Aeromonas fluvialis sp. nov., isolated from a Spanish river

Anabel Alperi,¹ Antonio J. Martínez-Murcia,² Arturo Monera,²
Maria J. Saavedra^{2,3} and Maria J. Figueras¹

Correspondence

Maria J. Figueras
mariajose.figueras@urv.cat

¹Unit of Microbiology, Rovira i Virgili University, IISPV, Sant Llorenç 21, 43201, Reus, Spain

²Molecular Diagnostics Center (MDC), Biomolecular Technologies S.L., and Miguel Hernández University, Orihuela E-03300, Alicante, Spain

³Department of Veterinary Sciences, CECAV-University of Trás-os-Montes e Alto Douro, Vila Real, Portugal

A Gram-stain-negative, facultatively anaerobic bacterial strain, designated 717^T, was isolated from a water sample collected from the Muga river, Girona, north-east Spain. Preliminary analysis of the 16S rRNA gene sequence showed that this strain belonged to the genus *Aeromonas*, the nearest species being *Aeromonas veronii* (99.5% similarity, with seven different nucleotides). A polyphasic study based on a multilocus phylogenetic analysis of five housekeeping genes (*gyrB*, *rpoD*, *recA*, *dnaJ* and *gyrA*; 3684 bp) showed isolate 717^T to be an independent phylogenetic line, with *Aeromonas sobria*, *Aeromonas veronii* and *Aeromonas allosaccharophila* as the closest neighbour species. DNA–DNA reassociation experiments and phenotypic analysis identified that strain 717^T represents a novel species, for which the name *Aeromonas fluvialis* sp. nov. is proposed, with type strain 717^T (=CECT 7401^T =LMG 24681^T).

The genus *Aeromonas* belongs to the class *Gammaproteobacteria* and to the family *Aeromonadaceae*, and includes facultatively anaerobic, Gram-negative, non-spore-forming, motile bacilli or coccobacilli that are oxidase- and catalase-positive, able to reduce nitrate to nitrite and are generally resistant to the vibriostatic agent O/129 (Abbott *et al.*, 2003; Martin-Carnahan & Joseph, 2005). Species of the genus *Aeromonas* are primarily inhabitants of aquatic environments often associated with fish and human diseases (Martin-Carnahan & Joseph, 2005; Figueras, 2005). The genus includes 19 species: *Aeromonas hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii*, *A. jandaei*, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. popoffii*, *A. simiae*, *A. molluscorum*, *A.*

bivalvium, the recently described *A. aquariorum* and ‘*A. tecta*’ and one unnamed DNA homology group, *Aeromonas* Group 501 (Harf-Monteil *et al.*, 2004; Miñana-Galbis *et al.*, 2004; Martin-Carnahan & Joseph, 2005; Miñana-Galbis *et al.*, 2007; Martínez-Murcia *et al.*, 2008; Demarta *et al.*, 2008). There is considerable evidence that the species *Aeromonas sharmiana* (Saha & Chakrabarti, 2006) may not belong to the genus *Aeromonas* (Martínez-Murcia *et al.*, 2007), while *Aeromonas culicicola* (Pidiyar *et al.*, 2002) and *A. hydrophila* subsp. *dhakensis* (Huys *et al.*, 2002) are potentially considered synonyms of *A. veronii* (Huys *et al.*, 2005) and *A. aquariorum* (Martínez-Murcia *et al.*, 2009), respectively.

The taxonomy of this genus is complex due to the high inter-species similarity of the 16S rRNA gene sequence, which ranges from 96.7 to 100% (Martínez-Murcia *et al.*, 2007), the overlap of biochemical profiles and a poor correlation between genotypic and phenotypic identification (Soler *et al.*, 2003; Figueras, 2005; Ormen *et al.*, 2005). Furthermore, the existence of microheterogeneities in the 16S rRNA gene sequence could generate misidentifications (Alperi *et al.*, 2008; Morandi *et al.*, 2005). This has recently been shown to occur between *A. media* and *A. hydrophila*, *A. caviae* and *A. trota*, and *A. veronii* and *A. jandaei* (Alperi *et al.*, 2008). Analysis based on the sequences of one or two housekeeping genes has proven to be a useful tool for inferring the taxonomy of the genus *Aeromonas* (Yañez *et al.*, 2003; Soler *et al.*, 2004; Küpfer *et al.*, 2006; Nhung

Abbreviations: ADH, arginine dihydrolase; LDC, lysine decarboxylase; MLPA, multi locus phylogenetic analysis; ODC, ornithine decarboxylase; VP, Voges–Proskauer test.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoD*, *recA*, *gyrB*, *dnaJ* and *gyrA* gene sequences of strain 717^T are FJ230078, FJ603453, FJ603457, FJ603455, FJ603454 and FJ603456 respectively.

