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Short Communication

Vibrio sp. ArtGut-C1, a polyhydroxybutyrate producer isolated from the gut of the aquaculture live diet *Artemia* (Crustacea)

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ABSTRACT

Background: *Vibrio* species display variable and plastic fitness strategies to survive and interact with multiple hosts, including marine aquaculture species that are severely affected by pathogenic Vibrios. The culturable *Vibrio* sp. strain ArtGut-C1, the focus of this study, provides new evidence of such phenotypic plasticity as it accumulates polyhydroxybutyrate (PHB), a biodegradable polymer with anti-pathogen activity, particularly in the marine larviculture phase. The strain was isolated from the gut of laboratory-reared *Artemia* individuals, the live diet and PHB carrier used in larviculture. Its main phenotypic properties, taxonomic status and genomic properties are reported based on the whole-genome sequencing.

Results: *Vibrio* sp. ArtGut-C1 yielded 72.6% PHB of cells' dry weight at 25°C. The genomic average nucleotide identity (ANI) shows it is closely related to *V. diabolicus* (ANI: 88.6%). Its genome contains 5,236,997-bp with 44.8% GC content, 3,710 protein-coding sequences, 96 RNA, 9 PHB genes functionally related to PHB metabolic pathways, and several genes linked to competing and colonizing abilities.

Conclusions: This culturable PHB-accumulating *Vibrio* strain shows high genomic and phenotypic variability. It may be used as a natural pathogen biocontrol in the marine hatchery and as a potential cell factory for PHB production.

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1. Introduction

The genus *Vibrio* is a highly diverse and versatile group of species widespread not only in marine coastal waters, sediments, estuaries, and salterns but also in marine hatchery facilities where pathogenic species cause vibriosis [1], a highly detrimental disease accounting for high mortality particularly in the larviculture phase (larval rearing) of marine fish, shrimp and shellfish species [2,3,4]. Inefficient disease control with antibiotics [5] is related to *Vibrio* plastic genomes, metabolism, and broad niche adaptation that translate into opportunistic fitness strategies allowing them to interact with different hosts in practically any natural and artificial (aquaculture) ecosystems [6,7]. In this context, it is worth mentioning *Vibrio's* ability to modulate their ecological relationships by communicating (e.g., *quorum sensing*) or by transferring via horizontal gene transfer capabilities often associated with particular mobile elements, such as genomic islands and conjugative plasmids [8] encoding antibiotic resistance, virulence and other traits [9,10]. *Vibrio* species are facultative pathogens that may quickly acquire virulence (pathogenicity islands) by natural transformation or vice-versa, i.e., pathogenicity islands can be silenced [11,12]. Such diversity explains why genomic, metabolic, virulence and ecological differences appear distributed at both intraspecific

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and interspecific levels, making it difficult to delineate species [13,14].

In contrast, *Vibrios* are pivotal components in the gut microbiome of a wide variety of marine species, establishing symbiotic interactions that contribute to nutrient acquisition and other critical functions of their hosts by providing enzymatic activities like chitinases, nitrogenases, and phosphatases, among others. Additionally, as revised by Leyton and Riquelme [15], diverse *Vibrio* species act as probiotics that prevent diseases, including inhibition of *Vibrio* pathogens, thus decreasing antibiotic dependence in aquaculture hatcheries. An example is the combined use of *Pseudoalteromonas* sp. PP107 and *Vibrio* sp. PP05 delivered through the live diet *Artemia* to protect spiny lobster larvae (*Panulirus ornatus*) against *Vibrio owensii* DY05 infection [16].

