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Marinobacter iranensis sp. nov., a slightly halophilic bacterium from a hypersaline lake

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Abstract

A novel halophilic bacterium, strain 71-i^T, was isolated from Inche-Broun hypersaline lake in Golestan province, in the north of Iran. It was a Gram-stain-negative, non-endospore forming, rod-shaped bacterium. It grew at 4–40°C (optimum 30°C), pH 6.0–11.0 (optimum pH 7.5) and with 0.5–15% (w/v) NaCl [optimum 3% (w/v) NaCl]. The results of phylogenetic analyses based on the 16S rRNA gene sequence comparison indicated its affiliation to the genus *Marinobacter* and the low percentage of identity with the most closely related species (97.5%), indicated its placement as a novel species within this genus. Digital DNA–DNA hybridization (dDDH) values and average nucleotide identity (ANI) analyses of this strain against closely related species confirmed its condition of novel taxon. On the other hand, the percentage of the average amino acid identity (AAI) affiliated strain 71-i^T within the genus *Marinobacter*. The DNA G+C content of this isolate was 57.7 mol%. The major fatty acids were $C_{16:0}$ and $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$. Ubiquinone-9 was the major isoprenoid quinone and diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) were the main polar lipids of this strain. On the basis of the phylogenomic and phenotypic (including chemotaxonomic) features, we propose strain 71-i^T (= IBRC M 11023^T = CECT 30160^T = LMG 29252^T) as the type strain of a novel species within the genus *Marinobacter*, with the name *Marinobacter iranensis* sp. nov. Genomic detections of this strain in various metagenomic databases indicate that it is a relatively abundant species in environments with low salinities (approximately 5% salinity), but not in hypersaline habitats with high salt concentrations.

INTRODUCTION

Halophilic microorganisms are classically categorized on the basis of their optimal growth at different salt concentrations. The two main groups are the extreme halophiles [optimal growth above 15% (w/v) NaCl] and moderate halophiles [optimal growth at 3–15% (w/v) NaCl]. In contrast, slight halophiles are able to grow optimally in media with 1–3% (w/v) NaCl [1].

Traditionally, the genus *Marinobacter* has been classified as part of the family *Alteromonadaceae*, within the order *Alteromonadales*, class *Gammaproteobacteria* [2], but recently a new family, *Marinobacteraceae* (order *Oceanospirillales*), has been proposed to include the genus *Marinobacter*, together with the genera *Mangrovitalea* and *Tamilnaduibacter* [3]. The genus *Marinobacter* was initially established by Gauthier *et al.* in 1992 [2], with *Marinobacter hydrocarbonoclasticus* as the type species, but as a result of instances of synonymy and an incorrect interpretation of the International Code of Nomenclature of Prokaryotes, *Marinobacter nauticus* is currently recognized as the new type species of the genus *Marinobacter* [4]. At the time of writing, this genus comprised 57 species with validly published names [5]. Cell of species of this genus are Gram-stain-negative, rod-shaped, motile, halophilic or halotolerant and aerobic or facultatively anaerobic. The DNA G+C content of the strains of species of the genus *Marinobacter* ranges from 52.7 to 63.5 mol% [5].

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Abbreviations: AAI, average amino acid identity; ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; DPG, diphosphatidylglycerol; GGDC, Genome-to-Genome Distance Calculator; MK, menaquinone; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGAP, NCBI Prokaryotic Genome Annotation Pipeline.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Marinobacter iranenesis* 71-i^T is MK101100 and that of its complete genome is JANCMW000000000.
One supplementary table and one supplementary figure are available with the online version of this article.

Most members of the genus *Marinobacter* have been isolated from saline environments, such as sea water and sediment, tidal flats, saline soil, solar salterns and salt lakes [6–12] but they have also been isolated from sites as diverse as associated with diatoms [13], dinoflagellates [14, 15] or sponges [16], from mangrove sediments [17], hydrothermal sediment [18], Antarctic environments [19–22] or from wine-barrel-decalcification wastewater [23]. Recently, 16S rRNA gene sequences related to this genus have also been reported in culture-independent studies in different habitats, such as an oil and gas reservoir [24], salt mines [25], associated with algae [26] or at Rio Tinto (Spain) [27].

During the course of the study of the prokaryotic diversity of hypersaline environments in Iran, different habitats were sampled and a novel bacterium, designated strain 71-i^T, was isolated in pure culture from Inche-Broun hypersaline lake, located in Golestan province, northern Iran. The sampling information and physico-chemical properties of the samples have been described by Rasooli *et al.* [28]. This strain was phylogenetically most closely related to species of the genus *Marinobacter*. In this paper we describe the isolation as well as the taxogenomic and phenoptypic characterization of this bacterium and we propose it as a novel species of the genus *Marinobacter*, with the name *Marinobacter iranensis* sp. nov.

ISOLATION AND ECOLOGY

Strain 71-i^T was isolated from a superficial water sample obtained in May 2014 from Inche-Broun hypersaline lake, Golestan province, northern Iran, (37°13' N, 54°30′ E). The salinity of the sample was 17.7% (w/v), pH 4.3 and temperature 33°C. Samples were collected in sterile containers, transported to the laboratory and plated under sterile conditions. The strain was isolated by serial dilution in 10% (w/v) salt solution, plating on marine medium 10% (MM10) after incubation at 34°C aerobically for 2 days. The MM10 medium contained (g l^{-1}): NaCl, 86; $MgCl_2$.6 H_2O, 12.6; Na_2SO_4, 3.24; $CaCl_2$, 1.8; KCl, 0.55; $NaHCO_3$, 0.16; ferric citrate, 0.1; KBr, 0.08; $SrCl_2$, 0.03; H_3BO_3, 0.02; Na_2HPO_4, 0.0008, Na_2SiO_3, 0004; NaF, 00024; NH_4NO3, 0.00016, peptone, 5; yeast extract, 1. The pH of the medium was adjusted to 7.5 with 1 M KOH, and 1.5% agar (Oxoid) was added to solidify the medium when necessary. Strain 71-i^T was routinely grown in the same MM10 medium and incubated aerobically at 34°C, using a rotary shaker for growth in liquid medium. The isolate was maintained at −80°C in MM10 liquid medium containing 20% (v/v) glycerol. *Marinobacter salarius* DSM 27081^T, *Marinobacter algicola* DSM 16394^T and *Marinobacter gudaonensis* DSM 18066^T were used as reference strains for comparative purposes in our study.

