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Arcobacter molluscorum sp. nov., a new species isolated from shellfish☆

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ABSTRACT

Nineteen bacteria isolates recovered from shellfish samples (mussels and oysters) showed a new and specific 16S rDNA-RFLP pattern with an *Arcobacter* identification method designed to recognize all species described up to 2008. These results suggested that they could belong to a new species. ERIC-PCR revealed that the 19 isolates belonged to 3 different strains. The sequence of the 16S rRNA gene of a representative strain (F98-3^T) showed 97.6% similarity with the closest species *Arcobacter marinus* followed by *Arcobacter halophilus* (95.6%) and *Arcobacter mytili* (94.7%). The phylogenetic analysis with the16S rRNA, *rpoB*, *gyrB* and *hsp60* genes placed the shellfish strains within the same cluster as the three species mentioned (also isolated from saline habitats) but they formed an independent phylogenetic line. The DDH results between strain F98-3^T and *A. marinus* (54.8% ± 1.05), confirmed that it represents a new species. Several biochemical tests differentiated the shellfish isolates from all other *Arcobacter* species. Although the new species was different from *A. mytili*, they shared not only the same habitat (mussels) but also the characteristic of being so far the only *Arcobacter* species that are simultaneously negative for urea and indoxyl acetate hydrolysis. All results supported the classification of the shellfish strains as a new species, for which the name *Arcobacter molluscorum* sp. nov. with the type strain F98-3^T is proposed (=CECT 7696^T = LMG 25693^T).

The genus *Arcobacter* was defined almost 20 years ago by Vandamme et al. with bacteria species that had previously been included in the genus *Campylobacter* [34–36]. Until 2005, the genus comprised six species: *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii*, *Arcobacter nitrofigilis* [8,34–36], *Arcobacter cibarius* [18] and *Arcobacter halophilus* [10]. However, since then the genus has been extended, with five additional species: *Arcobacter mytili* [2], *Arcobacter thereius* [17], *Arcobacter marinus* [21], *Arcobacter trophiarum* [7] and *Arcobacter defluvii* [6]. Furthermore, *Candidatus* Arcobacter sulfidicus is a potentially new species that has not yet been formally described [8,37].

The species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been recovered from faeces of livestock and humans [15,33,36]. To date, these are the only species that have been associated with gastrointestinal disease and bacteraemia in humans [15]. These three species have been isolated in association with mastitis and gastric ulcers in farm animals and, together with *A. thereius*, in animal abortions [15,17]. Food or water contaminated with *Arcobacter* is regarded as being the transmission routes to humans [15]. In this sense, the International Commission on Microbiological Specification for Foods [20] considered *A. butzleri* to be a serious hazard to human health.

In a survey on *Arcobacter* spp. from shellfish, harvested monthly between May and September 2009 at mussel farms in the Ebro delta in Catalonia (north-east Spain), 19 isolates (17 from mussels and 2 from oysters, Table S1) had the typical *Arcobacter* colony morphology in blood agar (small, translucent colourless or beige to off-white) and the same restriction pattern (551, 141–138, 72 nt, Fig. S1), which was different from those described for the other species using the 16S rDNA-RFLP *Arcobacter* identification method [2,3,6,14]. The isolation protocol involved an enrichment step in CAT broth followed by a passive filtration of an aliquot of the enrichment on blood agar plates as described by Collado et al. [3,4]. In the present study, however, plates were incubated in parallel under both aerobic and microaerobic conditions. Table S1 shows the specific origin of the isolates, indicating the atmospheric conditions (aerobic or microaerobic) from which they were recovered.

☆ The GenBank/EMBL/DDBJ accession numbers of the sequences of strain F98-3^T (=CECT 7696^T = LMG 25693^T) for the 16S rRNA, the *rpoB* the *gyrB* and the *hsp60* genes are FR675874, FR675877, FR675880 and FR675883, respectively. The 16S rRNA, the *rpoB* the *gyrB* and the *hsp60* genes sequences of strains F91 (FR675876, FR675879, FR675882, FR675885, respectively) and F101-1 (FR675875, FR675878, FR675881, FR675884, respectively) have also been deposited.

