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Short communication

Arcobacter bivalviorum sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish☆

Arturo Levican[a], Luis Collado[a,b], Carmen Aguilar[c], Clara Yustes[c], Ana L. Diéguez[d], Jesús L. Romalde[d], Maria José Figueras[a,*]

[a] *Unitat de Microbiologia, Departament de Ciències Mediques Bàsiques, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain*
[b] *Institute of Microbiology, Faculty of Sciences, Universidad Austral de Chile, Valdivia, Chile*
[c] *Agencia de Protecció de la Salut, Laboratori de Salut Pública de Tarragona, Spain*
[d] *Departamento de Microbiología y Parasitología, Centro de Investigaciones Biológicas (CIBUS)-Facultad de Biología, Universidad de Santiago de Compostela, Spain*

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ABSTRACT

A group of ten *Arcobacter* isolates (Gram negative, slightly curved motile rods, oxidase positive) was recovered from mussels (nine) and from clams (one). These isolates could not be assigned to any known species using the molecular identification methods specific for this genus (16S rDNA-RFLP and m-PCR). The aim of this study is to establish the taxonomic position of these isolates. The 16S rRNA gene sequence similarity of mussel strain F4^T to the type strains of all other *Arcobacter* species ranged from 91.1% to 94.8%. The species most similar to the clams' strain F67-11^T were *Arcobacter defluvii* (CECT 7697^T, 97.1%) and *Arcobacter ellisii* (CECT 7837^T, 97.0%). On the basis of phylogenetic analyses with 16S rRNA, *rpoB*, *gyrB* and *hsp60* genes, the mussel and clam strains formed two different, new lineages within the genus *Arcobacter*. These data, together with their different phenotypic characteristics and MALDI-TOF mass spectra, revealed that these strains represent two new species, for which the names *Arcobacter bivalviorum* (type strain F4^T = CECT 7835^T = LMG 26154^T) and *Arcobacter venerupis* (type strain F67-11^T = CECT 7836^T = LMG 26156^T) are proposed.

The genus *Arcobacter* belongs to the family *Campylobacteraceae* together with the genera *Campylobacter* and *Sulfurospirillum* [3,30]. It embraces species that have been isolated from a wide diversity of habitats and hosts [3,30–32]. Vandamme et al. created the genus in 1991 [31] and expanded it in 1992 [32] to 4 species i.e. *Arcobacter nitrofigilis* (the type species of the genus) recovered for the first time from roots of *Spartina alterniflora* [23], *Arcobacter cryaerophilus* from bovine abortion foetuses [24], *Arcobacter butzleri* from human faeces [19] and *Arcobacter skirrowii* from sheep faeces [32]. Since then, it has rapidly expanded and currently includes 9 additional species, i.e. *Arcobacter cibarius* recovered from chicken meat [16], *Arcobacter halophilus* from a hypersaline lagoon [7], *Arcobacter mytili*, *Arcobacter molluscorum* and *Arcobacter ellisii* from mussels [2,11,12], *Arcobacter thereius* from porcine abortions [17], *Arcobacter marinus* from a mix of seawater, seaweed and a starfish [20], *Arcobacter trophiarum* from faeces of fattening pigs [6] and *Arcobacter defluvii* from sewage [5]. Similarity of the 16S rRNA gene within the type strains of all the species of the genus ranges from 92.0% to 99.1% [3,12]. Phylogenetic analyses based on housekeeping genes such as *gyrB*, *rpoB*, and *hsp60* have been useful for delineating closely related species and have been used in the description of the latest species, showing a good agreement with the DNA–DNA hybridization results [2,5,6,9,11,12]. The existence of several other potentially new species from diverse environments can be inferred from the16S rRNA gene sequences deposited in public databases [3,9].

The importance of the genus *Arcobacter* lies in the fact that some species are considered emerging enteropathogens and potential zoonotic agents [3,30]. The frequent isolation of species of the genus from foods of animal origin and from water suggests that these are the transmission routes of these bacteria [3,4,14]. Regarding this, in a recent study in seafood, which is often eaten uncooked, 100% of the clams and 41% of the mussel samples were positive for *Arcobacter* spp. [4]. In that study, the isolates were identified using two molecular identification methods for *Arcobacter* spp. in parallel, a multiplex PCR (m-PCR) [18] and a 16S rDNA restriction fragment length polymorphism (16S rDNA-RFLP) [10], although one strain

☆ The GenBank/EMBL/DDBJ accession numbers of the sequences of strain F4^T, F67-11^T, for the 16S rRNA gene are FJ573217 and HE565359, and for the *rpoB*, *hsp60* and *gyrB* genes are HE565362–HE565364 and HE565374–HE565376, respectively. The sequences for 16S rRNA, *rpoB*, *hsp60* and *gyrB* genes for the strains F118-2, F118-3 and F118-4 had also been deposited (HE565357, HE565358, HE565365–HE565373 and HE575529).

