



Article

Comparative Genome Analysis of Piscine *Vibrio vulnificus*: Virulence-Associated Metabolic Pathways

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Abstract: Vibriosis caused by *Vibrio vulnificus* is a major problem in aquatic animals, particularly brown marble groupers (*Epinephelus fuscoguttatus*). *V. vulnificus* biotype I has recently been isolated and classified into subgroups Suku_G1, Suku_G2, and Suku_G3 according to the different types of virulence genes. In a previous study, we have shown that biotype I *V. vulnificus* strains were classified into three subgroups according to the different types of virulence genes, which exhibited different phenotypes in terms of growth rate and virulence. To gain insight into the different genetic features revealed by the potential virulence mechanisms of *V. vulnificus* in relation to a spectrum of pathogenesis, comparative genomic analyses of three biotype I *V. vulnificus* strains belonging to different subgroups (Suku_G1, Suku_G2, and Suku_G3) were performed. The *V. vulnificus* genome is composed of two circular chromosomes with average sizes of 3 Mbp and 1.7 Mbp that are evolutionarily related based on the analysis of orthologous genes. A comparative genome analysis of *V. vulnificus* revealed 5200 coding sequences, of which 3887 represented the core genome and the remaining 1313 constituted the dispensable genome. The most virulent isolate (Suku_G1) carries unique enzymes that are important for lipopolysaccharide (LPS) and capsular polysaccharide (CPS) synthesis, as well as flagellar glycosylation, and harbors another type of repeat in toxin (RTX) and bacterial defense mechanisms. The less virulent isolate (Suku_G2) shares enzymes related to CPS biosynthesis or flagellar glycosylation, while the avirulent isolate (Suku_G3) and a less virulent isolate (Suku_G2) share enzymes related to the production of rare sugars. Interestingly, the isolates from the three subgroups containing specific CMP-N-acetylneuraminate-producing enzymes that are correlated with their growth abilities. Collectively, these observations provide an understanding of the molecular mechanisms underlying disease pathogenesis and support the development of strategies for bacterial disease prevention and control.

Keywords: comparative genome; metabolic pathway; pathogenesis; *Vibrio vulnificus*; virulence factors associated pathogenesis

1. Introduction

Vibriosis is a bacterial disease caused by *Vibrio* spp., such as *V. vulnificus*, *V. harveyi*, *V. ordalii*, *V. splendidus*, and *V. salmonicida*, that affects diverse marine fish and shellfish, leading to worldwide economic losses in the aquaculture industry [1,2]. Groupers are marine fish

species with a mortality rate of approximately 66.7%, owing to vibriosis [3]. In Thailand, *V. vulnificus*, a Gram-negative halophilic bacterial pathogen [4], is a major bacterial species that causes diseases in the mariculture brown-marbled grouper (*Epinephelus fuscoguttatus*) [5].

In recent years, our laboratory isolated *V. vulnificus* biotype I bacteria from diseased brown-marbled groupers, which were classified into three subgroups (SUKU_G1, SUKU_G2, and SUKU_G3) based on the presence of virulence genes [6]. Importantly, all three STs show distinct pathogenicity, with the brown-marbled grouper, *V. vulnificus* SUKU_G1, exhibiting the highest virulence, whereas SUKU_G3 was non-virulent [6]. Moreover, the growth rate of *V. vulnificus* showed that SUKU_G1 and SUKU_G3 had the fastest growth rates compared to SUKU_G2 [6]. Sixteen common virulence genes were found in all the isolates, including *vvpE*, *gloB*, *tlh*, *Mann Hemo*, *ManIIA*, *rimO*, *vllY*, *ompU*, *vvhA*, *rtx*, *hlyIII*, *pilF*, *Mann TRAP*, *vcgC*, *16S rRNA type B*, and *nanA*, which are present in isolates classified as SUKU_G1. The SUKU_G2 isolates harbored four additional virulence genes (*YJ-like nab 1*, *YJ-like nab 2*, *cps*, and *sodA*), whereas SUKU_G3 isolates harbored three additional genes (*CM-like nab 1*, *CM-like nab2*, and *sodA*). Moreover, multilocus sequence typing (MLST) indicated that all isolated bacteria belonged to new sequence types (STs): SUKU_G1 (ST-595), SUKU_G2 (ST-596), and SUKU_G3 (ST-597).

Recently, whole-genome sequencing (WGS) has provided insights into antimicrobial resistance (AMR), metabolism, and the virulence of bacteria [7]. Pan-genomic reverse vaccinology compares the genomes of related bacterial strains to predict putative vaccine candidates and develop universal vaccines that are effective against different strains of the same pathogenic species [8,9], such as vaccines against *Escherichia coli* O157:H7 [10]. Moreover, synthetic biology and genome engineering can be used to develop novel diagnostic methods for infection detection, gain insight into antibiotic resistance mechanisms, and develop attenuated vaccine strains based on pathogenic bacterial genomes [7,11].

The genomes of *Vibrio* species typically have two chromosomes and mobile genetic elements, such as genomic islands (GI) and bacteriophages, that encode virulence and antibiotic resistance proteins [12]. Although all isolates of *V. vulnificus* were classified as biotype I, genomic information related to piscine pathogenesis is not available. Therefore, the genomes of the three subgroups, SUKU_G1, SUKU_G2, and SUKU_G3, were sequenced, and a comparative genome analysis was performed among the different strains to extend our understanding of the pathogenicity of *V. vulnificus* and related virulence factors at the genomic level.

2. Materials and Methods

2.1. Bacterial Strains

Three isolates of *V. vulnificus*, SUKU_G1, SUKU_G2, and SUKU_G3, were identified and characterized in Krabi, Thailand, between 2014 and 2017 [6]. Briefly, bacteria were isolated from the diseased groups and classified into three subgroups. The bacteria were cultured on Thiosulfate Citrate Bile Salt Sucrose agar to obtain single colonies. The sequence type of *V. vulnificus* in each group was verified using PCR testing of the virulence gene profile and an MLST analysis [6]. All isolated bacteria were stored at -80°C until further use.

2.2. WGS and Assembly

SUKU_G1, SUKU_G2, and SUKU_G3 were cultured in tryptic soy broth (Difco, Franklin Lakes, NJ, USA) containing 1.5% sodium chloride at 30°C , with shaking at 220 rpm for 16 h. Total genomic DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The genome was sequenced using Illumina SBS technology in 2017 (Macrogen, Seoul, Republic of Korea) and converted into raw data for bioinformatic analysis. Raw sequence read data were tested for quality control using FastQC [13]. The reads were assembled using the reference genome of the pathogenic *V. vulnificus* strain CMCP6 biotype I (GenBank assembly accession: GCA_000039765) using the Burrows-Wheeler Aligner [14]. Variant information

(single nucleotide polymorphisms (SNPs) and short indels) was obtained using SAMTools (<http://www.htslib.org>, accessed on 20 September 2017) through genomic alignment with the reference genome [15], and the aligned data were converted to FASTA format for further analysis. This whole-genome shotgun project was deposited in DDBJ/ENA/GenBank under accession numbers JAVKUT000000000 (SUKU_G1), JAVKUU000000000 (SUKU_G2), and JAVKUV000000000 (SUKU_G3). To construct the chromosome-level genome, the contigs were mapped and ordered into each chromosome of the reference genome using CONTIGuator (<https://contiguator.sourceforge.net>, accessed on 19 October 2021), a software tool for genome finishing, and the genome structure and contig profiling were visualized using the Artemis comparison tool [16,17].

2.3. Genome Annotation

The assembled genome was annotated with the Rapid Annotation Subsystem Technology (RAST) server v. 2.0 using the SEED framework (<http://rast.nmpdr.org/>, accessed on 25 October 2021) [18–20]. The virulence factor database (VFDB) was used to predict virulence factors based on BlastP search (<http://www.mgc.ac.cn/VFs/main.htm>, accessed on 30 October 2021) [21]. Genes potentially responsible for AMR in the *V. vulnificus* genome were identified using the Resistance Gene Identifier (RGI) with the Comprehensive Antibiotic Resistance Database (CARD) [22].