16S rRNA GENE PHYLOGENY

The method described by Marmur [29] was carried out for the extraction and purification of the genomic DNA from strain 71-i^T. The quality of the DNA was checked using 1% (w/v) agarose gel electrophoresis. DNA quantification was determined by spectrophotometry (DS-11 FX, DeNovix Technologies) and fluorometry (Qubit 3.0 Fluorometer, Thermofisher Scientific). The 16S rRNA gene was amplified by PCR [30] with the universal primers 16F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16R1488 (5′-CGGTTACCTTGTTAGGACTTCACC-3′) [31]. PCR products were purified using the commercial kit MEGAquick-spinTM Plus (INtRON Biotechnology) and were sequenced by StabVida (Caparica, Portugal) using the Sanger method with the same primers used for amplification and also the primers 16R343 (5′-ACTGCTGCCTCCCGTA-3′) and 16F530 (5′-GTGCCAGCAGCCGCGG-3′) in order to obtain and be able to assemble the complete sequence. Sequencing reactions were carried out using a BigDye terminator kit version 3.1 from Applied Biosystems.

The 16S rRNA gene sequence of strain 71-i^T was aligned with ChromasPro (Technelysium) software version 1.5 and deposited in GenBank/EMBL/DDBJ under the accession number MK101100. The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequences similarities were achieved using the EzBioCloud tool [32] and ARB software package [33]. The 16S rRNA gene sequence analysis and phylogenetic tree reconstructions were performed with the ARB software package [32]. Phylogenetic trees were reconstructed using three different methods: maximum-parsimony [34], neighbour-joining [35] and maximum-likelihood [36] algorithms integrated in the ARB software for phylogenetic inference. A bootstrap analysis (1000 replications) was performed to evaluate the robustness of the phylogenetic trees [37]. The 16S rRNA gene sequences from related species used for phylogenetic comparisons were obtained from the GenBank database.

The almost-complete 16S rRNA gene analysis of strain 71-i^T (1528 bp) indicated that it was closely related to members of the genus *Marinobacter*, with *Marinobacter salarius* R9SW1^T [38], *Marinobacter algicola* DG893^T [14], *Marinobacter gudaonensis* CGMCC 1.6294^T [39] and *Marinobacter vulgaris* F01^T [40] as the closest relatives, with 97.5%, 97.5%, 97.4% and 97.4% sequence similarity, respectively. The 16S rRNA gene sequence similarity with other species of the genus *Marinobacter* as well as species of other genera, such as *Tamilnaduibacter* [41] or *Mangrovitalea* [42] was always equal to or lower than 97.3% (Table 1). The 16S rRNA gene sequence of strain 71-i^T obtained by PCR was identical to that obtained from the genome. The 16S rRNA-based phylogenetic tree reconstructed using the maximum-parsimony method (Fig. 1) indicated that strain 71-i^T clustered with *Marinobacter vulgaris* F01^T, but it was placed in an independent branch, with a bootstrap value of 99%. This topology indicates that the novel strain 71-i^T could represent a novel species of the genus *Marinobacter*. In the same way, the topologies of phylogenetic trees inferred

Table 1. 16S rRNA gene sequence similarity, digital DDH, OrthoANI and AAI percentages between genomes of strain 71-i^T and strains of species of the genus *Marinobacter* and the other two genera of the family *Marinobacteriaceae*

