UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Luis Roberto Collado González

Taxonomy and Epidemiology of the Genus *Arcobacter*

Doctoral Thesis

Directed by Dr. Maria José Figueras
Departament de Ciències Mèdiques Bàsiques
Unitat de Biologia i Microbiologia

UNIVERSITAT ROVIRA I VIRGILI

Reus

2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI

Prof. Dr. Maria Jose Figueras
Unit of Biology and Microbiology
Faculty of Medicine and Health Sciences
University Rovira i Virgili
Sant Llorenc 21, 43201 REUS, SPAIN
Tel 0034 977759321, Fax 0034 977759322
mariajose.figueras@urv.cat

Dr. Maria José Figueras professor of the Department of Basic Health Sciences of the University Rovira i Virgili

CERTIFY THAT:

The present work entitled "Taxonomy and Epidemiology of the Genus *Arcobacter*" presented by Luis Collado González to obtain the degree of doctor by the University Rovira i Virgili, has been carried out under my supervision at the Unit of Microbiology of the Department of Basic Health Sciences, and that it fulfills the requirements to obtain the European Doctorate mention.

Reus, November 17, 2009.

Prof. Maria José Figueras

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

to my loved daughter Angela

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Acknowledgment

I cannot adequately express how grateful I am with Dr. Maria José Figueras (director of my thesis), for the opportunity to work with her. For all her advice, support and for the enthusiasm that she have transmitted to me and confidence during my research.

In addition, I would like to express special gratitude to all other professors of the Unit of Microbiology of URV, especially to Dr. Josep Guarro (Head of the Unit of Microbiology) for his advice, assistance and for the revision of some of the works included here. Also thanks to Dr. Fina Gene for useful discussions about this thesis.

I would like to sincerely thank to Dr. Luis Zaror (Universidad Austral de Chile) for his friendship and advice. He was the one that help me to get here. I want thank also to Dr. Heriberto Fernández (Universidad Austral de Chile), from who I learned so much about campylobacters.

I would like to express my gratitude to Dr. Wim Gaastra and Dr. Marc Wösten, from the Department of Infectious Diseases and Immunology, Utrecht University, not only for accepting me in their group for 3 month, but also for his scientific advice and encouragement. I would also like to thank all the members of the Utrecht research group who made me feel at home with their hospitality, specially to Irati Martinez (University of the Basque Country), who help me a lot in the laboratory just when I arrived and during the period she was there. I always remembered when you accompanied me to buy my second hand bicycle!!

I really appreciate the friendship and help from my labmates: Eduardo, Hugo, Shiva, Mabel, Carolina, Katihuska, Arturo, Fabiola, Jessica, Anabel, Monica, Mery, Cati, Nuria, Enrique, Marçal, Mar, Alexandra, Sarah, Margarita and Haybrig.

I am also deeply grateful to Maria Josep Solsona, Carmen Aguilar, and Maria Teresa Feliu from the “Laboratori de Salut Pública, Serveis Territorials de Salut de Tarragona, Spain”

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

and to Dr. Jesus Romalde from the University of Santiago de Compostela for providing the food samples for our research studies.

I would like to thank to Dr. Robert Madden (Queen's University of Belfast, UK), Dr. Kurt Houf (Ghent University, Belgium), Dr. Nalini Chinivasagam (Animal Research Institute, Queensland, Australia) and Dr. Maqsudul Alam (University of Hawaii, USA) for kindly providing *Arcobacter* strains which were included in our studies.

I owe my sincere thanks to my parents Maria and Luis that provide me their support especially during the very early years in my studies. I also acknowledge efforts of my younger brother Victor, who always have trust and help me.

I want to thank my wife Cecilia for her love and unconditional support that made my dream of postgraduate studies to finally come true. Thank you!!!!!!!!!!!!!!!!!!!!!

Finally, I want to acknowledge the financial support of my doctoral grant given by the Rovira i Virgili University as well as the possibility of carry out my work within the frame of the European projects EPIBATHE and HEALTHY-WATER in which prof. M. J. Figueras participated. I also acknowledge the grants from SEM and FEMS to participate in several congresses and finally the one obtained from Spanish Government which enabled me to expend three month at the Utrecht University, one of the requirements for the European Doctorate mention.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

EXAMINATION COMMITTEE

Prof. Dr. Alberto Ramos Cormenzana
Departament of Microbiology
University of Granada
Granada, Spain

Prof. Dr. Josep Guarro
Departament of Basic Health Sciences
Universitat Rovira i Virgili
Reus, Spain

Prof. Dr. Antonio Ventosa
Departament of Microbiology and Parasitology
University of Sevilla
Sevilla, Spain

Prof. Dr. Ramon Roselló-Móra
Departament de Recursos Naturals
Institut Mediterrani d'Estudis Avancats (CSIC-UIB)
Palma de Mallorca, Spain

Prof. Dr. Robert Madden
Food Science Department,
The Queen’s University of Belfast
Belfast, United Kingdom

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

EXTERNAL EVALUATOR

Prof. Dr. Kurt Houf
Department of Veterinary Public Health and Food Safety
Ghent University
Ghent, Belgium

Prof. Dr. Wim Gaastra
Department of Infectious Diseases and Immunology
Utrecht University
Utrecht, The Netherlands

SUBSTITUTES

Prof. Dr. Roger Stephan
Institute for Food Safety and Hygiene
University of Zurich
Zurich, Switzerland

Prof. Dr. Francisco Javier Pastor
Departament of Basic Health Sciences
Universitat Rovira i Virgili
Reus, Spain

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

CONTENTS

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

LIST OF FREQUENTLY USED ABBREVIATIONS

AEB	Arcobacter enrichment broth
AFLP	Amplified fragment length polymorphism
ASB	Arcobacter selective broth
ASM	Arcobacter selective medium
BA	Blood agar
CAT	cefoperazone-amphotericin B-teicoplanin
CVA	cephalotin-vancomycin–amphotericin B
DNA	deoxyribonucleic acid
DWTP	drinking water treatment plant
Ec	*Escherichia coli*
EMJH	Ellinghausen - McCullough - Johnson - Harris semisolid broth
ERIC	enterobacterial repetitive intergenic consensus
FC	fecal coliforms
HIV	Human immunodeficiency virus
IE	intestinal enterococci
JM	Johnson-Murano culture media
MLST	multilocus sequence typing
m-PCR	multiplex-PCR
MPN	most–probable–number
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PTP	person to person
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscopy
TEM	transmission electron microscopy
VBNC	viable but nonculturable
WWTP	wastewater treatment plant

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

1. INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

The Genus *Arcobacter*: A review of its taxonomy, epidemiology and importance

Luis Collado and Maria José Figueras

Submitted for publication

This introduction intercalates results derived from this doctoral thesis, because have been submitted for publication as an updated review of the genus *Arcobacter* to the Journal of Applied Microbiology.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.1 Abstract

This review updates the knowledge of the genus *Arcobacter* providing the most recent data on the taxonomy, species diversity, methods of detection, identification and on the role that these microbes can play as human and animal pathogens. Special emphasis is made on the studies that demonstrate the incidence of these microbes in food products and water as the routes of transmission. The recently discovered association between *Arcobacter* spp. and indicators of faecal pollution are presented as well as the water outbreaks in which they were involved. The findings provided by the complete genome of *Arcobacter butzleri* have revealed that the proteome of this species seems to be more closely related to members of the family *Helicobactereaceae* or the deep-sea vent *Epsilonproteobacteria* than to *Campylobacter.* Furthermore, virulence determinants homologous to those of *Campylobacter* were discovered such as the fibronectin binding proteins CadF and Cj1349, invasin protein CiaB, the virulence factor MviN, the phospholipase PldA and the TlyA hemolysin.

Data derived from the first MLST analysis of five species, including 374 strains and 7 housekeeping genes (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm* and *tkt*) show a great diversity of sequence types with no association with specific host or geographical regions. In conclusion, it is emphasized that studies are needed that comparatively evaluate the proposed methods of recovery and isolation. Furthermore, identification techniques that enable the 9 species presently accepted in the genus to be recognized should be applied to establish the true relevance of all species in human and veterinary medicine.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.2 INTRODUCTION

The genus *Arcobacter* has become increasingly important in recent decades because its members can act as emergent enteropathogens and/or potential zoonotic agents (Ho *et al*. 2006a, Snelling *et al*. 2006). This genus is considered an atypical group within the epsilon subdivision of the proteobacteria by the wide diversity of habitats and hosts where they can be found (Debruyne *et al*. 2008). Some *Arcobacter* species have been isolated from stool specimens of patients with diarrhoea and occasionally in association with bacteremia (Ho *et al*. 2006a). In animals, arcobacters have been implicated in abortions, mastitis and gastrointestinal disorders but have also been recovered from asymptomatic animals (Vandamme *et al*. 1992b; Van Driessche *et al*. 2003). However, the incidence of *Arcobacter* species is considered to be underestimated due mainly to limitations in current detection and identification methods (Vandenberg *et al*. 2004).

In recent years considerable progress has been made in understanding the taxonomy and pathogenicity of this group of microorganisms and two excellent and complete reviews were provided independently by Ho *et al*. and Snelling *et al*. in 2006. Those studies focussed on the presence of *Arcobacter* in food and water samples, isolation and detection techniques, the pathogenicity in animals and humans and the virulence factors described for the species accepted at that time. Since then important new contributions have been published such as the complete genome of *Arcobacter butzleri* from a human clinical strain, which revealed interesting information about the physiology and genetics of this organism (Miller *et al*. 2007). This is the most important and prevalent species of the genus and has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF 2002). Furthermore, significant advances in the taxonomy, routes of transmission and pathogenicity of this microorganism have been reported (Ho *et al*., 2008a,b; Collado *et al*. 2008, 2009a,b; Bücker *et al*. 2009; Houf *et al*. 2009; Kim *et al*. in press, among others) that justify the need for a reevaluation. In the present overview, special emphasis is made in the data obtained from the genome of *A. butzleri*, the description of novel *Arcobacter* species, advances in the transmission routes as well as the information obtained in recent water and food surveys using novel detection, identification and typing techniques.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.3 TAXONOMY

The genus *Arcobacter* was proposed by Vandamme *et al*. (1991) to accommodate two aerotolerant *Campylobacter* species: *Campylobacter cryaerophila* (now *Arcobacter cryaerophilus*) and *Campylobacter nitrofigilis* (now *Arcobacter nitrofigilis,* the genus type species). The former was isolated from diverse types of samples i.e., faeces, reproductive tract and aborted foetuses of several farm animals and from the milk of cows with mastitis (Neill *et al*. 1985). The latter species is a nitrogen-fixing bacterium isolated from roots and root-associated sediments of *Spartina alterniflora*, a salt marsh plant (McClung *et al*. 1983). The genus was amended and enlarged with the reclassification of *Campylobacter butzleri* (isolated from humans and animals with diarrhea), described by Kiehlbauch *et al*. (1991) as *Arcobacter butzleri* (Vandamme *et al*. 1992b), and with the description of *Arcobacter skirrowii*, recovered from faeces of lambs with diarrhoea, aborted porcine, ovine, and bovine foetuses and from the preputium of bulls. Two groups (named 1A and 1B or 1 and 2) were differentiated within *A. cryaerophilus* by their different restriction fragment length polymorphisms (RFLP) of the ribosomal RNA genes (Kiehlbauch *et al*. 1991), their whole-cell protein and fatty acid contents (Vandamme *et al*. 1992b) and by their amplified fragment length polymorphism (AFLP) profiles (On *et al*. 2003). Group 1B is currently more prevalent than 1A (Kabeya *et al*. 2003b; Son *et al*. 2007; Collado *et al*. 2009b). It remains to be clarified whether these two groups belong to two separate taxa (On *et al*. 2001; Vandamme *et al*. 2005). A very recent study has investigated the taxonomy of these two groups of *A. cryaerophilus* using AFLP and the sequences of the *cpn60* gene. The clusters obtained suggested that the nomenclature of the two groups (1 and 2) should be abandoned and that the current type strain of this species (LMG 24291^T) should be changed for LMG 10829 because it is more representative of this species (Debruyne *et al*. in press).

Two additional species were described in 2005; one of them, was *Arcobacter cibarius*, isolated from broiler carcasses in Belgium (Houf *et al*. 2005), and the other was *Arcobacter halophilus*, described on the basis of a unique strain recovered from a hypersaline lagoon in Hawaii (Donachie *et al*. 2005). The latter represents the first obligate halophilic *Arcobacter* spp.. Very recently, three new species have been added to the genus. *Arcobacter mytili*, isolated from mussels in Spain, which is the first species of the genus unable to hydrolyze indoxil-acetate, a hitherto undescribed feature among *Arcobacter* species (Collado *et al*. 2009a). *Arcobacter thereius* (Houf *et al*. 2009) has been isolated from liver and kidney of spontaneous porcine abortions and from duck's cloacal

ISBN: 978-84-693-0707-6 /DL:T-420-2010

samples (On *et al*. 2003). Finally, *Arcobacter marinus* (also reported on the basis of only one strain) has been isolated from seawater associated with starfish in Korea (Kim *et al*. in press). This new species enlarged the genus to nine validly published species. Table 1 summarizes the features of all the type strains of the currently accepted *Arcobacter* species.

Table 1. Accepted species in the genus *Arcobacter*

SPECIES	TYPE STRAIN	OTHER DESIGNATIONS	SOURCE	REFERENCES
A. nitrofigilis	LMG 7604	CI; ATCC 33309; CCUG 15893; CECT 7204	Roots from *Spartina alterniflora* (Canada)	McClung *et al*. 1983 Vandamme *et al*. 1991
A. cryaerophilus	LMG 9904	D2792; ATCC 43158; LMG 24291; LMG 7536	Brain, aborted bovine foetus (Ireland)	Neill *et al*. 1985 Vandamme *et al*. 1991
A. butzleri	LMG 10828	D2686; ATCC 49616; CCUG 30485; CIP 103493	Faeces, human with diarrhoea (USA)	Kiehlbauch *et al*. 1991 Vandamme *et al*. 1992b
A. skirrowii	LMG 6621	Skirrow 449/80; ATCC 51132; CCUG 10374; CIP 103538	Faeces, lamb with diarrhoea (Belgium)	Vandamme *et al*. 1992b
A. cibarius	LMG 21996	CECT 7203; CIP 108697; CCUG 48482	Broiler carcasses (Belgium)	Houf *et al*. 2005
A. halophilus	ATCC BAA 1022	LA31B; CIP 108450; CCUG 53805	Hypersaline lagoon (USA)	Donachie *et al*. 2005
A. mytili	CECT 7386	F2075; LMG 24559 CIP 110066	Mussels (Spain)	Collado *et al*. 2009a
A. thereius	LMG 24486	CCUG 56902; 16398	Pig abortion (Denmark)	Houf *et al*. 2009
A. marinus	JCM 15502	CL-S1; KCCM 90072	Seawater associated with starfish (Korea)	Kim *et al*. in press

ATCC, American Type Culture Collection, Rockville, Md; CCUG, Culture Collection of the University Göteborg, Göteborg, Sweden; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CIP, Collection Bactérienne de l'Institut Pasteur, Paris, France; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Gent, Belgium; JCM, Japan Collection of Microorganisms; KCCM, Korean Culture Center of Microorganisms, Seoul, Korea.

An obligate microaerophilic organism that oxidizes sulphides had been proposed as a potential novel species *Candidatus* Arcobacter sulfidicus (Wirsen *et al*. 2002). Several new species have been recently recognised by our group such us *Arcobacter valdiviensis* recovered from a chicken cloacal swab sample (Collado *et al*. submitted), *Arcobacter defluvii* isolated from wastewater and *Arcobacter molluscorum* recovered from mussels (Collado *et al*. in preparation) as well as two strains recovered from mussels and from pork meat that are waiting to be formally described and named. In fact, on the basis of 16S rRNA gene sequences deposited in public databases, one can infer the existence of several potentially novel *Arcobacter* species from very different hosts or habitats, i.e.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

sewage (Heylen *et al*. 2006), oysters (Romero *et al*. 2002), oil field environments (Sette *et al*. 2007), associated with cod larviculture (McIntosh *et al*. 2008) or with cyanobacterial mats (Taske *et al*. 1996). Although most of them are sequences from uncultured bacteria, it is clear that several new species will probably be proposed in the near future. Figure 1 shows the phylogenetic relationship among the presently accepted species on the basis of the 16S rRNA gene.

The taxonomy of the genus *Arcobacter*, as that of other bacterial genera, has been based on the analysis of the 16S rRNA gene (Wesley *et al*. 1995). The nine currently accepted species of *Arcobacter* show a similarity of interspecies levels of the 16S rRNA gene ranging from 92.0 to 98.8% (Fig 1). The higher value corresponds to the similarity between *A. cibarius* and *A. cryaerophilus*, and the lower one between *A. thereius* and *A. halophilus*. Some housekeeping genes have been investigated in *Arcobacter*, such as the *gyrA* (Albdelbaqi *et al*. 2007b) and *rpoB-rpoC* (Morita *et al*. 2004) to better differentiate the species and their phylogenetic relationships. However, only two recent studies have evaluated the phylogeny of the genus on the basis of housekeeping genes using all available type strains of the accepted species with the *rpoB* (Collado *et al*. 2009a) and *cpn60* genes (Debruyne *et al*. in press).

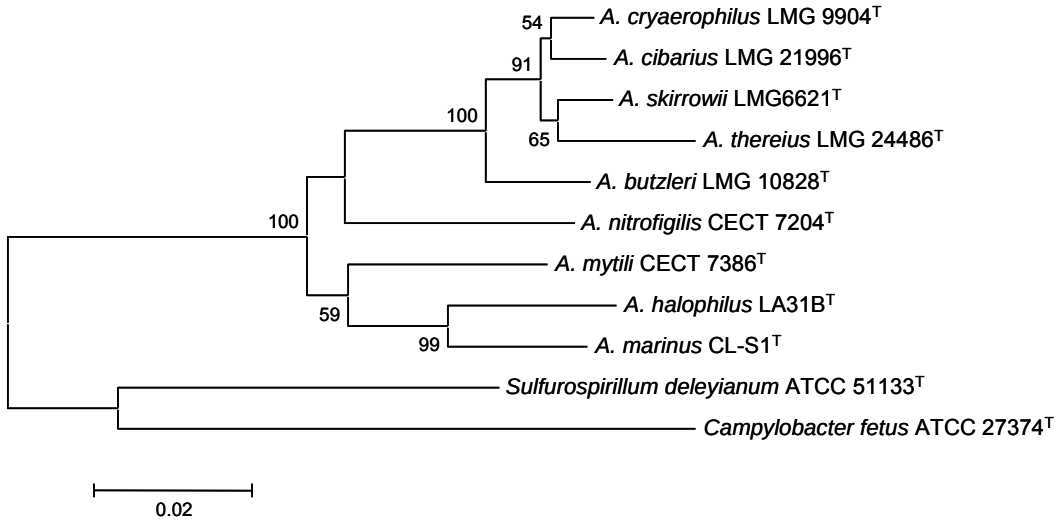


Figure 1 Neighbour-joining phylogenetic tree showing the relationship of the accepted *Arcobacter* species on the basis of the 16S rRNA gene. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

In *Arcobacter* taxonomy it is important to take into account that although this genus is included in the family *Campylobacteraceae*, together with the genera *Campylobacter* and *Sulfurospirillum* (Vandamme *et al*. 2005), recent data from the genome of *A. butzleri* showed an important number of genes involved in sulphur metabolism that were more in common with free-living taxa such as *Nitratiruptor*, *Sulfurovum* and *Sulfurimonas* (Miller *et al*. 2007). A phylogenetic study using 60 genes, taken from the available genome of *A. butzleri* and members of *Campylobactereaceae* and related bacteria, showed *Sulfurimonas denitrificans* as the species most closely related to *A. butzleri* (Debruyne *et al*. 2008). These data suggests that the inclusion of *Arcobacter* in the family *Campylobacteraceae* should be revised (On and Owen 2009).

Several genome projects are currently being developed in different *Arcobacter* species. In the last *Campylobacter*, *Helicobacter* and Related Organism Congress (CHRO 2009), it was reported that the ongoing genome sequencing of a bovine strain of *A. butzleri* showed a considerable divergence from the human strain RM4018 (Sloane *et al*. 2009). Furthermore, at the web site (http://www.ncbi.nlm.nih.gov/) of the National Center for Biotechnology Information (NCBI) it is possible to find at least two other ongoing genome sequencing projects, one for *A. nitrofigilis* DSM 7299 and other for *Candidatus* Arcobacter sulfidicus. Miller *et al*. (2009) used the genome of *A. butzleri* (Miller *et al*. 2007), and the draft genome of *A. halophilus* LA31B, to design Multilocus Sequence Typing (MLST) primers.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.4 ISOLATION AND DETECTION

Despite several media and procedures having been used to isolate *Arcobacter* in different samples, a standardized reference method has not so far been proposed. The first isolated *Arcobacter* species (at that time named a *Spirillum*/*Vibrio*-like organism) was recovered from aborted bovine foetuses using the Ellinghausen-McCullough-Johnson-Harris (EMJH) *Leptospira* medium (Ellis *et al*. 1977). Since then, several media and protocols which are summarized in Table 2 have been used to recover *Arcobacter* from different types of samples. One of the most used *Arcobacter* isolation protocols is based on the use of an enrichment broth supplemented with cefoperazone, amphotericin B and teicoplanin, known as CAT broth, followed by passive filtration of the broth through a 0.45 µm filter placed over blood agar (Atabay and Corry 1997). Later, Johnson and Murano (1999) proposed a new enrichment broth and isolation medium with cefoperazone and 5-fluorouracil as selective supplements, achieving a good recovery of *Arcobacter* and a strong inhibition of other bacteria. Another very popular method was designed after an antimicrobial susceptibility study by Houf *et al.* (2001), proposing an *Arcobacter* selective isolation protocol incorporating 5 antibiotics in the enrichment and plating media. This media was later modified by Van Driessche *et al.* (2003) for the isolation of these bacteria from animal faecal specimens, adding cycloheximide and increasing the novobiocin concentration. Recently, Scullion *et al*. (2004), using the methods of Houf *et al*. (2001) and Johnson and Murano (1999) in parallel, obtained 25% more positive samples from poultry than when the results obtained with each method independently were considered. Despite the availability of all those media to recover *Arcobacter*, there is a lack of consensus about what the ideal isolation method should be because few comparative studies have been carried out using all the described methods. Some of the recovery problems reported include the inhibition of some *Arcobacter* species when using certain antibiotics (Houf *et al*. 2001) and the insufficient inhibition of the accompanying microbiota (Fera *et al*. 2004). Generally, as indicated above, *Arcobacter* isolation includes an enrichment step in a broth containing several antibiotics (which usually takes 48h) followed by incubation in agar media (with or without antibiotics) for an additional period of 48 to 72h. However, it has been reported that the enrichment step reduces the diversity of species recovered in the later cultures in comparison with direct plating because it favours the fast-growing species (Houf *et al*. 2002). This may also affect their direct molecular detection from enrichment broth. Regarding this, Ho *et al*. (2006b) demonstrated that often only the predominant species are detected from the broth using the multiplex PCR (m-PCR) assay proposed by

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Houf *et al*. (2000). The direct detection from the CAT broth by m-PCR and the identification of isolates recovered by culture, after passive filtration of the broth on blood agar, produced more or less the same results in our studies (Collado *et al*. 2008, 2009b), which agrees with Houf *et al*. (2000). However, other authors (Fera *et al*. 2004; González *et al*. 2007) have found big discordances between molecular detection and culturing, which could be due to the protocol used for culturing that includes a short enrichment period (24 h) or to the use of a plating media with insufficient inhibition over other bacteria because no filtration was used (Collado *et al*. 2008). Moreover, missing co-existing strains or species may result from picking only a few colonies from the isolation plates (Ho *et al*. 2006a). Another poorly-explored aspect is the need or not for microaerophilic conditions for the initial recovery of *Arcobacter* from both clinical and environmental samples. Reviewing the available data in the literature, approximately 50% of the studies used aerobic conditions in the isolation procedures. Only one study used aerobic and microaerophilic conditions in parallel, but gave inconclusive results (González *et al*. 2007).

Several molecular detection methods have been developed for *Arcobacter*, aimed at improving sensivity and reducing the time required to carry out the experiments. At least three real-time PCRs, using the TaqMan (Brightwell *et al*. 2007), Fluorescence Resonance Energy Transfer (Abdelbaqi *et al*. 2007a) and SYBR Green (González *et al*. 2009) technology, respectively, have been designed. When two of the latter methods were compared with the conventional m-PCR (Houf *et al.* 2000) and applied to food and water samples, they provided a 2 log improvement in sensitivity (Brightwell *et al*. 2007; González *et al*. in press). On the other hand, a DNA Microarray method targeting housekeeping and virulence-associated genes have been developed for the detection of *A. butzleri*, *Campylobacter jejuni* and *Campylobacter coli* and have shown a high level of specificity and sensitivity (Quiñones *et al*. 2007).

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 2. Media and procedures used for *Arcobacter* isolation from different types of samples[a]

Method	Enrichment			Isolation		
	Semisolid or broth medium	Selective antibiotics (concentration)	Incubation conditions	Plating medium	Selective antibiotics (concentration) or procedure	Incubation conditions
Ellis *et al*. 1977	EMJH	5-fluorouracil (100 mg/l)	30ºC, 48-72h, mO_2	Blood agar	No antibiotics	30ºC, 48-72h, mO_2/O_2
De Boer *et al*. 1996	ASB	Cefoperazone (32 mg/l) Piperacillin (75 mg/l) Trimethoprim (20 mg/l) Cycloheximide (100 mg/l)	24ºC, 48h, O_2	ASM	Cefoperazone (32 mg/l) Piperacillin (75 mg/l) Trimethoprim (20 mg/l) Cycloheximide (100 mg/l)	24ºC, 48-72h, O_2
Collins *et al*. 1996	EMJH	5-fluorouracil (200 mg/l)	30ºC, 9 days, O_2	CVA	Cephalothin (20 mg/l) Vancomycin (10 mg/l) Amphotericin B (5 mg/l)	30ºC, 48-72h, mO_2
Atabay and Corry 1997	AEB[b]	Cefoperazone (8mg/l) Amphotericin B (10 mg/l) Teicoplanin (4 mg/)	30ºC, 48h, mO_2	Blood agar	No antibiotics Membrane filtration	30ºC, up to 7 days, O_2
Johnson and Murano 1999	JM	Cefoperazone (32 mg/l) 5-fluorouracil (200 mg/l)	30ºC, 48h, O_2	JM agar	Cefoperazone (32 mg/l)	30ºC, 48h, O_2
Houf *et al*. 2001[c]	Arcobacter broth	Cefoperazone (16 mg/l) Amphotericin B (10 mg/l) 5-fluorouracil (100 mg/l) Novobiocin (32 mg/l) Trimethoprim (64 mg/l)	28ºC, 48h, mO_2	Arcobacter plating medium	Cefoperazone (16 mg/l) Amphotericin B (10 mg/l) 5-fluorouracil (100 mg/l) Novobiocin (32 mg/l) Trimethoprim (64 mg/l)	30ºC, 24-72h, mO_2

[a]Adapted and completed from Scullion *et al*. 2004
[b]The AEB supplemented with cefoperazone, amphotericin and teicoplanin usually is called CAT broth.
[c]Van Driessche *et al*. 2003 modified the selective supplement of Arcobacter broth adding cycloheximide and increasing the novobiocin concentration.
EMJH, Ellinghausen-McCullough-Johnson-Harris semisolid medium; ASB, Arcobacter selective broth; ASM, Arcobacter selective medium; CVA, cephalotin, vancomycin and amphotericin B agar; AEB: Arcobacter enrichment broth; JM, Johnson-Murano broth; O_2, aerobic conditions; mO_2, microaerobic conditions

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.5 IDENTIFICATION

Arcobacter spp. show similar morphological characteristics to *Campylobacter* i.e., Gram negative, slender spirally curved rod, often S-shaped or helical cells, motile by means of a single polar unsheathed flagellum at one or both ends of the cells (Figure 2) (Debruyne *et al*. 2008). They can be differentiated from *Campylobacter* spp. by their ability to grow in air and at lower temperatures ranging from 15 to 30°C. In general, classical biochemical tests routinely used for the identification of clinical bacteria often yielded negative or variable results with *Arcobacter* species (Debruyne *et al*. 2008). On and Holmes (1991, 1992, 1996) standardized the inoculum and biochemical identification tests for campylobacters and from their results a set of tests useful for distinguishing *Arcobacter* species were proposed in Bergey's Manual (Vandamme *et al*. 2005). However, at least five novel *Arcobacter* species have been described since then. Table 3 shows the most useful biochemical tests to differentiate the nine currently accepted *Arcobacter* species.

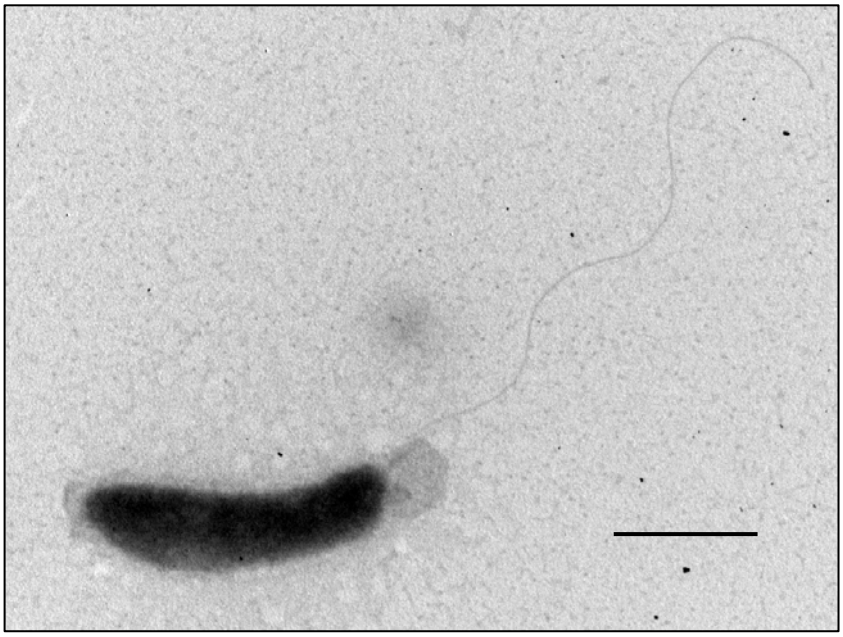


Figure 2 Image of *Arcobacter mytili* (strain F2075^T) as observed with transmission electron microscopy, negatively stained. Bar, 1 µm.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 3. Phenotypic characteristics of all the accepted *Arcobacter* species[a].

Characteristic	*A. butzleri*	*A. cryaerophilus*	*A. skirrowii*	*A. cibarius*	*A. thereius*	*A. nitrofigilis*	*A. mytili*	*A. halophilus*	*A. marinus*
Catalase activity	V	+	+	V	+	+	+	-	-
Urease activity	-	-	-	-	-	+	-	-	-
Nitrate reduction	+	+[b]	+	-	+	+	-	+	+
Indoxyl acetate hydrolysis	+	+	+	+	+	+	-	+	+
Growth in:									
O_2 at 37ºC	+	V	+	-	-	V	+	+	+
mO_2 at 37ºC	+	V	+	+	-	-	+	+	+
Growth on:									
Media w/1%glycine	-	-	-	-	+	-	+[d]	-	ND
4% (W/V) NaCl	-	-	+	-	-	+	+	+	+
Mac Conkey agar	+	V	-	+	V	-	+	-	ND
Minimal media	+	-[c]	-	+	+	-	-	-	ND
Resistance to:									
Cefoperazone (64 mgl^{-1})	+	+	+	+	+	-	-	-	-

[a]Data taken from On *et al*. 1996; Donachie *et al*. 2005; Houf *et al*. 2005, 2009; Collado *et al*. 2009a ; Kim *et al*. in press. +, ≥95% Strains positive; -, ≤11% strains positive; V, 12–94% strains positive. O_2, aerobic conditions; mO_2, microaerobic conditions; ND, not determined.
[b]Two of the four strains tested in Collado *et al*. (2009a) (LMG 9904T and LMG 9065) were negative.
[c]Two of the four strains tested in Collado *et al*. (2009a) (LMG 7537 and LMG 10241) were positive.
[d]Data from this study.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Due to the difficult phenotypic characterization of *Arcobacter* spp., several molecular methods have been designed for its identification at genus and species level (Table 4). The more globally used method is the m-PCR (targeting the 16S and 23S rRNA genes), developed for the simultaneous detection and identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* by Houf *et al*. (2000). Despite this method being very popular it produces misidentification of *A. nitrofigilis* with *A. skirrowii* and also confuses with the latter species the recently proposed with *A. mytili* (Collado *et al*. 2009a). Furthermore the recently described species *A. thereius* is also confused with *A. cryaerophilus* (Houf *et al*. 2009). Another m-PCR for the identification of species considered of medical importance (*A. butzleri*, *A. cryaerophilus* and *A. skirrowii*) was described by Kabeya *et al.* (2003a), a method that can differentiate the two DNA groups of *A. cryaerophilus* but has not gained much popularity. Furthermore, as has been commented before, several other molecular methods for detection and identification of *Arcobacter* have recently been described i.e. PCR-DGGE (Petersen *et al*. 2007), Real time-PCR (Abdelbaqi *et al*. 2007a, Brightwell *et al*. 2007) and DNA microarray assay (Quiñonez *et al*. 2007), though none of them allow the detection and/or identification of all the accepted *Arcobacter* species. A recently proposed method based on the 16S rDNA-RFLP patterns differentiated all the accepted species *A. butzleri*, *A. cryaerophilus*, *A. cibarius*, *A. skirrowii, A. nitrofigilis* and *A. halophilus* (Figueras *et al*. 2008). This method has not only been successfully used for the identification of more than 600 *Arcobacter* strains in several studies (Figueras *et al*., 2008; Collado *et al*. 2008, 2009b, submitted) but has also enabled us to recognize new *Arcobacter* species such as *A. mytili* (Collado *et al*. 2009a) and other new candidate species on the basis of the new RFLP patterns observed. This method can also differentiate the new species *A. marinus*, which also possesses a distinctive pattern observed after simulating the digestion of the 16S rRNA gene of this species with the *Mse*I enzyme. However, this method cannot differentiate the recently described species *A. thereius* because it produces the same pattern as *A. butzleri* (Collado *et al*. 2009b). This data demonstrates that until a new identification method is developed for the characterization of the species with common RFLP patterns, the best way to identify the strains is to use both methods (m-PCR and the 16S rDNA-RFLP) in parallel and to further sequence the 16S rRNA or the *rpoB* genes in cases of incongruent results. So far, in our hands this has been the only way to obtain accurate identification of all *Arcobacter* species.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 4. Comparison of molecular methods for *Arcobacter* spp. identification

Reference	Method	Gene(s) targeted	Specie(s) discriminated	Comments
Kiehlbauch *et al*. 1991	RFLP Southern blot	16S rRNA 23S rRNA	*A. butzleri*	Equal pattern for *A. cryaerophilus* and *A. skirrowii*
Cardarelli-Leite *et al*. 1996	PCR-RFLP	16S rRNA	*A. butzleri*	Equal pattern for *A. cryarophilus*, *A. skirrowii* and *A. nitrofigilis*
Harmon and Wesley 1997	Multiplex PCR	16S rRNA 23S rRNA	*Arcobacter* sp. *A. butzleri*	Equal pattern for *A. cryaerophilus* and *A. skirrowii*
Hurtado and Owen 1997	PCR-RFLP	23S rRNA	*A. butzleri* *A. nitrofigilis*	Equal pattern for *A. cryaerophilus* and *A. skirrowii*
Marshall *et al*. 1999	PCR-RFLP	16S rRNA	*A. butzleri* *A. cryaerophilus* *A. skirrowii*	
Houf *et al*. 2000	Multiplex PCR	16S rRNA 23S rRNA	*A. butzleri* *A. cryaerophilus* *A. skirrowii*	Confusion with some species has been reported by Figueras *et al* (2008), Collado *et al*. (2009b) and Houf *et al.* (2009).
Kabeya *et al*. 2003a	Multiplex PCR	23S rRNA	*A. butzleri* *A. cryaerophilus* 1A *A. cryaerophilus* 1B *A. skirrowii*	The concentration of the DNA has to be accurately adjusted to 20 ng/tube because any difference in the concentration of the template produces some nonspecific amplifications (Kabeya *et al*. 2003a)
Kärenlampi *et al*. 2004	PCR-RFLP	*groEL*	*A. butzleri*	No other *Arcobacter* species were tested
González *et al*. 2006	PCR-RFLP	16S rRNA 23S rRNA	*A. butzleri*	Equal pattern for *A. cryaerophilus* and *A. skirrowii*
Petersen *et al*. 2007	PCR-DGGE	16S rRNA	*A. cryaerophilus* 1B *A. nitrofigilis*	Equal pattern for *A. cryarophilus* 1A, *A. butzleri* and *A. skirrowii*

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 4. (continuation) Comparison of molecular methods for *Arcobacter* spp. identification

Reference	Method	Gene(s) targeted	Specie(s) discriminated	Comments
Brightwell *et al*. 2007	Real time PCR	*rpo*B/C 23S rRNA	*A. butzleri* *A. cryaerophilus*	Not tested in other studies
	Multiplex PCR	*rpo*B/C 23S rRNA	*A. butzleri* *A. cryaerophilus*	The m-PCR uses primers CRY1-CRY2 from Houf *et al*. (2000), for which an unspecific reaction has been reported (Houf *et al*. 2009).
Abdelbaqi *et al*. 2007a	Real time PCR	*gyr*A	*A. butzleri* *A. cryaerophilus* *A. cibarius* *A. nitrofigilis*	Failed to detect *A. skirrowii*
Figueras *et al*. 2008	RFLP	16S rRNA	*A. butzleri* *A. cryaerophilus* 1A *A. cryaerophilus* 1B *A. skirrowii* *A. cibarius* *A. nitrofigilis* *A. halophilus* *A. cibarius* *A. mytili*	The new species *A. thereius* (Houf *et al*. 2009) showed the same restriction pattern as *A. butzleri* (Collado *et al*. 2009b). A specific pattern was obtained for *A. marinus* after a computer simulation (Collado *et al*. unpublished data).
Pentimalli *et al*. 2009	PCR	*gyr*A 16S rRNA	*A. butzleri* *A. cryaerophilus* *A. skirrowii* *A. cibarius*	

RFLP, restriction fragment length polymorphisms; DGGE, denaturing gradient gel electrophoresis

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.6 GENOTYPING

Different methods have been applied for distinguishing one strain of *Arcobacter* from another, for studying transmission routes or for tracing sources of outbreaks, including several PCR methods such as the enterobacterial repetitive intergenic consensus (ERIC-PCR), the randomly amplified polymorphic DNA (RAPD-PCR) (Houf *et al*. 2002), AFLP (On *et al*. 2003, 2004) and the pulsed field gel electrophoresis PFGE (Hume *et al*. 2001). Each of these methods has advantages and disadvantages related to its typeability, reproducibility, simplicity, discriminatory power and its cost (Houf *et al*. 2002). The most used typing technique has been the ERIC-PCR, which has been successfully applied for investigating outbreaks (Vandamme *et al*. 1993), for the characterization of isolates from foods (Houf *et al*. 2002; Aydin *et al*. 2007) and for the differentiation of strains included in the description of novel *Arcobacter* species (Houf *et al*., 2005, 2009; Collado *et al*., 2009a). The first website database for MLST (http://pubmlst.org/arcobacter/) targeting seven genes of 374 strains belonging to five species of the genus (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius* and *A. thereius*), has recently been created by Miller *et al*. (2009). The site has information for primers and sequencing conditions for the seven genes (*aspA*, *atpA*, *glnA*, *gltA*, *pgm*, *tkt* and *glyA*) and provides a way to submit new sequences. However, the results of the MLST approach did not find any association of the sequence types with host or geographical sources, thus corroborating the high genetic diversity within the *Arcobacter* spp. reported by previous studies using other typing methods (Hume *et al*. 2001; Houf *et al*. 2002; On *et al*. 2003; Aydin *et al*. 2007). The wide variation in the genotypes may be due to multiple sources of contamination or, as has been suggested for *Campylobacter*, to their ability to incorporate exogenous DNA or to undergo genomic rearrangement by multiple recombination (Hume *et al*. 2001), but this has not yet been demonstrated in *Arcobacter* species.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.7 CLINICAL IMPORTANCE

1.7.1 *Arcobacter* in humans

Although the role of *Arcobacter* species in human diseases in not yet well established, *A. butzleri* and *A. cryaerophilus* have been associated with gastrointestinal diseases on several occasions (Vandamme *et al*. 1991, Vandenberg *et al*. 2004, Wybo *et al*. 2004). In those cases watery and persistent diarrhoea was the main symptom, in contrast with the *Campylobacter* intestinal infection associated mostly with bloody diarrhoea (Vandenberg *et al*. 2004). A reported outbreak in an Italian nursery and in a primary school showed that infections associated with *A. butzleri* in humans can be severe enough to require hospitalization (Vandamme *et al*. 1992a). So far, *A. skirrowii* has only been associated with human infections on two occasions, one affecting an elderly patient with chronic gastrointestinal disease (Wybo *et al*. 2004) and the other in a study that investigated the incidence of *Arcobacter* in stool specimens from patients (with and without HIV) in South Africa (Samie *et al*. 2007). Additionally, *A. butzleri* and *A. cryaerophilus* have also occasionally been reported to cause bacteraemia (On *et al*. 1995; Hseuh *et al*. 1997; Yan *et al*. 2000; Lau *et al*. 2002). In one of theses cases, *A. butzleri* was isolated from the blood of a neonate and clinical data indicated an in utero sepsis, suggesting a vertical or transplacental transmission (On *et al*. 1995).

In two independent studies performed in Belgium and France (Vandenberg *et al*. 2004; Prouzet-Mauléon *et al*. 2006), *A. butzleri* was the fourth most common *Campylobacter*-like organism recovered from human stools of patients with diarrhoea. Prevalence values reported for *Arcobacter* using culture methods range from 0.33% in South Africa (Lastovica and Le Roux 2001) to 2.4% in Thailand (Taylor *et al*. 1991). However, using molecular detection methods prevalence was higher, ranging from 1.2% in France (Abdelbaqi *et al*. 2007a) to 12.9% in South Africa (Samie *et al*. 2007). The few studies performed makes it impossible to establish if prevalence varies between developed and developing countries.

Only a few studies have reported the isolation of *Arcobacter* species from faeces of healthy people (Vandenberg *et al.* 2004; Houf and Stephan 2007; Samie *et al*. 2007). Regarding this, *A. cryaerophilus* was found in 1.4% of stool specimens from asymptomatic people working in a slaughterhouse environment in Switzerland (Houf and Stephan 2007). Another study conducted in Belgium, described human asymptomatic carriage of *A. butzleri* as being more frequent than for *C. jejuni* but without significant differences (Vandenberg *et al.*

ISBN: 978-84-693-0707-6 /DL:T-420-2010

2004). In South Africa, *Arcobacter* has also been detected from asymptomatic people (3%), but with a lower prevalence than from people suffering diarrhoea (12.9%) (Samie *et al*. 2007). In the latter study *Arcobacter* species were often recovered together with another pathogen such as *C. jejuni*, *C. coli*, *Campylobacter concisus* and *Helicobacter pylori*.

Although *Arcobacter* spp. are not currently considered microorganisms of major public health concern, increasing data suggests that their significance in human infections may be underestimated, mainly by the inappropriate detection and identification methods (Vandenberg *et al*. 2004; Snelling *et al*. 2006; Figueras *et al*. 2008). One of the major pitfalls is that the optimal conditions for recovery of *Arcobacter* from clinical specimens have not yet been determined. In fact, despite only some *A. butzleri* and *A. skirrowii* strains being able to grow at 42ºC (On *et al*. 1996), in most laboratories this is the only temperature used for isolation of campylobacters (Abdelbaqi *et al*. 2007a). Furthermore, the non-*C. jejuni/C. coli* and related organisms are rarely identified to the species level (Vandenberg *et al*. 2004). To enhance recovery and proper identification of *Arcobacter* spp. a standardized isolation and characterization protocol should be established to determine the true role and prevalence of these microbes in human disease.

Although it has not been clearly established if host predisposition factors such as age and immune status play a role in *Arcobacter* infections, it is probable that they do, as occurs with other microbes. Vandenberg *et al*. (2004), described infection in people with ages ranging from 30 days to 90 years in Belgium. Also, in India, *Arcobacter* has been isolated in 1.5% of the faeces of patients with diarrhoea affected by HIV and in 1% from those not affected by this disease (Kownhar *et al*. 2007).

1.7.2 *Arcobacter* in animals

Arcobacter has frequently been isolated from the intestinal tract and faecal samples of different farm animals, but it apparently only has the capacity to cause disease in some of them. The most serious effects of *Arcobacter* in animals include abortions, mastitis and diarrhoea (Logan *et al*. 1982; Vandamme *et al*. 1992b). Although *Arcobacter* has been associated several times with bovine abortion (Ellis *et al*. 1977; Neill *et al*. 1985; Fernández *et al*. 1995b), these bacteria have also been recovered from healthy bovine preputial sheath washings (Gill 1983) as well as from vaginal swabs of cows with no reproduction problems (Kabeya *et al*. 2003b). Association with porcine abortion, with sows with reproductive problems, and with preputial fluid of boars and fattening pigs has also been reported (Neill *et*

ISBN: 978-84-693-0707-6 /DL:T-420-2010

al. 1985; De Oliveira *et al*. 1997). *Arcobacter cryaerophilus* is the species predominantly associated with animal abortion while *A. butzleri* and *A. skirrowii* are less frequent (Schroeder-Tucker *et al*. 1996; De Oliveira *et al*. 1997; On *et al*. 2003). The recently described species *A. thereius* was also recovered from liver and kidney of spontaneous porcine abortions, but despite no other established abortifacient agents being detected, the pathogenic role of this recently described species remains unknown (Houf *et al*. 2009).

Logan *et al*. (1982) reported the isolation of an *Arcobacter* sp. isolate (then identified as an aerotolerant *Campylobacter*) from a milk sample during the course of an outbreak of mastitis in a dairy herd. In that study, four cows were experimentally intramammary infected with the outbreak strain and all of them developed an acute clinical mastitis that resolved spontaneously after 5 days. Among the strains included in the description of *A. cryaerophilus*, one was isolated from the milk of a cow with mastitis (Neill *et al*. 1985, Vandamme *et al*. 1992b). *Arcobacter butzleri* has been associated with enteritis and diarrhoea in pigs, cattle, and horses while *A. skirrowii* has been associated with diarrhoea and haemorrhagic colitis in sheep and cattle (Vandamme *et al*. 1992b; Ho *et al*. 2006a). *Arcobacter butzleri* is the only species isolated both from healthy non-human primates and from those with diarrhoea in several studies (Anderson *et al*. 1993; Higgins *et al*. 1999; Wesley *et al*. 2003; Stirling *et al*. 2008). In one of these studies, the histological examination of the enteric tissues of the infected animals revealed a chronic active colitis (Anderson *et al*. 1993). In other cases, the strains recovered showed a strong resistance to antibiotics (Higgins *et al*. 1999; Stirling *et al*. 2008). Despite this data, the significance of arcobacters as a pathogen in non-human primates has yet to be determined.

Most clinical cases affecting animals are restricted to mammals, although one study (Yildiz and Aydin 2006) reported the isolation of *A. cryaerophilus* from a naturally infected rainbow trout (*Oncorhynchus mykiss*). The pathogenicity of the recovered strain was demonstrated by *in vivo* experimental infection, causing the death of the fish that showed liver, kidney and intestine damage.

Faecal shedding of *Arcobacter* is well known in poultry i.e. chicken, ducks, turkeys, and domestic geese (Atabay *et al*. 2006, 2008). However, there have been no reports of association with diseases, which would indicate that poultry is a natural reservoir of *Arcobacter* species.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.7.3 Virulence Factors

Little is known about the pathogenic mechanisms or potential virulence factors of *Arcobacter* spp. However, the first studies performed showed that *A. cryaerophilus* had a toxigenic capacity because with the rat ileal loop test it was able to increase electrolytes and to accumulate fluids. Furthermore, it showed *in vitro* invasion of Hep-2 cells (Fernández *et al*. 1995a). Other studies have demonstrated that *A. butzleri* can invade and colonize piglets and chickens (Wesley *et al*. 1996; Wesley and Baetz 1999). The adhesion, invasion and toxygenic properties of *Arcobacter* species in different cell lines of human or animal origin corroborate those initial *in vivo* findings (Table 5). The mechanisms by which *A. butzleri* induces enteritis have been very recently reported and comprise an epithelial barrier dysfunction by a reduced expression of claudin-1,-5, and -8 as well as an induction of epithelial apoptosis, resulting in a leak flux mechanism and diarrhoea (Bücker *et al*. 2009). The induction of the expression of the proinflammatory cytokine interleukin-8, which is considered a major virulence factor in *H. pylori* and *Campylobacter* spp., has also been reported for *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* (Ho *et al*. 2007).

