Marinomonas gallaica sp. nov. and *Marinomonas atlantica* sp. nov., isolated from reared clams (*Ruditapes decussatus*)

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Three Gram-negative bacterial strains (Cmf 17.2^T, Rd 20.33 and Cmf 18.22^T) isolated from reared clams in Galicia were subjected to a taxonomic study, based on genetic and phenotypic characterization. Analysis of the 16S rRNA gene allowed the identification of the strains as members of the genus *Marinomonas*, sharing the highest similarity with *Marinomonas aquimarina* CECT 5080^T (97.8 %–98.5 % 16S rRNA gene sequence similarity). Phylogenetic analysis of the sequences showed that the three isolates formed two different groups distantly related to their closest relative, *M. aquimarina*. DNA–DNA hybridizations were performed to confirm the taxonomic position and the results were below the recommended threshold for species delimitation, specifically 44.5 % (Cmf 17.2^T with *M. aquimarina* CECT 5080^T) and 55 % (Cmf 18.22^{T}with *M. aquimarina* CECT 5080^T). Furthermore, the average nucleotide identity (ANIb, ANIm and OrthoANI) and *in silico* estimated DNA–DNA reassociation values among Cmf 17.2^T, Cmf 18.22^T and *M. aquimarina* CECT 5080^T were in all cases below the respective threshold for species differentiation. The estimated G+C content of the genomic DNA was found to be 45.3 % (Cmf 17.2^T) and 44.6 % (Cmf 18.22^T). The principal fatty acids of the strains were found to be summed feature 3 ($C_{16:1}$ ω7*c*/$C_{16:1}\omega$6*c*), summed feature 8 ($C_{18:1}\omega$7*c*/$C_{18:1}\omega$6*c*), $C_{16:0}$, $C_{12:0}$ and $C_{10:0}$ 3-OH. The results obtained on the characterization of the clam isolates indicate that they represent two novel species of the genus *Marinomonas*, for which the names *Marinomonas gallaica* sp. nov. (type strain Cmf 17.2^T=CECT 9049^T=LMG 29243^T) and *Marinomonas atlantica* sp. nov. (type strain Cmf 18.22^T=CECT 9050^T=LMG 29244^T) are proposed.

The genus *Marinomonas*, within the family *Oceanospirillaceae*, is composed of Gram-negative bacteria that are present in different marine environments such as the Mediterranean sea (Solano & Sanchez-Amat, 1999; Macián *et al.*, 2005), Black sea (Ivanova *et al.*, 2005) and Arctic Ocean (Zhang *et al.*, 2008). The genus was proposed by Van Landschoot & De Ley (1983) to accommodate two unusual species of the genus *Alteromonas*, *Alteromonas communis* and *Alteromonas vaga*. In recent years, the number of described species has increased and, at the time of writing, the genus *Marinomonas* comprises 24 recognized species (http://www.bacterio.net/marinomonas.html) after the reclassification of *Marinomonas basaltis* as a later heterotypic synonym of *Marinomonas communis* (Chimetto *et al.*, 2011). In this study we report the characterization of three strains belonging to the genus *Marinomonas* isolated from reared clams in Galicia (Spain) and determine their taxonomic position within the genus.

Bacterial isolates (Cmf 17.2^T, Rd 20.33 and Cmf 18.22^T) were obtained from healthy reared clams (*Ruditapes decussatus*) on the Galician coast (NW Spain). The type strain of

Abbreviations: ANI, average nucleotide identity; DDH, DNA-DNA hybridization; eDDH, estimated DNA-DNA hybridization; FAME, fatty acid methyl esters.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Marinomonas gallaica* Cmf 17.2^T and Rd 20.33, and *Marinomonas atlantica* Cmf 18.22^T are LN909520, LN909521 and LN909522, respectively. Genome sequence data of *Marinomonas aquimarina* CECT 5080^T, *Marinomonas atlantica* Cmf 18.22^T and *Marinomonas gallaica* Cmf 17.2^T are publicly available under the accession numbers LTAV00000000, LTAW00000000 and LTAX00000000, respectively.

A supplementary table available with the online Supplementary Material.

Marinomonas aquimarina CECT 5080^T was provided by the Spanish Collection of Type Cultures (CECT) and included in the study for taxonomic and phenotypic comparison. All strains were cultured on marine agar (MA; Difco) at 24 ±1 °C for 24 h. Cultures were maintained frozen at −80 °C in marine broth (MB; Difco) supplemented with 15 % (v/v) glycerol.