* Corresponding author at: Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus, Spain. Tel.: +34 977759321; fax: +34 977759322.
E-mail address: mariajose.figueras@urv.cat (M.J. Figueras).

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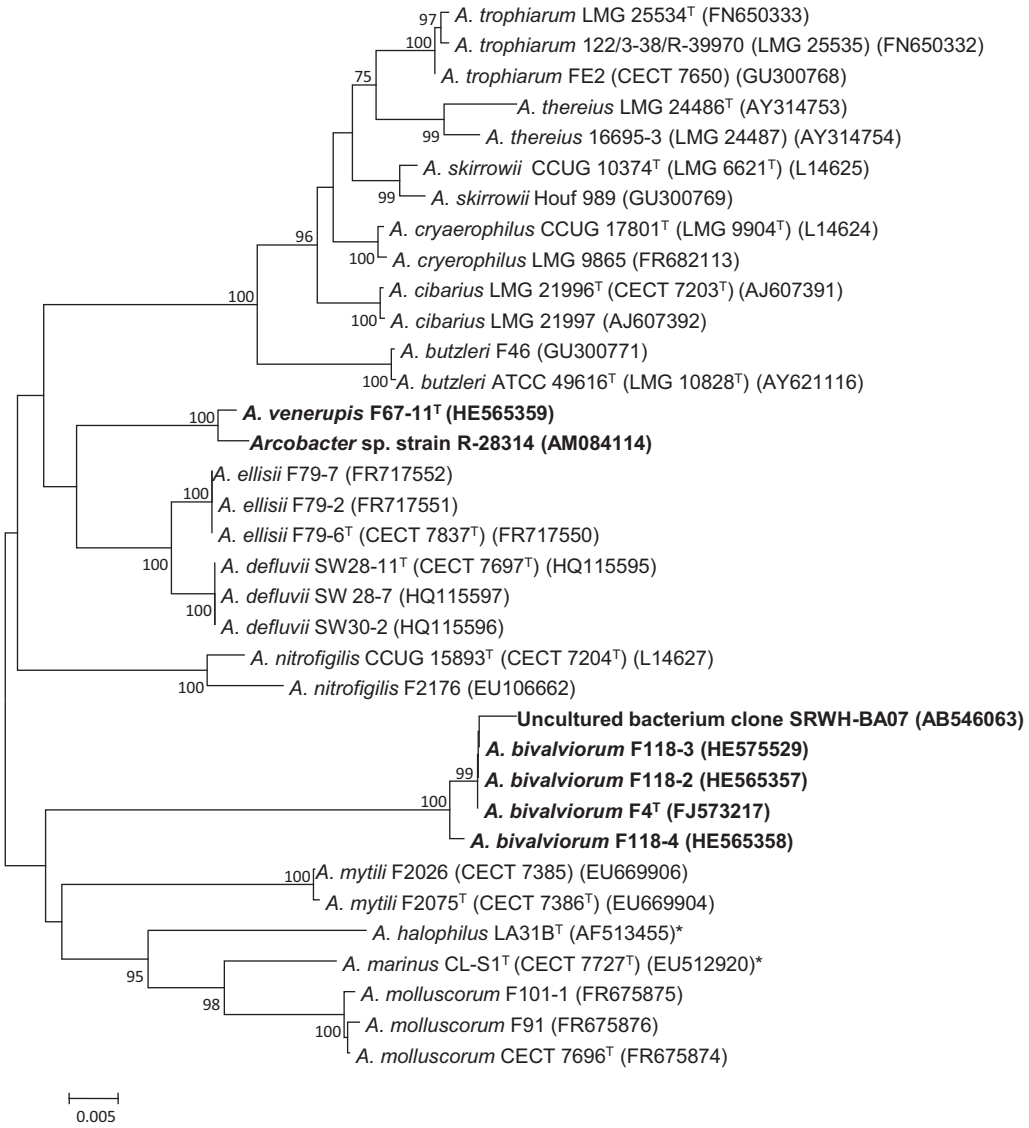

