

Arcobacter lacus sp. nov. and *Arcobacter caeni* sp. nov., two novel species isolated from reclaimed water

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Abstract

Two strains (RW43-9^T and RW17-10^T) recovered from secondary treated wastewater from the Wastewater Treatment Plant (WWTP) in Reus (Spain) were characterized by a polyphasic taxonomic study, showing evidence that they represented two novel *Arcobacter* species. Based on the 16S rRNA gene for strain RW43-9^T, the closest relative was *Arcobacter butzleri* LMG 10828^T (99.9% similarity), while for strain RW17-10^T it was *Arcobacter venerupis* CECT 7836^T (99.4%). Additionally, multilocus phylogenetic analysis of five concatenated housekeeping genes (*atpA*, *gyrA*, *gyrB*, *hsp60* and *rpoB*) showed that the two strains formed separate branches that are different from known *Arcobacter* species. Whole genome sequences of the two strains (RW43-9^T and RW17-10^T) were obtained and they were compared with those of the type strains of their nearest species. Using average nucleotide identity and *in silico* DNA–DNA hybridization gave values that were below 96 and 70%, respectively. These results clearly confirm that they represent novel species. Additionally, the phenotypic characterization of the strains allowed their differentiation from other species. Therefore, the strains are proposed as representing two novel species with the names *Arcobacter lacus* sp. nov. (type strain RW43-9^T=CECT 8994^T=LMG 29062^T) and *Arcobacter caeni* sp. nov. (type strain RW17-10^T=CECT 9140^T=LMG 29151^T).

The genus *Arcobacter* is composed of Gram-negative bacteria found in a wide range of habitats, and until recently was included in the family *Campylobacteraceae* [1]. The first two species used to describe the genus *Arcobacter* by Vandamme and co-workers in 1991 [2] were *Arcobacter cryaerophilus* and *Arcobacter nitrofigilis*, which were first described as aerotolerant campylobacters. However, in 2017, Waite *et al.* [3] reviewed the taxonomy of the epsilonproteobacteria and proposed the new family *Arcobacteraceae*, uniquely for the genus *Arcobacter*. The genus currently includes 27 species that have mainly been described from water-related environments and shellfish [4–7].

In a study that evaluated the presence of members of the genus *Arcobacter* in the inlet and outlet water of a wastewater treatment plant in the city of Reus (north-east Spain), two strains (RW43-9^T and RW17-10^T) were isolated from the secondary treated water at the entrance site of the tertiary lagooning treatment system [8]. Isolation was carried out using 200 ml of water sample by filtration through a

0.45 µm filter (Millipore). The filter was resuspended in 1 ml of distilled water and 100 µl of this resuspension was used to inoculate, by passive filtration (with a 0.45 µm pore diameter filter), the surface of blood agar plates (BD). After 30 min of passive filtration, the filter was removed and the plates were incubated at 30 °C under microaerobic conditions for 48 h. The resultant colonies were presumed to be of *Arcobacter* members because they were small and translucent. Then they were Gram-stained and tested for oxidase activity. Under the microscope, both isolates (RW43-9^T and RW17-10^T) were Gram-stain-negative curved rods and presented oxidase activity, in accordance with previous descriptions of the genus [2, 9]. The two isolates were genotyped using Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) using primers and conditions previously described [10]. Both isolates showed different ERIC-PCR patterns (data not shown), indicating that they represented two different strains. Identification was made using the sequences of the *rpoB* gene, which was amplified and sequenced with primers and conditions previously

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Abbreviations: ANI, average nucleotide identity; isDDH, *in silico* DNA–DNA hybridization; MLPA, multilocus phylogenetic analysis; TTC, 2,3,5-triphenyl-tetrazolium chloride; ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *atpA*, *gyrA*, *gyrB*, *hsp60* and *rpoB* gene sequences of strain RW43-9^T are LT629997, LT904795, LT904819, LT904807, LT904831 and LT904783, respectively; and for strain RW17-10^T they are LT629998, LT904796, LT904820, LT904808, LT904832 and LT904784, respectively. The accession numbers for the genomes of strains RW43-9^T and RW17-10^T are MUXE000000000 and MUXE000000000, respectively.