2.4. Mobile Genetic Element Prediction

Putative GI and prophages were predicted and compared using IslandCompare (<https://islandcompare.ca/>, accessed on 1 November 2021) [23] and PHAGE Search Tool Enhanced Release (PHASTER) (<https://phaster.ca/>, accessed on 1 November 2021), respectively [24]. The *V. vulnificus* genome was searched for clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system-associated locus using CRISPRminer (<http://www.microbiome-bigdata.com/CRISPRminer/index.php/Home/Index/help>, accessed on 3 November 2021) [25].

2.5. Comparative Whole-Genome Analysis

2.5.1. Average Nucleotide Identity (ANI)

The average nucleotide identity values between the three piscine *V. vulnificus* genomes and the reference genome (CMCP6) were measured using the BLAST + (ANIb) program on the JSpeciesWS server (<http://jspecies.ribohost.com/jspeciesws/>, accessed on 2 December 2021) [26].

2.5.2. Phylogenetic Tree Analysis

A phylogenetic tree was constructed from 3 piscine *V. vulnificus*, and 25 reference strains retrieved from the NCBI RefSeq database (Table S1). The core genome multilocus sequence typing (cgMLST) scheme was performed using SeqSphere+ (Ridom GmbH, Münster, Germany, <http://www.ridom.de/seqsphere/index.shtml>, accessed on 20 November 2024) with default parameters [27,28].

2.5.3. Prediction of Clusters of Orthologous Groups (COGs)

Clusters of orthologous groups are clusters of proteins that are orthologous across at least three lineages and correspond to ancient, conserved domains. Clusters of orthologous groups were annotated and compared before the gene ontology (GO) enrichment analysis for the *V. vulnificus* genomes using the online program OrthoVenn2 (<https://orthovenn2.bioinfotoolkits.net/start/db>, accessed on 4 December 2021) with default parameters (E-value 1×10^{-2} and inflation value of 1.5) [29].

2.5.4. Comparative Whole-Genome Visualization

Genome comparisons were performed among *V. vulnificus* strains using the BLAST Ring Image Generator (BRIG) [30]. A circular genomic map was drawn using the reference

genome (*V. vulnificus* strain CMCP6) on a local BLAST + basis, with parameters 70% lower, a 90% upper cut-off for identity, and an E-value of 10. The ring color gradients correspond to varying degrees of the BLAST match identity. Circular genomic maps also included information on GC skew and content.

2.6. Pan-Core Genome Analysis

The bacterial pan-genome analysis tool was used to analyze the pan-core genomes of the three subgroups of *V. vulnificus* [31]. The program embedded MUSCLE and rsvg-convert for sequence alignment and tree generation [32]. The sGnuplot 4.6.6 program was used to plot the results. Functions and pathways were analyzed using BLASTKOALA based on the Kyoto Encyclopedia of Genes and Genomes ontology assignments [33].

3. Results

3.1. Genome Properties of Three Subgroups of *V. vulnificus*

The total base reads from the three subgroups of *V. vulnificus* showed more than 90% GC content similarity among the three isolates (chromosome I: 46.6–46.8% and chromosome II: 47–47.3%). The highest number of SNPs in the genome was observed in SUKU_G1, followed by SUKU_G2 and SUKU_G3, compared with the reference genome (*V. vulnificus* strain CMCP6). Nucleotide insertions and deletions were the lowest in the SUKU_G2 genome, with 275 deleted sites and 303 inserted sites, whereas SUKU_G1 and SUKU_G3 showed similar numbers of deleted and inserted sites. Mapped read data were assembled from the genome of *V. vulnificus* into two circular chromosomes, with GC content of approximately 46% in chromosome I and 47% in chromosome II. G1 had 3.2 Mbp in chromosome I (large) and 1.8 Mbp in chromosome II (small), whereas G2 and G3 contained 3 Mbp in the large chromosome and 1.7 Mbp in the small chromosome (Table 1).

Table 1. Summary of assembly and annotation characteristics for *V. vulnificus*.

Chromosome	SUKU-G1		SUKU-G2		SUKU-G3	
	I	II	I	II	I	II
Total read (Mbp)	8.8		6.2		9.5	
Reference length (Mbp)			5.1			
Mapped sites (Mbp)	4.7 (91.75%)		4.6 (90.41%)		4.6 (91.5%)	
Number of contigs	207		209		66	
SNPs	67,907		66,806		63,273	
Insertion/deletion	300/340		275/303		306/344	
Chromosomes number	2		2		2	
Mapped contigs	66	31	68	31	40	1
Mapped length (Mbp)	3.2	1.8	3.0	1.7	3.0	1.7
GC content (%)	46.6	47	46.8	47.2	46.7	47.3
Number of coding sequences	3041	1629	2872	1638	2901	1536
Number of subsystems	371		368		367	
Number of RNA						
rRNA	1		-		-	
tRNA	48		37		62	

Genome annotation showed that G1 had the highest number (4670) of CDS, followed by 4510 in G2 and 4437 in G3. Approximately 30% of CDSs were functionally categorized into 367–371 subsystems. Among these subsystems, amino acids and derivatives, carbohydrates, and protein metabolism had the highest number of genes (Figure 1). SUKU_G2 had 20% fewer metabolism-related proteins than SUKU_G1 and SUKU_G3.

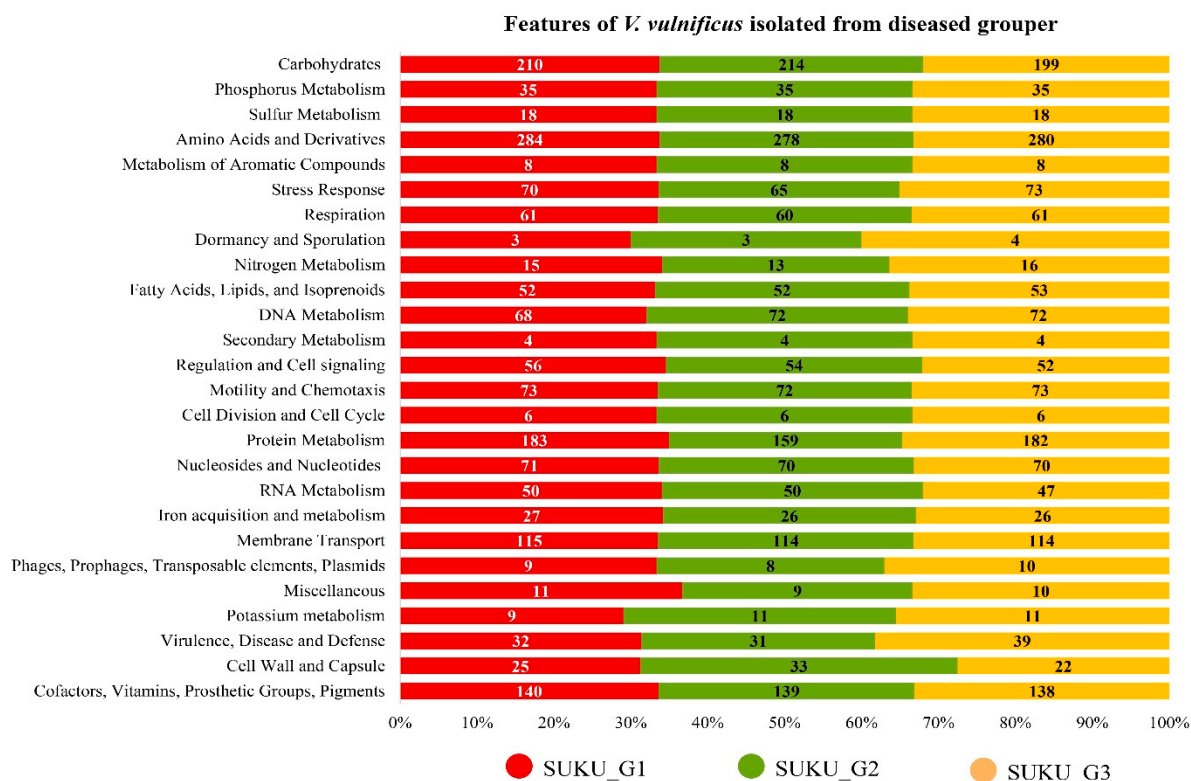


Figure 1. Functional annotation of predicted coding sequencing (CDs) from three strains of *V. vulnificus* (SUKU_G1, SUKU_G2, and SUKU_G3) isolated from diseased grouper using the SEED subsystem. Number represented the number of genes annotated within a particular function.