Electron micrographs of strain 717^T, data showing DNA–DNA relatedness and differences in 16S rRNA gene sequence between strain 717^T and type strains of closely related species, and trees derived from 16S rRNA gene sequences and MLPA showing the relationship of strain 717^T to all other described species of the genus *Aeromonas* are available as supplementary material with the online version of this paper.

et al., 2007; Sepe *et al.*, 2008). A comprehensive multi locus phylogenetic analysis (MLPA) based on seven housekeeping genes (*gyrB*, *gyrA*, *rpoD*, *dnaJ*, *dnaX*, *recA* and *atpD*), and including several strains of the 19 described species, demonstrated a robust phylogenetic frame that can be used for taxonomy, revealing a clear differentiation among closely related species of the genus *Aeromonas* whose positions had previously been questioned (unpublished results).

The present investigation was initiated to determine the taxonomic position of strain 717^T, a previously undescribed member of the genus *Aeromonas* that presented a new 16S rRNA gene RFLP pattern when using an RFLP protocol designed to differentiate all species of the genus *Aeromonas* described prior to 2000 (Figueras *et al.*, 2000). Furthermore, strain 717^T appeared as an independent phylogenetic line after analysis of the *rpoD* gene. In the present study, a polyphasic approach based on an MLPA of five genes (*gyrB*, *gyrA*, *rpoD*, *dnaJ* and *recA*), DNA–DNA reassociation experiments and phenotypic analysis was performed to establish the taxonomic allocation of strain 717^T. Results showed that it represents a novel species of the genus *Aeromonas*, for which the name *Aeromonas fluvialis* sp. nov. is proposed.

Strain 717^T was isolated from a water sample of the Muga river, Girona, north-east Spain, using the membrane filtration technique and ampicillin dextrin agar, as described previously (Borrell *et al.*, 1998). The strain was genetically confirmed as a member of the genus *Aeromonas* using the genus probe described by Chacón *et al.* (2002) and showed a new pattern with the 16S rRNA gene RFLP identification method (Figueras *et al.*, 2000).

The following phenotypic tests used for characterization of strain 717^T were selected from Abbott *et al.* (2003): catalase and oxidase activity, nitrate reduction, hydrogen sulphide production from cysteine, indole production, susceptibility to vibriostatic agent O/129 (150 µg), growth in nutrient broth at 0 and at 6 % NaCl, production of brown diffusible pigment (on TSA) and gas from D-glucose, Voges–Proskauer (VP) test, β-galactosidase activity, growth on MacConkey agar, hydrolysis of elastin, gelatin, DNA and urea, and presence of arginine dihydrolase (ADH), lysine decarboxylase (LDC) and ornithine decarboxylase (ODC) by the Moeller's method. Acid production from carbohydrates was determined in nutrient broth at a final concentration of 1 % (w/v), except for salicin that was at 0.5 % (w/v), supplemented with phenol red and one of the following substrates: sucrose, L-arabinose, cellobiose, lactose, raffinose, L-rhamnose, myo-inositol, D-mannitol, D-sorbitol, N-acetylglucosamine and salicin (Borrell *et al.*, 1998). These tests were performed at least twice and some of them (production of indole and hydrogen sulphide, VP test, β-galactosidase activity, presence of ADH, LDC and ODC, hydrolysis of gelatin and urea and acid production from D-mannitol, D-sorbitol, L-rhamnose, myo-inositol, sucrose and L-arabinose) were performed in parallel using

conventional methods and commercial identification kits (API20NE and API20E, bioMérieux). Finally, 49 carbohydrates for substrate fermentation/oxidation were tested by using API50CH (bioMérieux) following the manufacturer's instructions. Appropriate positive and negative controls were included. All tests were evaluated for 7 days and performed at 30 °C with the exception of those on *A. salmonicida*, which were tested at room temperature (± 25 °C). Type strains belonging to all species of the genus *Aeromonas* were evaluated under identical conditions to those for strain 717^T for all tests included in Table 1.

Isolate 717^T was found to be biochemically different from the other species of the genus *Aeromonas* because of its ability to produce acid from lactose and sucrose but not from L-arabinose or D-mannitol, together with the negative production of ADH, LDC (Table 1) and ODC. Acid production from lactose was negative with API50CH but positive on MacConkey agar and in nutrient broth.

