Halomonas titanicae sp. nov., a halophilic bacterium isolated from the RMS Titanic

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A Gram-negative, heterotrophic, aerobic, non-endospore-forming, peritrichously flagellated and motile bacterial strain, designated BH1T, was isolated from samples of rusticles, which are formed in part by a consortium of micro-organisms, collected from the RMS Titanic wreck site. The strain grew optimally at 30–37 °C, pH 7.0–7.5 and in the presence of 2–8 % (w/v) NaCl. We carried out a polyphasic taxonomic study in order to characterize the strain in detail. Phylogenetic analyses based on 16S rRNA gene sequence comparison indicated that strain BH1T clustered within the branch consisting of species of Halomonas. The most closely related type strains were Halomonas neptunia (98.6 % 16S rRNA sequence similarity), Halomonas variabilis (98.4 %), Halomonas boliviensis (98.3 %) and Halomonas sulfidaeris (97.5 %). Other closely related species were Halomonas alkaliphila (96.5 % sequence similarity), Halomonas hydrothermalis (96.3 %), Halomonas gomseomensis (96.3 %), Halomonas venusta (96.3 %) and Halomonas mendiana (96.2 %). The major fatty acids of strain BH1T were C18:1ω7c (36.3 %), C18:0 (18.4 %) and C19:0 cyclo ω8c (17.9 %). The DNA G+C content was 60.0 mol% (Tm).

Ubiquinone 9 (Q-9) was the major lipoquinone. The phenotypic features, fatty acid profile and DNA G+C content further supported the placement of strain BH1T in the genus Halomonas. DNA–DNA hybridization values between strain BH1T and H. neptunia CECT 5815T, H. variabilis DSM 3051T, H. boliviensis DSM 15516T and H. sulfidaeris CECT 5817T were 19, 17, 30 and 29 %, respectively, supporting the differential taxonomic status of BH1T. On the basis of the phenotypic, chemotaxonomic and phylogenetic data, strain BH1T is considered to represent a novel species, for which the name Halomonas titanicae sp. nov. is proposed. The type strain is BH1T (=ATCC BAA-1257T =CECT 7585T =JCM 16411T =LMG 25388T).

The family Halomonadaceae, originally proposed by Franzmann et al. (1988), belongs to the Gammaproteobacteria and currently comprises 10 genera: Halomonas (Vreeland et al., 1980; Dobson & Franzmann, 1996), Aidingimonas (Wang et al., 2009), Carnimonas (Garriga et al., 1998), Chromohalobacter (Ventosa et al., 1989), Cobetia (Arahal et al., 2002b), Halotalea (Ntougias et al., 2007), Kushneria (Sánchez-Porro et al., 2009), Modicisalibacter (Ben Ali Gam et al., 2007), Salinicola (de la Haba et al., 2010b) and Zymobacter (Okamoto et al., 1993). All members described so far in this family are able to grow at salinities between 5 and 10 % (w/v), except Zymobacter palmae (Okamoto et al., 1993). Taxonomically, the genus Halomonas is very heterogeneous and to date includes more than 60 species (http://www.bacterio.cict.fr/h/halomonas.html). The most recently described species, at the time of writing, are Halomonas ilicicola (Arenas et al., 2009), Halomonas fomitipodisoi (González-Domenech et al., 2009) and Halomonas zhanjiangensis (Chen et al., 2009). Arahal et al. (2002a) established that this genus is not monophyletic on the basis of 16S and 23S rRNA gene sequence analyses. A recent study (de la Haba et al., 2010) confirmed that the species of the genus Halomonas fall into two phylogenetic groups; however, several species do not fall clearly into either of the two groups and do not form a group themselves. All members of the Halomonadaceae are aerobes, although some Halomonas species have been shown to grow anaerobically with nitrate, nitrite or fumarate as an electron acceptor and with glucose on solid
media in sealed jars (Mata et al., 2002). They have been isolated from many different water and soil environments and mainly from saline, hypersaline or alkaline habitats. They are considered to be non-pathogenic; however, a human infection caused by Halomonas venusta, following a fish bite, has been reported (von Graevenitz et al., 2000).

