

Spongiibacter marinus gen. nov., sp. nov., a halophilic marine bacterium isolated from the boreal sponge *Haliclona* sp. 1

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Strain HAL40b^T was isolated from the marine sponge *Haliclona* sp. 1 collected at the Sula Ridge off the Norwegian coast and characterized by physiological, biochemical and phylogenetic analyses. The isolate was a small rod with a polar flagellum. It was aerobic, Gram-negative and oxidase- and catalase-positive. Optimal growth was observed at 20–30 °C, pH 7–9 and in 3% NaCl. Substrate utilization tests were positive for arabinose, Tween 40 and Tween 80. Enzyme tests were positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl- β -glucosaminidase. The predominant cellular fatty acid was C_{17:1 ω 8}, followed by C_{17:0} and C_{18:1 ω 7}. Analysis by matrix-assisted laser desorption/ionization time-of-flight MS was used to characterize the strain, producing a characteristic low-molecular-mass protein pattern that could be used as a fingerprint for identification of members of this species. The DNA G + C content was 69.1 mol%. Phylogenetic analysis supported by 16S rRNA gene sequence comparison classified the strain as a member of the class *Gammaproteobacteria*. Strain HAL40b^T was only distantly related to other marine bacteria including *Neptunomonas naphthovorans* and *Marinobacter daepoensis* (type strain sequence similarity >90%). Based on its phenotypic, physiological and phylogenetic characteristics, it is proposed that the strain should be placed into a new genus as a representative of a novel species, *Spongiibacter marinus* gen. nov., sp. nov.; the type strain of *Spongiibacter marinus* is HAL40b^T (=DSM 17750^T =CCUG 54896^T).

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Abbreviations: FAMES, fatty acid methyl esters; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HAL40b^T is AM117932.

A MALDI-TOF MS profile showing characteristic peaks and fatty acid composition data of *Spongiibacter marinus* HAL40b^T are available as supplementary material with the online version of this paper.

In the last few years, interest has focussed on the analysis of microbial populations in different marine habitats as well as on marine bacteria as sources of natural products (Dobretsov *et al.*, 2006; Piel, 2006; Taylor *et al.*, 2007). In addition to investigations of the bacterial assemblage from coastal regions (Kan *et al.*, 2006), the open sea (Ghiglione *et al.*, 2005), deep-sea areas (DeLong *et al.*, 2006) and from sediments (Edlund *et al.*, 2006), marine eukaryotes like algae (Crocchi *et al.*, 2006) and invertebrates (Holmström & Kjelleberg, 1999; Sfanos *et al.*, 2005) have also been targets for analysis.

Marine sponges have attracted attention specifically as they often harbour a diverse range of bacteria either resident in the mesohyl of the sponge or transiently within the channel system, where rapid exchange of surrounding water occurs. Microbial communities have been analysed in a number of marine sponges originating mostly from Mediterranean, tropical and temperate regions (Webster & Hill, 2001; Webb & Maas, 2002; Fieseler *et al.*, 2004; Taylor *et al.*, 2004); cold-water sponges (Althoff *et al.*, 1998; Thiel *et al.*, 2002; Webster *et al.*, 2004) have rarely been examined. Based on cultivation-independent methods, a wide spectrum of bacteria has been shown to be in close association with the sponge host with representatives encompassing the bacilli, high-G+C-containing Gram-positive bacteria, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, *Planctomycetes* and *Verrucomicrobia*, as well as proteobacteria (Webster *et al.*, 2001; Hentschel *et al.*, 2002). Cultivation and isolation of individual strains from sponges have revealed that proteobacteria are a major component of the bacterial community; in particular, gamma- and alphaproteobacteria have been found to be abundant (De Rosa *et al.*, 2000; Hentschel *et al.*, 2001; Lafi *et al.*, 2005).

Our research objects were boreal sponges of the class Demospongiae and, among them, the sponge *Haliclona* sp. 1 was collected from a depth of approximately 300 m at the Sula Ridge, a deep cold-water reef complex stretching along the mid-Norwegian coast. A number of bacteria (183; data not shown) was isolated from this sponge, most of which were affiliated to the classes *Gammaproteobacteria* and *Alphaproteobacteria* (Dieckmann *et al.*, 2005; and data not shown). The strain described here represents a taxon that has been isolated only from *Haliclona*, not from other cold-water sponge species, and is only distantly related to members of the *Gammaproteobacteria* with validly published names.

Sampling was performed with a manned submersible, enabling precise selection of the desired sponges with minimal damage to the reef. Sections of the sponge were homogenized, serially diluted in sterile seawater and spread on marine broth agar (MBA 2216; Difco). The plates were kept cold (8 °C) during the initial cultivation; purification to single colonies was performed at room temperature.

