Ph.D. Thesis

Genomic and ecological studies on novel *Arcobacter* isolated from abalone

（アワビから分離された新規アルコバクターに関する
遺伝学的および生態学的研究）

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Contents

List of abbreviations

Abbreviations	Explanation
16S rRNA-RFLP	16S ribosomal RNA restriction-fragment length polymorphism
AAI	Average amino-acid Identity
AFLP	Amplified fragment length polymorphism
ANI	Average nucleotide identity
ANIb	Average nucleotide identity calculation based on BLAST+
ANIm	Average nucleotide identity calculation based on MUMmer
APS	Aminopropyl-silane
APS	Adenosine phosphosulfate
CA	Cultivated abalone
CDSs	Coding sequences
CTAB	Cetyltrimethylammonium bromide
DDH	DNA-DNA hybridization
ddH2O	Deionized-destilled H2O
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
FISH	Fluorescent in situ hybridization
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGDC	Genome-to-genome distance calculator
KAAS	KEGG automatic annotation server
KO	KEGG orthology
LA plate	Lactate agar plate

(*continued*)

List of abbreviations (*cont.*)

Abbreviations	Explanation
m-PCR	Multiplex polymerase chain reaction
NADH	Nicotinamide adenine dinucleotide hydride
NADP	Nicotinamide adenine dinucleotide phosphate
OTUs	Operational Taxonomic Units
PAPS	3'-phosphoadenosine-5'phosphosulfate
PBS	Phosphate-buffered saline
POCP	Percentage of conserved proteins
qPCR	Quantitative polymerase chain reaction
RAPD-PCR	Randomly amplified polymorphic DNA polymerase chain reaction
RAST	Rapid annotations using subsystems technology
rTCA cycle	Reductive tricarboxylic acid cycle
RW	Rearing water
SCFA	Short-chain fatty acid
SDS	Sodium dodecyl sulfate
SOX system	Sulfur-oxidation system
TCA cycle	Tricarboxylic acid cycle

Background and objective

Arcobacter, formerly classified as *Campylobacter*, is a member of the class Epsilonproteobacteria, as proposed by Vandamme et al. (1991). Some *Arcobacter* bacteria have shown pathogenicity to humans, and thus many studies have focused on livestock. Species isolated from pork, broiler carcasses, cattle, ducks, human stool, or porcine abortions (Figure 1) include: *Arcobacter cryaerophilus* (Neill et al. 1985), *A. butzleri* (Kiehlbauch et al. 1991 and Vandamme et al. 1992), *A. skirrowii* (Vandamme et al. 1992), *A. cibarius* (Houf et al. 2005), *A. thereius* (Houf et al. 2009), *A. trophiarum* (De Smet et al. 2011), *A. defluvii* (Collado et al. 2011), *A. suis* (Levican et al. 2013), *A. cloacae* (Levican et al. 2013), *A. lanthieri* (Whiteduck-Léveillée et al. 2015), *A. faecis* (Whiteduck-Léveillée et al. 2016), *A. lacus* (Pérez-Cataluña et al. 2018b), and *A. caeni* (Pérez-Cataluña et al. 2018b). Among them, *A. butzleri, A. cryaerophilus*, and *A. skirrowii* are considered to be of clinical interest because they are associated with gastrointestinal disease and bacteremia in humans, and with reproduction disorders, mastitis, and gastric ulcers in farm animals (Ho et al. 2006). *Arcobacter thereius* was also isolated from porcine abortions, but the pathological potential of this species is still unknown (Houf et al. 2009). In contrast, other species have not been directly associated with animal or human diseases.

Recently, *Arcobacter* spp. have also been isolated from marine environments, such as seawater and coastal sediments, and from marine invertebrates (Figure 1). To date, 18 *Arcobacter* species from a total of 29 have been isolated from marine environments (Table 1), suggesting that this environment may be one of the main habitats for this genus. *Arcobacter* are found in bivalves (Collado et al. 2009b, Laishram et al. 2016, Levican et al. 2014, Salas-Massó et al. 2016). Romero et al. (2002) reported *Arcobacter* spp. are

widespread in the Chilean oyster in their analysis using 16S rRNA-RFLP. In addition to bivalves, it has been reported that *Arcobacter* spp. are found in European lobsters (Meziti et al. 2012) and abalone (Tanaka et al. 2004). These results suggest that *Arcobacter* spp. are widely distributed in marine invertebrates, and potentially indigenous bacteria may play some important role in the host. However, knowledge on the presence and diversity of *Arcobacter* associated with marine invertebrates including abalone is still lacking compared to pathogenic *Arcobacter*.

Therefore, at first, it was explored that the diversity and abundance of the genus *Arcobacter* in abalones, an important fishery resource inhabiting shallow water environments, in order to gain knowledge on *Arcobacter* spp. in coastal invertebrates. Also, isolation of *Arcobacter* strains was attempted using a newly established method and an already established one. Second, the genomic information of *Arcobacter* sp. LA11 isolated from abalones was analyzed to estimate the relationships between *Arcobacter* spp.

Table 1. List of *Arcobacter* spp. isolated from different marine environments

Source	Species	Reference
Roots of *Spartina alterniflora* and sediments from salt marshes	*A. nitrofigilis* CI	McClung et al. 1983
Water from hypersaline lagoon	*A. halophilus* LA31B	Donachie et al. 2005
Mussels, brackish water	*A. mytili* F2075	Collado et al. 2009a
Seawater, seaweeds and starfish	*A. marinus* CL-S1	Kim et al. 2010
Sewage	*A. defluvii* SW28-11	Collado et al. 2011b
Mussels	*A. ellisii* F79-6	Figueras et al. 2011b
Mussels and oysters	*A. molluscorum* F98-3	Figueras et al. 2011a
Clams	*A. venerupis* F67-11	Levican et al. 2012
Mussels	*A. bivalviorum* F4	Levican et al. 2012
Mussels and sewage	*A. cloacae* SW28-13	Levican et al. 2013
Estuarine sediment	*A. anaerophilus* JC84	Sasi Jyothsna et al. 2013
Mussels	*A. ebronensis* F128-2	Levican et al. 2015
Seawater	*A. aquimarinus* W63	Levican et al. 2015
Seawater	*A. acticola* AR-13	Park et al. 2016
Seawater	*A. pacificus* SW028	Zhang et al. 2016
Great scallop larvae and tank seawater	*A. lekithochrous* LFT 1.7	Diéguez et al. 2017
Abalone	*A. lekithochrous* MA5 (syn. *A. haliotis* MA5)	Tanaka et al. 2017
Sewage	*A. canalis* F138-33	Pérez-Cataluña et al. 2018a

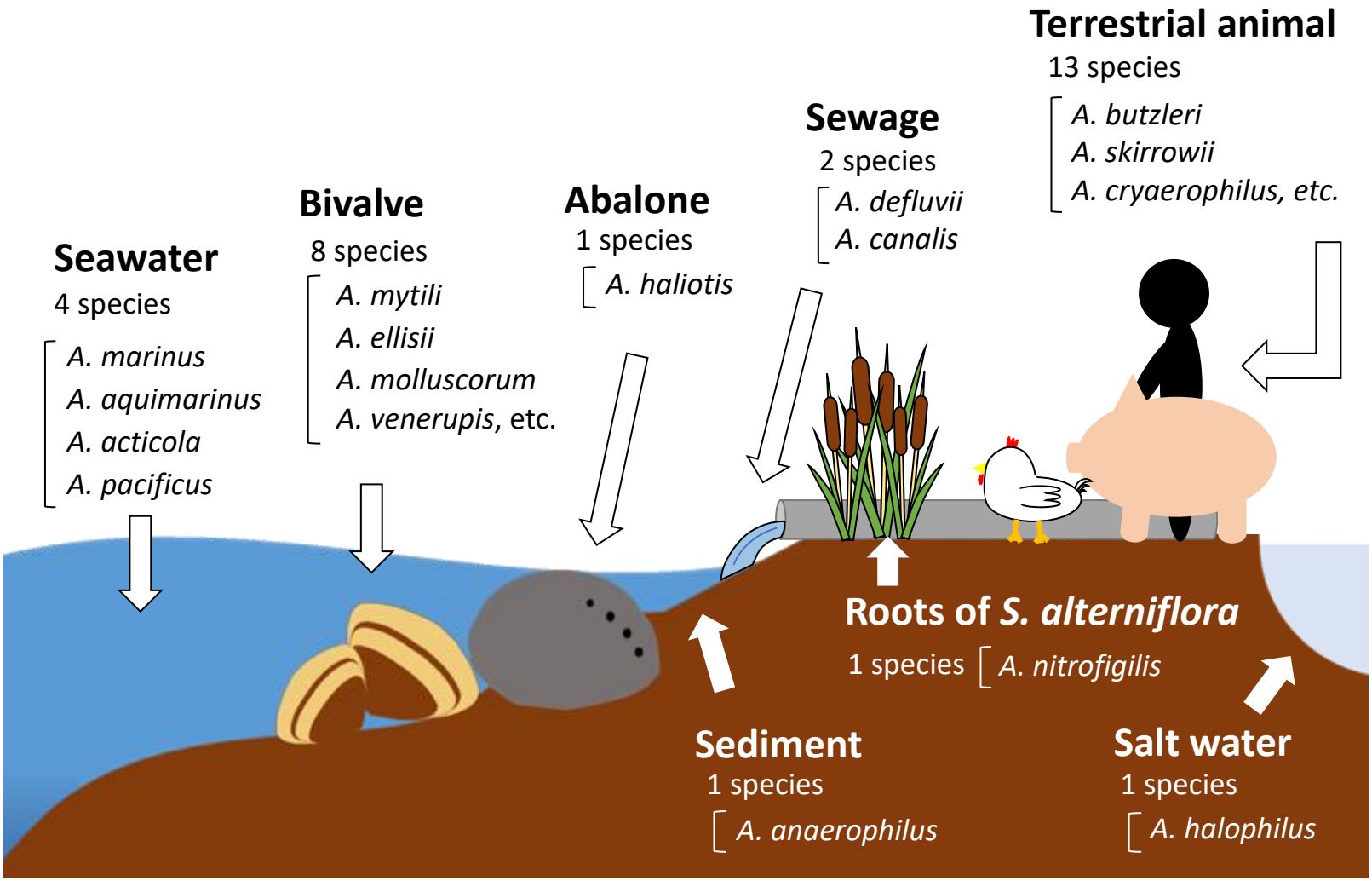

Figure 1. Described *Arcobacter* species in different habitats.

Chapter 1 Diversity, enumeration, and isolation of *Arcobacter* spp. in the giant abalone, *Haliotis gigantea*

1.1 Introduction

The marine environment is considered to be one of the main habitats of *Arcobacter* species. *Arcobacter* have been frequently reported in bivalves, and edible bivalves pose a high risk to human health due to the ability of viruses and bacteria to be entrapped by their filter-feeding (Potasman et al. 2002). Many reports have demonstrated high abundances of *Arcobacter* spp. in bivalves using various types of methods, such as m-PCR (Collado et al. 2009, Patyal et al. 2011, Levican et al. 2014, Šilha et al. 2015 and Mottola et al. 2016), 16S rRNA RFLP (Romero et al. 2002, Collado et al. 2009 and Mottola et al. 2016), and culture (Romero et al. 2002, Salas-Massó et al. 2016, Ottaviani et al. 2017, Rathlavath et al. 2017 and Zhang et al. 2019). *Arcobacter* has also been detected in the gastropod mollusk *Haliotis discus hannai* using a 16S rDNA clone library (Tanaka et al. 2004), and also isolated from *Haliotis gigantea* (Tanaka et al. 2017). These studies suggest that *Arcobacter* might also inhabit abalones in addition to bivalves. However, the diversity and abundance of *Arcobacter* in gastropods, including abalones, have not been clarified.

In this section, I explore the diversity and abundance of the genus *Arcobacter* in abalones, an important fishery resource inhabiting shallow water environments, in order to gain knowledge on *Arcobacter* spp. in marine invertebrates. Cultivation-independent methods, such as *Arcobacter*-specific clone libraries and FISH, were used. I also attempted to isolate *Arcobacter* strains using selective cultivation, and report here the genetic relationships between successfully isolated strains.

