Distribution, Phylogeny and Evolution of Clinical and Environmental *Vibrio vulnificus* Antibiotic-Resistant Genes

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ABSTRACT: *Vibrio vulnificus* is an emergent marine pathogen and is the cause of a deadly septicemia. However, the evolution mechanism of antibiotic-resistant genes (ARGs) is still unclear. Twenty-two high-quality complete genomes of *V. vulnificus* were obtained and grouped into 16 clinical isolates and 6 environmental isolates. Genomic annotations found 23 ARG orthologous genes, among which 14 ARGs were shared by *V. vulnificus* and other *Vibrio* members. Furthermore, those ARGs were located in their chromosomes, rather than in the plasmids. Phylogenomic reconstruction based on single-copy orthologous protein sequences and ARG protein sequences revealed that clinical and environmental *V. vulnificus* isolates were in a scattered distribution. The calculation of non-synonymous and synonymous substitutions indicated that most of ARGs evolved under purifying selection with the *Ka*/*Ks* ratios lower than one, while *h-ns, rsmA*, and *soxR* in several clinical isolates evolved under the positive selection with *Ka*/*Ks* ratios >1. Our result indicated that *V. vulnificus* antibiotic-resistant armory was not only confined to clinical isolates, but to environmental ones as well and clinical isolates inclined to accumulate beneficial non-synonymous substitutions that could be retained to improve competitiveness.

KEYWORDS: *Vibrio vulnificus*, antibiotic-resistant genes, clinical and environmental isolates, comparative genomics, phylogeny, *Ka*/*Ks* analysis

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Introduction

Vibrio vulnificus is an opportunistic pathogen for severe human infection,[1] and is one of the leading causes of non-Cholera, *Vibrio*-associated deaths globally.[2] Apart from clinical isolates, *V. vulnificus* inhabits a range of different environmental sources, including estuarine water, sediment and seafood produce.[3] Moreover. *V. vulnificus* isolates have been classified into 3 biotypes based on biochemical traits.[3] Biotype 1 as a human pathogen is the most common worldwide[4] and biotype 2 is regarded as an eel pathogen,[5] while biotype 3 is recorded as sharing biochemical properties of biotype 1 and 2.[6] The advances of sequencing and bioinformatic technology facilitate researchers to obtain *V. vulnificus* genomes. Recent studies elucidate genetic and evolutionary mechanisms of infections and pathogenesis of *V. vulnificus* at the genomic level.[2,7,8]

Antibiotic resistance in the *V. vulnificus* is a challenge that is associated with high morbidity and mortality.[9] Particularly, the presence of antibiotic-resistant genes (ARGs) in environmental isolates can be a huge risk to the public health.[10] However, the evolution mechanism of ARGs is still unclear. Moreover, the complete genomes can provide a more detailed gene information than the draft genomes.[11] In this study, the complete *V. vulnificus* genomes were obtained to demonstrate their ARGs distribution, phylogeny and evolutionary driving force, which could broaden our understandings of antibiotic-resistance in the *V. vulnificus*.

Materials and Methods

Collection of V. vulnificus and its relatives genomes

The complete genomes of *V. vulnificus* were obtained from the NCBI GenBank database.[12] In addition, other *Vibrio* type strain genomes were acquired from the gcType database[13] and their accession numbers in the NCBI GenBank database were listed in Table 1. Moreover, *Escherichia coli* ATCC 11775^T was used as an outgroup in the phylogenomic analysis. Detailed information for the complete genomes in this study was shown in Table 1.

Genomic quality estimation and annotations

The quality of obtained genomes was estimated by CheckM v1.10.3 with the typical workflow, and the genome with the completeness of >90% and contamination of <5%. were regarded as a high-quality genome as recommended by Bowers et al[14] Open reading frames (ORFs), rRNA and tRNA genes were annotated by using Prokka v.1.14.6[15] with the command

"–gram neg –kingdom Bacteria –gcode 11." Non-redundant 16S rRNA genes were generated by CD-HIT program v4.8.1[16] with the sequence identity of 99%. The classification of biotypes was analyzed based on the 16S rRNA gene phylogeny as described by Hoihuan et al[4].

The ARGs were annotated against the Comprehensive Antibiotic Resistance Database (CARD) by using BLASTP with an e-value ⩽ 10,[5] with an identity and query coverage thresholds of 50% and 50%.[17,18] In addition, those potential ARGs were double-checked by using the Resistance Gene Identifier (RGI) v.5.2.1[17] with the command. The potential of horizontal gene transfer was carried out by using BLASTP against the NCBI RefSeq select proteins database based on the best hit taxon.