Cell size, morphology and the presence of flagella were determined by electron microscopy (Supplementary Fig. S1a, b, available in IJSEM Online) following procedures described previously (Collado *et al.*, 2009).

The susceptibility of strain 717^T to 27 antibiotics was tested as described previously (Martínez-Murcia *et al.*, 2008), and the strain was classified as susceptible, intermediate, or resistant according to CLSI standards (CLSI, 2005). The following antibiotic-containing discs were obtained from Oxoid: piperacillin (PRL100), piperacillin plus tazobactam (TZP110), amoxycillin (AML10), amoxycillin plus clavulanic acid (AMC30), ticarcillin (TIC75), ticarcillin plus clavulanic acid (TIM85), cephalothin (KF30), cefoxitin (FOX), cefotaxime (CTX30), cefoperazone (CFP30), ceftazidime (CAZ30), ceftriaxone (CRO30), cefepime (FEP30), aztreonam (ATM30), imipenem (IMP10), gentamicin (CN10), kanamycin (K30), tobramycin (TOB10), amikacin (AK30), streptomycin (S), tetracycline (TE30), ciprofloxacin (CIP5), nalidixic acid (NA), fosfomycin (FOS), erythromycin (E15), trimethoprim/sulfamethoxazole (SxT25) and chloramphenicol (C30).

For phylogenetic study of the 16S rRNA and *rpoD* genes, strain 717^T was cultured on blood agar at 30 °C. DNA was extracted from a single colony by using InstaGene Matrix (Bio-Rad) following the manufacturer's instructions. Primers and conditions for amplification and sequencing of the 16S rRNA (1503 bp) and *rpoD* (820 bp) genes have been described previously (Martínez-Murcia *et al.*, 1992; Soler *et al.*, 2004). PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) and prepared for sequencing by using the BigDye Terminator v.1.1 cycle sequencing kit (Applied Biosystems). Amplified genes were sequenced with an ABI PRISM 310 genetic analyser (Applied Biosystems). Using the CLUSTAL W program, version 1.83 (Thompson *et al.*, 1994), the sequences obtained were independently aligned with sequences of the type and reference strains of all

Table 1. Key tests for phenotypic differentiation of strain 717^T from other species of the genus *Aeromonas*.

Taxa: 1, *Aeromonas hydrophila*; 2, *A. bestiarum*; 3, *A. salmonicida*; 4, *A. caviae*; 5, *A. media*; 6, *A. eucrenophila*; 7, *A. sobria*; 8, *A. veronii* biovar *sobria*; 9, *A. jandaei*; 10, *A. veronii* biovar *veronii*; 11, *A. schubertii*; 12, *A. trota*; 13, *A. encheleia*; 14, *A. allosaccharophila*; 15, *A. popoffii* [data for taxa 1–15 from Abbott *et al.* (2003), tests performed at 35 °C, with two exceptions* tested at 25 °C]; 16, *A. simiae* [data from Harf-Monteil *et al.* (2004)]; 17, *A. molluscorum* [data from Miñana-Galbis *et al.* (2004), tests performed at 25 °C]; 18, *A. bivalvium* [data from Miñana-Galbis *et al.* (2007), tests performed at 25–30 °C]; 19, *A. aquariorum* [data from Martínez-Murcia *et al.* (2008)]; 20, '*A. tecta*' [data from Demarta *et al.* (2008)]. All tests were performed at 30 °C unless specified and evaluated for 7 days. +, 85–100 % of strains positive; v, 16–84 % of strains positive; –, 0–15 % of strains positive; ND, no data available. Results for type strains are shown in parentheses (data from this study).