Fig. 1. Neighbour-joining tree based on 16S rRNA sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 nt. *Only type strain is available so far.

recovered from mussels (F4) could not be assigned to any known species [4]. This strain F4 appeared to be a potentially new phylogenetic line on the basis of its 16S rRNA gene sequence [4] but it remained undescribed while waiting for the isolation of other similar strains. In a more recent shellfish survey, eight *Arcobacter* isolates from mussels that were identified using the mentioned methods (m-PCR and 16S rDNA-RFLP) appeared to resemble strain F4. Furthermore, a strain recovered from a sample of clams could not be properly identified, either. The objective of the present study was to study the taxonomic position of strain F4 and the other nine isolates from mussels and clams.

Eight *Arcobacter* isolates from mussels (F118-2, F118-3, F118-4, F118-5, F118-6, F118-7, F118-8 and F118-9) were recovered from a sample collected from the Ebro delta, Catalonia (northeast Spain) in September 2010. Strain F4 was isolated from mussels in the same area in March 2007 [4], while strain F67-11^T was recovered from a sample of clams collected in January 2009 from Ferrol, Galicia (northwest Spain). All *Arcobacter* isolates were identified as such on the basis of their colony morphology on blood agar (small, translucent, beige to off-white) and their phenotypic characteristics (Gram negative, slightly curved, motile rods that produce oxidase activity) [4,5,11,12].

Considering that the 8 mussel isolates were recovered from the same sample and that isolate F4 also came from the same locality, all of them together with the clam isolate (F67-11^T) were genotyped using the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), as described previously [15]. The 8 mussel isolates showed only 3 distinctive ERIC patterns (for which isolates F118-2, F118-3 and F118-4 were chosen as the representatives) different from those shown by strain F4 (Fig. S1) and F67-11^T (data not shown).

Identification of the strains was initially attempted using two m-PCR [8,18] and the 16S rDNA-RFLP *Arcobacter* identification methods [10] in parallel. The mussel strains (F118-2, F118-3 and F118-4) behaved like strain F4 [4], i.e. they produced an amplicon of the expected size described for *A. cryaerophilus* with the m-PCR of Houf et al. [18] and the same new RFLP pattern of strain F4, which was different from the patterns shown by all other known *Arcobacter* spp. [2,5,10–12] (Figs. S2 and S3). In addition, strains F4^T (selected as the type), F118-2, F118-3 and F118-4 did not show any

