Marinobacter salinus sp. nov., a moderately halophilic bacterium isolated from a tidal flat environment

Sundas Rani,1 Hyeon-Woo Koh,1 Hongik Kim,2 Sung-Keun Rhee3 and Soo-Je Park1,∗

Abstract

Two Gram-stain-negative, aerobic, motile, halophilic, rod-shaped bacteria, designated Hb8T and Hb20, were isolated from a tidal flat environment located on the South-West Korean peninsula. The isolates grew at 10–37 °C, at pH 5.0–9.0 and in NaCl concentrations of 0.5–15 % (w/v; optimum, 3.0–6.0 %). Sequence analysis of the 16S rRNA indicated that the isolates belong to the genus Marinobacter and are most closely related to Marinobacter sediminum R65T (98.3 %), followed by Marinobacter lipolyticus SM19T, Marinobacter salsuginis SD-14BT and Marinobacter similis A3d10T. The overall 16S rRNA gene sequence similarity with these species was 97.9 %, but Hb8T and Hb20 showed 100 % sequence similarity with each other. DNA–DNA relatedness values of Hb8T and Hb20 suggested that these isolates represent a single species, while DNA–DNA relatedness values of the two novel isolates with M. sediminum DSM 27079T and M. similis DSM 15401T were only 21.3 and 22.9 %, respectively. The major fatty acids present in strain Hb8T were identified as C16:0, C16:1ω9c, C18:1ω9c, C18:0 3-OH and summed feature 3 (C16:1ω6c and/or C16:1ω7c). Ubiquinone-9 was the main respiratory quinone in both the novel strains. The polar lipids found to be present included diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, four unidentified phospholipids and five unidentified lipids. The genomic DNA G+C content of Hb8T and Hb20 was 54.5 mol%. Polyphasic analysis indicated that the two isolates are representatives of a novel species of the genus Marinobacter, for which the name Marinobacter salinus sp. nov. is proposed. The type strain is Hb8T (=KCTC 52255T=JCM 31416T).

The genus Marinobacter belongs to the family Alteromonadaceae, of the order Alteromonadales, class Gammaproteobacteria, and was first described by Gauthier et al. [1] to accommodate a hydrocarbon-degrading bacterium. At the time of writing, the genus Marinobacter comprises 40 species with validly published names (www.bacterio.net) isolated from a diverse range of environments including seawater [2, 3], marine sand [4], marine sediment [5], the brine–seawater interface [6], coastal hot springs, saline soil [7], wastewater from wine production [8] and even laboratory cultures from dinoflagellates [9]. Members of the genus Marinobacter are Gram-stain-negative, oxidase- and catalase-positive, halophilic and rod-shaped [1]. Several species of the genus Marinobacter (i.e. Marinobacter aquaeolei, Marinobacter maritimus and Marinobacter algicola) have been associated with aromatic and aliphatic hydrocarbon degradation [1, 2, 9–11]. This may indicate a functional role in the organic carbon cycle, as they constitute one of the dominant bacterial community groups in contaminated environments [12]. The objectives of this study were to classify two newly isolated marine bacteria obtained when studying the diversity of halophilic bacteria associated with the marine environment.

Strains Hb8T and Hb20 were isolated from tidal flat sediment samples collected at Gunsan (36° 0′ N 126° 43′ E). The pH of the sample was 7.2 and the salinity is 2.5 % (w/v). The sediments were placed in sterile conical tubes, serially diluted with natural sea water, filtered (0.22 μm pore size) and transferred to marine 2216 agar (MA) plates in duplicate. Plates were incubated at 25 °C for 7 days under aerobic conditions. Single colonies were transferred to new MA plates and two of these were selected and designated Hb8T and Hb20. These were cultured routinely on MA plates at 30 °C and maintained as glycerol suspensions (30 %, w/v) at −80 °C.