Eight supplementary figures are available with the online version of this article.

described [11]. The obtained *rpoB* gene sequences (622 bp) were aligned with the CLUSTAL W algorithm [12] and a phylogenetic tree was built with MEGA 7.0 [13] using the Maximum-Likelihood (ML) method [14]. The phylogenetic tree showed that the two strains were arranged into two different branches, separate from other species, *Arcobacter butzleri* being the nearest species to strain RW43-9^T and *Arcobacter suis* the nearest species to strain RW17-10^T (Fig. S1, available with the online version of this article). For complete phylogenetic analysis of these two strains, the 16S rRNA genes and the housekeeping genes *atpA*, *gyrA*, *gyrB* and *hsp60* were additionally amplified and sequenced [11]. Alignments were made with CLUSTAL W [12], phylogenetic trees were built as above with the Neighbour-Joining (NJ) [15, 16] and the ML methods for the 16S rRNA gene (Figs 1

and S1), multilocus phylogenetic analysis (MLPA) of the concatenated sequences of the five mentioned housekeeping genes (Figs 2 and S3) and with the ML method for the individual housekeeping genes (Figs S1 and S4–S7). The resulting trees showed that the two strains again formed independent branches from known *Arcobacter* species. In the MLPA tree, strain RW17-10^T also had *A. suis* CECT 7833^T as its nearest relative along with *Arcobacter venerupis* CECT 7836^T (Figs 2 and S3), while strain RW43-9^T had *A. butzleri* LMG 10828^T as its nearest relative.

16S rRNA gene sequence similarities (1413 bp) between the two potential new species and other species of the genus *Arcobacter* were calculated using MegAlign version 7.0.0 (DNASTAR). Strain RW43-9^T showed the highest similarity of 99.9% with *A. butzleri* LMG 10828^T, while the level of

