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TAXONOMIC DESCRIPTION
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Sagittula salina sp. nov., isolated from marine waste

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Abstract

A novel Gram-stain-negative, non-motile, halophilic bacterium designated strain M10.9X^T was isolated from the inner sediment of an aluminium can collected from the Mediterranean Sea (València, Spain). Cells of strain M10.9X^T were rod-shaped and occasionally formed aggregates. The strain was oxidase-negative and catalase-positive, and showed a slightly psychrophilic, neutrophilic and slightly halophilic metabolism. The phylogenetic analyses revealed that strain M10.9X^T was closely related to *Sagittula stellata* E-37^T and *Sagittula marina* F028-2^T. The genomic G+C content of strain M10.9X^T was 65.2 mol%. The average nucleotide identity and digital DNA–DNA hybridization values were 76.6 and 20.9 %, respectively, confirming its adscription to a new species within the genus *Sagittula*. The major cellular fatty acids were $C_{18:1}\,\omega7c/C_{18:1}\,\omega6c$ and $C_{16:0}$. The polar lipids consisted of phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid, an unidentified phospholipid and an unidentified lipid. According to the resuts of a polyphasic study, strain M10.9X^T represents a novel species of the genus *Sagittula* for which the name *Sagittula salina* sp. nov. (type strain M10.9X^T=DSM 112301^T=CECT 30307^T) is proposed.

The genus *Sagittula* was first described by González *et al*. in 1997 and reclassified within the *Rhodobacteraceae* family by Lee *et al*. in 2012 [1, 2]. At the time of writing, the genus *Sagittula* is composed of only two species: *Sagittula stellata* and *Sagittula marina*, both isolated from marine environments and promising strains with bioremediation capacities [1–3]. In the present research, we describe the polyphasic characterization of strain M10.9X^T, which was isolated from the inner sediment of an aluminium can during the study on the microbial diversity of marine waste. Anthropic residue distributed worldwide represent a major environmental problem and constitute new ecological niches which may harbour potential new microbial species.

Strain M10.9X^T was isolated from the inner sediment of a can collected from Malva-rosa beach, on the western Mediterranean Coast (València, Spain; 39° 27′ 48.3″ N 0° 19′ 07.6″ E), during a study of the microbial communities associated with marine waste residues [4]. The sediment was resuspended in PBS (1×, pH 7.4) and 50 µl was then spread on marine agar (MA; Laboratorios Conda S.A. Ref: 1059). The plates were incubated at 18 °C for a week. Strain isolation was carried out by restreaking on fresh media until pure cultures were obtained. Cell suspensions in marine broth (MB; Laboratorios Conda S.A. Ref: 1217) supplemented with 15% glycerol (v/v) were cryopreserved at −80 °C. A polyphasic approach was followed in order to determine the taxonomic status of strain M10.9X^T. After isolation, analysis of the 16S rRNA gene sequence in EzBioCloud revealed that *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were closely related to strain M10.9X^T. Therefore, these strains were selected as comparative strains. Unless otherwise specified, the reference strains *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T, from the DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany), and strain M10.9X^T were grown simultaneously on MA medium at 30 °C.

The phenotypic characterization of strain M10.9X^T was carried out after a week of growth at 30 °C. The Gram-staining test was performed with KOH 3% (w/v), recording viscosity as a positive result for Gram-negative bacteria [5, 6]. In order to test oxidase activity, a commercial oxidase test stick for microbiology (PanReac AppliChem) was used following the manufacturer's instructions. Hydrogen peroxide 30% (v/v) was used to test catalase activity, by recording bubble formation as a positive result