In comparison with *Campylobacter*, almost nothing is known about which *Arcobacter* genes are involved in the virulence mechanisms. The recently published sequence of the whole genome of *A. butzleri* (strain RM 4018, a derivate of the type strain ATCC 49616) revealed that this strain possesses some putative virulence determinants homologous to *C. jejuni* such as the genes coding for fibronectin binding proteins CadF and Cj1349, invasin protein CiaB, virulence factor MviN, phospholipase PldA and the TlyA hemolysin (Miller *et al*. 2007). It is still unknown if these putative virulence determinants are functional or if they have a similar role to their *Campylobacter* homologs (Miller *et al*. 2007). The genome sequence of *A. butzleri* does not present the genes that encode for the cytolethal distending toxin (CDT) (Miller *et al*. 2007), which produces citotoxicity on eukaryotic cells by breaking the double stranded DNA. This corroborates previous findings by Johnson and Murano (2002) who did not detect CDT genes by PCR in 6 *A. cryaerophilus* and 18 *A. butzleri* strains from different origins. Ho *et al.* (2008b) studied the flagella and demonstrated that *Arcobacter* possess two flagellin genes (*flaA* and *flaB*), only *flaA* being necessary for motility. With the publication of the genome of *A. butzleri* (Miller *et al*. 2007) and with the recent development of tools for construction of *Arcobacter* mutants (Ho *et al*. 2008b), it can be foreseen that new insights into the virulence of these microorganisms will soon be available.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 5. Summary of *in vitro* studies on the pathogenicity of *Arcobacter* species on different cell lines[a]

Species/Cell line	Origin of the strains	Adhesion	Invasion	Cytotoxicity	Reference
A. butzleri					
Hep-2	Sea water	6/17[b]	ND	ND	Carbone *et al*. 2003
	Human faeces	12/12	4/12	3/12	Vandenberg *et al*. 2004
	Zooplankton	4/4	ND	ND	Gugliandolo *et al*. 2008
Hela	River water	6/17	ND	ND	Carbone *et al*. 2003
	River water	1/8	0/8	ND	Mussmano *et al*. 1997
	River water	ND	ND	3/3	Johnson and Murano 2002
	Animal /Human	ND	ND	3/3	Johnson and Murano 2002
	Zooplankton	4/4	ND	ND	Gugliandolo *et al*. 2008
INT407	River water	1/8	0/8	ND	Mussmano *et al*. 1997
	River water	ND	ND	3/3	Johnson and Murano 2002
	Animal /Human	ND	ND	3/3	Johnson and Murano 2002
Vero	River water	ND	ND	17/18	Mussmano *et al*. 1997
	Sea water	ND	ND	5/17	Carbone *et al*. 2003
	Meats	ND	ND	76/80	Villarruel-Lopez *et al*. 2003
	Zooplankton	ND	ND	3/4	Gugliandolo *et al*. 2008
CHO	River water	ND	ND	17/18	Mussmano *et al*. 1997
Caco-2	Human blood	1/1	0/1	ND	Ho *et al*. 2007
IPI-2I	Human blood	1/1	0/1	ND	Ho *et al*. 2007
A. cryaerophilus					
Hep-2	Swine faeces	ND	1/1	ND	Fernández *et al*. 1995a
	Bovine foetus	ND	1/1	ND	Fernández *et al*. 1995a
	Human faeces	4/7	ND	ND	Houf and Stephan 2007
Hela	River water	ND	ND	3/3	Johnson and Murano 2002
	Animal /Human	ND	ND	3/3	Johnson and Murano 2002
INT407	River water	ND	ND	3/3	Johnson and Murano 2002
	Animal /Human	ND	ND	3/3	Johnson and Murano 2002
Vero	Meats	ND	ND	2/2	Villarruel-Lopez *et al*. 2003
Caco-2	Porcine/ovine	4/4	2/4	ND	Ho *et al*. 2007
Caco-2	Human faeces	2/7	ND	ND	Houf and Stephan 2007
IPI-2I	Porcine/ovine	4/4	1/4	ND	Ho *et al*. 2007
A. skirrowii					
Vero	Meats	ND	ND	17/19	Villarruel-Lopez *et al*. 2003
Caco-2	Porcine/ovine	2/2	0/2	ND	Ho *et al*. 2007
IPI-2I	Porcine/ovine	2/2	0/2	ND	Ho *et al*. 2007
A. cibarius					
Caco-2	Chicken carcass	1/1	0/2	ND	Ho *et al*. 2007
IPI-2I	Chicken carcass	1/1	0/2	ND	Ho *et al*. 2007

[a]Updated from Ho *et al*. (2006a)
[b]Number of positive samples/number of tested samples
ND, not determined

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.7.4 Antibiotic resistance

As occurs with *Campylobacter*, the majority of cases of clinical enteritis by *Arcobacter* are presumed to be self-limiting and do not require antimicrobial treatment. However, the severity or prolongation of symptoms may justify the use of antibiotic treatment (Skirrow and Blaser 2000). Antimicrobial susceptibility tests for *Arcobacter* species are not standarized (Vandenberg *et al*. 2006), and there have been few studies carried out using different methods i. e., E-test, agar dilution, disc agar diffusion or broth microdilution methods (Harraß *et al*. 1998; Houf *et al*. 2004, Fera *et al*. 2003; Vandenberg *et al*. 2006; Son *et al*. 2007). Results have shown that human, food and environmental strains of *A. butzleri* and *A. cryaerophilus* are generally susceptible to those drugs considered of choice for treating *Campylobacter* infections, such as erythromycin, ciprofloxacin, tetracycline and gentamicin (Vandenberg *et al*. 2006; Son *et al*. 2007). However, there are a high number of strains of *A. butzleri* resistant to other antibiotics such as clindamycin, azithromycin and/or nalidixic acid (Son *et al*. 2007). Fluroquinolones have been suggested for the treatment of infections produced by *A. butzleri* (Vandenberg *et al.* 2006), but, Son *et al*. (2007) suggested tetracycline for the treatment of *Arcobacter* infections in humans and animals. A genetic mechanism involved in the antibiotic resistance has recently been described, based on a mutation in the quinolone resistance-determining region of *gyrA* gene, which was detected in two *A. butzleri* and one *A. cryaerophilus* strains resistant to ciprofloxacin (Abdelbaqi *et al*. 2007b).

The *A. butzleri* strain (RM 4018) from which the complete genome was sequenced showed a remarkably high antibiotic resistance associated with the presence or absence of specific genes (Miller *et al*. 2007). In this sense the presence of the cat gene (encoding a chloramphenicol O-acetyltransferase) was related to chloramphenicol resistance, three putative β-lactamase genes or the *lrgAB* operon were associated with the β-lactam resistance and the absence of the *upp* gene (encoding for uracil phosphoribosyltransferase) with the 5-fluorouracil resistance (Miller et al. 2007). Transference of antibiotic resistance genes between *Arcobacter* species or between *Cambylobacter* and *Arcobacter* spp. has so far not been observed (Snelling *et al*. 2006).

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1.8 TRANSMISSION ROUTES

Although no direct connection has been established between consumption of *Arcobacter*-contaminated food or water and human illness, it is likely that transmission of arcobacters occurs via these routes (Miller *et al*. 2009). In fact, some drinking water outbreaks have involved the isolation of *Arcobacter* spp. both in the patients and/or in the contaminated water (Rice *et al*. 1999; Fong *et al*. 2007; Kopilovic *et al*. 2008). In addition, *Arcobacter* species have been considered a potential zoonotic agent due to their pathogenic role in humans and animals (Ho *et al*. 2006a). Direct transmission between these two groups has not yet been demonstrated.

1.8.1 Person-to person transmission

A person-to person (PTP) transmission of *A. butzleri* has been suggested in an outbreak of recurrent abdominal cramps in an Italian school (Vandamme *et al*. 1992a). The epidemiological data showed that all strains recovered from faecal samples of the infected patients had the same phenotype and genotype (Vandamme *et al*. 1993). Another possible PTP transmission, commented before, was associated with a neonate, presumably infected through the placenta with *A. butzleri* (On *et al*. 1995).

1.8.2 Consumption of contaminated water or foods

Several authors have suggested that contaminated water is one possible transmission route of arcobacters to animals and humans (Ho *et al*. 2006a and references therein). In fact, strains of *Arcobacter* isolated from drinking water treatment plants in Germany have shown the same serotypes as those observed from human isolates (Jacob *et al*. 1998). *Arcobacter* species have been recovered from several types of environmental waters i.e, river, lakes, groundwater and seawater (Jacob *et al*. 1993, Rice *et al*. 1999, Fera *et al*. 2004, Morita *et al*. 2004, Collado *et al.* 2008). It was recently hypothesized that *Arcobacter* species are autochthonous of aquatic environments (Fera *et al*. 2004), although a high prevalence of this bacteria has also been found in faeces of livestock animals (Van Driessche *et al*. 2003), and from their farm effluents (Chinivasagam *et al*. 2007). This could indicate that those are the sources of surface water contamination; however, this was not demonstrated. With that aim we initiated a study to understand if the presence of *Arcobacter* in water had an autochthonous nature or if it was due to faecal contamination. From our results we were able to demonstrate that *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* were significantly more

ISBN: 978-84-693-0707-6 /DL:T-420-2010

prevalent in waters that were faecally contaminated than in waters that were not. Furthermore, we demonstrated that these species entered seawater with the polluted freshwater (Collado *et al*. 2008). There, they could coexist with other autochthonous species like *A. marinus* or *A. halophilus* that had so far only been isolated in those environments.

Arcobacter have been associated with at least three waterborne outbreaks (Rice *et al*. 1999; Fong *et al*. 2007; Kopilovic *et al*. 2008). The first was an outbreak of gastroenteritis that occurred at a Girls Scout Camp in Idaho (with nausea, vomiting, abdominals crams and diarrhoea as the predominant symptoms) reported by Rice *et al*. (1999). *Arcobacter butzleri* was isolated from the ground-water used as the source of drinking water (well water) and it was assumed to be the source of the outbreak because at that time the automated chlorination system for the Camp drinking water had broken down (Rice *et al*. 1999). The second outbreak was reported in Ohio and had multiple etiologies (Fong et al. 2007), *Arcobacter* spp. being isolated in the most faecally polluted well samples. Finally, *A. cryaerophilus* and other different pathogens were isolated from stool samples of patients in Slovenia during an outbreak caused by contamination of the drinking water system after it had been connected to a new building (Kopilovic *et al*. 2008). All these outbreaks may be explained by the presence of faecal contamination as well as by the capacity of *Arcobacter* to adhere to different types of pipes and to form biofilms (Assanta *et al*. 2002). Although the susceptibility of *A. butzleri* to chlorine has been demonstrated (Rice *et al*. 1999; Moreno *et al*. 2004), it is still unknown if conventional procedures for drinking water treatment can effectively remove this bacterium as commented by Ho *et al*. (2006a). In a recent study carried out by our group we found that, although the species *A. butzleri* and *A. cryaerophilus* were very prevalent in the Llobregat River water (one of the main sources of drinking water production for the metropolitan area of Barcelona, Spain), these species were never detected or isolated from finished drinking water, which suggests clearly that water treatment is effective in removing *Arcobacter* species (Collado *et al*. submitted).

Van Driessche and Houf (2008) demonstrated that the capacity of *Arcobacter* species to survive in water is influenced by the presence of organic mater and temperature, showing that in optimal laboratory conditions *Arcobacter* can stay viable for up to at least 250 days. Moreover, it has been reported that *A. butzleri* has the capacity to become viable but nonculturable (VBNC) when induced experimentally (Fera *et al*. 2008). However, this VBNC state has not yet been shown to occur in *Arcobacter* in natural aquatic environments.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Food products of animal origin have also been suggested as an important potential transmission route of *Arcobacter* (Ho *et al*. 2006a). This hypothesis relies on the high prevalence of those microbes in the intestinal tract and faecal samples of healthy farm animals and in many retailed meat products (Hume *et al*. 2001; Van Driessche *et al*. 2003). It has been hypothesized that contamination of meat products by *Arcobacter* probably occurs when the faeces of contaminated animals comes into contact with the carcasses during the slaughtering process (Ohlendorf and Murano 2002; Van Driessche *et al*. 2007). Most studies on the prevalence of *Arcobacter* in foods are on poultry (with the highest prevalence), followed by pork, beef products (Kabeya *et al*. 2004; Lehner *et al*. 2005, Collado *et al.* 2009b and references therein) and raw milk (Scullion *et al*. 2006). Shellfish are other potential sources of infection according to the few existing studies (Maugeri *et al*. 2000; Fernández *et al*. 2001). Therefore, we have investigated 84 samples of shellfish (shrimp, mussels, clams and oysters) and demonstrated that clams and mussels can contain a high prevalence and a wide diversity of *Arcobacter* species (Collado *et al*. 2009b). This could have some public health importance considering that seafood is traditionally often eaten undercooked or raw. Studies on foods have shown that in general, *A. butzleri* is the most prevalent species followed by *A. cryaerophilus* and *A. skirrowii* as reviewed by Lehner *et al*. (2005) and further demonstrated in other recent studies (Scullion *et al*. 2006; Collado *et al*. 2009b; Pentimalli *et al*. 2009). Other, less commonly isolated species are *A. nitrofigilis* (Maugeri *et al*. 2000; Collado *et al*. 2009b), *A. mytili* and *A. thereius* (Collado *et al*. 2009b).

1.8.3 Contact with pets

Contact with pets' faeces or by licking are other potential transmission routes of *Arcobacter*. Very recently, Fera *et al.* (2009), using the m-PCR developed by Houf *et al*. (2000), reported a high prevalence of *A. butzleri* (77.6%) and *A. cryaerophilus* (34.1%) in oral swab samples of pet cats. They suggested that the presence of arcobacters in these pets may play a role in their dissemination in the domestic habitat. However, in a previous study conducted in Belgium, Houf *et al*. (2008) reported no isolation from oral swab or cat faeces. Despite that, in the latter study *A. cryaerophilus* was isolated from faeces (1.5%) and oral swabs (0.7%) of dogs while *A. butzleri* was only recovered from faecal samples (0.75%). A prevalence of 3.3% in faeces of dogs was reported by Fernández *et al.* (2008) in Chile. However, Aydin *et al.* (2007) in Turkey did not find any positive faecal samples from dogs.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

These differences in prevalence could be due to the different methods of isolation and detection of *Arcobacter* spp. used in those studies.

1.8.4 Wild animals as source of contamination

Few studies have been performed to determine the presence of *Arcobacter* species in wild animals, as indicated by Hamir *et al*. (2004), who reported *Arcobacter* spp. from intestinal samples of raccoons (*Procyon lotor*). They suggested that theses animals could play a significant role in the epidemiology of these bacteria, since they share with humans the urban or suburban environment. Moreover, *Arcobacter* have also been reported from other exotic or non-domesticated animals such as the Galapagos turtle, the white rhinoceros, the gazelle, the rhea and the alpaca (Wesley *et al*. 2003).

1.8.5 Transmission among animals

Vertical or transplacental *Arcobacter* transmission has been demonstrated from carrying sows to their offspring as well as the horizontal or post-natal infection of the piglets from their mothers, newcomers or the environment (Ho *et al*. 2006b).

Recently Ho *et al*. (2008a) found a high prevalence of *Arcobacter* in the intestinal content of poultry. In that study, the isolates recovered from the content of the gut and from the carcasses of the same flock had a similar genotype (using ERIC-PCR). In addition, it has been demonstrated that the intestinal tract and oviduct of breeding hens can be infected with *Arcobacter* (Lipman *et al*. 2008), although no evidence was found for transmission from hens to eggs.

1.9 CONCLUSIONS AND PERSPECTIVES

Recent studies have shown that *Arcobacter* species may play a role in human and animal diseases, and that faecally contaminated water and food products can be the transmission routes. However, data on the association between diarrhoea and *Arcobacter* in humans comes from just a few studies, as it does in bacteremia cases. In animals, apart from diarrhoea, cases of abortion and mastitis have been attributed to those microbes. More studies are needed to establish the epidemiological distribution of the infections produced by *Arcobacter*. Dedicated studies, using appropriate isolation or molecular detection methods, should be carried out in different parts of the world. Several isolation procedures and media

ISBN: 978-84-693-0707-6 /DL:T-420-2010

have been proposed for recovering *Arcobacter* from different types of samples. However, there are not enough studies that compare those methods systematically. In our hands, the use of an enrichment step in CAT broth followed by the passive filtration of the broth (0.45 µm filters) on blood agar is a method that has produced very similar results to those obtained with direct detection by m-PCR. Furthermore, the use of the 16S rDNA-RFLP identification method proposed by our group has allowed us to identify *A. skirrowii* and *A. nitrofigilis* in addition to the more classically prevalent species *A. butzleri* and *A. cryaerophilus* and enabled us to discover not only the recently published A. mytili, but other new species that are about to be published. An effort should be made to use detection and identification techniques that allow all the *Arcobacter* species to be recognized in order to know their true implication in human and veterinary medicine.

In relation to taxonomy, apart from the mentioned recognized new species it is clear that the use of molecular techniques will expand rapidly the number of species included in the genus. The inclusion of the genus *Arcobacter* in the *Campylobactereaceae* family has been questioned in the light of the new evidence derived from the completed genome of *A. butzleri*. When the new ongoing genome sequencing projects of *A. halophilus*, *A. butzleri* and *Candidatus* Arcobacter sulfidicus are finished, more information will probably be available to establish more accurately the taxonomic position of the genus *Arcobacter*. These new genomes will probably also complement the information on the virulence genes already derived from the available *A. butzleri* genome. Studies are needed on virulence genes and on their role in developing disease, and we expect some to appear in the near future.

1.10 REFERENCES

Abdelbaqi, K., Buissonniere, A., Prouzet-Mauleon, V., Gresser, J., Wesley, I., Megraud, F. and Menard, A. (2007a) Development of a real-time fluorescence resonance energy transfer PCR to detect *Arcobacter* species. J Clin Microbiol 45, 3015-3021.

Abdelbaqi, K., Menard, A., Prouzet-Mauleon, V., Bringaud, F., Lehours, P. and Megraud, F. (2007b) Nucleotide sequence of the *gyrA* gene of *Arcobacter* species and characterization of human ciprofloxacin-resistant clinical isolates. FEMS Immunol Med Microbiol 49, 337-345.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Anderson, K.F., Kiehlbauch, J.A., Anderson, D.C., McClure, H.M. and Wachsmuth, I.K. (1993) *Arcobacter* (*Campylobacter*) *butzleri*-associated diarrheal illness in a nonhuman primate population. Infect Immun 61, 2220-2223.

Assanta, M.A., Roy, D., Lemay, M.J. and Montpetit, D. (2002) Attachment of *Arcobacter butzleri*, a new waterborne pathogen, to water distribution pipe surfaces. J Food Prot 65, 1240-1247.

Atabay, H.I. and Corry, J.E. (1997) The prevalence of campylobacters and arcobacters in broiler chickens. J Appl Microbiol 83, 619-626.

Atabay, H.I., Unver, A., Sahin, M., Otlu, S., Elmali, M. and Yaman, H. (2008) Isolation of various *Arcobacter* species from domestic geese (*Anser anser*). Vet Microbiol 128, 400-405.

Atabay, H.I., Waino, M. and Madsen, M. (2006) Detection and diversity of various *Arcobacter* species in Danish poultry. Int J Food Microbiol 109, 139-145.

Aydin, F., Gumussoy, K.S., Atabay, H.I., Ica, T. and Abay, S. (2007) Prevalence and distribution of *Arcobacter* species in various sources in Turkey and molecular analysis of isolated strains by ERIC-PCR. J Appl Microbiol 103, 27-35.

Brightwell, G., Mowat, E., Clemens, R., Boerema, J., Pulford, D.J. and On, S.L. (2007) Development of a multiplex and real time PCR assay for the specific detection of *Arcobacter butzleri* and *Arcobacter cryaerophilus*. J Microbiol Methods 68, 318-325.

Bücker, R., Troeger, H., Kleer, J., Fromm, M. and Schulzke, J.D. (2009) *Arcobacter butzleri* Induces Barrier Dysfunction in Intestinal HT-29/B6 Cells. J Infect Dis 200, 756-764.

Carbone, M., Maugeri, T.L., Giannone, M., Gugliandolo, C., Midiri, A. and Fera, M.T. (2003) Adherence of environmental *Arcobacter butzleri* and *Vibrio* spp. isolates to epithelial cells in vitro. Food Microbiol 20, 611-616.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Cardarelli-Leite, P., Blom, K., Patton, C.M., Nicholson, M.A., Steigerwalt, A.G., Hunter, S.B., Brenner, D.J., Barrett, T.J. and Swaminathan, B. (1996) Rapid identification of *Campylobacter* species by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. J Clin Microbiol 34, 62-67.

Chinivasagam, H.N., Corney, B.G., Wright, L.L., Diallo, I.S. and Blackall, P.J. (2007) Detection of *Arcobacter* spp. in piggery effluent and effluent-irrigated soils in southeast Queensland. J Appl Microbiol 103, 418-426.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. and Figueras, M.J. (2009a) *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. Int J Syst Evol Microbiol 59, 1391-1396.

Collado, L., Fernández, H. & Figueras, M. J. *Arcobacter valdiviensis* sp. nov., isolated from a chicken cloacal swab sample in Valdivia, Chile. (submitted).

Collado, L., Guarro, J. and Figueras, M.J. (2009b) Prevalence of *Arcobacter* in meat and shellfish. J Food Prot 72, 1102-1106.

Collado, L., Inza, I., Guarro, J. and Figueras, M.J. (2008) Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. Environ Microbiol 10, 1635-1640.

Collado, L., Kasimir, G., Perez, U., Bosch, A., Pinto, R., Saucedo, G., Huguet, J. and Figueras, M. J. Occurrence and diversity of *Arcobacter* spp. along the Llobregat river catchment, at sewage effluents and in a drinking water treatment plant (submitted).

Collado, L., Levican, A., Perez, J. & Figueras, M. J. Description of two new species, *Arcobacter defluvii* sp. nov., isolated from sewage and *Arcobacter molluscorum* sp. nov., isolated from shellfisfh. (In preparation).

Collins, C.I., Wesley, I.V. and Murano, E.A. (1996) Detection of *Arcobacter* spp. in ground pork by modified plating methods. J Food Prot 59, 448-452.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

de Boer, E., Tilburg, J.J., Woodward, D.L., Lior, H. and Johnson, W.M. (1996) A selective medium for the isolation of *Arcobacter* from meats. Lett Appl Microbiol 23, 64-66.

de Oliveira, S.J., Baetz, A.L., Wesley, I.V. and Harmon, K.M. (1997) Classification of *Arcobacter* species isolated from aborted pig fetuses and sows with reproductive problems in Brazil. Vet Microbiol 57, 347-354.

Debruyne, L., Gevers, D. and Vandamme, P. (2008) Taxonomy of the Family *Campylobactereaceae*. In: *Campylobacter* (Third edition) eds Nachamkin, I., Szymanski, C. and Blaser, M. pp 3-25. Washington, DC: ASM press.

Debruyne, L., Houf, K., Douidah, L., De Smet, S. and Vandamme, P. (2009). Reassessment of the taxonomy of *Arcobacter cryaerophilus*. Syst Appl Microbiol (In press).

Donachie, S.P., Bowman, J.P., On, S.L. and Alam, M. (2005) *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. Int J Syst Evol Microbiol 55, 1271-1277.

Ellis, W.A., Neill, S.D., O'Brien, J.J., Ferguson, H.W. and Hanna, J. (1977) Isolation of *Spirillum*/*Vibrio*-like organisms from bovine fetuses. Vet Rec 100, 451-452.

Fera, M.T., La Camera, E., Carbone, M., Malara, D. and Pennisi, M.G. (2009) Pet cats as carriers of *Arcobacter* spp. in Southern Italy. J Appl Microbiol 106, 1661-1666.

Fera, M.T., Maugeri, T.L., Giannone, M., Gugliandolo, C., La Camera, E., Blandino, G. and Carbone, M. (2003) In vitro susceptibility of *Arcobacter butzleri* and *Arcobacter cryaerophilus* to different antimicrobial agents. Int J Antimicrob Agents 21, 488-491.

Fera, M.T., Maugeri, T.L., Gugliandolo, C., Beninati, C., Giannone, M., La Camera, E. and Carbone, M. (2004) Detection of *Arcobacter* spp. in the coastal environment of the Mediterranean Sea. Appl Environ Microbiol 70, 1271-1276.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Fera, M.T., Maugeri, T.L., Gugliandolo, C., La Camera, E., Lentini, V., Favaloro, A., Bonanno, D. and Carbone, M. (2008) Induction and resuscitation of viable nonculturable *Arcobacter butzleri* cells. Appl Environ Microbiol 74, 3266-3268.

Fernández, H., Eller, G., Paillacar, J., Gajardo, T. and Riquelme, A. (1995a) Toxigenic and invasive capacities: possible pathogenic mechanisms in *Arcobacter cryaerophilus*. Mem Inst Oswaldo Cruz 90, 633-634.

Fernández, H., Otth, L., Wilson, M., Rodríguez, R., Proboste, B., Saldivia, C. and Barría, P. (2001) Occurrence of *Arcobacter* sp. in river water, mussels and commercial chicken livers in southern Chile. Int J Med Microbiol 291,140.

Fernández, H., Rojas, X. and Gajardo, T. (1995b) First isolation in Chile of *Arcobacter cryaerophilus* from a bovine abortion. Arch Med Vet 27, 111-114.

Fernández, H., Vera, F. and Villanueva, M. P. (2008) *Arcobacter* and *Campylobacter* species in birds and mammals from Southern Chile. Arch Med Vet 39, 163-165.

Figueras, M.J., Collado, L. and Guarro, J. (2008) A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. Diagn Microbiol Infect Dis 62, 11-15.

Fong, T.T., Mansfield, L.S., Wilson, D.L., Schwab, D.J., Molloy, S.L. and Rose, J.B. (2007) Massive microbiological groundwater contamination associated with a waterborne outbreak in Lake Erie, South Bass Island, Ohio. Environ Health Perspect 115, 856-864.

Gill, K.P. (1983) Aerotolerant campylobacter strain isolated from a bovine preputial sheath washing. Vet Rec 112, 459.

González, A., Botella, S., Montes, R.M., Moreno, Y. and Ferrus, M.A. (2007) Direct detection and identification of *Arcobacter* species by multiplex PCR in chicken and wastewater samples from Spain. J Food Prot 70, 341-347.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

González, A., Moreno, Y., Gonzalez, R., Hernandez, J. and Ferrus, M.A. (2006) Development of a simple and rapid method based on polymerase chain reaction-based restriction fragment length polymorphism analysis to differentiate *Helicobacter*, *Campylobacter*, and *Arcobacter* species. Curr Microbiol 53, 416-421.

González, A., Suski, J. and Ferrus, M.A. (2009) Rapid and Accurate Detection of *Arcobacter* Contamination in Commercial Chicken Products and Wastewater Samples by Real-Time Polymerase Chain Reaction. Foodborne Pathog Dis. (in press).

Gugliandolo, C., Irrera, G.P., Lentini, V. and Maugeri, T.L. (2008) Pathogenic *Vibrio*, *Aeromonas* and *Arcobacter* spp. associated with copepods in the Straits of Messina (Italy). Mar Pollut Bull 56, 600-606.

Hamir, A.N., Sonn, R.J., Franklin, S. and Wesley, I.V. (2004) *Campylobacter jejuni* and *Arcobacter* species associated with intussusception in a raccoon (*Procyon lotor*). Vet Rec 155, 338-340.

Harmon, K.M. and Wesley, I.V. (1997) Multiplex PCR for the identification of *Arcobacter* and differentiation of *Arcobacter butzleri* from other arcobacters. Vet Microbiol 58, 215-227.

Harraß, B., Schwarz, S. and Wenzel, S. (1998) Identification and characterization of *Arcobacter* isolates from broilers by biochemical tests, antimicrobial resistance patterns and plasmid analysis. Zentralbl Veterinarmed B 45, 87-94.

Heylen, K., Vanparys, B., Wittebolle, L., Verstraete, W., Boon, N. and De Vos, P. (2006) Cultivation of denitrifying bacteria: optimization of isolation conditions and diversity study. Appl Environ Microbiol 72, 2637-2643.

Higgins, R., Messier, S., Daignault, D. and Lorange, M. (1999) *Arcobacter butzleri* isolated from a diarrhoeic non-human primate. Lab Anim 33, 87-90.

Ho, H.T., Lipman, L.J. and Gaastra, W. (2008a) The introduction of *Arcobacter* spp. in poultry slaughterhouses. Int J Food Microbiol 125, 223-229.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Ho, H.T., Lipman, L.J. and Gaastra, W. (2006a) *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent! Vet Microbiol 115, 1-13.

Ho, H.T., Lipman, L.J., Hendriks, H.G., Tooten, P.C., Ultee, T. and Gaastra, W. (2007) Interaction of *Arcobacter* spp. with human and porcine intestinal epithelial cells. FEMS Immunol Med Microbiol 50, 51-58.

Ho, H.T., Lipman, L.J., Wosten, M.M., van Asten, A.J. and Gaastra, W. (2008b) *Arcobacter* spp. possess two very short flagellins of which *flaA* is essential for motility. FEMS Immunol Med Microbiol 53, 85-95.

Ho, T.K., Lipman, L.J., van der Graaf-van Bloois, L., van Bergen, M. and Gaastra, W. (2006b) Potential routes of acquisition of *Arcobacter* species by piglets. Vet Microbiol 114, 123-133.

Houf, K., De Smet, S., Bare, J. and Daminet, S. (2008) Dogs as carriers of the emerging pathogen *Arcobacter*. Vet Microbiol 130, 208-213.

Houf, K., De Zutter, L., Van Hoof, J. and Vandamme, P. (2002) Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. Appl Environ Microbiol 68, 2172-2178.

Houf, K., Devriese, L.A., De Zutter, L., Van Hoof, J. and Vandamme, P. (2001) Development of a new protocol for the isolation and quantification of *Arcobacter* species from poultry products. Int J Food Microbiol 71, 189-196.

Houf, K., Devriese, L.A., Haesebrouck, F., Vandenberg, O., Butzler, J.P., van Hoof, J. and Vandamme, P. (2004) Antimicrobial susceptibility patterns of *Arcobacter butzleri* and *Arcobacter cryaerophilus* strains isolated from humans and broilers. Microb Drug Resist 10, 243-247.

Houf, K., On, S., Coenye, T., Debruyne, L., De Smet, S. and Vandamme, P. (2009) *Arcobacter thereius* sp. nov., isolated from pigs and ducks. Int J Syst Evol Microbiol.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Houf, K., On, S.L., Coenye, T., Mast, J., Van Hoof, J. and Vandamme, P. (2005) *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. Int J Syst Evol Microbiol 55, 713-717.

Houf, K. and Stephan, R. (2007) Isolation and characterization of the emerging foodborn pathogen *Arcobacter* from human stool. J Microbiol Methods 68, 408-413.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J. and Vandamme, P. (2000) Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiol Lett 193, 89-94.

Hsueh, P.R., Teng, L.J., Yang, P.C., Wang, S.K., Chang, S.C., Ho, S.W., Hsieh, W.C. and Luh, K.T. (1997) Bacteremia caused by *Arcobacter cryaerophilus* 1B. J Clin Microbiol 35, 489-491.

Hume, M.E., Harvey, R.B., Stanker, L.H., Droleskey, R.E., Poole, T.L. and Zhang, H.B. (2001) Genotypic variation among *Arcobacter* isolates from a farrow-to-finish swine facility. J Food Prot 64, 645-651.

Hurtado, A. and Owen, R.J. (1997) A molecular scheme based on 23S rRNA gene polymorphisms for rapid identification of *Campylobacter* and *Arcobacter* species. J Clin Microbiol 35, 2401-2404.

ICMSF (2002). Microorganisms in Foods 7 – Microbiological Testing in Food Safety Management. International Commission on Microbiological Specifications for Foods. New York: Kluwer Academic/Plenum.

Jacob, J., Lior, H. and Feuerpfeil, I. (1993) Isolation of *Arcobacter butzleri* from a drinking water reservoir in eastern Germany. Zentralbl Hyg Umweltmed 193, 557-562.

Jacob, J., Woodward, D., Feuerpfeil, I. and Johnson, W.M. (1998) Isolation of *Arcobacter butzleri* in raw water and drinking water treatment plants in Germany. Zentralbl Hyg Umweltmed 201, 189-198.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Johnson, L.G. and Murano, E.A. (2002) Lack of a cytolethal distending toxin among *Arcobacter* isolates from various sources. J Food Prot 65, 1789-1795.

Johnson, L.G. and Murano, E.A. (1999) Development of a new medium for the isolation of *Arcobacter* spp. J Food Prot 62, 456-462.

Kabeya, H., Kobayashi, Y., Maruyama, S. and Mikami, T. (2003a) One-step polymerase chain reaction-based typing of *Arcobacter* species. Int J Food Microbiol 81, 163-168.

Kabeya, H., Maruyama, S., Morita, Y., Kubo, M., Yamamoto, K., Arai, S., Izumi, T., Kobayashi, Y., Katsube, Y. and Mikami, T. (2003b) Distribution of *Arcobacter* species among livestock in Japan. Vet Microbiol 93, 153-158.

Kabeya, H., Maruyama, S., Morita, Y., Ohsuga, T., Ozawa, S., Kobayashi, Y., Abe, M., Katsube, Y. and Mikami, T. (2004) Prevalence of *Arcobacter* species in retail meats and antimicrobial susceptibility of the isolates in Japan. Int J Food Microbiol 90, 303-308.

Kärenlampi, R.I., Tolvanen, T.P. and Hanninen, M.L. (2004) Phylogenetic analysis and PCR-restriction fragment length polymorphism identification of *Campylobacter* species based on partial *groEL* gene sequences. J Clin Microbiol 42, 5731-5738.

Kiehlbauch, J.A., Plikaytis, B.D., Swaminathan, B., Cameron, D.N. and Wachsmuth, I.K. (1991) Restriction fragment length polymorphisms in the ribosomal genes for species identification and subtyping of aerotolerant *Campylobacter* species. J Clin Microbiol 29, 1670-1676.

Kim, H.M., Hwang, C.Y. and Cho, B.C. (2009) *Arcobacter marinus* sp. nov. Int J Syst Evol Microbiol. (In press).

Kopilovic et al., Ucakar, V., Koren, N., Krek, M. and Kraigher, A. (2008) Waterborne outbreak of acute gastroenteritis in a costal area in Slovenia in june and july 2008. Eurosurveillance 13, 1-3.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Kownhar, H., Shankar, E.M., Rajan, R., Vengatesan, A. and Rao, U.A. (2007) Prevalence of *Campylobacter jejuni* and enteric bacterial pathogens among hospitalized HIV infected versus non-HIV infected patients with diarrhoea in southern India. Scand J Infect Dis 39, 862-866.

Lastovica, A.J. and Le Roux, E. (2001) Efficient isolation of *Campylobacter upsaliensis* from stools. J Clin Microbiol 39, 4222-4223.

Lau, S.K., Woo, P.C., Teng, J.L., Leung, K.W. and Yuen, K.Y. (2002) Identification by 16S ribosomal RNA gene sequencing of *Arcobacter butzleri* bacteraemia in a patient with acute gangrenous appendicitis. Mol Pathol 55, 182-185.

Lehner, A., Tasara, T. and Stephan, R. (2005) Relevant aspects of *Arcobacter* spp. as potential foodborne pathogen. Int J Food Microbiol 102, 127-135.

Lipman, L., Ho, H. and Gaastra, W. (2008) The presence of *Arcobacter* species in breeding hens and eggs from these hens. Poult Sci 87, 2404-2407.

Logan, E.F., Neill, S.D. and Mackie, D.P. (1982) Mastitis in dairy cows associated with an aerotolerant campylobacter. Vet Rec 110, 229-230.

Marshall, S.M., Melito, P.L., Woodward, D.L., Johnson, W.M., Rodgers, F.G. and Mulvey, M.R. (1999) Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. J Clin Microbiol 37, 4158-4160.

Maugeri, T.L., Gugliandolo, C., Carbone, M., Caccamo, D. and Fera, M.T. (2000) Isolation of *Arcobacter* spp. from a brackish environment. New Microbiol 23, 143-149.

McIntosh, D., Ji, B., Forward, B.S., Puvanendran, V., Boyce, D. and Ritchie, R. (2008) Culture-independent characterization of the bacterial populations associated with cod (Gadus *morhua* L.) and live feed at an experimental hatchery facility using denaturing gradient gel electrophoresis. Aquaculture 275, 42-50.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Miller, W.G., Parker, C.T., Rubenfield, M., Mendz, G.L., Wosten, M.M., Ussery, D.W., Stolz, J.F., Binnewies, T.T., Hallin, P.F., Wang, G., Malek, J.A., Rogosin, A., Stanker, L.H. and Mandrell, R.E. (2007) The complete genome sequence and analysis of the epsilonproteobacterium *Arcobacter butzleri*. PLoS One 2, e1358.

Miller, W.G., Wesley, I.V., On, S.L., Houf, K., Megraud, F., Wang, G., Yee, E., Srijan, A. and Mason, C.J. (2009) First multi-locus sequence typing scheme for *Arcobacter* spp. BMC Microbiol 9, 196.

Moreno, Y., Alonso, J.L., Botella, S., Ferrus, M.A. and Hernandez, J. (2004) Survival and injury of *Arcobacter* after artificial inoculation into drinking water. Res Microbiol 155, 726-730.

Morita, Y., Maruyama, S., Kabeya, H., Boonmar, S., Nimsuphan, B., Nagai, A., Kozawa, K., Nakajima, T., Mikami, T. and Kimura, H. (2004) Isolation and phylogenetic analysis of *Arcobacter* spp. in ground chicken meat and environmental water in Japan and Thailand. Microbiol Immunol 48, 527-533.

Musmanno, R.A., Russi, M., Lior, H. and Figura, N. (1997) In vitro virulence factors of *Arcobacter butzleri* strains isolated from superficial water samples. New Microbiol. 20, 63–68.

Neill, S.D. CampbelL, J.N., O'Brien, J.J., Weatherup, I.S.T.C. and Ellis W. A. (1985) Taxonomic Position of *Campylobacter cryaerophila* sp. nov. Int J Syst Bacteriol 35, 342-356.

Ohlendorf, D.S. and Murano, E.A. (2002) Sensitivity of three methods used in the isolation of *Arcobacter* spp. in raw ground pork. J Food Prot 65, 1784-1788.

On, S.L. (2001) Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter* and related bacteria: current status, future prospects and immediate concerns. Symp Ser Soc Appl Microbiol (30), 1S-15S.

On, S.L. and Owen, R.J. (2009) International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of *Campylobacter* and related bacteria. Minutes of the

ISBN: 978-84-693-0707-6 /DL:T-420-2010

meetings, 3 and 4 September 2007, Rotterdam, Holland. Int J Syst Evol Microbiol 59, 197–199.

On, S.L., Atabay, H.I., Amisu, K.O., Coker, A.O. and Harrington, C.S. (2004) Genotyping and genetic diversity of *Arcobacter butzleri* by amplified fragment length polymorphism (AFLP) analysis. Lett Appl Microbiol 39, 347-352.

On, S.L., Harrington, C.S. and Atabay, H.I. (2003) Differentiation of *Arcobacter* species by numerical analysis of AFLP profiles and description of a novel *Arcobacter* from pig abortions and turkey faeces. J Appl Microbiol 95, 1096-1105.

On, S.L. and Holmes, B. (1992) Assessment of enzyme detection tests useful in identification of campylobacteria. J Clin Microbiol 30, 746-749.

On, S.L. and Holmes, B. (1991) Reproducibility of tolerance tests that are useful in the identification of campylobacteria. J Clin Microbiol 29, 1785-1788.

On, S.L., Holmes, B. and Sackin, M.J. (1996) A probability matrix for the identification of campylobacters, helicobacters and allied taxa. J Appl Bacteriol 81, 425-432.

On, S.L., Stacey, A. and Smyth, J. (1995) Isolation of *Arcobacter butzleri* from a neonate with bacteraemia. J Infect 31, 225-227.

Pentimalli, D., Pegels, N., Garcia, T., Martin, R. and Gonzalez, I. (2009) Specific PCR detection of *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii*, and *Arcobacter cibarius* in chicken meat. J Food Prot 72, 1491-1495.

Petersen, R.F., Harrington, C.S., Kortegaard, H.E. and On, S.L. (2007) A PCR-DGGE method for detection and identification of *Campylobacter*, *Helicobacter*, *Arcobacter* and related Epsilobacteria and its application to saliva samples from humans and domestic pets. J Appl Microbiol 103, 2601-2615.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Prouzet-Mauleon, V., Labadi, L., Bouges, N., Menard, A. and Megraud, F. (2006) *Arcobacter butzleri*: underestimated enteropathogen. Emerg Infect Dis 12, 307-309.

Quiñones, B., Parker, C.T., Janda, J.M.,Jr, Miller, W.G. and Mandrell, R.E. (2007) Detection and genotyping of *Arcobacter* and *Campylobacter* isolates from retail chicken samples by use of DNA oligonucleotide arrays. Appl Environ Microbiol 73, 3645-3655.

Rice, E.W., Rodgers, M.R., Wesley, I.V., Johnson, C.H. and Tanner, S.A. (1999) Isolation of *Arcobacter butzleri* from ground water. Lett Appl Microbiol 28, 31-35.

Romero, J., Garcia-Varela, M., Laclette, J.P. and Espejo, R.T. (2002) Bacterial 16S rRNA gene analysis revealed that bacteria related to *Arcobacter* spp. constitute an abundant and common component of the oyster microbiota (*Tiostrea chilensis*). Microb Ecol 44, 365-371.

Samie, A., Obi, C.L., Barrett, L.J., Powell, S.M. and Guerrant, R.L. (2007) Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: studies using molecular diagnostic methods. J Infect 54, 558-566.

Schroeder-Tucker, L., Wesley, I.V., Kiehlbauch, J.A., Larson, D.J., Thomas, L.A. and Erickson, G.A. (1996) Phenotypic and ribosomal RNA characterization of *Arcobacter* species isolated from porcine aborted fetuses. J Vet Diagn Invest 8, 186-195.

Scullion, R., Harrington, C.S. and Madden, R.H. (2006) Prevalence of *Arcobacter* spp. in raw milk and retail raw meats in Northern Ireland. J Food Prot 69, 1986-1990.

Scullion, R., Harrington, C.S. and Madden, R.H. (2004) A comparison of three methods for the isolation of *Arcobacter* spp. from retail raw poultry in Northern Ireland. J Food Prot 67, 799-804.

Sette, L.D., Simioni, K.C., Vasconcellos, S.P., Dussan, L.J., Neto, E.V. and Oliveira, V.M. (2007) Analysis of the composition of bacterial communities in oil reservoirs from a southern offshore Brazilian basin. Antonie Van Leeuwenhoek 91, 253-266.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Skirrow and Blaser, 2000 M.B. Skirrow and M.J. Blaser, Clinical aspects of *Campylobacter* infection. In: I. Nachamkin and M.J. Blaser, Editors, *Campylobacter* (2nd ed.), ASM, Washington (DC) (2000), pp. 69–88.

Sloane, Y., Winstanley, C., Hall, N., Ashelford, K., Leatherbarrow, H., Bennet, M. and Williams, N. (2009) The genome sequence of an *Arcobacter butzleri* isolate from cattle indicates considerable divergence from human isolates. 15th International Workshop on Campylobacter, Helicobacter and Related Organisms (CHRO). Niigata, Japan.

Snelling, W.J., Matsuda, M., Moore, J.E. and Dooley, J.S. (2006) Under the microscope: *Arcobacter*. Lett Appl Microbiol 42, 7-14.

Son, I., Englen, M.D., Berrang, M.E., Fedorka-Cray, P.J. and Harrison, M.A. (2007) Antimicrobial resistance of *Arcobacter* and *Campylobacter* from broiler carcasses. Int J Antimicrob Agents 29, 451-455.

Stirling, J., Griffith, M., Blair, I., Cormican, M., Dooley, J.S., Goldsmith, C.E., Glover, S.G., Loughrey, A., Lowery, C.J., Matsuda, M., McClurg, R., McCorry, K., McDowell, D., McMahon, A., Cherie Millar, B., Nagano, Y., Rao, J.R., Rooney, P.J., Smyth, M., Snelling, W.J., Xu, J. and Moore, J.E. (2008) Prevalence of gastrointestinal bacterial pathogens in a population of zoo animals. Zoonoses Public Health 55, 166-172.

Taylor, D.N., Kiehlbauch, J.A., Tee, W., Pitarangsi, C. and Echeverria, P. (1991) Isolation of group 2 aerotolerant *Campylobacter* species from Thai children with diarrhea. J Infect Dis 163, 1062-1067.

Teske, A., Sigalevich, P., Cohen, Y. and Muyzer, G. (1996) Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. Appl Environ Microbiol 62, 4210-4215.

Van Driessche, E. and Houf, K. (2008) Survival capacity in water of *Arcobacter* species under different temperature conditions. J Appl Microbiol 105, 443-451.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Van Driessche, E. and Houf, K. (2007) Discrepancy between the occurrence of *Arcobacter* in chickens and broiler carcass contamination. Poult Sci 86, 744-751.

Van Driessche, E., Houf, K., van Hoof, J., De Zutter, L. and Vandamme, P. (2003) Isolation of *Arcobacter* species from animal feces. FEMS Microbiol Lett 229, 243-248.

Vandamme, P., Dewhirst, F. E., Paster, B. J. and On, S. L. W. (2005) Genus II. *Arcobacter* Vandamme, Falsen, Rossau, Segers, Tytgat and De Ley 1991a, 99VP. In Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 2, pp. 1161–1165. Edited by D. J. Brenner, N. P. Kreig, J. T. Staley & G. M. Garrity. New York: Springer.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. and De Ley, J. (1991) Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. Int J Syst Bacteriol 41, 88-103.

Vandamme, P., Giesendorf, B.A., van Belkum, A., Pierard, D., Lauwers, S., Kersters, K., Butzler, J.P., Goossens, H. and Quint, W.G. (1993) Discrimination of epidemic and sporadic isolates of *Arcobacter butzleri* by polymerase chain reaction-mediated DNA fingerprinting. J Clin Microbiol 31, 3317-3319.

Vandamme, P., Pugina, P., Benzi, G., Van Etterijck, R., Vlaes, L., Kersters, K., Butzler, J.P., Lior, H. and Lauwers, S. (1992a) Outbreak of recurrent abdominal cramps associated with *Arcobacter butzleri* in an Italian school. J Clin Microbiol 30, 2335-2337.

Vandamme, P., Vancanneyt, M., Pot, B., Mels, L., Hoste, B., Dewettinck, D., Vlaes, L., van den Borre, C., Higgins, R. and Hommez, J. (1992b) Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. Int J Syst Bacteriol 42, 344-356.

Vandenberg, O., Dediste, A., Houf, K., Ibekwem, S., Souayah, H., Cadranel, S., Douat, N., Zissis, G., Butzler, J.P. and Vandamme, P. (2004) *Arcobacter* species in humans. Emerg Infect Dis 10, 1863-1867.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Vandenberg, O., Houf, K., Douat, N., Vlaes, L., Retore, P., Butzler, J.P. and Dediste, A. (2006) Antimicrobial susceptibility of clinical isolates of non-jejuni/coli campylobacters and arcobacters from Belgium. J Antimicrob Chemother 57, 908-913.

Villarruel-Lopez, A., Marquez-Gonzalez, M., Garay-Martinez, L.E., Zepeda, H., Castillo, A., Mota de la Garza, L., Murano, E.A. and Torres-Vitela, R. (2003) Isolation of *Arcobacter* spp. from retail meats and cytotoxic effects of isolates against vero cells. J Food Prot 66, 1374-1378.

Wesley, I.V. and Baetz, A.L. (1999) Natural and experimental infections of *Arcobacter* in poultry. Poult Sci 78, 536-545.

Wesley, I.V., Baetz, A.L. and Larson, D.J. (1996) Infection of cesarean-derived colostrum-deprived 1-day-old piglets with *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii*. Infect Immun 64, 2295-2299.

Wesley, I.V., Schroeder-Tucker, L., Baetz, A.L., Dewhirst, F.E. and Paster, B.J. (1995) Arcobacter-specific and Arcobacter butzleri-specific 16S rRNA-based DNA probes. J Clin Microbiol 33, 1691-1698.

Wesley, I.V., Schroeder-Tucker, L. and Franklin, S.L. (2003) Recovery of *Arcobacter* Spp. from Exotic Animal Species. Int J Med Microbiol 293, 57.

Wirsen, C.O., Sievert, S.M., Cavanaugh, C.M., Molyneaux, S.J., Ahmad, A., Taylor, L.T., DeLong, E.F. and Taylor, C.D. (2002) Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. Appl Environ Microbiol 68, 316-325.

Wybo, I., Breynaert, J., Lauwers, S., Lindenburg, F. and Houf, K. (2004) Isolation of *Arcobacter skirrowii* from a patient with chronic diarrhea. J Clin Microbiol 42, 1851-1852.

Yan, J.J., Ko, W.C., Huang, A.H., Chen, H.M., Jin, Y.T. and Wu, J.J. (2000) *Arcobacter butzleri* bacteremia in a patient with liver cirrhosis. J Formos Med Assoc 99, 166-169.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Yildiz, H. and Aydin, S. (2006) Pathological effects of *Arcobacter cryaerophilus* infection in rainbow trout (Oncorhynchus mykiss Walbaum). Acta Vet Hung 54, 191-199.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

2. INTEREST AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

ISBN: 978-84-693-0707-6 /DL:T-420-2010

As have been outlined in the previous chapter (review), some species of the genus *Arcobacter* are considered emergent bacterial pathogens, gastrointestinal symptomatology and bacteraemia being the main human related diseases, whereas in farm animals these bacteria are associated to abortion, diarrhoea and mastitis.