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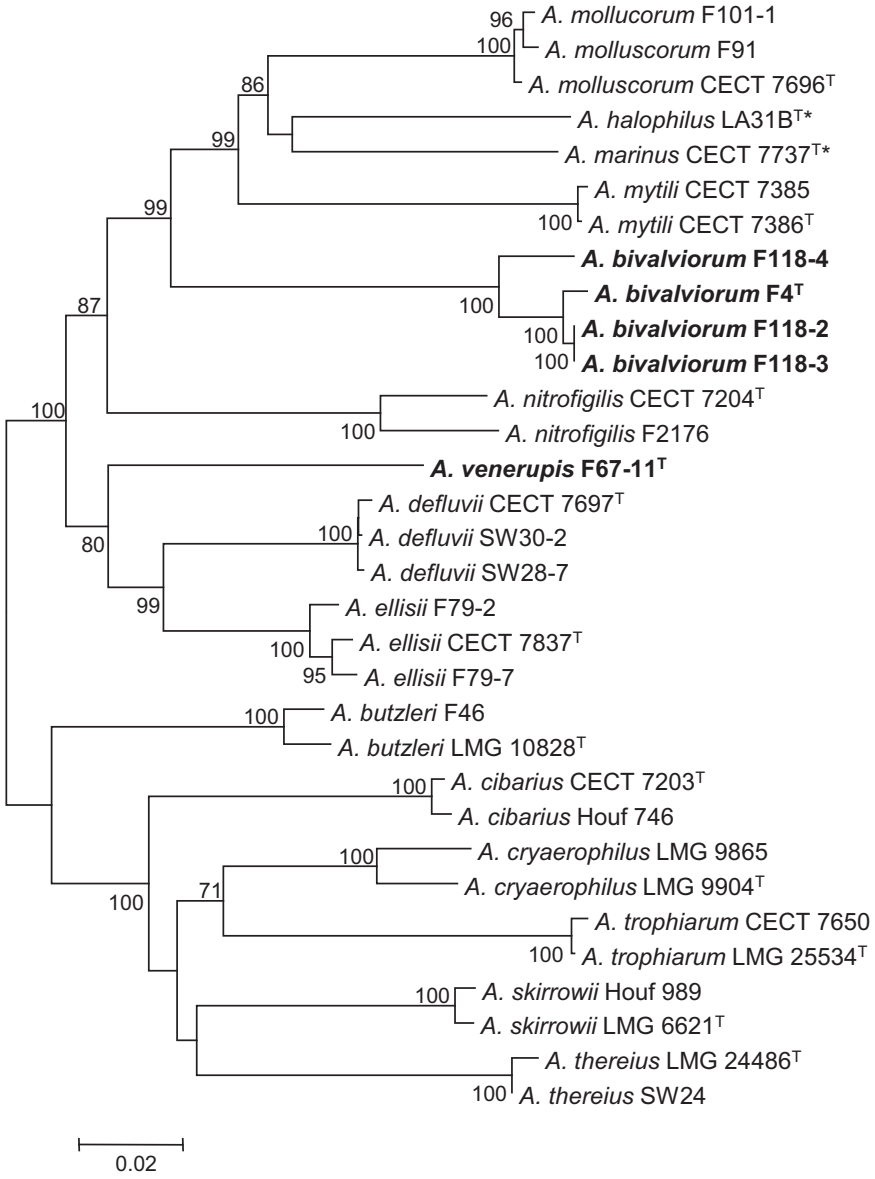

Fig. 2. Neighbour-joining tree based on the concatenated *hsp60, rpoB and gyrB* sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70%) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt. *Only type strain is available so far.

amplicon when tested with the new m-PCR designed to identify the most common *Arcobacter* species by Douidah et al. [8] (Fig. S2). The strain obtained from clams (F67-11^T) also showed different results depending on which identification method was used. With the m-PCR of Houf et al. [18] the strain showed an amplicon similar to the one described for *A. cryaerophilus* and one similar to that described for *A. butzleri* with the m-PCR of Douidah et al. [8] (Fig. S2). With the 16S rDNA-RFLP *Arcobacter* identification method [10], it showed a pattern (308, 243, 141, 138, 100, 52 nt) that could be confused with the one described for *A. marinus* [11] (308, 243, 138, 100, 52 nt) (Fig. S3).

The 16S rRNA, *rpoB*, *gyrB* and *hsp60* genes were amplified, sequenced and analysed as previously described [2,5,11]. The similarity of the 16S rRNA gene sequences was determined using EzTaxon software [1]. The 16S rRNA (1401 nt), *rpoB* (487 nt), *gyrB* (665 nt) and *hsp60* (555 nt) gene sequences were independently aligned using MEGA software version 5 [28] and CLUSTAL W [22]. Genetic distances were obtained using Kimura's two-parameter model [21] and clustered with the neighbour-joining [26], maximum parsimony and maximum likelihood methods using MEGA software version 5 [28]. The neighbour joining phylogenetic tree obtained with the 16S rRNA gene (Fig. 1), as well as those individually constructed with *rpoB*, *gyrB* and *hsp60* (Figs. S4–S6) or their concatenated sequences (*rpoB*, *gyrB* and *hsp60*, 1651 nt) (Fig. 2), showed that the group of mussel strains (F4^T, F118-2, F118-3 and F118-4) and the strain from clams (F67-11^T) belonged to two different, unknown phylogenetic lineages within the genus *Arcobacter*. The same results were obtained with the 16S rRNA gene and the concatenated sequences (*rpoB*, *gyrB* and *hsp60*) when other algorithms i.e. maximum parsimony and maximum likelihood were used (Figs. S7–S10).