Aquaculture is the fastest-growing food production sector, but its expansion and diversification will continue to be constrained by the outbreak of bacterial diseases such as vibriosis, preventing the industry from complying with the increasingly restrictive food security standards on the use of antibiotics [1]. Thus, finding diverse culturable marine bacteria [17], including some *Vibrio* sp. [18,19,20], able to accumulate the biodegradable polymer polyhydroxybutyrate (PHB) with proven anti-pathogen or anti-vibriosis activity on hatchery-reared marine species, offers a potentially eco-friendly approach to controlling diseases in aquaculture systems [21]. This carbon-storage, semi-crystalline, biodegradable polymer allows cell survival and replication under nutrient-limiting or stressful conditions such as excess of carbon source and nitrogen limitation [17,22,23]. The synthesis of PHB involves the reduction in Acetoacetyl-CoA, from the tricarboxylic acid cycle to hydroxybutanoyl-CoA by the NADH-dependent enzyme acetoacetyl-CoA reductase. Then the monomers are esterified and polymerized by the enzyme polyhydroxyalkanoate synthase (PHAS) type III, encoded by the *PhaC* gene. The antibacterial activity of both PHB-accumulating bacteria and the crystalline form of PHB is the consequence of its breakdown in short-chain fatty acids in the host gut that affect pH [24], bacterial richness [2] and enhances immunological defense [25,26]. A study using the experimental host–pathogen model *Artemia franciscana-Vibrio campbellii* revealed that PHB protects *Artemia* by enhancing the expression of the heat shock protein 70 (*Hsp70*), an effect associated with the generation of the protective innate immune response [22].

The beneficial effect of PHB is known in fish, shrimp and shellfish. In the European sea bass *Dicentrarchus labrax*, juveniles showed enhanced growth rate and intestine bacterial richness when the standard diet is supplemented with a commercial PHB [2]. Likewise, postlarvae of the giant tiger prawn *Penaeus monodon* fed a PHB-enriched live diet *Artemia* exhibited significantly higher survival than any other diet combination [27]. Another study [4] showed that enriching *Artemia* with PHB accumulating *Halomonas*, instead of crystalline PHB, improved the survival, growth, and robustness of the shrimp *Litopenaeus vannamei*. This study additionally highlighted the importance of this crustacean as a PHB carrier to deliver it directly to the gut of shrimp juveniles.

This study reports a cultivable PHB-accumulating *Vibrio* sp. strain isolated from the gut of laboratory-reared *A. franciscana* individuals (brine shrimp), conditions for bacterial growth and PHB production. The taxonomic status of the strain was assessed by whole-genome sequence comparison, considering tools such as Average Nucleotide Identity (ANI), 16S rRNA gene sequence comparison, and a phylogenetic comparison based on the sequence of the gene encoding for the enzyme polyhydroxyalkanoate synthase, the final step in the synthesis of polyhydroxyalkanoic acids. The main genomic features, including PHB and fitness-related genes, are compared to closely related species with reported genomes.

2. Materials and methods

2.1. Bacterial isolation and growing conditions

Artemia individuals collected in solar saltworks of Cahuil, central Chile (34°29′36.85″S–71°59′58.73″W) were brought alive to the laboratory, and conditioned to artificial rearing conditions: artificial sterile seawater (35 ppt of salinity), room temperature and fed with a microalgal mix (*Dunaliella* sp., *Tetraselmis* sp. and *Isocrhisis* sp.). For the complete gut extraction, individuals were washed and dissected in sterile artificial seawater under a stereoscopic microscope. Each whole isolated gut homogenized was serially diluted with 50 μL of each dilution plated in both Luria Bertani (LB) and modified Ramsay saline glucose culture medium (RSG3) with limited nitrogen content, then incubated at 20°C for 48 h. The four largest colonies growing on agar (15 g/L) plates with RSG3 medium containing (g/L) glucose (30), domestic salt (20), Na2HPO4 (3.5), K2HPO4 (1.5), (NH4)2SO4 (1), MgSO4 · 7H2O (0.2), CaCl2 · 2H2O (0.01), were selected and tested for PHB production with 1% Nile Blue A staining [28]. Afterward, a monoculture of the strain coded C1 was further checked due to its fastest growth and PHB production. Bacterial morphology considered Gram staining and the biochemical identification test kit API2 0NE (BioMérieux, Inc.). For bacterial visualization, cells were fixed at room temperature in 2.5% glutaraldehyde and embedded in Epon 812, acetone 1:1 overnight, and finally in pure resin. The polymerization time was 48 h at 60°C. Thin slices of 90 nm obtained with an ultramicrotome (Leica Ultracut R) were stained with 4% uranyl acetate in methanol for 1 min and citrate for 5 min. Cells were observed under the electronic transmission microscopy (TEM) using a Philips transmission microscope (Tecnai 12 at 80 kV). The isolated strain with average PBH production is deposited in the Chilean collection of Microbial Genetic Resources (http://www.cchrgm.cl/), access code RGM2263, managed by INIA-Quilamapu, Chillán, Chile.