3.2. Identification of Genomic Islands (GI)

All isolates had one GI region in chromosome I, whereas chromosome II from SUKU_G1, SUKU_G2, and SUKU_G3 had four, five, and one GI regions, respectively (Figure 2). SUKU_G1 and SUKU_G2 shared GI regions that contained the 2-amino-3-ketobutyrate coenzyme A ligase and L-threonine 3-dehydrogenase genes.

3.3. Prophage Identification

Prophages in bacterial chromosomes play an important role in lateral gene transfer, harboring virulence factors and pathogenicity islands [34–36]. The PHASTER web server was used to annotate the putative prophage sequences in the *V. vulnificus* genome. In all three subgroups, the putative *Vibrio* prophage vB_VpaM_MAR was found in all three subgroups. *V. vulnificus* isolates SUKU_G1 and SUKU_G3 shared the *Klebsiella pneumoniae* ST147 VIM1phi7.1 phage, whereas SUKU_G2 and SUKU_G3 shared the *Bacillus* phage phBC6A51 (Table 2). SUKU_G1 had two unique prophages, *Enterobacteria* phage mEp235 and *Vibrio* phage PV94, whereas *Vibrio* phages 12B12 and KSF 1phi were found only in SUKU_G2, and *Vibrio* phages VfO3K6 and VFJ were found only in SUKU_G3. However, the majority of CDS of the predicted prophages encoded hypothetical proteins; therefore, it is unclear whether these prophages contribute to the virulence of this bacterium.

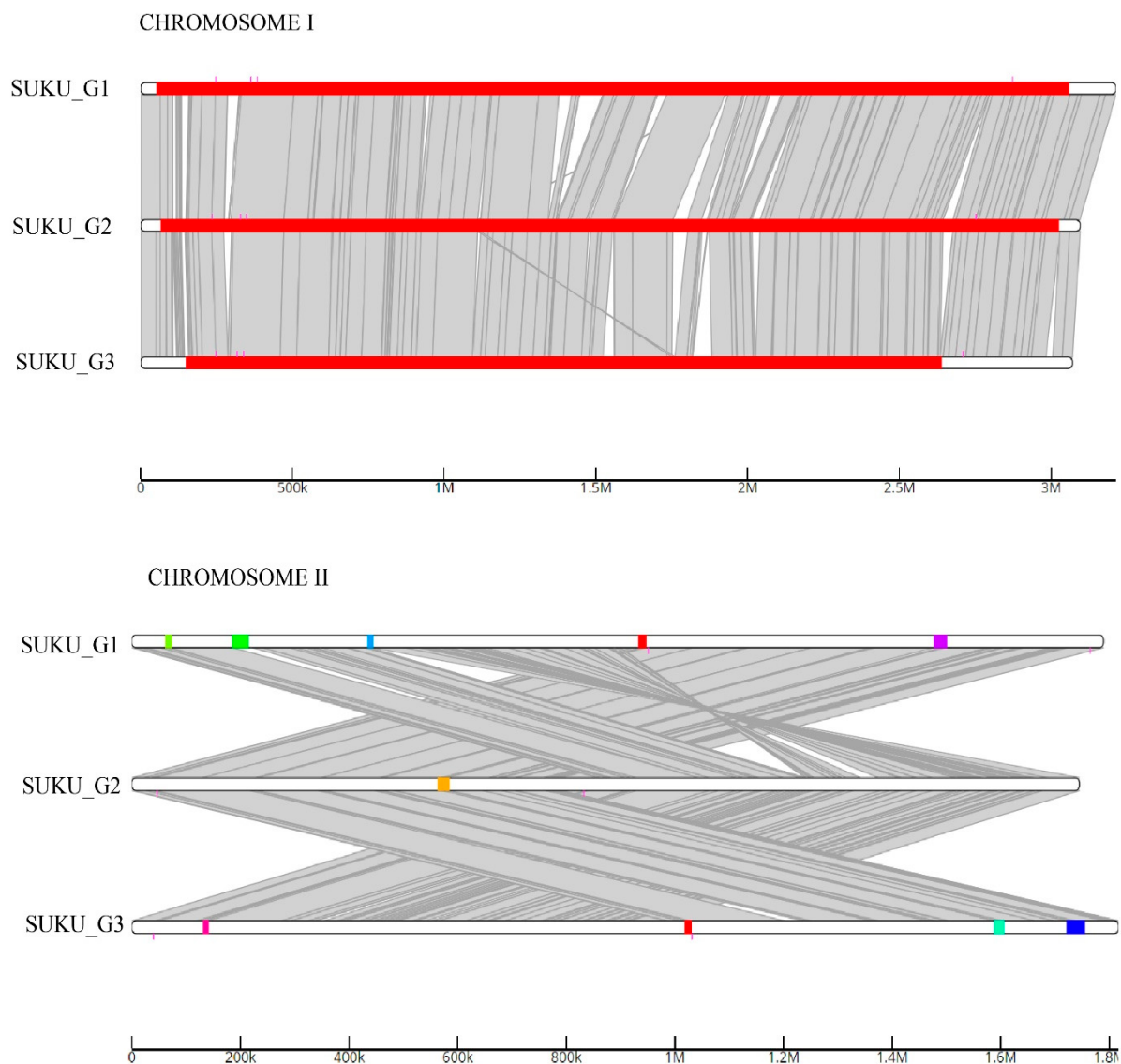


Figure 2. Comparative overview of genomic islands (GIs) found in the *V. vulnificus* genomes predicted using IslandCompare. Different box colors represent different clusters of GIs.

Table 2. Prophage identification from *V. vulnificus* genomes.

Predicted Prophage	SUKU_G1	SUKU_G2	SUKU_G3
Phage <i>Vibrio</i> vB	+	+	+
VpaM MAR	+	+	+
Phage <i>Klebsi</i> ST147	+	—	+
VIM1phi7.1	+	—	—
Phage <i>Enterobacter</i> mEp235	+	—	—
Phage <i>Vibrio</i> PV94	—	+	+
Phage <i>Bacillus</i>	—	+	—
phBC6A51	—	+	—
Phage <i>Vibrio</i> 12B12	—	—	+
Phage <i>Vibrio</i> KSF 1phi	—	—	+
Phage <i>Vibrio</i> Vfo3K6	—	—	+
Phage <i>Vibrio</i> VFJ	—	—	+

+, present; —, absent.

3.4. CRISPR Prediction

CRISPR has an immune function in bacteria and has been reported to be associated with virulence factors in some bacteria, such as *V. parahaemolyticus* [37]. To understand the role of CRISPR in the virulence and evolution of pathogens, CRISPR sequences and spacers were predicted. Only two CRISPR sequences were found in the SUKU_G1 genome, which is located on chromosomes I (2011365–2011478) and II (1251773–1251889), with one spacer at each site. The predicted genes in the 20-kb region and their detailed features are listed in Table S2.

3.5. Virulence Factors and AMR Profiles

Putative virulence factors involved in nutritional/metabolic pathways (heme biosynthesis and biotin synthesis), adherence (chitin-regulated pilus, mannose-sensitive HA, outer membrane protein U, Flp pili), type II and VI secretion system proteins, anti-phagocytosis (capsule), chemotaxis and motility, exotoxins, and exoenzymes (RTX, thermolabile hemolysin, leukocidin family pore-forming toxin (VvhA), and metalloproteinase) were observed in all three subgroups of *V. vulnificus* genomes (Table 3). Many putative virulence factors were observed in *V. cholerae*, *V. parahaemolyticus*, and other bacteria, including *Aeromonas* spp., *Acinetobacter* spp., *Haemophilus* spp., *Legionella* spp., *Pseudomonas* spp., and *E. coli*.