In the last two decades, increasing progress in the taxonomy and epidemiology of this microbe has been reported in a large number of publications. However, several questions related to its transmission routes, virulence factors or detection/identification procedures are still waiting to be answered.

One of the most important problems in *Arcobacter* research is the difficulty in characterizing the strains to the species level. The phenotypic characterization is complex and imprecise and the existing molecular methods do not enable identification of all the accepted species of the genus. In most cases, only detection and identification of the three *Arcobacter* species related to animals and human infections (*Arcobacter butzleri*, *A. cryaerophilus* and *A. skirrowii*) is carried out, the other species defined up to the beginning of this work in 2006 being totally ignored. This limitation hampers the possibility of establishing the true species diversity in clinical and environmental samples. The molecular detection and identification techniques as well as the taxonomy of this genus have mainly been based on the analysis of the 16S rRNA and 23S rRNA genes. Furthermore, the use of some housekeeping genes (*gyrA*, *rpoB-rpoC* and *cpn60*) has been introduced to the genus as a tool to better differentiate the species and their phylogenetic relationships. However, it seems necessary to evaluate the use of additional genes that could be also useful for the genus.

On the other hand, *Arcobacter* transmission routes are still not very clear because all potential sources have not been well explored. Regarding this, contaminated water has been suggested as one potential route of infection, but whether *Arcobacter* species are autochthonous organisms of environmental waters or if they are external contaminants has not been investigated. Furthermore, there has been no investigation into whether the water treatments used in the production of drinking water are effective in the inactivation or elimination of this microbe.

Foods of animal origins are other suggested transmission routes. However, the data available has concentrated on the prevalence of *Arcobacter* mainly in meat products from poultry, pork and beef origins. Few studies have been conducted regarding its prevalence in other less common types of meats as well as shellfish.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

This thesis aims to increase the knowledge of the taxonomy and epidemiology of the genus *Arcobacter* by studying those aspects poorly explored so far. The specific objectives of this thesis are the following:

1. To develop a flexible and easy-to-use molecular identification method for differentiating all the species included in this genus.

2. To determine the presence and prevalence of *Arcobacter* species in different types of environmental waters (river, lakes, seawater) and to try to understand whether its presence is of an autochthonous nature or is due to faecal contamination.

3. To investigate the genetic diversity of *Arcobacter* in the Llobregat River water used to produce drinking water and to determine if the water treatments are effective for removing this bacteria in the finished treated water.

4. To study the prevalence and distribution of *Arcobacter* species in several types of meats and shellfish.

5. To evaluate different housekeeping genes that may be useful for establishing the phylogenetic relationship of the *Arcobacter* spp. included in the genus.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

3. MATERIALS AND METHODS

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

3.1 Origin and isolation of strains

Type and reference strains

The type and reference strains as well as those received from other authors are detailed in the Tables 1 and 2. Theses strains and the one isolated in the different studies were stored frozen at -80°C in Tryptic Soy Broth (Becton Dickinson) supplemented with 15 % glycerol (Panreac). All strains were also freeze-dried for storage. Those dried strains were rehydrated with brain heart infusion broth (Becton Dickinson) and recovered on blood agar (BA) plates incubated at 30°C for 48-72 h under aerobic conditions when they were needed. Only *A. nitrofigilis* strains needed to be incubated in a microaerobic atmosphere using the gas generating kits CampyGen (Oxoid) or GasPak™ EZ Campy Container System (Becton Dickinson).

Table 1. Type and reference strains used in this study

***Arcobacter* species**	**Type Strain**	**Other designation**	**Source**	**Country**
A. butzleri	LMG 10828	ATCC 49616, CCUG 30485	Man, faeces	USA
A. cryaerophilus 1A	LMG 9904	CCUG 17801, LMG 7536	Aborted bovine fetus	Ireland
A. skirrowii	LMG 6621	ATCC 51132, CCUG 10374	Lamb with diarrhoea, faeces	
A. nitrofigilis	CECT 7204	ATCC 33309, LMG 7604	*Spartina alterniflora*, roots	Canada
A. cibarius	CECT 7203	CCUG 48482, LMG 21996	Broiler, skin	Belgium
A. halophilus	LA31B	ATCC BAA1022, CIP 108450	Water of hypersaline lagoon	USA
A. mytili	CECT 7386	LMG 24559 , F2075	Mussels	Spain
A. thereius	LMG 24486	CCUG 56902, 16389	Aborted bovine foetus	Denmark

***Arcobacter* species**	**Reference Strain**	**Other designation**	**Source**	**Country**
A. butzleri	LMG 11118	CCUG 30486	Man, faeces	Italy
A. cryaerophilus 1A	LMG 6622	CCUG 12018	Aborted swine foetus	Ireland
A. cryaerophilus 1A	LMG 9065	CCUG 12019	Aborted ovine foetus	
A. cryaerophilus 1A	LMG 9863	CCUG 17806	Aborted ovine foetus	Ireland
A. cryaerophilus 1A	LMG 9865	CCUG 17808	Aborted porcine foetus	Ireland
A. cryaerophilus 1A	LMG 9871	CCUG 17814, LMG 9905	Aborted bovine foetus	Ireland
A. cryaerophilus 1B	LMG 7537	CCUG 17805	Aborted ovine foetus	Ireland
A. cryaerophilus 1B	LMG 9861	CCUG 17802	Aborted bovine foetus	Ireland
A. cryaerophilus 1B	LMG 10229	Higgins 87-5154	Aborted porcine foetus	Canada
A. cryaerophilus 1B	LMG 10241	Higgins 88-3421R	Aborted porcine foetus	Canada
A. cryaerophilus 1B	LMG 10829	ATCC 49615, CDC D2610	Man, blood	Belgium
A. skirrowii	LMG 9911	Neill 02777	Aborted porcine foetus	Ireland
A. nitrofigilis	LMG 7547	CCUG 12022	*Spartina alterniflora*, roots	USA
A. mytili	CECT 7385	F2026	Mussels	Spain
A. thereius	LMG 24487	16695-3	Aborted bovine foetus	Denmark

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 2. Strains received from other authors

Arcobacter species	Strain	Source	Country	Received from
A. butzleri	AP6	Coastal water	Italy	Teresa Maugeri
A. butzleri	D		Ireland	Robert Madden
A. butzleri	I		Ireland	Robert Madden
A. cryaerophilus	A		Ireland	Robert Madden
A. cryaerophilus	B		Ireland	Robert Madden
A. cryaerophilus	C		Ireland	Robert Madden
A. cryaerophilus	G		Ireland	Robert Madden
A. cryaerophilus	H		Ireland	Robert Madden
A. skirrowii	989	Feces , cow	Belgium	Kurt Houf
A. skirrowii	994	Feces , pig	Belgium	Kurt Houf
A. skirrowii	E		Ireland	Robert Madden
A. skirrowii	F		Ireland	Robert Madden
A. cibarius	NC81	Pig effluent	Australia	Nalini Chinivasagam
A. cibarius	NC88	Pig effluent	Australia	Nalini Chinivasagam
A. cibarius	742	Poultry carcass	Belgium	Kurt Houf
A. cibarius	743	Poultry carcass	Belgium	Kurt Houf
A. cibarius	745	Poultry carcass	Belgium	Kurt Houf
A. cibarius	746	Poultry carcass	Belgium	Kurt Houf
A. cibarius	748	Poultry carcass	Belgium	Kurt Houf

Sample Collection

Water samples:

Water samples including in the **Study 4.2**, were collected from freshwater (river and lakes) and seawater bathing sites in Catalonia (north-east Spain). In this study sewage and sludge samples obtained from the waste water treatment plant (WWTP) of the city of Reus were also analysed. In the **Study 4.3**, water samples were taken along the year seasons in 12 sampling points of the Llobregat River catchment (one of most important source of drinking water of the metropolitan area of Barcelona, Spain) including 3 sites at a drinking water treatment plant. The water samples were taken in 2L sterile polypropylene bottles, chilled in ice and transported to the laboratory.

Food samples:

In **Study 4.4**, diverse types of meats (chicken, turkey, duck, pork, beef, rabbit, and sausage) and shellfish samples (mussel, clam, oyster and frozen shrimp) were obtained from retail markets in Catalonia. Shellfish samples also were obtained from local markets, with the exception of the mussels, which were harvested from a mussel farm at the Ebro River delta. Ten grams of each food sample were mixed with the Arcobacter enrichment broth and isolated with the protocols explained below.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Arcobacter isolation

Arcobacter spp. were isolated from water and food samples using the Arcobacter enrichment broth was either prepared as follows: peptone, 18 g/L; NaCl, 5 g/L and yeast extract, 1 g/L or purchased already prepared (Oxoid). In both cases the broth was supplemented with CAT (cefoperazone, amphotericin B and teicoplanin) (Oxoid). Incubation was performed at 30°C for 48 h under aerobic conditions. After enrichment, an aliquot (200 to 400 µl) of the broth was transferred onto the surface of a 0.45 µm membrane filter (Millipore) that had been placed on a Petri dish containing BA and was allowed to filter passively under ambient conditions for 30 min (Atabay and Corry 1997). Then, the filters were removed and the plates were incubated at 30°C for 48 h to 72 h under aerobic condition.

In the **Study 4.6**, the strain FE2 was isolated using the Arcobacter selective method designed by Houf *et al*. (2001). For this the Arcobacter enrichment broth was prepared with 28 g/L of Arcobacter broth (Oxoid), and the selective supplement, comprising amphotericin B (10 mg/L), cefoperazone (16 mg/L), 5-fluorouracil (100 mg/L), novobiocin (32 mg/L), and trimethoprim (64 mg/L). For the Arcobacter selective agar, the same mentioned reactive supplement was added to 12 g/L of Agar Technical no. 3 (Oxoid). After an incubation of 48 h at 28°C under microaerobic condition, an aliquot of the Arcobacter enrichment broth was streaked on Arcobacter selective agar and incubated under the mentioned conditions.

Bacterial indicator of faecal pollution analysis

The membrane filtration technique was used, in the **Study 4.2**, to determine faecal coliforms (FC), *Escherichia coli* (Ec) and intestinal enterococci (IE) using previously described methods (Figueras *et al*. 1994; 1996; Mates and Schaffer 1988).

In the **Study 4.3**, the membrane filtration technique was also employed for the detection of IE and *Clostridium perfringens* (Cp) as previously described (Figueras et al., 1996) using the ISO 7899-2 and ISO 6461-2 methods respectively. Detection of Total coliforms (TC) and Ec was done using the Colilert-18 method (Quanti-Tray, IDEXX Laboratories) according to the manufacturer's instructions. All the methods are accredited by ISO 17025 standard at the Agbar microbiology laboratory.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

3.2 Phenotypic characterization

Gram stain

Initially the shape and staining behaviour of all isolates obtained from different samples were evaluated by Gram stain (with 0.3% carbol fuchsin) as proposed for *Campylobacter* (Ursing *et al*. 1994).

Wet mount

The motility of cells was observed in young cultures by examining wet mounts in distilled water by phase-contrast microscopy.

Biochemical characterization

Phenotypic characterization of strains was performed using the biochemical identification scheme of Vandamme *et al*. (2005). Briefly, NaCl tolerance and susceptibility to cefoperazone were tested on nutrient broth no. 2 (Oxoid) supplemented with 5% whole sheep blood and 1.5% agar. The indoxyl acetate hydrolysis test was performed according to Mills and Gherna (1987) and confirmed using indoxyl acetate diagnostic tablets (IAC)-DIETABS (Rosco Diagnostica). The nitrate reduction was evaluated by the method of Cook (1950).

Electron microscopy

The cell size, morphology and presence of flagella in *Arcobacter* strains were determined with transmission electron microscopy (JEOL 1011) after negative staining with 2% (W/V) of phosphotungstic acid solution (pH 6.9) for 1 min. For scanning electron microscopy, pieces of agar containing the growing strains were fixed in 2.5% glutaraldehyde in phosphate buffer for 24 h. Subsequently, the samples were post-fixed in 1% osmium tetroxide for 2 h. After dehydration and critical point drying, specimens were mounted and coated with a thin layer of gold before examination in a JEOL JSM 6400.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

3.3 Molecular characterization

DNA extraction

Except for the DNA-DNA hybridization methods, the DNA extraction was performed using the InstaGene™ DNA Purification Matrix (Bio-Rad). The concentration of each DNA sample was determined using the GenQuant pro (Amersham Biosciences) at A_{260}.

Multiplex PCR

For identification at species level, an *Arcobacter* species-specific multiplex-PCR (m-PCR) assay was performed as described by Houf *et al*. (2000). Briefly, in a reaction mixture of 50 µl of final volume, composed of 2 µl of DNA, 5 µl of 10XPCR buffer, 1.5 U of Taq DNA polymerase (Invitrogen), 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), 1.3 mM of MgCl2, and 50 pmol of the primers ARCO, BUTZ, CRY1, CRY2, and 25 pmol of primer SKIR (Table 3). Amplified products were size-separated by electrophoresis in 2% agarose. Gels were stained with ethidium bromide and a UV light used for visualization. This m-PCR method produces a species-specific amplicon of 257 bp fragment for *A. cryaerophilus*, 401 bp fragment for *A. butzleri* and 641 bp fragment for *A. skirrowii*.

16S rDNA-RFLP

For each strain, 1026 bp of the 16S rRNA gene was amplified using the primers CAH16S1am and CAH16S1b (Table 3). Enzymatic digestion was performed by incubating 10 µl of the amplification product with 10 U of the enzyme *Mse*I (MBI Fermentas) and 2 µl of the 10X buffer R in a total volume of 30 µl. The reaction mixture was incubated at 65 °C for 3 h. Restriction fragments were separated on 15% polyacrylamide gel (ProtoGel) electrophoresis in Tris-Borate-EDTA (TBE) 1X buffer at 350 V for 5 h. The pBR322 DNA/BsuRI (*Hae*III) (MBI Fermentas) was used as a molecular weight marker. The gels were stained with ethidium bromide and photographed on a UV transilluminator.

DNA sequencing

16S rRNA gene

The nearly complete 16S rRNA gene (over 1400 bases) was amplified by PCR with a universal primer pair Anti I and S (Table 3). Sequencing of the purified (QIAquick PCR Purification Kit, Qiagen, Venlo, Netherlands) PCR product was performed using a BigDye

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. Sequence assembly was performed with AUTO ASSEMBLER (Applied Biosystems).

rpoB gene

The *rpoB* gene was amplified using the PCR primers CamrpoB-L and RpoB-R (Table 3) and the conditions that have been established for the genus *Campylobacter* (Korczak *et al*. 2006). The expected PCR product size (524 bp) was obtained for all strains, with additional unexpected bands in some cases. The bands of the expected size were purified from the agarose gel with the GFX PCR DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions. The PCR products were sequenced in both directions and in duplicate. The *rpoB* gene sequences were used to calculate the percentage nucleotide substitutions for a continuous stretch of 487 bp (positions 1552–2039 according to *E. coli* numbering).

gyrB gene

PCR amplifications were performed in a reaction mixture of 50 µl containing 1 µl of genomic DNA, 0.2 µM each dNTP, 0.2 µM each primer gyrB-Arc-7F and gyrB-Arc-14R (Table 3), 2 mM of MgCl2, 1 U Taq DNA polymerase (Invitrogen) and the buffer supplied with the enzyme. PCR conditions applied were 3 min at 94ºC followed by 35 cycles of 15 s at 94ºC, 30 s at 50ºC and 45 s at 72ºC, followed by 5 min at 72ºC. The PCR product was sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. The *gyrB* gene sequences were used to calculate the percentage nucleotide substitutions for a continuous stretch of 665 bp (positions 672–1342 according to *E. coli* numbering).

Sequence analysis

The obtained DNA sequences were aligned with the software CLUSTAL W (Thompson *et al*. 1994). Genetic distances were obtained using Kimura's two-parameter model (Kimura 1980) and evolutionary trees were constructed by the neighbour-joining method with the MEGA4 program (Tamura *et al*. 2007). The stability of each relationship was assessed by bootstrap analysis (1000 replicates).

ISBN: 978-84-693-0707-6 /DL:T-420-2010

DNA-DNA hybridization (method 1)

DNA was extracted using the procedure described by Wilson (1987). DDH was performed using the microplate method described by Ezaki *et al*. (1989), slightly modified by Goris *et al*. (1998), which involves immobilizing denatured (single-stranded) unlabeled DNA onto a "membrane", treating it so that additional DNA will not adsorb to it and then incubating it in the presence of labelled denatured DNA fragments. This analysis was performed by the BCCM/LMG (Belgian Coordinated Collection of Microorganisms/ Laboratorium voor Microbiologie from Universiteit Gent) identification Service for the **Study 4.5**.

DNA-DNA hybridization (method 2)

DNA extraction and labelling

DNA was extracted using the method of Marmur (1961). The reference DNA were labelled using DIG-11-dUTP and biotin-16-dUTP by using the nick-translation kit (Roche), incubating for 90 min. After labelling the DNA was precipitated with ethanol and resuspended in 200 ml sterile MilliQ water (Ziemke *et al*., 1998).

Hybridization

Fifteen µg of unlabelled DNA were mixed with 100–150 ng of labelled DNA, and filled to 72 µl with MilliQ water. This solution was denatured by boiling for 5 min and immediately chilled on ice. After a short spin of the DNA mixture, 28 µl of 1M phosphate buffer (PB) were added and mixed. The 100 µl hybridization mixture was covered with 100 µl of light mineral oil (Sigma) in order to avoid evaporation and volume changes during incubation. Finally, all the solutions were incubated for 16 h at either 30ºC below the melting point temperature (T_m) of the homologous (considered non-restrictive hybridization conditions). The T_m was calculated with the following formulae:

[T_m: (G+C+182.2)/2.44].

Separation of single and double strands

Single and double-stranded DNA were eluted on hydroxyapatite (HA) (Sigma). Prior to chain separation, HA was equilibrated with 0.14M PB. Two 50 µl aliquots of each single DDH mixture were transferred to two tubes containing equilibrated HA, respectively. The DDH solution was well mixed with the HA and incubated for 15 min at Tm-35ºC.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

During incubation, double-stranded DNA was bound to HA and after a centrifugation was possible to separate the single strands in a new tube. The HA was washed two additional times with 450 and 500 µl of 0.14M PB respectively and incubated at TM-35ºC, then the supernatant obtained after centrifugation was collected in the tubes of single stranded DNA. The HA pellet containing bound double-stranded DNA was well mixed with 200 µl 0.4MPB, and kept at room temperature (RT) for 1–2 min. Supernatant was collected after centrifugation (2 min at 13,000 rpm), and the pellet was washed again with 200 µl 0.4M PB. The final volume of double-stranded DNA was 400 µl. These final samples were denatured by boiling, and they were ice chilled prior to their detection on microtitre plates.

Detection on microtitre plates

Two hundred µl were transferred to a well of a streptavidin coated micro titreplate (Roche), and incubated for 2 h at RT. Wells were then washed with 1XPBS. In each well, 200 µl of the antibody mixture (anti-digoxygenin) were added and incubated for 1h at RT. Wells were then washed again with 1X PBS. Finally, 200 µl of coating buffer 1X with 1 mg/ml p-nitrophenylphosphate (Sigma) were added to each well and the plates were incubated at 37ºC. The colour development was measured at 405nm.

C+G content

For the determination of DNA G+C content, genomic DNA was prepared according to the procedure of Wilson (1987). The G+C content of each DNA sample was determined by three independent analyses using the HPLC technique (Mesbah et al., 1989). This analysis was performed by the BCCM/LMG (Belgian Coordinated Collection of Microorganisms/Laboratorium voor Microbiologie from Universiteit Gent) identification Service for **Study 4.5**.

ERIC-PCR

Each 50 µl PCR mixture was composed of 5 µl of 10X PCR buffer (Invitrogen), 5 U of Taq DNA polymerase, and a mixture of each dNTP at 0.2 mM. The primers ERIC 1R and ERIC 2 designed by Versalovic *et al*. (1991) (Table 3) were each used at concentrations of 25 pmol. The PCR consisted of 40 cycles of 94°C for 1 min, 25°C for 1 min, and 72°C for 2 min. Prior to cycling, samples were heated at 94°C for 5 min. The PCR products were size separated by electrophoresis of 8 µl portions of the reaction mixtures in ethidium bromide-stained 2% agarose gels with 1X TBE buffer for 2.5 h at 100 V. The

ISBN: 978-84-693-0707-6 /DL:T-420-2010

DNA profiles were visualized by UV transillumination and photographed. Gel images were saved as TIFF files, normalized with the GeneRuler™ 100bp DNA Ladder Plus (MBI Fermentas), and further analyzed by Bionumerics software, version 2.5 and 6.0 (Applied Maths). Patterns with at least one different band were considered as different genotypes.

Table 3. Primers used in this thesis

Method	Primer	Sequence 5' to 3'	Gene	Reference
m-PCR	ARCO	CGTATTCACCGTAGCATAGC	16S rRNA	Houf *et al*. 2000
	BUTZ	CCTGGACTTGACATAGTAAGAATGA	16S rRNA	Houf *et al*. 2000
	CRY1	TGCTGGAGCGGATAGAAGTA	23S rRNA	Houf *et al*. 2000
	CRY2	AACAACCTACGTCCTTCGAC	23S rRNA	Houf *et al*. 2000
	SKIR	GGCGATTTACTGGAACACA	16S rRNA	Houf *et al*. 2000
16SrDNA-RFLP	CAH16S1am	AACACATGCAAGTCGAACGA	16S rRNA	Figueras *et al*. 2008
	CAH16S1b	TTAACCCAACATCTCACGAC	16S rRNA	Marshall *et al*. 1999
ERIC-PCR	ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	Genome	Vesalovic *et al*. 1991
	ERIC 2	AAGTAAGTGACTGGGGTGAGCG	Genome	Vesalovic *et al*. 1991
16SrRNA	Anti I	AGAGTTTGATCATGGCTCAG	16S rRNA	Martínez-Murcia *et al*.1992
	S	GGTTACCTTGTTACGACTT	16S rRNA	Martínez-Murcia *et al*.1992
rpoB	CamrpoB-L	CCAATTTATGGATCAAAC	*rpoB*	Korczak *et al*. 2006
	RpoB-R	GTTGCATGTTNGNACCCAT	*rpoB*	Korczak *et al*. 2006
gyrB	gyrB-Arc-7F	GTTTAYCAYTTTGAAGGTGG	*gyrB*	Collado *et al*. In prep.
	gyrB-Arc-14R	CTAGATTTTTCAACATTTAAAAT	*gyrB*	Collado *et al*. In prep.

3.4 References

Atabay, H.I. and Corry, J.E. (1997) The prevalence of campylobacters and arcobacters in broiler chickens. J Appl Microbiol 83, 619-626.

Collado, L., Fernández, H. and Figueras, M. J. *Arcobacter valdiviensis* sp. nov., isolated from a chicken cloacal swab sample in Valdivia, Chile. (Submitted for publication).

Cook, G.T. (1950) A Plate Test for Nitrate Reduction. J Clin Pathol 3, 359-362.

Ezaki, T., Hashimoto, Y. and Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane

ISBN: 978-84-693-0707-6 /DL:T-420-2010

filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39, 224–229.

Figueras, M.J., Polo, F., Inza, I., and Guarro, J. (1994) Poor specificity of m-Endo and m-FC culture media for the enumeration of coliform bacteria in sea water. Lett Appl Microbiol 19: 446–450.

Figueras, M.J., Collado, L. and Guarro, J. (2008) A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. Diagn Microbiol Infect Dis 62, 11-15.

Figueras, M.J., Inza, I., Polo, F.L., Feliu, M.T. and Guarro, J. (1996) A fast method for the confirmation of fecal streptococci from M-enterococcus medium. Appl Environ Microbiol 62, 2177-2178.

Goris, J., Suzuki, K., De Vos, P., Nakase, T. and Kersters, K. (1998). Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. Can J Microbiol 44, 1148–1153.

Houf, K., De Zutter, L., Van Hoof, J. and Vandamme, P. (2002) Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. Appl Environ Microbiol 68, 2172-2178.

Houf, K., Devriese, L.A., De Zutter, L., Van Hoof, J. and Vandamme, P. (2001) Development of a new protocol for the isolation and quantification of *Arcobacter* species from poultry products. Int J Food Microbiol 71, 189-196.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J. and Vandamme, P. (2000) Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiol Lett 193, 89-94.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16, 111-120.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Korczak, B.M., Stieber, R., Emler, S., Burnens, A.P., Frey, J. and Kuhnert, P. (2006) Genetic relatedness within the genus *Campylobacter* inferred from *rpoB* sequences. Int J Syst Evol Microbiol 56, 937-945.

Marmur, J. (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3, 208-218.

Marshall, S.M., Melito, P.L., Woodward, D.L., Johnson, W.M., Rodgers, F.G. and Mulvey, M.R. (1999) Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. J Clin Microbiol 37, 4158-4160.

Martinez-Murcia, A.J., Benlloch, S. and Collins, M.D. (1992) Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. Int J Syst Bacteriol 42, 412-421.

Mates, A. and Schaffer, M. (1988) Quantitative determination of *Escherichia coli* from faecal coliforms in seawater. Microbios 53, 161-165.

Mesbah, M., Premachandran, U. and Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. Int J Syst Bacteriol 39, 159–167.

Mills, C.K. and Gherna, R.L. (1987) Hydrolysis of indoxyl acetate by *Campylobacter* species. J Clin Microbiol 25, 1560-1561.

On, S.L., Holmes, B. and Sackin, M.J. (1996) A probability matrix for the identification of campylobacters, helicobacters and allied taxa. J Appl Bacteriol 81, 425-432.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24, 1596-1599.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673-4680.

Ursing, J.B., Lior, H. and Owen, R.J. (1994) Proposal of minimal standards for describing new species of the family *Campylobacteraceae*. Int J Syst Bacteriol 44, 842-845.

Vandamme, P., Dewhirst, F. E., Paster, B. J. and On, S. L. W. (2005) Genus II. *Arcobacter* Vandamme, Falsen, Rossau, Segers, Tytgat and De Ley 1991a, 99VP. In Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 2, pp. 1161–1165. Edited by D. J. Brenner, N. P. Kreig, J. T. Staley & G. M. Garrity. New York: Springer.

Versalovic, J., Koeuth, T. and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19, 6823-6831.

Wilson, K. (1987). Preparation of genomic DNA from bacteria. In Current Protocols in Molecular Biology, pp. 241–245. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Greene Publishing and Wiley Interscience.

Ziemke, F., Hofle, M.G., Lalucat, J. and Rossello-Mora, R. (1998) Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. Int J Syst Bacteriol 48 Pt 1, 179-186.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

4. RESULTS AND DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

4.1 A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*.

Figueras, M. J., Collado, L. & Guarro, J.

Diagnostic Microbiology and Infectious Diseases (2008) 62, 11-15

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Available online at www.sciencedirect.com

Diagnostic Microbiology and Infectious Disease 62 (2008) 11–15

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

www.elsevier.com/locate/diagmicrobio

A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*

Maria José Figueras*, Luis Collado, Josep Guarro

Unitat de Microbiologia, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus, Spain

Received 23 May 2007; accepted 10 September 2007

Abstract

The genus *Arcobacter* that includes some species of clinical interest has been recently enlarged with the addition of several new species. The available molecular techniques for the characterization of *Arcobacter* spp. are unable to identify all the species included in this genus. We have developed a 16S rDNA-RFLP method able to separate the currently accepted 6 species of *Arcobacter*, including the 2 hybridization groups of *Arcobacter cryaerophilus*. The method based on the use of a pair of primers and of the endonuclease *Mse*I has been validated using 12 reference strains (including the type strains) and 75 fresh isolates. All isolates tested produced species-specific RFLP patterns. This easy-to-perform method allows a fast and reliable recognition of the members of this complex genus.

Keywords: *Arcobacter*; 16S rDNA-RFLP; Species identification

1. Introduction

The genus *Arcobacter* was erected in 1991 to accommodate the species *Arcobacter nitrofigilis*, the type species, and *Arcobacter cryaerophilus*, both previously included in *Campylobacter* (Vandamme et al., 1991). One year later, 2 more species, *Arcobacter butzleri* and *Arcobacter skirrowii*, were added (Vandamme et al., 1992), and recently, the genus has been enlarged to 6 species with the inclusion of *Arcobacter cibarius* and *Arcobacter halophilus* (Donachie et al., 2005; Houf et al., 2005). The species *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* have been associated with human and animal infections and are frequently isolated from meat products with the highest prevalence in chicken followed by pork and beef (Ho et al., 2006). *A. cibarius* was isolated for the 1st time from skin of broiler carcasses and recently from piggery effluents (Chinivasagam et al., 2007; Houf et al., 2005). *A. nitrofigilis* is a nitrogen-fixing bacterium associated with the roots of the salt marsh plant *Spartina alterniflora* (McClung et al., 1983). *A. halophilus* was isolated from a hypersaline lagoon in Hawaii (Donachie et al., 2005) and is so far the only halophilic species of the genus.

Like *Campylobacter*, the phylogenetic close genus, *Arcobacter* is considered an emerging foodborne pathogen, and untreated water and contaminated foods appear to be potential sources of infection (Ho et al., 2006). A recent survey of 205 samples of environmental waters (rivers, lakes, seawater, and sewage) performed in the northeast of Spain, using culturing techniques and an m-PCR identification method, detected the presence of *Arcobacter* spp. in approximately half of the samples (Collado et al., 2008). The phenotypic identification of the species of *Arcobacter* is problematic due to their low metabolic activity and the atypical reactions of some isolates (Atabay et al., 2006; On, 1996), and a proper and reliable identification scheme does not yet exist. Several DNA-based approaches have been described for characterizing some species of *Arcobacter*, that is, RFLP (Carderelli-Leite et al., 1996; Hurtado and Owen, 1997; Kiehlbauch et al., 1991; Marshall et al., 1999), sequencing of 16S rRNA (Lau et al., 2002), *rpoB–rpoC* (Morita et al., 2004) and *gyrA* (Abdelbaqi et al., 2007a) genes, m-PCR techniques (Brightwell et al., 2007; Harmon and Wesley, 1997; Houf et al., 2000; Kabeya et al., 2003), and real-time PCR (Brightwell et al., 2007). Recently, a microarray technique (Quiñones et al., 2007) and a real-time

* Corresponding author. Tel.: +34-977759321; fax: +34-977759322.
E-mail address: mariajose.figueras@urv.cat (M.J. Figueras).

0732-8893/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2007.09.019

fluorescence resonance energy transfer PCR (Abdelbaqi et al., 2007b) for the detection and identification of *Arcobacter* spp. were reported. So far, the most employed identification method is the m-PCR technique of Houf et al. (2000), which enables characterization of the 3 species of clinical interest. However, none of the abovementioned methods are able to discriminate the 6 currently accepted species. For this reason, the main objective of this study was to develop a rapid and inexpensive 16S rDNA-RFLP method for the identification of all the species of the genus.

2. Materials and methods

2.1. Bacterial strains

A total of 87 strains were included in the study (Table 1). They were the type strains of the 6 species of *Arcobacter*, a reference strain of *A. nitrofigilis*, 5 strains of *A. cibarius* received from Dr. Houf, and 75 fresh isolates recovered from a survey of 72 different samples. Strains were isolated and biochemically identified as *Arcobacter* using procedures described by Atabay et al. (2006). The m-PCR method (Houf et al., 2000) in parallel with the 16S rDNA-RFLP method proposed here were used for identification of all isolates.

2.2. Computer analysis of the 16s rRNA gene

The 16S rRNA gene sequences of the type strains of *Arcobacter* spp. were downloaded from the GenBank database (Table 2). A fragment of them (1026 bp) was analyzed with the NEBcutter V2.0 software (Vincze et al., 2003), available online (http://tools.neb.com/NEBcutter2/index.php), to select an endonuclease able to yield species-specific restriction patterns. The following 10 endonucleases, *Alu*I, *Mbo*I, *Hae*II, *Taq*I, *Dde*I, *Alw*NI, *Bsr*I, *Tse*I, *Mse*I, and *Hpa*I, were tested, but only *Mse*I achieved this goal.

2.3. PCR, endonuclease digestion, electrophoresis, and pattern analysis

DNA was extracted from isolates grown in blood agar at 26 °C for 1 to 3 days under aerobic conditions, with the exception of *A. nitrofigilis*, which was incubated in a microaerobic atmosphere using gas generating kits (CampyGen; Oxoid, Basingstoke, UK). The extraction was performed using the InstaGene™ DNA Purification Matrix (Bio-Rad Laboratories, Hercules, CA). For each strain, 1026 bp of the 16S rRNA gene were amplified using the primers and conditions developed by Marshall et al. (1999) with a base modification in the primer CAH 1a. (The 3rd base was substituted from T→C to make it more specific for *Arcobacter* spp.) Enzymatic digestion was performed by incubating 10 μL of the amplification product with 10 U of the enzyme *Mse*I (Tru1I) (MBI Fermentas, St. Leon-Rot, Germany) and 2 μL of the 10× buffer R in a total volume of 30 μL. The reaction mixture was incubated at 65 °C for 3 h. Restriction fragments were separated on 15% polyacrylamide gel (ProtoGel; National Diagnostics, Hessle, UK) electrophoresis in Tris-Borate-EDTA (TBE) 1× buffer at 350 V for 5 h. The pBR322 DNA/*Bsu*RI (*Hae*III) (MBI Fermentas) was used as a molecular weight marker. The gels were stained with ethidium bromide and photographed on a UV transilluminator.

To determine the reproducibility of the technique, a set of 15 randomly chosen isolates was checked twice by the RFLP method. Gel images were saved as TIFF files, normalized with the abovementioned molecular marker, and further analyzed by Bionumerics software, version 1.5 (Applied Maths, Kortrijk, Belgium). A band-matching tolerance of 0.75% was chosen, and similarity matrices of whole densitometric curves of the gel tracks were calculated using the pairwise Pearson's product–moment correlation coefficient (r value).

2.4. Enterobacterial repetitive intergenic consensus PCR

To avoid the use of duplicate strains, all isolates recovered from the same location and belonging to the same species (18 strains from sludge, 7 from sewage, 4 from freshwater, 6 from seawater, and 5 from mussels) were genotyped using the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) technique with the protocol described by Houf et al. (2002). Patterns with at least 1 different band were considered as different types (Houf et al., 2002).

2.5. Sequencing of 16S rRNA genes

When some strains showed incongruent identification with the 2 methods used (m-PCR and RFLP), or when a different pattern to the 1 defined for the type strains was obtained, the 16S rRNA gene was sequenced. The 16S rRNA gene of these strains was amplified using the universal primers and protocol described by Martínez-Murcia et al. (1992). The PCR products were purified using GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Sequence analysis was performed with an Applied Biosystem 310 DNA sequencer using the ABI Prism Dye Terminator cycle sequencing ready reaction kit. Sequence assembly was performed using the program Auto Assembler (PerkinElmer, Norwalk, CT).

3. Results and discussion

The results of the computational restriction fragment length analysis applied to the type and reference strains showed that only *Mse*I produced species-specific patterns for the *Arcobacter* spp. and for each of the 2 DNA groups 1A and 1B (Table 2) described within *A. cryaerophilus* (Kiehlbauch et al., 1991). Therefore, this enzyme was selected for the experimental analysis. Simulation using the 16S rRNA sequences of all the type strains of *Campylobacter* and *Helicobacter* showed that the patterns obtained for those species could never be mistaken with those of *Arcobacter*. This has also been confirmed experimentally

Table 1
Strains of *Arcobacter* used in this study

Strain	Species identify by:		Source
	16S rDNA-RFLP	m-PCR	
LMG 10828^T (CCUG 10373 ^{T})	*A. butzleri*	*A. butzleri*	Human feces (USA)
W9, W14, W26, W50, W73, W110	*A. butzleri*	*A. butzleri*	Seawater (Spain)
AP6	*A. butzleri*	*A. butzleri*	Seawater (Italy) received from Dr. Maugueri
W7, W8, W54, W61, W76, W77,	*A. butzleri*	*A. butzleri*	Freshwater (Spain)
W84, W95, W97, W120, W232	*A. butzleri*	*A. butzleri*	Freshwater (Spain)
S9-2, S28, S30, S46	*A. butzleri*	*A. butzleri*	Sludge (Spain)
F5, F2137-4, F2139, F2168	*A. butzleri*	*A. butzleri*	Mussels (Spain)
F15	*A. butzleri*	*A. butzleri*	Rabbit meat (Spain)
F10, F11	*A. butzleri*	*A. butzleri*	Broiler meat (Spain)
F14, F17, F21	*A. butzleri*	*A. butzleri*	Turkey meat (Spain)
F27	*A. butzleri*	*A. butzleri*	Duck meat (Spain)
LMG 9904^T (CCUG 17801 ^{T})	*A. cryaerophilus*	*A. cryaerophilus*	Kidney, aborted bovine fetus (Ireland)
S88, S89, S91-4, S9, S10, S11, S12,	*A. cryaerophilus*	*A. cryaerophilus*	Sludge (Spain)
S13, S15, S24, S29, S30-2, S31, S32	*A. cryaerophilus*	*A. cryaerophilus*	Sludge (Spain)
FE17	*A. cryaerophilus*	*A. cryaerophilus*	Chicken cloacal swab (Chile)
F2065, F2079	*A. cryaerophilus*	*A. cryaerophilus*	Turkey meat (Spain)
F2130	*A. cryaerophilus*	*A. cryaerophilus*	Beef meat (Spain)
F35-2, F36, F45	*A. cryaerophilus*	*A. cryaerophilus*	Mussels (Spain)
W37	*A. cryaerophilus*	*A. cryaerophilus*	Freshwater (Spain)
W12, W13, W79, W108	*A. cryaerophilus*	*A. cryaerophilus*	Seawater (Spain)
SW33, SW35, SW36, SW39, SW40,	*A. cryaerophilus*	*A. cryaerophilus*	Sewage (Spain)
SW42, SW44	*A. cryaerophilus*	*A. cryaerophilus*	Sewage (Spain)
LMG 6621^T (CCUG 10374 ^{T})	*A. skirrowii*	*A. skirrowii*	Lamb feces
989	*A. skirrowii*	*A. skirrowii*	Cow feces (Belgium) received from Dr. Houf
994	*A. skirrowii*	*A. skirrowii*	Pig feces (Belgium) received from Dr. Houf
F2108	*A. skirrowii*	*A. skirrowii*	Tusk meat (Spain)
S90-2	*A. skirrowii*	*A. skirrowii*	Sludge (Spain)
CECT 7204^T (CCUG 15893 ^{T})	*A. nitrofigilis*	*A. skirrowii*	Roots of *S. alterniflora* (Canada)
LMG 7547	*A. nitrofigilis*	*A. skirrowii*	Root-associated sediment of *S. alterniflora* (Canada)
F2176, F2173	*A. nitrofigilis*	*A. skirrowii*	Mussels (Spain)
F2026, F2075, F2076	*Arcobacter* sp.	*A. skirrowii*	Mussels (Spain)
CECT 7203^T (LMG 21996 ^{T})	*A. cibarius*	ND	Poultry carcass (Belgium)
742, 743, 745, 746, 748	*A. cibarius*	ND	Poultry carcass (Belgium) received from Dr. Houf
LA31B^T (CIP 108450 ^{T})	*A. halophilus*	*A. cryaerophilus/A. skirrowii*	Hypersaline lagoon (USA) received from Dr. Alam

LMG = Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Gent, Belgium; CECT = Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CCUG = Culture Collection of the University Göteborg, Göteborg, Sweden; CIP = Collection Bactérienne de l'Institut Pasteur, Paris, France; ND = not detected, because no amplicons were obtained; W = water; S = sludge; SW = sewage; F = food; FE = feces.

using several strains of *Campylobacter* and other unrelated genera (i.e., *Escherichia*, *Salmonella*, *Vibrio*, *Aeromonas*, *Pseudomonas*), although it is very improbable that strains of these unrelated genera can be mistaken with *Arcobacter*. All of them produced different patterns from those described for *Arcobacter* (Fig. 1). All the isolates typed with ERIC-PCR showed different patterns (data not shown), which confirmed them as belonging to different strains. The obtained RFLP patterns for 70 of the 75 fresh isolates investigated were identical to those predicted. Fig. 2 shows an example of the RFLP patterns. However, 5 isolates from mussels that were identified by the Houf's m-PCR method (Houf et al., 2000) as *A. skirrowii* on the basis of the presence of a 641-bp band characteristic of this species showed discordant results. Two of these strains showed the RFLP pattern of *A. nitrofigilis*, their identity being confirmed by sequencing ca. 1480 bp of the 16S rRNA gene (GenBank accession number: EU106661 and EU106662). The type and reference strains of *A. nitrofigilis* also showed the 641-bp band of *A. skirrowii*. These results disagree with Houf et al. (2000) who indicated that, for *A. nitrofigilis*, no amplicons should be obtained with the m-PCR technique. Interestingly, the other 3 remaining strains isolated from mussels showed a novel RFLP pattern (Fig. 2). In the phylogenetic tree inferred from the analysis of the 16S rRNA gene sequences, those strains grouped in a separate branch, close to *A. halophilus* (data not shown). The similarity of the sequences of those strains with the other species of the genus was 93% to 94%.

Of the 21 strains of *A. cryaerophilus* investigated, all except 1 produced the pattern of the subgroup 1B, which seems to demonstrate the higher prevalence of this subgroup. This agrees with the data reported by other authors (Schroeder-Tucker et al., 1996). All the strains of *A. cibarius* and *A. halophilus* tested produced the expected RFLP patterns. However, using the m-PCR method *A. halophilus* showed 2 amplicons of 257 and 641 bp, which coincided with those described by Houf et al. (2000) as characteristic of *A. cryaerophilus* and *A. skirrowii*,

Table 2
DNA fragments obtained after computer simulation of the digestion of the 16S rRNA gene (1026 bp) with the endonuclease *Mse*I

Species	Reference strain	GenBank accession no.	Presence of 16S rRNA gene RFLP fragments of the following size (bp):										
			551	548	519	434	395	365	243	216	143	138	101[a]
A. butzleri	CCUG 10373^T	L14626		X						X		X	
A. cryaerophilus 1A	CCUG 17801^T	L14624					X			X	X	X	
A. cryaerophilus 1B	CCUG 17802	AY314755						X		X	X	X	
A. skirrowii	CCUG 10374^T	L14625						X	X		X	X	
A. halophilus	LA31B^T	AF513455	X								X	X	X
A. nitrofigilis	CCUG 15893^T	L14627				X				X		X	X
A. cibarius	LMG 21996^T	AJ607391			X				X			X	

[a] Small-size bands below 100 bp were not resolved in the electrophoresis and not included in the table.

respectively. This is not surprising if we consider that the species *A. halophilus* was not yet known at the time the m-PCR method was described. *A. cibarius* did not show amplification with the m-PCR method as already indicated (Houf et al., 2005). Thirteen isolates of the latter species have recently been isolated from a piggery effluent and effluent-irrigated soil, and could only be identified after partial sequencing of the 16S rRNA gene (Chinivasagam et al., 2007). One of the advantages of the proposed RFLP method is that it is the 1st rapid molecular test able to identify *A. halophilus* and *A. cibarius*, which cannot be differentiated even by the most recently published methods (Brightwell et al., 2007; González et al., 2006). Although the m-PCR may distinguish the 3 most common species of *Arcobacter* (Houf et al., 2000), it has failed to separate *A. nitrofigilis* from *A. skirrowii*, which may hamper establishing the true prevalence of these species. The misidentification detected in our study between *A. nitrofigilis* and *A. skirrowii* could also have occurred in other studies that characterized *A. skirrowii* with the m-PCR method.

The RFLP patterns of the tested strains yielded a Pearson's correlation coefficient ≥96% and showed no variation when tested with different batches of DNA, which indicated that they were reproducible.

In conclusion, a method able to differentiate the 6 species of the genus *Arcobacter* using only a pair of primers and a single enzyme is described. This rapid and inexpensive technique can be performed in only 1 working day, is highly discriminatory, and is much more efficient and cost-effective than the classic phenotypic identification methods.

Acknowledgments

The authors thank Dr. Maqsudul Alam (University of Hawaii, Manoa, HI) for kindly providing the type strain of *A. halophilus* and Dr. Kurt Houf (Ghent University, Ghent, Belgium) for providing strains of *A. cibarius* and *A. skirrowii*.

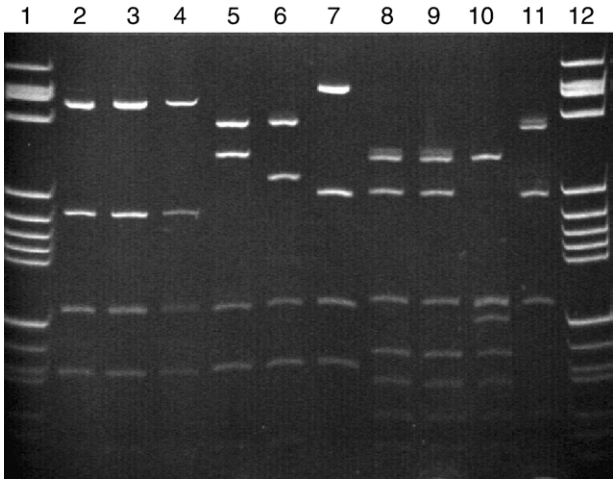


Fig. 1. Polyacrylamide gel showing the 16S rDNA-RFLP patterns, obtained by using the endonuclease *Mse*I. Lanes 1 and 12, pBR322 DNA/*Bsu*RI (*Hae*III) ladder; 2, *Aeromonas hydrophila* (CECT 839^T); 3, *Aeromonas caviae* (CECT 838^T); 4, *Vibrio cholerae* NO:01 (CECT 557); 5, *Salmonella typhimurium* (ATCC 14028); 6, *Escherichia coli* (CECT 744); 7, *Pseudomonas aeruginosa* (CECT 108); 8, *Campylobacter jejuni* (DSM 4688^T); 9, *Campylobacter coli* (DSM 4689^T); 10, *Campylobacter lari* (DSM 11375^T); 11, *Campylobacter fetus* (20402).

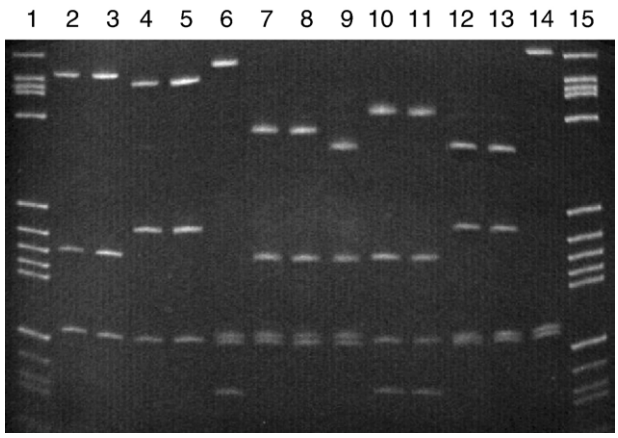


Fig. 2. Polyacrylamide gel showing the 16S rDNA-RFLP patterns, obtained by using the endonuclease *Mse*I. Lanes 1 and 15, pBR322 DNA/*Bsu*RI (*Hae*III) ladder; 2, *A. butzleri* (LMG 10828^T); 3, *A. butzleri* (W9); 4, *A. cibarius* (CECT 7203^T); 5, *A. cibarius* (748); 6, *A. halophilus* (LA31B^T); 7, *A. cryaerophilus* 1A (LMG 9904^T); 8, *A. cryaerophilus* 1A (F2065); 9, *A. cryaerophilus* 1B (F2079); 10, *A. nitrofigilis* (CECT 7204^T); 11, *A. nitrofigilis* (LMG 7547); 12, *A. skirrowii* (LMG 6621^T); 13, *A. skirrowii* (F2108); 14, *Arcobacter* sp. (F2075).

References

Abdelbaqi K, Ménard A, Prouzet-Mauleon V, Bringaud F, Lehours P, Mégraud F (2007a) Nucleotide sequence of the *gyr*A gene of *Arcobacter* species and characterization of human ciprofloxacin-resistant clinical isolates. *FEMS Immunol Med Microbiol* 49: 337–345.

Abdelbaqi K, Buissonnière A, Prouzet-Mauleon V, Gresser J, Wesley I, Mégraud F, Ménard A (2007b) Development of a real-time fluorescence resonance energy transfer PCR to detect *Arcobacter* species. *J Clin Microbiol* 45:3015–3021.

Atabay HI, Waino HI, Madsen M (2006) Detection and diversity of various *Arcobacter* species in Danish poultry. *Int J Food Microbiol* 109:139–145.

Brightwell G, Mowat E, Clemens R, Boerema J, Pulford DJ, On S (2007) Development of a multiplex and real time PCR assay for the specific detection of *Arcobacter butzleri* and *Arcobacter cryaerophilus*. *J Microbiol Methods* 68:318–325.

Carderelli-Leite P, Blom K, Patton CH, Nicholson M, Steigerwalt A, Hunter S, Brenner D, Barret T, Swaminathan B (1996) Rapid identification of *Campylobacter* species by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. *J Clin Microbiol* 34:62–67.

Chinivasagam HN, Corney BG, Wright LL, Diallo IS, Blackall PJ (2007) Detection of *Arcobacter* spp. in piggery effluent and effluent-irrigated soils in southeast Queensland. *J Appl Microbiol* 103:418–426.

Collado L, Inza I, Guarro J, Figueras MJ (2008) Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. *Environ Microbiol* 10:1635–1640.

Donachie SP, Bowman JP, On SLW, Alam M (2005) *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. *Int J Syst Evol Microbiol* 55:1271–1277.