Comparative genomic analysis and phylogenomic reconstruction

Based on the annotation, protein sequences translated from ORFs were compared by using OrthoFinder v.2.5.4[19] with the default setting. Single-copy were chosen into the following phylogenomic reconstruction as described previously.[20] In brief, each were aligned by MAFFT v.7.490[21] with the parameter "–auto." Aligned protein sequences were refined by using trimAL v.10.2rev59[22] with the parameter "-automated1" to remove spurious sequences or poorly aligned regions and then concatenated. The maximum-likelihood phylogenomic analysis were carried out by using IQ-TREE v.1.6.12[23] with the parameter "-st AA -m LG+F+R10 -bb 1000," and the best amino acid substitution model was also inferred by the same software with the parameter "-st AA -m MFP."

Evolutionary analysis of ARGs

Protein sequences of each group of ARGs were aligned as described in the phylogenomic analysis. And the best amino acid substitution model for those sequences were also estimated by using IQ-TREE v.1.6.12.[23]

The aligned codon sequences were generated based on converting a multiple sequence alignment of proteins and the corresponding DNA by using PAL2NAL v.14[24] And then, the resulting codon alignments were subjected to the calculation of synonymous and non-synonymous substitution (*Ka*/*Ks*) rates by KaKs_Calculator package v.1.2[25] through model selection and model averaging. Furthermore, the codon usages of those ARGs were summarized by using the sequence manipulation suite.[26]

Statistics and visualization

The regression analysis were performed by using the formulation *lm* implanted in R version.[27] The phylogenetic trees were visualized by using MEGA7 software[28] and edited by PowerPoint 2019 software (Microsoft Cooperation, Redmond, WA, USA). Other figures were generated by and PowerPoint 2019 software (Microsoft Cooperation, Redmond, WA, USA).

Results and Discussion

General genomic features and phylogenomic relationship of V. vulnificus

A collection of twenty-four *V. vulnificus* isolates and other 18 *Vibrio* type strains genomes were obtained in this study (Table 1). Genomic quality estimations indicated that all genomes were high-quality with the completeness of >90.0% and contamination of <5.0% (Table 1), except for *V. vulnificus* FORC_036 (completeness of 100.0% and contamination of 5.1%), *V. vulnificus* FORC_053 (completeness of 100.0% and contamination of 5.4%) and *V. cholerae* CECT 514^T (completeness of 55.3% and contamination of 0.8%). Based on isolation sources, twenty-two *V. vulnificus* isolates could be divided into 2 categories including clinical isolates(n = 16) isolated from hospital and patients,[1,29-32] and environmental isolates (n = 6) cultivated from estuarine, *Anguilla anguilla, Konosirus punctatus, Mya arenaria, Oreochromis* sp. etc.[33] The neighbor-joining phylogenetic analysis based on 16S rRNA gene sequences revealed that those strains were classified into 2 clades including biotype 1 clade (strains 2142-77, 93U204, CMCP6, FDAARGOS_119, FORC_009, FORC_016, FORC_017, FORC_077, MO6-24/O, Vv180806, VV2014DJH, and YJ016) and biotype 2 clade (strains 06-2410, 07-2444, 2015AW-0208, 2497-87, ATCC 27562, CECT 4999, Env1, FDAARGOS_663, FORC_037, and FORC_054), as shown in Supplemental Figure S1.

Genomic sizes of 22 *V. vulnificus* isolates varied from 4.95 to 5.36 Mbp, while their DNA $G+C$ contents were in a narrow range with 46.5% to 46.9% (Table 1). Commonly, their genomes constituted of 2 chromosomes, while several isolates genomes contained one plasmid as an accessory genetic material that was both present in the clinical (n = 3) and environmental (n = 4) isolates. Genomic annotation revealed those *V. vulnificus* isolates encoded 4447 to 5739 genes in their genomes. Their gene counts were mostly positively correlated with their genomic size ($r^2 = 0.96$, $P = 5.1e^{-15}$) except for the isolate 2015AW-0208, which harbored the most genes with the genomic size of 5.12 Mbp (Figure 1).