Colony morphology was analysed on MBA by stereomicroscopy. Colonies were 0.2–0.4 mm after 5 days of incubation at room temperature, beige, nearly transparent and possessed a circular convex shape and a shiny surface. Cellular morphology and motility were examined by phase-contrast microscopy from wet mounts of bacterial cells on agar-coated slides grown in liquid cultures for 2 days at room temperature and by transmission electron microscopy with a CM100 (Philips) equipped with a FastScan 1k × 1k CCD camera (TVIPS). Cells were grown for 5 days in sterile filtered marine broth, concentrated by centrifugation and either negatively stained by uranyl acetate according to Steven *et al.* (1988) (Fig. 1a) or embedded in epoxy resin according to Spurr (1969) for the

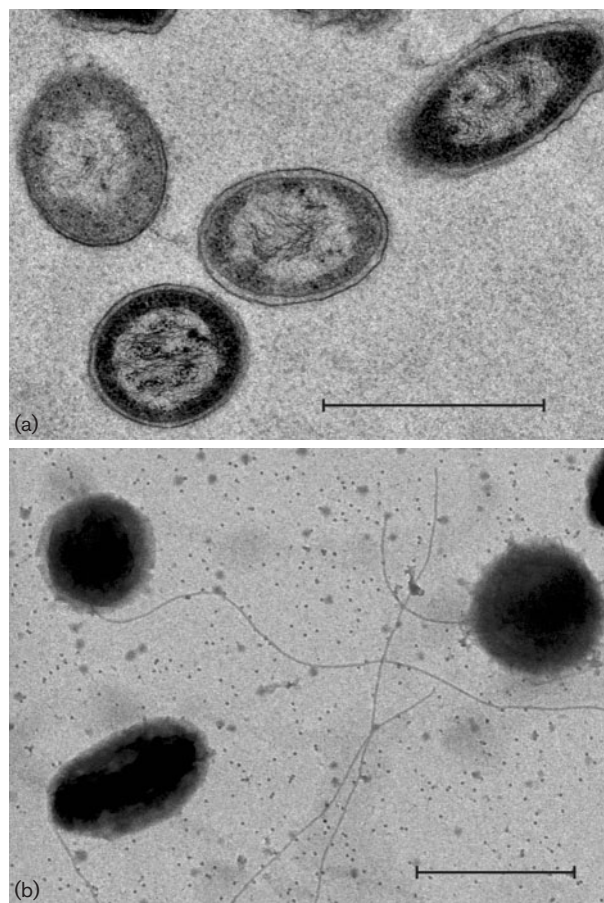


Fig. 1. Transmission electron micrographs of strain HAL40b^T. (a) Cell shape and nuclear material shown after ultrathin sectioning; (b) cell shape and single polar flagellum shown after negative staining with 0.8% uranyl acetate. Bars, 1 µm.

examination of ultrathin sections (Fig. 1b). The analysis revealed short, motile rods, 1.0–2.0 × 0.4–0.6 µm, with a single polar flagellum.

During the isolation campaign, whole-cell MS analysis [matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS] in the mass range 2–20 kDa was used to characterize and deduplicate strains and to identify unique isolates. Colonies of cells growing on MBA plates were transferred directly onto a stainless steel sample plate and mixed immediately with 1 µl matrix solution [10 mg 2,5-dihydroxybenzoic acid ml⁻¹ in acetonitrile/methanol/water (1:1:1) and 0.3% trifluoroacetic acid]. The sample/matrix mixture was allowed to air dry prior to analysis. MS measurements were performed on a Voyager DE-PRO time-of-flight MS (Applied Biosystems) according to Dieckmann *et al.* (2005). The SARAMIS software (AnagnosTec) was used for archiving and classification of mass spectra. Information on the SARAMIS system and database can be obtained from <http://www.anagnostec.eu>. The majority of peaks clustered between masses of 2 and

10 kDa. The method revealed several clusters of bacteria representing the taxonomic groups of gamma- and alphaproteobacteria, bacilli and sphingobacteria (Dieckmann *et al.*, 2005). Such a composition of the culturable microbial community is not unusual for a marine sponge (Hentschel *et al.*, 2001; Taylor *et al.*, 2007; and references therein). Sponges often harbour a huge mass of bacteria in their mesohyl, where they either reside permanently in parasitic, commensalistic or symbiotic relationships or are part of the quick turnover with the surrounding seawater. Strain HAL40b^T was detected on nutrient-rich medium as a very slowly growing gammaproteobacterium. With the methods used, its status in the sponge and its frequency of occurrence could not be determined. The MALDI-TOF MS intact-cell profile of HAL40b^T (see Supplementary Fig. S1 available in IJSEM Online) with its characteristic low-molecular-mass protein pattern was found to be unique when compared with mass data obtained from all other strains isolated during the campaign by using the SARAMIS software (data not shown).

