Description of Spongiibacter borealis sp. nov., isolated from Arctic seawater, and reclassification of Melitea salexigens Urios et al. 2008 as a later heterotypic synonym of Spongiibacter marinus Graeber et al. 2008 with emended descriptions of the genus Spongiibacter and Spongiibacter marinus

Gwang Il Jang,1 Chung Yeon Hwang,1 Han-Gu Choi,2 Sung-Ho Kang2 and Byung Cheol Cho1

1Microbial Oceanography Laboratory, School of Earth and Environmental Sciences and Research Institute of Oceanography, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-742, Republic of Korea
2Division of Polar Biology and Ocean Sciences, Korea Polar Research Institute, Get-Pearl Tower, Songdo Technopark, 7-50 Songdo-dong, Yeonsu-gu, Incheon 406-840, Republic of Korea

A Gram-negative, rod-shaped and motile strain, designated CL-AS9T, was isolated from polar seawater of the Arctic. Analysis of the 16S rRNA gene sequence of the strain showed an affiliation with the genus Spongiibacter, sharing 93.9% and 93.7% sequence similarities with the type strains of Spongiibacter tropicus CL-CB221T and Spongiibacter marinus HAL40bT, respectively. Phylogenetic analyses revealed that strain CL-AS9T formed a separate branch that was distinct from a clade comprising Spongiibacter marinus HAL40bT, Spongiibacter tropicus CL-CB221T and Melitea salexigens 5IX/A01/131T. Cells of the strain grew optimally at 20–25°C and pH 6.6–8.0 in the presence of 3–4% (w/v) sea salts. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and an unidentified aminophospholipid. The major quinone was ubiquinone 8. The major cellular fatty acids were C16:1v7c and/or iso-C15:0 2-OH (23.1%), C17:1v8c (22.1%) and C18:1v7c (15.6%). The genomic DNA G+C content was 53.6 mol%. Based on the phylogenetic, chemotaxonomic and phenotypic data presented, we propose the name Spongiibacter borealis sp. nov. with the type strain CL-AS9T (=KCCM 90094T =JCM 17304T) and the reclassification of Melitea salexigens as a later heterotypic synonym of Spongiibacter marinus. We also provide emended descriptions of the genus Spongiibacter and Spongiibacter marinus.

The genus Spongiibacter in the class Gammaproteobacteria was established by Graeber et al. (2008) with Spongiibacter marinus as the type species. At the time of writing, two species of the genus Spongiibacter have been reported from marine environments: Spongiibacter marinus was isolated from a boreal sponge (Graeber et al., 2008) and Spongiibacter tropicus from a marine Synechococcus culture (Hwang & Cho, 2009). Both species required NaCl for growth (1–7% and 1–9% for Spongiibacter marinus and Spongiibacter tropicus, respectively) and had the same temperature range of 10 to 40°C for growth (Graeber et al., 2008; Hwang & Cho, 2009). The major fatty acids of the two species were C17:1v8c and C18:1v7c (Hwang & Cho, 2009).

Eight months after the proposal of the genus Spongiibacter, the genus Melitea was proposed by Urios et al. (2008) with Melitea salexigens, a novel gammaproteobacterium isolated from sea surface layer. However, the close taxonomic relationship between the genera Spongiibacter and Melitea was not noticed until recently. During the present study, we recognized that Spongiibacter marinus HAL40bT and Melitea salexigens 5IX/A01/131T shared 99.2% 16S rRNA
gene sequence similarity. This similarity value is higher than the threshold of 97% (Rossello-Mora & Amann, 2001) or 98.7–99% (Stackebrandt & Ebers, 2006) above which DNA–DNA relatedness experiments should be mandatory for confirming separate species.

In this study, a marine bacterium was isolated and subjected to a polyphasic taxonomic analysis. In addition, *Spongiibacter marinus* HALA0b and *M. salseigen* 5IX/A01/131T were compared using a polyphasic taxonomic approach to clarify the taxonomic positions of the two species.