González A, Moreno Y, González R, Hernández J, Ferrús MA (2006) Development of a simple and rapid method based on polymerase chain reaction-based restriction fragment length polymorphism analysis to differentiate *Helicobacter*, *Campylobacter*, and *Arcobacter* species. *Curr Microbiol* 53:416–421.

Harmon KM, Wesley IV (1997) Multiplex PCR for the identification of *Arcobacter* and differentiation of *Arcobacter butzleri* from other arcobacters. *Vet Microbiol* 58:215–227.

Ho HT, Lipman LJ, Gaastra W (2006) *Arcobacter*, what is known about a potential foodborne zoonotic agent! *Vet Microbiol* 115:1–13.

Houf K, Tutenel A, De Zutter L, Van Hoof J, Vandamme P (2000) Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. *FEMS Microbiol Lett* 193: 89–94.

Houf K, De Zutter L, Van Hoof J, Vandamme P (2002) Assessment of the genetic diversity among *Arcobacter* isolated from poultry products by using two PCR-based typing methods. *Appl Environ Microbiol* 68:2172–2178.

Houf K, Stephen L, On W, Coenye T, Mast J, Van Hoof J, Vandamme P (2005) *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. *Int J Syst Evol Microbiol* 55:713–717.

Hurtado A, Owen RJ (1997) A molecular scheme based on 23 rRNA gene polymorphisms for rapid identification of *Campylobacter* and *Arcobacter* species. *J Clin Microbiol* 35:2401–2404.

Kabeya H, Koboyashi Y, Maruyama S, Mikami T (2003) One-step polymerase Caín reaction-based typing of *Arcobacter* species. *Int J Food Microbiol* 81:163–168.

Kiehlbauch JA, Plikaytis BD, Swaminathan B, Cameron DN, Wachsmuth IA (1991) Restriction fragment length polymorphisms in the ribosomal genes for species identification and subtyping of aerotolerant *Campylobacter* species. *J Clin Microbiol* 29:1670–1676.

Lau SK, Woo PC, Teng JL, Leung KW, Yuen KY (2002) Identification by 16S ribosomal RNA gene sequencing of *Arcobacter butzleri* bacteraemia in a patient with acute gangrenous appendicitis. *J Clin Pathol* 55:182–185.

Marshall SM, Melito PL, Woodward DL, Johnson WM, Rodgers FG, Mulvey R (1999) Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. *J Clin Microbiol* 37:4158–4160.

Martinez-Murcia AJ, Benlloch S, Collins MD (1992) Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA–DNA hybridizations. *Int J Syst Bacteriol* 42:412–421.

McClung CR, Patriquin DG, Davis RE (1983) *Campylobacter nitrofigilis* sp. nov., a nitrogen-fixing bacterium associated with roots of *Spartina alterniflora* Loisel. *Int J Syst Bacteriol* 33:605–612.

Morita Y, Maruyama S, Kabeya H, Boonmar S, Nimsuphan B, Nagai A, Kozawa K, Nakajima T, Mikami T, Kimura H (2004) Isolation and phylogenetic analysis of *Arcobacter* spp. in ground chicken meat and environmental water in Japan and Thailand. *Microbiol Immunol* 48:527–533.

On SL (1996) Identification methods for campylobacters, helicobacters, and related organisms. *Clin Microbiol Rev* 9:405–422.

Quiñones B, Parker CT, Janda Jr JM, Miller WG, Mandrell RE (2007) Detection and genotyping of *Arcobacter* and *Campylobacter* isolates from retail chicken samples by use of DNA oligonucleotide arrays. *Appl Environ Microbiol* 73:3645–3655.

Schroeder-Tucker L, Wesley IV, Kiehlbauch JA, Larson DJ, Thomas LA, Erickson GA (1996) Phenotypic and ribosomal RNA characterization *Arcobacter* species isolated from porcine aborted fetuses. *J Vet Diagn Invest* 8:186–195.

Vandamme P, Falsen E, Rossau R, Hoste B, Segers P, Tytgat R, De Ley J (1991) Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* 41:88–103.

Vandamme P, Vancanneyt M, Pot B, Mels L, Hoste B, Dewettinck D, Vlaes L, Van Den Borre C, Higgins R, Hommez J, Kersters K, Butzler JP, Gossens H (1992) Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* 42:344–356.

Vincze T, Posfai J, Roberts RJ (2003) NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Res* 31:3688–3691.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

4.2 Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution.

Collado, L., Inza, I., Guarro, J. & Figueras, M. J.

Environmental Microbiology (2008) 10, 1635-1640

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Environmental Microbiology (2008) **10**(6), 1635–1640 doi:10.1111/j.1462-2920.2007.01555.x

Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution

Luis Collado, Isabel Inza, Josep Guarro and Maria Jose Figueras*
Unitat de Microbiologia, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus, Spain.

Summary

We investigated the presence of *Arcobacter* spp. in 205 water samples of freshwater, seawater and sewage in Spain. These bacteria were present in 55.1% of the samples (113/205) and were significantly associated for the first time with bacterial indicators of fecal pollution. The dominant species in the positive samples was *Arcobacter butzleri* (94%) followed by *Arcobacter cryaerophilus* (30%) and *Arcobacter skirrowii* (1.8%).

The genus *Arcobacter* belongs to the family *Campylobactereaceae*, and includes six species: *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter nitrofigilis, Arcobacter skirrowii*, *Arcobacter cibarius* and *Arcobacter halophilus* (Donachie *et al*., 2005; Houf *et al*., 2005; Vandamme *et al*., 2005). *Arcobacter* is considered an emerging, potential food and waterborne pathogen (Ho *et al*., 2006; Samie *et al*., 2006). The species *A. butzleri* and *A. cryaerophilus* have been associated with diarrhoea and bacteremia in humans and with abortion, mastitis and diarrhoea in animals (Ho *et al*., 2006). *Arcobacter butzleri* is the most common species of the genus isolated from the stools of patients in Belgium and France (Ho *et al*., 2006) and from patients with gastrointestinal complains in South Africa (Samie *et al*., 2006). Although *A. skirrowii* has only been associated with chronic diarrhoea in an elderly patient (Wybo *et al*., 2004), it is frequently isolated from preputial fluids of bulls and feces of animals, including sheep and cattle (Vandamme *et al*., 2005). The epidemiology and pathogenic role of *Arcobacter* spp. in human disease is still unknown. Although it has been indicated that these bacteria are members of the microbiota of seawater (Wirsen *et al*., 2002; Fera *et al*., 2004), only a few authors have investigated their presence in water environments, i.e. seawater, rivers, ground waters, wastewater and drinking water reservoirs (Jacob *et al*., 1993; Rice *et al*., 1999; Moreno *et al*., 2003; Fera *et al*., 2004; Maugeri *et al*., 2004; Morita *et al*., 2004). These studies concluded that water may play an important role in the transmission of this microbe to humans and animals. In fact, in a very recent reported drinking water outbreak in Ohio, *Arcobacter* was recorded in the most fecally contaminated groundwater wells that provided potable water to the public (Fong *et al*., 2007). Water microbiology quality is based on the levels of indicators of fecal pollution as predictors of the presence of intestinal pathogenic microorganisms. However, nothing is known about the potential relationship between such indicators and the presence of *Arcobacter*. Therefore, the main objectives of this study were to determine the prevalence of *Arcobacter* species in environmental waters and try to establish its relationship with fecal contamination.

A total of 205 samples were investigated between May and August of 2006, 159 were from freshwater and seawater from bathing sites in Catalonia (north-east Spain), 27 from sludge and 19 from sewage (Table 1). Freshwater sites corresponded to rivers and lakes. Seawater sites included 28 beaches of excellent water quality and 17 that received different degrees of fecal pollution through contaminated freshwater streams. All those beaches had relatively small (few cm) tidal fluctuations characteristic of most Mediterranean beaches. Data on salinity and temperature were collected for all seawater stations. While temperature was quite stable at all the stations ranging from 20°C to 22°C throughout the study period, a mean salinity of 37.8‰ was recorded for the 28 beaches of excellent water quality and of 29.1‰ for the remaining 17 beaches reflecting the impact of freshwater. Sampling at bathing areas was performed at chest level two or three times throughout the study period. The majority of the sewage samples were taken at the Waste Water Treatment Plant of the city of Reus, treating water from c. one hundred thousand persons. Sludge had also the same origin.

For the detection and isolation of *Arcobacter* spp., 200 ml of each water sample were filtered using a 0.45 μm membrane filter (Millipore, Bedford, MA, USA). The filters were rolled and placed into tubes containing 9 ml of *Arcobacter* enrichment broth (peptone, 18 g; NaCl,

Received 30 March, 2007; accepted 5 December, 2007. *Correspondence. E-mail mariajose.figueras@urv.cat; Tel. (+34) 977759321; Fax (+34) 977759322.

Table 1. Prevalence of *Arcobacter* spp. in freshwater, seawater, sewage and sludge.

Type of sample (*n*)	N° positives samples (%)	Species obtained from samples (%)			
		A. butzleri	*A. cryaerophilus*	*A. butzleri* + *A. cryaerophilus*	*A. butzleri* + *A. skirrowii*
Rivers (29)	17 (58.6)	16 (94.1)[a]	1 (5.9)	–	–
Lakes (29)	8 (27.6)	8 (100)	–	–	–
Seawater (101)	43 (42.6)	36 (83.7)	3 (7.0)[b]	3 (7.0)[c]	1 (2.3)
Sewage (19)	19 (100)	5 (26.3)	–	14 (73.7)	–
Sludge (27)	26 (96.3)	12 (46.2)	3 (11.5)	10 (38.5)	1 (3.8)
Total (205)	113 (55.1)	77 (68.1)	7 (6.2)	27 (23.9)	2 (1.8)

a. In one sample the species was detected from the enrichment broth but not recovered by culture.
b. In one sample the species was isolated in culture but not detected from the broth.
c. In two samples both species were detected from the enrichment broth but only *A. butzleri* was recovered by culture.

5 g and yeast extract, 1 g) supplemented with CAT (cefoperazone, amphotericin B and teicoplanin) (Oxoid, Basingstoke, UK) and were incubated at 30°C for 48 h under aerobic conditions. After enrichment, 200 μl of the broth was transferred onto the surface of a 0.45 μm membrane filter that had been placed on a Petri dish containing blood agar and was allowed to filter passively under ambient conditions for 30 min (Atabay and Corry, 1997). Then, the filters were removed and the plates were incubated at 30°C for 48 h to 72 h under aerobic condition. Three or four suspicious colonies were picked up, and checked by Gram stain, oxidase and motility. Final identification of the presumptive isolates was made using the multiplex polymerase chain reaction (m-PCR) using the primers ARCO, BUTZ, CRY1, CRY2 and SKIR described by Houf and colleagues (2000). For isolation of arcobacters from sludge, 1 ml of sample was diluted in 9 ml of buffered peptone water. Aliquots of 300 μl were inoculated in *Arcobacter* enrichment broth and the same procedure described above was followed for culturing. Polymerase chain reaction detection of *Arcobacter* spp. from water and sludge was carried out using 400 μl of the *Arcobacter* enrichment broth after incubation using the same above-mentioned method.

A stream of fecally contaminated freshwater discharging in one of the beaches sampled was chosen for the quantification of *Arcobacter* and the sampling was performed three times (weekly) at four sites: (i) in the stream at *c.* 300 m before the seashore, (ii) at the point of entrance of the freshwater into the sea, (iii) in the seawater *c.* 10 m south from the discharge and (iv) in the seawater *c.* 200 m south from the discharge. Quantification was performed in duplicate using the most-probable-number (MPN) method (Chinivasagam *et al.*, 2007). However, in our study we chose *Arcobacter* enrichment broth supplemented with CAT and with the posterior passively filtration over a blood agar for isolation and confirmation. Concentration of *Arcobacter* and indicators at each site were recorded as mean values. Levels of nutrients (NO_3, NO_2, PO_4, SiO_4 and NH^+_4), salinity and chlorophyll-*a* were also evaluated at those four sites (Table 2).

The membrane filtration technique was used to determine fecal coliforms (FC), *Escherichia coli* (Ec) and intestinal enterococci (IE) using previously described methods (Figueras *et al.*, 1994; 1996; Mates and Schaffer, 1988).

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 11.5.5, Chicago, USA). The data did not follow a normal distribution and therefore the non-parametric test of Mann–Whitney *U*-Wilcoxon rank sum *W* was used for comparing the geometric means of the indicators bacteria in samples with and without *Arcobacter* and also for comparing the concentration of indicators in the different types of waters. Statistical significance was assessed at $P < 0.05$.

The results obtained are shown in Table 1. The number of samples positive for *Arcobacter* was 113 (55.1%). Overall, the presence of *Arcobacter* spp. was similar in fresh and in sea water (Table 1). However, among the freshwater samples a significant difference was shown

Table 2. Basic physicochemical characteristics of the water samples where *Arcobacter* have been enumerated.

Samples	NO_3	NO_2	NH^+_4	PO_4	SiO_4	Chl-*a*	Salinity
Freshwater	137.8[a]	1.7	15.3	3.1	63.3	1.7	0.3
Seashore outlet	54.5	1.3	14.9	1.8	25.1	1.3	29.1
10 m south	17.0	1.1	13.6	0.7	6.9	0.7	30.6
200 m south	6.2	1.1	7.3	0.4	5.4	0.6	34.3

All data of nutrients are expressed as μmol l^{-1}, chlorophyll-a as μg l^{-1} and salinity as ‰.
[a]Mean of three values.

Table 3. Concentrations of indicator bacteria in water samples of different origins in relation to the presence/absence of *Arcobacter*.

Origin (samples tested)	Indicator	Absence of *Arcobacter* Geometric mean	95% CI	Presence of *Arcobacter* Geometric mean	95% CI
Freshwater (58)	FC	43[a]	24–79	541[b]	135–2165
	Ec	25	11–56	200[b]	52–762
	IE	10	5–17	39[b]	16–94
Seawater (101)	FC	19	10–39	666[b]	253–1758
	Ec	42[c]	11–171	139	48–405
	IE	7	4–10	76[b]	37–156
Total (159)	FC	26	16–42	617[b]	284–1341
	Ec	32[c]	15–66	159[b]	70–358
	IE	8	6–11	60[b]	35–103

a. cfu/100 m.
b. Statistically significant in comparison to *Arcobacter* (–) samples ($P < 0.05$).
c. *Escherichia coli* was only analysed on the samples with FC above 100 cfu/100 ml.
FC, fecal coliforms; Ec, *E. coli*; IE, intestinal enterococci; CI, confidence interval.

between rivers and lakes-reservoirs (58.6% versus 27.6%, $P = 0.016$). *Arcobacter* was isolated from almost all sewage and sludge samples. The three species of *Arcobacter* of clinical significance were isolated in the study (Table 1), *A. butzleri* being the dominant species (94%) followed by *A. cryaerophilus* (30%). Both species were isolated together in 23.9% of the samples. *Arcobacter skirrowii* was only identified in combination with *A. butzleri* in one sample of seawater and one sample of sludge. The highest frequency of *A. butzleri* is a constant characteristic in many studies (Fera *et al*., 2004; Atabay *et al*., 2006; Ho *et al*., 2006; Samie *et al*., 2006).

The two methods we used for detecting and recovering arcobacters yielded similar results, only four water samples showed differences between m-PCR species detection and isolation by culture (Table 1). This concordance is similar to that found in another study also using both methods in parallel (Houf *et al*., 2000). However, Fera and colleagues (2004) had previously shown that *Arcobacter* detection by PCR was better than by culture when filtering the water through 0.22 μm membrane filters. In our view, the discordance observed by these authors is related to the culture methodology used, because despite the fact they used the same enrichment media as ours, the incubation they did was only of 24 h, after which they streaked the same media-containing agar. It is well known, that *Arcobacter* species have a low growth rate and therefore most of the studies used an enrichment step of 48 h (Atabay and Corry, 1997; Moreno *et al*., 2003). Furthermore, several authors have reported that the isolation media-containing CAT supplement is generally overgrown by different bacteria that hampers the *Arcobacter* detection (Atabay and Corry, 1997; Chinivasagam *et al*., 2007). This problem was solved by Atabay and Corry (1997) using the passive filtration method to inoculate the agar plate, as we did. To further explore methodological differences 25 additional seawater samples (data not included in the tables) were investigated using the same direct PCR detection from water samples, culture media and methods used by Fera and colleagues (2004) in parallel with those used in our study. These samples were taken again from locations where *Arcobacter* was previously not detected. Only one sample out of the 25 was positive by culture and by PCR detection either in the water or in the enrichment broth. This positive sample showed high levels of FC (2000 cfu/100 ml), Ec (364 cfu/100 ml) and IE (108 cfu/100 ml), while the indicators were not detected or were below 20 cfu/100 ml in the rest of the samples. Moreover, this PCR positive sample was also positive using our methods. However, the observed overgrowth using the culture media and procedure of Fera and colleagues (2004) hampered recovery of *Arcobacter* in this sample. Also other authors like González and colleagues (2007) in a survey of *Arcobacter* in wastewater found a high discrepancy between the molecular and culture methods. Again, in our view their poor recovery by culture could be due to the presence of interfering microbes, because only 5-fluorouracil was added to the *Arcobacter* broth, instead of the CAT supplement and posterior filtration used in our case and in other studies (Atabay and Corry, 1997).

Our study is the first to demonstrate the association between *Arcobacter* spp. and bacterial indicators of fecal pollution and has revealed that samples, from the different origins, that were positive for *Arcobacter* showed a significantly higher concentration of the three indicator organisms than the negative ones (Table 3). This also may explain the higher prevalence in rivers (more fecally polluted) than in lakes encountered in our study. Our results on the incidence of arcobacters in the different types of waters are difficult to compare with those of other authors because in most cases data on the magnitude of fecal pollution were not reported. The incidence of *Arcobacter* spp. found in 17 river water samples studied in Japan was relatively low (23%) (Morita *et al*., 2004) when compared with our results (58.6%). More similar results to

ours were those of Moreno and colleagues (2003) who isolated *Arcobacter* spp. in 40% of the samples from river and wastewater in Valencia (Spain). To our knowledge, our study is the first report on the isolation of *A. cryaerophilus* and *A. skirrowii* from seawater. Previously, Fera and colleagues (2004) already isolated *A. butzleri* in seawater, but not the two other mentioned species, which were only detected by PCR. These authors considered *Arcobacter* as belonging to the microbiota of seawater (Fera *et al*., 2004). However, based on the present study and on additional results of those authors on the abundance of *E. coli* and enterococci in their seawater samples (Maugeri *et al*., 2004), we think that the presence of *Arcobacter* has a fecal origin. In fact, in our study we never detected *Arcobacter* in seawater samples in the absence of fecal indicators and the incidence was very low 11.9% (12/101) when the concentration of all of the indicators was less than 100 cfu/100 ml ($P < 0.001$). Most of the samples positive for *Arcobacter* had levels of Ec and IE above the standards established in the new European Directive for the bathing waters (2006/7/EC). Another clear example of the correlation between the presence of arcobacters and the levels of fecal indicator can be found in a recent drinking waterborne outbreak in Ohio (Fong *et al*., 2007). In this outbreak that affected approximately 1.450 persons, the groundwater wells that provided potable water to public systems were studied for fecal indicators, coliphages and several enteropathogens. *Arcobacter* sp. was isolated from the samples with the highest fecal contamination. The high prevalence of *Arcobacter* found in our study in sewage and sludge samples seems to confirm this fecal origin. In an attempt to clarify if the presence of *Arcobacter* in seawater was linked to freshwater-contaminated inputs with this microbe, we have quantified *Arcobacter* by the MPN and a clear gradient was observed on the samples studied, from 3.7×10^5 MPN/100 ml at the freshwater stream, to 2.3×10^5 MPN/100 ml at the seashore area and to 5.2×10^4 MPN/100 ml at the seawater 10 m south from the discharge. It is significant that at the latter sampling site one of the three samples taken was negative but this sample had levels of FC, *E. coli* and IE of 2, < 1 and 2 cfu/100 ml respectively. None of the samples taken at the seawater 200 m south from the discharge were positive for *Arcobacter* or fecal indicators. These results indicated that *Arcobacter* entered the sea with the sewage polluted freshwater stream, which showed levels of FC, *E. coli* and IE of 9788, 4932 and 325 cfu/100 ml respectively. This was also confirmed by the high nutrient load of the fecally polluted stream (Table 2). The reported high incidence of *Arcobacter* spp. in porcine (43.9%), bovine (39.2%), ovine (16.1%) and equine (15.4%) livestock fecal samples (Van Driessche *et al*., 2003) could to some extent justify its presence in water. In a study in which the *Arcobacter* concentration in a piggery effluent was quantified, the levels varied from 1.1×10^8 to 6.5×10^5 MPN/100 ml (Chinivasagam *et al*., 2007). These results are similar to those found in our study. Previous studies in Italy revealed high densities of *Arcobacter* in sewage and sludge, and suggested that this microbe may be present in urban sewage through the inflows from slaughterhouses (Stampi *et al*., 1993; 1999). However, in our view, human feces may also contribute to its presence, either through the feces of patients infected with *Arcobacter* (Taylor *et al*., 1991; Ho *et al*., 2006) or from healthy carriers (Houf and Stephan, 2007). In our study all sewage samples from the wastewater treatment plant of our city, that treat mainly human sewage were positive for *Arcobacter*. It is possible that in the marine environment potential pathogenic *Arcobacter* species, from human and animal origin, as well as other marine autochthonous species like the sulfide-oxidizing nitrogen-fixing *Arcobacter* (Wirsen *et al*., 2002) may coexist.

It has been suggested that *Arcobacter* may be better adapted than *Campylobacter* to survive in aquatic environments owing to its higher resistance to high NaCl concentration, to low temperatures and its capacity to survive and grow in aerobic conditions (D'Sa and Harrison, 2005). In our study, marine salinity had no effect because *Arcobacter* could be recovered equally from all ranges of salinities (freshwater 43.1% and seawater 42.6%), with no significant differences ($P = 0.69$). However, the amount of nutrients associated with fecal contamination may also play a role favouring the survival and persistence of *Arcobacter* in the water environment. This can be supported by a recent study that has experimentally evaluated the survival of *Arcobacter* species in sterilized drinking water with the addition of organic matter (a mixture of sterile horse blood and cow's milk) and found that this organic matter favoured their survival up to 250 days, but regrowth was not observed (Van Driessche and Houf, 2007). Our results of quantification do not seem to indicate that *Arcobacter* is able to grow in seawater, but as it happens with fecal indicators, sediments may act as a reservoir together with plankton as already demonstrated (Fera *et al*., 2004; Maugeri *et al*., 2005).

In conclusion, the presence of *Arcobacter* spp. in environmental waters may be regarded as a potential risk for human health, considering that many of the environmental waters studied are used for bathing and recreational activities, and that their presence in marine waters may contaminate shellfish.

Acknowledgements

The authors would like to acknowledge the Agencia Catalana de l'Aigua, Generalitat de Catalunya for providing data on nutrients.

References

Atabay, H.I., and Corry, J.E. (1997) The prevalence of campylobacters and arcobacters in broiler chickens. *J Appl Microbiol* **83:** 619–626.

Atabay, H.I., Wainø, M., and Madsen, M. (2006) Detection and diversity of various *Arcobacter* species in Danish poultry. *Int J Food Microbiol* **109:** 139–145.

Chinivasagam, H.N., Corney, B.G., Wright, L.L., Diallo, I.S., and Blackall, P.J. (2007) Detection of *Arcobacter* spp. in piggery effluent and effluent-irrigated soils in southeast Queensland. *J Appl Microbiol* **103:** 418–426.

Donachie, S.P., Bowman, J.P., On, S.L.W., and Alam, M. (2005) *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus Arcobacter. *Int J Syst Evol Microbiol* **55:** 1271–1277.

D'Sa, E.M., and Harrison, M.A. (2005) Effect of pH, NaCl content, and temperature on growth and survival of *Arcobacter* spp. *J Food Protect* **68:** 18–25.

Fera, M.T., Maugeri, T.L., Gugliandolo, C., Beninati, C., Giannone, M., La Camera, E., and Carbone, M. (2004) Detection of *Arcobacter* spp. in the coastal environment of the Mediterranean sea. *Appl Environ Microbiol* **70:** 1271–1276.

Figueras, M.J., Polo, F., Inza, I., and Guarro, J. (1994) Poor specificity of m-Endo and m-FC culture media for the enumeration of coliform bacteria in sea water. *Lett Appl Microbiol* **19:** 446–450.

Figueras, M.J., Inza, I., Polo, F., Feliu, M.T., and Guarro, J. (1996) A fast method for the confirmation of fecal streptococci from M-Enterococcus medium. *Appl Environ Microbiol* **62:** 2177–2178.

Fong, T.T., Mansfield, L.S., Wilson, D.L., Schwab, D.J., Molloy, S.L., and Rose, J.B. (2007) Massive microbiological groundwater contamination associated with a waterborne outbreak in Lake Erie, South Bass Island, Ohio. *Environ Health Perspect* **115:** 856–864.

González, A., Botella, S., Montes, R.M., Moreno, Y., and Ferrús, M.A. (2007) Direct detection and identification of *Arcobacter* species by multiplex PCR in chicken and wastewater samples from Spain. *J Food Prot* **70:** 341–347.

Ho, H.T.K., Lipman, L.J.A., and Gaastra, W. (2006) *Arcobacter*, what is known about a potential foodborne zoonotic agent! *Vet Microbiol* **115:** 1–13.

Houf, K., and Stephan, R. (2007) Isolation and characterization of the emerging foodborn pathogen *Arcobacter* from human stool. *J Microbiol Methods* **68:** 408–413.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J., and Vandamme, P. (2000) Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri, Arcobacter cryaerophilus* and *Arcobacter skirrowii*. *FEMS Microbiol Lett* **193:** 89–94.

Houf, K., Stephen, L., On, W., Coenye, T., Mast, J., Van Hoof, J., and Vandamme, P. (2005) *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. *Int J Syst Evol Microbiol* **55:** 713–717.

Jacob, J., Lior, H., and Feuerpfeil, I. (1993) Isolation of *Arcobacter butzleri* from a drinking water reservoir in Eastern Germany. *Zentralbl Hyg Umweltmed* **193:** 557–562.

Mates, A., and Schaffer, M. (1988) Quantitative determination of *Escherichia coli* from faecal coliforms in seawater. *Microbios* **53:** 161–165.

Maugeri, T.L., Carbone, M., Fera, M.T., Herrera, G.P., and Gugliandolo, C. (2004) Distribution of potentially pathogenic bacteria as free living and plankton associated in a marine coastal zone. *J Appl Microbiol* **97:** 354–361.

Maugeri, T.L., Irrera, G.P., Lentini, V., Carbone, M., Fera, M.T., and Gugliandolo, C. (2005) Detection and enumeration of *Arcobacter* spp. in the coastal environment of the Straits of Messina (Italy). *New Microbiol* **28:** 117–182.

Moreno, Y., Botella, S., Alonso, J.L., Ferrús, M.A., Hernández, M., and Hernández, J. (2003) Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization. *Appl Environ Microbiol* **69:** 1181–1186.

Morita, Y., Maruyama, S., Kabeya, H., Boonmar, S., Nimsuphan, B., Nagai, A., *et al.* (2004) Isolation and phylogenetic analysis of *Arcobacter* spp. in ground chicken meat and environmental water in Japan and Thailand. *Microbiol Immunol* **48:** 527–533.

Rice, E.W., Rodgers, M.R., Wesley, I.V., Johnson, C.H., and Tanner, S.A. (1999) Isolation of *Arcobacter butzleri* from ground water. *Lett Appl Microbiol* **28:** 31–35.

Samie, A., Obi, C.L., Barret, L.J., Powell, S.M., and Guerrant, R.L. (2006) Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: studies using molecular diagnostic methods. *J Infect* **54:** 558–566.

Stampi, S., Varoli, O., Zanetti, F., and De Luca, G. (1993) *Arcobacter cryaerophilus* and thermophilic campylobacters in a sewage treatment plant in Italy: two secondary treatment compared. *Epidemiol Infect* **110:** 633–639.

Stampi, S., De Luca, G., Varoli, O., and Zanetti, F. (1999) Occurrence, removal and seasonal variation of thermophilic campylobacters and *Arcobacter* in sewage sludge. *Zentralbl Hyg Umweltmed* **202:** 19–27.

Taylor, D.N., Kiehlbauch, J.A., Tee, W., Pitarangsi, C., and Echeverria, P. (1991) Isolation of group 2 aerotolerant *Campylobacter* species from Thai children with diarrhea. *J Infect Dis* **163:** 1062–1067.

Vandamme, P., Dewhirst, F.E., Paster, B.J., and On, S.L.W. (2005) Genus II. *Arcobacter* Vandamme, Falsen, Rossau, Segers, Tytgat and De Ley 1991a, 99^{VP} emend Vandamme, Vancanneyt, Pot, Mels, Hoste, Dewettinck, Vlaes, Van Den Borre, Higgins, Hommez, Kersters, Butzler and Goossens 1992b, 335. In *Bergey's Manual of Systematic Bacteriology*, Vol. 2. Boone, D.R., Castenholz, R.W., and Garrity, G.M. (eds). New York, USA: Springer, pp. 1161–1165.

Van Driessche, E., and Houf, K. (2007) Survival capacity in water of *Arcobacter* species under different temperature conditions. In *Study of Possible Transmission Routes of Arcobacter Species in Livestock and Poultry*. Van Driessche, E. (ed.). (PhD Thesis). Ghent, Belgium: Ghent University, pp. 133–152.

Van Driessche, E., Houf, K., Van Hoof, J., De Zutter, L., and Vandamme, P. (2003) Isolation of *Arcobacter* species from animal feces. *FEMS Microbiol Lett* **229:** 243–248.

Wirsen, C.O., Sievert, S.M., Cavanaugh, C.M., Molyneaux, S.J., Ahmad, A., Taylor, L.T., *et al.* (2002) Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. *Appl Environ Microbiol* **68:** 316–325.

Wybo, I., Breynaert, J., Lauwers, S., Lindenburg, F., and Houf, K. (2004) Isolation of *Arcobacter skirrowii* from a patient with chronic diarrhea. *J Clin Microbiol* **42:** 1851–1852.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

4.3 Occurrence and diversity of *Arcobacter* spp. along the Llobregat river catchment, at sewage effluents and in a drinking water treatment plant.

Collado, L., Kasimir, G., Perez, U., Bosh, A., Pinto, R., Saucedo, G., Huguet, J. & Figueras, M.

Submitted for publication

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Occurrence and diversity of *Arcobacter* spp. along the Llobregat river catchment, at sewage effluents and in a drinking water treatment plant

Luis Collado[a], Georg Kasimir[b], Unai Perez[c], Albert Bosch[c] , Rosa Pinto[C], Gemma Saucedo[d], Josep M Huguet[d], and Maria Jose Figueras[a]

[a]Unitat de Microbiologia, Departament de Ciències Mediques Bàsiques, Facultat de Medicina i Ciències de la Salut. IISPV. Universitat Rovira i Virgili, Reus, Spain

[b]Austrian Comittee Danube Research, International Association of Danube Research, Vienna, Austria

[c]Enteric Virus Laboratory, Department of Microbiology, University of Barcelona, Spain

[d]Agbar Microbiology Laboratory, Barcelona, Spain

Keywords: *Arcobacter*, water, Llobregat River, ERIC-PCR

Corresponding author:
Maria José Figueras
Departament de Ciències Mèdiques Bàsiques
Facultat de Medicina i Ciències de la Salut, IISPV
Universitat Rovira i Virgili
Sant Llorenç 21
43201 Reus (Spain)
Phone: 34-977759321
Fax: 34-977759322
E-mail: mariajose.figueras@urv.cat

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Abstract

The predominance of *Arcobacter* species in faecally-contaminated environmental waters has previously been studied. However, the ability to eliminate *Arcobacter* during the water treatment processes that produce drinking water has been poorly studied. We have investigated the prevalence and diversity of *Arcobacter* spp. throughout the year at 12 sampling points in the Llobregat River catchment (Catalonia, Spain) including 3 sites at a drinking water treatment plant. Positive samples for *Arcobacter* spp., came predominantly from the most faecally polluted sites. Recovery rates from all sites were greater in the spring (91.7%) and summer (83.3%) than in autumn and winter (75.0% in both cases). Among the 339 colonies investigated, the most prevalent species by multiplex PCR and 16S rDNA restriction fragment length polymorphism were *Arcobacter butzleri* (81.1%), followed by *Arcobacter cryaerophilus* (18.6%) and *Arcobacter skirrowii* (0.3%). Isolates showed a high genetic heterogeneity with the enterobacterial repetitive intergenic consensus PCR. In fact, 90.8% (308/339) of the colonies had different genotypes i. e., 248 of them among the 275 isolates of *A. butzleri* and 60 among the 63 isolates of *A. cryaerophilus*. *Arcobacter* was never detected or isolated from finished drinking water, demonstrating that water treatment is effective in removing *Arcobacter* species.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Introduction

The genus *Arcobacter* was proposed by Vandamme et al. (1991) to accommodate bacteria first referred to as aerotolerant campylobacters. Currently, this genus includes nine species: *Arcobacter nitrofigilis* (the type species), *Arcobacter cryaerophilus* (with two subgroups, 1A and 1B), *Arcobacter butzleri*, *Arcobacter skirrowii* (Vandamme et al., 1991, 1992), *Arcobacter halophilus* (Donachie et al., 2005), *Arcobacter cibarius* (Houf et al., 2005), *Arcobacter mytili* (Collado et al., 2009a), *Arcobacter thereius* (Houf et al., 2009) and *Arcobacter marinus* (Kim et al., in press). Most of the species have been isolated from different types of environmental waters (Donachie et al., 2005; Collado et al., 2008; 2009a; Kim et al., in press). The species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* have been associated with animal and human infections (Ho et al., 2006), and it has been suggested that contaminated water can play an important role in the transmission of these microorganisms (Jacob et al., 1998; Ho et al., 2006). So far, three waterborne outbreaks have been associated with *Arcobacter* (Rice et al., 1999; Fong et al., 2007; Kopilović et al., 2008). The first occurred in a girl school camp in Idaho, where faecal contamination was detected in the drinking water (a drilled well) that was servicing the camp (Rice et al., 1999). *Arcobacter* was also isolated in an outbreak in South Bass Island (Ohio), where a massive microbiological groundwater contamination occurred (Fong et al., 2007). More recently, *A. cryaerophilus* was isolated from a stool sample from a patient in a multi-microbial waterborne outbreak reported in Slovenia (Kopilović et al., 2008).

Arcobacter have the capacity to adhere to and form biofilms in various pipe surfaces, such as stainless steel, copper, and plastic and therefore can colonize water distribution systems (Assanta et al., 2002). Despite this bacteria having shown to be susceptible to chlorine (Rice et al., 1999; Moreno et al., 2004), Ho et al. (2006) indicated that it is still unknown if conventional treatments at a drinking water treatment plant (DWTP) can effectively remove them. In a previous study we found that *Arcobacter* spp. are highly prevalent in faecally contaminated waters (Collado et al., 2008). However, the genetic diversity of these bacteria in surface water samples is unexplored. Furthermore, it is also unknown whether specific genotypes are able to persist in a water source or whether the prevalence of *Arcobacter* is seasonal.

The Llobregat River catchment (Catalonia, NE Spain) flows for 170 km from its source in the pre-Pyrenees mountains to the Mediterranean Sea, and is one of the main drinking water sources serving the metropolitan area of Barcelona. The river receives urban and industrial impacts from more than 30 sewage treatment plants (Céspedes et al., 2005).

ISBN: 978-84-693-0707-6 /DL:T-420-2010

The main objective of this study was to establish the prevalence and genetic diversity of *Arcobacter* spp. in the Llobregat River catchment, including sampling sites affected by wastewater, the point where water enters a drinking water treatment plant, from which we studied samples after carbon filtration and finished water.

Materials and Methods

Sampling sites

The sampling strategy covered a wide range of socio-geographical areas along the Llobregat River throughout the year. The samples were taken at five different times between February and September 2008, at the twelve sampling points (S1 to S12) indicated in Fig.1. Sampling point S1 was at the beginning of the catchment, before the river reaches the city of Berga (16.600 inhabitants), thus representing the “clean water reference site”. Sampling point S2 was at Balsareny (3.300 inhabitants), where a canal transports river water to the drinking water supply system of the city of Manresa (76.100 inhabitants). Sampling points S3 and S4 were in the Cardener River (one of the most important tributaries of the Llobregat) before and at the city of Manresa, respectively. S5 was in the inflow water of the wastewater treatment plant (WWTP) of Manresa, while S6 was the outflow of the WWTP to the Cardener River. Sampling point S7 was at the Llobregat river water as it passes through the city of Martorell (28.700 inhabitants), where the Llobregat receives another tributary, the Anoia River. Sampling point S8 was situated in the Riulet of Rubí, another tributary passing trough the city of Rubí (70.100 inhabitants). Sampling point S9 was the river water where it enters the Sant Joan Despí DWTP while S10 was semi-treated drinking water, after carbon filtration and S11 corresponded to the finished drinking water after chlorination (Fig. 2). Finally, sampling point S12 was in water from a canal of untreated sewage from St. Feliu city (42.700 inhabitants) that flows into the Llobregat River below the DWTP. At each point, the water samples were collected into 2L sterile polypropylene bottles and chilled with ice. Microbiological assays began on the same day of sampling.

Arcobacter detection and isolation procedure

For the detection and isolation of *Arcobacter* species, 200 ml of each water sample was filtered using a 0.45 µm membrane filter (Millipore Corp., Bedford, MA, USA). The filters were rolled and placed into tubes containing 9 ml of *Arcobacter* enrichment broth (Oxoid,

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Basingstoke, UK) supplemented with CAT (cefoperazone, amphotericin B and teicoplanin) (Oxoid) and incubated at 30°C for 48h under aerobic conditions. After enrichment, 200 µl of the broth was transferred onto the surface of a 0.45 µm membrane filter placed on blood agar media and allowed to filter passively under ambient conditions for 30 min (Collado et al., 2008). Then, the filters were removed and the plates were incubated at 30°C for 48h to 72h under aerobic conditions. From each positive sample, eight small colourless or beige to off-white translucent colonies were picked up, and were checked by Gram stain, oxidase and motility tests. Those tests enabled some of the selected colonies to be recognized as not belonging to *Arcobacter* (Table 1).

Arcobacter molecular identification and typing

DNA was extracted from all the investigated colonies using the InstaGene™ DNA Purification Matrix (Bio-Rad Laboratories, Hercules, CA). Final identification of the presumptive isolates was made by the multiplex PCR (m-PCR) of Houf et al. (2000) and using the 16S rDNA restriction fragment length polymorphism (16S rDNA-RFLP) method described by Figueras et al. (2008). The latter method consists of an amplification of 1026 bp of the 16S rRNA gene followed by digestion with endonuclease *Mse*I that produces a species-specific pattern for all *Arcobacter* species described up to 2008 (Figueras et al., 2008). All isolates were genotyped using the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) technique with the protocol described by Houf et al. (2002). The concentration of each DNA template was determined using the GenQuant *pro* (Amersham Biosciences, Cambridge, England) at A_{260} and adjusted to 25 ngμl^{-1}. Gel images were saved as TIFF files, normalized with the GeneRuler™ 100bp DNA Ladder Plus (Fermentas, MBI Fermentas, St. LeonRot, Germany), and further analyzed by Bionumerics software, version 2.5 (Applied Maths, Kortrijk, Belgium). Patterns with one or more different bands were considered different genotypes, as in previous studies (Houf et al., 2002; De Smet et al., in press).

Analyses of indicators of faecal pollution

The membrane filtration technique was employed for the detection of intestinal enterococci (IE) and *Clostridium perfringens* (Cp) as previously described (Figueras et al., 1996) using the ISO 7899-2 and ISO 6461-2 methods, respectively. Total coliforms (TC) and *Escherichia coli* (Ec) were analyzed using the Colilert-18 method (Quanti-Tray, IDEXX

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Laboratories) according to the manufacturer's instructions. All the methods are accredited by ISO 17025 standard at the Agbar microbiology laboratory.

Results and discussion

Prevalence and seasonality

Arcobacter spp. were detected in 80% of the 60 samples studied, from 11 of the 12 sampling points (Table 1). The finished drinking water was always negative and only on one occasion (April) was the sampling point after carbon filtration (S10) positive for *A. butzleri* (Table 2). This species was also the only one recovered at the cleanest reference site (S1) at the beginning of the catchment (Table 2). The greatest number of positive samples for *Arcobacter* coincided with the most faecally contaminated water samples based on of the results of the bacterial faecal indicators (TC, Ec, IE and Cp) (Table 2). Greater species diversity was also found at the most contaminated sampling points (Table 2). Furthermore all sites positive for *Arcobacter* (with the exception of S10) were coincidentally positive for the Noroviruses analyzed by the Enteric Virus Laboratory of the University of Barcelona. In contrast, the Hepatitis A virus was never detected on the five sampling dates studied (U. Perez et al. manuscript in preparation). These results confirm the association of *Arcobacter* with faecally polluted waters, described for the first time in a previous study (Collado et al., 2008). Recovery rates from all sites in our study appeared to be greater in the spring (91.7%) and summer (83.3%) than in autumn and winter (75.0% in both cases). Because of the limited number of samples taken in this study, further studies should confirm this apparent seasonality. Despite that, these results agree with the study of Stampi et al. (1999), which found that *A. butzleri* was more prevalent in sewage sludge samples during the spring/summer period in Italy, a country with very similar climatic conditions to Spain.

Among the 339 genetically identified *Arcobacter* colonies, *A. butzleri* was the most prevalent species (275/339, 81.1%), followed by *A. cryaerophilus* (63/339, 18.6%). All isolates of the latter species corresponded to group 1B, which is the predominant group detected in food studies (Collado et al., 2009b), but which has so far never been reported from water samples. In a previous study we found an *Arcobacter* prevalence of 58.6% in other rivers in Catalonia, were *A. butzleri* was also the dominating species (94.1%) over *A. cryaerophilus* (5.9%) (Collado et al., 2008). *Arcobacter skirrowii* was only isolated once in this study (in March at S5), this to our knowledge being the second report of this species

ISBN: 978-84-693-0707-6 /DL:T-420-2010

from river water, the first having been reported from a river water sample in Japan (Morita et al., 2004). A recent study in estuarine waters in Southern Italy found *A. cryaerophilus* and *A. butzleri* to be the prevailing species (Fera et al., in press).

The 16S rDNA-RFLP and the m-PCR methods produced the same identification results for all tested strains, contrary to previous results obtained in a food survey, where discordances were found between these two identification methods (Collado et al., 2009b). In that case, the m-PCR method misidentified the species *A. mytili* and *A. nitrofigilis* as *A. skirrowii* and the recently described *A. thereius* as *A. cryaerophilus*, but those species were not found in the present study. Interestingly, as indicated previously, *A. butzleri* was the only species found in the cleaner waters.

Our results indicate that the species *A. butzleri* and *A. cryaerophilus*, considered potential human pathogens, were very abundant in the Llobregat River water used to produce drinking water. Despite that, drinking water treatments effectively removed these microorganisms from the finished chlorinated water, even when carbon filtration had failed as a treatment barrier on 1 of the 5 occasions studied. At least two studies have described the high susceptibility of *Arcobacter* to chlorine (Rice et al., 1999; Moreno et al., 2004), which could explain why *Arcobacter* was not detected in the finished chlorinated water. Our results agree with previous data reported by Diergaardt et al. (2004) and Aydin et al. (2007), who did not find any *Arcobacter* in drinking water samples. Jacob et al. (1998), found *A. butzleri* and other isolates defined as *A. butzleri*-like in raw water and at treatment stages, using a biochemical protocol.

Genetic diversity

The 12 sampling sites along the river not only had more than one species but also had multiple genotypes, as revealed by the analysis of the ERIC-PCR profiles. In fact, 60 (95.2%) of 63 isolates of *A. cryaerophilus* were found to belong to different genotypes, as were 248 (90.2%) of the 275 isolates of *A. butzleri* (Table 1). After eliminating the redundant isolates**,** the prevalence of the species was 80.2% instead of 81.1% for *A. butzleri* and 19.4% instead 18.6% for *A. cryaerophilus*. Interestingly, the higher diversity of *A. butzleri* and *A. cryaerophilus* genotypes (ranging from 5 to 36 different genotypes) were found in sampling sites S4 to S9, which had a greater impact of urban sewage. In contrast, only two and five different genotypes were found at points S1 (reference clean site) and S11 (after carbon filtration), respectively. The same genotype was never isolated from different sampling points or sampling dates, which seems to indicate that the same

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Arcobacter isolates are not able to persist or predominantly colonize the Llobregat River catchment. González et al. (in press) also never detected the same *Arcobacter* genotype among the 33 wastewater samples studied. As suggested by other authors (Aydin et al., 2007), the detection of several different *Arcobacter* strains may indicate many sources of contamination. For instance, *Arcobacter* is frequently recovered from faeces of several animals or from contaminated environmental water samples (Van Driessche et al., 2005; Ho et al., 2006; Collado et al., 2008). However, genomic rearrangement, as a consequence of multiple recombination events, has been put forward as another explanation for the high genetic diversity in *Campylobacter* (On, 1998) and probably for *Arcobacter,* as suggested by Hume et al. (2001). To our knowledge, the genetic diversity of *Arcobacter* isolates recovered from environmental water samples has never been investigated, the present study being the first to report it. The ERIC-PCR has successfully been applied to *Arcobacter* isolates involved in a gastrointestinal outbreak (Vandamme et al. 1993), to those recovered from chicken and beef meats (Houf et al., 2002; Aydin et al., 2007; De Smet et al., 2009) or from other animal faecal samples (Van Driessche et al., 2005), and for typing strains included in the descriptions of novel *Arcobacter* species (Houf et al., 2005, 2009; Collado et al., 2009a). The genetic diversity reported in those studies ranged from 11 to 58.6% for the isolates of *A. butzleri* and from 43.2 to 100% for the isolates of *A. cryaerophilus* having different genotypes. Those results, together with those obtained in the present study, indicate an important genetic heterogeneity between the members of this genus. In fact, this was very recently evidenced by the results of the first Multilocus Sequence Typing method applied to the genus, in which Miller et al. (2009) found no association of sequence types (ST) with host or geographical source. They found that 59 (81.9%) of 72 isolates of *A. cryaerophilus* showed different ST as did 208 (75.6%) of the 275 genotyped isolates of *A. butzleri*. However, the set of strains investigated were all from food, animal and human samples but did not include water strains.

In conclusion, the results from the present study may contribute to the assessment of the epidemiology of this emerging foodborne pathogen, showing a high prevalence and large heterogeneity of *Arcobacter* species in faecally contaminated river water. However, treatment processes used to produce drinking water were adequate for removing *Arcobacter* species from the finished water.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Acknowledgment

This work was supported by funds from the European Commission for the HEALTHY WATER project (FOOD-CT-2006-036306). The authors are solely responsible for the content of this publication. It does not represent the opinion of the European Commission. The European Commission is not responsible for any use that might be made of data appearing therein. L. C. is indebted to Universitat Rovira i Virgili for a doctoral grant. We thank Elia Bernat for the preparation of Figure 2.

References

Assanta, M. A., Roy, D., Lemay, M. J., Montpetit, D., 2002. Attachment of *Arcobacter butzleri*, a new waterborne pathogen, to water distribution pipe surfaces. J. Food Prot. 65(8), 1240-1247.

Aydin, F., Gumussoy, K. S., Atabay, H. I., Ica, T., Abay, S., 2007. Prevalence and distribution of *Arcobacter* species in various sources in Turkey and molecular analysis of isolated strains by ERIC-PCR. J. Appl. Microbiol. 103(1), 27-35.

Céspedes, R., Lacorte, S., Raldua, D., Ginebreda, A., Barcelo, D., Pina, B., 2005. Distribution of endocrine disruptors in the Llobregat River basin (Catalonia, NE Spain). Chemosphere 61(11), 1710-1719.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P., Figueras, M. J., 2009a. *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. Int. J. Syst. Evol. Microbiol. 59(Pt 6), 1391-1396.

Collado, L., Guarro, J., Figueras, M. J., 2009b. Prevalence of *Arcobacter* in meat and shellfish. J. Food Prot. 72(5), 1102-1106.

Collado, L., Inza, I., Guarro, J., Figueras, M. J., 2008. Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. Environ. Microbiol. 10(6), 1635-1640.

De Smet, S., De Zutter, L., Van Hende, J., Houf, K., 2009. *Arcobacter* contamination on pre- and post-chilled bovine carcasses and in minced beef at retail. J. Appl. Microbiol. (In press)

Diergaardt, S. M., Venter, S. N., Spreeth, A., Theron, J., Brozel, V. S., 2004. The occurrence of campylobacters in water sources in South Africa. Water Res. 38(10), 2589-2595.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Donachie, S. P., Bowman, J. P., On, S. L., Alam, M., 2005. *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus Arcobacter. Int. J. Syst. Evol. Microbiol. 55(Pt 3), 1271-1277.

Fera, M. T., Gugliandolo, C., Lentini, V., Favaloro, A., Bonanno, D., La Camera, E., Maugeri, T. L. (2009) Specific detection of *Arcobacter* spp. in estuarine waters of Southern Italy by PCR and fluorescent in situ hybridization. Lett. Appl. Microbiol. (in press).

Figueras, M. J., Collado, L., Guarro, J., 2008. A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. Diagn. Microbiol. Infect. Dis. 62(1), 11-15.