The individual and concatenated trees of the *rpoB*, *gyrB* and *hsp60* genes showed that two of the mussel isolates F118-2 and F118-3 shared the same nucleotide sequences (Fig. 2 and Figs. S4–S6), despite showing different ERIC patterns (Fig. S1). In order to verify the latter result, the ERIC assay was repeated twice using different DNA extracts each time. The two assays showed different results because two different patterns were obtained on

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Table 1
Differential characteristics of *Arcobacter bivalviorum* and *Arcobacter venerupis* spp. nov. from other members of the genus. Taxa: 1, *Arcobacter bivalviorum* (*n* = 3); 2, *Arcobacter venerupis* (*n* = 1); 3, *A. nitrofigilis* (*n* = 4) [2,25]; 4, *A. cryaerophilus* (*n* = 19) [2,25]; 5, *A. butzleri* (*n* = 12) [25]; 6, *A. skirrowii* (*n* = 9) [25]; 7, *A. cibarius* (*n* = 15) [16]; 8, *A. halophilus* (*n* = 1) [7,12]; 9, *A. mytili* (*n* = 3) [2]; 10, *A. thereius* (*n* = 8) [17]; 11, *A. marinus* (*n* = 1) [12,20]; 12, *A. trophiarum* (*n* = 11) [6,12]; 13, *A. defluvii* (*n* = 8) [5]; 14, *A. molluscorum* (*n* = 3) [11]; 15, *A. ellisii* [12]. The specific responses for type strains were identical or expressed in brackets. Unless otherwise indicated: +, ≥ 95% strains positive; −, ≤11% strains positive; V, 12–94% strains positive; CO_2 indicates microaerobic conditions; CCDA: Campylobacter Charcoal Deoxycholate Agar; TTC: triphenyl tetrazolium chloride.

Characteristics	1	2	3	4	5	6	7	8[a]	9	10	11[a]	12	13	14	15
Growth in/on															
Air at 37 °C	+	−	V(−)	V(+)	+	+	−	+	+	−	+	−	+	+	+
CO_2 at 37 °C	+	+	−	V(+)	+	+	+	+	+	−	+	−	+	+	+
CO_2 at 42 °C	−	−	−	−	V(+)	−	−	−	+	−	−	−	+	+	+
0.5% (w/v) NaCl[b]	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+
4% (w/v) NaCl	+	−	+	−	−	+	−	+	+	−	+	−	−	+	−
1% (w/v) glycine	−	−	−	−	−	−	−	+	+	+	+	V(−)	−	−	−
MacConkey	−	+	−	V(−)	+	−	+	−	+	V(+)	−	V(+)[c]	+	+	V(+)
Minimal medium	−	+	−	−[d]	+	−	+	−	−	+	−	−[e]	+	−	+
0.05% safranin	−	−	−	+	+	+	+	−	−	+	+	V(+)	+	+	−
CCDA	−	+	−	+	+	+	V(−)	−	−	V(−)	−	+	+	−	+[f]
0.01% sodium deoxycholate	−	−	V(−)	V(+)	+	+	+	−	+	V(−)	−	+	+	+	+[f]
1% (w/v) oxgall	−	−	−	+	V(+)	+	+	−	+	−	−	+	+	+	−
0.04% TTC	−	−	−	+	+	V(−)	V(−)	−	−	V(−)	−	+	−	−	−
0.01% TTC	−	−	−	+	+	+	+	−	−	+	−	+	+	+	−
Resistance to															
Cefoperazone (64 mg l^{-1})	−	−	−	+	+	+	+	−	−	+	−	+	V(+)	+	−
Enzyme activity															
Catalase	+	+	+	+	V(+)	+	V(−)	−	+[g]	+	−	+	+[g]	+	+
Urease	−	+	+	−	−	−	−	−	−	−	−	−	+	−	V(−)
Nitrate reduction	−	+	+	+[h]	+	+	−	+	+[i]	+	+	−	+	+[j]	+
Indoxyl acetate hydrolysis	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+

[a] For these strains, the tests were carried out on media supplemented with 2% NaCl, with the exception of 0.5 and 4% (w/v) NaCl, catalase and indoxyl acetate hydrolysis [12].
[b] Growth on 0.5% (w/v) NaCl was carried out using nutrient medium supplemented with 5% sheep blood.
[c] Strains LMG 25534^T, LMG 25535 of *A. trophiarum* and strain FE2 (CECT 7650) of this species identified in our laboratory grew on MacConkey agar in contraposition with the 80% described for this species [6,12].
[d] Two (LMG 7537 and LMG 10241) of the four strains tested were positive [2].
[e] Test not evaluated by De Smet et al. [6] but tested by Figueras et al. [12].
[f] All strains grew weakly after 5 days of incubation [12].
[g] Weak reaction [2,5].
[h] Two (LMG 9904^T and LMG 9065) of the four strains tested were negative [2].
[i] Nitrate reduction was found to be positive for the 3 strains of *A. mytili* [12] in contradiction to our previously published data [2].
[j] Nitrate is reduced after 72 h and 5 days for all strains under microaerobic and aerobic conditions, respectively [12].

