**Marinobacter salarius** sp. nov. and **Marinobacter similis** sp. nov., Isolated from Sea Water

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**Abstract**

Two non-pigmented, motile, Gram-negative marine bacteria designated R9SW1ᵀ and A3d10ᵀ were isolated from sea water samples collected from Chazhma Bay, Gulf of Peter the Great, Sea of Japan, Pacific Ocean, Russia and St. Kilda Beach, Port Phillip Bay, the Tasman Sea, Pacific Ocean, respectively. Both organisms were found to grow between 4°C and 40°C, between pH 6 to 9, and are moderately halophilic, tolerating up to 20% (w/v) NaCl. Both strains were found to be able to degrade Tween 40 and 80, but only strain R9SW1ᵀ was found to be able to degrade starch. The major fatty acids were characteristic for the genus **Marinobacter** including C₁₆:₀, C₁₆:₁o7c, C₁₈:₁o9c and C₁₈:₁o7c. The G+C content of the DNA for strains R9SW1ᵀ and A3d10ᵀ were determined to be 57.1 mol% and 57.6 mol%, respectively. The two new strains share 97.6% of their 16S rRNA gene sequences, with 82.3% similarity in the average nucleotide identity (ANI), 19.8% similarity in the in silico genome-to-genome distance (GGD), 68.1% similarity in the average amino acid identity (AAI) of all conserved protein-coding genes, and 31 of the Karlin’s genomic signature dissimilarity. A phylogenetic analysis showed that R9SW1ᵀ clusters with **M. algicola** DG893ᵀ sharing 99.40%, and A3d10ᵀ clusters with **M. sediminum** R65ᵀ sharing 99.53% of 16S rRNA gene sequence similarities. The results of the genomic and polyphasic taxonomic study, including genomic, genetic, phenotypic, chemotaxonomic and phylogenetic analyses based on the 16S rRNA, gyrB and rpoD gene sequence similarities, the analysis of the protein profiles generated using MALDI-TOF mass spectrometry, and DNA-DNA relatedness data, indicated that strains R9SW1ᵀ and A3d10ᵀ represent two novel species of the genus **Marinobacter**. The names **Marinobacter salarius** sp. nov., with the type strain R9SW1ᵀ (= LMG 27497ᵀ = JCM 19399ᵀ = CIP 110588ᵀ = KMM 7502ᵀ) and **Marinobacter similis** sp. nov., with the type strain A3d10ᵀ (= JCM 19398ᵀ = CIP 110589ᵀ = KMM 7501ᵀ), are proposed.

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**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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**Introduction**

The genus **Marinobacter** (family Alteromonadaceae, order Alteromonadales, class Gammaproteobacteria) was created by Gauthier et al. for a hydrocarbon degrading bacterium. At the time of writing, the genus comprises 33 validly described species, http://www.bacterio.net/marinobacter.html [1], which accommodates Gram-negative, chemoheterotrophic and halophilic, rod-shaped bacteria [2,3]. The important role played by **Marinobacter** spp. in metabolizing hydrocarbons has long been noted, with **M. hydrocarbonoclasticus** [2], **M. aquaeolei** [4,5], **M. maritimus** [6], and **M. algicola** [7] having been characterized as being able to utilise aromatic and aliphatic hydrocarbons as their sole carbon and energy sources. It was also shown that bacteria of the genus **Marinobacter** are one of the dominant bacterial community groups constantly recovered from hydrocarbon polluted sites [8–10]. For example, it was recently demonstrated that **M. vannioni** was able to effectively degrade toluene, benzene, ethylbenzene, and p-xylene [11].

The objectives of this study were to classify two newly isolated marine bacteria; strain R9SW1ᵀ, which was derived from a water sample collected from Chazhma Bay (Gulf of Peter the Great, Sea of Japan, Pacific Ocean) during taxonomic studies of microbial communities developed in sea water contaminated by radionuclides [12]; and strain A3d10ᵀ, which was isolated from Port Philip Bay (the Tasman Sea, Pacific Ocean) during the course of polymer biodegradation studies [13]. The comparative taxonomic investigations of these bacteria, together with their close relatives,
Table 1. GenBank/EMBL/DBJ accession numbers of 16S rDNA, gyrB, rpoD and whole genome sequences for strains R9SW1T, A3d10T and phylogenetically related type strains and type species of the genus Marinobacter.

| Species name                                      | GenBank/EMBL/DBJ accession numbers                  |
|--------------------------------------------------|-----------------------------------------------------|
|                                                   | 16S rDNA    | gyrB     | rpoD     | whole genome |
| Strain R9SW1T                                     | K547705     | KF811464 | KF811478 | CP007152     |
| Strain A3d10T                                     | K547704     | KF811465 | KF811471 | CP007151     |
| M. algicola LMG 23835T                            | AY258110*   | KF811463 | KF811474 | -             |
| M. sediminum LMG 23833T                           | A6699270*   | KF811466 | KF811477 | -             |
| M. adhaerens CIP 110141T                          | AY241552*   | KF811467 | KF811473 | NC_017506*   |
| M. fluvimaris CIP 108615T                          | AYS17632*   | KF811468 | KF811475 | -             |
| M. salsuginis CIP 109893T                          | EF028328*   | KF811469 | KF811476 | -             |
| M. hydrocarbonoclasticus SP.17T                    | X67022*     | KF811470 | KF811472 | NC_017067*   |

*Accession numbers from previous publications.