1.2 Material and methods

1.2.1 Sample collection and DNA extraction

For the isolation of *Arcobacter* species, two isolation methods were used. In the first method, the bacterial mixture from abalone tissue was spread on an LA plate (lactate 5 g, yeast extract 0.1 g, agar 15.0 g, 75% artificial seawater 1000 mL). The LA plate was incubated at 25 °C for 3 days under aerobic or micro-aerobic conditions, and then presumed *Arcobacter* colonies (tiny and beige to off-white in color) were transferred onto Marine Agar 2216 (Difco, Detroit, MI, USA) with 100 μg/mL of vibriostatic agent O129 (2,4-diamino-6,7-diisopropyl pteridine) and incubated for 3 days at 25 °C under aerobic or micro-aerobic conditions. After incubation, *Arcobacter*-like colonies were subcultivated on Marine Agar 2216 and subjected to 16S rRNA gene sequence analysis. The internal organs, including the gut and gills, were collected from the abalones followed by the previously described method (Tanaka et al. 2004). To tear off bacterial cells from their host tissue, a beads beater was used on the condition of slower stroke and shorter time. Abalone specimens were pooled into each tube and homogenized using a beads beater (4200 rpm, 30 seconds; Tietech Co., Nagoya, Japan). Host tissues were removed from CA samples by quick centrifugation (1 s, 8000 g), and the supernatant was transferred to new tubes and centrifuged for 20 min at 15000 g to recover bacterial cells. RW samples were concentrated (50 ×) using 0.22 μm cellulose membrane filters (Advantec, Tokyo, Japan) and resuspended in sterile PBS (130 mM NaCl, 10 mM Na_2HPO_4/ NaH_2PO_4; pH 7.4). Aliquots of bacterial pellets thus obtained from CA and RW samples were subsequently used for FISH analysis and DNA extraction. Bacterial genomic DNA from each sample was extracted using Promega DNA purification system (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions.

1.2.2 PCR analysis, construction of *Arcobacter*-specific clone libraries and sequencing

The *Arcobacter* genus-specific primers, ARC94F primer (5'-TGCGCCACTTAGCTGACA-3') and ARC1446R primer (5'-TAGCATCCCCGCTTCGAATGA-3') (Harmon et al. 1996 and Snaidr et al. 1997) were used to amplify the *Arcobacter* 16S rRNA gene from each sample. PCR reaction mixtures contained 1× PCR reaction buffer, 200 μM dNTP, 5 pmol of each primer, 2.5 units Ex Taq polymerase (TaKaRa Biotechnology Corp., Kyoto, Japan), and 10-100 ng of DNA for a total volume of 50 μL. PCR reactions were performed using an iCycler (Bio-Rad Lab., Hercules, CA, USA). The amplification conditions were as follows: initial denaturation of 4 min at 95 ºC followed by 25 cycles of denaturation for 30 s at 95 ºC, primer annealing for 30 s at 55 ºC, and primer extension at 72 ºC for 1.5 min. This was followed by a final extension reaction at 72 ºC for 7 min. Distilled H_2O was used as the template for negative controls; these produced no PCR product, indicating the absence of contaminating DNA in reactions and reagents. The 16S rRNA gene amplicons were purified by the PCR Preps DNA Purification System (Promega Corp.) according to the manufacturer's instructions, and subsequently ligated into the TOPO TA cloning vector (Invitrogen Corp., Carlsbad, CA, USA). Ligation products were transformed into Escherichia coli One Shot TOP10 cells (Invitrogen Corp.) and screened for plasmid insertions by following the manufacturer's instructions. Plasmid DNA containing insertions was sequenced with the ARC94F primer using the Sanger method with an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). Chromatograms of DNA sequences were examined using Chromas v2.3.3 (Technelysium Pty Ltd., South Brisbane, Australia). All sequences were examined for chimerism using a chimeric sequences detection tool, Bellerophon (Huber et al. 2004).

1.2.3 Fluorescent in situ hybridization

The total number of *Arcobacter* cells in abalone samples was counted using the FISH method (Kepner and Pratt 1994). Aliquots of bacterial pellets obtained from CA and RW samples were rinsed by 600 μL of sterile PBS and centrifuged at 12,000 g for 5 min, then removed supernatant. These procedures were performed two times. Samples were fixed by adding one volume of PBS to three volumes of 4% paraformaldehyde/PBS, and incubating at 4 °C for 3 h. After centrifugation, the fixative was removed, and the bacterial pellet was washed twice with PBS. The washed cells were mixed with 1 × PBS and 96% EtOH (1:1) and stored at -20 °C (Roller et al. 1994). Three micro little of fixed-cell suspension was spread on the well of an APS-coated 8-well slide (Matsunami, Japan). The slides were air-dried and dehydrated by successive immersion in 50, 80, and 99.5%. Hybridization was performed based on a previous study by Ootsubo et al. (2003), with modifications. A 5'-end TAMRA-labeled ARC94F probe (5'-TGCGCCACTTAGCTGACA-3'; Sigma-Aldrich Corp, St. Louis, MO; Moreno et al. 2003) was designed to target the 16S rRNA of *Arcobacter* spp., and a 5'-end FITC -labeled EUB338 probe (5'-GCTGCCTCCCGTAGGAGT-3'; Sigma-Aldrich Corp; Amann et al. 1990) to target the 16S rRNA gene of most members of the domain Bacteria. Prior to hybridization, each probe was added to pre-warmed hybridization buffer [0.9 M NaCl, 20% formamide, 20 mM Tris-HCl (pH 7.4) and 0.1% SDS] to a final concentration of 10 pM. The probe solution was spread on each hybridization well, and the slides incubated at 46 °C for 3 h in an MHS-2000 hybridization oven (EYELA, Tokyo, Japan). After hybridization, each well was washed twice with washing buffer [20 mM Tris-HCl (pH 7.4), 180 mM NaCl and 0.01% SDS], rinsed with ddH_2O and air-dried. An epifluorescence light microscope (Eclipse 400; Nikon Corp., Tokyo, Japan), was used for

observing the stained cells. Due to insufficient concentration of rearing water, detecting the mean±SE from RW samples was unable.

1.2.4 Isolation of *Arcobacter* spp.

In the first isolation method, after pre-culture using LA plates without vibriostatic O129 under aerobic or micro-aerobic conditions, the total numbers of colonies were 117 or 222 isolated from *H. discus*, respectively. All isolations were translated to LA plates with vibriostatic O129 and incubated at 25 °C for 3 days. After incubation, 45 or 143 colonies grew under aerobic or micro-aerobic conditions, respectively. *Arcobacter* colonies (tiny and beige to off-white in color) were selected from grown colonies, and then colony PCR was performed to identify them. Colony PCR subsequently confirmed that one colony (strain LA11) of the selected *Arcobacter*-like colonies for each sample was affiliated with *Arcobacter* spp. (LC180340). The 16S rRNA gene sequence of LA11 showed high similarity (97.7%) with uncultured epsilon proteobacterium clone OMEGA_pl_ Cons_1(6) (EU052236) (Table 4). Since the described *Arcobacter* species most similar to strain LA11 was *A. anaerophilus* strain DSM 24636 (95.7%), this suggests a potentially new species. The second isolation method was followed by Salas-Massó et al. (2016), but the media was slightly modified by changing 2.5% NaCl to artificial seawater. The bacterial mixture from three abalones was diluted ten times using sterile Daigo's Artificial Seawater SP (Nihon Pharmaceutical Co., Tokyo, Japan) and 100 µL of the diluted mixture was inoculated into *Arcobacter* Broth (Oxoid Ltd., Hampshire, UK, USA) with C.A.T. supplement [cefoperazone at 8 mg/L, amphotericin B at 10 mg/L and teicoplanin at 4 mg/L] (Oxoid Ltd., Atabay and Corry 1997) suspended in 75% Daigo's Artificial Seawater SP instead of distilled water with 2.5% NaCl. The culture solutions were incubated for 48 or 96 h at 15 °C (sample

codes: 15T48H and 15T96H, respectively) or 25 °C (sample codes: 25T48H and 25T96H) under aerobic conditions. After cultivation, 200 μL of post-cultured broth was pipetted onto the surface of polycarbonate membrane filters (pore size, 0.4 μm: Merck Millipore, Burlington, MA) placed on Marine Agar 2216. The plates were incubated at room temperature for 30 min to allow passive filtration (Atabay and Corry 1997). Next, the filters were carefully removed and the flow-through was spread on Marine Agar 2216. The media was incubated at the same temperature and time as the primary culture. After cultivation, presumed *Arcobacter* colonies were selected and applied to colony PCR in the same way as *Arcobacter*-specific clone libraries using ARC94F and ARC1446R primers. Positive PCR products were sequenced using standard Sanger sequencing.

1.2.5 Cluster analysis of the bacterial community structure

For each sample, sequences were aligned and grouped in OTUs with >97 % sequence identity (Stackebrandt and Goebel 1994). Homology searches were performed using sequences of approximately 700 bp and the highest homology sequences with each OTUs were chosen as the closest relatives. All BLASTn searches were performed with the default parameters available through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Multiple alignments and calculation of distant matrixes were performed by CLUSTAL W (Thompson et al. 1994), using MEGA 7.0 (Kumar et al. 2016). A phylogenetic tree was constructed using the maximum-likelihood method of MEGA 7.0, with 1000 replicates in the bootstrap analysis and Kimura's two-parameter model (Kimura 1980). Distances were estimated with the Jukes-Cantor correction.

1.3 Results

1.3.1 *Arcobacter*-specific clone libraries

To perform a comprehensive search for *Arcobacter* spp. from abalone and their surrounding seawater, I established *Arcobacter*-specific 16S rRNA gene clone libraries. A total of 120 and 30 clones were obtained in our study from CA and RW samples, respectively (Table 2). Among these clones, *Arcobacter* sequences were observed as twelve OTUs from CA and 7 OTUs from RW.

The 16S rRNA genes identified from abalone (CA) using the *Arcobacter*-specific clone library did not show high similarity to known species within the NCBI database (Figure 2). The most abundant genotype in CA was CA11 (25 clones, accession number: LC133145), which had a high similarity score of 99.2% to uncultured bacterium clone SF-July-74 (HM591442). CA1 (LC133157) had 100% similarity to bacterium clone KSTye-VF1-B-003 (JQ611206) collected from venting fluid in a yellow vent off Kueishan Island, while CA3 and CA12 (LC133141 and LC133148) each had a similarity of 98.8% and 100% to uncultured bacterium clone SF-July-156 (HM591463) and epsilon-proteobacterium Yb-F (AB496655) collected from seawater. CA3 did not cluster with any sequences. CA4, CA5, CA8 and CA9 (LC133159, LC133146, LC133160 and LC133142) were assigned to uncultured bacteria collected from marine environments: CA4 and CA5 showed similarity of 98.9% and 99.5% to uncultured *Arcobacter* sp. clones DVASD_D318 and DVBSW_D345, respectively (KF463610 and KF722009) from a marine coastal ecosystem, CA8 showed 97.2% similarity to uncultured bacterium clone TopBa31 (EF999357) from Pearl River Estuary sediments, and CA9 showed 95.2% similarity to uncultured epsilon-proteobacterium clone AT-pp13 (AY225610) from pumice fragments exposed to a Mid-Atlantic Ridge vent. CA6 and CA7 (LC133158 and LC133140) were assigned to uncultured bacterium clone AJ-U-CD-41(H) (JX170315)

isolated from the intestine of a sea cucumber, *Apostichopus japonicas*, with 98.4% and 100% similarity, respectively. Finally, CA2 and CA10 (LC133161 and LC133156) were closely affiliated with an isolate or a clone collected from protists. CA2 had 98.6% similarity to *Arcobacter* sp. EP1 (LT629996) isolated from the epibiont of unicellular protists, and CA10 had 99.6% similarity to uncultured epsilon-proteobacterium clone PI_4z10e from coastal bacterioplankton sampled at Plum Island Sound Estuary.