Coastal seawater was collected near the Korean Arctic Dasan station at Svalbard in April 2010. An aliquot (100 µl) of seawater was spread on various media including marine agar 2216 (MA, Difco) and incubated under aerobic conditions at 4–20 °C for a month. Among 56 colonies isolated and identified by 16S rRNA gene sequencing (data not shown), only one strain (designated CL-AS9T) was affiliated with the genus *Spongiibacter*. Strain CL-AS9T has been subsequently purified on fresh MA. Strain CL-AS9T was maintained both on MA at 20 °C and in marine broth 2216 (MB, Difco) supplemented with 30% (v/v) glycerol at −80 °C.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by a boiling method (Englen & Kelley, 2000). The crude extract served as the DNA template for PCR, which included *Tag* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). The PCR product was purified by using the *AccuPrep* PCR purification kit (Bioneer) and direct sequence determination of the purified 16S rRNA gene was performed using sequencing primers (27F, 518F, 800R and 1492R; Lane, 1991; Anzai et al., 1997) with an Applied Biosystems automated sequencer (ABI3730XL) at Macrogen, Seoul, Korea. The almost complete 16S rRNA gene sequence of strain CL-AS9T (1429 bp) was obtained and compared with available 16S rRNA gene sequences in GenBank using *BLASTN* searches (Altschul et al., 1990). The 16S rRNA gene sequence of strain CL-AS9T (1429 bp) was aligned by the *jPHYDIT* program (Jeon et al., 2005) with the sequences of the type strains of other phylogenetically related species obtained from GenBank and Ribosomal Database Project II (Cole et al., 2007). Accurate multiple alignment was made manually according to the 16S rRNA secondary structure information implemented in the *jPHYDIT* program. Phylogenetic trees were obtained by use of the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications for the neighbour-joining and maximum-parsimony methods and 100 replications for the maximum-likelihood method. Phylogenetic analyses were carried out using *MEGA 4* (Tamura et al., 2007) and *PAUP*

4.0 (Swofford, 1998). Likelihood parameters were estimated by using the hierarchical ratio test in *MODELTEST*, version 3.04 (Posada & Crandall, 1998).

Morphological and physiological tests were performed as follows. Gram-staining was performed as described by Smibert & Krieg (1994). Unless otherwise specified, all biochemical characteristics of strain CL-AS9T and *Spongiibacter tropicus* CL-CB221T were based on cultures grown on MA at 25 °C. Motility of the cells was assessed by the hanging drop method (Skerman, 1967) with cells grown in MB for 5 days. Cellular morphology and the presence of flagella were observed using transmission electron microscopy (EX2; JEOL). Anaerobic growth was checked on MA by using the GasPak anaerobic system (BBL) at 25 °C for 3 weeks. The temperature range for growth was examined on the basis of colony formation on MA incubated at 4 °C and temperatures ranging from 5 to 45 °C, using increments of 5 °C. The pH range (pH 4.1–9.9, using increments of approximately 1 pH unit) for growth in MB was determined by assessing changes in optical density at a wavelength of 600 nm (OD600) over an incubation period of 10 days at 25 °C; prior to autolysis of the medium, its pH was adjusted using 1 M NaOH and 1 M HCl solutions. Salt tolerance of strain CL-AS9T was determined by assessing changes in OD600 in synthetic ZoBell broth (Bacto peptone, 5 g; yeast extract, 1 g; ferric citrate 0.1 g; distilled water, 1 l) with varying concentrations (0–10% in increments of 1%, and 15%, w/v) of NaCl and sea salts (Sigma) at 25 °C.