Genomic DNA was extracted from 24 h cultures on MA for *in vitro* amplification of the 16S rRNA gene using the Insta-Gene Matrix (Bio-Rad) following the manufacturer's protocol. Sequences for reference strains were retrieved from the GenBank/EMBL/DDBJ databases. Sequence analyses were performed using the DNASTAR Lasergene SEQMAN program. Sequence similarities of the 16S rRNA gene were determined using the EzTaxon-e server (www.eztaxone.ezbiocloud.net) (Kim *et al.*, 2012) and the BLASTN program, and checked with the Species Living Tree Project (LTP) database (www.arb-silva.de/living-tree) that has frequently been used for delineating bacterial species due to its high quality sequences (Yarza *et al.*, 2008, 2014). Sequences were aligned using CLUSTALW tool (Larkin *et al.*, 2007), and phylogenetic trees were reconstructed using the neighbour-joining (NJ) and maximum-likelihood (ML) algorithms in MEGA software package version 6.06 (Tamura *et al.*, 2011). Distance matrices were calculated by using Kimura's two-parameter correction and stability of the groups was estimated by bootstrap analysis (1000 replicates) using MEGA version 6.06 (Tamura *et al.*, 2011).

Analysis of the 16S rRNA gene sequence revealed that the three strains belonged to the genus *Marinomonas*, showing the highest sequence similarity with the type strains of *M. aquimarina* (97.8 %–98.5 %), *Marinomonas ostreistagni* (97.3 %–97.4 %), *Marinomonas fungiae* (97.2 %–97.3 %), and *Marinomonas brasilensis* (97.1 %–97.2 %). Similarity between strains Cmf 17.2^T and Rd 20.33 was 99.5 %, and between Cmf 17.2^T and Cmf 18.22^T was 97.9 %. According to the LTP database, sequence similarities ranging between 94.5 % and 98.7 % suggest that the microbial strain can be classified as a member of a given genus, whereas higher similarities would be indicative of belonging to a given species (Yarza *et al.*, 2008). Phylogenetic analysis based on the 16S rRNA gene sequences, made with NJ and ML approaches, showed that the clam isolates form two different groups within the genus (Fig. 1). Strains Cmf 17.2^T and Rd 20.33 clustered together in an independent branch from strain Cmf 18.22^T and *M. aquimarina* CECT 5080^T, suggesting that these isolates may represent two novel species. Thus, Cmf 17.2^T and Cmf 18.22^T were selected as representative strains for further analyses.

High Pure PCR Template Preparation kit (Roche) was employed for isolation of genomic DNA for whole-genome sequencing and DNA–DNA hybridization (DDH) experiments. The genomes of strains Cmf 17.2^T and Cmf 18.22^T were sequenced at Sistemas Genómicos (Valencia, Spain) using Illumina paired-end sequencing technology. The reads were trimmed using Trimmomatic 0.32 (Bolger *et al.*, 2014). Genome assembly was performed using SPAdes 3.6.1 (Nurk *et al.*, 2013). The G+C content of the chromosomal DNA was calculated on the basis of its whole-genome sequence. The ANIb, ANIm and OrthoANI values were calculated as described by Richter & Rosselló-Móra (2009) using JSpecies (V1.2.1), and Lee *et al.* (2015). Estimated DNA–DNA hybridization (eDDH) value was determined between these two strains using the genome-to-genome distance calculator (GGDC2.1) (Auch *et al.*, 2010a, b; Meier-Kolthoff *et al.*, 2013). The DNA G+C content was estimated according to the draft genome of strains Cmf 17.2^T and Cmf 18.22^T, and was found to be 45.3 % and 44.6 % respectively, which are in the range of the genus (i.e., 41–50 %) (Espinosa *et al.*, 2010). Average nucleotide identity (ANI) and eDDH calculations were performed to elucidate genome similarities and species assignation of strains Cmf 17.2^T and Cmf 18.22^T. The values obtained using these indexes (Table 1) were below the respective thresholds (95–96 % ANI and 70 % eDDH) indicating that these strains represent two novel species of the genus. Additionally, OrthoANI (Lee *et al.*, 2015) values showed similarities of 76.26 % between *M. aquimarina* CECT 5080^T and Cmf 17.2^T, 76.01 % between *M. aquimarina* CECT 5080^T and Cmf 18.22^T and 90.75 % between strains Cmf 17.2^T and Cmf 18.22^T.

