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ORIGINAL PAPER

IS*CR2* is associated with the dissemination of multiple resistance genes among *Vibrio* spp. and *Pseudoalteromonas* spp. isolated from farmed fish

Yixiang Xu[1,2] · Chao Wang[1] · Gang Zhang[1] · Jingjing Tian[1] · Ying Liu[3] · Xihui Shen[2] · Jie Feng[1]

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Abstract 58 multiresistant strains representing diverse genera were isolated from farmed fish in an aquaculture facility. Resistant rates of strains harboring IS*CR*2, an insertion sequence type element, were higher than those in which this element was absent. Full genome sequencing of a *Vibrio* isolate containing IS*CR*2 confirmed that it is associated with multiple resistance genes, many of which are of clinical relevance. We describe the structural variation within IS*CR*2, and its distribution throughout multiple diverse aquatic genera, including *Vibrio, Shewenalla, Pseudoalteromonas* and *Psychrobacter*, suggesting the potential role of IS*CR*2 in disseminating antibiotic resistance. We also observe, and experimentally verify, a novel macrolide resistance gene that is also associated with IS*CR*2.

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Y. Xu and C. Wang contributed equally to this work.

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✉ Xihui Shen
xihuishen@nwsuaf.edu.cn

✉ Jie Feng
fengj@im.ac.cn

[1] State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

[2] College of Life Sciences, Northwest A&F University, Yangling 712100, Shaanxi, China

[3] Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China

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Introduction

The discovery of antimicrobial agents was one of the great triumphs of the twentieth century. However, we have moved from the age of antibiotics to the age of antibiotic resistance. The widespread dissemination of antibiotic resistance genes (ARGs) is a serious challenge in clinical settings (Arias and Murray 2009). More worrisome is the increased occurrence of multidrug-resistant pathogens harbouring multiple resistance genes (sometimes dozens). Horizontal gene transfer (HGT) has been responsible for the development of resistance, particularly multidrug-resistance in pathogens (Dzidic and Bedeković 2003). Mobile genetic elements, including plasmids, transposons, integrons, and integrative conjugative elements, are vehicles for HGT. Recently a novel group of mobile elements gained attention for their links to multiple resistance genes. These are IS*CR* (insertion sequence common region) elements.

IS*CR* elements accommodate a putative transposase that contains the signature motifs, particularly tyrosine 253 of IS91 transposase, involved in rolling circle replication (RC). IS*CR* elements also contain a consensus sequence that is strikingly similar to the *ori*IS consensus sequence of IS91-family elements, and the IS*CR* group is proposed as a subgroup of IS91-like elements (Toleman et al. 2006). IS*CR* elements are classified based on transposase phylogeny, and IS*CR*1 and IS*CR*2 are the most prevalent elements. IS*CR*1 is characterised by its location; it is almost always found in complex class 1 integrons beside the same truncated version of 3′-CS, which was found to be able to self-excise to become

a free form (Li et al. 2014). IS*CR*2 transposase shares 65% amino acid identity with IS*CR*1 and has never been found associated with class 1 integrons. The element was found in SXT of *Vibrio cholerae*, which is an integrating conjugative element, and has also been found on numerous plasmids (Toleman and Walsh 2008). IS*CR*2 is frequently adjacent to a truncated phosphoglucosamine mutase gene (Δ*glmM*) followed by *sul2*, for example, on plasmid pRVS1 of *V. salmonicida*, on a plasmid of *Salmonella enterica*, and on the chromosome of *Shigella flexneri* (Toleman and Walsh 2008). More interestingly, multiple ARGs have been found between IS*CR*2 and *sul2*. IS*CR*2 has been found adjacent to *floR, tetA, tetR, strB, strA*, and *sul2* in plasmids of *Escherichia coli* isolated from multiple animal sources (Fernández-Alarcón et al. 2011). The genes *tetR* and *tetY* replace *tetA* and *tetR* in a plasmid of *Aeromonas bestiarum* isolated from freshwater (Gordon et al. 2008). The genes *mphG* and *mefC* were located between IS*CR*2 and *sul2* in a plasmid of *Photobacterium damselae* isolated from a marine aquaculture environment (Nonaka et al. 2012). These ARGs confer resistance to sulfonamides, chloramphenicol, tetracycline, aminoglycosides, and erythromycin, which are used more frequently as veterinary and aquaculture medicines than as human medicines.

Oxytetracycline, florfenicol, sarafloxacin, erythromycin, and tribrissen, a combination of sulfonamide and trimethoprim, are antibiotics authorised for use in aquaculture (Kümmerer 2009). Tetracycline and sulfonamide resistance genes have been identified in fish farms throughout the world (Akinbowale et al. 2007; Shah et al. 2014; Xiong et al. 2015). The florfenicol resistance gene *floR* has been found in diverse bacteria, such as *Enterobacter aerogenes, Kluyvera ascorbata, Citrobacter freundii*, and *Pseudomonas alcaligenes* isolated from Chilean salmon farms (Fernández-Alarcón et al. 2010). Tetracycline, sulfonamide, and florfenicol resistance genes are associated with IS*CR*2. However, the role of IS*CR*2 in the dissemination of ARGs in fish farms is still unclear.

