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

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ABSTRACT

Objectives: This study examined the role of resistance-nodulation-cell division (RND) efflux pumps in resistance to first-generation and third-generation cephalosporins, and the potential contribution to increased virulence in two *Vibrio* isolates from the gut microbiota of a forage-feeder fish.
Methods: Phenotypic MIC testing was performed in the presence and absence of an RND efflux pump inhibitor, phenylalanine-arginine-beta-napthylamide (PAβN). Genomes of the two *Vibrio* spp. were compared to characterise RND efflux pump gene homologs.
Results: The study identified 13 and 12 RND operons, respectively, in *Vibrio* spp. T21 and T9, with *Vibrio* sp. T21 containing an additional RND operon compared with other *V. parahaemolyticus* strains. Both the inner-membrane protein (IMP) and the membrane facilitator protein (MFP) sequences of this operon were homologous to VexD and VexC, respectively, which is an RND operon in *Vibrio cholerae*. More generally, the other RND proteins in these strains showed homology to RND efflux pumps characterised in *Escherichia coli* and *Vibrio cholerae*. Decreased resistance to cefoperazone and cephradine was observed in *Vibrio* sp. T21, and to cefoperazone and cefsulodin in *Vibrio* sp. *T9* in the presence of PaβN. The RND pumps may also mediate transport of kanamycin.
Conclusions: By analysing the genomes of two *Vibrio* spp. isolated from the mummichog fish gut, RND efflux pump-mediated resistance to first-generation and third-generation cephalosporins was discovered in these strains. This work highlights the need for further research into this unique *Vibrio* spp. operon and, more generally, RND efflux pumps in *Vibrio* spp., as *Vibrio* spp. often cause seafood-borne illness.

1. Introduction

Vibrios — commonly associated with marine animals including fish, shellfish, and shrimp — are ubiquitous in aquatic environments. Some *Vibrio* spp. cause disease in humans, including *Vibrio parahaemolyticus* (*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 vibriosis [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 1990–2010 [2]. Cephalosporin antibiotics in combination with doxycycline are currently recommended for treatment of vibriosis [3], despite reports of increased cephalosporin resistance [4].

With approximately 90% of global aquaculture production being 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 excessively used 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.

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Efflux pumps are distributed amongst all domains of life and their genetic organisation and structural features are well-conserved [7]. The resistance-nodulation-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]. AcrAB is one of the most well-studied RND systems — characterised in *Escherichia coli* (*E. coli*) and broadly distributed among Enterobacteriaceae — that exports dyes, detergents, several antibiotics, and organic solvents [8]. In addition, RND efflux pumps are involved in cell-to-cell communication, host colonisation via the export of bile salts [8], and virulence [9]. Such efflux pumps mediate resistance to β-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 β-lactamases) [11]. The role of efflux pumps in *Vibrio* spp. is important to understand, as efflux pumps contribute to AMR, virulence, and host colonisation.

Two *Vibrio* strains (closely related to *V. parahaemolyticus* and *V. antiquarius*) isolated from the gut of the mummichog fish (*Fundulus heteroclitus*) were recently described [12], which is 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 β-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 *Vibrio antiquarius*, it is genetically related to other pathogenic *Vibrio* spp. [14]. With global increases in resistance to cephalosporins [4], it is important to understand the mechanisms of AMR in *Vibrio* spp. This study aimed to examine the roles of RND efflux pumps in resistance to first-generation and third-generation cephalosporins, and 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 multilocus sequence analysis (MLSA), which was performed on partial 16S rRNA, *gyrB, pyrH, recA,* and *rpoD* genes from *Vibrio* sp. T9 and compared with 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), which is an automated microbiology growth curve analysis system. Pre-cultures were grown on tryptic soy agar media overnight at 28 °C. Colonies were suspended in tryptic soy broth media to a McFarland standard of ca. 1.5 measured using the DensiCheck system. Ca. 4.5×10^6 CFU/mL were inoculated into Bioscreen C honeycomb plates containing serial dilutions of PAβN (0–100 μg/mL) in DMSO. Growth rates (μ) 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 visualised in Microsoft Excel (Fig. S1).

2.3. Liquid MICs in presence of phenylalanine-arginine-beta-napthylamide

Minimum inhibitory concentrations of various cephalosporin antibiotics and kanamycin were determined using the established broth microdilution method, according Clinical Laboratory Standards Institute (CLSI) guidelines, using microtiter plates [19] in the presence or absence of the efflux pump inhibitor phenylalanine-arginine-beta-napthylamide (PaβN). This concentration of PAβN was experimentally determined to not affect organism growth (Fig. 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 μg/mL PAβN. The plates were incubated for 24 h at 28 °C before interpretation. MIC changes of four-fold or greater were considered significant [20].