Materials and Methods

Isolation procedures, bacterial strains, and growth conditions

Strain R9SW1T was isolated from a sea water sample collected from Chazhma Bay in the Sea of Japan, Pacific Ocean, in 2000. Water sample collection was within the research program funded by the Federal Agency for Science of the Ministry of Education and Science of the Russian Federation, grant 2–2.16 and by the Russian Foundation for Basic Research and grant ‘Molecular and Cell Biology’ from the Presidium of the Russian Academy of Sciences, grant 02-04-48211”. The specific location of the studies (GPS coordinates) was 42°30′ 38″ N 132°22′ 02″ E. The permit issued by the Department of Marine Expeditions, Ministry of Education and Science of the Russian Federation. Strain A3d10T was isolated from a sea water sample collected one metre below the water surface in Port Philip Bay, the Tasman Sea, Pacific Ocean, in 2008. Sea water collected from St Kilda Beach which is a publicly accessible beach area in Melbourne, not part of any protected area of land or sea. Furthermore, the field studies not involve endangered or protected species. The specific location of the studies (GPS coordinates) was 37°51′50″S 144°58′55″E. The sample handling and isolation procedures used were as previously described [12,13]. Samples were plated on marine agar 2216 (BD, USA) and incubated aerobically at approximately 22–25°C for 5, 7 or 10 days. The isolation and purification procedure has been described elsewhere [14,15]. Ten type strains of the Marinobacter species were obtained from various culture collections and used as the reference strains; M. lipolyticus CIP 107627T, M. gudovensis CIP 109531T, M. adhaerens CIP 110141T, M. salsuginis CIP 109893T and M. fluvimaris CIP 108615T were obtained from Collection de l’Institut Pasteur (CIP) culture collection, M. algicola LMG 23835T, M. guineae LMG 24048T and M. sediminum LMG 23833T were obtained from The Belgian Co-ordinated Collections of Micro-organisms (BCCM/ LMG), M. gosengensis KCTC 12515T was obtained from Korean Collection for Type Cultures (KCTC) and M. xestospongiae JCM 17469T was obtained from RIKEN BRC-Japan Collection of Macroorganisms (JCM). The type species of the genus, M. hydrocarbonoclasticus SP. 17T was kindly provided by Dr. Stan-Lotter. Strains were stored at −80°C in marine broth 2216 (BD, USA) that had been supplemented with 20% (v/v) glycerol.

16S rDNA, gyrB, rpoD sequencing and phylogenetic analysis

Genomic DNAs were isolated using a Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer’s specifications. The 16S rRNA gene sequences for strains R9SW1T and A3d10T were extracted from the whole genome sequences [16] while gyrB and rpoD genes were amplified using primers (see Supporting Information, Table S1 in File S1) that have been previously described [17,18]. The 16S rRNA gene sequences of validly described Marinobacter species were retrieved from GenBank and aligned using the CLUSTAL W program [19]. Evolutionary phylogenetic trees were constructed using the neighbour-joining [N] [20], maximum-likelihood (ML) [21] and maximum-parsimony (MP) [22] algorithms. Genetic distances were calculated using Kimura’s two-parameter model [23] by using the MEGA 5 software [24]. The GenBank/EMBL/DBJ accession numbers of 16S rRNA gene, gyrB, rpoD and whole genome sequences were presented as in Table 1.

MALDI-TOF MS analysis

The sample preparation and MALDI-TOF MS analysis was carried out according to the techniques described elsewhere [25]. Briefly, 5 μL of the cultures grown overnight were transferred into microcentrifuge tubes and subjected to ethanol and formic acid protein extraction. One μL aliquots of the supernatant were transferred onto the MALDI target plate and air dried at room temperature, followed by the addition of 1 μL of matrix solution, then air dried. Samples were then subjected to analysis using a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) equipped with a 60 Hz nitrogen laser. Spectra were recorded in the positive linear mode for the mass range of 2,000 to 20,000 Da at the maximum laser frequency. The raw spectra were then analysed using the MALDI Biotyper 3.0 software package (Bruker Daltonik GmbH, Bremen, Germany) under the default settings. Measurements were performed via the automatic mode, without any user intervention.

GC content and DNA-DNA hybridization

The GC content of strains R9SW1T and A3d10T was calculated on the basis of their whole genome sequences [16,26], and these
have been deposited at GenBank/EMBL/DDBJ under the accession number of CP007152 and CP007151, respectively. The DNA-DNA hybridizations between strains R9SW1 T and *M. adhaerens* HP15 T, and strains A3d10 T and *M. sediminum* LMG 23033 T, were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) identification service, where cells were initially disrupted using a Constant Systems TS 0.75 KW (IUL Instruments, Germany), followed by purification of the extracted DNA in the crude lysate form by chromatography on hydroxyapatite as described by Cashion et. al. (1977) [27]. DNA-DNA hybridization was carried out in duplicate using a 2× saline sodium citrate (SSC) buffer with 5% formamide as described by De Ley et al. [28], with consideration of the modifications described by Huss et. al. (1983) [29], using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6×6 multi-cell changer and a temperature controller with an *in-situ* temperature probe (Varian).