In the rearing water samples, RW5 (LC133151) showed 98.5% sequence similarity to *A. pacificus* strain SW028 isolated from seawater (JN118552), and RW10 (LC133153) had a 97.4% high homology to *A. bivalviorum* strain F4 isolated from bivalves (FJ573217). RW1 (LC133149) showed a sequence similarity of 98.7% to uncultured bacterium clone HF071 (JX391310) detected from marine sediment. RW4 (LC133150) was affiliated with uncultured epsilon-proteobacterium clone PI_4z7d in a coastal bacterioplankton sample from Plum Island Sound Estuary, at 98.3% similarity. RW17 (LC133154) was closely related to uncultured bacterium clone C13W_197 (HM057704) collected from seawater, at 98.8% similarity. RW6 (LC133152) showed 97.9% similarity to uncultured bacterium clone SF-July-156 (HM591463), and RW20 (LC133155) was closely related at 99.8% similarity to uncultured marine bacterium clone B-Alg40 (HM437504) detected from the surface of algae.

1.3.2 Detection of ARC94F-positive cells by FISH

The amount of *Arcobacter* spp. in homogenized cultured abalone and rearing water samples from Minami-ise, Mie, Japan, was determined by FISH. From the abalone samples (n=9), with a total bacteria count of $1.18 \pm 0.71 \times 10^7$ cells/g, the number of ARC94F-positive bacteria was $8.06 \pm 0.05 \times 10^5$ cells/g, dominating the total bacteria count at 6.96 ± 0.72% (Table 3). In the abalone rearing water (n=2), the total bacteria

count for each sample was 2.76×10^4 cells/mL and 4.63×10^4 cells/mL (the average cells, 3.70×10^4 cells/mL), respectively. Among these, the number of ARC94F-positive bacteria were 1.33×10^3 and 1.08×10^3 cells/mL (the average cells, 1.21×10^3 cells/mL), dominating 4.8% or 2.3% of the total bacteria (the average rate, 3.55%), respectively. It was observed that ARC94F-positive cells colonized on the abalone tissue (Figure 3).

1.3.3 Isolation of *Arcobacter* spp.

In the first isolation method, after pre-culture using LA plates without vibriostatic O129 under aerobic or micro-aerobic conditions, the total numbers of colonies were 117 or 222 isolated from *H. discus*, respectively. All isolations were translated to LA plates with vibriostatic O129 and incubated at 25 °C for 3 days. After incubation, 45 or 143 colonies grew under aerobic or micro-aerobic conditions, respectively. *Arcobacter* colonies (tiny and beige to off-white in color) were selected from grown colonies, and then colony PCR was performed to identify them. Colony PCR subsequently confirmed that one colony (strain LA11) of the selected *Arcobacter*-like colonies for each sample was affiliated with *Arcobacter* spp. (LC180340). The 16S rRNA gene sequence of LA11 showed high similarity (97.7%) with uncultured epsilon proteobacterium clone OMEGA_pl_ Cons_1(6) (EU052236) (Table 4). Since the described *Arcobacter* species most similar to strain LA11 was *A. anaerophilus* strain DSM 24636 (95.7%), this suggests a potentially new species.

In the second isolation method, when incubation of samples from natural *H. gigantea* collected in Mie, Japan, was performed for 48 hours and 96 hours at 15 °C or 25 °C (sample codes: 15T48H, 25T48H, 15T96H, or 25T96H). For the samples incubated at 15 °C or 25 °C for 48 h, a few colonies were isolated, but they were not identified as *Arcobacter* spp. In contrast, the total numbers of colonies were 871 and 338, respectively,

when the incubation time was extended for 96 hours. Upon further selection, twelve and eight colonies from the 15T96H and 25T96H samples were presumed to be *Arcobacter* colonies. Colony PCR subsequently confirmed that five of the selected *Arcobacter*-like colonies for each sample were affiliated to *Arcobacter* spp. Finally, colonies with sequence similarity of >97% were grouped and given a similar designation. I obtained four strains designated as 15T96H-1, one strain designated as 15T96H-2 from the 15T96H sample, and five strains designated as 25T96H-1 from the 25T96H sample (Table 4). The 16S rRNA gene sequences of 15T96H-1 and 15T96H-2 showed high similarity (98.2% and 98.3%, respectively) with uncultured bacterium clone HglApr921 (JX016315). Since both these strains have low homology with other isolated *Arcobacter* species (93.3-94.3% similarity), they suggest potential new species. On the other hand, all strains of 25T96H-1 showed similarity ranging from 99.2 to 100% with *A. marinus*.

1.4 Discussion

Thus far, research on *Arcobacter* has mainly been focused on detection since several *Arcobacter* species are pathogenic to humans (Houf et al. 2000, Ho et al. 2006 and Collado and Figueras 2011). Meat and seafood are the most common sources of *Arcobacter* reported (Atabay et al. 1997, Houf et al. 2002, Hume et al. 2001, Romero et al. 2002, Collado et al. 2009b, Levican et al. 2014, Salas-Massó et al. 2016, Mottola et al. 2016, Rathlavath et al. 2017a and 2017b and Vicente-Martins et al. 2018). *Arcobacter* is also detected or isolated from seawater, sewage and drinking water, and these environments are considered important as they could be one of the possible routes of transmission of *Arcobacter* to human and animal intestinal tracts (Collado and Figueras 2011). Various methods including ERIC-PCR, RAPD-PCR (Houf et al. 2002), and AFLP (On et al. 2003 and 2004) have been used to detect and elucidate the transmission routes or to trace the sources of *Arcobacter* outbreaks (Collado and Figueras 2011). m-PCR that can detect multiple species simultaneously has also been used (Houf et al. 2000, Brightwell et al. 2007 and Khan et al. 2017). ERIC-PCR in particular has been successfully applied to outbreak investigations (Vandamme et al. 1993) in food. Although these methods have advantages due to its simplicity and cost, they detect only a specific species from isolated strains or from mixed cultures based on specific culture conditions (González et al. 2017).

To prevent bias resulting from culture-dependent methods in this study, *Arcobacter*-specific clone libraries was used to directly identify 16S rRNA gene sequences. Twelve OTUs relating to *Arcobacter* were detected from abalones using *Arcobacter*-specific clone libraries (Table 2), all clustered with previously-reported *Arcobacter* sequences (Figure 2). All the OTUs showed similarity to 16S rRNA genes detected from marine environments such as marine invertebrates or seawater, but not from

those identified from terrestrial sources such as poultry. Furthermore, these sequences are not classified with any other known *Arcobacter* species. Interestingly, using our analytical approach, the samples from abalones also did not show the presence of pathogenic *Arcobacter* spp., *A. butzleri, A. skirrowii* and *A. cryaerophilus*, commonly isolated or detected from bivalves. Bivalves such as mussels and clams are filter feeders that feed plankton using gills, while abalones feed on brown algae. Thus, they have a more developed digestive system compared to bivalves. The result suggests that gastropods such as abalone may not be host or harbor pathogenic *Arcobacter* species, perhaps due to their different feeding habits and digestive system.

In this study, I employed the ARC94F *Arcobacter*-specific probe for FISH against cells isolated from abalone for detection and quantification. This ARC94F probe has been used for specific counting of genus *Arcobacter* in seawater (Moreno et al. 2003, Fera et al. 2008 and 2010). The ratio of ARC94F-positive cells suggested that *Arcobacter* might be a common bacterial genus in abalones (6.96%, Table 3). *H. gigantea* appears to be a habitat for *Arcobacter* species, but the role and effect on their hosts are still unclear.

Four phenotype 11 strains related to *Arcobacter* were isolated from abalone. Strain LA11 was isolated using LA plates. The medium contained lactate as the lone carbon source because *A. haliotis* strain MA5 isolated from abalone can grow with lactate as the only carbon source (Tanaka et al. 2017). However, *Vibrio* spp. are also able to grow and be isolated in that condition. For that reason, medium supplemented with an antibiotic against *Vibrio* was used for screening. Although the only isolated strain using the above method was LA11, the strain clustered with sequences of CA2 and CA11, which are the most frequently detected using *Arcobacter*-specific clone libraries. This suggests that the dominant *Arcobacter* in cloning was successfully isolated. The 16S rRNA gene sequence of strain LA11 was most closely related to *A. anaerophilus* as a result of homology

analysis of BLAST, but the phylogenetic tree showed that LA11 grouped into the same cluster with *A. bivalviorum*. This is probably due to the short sequence reads used for the phylogenetic analysis.

Regarding the cultivation of *Arcobacter* spp., several conditions have been introduced, such as altering the NaCl concentration (Salas-Massó et al. 2016) or the requirement of sea salts (Diéguez et al. 2017). Hence, artificial sea salt was used instead of NaCl and added more than 2.5% sea salt during isolation. In addition, the incubation temperature used for *Arcobacter* isolation was set to 15 or 25 °C, which are closer to the seawater temperatures of the natural habitat of the abalones at Ise Bay, Mie prefecture, against common methods (30 to 37°C: Vandamme et al. 1991, Houf et al. 2000, Collado et al. 2009b, Merga et al. 2011, Salas-Massó et al. 2016 and 2018, Laishram et al. 2016, González et al. 2017). As a result, ten *Arcobacter* isolates (five known and five novels) were recovered from samples 15T96H and 25T96H. These were classified into three genotypes based on 97% sequence similarity (15T96H-1, 15T96H-2 and 25T96H-1). The 16S rRNA gene of 25T96H-1 had a high similarity of 99.2-100% to *A. marinas*, which has been isolated from a mixture of seawater and starfish (Kim et al. 2010). In contrast, the isolates 15T96H-1 and 15T96H-2 had no closely related sequences in all other known *Arcobacter* isolates. Including comparisons with uncultured clones, the 16S rRNA genes of 15T96H-1 or 1596H-2 have related to an uncultured clone detected from the Pacific oyster *Crassostrea gigas*, an invertebrate living in shallow water (Madigan et al. 2014) and marine bulk water (Teeling et al. 2012). Both 15T96H-1 and 15T96H-2 were isolated only at the 15 °C incubation temperature. From these observations, I believe that 15T96H-1 and 15T96H-2 will be able to be isolated from various marine invertebrates with sea salt medium at lower temperatures. In terms of incubation time, no *Arcobacter* species were isolated within two days of incubation. This implies that when incubation

temperature is lower than 37 °C, *Arcobacter* requires more than 96 hours to grow before colonies can be detected. There are still many species of *Arcobacter* detectable by molecular methods in abalones that are not cultivable. I feel that isolation methods should be improved to obtain these uncultured *Arcobacter* species.

In conclusion, I succeeded in detecting several new *Arcobacter* genotypes from abalone using *Arcobacter*-specific 16S rRNA gene libraries. Furthermore, since most of the clones showed low similarity with other known *Arcobacter* spp. and no pathogenic *Arcobacter* were detected or isolated from abalone, I need further investigations for uncultured *Arcobacter* spp. which remains to be determined in *Haliotis gigantea*.

