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

Nan Geng1, Guojin Sun1, Wen-Jia Liu2, Bin-Cheng Gao2, Cong Sun2,3, Cundong Xu1, Ertian Hua1 and Lin Xu2,3

1Key Laboratory for Technology in Rural Water Management of Zhejiang Province, Zhejiang University of Water Resources and Electric Power, Hangzhou, People’s Republic of China.
2College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou, People’s Republic of China. 3Zhejiang Sci-Tech University Shaoxing Academy of Biomedicine Co., Ltd, Shaoxing, People’s Republic of China.

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 aoxR 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

Introduction

Vibrio vulnificus is an opportunistic pathogen for severe human infection, and is one of the leading causes of non-Cholera, Vibrio-associated deaths globally.1 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 worldwide4 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 database13 and their accession numbers in the NCBI GenBank database were listed in Table 1. Moreover, Escherichia coli ATCC 117757 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.1.14.615 with the command

Creative Commons Non Commercial CC BY-NC. This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/hom/open-access-at-sage).
“-gram neg –kingdom Bacteria –code 11.” Non-redundant 16S rRNA genes were generated by CD-HIT program v4.8.116 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.5.2.117 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.2.5.419 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.7,49021 with the parameter “–auto.” Aligned protein sequences were refined by using trimAL v.10.2rev5922 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.1.6.1223 with the parameter “-st AA -m MFP .” The aligned codon sequences were generated based on converting a multiple sequence alignment of proteins and the corresponding DNA by using PAL2NAL v.1424 And then, the resulting codon alignments were subjected to the calculation of synonymous and non-synonymous substitution (Ks/Ka) rates by KaKs_Calculator package v.1.225 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 formulaion lm implanted in R version.27 The phylogenetic trees were visualized by using MEGA7 software28 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 5147 (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 (r² = 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.3,34 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 27582' | GCA_002224265.1         | Two chromosomes                    | 5007,160         | 46.7              | 100.0            | 0.3              |
| V. vulnificus VV2014D.JH | 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_009763005.1         | 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)
| ISOLATE         | ACCESSION NUMBER | GENETIC MATERIALS          | GENOMIC SIZE (BP) | G + C CONTENT (%) | COMPLETENESS (%) | CONTAMINATION (%) |
|----------------|------------------|-----------------------------|-------------------|-------------------|------------------|------------------|
| *V. vulnificus* | GCA_014107515.1  | Two chromosomes, One plasmid | 5356,494          | 46.6              | 100.0            | 0.1              |
| *V. vulnificus* | GCA_014211935.1  | Two chromosomes             | 5032,819          | 46.8              | 100.0            | 0.1              |
| *V. alfaeensis* | GCA_003544875.1  | Two chromosomes, One plasmid | 4910,231          | 44.2              | 99.4             | 0                |
| *V. alginolyticus* | GCA_000354175.2 | Two chromosomes            | 5146,637          | 44.7              | 99.5             | 0.2              |
| *V. aphrogenes* | GCA_002157735.2  | Two chromosomes             | 3375,144          | 42.1              | 95.6             | 0.9              |
| *V. azureus*    | GCA_002849855.1  | Two chromosomes, Two plasmids | 4833,901         | 42.3              | 99.0             | 0.2              |
| *V. campbellii* | GCA_002163755.1  | Two chromosomes, One plasmid | 5178,103          | 45.1              | 99.9             | 0.4              |
| *V. casei*      | GCA_002218025.2  | Two chromosomes, Three plasmid | 4140,771         | 40.7              | 96.3             | 1.0              |
| *V. cholerae*   | GCA_013155105.1  | Two chromosomes             | 4100,705          | 47.2              | 55.3             | 0.9              |
| *V. cideii*     | GCA_009763805.1  | Two chromosomes             | 4750,222          | 47.9              | 97.2             | 0                |
| *V. fluvialis*  | GCA_001558415.2  | Two chromosomes             | 4827,733          | 49.9              | 99.9             | 0.9              |
| *V. hyugaensis* | GCA_002906655.1  | Two chromosomes             | 5612,082          | 45.0              | 100.0            | 0.3              |
| *V. natriegens* | GCA_001456255.1  | Two chromosomes             | 5175,153          | 45.1              | 100.0            | 2.8              |
| *V. panulinii*  | GCA_009938205.1  | Two chromosomes, One plasmid | 4855,939         | 45.2              | 99.7             | 1.5              |
| *V. parahaemolyticus* | GCA_001558495.2 | Two chromosomes            | 5152,461          | 45.3              | 100.0            | 0.1              |
| *V. ponticus*   | GCA_009938225.1  | Two chromosomes, One plasmid | 4796,932         | 44.8              | 98.3             | 2.0              |
| *V. rumoiensis* | GCA_002218045.2  | Two chromosomes, Two plasmids | 4207,152         | 42.3              | 95.6             | 0.5              |
| *V. tritonius*  | GCA_001547935.1  | Two chromosomes             | 5221,926          | 43.9              | 98.1             | 3.2              |
| *V. tubiashii*  | GCA_000772105.1  | Two chromosomes, Four plasmids | 5540,337        | 45.0              | 100.0            | 0.8              |
| *Escherichia coli* | 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. 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 11775\(^T\) 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-A, 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