Figueras, M. J., Inza, I., Polo, F. L., Feliu, M. T., Guarro, J., 1996. A fast method for the confirmation of fecal streptococci from M-enterococcus medium. Appl. Environ. Microbiol. 62(6), 2177-2178.

Fong, T. T., Mansfield, L. S., Wilson, D. L., Schwab, D. J., Molloy, S. L., Rose, J. B., 2007. Massive microbiological groundwater contamination associated with a waterborne outbreak in Lake Erie, South Bass Island, Ohio. Environ. Health Perspect. 115(6), 856-864.

González, A., Suski, J., Ferrus, M. A. (2009) Rapid and Accurate Detection of *Arcobacter* Contamination in Commercial Chicken Products and Wastewater Samples by Real-Time Polymerase Chain Reaction. Foodborne Pathog. Dis. (In press).

Ho, H. T., Lipman, L. J., Gaastra, W., 2006. *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent! Vet. Microbiol. 115(1-3), 1-13.

Houf, K., On, S. L., Coenye, T., Debruyne, L., De Smet, S., Vandamme, P., 2009. *Arcobacter thereius* sp. nov., isolated from pigs and ducks. Int. J. Syst. Evol. Microbiol. 59(Pt 10), 2599-2604.

Houf, K., On, S. L., Coenye, T., Mast, J., Van Hoof, J., Vandamme, P., 2005. *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. Int. J. Syst. Evol. Microbiol. 55(Pt 2), 713-717.

Houf, K., De Zutter, L., Van Hoof, J., Vandamme, P., 2002. Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. Appl. Environ. Microbiol. 68(5), 2172-2178.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J., Vandamme, P., 2000. Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiol. Lett. 193(1), 89-94.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Hume, M. E., Harvey, R. B., Stanker, L. H., Droleskey, R. E., Poole, T. L., Zhang, H. B., 2001. Genotypic variation among *Arcobacter* isolates from a farrow-to-finish swine facility. J. Food Prot. 64(5), 645-651.

Jacob, J., Woodward, D., Feuerpfeil, I., Johnson, W. M., 1998. Isolation of *Arcobacter butzleri* in raw water and drinking water treatment plants in Germany. Zentralbl. Hyg. Umweltmed. 201(2), 189-198.

Kim, H. M., Hwang, C. Y., Cho, B. C., 2009. *Arcobacter marinus* sp. nov. Int. J. Syst. Evol. Microbiol. (In press).

Kopilovic et al., Ucakar, V., Koren, N., Krek, M., Kraigher, A., 2008. Waterborne outbreak of acute gastroenteritis in a costal area in Slovenia in june and july 2008. Eurosurveillance 13, 1-3.

Miller, W. G., Wesley, I. V., On, S. L., Houf, K., Megraud, F., Wang, G., Yee, E., Srijan, A., Mason, C. J., 2009. First multi-locus sequence typing scheme for *Arcobacter* spp. BMC Microbiol. 9, 196.

Moreno, Y., Alonso, J. L., Botella, S., Ferrus, M. A., Hernandez, J., 2004. Survival and injury of *Arcobacter* after artificial inoculation into drinking water. Res. Microbiol. 155(9), 726-730.

Morita, Y., Maruyama, S., Kabeya, H., Boonmar, S., Nimsuphan, B., Nagai, A., Kozawa, K., Nakajima, T., Mikami, T., Kimura, H., 2004. Isolation and phylogenetic analysis of *Arcobacter* spp. in ground chicken meat and environmental water in Japan and Thailand. Microbiol. Immunol. 48(7), 527-533.

On, S. L., 1998. In vitro genotypic variation of *Campylobacter coli* documented by pulsed-field gel electrophoretic DNA profiling: implications for epidemiological studies. FEMS Microbiol. Lett. 165(2), 341-346.

Rice, E. W., Rodgers, M. R., Wesley, I. V., Johnson, C. H., Tanner, S. A., 1999. Isolation of *Arcobacter butzleri* from ground water. Lett. Appl. Microbiol. 28(1), 31-35.

Stampi, S., De Luca, G., Varoli, O., Zanetti, F., 1999. Occurrence, removal and seasonal variation of thermophilic campylobacters and *Arcobacter* in sewage sludge. Zentralbl. Hyg. Umweltmed. 202(1), 19-27.

Van Driessche, E., Houf, K., Vangroenweghe, F., De Zutter, L., Van Hoof, J., 2005. Prevalence, enumeration and strain variation of *Arcobacter* species in the faeces of healthy cattle in Belgium. Vet. Microbiol. 105(2), 149-154.

Vandamme, P., Giesendorf, B. A., van Belkum, A., Pierard, D., Lauwers, S., Kersters, K., Butzler, J. P., Goossens, H., Quint, W. G., 1993. Discrimination of epidemic and sporadic

ISBN: 978-84-693-0707-6 /DL:T-420-2010

isolates of *Arcobacter butzleri* by polymerase chain reaction-mediated DNA fingerprinting. J. Clin. Microbiol. 31(12), 3317-3319.

Vandamme, P., Vancanneyt, M., Pot, B., Mels, L., Hoste, B., Dewettinck, D., Vlaes, L., van den Borre, C., Higgins, R., Hommez, J., 1992. Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. Int. J. Syst. Bacteriol. 42(3), 344-356.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R., De Ley, J., 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. Int. J. Syst. Bacteriol. 41(1), 88-103.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 1. Prevalence and genetic diversity of *Arcobacter* species isolated from the Llobregat River catchment

Simples			Nº of identified colonies (*n*)		Identified species and genotypes					
					A. butzleri		*A. cryaerophilus*		*A. skirrowii*	
Sampling month	Points (*n*)[1]	Positives (%)	Any *Arcobacter* spp.	Other bacteria	N° colonies	ERIC types	N° colonies	ERIC types	N° colonies	ERIC types
February	12	9 (75.0)[1]	65	7	39	33	26	26		
March	12	9 (75.0) [1]	63	9	55	49	7	7	1	1
April	12	11 (91.7)[2]	71	17	67	57	4	4		
August	12	10 (83.3)[3]	75	5	69	65	6	3		
September	12	9 (75.0) [1]	65	7	45	44	20	20		
Total	60	48 (80.0)	339	45	275	248	63	60	1	1

[1]S1 (Llobregat River Water at the beginning of the catchment, representing the “clean water reference site”, S10 (water after carbon filtration at the DWTP) and S11 (the finished drinking water) were negative for *Arcobacter*
[2] S11 was negative for *Arcobacter*
[3] S10 and S11 were negative for *Arcobacter*
ERIC: enterobacterial repetitive intergenic consensus

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 2. Relationship between *Arcobacter* positive samples in the Llobregat River catchment and the mean concentrations of bacteria indicators of faecal pollution.

Sampling Points[1]	*Arcobacter* positive samples (%)	*Arcobacter* isolated species	Mean concentration of bacterial faecal indicators				Positive samples (%) for Noroviruses
			Total coliforms MPN/100ml	*E. coli* MPN/100ml	Intestinal enterococci CFU/100ml	*C. perfringens* CFU/100ml	
S1	40	Ab	$1.9x10^2$	$2.0x10^0$	$3.0x10^0$	$1.3x10^1$	20
S2	100	Ab-Ac	$4.8x10^3$	$8.5x10^2$	$3.0x10^2$	$6.5x10^2$	80
S3	100	Ab-Ac	$2.7x10^4$	$2.7x10^3$	$9.0x10^2$	$2.9x10^3$	80
S4	100	Ab-Ac	$4.0x10^4$	$8.6x10^3$	$1.7x10^3$	$3.2x10^3$	80
S5	100	Ab-Ac-As	$2.4x10^8$	$1.0x10^7$	$5.4x10^6$	$2.1x10^6$	100
S6	100	Ab-Ac	$1.0x10^5$	$2.4x10^4$	$1.3x10^4$	$6.8x10^3$	100
S7	100	Ab-Ac	$4.3x10^4$	$8.3x10^3$	$2.2x10^3$	$4.4x10^3$	80
S8	100	Ab-Ac	$3.3x10^5$	$5.8x10^4$	$1.0x10^4$	$8.3x10^3$	80
S9	100	Ab-Ac	$1.5x10^5$	$1.1x10^4$	$2.7x10^3$	$1.2x10^4$	80
S10	20	Ab	$4.0x10^0$	ND	ND	$1.0x10^0$	0
S11	0	ND	ND	ND	ND	ND	0
S12	100	Ab-Ac	$3.3x10^6$	$4.7x10^5$	$4.7x10^4$	$1.2x10^4$	100

[1]S1, Llobregat River Water at the beginning of the catchment, representing the “clean water reference site”; S2, Balsareny; S3, Cardener River; S4, Cardener riverwater at Manresa; S5, inflow water of the WWTP of Manresa; S6, outflow of the WWTP to the Cardener river; S7, Llobregat River water at Martorell; S8, at the Riera de Rubi; S9, river water entering the DWTP in Sant Joan Despí; S10, water after carbon filtration at the DWTP; S11, the finished drinking water; S12, untreated sewage which is flowing into the Llobregat River after the DWTP.
Ab, *A. butzleri*; Ac, *A. cryaerophilus*; As, *A. skirrowii*; ND, Not Detected

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

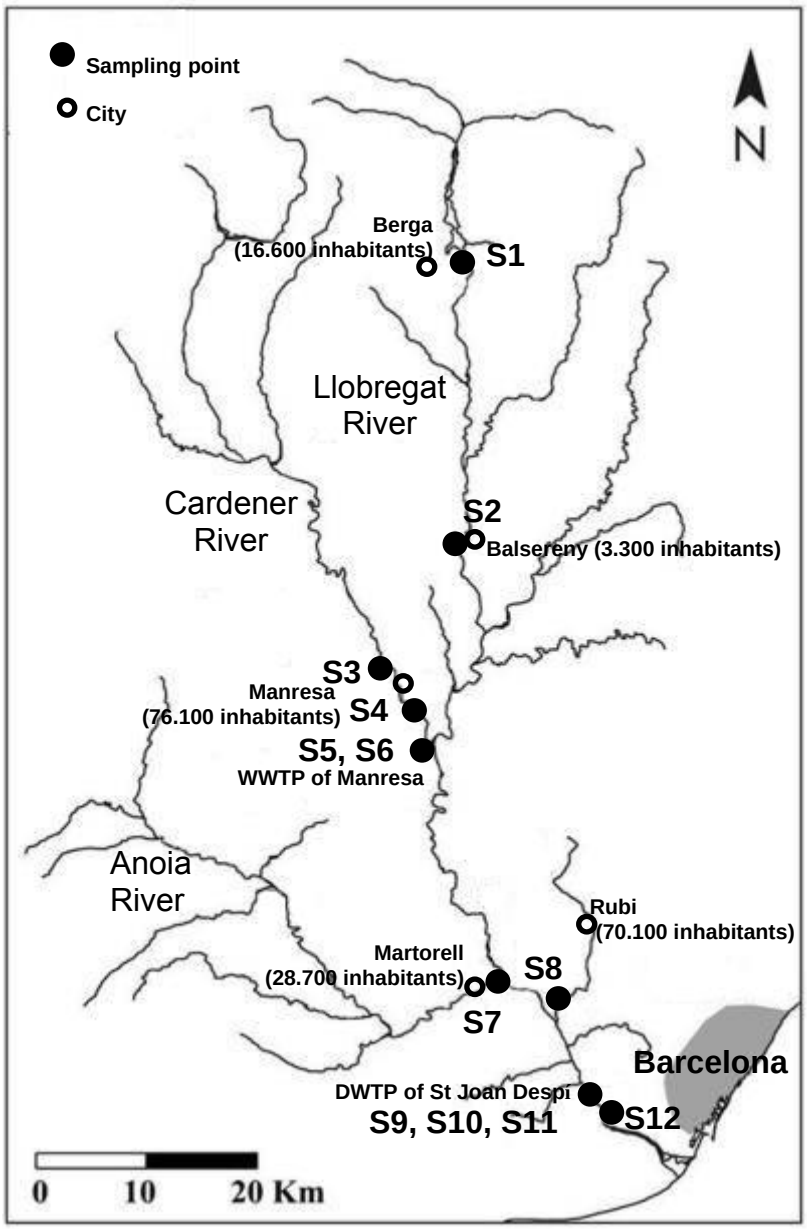


Fig. 1. Llobregat River catchment showing sampling points S1 to S12. WWTP, wastewater treatment plant; DWTP, drinking water treatment plant. Image adapted from Céspedes et al. (2005)

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Drinking Water Treatment Plant of Sant Joan Despí

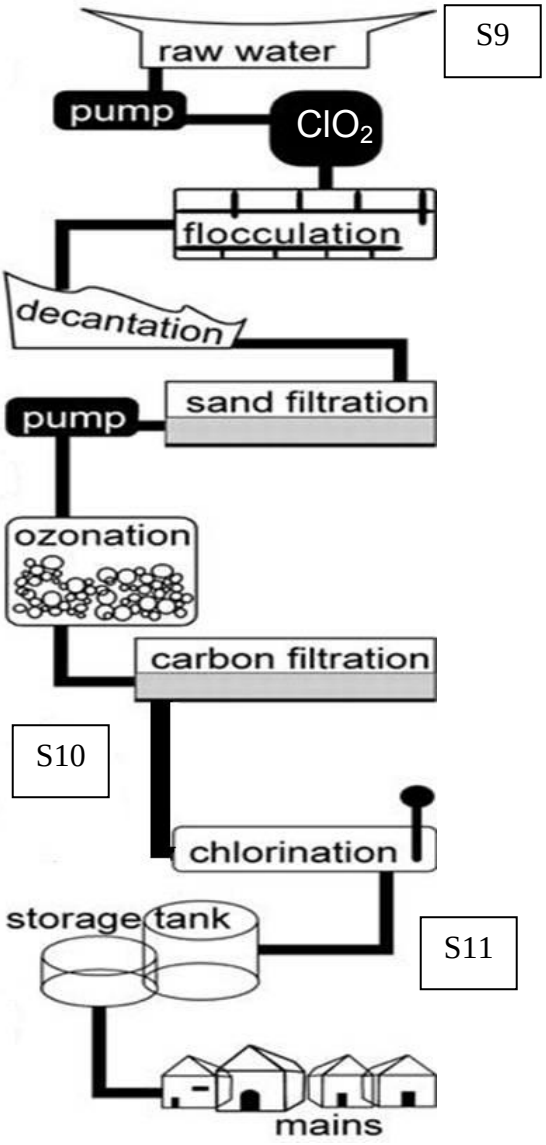


Fig. 2. Water treatment flowchart at the Sant Joan Despí drinking water treatment plant showing sampling points S9 (raw river water), S10 (water after carbon filtration) and S11 (after chlorination)

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

4.4 Prevalence of *Arcobacter* in meat and shellfish.

Collado, L., Guarro, J. & Figueras, M. J.

Journal of Food Protection (2009) 72, 1102-1106

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Journal of Food Protection, Vol. 72, No. 5, 2009, Pages 1102–1106

Research Note

Prevalence of *Arcobacter* in Meat and Shellfish

LUIS COLLADO, JOSEP GUARRO, AND MARIA JOSÉ FIGUERAS*

Unitat de Microbiologia, Departament de Ciències Mediques Bàsiques, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain

MS 08-555: Received 5 November 2008/Accepted 9 January 2009

ABSTRACT

Arcobacter is considered an emergent foodborne and waterborne enteropathogen. However, its prevalence in foods of animal origin is only partially known, because most studies have been concentrated on poultry, pork, and beef, and methods applied do not allow identification of all currently accepted *Arcobacter* species. We investigated the prevalence of *Arcobacter* in 203 food samples, 119 samples of seven different types of meats and 84 samples of four types of shellfish. Isolates were identified in parallel by using a published multiplex PCR method and a recently described 16S rDNA restriction fragment length polymorphism method that allows all currently accepted *Arcobacter* species to be characterized. The global prevalence of *Arcobacter* was 32%; it was highest in clams (5 of 5 samples, 100%) and chicken (9 of 14 samples, 64.3%) followed by pork (9 of 17 samples, 53.0%), mussels (23 of 56 samples, 41.1%), and duck meat (2 of 5 samples, 40.0%). Turkey meat and beef had a similar recovery rate (10 of 30 samples, 33.3%; 5 of 16 samples, 31.3%; respectively), and rabbit meat had the lowest rate (1 of 10 samples, 10.0%). No arcobacters were found in oysters, frozen shrimps, or sausages. This food survey is the first in which five of the seven accepted *Arcobacter* species have been isolated. *Arcobacter butzleri* was the most prevalent species (63.0% of isolates) followed by *Arcobacter cryaerophilus* (26.6%), *Arcobacter mytili* (4.7%), *Arcobacter skirrowii* (3.1%), and *Arcobacter nitrofigilis* (3.1%). Three (4.7%) of the isolates were classified as belonging to three potentially new phylogenetic lines. Our results indicated that *Arcobacter* species are widely distributed in the food products studied.

The genus *Arcobacter* (previously known as a group of aerotolerant campylobacters) has gained increased attention as an emergent waterborne and foodborne enteropathogen *(2, 12).* Currently, the genus contains seven species: *Arcobacter butzleri, Arcobacter cryaerophilus* (with two DNA groups, 1A and 1B), *Arcobacter skirrowii, Arcobacter nitrofigilis (34), Arcobacter cibarius (14), Arcobacter halophilus (7),* and *Arcobacter mytili (3). A. butzleri, A. cryaerophilus,* and *A. skirrowii* have been associated with gastrointestinal disease and bacteremia in humans and with abortion and diarrhea in animals *(12).* Nevertheless, information on their pathogenicity and virulence factors is limited *(12). A. butzleri* is the most commonly isolated species and has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods *(16). Arcobacter* species have been isolated frequently from drinking water reservoirs *(17)* and recently from fecally contaminated bathing waters *(4).* These bacteria also are present in feces of livestock *(35)* and in poultry, beef, and pork meats *(2, 20, 32)*; however, few studies have been conducted with other types of meats. Despite the ability of molluscs to concentrate bacterial pathogens, the prevalence of *Arcobacter* in shellfish has rarely been studied *(8, 23).*

In previous studies, the molecular method used *(15)* allowed the identification of only three of the seven species included in the genus: *A. butzleri, A. cryaerophilus,* and *A. skirrowii.* Recently, we have published a fast molecular method, a 16S rDNA restriction fragment length polymorphism (RFLP) assay, that enables the identification of all the currently accepted *Arcobacter* species *(9),* including the new species *A. mytili (3).* The main objective of this study was to use this identification method to investigate the prevalence and diversity of the *Arcobacter* species in different meat and shellfish samples to establish the true importance of these emergent enteropathogens in common foods. The results of the 16S rDNA–RFLP assay were compared with those obtained with the commonly used multiplex PCR (m-PCR) method of Houf et al. *(15).*

MATERIALS AND METHODS

Samples and isolation procedure. A total of 203 samples were studied: 119 meat samples from retail markets in Catalonia (northeastern Spain) obtained between June 2006 and March 2008 (14 chicken, 30 turkey, 5 duck, 17 pork, 16 beef, 10 rabbit, and 27 sausage) and 84 shellfish samples (56 mussel, 5 clam, 6 oyster, and 17 frozen shrimp). Shellfish samples also were obtained from local markets, with the exception of the mussels, which were harvested from a mussel farm at the Ebro River delta.

Within 24 to 48 h of collection, 10-g samples of the meat or shellfish were each homogenized with 90 ml of *Arcobacter* enrichment broth supplemented with cefoperazone, amphotericin B, and teicoplanin (Oxoid, Basingstoke, UK) in tightly sealed stomacher bags. After incubation for 48 h at 30°C under aerobic conditions, 200 μl of the broth was inoculated onto blood agar plates following the procedure described by Collado et al. *(4).* Four

* Author for correspondence. Tel: 34-977759321; Fax: 34-977759322; E-mail: mariajose.figueras@urv.cat.

TABLE 1. *Prevalence and diversity of* Arcobacter *species from foods*[a]

Sample type	No. of samples	No. (%) of samples positive for:							
		Any *Arcobacter*	*A. butzleri*	*A. cryaerophilus*	*A. mytili*	*A. skirrowii*	*A. nitrofigilis*	*A. skirrowii* like[b]	Other arcobacters
Mussels	56	23 (41.1)[c]	10 (43.5)	8 (35.0)	3 (13.0)		2 (9.0)		1 (4.3)
Oysters	6								
Clams	5	5 (100)[d]	2 (40.0)	4 (80.0)		1 (20.0)			
Shrimp	17								
Chicken meat	14	9 (64.3)	9 (100)						
Turkey meat	30	10 (33.3)	8 (80.0)	2 (20.0)					
Duck meat	5	2 (40.0)	1 (50.0)	1 (50.0)					
Pork	17	9 (53.0)	6 (66.7)			1 (11.1)		1 (11.1)	1 (11.1)
Rabbit meat	10	1 (10.0)	1 (100)						
Beef	16	5 (31.3)	3 (60.0)	2 (40.0)					
Sausage	27								
Total	203	64 (32.0)	40 (62.5)	17 (26.6)	3 (4.7)	2 (3.1)	2 (3.1)	1 (1.6)	2 (3.1)

[a] Identification by 16S rDNA–RFLP assay.
[b] A possible new species suggested by On et al. *(27).*
[c] In one sample, both *A. butzleri* and *A. cryaerophilus* were present.
[d] In one sample, *A. butzleri, A. cryaerophilus,* and *A. skirrowii* were present.

small colorless or beige to off-white translucent colonies were selected from each plate and transferred to blood agar to obtain pure cultures.

Detection and identification of *Arcobacter*. For the detection of *Arcobacter,* 400 μl of the incubated enrichment broth was used for the m-PCR using primers and conditions described by Houf et al. *(15)* for detection and identification of *A. butzleri, A. cryaerophilus,* and *A. skirrowii.* In parallel, colonies were submitted to Gram staining and oxidase and motility tests. Final identification of the colonies was made using the m-PCR *(15)* and a recently described 16S rDNA–RFLP *Arcobacter* identification method, which consists of amplification of 1,026 bp of the 16S rRNA gene and digestion with the *Mse*I endonuclease *(9).* When the four isolates on each plate were identified as the same species, only one was recorded for the analysis of prevalence. When the identification methods gave different results, the 16S rRNA gene was sequenced to establish correct identification using the universal primers developed by Martinez-Murcia et al. *(22).* The generated sequences were then compared with those deposited in GenBank using BlastN (NCBI, Bethesda, MD), and a phylogenetic tree was constructed using the MEGA4 software (Sudhir Kumar, Center for Evolutionary Functional Genomics, Tempe, AZ).

Reference strains. The type strains of *A. butzleri* (LMG 10828), *A. cryaerophilus* (LMG 9904), *A. skirrowii* (LMG 6621), *A. halophilus* (LA31B), *A. cibarius* (CECT 7203), *A. nitrofigilis* (CECT 7204), and *A. mytili* (CECT 7386) were used as controls.

Statistical analyses. Fisher's exact test was used to compare the prevalence of *Arcobacter* in meat and shellfish samples. Statistical analyses were performed using the Statistical Package for Social Sciences (v. 15.0, SPSS Inc., Chicago, IL). Statistical significance was assessed at $P < 0.05$.

RESULTS AND DISCUSSION

The results on the prevalence and diversity of the *Arcobacter* species are summarized in Table 1. *Arcobacter* spp. were present in 64 (32.0%) of the samples; they were most prevalent in clams (5 of 5 samples, 100%) and chicken (9 of 14 samples, 64.3%) followed by pork (9 of 17 samples, 53.0%), mussels (23 of 56 samples, 41.1%), and duck meat (2 of 5 samples, 40.0%). Turkey meat and beef had similar prevalences (10 of 30 samples, 33.3%; 5 of 16 samples, 31.3%; respectively), and rabbit meat had the lowest prevalence (1 of 10 samples, 10.0%). In oysters, frozen shrimp, and sausages, no arcobacters were recovered or detected. No significant differences were found between meat and shellfish samples ($P = 0.38$). Although 59 of the 64 culture-positive samples were positive by m-PCR assay (from enrichment broth cultures), the other five (7.8%) were negative by m-PCR, possibly because of PCR inhibition caused by coarse particles and solidified fat, as previously described *(15).*

Discrepancies between the identification methods. The 16S rDNA–RFLP assay and the m-PCR method produced the same identity results for 59 of 67 strains (88.1% agreement). Of the eight isolates that yielded dissimilar results, five were identified as *A. skirrowii* and three were identified as *A. cryaerophilus* based on the m-PCR results. However, of those five *A. skirrowii* isolates, two had the characteristic RFLP pattern of *A. nitrofigilis* and three had the pattern of the recently proposed species *A. mytili (3).* This discrepancy occurred because when the m-PCR method was designed, the strain of *A. nitrofigilis* tested did not produce any amplification *(15),* and at that time *A. mytili* was unknown. Of the three strains that were identified as *A. cryaerophilus* by m-PCR assay, two strains (F4 and F41), isolated from mussels and pork samples, respectively, were assigned to two potentially new phylogenetic lines on the basis of their 16S rDNA–RFLP patterns and 16S rRNA gene sequences. The phylogenetic tree derived from this gene shows these strains as independent branches (Fig. 1). The remaining strain (F61, from pork) had a restriction pattern identical to that of *A. butzleri,* but the analysis of its 16S rRNA gene sequence (Fig. 1) indicated a 99.8% sim-

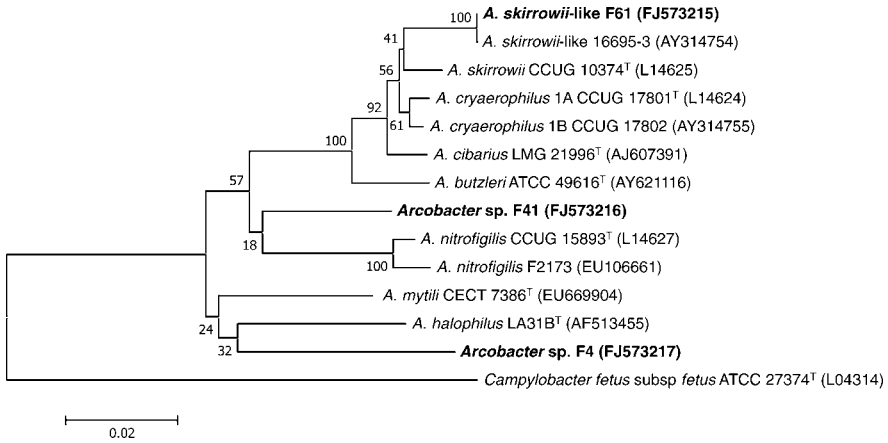


FIGURE 1. *Neighbor-joining phylogenetic tree showing the relationship between the three potentially novel species and the other members of the genus* Arcobacter *based on 1,409 nucleotides from the 16S rRNA gene. The stability of the groupings was estimated by bootstrap analysis (1,000 replications).*

ilarity with a potential new *Arcobacter* species recovered from porcine abortion material in Denmark *(27).* These data agree with those from other studies in which some isolates could not be identified to species *(31, 32),* indicating a diversity of potentially new *Arcobacter* species in foods. The misidentification problems that occurred here with the m-PCR method *(15)* were previously discussed when the 16S rDNA–RFLP identification method was proposed *(9).* The application of the 16S rDNA–RFLP method in this work, apart from being a reliable identifier of all known *Arcobacter* spp., has been very useful for recognizing potential new species since new RFLP patterns have been obtained.

Prevalence of *Arcobacter* species. *A. butzleri* was the most prevalent species in this study (40 of 64 samples, 62.5%), followed by *A. cryaerophilus* (17 of 64 samples, 26.6%), *A. mytili* (3 of 64 samples, 4.7%), and *A. skirrowii* and *A. nitrofigilis* (the same prevalence: 2 of 64 samples, 3.1%). *A. skirrowii, A. butzleri,* and *A. cryaerophilus* were detected in one sample of clams, and *A. butzleri* and *A. cryaerophilus* were detected in one mussel sample (Table 1). *A. butzleri* was the most common species recovered from most types of meats and shellfish, except in clams, where *A. cryaerophilus* prevailed. *A. butzleri* is an emergent foodborne pathogen recently classified as a serious hazard to human health *(16),* and high prevalence was found in all studies listed in Table 2. The 16S rDNA–RFLP identification method used here enabled the differentiation of the two DNA groups (1A and 1B) defined for *A. cryaerophilus.* Only one strain recovered from turkey meat belonged to group 1A, and the rest belonged to group 1B. This tendency to recover group 1B more frequently was reported in a study of broiler chicken carcasses *(33).*

In the present study, the new species *A. mytili* was recovered only from mussels, although in general it had a prevalence similar to that of *A. skirrowii* and *A. nitrofigilis.* The three isolates of this new species were recovered from three different mussel samples obtained on two different sampling dates. However, the *A. mytili* strains belonged to only two different genotypes, as determined by an enterobacterial repetitive intergenic consensus PCR assay *(3).* The true prevalence of *A. mytili* and *A. nitrofigilis* probably is being underestimated because the commonly used m-PCR method *(15)* wrongly identifies these species as *A. skirrowii.*

The infrequent recovery of *A. skirrowii* (4.7%) in this study is in agreement with the results of previous studies of meat samples *(13, 19).*

The only two *A. nitrofigilis* isolates recovered in the present study were from mussels and were identified on the basis of the 16S rDNA–RFLP assay, 16S rRNA gene sequencing (GenBank accession nos. EU106661 and EU106662), and urease activity, which is characteristic of this species *(24).* Originally, *A. nitrofigilis* was isolated from the roots of the salt marsh plant *Spartina alterniflora,* which grows in brackish environments *(24).* The Ebro River delta is a brackish environment, so it is not surprising that we isolated this species from the mussels farmed in this area. To our knowledge, since the description of *A. nitrofigilis* by McClung et al. in 1983 *(24)* only one group of researchers has isolated and phenotypically identified this species *(23).* From that study, it was not possible to determine the prevalence or the number of isolates or whether the isolates were recovered from mussels or water. The present study is the first since 1983 that has genetically identified *A. nitrofigilis.*

Prevalence of *Arcobacter* in different types of meats. Several factors, i.e., different geographical regions, hygienic conditions, and isolation methods, can affect comparison of our results with those of previous studies. However, the prevalence of *Arcobacter* in chicken meat (64.3%) is consistent with prevalences found in previous studies, which ranged from 20 to 73% (Table 2). However, when chicken skin and carcass samples were analyzed, the prevalence was 100% *(2).* The results reported by Zanetti et al. *(39)* were not considered in Table 2 because *Arcobacter* was not recovered from sausages, poultry, or turkey meats and only one beef sample was positive for *Arcobacter.* This result probably is linked to the inappropriate isolation protocols used in that study. The present study is the first in which the prevalence of *Arcobacter* in duck and turkey meats (40 and 33.3%, respectively) has been evaluated. The only previous studies of *Arcobacter* prevalence in these products have been conducted with duck carcasses (80%) *(29)* and turkey carcasses (93%) *(1)* or mixtures of turkey skeletal

TABLE 2. *Reported prevalence of* Arcobacter *in meats and shellfish*[a]

Sample type	No. of samples studied	% positive samples	Identification method	Country	Reference
Chicken	50	38.0	Biochemical identification	Thailand	*37*
	94	62.0	m-PCR *(15)*	Ireland	*32*
	100	23.0	m-PCR *(18)*	Japan	*19*
	51	59.0	m-PCR *(18)*	Japan, Thailand	*25*
	22	73.0	m-PCR *(15)*	Australia	*30*
	15	20.0	PCR *(11)*	United States	*36*
	52	65.4	m-PCR *(15)*	Belgium	*13*
	14	50.0	m-PCR *(15)*	Belgium	*15*
	220	24.1	Biochemical identification	The Netherlands	*6*
Pork	50	16.0	Biochemical identification	Thailand	*37*
	101	35.0	m-PCR *(15)*	Ireland	*32*
	100	7.0	m-PCR *(18)*	Japan	*19*
	21	29.0	m-PCR *(15)*	Australia	*30*
	45	51.1	PCR *(11)*	United States	*36*
	200	32.0	PCR *(11)*	United States	*26*
	194	0.5	Biochemical identification	The Netherlands	*6*
	299	55.8	DNA Probe *(38)*	United States	*5*
Beef	80	15.0	m-PCR *(15)*	Ireland	*10*
	50	28.0	Biochemical identification	Thailand	*37*
	108	34.3	m-PCR *(15)*	Ireland	*32*
	90	2.2	m-PCR *(18)*	Japan	*19*
	32	22.0	m-PCR *(15)*	Australia	*30*
	97	5.1	m-PCR *(15)*	Turkey	*28*
	45	28.9	PCR *(11)*	United States	*36*
	68	1.5	Biochemical identification	The Netherlands	*6*
Mussels	80	35.0	Biochemical identification	Chile	*8*
	NS	NS	Biochemical identification	Italy	*23*

[a] Only meat products (not carcasses, viscera, or skin) were considered. NS, not specified.

tissue and skin (77%) *(21).* In our study, *Arcobacter* prevalence in pork (53.0%) was higher than that found in other studies, which ranged from 7 to 35% (Table 2), and is similar to the 51.1 and 55.8% reported by Villarruel-López et al. *(36)* and Collins et al. *(5),* respectively.

In beef, the *Arcobacter* prevalence found in this study (31.3%) is consistent with the findings of other authors (Table 2). Our study is the first to find *A. butzleri* (10.0%) in rabbit meat. No arcobacters were detected in sausages, in agreement with results of a previous study *(39).* However, this finding is surprising considering the high prevalence of *Arcobacter* in pork (Table 2).

Prevalence of *Arcobacter* in shellfish. There are few reports of *Arcobacter* in shellfish, although these bacteria have been detected in oysters, as determined by analysis of 16S rDNA sequences *(31). Arcobacter* was not found in oysters in the present study, and although only five clam samples were investigated, all of them were positive for *Arcobacter.* This finding is important because all of the species recovered from this type of shellfish have been implicated in gastrointestinal diseases *(12). A. butzleri* and *A. nitrofigilis* have been recovered previously (prevalence not specified) from mussels and brackish water in Italy *(23).* In that study, the water quality of the samples associated with mussels was very poor, with high levels of fecal coliforms, confirming previous findings that most *Arcobacter* species enter the marine environment through sewage pollution *(3).* This pollution effect also may explain the presence of *Arcobacter* in other shellfish. In our study the prevalence of *Arcobacter* in mussels was high (23 of 56 samples, 41.1%), and four species and a potential new species were also recovered. A study conducted in Chile revealed a similar prevalence of *Arcobacter* in mussels (35%) *(8).*

This survey of several types of meat and shellfish revealed that about one-third of the samples were contaminated with several species of *Arcobacter.*

Note added in proof. After acceptance of this manuscript, *Arcobacter thereius* sp. nov. was described (Houf et al., *Int. J. Syst. Evol. Microbiol.,* in press), including strains termed *A. skirrowii*–like by On et al. *(27)*; therefore, the so referred strain recovered in our study (Table 1 and Fig. 1) belongs to this new species.

ACKNOWLEDGMENTS

The authors thank M. Josep Solsona, Carmen Aguilar, and Ma Teresa Feliu (Laboratori de Salut Pública, Serveis Territorials de Salut de Tarragona, Spain) for helpful collaboration.

REFERENCES

1. Andersen, M. M., I. V. Wesley, E. Nestor, and D. W. Trampel. 2007. Prevalence of *Arcobacter* species in market-weight commercial turkeys. *Antonie Leeuwenhoek* 92:309–317.
2. Atabay, H. I., M. Waino, and M. Madsen. 2006. Detection and diversity of various *Arcobacter* species in Danish poultry. *Int. J. Food Microbiol.* 109:139–145.

3. Collado, L., I. Cleenwerck, S. Van Trappen, P. De Vos, and M. J. Figueras. 2009. *Arcobacter mytili* sp. nov., an indoxyl acetate hydrolysis negative bacterium isolated from mussels. *Int. J. Syst. Evol. Microbiol.*, in press.
4. Collado, L., I. Inza, J. Guarro, and M. J. Figueras. 2008. Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. *Environ. Microbiol.* 10:1635–1640.
5. Collins, C. I., I. V. Wesley, and E. A. Murano. 1996. Detection of *Arcobacter* spp. in ground pork by modified plating methods. *J. Food Prot.* 59:448–452.
6. de Boer, E., J. J. Tilburg, D. L. Woodward, H. Lior, and W. M. Johnson. 1996. A selective medium for the isolation of *Arcobacter* from meats. *Lett. Appl. Microbiol.* 23:64–66.
7. Donachie, S. P., J. P. Bowman, S. L. On, and M. Alam. 2005. *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter. Int. J. Syst. Evol. Microbiol.* 55:1271–1277.
8. Fernández, H., L. Otth, M. Wilson, R. Rodríguez, B. Proboste, C. Saldivia, and P. Barría. 2001. Occurrence of *Arcobacter* sp. in river water, mussels and commercial chicken livers in southern Chile. *Int. J. Med. Microbiol.* 291:140.
9. Figueras, M. J., L. Collado, and J. Guarro. 2008. A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter. Diagn. Microbiol. Infect. Dis.* 62:11–15.
10. Hamill, S., S. D. Neill, and R. H. Madden. 2008. A comparison of media for the isolation of *Arcobacter* spp. from retail packs of beef. *J. Food Prot.* 71:850–854.
11. Harmon, K. M., and I. V. Wesley. 1996. Identification of *Arcobacter* isolates by PCR. *Lett. Appl. Microbiol.* 23:241–244.
12. Ho, H. T., L. J. Lipman, and W. Gaastra. 2006. *Arcobacter,* what is known and unknown about a potential foodborne zoonotic agent! *Vet. Microbiol.* 115:1–13.
13. Houf, K., L. A. Devriese, L. De Zutter, J. Van Hoof, and P. Vandamme. 2001. Development of a new protocol for the isolation and quantification of *Arcobacter* species from poultry products. *Int. J. Food Microbiol.* 71:189–196.
14. Houf, K., S. L. On, T. Coenye, J. Mast, J. Van Hoof, and P. Vandamme. 2005. *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. *Int. J. Syst. Evol. Microbiol.* 55:713–717.
15. Houf, K., A. Tutenel, L. De Zutter, J. Van Hoof, and P. Vandamme. 2000. Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri, Arcobacter cryaerophilus* and *Arcobacter skirrowii. FEMS Microbiol. Lett.* 193:89–94.
16. International Commission on Microbiological Specifications for Foods. 2002. Microorganisms in foods. 7. Microbiological testing in food safety management. Kluwer Academic–Plenum Publishers, New York.
17. Jacob, J., H. Lior, and I. Feuerpfeil. 1993. Isolation of *Arcobacter butzleri* from a drinking water reservoir in eastern Germany. *Zentbl. Hyg. Umweltmed.* 193:557–562.
18. Kabeya, H., Y. Kobayashi, S. Maruyama, and T. Mikami. 2003. One-step polymerase chain reaction–based typing of *Arcobacter* species. *Int. J. Food Microbiol.* 81:163–168.
19. Kabeya, H., S. Maruyama, Y. Morita, T. Ohsuga, S. Ozawa, Y. Kobayashi, M. Abe, Y. Katsube, and T. Mikami. 2004. Prevalence of *Arcobacter* species in retail meats and antimicrobial susceptibility of the isolates in Japan. *Int. J. Food Microbiol.* 90:303–308.
20. Lehner, A., T. Tasara, and R. Stephan. 2005. Relevant aspects of *Arcobacter* spp. as potential foodborne pathogen. *Int. J. Food Microbiol.* 102:127–135.
21. Manke, T. R., I. V. Wesley, J. S. Dickson, and K. M. Harmon. 1998. Prevalence and genetic variability of *Arcobacter* species in mechanically separated turkey. *J. Food Prot.* 61:1623–1628.
22. Martinez-Murcia, A. J., S. Benlloch, and M. D. Collins. 1992. Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. *Int. J. Syst. Bacteriol.* 42:412–421.
23. Maugeri, T. L., C. Gugliandolo, M. Carbone, D. Caccamo, and M. T. Fera. 2000. Isolation of *Arcobacter* spp. from a brackish environment. *New Microbiol.* 23:143–149.
24. McClung, C. R., D. G. Patriquin, and R. E. Davis. 1983. *Campylobacter nitrofigilis* sp. nov., a nitrogen-fixing bacterium associated with roots of *Spartina alterniflora. Int. J. Syst. Bacteriol.* 33:605–612.
25. Morita, Y., S. Maruyama, H. Kabeya, S. Boonmar, B. Nimsuphan, A. Nagai, K. Kozawa, T. Nakajima, T. Mikami, and H. Kimura. 2004. Isolation and phylogenetic analysis of *Arcobacter* spp. in ground chicken meat and environmental water in Japan and Thailand. *Microbiol. Immunol.* 48:527–533.
26. Ohlendorf, D. S., and E. A. Murano. 2002. Prevalence of *Arcobacter* spp. in raw ground pork from several geographical regions according to various isolation methods. *J. Food Prot.* 65:1700–1705.
27. On, S. L., C. S. Harrington, and H. I. Atabay. 2003. Differentiation of *Arcobacter* species by numerical analysis of AFLP profiles and description of a novel *Arcobacter* from pig abortions and turkey faeces. *J. Appl. Microbiol.* 95:1096–1105.
28. Öngör, H., B. Cetinkaya, M. N. Acik, and H. I. Atabay. 2004. Investigation of arcobacters in meat and faecal samples of clinically healthy cattle in Turkey. *Lett. Appl. Microbiol.* 38:339–344.
29. Ridsdale, J. A., H. I. Atabay, and J. E. L. Corry. 1998. Prevalence of campylobacters and arcobacters in ducks at the abattoir. *J. Appl. Microbiol.* 85:567–573.
30. Rivas, L., N. Fegan, and P. Vanderlinde. 2004. Isolation and characterisation of *Arcobacter butzleri* from meat. *Int. J. Food Microbiol.* 91:31–41.
31. Romero, J., M. Garcia-Varela, J. P. Laclette, and R. T. Espejo. 2002. Bacterial 16S rRNA gene analysis revealed that bacteria related to *Arcobacter* spp. constitute an abundant and common component of the oyster microbiota (*Tiostrea chilensis*). *Microb. Ecol.* 44:365–371.
32. Scullion, R., C. S. Harrington, and R. H. Madden. 2006. Prevalence of *Arcobacter* spp. in raw milk and retail raw meats in Northern Ireland. *J. Food Prot.* 69:1986–1990.
33. Son, I., M. D. Englen, M. E. Berrang, P. J. Fedorka-Cray, and M. A. Harrison. 2007. Prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. *Int. J. Food Microbiol.* 113:16–22.
34. Vandamme, P., M. Vancanneyt, B. Pot, L. Mels, B. Hoste, D. Dewettinck, L. Vlaes, C. van den Borre, R. Higgins, and J. Hommez. 1992. Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. *Int. J. Syst. Bacteriol.* 42:344–356.
35. Van Driessche, E., K. Houf, J. van Hoof, L. De Zutter, and P. Vandamme. 2003. Isolation of *Arcobacter* species from animal feces. *FEMS Microbiol. Lett.* 229:243–248.
36. Villarruel-López, A., M. Marquez-Gonzalez, L. E. Garay-Martinez, H. Zepeda, A. Castillo, L. Mota de la Garza, E. A. Murano, and R. Torres-Vitela. 2003. Isolation of *Arcobacter* spp. from retail meats and cytotoxic effects of isolates against Vero cells. *J. Food Prot.* 66: 1374–1378.
37. Vindigni, S. M., A. Srijan, B. Wongstitwilairoong, R. Marcus, J. Meek, P. L. Riley, and C. Mason. 2007. Prevalence of foodborne microorganisms in retail foods in Thailand. *Foodborne Pathog. Dis.* 4:208–215.
38. Wesley, I. V., L. Schroeder-Tucker, A. L. Baetz, F. E. Dewhirst, and B. J. Paster. 1995. *Arcobacter*-specific and *Arcobacter butzleri*–specific 16S rRNA-based DNA probes. *J. Clin. Microbiol.* 33:1691–1698.
39. Zanetti, F., O. Varoli, S. Stampi, and G. De Luca. 1996. Prevalence of thermophilic *Campylobacter* and *Arcobacter butzleri* in food of animal origin. *Int. J. Food Microbiol.* 33:315–332.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

4.5 *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. & Figueras, M. J.

International Journal of Systematic and Evolutionary Microbiology
(2009) 59, 1391-1396

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

International Journal of Systematic and Evolutionary Microbiology (2009), **59**, 1391–1396 DOI 10.1099/ijs.0.003749-0

Arcobacter mytili sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels

Luis Collado,[1] Ilse Cleenwerck,[2] Stefanie Van Trappen,[2] Paul De Vos[2] and Maria Jose Figueras[1]

Correspondence
Maria Jose Figueras
mariajose.figueras@urv.cat

[1]Unitat de Microbiologia, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus, Spain

[2]BCCM/LMG Bacteria Collection and Laboratory of Microbiology, Faculty of Sciences, Ghent University, K. L. Ledeganckstraat 35, 9000 Ghent, Belgium

Three *Arcobacter* isolates, recovered from mussels (genus *Mytilus*), and one isolate from brackish water in Catalonia (north-east Spain) showed a novel pattern using a recently described identification method for members of the genus *Arcobacter*, 16S rRNA gene RFLP. Enterobacterial repetitive intergenic consensus PCR fingerprinting demonstrated that the three isolates from mussels belonged to two genotypes and that the fourth isolate from water belonged to a third genotype. Analysis of the 16S rRNA and *rpoB* gene sequences showed that the new isolates formed a separate lineage within the genus *Arcobacter*. This was also confirmed by the low DNA–DNA relatedness values (16–30 %) of the isolates with the type strains of recognized *Arcobacter* species. Hydrolysis of indoxyl acetate, a characteristic trait for all species of the genus *Arcobacter*, was negative for the novel isolates. The susceptibility of the novel isolates to cefoperazone, together with the lack of urease production and nitrate reduction, further enabled them to be differentiated from recognized *Arcobacter* species based on physiological characteristics. Genotypic and phenotypic characteristics indicated that the new isolates represent a novel species of the genus *Arcobacter*, for which the name *Arcobacter mytili* sp. nov. is proposed, with the type strain F2075^T (=CECT 7386^T =LMG 24559^T). The DNA G+C content of strain F2075^T was 26.9 mol%.

In 1991, Vandamme and colleagues reclassified the 'aerotolerant campylobacters' in the genus *Arcobacter*, including *Arcobacter nitrofigilis* and *Arcobacter cryaerophilus* (Vandamme *et al.*, 1991). The genus was emended a year later with the addition of *Arcobacter butzleri* and *Arcobacter skirrowii* (Vandamme *et al.*, 1992). Two more novel species have since been described, *Arcobacter cibarius*, isolated from broiler carcasses in Belgium (Houf *et al.*, 2005) and *Arcobacter halophilus*, isolated from a hypersaline lagoon in Hawaii (Donachie *et al.*, 2005) and the genus currently comprises six species. Moreover, an autotrophic, obligate microaerophilic sulfide-oxidizing bacteria named '*Candidatus* Arcobacter sulfidicus' of marine origin was described in 2002 as a possible additional novel taxon (Wirsen *et al.*, 2002).

The type species of the genus, *A. nitrofigilis*, was first recovered from the roots of *Spartina alterniflora*, a salt marsh plant (McClung *et al.*, 1983), and since then there have been few reports on this species (Figueras *et al.*, 2008). *A. butzleri* is the most common species in environmental water, food and clinical samples (Ho *et al.*, 2006). In fact, this species was ranked as the fourth most common campylobacterium isolated from human faeces in two independent studies performed in Belgium and France (Vandenberg *et al.*, 2004; Prouzet-Mauléon *et al.*, 2006). Recently, this species was considered to be a serious hazard to human health by the International Commission on Microbiological Specification for Foods (ICMSF, 2002). *A. cryaerophilus*, the second most commonly isolated species of the genus, has been recovered from cases of diarrhoea and bacteraemia in humans, as well as from the meat of several animals (Ho *et al.*, 2006). This species was also recovered from faeces of 1.4 % of healthy people (Houf & Stephan, 2007). *A. skirrowii* is usually isolated from

Abbreviations: ERIC-PCR, Enterobacterial repetitive intergenic consensus PCR; m-PCR, multiplex PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *rpoB* sequences of strain F2075^T are EU669904 and EU669901, respectively.

Supplementary figures showing the ERIC-PCR patterns and a phylogenetic tree based on *rpoB* gene sequences for species of the genus *Arcobacter* are available with the online version of this paper.

preputial fluids of bulls and faeces of animals, including sheep and cattle (Vandamme *et al.*, 2005). It has also been associated with chronic diarrhoea in an old man (Wybo *et al.*, 2004) and has been recently detected in humans with and without diarrhoea in South Africa (Samie *et al.*, 2007). *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are frequently isolated from animal faeces (Van Driessche *et al.*, 2003) and have recently been associated with faecally polluted environmental waters (Collado *et al.*, 2008). Although the epidemiology of *Arcobacter* species is not clear, it has been suggested that water and foods are the transmission route of arcobacters to humans (Ho *et al.*, 2006).

As part of a study on the prevalence of arcobacters in meat and shellfish products in Catalonia (north-east Spain), three isolates (F2026, F2075^T and F2076) were recovered from mussels (genus *Mytilus*) in 2006 and an additional strain (T234) was isolated from brackish water in 2008. Using a recently proposed *Arcobacter* species identification method, 16S rRNA gene RFLP, these isolates showed a specific pattern that was different from those defined for the six recognized species of the genus (Figueras *et al.*, 2008). In the present study, a polyphasic approach was used to establish the taxonomic position of these novel isolates. For this purpose, phylogenetic analyses of the 16S rRNA and *rpoB* gene sequences, DNA–DNA hybridization experiments, DNA G+C content determination, enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) genotyping and phenotypic analysis were performed. Based on the results obtained, we propose the allocation of these isolates to a novel species of the genus *Arcobacter*.

