Arcobacter defluvii sp. nov., isolated from sewage samples

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A study employing a polyphasic taxonomic approach was undertaken to clarify the position of 12 isolates recovered from sewage samples. These isolates were recognized as a potential novel species because a new and specific pattern was produced with the 16S rRNA-RFLP *Arcobacter* identification method. The sequences of the 16S rRNA gene not only supported the classification of these novel strains as members of the genus *Arcobacter*, but also showed that they formed a separate phylogenetic line. Strain SW28-11[T], chosen as the representative of these strains, showed 16S rRNA gene sequence similarity of 95.6 % with the closest related species *Arcobacter nitrofigilis*. The phylogenetic position of the novel strains was further confirmed by analysis of the housekeeping genes *hsp60*, *rpoB* and, for the first time, *gyrB*. The latter proved to be an excellent additional gene for establishing the phylogeny of this genus. These data, together with phenotypic characterization, revealed that this group of isolates represent a novel species of the genus *Arcobacter*. The name *Arcobacter defluvii* sp. nov., is proposed, with the type strain SW28-11[T] (=CECT 7697[T]=LMG 25694[T]).

The genus *Arcobacter* is an unusual taxon within the class *Epsilonproteobacteria* in that it contains both pathogenic and free-living species that are found in a wide range of environments (Debruyne *et al.*, 2008). At the time of writing, this genus comprises ten recognized species: *Arcobacter butzleri*, *Arcobacter cryaerophilus* (with two subgroups 1A and 1B), *Arcobacter skirrowii*, *Arcobacter nitrofigilis* (Vandamme *et al.*, 1992), *Arcobacter cibarius* (Houf *et al.*, 2005), *Arcobacter halophilus* (Donachie *et al.*, 2005), and the recently described species *Arcobacter mytili* (Collado *et al.*, 2009a), *Arcobacter thereius* (Houf *et al.*, 2009), *Arcobacter marinus* (Kim *et al.*, 2010) and *Arcobacter trophiarum* (De Smet *et al.*, 2011). Another novel species, *A. molluscorum* has been described recently (Figueras *et al.*, 2011). A *Candidatus* taxon, 'Candidatus Arcobacter sulfidicus' has also been described (Wirsen *et al.*, 2002; Debruyne *et al.*, 2008).

The species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are associated with gastrointestinal disease and bacteraemia in humans and with reproduction disorders, mastitis and gastric ulcers in farm animals (Ho *et al.*, 2006). The latter two species can also be recovered from the faeces of healthy livestock (van Driessche *et al.*, 2003; Vandamme *et al.*, 2005). *A. thereius* was isolated from samples from porcine abortions, but the role of this species in pathogenicity is still unknown (Houf *et al.*, 2009). The other species of the genus have not so far been directly related to animal or human diseases (Ho *et al.*, 2006).

In relation to transmission routes, it has been demonstrated that *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are very prevalent in meat products and shellfish (Collado *et al.*, 2009b) and in faecally polluted environmental waters, sewage and sludge (Collado *et al.*, 2008). A high genetic diversity of these species has been found in river water with different degrees of sewage pollution (Collado *et al.*, 2010) and in wastewater samples (González *et al.*, 2010).

In a survey carried out in Catalonia (north-east Spain), a group of isolates recovered from sewage samples was studied using a polyphasic taxonomic approach, including genotyping by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), phylogenetic analysis of the 16S rRNA, *hsp60*, *rpoB* and *gyrB* gene sequences and phenotypic characterization. Based on the results obtained, it is proposed that this group of isolates represent a novel species of the genus *Arcobacter*.