Table 3. Potential virulence factor profiles of the three *V. vulnificus* strains predicted by the virulence factor database.

Classification	Virulence Factors	Related Genes	Chr.
Nutritional/ Metabolic factor	Heme biosynthesis (<i>Haemophilus</i>)	<i>hemA, hemB, hemC, hemD, hemE, hemG, hemH, hemL, hemM, hemN, hemX, hemY</i>	I
	CcmC (<i>Legionella</i>)	<i>ccmA, ccmB, ccmC, ccmE, ccmF</i>	I
	Biotin synthesis (<i>Francisella</i>)	<i>bioA, bioB, bioD, bioF</i>	I
	Vibriobactin/ Vulnibactin	<i>vibA, vibB, vibC, vibE, vibF, vibH, viuA, viuB, BJE04_RS21740, BJE04_RS21750, BJE04_RS21780, BJE04_RS21785, BJE04_RS21790</i>	II
	VctPDGC system	<i>vctP, vctD, vctG, vctC</i>	II
	Aerobactin (<i>Escherichia</i>)	<i>iutA</i>	II
	Heme receptor	<i>hutA, hutR</i>	II
	HuvA (<i>Listonella</i>)	<i>huvB, huvC, huvD, huvX, hucZ</i>	II
	ChiRP	<i>pilA, pilB, pilC, pilD/vcpD, pilM, pilN, pilO, pilP, pilQ, pilT, PilT/PilU, VV1_RS01710, VV1_RS01705, VV1_RS01700,</i>	I
	Mannose-sensitive HA	<i>mshA-N</i>	I
Adherence	Immunogenic lipoprotein A	<i>IlpA</i>	I
	Outer membrane protein U	<i>ompU</i>	I
	Flp type IV pili (<i>Aeromonas</i>)	<i>flpA-L, flp1</i>	I
	Accessory colonization factor	<i>acfB, acfD</i>	I
	Heat shock protein 60	<i>hspB</i>	I,II
	N-acetylglucosamine-binding protein	<i>gbpA</i>	II
	Flp pili	<i>VV1_RS15585, VV1_RS15590, VV1_RS15595, VV1_RS15600, VV1_RS15605, VV1_RS15610, VV1_RS15615, VV1_RS15620, VV1_RS15625, VV1_RS15630, VV1_RS15635, VV1_RS15640</i>	II
Effector delivery system	Eps T2SS	<i>epsC-N, VV1_RS03010</i>	I
	T6SS HSI-I (<i>Pseudomonas</i>)	<i>vgrG1a, hcp1, clpV1, hsiH1/tssG, hsiG1/tssF, hsiF1/tssE, hsiE1, hsiC1/vipB/tssC, hsiB1/vipA/tssB, hsiA1, pppA, tagF/pppB, icmF1/tssM1, dotU1, hsiJ1, lip1, fha1, ppkA</i>	II

Table 3. Cont.

Classification	Virulence Factors	Related Genes	Chr.
Immune modulation	LOS (<i>Haemophilus</i>)	<i>htrB, waaQ, lgtF, kdkA, msbA, msbB, kpsF, kdsA, gmhA/lpcA, opsX/rfaC, rfaD, rfaE, lpxA-E, lpxH, lpxK, orfM, manB/yhxB</i>	I
	LOS	<i>manB/yhxB</i>	II
	LPS (<i>Pseudomonas</i>)	<i>hisH2, hisF2, waaA, waaF</i>	I
	Capsule (<i>Klebsiella</i>)	<i>gndA</i>	I
	Capsule (<i>Francisella</i>)	<i>rpe</i>	I
	Capsular polysaccharide	<i>wbfB, wbfC, wbpM, ugd, wza-c</i>	I
	Exopolysaccharide (<i>Haemophilus</i>)	<i>cpsA-D, cpsF, cpsH-J</i>	II
Chemotaxis and motility	Flagella	<i>mrsA/glmM, pgi</i>	I
		<i>manA</i>	II
Regulation	Two-component system (<i>Acinetobacter</i>) GacS/GacA two-component system (<i>Pseudomonas</i>)	<i>cheA, cheB, cheR, cheV, cheW, cheY, cheZ, flgA-O, flgT, flaA-E, flaG, flal, flhA, flhB, flhF, fleN/flhG, flrA, fleS/flrB, fleR/flrC, fliA, fliD-S, motA, motB, motX, motZ</i>	I
		<i>bfmR, bfmS</i>	I
		<i>GacS, GacA</i>	I
Biofilm	Alginate (<i>Pseudomonas</i>)	<i>mucA-D, mucP, algB, algU, algR, algW, algZ</i>	I
	VPS	<i>vpsE, vpsG, vpsH, vpsI, vpsU</i>	I
	AI-2	<i>luxS</i>	I
Stress survival	catalase-peroxidase (<i>Legionella</i>)	<i>katB</i>	I
	superoxide dismutase (<i>Legionella</i>)	<i>katA</i>	II
		<i>sodB</i>	I
Exotoxin	Clp protease proteolytic (<i>Listeria</i>)	<i>sodCI</i>	II
	Thermolabile hemolysin	<i>clpP</i>	I
	Leukocidin family pore-forming toxin	<i>tlh</i>	II
Exoenzyme	Repeat in toxin	<i>vvhA</i>	II
	Metallopeptidase	<i>rtxA-E, rtxH</i>	II
		<i>hap/vvp</i>	II

CcmC, cytochrome c maturation; ChiRP, chitin-regulated pilus; T6SS, type 6 secretion system; LOS, lipooligosaccharides; LPS, lipopolysaccharides; VPS, Vibrio polysaccharide; AI-2, cholerae autoinducer-2; Eps, extracellular protein secretion.

V. vulnificus strain Suku_G1 has a unique pathway for the production of CMP-pseudaminic acid (CMP-Pse5Ac7Ac) (*Pse* gene) and LPS transport system (*wzx* and *wzy*), whereas the Suku_G2 genome contains genes involved in the production of dTDP-4-amino-4,6-di-deoxy-D-galactose related to capsular polysaccharide biosynthesis. Different sets of genes involved in the capsular polysaccharide biosynthesis pathways, UDP-N-acetyl-D-mannosaminouronate (UDP-ManNAcA), UDP-N-acetyl-alpha-D-quinovosamine (UDP-QuiNAc), and UDP-N-acetyl-alpha-D-fucosamine (UDP-D-FucNAc), were observed in Suku_G1 and Suku_G2, whereas Suku_G2 and Suku_G3 contained genes involved in the CMP-N,N'-diacetyllegionamine (CMP-leg5Ac7Ac) and UDP-N-acetyl-D-galactosaminuronic acid (UDP-GalNAcA) biosynthesis pathways (Table 4).

Table 4. Different potential virulence genes among three subgroups of *V. vulnificus*.

