EPIDEMIOLOGY AND TAXOGENOMICS OF THE GENUS ARCOBACTER

Alba Pérez Cataluña

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EPIDEMIOLOGY AND TAXOGENOMICS OF THE GENUS *ARCOBACTER*

Alba Pérez-Cataluña

Doctoral Thesis
2018

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EPIDEMIOLOGY AND TAXOGENOMICS OF THE GENUS *ARCOBACTER*

DOCTORAL THESIS

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The present work entitled: "Epidemiology and taxogenomics of the genus *Arcobacter*" prepared by Alba Pérez Cataluña to obtain the degree of doctor by the University *Rovira i Virgili*, have been carried out under my supervision at the Unit of Microbiology of the Department of Basic Health Sciences, and that it fulfils the requirements to obtain the International Doctorate mention.

Reus, July 3, 2018.

Dra. María José Figueras Salvat

To my father

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FREQUENTLY USED ABBREVIATIONS

AAI	Average Aminoacid Identity
AFLP	Amplified Fragment Length Polymorphism
AMC	Amoxicilin/Clavulate
ANI	Average Nucleotide Identity
ARDB	Antibiotic Resistance Database
ARG-ANNOT	Antibiotic Resistance Gene-Annotation database
ATCC	American Type Culture collection
BLAST	Basic Local Alignment Search Tool
CAI	Codon Adaptation Index
CARD	Comprehensive Antibiotic Resistance Database
CAT	Cefoperazone-Amphotericin B-Teicoplanin (antibiotic supplement)
CCDA	Campylobacter Cefoperazone Deoxycholate Agar
CCUG	Culture Collection, University of Göteborg, Sweden
CDC	Center for Disease control, Culture Collection
CECT	*Colección Española de Cultivos Tipo*
CIN	Cefsulodin-Irgasan-Novobiocin agar
CIP	Collection of Institute Pasteur
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
DDH	DNA-DNA Hibridization
DSM	*Deutsche Sammlung von Microorganisme und Zellkulturen GmbH,* German Culture Collection
DPRB	Dissimilatory Perchlorate-Reducing Bacteria
ECDC	European Centre for Disease Prevention and Control
EMEA	European Medicines Agency
ERIC	Enterobacterial Repetitive Intergenic Consensus
E	Erythromycin
GGCD	Genome-to-Genome Distance Calculator
GM	Gentamycin
HJXXIII	Hospital Juan XXIII
HMM	Hidden Markov Models
HSJR	Hospital Sant Joan de Reus
HUMS	Hospital Miguel Servet
ICMSF	International Commission on Microbiological Specifications for Foods
*is*DDH	*in silico* DNA-DNA hybridization
KCTC	Korean Collection of Type Cultures
LMG	*Laboratorium voor Microbiologie, Universiteit Gent,* Belgium Culture Collection
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MCL	Markov Cluster Algorithm
MIC	Minimum Inhibitory concentration
ML	Maximum Likelihood
MLPA	Multilocus Phylogenetic Analysis
MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
MPN	Most Probably Number
NCTC	National Collection of Type Cultures, England
m-PCR	multiplex-PCR
NCBI	National Center for Biotechnology Information
NJ	Neighbor-Joining

PCA	Principal Component Analysis
PFGE	Pulsed Field Gel Electrophoresis
PGAAP	NCBI Prokaryotic Genome Automatic Annotation Pipeline
POCP	Percentage Of Conserved Proteins
QRDR	Quinolone resistance-determining region
RAPD	Random Amplified Polymorphic DNA
RAST	Rapid Annotation Subsystem Technology
RFLP	Restriction Fragment Length Polymorphism
RSCU	Relative Synonymous Codon Usage
SDA	Split Decomposition Analysis
ST	Sequence Type
TEM	Transmission Electron Microscopy
TLR-4	Toll-Like Receptor 4
TTC	Triphenyl Tetrazolium Chloride
VFDB	Virulence Factors of Pathogenic Bacteria Database
WWTP	Wastewater Treatment Plant

1. INTRODUCTION

The genus *Arcobacter* comprises Gram-negative bacteria with curved shapes and oxidase activity. The species *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii* and *Arcobacter thereius* have been considered as emergent enteropathogens for humans and animals (Collado and Figueras, 2011). The bacteria of this genus have a worldwide distribution and can be isolated from a broad range of habitats and hosts (Collado and Figueras, 2011; Merga et al., 2014; Hsu and Lee, 2015, and references therein). The most important reservoirs for these bacteria are farm animals, wastewater, marine waters and shellfish. It has been demonstrated that the presence of *Arcobacter* in water correlates with the presence of faecal contamination, and wastewater always show presence of this microbe as signature (Collado et al., 2008; McLellan et al., 2010; Fisher et al., 2014). The colonization of *Arcobacter* in other habitats such as drinking water, irrigation water, ready-to-eat vegetables and other processed food products occur through faecal contamination (Collado and Figueras, 2011; Merga et al., 2014; Fernández et al., 2015; Hsu and Lee, 2015; Ferreira et al., 2017, and references therein). Regarding the prevalence in food products of animal origin, the highest prevalence in meat products occur in poultry and pork. In animals, *Arcobacter* can produce abortions and mastitis, among other disorders. In humans, produces diarrhoea as the main disease, but can also produce bacteraemia, being *A. butzleri* the most prevalent species (ICMSF, 2002; Collado and Figueras, 2011 and references therein).

1.1 Taxonomy

1.1.1 Taxonomic classification

The genus *Arcobacter* was proposed in 1991 to classify two species (*A. cryaerophilus* and *Arcobacter nitrofigilis*) previously considered atypical campylobacters (Vandamme et al., 1991). The differential characteristics between *Campylobacter* and *Arcobacter* is the ability of the latter to grow at lower temperatures, i.e. 15°C-30°C (Vandamme et al., 1991, 1992). Initially, these two genera were ascribed to the family *Campylobacteraceae,* order *Campylobacterales* (Collado and Figueras, 2011). However, in 2017, Waite and co-workers analysed the class *Epsilonproteobacteria* based on their phylogenetic relationship with the 16S and 23S rRNA genes and with 120 single-copy proteins. These authors proposed a reclassification of the *Epsilonproteobacteria* and *Desulfurellales* within the new phylum *Epsilonbacteraeota*, without specific relation with the other Proteobacteria, and the creation of the new family *Arcobacteraceae* that only comprises the genus *Arcobacter* (Waite et al., 2017). They also showed that *Arcobacter* was related with the genera *Sulfurimonas* and *Sulfuricurvum* (Waite et al., 2017), as evidenced by other studies (Miller et al., 2007; Roalkvam et al., 2015). In 1992, two new species were added to the genus: *A. skirrowii*, isolated from animal origin and from aborted cattle foetuses; and *A. butzleri* from human and animal origin (Vandamme et al., 1992). Two new species were incorporated in 2005, *Arcobacter halophilus* isolated from a hypersaline lagoon (Donachie et al., 2005) and *Arcobacter cibarius* from broiler carcasses (Houf et al., 2005). Since then, the number of species has increased very fast (Figure 1) and nowadays the genus is composed by 27 species (Figure 2) (Diéguez et al., 2017; Tanaka et al., 2017; and http://www.bacterio.net/arcobacter.html).

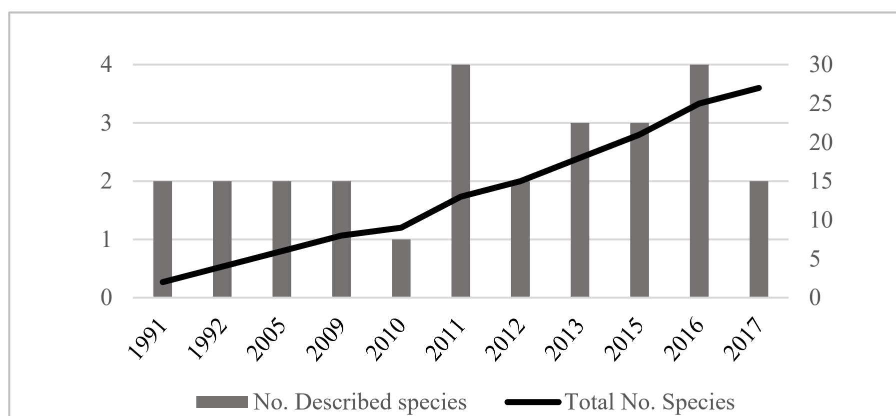

Figure 1. Number of described species (bars) and accumulated total number of species (line) from 1991 to 2017.

1.1.2 Morphology and biochemical characteristics

Arcobacter species are Gram-stain-negative curved or helical rod-shaped cells, non-spore forming, and motile by a polar flagellum (Vandamme et al., 1992). Cells are 0.1-0.9 µm wide and 0.5-3.6 µm long (Vandamme et al., 1992; Kim et al., 2010; Zhang et al., 2016). These characteristics are very similar to *Campylobacter* spp. However, most of the *Arcobacter* species can grow in the presence of at least 5% oxygen and in a range of temperatures from 15ºC to 42ºC, being 30ºC in microaerobiosis the optimal conditions to grow. Despite that, some studies showed the same incidence of *Arcobacter* isolated from diarrhoeic human faeces at 25ºC and at 37ºC (Kownhar et al., 2007; Van den Abeele et al., 2014). Additionally, Levican et al. (2016) evidenced that no significant differences in species recovery from wastewater were found comparing the use of microaerobic or aerobic conditions for incubation. Two atypical species have been described in *Arcobacter*: the obligate anaerobic species *A. anaerophilus* (Sasi Jyothsna et al., 2013), and the obligate halophile species *A. halophilus* that needs the presence of at least 2% NaCl to grow (Donachie et al., 2005).

Arcobacter are considered fastidious bacteria because they grow and replicate slowly requiring between 48 and 72 hours. The only exception is the species *A. butzleri*, that only need 24 hours to grow. This characteristic of *A. butzleri* along with the fact that when using an enrichment step during culturing the latter is the dominating species can be the reasons that explains its higher prevalence that can mask the incidence of other species (Houf et al., 2002; Collado and Figueras, 2011; Levican et al., 2016). One of these species is *A. cryaerophilus*, that has been reported as the most prevalent in wastewater using a metagenomic analysis targeting the 16S rRNA gene (Fisher et al., 2014).

1.1.3 Identification

The species of *Arcobacter* have been described following the minimal standards for the phenotypical characterization that were proposed in 1994 by Ursing et al. and were recently updated by On and co-workers (2017). Despite that, the identification to the species level using this approach is difficult due to the variable behaviour of the strains. The classical tool used in bacterial

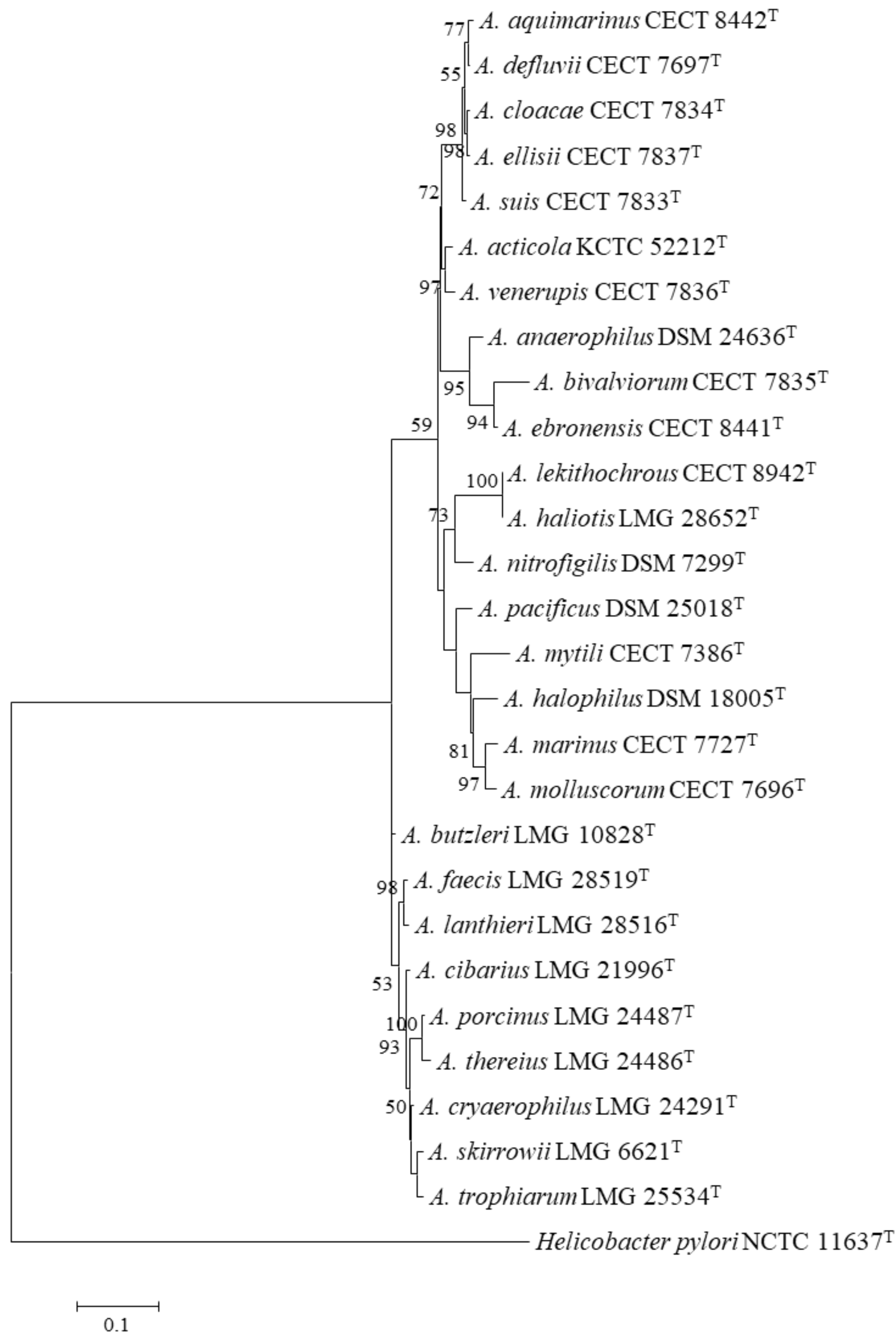

Figure 2. Maximum-Likelihood tree based on the 16S rRNA gene sequences (1417 bp) showing the phylogenetic position of the 27 described species of the genus *Arcobacter*. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar, 1 substitutions per 10 nt.

taxonomy has been the 16S rRNA gene and this gene shows a wide range of similarity between the type strains of the described *Arcobacter* species (91.2% to 99.6%) (Levican et al., 2013a; Diéguez et al., 2017). The high percentage of similarity of this gene (>99%) between the species *Arcobacter ellisii* and *Arcobacter cloacae* demonstrated that it has a low resolution power between certain species (Levican et al., 2013a). Molecular identification methods developed for *Arcobacter*, including m-PCRs (Houf et al., 2000; Douidah et al., 2010) and 16S rDNA-RFLP (Figueras et al., 2012) have helped to the expansion of the genus. However, these methods showed also certain disadvantages, as demonstrated by Levican and Figueras (2013). The latter authors concluded that the use of methods that targets the 16S or 23S rRNA genes can produce misidentifications due to the lower resolution power of these genes for certain species commented above. In 2017, Khan et al. developed a m-PCR method for the identification of 6 *Arcobacter* species. This method included the detection of *A. skirrowii* with the primers described by Houf et al. (2000) but with modifications of the PCR amplification protocol, and new primers targeting housekeeping genes for the identification of *Arcobacter lanthieri*, *A. butzleri*, *A, cryaerophilus*, *A. cibarius* and *Arcobacter faecis* (Khan et al., 2017). However, the study only included 17 of the 27 described species at the moment of publication and the validation with the remaining species is still pending to be tested.

The most accurate identification method are the ones that use the sequences of housekeeping genes, such as *rpoB* (Levican Asenjo, 2013), *hsp60* (Levican Asenjo, 2013) and *gyrB* (Collado et al., 2011), to perform a phylogenetic tree with representative strains of all the described species of the genus. The use of *rpoB* gene allowed us to identify *Arcobacter* strains uncovered by *Campylobacter* (Figueras et al., 2014). Furthermore, the concatenation of five housekeeping genes (*gyrA*, *atpA*, *rpoB*, *gyrB* and *hsp60*), known as Multilocus Sequence Analysis (MLSA) or Multilocus Phylogenetic Analysis (MLPA), has been used for the new species description providing a more robust separation of the species than the 16S rRNA gene (Levican et al., 2015; Diéguez et al., 2017). Moreover, the low percentages of similarities (<95%) found between some of the species of the genus evidenced that a deep revision of the taxonomy is needed, as mentioned in the description of *Arcobacter lekithochrous* (Diéguez et al., 2017).

Other techniques for the identification and quantification of *Arcobacter* have been developed, such as real-time PCR (qPCR) methods. These methods target the 16S and 23S rRNA genes (Lee et al., 2012; Hausdorf et al., 2013), the housekeeping gene *hsp60* (De Boer et al., 2013) and the *qhnDH* gene, that encodes the gamma subunit of a quinohemoprotein amine dehydrogenase (Webb et al., 2016) for the detection of *A. butzleri* from stool samples. However, so far the existence of possible interferences with other new species has not yet been studied. Additionally, some of these methods showed that *Arcobacter* can uncover *Campylobacter*, as demonstrated by Banting et al. (2016). One of the most fast, cheap and reproducible method for the identification of bacteria is the Matrix- Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry method (Alispahic et al., 2010; Levican et al., 2012, 2015). This method has helped to recognize *Arcobacter* in the clinical setting (Figueras et al., 2014). However, this method and its results depends on the species and strain included within the database that in some cases are limited, as occurs in the Biotyper database of Bruker or the one of the bioMérieux system which was unable to identify a strain of *A. butzleri* isolated from a bacteraemia case (Arguello et al., 2015).

1.2 Biology and Ecology

The higher persistence of *Arcobacter* in contrast with the persistence of *Campylobacter* in the environment can be due to the capacity of *Arcobacter* to grow at ambient temperature in aerobiosis, and to tolerate the presence of NaCl (Cervenka, 2007; Collado and Figueras, 2011; Hsu and Lee, 2015). Moreover, the genome of *A. butzleri* RM4018 is more similar to the *Helicobacteraceae* bacteria *Sulfurimonas denitrificans* and *Wolinella succinogenes*, and to *Sulfurovum* and *Nitratiruptor*, a deep-sea vent bacterium (Miller et al., 2007). This relationship of *Arcobacter* with the genus *Sulfurimonas* was also evidenced at genomic level in the study of Waite et al. (2017). Additionally, the genome of *A. butzleri* RM4018 shows characteristics related with signal transduction, adaptation, DNA repair, chemotaxis and respiration, that allow this bacterium to grow and survive in different environmental conditions (Miller et al., 2007).

Bacteria of the genus *Arcobacter* shows a high abundance (> 10^6 MPN/100mL) in waters with a high level of faecal contamination from humans and animals (Maugeri et al., 2000; Collado et al., 2008; Fernandez-Cassi et al., 2016). However, the low abundance of *Arcobacter* in human faeces does not justify their high densities in sewage and wastewater, that have been related with the capacity of this bacteria to multiply in the sewerage system (McLellan et al., 2010; Fisher et al., 2014; Banting and Figueras, 2017). Abundance data along with the genomic characteristics of *Arcobacter* indicates that these bacteria are free-living organisms that can adapt and replicate in different environments (Miller et al., 2007; McLellan et al., 2010; Wesley and Miller, 2010).

The isolation of *Arcobacter* from faeces of symptomatic and asymptomatic individual and from livestock suggest that *Arcobacter* species can be considered as opportunistic pathogens (On et al., 2002; Houf et al., 2007; Collado et al., 2014; Webb et al., 2016).

1.3 Epidemiology

The routes of transmission of *Arcobacter* have been related with the consumption of *Arcobacter*-contaminated water or food (Collado and Figueras, 2011; Hsu and Lee, 2015; Ramees et al., 2017). This way of transmission was evidenced in 4 outbreaks, 3 related with the consumption of faecal contaminated water and one with the consumption of chicken (Collado and Figueras, 2011; Ferreira et al., 2015; and references therein). Additionally, *Arcobacter* has been detected in restaurant meals with a higher prevalence than other typical food-associated bacteria as *Salmonella* or *Campylobacter* (Collado and Figueras, 2011 and references therein). This potential route has been also demonstrated with the detection of *Arcobacter* in different types of vegetables (González and Ferrús, 2011; Hausdorf et al., 2013; Fernandez-Cassi et al., 2016; Mottola et al., 2016a) and can be related with the use of reclaimed water for irrigation because the prevalence of *Arcobacter* in this type of water ranges from 66.6% to 100% (Levican et al., 2016). Another route for *Arcobacter* dissemination is related with the person-to-person transmission (Collado and Figueras, 2011). This mechanism was described in an outbreak that occurred in an Italian school (Vandamme et al., 1992) where all the isolates recovered from the faeces samples showed the same genotype and phenotype. A case of person to person transmission was also observed in an infection of a neonate with *A. butzleri* that could be acquired by the contact with the mother's placenta (On et al., 1995).

For the study of the epidemiology of *Arcobacter*, different molecular tools have been developed. Some of these methods are the Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), the Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR), the Amplified Fragment Length Polymorphism (AFLP), the Pulsed-Field Gel Electrophoresis (PFGE) and the Multi-Locus Sequence Typing (MLST) scheme. The MLST methods have been used in many different bacteria genera and in the case of *Arcobacter* the seven housekeeping genes used in the analysis were proposed in 2009 by Miller et al. and primers and conditions are available at the specific database (https://pubmlst.org/arcobacter/). Investigators can deposit their sequences in this database, compare them with the ones already deposited to find out if the gene alleles of the analysed strains are new or already known, and determine the sequence type (ST). At the time of writing the different origins of the available sequences at this database were the following: 44.9% of the sequences were isolated from animals, 34.3% from food, 14.8% from humans and 4.2% from environmental waters.

All the above mentioned genotyping methods presented advantages and disadvantages (Collado and Figueras, 2011; González et al., 2012; Ferreira et al., 2017a). The most extended method for genotyping is the ERIC-PCR method (Houf et al., 2002), because it easy to perform, do not requires expensive equipment and has a highly reproducibility. Despite that, this method does not have the high discriminatory power obtained with the PFGE, however the PFGE method is more complex than the ERIC-PCR. The MLST technique has demonstrated a high discriminatory power and reproducibility, along with the existence of a database that helps investigators to compare their results. The major problem of the latter technique is the high cost because it needs the sequencing of seven housekeeping genes, a technique that is not available in all the laboratories, and that the comparison of the results depends on the variability of sources available at the database (González et al., 2012; Ferreira et al., 2017a).

1.4 Distribution and prevalence

1.4.1 Arcobacter in water

As mentioned in the beginning of this thesis, *Arcobacter* can be found in different types of water including drinking water, rivers, lakes, seawater, wastewater, and water used in food chains (See section 1.4.2) (Hsu and Lee, 2015; Çelik and Ünver, 2015; Levican et al., 2016; Salas-Massó et al., 2016; Talay et al., 2016; Rathlavath et al., 2017). The highest prevalence of *Arcobacter* in water samples have been reported in wastewater (70.9-100%) (Collado et al., 2008; McLellan et al., 2010; Fisher et al., 2014, 2015; Hsu and Lee, 2015; Fernandez-Cassi et al., 2016; Levican et al., 2016; Banting and Figueras, 2017) (Figure 3). In water the presence of *Arcobacter* has been related with faecal contamination (Collado et al., 2008; McLellan et al., 2010; Fisher et al., 2014, 2015; Fernandez-Cassi et al., 2016; Banting and Figueras, 2017). Despite the high concentration of *Arcobacter* in wastewater and sewage, it has been demonstrated that the disinfection treatments used for drinking water production are effective in reducing and eliminating the populations of *Arcobacter* (Collado et al., 2010) and the same occurs during wastewater treatment (Fernandez-Cassi et al., 2016; Banting and Figueras, 2017). However, Webb et al. (2016) demonstrated that viable *A. butzleri* are able to survive wastewater treatment, including UVB irradiation, which may lead to increased density and genetic diversity of this suspected pathogen in environmental waters

via wastewater effluent discharge. One interesting aspect related with the prevalence of *Arcobacter* in water is the one related with prevalence of *A. butzleri* and *A. cryaerophilus*. The use of isolation methods that involve an enrichment step showed *A. butzleri* as the most prevalent species, while the use of direct plating enhanced the recovery of *A. cryaerophilus* (Levican et al., 2016). Additionally, when this samples are analysed using metagenomics targeting the 16S rRNA gene, the most prevalent species is *A. cryaerophilus* (Fisher et al., 2015). These results evidenced that the employed culture method influences the recovery of the different species and may produce a bias on the prevalence of *Arcobacter* species (Levican et al., 2016; Salas-Massó et al., 2016). Moreover, there is a different detection of the described *A. cryaerophilus* subgroups (1A and 1B) in wastewater depending on the water temperature (Fisher et al., 2014). In that sense, when the water temperature is higher than 20ºC, the number of reads belonging to subgroup 1B is higher than the ones of the subgroup 1B. This was also demonstrated by culture using direct plating, because the higher percentage of isolation corresponded to *A. cryaerophilus* subgroup 1B in wastewater samples from the Waste Water Treatment Plant (WWTP) of Reus (data not published).

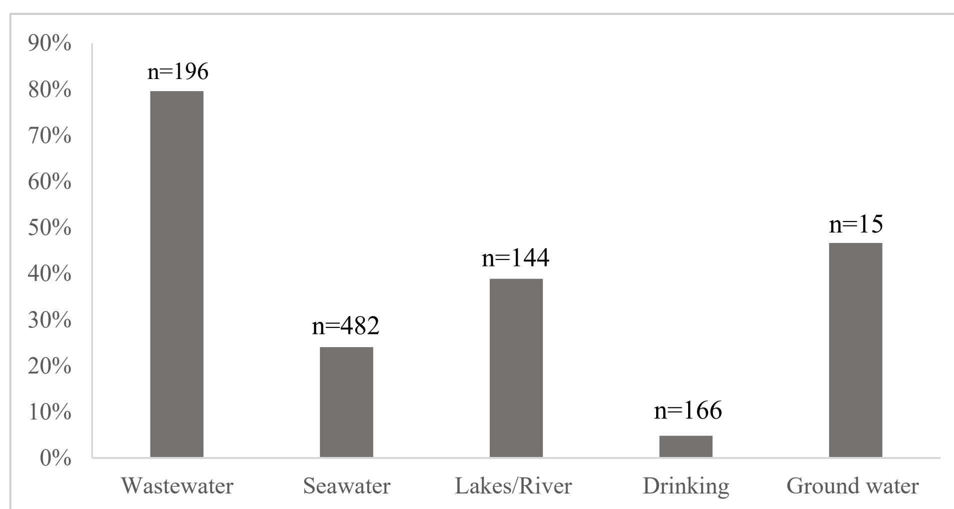

Figure 3. Percentage of positive samples for *Arcobacter* species in different water sources.

Data extracted from Hsu and Lee (2015) and updated with Aydin et al. (2007), Çelik and Ünver (2015), Levican et al. (2016), Salas-Massó et al. (2016), Talay et al. (2016) and Rathlavath et al. (2017).

1.4.2 Arcobacter in food

The distribution and prevalence of *Arcobacter* in food products was reviewed by Hsu and Lee (2015) and updated in Salas-Massó et al. (2018) and in this thesis (Laishram et al., 2016; Barboza et al., 2017a; Ferreira et al., 2017b; González et al., 2017; Leoni et al., 2017; Morejón et al., 2017; Ottaviani et al., 2017; Rathlavath et al., 2017b; Oliveira et al., 2018; Vicente-Martins et al., 2018). The presence of *Arcobacter* in meat products have been widely studied and showed that the percentage of positive samples ranged from 38.9% in poultry products to 10.0% in rabbit meat (Figure 4) (Barboza et al., 2017a; Oliveira et al., 2018; Salas-Massó et al., 2018; Vicente-Martins et al., 2018). These results correlated with the ones obtained in food processing environments, where the higher prevalence of *Arcobacter* was also observed in poultry processing plants (42.3%, Figure 5) (Ferreira et al., 2017b; Salas-Massó et al., 2018). *Arcobacter* can be also found in higher

percentage in seafood, mostly in shellfish, with a 35% of positive results (Figure 4) (Laishram et al., 2016; Leoni et al., 2017; Morejón et al., 2017; Ottaviani et al., 2017; Rathlavath et al., 2017b; Salas-Massó et al., 2018; Vicente-Martins et al., 2018). Vegetables also shows a high prevalence of this bacterium (González et al., 2017; Salas-Massó et al., 2018), even in ready-to-eat vegetables (Salas-Massó et al., 2018; Vicente-Martins et al., 2018). In this source, the positive samples for *Arcobacter* is around 24% (Figure 4). In milk products the percentage of positive samples for *Arcobacter* was 46.1% (Figure 4) (Wesley and Miller, 2010).

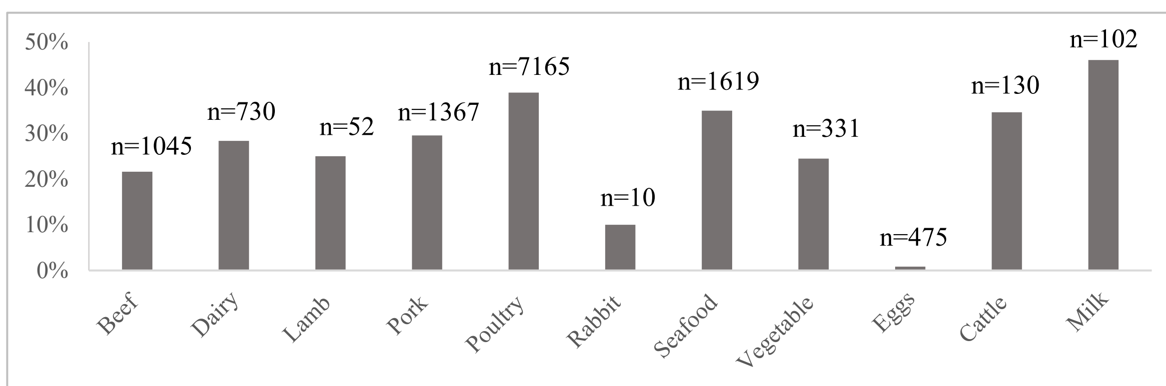

Figure 4. Percentage of positive samples for *Arcobacter* species in different food sources.
Data extracted from the reviews of Hsu and Lee (2015) and Salas-Massó et al. (2018), and from Aydin et al. (2007), Laishram et al. (2016), Barboza et al., (2017a), Ferreira et al. (2017), González et al. (2017), Leoni et al. (2017), Morejon et al. (2017), Oliveira et al. (2018), Otaviani et al. (2017), Rathlavath et al. (2017), and Vicente-Martins et al. (2017).

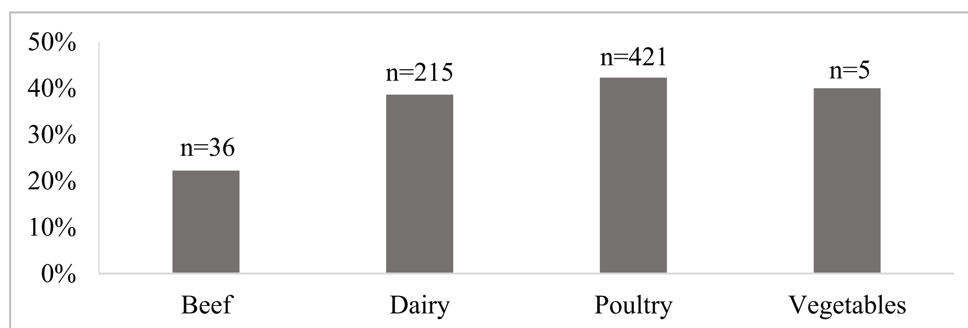

Figure 5. Percentage of positive samples for *Arcobacter* species in different food processing plants.
Data extracted from Salas- Massó et al. (2018) adding missing data from Ferreira et al. (2017).

1.4.3 Arcobacter in animals

The prevalence of *Arcobacter* in animals have been studied mostly from their faeces (Aydin et al., 2007; Fernández et al., 2007; Ho et al., 2008a; Patyal et al., 2011; Wesley and Schroeder-Tucker, 2011; Gilbert et al., 2014; Salas-Massó et al., 2018), but also from mouth washes (Houf et al., 2008; Fera et al., 2009); blood or lymph (Fera et al., 2009); viscera (Salas-Massó et al., 2018; Wesley and Schroeder-Tucker, 2011), among others; and is represented in Figure 6. All the 40 caimans studied in Brazil were positive for *Arcobacter*, as well as horses and wild animals (Wesley

and Schroeder-Tucker, 2011; Oliveira et al., 2017). For the raccoons faeces (n=10) analysed by Hamir et al. (2004), 60% were positive for *Arcobacter*. Regarding the domestic dogs and cats, the higher prevalence was detected in mouth washes of cats (78.8%) (Fera et al., 2009), while the presence in faeces and mouth washes of dogs were 2.6% (Aydin et al., 2007; Fernández et al., 2007; Houf et al., 2008). Studies of faeces of farm animals showed that *Arcobacter* was more prevalent in cattle, with 38.7% of positive samples (Aydin et al., 2007; Fernández et al., 2007; Salas-Massó et al., 2018) and in pig faeces with 38.1% positive (Salas-Massó et al., 2018), while in sheep the percentage was 10.4% (Aydin et al., 2007; Salas-Massó et al., 2018). Interestingly, the presence of *Arcobacter* in poultry faeces (Chickens, turkeys and geese) was lower than 10% (Aydin et al., 2007; Fernández et al., 2007; Ho et al., 2008a; Patyal et al., 2011; Salas-Massó et al., 2018). The percentage of positive samples in birds and reptiles were 21.1% and 19.9%, respectively (Fernández et al., 2007; Gilbert et al., 2017; Salas-Massó et al., 2018).

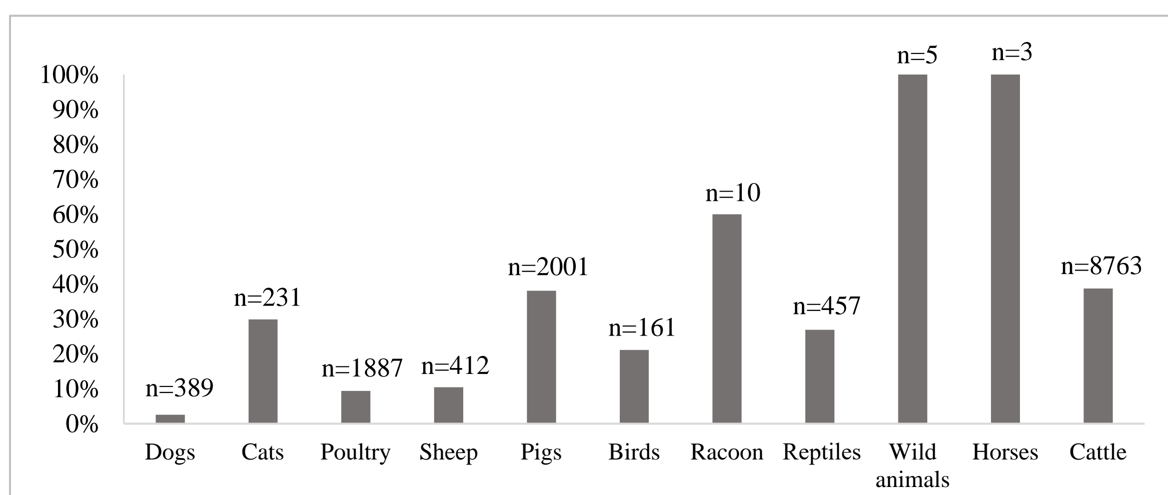

Figure 6. Percentage of positive samples for *Arcobacter* species in different animals.
Data extracted from Aydin et al. (2007), Fernández et al. (2007), Ho et al. (2008), Houf et al. (2008), Fera et al. (2009), Patyal et al. (2011), Wesley and Schroeder-Tucker (2011), Gilbert et al., (2017); Oliveira et al. (2017), and Salas-Massó et al. (2018).

1.5 Clinical Features

1.5.1 Clinical diagnostic in humans

The genus *Arcobacter* have been considered as an emergent pathogen. In human, bacteria of this genus can produce diarrhoea (Collado and Figueras, 2011; Ferreira et al., 2015; Hsu and Lee, 2015, and references therein; Webb et al., 2016) but a clear picture of the relationship with human disease is still poorly known due to the lack of routine screening of this bacteria in the clinical laboratories. In Table 1 are summarized the cases associated with *Arcobacter*, reviewed by Figueras et al. (2014) and Salas-Massó et al. (2018) and updated with Barboza et al. (2017b). These cases are mostly associated to *A. butzleri* and *A. cryaerophilus*, but some cases related with *A. skirrowii* and *A. thereius* have been reported (Wybo et al., 2004; Van den Abeele et al., 2014).

Some studies analysed the prevalence of *Arcobacter* in faeces of patients with diarrhoea (Table 1) and the prevalence by culture methods ranges from 0.1% in Belgium and France

(Vandenberg et al., 2004) to 1.4% in Chile (Collado et al., 2013). However, when molecular techniques are compared with culture methods, the prevalence is higher, as demonstrated by Webb et al. (2016). The percentage of positive samples for *A. butzleri* using culture methods was 0.8%, while 56.7% positive samples were found using molecular methods (Webb et al., 2016). These results indicate that the use of culturing methods instead of molecular ones can underestimate the prevalence of this pathogen (Collado and Figueras, 2011; Figueras et al., 2014), as occurred in samples from other sources.

The most common clinical feature associated to *Arcobacter* infections in humans is the appearance of an acute and watery diarrhoea along with abdominal pain. Sometimes, these symptoms are accompanied by vomiting and fever, as well as the presence of bloody diarrhoea but these complications appeared in low proportions (Table 1). The co-infections of *Arcobacter* with other pathogens are low, and in 13.7% to 50% of the cases there is no underlying disease (Table 1). Bacteria of this genus can also produce extra intestinal infections, being bacteraemia cases the most common in patients with underlying conditions (Table 1). Usually, the infections by *Arcobacter* are self-limiting but when the symptoms are prolonged or severe the use of antibiotics is important, see section 1.5.4. (Table 1). In these cases, when the unique bacteria isolated from faeces is *Arcobacter*, the patient recovery using antibiotics could indicate that this bacterium is the etiological agent.

There is no standardized method for the detection and identification of *Arcobacter* at the hospital laboratories. In these services, the isolation is carried out with media used for the recovery of Campylobacter, as Campylosel agar (bioMèrieux, Barcelona, Spain) or in the Yersinia Selective Agar (CIN agar, Cefsulodin-Irgasan-Novobiocin, BD, Madrid, Spain). The differentiation of *Arcobacter* from *Campylobacter* can be assessed testing the aerotolerance and the ability to grow at 15ºC, 25ºC and 37ºC (Collado and Figueras, 2011). Despite that, the use of phenotypic methods can report wrong results, where *Arcobacter* is covered by *Campylobacter* (Vandenberg et al., 2004; Figueras et al., 2014). The introduction of the MALDI-TOF technique for the identification of bacterial isolates in the clinical laboratories has helped in some cases to the correct identification of *Arcobacter* (Alispahic et al., 2010; Figueras et al., 2014). Additionally, the use of housekeeping genes as the *rpoB* gene for the identification of the strains in the research laboratory, helped clinicians to improve the identification of *Arcobacter* (Figueras et al., 2014).

1.5.2 Clinical diagnostic in animals

The clinical importance of *Arcobacter* in animals has been reviewed by Collado and Figueras (2011) and more recently by Ramees et al. (2017). Despite *Arcobacter* spp. have been isolated from different animals, the capacity of this bacteria to produce disease seems only restricted to a certain type of farm animals (Ho et al., 2006). Species of this genus have been isolated mostly from faeces and from the intestinal tracts in healthy animals, and also from bovine preputial sheath (Gill, 1983) and from vaginal swabs of cows (Kabeya et al., 2003). The most important diseases produced by *Arcobacter* in animals are abortions, mastitis and diarrhoea (Collado and Figueras, 2011; Ramees et al., 2017, and references therein). Furthermore, during a mastitis outbreak in cows, the isolated strains were used to re-infect cows producing the same effects in these animals, demonstrating therefore *Arcobacter* as the etiological agent of the mastitis (Logan et al., 1982). The role of *Arcobacter* in animal diseases have also been tested in rats, producing watery diarrhoea in

adults and necrosis in the intestine and the liver of neonatal albino rats (Adesiji, 2010; Adesiji et al., 2012). Another study of experimental infections was carried out with zebrafish (*Danio rerio*) in which *Arcobacter* produced inflammation, necrosis and viscera congestions (Açik et al., 2016). The latter study suggested that zebrafish can be used as a model of infection for *Arcobacter* (Açik et al., 2016). Regarding the species related with animal disease, the most commonly isolated species was *A. cryaerophilus*, mostly in porcine abortions (Neill et al., 1985; de Oliveira et al., 1997) and in mastitis (Logan et al., 1982). *Arcobacter butzleri*, *A. skirrowii* and *A. thereius* have also been associated sporadically with animal diseases (de Oliveira et al., 1997; On et al., 2003). In the case of *A. butzleri*, this species has been associated with diarrhoea and enteritis in several farm animals, as horses, pigs and cattle, while *A. skirrowii* has been reported in haemorrhagic colitis and diarrhoea in sheep and cattle (Vandamme et al., 1992; Ho et al., 2006). Other animals that showed infections by *Arcobacter* were non-human primates (Kiehlbauch et al., 1991; Anderson et al., 1993; Higgins et al., 1999) with the isolation of *A. butzleri*, and also rainbow trout (*Oncorhynchus mykiss*) were the reported species was *A. cryaerophilus* (Yildiz and Aydin, 2006).

1.5.3 Treatment

Currently official protocols for the treatment of *Arcobacter* infections does not exist, as indicated in many studies (Collado and Figueras, 2011; Ferreira et al., 2015; Van den Abeele et al., 2016). The first treatment used for gastrointestinal infections is the fluid therapy, used with many enteropathogen disorders. Only when the disease is severe or prolonged in time, or when the patient has an underlying disease, the treatment involves the use of antibiotics (Collado and Figueras, 2011). However, the use of antibiotics is common in enteritis produced by *Arcobacter* due to the chronic characteristics, and it is necessary in bacteraemia cases (Table 1). Between the antibiotics used, the most common is the use of macrolides (erythromycin) and fluoroquinolones (ciprofloxacin or ofloxacin) in gastrointestinal cases, and in less proportion tetracyclines (doxycycline). As reported by Figueras et al. (2014), a case of acute bloody and watery diarrhoea produced by *A. cryaerophilus* was treated successfully with amoxicillin/clavulanic acid. Cases of bacteraemia have been treated with β-lactams antibiotics (Yap et al., 2013), cephalosporins of second generation (Lau et al., 2002), or combinations of two antibiotics (On et al., 1995; Hsueh et al., 1997; Yan et al., 2000; Arguello et al., 2015) as show in Table 1. In all the cases of bacteraemia the patients showed an underlying disease.

1.5.4 Antibiotic resistance

In 2015, Ferreira and co-workers reviewed the in vitro antimicrobial susceptibility of the clinical *Arcobacter* species i.e. *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* using isolates from poultry, livestock and shellfish. The most interesting results were the high number of strains that showed resistance to ampicillin, nalidixic acid, ciprofloxacin, erythromycin or gentamicin, reaching in some cases a 100% of resistance (Ferreira et al., 2015, and references therein). Similar results were obtained by Van den Abeele et al. (2016) with strains isolated from humans in Belgium. The latter authors suggested that treatments of gastrointestinal disease produced by *Arcobacter* should be done with tetracyclines, instead of fluoroquinolones and macrolides (Van der Abeele et al., 2016). However, the antimicrobial susceptibility studies performed with *Arcobacter* followed

different methodologies and the comparison of the results should be done carefully (Ferreira et al, 2015). In fact, the contradictory results obtained between the commonly used treatments at the clinical setting and the observed resistances to the antibiotics used in those treatments in *in vitro* studies evidences the needed for a standardized protocol for the treatment of *Arcobacter* infections.

The resistance to quinolones and their molecular mechanisms in *Arcobacter* were firstly studied by Abdelbaqui et al. (2007) in human clinical isolates in France. In this study, the *gyrA* gene of these clinical strains was studied and it was discovered the existence of mutations in the quinolone resistance-determining region (QRDR) associated to strains that showed resistance to quinolones (Abdelbaqui et al., 2007). These mutations were situated in the position 254 of the *gyrA* gene and produced an amino acid substitution (Thr-85-Ile) in the GyrA protein. This mutation had also been found in other studies of *Arcobacter* (Van den Abeele et al., 2016; González et al., 2017; Ferreira et al., 2018). In the study of Ferreira et al. (2018), where ciprofloxacin mutants where inducted at the laboratory, another point mutation was described for the first time in the position 265 of the *gyrA* gene, corresponding to an amino acid substitution (Asp-89-Tyr) in the GyrA protein. This new mutation decreased the MIC value for ciprofloxacin to 64mg/L, while the mutation at position 254 or the presence of the two mutations presented a MIC value higher than 256mg/L (Ferreira et al., 2018).

The molecular analysis of the genome of *A. butzleri* RM4018 showed that it presents genes related with resistance to chloramphenicol (*cat* gene) and β-lactam antibiotics (three putative β-lactamases and the operon *lrgAB*) (Miller et al., 2007). Additionally, the analysis of a metagenome of *A. cryaerophilus* from sewage showed that this species had a high capacity to accumulate antibiotic resistance genes, mostly corresponding to genes that confers resistance to macrolides, fluoroquinolones, aminocoumarin and vancomycin (Millar and Raghavan, 2017).

1.6 Pathogenesis

1.6.1 Adhesion, invasion and cytotoxicity

Not much is known about the mechanisms used in the pathogenesis of *Arcobacter*. However, the pathogenesis of *Arcobacter* has been studied with the analysis of nine putative virulence genes (*cadF*, *cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *hecA*, *hecB* and *irgA*). These genes have been found in the genome of *A. butzleri* RM4018 and have been associated to pathogenesis in other taxa (Collado and Figueras, 2011; Douidah et al., 2012; Ferreira et al., 2015, and references therein). The genes *cadF* and *cj1349* are related with cell adhesion by the production of two fibronectin binding proteins (CadF and Cj1349); *ciaB* encodes the invasion protein CiaB, while the *mviN* gene codifies a protein related with peptidoglycan biosynthesis homologue of MviN in *Escherichia coli*; the *pldA* and the tlyA genes codify a phospholipase with haemolytic activity and an haemolysin, respectively, while the *hecB* gene is related with the haemolysis activation; the *hecA* gene produces an adhesin of the filamentous hemagglutinin family and the gene *irgA* codifies an outer membrane protein regulated by iron (IrgA). The analysis of these genes was performed by PCR with primers developed from the sequences present in the genome of *A. butzleri* RM4018 by Douidah et al. (2012). In the study of Douidah et al. (2012), the nine virulence genes were simultaneously present in 14.3% of the *A. butzleri* strains (n=192). Since this first study, several ones have been published and are summarized in Table 2. These studies showed that six of these

Table 1. Cases of *Arcobacter* infection. Adapted from Salas-Massó et al., 2018 and updated with the study of Barboza et al., 2017b*.

					Antimicrobial susceptibility											
Country	**Patients sex/age**	**Duration**	**Presentation/Duration**	**Species**	**E**	**GN**	**CIP**	**AMP**	**CLR**	**TET**	**AMG**	**Other susceptibilities**	**Other resistances**	**Treatment**	**Recovery**	**Underlying disease**
Intestinal																
Australia	M/35y	6 months	Chronic diarrhea and abdominal pain. Coinfection: *Iodamoeba butchlii* + *Entamoeba coli*	*Ac*				NS						NS	NS	None
Belgium	M/73y	2 months	Chronic diarrhea	*As*				NS						None	10 days	Prosthetic aortic heart valve
Chile	M/2y	3 months	Chronic mucous diarrhea and	*Ab*	S	S	S	R	R	R	NS			E	10 days	None
	F/1y	4 months	Chronic diarrhea with abdominal cramps and pain		S	S	S	R	R	R	NS			E	10 days	None
Germany	M/48y	12 days	Acute watery diarrhea and abdominal cramps	*Ab*	S	NS	NS	R	NS	S	S	QUI	MZL, AMC, CEPH, SXT	OFX	3 days	Type 1 diabetes mellitus
	F/52y	3 weeks	Chronic diarrhea and abdominal cramps	*Ab*	S	NS	NS	R	NS	S	NS		MZL, AMC, CEPH, QUI	DXC	2 days	Alcohol abuse, hyperuricemia
Italy	4M & 6F 3y - 7y	NS	Abdominal pain and occasional vomiting with no diarrhea or fever	*Ab*				NS						None	5-10 days	None
Spain	M/26y	3 weeks	Persistent bloody and watery diarrhea	*Ac*	R	S	R			NS		AMC		AMC	8days	Acute gastroenteritis 4 months earlier
Turkey	M/30y	NS	Acute watery diarrhea, abdominal pain, nausea and sweating	*Ab*	S	NS	S			NS		AMK, DXC, PIPT, LEV, NA	CFR, CD	CIP	2 days	None
Costa Rica*	F/27y	2 months	Chronic diarreah of 2 months that changed to bloody watery diarrhea	*Ac*				NS						None	NS	None
Extraintestinal																
China	F/63y		Peritonitis (fever, abdominal pain)	*A.* sp				NS						CFZ + LEV, TIC	15 days	End stage renal-failure
Hong	F/69y		Bateremia (fever)	*Ab*				NS						CFR + MET	3 days	Gangrenous appendicitis
Taiwan	F/72y		Bateremia (fever, hematogenous pneumonia, purulent sputum, stool loose	*Ac*	S	NS	S	S	S	R	NS	AMC, CFZ, CTX, CTZ, AZT, CLA, TOB	CFZ, MIN, SXT, RIF	CTZ + TOB	14 days	Chronic renal failure
	M/60y		Bacteremia (fever)	*Ab*		NS		S		NS		AMC, CLA	CPH, CFR, CTX	CFR	4 days	Chronic hepatitis B and liver cirrhosis.
UK	Neonate		Bacteremia (hypotension, hypothermia, hypoglycemia)	*Ab*				NS					AMX, PIP, CFR, CAZ, CTZ, AMC,SXT	P + CTX	6 days	Placenta previa, prenatal bleeding and delivery at 26th week
USA	M/85		Bacteremia (fever, hypotension)	*Ab*				NS						VAN + PIPT	3 days	Chronic lymphocytic leukemia (CLL)

NS: Not specified; *Ac*: *A. cryaerophilus*; *Ab*: *A. butzleri*; *As*: *A. skirrowii*; AMG: Aminoglycosides; AMK: Amikacin; AMP: Ampicillin; AMX: Amoxicillin; AMC: Amoxicillin/clavulanic acid; CAZ: Ceftazidime; CD: Clindamycin; CEPH: Cephalosporin (any); CFR: Cefuroxime; CFZ: Cefazolin; CIP: Ciprofloxacin; CLR: Chloramphenicol; CTR: Ceftriaxone; CTZ: Ceftizoxime; DXC: Doxycycline; E: Erythromycin; GN: Gentamicin; LEV: Levofloxacin; MIN: Minocycline; NA: Nalidixic acid; OFX: Ofloxacin; P: Penicillin; PIP: Piperacillin; PIPT: Piperacillin/tazobactam; QUI: Quinolones (any); SXT: Trimethoprim-sulfamethoxazole; TE: Tetracycline; TIC: Ticarcillin/clavulanic acid; TOB: Tobramycin; VAN: Vancomycin.

genes (*cadF*, *cj1349*, *ciaB*, *mviN*, *pldA* and *tlyA*) are present in all the *A. butzleri* strains isolated from animals, humans and food processing plants (Karadas et al., 2013; Tabatabaei et al., 2014; Sekhar et al., 2017). These six genes also showed high prevalence in foods (Karadas et al., 2013; Tabatabaei et al., 2014; Jribi et al., 2017; Sekhar et al., 2017; Oliveira et al., 2018), fish (Rathlavath et al., 2017a), water (Karadas et al., 2013; Rathlavath et al., 2017a), vegetables (Mottola et al., 2016a), and in dairy plants and farms (Piva et al., 2017) (Table 2). The lower prevalence of these six genes (Table 2) was detected in shellfish isolates of *A. butzleri* (Collado et al., 2014; Mottola et al., 2016b; Rathlavath et al., 2017a). The other three genes (*hecA*, *hecB* and *irgA*) showed low prevalence in all the isolation sources (Table 2). In *A. cryaerophilus,* the genes that were present in all the tested strains were the *ciaB* gene in isolates recovered from food processing plants, the *mviN* gene in isolates from shellfish, and the *cadF* and *mviN* genes in isolated from vegetables (Collado et al., 2014; Tabatabaei et al., 2014; Mottola et al., 2016a, 2016b) (Table 2). Interestingly, in vegetables only the latter mentioned genes (*cadF* and *mviN*) were detected. However, the number of isolates studied was very low (n=4) (Mottola et al., 2016a). The other genes showed prevalence ranging from 0% to 95.6%, being the most prevalent the *ciaB* gene in animal (94.6%) and food (93.2%) strains, and the *mviN* gene in humans (95.6%) (Table 2). In the case of the species *A. skirrowii*, the analysed genes showed an incidence that ranged between 0% and 96.5% (Table 2), being the *ciaB* gene the most prevalent in animal strains (Douidah et al., 2012; Tabatabaei et al., 2014; Sekhar et al., 2017). The low prevalence of most of the genes in *A. cryaerophilus* and *A. skirrowii* can be due to the absence of the gene or to the presence of some heterogeneities in the primer binding sites of the gene sequence that do not allow the amplification (Douidah et al., 2012). However, all these studies were only focused in the presence or absence of the mentioned genes, and nothing is known about the functions of expression of them and its similarity with these proteins in other genera (Collado and Figueras, 2011).

Another important virulence factor is the presence of flagellum, that allows the bacteria to infect and invade cells and is also related with chemotaxis (Ho et al., 2008b; Miller and Parker, 2011). Ho et al. (2008b) studied the role of the flagellin genes *flaA* and *flaB* in five *Arcobacter* species and demonstrated that only the gen *flaA* is essential for the motility in one mutant strain of *A. butzleri*. The genome of *A. butzleri* RM4018 shows the presence of all the genes related with the flagella structure, however, genes *flgM* or *rpoN*, responsible of the transcription regulation in other Epsilonproteobacteria were not present in this genome (Miller et al., 2007). Nonetheless, the functions of these missing genes could be carried out by extracytoplasmic sigma factors present in the genome of *A. butzleri* RM4018 (Miller and Parker, 2011).

The potential of *Arcobacter* species to adhere, invade and produce cytotoxicity has been reviewed by Collado and Figueras (2011) and update by Ferreira et al. (2015). These reviews evidenced that the most common interactions between *Arcobacter* and the different cell lines tested were adherence and cytotoxicity, occurring in the 71.3% and 88.5% of the tested strains, respectively (Ferreira et al., 2015). The capacity of *Arcobacter* to invade cells was lower (47.3%)

Table 2. Presence of virulence genes in *Arcobacter* species from different sources.

Species	Source	*cadF*	*ciaB*	*cj1349*	*mviN*	*pldA*	*tlyA*	*hecA*	*hecB*	*irgA*
A. butzleri	**Animals**[a, b, c]	139/139 (100)	139/139 (100)	139/139 (100)	139/139 (100)	139/139 (100)	139/139 (100)	31/109 (28.4)	59/109 (54.1)	28/109 (25.7)
	Human[a, c, d]	96/96 (100)	96/96 (100)	96/96 (100)	96/96 (100)	96/96 (100)	96/96 (100)	19/89 (21.3)	59/89 (66.3)	33/89 (37.1)
	Food processing[b]	36/36 (100)	36/36 (100)	36/36 (100)	36/36 (100)	36/36 (100)	36/36 (100)			
	Food[b, c, d, e, f]	138/149 (92.6)	146/149 (98.0)	146/149 (98.0)	149/149 (100)	148/149 (99.3)	147/149 (98.6)	34/93 (36.5)	44/93 (47.3)	31/93 (33.3)
	Shellfish[g, h, i]	90/114 (79.0)	88/114 (77.2)	58/114 (50.9)	89/102 (87.2)	76/102 (74.5)	79/102 (77.4)	26/114 (22.8)	24/102 (23.5)	31/114 (27.2)
	Fish[i]	75/81 (92.6)	81/81 (100)	81/81 (100)	81/81 (100)	79/81 (97.5)	78/81 (96.3)	8/81 (9.9)	15/81 (18.5)	12/81 (14.8)
	Water[d, i]	33/35 (94.3)	32/35 (91.4)	34/35 (97.1)	35/35 (100)	33/35 (94.3)	34/35 (97.1)	10/35 (28.6)	10/35 (28.6)	12/35 (34.3)
	Vegetables[j]	36/40 (90.0)	40/40 (100)	40/40 (100)	40/40 (100)	40/40 (100)	40/40 (100)	12/40 (30.0)	28/40 (70.0)	0/40 (0)
	Dairy plants[k]	174/178 (97.7)	178/178 (100)	176/178 (98.9)	175/178 (98.3)	175/178 (98.3)	178/178 (100)	46/178 (25.8)	33/178 (18.5)	30/178 (16.8)
	Farm[k]	30/34 (88.2)	34/34 (100)	30/34 (88.2)	34/34 (100)	31/34 (91.2)	30/34 (88.2)	0/34 (0)	1/34 (2.9)	0/34 (0)
A. cryaerophilus	**Animals**[a, b, c]	43/112 (38.4)	106/112 (94.6)	62/112 (55.3)	102/112 (91.1)	36/112 (32.1)	45/112 (40.2)	3/91 (3.3)	5/91 (5.5)	2/91 (2.2)
	Humans[a, l, m]	15/24 (62.5)	22/24 (91.6)	17/24 (70.8)	22/23 (95.6)	14/23 (60.9)	12/23 (52.2)	2/24 (8.3)	8/23 (34.8)	1/24 (4.2)
	Food processing[b]	9/13 (69.2)	13/13 (100)	5/13 (38.5)	11/13 (84.6)	4/13 (30.8)	5/13 (38.5)			
	Food[b, c, e, f]	26/44 (59.1)	41/44 (93.2)	30/44 (68.2)	37/44 (84.1)	27/44 (61.4)	26/44 (59.1)	12/25 (48.0)	6/25 (24.0)	15/25 (60.0)
	Shellfish[g, h]	4/25 (16.0)	22/25 (88.0)	3/25 (12.0)	21/21 (100)	7/21 (33.3)	14/21 (66.7)	6/25 (24.0)	0/21 (0)	0/25 (0)
	Vegetables[j]	4/4 (100)	0/4 (0)	0/4 (0)	4/4 (100)	0/4 (0)	0/4 (0)	0/4 (0)	0/4 (0)	0/4 (0)
A. skirrowii	**Animals**[a, b, c]	20/58 (34.5)	56/58 (96.5)	21/58 (36.2)	26/58 (44.8)	18/58 (31.0)	20/58 (34.5)	3/43 (7.0)	10/43 (23.5)	0/43 (0)
	Food processing[b]	3/5 (60.0)	5/5 (100)	3/5 (60.0)	4/5 (80.0)	1/5 (20.0)	3/5 (60.0)			
	Food[b, c]	4/7 (57.1)	5/7 (71.4)	4/7 (57.1)	5/7 (71.4)	1/7 (14.3)	2/7 (28.6)			
	Shellfish[g]	2/21 (9.5)	15/21 (71.4)	5/21 (23.8)	13/21 (62.0)	4/21 (19.0)	11/21 (52.4)	1/21 (4.8)	1/21 (4.8)	0/21 (0)
Arcobacter spp.*	**Animals**[n, o]	39/48 (81.2)	40/48 (83.3)	38/48 (79.2)	41/48 (85.4)	39/48 (81.2)	39/48 (81.2)	21/48 (43.7)	18/48 (37.5)	1/48 (2.1)
	Food[n]	3/15 (20.0)	6/15 (40.0)	2/15 (13.3)	8/15 (5.3)	1/15 (6.7)	8/15 (5.3)	1/15 (6.7)	4/15 (26.7)	1/15 (6.7)

[a]Douidha et al. (2012); [b]Tabatabaei et al. (2014); [c]Sekhar et al. (2017); [d]Karadas et al. (2013); [e]Oliveira et al. (2018); [f]Jibri; [g]Collado et al. (2014); [h]Mottola et al. (2016b); [i]Rathlavath et al. (2017); [j]Mottola et al. (2016[a]); [k]Piva et al. (2017); [l]Barboza et al. (2017b); [m]Figueras et al. (2014); [n]Barboza et al. (2017a); [o]Oliveira et al. (2017). *Species not specified in the studies.

than the other two interactions (Ferreira et al., 2015). However, the capacity of *Arcobacter* to adhere,invade and produce cytotoxicity depends on the strains and the cell line used (Collado and Figueras, 2011). In a study of Levican et al. (2013b) in which the interactions of 16 *Arcobacter* species with the human carcinoma cells Caco-2 were tested, 87.5% of the species showed capacity of cellular adhesion (with the exception of *A. bivalviorum* and *A. aquimarinus*) and 62.5% of the species invaded the cells. Regarding the ability to produce cytotoxicity, several studies demonstrated the ability of *Arcobacter* to produce toxicity on African green monkey kidney (Vero) and Chinese hamster ovary (CHO) cells (Collado and Figueras, 2011 and references therein). Despite that, a cytolethal distending toxin (CDT) similar to the one of *Campylobacter* have not been found in *Arcobacter* (Johnson and Murano, 2002; Miller et al., 2007), evidencing that another toxin could be the agent that produces this cytotoxicity.

1.6.2 Host immune response

Interactions of *Arcobacter* spp. with the host immune response had been reviewed by Ferreira et al. (2015). The complement system, that helps to remove microorganisms and cells affected during infection processes, showed activity against *A. butzleri*. However, sensitive levels depend on the *Arcobacter* strain origin, being strains isolated from bacteraemia more resistant than strains from other origins (Ferreira et al., 2015). Regarding the production of defensins that decrease the bacterial populations, Veldhuizen et al. (2006) demonstrated that in experimental infections with *A. cryaerophilus* in porcine intestine cell lines (IPI-21) no expression of these peptides was observed. In a study developed by Ho et al. (2007), the species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* were tested for cytokine responses of human (Caco-2) and porcine (IPI-21) epithelial cells. This study demonstrated that the infection by these *Arcobacter* species induced the production of the cytokine Interleukin-8 (IL-8) and the consequent inflammatory process (Ho et al., 2007). The Toll-Like Receptor (TLR-4), a transmembrane receptor of macrophages and dendritic cells related with innate and adapted immunity, has been widely studied in *A. butzleri* (Gölz et al., 2016a, and references therein). These studies used gnobiotic or germ-free mice and demonstrated that the immune response, both local and systemic, is dependent of the TLR-4 expression (Gölz et al., 2015a) and that the union of lipopolysaccharides (LPS) and lipooligosaccharides (LOS) to the membrane receptor TLR-4 produced the increment of the inflammatory response (Heimesaat et al., 2015a). Furthermore, the absence of TLR-4 receptors produced a diminution in the apoptosis of colonic cells (Gölz et al., 2015b) and in the number of immune response cells (Heimesaat et al., 2015b). The presence or absence of TLR-4 receptors produces changes in the expression of genes related with inflammatory and regulatory immune responses (Gölz et al., 2016b; Heimesaat et al., 2016). The ability of *Arcobacter* to adhere and invade epithelial cells, along with the production of cytotoxicity and inflammatory response can be the mechanisms used by the bacteria for the production of illness. Additionally, Bücker et al. (2009) demonstrated the ability of *A. butzleri* to decrease the expression of claudin-1, -5 and -8 in tight junctions that produced the destruction of the epithelial barrier and the increase in paracellular transport. This mechanism is related with the leak efflux diarrhoea produced by *Arcobacter*, as schematized by Collado and Figueras (2011) (Figure 3).

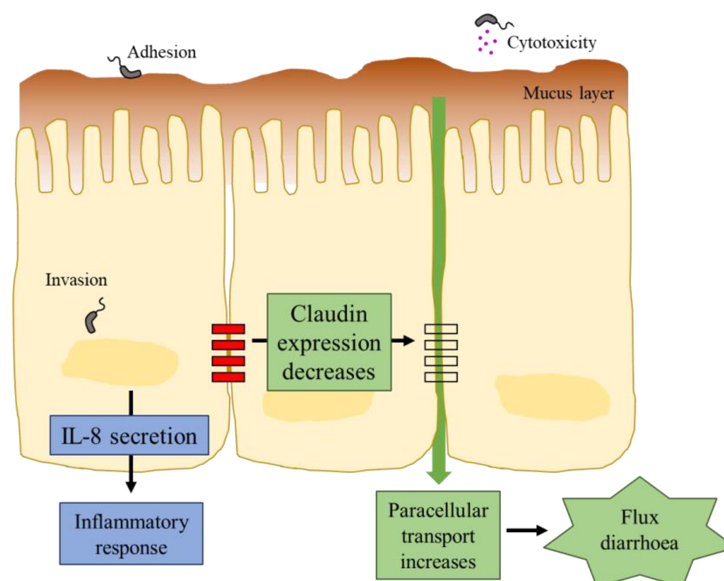

Figure 3. Mechanisms in the pathogenesis of *Arcobacter* in intestinal epithelial cells. Adapted from Collado and Figueras (2011).

1.6.3 Animal models

Several studies investigated the *Arcobacter* infections in different animal models, as piglets, chickens and turkeys (Wesley et al., 1996; Wesley and Baetz, 1999), mice (Gölz et al., 2016a; and references therein) and zebrafish (Açik et al., 2016). The most important results of these studies were the demonstration that the virulence of *Arcobacter* depends of the infected host and the species studied. For example, Wesley et al. (1996) tested the infection of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* in a one-day-old caesarean-derived colostrum-deprived piglets. In this study *A. butzleri* exhibit the longer bacteria shedding and the most invasiveness, while *A. cryaerophilus* and *A. skirrowii* did not show these activities (Wesley et al., 1996). This behaviour of *A. butzleri* was not present in chickens or turkeys, however this species colonized and even killed Beltsville white turkeys (Wesley and Baetz, 1999). The behaviour of *A. cryaerophilus* in orally infected Rainbow trout (*Oncorhynchus mykiss*) was quite different. In these studies, the bacteria produced degeneration in the respiratory system and viscera haemorrhage, inflammation of intestine and spleen, and finally produced the death of the host (Yildiz and Aydin, 2006). Açik et al. (2016) tested *A. butzleri* infection in Zebrafish (*Danio rerio*) using intraperitoneal and immersion infections and showed microscopic lesions in some organs and tissues, however, macroscopic lesions or clinical symptoms were not observed.

1.7 Genomics

A total of 48 *Arcobacter* genomes and 19 metagenomes were publicly available at the GenBank database up to 11th May, 2018 (https://www.ncbi.nlm.nih.gov/genome/?term=arcobacter) and are summarized in Table 3. From them, 7 genomes are at complete status, 4 belonging to *A. butzleri* (RM4018, NCTC 12481^T, 7h1h and ED-1 strains), one to *A. nitrofigilis* DSM 7299^T and 2 to *Arcobacter* sp. (L and LPB0137 strains). Only in 5 of these genomes (*A. butzleri*, *A. nitrofigilis*, *A. anaerophilus*, *A. thereius* and *Arcobacter* sp. CAB) an almost complete functional analysis had been

performed (Miller et al., 2007; Pati et al., 2010; Carlström et al., 2013; Roalkvam et al., 2015; Rovetto et al., 2017), and 6 have been published as genome announcements only describing the genomic characteristics obtained after their annotation (Toh et al., 2011; Merga et al., 2013; Adam et al., 2014a, 2014b, 2014c; Mizutani and Tanaka, 2017) (Table 3). The genome of *A. butzleri* RM4018, isolated from a clinical human sample, was the first published genome of *Arcobacter* (Miller et al., 2007). Analyses performed on this genome evidenced that this strain possessed pathways and loci characteristic of free-living organisms and presented sulphur metabolism genes typical of *Nitratiruptor*, *Sulfurovum* and *Sulfurimonas* (Miller et al., 2007). These characteristics suggested that a re-evaluation of the taxonomy of the genus *Arcobacter* was needed. In 2017, Waite and co-workers analysed the genomes of several taxa evidencing that the proteome of the genus *Arcobacter* is more related to *Sulfurimonas*, *Sulfuricurvum* and *Thiovulum*, in agreement with the result obtained by Miller et al. (2007). Additionally, the genome of *A. butzleri* RM4018 also showed putative virulence genes homologous to the ones described for *Campylobacter* evidencing the potential pathogenicity of this species (Miller et al., 2007). The second published genome was the one of *A. nitrofigilis* DSM7299^T but in this paper, they did not analyse the relationship of the genes present with the physiological characteristics of this species (Pati et al., 2010). The latter species is the one with the biggest genome (3.19 Mb) when compared with the genomes of *A.butzleri* RM4018 (2.34 Mb) or *Campylobacter jejuni* (1.64 Mb). It has been considered that the size could be an indicative of the adaptation of *A. nitrofigilis* to the environment (Miller and Parker, 2011).

In 2013, Carlström et al. published the genome of *Arcobacter* sp. CAB isolated from marine sediment of Berkeley Marina (Berkeley, CA, USA) that was the first *Arcobacter* dissimilatory perchlorate-reducing bacteria (DPRB), being also the first DPRB *Epsilonproteobacteria* isolated in a pure culture. Furthermore, the halophilic character of the strain CAB, was not observed in other DPRB, allowing the study of this physiological trait in a new environmental background (Carlström et al., 2013). The analysis of the (per)chlorate reduction island showed that the strain CAB did not show the essential gene (*pcrC*) that encodes for the c-type cytochrome, evidencing a new metabolic pathway within the DPRB (Carlström et al., 2013). The low percentage of similarity of the 16S rRNA gene (94%) of the CAB genome and the ability to oxidize fructose and catechol are characteristics absent in other *Arcobacter* species, indicating that the CAB strain belongs not only to a new *Arcobacter* species as suggested by the authors (Carlström et al., 2013), but also to a potential new genus.