Type strain	Genome accession numbers	16S rRNA (%)	OrthoANI (%)	Digital DDH (%)	AAI (%)
Marinobacter salarius R9SW1^T	NZ_CP007152.1	97.5	83.8	27	88.3
Marinobacter algicola DG893^T	NZ_ABCP00000000.1	97.5	83.6	27	88.6
Marinobacter gudaonensis CGMCC 1.6294^T	NZ_FOYV00000000.1	97.4	76.6	20	78.4
Marinobacter vulgaris F01^T	NZ_VMBE00000000.1	97.4	82.4	25	85.8
Marinobacter nauticus DSM 50418^T	NZ_RBJB00000000.1	96.4	75.5	20	75.3
Marinobacter adhaerens HP15^T	NZ_CP076686.1	97.1	76.4	20	77.5
Marinobacter alexandrii LZ-8^T	NZ_SWKM00000000.1	*95.9*	76.2	24	76.4
Marinobacter antarcticus CGMCC 1.10835^T	NZ_FRAQ00000000.1	*95.2*	74.8	20	76.3
Marinobacter aromaticivorans D15-8P^T	NZ_NIHD00000000.1	96.0	74.9	19	76.0
Marinobacter bohaiensis T17^T	NZ_QGEH00000000.1	*95.5*	73.4	20	68.2
Marinobacter bryozoorum DSM 15401^T	NZ_JAKZAH000000000.1	96.0	74.5	20	70.3
Marinobacter changyiensis CLL7-20^T	NZ_VZZZ00000000.1	96.2	74.5	20	74.0
Marinobacter confluentis HJM-18^T	NZ_SRPF00000000.1	96.5	76.4	20	78.3
Marinobacter daepoensis DSM 16072^T	NZ_ATWI00000000.1	*94.7*	74.3	19	74.3
Marinobacter daqiaonensis YCSA40^T	NZ_JAAFYR000000000.1	95.6	74.6	20	71.2
Marinobacter denitrificans JB02H27^T	NZ_VMHN00000000.1	97.1	82.5	25	86.5
Marinobacter excellens LAMA 842	NZ_LOCO00000000.1	*94.2*	75.3	20	75.4
Marinobacter flavimaris KCTC 12185^T	NZ_QRDH00000000.1	96.6	76.6	20	78.0
Marinobacter fonticola CS412^T	NZ_CP043042.1	*95.3*	73.0	21	69.0
Marinobacter fuscus NH169-3^T	NZ_PXNP00000000.1	*95.3*	74.8	20	73.4
Marinobacter goseongensis KCTC 12515^T	NZ_JAKZAI000000000.1	96.7	77.1	21	78.4
Marinobacter guineae M3B^T	NZ_NTFI00000000.1	96.8	76.9	21	78.6
Marinobacter halodurans YJ-S3-2^T	NZ_SJDL00000000.1	*95.4*	73.9	20	68.4
Marinobacter halophilus JCM 30472^T	NZ_PXNN00000000.1	*95.2*	75.3	20	75.0
Marinobacter halotolerans NBRC 110910^T	NZ_VMHP00000000.1	96.2	77.1	21	78.9
Marinibacter koreensis DSM 17924^T	NZ_JAKZAJ000000000.1	96.3	75.2	20	76.1
Marinobacter lipolyticus SM19^T	NZ_ASAD00000000.1	97.3	77.1	21	79.4
Marinobacter litoralis SW-45^T	NZ_QMDL00000000.1	*93.9*	73.4	19	74.0
Marinobacter lutaoensis T5054^T	NZ_MSCW00000000.1	95.6	75.3	19	73.7
Marinobacter mangrovi CHFG3-1-5^T	NZ_JAERVO000000000.1	*95.7*	73.6	20	68.6
Marinobacter maritimus CK 47^T	NZ_VCGW00000000.1	96.0	74.2	19	75.3
Marinobacter maroccanus N4^T	NZ_PSSX00000000.1	96.9	76.5	20	78.4
Marinobacter mobilis CGMCC 1.7059^T	NZ_FNNE00000000.1	95.9	74.1	19	71.8
Marinobacter nitratireducens AK21^T	NZ_ANIE00000000.1	95.8	75.1	19	76.2
Marinobacter oulmenensis DSM 22359^T	NZ_JACHFE000000000.1	*95.6*	75.2	20	72.8
Marinobacter orientalis W62^T	NZ_SRZX00000000.1	*96.6*	82.7	26	85.3
Marinobacter panjinensis PJ-16^T	NZ_SZYH01000000	97.2	83.6	26	87.0
Marinobacter pelagius CGMCC 1.6775^T	NZ_FOUR00000000.1	96.6	76.6	21	76.9
Marinobacter persicus IBRC M 10445^T	NZ_FOSC00000000.1	96.0	75.4	20	72.9

Continued

Table 1. Continued

Type strain	Genome accession numbers	16S rRNA (%)	OrthoANI (%)	Digital DDH (%)	AAI (%)
Marinobacter profundi PWS21^T	NZ_NTFH00000000.1	96.7	76.1	21	76.1
Marinobacter psychrophilus 20041^T	NZ_CP011494.1	*95.5*	73.1	20	71.1
Marinobacter salexigens HJR7^T	NZ_NIHC00000000.1	*94.9*	74.0	19	75.0
Marinobacter salicampi ISL-40^T	NZ_JAAMPF000000000.1	96.2	73.8	20	71.8
Marinobacter salinexigens ZYF650^T	NZ_VTUU00000000.1	96.0	74.6	19	75.4
Marinobacter salinus Hb8^T	NZ_CP017715.1	96.8	75.6	20	77.5
Marinobacter salsuginis SN-3	NZ_BGZH00000000.1	96.4	76.4	20	78.3
Marinobacter santoriniensis NKSG1^T	NZ_APAT00000000.1	96.0	75.2	20	75.7
Marinobacter sediminum R65^T	NZ_JAEMQH000000000.1	96.4	75.8	20	77.1
Marinobacter segnicrescens CGMCC 1.6489^T	NZ_FOHZ00000000.1	96.6	74.3	20	70.1
Marinobacter shengliensis SL013A34A2^T	NZ_PXNO00000000.1	*95.2*	75.1	20	75.3
Marinobacter similis A3d10^T	NZ_CP007151.1	96.6	75.9	20	76.1
Marinobacter vinifirmus FB1^T	NZ_NEFY00000000.1	95.0	76.3	21	74.6
Marinibacter xestospongiae JCM 17469^T	NZ_JAKZAK000000000.1	96.0	74.8	20	70.9
Marinobacter zhanjiangensis KCTC 22280^T	NZ_BMXV00000000.1	96.1	74.6	20	70.0
Marinobacter zhejiangensis CGMCC 1.7061^T	*NZ_FOUE00000000.1*	*95.5*	73.7	19	71.5
Mangrovitalea sediminis M11-4^T	*NZ_NTLB00000000.1*	*93.7*	70.4	19	60.2
Tamilnaduibacter salinus Mi-7^T	*NZ_NMPM00000000.1*	*93.2*	72.0	19	66.5

*Numbers in black indicate that the calculation of pairwise 16S rRNA gene sequences similarities were achieved using the EzBioCloud tool [31] and numbers in italics indicate that the calculation was performed with the ARB software package [32].

using the neighbour-joining and maximum-likelihood algorithms were highly similar to that of the tree obtained using the maximum-parsimony method (Fig. 1).

