



**HAL**  
open science

# Genome-facilitated discovery of RND efflux pump-mediated resistance to cephalosporins in *Vibrio* spp. isolated from the mummichog fish gut

Nicole A Lloyd, Sylvie Nazaret, Tamar Barkay

## ► To cite this version:

Nicole A Lloyd, Sylvie Nazaret, Tamar Barkay. Genome-facilitated discovery of RND efflux pump-mediated resistance to cephalosporins in *Vibrio* spp. isolated from the mummichog fish gut. *Journal of Global Antimicrobial Resistance*, 2019, 19, pp.294-300. 10.1016/j.jgar.2019.05.006 . hal-02335501

**HAL Id: hal-02335501**

**<https://hal.science/hal-02335501>**

Submitted on 28 Oct 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

Title: Genome-facilitated discovery of RND efflux pump-mediated resistance to cephalosporins in *Vibrio* spp. isolated from the mummichog fish gut

Authors: Nicole A. Lloyd, Sylvie Nazaret, Tamar Barkay



PII: S2213-7165(19)30116-X  
DOI: <https://doi.org/10.1016/j.jgar.2019.05.006>  
Reference: JGAR 929

To appear in:

Received date: 31 August 2018  
Accepted date: 6 May 2019

Please cite this article as: Lloyd NA, Nazaret S, Barkay T, Genome-facilitated discovery of RND efflux pump-mediated resistance to cephalosporins in *Vibrio* spp. isolated from the mummichog fish gut, *Journal of Global Antimicrobial Resistance* (2019), <https://doi.org/10.1016/j.jgar.2019.05.006>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Genome-facilitated discovery of RND efflux pump-mediated resistance to cephalosporins in *Vibrio* spp. isolated from the mummichog fish gut**

Nicole A. Lloyd<sup>a1\*</sup>, Sylvie Nazaret<sup>b</sup>, Tamar Barkay<sup>a</sup>

<sup>a</sup>Department of Biochemistry and Microbiology, Rutgers University, 76 Lipman Drive, New Brunswick, NJ, 08901 USA

<sup>b</sup>UMR 5557 Ecologie Microbienne, CNRS, INRA, VetagroSup, UCBL, Université de Lyon, 43 Boulevard du 11 Novembre, F-69622 Villeurbanne, France

**\*Corresponding author:** <sup>1</sup> Present address: Department of Laboratory Medicine, Clinical Center, National Institutes of Health, 10 Center Drive, Bethesda, MD, 20892 USA, E-mail: nicole.lloyd@nih.gov

**Highlights:**

- RND efflux pumps mediate cephalosporin resistance in *Vibrio* from the fish gut

- *Vibrio* harbor additional RND pumps with high homology to *V. cholerae*
- RND pumps have potential for increased virulence

## Abstract

**Objectives:** This study examines the role of RND efflux pumps in resistance to first- and third-generation cephalosporins as well as the potential contribution to increased virulence in two *Vibrio* isolates from the gut microbiota of a forage-feeder fish.

**Methods:** We performed phenotypic MIC testing in the presence and absence of an RND efflux pump inhibitor, phenylalanine-arginine-beta-naphthylamide (PA $\beta$ N), and further compared the genomes of the two *Vibrio* spp. to characterized RND efflux pump gene homologs.

**Results:** We identified 13 and 12 RND operons, respectively, in *Vibrio* spp. T21 and T9, with *Vibrio* sp. T21 containing an additional RND operon compared to other *V. parahaemolyticus* strains. Both the inner-membrane protein (IMP) and the membrane facilitator protein (MFP) sequences of this operon are homologous to VexD and VexC, respectively, an RND operon in *V. cholerae*. More generally, the other RND proteins in our strains show homology to RND efflux pumps characterized in *E. coli* and *V. cholerae*. In

*Vibrio* sp. T21, we observed decreased resistance to cefoperazone and cephradine, and in *Vibrio* sp. T9, decreased resistance to cefoperazone and cefsulodin in the presence of PA $\beta$ N. RND pumps may also mediate transport of kanamycin.

**Conclusions:** By analyzing the genomes of two *Vibrio* spp. isolated from the mummichog fish gut we discovered RND efflux pump-mediated resistance to first- and third-generation cephalosporins in these strains. This work highlights the need for further research into this unique *Vibrio* sp. operon, and more generally, RND efflux pumps in *Vibrio* species, as *Vibrio* often cause seafood-borne illness.

**Keywords:** RND, *Vibrio*, cephalosporin resistance, efflux pumps

## 1. Introduction

Vibrios, commonly associated with marine animals including fish, shellfish, and shrimp, are ubiquitous in aquatic environments. Some species of *Vibrio* cause disease in humans, including *V. parahaemolyticus* which is among the leading causes of seafood-borne infections in the United States (1). Cases of Vibrionosis are currently rising in the United States, with the latest major outbreak reported in 2013. Because antibiotics are commonly

used to treat Vibrionosis (2), the rise of antimicrobial resistance (AMR) among pathogenic *Vibrio* spp. is a major public health concern.

Surveillance data provided by the United States Centers for Disease Control and Prevention show that quinolones and cephalosporins were the most commonly prescribed antibiotics for Vibrionosis in the United States between 1990 and 2010 (2). Cephalosporin antibiotics, in combination with doxycycline, are currently recommended for treatment of Vibrionosis (3) despite reports of increased cephalosporin resistance (4).

With approximately 90% of global aquaculture production in Asia, it is concerning that numerous seafood-borne outbreaks in several Asian countries have been attributed to *Vibrio* contamination of seafood (5). In aquaculture practices, antibiotics are used excessively without regulation to prevent and treat infections assuring continuous production of seafood (5). It is estimated that up to 80% of antibiotics enter the environment unaltered (6), contributing to the selection of resistant organisms. The overuse of antibiotics in aquaculture potentially poses a great risk to human health, as exposure to multi-drug resistant (MDR) bacteria is possible via consumption of seafood and exposure to recreational water.