**Genome comparison and genomic signatures analyses**

Complete genome sequences for only two validly described species of *Marinobacter, M. hydrocarbonoclasticus* ATCC 49840 T [30] and *M. adhaerens* HP15 T [31], which have previously been assembled, were used in this study for genomic analysis. The fully sequenced and assembled genomes of both these species were retrieved from GenBank, and compared to those of R9SW1 T and A3d10 T. Genome comparison between strains R9SW1 T, A3d10 T, *M. adhaerens* HP15 T and *M. hydrocarbonoclasticus* ATCC 49840 T was carried out using reciprocal BLAST analysis, according to the method described by Goris et al. [32]. A map of the percentage identity between each of *M. adhaerens* HP15 T, R9SW1 T and A3d10 T to the type species was generated using the BLAST Ring Image Generator (BRIG) software [33]. The in-silico genome-to-genome distance (GGD) between the four strains was also calculated using the genome-to-genome distance calculator 2.0 (GGDC) provided by DSMZ, http://ggdc.dsmz.de [34,35]. The average amino acid identity (AAI) of all conserved protein-coding genes was calculated as previously described [36]. The conserved genes between a pair of genomes were determined by whole-genome pairwise sequence comparison using the BLAST algorithm release 2.2.5 [37] using a minimum cut-off of 40% identity and 70% of the length of the query gene. The difference in genome signature between two individual sequences is expressed in terms of the Karlin’s genomic signature dissimilarity (*δ*), which was calculated by dividing the genomic dinucleotide frequencies with the corresponding mononucleotide content using the equation described by Karlin et al. [38]. Phylogenomic relationship between the four strains were also elucidated using Mauve multiple alignment software (v2.3.1) [39] and ClonalFrame software v1.2 [40], with *Alteromonas* sp. DE [41] used as an outgroup.

Genotype to phenotype analyses of a few distinctive phenotypes were also carried using the whole genome sequences of strains R9SW1 T, A3d10 T, *M. hydrocarbonoclasticus* ATCC 49840 T and *M. adhaerens* HP15 T using the methods as previously described [42].

**Physiological and biochemical analysis**

Six reference type strains, along with strains R9SW1 T and A3d10 T, were used for the phenotypic and biochemical tests (Table 2). The cell morphology and motility were determined using scanning electron and light microscopies. Gram stain reaction, catalase (5% H₂O₂) and starch hydrolysis analyses were performed according to the method described by Smibert and Krieg (1994) [43]. Determination of the oxidative activity was performed using Bactident oxidase strips (Merck Millipore, Germany). The capacity of the strains to oxidize and to ferment α-glucose and lactose was carried out according to the method described by Smibert and Krieg (1994) [43], using a modified semi-solid medium containing: 9.4 g L⁻¹ O/F medium (Oxoid, UK), 20 g L⁻¹ Sea Salt (Sigma-Aldrich, USA) and 1% carbohydrate. The strains were incubated at 30°C and the results were obtained after 48 hours. The temperature and pH tolerance ranges were determined via marine agar growth tests subjected to different temperature (4, 10, 15, 20, 25, 30, 37, 40 and 45°C) and pH (4, 6, 7, 8, 9 and 11, adjusting the pH with HCl and NaOH) conditions. The NaCl tolerance was determined using different concentrations of NaCl (0, 0.5, 1, 5, 6, 10, 15, 20 and 25%) in modified salinity agar (SA) containing: 5 g L⁻¹ peptone, 1 g L⁻¹ yeast extract, 0.1 g L⁻¹ ferric citrate, 3.24 g L⁻¹ magnesium sulphate (MgSO₄), 0.55 g L⁻¹ dipotassium phosphate (K₂HPO₄), 15 g L⁻¹ agar, and the respective NaCl concentration, each at a pH of 7.6±0.2. Plates were incubated under optimal temperature conditions and the results were recorded daily for 7 days.

The susceptibility of the bacteria to antibiotics was tested using modified media containing: 21 g L⁻¹ Mueller-Hinton medium (Oxoid, UK), 7.5% Sea salt and 15 g L⁻¹ bacteriological agar (Agar No. 1, Oxoid, UK). The antibiotics tested were penicillin G (10 μg), chloramphenicol (30 μg), streptomycin (10 μg), tetracycline (30 μg), ampicillin (10 μg) and oxacillin (1 μg). The strains were incubated under optimal temperature conditions and results were obtained after 24 hours of incubation.

