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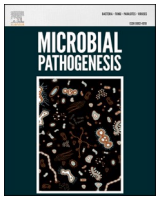

Characterization of the genome and cell invasive phenotype of *Vibrio diabolicus* Cg5 isolated from mass mortality of Pacific oyster, *Crassostrea gigas*

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ABSTRACT

Vibrio is an important group of aquatic animal pathogens, which has been identified as the main pathogenic factor causing mass summer mortality of *Crassostrea gigas* in northern China. This study aims to investigate the potential pathogenic mechanisms of *Vibrio* Cg5 isolate in *C. gigas*. We sequenced and annotated the genome of *Vibrio* Cg5 to analyze potential virulence factors. The gentamicin protection assays were performed with *C. gigas* primary cells to reveal the cell-invasive behavior of Cg5. The genome analysis showed that Cg5 was a strain of human disease-associated pathogen with multiple antibiotic resistance, and four virulence factors associated with intracellular survival were present in the genome. The gentamicin protection assays showed that Cg5 could potentially invade the cells of *C. gigas*, indicating that Cg5 could be a facultative intracellular pathogen of *C. gigas*. These results provide insights into the pathogenic mechanism of *V. diabolicus*, an emerging pathogenic *Vibrio* on aquatic animals, which would be valuable in preventing and controlling diseases in oysters.

1. Introduction

Vibrio is a genus of gram-negative bacteria widely distributed in estuaries and oceans [1]. It has been reported that various *Vibrio* can infect aquatic animals and cause serious economic losses [2,3]. In human, *V. cholerae* [4], *V. vulnificus* [5], and *V. parahaemolyticus* [6] have been reported as important pathogens. Moreover, the species such as *V. parahaemolyticus* and *V. vulnificus* have been stated to cause foodborne infection via consumption of raw or undercooked shellfish to cause human disease [7,8]. Some pathogenic bacteria can be accumulated in oysters because these animals are filter-feeding organisms that ingesting food and microorganisms from the aquatic environment.

The cell invasion is an important mechanism by which pathogenic bacteria cause damage to the host tissues [9]. The process of pathogenic microorganisms invading host cells starts by their entry into the host cell, followed by surviving, replicating, and finally exiting the host cell [10]. Several *Vibrio* species, including *V. parahaemolyticus* [11], *V. splendidus* [12], and *V. cholerae* [13] have been reported to invade host cells via various mechanisms. The cell invasion promotes the proliferation of *Vibrio* in the host, resulting in an increased *Vibrio* loads within the host via various mechanisms [13].

The Pacific oyster (*Crassostrea gigas*), as one of the most important aquaculture species, has been introduced to various countries for aquaculture due to its fast growth and high environmental adaptability [14]. However, due to the combined effects of the environment, pathogens, and physiology of this species, mass mortalities have been recorded in many countries during summer season [15,16]. The *Ostreid herpesvirus* (OsHV-1) and *Vibrio* are the most reported pathogens that cause mass summer mortality of *C. gigas* [17]. In our previous studies, multiple pathogenic *Vibrio* have been isolated from moribund *C. gigas* collected from aquaculture areas undergoing mass summer mortality in northern China. One of the most virulent pathogens was identified as *Vibrio* Cg5 [18,19]. However, the pathogenic mechanisms of *Vibrio* Cg5 remains largely unknown. In this study, we aim to investigate the potential pathogenic mechanisms of *Vibrio* Cg5 isolate in *C. gigas*. We sequenced and annotated the genome of *Vibrio* Cg5 to analyze potential virulence factors. The gentamicin protection assays were also performed with *C. gigas* primary cells to reveal the cell-invasive behavior of *Vibrio* Cg5.

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Table 1
Genomic features of *Vibrio* Cg5 strains.

Genome of Cg5	Numbers
Genome size (bp)	4,987,598
G + C (%)	44.94
Numbers of contigs	131
Numbers of scaffolds	35
L50	1
N50	4,954,623
Numbers of tRNA	82
Numbers of rRNA	5

2. Materials and methods

2.1. Bacteria strain

The *Vibrio* Cg5 was the *Vibrio* strain screened from moribund oysters, which was identified to be a highly pathogenic strain for *C. gigas* [18, 19]. The Cg5 strain was stored in a medium containing 25% glycerol (v/v) at −80 °C. After the strains were removed from the ultra-low temperature refrigerator, they were incubated in 10 mL 2216E liquid medium for 8 h (25 °C, 150 rpm). The bacterial solution was spread in TCBS agar medium using an inoculating loop and incubated at 25 °C overnight. Single colonies were picked with an inoculation loop into 100 mL of 2216E liquid medium for 8 h (25 °C, 150 rpm). Bacterial concentrations were determined using a UV spectrophotometer.