2.2. PHB production and DNA extraction

The kinetic of the growth curve in RSG3 of strain C1 began with the addition in triplicate of a 50 ml inoculum of bacteria to 750 ml of RSG3 broth [29] at 25°C for 24 h. Bacterial growth was assessed by taking three aliquots of 1-ml at 0, 3, 5, 6, 7, 8, 9, 15, and 24 h after the culture began, each one checked in a Jasco V-630 spectrophotometer (560 nm). For quantifying the amount of PHB produced during the log phase, a 50 ml inoculum of bacteria cultivated in RSG3 broth was added to 1-L Erlenmeyer flasks with 500 ml of RSG3 and then incubated at 25°C in a shaker at 100 rpm for 24 h. The bacterial culture was centrifuged at 5000 rpm for 10 min, and the pellet was lyophilized to estimate the culture dry weight. The PHB was isolated by digesting the dry pellet with 50% sodium hypochlorite at 30°C for 90 min, followed by two washes in distilled water and two in methanol. The yield of PHB was estimated as percentage of cell dry weight. The PHB obtained was identified comparing the FTIR pattern (Fourier Transform Infrared Spectroscopy) to a control sample of Poly (R)-3-hydroxybutyric acid (Sigma-Aldrich, CAT363502).

For DNA extraction, 10 ml of bacteria picked from the log phase culture was grown in LB broth at 25°C for 16 h, to avoid that PHB production (bacteria do not produce PHB in LB) interfere with DNA extraction. This culture was centrifuged at 5,000 × g for 10 min at 4°C, the pellet was washed with sterile water twice and lyophilized. This lyophilized pellet was processed by Macrogen Korea (Seoul, Korea) for DNA extraction and sequencing.

2.3. Genomic DNA sequencing and assembly

Genome sequencing of this bacterium considered the Illumina HiSeq 2000 Sequencing Platform (Illumina Inc., CA, USA) and a 100 bp paired-end approach. The quality of reads was checked and trimmed using FastQC and Trimmomatic v0.35 bioinformatic tools [30], k-mer analysis used Jellyfish [31]. Clean reads were de novo assembled into contigs and joined into scaffolds using SOAPdenovo2 [32], A5-MiSeq [33] pipelines, and Geneious software v2019.2.1 [34]. The sequence of this draft genome containing 1,034,318,578 nucleotides ended up in 10,240,778 filtered clean reads (129-fold genome coverage) mapped into 60 scaffolds.

2.4. Taxonomic affiliation and genome annotation.

The taxonomic status of ArtGut-C1 was evaluated by comparing the ANI [35] to related *Vibrio* species with sequenced genomes using the ANI Calculator Server [36] and the OrthoANI algorithm [37]. The comparison of the 1,330-bp 16S rRNA gene sequence considered the BLAST searcher from the NCBI Server and the RDP database. Additionally, a phylogenetic based on the 1,669 bp fragment of the polyhydroxyalkanoate acid synthase type III gene (*PhaC*) sequence comparison considered MEGA 7 [38], with a bootstrap test of 1,000 replicates using both the neighbour-joining and maximum likelihood approaches based on the Tamura-Nei model.

Several databases were searched for the functional annotation of coding sequences. For example, identification of rRNA and tRNA genes considered RNAmmer 1.2 Server [39] and tRNA scan-SE 2.0 Server [40], respectively. The open reading frames (ORFs) and functional annotations of translated ORFs used GeneMarkS+ Server v4.28 [41] and the RAST Server v2.0 [42]. Predicted genes were categorized in functional gene subsystems based on their taxonomic affiliation through SEED Viewer v2.0 [43].