Efflux pumps are distributed amongst all domains of life and their genetic organization and structural features are well-conserved (7). The

Resistance Nodulation and cell Division (RND) superfamily of efflux pumps plays a prominent role in AMR in Gram-negative clinical bacterial isolates (7). They consist of a tripartite system including an outer membrane protein (OMP), a membrane fusion protein (MFP), and an inner membrane protein (IMP) (7). One of the most well-studied RND systems, AcrAB, characterized in *Escherichia coli* and broadly distributed among the Enterobacteriaceae, exports dyes, detergents, several antibiotics, and organic solvents (8). In addition, RND efflux pumps are involved in cell-to-cell communication, host colonization via the export of bile salts (8), and virulence (9). Such efflux pumps mediate resistance to the  $\beta$ -lactam cephalosporins via the *mexAB* operon in *Pseudomonas* spp. and the *acrAB* operon in *E. coli* (7). Resistance is enhanced when these genes are over-expressed in combination with other factors such as porin deficiency and chromosomal resistance genes (10), e.g., those encoding various classes of  $\beta$ -lactamases (11). The role of efflux pumps in *Vibrio* spp. is important to understand, as efflux pumps contribute to AMR, virulence, and host colonization.

We previously described two *Vibrio* strains (closely related to *V. parahaemolyticus* and *V. antiquarius*) isolated from the gut of the mummichog fish (*Fundulus heteroclitus*) (12), an environment with an

abundance of *Vibrio* spp. (13). The strains exhibited resistance to penicillins, cephalosporins, aminoglycosides, and in one case, to a fluoroquinolone (12). Their genomes contained class A, class B, and class C  $\beta$ -lactamase gene homologs, along with many efflux-pump gene homologs (12). The importance of these findings stems from the known role of *V. parahaemolyticus* as an opportunistic pathogen, and while not much is known about the potential pathogenicity of *V. antiquarius*, it is genetically-related to other pathogenic *Vibrio* spp. (14). With resistance to cephalosporins increasing globally (4), it is important to understand the mechanisms of AMR in *Vibrio* spp. The aim of this paper is to examine the roles of RND efflux pumps in resistance to first- and third-generation cephalosporins as well as in increased virulence in two *Vibrio* spp. from the gut microbiota of a forage-feeder fish.

## 2. Methods

### 2.1 Isolation and Species Determination

*Vibrio* spp. were isolated from mummichog gut microbiota (15). The taxonomic identity of the isolates was determined based on the average nucleotide identity (ANI) (16) and confirmed by multi-locus sequence analysis (MLSA) that was performed on partial 16S rRNA, *gyrB*, *pyrH*, *recA*,

and *rpoD* genes from *Vibrio* sp. T9 and compared to reference MLSA patterns of these genes in *Vibrio* spp. (17).

## 2.2 Effect of Efflux Inhibition on Growth Rates

Growth was monitored using the Bioscreen C (Oy Growth Ab Ltd), an automated microbiology growth curve analysis system. Pre-cultures were grown on tryptic soy agar (TSA) media overnight at 28°C. Colonies were suspended in tryptic soy broth (TSB) media to a McFarland standard of ~1.5 measured using the DensiCheck system. Approximately  $4.5 \times 10^6$  CFU/mL were inoculated into Bioscreen C honeycomb plates containing serial dilutions of PA $\beta$ N (0 – 100  $\mu$ g/mL) in DMSO. Growth rates ( $\mu$ ) of the triplicate wells from the Bioscreen C assays were calculated from growth curves using the grofit package for R (18). The averages and standard deviations of the growth rates were visualized in Microsoft Excel (Figure S1).

## 2.3 Liquid MICs in Presence of Phenylalanine-Arginine-Beta-Naphthylamide (PA $\beta$ N)

Minimum inhibitory concentrations of various cephalosporin antibiotics and kanamycin were determined using the established broth

microdilution method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) using microtiter plates (19) in the presence or absence of the efflux pump inhibitor PA $\beta$ N. This concentration of PA $\beta$ N was experimentally determined to not affect growth of the organisms (Figure S1). Briefly, overnight (Mueller-Hinton agar at 28°C) cultures were diluted to a 0.5 McFarland standard as measured by the DensiCheck (bioMérieux, Craponne, France) system. The cultures were then used to inoculate a microtiter plate containing serial dilutions of antibiotics in Mueller-Hinton broth with and without 50  $\mu$ g/mL PA $\beta$ N. The plates were incubated for 24 h at 28°C before interpretation. MIC changes of 4-fold or greater were considered significant (20).

#### *2.4 Identification of Putative RND Efflux Genes in the Genomes of Vibrio spp. T9 and T21*

RND efflux gene sequences characterized by Matsuo *et al.* (21) were extracted from the *V. parahaemolyticus* RIMD 2210633 genome sequence and used to create a blast database. This database was then used to blast search (22) our *Vibrio* spp. genome sequences for homologs. We further manually searched upstream and downstream of each RND gene hit to locate additional RND gene homologs. To determine relatedness of the

putative RND proteins in our *Vibrio* spp., we aligned them with translated reference genes to obtain % identity values. The following reference genes were used: *acrA*, *acrB*, and *acrD* from *E. coli* O157:H7 strain Sakai (NC\_002695); *mexA* and *mexB* from *P. aeruginosa* PAO1 (NC\_002516), and *vexA*, *vexB*, *vexC*, *vexD*, *vexK*, from *V. cholerae* O1 biovar El Tor strain N1696 (NC\_002505 and NC\_002506).

### 2.5 Predicted Pathogenicity

Genome sequences were uploaded to Pathogen Finder version 1.1 to predict pathogenicity from whole genome sequences, accessed in June 2018 (23).

### 2.6 Virulence Testing

Virulence of *Vibrio* spp. was determined as described by Froquet *et al.* (24) using the amoebal model, *Dictyostelium discoideum*. Overnight bacterial cultures were adjusted to an OD<sub>600nm</sub> of 1.5 by dilution in LB. One mL of each adjusted bacterial suspension was plated on SM Agar (Formedium, Hustanton, United Kingdom) medium. The plates were allowed to dry for one hour to obtain a dry bacterial layer. Cells of *D. discoideum* were washed twice in PAS (Page's amoeba saline) buffer by

centrifugation at 1 000 g for 10 minutes. The amoebal suspension was adjusted to  $2 \times 10^6$  cells/mL and serial dilutions were performed in order to obtain a final concentration of 7 812 cells/mL (in the most dilute sample). Five  $\mu$ L of each serial dilution of *D. discoïdeum* (containing 39 – 14 000 cells per 5  $\mu$ L aliquot) was spotted on the bacterial lawn. Plates were incubated at 22.5°C for five days and, appearance of phagocytic plaques was checked at the end of the incubation time. This assay was performed in triplicate. Strains of *P. aeruginosa* PT5 and *K. pneumoniae* KpGe were used as negative (virulent *P. aeruginosa* PT5 should be non-permissive for *D. discoïdeum* growth) and positive (*K. pneumoniae* KpGe, a non-virulent strain, should be permissive for *D. discoïdeum*) controls, respectively, in each assay. Three categories of response were defined to interpret the results: non-virulent bacteria (fewer than 400 amoebae cells were sufficient for lysis plaque formation), low-virulent (400-2500 amoebae for lysis plaque formation) and virulent (more than 2500 amoebae).