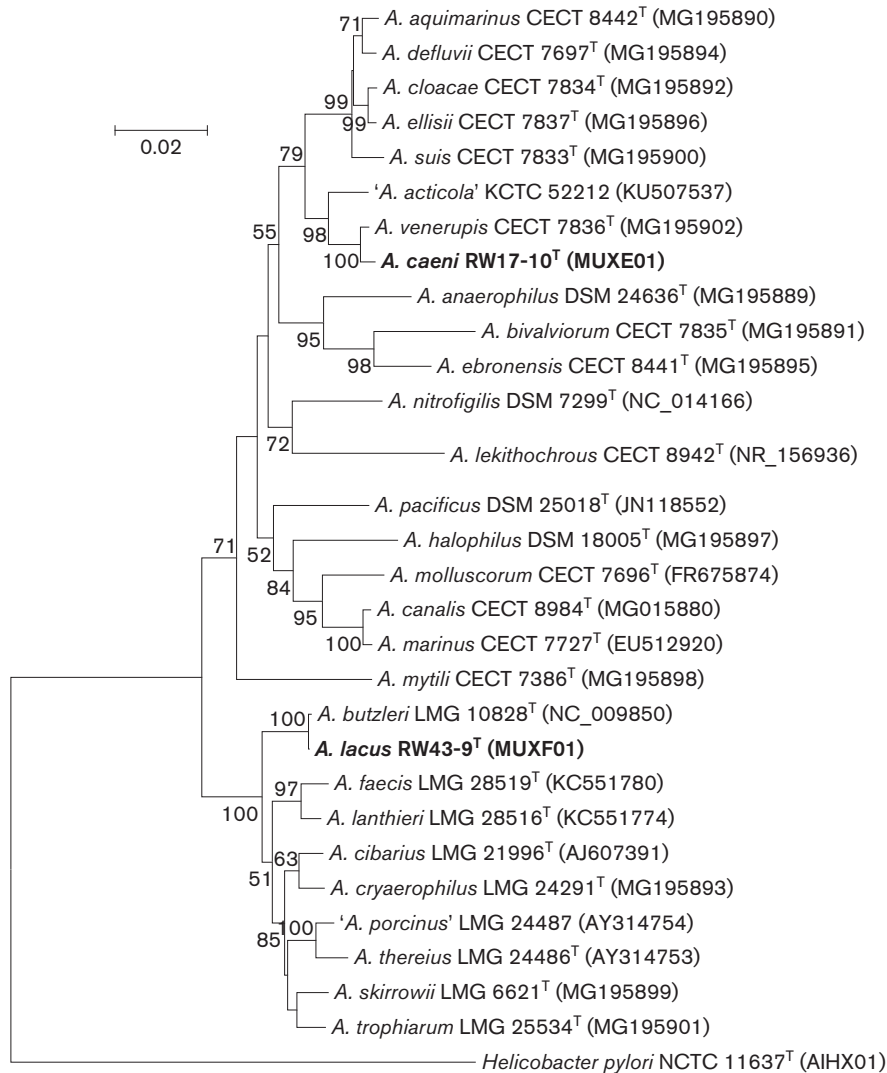


Fig. 1. NJ tree based on 16S rRNA gene sequences (1406 bp) showing the phylogenetic position of strains RW43-9^T and RW17-10^T within the genus *Arcobacter*. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

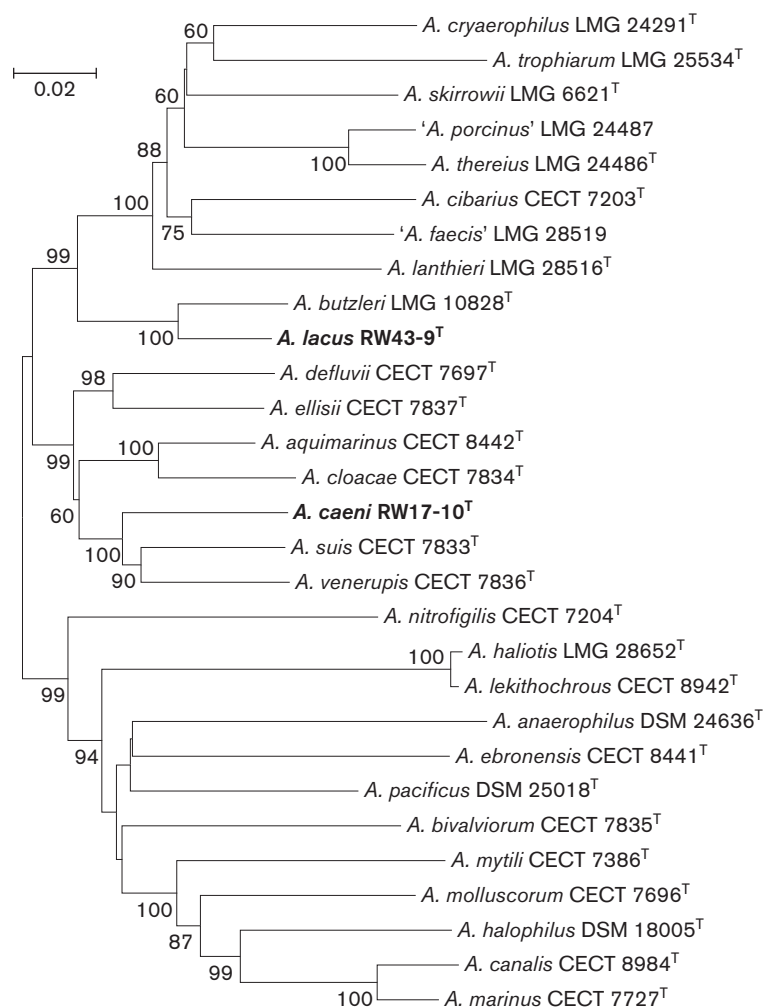


Fig. 2. NJ tree based on the concatenated sequences of the *atpA*, *gyrB*, *hsp60*, *rpoB* and *gyrA* (3061 bp) genes showing the phylogenetic position of strains RW43-9^T and RW17-10^T within the genus *Arcobacter*. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

similarity with other species of the genus ranged from 91.2% with *Arcobacter bivalviorum* CECT 7835^T to 97.9% with 'Arcobacter faecis' LMG 28519 and *Arcobacter lanthieri* LMG 28516^T. The highest level of similarity for strain RW17-10^T was 99.4% with *A. venerupis* CECT 7836^T, but with *A. suis* CECT 7833^T was only 97.7%. Similarities to the other species of the genus ranged from 92.9% with *A. bivalviorum* CECT 7835^T to 98.2% with 'Arcobacter acticola' KCTC 52212.

