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Genomic Diversity of Arcobacter spp. Isolated from Surface Waters

Rodrigo Alonso, Irati Martínez-Malaxetxebarria*, Cecilia Girbau, Sandra Carmona, Héctor Velasco and Aurora Fernández-Astorga

Department of Immunology, Microbiology and Parasitology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Vitoria-Gasteiz, Spain

Research Article

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***For Correspondence**

Irati Martínez-Malaxetxebarria, Department of Immunology, Microbiology and Parasitology. Faculty of Pharmacy. University of the Basque Country (UPV/EHU). Vitoria-Gasteiz, Spain, Tel: +34 945 013471.

E-mail: irati.martinez@ehu.eus

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ABSTRACT

A total of 45 non-clonal *Arcobacter* isolates recovered from wastewater and river water samples collected around Vitoria-Gasteiz, North of Spain, were investigated. Our findings confirmed *A. butzleri* as the most predominant species, although *A. cryaerophilus* and *A. lanthieri* were identified also. Eight isolates showed discordant results with the identification m-PCRs. Based on the rpoB gene sequence, two of these eight isolates turned to be *A. lanthieri*. However, the six remaining seemed to be possible new species needing additional analysis. The occurrence of virulence-associated genes was significantly (P<0.05) higher in *A. butzleri*. Seven of the ten virulence-associated genes investigated (*ciaB, mviN, tlyA, cadF, pldA, cj1349* and *hecA*) were present in a high number of isolates. Among them, *hecA* gene was significantly (P<0.05) related to isolates from wastewater. MLST showed a high rate of genetic diversity for *A. butzleri* isolates. However, there were no signs of relationships between STs and the presence of virulence related genes. In conclusion, *Arcobacter* population in surface waters may be more varied in species than detected because most of the enrichment media and molecular techniques are designed based on *A. butzleri* and this may be biasing the information in favour of this species. Additionally, we found a possible relationship between the content of virulence-associated genes and the origin of the isolates.

INTRODUCTION

Arcobacter species, which are considered zoonotic agents, have become particularly relevant in the last few years due to the increase of their association with human infections [1]. In the recent years the number of described species has increased substantially, even though this genus was first recognized over 25 years ago [2]. The genus *Arcobacter* includes an increasing number of species, being *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* the species commonly associated with human infection [3].

Arcobacter spp. have been detected in human and livestock stools [4], wildlife [5], foods [6] and in several water sources including drinking [7], recreational [8], and surface waters, as well as untreated and treated wastewater, which could indicate a possible ineffective wastewater treatment [9-11]. In addition, *Arcobacter* spp. outbreaks have been associated with human exposure to faecal-contaminated drinking water [12], consumption of contaminated raw or undercooked food of animal origin [1], and even with direct contact with animals. However, from an epidemiological point of view, its reservoirs and transmission routes to humans are poorly verified. Epidemiological investigation of *Arcobacter* spp., including typing-based surveillance, is claimed to elucidate the modes of transmission by which this emergent pathogen moves from the environment to potential hosts [11,13].

Molecular typing methods are suitable for tracing epidemiologically related isolates, which is of interest on epidemiological surveillance and prevention and control of infection. Different genotyping techniques have been used to evaluate the diversity

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of *Arcobacter* isolates, including ERIC-PCR [14], PFGE [15], MLST [16], or CGF [10]. Although no standard typing method has been proposed, MLST has become a robust tool for studies on global epidemiological and molecular evolution of *Arcobacter* spp. [16–18].

The virulence mechanisms of *Arcobacter* spp. are also poorly verified. Although numerous studies have investigated the presence of putative virulence genes in *Arcobacter*, most of them were focused on human, animal or food derived isolates [19–21]. As far as we know just two works have been conducted to analyse the occurrence of these genes on isolates from environmental samples. The first one was held with isolates derived from different environmental sites throughout the dairy chain [22] and the second one with isolates derived from water samples [23].

With this background, research on *Arcobacter* spp. and water sources is essential to elucidate the role of transmission, ecological characteristics and zoonotic risks of this pathogen. The aim of the present study was to confirm the presence of *Arcobacter* spp. in surface water samples recovered from different locations in Vitoria-Gasteiz, North of Spain, and to evaluate its genetic diversity, including the detection of putative virulence genes.