DDH experiments were undertaken between the strains Cmf 17.2^T and Cmf 18.22^T against the type strain of the species with highest similarity in the 16S rRNA gene, *M. aquimarina* CECT 5080^T. DDH experiments were performed with the hydroxyapatite/microtitre plate method (Ziemke *et al.*, 1998) using a hybridization temperature (Tm) of 50 °C. Reciprocal reactions (i.e. A×B and B×A) were performed and were generally within the limits of this method (Goris *et al.*, 1998). Results obtained from DDH experiments between Cmf 17.2^T and Cmf 18.22^T with *M. aquimarina* CECT 5080^T confirmed their separate status at the species level since the reassociation values were always below the recommended threshold; 44.5 % reassociation value for Cmf 17.2^T with *M. aquimarina* CECT 5080^T and 55 % reassociation value for Cmf 18.22^T with *M. aquimarina* CECT 5080^T. The reassociation value between strains Cmf 17.2^T and Cmf 18.22^T was 45.5 %.

The three clam isolates and *M. aquimarina* CECT 5080^T were subjected to the following phenotypic tests (MacFaddin, 1993): cell morphology and motility, Gram stain, oxidase, catalase, oxidation/fermentation test, gas and acid production from glucose, indole, methyl red, Voges–Proskauer reaction, utilization of citrate, Thornley's arginine dihydrolase test, Moeller's arginine dihydrolase and lysine and ornithine decarboxylases, nitrate reduction, and hydrolysis of gelatin, Tween 80, amylase and aesculin. Salt tolerance tests were performed on Basal medium agar [BMA: neopeptone (4 g l^{-1}), yeast extract (1 g l^{-1}) and bacteriological agar (15 g l^{-1})] supplemented with 0, 0.5, 1, 3, 6, 8 and 10 % NaCl. Growth at different temperatures (4, 20, 25, 30, 37 and 44 °C) and pH (4–10) were also determined. All media were supplemented with 1 % NaCl when required. Additional