### 3. Results

#### 3.1 Taxonomic Information

*Vibrio* sp. T9 was determined to be closely related to *V. antiquarius* EX25, based on an ANI value of 98.3% and 94.5% DNA identity in the

MLSA; values of >95% in each assay delineate the same species (25).

*Vibrio* sp. T21 was identified as closely related to *V. parahaemolyticus* FORC 018 with an ANI value of 98.2% (12) (Table 1).

### 3.2 Effect of PA $\beta$ N on Antibiotic Resistance Profiles

To assess the possible contribution of RND efflux pumps to drug resistance, we tested the MIC of five different cephalosporins in the presence and absence of 50  $\mu$ g/mL of the efflux pump inhibitor PA $\beta$ N. Both *Vibrio* strains showed sensitivity to cefoperazone in the presence of the inhibitor, with *Vibrio* sp. T9 showing a 64-fold decrease in MIC and *Vibrio* sp. T21 showing a 128-fold decrease in MIC (Table 2). *Vibrio* sp. T21 showed a 32-fold decrease in MIC also to cephradine, while *Vibrio* sp. T9 showed a 4-fold decrease in MIC to cefsulodin. Both strains had no change in MIC to ceftazidime, a third-generation drug used in treatments against *Pseudomonas* and *Enterobacteriaceae*. Regarding cefazolin, a very hydrophobic molecule, shown not to enter the cell in *E. coli* (26), we saw a slight (4-fold) decrease in MIC in *Vibrio* sp. T21 and an 8-fold increase in *Vibrio* sp. T9 (Table 2). Strain T21 exhibited an 8-fold reduced sensitivity to kanamycin, an aminoglycoside, with MIC decreasing from 8 to 1  $\mu$ g/mL.

The corresponding decrease in strain T9 was 4-fold from 1 to 0.25 µg/mL kanamycin (Table 2).

### 3.3 Inventory of Putative RND Efflux Gene Homologs

We identified 12 putative RND operons in *Vibrio* sp. T9 (Figure 1 b), consistent with the number and operon structure in *V. antiquarius* EX25 (NC\_013456, NC\_013457) (Table S1), a reference strain. The % identity values of *Vibrio* sp. T9 compared to the proteins described by Matsuo *et al.* (21) were between 85 and 100% (Table 3 a), indicating strong homology between the two strains. However, one of the potential RND IMPs (CBX98\_02130) showed very low homology to the *V. parahaemolyticus* RIMD 2210633 pumps, and instead showed high homology (85%) to VexK from *V. cholerae* (Table 3 a).

In *Vibrio* sp. T21, we identified 13 RND operons, one additional compared to those described by Matsuo *et al.* (21) in *V. parahaemolyticus* RIMD 2210633 (21) (Figure 1 a). The % identity values between the putative proteins and reference proteins are reported in Table 3 b.

The *Vibrio* sp. T21 MFP and IMP efflux pump protein homologs had between 97-100% identity to pumps described by Matsuo *et al.* (21), except for the additional operon. The novel IMP (gene locus CCD93\_22990)

showed 86% amino acid identity by pairwise alignment to *V. cholerae* VexD (gene locus VC1756), and the MFP located upstream (gene locus CCD93\_22985) showed 70% amino acid identity to VexC (gene locus VC1757) (Table 4).

To locate orthologs, we compared the efflux pump proteins to pumps characterized in reference organisms. Both *Vibrio* spp. contained proteins orthologous to the AcrAB and MexAB efflux pumps from *E. coli* and *P. aeruginosa* (Table 3 a-b), two systems known to affect cephalosporin resistance (10). Both *Vibrio* spp. also harbored orthologs of VexAB, a typical RND broad spectrum efflux pump shown to be effective against antibiotics and detergents (9). Both *Vibrio* strains contained VmeB, presumably an ortholog (58% identity) to AcrD, an *E. coli* IMP with activity against aminoglycosides (10) (Table 3 a-b).

### 3.4 Virulence

Based on the genome sequences, Pathogen Finder (23) predicts both organisms as potential human pathogens with *Vibrio* sp. T21 and *Vibrio* sp. T9 having a 71.9% and 67.7% probability of being a human pathogen, respectively (Table 1). However, both *Vibrio* strains showed non-virulent properties when tested with the *D. discoïdeum* virulence assay described

above, as fewer than 400 amoebae were needed to form lysis plaques for each strain.

#### 4. Discussion

We hypothesized that RND efflux pumps contributed to phenotypic resistance to multiple classes of antibiotics in two *Vibrio* spp. isolates. Here, we report on the genome-facilitated discovery of efflux pump-mediated AMR in these strains. While the two strains were isolated from the same environment, the gut of a forage-feeder fish, and are both members of the *Vibrio* genus, their resistance phenotypes in the presence of an efflux pump inhibitor were dissimilar, indicating varying levels of RND efflux pump involvement in AMR. Our findings suggest that resistance to first- and third-generation cephalosporins in these *Vibrio* spp. is mediated by RND efflux pumps. Genome sequences revealed that *Vibrio* sp. T9 contained 12 and *Vibrio* sp. T21 contained 13 RND operons with homology to pumps characterized in *V. parahaemolyticus* RIMD 2210633. Furthermore, *Vibrio* sp. T21 harbored an additional RND operon, with strong homology to the *V. cholerae* efflux pump VexCD, suggesting that this organism has the potential for increased virulence.

Comparisons between environmental and clinical strains of *Vibrio* show that environmental strains had a more diverse antibiotic resistance profile (27) suggesting the importance of understanding the evolution of resistance among environmental strains and transmission to pathogens of clinical importance. Importantly, both *Vibrio* spp. strains showed a significant decrease (64- to 128-fold) in MIC to cefoperazone (Table 2), a third-generation cephalosporin, when tested with PA $\beta$ N. Resistance to cephalosporins via the AcrAB RND system in *E. coli* has been well-characterized (28). Both *Vibrio* spp. T9 and T21 harbored VmeB protein homologs which were 63% identical to *E. coli* AcrB (Table 3 a-b); others have characterized VmeAB as an orthologue of AcrAB in *E. coli* (29), providing support for our hypothesis of RND involvement in cephalosporin resistance. Third-generation cephalosporins, to which resistance is emerging world-wide (4), are currently part of the recommended treatment for Vibrionosis (3). These results suggest that RND efflux pumps among fish gut microbiota, and more broadly, in aquatic environments, may contribute to the emergence of resistance to third-generation cephalosporin drugs.