The ability of the strains to oxidise a range of organic substrates was investigated using a 96-well Biolog GN2 microplate (Biolog, USA), in triplicate. Inoculates were prepared by suspending culture that had been grown overnight into 3% (w/v) saline solution, then adjusting the density of the suspension to McFarland standard no. 1, followed by pipetting 150 μL aliquots of the suspension into each well. All the plates were incubated at 30°C and results were manually obtained after 24 h and 48 h. Enzymatic tests were performed using API ZYM test strips (bioMérieux, France) in two individual experiments. Inoculates were prepared by suspending culture that had been grown overnight into 3% (w/v) saline solution and adjusting the density to McFarland standard no. 5. A Microbact 24E Gram-negative identification system (Oxoid, UK) was also used to test other biochemical reactions, namely: lysine and ornithine decarboxylase; H₂S production; glucose, mannitol and xylose fermentation; hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG); indole production; urea hydrolysis; acetoin production (Voges-Proskauer reaction); citrate utilisation; production of indolepyruvate; gelatin liquefaction; malonate inhibition; inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose and salicin fermentation; and arginine dihydrolase. All tests were carried out according to the manufacturer’s specifications unless otherwise stated.

**Fatty acids analysis**

Fatty acid [FA] methyl esters were prepared as described elsewhere [44]. The resulting fatty acid methyl esters were analysed using a Shimadzu GC-14A gas chromatograph with a flame ionization detector, using both a nonpolar SPB-5 fused-silica column (30 m x 0.25 mm i.d.) at 210°C and a polar Supelcowax-10 fused-silica column (30 m x 0.25 mm i.d.) at 200°C.
Table 2. Differential characteristics between strains R9SW1, A3d10, their close phylogenetic neighbors and type species of the genus *Marinobacter*.

| Characteristics                  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 |
|----------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Cell length (µm)                 | 1.9–3.2 | 1.6–2.5 | 1.3–2.1 | 1.8–2.5 | 2.0–4.0 | 1.7–2.4 | 1.5–3.0 | 2.5–3.5 | 1.2–1.8 | 1.6–2.0 | 2.0–2.5 | 1.4–4.0 | 2.0–3.0 |
| Cell width (µm)                  | 0.40–0.72 | 0.45–0.55 | 0.40–0.45 | 0.3–0.4 | 1.0 | 0.6–0.8 | 0.6–0.9 | 0.3–0.5 | 0.3–0.5 | 0.5–0.8 | 0.6–0.8 | 0.4 | 0.3–0.6 |
| Growth temperature (°C)          | 4–40 | 5–40 | 4–40 | 4–42 | 10–45 | 4–45 | 4–45 | 15–40 | 10–45 | 10–37 | 15–42 | 4–42 | 10–45 |
| pH range                         | 6–9 | 5–10 | 6–9 | ND | 6.5–9.5 | 5.5–10.0 | >5.5 | 5.0–10.0 | 6.0–9.5 | 5.3–9.3 | 5.0–10.0 | 5.0–9.5 | 6–9.5 |
| Salinity range (% w/v)           | 0.5–20 | 1–12 | 0.5–20 | 0.5–18 | 1–20 | 0.5–20 | 1–20 | 1–15 | 0–15 | 1–25 | 0.5–60 | 1–15 | 1–20 |
| Nitrate reduction                | - | + (-) | + | + | + | + | - | + | + | ND | + | + | + |
| Nitrite reduction                | + | (-) | + | (+) | + | (-) | - | - | - | - | - | ND | - |
| Hydrolysis of starch             | + | + | + | w | - | w | - | ND | ND | ND | - | - | - |
| Indole production                | - | - | - | - | - | - | - | ND | ND | + | - | - | - |
| Fermentation of:                 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Glucose                          | - | - | w | w | - | w | - | ND | ND | ND | + | + | - |
| Lactose                          | - | - | - | - | - | - | w | - | ND | ND | ND | - | - |
| Utilisation of:                  |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Glycogen                         | + | + | + | - | - | - | - | + | ND | - | ND | ND | - |
| Mono-methyl-succinate            | + | - | + | (+) | + | + | w | - | ND | - | ND | ND | + |
| γ-Hydroxy-butyric acid           | + | + | + | - | - | w (+) | w | - | ND | - | ND | ND | - |
| Succinic acid                    | + | - (+) | - | - | + | w | - (+) | - | + | + | ND | + |
| α-Glutamic acid                  | + | + | w | (-) | + | + | (+) | - | - | - | + | ND | + |
| α-Phenylalanine                  | - | + | - | - | - | (-) | - | - | ND | - | ND | ND | - |
| α-Serine                         | + | - | - | - | - | (-) | - | - | ND | - | ND | ND | - |
| Glycerol                         | + | - (+) | - | - | w (+) | (+) | - | - | + | + | + | - | - |
| DNA G+C content (mol%)           | 57.1 | 55.0 | 57.6 | 56.5 | 55.9 | 56.9 | 58.0 | 57.0 | 57.9 | ND | 57.1 | 57.1 | 52.7 |