Table 2. 16S rRNA gene sequences identified in the clone library from abalone or seawater

Samples	OTUs (accession number)	No. of clones	Highest similarity sequence (accession number)	Identity (%)
Abalone (CA)	CA1 (LC133157)	16	Uncultured bacterium clone KSTye-VF1-B-003 (JQ611206)	100
	CA2 (LC133161)	3	*Arcobacter* sp. EP1 (LT629996)	98.6
	CA3 (LC133141)	17	Uncultured bacterium clone SF-July-156 (HM591463)	98.8
	CA4 (LC133159)	3	Uncultured *Arcobacter* sp. clone DVASD_D318 (KF463610)	98.9
	CA5 (LC133146)	3	Uncultured *Arcobacter* sp. clone DVBSW_D345 (KF722009)	99.5
	CA6 (LC133158)	3	Uncultured bacterium clone AJ-U-CD-41(H) (JX170315)	98.4
	CA7 (LC133140)	20	Uncultured bacterium clone AJ-U-CD-41(H) (JX170315)	100
	CA8 (LC133160)	1	Uncultured bacterium clone TopBa31 (EF999357)	97.2
	CA9 (LC133142)	24	Uncultured epsilon-proteobacterium clone AT-pp13 (AY225610)	95.2
	CA10 (LC133156)	4	Uncultured epsilon-proteobacterium clone PI_4z10e (AY580424)	99.6
	CA11 (LC133145)	25	Uncultured bacterium clone SF-July-74 (HM591442)	99.2
	CA12 (LC133148)	1	Epsilon-proteobacterium Yb-F (AB496655)	100
Rearing water (RW)	RW1 (LC133149)	12	Uncultured bacterium clone HF071 (JX391310)	98.7
	RW4 (LC133150)	4	Uncultured epsilon-proteobacterium clone PI_4z7d (AY580420)	98.3
	RW5 (LC133151)	4	*Arcobacter pacificus* SW028 (JN118552)	98.5
	RW6 (LC133152)	3	Uncultured bacterium clone SF-July-156 (HM591463)	97.9
	RW10 (LC133153)	4	*Arcobacter bivalviorum* F4 (FJ573217)	97.4
	RW17 (LC133154)	2	Uncultured bacterium clone C13W_197 (HM057704)	98.8
	RW20 (LC133155)	1	Uncultured marine bacterium clone B-Alg40 (HM437504)	99.8

Table 3. Total and *Arcobacter* bacterial counts from abalones or in rearing water by direct microscopy

	Abalone (n=9, mean±SE)	Rearing water (n=2, mean)
EUB338 (cells/g or mL)	$1.18 \pm 0.71 \times 10^7$	3.70×10^4
ARC94 (cells/g or mL)	$8.06 \pm 0.05 \times 10^5$	1.21×10^3
Rate of *Arcobacter* (% of total bacterial count)	6.96 ± 0.72	3.55

Table 4. 16S rRNA gene sequences identified in the isolation from abalone using two types isolation methods

Strains (accession number)	**No. of isolates**	**Highest similarity sequence (accession number)**	**Identity (%)**
LA11 (LC180340)	1	Uncultured epsilon proteobacterium clone OMEGA_pl_ Cons_1(6) (EU052236)	97.7
15T96H-1 (LC457972)	4	Uncultured bacterium clone HglApr921 (JX016315)	98.2
15T96H-2 (LC457973)	1	Uncultured bacterium clone HglApr921 (JX016315)	98.3
25T96H-1 (LC457974)	5	*Arcobacter marinus* strain CL-S1 (EU512920)	100

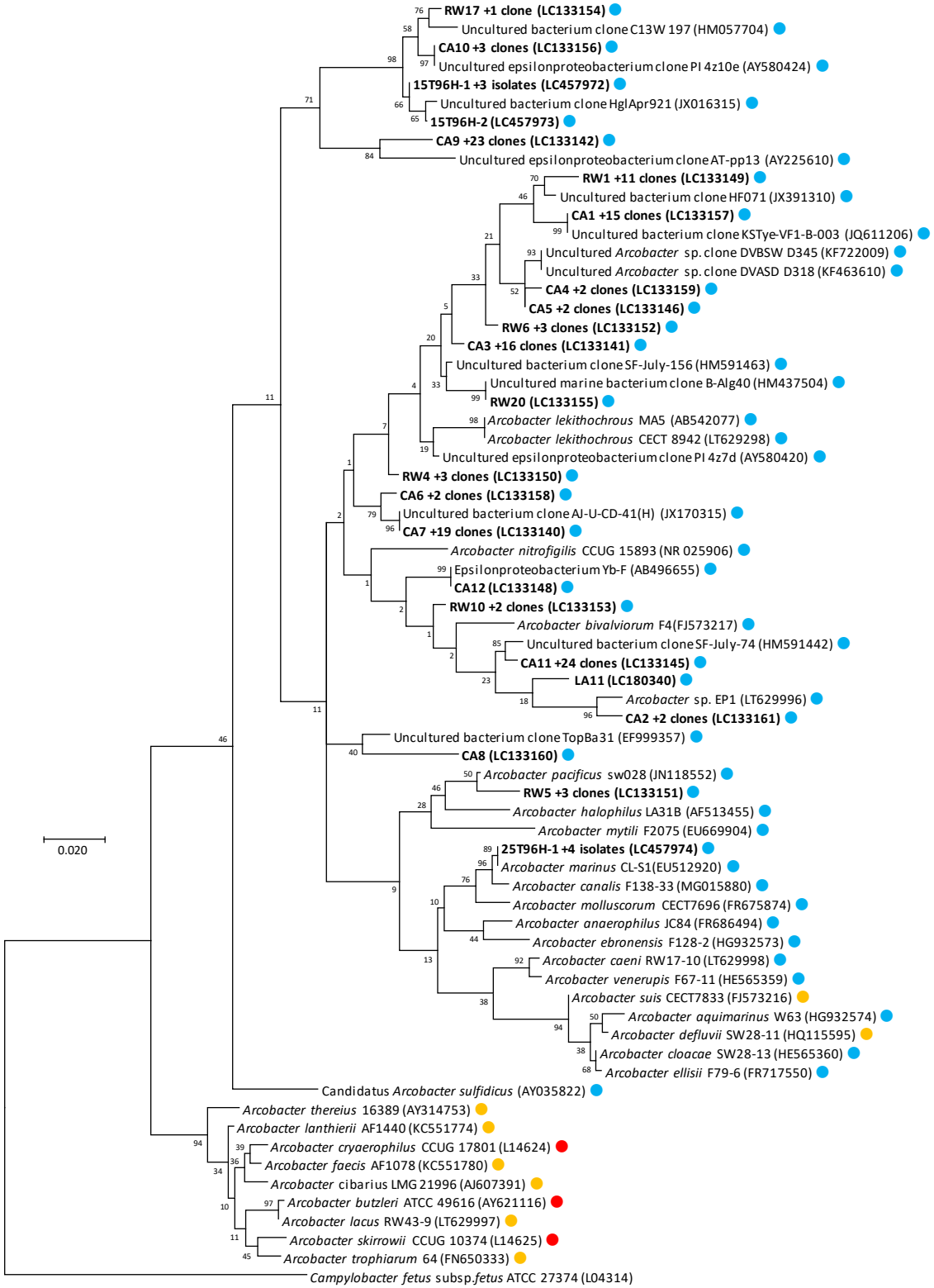

Figure 2. Legend was noted next page.

Figure 2. 16S rRNA gene-based phylogenetic tree of *Arcobacter* spp. from abalone and environmental samples. Circles colors indicate origins or pathogenicity of *Arcobacter* spp. as follows: blue, marine habitats, orange, terrestrial environments and red, pathogenic species. The tree was generated using the maximum likelihood method with 1,000 replicates in the bootstrap analysis. The distances were estimated with the Jukes-Cantor correction. The tree was rooted with Campylobacter fetus subsp. fetus ATCC 27374, and gene sequences are followed by GenBank accession numbers in parentheses. Scale bar represents 2% sequence divergence.

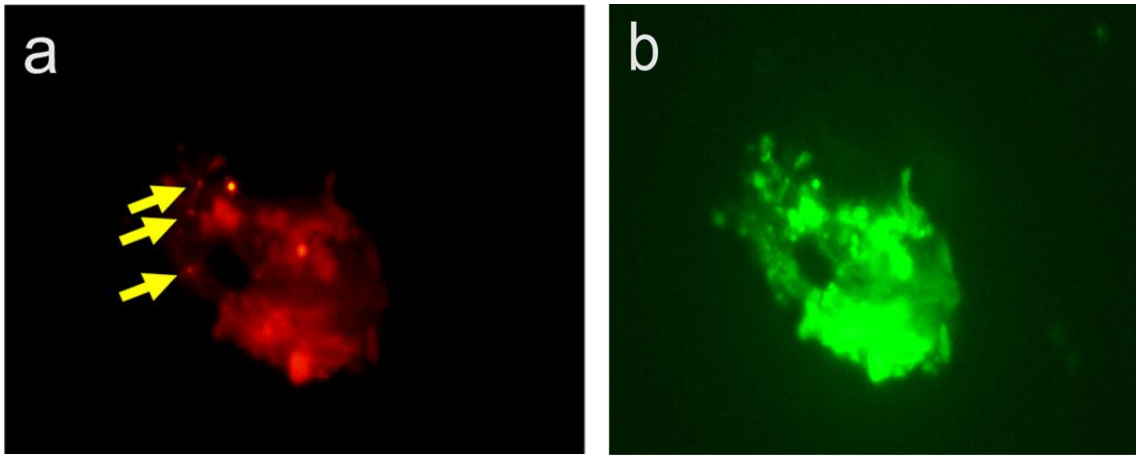

Figure 3. FISH photograph using (a) probe ARC94F and (b) probe EUB338, showing positive cells attached to abalone tissues. Yellow arrows indicate *Arcobacter*-stained cells.

Chapter 2 Genomic characterization of *Arcobacter* sp. LA11 isolated from abalones

2.1 Introduction

The class Epsilonproteobacteria, in which the genus *Arcobacter* is classified, is widely known for its pathogenic genera *Campylobacter*, *Helicobacter*, and to a lesser extent *Arcobacter* in terrestrial environments (Gilbreath et al. 2011). *Campylobacter* spp. are a major cause of acute gastroenteritis in a variety of animals, including humans, with *Campylobacter jejuni* and *Campylobacter coli* responsible for the majority of cases (Altekruse et al. 1999, Janssen et al. 2008, Skirrow and Blaser 1995). *Helicobacter* spp. can be divided into two major categories, gastric and non-gastric species. *Helicobacter pylori* is a gastric species that is the major pathogen among all *Helicobacter* spp. and is commonly associated with outcomes that range from asymptomatic carriage and mild gastritis to more severe diseases such as peptic ulcer disease and gastric cancer (Blaser et al 1995, Blaser 1998, Ernst and Gold 2000, Parsonnet et al. 1991 and 1994, Swisher and Barbati 2007, Talley et al. 1991). *Arcobacter* spp. include three potential human pathogens, *A. butzleri, A. cryaerophilus*, and *A. skirrowii*, and they have been isolated from cases of gastroenteritis, endocarditis, peritonitis, and bacteraemia as well as from healthy humans (Ho et al. 2006, Collado and Figueras 2011). The habitat and clinical symptoms of these *Arcobacter* species are similar to pathogenic *Campylobacter* species, and they belong to the same family, Campylobacteriaceae, as *Campylobacter*. However, the potential pathogenic *Arcobacter* spp. are only 3 out of 29 species that have been described.

In contrast, some members of Epsilonproteobacteria also inhabit hydrothermal vents, and they often associate as ecto-, endo-, or epi-symbionts with diverse invertebrate

animals including polychaete worms (*Alvinella pompejana*) (Haddad et al. 1995), shrimp (*Alvinocaris longirostris*) (Tokuda et al. 2008), crabs (*Shinkaia crosnieri*) (Fujiyoshi et al. 2015), and gastropods (*Alviniconcha* spp.) (Suzuki et al. 2005 and 2006, Urakawa et al. 2005). The members of this group are considered as symbionts that provide organic carbon compounds by carbon fixation and detoxify hydrogen sulfide.

The members of genus *Arcobacter* have been referred to as heterotrophs; however, some of them have autotrophic activities. Wirsen et al. reported in 2002 that Candidatus "*Arcobacter sulfidicus*" isolated from the coastal environment is a lithoautotrophic sulfur oxidizer. This bacterium contains activities of the key enzymes of the reductive tricarboxylic acid cycle (rTCA) for autotrophic CO_2 fixation, but no activities of key enzymes of other CO_2 fixation pathways, such as the Calvin cycle, the reductive acetyl-coenzyme A pathway, or the 3-hydroxypropionate cycle (Hügler et al. 2005). Some species of *Arcobacter* living in the marine environment have similar genes to Epsilonproteobacteria, which is a symbiont of invertebrates on the deep-sea floor. Furthermore, there are some studies showing that *Arcobacter* has symbiotic relationships with some hosts. Hamman et al. reported in 2016 that the association of *Lenisia limosa*, a free-living unicellular protist, with *Arcobacter* is driven by the transfer of hydrogen and is mutualistic, providing benefits to both partners. In the same report, proteomics revealed that the presence of *Lenisia* stimulates the expression of known 'virulence' factors by *Arcobacter*, and these proteins may act for mutual benefit. However, it has remained unclear what function these proteins have except for colonization. There is little research on host-*Arcobacter* mutualism, and even when they are in a symbiotic relationship, it has not been clarified what functions *Arcobacter* have.

From this point of view, the genomic information of strain LA11 isolated from abalone was analyzed to estimate the relationship between *Arcobacter* sp. LA11 and host

abalones. Furthermore, it was examined whether strain LA11 has key genes related to symbiosis and pathogenicity.

