Idiomarina aquatica sp. nov., a moderately halophilic bacterium isolated from Spanish salterns

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SN-14^T is HF954116.

Four bacterial strains, SN-14^T, SN-4, M6-46 and M6-58B, were isolated from water of ponds of two salterns located in Huelva (Spain). They were Gram-staining-negative, aerobic and slightly curved rods. Phylogenetic analysis based on 16S rRNA gene sequences indicate that the four strains belong to the genus *Idiomarina*, being the most closely related species *Idiomarina fontislapidosi* F23^T (98.4-98.0 % sequence similarity), *Idiomarina seosinensis* CL-SP19^T (98.3-98.0 %), *Idiomarina piscisalsi* TPS4-2^T (97.9-97.4 %), *Idiomarina baltica* OS145^T (97.5-97.4 %) and *Idiomarina zobellii* KMM 231^T (97.6-97.0 %). The similarity with the type species of the genus, *Idiomarina abyssalis* KMM 227^T was 97.2-96.7 %. The novel strains exhibited optimal growth at 5-10 % (w/v) total salts, pH 7 and at 37 ºC. The major fatty acids of strain SN-14^T were iso-$C_{15:0}$ (30.4 %), iso-$C_{17:0}$ (10.7 %), $C_{18:1}$ *ω7c/*$C_{18:1}$ *ω6c* (7.3 %), $C_{16:0}$ (7.1 %) and iso-$C_{17:1}$ *ω9c/*$C_{16:0}$ *10 methyl* (7.0 %). The DNA G+C content range was 47.6 to 50.8 mol%. The level of DNA-DNA relatedness between strain SN-14^T and *I. fontislapidosi* F23^T was 13 %, while those between strain SN-14^T and the other four new isolates were between 77 and 99 %. These data demonstrated that the four isolates constitute a new species of the genus *Idiomarina*. Based on the phylogenetic, genotypic, phenotypic and chemotaxonomic data, the four strains represent a novel species of the genus *Idiomarina*, for which the name *Idiomarina aquatica* sp. nov. is proposed. The type strain is SN-14^T (= CCM 8471^T = CECT 8360^T = LMG 27613^T).

The genus *Idiomarina* was first proposed by Ivanova *et al.* (2000) and the genus *Pseudidiomarina* was later established by Jean *et al.* (2006). Both genera belong to the family *Idiomarinaceae*, within the order *Alteromonadales*, class *Gammaproteobacteria* in the phylum *Proteobacteria*. In 2009 Taborda *et al.* proposed that the species classified in the genus *Pseudidiomarina* should be transferred to the genus *Idiomarina*, due to the inability to distinguish both genera from each other using the phenotypic or chemotaxonomic characteristics examined. At the time of writing the genus *Idiomarina* comprises 24 species with validly published names (Parte, 2014). Most of these species were isolated from seawater samples and sea salt evaporation ponds. The species of the genus *Idiomarina* stain Gram-negative, are motile rods, colonies are non-pigmented or are slightly yellowish-coloured; NaCl is required for growth, showing a range between 0.5 and 25 % (w/v) NaCl and an optimum growth in media containing from 1 to 10 % NaCl. They are strictly aerobic, catalase and oxidase positive. Ubiquinone 8 is the major respiratory quinone. The DNA G+C content ranges from 45 to 54 mol% (Taborda *et al.*, 2009).

In this study, we describe the isolation and taxonomic characterization of four novel moderately halophilic bacteria from two salterns located in Huelva (Spain) and the data suggest that they constitute a novel species of the genus *Idiomarina*. The characterization of these strains was achieved by following a polyphasic approach, including conventional phenotypic features, chemotaxonomic data (polar lipid, fatty acid and quinone composition) and molecular analysis (16S rRNA gene sequence similarity and DNA-DNA hybridization).

Strains SN-14^{T} and SN-4 were isolated from a water pond of Isla Cristina saltern and strains M6-46 and M6-58B were isolated from water of a pond of Aragonesas saltern, both located in Huelva, in Southwest Spain. The isolation medium was modified from