Strains: 1, strain R9SW1; 2, *M. algicola* LMG 23835T; 3, strain A3d10; 4, *M. sediminum* LMG 23833T; 5, *M. saluginis* CIP 109893T; 6, *M. adhaerens* CIP 110141T; 7, *M. flavimaris* CIP 108615T; 8, *M. lipolytica* SM19T; 9, *M. gudaonensis* SL014B61AT; 10, *M. goseongensis* En6T; 11, *M. xestospongiae* UST090418-1611T; 12, *M. guineae* MO8T; 13, *M. hydrocarbonoclasticus* SP.1T.  
Data for nitrate and nitrite reduction, starch hydrolysis, fermentation, indole and acid production, organic substrates utilisation, and enzyme activities for strains R9SW1, *M. algicola* LMG 23835T, A3d10, *M. sediminum* LMG 23833T, *M. saluginis* CIP 109893T, *M. adhaerens* CIP 110141T, *M. flavimaris* CIP 108615T and *M. hydrocarbonoclasticus* SP.1T are from this study. The data in brackets are from previously published work [2,7,62–70].  
+ , Positive; - , Negative; w, Weak reaction; ND, No Data available.  
doi:10.1371/journal.pone.0106514.t002
Results and Discussion

Analysis of the complete 16S rRNA gene sequences of strains R9SW1T and A3d10T revealed that both strains are grouped with species of the genus *Marinobacter*, with the sequence similarity between strains R9SW1T, A3d10T and all validly described *Marinobacter* species being in the range of 93.84–99.40% and 96.91–99.53%, respectively. The two new strains, R9SW1T and A3d10T shared 97.6% of their 16S rRNA gene sequences, however, phylogenetic analysis showed that they cluster separately forming two different clusters, one with *M. algicola* DG893T and another with *M. sediminum* R65T, where both clusters were supported by the bootstrap value of 99% and 100% in both the NJ and ML methods (Figure 1A and Figure S1 in File S1). The highest 16S rRNA gene sequence similarity between strain R9SW1T and *M. algicola* DG893T was found to be 99.40% (*M. algicola* DG893T), whilst strain A3d10T displays the highest 16S rRNA gene sequence similarity with *M. sediminum* LM23833T (99.53%).

Due to the high 16S rRNA gene sequence similarity between strains R9SW1T and *M. algicola* DG893T, and between A3d10T and *M. sediminum* R65T, an extended phylogenetic analysis based on *gyrB* and *rpoD* genes was carried out. The use of housekeeping genes in phylogenetic analysis can be beneficial, in that it overcomes the possibility of the presence of nucleotide polymorphisms in the 16S rRNA gene [45,46]. Two genes, *gyrB* and *rpoD*, were selected, since they have been previously reported to be excellent marker genes and sufficient for the identification and classification of various groups of microorganism [25,47–49]. *M. salarius* sp. nov. and *Marinobacter similis* sp. nov.

Figure 1. Neighbour-joining phylogenetic tree showing the taxonomic position of strains R9SW1T and A3d10T according to their (A) 16S rRNA, (B) *gyrB* and (C) *rpoD* gene sequences. Numbers at branching points are percentage bootstrap values based on 1000 replications, with only values above 50% are shown. Scale bar represents 0.005/0.02 substitutions per nucleotide position. The Maximum-likelihood (ML) and maximum Parsimony (MP) algorithms were also used for tree construction, where branches in agreement with ML and MP methods were marked with + and X respectively.

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values than the gyrB and rpoD sequence similarities of strains R9SW1<sup>T</sup>, A3d10<sup>T</sup> and their closest relatives. The sequence similarities for gyrB and rpoD between strains R9SW1<sup>T</sup> and A3d10<sup>T</sup> were significantly lower than the values mentioned above, i.e., 61.6% for gyrB and 78.2% for rpoD, suggesting distinct standing of new strains on the species level.

In order to further assess the taxonomic affiliation of the two new bacteria, a comparative analysis of the total protein profiles was performed using MALDI-TOF mass spectrometry (Figure 2). The results are in agreement with the phylogenetic analyses, clearly indicating that strain R9SW1<sup>T</sup> is clustering with *M. algicola* LMG 23835<sup>T</sup>, and strain A3d10<sup>T</sup> is clustering with *M. sediminum* LMG 23833<sup>T</sup> with a critical distance level below 500. As suggested in the previously reported studies, clustering below the distance level of 500 can be considered as reliable clustering [53,54], which was also in agreement with the recent studies on *Alderomonas* spp., where the clustering within the distance level of 500 was shown to be able to differentiate the closely related *Alderomonas* species [25,53]. Hence, the results of this study confirmed the separate species affiliation of the two new isolates within the genus *Marinobacter*. Also, the clusters of both strains R9SW1<sup>T</sup> and A3d10<sup>T</sup> with their nearest neighbour were stable, but exceeded the minimum differences between existing species, e.g., the distance level between species in both clusters were greater than those within a cluster that contained *M. gudnaensis* CIP 109534<sup>T</sup>, *M. adhaerens* CIP 110141<sup>T</sup>, *M. salarius* CIP 109093<sup>T</sup>, and *M. flavimaris* CIP 108615<sup>T</sup>; so does the position of strains R9SW1<sup>T</sup> and A3d10<sup>T</sup> resulting in different clusters in the MALDI dendrogram, provide evidence of the distinctive standing of two new bacteria.