2.2. Genome sequencing and annotation of Cg5

The DNA of Cg5 was extracted using the traditional phenol-chloroform-isoamyl alcohol extraction protocol [20], and whole-genome shotgun sequencing of Cg5 was performed on the NovaSeq 6000 sequencer (Illumina) for 150bp paired-end reads. The raw data were processed using Trimmomatic [21] and assembled by Unicycler [22]. The assembly were evaluated using QUAST [23], and the contigs were assembled into longer scaffolds using Medusa [24].

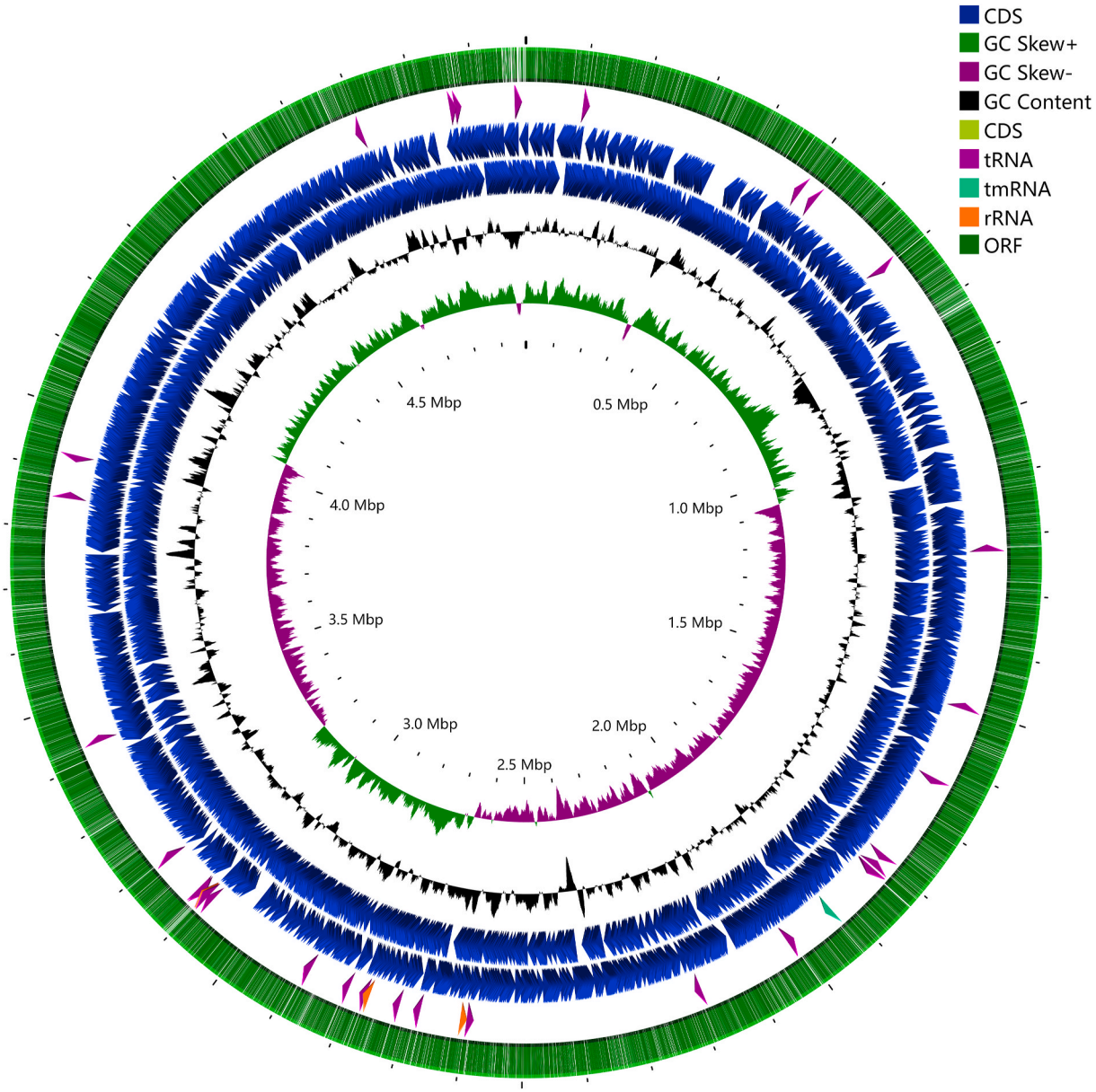

Fig. 1. Features of Cg5 genome. From inside to outside, the scale of genome, GC skew, GC content, coding sequences (CDS) on the forward strand, CDS on the reverse strand, RNAs, open reading frames (ORFs). The Cgviewer was utilized to produce this figure.

