Spongiibacter tropicus sp. nov., isolated from a *Synechococcus* culture

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Two Gram-staining-negative, rod-shaped and non-motile strains, designated CL-CB221^T and CL-CB467, were isolated from a *Synechococcus* culture derived from tropical surface water of the Pacific Ocean. The 16S rRNA gene sequences of the two strains were identical, and it was found that they belonged to the class *Gammaproteobacteria*, with *Spongiibacter marinus* HAL40b^T as their closest relative (similarity of 96.3 %). Both strains grew optimally at 30–35 °C and pH 7–8 in the presence of 3–4 % (w/v) NaCl. The major cellular fatty acids were C_{18:1}ω7c, C_{17:1}ω8c, C_{16:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH). The genomic DNA G + C contents were 57.7 and 57.8 mol%, respectively. DNA–DNA hybridization experiments revealed high values (97 ± 2 %) for relatedness between strains CL-CB221^T and CL-CB467, which suggested that these two strains belong to a single species. Based on the phylogenetic, chemotaxonomic and phenotypic data presented, it is proposed that strains CL-CB221^T and CL-CB467 represent a novel species of the genus *Spongiibacter*, for which the name *Spongiibacter tropicus* sp. nov. is proposed. The type strain is CL-CB221^T (=KCCM 90065^T =DSM 19543^T).

The genus *Spongiibacter* was established by Graeber *et al.* (2008) with *Spongiibacter marinus* as the type species for a motile marine bacterium that was isolated from a boreal sponge and required NaCl for growth (1–7 %, w/v). At the time of publication, *Spongiibacter marinus* HAL40b^T was found to be phylogenetically distantly related to the genera *Neptunomonas* and *Marinobacter* in the class *Gammaproteobacteria* with low 16S rRNA gene sequence similarities (about 90 %). The major fatty acids (C_{17:1}ω8, C_{17:0} and C_{18:1}ω7) of *Spongiibacter marinus* HAL40b^T supported the proposal of a novel species within a novel genus (Graeber *et al.*, 2008). In this study, two strains isolated from a *Synechococcus* culture were subjected to a polyphasic taxonomic analysis and allocated to the genus *Spongiibacter*.

A *Synechococcus* culture was derived from tropical surface water of the Pacific Ocean and maintained by transferring a small quantity of the culture to f/2 medium (Guillard & Ryther, 1962) supplemented with ammonium chloride (final concentration 100 μM). The culture was incubated at 25 °C under approx. 20 μE m⁻² s⁻¹ (a 14 h light/10 h dark cycle). To isolate bacteria in the *Synechococcus* culture, an aliquot (30 μl) of the culture in the exponential growth phase was spread on a marine agar 2216 (MA; Difco) plate,

and the plate was aerobically incubated at 25 °C for 1 week. Strains CL-CB221^T and CL-CB467 were isolated and subsequently streaked onto fresh MA plates. The purification procedure was repeated four times. The strains were maintained on MA at 4 °C and preserved in marine broth 2216 (MB; Difco) supplemented with 30 % (v/v) glycerol at –80 °C.

For 16S rRNA gene sequence amplification by PCR, DNA was extracted from single colonies by a boiling method (Englen & Kelley, 2000) and the crude extract served as the template for amplification with *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). PCR products were purified with the AccuPrep PCR purification kit (Bioneer) and direct sequence determination was performed with an automated sequencer (ABI 3730xl; Applied Biosystems) at Macrogen (Seoul, Korea). The almost-complete 16S rRNA gene sequences of strains CL-CB221^T (1406 bp) and CL-CB467 (1409 bp) were obtained and compared with available 16S rRNA gene sequences in GenBank using BLASTN searches (Altschul *et al.*, 1990). The sequences were manually aligned with those of related species in the class *Gammaproteobacteria*, obtained from the GenBank and Ribosomal Database Project II (Cole *et al.*, 2007) databases, using known 16S rRNA secondary-structure information. Phylogenetic trees were obtained by use of 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

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains CL-CB221^T and CL-CB467 are respectively EF988653 and EF988654.

A supplementary table showing cellular fatty acid compositions is available with the online version of this paper.

robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications for the neighbour-joining and the maximum-parsimony methods and 100 replications for the maximum-likelihood method. Alignment analysis was carried out using the jPHYDIT program (Jeon *et al.*, 2005), and phylogenetic analyses were carried out using MEGA 4 (Tamura *et al.*, 2007) and PAUP 4.0 (Swofford, 1998). Likelihood parameters were estimated by using 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 strains CL-CB221^T and CL-CB467 and *Spongiibacter marinus* HAL40b^T were determined using cultures grown on MA at 30 °C. Motility of the cells was assessed by the hanging-drop method (Skerman, 1967) with cells grown in MB for 3 days. Cellular morphology and the presence of flagella were observed using transmission electron microscopy (EX2; JEOL). Anaerobic growth was checked on MA and ZOF medium (Lemos *et al.*, 1985) supplemented with agar (1.5 %) by using the GasPak anaerobic system (BBL) at 30 °C for 15 days. The temperature range for growth was examined on the basis of colony formation on MA incubated at temperatures ranging from 5 to 45 °C (in increments of 5 °C). The pH range for growth was determined by assessing changes in OD₆₀₀ in pH-buffered MB (pH 5–11, in increments of 1 pH unit) (Manaia *et al.*, 2003; Yumoto *et al.*, 2004) at 30 °C for up to 7 days. The tolerance of strains CL-CB221^T and CL-CB467 to NaCl was determined by assessing changes in OD₆₀₀ in synthetic ZoBell broth (l⁻¹ distilled water: 5 g Bacto peptone; 1 g yeast extract; 0.1 g ferric citrate) with 0–10 % (in increments of 1 %) and 15 % NaCl at 30 °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 (l⁻¹ distilled water: 24 g NaCl; 10.9 g MgCl₂·6H₂O; 4 g Na₂SO₄; 1.5 g CaCl₂·2H₂O; 0.7 g KCl; 0.2 g NaHCO₃; 0.1 g KBr; 0.027 g H₃BO₃; 0.03 g SrCl₂·6H₂O; 0.003 g NaF; Lyman & Fleming, 1940). Carbon utilization was tested using the basal broth medium supplemented with yeast extract (l⁻¹ distilled water: 23.6 g NaCl; 0.64 g KCl; 4.53 g MgCl₂·6H₂O; 5.94 g MgSO₄·7H₂O; 1.3 g CaCl₂·2H₂O; 0.2 g NaNO₃; 0.2 g NH₄Cl; 0.05 g yeast extract; Bruns *et al.*, 2001) containing 0.4 % carbon source. Growth was monitored by OD₆₀₀ using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech). OD₆₀₀ was measured every week for

about 1 month. Carbon utilization was scored as negative when the growth rate was equal to or less than that in the negative control with no carbon source.

Fatty acid methyl esters in whole cells of strains CL-CB221^T and CL-CB467 and *Spongiibacter marinus* HAL40b^T grown on MA at 30 °C for 3 days were analysed by gas chromatography according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center of Microorganisms (Seoul, Korea). Genomic DNA of strains CL-CB221^T and CL-CB467 and *Spongiibacter marinus* HAL40b^T was extracted by the method of Marmur (1961) and G+C contents were determined by HPLC (HP 100; Hewlett Packard) of deoxyribonucleosides as described by Mesbah *et al.* (1989). Lambda phage DNA was used as a standard and the genomic DNA of *Salinisphaera shabanensis* E1L3A^T (62 mol%; Antunes *et al.*, 2003) and *Maribacter arcticus* KOPRI 20941^T (36 mol%; Cho *et al.*, 2008) was used as references. The relatedness of genomic DNA between strains CL-CB221^T and CL-CB467 was determined by dot-blot hybridization as described by Choi *et al.* (2006). The experiment was repeated on different days.

The 16S rRNA gene sequences of strains CL-CB221^T and CL-CB467 were identical. Phylogenetic analyses based on the 16S rRNA gene sequence showed that both strains belonged to the class *Gammaproteobacteria* (Fig. 1). They were most closely related to *Spongiibacter marinus* HAL40b^T, with 96.3 % similarity, and no other species in the class *Gammaproteobacteria* shared more than 92 % sequence similarity with the isolates. The tree topologies inferred from three tree-making algorithms showed that strains CL-CB221^T and CL-CB467 formed a robust cluster with the genus *Spongiibacter* (Fig. 1). This grouping was supported by high bootstrap values (neighbour-joining, 100 %; maximum-parsimony and maximum-likelihood, 99 %). Thus, it is clear that our isolates belong to the genus *Spongiibacter*. However, the low similarity value (96.3 %) between the 16S rRNA gene sequences of the two isolates and *Spongiibacter marinus* HAL40b^T indicated that strains CL-CB221^T and CL-CB467 represent a novel species in the genus (Rosselló-Mora & Amann, 2001).

Strains CL-CB221^T and CL-CB467 gave identical results for morphological, physiological and biochemical characteristics (except for the utilization of succinate as a sole carbon source) and the results are given in the species description and Table 1. The genomic DNA G+C contents were 57.7 and 57.8 mol%, respectively. DNA–DNA hybridization experiments revealed high values (97 ± 2 %) for relatedness between strains CL-CB221^T and CL-CB467. DNA–DNA hybridization, phenotypic and chemotaxonomic characteristics revealed that they belonged to the same species. The fatty acid profiles were generally similar between the two strains (Supplementary Table S1, available in IJSEM Online); dominant fatty acids for the strains were C_{18:1ω7c} (27.3–31.5 %), followed by C_{17:1ω8c} (14.3–15.3 %), C_{16:0} (12.9–14.4 %) and summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 11.4–16.2 %).