2.2 Material and methods

2.2.1 Growth conditions and genomic DNA extraction from LA11

Strain LA11 was cultivated at 25 °C in 50 mL of Marine Broth 2216 (Difco, Detroit, MI, USA) for 3 days. Genomic DNA was extracted from strain LA11 with the CTAB method as described by Porebski et al. (1997). Total DNA obtained was subjected to quality control by agarose gel electrophoresis and quantified by a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and Quant-iT dsDNA BR Assay Kit (Invtrogen Corp., Carlsbad, CA, USA).

2.2.2 Genome sequencing, assembly, and functional annotation

The genome of strain LA11 was sequenced using the Illumina HiSeq 2500 platform with a HiSeq SBS kit version 4-HS (Illumina Inc., San Diego, CA, USA). The DNA sample was used to produce a pair-end sequencing library with an average size of 100 bp. The reads were then assembled in k-mer lengths of 81, 83, 85, 87, 89, 91, 93, 95, 97, or 99 using Edena ver. 3 software (Hernandez et al. 2008). The assembled genome was annotated with the RAST server (http://rast.nmpdr.org/) (Aziz et al. 2008) and KAAS (http://www.genome.jp/kegg/kaas/) (Moriya et al. 2007).

2.2.3 Comparative genome analysis

Six species in the genus *Arcobacter* were chosen as representative strains to compare with strain LA11, and their genomes were obtained from the NCBI database: *A. lekithochrous* LFT 1.7 (heterotypic synonym of *A. haliotis* MA5; MKCO00000000), *A. nitrofigilis* DSM 7299 (NC_014166), *A. bivalviorum* LMG 26154 (CP031217), *A. halophilus* CCUG 53805 (CP031218), *A. defluvii* CECT 7697 (NXIH00000000), and *A. cryaerophilus* L406 (LRUV00000000). In order to ensure the correct assignation at the

species level of strain LA11, taxonomic comparative analysis was performed in *in silico* DDH and ANI (Camacho et al. 2009). The *in silico* DDH values between the genomes of strain LA11 and other *Arcobacter* spp. were calculated using the GGDC 2.1 online server with default settings (http://ggdc.dsmz.de/ggdc.php/) (Meier-Kolthoff et al. 2013). The ANI values were measured based on ANIb (Camacho et al. 2009) and ANIm (Kurtz et al. 2004) using the JSpeciesWS online server with default settings (http://jspecies.ribohost.com/jspeciesws/) (Richter et al. 2016). The AAI via functional gene similarities from RAST Sequence-Based Comparison Tool was calculated with the Lycoming College Newman Lab AAIr Calculator (http://lycofs01.lycoming.edu/~newman/AAI/) (Krebs et al. 2013). POCP was calculated according to the method described by Qin et al. (2014) with the number of conserved proteins obtained from BLASTP. In order to search unique genes of strain LA11, comparative genomics was performed. The genomes of *Arcobacter* spp. were uploaded to the RAST server, and each genome was compared using the compare genome tool in the same server. The genomes of *Arcobacter* spp. were also uploaded to KAAS and compared to each KO number obtained from KAAS using the Upset diagram generated by the UpSetR package (version 1.3.3) (Lex et al. 2014) instead of the traditional Venn diagram.

2.2.4 Searching for putative virulence genes in the NCBI database

Miller et al. reported that *A. butzleri* RM4018 has ten putative virulence genes: *ciaB* (encodes *Campylobacter* invasive antigen B), *mviN* (required for peptidoglycan biosynthesis), *pldA* (encodes an outer membrane phospholipase A associated with the lysis of erythrocytes), *tlyA* (encodes a hemolysin), *irgA* (encodes an iron-regulated outer membrane protein), *hecA* (encodes a filamentous hemagglutinin), *hecB* (encodes a

hemolysin activation protein), *cjl349* and *cadF* (encoding fibronectin-binding proteins), and *iroE* (encodes a periplasmic enzyme for iron acquisition) (Miller et al. 2007). These putative virulence genes were searched for in the genome sequences of strain LA11 and six described *Arcobacter* spices using BLAST searches on the NCBI database and RAST server.

2.3 Results

2.3.1 Genomic information of *Arcobacter* sp. LA11

A total of 52,749,226 paired-end reads comprising 5,274,922,600 bp were obtained. Sequences were pooled and *de novo* assembled using Edena version 3 (Hernandez et al. 2008) to reveal a total of 3,098,976 bp with an average G+C content of 27.9% and consisting of 53 contigs. Automated annotation was performed using the KAAS and RAST annotation servers (Aziz et al. 2008) with the SEED viewer (Overbeek et al. 2005) (Figure 4). In addition, rRNA coding and tRNA coding were identified by RNAmmer version 1.2 (Lagesen et al. 2007) and tRNAscan-SE (Schattner et al. 2005). In KAAS annotation, 1556 KO assignments were obtained from amino acid sequences of *Arcobacter* sp. LA11. From the RAST server, the draft genome contains 3,012 CDSs, of which 1,339 CDSs (45%) were classified in 353 subsystems, while 1,673 CDSs (55%) were uncategorized. In addition, 45 predicted noncoding RNAs, including two rRNA genes (SSU:1, LSU:1), 42 tRNA genes for 20 amino acids, and one pseudo-tRNA gene were annotated by the RAST annotation server. However, RNAmmer version 1.2 predicted three rRNA genes (SSU:1, LSU:1, 5S:1) and tRNAscan-SE predicted 43 tRNA genes. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under accession number BDIR00000000. The version described in this paper is the first version, BDIR01000000.

2.3.2 Central metabolism of *Arcobacter* sp. LA11

The central metabolic map of the *Arcobacter* sp. LA11 genome is shown in Figure 5. Regarding glycolysis and gluconeogenesis, the genome of *Arcobacter* sp. LA11 encodes gluconeogenesis genes, but this bacterium is apparently unable to utilize hexose as the sole source of carbon and energy because of the absence of both enzymes 6-

phosphofructokinase (*pfk*) and NAD-dependent GAPDH. On the other hand, the presence of genes of the Entner-Doudoroff pathway indicated that this pathway is used in place of glycolysis. Some genes encoding the pentose phosphate pathway are present, but any 6-P-gluconate produced is further dehydrated to 2-keto-3-deoxy-6-P-gluconate via the Entner-Doudoroff pathway rather than being oxidized to ribulose-5-phosphate, because 6-phosphogluconate dehydrogenase is absent. Genes encoding a complete TCA cycle are central to carbon metabolism. *Arcobacter* sp. LA11 has key genes for utilizing SCFA such as lactate (*ldh*), acetate (*ack*), and pyruvate (*por*). However, the gene for phosphoenolpyruvate synthase (*pps*) is not present. Acetyl-CoA can enter the TCA cycle by citrate synthase (*glt*) or malate synthase (*glc*) or be included in various anabolic pathways. Some genes of the rTCA cycle, which is a carbon-fixation pathway, were found in the draft genome of strain LA11, including malate dehydrogenase (*mdh*), fumarate hydratase (*fumABC*), succinyl-CoA synthetase (*sucCD*), 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase (*korABCD*), isocitrate dehydrogenase (*icd*), aconitate hydratase (*acnAB*), and pyruvate:ferredoxin oxidoreductase (*por*). However, ATP citrate lyase is not present. The *Arcobacter* sp. LA11 genome encodes various amino acid biosynthesis genes, including genes for glutamine, glutamate, aspartate, and asparagine biosynthesis (*gltBD*, *gdhA*, *aspAC* and *glnASN*), histidine biosynthesis (*hisABCDEFGHI*), arginine biosynthesis (*argABCDFGHJ*), methionine biosynthesis (*metEH*), threonine biosynthesis (*thrABC*, *asd*, and *hom*), lysine biosynthesis (*DAPDC*), cysteine biosynthesis (*cysCDEHK* and *SP*), leucine biosynthesis (*IPMSY, IPMDL, IPMDS, IMPDH,* and *lta*), tryptophan synthesis (*trpABCDE* and *padAa-Ab-Ac*), proline synthesis (*proABC* and *comER*), glycine biosynthesis (*glyA*), alanine biosynthesis (*iscS, alaR,* and *ilvE*) and serine biosynthesis (*serABC* and *glyA*).

2.3.3 Energy metabolism of *Arcobacter* sp. LA11

Genes for aerobic respiratory chain complex I–IV were found in the draft genome of *Arcobacter* sp. LA11, including NADH ubiquinone oxidoreductase *(nuoABCDEFG HIJKLMN*; complex I), fumarate reductase (*frdABCD*; complex II) as a homologous enzyme of succinate dehydrogenase, cytochrome bc1 complex (*petABC*; complex III), and cytochrome c oxidoreductase (*ccoNOPQ*; complex IV). Hydrogen is also utilized as an electron donor, and the draft genome of strain LA11 contains genes encoding several hydrogenases as Ni/Fe hydrogenase (*hydABC*), a Ni/Fe uptake hydrogenase (*hyaAB* and *hupUV*), an NADP-reducing hydrogenase (*hndABCD*), and a cytoplasmic NAD^+ reducing hydrogenase (*hoxFHUY*). The draft genome revealed that strain LA11 can use nitrate for respiration with periplasmic nitrate reductase complex (*napAGHBFLD*). Additionally, strain LA11 has a denitrification pathway, including nitrite reductase (*nirFJNS*), nitric oxide reductase (*norBC*), and nitrous oxide reductase (*nosDFLYZ*). Also present is the *nif* gene cluster (*nifDEHKNTXZ*), which is involved in nitrogen fixation. Strain LA11 encodes gene clusters for the sulfur compound oxidation system (*soxABCDXYZ*). In addition to *sox*, strain LA11 has periplasmic sulfide-quinone oxidoreductases (*sqr*) that catalyze the oxidation of sulfide to elemental sulfur. Strain LA11 also has thiosulfate reductase/polysulfide reductase chain A (*psrA*), but strain LA11 lacks *psrB* and *psrC*. However, the gene cluster for assimilatory sulfate reduction was found in the draft genome of *Arcobacter* sp. LA11 (*cysCDH*, and *sir*).

2.3.4 Taxonomic position of *Arcobacter* sp. LA11 based on comparative genomics

The genome sequence of *Arcobacter* sp. LA11 was compared to six related species (Table 5). The highest 16S rRNA gene identification score of *Arcobacter* sp. LA11 showed 95.3% identity with *A. bivalviorum* LMG 26154. Additionally, in almost all *in*

silico DDH and ANI analyses, *Arcobacter* sp. LA11 shared the highest identity value with *A. bivalviorum* LMG 26154 among other *Arcobacter* species: the GGDC score was 21.4% and the ANIb score was 77.62%. However, these assessed values were clearly below the boundary of threshold for species delineation: GGDC: <70% (Meier-Kolthoff et al. 2013) and ANIb: <95–96% (Camacho et al. 2009). However, in ANIm analysis, *Arcobacter* sp. LA11 shared the highest value with *A. lekithochrous* LFT 1.7 in the genus *Arcobacter,* although the identity value was also lower than the threshold level for new species delimitation (Kurtz et al. 2004). When the AAI values range between 60 and 80%, these species belong to the same genus (Pérez-Cataluña et al. 2018c). The AAI values between strain LA11 and other *Arcobacter* species were 64.9-74.0%, which were within that range. The POCP values obtained in this study ranged between 58.8 and 72.3%, which is also on the lower edge for new genus threshold (50%) (Qin et al. 2014).

2.3.5 Comparative genomics

In KAAS, genome analyses of *A. lekithochrous* LFT 1.7, *A. nitrofigilis* DSM 7299, *A. bivalviorum* LMG 26154, *A. holophilus* CCUG 53805, *A. defluvii* CECT 7697, and *A. cryaerophilus* L406 resulted in 1649, 1658, 1391, 1446, 1447, and 1154 annotated genes (KO numbers), respectively. Duplicate genes in each genome were removed from the dataset leaving *A. lekithochrous*, *A. nitrofigilis*, *A. bivalviorum*, *A. holophilus*, *A. defluvii*, and *A. cryaerophilus* with 1349, 1351, 1180, 1231, 1235, and 1049 unique genes, respectively. Among the obtained genes, 876 were common for all seven genomes in the UpSet plot (Figure 6), and 51 were found only in the genome of LA11 (Table 6). Meanwhile, from the RAST server, genome analyses of *A. lekithochrous*, *A. nitrofigilis*, *A. bivalviorum*, *A. holophilus*, *A. defluvii,* and *A. cryaerophilus* resulted in 1495, 1468, 715, 767, 779, and 606 annotated genes, respectively. The comparison tool in RAST

showed that LA11 had 34 unique genes (Table 7). Various functional genes were found in LA11 as unique genes; however, most were part of incomplete pathways. As a consequence, these approaches revealed that *Arcobacter* sp. LA11 has aromatic amino acid interconversions with the aryl acids pathway and cardiolipin synthesis as unique forms of metabolism. Furthermore, strain LA11 has a *Tad* (tight adherence) gene cluster (*tadABCZ*), although *A. bivalviorum* also has *tadAB* genes (Kachlany et al. 2000 and Schreiner et al. 2003).