Oxidase and catalase tests were performed according to the protocols described by Smibert & Krieg (1994). Hydrolysis of DNA, gelatin, starch and Tweens 40 and 80, ornithine and lysine deaminase activities, and Voges–Proskauer and methyl red tests were determined according to Hansen & Sørheim (1991). H2S production was tested as described by Bruns et al. (2001). In addition, other enzyme activities were assayed in duplicate using the API ZYM and API 20NE kits (bioMérieux) according to the manufacturer’s instructions, except that the cell suspension was prepared using artificial seawater (NaCl, 24 g; MgCl2, 6H2O, 10.9 g; Na2SO4, 4 g; CaCl2, 2H2O, 1.5 g; KCl, 0.7 g; NaHCO3, 0.2 g; KBr, 0.1 g; H2BO3, 0.027 g; SrCl2, 6H2O, 0.03 g; NaF, 0.003 g; distilled water, 1 l; Lyman & Fleming, 1940). Carbon utilization was tested using the basal broth medium supplemented with yeast extract (NaCl, 23.6 g; KCl, 0.64 g; MgCl2, 6H2O, 4.53 g; MgSO4, 7H2O, 5.94 g; CaCl2, 2H2O, 1.3 g; NaNO3, 0.2 g; NH4Cl, 0.2 g; yeast extract, 0.05 g; distilled water, 1 l; Bruns et al., 2001) containing 0.4% carbon source. Growth was monitored by measuring OD600 using a spectrophotometer (Ultraspex 2000; Pharmacia Biotech) twice a week for approximately one month. Carbon utilization was scored as negative when growth rate was equal to or less than that in the negative control with no carbon source.

Polar lipids were extracted using the procedures described by Minnikin et al. (1984) and identified by two-dimensional
TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). The quinone system was determined according to Minnikin et al. (1984) and analysed by HPLC as described by Collins (1985) using Salinisphaera dokdonensis CL-ES53T (Bae et al., 2010) as a reference strain. The fatty acid methyl esters in whole cells of strain CL-AS9T grown on MA at 30 °C for 3 days were analysed by using gas chromatography according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center of Microorganisms (KCCM) in Seoul, Korea. The genomic DNA G+C content of strain CL-AS9T was analysed by HPLC (HP 100; Hewlett Packard) analysis of deoxyribonucleosides as described by Mesbah et al. (1989), after DNA was extracted by the method of Marmur (1961). Lambda phage DNA was used for calibration and the genomic DNA of Spongiibacter tropicus CL-CB221T as a reference.

To compare characteristics of Spongiibacter marinus HAL40bT and M. salexigens 5IX/A01/131T, the following experiments were performed for both strains at the same time. Unless otherwise specified, all characteristics of Spongiibacter marinus HAL40bT and M. salexigens 5IX/A01/131T were based on cultures grown on MA at 30 °C for 3 days, and the same methods were employed as described above. Genomic DNA–DNA relatedness was determined by dot-blot hybridization (Kim et al., 2007). Pre-hybridization, hybridization (temperature of 45.8 °C) and detection were performed by using a DIG labelling and detection kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The DNA–DNA hybridization experiment was repeated on two different days. Quinone system and polar lipids were determined as described above. Fatty acid methyl esters were analysed for cells of M. salexigens 5IX/A01/131T grown on MA at 30 °C for 3 days, which were identical to conditions employed for fatty acid analysis of other species of the genus Spongiibacter (Hwang & Cho, 2009; this study). Physiological tests (i.e. temperature, pH and NaCl ranges for growth) and all of the above biochemical tests were performed for Spongiibacter marinus HAL40bT and M. salexigens 5IX/A01/131T.

Sequence analysis of the 16S rRNA gene showed that strain CL-AS9T belonged to the class Gammaproteobacteria (Fig. 1). Strain CL-AS9T was most closely related to Spongiibacter tropicus CL-CB221T (93.9 % similarity) and next to Spongiibacter marinus HAL40bT (93.7 % similarity) and Melitaea salexigens 5IX/A01/131T (93.7 % similarity); no other species in the class Gammaproteobacteria shared more than 92 % sequence similarity with strain CL-AS9T.