HM medium, previously described by Ventosa *et al.* (1982) and contained (g l^{-1}): NaCl, 117; $MgCl_2.6H_2O$, 19.5; $MgSO_4.7H_2O$, 30.5; $CaCl_2$, 0.5; KCl, 3; $NaHCO_3$, 0.1; NaBr, 0.35; yeast extract, 0.05, solidified with 1.8 % agar (BD). The pH of this medium was adjusted to pH 7,5 with 1 M KOH. The strains were isolated by plating 0.1 ml of the water samples on this medium after incubation under aerobic conditions at 37 ºC. The strains were subsequently purified three times by plating on the same medium. The strains were routinely grown in SW 7.5 % medium at 37 ºC. The composition of this medium was the following: (g l^{-1}): NaCl, 58.5; $MgCl_2.6H_2O$, 9.75; $MgSO_4.7H_2O$, 15.25; $CaCl_2$, 0.25; KCl, 1.5; $NaHCO_3$, 0.05; NaBr, 0.175 and yeast extract, 5. The pH of this medium was adjusted to 7.5. The strains were maintained on SW 7.5 % medium and at -80 ºC supplemented with 30 % (v/v) glycerol. The type strains *I. fontislapidosi* $F23^T$, *I. abyssalis* CIP 107408^T, *I. baltica* DSM 15154^T, *I piscisalsi* NBRC 108617^T, *I. seosinensis* CIP 108665^T and *I. zobellii* DSM 15924^T were used as reference strains for comparison in our study.

Cell morphology and motility were examined by phase-contrast microscopy (Olympus CX41) from exponentially growing cultures. Growth range and optimal were determined at different NaCl concentrations (0.5, 3, 5, 7.5, 10, 15, 20 and 25 %, w/v) on SW medium at pH 7.5. To determine the optimal and range of temperature and pH for growth of strains, broth cultures were incubated at temperatures of 5-45 ºC at intervals of 5 ºC and from 35 to 40 ºC in increments of 1 ºC and at pH 4-10.5 at intervals of 0.5 pH units. Growth was determined by monitoring the optical density at 600 nm using a spectrophotometer. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 solution. Oxidase activity was examined with 1 % (v/v) tetramethyl-p-phenylenediamine (Kovacs, 1956). Growth under anaerobic conditions was determined by incubation in an anaerobic jar using Anaerogen (Oxoid) to generate anaerobic

atmosphere and an anaerobic indicator (Oxoid) in SW 7.5 % solid medium. Hydrolysis of casein, DNA, gelatin, starch, Tween 80 and aesculin, nitrate and nitrite reduction, Simmons' citrate, selenite reduction, Voges-Proskauer and methyl red tests, oxidation-fermentation from carbohydrates, production of indole and phosphatase, urease and phenylalanine deaminase activities were determined as described by Cowan & Steel (1977) with the addition of 7.5 % total salts to the medium (Ventosa *et al.*, 1982; Quesada *et al.*, 1984). H_2S production was tested in SW 7.5 % medium supplemented with 0.05 % (w/v) sodium thiosulfate; with a paper strip impregnated with lead-acetate placed in the neck of the tube (Clarke, 1953). Acid production from carbohydrates was determined using a phenol red base supplemented with 1 % carbohydrate and SW 7.5 % medium. For determination of the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources, the classical medium of Koser (1923) as modified by Ventosa *et al.* (1982) was used. This medium contained (l^{-1}): 75 g NaCl, 2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1 g KNO_3, 1 g $(NH_4)_2HPO_4$, 0.5 g KH_2PO_4 and 0.05 g yeast extract (BD). Substrates were added as filter-sterilized solutions to give a final concentration of 1 g l^{-1}, except for carbohydrates, which were used at 2 g l^{-1}. When the substrate was an amino acid, it was tested as carbon, nitrogen and energy source, and the basal medium was therefore prepared without KNO_3 and $(NH_4)_2HPO_4$.

Cells of the new isolates were motile, slightly curved rods, stained Gram-negative and were strictly aerobic. They were moderately halophilic, growing at 3-15 % (w/v) NaCl, with optimal growth at 7.5 % (w/v) NaCl; they were not able to grow in the absence of NaCl. The temperature range for growth was 5 to 40 ºC, with optimal growth at 37 ºC. The pH range for growth was 5-10 and the optimal growth was at pH 7.0. Other morphological, physiological, biochemical and nutritional characteristics of strains SN-14^T, SN-4, M6-46 and M6-58B are given in the species description and Table 1.

The genomic DNA of the four strains was isolated and purified using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the forward primer 16F27 and the reverse primer 16R1488 (Márquez et al., 2008). Direct sequence determination of the PCR-amplified DNA was carried out using an automatic DNA sequencer (ABI 3139XL, Applied Biosystems). The 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig *et al.*, 2004). The 16S rRNA gene sequence was aligned with the published sequences from closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were constructed using three different methods: maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms integrated in the ARB software for phylogenetic inference. Bootstrap analysis was based on 1000 resamplings (Felsenstein, 1985). The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1.