GENOME CHARACTERIZATION AND KEY FEATURES

The genomic DNA extracted by the Marmur [29] method described in the previous section was used to obtain the draft genome sequence of strain 71-i^T, using a whole-genome shotgun strategy with a NovaSeq 6000 platform (Illumina) and 150 bp paired-end sequencing reads (Novogene Europe, Cambridge, United Kingdom). For *de novo* assembly of the reads Spades v.3.13.0 [43] was used. The bioinformatic software CheckM v1.0.5 [44] and Quast v2.3 [45] were utilized to assess the quality of the final contigs. The genome sequence of strain 71-i^T was annotated using the prokaryotic genome annotation pipeline (PGAP) [46] and deposited in GenBank/EMBL/DDBJ under the accession number JANCMW000000000. For the phylogenetic core genome analysis reconstruction all-versus-all BLAST search [47] was used for comparisons of all predicted protein-coding genes annotated from each available genome. MUSCLE [48] was used for the individual alignment of the core orthologous genes with diagonal optimization and adjusting to the maximum number of iterations (default values for the other parameters). The phylogenomic tree was reconstructed by using FastTree v.2.1 (approximately maximum-likelihood) [49].

The genomic indexes average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) between the genome of strain 71-i^T and the available genomes of the species of the genera *Marinobacter*, *Mangrovitalea* and *Tamilnaduibacter* (the only three genera of the new family *Marinobacteraceae*) were calculated by using OAT software v0.93.1 [50] and the Genome-to-Genome Distance Calculator (GGDC) [51] website, using formula 2 [52], respectively. Also, the average amino acid identity (AAI) was estimated using AAI CompareM programme [https://github.com/dparks1134/CompareM].

The main features of the draft genomes of strain 71-i^T and its closest related species are shown in Table 2. The draft genome of strain 71-i^T was *de novo* assembled in a total of 39 contigs. The sequencing coverage depth of the entire genome was 369× with a N50 value of 266694 bp. These genome features are in accordance with the minimal standards for the use of genome data for the taxonomy of prokaryotes [53]. The genome size of strain 71-i^T was 4.7 Mb, almost identical to those of *Marinobacter salarius*

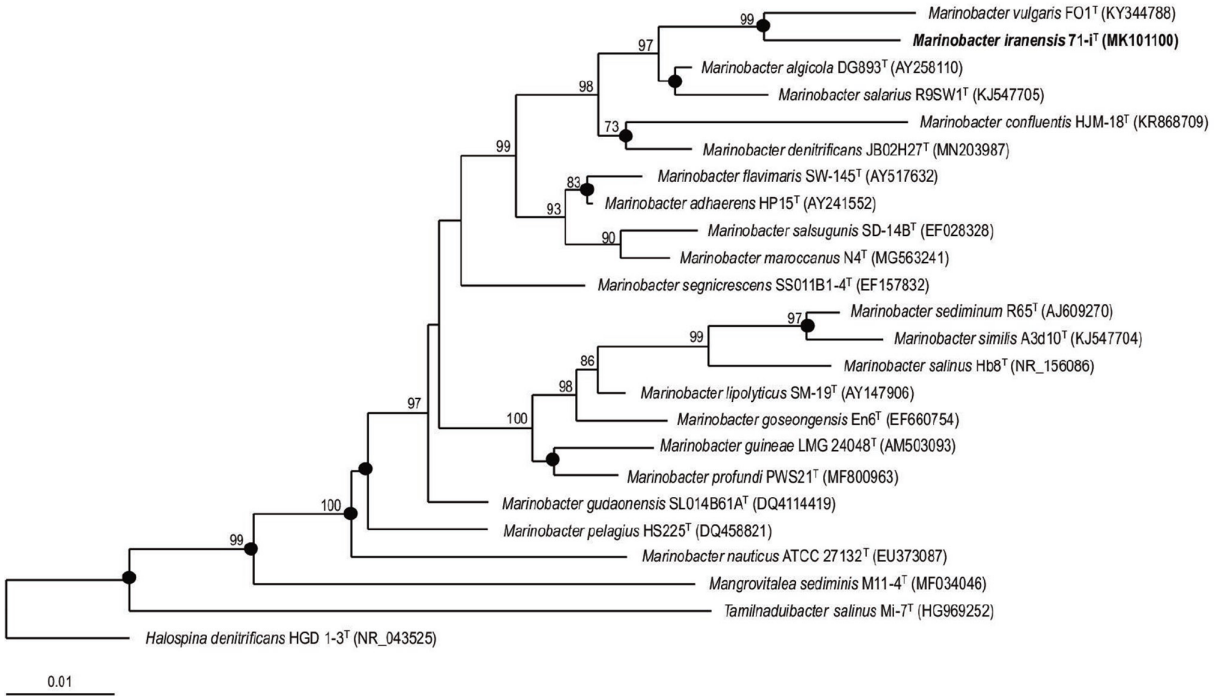

Fig. 1. Maximum-parsimony phylogenetic tree based on the 16S rRNA gene sequence comparison showing the phylogenetic position of strain 71-i^T and the closely related species of the genus *Marinobacter* and other related genera. Sequence accession numbers are shown in parenthesis. Bootstrap values higher than 70% are indicated at branch-points. Filled circles indicate that the corresponding nodes were also obtained in the trees reconstructed with the neighbour-joining and maximum-likelihood algorithms. *Halospina denitrificans* HGD 1-3^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

R9SW1^T and *Marinobacter algicola* DG893^T. The DNA G+C content (57.7 mol%) and the number of rRNAs (3) and tRNAs (50) also were similar to those of the other species of the genus *Marinobacter* (Table 2).

In addition, a reconstruction of the phylogenetic core genome analysis was performed. The phylogenomic tree reconstruction (Fig. 2) based on the alignment of 1350 translated proteins shared by all strains revealed unequivocally that strain F71-i^T formed a clade with *Marinobacter salarius* R9SW1^T and *Marinobacter algicola* DG893^T, with a bootstrap value of 100%, but clustered in a different branch, well separated from them with a bootstrap value of 100%, reinforcing its identification as a novel taxon within the genus *Marinobacter*.