Twelve isolates were recovered from the wastewater treatment plant (WWTP) in the town of Reus (north-east Spain) in April 2009. Sewage samples were taken from the inflow to the treatment plant (IW) and during treatment at the primary sedimentation tanks (PS1), the secondary biological treatment (SBT), the secondary sedimentation tanks (SS2) and from the outflow of treated water (with secondary treatment) from the WWTP. The isolation methodology was the same as previously described by Collado et al. (2008), although this time the samples were analysed in parallel by both direct plating and after enrichment. The origin of these isolates and their specific isolation after direct plating or enrichment are detailed in Supplementary Table S1 (available in IJSEM Online). The 12 new isolates were recovered from all points except from the outflow treated water of the WWTP and showed the colony morphology typical of members of the genus Arcobacter on sheep blood agar, i.e. small, translucent colourless or beige to off-white. All isolates appeared as Gram-negative, slightly curved rods under examination with a light microscope. These isolates were further recognized as belonging to the genus Arcobacter by their capacity to grow under aerobic conditions and at 30 °C, their inability to use carbohydrates and their production of oxidase. Additionally, this group of isolates was suspected to belong to a potential novel species because they showed a new and common restriction pattern that was different from those seen for the other species of the genus according to the 16S rRNA-RFLP Arcobacter identification method (Figueras et al., 2008) (see Supplementary Fig. S1).

To avoid studying duplicates of the same strain, all isolates were genotyped by ERIC-PCR using primers and conditions as described previously (Houf et al., 2002). DNA patterns that differed by one or more DNA fragments were considered to be different genotypes (Houf et al., 2002; Collado et al., 2010). Gel images were saved as TIFF files, normalized with the GeneRuler 100nt Plus Ladder (Fermentas) and further analysed by Bionumerics software, version 6.1 (Applied Maths). A dendrogram was constructed using the Jaccard similarity coefficient with a band-matching tolerance of 2 % and the cluster analysis of similarity matrices was calculated with the unweighted pair group method with arithmetic mean (UPGMA). This typing method generated different patterns for the eight new isolates (see Supplementary Fig. S2 in IJSEM Online). Three coincident genotypes were indistinctly found in isolates recovered from different sampling points (IW, PS1, SBT and SS2) of the WWTP. Strains belonging to the same genotype, strains SW28-9 and SW29-4 or strains SW28-10, SW29-3 and SW30-1, were recovered using the two isolation procedures described (see Supplementary Table S1). A molecular and phenotypic characterization was performed following the proposals for the minimal standards for describing novel species of the family Campylobacteraceae (Ursing et al., 1994).

In order to establish the taxonomic position of the new isolates, the 16S rRNA and rpoB genes were sequenced for strains SW28-7, SW28-11^T and SW30-2 (chosen as representatives of the group) as described by Collado et al. (2009a). For sequencing the hsp60 gene, the conditions were as described by Debruyne et al. (2010). In the present study, the taxonomic value of the gyrB gene (encoding the B subunit of DNA gyrase) was also assessed as a complementary phylogenetic and identification marker for the genus Arcobacter. For the primer design of the gyrB gene, a preliminary sequence (1100 bp) for all type strains of the genus Arcobacter was obtained with the degenerate primers UP1 and UP2r as described by Yamamoto & Harayama (1995). On the basis of these sequences, the primers gyrB-Arc-7F (5′-GTTTAYCAYTT-TGAAGGTGG-3′) and gyrB-Arc-14R (5′-CTAGATTTT-TCAACATTTAAAAT-3′) were designed, which enabled the amplification of a 722 bp fragment from the gyrB gene of species of the genus Arcobacter. These primers were also used for sequencing. Both the design of the primers and the sequencing were performed by the Molecular Diagnostics Center, Orihuela, Spain. The specific PCR amplifications for the gyrB gene were conducted after extracting the DNA from a single colony with the InstaGene Matrix (Bio-Rad Laboratories) following the manufacturer's instructions in a 50 µl reaction mix containing 1 µl genomic DNA, 0.2 µM of each dNTP, 0.2 µM of each primer, 2 mM MgCl$_2$, 1 U Taq DNA polymerase (Invitrogen) and the buffer supplied with the enzyme. PCR conditions applied were 3 min at 94 °C followed by 35 cycles of 15 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C, followed by 5 min at 72 °C. The PCR product was sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. Phylogenetic analyses were performed after multiple alignments of sequences by CLUSTAL W (Thompson et al., 1994), using MEGA software version 4 (Tamura et al., 2007), with Kimura's two-parameter model (Kimura, 1980) and clustering with the neighbour-joining method (Saitou & Nei, 1987). The percentage nucleotide substitutions for a continuous stretch of 665 bp, corresponding to approximately 29 % of the coding region (672–1342 bp according to Escherichia coli numbering) was calculated from the gyrB gene sequences obtained. The sequences of all genes (16S rRNA, rpoB, hsp60 and gyrB) of two representative strains for each species, either determined in previous studies or sequenced in the present study, were included in the phylogenetic analysis. For A. trophiarum, apart from the type and a reference strain (LMG 25534^T and LMG 25535), an additional identified isolate, FE2 (=CECT 7650), was evaluated on the basis of its 99.8 % similarity for the 16S rRNA gene (M. J. Figueras, L. Collado, A. Levican & H. Fernández, unpublished results).