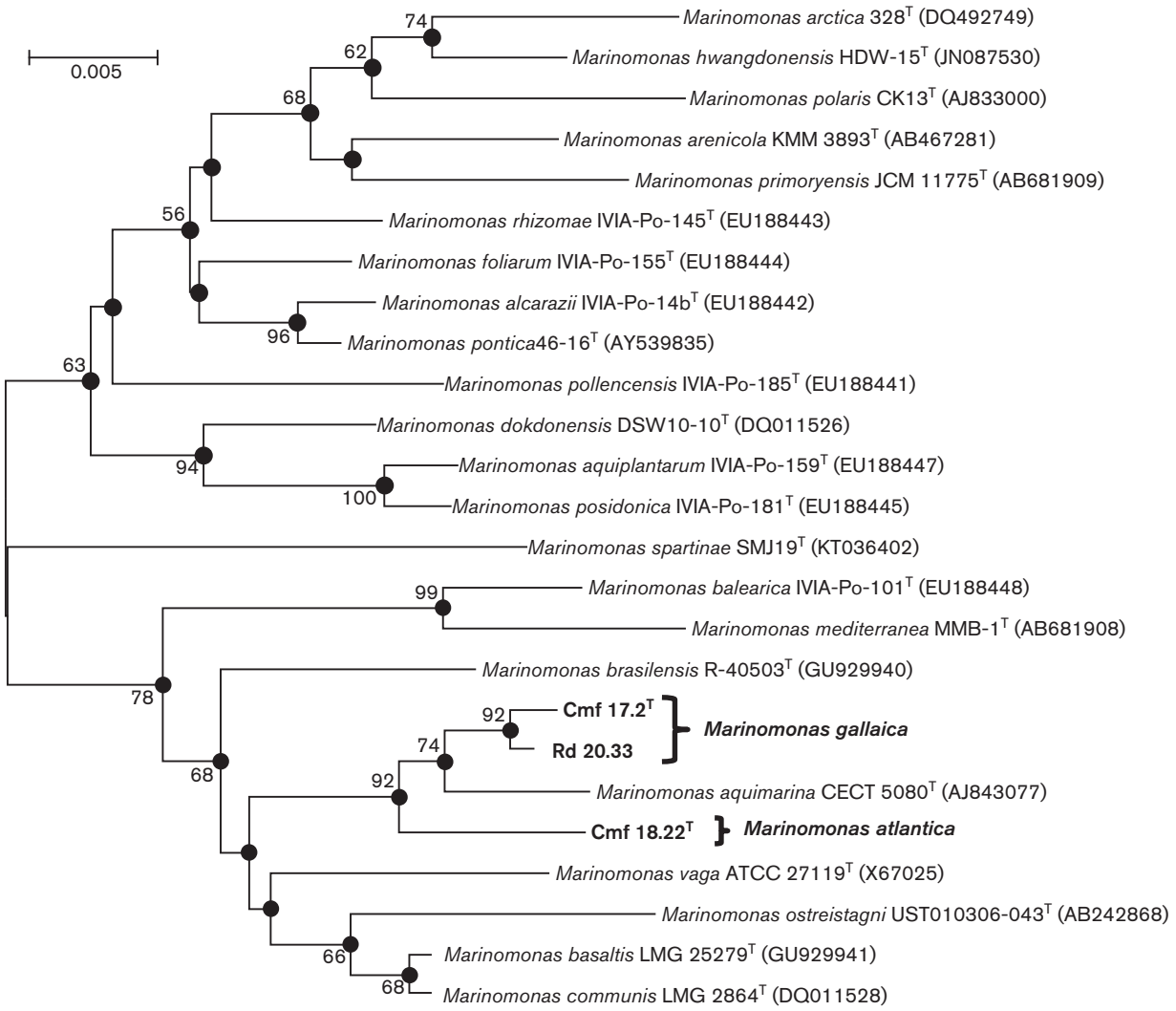

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships of *Marinomonas gallaica* sp. nov. and *Marinomonas atlantica* sp. nov. within the genus *Marinomonas*. Filled circles indicate nodes that were also recovered in maximum-likelihood (HKY+G+I parameter) tree based on the same sequences. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar, 0.005 substitutions per nucleotide position.

phenotypic characteristics were determined using API ZYM strips (bioMérieux) and Biolog GN2 plates, according to the manufacturers' instructions. Furthermore, utilization of additional sugars, alcohols and organic acids as sole carbon source was determined on plates of basal medium agar (Baumann 1981) supplemented with 1 % (w/v) of the corresponding organic compound. The following substrates were tested: D-ribose, arabinose, D-xylose, D-glucose, fructose, D-galactose, trehalose, D-mannose, L-rhamnose, maltose, cellobiose, sucrose, lactose, melibiose, salicin, amygdalin, gluconic acid, D-mannitol, sorbitol, *myo*-inositol, glycerol, sodium acetate, propionic acid, citric acid, pyruvate, lactic acid, trans-aconitic acid, succinic acid, glycine, L-leucine, L-serine, threonine, glutamic acid, D-alanine, arginine, tyrosine, ornithine, citrulline, amino-*N*-butyric acid, aspartic acid, L-histidine, lysine, putrescine, 3-hydroxybutyric acid, *N*-acetyl-D-glucosamine, fumaric acid, malic acid and D-saccharic acid.

Bacterial cells were straight rods and cell motility was observed. The isolates grow unpigmented on MA plates with small regular colonies. All strains are oxidase- and catalase-positive with aerobic metabolism. The presence of salt is required for growth. Produced enzymatic activities are listed in the species descriptions and the differences among the three strains are shown in Table S1 (available in the online Supplementary Material). In addition, Biolog GN2 results showed several differences among the clam isolates (Table S1 and species description). Several phenotypic characteristics allow the discrimination of the two different genomic groups from *M. aquimarina* such as growth temperature range

Table 1. Results of ANI calculations (%) using JSpecies and estimated DDH (%) using GGDC 2.1

Index	*M. gallaica* sp. nov. Cmf 17.2^T	*M. atlantica* sp. nov. Cmf 18.22^T	*M. aquimarina* CECT 5080^T
ANIb			
Cmf 17.2^T		90.38	75.95
Cmf 18.22^T	90.49		75.58
M. aquimarina CECT 5080^T	76.04	75.70	
ANIm			
Cmf 17.2^T		91.58	83.60
Cmf 18.22^T	91.58		83.40
M. aquimarina CECT 5080^T	83.61	83.41	
eDDH			
Cmf 17.2^T		42.60	19.80
Cmf 18.22^T	42.60		19.60
M. aquimarina CECT 5080^T	19.80	19.60	

(*M. aquimarina* CECT 5080^T grows at 40 °C but not at 4 °C), and growth in the presence of D-mannitol and glycerol. Moreover, the clam isolates can be distinguished from each other on the ability to use L-arabinose and D-galactose - activities present in Cmf 18.22^{T}but absent in Cmf 17.2^T and Rd 20.33. On the other hand, Cmf 17.2^T and Rd 20.33 are capable of using sucrose, glycine, and lysine but Cmf 18.22^T is not able to use these compounds (Table 2).