For the phylogenetic analysis of strains Hb8T and Hb20, DNA was extracted using a commercial genomic DNA extraction kit (Geneall). 16S rRNA genes were amplified by PCR using a universal bacterial primer set 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [13], and rpoD and

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seca genes were amplified using primers previously described (see [14, 15]). Purified PCR products were sequenced by Macrogen using primers 27F, 338F, 786R and 1492R [13]. Almost complete Hb8 and Hb20 16S rRNA gene sequences (~1460 bp) were obtained by assembling the sequences with SeqMan software (DNASTAR), and the sequences were compared with 16S rRNA gene sequences of related taxa obtained from the GenBank database and the ExTaxon server [16]. Sequence alignments were performed with CLUSTAL X [17]. Gaps were edited using the BioEdit program [18] and evolutionary distances were calculated using the Kimura two-parameter model [19]. Phylogenetic trees were reconstructed based on neighbour-joining [20], minimum-evolution [21] and maximum-parsimony methods using MEGA 7 [22]. Bootstrap analysis was performed based on 1000 resampled datasets using programs within the software. The sequence similarity between Hb8 and Hb20 16S rRNA genes was 100 %, and phylogenetic analysis strongly indicated that these strains belong to the genus Marinobacter (Fig. 1A and S1, available in the online Supplementary Material). The strains are related most closely to Marinobacter sediminum R65T (98.3 % 16S rRNA gene sequence similarity), Marinobacter similis A3d10T (97.9 %), Marinobacter lipolyticus SM19T (97.9 %), Marinobacter salugininis SD-14B1T (97.9 %), Marinobacter gaseoengensis En6T (97.7 %), Marinobacter gudaoensis SL014B61A1T (97.5 %), Marinobacter xestospongiae UST090418-1611T (97.5 %), Marinobacter adhaerens HP15T (97.5 %), and Marinobacter mobilis CN46T (97.0 %). Phylogenetic analysis of seca and rpoD genes further indicated that the strains belong to the genus Marinobacter, and revealed that they are highly related to M. similis A3d10T (81.1, 81.1, 89.0 and 90.5 % sequence similarity at the nucleotide and amino acid level with the seca gene and SecA protein, respectively) and M. sediminum R65T (79.7, 80.1, 85.4 and 85.4 % for rpoD and RpoD, respectively) with 100 % bootstrap values (Fig. 1). The two type strains M. sediminum R65T (DSM 27079) and M. similis A3d10T (DSM 15400) were selected as reference strains for comparison with the novel strains based on 16S rRNA gene sequence similarities and phylogenetic positions, and were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Unless stated otherwise, all reference strains were grown on MA or marine broth (MB) medium under optimal culture conditions [15, 23].

For the analysis of cellular fatty acids, strains Hb8 and Hb20, and reference strains, were cultivated in MB, and harvested during the late exponential phase of growth. Fatty acid methyl esters were prepared and extracted according to the protocol published in the Sherlock Microbial Identification System (MIDI), and profiles were determined according to the MID/Hewlett Packard Microbial Identification System using a GC 6890N and 7683 auto sampler (Agilent Technologies), according to the manufacturer’s instructions [24]. Polar lipids were extracted from freeze-dried cells (100 mg) derived from the isolated strains and analyzed as described by Komagata and Suzuki [25].

The most abundant cellular fatty acids in strains Hb8T and Hb20 were C16:0, C16:1ω9c, C18:1ω9c and summed feature 3 (comprising C16:1ω6c and/or C16:1ω7c). However, the C18:1ω6 3-OH hydroxyl fatty acid was abundant only in Hb8T, as it was absent from Hb20 and from the reference strains. Full fatty acid profiles are given in Table S1. Although the overall profile, with C16:0 as a major component, was common to members of the genus Marinobacter, the relative compositions of major fatty acids differed. For example, C16:1ω9c was more abundant in the newly identified strains than in the reference strains. The total amount of fatty acids calculated to be present in Hb8T, Hb20, M. similis DSM 15400T and M. sediminum DSM 27079T were 97.22, 97.78, 97.55 and 97.67, respectively. Fatty acid profiling strongly indicated that Hb8T and Hb20 represent a species that is distinct from other species of the genus Marinobacter with validly published names.