The almost-complete 16S rRNA gene sequence of strains SN-14^T (1460 bp), SN-4 (1463 bp), M6-46 (1506 bp) and M6-58B (1477 bp) was obtained and used for initial BLAST searches in GenBank and for phylogenetic analysis. The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon-e server (http://www.eztaxon-e.ezcloud.net) (Kim *et al.,* 2012). The 16S rRNA gene sequence analysis showed that strains SN-14^T, SN-4, M6-46 and M6-58B were members of the genus *Idiomarina*. Their closest relatives were *Idiomarina fontislapidosi* F23^T (98.4-98.0 % sequence similarities), *Idiomarina seosinensis* CL-SP19^T (98.3-98.0 % sequence similarities),

Idiomarina piscisalsi TPS4-2^T (97.9-97.4 % sequence similarities), *Idiomarina baltica* OS145^T (97.5-97.4 % sequence similarities), and *Idiomarina zobellii* KMM 231^T (97.6-97.0 % sequence similarities). On the other hand, the 16S rRNA gene sequence similarity with the type species of the genus, *Idiomarina abyssalis* KMM 227^T was 97.2-96.7 %. Phylogenetic analysis using the maximum-parsimony algorithm revealed that the four strains formed a separate lineage within the genus *Idiomarina*. The phylogenetic position of these strains was also confirmed by trees generated using the neighbour-joining and maximum-likelihood algorithms (Fig. 1). The four strains were included within the rRNA group 1 of the genus *Idiomarina* (Taborda *et al.*, 2009).

The G+C content of the genomic DNA was determined from the midpoint value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) by using the equation of Owen & Hill (1979). The DNA G+C content of strains SN-14^T, SN-4, M6-46 and M6-58B was estimated to be 49.4, 47.6, 48.6, and 50.8 mol%, respectively. These values are within the DNA G+C range reported for the genus *Idiomarina* (45 to 54 mol%) (Taborda *et al.,* 2009). DNA–DNA hybridization studies were performed by the competition procedure of the membrane method (Johnson, 1994), described in detail by Arahal *et al.* (2001a, 2001b). The hybridization temperature used was 49.6 °C, which is within the limit of validity for the filter method (De Ley & Tijtgat, 1970), and the percentage of hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicate. DNA–DNA hybridization between strain SN-14^T and SN-4, M6-46 and M6-58B was 99, 83 and 77 %, respectively, indicating that the four strains are members of the same species. However, the DNA–DNA hybridization between strain SN-14^T and *I. fontislapidosi* F23^T, *I. seosinensis* CIP 108665^T, *I. piscisalsi* NBRC 108617^T, *I. baltica* DSM 15154^T, *I. zobellii* DSM 15924^T and *I. abyssalis* CIP 107408^T was 13, 7, 12, 6, 15 and 17 %, respectively. These levels

of DNA–DNA hybridization with respect to the type strains of the phylogenetically most closely related species are significantly lower than the 70 % threshold value recommended for the delineation of new species (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

For the analysis of the fatty acids, the cells of strain SN-14^T and the type strain of the most closely related species were grown on marine agar (BD), at 28 °C obtained in the late-exponential growth phase. The whole-cell composition of fatty acids was determined by GC using the MIDI Microbial Identification System (Sasser, 1990). The fatty acids composition was obtained with a gas chromatograph Agilent 6850 using the database TSBA6 (MIDI, 2008). These analyses were carried out by the CECT culture collection (Spain). Analysis of the respiratory quinones and polar lipids of strain SN-14^T and *I. fontislapidosi* F23^T were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Cell biomass for these analyses was obtained by growth of the strains on marine agar (BD) at 37 ºC.

The major fatty acids of strain SN-14^T were iso-$C_{15:0}$ (30.4 %), iso-$C_{17:0}$ (10.7 %), $C_{18:1}$ *ω7c/*$C_{18:1}$ *ω6c* (7.3 %), $C_{16:0}$ (7.1 %) and iso-$C_{17:1}$ *ω9c/*$C_{16:0}$ *10-methyl* (7.0 %). The fatty acid composition is similar to those of the related species of *Idiomarina*, except for the presence of summed feature 9: iso-$C_{17:1}$ *ω9c/* $C_{16:0}$ *10-methyl* (Table 2). Strain SN-14^T exhibited a polar lipid profile consisting of phosphatidylglycerol, phosphatidylethanolamine, two phospholipids and a phosphoaminoglycolipid (atypical sugar). This profile is similar to those reported for species of the genus *Idiomarina* (Taborda *et al.*, 2009). Ubiquinone 8 was the only respiratory quinone detected in strain SN-14^T in accordance with the lipoquinone determined for the family *Idiomarinaceae* and the species of the genus *Idiomarina* (Taborda *et al.*, 2009).