![Graph showing genomic size and gene counts of Vibrio vulnificus.](image1)

![Graph showing maximum-likelihood phylogenetic tree.](image2)
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 al42 |
| OG0000118 and OG0000942 | MsbA     | 3003950        | Antibiotic efflux | Nitroimidazole antibiotic | Singh et al43 |
| OG0000191          | CatB9       | 3002681        | Antibiotic inactivation | Phenicol antibiotic | Heidelberg et al44 |
| OG0000210          | PmrE        | 3003577        | Antibiotic target alteration | Peptide antibiotic | Lee et al45 |
| OG0000223          | H-NS        | 3000676        | Antibiotic efflux | Tetracycline antibiotic, penam, macrolide antibiotic, fluoroquinolone antibiotic, cephalexin, cephalosporin | Nishino and Yamaguchi46 |
| OG0000702          | RsmA        | 3005069        | Antibiotic efflux | Phenicol antibiotic, diaminopyrimidine antibiotic, fluoroquinolone antibiotic | Mulcahy et al47 |
| OG0001054          | YajC        | 3005040        | Antibiotic efflux | Tetracycline antibiotic, penam, phenicol antibiotic, rifamycin antibiotic, cephalexin, cephalosporin, glycolcycline, fluoroquinolone antibiotic, triclosan | Rundell et al48 |
| OG0001146          | DfrA3       | 3003105        | Antibiotic target replacement | Diaminopyrimidine antibiotic | Brolund et al49 |
| OG0001216          | SoxR        | 3004107        | Antibiotic efflux, Antibiotic target alteration | Tetracycline antibiotic, fluoroquinolone antibiotic, glyclcycline, cephalosporin, phenicol antibiotic, rifamycin antibiotic, penam, disinfecting agents and intercalating dyes, triclosan, acridine dye | Sakhtah et al50 |
| OG0001389          | CRP         | 3000518        | Antibiotic efflux | Macrolide antibiotic, penam, fluoroquinolone antibiotic | Nishino et al51 |
| OG0001739 and OG0014842 | Tet35     | 3000481        | Antibiotic efflux | Tetracycline antibiotic | Teo et al52 |
| OG0002135          | ArtR        | 3000838        | Antibiotic efflux | Disinfecting agents and intercalating dyes, fluoroquinolone antibiotic, acridine dye | Fournier et al53 |
| OG0002643          | QnrVC1      | 3002799        | Antibiotic target protection | Fluoroquinolone antibiotic | Fonseca et al54 |
| OG0003164          | MacB        | 3000535        | Antibiotic efflux | Macrolide antibiotic | Xu et al55 |
| OG0003616          | VarG        | 3004289        | Antibiotic inactivation | Carbapenem | Lin et al56 |
| OG0006136          | MexK        | 3003693        | Antibiotic efflux | Macrolide antibiotic, tetracycline antibiotic, triclosan | Chuanchuen et al57 |
| OG0006781          | FosC2       | 3002874        | Antibiotic inactivation | Fosfomycin | Wachino et al58 |
| OG0014873          | PmpM        | 3004077        | Antibiotic efflux | Aminoglycoside antibiotic, fluoroquinolone antibiotic, benzalkonium chloride | He et al59 |
| OG0015064          | TetT        | 3000193        | Antibiotic target protection | Tetracycline antibiotic | Clermont et al60 |