Sequence determination of strain HAL40b^T was done by amplification of the 16S rRNA gene region by PCR using primers 16Sf and 16Sr. Standard primers 357F, 530R, 1100F, 1230F and 1390R were used for sequencing the nearly full-length 16S rRNA gene (1452 bp) according to *Escherichia coli* standard positions (Brosius *et al.*, 1978). Sequence data were analysed with an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Phylogenetic affiliation was performed with the ARB software (Ludwig *et al.*, 2004), in addition to sequence comparison to the EMBL/GenBank nucleotide sequence databases. The phylogenetic tree was calculated with 16S rRNA gene sequences (1409 bp; *E. coli* positions 51–1460) by using the neighbour-joining (Jukes–Cantor correction), maximum-parsimony and maximum-likelihood methods implemented in ARB. Different filters were used for calculations. Data

revealed that the strain belonged to the class *Gammaproteobacteria*. This class represents a large phylogenetic group of species found in most marine environments, which are generally well represented in culturing assays. The sequence was aligned against those of a number of close relatives identified from a BLAST query of the GenBank database. Highest sequence similarities (99 %) were found to a planktonic strain RED77 (Pinhassi & Berman, 2003; GenBank accession no. AY136133), isolated from Red Sea dilution cultures (with only 770 bp available for alignment) and to an isolate from the sea surface microlayer obtained from a coastal environment (Agogué *et al.*, 2005a, b; GenBank accession no. AY576729). An epibiotic bacterium originating from diatoms in natural coastal waters showed 96 % sequence similarity (Kaczmarek *et al.*, 2005; GenBank accession no. AY548769). The characterizations of these bacteria were restricted to only morphological and/or phylogenetic analysis; no biochemical or physiological data were determined. Also, 96 % similarity was described for strain NEP4, which was isolated from marine coastal sediment (Maeda *et al.*, 2005; GenBank accession no. AB212803). Lower similarity (91 %) was found to a marine strain with specific degrading capacities (GenBank accession no. AB086227). Uncultured bacterial sequences (GenBank accession nos DQ395870, AF468306 and DQ906761) from marine habitats clustered with sequence similarities of 90–93 %. A number of *Pseudomonas* strains were distantly affiliated to HAL40b^T, with sequence similarities of 89 %. The closest relatives to strain HAL40b^T with validly published names included the type strains of *Marinobacter daeopoensis* (Yoon *et al.*, 2004) and *Neptunomonas naphthovorans* (Hedlund *et al.*, 1999), with sequence similarities of about 90 %. A phylogenetic tree was constructed on the basis of these sequences (Fig. 2), revealing a branch for HAL40b^T that was separate from these two type strains and from the cluster of *Pseudomonas* strains.

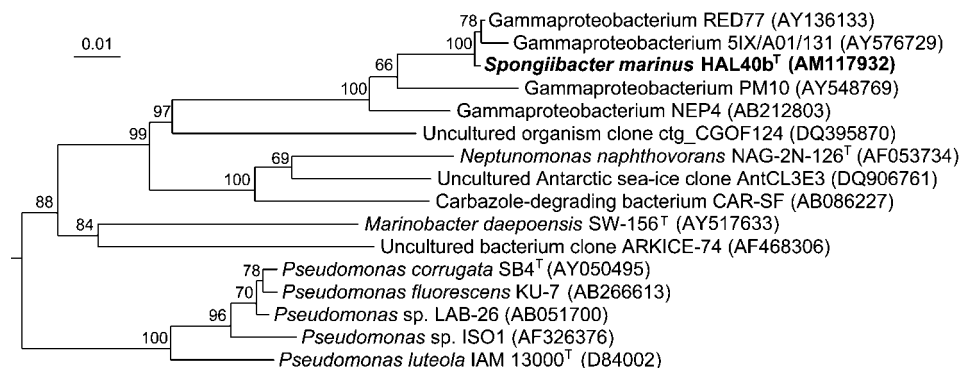


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of strain HAL40b^T and related gammaproteobacteria. The tree was calculated with the maximum-parsimony method. The short sequence of strain RED77 (770 bp) was added to the calculated tree by the quick add function of ARB. Numbers given at branch nodes indicate bootstrap values (%) for 1000 replicates. Accession numbers of the sequences are shown. Bar, 1 % sequence divergence. *Thermotoga maritima* MSB8^T (GenBank accession no. M21774) was used as an outgroup (not shown).