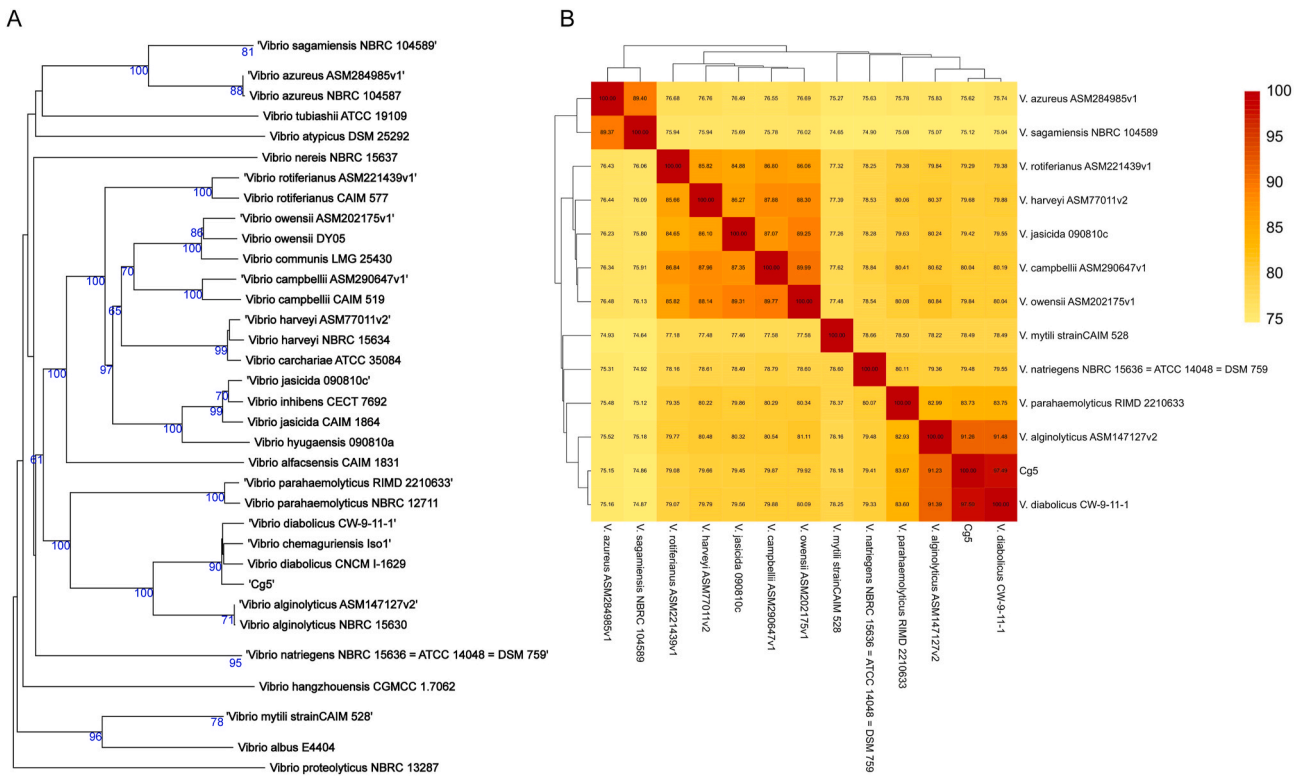

Fig. 2. Species identification of Cg5 based on whole-genome sequence. (A) Phylogenetic tree based on digital DNA-DNA hybridization. Whole-genome sequences of Cg5 and other representative *Vibrio* genomes were analyzed by pairwise comparison against a set of best-matching type-strains genome sequences using the Type Strain Genome Server (TYGS). The numbers indicate Genome Blast Distance Phylogeny (GBDP) pseudo-bootstrap support values > 60% from 100 replications. (B) Heatmap presentation of average nucleotide identity (ANI) of the 12 *Vibrio* species.

Open reading frame (ORF) prediction and genome annotation were carried out using the RAST (Rapid Annotation using Subsystem Technology) pipeline [25] and prokka [26]. The tRNA and rRNA-encoding genes were performed with tRNAscan [27] and RNAmmer [28] respectively. The Cgviewer was used to visualize the obtained data [29]. The BlastKOALA was used for the functional annotation of protein-coding genes using the KEGG Annotation [30]. The R package clusterProfiler [31] was used to analyze the Gene Ontology (GO) enrichment of the Cg5 genome.