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Keywords: *Sagittula*; marine waste; new species; *Rhodobacteraceae*; *Alphaproteobacteria*.
Abbreviations: ANIb, average nucleotide identity; dDDH, digital DNA–DNA hybridization; MA, marine agar; MB, marine broth; ML, maximum-likelihood; NJ, neighbour-joining.
The 16S rRNA gene sequence of strain M10.9X^T has been deposited in DDBJ/ENA/GenBank under the accession number MW785249. The genomic sequence of strain M10.9X^T has been deposited under the DDBJ/ENA/GenBank accession number JAGISH000000000.
Two supplementary tables and one supplementary figure are available with the online version of this article.
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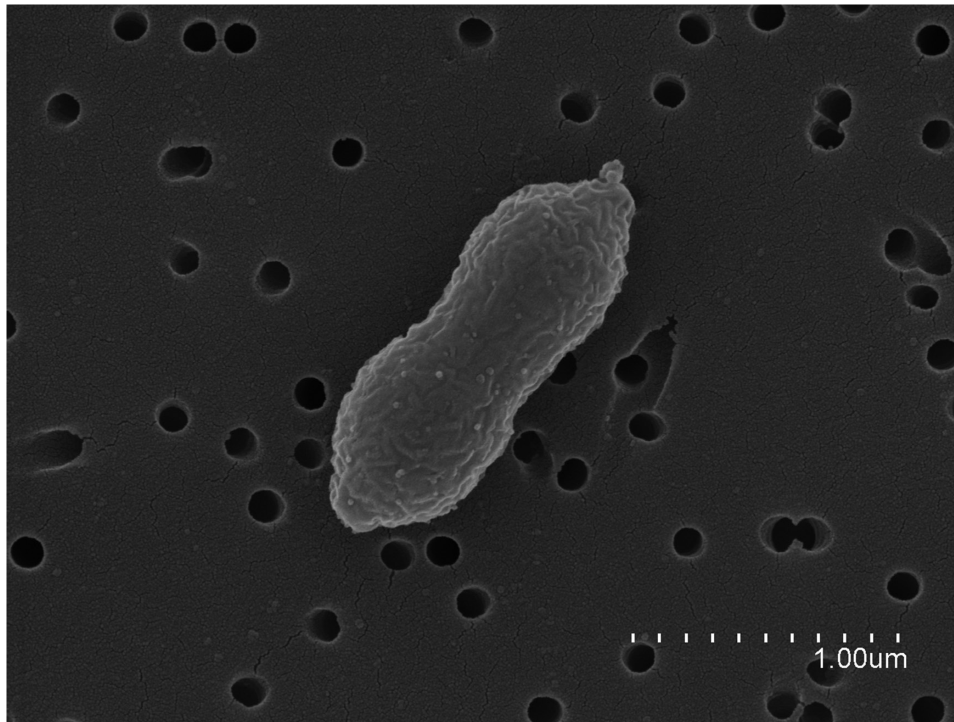

Fig. 1. Field emission scanning electron microscope image showing the general morphology of a cell of strain M10.9X^T after growth in marine broth at 30 °C, 180 r.p.m. overnight. Size bar corresponds to 1 µm.

[5]. The motility of the strain was studied using the hanging-drop method [7]. Growth at different temperatures (4, 10, 16, 20, 24, 30, 37, 40 and 42 °C) and salt tolerance was checked on Zobell agar medium that contained the following ingredients (g l^{-1} in modified artificial seawater): Bacto peptone, 5; yeast extract,1; ferric citrate, 0.1; and Bacto agar, 15. The modified artificial seawater contained (g l^{-1}): NaCl, 0–100; $MgSO_4 \cdot 7H_2O$, 5.94; $MgCl_2 \cdot 6H_2O$, 0.64; KCl, 4.53; and $CaCl_2$, 1.3 [2]. Growth at different pH values (pH 5.0–10.0 at intervals of 0.5 pH units) was tested by culturing the strain in MB buffered with MES (pH 5.0–6.5), HEPES (7.0–8.5) and CHES (9.0–10.0) at a final concentration of 10 mM. The ability to grow under anaerobic conditions was determined with the BD GasPak EZ pouch system (Becton, Dickinson and Company). Growth under microaerophilic conditions was also tested using the BD GasPak EZ pouch system. Carbon source assimilation and enzymatic activities were assessed using API 20 NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions, replacing saline solution 0.9% with 3.5 % (w/v) sea salts solution (Sigma-Aldrich; Ref: S9883-500G) for cell suspension preparation. GEN III MicroPlates (Biolog) were also used to test carbon source assimilation.