Clinical and environmental *V. vulnificus* isolates were in a scattered distribution as indicated by the maximum-likelihood phylogenomic tree, and they were also clustered into 2 clades classified as biotype 1 and 2 clade (Figure 2), which was identical with the phylogenetic reconstruction based on 16S rRNA gene sequences. Moreover, the absence/presence of plasmid(s) did not affect their phylogenetic relationship either (Figure 2). Our phylogenomic reconstruction is similar with those of others about *V. vulnificus*.[2,8,34] The recent phylogenomic reconstructions of *V. vulnificus* indicated that its isolates were divided into 4 or 5 clades, among which nearly 90% of isolates were clustered into 2 clades.[2,34] The biotype 1 clade appeared to contain a significantly higher proportion of clinical isolates, while

Table 1. Genomic attributes of *Vibrio* genomes in this study.

ISOLATE	ACCESSION NUMBER	GENETIC MATERIALS	GENOMIC SIZE (BP)	*G* + *C* CONTENT (%)	COMPLETENESS (%)	CONTAMINATION (%)
V. vulnificus YJ016	GCA_000009745.1	Two chromosomes, One plasmid	5260,086	46.7	100.0	0.1
V. vulnificus CMCP6	GCA_000039765.1	Two chromosomes	5126,696	46.7	100.0	0.2
V. vulnificus MO6-24/O	GCA_000186585.1	Two chromosomes	5007,768	46.9	100.0	0
V. vulnificus 93U204	GCA_000746665.1	Two chromosomes, One plasmid	5127,345	46.7	100.0	0.4
V. vulnificus FORC_009	GCA_001433435.1	Two chromosomes	5060,705	46.7	100.0	0.5
V. vulnificus FDAARGOS_119	GCA_001558515.2	Two chromosomes	4978,797	46.9	100.0	0.1
V. vulnificus FORC_016	GCA_001653775.1	Two chromosomes	5072,369	46.7	100.0	0.5
V. vulnificus FORC_017	GCA_001675245.1	Two chromosomes, One plasmid	5229,231	46.6	100.0	0.3
V. vulnificus FORC_036	GCA_002117205.1	Two chromosomes, One plasmid	6067,960	45.5	100.0	5.1
V. vulnificus FORC_037	GCA_002204915.1	Two chromosomes, One plasmid	5117,890	46.8	100.0	0.2
V. vulnificus CECT 4999	GCA_002215135.1	Two chromosomes, One plasmid	5163,135	46.5	100.0	0.1
V. vulnificus ATCC 27562^T	GCA_002224265.1	Two chromosomes	5007,160	46.7	100.0	0.3
V. vulnificus VV2014DJH	GCA_002850455.1	Two chromosomes	5074,562	46.8	99.7	0
V. vulnificus FORC_054	GCA_002863725.1	Two chromosomes, One plasmid	5120,766	46.7	100.0	0.4
V. vulnificus Env1	GCA_003047125.1	Two chromosomes	4954,048	46.7	99.9	0.6
V. vulnificus FORC_053	GCA_003522555.1	Three chromosomes	6019,009	45.4	100.0	5.4
V. vulnificus FORC_077	GCA_004319645.1	Two chromosomes	5018,260	46.9	100.0	0.3
V. vulnificus FDAARGOS_663	GCA_008693685.1	Two chromosomes	4974,815	46.7	100.0	0.3
V. vulnificus 2142-77	GCA_009665475.1	Two chromosomes	5079,985	46.8	99.9	0
V. vulnificus 2015AW-0208	GCA_009763305.1	Two chromosomes	5125,419	46.5	97.7	0.7
V. vulnificus 06-2410	GCA_009764095.1	Two chromosomes	4996,741	46.8	100.0	0.4
V. vulnificus 07-2444	GCA_009764115.1	Two chromosomes	5226,423	46.5	100.0	0.3

(Continued)

Table 1. (Continued)