2.3. Species identification based on whole genome sequence

The genome sequences of representative strains of other species from the *Vibrio harveyi* group were downloaded from the National Center for Biotechnology Information (NCBI) and used for phylogenetic analysis with *Vibrio* Cg5. The JspeciesWS was used to calculate the average nucleotide identity (ANI) between two genomes of *Vibrio* Cg5 and other *Vibrio* species [32]. The Type Strain Genome Server (TYGS) was used to identify digital DNA-DNA hybridization (dDDH) between *Vibrio* Cg5 and other *Vibrio* species [33].

2.4. Prediction of virulence factors and antibiotic resistance

The virulence factor-related genes in Cg5 were predicted using the Database of Virulence Factors of Pathogenic Bacteria (VFDB) [34]. The Pathogenfinder was used to predict whether Cg5 could cause human disease [35]. The antibiotic resistance of Cg5 was predicted by the Comprehensive Antibiotic Resistance Database (CARD) [36]. The genomic islands (GIs) were predicted through the IslandViewer 4 website [37], and the Cgviewer was used to visualize this information.

2.5. Acquisition of primary cells from C. gigas

Oysters were dissected with sterile scalpels and forceps to obtain the gills, digestive glands, adductor muscles and mantle. These tissues were sectioned and digested with trypsin for 15 min. After the centrifugation process (300 g, 10 min, 25 °C), these tissues were resuspended with dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and then filtered through a 40 μm cell-strainer. The hemolymph of the oyster was collected with a 1-mL syringe. After centrifugation (300 g, 10 min, 25 °C), hemocytes were resuspended with DMEM supplemented with 10% FBS, and then filtered through a 40 μm cell-strainer. These filtered cells were transferred to 24-well plates, and a final concentration of 100 U/mL Penicillin and 100 μg/mL Streptomycin were added to these cells. After 24 h of incubation with 5% CO_2 at 25 °C, these cells were washed without antibiotics, and cultured in L-15 medium in 24-well plates at 25 °C.

2.6. Cell invasion assay

The cell invasion assay was performed as described by Wang et al. [38], with minor modifications. Briefly, the cultured *Vibrio* Cg5 was washed with phosphate buffer saline (PBS) three times and the concentration of Cg5 was adjusted to 5×10^8 CFU/mL. Each tissue of primary cells was infected with *Vibrio* Cg5 at a multiplicity of infection (MOI) of 10:1, respectively. After 3 h of bacterial infection, the cells were placed into a medium containing 100 μg/mL of gentamicin and incubated for 2 h. The cells were lysed with 1% (v/v) Triton X-100 in PBS and plated on TCBS agar medium to calculate the number of *Vibrio* invading the cells.

After centrifugation (300 g, 10 min, 25 °C), the cells were resuspended by 4% paraformaldehyde for 20 min. Then the cells were washed with PBS and plated on the slide. The cells were observed under the