In order to confirm whether strain 71-i^T represents a novel species within the genus *Marinobacter*, the genomic parameters OrthoANI and dDDH were estimated. The OrthoANI values between strain 71-i^T, *Marinobacter salarius* R9SW1^T, *Marinobacter*

Table 2. General features of the genomes of strain 71-i^T and type strains of related species of the genus *Marinobacter*

Feature	Strain 71-i^T	*Marinobacter salarius* R9SW1^T	*Marinobacter algicola* DG893^T	*Marinobacter gudaonensis* CGMCC 1.6294^T	*Marinobacter vulgaris* F01^T
Size (bp)	4694622	4616530	4413000	3839020	3803850
Contigs	39	1	104	11	24
DNA G+C content (mol%)	57.7	57.2	57.0	59.9	57.7
N_{50} (bp)	266694	4616532	79366	2894534	650725
Coverage	369×	27×	83×	228×	100×
Genes	4507	4462	4093	3434	3529
rRNA	3	3	7	10	5
tRNA	50	44	45	45	46

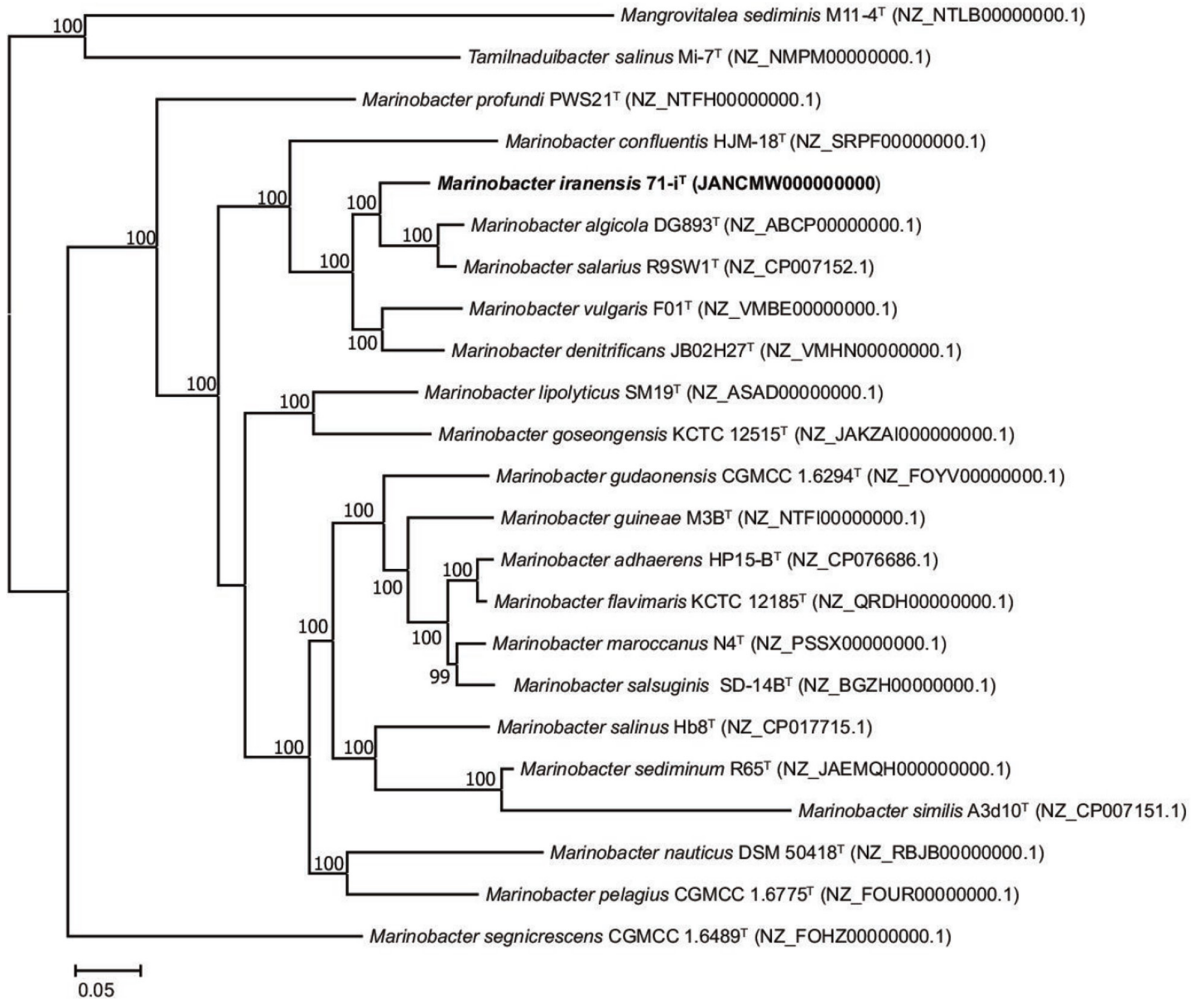

Fig. 2. Phylogenomic tree based on the core orthologous genes of strain 71-i^T and related species based on the neighbour-joining algorithm. This tree was obtained after the alignment of 1350 shared translated proteins of their genomes. Bootstrap values higher than 70% are indicated at branch-points. Bar, 0.05 substitutions per nucleotide position.

algicola DG893^T, *Marinobacter gudaonensis* CGMCC 1.6294^T and *Marinobacter vulgaris* F01^T were 83.8%, 83.6%, 76.6% and 82.4%, respectively (Table 1), while the dDDH values between these strains were 27%, 27%, 20% and 25%, respectively (Table 1). The values of OrthoANI and *digital* DDH between strain 71-i^T and the type species of the genus *Marinobacter nauticus* were 75.5% and 20%, respectively (Table 2). All these percentages of OrthoANI and dDDH are lower than 95–96% and 70%, respectively, which are the defined cut-off limits for species delineation [50, 54–56], and they confirm that strain 71-i^T is genotypically distinct from any previously described species and should be assigned to a different species.