Using the power of genome sequencing, we report on the number of RND protein homologs located in each strain and infer function based on

homology. *Vibrio* species are known to contain between six (*V. cholerae*) (30) and 12 (*V. parahaemolyticus*) (21) RND efflux pump operons. The genetic structure of the putative RND efflux pumps in both *Vibrio* spp. T9 and T21 agrees with others' reports (21). *Vibrio* sp. T9 contained 12 potential RND operons, and almost all of those proteins showed homology ( $\geq 85\%$  identity) with *V. parahaemolyticus* RIMD 2210633 pumps (Table 3 a-b). The only exception was one *Vibrio* sp. T9 IMP (CBX98\_02130) that was most similar to VexK of *V. cholerae*. VexK specifies resistance to bile acids, detergents, and potentially is required for pathogenesis in *V. cholerae* (31), suggesting *Vibrio* sp. T9 may have increased virulence properties. Others have commented on the potential pathogenicity of the *V. antiquarius* species, noting that the genome encodes for pathogenicity factors in other *Vibrio* spp. (14). Although virulence testing in an amoeba model suggested no virulence phenotype, some features in the genome of *Vibrio* sp. T9 do suggest pathogenicity as determined by the 67.7% probability of being a human pathogen predicted by Pathogen Finder (23) (Table 1), which is slightly greater than the score of 62.9% predicted for *V. antiquarius* EX25. *Vibrio* sp. T21 contained 13 RND operons, one additional operon with high amino acid sequence homology (70% in MFP; 86% in IMP) to VexCD from *V. cholerae* (Table 4) compared to *V.*

*parahaemolyticus* RIMD 2210633 (Figure 1 a). Notably, *Vibrio* sp. T9 did not contain a homolog to *V. cholerae* VexD (Table 3 a).

In *Vibrio* sp. T21, PA $\beta$ N decreased the MIC of cephadrine (a first-generation cephalosporin) by 32-fold, while in *Vibrio* sp. T9, it had no effect on sensitivity (Table 2). To explain this discrepancy, we speculate that either i) the additional RND operon (VexCD-like) in *Vibrio* sp. T21 contributes to this resistance phenotype or ii) the *V. parahaemolyticus* RIMD 2210633 VmeI protein, lacking in *Vibrio* sp. T9, may be necessary for RND efflux pump-mediated resistance to cephadrine. However, to date VexCD-mediated antibiotic resistance has not been reported in *V. cholerae* (32). VexCD is known to be involved in virulence, as it pumps out bile acids, which aids in host colonization (32). Bile acids, possibly one of the primary substrates for RND efflux pumps, are part of the antimicrobial barrier in enteric environments (8). Much like *V. cholerae* VexCD, the VmeAB and VmeCD proteins in *V. parahaemolyticus* RIMD 2210633, homologs of which were identified in both our *Vibrio* spp. (Table 3 a-b) are also involved in the export of bile acids (21).

Others have discussed the contributions of RND efflux pumps not only to antibiotic resistance, but also to increased virulence and pathogenicity (8). For example, bile enhances expression of virulence

genes in *V. parahaemolyticus* (33). In *V. cholerae*, RND *vex* operons are required for colonization of infant mouse intestinal tracts (30) and, importantly, bile can induce regulators of VexCD, which can lead to overexpression (34). While a Pathogen Finder survey of the *Vibrio* sp. T21 genome predicted a 71.9% chance that this organism was pathogenic, the results of the amoebal virulence test defined the strain as non-virulent. This result could indicate that the strain may not be digested by the amoeba, or the virulence may be mediated by a set of mechanisms that would manifest in a different organism, e.g. a rodent model (24). Overall, we conclude that the additional RND operon (Table 4) may help *Vibrio* sp. T21 survive in its niche of the fish gut, an environment rich in cholate and taurocholate (35). Clearly, the exact role of the *V. cholerae*-like RND system, and the other RND systems, in AMR and virulence, will require *in vivo* experimentation.

In some cases, RND efflux pumps were shown to not affect the resistance phenotype. For the first-generation cephalosporin ceftazidime, a drug typically used in the treatment of serious Gram-negative infections, we saw no change in MIC upon addition of PA $\beta$ N in either *Vibrio* strain. Similarly, for the third-generation cefsulodin, a drug targeting *Pseudomonas* spp., there was no change in MIC in *Vibrio* sp. T21, and only a minimal effect in *Vibrio* sp. T9. Both strains exhibited clinical-levels of resistance to

these drugs, suggesting that resistance phenotypes are likely attributed to other mechanisms, e.g. degradation of the drugs by  $\beta$ -lactamases encoded by the genomes (12).

*Vibrio* spp. T9 and T21 exhibited contrasting resistance phenotypes to cefazolin upon PA $\beta$ N addition, with an 8-fold increase in MIC in *Vibrio* sp. T9 and a 4-fold decrease in *Vibrio* sp. T21. We note that both strains were cefazolin sensitive (19) with a maximum MIC of only 4  $\mu$ g/mL. Others have shown PA $\beta$ N reduces resistance to cephalosporins in *E. coli*, speculated to be due to interactions between PA $\beta$ N and other substrates within the binding site (36). Further, cefazolin is a hydrophilic molecule and is known to not be effluxed by AcrB in *E. coli* (26). Therefore, resistance to these two drugs are likely not affected by RND efflux pumps in these *Vibrio* strains.

Aminoglycosides are an important class of antibiotics known to be counter-acted by RND efflux pumps such as AcrD in *E. coli* (28). In *Vibrio* spp. T21 and T9, we observed an 8- and 4-fold decrease, respectively, in MIC to kanamycin. However, the starting MIC values would be considered sensitive according to Bier *et al.* (37). Both *Vibrio* spp. contained proteins with some homology to AcrD (58% and 59% identity) (Table 3 a-b), which could potentially mediate efflux, a hypothesis that will need to be addressed by further testing. Interestingly, when RND efflux pump operons were

individually knocked out in *V. parahaemolyticus* RIMD 2201633, its MIC to kanamycin remained unchanged (21). Our results suggest that RND efflux pumps may be involved in efflux of kanamycin in the fish gut *Vibrio* spp. described here.