2.3.6 Virulence genes

The 10 virulence genes in *Arcobacter* sp. LA11 and 6 in described *Arcobacter* spices are reported in Table 8. All strains used in this study had *ciaB* and *myiN* genes, while c*jl349*, *porA*, and *irgA* genes were not found in all of them. From *Arcobacter* sp. LA11, *A. lekithochrous* LFT 1.7, and *A. cryaerophilus* L406, the same virulence genes were observed. *A. bivalviorum* LMG 26154 and *A. halophilus* F166-45 have the same virulence *cadF*, *ciaB*, *mviN*, *pldA*, and *tlyA* genes. *hecA*, *hecB*, and *tlyA* genes were observed in *A. nitrofigilis* DSM 7299 in addition to *ciaB* and *myiN* genes. *A. defluvii* CECT 7697 had *hecA* and *iroE* genes.

2.4 Discussion

A preliminary consideration of the taxonomic position of *Arcobacter* sp. LA11 was performed using 16S rRNA sequencing and *in silico* DDH, ANI, AAI, and POCP analyses. The assessment values from 16S rRNA sequencing and *in silico* DDH and ANI analysis showed that strain LA11 shares significantly lower values with six *Arcobacter* spp. Therefore, in order to ensure the position of stain LA11 at the genus level, the AAI and the POCP analyses were conducted. The indices obtained from both analyses were within the threshold. Consequently, this was suggested that strain LA11 is a new species that belongs to the same genus as the compared species.

In this study, genome analysis revealed a versatile metabolism of *Arcobacter* sp. LA11 that was isolated form abalone. In the draft genome of strain LA11, kinase genes in the glycolysis pathway were not detected. This indicates that *Arcobacter* sp. LA11 cannot utilize glucose. Although the kinases may not have been detected by chance due to using a draft genome, the inability to utilize hexoses as the sole source of carbon and energy is a characteristic often found in *Arcobacter* species (Roalkvam et al. 2015). *Arcobacter* sp. LA11 can use lactate, acetate, and pyruvate as sole carbon sources instead of glucose.

The rTCA cycle leads to the fixation of CO_2 and to the synthesis of acetyl coenzyme A. Recent reports have demonstrated that pure cultures of Epsilonproteobacteria and *Aquificae*, which include representatives of hydrothermal vent bacteria, fix carbon dioxide via the rTCA cycle (Ferrera et al. 2007). There are three essential key enzymes to run the rTCA cycle: ATP citrate lyase, 2-oxoglutarate ferredoxin oxidoreductase, and fumarate reductase. Some genes of reductive TCA were found in the genome of *Arcobacter* sp. LA11, but ATP citrate lyase was not found in the draft genome. Strain LA11 may lack the ability to grow autotrophically that it once had.

Genome analysis showed that *Arcobacter* sp. LA11 presumably uses hydrogen sulfide, thiosulfate, or H_2 as electron donors, and oxygen, sulfate, or nitrate as electron acceptors. Oxidizing hydrogen sulfide has also been reported as an ability of symbionts. Sulfide is a potent toxin to humans and most animals because it inhibits mitochondrial cytochrome c oxidase at micromolar concentrations (Nicholls 1975). In most members of *Arcobacter*, including pathogens, electron transfer from sulfide to quinone is catalyzed by the membrane-bound flavoprotein sulfide:quinone oxidoreductase (*sqr*). Although *sqr* was not found in the genome of *Arcobacter* sp. LA11 using the RAST server, the gene was found in strain LA11 using KAAS. Oxidation of thiosulfate, which requires an organic carbon source, may be catalyzed by the SOX system. The genes encoding the SOX system are often found in Epsilonproteobacteria including *Arcobacter* spp. Hydrogen gas is also an important energy source for many bacteria. Hydrogenase is the enzyme that catalyzes the reversible oxidation of hydrogen to protons and electrons. LA11 has four types of hydrogenases, including Ni/Fe hydrogenase (*hydABC*), a Ni/Fe uptake hydrogenase (*hyaAB* and *hupUV*), an NADP-reducing hydrogenase (*hndABCD*), and a cytoplasmic NAD^+ reducing hydrogenase (*hoxFHUY*). Most Epsilonproteobactia have Ni/Fe hydrogenase, while a cytoplasmic NAD^+ reducing hydrogenase is not found in the genomes of LA11 and *Arcobacter* spp., except for *A. anaerophilus* (Roalkvam et al. 2015). Additionally, genes encoding an NADP-reducing hydrogenase are not found in Epsilonproteobacteria species in the KEGG database. LA11 could respire with nitrate or sulfate instead of oxygen. In nitrate respiration and denitrification pathways, nitrate is successively reduced to nitrite, N oxides (NO and N_2O), and dinitrogen (N_2) with denitrification. The genome of LA11 encodes complete copies of nitrate respiration and denitrification pathway genes as the periplasmic nitrate reductase complex (*napAGHBFLD*), nitrite reductase (*nirSNJF*), nitric oxide reductase (*norCB*), and nitrous

oxide reductase (*nosYZDFL*). Furthermore, I confirmed that *Arcobacter* sp. LA11 is able to denitrify in a culture medium with nitrate (data not shown). Sulfur is needed by all organisms for the biosynthesis of several essential compounds like amino acids (cysteine and methionine), vitamins (biotin and thiamin), and prosthetic groups (Fe-S clusters). In order to synthesize these compounds, bacteria usually reduce sulfate to sulfide and then incorporate sulfide in various sulfur-containing metabolites (Le Faou et al. 1990). The genome of strain LA11 revealed that sulfate is activated by an assimilatory sulfate reduction (*cysND*) to yield APS (Pinto et al. 2004). APS is converted to PAPS by a bifunctional enzyme (*cysC*) (Pinto et al. 2004) and subsequently reduced to sulfite by phosphoadenosine phosphosulfate reductase (*cysH*) (Berendt et al. 1995 and Bick et al. 2000). The last step of the pathway is the reduction of sulfite to sulfide by sulfite reductase (ferredoxin) (*sir*) (Bork et al. 1998).

Comparative genomics showed that *Arcobacter* sp. LA11 has aromatic amino acid interconversions with the aryl acids pathway and cardiolipin synthesis as unique forms of metabolism. Aromatic amino acid interconversions with the aryl acids pathway is usually found in anaerobes. This system interconverts aromatic amino acids (tryptophan, tyrosine, and phenylalanine) with aryl acids (indoleacete, hydroxyphenylacetate, and phenylacetate). This pathway is biosynthetically used in many methanogens and other organisms and is involved in amino acid catabolism in *Pyrococcus* and others (Porat et al. 2004). The biosynthetic pathway of cardiolipin (diphosphatidylglycerol) is a phospholipid synthetic pathway (Tan et al. 2012). Christie and Han (2012) suggested that cardiolipin is a unique phospholipid with a dimeric structure and four acyl groups potentially carrying two negative charges.

Some putative virulence genes were detected in the genomes of *Arcobacter* sp. LA11 and other *Arcobacter* spp. However, it has not been elucidated whether these genes

are functional or have similar roles in the pathogenic process as their respective homologues (Miller et al. 2007). *Arcobacter* sp. LA11 has *ciaB* and *mviN* genes. The *ciaB* gene encodes a 73-kDa secreted protein denominated *Campylobacter* invasive antigen B invasin (*ciaB*), which is involved in exporting other *cia* proteins and in the bacterial invasion of host cells (Konkel et al. 1999). Medina et al. reported that *A. butzleri* increases the transcription of the *ciaB* gene before it becomes established as an endocytobiont of *Acanthamoeba castellanii*, a free-living amoeba found in environmental matrices such as soil and water, and the internalization of *A. butzleri* by *A. castellanii* requires the previous delivery of *cia*-like effectors into the amoebic cytosol (Medina et al. 2019). Strain LA11 may be also an endosymbiont like *A. butzleri* and may have a *ciaB* gene to enable entry into host cells. *MviN* (*murJ*) is required for peptidoglycan biosynthesis (Miller et al., 2007), and peptidoglycan serves as an anchor for virulence factors and cellular structures (Dramsi et al. 2008). However, Ruiz (2008) reported that the function of *mviN* is still unknown. The *mviN* (or *murJ*) gene had been misnamed as a gene encoding a factor required for mouse virulence, but it was later found that the mutation responsible for the virulence phenotype maps to nearby genes responsible for flagellum biogenesis (Schmitt et al. 1994). Therefore, having a *mviN* gene does not mean that *Arcobacter* sp. LA11 is a pathogen.

Table 5. Genomic similarities (%) between strain LA11 and representative *Arcobacter* species obtained from different genomic indexes analyzed

	A. bivalviorum **LMG 26154**	***A. lekithochrous*** **LFT 1.7**	***A. nitrofigilis*** **DSM7299**	***A. halophilus*** **CCUG 53805**	***A. defluvii*** **CECT 7697**	***A. cryaerophilus*** **L406**
16S rDNA	95.3	94.6	94.4	93.6	93.8	92.4
GGDC	21.4	20.3	19.5	19.0	19.3	19.2
ANIb	77.6	76.0	74.9	74.9	75.6	73.2
ANIm	83.6	84.0	83.4	83.2	83.0	82.2
AAI	74.0	70.8	68.1	68.6	68.4	64.9
POCP	72.3	71.6	66.5	69.1	66.7	58.8

Table 6. List of unique genes in *Arcobacter* sp. LA11 annotated using KAAS

KO no.	Enzymes	KO no.	Enzymes
K00179	*iorA*; indolepyruvate ferredoxin oxidoreductase, alpha subunit [EC:1.2.7.8]	K05569	*mnhE*, *mrpE*; multicomponent Na+:H+ antiporter subunit E
K00180	*iorB*; indolepyruvate ferredoxin oxidoreductase, beta subunit [EC:1.2.7.8]	K05571	*mnhG*, *mrpG*; multicomponent Na+:H+ antiporter subunit G
K00185	prokaryotic molybdopterin-containing oxidoreductase family, membrane subunit	K05916	*hmp*, *YHB1*; nitric oxide dioxygenase [EC:1.14.12.17]
K00632	*fadA*, *fadI*; acetyl-CoA acyltransferase [EC:2.3.1.16]	K06132	*clsC*; cardiolipin synthase C [EC:2.7.8.-]
K00899	*mtnK*; 5-methylthioribose kinase [EC:2.7.1.100]	K06888	uncharacterized protein
K00934	arginine kinase [EC:2.7.3.3]	K06996	uncharacterized protein
K00991	*ispD*; 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase [EC:2.7.7.60]	K07319	*yhdJ*; adenine-specific DNA-methyltransferase [EC:2.1.1.72]
K01560	2-haloacid dehalogenase [EC:3.8.1.2]	K07387	metalloprotease [EC:3.4.24.-]
K01715	*crt*; enoyl-CoA hydratase [EC:4.2.1.17]	K07511	*ECHS1*; enoyl-CoA hydratase [EC:4.2.1.17]
K01834	*PGAM*, *gpmA*; 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase [EC:5.4.2.11]	K07679	*evgS*, *bvgS*; two-component system, *NarL* family, sensor histidine kinase *EvgS* [EC:2.7.13.3]
K01854	*glf*; UDP-galactopyranose mutase [EC:5.4.99.9]	K07755	AS3MT; arsenite methyltransferase [EC:2.1.1.137]
K02230	*cobN*; cobaltochelatase *CobN* [EC:6.6.1.2]	K08351	*bisC*; biotin/methionine sulfoxide reductase [EC:1.-.-.-]
K02424	*fliY*, *tcyA*; L-cystine transport system substrate-binding protein	K08963	*mtnA*; methylthioribose-1-phosphate isomerase [EC:5.3.1.23]

(*continued*)

