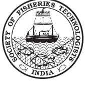 *Fishery Technology* 61 (2024) : 53 - 62

Whole Genome Sequencing of *Vibrio alginolyticus*: Insights into Virulence and Antimicrobial Resistance

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Abstract

Vibrio alginolyticus is a significant pathogen causing mortality and economic losses in mariculture and illness associated with human gastrointestinal disorders. This paper presents genome characteristics, comparative genome analysis, virulence factors, and antimicrobial resistance of a virulent *V. alginolyticus* strain (CMFRI-JF260912) isolated from diseased Asian seabass. A genome size of 5.05Mb consist of two circular chromosomes were revealed following genome sequencing using the Illumina HiSeq pipeline. Comparative analysis with other *Vibrio* species showed a close phylogenetic distance between CMFRI-JF260912 and *Vibrio natriegens*. Various virulence factors were identified, including genes related to adhesion, biofilm formation, quorum sensing, and toxin production. Four antimicrobial resistance genes namely tetracycline efflux Na+/H+ antiporter, Type B chloramphenicol O-acetyltransferase, carbenicillin-hydrolyzing class A beta-lactamase (CARB-42) and an oxytetracycline resistance phosphoribosyltransferase were also found. These findings enhance our understanding of the pathogenesis and antimicrobial resistance mechanisms of *V. alginolyticus* and offer valuable insights for mitigating *Vibrio* infections in aquaculture systems.

Keywords: *Vibrio alginolyticus,* genome sequence, virulence factors, antimicrobial resistance

Introduction

Vibrio alginolyticus is a gram-negative, halophilic, facultative anaerobic, and motile bacterium with a global distribution (Guo et al., 2018). It has been recorded from various environments, including marine and brackish water habitats (Lajnef et al., 2012). Although *V. alginolyticus* was initially considered part of the standard marine flora (Carli et al., 1993), presently it is recognized as a significant pathogen causing severe infections in cultured marine animals, including trout (Austin et al., 1993), grouper (Lee, 1995), seabream (Balebona et al., 1998), European seabass (Kahla-Nakbi et al., 2006), Asian seabass (Sharma et al., 2012) and cobia (Kumar et al., 2014), resulting in substantial economic losses. Additionally, it has been identified as an opportunistic pathogen in humans, particularly associated with wound or ear infections and gastrointestinal disorders (Slifka et al., 2017). Clinical manifestations of vibriosis in marine organisms often include haemorrhage, necrosis, ulcers, and sepsis, which have been attributed to the production of various virulence factors including siderophores, haemolysins, and proteases (Bluford et al., 2017). Several strains of *V. alginolyticus* harbour virulent genes derived from pathogenic *V. parahaemolyticus* and *V. cholerae* (Broberg et al., 2011; Gobarah et al., 2022). Majority of the environmental and clinical strains of *V. alginolyticus* were found to be resistant to antibiotics, especially beta-lactams, which is significant in the light of treatment of animal/human infections (Hernández-Robles et al., 2016), threatening aquaculture and public health (Ye et al., 2008; Lajnef et al., 2012). Horizontal gene transfer (HGT) plays a critical role in the acquisition of virulence factors and resistance genes, serving as a major driving force for the emergence and evolution of antibiotic-resistant strains (Wang et al., 2016). Therefore, it is crucial to comprehend the genomic features and virulence factors of *V. alginolyticus* to gain insights into its pathogenicity and antibiotic resistance mechanisms.

The present study analyses the genome of *V. alginolyticus* isolated from diseased Asian seabass (*Lates calcarifer*) in order to identify the genomic

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features associated with virulence and antimicrobial resistance.

Materials and Methods

Vibrio alginolyticus (CMFRI-JF260912) used for the present study was originally isolated from diseased cage-cultured Asian seabass in Karwar and deposited in the ICAR-CMFRI microbial repository (Sharma et al., 2012). The retrieved isolate was grown on Tryptone Soya Broth (TSB, Himedia) supplemented with 2% NaCl for 24 hours at 30°C with mild shaking. Genomic DNA was extracted using the HiPurA™ 96 Bacterial Genomic DNA Purification Kit (Himedia). The quantity and quality of the genomic DNA were assessed using a Biophotometer (Eppendorf). Whole genome sequencing was performed using Illumina HiSeq™ 2500 platform (Illumina) with a library containing 2×100 paired-end sequencing (Agrigenome, India).