Materials and Methods

Sample Collection

A total of ten samples including wastewater (n=7) and river water (n=3) collected between October 2010 and January 2011 were analysed. River water samples were taken from the Zadorra River that surrounds the city of Vitoria-Gasteiz (North of Spain), and wastewater samples were collected from the Crispijana wastewater treatment plant located approximately 6 km West of Vitoria-Gasteiz. Samples were collected using sterile plastic bottles, transported to the laboratory and processed immediately. Four litters of river water and 500 mL of wastewater were sequentially filtered through membrane filters with decreasing pore size: 20, 5, 3, 0.8, 0.45, and 0.22 µm filters (Millipore, Massachusetts, USA).

Isolation of Arcobacter

The 0.45 µm and 0.22 µm filters were enriched in 10 mL *Arcobacter* Broth (Oxoid, Basingstoke, Hampshire, UK) with CAT supplement (Oxoid) and incubated aerobically at 30°C for 24 h. After enrichment, membrane filtration technique was applied using 0.45 µm pore size nitrocellulose membrane filters (Millipore) as previously described [6]. The plates were incubated at 30°C for a maximum of 7 days under aerobic conditions, and checked every 24 h for the presence of suspect *Arcobacter* colonies. Three to four suspect colonies (small, smooth, and translucent or whitish) were selected from each plate and sub-cultured, at least three times, by streaking on Columbia agar blood plates (Oxoid). Those isolates presenting a curved to spiral shape and motility under microscopic examination were subjected to PCR identification.

Genomic DNA Isolation

DNA was isolated from broth cultures using the PrepMan™ Ultra reagent (Applied Biosystems, FosterCity, CA, USA) according to the manufacturer's specifications. The concentration was determined spectrophotometrically (NanoDrop-Thermo Fisher Scientific, Massachusetts, USA), diluted to 20 ng/µl and stored at −20°C.

Identification of Arcobacter spp. Isolates

In order to confirm the isolates as *Arcobacter* spp., a genus-specific PCR was performed using the primer pair described by Bastyns et al. [24]. The isolates were identified at species level by two multiplex-PCR (m-PCR) methods. The m-PCR described by Houf et al. [25] was performed to identify the species *A. butzleri*, *A. skirrowii* and *A. cryaerophilus*. Additionally, the isolates were subjected to the multiplex-PCR assay specific for *A. butzleri*, *A. cibarius, A. cryaerophilus, A. skirrowii* and *A. thereius* described by Douidah et al. [26]. DNA from reference strains and deionized water were used as positive and negative controls, respectively. When an isolate showed incongruent identification by both m-PCRs, the 16S rRNA-RFLP (RFLP-PCR) assay described by Figueras et al. [27] was performed.

Enterobacterial Repetitive Intergenic Consensus Sequence PCR

In order to avoid the use of duplicate strains, all isolates recovered from the same water sample were genotyped using the ERIC-PCR technique with the primers and conditions described by Houf et al. [14]. Patterns with at least one different band were considered as different genotypes.

Sequencing of the rpoB Gene

The *rpoB* gene was sequenced in those cases where the m-PCR results were inconsistent with the results obtained by PCR-RFLP. The *rpoB* gene was amplified as previously described Lane [28].

The PCR products were purified using NucleoSpin® Gel and PCR Clean up (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The amplicons were sequenced bidirectionally by Sistemas Genómicos (Valencia, Spain).

Sequenced-based phylogenetic analysis was performed for taxonomic classification of some isolates within the genus *Arcobacter*. For phylogenetic analysis, the *rpoB* sequences were analyzed and compared by multiple sequence alignment and phylogenetic

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analysis with known reference species of the genus *Arcobacter*. Clustal W and Mega 6 software were used for multiple sequence alignment, calculating genetic distances, and clustering using the neighbor-joining algorithm to construct the phylogenetic tree.