For fatty acids analysis, strains M10.9X^T, *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were grown on MA at 30 °C for 48 h. The analysis was carried out following the protocol recommended by the MIDI Microbial Identification System (version 6.1) [8, 9]. Fatty acids were analysed on an Agilent 6850 gas chromatography system and using the TSBA6 method following the manufacturer's instructions.

DNeasy PowerSoil kit (Qiagen) was used for genomic DNA extraction according to the manufacturer's instructions but incubating at 65 °C after adding C1 reagent. Whole 16S rRNA gene PCR was carried out with universal primers 8F (5′-AGAGTTTGATC-CTGGCTCAG-3′) [10] and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [11] following the protocol described by Molina-Menor *et al.* [12]. MEGA X software version 10.1.7 was used to reconstruct a phylogenetic tree based on the 16S rRNA gene sequences. The trees were reconstructed by the maximum-likelihood (ML) [13] and neighbour-joining (NJ) [14] methods. For the ML and the NJ trees, the T92 +G+I evolutionary model and the Kimura two-parameter model were used, respectively. Bootstrap analysis was used in order to assess the reliability of the branch patterns based on 500 and 1000 replicates, respectively, for the ML and NJ trees [15].

The draft genome of strain M10.9X^T was sequenced with an Illumina NovaSeq 6000 system (2×150 bp paired-end sequencing). The genomic DNA was randomly fragmented by sonication. DNA fragments were then end polished, A-tailed, and ligated with the full-length adapters of Illumina sequencing. PCR amplification was carried out with P5 and indexed P7 oligos. An AMPure XP system was used to purify PCR products as the final construction of the libraries. The size distribution of the libraries was

Table 1. Differential phenotypic characteristics between strain M10.9X^T and other members of the genus *Sagittula*

Strains: 1, M10.9X^T; 2, *S. marina* DSM 102235^T; 3, *S. stellata* DSM 11524^T. Data for reference strains were analysed in parallel in the present study. +, Positive; −, negative. All strains are positive for alkaline phosphatase, esterase (C4), leucine arylamidase, esterase lipase (C8), valine arylamidase and naphthol-AS-BI-phosphydrolase, D-glucose, D-mannose, D-mannitol, maltose, potassium gluconate and malic acid. All strains are negative for lipase (C14), cystine arylamidase, trypsin, α-chymotripsin, α-galactosidase, *β*-galactosidase, α-manosidase, α-fucosidase, nitrate reduction and indole production.

Characteristic	1	2	3
Source	Inner sediment of aluminium can	Sea water	Coastal environment
G+C content (mol%)	65.2	61.6	65.0
Growth at/in:			
Temperature range (°C)	16–37	16–37	16–40
pH range	5.5–9.0	5.5–9.0	6.5–9.0
Salt tolerance (%, w/v)	1.0–5.0	1.0–8.0	1.0–8.0
Carbon source utilization (API 20NE):			
L-Arabinose	+	−	−
N-Acetyl-glucosamine	+	+	−
Adipic acid	+	+	−
Phenylacetic acid	−	+	+
Enzymatic activity (API 20NE):			
Urease	−	−	+
Gelatin	−	+	+
Enzymatic activity (API ZYM):			
Acid phosphatase	−	+	+
α-Glucosidase	−	−	+
β-Glucosidase	−	−	+
N-Acetyl-*β*-glucosaminidase	+	−	−

checked by an Agilent 2100 Bioanalyzer (Agilent Technologies), and quantified by real-time PCR. The quality of the sequence reads was assessed with the FastQC tool (version 0.11.5) [16]. The '--isolate' mode in SPAdes (version 3.14.1) [17] was used for genome assembly of paired reads. Assembly statistics were calculated with QUAST (version 5.0.2) [18]. Completeness and contamination levels were evaluated with CheckM (version 1.1.3) [19]. Genome annotation was carried out using the RAST tool kit [20] integrated in PATRIC version 3.6.8. In order to identify the closest type strains of strain M10.9X^T and to calculate the dDDH genomic index, the draft genome was uploaded to TYGS [21]. Average nucleotide identity (ANIb) values were calculated with JSpecies [22] according to BLAST between genome pairs. The phylogenomic tree reconstruction based on a multiple alignment of a set of 92 housekeeping genes was conducted with UBCG (version 3.0) [23]. FastTree was used to infer the phylogenetic relationships. The reliability of the branch patterns was assessed using bootstrap analysis based on 100 replicates as well as with gene support indices.