To further confirm that the two strains represented new taxa, we sequenced the genomes of strains RW43-9^T (MUXF00000000) and RW17-10^T (MUXE00000000), as well as those of *A. venerupis* CECT 7836^T (NREP00000000) and *A. suis* CECT 7833^T (NREO00000000) because they were the closest species on the basis of MLPA. Sequencing was carried out with the MiSeq platform (Illumina), giving a sequencing depth of coverage >100× for all the genomes.

Genomes were assembled with SPAdes [17, 18] and annotated with the Rapid Annotation of microbial genomes using Subsystems Technology (RAST) [19, 20]. The genomes of strains RW43-9^T and RW17-10^T were compared with their nearest species based on average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (*is*DDH) calculated with the OrthoANI [21] and GGDC [22] software, respectively. The values of ANI and *is*DDH between each strain and their nearest species were below 96 and 70%, respectively (Table 1). These results, along with those obtained based on the phylogenetic analyses, indicated that the two strains (RW43-9^T and RW17-10^T) represented two novel species of the genus *Arcobacter*.

The G+C content values were 27.2% for the genome of strain RW43-9^T and 26.8% for the genome of strain RW17-10^T, values which are within the 26.6–31.9% range described for other *Arcobacter* species [23, 24].

Table 1. Results (%) of ANI (bold, lower left) and *isDDH* (italics, upper right) between the genomes of strains RW43-9^T and RW17-10^T and the genomes of the most closely related species

Values of ANI and *isDDH* below 96 and 70 %, respectively, indicate that the genomes correspond to different species.

		1	2	3	4	5
1	RW43-9 ^T		55.8	23.3	23.2	23.5
2	<i>A. butzleri</i> RM4018 ^T	94.3		23.2	23.3	23.4
3	RW17-10 ^T	80.6	80.3		30.3	34.4
4	<i>A. venerupis</i> CECT 7836 ^T	79.9	80.0	85.8		31.8
5	<i>A. suis</i> CECT 7833 ^T	80.9	80.8	87.9	86.7	

The strains were characterized phenotypically following the recommended minimal standards for the description of new *Campylobacteraceae* taxa described by Ursing *et al.* [25] and updated by On *et al.* [26]. This characterization included morphology of the colonies, growth at different temperatures and atmospheric conditions, biochemical properties and resistance to antimicrobials [i.e. nalidixic acid (30 mg l⁻¹), cephalothin (30 mg l⁻¹) and cefoperazone (64 mg l⁻¹)]. Each phenotypic characteristic was tested at least twice in blood agar for the two new strains (RW43-9^T and RW17-10^T) along with the type strains of the closest related species, *A. butzleri* LMG 10828^T, *A. venerupis* CECT 7836^T and *A. suis* CECT 7833^T, and positive and negative controls for each test. To prepare the inoculum for phenotypic tests, *A. butzleri*, *A. suis* and *A. venerupis* were cultured from frozen glycerinated cultures in blood agar at 30 °C for 48 h. A total of 39 tests were carried out, i.e. 12 tests of growth conditions (i.e. 22–25 °C, 30, 37 and 42 °C under aerobic, microaerobic and anaerobic conditions) and 27 tests for biochemical properties [i.e. oxidase, catalase and urease activity, nitrate reduction and glucose fermentation on triple-sugar iron agar (TSI), indoxyl acetate hydrolysis, casein, lecithin and starch, growth in media supplemented with 2 and 4 % (w/v) NaCl, 1 % oxgall, 0.1 % sodium deoxycholate, 1 % glycine, 0.05 % safranin, 0.005 % basic fuchsin, 0.0005 % crystal violet, 0.001 % brilliant green, 0.1, 0.01 and 0.04 % 2,3,5-triphenyltetrazolium chloride (TTC), and growth on charcoal cefoperazone deoxycholate agar (CCDA), minimal media and MacConkey agar]. The colony morphology of the strains was assessed after culturing on blood agar at 30 °C under aerobiosis for 48 h. The morphology of the bacteria, the presence of flagella and cell sizes were evaluated with a JEOL 1011-TEM transmission electron microscope. For this, cells grown on blood agar at 30 °C for 48 h were fixed in 2 % glutaraldehyde/0.1 % phosphate buffer for 30 min and suspended cells were transferred to a copper grid and negatively stained with 2 % phosphotungstic acid (pH 7.5). Under the electron microscope, both strains showed the presence of a polar flagellum (Fig. S8). Additionally, both strains showed motility when viewed under a phase contrast microscope. Phenotypic evaluation indicated that strain RW43-9^T could be differentiated from *A. butzleri* LMG 10828^T on the basis of four tests. Strain

