming from the cold).

Cells are Gram-negative, psychrophilic, chemo-organotrophic, strictly aerobic, motile by means of a single polar flagellum and positive for catalase and cytochrome oxidase. Nitrate is reduced to nitrite and nitrite is reduced to ammonia (assimilated). No growth occurs at NaCl concentrations below 1% or above 5%. Major fatty acids (>10%) are C₁₆:₀ and C₁₆:₁ω₇c. The isoprenoid quinone is Q-8. Polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidyglycerol and phosphatidymethyl ether phospholipid. The DNA
The type strain is strain BJ-1T (=JCM 30103T=DSM 28701T), isolated from gill tissue of the deep-sea hydrothermal vent mussel Bathymodiolus japonicus collected at the Ihey North hydrothermal field in the Okinawa Trough, Japan.

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References