The lithoautotrophic metabolism was also described two years later in *A. anaerophilus* IR-1 by Roalkvam et al. (2015). This species used tryptone, reduced ferric iron citrate and showed the presence of genes that encodes NAD^+-reducing hydrogenase. This strain was isolated from the Utsira aquifer (Utsira, Norway) and showed a metabolic profile similar to *Nautiliales*, *Sulfurovum* and *Sulfurimonas*, in agreement with previous results of proteomic data (Miller et al., 2007; Waite et al., 2017). In this study, Roalkvam et al. (2015) compared the genome of *A. anaerophilus* IR-1 with the genomes of *A. butzleri* RM4018 and *A. nitrofigilis* DSM 7299^T and showed that the free-living strains (*A. anaerophilus* IR-1 and *A. nitrofigilis* DSM 7299^T) had common pathways for central carbon metabolism, nitrogen fixation and sulphur reduction that were absent in the genome of *A. butzleri* RM4018 (Roalkvam et al., 2015). Unlike the other two genomes, the genome of *A. anaerophilus* IR-1 did not have genes for nitrite reduction, that could indicate a good tolerance to high nitrite levels (Roalkvam et al., 2015).

Table 3. Genomes and metagenomes available at GenBank database (extracted from prokaryotes database 11th May, 2018).

Organism	Strain	Size (Mb)	GC (mol%)	Scaffolds	Genes	Proteins	Status
A. anaerophilus	IR-1	3.25	30.2	7	3421	3024	Cn
A. butzleri	NCTC 12481^T	2.35	27.1	1	2333	2253	Cp
A. butzleri	7h1h	2.25	27.1	1	2266	2176	Cp
A. butzleri	ED-1	2.26	27.1	1	2224	2146	Cp
A. butzleri	JV22	2.30	26.3	22	2428	2381	Sc
A. butzleri	L348	2.47	27.1	176	2570	2411	Sc
A. butzleri	L349	2.28	27.0	72	2282	2198	Sc
A. butzleri	L350	2.29	26.9	90	2260	2165	Sc
A. butzleri	L351	2.29	27.0	95	2259	2167	Sc
A. butzleri	L352	2.23	27.0	62	2261	2177	Cn
A. butzleri	L353	2.15	26.9	67	2202	2110	Sc
A. butzleri	L354	2.22	26.9	91	2238	2152	Sc
A. butzleri	L355	2.22	27.1	71	2250	2143	Sc
A. butzleri	RM4018	2.34	27.0	1	2332	2256	Cp
A. cibarius	LMG 21996^T	2.20	27.1	44	2211	2110	Cn
A. cryaerophilus	L406	2.02	27.4	64	2092	2020	Sc
A. cryaerophilus	L397	2.31	27.0	96	2355	2246	Cn
A. cryaerophilus	L401	2.18	27.1	83	2190	2117	Sc
A. cryaerophilus	L398	2.03	27.2	67	2083	2002	Sc
A. cryaerophilus	L400	2.21	27.3	88	2245	2138	Sc
A. cryaerophilus	L399	2.10	27.4	91	2195	2100	Sc
A. faecis	AF1078^T	2.50	27.2	53	2490	2376	Sc
A. haliotis	LMG 28652	3.50	28.2	82	-	-	Cn
A. lanthieri	AF1440	2.29	26.7	29	2289	2190	Cn
A. lanthieri	AF1581	2.26	26.8	22	2286	2186	Sc
A. lanthieri	AF1430^T	2.24	26.4	27	2240	2170	Sc
A. lekithochrous	LFT 1.7^T	3.61	28.6	436	3394	3316	Cn
A. marinus	SH-4D_Col1	2.82	27.1	69	2768	2663	Sc
A. marinus	MARC-MIP3H16	2.90	27.2	70	2908	2845	Sc
A. nitrofigilis	DSM 7299^T	3.19	28.4	1	3170	3086	Cp
A. porcinus	213	1.78	27.1	11	1868	1813	Cn
A. porcinus	216	1.78	27.1	12	1865	1809	Cn
A. porcinus	117434	1.97	27.0	9	2042	1978	Cn
A. porcinus	DU19	1.88	27.0	21	1946	1881	Cn
A. skirrowii	L403	1.81	27.8	36	1879	1810	Cn
A. skirrowii	L402	1.87	27.8	51	1950	1880	Cn
A. skirrowii	L404	1.87	27.9	83	1959	1865	Cn
A. skirrowii	L405	1.73	28.0	41	1810	1746	Sc
A. thereius	452	1.97	26.7	17	2030	1971	Cn
A. thereius	LMG24487^T	2.14	27.0	62	2224	2112	Sc
A. thereius	440	1.93	26.9	8	1969	1916	Sc
A. thereius	DU22	2.01	26.8	17	2048	1983	Sc
A. thereius	LMG 24486	1.91	27.0	1	1953	1883	Sc
Arcobacter sp.	AF1028	2.41	27.2	46	2394	2285	Sc
Arcobacter sp.	L	2.95	26.6	2	2923	2825	Cp
Arcobacter sp.	LA11	3.10	27.9	53	3044	2961	Cn
Arcobacter sp.	LPB0137	2.87	27.7	1	2816	2698	Cp
Metagenomes							
A. cryaerophilus	AZT-1	1.85	28.0	456	2113	1503	Sc
Arcobacter sp.	CPC309	2.91	27.3	170	2871	2814	Cn
Arcobacter sp.	CPC16	2.68	27.4	161	2672	2597	Cn
Arcobacter sp.	NORP36	3.32	28.4	36	3232	3096	Cn
Arcobacter sp.	NORP14	3.15	34.8	60	3186	3060	Cn
Arcobacter sp.	DOLZORAL124_29_21	1.19	29.2	128	1267	1213	Cn
Arcobacter sp.	BM504	2.68	29.0	12	2771	2562	Sc
Arcobacter sp.	BM102	3.49	28.1	42	3495	3365	Sc
Arcobacter sp.	31_11_sub10_T18	1.96	30.5	198	2021	1938	Sc
Arcobacter sp.	EP1	3.01	31.9	229	-	-	Sc
Arcobacter sp.	UBA1348	2.84	26.8	46	-	-	Sc
Arcobacter sp.	UBA2431	2.85	26.8	35	-	-	Sc
Arcobacter sp.	UBA2434	2.84	26.8	38	-	-	Sc
Arcobacter sp.	UBA2438	2.83	26.8	41	-	-	Sc
Arcobacter sp.	UBA2443	2.76	26.9	63	-	-	Sc
Arcobacter sp.	UBA4038	2.07	27.0	52	-	-	Sc
Arcobacter sp.	UBA6788	2.39	26.7	199	-	-	Sc
Arcobacter sp.	UBA6789	2.11	26.3	97	-	-	Sc
Arcobacter sp.	UBA7394	2.54	27.6	268	-	-	Sc

Cn, contig; Cp, complete; Sc, scaffold

The most deeply study comparing several genomes was recently performed by Rovetto et al (2017), in which authors completely sequenced the genome of *A. thereius* LMG 24486^T and compared it with 8 more genomes of *A. thereius*. Comparative analyses showed that the genomes are very similar, however minor differences can be found between genomes from different sources i.e. cloacal swab of duck and pig faeces (Rovetto et al., 2017). The presence of virulence genes and antibiotic resistance genes evidenced the pathogenic role of *A. thereius* and its consideration as emergent pathogen (Rovetto et al., 2017). The genome of *A. thereius* LMG 24486^T showed one cluster for thiamine autotrophy that is not present in other *Arcobacter* species (Rovetto et al., 2017; Miller et al., 2007; Pati et al., 2010). The comparison of this genome with the genome of *A. butzleri* RM4018 evidenced the presence in the genome of *A. thereius* LMG 24486^T of the type I and II restriction endonucleases and the ectoine biosynthesis pathway, while the genes involved in urease degradation where absent in this genome (Rovetto et al., 2017). All these studies evidenced differences between the species considered as human and animal pathogens and the ones considered as free-living bacteria. Thus, a deeply phylogenetic and phenotypic analysis involving all the described *Arcobacter* species must be performed to improve our knowledge and understanding of this diverse genus.

1.7.1 Genomic indexes used in taxonomy

The use of the DNA-DNA hybridization (DDH) has been widely used for species delineation (Figueras et al., 2011), however, this methodology is time consuming and produces experimental errors (Richter and Rosselló-Móra, 2009; Figueras et al., 2011). The disadvantages of the DDH technique along with the increasing genome sequencing, encouraged researchers to develop other genomic analyses or indexes that involves bioinformatic comparisons of the genomes. These new tools show a higher reproducibility and produce more accurate similarity results. The calculation of the *in silico* DNA-DNA hybridization (*is*DDH) described by Meier-Koltoff et al (2013) is the most closely related to the experimental DDH and can be easily performed using the Genome-to-Genome Distance Calculator (GGDC). In 2009, Richter and Roselló-Mora proposed the Average Nucleotide Identity (ANI) for species delineation with a cut-off value of 95-96%. There are several tools to calculate the ANI value like JSpecies (Richter and Rosselló-Móra, 2009), OrthoANI (Lee et al., 2016) or Kostas lab calculator (Goris et al., 2007; Rodrguez and Konstantinidis, 2014).

Despite the parameters to describe new bacterial species are well defined, the criteria to describe new genera are limited to a value lower than 95% for the 16S rRNA gene similarity and a G+C contents differing in more than 10% (Rosselló-Móra and Amann, 2001; Yarza et al., 2008, 2014; Tindall et al., 2010). Furthermore, the ANI and *is*DDH used for species delineation are considered not useful for genera description (Konstantinidis and Tiedje, 2005; Goris et al., 2007; Richter and Rosselló-Móra, 2009; Qin et al., 2014; Chun et al., 2018). The last years, new genomic indexes have been proposed for genus delineation like the Average Aminoacid Identity (AAI) with values of 60-80% between genomes of the same genera and the Percentage Of Conserved Proteins (POCP) with values above 50% (Konstantinidis and Tiedje, 2007; Luo et al., 2014; Qin et al., 2014). Another parameter used for evolutionary and ecological links between species has been the Relative Synonymous Codon Usage (RSCU) (Ma et al., 2015; Farooqi et al., 2016).

1.7.2 Bioinformatic tools

Several tools have been developed for the genome characterization at functional and metabolic levels including software and database that analyse the presence of virulence and resistant genes in the studied genome. For virulence gene detection the most commonly used databases are the Virulence Factors of Pathogenic Bacteria Database (VFDB) (Chen et al., 2005), Victors Database (University of Michigan, USA) and PATRIC_VF (Wattam et al., 2017). Antibiotic resistance genes detection can be performed with the Antibiotic Resistance Database (ARDB) (Liu and Pop, 2009) and the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017). An advantage of these databases is that they can be used trough the Specialty Genes tool available at the PATRIC server (Wattam et al., 2017), that is a user-friendly web page with tools for deep genomic analyses. Another database for antibiotic resistance genes detection is the Antibiotic Resistance Gene-Annotation database (ARG-ANNOT) (Gupta et al., 2014) that is periodically updated. This database must be used with the BLASTp tool (Boratyn et al., 2013). Additionally, the annotation by the Rapid Annotation Subsystem Technology (RAST) (Aziz et al., 2008) allows to detect several genes related with virulence and antibiotic resistance.

The functional and metabolic pathways can be derived from the genomic information using the Functional Comparison Tool available in the Seed Viewer (Overbeek et al., 2014) and that allows the comparison of the annotated proteins with RAST (Aziz et al., 2008) between two genomes by the reconstruction of their metabolic pathways. For the phenotypic characterization of the genomes, Weimann et al. (2016) developed the software Traitar. This software derives 67 phenotypic traits from information extracted from the Global Infectious Disease and Epidemiology Online Network (GIDEON) and from the Bergey’s Systematic Bacteriology (Goodfellow et al., 2012).

2. INTEREST AND OBJECTIVES

Four different species of *Arcobacter* have been related with human illness, being *A. butzleri* the most prevalent and with less prevalence the species *A. cryaerophilus*, *A. skirrowii* and *A. thereius*. In fact, *A. butzleri* represents one of the most common *Campylobacteraceae* species isolated from stool specimens of patients with acute enteritis. The typical presentation of an *Arcobacter* infection is watery non-bloody diarrhoea, but cases of bacteraemia and peritonitis can also be found in the literature. Despite the species *A. butzleri* have been described as an emergent pathogen a few studies about their epidemiology and genotypes have been developed. Moreover, the routes that the bacteria follows to produce infections in animal and humans is still poorly known. Different genotyping techniques have been developed, such as AFLP, ERIC-PCR, MLST, etc. to analyse the epidemiological relationships between *Arcobacter* strains. Additionally, no specific treatments for cases of *Arcobacter* infections have been proposed despite different antimicrobials have been used in several studies. The lack of a specific recommended treatment along with the increasing number of antibiotic resistance mechanisms to different antibiotics evidences the needing for performing more studies using clinical strains that may help to clarify these aspects.

Regarding the taxonomy of *Arcobacter*, the genus has been enlarged from the two species that included the original description in 1991 to 27 species in 2018. This increment has been produced by different reasons. One of them is the utilization of new molecular techniques to analyse the phylogeny, such as the use of housekeeping genes or genomic information. Another reason has been the development of new isolation protocols including 2.5% of NaCl in the enrichment step and using marine agar for the isolation of the strains. Finally, the study of new environments such as new types of shellfish and Antarctic mammals have also increased the number of known species. Another taxonomic problem is the one related with the species *A. cryaerophilus* that has been classically differentiated in two DNA-DNA hybridization subgroups (1A and 1B) but that recent studies concluded that the nomenclature of these subgroups should be abandoned. Nonetheless, the use of genomic information can allow us to understand better the relationships between these subgroups.

An important aspect in the taxonomical field is that the similarities between the 16S rRNA gene of the *Arcobacter* species ranged from 91.2% to 99.6%, evidencing two main aspects: i) the 16S rRNA gene do not have enough resolution power to differentiate some *Arcobacter* species i.e. the 99.6% of similarity between *A. cloacae* and *A. ellisii*; and ii) the low similarities below the proposed cut-off for genus boundary (<95%) could represent that these species belong to a different genus. Apart from the 16S rRNA similarity mentioned above and a difference in G+C content higher than 10%, there is no standardized criteria for genus delimitations. However, the introduction in taxonomic studies that compares genomes either using different indexes (ANI, *is*DDH etc) or using phylogenomic studies of core genes may provide new tools for the potential delineation of genera that could be tested in *Arcobacter*. The increment in the number of described species along with the proposals of new families and phyla related with *Arcobacter* evidence that the taxonomy of this genus should be reviewed using the new commented technologies. The present study intends to analyse the taxonomy of *Arcobacter* using a polyphasic approach including genomic information and to increase the knowledge about the epidemiology and virulence potential of clinical strains recovered from human infections.

To achieve these goals, we developed the following specific objectives:

1. To characterize the epidemiological relationship of clinical strains isolated from the faeces of patients from three Spanish hospitals using the MLST approach and to screen them for their antibiotic susceptibility and for the presence of virulence genes.

2. To determine, using a polyphasic taxonomic approach, including genomic information, if seven *Arcobacter* isolates obtained from different sources can be considered new species.

3. To perform a polyphasic re-evaluation of the taxonomic diversity of *Arcobacter cryaerophilus* including genomic analyses.

4. To re-assess the taxonomy of the genus *Arcobacter* using phylogenetic and genomic analyses including several genomic indexes and a phenotypic characterization.

3. MATERIALS AND METHODS

3.1 Strains used and isolation

The strains used in this thesis are shown in Supp. Table S1. A total of 78 strains corresponds to field isolates, including strains from human clinical samples, from wastewater, mammals, shellfish, etc; and 36 strains were obtained from culture collections, including reference strains (n=10) and the type strains (n=26) of all the described *Arcobacter* species (Supp. Table S1). The other 25 strains corresponded to genomes obtained from different databases (n=16) or from strains isolated in other studies (n=9).

3.1.1 Strains isolated from human faeces

A total of 28 clinical strains isolated from human faeces were used in the study 4.1. These strains were isolated from human faeces of patients from three different Spanish hospitals i.e. Hospital Sant Joan de Reus (HSJR, n=5), Hospital Juan XXIII de Tarragona (HJXXIII, n=22) and Hospital Miguel Servet of Zaragoza (HUMS, n=1). All strains were isolated from Yersinia Selective Agar (CIN agar, Cefsulodin-Irgasan-Novobiocin, BD, Madrid, Spain) or Campylosel agar (bioMérieux, Barcelona, Spain) and identified at the hospital laboratories using phenotypical tests or matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) with the Ultraflex instrument, that uses MALDI BIOTYPER 2.0 software (Bruker Daltonics, Bremen, Germany). Strains identified as *Campylobacter* or *Arcobacter* were sent to our laboratory at the University Rovira i Virgili (Reus, Spain) for further analysis. All the received strains were subcultured in Blood Agar (Merk, Madrid, Spain) and incubated at 30ºC for 24–48 h.

3.1.2 Strains isolated from shellfish and water

The strains used in the study 4.2 and several strains of the study 4.6 (Supp. Table S1) were isolated from shellfish and water samples. Shellfish samples were obtained from local fishermen of Ebro delta (Spain), while water samples were obtained from a channel contaminated with untreated human sewage from Poble Nou town (Spain) were the shellfish were exposed for 4 days. The isolation was performed as previously described by Salas-Massó et al. (2016) for shellfish samples and with modification of the protocol described by Collado et al. (2009) for water samples (Salas-Massó et al., 2016). Briefly, 10 g of shellfish flesh and intervarval liquid were resuspended in 90 ml of *Arcobacter* broth supplemented with CAT (cefoperazone [8 µg/ml], teicoplanin [4 µg/ml], and amphotericin B [10 µg/ml) and with 2.5% (w/v) of NaCl (Salas-Massó et al., 2016). For the water samples, 200 ml of the sample was concentrated by filtration using a 0.45 µm nitrocellulose membrane filter (Millipore, Darmstadt, Germany) and the filter was resuspended in 9 ml of Arcobacter-CAT broth supplemented with 2.5% (w/v) of NaCl. Samples were incubated on marine agar at 30ºC in aerobiosis for 48 h. After incubation time, both the water and shellfish samples were processed in the same way: 200 µl of Arcobacter-CAT broth was inoculated by passive filtration in marine agar through 0.45 µm filter (Salas-Massó et al., 2017). Marine agar plates were incubated at 30º for 48 hours in aerobiosis. Colonies with similar morphology to *Arcobacter*, i.e. small, circular, translucent and beige to off-white colonies, with entire margins and without swarming activity, were subcultured in marine agar plates for subsequent analyses.

3.1.3 Strains isolated from wastewater

The wastewater samples used in the study 4.3. were collected from the inlet of the lagooning system (tertiary treatment) of the WWTP of Reus city (Spain). The samples were collected in sterile bottles of 2 L and 200 ml of the sample was concentrated by filtration through 0.45 µm nitrocellulose membrane filter. Filters were resuspended in 1 ml of distilled water and mixed in a vortex. From this resuspension, 200 µl of the distilled water was plated by passive filtration through a 0.45 filter onto blood agar plates. Plates were incubated at 30ºC for 48 hours in aerobiosis. Colonies with similar morphology to *Arcobacter* as described above were subcultured in blood agar plates for subsequent analyses.

3.1.4 Strains isolated from mammals

During a Spanish expedition to the Antarctic peninsula in the austral summer of 2010, one isolate (AHV-9/2010) was recovered from the rectal swab of an elephant seal (*Mirounga leonina*) from the Avian island. The swab was introduced in FBP media (Gorman and Adley, 2004) supplemented with 0.5% active charcoal (Sigma Ltd) and conserved at -20ºC until the identification at the laboratory. The swab and 100 µl of the shipping media were added to 10 ml of Campylobacter enrichment broth (Lab M) with 5% horse blood (Oxoid) and supplemented with CAT (cefoperazone [8 µg/ml], teicoplanin [4 µg/ml], and amphotericin B [10 µg/ml]). The broth was incubated at 37°C for 5 days in microaerobiosis using CampyGen sachets (Oxoid). After 48 hours and 5 days of incubation, an aliquot of 100 µl was plated onto mCCDA agar plates and incubated at 37°C for 72 h in a microaerobic atmosphere. In addition, a 47 mm diameter cellulose membrane with 0.60 µm pores was placed on the surface of a Columbia agar (Oxoid) with 5% defibrinated sheep blood. Eight to ten drops of enrichment broth (200 µl) were placed onto the surface of the membrane at 48 hours and 5 days of incubation. The membrane was left for 20 to 30 min on the agar surface at room temperature until all the fluid had passed through. The plates were incubated as described above for 5 days to isolate the less common, slower growing species. Presumptive *Arcobacter* colonies as described before for the other isolation sources were only detected in Columbia agar. These colonies were isolated on blood agar for subsequent analyses.

3.2 DNA extraction and genotyping

Genomic DNA for genotyping and phylogenetic analyses was extracted from pure cultures using the InstaGene™ DNA Purification Matrix (Bio-Rad, Hercules CA, USA) following manufacturer instructions. All the strains isolated from the different sources were genotyped using the ERIC-PCR (Houf et al., 2000) in order to avoid working with clones. Additionally, clinical strains were also genotyped with the MLST method described by Miller et al. (2009). For this genotyping method, the seven housekeeping genes (*aspA*, *atpA, glnA, gltA*, *glyA*, *pgm* and *tkt*) included in the *Arcobacter* scheme of the PubMLST database (http://pubmlst.org/arcobacter/) were amplified and sequenced using primers and protocols previously described (Miller et a., 2007, http://pubmlst.org/arcobacter/). The clustering analysis of the strains was performed with a phylogenetic analysis using the clinical strains deposited in the database of the Arcobacter scheme. Sequences were aligned with ClustalW (Larkin et al., 2007) and a Neighbor-Joining (NJ) tree (Kimura, 1980; Saitou and Nei, 1987) was constructed using MEGA v6.0 (Tamura et al., 2013). Recombination and selection tests were performed using s.t.a.r.t.2 software (https://pubmlst.org/software/analysis/start2/). Primers and conditions used for the two genotyping methods are shown in Table 4.

3.3 Identification

The first approach for the identification of the strains of the study 4.2 at species level was performed using four different molecular approaches: three different m-PCR (Houf et al., 2000; Douidah et al., 2010; Khan et al., 2017) and the 16S rRNA gene RFLP (Figueras et al., 2012). Primers used are summarized in Table 4. For the identification at species level in the other studies, the *rpoB* housekeeping gene was analysed. Amplification and sequencing of this housekeeping gene was performed using primers and conditions described by Levican Asenjo (2013) (Table 4). Resulting sequences were aligned with the other described species of the genus using ClustalW algorithm (Larkin et al., 2007) implemented in MEGA v6.0 (Tamura et al., 2013). Phylogenetic analyses were performed with the Maximum Likelihood (ML) method (Nei and Kumar, 2000) with MEGA v6.0 (Tamura et al., 2013).

Table 4. Primers used for the genotyping and identification of *Arcobacter* isolates.

Method	Forward Primer	Reverse primer	Target	Size (bp)
Genotyping				
ERIC[a, b]	ERIC 1R	ERIC 2	Genome	NA
MLST[c]	aspABF	aspABR	*aspA*	477
	atpABF	atpABR	*atpA*	489
	glnABF	glnABR	*glnA*	474
	gltABF	gltABR	*gltA*	429
	glyABF	glyABR	*glyA1*	507
	pgmABF1	pgmABR	*pgm*	503
	tktABF	tktABR	*tkt*	462
	aspACF2	aspACR2	*aspA*	477
	atpACF	atpACR	*atpA*	489
	glnACF2	glnACR1	*glnA*	474
	gltACF	gltACR	*gltA*	429
	glyACF	glyACR	*glyA1*	507
	pgmACF1	pgmACR	*pgm*	503
	tktACF	tktACR2	*tkt*	462
Identification				
m-PCR 1[d]	BUTZ	ARCO	16S rRNA	401
	SKIRR	ARCO	16S rRNA	641
	CRY1	CRY2	23S rRNA	257
m-PCR 2[e]	ArcoF	ButR	23S rRNA	2061
	ArcoF	TherR	23S rRNA	1590
	ArcoF	CibR	23S rRNA	1125
m-PCR 2[e]	ArcoF	SkiR	23S rRNA	198
	GyrasF	GyrasR	*gyrA*	395
	hsp60F	hsp60R	*hsp60*	
m-PCR 3[d, f]	SKIRR	ARCO	16S rRNA	641
	GyrB-F	BGyrB-R	*gyrB*	461

(Continue...)

Method	Forward Primer	Reverse primer	Target	Size (bp)
m-PCR 3[d, f]	Cpn60-F	Cpn60-R	*cpn60*	372
	GyrAcry-F	GyrAcry-R	*gyrA*	262
	RpoB-F	RpoB-R	*rpoB*	152
	GyrAcib-F	GyrAcid-R	*gyrA*	72
16S rDNA-RFLP[g, h]	CAH16S1a	CAH16S1b	16S rRNA	1026
16S rRNA gene[i]	Anti 1	S	16S rRNA	1500
Housekeeping genes[j]				
rpoB	rpoB-Arc15F	rpoB-Arc24R	*rpoB*	900
atpA	atpA-Arc5F	atpA-Arc12R	*atpA*	751
gyrA	gyrA-Arc4F	gyrA-Arc13R	*gyrA*	1014
gyrB	gyrB-Arc-7F	gyrB-Arc-14R	*gyrB*	722
hsp60	cpn60-Arc2F	cpn60-Arc8R	*hsp60*	570

[a]Versalovic et al. (1991); [b]Houf et al. (2002), [c]Miller et al. (2009), [d]Houf et al. (2000), [e]Douidah et al. (2010), [f]Khan et al. (2017), [g]Figueras et al. (2008); [h]Marshall et al (1999), [i]Martínez-Murcia et al. (1992), [j]Levican Asenjo (2013)

For strains that clustered in a different branch from the known *Arcobacter* species, the 16S rRNA gene was amplified using primers and conditions described by Martínez-Murcia et al. (1992) (Table 4) and the phylogenetic analysis was performed with the NJ method (Kimura, 1980; Saitou and Nei, 1987) and the ML method (Nei and Kumar, 2000) with MEGA v6.0 (Tamura et al., 2013). Furthermore, to know the exact taxonomic position of the strains, a Multilocus Phylogenetic Analysis (MLPA) was performed using the concatenated sequences of the five housekeeping genes *atpA*, *gyrA*, *gyrB, hsp60* and *rpoB.* These housekeeping genes were amplified as described by Levican Asenjo (2013) (Table 4). Phylogenetic analyses of the individual housekeeping genes were performed with the (NJ) (Kimura, 1980; Saitou and Nei, 1987) and the ML (Nei and Kumar, 2000) methods.

3.4 Characterization of virulence factors and antimicrobial resistance

In the study 4.1., the presence of virulence genes and the antibiotic resistance were studied. For the detection of the virulence genes, a m-PCR described by Douidah et al. (2012) was used. With this method, the presence of nine putative genes (*cadF*, *ciaB*, *cj1349*, *hecA*, *hecB*, *irgA*, *mviN*, *pldA*, and *tlyA*) related with *Campylobacter* virulence can be detected (Douidah et al., 2012). Primers and conditions used for this analysis are shown in Table 5. *Arcobacter butzleri* LMG 10828^T was used as a positive control and water as a negative control. PCR products were detected on 2% agarose gels prepared in 1X Tris-Borate-EDTA buffet and stained with RedSafe (INtRON Biotechnology, Lynnwood, WA, USA) at 80V for 90 min, using a 100bp ladder (Thermo Fisher Scientific, Madrid, Spain) as a weight marker. The amplification products were sequenced with the same primers used for the amplification to ensure that the amplicons belonged to the expected genes.

Table 5. Primers described by Douidah et al. (2012) for the detection of virulence genes.

Target	Forward Primer	Reverse primer	Size (bp)
cadF	cadF-F	cadF-R	283
ciaB	ciaB-F	ciaB-R	284
cj1349	cj1349-F	cj1349-R	659
irgA	irgA-F	irgA-R	437
hecA	hecA-F	hecA-R	537
hecB	hecB-F	hecB-R	528
mviN	mviN-F	mviN-R	294
pldA	pldA-F	pldA-R	293
tlyA	tlyA-F	tlyA-R	230

For the antibiotic susceptibility tests the disk diffusion method was used, following recommendations of the Clinical and Laboratory Standards Institute for *Campylobacter* with some modifications (CLSI, 2015). The BBL Sensi-Disc Susceptibility Test Discs (BD, Madrid, Spain) used contained amoxicillin/clavulanate (20/10 µg), erythromycin (15 µg), gentamycin (10 µg) tetracycline (30 µg) and ciprofloxacin (5 µg). For the susceptibility test, 100 µl of a standardized culture containing 10^6 c.f.u. were plated in Blood Agar (Difco) and each disc was placed on top of the agar. Plates were incubated at 30 ºC in air and the inhibition zones were measured at 24, 48 and 72 h. Each experiment was repeated twice in parallel.

3.5 Genomics

3.5.1 DNA extraction and sequencing

A total of 45 genomes were sequenced in our laboratory, 32 from known species (Supp. Table S1). Total genomic DNA was extracted from pure cultures grown in blood agar or marine agar, depending on the requirements, with the Easy-DNA™ gDNA Purification kit (Invitrogen, Madrid, Spain) following manufacturer's instructions. The extraction and structural quality of the extracted DNA was checked by electrophoresis of 10 µl of DNA sample in a 1.5% agarose gel. The total amount of DNA was quantified using Qubit™ with the dsDNA Broad Range Assay kit (Invitrogen) and adjusted to 2ng/µl. For the genome sequencing, paired-end libraries were constructed from 50 ng of DNA with the Nextera DNA Library Preparation kit (Illumina, Lisbon, Portugal). Resulting libraries were sequenced with the MiSeq platform of Illumina generating 300x2 paired-end reads. After reads cleaning, the sequences were assembled using SPAdes (Bankevich et al., 2012) and the CGE assembler (Larsen et al., 2012) in order to select the better assembly. To ensure that the sequenced genomes corresponded to the strain of interest, the five housekeeping genes used in the MLSA analysis performed in the identification (See section 3.3) were extracted from each genome and compared with the Sanger sequences originally obtained for the first identification of the strain. The no contamination and correct strain confirmation occurred when a single and identical copy of these genes are obtained. Assembled genomes were screened for eukaryotic and prokaryotic sequences and for adaptors, using BLASTn (Boratyn et al., 2013) and VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/), respectively. Before the genome deposit at GenBank, contigs with less than 200 bp were eliminated.

3.5.2 Genome annotation

The assembled genomes were annotated using three different annotation software in order to avoid miss identifications and to reduce the number of non-annotated CDS. Some of this software were also used specifically for some analyses, as the use of Prokka annotation (Seemann, 2014) for Traitar (Weimann et al., 2016) and Roary (Page et al., 2015) software or the use of RAST annotation (Aziz et al., 2008) for the Functional Comparison tool. The annotations systems used were the Prokka v1.11 software (Seemann, 2014) using the prediction tool Prodigal V2.6 (Hyatt et al., 2010), ARAGORN v1.2 (Laslett et al., 2004) and Barnap v0.6 (http://www.vicbioinformatics.com/software.barrnap.shtml) for the annotation of rRNA genes, and the Coding sequences were also annotated with RAST (Aziz et al., 2008), the PATRIC server v3.5.2. (Wattam et al., 2017) and the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP). The latter annotation was deposited in GenBank and its publicly available.

3.5.3 Genomic characterization: phenotype, metabolism and pathogenesis.

For the virulence and antibiotic resistance genes on the genomes of the study 4.5, the databases available at the Special genes tool of PATRIC were used (Virulence Factors of Pathogenic Bacteria Database (VFDB) (Chen et al., 2005), Victors Database (University of Michigan, USA), PATRIC_VF (Wattam et al., 2017), Antibiotic Resistance Database (ARDB) (Liu and Pop, 2009) and Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017)). Additionally, the Antibiotic Resistance Gene-Annotation database (ARG-ANNOT) (Gupta et al., 2014) was also used with BLASTp algorithm (Boratyn et al., 2013). The functional and metabolic characterization of the genomes of the studies 4.5 and 4.6 was performed with the functional Comparison Tool of the Seed Viewer (Overbeek et al., 2014) using the annotations obtained with RAST (Aziz et al., 2008). Furthermore, the metabolic routes were inferred using the Traitar software (Weimann et al., 2016) from the protein coding gene files obtained after the genome annotation with Prokka v1.2 (Seemann, 2014).

3.5.4 Genomic indexes

Several genomic indexes were calculated between the genomes in the studies 4.2, 4.3, 4.4, 4.5 and 4.6. The Average Nucleotide Identity (ANI) was calculated with OrthoANI (Lee et al., 2016) (Studies from 4.2 to 4.5) and with the JSpeciesWS software (Richter et al., 2016) (Study 4.6), while the *in silico* DNA-DNA hybridization (*is*DDH) was calculated in all the mentioned studies with the GGDC software (Meier-Kolthoff et al., 2013). Additionally, in the study 4.6 other three indexes were calculated. The Average Aminoacid Identity (AAI) was calculated from the output files obtained with the Sequence-Based Comparison Tool of RAST with the Lycoming College Newman Lab AAIr calculation tool (http://lycofs01.lycoming.edu/~newman/AAI/). The Percentage Of Conserved Proteins (POCP) was calculated with BLASTp (Boratyn et al., 2013) as described by Qin et al. (2014), that considers a conserved protein when it covers an aligned region higher than 50% and shows an e-value lower than 1e-5 and a percentage of identity higher than 40% (Qin et al., 2014). Finally, the Relative Synonymous Codon Usage (RSCU) (Ma et al., 2015; Farooqi et al., 2016) was computed with the Codon Adaptation Index (CAI) (Sharpl and Li, 1987) using the CAI-calc webserver (Puigbò et al., 2008). The differences in the RSCU were statistically analysed by multinomial regression using the R package (R Core Team, 2015). Results of RSCU were represented by a Principal Component Analysis (PCA) with R package and

visualized with ggplot2 and ggfortify (Wickham, 2009; Horikoshi and Tang, 2015; Tang et al., 2016).

3.5.5 Phylogenetic analysis

A phylogenetic analysis was performed for the genomes used in the studies 4.5 and 4.6. The phylogenetic relationship of the 13 representative genomes of *A. cryaerophilus* (LMG 24291^T, LMG 10229^T, LMG 9861, LMG 9065^T, LMG 9871, LMG 29976^T, and LMG 10210) analysed in the study 4.5 was performed with the pipeline implemented in the PATRIC (Wattam et al., 2017) server using the ML estimation (Nei and Kumar, 2000) with RAxML (Stamatakis, 2014) taking as outgroup the genome of *A. trophiarum* LMG 25534^T. The PATRIC pipeline constructs a phylogeny with the homologous proteins identified by BLASTp (Boratyn et al., 2013) and clusters these proteins with the Markov Cluster Algorithm (MCL) (Dongen, 2000). After that, an alignment of the protein set is performed using MUSCLE (Edgar, 2004), and the Hidden Markov Models (HMM) were constructed with HMMER tools (Eddy, 1998).

The genomes used in the study 4.6 were phylogenetically analysed using 13 housekeeping genes extracted from the genomes and studying the core genome. For the core genome analysis, the software Roary (Page et al., 2015) was used to extract and align the core genome shared by the 55 genomes used. For this analysis, a cut-off of 80% was used for the BLASTp search. Both phylogenies were represented with the Split Decomposition Analysis (SDA) as described by Sawabe et al. (2007) with the program SplitsTree v4.14.2 (Huson and Bryant, 2006) using the neighbor net drawing (Bandelt and Dress, 1992) and Jukes-Cantor correction (Jukes and Cantor, 1969).

3.6 Phenotypical characterization

The recommendations in the minimal standards for describing new taxa of the family *Campylobacteraceae* (Ursing et al., 1994; On t al., 2017) were used for the colony morphology, the temperature and atmospheric conditions for growth, the analysis of the biochemical properties and the resistance to antimicrobial agents. All tests were carried out at least twice for the strains considered as potential new species, from the nearest species recognized after the phylogenetic analyses and for the species used as positive and negative controls. In some cases (see results from studies 4.2 to 4.4), the phenotypic characteristics of the nearest species were extracted from the literature. The morphology of the colonies was analysed from the growth obtained at 30ºC on marine agar or blood agar for 24-72 hours, depending on the requirements of the strains. A total of 39 tests were carried out, comprising tests for growth conditions (n=12) and biochemical properties (n=27). Growth conditions were evaluated on the required culture media at 22–25ºC, 30ºC, 37ºC and 42ºC in aerobiosis, microaerobiosis and anaerobiosis. The biochemical properties were evaluated at 30ºC on blood agar or blood agar supplemented with 2% NaCl, depending on the strain tested, for each condition. The biochemical properties tested included catalase, oxidase, and urease activity, nitrate reduction, glucose fermentation and H_2S production in triple-sugar iron agar, hydrolysis of casein, lecithin, starch and indoxyl acetate, growth in media supplemented with 4% NaCl, 0.05% safranin, 0.005 fuchsine, crystal violet, brilliant green, 0.1, 0.01 and 0.04% triphenyl tetrazolium chloride (TTC), 1% oxgall, 0.1% sodium deoxycholate, 1% glycine, and growth in charcoal cefoperazone deoxycholate agar (CCDA), minimal media and MacConkey agar.

For the antibiotic resistance, the tested antibiotics were nalidixic acid (30 µg l-1), cephalothin (30 µg l-1) and cefoperazone (64 mg l-1).

3.6.1 Transmission Electron Microscope

Bacterial cells were observed at the Transmission Electron Microscope (TEM) JEOL 1011 for the study of the morphology, the cell size and the presence of flagella. Cells growth on the required media were fixed in 2% glutaraldehyde in 0.1% phosphate buffer for 30 min. The suspension was mounted in a copper grid and the excess of liquid was absorbed through the grid using a cellulose filter positioned in the bottom of the grid. The preparation was negatively stained with 2% phosphotungstic acid (pH 7.5) for 1 min and the images were obtained at 100V. The cell measurements were take on the images using ImageJ software (National Institutes of Health, USA).

4. RESULTS

4.1 Antimicrobial susceptibility, virulence potential and sequence types associated with *Arcobacter* strains recovered from human faeces. (2017) Pérez-Cataluña, A., Tapiol, J., Benavent, C., Sarvis, C., Gómez, F., Martínez, B., Terrón-Puig, M., Recio, G., Vilanova, A., Pujol, I., Ballester, F., Rezusta, A., Figueras M.J. *J Med. Microbiol.* 66:1–8.

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Antimicrobial susceptibility, virulence potential and sequence types associated with *Arcobacter* strains recovered from human faeces

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Abstract

Purpose. The genus *Arcobacter* includes bacteria that are considered emergent pathogens because they can produce infections in humans and animals. The most common symptoms are bloody and non-bloody persistent diarrhea but cases with abdominal cramps without diarrhea or asymptomatic cases have also been described as well as cases with bacteremia. The objective was to characterize *Arcobacter* clinical strains isolated from the faeces of patients from three Spanish hospitals.

Methodology. We have characterized 28 clinical strains (27 of *A. butzleri* and one of *A. cryaerophilus*) isolated from faeces, analysing their epidemiological relationship using the multilocus sequence typing (MLST) approach and screening them for their antibiotic susceptibility and for the presence of virulence genes.

Results/Key findings. Typing results showed that only one of the 28 identified sequence types (i.e. ST 2) was already present in the MLST database. The other 27 STs constituted new records because they included new alleles for five of the seven genes or new combinations of known alleles of the seven genes. All strains were positive for the *ciaB* virulence gene and sensitive to tetracycline. However, 7.4 % of the *A. butzleri* and *A. cryaerophilus* strains showed resistance to ciprofloxacin.

Conclusion. The fact that epidemiological unrelated strains show the same ST indicates that other techniques with higher resolution should be developed to effectively recognize the infection source. Resistance to ciprofloxacin, one of the antibiotics recommended for the treatment of *Arcobacter* intestinal infections, demonstrated in 10.7 % of the strains, indicates the importance of selecting the most appropriate effective treatment.

INTRODUCTION

The genus *Arcobacter,* considered closely related to the genus *Campylobacter*, belongs to the family *Campylobacteraceae* [1, 2]. The differentiation between *Campylobacter* and *Arcobacter* is based on the capacity of the species of the latter genus to grow at lower temperatures and to tolerate oxygen [1]. Recently, a new classification has been proposed with a description of the new family *Arcobacteraceae* and *Epsilonbacteria* being raised to the phylum level with the new name *Epsilonbacteraeota* [3]. This reclassification was supported by a phylogenetic inference with the 16S and 23S rRNA genes as well as with 120 concatenated conserved proteins extracted from 628 *Epsilonbacteria* genomes and 33 population genomes from metagenome datasets [3]. Since the first description of the genus *Arcobacter* with species previously considered atypical aerotolerant campylobacters in 1991 and 1992 [1, 4], the genus has evolved very fast and currently, in 2017, it includes a total of 26 species [5–7]. *Arcobacter* spp. have been isolated worldwide from various sources including food products mainly of animal origin like meat, milk, as well as from vegetables, shellfish, etc. [2, 8].

In Belgium and France, *Arcobacter* is the fourth most common bacteria belonging to the family *Campylobacteraceae*

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Keywords: *Arcobacter butzleri*; *Arcobacter cryaerophilus*; MLST; virulence genes; antibiotic resistance.
Abbreviations: LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium Culture Collection; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MLST, multilocus sequence typing; NJ, neighbour joining; ST, sequence type.

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isolated from the faeces of patients with enteric disease, and the third most prevalent in South Africa [9–11]. Bacteria of this genus are considered emergent pathogens according to the International Commission on Microbial Specifications for Foods [12] because they can produce infections in humans and animals ([2, 13] and references therein). Four species, i.e. *A. butzleri*, *A. cryaerophilus*, *A. thereius* and *A. skirrowii,* have so far been related to human infections [11, 13, 14]. Among the cases reported, the most common symptoms are bloody and non-bloody persistent diarrhea but cases with abdominal cramps without diarrhea or asymptomatic cases have also been described as well as cases with bacteremia [11, 13–16]. Cases of person-to-person transmission have been reported but the most common routes have been related to the consumption of contaminated water, raw or poorly cooked vegetables, shellfish, etc. [2, 13]. *Arcobacter* have also been associated with foodborne and waterborne outbreaks [2, 17, 18].

In 2009, Miller and co-workers developed an *Arcobacter* multilocus sequence typing (MLST) scheme and PubMLST database (http://pubmlst.org/arcobacter/) based on seven housekeeping genes to determine the epidemiological relationship of the studied isolates [19]. Since then this epidemiological typing method has been used in several studies [20–24]. However, at the time of writing, only 14.8 % (127/859) of the isolates deposited in the PubMLST database were from human origin. Considering the limited number of sequences from clinical sources, it is important to continue studying these kinds of isolates to create a more robust database that can be used in epidemiological studies of the genus. Therefore, the aim of this work was to characterize clinical strains isolated from the faeces of patients from three Spanish hospitals, analysing their epidemiological relationship using the MLST approach and screening them for their antibiotic susceptibility and for the presence of virulence genes.

METHODS

Strain isolation

A total of 28 strains recovered from faeces were obtained from three different Spanish University hospitals between 2013 and 2017. One strain came from the University Hospital Miguel Servet (prefix strain HUMS) from the city of Zaragoza, five from the University Hospital Sant Joan de Reus (prefix strain HSJR) from Reus and 22 strains were received from the University Hospital Joan XXIII (prefix strain HJXXIII) from Tarragona. All strains came from patients with gastrointestinal symptoms except one that was isolated from a healthy patient during a medical check-up after returning from Africa (HSJR-6). All strains were isolated from Yersinia Selective Agar (CIN agar, Cefsulodin-Irgasan-Novobiocin, BD, Madrid, Spain) or Campylosel agar (bioMèrieux, Barcelona, Spain). Strains were identified at the hospital laboratories using phenotypical tests or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) with the Ultraflex instrument, that uses MALDI BIOTYPER 2.0 software (Bruker Daltonics, Bremen, Germany). Strains identified as *Campylobacter* or *Arcobacter* were sent to our laboratory at the University Rovira i Virgili (Reus, Spain) for further analysis. All strains were subcultured in Blood Agar (Merk, Madrid, Spain) and incubated at 30 °C for 24–48 h from where the DNA from pure cultures was extracted using InstaGene DNA Purification Matrix (Bio-Rad Laboratories, Hercules, CA, USA). Each strain was genotyped with the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), using primers and conditions previously described [25] to find potential clones. In order to verify the species identity, the *rpoB* gene was sequenced using primers (rpoB-Arc-F/rpoB-Arc-R) and conditions described by Levican [26]. The amplification product was confirmed by electrophoresis in a 1.5 % agarose gel with 3.5 µl of RedSafe (INtRON Biotechnology, Lynnwood, WA, USA) nucleic acid staining solution at 100 V for 45 min. The PCR products were diluted to a final concentration of 75 ng μl^{-1} and sequenced by Macrogen Europe. The obtained *rpoB* sequences were aligned using CLUSTALW [27] with those of all type strains of all of the known *Arcobacter* species included in our bona fide in-house database using MEGA v6.0 [28]. A phylogenetic analysis was used to identify the strains at species level using the neighbour joining (NJ) method [29, 30].

Multilocus sequence typing

The seven housekeeping genes (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm* and *tkt*) included in the *Arcobacter* scheme of the PubMLST database (http://pubmlst.org/arcobacter/) were amplified and sequenced using primers and protocols described there and published by Miller *et al.* [19]. The allele and sequence type (ST) assignments of the studied isolates were obtained using the database where they were all deposited (http://pubmlst.org/arcobacter/). Recombination and selection tests were performed using s.t.a.r.t.2 software (https://pubmlst.org/software/analysis/start2/). To analyse the clustering of our strains with the clinical strains isolated from humans and deposited in the PubMLST database, sequences of the seven genes were aligned with CLUSTALW [27] and a phylogenetic tree was constructed using the NJ algorithm [29, 30] with MEGA 6.0 [28]. Type strains of *A. thereius* and *A. cibarius* and the strain of *A. skirrowii* LMG 10238 were used as the outgroup (Fig. 1).

Detection of virulence genes

The DNA from each strain was also screened for the presence of five virulence genes (*ciaB*, *cadF*, *cj1349*, *hecA* and *irgA*) using the PCR primers and conditions described by Douidah *et al.* [31], using strain *A. butzleri* LMG 10828^T as a positive control and water as a negative control. Amplification products were analysed on 2 % agarose gels at 80 V for 90 min using a 100 bp ladder as a weight marker (Thermo Fisher Scientific, Madrid, Spain). Gels were prepared in buffer 1x Tris-Borate-EDTA (TBE) and stained with RedSafe (INtRON Biotechnology, Lynnwood, WA, USA). Image capture was performed using Gel Doc XR System (Bio-Rad, Madrid, Spain). To ensure that the

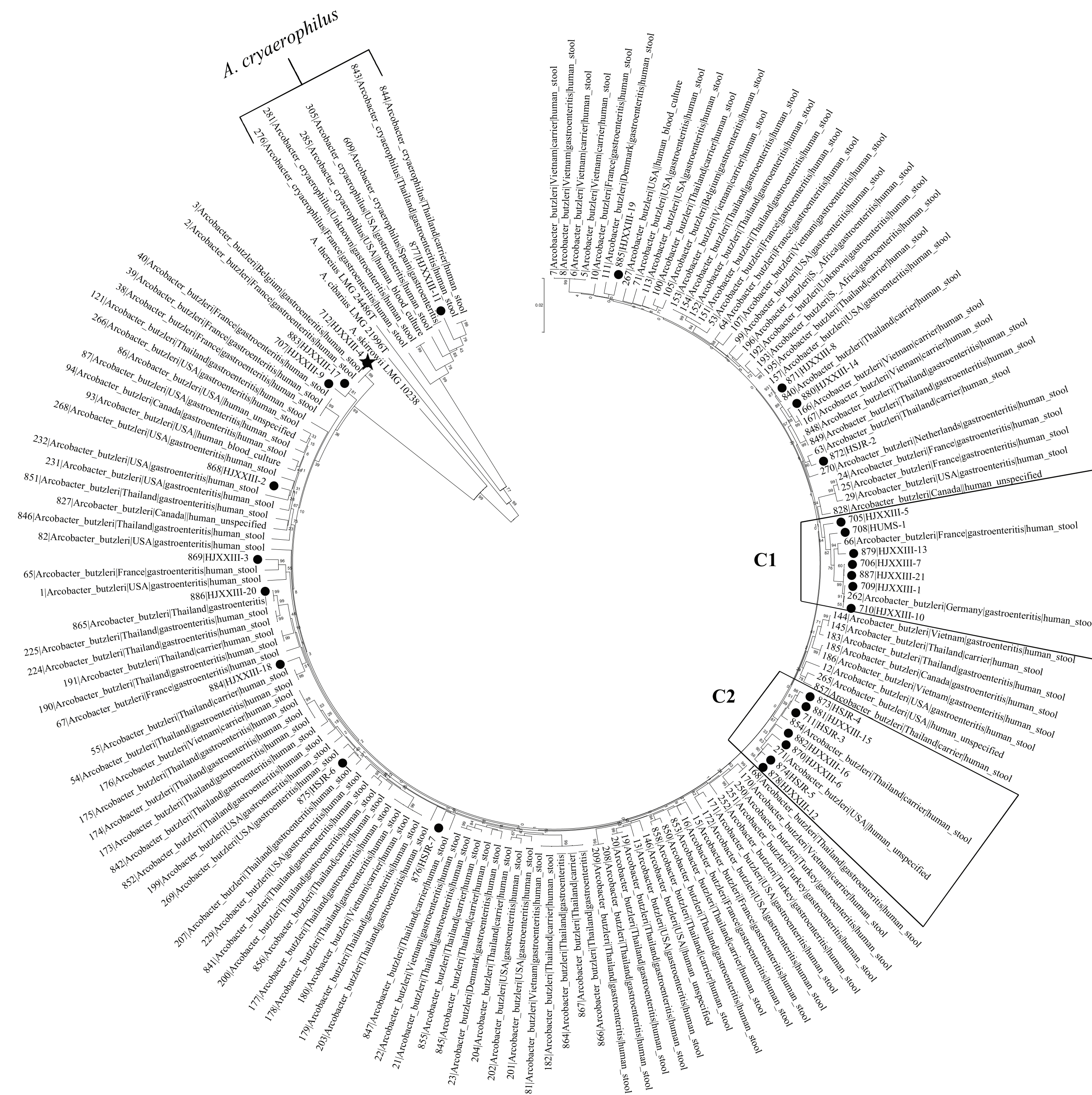

Fig. 1. NJ tree showing the distribution of the Spanish STs (black dot and black star) among the human clinical strains of *A. butzleri* and *A. cryaerophilus* available in the PubMLST database. Notice that the Spanish strain (HJXXX-4) assigned to ST2 (black star) cluster with the only two ST2 strains of the database that corresponded to gastrointestinal cases from France and Belgium. Two groups of seven Spanish strains each cluster together with other STs of the database (C1 and C2).

amplification products belonged to the expected virulence genes the PCR products were sequenced with the same primers used in the amplification.

Antibiotic resistance

The antibiotic susceptibility of each strain was assayed using the disk diffusion method following recommendations of the Clinical and Laboratory Standards Institute for *Campylobacter* with some modifications [32]. Tested antibiotics and concentrations were amoxicillin/clavulanate (20/10 µg), erythromycin (15 µg), gentamycin (10 µg) tetracycline (30 µg) and ciprofloxacin (5 µg) using BBL Sensi-Disc Susceptibility Test Discs (BD, Madrid, Spain). For each strain,

100 µl of a standardized inoculum containing 10^6 c.f.u. were plated in Blood Agar and each disc was placed on top of the agar. Plates were incubated at 30 °C in air and the inhibition zones were measured at 24, 48 and 72 h. Each experiment was repeated twice in parallel.

RESULTS AND DISCUSSION

Strain identification and multilocus sequence typing

The 28 strains received at the Unversitat Rovira I Virgili were identified at the hospitals as *A. butzleri* (*n*=22), *Arcobacter* sp. (*n*=5) and *Campylobacter* (*n*=1). The *rpoB* gene analysis showed that 27 strains belonged to *A. butzleri* and one to *A. cryaerophilus* (HSJXII-11) (data not shown). These results are in agreement with the *Arcobacter* spp. of human origin present in the PubMLST database dominated by the species *A. butzleri* (94.6 %) and *A. cryaerophilus* (5.4 %) (http://pubmlst.org/arcobacter/). The 22 strains identified with MALDI-TOF as *A. butzleri* at the hospitals were correctly assigned at species level.

The MLST alleles and ST found for the 28 strains are listed in Table 1 and their relationship with the ST of other strains of human origin included in the database is shown in Fig. 1. Only one strain (HJXXIII-4) belonged to the already known ST 2 (*aspA*-2; *atpA*-2; *glnA*-10; *gltA*-10; *glyA*-10; *pgm*-11; *tkt*-10) available in the database from only two clinical strains from France and Belgium related to gastrointestinal cases (Fig. 1). The fact that apparently epidemiologically unrelated clinical strains show the same ST, as has been previously reported for the *Legionella pneumophila* MLST schemes, indicates that this approach does not show enough resolution to discriminate the infection source, especially for worldwide distributed STs [33]. The other 27 STs were all new and therefore our study increased the number of clinical STs in the database, from 77 (at the time of writing) to 104. However, of these new STs the majority (20/27,

Table 1. MLST results of the 28 clinical strains with allelic profiles according to the *Arcobacter* MLST database

The new alleles and the resulting new ST are in bold. Strain HJXXIII-11, reference 877 at the PubMLST database is the one corresponding to *A. cryaerophilus*.

Strain	PubMLST ID	aspA	atpA	glnA	gltA	glyA	pgm	tkt	ST
HUMS-1	**708**	30^{a-d}	5^{a-d}	1^{a-e}	65^{a}	**517**	$16^{a,b,d}$	$9^{a,b,d,e}$	**483**
HSJR-2	**872**	6^{a-d}	34^{a-d}	1^{a-e}	12^{a-d}	**522**	$50^{,b,d}$	55^{a-d}	**493**
HSJR-3	**711**	80^{a}	67^{a}	49^{a}	12^{a-d}	47^{a}	44^{a}	33^{a-d}	**485**
HSJR-4	**873**	80^{a}	67^{a}	49^{a}	12^{a-d}	**524**	$2^{a,b,d}$	33^{a-d}	**494**
HSJR-5	**874**	80^{a}	67^{a}	49^{a}	$25^{a,d}$	171^{a}	**265**	$26^{a,d}$	**495**
HSJR-6	**875**	$38^{a,b}$	30^{a-c}	11^{a-d}	20^{b-d}	**518**	**264**	**208**	**496**
HSJR-7	**876**	39^{a-c}	33^{a-d}	2^{a-d}	128^{b}	**595**	**317**	4^{a-d}	**642**
HJXXIII-1	**709**	73^{a-e}	12^{a-e}	1^{a-e}	$9^{a,d,e}$	**517**	$10^{a,d,e}$	$9^{a,b,d,e}$	**484**
HJXXIII-2	**868**	**237**	45^{b}	26^{a-e}	$48^{a,b,d}$	$113^{b,e}$	85^{b}	**205**	**490**
HJXXIII-3	**869**	$8^{a,e}$	8^{a-e}	137^{c}	$166^{c,e}$	**519**	19^{d}	204^{c}	**491**
HJXXIII-4	**712**	$2^{a,b,d}$	2^{a-d}	10^{a}	10^{a}	10^{a}	11^{a-d}	$10^{a,b,d}$	2^{a}
HJXXIII-5	**705**	30^{a-d}	5^{a-d}	7^{a-d}	$26^{a,d}$	125^{d}	102^{a-e}	$58^{b,d,e}$	**470***
HJXXIII-6	**870**	80^{a}	67^{a}	49^{a}	$25^{a,d}$	**523**	**266**	$9^{a,b,d,e}$	**492**
HJXXIII-7	**706**	73^{a-e}	12^{a-e}	$30^{a,d}$	$9^{a,d,e}$	220^{e}	$10^{a,d,e}$	179^{d}	**478***
HJXXIII-8	**871**	30^{a-d}	5^{a-d}	5^{a-e}	30^{a-d}	**525**	35^{a-d}	4^{a-d}	**497**
HJXXIII-9	**707**	$24^{a,d}$	23^{a-d}	22^{d}	$25^{a,d}$	10^{a}	$86^{b,d}$	$26^{a,d}$	**482***
HJXXIII-10	**710**	73^{a-e}	12^{a-e}	1^{a-e}	65^{a}	**517**	$10^{a,d,e}$	$9^{a,b,d,e}$	**486**
HJXXIII-11	**877**	**260**	$81^{b,d}$	$61^{a,b,d}$	**177**	**560**	319^{f}	**227**	**638**
HJXXIII-12	**878**	80^{a}	67^{a}	49^{a}	$25^{a,d}$	171^{a}	**290**	$26^{a,d}$	**639**
HJXXIII-13	**879**	30^{a-d}	5^{a-d}	5^{a-e}	65^{a}	44^{a}	$10^{a,d,e}$	$9^{a,b,d,e}$	**640***
HJXXIII-14	**880**	30^{a-d}	5^{a-d}	9^{a-d}	30^{a-d}	**559**	35^{a-d}	4^{a-d}	**641**
HJXXIII-15	**881**	80^{a}	67^{a}	49^{a}	12^{a-d}	**524**	**321**	33^{a-d}	**643**
HJXXIII-16	**882**	80^{a}	67^{a}	49^{a}	23^{a-d}	**524**	30^{d}	$23^{b,d}$	**679**
HJXXIII-17	**883**	$24^{a,d}$	23^{a-d}	22^{d}	$25^{a,d}$	$56^{a,b}$	$86^{b,d}$	$26^{a,d}$	**644***
HJXXIII-18	**884**	73^{a-e}	12^{a-e}	1^{a-e}	$9^{a,d,e}$	385^{e}	263^{e}	14^{a-e}	**645***
HJXXIII-19	**885**	$15^{b,d,e}$	$10^{a,b,d,e}$	$34^{a,d}$	23^{a-d}	441^{d}	**368**	$51^{a,d}$	**677**
HJXXIII-20	**886**	$50^{a,d}$	$40^{a,d}$	$19^{a,d}$	12^{a-d}	165^{d}	$68^{a,d}$	$48^{a,d}$	**678***
HJXXIII-21	**887**	73^{a-e}	12^{a-e}	1^{a-e}	$9^{a,d,e}$	220^{e}	$10^{a,d,e}$	179^{d}	**676***

*New ST resulting from new combinations of known alleles. Source of isolation for the already existing alleles in the database are: *a*, human; *b*, animals; *c*, environmental water; *d*, food; *e*, shellfish; *f*, unknown.

74 %) were generated by the incorporation of new alleles for five of the seven genes (*glyA* 12 new alleles, *pgm* 7, *tkt* 3, *aspA* 2 and *gltA* 1) as shown in Table 1.

Genes *atpA* and *glnA* did not show any new allele. In the remaining strains (7/27, 25 %), the new STs were due to the presence, in our strains, of new combinations of known alleles. All of the known alleles were previously isolated from humans, among other sources as indicated in Table 1. In relation to the other strains and as expected, the *A. cryaerophilus* strain (HJXXIII-11, 877) was the most different, with four of the seven genes presenting new alleles, including one for the *gltA* gene, for which none of the *A. butzleri* strain showed any new allele (Table 1). The number of different alleles found for the 28 strains and for each of the seven genes was gene-specific, the genes *pgm* (22 alleles) and *glyA* (21 alleles) being the most diverse. The high diversity of *glyA* and *pmg* agrees with these being the genes with more alleles in the database (http://pubmlst.org/arcoabcter/) with 425 and 286, respectively.

When the composition of the allelic profiles was analysed in our strains, an apparent association between the alleles *aspA*-80, *atpA*-67 and *glnA*-49 known only from human origin was observed (Table 1). This was suggested by the fact that the combination of these three alleles occurred in seven of our strains (25 %, Table 1) and also in isolate 271 of the database that was also isolated from humans. None of the other strains in the database showed these allele combinations nor the alleles *atpA*-67 and/or *gln*A-49, and the allele *aspA*-80 was only found in another strain (strain 270, LMG 15577) recovered from the stool of a patient with gastroenteritis. These findings support the hypothesis that these alleles could be a human signature. Further studies would be required to confirm this observation.

The number of alleles, the synonym substitutions (d_S) and the non-synonymous substitutions (d_N) and the polymorphic sites for each locus are listed in Table 2. In fact, genes *pgm* and *glyA* that showed a higher number of alleles, i.e. 22 and 21, respectively, were also the genes that showed more polymorphic sites (Table 2). Several studies have evaluated the ratio of non-synonymous to synonymous evolutionary substitutions (d_N/d_S) of the seven genes included in the MLST approaches of several genera including *Arcobacter* [24, 34–37]. In our study, the ratios ranged from 0.0000 for the *gltA* gene to 0.1379 for the *glyA* gene. These results (d_N/d_S<1) evidenced that a negative selection occurs within these genes, in agreement with the *A. butzleri* data obtained by De Cesare *et al.* [24] with d_N/d_S ratios ranging between 0.0000 for *gltA* to 0.1246 for *atpA* among the seven loci.

Table 2. Results of recombination and selection tests for each locus

Locus	Alleles	Polymorphic sites	d_N	d_S	d_N/d_S
aspA	11	20	0.0009	0.0585	0.0161
atpA	13	23	0.0039	0.0424	0.0923
glnA	16	20	0.0015	0.0365	0.0402
gltA	14	13	0.0000	0.0473	0.0000
glyA	21	45	0.0086	0.0624	0.1379
pgm	22	38	0.0016	0.0836	0.0187
tkt	15	21	0.0014	0.0610	0.0226

Results of negative selection have also been observed in other MLST schemes using different loci for *Leptospira* species [34], *Lactococcus lactis* [36] or *Mycoplasma hominis* [37] with results of d_N/d_S in most of the cases below 0.1. The only exception is the MLST study for *Staphylococcus lugdunensis* where four of the seven genes showed d_N/d_S ratios higher than 0.2, but there was no evidence of positive selection when other selection tests (i.e. Tajima's test) were performed [35].

Epidemiological analysis

A phylogenetic analysis (Fig. 1) was performed comparing the 28 STs from Spain with all those from human origin (n=132) present in the MLST database, at the time of writing. This comparison showed that half of the Spanish STs (n=14) grouped randomly with the STs already included in the database, while the other half (14, 50 %) formed two clusters (Fig. 1). Cluster 1 (C1) included seven Spanish STs [i.e. six from the same hospital (HJXXIII) not related in time and the ST isolated from the city of Zaragoza] and two STs from other European countries (one from Germany and one from France). However, cluster 2 (C2) grouped seven Spanish STs, recovered from two hospitals [i.e. HSJR (n=3) and HJXXIII (n=4)], with two STs from strains from the USA and Thailand (Fig. 1). Apart from these associations, no other ones could be observed.

Virulence genes and antibiotic susceptibility

The pathogenic mechanisms of *Arcobacter* spp. are relatively poorly known [2]. However, the prevalence of nine putative virulence genes (*cadF*, *cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *hecA*, *hecB* and *irgA*) have been screened for using the PCR described by Douidah *et al.* [31]. The primers in the latter study were developed on the basis of only the sequences of these genes being retrieved from the genome of strain *A. butzleri* (ATCC49616^T). However, only five genes (*cadF*, *cj1349*, *ciaB*, *hecA* and *irgA*) have shown to be more abundant [31] and are those that have been previously studied [38, 39] and analysed in this study. Genes *cadF* and *cj1349* encode two fibronectin-binding proteins (CadF and Cj1349); *ciaB* encodes the invasion protein CiaB; the *hecA* gene encodes for an adhesin of the filamentous haemagglutinin family and the gene *irgA* encodes the iron-regulated outer membrane protein IrgA. The PCR results for the five virulence genes found in our study are shown in Table 3. In the *A. cryaerophilus* strain only the gene *ciaB* was detected and the absence of the other genes in this species can be due to the absence of them or to the heterogeneity of the gene sequences which may hamper the amplification [31, 38]. Genes *ciaB* and *cj1349* were present in all *A. butzleri* strains tested while gene *cadF* was detected in 96.4 % of the strains. Genes *hecA* and *irgA* were only detected in 3.5 % (n=1) and 7.1 % (n=2) of the strains, respectively. Interestingly, the

Table 3. Presence of virulence genes and antibiotic susceptibility of the 28 clinical strains

	Virulence genes					Antibiotic susceptibility				
	ciaB	cadF	cj1349	hecA	irgA	GM	AMC	E	TE	CIP
HUMS-1	+	+	+	–	–	S	S	S	S	S
HSJR-2	+	+	+	–	–	S	S	S	S	S
HSJR-3	+	+	+	–	–	S	S	S	S	S
HSJR-4	+	+	+	–	–	S	S	S	S	S
HSJR-5	+	+	+	–	–	S	S	S	S	S
HSJR-6	+	+	+	–	–	S	R	S	S	S
HSJR-7	+	+	+	+	–	S	S	S	S	S
HJXXIII-1	+	+	+	–	–	S	S	S	S	S
HJXXIII-2	+	+	+	–	+	R	R	S	S	S
HJXXIII-3	+	+	+	–	–	S	R	S	S	S
HJXXIII-4	+	+	+	–	–	S	R	S	S	S
HJXXIII-5	+	+	+	–	–	S	S	S	S	S
HJXXIII-6	+	+	+	–	–	S	S	S	S	S
HJXXIII-7	+	+	+	–	–	S	S	R	S	S
HJXXIII-8	+	+	+	–	–	S	S	S	S	S
HJXXIII-9	+	+	+	–	–	S	S	S	S	S
HJXXIII-10	+	–	+	–	–	S	S	S	S	S
HJXXIII-11	+	–	–	–	–	S	S	S	S	R
HJXXIII-12	+	+	+	–	–	S	S	S	S	S
HJXXIII-13	+	+	+	–	–	S	S	S	S	S
HJXXIII-14	+	+	+	–	–	S	S	S	S	S
HJXXIII-15	+	+	+	–	–	S	S	S	S	R
HJXXIII-16	+	+	+	–	–	S	S	S	S	S
HJXXIII-17	+	+	+	–	–	S	R	S	S	S
HJXXIII-18	+	+	+	–	–	S	S	S	S	S
HJXXIII-19	+	–	+	–	–	S	S	S	S	S
HJXXIII-20	+	+	+	–	+	S	S	S	S	R
HJXXIII-21	+	+	+	–	–	S	S	S	S	S

GM, Gentamycin; AMC, Amoxicillin/Clavulate; E, Erythromycin; TE, Tetracycline; CIP, Ciprofloxacin; R, Resistant; S, Sensitive.

only strain that was positive for the *hecA* gene was the one isolated from the healthy patient coming from Africa. The detected genes in our study are in concordance with those found from human strains by Karadas *et al.* [40] or for strains from different sources (animals, foods…) studied by Tabatabaei *et al.* [41]. However, in those studies the *cadF* gene was detected in all of the tested *A. butzleri* strains, in contrast with the studies of Levican *et al.* [38] and Collado *et al.* [42] in which they reported a lower presence of this gene in strains isolated from shellfish and sewage.

The susceptibility results (Table 3) showed that nine of the 28 tested strains (32.1 %) were resistant to at least one of the analysed antibiotics. The strain *A. cryaerophilus* HSJXXIII-11 and 7.4 % of the *A. butzleri* strains showed resistance to ciprofloxacin. Previous studies also showed resistance to this fluoroquinolone by strains of *Arcobacter* spp. of human clinical origin [43, 44]. However, ciprofloxacin has previously been recommended for the treatment of *Arcobacter* intestinal infections. The highest resistance detected among our strains (17.9 %) was for amoxicillin combined with the β-lactamases inhibitor clavulanic acid. Resistance to β-lactam antibiotics has been reported previously [45–47].

In our study only one strain of *A. butzleri* showed resistance to erythromycin (3.6 %) and the same occurred for gentamycin. However, Vandenberg *et al.* [48] found 21.3 % of the strains were resistant to erythromycin and none of them to gentamycin. Interestingly, none of our strains showed resistance to tetracycline which is another drug recommended for intestinal infections [2, 39]. The latter seems to be the most effective treatment due to the high susceptibility observed among clinical strains [44, 48, 49].

Conclusions

In the present study 28 clinical *Arcobacter* strains were analysed using a MLST approach and only one ST (ST 2) was already present in the database and the other 27 constituted new records. These resulted from the presence of new alleles in five of the seven analysed loci, evidencing a high diversity

among the clinical strains. More studies are needed to clarify the epidemiology and the molecular relationships among the different *Arcobacter* strains around the world. Most importantly, clinicians should be alerted to the acquisition of resistances to ciprofloxacin, one of the recommended treatments for intestinal *Arcobacter* infections. Furthermore, antibiotic susceptibility tests of the recovered strains should be performed before selecting any empirical treatment to ensure the use of the most effective antimicrobials and to prevent the development of more resistant clinical strains.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Arcobacter canalis sp. nov., isolated from a water canal contaminated with urban sewage

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Abstract

Four bacterial strains recovered from shellfish (*n*=3) and from the water (*n*=1) of a canal contaminated with urban sewage were recognized as belonging to a novel species of the genus *Arcobacter* (represented by strain F138-33^T) by using a polyphasic characterization. All the new isolates required 2 % NaCl to grow. Phylogenetic analyses based on 16S rRNA gene sequences indicated that all strains clustered together, with the most closely related species being *Arcobacter marinus* and *Arcobacter molluscorum*. However, phylogenetic analyses using the concatenated sequences of housekeeping genes (*atpA*, *gyrB*, *hsp60*, *gyrA* and *rpoB*) showed that all the novel strains formed a distinct lineage within the genus *Arcobacter*. Results of *in silico* DNA–DNA hybridization and the average nucleotide identity between the genome of strain F138-33^T and those of the closely related species *A. marinus* and other relatively closely related species such as *A. molluscorum* and *Arcobacter halophilus* were all below 70 and 96 %, respectively. All the above results, together with the 15 physiological and biochemical tests that could distinguish the newly isolated strains from the closely related species, confirmed that these strains represent a novel species for which the name *Arcobacter canalis* sp. nov. is proposed, with the type strain F138-33^T (=CECT 8984^T=LMG 29148^T).

The taxonomy of the class *Epsilonproteobacteria* has recently been reviewed by Waite *et al.* [1] on the basis of 16S and 23S rRNA genes as well as 120 single-copy marker proteins. The authors proposed that the class *Epsilonproteobacteria* together with the order *Desulfurellales* should be considered a new phylum, *Epsilonbacteraeota*. In this new phylum, the genus *Arcobacter* within the order *Campylobacterales* has been proposed as the only known member of the new family *Arcobacteraceae*. This genus comprises Gram-stain-negative, campylobacter-like bacteria that are able to grow at low temperatures and under aerobic conditions, these being the features that differentiate the genus *Arcobacter* from the genus *Campylobacter* [2, 3]. Since the description of the genus *Arcobacter* in 1991 [2], 26 member species have been described [4–6].