In order to confirm the separate species standing of these two strains, a DNA-DNA hybridization experiment was conducted. DNA-DNA relatedness between strain R9SW1<sup>T</sup> and *M. algicola* LMG 23835<sup>T</sup> was found to be 63.05±1.55%, and between strain A3d10<sup>T</sup> and *M. sediminum* LMG 23833<sup>T</sup> was found to be 67.60±1.3%. Both of these relatedness values are below the 70% cut-off value generally recommended for species differentiation [56]. Recently, information of whole genome sequences have been recommended to be integrated into bacterial systematics [57–59]. In this study, whole genome sequences of strains R9SW1<sup>T</sup>, A3d10<sup>T</sup>, *M. adhaerens* HP15<sup>T</sup> and *M. hydrocarbonoclasticus* ATCC 49840<sup>T</sup> were visually compared using BLAST (Figure S2 in File S1) and the average nucleotide identity (ANI), genome-to-genome distance (GGD), average amino acid identity (AAI), and the Karlin’s genomic signature dissimilarity (d<sup>k</sup>) between the four strains were calculated, the results of which are presented in Table 4. Due to the lack of the availability of the assembled, whole genome sequences for validly named *Marinobacter* species, genomic signatures between strains R9SW1<sup>T</sup>, A3d10<sup>T</sup> and validly described *Marinobacter* species can only be performed using those of *M. adhaerens* HP15<sup>T</sup> [31] and *M. hydrocarbonoclasticus* ATCC 49840<sup>T</sup> [30]. As can be seen from the information presented in Table 4, the ANIs between the four strains were in the range of 82.3–83.3%, which is significantly lower than the suggested threshold range of 95–96% [58,60]; the GGDs were calculated to be in the range of 19.8–20.7% which is lower than the cut-off value of 70% [61]; the AAI and Karlin signature dissimilarity values for the four strains were in the range of 68.1–77.6% and 31–36 respectively, each of which fall outside the range to be considered as same species [42,61]; and thus again indicating that strains R9SW1<sup>T</sup> and A3d10<sup>T</sup> can be considered as two novel species of the genus *Marinobacter*. The distinct standing of strains R9SW1<sup>T</sup> and A3d10<sup>T</sup> can also be confirmed by the phylogenomic

### Table 3.

| Similarity of gyrB and rpoD genes (%) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-------------------------------------|---|---|---|---|---|---|---|---|
| 1. M. adhaerens CIP 110141<sup>T</sup> | 100/100 | | | | | | | |
| 2. M. algicola LMG 23835<sup>T</sup> | 100/100 | 78.0/81.2 | | | | | | |
| 3. M. adhaerens CIP 108615<sup>T</sup> | 100/100 | 100/100 | 77.8/81.0 | | | | | |
| 4. M. hydrocarbonoclasticus ATCC 49840<sup>T</sup> | 100/100 | 100/100 | 100/100 | 100/100 | | | | |
| 5. M. flavimaris CIP 109893<sup>T</sup> | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | | | |
| 6. M. adhaerens HP15<sup>T</sup> | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | | |
| 7. Marinobacter sp. A3d10<sup>T</sup> | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 |
| 8. Marinobacter sp. R9SW1<sup>T</sup> | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 | 100/100 |

The distinct standing of strains R9SW1<sup>T</sup> and A3d10<sup>T</sup> can also be confirmed by the phylogenomic
relationship analysis using the core proteome of the genomes from the four strains (Figure 3).

The major features of the genomes of strains R9SW1T and A3d10T were identified as described elsewhere [16]. Briefly, they are 4,616,332 bp and 3,975,896 bp in size, composed of 99 and 29 contigs, both have 3 rRNAs, and 44 and 46 tRNAs, for strains R9SW1T and A3d10T, respectively. The DNA G+C content of strains R9SW1T and A3d10T were found to be 57.1 and 57.6 mol%, respectively (Table 2), the values which are consistent with those of the genus Marinobacter.

Both bacteria were found to be Gram-negative, aerobic, motile by means of a single flagellum and rod-shaped with the size of 1.9–3.2×0.40–0.72 μm for strain R9SW1T and 1.3–2.1×0.40–0.45 μm for strain A3d10T (Figure S3 in File S1). The catalase and oxidase tests were found to be positive, H₂S and indole tests were found to be negative. It can be seen that strain R9SW1T can be clearly differentiated from M. algicola by its inability to utilise mono- methyl succinate and L-serine, its inability to utilise L-phenylalanine and the absence of lipase (C14); while strain A3d10T can be clearly differentiated from M. sediminum by its inability to reduce nitrite, its ability to utilise glycogen, γ-hydroxybutyric acid and α-glutamic acid, and its weak activities for valine aminopeptidase and cystine aminopeptidase. The major phenotypic difference between strains R9SW1T and A3d10T are nitrate reduction, hydrolysis of starch, fermentation of D-glucose, and their utilisation of dextrin, D-fructose, maltose, acetic acid, propionic acid, succinic acid, L-serine and glycerol. Other phenotypic characteristics which differentiate the two novel strains from each other and their closest phylogenetic neighbours are shown in Table 2, Table S2 in File S1, and in their respective species descriptions. Both strains were found to be sensitive to penicillin G (10 μg), chloramphenicol (30 μg), and ampicillin (10 μg), and resistant to streptomycin (10 μg) and tetracycline (30 μg). The fatty acid composition of strains R9SW1T and A3d10T are shown in Table S3 in File S1, where the predominant