RND efflux pumps support bacterial life in enteric environments, confer resistance to antibiotics, and play a role in pathogenicity. Here, we report on the potential involvement of such pumps in cephalosporin resistance in two *Vibrio* strains. We show that each *Vibrio* harbors a set of RND efflux genes, including pumps with homology to those in *V. cholerae*, suggesting a possible contribution to virulence. Could RND efflux pumps be partly responsible for the reported increase in *Vibrio* infections observed in recent years in the United States (1)? The increase in AMR and virulence may be related if altered expression of RND efflux pumps led to increased bile and antibiotic resistance, as suggested by our genome analyses. If these MDR strains survive in the fish gut, they are likely able to live also in the human gut, as the composition of bile acids are similar in the two environments (35).

This work demonstrates the connection between RND efflux pumps and AMR in commensal fish gut bacteria. It has implications for antibiotic usage in aquaculture, as *V. parahaemolyticus* is one of the leading causes

of seafood-borne gastroenteritis (1). A recent study on the adaptation of microbial populations to antibiotic treatment in a high-intensity catfish production system showed that 21 antibiotic resistant genes were under selection, with a majority of them classified as efflux pumps (38). The overuse of antibiotics will not only contribute to the spread of resistance by applying selective pressure in an environment that is highly conducive to genetic exchange and microbial growth, but also by selection for the over-expression of efflux pumps, which can, in turn, lead to populations with increased pathogenic potential.

Efflux pumps are central to microbial life in any environment as they may modulate gene expression during biofilm formation (39) and impact virulence factor production and adaptive responses to environmental challenges (40). While the cited examples are from human pathogens, the importance of efflux pumps extends far beyond, as efflux pumps mediate resistance to toxic metals, and biofilms are a major mode of microbial life in nature. We suggest that the microbial ecology of efflux pumps is an area of needed research not only for their contribution to AMR and pathogenicity but also for their general role in modulating adaptation and facilitating life in diverse environments.

## **Declarations**

**Funding:** This project was supported with funding from the New Jersey Water Resources Research Institute. This material is based upon research supported by the Chateaubriand Fellowship of the Office for Science & Technology of the Embassy of France in the United States.

**Ethical Approval:** Not required

**Competing Interests:** None

### **Acknowledgements**

The platform PARMIC (UMR 5557 Ecologie Microbienne) and associated technicians are also acknowledged for providing equipment, facilities, and valuable advice.

## References

1. Elmahdi S, DaSilva LV and Parveen S. *Antibiotic resistance of Vibrio parahaemolyticus and Vibrio vulnificus in various countries: A review.* Food Microbiol. 2016. 57:128-134.
2. Wong KC, Brown AM, Luscombe GM, Wong SJ and Mendis K. *Antibiotic use for Vibrio infections: important insights from surveillance data.* BMC Infect. Dis. 2015. 15; 226:1-9.
3. Centers for Disease Control. *Management of Vibrio vulnificus wound infections after a disaster.* 2017; Available from: <https://www.cdc.gov/disasters/disease/vibriofaq.html>.
4. Obaidat MM, Salman AEB and Roess AA. *Virulence and antibiotic resistance of Vibrio parahaemolyticus isolates from seafood from three developing countries and of worldwide environmental, seafood, and clinical isolates from 2000 to 2017.* J. Food Prot. 2017. 80; 12:2060-2067.
5. Letchumanan V, Pusparajah P, Tan LT, Yin WF, Lee LH and Chan KG. *Occurrence and antibiotic resistance of Vibrio parahaemolyticus from shellfish in Selangor, Malaysia.* Front. Microbiol. 2015. 6; 1417.
6. Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dolz H, Millanao A and Buschmann AH. *Antimicrobial use in aquaculture re-examined:*

- its relevance to antimicrobial resistance and to animal and human health.* Environ. Microbiol. 2013. 15; 7:1917-1942.
7. Hernando-Amado S, Blanco P, Alcalde-Rico M, Corona F, Reales-Calderón JA, Sánchez MB and Martínez JL. *Multidrug efflux pumps as main players in intrinsic and acquired resistance to antimicrobials.* Drug Resist. Updat. 2016. 28:13-27.
  8. Blanco P, Hernando-Amado S, Reales-Calderon J, Corona F, Lira F, Alcalde-Rico M, Bernardini A, *et al.* *Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants.* Microorganisms. 2016. 4; 1:1-19.
  9. Bina JE, Provenzano D, Wang C, Bina XR and Mekalanos JJ. *Characterization of the Vibrio cholerae vexAB and vexCD efflux systems.* Arch. Microbiol. 2006. 186; 3:171-181.
  10. Li XZ, Plesiat P and Nikaido H. *The challenge of efflux-mediated antibiotic resistance in gram-negative bacteria.* Clin. Microbiol. Rev. 2015. 28; 2:337-418.
  11. Bush K and Jacoby GA. *Updated functional classification of  $\beta$ -Lactamases.* Antimicrob. Agents Chemother. 2010. 54; 3:969-976.
  12. Lloyd NA, Nazaret S and Barkay T. *Whole genome sequences to assess the link between antibiotic and metal resistance in three*

- coastal marine bacteria isolated from the mummichog gastrointestinal tract*. Mar. Pollut. Bull. 2018. 135:514-520.
13. Givens CE, Ransom B, Bano N and Hollibaugh JT. *Comparison of the gut microbiomes of 12 bony fish and 3 shark species*. Mar. Ecol. Prog. Ser. 2015. 518:209-223.
  14. Hasan NA, Grim CJ, Lipp EK, Rivera ING, Chun J, Haley BJ, Taviani E, et al. *Deep-sea hydrothermal vent bacteria related to human pathogenic Vibrio species*. Proc. Natl. Acad. Sci. 2015. 112; 21:E2813-E2819.
  15. Lloyd NA, Janssen SE, Reinfelder JR and Barkay T. *Co-selection of mercury and multiple antibiotic resistances in bacteria exposed to mercury in the Fundulus heteroclitus gut microbiome*. Curr. Microbiol. 2016. 73; 6:834-842.
  16. Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, Seo H and Chun J. *Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies*. Int. J. Syst. Evol. Microbiol. 2017. 67; 5:1613-1617.
  17. Pascual J, Macian MC, Arahal DR, Garay E and Pujalte MJ. *Multilocus sequence analysis of the central clade of the genus Vibrio*