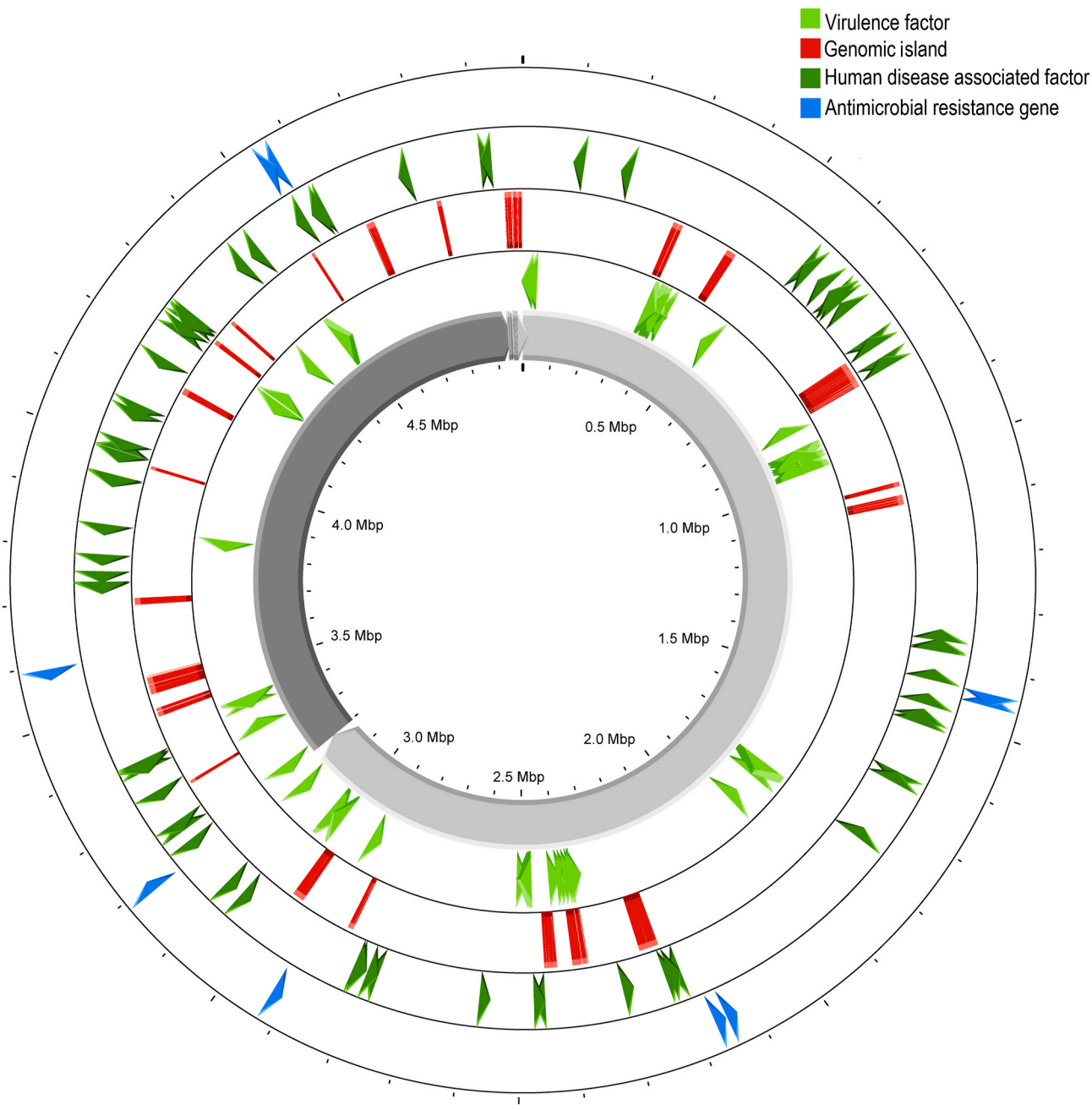

Fig. 3. Prediction of virulence factors in Cg5 genome. From inside to outside, the scale of genome, genome of Cg5, virulence factors predicted by Virulence Factors of Pathogenic Bacteria (VFDB), genomic islands, human disease associated factors, antimicrobial resistance genes. The Cgviewer was utilized to produce this figure.

microscope (1000 ×) after staining with a modified Giemsa staining solution for 30 min.

2.7. Statistical analysis

All data were analyzed for statistical significance with R. Results are displayed as the mean ± Standard Error of the Mean (SEM) of each experimental condition. The normality and homoscedasticity of these data were confirmed by Shapiro-Wilk test. If these data were consistent with normality and homoscedasticity, one-way analysis of variance (ANOVA) was used to analyze these data. Otherwise, Mann-Whitney *t*-test was used to analyze these data. Differences were considered significant when $p < 0.05$. Data is presented as the mean ± standard deviation.

3. Results

3.1. General features of Vibrio Cg5 genome

The draft genome of Cg5 is composed of 4,987,598 base pairs (bp) with a GC content of 44.94%. A total of 4505 protein-coding sequences (CDS) including five rRNAs and 82 tRNAs were annotated (Table 1, Fig. 1).

3.2. The Vibrio Cg5 is re-identified as V. diabolicus

The phylogenetic tree constructed using TYGS indicates that Cg5 clusters with *V. diabolicus* (Fig. 2A). The corresponding heatmap, based on ANI values, shows that Cg5 shares a 97.50% ANI value with *V. diabolicus*, which surpasses the standard 95% threshold used for

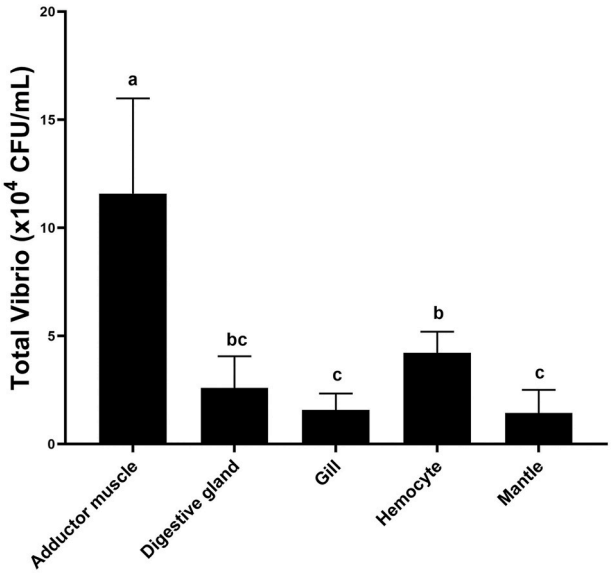

Fig. 4. Invasion of *Vibrio* Cg5 in primary cells of *C. gigas*. Primary cells were obtained from different tissues of oyster and cultured in a cell culture incubator containing 5% CO_2 at 25 °C. Different letters indicate significant differences in bacterial loads in cells of the two tissues.

species identification. (Fig. 2B).