ISOLATE	ACCESSION NUMBER	GENETIC MATERIALS	GENOMIC SIZE (BP)	*G* + *C* CONTENT (%)	COMPLETENESS (%)	CONTAMINATION (%)
V. vulnificus Vv180806	GCA_014107515.1	Two chromosomes, One plasmid	5356,494	46.6	100.0	0.1
V. vulnificus 2497-87	GCA_014211935.1	Two chromosomes	5032,819	46.8	100.0	0.1
V. alfacsensis CAIM 1831^T	GCA_003544875.1	Two chromosomes, One plasmid	4910,231	44.2	99.4	0
V. alginolyticus ATCC 17749^T	GCA_000354175.2	Two chromosomes	5146,637	44.7	99.5	0.2
V. aphrogenes CA-1004T	GCA_002157735.2	Two chromosomes	3375,144	42.1	95.6	0.9
V. azureus LC2-005^T	GCA_002849855.1	Two chromosomes, Two plasmids	4833,901	42.3	99.0	0.2
V. campbellii ATCC 25920^T	GCA_002163755.1	Two chromosomes, One plasmid	5178,103	45.1	99.9	0.4
V. casei DSM 22364^T	GCA_002218025.2	Two chromosomes, Three plasmid	4140,771	40.7	96.3	1.0
V. cholerae CECT 514^T	GCA_013155105.1	Two chromosomes	4100,705	47.2	55.3	0.9
V. cidicii 2756-81^T	GCA_009763805.1	Two chromosomes	4750,222	47.9	97.2	0
V. fluvialis ATCC 33809^T	GCA_001558415.2	Two chromosomes	4827,733	49.9	99.9	0.9
V. hyugaensis 090810a^T	GCA_002906655.1	Two chromosomes	5612,082	45.0	100.0	0.3
V. natriegens ATCC 14048^T	GCA_001456255.1	Two chromosomes	5175,153	45.1	100.0	2.8
V. panuliri JCM 19500^T	GCA_009938205.1	Two chromosomes, One plasmid	4855,939	45.2	99.7	1.5
V. parahaemolyticus ATCC 17802^T	GCA_001558495.2	Two chromosomes	5152,461	45.3	100.0	0.1
V. ponticus DSM 16217^T	GCA_009938225.1	Two chromosomes, One plasmid	4796,932	44.8	98.3	2.0
V. rumoiensis FERM P-14531^T	GCA_002218045.2	Two chromosomes, Two plasmids	4207,152	42.3	95.6	0.5
V. tritonius JCM 16456^T	GCA_001547935.1	Two chromosomes	5221,926	43.9	98.1	3.2
V. tubiashii ATCC 19109	GCA_000772105.1	Two chromosomes, Four plasmids	5540,337	45.0	100.0	0.8
Escherichia coli ATCC 11775^T	GCA_003697165.1	One chromosome, One plasmid	5034,833	50.6	99.9	0.4

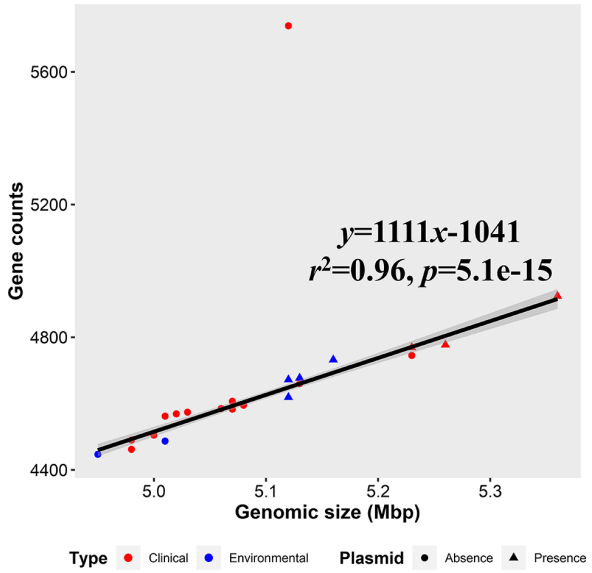

Figure 1. Genomic size and gene counts of *Vibrio vulnificus*. Red and blue indicated clinical and environmental isolates, respectively. Circle and triangle represented absence and presence of the plasmid, respectively.

biotype 2 clade contained both of clinical and environmental isolates, that was also reported by López-Pérez et al[8] Despite the genomic divergence among clusters, a distinct pattern linking strain phylogeny, source of isolation, and virulent capabilities was not identified. This mixed distribution of clinical and environmental *V. vulnificus* isolates suggested that the genomic difference between 2 groups was subtle, which was also detected in other pathogens, such as *Escherichia coli*,[35] *Legionella pneumophila*,[36] *Pseudomonas aeruginosa*,[37] and *Pseudomonas putida*.[38] Moreover, this distribution indicated *V. vulnificus* antibiotic-resistant armory was not only confined to clinical isolates, but to environmental ones as well.