In a recent survey on the prevalence of members of the genus *Arcobacter* in water and shellfish samples, 27 isolates were recovered (13 from mussels, 12 from oysters and 2 from water) with the typical colony morphology (small, translucent, beige to pale orange) of the genus *Arcobacter* on marine agar [7]. The samples came from a canal that receives untreated urban sewage from the village of Poble Nou (40° 38′ 30.8″ N 0° 41′ 37.2″ E), to which the shellfish were exposed for 72 h [7]. The isolation protocol involved an enrichment step in Arcobacter CAT (cefoperazone, amphotericin B and teicoplanin) broth supplemented with 2.5 % NaCl, followed by sub-culturing on marine agar at 30 °C under aerobic and microaerobic conditions [7]. All the 27 presumptive isolates of the genus *Arcobacter* were Gram-stain-negative, slightly curved rods with oxidase activity, as previously described for this genus [2, 3]. All isolates were genotyped with Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) using previously described primers and conditions [8] to recognize potential clones [9]. Patterns that differed by one or more bands were considered different genotypes, as in other studies [3, 8]. Among the 13 isolates from mussels, 12 isolates from oysters and 2 from water, only four different ERIC-genotypes were recognized, represented by strains F190-2IL33 from mussels, F138-33^T and F181-1F33 from oysters and

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Keywords: *Arcobacter*; *A. canalis*; shellfish; MLPA; 16S rRNA; ANI; *is*DDH.
Abbreviations: ANI, average nucleotide identity; *is*DDH, in silico DNA-DNA hybridization; MLPA, multilocus phylogenetic analysis; m-PCR, multiplex PCR; TEM, transmission electron microscope.
†These authors contributed equally to this work.
The GenBank/EMTBL/DDBJ accession numbers for the 16S rRNA, *atpA*, *gyrB*, *hsp60*, *gyrA* and *rpoB* gene sequences of strain F138-33^T are MG015880, LT903675, LT903676, LT903678, LT903677 and LT903674, respectively. The accession number for the whole-genome sequence is NWVW00000000.
Nine supplementary figures are available with the online version of this article.

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W110-33 from water. The characterization of the four strains was initially attempted using identification methods specific for the genus *Arcobacter*, i.e. two multiplex-PCR (m-PCR) methods [10, 11] and one RFLP analysis of the 16S rRNA gene [12]. The four strains produced two amplicons of the expected size described for *Arcobacter cryaerophilus* and *Arcobacter skirrowii* with the m-PCR of Houf *et al.* [10]. However, with the m-PCR of Douidah *et al.* [11], the strains produced an amplicon corresponding to *Arcobacter butzleri*. By contrast, with the 16S rRNA gene RFLP identification method [12], the four strains produced the same pattern described for *Arcobacter marinus* using *Mse*I and *Mnl*I endonucleases. Considering the contradictory results, the *rpoB* (621 bp) genes of the four strains were sequenced using primers and conditions described previously [13, 14]. The phylogenetic tree reconstructed with the maximum-likelihood method [15] showed that the four strains clustered together forming a differential branch closely related to *A. marinus* (Fig. S1, available in the online version of this article). In order to further investigate these findings, the 16S rRNA gene and the housekeeping genes *atpA*, *gyrB*, *gyrA* and *hsp60* were also amplified and sequenced as described previously [16]. Alignments were performed using MEGA 6.0 [17] with the ClustalW algorithm [18]. The phylogenetic trees were reconstructed using the neighbour-joining [19, 20] (Figs 1 and 2) and/or the maximum-likelihood methods [15]. The trees reconstructed with the latter method with the five individual gene sequences and with the concatenated sequences are shown in Figs S1 to S7.

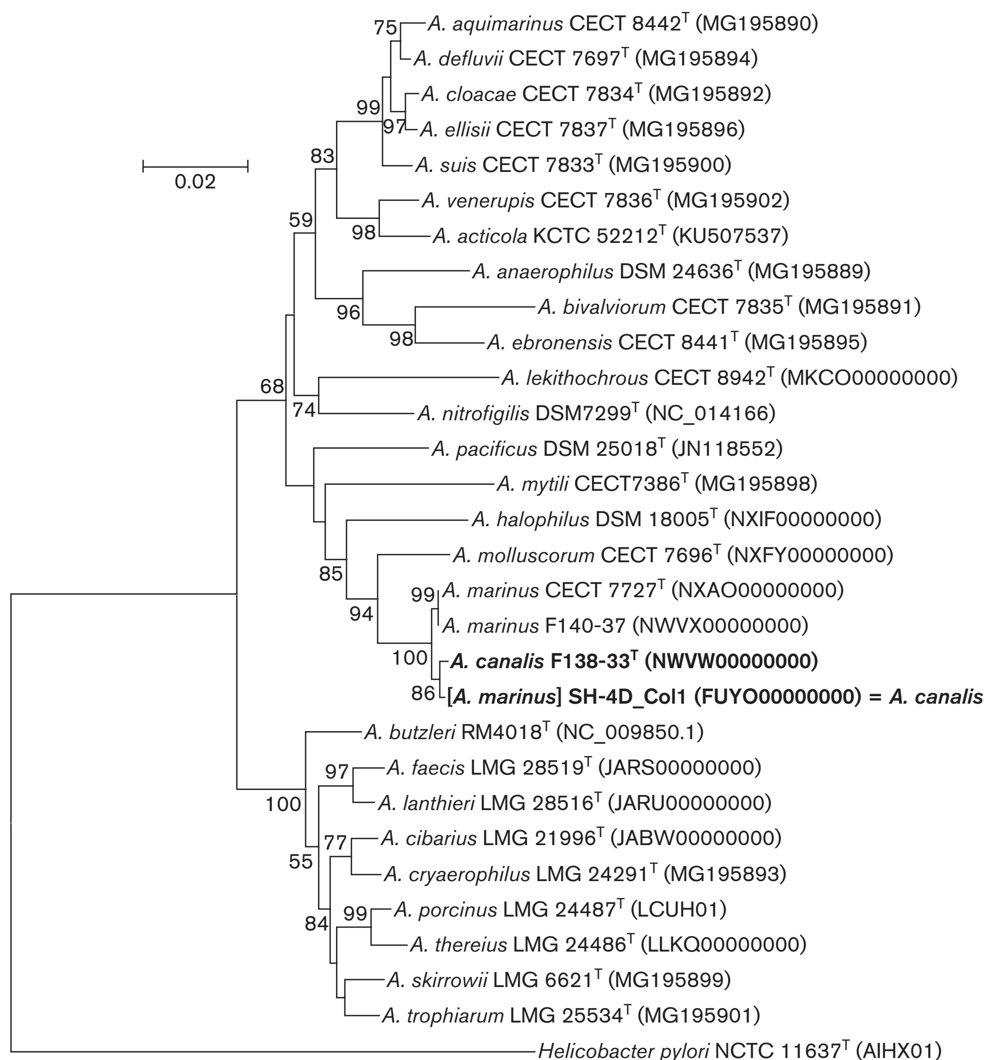

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1427 bp) showing the phylogenetic position of *A. canalis* sp. nov. within the genus *Arcobacter*. Notice that the genome deposited at the NCBI as [*A. marinus*] SH-4D_Col1 does not belong to the species *A. marinus* because it clusters with *A. canalis* sp. nov. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

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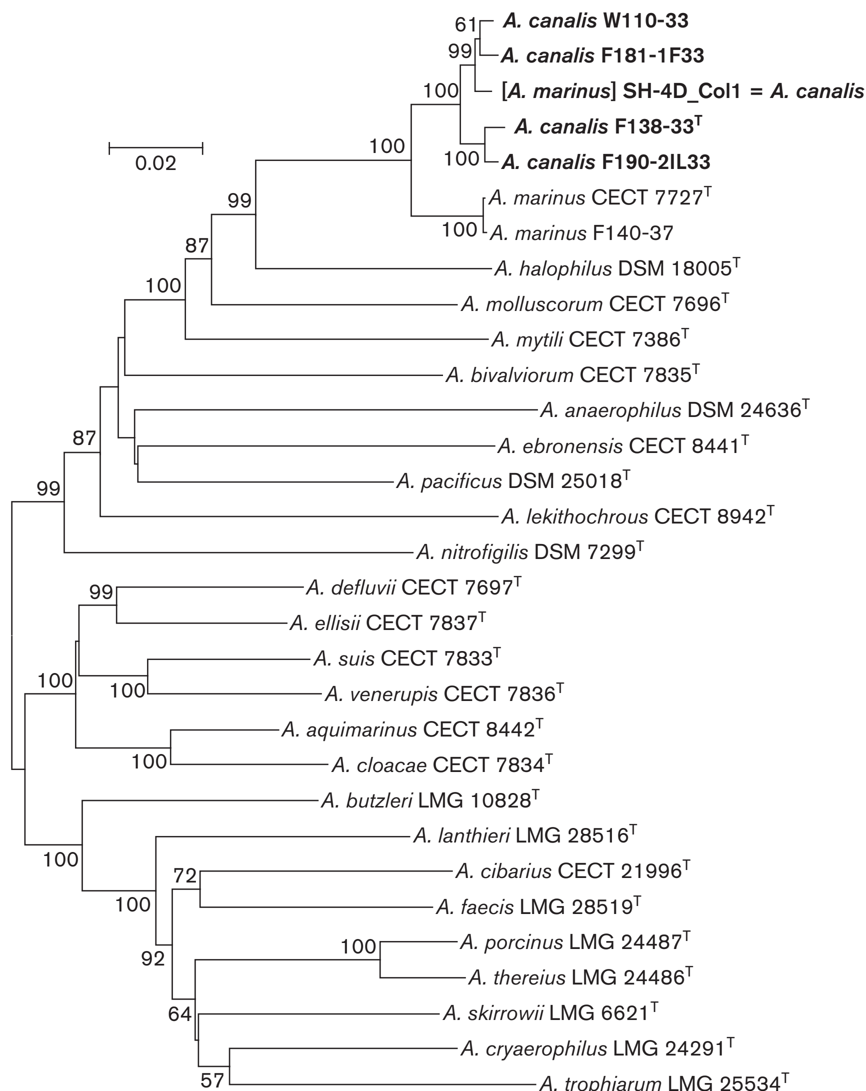

Fig. 2. Neighbour-joining tree based on the concatenated sequences of *atpA*, *gyrB*, *hsp60*, *rpoB* and *gyrA* (3039 bp) genes showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Notice that the genome deposited at the NCBI as [*A. marinus*] SH-4D_Col1, from where the gene sequences were extracted, does not belong to the species *A. marinus* because it clusters with *A. canalis* sp. nov. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.

The 16S rRNA gene of strain F138-33^{T} (chosen as a representative of the four strains) clustered in the phylogenetic tree with the type strain of the species *A. marinus*, CECT 7727^{T}, and with a sequence that came from a genome deposited at the NCBI as *A. marinus* SH-4D_Col1 (FUYO00000000) (Fig. 1). In addition, the strain *A. marinus* F140-37, isolated in a previous study [7], also clustered in the group. The similarity of the 16S rRNA genes between the potential novel species represented by strain F138-33^{T} (1503 bp) and the *A. marinus* strains CECT 7727^{T} and F140-37 was 99.7 %, while with *Arcobacter molluscorum* CECT 7696^{T} and *Arcobacter halophilus* DSM 18005^{T} it was much lower, i.e. 97.6 and 96.8 %, respectively. Interestingly, a 16S rRNA gene sequence similarity of 99.8 % was obtained between strain F138-33^{T} and the gene extracted from the genome of *A. marinus* SH-4D_Col1. The multilocus phylogenetic analysis (MLPA) performed with the concatenated sequences of the five housekeeping genes (*atpA*, *gyrB*, *hsp60*, *gyrA* and *rpoB*, 3039 bp) confirmed that the new cluster formed by the four newly isolated strains (F138-33^{T}, W110-33, F181-1F33 and F190-2IL33) represented a well separated linage from the species *A. marinus* (Fig. 2). Notably, this new cluster also included the concatenated sequences of the five genes mentioned that were obtained from the genome labelled *A. marinus* SH-4D_Col1 (FUYO00000000). This, together with the 99.8 % sequence similarity observed with

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strain F138-33^T, indicated that the genome SH-4D_Col1 belongs to the novel species.

In order to further confirm the latter finding and that we were dealing with a novel species, the genomes of five strains i.e. F138-33^T (NWVW00000000); *A. marinus* strains F140-37 (NWVX00000000) and CECT 7727^T (NXAO00000000); *A. halophilus* DSM 18005^T (NXIF00000000) and *A. molluscorum* CECT 7696^T (NXFY00000000) were obtained using the MiSeq Illumina platform and assembled with SPAdes software [21]. The average nucleotide identity (ANI) and the *in silico* DNA–DNA hybridization (*is*DDH) values were used to compare the genomes of strain F138-33^T and the species *A. marinus*, *A. molluscorum* and *A. halophilus* using OrthoANI [22] and GGDC [23] software, respectively. Table 1 shows that the ANI and *is*DDH values obtained between the genome of the newly proposed species (represented by F138-33^T) and those of *A. marinus* (strains CECT 7727^T, F140-37 and SH-4D_Col1), *A. molluscorum* CECT 7696^T and *A. halophilus* DSM 18005^T were all lower than 96 and 70 %, respectively, with the only exception being the values obtained with the genome of *A. marinus* SH-4D_Col1, which were 97.7 and 79.3 %, respectively (Table 1). In general, for separation of species, an ANI below 95–96 % [22] and an *is*DDH below 70 % [23] have been recommended. However, for the genus *Arcobacter*, ANI values above 96 % were shown to be the ones that correlated better with *is*DDH results above 70 % [5]. The ANI and *is*DDH results confirmed that the genome of *A. marinus* SH-4D_Col1 belongs to the novel species as shown by the phylogeny of the 16S rRNA gene and MLPA (Figs 1, 2). Therefore, the name of the genome sequence *A. marinus* SH-4D_Col1 should be changed in the NCBI database to *Arcobacter canalis* SH-4D_Col1 because this genome corresponds to the proposed novel species. It is clear that before the description of our novel species, the most closely related species to SH-4D_Col1 in the databases was *A. marinus* CECT 7727^T, with a 16S rRNA gene sequence similarity of 99.51 % (1417 bp). However, if identification had been done with the *rpoB* phylogeny, a branch separated from *A. marinus* (Fig. S1) could have been detected indicating that genome SH-4D_Col1 represented a distinct taxon. The genomic information derived from ANI and the *is*DDH was shown to have a higher resolution than that from the 16S rRNA gene for differentiating new species of the genus *Arcobacter*.

The DNA G+C content of strain F138-33^T was 27.5 %, in agreement with the values for *A. marinus* (27 %) and other species of the genus *Arcobacter*, which range between 26.6 and 31.9 % [24, 25].

The colony morphology, temperature and atmospheric conditions for growth as well as the biochemical properties and resistance to antimicrobial agents were evaluated following the recommendations in the minimal standards for describing new taxa of the family *Campylobacteraceae* [26], which were recently updated [27]. All tests were carried out at least twice for the four newly isolated strains and for the type strains of *A. marinus* CECT 7727^T and *A. molluscorum* CECT 7696^T and the strains of the species used as positive and negative controls for each test. Phenotypical characteristics for *A. halophilus* DSM 18005^T were derived from the description of the species [28] and from the recent re-evaluation of this species done in our laboratory [7]. Colony morphology was analysed from the growth obtained on marine agar at 30 °C under aerobic conditions for 48 h. A total of 39 tests were carried out, 12 testing growth conditions and 27 testing biochemical properties. Growth conditions were evaluated on marine agar at 22–25 °C, 30 °C, 37 and 42 °C in three different atmospheres: aerobiosis, microaerobiosis and anaerobiosis. The biochemical properties were evaluated at 30 °C on blood agar supplemented with 2 % NaCl for each condition. The biochemical properties tested included oxidase, catalase and urease activity, nitrate reduction, glucose fermentation in triple-sugar iron agar, hydrolysis of indoxyl acetate, casein, lecithin and starch, growth in media supplemented with 0.5 and 4 % NaCl, 1 % oxgall, 0.1 % sodium deoxycholate, 1 % glycine, 0.05 % safranin, 0.005 fucsine, crystal violet, brilliant green, 0.1, 0.01 and 0.04 % triphenyl tetrazolium chloride, and growth in charcoal cefoperazone deoxycholate agar (CCDA), minimal media and MacConkey agar. Resistance to nalidixic acid (30 $\mu g\ l^{-1}$), cefalotin (30 $\mu g\ l^{-1}$) and cefoperazone (64 $mg\ l^{-1}$) was also tested using TSA supplemented with 2 % NaCl and with each antimicrobial. The bacterial morphology, cell size and the presence of flagella were determined using a transmission

Table 1. Results (percentages) of ANI and *is*DDH between the genome of *A. canalis* sp. nov. F138-33^T and those of the most closely related species

The ANI values are displayed in bold type (down-left) and the *is*DDH values are in italics (up-right); values below 96 and 70 % indicate that the genomes belong to different species. Notice that ANI and *is*DDH values show that the genome [*A. marinus*] SH-4D_Col1 does not belong to this species* but to *A. canalis* sp. nov.,† in agreement with the phylogenetic results of Fig. 2.

		DNA G+C content (mol%)	1	2	3	4	5	6
1	*A. canalis* sp. nov. F138-33^T	27.3	***	*79.3†*	*63.6*	*63.5*	*22.8*	*30.4*
2	[*A. marinus*] SH-4D_Col1	27.1	**97.7†**	***	*63.3**	*63.8**	*23.0*	*30.5*
3	*A. marinus* CECT 7727^T	27.0	**95.4**	**95.3***	***	*93.9*	*22.9*	*31.3*
4	*A. marinus* F140-37	27.0	**95.4**	**95.4***	**99.2**	***	*22.8*	*30.7*
5	*A. molluscorum* CECT 7696^T	26.1	**80.3**	**80.5**	**80.0**	**80.2**	***	22.8
6	*A. halophillus* DMS 18005^T	27.4	**86.2**	**86.2**	**86.6**	**86.3**	**80.2**	***

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electron microscope (TEM), JEOL 1011. Cells were grown for 24 h in marine agar and suspended for fixation in 2 % glutaraldehyde in 0.1 % phosphate buffer for 30 min. The suspension was mounted in a copper grid and negatively stained with 2 % phosphotungstic acid (pH 7.5) for 1 min. Strain F138-33^T showed a single polar flagellum under the TEM (Fig. S8), and all the newly isolated strains showed motility under the phase contrast microscope. Fifteen tests could be considered diagnostic because they enabled differentiation of the four novel strains, F138-33^T, W110-33, F181-1F33 and F190-2IL33, from the most closely related species, *A. marinus*, and also from *A. molluscorum* and *A. halophilus* (Table 2). Differentiation from *A. marinus* was based on the capacity of the novel species to grow in marine agar at 42 °C under microaerobiosis, with 2 % NaCl and in media with 0.05 % safranin or crystal violet supplemented with NaCl and an inability to produce H_2S in triple-sugar iron media supplemented with NaCl, to reduce nitrate or to grow with 0.1 % oxgall (Table 2). Phenotypical differentiation of the novel species from the species *A. molluscorum* was based on the inability of the novel species to grow with 0.5 % NaCl, 0.1 % sodium deoxycholate, 1 % oxgall or 0.01 % triphenyl tetrazolium chloride, to resist cefoperazone (64 mg l^{-1}) or to reduce nitrate. Another differential characteristic between *A. molluscorum* and *A. canalis* sp. nov. was the capacity of the novel species to grow at 22–25 °C, 30 and 37 °C under anaerobiosis, to grow in minimal medium and to produce H_2S in triple-sugar iron media supplemented with NaCl. Differentiation between the novel species and *A. halophilus* could be demonstrated because the latter does not grow in marine agar at 42 °C under microaerobiosis, or in media supplemented with 0.05 % safranin or crystal violet, or minimal media or MacConkey agar, among several other differential characteristics, which are listed in Table 2.

The six genomes studied (F138-33^T, F140-37, SH-4D-Col1, CECT 7727^T, CECT 7696^T and DSM 18005^T) were annotated using RAST [29], and genes encoding for polar lipid syntheses were searched for. All the genomes possessed the genes that encode phosphatidylglycerolphosphatase A (*pspA*, EC3.1.3.27) and phosphatidase cytidylyltransferase (*cdsA*, EC 2.7.7.41), related with the synthesis of phosphatidylglycerol. Additionally, the genomes also contained the gene phosphatidylserine descarboxilase (*psd*, EC4.1.1.65) involved in the synthesis of phosphatidylethanolamine. These results agree with the polar lipids found experimentally in other species of the genus *Arcobacter*, such as *Arcobacter pacificus*, *Arcobacter acticola* and *Arcobacter haliotis* [4, 30, 31].

Considering that during the preparation of this paper an additional m-PCR method for the characterization of six species of the genus *Arcobacter* was described by Khan *et al.* [32], the method was tested on the type strain F138-33^T of the novel species and in parallel on *A. marinus* CECT 7727^T, *A. molluscorum* CECT 7696^T and *A. halophilus* DSM 18005^T. Strain F138-33^T and the type strains of the other three species showed an amplicon of 654 bp, which was similar to the one expected for *A. skirrowii* according to Khan *et al.* [32]. Results obtained with this method and with the m-PCRs of Houf *et al.* [10] and Douidah *et al.* [11] and the 16S rRNA gene RFLP [12] are shown in Fig. S9.

This study has demonstrated the existence of a novel *Arcobacter* species, for which the name *A. canalis* sp. nov. is proposed.

Table 2. Differential characteristics of *Arcobacter canalis* sp. nov. and type strains of the most closely related species of the genus *Arcobacter*

Taxa: 1, *Arcobacter canalis* sp. nov. (*n*=4); 2, *A. marinus* CECT 7727^T; 3, *A. molluscorum* CECT 7696^T; 4, *A. halophilus* DSM 18005^T. Unless otherwise indicated: +, ≥95 % strains positive; −, ≤11 % strains positive; V, 12–94 % strains positive; ND, not determined. All strains show catalase activity and to grow they require the media to be supplemented with 2 % NaCl. In addition, all strains grow in TSA with 4 % NaCl and in marine agar under aerobiosis and microaerobiosis at 22–25 °C, 30, 37 and 42 °C. None of the strains grow under anaerobiosis at 42 °C, in TSA with 1 % glycine, 0.01–0.1 % triphenyl tetrazolium chloride (TTC), nor in CCDA. None of the strains hydrolyse starch, casein or lecithin nor show urease activity or resistance to nalidixic acid.

Characteristic	1	2	3	4*
Growth at/with/on:				
42 °C (microaerobic)†	+	−	+	−
22–25 °C (anaerobiosis)†	+	+	−	+
30 °C (anaerobiosis)†	+	+	−	+
37 °C (anaerobiosis)†	+	+	−	+
0.5 % NaCl (w/v)	−	−	+	−
2 % NaCl (w/v)	+	−	+	+
0.05 % Safranin‡	+	−	+	−
0.005 % Basic fuchsine‡	V	−	+	−
Crystal violet‡	+	−	+	−
Brilliant green‡	V	−	−	ND
0.1 % Sodium deoxycholate‡	−	−	+	−
1 % (w/v) Oxgall‡	−	+	+	ND
0.01 % TTC‡	−	−	+	ND
Minimal medium‡	+	+	−	−
MacConkey‡	V	+	+	−
Triple-sugar iron+NaCl	+	−	−	−
Resistance to:				
Cefoperazone (64 mg l^{-1})‡	−	−	+	−
Cefalotin (30 mg l^{-1})‡	V	−	+	−
Enzyme activity				
Nitrate reduction‡	−	+	+	+
Indoxyl acetate hydrolysis§	−	+	−	+
Catalase	+	−	+	−

*Information extracted from Donachie *et al.* [28] and Salas-Massó *et al.* [7].

†These tests were carried out on marine agar.

‡These tests were carried out on TSA supplemented with 2 % NaCl.

§This test was performed under aerobic and microaerophilic conditions and produced the same results, except for *A. marinus* CECT 7727^T, which was only positive under microaerophilic conditions.

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DESCRIPTION OF *ARCOBACTER CANALIS* SP. NOV.

Arcobacter canalis (ca.na′lis. L. gen. n. *canalis* of a canal).

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.2–0.4 µm wide and 1.2–2.8 µm long. They are motile by a single polar flagellum. Colonies on marine agar incubated under aerobic conditions at 30 °C for 48 h are 2–4 mm in diameter, pale yellow to pale orange, circular with entire margins, convex and non-swarming. Pigments are not produced in marine agar. Strains grow on marine agar at 22–25, 30 and 37 °C under aerobic, microaerobic and anaerobic conditions, and at 42 °C under aerobic and microaerobic conditions. After 48 h, colonies are smaller at 37 and 42 °C than at room temperature or 30 °C. No growth is observed in blood agar at the different temperatures tested nor under the different atmospheric conditions. Produces oxidase and catalase activity. Does not hydrolyse indoxyl acetate, urea, casein, lecithin or starch. Not able to produce acid from glucose by oxidization or fermentation, but produces hydrogen sulphide in triple-sugar iron agar medium, and is not able to reduce nitrate. Under aerobic conditions at 30 °C, grows on minimal medium with 2 % NaCl and on nutrient medium supplemented with 5 % sheep blood and 2 % NaCl containing 0.05 % safranin or 0.005 % crystal violet. Additionally, strains W110-33 and F181-1F33 grow on 0.005 % basic fuchsine, 0.001 % brilliant green and MacConkey agar supplemented with 2 % NaCl, while the other strains (F138-33^T and F190-2IL33) are unable to grow in these media. No growth occurs on campylobacter charcoal deoxycholate agar (CCDA), on nutrient medium supplemented with 5 % sheep blood containing 0.5 % NaCl, 1 % oxgall, 0.1 % sodium deoxycholate, 1 % glycine or 0.01–0.1 % 2,3,5-triphenyltetrazolium chloride. Strains W110-33 and F181-1F33 are resistant to 30 µg cefalotin l^{-1} while strains F138-33^T and F190-2IL33 are susceptible. All strains are susceptible to 64 mg cefoperazone l^{-1} and 30 µg nalidixic acid l^{-1}.

The type strain is F138-33^T (=CECT 8984^T=LMG 29148^T), isolated from oysters exposed for 72 h to untreated urban sewage in Poble Nou canal, Catalonia, Spain.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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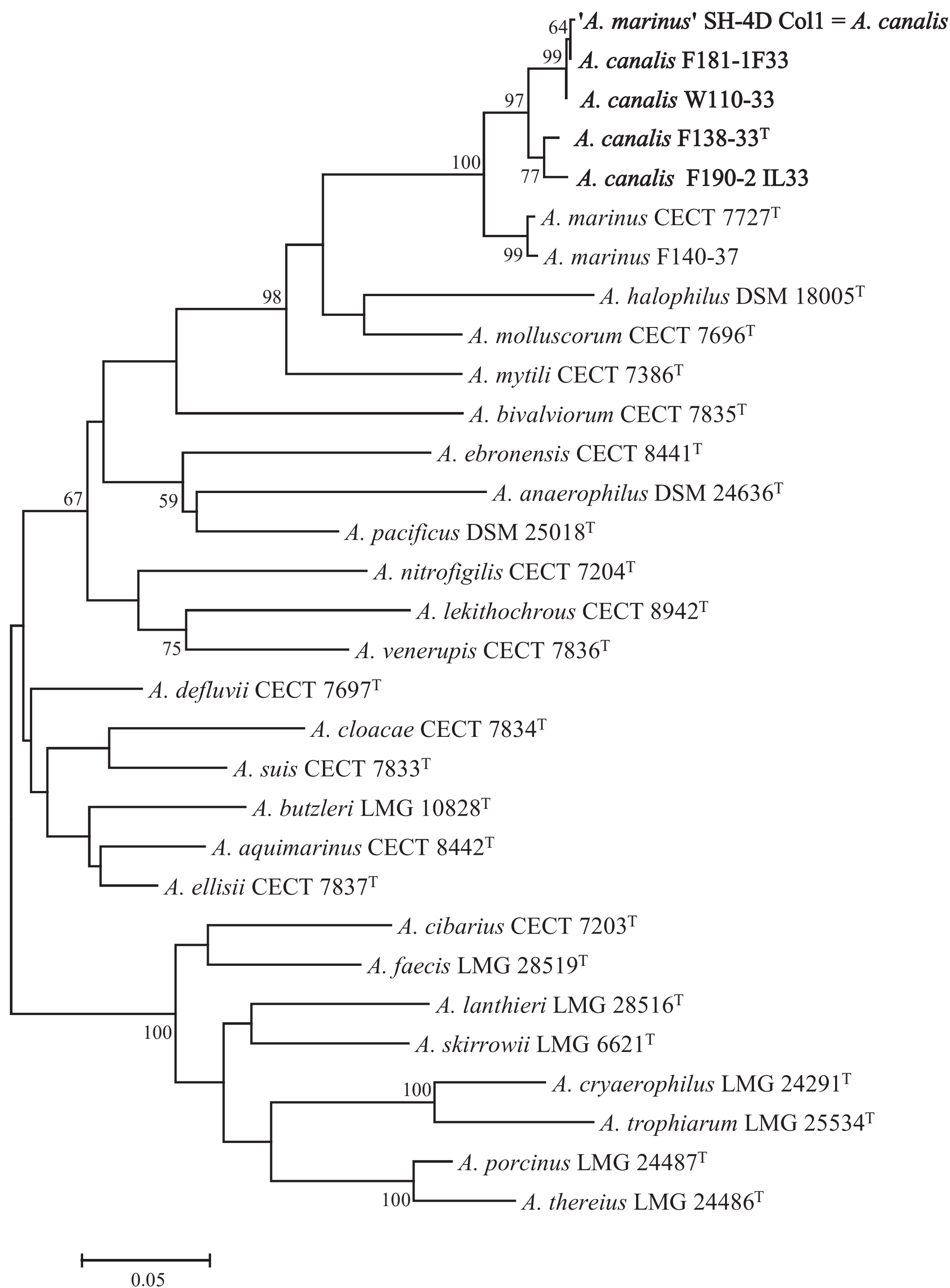
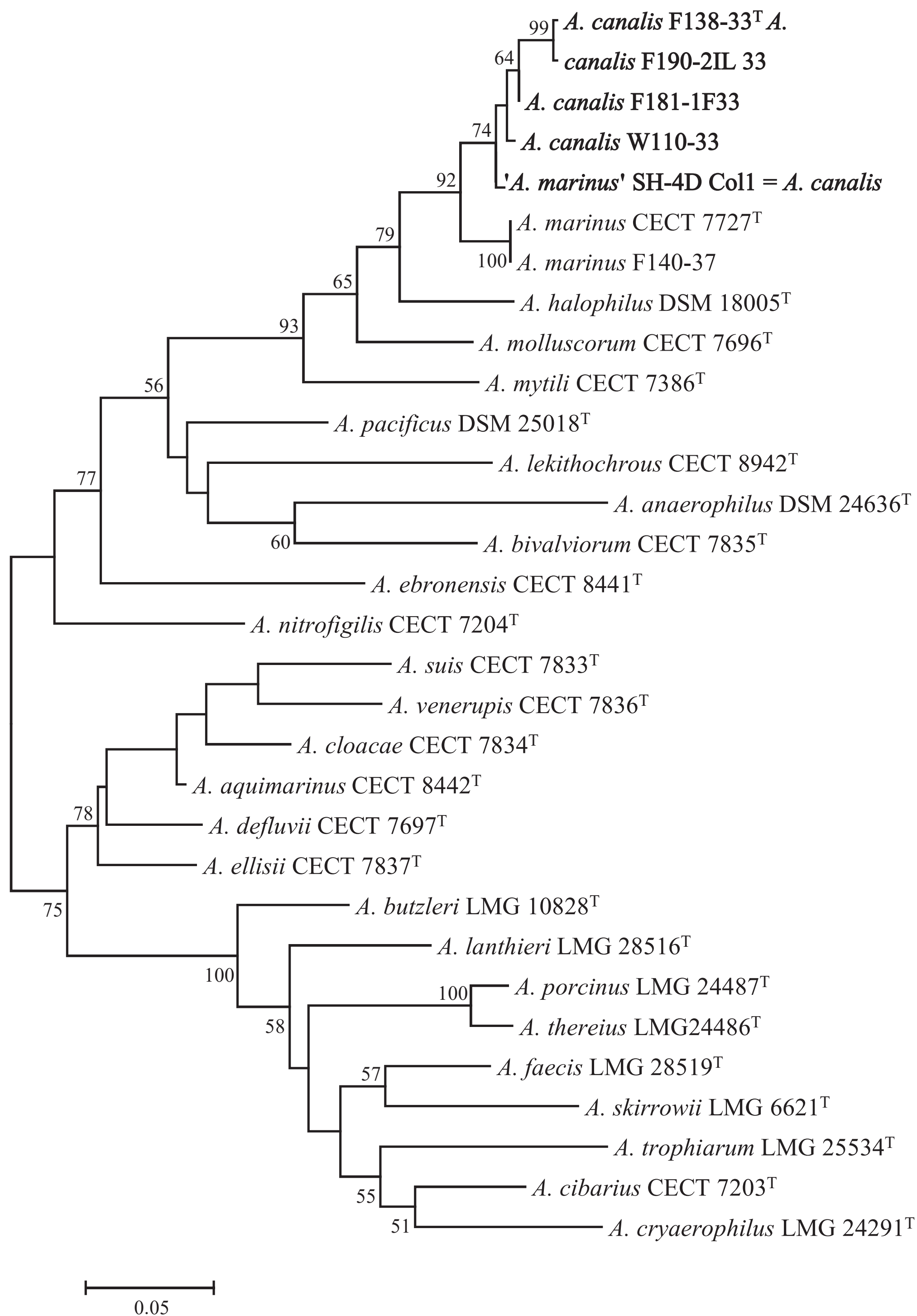
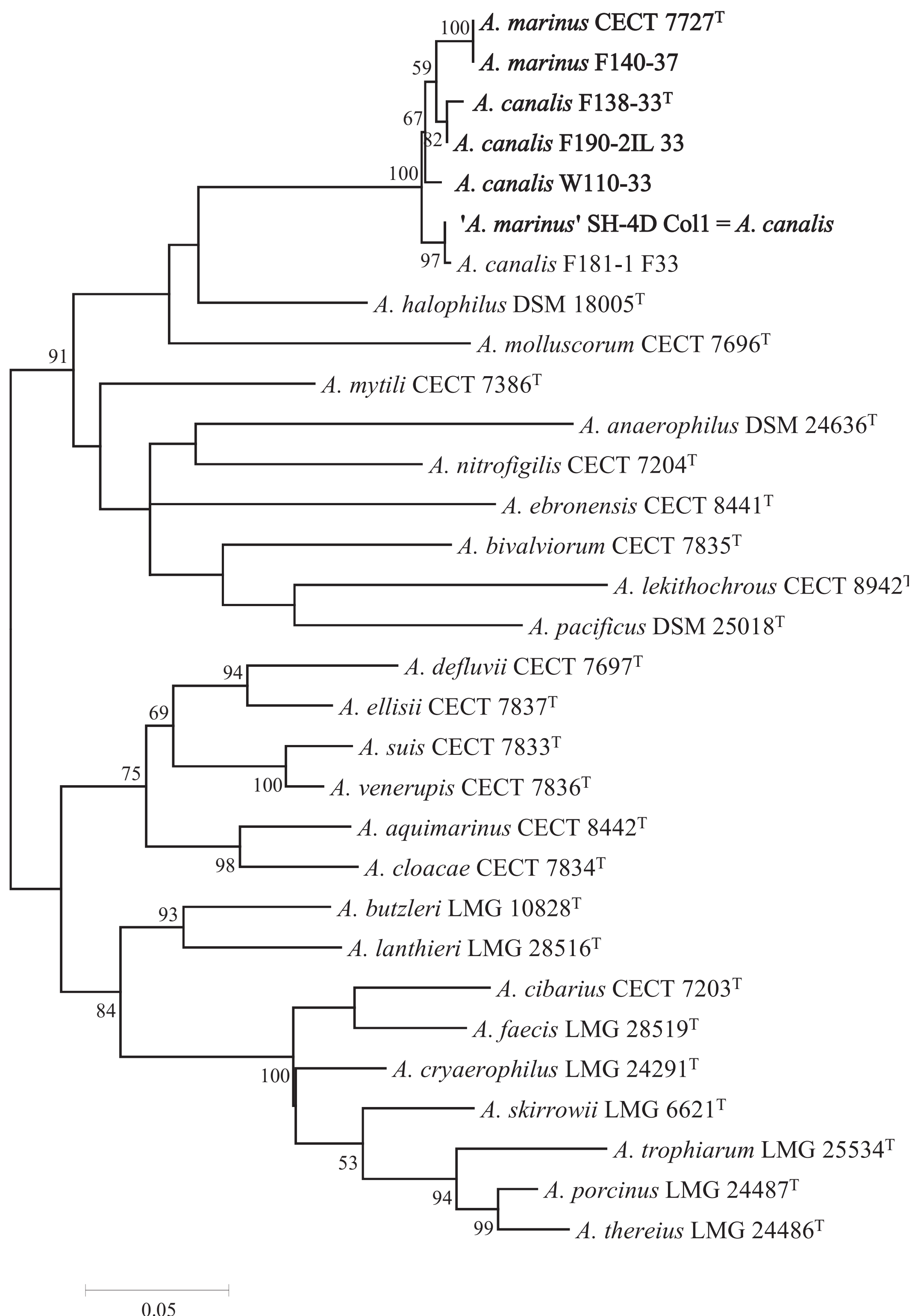


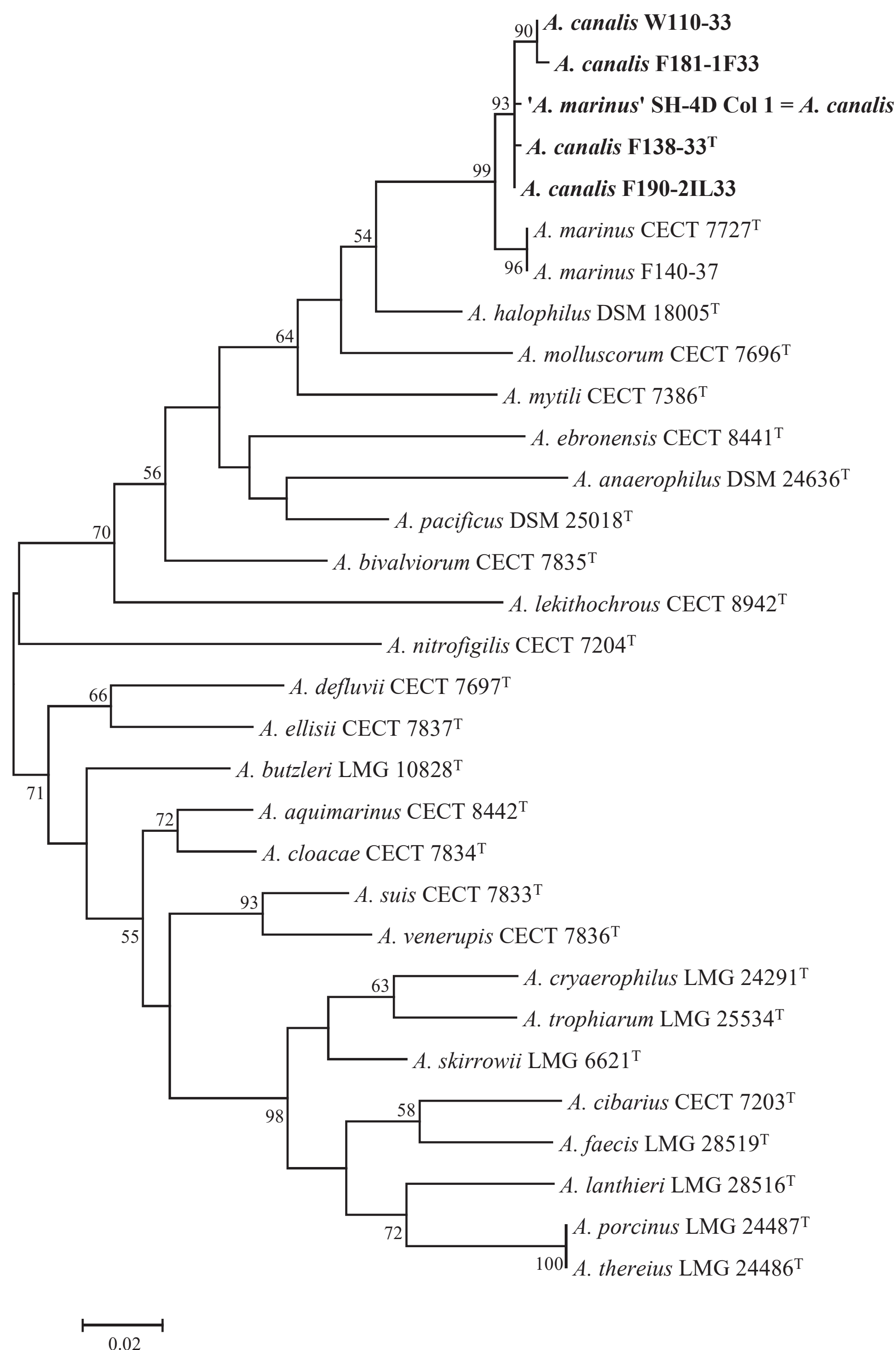
Supplementary figure S1. Maximum Likelihood tree (model GTR+G+I) based on the *rpoB* gene sequence (619 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



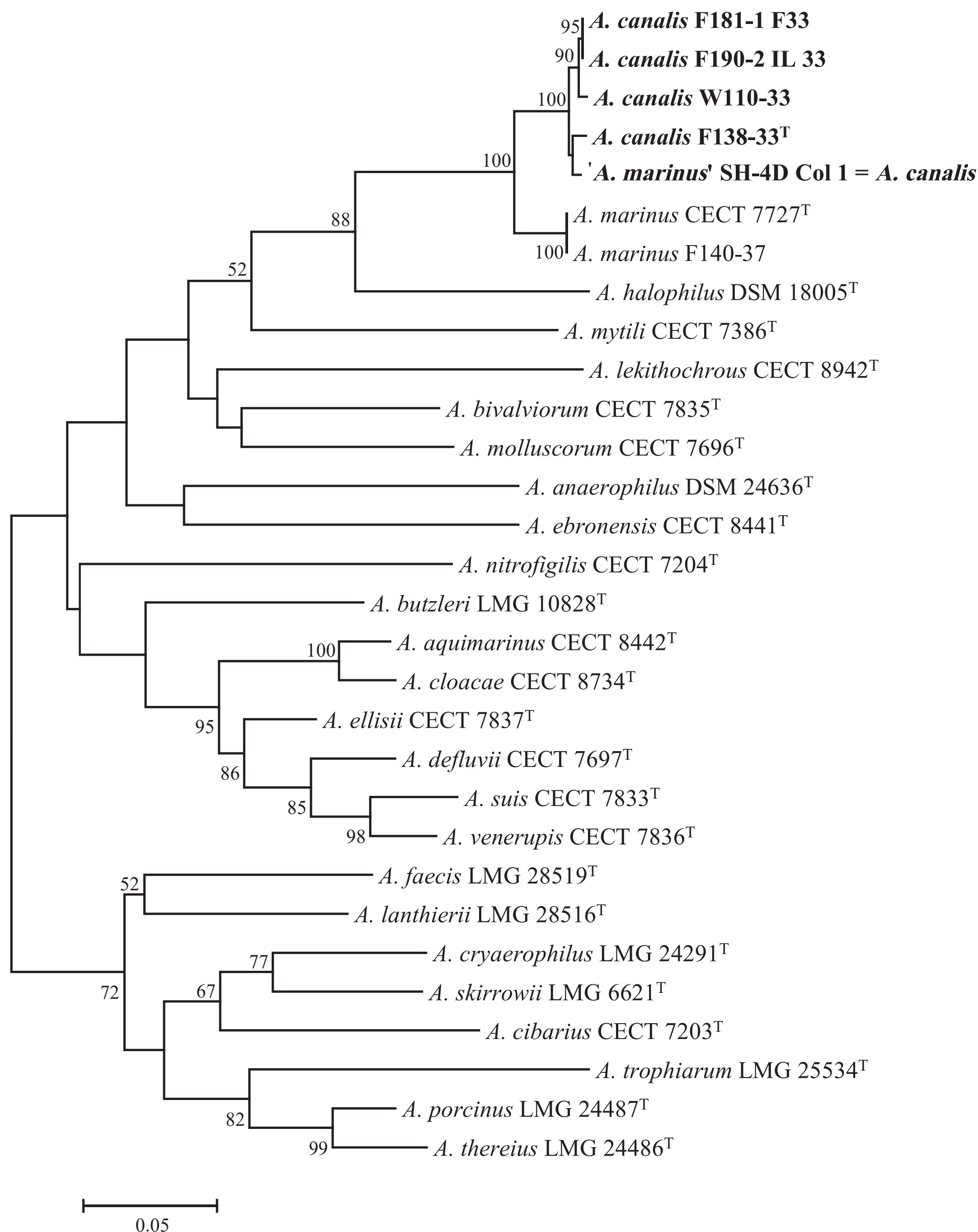
Supplementary figure S2. Maximum Likelihood tree (model GTR+G) based on the *gyrB* gene sequence (617 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



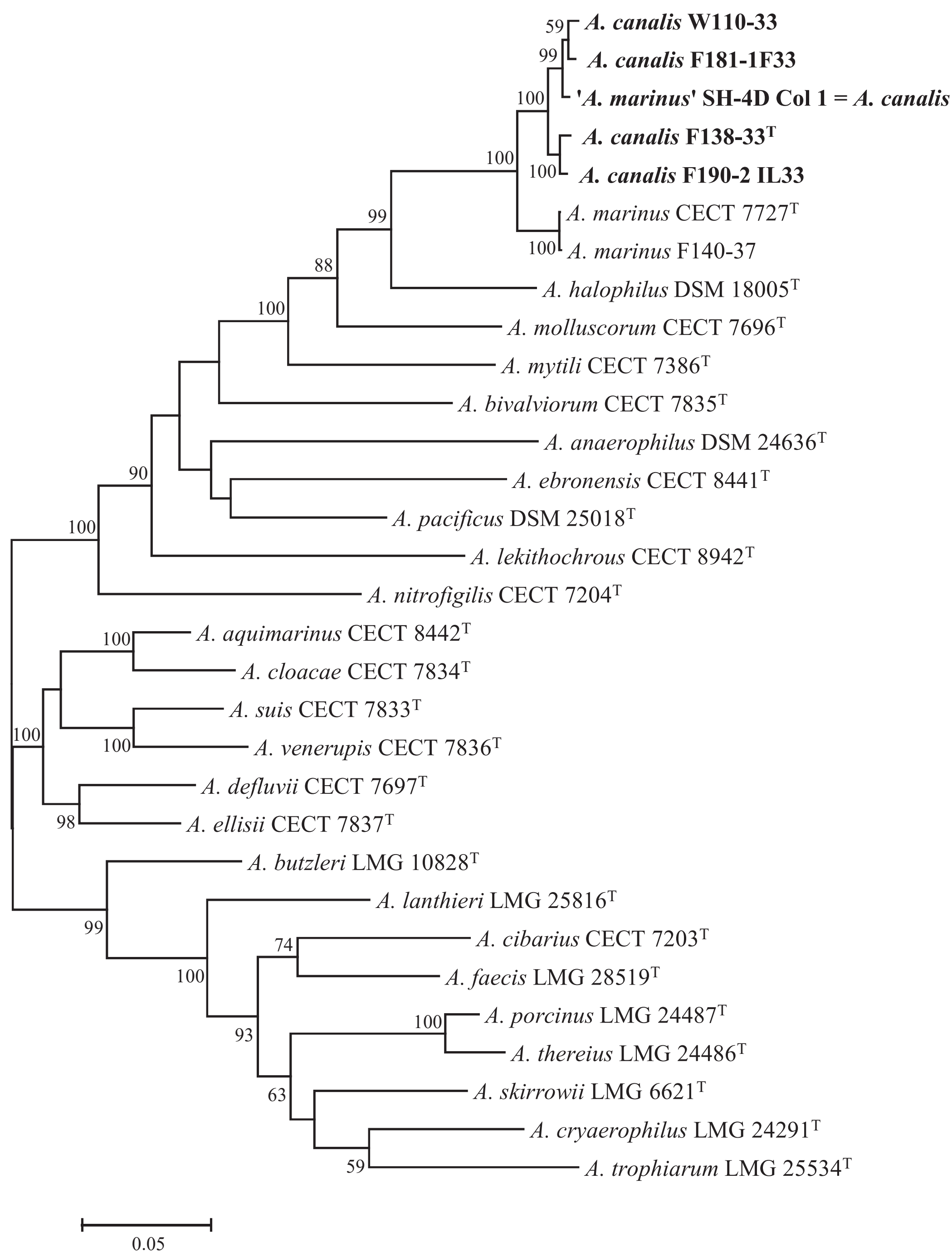
Supplementary figure S3. Maximum Likelihood tree (model GTR+G+I) based on the *hsp60* gene sequence (545 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



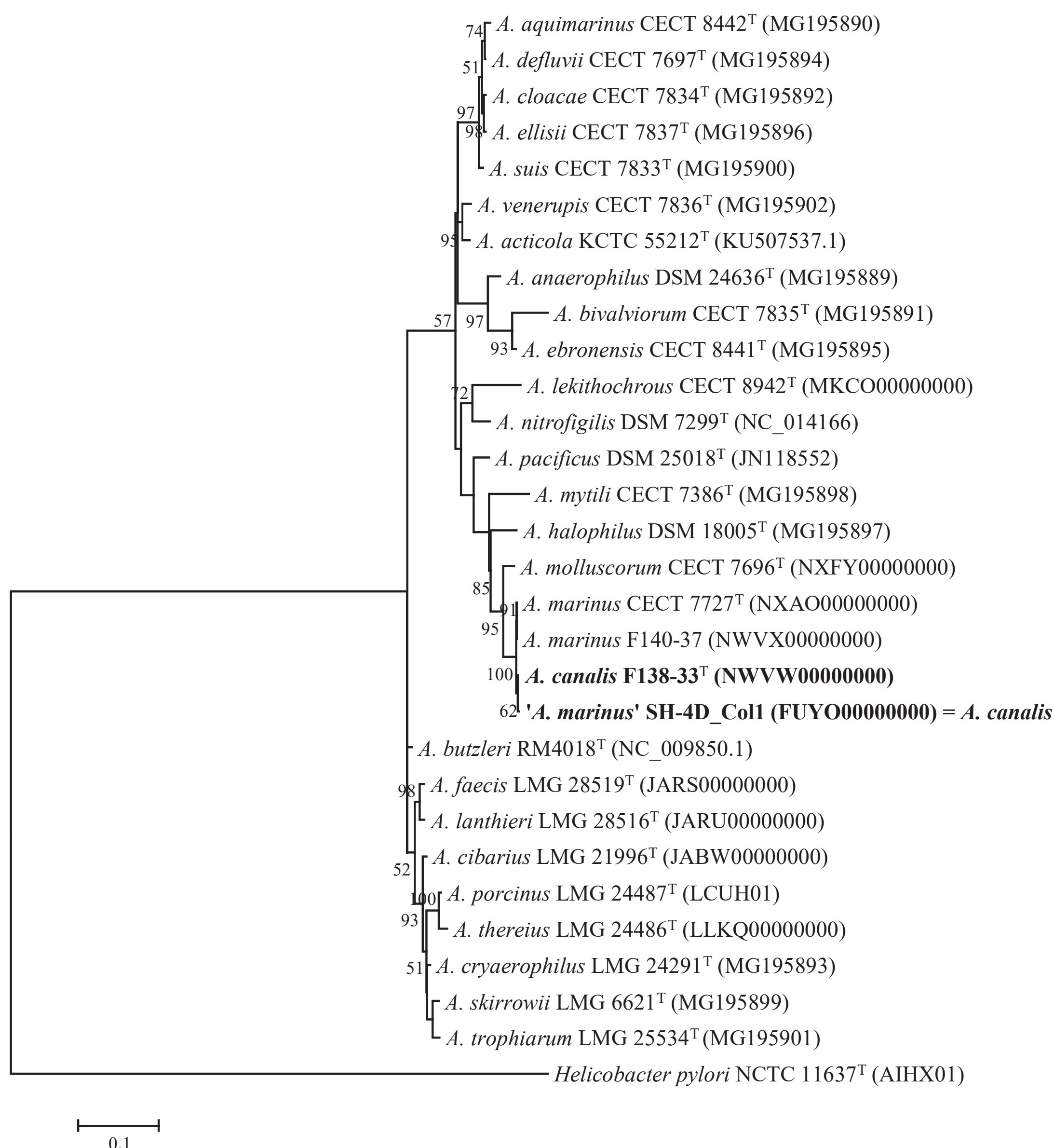
Supplementary figure S4. Maximum Likelihood tree (model GTR+G) based on the *atpA* gene sequence (613 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt



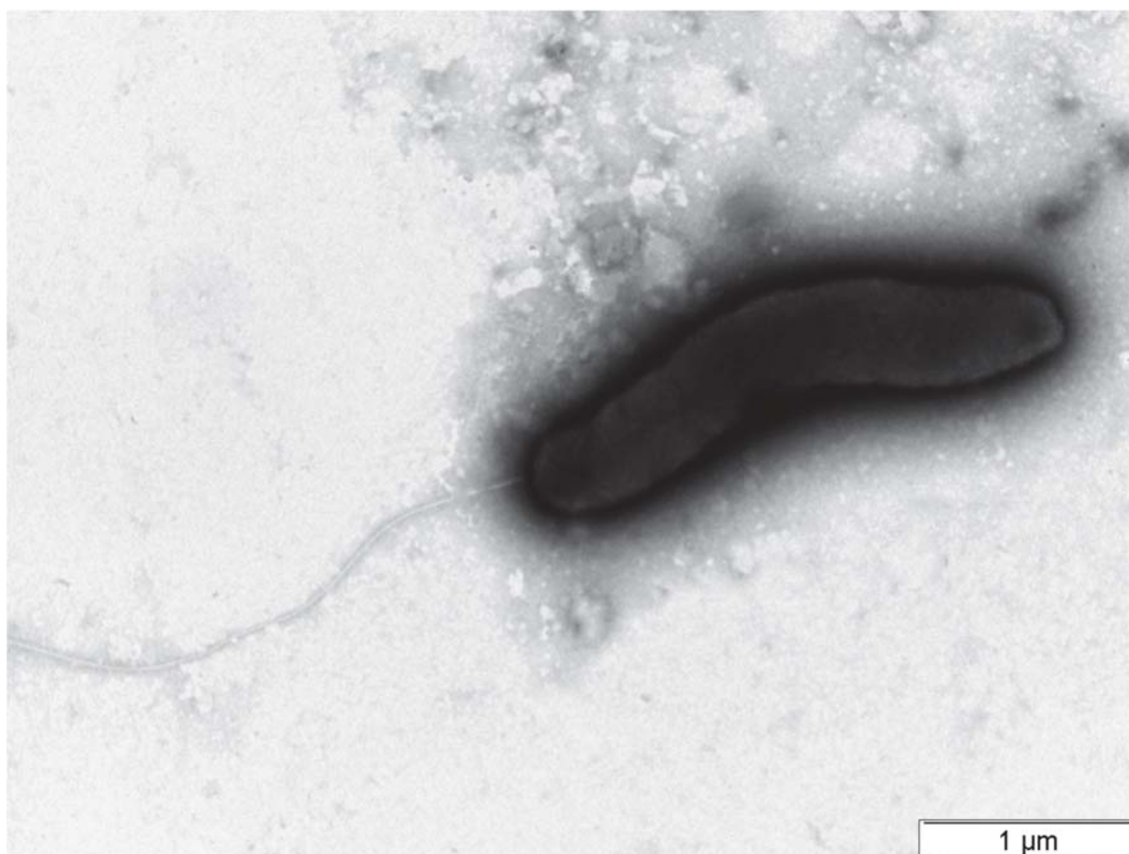
Supplementary figure S5. Maximum Likelihood tree (model GTR+G+I) based on the *gyrA* gene sequence (647 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. *Arcobacter pacificus* has not been added to the phylogeny because the gene is not available. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt



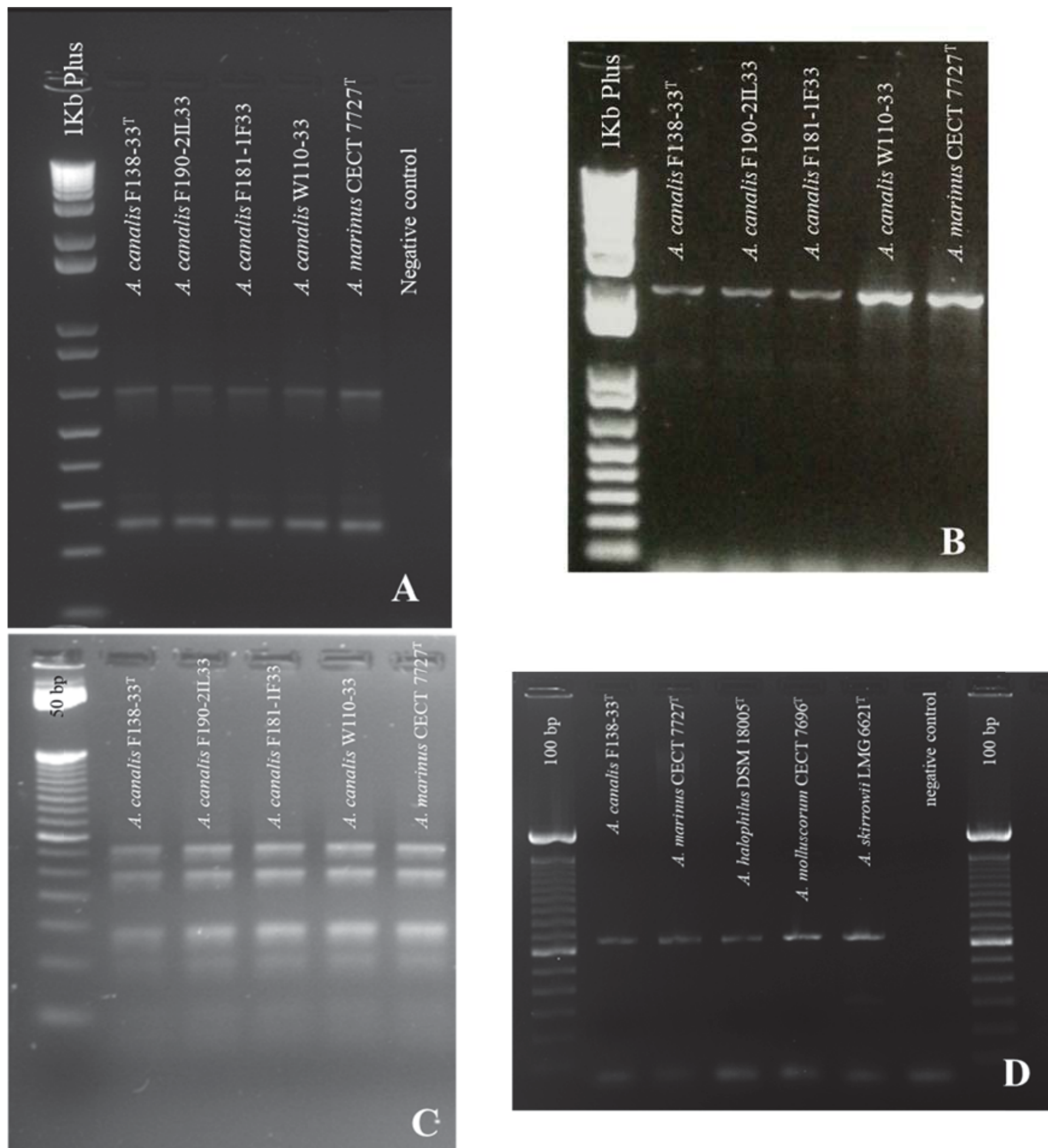
Supplementary figure S6. Maximum Likelihood tree based on the concatenated sequences of *atpA*, *gyrB*, *hsp60*, *rpoB* and *gyrA* (3041 bp) genes showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. The region of the *gyrA* gene of *A. pacificus* available at the NCBI database is not the same of the other *Arcobacter* species and was not added to the phylogenetic analysis. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.



Supplementary figure S7. Maximum Likelihood tree (model GTR+G+I) based on the 16S rRNA gene sequence (1427 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 10 substitutions per 100 nt



Supplementary figure S8. Transmission electron microscopy image of a cell of the strain F138-33^T negatively stained. Bar, 1 μm.



Supplementary figure S9. Results obtained for the new species *A. canalis* with four molecular methods (A-D) used for the identification of *Arcobacter* spp. [10-12, 32]: A, The amplicons obtained for the four strains of *A. canalis* with the mPCR of Houf *et al*. [10] were of the expected sizes described for *A. cryaerophilus* (257 bp) and *A. skirrowii* (641bp); B, The amplicon of the four strains was of the size described for *A. butzleri* (1590 bp) with m-PCR of Douidah *et al*. [11]; C, The 16S rDNA-RFLP patter obtained for the four strains was identical to the one described for *A. marinus* [12]; Notice that the latter species produced identical amplicons than *A. canalis* with the A, B mPCR methods [10, 11]. D. With the mPCR of Khan *et al*. [32] the type strain of *A. canalis* showed, as occurred with the nearest species *A. marinus*, *A. molluscorum* and *A. halophilus*, an amplicon of the size (654bp) described for *A. skirrowii* [32]. Ladders from Invitrogen A and B 1Kb plus, C 50 bp and D 100 bp

4.3 *Arcobacter lacus* sp. nov. and *Arcobacter caeni*, two new species isolated from wastewater. Pérez-Cataluña, A., Salas-Massó, N., and Figueras, M.J. *Int. J. Syst. Evol. Microbiol.* (Under Review)

Arcobacter lacus sp. nov. and _Arcobacter caeni_ sp. nov., two new species isolated from reclaimed water.

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The GenBank/EMTBL/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 the strain RW17-10^T they are LT629998, LT904796, LT904820, LT904808, LT904832 and LT904784, respectively. The accession number for the genomes of the strains RW43-9^T and RW17-10^T are MUXF00000000 and MUXE00000000, respectively.

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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 polyphasic taxonomy, showing evidence that they were two new *Arcobacter* species. Based on the 16S rRNA gene for strain RW43-9^T, the closest species was *A. butzleri* LMG 10828^T (99.9% similarity), while for strain RW17-10^T it was *A. venerupis* CECT 7836^T (99.4%). Additionally, the multilocus phylogenetic analysis of five concatenated housekeeping genes (*atpA*, *gyrA*, *gyrB*, *hsp60* and *rpoB*) showed that the two strains formed separated branches that are different from the known *Arcobacter* species. The whole genome sequence of both strains (RW43-9^T and RW17-10^T) were obtained and they were compared with the genomes of the type strains of their nearest species. Using the Average Nucleotide Identity and *in silico* DNA-DNA hybridization results were below 96% and 70%, respectively. These results clearly confirm that they represent new species. Additionally, the phenotypic characterization of the strains allows their differentiation from other species. Therefore, the two strains were proposed as new 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).

Keywords: *Arcobacter lacus*, *Arcobacter caeni*, reclaimed water, MLPA, 16S rRNA, ANI, *is*DDH

Abbreviations: MLPA, Multilocus Phylogenetic Analysis; ANI, Average Nucleotide Identity; isDDH, in silico DNA-DNA hybridization; TEM, Transmission Electron Microscope.

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 coworkers 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 ε-proteobacteria 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 *Arcobacter* on the inlet and outlet water of a wastewater treatment plant in the city of Reus (North-East of Spain) two strains (RW43-9^T and RW17-10^T) were isolated from the secondary treated water at the entrance site of the tertiary treatment system by lagooning [8]. Isolation was carried out with a prior concentration of 200 ml of water sample by filtration through a 0.45 µm filter (Millipore, Darmstadt, Germany). 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, Madrid, Spain). After 30 minutes of passive filtration, the filter was removed and the plates were incubated at 30ºC in microaerobic conditions for 48 hours. The colonies were presumed to be *Arcobacter* 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 the Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) using primers and conditions previously described [10]. Both isolates showed different ERIC-PCR patterns (data not shown) evidencing 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 described [11]. The obtained *rpoB* sequences (622 bp) were aligned with the ClustalW algorithm [12] and a phylogenetic tree was built with MEGA 6.0 [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, the species *Arcobacter butzleri* being the nearest species to the strain RW43-9^T and *Arcobacter suis* the nearest species to the strain RW17-10^T (Fig. S1). To perform a 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 ClustalW [12], phylogenetic trees were built as above with the Neighbour- Joining (NJ) [15,16] and the ML methods for the 16S rRNA (Fig. 1, Fig. S2), and the Multilocus Phylogenetic Analysis (MLPA) of the concatenated sequences of the five mentioned housekeeping genes (Fig. 2, Fig.

S3) and with the ML method for the individual housekeeping genes (Figs. S1 and S4 to S7). The resulting trees showed that the two strains again formed independent branches from known *Arcobacter* species. In the MLPA, the strain RW17-10^{T} also showed the species *A. suis* CECT 7833^{T} as a nearest species along with *A. venerupis* CECT 7836^{T} (Fig. 2, Fig. S3), while the strain RW43-9^{T} showed *A. butzleri* LMG 10828^{T} as its nearest one.

The 16S rRNA gene similarities (1413 bp) between the two potential new species and the other species of the genus were calculated using MegAlign version 7.0.0 (DNASTAR®, Madison, WI). Strain RW43-9^{T} showed the highest similarity of 99.9% with the species *A. butzleri* LMG 10828^{T}, while the similarity with the other species of the genus ranged from 91.2% with *A. bivalviorum* CECT 7835^{T} to 97.9% with *A. faecis* LMG 28519^{T} and *A. lanthieri* LMG 28516^{T}. The highest similarity of strain RW17-10^{T} was 99.4% with *A. venerupis* CECT 7836^{T}, while 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 *A. acticola* KCTC 52212^{T}.

To further confirm that the two strains represented new taxa, we sequenced the genomes of the strains RW43-9^{T} (MUXF00000000) and RW17-10^{T} (MUXE00000000), as well as those of the type strains of the species *A. venerupis* CECT 7836^{T} (NREP00000000) and *A. suis* CECT 7833^{T} (NREO00000000) because they were the closest species on the basis of the MLPA. Sequencing was carried out with the MiSeq platform (Illumina, Lisbon, Portugal) obtaining a sequencing depth of coverage >100X 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 the strains RW43-9^{T} and RW17-10^{T} were compared with their nearest species using the Average Nucleotide Identity (ANI) and the *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 with the phylogenetic analyses evidenced that the two strains (RW43-9^{T} and RW17-10^{T}) represented two new species of the genus *Arcobacter*.

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

The phenotype was characterized 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 nearest species *A. butzleri* LMG 10828^T, *A. venerupis* CECT 7836^T and *A. suis* CECT 7833^T, and the positive and negative controls for each test. A total of 39 tests were carried out, i.e. 12 tests of growth conditions (i.e. 22-25ºC, 30ºC, 37ºC and 42ºC in aerobic, microaerobic and anaerobic conditions) and 27 tests for biochemical properties i.e. oxidase, catalase, and urease activity, nitrate reduction and glucose fermentation in triple-sugar iron agar (TSI), indoxyl acetate hydrolysis, casein, lecithin and starch, growth in media supplemented with 2% and 4% NaCl, 1% oxgall, 0.1% sodium deoxycholate, 1% glycine, 0.05% safranin, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green, 0.1, 0.01 and 0.04% 2,3,5-triphenyltetrazolium chloride, growth in charcoal cefoperazone deoxycholate agar (CCDA), minimal media and MacConkey agar). The colony morphology of the strains was assessed after culturing on blood agar at 30ºC in aerobiosis for 48h. The morphology of the bacteria, the presence of flagella and cell sizes were evaluated with the transmission electron microscope JEOL 1011-TEM. For the latter analysis, cells grown on blood agar at 30ºC for 48 hours were fixed in 2% glutaraldehyde-0.1% phosphate buffer for 30 minutes and suspended cells were transferred to a copper grid and negative stained with 2% phosphotungstic acid (pH 7.5). Under the TEM, both strains showed the presence of a polar flagellum (Fig. S8). Additionally, both strains showed motility using the phase contrast microscope. Phenotypic evaluation indicated that strain RW43-9^T could be differentiated from the species *A. butzleri* LMG 10828^T on the basis of four tests. In this sense, strain RW43-9^T showed growth in the presence of nalidixic acid (30mg l^{-1}), but no growth was observed on TTC 0.04% or on blood agar at 37ºC and 42ºC in 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 in crystal violet and minimal medium; and the ability of RW17-10^T to grow in media with safranine, to produce hydrogen sulphide from TSI agar and to show resistance to cefoperazone (64 mg l^{-1}) (Table 2). Additionally, 6 tests (Table 2) differentiated strain RW17-10^T from the species *A. venerupis* CECT 7836^T: the inability of the strain RW17-10^T to grow at 37ºC in microaerobiosis and anaerobiosis, in media with 2% of NaCl, basic fuchsine, brilliant green, and to produce urease activity. In addition, strain RW17-10^T can be differentiated from *A. suis* CECT 7833^T because the latter does not grow in 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 genome of the two strains annotated with RAST [19,20] showed two genes involved in the synthesis of phosphatidilglycerol i.e. phosphatidylglycerolphosphatase A (*pspA*, EC3.1.3.27) and phosphatidase cytidylyltransferase (*cdsA*, EC 2.7.7.41); and one gene related with the synthesis of phosphatidylethanolamine, the gene phosphatidylserine descarboxilase (*psd*,

EC4.1.1.65). These polar lipids have been found in other *Arcobacter* species using experimental detection by two-dimensional Thin-Layer Chromatography [4,5,27].

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 30ºC in aerobiosis for 48 hours are 2-4 mm in diameter, beige to off/white, convex, circular with entire margins and non-swarming. Pigments or hemolysis are not produced on blood agar. The strain grows on blood agar at 22-25ºC, and 30ºC in aerobiosis, anaerobiosis and microaerobiosis; at 37ºC in aerobiosis and microaerobiosis and at 42ºC in microaerobiosis. No growth occurs at 37º C in anaerobiosis or at 42º C in aerobiosis and anaerobiosis. Produces oxidase and catalase activities and reduces nitrates. Indoxyl acetate is hydrolysed, but not urea, casein, lecithin or starch. The strain is able to produce hydrogen sulphide in TSI but not to produce acid from glucose in this media. Growth occurs in media with 2% NaCl, 1% Oxgall, 0.1% sodium deoxycholate, 0.05% safranin, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green, 0.01% TTC, in CCDA and MacConkey agars, and in minimal medium. No growth occurs in media with 4% NaCl, 1% glycine, or in 0.1% or 0.04% TTC. The strain shows resistance 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 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 30ºC in aerobiosis for 48 hours are 1-3 mm in diameter, beige to off-white, convex, circular with entire margins and non-swarming. Pigments or hemolysis are not produced on blood agar. The strains grow on blood agar at 22-25ºC and 30ºC in aerobiosis, anaerobiosis and microaerobiosis; no growth was observed at the other temperatures and atmospheres tested. The strain produces oxidase and catalase activity, reduces nitrates, indoxyl

acetate is hydrolysed, but not urea, casein, lecithin or starch. The strain is able to produce hydrogen sulphide in TSI agar but not to produce acid from glucose in this media. Growth occurs in media with 0.05% safranin, and in CCDA and MacConkey agars. No growth is observed in media with 2% and 4% NaCl, 1% oxgall, 0.1% sodium deoxycholate, 1% glycine, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green, 0.01%, 0.04% and 0.1% TTC, and in minimal medium. Strain is 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 from secondary treated wastewater at the WWTP in Reus, Spain.