- by using the 16S rRNA, recA, pyrH, rpoD, gyrB, rctB and toxR genes.*  
Int. J. Syst. Evol. Microbiol. 2010. 60; 1:154-165.
18. Kahm M, Hasenbrink G, Lichtenberg-Frate H, Ludwig J and Kschischo M. *Grofit: Fitting biological growth curves with R.* J. Stat. Softw. 2010. 33; 7:1-21.
  19. CLSI. *M07-A9: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition.* 2012. p. 1-88.
  20. Bina XR, Philippart JA and Bina JE. *Effect of the efflux inhibitors 1-(1-naphthylmethyl)-piperazine and phenyl-arginine- $\beta$ -naphthylamide on antimicrobial susceptibility and virulence factor production in Vibrio cholerae.* J. Antimicrob. Chemother. 2008. 63; 1:103-108.
  21. Matsuo T, Nakamura K, Kodama T, Mikami T, Hiyoshi H, Tsuchiya T, Ogawa W, *et al.* *Characterization of all RND-type multidrug efflux transporters in Vibrio parahaemolyticus.* Microbiologyopen. 2013. 2; 5:725-742.
  22. Madden T. *Chapter 16: The BLAST Sequence Analysis Tool*, in *The NCBI Handbook*. 2002, National Center for Biotechnology Information. p. 1-15.

23. Cosentino S, Voldby Larsen M, Møller Aarestrup F and Lund O. *PathogenFinder - Distinguishing friend from foe using bacterial whole genome sequence data*. PLoS ONE. 2013. 8; 10:e77302.
24. Froquet R, Lelong E, Marchetti A and Cosson P. *Dictyostelium discoideum: a model host to measure bacterial virulence*. Nat. Protoc. 2008. 4:25.
25. Steinum TM, Karatas S, Martinussen NT, Meirelles PM, Thompson FL and Colquhoun DJ. *Multilocus sequence analysis of close relatives Vibrio anguillarum and Vibrio ordalii*. Appl. Environ. Microbiol. 2016. 82; 18:5496-504.
26. Nagano K and Nikaido H. *Kinetic behavior of the major multidrug efflux pump AcrB of Escherichia coli*. Proc. Natl. Acad. Sci. 2009. 106; 14:5854-5858.
27. Shaw KS, Rosenberg Goldstein RE, He X, Jacobs JM, Crump BC and Sapkota AR. *Antimicrobial susceptibility of Vibrio vulnificus and Vibrio parahaemolyticus recovered from recreational and commercial areas of Chesapeake Bay and Maryland coastal bays*. PLoS ONE. 2014. 9; 2:e89616.
28. Anes J, McCusker MP, Fanning S and Martins M. *The ins and outs of RND efflux pumps in Escherichia coli*. Front. Microbiol. 2015. 6; 587.

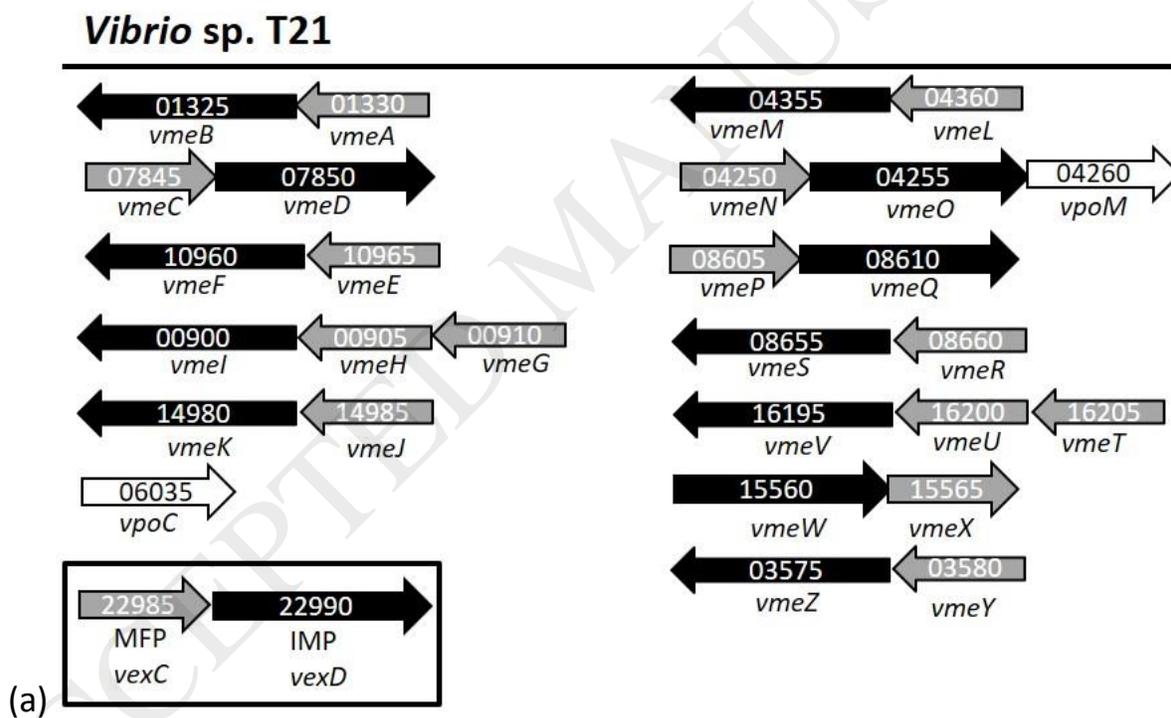
29. Matsuo T, Hayashi K, Morita Y, Koterasawa M, Ogawa W, Mizushima T, Tsuchiya T, *et al.* *VmeAB, an RND-type multidrug efflux transporter in Vibrio parahaemolyticus.* Microbiology. 2007. 153; 12:4129-4137.
30. Bina XR, Provenzano D, Nguyen N and Bina JE. *Vibrio cholerae RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine.* Infect. Immun. 2008. 76; 8:3595-3605.
31. Taylor DL, Bina XR and Bina JE. *Vibrio cholerae vexH Encodes a multiple drug efflux pump that contributes to the production of cholera toxin and the toxin co-regulated pilus.* PLoS ONE. 2012. 7; 5:e38208.
32. Rahman MM, Matsuo T, Ogawa W, Koterasawa M, Kuroda T and Tsuchiya T. *Molecular cloning and characterization of all RND-Type efflux transporters in Vibrio cholerae Non-O1.* Microbiol. Immunol. 2007. 51; 11:1061-1070.
33. Gotoh K, Kodama T, Hiyoshi H, Izutsu K, Park KS, Dryselius R, Akeda Y, *et al.* *Bile acid-induced virulence gene expression of Vibrio parahaemolyticus reveals a novel therapeutic potential for bile acid sequestrants.* PLoS ONE. 2010. 5; 10:e13365.