The morphological characteristics of strain M10.9X^T were analysed through scanning electron microscopy. A fresh overnight culture of strain M10.9X^T in MB was centrifuged and cells were fixed by resuspending them in 1 ml Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer; pH 7.4) overnight. Cells were harvested by centrifugation at 4 °C for 10 min at 7500 r.p.m. and washed with sterile deionized water and a series of ethanol solutions (30, 50, 70, 90 and 100%; Pan Reac, AppliChem). Samples were filtered through a polycarbonate membrane filter with a 0.1 µm pore size (Filter-Lab PC; MPC0010013N) and incubated for 48 h in a desiccator prior to embedding them in resin using carbon tape. Sputtering was done using gold/palladium particles and samples were examined under the field emission scanning electron microscope (Hitachi S4800; at SCSIE, University of Valencia).

Cells of strain M10.9X^T were aerobic, oxidase-negative, catalase-positive, Gram-stain-negative, non-motile, rod-shaped (approximately 0.5 µm in diameter and 1.0–2.0 µm in length), exhibited polarity (Fig. 1), and occasionally aggregated. Colonies on MA

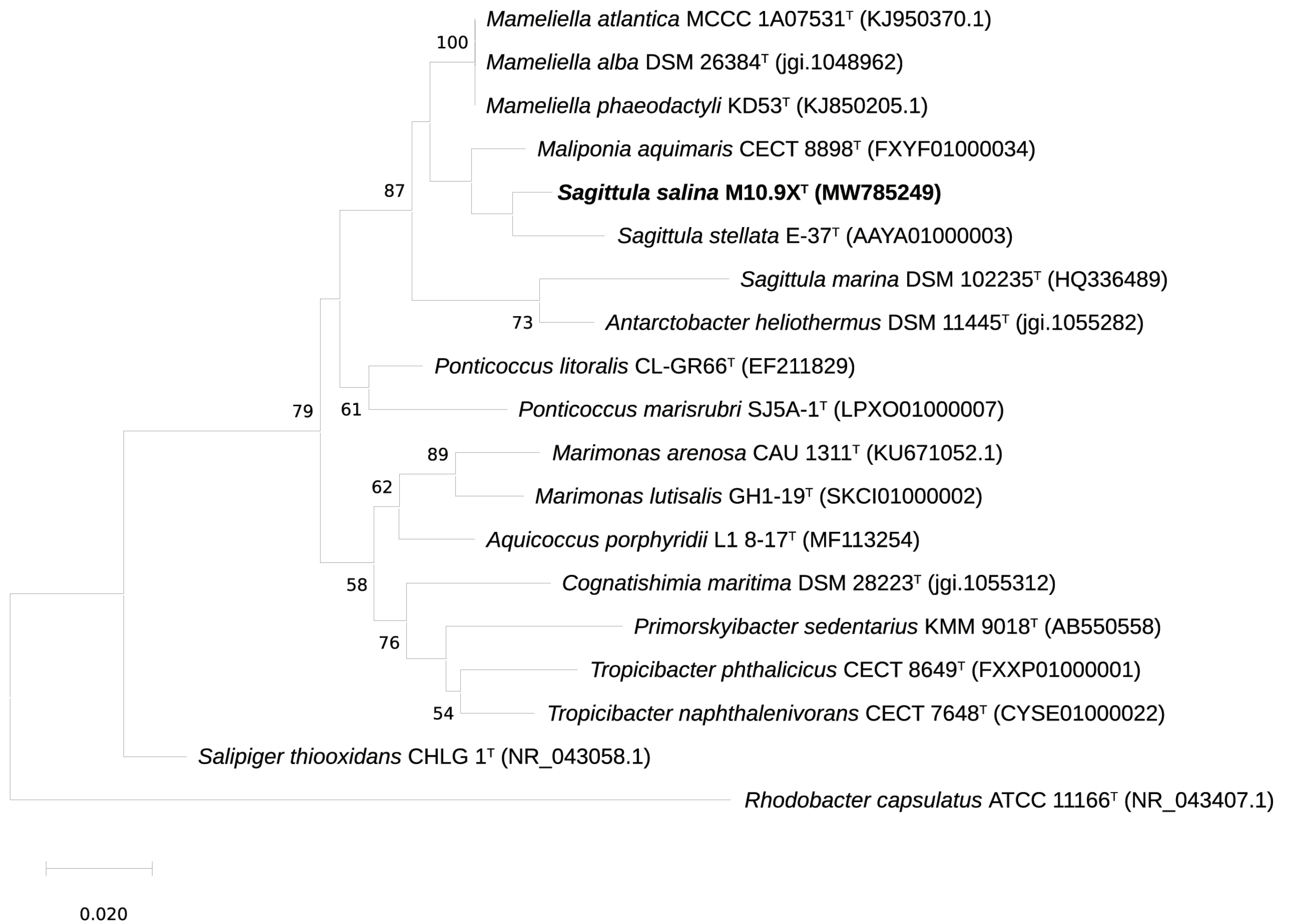

Fig. 2. Maximum-likelihood phylogenetic tree showing the relationship between strain M10.9X^T and other members of the family *Rhodobacteraceae* based on 16S rRNA gene sequences. The evolutionary model of nucleotide substitution used was the T92 +G+I model. Numbers at the nodes indicate bootstrap percentages based on 500 replicates (values below 50% are not indicated). Scale bar, 0.020 substitutions per nucleotide position. *Rhodobacter capsulatus* ATCC 11166^T (NR_043407.1) was used as an outgroup.

medium were light cream, circular, convex, had entire margins, smooth and displayed a diameter of 1–2 mm after 5 days of incubation at 30 °C.