(Continued)
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 isolate 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, \( \beta \)-lactam combination agent, lipopeptide, macrolide, nitrofurans, penicillin and phenicols, which were consistent with those ARGs found in their genomes (Table 2). Furthermore, the genomes of environmental isolates 93U204, ATCC 27562T, 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.

### 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\textsuperscript{61} |
| OG0015104          | TetWNW      | 3004442        | Antibiotic target protection | Tetracycline antibiotic | Leclercq et al\textsuperscript{62} |

Figure 3. Distribution of antibiotic-resistant genes in the \( V. vulnificus \) genomes.
(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\(^7\), 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. Moreover, metals and antibiotic pollutants 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. 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-sns, 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.

ORCID iD
Lin Xu https://orcid.org/0000-0003-2602-9875

Supplemental material

For this article is available online.

REFERENCES

1. Chen C-Y, Wu K-M, Chang Y-C, et al. Comparative genome analysis of Vibrio vulnificus, a marine pathogen. Genome Res. 2003;13:2577-2587.
2. López-Pérez M, Jayakumar JM, Grant TA, Zaragoza-Solas A, Cabello-Yeves PJ, Almagro-Moreno S. Ecological diversification reveals routes of pathogen emergence in endemic Vibrio vulnificus populations. Proc Natl Acad Sci U S A. 2021;118:e2103470118.
3. Baker-Austin C, Oliver JD. Vibrio vulnificus: new insights into a deadly opportunistic pathogen. Environ Microbiol. 2018;20:423-430.
4. Hooban A, Sooson P, Bunnlpatan P, et al. Molecular genotyping and phenotyping of Vibrio vulnificus isolated from diseased, brown-marbled gizzard raw dotted gizzard shad. Gut Pathog. 2016;8:22.
5. Li, W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006;22:1658-1659.
6. BIOinformatics. 2020:24:D517-D525.
7. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006;22:1658-1659.
8. BIOinformatics. 2020:24:D517-D525.
9. Frieri M, Kumar K, Bourtin A. Antibiotic resistance. J Infect Public Health. 2017;10:369-378.
10. Zhu Y-G, Johnson TA, Su JQ, et al. Diverse and abundant antibiotic resistance genes in Chinese wine strains. Proc Natl Acad Sci U S A. 2013;110:3435-3440.
11. Land M, Hauser L, Jun SR, et al. Insights from 20 years of bacterial genome sequencing. Funct Integr Genomics. 2015;15:144-161.
12. Benson DA, Cavanaugh M, Clark K, et al. GenBank. Nucleic Acids Res. 2012;41:D36-D42.
13. Shi W, Sun Q, Fan G, et al. gcType: a high-quality type strain genome database for microbial phylogenetic and functional research. Nucleic Acids Res. 2021;49:D705-D709.
14. Bowers RM, Kyrpides NC, Stepanaukas R, et al. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MGAG) of bacteria and archaea. Nat Biotechnol. 2017;35:725-731.
15. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30:2068-2069.
16. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006;22:1658-1659.
17. Koike T. Vibrio vulnificus: new insights into a deadly opportunistic pathogen. Int J Syst Evol Microbiol. 2011;61:3-10.
18. Alcock BP, Raphenya AR, Lau TTY, et al. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res. 2020;48:D517-D525.
19. Abschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403-410.
20. Vizcaíno JA, Querol M, Cervera R, et al. The bioCyc collection of metabolic and molecular pathway databases. Nucleic Acids Res. 2013;41:D360-D367.
21. Zhang Z, Li J, Zhao X-Q, Wang J, Wong G-K, Yu J. KaKs_Calculator: calculating Ka and Ks through model selection and model averaging. Genom Proteom Bioinform. 2006;4:259-263.
22. Stothard P. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Bioinformatics. 2000;28:1102, 1104.
23. Hulka R, Gentleman R. Ks: a language for data analysis and graphics. J Comput Graph Stat. 1996;5:299-314.
24. Gumus K, Chen J, Grant TA, Zaragoza-Solas A, Cabello-Yeves P. V. vulnificus: new insights into a deadly opportunistic pathogen. Environ Microbiol. 2020;33:2941-2950.
25. Kusum S, Stecher G, Tamura K. MEGAT: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870-1874.
26. Kapil V, Joseph N, Krishna K, et al. A comparative study of coastal and clinical V. vulnificus isolates for drug targeting and discovery. Mol Syst Biol. 2011;7:460.
40. San Millan A. Evolution of plasmid-mediated antibiotic resistance in the clinical context. Trends Microbiol. 2018;26:978-985.