The 16S rRNA gene sequences obtained for the new strains (SW28-7, SW28-11^T and SW30-2) were compared with public databases using the EzTaxon tool (Chun et al., 2007). The 16S rRNA gene sequence of strain SW28-11^T

showed similarity values of between 93.8 and 95.6 % with the sequences of the type strains of all species, including *A. marinus* (Kim *et al.*, 2010), *A. trophiarum* (De Smet *et al.*, 2011) and *A. molluscorum* (Figueras *et al.*, 2011). The highest value (95.6 %) was obtained with the type strain of *A. nitrofigilis*, which is the type species of the genus. The 16S rRNA gene similarity between strain SW28-11^T and the other strains (SW28-7 and SW30-2) was >99.7 %, confirming that they belonged to the same species. The phylogenetic tree constructed using 16S rRNA gene sequences (Fig. 1) showed these sewage strains as an independent phylogenetic line within the genus *Arcobacter* clustering with *A. nitrofigilis* with a low bootstrap value of 53 % (data not shown).

The tree constructed using the *gyrB* gene sequence data was useful in delineating all recognized species of the genus *Arcobacter* and the new strains as independent phylogenetic lines (Supplementary Fig. S3), in agreement with the phylogenies derived from the 16S rRNA (Fig. 1), *hsp60* (Supplementary Fig. S4) and *rpoB* genes (Supplementary Fig. S5). Furthermore the inter-species rate of nucleotide substitutions for the *gyrB* gene was over 9.5 %, while the intra-species variation (on the basis of two strains of each species investigated) ranged from 0.2 to 8.6 % and was comparable to the values previously described for the *rpoB* gene for members of the genus *Arcobacter* (Collado *et al.*, 2009a). This indicated that the *gyrB* is an excellent additional gene for inferring the phylogeny of members of the genus *Arcobacter*. The phylogenetic trees constructed with the concatenated sequences of the *hsp60*, *rpoB* and *gyrB* genes also revealed that the novel strains belonged to a new phylogenetic line within this genus, reinforcing their phylogenetic position close to the group of environmental members of the genus *Arcobacter* that includes *A. nitrofigilis* (Fig. 2).

DNA–DNA hybridization (DDH) studies were performed only between strain SW28-11^T and SW31-1 as these strains showed 16S rRNA gene sequence similarities of <97 % with other species of the genus *Arcobacter* and therefore the overall genomic relatedness was considered to be less than 70 % (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006). DNA was extracted using the method described by Marmur (1961) and DDH was conducted according to the method described by Urdiain *et al.* (2008). Renaturation was performed under optimal conditions at 57 °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. DDH values were determined three times for both direct and reciprocal reactions. Mean DDH relatedness % ($\pm$SD) between strains SW28-11^T and SW31-1 was 80.4 % ($\pm$5.48), confirming that these strains belonged to the same species.