Tree topologies inferred from three tree-making algorithms showed that strain CL-AS9T formed a separate branch that was distinct from a clade (hereafter referred to as the Spongiibacter clade) comprising Spongiibacter tropicus, Spongiibacter marinus and M. salexigens (Fig. 1). Thus, the low sequence similarities (93.7–93.9 %) between strain CL-AS9T and species with validly published names, and the phylogenetic position of strain CL-AS9T, indicated that our strain represents a novel species in the genus Spongiibacter.

The morphological, physiological and biochemical characteristics of strain CL-AS9T are given in the species description and Table 1. The major fatty acids of strain CL-AS9T were summed feature 3 (C16:1ω7c and/or iso-C15:0 2-0H; 23.1 %), C17:1ω8c (22.1 %) and C18:1ω7c (15.6 %). The presence of a high amount of summed feature 3 and similar amounts of C17:1ω8c and C18:1ω7c could clearly differentiate strain CL-AS9T from other species of the genus Spongiibacter and M. salexigens (Table 1). Other fatty acids of strain CL-AS9T are shown in Supplementary Table S1 (available in IJSEM Online). The major polar lipids of strain CL-AS9T were diphosphatidylglycerol, phosphatidylglycerol and an unidentified aminophospholipid (Supplementary Figure S1). The absence of two unidentified aminophospholipids (APL2 and APL4) could differentiate strain CL-AS9T from Spongiibacter marinus and M. salexigens (Supplementary Figure S1). The genomic DNA G+C content of strain CL-AS9T was 53.6 mol%, which

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**Fig. 1.** Neighbour-joining tree showing the phylogenetic positions of strain CL-AS9T and related members of the class Gammaproteobacteria on the basis of 16S rDNA gene sequence. Only bootstrap values above 60 % are shown (1000 resamplings) at branching points. Solid circles indicate that the corresponding nodes were also obtained in the maximum-likelihood and maximum-parsimony trees. Thermotoga maritima MSB8T (M21774) was used as an outgroup. Bar, 0.02 nucleotide substitutions per site.
is lower (>3.6 mol%) than those of members of the *Spongiibacter* clade (Table 1).

Furthermore, strain CL-AS9T can be differentiated from other species in the *Spongiibacter* clade by the ability to grow at 4–5 °C, inability to grow at 35 °C or above and inability to grow in the presence of NaCl as sole salt (Table 1). Strain CL-AS9T can be distinguished from *Spongiibacter tropicus* by the ability to hydrolyse Tween 40. Strain CL-AS9T can be distinguished from *Spongiibacter marinus* and *M. salexigens* by the inability to produce lipase (C14) and leucine arylamidase, and the ability to utilize pyruvate as sole carbon source (Table 1).

To determine the taxonomic positions of *Spongiibacter marinus* HAL40bT and *M. salexigens* 5IX/A01/131T, a polyphasic approach was employed. Phylogenetic analyses of 16S rRNA gene sequences of both type strains revealed that they formed a robust clade within the genus *Spongiibacter* (Fig. 1) with a similarity of 99.2%. This grouping was supported by high bootstrap values (neighbour-joining, 100%; maximum-likelihood, 100%; and maximum-parsimony, 99%). The level of DNA–DNA relatedness between *Spongiibacter marinus* HAL40bT and *M. salexigens* 5IX/A01/131T was 83±5% (reciprocal 85±4%), indicating that they belonged to the same species (Rossello-Mora & Amann, 2001). Fatty acid patterns were similar between *Spongiibacter marinus* HAL40bT and *M. salexigens* 5IX/A01/131T (Supplementary Table S1). The major quinone was ubiquinone 8 (Q-8). The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and unidentified aminophospholipid APL1 for both species (Supplementary Figure S1). Phenotypic characteristics were mostly identical (32 of 33 enzyme activities and 17 of 20 sole carbon source utilization) between *Spongiibacter marinus* HAL40bT and *M. salexigens* 5IX/A01/131T.