RW43-9^T showed growth in the presence of nalidixic acid (30 mg l⁻¹), but no growth was observed on 0.04 % TTC or on blood agar at 37 and 42 °C under anaerobic conditions (Table 2). Five tests differentiated strain RW17-10^T from its nearest species *A. suis* CECT 7833^T and *A. venerupis* CECT 7836^T: the inability of RW17-10^T to grow with crystal violet and on minimal medium; and the ability of RW17-10^T to grow in media with safranin, to produce hydrogen sulphide from TSI agar and to show resistance to cefoperazone (64 mg l⁻¹) (Table 2). Additionally, six tests (Table 2) differentiated strain RW17-10^T from *A. venerupis* CECT 7836^T: the inability of strain RW17-10^T to grow at 37 °C under microaerobiosis and anaerobiosis, in media with 2 % NaCl, basic fuchsin or brilliant green, and no urease activity. In addition, strain RW17-10^T could be differentiated from *A. suis* CECT 7833^T because the latter does not grow on CCDA media, it is susceptible to cephalothin (30 mg l⁻¹) and shows growth in media with 0.01 % TTC (Table 2). Regarding polar lipids, the genomes of the two strains annotated with RAST [19, 20] showed two genes involved in the synthesis of phosphatidylglycerol, i.e. phosphatidylglycerolphosphatase A (*pspA*, EC3.1.3.27) and phosphatidase cytidyltransferase (*cdsA*, EC 2.7.7.41), one gene related to the synthesis of phosphatidylethanolamine, and the gene phosphatidylserine decarboxylase (*psd*, EC4.1.1.65). These polar lipids have been found in other *Arcobacter* species using experimental detection by two-dimensional TLC [4, 5, 27]. Additionally, virulence and resistance genes present in the genome of *A. butzleri* RM4018 [28] were searched for in the genome of strain RW43-9^T. The latter genome showed the same virulence and resistance genes described in the genome of *A. butzleri* RM4018, including *ciaB*, *cadF*, *irgA* and *tlyA* genes related to virulence and the *lrgAB* operon and a β -lactamase with antibiotic resistance [28]. The only exceptions were the absence of the *cat* resistance gene in the genome of RW43-9^T and the presence of the MCR-1 gene that confers resistance to polymyxin and of the *gyrA* mutation Thr-85-Ile [29] responsible for resistance to ciprofloxacin that were not present in the genome of *A. butzleri* RM4018.

Therefore, on the basis of the data presented, the strains are proposed as representing two novel species with the names *Arcobacter lacus* sp. nov. and *Arcobacter caeni* sp. nov.

Table 2. Differential characteristics between strains RW43-9^T and RW17-10^T and the type strains of the most closely related species of the genus *Arcobacter*

Strains: 1, RW43-9^T; 2, *A. butzleri* LMG 10828^T; 3, RW17-10^T; 4, *A. venerupis* CECT 7836^T; 5, *A. suis* CECT 7833^T. Unless otherwise indicated data are from the present study: +, ≥95 % strains positive; –, ≤11 % strains positive; v, 12–94 % strains positive.

Characteristic	1	2*	3	4†	5‡
Growth at/by/on:					
37 °C (aerobiosis)	+	+	–	–	–
37 °C (microaerobic)	+	+	–	+	–
42 °C (microaerobic)	+	v	–	–	–
37 °C (anaerobic)	–	+	–	+	–
42 °C (anaerobic)	–	+	–	–	–
2 % NaCl	+	+	–	+	–
4 % NaCl	–	–	–	–	–
0.1 % Sodium deoxycholate	+	+	–	–	+
0.05 % Safranin	+	+	+	–	–
0.005 % Basic fuchsin	+	+	–	+	–
0.0005 % Crystal violet	+	+	–	+	+
0.001 % Brilliant green	+	+	–	+	–
0.01 % TTC	+	+	–	–	+
0.04 % TTC	–	+	–	–	–
CCDA	+	+	+	+	–
Minimal medium	+	+	–	+	+
Tripe-sugar iron	+	+	+	–	–
Resistance to:					
Cefoperazone (64 mg l ⁻¹)	+	+	+	–	–
Cephalothin (30 mg l ⁻¹)	+	+	+	+	–
Nalidixic acid (30 mg l ⁻¹)	+	–	+	+	+
Nitrate reduction	+	+	+	+	+
Urease	–	–	–	+	–
Indoxyl acetate hydrolysis	+	+	+	+	+