**Table 4.** The genomic signatures between strains R9SW1T, A3d10T, M. adhaerens HP15T and M. hydrocarbonoclasticus ATCC 49840T.

| Genomic signatures | 1 | 2 | 3 | 4 |
|--------------------|---|---|---|---|
| 1. M. hydrocarbonoclasticus ATCC 49840T | 20.1/35 | 20/31 | 19.8/32 |
| 2. M. adhaerens HP15T | 83.1/74.5 | 20.2/36 | 20.7/35 |
| 3. Strain R9SW1T | 82.3/69.5 | 82.7/72.6 | 19.8/31 |
| 4. Strain A3d10T | 82.5/72.7 | 83.3/77.6 | 82.3/68.1 |

Data in the lower triangular corresponds to ANI/AAI (%) and data in the upper triangular corresponds to GGD (%)/Karlin signature.
fatty acids were identified as being C16:0, C16:1ω7c, C18:1ω9c and C11:1ω7c.

The genotype to phenotype analyses were also carried out based on the whole genome sequences of the four strains, the results of which are presented in Table 5. It can be seen that of the results of physiological and biochemical tests match when comparing the *m. silico* results, however a few discrepancies are noted. A similar level of deviation previously reported in the case of *Vibrio* species and it was suggested that expression of certain genes may be restricted by stop codon, repressor genes, regulatory proteins, global regulators, genome coverage or sequencing errors [42].

In summary, the comparative genomic and phylogenetic analysis based on the full-length of 16S rRNA gene sequence similarities, pheno- and chemotaxonomic properties revealed that strains R9SW1T and A3d10T can be affiliated to the genus *Marinobacter*. A further dual-locus sequence analysis based on gyrB and rpoD gene sequence similarities, the comparative analysis of whole cells protein profiles based on MALDI-TOF mass spectrometry analysis, their phenotypic characteristics and their DNA-DNA hybridization values below 70% confirmed that strains R9SW1T and A3d10T should be classified as two novel species of the genus *Marinobacter* for which the name *Marinobacter salarius* sp. nov. and *Marinobacter similis* sp. nov. are proposed.

**Description of Marinobacter salarius sp. nov.**

*Marinobacter salarius* (*sa.la.riu.s*, L. masc. adj., salarius, of or belonging to salt, pertaining to salt tolerance)

Cells are Gram-negative rods (approximately 1.9–3.2×0.40–0.72 μm). Motile by means of a single polar flagellum. Colonies on marine agar are semi-translucent, non-pigmented, circular to slightly irregular (0.8–1.0 mm) and smooth after 48 hours of incubation. Colonies turn to creamy in colour with increasing incubation time. Growth occurs at 4°C–40°C (optimum, 25°C–30°C), between pH 6–9 (optimum, pH 7.5) and in the presence of 0.5–20% (w/v) NaCl. No growth was observed at 0 or 25% (w/v) NaCl. Catalase and oxidase tests are positive. Starch, Tween 40 and 80 are positive, while nitrate and nitrite reduction are negative. Indole, lysine decarboxylase, ornithine decarboxylase, β-galactosidase, tryptophan deaminase, gelatinase, arginine dihydrolase, acetoin, urea and H2S are not produced. Acid is not produced from glucose, mannitol, xylose, inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose and salcin. According to API ZYM, strain R9SW1T is positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase and N-acetyl-β-glucosaminidase weakly positive for acid phosphatase, naphthol-AS-BI-phosphohydrolase and 2-glucosidase; negative for lipase (C14), tryspin, 2-chymotrypsin, 2-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetylgalactosamine and 2-fucosidase. Positive for the utilization of dextrin, glycogen, 13-fructose, maltose, methyl-pyruvate, mono-methyl-succinate, acetic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, β-lactamic acid, propionic acid, succinic acid, 1-glutamic acid, 1-proline, 1-serine and glyceral; weakly positive for the utilization of 1-alaniamide, 1-alanine, 1-alanine and 1-leucine; negative for the utilization of 2-cycloexetin, N-acetyl-β-glucosaminase, N-acetyl-β-glucosamine, adonitol, 1-arabino1, 1-arabinbio1, 1-xylobio1, 2-glucose, 2-galactose, gentiobiose, 2-3-glucose, 2-mannose, 2-lactose, lactulose, 2-mannitol, 2-mannose, 2-melibiose, 2-β-methy1-2-glucoside, 2-psicose, 2-raffinose, 2-thaumatin, 2-sorbitol, sucrose, 2-trehalose, turanose, xylitol, cis-acetic acid, citric acid, formic acid, β-galactosaminic acid lactone, 2-galacturonic acid, 2-glucosaminic acid, 2-glucosuronic acid, β-hydroxybutyric acid, β-hydroxyphenylacetic acid, itaconic acid, 2-ketogluaric acid, 2-ketobutyric acid, 2-ketovaleric acid, malonic acid, quinic acid, 2-saccaric acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamidase, 2-danlyl-glucine, 1-asparagine, 1-aspartic acid, 2-glutamic acid, 1-thistidine, hydroxyl-2-proline, 1-ornithine, 1-phenylalanine, 1-proglutamic acid, 1-serine, 1-threonine, 1-malicarnitine, γ-aminoacetic acid, uracil acid, inosine, uridine, thymidine, phenylhydrimine, putresine, 2-aninoethanol, 2,3-butanol, 2-n-gluceraldehyde, 2-phosphoglucose and glucose-6-phosphate as the sole carbon and energy source. The main cellular fatty acids are C16:0, C16:1ω7c, C18:1ω9c and C11:1ω7c. The G+C content of the type strain is 57.1 mol%. The type strain is R9SW1T (≡ LMG 27497T = JCM 19399T = CIP 110308T = KMM 7502T), isolated from sea water from Chazhma Bay in the Sea of Japan, Pacific Ocean. The accession number for the whole genome sequence of strain R9SW1T is CP007152.