2.4. Identification of putative RND efflux genes in the genomes of Vibrio *spp. T9 and T21*

The RND efflux gene sequences characterised 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] the current *Vibrio* spp. genome sequences for homologs. There was further manual searching upstream and downstream of each RND gene hit to locate additional RND gene homologs. To determine relatedness of the putative RND proteins in the current *Vibrio* spp., they were aligned 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 *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 (NC_002516); and *vexA*, *vexB*, *vexC*, *vexD*, and *vexK* from *Vibrio 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, which was 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 discoïdeum* (*D. discoïdeum*). Overnight bacterial cultures were adjusted to an OD_{600nm} of 1.5 by dilution in LB. Then, 1 mL of each adjusted bacterial suspension was plated on SM Agar (Formedium, Hustanton, United Kingdom) medium. The plates were allowed to dry for 1 h to obtain a dry bacterial layer. Cells of *D. discoïdeum* were washed twice in Page's amoeba saline buffer by centrifugation at $1000 \times g$ for 10 min. The amoebal suspension was adjusted to 2×10^6 cells/mL and serial dilutions were performed in order to obtain a final concentration of 7812 cells/mL (in the most dilute sample). Finally, 5 μL of each serial dilution of *D. discoïdeum* (containing 39–14 000 cells/5 μL aliquot) were spotted on the bacterial lawn. Plates were incubated at 22.5 °C for 5 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 *Klebsiella pneumoniae* (*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 (<400 amoebae cells were sufficient for lysis plaque formation), low-virulent (400–2500 amoebae for lysis plaque formation) and virulent (>2500 amoebae).

3. Results

3.1. Taxonomic information

Vibrio sp. T9 was determined to be closely related to *Vibrio 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 being closely related to *V. parahaemolyticus* FORC 018 with an ANI value of 98.2% [12] (Table 1).

3.2. Effect of PAβN on antibiotic resistance profiles

To assess the possible contribution of RND efflux pumps to drug resistance, the MIC of five different cephalosporins was tested in the presence and absence of 50 μg/mL of the efflux pump inhibitor PAβN. 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 also showed a 32-fold decrease in MIC to cephradine, while *Vibrio* sp. T9 showed a four-fold decrease in MIC to cefsulodin. Both strains had no change in MIC to ceftazidime, which is a third-generation drug used in treatments against Pseudomonas and Enterobacteriaceae. Cefazolin, a very hydrophobic molecule shown not to enter the cell in *E. coli* [26], resulted in a slight (four-fold) decrease in the MIC of *Vibrio* sp. T21 and an eight-fold increase in *Vibrio* sp. T9 (Table 2). Strain T21 exhibited an eight-fold reduced sensitivity to kanamycin, an aminoglycoside, with MIC decreasing from 8 to 1 μg/mL. The corresponding decrease in strain T9 was four-fold from 1 to 0.25 μg/mL kanamycin (Table 2).

3.3. Inventory of putative RND efflux gene homologs

This study identified 12 putative RND operons in *Vibrio* sp. T9 (Fig. 1b), which was 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 with the proteins described by Matsuo et al. [21] were between 85–100% (Table 3a), 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 3a).

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

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, the efflux pump proteins were compared with pumps characterised in reference organisms. Both *Vibrio* spp. contained proteins orthologous to the AcrAB and MexAB efflux pumps from *E. coli* and *P. aeruginosa* (Tables 3a and 3b), two systems known to affect cephalosporin resistance [10]. Both *Vibrio* spp. also harboured 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] (Tables 3a and 3b).

3.4. Virulence

Based on the genome sequences, Pathogen Finder [23] predicted 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 <400 amoebae were needed to form lysis plaques for each strain.

4. Discussion

It was hypothesised that RND efflux pumps contribute to phenotypic resistance to multiple classes of antibiotics in two *Vibrio* spp. isolates. This study reported 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 both are 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. These findings suggest that resistance to first-generation 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 characterised in *V. parahaemolyticus* RIMD 2210633. Furthermore, *Vibrio* sp. T21 harboured 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-fold to 128-fold) in MIC to cefoperazone (Table 2), a

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

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

Abbreviations: ANI, average nucleotide identity; MLSA, multilocus sequence analysis.
[a] As determined by Pathogen Finder [23].

Table 2
Effect of efflux pump inhibition (50 ug/mL PAβN) on cephalosporin and aminoglycoside resistance in *Vibrio* spp. T9 and T21.