Strain M10.9X^T was able to grow between 16 and 37 °C (optimum at 30 °C) and showed NaCl tolerance up to 5.0% (w/v). Strain M10.9X^T was not able to grow on the media without salt. This strain can, therefore, be considered as halophilic based on the salt requirement shown for optimum growth [24, 25], which is in accordance with the data reported for other species within this genus. Strain M10.9X^T was able to grow at pH 5.5–9.0 (optimum at pH 6.0–7.5).

Strain M10.9X^T was negative for acid phosphatase and gelatin hydrolysis, in contrast to *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T, which were positive for these activities. Moreover, strain M10.9X^T was positive for *N*-acetyl-β-glucosaminidase, whereas *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T showed a negative response to it. In contrast to strains M10.9X^T and *S. marina* DSM 102235^T, *S. stellata* DSM 11524^T showed a positive response to urease, α- and β-glucosidase. In API 20 NE strips, all three strains were able to assimilate D-glucose, D-mannose, D-mannitol, maltose, potassium gluconate and malic acid. All three strains were negative for the assimilation of capric acid and trisodium citrate. The utilization of L-arabinose was only positive for strain M10.9X^T, while *N*-acetyl-glucosamine and adipic acid were utilized by strain M10.9X^T and *S. marina* DSM 102235^T. Moreover, strain M10.9X^T could not assimilate phenylacetic acid (Table 1). Biolog GENIII MicroPlates revealed that strain M10.9X^T was able to oxidize 48 carbon sources after 72 h, in contrast to strains *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T, which were able to oxidize 67 and 69 carbon sources, respectively (Table S1, available in the online version of this article). This suggests that *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T display a more polytrophic metabolism than strain M10.9X^T.