Multilocus Sequence Typing (MLST)

MLST was carried out according to the method of Miller et al. [16]. Allele numbers and sequence types (STs) were assigned using the PubMLST database (http://pubmlst.org/*arcobacter*/). New alleles and STs were submitted to the database curator to be assigned new allele or ST numbers. The relationships between sequence types were investigated by concatenating the allele sequences comprising each unique ST. Dendrograms were constructed using the neighbor-joining method with the Kimura 2-parameter distance estimation method. Cluster analyses were performed using MEGA 6 [29]. Polymorphic sites and dN/dS ratios were determined using START2 program [30].

Detection of Putative Virulence Genes

The presence of ten putative virulence genes (*cadF, cj1349, ciaB, mviN, pldA, tlyA, irgA, hecA, hecB and iroE*) was detected using the primers and conditions designed by Douidah et al. [19] and Karadas et al. [31]. The amplification products were separated by electrophoresis in 2% agarose gels stained with GelRed (Biotium, Hayward, CA, USA). To analyse the association of the putative virulence genes in *Arcobacter* strains with their biological source, the Chi-square test and Fisher's exact test were performed with the SPSS statistical software (SPSS Inc., Chicago, IL, USA). A P value of<0.05 was considered statistically significant.

Results

Detection of Arcobacter spp. in Surface Waters

Arcobacter spp. was isolated from a total of 10 surface water samples including wastewater and river water. Based on the genus-specific PCR, 77 isolates were identified as *Arcobacter* yielding a 331-bp fragment. This included 61 isolates from wastewaters and 16 from river waters. All isolates recovered from the same water sample were subjected to ERIC-PCR to avoid the inclusion of identical strains in the study of genetic variability. After that, only 45 isolates showed different ERIC-genotype, 33 from wastewater and 12 from river water.

Following the m-PCRs of Houf et al. [25] and Douidah et al. [26], 37 isolates (82%) were clearly identified, 36 as *A. butzleri* (25 from wastewater, and 11 from river water) and one as *A. cryaerophilus* (from river water). However, eight wastewater isolates (18%) showed discordant results. All of them generated an amplicon of the same size expected for *A. butzleri* (401 bp) with the m-PCR of Houf et al. [25]. However, with that developed by Douidah et al. [26], five (FW-4, FW-8, FW-53, FW-54, FW-61) generated an amplicon of the same size expected for *A. skirrowii* (198 bp), two isolates (FW-34, FW-40) gave two amplicons of the same sizes expected for *A. skirrowii* (198 bp) and *A. cibarius* (1125 bp), and one isolate (FW-59) gave two amplicons of the same sizes expected for *A. butzleri* (2061 bp) and *A. skirrowii* (198 bp). When subjected to 16S rRNA-RFLP assay, these eight discordant isolates produced a band pattern similar to that of *A. trophiarum* (578, 256, and 175 bp) after digestion with endonuclease Bfa. Nevertheless, when species-specific PCR for *A. trophiarum* [32] was performed no specific amplicon was obtained. Owing to these discordant results they were subjected to further analyses based on sequenced-based phylogenetic analysis.

Discordant Isolates from Wastewater

Sequenced-based phylogenetic analysis by *rpoB* gene sequence comparison was performed for taxonomic classification of the eight discordant isolates. The sequences of the *ropB* gene from some of these isolates were deposited in GenBank under accession numbers: KY002770 (FW-34); KY002772 (FW-40); KY002771 (FW-4); KY002773 (FW-53); KX962637 (FW-54); KY002769 (FW-61); and KX944697 (FW-59). The phylogenetic tree obtained is shown in **Figure 1**. The sequence of the *rpoB* gene of both FW-34 and FW-40 isolates showed a 99.84% and 99.52% similarity percentage, respectively, with that of *A. lanthieri* type strain AF1440T. In addition, the phylogenetic tree grouped both isolates together with *A. lanthieri* in the same cluster. According to these results, FW-34 and FW-40 isolates were identified as *A. lanthieri*.