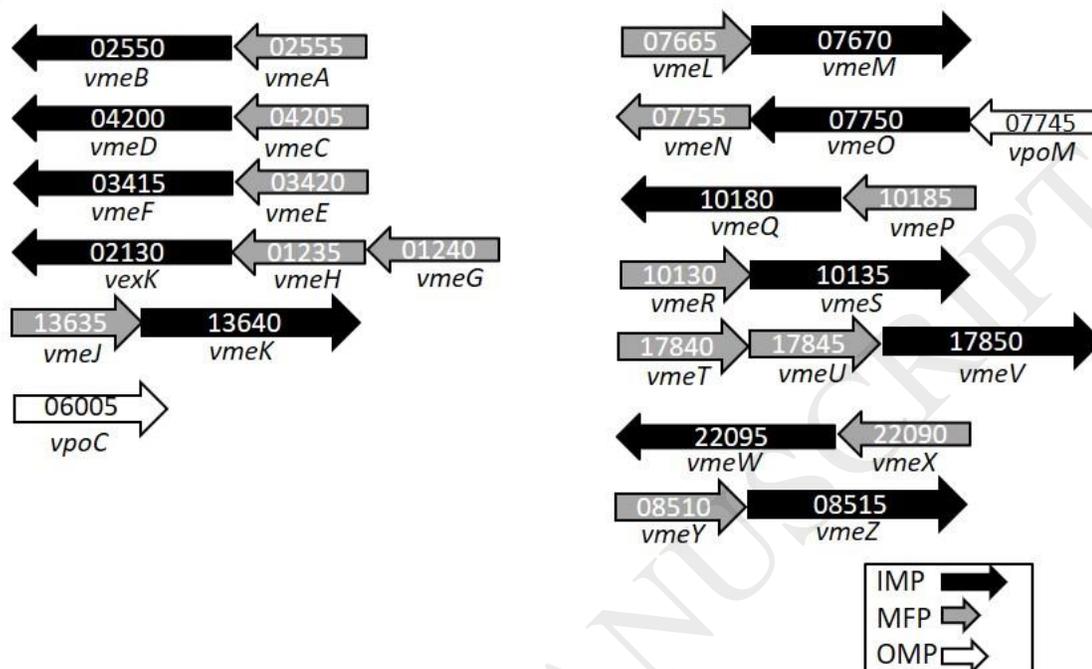
34. Cerda-Maira FA, Ringelberg CS and Taylor RK. *The bile response repressor BreR regulates expression of the Vibrio cholerae breAB efflux system operon*. J. Bacteriol. 2008. 190; 22:7441-52.
35. Honkanen RE and Patton JS. *Bile salt absorption in killifish intestine*. Am. J. Physiol. Gastrointest. Liver Physiol. 1987. 253; 6:G730-G736.
36. Kinana AD, Vargiu AV and Nikaido H. *Some ligands enhance the efflux of other ligands by the Escherichia coli multidrug pump AcrB*. Biochemistry. 2013. 52; 46:8342-8351.
37. Bier N, Schwartz K, Guerra B and Strauch E. *Survey on antimicrobial resistance patterns in Vibrio vulnificus and Vibrio cholerae non-O1/non-O139 in Germany reveals carbapenemase-producing Vibrio cholerae in coastal waters*. Front. Microbiol. 2015. 6; 1179:1-11.
38. Zeng Q, Tian X and Wang L. *Genetic adaptation of microbial populations present in high-intensity catfish production systems with therapeutic oxytetracycline treatment*. Sci. Rep. 2017. 7:1-13.
39. Sakhtah H, Koyama L, Zhang Y, Morales DK, Fields BL, Price-Whelan A, Hogan DA, et al. *The Pseudomonas aeruginosa efflux pump MexGHI-OpmD transports a natural phenazine that controls gene expression and biofilm development*. Proc. Natl. Acad. Sci. 2016. 113; 25:E3538-E3547.

40. Bina XR, Howard MF, Taylor-Mulneix DL, Ante VM, Kunkle DE and Bina JE. *The Vibrio cholerae RND efflux systems impact virulence factor production and adaptive responses via periplasmic sensor proteins*. PLoS Pathog. 2018. 14; 1:e1006804.

## Figure captions

**Figure 1 a-b** – Operon structure and gene loci of RND efflux pumps gene homologs identified in *Vibrio* sp. T21 (a) and *Vibrio* sp. T9 (b). Genes designations are based on the protein with the highest % identity compared to reference proteins in *V. parahaemolyticus* (*vme*) or *V. cholerae* (*vex*). The additional operon identified in *Vibrio* sp. T21 not found in *V. parahaemolyticus* RIMD 2210633 is enclosed in the black box.



***Vibrio* sp. T9**

(b)

## Tables

**Table 1** – Taxonomic determination of *Vibrio* strains described in this study

	<b><i>Vibrio</i> sp. T9</b>	<b><i>Vibrio</i> sp. T21</b>
Most closely related organism and accession number	<i>Vibrio antiquarius</i> EX25 (NC_013456, NC_013457)	<i>Vibrio parahaemolyticus</i> FORC 018 (NZ_CP013826, NZ_CP013827)
ANI (%)	98.3	98.2
MLSA (%)	94.5	Not tested
Probability of being a human pathogen (%) <sup>1</sup>	67.7	71.9

<sup>1</sup> as Determined by Pathogen Finder (23).

**Table 2** – Effect of efflux pump inhibition (50  $\mu\text{g/mL}$  PA $\beta$ N) on cephalosporin and aminoglycoside resistance in *Vibrio* spp. T9 and T21.