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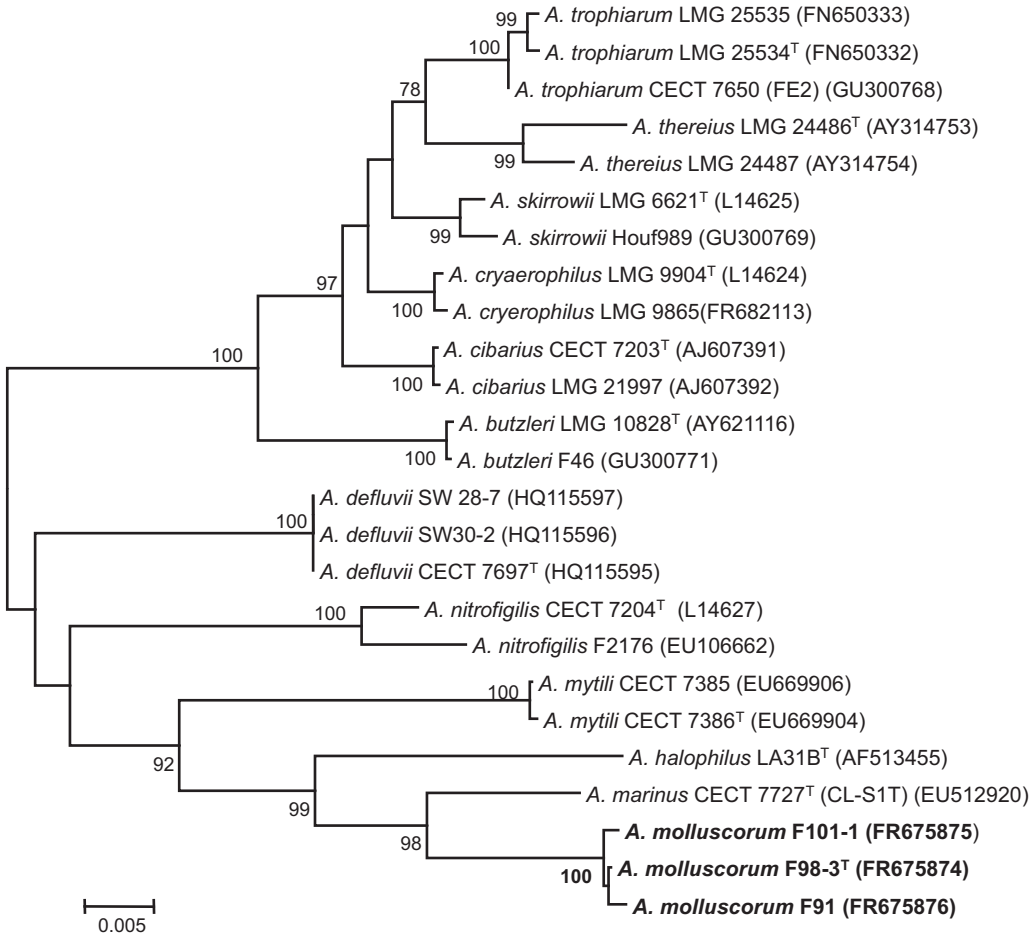

Fig. 1. Neighbour-joining tree based on 16S rRNA sequences (1401 nt) showing the phylogenetic position of *Arcobacter molluscorum* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 nt.

Under a light microscope, all isolates were seen to be Gram-negative slightly curved rods. Isolates were identified with the aforementioned 16S rDNA-RFLP and two multiplex-PCR (m-PCR) identification methods for *Arcobacter* spp. [11,14,19]. With the m-PCR described by Houf et al. [19], which specifically targeted the 16S rRNA gene of the species *A. butzleri* and *A. skirrowii* and the 23S rRNA gene of *A. cryaerophilus*, the shellfish isolates generated two amplification products (Fig. S1) of the same size expected for *A. cryaerophilus* (257 nt) and *A. skirrowii* (641 nt). However, no amplicons were obtained with the m-PCR assay recently developed by Douidah et al. [11] for *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius* and *A. thereius* (data not shown). The new RFLP pattern obtained together with m-PCR results suggested that these isolates could belong to a potential new *Arcobacter* species.

All 19 shellfish isolates were genotyped with enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) using previously described primers and conditions [16] to recognize potential clones [13]. Patterns that differed by one or more bands were considered different genotypes, as in other studies [5,16]. Only three different genotypes were obtained (represented by strains F98-3^T, F101-1 and F91) among the 19 shellfish isolates (Fig. S2). As shown in Fig. S2, the ERIC-PCR patterns of two isolates (F101-1 and F101-2) recovered from oysters and of five (F82-1, F83-1, F90-1, F90-5 and F90-8) recovered from mussels, harvested on different days, were identical. This indicates that the same genotype may persist in different types of shellfish.

The molecular and phenotypic characterization carried out to establish the taxonomic position of the three shellfish strains followed the requirements described in the proposed minimal standards for describing new species of *Arcobacter* [32].

The 16S rRNA and *rpoB* genes were sequenced with the same primers and in the same conditions used in the description of *A. mytili* [2], while for the *hsp60* gene the primers and conditions where those described by Debruyne et al. [9]. Primers and sequences for the *gyrB* gene were designed and performed at the Molecular Diagnostic Center (MDC), Orihuela, Spain, as described by Collado et al. [6]. The EzTaxon server was used to compare the 16S rRNA gene sequences obtained with those deposited in GenBank [1]. Phylogenetic analyses were performed after multiple alignments of sequences by CLUSTAL W [30], using MEGA (Molecular Evolutionary Genetics Analysis) software version 4 [29], with the Kimura's two-parameter model [22] and clustering with the neighbour-joining method [25]. The alignments of the *rpoB* the *gyrB* and *hsp60* gene sequences were also concatenated (1577 nt) and used for tree reconstruction.

The 16S rRNA gene sequence (1441 nt) of the chosen representative shellfish strain F98-3^T showed similarity values of 99.8% and 99.9% with the other strains F101-1 and F91, respectively. The sequence similarities of the 16S gene of strain F98-3^T with the type strains of all other *Arcobacter* species ranged from 92.4 to 97.6%. Values were highest for *A. marinus* (97.6%) followed by *A. halophilus* (95.6%) and *A. mytili* (94.7%). The similarity with *A. marinus* was slightly above the classical threshold of 97.0% [28] at which DNA–DNA reassociation experiments are recommended, but clearly below the new range (>98.7–99%) at which these experiments should be mandatory [26,27]. DNA–DNA hybridization (DDH) experiments were performed between the strain F98-3^T and