An alternative to ANI for more distantly related genomes is the average amino acid identity (AAI). In this case, to confirm that strain 71-i^T and all the species of the genus *Marinobacter* are properly assigned to this genus, the AAI values between them were calculated as described previously. The AAI values between strain 71-i^T and all members of the genus *Marinobacter* ranged between 68.2 and 88.6%, (Table 1), these values are above the threshold considered for species of the same genus (65%) [57–59]. The AAI values between strain 71-i^T and *Tamilnaduibacter salinus* Mi-7^T and *Mangrovitalea sediminis* M11-4^T were 66.5% and 60.3% respectively (Table 1). The AAI values between strain 71-i^T and *Tamilnaduibacter salinus* Mi-7^T was slightly above the cutoff percentage, but, based on the topology of the core-genome tree, it is evident that they belong to two different genera.

PHYSIOLOGY AND CHEMOTAXONOMY

Colony morphology was observed by culturing the isolate on MM10 medium after 48 h of incubation at 30°C, and Gram staining was performed according to the Burke method [60]. The morphology of the cells in exponential phase was examined

using a BX51 optical microscope (Olympus) equipped with phase-contrast optics. The wet-mount method was used to examine motility [60]. The anaerobic growth of strain 71-i^T was tested in the presence of N_2 in an anaerobic chamber in MM10 medium plates. Broth cultures in MM10 medium were prepared and incubated at 4 and 15–45°C (at intervals of 5°C) to determine the range and optimum temperature for strain growth, and MM10 medium with different pH values of 4–11 (at intervals of 0.5 units) were used to evaluate the pH range and optimum, with the flask incubated in a shaker incubator at 150 rpm. The pH was adjusted by adding 50 mM sodium acetate/acetic acid (pH 4.0–6.0), Tris/HCl (pH 6.5–8.5), and glycine/sodium hydroxide (pH 9–11) buffers to the media. The range and optimal NaCl concentration for growth was determined using MM10 without NaCl as the base medium, to which increasing concentrations of NaCl were added at intervals of 1% (w/v) NaCl up to-20% (w/v) NaCl.

Catalase and oxidase activity were examined by the methods of Krieg and Padgett [61] in a medium with 3% (w/v) NaCl. Hydrolysis of Tweens 20 and 80 was detected as described by Gutiérrez and González [62]. Hydrolysis of gelatin, casein and starch and urease activity were examined according to the methods of Mata *et al.* [63]. Acid production from carbohydrates was determined by the method of Leifson [64]. Nitrate reduction, indole production, arginine dihydrolase, β-glucosidase and β-galactosidase activity were assayed using API 20NE strips (bioMérieux) according to manufacturer's instructions. The method recommended by Ventosa *et al.* [65], was used to investigate the use of organic compounds as their sole carbon and energy source. Antibiotic susceptibility tests were done on Muller–Hinton agar medium with 3% (w/v) NaCl. The bacterial suspension with 1.5×10^6 c.f.u. ml^{-1} and (HiMedia) antimicrobial discs were used for evaluation. The inhibition zone after 48 h incubation at 30°C was interpreted according to the manufacturer's manual [66].

Cell biomass for fatty acids determination was obtained by cultivation on Marine Agar (Difco) supplemented with 3% (w/v) NaCl at pH 8 and 25°C. Cells were harvested in the mid-exponential growth phase. The whole-cell fatty acid composition of strain 71-i^T was determined using the MIDI, Version 6.1; Identification Library TSBA40 4.1; Microbial ID. Extracts were analysed using a model HP6890A gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector as described by Kämpfer and Kroppenstedt [67]. Fatty acid peaks were identified using the TSBA40 database. The determination of the fatty acids of *Marinobacter salarius* JCM 19399^T; *Marinobacter algicola* DSM 16394^T and *Marinobacter vulgaris* FO1^T had also been carried out under the same conditions [40], so these results could be compared. Lyophilized cells used to extract the polar lipids and quinones were obtained by growing strain 71-i^T on MM10 liquid medium at 30°C for 48 h. After that, the biomass was harvested by centrifugation for 20 min at 2600 *g*, washed twice with distilled water, and used for the analysis. The methods described by Minnikin *et al.* [68] were used.

Cells of strain 71-i^T were rod-sharped with a size of 0.2 µm (width) by 0.8 to 1.9 µm. They were motile and Gram-stain-negative. Colonies on MM10 were circular, with entire margins, cream-pigmented, translucent, smooth, convex and 0.2–0.5 mm in diameter. Facultatively anaerobic, able to grow under anaerobic conditions. The temperature range for growth of strain 71-i^T was found to be 4–40°C, with an optimum at 25–30°C, and the pH range was pH 6–11 with the optimum at pH 7.5. The strain grew at NaCl concentrations of 0.5–15% (w/v), with optimal growth at 3% (w/v) NaCl. This result indicates that this strain is a slightly halophilic bacterium.

Catalase and oxidase activities were present. The strain could hydrolyze Tweens 20 and 80 but not casein, gelatin or starch. Strain 71-i^T was sensitive to chloramphenicol (30 µg), neomycin (30 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), streptomycin (10 µg), tobramycin (10 µg), tetracycline (30 µg), ampicillin (10 µg), polymyxin-B (300 units), cefoxitin (30 µg) and gentamicin (30 µg), but resistant to bacitracin (10 units), erythromycin (10 µg), novobiocin (30 µg), kanamycin (30 µg), penicillin G (10 units), amikacin (30 µg) and amoxicillin (30 µg). Other features of strain 71-i^T and the differential characteristics between this strain and the closely related species *Marinobacter salarius* DSM 27081^T, *Marinobacter algicola* DSM 16394^T and *Marinobacter gudaonensis* DSM 18066^T are given in the species description and Table 3.