General features and Minimum Information about the Genome Sequence (MIGS) are presented in Table 1.

The fastq files obtained from sequencing were preprocessed before assembly by trimming the adapter sequences and filtering out reads with an average quality score of less than 30 in any of the paired-end reads. Unique reads were obtained using Fastuniq (https://sourceforge.net/projects/fastuniq/files/). *De novo* assembly was performed using three different assemblers: Spades, ABySS, and Velvet. Among the assemblies generated, the Spades assembly (http://bioinf.spbau.ru/spades) with default k-mer sizes showed superior statistics and was selected for further downstream analysis. Genes were predicted from the Spades assembled contigs using the Glimmer software (https://ccb.jhu.edu/software/glimmer/). The predicted genes were then annotated through a series of steps involving comparison with the Uniprot database using the BLASTX program (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/) and organism annotation using BLASTX results (https://www.blast2go.com/). During the comparison with the Uniprot database using BLASTX, an E-value cutoff of 10^{-3} was applied. The best BLASTX hit was selected based on the query coverage, identity, similarity score, and gene description.

In order to predict the potential virulence factors of the *V. alginolyticus* strain, the essential local alignment search tool (BLAST) was used in conjunction with the Virulence Factor Database (VFDB; http://www.mgc.ac.cn/VFs/main.htm) (Chen et al., 2016). Further, queries of the contigs were performed using Resfinder (Zankari et al., 2012) and CARD (McArthur et al., 2013) to investigate the presence of antimicrobial resistance genes. The identification of tRNAs and rRNAs was conducted using tRNAscan-SE and rRNAmmer (Lagesen et al., 2007; Lowe & Eddy, 1997) respectively. Functional annotation of the protein-coding genes was performed using Gene Ontology (GO, http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/). Visualization of the chromosomes was carried out using Proksee (https://proksee.ca/) online tool.

Results and Discussion

Through Illumina HiSeq pipeline, a total of 5,261,019 high-quality paired reads were generated. The combined sequencing analysis revealed that the genome of *V. alginolyticus* (CMFRI-JF260912) consisted of two circular chromosomes. Chromosome I was 3,287,226 bp in length, with a GC content of 54.5%, and contained 3,790 predicted open reading frames (ORFs) (Fig. 1a). Chromosome II was 1,764,503 bp in size, with a GC content of 50.69%, and contained 2,065 predicted ORFs (Fig. 1b). The genome assembly resulted in the prediction of 4,510 genes.

To identify the position of the present isolate of *V. alginolyticus* in relation to other *Vibrio* species, a comprehensive phylogenetic tree, showcasing the evolutionary relationships among various *Vibrio* species, was constructed using single-copy orthologous genes. This approach provided a detailed representation of their evolutionary relationships. The tree was based on the complete genome sequences and included 10 orthologous genomes of different *Vibrio* species, as well as an out group represented by *Photobacterium damselae* subsp. *damselae.* Present strain shows same phylogenetic composition with *V. alginolyticus* (ATCC 33787). Notably, the phylogenetic distance between *V. alginolyticus* and *Vibrio natriegens* was found to be remarkably close (Fig. 2).

Out of the total genes examined, 39.02% (1760 genes) were successfully annotated using the GO database, while 59.44% (2681 genes) were annotated using the KEGG database. Among the annotated genes from the GO database, the most prevalent functions were associated with transcription (9.4%),

Table 1. General features of *V. alginolyticus* and MIGS mandatory information

Items	Description
General Features	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Vibrionales Family: *Vibrionaceae* Genus: *Vibrio* Species: *Vibrio alginolyticus*
Gram stain	Negative
Cell shape	Curved rod
Pigmentation	Non-pigmented
Temperature	4–40°C (optimum 30-35°C)
pH	6.0–9.0 (optimum 7.6–8.0)
salinity	0–7% (optimum 2%–5%)
Motility	Motile by means of flagella
MIGS data	
Investigation type	Bacteria-archaea
Project name	Genome sequence of *Vibrio alginolyticus* CMFRI - JF260912
Location	latitude: 14.806335′N; longitude: 740110880′E
Depth	NA
Geographical location name	INDIA: Karnataka: Karwar
Collection date	Before 2011-03-20
Environmental biome	Marine (ENVO: 00000447)
Environmental feature	Free living
Environmental material	Water (ENVO: 00,002,006)
Environmental package	Missing
Reference biomaterial	Not available
Source material identifier	CMFRI-JF260912
Biotic relationship	Free-living
Trophic level	Chemoorganotrophic
Relationship to oxygen	Facultative anaerobic
Genome attribute	
Sequencing technology	Illumina-Hiseq 2500
Assembly method	SPAdes
Assembly name	SPAdes v. 3.13.0
Finishing strategy	Complete; 60× coverage, 104 contigs
Genome size	5,261,019
Genome coverage	60×
G + C content (%)	44.68
Protein coding sequences	4510
rRNAs	1, 2, 3 (5S, 16S, 23S)
tRNAs	43
Number of replicons	2
Extra chromosome elements	0
Annotation source	GenBank
GenBank accession number	JAUJYY000000000
KEGG annotated proteins	2681