Mussel samples were taken from the Ebro river delta (north-east Spain) on 29 July (one sample) and 21 September 2006 (two samples) and were processed by homogenizing 10 g of mussel flesh with 90 ml *Arcobacter* enrichment broth (Oxoid) supplemented with CAT comprising (mg l^{-1}) cefoperazone (8), amphotericin B (10) and teicoplanin (4). After incubation for 48 h at 30 °C under aerobic conditions, 200 µl broth was inoculated on a blood agar (BA) plate following the procedure described by Collado *et al.* (2008). Small, colourless or beige-to-off-white, translucent colonies, the characteristic form of colonial growth for members of the genus *Arcobacter*, were selected from each sample for genetic identification. This was performed using a multiplex PCR (m-PCR) for simultaneous detection of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Houf *et al.*, 2000) and a 16S rRNA gene RFLP method, which consists of an amplification of 1026 bp of the 16S rRNA gene and posterior digestion with *Mse*I endonuclease to obtain restriction patterns that enable characterization of the six recognized species (Figueras *et al.*, 2008). Three mussel isolates, one recovered from the July sample (F2026) and one from each of the two September samples (F2075^T and F2076), were identified as *A. skirrowii* with the m-PCR method due to the presence of a 653 bp band on the agarose gel that could not be differentiated from the 641 bp band that is typical of *A. skirrowii*. However, the isolates showed a novel RFLP pattern (650/143/138 bp) that did not correspond to any of the recognized *Arcobacter* species (Figueras *et al.*, 2008). One additional isolate (T234) was recovered (June 2008) from brackish water (salinity 14.4 ‰) of the Ebro river delta using the isolation procedure described by Collado *et al.* (2008). This isolate was also identified as *A. skirrowii* with m-PCR and showed the same RFLP pattern as the mussel isolates.

To investigate the genetic relatedness of the four isolates, ERIC-PCR (Houf *et al.*, 2002) was performed. Isolates F2075^T and F2076, despite having been isolated from different samples, were considered to have the same genotype on the basis of sharing the same ERIC-PCR pattern. This pattern was clearly different from those of isolates F2026 and T234 (see Supplementary Fig. S1, available in IJSEM Online).

The 16S rRNA genes of the four isolates (around 1460 bp) were amplified according to Martínez-Murcia *et al.* (1992) and sequenced in both directions using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. Sequence assembly was performed with AUTO ASSEMBLER (Applied Biosystems). In addition, the *rpoB* genes of the four novel isolates and 11 *Arcobacter* strains [two strains for each recognized species of the genus *Arcobacter*, with the exception of *A. halophilus* (see Supplementary Table S1)], were amplified using the PCR primers CamrpoB-L and RpoB-R and the conditions that have been established for use with the genus *Campylobacter* (Korczak *et al.*, 2006). The expected PCR product size (524 bp) was obtained for all strains, with additional unexpected bands in some cases. The bands of the expected size were purified from the agarose gel with the GFX PCR DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions. The PCR products were sequenced in both directions and in duplicate. The *rpoB* gene sequences were used to calculate the percentage nucleotide substitutions for a continuous stretch of 487 bp (positions 1552–2039 according to *Escherichia coli* numbering).

Both sets of sequence data, for the 16S rRNA and *rpoB* genes, from strains F2075^T and F2076 were identical and confirmed the ERIC-PCR results. Therefore, strain F2076 was not subjected to further phylogenetic or phenotypic analysis.

Using sequences obtained from this study and from GenBank, separate alignments of 16S rRNA gene sequences (1409 bp) and *rpoB* sequences (487 bp) were performed with CLUSTAL W (Thompson *et al.*, 1994). Genetic distances were obtained using Kimura's two-parameter model (Kimura, 1980) and evolutionary trees were constructed by the neighbour-joining method with the MEGA4 program (Tamura *et al.*, 2007). The stability of each relationship was assessed by bootstrap analysis (1000 replicates).

In both of the phylogenetic trees derived from the 16S rRNA and *rpoB* gene sequences (Fig. 1 and Supplementary

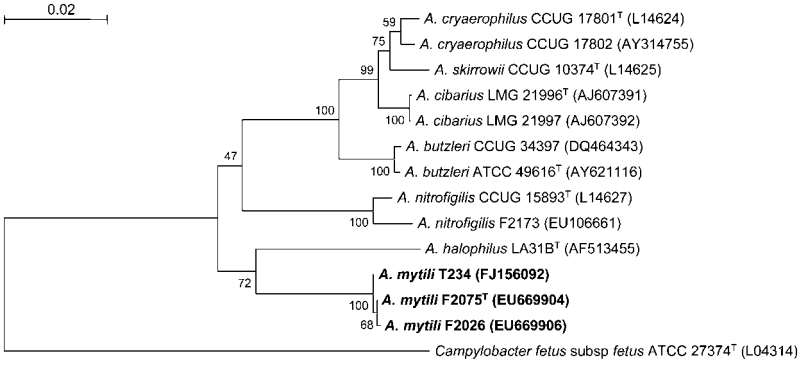


Fig. 1. Neighbour-joining phylogenetic tree showing the relationship of *Arcobacter mytili* sp. nov. with other *Arcobacter* species on the basis of 1409 nucleotides from the 16S rRNA gene. Numbers at nodes represent percentage bootstrap values (1000 replications). Bar, 2 substitutions per 100 nt.

Fig. S2), three of the novel strains (F2026, F2075^T and T234) formed a distinct clade with *A. halophilus* LA31B^T. The 16S rRNA gene sequence of strain F2075^T was compared with the sequences of type strains deposited in GenBank using both BLASTN (Altschul *et al.*, 1990) and EZTAXON (Chun *et al.*, 2007) and it showed the highest similarity values with the type strains of the six *Arcobacter* species: *A. halophilus*, 94.8 %; *A. nitrofigilis*, 93.8 %; *A. butzleri*, 93.6 %; *A. cibarius*, 93.3 %, *A. cryaerophilus*, 93.1 %, and *A. skirrowii*, 92.8 %. The levels of similarity to *Campylobacter* species were below 87.9 %. The 16S rRNA gene sequence of strain F2075^T showed 99.9 and 99.8 % sequence similarities with those of strains F2026 and T234, respectively. The *rpoB* gene sequence similarities between strain F2075^T and each *Arcobacter* type strain were 88.0 %, 86.7 %, 85.8 %, 83.0 %, 82.1 % and 81.9 % for *A. halophilus*, *A. butzleri*, *A. nitrofigilis*, *A. cryaerophilus*, *A. cibarius* and *A. skirrowii*, respectively. The inter-species rate of nucleotide substitutions for the *rpoB* gene was over 10.7 %, while the intra-species variation ranged from 0.2 to 5.3 %, with the sequences from *A. skirrowii*, *A. butzleri* and the novel isolates being at the lower range of intra-species variability (see Supplementary Fig. S2). In a recent study that analysed the relationship between *rpoB* gene sequence similarity and DNA–DNA hybridization for 230 bacteria, a DNA–DNA relatedness value of more than 70 % correlated with a *rpoB* gene sequence similarity of 97.7 %, and this was proposed as the cut-off value for species delineation (Adékambi *et al.*, 2008). The *rpoB* gene sequence similarities between strain F2075^T and strains F2026 and T234 were 99.8 and 99.6 %, respectively, which are clearly above this cut-off value.

For DNA–DNA hybridization experiments and for the determination of DNA G+C content, genomic DNA was prepared according to the procedure of Wilson (1987) with the modification by Cleenwerck *et al.* (2002). DNA–DNA hybridizations were performed at 32 °C according to a modification (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the method described by Ezaki *et al.* (1989). Reciprocal reactions were performed for every comparison pair and the variation found was within the limits of this method (Goris *et al.*, 1998). The DNA–DNA relatedness values reported are the means of a minimum of four hybridizations. The DNA–DNA relatedness value obtained for strain F2075^T with *A. halophilus* LA31B^T, the most closely related species on the basis of the 16S rRNA and *rpoB* gene sequences, was 30 %, while values with all other recognized *Arcobacter* species were 23 % or below (Table 1). Although these DNA–DNA hybridization values may seem low, they are not low in comparison with those recently published in the description of *A. halophilus*, which ranged between 4 and 12 % with the other recognized *Arcobacter* species (Donachie *et al.*, 2005). The G+C content of each DNA sample was determined by three independent analyses using the HPLC technique (Mesbah *et al.*, 1989). The DNA G+C content of strain F2075^T was 26.9 mol%, which is within the previously defined range of 26.8–35 mol% for the genus (Donachie *et al.*, 2005; Houf *et al.*, 2005; Vandamme *et al.*, 2005).

Phenotypic characterization of strains F2026, F2075^T and T234 was performed using the biochemical identification scheme of Vandamme *et al.* (2005) (Table 2). NaCl tolerance and susceptibility to cefoperazone were tested on nutrient broth no. 2 (Oxoid) supplemented with 5 % whole sheep blood and 1.5 % agar. The indoxyl acetate hydrolysis test was performed according to Mills & Gherna (1987) and confirmed using indoxyl acetate diagnostic tablets (IAC)-DIETABS (Rosco Diagnostica). All tests were conducted at least twice. The novel isolates were biochemically different from the recognized species of the genus

Table 1. DNA–DNA relatedness between strain F2075^T and the type strains of other *Arcobacter* species

Results are expressed as the mean ± standard deviation.

Strain	DNA–DNA relatedness with strain F2075^T (%)
A. halophilus LA31B^T	30±12
A. butzleri LMG 10828^T	23±1
A. skirrowii LMG 6621^T	19±8
A. nitrofigilis CECT 7204^T	16±2
A. cibarius CECT 7203^T	16±6
A. cryaerophilus LMG 9904^T	16±7

Table 2. Differential characteristics of *Arcobacter mytili* sp. nov. and other species of the genus *Arcobacter*

Species: 1, *A. mytili* sp. nov. (n=3, data from this study); 2, *A. nitrofigilis* (n=4, this study); 3, *A. halophilus* (n=1); 4, *A. cibarius* (n=15); 5, *A. cryaerophilus* (n=19; four strains were retested in this study); 6, *A. butzleri* (n=12); 7, *A. skirrowii* (n=9). Data taken from previous studies (On *et al.*, 1996; Donachie *et al.*, 2005; Houf *et al.*, 2005) unless otherwise indicated. +, >95 % Strains positive; −, <11 % strains positive; v, 12–94 % strains positive.

Characteristic	1	2	3	4	5	6	7
Growth condition							
Air at 25 °C	+	+	+	v	+	+	+
MacConkey agar	+	−	−	+	v	+	−
Minimal media	−	−	−	+	−*	+	−
NaCl 4 % (w/v)	+	+	+	−	−	−	+
Cefoperazone (64 mg l^{-1})	−	−	−	+	+	+	+
Enzyme activity							
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	−	v	+	v	+
Urease	−	+	−	−	−	−	−
Nitrate reduction	−	+	+	−	+†	+	+
Indoxyl acetate hydrolysis	−	+	+	+	+	+	+

*Two of the four strains tested in this study (LMG 7537 and LMG 10241) were positive.
†Two of the four strains tested in this study (LMG 9904^T and LMG 9065) were negative.

Arcobacter in that they did not hydrolyse indoxyl acetate (Table 2). Since the studies of On *et al.* (1996) demonstrated that *A. nitrofigilis* was able to hydrolyse indoxyl acetate, which contradicted previous data (Mills & Gherna, 1987), all species of the genus *Arcobacter* have been considered to be indoxyl-acetate-hydrolysis-positive. In the present study, using four strains of *A. nitrofigilis* (CECT 7204^T, LMG 7547, F2173 and F2176), the results of On *et al.* (1996) were corroborated as, despite the reactions being slower and weaker than that observed for the other species, they were clearly positive. The susceptibility to cefoperazone (64 mg l^{-1}) differentiated the novel isolates from *A. butzleri*, *A. cibarius*, *A. cryaerophilus* and *A. skirrowii*. The lack of urease activity enabled the novel strains to be differentiated from *A. nitrofigilis*. The growth in MacConkey agar and the inability to reduce nitrate to nitrite differentiated the novel strains from *A. halophilus*. In addition, strains F2026, F2075^T and T234 differed from the other *Arcobacter* speicies by their fast growth in BA (growth was observable after 24 h incubation in aerobic conditions).

Motility was observed in young cultures by examining wet mounts in nutrient broth no. 2 by phase-contrast microscopy. Cell size, morphology and presence of flagella (Fig. 2) were determined with transmission electron microscopy (JEOL 1011) after negative staining with 2 % (w/v) phosphotungstic acid solution (pH 6.9) for 1 min and with scanning electron microscopy after fixing pieces of agar containing cells of

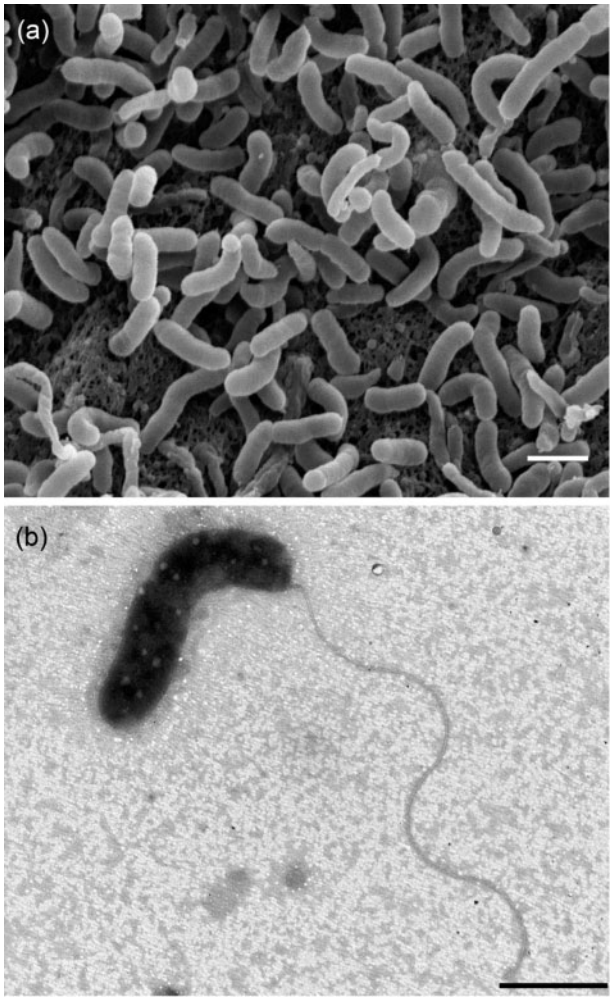


Fig. 2. Images of cells of strain F2075^T as observed with scanning electron microscopy (a) and transmission electron microscopy, negatively stained (b). Bars, 1 μm.

growing strain F2075^T in 2.5 % glutaraldehyde in phosphate buffer for 24 h. Subsequently, the samples were post-fixed in 1 % osmium tetroxide for 2 h. After dehydration and critical-point drying, specimens were mounted and coated with a thin layer of gold before examination with a JEOL JSM 6400 scanning electron microscope.

The data presented here support the suggestion that the four novel strains belong to a previously unrecognized species of the genus *Arcobacter*, for which the name *Arcobacter mytili* sp. nov. is proposed.

The recently described 16S rRNA gene RFLP *Arcobacter* identification protocol (Figueras *et al.*, 2008) is currently the only fast method that enables the differentiation of these four novel strains from the rest of the species of the genus on the basis of specific restriction patterns. In contrast, the m-PCR method (Houf *et al.*, 2000) misidentifies the four novel strains as *A. skirrowii*.

Description of *Arcobacter mytili* sp. nov.

Arcobacter mytili (my′ti.li. L. gen. n. *mytili* of a mussel, from the genus name *Mytilus*, from which the species was first isolated).

Cells are Gram-negative, non-encapsulated, non-spore-forming, slightly curved rods, some S-shaped, 0.4–0.6 μm wide and 1–3 μm long. Motile by means of a single polar flagellum. Colonies on BA incubated in aerobic conditions at 30 °C for 48 h are 2–4 mm in diameter, beige to off-white, circular with entire margins, convex and non-swarming. Pigments are not produced. All strains grow on BA at room temperature (18–22 °C) and at 30 and 37 °C under aerobic or microaerobic culture conditions with no significant differences. Under aerobic conditions, all strains grow at 30 °C on MacConkey agar and on media containing 2.0–4.0 % (w/v) NaCl. No growth is obtained on casein, minimal medium or media containing 64 mg cefoperazone l^{-1}. Weak growth is obtained in anaerobic conditions at 30 °C and in aerobic conditions at 42 °C, and no growth is observed at 4 °C. Oxidase-positive and weakly catalase-positive. Strains are not haemolytic and do not hydrolyse indoxyl acetate. Urease is not produced and nitrate is not reduced. Hydrogen sulfide is not produced in triple-sugar iron agar medium.

The type strain, F2075^T (=CECT 7386^T=LMG 24559^T), was isolated from mussels from Catalonia, Spain. The DNA G+C content of the type strain is 26.9 mol%.

Acknowledgements

The BCCM/LMG bacteria collection is supported by the Prime Minister's Services–Federal Office for Scientific, Technical and Cultural Affairs, Belgium. We thank Laboratori de Salut Pública, Serveis Territorials de Salut de Tarragona for helpful collaboration and Dr Maqsudul Alam (University of Hawaii, Manoa, HI) and Dr Kurt Houf (Ghent University, Belgium) for kindly providing strains. This work was supported by funds from the European Commission for the HEALTHY WATER project (FOOD-CT-2006-036306). The authors are solely responsible for the content of this publication. It does not represent the opinion of the European Commission. The European Commission is not responsible for any use that might be made of data appearing therein.

References

Adékambi, T., Shinnick, T. M., Raoult, D. & Drancourt, M. (2008). Complete *rpoB* gene sequencing as a suitable supplement to DNA–DNA hybridization for bacterial species and genus delineation. *Int J Syst Evol Microbiol* **58**, 1807–1814.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D. & Swings, J. (2002). Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. *Int J Syst Evol Microbiol* **52**, 1551–1558.

Collado, L., Inza, I., Guarro, J. & Figueras, M. J. (2008). Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. *Environ Microbiol* **10**, 1635–1640.

Donachie, S. P., Bowman, J. P., On, S. L. & Alam, M. (2005). *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. *Int J Syst Evol Microbiol* **55**, 1271–1277.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Figueras, M. J., Collado, L. & Guarro, J. (2008). A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. *Diagn Microbiol Infect Dis* **62**, 11–15.

Goris, J., Suzuki, K., De Vos, P., Nakase, T. & Kersters, K. (1998). Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. *Can J Microbiol* **44**, 1148–1153.

Ho, H. T. K., Lipman, L. J. & Gaastra, W. (2006). *Arcobacter*, what is known about a potential foodborne zoonotic agent! *Vet Microbiol* **115**, 1–13.

Houf, K. & Stephan, R. (2007). Isolation and characterization of the emerging foodborn pathogen *Arcobacter* from human stool. *J Microbiol Methods* **68**, 408–413.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J. & Vandamme, P. (2000). Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. *FEMS Microbiol Lett* **193**, 89–94.

Houf, K., De Zutter, L., Van Hoof, J. & Vandamme, P. (2002). Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. *Appl Environ Microbiol* **68**, 2172–2178.

Houf, K., Stephen, L., On, W., Coenye, T., Mast, J., Van Hoof, J. & Vandamme, P. (2005). *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. *Int J Syst Evol Microbiol* **55**, 713–717.

ICMSF (2002). *Microorganisms in Foods 7 – Microbiological Testing in Food Safety Management*. International Commission on Microbiological Specifications for Foods. New York: Kluwer Academic/Plenum.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Korczak, B. M., Stieber, R., Emler, S., Burnens, A. P., Frey, J. & Kuhnert, P. (2006). Genetic relatedness within the genus *Campylobacter* inferred from *rpoB* sequences. *Int J Syst Evol Microbiol* **56**, 937–945.

Martínez-Murcia, A. J., Benlloch, S. & Collins, M. D. (1992). Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. *Int J Syst Bacteriol* **42**, 412–421.

McClung, C. R., Patriquin, D. G. & Davis, R. E. (1983). *Campylobacter nitrofigilis* sp. nov., a nitrogen-fixing bacterium associated with roots of *Spartina alterniflora* Loisel. *Int J Syst Bacteriol* **33**, 605–612.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Mills, C. K. & Gherna, R. L. (1987). Hydrolysis of indoxyl acetate by *Campylobacter* species. *J Clin Microbiol* **25**, 1560–1561.

On, S. L., Holmes, B. & Sackin, M. J. (1996). A probability matrix for the identification of campylobacters, helicobacters and allied taxa. *J Appl Bacteriol* **81**, 425–432.

Prouzet-Mauléon, V., Labadi, L., Bouges, N., Menard, A. & Megraud, F. (2006). *Arcobacter butzleri*: underestimated enteropathogen. *Emerg Infect Dis* **12**, 307–309.

Samie, A., Obi, C. L., Barrett, L. J., Powell, S. M. & Guerrant, R. L. (2007). Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: Studies using molecular diagnostic methods. *J Infect* **54**, 558–566.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. & De Ley, J. (1991). Revision of *Campylobacter*, *Helicobacter* and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* **41**, 88–103.

Vandamme, P., Vancanneyt, M., Pot, B., Mels, L., Hoste, B., Dewettinck, D., Vlaes, L., van den Borre, C., Higgins, R. & other authors (1992). Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* **42**, 344–356.

Vandamme, P., Dewhirst, F. E., Paster, B. J. & On, S. L. W. (2005). Genus II. *Arcobacter* Vandamme, Falsen, Rossau, Segers, Tytgat and De Ley 1991a, 99^{VP}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, pp. 1161–1165. Edited by D. J. Brenner, N. P. Kreig, J. T. Staley & G. M. Garrity. New York: Springer.

Vandenberg, O., Dediste, A., Houf, K., Ibekwem, S., Souayah, H., Cadranel, S., Douat, N., Zissis, G., Butzler, J. P. & Vandamme, P. (2004). *Arcobacter* species in humans. *Emerg Infect Dis* **10**, 1863–1867.

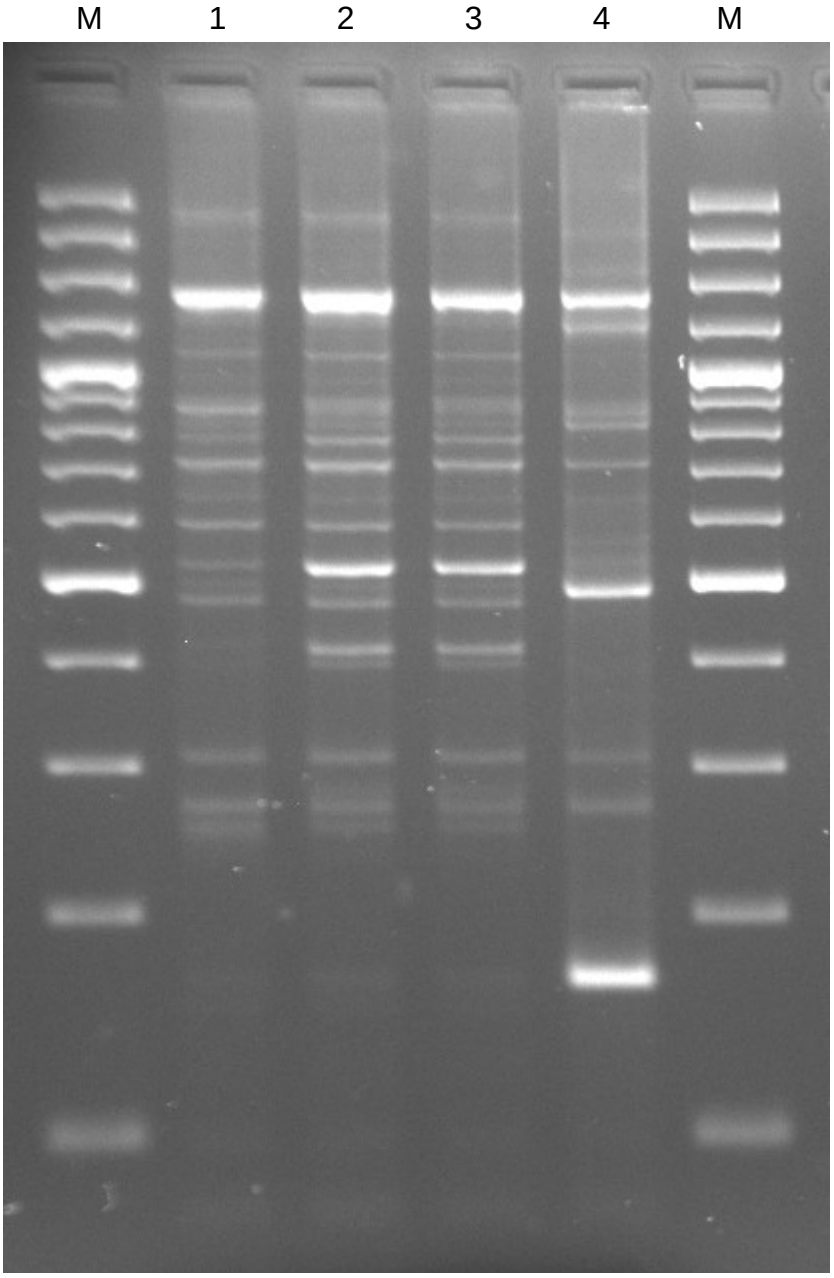
Van Driessche, E., Houf, K., Van Hoof, J., De Zutter, L. & Vandamme, P. (2003). Isolation of *Arcobacter* species from animal feces. *FEMS Microbiol Lett* **229**, 243–248.

Wilson, K. (1987). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 241–245. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Greene Publishing and Wiley Interscience.

Wirsen, C. O., Sievert, S. M., Cavanaugh, C. M., Molyneaux, S. J., Ahmad, A., Taylor, L. T., DeLong, E. F. & Taylor, C. D. (2002). Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. *Appl Environ Microbiol* **68**, 316–325.

Wybo, I., Breynaert, J., Lauwers, S., Lindenburg, F. & Houf, K. (2004). Isolation of *Arcobacter skirrowii* from a patient with chronic diarrhea. *J Clin Microbiol* **42**, 1851–1852.

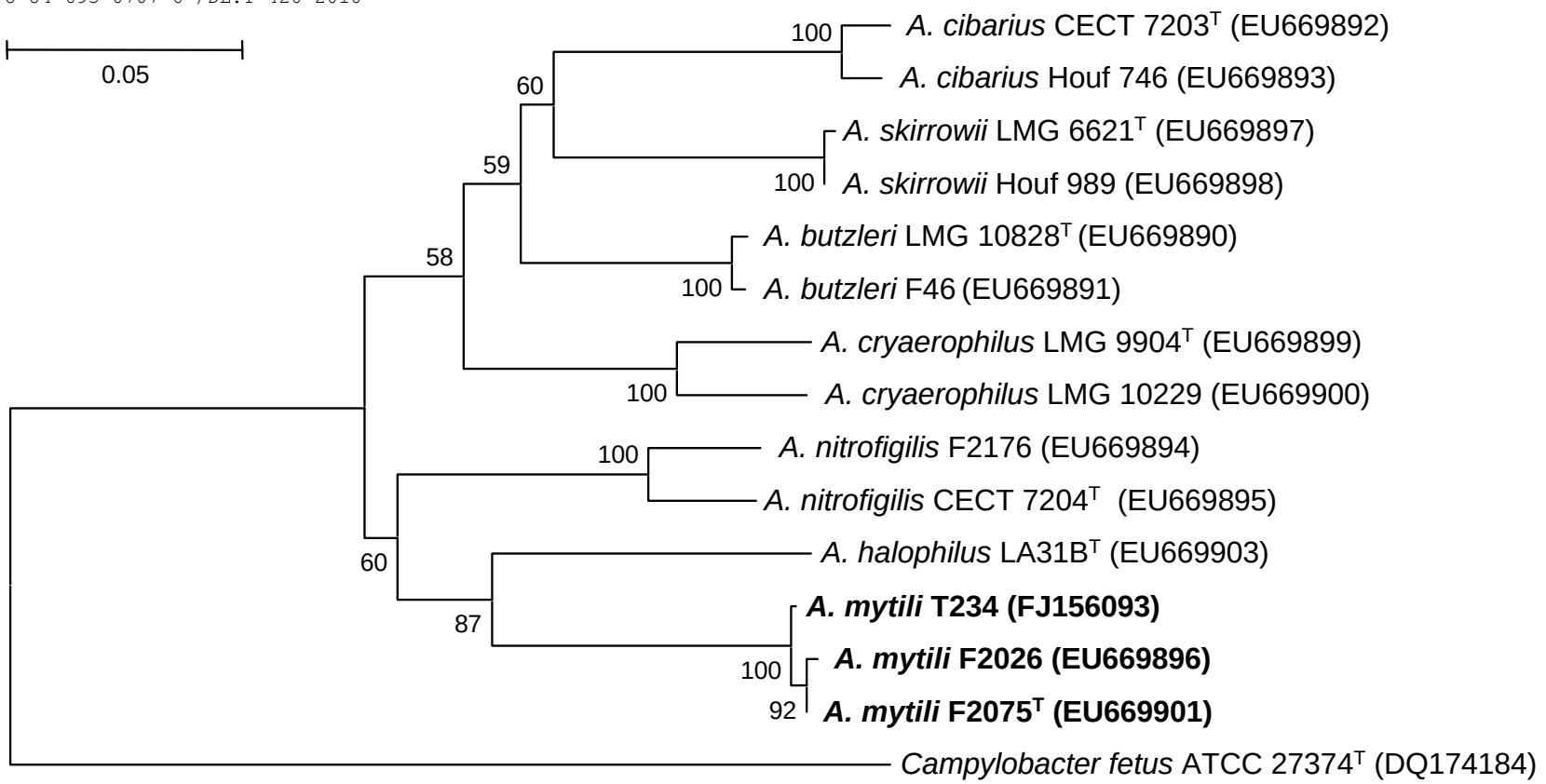
UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010



Supplementary Fig. S1. Agarose gel showing ERIC-PCR patterns of *Arcobacter mytili* sp. nov. Lanes: 1, F2026; 2, F2075^T; 3, F2076; 4, T234. M, GeneRuler 100 bp Plus DNA Ladder (Fermentas), fragment sizes (bp) from top to bottom: 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100. Note that isolate F2076 is considered the same genotype as F2075^T.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. & Figueras, M. J. (2009). *Arcobacter mytili* sp. nov., an indoxyl acetate hydrolysis-negative bacterium isolated from mussels. *Int J Syst Evol Microbiol* **59**, 1391–1396.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010



Supplementary Fig. S2. Neighbour-joining phylogenetic tree showing the relationship of *Arcobacter mytili* sp. nov. with other species of the genus *Arcobacter* on the basis of 487 nucleotides from the *rpoB* gene. Numbers at nodes represent percentage bootstrap values (1000 replications). Bar, 5 substitutions per 100 nt.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. & Figueras, M. J. (2009). *Arcobacter mytili* sp. nov., an indoxyl acetate hydrolysis-negative bacterium isolated from mussels. *Int J Syst Evol Microbiol* **59**, 1391–1396.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Supplementary Table S1. Species and strains used in this study

CECT, Colección Española de Cultivos Tipo.

Strains	Source	Country	GenBank accession no.	
			16S rRNA	*rpoB*
A. mytili				
F2026 (=CECT 7385)	Mussels	Spain	EU669906	EU669896
F2075^T (=CECT 7386^T)	Mussels	Spain	EU669904	EU669901
F2076	Mussels	Spain	EU669905	EU669902
T234	Brackish water	Spain	FJ156092	FJ156093
A. nitrofigilis				
CECT 7204^T (=CCUG 15893^T)	Roots of *Spartina alterniflora*	Canada	L14627*	EU669895
LMG 7547	Root-associated sediment of *S. alterniflora*	Canada		
F2173	Mussels	Spain		
F2176	Mussels	Spain	EU106662*	EU669894
A. halophilus				
LA31B^T	Hypersaline lagoon	USA	AF513455*	EU669903
A. cibarius				
CECT 7203^T (=LMG 21996^T)	Poultry carcass	Belgium	AJ607391*	EU669892
LMG 21997	Poultry carcass	Belgium	AJ607392*	
Houf 746	Poultry carcass	Belgium		EU669893
A. cryaerophilus				
LMG 9904^T (=CCUG 17801^T)	Aborted bovine foetus	Ireland	L14624*	EU669899
LMG 9065	Aborted ovine foetus			
LMG 7537	Aborted ovine foetus			
LMG 10241	Aborted porcine foetus	Canada		
LMG 10229	Aborted porcine foetus	Canada		EU669900
CCUG 17802	Aborted bovine foetus	Ireland	AY314755*	
A. butzleri				
LMG 10828^T (=ATCC 49616^T)	Human faeces	USA	AY621116*	EU669890
F46	Pork meat	Spain		EU669891
CCUG 34397	Bovine	Argentina	DQ464343*	
A. skirrowii				
LMG 6621^T (=CCUG 10374^T)	Lamb faeces		L14625*	EU669897
Houf 989	Cow faeces	Belgium		EU669898
Campylobacter fetus				
ATCC 27374^T	Aborted ovine foetus		L04314*	DQ174184*

*Sequences retrieved from GenBank.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. & Figueras, M. J. (2009). *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. *Int J Syst Evol Microbiol* **59**, 1391–1396.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

4.6 *Arcobacter valdiviensis* sp. nov., isolated from a chicken fecal sample in Valdivia, Chile.

Collado, L., Fernández, H. & Figueras, M. J.

Submitted for publication

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Arcobacter valdiviensis sp. nov., isolated from a chicken cloacal swab sample in Valdivia, Chile

Luis Collado[1,2], Heriberto Fernández[2] & Maria José Figueras[1]

[1]Unitat de Microbiologia, Departament de Ciències Mediques Bàsiques, Facultat de Medicina i Ciències de la Salut. IISPV. Universitat Rovira i Virgili, Reus, Spain.
[2]Instituto de Microbiología Clínica, Facultad de Medicina, Universidad Austral de Chile, Valdivia, Chile.

Corresponding author:
Maria José Figueras
Departament de Ciències Mèdiques Bàsiques
Facultat de Medicina i Ciències de la Salut, IISPV
Universitat Rovira i Virgili
Sant Llorenç 21
43201 Reus (Spain)
Phone: 34-977759321
Fax: 34-977759322
E-mail: mariajose.figueras@urv.cat

ISBN: 978-84-693-0707-6 /DL:T-420-2010

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB* and *gyrB* genes of the strain FE2^T are GU300768, GU291973 and GU291957, respectively.

ABSTRACT

A Gram negative, slightly curved rod-shaped isolate (FE2^T), recovered from a chicken cloacal swab sample was subjected to a polyphasic taxonomic study. Phylogenetic analyses based on the 16S rRNA gene sequence indicated that this bacterium belongs to the genus *Arcobacter*, forming an independent phylogenetic line closely related to *Arcobacter skirrowii* and *Arcobacter cryaerophilus* with sequence similarities of 98.8% and 98.6%, respectively. This was further confirmed by a phylogenetic analysis based on the B-subunit of RNA polymerase (*rpoB*) and the B-subunit of DNA gyrase (*gyrB*) genes. The DNA-DNA relatedness values obtained comparing the new strain with type strains of the more related *Arcobacter* species were below the 70% threshold for species delineation. Phenotypically, the new isolate can be distinguished from all recognized *Arcobacter* species by its resistance to cefoperazone, its inability to reduce nitrates and its capacity to grow in media with 1% glycine.

The name *Arcobacter valdiviensis* sp. nov. is proposed, with the type strain FE2^T (= CECT XXX^T = LMG XXXX^T).

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

The genus *Arcobacter* includes bacteria previously known as aerotolerant campylobacters (Vandamme *et al*., 1991) and belongs to the family *Campylobacteraceae*, being originally defined with the species: *Arcobacter butzleri*, *Arcobacter cryaerophilus* (with two subgroups 1A and 1B), *Arcobacter skirrowii* and *Arcobacter nitrofigilis* (Vandamme *et al*., 1991, 1992). Since then, five new species have been described: *Arcobacter cibarius* (Houf *et al*., 2005), *Arcobacter halophilus* (Donachie *et al*., 2005), *Arcobacter mytili* (Collado *et al*., 2009a), *Arcobacter thereius* (Houf *et al*., 2009) and *Arcobacter marinus* (Kim *et al*., in press). Additionally, the *Candidatus* Arcobacter sulfidicus represents a possible new taxon but has not yet been formally described (Wirsen *et al*., 2002; Debruyne *et al*., 2008). The genus *Arcobacter* has become increasingly important over recent decades as an emergent enteropathogen and as a potential zoonotic agent (Ho *et al*. 2006). Poultry is considered one of the most important reservoirs of arcobacters and contamination of the animal carcasses during slaughter has been suggested as well as the capacity of these microorganisms to inhabit the chicken intestine (Ho *et al*., 2008 and reference there in). Of the recognized species, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius* and recently *A. thereius* have been reported from carcasses or meat from products of poultry and livestock (Debruyne *et al*., 2008; Collado *et al*., 2009b). All these species share a common association with animal and/or humans different from the rest of the species, which are generally considered free-living or environmental (Ho *et al*., 2006).

In the present study, a new *Arcobacter* isolate ($FE2^T$) recovered from a chicken cloacal swab sample was subjected to a polyphasic approach including a phylogenetic analysis by the 16SrRNA, *rpoB*, and *gyrB* genes, DNA–DNA hybridization and a phenotypic characterization, in order to determine its taxonomic position. Based on the reported findings, we proposed and describe this strain as a novel species within the genus *Arcobacter*.

During a study on the prevalence of *Arcobacter* in animal and human faecal samples carried out in the city of Valdivia, Southern Chile, in 2005 (Collado *et al*., unpublished data), an isolate (FE2) recovered from a chicken cloacal swab sample using the Arcobacter selective media and procedure described by Houf *et al*. (2001), was initially identified as *A. cryaerophilus* with a species-specific multiplex PCR (m-PCR) (Houf *et al*., 2000). However, this isolate showed a restriction pattern identical to *A. butzleri* with the Restriction Fragment Length Polymorphism (RFLP) *Arcobacter* spp. identification method

ISBN: 978-84-693-0707-6 /DL:T-420-2010

described by Figueras *et al*. (2008) and a pattern of *A. skirrowii* with the RFLP identification method of Marshall *et al*. (1999).

A nearly complete 16S rRNA gene sequence of strain FE2^T (1444 bp) as well as 524 bp of the *rpoB* gene were amplified and sequenced as described by Collado *et al*. (2009a). In the present study we also asses the taxonomic value of the *gyr*B gene (encoding the B-subunit of DNA gyrase) as a complementary phylogenetic and identification marker for the genus *Arcobacter*. For the *gyrB* gene primer design, a preliminary sequence (1100 bp) of all *Arcobacter* type strains was obtained with the degenerate primers UP1 and UP2r described by Yamamoto & Harayama (1995). On the basis of this sequence the primers gyrB-Arc-7F (5´-GTTTAYCAYTTTGAAGGTGG-3´) and gyrB-Arc-14R (5´-CTAGATTTTTCAACATTTAAAAT-3´) were designed, which enabled the amplification of a 722 bp fragment from the *gyrB* gene of *Arcobacter* species. Those primers were also used for sequencing. The percentage nucleotide substitutions for a continuous stretch of 665 bp, corresponding to approximately 29% of the coding region (672–1342 bp of the *Escherichia coli* numbering) was calculated from the obtained *gyrB* sequences using MEGA 4 software (Tamura *et al*., 2007). PCR amplifications were performed in a reaction mixture of 50 µl containing 1 µl of genomic DNA, 0.2 µM of each dNTP, 0.2 µM of each primer, 2 mM of MgCl2, 1 U Taq DNA polymerase (Invitrogen) and the buffer supplied with the enzyme. PCR conditions applied were 3 min at 94ºC followed by 35 cycles of 15 s at 94ºC, 30 s at 50ºC and 45 s at 72ºC, followed by 5 min at 72ºC. The PCR product was sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. The only species that could not be evaluated with the *rpoB* and *gyrB* genes was *A. marinus*, since the type strain of this species is not yet available from the Asian culture collections.

Alignment of the 16S rRNA, *rpoB* and *gyrB* gene sequences was performed with the CLUSTAL W program (Thompson *et al*., 1994). Phylogenetic trees were constructed with the MEGA 4 software (Tamura *et al*., 2007), by using the neighbour-joining method (Saitou & Nei, 1987) with Kimura's two-parameter calculation model (Kimura, 1980) and the stability of the groupings was estimated by bootstrap analysis (1000 replications). Similarity values between the 16S rRNA gene sequence of strain FE2^T with the type strains of all accepted arcobacters were calculated with the EzTaxon program (Chun *et al*., 2007). The values obtained between strain FE2^T and all the accepted *Arcobacter* species

ISBN: 978-84-693-0707-6 /DL:T-420-2010

ranged from 92.7 to 98.8%. Individual similarity obtained for this new strain with the different species was as follows: *A. skirrowii* (98.8%), *A. cryaerophilus* (98,6%), *A. thereius* (98.1%), *A. cibarius* (97.8%), *A. butzleri* (96.8%), *A. nitrofigilis* (94.0%), *A. mytili* (93.1%), *A. marinus* (92.9%), and *A. halophilus* (92.7%). The phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) clearly indicate that strain FE2^T is related to members of the genus *Arcobacter* and group within the same cluster as *A. thereius* and *A. skirrowii* with a low bootstrap value (58%). However, in the *rpoB* phylogenetic tree, this strain clusters with *A. cryaerophilus* (see Supplementary Fig. S1, available in IJSEM Online) and a close position with the latter species was also observed when the phylogeny was based on the *gyrB* gene (Supplementary Fig. S2). The concatenated phylogenetic tree constructed with *rpoB* and *gyrB* reinforced the position of FE2^T close to *A. cryaerophilus* and demonstrated that this strain represents a new phylogenetic line for the genus *Arcobacter* (Fig. 2).
The *gyrB* gene inter-species rate of nucleotide substitutions was over 9.5% while the intra-species variation ranged from 0.2 to 8.6%, being comparable to the values previously described for the *rpoB* gene in *Arcobacter* species (Collado *et al*. 2009a).

DNA-DNA hybridization (DDH) studies were performed between strain FE2^T and the type strains of the closest phylogenetic species that showed ≥ 97% 16S rRNA sequence similarity as recommended by Stackebrandt and Goebel (1994). DNA was prepared as described by Marmur *et al*. (1961). Hybridization reactions were carried out with the microplate method described by Ziemke *et al*. (1998) and Urdiain *et al*. (2008), using a Kinetic-QCL plate reader (Bio-Whitaker) for the fluorescence measurements. The hybridization temperature was 57°C. Values of DDH were determined at least three times for both direct and reciprocal reactions (e.g. A x B and B x A) for any given strain pair, as described previously (Alperi *et al*., 2009). The strain FE2^T showed a DDH values of 59% with *A. thereius* (LMG 24486^T), 58% with *A. cryaerophilus* (LMG 9904^T), 58% with *A. cibarius* (CECT 7203^T) and 45% with *A. skirrowii* (LMG 6621^T). All these values below 70% corroborates that FE2^T represents a novel species within the genus *Arcobacter*.

Phenotypic characterization of strain FE2^T was evaluated using over 20 tests including growth conditions described as useful in distinguishing the nine *Arcobacter* accepted species (Vandamme *et al*., 2005; On *et al*., 1996; Collado *et al*., 2009a). Type strains of all species except *A. marinus* were used as controls. Aerobic growth was evaluated at room temperature (18-22ºC), 30, 35 and 37ºC on BA for 72h. Microaerobic and anaerobic

ISBN: 978-84-693-0707-6 /DL:T-420-2010

growth were evaluated on BA at 30ºC for 72h using the GasPak™ Campy Container Systems (Becton Dickinson) and the AnaeroGen™ (Oxoid), respectively. Motility was observed in 48h cultures by examining wet mounts in broth no. 2 (Oxoid) by phase-contrast microscopy. The morphology, cell size, and presence of flagella of strain FE2^T were determined by Electron Microscopy (Supplementary Fig. S3) following procedures described previously (Collado *et al*., 2009a). Table 1 shows the most important phenotypic differential characteristics of strain FE2^T from the other *Arcobacter* species. The strain FE2^T showed resistance to cefoperazone (64 mg l^{-1}) a characteristic that differentiates this new candidate species from *A. halophilus*, *A. mytili* and *A. nitrofigilis*. The inability to reduce nitrate differentiates this strain from *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. thereius*. Finally FE2^T, can be discriminated from *A. cibarius* by its capacity to grow in media with 1% glycine. Under aerobic conditions, strain FE2^T grows well in BA from 18 to 30ºC (optimal growth) but a weak growth was observed at 35ºC. Although this strain was isolated from chicken cloacal samples it was unable to grow at 37ºC on BA under laboratory conditions. However, this problem has been described previously for *A. thereius* (Houf *et al*., 2009), and could be explained by the different conditions offered by the host organism when compare to those in culture media.

In conclusion, the present study demonstrates the existence of a novel *Arcobacter* species, for which we propose the name *Arcobacter valdiviensis* sp. nov.

Description of *Arcobacter valdiviensis* sp. nov.

Arcobacter valdiviensis (val.di.vien´sis. L. adj. valdiviensis referring to Valdivia, a Chilean city, where the type strain was isolated).

Cells are Gram-negative slightly curved rods, non-encapsulated, non-spore forming, and are 0.3-0.5 μm wide and 1.3-2 μm long. Non-spherical or coccoid cells were observed in old cultures. Motile by a single polar flagellum. Colonies on blood agar incubated in aerobic conditions at 30°C for 72h are 2mm in diameter, translucent to off-white, circular with entire margins and convex. No swarming on solid media was noted. Pigments are not produced. The strain grows on blood agar at room temperature (18-22°C), and at 30°C under aerobic or microaerobic culture conditions with no significant differences, but does not grow at 37°C. Weak growth was observed at 35ºC on aerobiosis and in anaerobic conditions at 30°C. No haemolysis is seen on blood agar. The strain showed oxidase and

ISBN: 978-84-693-0707-6 /DL:T-420-2010

catalase activity and hydrolysed indoxyl acetate. Urease is not produced and nitrate is not reduced. Hydrogen sulfide is not produced in triple-sugar iron agar medium. Under aerobic conditions the strain grows weakly at 30°C on MacConkey agar, on media containing 64 mg cefoperazone l^{-1} and on minimal medium. No growth was obtained on media containing 2.0-4.0 % (w/v) NaCl.

The type strain is FE2^T (=CECT XXXX^T =LMG XXXXX^T), isolated from a chicken cloacal swab sample in Valdivia, Chile.

Acknowledgements

This work was partially supported by FONDECYT Grants 1030245. This work was also supported by funds from the European Commission for the HEALTHY WATER project (FOOD-CT-2006-036306). The authors are solely responsible for the content of this publication. It does not represent the opinion of the European Commission. The European Commission is not responsible for any use that might be made of data appearing therein.

L. C. is indebted to Universitat Rovira i Virgili for his doctoral grant.

REFERENCES

Alperi, A., Martinez-Murcia, A. J., Ko, W. C., Monera, A., Saavedra, M. J. & Figueras, M. J. (2009). *Aeromonas taiwanensis* sp. nov. and *Aeromonas sanarellii* sp. nov., two new clinical species from Taiwan. Int J Syst Evol Microbiol (In press)

Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol **57**, 2259-2261.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. & Figueras, M. J. (2009a). *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. Int J Syst Evol Microbiol **59**, 1391-1396.

Collado, L., Guarro, J. & Figueras, M. J. (2009b). Prevalence of *Arcobacter* in meat and shellfish. J Food Prot **72**, 1102-1106.

Debruyne, L., Gevers, D. & Vandamme, P. (2008). Taxonomy of the Family *Campylobactereaceae*. In *Campylobacter*, 3rd edn. pp 3-25. Edited by Nachamkin, I., Szymanski, C. and Blaser, M. Washington, DC: ASM press.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Donachie, S. P., Bowman, J. P., On, S. L. & Alam, M. (2005). *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. Int J Syst Evol Microbiol **55**, 1271-1277.

Figueras, M. J., Collado, L. & Guarro, J. (2008). A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. Diagn Microbiol Infect Dis **62**, 11-15.

Ho, H. T., Lipman, L. J. & Gaastra, W. (2008). The introduction of *Arcobacter* spp. in poultry slaughterhouses. Int J Food Microbiol **125**, 223-229.

Ho, H. T., Lipman, L. J. & Gaastra, W. (2006). *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent! Vet Microbiol **115**, 1-13.

Houf, K., Devriese, L. A., De Zutter, L., Van Hoof, J. & Vandamme, P. (2001). Development of a new protocol for the isolation and quantification of *Arcobacter* species from poultry products. Int J Food Microbiol **71**, 189-196.

Houf, K., On, S., Coenye, T., Debruyne, L., De Smet, S. & Vandamme, P. (2009). *Arcobacter thereius* sp. nov., isolated from pigs and ducks. Int J Syst Evol Microbiol **59**, 2599-2604.