Conflict of Interest

The authors have no conflict of interest to declare.

Acknowledgements

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Figure legends

Figure 1. Neighbour-Joining tree based on 16S rRNA gene sequences (1406 bp) showing the phylogenetic position of *A. lacus* sp. nov. RW43-9^T and *A. caeni* sp. nov. 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.

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

Table 1. Results (in percentage) of ANI (bold, down-left) and *is*DDH (italics, up-right) between the genomes of *A. lacus* sp. nov. RW43-9^T and *A. caeni* sp. nov. RW17-10^T and the genomes of the most closely related species. Values of ANI and *is*DDH below 96% and 70%, respectively, indicate that the genomes correspond to different species.

	Species	1	2	3	4	5
1	***A. lacus*** **sp. nov. RW43-9^T**	***	*55.8*	*23.3*	*23.2*	*23.5*
2	***A. butzleri*** **RM4018^T**	**94.3**	***	*23.2*	*23.3*	*23.4*
3	***A. caeni*** **sp. nov. RW17-10^T**	**80.6**	**80.3**	***	*30.3*	*34.4*
4	***A. venerupis*** **CECT 7836^T**	**79.9**	**80.0**	**85.8**	***	*31.8*
5	***A. suis*** **CECT 7833^T**	**80.9**	**80.8**	**87.9**	**86.7**	***

Table 2. Differential characteristics of *Arcobacter lacus* sp. nov. RW43-9^T, *A. caeni* sp. nov. RW17-10^T and type strains of the most closely-related species of the genus *Arcobacter*.

Taxa: 1, *Arcobacter lacus* RW43-9^T sp. nov.; 2, *A. butzleri* LMG 10828^T; 3, *A. caeni* RW17-10^T; 4, *A. venerupis* CECT 7836^T; 5, *A. suis* CECT 7833^T. Unless otherwise indicated: +, ≥95% strains positive; −, ≤11% strains positive; V, 12–94% strains positive.

Characteristic	*A. lacus*	*A. butzleri**	*A. caeni*	*A. venerupis*†	*A. suis*‡
Growth at/by/on:					
37°C (aerobiosis)	+	+	-	-	-
37°C (microaerobic)	+	+	-	+	-
42°C (microaerobic)	+	V	-	-	-
37°C (anaerobic)	-	+	-	+	-
42°C (anaerobic)	-	+	-	-	-
NaCl 2%	+	+	-	+	-
NaCl 4%	-	-	-	-	-
0.1% Sodium deoxycholate	+	+	-	-	+
0.05% Safranin	+	+	+	-	-
0.005% Basic fuchsine	+	+	-	+	-
0.0005% Crystal violet	+	+	-	+	+
0.001% Brilliant green	+	+	-	+	-
0.01% TTC	+	+	-	-	+
0.04% TTC	-	+	-	-	-
CCDA	+	+	+	+	-
Minimal medium	+	+	-	+	+
Tripe-sugar iron	+	+	+	-	-
Resistance to:					
Cefoperazone (64mg l^{-1})	+	+	+	-	-
Cephalothin (30mg l^{-1})	+	+	+	+	-
Nalidixic Acid (30mg l^{-1})	+	-	+	+	+
Enzyme activity:					
Nitrate reduction	+	+	+	+	+
Urease	-	-	-	+	-
Indoxyl acetate hydrolysis	+	+	+	+	+

* Vandamme *et al*., 1992
† Levican *et al*., 2012
‡ Levican *et al*., 2013

Figure 1

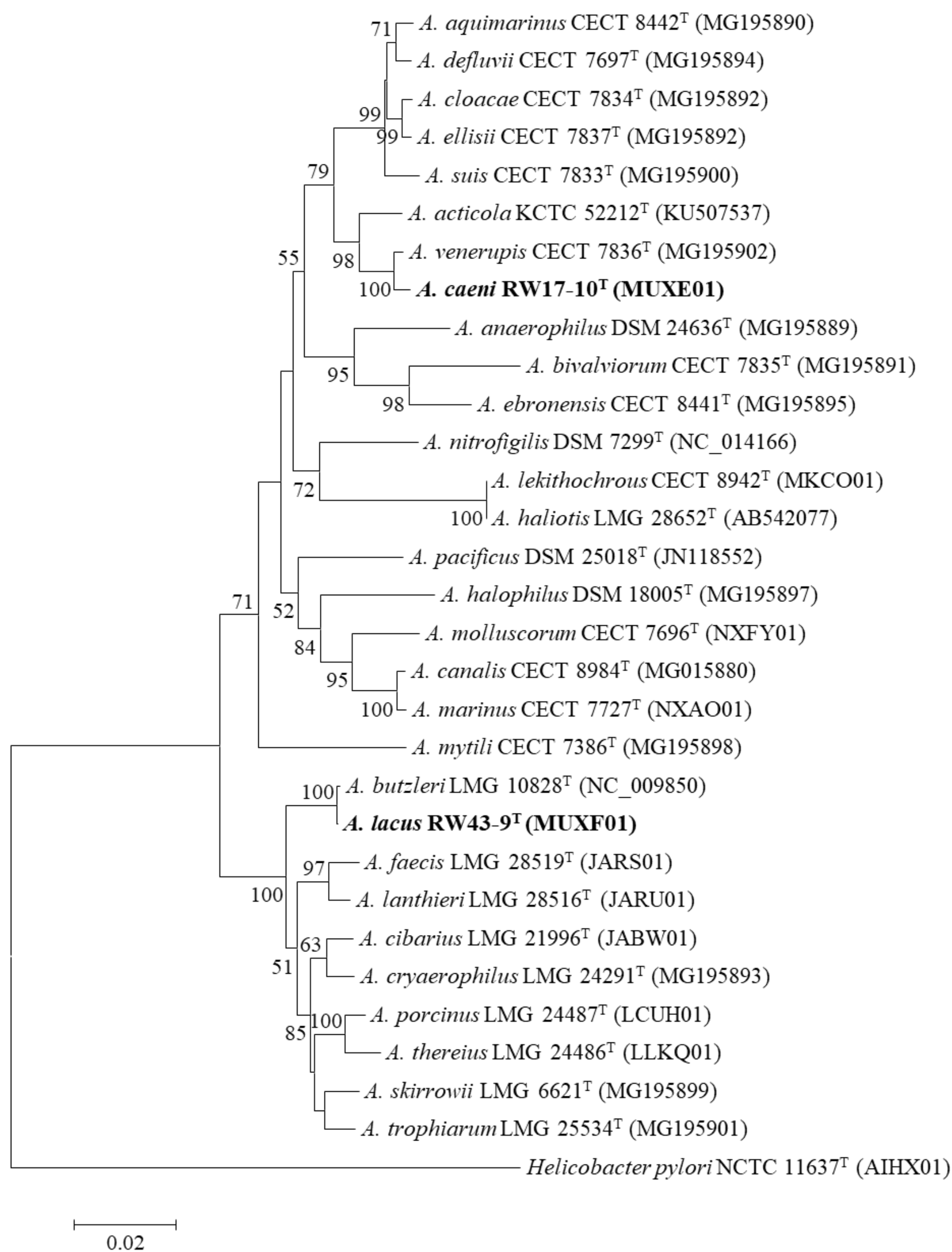
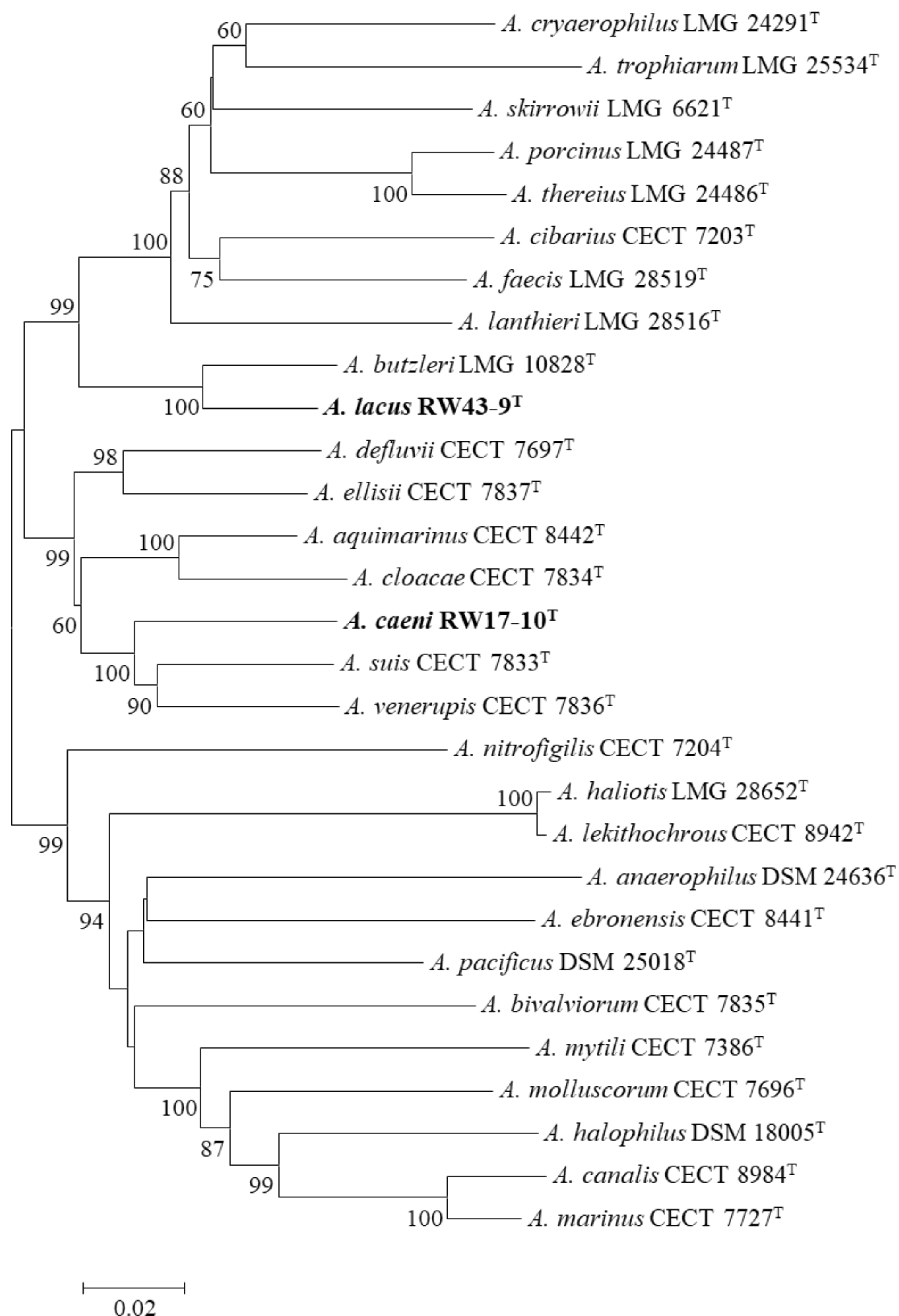
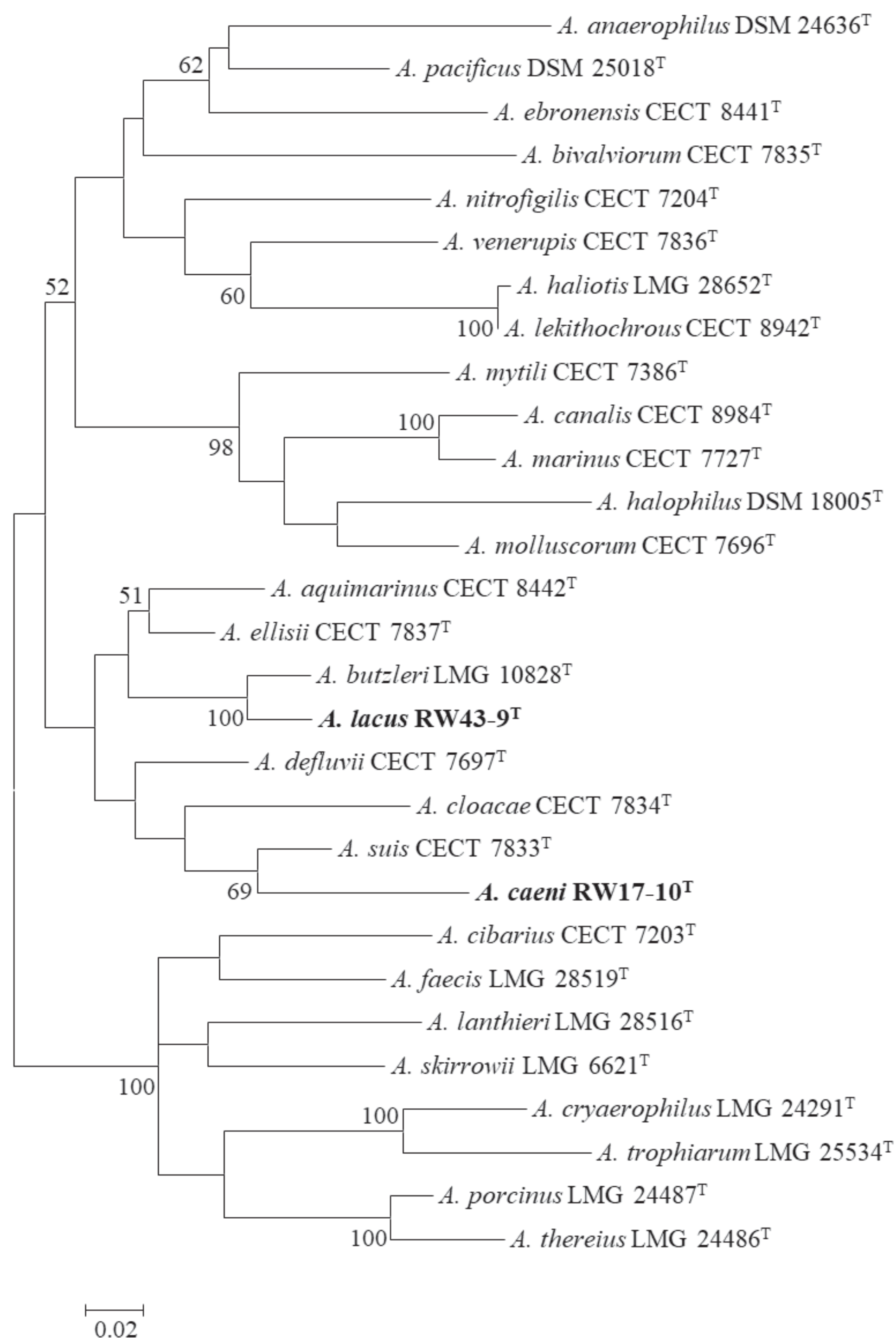
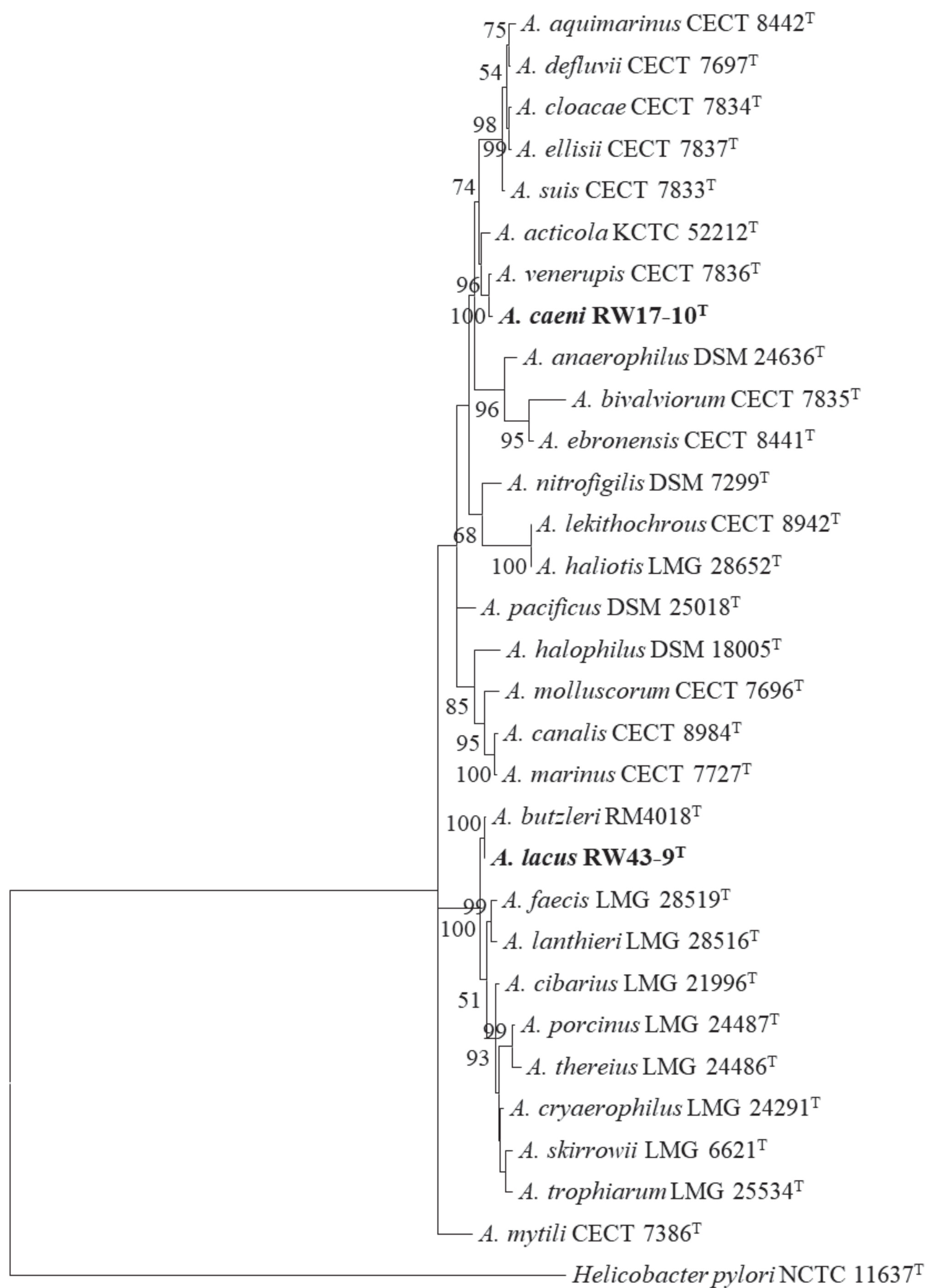


Figure 2

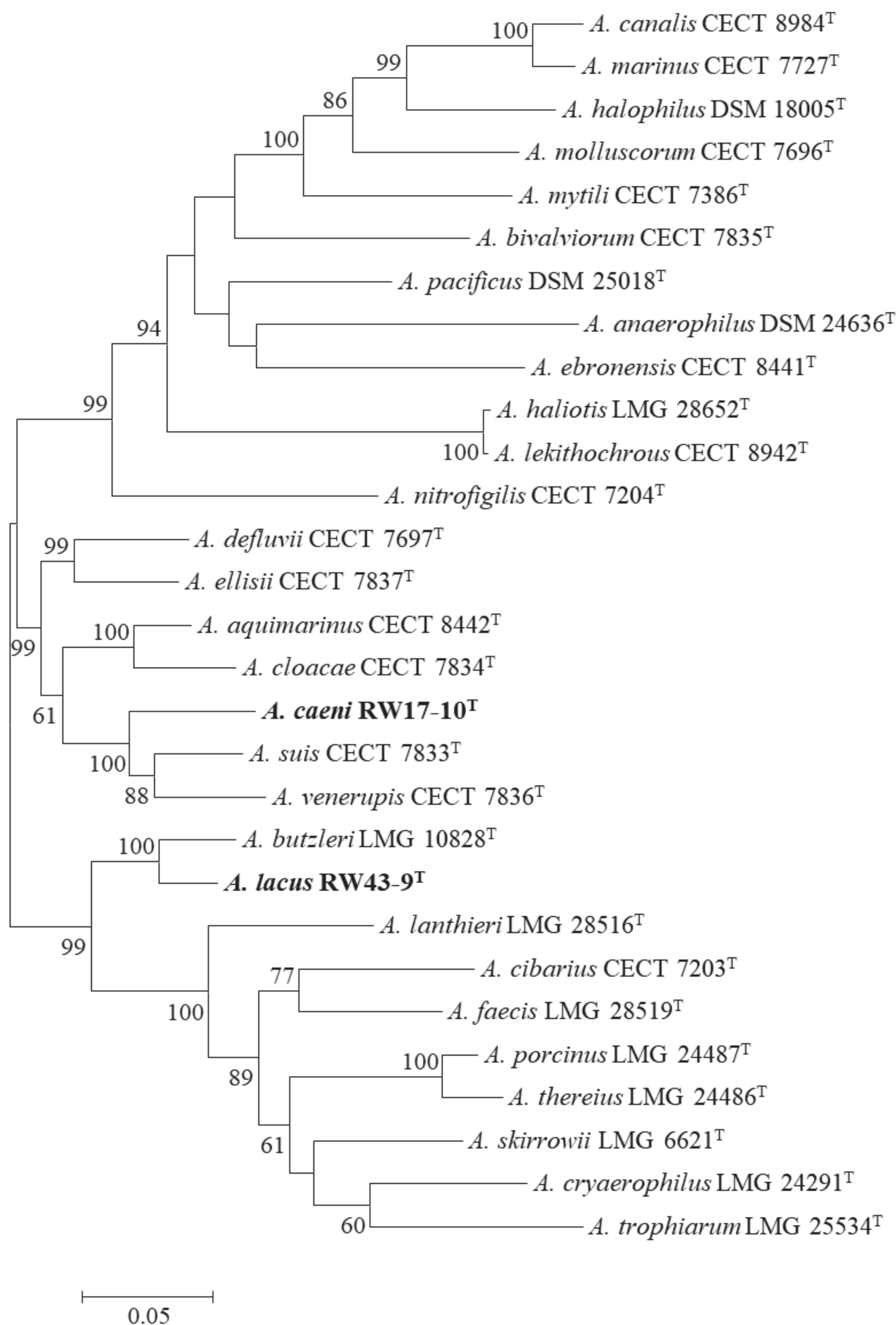




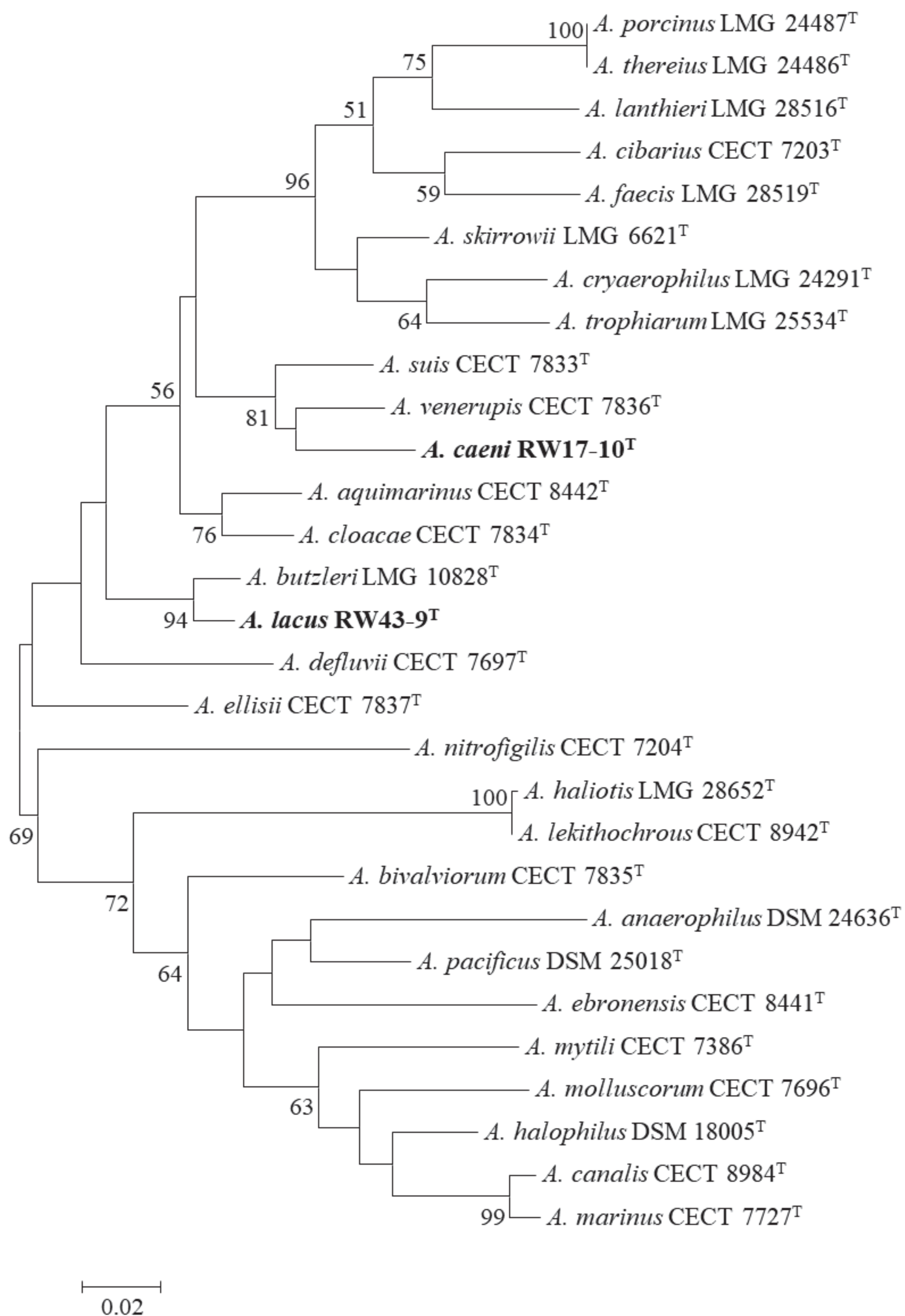
Supplementary figure S1. Maximum Likelihood tree (model GTR+G+I) based on the *rpoB* gene sequence (622 bp) showing the phylogenetic position of *A. lacus* sp. nov. RW43-9^{T} and *A. caeni* sp. nov. RW17-10^{T} within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.



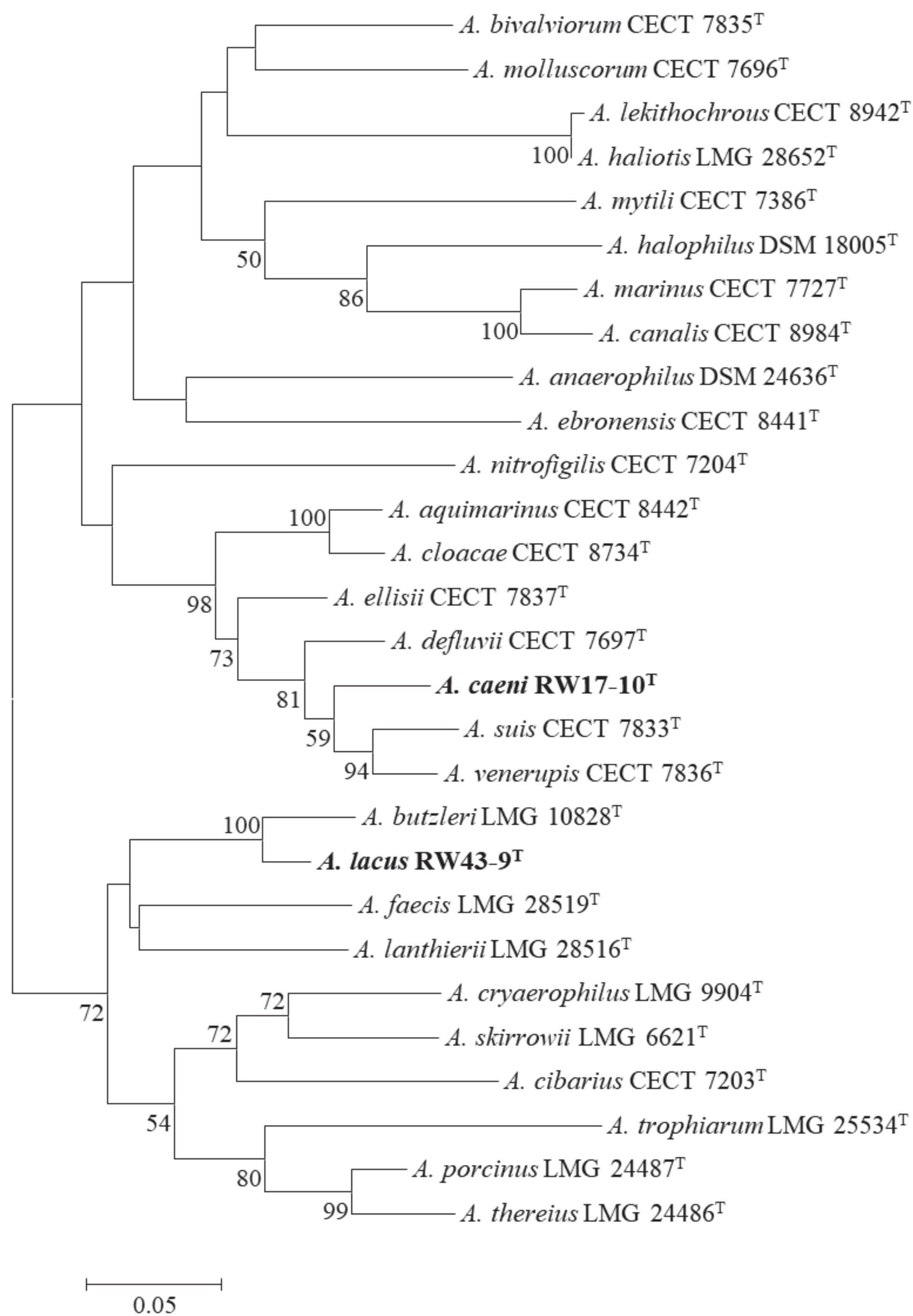
Supplementary figure S2. Maximum Likelihood tree (model GTR+G+I) based on the 16S rRNA gene sequence (1427 bp) showing the phylogenetic position of *A. lacus* sp. nov. RW43-9^T and *A. caeni* sp. nov. RW17-10^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at thenodes of the tree. Bar indicates 10 substitutions per 100 nt



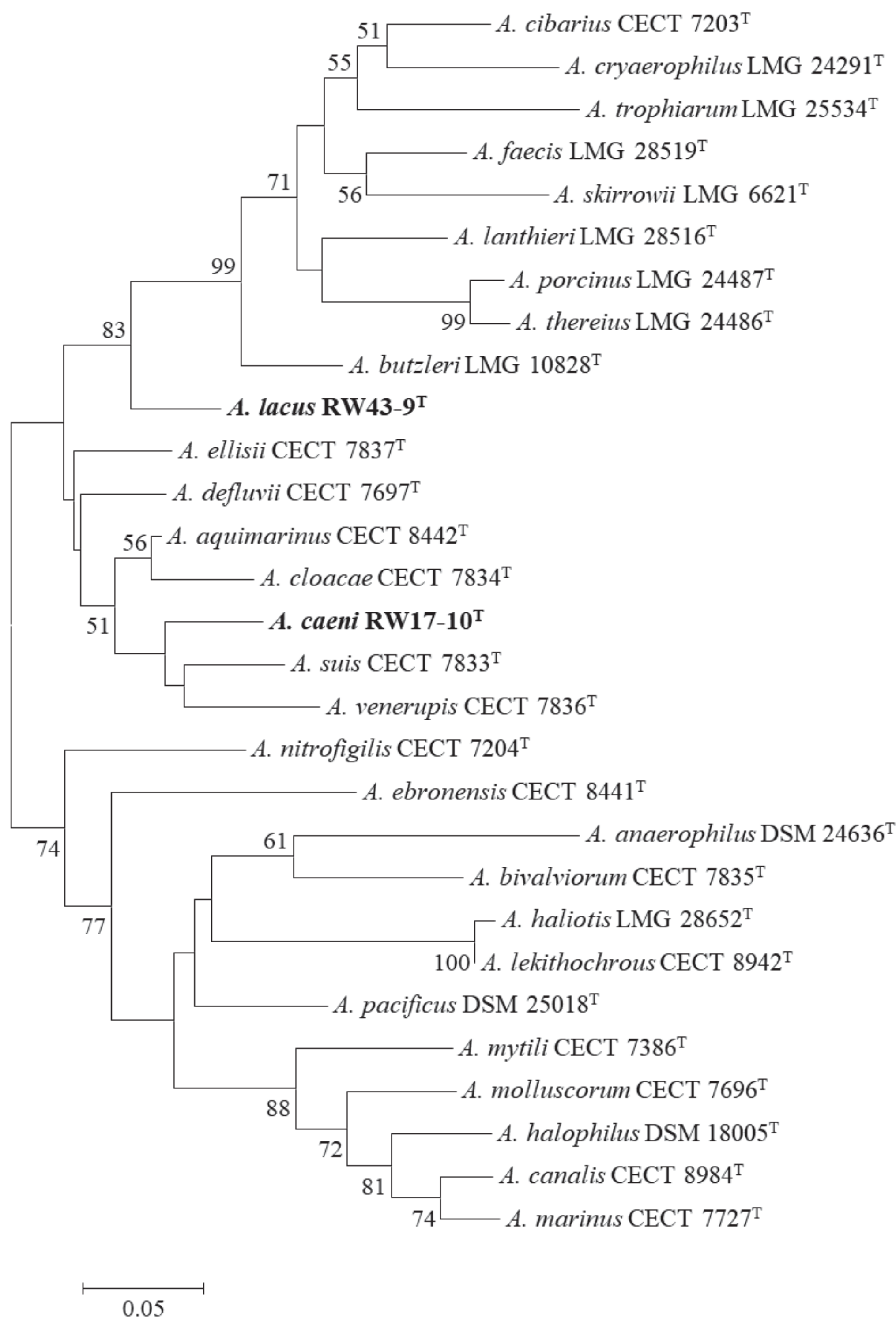
Supplementary figure S3. Maximum Likelihood tree (GTR+G+I) based on the concatenated sequences of *atpA*, *gyrB*, *hsp60*, *rpoB* and *gyrA* (3103 bp) genes showing the phylogenetic position of *A. lacus* sp. nov. RW43-9^T and *A. caeni* sp. nov. RW17-10^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



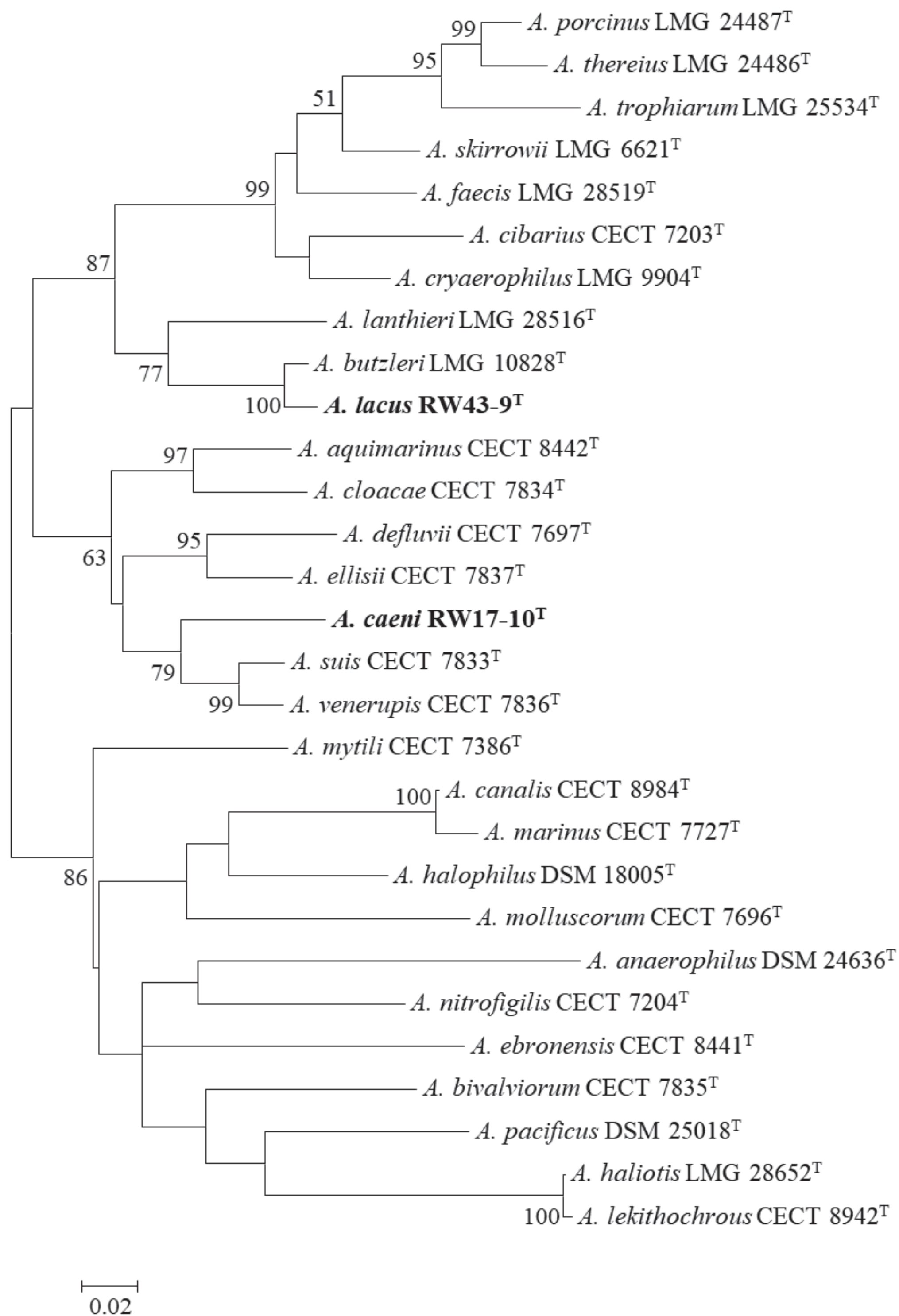
Supplementary figure S4. Maximum Likelihood tree (model GTR+G+I) based on the *atpA* gene sequence (612 bp) showing the phylogenetic position of *A. lacus* sp. nov. RW43-9^T and *A. caeni* sp. nov. RW17-10^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.



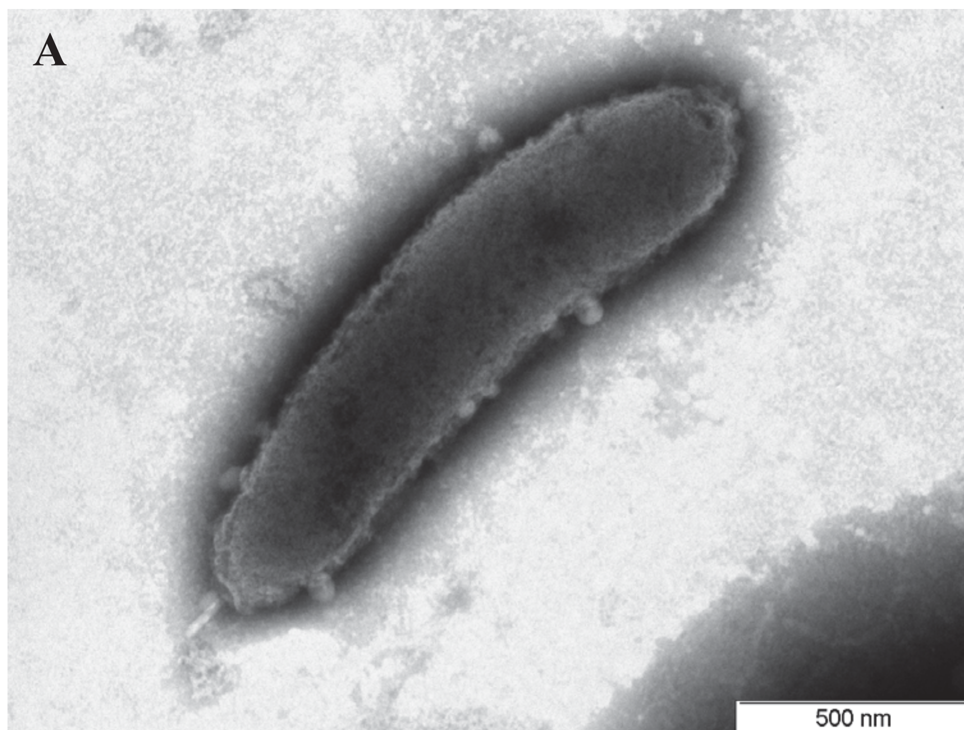
Supplementary figure S5. Maximum Likelihood tree (model GTR+G+I) based on the *gyrA* gene sequence (647 bp) showing the phylogenetic position of *A. lacus* sp. nov. RW43-9^T and *A. caeni* sp. nov. RW17-10^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.

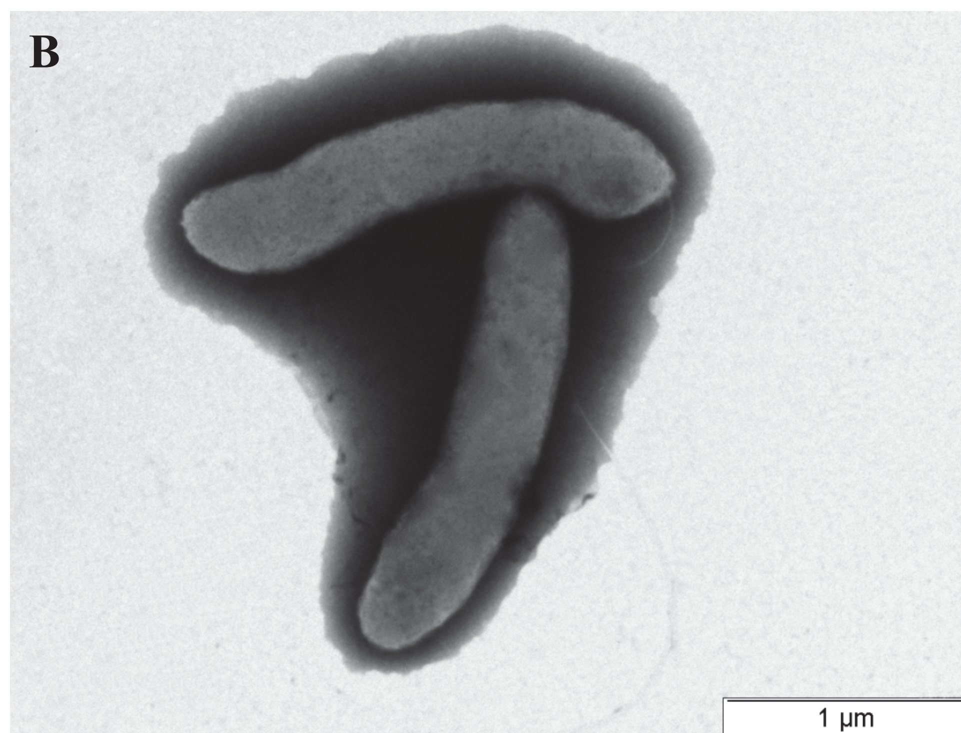


Supplementary figure S6. Maximum Likelihood tree (model GTR+G) based on the *gyrB* gene sequence (616 bp) showing the phylogenetic position of *A. lacus* sp. nov. RW43-9^T and *A. caeni* sp. nov. RW17-10^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



Supplementary figure S7. Maximum Likelihood tree (model GTR+G+I) based on the *hsp60* gene sequence (595 bp) showing the phylogenetic position of *A. lacus* sp. nov. RW43-9^T and *A. caeni* sp. nov. RW17-10^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.





Supplementary figure S8. Transmission electron microscopy images of the new *Arcobacter* species with negative staining using phosphotungstic acid. A, image of *A. lacus* sp. nov. RW43-9^T, bar 500nm; B, image of *A. caeni* sp. nov. RW17-10^T, bar 1 µm.

4.4 *Arcobacter miroungae* sp. nov., a new species isolated from southern elephant seal (*Mirounga leonina*). Pérez-Cataluña, A., Salas-Massó, N., García-Peña, F., Romalde, J., and Figueras, M.J. (In preparation)

Arcobacter miroungae sp. nov., a new species isolated from southern elephant seal (_Mirounga leonina_).

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The GenBank/EMTBL/DDBJ accession numbers for the 16S rRNA, *atpA*, *gyrA*, *gyrB*, *hsp60*, and *rpoB* gene sequences of strain AHV-9/2010^T are LT904640, LT904794, LT904818, LT904806, LT904830 and LT904782, respectively. The accession number for the genome of the strain AHV-9/2010^T is PDKH00000000.

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Abstract

The bacterial strain AHV-9/2010^T recovered from a rectal swab of one elephant seal (*Mirounga leonina*) was identified as belonging to the genus *Arcobacter* and showed to be a potential new species. A polyphasic characterization of the strain evidenced that it belongs to a novel species of the genus *Arcobacter*. The phylogenetic analysis performed with the 16S rRNA gene showed that the strain represented a new linage being *Arcobacter skirrowii* LMG 6621^T its closest species. Genomic analyses were performed between the genomes of the strain AHV-9/2010^T and *A. skirrowii* LMG 6621^T using the Average Nucleotide Identity (ANI) and the *in silico* DNA-DNA hybridization (*is*DDH). Results obtained with this two similarity indexes were below 95 and 70% cutoff values. This results along with the phylogenetic analyses and the 10 differential phenotypical characteristics with its closest species *A. skirrowii* confirmed that the strain AHV-9/2010^T belong to anew Arcobacter species for which the name *Arcobacter miroungae* sp. nov. is proposed, with the type strain AHV-9/2010^T (= LMG 29975^T= CECT 9299^T).

Keywords: *Arcobacter miroungae*, Antarctic Peninsula, *Mirounga leonina*, MLPA, 16S rRNA, ANI, *is*DDH

Abbreviations: MLPA, Multilocus Phylogenetic Analysis; ANI, Average Nucleotide Identity; isDDH, in silico DNA-DNA hybridization; TEM, Transmission Electron Microscope.

The genus *Arcobacter* was described by Vandamme *et al.* [1] with two species firstly ascribed to the aerotolerant campylobacters group. In 2017, Waite and coworkers [2] reviewed the taxonomy of the *Epsilonproteobacteria* using more than 4,000 genomes and proposed the new phylum *Epsilonbacteraeota* composed by the class *Epsilonbacteria* and the order *Desulfurellales*. In this new reclassification, *Arcobacter* was included in the new family *Arcobacteraceae* [2]. The genus *Arcobacter* is composed by Gram-stain-negative bacteria which grows at low temperatures and in the presence of oxygen. The latter two characteristics are the ones that differentiate *Arcobacter* from *Campylobacter* [1]. At the time of writing, the genus is composed by 27 species [3–5].

During a Spanish expedition to the Antarctic peninsula in the austral summer of 2010, one isolate (AHV-9/2010) was recovered from a rectal swab of an elephant seal (*Mirounga leonina*) from the Avian Island. The swab was introduced in FBP media [6] with 0.5% active charcoal (Sigma Ltd, Scotland, UK) and frozen at -20º C until analysis. The swab and 100 µl of the shipping media were added to 10 ml of *Campylobacter* enrichment broth (Lab M, Lancashire, UK) with 5% laked horse blood (Oxoid, Hampshire, UK) and CAT (cefoperazone [8 µg/ml], teicoplanin [4 µg/ml], and amphotericin B [10 µg/ml]) supplement (Oxoid) at 37°C. The broth was incubated at 37°C for 5 days in 3.5-liter anaerobic containers using CampyGen sachets (Oxoid). A 47 mm diameter cellulose membrane with 0.60 µm pores was placed on the surface of an Columbia agar (Oxoid) with 5% defibrinated sheep blood. Eight to ten drops of enrichment broth (200 µl) were placed onto the surface of the membrane at 48 hours and 5 days of incubation. The membrane was left for 20 to 30 min on the agar surface at room temperature until all of the fluid had passed through. The plates were incubated as described above, but for a 5 days period of time to isolate the less common, slower growing species.

Presumptive *Arcobacter* colonies (small, translucent, beige to pale white) were detected only on Columbia agar plate and these colonies were observed under the optical microscope after a gram staining, and oxidase activity was tested. Cells were gram-stain-negative rods, with S shapes and presented oxidase activity [1,7]. To determine the identification of the strain AHV-9/2010^T to the species level, the *rpoB* gene (622 bp) was amplified and sequenced as described by Levican [8]. This gene was aligned with the type strains of the other described species of the genus using ClustalW [9] implemented in Mega 6.0 [10]. A phylogenetic analysis was performed using the Maximum likelihood (ML) method [11]. Phylogenetic analysis (Figure S1) showed that the strain AHV-9/2010^T formed a distinct branch near to *Arcobacter skirrowii* LMG 6621^T. In order to know the exact taxonomic position of the strain AHV-9/2010^T, the 16S rRNA gene (1406 bp) and the housekeeping genes *atpA*, *gyrA*, *gyrB* and *hsp60* were amplified and sequenced as previously described [8]. The 16S rRNA gene, the five housekeeping genes and the concatenated sequences of these

five housekeeping genes (*rpoB*, *atpA*, *gyrA*, *gyrB* and *hsp60*, 3102 bp) were aligned as described above for the *rpoB* gene. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) [12,13] and the ML [11] algorithms for the concatenated sequences and for the 16S rRNA gene; and the ML method was used for the individual houskeeping genes trees. All the analyzed trees (Figs. 1 and 2, Supplementary Figs. S1-S7) showed that the strain AHV-9/2010^T represents a potential new species, being its nearest species *A. skirrowii* LMG 6621^T. The 16S rRNA gene similarities was calculated with MegAlign version 7.0.0 (DNASTAR®, Madison, WI). The highest percentage of similarity of the 16S rRNA gene of *A. miroungae* sp. nov. AHV-9/2010^T was 99.4% with *A. skirrowii* LMG 6621T. The percentage of similarity with the other species of the genus ranged from 91.2% with *Arcobacter bivalviorum* CECT 7835^T to 98.6% with *Arcobacter trophiarum* LMG 25534^T.

To confirm that the new strain belongs to a novel species, the genomes of *A. miroungae* sp. nov. AHV-9/2010^T (PDKH00000000) and *A. skirrowii* LMG 6621^T (NXIC00000000) were sequenced with MiSeq platform of Illumina and assembled with SPAdes v3.9.0. [14]. The obtained genomes were annotated with the Rapid Annotation of microbial genomes using Subsystems Technology (RAST) [15]. Genomes were compared using the Average Nucleotide Identity (ANI) and the *in silico* DNA-DNA hybridization (*is*DDH), using the software OrthoANI [16] and GGDC [17], respectively. The values obtained between the genomes of *A. miroungae* sp. nov. AHV-9/2010^T and *A. skirrowii* LMG 6621^T were 94.5% for ANI and 61.0% for *is*DDH. The values below the cutoff of 95% for ANI and 70% for isDDH evidenced that *A. miroungae* sp. nov. AHV-9/2010^T represents a new species of the genus *Arcobacter* [17,18]. Additionally, both genomes showed the presence of genes related with phosphatidylglycerol synthesis, as phosphatidylglycerolphosphatase A (*pspA*, EC3.1.3.27) and phosphatidase cytidylyltransferase (*cdsA*, EC 2.7.7.41); as well as the gene phosphatidylserine descarboxilase (*psd*, EC4.1.1.65) for the synthesis of phosphatidylethanolamine. These genes had also been found by chromatographic methods in *Arcobacter pacificus*, *Arcobacter acticola* and *Arcobacter haliotis* [4,19,20]; and in the genome of the type strains of *Arcobacter canalis*, *Arcobacter molluscorum*, *Arcobacter marinus*, *Arcobacter halophilus* and *A. cryaerophilus* [5,21].

To complete the description of this new species, a phenotypical characterization was performed following the minimal standards proposed for the family *Campylobacteraceae* [22,23]. The presence of flagella was studied using the Transmission Electron Microscope (TEM) JEOL 1011 (Figure S8). Strain was grow in blood agar at 30ºC in aerobiosis for 48 hours and cells were prepared for the TEM analysis as previously described [5]. Tests were assayed at least twice for the strain *A. miroungae* sp. nov. AHV-9/2010^T and its closest

species *A. skirrowii* LMG 6621^T. The phenotypical results obtained for *A. skirrowii* LMG 6621^T were extracted from the species description [24] and from results obtained in our laboratory. Morphology of the colonies were studied from cultures on blood agar at 30ºC for 48 hours. The phenotypic characterization was performed with 39 tests, including 12 tests for growth conditions in different temperatures and atmospheres, and 27 tests for phenotypic characteristics. The 27 phenotypical tests included: growth in the presence of different compounds (2% and 4% NaCl, 1% oxgall, 0.1% sodium deoxycholate, 1% glycine, 0.01 to 0.1% of triphenyl tetrazolium chloride), antibiotics (cefoperazone 64 mg l^{-1}, cephalothin 30 mg l^{-1}, nalidixic acid 30 mg l^{-1}) and dyes (0.05% safranine, 0.005% fuchsine, 0.0005% crystal violet, 0.001% brilliant green), growth in different media (CCDA, McConkey agar, minimal media), hydrolysis of starch, casein, lecithin and indoxyl acetate, production of H_2S and sugar fermentation on triple sugar iron agar, nitrate reduction in Cook Nitrate agar, and enzymatic activity (catalase, oxidase and urease). The phenotypical characteristics that allows the differentiation between *A. miroungae* sp. nov. AHV-9/2010^T and its closest species *A. skirrowii* LMG 6621^T are summarized in Table 1. Differential characteristics were the ability of *A. miroungae* sp. nov. AHV-9/2010^T to grow in minimal media, the production of H_2S from triple sugar iron agar and the resistance to nalidixic acid (30 mg l^{-1}), and the inability to grow on blood agar in microaerobiosis at 42ºC and anaerobiosis at 30ºC and 42ºC, in the presence of 4% NaCl, 0.01% TTC and on CCDA agar. Other differential characteristics but with variable results of *A. skirrowii* were the inability of *A. miroungae* sp. nov. AHV-9/2010^T to growth in the presence of 1% glycine.

Results obtained in this study evidenced that the strain AHV-9/2010^T belongs to a novel species of the genus *Arcobacter*, for which the name *A. miroungae* sp. nov. in proposed.

Description of *Arcobacter miroungae* sp. nov.

Arcobacter miroungae (*miroun´gae*. N.L. gen. n. miroungae of *Mirounga*, named because the organism was isolated from the southern elephant seal, *Mirounga leonina*.)

Cells are gram-negative staining, curved rods with a single polar flagellum, non-encapsulated and non-spore forming with 0.2-0.5 µm wide and 1.2-1.9 µm long. Cell growth on blood agar for 48 hours at 30ºC in aerobic conditions are 1-3 mm in diameter, pale white, circular with entire margins, convex and non-swarming. Pigments are not produced on blood agar. Strain grows on blood agar in aerobiosis and microaerobiosis at 22-25C, 30ºC and 37ºC, but not at 42ºC. In anaerobiosis growth only occurs at 37ºC. Produces oxidase and catalase activities, but not urease activity. Strain hydrolyses indoxyl acetate but not casein, lecithin or starch. Produces

H_2S from triple sugar iron agar and reduces nitrates on Cook nitrate agar. Growth occurs in blood agar supplemented with 0.1% sodium deoxycholate, 0.05% safranine, 0.005% fuchsine, 0.0005% crystal violet. Growth not occurs in media supplemented with 2% NaCl, 4% NaCl, 1% oxgall, 1% glycine, 0.001 brilliant green nor 0.01-0.1% TTC. Strain grows in minimal media but not in CDCA or McConkey agars. Strain is susceptible to cefoperazone (64 mg l^{-1}) and cephalothin (30 mg l^{-1}) and showed resistance to nalidixic acid (30 mg l^{-1}).

The type strain is AHV-9/2010^T (= LMG 29975^T= CECT 9299^T) isolated from a rectal swab of a southern elephant seal (*Mirounga leonina*) in the Avian Island (Antarctic Peninsula).

Conflict of Interest

The authors have no conflict of interest to declare.

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Figure legends

Figure 1. Neighbour-Joining tree based on 16S rRNA gene sequences (1406 bp) showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^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.

Figure 2. Neighbour-Joining tree based on the concatenated sequences of *atpA*, *gyrB*, *hsp60*, *rpoB* and *gyrA* (3061 bp) genes showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^T within the genus *Arcobacter*. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.

Table 1. Differential characteristics of *Arcobacter miroungae* sp. nov. AHV-9/2010^T and its closely related species *A. skirrowii* LMG 6621^T.

Characteristic	***A. miroungae* sp. nov.** AHV-9/2010^T	***A. skirrowii***
Growth at/with/on:		
42°C (microaerobic)	-	+
30°C (anaerobic)	-	+
42°C (anaerobic)	-	+
4% NaCl	-	+
1% glycine	-	V (+)*
0.01% TTC	-	+
CCDA	-	+
Minimal medium	+	-
Triple-sugar iron	+	-*
Resistance to:		
Nalidixic acid (30 mg l^{-1})	+	-*

*Data extracted from the *A. skirrowii* description (n=18) (Vandamme et al., 1992).

Figure 1

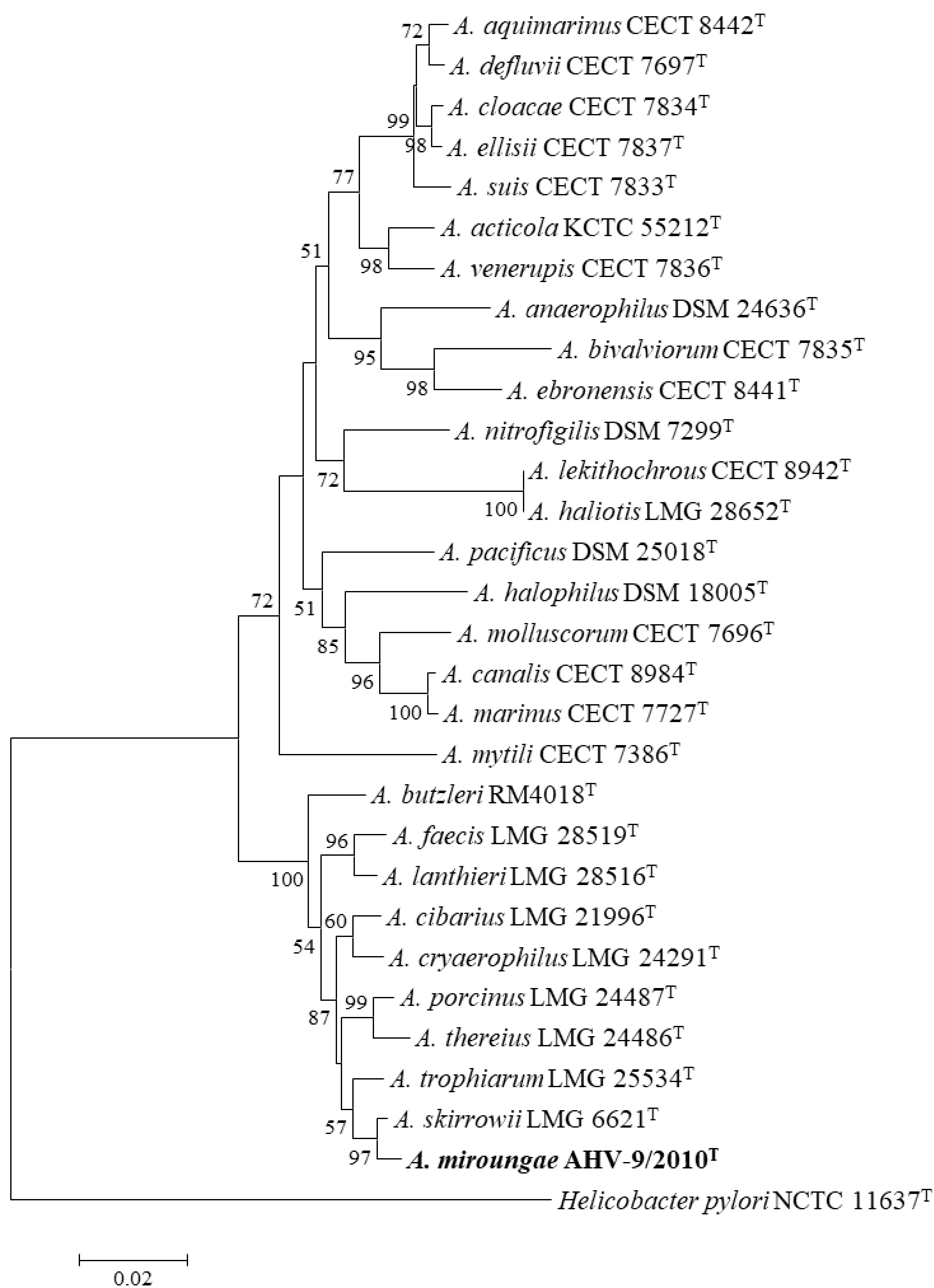
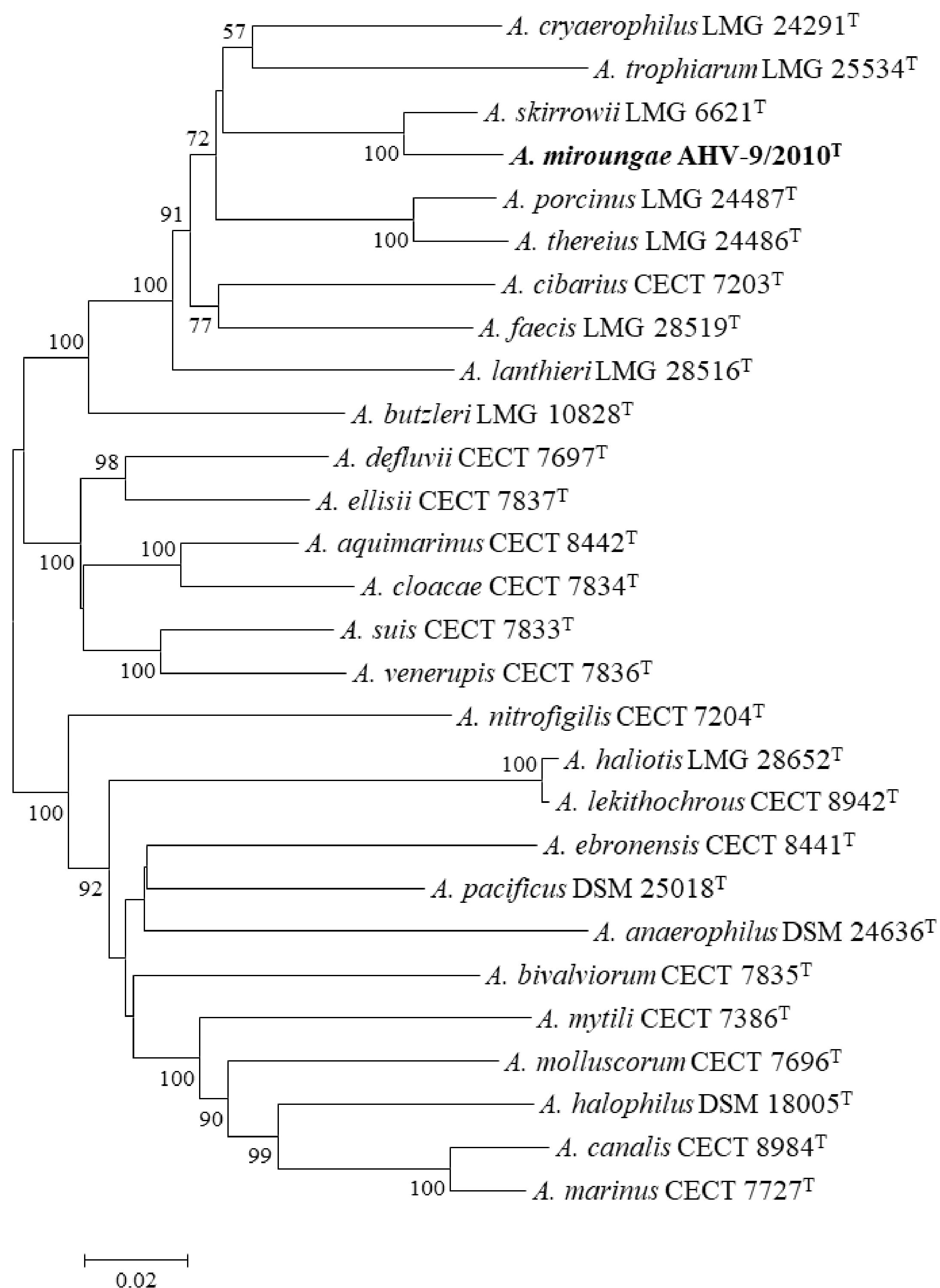
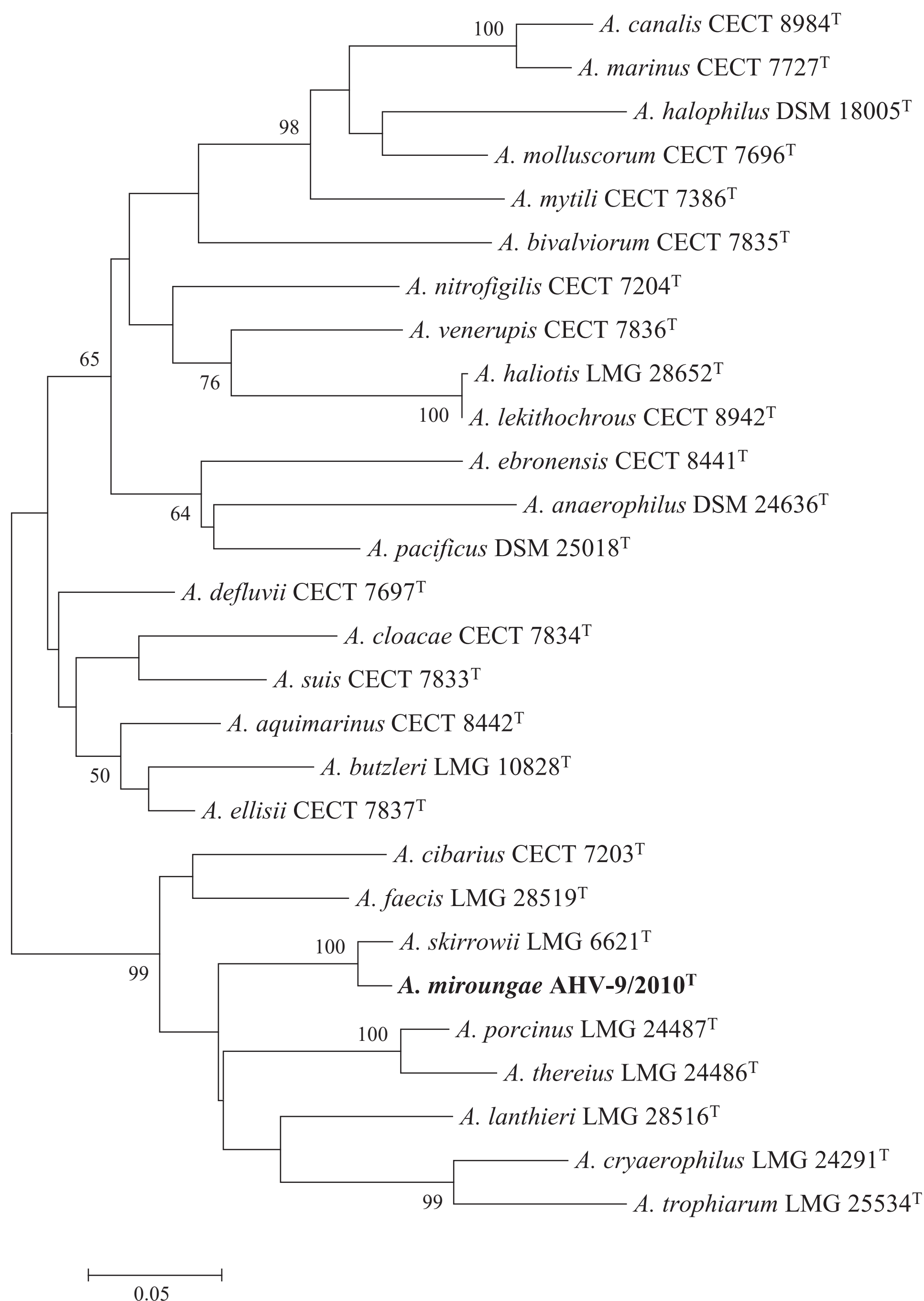
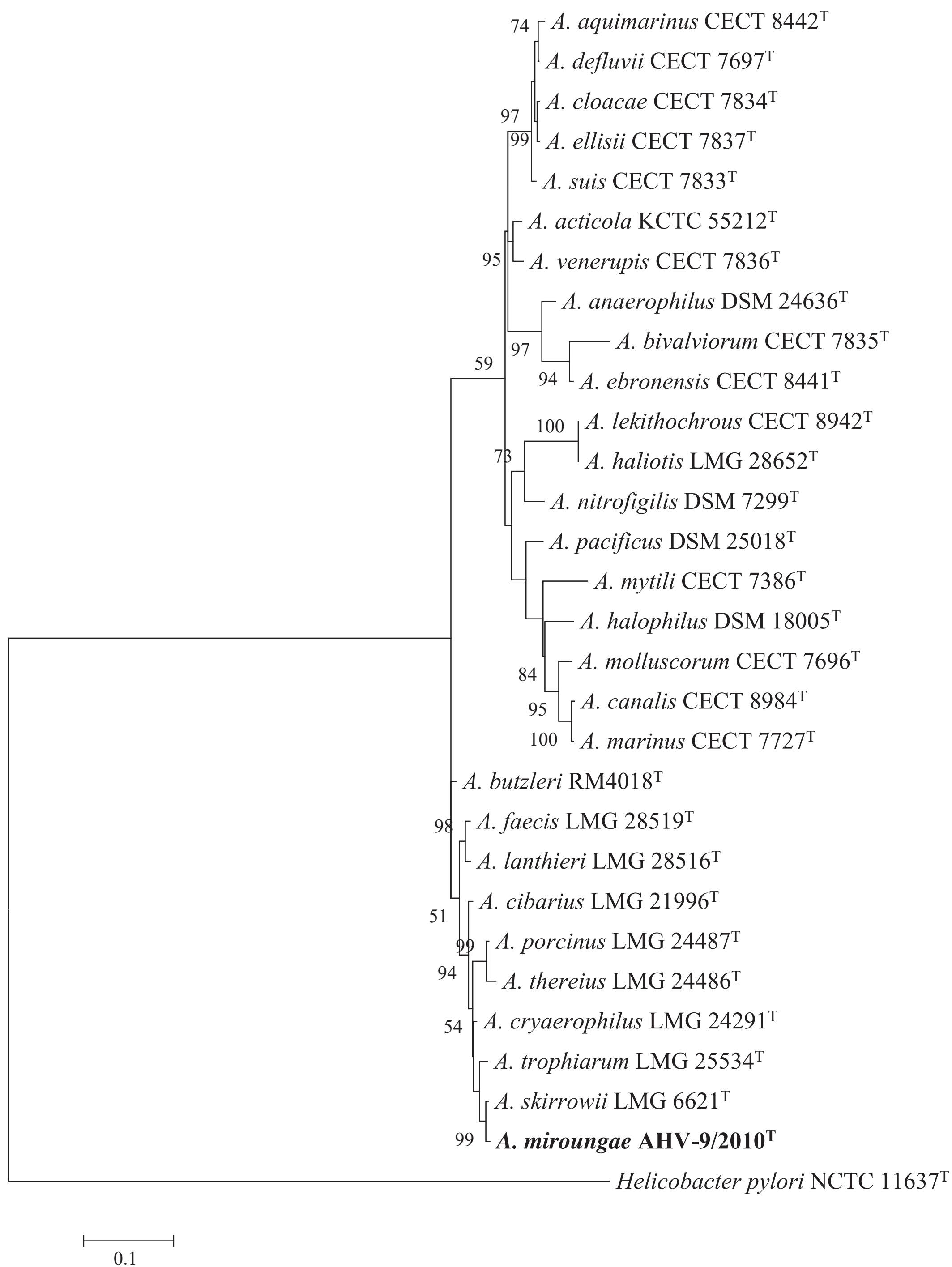