3. Results and discussion

3.1. Bacterial characterization and PHB production

The dissected gut of each *Artemia* individual contained an average of 2,693 ± 721 CFU. Bacterial cells of the selected PHB strain C1 were Gram-negative, motile, non-spore-forming (Fig. 1a, b), and tested positive for urease, D-glucose, sucrose, glycerol, fumaric acid, pyruvic acid, L-arginine, esculin ferric citrate, ovine gelatine and 4-nitrofenil-Bd-galactopyranoside; and negative for sodium acetate, citrate, ethanol, glutamic acid, succinic acid, nitrate, and L-tryptophan. Although bacterial growth occurred in both LB and RSG3 media, only cells growing in RSG3 accumulated PHB (Fig. 1c, d) and reached concentrations as high as 72.6 ± 4.47 % cells' dry weight at the stationary phase, 16 h after adding the inoculum (Fig. 1e). The PHB extracted was identified as its FTIR pattern was highly similar to that of poly (R)-3-hydroxybutyric acid (Sigma-Aldrich, CAT363502), the standard used (Fig. 1f). The PHB yield of this bacterium is higher to the amount of ~50% (dry cell weight) reported for different PHB-producer bacteria, including some *Vibrio* sp. [19,23], *Neptunomonas* sp. [18] and *Halomonas* sp. [4].

3.2. Taxonomic affiliation

The draft genome of ArtGut-C1 was closely similar to three related *Vibrio* species with reported genomes, depending on the identification tool considered. The ANI, the most reliable new standard for species delineation [44], showed 88.5% symmetrical identity with *V. diabolicus* type strain CNCMI-1629 (GenBank accession no. **GCA_001048675**), isolated from deep marine vents [45,46]. However, this value is lower than the estimated optimal threshold (95–96%) for species delimitation [13]. The 16S rRNA gene sequence comparison (1,330-bp) confirmed similarity (99%) to *V. diabolicus* strain LMG 3418 (GenBank accession no. **CP014133**), but this time similarity was over the optimal threshold (98.65%) for species demarcation with this marker [13]. The same similarity existed with *V. alginolyticus* strain J207 (GenBank accession no. **CP014041**) and *V. antiquarius* strain EX25 (GenBank accession no. **CP001806**), the latter also isolated from deep-sea hydrothermal vents [47]. Ideally, a concatenated threshold should exist for both estimators so that the 98.65% similarity of the 16S rRNA gene should correspond to the 95–96% ANI threshold. Our comparative analysis does not show such correspondence between both markers. However, we take this relationship with care as it depends on the whole-genome data applied [44], and because the refinement of this draft sequence may change the observed similarity percentages, likewise, the 16S rRNA gene sequence comparison tends to show low interspecies resolution, according to Sawabe et al. [14].

The phylogenetic picture based on the comparison of the 1,669-bp *PhaC* gene sequence (Fig. 2), shows that strain ArtGut-C1 shares the same clade with *V. diabolicus* type strain CNCMI-1629 and *V. antiquarius* EX25, whereas *V. alginolyticus* appears more distant. *Artemia* pathogens such as *V. harveyi* and *V. campbellii* cluster far away from ArtGutC1. This phylogeny is similar to that reported using eight housekeeping genes [14] that show *V. antiquarius* and *V. alginolyticus* in the same cluster sharing a common ancestor in the phyletic branch of *V. parahaemolyticus*.

The difficulty to reliably assign a species name to strain ArtGut-C1 is a common problem of *Vibrio* taxonomy due to their imprinted genomic plasticity resulting from the variety of environmental conditions and selection forces they have experienced [13]. For such a reason, closely related species with relatively similar though variable genomes, as shown in Table 1, express a variety of phenotypic-metabolic traits. The current variety of biochemical and phenotypic tests available help in this regard [6,9,10].

The ability to accumulate PHB is a distinctive phenotypic trait of ArtGut-C1. Although *V. diabolicus*, *V. antiquarius* and *V. alginolyticus* could potentially produce the polymer as they also have PHB genes, this needs confirmation yet. Finally, we subscribe to the statement that good-quality markers for taxonomic identification should integrate different domains of *Vibrio* biology, from genomics to ecology as species signatures may be critically distributed along the genome (genes, amino acids in proteins) [13].

Because this bacterium was isolated from the brine shrimp *Artemia* gut, the name ArtGut-C1 acknowledges this different environment. The draft genome sequence is deposited in GenBank (accession no. **GCA_002105475**).