Houf, K., On, S. L., Coenye, T., Mast, J., Van Hoof, J. & Vandamme, P. (2005). *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. Int J Syst Evol Microbiol **55**, 713-717.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J. & Vandamme, P. (2000). Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiol Lett **193**, 89-94.

Kim, H. M., Hwang, C. Y. & Cho, B. C. *Arcobacter marinus* sp. nov. Int J Syst Evol Microbiol (In press).

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol **16**, 111-120.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms, J Mol Biol **3**, 208-218.

Marshall, S. M., Melito, P. L., Woodward, D. L., Johnson, W. M., Rodgers, F. G. & Mulvey, M. R. (1999). Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. J Clin Microbiol **37**, 4158-4160.

On, S. L., Holmes, B. & Sackin, M. J. (1996). A probability matrix for the identification of campylobacters, helicobacters and allied taxa. J Appl Bacteriol **81**, 425-432.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4**, 406-425.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociations and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol **44**, 846-849

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24**, 1596-1599.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22**, 4673-4680 4673-4680 .

Urdiain, M., Lopez-Lopez, A., Gonzalo, C., Busse, H. J., Langer, S., Kampfer, P. & Rossello-Mora, R. (2008). Reclassification of *Rhodobium marinum* and *Rhodobium pfennigii* as *Afifella marina* gen. nov. comb. nov. and *Afifella pfennigii* comb. nov., a new genus of photoheterotrophic *Alphaproteobacteria* and emended descriptions of *Rhodobium*, *Rhodobium orientis* and *Rhodobium gokarnense*. Syst Appl Microbiol **31**, 339-351.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. & De Ley, J. (1991). Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. Int J Syst Bacteriol **41**, 88-103.

Vandamme, P., Vancanneyt, M., Pot, B.& other authors (1992). Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. Int J Syst Bacteriol **42**, 344-356.

Vandamme, P., Dewhirst, F. E., Paster, B. J. & On, S. L. W. (2005). Genus II. *Arcobacter* Vandamme, Falsen, Rossau, Segers, Tytgat and De Ley 1991a, 99^{VP}. In Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 2, pp. 1161–1165. Edited by D. J. Brenner, N. P. Kreig, J. T. Staley & G. M. Garrity. New York: Springer.

Wirsen, C. O., Sievert, S. M., Cavanaugh, C. M., Molyneaux, S. J., Ahmad, A., Taylor, L. T., DeLong, E. F. & Taylor, C. D. (2002). Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. Appl Environ Microbiol **68**, 316-325.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Yamamoto, S. & Harayama, S. (1995). PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl Environ Microbiol **61**, 1104-1109.

Ziemke, F., Hofle, M. G., Lalucat, J. & Rossello-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. Int J Syst Bacteriol **48**, 179-186

ISBN: 978-84-693-0707-6 /DL:T-420-2010

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

Arcobacter species: 1, *A. valdiviensis* (*n*=1) (data from this study); 2, *A. cryaerophilus* (*n*=19); 3, *A. skirrowii* (*n*=9); 4, *A. cibarius* (*n*=15); 5, *A. thereius* (*n*=8); 6*, A. butzleri* (*n*=12); 7, *A. halophilus* (*n*=1); 8, *A. nitrofigilis* (*n*=4); 9 *A. mytili* (*n*=3); 10, *A. marinus* (*n*=1). Data taken from previous studies (On *et al*., 1996; Donachie *et al*., 2005; Houf *et al*., 2005, 2009, Collado *et al*., 2009a and Kim *et al*., 2009) unless otherwise indicated. +, ≥ 95% strains positive; -, ≤11% strains positive; V, 12-94% strains positive.

Characteristic	1	2	3	4	5	6	7	8	9	10
Catalase activity	+	+	+	V	+	V	-	+	+	-
Urease activity	-	-	-	-	-	-	-	+	-	-
Nitrate reduction	-	+[a]	+	-	+	+	+	+	-	+
Indoxyl acetate hydrolysis	+	+	+	+	+	+	+	+	-	+
Growth in:										
Air at 37°C	-	V	+	-	-	+	+	V	+	+
CO_2 at 37ºC	-	V	+	+	-	+	+	-	+	+
4% (W/V) NaCl	-	-	+	-	-	-	+	+	+	+
Growth on:										
Media w/1% glycine	+	-	-	-	+	-	-	-	+	ND
Mac Conkey agar	+	V	-	+	V	+	-	-	+	ND
Minimal media	+	-[b]	-	+	+	+	-	-	-	ND
Resistence to:										
Cefoperazone (64mg l^{-1})	+	+	+	+	+	+	-	-	-	ND

ND, not determined; CO_2 indicates microaerobic conditions.
[a]Two (LMG 9904T and LMG 9065) of the four strains tested in Collado *et al*. (2009a) were negative.
[b]Two (LMG 7537 and LMG 10241) of the four strains tested in Collado *et al*. (2009a) were positive.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

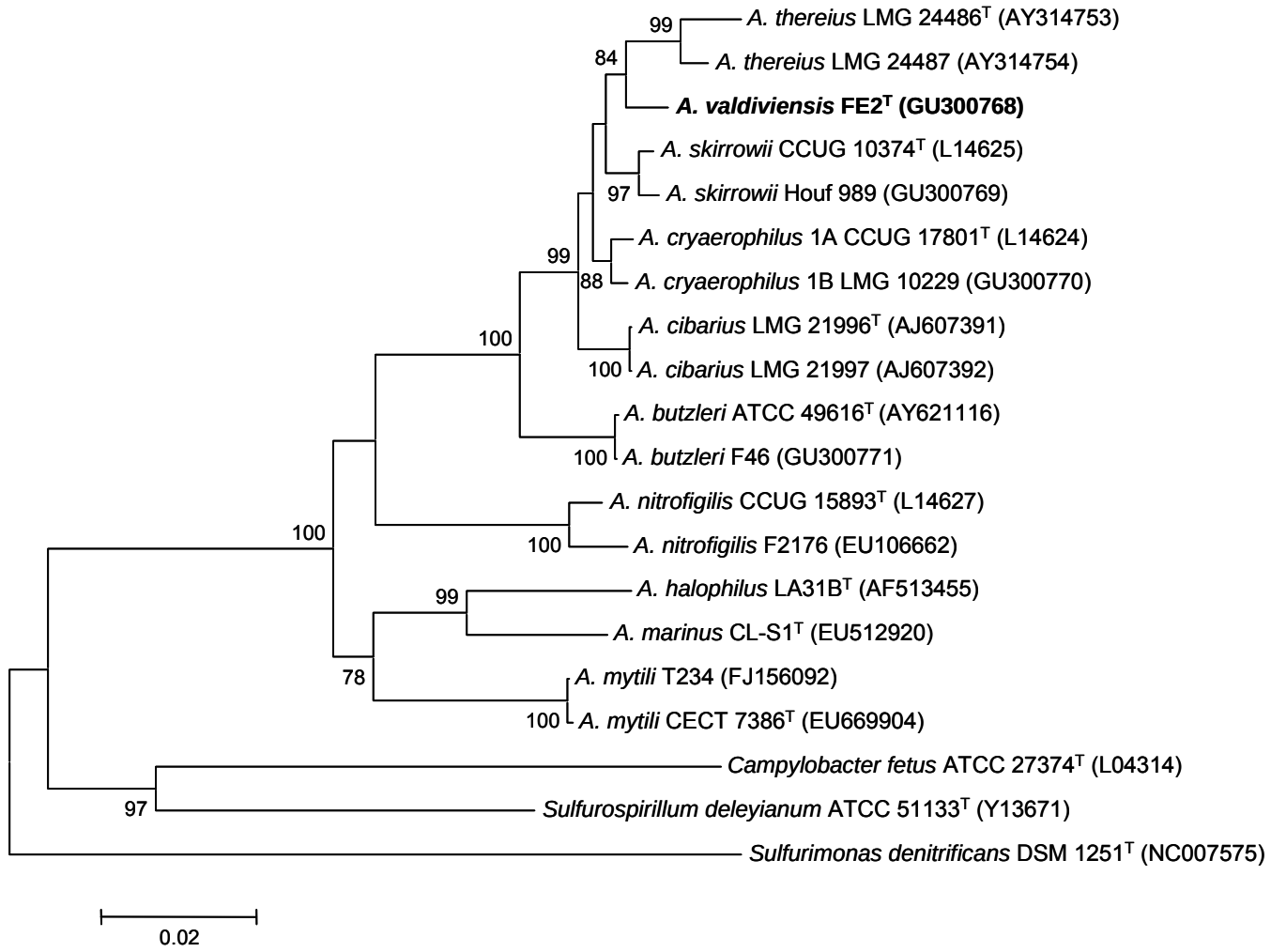


Fig. 1. Neighbour-joining tree based on 16S rRNA sequences (1405bp) showing the phylogenetic position of *Arcobacter valdiviensis* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

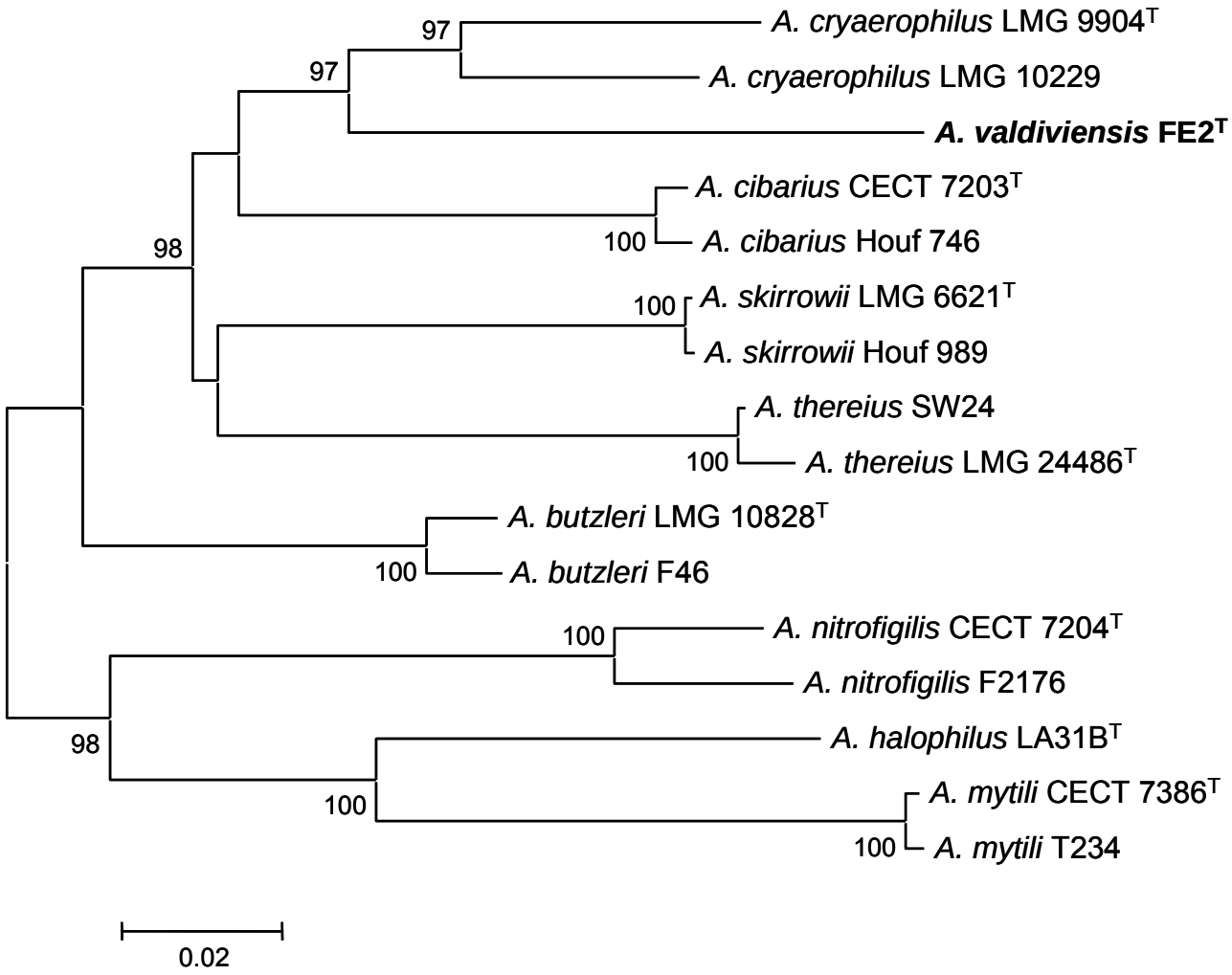
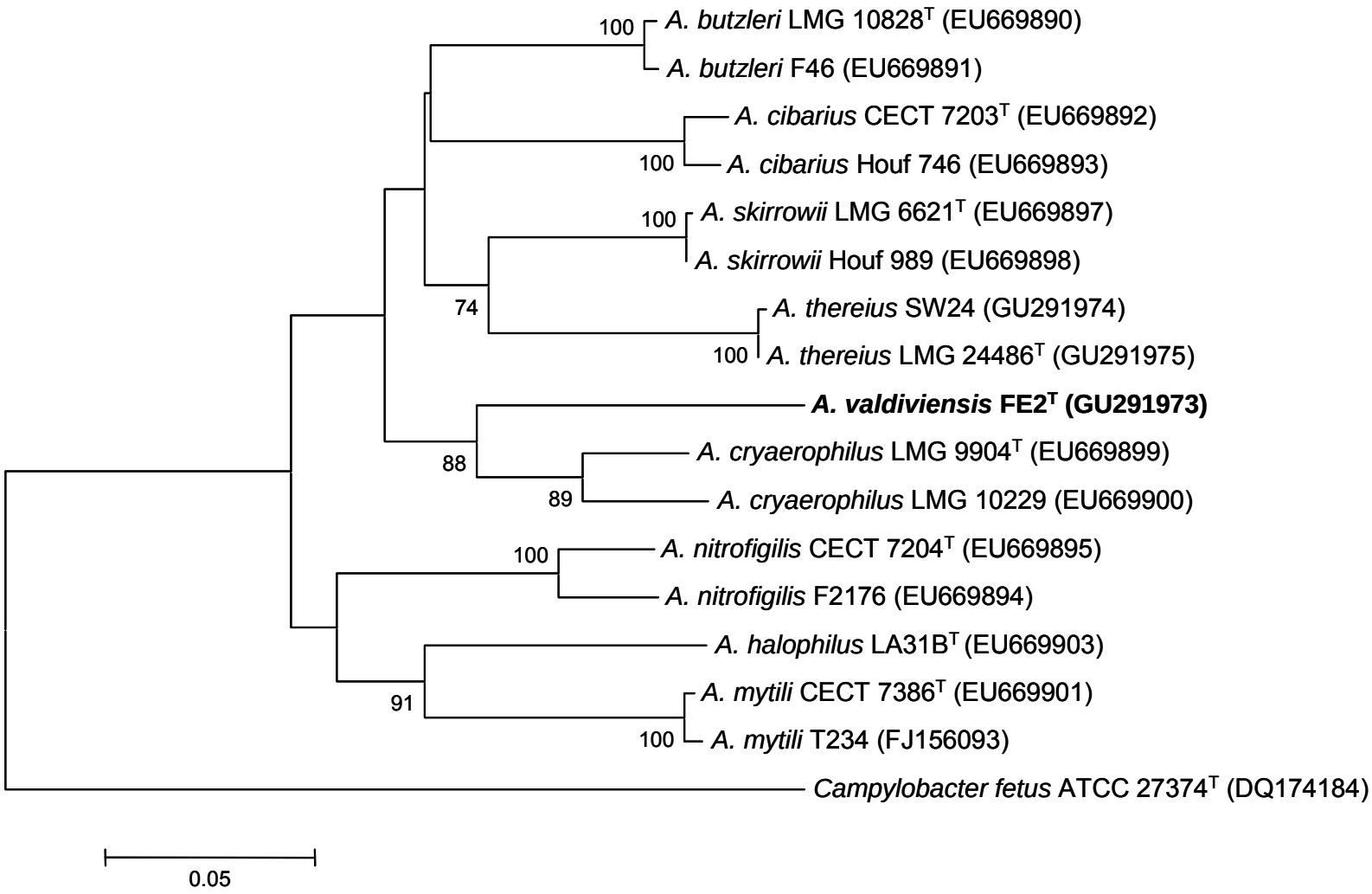


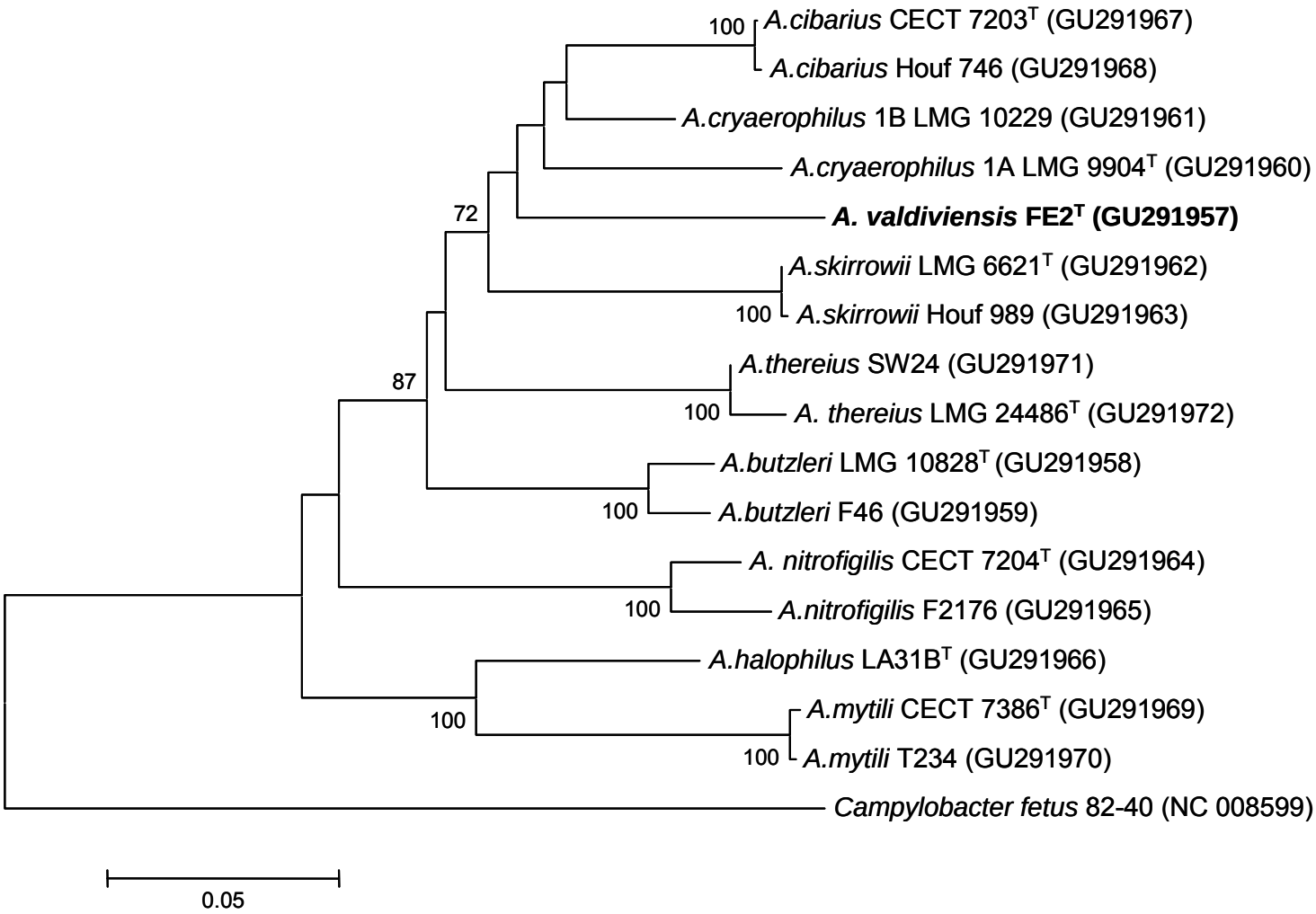
Fig. 2. Neighbour-joining tree based on the concatenated *rpoB* (487bp) and *gyrB* (665bp) sequences showing the phylogenetic position of *Arcobacter valdiviensis* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010



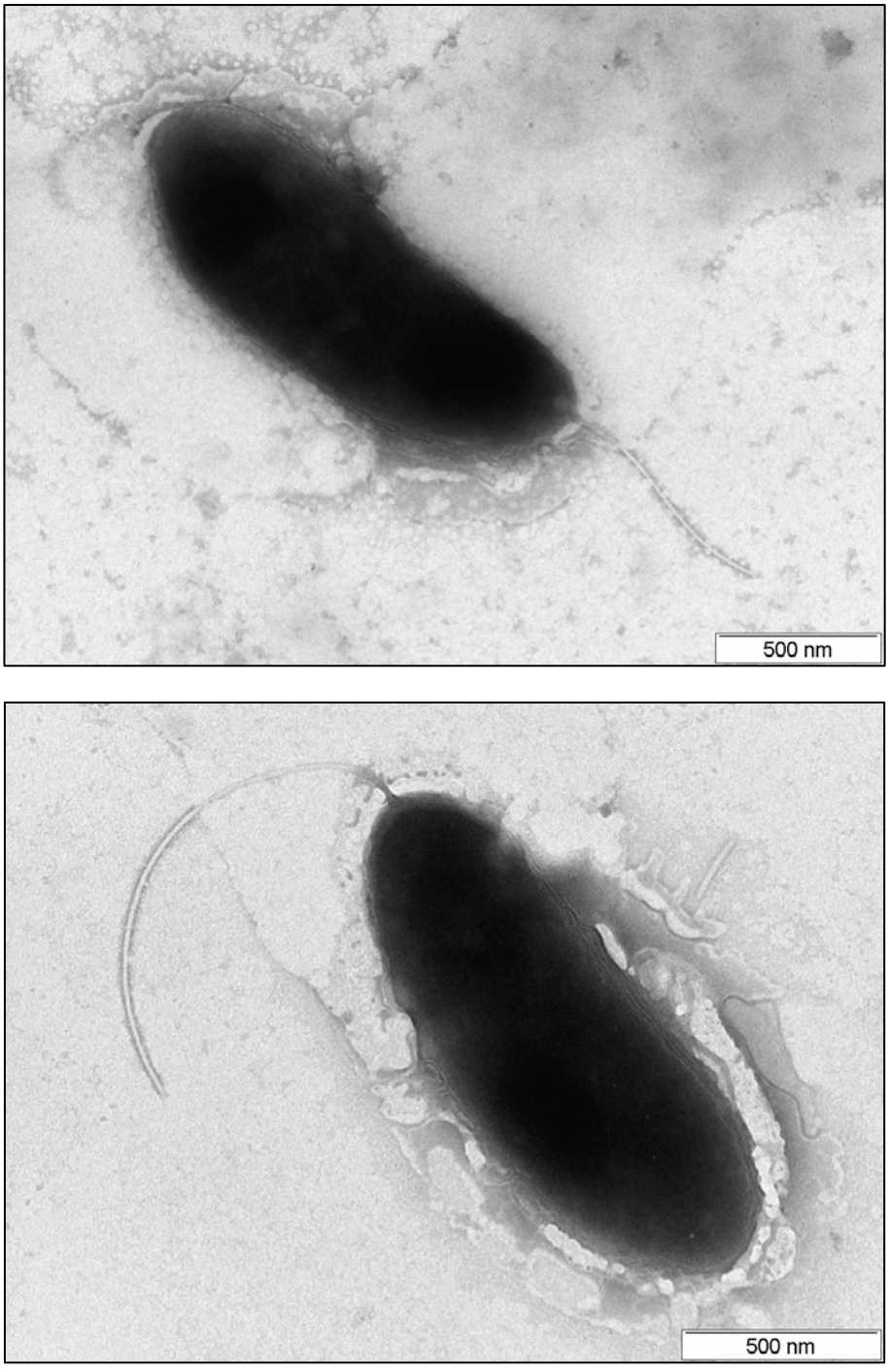
Supplementary Fig. S1. Neighbour-joining tree based on *rpoB* sequences (487bp) showing the phylogenetic position of *Arcobacter valdiviensis* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 100 nt.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010



Supplementary Fig. S2. Neighbour-joining tree based on *gyrB* sequences (665bp) showing the phylogenetic position of *Arcobacter valdiviensis* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 100 nt.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010



Supplementary Fig. S3. Images of cells of strain FE2^T as observed with transmission electron microscopy, negatively stained.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

4.7 Description of two new species, *Arcobacter defluvii* sp. nov., isolated from sewage and *Arcobacter molluscorum* sp. nov., isolated from shellfish

Collado, L., Levican, A., Perez, J. & Figueras, M. J.

In preparation

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Description of two new species, *Arcobacter defluvii* sp. nov., isolated from sewage and *Arcobacter molluscorum* sp. nov., isolated from shellfish

Luis Collado, Arturo Levican, Jessica Perez & Maria José Figueras

Unitat de Microbiologia, Departament de Ciències Mediques Bàsiques, Facultat de Medicina i Ciències de la Salut. IISPV. Universitat Rovira i Virgili, Reus, Spain.

Corresponding author:
Maria José Figueras
Departament de Ciències Mèdiques Bàsiques
Facultat de Medicina i Ciències de la Salut, IISPV
Universitat Rovira i Virgili
Sant Llorenç 21
43201 Reus (Spain)
Phone: 34-977759321
Fax: 34-977759322
E-mail: mariajose.figueras@urv.cat

ISBN: 978-84-693-0707-6 /DL:T-420-2010

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene of the strains SW28-11^T and F98-2^T are XXXX and XXXX, those for the *rpoB* gene sequence are XXXX and XXXX and those for the *gyrB* gene sequence are XXXX and XXXX, respectively.

ABSTRACT

A polyphasic study was undertaken to clarify the taxonomic position of 6 isolates recovered from sewage and 17 recovered from shellfish samples. The two groups of isolates were recognized as two potential novel species because each group produced a new and specific pattern using the 16S rDNA-RFLP *Arcobacter* identification method. The analysis of 16S rDNA data not only supported the classification of these strains in the genus *Arcobacter* but also showed that they formed two separate phylogenetic lines. The strain SW28-11^T representative of the isolates recovered from sewage showed a 16S rRNA sequence similarity of 95.6% with respect to the closest species *Arcobacter nitrofigilis*. Strain F98-2^T representative of the strains recovered from shellfish showed 97.6% similarity with *Arcobacter marinus*, the closest related species. The phylogenetic position of those strains was further evaluated with the analysis of the housekeeping genes *rpoB* and *gyrB*. This data together with the phenotypic characterization and genotyping with ERIC-PCR revealed that these two groups of isolates are novel *Arcobacter* species represented by 6 and 3 different strains. The names *Arcobacter defluvii* sp. nov., is proposed for the sewage strains while *Arcobacter molluscorum* sp. nov. is proposed for the shellfish strains, with the type strains SW28-11^T (=CECT XXX^T =LMG XXX^T) and F98-2^T (=CECT XXX^T =LMG XXX^T), respectively.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

The genus *Arcobacter* is an unusual taxon within the *Epsilonproteobacteria* in that it contains both pathogenic and free-living species found in a wide range of environments (Debruyne *et al*., 2008). At the time of writing, this genus comprises ten established species: *Arcobacter butzleri*, *Arcobacter cryaerophilus* (with two DNA groups 1A and 1B), *Arcobacter skirrowii*, *Arcobacter nitrofigilis* (Vandamme *et al*., 1991, 1992), *Arcobacter cibarius* (Houf *et al*., 2005)*, Arcobacter halophilus* (Donachie *et al*., 2005), and the recently described species *Arcobacter mytili* (Collado *et al*., 2009a), *Arcobacter thereius* (Houf et al., 2009), *Arcobacter marinus* (Kim *et al*., in press) and *Arcobacter valdiviensis* (Collado *et al*., submitted). The *Candidatus* Arcobacter sulfidicus is a potentially novel species that has not yet been formally described (Wirsen *et al*., 2002: Debruyne *et al*. 2008).

The species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are associated with gastrointestinal disease and bacteremia in humans and with reproduction disorders, mastitis and gastric ulcers in farm animals (Ho *et al*., 2006). Those species can also be recovered from faeces of healthy livestock and humans (Vandamme *et al*. 2005). *A. thereius* was isolated from porcine abortion, although the pathogenic role of this species is still unknown (Houf *et al*. 2009). The other species have never been directly related to animal or human diseases (Collado and Figueras. Review in preparation).

In two surveys carried out in parallel in Catalonia (north-east Spain), 2 groups of isolates recovered from sewage and shellfish samples respectively were subjected to a polyphasic approach including genotyping by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), phylogenetic analysis of the 16S rRNA, *rpoB* and *gyrB* gene sequences, and phenotypic characterization. Based on the obtained results, we propose allocating these two groups of isolates into two novel *Arcobacter* species.

The isolates SW28-9, SW28-10, SW28-11^T, SW29-3, SW30-3 and SW31-1 were recovered from a sewage treatment plant in the city of Reus (northeast Spain) in April 2009. Furthermore, the Isolates F80, F82-1, F82-2, F83-1, F90-1, F90-2, F90-4, F90-5, F90-6, F91, F92, F98-2^T, F98-3, F98-4 and F99-2 were recovered from mussel samples while the isolates F101-1 and F101-2 were recovered from oysters. All the shellfish were harvested from a shellfish farm at the Ebro River Delta from May to September 2009. Table 1 summarizes the date and type of sample as well as the isolation culture conditions for all the isolates. The isolation methodology for the sewage and mussel samples was the same as previously described by Collado *et al*. (2008, 2009b). However, the sewage samples were isolated this time both in parallel by direct plating and after enrichment. The

ISBN: 978-84-693-0707-6 /DL:T-420-2010

only variation in relation to the protocol for shellfish was that the samples after enrichment and filtration on the blood agar plates were incubated in parallel under aerobic and microaerobic conditions. The new isolates showed the typical *Arcobacter* colony morphology in blood agar i.e., small size, colourless and translucent. All isolates were Gram-negative slightly curved rods under light microscopic examination. The two groups of isolates were recognized as two potential new species because they showed new restriction patterns (Fig. 1) from those described for the rest of species of the genus with 16S rDNA-RFLP *Arcobacter* identification method (Figueras *et al*., 2008). With the multiplex PCR (m-PCR) described by Houf *et al*. (2000) the mussel isolates produced a double amplification product with a size specific to *A. skirrowii* (257 bp) and *A. cryaerophilus* (641 bp), while only two sewage isolates (SW28-9 and SW28-10) produced an amplicon of 257 and 401 bp, respectively (data not shown) with the m-PCR.

To avoid studying duplicate isolates of the same strain, all isolates were genotyped with ERIC-PCR using primers and conditions described previously (Houf *et al*. 2002). DNA patterns that differed in one or more DNA-fragments were considered different genotypes (Houf *et al*., 2002). This typing method generated different patterns for all sewage isolates (Fig. 2). From the 17 shellfish isolates it was possible to recognize 3 different genotypes. Interestingly as showed in Fig. 2, the isolates recovered from oysters showed an identical ERIC-PCR pattern than mussel isolates (F82-1, F83-1, F90-1 and F90-5). Therefore were choose 3 representatives strains, each of them, with different genotypes (F83-1, F91 and F98-2) and 1 isolate from oysters (F101-1) for further molecular and phenotypic characterization.

In order to establish their taxonomic position, the 16S rRNA, *rpoB* and *gyrB* genes were sequenced as described by Collado *et al*. (2009a, submitted). The obtained 16S rRNA gene sequences were compared with public database using EzTAxon tool (Chung *et al*., 2007). The sequence of SW28-11^T representative of the sewage strains showed a 95.6% similarity with the type strain of *A. nitrofigilis* while F98-2^T strain from shellfish showed a 97.6% with *A. marinus*. Phylogenetic analyses were performed using the MEGA (Molecular Evolutionary Genetics Analysis) software version 4 (Tamura *et al*., 2007) after multiple alignment of sequences by CLUSTAL W. Distances were calculated according to the Kimura two-parameter model (Kimura 1980) and clustering with the neighbour-joining method (Saito & Nei 1987). The 16S rRNA gene phylogenetic trees (Fig. 4) showed the sewage strains (SW28-11 and SW31-1) and the shellfish strains (F83-1, F91 and F98-2)

ISBN: 978-84-693-0707-6 /DL:T-420-2010

as independent phylogenetic lines within the genus *Arcobacter*. Strains SW28-11^T showed a 16S rRNA sequence similarity values ranged between 91.8 to 95.6% while the strain F98-2^T showed similarity values ranging from 92.4 to 97.6%. A low level of sequence similarity (95%) was found between SW28-11 and F98-2. The phylogenetic trees constructed with the housekeeping genes revealed a similar branching than 16S rRNA gene (Fig. 5 and 6).

The procedures followed for phenotypic characterization were performed by using the recommended media and methods described previously (On *et al*., 1996; Vandamme *et al*. 2005). Motility was observed in young cultures by examining wet mounts in broth by phase-contrast microscopy. The cell size, morphology and presence of flagella were determined with electron microscopy following procedures described in a previous study (Collado *et al*., 2009a). Phenotypically the two new strains could be differentiated from other *Arcobacter* species with a combination of biochemical test, which are detailed in Table 2.

The strain F98-2^T from mussels showed a similarity of the 16S rRNA gene of 97.6% with *A. marinus* (CL-S1^T) that could justify the need for DNA-DNA hybridization among these strains according to Stackebrandt & Goebel (1994). However, it was not possible those experiments because the type strain of *A. marinus* it is not yet available from the culture collections due to the fact that the study is still in press. Despite that the phylogeny derived from 16S rRNA gene clearly differentiates theses species as independent phylogenetic lines (Fig. 3). Moreover, this two species show different biochemical response to catalase, reduction of nitrate and hydrolysis of indoxyl acetate test. Furthermore, the mussels strains can also be differentiated from *A. marinus* by 16S rDNA-RFLP pattern after an informatic simulation.

In conclusion, on the basis of the phylogenetic relationships and phenotypic characteristics, this study revealed the existence of two novel *Arcobacter* species, *A. defluvii* (type strain CECT XXX, LMG XXX) and *A. molluscorum* (type strain CECT XXX, LMG XXXX). The 16S rDNA-RFLP identification method (Figueras *et al*., 2008), is a reliable and fast technique for the characterization of this two new *Arcobacter* species giving novel species-specific digestion patterns of (407/243/141/138 bp) for *A. defluvii*, and (551/141/138/72 bp) for *A. molluscorum* as shown in Fig. 1.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Description of *Arcobacter defluvii* sp. nov.

Arcobacter defluvii (*de.flu´vi.i*. L. neut. n. *defluvium* sewage; L. gen. n. *defluvii* of sewage.). Cells are Gram-negative slightly curved rods, in S-shape, non-encapsulated, non-spore forming, and are 0.3-0.5 μm wide and 1-2 μm long. Motile by a single polar flagellum. Colonies on blood agar incubated in aerobic conditions at 30°C for 48h are 2 - 4 mm in diameter, beige to off-white, circular with entire margins, convex, and non-swarming. Pigments are not produced. All the strains grow both on blood agar at room temperature (18-22°C), 30°C and 37°C under aerobic or microaerobic culture conditions with no significant differences. Weak growth is obtained in anaerobic conditions at 30°C and in aerobic conditions at 42°C. No haemolysis is seen on blood agar. Strains produce oxidase and show a weak catalase activity and hydrolyse indoxyl acetate. Urease is produced and nitrate is reduced. Hydrogen sulfide is not produced in triple-sugar iron agar medium. Under aerobic conditions all the strains grow at 30°C on MacConkey agar and on minimal medium. A 67% of strains growth on media containing 64 mg cefoperazone l^{-1}. No growth is obtained on media containing 2.0-4.0 % (w/v) NaCl or 1% of glycine.

The type strain is SW28-11^T (=CECT XXXX^T =LMG XXXXX^T), isolated from raw sewage waste water from the treatment plant of the city of Reus, Spain. *A. defluvii* strains SW29-3 and SW31-1 have been deposited in the Spanish culture collection (CECT).

Description of *Arcobacter molluscorum* sp. nov.

Arcobacter molluscorum (mol.lus.co´rum. N.L pl. n. *Mollusca* a zoological phylum; N.L. gen. Pl. n. *molluscorum* of molluscs classified in the phylum *Mollusca*).

Cells are Gram-negative, slightly curved rods, non-encapsulated, non-spore forming, and are 0.3-0.5 μm wide and 1-3.4 μm long, some cells form filaments up to 7 μm long. Motile by a single polar flagellum. Colonies on blood agar incubated in aerobic conditions at 30°C for 48h are 2 - 4 mm in diameter, beige to off-white, circular with entire margins, convex, and non-swarming. Pigments are not produced. All the strains grow both on blood agar at room temperature (18-22°C), 30°C and 37°C under aerobic or microaerobic culture conditions with no significant differences. Weak growth is obtained in anaerobic conditions at 30°C and in aerobic conditions at 42°C. No haemolysis is seen on blood agar. Strains produce oxidase and catalase activity but not hydrolyse indoxyl acetate. Nitrate is reduced but urease is not produced. Hydrogen sulfide is not produced in triple-sugar iron agar

ISBN: 978-84-693-0707-6 /DL:T-420-2010

medium. Under aerobic conditions all the strains grow at 30°C on MacConkey agar and on media containing 2.0-4.0 % (w/v) NaCl or media with 64 mg cefoperazone l^{-1}. No growth is obtained on minimal medium and media containing 1% glycine.

The type strain is F98-3^T (=CECT XXXX^T =LMG XXXXX^T), isolated from a mussles sample from the Ebro River Delta, Spain. *A. molluscorum* strains F99-2 and F101-1 have been deposited in the Spanish culture collection (CECT).

REFERENCES

Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol **57**, 2259-2261.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. & Figueras, M. J. (2009a). *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. Int J Syst Evol Microbiol **59**, 1391-1396.

Collado, L., Fernandez, H., & Figueras, M. J. *Arcobacter valdiviensis* sp. nov. isolated from a chicken cloacal swab in Valdivia, Chile. Int J Syst Evol Microbiol (Submitted.)

Collado, L., & Figueras, M. J. The genus *Arcobacter*: A review of its taxonomy, epidemiology and importante (Submitted).

Collado, L., Guarro, J. & Figueras, M. J. (2009b). Prevalence of *Arcobacter* in meat and shellfish. J Food Prot **72**, 1102-1106.

Collado, L., Inza, I., Guarro, J. & Figueras, M. J. (2008). Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. Environ Microbiol **10,** 1635–1640.

Debruyne, L., Gevers, D. and Vandamme, P. (2008). Taxonomy of the Family *Campylobactereaceae*. In: Campylobacter (Third edition) eds Nachamkin, I., Szymanski, C. and Blaser, M. pp 3-25. Washington, DC: ASM press.

Donachie, S. P., Bowman, J. P., On, S. L. & Alam, M. (2005). *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. Int J Syst Evol Microbiol **55**, 1271-1277.

Figueras, M. J., Collado, L. & Guarro, J. (2008). A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. Diagn Microbiol Infect Dis **62**, 11-15.

Ho, H. T., Lipman, L. J. & Gaastra, W. (2006). *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent! Vet Microbiol **115**, 1-13.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Houf, K., On, S., Coenye, T., Debruyne, L., De Smet, S. & Vandamme, P. (2009). *Arcobacter thereius* sp. nov., isolated from pigs and ducks. Int J Syst Evol Microbiol **59**, 2599-2604.

Houf, K., On, S. L., Coenye, T., Mast, J., Van Hoof, J. & Vandamme, P. (2005). *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. Int J Syst Evol Microbiol **55**, 713-717.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J. & Vandamme, P. (2000). Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiol Lett **193**, 89-94.

Houf, K., De Zutter, L., Van Hoof, J. & Vandamme, P. (2002). Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. Appl Environ Microbiol **68**, 2172-2178.

Kim, H. M., Hwang, C. Y. & Cho, B. C. (2009). *Arcobacter marinus* sp. nov. Int J Syst Evol Microbiol. (In press).

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol **16**, 111-120.

On, S. L., Holmes, B. & Sackin, M. J. (1996). A probability matrix for the identification of campylobacters, helicobacters and allied taxa. J Appl Bacteriol **81**, 425-432.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4**, 406-425.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24**, 1596-1599.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. & De Ley, J. (1991). Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. Int J Syst Bacteriol **41**, 88-103.

Vandamme, P., Vancanneyt, M., Pot, B.& other authors (1992). Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. Int J Syst Bacteriol **42**, 344-356.

Vandamme, P., Dewhirst, F. E., Paster, B. J. & On, S. L. W. (2005). Genus II. *Arcobacter* Vandamme, Falsen, Rossau, Segers, Tytgat and De Ley 1991a, 99VP. In Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 2, pp. 1161–1165. Edited by D. J. Brenner, N. P. Kreig, J. T. Staley & G. M. Garrity. New York: Springer.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Wirsen, C. O., Sievert, S. M., Cavanaugh, C. M., Molyneaux, S. J., Ahmad, A., Taylor, L. T., DeLong, E. F. & Taylor, C. D. (2002). Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. Appl Environ Microbiol **68**, 316-325.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 1. Features of all isolates recovered from sewage and shellfish samples

Isolate	Sample	Isolation date (month/day/year)	Isolation procedure	Proposed species
F80	Mussels	5/19/2009	Aerobic conditions	*A. molluscorum*
F82-1	Mussels	6/23/2009	Aerobic conditions	*A. molluscorum*
F82-2	Mussels	7/7/2009	Microaerobic conditions	*A. molluscorum*
F83-1	Mussels	7/8/2009	Microaerobic conditions	*A. molluscorum*
F90-1	Mussels	7/8/2009	Aerobic conditions	*A. molluscorum*
F90-2	Mussels	7/8/2009	Aerobic conditions	*A. molluscorum*
F90-4	Mussels	7/8/2009	Microaerobic conditions	*A. molluscorum*
F90-5	Mussels	7/8/2009	Microaerobic conditions	*A. molluscorum*
F90-6	Mussels	7/8/2009	Aerobic conditions	*A. molluscorum*
F91	Mussels	7/14/2009	Aerobic conditions	*A. molluscorum*
F92	Mussels	7/14/2009	Aerobic conditions	*A. molluscorum*
F98-2	Mussels	9/1/2009	Aerobic conditions	*A. molluscorum*
F98-3	Mussels	9/1/2009	Aerobic conditions	*A. molluscorum*
F98-4	Mussels	9/1/2009	Aerobic conditions	*A. molluscorum*
F99-2	Mussels	9/8/2009	Aerobic conditions	*A. molluscorum*
F101-1	Oysters	9/29/2009	Microaerobic conditions	*A. molluscorum*
F101-2	Oysters	9/29/2009	Aerobic conditions	*A. molluscorum*
SW28-9	Sewage	4/14/2009	After enrichment	*A. defluvii*
SW28-10	Sewage	4/14/2009	After enrichment	*A. defluvii*
SW28-11	Sewage	4/14/2009	After enrichment	*A. defluvii*
SW29-3	Sewage	4/14/2009	Direct	*A. defluvii*
SW30-3	Sewage	4/14/2009	Direct	*A. defluvii*
SW31-1	Sewage	4/14/2009	After enrichment	*A. defluvii*

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 2. Differential characteristics of *Arcobacter defluvii* sp nov., and *Arcobacter molluscorum* sp. nov., and other members of the genus.