The cellular fatty acids profile of strain 71-i^T was characterized by the fatty acids $C_{16:0}$ (25.0%) and $C_{16:1}\omega7c/C_{16:1}\omega6c$ (20.4%) as the major fatty acids. The fatty acids profile of this strain was similar to those of other type strains of species of the genus *Marinobacter*. However, the percentages of these fatty acids were different from those obtained for other phylogenetically related species [40]. Strain 71-i^T presented a small percentage of $C_{16:1}\omega9c$, not detected in the rest of the related strains used for comparison (Table S1, available in the online version of this article).

For the chemotaxonomic characterization of strain 71-i^T, the total lipids and quinones were determined. High performance thin layer chromatography (HPTLC) of the polar lipids (Fig. S1) revealed that the polar lipid profile of strain 71-i^T consisted of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and aminoglycophospholipid (AGPL). With respect to the presence of quinones, ubiquinone-9 (Q9) was determined to be the main respiratory quinone.

On the basis of the evidence obtained from the phylogenetic and phylogenomic analyses and the OrthoANI, dDDH and AAI genomic indexes, it is concluded that strain 71-i^T should be considered to represent a novel species within the genus *Marinobacter*.

Table 3. Differential characteristics between strain 71-i^T and the phylogenetically related type strains of species of the genus *Marinobacter*

Taxa: 1, strain 71-i^T; 2, *Marinobacter salarius* DSM 27081^T; 3, *Marinobacter algicola* DSM 16394^T; 4, *Marinobacter gudaonensis* DSM 18066^T. Data are from this study except where indicated. +, Positive; –, negative; w, weakly positive; ND, not determined.

Feature	1	2	3	4
Colony pigmentation	Cream	Cream	Cream	Transparent
pH range	6.0–11.0	6.0–9.0*	5.0–10.0†	6.0–9.5
Temperature range (°C)	4–40	4-40*	5-40†	10-45‡
Optimum temperature (°C)	30	25-30*	25-30†	ND
Salinity range (%, w/v)	0.5–15	0.5-20*	1-12†	0-15‡
Nitrate reduction	+	–	–	+
Hydrolysis of starch	–	+	+	+
Urease activity	–	–	+	–
Arginine dihydrolase	–	–	+	ND
Utilization of:				
D-glucose	+	–	+	+
Sorbitol	w	–	–	+
Sucrose	+	–	–	+
Succinate	–	–	+	+
Leucine	–	–	+	ND
L-serine	–	+	–	ND
L-glutamic acid	–	+	+	–

*Data from Ng *et al.* [38].
†Data from Green *et al.* [14].
‡Data from Gu *et al.* [39].

GENOMIC FRAGMENT RECRUITMENT ANALYSIS

To evaluate the presence or abundance in saline or hypersaline habitats of strains related (at the species level) to strain 71-i^T, genome fragment recruitments with environmental metagenomic datasets were performed. Genome contigs were concatenated and all the 16S rRNA gene sequences present were masked. BLASTN (with the cut-offs: Alignment length ≥ 30 nt, identity > 95%, E-value ≤1×10^{-5}) was used in order to align the metagenomic quality-filtered shotgun reads against the strain 71-i^T genome. Best-hits BLASTN results obtained were used to construct the figures. To show that strain 71-i^T and strains related at the species level are present or abundant in natural habitats, genome fragment recruitment with several environmental metagenomic datasets from habitats with different salinities were performed. Results are shown in Fig. 3.

Recruitment plots with respect to six metagenomic datasets obtained from different habitats, ordered by salinity concentration were obtained. They corresponded to two different hypersaline environments: Santa Pola saltern, located in East Spain (SS13 with 13%, SS19 with 19%, SS33 with 33% and SS37 with 37% salinity, respectively) and the hypersaline Lake Meyghan in Iran (LM5 with 5% and LM30 with 30% salinity, respectively) [69–71]. In both type of environments, the presence and abundance of strain 71-i^T in the metagenomes decreased with increasing salinity, indicating that strain 71-i^T prefers to inhabit ecosystems with low salinities (approximately 5%), and thus these results are congruent with the laboratory data, which indicated that the novel isolate is slightly halophilic, unlike other moderately or extremely halophilic species of the genus *Marinobacter*.

As supported by numerous studies, many prokaryotes remain to be discovered and described, and great efforts must continue to isolate them in pure culture. In this work we describe the course of a biodiversity study at Inche-Broun hypersaline lake (Golestan province, northern Iran), that allowed us to isolate a novel halophilic bacterium, strain 71-i^T, phylogenetically related to the genus *Marinobacter*. It was characterized using both genomic and classical taxonomic methods in order to determine its precise affiliation. In the light of the results of the polyphasic taxogenomic study it is concluded that strain 71-i^T represents a novel species within the genus *Marinobacter*, for which the name *Marinobacter iranenesis* sp. nov. is proposed, whose description is given below. Metagenomic fragment recruitment indicated that this novel taxon prefers to inhabit environments with low salinities (5% salinity).