3.3. Genome properties

This draft genome of *Vibrio* sp. strain ArtGut-C1 contains 5,236,997-bp with 44.8% GC content. The annotated sequences are distributed in 546 subsystem pathways predicted to contain 96 total RNA genes and 4,744 protein-coding sequences, of which 3,710 have predicted functions. Table 1 compares *Vibrio* sp. strain ArtGut-C1 to closely related *Vibrio* species, the main difference being a higher number of protein-coding genes and fewer rRNA genes. According to functional annotations, ArtGut-C1 encodes 9 PHB genes functionally involved in PHB metabolic pathways, whereas 232 genes with putative adaptive capacities distribute in different functional subsystems. For example, Table 1 shows the number of genes (in parenthesis) in selected subsystems such as adhesion (1), invasion and intracellular resistance (13), regulation of virulence (9), DNA uptake and competence (8). The number of genes is similar for all the species. However, ArtGut-C1 differs from the others as it shows the highest number of genes contained

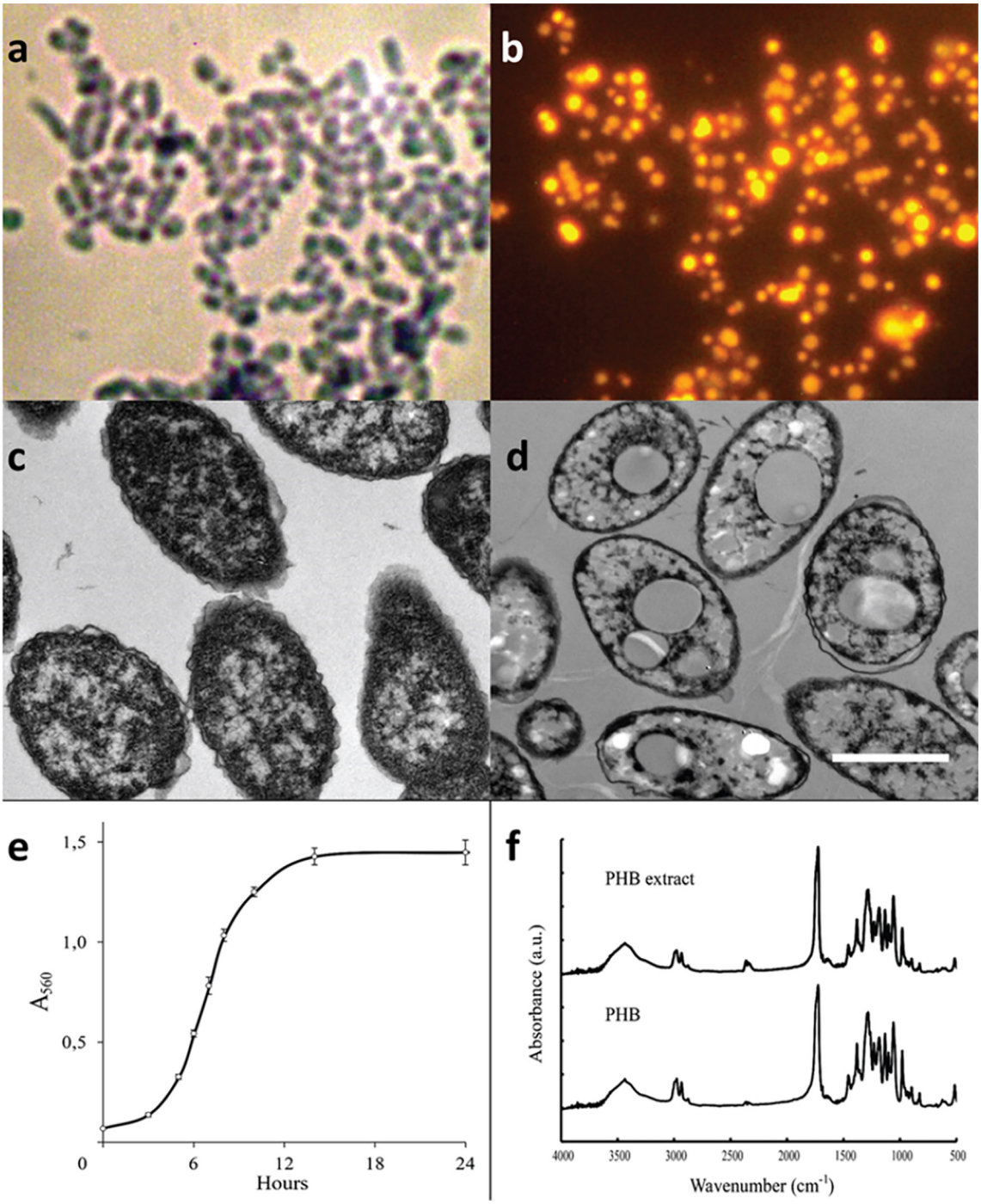

Fig. 1. General features of *Vibrio* sp. ArtGut-C1 cells. (a) transmission and (b) fluorescence micrographs of Nile Blue A staining of cells polyhydroxybutyrate (PHB) positives cultured in RSG3 medium, (c) transmission micrographs of bacterial cells grown in LB medium without cytoplasmic PHB inclusions and (d) bacterial cells grown in RSG3 medium with cytoplasmic PHB inclusions, bar represents 500 nm; (e) growing curve in RSG3 medium culture during 24 h at 25°C and 100 rpm of agitation and (f) FTIR for PHB extracted from *Vibrio* sp. ArtGut-C1 and for PHB standard.

in subsystem resistance to antibiotics and toxic compounds (77), regulation and cell signaling (77), and *quorum sensing* and biofilm formation (19). It is worth noting that ArtGut-C1 does not seem to contain gene clusters for toxins and superantigens. Although Vibrios have two chromosomes, the number was not determined as this is a draft genome sequence; thus, a confirmatory study with ideally larger fragments, like those produced by other sequencing technologies (PacBio and Oxford Nanopore sequencers), should provide a more reliable outcome.

Besides the fact that *Artemia* is commonly used as a PHB carrier in aquaculture, particularly in fish and shrimp hatcheries, it is also a standard model for studying host-pathogen relationships in aquaculture, which allowed to experimentally demonstrate that *Artemia* is protected by PHB when challenged with pathogenic *Vibrio* spp. [5,25,48]. In this context, *Vibrio* sp. ArtGut-C1 is a novel finding that may boost the therapeutic role this crustacean plays to improve the performance of aquaculture species. We cannot at this stage assure that ArtGut-C1 is a gut resident, but the gut