		Cephalosporins										Aminoglycoside	
		First-generation				Third-generation							
		Cefazolin		Cephradine		Cefoperazone		Cefsulodin		Ceftazidime		Kanamycin	
Vibrio sp. T9	PaβN[a]	–	+	–	+	–	+	–	+	–	+	–	+
	MIC (μg/mL)	0.5	4 (**8**)[b]	8	8	64	1 (**64**)	64	16 (**4**)	64	64	1	0.25 (**4**)
Vibrio sp. T21	PAβN	–	+	–	+	–	+	–	+	–	+	–	+
	MIC (μg/mL)	4	1 (**4**)	64	2 (**32**)	64	0.5 (**128**)	64	64	64	64	8	1 (**8**)

[a]Presence (+) or absence (–) of 50 μg/mL phenylalanine-arginine-beta-napthylamide (PaβN).
[b]Fold changes in MIC are reported in bold and parentheses.

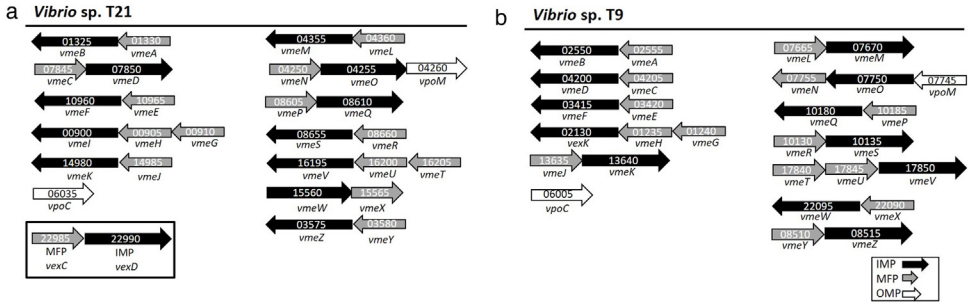

Fig. 1. (a and b) Operon structure and gene loci of RND efflux pumps gene homologs identified in *Vibrio* spp. T21 (a) and T9 (b). Genes designations are based on the protein with the highest % identity compared with reference proteins in *Vibrio parahaemolyticus* (*vme*) or *Vibrio cholerae* (*vex*).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

third-generation cephalosporin, when tested with PAβN. Resistance to cephalosporins via the AcrAB RND system in *E. coli* has been well characterised [28]. Both *Vibrio* spp. T9 and T21 harboured VmeB protein homologs, which were 63% identical to *E. coli* AcrB (Tables 3a and 3b); others have characterised VmeAB as an orthologue of AcrAB in *E. coli* [29], providing support for the hypothesis of RND involvement in cephalosporin resistance. Third-generation cephalosporins, to which resistance is emerging globally [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, this study reports 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 other reports [21]. *Vibrio sp.* T9 contained 12 potential RND operons, and almost all of those proteins showed homology (≥85% identity) with *V. parahaemolyticus* RIMD 2210633 pumps (Tables 3a and 3b). 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 is potentially required for pathogenesis in *V. cholerae* [31], suggesting that *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 with *V. parahaemolyticus* RIMD 2210633 (Fig. 1a). Notably, *Vibrio* sp. T9 did not contain a homolog to *V. cholerae* VexD (Table 3a).

In *Vibrio* sp. T21, PAβN decreased the MIC of cephradine (a first-generation cephalosporin) by 32-fold, while it had no effect on sensitivity in *Vibrio* sp. T9 (Table 2). To explain this discrepancy, it was speculated 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 cephradine. 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 colonisation [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 current *Vibrio* spp. (Tables 3a and 3b), are also involved exporting 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 colonisation 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 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, it was

concluded that the additional RND operon (Table 4) may help *Vibrio* sp. T21 survive in its niche of the fish gut, which is 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, no change was seen in MIC upon addition of PAβN 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 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 β-lactamases encoded by the genomes) [12].

On PAβN addition, *Vibrio* spp. T9 and T21 exhibited contrasting resistance phenotypes to cefazolin, with an eight-fold increase in MIC in *Vibrio* sp. T9 and a four-fold decrease in *Vibrio* sp. T21. It was noted that both strains were cefazolin sensitive [19] with a maximum MIC of 4 μg/mL. Others have shown that PAβN reduces resistance to cephalosporins in *E. coli*, which is speculated to be due to interactions between PAβN 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 is likely not affected by RND efflux pumps in these *Vibrio* strains.

Aminoglycosides are an important class of antibiotics known to be counteracted by RND efflux pumps such as AcrD in *E. coli* [28]. In the current *Vibrio* spp. T21 and T9, eight-fold and four-fold decreases, respectively, were observed 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) (Tables 3a and 3b), 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]. The current 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. This study reported on the potential involvement of such pumps in cephalosporin resistance in two *Vibrio* strains. It showed that each *Vibrio* harbours 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

Table 3a
Homology of RND efflux pump MFP (grey), IMP (black), and OMP (white) proteins from *Vibrio* sp. T9 compared with reference proteins from *Vibrio parahaemolyticus*[1], *Escherichia coli*[2], *Pseudomonas aeruginosa*[3], and *Vibrio cholerae*[4].