Recently some other Halomonas strains have also been recognized as human pathogens, causing infections and contamination in a dialysis centre (Stevens et al., 2009). The exhaustive taxonomic characterization of these isolates has permitted them to be described as three novel species of Halomonas: Halomonas stevensii, Halomonas hamiltonii and Halomonas johnsoniae (Kim et al., 2010). In this paper, we describe the features of a novel strain that constitutes a Halomonas johnsoniae and Halomonas hamiltoniae. In this paper, we describe the features of a novel strain that constitutes a Halomonas johnsoniae.

The isolate was obtained from rusticle samples collected during the Akademik Keldysh expedition in 1991, at the site of the wreck. The term ‘rusticles’ is used to describe bioconcretions structures that look like icicles but are made up of rust (Ballard, 1989; MacInnis, 1992). These rusticle pieces were removed from the hull using the articulated arm of the Mir-2 submersible. They were transferred to plastic collection bags and transported aseptically to the surface. The samples were stored under dark, vacuum-sealed conditions at 4 °C. The isolation of strain BH1T was carried out by streaking a sample from the rusticles on Bacto marine agar 2216 medium (Difco) three times in repetition, resulting in a single colony. The pure culture was then kept in a cold chamber and subcultured as needed until gene sequencing information could be obtained (Kaur, 2004; Wells & Mann, 1997). For comparative purposes, the following culture collection strains were also used in this study: Halomonas neptunia CECT 5815T, Halomonas variabilis DSM 3051T, Halomonas boliviensis DSM 15516T and Halomonas sulfidaeris CECT 5812T. These bacteria were cultured on 10% MH medium that had the following composition (% w/v): NaCl (8.1), MgCl2 (0.7), MgSO4 (0.96), CaCl2 (0.036), KCl (0.2), NaHCO3 (0.006), NaBr (0.0026), proteose peptone (Difco) (0.5), yeast extract (Difco) (1.0), glucose (0.1) and agar (1.5) (Ventosa et al., 1982).

To characterize strain BH1T phenotypically, we followed the recommended minimal standards for describing new taxa of the family Halomonadaceae (Aralah et al., 2007). Macroscopic properties were determined using the classical characterization of colony appearance. All biochemical tests were carried out in the presence of NaCl (8.1%, w/v) and at 37 °C, unless stated otherwise. Catalase activity was determined by adding a 1% (w/v) H2O2 solution to colonies on SW10 agar (Sánchez-Porro et al., 2009). The oxidase test was performed using the Dry Slide Assay (Difco). Hydrolysis of casein, DNA, aesculin and tyrosine, reduction of selenite, the Voges–Proskauer, methyl red and oxidation-fermentation tests, gluconate oxidation, production of indole and β-galactosidase, lysine and ornithine decarboxylase, phenylalanine deaminase and phosphatase activities were determined as described by Cowan & Steel (1965) with the addition of 10% total salts to the medium (Ventosa et al., 1982; Quesada et al., 1984). The production of exopolysaccharide (EPS) and respiration on fumarate, nitrate and nitrite were determined as described by Mata et al. (2002). Production of poly-β-hydroxyalkanoate was determined according to Cowan & Steel (1965). Acid production from carbohydrates was determined using a phenol red base supplemented with 1% carbohydrate and SW10 medium (Ventosa et al., 1982). Optimal conditions for growth were determined by growing the strain in SW medium at 0, 0.5, 2, 5, 8, 10, 12.5, 15, 17.5, 20, 25 and 30% (w/v) NaCl and at 4, 15, 20, 25, 30, 37, 40 and 45 °C. The pH range for growth of the isolate was tested in SW10 medium adjusted to pH 4, 5, 5.5, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10 with the addition of the appropriate buffer to each medium (Sánchez-Porro et al., 2009). For determination of the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources, the classical medium of Koser (1923) as modified by Ventosa et al. (1982) was used, containing (1L−1): 75 g NaCl, 2 g KCl, 0.2 g MgSO4.7H2O, 1 g KNO3, 1 g (NH4)2HPO4, 0.5 g KH2PO4 and 0.05 g yeast extract (Difco). Substrates were added as filter-sterilized solutions to give a final concentration of 1 g L−1, except for carbohydrates, which were used at 2 g L−1. When the substrate was an amino acid, it was tested as carbon, nitrogen and energy source, and the basal medium was therefore prepared without KNO3 and (NH4)2HPO4. Susceptibility to antimicrobial compounds was determined according to the conventional Kirby–Bauer method (Bauer et al., 1966). In addition, the ultrastructure of strain BH1T, grown on marine agar (Difco), was studied under a transmission electron microscope after fixation in osmium tetroxide, and double staining with lead citrate and uranyl acetate (Kaur, 2004; Kaur & Mann, 2004). Unfixed bacterial cells were observed after negative staining with uranyl acetate (Kaur, 2004; Kaur & Mann, 2004). Transmission electron micrographs of strain BH1T are available as supplementary material with the online version of this paper and scanning electron micrographs are depicted in New Life on the Titanic (Mann et al., 1999). The results obtained are detailed in the species description and in Table 1.