integral component of membrane (42.43%), and ATP binding (8.1%) (Fig. 3). The KEGG database annotations revealed a higher abundance of genes linked to biosynthesis of carbohydrate metabolism (10.66%), amino acid metabolism (9.4%), metabolism of cofactors and vitamins (6.7%), and membrane transport (6.67 %) (Fig. 4).

The putative virulence elements in *V. alginolyticus* analyzed by querying the VFDB database are depicted in Table 2. Eighty percent of the previously reported virulence factors of *V. alginolyticus* were identified in the present strain. The genes for Type II (*T2SS*) and Type III secretion systems (*T3SS*), *LuxS* gene, and *adeG* gene were located on chromosome I, while the *tlh* gene was located on chromosome II.

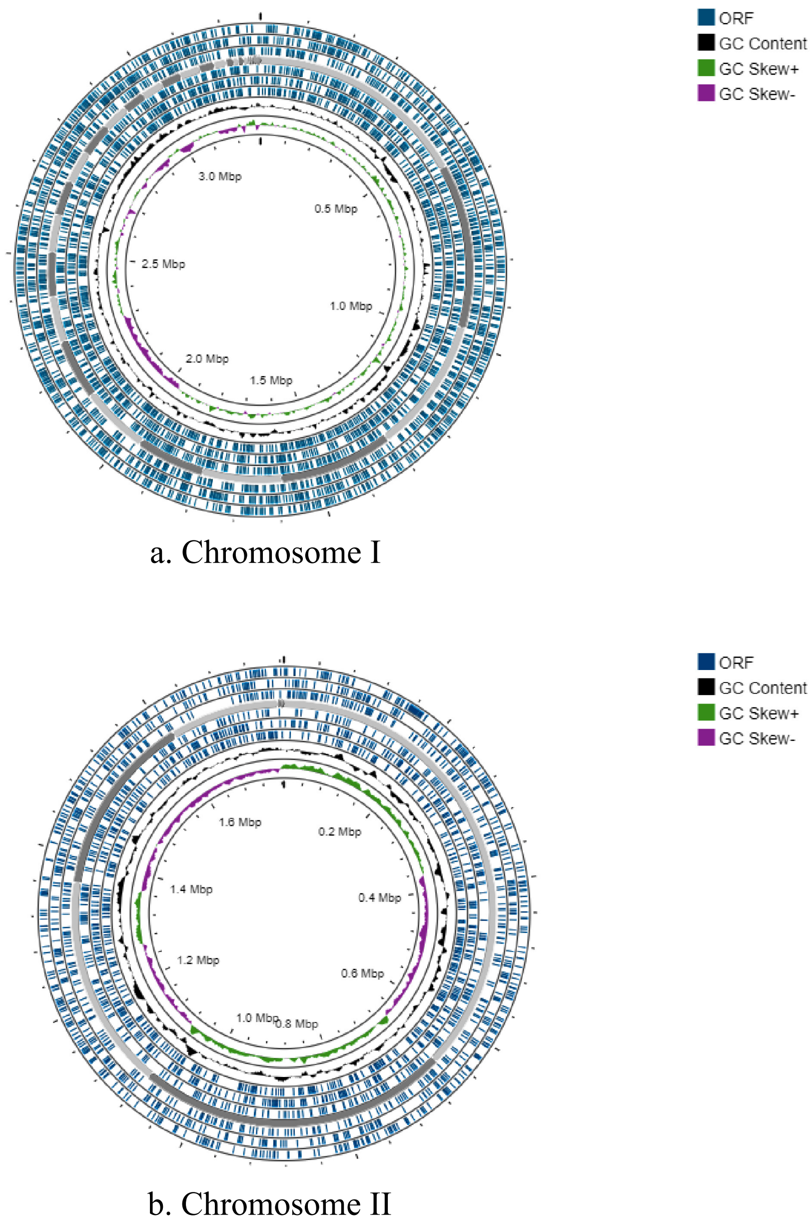

Fig. 1. The draft circular genome of *V. alginolyticus* consists of two chromosomes, chromosome I (a) and chromosome II (b). The coding sequences are distributed in blue boxes, with the plus strand in the first circle and the minus strand in the second circle. The third circle shows the mean gene centered G+C content (black plot) and GC skew (G “ C (green))/ (G + C (purple).

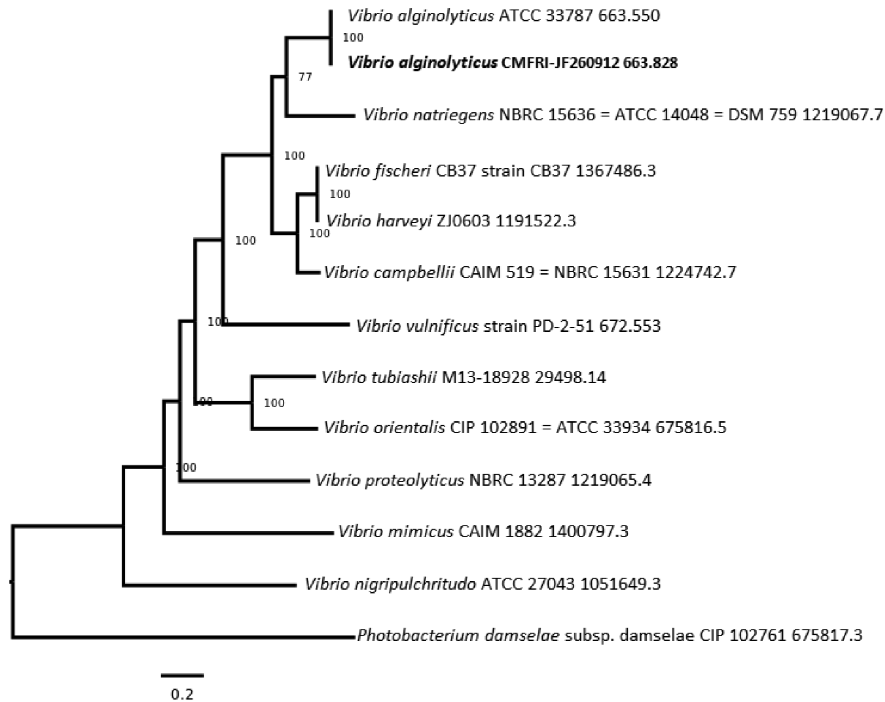

Fig. 2. Phylogenetic relationship of *V. alginolyticus* with related species of Vibrios, based on the complete genome sequences. Bootstrap values are indicated at the nodes. Scale bar indicates 0.02 substitutions per sequence position.