Virulence Factor	Gene Function	Gene	G1	G2	G3
LPS	DegT/DnrJ/EryC1/StrS family aminotransferase	<i>wecE</i>	/	/	
	acetyltransferase	<i>pglD</i>		/	/
	O-antigen translocase	<i>wzx</i>	/		
	B-band O-antigen polymerase	<i>wzy</i>	/		
	Vi polysaccharide biosynthesis				
	UDP-N-acetylglucosamine C-6 dehydrogenase TviB	<i>tviB</i>			/
Capsule	UDP-N-acetylglucosamine 4,6-dehydratase	<i>PseB</i>	/		
	UDP-4-amino-4, 6-dideoxy-N-acetyl-beta-L-altrosamine transaminase	<i>PseC</i>	/		
	pseudaminic acid biosynthesis PseA protein	<i>PseA</i>	/		
	pseudaminic acid cytidyltransferase	<i>PseF</i>	/		
	UDP-2,4-diacetamido-2,4, 6-trideoxy-beta-L-altropyranose hydrolase	<i>PseG</i>	/		
	pseudaminic acid synthase	<i>PseI</i>	/		
	Vi polysaccharide biosynthesis				
	UDP-N-acetylglucosamine C-6 dehydrogenase TviB	<i>tviB</i>	/		
	MviM protein	<i>mviM</i>	/		
	WbbJ protein	<i>wbbJ</i>	/		
	WecE protein	<i>wecE</i>	/		
	UDP-N-acetylglucosamine 4,6-dehydratase	<i>pglF</i>		/	/
	LegC family aminotransferase	<i>pglE</i>		/	/
	N,N'-diacetyllegionaminate synthase (O-linked)	<i>legI</i>		/	/
	UDP-N-acetylglucosamine 2-epimerase	<i>legG</i>		/	/
	acylneuraminate cytidyltransferase family	<i>legF</i>		/	/
	Vi polysaccharide biosynthesis				
	UDP-N-acetylglucosaminuronic acid C-4 epimerase TviC	<i>tviC</i>	/		/
Capsular polysaccharide	dTDP-glucose 4,6-dehydratase	<i>rmlB</i>		/	
	glucose-1-phosphate thymidyltransferase	<i>rmlA</i>		/	
	NAD-dependent epimerase/dehydratase family protein	<i>wbpK</i>	/	/	
	sugar transferase	<i>wbfU</i>	/	/	
	UDP-N-acetylglucosamine 2-epimerase (non-hydrolyzing)	<i>wbjD/wecB</i>	/	/	
	UDP-N-acetyl-D-mannosamine dehydrogenase	<i>wecC</i>	/	/	
	sugar transferase	<i>wcaJ</i>			/

Based on RGI with CARD, 49 drug classes were included in the antibiotic resistance gene prediction program. With the default settings (perfect and strict), seven drug classes were found in these three subgroups, including cephamycin, cephalosporin, carbapenem, tetracycline, penam, fluoroquinolone, and macrolides (Table S3).

3.6. Phylogenetic Tree Analysis

Based on the ANI values, the close relationship among all piscine *V. vulnificus* samples in this study was supported by % ANI values higher than 98% (Table S4). Suku_G3 was most closely related to the reference strain CMCP6. A phylogenetic tree was analyzed based on core genome multilocus sequence typing, which found that Suku_G2 and Suku_G3 belong to *V. vulnificus* strains YJ016 and CMCP6, respectively, while Suku_G1 is related to *V. vulnificus* strain 2142-77. The outgroups, *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*, showed distinct distances from *V. vulnificus* (Figure 3).

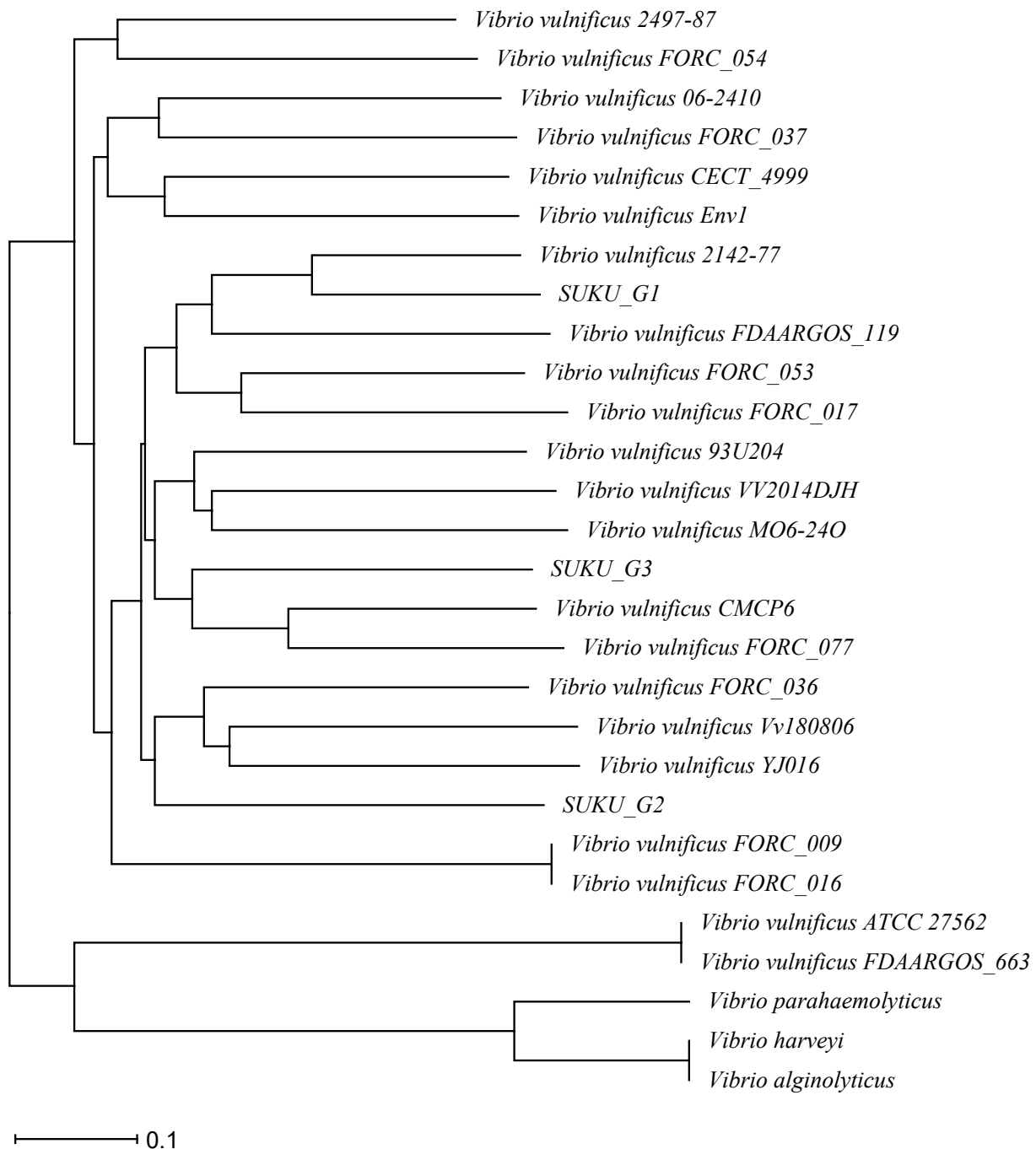


Figure 3. Phylogenetic tree constructed based on core genome multilocus sequence typing from *Vibrio* spp. (*V. vulnificus*, *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*).

To identify gene similarities and differences between the subgroups and CMCP6, a circular genomic map was constructed using BRIG, which revealed a high level of nucleotide identity among the three subgroups. Chromosomal architecture confirmed nucleotide conservation within certain regions of variation among the subgroups (Figure 4).