Chemotaxonomic features were studied by the analyses of fatty acid methyl esters (FAME). FAME were extracted and prepared from 24 h cultures on MA incubated at 24±1 °C as described by Sasser (1990) according to the MIDI Microbial Identification System. In all strains the principal fatty acids were found to be summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$), summed feature 8 ($C_{18:1}\omega7c/C_{18:1}\omega6c$), $C_{16:0}$, $C_{12:0}$ and $C_{10:0}$ 3-OH (Table 3). FAME profiles also showed differences in the percentages of the main fatty acid among the three type strains, such as in summed feature 3 or $C_{10:0}$ 3-OH.

In summary, the taxonomic study of the three clam isolates supports the proposal of two novel species of the genus *Marinomonas*, for which the names *Marinomonas gallaica* sp. nov. and *Marinomonas atlantica* sp. nov. are proposed with Cmf 17.2^T and Cmf 18.22^{T}as respective type strains.

Description of *Marinomonas gallaica* sp. nov.

Marinomonas gallaica (gal.la′i.ca L. fem. adj. *gallaica* pertaining to Gallaecia, the north-western region of Spain).

Table 2. Differential characteristics among the two proposed species *Marinomonas gallaica* sp. nov. and *Marinomonas atlantica* sp. nov. and the closest relative *M. aquimarina*

Taxa: 1, *M. gallaica* sp. nov. (Cmf 17.2^T and Rd 20.33); 2, *M. atlantica* sp. nov. (Cmf 18.22^T); 3, *M. aquimarina* (CECT 5080^T). All data were obtained from this study. W, Weak reaction.

Characteristic	1	2	3
Growth at:			
4 °C	+	+	–
40 °C	–	–	+
Growth with:			
L-Arabinose	–	+	–
D-Galactose	–	+	–
Sucrose	+	–	–
D-Mannitol	+	+	–
Glycerol	+	+	–
Glycine	+	–	+
Lysine	+	–	–
Putrescine	+	+	W
Aspartic acid	+	–	+

Table 3. Fatty acid contents (%) of *Marinomonas gallaica* sp. nov. Cmf 17.2^T, *Marinomonas atlantica* sp. nov. Cmf 18.22^T and *M. aquimarina* CECT 5080^T

Strains: 1, *M. gallaica* sp. nov. Cmf 17.2^T; 2, *M. atlantica* sp. nov.Cmf 18.22^T; 3, *M. aquimarina* CECT 5080^T. All data are from the present study. Strains were grown on MA plates at 24 °C for 24 h.

Fatty acid	1	2	3
Summed features*			
3	24.96	27.73	22.94
8	44.45	45.10	47.53
$C_{10:0}$	2.89	1.90	2.83
$C_{10:0}$ 3-OH	5.88	5.01	3.03
$C_{12:0}$	4.14	3.53	2.95
$C_{14:0}$	2.21	2.13	1.71
$C_{16:0}$	12.72	11.50	15.94
$C_{18:0}$	1.55	2.30	1.51

*Summed features are groups of two or more fatty acids that could not be separated using the MIDI system. Summed feature 3 contained $C_{16:1}\omega7c/C_{16:1}\omega6c$; Summed feature 8 contained $C_{18:1}\omega7c/C_{18:1}\omega6c$.