Genomic DNA from strain BH1T was prepared using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the forward primer 16F27 [5′-AGAGTTTGATCACGCTGCTCAG-3′] and the reverse primer 16R1488 [5′-CGGTATTACCTTGTTAGACTCAG-3′]. Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer model ABI 3130XL (Applied Biosystems). 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig et al., 2004). The 16S rRNA gene sequence was aligned with the published sequences of closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were constructed using three
Table 1. Characteristics that distinguish strain BH1\textsuperscript{T} from related species of the genus *Halomonas*

Strains: 1, BH1\textsuperscript{T} (data from this study); 2, *H. neptunia* CECT 5815\textsuperscript{T} (Kaye et al., 2004); 3, *H. variabilis* DSM 3051\textsuperscript{T} (Fendrich, 1988; Kaye et al., 2004); 4, *H. boliviensis* DSM 15516\textsuperscript{T} (Quillaguamán et al., 2004); 5, *H. sulfidaeris* CECT 5817\textsuperscript{T} (Kaye et al., 2004). Data for reference strains were taken from the studies listed unless indicated.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.5-0.8 × 1.5-6.0</td>
<td>0.9 × 1.5-2.0 *</td>
<td>0.9 × 1.5-2.0 *</td>
<td>0.4-0.6 × 1.1-3.4</td>
<td>0.9 × 1.2-2.0 *</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NaCl range (% w/v)</td>
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<td>0.5-27</td>
<td>7-29</td>
<td>0-25</td>
<td>0.5-24</td>
</tr>
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<td>NaCl optimum (% w/v)</td>
<td>2-80</td>
<td>2-3</td>
<td>10</td>
<td>5</td>
<td>2-3</td>
</tr>
<tr>
<td>pH range</td>
<td>5.5-9.5</td>
<td>5-12</td>
<td>6-9.0</td>
<td>6.0-11.0</td>
<td>5-10</td>
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<tr>
<td>Temperature range (°C)</td>
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<td>-1-35</td>
<td>15-37</td>
<td>0-45</td>
<td>-1-35</td>
</tr>
<tr>
<td>Temperature optimum (°C)</td>
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<td>30</td>
<td>33</td>
<td>25-30</td>
<td>20-35</td>
</tr>
<tr>
<td>Selenite reduction</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>Acid production from:</td>
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<tr>
<td>L-arabinose</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Maltose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-mannose</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D-xylose</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>60.0</td>
<td>57.3</td>
<td>61.0</td>
<td>51.4</td>
<td>56.0</td>
</tr>
</tbody>
</table>

\*Data not reported in the original description and obtained in this study.

\*Data from this study; reported different reactions were in the original description.

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different algorithms integrated in the ARB software for phylogenetic inference: neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971). The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database.

The almost-complete 16S rRNA gene sequence (1453 bp) of strain BH1\textsuperscript{T} was obtained and used for initial BLAST searches in GenBank and for phylogenetic analysis. The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (http://www.eztaxon.org; Chun et al., 2007). Phylogenetic analysis based on the neighbour-joining algorithm revealed that strain BH1\textsuperscript{T} was included in the cluster of species of the genus *Halomonas* (Fig. 1). The most closely related type strains were *H. neptunia* (98.6 % 16S rRNA sequence similarity), *H. variabilis* (98.4 %), *H. boliviensis* (98.3 %) and *H. sulfidaeris* (97.5 %). Other closely related type strains were *Halomonas alcaliphila* (96.5 % sequence similarity), *Halomonas hydrothermalis* (96.3 %), *Halomonas gomseomensis* (96.3 %), *H. venusta* (96.3 %) and *Halomonas meridiana* (96.2 %). The maximum-likelihood and maximum-parsimony methods resulted in highly similar tree topologies (Supplementary Fig. S1), confirming the phylogenetic cluster formed by strain BH1\textsuperscript{T} and species of the genus *Halomonas*. Based on these data, the new isolate would belong to group 2 of the two phylogenetic groups defined for the genus *Halomonas* (Arahal et al., 2002a; de la Haba et al., 2010a).