Distribution of ARGs in the V. vulnificus

Twenty-three ARG orthologous proteins classified into 5 resistance mechanisms including antibiotic efflux, antibiotic inactivation, antibiotic target alteration, antibiotic target protection and antibiotic target replacement, were annotated in the genomes of *V. vulnificus* (Table 2). Besides, 2 ARGs annotated as ATP-dependent lipid A-core flippase MsbA and tetracycline efflux Na^+/H^+ antiporter family transporter Tet35 were compared into 2 different orthologous genes. Those ARGs were located in the *V. vulnificus* chromosomes, rather than in the plasmid, which was not common in other pathogens.[39,40] Furthermore, core and accessory ARGs showed highest sequence identities with *V. vulnificus* or other *Vibrio* species (Supplemental Table S1), indicating that the low horizontal gene transfer frequencies of those ARGs.[41]

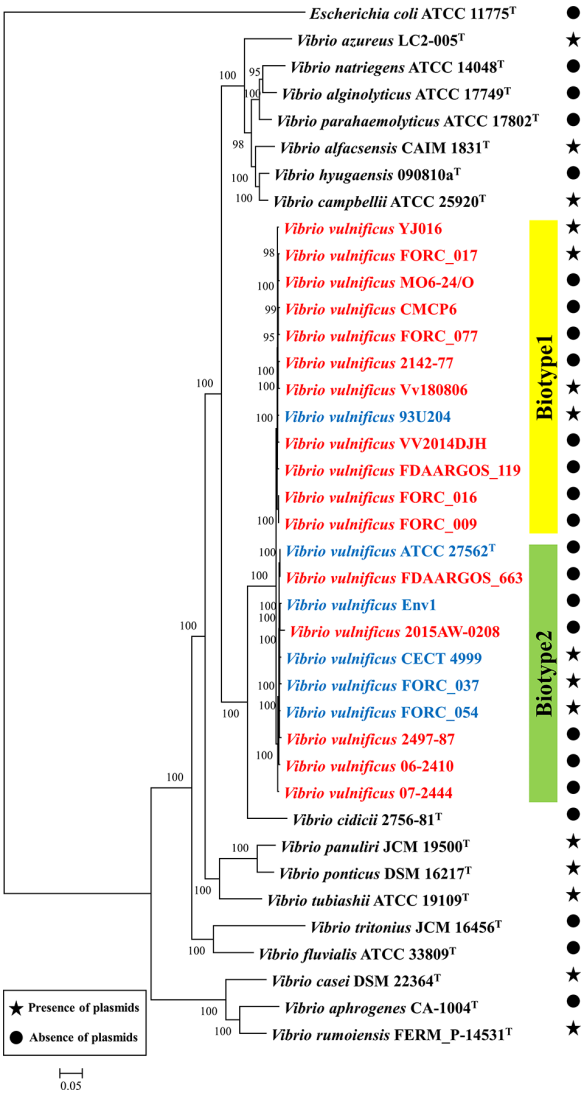

Figure 2. The maximum-likelihood phylogenetic tree based on single-copy orthologous protein sequences. Red and blue indicate clinical and environmental isolates, respectively. *Escherichia coli* ATCC 11775T was used as an outgroup.

Among those ARGs, 61% (14/23) of them shared by *V. vulnificus* and other *Vibrio* type strains were chloramphenicol acetyltransferase CatB9, translational regulators CRP and RsmA, dihydrofolate reductase Dfr-A3, fosfomycin resistance phosphotransferase FosC2, histone-like nucleoid structuring protein H-NS, ABC-type macrolide antibiotic exporter MacB, mobile colistin resistance phosphoethanolamine transferase MCR-9, ATP-dependent lipid A-core flippase MsbA, polymyxin resistance phosphoethanolamine transferase PmrE, quinolone resistance protein QnrVC1, redox-sensitive transcriptional activator SoxR, tetracycline efflux Na^+/H^+ antiporter family transporter Tet35 and AcrAB-TolC multidrug efflux pump YajC (Figure 3). Furthermore, the metallo-beta-lactamase VarG were exclusively in all of *V. vulnificus* isolates, other than the isolate VV2014DJH. Other exclusive genes

Table 2. Detailed information of antibiotic-resistant genes annotated in the *V. vulnificus* genomes.