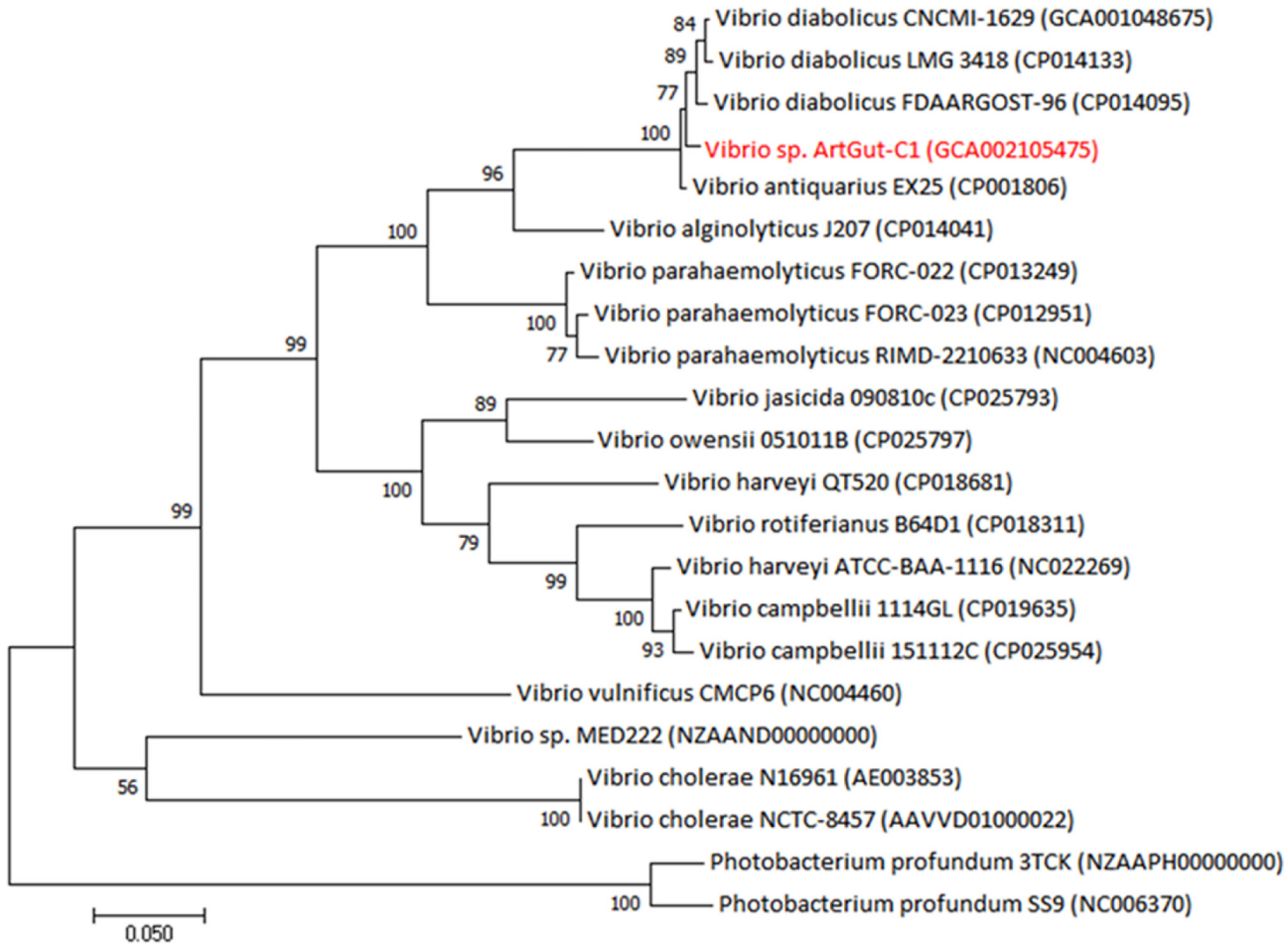

Fig. 2. Phylogenetic relationship of *Vibrio* sp. ArtGut-C1 (red) with 19 closely related *Vibrio* species. The optimal tree displayed was inferred using neighbour-joining over 1,669 positions of polyhydroxylalkanoic acid synthase gene sequences (*PhaC*) as final dataset, eliminating positions containing gaps and missing data. The tree is drawn to scale, branch lengths measured in the number of substitutions per site and distances computed using Maximum Composite Likelihood. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches.

Table 1
General genomic features of *Vibrio* sp. strain ArtGut-C1 compared to closely related species. Only functional subsystems related to PHB production and some colonizing abilities are considered.

Attribute	*Vibrio* sp. ArtGut-C1	*V. diabolicus* CNCM I-1629	*V. diabolicus* LMG 3418	*V. antiquarius* EX25	*V. alginolyticus* J207
Genome size (bp)	5,236,997	5,132,517	5,131,801	5,089,025	5,268,677
Number of chromosomes	–	–	2	2	2
DNA G + C content (%)	44.8	44.8	44.9	44.9	44.6
Total genes	4,842	4,788	4,772	4,673	4,909
Protein coding genes	4,744	4,712	4,612	4,515	4,743
Genes connected to functional subsystems	3,710	3,690	3,131	3,177	3,152
RNA genes	96	76	160	158	166
rRNA genes	5	9	34	34	37
tRNA genes	91	67	126	124	129
Number of genes in functional subsystems					