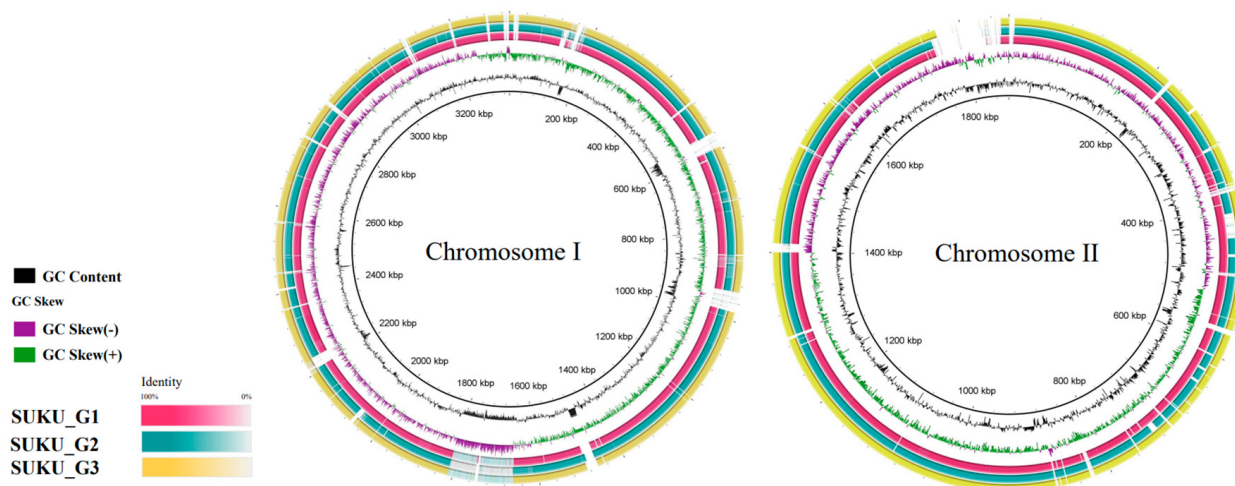


Figure 4. Comparative overview of *V. vulnificus* chromosomes of SUKU_G1 (pink), SUKU_G2 (green), and SUKU_G3 (yellow). Colored circles represent the genomes of each sequenced subgroup. The black circle represents the GC content, and the innermost circle represents the reference genome (CMCP6). The identity among the three subgroups is indicated by the color gradient.

3.7. Orthologous Gene Analysis

An orthologous cluster gene analysis identified 3974 orthologous clusters shared among the three subgroups of *V. vulnificus*. SUKU_G1 and SUKU_G3 had the highest number of shared gene clusters ($n = 100$), followed by SUKU_G1 and SUKU_G2 ($n = 95$), indicating a close evolutionary relationship. SUKU_G1 contained the highest number of unique gene clusters ($n = 11$). One gene cluster was annotated as the histidine biosynthesis process (GO: 0000105). SUKU_G3 had the lowest number of unique gene clusters ($n = 4$), one of which was annotated as the DNA restriction-modification system GO:0009307 (Figure S1).

3.8. Pan-Core Genome Analysis

The pan-genome is the entire genome of a strain and is composed of a core genome and a dispensable genome. Core genes were present in all strains, whereas dispensable genes appeared only in a subset of strains. An analysis of the *V. vulnificus* pan-genome revealed 5200 CDS, of which 3887 were in the core genome, and the remaining 1313 constituted the dispensable genome, which corresponded to a circular genomic map representing the conservation and variation areas. (Figure 5). The dispensable genome was divided into 237 genes shared by the two strains and 1076 strain-specific genes. SUKU_G1 had the highest number of unique genes (475), whereas SUKU_G3 had the lowest (262). The core genome of *V. vulnificus* strains isolated from diseased groupers accounted for 74.75% of the average genome, indicating highly conserved genomic features. The majority of the core genes (64.6%) belonged to four main categories: metabolism, genetic information processing, signaling, and cellular processes, whereas accessory and unique genes were mainly categorized as unknown genes (Figure 6).

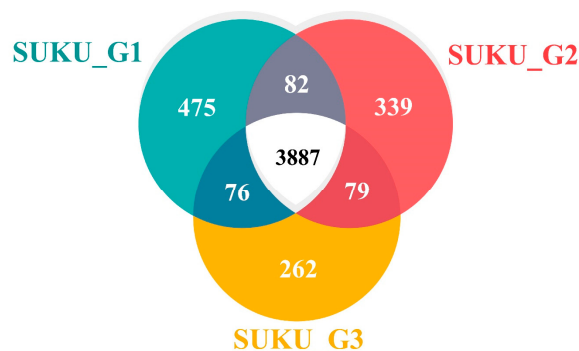


Figure 5. Venn diagram showing the core gene (white) and dispensable gene shared among the three subgroups (gray; SUKU_G1/SUKU_G2, dark red; SUKU_G2/SUKU_G3, and dark blue; SUKU_G1/SUKU_G3) and unique genes (green; SUKU_G1, red; SUKU_G2, and yellow; SUKU_G3) (right). Distribution of orthologous groups (functionally annotated or not) in different parts of the pan-genome based on the KEGG database (left).

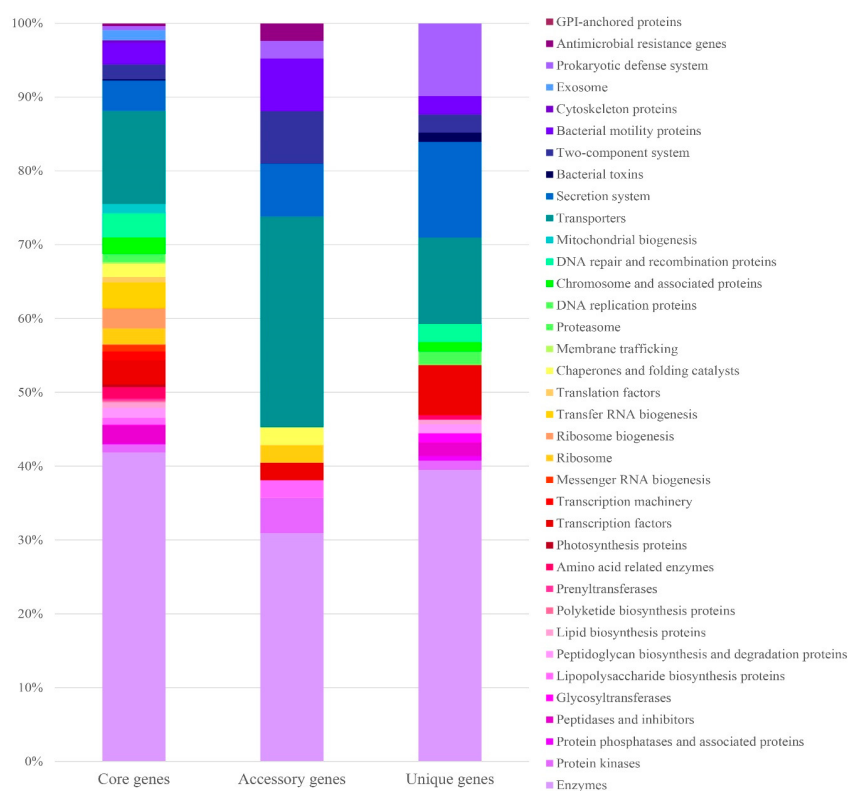


Figure 6. Distribution of functional classification of genes in the pan-genome of *V. vulnificus* samples. Colored bars represent the proportion of functional from core genes, accessory genes, and unique genes compared among three subgroups.

From the core genome, 1052 genes were categorized as metabolic enzymes from various pathways, such as carbohydrate, protein, lipid, and nucleotide metabolism, which normally function in energy supply; 615 genes were involved in genetic information processing, and the remaining 616 genes were involved in environmental information processing. Dispensable genes shared between strains were partially annotated as follows: 21.9% between SUKU_G1 and SUKU_G2 (82 genes), 13.1% between SUKU_G1 and SUKU_G3 (76 genes), and 22.7% between SUKU_G2 and SUKU_G3 (79 genes). These dispensable genes were involved in metabolic pathways, genetic information processing, transport, secretion systems, and prokaryotic defense systems. Specific genes from SUKU_G1 and SUKU_G2 were closely related, sharing a higher number of genes in the

dispensable genome. Among the unique genes, 162 were functionally assigned, and 914 remained unknown. *V. vulnificus* strain SUKU_G1 had the highest number of unique genes, whereas SUKU_G3 had the lowest.