Characteristic	717 ^T	1	2	3	4	5	6	7*	8	9	10	11	12	13	14	15*	16	17	18	19	20
Presence of:																					
ADH	–	+(+)	+(+)	v(+)	+(+)	v(+)	v(+)	–(+)	+(+)	+(+)	–(–)	+(+)	+(+)	v(+)	v(+)	+(+)	+(+)	+(+)	–(+)	+(+)	+(+)
LDC	–	+(+)	v(–)	v(+)	–(–)	–(–)	–(–)	+(+)	+(+)	+(+)	+(+)	v(–)	+(+)	–(–)	+(+)	–(–)	+(+)	–(–)	+(+)	+(+)	v(+)
Hydrolysis of:																					
Gelatin	–	+(+)	v(+)	+(+)	+(+)	v(–)	+(+)	–(–)	+(+)	+(+)	+(+)	+(+)	+(+)	–(–)	+(+)	+(+)	ND(–)	ND(–)	+(+)	+(+)	ND(–)
Acid from:																					
Sucrose	+	+(+)	+(+)	+(+)	+(+)	+(+)	v(+)	+(+)	+(+)	–(+)	+(+)	–(–)	v(–)	v(+)	+(+)	–(+)	+(+)	+(+)	+(+)	+(+)	–(–)
Lactose	+	v(–)	–(–)	+(–)	v(–)	v(+)	–(+)	–(–)	–(–)	–(–)	v(–)	–(–)	–(–)	–(–)	–(–)	–(–)	–(–)	–(–)	–(–)	–(–)	–(–)
D-Mannitol	–	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	–(–)	v(+)	+(+)	+(+)	+(+)	–(–)	+(+)	+(+)	+(+)	ND(+)
L-Arabinose	–	v(+)	+(+)	+(+)	+(+)	+(+)	v(+)	–(–)	–(+)	–(–)	–(–)	–(–)	–(–)	–(–)	v(+)	v(+)	–(–)	+(+)	+(+)	–(–)	–(–)
Salicin	+	v(–)	v(+)	v(–)	v(+)	v(+)	v(+)	–(–)	–(–)	–(–)	+(+)	–(–)	–(–)	–(+)	–(–)	–(–)	–(–)	ND(+)	+(+)	+(+)	v(–)

members of the genus *Aeromonas* available in GenBank. Genetic distances and clustering were determined using Kimura's two-parameter model (Kimura, 1980) and evolutionary trees were reconstructed by the neighbour-joining (16S rRNA and *rpoD* genes) and maximum-parsimony (16S rRNA gene) methods (Saitou & Nei, 1987) using the MEGA4 program (Tamura *et al.*, 2007). Stability of the relationships was assessed by the bootstrap method (1000 replicates).

The MLPA was performed on the basis of the *gyrB* (923 bp), *rpoD* (652 bp), *recA* (600 bp), *dnaJ* (800 bp) and *gyrA* (709 bp) genes at the Molecular Diagnostic Center (MDC), Orihuela, Spain (unpublished results).

Sequence analysis of the 16S rRNA gene of strain 717^T showed the existence of polymorphism (Supplementary Table S1) at seven positions (0.46%), all of them within the V3 region. This may hamper correct distinction from the nearest species, *A. veronii* (Alperi *et al.*, 2008). The 16S rRNA gene phylogenetic trees (Fig. 1 and Supplementary Fig. S2) showed strain 717^T as an independent phylogenetic line within the genus *Aeromonas*. The 16S rRNA gene sequence similarity between strain 717^T and other species of the genus *Aeromonas* ranged from 97.6% to 99.5%, corresponding to 36–7 bp differences, values within the range (96.7–100%) described for this genus (Saavedra *et al.*, 2006; Martínez-Murcia *et al.*, 2007). The most similar species on the basis of 16S rRNA gene sequences were determined to be *A. veronii* (99.5%), with 7 bp differences, *A. allosaccharophila* (99.4%), with 9 bp differences, followed by *A. jandaei* (99.1%), with 13 bp differences. This high similarity is very common within the genus *Aeromonas*; in fact only one species (*A. simiae*) of the 19 presently accepted in the genus shows 16S rRNA gene sequence similarities below 97%.

The *rpoD* phylogenetic tree showed strain 717^T as an independent phylogenetic line, with *A. veronii* and *A. allosaccharophila* being the phylogenetically closest neighbours (94.6% and 94.3% similarity corresponding to 43 bp and 47 bp differences, respectively) (data not shown). These values were below the intra-species similarities value of 97% obtained from *rpoD* phylogeny in the genus *Aeromonas* (Soler *et al.*, 2004; Alperi *et al.*, 2008). Alignment of the *rpoD* sequences of strain 717^T revealed deletion of a single triplet at the same position as that described for *A. trota* CECT 4255^T (GenBank accession no. AY169344) (Soler *et al.*, 2004).

The MLPA tree showed strain 717^T within the cluster of *A. veronii*, *A. allosaccharophila* and *A. sobria*, representing an independent branch (Supplementary Fig. S3). In contrast to that observed with the other genes (16S rRNA and *rpoD*), the MLPA tree revealed strain 717^T to be more distant from *A. veronii* and *A. allosaccharophila*, with *A. sobria* being the phylogenetically closest neighbour.