Table 6. (*cont.*)

KO no.	Enzymes	KO no.	Enzymes
K02488	*pleD*; two-component system, cell cycle response regulator [EC:2.7.7.65]	K09678	*HS3ST4*; [heparan sulfate]-glucosamine 3-sulfotransferase 4 [EC:2.8.2.-]
K02614	*paaI*; acyl-CoA thioesterase [EC:3.1.2.-]	K09931	uncharacterized protein
K02616	*paaX*; phenylacetic acid degradation operon negative regulatory protein	K09946	uncharacterized protein
K02617	*paaY*; phenylacetic acid degradation protein	K09964	uncharacterized protein
K03409	*cheX*; chemotaxis protein *CheX*	K11354	*cph1*; two-component system, chemotaxis family, sensor kinase *Cph1* [EC:2.7.13.3]
K03576	*LysR* family transcriptional regulator, regulator for *metE* and *metH*	K13695	*nlpC*; probable lipoprotein *NlpC*
K03748	*sanA*; *SanA* protein	K13730	*inlA*; Bacterial invasion of epithelial cells
K03810	*mviM*; virulence factor	K14623	*dinD*; DNA-damage-inducible protein D
K04853	*CACNA1F*, CAV1.4; voltage-dependent calcium channel L type alpha-1F	K15866	*paaG*; 2-(1,2-epoxy-1,2-dihydrophenyl)acetyl-CoA isomerase [EC:5.3.3.18]
K04940	*odh*; opine dehydrogenase [EC:1.5.1.28]	K20023	*talrD*, *galrD*; L-talarate/galactarate dehydratase [EC:4.2.1.156 4.2.1.42]
K05566	*mnhB*, *mrpB*; multicomponent Na+:H+ antiporter subunit B	K21344	*rfaE1*; D-glycero-beta-D-manno-heptose-7-phosphate kinase [EC:2.7.1.167]
K05567	*mnhC*, *mrpC*; multicomponent Na+:H+ antiporter subunit C	K23779	*sutR*; XRE family transcriptional regulator, regulator of sulfur utilization
K05568	*mnhD*, *mrpD*; multicomponent Na+:H+ antiporter subunit D		

Table 7. List of unique genes in *Arcobacter* sp. LA11 annotated using RAST server

Category	Subsystem	Role
Amino Acids and Derivatives	Aromatic amino acid interconversions with aryl acids	Amino acid-binding ACT
		Indolepyruvate oxidoreductase subunit *IorA* (EC 1.2.7.8)
		Indolepyruvate oxidoreductase subunit *IorB* (EC 1.2.7.8)
		Phenylacetate-coenzyme A ligase (EC 6.2.1.30)
Carbohydrates	Entner-Doudoroff Pathway	Phosphoglycerate mutase (EC 5.4.2.1)
	Pyruvate metabolism I: anaplerotic reactions, PEP	Oxaloacetate decarboxylase gamma chain (EC 4.1.1.3)
Clustering-based subsystems	CBSS-316407.3.peg.1371	Putative lipase in cluster with Phosphatidate cytidylyltransferase
		Ser/Thr and Tyr protein phosphatase (dual specificity)
	CBSS-257314.1.peg.752	Adenine-specific methyltransferase (EC 2.1.1.72)
	CBSS-272569.1.peg.3198	PH adaptation potassium efflux system protein B1
		PH adaptation potassium efflux system protein C
Cofactors, Vitamins, Prosthetic-Groups, Pigments	Menaquinone and Phylloquinone Biosynthesis	O-succinylbenzoate synthase (EC 4.2.1.113)
DNA Metabolism	DNA structural proteins, bacterial	Chromosome partition protein smc
Fatty Acids, Lipids, and Isoprenoids	Cardiolipin synthesis	Cardiolipin synthetase (EC 2.7.8.-)

(*continued*)

Table 7. (*cont.*)

Category	Subsystem	Role
Iron acquisition and metabolism	Hemin transport system	Periplasmic hemin-binding protein
Membrane Transport	Widespread colonization island	Type II/IV secretion system ATPase *TadZ*/*CpaE*, associated with Flp pilus assembly
Metabolism of Aromatic Compounds	Aromatic Amin Catabolism	Aldehyde dehydrogenase (EC 1.2.1.3), *PaaZ*
	Gentisate degradation	Fumarylacetoacetate hydrolase family protein
Phages, Prophages, Transposable elements, Plasmids	Phage lysis modules	Phage endolysin
Protein Metabolism	Proteolysis in bacteria, ATP-dependent	Putative ATP:guanido phosphotransferase *YacI* (EC 2.7.3.-)
Respiration	Anaerobic respiratory reductases	polysulfide reductase, subunit A
		Thiosulfate reductase electron transport protein *phsB*
	trimethylamine N-oxide (TMAO) reductase	Chaperone protein *TorD*
	Biogenesis of c-type cytochromes	Cytochrome c-type biogenesis protein *ResA*
RNA Metabolism	Group II intron-associated genes	Retron-type RNA-directed DNA polymerase (EC 2.7.7.49)
	RNA methylation	LSU m5C1962 methyltransferase *RlmI*
Stress Response	Osmoregulation	Outer membrane protein A precursor
	Glutathione: Non-redox reactions	Lactoylglutathione lyase (EC 4.4.1.5)

(*continued*)

Table 7. (*cont.*)

Category	Subsystem	Role
Stress Response	Oxidative stress	bacteriophytochrome heme oxygenase *BphO*
		Phytochrome, two-component sensor histidine kinase (EC 2.7.3.-)
Sulfur Metabolism	Sulfur oxidation	Sulfide dehydrogenase [flavocytochrome C] flavoprotein chain precursor (EC 1.8.2.-)
Virulence, Disease and Defense	Beta-lactamase	Beta-lactamase (EC 3.5.2.6)
		Beta-lactamase class C and other penicillin binding proteins
	Mercuric reductase	Mercuric ion reductase (EC 1.16.1.1)

Table 8. List of putative virulence genes based on the NCBI database

	***Arcobacter* sp.**	***A. lekithochrous***	***A. nitrofigilis***	***A. bivalviorum***	***A. halophilus***	***A. defluvii***	***A. cryaerophilus***
	LA11	**LFT 1.7**	**DSM 7299**	**LMG 26154**	**F166-45**	**CECT 7697**	**L406**
cadF	-	-	-	+	+	-	-
cjl349	-	-	-	-	-	-	-
ciaB	+	+	+	+	+	+	+
mviN (murJ)	+	+	+	+	+	+	+
pldA	-	-	-	+	+	-	-
tlyA	-	-	+	+	+	-	-
porA	-	-	-	-	-	-	-
irgA	-	-	-	-	-	-	-
hecA	-	-	+	-	-	+	-
hecB	-	-	+	-	-	-	-
iroE	-	-	-	-	-	+	-

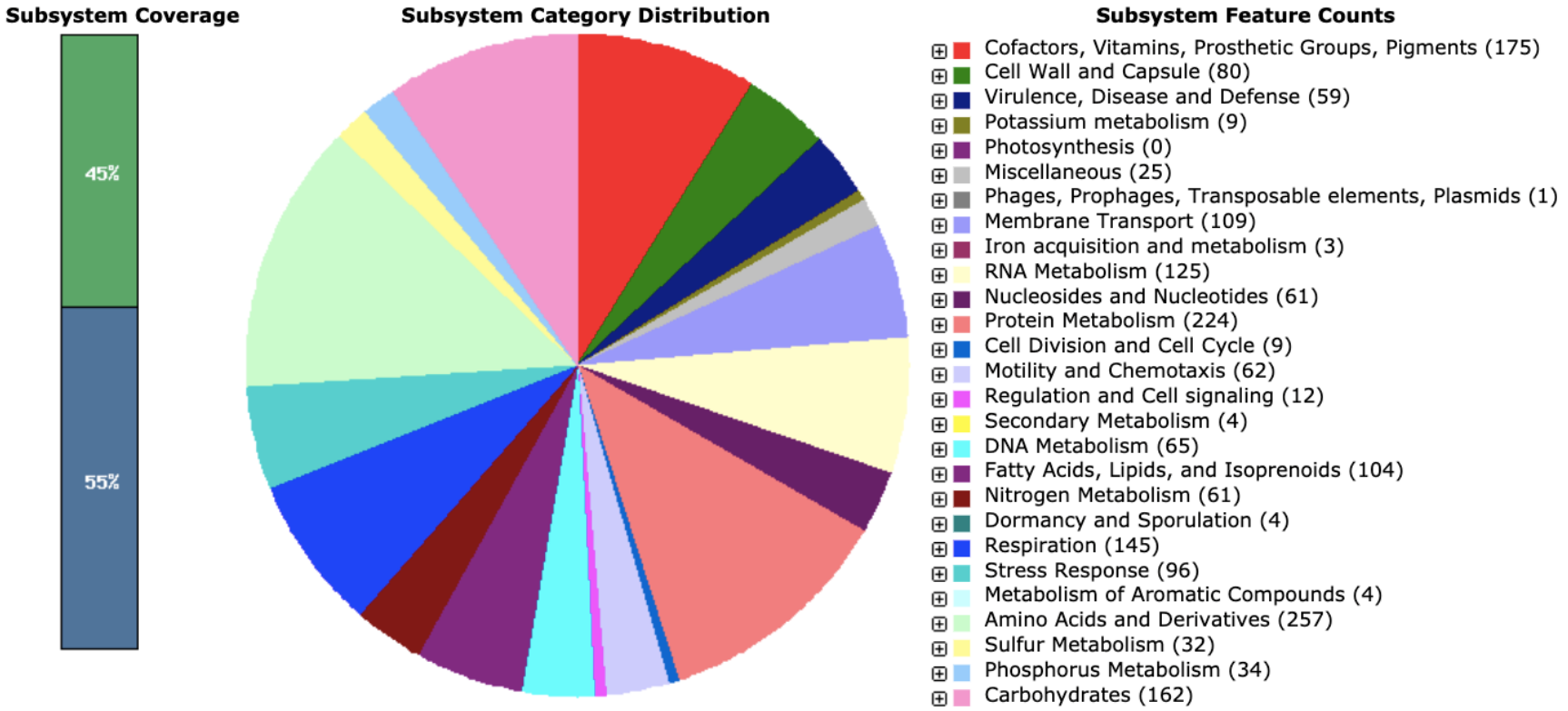

Figure 4. Subsystem information of the genome of *Arcobacter* sp. LA11 (annotated using the RAST server). In total, 26 subsystems were observed from genome sequencing. Annotated genes covered 45% of total encoded genes. A photosynthesis gene system was not observed.