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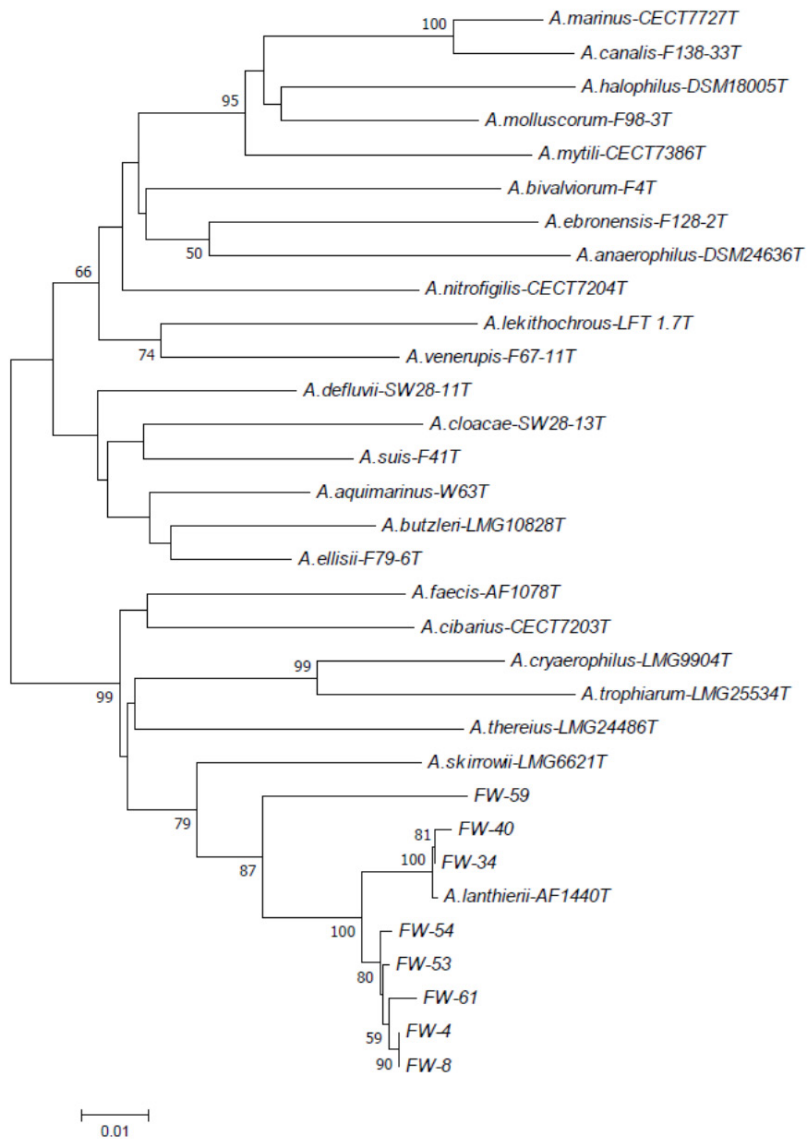

Figure 1: Neighbour-joining tree based on *rpoB* sequences (621 nt) showing the phylogenetic position of the discordant isolates within the genus *Arcobacter*. Bootstrap values (≥ 50%) based on 1000 replications are shown at the nodes of the tree. Bar, 1 substitutions per 100 nt.

The remaining six isolates showed close similarities with *A. lanthieri* (92.58 to 97.91%) according to the sequence of the *rpoB* gene, but also with *A. skirrowii* (90.66 to 91.30%) and *A. faecis* (87.12 to 88.24%) type strains. The *rpoB* gene phylogenetic tree showed that, although closely related to *A. lanthieri*, isolates FW-4, FW-8, FW-53, FW-54, and FW-61 were separated from this species in a different cluster, and that the isolate FW-59 formed an independent line from the other species of the genus *Arcobacter* (**Figure 1**).

Overall, the results derived from the sequenced-based phylogenetic analysis based on *rpoB* genes suggest that isolates FW-4, FW-8, FW-53, FW-54, and FW-61 are a closed group separated from isolate FW-59, related to A. *lanthieri* but belonging to different undescribed species of the genus *Arcobacter*. To confirm this hypothesis we have initiated polyphasic analyses for describing the possible novel species, which are in a preliminary level.

Genotyping by MLST

A total of 33 STs were identified among the 36 *A. butzleri* isolates analysed by MLST (**Table 1**). Overall, 26 STs (78.8%) were previously unreported and derived from new allele's sequences (n=19) or new combinations of known alleles (n=7). Most of the new STs (23/26, 88.5%) were represented by a single isolate, and only three STs by two isolates.