Cells are Gram-stain-negative, straight-rod-shaped and motile. Colonies on MA are dull white with regular edges and are non-pigmented. Obligately aerobic. Catalase- and oxidase-positive. Does not reduce nitrates to nitrites nor produce N_2 gas. Negative for production of indole, arginine dihydrolase, and lysine and ornithine decarboxylases. Negative for hydrolysis of Tween 80, aesculin, starch and gelatin. Growth occurs in media with 1–10 % NaCl (optimum 3 %). Growth is observed from 4 °C to 37 °C, with optimum growth at 24 °C. The pH for growth ranges from pH 5 to pH 10 (optimum pH 8). According to API ZYM tests, positive for alkaline and acid phosphatases, leucine arylamidase, napthol-AS-BI-phosphohydrolase and α-glucosidase, and negative for all other enzymatic activities [strain Rd 20.33 also positive for esterase (C4)]. The following compounds are used as sole carbon source on BMA: D-glucose, fructose, D-mannose, gluconic acid, D-mannitol, glycerol, sodium acetate, propionic acid, citric acid, pyruvate, lactic acid, succinic acid, L-serine, glutamic acid, D-alanine, arginine, ornithine, citrulline, amino-n-butyric acid, L-histidine, lysine, putrescine, fumaric acid and malic acid. Also utilizes the following compounds on Biolog GN2 microplates: dextrin, glycogen, Tween 40, D-fructose, acetic acid, *cis*-aconitic acid, formic acid, α-ketoglutaric acid, DL-lactic acid, bromo-succinic acid, succinic acid, L-alaninamide, D-alanine, L-alanine, hydroxyl-L-proline, L-ornithine, L-phenylalanine, L-proline, inosine, uridine, putrescine, L-arabinose, maltose, D-mannitol, turanose, methyl pyruvate, monomethyl succinate, succinic acid, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-aspartic acid, L-aspartic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, γ-aminobutyric acid, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Weak reactions were observed for the following compounds: D-psicose, thymidine, quinic acid, D-saccharic acid and DL-carnitine. The main fatty acids are summed feature 3 ($C_{16:1}\omega7c$/$C_{16:1}\omega6c$), summed feature 8 ($C_{18:1}\omega7c$/$C_{18:1}\omega6c$), $C_{16:0}$, $C_{10:0}$ 3-OH and $C_{12:0}$.

The type strain, Cmf 17.2^T (=CECT 9049^T=LMG 29243^T), was isolated from clam, *Ruditapes decussatus*, in Galicia, north-west Spain (43° 7′ 46″ N 9° 11′ 1″ W). The G+C content of the genome of the type strain is 45.3 %. An additional strain of the species is Rd 20.33,

Description of *Marinomonas atlantica* sp. nov.

Marinomonas atlantica (at.lan′ti.ca L. fem. adj. *atlantica* Atlantic, from the Atlantic Ocean).

Cells are Gram-stain-negative, straight-rod-shaped and motile. Colonies on MA are dull white with regular edges and are non-pigmented. Obligately aerobic. Catalase- and oxidase-positive. Does not reduce nitrates to nitrites nor produce N_2 gas. Negative for production of indole, arginine dihydrolase, and lysine and ornithine decarboxylases. Negative for hydrolysis of Tween 80, aesculin, starch and gelatin. Growth occurs in media with 1–10 % NaCl (optimum 3 %). Growth is observed from 4 °C to 37 °C, with optimum growth at 24 °C. The pH for growth ranges from pH 5 to pH 10 (optimum pH 8). The following reactions were positive on the API ZYM strips: alkaline and acid phosphatases, esterase (C4), leucine arylamidase and α-glucosidase; negative for all other enzymatic activities. The following compounds are used as sole carbon source on BMA: arabinose, D-glucose, fructose, D-galactose, D-mannose, gluconic acid, D-mannitol, glycerol, sodium acetate, propionic acid, citric acid, pyruvate, lactic acid, succinic acid, L-serine, glutamic acid, D-alanine, arginine, ornithine, citrulline, amino-n-butyric acid, L-histidine, 3-hydroxybutyric acid, fumaric acid and malic acid. Utilizes the following compounds on Biolog GN2 microplates: formic acid, DL-lactic acid and L-arabinose. Weak reactions were observed for the following compounds: Tween 40, inosine, udiridine, thymidine, phenyethylamine, putrescine, monomethyl succinate, L-asparagine, L-glutamic acid, succinic acid, L-serine, 2-aminoethanol, 2, 3-butanediol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. The main fatty acids are summed feature 3 ($C_{16:1}\omega7c$/$C_{16:1}\omega6c$), summed feature 8 ($C_{18:1}\omega7c$/C $_{18:1}\omega6c$), $C_{16:0}$, $C_{10:0}$ 3-OH and $C_{12:0}$.

The type strain, Cmf 18.22^T (=CECT 9050^T=LMG 29244^T), was isolated from clam, *Ruditapes decussatus*, in Galicia, north-west Spain (43° 7′ 46″ N 9° 11′ 1″ W). The G+C content of the genome of the type strain is 44.6 %.

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