The G+C content of the genomic DNA was determined from the midpoint value (T\textsubscript{m}) of the thermal denaturation profile (Marmur & Doty, 1962) by using the equation of Owen & Hill (1979). Cell biomass for analysis of cell-wall peptidoglycan, isoprenoid quinones and polar lipids was obtained by cultivation on 5 % HM medium at 30 °C. The cell-wall peptidoglycan type, polar lipids and respiratory lipoquinone of strain BH1\textsuperscript{T} were analysed by the Identification Service of the DSMZ (Braunschweig, Germany) and Dr Brian Tindall. For analysis of the fatty acids, the cells were grown on marine agar (Difco) with 5 % NaCl, pH 7.2, at 30 °C for 48 h. The whole-cell composition of fatty acids was determined by GC (Kämpfer & Kroppenstedt, 1996; Miller, 1982) using the Microbial Identification System (MIDI, Microbial ID). This analysis was carried out by the Identification, Characterization and Molecular Typing Service of the BCCM/LMG Bacteria Collection (Gent, Belgium).

The G+C content of the DNA of strain BH1\textsuperscript{T} was 60.0 mol%. This value is similar to those reported for closely related species of *Halomonas* (Table 1). The polar lipids detected included three phospholipids, two aminophospholipids and a phosphoglycolipid. Strain BH1\textsuperscript{T} contained ubiquinone 9 (Q-9) as the major lipoquinone (96 %), a minor amount of ubiquinone 8 (Q-8) (4 %) and peptidoglycan cell-wall-type A1\ Asc, with meso-diaminopimelic acid as diagnostic diamino acid. These features are typical of the characteristics defined for the genus *Halomonas* (Arahal & Ventosa, 2006; Arahal et al., 2007). Strain BH1\textsuperscript{T} had a fatty acid profile similar to those described for the type strains of the closely related *Halomonas* species (Table 2). The predominant fatty acids were C\textsubscript{18:1}ω7c (36.3 %), C\textsubscript{16:0} (18.4 %) and C\textsubscript{19:0} cyclo ω8c (17.9 %).
DNA–DNA hybridization studies were performed by the competition procedure of the membrane method (Johnson, 1994), described in detail by Arahal et al. (2001a, b). The hybridization temperature used was 56.0 °C, which is within the limit of validity for the filter method (De Ley & Tijtgat, 1970), and the percentage of hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicate. DNA–DNA hybridization between strain BH1T and H. neptunia CECT 5815T, H. variabilis DSM 3051T, H. boliviensis DSM 15516T and H. sulfidaeris CECT 5817T was 19, 17, 30 and 29 %, respectively. These levels of DNA–DNA hybridization are low enough to classify strain BH1T as representing a genotypically distinct species (Stackebrandt & Goebel, 1994; Stackebrandt et al., 2002).

Overall, our results show clearly that strain BH1T is phylogenetically related to other species of the genus Halomonas; however, there are important differences in its phenotypic and chemotaxonomic features and the DNA–DNA hybridization studies confirmed its distinct species status. On the basis of these data, we propose that the novel strain BH1T represents a novel species of the genus Halomonas, for which the new name Halomonas titanicae sp. nov. is proposed. The characteristics that differentiate strain BH1T from related species of the genus Halomonas are summarized in Table 1.

**Description of Halomonas titanicae Mann, Kaur, Sánchez-Porro and Ventosa sp. nov.**

Halomonas titanicae (t.i.ta.ni.ca.e. N.L. fem. n. titanica the ship Titanic; N.L. fem. gen. n. titanicae of or from the ship Titanic).