The reported phylogenetic, phenotypic, genotypic and chemotaxonomic data clearly indicate that the four strains constitute a single taxon and they represent a novel species of the genus *Idiomarina*, for which we propose the name *Idiomarina aquatica* sp. nov.

Description of *Idiomarina aquatica* sp. nov.

Idiomarina aquatica (a.qua'ti.ca L. fem. adj. *aquatica*, living or found in the water, aquatic).

Cells are Gram-staining-negative, motile, slightly curved rods, with 0.4 x 1.25-2.1 µm, occurring as single cells. Non-endospore-forming. Colonies are circular, entire, smooth, convex, cream and 1.5-2.5 mm in diameter on SW 7.5 % agar medium after 48 h of incubation at 37 ºC. Strictly aerobic. Moderately halophilic, growing at 3-15 % (w/v) NaCl, with optimal growth at 7.5 % (w/v) NaCl. No growth occurs in the absence of NaCl. The temperature range for growth is 5-40 ºC, with optimal growth at 37 ºC. The pH range for growth is 5-10 and the optimal growth is at pH 7.0. Catalase and oxidase positive. Gelatin, Tween 80, DNA and aesculin are hydrolyzed but casein and starch are not. Nitrate and nitrite are reduced (except strain M6-46). Acid is not produced from D-arabinose, D-fructose, D-glucose, D-galactose, lactose, D-mannose, melezitose, melibiose, rafinose, ribose, sucrose, D-xylose, D- mannitol, sorbitol, xylitol, amygdalin, arbutin, citrulline and inulin. Indole or H_2S are not produced. Methyl red, Voges-Proskauer, Simmons' citrate and phenylalanine deaminase tests are negative. Selenite is reduced. Phosphatase and urease are positive. Propionate is used as sole source of carbon and energy. The following compounds are not utilized as sole source of carbon and energy: D-arabinose, D-cellobiose, fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-melezitose, D-melibiose, L-raffinose, ribose, salicin, sucrose,

D-trehalose, ethanol, glycerol, *myo*-inositol, D-mannitol, methanol, D-sorbitol, xylitol, benzoate, citrate, formate, hippurate, malate, succinate and tartrate. The following compounds are utilized as sole source of carbon, nitrogen and energy: glutamine, and valine. The following compounds are not utilized as sole source of carbon, nitrogen and energy: L.cysteine and L.methionine. The major cellular fatty acids were iso-$C_{15:0}$, iso-$C_{17:0}$, $C_{18:1}$ *ω7c/*$C_{18:1}$ *ω6c*, $C_{16:0}$ and iso-$C_{17:1}$ *ω9c/*$C_{16:0}$ *10 methyl*. The respiratory isoprenoid quinone is ubiquinone 8 (Q-8). The polar lipid profile consists of phosphatidylglycerol, phosphatidylethanolamine, two phospholipids and a phosphoaminoglycolipid. The DNA G+C content is 47.6-50.8 mol% (Tm).

The type strain is SN-14^T (= CCM 8471^T = CECT 8360^T = LMG 27613^T), isolated from the water of a pond of Isla Cristina saltern, Huelva (Spain). The genomic DNA G+C content of the type strain is 49.4 mol% (Tm).

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323 **Table 1.** Differential characteristics of *Idiomarina aquatica* sp. nov. (strains SN-14^T,
324 SN-4, M6-46 and M6-58B) and related species of the genus *Idiomarina*.

325 Strains: 1, SN-14^T; 2, SN-4; 3, M6-46; 4, M6-58B; 5, *Idiomarina fontislapidosi* F23^T
326 and 6, *Idiomarina abyssalis* KMM 277^T. Data were obtained in this study unless
327 mentioned otherwise. +, Positive, -, negative.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
Cell morphology	Curved rods	Curved rods	Curved rods	Curved rods	Slightly curved rods[a]	Rod-shaped[b]
Colony pigmentation	Cream	Cream	Cream	Cream	Cream[a]	Light yellowish[b]
Cell size (µm)	1.25-2.1 x 0.4	0.8-1.7 x 0.4	0.8-1.7 x 0.4	1.25-2.1 x 0.4	3.0-4.0 x 0.75[a]	1-1.8 x 0.7-0.9[b]
NaCl range (%, w/v)	3.0-15.0	3.0-15.0	3.0-15.0	3.0-15.0	0.5-25.0[a]	0.6-15.0[b]
NaCl optimum (%, w/v)	7.5	7.5	7.5	7.5	3.0-5.0[a]	3.0[b]
Temperature range (ºC)	5-40	5-40	5-40	5-40	4-45[a]	4-30[b]
Temperature optimum (ºC)	37	37	37	37	32[a]	20-22[b]
pH range	5-10	5-10	6-10	6-10	5-10[a]	5.5-9.5[b]
pH optimum	7.0	7.0	7.0	7.0	7-8a	7.5-8[b]
Nitrate reduction	+	+	-	+	-	+
Nitrite reduction	+	+	-	+	-	+