Polar lipids detected included diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), four unidentified phospholipids (PLs) and five unidentified lipids (ULs; Fig. S2). Two types of amino lipids (AL1, AL2) were only observed in M. similis DSM15400T. The results are consistent with patterns reported previously for some type strains of species of the genus Marinobacter [5, 26].

Quinones were extracted with a chloroform/methanol mixture (2:1, v/v), evaporated under vacuum and reextracted three times with n-hexane/water (1:1, v/v). They were then concentrated and applied to a Sep-Pak Plus silica column (Waters). Quinone components were separated and identified by reversed-phase HPLC equipped with a photodiode array detector and internal and external quinone standards, as described by Hiraishi et al. [27]. Similarly to strains of species of the genus Marinobacter with validly published names, strains Hb8 and Hb20 also use ubiquinone 9 (Q-9) as the major respiratory quinone. Marinobacter lutoensis contains ubiquinone-8 and so is an exception [28].

Chromosomal DNA extracted for 16S rRNA gene amplification was used for the determination of G+C contents following removal of DNA by incubation with a mixture of RNase A and T1 (20 U ml−1 each) at 30 °C for 1 h. The G+C content of chromosomal DNA was determined according to Gonzalez and Saiz-Jimenez [29] and was 54.5 mol% for both Hb8T and Hb20, which is within the expected range for species of the genus Marinobacter. The G+C content for the genus Marinobacter has been reported to vary from 54.0 to 63.5 mol% [4, 28].

DNA–DNA hybridization experiments were carried out with strains Hb8T and Hb20, according to the method described by Ezaki et al. [30]. Genomic DNA was extracted using a genomic DNA extraction kit (Geneall) and used as probe DNA after biotinylation with photobiotin and hybridisation with single-stranded unlabelled chromosomal DNA fragments from experimental or test strains. Mean values from three independent DNA–DNA hybridization experiments were
obtained, and the DNA-DNA relatedness between Hb8\(^T\) and Hb20, *M. sediminum* DSM 27079\(^T\) and *M. similis* DSM 15400\(^T\) was ~99 %, 21.45 and 22.92 %, respectively.

Gram staining was performed using a BD Gram stain kit according to the manufacturer’s instructions (Becton-Dickinson). Cell morphology, cell size and the presence of flagella were determined at the Korean Basic Science Institute by transmission electron microscopy (Tecnai G2 Sprite; FEI) following negative staining with 1 % (w/v) phosphotungstic acid. Motility was tested by the hanging-drop method, while anaerobic growth was tested using the BD GasPak 145 EZ Gas Generating Pouch System over a period of 2 weeks. Catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide solution, and oxidase activity was determined using 1 % (w/v) tetramethyl p-phenylenediamine (Merck), as described previously [31]. Cells of variable size (1.5–2.80 x 0.5–0.7 \(\mu\)m) were observed, 

![Fig. 1. Neighbour-joining phylogenetic tree showing the taxonomic positions of strains Hb8\(^T\) and Hb20 according to (a) their 16S rRNA, (b) their secA and (c) their rpoD gene sequences. Numbers at branching points are percentage bootstrap values based on 1000 replications, with only values above 60 % shown. The scale bar represents 0.01/0.02 substitutions per nucleotide position. Minimum-evolution and maximum-parsimony algorithms were also used for tree reconstruction, and branches that were in agreement using these methods are marked with a dash.](https://www.microbiologyresearch.org/)

**Fig. 1.** Neighbour-joining phylogenetic tree showing the taxonomic positions of strains Hb8\(^T\) and Hb20 according to (a) their 16S rRNA, (b) their secA and (c) their rpoD gene sequences. Numbers at branching points are percentage bootstrap values based on 1000 replications, with only values above 60 % shown. The scale bar represents 0.01/0.02 substitutions per nucleotide position. Minimum-evolution and maximum-parsimony algorithms were also used for tree reconstruction, and branches that were in agreement using these methods are marked with a dash.
but flagella were only seen to be associated with cells of strain Hb8\textsuperscript{T} by transmission electron microscopy (Fig. S3). Endospores were not observed. Hb8\textsuperscript{T} and Hb20 cells were, therefore, Gram-stain-negative, aerobic, and oxidase- and catalase-positive.