ORTHOLOGOUS GENES	ANNOTATIONS	CARD ACCESSION	RESISTANCE MECHANISM	DRUG CLASS	REFERENCE
OG0000070	MCR-9.1	3004684	Antibiotic target alteration	Peptide antibiotic	Carroll et al[42]
OG0000118 and OG0000942	MsbA	3003950	Antibiotic efflux	Nitroimidazole antibiotic	Singh et al[43]
OG0000191	CatB9	3002681	Antibiotic inactivation	Phenicol antibiotic	Heidelberg et al[44]
OG0000210	PmrE	3003577	Antibiotic target alteration	Peptide antibiotic	Lee et al[45]
OG0000223	H-NS	3000676	Antibiotic efflux	Tetracycline antibiotic, penam, macrolide antibiotic, fluoroquinolone antibiotic, cephamycin, cephalosporin	Nishino and Yamaguchi[46]
OG0000702	RsmA	3005069	Antibiotic efflux	Phenicol antibiotic, diaminopyrimidine antibiotic, fluoroquinolone antibiotic	Mulcahy et al[47]
OG0001054	YajC	3005040	Antibiotic efflux	Tetracycline antibiotic, penam, phenicol antibiotic, rifamycin antibiotic, cephalosporin, glycylcycline, fluoroquinolone antibiotic, triclosan	Rundell et al[48]
OG0001146	DfrA3	3003105	Antibiotic target replacement	Diaminopyrimidine antibiotic	Brolund et al[49]
OG0001216	SoxR	3004107	Antibiotic efflux, Antibiotic target alteration	Tetracycline antibiotic, fluoroquinolone antibiotic, glycylcycline, cephalosporin, phenicol antibiotic, rifamycin antibiotic, penam, disinfecting agents and intercalating dyes, triclosan, acridine dye	Sakhtah et al[50]
OG0001389	CRP	3000518	Antibiotic efflux	Macrolide antibiotic, penam, fluoroquinolone antibiotic	Nishino et al[51]
OG0001739 and OG0014842	Tet35	3000481	Antibiotic efflux	Tetracycline antibiotic	Teo et al[52]
OG0002135	ArlR	3000838	Antibiotic efflux	Disinfecting agents and intercalating dyes, fluoroquinolone antibiotic, acridine dye	Fournier et al[53]
OG0002643	QnrVC1	3002799	Antibiotic target protection	Fluoroquinolone antibiotic	Fonseca et al[54]
OG0003164	MacB	3000535	Antibiotic efflux	Macrolide antibiotic	Xu et al[55]
OG0003616	VarG	3004289	Antibiotic inactivation	Carbapenem	Lin et al[56]
OG0006136	MexK	3003693	Antibiotic efflux	Macrolide antibiotic, tetracycline antibiotic, triclosan	Chuanchuen et al[57]
OG0006781	FosC2	3002874	Antibiotic inactivation	Fosfomycin	Wachino et al[58]
OG0014873	PmpM	3004077	Antibiotic efflux	Aminoglycoside antibiotic, fluoroquinolone antibiotic, benzalkonium chloride	He et al[59]
OG0015064	TetT	3000193	Antibiotic target protection	Tetracycline antibiotic	Clermont et al[60]

(Continued)

Table 2. (Continued)

ORTHOLOGOUS GENES	ANNOTATIONS	CARD ACCESSION	RESISTANCE MECHANISM	DRUG CLASS	REFERENCE
OG0015076	RpoB2	3000501	Antibiotic target alteration, antibiotic target replacement	Rifamycin antibiotic	Ishikawa et al[61]
OG0015104	TetWNW	3004442	Antibiotic target protection	Tetracycline antibiotic	Leclercq et al[62]