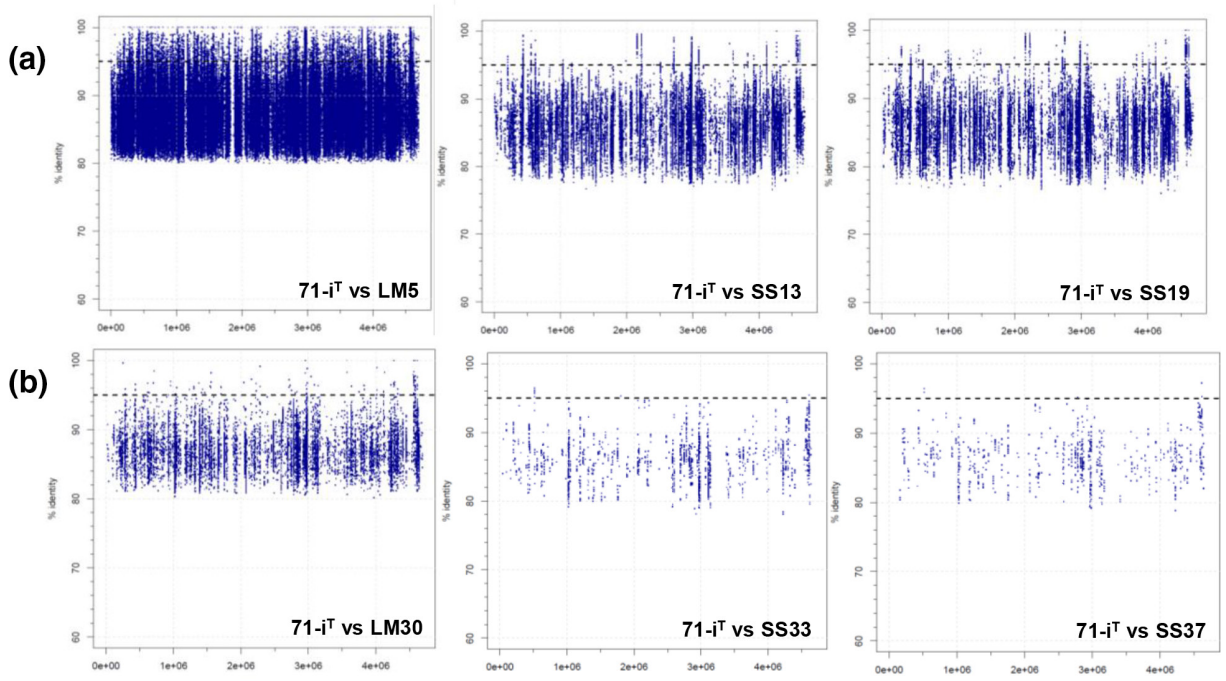

Fig. 3. Recruitment plots of strain 71-i^T against the metagenomic datasets: (a) LM5, SS13 and SS19; (b) LM30, SS33 and SS37. In each panel the Y axis represents the identity percentage and the X axis represents the genome length. A restrictive cut-off 95% of nucleotide identity in at least 30 bp of the metagenomic read was used. The black dashed lines show the threshold for the presence of the same species (95% identity). Abbreviations: LM5, Metagenome from Lake Meyghan (Iran) with 5% salinity (ERS1455389) [71]; SS13, Metagenome from Santa Pola saltern (Spain) with 13% salinity (SRX328504) [70]; SS19, Metagenome from Santa Pola saltern (Spain) with 19% salinity (SRX090228) [69]; LM30, Metagenome from Lake Meyghan (Iran) with 30% salinity (ERS1455391) [71]; SS33, Metagenome from Santa Pola saltern (Spain) with 33% salinity (SRX347883) [70]; SS37, Metagenome from Santa Pola saltern (Spain) with 37% salinity (SRX090229) [69].

DESCRIPTION OF *MARINOBACTER IRANENSIS* SP. NOV.

Marinobacter iranensis (i.ran.en'sis. N.L. masc. adj. *iranensis* referring to Iran, the country from where the type strain was isolated).

Cells are Gram-stain-negative, motile rods, with 0.2 µm width and 0.8–1.9 µm length. Facultative anaerobe. Endospores are not produced. Colonies are cream-coloured, circular, with entire margins, translucent, smooth, convex and 0.2–0.5 mm in diameter on MM10 medium after 48 h of incubation at 30°C. Slightly halophilic, able to grow over a wide range of NaCl concentrations from 0.2–0.5 to 15% (w/v) NaCl, with optimal growth at 3% (w/v) NaCl. Grows at a pH range of 6.0–11.0 and from 4 to 40°C, with optimal growth at pH 7.5 and at 30°C. Catalase- and oxidase-positive. Tweens 20 and 80 are hydrolyzed, while casein, gelatin and starch are not. Nitrate is reduced to nitrite. Indole and H_2S are not produced. Urease, β-galactosidase, and arginine dihydrolase tests are negative. Acid is not produced from arabinose, D-fructose, galactose, D-glucose, lactose, raffinose, rhamnose, ribose, salicin, sucrose, D-xylose, adonitol, inositol, D-mannitol and sorbitol. The following compounds are utilized as sole sources of carbon and energy: D-fructose, D-glucose, maltose, sucrose, glycerol, D-mannitol, sorbitol and dextrin but not D-galactose, lactose, mannose, xylose, citrate, lactate, succinate and L-glutamic acid. L-alanine, L-phenylalanine, and L-proline are used as sole carbon, nitrogen and energy sources, but not L-leucine and L-serine. The major polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). The only isoprenoid quinone is Q9 and $C_{16:0}$ and $C_{16:1}\omega7c$/$C_{16:1}\omega6c$ are the major fatty acids.

The type strain is 71-i^T (= IBRC M 11023^T = CECT 30160^T = LMG 29252^T), isolated from Inche-Broun hypersaline lake in Iran. The DNA G+C content of the type strain is 57.7 mol% (genome).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Marinobacter iranenesis* 71-i^T is MK101100 and that of the complete genome is JANCMW000000000.

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Author contributions

S. R. performed the isolation of the strain. S. R., S. M., M. S. A, M. M. N. and C.S-P. performed the phenotypic and genomic characterization. C.S-P., A.V., S. R. and M. A. A. prepared the draft manuscript and the tables and figures. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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