The procedures for phenotypic characterization included the recommended media and methods as described previously (Ursing *et al.*, 1994; On *et al.*, 1996; Vandamme *et al.*, 2005). All tests were conducted at least twice with positive and negative controls for the eight new strains and for the type strains of all species of the genus *Arcobacter*. However, as previously mentioned, an additional strain, FE2, of *A. trophiarum* (M. J. Figueras, L. Collado, A. Levican & H. Fernández, unpublished results) was also tested. Motility was observed from young cultures in broth by examining wet mounts by phase-contrast microscopy. Cell size, morphology and presence of flagella were determined by electron microscopy following the procedures described by Collado *et al.* (2009a). The
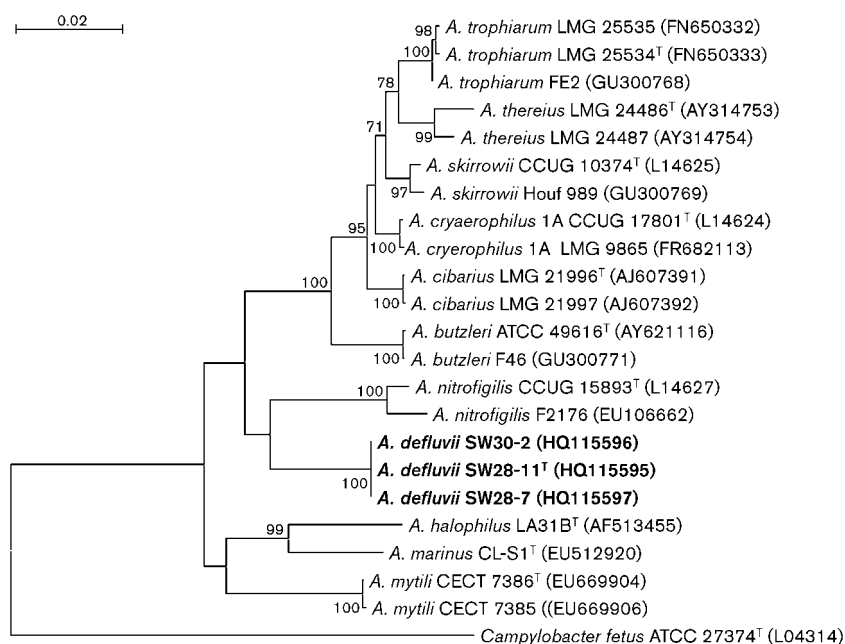
Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of *Arcobacter defluvii* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 bp.
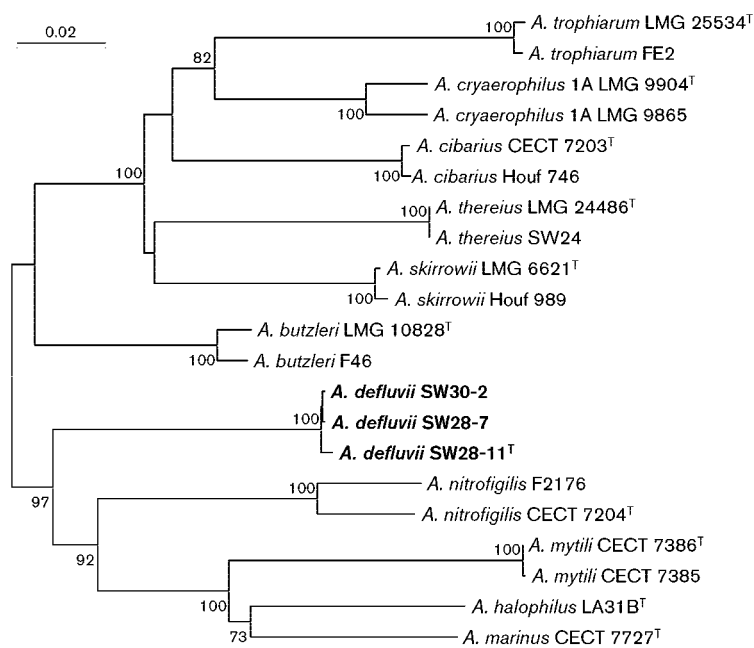
Fig. 2. Neighbour-joining tree based on the concatenated *hsp60*, *rpoB* and *gyrB* gene sequences showing the phylogenetic position of *Arcobacter defluvii* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 bp.

isolates showed a typical cell size and morphology for members of the genus *Arcobacter*, as well as the presence of a single polar flagellum (Supplementary Fig. S6). Phenotypically, the new strains (*n*=8) could be differentiated from all other species of the genus *Arcobacter* by several specific tests as shown in Table 1. The easiest key diagnostic test was the ability to produce urease, which was characteristic only for the new strains and for *A. nitrofigilis*. The novel strains could be distinguished from *A. nitrofigilis* by their ability to grow in MacConkey agar and minimal medium and inability to grow in media containing 4 % (w/v) NaCl. Phenotypic differentiation from *A. butzleri*, the most commonly isolated species, was possible by the novel strains' ability to produce urease and inability to grow in nutrient agar medium containing 0.04 % 2,3,5 triphenyl tetrazolium chloride (TTC).