**Description of Marinobacter similis sp. nov.**

*Marinobacter similis* (*si.mi.lys*, L. masc. adj., similis, like, resembling, similar, pertaining to close similarity with other species)

Cells are Gram-negative rods (approximately 1.3 – 2.1×0.40 – 0.45 μm). Motile by means of a single polar flagellum. Colonies on marine agar are semi-translucent, non-pigmented, circular to slightly irregular (0.5 – 1.0 mm) and smooth after 48 hours of incubation. Colonies turn to creamy in colour with increasing incubation time. Growth occurs at 4°C - 40°C (optimum, 25°C - 30°C), between pH 6 to 9 (optimum, pH 7.5) and in the presence of 0.5–20% (w/v) NaCl. No growth was observed at 0 or 25% (w/v) NaCl. Catalase and oxidase tests are positive. Tween 40 and 80 are positive, while starch is not. Nitrate is reduced but not nitrite. Indole, lysine decarboxylase, ornithine decarboxylase, β-galactosidase, tryptophan deaminase, gelatinase, arginine dihydrolase, acetoin, urea and H2S are not produced. Acid is not produced from glucose, mannitol, xylose, inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose and salcin. According to API ZYM, strain A3d10T is positive for alkaline phosphatase, esterase (C4), esterase lipase (C8),

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**Figure 3. Phylogenomic tree of strains R9SW1T, A3d10T, M. hydrocarbonoclasticus ATCC 49840T and M. adhaerens HP15T constructed using concatenated sequence of the core proteome (544,643 bp) of the genomes.** Alteromonas sp. DE was used as outgroup.
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Table 5. Comparative identification of phenotypic characteristics based on genomic analysis and physiological and biochemical tests.

|                        | Strain R9SW1<sup>T</sup> | Strain A3d10<sup>T</sup> | M. hydrocarbonoclasticus ATCC 49840<sup>T</sup> | M. adhaerens HP15<sup>T</sup> |
|------------------------|--------------------------|--------------------------|-----------------------------------------------|-----------------------------|
|                        | In vitro | In silico | In vitro | In silico | In vitro | In silico | In vitro | In silico |
| Nitrate reduction       | -        | +         | +        | +         | +         | +         |          |          |
| Nitrite reduction       | -        | -         | -        | -         | -         | +         |          |          |
| Hydrolysis of starch    | +        | -         | -        | -         | -         | -         |          |          |
| Indole production       | -        | -         | -        | -         | -         | -         |          |          |
| D-Glucose              | -        | -         | w        | -         | -         | -         |          | w         |
| Lactose                | -        | -         | -        | -         | -         | -         |          | w         |
| Glycogen               | +        | -         | +        | -         | -         | +         |          | +         |
| Mono-methyl-succinate  | +        | +         | +        | +         | +         | +         | +         | +         |
| γ-Hydroxy-butyric acid  | +        | -         | +        | -         | -         | -         | w         | +         |
| Succinic acid           | +        | +         | -        | -         | +         | +         | w         | +         |
| L-Glutamic acid         | +        | +         | +        | +         | +         | +         |          | +         |
| L-Phenylalanine         | -        | -         | -        | -         | -         | -         |          | -         |
| L-Serine               | +        | +         | -        | -         | -         | -         |          | -         |
| Glycerol               | +        | +         | -        | -         | -         | -         |          | +         |

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leucine arylamidase, naphthol-AS-BI-phosphohydrolase, and N-acetyl-β-glucosaminidase; weakly positive for lipase (C14), valine arylamidase, cystine arylamidase and acid phosphatase; negative for trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-glucosidase, α-mannosidase and α-fucosidase. Positive for the utilization of, glycerol, methyl-β-pyruvate, mono-methyl-succinate, β-hydroxybutyric acid, γ-hydroxybutyric acid, α- and β-malic acid, α, β-galactose, α, β-glucose, α, β-mannose, α, β-galactoside, α, β-glucosamine, α, β-galactosamine, α, β-galactosylglucosamine, and α, β-proline; negative for the utilization of 2-cycloexetrin, dextrin, N-acetyl-β-glucosamine, N-acetyl-β-glucosaminidase, adonitol, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arabinose, α-arbin...
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