Arcobacter species: 1, *A. defluvii* (n=6); 2, *A. molluscorum* (n=3) (data from this study); 3, *A. valdiviensis* (*n*=1); 4, *A. cryaerophilus* (*n*=19); 5, *A. skirrowii* (*n*=9); 6, *A. cibarius* (*n*=15); 7, *A. thereius* (*n*=8); 8, *A. butzleri* (*n*=12); 9, *A. halophilus* (*n*=1); 10, *A. nitrofigilis* (*n*=4); 11, *A. mytili* (*n*=3); 12, *A. marinus* (*n*=1). Data from On *et al*., 1996; Donachie *et al*., 2005; Houf *et al*., 2005, 2009, Collado *et al*., 2009a, submitted and Kim *et al*., 2009.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Catalase activity	+	+	+	+	+	V	+	V	-	+	+	-
Urease activity	+	-	-	-	-	-	-	-	-	+	-	-
Nitrate reduction	+	-	-	+	+	-	+	+	+	+	-	+
Indoxyl acetate hydrolysis	+	-	+	+	+	+	+	+	+	+	-	+
Growth in:												
Air at 37°C	+	+	-	V	+	-	-	+	+	V	+	+
CO_2 at 37ºC				V	+	+	-	+	+	-	+	+
4% (W/V) NaCl	-	+	-	-	+	-	-	-	+	+	+	+
Growth on:												
Media w/1% glycine	-	-	+	-	-	-	+	-	-	-	+	ND
Mac Conkey agar	+	+	+	V	-	+	V	+	-	-	+	ND
Minimal media	+	-	-	-	-	+	+	+	-	-	-	ND
Resistence to:												
Cefoperazone (64mg l^{-1})	V	+	+	+	+	+	+	+	-	-	-	ND

Symbols: +, ≥ 95% strains positive; -, ≤11% strains positive; V, 12-94% strains positive.
ND, not determined; CO_2 indicates microaerobic conditions.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

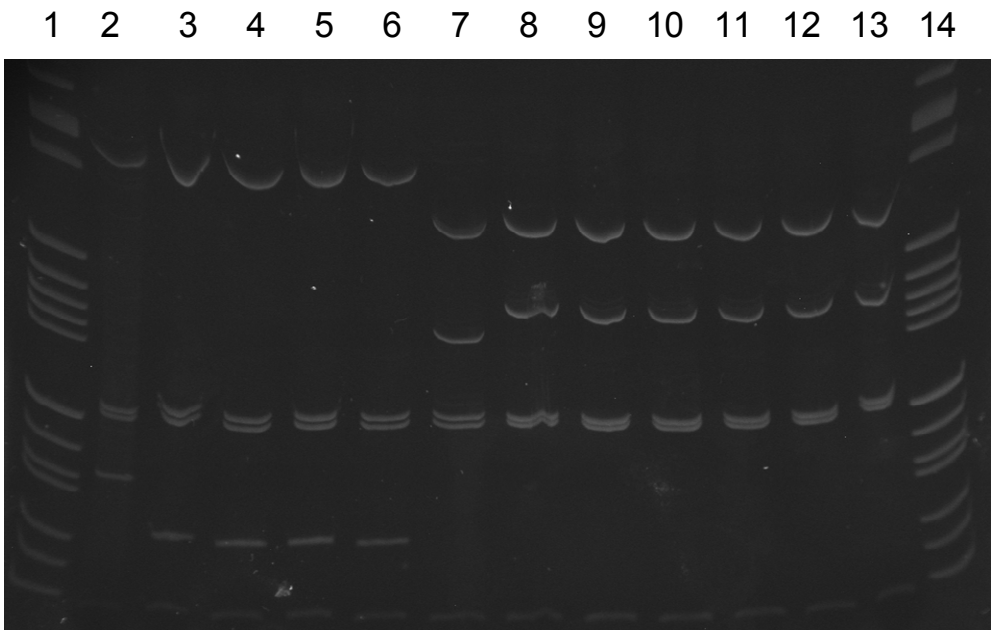


Fig. 1 Polyacrylamide gel showing the 16S rDNA-RFLP patterns, Lanes: 1 and 14 pBR322 DNA/*Bsu*RI (*Hae*III) ladder (Fermentas); 2, *A. halophilus* (LA31B^T); 3, F83-1; 4, F91; 5, F98-2; 6, F101-1; 7, *A. cryaerophilus* (LMG 9904^T); 8, SW28-9; 9, SW28-10; 10, SW28-11; 11, SW29-3; 12; SW30-3; 13, SW31-1.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

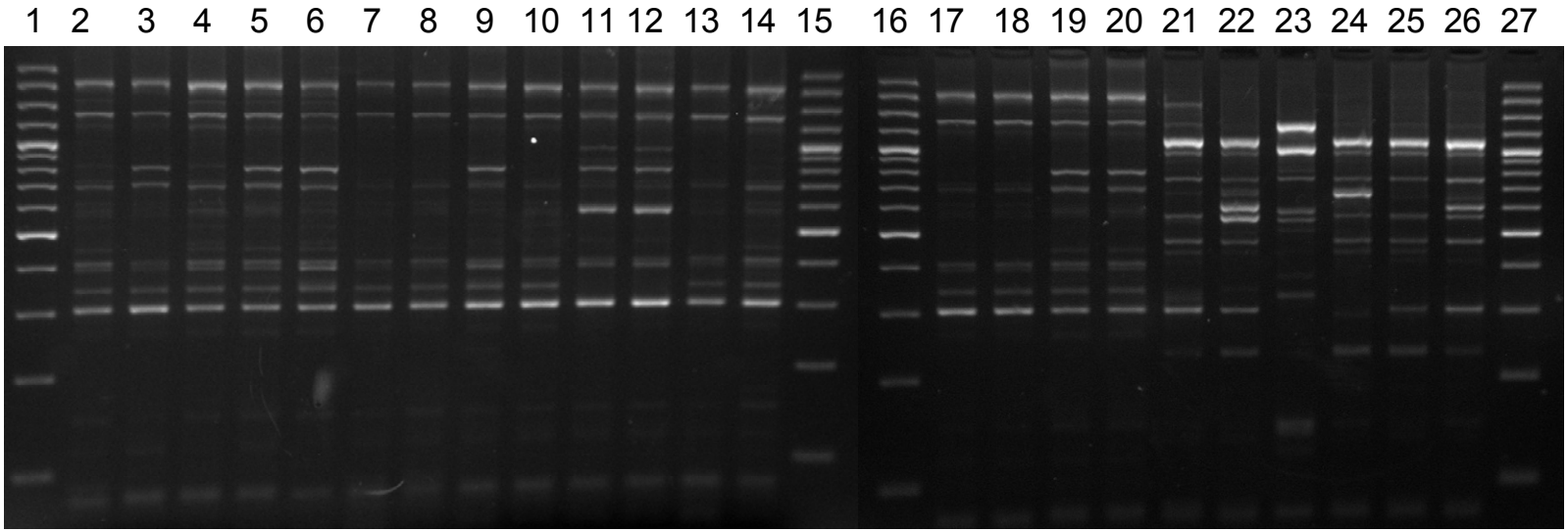


Fig. 2 Agarose gel showing ERIC-PCR patterns of shellfish and sewage isolates. Lanes: 1, 15, 16 and 27 GeneRuler 100 bp Plus DNA Ladder (Fermentas); 2 F80; 3, F82-1; 4, F82-2; 5, F83-1; 6, F90-1; 7, F90-2; 8, F90-4; 9, F90-5; 10, F90-6; 11, F91; 12, F92; 13, F98-2; 14, F98-3; 17, F98-4, 18, F99-2; 19, F101-1, 20, F101-2; 21, SW28-9; 22; SW28-10; 23, SW28-11; 24, SW29-3; 25, SW30-3; 26, SW31-1.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

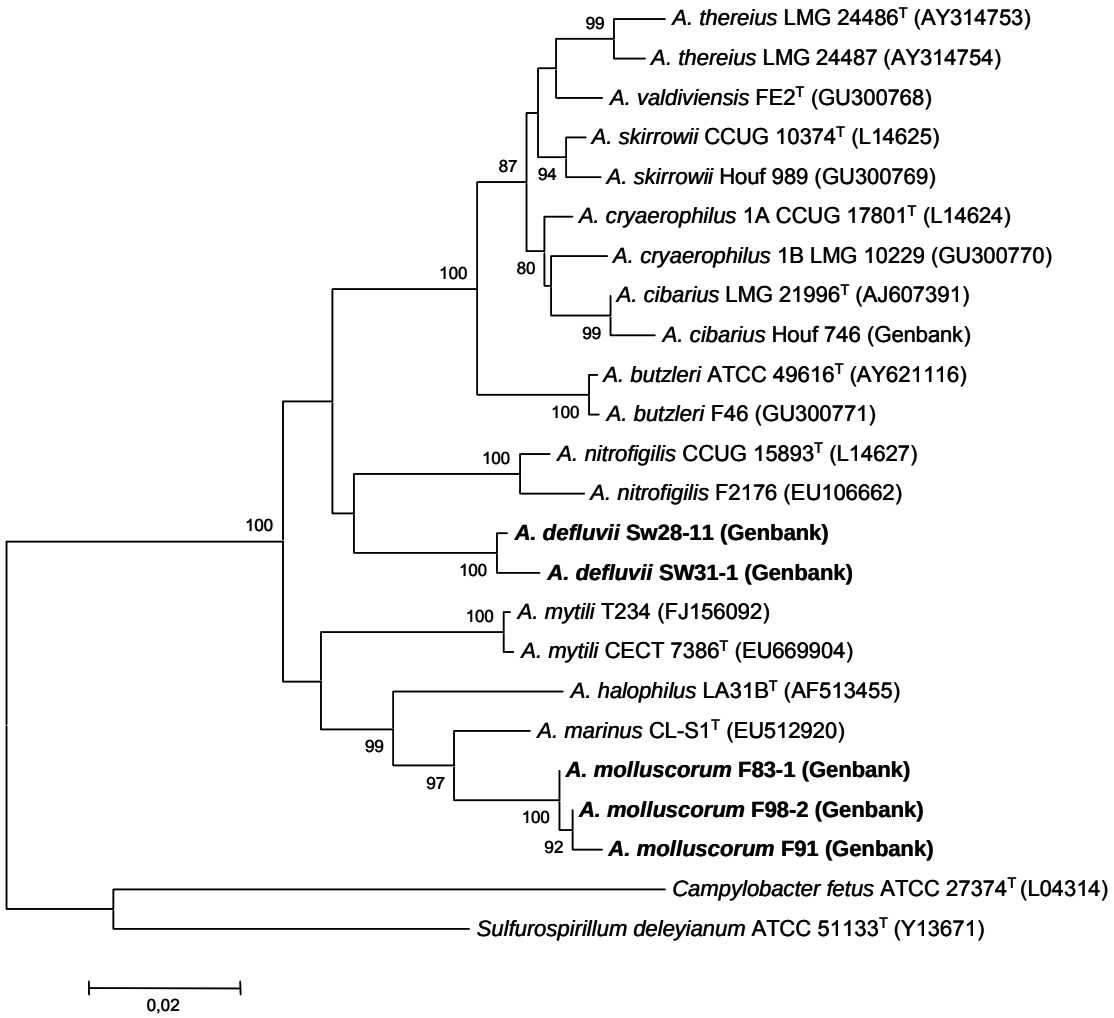


Fig. 3. Neighbour-joining tree based on 16S rRNA sequences (1405bp) showing the phylogenetic position of *Arcobacter defluvii* sp. nov. and *Arcobacter molluscorum* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

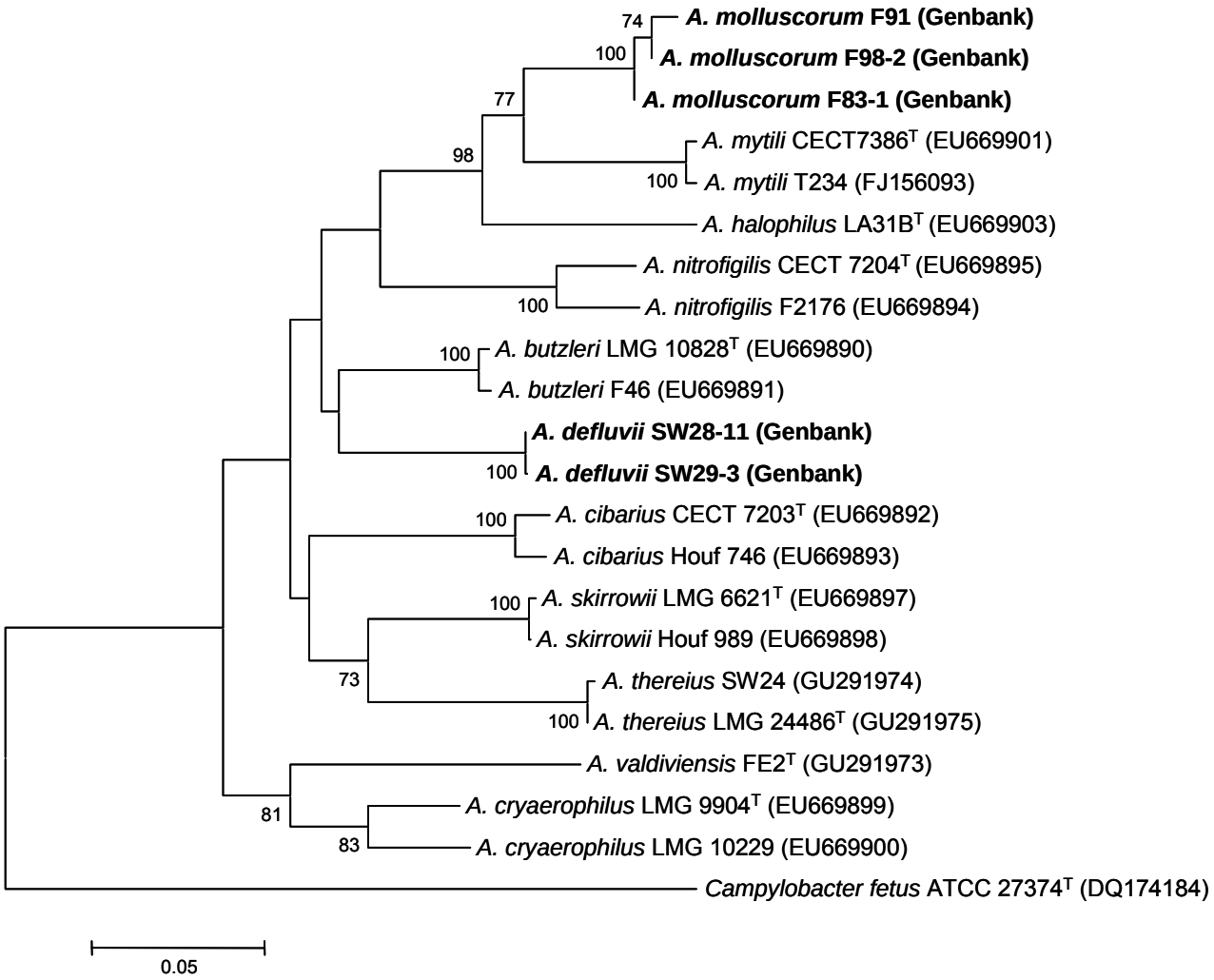


Fig. 4. Neighbour-joining tree based on *rpoB* sequences (487bp) showing the phylogenetic position of *Arcobacter defluvii* sp. nov. and *Arcobacter molluscorum* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 100 nt.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

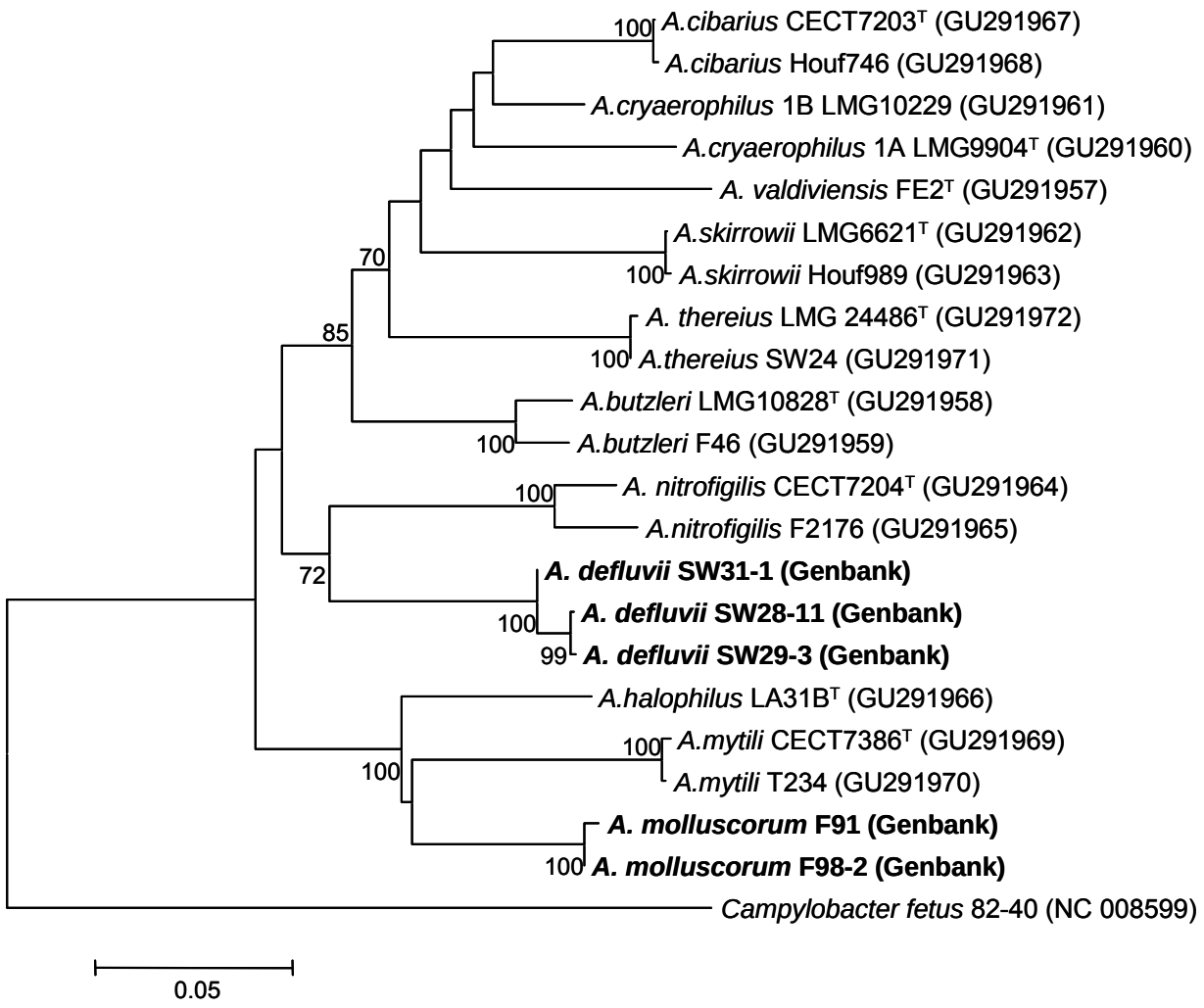


Fig. 5. Neighbour-joining tree based on *gyrB* sequences (665bp) showing the phylogenetic position of *Arcobacter defluvii* sp. nov. and *Arcobacter molluscorum* sp. nov within the genus *Arcobacter*. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 100 nt.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

5. SUMMARISING DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

The genus *Arcobacter* currently comprises nine species. Some of them are associated with human and animal diseases, whereas others have been isolated from healthy animals and environmental samples. The **Introductory Review (1.1)** presented in this thesis emphasizes the need for further research on the detection and identification of *Arcobacter* spp. as well as on the transmission routes. Our studies have enlarged the knowledge of this genus, providing a new identification method that has been used to establish the prevalence of this microorganism in foods of animal origin and in environmental waters. Furthermore, from the food survey new potential arcobacters were recognized that, after polyphasic taxonomic studies, were verified to represent new *Arcobacter* species. This section is a summarizing discussion of the results obtained in this doctoral thesis.

5.1 The need for a new *Arcobacter* identification method

Although several molecular identification methods for *Arcobacter* have been described (revised in the **Introductory Review 1.1**), none of them enabled identification of the 6 species included in this genus at the time this study was initiated. In **Study 4.1** we report a new, fast and inexpensive molecular identification technique based on an initial amplification by PCR of a fragment (1026bp) of the 16S rRNA gene followed by digestion using the *Mse*I endonuclease, generating different species-specific restriction patterns (Figueras *et al*. 2008). The proposed method was validated using 12 reference strains (including 6 type and 6 reference strains) and 75 field isolates. In later studies this method was used for the characterization of 502 additional *Arcobacter* strains (included in **Studies 4.2**, **4.3** and **4.4**) and 11 additional strains received from other authors (see Table 2 in Materials and Methods). The 16S rDNA-RFLP method has shown to be useful for the identification of the species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. nitrofigilis*, *A. cibarius* and *A. halophilus* and to enable recognition of new *Arcobacter* species like *A. mytili*, *A. defluvii* and *A. molluscorum* (proposed in **Studies 4.5** and **4.7**). The latter were recognized as potential new species because they produce different 16S rDNA-RFLP patterns from those previously described for known species. This new pattern may be expected if the digested sequence belongs to a new *Arcobacter* species or if the restriction sites in known species are affected by intraspecies nucleotide diversity or microheterogeneities (Figueras *et al*. 2000; Alperi *et al*. 2009). The available genome of *A.*

ISBN: 978-84-693-0707-6 /DL:T-420-2010

butzleri RM 4018 (Miller *et al*. 2007) presents five copies of the 16S rRNA gene with an identical sequence, suggesting that microheterogeneities are uncommon in the genus in comparison with what occurs in other genera (Alperi *et al*. 2009, and reference therein). In fact, of the 32 16S rDNA sequenced strains in this study, we only observed microheterogeneities on the 16S rRNA gene in one strain.

Since the publication of the 16S rDNA-RFLP method and during the development of this thesis other new *Arcobacter* species have been published by other authors: *A. thereius* (Houf *et al*. 2009) and more recently *A. marinus* (Kim *et al*. 2009). The 16S rDNA-RFLP enables differentiation of *A. marinus* from the other species on the basis of a new RFLP pattern obtained after a computer simulation. However, this could not be evaluated experimentally with the type strain of this species because it is not yet available from the culture collections as the study of Kim *et al*. (2009) is still in press. In relation to the new species *A. thereius*, the same RFLP pattern as *A. butzleri* is produced as happens with one of the proposed new species *A. valdiviensis*. These common patterns are generated as a result of the same 16S rRNA sequences for this new species at the targeted restriction sites of the *Mse*I enzyme. So it is clear again that there is a need for designing a new identification method for these species. However, *A. thereius* can be differentiated from *A. butzleri* by the ability of *A. thereius* to grow in media with 1% glycine (Houf *et al*. 2009). The new proposed species *A. valdiviensis* can be also differentiated from *A. butzleri* by its growth in media with glycine but also by its incapacity to grow in minimal media and to reduce nitrate. This latter test is also useful to differentiate *A. thereius* from *A. valdiviensis*.

Despite the common pattern observed with the 16S rDNA-RFLP method for *A. butzleri*, *A. thereius* and *A. valdiviensis*, our method is currently the molecular technique available (different from sequencing) with the highest capacity to discriminate *Arcobacter* species. The method can be highly useful in future studies for determining the true incidence of some species not detected or identified with the available methods (*A. cibarius*, *A. halophilus*, *A. nitrofigilis*, *A. mytili* and *A. marinus*). In fact, in our studies we could isolate *A. nitrofigilis* from mussel samples, this being the first record of this species since its description from roots and root-associated sediments of a salt marsh plant (McClung *et al*. 1983). Since then only Maugeri et al. (2000) has isolated and phenotypically identified this species, from a brackish environment.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

An unexpected result observed during the design and development of our RFLP method was that several strains previously characterized with the m-PCR of Houf *et al*. (2000) showed incongruent results with the RFLP method. After further characterization by sequencing of the 16S rRNA gene we were able to recognize that the m-PCR misidentified those strains (Table 1). For instance, we have demonstrated for the first time that with the m-PCR *A. nitrofigilis* could be confused with *A. skirrowii* because it produced an amplificon of the same size (641 bp) expected for the latter species. In fact, our results contradict those of Houf *et al*. (2000) that indicate that no amplicon should be expected for *A. nitrofigilis* with the m-PCR method they proposed. Another three strains obtained from mussels also showed the same band expected for *A. skirrowii* with the m-PCR. Finally, after sequencing, these strains belonged to the newly discovered species *A. mytili*. We also found that the type strain of *A. halophilus* (Donachie *et al*. 2005), a species described after the m-PCR was published (Houf *et al*. 2000) produces two amplicons (257 y 641bp) that correspond with an individual amplicon expected for *A. cryaerophilus* and *A. skirrowii* respectively (Figueras *et al*. 2008). On the other hand, *A. molluscorum*, proposed in the present thesis, showed the amplicons commented above for *A. halophilus* with m-PCR. However, both species, *A. halophilus* and *A. molluscorum*, show species-specific RFLP patterns with the 16S rDNA-RFLP method (Table 1).

Very recently in the description of *A. thereius* (Houf *et al*. 2009), it was also recognized that with the m-PCR method (Houf *et al*. 2000) an amplicon of the same size (257 bp) as that described for *A. cryaerophilus* was obtained for this new species. We also found this amplicon in one strain recovered from a pork meat sample that produced the pattern of *A. butzleri* with the RFLP method. However, the sequence of the 16S rRNA gene of this strain demonstrated that it belonged to the recently proposed species *A. thereius*. This strain corresponds to the first record of this species from pork meat and the first finding of this new species since its description (Collado *et al*. 2009b). A similar m-PCR fragment size (257 bp) has been found for the proposed species *A. valdiviensis* and for two other potential novel species (F4 and F41) waiting to be formally described and named. However, the 16S rDNA-RFLP patterns of these strains are different from the ones described. All this data demonstrates that until a new identification method is developed for the characterization of the species with common RFLP patterns, the best way to identify the strains is to use both methods in parallel (m-PCR and the 16S rDNA-RFLP) and to further sequence the 16S rRNA gene in cases of incongruent results. So far this is

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

the only way to obtain an accurate identification of all *Arcobacter* species. Table 1 summarizes the above discussion providing the results of the identification obtained with the m-PCR (Houf *et al*. 2000) and 16S rDNA-RFLP methods (Figueras *et al*. 2008) for all the accepted *Arcobacter* species and the ones proposed in the present thesis.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

Table 1. Results of the comparison of the m-PCR and 16S rDNA-RFLP identification methods in all species of the genus *Arcobacter* and of the candidate new species

Species (type strains)	m-PCR			16S rDNA-RFLP		
	Amplicon (bp)	Correct species identification	Species identification	Restriction fragments	Specific pattern	Species identification
A. butzleri	401	Yes	*A. butzleri*	548/216/138[a]	Yes	*A. butzleri*
A. cryaerophilus 1A	257	Yes	*A. cryaerophilus*	395/216/143/138	Yes	*A. cryaerophilus* 1A
A. cryaerophilus 1B	257	Yes	*A. cryaerophilus*	365/216/143/138	Yes	*A. cryaerophilus* 1B
A. skirrowii	641	Yes	*A. skirrowii*	395/243/143/138	Yes	*A. skirrowii*
A. halophilus	257 and 641	No	*A. cryaerophilus*/ *A. skirrowii*	551/143/138/101	Yes	*A. halophilus*
A. nitrofigilis	641	No	*A. skirrowii*	434/216/138/101	Yes	*A. nitrofigilis*
A. cibarius	NA			519/243/138	Yes	*A. cibarius*
A. mytili	641	No	*A. skirrowii*	650/143/138	Yes	*A. mytili*
A. thereius	257	No	*A. cryaerophilus*	548/216/138	No	*A. butzleri*
A. marinus[b]	ND			308/243/138[a]	Yes	*A. marinus*
A. valdiviensis	257	No	*A. cryaerophilus*	548/216/138	No	*A. butzleri*
A. defluvii				434*/243/143/138	Yes	*A. defluvii*
A. molluscorum	257 and 641	No	*A. cryaerophilus*/ *A. skirrowii*	551/143/138/72	Yes	*A. molluscorum*

[a]RFLP pattern obtained experimentally and after an computer simulation

[b]RFLP pattern obtained only after an computer simulation

NA, not amplified; ND, not determined

m-PCR (Houf *et al*. 2000) and 16S rDNA-RFLP (Figueras *et al*. 2008)

ISBN: 978-84-693-0707-6 /DL:T-420-2010

5.2 Are *Arcobacter* species members of the seawater bacterial flora?

Arcobacters have been isolated from different types of environmental waters with prevalence ranging from 0 to 83% (Jacob *et al*. 1998; Rice *et al*. 1999; Diergaardt *et al*. 2004; Maugeri *et al*. 2000, 2004; Fera *et al*. 2004; Donachie *et al*. 2005), and it has been suggested that *Arcobacter* species could be members of the bacterial flora of seawater (Fera *et al*. 2004). However, in **Study 4.2** we have demonstrated, for the first time, an association between *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* and bacterial indicators of faecal pollution. These species were significantly more prevalent in contaminated river, lakes and sea waters than in non-polluted waters. Furthermore, almost all samples from sewage and sludge were positive for *Arcobacter*. All this indicated that the presence of *Arcobacter* in environmental waters is associated to faecal pollution (Collado *et al*. 2008). Moreover, and in order to clarify if *Arcobacter* spp. present in seawater has a contaminated freshwater origin, we have quantified *Arcobacter* using the most-probable-number (MPN) in a bathing area contaminated by a freshwater stream. A clear concentration gradient of *Arcobacter* was observed from the fresh water stream (3.7×10^5 MPN/100 ml) to the seawater (5.2×10^4 MPN/100 ml), with no detection at 200 m south of the freshwater discharge (Collado *et al*. 2008). The concentrations of *Arcobacter* found in our study were similar to the levels found in piggery effluents ranging from 6.5×10^5 to 1.1×10^8 MPN/100 ml (Chinivasagam *et al*. 2007). Our results indicate that the *Arcobacter* species isolated in water have a human and/or animal faecal origin. However, other species, like *A. marinus*, *A. halophilus* and *Candidatus* Arcobacter sulfidicus, could be autochthonous of aquatic environment. The conclusion of our work could have an ecological and epidemiological implication, since environmental waters have been suggested as one of the most important transmission routes to animals and humans. In fact, in some recently reported waterborne outbreaks associated with *Arcobacter*, the drinking water was also always faecally contaminated (Rice *et al*. 1999; Fong *et al*. 2007; Kopilovic *et al*. 2008), confirming the results from **Study 4.2**.

Arcobacter have been investigated in drinking water systems in some studies (Jacob *et al*. 1998; Diergaardt *et al*. 2004; Aydin *et al*. 2007); however, according to the review by Ho *et al*. (2006), it is still unknown whether or not classical treatments used in the production of drinking water are effective in the elimination of *Arcobacter* species. In **Study 4.3** we have investigated the incidence of *Arcobacter* along the Llobregat River catchment, one of the main sources of drinking water to the metropolitan area of

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Barcelona, Spain. In this study, we have demonstrated again that *Arcobacter* was associated with the most faecally contaminated sites of the river. Furthermore, all the sites positive for *Arcobacter* were coincidentally positive for the several enteroviruses investigated by the group of Enterics Viruses of the Barcelona University (U. Perez, Manuscript in preparation), agreeing with the results obtained from water samples in a waterborne outbreak related to a massive microbiological groundwater contamination in Ohio (Fong *et al*. 2007). In our study, we have also demonstrated that classical treatment procedures for producing drinking water are effective in the elimination of theses microorganisms. The results obtained showed a high prevalence of *A. butzleri* (81.1%) and *A. cryaerophilus* (17.6%). Furthermore, we have investigated for the first time the genetic diversity of the isolates recovered from the river water, since so far, despite several studies investigating the genetic diversity of *Arcobacter* isolates (Houf *et al*. 2002 and references therein), none of them have included water isolates. Our results showed that among the 339 investigated strains we found 309 genotypes. Never the same genotype was recovered from different sampling points or dates. These results are in agreement with the high genetic heterogeneity of sequence types also encountered in the first multilocus sequence typing (MLST) study performed using seven housekeeping genes in *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius* and *A. thereius* (Miller *et al*. 2009). However, in the set of strains used for the *Arcobacter* MLST study no strains from water were included. This high genetic diversity suggests that the same *Arcobacter* genotype cannot colonize the river water and that the strains may come from different sources. Other reasons that could explain this diversity of genotypes is the genomic rearrangement observed in *Campylobacter* spp. (Hume *et al*. 2001). To our knowledge, our work (**Study 4.3**) is the first to report the genetic diversity of arcobacters recovered from environmental water.

5.3 The spread of *Arcobacter* species in different types of meats and shellfish

Food of animal origin has also been considered a potential transmission route of *Arcobacter*. However, the available surveys on the presence of *Arcobacter* in foods have been concentrated in poultry, pork and beef meats (reviewed by Lehner *et al*. 2005), the presence of this microorganism being almost unknown in other types of food like shellfish or other types of meat products. In **Study 4.4** we have demonstrated a high prevalence of

ISBN: 978-84-693-0707-6 /DL:T-420-2010

A. butzleri, *A. cryaerophilus* and *A. skirrowii* in several types of meats (chicken, turkey, duck, pork, beef and rabbits), and interestingly also in shellfish (mussels and clams). Moreover, as indicated previously in our study we isolated the recently described species *A. thereius* from pork meat for the first time. Additionally, as result of this study, the molecular identification method described by us enable a potential new species isolated from mussels to be recognized, which was confirmed with a polyphasic approach as the new species *A. mytili* (described in **Study 4.5**). Another two potential novel *Arcobacter* species (at the moment, waiting to be formally described and named) were found from mussels and pork meat, respectively.

In this food survey we found a 88.1% of concordance in the identification using our method (16S rDNA-RFLP) and the m-PCR method described by Houf *et al*. (2000) in parallel. The eight isolates with dissimilar identification were the 3 strains later identified as *A. mytili* and 2 as *A. nitrofigilis* (identified as *A. skirrowii* with m-PCR), 1 strain of *A. thereius* (identified as *A. butzleri* with 16S rDNA-RFLP and *A. cryaerophilus* with m-PCR) and the two potential novel species commented before.

5.4 Novel bacteria taxa within the genus *Arcobacter*

As commented earlier, the 16S rDNA-RFLP method enabled detection of a new restriction pattern for some isolates (F2026, F2075 and F2076) recovered from mussels samples that had been previously identified as *A. skirrowii* by the m-PCR of Houf *et al*. (2000). After that, an additional isolate (T234) obtained from a brackish water sample showed the same characteristics. A polyphasic taxonomic study including 16S rRNA and *rpo*B gene analysis, DNA-DNA hybridization, G+C content, genotyping by ERIC-PCR and biochemical characterization enabled those isolates to be defined as a new species named *Arcobacter mytili* sp. nov (**Study 4.5**). This proposed novel species was found to be unable to hydrolyze the indoxil-acetate, a trait that can differentiate it from the other *Acobacter* species. Therefore, in order to improve the phenotypic characterization, this biochemical test must be considered in future biochemical identification studies.

The taxonomy and phylogeny of the genus *Arcobacter*, as in other bacterial genera, has been based mainly on the 16S rRNA gene analysis. The 16S rRNA gene similarity values reported a range of between 93 and 98% among the species *A. butzleri*,

ISBN: 978-84-693-0707-6 /DL:T-420-2010

A. cryaerophilus, *A. skirrowii* and *A. nitrofigilis* (Wesley *et al*. 1995). Very similar values (93 to 99%) were found when we compared all the currently accepted species (**Introductory Review 1.1**). However, in recent years some housekeeping genes have been analyzed in *Arcobacter* as a complementary support to the 16S rRNA gene. Morita *et al*. (2004) used the *rpo*B-*rpo*C genes (encoding the β and β' subunits of DNA-dependent RNA polymerase) using reference and field strains to assess the phylogeny of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. On the other hand, Abddelbaqi *et al*. (2007) obtained the complete sequences of the *gyr*A gene (encoding for the A subunit of the DNA gyrase) of the species *A. butzleri*, *A. cryaerophilus*, *A. cibarius* and *A. skirrowii* and demonstrated that this gene shows a higher resolution than the 16S gene for discriminating the 5 studied strains of *A. butzleri*. Other studies conducted with different taxonomic and phylogenetic objectives in members of the *Epsilonproteobacteria* have analyzed the *cpn60* gene (also known as the 60 kDa chaperonin, *groEL* or *hsp60*) in reference strains of *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis* (Kärenlampi *et al*. 2004; Hill *et al*. 2006). Very recently, Debruyne *et al*. (In press) evaluated the phylogeny of the genus *Arcobacter* on the basis of the *cpn60* gene including several representative strains of accepted species. The latter study not included the recently proposed *A. marinus* and concluded that the differentiation of the two subgroups 1A and 1B of *A. cryaerophilus* does not seem justified on the basis of this gene.

The ad hoc committee for the re-evaluation of the species definition in bacteriology recommends evaluating at least five housekeeping genes (universally distributed, present as single copies and located at distinct chromosomal loci) for genomically circumscribing the taxon species and differentiating it from neighbouring species (Stackebrandt *et al*. 2002). Regarding this, the description of *A. mytili* was the first one to include the analysis of one housekeeping gene (*rpoB* gene encoding the β-subunit of RNA polymerase). This gene was selected because it had been very useful for the identification of the species of *Campylobacter* and for the phylogenetic analysis of the genus (Korczak *et al*. 2006). The phylogenetic analysis of *Arcobacter* we performed included two strains of all the species accepted at that time: *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. nitrofigilis*, *A. halophilus*, *A. cibarius* and the new *A. mytili* and showed, in agreement with the 16S rRNA gene phylogeny, that the new species *A. mytili* clustered with *A. halophilus* (Collado *et al*. 2009a).

ISBN: 978-84-693-0707-6 /DL:T-420-2010

After the description of *A. mytili*, two new species have been described: *A. thereius* isolated from pig and duck (Houf *et al*. 2009) and *A. marinus* isolated from seawater associated with starfish in Korea (Kim *et al*. 2009). The description of *A. marinus* also included the analysis of the housekeeping gene *gyr*A, this being the second formal description of an *Arcobacter* species to include the analysis of one housekeeping gene.

Besides the *rpoB* gene, we have also explored the usefulness of the *gyrB* gene (encoding for the β subunit of the DNA gyrase) as another novel universal phylogenetic marker for *Arcobacter*. This gene has proven to be an excellent marker for establishing the phylogeny of *Arcobacter* spp.

An additional novel *Arcobacter* species proposed in the present thesis is *Arcobacter valdiviensis*, which was recovered in 2005 from chicken cloacal swabs in the Chilean city of Valdivia (**Study 4.6**). Strain FE2^T was identified as three different species depending on the method used [as *A. cryaerophilus* using the m-PCR (Houf *et al*. 2000), as was *A. butzleri* with the 16S rDNA-RFLP (Figueras *et al*. 2008) and as *A. skirrowii* with the RFLP method of Marshall *et al*. (1999)]. After a polyphasic study of this strain we recognized it as a novel *Arcobacter* species. The ecological and pathogenic role of *A. valdiviensis* sp. nov. is currently unknown. However, this novel taxon is phylogenetically related to some species, like *A. cryaerophilus* and A*. skirrowii*, which are related to animal and human diseases (Ho *et al*. 2006). Furthermore, the origin of this strain from cloacal swabs could reinforce previous data of Ho *et al*. (2008), who suggest that *Arcobacter* species are commonly carried in the intestinal content of chickens, contradicting previous studies (Aydin *et al*. 2007; Van Driessche and Houf 2007).

Another two additional species proposed by our group are *Arcobacter defluvii* and *Arcobacter molluscorum* (**Study 4.7**). *A. defluvii* was isolated from samples from the sewage treatment plant in the city of Reus (Spain) and was recognized as a potential new species because it showed a distinctive 16S rDNA-RFLP restriction pattern that differed from the ones described for the other *Arcobacter* spp. (Figueras *et al*. 2008). However, with the m-PCR of Houf *et al*. (2000) it was identified as *A. cryaerophilus*. This species is phylogenetic closely related to *A. nitrofigilis* on the basis of the 16S rRNA gene and like this species is able to reduce nitrate. On the other hand, strains of *A. molluscorum* were recovered from mussels and also showed a new 16S rDNA-RFLP pattern. As commented before, with the m-PCR (Houf *et al*. 2000), these strains give two amplicons (257 and 647

ISBN: 978-84-693-0707-6 /DL:T-420-2010

bp) corresponding to the species–specific fragment size reported for *A. cryaerophilus* and *A. skirrowii,* respectively.

In order to summarize the phylogenetic relationship of the accepted *Arcobacter* species and of the ones proposed in the present thesis (A. valdiviensis, A. defluvii and A. molluscorum) together with the two strains (F4 and F41), which are also considered potential novel species, we have constructed a phylogenetic tree on the basis of the 16S rRNA gene (Fig 1). In this tree, we have also included one strain isolated from a sewage sample (strain F67-11) that showed to be closely related to one 16S rRNA sequence deposited in GenBank (accession number AM084114) and that corresponded to one strain obtained from activated sludge by the group directed by Dr. Paul de Vos from the Laboratory of Microbiology of the Ghent University (LMG). The derived phylogenetic tree clearly shows that all these isolates constitute new taxa.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

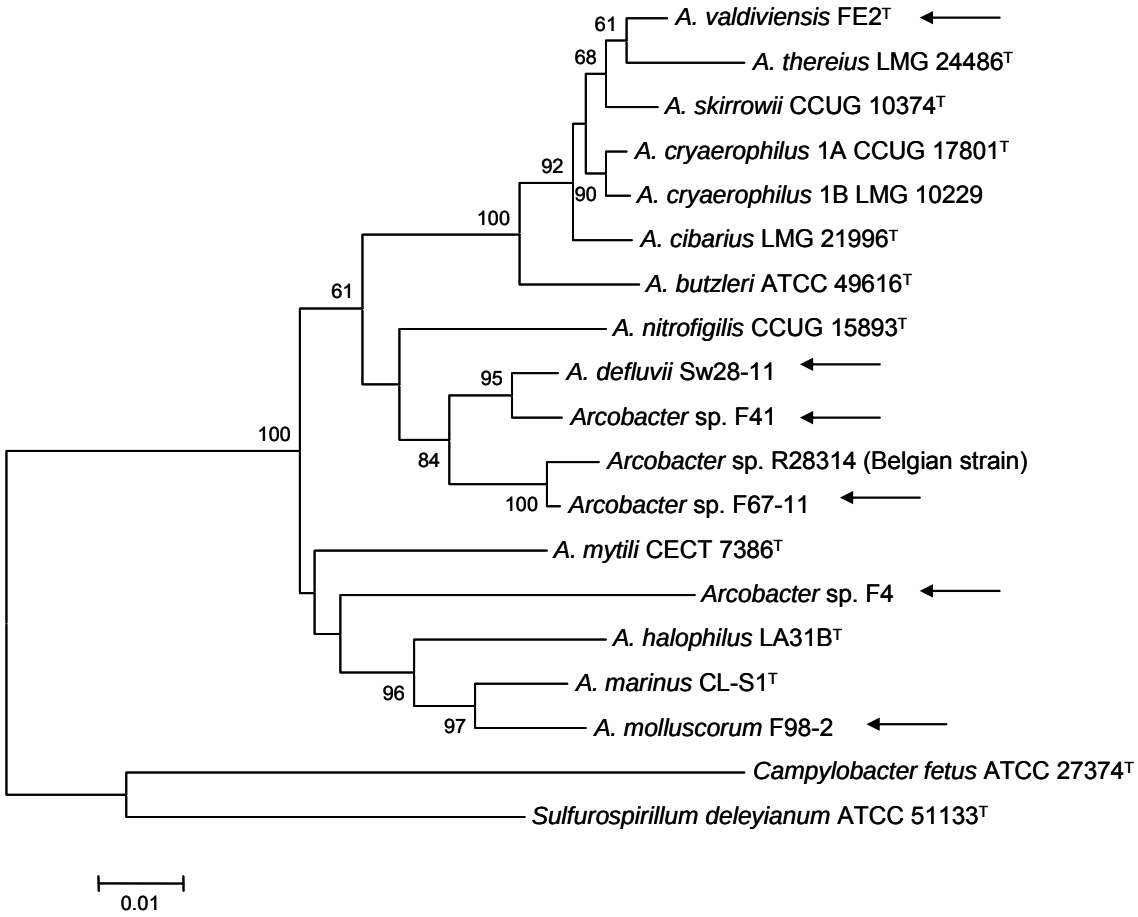


Fig. 1. Neighbour-joining tree based on 16S rRNA sequences (1420bp) showing the phylogenetic position of the proposed and potential new species (arrows) in relation to all the presently accepted *Arcobacter* species. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar, 1 substitution per 100 nt.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

5.5 References

Abdelbaqi, K., Menard, A., Prouzet-Mauleon, V., Bringaud, F., Lehours, P. & Megraud, F. (2007). Nucleotide sequence of the *gyrA* gene of *Arcobacter* species and characterization of human ciprofloxacin-resistant clinical isolates. FEMS Immunol Med Microbiol 49, 337-345.

Alperi, A., Martinez-Murcia, A.J., Ko, W.C., Monera, A., Saavedra, M.J. and Figueras, M.J. (2009). *Aeromonas taiwanensis* sp. nov. and *Aeromonas sanarellii* sp. nov., two new clinical species from Taiwan. Int J Syst Evol Microbiol. (In press).

Christensen, H., Bisgaard, M., Frederiksen, W., Mutters, R., Kuhnert, P. & Olsen, J. E. (2001). Is characterization of a single isolate sufficient for valid publication of a new genus or species? Proposal to modify recommendation 30b of the Bacteriological Code (1990 Revision). Int J Syst Evol Microbiol 51, 2221-2225.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. & Figueras, M. J. (2009a). *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. Int J Syst Evol Microbiol 59, 1391-1396.

Collado, L., Fernández, H. & Figueras, M. J. *Arcobacter valdiviensis* sp. nov., isolated from a chicken cloacal swab sample in Valdivia, Chile. (submitted).

Collado, L. & Figueras, M. J. The genus *Arcobacter*: A review of this taxonomy, epidemiology and importance. (submitted).

Collado, L., Guarro, J. & Figueras, M. J. (2009b). Prevalence of *Arcobacter* in meat and shellfish. J Food Prot 72, 1102-1106.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Collado, L., Inza, I., Guarro, J. & Figueras, M. J. (2008). Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. Environ Microbiol 10, 1635-1640.

Collado, L., Levican, A., Perez, J. & Figueras, M. J. Description of two new species, *Arcobacter defluvii* sp. nov., isolated from sewage and *Arcobacter molluscorum* sp. nov., isolated from shellfisfh. (In preparation).

Diergaardt, S. M., Venter, S. N., Spreeth, A., Theron, J. & Brozel, V. S. (2004). The occurrence of campylobacters in water sources in South Africa. Water Res 38, 2589-2595 2589-2595 .

Donachie, S. P., Bowman, J. P., On, S. L. & Alam, M. (2005). *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. Int J Syst Evol Microbiol 55, 1271-1277.

D'Sa, E. M. & Harrison, M. A. (2005). Effect of pH, NaCl content, and temperature on growth and survival of *Arcobacter* spp. J Food Prot 68, 18-25.

Felis, G. E. & Dellaglio, F. (2007). On species descriptions based on a single strain: proposal to introduce the status species proponenda (sp. pr.). Int J Syst Evol Microbiol 57, 2185-2187.

Fera, M. T., Maugeri, T. L., Gugliandolo, C., Beninati, C., Giannone, M., La Camera, E. & Carbone, M. (2004). Detection of *Arcobacter* spp. in the coastal environment of the Mediterranean Sea. Appl Environ Microbiol 70, 1271-1276.

Figueras, M. J., Collado, L. & Guarro, J. (2008). A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. Diagn Microbiol Infect Dis 62, 11-15.

Harraß, B., Schwarz, S. & Wenzel, S. (1998). Identification and characterization of *Arcobacter* isolates from broilers by biochemical tests, antimicrobial resistance patterns and plasmid analysis. Zentralbl Veterinarmed B 45, 87-94.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Hill, J. E., Paccagnella, A., Law, K.& other authors (2006). Identification of *Campylobacter* spp. and discrimination from *Helicobacter* and *Arcobacter* spp. by direct sequencing of PCR-amplified *cpn60* sequences and comparison to cpnDB, a chaperonin reference sequence database. J Med Microbiol 55, 393-399.

Ho, H. T., Lipman, L. J. & Gaastra, W. (2008). The introduction of *Arcobacter* spp. in poultry slaughterhouses. Int J Food Microbiol 125, 223-229.

Ho, H. T., Lipman, L. J. & Gaastra, W. (2006). *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent! Vet Microbiol 115, 1-13.

Houf, K., On, S. L., Coenye, T., Debruyne, L., De Smet, S. & Vandamme, P. (2009). *Arcobacter thereius* sp. nov., isolated from pigs and ducks. Int J Syst Evol Microbiol 59, 2599-2604 2599-2604 .

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J. & Vandamme, P. (2000). Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiol Lett 193, 89-94.

Kabeya, H., Kobayashi, Y., Maruyama, S. & Mikami, T. (2003). One-step polymerase chain reaction-based typing of *Arcobacter* species. Int J Food Microbiol 81, 163-168.

Kim, H. M., Hwang, C. Y. & Cho, B. C. (2009). *Arcobacter marinus* sp. nov. Int J Syst Evol Microbiol (In press).

Korczak, B. M., Stieber, R., Emler, S., Burnens, A. P., Frey, J. & Kuhnert, P. (2006). Genetic relatedness within the genus *Campylobacter* inferred from *rpoB* sequences. Int J Syst Evol Microbiol 56, 937-945.

Lehner, A., Tasara, T. & Stephan, R. (2005). Relevant aspects of *Arcobacter* spp. as potential foodborne pathogen. Int J Food Microbiol 102, 127-135.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

Marshall, S. M., Melito, P. L., Woodward, D. L., Johnson, W. M., Rodgers, F. G. & Mulvey, M. R. (1999). Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. J Clin Microbiol 37, 4158-4160 4158-4160 .

Maugeri, T. L., Carbone, M., Fera, M. T., Irrera, G. P. & Gugliandolo, C. (2004). Distribution of potentially pathogenic bacteria as free living and plankton associated in a marine coastal zone. J Appl Microbiol 97, 354-361.

Maugeri, T. L., Gugliandolo, C., Carbone, M., Caccamo, D. & Fera, M. T. (2000). Isolation of *Arcobacter* spp. from a brackish environment. New Microbiol 23, 143-149.

Miller, W. G., Wesley, I. V., On, S. L., Houf, K., Megraud, F., Wang, G., Yee, E., Srijan, A. & Mason, C. J. (2009). First multi-locus sequence typing scheme for *Arcobacter* spp. BMC Microbiol 9, 196.

Morita, Y., Maruyama, S., Kabeya, H.& other authors (2004). Isolation and phylogenetic analysis of *Arcobacter* spp. in ground chicken meat and environmental water in Japan and Thailand. Microbiol Immunol 48, 527-533.

Rice, E. W., Rodgers, M. R., Wesley, I. V., Johnson, C. H. & Tanner, S. A. (1999). Isolation of *Arcobacter butzleri* from ground water. Lett Appl Microbiol 28, 31-35.

Stackebrandt, E., Frederiksen, W., Garrity, G. M.& other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52, 1043-1047.

Van Driessche, E. & Houf, K. (2008). Survival capacity in water of *Arcobacter* species under different temperature conditions. J Appl Microbiol 105, 443-451.

Van Driessche, E. and Houf, K. (2007) Discrepancy between the occurrence of *Arcobacter* in chickens and broiler carcass contamination. Poult Sci 86, 744-751.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

6. CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

ISBN: 978-84-693-0707-6 /DL:T-420-2010

1. The proposed 16S rDNA-RFLP *Arcobacter* identification method, apart from being fast and inexpensive, is so far the only method that enables simultaneous characterization of six species of the genus.

Using the 16S rDNA-RFLP method we have identified, for the first time since its description, *Arcobacter nitrofigilis* from mussels, a species so far only known from plant roots, root-associated sediments and from a brackish environment.

Out of the 600 isolates investigated we have been able to recognize new RFLP patterns that corresponded to the new species *Arcobacter mytili* (formally accepted) and to 4 potential new species.

2. The discovered new species *A. mytili* was recovered from mussels and brackish water and is the only species of the genus that does not hydrolyze indoxil-acetate. This biochemical test should be considered a key phenotypic characteristic in future phenotypic studies.

3. The other proposed new species with new RFLP patterns are: *Arcobacter defluvii* isolated from wastewater, *Arcobacter molluscorum* recovered from mussels as well as two strains, F4 recovered from mussels and F41 from pork meat, that are waiting to be formally described and named.

4. The application of the 16S rDNA-RFLP method in parallel with the commonly used m-PCR method for the identification of *Arcobacter* revealed that the latter method may produce misidentification for some species. Therefore the m-PCR method should be interpreted carefully and in combination with the 16S rDNA-RFLP method in order to achieve a correct identification.

5. The incongruent results found between the two identification methods (16S rDNA-RFLP and m-PCR) lead us to identify, after sequencing the 16S rRNA gene, *Arcobacter thereius* for the first time from pork meat samples. This is the first report of this species since its description. These discordances also helped to recognize the new candidate species *A. valdiviensis* isolated from a chicken cloacal swab.

ISBN: 978-84-693-0707-6 /DL:T-420-2010

6. The 16S rDNA-RFLP method cannot differentiate *A. valdiviensis* or the recently accepted new species *A. thereius* from *Arcobacter butzleri.* A new protocol needs to be developed to differentiate these species. Despite that, the use of phenotypic tests could help to properly identify these species.

7. We have demonstrated that the presence of *Arcobacter* in environmental waters (rivers, lakes, seawater, and sewage) and in sludge samples correlates with high levels of faecal pollution. This strongly supports that *Arcobacter* species found in environmental waters come from human and/or animal faecal contamination, despite some species like *Arcobacter halophilus* and *Arcobacter marinus* maybe having an aquatic origin.

8. We have conducted the first study to investigate the genetic diversity of *Arcobacter* isolates recovered from river water along the different seasons. We demonstrated that isolates of *A. butzleri* and *A. cryaerophilus* from the Llobregat River shows a very high genetic diversity. This data is in agreement with the very high diversity of theses species in other studies and in the recently published MLST analysis that did not include water strains.

9. The species *A. butzleri* and *A. cryaerophilus* were very prevalent in the Llobregat River water used to produce drinking water. However, these species were never detected or isolated from the drinking water produced, which demonstrates that water treatments are effective in removing *Arcobacter* species.

10. A higher prevalence of *Arcobacter* was found in the Llobregat River water in spring/summer (91.7/83.3%) versus autumn/winter (75.0% in both cases), suggesting a seasonal distribution.

11. It was demonstrated that *Arcobacter* species are widespread in different types of meats (chicken, beef, pork, rabbits, turkey and duck) and in shellfish (clams and mussels).

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND EPIDEMIOLOGY OF THE GENUS ARCOBACTER
Luis Roberto Collado González
ISBN: 978-84-693-0707-6 /DL:T-420-2010

12. Considering that shellfish is consumed almost uncooked, the high prevalence of *Arcobacter* spp. found in mussels (41%) and clams (100%) could suggest this as another route of transmission to humans.

13. The *rpoB* and *gyrB* housekeeping genes have been employed for the first time to establish the phylogenetic relationships of the new candidate species with the rest of species of the genus. These genes have proven to be excellent additional tools for this purpose.