Isolates	aspA	atpA	glnA	gltA	glyA	pgm	tkt	ST
Waste water								
FW-3	23	7	17	19	496	11	65	462

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FW-6	20	5	1	23	502	111	40	463
FW-10	39	33	26	140	457	38	50	464
FW-11	30	5	5	30	120	35	4	119
FW-12	27	25	7	2	102	31	29	114
FW-14	60	15	24	27	102	62	20	465
FW-15	213	44	24	15	124	55	20	466
FW-16	31	12	1	44	497	7	33	467
FW-19	32	17	17	12	67	22	65	468
FW-23	23	5	45	37	498	56	20	469
FW-24	31	12	1	44	497	7	33	467
FW-25	213	140	2	129	499	109	4	471
FW-26	5	5	5	44	8	102	2	472
FW-27	47	7	138	165	509	261	31	473
FW-28	81	62	128	148	119	244	183	474
FW-29	231	133	51	23	510	4	160	475
FW-30	20	12	11	11	511	87	178	476
FW-32	5	34	9	15	512	102	2	477
FW-33	5	5	5	44	8	102	2	472
FW-42	3	30	16	23	516	83	31	461
FW-44	20	12	11	19	513	127	88	479
FW-47	3	30	16	23	516	83	31	461
FW-57	232	8	137	166	514	262	204	480
FW-62	23	5	24	44	80	35	55	97
FW-63	72	30	43	20	515	108	30	481
River Water								
RW-3	245	5	5	23	535	272	212	498
RW-4	246	141	26	37	536	38	79	499
RW-5	57	168	43	2	6	17	4	500
RW-6	59	169	147	55	537	273	213	501
RW-7	60	15	15	55	538	89	61	502
RW-8	20	7	20	15	186	8	14	78
RW-9	214	62	128	148	465	244	186	406
RW-10	73	2	11	44	146	111	40	195
RW-12	243	166	137	171	539	270	210	503
RW-13	6	23	4	176	540	111	143	504
RW-14	60	15	15	54	183	89	61	179
Boldface entries represent novel alleles or STs.								

Table 1: MLST typing data of *A. butzleri* isolated from surface water.

A total of 199 alleles were identified across all seven loci, ranging from 20 alleles at *atpA, glnA* and *gltA* to 32 at *glyA*. Overall, 67 out of the 199 (33.7%) alleles were previously unreported, ranging their frequency at a locus from 10% (*glnA*) to 56.3% (*glyA*). Allelic density (number of alleles/number of strains) ranged from approximately 35% at the *glnA* locus to 69% at the *glyA* locus (Table 2).

Locus	No. of alleles	No.(%) of new alleles	Allelic density	No. (%) of variable sites	d_N/d_s
aspA	24	5 (20,8)	66,7	34 (7,1)	0,011
atpA	20	3 (15,0)	55,6	44 (9,0)	0,070
glnA	20	2 (10,0)	55,6	23 (4,9)	0,034
gltA	20	4 (20,0)	55,6	14 (3,3)	0,000
glyA	32	18 (56,3)	88,9	45 (8,9)	0,101

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pgm	26	5 (19,2)	72,2	42 (8,3)	0,018
tkt	24	4 (16,7)	66,7	24 (5,1)	0,004

Table 2: Allelic diversity among 36 *A. butzleri* isolates from surface water.

The most variable locus was atpA with 9.0% variable sites and the least variable locus was gltA with only 4.9% variable sites. The dN/dS ratios varied across the seven loci, ranging from 0.000 for gltA to 0.101 for glyA. These ratios are in agreement with those reported by other authors [17,18,16]. A neighbor joining tree of the concatenated sequences (*aspA, atpA, glnA, gltA, glyA, pgm, tkt*; 3341 bp) from the unique STs (n=33) was generated by using MEGA v6 software. This phylogenetic analysis resulted in a tree that although not very robust (few bootstrap values above 50) showed no clustering of isolates.