3.3. Functional genome annotation and metabolic pathway mapping

A total of 4651 coding sequences were predicted in Cg5 with 34% of which being covered by the subsystem (Table S1). Furthermore, 58 genes related to virulence, disease, and defense were identified, including 13 genes linked to invasion and intracellular resistance (Fig. S1). Functional annotation conducted using BlastKOALA from the KEGG database revealed that 59.17% (2752) of the total 4651 coding sequences showed homology with KEGG orthologs (Table S2). The distribution of functional gene categories is shown in Fig. S2, with 27 genes being classified in human disease-related pathways. In addition, the most enriched GO terms included nucleic acid binding, nucleotide binding, and rRNA modification (Fig. S3). There were 26 genomic islands (GIs) predicted in the Cg5 genome, of which the largest GI involves acetyltransferase and toxin-antitoxin system, and the second largest GI contains a variety of genes related to carbohydrate utilization (Fig. 3, Table S4).

3.4. The Vibrio Cg5 could be a new potential zoonotic pathogen with multiple antibiotic resistance

The antibiotic resistance genes encoding β-lactamase, specifically blaCARB-42, were predicted in a genomic island in Cg5 genome (Table S3). The Cg5 also displayed resistance to three antibiotic classes, which include ampicillin, amoxicillin, and piperacillin (Table S4). Moreover, Cg5 was predicted to be resistant to tetracycline, macrolide, fluoroquinolone, and penam antibiotic classes through various mechanisms, including antibiotic efflux, antibiotic inactivation, antibiotic target alteration resistance mechanism (Table 3). Notably, the Cg5 was predicted to be a human pathogen with a possibility of 64.1% by Pathogenfinder. A total of 74 genes associated with human diseases were predicted, including 52 genes that matched with pathogenic families and 22 genes that matched with non-pathogenic families (Table S5, Fig. 3).

3.5. The virulence factors of Cg5

The virulence factors of Cg5 as predicted by VFDB was provided in Table S6 and Fig. 3. We predicted a total of 151 virulence factors, which were divided into several subcategories, including adherence (14), antiphagocytosis (19), chemotaxis and motility (55), iron uptake (8), quorum sensing (2), and secretion (49) toxin and endotoxin (2), and others (2). Moreover, there were four related to intracellular survival, including Capsule (*Klebsiella*), T4SS effectors (*Coxiella*), LOS (*Haemophilus*), and O-antigen (*Yersinia*), which suggest that Cg5 could be a facultative intracellular pathogen.

Table 2
The dDDH values of Cg5 with other *Vibrio* species belong to *Vibrio harveyi* group.

Query strain	Subject strain	dDDH (%)
Vibrio Cg5	*V. diabolicus* CNCM I-1629	81.4
Vibrio Cg5	*V. alginolyticus* NBRC 15630	44.9
Vibrio Cg5	*V. parahaemolyticus* NBRC 12711	27.4
Vibrio Cg5	*V. owensii* DY05	24
Vibrio Cg5	*V. communis* LMG 25430	24
Vibrio Cg5	*V. campbellii* CAIM 519	23.9
Vibrio Cg5	*V. carchariae* ATCC 35084	23.9
Vibrio Cg5	*V. inhibens* CECT 7692	23.6
Vibrio Cg5	*V. rotiferianus* CAIM 577	23.6
Vibrio Cg5	*V. harveyi* NBRC 15634	23.6
Vibrio Cg5	*V. jasicida* CAIM 1864	23.5
Vibrio Cg5	*V. natriegens* NBRC 15636	23.3
Vibrio Cg5	*V. mytili* CAIM 528	22.4
Vibrio Cg5	*V. azureus* NBRC 104587	21.7
Vibrio Cg5	*V. sagamiensis* NBRC 104589	21.2