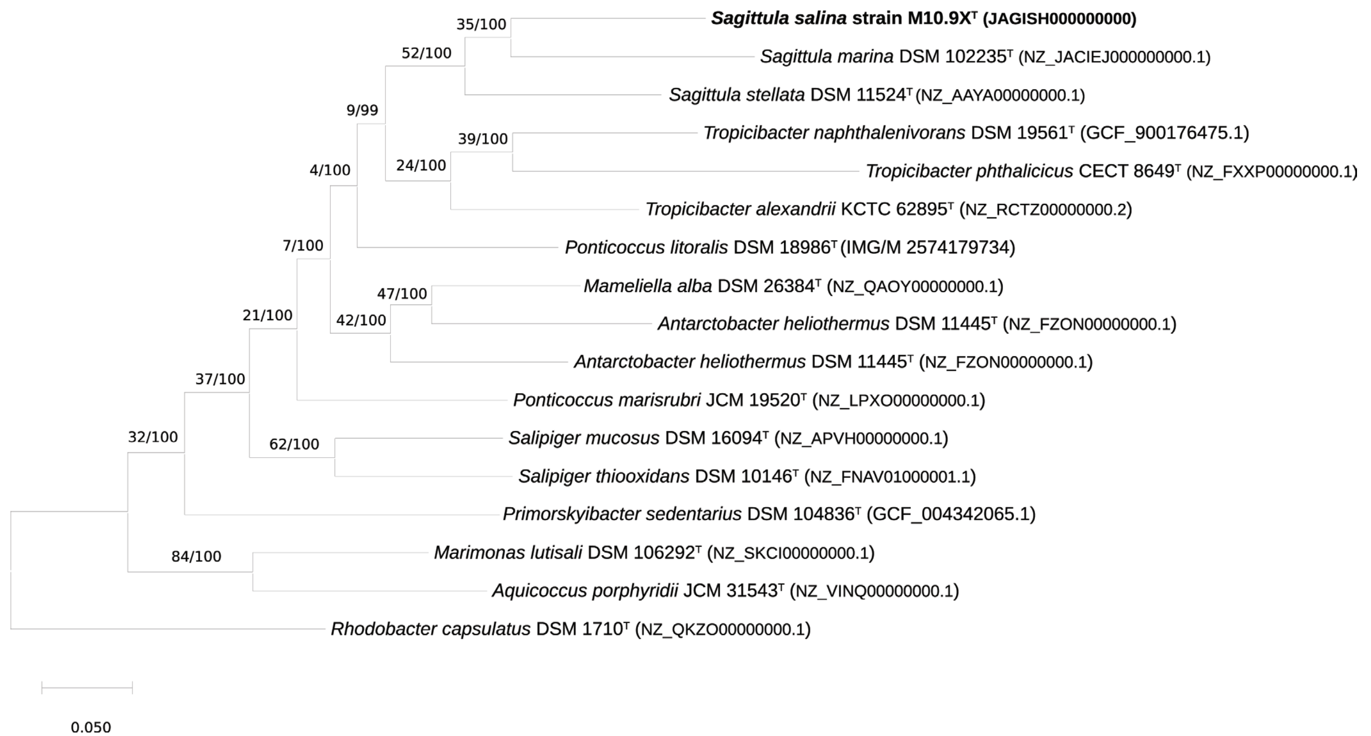

Fig. 3. Phylogenomic tree showing the position of strain M10.9X^T among other members of the family *Rhodobacteraceae* based on a multiple alignment of a set of 92 housekeeping genes. Bootstrap analysis was carried out based on 100 replicates. Gene support indices (max. value: 92) and percentage bootstrap values (max. value: 100) are given at nodes. Scale bar shows 0.050 substitutions per site. *Rhodobacter capsulatus* DSM 1710^T was considered as an outgroup.

The almost-complete 16S rRNA gene sequence (1410 bp) of strain M10.9X^T was obtained and deposited in the DDBJ/ENA/GenBank under the accession number MW785249. According to EzBioCloud database (www.ezbiocloud.net), the closest type strains to strain M10.9X^T were *Sagittula stellata* E-37^T, *Maliponia aquimaris* CECT 8898^T, *Mameliella alba* DSM 26384^T, *Ponticoccus litoralis* CL-GR66^T and *Sagittula marina* F028-2^T with 97.81, 97.74, 97.37, 96.34 and 96.22 % of similarity, respectively.

The phylogenetic trees based on the ML and NJ methods showed that strain M10.9X^T was closely related to *S. stellata* E-37^T (Fig. 2 and S1). Although the relationship between the reference strains and *S. marina* DSM 102235^T was not supported by high bootstrap values due to the low resolution of the 16S rRNA gene sequence, the phylogenomic inference confirmed the inclusion of strain M10.9X^T within the clade of *Sagittul*a (Fig. 3).