On the basis of the phylogenetic relationships and phenotypic characteristics, this study has revealed the existence of a novel species of the genus *Arcobacter*, for which the name *Arcobacter defluvii* sp. nov. is proposed. The species-specific RFLP pattern (407/243/ and a single band of 141–138 bp) observed for these new strains (Supplementary Fig. S1), obtained after the digestion of 1026 bp of the 16S rRNA gene with the *Mse*I enzyme (Figueras *et al.*, 2008), was identical to the pattern obtained after a computational restriction (*Mse*I) simulation of the 16S rRNA gene sequences. Therefore, the 16S rRNA-RFLP identification method (Figueras *et al.*, 2008), was proved to be a reliable and fast technique for the characterization of this novel species, and has been established as a useful method to differentiate all species of the genus *Arcobacter*, with the exception of *A. butzleri* from *A. thereius* (Collado *et al.*, 2009b). The novel strains described in this paper produce an amplicon of almost the same size (230 bp) as

that produced by *A. cryaerophilus* (257 bp) with the multiplex-PCR (m-PCR) of Houf *et al.* (2000) (Supplementary Fig. S7). After sequencing, this amplicon corresponded to the region of the 16S rRNA gene between nucleotides 1130 and 1357 (data not shown). With the m-PCR proposed recently by Douidah *et al.* (2010), the new strains showed an amplicon of the same size expected for *A. butzleri* (Supplementary Fig. S8). Consequently, these methods are not useful since they could identify the novel species as members of existing species and this may hamper efforts to establish the true prevalence of this novel species.

In a previous study, all sewage samples from the same WWTP were positive for the presence of members of the genus *Arcobacter* and showed a great diversity of species (Collado *et al.*, 2008). Apart from the demonstrated association of members of the genus *Arcobacter* with faecal pollution in that study, it is clear that sewage may be an important reservoir for species of this genus. In a recent study investigating the population structure of micro-organisms in the inflow sewage water to WWTPs in Milwaukee (USA) using pyrosequencing of hypervariable regions of the 16S rRNA genes, a great number of sequences of members of the genus *Arcobacter* were found, contrary to the few detected in surface waters (McLellan *et al.*, 2010). These authors indicated that further work is needed to determine if these micro-organisms are residents growing in sewer systems. It remains to be confirmed whether *Arcobacter defluvii* sp. nov. could have a human origin. This is highly probable, considering that the WWTP from which the strains were isolated treated mainly human sewage, although the alternative hypothesis should not be ignored.

A recent BLASTN search showed that the 16S rRNA gene sequence of the type strain (SW28-11^T) of *A. defluvii*

Table 1. Differential characteristics of *Arcobacter defluvii* sp. nov., and other members of the genus *Arcobacter*

Taxa: 1, *A. defluvii* sp. nov. ($n=8$, data from this study); 2, *A. nitrofigilis* ($n=4$) (On *et al.*, 1996; Collado *et al.*, 2009a); 3, *A. cryaerophilus* ($n=19$) (On *et al.*, 1996); 4, *A. butzleri* ($n=12$) (On *et al.*, 1996); 5, *A. skirrowii* ($n=9$) (On *et al.*, 1996); 6, *A. cibarius* ($n=15$) (Houf *et al.*, 2005); 7, *A. halophilus* LA31B[T] (Donachie *et al.*, 2005); 8, *A. mytili* ($n=3$) (Collado *et al.*, 2009a); 9, *A. thereius* ($n=8$) (Houf *et al.*, 2009); 10, *A. marinus* CL-S1[T] (Kim *et al.*, 2010); 11, *A. trophiarum* ($n=11$) (De Smet *et al.*, 2011); 12, *A. molluscorum* ($n=3$) (Figueras *et al.*, 2011). Unless otherwise indicated: +, $\geqslant95\%$ strains positive; −, $\leqslant11\%$ strains positive; v, 12–94 % strains positive; ND, not determined; CO_2 indicates microaerobic conditions.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Growth at/in												
Air at 37 °C	+	v	v	+	+	−	+	+	−	+	−	+
CO_2 at 37 °C	+	−	v	+	+	+	+	+	−	+	−	+
4 % (w/v) NaCl	−	+	−	−	+	−	+	+	−	+	+*	+
Growth on:												
1 % (w/v) glycine	−	−	−	−	−	−	+†	+	+	+†	v‡	−
MacConkey	+	−	v	+	−	+	−	+	v	−†	v*	+
Minimal media	+	−	−§	+	−	+	−	−	+	−	−‡	−
0.04 % TTC	−	−	+	+	v	v	−	−	v	ND	ND	−
Resistance to:												
Cefoperazone (64 mg l^{-1})	v	−	+	+	+	+	−	−	+	−†	+	+
Enzyme activity												
Catalase	+‖	+	+	v	+	v	−	+	+	−	+	+
Urease	+	+	−	−	−	−	−	−	−	−	−	−
Nitrate reduction	+	+	+¶	+	+	−	+	−	+	+	−	+#
Indoxyl acetate hydrolysis	+	+	+	+	+	+	−	+	+	+	−	−