Hydrolysis of casein	-	-	-	-	+	-
Simmons' citrate	-	-	-	-	+	+
H_2S production	-	-	-	-	+	-
Utilization of:						
Maltose	-	-	+	-	-	-
Raffinose	-	-	+	-	-	-
Citrate	-	-	+	-	-	-
Fumarate	+	+	+	-	+	+
Hippurate	-	-	-	-	+	-
L-Glutamine	+	+	+	-	+	+
L-Threonine	-	-	+	-	-	-
L-Valine	+	-	-	-	-	-
DNA G+C content (mol%)	49.4	47.6	48.6	50.8	46.0[a]	50.4[b]

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329 [a] Data from Martínez-Cánovas *et al.* (2004); [b] Data from Ivanova *et al.* (2000).

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Table 2. Cellular fatty acid composition of strain SN-14^T and closely related species of the genus *Idiomarina.*

Strains: 1, SN-14^T, 2, *Idiomarina fontislapidosi* F23^T; 3, *Idiomarina abyssalis* KMM 277^T. All data were obtained using the same growth conditions (marine agar, 28 ºC, late-exponential growth phase). Data from this study, except for *I. abyssalis*. Values are percentages of the total cellular fatty acids. Only fatty acids amounting to at least 1.0 % of the total cellular fatty acids of at least one of the strains are shown. -, Not detected or < 1.0 %.

Fatty acid	**1**	**2**	**3**[a]
$C_{10:0}$ 3-OH	1.5	2.1	-
iso-$C_{11:0}$	3.5	3.6	-
iso-$C_{11:0}$ 3-OH	4.5	4.2	-
iso-$C_{12:0}$ 3-OH	-	1.1	-
iso-$C_{13:0}$	-	1.3	1.0
iso-$C_{13:0}$ 3-OH	5.2	5.1	-
$C_{14:0}$	1.4	-	-
$C_{15:1}$ *ω8c*	-	-	1.3
iso-$C_{15:1}$F	2.1	1.6	2.3
iso-$C_{15:0}$	30.4	32.8	33.7
$C_{16:0}$	7.1	8.7	6.3

Fatty acid	**1**	**2**	**3**[a]
Summed feature 3*	5.4	7.6	7.8
Summed feature 9*	7.0	-	-
iso-$C_{17:0}$	10.7	9.6	11.9
$C_{17:1}$ *ω6c*	-	-	1.5
$C_{17:1}$ *ω8c*	1.3	1.5	-
$C_{17:0}$ cyclo	2.8	1.4	-
$C_{17:0}$	2.8	2.4	-
Summed feature 8*	7.3	4.2	6.7
$C_{18:0}$	3.4	2.5	1.8
$C_{18:1}$ *ω9c*	-	-	1.4

*Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 3 comprised $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*; summed feature 8 comprised $C_{18:1}$ *ω7c*/ $C_{18:1}$ *ω6c*; summed feature 9 comprised iso-$C_{17:1}$ *ω9c* and/or $C_{16:0}$ *10-methyl*.

[a] Data from Ivanova *et al.* (2000).

Legend to figure

Fig. 1. Maximum-parsimony phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between *Idiomarina aquatica* (strains SN-14^T, SN-4, M6-46 and M6-58B), related species of the genus *Idiomarina* and other related genera. Filled circles indicate nodes that were also recovered in neighbor-joining and maximum-likelihood trees based on the same sequences. Numbers at nodes are levels of bootstrap support (percentages) based on analyses of 1000 resampled datasets; only values >70 % are shown. The sequence of *Agarivorans albus* MKT 106^T was used as outgroup. Bar, 0.01 nucleotide changes per position. The GenBank/EMBL/DDBJ accession number of each sequence is shown in parenthesis.

Figure

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