The three subgroups of *V. vulnificus* had variable O-antigens that may be synthesized through different pathways that reflected their virulence. The *V. vulnificus* strain SUKU_G1 genome contained unique pathways for the synthesis of UDP-2,3-dideoxy-2-acetamido-3-acetamidino-mannuronic acid (UDP-ManNAc3NAmA) and CMP-Pse5Ac7Ac. UDP-ManNAc3NAmA is an unusual sugar and is synthesized by enzymes encoded by *wbpB*, *wbpE*, and *wbpD*; CMP-Pse5Ac7Ac is synthesized by enzymes encoded by *pseB*, *pseC*, *pseG*, *pseI*, and *pseF*. Subgroups SUKU_G1 and SUKU_G2 shared three pathways to produce UDP-ManNAcA, which is synthesized by enzymes encoded by *wecB* and *wecC*, and UDP-QuiNAc and UDP-D-FucNAc, which are synthesized by enzymes encoded by *wpmM*, *wbpV*, and *wbpK*, respectively. The *V. vulnificus* strain SUKU_G2 and SUKU_G3 strains contained CMP-leg5Ac7Ac and UDP-GalNAcA, which are synthesized by enzymes encoded by *pglF*, *pglE*, *pglD*, *legG*, *legI*, *legF*, and *wbpP* and *wbpO*, respectively. Moreover, the gene that encodes the enzymes for the synthesis of CMP-N-acetylneuraminate (CMP-Neu5Ac) was different among the three subgroups; SUKU_G1 had a unique sequence, whereas SUKU_G2 and SUKU_G3 had genes similar to YJ-like nab and CM-like nab (Figure 7).

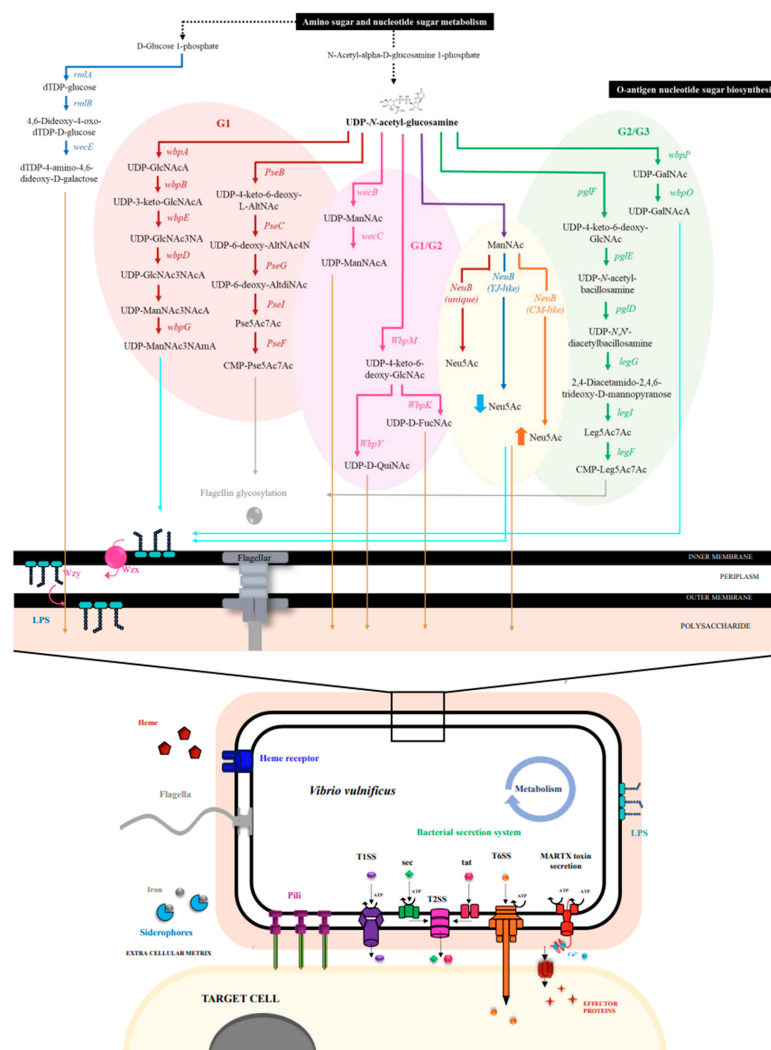


Figure 7. Purposed metabolic pathways associated with virulence and pathogenicity of *V. vulnificus* biotype I. The different arrow colors represent the presence of pathways found in different subgroups: red, G1; blue, G2; orange, G3; pink, G1/G2; and green, G2/G3.

4. Discussion

Recently, many genomes of *V. vulnificus* biotype I have been isolated from humans, including strains YJ016, CMCP6, FORC_017, and MO6-24/O [38–41]. In fish, only *V. vulnificus* isolated from tilapia (strain 93U204) has been recorded [42], and none of the *V. vulnificus* genomes from groupers were present in any database. Thus, the WGSs of the three novel *V. vulnificus*, Suku_G1, Suku_G2, and Suku_G3, isolated from groupers, exhibited different phenotypic and genotypic characteristics. All strains harbored two circular DNA chromosomes, which is consistent with the genomes of other *Vibrio* spp. [43]. The average nucleotide identity value is considered the best method for delineating bacterial species at the genome level and should be higher than 95% [44]. All grouper *V. vulnificus* and references shared an ANI value of over 98%, indicating that these strains belonged to the same species. The *V. vulnificus* strain Suku_G3 had a higher ANI value and showed genetic relationships for strain CMCP6, which is in agreement with the presence of the CM-like *nab 1* and CM-like *nab2* alleles [6].

Antibiotic resistance, the CRISPR-Cas system, and virulence are important for bacterial survival; antibiotic resistance helps bacteria to deactivate drugs, CRISPR-Cas is an adaptive immune system, and virulence factors can establish pathogen infection. An antibiotic resistance analysis revealed that all subgroups carried antibiotic resistance genes to macrolides, fluoroquinolones, beta-lactams, and tetracycline with different resistance mechanisms, which related to our previous finding that Suku_G1, Suku_G2, and Suku_G3 were multidrug-resistant strains [6]. However, *V. vulnificus* strains Suku_G1 and Suku_G3 were found to be sensitive to tetracycline (30 µg), whereas Suku_G3 was susceptible to oxytetracycline (30 µg) [6]. The limitations of predicting resistance phenotypes from genotypic data may occur at the genetic level, as they are dependent on the mutations, copy number, and control of gene expression levels, which confer different susceptibility profiles [45,46].

Virulence factors are bacteria-associated molecules required for pathogenicity in host cell infection, including attachment, immunosuppression, and immune evasion [47]. The idea that “all subgroups carried antibiotic resistance genes to identification of virulence factors” is essential for understanding diseases, particularly when comparing virulent and avirulent subgroups. These bacteria can cause diseases through numerous virulence factors predicted by GI, VFDB, and pan-core genome analyses. An essential component of bacterial pathogens is the secretion of virulence factors in eukaryotic cells. Secretory proteins are translocated across the cytoplasmic membrane via either the general secretion pathway (Sec pathway) or twin-arginine translocation (Tat pathway), both of which are found in the core genome [48,49]. The pathogenic bacterium *V. parahaemolyticus* exports toxins through the Sec machinery to the periplasm, followed by secretion across the outer membrane via the type II secretion system (T2SS) [50]. Virulence factors from Gram-negative bacteria are transported outside the cell and, in some cases, directly into target cells via the secretion system. Secretion systems, including types I, II, and VI (T1SS, T2SS, and T6SS, respectively), are present in the core genome. Type I secretion systems export small molecules, such as antibiotics and toxins, out of the cell, as in *V. cholerae*, which uses its T1SS to secrete the MARTX toxin, thereby causing host cell rupture [51,52]. Type II secretion systems secrete a wide variety of folded exoproteins, including proteases, lipases, phospholipases, and toxins [53,54]. In *V. vulnificus*, T2SS is required for pathogenicity through the mediation of VvhA and VvpE secretion into the host cells [55]. Finally, type VI secretion systems are associated with bacterial toxin protein injection machinery for virulence in host cells [56].