The draft genome of strain M10.9X^T consisted of 147 contigs (4800470 bp total length). The genomic G+C content was 65.2 mol%. There were 4717 predicted coding sequences, of which 3189 were predicted as proteins with functional assignments. The completeness value and contamination level of the draft genome were 99.7 and 1.3 %, respectively. This contamination value is low enough to consider the draft genome for further analysis.

The strain M10.9X^T 16S rRNA gene sequence was checked by using the BLAST tool (BLASTN) integrated in PATRIC version 3.6.12 and compared with the 16S rRNA gene sequence retrieved from the complete genome sequence; the result was 100 % similarity.

The phylogenetic trees based on the ML and NJ methods showed that strain M10.9X^T was closely related to *S. stellata* E-37^T (Fig. 2 and S1). The 16S rRNA gene based phylogenetic inference revealed that the genus *Sagittula* did not form a monophyletic group, independently from the algorithm used, as *S. marina* showed a paraphyletic position in both ML and NJ trees. Although the relationship between the reference strains and *S. marina* DSM 102235^T was not supported by high bootstrap values due to the low resolution of the 16S rRNA gene sequence, the phylogenomic inference confirmed the inclusion of strain M10.9X^T within the clade of *Sagittul*a (Fig. 3).

A phylogenomic tree based on a set of 92 housekeeping gene sequences was reconstructed in order to obtain a more accurate phylogenetic inference of strain M10.9X^T. Strain M10.9X^T clustered with *S. marina* DSM 102235^T. All three *Sagittula* strains formed a monophyletic group, which was supported by high bootstrap and Gamma distribution with Invariant sites (G+I) values.

Table 2. Fatty acid analysis (%) of strain M10.9X^T and the strains of the genus *Sagittula*

Strains: 1, M10.9X^T; 2, *S. marina* DSM 102235^T; 3, *S. stellata* DSM 11524^T. TR, <1.0%; –, not detected. Summed feature 3 was TR for all the strains.

Fatty acid	1	2	3
Saturated			
$C_{16:0}$	11.11	7.26	12.65
$C_{18:0}$	1.34	1.67	2.16
$C_{19:0}$ cyclo $\omega 8c$	–	–	5.57
Unsaturated			
$C_{18:1}$ $\omega 7c$ 11-methyl	–	4.67	3.42
Hydroxylated			
$C_{12:0}$ 2-OH	–	TR	TR
$C_{12:1}$ 3-OH	2.45	3.32	3.27
Summed features*			
8	84.37	81.67	71.96

*Groups of fatty acids that cannot be resolved reliably from another fatty acid by the chromatographic system were labelled as 'summed features'. Summed feature 3 corresponds to $C_{16:1}$ $\omega 7c$/$C_{16:1}$ $\omega 6c$. Summed feature 8 corresponds to $C_{18:1}$ $\omega 7c$/$C_{18:1}$ $\omega 6c$.

The dDDH and ANIb values between strain M10.9X^T and other type strains of the genus *Sagittula* were compared (Table 2). The dDDH values of strain M10.9X^T against *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were 20.9 and 20.2%. The ANIb values of strain M10.9X^T against *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were 76.6, and 75.6%, respectively. These values are in accordance with the threshold established to consider strain M10.9X^T as a new species, which are 70% [26] and 95% [27], respectively, for dDDH and ANIb.

Analysis of polar lipids for strain M10.9X^T was carried out by the Identification Service, DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Germany). Strain M10.9X^T was able to synthesize phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid, an unidentified phospholipid and an unidentified lipid. The polar lipid profile of strain M10.9X^T is in accordance with the data previously reported by Lee *et al.* [2] for *S. marina* F028-2^T, which was also able to synthesize phosphatidylglycerol, phosphatidylethanolamine, two unidentified aminolipids, an unidentified phospholipid and six unidentified lipids.

The major fatty acid of strain M10.9X^T was summed feature 8 ($C_{18:1}$ $\omega 7c$/$C_{18:1}$ $\omega 6c$; 84.4%). Moreover, there was also a large amount of $C_{16:0}$ (11.1%). This is in accordance with the profiles displayed by other species within the genus *Sagittula*, which were also dominated by summed feature 8 and $C_{16:0}$ (Table 2). This result supports the adscription of strain M10.9X^T to the genus *Sagittula*.