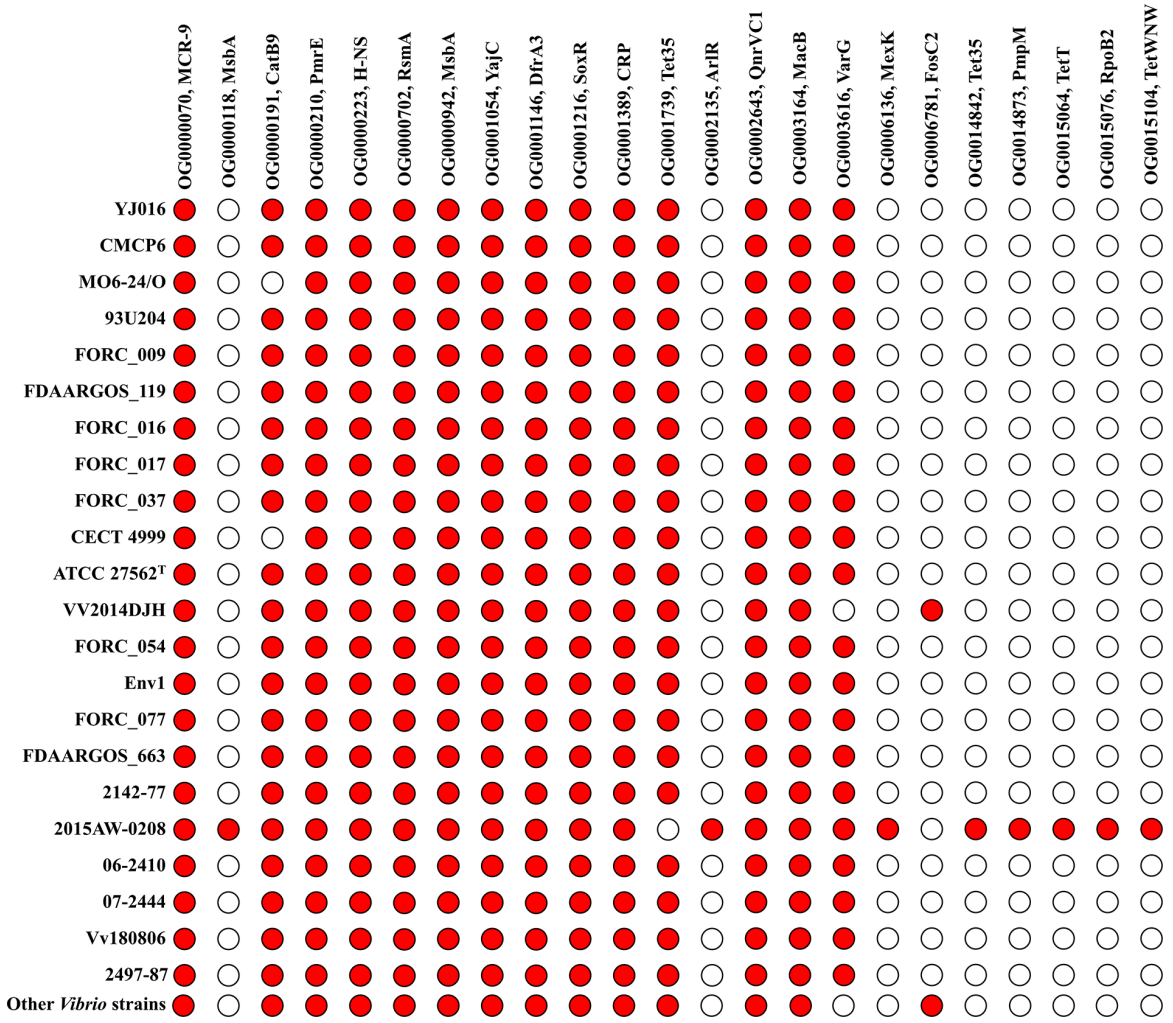

Figure 3. Distribution of antibiotic-resistant genes in the *V. vulnificus* genomes.

encoding the response regulator ArlR, multidrug efflux RND transporter permease subunit MexK, ATP-dependent lipid A-core flippase MsbA, H^+-coupled multidrug efflux pump PmpM, rifampin-resistant beta-subunit of RNA polymerase RpoB2, tetracycline efflux Na^+/H^+ antiporter family transporter Tet35, tetracycline-resistant ribosomal protection proteins TetT and TetWNW were mostly in the isoalte 2015AW-0208, which showed genomic differences compared with other *V. vulnificus* isolates.

Antibiotic resistance determinations revealed that *V. vulnificus* could resist various antibiotics including fluoroquinolones, β-lactam combination agent, lipopeptide, macrolide, nitrofurans, penicillin and phenicols,[63-66] which were consistent with those ARGs found in their genomes (Table 2). Furthermore, the genomes of environmental isolates 93U204, ATCC 27562^T, CECT 4999, Env1, FORC_037, and FORC_054 encoded 13 or 14 ARG orthologous genes, which were similar with the ARGs profile of clinical isolates