Two major factors of virulence, capsular polysaccharide (CPS) and lipopolysaccharide (LPS), are differentially presented in different *V. vulnificus* subgroups. Capsular polysaccharides are components of the capsules that are used to avoid phagocytosis by host defense cells [57,58]. Lipopolysaccharide is an important pyrogen in *V. vulnificus* that causes endotoxic shock [59,60]. Both CPS and LPS are composed of flexible sugar polymers (O-antigens). Therefore, the types of sugars produced by each isolate may vary and may be related to their pathogenesis. The three subgroups of *V. vulnificus* have variable O-antigens,

which may be synthesized through different pathways and reflect their virulence. The *V. vulnificus* strain Suku_G1 genome contains unique pathways for the production of UDP-ManNAc3NAcA and CMP-Pse5Ac7Ac. An unusual sugar, UDP-ManNAc3NAcA, is found in the LPS of *Pseudomonas aeruginosa* PAO1 and is synthesized by *wbpB*-, *wbpE*-, and *wbpD*-encoded enzymes [61]. The CMP-Pse5Ac7Ac is sequentially transferred to flagellin monomers, and glycosylated flagellin monomers are secreted into the filament tip. Pseudaminic acids are present on the surface of pathogenic bacteria and are involved in flagellar glycosylation, which is necessary for the modification of flagellar assembly, bacterial motility, and colonization and, hence, the virulence of gastrointestinal pathogens [62–64].

The *V. vulnificus* Suku_G1 and Suku_G2 strains share three pathways for the production of UDP-ManNAcA, UDP-QuiNAc, and UDP-D-FucNAc. These sugars were discovered in CPS or LPS of various Gram-negative bacteria [62,65–69]. The *V. vulnificus* Suku_G2 and Suku_G3 strains had the CMP-leg5Ac7Ac and UDP-GalNAcA biosynthesis pathways, respectively. Legionaminic acids are critical O-linked modifications of Fla proteins and are essential for flagellar assembly, which is involved in the pathogenesis of Gram-negative bacteria [62]. UDP-GalNAcA is a component of the B-band O-antigen of *P. aeruginosa* [70].

Nucleotide sugar, CMP-Neu5Ac, is associated with lipooligosaccharides, which have been reported to contain terminal sialic acids in pathogenic bacteria, such as *Neisseria meningitidis* and *N. gonorrhoeae* [71,72]. The finding of unique genes correlated with our previous multiplex PCR results showed that the YJ-like nab allele demonstrated a low expression of NulOs in Suku_G2, and the CM-like nab allele demonstrated a high expression of NulOs in Suku_G3, as well as another sequence in Suku_G1 [6]. Taken together, *V. vulnificus* strain Suku_G1 has various sugar biosynthesis pathways, particularly the UDP-ManNAc3NAcA and CMP-Pse5Ac7Ac pathways, that are involved in pathogenesis.

Toxins are notable virulence factors of *Vibrio* spp. All *V. vulnificus* strains from groupers contained hemolysin, protease, and MARTX and its transport system, including the RtxBDE channel and TolC. Thermolabile hemolysin and hemolysin III proteins are putative pathogenic factors in many bacterial pathogens and are involved in bacterial bloodstream invasion [73]. Haemolysin may cause vasodilation, leading to hypotensive septic shock [74]. Exfoliative toxins A/B (chymotrypsin family of serine proteases) and collagenase can induce hemorrhagic damage and enhance vascular permeability and edema [75]. MARTX is a significant exotoxin that delivers various effector domains to host cells, ultimately inducing cell death [76]. It forms pores in the host cell membrane, facilitating the translocation and release of effector domains into the cells. The MARTX toxin identified in Suku_G1 contains unique repeat domains and a galactose-binding-like domain, enabling binding to specific ligands on the host cell membrane [77–80]. This, coupled with the presence of RTX toxin determinant A and related Ca²⁺-binding proteins in Suku_G1, as well as the presence of T6SS proteins, can contribute to its high virulence. Zonula occludens toxin, an enterotoxin that increases intestinal permeability, was discovered in Suku_G2 and Suku_G3, with different sequences that may function in pathogenesis.

Other minor virulence-related genes, such as *Vibrio*-associated phages, were observed among the three subgroups (vB_VpaM_MAR) and have also been observed in other *Vibrio* species in aquaculture. It should be noted that phage transfer may occur in aquatic animals [81]. The CRISPR-Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements, such as those present within plasmids and phages, thereby providing a form of acquired immunity [82]. Only *V. vulnificus* strain Suku_G1 contained CRISPR arrays that were identified as the orphan type, consisting only of palindromes and spacers without any *cas* genes. Quorum sensing is a cell-to-cell communication process that involves the production and release of extracellular compounds at high cell densities using autoinducers (AIs). The gene for AI-2 biosynthesis, *LuxS*, was found in our *V. vulnificus* strains and was present in all strains of *V. vulnificus*, regardless of biotype and serovar [83]. Outer membrane vesicles (OMVs) were produced by many pathogenic bacteria, including *V. vulnificus*. These vesicles are related to bacterial–host interactions, specifically their pathogenesis, as they modulate immune responses, contribute to biofilm formation, and

deliver virulence factors to host cells, such as VvhA, which induces cytotoxicity [84–86]. Interestingly, the organization of *V. vulnificus* OMVs is affected by CPS expression [87].

Taken together, the *V. vulnificus* strain Suku_G1 strain carried several genes related to virulence and pathogenesis, including those involved in the T6SS mechanism, the O-antigen nucleotide sugar biosynthesis pathway, and RTX toxin. The results of this study were consistent with our previous result that Suku_G1 showed the fastest growth and highest pathogenicity ($LD_{50} = 1.88 \times 10^6$ CFU.mL⁻¹). In contrast, the *V. vulnificus* Suku_G3 strain had the same growth rate but no virulence effect on the fish. The *V. vulnificus* Suku_G2 strain had less pathogenicity ($LD_{50} = 7.63 \times 10^6$ CFU.mL⁻¹) than Suku_G1 and had the slowest growth. Therefore, we proposed a pathogenic mechanism among these three subgroups of *V. vulnificus* biotype I, showing a correlation between the bacterial growth rate and pathogenicity.

A comparative genome analysis revealed that different virulence mechanisms among the three subgroups of *V. vulnificus* biotype I were correlated with pathogenicity. Understanding the virulence mechanisms has led to the development of prevention methods based on conserved virulence factors. The conserved features of virulent, less virulent, and avirulent strains are useful for vaccine design to prevent all types of this pathogen. Moreover, synthetic biology and genome engineering can be used to develop a novel diagnosis of infection using the different genes among the three subgroups, to understand antibiotic resistance mechanisms, and to develop attenuated vaccine strains from pathogenic bacterial genomes using the conserved phages found in this study.

5. Conclusions

This comparative genome analysis provides detailed insights into the diverse metabolic pathways of biotype I *V. vulnificus* strains. By combining the metabolic pathways with the pathogenicity of a strain, the virulence-related pathways could elucidate the potential virulence strategy of the bacteria. Hence, this alternated metabolic pathway, which both increases and decreases metabolites, as well as changes metabolism could be affected by different environments or specific triggers that co-opt the virulence of bacteria. This system is known as bacterial virulence programming, which results in the enhancement of bacterial virulence, including changes in bacterial characteristics or properties, such as from acute to chronic infection, from local to systemic infection, and from infection to colonization [88]. Taken together, this is the first report of the association between bacterial pathogenicity and virulent pathways in piscine *V. vulnificus* that not only provides an understanding of relevant metabolic and virulence pathways but also supports the design of disease prevention and control methods. The results of this study provide insights into the genetic differences among the three subgroups. The main virulence factors associated with pathogenesis may be useful for controlling this virulent pathogen in brown-marbled groupers.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/microorganisms12122518/s1>: Figure S1: Venn diagram showed the distribution of shared and unique orthologous gene clusters among *V. vulnificus* samples by OrthoVenn2. Figure S2: Subsystem category distribution based on RAST annotation server. Table S1: Reference genomes of *V. vulnificus* and other *Vibrio* species. Table S2: The predicted genes in the 20-kb regions and detailed features from CRISPR prediction. Table S3: Antibiotic resistance profile predicted from CARD. Table S4: ANI value between piscine *V. vulnificus* and strain CMCP6. The asterisk (*) represents the ANI value compared to itself.

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