According to the results of the phenotypic, genomic, and phylogenetic analyses carried out in the present study, there is evidence to support the inclusion of strain M10.9X^T as a new member of the genus *Sagittula*, for which the name *Sagittula salina* sp. nov. is proposed.

DESCRIPTION OF *SAGITTULA SALINA* SP. NOV.

Sagittula salina (sa.li'na. N.L. fem. adj. *salina*, salty, referring to the marine environment from which the strain was isolated).

Colonies are light cream, circular, convex, entire, smooth and 1–2 mm in diameter after 5 days of incubation at 30 °C. Cells are Gram-stain-negative, non-motile, rod-shaped (approximately 0.5 µm in diameter and 1.0–2.0 µm in length), exhibit polarity and occasionally aggregate. This species grows under aerobic and microaerophilic conditions, but no growth is observed in anaerobiosis. Growth occurs at 16–37 °C (optimum, 30 °C), pH 5.5–9.0 (optimum, pH 6.0–7.5), and tolerates 1.0–5.0% (w/v) NaCl. Alkaline phosphatase, esterase (C4), leucine arylamidase, esterase lipase (C8), valine arylamidase, naphthol-AS-BI-phosphydrolase, *N*-acetyl-*β*-glucosaminidase and aesculin hydrolysis are detected. Lipase (C14), cystine arylamidase, trypsin, α-chymotripsin, acid phosphatase, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase, α-manosidase, α-fucosidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, aesculin hydrolysis, gelatinase and urease are not detected. In API 20 NE tests, positive for the assimilation of D-glucose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, maltose, potassium gluconate, malic acid, L-arabinose and adipic acid; and negative for the assimilation of capric acid, trisodium citrate and phenylacetic acid. Using Biolog GENIII MicroPlates, positive for the utilization of raffinose, α-D-glucose, pectin,

D-mannose, D-mannitol, D-galacturonic acid, methyl pyruvate, γ-amino-butyric acid, maltose, D-fructose, D-arabitol, L-alanine, L-galactonic acid lactone, D-lactic acid methyl ester, α-hydroxy-butyric acid, trehalose, methyl *β*-D-glucoside, D-galactose, *myo*-inositol, L-arginine, D-gluconic acid, L-lactic acid, *β*-hydroxy-D,L-butyric acid, cellobiose, D-salicin, 3-methyl-D-glucoside, glycerol, D-glucuronic acid, gentiobiose, *N*-acetyl-D-glucosamine, D-fucose, D-glucose-6-PO_4, glucuronamide, α-keto-glutaric acid, sucrose, *N*-acetyl-*β*-D-mannosamine, L-fucose, D-fructose-6PO_4, D-malic acid, propionic acid, turanose, L-rhamnose, quinic acid, L-malic acid, inosine, L-serine, D-saccharic acid and bromo-succinic acid; and negative for the utilization of D-sorbitol, gelatin, p-hydroxy-phenylacetic acid, Tween 40, dextrin, lactose, glycyl-L-proline, melibiose, L-aspartic acid, citric acid, α-keto-butyric acid, L-glutamic acid, acetoacetic acid, L-histidine, mucic acid, *N*-acetyl-D-galactosamine, D-aspartic acid, L-pyroglutamic acid, acetic acid, stachyose, *N*-acetyl neuraminic acid, D-serine and formic acid. The major cellular fatty acids are $C_{18:1}\ \omega7c/C_{18:1}\ \omega6c$ and $C_{16:0}$. The polar lipids consist of phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid, an unidentified phospholipid and an unidentified lipid. The type strain, M10.9X^T (DSM 112301^T=CECT 30307^T), was isolated from the inner sediment of an aluminium can collected from the western Mediterranean coast (Malva-rosa beach, València, Spain). The DNA G+C content of the type strain is 65.2mol%.

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Author contributions
À.V.V., E.M.M. and M.P. carried out the sampling. L.S., E.M.M. and À.V.V. performed the experimental procedures. J.Pa., supervised the experimental work and the bioinformatic analysis. L.S., E.M.M., À.V.V., J.Pa., J.Pe. and M.P. analysed the results as well as wrote and approved the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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