Polyhydroxybutyrate (PHB) metabolic pathways	9	9	9	8	9
Adhesion	1	1	1	1	1
Toxins and superantigens	–	4	–	–	–
Bacteriocins, ribosomally synthesized antibacterial peptides	10	10	8	8	11
Resistance to antibiotics and toxic compounds	77	75	70	69	69
Invasion and intracellular resistance	13	12	13	13	13
Regulation and cell signalling	77	77	59	61	63
Quorum sensing and biofilm formation	19	18	15	15	15
Regulation of virulence	9	9	9	9	10
Programmed cell death and toxin-antitoxin systems	9	12	12	8	11
DNA uptake and competence	8	8	8	8	8

microbiota of *Artemia*, fish and shrimp is related to fitness. Another possibility is that *Artemia* could offer temporary refuge for *Vibrio* spp. to persist. Indeed, copepods (common host), amphipods and other crustaceans are reported as reservoirs of *Vibrio* species in the marine environment [9]. Although *Artemia* is not properly an inhabitant of the marine environments [49], it has wide salinity tolerance to either natural or artificial seawater. Finally, this knowledge also should facilitate its biotechnological use as a cell factory for PHB production and should provide insights about how metabolic pathways are regulated to manipulate them, or as a source of PHB production genes.

Conflicts of interest

The authors declare no conflicts of interest.

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