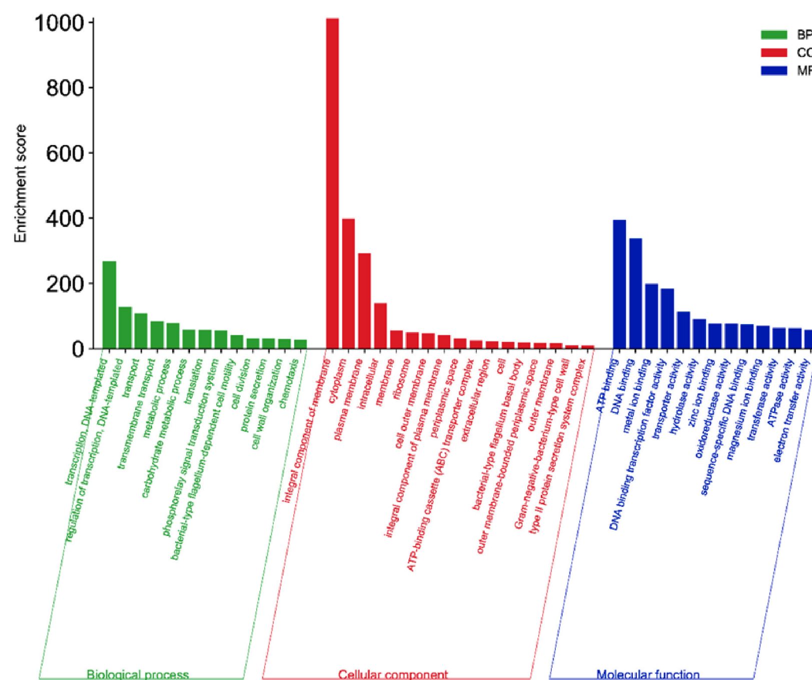

Fig. 3. Gene Ontology annotation of *V. alginolyticus*, the number of genes annotated to the subset GO terms, primarily biological Process (BP), cellular components (CC) and molecular functions (MF)

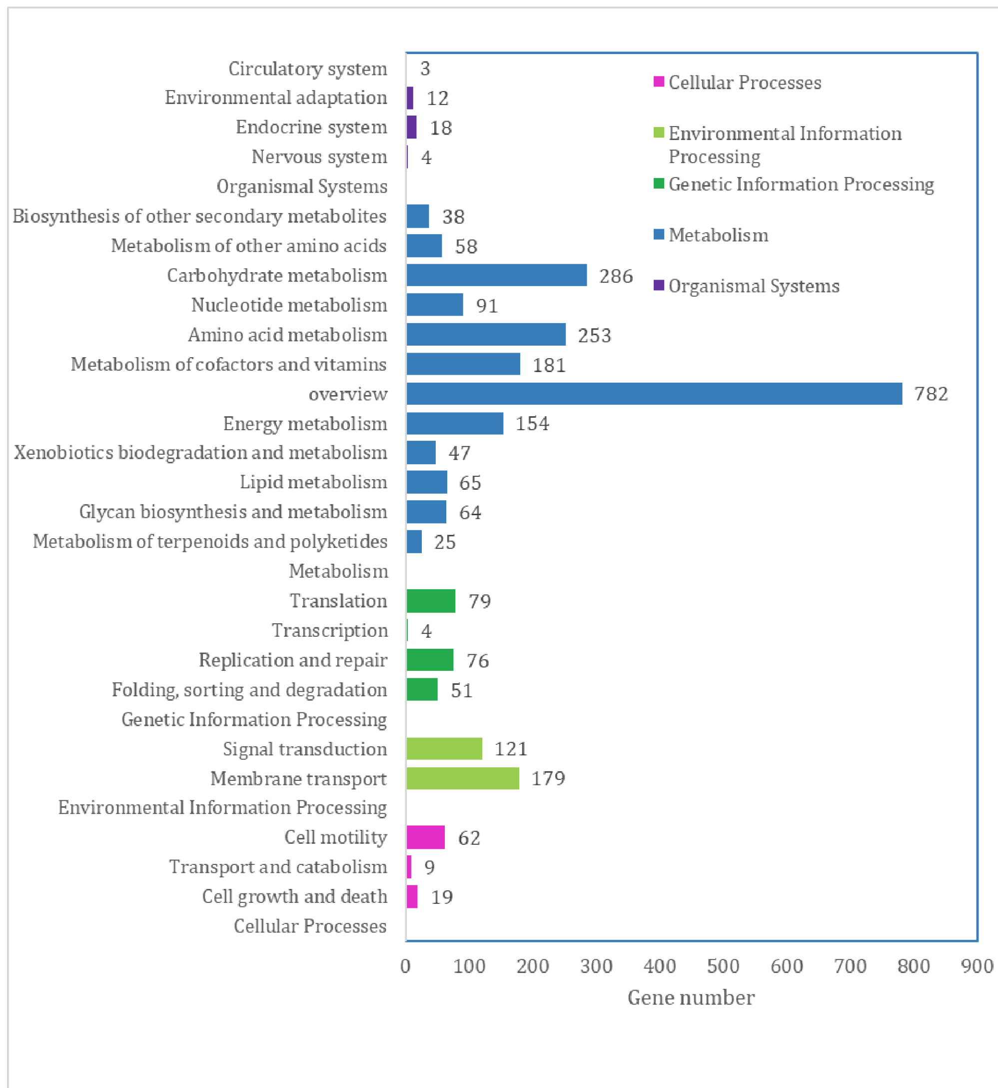

Fig. 4. The KEGG Pathway annotations of genes; the secondary classification of biological pathways is represented by the y-coordinate, while the x-coordinate denotes the number of genes; different colours are utilized to distinguish the primary classification of biological pathways.