*Strain FE2 (=CECT 7650) of this species was unable to grow in media with 4 % NaCl, but grew in MacConkey agar.

†Data from (Figueras *et al.*, 2011), all tested in media supplemented with 2 % NaCl.

‡Test not evaluated by De Smet *et al.* (2011) and derived from our study (Figueras *et al.*, 2011).

§Two strains (LMG 7537 and LMG 10241) of four strains tested were positive (Collado *et al.*, 2009a).

‖Weak reaction.

¶Two strains (LMG 9904[T] and LMG 9065) of four strains tested were negative (Collado *et al.*, 2009a).

#Nitrate is reduced after 72 h and 5 days for all strains under microaerobic and aerobic conditions, respectively.

sp. nov. showed 99 % similarity with the sequence of a strain named *Arcobacter*-L (GenBank accession no. FJ968635). Strain SW28-11[T] and *Arcobacter*-L also clustered together in a phylogenetic tree (data not shown). Strain *Arcobacter*-L was isolated as a part of a microbial fuel cell community that generated electricity (Fedorovich *et al.*, 2009). It remains to be investigated whether strains of *A. defluvii* sp. nov. could have a similar biotechnological potential.

Description of *Arcobacter defluvii* sp. nov.

Arcobacter defluvii (de.flu′vi.i. L. neut. n. *defluvium* sewage; L. gen. n. *defluvii* of sewage).

Cells are Gram-negative, slightly curved rods, non-encapsulated, non-spore-forming and 0.3–0.5 μm wide and 1–2 μm long. Motile by a single polar flagellum. Colonies on blood agar incubated in aerobic conditions at 30 °C for 48 h are 2–4 mm in diameter, beige to off-white, circular with entire margins, convex and non-swarming. Pigments are not produced. Grows on blood agar at room temperature (18–22 °C), 30 °C and 37 °C under aerobic or microaerobic culture conditions with no significant differences. Weak growth is obtained both in anaerobic

conditions at 30 °C and in aerobic conditions at 42 °C. No haemolysis is observed on TSA supplemented with 5 % sheep blood. Produces oxidase and shows a weak catalase activity. Hydrolyses indoxyl acetate. Does not hydrolyse casein, lecithin or starch. Urea is hydrolysed and nitrate is reduced. Hydrogen sulphide is not produced in triple-sugar–iron agar. Under aerobic conditions, grows at 30 °C on MacConkey agar, on minimal media, on *Campylobacter* charcoal deoxycholate agar and on media containing: 2 % NaCl, 0.1 % sodium deoxycholate, 1 % oxgall, 0.05 % safranin, 0.005 % crystal violet, 0.005 % basic fuchsin and 0.001 % brilliant green. Three of the eight strains (37.5 %), including the type strain, grow on media containing 64 mg cefoperazone l^{-1}. Growth is not obtained on media containing 1 % glycine, 4 % (w/v) NaCl or 0.04 % TTC.

The type strain, SW28-11[T] (=CECT 7697[T]=LMG 25694[T]), was isolated from raw sewage from the wastewater treatment plant in the town of Reus, Spain.

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