one occasion and the same pattern on the other (data not shown). On the basis of these new results and on the equal sequences obtained with *rpoB*, *gyrB* and *hsp60* genes, these two isolates were considered a single strain and F118-2 was used for further analyses. This is the first time that we have seen a changing ERIC PCR pattern, and this should be taken into account in future studies for strains that show very similar ERIC PCR patterns.

The 16S rRNA gene sequences of mussel strains F4^T, F118-2 and F118-4 (1401 nt) showed a similarity that ranged from 99.6% to 100%. Direct and reverse DDH experiments were carried out between two of these strains (F4^T and F118-2) as described previously [5] and the results (82.3 ± 11.4%) confirmed that they belonged to the same new species. The 16S rRNA gene sequence similarity of the 3 mussel strains (F4^T, F118-2 and F118-4) with the clam strain F67-11^T was 93.0%, while the similarity of strain F4^T with all *Arcobacter* species ranged from 91.1% with *A. cryaerophilus* (LMG 9904^T) to 94.8% with *A. defluvii* (CECT 7697^T). All these results were relatively low and far below the 97% threshold above which DNA–DNA hybridization (DDH) experiments with other known species should be carried out [9,27].

The 16S rRNA gene sequence similarities between the strain F67-11^T from clams and the type strains of all *Arcobacter* spp. ranged from 93.2% with *A. mytili* (CECT 7386^T) to 97.1% with *A. defluvii* (CECT 7697^T), followed by *A. ellisii* (CECT 7837^T) with 97.0% and *A. nitrofigilis* (CECT 7204^T) with 96.0%. The species *A. defluvii* (CECT 7697^T) and *A. ellisii* (CECT 7837^T) were selected for DDH experiments with strain F67-11^T because were also its closest neighbours in the phylogenetic trees (Figs. 1 and 2, Figs. S5, S6 and S8). The mean results obtained from direct and reverse DNA–DNA reassociation were 56.6% (±4.5) and 63.4% (±1.5), respectively, confirming that the strain F67-11^T from clams belongs to a new and different species. A BlastN analysis of the 16S rRNA sequence of strain F67-11^T, showed a 99.5% similarity with the deposited sequence of strain R-28314 (1464 nt; GenBank AM084114) from a denitrifying bacterium isolated from activated sludge at a waste water treatment plant in Ghent (Belgium) [13]. When this sequence was added to the 16S rRNA phylogenetic tree, it clustered with a 100% bootstrap with strain F67-11^T (Fig. 1). We have tried to obtain this strain in order to add it to our study, but unfortunately it was not alive anymore (de Vos, personal communication). A similar analysis was carried out with the representative of the mussel strains (F4^T) and it showed a 99.6% similarity with the sequence of an uncultured bacterium clone SRWH-BA07 (1459 nt, GenBank AB546063) from subsurface crude oil deposits in Japan. This sequence (AB546063) was included in the 16S rRNA gene phylogenetic tree and it clustered together with mussel strains (Fig. 1). These results confirm that these two new species can be found in other geographical regions and habitats.

Phenotypic characterization was carried out using the standardized test recommended for this genus in the minimal standards for describing new species of the family *Campylobacteraceae* [29] and in other *Arcobacter* publications [5,9,11,12,25,30–32]. Parallel testing was carried out with all the type strains of the species of the genus, using appropriate positive and negative controls. When examined with the transmission electron microscope [2], the cell size and morphology of the strains was determined as well as the presence of a single polar flagellum (data not shown). Phase-contrast microscopy and wet mounts were used to confirm motility. Table 1

shows the key distinctive characteristics between the new strains and the other *Arcobacter* spp. The 3 mussel strains (F4^T, F118-2 and F118-4) showed the same phenotypic response and could be differentiated from the rest of the species of the genus, including strain F67-11^T, with at least 3 tests. The ability of the mussel strains to grow on media containing 0.5–4% (w/v) NaCl was a common characteristic they shared with *A. nitrofigilis*, *A. skirrowii*, *A. mytili* and *A. molluscorum*, but they could be differentiated from these species by their inability to reduce nitrate, among other tests (Table 1). Therefore, nitrate reduction and growth on NaCl can be two useful, initial key characteristics that differentiate this new mussel species from the other *Arcobacter* species. Strain F67-11^T from clams could be differentiated from *A. ellisii*, its closest phenotypic species, by its inability to grow in aerobic conditions at 37 °C or on media containing 0.1% sodium deoxycholate, and from the other *Arcobacter* species with at least 5 different tests.