Figure 2

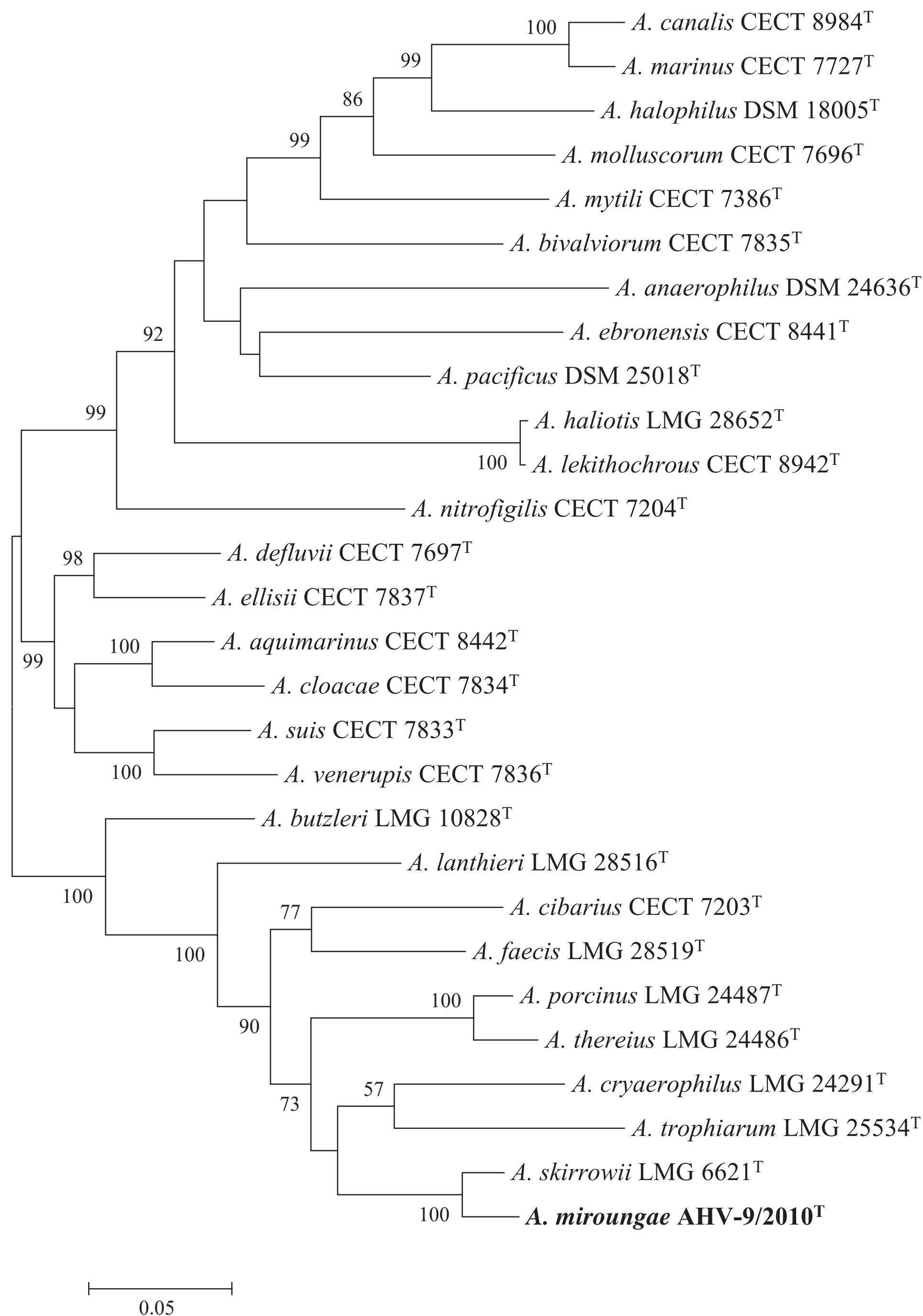




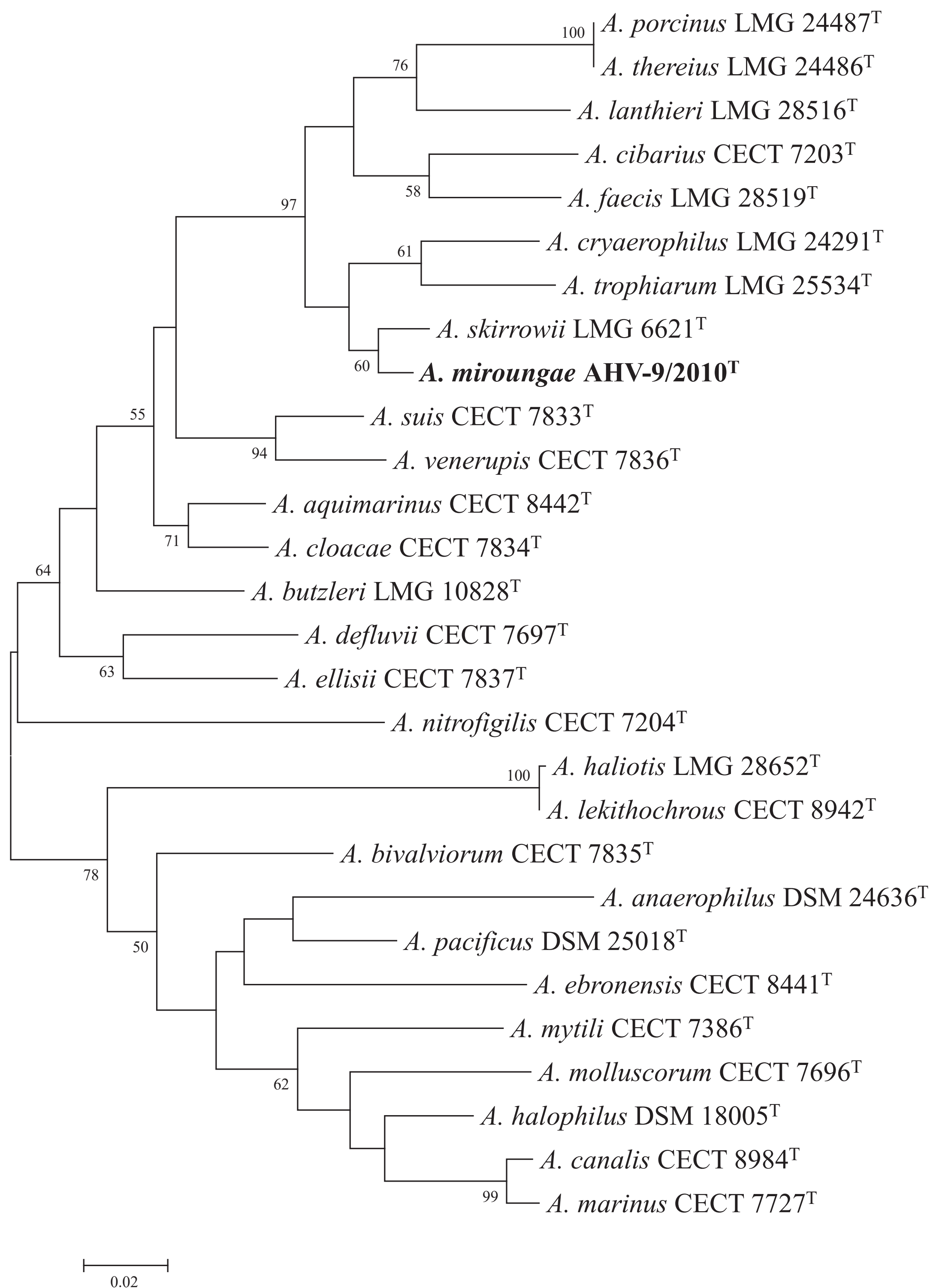
Supplementary figure S1. Maximum Likelihood tree (model GTR+G+I) based on the *rpoB* gene sequence (622 bp) showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



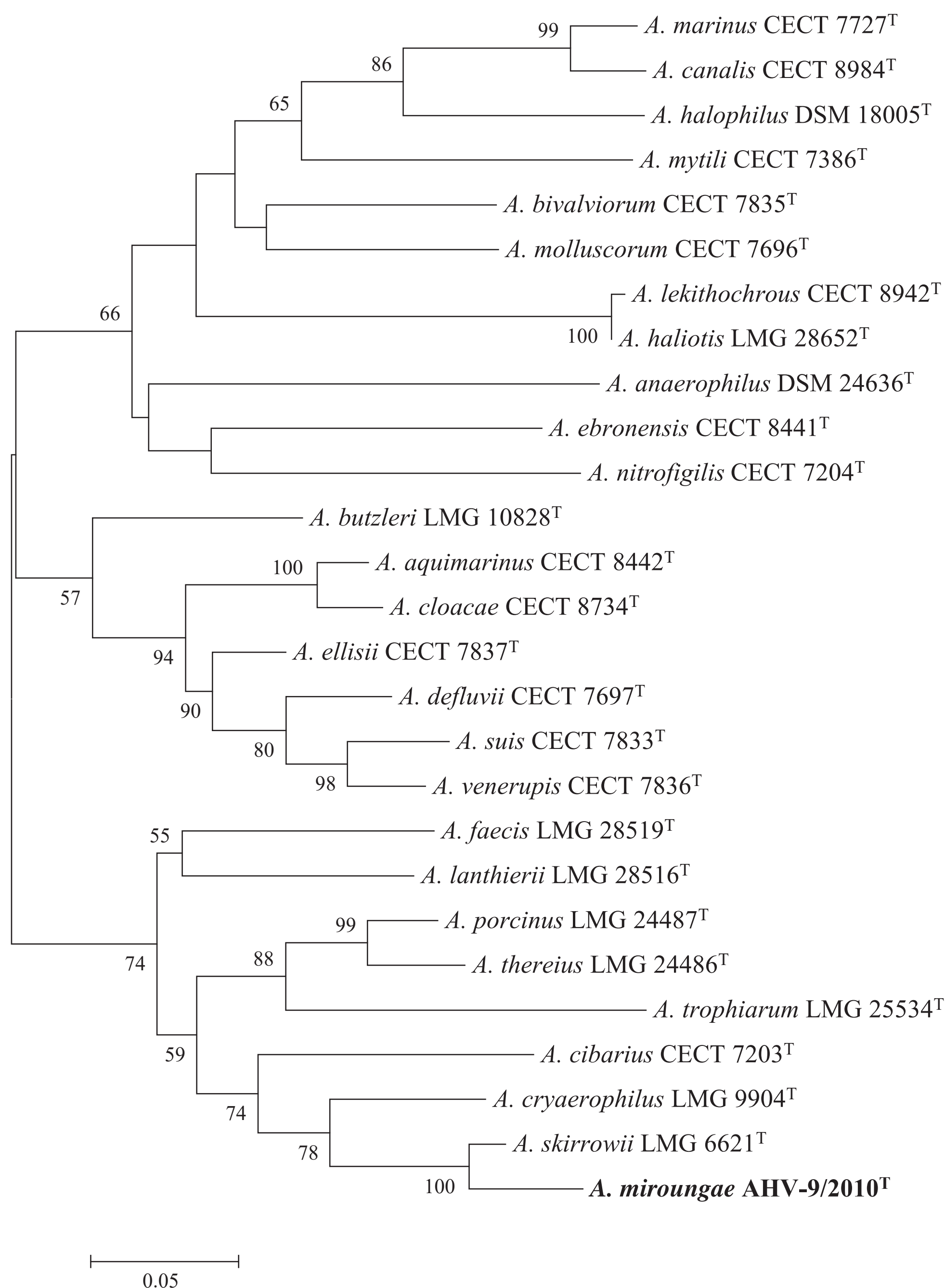
Supplementary figure S2. Maximum Likelihood tree (model GTR+G+I) based on the 16S rRNA gene sequence (1417 bp) showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at thenodes of the tree. Bar indicates 10 substitutions per 100 nt.



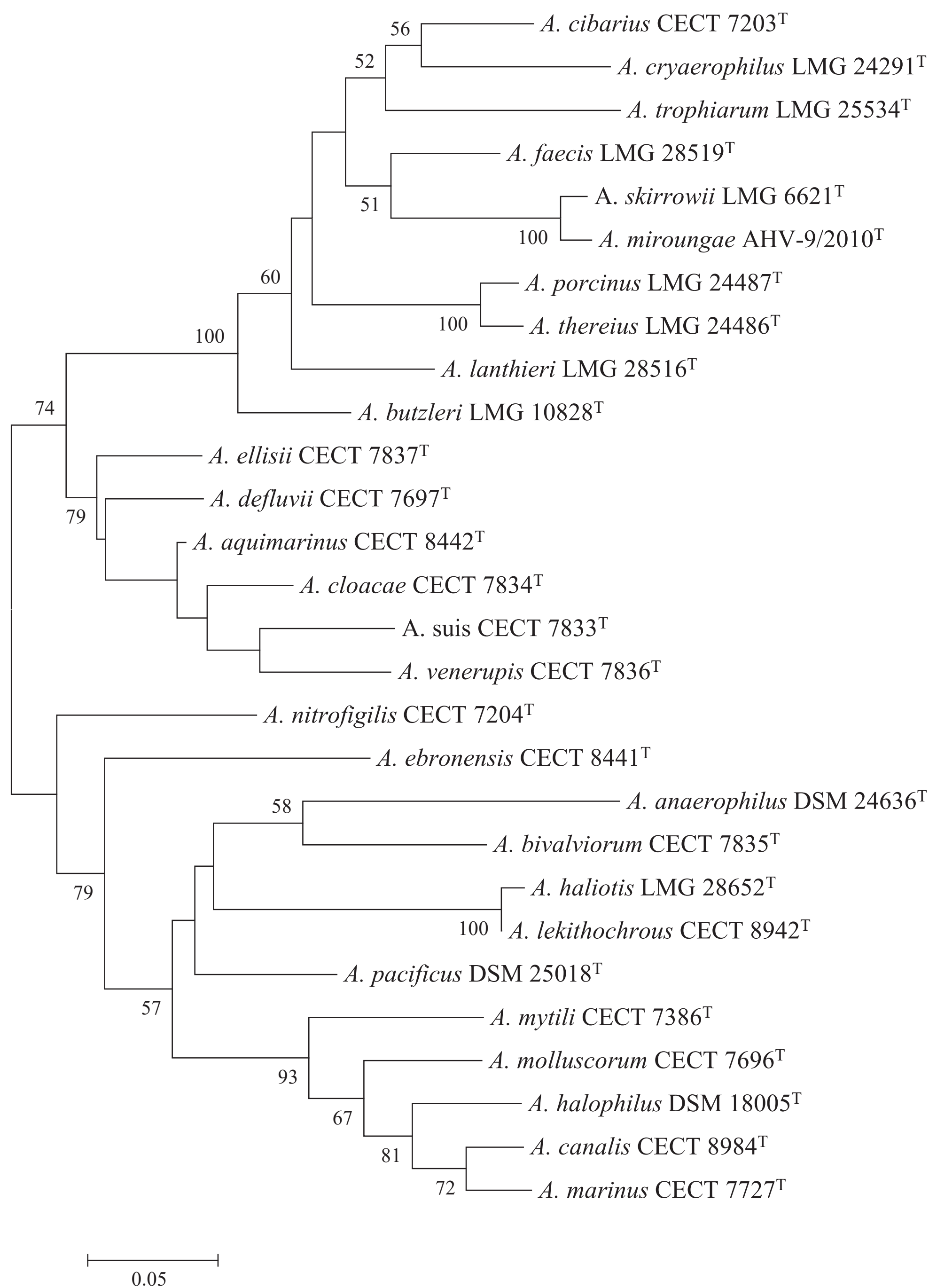
Supplementary figure S3. Maximum Likelihood tree (GTR+G+I) based on the concatenated sequences of *atpA*, *gyrB*, *hsp60*, *rpoB* and *gyrA* (3103 bp) genes showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



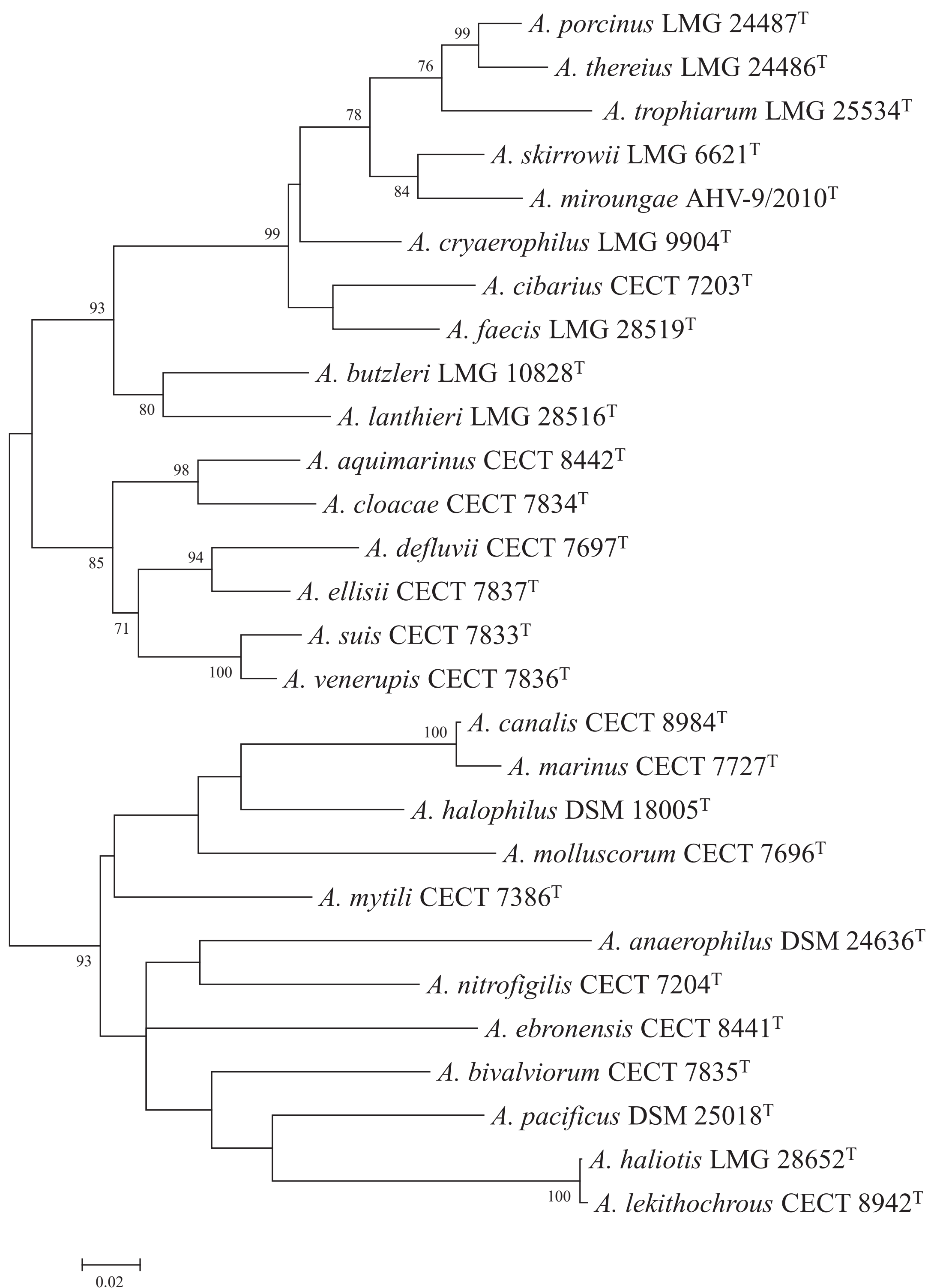
Supplementary figure S4. Maximum Likelihood tree (model GTR+G+I) based on the *atpA* gene sequence (612 bp) showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.



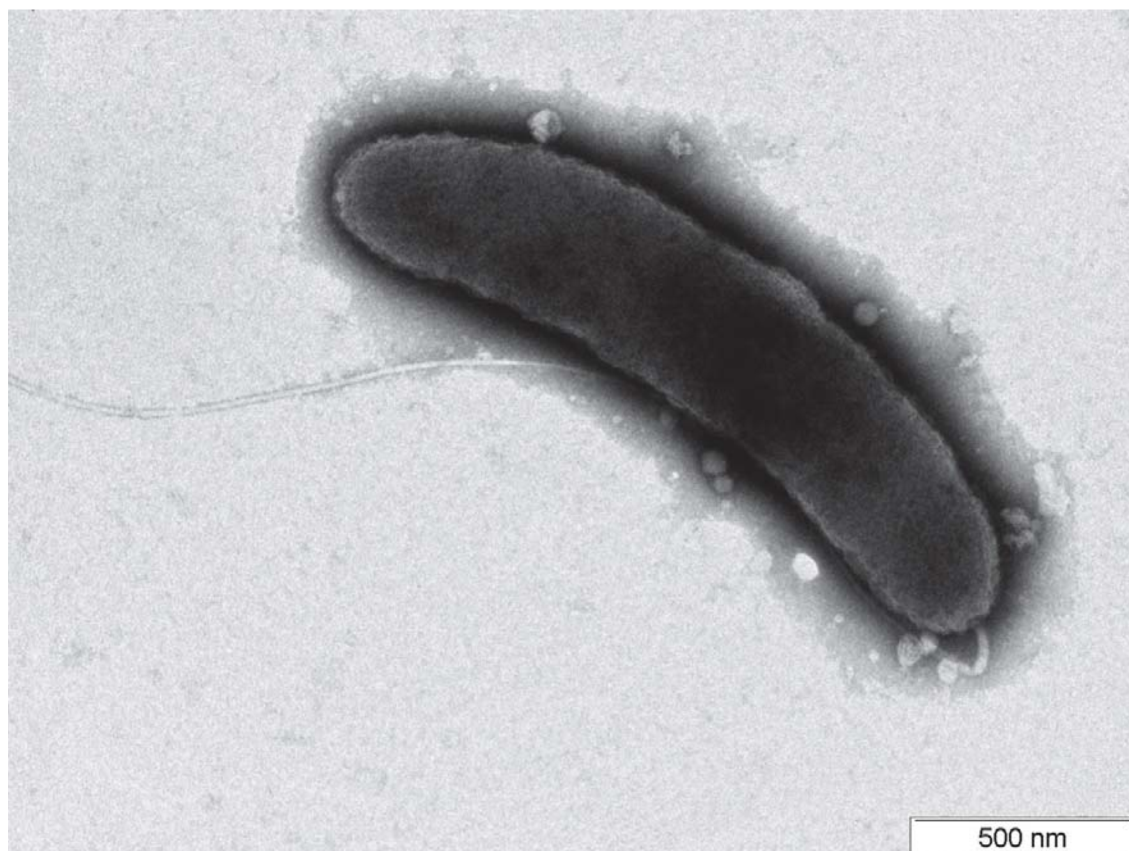
Supplementary figure S5. Maximum Likelihood tree (model GTR+G+I) based on the *gyrA* gene sequence (686 bp) showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^{T} within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



Supplementary figure S6. Maximum Likelihood tree (model GTR+G) based on the *gyrB* gene sequence (618 bp) showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



Supplementary figure S7. Maximum Likelihood tree (model GTR+G+I) based on the *hsp60* gene sequence (595 bp) showing the phylogenetic position of *A. miroungae* sp. nov. AHV-9/2010^T within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.



Supplementary figure S8. Transmission electron microscopy images of a cell of *A. miroungae* sp. nov AHV-9/2010^{T} with negative staining using phosphotungstic acid Bar, 500nm.

4.5 A polyphasic and taxogenomic evaluation uncovers *Arcobacter cryaerophilus* as a species complex that embraces four genomovars. (2018) Pérez-Cataluña, A., Collado, L., Salgado, O., Lefiñanco, V. and Figueras, M.J. *Front. Microbiol*. 9:805.

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A Polyphasic and Taxogenomic Evaluation Uncovers *Arcobacter cryaerophilus* as a Species Complex That Embraces Four Genomovars

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The species *Arcobacter cryaerophilus* is found in many food products of animal origin and is the dominating species in wastewater. In addition, it is associated with cases of farm animal and human infectious diseases,. The species embraces two subgroups i.e., 1A (LMG 24291^T = LMG 9904^T) and 1B (LMG 10829) that can be differentiated by their 16S rRNA-RFLP pattern. However, some authors, on the basis of the shared intermediate levels of DNA-DNA hybridization, have suggested abandoning the subgroup classification. This contradiction indicates that the taxonomy of this species is not yet resolved. The objective of the present study was to perform a taxonomic evaluation of the diversity of *A. cryaerophilus.* Genomic information was used along with a Multilocus Phylogenetic Analysis (MLPA) and phenotypic characterization on a group of 52 temporally and geographically dispersed strains, coming from different types of samples and hosts from nine countries. The MLPA analysis showed that those strains formed four clusters (I–IV). Values of Average Nucleotide Identity (ANI) and *in silico* DNA-DNA Hybridization (*is*DDH) obtained between 13 genomes representing strains of the four clusters were below the proposed cut-offs of 96 and 70%, respectively, confirming that each of the clusters represented a different genomic species. However, none of the evaluated phenotypic tests enabled their unequivocal differentiation into species. Therefore, the genomic delimited clusters should be considered genomovars of the species *A. cryaerophilus.* These genomovars could have different clinical importance, since only the cluster I included strains isolated from human specimens. The discovery of at least one stable distinctive phenotypic character would be needed to define each cluster or genomovar as a different species. Until then, we propose naming them "*A. cryaerophilus gv. pseudocryaerophilus*" (Cluster I = LMG 10229^T), "*A. cryaerophilus gv. crypticus*" (Cluster II = LMG 9065^T), "*A. cryaerophilus gv. cryaerophilus"* (Cluster III = LMG 24291^T) and "*A. cryaerophilus gv. occultus*" (Cluster IV = LMG 29976^T).

Keywords: ***Arcobacter cryaerophilus*, *is*DDH, ANI, MLPA, genomovar**

INTRODUCTION

The genus *Arcobacter,* within the family *Campylobacteraceae*, was proposed by Vandamme et al. (1991) to reclassify two species that were, at that time, assigned to the genus *Campylobacter* i.e., *Campylobacter nitrofigilis* (*Arcobacter nitrofigilis*, that was selected as the representative or the type species of the genus) and *Campylobacter cryaerophila* (now *Arcobacter cryaerophilus*). The phenotypic characteristics that differentiate *Campylobacter* and *Arcobacter* are the ability of the latter to grow in aerobic conditions and at lower temperatures (Vandamme et al., 1991; Collado and Figueras, 2011).

Using more than 4000 genomes, Waite et al. (2017) recently analyzed the 16S and 23S rRNA genes and 120 protein sequences and as a result they moved the *Epsilonproteobacteria* to the phylum level with the name Epsilonbacteraeota. In addition, they created a new family *Arcobacteraceae* that includes only the genus *Arcobacter*. Currently, the genus *Arcobacter* includes 27 species (Park et al., 2016; Whiteduck-Léveillée et al., 2016; Diéguez et al., 2017; Figueras et al., 2017; Tanaka et al., 2017; Pérez-Cataluña et al., 2018), four of which have been linked with human disease: *Arcobacter butzleri*, *A. cryaerophilus*, *A. thereius,* and *A. skirrowii* (Collado and Figueras, 2011; Figueras et al., 2014; Ferreira et al., 2015). The species *A. cryaerophilus* has been found in many food products of animal origin (like poultry, pork, lamb, and seafood and in dairy food processing facilities (Collado et al., 2008; Collado and Figueras, 2011).

On the basis of the different Restriction Fragment Length Polymorphism (RFLP) of the 16S and 23S rRNA genes, Kiehlbauch et al. (1991) and Vandamme et al. (1992) divided the species *A. cryaerophilus* into two subgroups, subgroup 1 or 1A and subgroup 2 or 1B (from here on we will call them subgroups 1A and 1B), represented by strains LMG 24291^{T} (=LMG 9904^{T}) and LMG 10829, respectively. Additionally, it was demonstrated that the two subgroups showed different whole-cell protein and fatty acid contents (Vandamme et al., 1992) and clustered apart by their Amplified Fragment Length Polymorphism (AFLP) patterns (On et al., 2003). A 16S rDNA-RFLP identification method established the separation of the subgroups on the basis of their restriction patterns (Figueras et al., 2008). Despite strains belonging to both subgroups having been found at the same time in animal and human clinical samples and in food products, 1B is generally much more frequently found than 1A (Collado and Figueras, 2011 and references therein). In 2010, Debruyne et al. (2010) reassessed the taxonomy of these two subgroups of *A. cryaerophilus* using 59 strains isolated mainly from aborted animals (74% of the strains) and human faces (19%). The clustering of the strains obtained by AFLP and by the phylogenetic analysis of the *cpn60* gene, together with the shared intermediate levels of DNA-DNA hybridization observed between the strains lead the authors to conclude that despite *A. cryaerophilus* having a complex taxonomy, the subgroup nomenclature should be abandoned (Debruyne et al., 2010). Furthermore, it was considered that the type strain (LMG 24291^{T} = LMG 9904^{T}) of *A. cryaerophilus* was not representative of the species because it corresponded with the less abundant 1A subgroup. They therefore proposed that it should be changed for the strain LMG 10829, representative of subgroup 1B (Debruyne et al., 2010). However, a recent metagenomic analysis of *Arcobacter* populations recovered from sewage samples of the wastewater treatment plant in the city of Reus (Spain) and from various cities of the United States gave evidence that both *A. cryaerophilus* subgroups (1A and 1B) were dominating in this environment (Fisher et al., 2014). In addition, a different prevalence of the two *A. cryaerophilus* subgroups was found depending on the wastewater temperature, 1B dominating in wastewater samples with temperatures above 20°C. Fisher et al. (2014) concluded that this finding is relevant because understanding the ecological factors that affect the fate of *Arcobacter* spp. in wastewater may help to better understand the risks associated with these emerging pathogens. The latter study showed that both subgroups of *A. cryaerophilus* were abundant and represented two different ecotypes. Therefore, based on those findings, a new polyphasic re-evaluation of the taxonomic diversity of this species is required. The aim of the present study was to investigate the taxonomy of *A. cryaerophilus,* evaluating strains from 9 different countries recovered from wastewater, different types of shellfish, human faces and various types of animal samples (feces, various viscera from fetuses, uterus, and milk). To our knowledge, this is the most diverse collection of strains of this species studied so far. The polyphasic study involved a phylogenetic analysis of the sequences of the 16S and 23S rRNA genes and of several housekeeping genes, an analysis of 13 genomes (7 of which were obtained in this study) from a representative strains and a phenotypic characterization.

Abbreviations: LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium Culture Collection; MLPA, Multilocus Phylogenetic Analysis; ANI, Average Nucleotide Identity; *is*DDH, *in silico* DNA-DNA hybridization.

MATERIALS AND METHODS

Strains Used in This Study

The study included a total of 52 strains that were widely distributed, both geographically and by the type of sample from which they were isolated that, included different host species (humans, pigs, cow, deer, clams, etc.) and environments (water, milk, reclaimed water etc.) as show in **Table 1**. Six strains possessed their genomes available at the GenBank database, 36 were field isolates from different sources and countries collected over a broad time frame (1985–2013) and 10 strains were from the BCCM/LMG Bacteria Culture Collection (**Table 1**). Among the latter was the type strain of *A. cryaerophilus* LMG 24291^{T} that corresponds to subgroup 1A and the reference strain LMG 10829 of the subgroup 1B (**Table 1**). The 46 strains were re-evaluated or ascribed to subgroups 1A or 1B using the 16S rDNA-RFLP method described by Figueras et al. (2008, 2012). The method consists of the digestion of an amplified fragment (1026 bp) of the 16S rRNA gene with the enzyme *Mse*I, which produces a pattern with different band sizes for subgroup 1A (395, 216, 143, 138 bp) and for subgroup 1B (365, 216, 143, and 138 bp). The RFLP patterns of the six genomes from the GenBank database (genomes L397 to L401 and L406) were obtained by an *in silico* simulation of the enzymatic digestion using GeneQuest

TABLE 1 | Strains used ($n = 52$) in this study included field isolates, the type and reference collection strains of the species *A. cryaerophilus* and genomes from the NCBI database[a] and 7 obtained in this study[b] (accession numbers in **Table 2**).

Country	Strain	Source	Isolation year	16S-RFLP Pattern	Cluster
Brazil	F196	Aborted porcine fetus	1997	1B	I
Brazil	UF1T	Uterus, sow	1997	1B	I
Brazil	UF2T	Uterus, sow	1997	1B	I
Brazil	UPER3	Uterus, sow	1997	1B	I
Canada	LMG 10229[b]	Kidney, aborted porcine fetus	1990	1B	I
Canada	LMG 10241	Kidney, aborted porcine fetus	1990	1B	I
Canada	LMG 10210[b]	Aborted bovine fetus	1990	1B	IV
Canada	L397[a]	Wastewater	2008	1B	I
Canada	L398[a]	Water	2008	1B	I
Canada	L399[a]	Wastewater	2008	1B	I
Canada	L400[a]	Wastewater	2008	1B	I
Canada	L401[a]	Goose feces	2009	1B	I
Canada	L406[a]	Water	2008	1B	I
Chile	AB3A	Abomasum, aborted bovine fetus	2011	1B	I
Chile	AB74A	Abomasum, aborted bovine fetus	2013	1B	I
Chile	AO2A	Lungs, aborted ovine fetus	2011	1B	I
Chile	AL 20-1	Clam	2011	1B	II
Chile	CV-152	Feces, deer	2013	1A	III
Chile	CV-2101	Feces, deer	2013	1A	III
Chile	EMU-3	Feces, emu	2013	1A	III
Chile	FE7	Feces, chicken	2005	Abutz	II
Chile	HHS 118A	Feces, asymtomatic human	2013	1B	I
Chile	HHS 133A	Feces, asymtomatic human	2013	1B	I
Chile	HHS 188A	Feces, asymtomatic human	2013	1B	I
Chile	HHS 191A	Feces, asymtomatic human	2013	1B	I
Chile	HHS 205A	Feces, asymtomatic human	2013	1B	I
Chile	MC 2-2	Surf clam	2011	NP	I
Chile	MCV 42-1	Feces, cow	2011	1B	I
Chile	ME 15-4	Mussel	2011	Abutz	II
Chile	NAV 15-1	Razor clam	2011	1A	IV
Chile	NAV12-2	Razor clam	2011	NP	I
Chile	NB14A	Jejunum, calf	2011	1B	I
Costa Rica	14 PHA	Viscera, chicken	2011	1B	I
Costa Rica	20 PHF	Viscera, chicken	2011	1B	I
Ireland	LMG 24291[Tb]	Brain, aborted bovine fetus	1985	1A	III
Ireland	LMG 9065[b]	Placenta, aborted ovine fetus	1989	1A	II
Ireland	LMG 9861[b]	Peritoneum, aborted bovine fetus	1990	1B	I
Ireland	LMG 9863[b]	Placenta, aborted ovine fetus	1990	Abutz	II
Ireland	LMG 29976[b]	Eye, aborted porcine fetus	1990	1A	IV
Ireland	LMG 9871[b]	Kidney, aborted bovine fetus	1990	Abutz	II
Italy	284/1	Cow milk	2012	1B	I
Italy	BUF3	Buffalo milk	2012	1B	I
Italy	FEBU4	Feces, buffalo	2012	1B	I
New Zealand	8749401	Diarrhoeic feces, human	2008	1B	I
New Zealand	8756347	Diarrhoeic feces, human	2008	1B	I
Spain	8122333	Diarrhoeic feces, human	2012	1B	I
Spain	RW15-1	Reclaimed water	2013	1A	IV
Spain	RW17-4	Reclaimed water	2013	1A	IV
Spain	RW25-5	Reclaimed water	2013	1A	I
Spain	RW33-8	Reclaimed water	2013	1A	I
Spain	RW45-3	Reclaimed water	2013	1A	IV
USA	LMG 10829	Human blood	1990	1B	I

software (DNASTAR, USA). When a different pattern from that expected for *A. cryaerophilus* was obtained, it was compared with those patterns described for the type strains of all the *Arcobacter* species by Figueras et al. (2008, 2012). In addition the identity of the strains were confirmed by sequencing the *rpoB* gene using primers and conditions described in other studies (Collado et al., 2009; Levican et al., 2015).

Phylogenetic Analysis

A Multilocus Phylogenetic Analysis (MLPA) was carried out by amplifying and sequencing 4 housekeeping genes (*gyrB, rpoB, atpA*, and *cpn60*) following protocols described by Levican Asenjo (2013). In addition, these genes and the 16S and 23S rRNA genes were extracted from the 7 obtained genomes and from the 6 downloaded from the GenBank database. Accession number or locus tag of each gene and strain are show in Supplementary Table S1. Genes were aligned (Supplementary Figure S4) using CLUSTALW (Larkin et al., 2007) implemented in MEGA 6 software (Tamura et al., 2013). The same software was used for the phylogenetic analysis using Neighbor-Joining (NJ) algorithm (Kimura, 1980; Saitou and Nei, 1987) and the bootstrap support for individual nodes was calculated with 1,000 replicates.

Whole Genome Sequencing and Analysis

The genome sequence of the type strain of *A. cryaerophilus* (LMG 24291^T) and of six additional strains (LMG 10229^T, LMG 9861, LMG 9065^T, LMG 9871, LMG 29976^T, and LMG 10210) representative of the different MLPA clusters were obtained in the present study using Illumina MiSeq platform (San Diego, CA, USA). The genomic DNA was extracted from pure cultures using the Easy-DNA™ gDNA Purification kit (Invitrogen, Madrid, Spain). Genomic libraries were prepared with the Nextera® XT DNA Sample Preparation Kit (Illumina) following manufacturer's instructions. Genome assembly was carried out with the SPAdes 3.9 (Nurk et al., 2013) and the CGE assemblers (Larsen et al., 2012) and the best results were selected for further analysis. Assembled genomes were annotated using Prokka v1.11 software (Seemann, 2014). Additionally, the protein-encoding sequences (CDS) were annotated using the Rapid Annotation Subsystem Technology (RAST) (Aziz et al., 2008) and the PATRIC server v3.5.2. (Wattam et al., 2017). The general characteristics derived from the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) and described for the 13 genomes (6 from the GenBank database and 7 from this study) were: genome size (Mb), number of contigs, N50 (bp), G+C content (%) and the number of predicted CDS. Furthermore, the genomes were compared by the Average Nucleotide Identity (ANI) and the *in silico* DNA-DNA hybridization (*is*DDH) indices using OrthoANI (Lee et al., 2015) and Genome-to-Genome Distance Calculator software (Meier-Kolthoff et al., 2013), respectively.

Additionally, a phylogenetic analysis of the 13 genomes (LMG 24291^T, LMG 10229^T, LMG 9861, L397-L401, L406, LMG 9065^T, LMG 9871, LMG 29976^T, and LMG 10210) was carried out using the Maximum Likelihood estimation using RAxML (Stamatakis, 2014) with the pipeline implemented in the PATRIC server (Wattam et al., 2017). The genome of *A. trophiarum* LMG 25534^T was used as outgroup. As a first step, the phylogeny was constructed using a set of homologous proteins identified with BLASTp (Boratyn et al., 2013) and clustered with the Markov Cluster Algorithm (MCL) (Dongen, 2000). The second step was an alignment of the protein set using MUSCLE (Edgar, 2004) and the Hidden Markov Models (HMM) were constructed with HMMER tools (Eddy, 1998).

Virulence and Antibiotic Resistance Genes

Virulence genes were searched by BLASTn analysis with default parameters using the Virulence Factors of Pathogenic Bacteria Database (VFDB) (Chen et al., 2005), Victors Database (University of Michigan, USA) and PATRIC_VF (Wattam et al., 2017). Antibiotic resistance genes were searched using the Antibiotic Resistance Database (ARDB) (Liu and Pop, 2009) and the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017). The five mentioned databases are included at the Specialty Genes tool available at the PATRIC server (Wattam et al., 2017). Furthermore, the Antibiotic Resistance Gene-Annotation database (ARG-ANNOT) (Gupta et al., 2014) was also used to search antibiotic resistance genes by BLASTp analysis using default parameters and the database ARG-ANNOT AA V3 (March 2017). Virulence and resistance mechanisms were also searched for with RAST (Aziz et al., 2008) and PATRIC servers (Wattam et al., 2017). Additionally, genes related with the virulence of *Arcobacter* (Collado and Figueras, 2011; Douidah et al., 2012; Levican et al., 2013a) were searched for with BLASTn using sequences obtained from GenBank and from the annotated *Arcobacter* genomes of *A. butzleri* RM4018, *A. nitrofigilis* DSM 7299 and *Arcobacter* sp. L. The genes studied were *cadF* and *cj1349*, which encode two fibronectin binding proteins; *ciaB* encodes the invasion protein CiaB, *mviN* gene related to peptidoglycan synthesis; *pldA* gene encodes a phospholipase; *tlyA* gene codifies for a hemolysine; *hecB* related to hemolysis activation; *hecA* gene that encodes an adhesion protein and finally the gene *irgA* that codifies an iron-regulated outer membrane protein (Collado and Figueras, 2011; Douidah et al., 2012; Levican et al., 2013a). The accession number or locus tag of those genes are show in Supplementary Table S2. A phylogenetic analysis was conducted using the three virulence genes (*cj1349*, *mviN*, and *pldA*) present in all the studied genomes to evaluate their genetic relatedness and evolution.

Comparison of the Genome Derived Metabolic and Phenotypic Information

The genomes of the seven representative strains from each cluster (LMG 10229^T, LMG 9861, LMG 9065^T, LMG 9871, LMG 24291^T, LMG 29976^T, and LMG 10210) were compared using the Functional Comparison Tool implemented in the Seed Viewer (Overbeek et al., 2014). This software uses the protein sequences of each compared genome annotated with RAST (Aziz et al., 2008) and reconstructs the metabolic pathways. On the other hand, the phenotypic traits derived from each genome were obtained with Traitar software (Weimann et al., 2016) using the protein annotations obtained with Prokka v1.2 (Seemann, 2014). This software infers phenotypic traits using

data from the Global Infectious Disease and Epidemiology Online Network (GIDEON) and from the Bergey's Systematic Bacteriology (Goodfellow et al., 2012). The software works with a total of 67 traits that embrace different microbiological or biochemical characteristics involved in enzyme activity, growth, oxygen requirements, morphology, and hydrogen sulfide production (Weimann et al., 2016).

Phenotypic Characterization

Phenotypic characterization of the 46 strains included 9 tests recommended in the guidelines for defining new species of the family *Campylobacteraceae* (Ursing et al., 1994; On et al., 2017) and 7 additional tests used in the description of other *Arcobacter* spp. (Donachie et al., 2005; Houf et al., 2005). Most of these tests were chosen using as

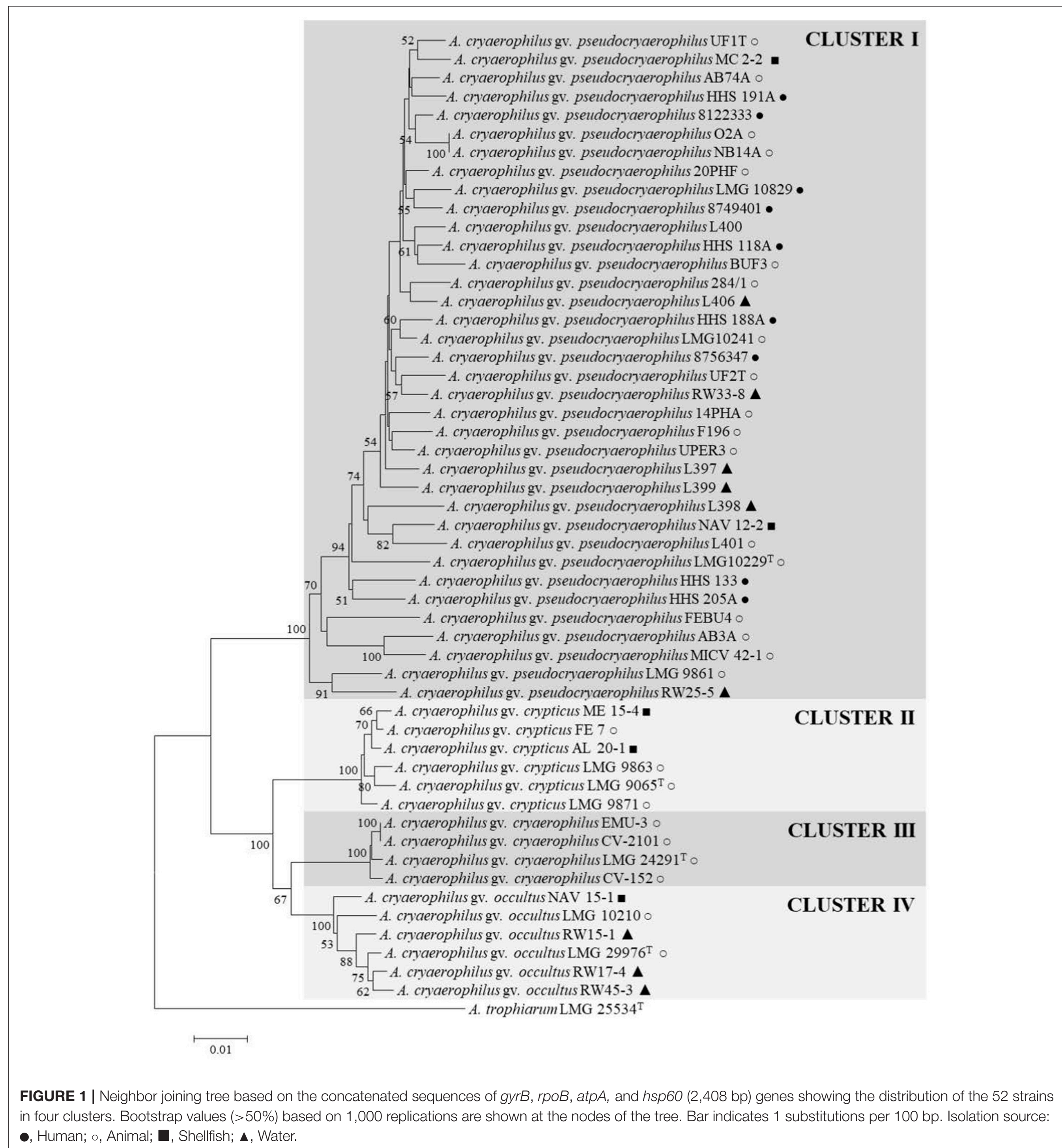

FIGURE 1 | Neighbor joining tree based on the concatenated sequences of *gyrB*, *rpoB*, *atpA,* and *hsp60* (2,408 bp) genes showing the distribution of the 52 strains in four clusters. Bootstrap values (>50%) based on 1,000 replications are shown at the nodes of the tree. Bar indicates 1 substitutions per 100 bp. Isolation source: ●, Human; ○, Animal; ■, Shellfish; ▲, Water.

a criterion the biochemical tests that gave variable results for both *A. cryaerophilus* subgroups in the previous study by On (1996), in which a total of 67 phenotypic tests were analyzed from 9 and 10 strains of subgroups 1A and 1B, respectively. Growth conditions on blood agar were tested (BD Difco, NJ, USA) at 37° and 42°C at three different atmospheres: aerobic, microaerobic ,and anaerobic conditions. The biochemical properties were tested at 30°C in aerobic conditions for the 46 strains using positive and negative controls in parallel for each specific test. To evaluate inter laboratory reproducibility, the strains LMG 9065, LMG 9861, LMG 9871, LMG 10229 and LMG 24291^{T}were tested in parallel in two different laboratories in different countries (Chile and Spain).

RESULTS AND DISCUSSION

Molecular Identification and Phylogeny

Table 1 shows that 46 of the 52 strains gave RFLP patterns defined by Figueras et al. (2008) for *A. cryaerophilus* and 4 showed the one for *A. butzleri* (FE7, ME15-4, LMG 9863, and LMG 9871). However, strains NAV12-2 and MC2-2 produced a new RFLP pattern different to the described ones (Figueras et al., 2008, 2012). From the 46 strains that gave the pattern of *A. cryaerophilus*, 34 gave the pattern of the subgroup 1B (including the in *silico* simulated patterns obtained from the 16S rRNA genes of the 6 GenBank genomes L397- L401 and L406) and 12 the one of the subgroup 1A. This demonstrated once more that subgroup 1B is more abundant than 1A, in agreement

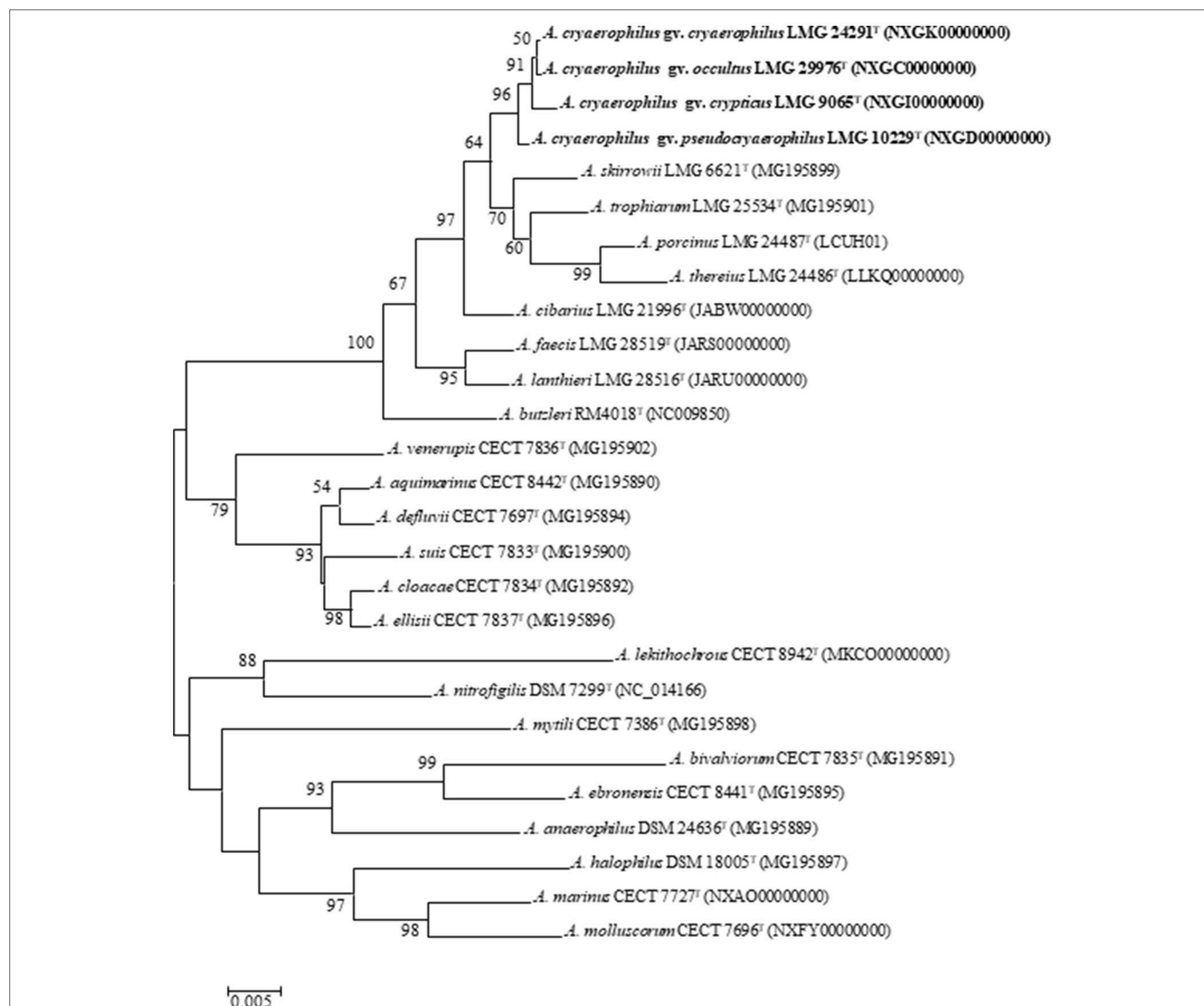

FIGURE 2 | Neighbor joining tree based on 16S rRNA (1,496 bp) sequences showing the phylogenetic position of the representative strains of the four clusters of *A. cryaerophilus* within the genus *Arcobacter*. Bootstrap values (>50%) based on 1,000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1,000 bp.

with results of previous studies (Debruyne et al., 2010; Collado and Figueras, 2011; Fisher et al., 2014). As Figueras et al. (2012) explained when describing the 16S rDNA-RFLP identification method, different RFLP patterns from those expected for the *Arcobacter* spp. can obtained for new species or might be due to the existence of a mutation on the targeted site of the endonucleases in a known species. The former occurred for instance in *A. mytili* (Collado et al., 2009) and *A. molluscorum* (Figueras et al., 2011a) among other species (Figueras et al., 2011b; Levican et al., 2012, 2013b, 2015). Mutations at the binding site of the endonuclease *MseI* were described in the strains LMG 9863 and LMG 9871 (used in this study, **Table 1**), but in this case instead of resulting in a new pattern they were responsible for generating the pattern for *A. butzleri* instead of *A. cryaerophilus* (Figueras et al., 2012).

The MLPA with the concatenated sequences (2,408 bp) of the four housekeeping genes (*gyrB*, *rpoB*, *atpA,* and *cpn60*) of the 52 strains showed that they grouped into four main clusters (**Figure 1**). Cluster I had 36 strains, most of them (88.8%) from the subgroup 1B, and included the reference strain for the 1B subgroup LMG 10829. The other four strain of this cluster presented the pattern of subgroup 1A ($n = 2$) and a different pattern to those described ($n = 2$). Cluster II ($n = 6$) corresponded to the four strains that showed a 16S rDNA-RFLP pattern similar to the one described for *A. butzleri* (Figueras et al., 2008) and two other strains with the patterns for the subgroups 1A and 1B. Cluster III, included the type strain of *A. cryaerophilus* LMG 24291^T and three field isolates from Chilean animals all belonging to the subgroup 1A, and Cluster IV comprised six strains, mostly from subgroup 1A ($n = 5$). Interestingly, strains recovered from human specimens belonged exclusively to Cluster I, suggesting potential host specificity because strains associated with farm animal abortions were present in the four clusters (**Figure 1**).

A representative type strain was selected from each cluster (I–IV) for further analysis and for constructing a 16S rRNA gene phylogenetic tree (**Figure 2**). The tree showed that the four strains formed separated branches, strains LMG 24291^T and LMG 29976^T being the nearest ones. The percentage of similarity of the 16S rRNA gene between the type strains ranged from 99.5% between strains LMG 10229^T (Cluster I) and LMG 9065^T (Cluster II) to 99.9% between the original type strain of *A. cryaerophilus* LMG 24291^T (Cluster III) and the representative strain of Cluster IV (LMG 29976^T). These results agree with what occurs between other species of *Arcobacter,* such as *A. ellisii* and *A. cloacae* (Figueras et al., 2011b; Levican et al., 2013b), where the 16S rRNA gene does not have enough resolution to differentiate the species. The phylogeny of the 23S rRNA gene (Supplementary Figure S1) and the one carried out with the concatenated sequences of the two rRNA genes (Supplementary Figure S2) presented the same topology shown with the 16S rRNA gene (**Figure 2**) and confirmed that the strains of Cluster III are more closely related to Cluster IV than to the other clusters.

Genome Analysis

The characteristics of the 13 compared genomes (8 representatives of Cluster I, two of clusters II and IV and

TABLE 2 | Characteristics of the 13 genomes from representative strains from each of the clusters.

Features	CLUSTERS												
	I								II		III	IV	
	LMG 10229^T (NXGD01[a])	LMG 9861 (NXGJ01[a])	L397 (LRUQ01[a])	L398 (LRUR01[a])	L399 (LRUS01[a])	L400 (LRUT01[a])	L401 (LRUU01[a])	L406 (LRUV01[a])	LMG9065^T (NXGI01[a])	LMG9871 (NXGH01[a])	LMG 24291^T (NXGK01[a])	LMG 29976^T (NXGK01[a])	LMG 10210 (NXGE01[a])
Deep Coverage	25X	37X	180X	129X	77X	106X	133X	190X	74X	19X	196X	187X	160X
Size (Mb)	2.06	2.02	2.31	2.03	2.10	2.20	2.17	2.02	2.05	2.08	2.05	2.19	2.26
Contigs	27	32	96	71	92	92	85	65	56	180	91	322	70
N50 (Kb)	199	109	56	64	54	54	58	64	138	38	54	241	355
G+C%	27.3	27.6	27.0	27.2	27.4	27.3	27.1	27.4	27.3	28.2	27.2	30.0	27.6
Genes (Total)	2,134	2,074	2,373	2,100	2,214	2,258	2,205	2,102	2,139	2,237	2,141	2,475	2,346
CDS (Coding)	2,071	2,000	2,246	2,002	2,100	2,138	2,117	2,020	2,070	2,136	2,081	2,288	2,255
Genes (RNA)	46	52	33	31	38	37	30	34	54	50	49	59	59
tRNAs	36	40	27	25	30	29	25	29	38	40	40	46	43
ncRNAs	2	3	3	2	3	2	2	2	2	2	3	3	3

[a]*Genome accession number; ncRNAs, non-coding RNAs.*

one of Cluster III) are shown in **Table 2**. The quality of the genome sequences was in general in agreement with the minimal standards established for the use of genome data for taxonomical purposes, that embraces characteristics of the sequencing and assembly of the genomes like the depth of coverage, the value of N50 and the number of contigs (Chun et al., 2018). The

TABLE 3 | Results of Average Nucleotide Identity (ANI) and *in silico* DNA-DNA hibridization (*is*DDH) between representative genomes of the four clusters.

	CLUSTER												
	I								II		III	IV	
	LMG 10229^T	LMG 9861	L397	L398	L399	L400	L401	L406	LMG 9065^T	LMG 9871	LMG 24291^T	LMG 10210	LMG 29976^T
Cluster I													
LMG 10229^T		74.7	74.3	72.3	73.5	73.5	71.9	75.7	50.3	50.4	50.1	63.7	62.0
LMG 9861	**97.2**		69.6	69.6	68.7	70.3	69.7	72.3	50.2	49.6	49.7	63.0	62.1
L397	**97.1**	**96.6**		73.5	75.3	78.4	71.9	82.1	49.3	49.0	49.2	60.1	59.9
L398	**96.9**	**96.6**	**97.1**		78.4	74.9	87.7	75.3	49.7	50.3	49.3	60.1	59.7
L399	**97.1**	**96.6**	**97.3**	**97.7**		75.3	79.0	75.8	49.8	49.5	49.5	60.2	59.2
L400	**97.1**	**96.7**	**97.7**	**97.2**	**97.3**		74.1	83.4	49.1	49.3	49.1	60.3	59.7
L401	**96.9**	**96.6**	**97.1**	**98.6**	**97.7**	**97.1**		74.6	49.9	49.9	50.5	60.7	59.5
L406	**97.3**	**96.9**	**98.1**	**97.3**	**97.4**	**98.2**	**97.2**		20.1	50.0	49.7	61.7	60.5
Cluster II													
LMG 9065^T	**93.1**	**93.1**	**92.9**	**93.1**	**93.1**	**92.9**	**93.1**	**93.0**		81.4	56.1	51.1	51.7
LMG 9871	**93.1**	**93.1**	**92.9**	**93.1**	**93.0**	**92.9**	**93.1**	**93.0**	**98.1**		49.7	51.0	52.2
Cluster III													
LMG 24291^T	**92.9**	**92.8**	**92.6**	**92.6**	**92.7**	**92.5**	**92.9**	**92.9**	**94.3**	**94.4**		52.3	54.4
Cluster IV													
LMG 10210	**95.7**	**95.8**	**95.2**	**95.2**	**95.3**	**95.3**	**95.2**	**95.5**	**93.5**	**93.4**	**93.4**		73.4
LMG 29976^T	**95.2**	**95.5**	**95.1**	**94.9**	**94.9**	**95.1**	**94.9**	**95.1**	**93.5**	**93.5**	**93.9**	**97.1**	

Values in bold in the lower triangle corresponds to ANI and in the upper triangle to isDDH.

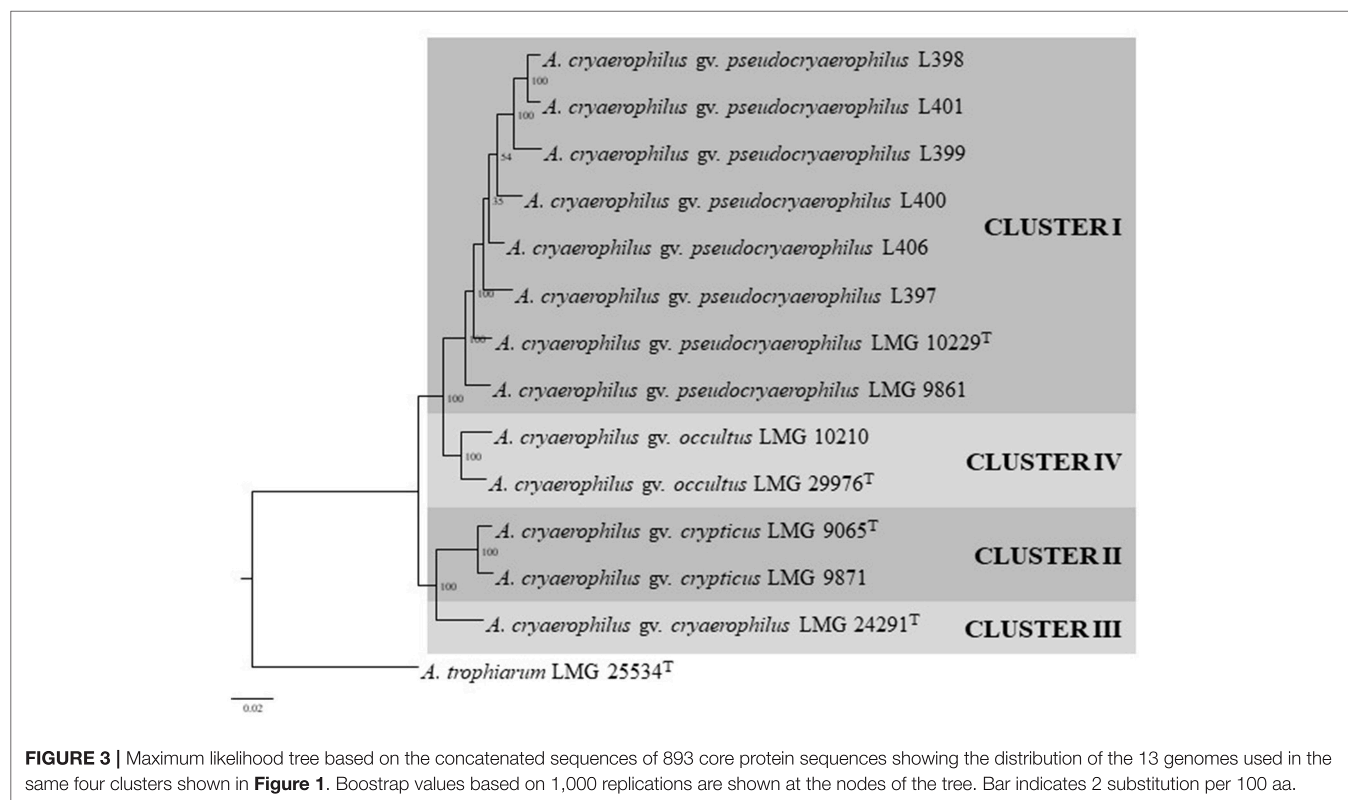

FIGURE 3 | Maximum likelihood tree based on the concatenated sequences of 893 core protein sequences showing the distribution of the 13 genomes used in the same four clusters shown in **Figure 1**. Boostrap values based on 1,000 replications are shown at the nodes of the tree. Bar indicates 2 substitution per 100 aa.

exceptions were the genome sequence data of strains LMG 10229^T, LMG 9871, and LMG 9861 that presented a depth of coverage lower than 50X proposed in the standards (**Table 2**). Globally, the genomic characteristics of the 13 compared genomes shown in **Table 2** were very similar, with sizes that did not differ in more than 0.29 Mb, with a %mol G+C content ranging between 27.0 and 30.0% and with a number of coding sequences or CDS of around 2000 (**Table 2**). The G+C values were in agreement with those (24.6–31%) described in the recent emended description of the genus *Arcobacter* (Sasi Jyothsna et al., 2013). **Table 3** shows the results from the calculated overall genome related taxonomical indices i.e., ANI and *is*DDH. For species delineation the generally accepted ANI and isDDH boundary values are 95–96 and 70%, respectively (Goris et al., 2007; Richter and Rossello-Mora, 2009; Meier-Kolthoff et al., 2013; Chun et al., 2018). However, for the genus *Arcobacter,* ANI values above 96% were the ones that better correlated with *is*DDH results above 70% in previous studies (Figueras et al., 2017; Pérez-Cataluña et al., 2018) in agreement with what happens in other genera (Beaz-Hidalgo et al., 2015; Figueras et al., 2017; Liu et al., 2017). The ANI values of the representative strains from each of the four different clusters were below the 96% cut-off indicating that the compared genomes belonged to different species, while the intra-cluster ANI values ranged from 96.6 to 98.6%. The *is*DDH results of <70% found between strains of the four clusters confirmed as the ANI results did that each cluster represented an independent species. The core genome phylogenetic tree inferred from 893 protein sequences of the

TABLE 4 | Antibiotic resistant genes and virulence factors.

	CLUSTERS												
	I								II		III	IV	
	1	2	3	4	5	6	7	8	9	10	11	12	13
ANTIBIOTIC RESISTANCE													
Multidrug efflux pumps													
CmeABC system[a]	+	+	+	+	+	+	+	+	+	+	+	+	+
MFS Superfamily[a]	−	+	−	+	+	+	+	+	+	−	−	−	−
Macrolids													
MacAB−TolC[a]	+	+	+	+	+	+	+	+	+	+	+	+	+
Quinolones													
gyrA mutation	−	−	−	−	−	−	−	−	−	−	−	−	−
23S rRNA mutations	−	−	−	−	−	−	−	−	−	−	−	−	−
OqxB[b]	+	+	+	+	+	+	+	+	+	+	+	+	+
β-lactamics													
β-lactamase[a]	−	−	−	−	−	−	−	+[d]	−	−	−	−	−
Colistin													
Mcr−1[b]	+	−	+	+	+	+	+	+	+	−	+	−	+
Mcr−2[b]	+	−	+	+	+	+	+	+	+	−	+	−	+
Acriflavin resistance[a]	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin/Spectinomycin[a]	−	−	−	−	−	−	−	−	−	−	−	+	−
VIRULENCE FACTORS													
Invasion													
ciaB[c]	+	+	+	+	+	+	+	+	+	+	+	+	+
mviN[c]	+	+	+	+	+	+	+	+	+	+	+	+	+
Adhesion													
cj1349[c]	+	+	+	+	+	+	+	+	+	+	+	+	+
cadF[c]	−	−	−	−	−	−	−	−	−	−	−	−	−
Filamentous hemmaglutinin													
hecA[c]	−	−	−	−	−	−	−	−	−	−	−	−	−
Hemolysis													
hecB[c]	−	−	−	−	−	−	−	−	−	−	−	−	−
tlyA[c]	−	−	−	−	−	−	−	−	+	−	+	−	−
Outer membarne protein													
irgA[c]	−	−	−	+	−	−	+	−	−	−	−	−	−
Phospholipase													
pldA[c]	+	+	+	+	+	+	+	+	+	+	+	+[e]	+

1, A. cryaerophilus gv. pseudocryaerophilus LMG 10229^T; 2, A. cryaerophilus gv. pseudocryaerophilus LMG 9861; 3, A. cryaerophilus gv. pseudocryaerophilus L397; 4, A. cryaerophilus gv. pseudocryaerophilus L398; 5, A. cryaerophilus gv. pseudocryaerophilus L399; 6, A. cryaerophilus gv. pseudocryaerophilus L400; 7, A. cryaerophilus gv. pseudocryaerophilus L401; 8, A. cryaerophilus gv. pseudocryaerophilus L406; 9, A. cryaerophilus gv. crypticus LMG 9065^T; 10, A. cryaerophilus gv. crypticus LMG 9871; 11, A. cryaerophilus gv. cryaerophilus LMG 24291^T; 12, A. cryaerophilus gv. occultus LMG 29976^T; 13, A. cryaerophilus gv. occultus LMG 10210. [a]RAST/PATRIC results, [b]ARG–ANNOT results, [c]BLASTn of virulence genes results (See Supplementary Table S2), [d]β-lactamase class D, [e]Phospholipase A and C.