*Data from Vandamme *et al.* (1992).

†Data from Levican *et al.* (2012).

‡Data from Levican *et al.* (2013).

DESCRIPTION OF *ARCOBACTER LACUS* SP. NOV.

Arcobacter lacus (la'cus. L. gen. n. *lacus* of a lake or pond, referring to the isolation of the type strain).

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.2–0.3 µm wide and 1.5–1.8 µm long. Motile by a single polar flagellum. Colonies grown on blood agar at 30 °C under aerobiosis for 48 h are 2–4 mm in diameter, beige to off-white, convex, circular with entire margins and non-swarming. Pigments or haemolysis are not produced on blood agar. Grows on blood agar at 22–25 °C, and at 30 °C under aerobiosis, anaerobiosis and microaerobiosis, at 37 °C under aerobiosis and microaerobiosis and at 42 °C under microaerobiosis. No growth occurs at 37 °C under anaerobiosis or at 42 °C under aerobiosis and anaerobiosis. Produces oxidase and catalase activities and reduces nitrates. Indoxyl acetate is hydrolysed, but urea, casein, lecithin and starch are not. Produces hydrogen

sulphide on TSI agar but does not produce acid from glucose on this medium. Growth occurs in media with 2 % (w/v) NaCl, 1 % Oxgall, 0.1 % sodium deoxycholate, 0.05 % safranin, 0.005 % basic fuchsin, 0.0005 % crystal violet, 0.001 % brilliant green and 0.01 % TTC, on CCDA and MacConkey agars, and in minimal medium. No growth occurs in media with 4 % NaCl, 1 % glycine, or 0.1 or 0.04 % TTC. Resistant to nalidixic acid (30 mg l⁻¹), cephalothin (30 mg l⁻¹) and cefoperazone (64 mg l⁻¹).

The type strain is RW43-9^T (=CECT 8994^T=LMG 29062^T), isolated in 2014 from secondary treated wastewater at the WWTP in Reus, Spain.

DESCRIPTION OF *ARCOBACTER CAENI* SP. NOV.

Arcobacter caeni (cae'ni. L. gen. n. *caeni* of sludge, indicating the origin of the species from water contaminated with sewage).

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.2–0.4 µm wide and 2.0–2.5 µm long. Motile by a single polar flagellum. Colonies grown on blood agar at 30 °C under aerobiosis for 48 h are 1–3 mm in diameter, beige to off-white, convex, circular with entire margins and non-swarming. Pigments or haemolysis are not produced on blood agar. Grows on blood agar at 22–25 and 30 °C under aerobiosis, anaerobiosis and microaerobiosis; no growth is observed at the other temperatures and atmospheres tested. Produces oxidase and catalase activity, and reduces nitrates; indoxyl acetate is hydrolysed, but not urea, casein, lecithin or starch. Produces hydrogen sulphide on TSI agar but does not to produce acid from glucose on this medium. Growth occurs in media with 0.05 % safranin, and on CCDA and MacConkey agars. No growth is observed in media with 2 and 4% (w/v) NaCl, 1 % Oxgall, 0.1 % sodium deoxycholate, 1 % glycine, 0.005 % basic fuchsin, 0.0005 % crystal violet, 0.001 % brilliant green, and 0.01, 0.04 and 0.1% TTC, or in minimal medium. Resistant to nalidixic acid (30 mg l⁻¹), cephalothin (30 mg l⁻¹) and cefoperazone (64 mg l⁻¹).

The type strain is RW17-10^T (=CECT 9140^T=LMG 29151^T), isolated in 2013 from secondary treated wastewater at the WWTP of the city of Reus, Spain.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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