Four genes identified in the *V. alginolyticus* genome exhibited significant homology to well-characterized antimicrobial resistance genes from the NCBI AMRfinderplus reference database (asm_accession: GCA_030487685.1). These included a tetracycline efflux Na+/H+ antiporter family transporter (Tet 35) with a sequence identity of 96.06%, a type B chloramphenicol O-acetyltransferase with 99.09% identity, a carbenicillin-hydrolyzing class A beta-lactamase (CARB-42) with 100% identity, and an oxytetracycline resistance phosphoribosyltransferase domain-containing protein (Tet 34) with 92.21% identity.

The present study unveiled the genome characteristics, presence of virulence factors, and antimicrobial resistance genes in a virulent strain *of V. alginolyticus* (CMFRI-JF260912). A comparative genome analysis was done based on the complete genome sequences. Interestingly, the phylogenetic distance between *V. alginolyticus* and *Vibrio natriegens* was found to be remarkably close. The presence of virulence factors is crucial for the pathogenicity of the bacteria. Virulence factors responsible for all the five steps involved in the pathogenic invasion, namely adhesion, invasion, colonization, proliferation, and production of toxins were found in the

Table 2. Predicted major genomic features of *V. alginolyticus*

Virulence factors Adherence	Annotation	Chromosome
MshA, mshC, mshD, mshE, MshF, mshG, mshH, mshI, MshJ, mshAK, mshL, mshM, MshN	Mannose-sensitive hemagglutinin (MSHA type IV pilus)	Chromosome I
PilA, pilB, pilC, pilD	Type IV pilus	Chromosome I
Antiphagocytosis		
wbfV/wcvB, wecA	Capsular polysaccharide	Chromosome I
cpsA, cpsB, cpsC, cpsD, cpsE, cpsF, cpsG, cpsH, cpsI, cpsJ, wza, wzc	Capsular polysaccharide	Chromosome II
Chemotaxis and motility		
CheA, cheB, cheR, cheV, cheW, cheY, cheZ, filM, flaA, flab, flaD, flaE, flag, flaI, flgA, flgB, flgC, flgD, flgE, flgF, flgG, flgH, flgI, flgJ, flgK, flgL, flgM, flgN, flhA, flhB, flhF, flhG, fliA, fliD, fliE, fliF, fliG, fliH, fliI, fliJ, fliK, fliL, fliN, fliO, flip, fliQ, fliR, fliS, flrA, flrB, flrC, motA, motB, motX, motY	flagella	Chromosome I
Iron uptake		
irgA	Enterobactin receptors	Chromosome I
vctA		Chromosome II
HutA, hutR	Heme receptors	Chromosome II
VctC, vctD, vctG, vctP	Periplasmic binding protein-dependent ABC transport systems	Chromosome II
Quorum sensing		
luxS	Autoinducer-2	Chromosome I
Secretion system		
EpsC, epsE, epsF, epsG, epsH, epsI, EpsJ, epsK, epsL, epsM, epsN, gspD	EPS type II secretion system	Chromosome I
VopQ, vopR, vopS	T3SS1 secreted effectors	Chromosome I
sycN, tyeA, vcrD, vcrG, vcrH, vcrR, vcrV, virF, virG, vopB, vopD, vopN, vscA, vscB, vscC, vscD, vscF, vscG, vscH, vscI, vscJ, vscK, vscL, vscN, vscO, vscQ, vscR, vscS, vscT, vscU, vscX, vscY, vxsC	T3SS1	Chromosome I
Biofilm formation		
adeG	AdeFGH efflux pump/transport autoinducer (Acinetobacter)	Chromosome I
Colonization and immune evasion		
kpsF	Capsule biosynthesis and transport (Campylobacter)	Chromosome I
Toxin		
tlh	Thermolabile hemolysin	Chromosome II

sequenced strain. Almost 80% of the previously reported virulence factors of *V. alginolyticus* were observed in the present strain, indicating its potential for pathogenic invasion. Wang et al. (2016) reported the presence of multiple virulence factors such as microbial collagenase, leukocidin, hemolysin, RTX toxin-related calcium-binding protein, and zonular occludens toxin on the chromosomes and plasmids of *V alginolyticus* isolated from seawater. The authors also reported two *T6SS* gene clusters in one of the two chromosomes.