Overall, the phylogenetic, chemotaxonomic and phenotypic data obtained in this study indicate that strain CL-AS9T should be assigned to a novel species in the genus *Spongiibacter*, for which the name *Spongiibacter borealis* sp. nov. is proposed. In addition, it is proposed to unite the species *Melitea salexigens* Urios et al. 2008 and *Spongiibacter marinus* Graeber et al. 2008. According to Rules 38, 42 and 24b(2) of the Bacteriological Code (Lapage et al., 1992), the name *Spongiibacter marinus* has

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**Table 1. Selected characteristics that differentiate strain CL-AS9T from other species in the *Spongiibacter* clade**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Arctic seawater</td>
<td><em>Synechococcus</em> culture</td>
<td>Marine sponge</td>
<td>Seawater</td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>5.8–9.3 (6.6–8.0)</td>
<td>6–10 (7–8)</td>
<td>6.5–9.5 (7–9)</td>
<td>6–10 (8)</td>
</tr>
<tr>
<td>Growth in the presence of NaCl as sole salt*</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of Tween 40*</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>API ZYM test*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N-acetyl-β-D-glucosaminidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Esterase (C4)</td>
<td>+</td>
<td>W</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Lipase (C14)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Arabinose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>W</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Major fatty acids (%)</td>
<td>7.4</td>
<td>12.9–14.4</td>
<td>3.2</td>
<td>3.9</td>
</tr>
<tr>
<td>C16:0</td>
<td>7.2</td>
<td>4.4–4.6</td>
<td>11.2</td>
<td>17.3</td>
</tr>
<tr>
<td>C17:0</td>
<td>22.1</td>
<td>14.7–15.3</td>
<td>44.1</td>
<td>35.8</td>
</tr>
<tr>
<td>C18:1ω8c</td>
<td>15.6</td>
<td>31.2–31.5</td>
<td>6.8</td>
<td>5.9</td>
</tr>
<tr>
<td>C18:1ω7c</td>
<td>2.1</td>
<td>2.1–2.5</td>
<td>6.3</td>
<td>6.5</td>
</tr>
<tr>
<td>Summed feature 3 (C16:1ω7c / iso-C15:0 2-OH)</td>
<td>23.1</td>
<td>11.4–12.5</td>
<td>6.3</td>
<td>4.8</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>53.6</td>
<td>57.7</td>
<td>69.1 (60.6)</td>
<td>57.2</td>
</tr>
</tbody>
</table>

*Data for *Spongiibacter marinus* HAL40bT and *Melitea salexigens* 5IX/A01/131T were obtained from this study.
†Data in parentheses are from Urios et al. (2008).
‡Data from Hwang & Cho (2009).
priority and hence should be used for the unified taxon, with *Melitea salexigens* as a later heterotypic synonym.

**Emended description of Spongiibacter Graeber et al. 2008**

The characteristics of the genus are as described by Graeber *et al.* (2008), with the following amendment. Motility is variable. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and an unidentified aminophospholipid. The major quinone is Q-8.

**Emended description of Spongiibacter marinus Graeber et al. 2008**

The characteristics of the species are as described by Graeber *et al.* (2008) with the following amendment. Motility is dependent, but acetate, arabinose, cellobiose, citrate, fructose, galactose, glycerol, glucose and mannose as sole carbon source is strain with *Melitea salexigens* priority and hence should be used for the unified taxon, Emended description of *Spongiibacter*.

The major quinone is ubiquinone 8. The major cellular fatty acids are C\(_{16:0}\)-7c and/or iso C\(_{15:0}\)-2-0H, C\(_{17:1}\)-08c and C\(_{18:1}\)-07c. The DNA G+C content of the type strain is 53.6 mol%.

The type strain, CL-AS9\(^T\) (=KCCM 90094\(^T\) =JCM 17304\(^T\)), was isolated from Arctic seawater.

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