Putative Virulence Genes

The incidence and distribution of ten virulence-associated genes was investigated in all 45 non-clonal isolates by PCR assays (Table 3). Overall, a high occurrence of the genes *ciaB* (100%), *mviN* (87%), *tlyA* (87%), *cadF* (80%), pldA (78%), cj1349 (73%) and *hecA* (60%) was observed, in contrast to *hecB* (22%), *irgA* (4%) and *iroE* (18%) genes which were less frequent. All the putative virulence genes were detected among all the 45 isolates, although the number of genes per isolate was variable. Thirty-five isolates (77.8%) harboured more than a half of the genes assayed each one, while two isolates (4.4%) harboured all ten genes and just one isolate (2.2%) only one gene. *A. butzleri* harboured a significant (P<0.05) higher number of putative virulence genes compared with the non-buzleri isolates. In addition, some genes were detected only in *A. butzleri* isolates. That was the case of the genes *cj1349, hecB, irgA, pldA* and *iroE*.

Table 3: Presence of putative virulence genes in *Arcobacter* spp. isolated from water.

Species	Source	No. strains	No. strains (%) generating specific gene amplicon									
			cadF	ciaB	cj1349	hecA*	hecB	irgA	mviN	pldA	tlyA	iroE
A. butzleri	Wastewater	25	24 (96)	25 (100)	22 (88)	18 (72)	5 (20)	0 (0)	25 (100)	24 (96)	25 (100)	5 (20)
	River water	11	11 (100)	11 (100)	11 (100)	4 (36.4)	5 (45.5)	2 (18.2)	11 (100)	11 (100)	11 (100)	3 (27.3)
A. cryaerophilus	*River water*	1	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)
A. lanthieri	Waste water	2	0 (0)	2 (100)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Arcobacter spp.	Wastewater	6	1 (16.7)	6 (100)	0 (0)	3 (50)	0 (0)	0 (0)	2 (33.3)	0 (0)	2 (33.3)	0 (0)
	TOTAL	45	36 (80)	45 (100)	33 (73)	27 (60)	10 (22)	2 (4)	39 (87)	35 (78)	39 (87)	8 (18)
	Waste water	33	25 (75.8)	33 (100)	22 (66.7)	23 (69.7)*	5 (15.2)	0 (0)	27 (81.8)	24 (72.7)	27 (81.8)	5 (15.2)
	River water	12	11 (91.7)	11 (91.7)	11 (91.7)	4 (33.3)	5 (41.7)	2 (16.7)	11 (91.7)	11 (91.7)	11 (91.7)	3 (25.2)

Regarding to the association of the putative virulence genes with the source of isolation it was noteworthy that the gene *hecA* was significantly (P<0.05) more prevalent among the isolates from wastewater than those from river water (**Table 3**). For the other nine genes assayed, although the differences among the sources were sometimes important, statistical analysis showed no relationship between the source of isolation and the prevalence of the virulence-associated genes.

In comparisons between genotype and putative virulence genes, no relation was observed among *A. butzleri* isolates, according to the MLST and virulotyping data. In fact, closely related isolates may possess different virulence-associated genes. This was the case of two isolates which share the ST461 and those which share the ST467, but with different virulotyping scheme each pair.

DISCUSSION

Since ICFMS [33] categorized *Arcobacter* as an emerging foodborne and zoonotic pathogen, little progress has been made in the epidemiology and pathogenesis of this bacterium. The difficulty of recovering *Arcobacter* from complex matrices, along with the lack of its routine detection in most hospital laboratories, leads to the scarcity of scientific contribution on this pathogen compared to other close genera such as Campylobacter [21]. All this contributes to the fact that *Arcobacter* is rarely considered responsible of human disease, which infection causes gastroenteritis similar to that produced by Campylobacter.

The presence of *Arcobacter* in waters has been mainly related to *A. butzleri*, because this is the most commonly identified