Fig. 1. Neighbour-joining tree derived from 16S rRNA gene sequences for strains CL-CB221^T and CL-CB467 and related members in the class Gammaproteobacteria. The sequence of *Thermotoga maritima* MSB8^T (GenBank accession no. M21774) was used as the outgroup (not shown). Bootstrap values ($\geq 60\%$) based on 1000 resamplings are shown as percentages at branch nodes. The solid circle indicates that the corresponding node was also recovered in the maximum-parsimony and maximum-likelihood trees. Bar, 0.02 substitutions per nucleotide position.

Certain chemotaxonomic and phenotypic characteristics can differentiate strains CL-CB221^T and CL-CB467 from the phylogenetically closest species, *Spongiibacter marinus*: the ability of the former strains to utilize pyruvate as a sole carbon source and inability to produce *N*-acetyl- β -glucosaminidase, leucine arylamidase and lipase (C14), to hydrolyse Tween 40 and to utilize arabinose (Table 1).

Overall, the phylogenetic, chemotaxonomic and phenotypic data obtained in this study indicate that strains CL-CB221^T and CL-CB467 should be assigned to a novel species in the genus *Spongiibacter*, for which the name *Spongiibacter tropicus* sp. nov. is proposed.

Description of *Spongiibacter tropicus* sp. nov.

Spongiibacter tropicus [tro'pi.cus. L. masc. adj. *tropicus* tropical, of or pertaining to the tropic(s), relating to its isolation from a tropical ocean].

Gram-staining-negative, strictly aerobic, non-motile, rods approximately 0.4–0.5 μm wide and 0.9–2.0 μm long. After 5 days on marine agar plates at 30 °C, colonies are creamy, circular and convex, and approximately 1 mm in diameter. Grows at 10–40 °C (optimum 30–35 °C), pH 6–10 (optimum pH 7–8) and with 1–9% (w/v) NaCl (optimum 3–4%). Positive for oxidase and catalase. Negative for ornithine and lysine deaminase activities, Voges–Proskauer and methyl red tests. Tween 80 is hydrolysed, but DNA, gelatin, starch and Tween 40 are not. H₂S is not produced. According to API ZYM, positive for acid and alkaline phosphatases, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase, but negative for *N*-acetyl- β -glucosaminidase, α -chymotrypsin, cystine arylamidase, α -fucosidase, α - and β -galactosidases, α - and β -glucosidases, β -glucuronidase, leucine arylamidase, lipase (C14), α -mannosidase, trypsin and valine arylamidase. According to API 20NE, positive for aesculin hydrolysis, but negative for arginine dihydrolase, β -galactosidase

Table 1. Selected characteristics that differentiate strains CL-CB221^T and CL-CB467 from *Spongiibacter marinus* HAL40b^T

Data were taken from this study and Graeber *et al.* (2008). Congruent results were obtained for strains CL-CB221^T and CL-CB467 unless stated. +, Positive; w, weak; –, negative.

Characteristic	CL-CB221 ^T	<i>Spongiibacter marinus</i> HAL40b ^T
Isolation source	<i>Synechococcus</i> culture	Marine sponge
Cell size (width $\times$ length) (μm)	0.4–0.5 $\times$ 0.9–2.0	0.4–0.6 $\times$ 1–2
Motility	–	+
Conditions for growth		
Temperature [optimum] (°C)	10–40 [30–35]	10–40 [20–30]
pH [optimum]	6–10 [7–8]	6.5–9.5 [7–9]
NaCl tolerance [optimum] (%)	1–9 [3–4]	1–7 [3]
Hydrolysis of Tween 40	–	+
API ZYM tests		
<i>N</i> -Acetyl- β -glucosaminidase	–	+
Leucine arylamidase	–	+
Lipase (C14)	–	+*
Utilization of:		
Arabinose	–	+
Pyruvate	+	–
Succinate	– (w)†	–
DNA G + C content (mol%)	57.7 (57.8)†	69.1‡

*From this study.

†Result for strain CL-CB467 in parentheses.

‡Data from Graeber *et al.* (2008); a value of 60.6 mol% was obtained in this study.

(PNPG) and gelatinase, glucose fermentation, indole production, nitrate reductase and urease. Pyruvate is utilized as a sole carbon source, but acetate, arabinose, L-arginine, cellobiose, citrate, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, lactose, mannitol, D-mannose, L-ornithine, L-rhamnose, D-salicin, sorbitol, succinate (weak in strain CL-CB467) and xylose are not utilized. The major cellular fatty acids are C_{18:1}ω7c, C_{17:1}ω8c, C_{16:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH). The DNA G+C content of the two known strains is 57.7–57.8 mol%.

The type strain is CL-CB221^T (=KCCM 90065^T =DSM 19543^T), isolated from a *Synechococcus* culture. Strain CL-CB467 is a second strain of the species.

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