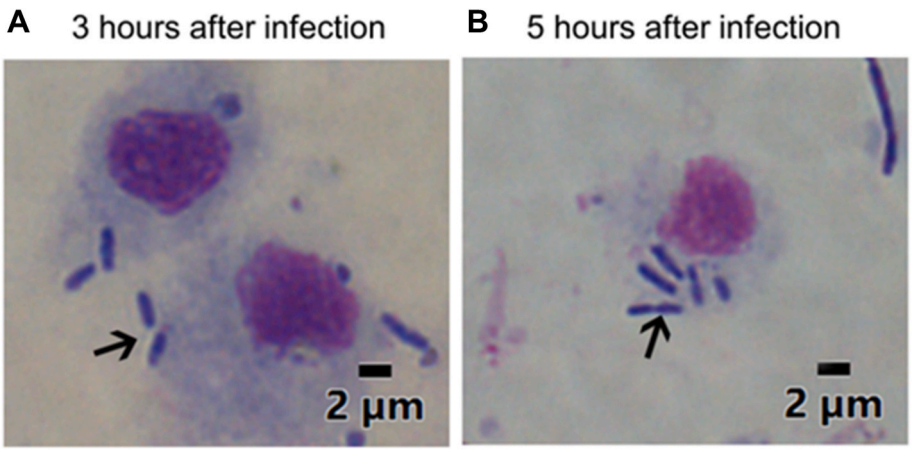

Fig. 5. Invasion of *Vibrio* Cg5 in cells as observed by modified Giemsa staining. (A) *C. gigas* gill primary cells 3 h after bacterial infection. (B) *C. gigas* gill primary cells 5 h after bacterial infection. Black arrows indicate bacteria.

Table 3
Antimicrobial resistance (AMR) genes detected in Cg5.

Best Hit ARO	AMR Gene Family	Model type	Drug Class	Resistance Mechanism
TxR	ATP-binding cassette (ABC) antibiotic efflux pump	protein homolog model	tetracycline antibiotic	antibiotic efflux
CRP	resistance-nodulation-cell division (RND) antibiotic efflux pump	protein homolog model	macrolide antibiotic; fluoroquinolone antibiotic; penam	antibiotic efflux
adeF	resistance-nodulation-cell division (RND) antibiotic efflux pump	protein homolog model	fluoroquinolone antibiotic; tetracycline antibiotic	antibiotic efflux
adeF	resistance-nodulation-cell division (RND) antibiotic efflux pump	protein homolog model	fluoroquinolone antibiotic; tetracycline antibiotic	antibiotic efflux
CARB-42	CARB beta-lactamase	protein homolog model	penam	antibiotic inactivation
Escherichia coli parE conferring resistance to fluoroquinolones	fluoroquinolone resistant parE	protein variant model	fluoroquinolone antibiotic	antibiotic target alteration

3.6. The Vibrio Cg5 was capable to invade cells of C. gigas

The *Vibrio* Cg5 can invade various tissue cells, but the invasion capability was different among various tissue cells. Among the tissues, the *Vibrio* Cg5 was found to be the most abundant in the adductor muscle, with bacterial loads being significantly higher than that in other tissues (Fig. 4). The modified Giemsa staining was used to determine Cg5 invasion of cells in *C. gigas*. After 3 h of infection, there were *Vibrio* being observed to enter the cells and some adhered to the cell surface. After incubation with gentamicin for 2 h, the surface-adherent *Vibrio* were killed and the number of intracellular *Vibrio* increased (Fig. 5).

4. Discussion

Various *Vibrio* species were isolated from moribund *C. gigas* that had suffered mass summer mortality in northern China, among which *Vibrio* Cg5 was the most virulent [18,19]. In order to reveal the virulence mechanism of *Vibrio* Cg5, we sequenced the whole genome of the *Vibrio* Cg5. Based on the genome, the Cg5 was re-identified as *V. diabolicus*, which had previously been identified as *V. alginolyticus* by 16S rRNA and gyrB sequences [18]. The dDDH value between Cg5 and *V. alginolyticus* was 44.9%, which is below the 70% threshold value used to identify species (Table 2). Moreover, the phylogenetic tree constructed based on the TYGS results showed that Cg5 was clustered unambiguously with *V. diabolicus*. Furthermore, the heatmap constructed based on ANI showed that the ANI value between Cg5 and *V. alginolyticus* was 91.26%, which was below the 95% threshold used for species identification while the ANI value between Cg5 and *V. diabolicus* was 97.50%, exceeding the threshold for species identification. According to a previously published study, the dDDH was considered as the reliable method for accurate species identification within *Vibrio harveyi* clade [39]. Together, both ANI and dDDH analysis showed that Cg5 should be classified as *V. diabolicus*. The *V. diabolicus* was first isolated from deep-sea hydrothermal vent, Polychaete annelid, *Alvinella pompejana* [40]. Several recent studies have reported that *V. diabolicus* is harmful to aquaculture species [41,42], while there is no report that human beings can be infected with *V. diabolicus*.