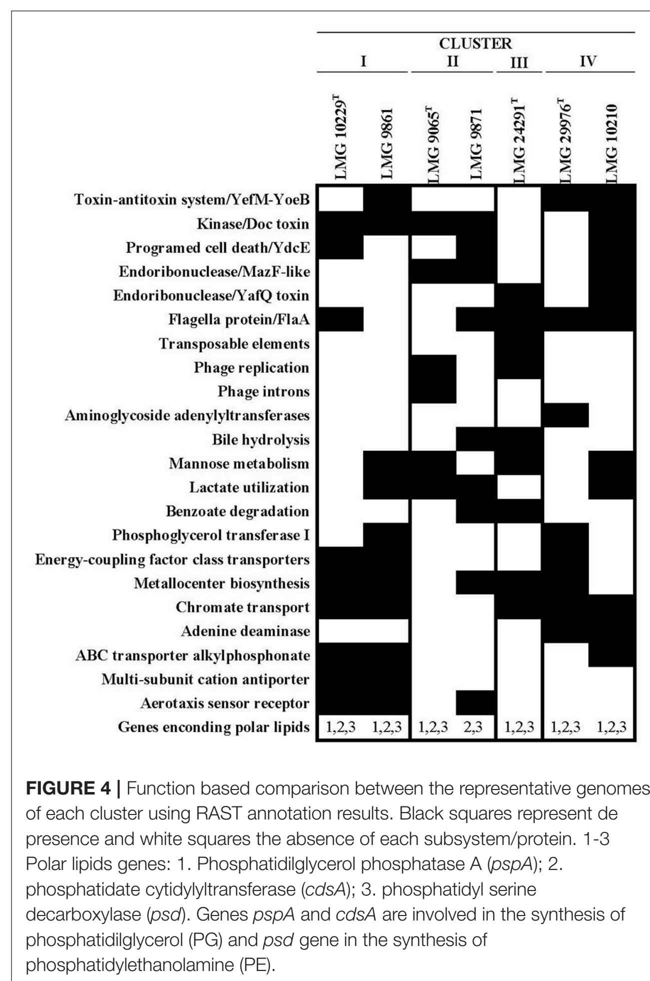

FIGURE 4 | Function based comparison between the representative genomes of each cluster using RAST annotation results. Black squares represent de presence and white squares the absence of each subsystem/protein. 1-3 Polar lipids genes: 1. Phosphatidilglycerol phosphatase A (*pspA*); 2. phosphatidate cytidylyltransferase (*cdsA*); 3. phosphatidyl serine decarboxylase (*psd*). Genes *pspA* and *cdsA* are involved in the synthesis of phosphatidilglycerol (PG) and *psd* gene in the synthesis of phosphatidylethanolamine (PE).

13 genomes obtained with PATRIC showed that the genomes also grouped into four different well-supported clusters with bootstraps of 100% (**Figure 3**). Interestingly, clusters IV and I formed a separate branch from clusters II and III. This indicates that the proteins of the genomes of clusters I and IV are more similar than the nucleotide sequences, and this was also in agreement with the higher values observed with ANI and *is*DDH for these two clusters.

Virulence and Antibiotic Resistance Genes

Of the different methods and databases used for recognizing virulence factors (Victors, VFDB and PATRIC_VF) none of them were useful for recognizing virulence genes. There were only a few exceptions. The phospholipase C identified with the databases PATRIC_VF and Victors in the genome of the strain LMG 29976^{T} (Cluster IV). The enzyme UDP-N-acetylglucosamine 4-6 dehidratase involved in flagelline glycosylation and identified with the VFDB database in the genomes L397 and L399 (Cluster I). Finally, the Pspa protein (EC 2.3.1.41), essential for gluconeogenesis, identified using PATRIC_VF in the genome LMG 9871 of Cluster II (**Table 4**). However the BLASTn carried out for the detection of virulence genes showed the presence of different genes related with adhesion (*cj1349*), invasion (*ciaB* and *mviN*) and phospholipase activity (*pldA*) (**Table 4**). None of the genomes showed the *cadF*, *hecA* and *hecB* genes that encode a fibronectin binding protein, an adhesion protein and a factor for hemolysis activation, respectively. These results agree with those obtained for the genome of *A. thereius* LMG 24486^{T} (Rovetto et al., 2017). The *irgA* and *tlyA* genes that encode an iron-regulated outer membrane protein and a hemolysine, respectively, were the only ones found i.e. the gene *irgA* in the genomes L398 and L401 (Cluster I); the gene *tlyA* in LMG 24291^{T} (Cluster III) and LMG 9065^{T} (Cluster II). The phylogenetic analysis of the concatenated sequences of the four virulence genes present in all the genomes (*cj1349*, *mviN*, *pldA* and *ciaB*) formed the same four clusters (Supplementary Figure S3). However, the distribution of the clusters was similar to the one obtained with the core genome tree (**Figure 3**), where clusters I and IV formed a separated branch from clusters II and III.

Regarding the presence of antibiotic resistant mechanisms, all the genomes showed the cmeABC multidrug efflux pump, the MacAB-TolC system for macrolide resistance, the *oxqB* gene related with quinolone resistance and genes related with the resistance to acriflavine. Resistance to colistin by the genes *mcr-1* and *mcr-2* were present in 85% of the genomes. The genome L406 was the only one that possessed a β-lactamase gene of class D. Resistance to β-lactamic compounds have been reported in other studies (Atabay and Aydin, 2001; Fera et al., 2003) and the same β-lactamase gene is present in the genome of *A. butleri* RM4018. However, this gene is absent in the genome of *A. thereius* LMG 24486^{T} (Rovetto et al., 2017). The genome LMG 29976 was the only one that presented genes for the resistance to streptomycin/spectomycin. The susceptibility of *A. cryaerophilus* to streptomycin has been previously demonstrated (Kabeya et al., 2004; Rahimi, 2014). However, this is the first report that show the presence of resistance genes to this antimicrobial compound. Mutations on the 23S rRNA (Ren et al., 2011) and the *gyrA* gene

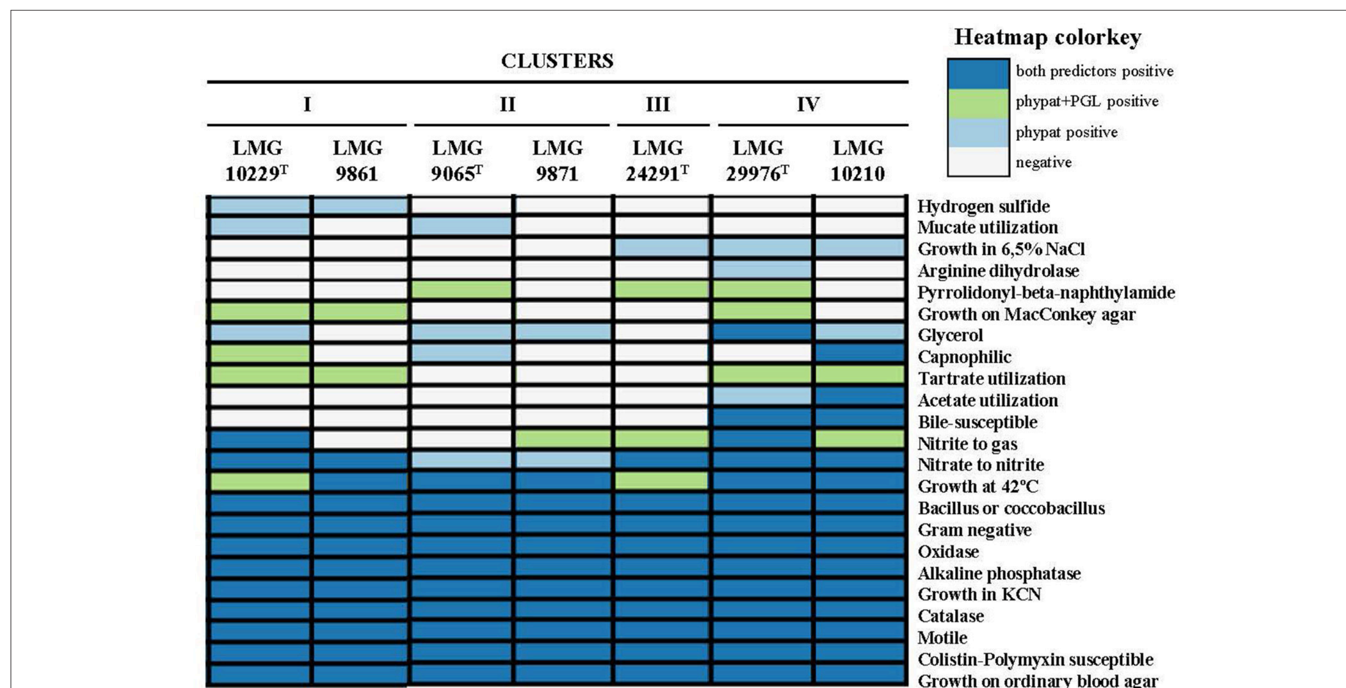

FIGURE 5 | Phenotypic inference using Traitar software for the representative genomes of each cluster. The software uses two prediction models, the phypat model (predicts the presence/absence of proteins found in the phenotype of 234 bacterial species) and a combination of phypat+PGL models (uses the information of phypat combined with the information of the acquisition or loss of protein families and phenotypes through the evolution), to determine the phenotypic characteristics.

(Carattoli et al., 2002) for erythromycin and quinolone resistance were not detected, despite *gyrA* mutations have been found in some quinolone-resistant *A. cryaerophilus* strains (Abdelbaqi et al., 2007; Van den Abeele et al., 2016).

Functional and Phenotypic Inference

Several subsystems where found to be characteristic of each Cluster on the basis of the functional-based comparison between the representative genomes (**Figure 4**). Cluster I genomes (LMG 10229^T and LMG 9861) carry specifically multi-subunit cation antiporters [Na(+) H(+) cation antiporter ABCDEFG] whose function includes sodium tolerance and pH homeostasis in an alkaline environment (Ito et al., 2017). Cluster II genomes (LMG 9065^T and LMG 9871) were the only ones that did not show the chromate transport protein ChrA, which confers resistance to chromate compounds present in the other studied genomes. Cluster III (LMG 24291^T) was the only one that presented transposable elements as the putative transposase TniA and the Nucleotide Triphosphate binding protein TniB. Finally, the enzyme Adenosine deaminase (EC 3.5.4.4) involved in purine metabolism was only detected in Cluster IV genomes (LMG 29976^T and LMG 10210).

From the 67 phenotypic inferred traits analyzed with Traitar 11(16.4%) were found in all the analyzed genomes while 12 were only found in some of them (**Figure 5**). The genomes of Cluster I (LMG 10229^T and LMG 9861) were predicted to produce hydrogen sulfide while those of Cluster IV (LMG 29976^T and LMG 10210) showed acetate utilization and bile susceptibility. However, none of these characteristics have been observed when they have been tested in the laboratory on those strains. This might be due to the inability to reproduce the necessary conditions in the laboratory for the expression of these features. None of the other nine traits recognized in some genomes enabled us to differentiate between the IV Clusters.

Phenotypical Characterization

Table 5 shows the phenotypical results obtained from the strains of each of the four clusters. In agreement with what was found in previous studies where phenotypic test did not differentiate between subgroups 1A and 1B (Neill et al., 1985; Vandamme et al., 1992; On, 1996), none of the performed phenotypic tests enabled to clearly distinguish strains from each of the four phylogenetic clusters. Most of the tests gave variable results except for Cluster IV. However, this might be due to the small number of strains ($n = 2$) analyzed in this group. Considering these results, each of the three genetically recognized new species (clusters I, II, and IV) should be considered a different genomovar (gv.) of the species *A. cryaerophilus.* A genomovar is a well-delimited group of strains that correspond to a new species by genomic information but that cannot be phenotypically differentiated (Ursing et al., 1995). Cluster III represents the original species *A. cryaerophilus* because it embraces the type strain of the species. The value of the phenotypic characterization has already been questioned considering the lack of reproducibility of results between laboratories and some authors have suggested it is now time to base the description of new taxa on the genome sequence analysis (Moore et al., 2010; Sutcliffe, 2015). According to Sutcliffe (2015), phenotypic characterization is harder to evaluate nowadays than the genotype. Considering that genomic characterization is objective and reproducible, we agree with Sutcliffe (2015) that we should be able to define species on the basis of genetic characters like the ones evaluated in this study. This will favor the faster discover of the large number of taxa waiting to be described (Sutcliffe, 2015). However, this will require a modification of the Bacteriological Code, which we hope will happen in the near future.

TABLE 5 | Phenotypic characteristics of the four clusters.

Characteristics	1	2	3	4
Growth in/on				
Air at 37°C	+	V(+)	+	−
Microaerobiosis at 37°C	+	V(+)	V(+)	−
Anaerobiosis at 37°C	V(+)	V(−)	V(−)	−
Air at 42°C	V(−)	−	−	−
2% (w/v) NaCl	V(+)	+	V(+)	+
3% NaCl	V(−)	−	−	−
4% (w/v) NaCl	−	−	−	−
1% bilis	+	V(+)	V(+)	+
1.5% bilis	+	+	V(+)	+
2% bilis	+	+	V(+)	+
1% (w/v) glycine	V(−)	−	−	−
0.1% sodium deoxycholate	V(+)	V(+)	V(+)	+
MacConkey	V(+)	+	V(+)	+
$CdCl_2$	V(−)	V(−)	V(−)	V
Resistance to				
Cefoperazone (64 mg/L)	V(+)	+	+	+
Enzyme activity				
Catalase	+	+	+	+

Taxa: 1, A. cryaerophilus gv. pseudocryaerophilus (n = 27) [Cluster I]; 2, A. cryaerophilus gv. crypticus (n = 5) [Cluster II]; 3, A. cryaerophilus gv. cryaerophilus(n = 3) [Cluster III]; 4, A. cryaerophilus gv. occultus (n = 2) [Cluster IV]. The specific responses for type strains were coincidental or expressed in brackets. Unless otherwise indicated: +, ≥95% strains positive; −, ≤11% strains positive; V, variable; (), main result of the strains; CO_2 indicates microaerobic conditions.

CONCLUSION

The phylogenetic and genomic analysis showed that the strains of the species *A. cryaerophilus* represent four separated species. In addition, phenotypical and functional traits were in evidence for the genomes selected as representative of each cluster. Despite all the results, phenotypic characterization carried out at the laboratory showed a high inter- and intra-cluster variability that did not allow us to determine specific phenotypic characteristics or therefore to define the three uncovered clusters as three new species. Following current bacterial taxonomic rules, we will not be able to define these species until we find phenotypical characteristics that allow us to discriminate the three new species from each other

and from the species *A. cryaerophilus*. Therefore, we describe them as four genomovars with the names "*A. cryaerophilus gv. pseudocryaerophilus*" (pseu.do.cry.a.e.ro'phi.lus. Gr. adj. *pseudês* false, N.L. masc. adj. *cryaerophilus* specific epithet of an *Arcobacter* species; N.L. masc. adj. *pseudocryaerophilus* false *cryaerophilus;* Cluster I = LMG 10229^{T}), "*A. cryaerophilus gv. crypticus*" (cryp'ti.cus. L. masc. adj. *Crypticus* hidden; Cluster II = LMG 9065^{T}), *A. cryaerophilus gv. cryaerophilus* (Cluster III = LMG 24291^{T}) and "*A. cryaerophilus gv. occultus*" (oc.cul$'$tus. L. adj. *occultus* occulted, hidden; Cluster IV = LMG 29976^{T}). Unfortunately, the phenotype derived from the genome could not be reproduced in the laboratory, either. This might be due to the inability to mimic *in vitro* the conditions for the expression of these pathways or characteristics. The phenotypic characterization limits a proper description and it might be considered an important shortcoming in the genomic era in which all the molecular and genomic data leave no doubts about the existence of four different species among the investigated *A. cryaerophilus* strains.

AUTHORS CONTRIBUTIONS

LC and MF: designed the work; LC and OS: carried out the phylogenetic analysis; VL and AP-C: carried out the phenotypic characterization of the strains; AP-C: carried out the genome sequencing and analysis; LC, MF, and AP-C: wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00805/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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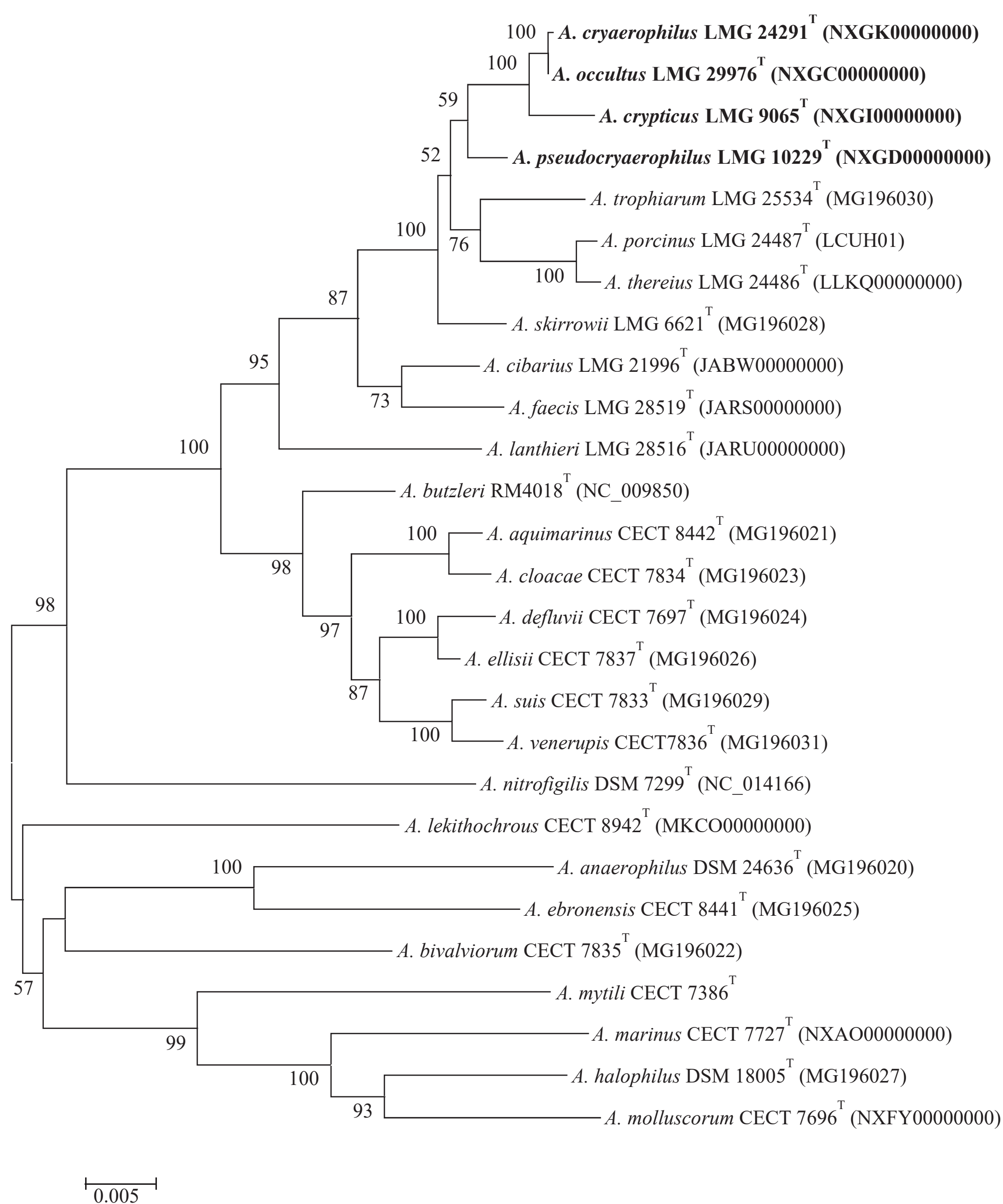
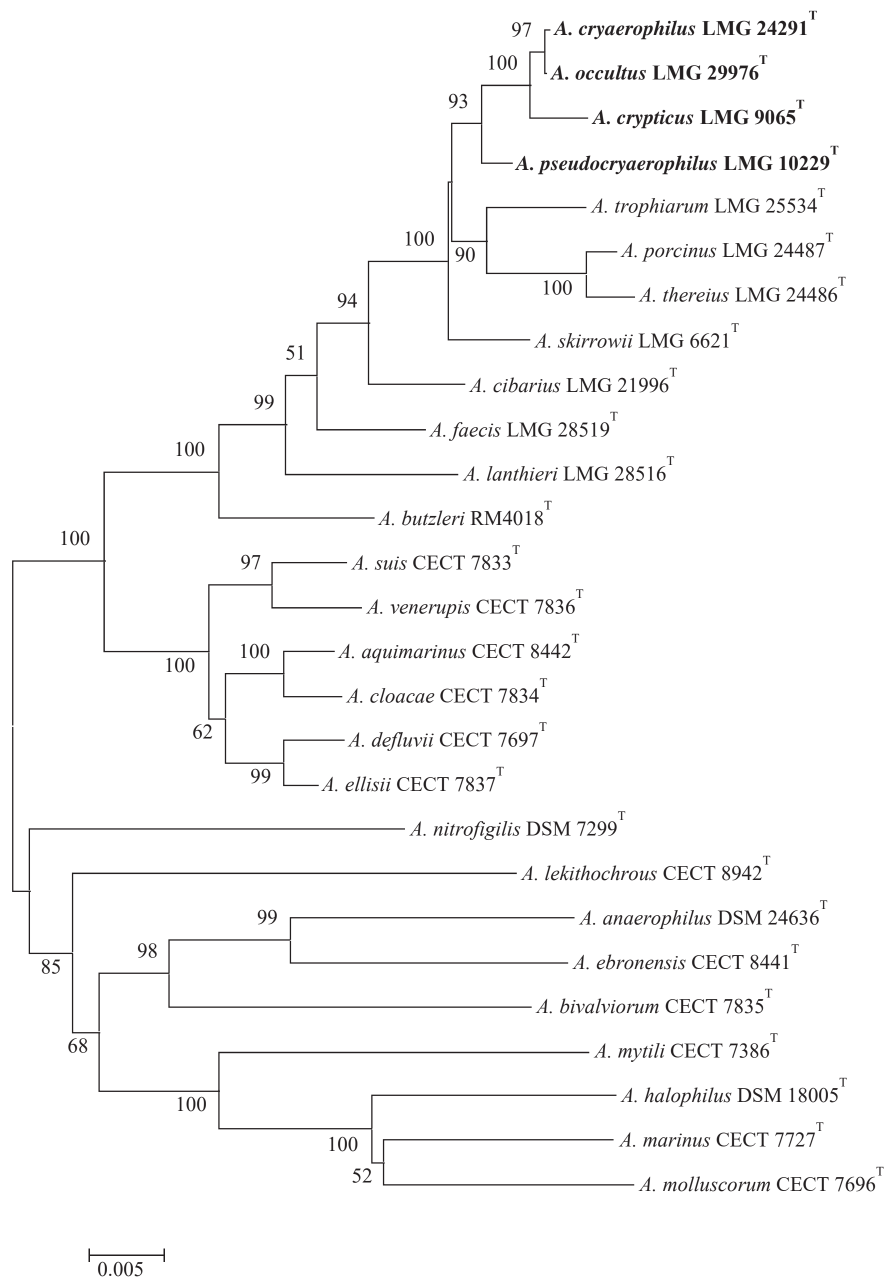
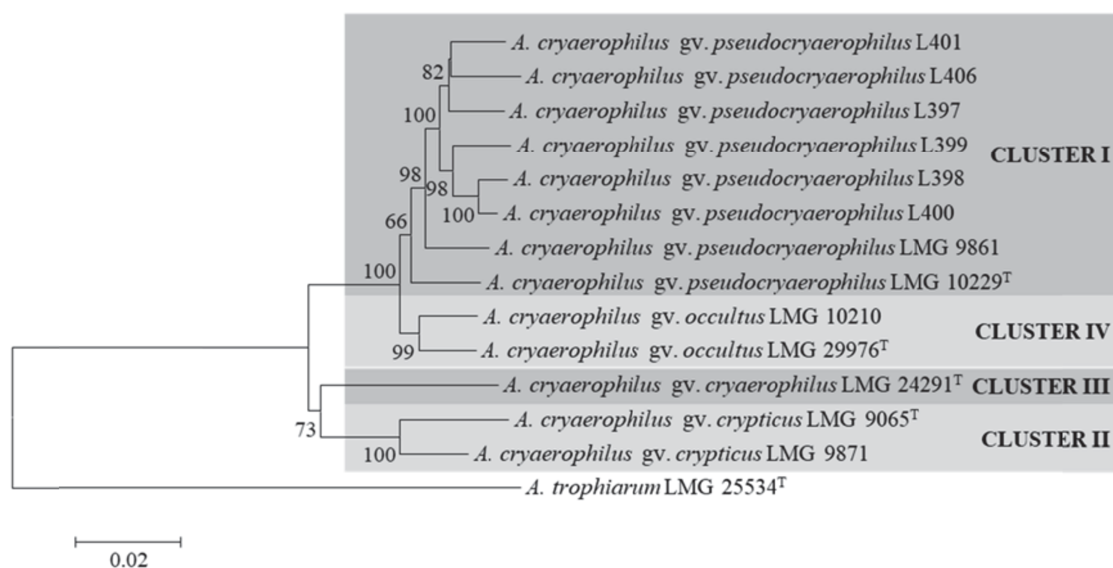


Supplementary Figure S1. Neighbour joining tree based on 23S rRNA (2857 bp) sequences showing the phylogenetic position of the three new species in relation with *A. cryaerophilus* and within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 bp.



Supplementary Figure S2. Neighbour joining tree based on the concatenated sequences of the 16S and 23S rRNA genes (4353 bp) sequences showing the phylogenetic position of the three new species within the genus *Arcobacter* and in relation with *A. cryaerophilus*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 bp.



Supplementary Figure S3. Neighbor-joining tree based on the concatenated sequences of the four virulence genes *cj1349*, *mviN*, *pldA* and *ciaB* (5386 bp) found in the 13 genomes. Notice the same four clusters observed in Fig. 1. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 bp.

Supplementary Table S1. Accession number or locus tag* of the housekeeping genes and genomes used in the study.

Strain	Accession number or Locus tag*					
	atpA	***gyrB***	***hsp60***	***rpoB***	**Genome**	**SRA**
LMG 24291^T	JF803015	JF803152	JF803062	JF803222	NXGK01	SRR6805241
LMG 9065	CJ670_02570*	CJ670_00435*	CJ670_06770*	CJ670_10580*	NXGI01	SRR6805237
LMG 9861	JF803017	HE565384	JF803061	JF803224	NXGJ01	SRR6805238
LMG 10229	JF803018	GU291961	JF803050	JF803225	NXGD01	SRR6805240
LMG 10210	CJ673_01840*	CJ673_04040*	FN555372	CJ673_08295*	NXGE01	SRR6805236
LMG 29976	JF803016	FR682117	JF803060	JF803223	NXGC01	SRR6805239
LMG 9871	CJ671_01645*	CJ671_06060*	FN555370	CJ671_03945*	NXGH01	SRR6805235
LMG 10241	JF803019	HE565383	JF803051	JF803226		
LMG 9863	KM365529	KM365481	KM365545	KM365513		
LMG 10829	MH059956	MH059992	DQ059481	MH060011		
L397	HCJBBJIN_01690*	HCJBBJIN_00726*	HCJBBJIN_01875*	HCJBBJIN_01069*	LRUQ01	NA
L398	IGHLCDKC_01788*	IGHLCDKC_00483*	IGHLCDKC_00603*	IGHLCDKC_01215*	LRUR01	NA
L399	LIEKBJFB_01644*	LIEKBJFB_00758*	LIEKBJFB_00350*	LIEKBJFB_01243*	LRUS01	NA
L400	PJKEJCAK_01809*	PJKEJCAK_01240*	PJKEJCAK_01056*	PJKEJCAK_01450*	LRUT01	NA
L401	KPFDGGMD_02061*	KPFDGGMD_01453*	KPFDGGMD_01541*	KPFDGGMD_01087*	LRUU01	NA
L406	EINDKKGD_01567	EINDKKGD_00477*	EINDKKGD_00519*	EINDKKGD_00435*	LRUV01	NA
F196	KM365530	KM365482	KM365546	KM365514		
8122333	KM365541	KM365493	KM365557	KM365525		
8749401	MH059957	MH059993	MH059974	MH060012		
8756347	MH059958	MH059994	MH059975	MH060013		
14 PHA	KM365534	KM365486	KM365550	KM365518		
20 PHF	KM365535	KM365487	KM365551	KM365519		
284/1	MH059959	MH059995	MH059976	MH060014		
AB3A	KM365536	KM365488	KM365552	KM365520		
AB74A	KM365538	KM365490	KM365554	KM365522		
AL 20-1	KM365542	KM365494	KM365558	KM365526		
AO2A	KM365537	KM365489	KM365553	KM365521		
BUF3	MH059960	MH059996	MH059977	MH060015		
CV-152	MH059962	MH059998	MH059979	MH060017		
CV-2101	MH059963	MH059999	MH059980	MH060018		
EMU-3	MH059964	MH060000	MH059981	MH060019		
FE7	MH059965	MH060001	MH059982	MH060020		
FEBU4	MH059961	MH059997	MH059978	MH060016		
HHS 118A	MH059966	MH060002	MH059983	MH060021		
HHS 133A	KM365539	KM365491	KM365555	KM365523		
HHS 188A	MH059967	MH060003	MH059984	MH060022		
HHS 191A	MH059968	MH060004	MH059985	MH060023		
HHS 205A	KM365540	KM365492	KM365556	KM365524		
MC 2-2	KM365543	KM365495	KM365559	KM365527		
MCV 42-1	MH059969	MH060005	MH059986	MH060024		
ME 15-4	MH059970	MH060006	MH059987	MH060025		
NAV 15-1	MH059971	MH060007	MH059988	MH060026		
NAV12-2	MH059972	MH060008	MH059989	MH060027		
NB14A	MH059973	MH060009	MH059990	MH060028		
RW15-1	LT986689	LT986694	LT986699	LT986684		
RW17-4	LT986690	LT986695	LT986700	LT986685		
RW25-5	LT986691	LT986696	LT986701	LT986686		
RW33-8	LT986692	LT986697	LT986702	LT986687		
RW45-3	LT986693	LT986698	LT986703	LT986688		
UF1T	KM365531	KM365483	KM365547	KM365515		
UF2T	KM365532	KM365484	KM365548	KM365516		
UPER3	KM365533	KM365485	KM365549	KM365517		

Supplementary Table S2. Accession numbers[a] or locus tag[b] of the virulence genes used in the in-house database.

Genes	Accession numbersa / Locus tag*			
ciaB	HF935054[a]	HF935053[a]	HF935047[a]	HF935046[a]
mviN	ABU_RS04395[b]	ABLL_RS05675[b]	ARNIT_RS10565[b]	
cj1349	HF935058[a]	HF935063[a]	HF935061[a]	
cadF	HF935040[a]	HF935042[a]	HF935041[a]	
hecA	HF935065[a]	HF935064[a]	HF935066[a]	
hecB	ABU_RS04705[b]	ABLL_RS04490[b]	ARNIT_RS15685[b]	
tlyA	ABLL_RS06845[b]	ABU_RS06800[b]		
irgA	HF935067[a]			
pldA	ABU_RS04310[b]	ABLL_RS05545[b]	ARNIT_RS10720[b]	

4.6 Revisiting the taxonomy of the genus *Arcobacter*: getting order from the chaos.

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Revisiting the Taxonomy of the Genus *Arcobacter*: Getting Order From the Chaos

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Since the description of the genus *Arcobacter* in 1991, a total of 27 species have been described, although some species have shown 16S rRNA similarities below 95%, which is the cut-off that usually separates species that belong to different genera. The objective of the present study was to reassess the taxonomy of the genus *Arcobacter* using information derived from the core genome (286 genes), a Multilocus Sequence Analysis (MLSA) with 13 housekeeping genes, as well as different genomic indexes like Average Nucleotide Identity (ANI), *in silico* DNA–DNA hybridization (*is*DDH), Average Amino-acid Identity (AAI), Percentage of Conserved Proteins (POCPs), and Relative Synonymous Codon Usage (RSCU). The study included a total of 39 strains that represent all the 27 species included in the genus *Arcobacter* together with 13 strains that are potentially new species, and the analysis of 57 genomes. The different phylogenetic analyses showed that the *Arcobacter* species grouped into four clusters. In addition, *A. lekithochrous* and the candidatus species '*A. aquaticus*' appeared, as did *A. nitrofigilis,* the type species of the genus, in separate branches. Furthermore, the genomic indices ANI and *is*DDH not only confirmed that all the species were well-defined, but also the coherence of the clusters. The AAI and POCP values showed intra-cluster ranges above the respective cut-off values of 60% and 50% described for species belonging to the same genus. Phenotypic analysis showed that certain test combinations could allow the differentiation of the four clusters and the three orphan species established by the phylogenetic and genomic analyses. The origin of the strains showed that each of the clusters embraced species recovered from a common or related environment. The results obtained enable the division of the current genus *Arcobacter* in at least seven different genera, for which the names *Arcobacter*, *Aliiarcobacter* gen. nov., *Pseudoarcobacter* gen. nov., *Haloarcobacter* gen. nov., *Malacobacter* gen. nov., *Poseidonibacter* gen. nov., and Candidate '*Arcomarinus*' gen. nov. are proposed.

Keywords: ***Arcobacter*, *Aliiarcobacter* gen. nov., *Pseudoarcobacter* gen. nov., *Haloarcobacter* gen. nov., *Malacobacter* gen. nov., *Poseidonibacter* gen. nov., taxonomic criteria**

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INTRODUCTION

The genus *Arcobacter* was created by Vandamme et al. (1991) to accommodate Gram-negative, curved-shaped bacteria belonging to two species *Campylobacter cryaerophila* (now *Arcobacter cryaerophilus*) and *Campylobacter nitrofigilis* (now *A. nitrofigilis*), considered atypical campylobacters due to their ability to grow at lower temperatures (15°C–30°C) and without microaerophilic conditions (Vandamme et al., 1991). The latter species was selected as the type species for the new genus (Vandamme et al., 1991). One year later the genus was enlarged with the addition of two new species, *A. skirrowii* with an animal origin being isolated from aborted ovine, porcine and bovine fetuses, and from lambs with diarrhea, and *A. butzleri,* which was recovered from cases of human and animal diarrhea (Vandamme et al., 1992). Another two new species were incorporated into the genus in 2005. *A. halophilus* was isolated from water from a hypersaline lagoon in Hawaii (Donachie et al., 2005), and *A. cibarius* was isolated from broiled carcasses in Belgium (Houf et al., 2005). These species were assigned to the genus *Arcobacter* on the basis of the 16S rRNA gene similarity (94% and 95% for *A. nitrofigilis* with *A. halophilus* and *A. cibarius,* respectively). However, these values are equal, or even below, the cut-off of 95% for genus definition (Rosselló-Mora and Amann, 2001; Yarza et al., 2008, 2014; Tindall et al., 2010).

From 2009 onward, new species were being described year-by-year, reaching a total number of 27 in 2017. In some of these descriptions, the similarity of the 16S rRNA gene was the decisive character for taxonomic assignation at genus level, although phylogeny based on housekeeping genes (*rpo*B first and then *gyr*B and *hsp*60) was also included as additional, more discriminatory tools for the species (Collado et al., 2009a, 2011; De Smet et al., 2011). Using this approach, *A. molluscorum*, *A. ellisii*, *A. defluvii*, or *A. bivalviorum* were defined, among others (Collado et al., 2009a, 2011; Figueras et al., 2011a,b; Levican et al., 2012), which showed 16S rRNA similarities ranging from 91.1 to 94.7%, not supporting their common affiliation. On the other hand, the most closely related species, which showed a similarity of 99.1% were *A. ellisii* and *A. defluvii* (Collado et al., 2011), giving evidence for the first time of the poor resolution of the 16S rRNA gene for separating closely related species in the genus *Arcobacter*. However, the phylogenetic analysis based on the concatenated sequences of *gyr*B, *rpo*B, and *cpn*60 genes, together with the DNA–DNA hybridization results, clearly supported the existence of these two differentiated taxa (Figueras et al., 2011a). Also in 2011, *A. trophiarum* was discovered from the intestinal tract of healthy fattening pigs, which interestingly showed the closest similarities (≥97.4%) with the other species also recovered from humans or animals, i.e., *A. cryaerophilus*, *A. thereius*, *A. cibarius,* or *A. skirrowii* (De Smet et al., 2011; Figueras et al., 2014; Van den Abeele et al., 2014).

In 2013, the species *A. cloacae* and *A. suis* were described, using a Multilocus Sequence Analysis (MLSA) approach including five housekeeping genes (Levican et al., 2013) for the first time. Simultaneously, and due to the highest 16S rRNA gene similarity with *A. marinus* (95.5%), the species *A. anaerophilus* was incorporated to the genus (Sasi-Jyothsna et al., 2013). However, this species showed atypical characteristics, including lack of motility and obligate anaerobic metabolism, which led to the original description of the genus *Arcobacter* being emended (Sasi-Jyothsna et al., 2013). The most recently described species from shellfish are *A. lekithochrous*, *A. haliotis*, and *A. canalis* (Diéguez et al., 2017; Tanaka et al., 2017; Pérez-Cataluña et al., 2018a). The first one included several isolates recovered from scallop larvae and from tank seawater of a Norwegian hatchery (Diéguez et al., 2017), the second species came from an abalone of Japan (Tanaka et al., 2017) and the third from oysters submerged in a water channel contaminated with wastewater (Pérez-Cataluña et al., 2018a). However, Diéguez et al. (2018) evidenced that the species *A. haliotis* is a later heterotypic synonym of *A. lekithochrous*. Additionally, the low 16S rRNA gene similarity of *A. lekithochrous* with the known *Arcobacter* species (91.0–94.8%) found in the *A. lekithochrous* description made Diéguez et al. (2017) suggest that certain species might belong to other genera and recommend that a profound revision of the genus might clarify the taxonomy.

On the other hand, adding 2.5% NaCl to the enrichment medium and subculturing on marine agar, Salas-Massó et al. (2016) recognized seven potential new species from water and shellfish (mussels and/or oysters), and recovered new isolates of *A. halophilus* and *A. marinus* of which only the type strains had been known. In addition, during the characterization of the most recently described species *A. canalis* (Pérez-Cataluña et al., 2018a) and when trying to define the seven mentioned new species, we observed that the *Arcobacter* species formed several different clusters distant enough to suspect they might correspond to different genera, in agreement with Diéguez et al. (2017).

There are clear criteria for describing new bacterial species (Tindall et al., 2010; Figueras et al., 2011a,b). However, the description of a genus is usually based on a cut-off of <95% similarity in the 16S rRNA gene sequence, and a G+C (% mol) content differing by more than 10% (Rosselló-Mora and Amann, 2001; Yarza et al., 2008; Tindall et al., 2010; Yarza et al., 2014). Nowadays, genomic data like the Average Nucleotide Identity (ANI) and the *in silico* DNA–DNA hybridization (*is*DDH) are used to define bacterial species, although have not yet been fully explored for delineating genera (Konstantinidis and Tiedje, 2005; Goris et al., 2007; Richter and Rosselló-Móra, 2009; Qin et al., 2014; Chun et al., 2018).

A percentage of Average Amino-acid Identity (AAI) ranging from 60 to 80% between the compared genomes of species or strains and a Percentage of Conserved Proteins (POCPs) above 50% has been proposed if they are to belong to the same genus (Konstantinidis and Tiedje, 2005; Qin et al., 2014). Finally, the Relative Synonymous Codon Usage (RSCU) has also been used by some authors to infer evolutionary and ecological links among bacterial species (Ma et al., 2015; Farooqi et al., 2016).

Very recently, Waite et al. (2017) carried out a comparative genomic analysis of the class *Epsilonproteobacteria.* Using 16S and 23S rRNA, 120 single-copy marker proteins and AAI analysis they proposed its reclassification as the new phylum Epsilonbacteraeota. In that study, Waite et al. (2017) also proposed a reclassification of the genus *Arcobacter* as a new

Family *Arcobacteraceae*, within the class *Campylobacteria*, order *Campylobacterales*. One weakness of this study, specifically regarding the genus *Arcobacter*, is that only seven validated species were included in the analysis. The new family therefore comprised only the genus *Arcobacter*. However, these findings also support the need for a clarification of the taxonomy of the current genus *Arcobacter*.

The rise of genome sequencing has dramatically changed the landscape of systematics of prokaryotes, improving different aspects such as the identification of species, the functional characterization for resolving taxonomic groups, and the resolution of the phylogeny of higher taxa (Whitman, 2015). It seems clear that the incorporation of genomics into the taxonomy will boost its credibility providing reproducible, reliable, highly informative means to infer phylogenetic relationships among prokaryotes, and avoiding unreliable methods and subjective difficult-to-replicate data (Chun and Rainey, 2014; Chun et al., 2018).

Within this modern taxonomy context, the objective of the present study was to reassess the taxonomy of the known and newly recognized *Arcobacter* species by using a MLSA of 13 housekeeping genes, the whole genome sequences and the derived genomic analysis. The latter analysis included ANI, *is*DDH, AAI, POCP, and RSCU of all *Arcobacter* type strains. In addition, phylogenies based on 16S and 23S rRNA gene sequences were also performed with comparative purposes. The new taxonomic criteria were stable when including whole genome sequences of a second strain of each species or of unassigned sequences obtained from the public databases.

MATERIALS AND METHODS

Bacterial Strains

All 27 valid species included in the genus *Arcobacter* have been studied. They are represented by 39 strains, and 13 strains that are potentially new species (**Table 1**). Furthermore, 50 genomes of *Arcobacter* strains identified at species level were investigated, 39 of which were obtained in our laboratory (27 from known species and 13 from potentially new species) and the others from the public databases[1,2]. Five genomes that had been deposited as *Arcobacter* sp. in the databases were also included in the study. If there was more than one strain of a known *Arcobacter* species, two representative genomes for each species were included in the analysis. The only exceptions were: *A. acticola* (Park et al., 2016) and *A. pacificus* (Zhang et al., 2015), whose taxonomic positions were only inferred by the phylogenetic analysis of the 16S rRNA gene sequences published in their species descriptions, together with a MLSA of three housekeeping genes (*atp*A, *gyr*B, and *rpo*B) for *A. pacificus* (Zhang et al., 2015; Park et al., 2016). The strains considered potentially new species, and named hereafter as 'candidate species,' had been recognized with an MLSA analysis of five housekeeping genes (*atp*A, *gyr*A, *gyr*B, *hsp*60, and *rpo*B) (data not shown).

[1] https://www.ncbi.nlm.nih.gov/genome/

[2] https://gold.jgi.doe.gov/

Culturing for genome sequencing was carried out either on blood agar (DIFCO, Madrid, Spain) or marine agar (Scharlau, Sentmenat, Spain) at 30°C in aerobiosis for 24–72 h, depending on the requirements. DNA was extracted using Easy-DNA™ gDNA Purification kit (Invitrogen, Madrid, Spain) following the manufacturer's instructions. The integrity of the DNA was evaluated by electrophoresis of 10 μl of the sample in a 1.5% agarose gel. The total amount of DNA was quantified using Qubit™ with the dsDNA Broad Range Assay kit (Invitrogen). Paired-end libraries were constructed with 50 ng of DNA using Nextera DNA Library Preparation Kit (Illumina, Lisbon, Portugal) and sequenced with MiSeq platform (Illumina). Sequencing generated 2 × 300 bp paired-end reads. Clean reads were assembled with SPAdes (Nurk et al., 2013) and the CGE assembler (Larsen et al., 2012) in order to select the better assembly. Before depositing the genomes in the NCBI database, FASTA files were screened for eukaryotic and prokaryotic sequences using BLASTn, and for adaptors with VecScreen standalone software[3]. The five housekeeping genes used in the first MLSA analysis (*atp*A, *gyr*A, *gyr*B, *hsp*60, and *rpo*B) were extracted from each genome and compared with the Sanger sequences of these genes obtained originally for the identification of the strain. The existence of a single and identical copy of these genes confirmed that the genomes were not contaminated and belonged to the correct strain. Finally, contigs were deleted if they had less than 200 bp. The genomes were deposited in the GenBank database and **Table 1** lists the accession numbers.

The 55 genomes were annotated with a local installation of Prokka v1.2 (Seemann, 2014) using an e-value of 1e-06. The annotation was performed with Prokka, with the prediction tools Prodigal v2.6 (Hyatt et al., 2010) and ARAGORN v1.2 (Laslett and Canback, 2004). The prediction tool Barrnap v0.6[4] included in Prokka v1.2 was used for the annotation of rRNA genes. Coding sequences (CDS) were annotated, combining the Rapid Annotation Subsystems Technology (RAST) (Overbeek et al., 2014) using the *classic RAST* scheme and the Annotation Tools of PATRIC server (Wattam et al., 2017). The characteristics of each genome (i.e., N50, number of contigs, number of CDS, G+C content) were obtained from NCBI annotations.

Analysis of Housekeeping Genes, Ribosomal Genes, and Core Genome

Thirteen housekeeping genes (*atp*A, *atp*D, *dna*A, *dna*J, *dna*K, *fts*Z, *gyr*A, *hsp*60, *rad*A, *rec*A, *rpo*B, *rpo*D, and *tsf*) were obtained from the genomes using BLASTn search. Sequence similarities of housekeeping genes were determined using the MegAlign program (DNASTAR®, Madison, WI, United States). Genes were aligned using ClustalW (Larkin et al., 2007) and phylogenies based on individual genes and on the concatenated sequences was constructed with MEGA version 6.0 (Tamura et al., 2013) using the Neighbor-Joining (NJ) and Maximum-Likelihood (ML) algorithms.

[3] ftp://ftp.ncbi.nlm.nih.gov/blast/demo/

[4] http://www.vicbioinformatics.com/software.barrnap.shtml

The phylogenetic analysis of the core genome was assessed with the Roary software (Page et al., 2015) using 80% as cut-off for the BLASTp search. The core genome alignment was extracted with the latter software and the phylogeny was inferred using SplitsTree version 4.14.2 as described in Sawabe et al. (2007) using SplitsTree version 4.14.2, with a neighbor net drawing and Jukes-Cantor correction (Bandelt and Dress, 1992; Huson and Bryant, 2005).

Furthermore, the 16S and 23S rRNA genes of each genome were obtained using RNammer (Lagesen et al., 2007). In some cases, 16S rRNA gene sequences were obtained in our laboratories by Sanger sequencing or from the GenBank. The similarity of the 16S rRNA genes was calculated using MegAlign version 7.0.0 (DNASTAR®, Madison, WI, United States). Phylogenetic trees were reconstructed with MEGA version 6.0 (Tamura et al., 2013) also using the NJ and ML algorithms. Alignments obtained for both genes were visually analyzed in order to localize signature sequences for strains or groups of strains.

Genomic Indices

In order to ensure the correct assignation at species level of each analyzed genome, the ANI and the *is*DDH were calculated between all the genomes (Konstantinidis and Tiedje, 2005; Richter and Rosselló-Móra, 2009; Qin et al., 2014). The ANIb was calculated using JSpeciesWS (Richter et al., 2016), the resulting matrix was clustered and visualized using ggplot2 2.2.1 package (Wickham, 2009) and the *is*DDH was calculated with the GGDC software using results obtained with the formula 2 (Meier-Kolthoff et al., 2013). Two other indices (AAI and POCP) described for genus classification (Konstantinidis and Tiedje, 2005; Luo et al., 2014; Qin et al., 2014) were calculated among the genomes that corresponded to the type strains of the accepted species and the reference strains of the candidate species. The AAI was calculated with the Lycoming College Newman Lab AAIr Calculator[5] using the Sequence-Based Comparison Tools output file from RAST (Overbeek et al., 2014). The POCP was determined as described by Qin et al. (2014) using the following parameters to consider a peptide as a conserved protein: an e-value lower than 1e-5 and an identity percentage higher than 40% from an aligned region higher than 50%.

Finally, the RSCU was computed using the Codon Adaptation Index (CAI) developed by Sharp and Li (1987) through the CAIcal web-server (Puigbò et al., 2008). Statistical differences in the RSCU were assessed by a multinomial regression approach using the R software environment (R Core Team, 2015). The principal component analysis (PCA) was performed by the R software environment (R Core Team, 2015, and visualized using ggplot2 2.2.1 and ggfortify 0.4.4 (Wickham, 2009; Horikoshi and Tang, 2015; Tang et al., 2016) or pca3d 0.10 (Weiner, 2017) packages.

Phenotypic Analysis and Metabolic Inference

Phenotypic characterization of each described species was obtained from this study, from the original descriptions or from the summary published by On et al. (2017). For the potentially new *Arcobacter* species, the phenotype was characterized following the recommended minimal standards described for new taxa of the family *Campylobacteraceae* (Ursing et al., 1994; On et al., 2017) and with complementary tests used in the description of other *Arcobacter* species (Levican et al., 2013).

Inference of the metabolic routes from the genome sequences was performed with the software package Traitar (Microbial Trait Analyzer) (Weimann et al., 2016), using the protein coding genes files obtained with Prokka v1.2 (Seemann, 2014). Traitar software is based on phenotypic data extracted from the Global Infectious Disease and Epidemiology Online Network (GIDEON) and Bergey's Systematic Bacteriology. The software uses two prediction models: the phypat classifier, which predicts the presence/absence of proteins found in the phenotype of 234 bacterial species; and the phypat+PGL classifier, which uses the same information as the phypat combined with the information of the acquisition and loss of protein families and phenotypes during evolutive events. A total of 67 traits available within the software, related to oxygen requirement, enzymatic activities, proteolysis, antibiotic resistance, morphology and motility and the use of different carbon sources, were tested and the combined results of the two predictors were analyzed using a heat map.

RESULTS AND DISCUSSION

Strains and Genomes

All the 27 species currently included in the genus *Arcobacter* and 13 candidate species have been investigated in the present study, which has analyzed 55 genomes, 16 of them from the public databases and 39 sequenced in this study (**Tables 1**, **2**). It was not possible to analyze the genomes from *A. acticola* and *A. pacificus* because we were unable to get the type strains of the species. The contigs obtained and the N50 values complied with the recently proposed minimal standards for the use of genomes in taxonomic studies (Chun et al., 2018). The genome size ranged from 1.81 Mb for *A. skirrowii* F28 to 3.60 Mb for *A. lekithochrous* CECT 8942^T (**Table 2**). The G+C content ranged from 26.1% in *A. molluscorum* CECT 7696^T to 34.9% in '*A. aquaticus*' W112-28. The G+C values agree with the range from 24.6% (which corresponded to the type strain of *A. anaerophilus*) to 31% indicated for the genus *Arcobacter* in the recent emended description by Sasi-Jyothsna et al. (2013). Interestingly, 26 genomes (47.3%) showed the presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and CRISPR-associated genes, related with the immune response of the bacteria.

Taxonomic and Phylogenetic Analysis

Similarities in the 16S rRNA gene sequences among type and representative strains of the different *Arcobacter* species (all the 27 species currently included in the genus and the 13 new candidate species) showed a wide range of values (**Supplementary Tables S1, S4**). They ranged from 90.8% (observed between *A. anaerophilus* and *A. faecis*) to 99.9% (between *A. butzleri and* '*A. lacus*'). The lower range of

[5] http://lycofs01.lycoming.edu/~newman/AAI/

TABLE 1 | Strains used in this study, source of isolation and accession numbers of the available genomes.

Species	Strain	Source	Acc. No. Genome	Species	Strain	Source	Acc. No. Genome
A. acticola	KCTC 52212^{T}	Seawater	NA[a]	*A. mytili*	T234	Seawater	PDJW00[b]
A. anaerophilus	DSM 24636^{T}	Estuarine sediment	PDKO00[b]	*A. nitrofigilis*	DSM7299^{T}	Marshland plant	NC014166[c]
	IR-1	Utsira aquifer	NZ_JXXG00[c]	*A. pacificus*	DSM 25018^{T}	Seawater	NA[a]
A. aquimarinus	CECT 8442^{T}	Mediterranean Sea	NXIJ00[b]	*A. skirrowii*	LMG 6621^{T}	Diarrheic lamb	NXIC00[b]
A. bivalviorum	CECT 7835^{T}	Mussels	PDKM00[b]		F28	Wild pig	PDJT00[b]
	F118-4	Mussels	PDKL00[b]	*A. suis*	CECT 7833^{T}	Pork meat	NREO00[b]
A. butzleri	RM4018^{T}	Human (Clinical)	NC_009850[c]	*A. thereius*	LMG 24486^{T}	Aborted pig foetus	LLKQ01[c]
	ED1	Microbial fuel cell	NC_017187[c]		DU22	Duck cloaca	LCUJ01[c]
A. canalis	F138-33	Oyster PNC[e]	NWVW01[b]	*A. trophiarum*	LMG 25534^{T}	Piglet feces	PDKD00[b]
	SH-4D_Col1	Unknown	FUYO00[c]		CECT 7650	Chicken cloacal swab	PDJS00[b]
A. cibarius	LMG 21996^{T}	Broiler, skin	NZ_JABW00[c]	*A. venerupis*	CECT 7836^{T}	Clams	NREP00[b]
A. cloacae	CECT 7834^{T}	Sewage	NXII00[b]	*Arcobacter* sp.	L	Microbial fuel cell	NC_017192[c]
	F26	Mussels	PDJZ00[b]		AF1028	Human feces	JART01[c]
A. cryaerophilus	LMG 24291^{T}	Aborted bovine foetus	NXGK00[b]		CAB	Marine	Go0012496[d]
A. defluvii	CECT 7697^{T}	Sewage	NXIH00[b]		LA11	Marine	BDIR01[c]
A. ebronensis	CECT 8441^{T}	Mussels	PDKK00[b]		LPB0137	Environmental	CP019070[c]
	CECT 8993	Seawater	PDKJ00[b]				
A. ellisii	CECT 7837^{T}	Mussels	NXIG00[b]	'*A. aquaticus*'	W112-28	Freshwater PNC[e]	PDKN00[b]
A. faecis	LMG 28519^{T}	Human septic tank	NZ_JARS00[c]	'*A. caeni*'	RW17-10	Recycled wastewater	MUXE00[b]
A. halophillus	DSM 18005^{T}	Hypersaline lagoon	PDJY00[b]	'*A. hispanicus*'	FW-54	Wastewater	PDKI00[b]
	F166-45	Oyster PNC[e]	PDJY00[b]	'*A. lacus*'	RW43-9	Recycled wastewater	MUXF00[b]
A. lanthieri	LMG 28516^{T}	Pig manure	JARU01[c]	'*A. mediterraneus*'	F156-34	Mussels Alfacs Bay	NXIE00[b]
	LMG 28517	Dairy cattle manure	JARV01[c]	'*A. miroungae*'	9Ant[f]	Cloaca elephant seal	PDKH00[b]
A. lekithochrous	CECT 8942^{T}	Great scallop larvae	NZ_MKCO00[b]	'*A. neptunis*'	F146-38	Mussels Alfacs Bay	PDKG00[b]
	LMG 28652	Abalon	PZYW00[c]	'*A. porcinus*'	LMG 24487^{T}	Aborted pig foetus	LCUH01[c]
A. marinus	CECT 7727^{T}	Seawater	NXAO01[b]	'*A. ponticus*'	F161-33	Cockle Alfacs Bay	PDKF00[b]
	F140-37	Clams Alfacs Bay	NWVX01[b]	'*A. salis*'	F155-33	Oyster PNC[e]	PDKE00[b]
A. molluscorum	CECT 7696^{T}	Mussels	NZ_NXFY00[b]	'*A. viscosus*'	F142-34[g]	Mussels PNC[e]	PDKC00[b]
	F91	Mussels	PDJX00[b]	'*A. vitoriensis*'	FW59[g]	Wastewater	PDKB00[b]
A. mytili	CECT 7386^{T}	Mussels	NXID00[b]	*Arcobacter* sp.	F2176	Mussels	PDJV00[b]

[a]Genome not available; [b]Genome sequenced in this study; [c]Genome obtained from NCBI database; [d]Genome obtained from JGI Gold atabase; [e]PNC means PobleNou Channel, which is a freshwater channel heavily (geometric mean of E. coli counts 4.1 × 10^{4} c.f.u./100ml) contaminated with wastewater where shellfish were exposed for 72h (Salas-Massó et al., 2016, 2018). [f]This strain was obtained from F.J. García from the Laboratorio Central de Veterinaria de Algete, MAGRAMA, Madrid, Spain; [g]These strains were recovered at the Faculty of Pharmacy, University of the Basque Country (UPV-EHU), Vitoria-Gasteiz, Spain, by R. Alonso, I. Martinez-Malaxetxebarria and A. Fernández-Astorga.

similarity (90.8%) is due to the fact that those species, as occurred with others, were assigned within the genus based on the premise that 16S rRNA gene similarity was higher with any type strain of *Arcobacter* than with other taxa. However, in some cases being below the 95% cut-off value for genus delimitation (Rosselló-Mora and Amann, 2001; Yarza et al., 2008; Tindall et al., 2010; Figueras et al., 2011a,b). It is interesting to point out that 16S rRNA gene sequence similarities among *A. nitrofigilis*, the type species of the genus, and the other described species ranged from 93.2% (with *A. thereius*) to 95.9% (with *A. venerupis*). Furthermore, *A. nitrofigilis* showed higher similarities than the threshold value of 95% with only seven species (*A. acticola*, '*A. caeni*,' *A. cloacae*, *A. defluvii*, *A. ellisii*, *A. suis*, and *A. venerupis*) out of the 27 accepted species. In any case, from the analysis of the similarities in the 16S rRNA gene sequences among the *Arcobacter* species it is clear that this gene has limited value and that other approaches available in the genomic era of taxonomy are needed for their study.

Phylogenetic analysis based on the core genome made up of 286 genes (**Figure 1** and **Supplementary Table S5**) and also on the concatenated sequences of 13 housekeeping genes of the representative *Arcobacter* strains (**Figure 2**) revealed that the *Arcobacter* species could be grouped into 4 major monophyletic clusters. Cluster 1, comprised seven validated species: *A. butzleri*, *A. cibarius*, *A. cryaerophilus*, *A. lanthieri*, *A. skirrowii*, *A. thereius*, and *A. trophiarum*, together with *A. faecis* (species described but not validated yet) and five candidate taxa '*A. hispanicus*,' '*A. lacus*,' '*A. miroungae*,' '*A. porcinus*,' and '*A. vitoriensis*' (**Figure 1**). Cluster 2 embraced the species *A. aquimarinus*, *A. cloacae*, *A. defluvii*, *A. ellisii*, *A. suis*, and *A. venerupis*, as well as the non-validated *A. acticola* and the candidatus '*A. caeni*.' Cluster 3 included five species, *A. canalis*, *A. halophilus*, *A. marinus*, *A. molluscorum*,

TABLE 2 | Genome characteristics and annotation results. Source of whole genome sequences as indicated in **Table 1**.

Species	No. Contigs	N50 (Kb)	CDS (Total)	CDS (Coding)	RNA Genes	tRNAs	ncRNAs	CRISPR Arrays	G+C (%)	Size (Mb)
A. anaerophilus DSM 24636^T	40	186	2,938	2,922	45	40	2	1	29.9	2.98
A. anaerophilus IR1	7	1,179	3,360	3,024	61	47	2	3	30.2	3.25
'*A. aquaticus*' W112-28^T	20	370	2,500	2,487	55	45	3	0	34.9	2.53
A. aquimarinus CECT 8442^T	68	75	2,473	2,463	46	42	2	0	26.6	2.46
A. bivalviorum CECT 7835^T	179	461	2,786	2,728	50	41	3	0	28.2	2.75
A. bivalviorum F118-4	26	209	2,652	2,652	47	38	3	0	28.1	2.71
A. butzleri RM4018^T	1	–	2,261	2,256	71	54	2	0	27.0	2.34
A. butzleri ED1	1	–	2,151	2,145	71	54	2	0	27.1	2.26
'*A. caeni* RW17-10^T	59	123	2,357	2,337	58	51	3	0	27.1	2.42
A. canalis CECT8984^T	50	166	2,733	2,720	53	48	2	1	27.3	2.78
A. canalis SH-4D_Col1	69	72	2,716	2,663	63	52	2	1	27.1	2.82
A. cibarius LMG 21996^T	44	119	2,156	2,110	68	46	2	0	27.1	2.20
A. cloacae CECT 7834^T	135	135	2,826	2,795	58	51	2	3	26.8	2.78
A. cloacae F26	40	218	2,470	2,459	53	44	2	1	26.9	2.51
A. cryaerophilus LMG 24291^T	91	54	2,092	2,081	49	40	3	0	27.2	2.06
A. defluvii CECT 7697^T	80	166	2,921	2,894	57	49	2	2	26.3	2.94
A. ebronensis CECT 8441^T	103	188	3,089	3,072	47	39	3	1	29.2	3.15
A. ebronensis W129-34	126	217	3,206	3,171	46	40	3	2	29.2	3.23
A. ellisii CECT 7837^T	135	177	2,875	2,840	64	52	2	1	26.9	2.80
A. faecis LMG 28519^T	55	127	2,429	2,376	76	53	2	1	27.2	2.50
A. halophilus DSM 18005^T	111	56	2,677	2,660	54	46	3	2	27.4	2.75
A. halophilus F166-45	90	56	2,879	2,864	59	51	2	2	27.0	2.96
'*A. hispanicus*' FW54^T	76	148	2,228	2,207	46	40	3	1	26.4	2.21
'*A. lacus*' RW43-9^T	24	295	2,194	2,182	47	40	2	0	26.8	2.22
A. lanthieri LMG 28516^T	29	466	2,223	2,190	73	52	3	1	26.7	2.29
A. lanthieri AF1581	24	353	2,199	2,186	88	57	3	0	26.8	2.26
A. lekithochrous CECT 8942^T	436	343	3,628	3,316	88	75	3	0	28.6	3.61
A. lekithochrous LMG 28652	82	343	3,499	3,330	61	55	3	0	28.2	3.50
A. marinus CECT 7727^T	162	54	2,809	2,781	55	50	2	0	27.0	2.87
A. marinus F140-37	76	67	2,725	2,652	59	48	2	0	27.0	2.78
'*A. mediterraneus*' F156-34^T	29	689	2,769	2,750	47	41	3	1	27.3	2.83
'*A. miroungae*' 9Ant^T	35	363	1,868	1,847	46	41	2	1	28.1	1.84
A. molluscorum CECT 7696^T	117	121	2,746	2,736	58	49	3	6	26.1	2.76

(Continued)

TABLE 2 | Continued

Species	No. Contigs	N50 (Kb)	CDS (Total)	CDS (Coding)	RNA Genes	tRNAs	ncRNAs	CRISPR Arrays	G+C (%)	Size (Mb)
A. molluscorum F91	240	150	2,951	2,889	71	58	3	2	26.3	2.89
A. mytili CECT 7386^T	126	70	2,950	2,934	58	48	3	1	26.3	2.97
A. mytili T234	145	37	2,735	2,723	54	48	3	0	26.4	2.77
'*A. neptunis*' F146-38^T	36	267	2,627	2,614	57	45	3	0	27.1	2.65
A. nitrofigilis DSM 7299^T	1	–	3,101	3,086	69	55	2	1	28.4	3.19
'*A. ponticus*' F161-33	24	597	2,632	2,621	46	36	3	0	28.1	2.74
'*A. porcinus*' LMG 24487^T	70	123	2,186	2,112	47	41	2	0	27.0	2.14
'*A. salis*' F155-33^T	153	169	2,932	2,904	50	43	3	0	29.0	2.93
A. skirrowii LMG 6621^T	62	306	2,029	2,006	48	42	2	2	27.7	1.97
A. skirrowii F28	110	40	1,911	1,897	46	41	2	0	27.8	1.81
A. suis CECT 7833^T	122	142	2,646	2,613	57	52	2	0	27.3	2.62
A. thereius LMG 24486^T	2	1,039	1,896	1,883	57	46	2	3	27.0	1.91
A. thereius DU22	19	252	2,006	1,983	47	42	2	1	26.8	2.01
A. trophiarum CECT 7650	37	152	1,911	1,894	48	37	3	0	28.0	1.90
A. trophiarum LMG 25534^T	266	86	2,167	2,071	49	41	3	0	29.4	2.00
A. venerupis CECT 7836^T	234	182	3,319	3,267	64	52	2	0	28.0	3.28
'*A. viscosus*' F142-34^T	82	65	2,772	2,756	55	48	3	1	26.6	2.79
'*A. vitoriensis*' FW59^T	144	179	2,617	2,570	53	46	2	0	27.4	2.58
Arcobacter sp. CAB	367	20	3,596	3,392	NA	31	NA	NA	28.2	3.48
Arcobacter sp. F2176	99	178	3,212	3,186	67	57	2	0	28.1	3.27
Arcobacter sp. LA11	53	229	3,006	2,961	49	43	3	0	27.9	3.10
Arcobacter sp. LPB0137	1	–	2,731	2,698	85	64	2	0	27.7	2.87
Arcobacter sp. L[a]	1	–	2,847	2,834	73	56	2	1	26.6	2.95
Arcobacter sp. AF1028[b]	46	148	2,336	2,285	71	51	2	1	27.2	2.41

[a]*Genome sequenced in this study;* [b]*Genome obtained from NCBI database;* [c]*Genome obtained from JGI Gold database. Our results show that these strains belong to the species.* [d]*A. defluvii and* [e]*A. faecis.*

and *A. mytili*, together with two candidates, '*A. neptunis*' and '*A. viscosus.*' Finally, Cluster 4 included the species *A. anaerophilus*, *A. bivalviorum*, and *A. ebronensis*, as well as the candidates '*A. mediterraneus,*' '*A. ponticus,*' and '*A. salis.*' The split decomposition network analysis of the core genome showed that the species *A. lekithochrous* CECT 8942^T and *A. nitrofigilis* DSM 7299^T appeared as orphan species. Furthermore, with this analysis the candidatus '*A. aquaticus*' W112-28 also appeared in a separate branch near to *A. nitrofigilis* DSM 7299^T. On the other hand, both analyses, MLSA and core genome, confirmed the existence of two sub-clusters in Cluster 1 (again *A. butzleri* and '*A. lacus*' were located in the most distant branch within the cluster), and also two subgroups could be observed in Cluster 4, one comprising the species *A. anaerophilus* and *A. ebronensis*, and the other including the rest of species within this cluster (**Figures 1**, **2**). All the clusters and sub-clusters showed a similarity in the concatenated sequences of the 13 housekeeping genes higher than 85% (**Figure 2**).

Phylogenies based on the 16S and 23S rRNA gene sequences, undertaken with the NJ and ML approachesserealso constructed with comparative purposes. 16S rRNA based tree showed also the four major clusters although less defined (**Supplementary Figure S1A**). Species within Cluster 1, showed 16S rRNA gene sequence similarities ranging from 96.1 to 99.9%. Cluster 2 yielded similarities among species for the 16S rRNA gene between 96.7 and 99.6%, whereas within Cluster 3 ranged between 93.0 and 99.1%. Finally, Cluster 4 included species with a range of 16S rRNA sequence similarity from 94.0 to 99.5%. With the exception of Cluster 3, similarity values within the clusters (>94–95%) were within the classical boundaries for genus assignation in bacterial taxonomy (Rosselló-Mora and Amann, 2001; Yarza et al., 2008, 2014; Tindall et al., 2010; Figueras et al., 2011a,b). Our results agree with those from a recent study by Yarza et al. (2014), who investigated 568 taxa and described a threshold in 16S rRNA sequence identity of 94.5% for genus delineation.

Similar groups and topology, with only minor differences, were obtained when the 23S rRNA gene sequences were used to analyze the phylogeny of the genus (**Supplementary Figure S2**). In this analysis, the recently described species *A. acticola*, and *A. pacificus* could not be included because of the unavailability of the type strains and/or whole genome sequences. The same four major clusters formed in the 23S rRNA gene phylogenetic tree, and the species *A. lekithochrous* and *A. nitrofigilis* appeared also as orphan species (**Supplementary Figure S2**). Within Cluster 1 two subgroups could also be obtained, differentiating the species *A. butzleri* and '*A. lacus*' from the rest of the species. Similarly, the species *A. anaerophilus* and *A. ebronensis* formed a differentiated subgroup in Cluster 4.

The visual analysis of the alignments obtained with the sequences of the 16S and 23S rRNA genes allowed the localization of signature motifs, especially in the 16S rRNA gene, for the different clusters established in the phylogenetic analysis. In these sequences, a total of 16 locations were found, presenting nucleotide combinations characteristic for the clusters (**Supplementary Figure S3**). Some of these motifs were located in helix regions as interactions with proteins of the ribosomal 30S subunit, such as helix 21 (region V4) or helix 28/44 (region V9), and therefore had a considerable level of protection against mutations (Adilakshmi et al., 2008; Kitahara et al., 2012). There are some studies on the presence of signature regions with taxonomic/phylogenetic implications in the ribosomal genes (Martínez-Murcia et al., 1992, 2007; Ue et al., 2011;Řeháková et al., 2014; Martínez-Murcia and Lamy, 2015). Some regions with signature motifs detected in the present study have also shown implications for phylogenetic analysis in cyanobacteria, including regions H15, H17, H21, H22-H23, H41, and H44 (Řeháková et al., 2014). A tree was also constructed weighting such positions (**Supplementary Figure S1B**), which allowed a better definition of the main clusters observed with the whole 16S rRNA sequences although, as expected, differentiation among species within each cluster was lower. Two sub-clusters were observed in Cluster 1, where the species *A. butzleri* and '*A. lacus*' grouped into a well-differentiated branch with respect to the other species in the cluster (**Supplementary Figure S1B**). In this analysis, *A. pacificus* was clearly located in the Cluster 3, whereas in Cluster 4, *A anaerophilus* was the borderline species, while *A. ebronensis* and '*A. mediterraneus*' were located in an independent branch (**Supplementary Figure S1B**). Therefore, the signature motifs described here might be a new tool for identification of the different clusters and/or genus.

Genomic Indices

The results of the calculations of the ANI and the *is*DDH among the 36 studied genomes are given in the **Supplementary Table S2** and **Supplementary Figure S4**. The results of the ANI and *is*DDH calculations showed that the genomes grouped into the same clusters observed by the analyses of the MLSA of the 13 housekeeping and core genes (**Figures 1**, **2**). Ranges of ANI within each cluster were from 75.2 to 95.4%, whereas *is*DDH values were between 19.5 and 65.4% (**Figure 2** and **Table 3**). These results confirm the phylogenetic analysis for the 13 new candidate species because all of them showed ANI and *is*DDH values of <96% and <70%, respectively, which are the cut-off values proposed for the delineation of new species (Konstantinidis and Tiedje, 2005; Goris et al., 2007; Richter and Rosselló-Móra, 2009; Figueras et al., 2017). As discussed in other studies, the ANI and *is*DDH indices provided reliable information for the delineation of *Arcobacter* species and are also included in the minimal guidelines to define species using genomes (Whiteduck-Léveillée et al., 2015, 2016; Figueras et al., 2017; Chun et al., 2018). Although those indices are not considered useful for delimiting genera, each of the four clusters showed values that ranged between 75.2 and 81.8% as their lowest ANI, which might be the suitable range for separating different, closely related genera. These values are relatively similar to those reported by Qin et al. (2014) that found 68–82% interspecies ANI values among the genera that they studied. Values of ANI obtained for the candidate species '*A. aquaticus*' were lower than the other results, from 70.0% with *A. cryaerophilus* LMG 24291^T to 71.9% with *A. bivalviorum* CECT 7835^T and more in line with the Qin et al. (2014) results of 68% (**Supplementary Table S2**). In the case of the *is*DDH the lower values among species in the same cluster ranged between 19.5 and 24.8%, and again these might be the levels associated to different genera.