To test tolerance to salinity, strains were grown in modified artificial marine [32] or MB medium containing 0–20 % (w/v) NaCl (0, 0.5, 1, 3, 5, 7, 9, 11, 15, 17, 19, 21, 23 and 25 %). Cultures were incubated at 30 °C for 2 weeks. Strains Hb8\textsuperscript{T} and Hb20 required sodium ions for growth and grew in the presence of 1–15 % and 0.5–15 % (w/v) NaCl, respectively. The optimal NaCl concentration was 5 % (w/v). Growth at pH 4.5–10 (0.5 pH unit increments) was determined in MB at 30 °C for 3 weeks following adjustment of the pH with sodium hydroxide and hydrochloric acid. Buffer components were as follows: 10 mM homo-PIPES (pH 4.5–5.0), 10 mM MES (pH 5.0–6.5), 10 mM Bis-tris propane (pH 7.0–8.5) or 10 mM CAPS (pH 9.0–10.0) [33]. Both strains grew at pH 5.0–9.0, but not below 4.5 or above 9.5, and a pH of 8.0 was optimal. The temperature range for growth was determined by incubating cultures at 5, 10, 20, 25, 30, 37 and 50 °C for 2...
Table 1. Differential phenotypic characteristics of strains Hb8T and Hb20 compared with type strains of species of Marinobacter with validly published names

1, Hb8T; 2, Hb20; 3, M. similis DSM 15400T; 4, M. sediminum DSM 27079T. All data are from the present study. All strains were oxidase- and catalase-positive, and positive for esterase, arabinose, mannose, mannitol, N-acetyl glucosamine, maltose and gluconate. Strains displayed a weak response in acid phosphatase, α-galactosidase, α-glucosidase, mannosidase and fucosidase tests, and are negative for dextrin, d-maltose, d-trehalose, d-cellobiose, gentiobiose, sucrose, α-d-lactose, d-melibiose, β-methyl-d-glucoside, d-salicin, α-d-glucose, α-d-mannose, d-fructose, d-galactose, 3-methyl glucose, d-sorbitol, d-arabitol, myoinositol, d-aspartic acid, L-arginine, L-aspartic acid, L-histidine, L-serine, d-galacturonic acid, L-galactonic acid lactone, d-gluconic acid, d-glucuronic acid, glucuronamidase, d-saccharic acid, p-hydroxyphenylacetic acid, l-lactic acid, α-ketoglutaric acid, d-malic acid, naldixic acid, γ-aminobutyric acid and indole production. +, Positive; −, negative; w, weakly positive; ND, no data available.

<table>
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<tr>
<th>Characteristic</th>
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<th>3</th>
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<td>1.3–2.1*</td>
<td>1.8–2.5†</td>
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<td>Cell width (µm)</td>
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<td>0.5–20*</td>
<td>0.5–18†</td>
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<td>54.5</td>
<td>57.6</td>
<td>56.5</td>
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</table>

*Data from Ng et al. [15].
†Data from Romanenko et al. [23].

Weeks on MA and MB, and both strains grew at 10–37°C. The optimum growth temperature was 30–37°C and 30°C for Hb8T and Hb20, respectively. Utilization tests for various carbon sources and enzyme activity tests were performed using API 20NE and ZYME (bioMérieux), and GEN III Microplate systems (Biolog), according to the manufacturer’s instructions. The selected reference strains were also tested under the same conditions, and some differences in the utilization and hydrolysis of substrates were observed between strains Hb8T and Hb20. Specifically, strain Hb8T was positive for the utilization of l-glutamic acid, l-pyroglutamic acids, bromosuccinic acid, acetoacetic acid and aztreonam, and this strain also hydrolyzed gelatine and displayed β-galactosidase activity, but was negative for esterase, lipase and β-glucosidase activity. However, strain Hb20 was only positive for N-acetyl-β-glucosaminidase and esterase activity.