Gram-negative bacteria utilize specialized translocation systems known as Secretory Systems (SSs) to transport proteins across the outer membrane and facilitate bacterial invasion (Green & Mecsas, 2016). In the present study, the Type II and Type III Secretion Systems were located on chromosome I. The *T2SS* functions as a multi-protein complex facilitating the secretion of hydrolytic enzymes and toxins, contributing to the degradation of host tissues and evasion of host immune responses (Korotkov & Sandkvist, 2019). *T3SS*, directly delivers effector proteins into host cells, enabling colonization and survival within them (Coburn et al., 2007). The regulation of *T2SS* and *T3SS* genes is responsive to specific environmental conditions and transcriptional factors (Ramamurthy et al., 2020). Both systems play crucial roles in the virulence of *V. alginolyticus*, with *T2SS* facilitating nutrient acquisition and immune evasion, while *T3SS* promoting host cell invasion and intracellular survival (Zhao et al., 2018). Understanding these virulence factors may aid in developing targeted interventions against this pathogenic strain. The occurrence of *T2SS* and *T3SS*s in *V. alginolyticus* and other *Vibrio* species suggests their potential involvement in fish-bacteria interactions across diverse habitats. The *LuxS* QS system has been implicated in regulating the expression of virulence factors in various pathogenic bacteria. *LuxS* gene located on chromosome I, modulates various virulence factors like protease, extra polymeric substances, and biofilms (Ye et al., 2008). *LuxS* is responsible for producing an autoinducer molecule, AI-2, which plays an important role in quorum sensing (Xu et al., 2006). AI-2, a furanosyl borate diester, is synthesized by *LuxS* synthase and serves as a signal for intra-species and inter-species communication (Jensen et al., 2013). These AIs activate the transcription of *tdh* and the biofilm formation related genes by binding to the receptors (*luxN, luxQP, CqsS*) on *Vibrio* surface (Wang et al., 2013). The *adeG* gene positioned on chromosome I is generally associated with biofilm formation, an essential survival mechanism for pathogenic bacteria, contributing to their ability to persist in various environments (Liu et al., 2023). It enhances the pathogen's adhesion to host surfaces, which is a critical initial step in the infection process (Chen et al., 2008).

Hemolysin, secreted as an extracellular product, is considered the most crucial and widely distributed virulence component among pathogenic vibrios (Zhang & Austin, 2005). The *tlh* gene located on chromosome II, encoding a thermolabile hemolysin, may also contribute to the present strain's pathogenicity. Previous studies have demonstrated apoptotic events in human vascular endothelial cells and rat cells following exposure to hemolytic fractions from *V. vulnificus* and *V. parahaemolyticus*, respectively (Naim et al., 2001).

The present investigation also revealed the presence of AMR genes, conferring resistance to tetracycline, chloramphenicol, carbenicillin, and oxytetracycline. There have been several reports of resistance of Vibrios to multiple antimicrobial classes such as ampicillin, penicillin and tetracycline (Zanetti et al., 2001; Dutta et al., 2021). Earlier studies revealed 22 AMR genes, including genes associated with multidrug-resistant efflux pumps in the draft genome of *V. alginolyticus* OS1T-47 (Yasir et al., 2020). The availability of genomic sequences, in conjunction with our preliminary findings on virulence factors, will substantially contribute to the comprehension of pathogenesis and antimicrobial resistance mechanisms in *Vibrio* species within marine aquaculture systems.

This study provides a comprehensive understanding of the genome characteristics, the presence of virulence factors and AMR genes in *V. alginolyticus*. The knowledge gained may enhance our understanding of the pathogenicity and antimicrobial resistance mechanisms in *V. alginolyticus*, which is valuable for developing targeted interventions to mitigate *Vibrio* infections in aquaculture systems. The findings presented here serve as a foundation for future studies focusing on the molecular mechanisms underlying *Vibrio* pathogenesis and developing effective strategies to prevent *Vibrio* infections in aquatic environments.

Accession numbers of nucleotide sequence: The complete genome sequences of the chromosome have been deposited at GenBank under the

BioProject: PRJNA992271 with accession numbers: JAUJYY010000001-JAUJYY010000104 (BioSample Id: SAMN36346887; SRA Id: SRR25383678)

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