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiles of the new strains F4^T, F118-2, F118-4 and F67-11^T and other representative strains (n = 42) of all *Arcobacter* spp. (Table S1) were obtained using the Voyager DE STR (Applied Biosystems, Foster city, USA) as previously described [12]. The MALDI-TOF mass spectra derived dendrogram showed that the strains F4^T, F118-2 and F118-4 clustered together and separately from all accepted species of the genus *Arcobacter* as did strain F67-11^T (Fig. 3). This represents the most complete MALDI-TOF analysis of the genus performed so far.

On the basis of the data obtained from the genetic and phenotypic characterization, this study has shown the existence of two new *Arcobacter* species, for which the names *Arcobacter bivalviorum* (type strain F4^T = CECT 7835^T = LMG 26154^T), and *Arcobacter venerupis* (type strain F67-11^T = CECT 7836^T = LMG 26156^T) are proposed. Using the identification method proposed by Figueras et al. [10], the new species *A. bivalviorum* can be clearly differentiated from the other *Arcobacter* spp. by its new specific 16S rDNA-RFLP pattern (Fig. S3). However, in the case of *A. venerupis*, attention should be paid to a thicker RFLP band of 141–138 nt that is the only difference from the pattern obtained for *A. marinus*, that shows only a thinner band (Fig. S3). As has already been commented, misidentifications will occur with the m-PCR methods [8,18] because the two new species will produce the typical *A. cryaerophilus* amplicon with the m-PCR of Houf et al. [18] and either no amplicon or the one of *A. butzleri* with the m-PCR of Douidah et al. [8] (Fig. S2).

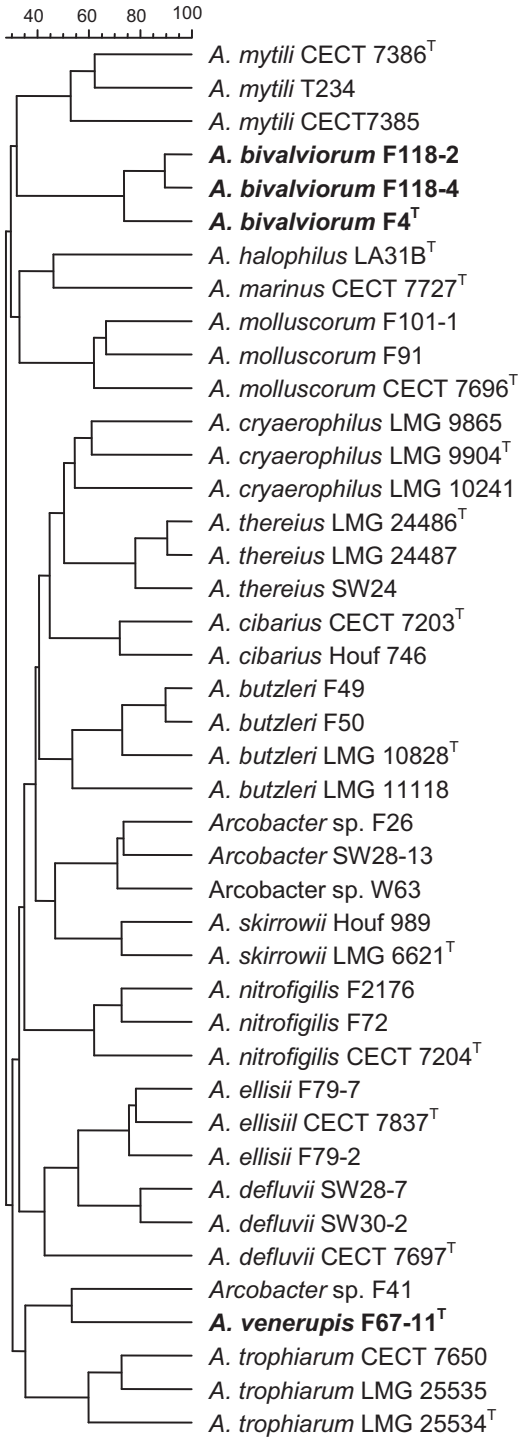

Fig. 3. Dendrogram comparing the MALDI-TOF profile similarities of strains of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. with those of representative strains of all Arcobacter species using UPGMA algorithm. The scale above the dendrogram gives percent matching mass signals between individual strains.