	Percent amino acid identity against:							Designation†
Gene locus in *Vibrio* sp. T9*	VmeA-Z[1]	AcrA or AcrB[2]	MexA or MexB[3]	VexA or VexB[4]	VexC or VexD[4]	AcrD[2]	VexK[4]	
CBX98_02555	88	46	45	22	23			VmeA
CBX98_02550	93	63	60	28	20	59		VmeB
CBX98_04205	95	22	21	86	24			VmeC
CBX98_04200	98	30	31	88	21	30		VmeD
CBX98_03420	93	21	21	23	23			VmeE
CBX98_03415	98	29	29	34	21	29		VmeF
CBX98_02140	87	21	25	17	26			VmeG
CBX98_02135	86	19	20	18	26			VmeH
CBX98_02130	low %	23	23	22	16	22	85	VexK
CBX98_13635	85	18	21	19	18			VmeJ
CBX98_13640	95	21	21	20	21	23		VmeK
CBX98_07665	94	18	18	16	15			VmeL
CBX98_07670	95	18	19	18	17	19		VmeM
CBX98_07755	99	17	16	18	21			VmeN
CBX98_07750	99	18	20	21	35	18		VmeO
CBX98_07745	98	nt	nt	nt	nt			VpoM
CBX98_10185	100	30	30	21	20			VmeP
CBX98_10180	99	39	38	31	20	36		VmeQ
CBX98_10130	93	11	12	13	13			VmeR
CBX98_10135	95	20	20	23	21	20		VmeS
CBX98_17840	85	19	20	20	28			VmeT
CBX98_17845	88	21	21	20	26			VmeU
CBX98_17850	94	21	22	21	40	20		VmeV
CBX98_22095	99	20	21	19	20	21		VmeW
CBX98_22090	99	20	16	17	23			VmeX
CBX98_08510	93	31	27	22	23			VmeY
CBX98_08515	97	39	40	31	20	36		VmeZ
CBX98_06005	91	‡nt	nt	nt	nt			VpoC

*Genes are listed according to their operonic affiliation and background colouring corresponds to those used in Fig. 1.
†Names were designated based on reference protein with highest % identity.
‡nt, not tested.
Abbreviations: IMP, inner-membrane protein; MFP, membrane facilitator protein; OMP, outer membrane protein.

Table 3b
Homology of RND efflux pump MFP (grey), IMP (black), and OMP (white) proteins from *Vibrio* sp. T21 compared with reference proteins from *Vibrio parahaemolyticus*[1], *Escherichia coli*[2], *Pseudomonas aeruginosa*[3], and *Vibrio cholerae*[4].

Gene locus in *Vibrio* sp. T21*	Percent amino acid identity against: VmeA-Z[1],	AcrA or AcrB[2]	MexA or MexB[3]	VexA or VexB[4]	VexC or VexD[4]	AcrD[2]	Designation†
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_04260	98	nt	nt	nt	nt		VpoM
CCD93_08605	100	31	30	21	21		VmeP
CCD93_08610	100	39	38	31	20	36	VmeQ
CCD93_08660	100	12	12	13	11		VmeR
CCD93_08665	100	21	19	21	22	19	VmeS
CCD93_16205	99	20	19	21	27		VmeT
CCD93_16200	99	19	17	15	25		VmeU
CCD93_16195	100	20	20	20	41	20	VmeV
CCD93_15560	100	20	21	20	20	21	VmeW
CCD93_15565	99	19	17	18	23		VmeX
CCD93_03580	100	31	26	22	25		VmeY
CCD93_03575	99	39	40	31	20	37	VmeZ
CCD93_22985	low %	20	19	20	70		VexC
CCD93_22990	low %	21	21	20	86	21	VexD
CCD93_06035	92	‡nt	nt	nt	nt		VpoC

*Genes are listed according to their operonic affiliation and background colouring corresponds to those used in Fig. 1.
†Names were designated based on reference protein with highest % identity.
‡nt, not tested.
Abbreviations: IMP, inner-membrane protein; MFP, membrane facilitator protein; OMP, outer membrane protein.

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

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

Abbreviations: nt, not tested.

efflux pumps led to increased bile and antibiotic resistance, as suggested by the genome analyses. If these MDR strains survive in the fish gut, they are likely able to also live 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 the 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. It is suggested that the microbial ecology of efflux pumps is a much-needed area of 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.

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Competing interests

None.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.05.006.

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