Temperature range and optimum for growth of strain HAL40b^T were tested at 2–45 °C in marine broth (Difco). The pH range and optimum for growth were determined at pH 5–10 at 30 °C in a medium containing peptone (1 g l⁻¹), yeast extract (5 g l⁻¹) and 3% (w/v) NaCl. Salt concentration range and optimum for growth were investigated in 0–9% NaCl at 30 °C. Comparative analysis of HAL40b^T with *M. daeponensis* and *N. naphthovorans* revealed a number of differences (Table 1). Cells of HAL40b^T were remarkably smaller and the temperature and pH growth profiles differed greatly. Growth of strain HAL40b^T occurred at 10–40 °C and was best at 20–30 °C. The pH range for growth was 6.5–9.5 and maximum growth rates were observed at pH 7–9. Growth was observed in media containing NaCl concentrations of 1–7%, with maximum growth at 3% NaCl, highlighting the marine origin of the bacterium. However, the salt tolerance was higher for *M. daeponensis* (18% NaCl).

Tests for the oxidation of various substrates were carried out using microplates (Biolog) for Gram-negative bacteria and API 20E and API ZYM test kits (bioMérieux) according to the manufacturers' recommendations. After a growth period of 4 days in marine broth at 18 °C, cells were resuspended in artificial seawater (Lyman & Fleming, 1940) for the Biolog test and in 0.6% NaCl for the API ZYM and API 20E kits. Test strips from API ZYM and API 20E kits were read after 2 days and Biolog plates were inoculated for up to 5 days. Catalase and oxidase activities were tested with test strips from Merck, according to the manufacturer's manual. Tests were performed in duplicate. HAL40b^T showed positive activity for alkaline and acid phosphatase, esterase (C4), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase,

N-acetyl- β -glucosaminidase and esterase lipase (C8). The strain was able to use arabinose, Tween 40 and Tween 80 for growth. HAL40b^T, *M. daeponensis* and *N. naphthovorans* were all Gram-negative, motile by means of a single polar flagellum and oxidase- and catalase-positive. They showed similar behaviour in the API ZYM, API 20E and Biolog tests (Table 1). However, limited substrate and enzyme data were available for *N. naphthovorans*, which made a detailed biochemical comparison with this strain impossible. Some physiological data were available for strain NEP4 (Maeda *et al.*, 2005). This strain was also Gram-negative, motile, and oxidase- and catalase-positive and showed similar growth to HAL40b^T at pH 7–9. In contrast to strain HAL40b^T, strain NEP4 grew at a higher temperature range (20–40 °C) and was able to utilize acetate, pyruvate, propionate, succinate, lactate, tetraethylene glycol and polyethylene glycol 300 as sole carbon sources.

Anaerobic growth was tested on MBA in glass tubes under anaerobic conditions (80% N₂/20% CO₂) and by using the Anaerocult System (Merck). Growth of HAL40b^T was strictly aerobic, although the strain survived for 3 weeks without gaseous oxygen and resumed growth after aeration.

Analysis of the DNA G+C content was done at the DSMZ (Braunschweig, Germany). Calibration of the method was performed using non-methylated lambda-DNA possessing a known G+C content of 49.9 mol% (Mesbah *et al.*, 1989), as well as three genomic DNAs for which complete genome sequences have been published, including *Bacillus subtilis* DSM 402 (G+C content 43.5 mol%), *Xanthomonas campestris* pv. *campestris* DSM 3586^T (65.1 mol%) and *Streptomyces violaceoruber* DSM 40783 (72.1 mol%).

Table 1. Phenotypic and physiological characteristics of strain HAL40b^T and the type strains of two related organisms

Data for *M. daeponensis* SW-156^T and *N. naphthovorans* NAG-2N-126^T were taken from Yoon *et al.* (2004) and Hedlund *et al.* (1999), respectively. Cells of all three strains were motile short rods, positive for alkaline and acid phosphatase, oxidase and catalase. HAL40b^T and *M. daeponensis* SW-156^T are positive for esterase (C4), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl- β -glucosaminidase activities and hydrolysis of Tween 40 and Tween 80; these data were not reported for *N. naphthovorans* NAG-2N-126^T. ND, No data available.