The data presented demonstrate that strains Hb8T and Hb20 are closely related to members of the genus Marinobacter with validly published names, but that they possess some unique features. Phylogenetic tree analysis of 16S rRNA, rpoD and secA genes, based on neighbour-joining, minimum-evolution and maximum-parsimony algorithms indicated that the two novel strains form their own robust cluster within the Marinobacter genus. In addition, these strains could be differentiated from members of other genera based on major fatty acids, DNA G+C contents and their moderate halophilic properties (Table 1). Low levels of DNA–DNA relatedness (~23.0%) and lack of esterase and lipase activity, together with very strong β-galactosidase activity and strong utilization of propionic acid, lactic acid and d-malic acid as carbon and energy sources, distinguish the novel strains from other members of the genus.
**Marinobacter.** Sequence similarities between Hb8\(^T\), Hb20 and the type strain of their closest phylogenetic relative (*M. sediminum* DSM 27079\(^T\); 98.3% 16S rRNA gene sequence similarity) clearly confirms that the isolates represent a novel species [34, 35] of the genus *Marinobacter*, for which the name *Marinobacter salinus* sp. nov. is proposed.

**DESCRIPTION OF MARINOBACTER SALINUS SP. NOV.**


Gram-stain-negative, aerobic and motile with a polar flagellum. Young cultures contain rod-like cells (1.5–2.8 \(\times\) 0.5–0.7 \(\mu\)m), and cells grown on MA for 42 h at 30 °C form circular, smooth, elevated, transparent and non-pigmented colonies that turn creamy after a longer incubation time. Cells are moderately halophilic; growth occurs in the presence of NaCl concentrations of 1–15 % (w/v), with optimum growth at 5 %, but growth does not occur in the complete absence of salt. Growth occurs at pH 5.0–9.0 and 10–37 °C, with optimal growth at pH 8 and 37 °C. Cells are oxidase- and catalase-positive, reduce nitrate to nitrite and do not form indole. Glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, malate, citrate and phenyl acetate are all assimilated, but urease activity and indole production are not observed. Esterase (C4) and \(\beta\)-galactosidase activity is seen, but trypsin, \(\beta\)-glucosidase, \(N\)-acyl-\(\beta\)-glucosaminidase, alkaline phosphatase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-Bl-phosphohydrolase, \(\alpha\)-galactosidase, \(\beta\)-galactosidase, \(\alpha\)-glucuronidase, \(\beta\)-mannosidase and \(\alpha\)-fucosidase activities are not detected. L-Glutamic acid, L-proglutamic acid, L-lactic acid, D-malic acid, L-malic acid, bromosuccinonic acid, \(\beta\)-hydroxy-DL-butyric acid, acetooacetic acid, propionic acid and acetic acid are utilised as carbon and energy sources for growth. The most abundant cellular fatty acids are C\(_{16:0}\), C\(_{16:1}\omega 9c\), C\(_{18:1}\omega 9c\), C\(_{18:0}\) 3-0H and summed feature 3 (C\(_{16:1}\omega 6c\) and/or C\(_{16:1}\omega 7c\)), and ubiquinone-9 is the major respiratory quinone. Polar lipids include diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, four unidentified phospholipids and five unidentified lipids.

The type strain, Hb8\(^T\) (=KCTC 52255\(^T\)=JCM 31416\(^T\)) and strain Hb20 (=KCTC 52254=JCM 31417), were isolated from a tidal flat environment in Gunsan, South Korea. The DNA G + C content of the type strain is 54.51 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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