DNA–DNA hybridization studies were performed between strain 717^T and the type strains of the phylogenetically closest species, *A. sobria*, *A. allosaccharophila*, *A. veronii* and *A. jandaei*, as well as with the unrelated species *A. molluscorum*. DNA was extracted using the method described by Marmur (1961) and DNA–DNA hybridization was conducted using the methods described by Ziemke *et al.* (1998) and Urdiain *et al.* (2008). Renaturalization was performed under optimal conditions at 70 °C, single- and double-stranded DNA molecules were separated by the use of hydroxyapatite and colour development was measured at 405 nm using a Bio Whittaker Kinetic-QCL microplate reader. Values of DNA–DNA reassociation were determined at least three

Fig. 1. Unrooted phylogenetic tree derived from 16S rRNA gene sequences showing relationships of strain 717^T to all other described species of the genus *Aeromonas*. The tree was reconstructed using the neighbour-joining method. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates). Bar, 0.002 substitutions per nucleotide position. *Sequence identical to respective type strains.

times for both direct and reciprocal reactions (e.g. $A \times B$ and $B \times A$) for any given strain pair.

Mean DNA–DNA hybridization levels between strain 717^T and the type strains *A. sobria* CECT 4245^T, *A. allosaccharophila* CECT 4199^T, *A. veronii* CECT 4257^T and *A. jandaei* CECT 4228^T were 54 %, 61 %, 66 % and 40 %, respectively (Supplementary Table S2). All were below the 70 % limit for species delineation (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), but some were above 60 % in concordance with other species of the genus *Aeromonas* (Huys *et al.*, 1997; Nhung *et al.*, 2007). Despite DNA–DNA reassociation being considered to give information on the DNA similarity between entire bacterial genomes, it has been criticized because of the high number of experimental errors, lack of reproducibility and failure to generate collective databases (Rosselló-Mora, 2006). Moreover, DNA–DNA reassociation values do not provide any information concerning phylogenetic relationships (Harayama & Kasai, 2006), in contrast to the phylogenetic reconstruction with the MLPA performed previously for members of the genus *Aeromonas* (unpublished results) and applied in the present study. The polyphasic approach using 16S rRNA gene sequences, MLPA, DNA–DNA reassociation results and phenotypic characterization all clearly differentiated strain 717^T from the remaining species of the genus *Aeromonas*.

Description of *Aeromonas fluvialis* sp. nov.

Aeromonas fluvialis (flu.vi.a'lis. L. fem. adj. *fluvialis* belonging to a river).

Cells are straight, Gram-stain-negative, non-spore-forming rods, non-encapsulated, mobile by single polar flagella, 0.6–0.7 µm wide and 3–3.5 µm long, oxidase- and catalase-positive, reduce nitrate to nitrite and are resistant to vibriostatic agent O/129. Colonies on TSA are 4–5 mm in diameter, opaque, circular and beige in colour after 48 h at 30 °C. No brown diffusible pigment is produced on TSA at room temperature or 30 °C. Optimal growth occurs at 30 °C after 24 h in TSB. Grows at 37 °C but not at 4 °C or 40 °C on TSA. No haemolysis observed on sheep blood agar at 30 °C. Grows on MacConkey agar and on TSB without NaCl but not with 6 % NaCl. Optimal growth at pH 9 after 24 h on TSB but no growth at pH 4.5. Produces indole from tryptophan and gas from glucose, positive for β-galactosidase activity and able to use citrate. Negative for production of hydrogen sulphide from cysteine, VP test and hydrolysis of aesculin, gelatin and elastin. No DNase, urease or L-tryptophan deaminase activity. Does not produce ADH, LDC or ODC. Able to use D-mannose, D-glucose, N-acetylglucosamine, maltose, potassium gluconate and malic acid as sole carbon and energy sources, but not L-arabinose, D-mannitol, capric acid, adipic acid, phenylacetic acid or trisodium citrate. Acid is produced from glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, lactose, N-acetylglucosamine, salicin, cellobiose, maltose, sucrose, trehalose, starch, glycogen, gentio-

biose and potassium gluconate, but not from erythritol, L- or D-arabinose, L- or D-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, aesculin, melibiose, inulin, melezitose, raffinose, xylitol, turanose, D-lyxose, D-tagatose, L- or D-fucose, L- or D-arabitol, potassium 2-ketogluconate or potassium 5-ketogluconate. Resistant to amoxycillin, amoxycillin plus clavulanic acid and ticarcillin, intermediately resistant to ticarcillin plus clavulanic acid and susceptible to the rest of the antimicrobials tested. The API20E and API20NE profiles obtained for strain 717^T were 1240024 and 7062744 respectively.

The type strain is 717^T (=CECT 7401^T =LMG 24681^T), isolated from water of the Muga river, Girona, north-east Spain.

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