The *Vibrio* Cg5 was predicted to be a potential pathogen related to human diseases with multiple antibiotic resistance. It shared the majority of the pathogenic family genes with *V. parahaemolyticus* which suggested that Cg5 may have similar mode of infection to *V. parahaemolyticus*. Consumption of raw or undercooked seafood was reported to cause infection by *V. parahaemolyticus*, a foodborne pathogen that is responsible for approximately 50–70% of cases of diarrhea [7,43]. Moreover, the multiple antimicrobial resistance of *V. diabolicus* posed a challenge for the treatment of vibriosis caused by *V. diabolicus*. However, further investigations were needed to evaluate the risk of *V. diabolicus* to human.

The prediction of virulence factors provided an insight into the potential pathogenic mechanism of Cg5. The Cg5 was predicted to possess virulence factors related to flagella, capsule, adhesion and secretion system which enable it to survive in the environment, adhere to host cells, and ultimately inject toxins into the host cells, leading to host diseases [43–45]. Notably, a total of 36 virulence factors encoding type III secretory system (T3SS) were annotated in the Cg5 genome. The T3SS is a crucial virulence factor in various bacteria, which enables bacterial invasion into non-phagocytic cells [45,46]. Meanwhile, the presence of T3SS enables the extracellular pathogen *Yersinia* to survive and replicate in phagocytes [47]. Additionally, four virulence factors predicted by VFDB in this study were previously reported in other species. The capsule protects *Klebsiella pneumoniae* from being cleared by host immune response, which is necessary for the virulence of *K. pneumoniae* [48]. The type IV secretion system (T4SS) is necessary for the pathogenesis of the obligate intracellular pathogen *C. burnetiid*, which enables *C. burnetii* to replicate in the vacuole, thus prevent immune responses [48]. The phosphorylcholine (ChoP) LOS glycoforms can mediate the invasion of non-typeable *Haemophilus influenzae* (NTHi) into bronchial cells (Swords et al., 2000). The O-antigen is critical for *Yersinia* virulence, which enables *Yersinia* to colonize epithelial cells [49]. Taken together, we speculate that Cg5 could be a facultative intracellular pathogen.

Previous studies had shown that the invasion of *V. parahaemolyticus* and *V. cholerae* to nonphagocytic HeLa cells was mediated by Type III Effector VopC [11]. In addition, a previous study showed that *V. splendidus* LGP32 that lacking Type III Effector VopC could invade *C. gigas* immune cells and survive in the cell. The OmpU porins was shown to be essential for the invasion of LGP32 [12]. Similarly, the *V. ordalii* was also reported to invade the host cells by unclear mechanisms [50]. These studies suggested that cell invasion may be widespread in *Vibrio* species. Cell invasion promotes the survival of *Vibrio* in the host, while intracellular *Vibrio* proliferates and escapes from the cell, which leads to the accumulation of pathogenic *Vibrio* in the host and the environment. Gentamicin protection assay is a method to quantify the ability of pathogenic bacteria to invade eukaryotic cells [51]. Gentamicin protection assay showed that Cg5 could invade the cells of *C. gigas*, and the invasion rate was significant different among various tissues. These results provided preliminary evidence that *Vibrio* Cg5 is a facultative intracellular pathogen, while the mechanisms of cell invasion and the impact on the virulence of *Vibrio* Cg5 require further investigation.

5. Conclusion

The whole genome of a highly virulent *Vibrio* Cg5 isolated from moribund *C. gigas* was sequenced in this study. Based on genome sequence, the Cg5 was re-identified as *V. diabolicus* by ANI and dDDH, while it was previously identified as *V. alginolyticus* by 16S rRNA and gyrB sequences. The Cg5 was predicted to be a human pathogen with multiple antibiotic resistance. Four virulence factors associated with cell invasion and intracellular survival were predicted in the genome of Cg5. The gentamicin protection assay of primary oyster cells suggested that

Cg5 was a facultative intracellular pathogen. Overall, this study sequenced and characterized the genome of a pathogen present in oysters, determined its extensive antibiotic resistance and characteristics of cell invasion, which can be valuable in preventing and controlling diseases in oysters.

CRediT authorship contribution statement

Hebing Wang: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Chenyu Shi:** Software, Formal analysis, Data curation. **Ben Yang:** Investigation, Formal analysis, Data curation. **Qi Li:** Supervision, Resources, Funding acquisition. **Shikai Liu:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2023.106466.

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