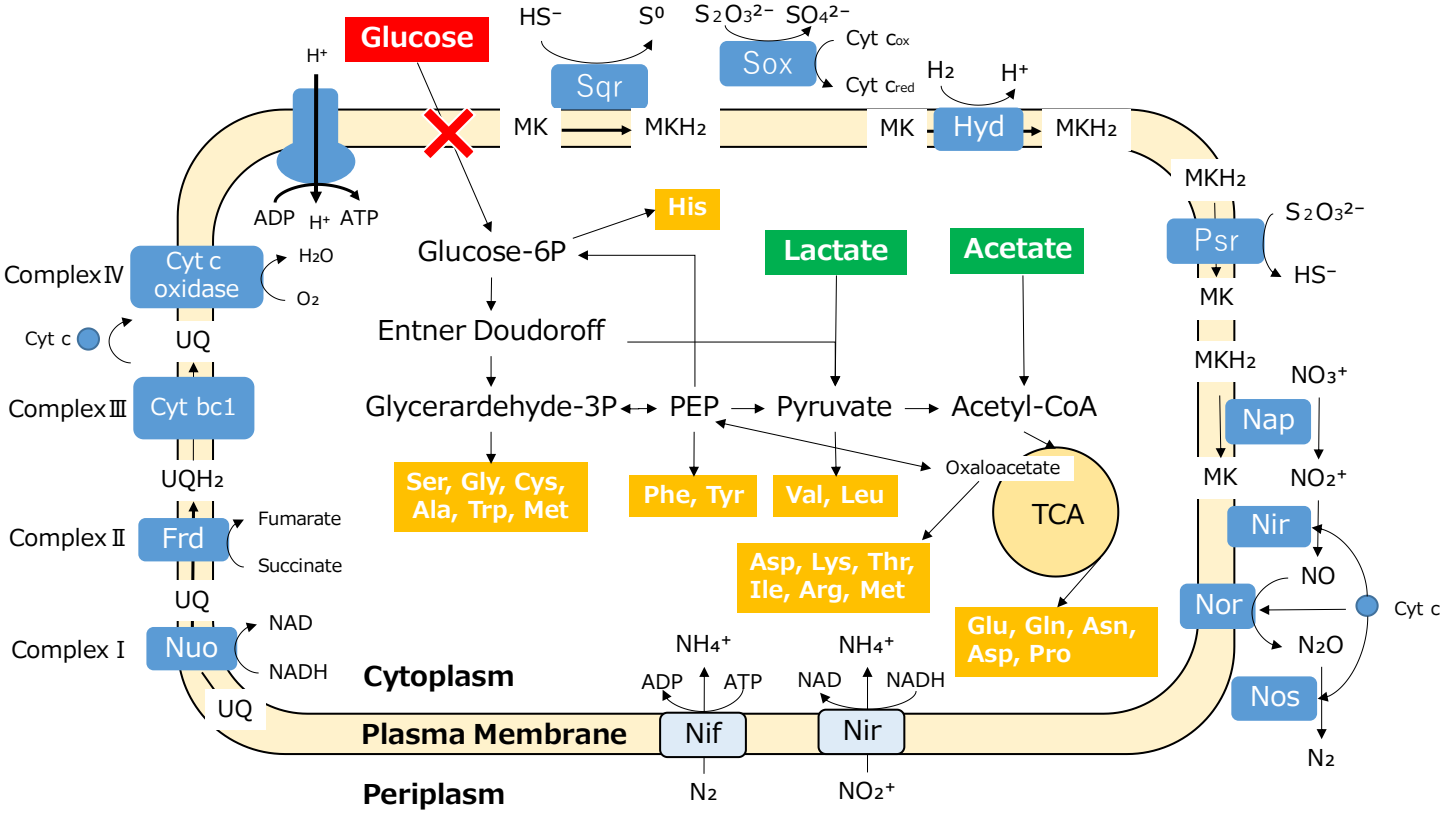

Figure 5. Central metabolism of *Arcobacter* sp. LA11. Cyt, cytochrome; Frd, fumarate reductase; Nuo, NADH ubiquinone oxidoreductase; Sqr, sulfide quinone oxido-reductases; Sox, sulfur compounds oxidation system; Hyd, Ni/Fe hydrogenase; Psr, polysulfide reductase; Nap, periplasmic nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; Nif, nitrogen fixation.

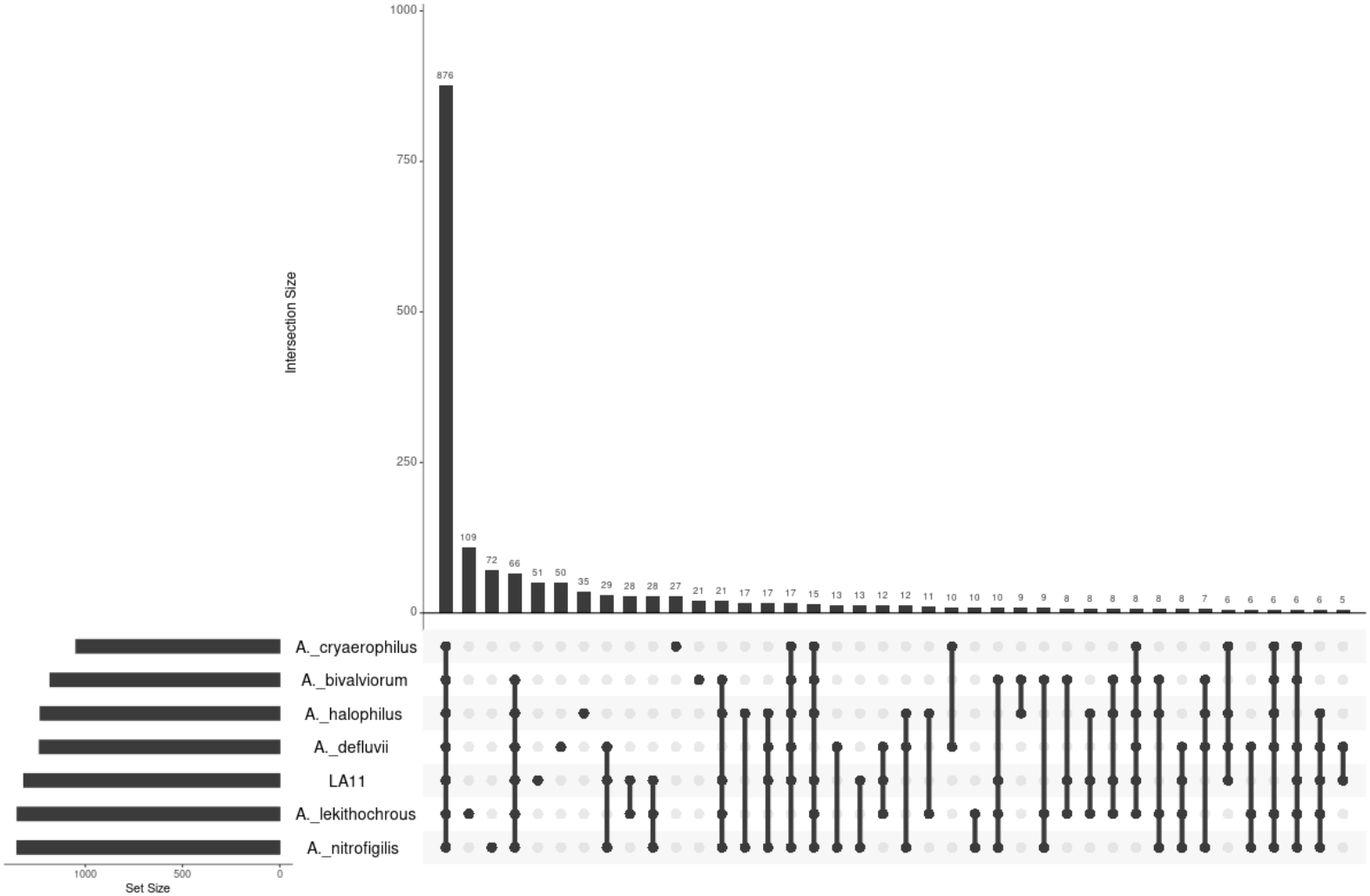

Figure 6. UpSet plot showing comparative genomics using KO lists of seven *Arcobacter* species obtained from the KAAS server.

General discussion

Arcobacter has been frequently detected in and isolated from bivalves, but there is very little information on the genus *Arcobacter* in the abalone, an important fishery resource. In the present study, I investigated the genetic diversity and abundance of bacteria from the genus *Arcobacter* in the Japanese giant abalone *Haliotis gigantea* using molecular methods such as *Arcobacter*-specific clone libraries and FISH analysis. Furthermore, I isolated the *Arcobacter* species detected. Twelve genotypes of clones were obtained from *Arcobacter*-specific clone libraries. These sequences are not classified with any other known *Arcobacter* species including pathogenic *Arcobacter* spp., *A. butzleri*, *A. skirrowii, or A. cryaerophilus*, which are commonly isolated or detected from bivalves. These results suggested that abalones have different microbiota from bivalves.

From the FISH analysis, it was observed that ARC94F-positive cells, presumed to be *Arcobacter*, accounted for $8.06 \pm 0.05 \times 10^5$ cells/g (6.96 ± 0.72% of all EUB338-positive cells). Although the number of symbiotic bacterial cells might be different depending on the role of the bacteria, *Vibrio midae* SY9, a symbiotic bacterium has the function as protein digestion in the digestive tract of *Haliotis midae*, has been reported to make a significant contribution at 10^6 cfu/g gut materials (Macey and Coyne, 2006). According to the results of the FISH analysis in this study, ARC94F-positive cells was not detected up to 10^6 cells/ g, but the amount was close to that value. Additionally, it has been observed that ARC94F-positive cells colonized on the host tissue, and the genome of strain LA11 isolated from abalone has a *Tad* (tight adherence) gene cluster (Kachlany et al. 2000 and Schreiner et al. 2003) and *ciaB* (*Campylobacter* invasive antigen B) gene (Konkel et al. 1999 and Medina et al. 2019). These data indicate that abalones carry *Arcobacter* as a common bacterial genus that includes uncultured species and *Arcobacter*

colonizes on/in their host tissue, although it is still unclear which region of abalone the bacteria inhabit.

From culturing, four genotypes of *Arcobacter* were isolated from abalones. One genotype had a similarity of 99.2-100.0% to the 16S rRNA gene of *A. marinus*, while the others showed only 93.3-94.3% similarity to other *Arcobacter* species. Among the isolations, strain LA11 has a similar sequence to CA11, which was most frequently detected using *Arcobacter*-specific clone libraries. Therefore, strain LA11 was assumed to be the dominant species in *Arcobacter*, and the genome of strain LA11 was analyzed.

The genome sequence of strain LA11 was obtained using paired-end sequencing on an Illumina HiSeq 2500. The sequencing data revealed a total of 3,098,976 bp with an average G+C content of 27.9% that consisted of 53 contigs. Taxonomic comparative genomics of the *Arcobacter* strains, strain LA11, *A. lekithochrous* LFT 1.7 (a heterotypic synonym of *A. haliotis* MA5), *A. nitrofigilis* DSM 7299, *A. bivalviorum* LMG 26154, *A. holophilus* CCUG 53805, *A. defluvii* CECT 7697, and *A. cryaerophilus* L406, revealed that strain LA11 is candidates new species because the genome of strain LA11 shares low identity value with the most closely related species, *A. bivalviorum* LMG 26154; the GGDC score was 21.4%, and the ANIb score was 77.62%. In AAI (Pérez-Cataluña et al. 2018c) and POCP analysis (Qin et al. 2014) for the determination of a new genus, the identity values between strain LA11 and other *Arcobacter* species showed within the range of the same genus. Therefore, strain LA11 is considered to be a new species of the genus *Arcobacter*.

The draft genome of strain LA11 revealed the presence of various pathways. The genome of strain LA11 encoded the Entner-Doudoroff pathways and the TCA cycle, while the pentose phosphate pathway and the reductive tricarboxylic acid (rTCA) cycle, which is a carbon-fixation pathway, was incomplete. Strain LA11 also has key genes for

utilizing SCFA. Genes encoding the biosynthesis of fewer than 16 amino acids were found in strain LA11. Additionally, complete denitrification and a *nif* gene cluster were also found. Recently, it has been reported that Gammaproteobacteria living in the gills of coastal bivalves fix nitrogen from the atmosphere and assimilate ammonia converted from nitrogen into amino acids (Petersen et al. 2016 and König et al. 2016). Strain LA11 has genes for nitrogen fixation and amino acid synthesis pathways. Additionally, the strain was able to utilize organic acids as the sole source of carbon and energy. In the abalone gut, *Vibrio* spp. ferments organic acids, especially acetate, from alginate of brown algae, providing organic acids to its hosts (Sawabe et al. 2003). Electron acceptors utilized by most Epsilonproteobacteria, such as oxygen, nitrate, and sulfate, were also used by strain LA11. Moreover, strain LA11 has the *sqr* gene that detoxifies hydrogen sulfide, which is harmful to the host. The oxidation of sulfide to elemental sulfur is one of characteristic metabolism of Epsilonproteobacteria in symbiosis with invertebrates in deep-sea hydrothermal vents (Nakagawa and Takaki 2009). These symbionts provide organic acids for their hosts by fixing carbon dioxide with hydrogen sulfide and/or hydrogen as energy sources (Dubilier et al. 2008). Strain LA11 has genes utilizing hydrogen sulfide and hydrogen as energy sources like symbiotic Epsilonproteobacteria. However, the gene cluster of the rTCA cycle for carbon fixation was incomplete in the genome of strain LA11. Therefore, strain LA11 may synthesize amino acids from nitrogen and SCFAs and produce nutrition for its host. Strain LA11, which is associated with abalones, appears to have a different symbiotic relationship from symbiotic Epsilonproteobacteria inhabiting the deep-sea floor.

I hope this study promotes research into the genomic and ecological characterization of *Arcobacter* species and other Epsilonproteobacteria.

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