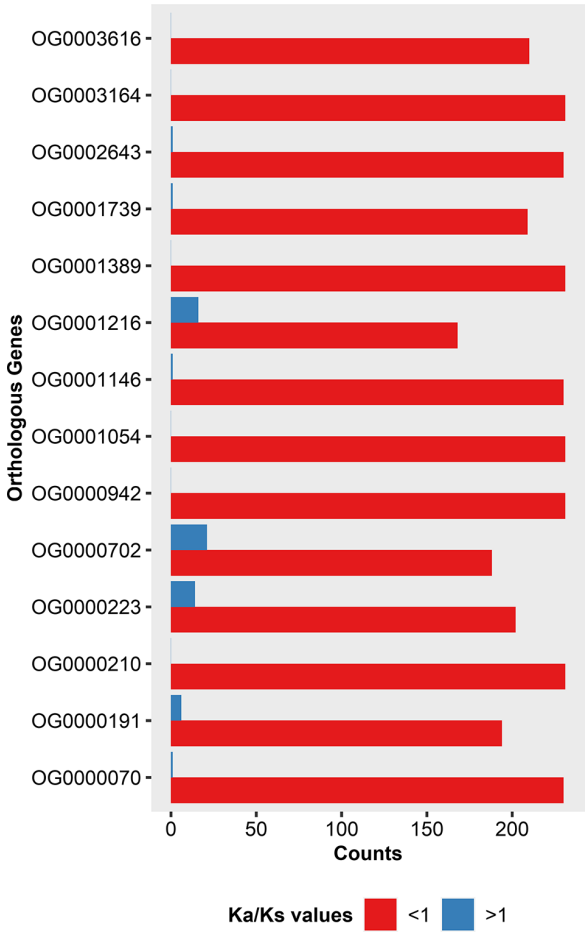

Figure 4. *Ka/Ks* ratios of each orthologous ARG in the *V. vulnificus*.

(Figure 3). Therefore, environmental *V. vulnificus* isolates may serve as reservoirs for transmission of their antibiotic resistance.

Evolution of ARGs in the V. vulnificus

Except for FosC2 only annotated in the *V. vulnificus* VV2014DJH and *V. campbellii* ATCC 25920^T, other 13 shared ARG orthologous proteins and VarG were processed into phylogenetic analysis. *V. vulnificus* isolates were clustered into an independent clade, which was separated from other *Vibrio* strains, of phylogenetic trees based on most ARG orthologous proteins including CatB9, CRP, Dfr-A3, H-NS, MacB, MCR-9, MsbA, QnrVC1, SoxR, Tet35, and YajC (Supplemental Figure S1). However, *V. vulnificus* isolates were in a scattered distribution in the phylogenetic trees of PmrE and RsmA, that were classified into antibiotic target alteration or antibiotic efflux resistance mechanisms. Clinical and environmental isolates in those scattered distribution phylogenetic trees were still mixed (Supplemental Figure S1), demonstrating that environmental ones had a similar evolutionary history with clinical ones which were also observed in other pathogens.[67,68]

The calculation of non-synonymous and synonymous substitutions indicated that most of ARGs evolved under purifying selection with the *Ka/Ks* ratios lower than one[69] (Figure 4). While several pairwise gene sequences of ARGs had the *Ka/Ks* ratios higher than 1 indicating that those genes evolved under positive selection[70] (Figure 4), especially for those antibiotic efflux ARG proteins H-NS, RsmA, and SoxR among which the group of $Ka/Ks > 1$ accounted for 6.5% to 10.0%. And those high *Ka/Ks* ratios were mostly confined to isolates 2015AW-0208, FDAARGOS_119, FDAARGOS_663, VV2014DJH, and YJ016, which were all isolated from clinical sources. Compared with environmental sources, clinical settings, where most antibiotics are prescribed, are hypothesized to serve as a major hotspot,[71] that forced several beneficial non-synonymous substitutions could be retained to improve competitiveness.

Conclusions

With regard to uptake of antibiotic resistance factors, marine environments with highly variable ecological niches provide an unrivaled gene pool with a diversity that considerably exceeds that of the human and marine animal microbiota. Indeed, the most remarkable feature of marine microbiome is its enormous diversity, providing numerous genes that potentially could be acquired and used by pathogens to counteract the effect of antibiotics.[72] Moreover, metals and antibiotic pollutions co-selecting for antibiotic-resistant strains via cross-resistance or co-resistance should also be taken into consideration seriously to retard the rapid evolutionary expansion and spread of antibiotic resistance factors.[73] Clinical and environmental *V. vulnificus* isolates were in a scatter distribution based on the genomic size and constitutes as well as the phylogenomic relationship. Genomic annotation and comparative genomic analysis also indicated that this mixed distribution of ARGs in clinical and environmental isolates. Unexpectedly, those ARGs were located in their chromosomes, rather than in the plasmids of them, suggesting that those genes were conserved in the *V. vulnificus*. The calculation of non-synonymous and synonymous substitutions indicated that most of ARGs evolved under purifying selection with the *Ka*/*Ks* ratios lower than one, while h-ns, rsmA and soxR in several clinical isolates evolved under the positive selection with *Ka*/*Ks* ratios >1. Therefore, *V. vulnificus* antibiotic-resistant armory was not only confined to clinical isolates, but to environmental ones as well and clinical isolates inclined to accumulate beneficial non-synonymous substitutions that could be retained to improve their competitiveness.

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Supplemental Material

Supplemental material for this article is available online.

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