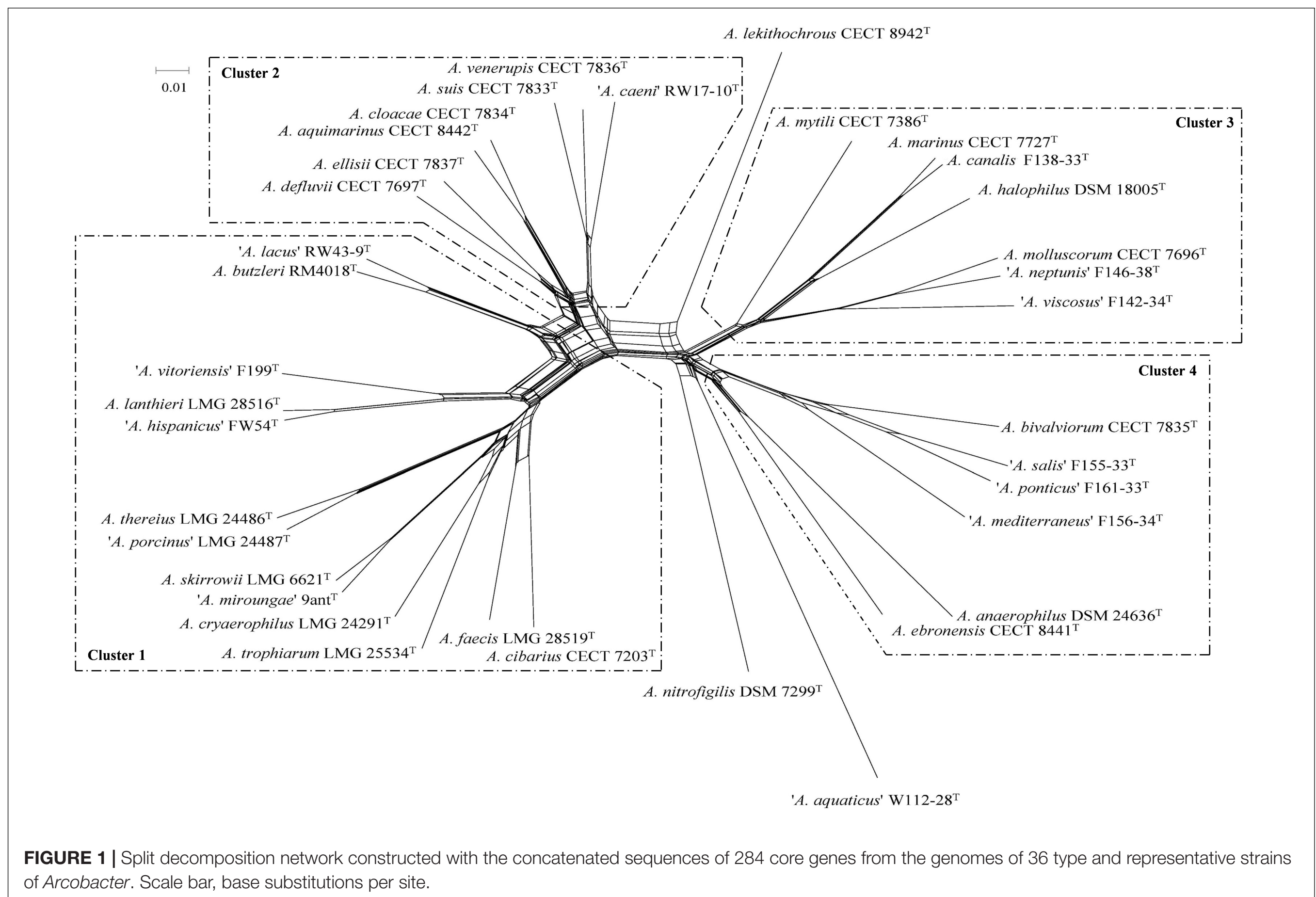

FIGURE 1 | Split decomposition network constructed with the concatenated sequences of 284 core genes from the genomes of 36 type and representative strains of *Arcobacter*. Scale bar, base substitutions per site.

With the aim of confirming if the clusters observed might represent different genera, as suggested by the phylogenetic analyses, the similarity indices AAI and POCP were also calculated (**Supplementary Table S3**). In agreement with the 60–80% AAI that have been described for species belonging to the same genus (Konstantinidis and Tiedje, 2005) all our clusters showed lower ranges of between 67.6 to 80.3% (**Table 3**). All the clusters also complied with the POCP proposed for genus separation above 50% (Luo et al., 2014; Qin et al., 2014) because as shown in **Table 3** all clusters showed the lowest values from 67.0 to 75.4%.

It is widely known that synonymous codon usage varies among organisms and that it is related to differences in G+C content, replication strand skew, or gene expression (Suzuki et al., 2008; Farooqi et al., 2016). The interaction of these factors may vary among species depending on their evolutionary process (Ma et al., 2015). It has also been suggested that the extent of codon usage bias plays a role in the adaptation of prokaryotic organisms to their environments and lifestyles (Botzman and Margalit, 2011). To analyze the overall codon usage trends of the *Arcobacter* species, the frequencies of the different codons were obtained from the whole genomes and the RSCU was computed using the CAI, which is a useful tool for estimating codon usage bias (Ma et al., 2015; Farooqi et al., 2016). A first finding was that all the *Arcobacter* species presented a preferential use of the codons finishing in A or T (**Supplementary Figure S5**), which might be expected due to their low G+C% content. The characteristic pattern showed by *A. aquaticus* is noteworthy (**Supplementary Figure S5**), which supports its differentiation from the other species in Cluster 3 as well as its unique taxonomy. Such difference was the only statistically significant ($p < 0.05$) in the multinomial regression analysis carried out.

Next, the codon usage trends were analyzed by PCA to reveal possible evolutionary relationships. Interestingly, different groups of strains could be observed in the three-dimensional graphic (**Figure 3**), which correlated with those clusters established in the different phylogenetic analyses, as shown above. As reported previously for different species of *Mycoplasma* (Marenda et al., 2005; Ma et al., 2015), PCA provides an additional pathway to investigate the evolutionary direction of the *Arcobacter* species. In addition, similarities in the synonymous codon usage patterns might reflect similar lifestyles (pathogenic vs. non-pathogenic) and adaptation to certain environments (marine water, shellfish, etc.).

Metabolic Inference and Phenotypic Analysis

Phylogenetic and genomic analysis confirmed the existence of four clusters among the validated and candidate *Arcobacter*

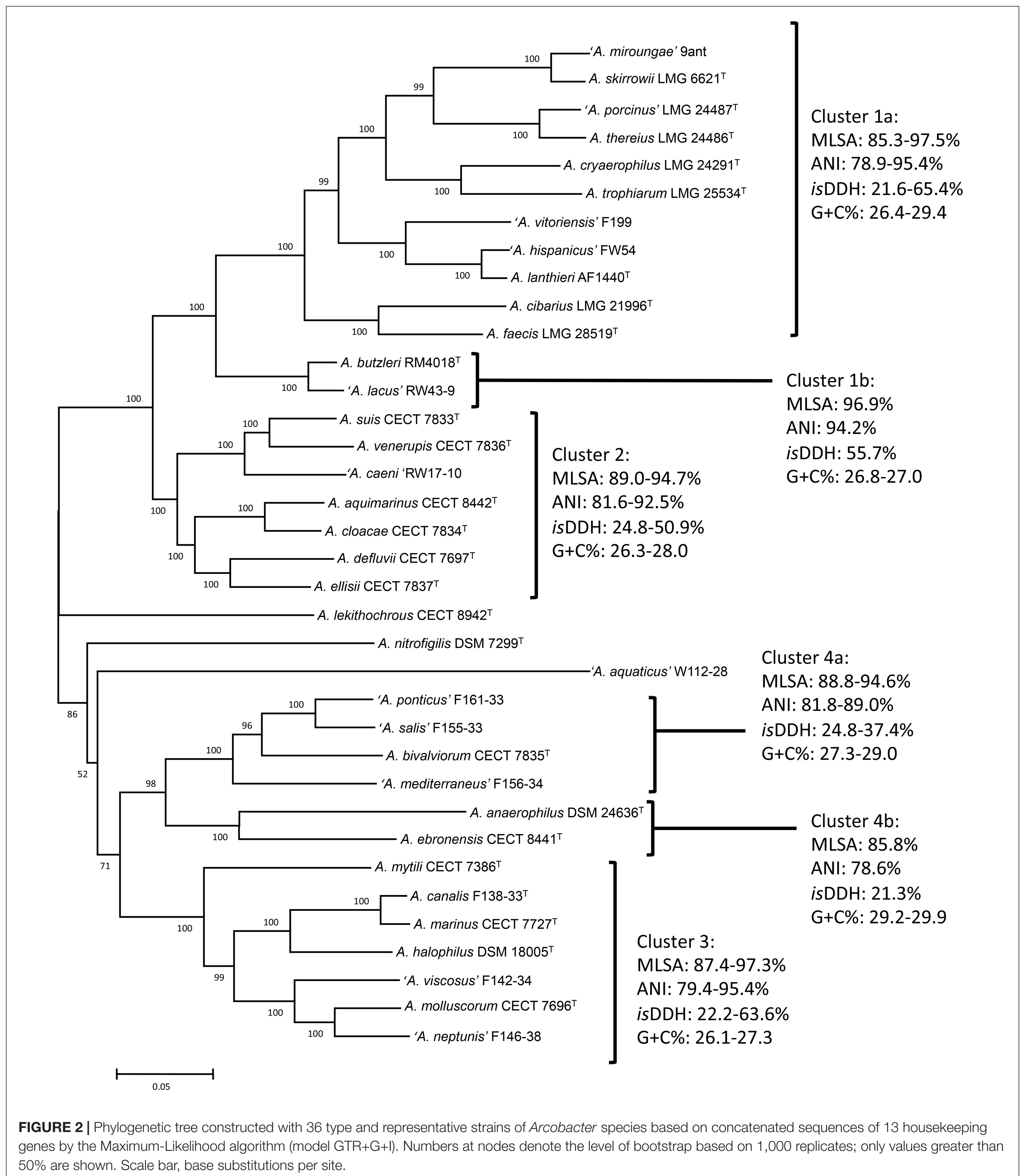

FIGURE 2 | Phylogenetic tree constructed with 36 type and representative strains of *Arcobacter* species based on concatenated sequences of 13 housekeeping genes by the Maximum-Likelihood algorithm (model GTR+G+I). Numbers at nodes denote the level of bootstrap based on 1,000 replicates; only values greater than 50% are shown. Scale bar, base substitutions per site.

species, which comply with the cut-off values established for the differentiation of independent genera. A thorough phenotypic analysis was therefore carried out to determine if the description of new taxa at genus level was possible or if such clusters were only clades or genomovars within the genus *Arcobacter*. In fact, this is what has occurred in a recent polyphasic study of 52 *A. cryaerophilus* strains (including genome information) in which, despite four different genomospecies

TABLE 3 | Intra-cluster similarities (%) obtained for the 16S rRNA gene and for the different genomic indexes analyzed.

	16S RNA gene		MLSA		ANI		*is*DDH		AAI		POCP		G+C% (mol)	
Cluster 1a	96.8–99.8	96.1–99.9	85.3–97.5	85.3–97.5	78.9–95.4	77.7–95.4	21.6–65.4	20.5–65.4	72.5–95.0	71.5–95.0	68.2–95.6	67.0–95.6	26.4–29.4	26.4–29.4
Cluster 1b	99.9		96.9		94.2		55.7		93.7		84.3		26.8–27.0	
Cluster 2	96.7–99.6		89.0–94.7		81.6–92.5		24.8–50.9		73.1–93.5		71.7–87.5		26.3–28.0	
Cluster 3	94.2–99.1		87.4–97.3		79.4–95.4		22.2–63.6		67.6–95.7		75.4–91.4		26.1–27.3	
Cluster 4a	96.6–99.5	94.0–99.5	88.8–94.6	85.8–94.6	81.8–89.0	75.2–89.0	24.8–37.4	19.5–37.4	80.3–83.4	68.7–83.4	74.4–90.7	71.6–90.7	27.3–29.0	27.3–29.9
Cluster 4b	96.9		85.8		78.6		21.3		78.4		77.9		29.2–29.9	

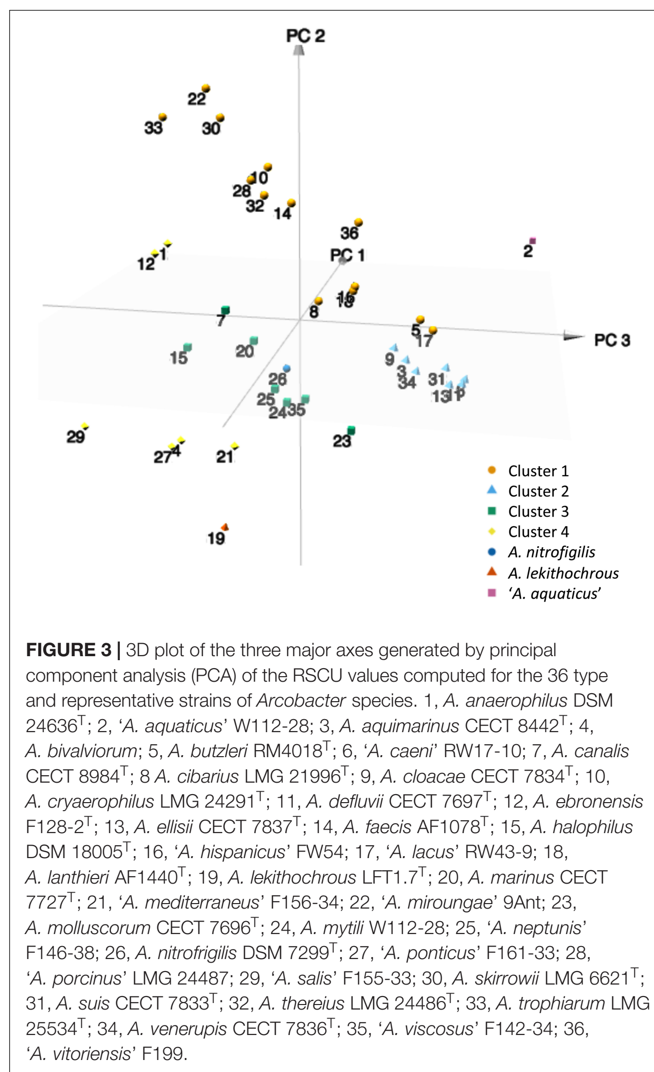

FIGURE 3 | 3D plot of the three major axes generated by principal component analysis (PCA) of the RSCU values computed for the 36 type and representative strains of *Arcobacter* species. 1, *A. anaerophilus* DSM 24636^T; 2, '*A. aquaticus*' W112-28; 3, *A. aquimarinus* CECT 8442^T; 4, *A. bivalviorum*; 5, *A. butzleri* RM4018^T; 6, '*A. caeni*' RW17-10; 7, *A. canalis* CECT 8984^T; 8 *A. cibarius* LMG 21996^T; 9, *A. cloacae* CECT 7834^T; 10, *A. cryaerophilus* LMG 24291^T; 11, *A. defluvii* CECT 7697^T; 12, *A. ebronensis* F128-2^T; 13, *A. ellisii* CECT 7837^T; 14, *A. faecis* AF1078^T; 15, *A. halophilus* DSM 18005^T; 16, '*A. hispanicus*' FW54; 17, '*A. lacus*' RW43-9; 18, *A. lanthieri* AF1440^T; 19, *A. lekithochrous* LFT1.7^T; 20, *A. marinus* CECT 7727^T; 21, '*A. mediterraneus*' F156-34; 22, '*A. miroungae*' 9Ant; 23, *A. molluscorum* CECT 7696^T; 24, *A. mytili* W112-28; 25, '*A. neptunis*' F146-38; 26, *A. nitrofrigilis* DSM 7299^T; 27, '*A. ponticus*' F161-33; 28, '*A. porcinus*' LMG 24487; 29, '*A. salis*' F155-33; 30, *A. skirrowii* LMG 6621^T; 31, *A. suis* CECT 7833^T; 32, *A. thereius* LMG 24486^T; 33, *A. trophiarum* LMG 25534^T; 34, *A. venerupis* CECT 7836^T; 35, '*A. viscosus*' F142-34; 36, '*A. vitoriensis*' F199.

being recognized, the phenotypic characterization did not allow their differentiation into separate species and were therefore considered genomovars (Pérez-Cataluña et al., 2018a).

Phenotypic inference using Traitar confirmed the lack of reaction of *Arcobacter* species to most of the tests commonly used for bacterial identification (**Supplementary Figure S6**). Thus, all the type and representative strains rendered negative results, regardless of the predictor employed, for use as the sole carbon source of sugars (D-Mannitol, D-Mannose, Salicin, or Trehalose, among others) and carboxylic acids (Citrate or Malonate). Such results have been previously reported in the original descriptions of the species (see review of On et al., 2017). On the other hand, there was some incongruence between results from Traitar and those obtained by classical characterization for some tests, including growth on MacConkey agar or urea hydrolysis (data not shown). A possible explanation is related with the macro-accuracy of the predictors employed in the Traitar analysis (82.6–85.5%), as reported in the original description of the microbial trait analyzer (Weimann et al., 2016). The fact that

some of the *Arcobacter* species studied are halophilic cannot be ignored, since some of the media usually employed in the wet-lab characterization are developed for non-halophilic microorganisms.

The heat maps built from the combined results of both predictors in the Traitar analysis revealed the existence of similarity groups regarding the metabolic characteristics of the *Arcobacter* type strains (**Supplementary Figure S6**). In most case, clustering of strains supported the groups obtained with genomic tools, although some incongruence was also observed, such as for *A. butzleri* (better related here to *A. defluvii, A. ellisii* or *A. cloacae*), *A. mytili* (closest Traitar species '*A. caeni*') or *A. venerupis* (forming a branch with *A. ebronensis* and '*A. ponticus*'). In any case, Traitar might be helpful as a first-step method for phenotypic inference, although further verification should be made, especially in environmental bacterial species with special growth requirements (i.e., halophilic conditions).

A deep review of the characteristics reported in the original descriptions of the *Arcobacter* species, together with results obtained in our respective laboratories, allowing phenotypic traits to differentiate the clusters established by the phylogenetic and genomic analyses (**Table 4**). Growth at 37°C in microaerophilic condition, the halophilic character, the ability to grow in presence of glycine, safranin, oxgall, or triphenyltetrazolium chloride (TTC), the presence of some enzymatic activities, such as catalase, urease or indoxyl acetate hydrolysis, and resistance to cefoperazone among others, were the main differentiating traits. Most of these characters are included in the minimal standards for describing new species in the families *Campylobacteraceae* and *Helicobacteraceae* (On et al., 2017), and they should, therefore, also be maintained for the new family *Arcobacteraceae* proposed by Waite et al. (2017), once this taxonomical change is validated. The phenotypic differentiation proposed in **Table 4** enabled to further describe the new genera that corresponded to the different clusters of *Arcobacter* species determined in the present study.

Stability of the Genomic-Based Clustering

In order to test the stability of the new taxonomical scheme proposed, we analyzed the whole genome sequences using second strains from each species or from unassigned sequences obtained from the public databases. That analysis is shown in **Supplementary Figure S7** and included 55 genomes. These new phylogenetic analyses of the core genome also using a Split network showed that the four clusters were maintained, but the two clusters (Clusters 3 and 4) that include species able to grow in media containing 2.5% NaCl appeared in the right place (**Supplementary Figure S7**). The genome of *Arcobacter* sp. LPB0137 obtained from the NCBI database grouped with the species *A. lekithochrous* CECT 8942^T, while the genomes *Arcobacter* sp. LA11 and CAB grouped together in a separate branch near to Cluster 4. Interestingly, the ANI and *is*DDH values of 91.4% and 45.8% between strain F2176, previously identified as *A. nitrofigilis* (Figueras et al., 2008), and the type strains of this species along with the phylogenetic position (**Supplementary Figure S7**), revealed that this strain belonged to another potentially new species. Furthermore, strains L and AF1028, deposited at the NCBI database as *Arcobacter* sp. were identified as *A. defluvii* and *A. faecis*, respectively, because they clustered with the type strains of those species (**Supplementary Figure S7**). This was also confirmed by the ANI and *is*DDH results being above 96% and 70%, respectively.

Collado and Figueras (2011), in their review about the epidemiology and clinical significance of the genus *Arcobacter*, reported that these bacteria should be considered quite atypical within the class *Epsilonproteobacteria* because of the great diversity of hosts and habitats from which they have been isolated. In order to show if the clusters obtained have a relationship with their ecological habitat, the origin of each strain is also given in **Supplementary Figure S7**. Despite the fact that only two strains from each species were included in the analysis, each of the clusters embraced species that had been recovered from common or related origins. Cluster 1 included by strains isolated from humans and animals, from wastewater and from broiler skin (*A. cibarius* CECT 7203^T). The fact that some strains isolated from wastewater that was contaminated by humans or animal excreta, gives evidence of the relationship of these sources. This finding agrees with the high abundance of *Arcobacter* in wastewater and in water contaminated with fecal pollution (Collado et al., 2008, 2010). Among the species of Cluster 1, both by metagenomics analysis or direct plating without enrichment (Fisher et al., 2014; Levican et al., 2016), the species *A. cryaerophilus* was the prevalent species in wastewater, while the species *A. butzleri* is normally predominant in studies that investigate water and food samples of animal origin, such as different types of meats using an enrichment step (Collado et al., 2009b; Collado and Figueras, 2011; Hsu and Lee, 2015; and references therein). So far, only the species *A. cryaerophilus*, *A. thereius*, *A. trophiarum, A. cibarius* or *A. skirrowii* have been recovered from humans or animals (De Smet et al., 2011; Figueras et al., 2014; Van den Abeele et al., 2014) and all these species are as commented in the same cluster.

Cluster 2 included strains from different origins but was dominated by species that came from wastewater, shellfish or food products. In this sense, *A. defluvii* CECT 7697^T and '*A. caeni*' RW17-10 were isolated from wastewater, while the strain *A. defluvii* L was recovered from a microbial fuel cell. Strains of *A. defluvii* have also been recovered from shellfish in other studies (Levican et al., 2014; Salas-Massó et al., 2016). The strain *A. suis* CECT 7833^T was isolated from pork meat, but other isolates have also been obtained from buffalo milk in Italy (Levican et al., 2013; Giacometti et al., 2015). The other five strains in the cluster were isolated from shellfish, wastewater and seawater (**Table 1** and **Supplementary Figure S7**). The other two clusters (Clusters 3 and 4) included strains isolated from seawater shellfish giving evidence of the marine origin of these clusters. The orphan species (*A. nitrofigilis* DSM7299^T, *A. lekithochrous* CECT 8942^T, and '*A. aquaticus*' W112-28) also corresponded

TABLE 4 | Differential phenotypic traits among the different clusters of *Arcobacter* species obtained on the basis of the characteristics of the type and representative strains of the species included in each group.

Test	*A. nitrofigilis*	Cluster 1	Cluster 2	Cluster 3	Cluster 4	*A. lekithochrous*	*A. aquaticus*
Growth at/on							
CO_2 37°C	–	V	V	+	V	–	+
0.5% NaCl	–	+[a]	+	V	–[b]	–[c]	+
4% NaCl	+	–	–	+	+	–	–
1% Glycine	–	V	–	V	V	–	–
0.05% Safranin	–	+	V	V	V	+	+
0.04 TTC	–	V	–	–	–	+	–
1% Oxgall	–	V	V	–	–[d]	–	–
CCDA	–	V	V	–[e]	–	+	+
Enzymatic activities							
Catalase	–	+[f]	+	V	V	+	–
Urease	+	–	V	–	–[d]	–	–
Indoxyl acetate hydrolysis	+	+[f]	+	V	V	–	–
Nitrate reduction	+	V	+	–[g]	V	–	–
Resistance to cefoperazone (64 mg/l)	ND	V	–	V	–	–	+

+, positive result; –, negative result; V, variable result in all the species of the cluster; [a]With the exception of A. skirrowii; [b]With the exception of A. pacificus; [c]A. lekithochrous needs sea salts to grow; [d]With the exception of A. ebronensis; [e]With the exception of A. molluscorum; [f]With the exception of A. cibarius; [g]With the exception of A. anaerophilus. ND, not determined.

to strains isolated from marine environments and their phylogenetic position was close to the two marine clusters (3 and 4).

As indicated in the review by Collado and Figueras (2011), there are many uncultured or not-yet-described species of *Arcobacter*, which have been recognized on the basis of nearly full-length 16S rRNA gene sequences, and which probably outnumber those species that were already known at that time. Their hosts and/or habitats are very diverse and include cod larvae, cyanobacterial mats, activated sludge, tidal and marine sediments, estuarine and river water, plankton, coral, tubeworms, snails, etc. (Collado et al., 2011; and references therein). In the near future new species can be expected to emerge that will reinforce the value of the different genera proposed in this study.

CONCLUSION

Genomic information obtained through next-generation sequencing leads to great advances in the systematics of prokaryotes (Whitman, 2015), not only to the general understanding of prokaryotic biology but also for the resolution of the phylogeny of taxa higher than species. Single gene phylogeny, including 16S rRNA gene, has often limitations that analysis of complete genome sequences can overcome. The study aims to use this modern taxonomy approach to clarify the relationships of the diverse *Arcobacter* species.

The results obtained in the present study confirmed the opinion of some authors on the need for a clarification of the taxonomy of the genus *Arcobacter*. The phylogenetic analyses derived from the MLSA of 13 genes and of the core genome as well as the existence of signature regions in the 16S rRNA gene have shown, together with the genomic indexes ANI (75.2–81.8%), *is*DDH (19.5–24.8%), AAI (67.6–80.3%), and POCP (67.0–75.4%), to be useful tools for delimiting several genomic and phylogenetic groups within this genus. The intra-genus ranges and cut-off values established here might also be helpful for future taxonomic studies in other bacterial groups.

Such genomic variability, together with the determination of combinations of differentiating phenotypic traits allowed the division of the current genus *Arcobacter* in at least six different genera for which the names *Aliiarcobacter* gen. nov., *Pseudoarcobacter* gen. nov., *Haloarcobacter* gen. nov., *Malacobacter* gen. nov., and *Poseidonibacter* gen. nov. are proposed. In addition, the candidate species '*A. aquaticus*' also constitutes a new genus for which the name Candidate '*Arcomarinus*' gen. nov. is proposed, although such proposal should be formulated in parallel to the formal description of the species.

According to Tindall et al. (2010) "*the type strain of a genus is the most important reference organism to which a novel species has to be compared.*" In the case of the genus *Arcobacter*, the type species has rarely been isolated (Collado et al., 2009b; Toh et al., 2011; Levican et al., 2016; Salas-Massó et al., 2016) and in fact, all the analyses show that *A. nitrofigilis* is an orphan species and the only representative of the genus *Arcobacter*, for which an emended description is provided.

The other genera are described here while taking into account the species validated at the time of writing but with the confidence that the formal description of the candidate species would fit in such descriptions. Thus, the genus *Aliiarcobacter* gen. nov. is described comprising seven species *Aliiarcobacter cryaerophilus* comb. nov., *A. butzleri* comb. nov., *A. skirrowii* comb. nov., *A. cibarius* comb. nov., *A. thereius* comb. nov., *A. trophiarum* comb. nov., *A. lanthieri* comb. nov., and *A. faecis* comb. nov. On the other hand, the genus *Pseudoarcobacter* gen. nov. includes

the species *Pseudoarcobacter defluvii* comb. nov., *P. ellisii* comb. nov., *P. venerupis* comb. nov., *P. cloacae* comb. nov., *P. suis* comb. nov., *P. aquimarinus* comb. nov., and *P. acticola* comb. nov. Four species, *Malacobacter halophilus* comb. nov., *M. mytili* comb. nov., *M. marinus* comb. nov., *M. molluscorum* comb. nov., and *M. pacificus* comb. nov. are compiled in the new genus *Malacobacter* gen. nov., whereas the genus *Haloarcobacter* gen. nov. comprises three species *Haloarcobacter bivalviorum* comb. nov., *H. anaerophilus* comb. nov., and *H. ebronensis* comb. nov. Finally, the genus *Poseidonibacter* gen. nov. has a unique species *Poseidonibacter lekithochrous* comb. nov.

Emended Description of the Genus *Arcobacter* Vandamme et al., 1991 emend. Vandamme et al., 1992 and Sasi-Jyothsna et al., 2013

Arcobacter (Ar'co.bac.ter. L. n. *arcus*, bow; Gr. n. *bacter*, rod; M. L. masc. n. *Arcobacter*, bow-shaped rod).

Cells are Gram-negative, curved rods 0.2–0.9 μm in diameter and 1–3 μm long. Coccoid bodies are found in old cultures but are not rapidly produced under aerobic conditions. Motile with a rapid corkscrew motion. Each cell possesses a single polar flagellum. Does not swarm. Chemoorganotrophic. Utilizes organic and amino acids as carbon sources, but not carbohydrates. Respiratory metabolism with oxygen as the terminal electron acceptor; anaerobic growth with aspartate and fumarate, but not with nitrate. Nitrate usually reduced to nitrite. Requires NaCl for growth. Grows at temperatures of 10°C–35°C but not at 42°C. Catalase, oxidase, urease, and nitrogenase positive. Phosphatase, sulfatase and indole negative. Does not hydrolyze esculin, casein, DNA, gelatine, hippurate or starch. Fluorescent pigments are not produced. Unable to grow with glycine (1% wt/vol), safranin (0.05% wt/vol), oxgall (1% wt/vol), or 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol). Positive for the hydrolysis of indoxyl acetate. Poly-β-hydroxybutyrate not produced.

The base composition of the DNA is 28.1–28.4% G+C as determined from the genomes.

The type species is *Arcobacter nitrofigilis.*

Description of *Aliiarcobacter* gen. nov.

Aliiarcobacter (A.li.i.ar.co.bac'ter, L. pronoun *alius* other, another; N.L. masc. n. *Arcobacter* a bacterial generic name; N.L. masc. n. *Aliiarcobacter* the other *Arcobacter*).

Cells are Gram-negative, curved rods 0.2–0.5 μm in diameter and 1–3 μm long. Motile by single polar flagellum. Does not swarm. Chemoorganotrophic. Oxidase and catalase positive. No growth occur at 4% NaCl. Growth occurs at 15°C–42°C. Carbohydrates are not fermented. Nitrate usually reduced to nitrite. Positive for the hydrolysis of indoxyl acetate and negative for urease. Growth does not occur in the presence 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol) or glycine (1% wt/vol). Some species may grow in the presence of safranin (0.05% wt/vol) or oxgall (1% wt/vol). Fluorescent pigments are not produced. Some species are sensitive to cefoperazone (64 mg/l). Range of DNA G+C content is 26.4–29.4 mol%.

The type species is *Aliiarcobacter cryaerophilus.*

Description of *Aliiarcobacter cryaerophilus* comb. nov.

Basonym: *Campylobacter cryaerophila* Neill et al., 1985.

Other synonym: *Arcobacter cryaerophilus* Vandamme et al., 1991.

The description is the same given by Neill et al. (1985). The type strain is A169/B^T (= NCTC 1185^T = ATCC 43158^T).

Description of *Aliiarcobacter butzleri* comb. nov.

Basonym: *Campylobacter butzleri* Kiehlbauch et al., 1991.

Other synonym: *Arcobacter butzleri* Vandamme et al., 1992.

The description is the same given by Vandamme et al. (1992). The type strain is LMG 10828^T (= CDC D2686^T = ATCC 49616^T).

Description of *Aliiarcobacter skirrowii* comb. nov.

Basonym: *Arcobacter skirrowii* Vandamme et al., 1992.

The description is the same given by Vandamme et al. (1992). The type strain is Skirrow 449/80^T (= LMG 6621^T = CCUG 10374^T).

Description of *Aliiarcobacter cibarius* comb. nov.

Basonym: *Arcobacter cibarius* Houf et al., 2005.

The description is the same given by Houf et al. (2005). The type strain is LMG 21996^T (= CCUG 48482^T).

Description of *Aliiarcobacter thereius* comb. nov.

Basonym: *Arcobacter thereius* Houf et al., 2009.

The description is the same given by Houf et al. (2009). The type strain is LMG 24486^T (= CCUG 56902^T).

Description of *Aliiarcobacter trophiarum* comb. nov.

Basonym: *Arcobacter trophiarum* De Smet et al., 2011.

The description is the same given by De Smet et al. (2011). The type strain is 64^T (= LMG 25534^T = CCUG 59229^T).

Description of *Aliiarcobacter lanthieri* comb. nov.

Basonym: *Arcobacter lanthieri* Whiteduck-Léveillée et al., 2015.

The description is the same given by Whiteduck-Léveillée et al. (2015). The type strain is AF1440^T (= LMG 28516^T = CCUG 66485^T).

Description of *Aliiarcobacter faecis* comb. nov.

Basonym: *Arcobacter faecis* Whiteduck-Léveillée et al., 2016.

The description is the same given by Whiteduck-Léveillée et al. (2016). The type strain is AF1078^T (= LMG 28519^T = CCUG 66484^T).

Description of *Pseudoarcobacter* gen. nov.

Pseudoarcobacter (Pseu.do.ar.co.bac'ter, Gr. adj. *pseudes*, false; N.L. masc. n. *Arcobacter* a bacterial generic name; N.L. masc. n. *Pseudoarcobacter*, false *Arcobacter*).

Gram-negative, cells are rod shaped and motile. Cell size 0.2–0.9 μm in diameter and 0.4–2.2 μm long. Some species may present cells up to 10 μm in length. Oxidase and catalase positive. No growth occurs at 4% NaCl. Growth occurs at 15–37°C, but not at 42°C. Carbohydrates are not fermented. Reduce nitrate to nitrite. Positive for the hydrolysis of indoxyl acetate. Some species may hydrolyze urea. Growth does not occur in the presence 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol) or glycine (1% wt/vol). Some species may grow in the presence of safranin (0.05% wt/vol) or oxgall (1% wt/vol). Sensitive to cefoperazone (64 mg/l). Range of DNA G+C content is 26.3–28.0 mol%.

The type species is *Pseudoarcobacter defluvii.*

Description of *Pseudoarcobacter defluvii* comb. nov.

Basonym: *Arcobacter defluvii* Collado et al., 2011.

The description is the same given by Collado et al. (2011). The type strain is SW28-11^T (= CECT 7697^T = LMG 25694^T).

Description of *Pseudoarcobacter ellisii* comb. nov.

Basonym: *Arcobacter ellisii* Figueras et al., 2011b.

The description is the same given by Figueras et al. (2011b). The type strain is F79-6^T (= CECT 7837^T = LMG 26155^T).

Description of *Pseudoarcobacter venerupis* comb. nov.

Basonym: *Arcobacter venerupis* Levican et al., 2012.

The description is the same given by Levican et al. (2012). The type strain is F67-11^T (= CECT 7836^T = LMG 26156^T).

Description of *Pseudoarcobacter cloacae* comb. nov.

Basonym: *Arcobacter cloacae* Levican et al., 2013.

The description is the same given by Levican et al. (2013). The type strain is SW28-13^T (= CECT 7834^T = LMG 26153^T)

Description of *Pseudoarcobacter suis* comb. nov.

Basonym: *Arcobacter suis* Levican et al., 2013.

The description is the same given by Levican et al. (2013). The type strain is F41^T (= CECT 7833^T = LMG 26152^T).

Description of *Pseudoarcobacter aquimarinus* comb. nov.

Basonym: *Arcobacter aquimarinus* Levican et al., 2015.

The description is the same given by Levican et al. (2015). The type strain is W63^T (= CECT 8442^T = LMG 27923^T).

Description of *Pseudoarcobacter acticola* comb. nov.

Basonym: *Arcobacter acticola* Park et al., 2016.

The description is the same given by Park et al. (2016). The type strain is AR-13^T (= KCTC 52212^T = NBRC 112272^T).

Description of *Malacobacter* gen. nov.

Malacobacter (Ma.la.co.bac'ter; Gr. n. *malaco*, soft, with soft boy, mollusc; Gr. n. *bacter*, rod; N.L. masc. n. *Malacobacter*, bacteria isolated from molluscs).

Gram-negative, cells are rod shaped and motile. Cell size 0.1–0.6 μm wide and 0.5–3.6 μm long. Oxidase positive and catalase variable among species. Halophilic, no growth can be obtained without NaCl and capable to grow up to 4% NaCl. Growth occurs at 15°C–37°C. Does not grow at 37°C in microaerophilic conditions nor at 42°C in anaerobiosis. Carbohydrates are not fermented. Does not reduce nitrate to nitrite. Negative for the hydrolysis of urea. Some species may hydrolyze indoxyl acetate. Growth does not occur in the presence of oxgall (1% wt/vol) or 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol). Some species may grow in the presence of glycine (1% wt/vol) or safranin (0.05% wt/vol). Sensitive to cefoperazone (64 mg/l) variable among species. Range of DNA G+C content is 26.1–27.3 mol%.

The type species is *Malacobacter halophilus.*

Description of *Malacobacter halophilus* comb. nov.

Basonym: *Arcobacter halophilus* Donachie et al., 2005.

The description is the same given by Donachie et al. (2005). The type strain is LA31B^T (= ATCC BAA-1022^T = CIP 108450^T).

Description of *Malacobacter mytili* comb. nov.

Basonym: *Arcobacter mytili* Collado et al., 2009a.

The description is the same given by Collado et al. (2009a). The type strain is F2075^T (= CECT 7386^T = LMG 24559^T).

Description of *Malacobacter marinus* comb. nov.

Basonym: *Arcobacter marinus* Kim et al., 2010.

The description is the same given by Kim et al. (2010), with the exception of variable result among strains for the hydrolysis of the indoxyl-acetate under microaerobic conditions (Salas-Massó et al., 2016). The type strain is CL-S1^T (= KCCM 90072^T = JCM 15502^T).

Description of *Malacobacter canalis* comb. nov.

Basonym: *Arcobacter canalis* Pérez-Cataluña et al., 2018b.

The description is the same given by Pérez-Cataluña et al. (2018b). The type strain is F138-33^T (= CECT 8984^T = LMG 29148^T).

Description of *Malacobacter molluscorum* comb. nov.

Basonym: *Arcobacter molluscorum* Figueras et al., 2011a.

The description is the same given by Figueras et al. (2011a). The type strain is F98-3^T (= CECT 7696^T = LMG 25693^T).

Description of *Malacobacter pacificus* comb. nov.

Basonym: *Arcobacter pacificus* Zhang et al., 2015.

The description is the same given by Zhang et al. (2015). The type strain is SW028^T (= DSM 25018T = JCM 17857^T = LMG 26638^T).

Description of *Haloarcobacter* gen. nov.

Haloarcobacter (Ha.lo.ar.co.bac'ter, Gr. n. *halo*, salt; N.L. masc. n. *Arcobacter*, a bacterial generic name; N.L. masc. n. *Haloarcobacter, Arcobacter* salt loving).

Gram-negative, cells are rod shaped and motile. Cell size 0.1–0.5 μm in diameter and 0.9–2.5 μm in length. Oxidase positive and catalase variable among species. Halophilic, growth can be obtained within the range of 0.5% (variable among species) and up to 4% NaCl. Growth occurs at 15–42°C. Growth at 37°C in microaerophilic conditions or at 42°C in anaerobiosis variable among species. Carbohydrates are not fermented. Some species may reduce nitrate to nitrite. Negative for the hydrolysis of urea (with the exception of *H. ebronensis*). Some species may hydrolyze indoxyl acetate. Growth does not occur in the presence of oxgall (1% wt/vol) (with the exception of *H. molluscorum*) or 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol). No growth on CCDA. Some species may grow in the presence of glycine (1% wt/vol) or safranin (0.05% wt/vol). Sensitive to cefoperazone (64 mg/l). Range of DNA G+C content is 27.3–29.9 mol%.

The type species is *Haloarcobacter bivalviorum.*

Description of *Haloarcobacter bivalviorum* comb. nov.

Basonym: *Arcobacter bivalviorum* Levican et al., 2012.

The description is the same given by Levican et al. (2012). The type strain is F4^T (= CECT 7835^T = LMG 26154^T).

Description of *Haloarcobacter anaerophilus* comb. nov.

Basonym: *Arcobacter anaerophilus* Sasi-Jyothsna et al., 2013.

The description is the same given by Sasi-Jyothsna et al. (2013). The type strain is JC84^T (= KCTC 15071^T = MTCC 10956^T = DSM 24636^T).

Description of *Haloarcobacter ebronensis* comb. nov.

Basonym: *Arcobacter ebronensis* Levican et al., 2015.

The description is the same given by Levican et al. (2015). The type strain is F128-2^T (= CECT 8441^T = LMG 27922^T).

Description of *Poseidonibacter* gen. nov.

Poseidonibacter (Po.se.i.do.ni.bac'ter, Gr. n. *Poseidon*, God of the sea; Gr. n. *bacter*, rod; N.L. masc. n. *Poseidonibacter* referring to the marine habitat of this bacteria).

Gram-negative, cells are rod shaped and motile. Oxidase and catalase positive. Halophilic, no growth can be obtained without seawater or the addition of combined marine salts to the medium. Growth occurs at 15°C–25°C, but not at 37°C or 42°C. Range of pH for growth is 6–8. Carbohydrates are not fermented. Reduce nitrate to nitrite. Negative for the hydrolysis of indoxyl acetate and urea. Growth occurs in the presence of safranin (0.05% wt/vol), and 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol), but not in the presence of glycine (1% wt/vol) sensitive to cefoperazone (30 μg). Possess ubiquinone MK-6 as a respiratory quinone. DNA G+C content is 28.7 mol%.

The type species is *Poseidonibacter lekithochrous*.

Description of *Poseidonibacter lekithochrous* comb. nov.

Basonym: *Arcobacter lekithochrous* Diéguez et al., 2017.

The description is the same given by Diéguez et al. (2017). The type strain is LFT1.7^T (= CECT 8942^T = DSM 100870^T).

AUTHOR CONTRIBUTIONS

MF and JR designed the work. AP-C, NS-M, and AD performed the phenotypic and phylogenetic experiments. AP-C and SB carried out the genome sequencing and analysis. AP-C, AL, and JR performed the bioinformatic work. JR, MF, AP-C, and AD wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02077/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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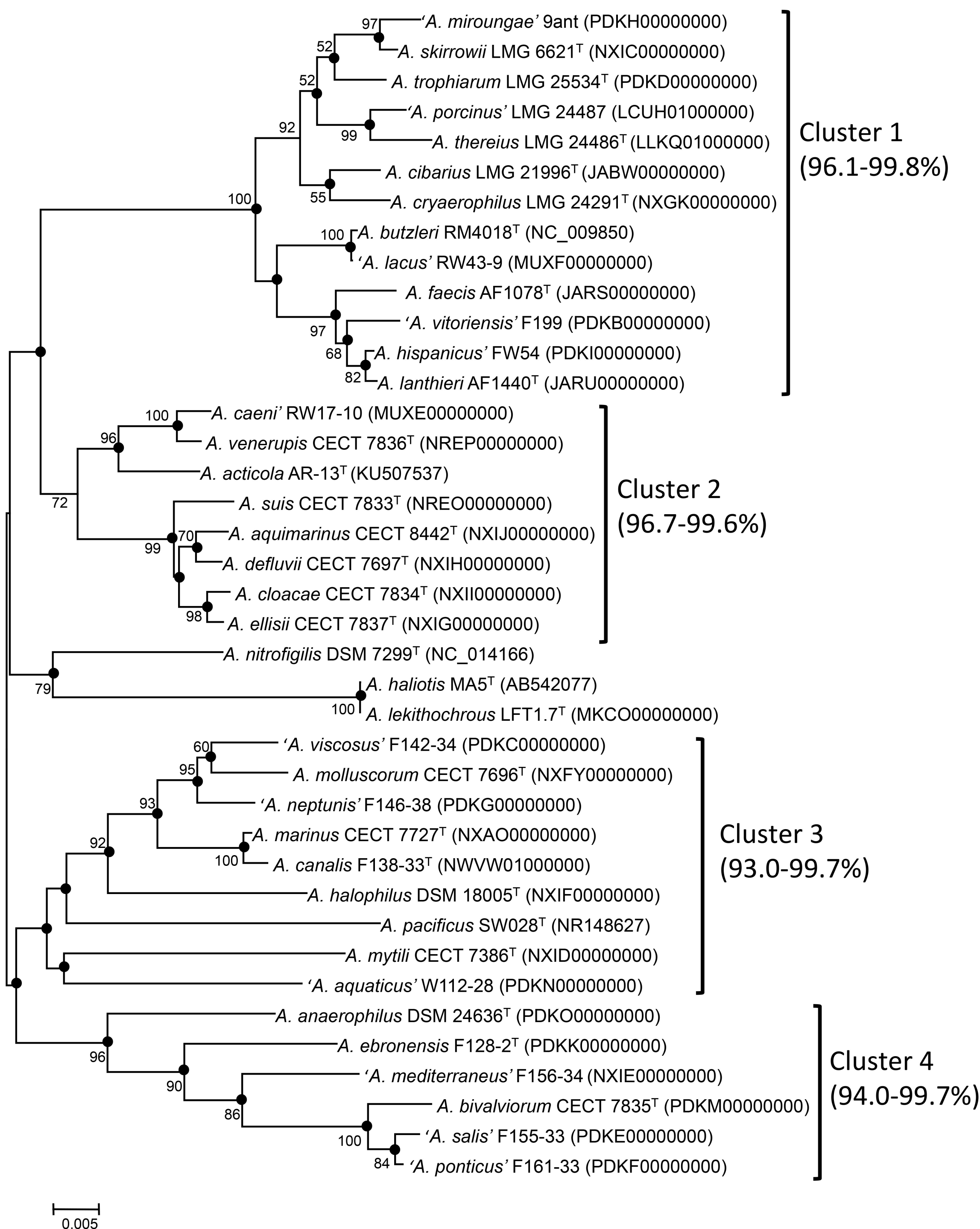
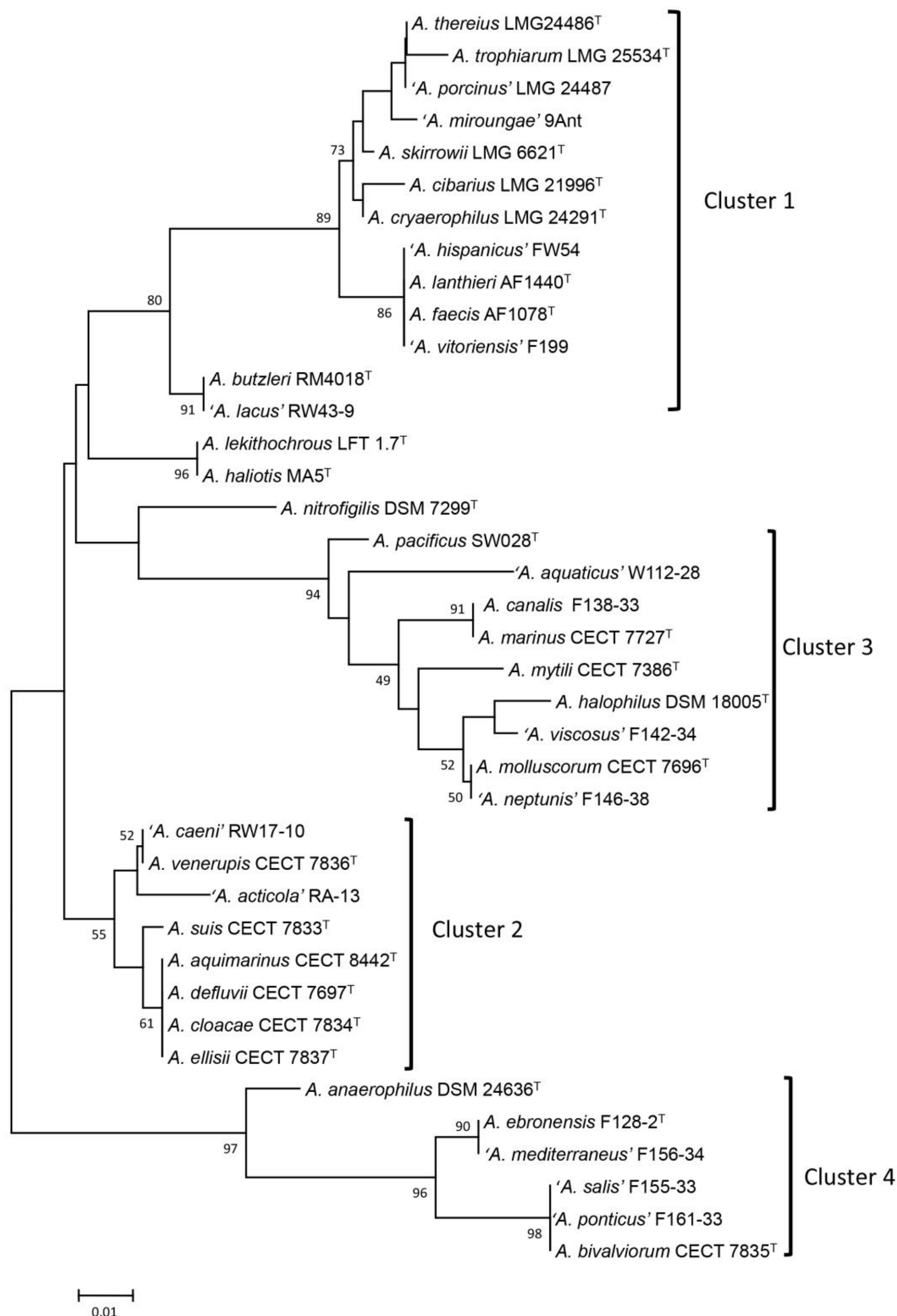
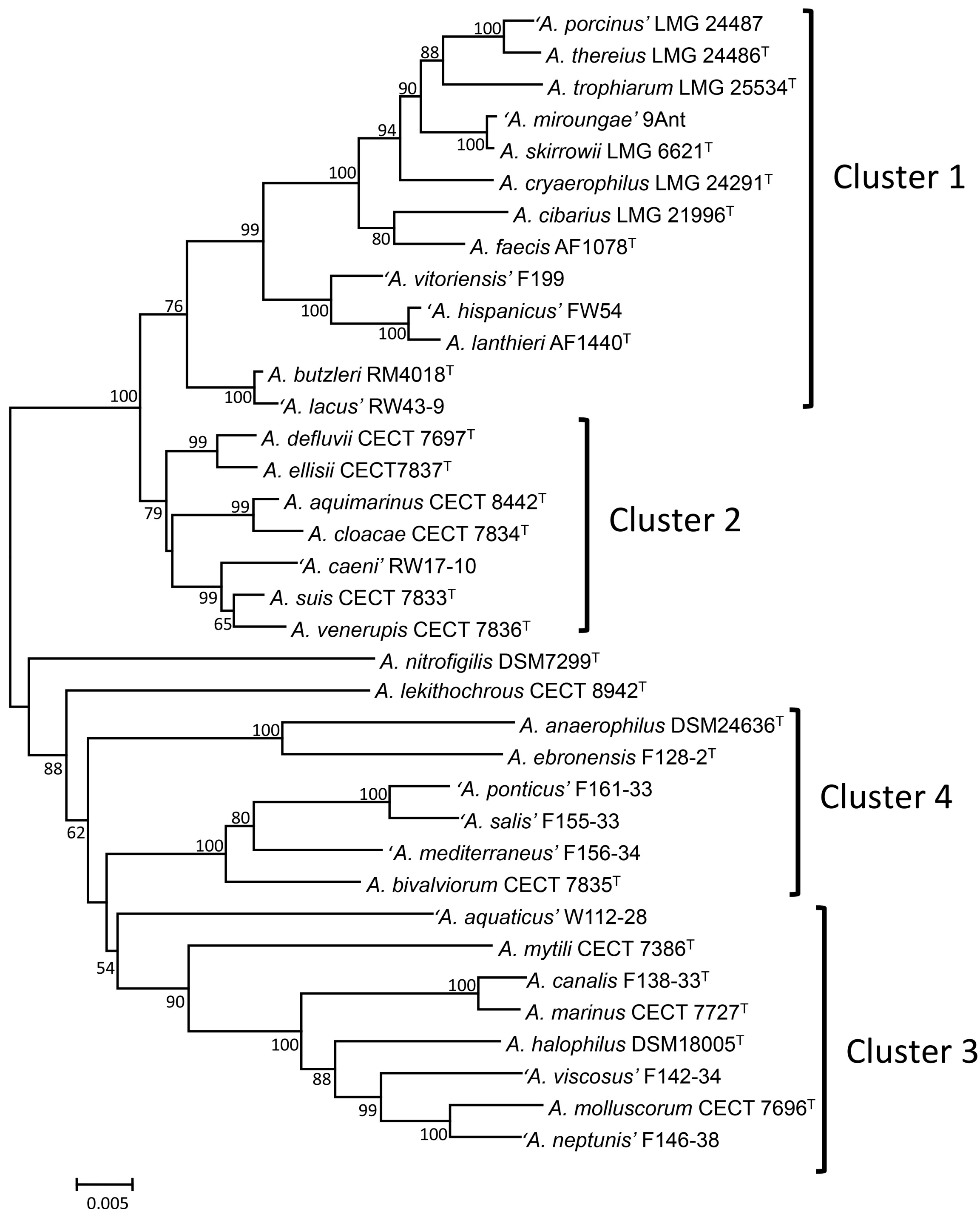
Supplementary Figure S1A.- Phylogenetic tree constructed with the near complete sequences (1450 nt) of the 16S rRNA gene of 36 type and representative strains of *Arcobacter* species by the Maximum-Likelihood algorithm (model GTR+G+I). Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the nodes. Scale bars indicate the number of substitutions per nucleotide position. Bold circles indicate that corresponding nodes were coincident in the tree generated with Neigbour-Joining algorithm. Brackets indicate the similarity range for 16S rRNA gene sequences. The cluster names in the tree are based in the phylogenetic results obtained from MLSA and core genome analyses. During the preparation of this article *A. haliotis* was confirmed as a later heterotypic synonym of *A. lekithochrous*.



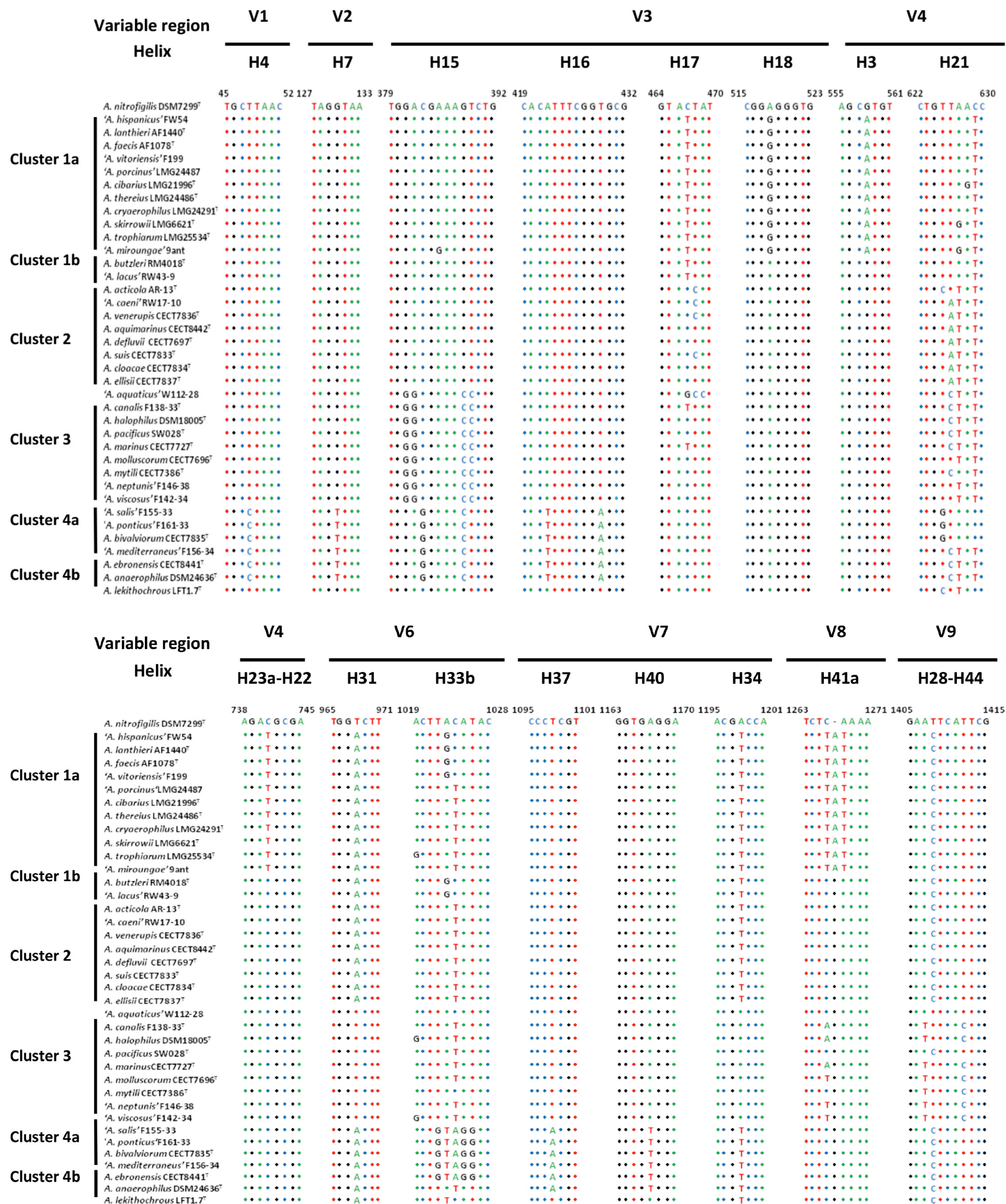
Supplementary Figure S1B.- Tree constructed with the concatenated signature motifs of the 16S rRNA gene for the different clusters. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the nodes. Scale bars indicate the number of substitutions per nucleotide position. The cluster names in the tree are based in the phylogenetic results obtained from MLSA and core genome analyses. During the preparation of this article *A. haliotis* was confirmed as a later heterotypic synonym of *A. lekithochrous*.



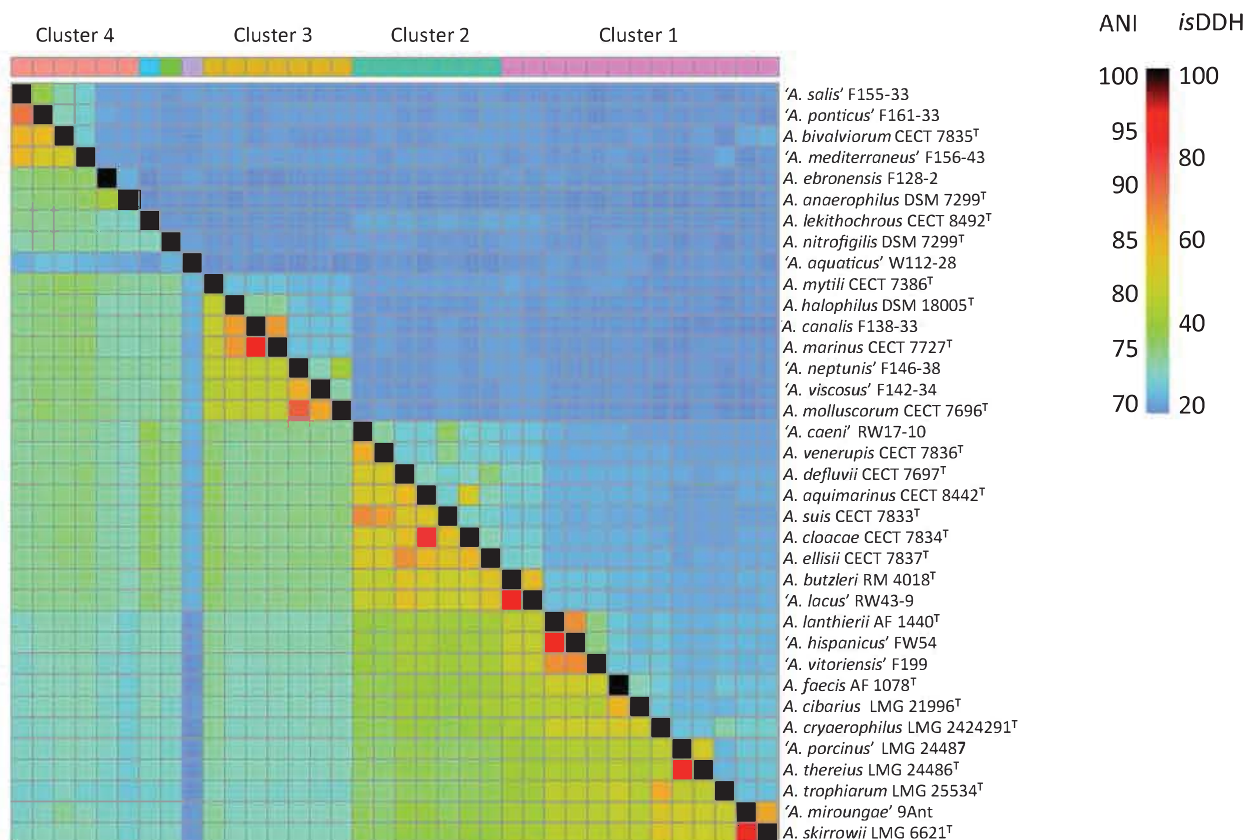
Supplementary Figure S2.- Neighbour joining phylogenetic tree constructed with the 23S rRNA gene sequences (2948 bp) of the type and representative strains of 36 species of *Arcobacter*. Numbers at the nodes indicated bootstrap values >50% obtained by repeating the analysis 1000 times. Scale bar indicates the number of substitutions per nucleotide position. The cluster names in the tree are based in the phylogenetic results obtained from MLSA and core genome analyses.



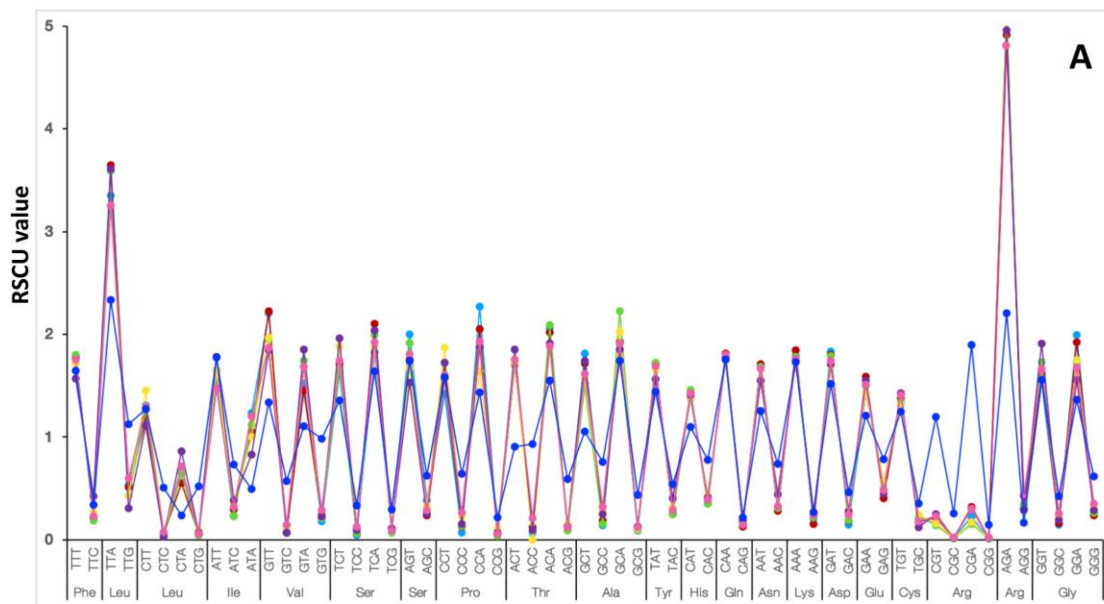
Supplementary Figure S3.- Group specific 16S rRNA gene signatures differentiating the type species of the genus, *Arcobacter nitrofigilis* DSM 7299T and other species of the genus. The numbers at the top give the position in the gene. The location of the signature motifs in the different variable regions and helix of the 16S rRNA gene secondary structure are also indicated. *Escherichia coli* sequence was used as reference to enumerate the nucleotide postitions (Adilakshmi et al., 2008).



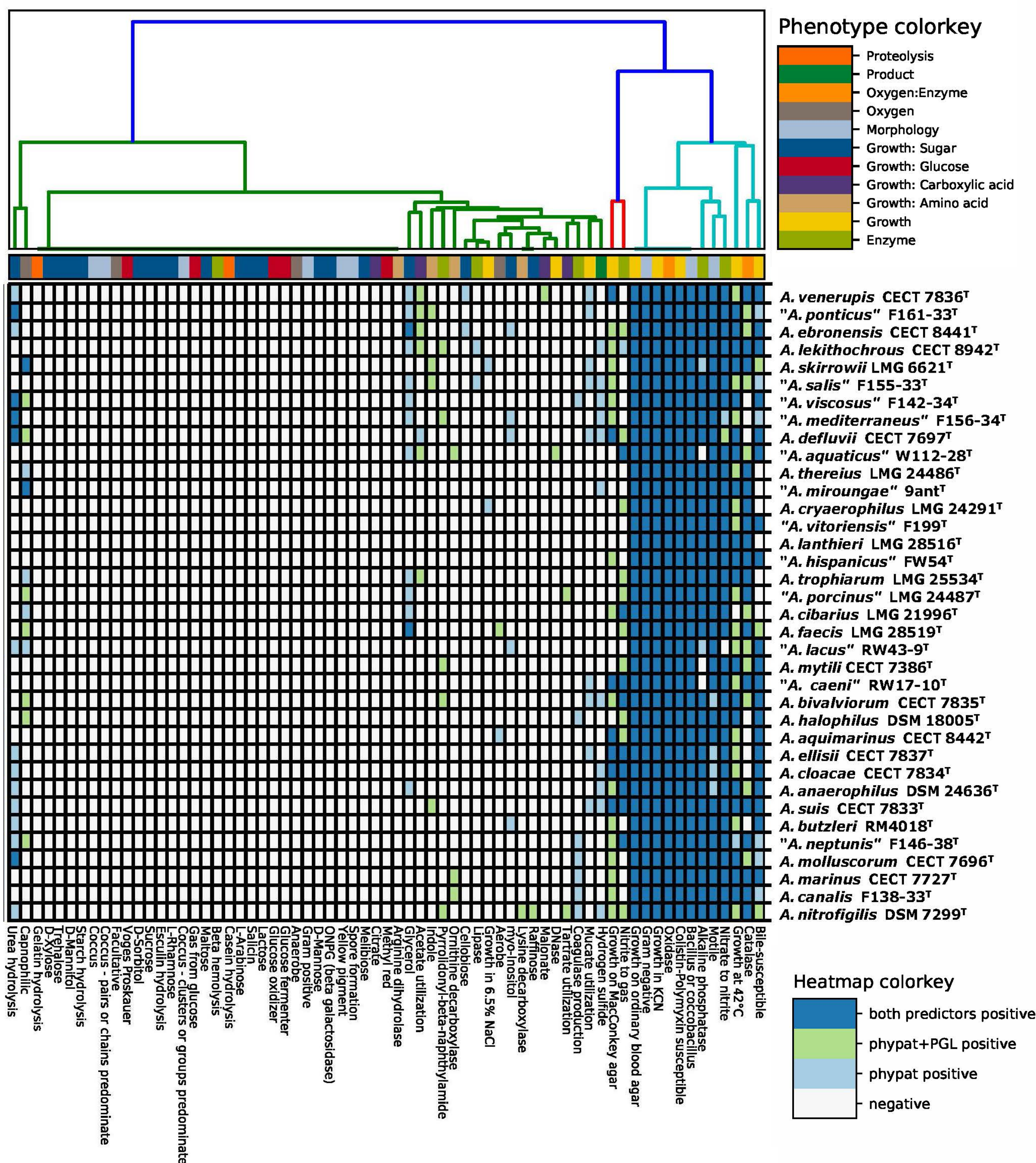
Supplementary Figure S4.- Heatmap representing the similarities (%) among the *Arcobacter* species obtained for ANI (left-down) and isDDH (up-right) indexes.



Supplementary Figure S5.- Comparison of the RSCU data of the 59 synonymous codon among the different clusters and orphan species of *Arcobacter*. Lines: blue, cluster 1; red, cluster 2; green, cluster 3; yellow, cluster 4; purple, *A. lekithochrous*; pink, *A. nitrofigilis*; dark blue, *A. aquaticus*.



Supplementary Figure S6.- Heatmap obtained with the phenotypic predictor tool Traitar for the type and representative strains of *Arcobacter* species. The origin of the phenotypes prediction (Traitar phypat and/or phypat+PGL classifier) determines the color of the heatmap entries.



Supplementary figure S7.- Phylogenetic network of the 57 analyzed genomes based on the concatenated sequences of core genes. Scale bar, base substitutions per site.