41. Bonham KS, Wolfe BE, Dutton RJ. Extensive horizontal gene transfer in cheese-associated bacteria. *elife*. 2017;6:e21444.

42. Carroll LM, Gaballa A, Guldmann C, Sullivan G, Henderson LO, Wiedmann M. Identification of novel mobilizable tetracycline resistance gene mstA in a multi-drug-resistant, colistin-susceptible *Salmonella enterica* serotype typhimurium isolate. *mBio*. 2019;10:e00853-19.

43. Singh H, Velamakanni S, Deery MJ, Howard J, Wei SL, van Veen HW. ATP-dependent substrate transport by the ABC transporter MshA is proton-coupled. *Nat Commun*. 2016;7:12387.

44. Heidelberg JF, Eisen JA, Nelson WC, et al. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature*. 2000;406:477-483.

45. Lee H, Hsu F-F, Turk J, Grosman EA. The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J Bacteriol*. 2004;186:4124-4133.

46. Nishino K, Yamaguchi A. Role of histone-like protein H-NS in multidrug resistance of *Escherichia coli*. *J Bacteriol*. 2004;186:1423-1429.

47. Mulcahy H, O'Callaghan J, O'Grady EP, Adams C, O'Gara F. The post-erythromycin-resistant phenotype in *Streptococcus pneumoniae*. *Environ Microbiol*. 2006;8:3012-3015.

48. Rundell EA, Commodore N, Goodman AL, Kazmierczak BI. A screen for *Enterococcus faecalis* promoters: identification of novel promoters associated with superintegron repeats in *Vibrio cholerae* var *cholerae* O1. *Vibrio cholerae*. *Antimicrob Agents Chemother*. 2016;7:12387.

49. Sakhtah H, Koyama L, Zhang Y, et al. The *pmrC* gene mediates staphylococcal resistance to glycopeptides. *J Bacteriol*. 2020;202:e00682-19.

50. Teo JW, Tan TM, Poh CL. Genetic determinants of tetracycline resistance in *Vibrio parahaemolyticus*. *Antimicrob Agents Chemother*. 2019;63:e00853-19.

51. Nishino K, Senda Y, Yamaguchi A. CRP regulator modulates multidrug resistance of *Escherichia coli* by repressing the mdtEF multidrug efflux genes. *J Antibiot*. 2002;56:1038-1045.

52. Fournier B, Aras R, Hooper DC. Expression of the multidrug resistance transporter NorA from *Staphylococcus aureus* is modified by a two-component regulatory system. *J Bacteriol*. 2000;182:4119-4125.

53. Fonseca EL, Dos Santos Freitas F, Vieira VV, Vicente AC. New *qnr* gene cas-settes associated with superintegron repeats in *Vibrio cholerae* O1. *Emerg Infect Dis*. 2008;14:1129-1131.