Description of *A. bivalviorum* sp. nov.

A. bivalviorum (bi.val.vi'o.rum. N.L. pl. neut.n. Bivalvia, scientific name of a class of molluscs; N.L. neut. gen. pl. n. bivalviorum, of bivalves of the class Bivalvia).

Cells of strains F4^T, F118-2, F118-4 are Gram-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.3–0.5 μm wide and 0.9–2.0 μm long. They are 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. All the strains grow on blood agar at room temperature (18–22 °C), 30 °C and 37 °C but not at 42 °C under both aerobic and microaerobic conditions, however, growth is weak under anaerobic conditions at 30 °C. No haemolysis is observed on TSA medium supplemented with 5% sheep blood. Strains produce oxidase and catalase activity; hydrolyse indoxyl acetate but not casein, lecithin or starch; do not reduce nitrate or produce urease. Hydrogen sulphide is not produced in triple-sugar iron agar medium. Under aerobic conditions at 30 °C all the strains grow on Marine agar and on nutrient medium (0.5%, w/v NaCl) supplemented with 5% sheep blood and also on this medium containing 2% or 4% (w/v) NaCl. No growth occurs on minimal medium, Campylobacter Charcoal Deoxycholate Agar (CCDA), MacConkey agar or on nutrient media supplemented with 5% sheep blood containing 1% glycine; 0.1% sodium deoxycholate; 1% oxgall; 0.01%, 0.04% or 0.1% 2,3,5 triphenyl tetrazolium chloride (TTC); 0.001% brilliant green; 0.05% safranin; 0.0005% crystal violet; 0.005% basic fuchsin and medium with 64 mg l^{-1} cefoperazone.

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The type strain is F4^T (=CECT 7835^T = LMG 26154^T) isolated from mussels of the Ebro Delta, Spain.

Description of *A. venerupis* sp. nov.

A. venerupis (ve.ne.ru'pis. N.L. n. Venerupis, scientific generic name of Marine bivalve molluscs; N.L. gen. n. venerupis of Venerupis, isolated from the clam species *Venerupis pullastra*).

Cells of strain F67-11^T are Gram-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.3–0.6 μm wide and 0.9–2.2 μm long. It is motile by a single polar flagellum. Colonies on blood agar incubated under aerobic conditions at 30 °C for 48–72 h are 1–3 mm in diameter, beige to off-white, circular with entire margins, convex, and non-swarming. Pigments are not produced. The strain grows on blood agar at room temperature (18–22 °C), 30 °C and 37 °C but not at 42 °C under microaerobic conditions. Under aerobic conditions, it grows well at room temperature (18–22 °C) and 30 °C but not at 37 °C or 42 °C. No growth is observed under anaerobic conditions at 30 °C. No haemolysis is observed on TSA medium supplemented with 5% sheep blood. Strain produces oxidase, catalase and urease activity, reduces nitrate and hydrolyses indoxyl acetate but not casein, lecithin or starch. Hydrogen sulphide is not produced in triple-sugar iron agar medium. Under aerobic conditions at 30 °C the strain F67-11^T grows on minimal medium, MacConkey agar, Marine agar or CCDA medium and on nutrient medium (0.5%, w/v NaCl) supplemented with 5% sheep blood and also on this medium containing 2% (w/v) NaCl. No growth occurs on nutrient medium supplemented with 5% sheep blood containing 4% (w/v) NaCl; 1% glycine; 0.1% sodium deoxycholate; 1% oxgall; 0.01%, 0.04% or 0.1% 2,3,5 TTC; 0.001% brilliant green; 0.05% safranin; 0.0005% crystal violet; 0.005% basic fuchsin and medium with 64 mg l^{-1} cefoperazone.

The type strain is F67-11^T (=CECT 7836^T = LMG 26156^T) isolated from a sample of clams from the locality of Ferrol, Galicia, Spain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2012.01.002.

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