Table S1. Similarities (%) in the 16S rRNA gene (down-left) and 23S rRNA gene (up-right) among type and representative strains of the different *Arcobacter* species.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	*A. salis* ' F155-33T	*	99.0	97.3	97.2	94.8	93.3	94.6	94.3	94.3	94.6	95.4	94.0	93.9	93.8	94.3	94.1	94.2	94.3	94.0	93.9	94.1	94.0	94.2	93.5	93.5	93.2	93.0	92.8	93.0	93.0	92.6	93.0	93,0	92.7	93.0	93.1
2	*A. ponticus* ' F161-33T	99.5	*	97.2	97.4	95.1	93.5	94.4	94.1	94.5	94.6	95.6	94.2	94.3	93.9	94.4	94.2	94.1	94.2	94.1	93.9	94.1	93.8	94.3	93.6	93.6	93.1	92.9	92.8	93.1	93.0	92.5	92.9	93.0	92.6	93.0	93.1
3	*A. bivalviorum* CECT 7835^T	98.9	98.6	*	97.3	95.7	94.1	95.1	94.1	95.0	94.5	95.2	93.9	93.9	94.4	94.4	93.9	95.2	94.8	95.2	94.9	94.9	94.8	95.1	94.4	94.4	93.8	93.7	93.6	93.4	93.7	93.7	93.8	93.9	93.7	93.7	93.8
4	*A. mediterraneus* ' F156-34T	96.8	96.9	96.6	*	95.5	93.6	95.5	94.0	95.1	94.4	95.3	94.3	94.4	94.7	95.0	94.2	94.9	94.7	95.1	94.6	94.8	94.5	94.8	94.3	94.1	93.6	93.7	93.5	93.5	93.4	93.2	93.3	93.4	93.2	93.2	93.3
5	*A. ebronensis* CECT 8441^T	96.4	96.3	96.7	95.8	*	96.9	94.6	93.6	93.7	93.7	94.3	93.6	93.5	94.2	94.2	93.8	94.5	94.6	95.0	94.6	94.7	94.5	94.9	94.8	95.0	94.2	93.8	94.0	94.4	94.5	94.1	93.9	94.0	93.7	94.2	94.3
6	*A. anaerophilus* DSM 24636^T	94.0	94.1	94.6	94.8	96.6	*	93.5	93.3	93.1	92.4	93.5	92.8	92.5	93.5	93.1	93.3	93.1	93.1	93.1	92.8	93.2	92.9	93.0	92.9	92.9	92.1	92.3	92.3	92.0	92.1	92.5	92.5	92.7	92.3	92.3	92.4
7	*A. lekithochrous* CECT 8942^T	93.0	93.4	92.9	93.5	92.8	93.8	*	94.2	94.6	93.2	94.5	94.3	94.2	93.6	93.9	93.6	95.0	95.2	94.9	94.8	95.2	94.6	94.9	94.5	94.4	93.7	94.1	93.8	93.1	93.2	93.7	93.9	93.9	93.6	93.8	93.8
8	*A. nitrofigilis* DSM 7299^T	93.3	93.4	93.4	93.8	93.8	94.6	95.1	*	95.1	94.4	94.8	95.0	94.9	95.2	95.2	94.9	95.1	94.3	94.8	94.6	94.7	94.8	94.6	94.4	94.3	94.2	94.5	93.9	93.8	93.7	94.0	93.9	94.0	93.8	94.1	94.1
9	*A. aquaticus* ' W112-28T	92.4	92.7	93.0	93,0	93.8	95.1	94.0	94.9	*	94.9	95.1	94.3	94.3	94.9	94.7	94.8	94.1	94.1	94.5	93.7	94.4	93.7	94.6	94.0	93.9	93.3	93.3	93.1	93.0	93.2	93.0	93.2	93.4	93.1	92.9	93,0
10	*A. mytili* CECT 7386^T	92.8	92.8	92.8	92.6	93.6	93.5	93.0	94,0	94.7	*	95.2	94.6	94.9	94.6	94.2	95.0	93.8	94.0	93.9	93.6	94.2	93.5	94.4	93.9	93.9	92.9	92.9	93.3	92.7	92.5	92.6	92.3	92.2	92.2	92.9	92.9
11	*A. halophilus* DSM 18005^T	93.3	93.3	93.6	93.4	93.8	94.6	92.9	94.5	94.2	94.7	*	96.8	96.8	96.9	97.0	97.3	93.5	94.0	93.7	93.4	93.9	93.1	94.0	93.5	93.5	92.4	92.4	92.6	92.4	92.4	92.5	92.7	92.8	92.6	92.7	92.8
12	*A. canalis* ' F138-33T	92.8	92.9	93.0	93.1	94.0	95.6	93.0	94.6	95.4	94.4	96.4	*	99.2	95.9	96.2	96.3	93.1	93.7	93.6	93.5	93.7	93.1	93.8	93.6	93.5	92.4	92.6	92.9	92.1	91.7	92.2	92.6	92.7	92.8	92.4	92.4
13	*A. marinus* CECT 7727^T	93.3	93.1	93.0	93.7	94.0	95.6	92.7	94.6	95.4	94.2	96,0	99.1	*	95.8	96.0	96.2	93.4	93.7	93.7	93.5	94.0	93.0	93.9	93.6	93.5	92.4	92.7	92.9	92.2	91.8	92.4	92.5	92.7	92.8	92.5	92.6
14	*A. neptunis* ' F146-38T	93.0	93.0	93.2	93.2	94.2	95.6	93.2	95,0	95.8	95.0	96.3	97.7	97.5	*	97.8	98.6	93.5	93.6	94.1	93.5	93.7	93.2	93.7	93.4	93.4	92.5	92.6	92.6	92.4	92.1	92.4	92.5	92.5	92.7	92.6	92.7
15	*A. viscosus* ' F142-34T	92.4	92.6	92.8	93.2	93.6	95.2	93.0	94.8	95.8	94.4	96.0	97.1	97,0	98.6	*	97.4	93.5	93.5	93.9	93.7	93.6	93.4	93.6	93.5	93.5	92.3	92.3	92.6	92.3	92.3	92.4	92.6	92.7	92.8	92.6	92.7
16	*A. molluscorum* CECT 7696^T	92.8	92.8	93.1	93,0	93.7	95.2	93.1	94.6	95.8	94.8	95.8	97.5	97.3	98.4	98.5	*	92.8	93.4	93.2	93.1	93.5	92.8	93.4	93.0	93.1	92.4	92.4	92.4	92.4	92.1	92.3	92.5	92.5	92.8	92.6	92.7
17	*A. caeni* ' RW17-10T	92.8	93.0	93.2	93.8	94.3	95.5	94.6	95.9	95.8	93.4	94,0	95.1	95.0	95.6	95.4	95.4	*	98.7	98.2	97.9	99.1	98.3	97.9	97.1	97.0	96.0	96.3	96.3	95.7	95.6	96.1	95.2	95.2	95.2	95.4	95.5
18	*A. venerupis* CECT 7836^T	93.0	93.2	93.3	93.9	94.2	95.4	94.4	96.0	95.8	93.5	93.9	94.9	94.8	95.6	95.5	95.5	99.3	*	98.4	98.3	99.3	98.0	98.6	97.7	97.5	96.0	96.3	96.7	95.8	95.4	96.0	95.2	95.2	95.2	95.4	95.4
19	*A. defluvii* CECT 7697^T	94.7	95.1	95.0	94.6	96.0	95.6	94.2	95.8	94.8	93.8	94.6	94.9	94.7	95.2	94.9	95.0	97.0	97.0	*	98.5	98.5	98.1	99.4	98.3	98.1	96.4	96.7	97.0	95.9	95.6	95.7	95.3	95.4	95.4	95.5	95.5
20	*A. aquimarinus* CECT 8442^T	95.0	95.3	95.3	94.9	95.9	95.6	94.4	95.7	94.6	94.0	94.4	94.6	94.4	95.2	94.7	94.8	96.8	96.7	99.4	*	98.3	99.4	98.3	98.3	98.1	96.4	96.7	97.3	96.2	95.7	95.9	95.6	95.6	95.5	95.7	95.7
21	*A. suis* CECT 7833^T	94.4	94.6	94.5	94.6	95.7	95.2	94.0	95.3	95.0	93.6	94.4	94.7	94.5	95.1	94.7	94.8	97.7	97.7	99.0	98.7	*	98.1	98.6	97.8	97.7	96.3	96.6	97.0	96.0	95.7	96.2	95.4	95.5	95.4	95.5	95.6
22	*A. cloacae* CECT 7834^T	94.4	94.8	94.6	94.4	95.8	95.2	94.3	95.6	94.5	94.2	94.4	94.8	94.6	95.0	94.6	94.7	96.8	97.0	99.2	98.9	98.9	*	97.9	97.8	97.6	96.4	96.7	96.8	96.1	95.9	95.9	95.6	95.6	95.5	95.7	95.7
23	*A. ellisii* CECT 7837^T	94.6	95.0	94.7	94.6	95.8	95.3	94.4	95.8	94.6	94.1	94.6	94.4	94.2	95.2	94.8	94.8	96.8	97.0	99.2	98.9	98.9	99.6	*	98.6	98.5	96.7	97.0	97.3	96.2	95.8	96.0	95.6	95.7	95.7	95.9	96.0
24	*A. butzleri* RM4018^T	91.6	91.9	91.6	92.6	93.0	93.8	93.6	94.4	93.0	94.0	93.2	93.1	93.0	93.4	93.0	92.8	94.8	94.9	95.0	95.2	94.6	95.1	95.4	*	99.7	97.1	97.4	98.1	97.0	96.3	96.4	96.4	96.3	96.1	96.5	96.5
25	*A. lacus* ' RW43-9T	91.7	92.0	91.6	92.7	93.1	93.9	93.6	94.4	93.0	94.0	93.3	93.2	93.0	93.5	93.0	92.9	94.9	95.0	95.1	95.2	94.6	95.1	95.4	99.9	*	97.3	97.4	98.0	97.2	96.5	96.3	96.3	96.2	96.0	96.6	96.6
26	*A. lanthierii* LMG 28516^T	91.5	91.8	91.8	92.4	93.0	93.7	92.9	94.1	92.4	94.0	93.2	93.1	93.0	93.1	92.8	92.6	94.4	94.2	94.9	94.8	94.3	94.6	94.5	97.9	98,0	*	99.6	98.7	97.7	96.9	96.5	96.5	96.5	96.4	97.1	97.1
27	*A. hispanicus* ' FW54T	91.6	91.9	92.0	92.4	93.0	93.6	92.9	94.1	92.4	93.9	93.1	93.0	92.9	93.0	92.9	92.5	94.6	94.4	94.9	94.8	94.4	94.6	94.5	97.9	97.9	99.8	*	99.1	97.4	96.5	96.8	96.7	96.7	96.7	97.1	97.1
28	*A. vitoriensis* ' FW59T	91.6	91.9	91.4	92.4	92.8	93.9	93.1	94.3	92.4	93.8	93.1	93.2	93.1	93.0	92.8	92.5	94.2	94.0	94.7	94.6	94.1	94.3	94.3	97.7	97.8	99.3	99.1	*	97.4	96.5	96.6	96.5	96.5	96.4	96.9	96.9
29	*A. faecis* LMG 28519^T	91.1	91.4	91.6	91.9	92.6	93.5	92.7	94.0	92.2	93.7	92.9	93.0	92.9	92.9	92.7	92.4	94.5	94.2	94.6	94.6	94.2	94.3	94.3	97.9	98,0	99.1	99.1	98.6	*	98.5	97.4	97.5	97.2	97.6	98.0	98.0
30	*A. cibarius* LMG 21996^T	91.5	91.9	91.5	92.2	92.5	93.2	92.5	93.9	92.4	93.4	92.5	92.4	92.2	93.0	92.7	92.6	94.7	94.8	94.8	95.0	94.6	95.0	95.2	97.7	97.7	98.1	98.2	97.6	98.1	*	98.0	97.6	97.8	97.3	98,0	97.9
31	*A. cryaerophilus* LMG 24291^T	91.2	91.4	91.2	92.0	92.1	93.0	92.0	93.8	92.6	93.2	92.5	92.3	92.1	92.8	92.6	92.5	95,0	94.8	94.5	94.7	94.5	94.5	94.6	97.3	97.3	97.6	97.7	97.1	97.8	98.8	*	98.0	98.2	98.3	98.5	98.5
32	*A. porcinus* ' LMG 24487T	91.7	92.1	91.8	92.3	92.6	93.1	92.3	93.8	92.7	93.1	92.5	92.8	92.7	92.9	92.6	92.7	94.7	94.4	94.7	94.6	94.4	94.7	94.6	96.5	96.6	97.5	97.5	96.9	97.1	97.8	98.1	*	99.5	98.5	98.6	98.5
33	*A. thereius* LMG 24486^T	91.7	91.8	91.7	92.2	92.6	92.6	92.1	93.4	92.5	93.2	92.2	92.8	92.5	92.7	92.5	92.5	94.3	94,0	94.4	94.4	94.2	94.5	94.4	96.1	96.1	97.3	97.5	96.8	96.9	97.5	97.6	99.0	*	98.2	98.3	98.3
34	*A. trophiarum* LMG 25534^T	91.6	91.9	91.7	92.2	92.8	93.1	92.1	94.0	93.0	93.2	92.9	92.7	92.6	93.1	93.1	93,0	94.9	94.7	94.9	95.0	94.4	94.8	94.8	96.9	97.0	97.5	97.7	97.2	97.3	97.9	98.5	98.4	98.2	*	98.5	98.5
35	*A. miroungae* ' 9AntT	91.0	91.4	91.4	91.6	92.4	92.6	91.8	93.8	92.7	93.1	92.4	92.6	92.4	92.8	92.6	92.6	94.4	94.2	94.5	94.7	94.2	94.6	94.5	96.6	96.6	97.5	97.7	96.8	97.3	98.0	98.3	97.9	98.1	98.7	*	99.9
36	*A. skirrowii* LMG 6621^T	91.2	91.4	91.5	91.8	92.6	93.2	92.0	94.1	92.8	93.1	92.6	92.7	92.6	93.1	92.8	92.8	94.6	94.5	94.6	94.8	94.2	94.8	94.6	96.9	96.9	97.9	97.9	97.2	97.7	98.2	98.5	98.1	97.7	98.7	99.4	*

Table S2. Values (%) of ANI (down-left) and *is* DDH (up-right) among type and representative strains of the different *Arcobacter* species

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	***A. salis*' F155-33T**	*	37.4	29.4	25.3	19.7	19.5	19.5	19.0	19.6	19.5	19.7	19.1	19.2	19.3	19.3	19.4	19.4	19.3	19.1	19.1	19.5	19.2	19.6	19.4	19.3	19,0	18.9	18.5	19.1	18.8	18.3	18.8	18.8	18.6	18.8	18.7
2	***A. ponticus*' F161-33T**	89.0	*	28.2	25.6	20.3	19.6	19.7	19.4	19.3	19.8	19.8	19.4	19.6	19.6	19.9	20.0	19.7	19.5	19.2	19.2	19.7	19.3	19.5	19.6	19.6	19.1	18.9	18.5	19.0	19.2	18.8	19.0	19.0	18.7	18.9	18.6
3	***A. bivalviorum* CECT 7835T**	84.9	84.2	*	24.8	20.0	19.7	19.8	19.2	18.8	20.1	20.0	19.4	19.7	19.4	19.2	19.4	19.4	19.7	19.4	19.4	19.9	20,0	19.6	19.7	19.7	19.1	19.1	19.0	19.2	19.8	19.4	19.2	19.5	18.6	20.5	21.6
4	***A. mediterraneus*' F156-34T**	84.8	82.2	81.8	*	19.9	20.0	19.4	19.3	18.7	19.5	19.9	19.7	20.1	19.5	19.7	19.5	19.5	19.4	19.6	19.5	19.8	19.4	19.5	19.4	19.6	19.2	18.8	18.8	19.6	19.3	18.7	18.5	19.0	20.7	18.6	18.9
5	***A. ebronensis* CECT 8441T**	75.6	76.2	76.5	76.1	*	21.3	18.3	18.8	19.8	19.2	18.9	18.4	18.5	19.2	18.9	19.2	18.9	18.9	19.0	18.9	19.1	18.9	18.9	19.5	19.2	19.6	18.8	18.7	19.0	18.7	18.7	18.7	18.8	18.3	18.8	18.8
6	***A. anaerophilus* DSM 24636T**	75.2	75.7	75.7	76.4	78.6	*	18.4	19.0	18.7	18.9	18.6	18.8	18.7	19.1	18.7	18.8	18.8	19.1	18.9	18.5	18.8	18.4	18.5	19.3	19.3	18.8	18.4	18.6	18.8	18.6	18.5	18.5	18.9	18.5	18.2	19.0
7	***A. lekithochrous* CECT 8942T**	75.2	74.9	75.2	74.8	73.5	73.2	*	19.0	19.3	19.1	19.0	18.8	18.9	19.0	18.9	18.8	20.6	20.5	20.3	20.1	20.6	20.2	20.5	20.5	20.3	19.8	19.2	19.3	19.3	19.3	18.9	18.8	19.4	19.2	18.9	18.9
8	***A. nitrofigilis* DSM 7299T**	74.4	74.8	74.6	74.6	74.4	73.7	73.7	*	19.8	19.3	19.2	19.2	19.3	18.9	19.3	19.5	19.5	20.0	19.5	19.3	19.4	19.4	19.4	19.7	19.5	19.2	18.8	19.0	19.5	19.4	18.9	18.6	18.8	18.6	19.2	19.2
9	***A. aquaticus*' W112-28T**	71.3	71.8	71.9	71.6	71.4	71.1	70.4	71.5	*	17.8	18.9	18.6	18.6	18.5	18.7	18.5	18.6	18.8	18.8	18.3	19.4	18.1	18.3	19.3	19.2	19.6	18.7	19.0	20.0	19.6	18.6	18.7	19.3	19.4	18.8	18.4
10	***A. mytili* CECT 7386T**	75.4	75.9	76.3	76.1	75.1	74.6	74.8	74.9	71.6	*	23.0	23.0	22.8	22.7	22.2	22.7	19.6	19.6	19.8	19.8	20.0	20.1	19.7	20.3	20.2	19.8	19.3	19.6	19.5	19.3	19.1	19.1	19.3	19.1	19.3	19.4
11	***A. halophilus* DSM 18005T**	75.2	75.6	75.9	75.8	74.1	74.0	74.2	74.2	71.3	80.7	*	30.4	31.3	22.9	22.7	22.8	19.2	19.3	19.3	19.4	19.2	19.4	19.5	19.6	19.4	19.7	19.3	19.2	19.1	19.1	18.7	18.7	19.2	18.5	18.7	18.8
12	***A. canalis*' F138-33T**	75.1	75.5	75.9	75.7	74.3	74.2	74.3	74.6	71.2	80.5	86.3	*	63.6	22.8	22.8	22.8	19.4	19.7	19.1	19.1	19.5	19.2	19.5	19.5	19.3	19.1	19,0	19.3	18.7	19.1	18.8	18.6	18.5	18.6	18.6	18.6
13	***A. marinus* CECT 7727T**	75.4	75.6	75.7	76.1	74.2	73.9	74.3	74.6	70.9	80.3	86.6	95.4	*	22.8	22.9	22.9	19.3	19.5	19.0	19.4	19.5	19.3	19.5	19.8	19.4	19.2	18.9	19,0	18.7	19.1	18.3	18.6	18.7	19.1	18.7	18.8
14	***A. neptunis*' F146-38T**	75.3	75.8	75.8	75.9	74.9	74.5	74.6	74.7	71.3	80.1	80.6	80.5	80.3	*	29.1	40.4	19.2	19.2	19.4	19.4	19.6	19.4	19.6	19.7	19.6	19.1	19,0	18.9	18.8	19,0	18.7	19.1	19.0	18.4	18.7	18.7
15	***A. viscosus*' F142-34T**	75.2	75.7	75.5	75.9	74.7	74.3	74.4	75.2	71.4	79.4	79.9	80.4	80.3	85.3	*	29.4	19.3	19.2	19.2	19.2	19.5	19.2	19.1	20.1	19.9	19.1	18.7	19,0	19.1	19.3	18.6	18.7	19.0	18.4	18.5	18.9
16	***A. molluscorum* CECT 7696T**	75.1	76.0	75.7	75.8	75.1	74.3	74.6	75.1	71.5	80.1	80.2	80.4	80.4	90.3	85.6	*	19.2	19.5	19.4	19.4	19.7	19.2	19.3	19.8	19.7	19.1	19.2	18.8	19.0	19.3	18.5	18.6	19.1	18.2	18.6	18.7
17	***A. caeni*' RW17-10T**	74.8	75.0	75.2	75.3	74.3	73.6	76.8	74.8	71.4	75.8	74.9	75.2	75.2	75.1	74.9	75.0	*	30.2	24.8	24.9	34.4	25.2	24.9	23.1	23.2	21.1	20.7	20.9	20.5	20.2	20.2	19.9	19.9	19.8	19.8	19.8
18	***A. venerupis* CECT 7836T**	74.5	74.9	75.1	75.0	74.2	73.6	76.2	75.7	71.1	75.8	74.6	75.1	74.9	75.1	75.1	75.2	85.3	*	24.9	24.8	31.8	24.8	25.0	23.3	23.2	20.8	20.9	20.9	20.5	20.4	20.6	19.5	20.0	19.7	19.9	19.9
19	***A. defluvii* CECT 7697T**	74.8	75.2	75.2	75.7	74.6	73.9	76.2	74.9	71.4	76.5	75.0	75.2	75.2	75.2	74.9	75.3	82.2	81.9	*	27.8	25.3	27.4	33.7	26.1	26.6	21.7	21.6	21.9	21.3	21.1	20.9	20.3	20.2	20.4	20.8	20.7
20	***A. aquimarinus* CECT 8442T**	74.8	75.1	75.6	75.5	74.5	73.6	76.1	74.8	71.1	76.3	75.0	75.3	75.2	75.4	75.3	75.5	82.1	81.8	84.3	*	25.7	50.9	28.2	24.8	24.6	21.7	21.5	21.6	21.1	21.2	20.7	20.2	20.1	20.1	20.6	20.7
21	***A. suis* CECT 7833T**	74.7	75.1	75.5	75.5	74.3	74.0	76.3	75.2	71.3	76.1	74.9	75.2	75.1	75.5	75.4	75.8	87.5	86.3	82.6	82.7	*	26.1	25.7	23.4	23.5	21.1	21.0	21.1	20.5	20.4	20.2	19.7	19.8	19.6	20.0	20.0
22	***A. cloacae* CECT 7834T**	74.8	75.1	75.7	75.5	74.2	74.0	76.0	74.8	71.3	76.2	75.1	75.3	75.3	75.3	75.3	75.6	82.2	81.6	83.7	92.5	83.0	*	28.8	24.5	24.6	21.5	21.5	21.5	21.4	21.3	20.9	20.0	20.1	20.0	20.7	20.9
23	***A. ellisii* CECT 7837T**	74.9	75.3	75.6	75.5	74.5	74.1	76.3	74.9	71.2	76.5	75.4	75.5	75.4	75.6	75.4	75.7	82.2	82.1	87.3	84.5	83.0	84.6	*	26.0	26.4	21.8	21.5	21.7	21.2	21.6	21.2	20.2	20.3	20.3	20.7	20.9
24	***A. butzleri* RM4018T**	74.9	75.1	75.1	75.4	74.6	73.8	75.6	74.9	71.4	76.3	74.8	75.0	75.1	75.2	75.3	75.2	80.4	80.0	82.8	81.7	80.8	81.4	82.7	*	55.7	22.8	22.8	23.3	22.4	22.0	21.6	20.7	20.6	20.7	21.3	21.5
25	***A. lacus*' RW43-9T**	75.0	74.9	75.3	75.2	74.6	74.0	75.7	74.7	71.2	75.9	75.2	75.0	75.1	75.6	75.2	75.3	80.5	80.0	83.2	81.5	80.9	81.6	82.9	94.2	*	22.9	22.9	23.2	22.3	21.7	21.2	20.6	20.5	20.6	20.8	21.2
26	***A. lanthierii* LMG 28516T**	73.5	73.9	74.2	74.2	73.6	72.8	74.0	73.8	70.5	74.8	74.0	74.0	74.0	74.1	74.1	74.3	77.7	77.0	78.6	78.2	77.9	78.0	78.5	80.4	80.4	*	65.4	33.2	23.7	22.5	23.4	21.7	21.9	22.6	22.1	22.0
27	***A. hispanicus*' FW54T**	73.6	74.0	74.4	74.3	73.6	73.2	73.9	73.8	70.4	75.2	74.1	74.2	74.2	74.3	74.3	74.6	77.7	77.1	78.7	78.4	77.7	78.2	78.5	80.6	80.5	95.4	*	33.0	23.8	22.7	23.4	21.7	21.8	22.6	22.0	21.9
28	***A. vitoriensis*' FW59T**	73.2	73.7	74.1	73.9	73.2	72.7	73.7	73.5	70.2	74.9	73.7	73.8	73.9	74.0	74.1	74.3	77.6	77.0	78.5	78.3	77.8	78.1	78.5	80.7	80.7	87.1	87.4	*	23.7	22.8	23.6	21.6	21.4	22.4	21.9	22.3
29	***A. faecis* LMG 28519T**	73.4	73.8	73.7	74.0	73.4	72.4	73.7	73.7	70.2	75.0	73.6	73.9	74.0	73.8	74.1	73.8	77.1	76.6	77.7	77.7	77.3	77.7	78.0	79.4	79.4	81.1	81.2	81.0	*	28.5	25.1	22.7	22.6	24.0	23.3	23.5
30	***A. cibarius* LMG 21996T**	73.3	73.8	73.9	74.1	73.0	72.6	73.9	73.2	70.3	74.5	73.6	73.7	73.6	73.9	73.7	73.9	77.1	76.6	77.9	77.9	77.1	78.1	78.4	79.1	79.0	79.6	79.7	79.5	84.6	*	24.1	22.3	22.3	22.7	23.2	23.2
31	***A. cryaerophilus* LMG 24291T**	73.1	73.5	73.6	73.6	73.3	72.5	73.4	73.4	70.0	74.8	73.5	73.8	73.8	73.9	73.9	73.7	76.7	76.3	77.6	77.3	76.7	77.5	77.7	78.7	78.9	81.0	80.9	80.9	82.0	81.2	*	22.9	22.8	29.8	25.2	26.0
32	***A. porcinus*' LMG 24487T**	73.3	73.5	73.9	73.6	73.5	72.6	73.5	73.2	70.2	74.5	73.6	73.7	73.6	73.8	73.7	73.8	76.4	75.8	76.7	76.7	76.2	76.4	76.6	77.9	77.9	79.2	79.3	79.1	79.8	79.3	80.3	*	51.1	22.6	23.7	23.9
33	***A. thereius* LMG 24486T**	73.3	73.5	73.7	73.6	73.1	72.6	73.4	73.4	70.1	74.4	73.7	73.9	73.7	73.8	73.8	73.9	76.1	75.7	76.8	76.8	76.0	76.3	76.6	78.0	77.8	79.2	79.2	79.1	79.6	78.9	79.9	93.3	*	22.5	23.5	23.6
34	***A. trophiarum* LMG 25534T**	72.7	72.9	73.3	72.9	72.9	72.2	72.9	72.8	70.0	73.8	73.3	73.3	73.2	73.4	73.1	73.1	75.8	75.5	76.5	76.3	76.0	76.3	76.6	77.7	77.9	79.9	80.1	80.2	80.9	79.5	85.5	80.1	79.9	*	23.0	23.2
35	***A. miroungae*' 9AntT**	73.1	73.4	74.8	73.6	73.3	72.8	73.2	73.3	70.3	74.6	73.5	73.8	73.8	73.8	73.7	73.9	76.4	76.0	77.3	77.2	76.5	77.0	77.1	78.4	78.3	79.5	79.4	79.6	80.5	79.8	82.4	81.5	81.3	80.6	*	61.0
36	***A. skirrowii* LMG 6621T**	73.48	73.7	73.9	73.9	73.39	72.7	73.5	73.3	70.3	74.7	73.7	73.8	73.8	73.7	73.8	73.6	76.4	76.1	77.0	77.1	76.6	77.2	77.4	78.5	78.6	79.6	79.6	79.7	80.2	80.1	82.8	81.5	81.2	80.6	94.9	*

Table S3. Values (%) of AAI (down-left) and POCP (up-right) among type and representative strains of the different *Arcobacter* species

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	*A. salis* ' F155-33T	*	90.68	86.76	74.4	71.6	72.16	68.5	65.82	66.94	68.29	69.84	69.88	69.12	69.16	68.85	68.7	64.91	64.12	62.97	68.3	65.93	64.96	64.8	63.97	61.97	60.8	61.15	60.33	60.17	60.32	60.42	56.46	58.79	59.62	57.97	59.22
2	*A. ponticus* ' F161-33T	82.86	*	85.07	77.6	72.19	72.16	67.48	68.67	67.58	68.19	69.48	70.08	69.52	69.6	69.54	69.59	67,0	67.4	65.27	68.67	67.8	67.06	66.88	67.32	63.76	63.69	63.17	62.02	60.44	62.56	62.15	57.59	60.58	61.47	60.58	61.76
3	*A. bivalviorum* CECT 7835^{T}	83.38	82.98	*	77.65	71.91	72.09	69.09	67.12	67.47	68.61	69.73	69.97	69.29	69.41	69.12	69.1	69.07	65.93	65.44	70.67	68.11	67.5	67.42	66.4	64.37	63.72	63,0	62.82	61.58	63.79	64.81	59.06	61.61	63.9	61.2	62.08
4	*A. mediterraneus* ' F156-34T	80.51	80.63	80.34	*	72.14	73.06	65.07	65.21	66.92	69.16	69.32	69.93	69.86	68.93	69.27	68.87	64.7	65.75	67.06	68.79	64.57	66.57	64.78	64.48	64.34	63.58	64.25	62.86	62.1	61.05	61.43	57.47	60.1	60.8	58.66	59.49
5	*A. ebronensis* CECT 8441^{T}	69.63	73.15	72.39	69.81	*	77.87	62.51	70.29	66.79	67.01	67.85	67.97	67.17	68.44	68.29	68.39	63.11	67.54	65.16	64.72	66.58	62.78	65.55	62.24	59.98	58.38	58.42	58.12	57.72	57.31	57.54	53.71	56.27	57.28	56.05	57.27
6	*A. anaerophilus* DSM 24636^{T}	68.7	71.33	71.37	72.63	78.44	*	62.59	66.72	67.8	68.17	68.2	68.93	67.94	69.42	69.12	68.85	63.9	66.58	68.77	65.79	65.76	63.61	66.29	62.98	63.29	58.45	59.59	58.04	59.68	59.32	58.46	54.84	57.35	58.17	57.36	57.53
7	*A. lekithochrous* CECT 8942^{T}	68.61	67.8	68.14	67.34	66.32	66.71	*	63.13	64.77	67.93	67.52	68.42	67.51	67.17	67.07	67.11	65.36	64.75	62.63	65.93	63.73	64.12	63.82	58.88	56.86	56.1	56.41	55.44	56.32	57.77	56.29	52.02	53.95	56.03	52.98	53.89
8	*A. nitrofigilis* DSM 7299^{T}	67.63	68.08	67.34	67.48	68.24	67.99	66.25	*	66.82	67.01	66.75	67.35	66.95	67.11	68.26	67.6	62.82	67.2	64.5	64.01	65.62	61.39	64.12	60.83	58.7	56.83	56.39	56.58	55.89	56.41	57.21	52.64	54.62	55.54	54.09	55.69
9	*A. aquaticus* ' W112-28T	67.64	70,0	72.16	68.61	68.36	66.5	62.41	65.69	*	68.11	66.79	66.79	65.79	66.63	66.85	66.62	66.76	64.1	64.74	66.65	66.29	63.21	65.13	64.97	63.73	61.98	61.37	60.71	59.46	60.2	61.32	56.69	59.87	62.28	59.32	59.72
10	*A. mytili* CECT 7386^{T}	70.14	70.49	73.65	66.26	66.56	65.15	66.16	63.46	68.28	*	77.87	75.4	76.53	77.12	76.06	76.39	65.38	63.11	62.39	66.65	64.17	63.7	64.09	62.73	60.82	59.32	58.76	59.41	58.14	59.61	60.58	54.68	57.37	60.46	56.86	58.06
11	*A. halophilus* DSM 18005^{T}	72.65	75.14	75.68	69.21	69.01	68.6	66.05	66.97	68.82	67.64	*	84.58	88.88	78.16	77.4	78.1	67.53	64.94	65.19	68.89	67.06	66.6	66.81	65.41	62.34	63.02	62.6	61.57	61.24	63,0	63.01	58.02	61.01	61.29	60.82	62.01
12	*A. canalis* ' F138-33T	69.88	72.72	72.58	68.36	66.46	66.87	64.46	65.07	66.67	67.93	86.52	*	86.66	78.12	77.94	77.78	64.68	64.65	63.87	68.39	65.68	65.62	64.97	64.56	60.79	61.57	61.26	60.57	60.01	61.62	61.1	57.81	60.5	60.11	60.16	61.41
13	*A. marinus* CECT 7727^{T}	71.98	74.45	74.63	70.05	68.41	68.52	66.56	66.79	68.55	68.65	87.48	95.68	*	77.68	77.32	77.53	66.48	65.16	64.38	68.18	66.53	65.56	65.64	64.79	61.26	62.02	61.46	61.18	60.82	61.71	62.02	57.52	60.89	60.8	60.57	62.12
14	*A. neptunis* ' F146-38T	70.03	73.24	72.27	67.79	71.72	71.53	65.33	68.4	68.34	74.13	80.22	77.06	78.3	*	85.04	91.36	66.33	68.31	67.36	67.92	68.38	65.9	69.38	65.43	63.44	61.01	61.23	60.1	60.39	62.3	61.79	57.32	60.4	60.44	59.84	61.49
15	*A. viscosus* ' F142-34T	70.07	73.17	71.95	69.46	71.24	70.22	64.47	70.64	68.52	72.45	78.76	78.94	78.46	85.62	*	85.44	64.61	67.4	66.19	67.13	67.17	64.28	66.15	64.48	61.84	59.78	59.7	59.4	58.56	60.36	60.8	55.82	58.62	59.31	58.75	60.45
16	*A. molluscorum* CECT 7696^{T}	68.28	73.06	71.3	67.76	71.64	70.78	63.8	68.94	68.01	72.31	79.15	77.71	79.15	87.12	87.6	*	65.45	67.9	66.72	67.22	67.76	65.44	67.1	65.64	62.56	60.92	61.16	59.79	59.73	61.35	60.56	56.72	59.45	59.29	58.9	61.03
17	*A. caeni* ' RW17-10T	66.92	67.23	67.69	67.6	67.11	67.11	70.35	67.04	66.19	67.64	66.68	67.72	66.79	67.62	67.19	66.97	*	84.55	72.22	75.57	87.53	73.39	75.63	73.09	71.06	68.1	67.99	66.17	68.61	71.24	69.69	63.75	66.85	70.32	66.63	66.61
18	*A. venerupis* CECT 7836^{T}	67.21	67.22	67.41	67.13	66.83	67.36	70.53	68.31	65.7	68.11	67.1	68.12	67.33	67.51	67.69	67.42	73.14	*	76.15	72.08	86.21	71.7	77.26	70.46	67.1	64.02	63.6	63.68	63.24	64.33	63.06	58.06	60.49	61.17	59.31	60.84
19	*A. defluvii* CECT 7697^{T}	67.63	67.53	67.99	68.24	66.99	67.45	70.14	66.39	66.38	68.15	67.21	67.53	67.37	67.38	67.29	67.01	79.61	79.61	*	82.81	80.68	82.85	78.93	70.41	65.59	65.69	67.33	64.16	66.45	69.24	64.65	60.2	63.02	65.3	62.79	63.73
20	*A. aquimarinus* CECT 8442^{T}	68.09	67.84	68.34	68.55	66.98	67.21	70.78	66.52	65.99	68.17	67.75	67.77	67.79	67.55	68.17	67.3	79.64	79.23	77,0	*	80.41	82.33	77.73	75.48	72.13	70.2	70.81	70.12	70.37	73.44	71.36	64.44	67.35	70.18	67.42	68.71
21	*A. suis* CECT 7833^{T}	67.1	67.45	67.36	67.71	68.02	67.42	70.42	67.41	66.31	68.29	67.28	67.84	66.83	67.85	67.51	67.83	79.9	78.32	76.49	76.76	*	73.61	78.68	76.07	73.34	69.03	68.5	68.37	68.28	70.25	68.73	62.54	65.98	67.44	65.34	66.58
22	*A. cloacae* CECT 7834^{T}	67.66	67.75	68.02	68.23	66.9	67.14	70.51	66.55	65.89	68.61	67.35	67.93	67.5	67.18	67.24	67.16	79.58	79.23	75.45	93.53	81.01	*	79.22	72.02	68.58	67.62	68.23	66.84	68.47	72.29	68.42	62.42	65.69	66.51	64.9	66.48
23	*A. ellisii* CECT 7837^{T}	67.55	67.81	68.02	67.81	67.32	67.63	70.63	66.82	66.3	68.5	67.43	67.72	67.19	68.05	67.56	67.42	80.01	80.1	87.03	83.23	81.19	83.47	*	71.99	69.21	65.9	66.01	64.57	67.23	71.2	67.56	62.15	64.01	65.68	63.13	65.14
24	*A. butzleri* RM4018^{T}	66.93	67.15	66.9	67.03	66.37	66.55	67.91	66.01	65.75	67.23	66.22	66.52	66.36	66.76	66.49	66.8	76.18	75.76	79.54	77.74	77.03	77.6	79.28	*	84.28	78.65	78.12	78.79	73.53	75,0	75.96	68.44	73.1	74.71	71.46	73.16
25	*A. lacus* ' RW43-9T	66.72	67.05	67.31	67.67	66.62	66.96	68.05	65.93	65.55	66.97	66.41	66.72	66.14	66.96	66.77	66.57	75.89	75.68	79.97	77.74	77.12	72.72	79.49	93.72	*	76.48	76.7	75.5	72.45	72.91	73.93	67,0	71.01	74.17	69.36	70.34
26	*A. lanthierii* LMG 28516^{T}	64.88	64.68	65.5	65.43	64.92	64.67	65.63	64.47	63.89	65.2	64.46	65.08	64.68	64.93	64.47	64.48	71.98	70.75	72.44	72.9	71.98	72.21	73.16	76.39	76.33	*	95.57	84,0	76.77	73.79	76.61	71.26	76.48	78.09	72.96	74.02
27	*A. hispanicus* ' FW54T	65,0	64.49	65.53	65.28	65.09	64.86	66.02	64.28	63.7	65.19	64.77	65.05	64.63	64.87	64.55	64.72	71.99	71.04	72.82	73.02	72.19	72.83	73.15	76.62	76.65	88.62	*	83.93	77.05	75.49	76.68	71.21	76.52	79.88	73.65	74.73
28	*A. vitoriensis* ' FW59T	64.65	64.92	65.5	65.14	64.53	64.35	65.42	63.9	63.49	65.01	64.73	64.87	64.64	64.43	64.57	64.58	71.58	71.07	72.52	72.27	72.3	72.03	72.55	76.87	77.14	87.23	86.97	*	74.94	71.7	76.09	69.89	74.12	75.47	71.58	72.66
29	*A. faecis* LMG 28519^{T}	64.82	64.76	65.06	65.23	64.2	64.05	66.27	63.52	63.22	65.37	64.44	65.17	64.5	64.06	63.91	64.13	70.84	70.67	72.42	72.66	71.72	72.28	72.5	74.94	74.8	77.52	77.61	77.67	*	84.34	73.6	69.4	71.73	73.52	68.15	71.56
30	*A. cibarius* LMG 21996^{T}	64.96	64.93	65.45	65.19	64.58	65.03	66.56	63.95	64.1	65.59	65.15	65.24	64.77	64.9	64.53	64.61	70.95	70.33	72.89	73.01	71.83	73.02	73.13	74.88	74.74	76.19	76.14	75.98	75.76	*	76.44	68.46	71.48	76.55	72.83	74.27
31	*A. cryaerophilus* LMG 24291^{T}	64.21	65.01	64.93	64.76	64.01	64.25	65.42	63.47	63.35	65.2	64.41	64.7	64.31	64.39	64.28	64.46	69.73	69.81	71.77	71.63	70.93	72.35	71.72	74.62	74.06	78.47	78.29	78.32	78.18	78.2	*	71.45	75.54	81.81	78.92	79.37
32	*A. porcinus* ' LMG 24487T	64.64	64.1	64.9	64.48	63.85	63.91	64.79	63.53	62.83	64.4	64.17	64.36	64.25	63.54	63.67	63.78	68.74	67.9	68.95	69.74	69.22	68.91	69.43	72.01	71.72	74.64	74.47	75.03	74.58	73.73	76.24	*	84.46	76.51	79.14	79.19
33	*A. thereius* LMG 24486^{T}	64.47	63.66	64.88	64.35	63.86	63.93	64.86	63.54	62.68	64.48	63.94	64.71	64.13	64.11	63.97	64.04	68.93	68.07	68.97	69.69	69.43	68.8	69.4	71.87	71.47	74.5	74.74	75.04	74.35	74.14	76.25	93.15	*	76.4	78.41	78.6
34	*A. trophiarum* LMG 25534^{T}	64.35	64.69	65.18	64.65	64.07	64.28	65.44	63.95	63.16	64.58	64.26	64.48	64.15	64.11	64.01	63.99	69.87	69.42	71.21	71.15	70.55	71.06	71.3	73.67	73.46	77.92	77.6	77.95	77.43	76.62	85,0	72.45	77.37	*	77.28	77.1
35	*A. miroungae* ' 9AntT	64.68	64.65	65.55	65.15	64.82	64.56	65.4	64.2	63.57	65.25	64.89	65.05	65.05	64.62	64.54	64.27	69.8	69.44	71.37	74.39	70.1	71.35	71.13	73.57	73.22	75.49	75.45	76.13	75.68	75.89	76.87	76.51	81.36	77.15	*	85.35
36	*A. skirrowii* LMG 6621^{T}	65.49	65.48	65.77	65.25	64.69	64.64	66,0	64.03	63.64	65.5	64.78	65.33	64.55	64.35	64.5	64.31	69.74	69.28	70.84	71.14	70.27	71.19	71.3	74.06	73.95	75.7	75.59	76.51	75.6	76.25	77.05	77.38	81.44	75.96	94.99	*

Table S4. Similarities (%) in the 16S rRNA gene among type species of each new described genus and the other genera of the family Campylobacteraceae.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13
1	***Arcobacter nitrofigilis* DSM 7299^T**	*												
2	***Aliiarcobacter cryaerophilus* LMG 24291^T**	93.8	*											
3	***Pseudoarcobacter defluvii* CECT 7697^T**	95.8	94.5	*										
4	***Haloarcobacter bivalviorum* CECT 7835^T**	93.4	91.2	95.0	*									
5	***Malacobacter halophilus* DSM 18005^T**	94.5	92.5	94.6	93.6	*								
6	***Poseidonibacter lekithochrous CECT 8942^T***	95.1	92.0	94.2	92.9	92.9	*							
7	***Arcomarinus aquaticus'* W112-28**	94.9	92.6	94.8	93.0	94.2	94.0	*						
8	***Campylobacter fetus* subsp. *fetus* ATCC 273**	85.4	85.9	85.7	86.1	85.2	85.7	86.9	*					
9	***Sulfurospirillum deleyianum* DSM 6946^T**	87.8	87.7	88.3	87.1	86.7	88.3	88.7	88.9	*				
10	***Thiovulum* sp.**	84.2	84.6	83.9	84.3	84.1	84.5	83.9	83.9	84.4	*			
11	***Sulfuricurvum kujiense* DSM 16994^T**	85.1	85.1	85.7	84.8	85.1	85.3	85.4	86.1	87.9	87.8	*		
12	***Sulfurimonas autotrophica* DSM 16294^T**	84.7	85.7	85.2	84.7	85.9	84.6	85.9	85.5	86.9	86.7	89.7	*	
13	***Helicobacter pylori* NCTC 11637^T**	83.8	85.1	84.6	84.3	84.7	84.1	84.1	85.1	84.2	85.1	86.7	85.6	*

5. GENERAL DISCUSSION

5.1 Identification and epidemiology of *Arcobacter* clinical strains.

There exist several methods for the identification of *Arcobacter* strains at species level summarized with their limitations in section 1.1.3. For instance, the use of methods like several m-PCRs that target the 16S and 23S rRNA genes (Houf et al., 2000; Douidah et al., 2010; Khan et al., 2017) can produce misidentifications due to the high similarity of these genes in some species (Levican and Figueras, 2013). Due to this high similarity, these m-PCR methods produced the same patterns in different species (Levican and Figueras, 2013). Two of the most accurate methods for the identification at species level are the use of MALDI-TOF (Alispahic et al., 2010; Levican et al., 2012, 2015) and the use of *rpoB* gene phylogenies (Collado, 2010; Figueras et al., 2014). However, as commented in the introduction, the successfully identification obtained with MALDI-TOF depends on the number of well characterised strains included in the database of the system that in the case of *Arcobacter*, can be insufficient to identify some strains (Arguello et al., 2015). In this thesis, 28 clinical strains of *Arcobacter*, isolated from human faeces, were studied. Identification of these strains was firstly attempted using phenotypic tests (n=6) or MALDI-TOF (n=22). These methods identified the strains as *A. butzleri* (n=22), *Arcobacter* sp. (n=5) and *Campylobacter* sp. (n=1). The second identification was performed with the phylogeny of the *rpoB* gene that enabled to confirm or identify to the species level each of the strains. Results showed that the strains identified using MALDI-TOF were correctly ascribed to the species *A. butzleri* (n=22), evidencing the usefulness of this technique (Alispahic et al., 2010; Levican et al., 2012, 2015; Figueras et al., 2014). Additionally, the identification of one strain as *Campylobacter* demonstrated that the use of phenotypic test for *Arcobacter* identification can produce misidentification. Furthermore, this thesis confirmed that the use of the *rpoB* gene is an excellent tool for the identification of the species level, as demonstrated in other studies (Collado, 2010; Figueras et al., 2014).

The first objective of this thesis was to analyse the epidemiological relationship between clinical strains of *Arcobacter* from human origin. As commented in the introduction (see section 1.3.), several tools have been widely used in bacterial epidemiology. In this thesis, we tested the MLST methodology for the analysis of clinical strains of *Arcobacter* recovered from human faeces. From the 28 analysed strains, only one strain showed a Sequence Type (ST 2) already present in the database and that corresponded interestingly to two human clinical strains isolated from France and Belgium. These three strains were apparently unrelated, evidencing that the MLST approach lack resolution to discriminate epidemiologically related strains involved in an infection process. This fact has been also evidenced in the case of the MLST scheme of *Legionella pneumophila* (Quero et al., 2016). Therefore, new methodologies must be developed to analyse the epidemiological relationships between *Arcobacter* strains. The other 27 strains showed new alleles and new ST (Study 4.1, Table 1), that contributed increasing the number of clinical STs of the MLST database in 26%. Most of these new STs were due to the discovering of new alleles in five of the seven genes of the MLST scheme. However, some of the new STs were produced by new combinations of known alleles (25% of the STs). An important aspect of these new STs is the presence of an apparent association between the alleles *aspA*-80, *atpA*-67 and *gln*-49. This combination occurred in 25% of our strains (Study 4.1, Table 1) and also appeared in one strain deposited in the MLST database, all of them from human clinical origin. Thus, this allele combination could be considered as a human signature, but more studies would be required to confirm this hypothesis.

The phylogenetic analysis (study 4.1, Figure 1) performed with all the *Arcobacter* strains from human origin present in the database (n=132) showed that 50% of our STs grouped randomly among the other clinical STs present in the database. However, the other 50% of our STs formed two clusters. Cluster 1 (study 4.1, Figure 1) was composed by 7 of our STs and by STs from other European countries. The other STs (n=7) grouped in the Cluster 2 with strains isolated from USA and Thailand (study 4.1, Figure 1).

5.2 Antibiotic resistance in clinical strains of *Arcobacter*

The number of available treatments for bacterial infections using antibiotics has increased since the description of the first antibiotic. However, the overuse or inadequate use of antibiotic treatments has produced an increasing of the bacterial resistance to these drugs. This resistance is directly related with morbidity and mortality, implicating around 25,000 deaths in Europe (ECDC/EMEA report, 2009). These results evidenced the needed for standardizing and controlling the use of antibiotics in the hospital and community environments. In the case of *Arcobacter*, there is no standardized protocol for the treatment of infections produced by this bacterium. Most of the *Arcobacter* infections are self-limiting but in some cases, when the infection is prolonged in time and the symptoms get worse, or the patient has an overlaying disease, the use of antibiotics is essential (Collado and Figueras, 2011). Several studies suggested the use of fluoroquinolones or tetracyclines for the treatment of *Arcobacter* (Fera et al., 2003; Vandenberg et al., 2006; Son et al., 2007). However, Van den Abeele et al. (2016) recommended the use of tetracyclines instead of fluoroquinolones or macrolides. Regarding the antimicrobial resistance in *Arcobacter*, in the review of Ferreira et al. (2015) and in the study of Van den Abeele et al. (2016), authors showed that a high number of *Arcobacter* strains were resistant to ampicillin, nalidixic acid, ciprofloxacin, erythromycin or gentamicin. As a part of the first objective of this thesis the antibiotic resistance of 28 strains was tested using the disc diffusion method against 5 antibiotics (amoxicillin/clavulanate (20/10 μg), erythromycin (15 μg), gentamycin (10 μg) tetracycline (30 μg) and ciprofloxacin (5 μg). Results (study 4.1, Table 3) showed that 32.1% of the strains were resistant to at least one of the tested antibiotics. These resistances corresponded to ciprofloxacin (fluoroquinolone), and occurred in 7.4% of the strains of *A. butzleri* and in the only strain of *A. cryaerophilus*. These results evidenced that this drug should not be use for the treatment of *Arcobacter* as suggested before (Collado and Figueras, 2011). An important finding of this work is that none of the strains showed resistance to tetracycline, as occurred in previous studies (Vandenberg et al., 2006; Van den Abeele et al., 2016; Šilha et al., 2017). These results suggest that this antibiotic can be used as treatment, in line with what was propose by other authors (Collado and Figueras, 2011; Ferreira et al., 2015; Van der Abeele et al., 2016).

5.3 Description of new *Arcobacter* species: from the 16S rRNA gene to the genome analysis

The second objective of this thesis was the characterization of several strains isolated from different sources considered as potential new species. These strains were isolated from reclaimed water (RW43-9, RW17-10), mussels (F190-2IL33), oysters (F138-33, F181-1F33), water (W110-33), and Antarctic mammals (AHV-9/2010). Additionally, and regarding the different prevalence of *A. cryaerophilus*, the third objective was to reassess the taxonomy of this species.

The classical tools used in taxonomic studies have been the 16S rRNA gene (Figueras et al., 2011). However, in the case of *Arcobacter*, this gene shows a low resolution power for certain species evidenced by the high percentage of similarity between the species *A. ellisii* and *A. cloacae* (Levican et al., 2013a). In this thesis, again this was shown by the similarity observed between the species *A. butzleri* and the new described species *A. lacus* sp. nov. of 99.9%. In addition, the lower value of the 16S rRNA gene similarity within the genus i.e. 91.2% is below the 95% cut-off stablished for genus delineation (Figueras et al., 2011). This evidences that this genus could be composed by cryptic genera, being this problem another of the research objectives of this Thesis.

As explained in the introduction the use of m-PCRs and 16S rDNA-RFLP methods targeting the 16S and 23S rRNA genes for the identification of *Arcobacter* spp. can produce either new unexpected patterns, patterns shared with different species and the combination of the different identification methods in parallel can produce contradictory results. This is the case for the strains isolated from shellfish and waters included in the study 4.2 and also for the strains of the study 4.3 RW43-9[a] and RW17-10[b]. Due to these discordant results, we decided to leave aside these identification techniques and use instead the phylogenetic analysis of the *rpoB* gene (studies 4.4 and 4.5). This gene showed a higher resolution for species delineation, even for species with a high percentage of similarity based on the 16S rRNA gene, as in the case of the strain RW43-9 with *A. butzleri* (see study 4.3, Supplementary Figure S1).

All the species descriptions included in these PhD Thesis were performed using also the information derived from the genomes. This aspect was most important in the case of species described with only one strain, because this became a mandatory requirement when a single strain is available as indicated in the editorial note published in the International Journal of Systematic and Evolutionary Microbiology in December 2017 and also by the Systematic and Applied Microbiology journal as indicated in the instructions to the authors. The use of genomic information in species description included the calculation of similarity indexes between the new species and their nearest ones. For these analyses, the ANI and the *is*DDH were used. The values stablished for these two indexes to ascribe strains to different species were below 95-96% and 70%, respectively (Richter and Rosselló-Mora, 2009; Meier-Koltoff et al., 2013). In the case of *Arcobacter*, results of these indexes showed that the cut-off value of 96% for ANI is the one that better correlates with *is*DDH values lower than 70%.

In the case of *A. cryaerophilus*, the phylogenetic analyses based on the *rpoB* gene along with the one derived from the concatenation of the sequence of the latter gene with other four housekeeping genes (*atpA*, *gyrA*, *gyrB* and *hsp60*) showed that the strains grouped in four clusters. Additionally, the calculation of the ANI and *is*DDH between representative genomes of each of the four clusters showed values below the cut-off of 96% and 70%, respectively. These results evidenced that indeed the four clusters could represent different species. For the description of new species the existence of at list one differential phenotypic characteristic from the know species is required (Stackebrandt et al., 2002; Figueras et al., 2011). Because that, several phenotypic tests including the ones obtained from the genomic information were performed. Despite that, any differential characteristic was found and moreover, the strains showed a high variability of response

[a] Pattern of *A. butzleri* with the m-PCR of Houf et al. (2000) and unknown pattern with the 16S-rDNA RFLP method of Figueras et al. (2012).
[b] Patterns of *A. cryaerophilus* and *A. skirrowii* with the m-PCR of Houf et al. (2000) and unknown pattern with the 16S-rDNA RFLP method of Figueras et al. (2012).

to these tests, both intra- and inter-clusters. Thus, regarding the current bacterial taxonomic guidelines, we were not able to define these clusters as new species. For that reason, the four clusters were considered as genomovars because they were well-limited genomic groups that could not be phenotypically differentiated. Further studies must be performed in order to find a differential characteristic that will allow describing each genomovar as a novel species. Furthermore, results of this study showed that the phenotypic characterization of strains required to describe new species is sometimes limiting the taxonomic classification and represents an important shortcoming for species description, especially when molecular and genomic data clearly demonstrate that the strains belong to different species.

5.4 Bioinformatic tools in the genomic era

Year by year, the number of available genomes and the facilities to sequence these genomes are increasing (https://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/prokaryotes.txt; Chun et al., 2018). Information extracted from genomes can be a helpful tool to understand and classify organisms and to elucidate new metabolic pathways and functionalities within the prokaryotes (Whitman, 2015; Chun et al., 2018). As a part of the characterization performed on the clusters formed by the studied *A. cryaerophilus* strains in the study 4.5, the genomes of 13 representative strains were analysed. For this analysis, genomes were screened for antibiotic resistance genes, virulence factors, metabolic pathways and functional characteristics. As explained in the introduction (see section 1.7), there are several tools for the identification of antibiotic resistance genes and virulence factor using different databases. In study 4.5, the antibiotic resistance genes were searched using the following databases: Antibiotic Resistance Database (ARDB) (Liu and Pop, 2009), the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017), and the Antibiotic Resistance Gene-Annotation database (ARG-ANNOT) (Gupta et al., 2014). The databases used for virulence gene detection were: Virulence Factors of Pathogenic Bacteria Database (VFDB) (Chen et al., 2005), the Victors Database (University of Michigan, USA), and the PATRIC_VF (Wattam et al., 2017). Additionally, detection of these features was also performed with the annotation of the genomes using RAST (Aziz et al., 2008) and PATRIC (Wattam et al., 2017). Results obtained with these tools showed that, although some of these tools can detect the presence of virulence and resistance genes, the correct identification depends on the composition of the dataset, being limited to the species or genus that are present in these databases. For that reason, a negative result obtained for a certain gene or loci, must be checked with an independent analysis performing a BLASTn or BLASTp (Boratyn et al., 2013) search using a known gene present in other species of the genus. Additionally, in the study 4.5, two bioinformatic tools were used to extract the functional and metabolic information derived from the genomes: The Functional Comparison Tool from the Seed Viewer (Overbeek et al., 2014) for the functional analysis and Traitar software (Weimann et al., 2016) for the metabolic pathways and the phenotypic traits. The obtained results were used to compare the genomes and to find a phenotypical characteristic that allow differentiation between the *A. cryaerophilus* genomovars. Despite some differences were obtained after the genomic analyses, these results were not confirmed when tested experimentally at the laboratory. These contradictory results could be due to the inability to mimic the conditions that the bacteria need to express these features at the laboratory.

5.5 Taxogenomics of the genus *Arcobacter*

In the study 4.6, the phylogenies performed with all the described species of this genus, using both 16S rRNA gene and Multilocus Phylogenetic Analysis with five housekeeping genes, showed a distribution of the species in four main clusters. Additionally, the genomes of all the described *Arcobacter* species (with the exception of *A. acticola* and *A. pacificus*) were studied in order to reassess the taxonomy of the genus. This analysis was performed in a polyphasic approach, using genomic phylogenies along with the calculation of several genomic indexes and the analysis of phenotypic information. Results showed that only the species *A. nitrofigilis* remained in the genus *Arcobacter,* that was composed by at least other additional 6 genera. The separation of the genera was assessed on the basis of the following ranges of the genomic indexes: 75.2-81.8% for ANI, 19.5-24.8% for *is*DDH, 67.6-80.3% for AAI, and 67.0-75.4% for POCP. Despite previous studies stablished that the ANI and *is*DDH values are not useful for genera delineation (Konstantinidis and Tiedje, 2005; Goris et al., 2005; Richter and Rosselló-Móra. 2009; Qin et al., 2014; Chun et al., 2018) results of study 4.6 evidenced that these indexes can be used to differentiate *Arcobacteraceae* family. In this sense, the cut-off values for ANI and isDDH of 75-82% and 19-25%, respectively, could be considered the low values within a genus of the Family *Arcobacteraceae* (Waite et al., 2017). As in the case of ANI and *is*DDH values, lower values obtained with the POCP and AAI indexes in each of the clusters could be considered the ranges for the differentiation of the closely related genera studied. Values for AAI and POCP are in agreement with the proposed cut-offs of 60-80% and 50%, respectively (Konstantinidis and Tiedje, 2005; Lou et al., 2014; Qin et al., 2014). Regarding the RSCU results, the species *A. aquaticus* showed a different codon usage pattern in relation to the ones obtained for the other *Arcobacter* species and this difference was the only that presented statistical significance ($p<0.05$) using a multinomial regression analysis. These results evidenced that the candidate species *A. aquaticus* represents a different genus, for which the name Candidate '*Arcomarinus'* is proposed. The other species of *Arcobacter* showed a preferential use of A or T codons, as could be expected by the G+C contents. The PCA performed with results of codon usage showed that different groups were produced that correlated with the 6 clusters showed with the other analyses. PCA results evidenced that these analyses are useful for the study of the evolutionary relationships among the species analysed as occurred in other genera (Marenda et al., 2005; Ma et al., 2015).

6. CONCLUSIONS

1. The fact that epidemiological unrelated *A. butzleri* strains show the same ST indicates that other techniques with higher resolution should be developed to effectively recognize the epidemiological related specially those involved in infection process.

2. The demonstrated resistance of 10.7% of the clinical strains of *A. butzleri* to ciprofloxacin, one of the antibiotics recommended for the treatment of the intestinal infections produced by these bacteria indicates the importance of selecting in each case the most effective treatment.

3. Four new *Arcobacter* species have been described for which the names *A. canalis*, *A. lacus*, *A. caeni* and *A. miroungae* are proposed.

4. Genomic analyses evidenced the existence of 10 potential new species that are pending to be completely described.

5. The genomic and polyphasic analysis of the species *A. cryaerophilus* evidenced the existence of four cryptic genomovars within this species for which the names *A. cryaerophilus* gv. *pseudocryaerophilus*, *A. cryaerophilus* gv. *crypticus*, *A. cryaerophilus* gv. *cryaerophilus*, and *A. cryaerophilus* gv. *occultus* are proposed.

6. The taxogenomic analysis of the genus *Arcobacter* along with the phenotypical characterization allowed the division of the genus in at least six different genera, for which the names *Aliiarcobacter* gen. nov., *Pseudoarcobacter* gen. nov., *Haloarcobacter* gen. nov., *Malacoarcobacter* gen. nov., *Poseidoniarcobacter* gen. nov., and *Arcomarinus* gen. nov., have been proposed. Those genera embrace all the previously defined *Arcobacter* species, with the exception of *A. nitrofigilis*.

7. The ANI, the *is*DDH, the AAI and the POCP values obtained after the taxogenomic analysis enabled to delineate the mentioned new genera among each other and from the genus *Arcobacter* using the following cut-off values: 75.2-81.8% for ANI, 19.5-24.8% for *is*DDH, 67.6-80.3% for AAI, and 67.0-75.4% for POCP.

8. The study of the RSCU using a Principal Component Analysis clustered the species within the 7 genera.

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Supplementary Table S1. Strains used in this thesis

Species	Strain	Source	Country	Genome	Study
A. acticola	KCTC 52212^T	Sea water	South Korea	NA	4.2-4.4, 4.6
A. anaerophilus	DSM 24636^T	Estuarine Sediment	India	URV	4.2-4.4, 4.6
	IR-1	Utsira aquifer	Norway	NCBI	4.6
A. aquimarinus	CECT 8442^T	Mediterranean Sea	Spain	URV	4.2-4.4, 4.6
A. bivalviorum	CECT 7835^T	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.2-4.4, 4.6
	F118-4	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.6
A. butzleri	RM4018^T	Clinical (Human)	USA	NCBI	4.2-4.4, 4.6
	ED1	Microbial fuel cell	Japan	NCBI	4.6
	HJXXIII-1	Human stool	Spain	NA	4.1
	HJXXIII-10	Human stool	Spain	NA	4.1
	HJXXIII-12	Human stool	Spain	NA	4.1
	HJXXIII-13	Human stool	Spain	NA	4.1
	HJXXIII-14	Human stool	Spain	NA	4.1
	HJXXIII-15	Human stool	Spain	NA	4.1
	HJXXIII-16	Human stool	Spain	NA	4.1
	HJXXIII-17	Human stool	Spain	NA	4.1
	HJXXIII-18	Human stool	Spain	NA	4.1
	HJXXIII-19	Human stool	Spain	NA	4.1
	HJXXIII-2	Human stool	Spain	NA	4.1
	HJXXIII-20	Human stool	Spain	NA	4.1
	HJXXIII-21	Human stool	Spain	NA	4.1
	HJXXIII-3	Human stool	Spain	NA	4.1
	HJXXIII-4	Human stool	Spain	NA	4.1
	HJXXIII-5	Human stool	Spain	NA	4.1
	HJXXIII-6	Human stool	Spain	NA	4.1
	HJXXIII-7	Human stool	Spain	NA	4.1
	HJXXIII-8	Human stool	Spain	NA	4.1
	HJXXIII-9	Human stool	Spain	NA	4.1
	HSJR-2	Human stool	Spain	NA	4.1
	HSJR-3	Human stool	Spain	NA	4.1
	HSJR-4	Human stool	Spain	NA	4.1
	HSJR-5	Human stool	Spain	NA	4.1
	HSJR-6	Human stool	Spain	NA	4.1
	HSJR-7	Human stool	Spain	NA	4.1
	HUMS-1	Human stool	Spain	NA	4.1
A. cibarius	LMG 21996^T	Broiler, skin	Belgium	NCBI	4.2-4.4, 4.6
A. cloacae	CECT 7834^T	Sewage	Spain	URV	4.2-4.4, 4.6
	F26	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.6
A. cryaerophilus	LMG 24291^T	Aborted bovine foetus, brain	Ireland	URV	4.2-4.4, 4.6
	LMG 10229	Aborted porcine foetus, kidney	Canada	URV	4.5
	LMG 10210	Aborted bovine foetus	Canada	URV	4.5
	LMG 10241	Kidney, aborted porcine foetus	Canada	NA	4.5

(... Continue)

Species	Strain	Source	Country	Genome	Study
A. cryaerophilus	LMG 9065	Aborted ovine foetus, placenta	Ireland	URV	4.5
	LMG 9861	Aborted bovine foetus, peritoneal fluid	Ireland	URV	4.5
	LMG 9863	Placenta, aborted ovine foetus	Ireland	NA	4.5
	LMG 29976	Aborted porcine foetus, eye	Ireland	URV	4.5
	LMG 9871	aborted bovine foetus, kidney	Ireland	URV	4.5
	LMG 10829	Human blood	USA	NA	4.5
	L397	Untreated city wastewater	Canada	NCBI	4.5
	L398	Wastewater outfall	Canada	NCBI	4.5
	L399	Biologically/UV treated city wastewater	Canada	NCBI	4.5
	L400	Biologically treated city wastewater	Canada	NCBI	4.5
	L401	Goose faeces	Canada	NCBI	4.5
	L406	Impoundment reservoir	Canada	NCBI	4.5
	F196	Aborted porcine foetus	Brazil	NA	4.5
	UF1T	Uterus, sow	Brazil	NA	4.5
	UF2T	Uterus, sow	Brazil	NA	4.5
	UPER3	Uterus, sow	Brazil	NA	4.5
	AB3A	Abomasum, aborted bovine foetus	Chile	NA	4.5
	AB74A	Abomasum, aborted bovine foetus	Chile	NA	4.5
	AO2A	Lungs, aborted ovine foetus	Chile	NA	4.5
	AL 20-1	Clam	Chile	NA	4.5
	CV-152	Faeces, deer	Chile	NA	4.5
	CV-2101	Faeces, deer	Chile	NA	4.5
	EMU-3	Faeces, emu	Chile	NA	4.5
	FE7	Faeces, chicken	Chile	NA	4.5
	HHS 118A	Faeces, asymptomatic human	Chile	NA	4.5
	HHS 133A	Faeces, asymptomatic human	Chile	NA	4.5
	HHS 188A	Faeces, asymptomatic human	Chile	NA	4.5
	HHS 191A	Faeces, asymptomatic human	Chile	NA	4.5
	HHS 205A	Faeces, asymptomatic human	Chile	NA	4.5
	MC 2-2	Surf clam	Chile	NA	4.5
	MCV 42-1	Faeces, cow	Chile	NA	4.5
	ME 15-4	Mussel	Chile	NA	4.5
	NAV 15-1	Razor clam	Chile	NA	4.5
	NAV12-2	Razor clam	Chile	NA	4.5
	NB14A	Jejunum, calf	Chile	NA	4.5
	14 PHA	Viscera, chicken	Costa Rica	NA	4.5
	20 PHF	Viscera, chicken	Costa Rica	NA	4.5
	284/1	Cow milk	Italy	NA	4.5
	BUF3	Buffalo milk	Italy	NA	4.5
	FEBU4	Faeces, buffalo	Italy	NA	4.5
	8749401	Diarrhoeic faeces, human	N. Zealand	NA	4.5
	8756347	Diarrhoeic faeces, human	N. Zealand	NA	4.5
	8122333	Diarrhoeic faeces, human	Spain	NA	4.5

(... Continue)

Species	Strain	Source	Country	Genome	Study
A. cryaerophilus	RW15-1	Reclaimed water	Spain	NA	4.5
	RW17-4	Reclaimed water	Spain	NA	4.5
	RW25-5	Reclaimed water	Spain	NA	4.5
	RW33-8	Reclaimed water	Spain	NA	4.5
	RW45-3	Reclaimed water	Spain	NA	4.5
	HJXXIII-11	Human stool	Spain	NA	4.1
A. defluvii	CECT 7697^T	Sewage	Spain	URV	4.2-4.4, 4.6
A. ebronensis	CECT 8441^T	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.2-4.4, 4.6
	W129-99	Wastewater	Spain	URV	4.6
A. ellisii	CECT 7837^T	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.2-4.4, 4.6
A. faecis	AF1078^T	Human waste septic tank	Canada	NCBI	4.2-4.4, 4.6
A. halophilus	DSM 18005^T	Hypersaline lagoon	USA	URV	4.2-4.4, 4.6
	F166-45	Oyster (*Crassostrea gigas*)	Spain	URV	4.6
A. lanthieri	AF1440^T	Pig manure	Canada	NCBI	4.2-4.4, 4.6
	AF1581	Dairy cattle manure	Canada	NCBI	4.6
A. lekithochrous	CECT 8942^T	Great scallop (*Pecten maximus*) larvae	Norway	NCBI	4.2-4.4, 4.6
	LMG 28652	Abalon (*Haliotis gigantea*)	Japan	NCBI	4.6
A. marinus	CECT 7727^T	East Sea	Korea	URV	4.2-4.4, 4.6
	F140-37	Clam (*Ruditapes philippinarum*)	Spain	URV	4.6
	SH-4D_Col1	Unknown	USA	NCBI	4.2
A. molluscorum	CECT 7696^T	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.2-4.4, 4.6
	F91	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.6
A. mytili	CECT 7386^T	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.2-4.4, 4.6
	T234	Brackish water	Spain	URV	4.6
A. nitrofigilis	DSMZ 7299^T	Roots of *Spartina alterniflora*	Canada	NCBI	4.2-4.4, 4.6
	F2176	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.6
A. pacificus	DSM 25018^T	Sea water	China	NA	4.2-4.4, 4.6
A. porcinus	LMG 24487^T	Aborted pig foetus, kidney and liver	Denmark	NCBI	4.2-4.4, 4.6
A. skirrowii	F28	wild pig	Spain	URV	4.6
	LMG 6621^T	Diarrheic lamb	UK	URV	4.2-4.4, 4.6
A. suis	CECT 7833^T	Pork meat	Spain	URV	4.2-4.4, 4.6
A. thereius	DU22	Duck cloaca	Denmark	NCBI	4.6
	LMG 24486^T	Aborted pig foetus, kidney and liver	Denmark	NCBI	4.2-4.4, 4.6
A. trophiarum	CECT 7650	Chicken cloacal swab	Chile	URV	4.6
	LMG 25534^T	Piglet faeces	Belgium	URV	4.2-4.6
A. venerupis	CECT 7836^T	Clams	Spain	URV	4.2-4.4, 4.6
***Arcobacter* sp.**	F138-33	Oyster (*Crassostrea gigas*)	Spain	URV	4.2, 4.6
	F181-1F33	Oyster (Crassostrea gigas)	Spain	NA	4.2
	F191-2IL33	Mussels (Mytilus galloprovincialis)	Spain	NA	4.2
	W110-33	Sea water	Spain	NA	4.2
	RW17-10	Reclaimed water	Spain	URV	4.3, 4.6
	RW43-9	Reclaimed water	Spain	URV	4.3, 4.6
	9Ant	Rectal swab *Mirounga leonina*	Antarctic Sea	URV	4.4, 4.6

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Species	Strain	Source	Country	Genome	Study
***Arcobacter* sp.**	AF1028	Human stool	Canada	NCBI	4.6
	CAB	Marine	USA	JGI	4.6
	F151-37	Oyster (*Crassostrea gigas*)	Spain	URV	4.6
	F155-33	*Oyster (Crassostrea gigas)*	Spain	URV	4.6
	F156-34	Mussels (*Mytilus galloprovincialis*)	Spain	URV	4.6
	F161-33	Cockle (*Cerastoderma edule*)	Spain	URV	4.6
	FW-54	Wastewater	Spain	URV	4.6
	FW59	Water	Spain	URV	4.6
	L	Microbial fuel cell	Japan	NCBI	4.6
	LA11	Marine	Japan	NCBI	4.6
	LPB0137	Environmental	Korea	NCBI	4.6
	W112-28	Wastewater	Spain	URV	4.6

NA, no available; URV, sequenced for this thesis; NCBI, GenBank, JGI, Joint Genome Institute.

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