54. Xie Y, Sun S-H, Nan KH, et al. Crystal structure of the periplasmic region of *MacB*, a noncanonical ABC transporters. *Biochemistry*. 2009;48:5218-5225.

55. Lin HTY, Massam-Wu T, Lin C-P, et al. The *Vibrio cholerae* var *cholerae* var regulon encodes a metallo-β-lactamase and an antibiotic efflux pump, which are regulated by VarR, a LysR-type transcription factor. *PLoS One*. 2017;12:e0184255.

56. Chuanchuen R, Narasaki CT, Schweizer HP. The *mesK* efflux pump of *Pseudomonas aeruginosa* requires *OpfM* for antibiotic efflux but not for efflux of triclosan. *J Bacteriol*. 2002;184:5036-5044.

57. Wachino J, Yamane K, Suzuki S, Kimura K, Arakawa Y. Prevalence of fosfomycin resistance among CTX-M-producing *Escherichia coli* clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes. *Antimicrob Agents Chemother*. 2015;59:3601-3604.

58. Ge GX, Kuroda T, Mima T, Morita Y, Mizushima T, Tsuchiya T. An H+/coupled multidrug efflux pump, PmpM, a member of the MATE family of transporters, from *Pseudomonas aeruginosa*. *J Bacteriol*. 2004;186:262-265.

59. Clermont D, Cheneau O, De Cespedes G, Horaud T. New tetracycline resistance determinants coding for ribosomal protection in streptococci and nucleotide sequence of tet(T) isolated from *Streptococcus pyogenes* A498. *Antimicrob Agents Chemother*. 1997;41:114-116.

60. Ishikawa J, Chiha K, Kuriha T, Satoh H. Contribution of *opdB*2 RNA polymerase beta subunit gene to rifampin resistance in *Nocardia species*. *Antimicrob Agents Chemother*. 2006;50:1342-1346.

61. Leclercq SO, Wang C, Zhu Y, et al. Diversity of the tetracycline mobilome within a Chinese pig manure sample. *Appl Environ Microbiol*. 2018;82:6454-6462.

62. Gxalo O, Digban TO, Igere BE, Olupade OA, Ohok AI, Nwodo UU. Virulence and antibiotic resistance characteristics of *Fibrobacter isolates* from rustic environmental freshwaters. *Front Cell Infect Microbiol*. 2019;8:9233.

63. Pal C, Asiani K, Arya S, et al. Metal resistance and its association with antibiotic resistance. *Front Cell Infect Microbiol*. 2021;12:e0184255.

64. Elmahdi S, DaSilva LV, Parveen S. Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: a review. *Food Microbiol*. 2016;57:128-134.

65. Yang Y, Higgins CH, Rehman I, et al. Genomic diversity, virulence, and antimicrobial resistance of *Klebsiella pneumoniae* strains from cows and humans. *Appl Environ Microbiol*. 2019;85:e02654-18.

66. Carvalho MJ, Martinez-Murcia A, Esteves AC, Correia A, Saavedra MJ. Phylogenetic diversity, antibiotic resistance and virulence traits of *Aeromonas spp.* from untreated waters for human consumption. *Int J Food Microbiol*. 2012;159:230-239.

67. Hurst LD. The *Ka/Ks* ratio: diagnosing the form of sequence evolution. *Trends Genet*. 2002;18:486-487.

68. Vitti JJ, Grossman SR, Ceberti PC. Detecting natural selection in genomic data. *Annu Rev Genet*. 2013;47:97-120.

69. Kunihikanan S, Thomas CJ, Franks AE, Mahadevaiah S, Kumar S, Petrovski S. Environmental hotspots for antibiotic resistance genes. *Microbiol Open*. 2021;10:e1917.

70. Larson DGJ, Flach C-F. Antibiotic resistance in the environment. *Nat Rev Microbiol*. 2022;20:257-269.

71. Paš C, Asiani K, Arya S, et al. Metal resistance and its association with antibiotic resistance. *Adv Microb Physiol*. 2017;70:261-313.