Cells are rods, 0.5–0.8 x 1.5–6.0 μm, occur singly or in pairs, are motile by peritrichous flagella (Supplementary Fig. S2), do not form endospores and are Gram-negative (Supplementary Fig. S3). The colonies are circular, smooth, convex, white–cream and 1.0–1.2 mm in diameter after 3 days of incubation on 10 % MH medium at 37 °C. In liquid medium, cells grow on the surface of the medium. Moderately halophilic and capable of growing in salt concentrations between 0.5 and 25 % (w/v) NaCl; no growth in the absence of NaCl. Optimal growth is at 2–8 % (w/v) NaCl. The temperature range for growth is 4–42 °C, with optimal growth at 30–37 °C. Growth occurs at pH 5.5–9.5 and is optimal at pH 7.0–7.5. Chemo-organotrophic. Metabolism is respiratory. Catalase and oxidase are produced. Acid is produced from D-galactose, D-glucose and D-fructose but not from D-arabinose, maltose, D-mannose, glycerol, D-mannitol, trehalose or D-xylene.
Table 2. Cellular fatty acid content of strain BH1T and the closely related Halomonas species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C10:0</td>
<td>2.3</td>
<td>2.0</td>
<td>1.1</td>
<td>2.5</td>
<td>1.4</td>
</tr>
<tr>
<td>C12:0</td>
<td>1.8</td>
<td>1.5</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>C12:0 3-OH</td>
<td>10.2</td>
<td>12.3</td>
<td>8.9</td>
<td>8.4</td>
<td>8.2</td>
</tr>
<tr>
<td>C16:0</td>
<td>18.4</td>
<td>16.1</td>
<td>21.7</td>
<td>18.0</td>
<td>12.2</td>
</tr>
<tr>
<td>C16:1ω7c</td>
<td>4.8</td>
<td>4.19</td>
<td>4.1</td>
<td>12.8</td>
<td>15.0</td>
</tr>
<tr>
<td>C17:0 ω7c</td>
<td>6.7</td>
<td>5.9</td>
<td>12.5</td>
<td>3.4</td>
<td>0.9</td>
</tr>
<tr>
<td>C18:0 3-OH</td>
<td>36.3</td>
<td>39.1</td>
<td>14.2</td>
<td>41.0</td>
<td>56.5</td>
</tr>
<tr>
<td>C19:0 ω7c</td>
<td>17.9</td>
<td>17.1</td>
<td>33.2</td>
<td>9.6</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Aesculin is hydrolysed but gelatin, starch, casein, Tween 80, DNA and tyrosine are not. Nitrate is reduced but nitrite is not. The methyl red test, indole production, Voges–Proskauer reaction, urease and phenylalanine deaminase production are negative. β-Galactosidase–positive (ONPG test). Selenite is reduced. Glucanate is not oxidized. The following compounds are utilized as sole carbon and energy sources: acetate, benzoate, cellobiose, citrate, D-fructose, fumarate, D-galactose, D-glucose, glyceral, myo-inositol, lactose, D-mannitol, propionate, raffinose, D-sorbitol, succrose, trehalose, xylitol and D-xylene. The following are not utilized as sole carbon and energy sources: α-arabinose, butanol, dulcitol, ethanol, formate, D-fucose, maltose, D-mannose, melibiose, methanol, propanol, ribose, starch, sucinate and valerate. The following compounds are utilized as sole carbon, nitrogen and energy sources: L-alanine, L-arginine, glutamic acid, L-ornithine, L-phenylalanine and tryptophan. The following compounds are not utilized as sole carbon, nitrogen and energy sources: aspartic acid, L-cysteine, L-methionine and L-serine. Cells are sensitive to (μg per disc) chloramphenicol (30), erythromycin (15) and trimethoprim/sulfamethoxazole (1.25/23.75). Resistant to (μg per disc, unless specified otherwise): cephalothin (30), bacitracin (10), kanamycin (30), nalidixic acid (30), neomycin (30), penicillin (10 U) and vancomycin (30).

The major fatty acids are C18:1ω7c, C16:0, C19:0 cyclo ω8c. Minor fatty acids are C12:0 3-OH, C17:0 cyclo, C16:1ω7c and C12:0. The polar lipids detected include three phospholipids, two aminophospholipids and a phosphoglycolipid. The ubiquinones detected are Q-9 (96%) and Q-8 (4%). The peptidoglycan type is A1γ. The peptidoglycan type is A1γ, with meso-diaminopimelic acid as the diagnostic diamino acid. The DNA G+C content of the type strain BH1T is 60.0 mol% (Tm method).

The type strain is BH1T (=ATCC BAA-1257T =CECT 7585T =ICM 16411T =LMG 25388T), isolated from a rusticle sample obtained from the RMS Titanic.

Acknowledgements

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