		Cephalosporins										Aminoglycoside	
		First-generation				Third-generation						Kanamycin	
		Cefazolin		Cephra dine		Cefopera zone		Cefsulo din		Ceftazid ime			
<i>Vibrio</i> sp. T9	PA $\beta$ N *	-	+	-	+	-	+	-	+	-	+	-	+
	MIC ( $\mu\text{g/mL}$ )	0.5	<b>4 (8)</b> <sup>‡</sup>	8	8	64	<b>1 (64)</b>	64	<b>16 (4)</b>	64	64	1	<b>0.25 (4)</b>
<i>Vibrio</i> sp. T21	PA $\beta$ N	-	+	-	+	-	+	-	+	-	+	-	+
	MIC ( $\mu\text{g/mL}$ )	4	<b>1 (4)</b>	64	<b>2 (32)</b>	64	<b>0.5 (128)</b>	64	64	64	64	8	<b>1 (8)</b>

\* Presence (+) or absence (-) of 50  $\mu\text{g/mL}$  PA $\beta$ N

<sup>‡</sup> Fold changes in MIC are reported in bold and parentheses

**Table 3a** – Homology of RND efflux pump MFP (grey), IMP (black), and OMP (white) proteins from *Vibrio* sp. T9 compared to reference proteins from *V. parahaemolyticus*<sup>1</sup>, *E. coli*<sup>2</sup>, *P. aeruginosa*<sup>3</sup>, and *V. cholerae*<sup>4</sup>.

Gene Locus in <i>Vibrio</i> sp. T9*	Percent Amino Acid Identity Against:							Designation†
	Vme A-Z <sup>1</sup>	Acr A or B <sup>2</sup>	Mex A or B <sup>3</sup>	Vex A or B <sup>4</sup>	Vex C or D <sup>4</sup>	Acr D <sup>2</sup>	Vex K <sup>4</sup>	
CBX98_02 555	88	46	45	22	23			VmeA
CBX98_02 550	93	63	60	28	20	59		VmeB
CBX98_04 205	95	22	21	86	24			VmeC
CBX98_04 200	98	30	31	88	21	30		VmeD
CBX98_03 420	93	21	21	23	23			VmeE
CBX98_03 415	98	29	29	34	21	29		VmeF
CBX98_02 140	87	21	25	17	26			VmeG
CBX98_02 135	86	19	20	18	26			VmeH
CBX98_02 130	low %	23	23	22	16	22	85	VexK
CBX98_13 635	85	18	21	19	18			VmeJ
CBX98_13 640	95	21	21	20	21	23		VmeK
CBX98_07 665	94	18	18	16	15			VmeL
CBX98_07 670	95	18	19	18	17	19		VmeM
CBX98_07 755	99	17	16	18	21			VmeN

CBX98_07 750	99	18	20	21	35	18	VmeO
CBX98_07 745	98	nt	nt	nt	nt		VpoM
CBX98_10 185	100	30	30	21	20		VmeP
CBX98_10 180	99	39	38	31	20	36	VmeQ
CBX98_10 130	93	11	12	13	13		VmeR
CBX98_10 135	95	20	20	23	21	20	VmeS
CBX98_17 840	85	19	20	20	28		VmeT
CBX98_17 845	88	21	21	20	26		VmeU
CBX98_17 850	94	21	22	21	40	20	VmeV
CBX98_22 095	99	20	21	19	20	21	VmeW
CBX98_22 090	99	20	16	17	23		VmeX
CBX98_08 510	93	31	27	22	23		VmeY
CBX98_08 515	97	39	40	31	20	36	VmeZ
CBX98_06 005	91	‡nt	nt	nt	nt		VpoC

\* Genes are listed according to their operonic affiliation and background coloring corresponds to those used in Figure 1.

† Names were designated based on reference protein with highest % identity.

‡nt = not tested

**Table 3b** – Homology of RND efflux pump MFP (grey), IMP (black), and OMP (white) proteins from *Vibrio* sp. T21 compared to reference proteins from *V. parahaemolyticus*<sup>1</sup>, *E. coli*<sup>2</sup>, *P. aeruginosa*<sup>3</sup>, and *V. cholerae*<sup>4</sup>.

Gene Locus in <i>Vibrio</i> sp. T21*	Percent Amino Acid Identity Against:						Designation†
	VmeA-Z <sup>1</sup> ,	AcrA or AcrB <sup>2</sup>	MexA or MexB <sup>3</sup>	VexA or VexB <sup>4</sup>	VexC or VexD <sup>4</sup>	AcrD <sup>2</sup>	
CCD93_01330	100	46	41	23	24		VmeA
CCD93_01325	100	63	61	29	20	58	VmeB
CCD93_07845	94	23	19	79	22		VmeC
CCD93_07850	97	30	30	88	20	29	VmeD
CCD93_01965	99	20	19	24	23		VmeE
CCD93_01960	100	28	29	35	21	29	VmeF
CCD93_00910	100	20	23	20	28		VmeG
CCD93_00905	99	17	19	18	27		VmeH
CCD93_00900	100	20	19	21	43	20	VmeI
CCD93_14985	99	18	20	19	18		VmeJ
CCD93_14980	100	21	20	20	21	22	VmeK
CCD93_04360	100	17	17	17	16		VmeL
CCD93_04355	100	18	19	18	17	18	VmeM
CCD93_04250	99	16	16	18	20		VmeN
CCD93_04255	100	18	20	20	36	18	VmeO

CCD93_042 60	98	nt	nt	nt	nt		VpoM
CCD93_086 05	100	31	30	21	21		VmeP
CCD93_086 10	100	39	38	31	20	36	VmeQ
CCD93_086 60	100	12	12	13	11		VmeR
CCD93_086 65	100	21	19	21	22	19	VmeS
CCD93_162 05	99	20	19	21	27		VmeT
CCD93_162 00	99	19	17	15	25		VmeU
CCD93_161 95	100	20	20	20	41	20	VmeV
CCD93_155 60	100	20	21	20	20	21	VmeW
CCD93_155 65	99	19	17	18	23		VmeX
CCD93_035 80	100	31	26	22	25		VmeY
CCD93_035 75	99	39	40	31	20	37	VmeZ
CCD93_229 85	low %	20	19	20	70		VexC
CCD93_229 90	low %	21	21	20	86	21	VexD
CCD93_060 35	92	‡nt	nt	nt	nt		VpoC

\* Genes are listed according to their operonic affiliation and background coloring corresponds to those used in Figure 1.

†Names were designated based on reference protein with highest % identity.

‡nt = not tested

**Table 4** – RND genes identified in *Vibrio* sp. T21, not present in *V. parahaemolyticus* RIMD 2210633

<b>Gene Locus in <i>Vibrio</i> sp. T21</b>	<b>Role in RND efflux pump</b>	<b>Length (nt)</b>	<b>%GC</b>	<b><i>V. cholerae</i> homolog (NC_002505)</b>	<b>% Identity (AA) by pairwise alignment</b>
CCD93_22985	MFP	1038	44	VexC (VC1757)	70
CCD93_22990	IMP	3045	47.6	VexD (VC1756)	86