Characteristic	HAL40b ^T	<i>M. daeponensis</i> SW-156 ^T	<i>N. naphthovorans</i> NAG-2N-126 ^T
Colony morphology	Circular, beige, 0.2–0.4 mm*	Cream, smooth, circular to slightly irregular, low-convex	Beige or light brown
Cell size (μ m)	1.0–2.0 \times 0.4–0.6	1.5–3.0 \times 0.6–0.8	2.0–3.0 \times 0.7–0.9
Temperature range for growth (optimum) (°C)	10–40 (20–30)	No growth at 4; maximum 45 (30–37)	4–24 (ND)
pH range for growth (optimum)	6.5–9.5 (7–9)	Minimum 5.5 (7–8)	6.5–8.5 (7.5)
Salt tolerance for growth (optimum) (% w/v)	1–7 (3)	Maximum 18 (2–6)	1.75–7 (ND)
Esterase lipase (C8)	+	+	–
Utilization of arabinose	+	ND	+
DNA G+C content (mol%)	69	57	46

*Diameter after 5 days incubation at room temperature.

The DNA G+C content of isolate HAL40b^T was 69.1 mol% and, thus, clearly differed from those of both *M. daeipoensis* SW-156^T (57 mol%) and *N. naphthovorans* NAG-2N-126^T (46 mol%).

Whole-cell fatty acid methyl esters (FAMES) were obtained from washed cells by extraction and transesterification of 20 mg freeze-dried cells with a mixture of trimethylchlorosilane/methanol (1:8, v/v; 1 h, 75 °C) and re-extraction with n-hexane. Compounds were analysed by GC and combined GC-MS. Quantification of individual compounds was achieved by adding n-heneicosanoic acid methyl ester (C_{21:0}) as an internal standard at a known concentration prior to GC analysis. FAMES were identified by comparison of mass spectra and retention times with published data and/or reference compounds. Double-bond positions of monounsaturated FAMES were determined from their dimethyldisulfide derivatives (Buser *et al.*, 1983). Hydroxy fatty acids were analysed as their trimethylsilyl ether derivatives. For structural elucidations, monounsaturated FAMES were converted to their saturated structural analogues by hydrogenation. Twenty-five fatty acids were present in the cell hydrolysate of HAL40b^T in relative amounts of >0.1% of total fatty acids and contained 11–19 carbon atoms (Supplementary Table S1). The most abundant fatty acid by far was heptadec-8-enoic acid (C_{17:1}ω8; 51.7% total fatty acids) followed by heptadecanoic acid (C_{17:0}; 9.6%) and octadec-7-enoic acid (C_{18:1}ω7; 7.8%). Strain HAL40b^T exhibited a whole-cell fatty acid composition that was uncommon amongst members of the class *Gammaproteobacteria*. Detailed fatty acid distribution patterns of the closest phylogenetic relatives (>90% similarity) of HAL40b^T have been reported for only *M. daeipoensis* SW-156^T so far. The two strains differed strongly in their fatty acid profiles; the major components found in *M. daeipoensis* SW-156^T were C_{16:0} (24.8%) and C_{18:1}ω9 (24.3%). Thus, the whole-cell fatty acid composition of strain HAL40b^T corroborates the proposal of a novel species within a new genus.

Results of these phenotypic, biochemical and physiological analyses, together with the phylogenetic differences, clearly indicate that strain HAL40b^T can be assigned to the *Gammaproteobacteria*, but not to a previously described bacterial genus in this class. It is proposed that it represents a novel species within a new genus: *Spongiibacter marinus* gen. nov., sp. nov.

Description of *Spongiibacter* gen. nov.

Spongiibacter (Spon.gi.i.bac' ter. L. fem. n. *spongia* sponge; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Spongiibacter* a rod-shaped bacterium isolated from a sponge).

Gram-negative, rod-shaped bacteria. Motile by means of a polar flagellum. The genus belongs to the class *Gammaproteobacteria* based on phylogenetic analysis of 16S rRNA gene sequences. Oxidase reaction is positive and catalase reaction weakly positive. Strictly aerobic. Halophilic. The type species is *Spongiibacter marinus*.

Description of *Spongiibacter marinus* sp. nov.

Spongiibacter marinus (ma.ri'nus. L. masc. adj. *marinus* of the sea, marine).

Characteristics are as above for the genus plus the following data. Cells are 1.0–2.0 × 0.4–0.6 μm. Grows at 10–40 °C and pH 6.5–9.5, with optimum growth at 20–30 °C and pH 7–9. NaCl is required for growth; grows at 1–7% NaCl, with optimum growth at 3% NaCl. Positive for alkaline phosphatase, esterase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities. Hydrolyses Tween 40 and Tween 80. Utilizes arabinose as a carbon source. The predominant cellular fatty acid is C_{17:1}ω8.

The type strain is HAL40b^T (=DSM 17750^T =CCUG 54896^T). The DNA G+C content of the type strain is 69.1 mol%.

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