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

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Strain HAL40bT 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-b-glucosaminidase. The predominant cellular fatty acid was C17:1ω8, followed by C17:0 and C18:1ω7. 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 HAL40bT 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 HAL40bT (=DSM 17750T =CCUG 54896T).

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 (Crocì et al., 2006) and invertebrates (Holmström & Kjelleberg, 1999; Sfanos et al., 2005) have also been targets for analysis.

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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 HAL40bT is AM117932.
A MALDI-TOF MS profile showing characteristic peaks and fatty acid composition data of Spongiibacter marinus HAL40bT are available as supplementary material with the online version of this paper.
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 x 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 examination of ultrathin sections (Fig. 1b). The analysis revealed short, motile rods, 1.0–2.0 x 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 sphenobacteria (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 HAL40bT 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 HAL40bT (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 HAL40bT 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 (Pinhasi & 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 (Agogue et al., 2005a, b; GenBank accession no. AY576729). An epibiotic bacterium originating from diatoms in natural coastal waters showed 96 % sequence similarity (Kaczmarska 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 HAL40bT, with sequence similarities of 89 %. The closest relatives to strain HAL40bT with validly published names included the type strains of Marinobacter daepoensis (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 HAL40bT that was separate from these two type strains and from the cluster of Pseudomonas strains.
Temperature range and optimum for growth of strain HAL40bT 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 HAL40bT with M. daepoensis and N. naphthovorans revealed a number of differences (Table 1). Cells of HAL40bT were remarkably smaller and the temperature and pH growth profiles differed greatly. Growth of strain HAL40bT 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. daepoensis (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. HAL40bT 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. HAL40bT, M. daepoensis 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 HAL40bT at pH 7–9. In contrast to strain HAL40bT, strain NEP4 grew at a higher temperature range (20–40 °C) and was able to utilize acetate, pyruvate, propionate, succinate, lactate, tetraethylglycol 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 HAL40bT 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 HAL40bT and the type strains of two related organisms

Data for M. daepoensis 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. HAL40bT and M. daepoensis 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.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HAL40bT</th>
<th>M. daepoensis SW-156 T</th>
<th>N. naphthovorans NAG-2N-126 T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony morphology</td>
<td>Circular, beige, 0.2–0.4 mm*</td>
<td>Cream, smooth, circular to slightly irregular, low-convex</td>
<td>Beige or light brown</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>1.0–2.0 × 0.4–0.6</td>
<td>1.5–3.0 × 0.6–0.8</td>
<td>2.0–3.0 × 0.7–0.9</td>
</tr>
<tr>
<td>Temperature range for growth</td>
<td>10–40°C (20–30°C)</td>
<td>No growth at 4; maximum 45 (30–37°C)</td>
<td>4–24 (ND)</td>
</tr>
<tr>
<td>(optimum) (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>6.5–9.5 (7–9)</td>
<td>Minimum 5.5 (7–8)</td>
<td>6.5–8.5 (7.5)</td>
</tr>
<tr>
<td>Salt tolerance for growth (optimum)</td>
<td>1–7 (3)</td>
<td>Maximum 18 (2–6)</td>
<td>1.75–7 (ND)</td>
</tr>
<tr>
<td>(% w/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esterase lipase (C8)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Utilization of arabinose</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>69</td>
<td>57</td>
<td>46</td>
</tr>
</tbody>
</table>

* Diameter after 5 days incubation at room temperature.
The DNA G+C content of isolate HAL40bT was 69.1 mol% and, thus, clearly differed from those of both M. daepoensis SW-156T (57 mol%) and N. naphthovorans NAG-2N-126T (46 mol%).

Whole-cell fatty acid methyl esters (FAMEs) were obtained from washed cells by extraction and transterification 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 (C21:0) 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. Twenty-five fatty acids were present in the cell hydrolysate of HAL40bT 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 (C17:1ω9; 51.7% total fatty acids) followed by heptadecanoic acid (C17:0; 9.6%) and octadec-7-enoic acid (C18:1ω7; 7.8%). Strain HAL40bT 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 HAL40bT have been reported for only M. daepoensis SW-156T so far. The two strains differed strongly in their fatty acid profiles; the major components found in M. daepoensis SW-156T were C16:0 (24.8%) and C18:1ω9 (24.3%). Thus, the whole-cell fatty acid composition of strain HAL40bT 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 HAL40bT 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 marinus gen. nov., 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 C17:1ω9. The type strain is HAL40bT (=DSM 17750T =CCUG 54896T). The DNA G+C content of the type strain is 69.1 mol%.

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References