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species among isolates of water origin. Our results confirm these findings, since 80% of the isolates were identified as *A. butzleri*, being this species the most predominant in both wastewater and river water. This predominance could be due to the enrichment media usually employed for the recovery of *arcobacter*s, which notably favour the isolation of *A. butzleri* [11,34]. Other reason that has been suggested to explain this predominance is their greater survival capacity to wastewater treatments, which would displace other bacterial populations [10,35]. We also point out the difficulty of identifying other non-butzleri species. Surprising species diversity was detected in this study among non-butzleri isolates: four different species out of nine isolates. Similar results were published by other authors such as Jalava et al. [12] and Gonzalez et al. [36]. All this leads us to consider that the *Arcobacter* population in water, and possibly in other environments, may be much more varied in species than detected. In fact, in line with the recent work of Talay et al. [7], we found a number of isolates that showed discordant results between the most commonly used m-PCRs for *Arcobacter* species identification. Although very useful and verified, these two m-PCRs [25,26] do not identify, or misidentify, the new *Arcobacter* species detected. Therefore, to identify these isolates, both m-PCRs should be used simultaneously and the discordant results should be studied, at least, by sequencing genes like *rpoB*. The description of new species requires long and costly polyphasic studies so these discordant isolates usually remain as *Arcobacter* spp. However, new species are increasingly being described, which shows that *Arcobacter* is still a genus with growing number of species. It would therefore be of great interest to have accurate culture media and/or identification tests to facilitate the recovery and identification of these species other than *A. butzleri*.

The presence of *Arcobacter* in surface waters is of great concern from the epidemiological point of view. The subsequent use of these waters as irrigation or recreational waters increases the risk of contact and/or infection by *Arcobacter*, for both humans and animals. In fact, several water-related outbreaks have been described although in some cases it was not possible to isolate the agent from the patients [12]. This risk is increased considering that the isolates could harbor virulence related genes. According to our data, all surface water isolates examined carried some putative virulence gene, highlighting significantly ($P<0.05$) *A. butzleri* which contained the largest number of them. However, these results could again be biased because the primers used for the detection of these genes were designed from the genome of *A. butzleri*. Therefore, as we already indicated in a previous study [20] it will be necessary to design more specific primers for the non-butzleri species in order to elucidate whether *A. butzleri* is really accumulating or not more putative virulence genes than other species of *Arcobacter*. In agreement with other studies focused on the prevalence of the putative virulence genes among *A. butzleri* isolates from different hosts and environments [20-23,37], the most frequently detected genes were *ciaB* (100%), *mviN* (87%), *tlyA* (87%), *cadF* (80%), *pldA* (78%) and *cj1349* (73%). These six genes, mainly related to adhesion and invasion mechanisms, were used to define the pathotype (P-type) 5, by Piva et al. [22], who suggest that this P-type allows *A. butzleri* to remain competitively in circulation in different hosts. The presence of P-type 5 would explain, in turn, the greater persistence of this species in different environmental sites. Our results support this hypothesis and also point out to a possible relationship between the content of putative virulence genes and the origin of the isolates. Such is the case of the *hecA* gene, involved in the adhesion to cells, significantly ($P<0.05$) more abundant among the wastewater isolates and present in more species than *A. butzleri*. This is not the first time that such an association is referred. In previous works, we [20] and other authors [19] found the *hecA* gen to be significantly ($P<0.05$) related to *A. butzleri* strains isolated from clams. Other authors such as Laishram et al. [23] suggest that strains from fecal or food origin carry a greater proportion of virulence genes compared to environmental strains. However, our results only partially support this hypothesis. Despite not being statistically significant all genes but *ciaB* and *hecA*, were present in a greater proportion in river water isolates compared to those from wastewater, where fecal matter is abundant.

The tree obtained from the MLST analysis, although not robust, revealed a high rate of genetic diversity in the *A. butzleri* population, isolated from both wastewater and river water. No clusters of the isolates were observed in the tree, which is indicative of a dispersed population. In addition, a large number of alleles and new STs were identified, which is consistent with other studies [11,17,18,]. It must be taken into account that analyses of higher numbers of isolates from different sources, including water, are necessary for more accurate assignments from the data base. No relationships between STs and the presence of virulence-associated genes were observed, probably due to the low number of strains examined in this study.

In summary, we have investigated the occurrence and genetic diversity of a population of *Arcobacter* from wastewater and river water, including the presence of virulence-associated genes. Our results confirmed *A. butzleri* as the most predominant species and the one containing a significantly ($P<0.05$) higher number of putative virulence genes as well as a high rate of genetic variability. At least one gene, *hecA*, could be significantly ($P<0.05$) related to the source of isolation, wastewater, although none of the genes could be related to a particular ST. Nevertheless all that warrants further research because most of the enrichment media and molecular techniques are based on *A. butzleri* and this may be biasing the information in favour of this species.

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