In this study, we examined multidrug resistant strains isolated from fish that feed in recirculating aquaculture systems (RASs), in which water flows from a fish tank through a treatment process and is then returned to the tank, and fish can be grown at high density under controlled environmental conditions (Hambly et al. 2015). Our results demonstrated that IS*CR*2 is associated with tetracycline, sulfonamide, florfenicol, aminoglycosides, and erythromycin resistance genes among *Vibrio* spp., *Pseudoalteromonas* spp., and other aquatic bacteria, suggesting that IS*CR*2 plays an important role in the dissemination of ARGs in fish farms.

Materials and methods

Bacterial strains, growth conditions, and MIC determinations

Bacteria were isolated from the necrotic skin, liver, and spleen of infected yellow groupers ($n=20$) from RASs located in Tianjin, northern China, in September and November 2013. The strains were cultured on modified 2216E agar (1 L seawater, 5.0 g fish peptone, 1.0 g yeast extract, 0.1 g iron phosphate, and 5.0 g agar, pH 7.6–7.8) at 25 °C. *E. coli* K-12 JW0451-2, a derivative of *E. coli* K-12 that is susceptible to erythromycin due to inactivation of the *acrB* pump (Baba et al. 2006), was used as a host for constructed plasmids. The strain was grown on Luria–Bertani agar (Oxoid, Wesel, Germany) plates at 37 °C. The minimum inhibitory concentrations (MICs) for strains were determined using broth microdilution assays, as recommended by the Clinical and Laboratory Standards Institute (CLSI; http://www.clsi.org/). Primers 27F and 1492R (Weisburg et al. 1991) were used for bacterial 16S rRNA gene amplification and sequencing for phylogenetic analysis of isolates.

Genome sequencing and analysis

The genome of *Vibrio* sp. L-85 was sequenced on an Illumina HiSeq 2000. Genomic DNA was used to construct the sequencing libraries according to the manufacturer's recommended protocols (Illumina), and paired-end shotgun sequencing was performed. After the shotgun stage, 1.92 gigabytes of raw data were obtained. Low quality reads and adapters were filtered using the Trim Galore software. The Velvet program was used to assemble genomes with an optimized k-mer of 49. A total of 514 contigs were obtained. The Glimmer software was used to predict open reading frames (ORFs) of the assembled genome with default parameters. The annotation of ORFs was based on BLAST searches against a number of bioinformatics databases, including Uniprot (http://www.uniprot.org/), KEGG (http://www.genome.jp/kegg/genes.html), Swissprot (http://www.expasy.ch/sprot/), and Refseq (http://www.ncbi.nlm.nih.gov/refseq/). In addition, the InterProScan software (http://www.ebi.ac.uk/Tools/InterProScan/) was used to search its domain architectures.

The contigs assembled by Velvet were subjected to a BLAST search against the non-redundant nucleotide database of NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/) using the BLASTn software (E value cutoff of 10^{-10}). In addition, BLASTP analysis with cutoff values of 10^{-5} (E value) and 30% similarity for orthologues was performed against the Antibiotic Resistance Genes Database (ARDB; http://ardb.cbcb.umd.edu/index.html).

Construction of recombinant plasmid carrying the putative macrolide resistance gene

To confirm the antibiotic resistance effectiveness of the ORF that shared similarities with reported resistance genes, PCR product was cloned into the multiple cloning sites of the pUC18 vector. The designed primers are listed in Table S.1 in the supplemental material. *E. coli* K-12 JW0451-2 cells were transformed with these recombinant plasmids, and clones were selected by blue-white screening on Luria–Bertani agar plates supplemented with 100 mg L^{-1} ampicillin. Transformants were confirmed to contain inserted fragments by PCR. Resistance of the transformants to erythromycin, clarithromycin, azithromycin, spiramycin, and midecamycin was determined using the microdilution method with induction by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), as recommended by CLSI (http://www.clsi.org/).

Resistance genes and IS*CR2* gene detection

The presence of the transposase gene of IS*CR2* in bacterial isolates was determined by PCR using the primers listed in Table S.1. PCR amplification was performed in 50-μL reaction volumes with the following program: 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The arrangements of IS*CR2* and *sul2* were amplified with the primers IS*CR2*F and *sul2*R, listed in Table S.1, with a longer extension time of 3–11 min.

Accession numbers

Genomic sequences of the *Vibrio* sp. L-85 in this study were deposited in GenBank under accession numbers PRJNA305262. Genomic sequences of six sequences arrangement types of IS*CR2* plus ARGs in this study were deposited in GenBank under accession numbers KU306390 to KU306395.

Results and discussion

IS*CR2* is distributed widely among multiresistant strains isolated from fish

A total of 58 strains were isolated from the necrotic skin, liver, and spleen of diseased yellow grouper. Amplification and sequencing of 16S rRNA genes from the isolates were performed. All sequences showed high similarities to known species and all strains were identified to at least the genus level. The most abundant bacterial group was *Pseudoalteromonas* spp. isolates (27 strains), followed by *Vibrio* spp. isolates (20 strains). Other isolates belonged to the genera *Halomonas, Marinobacter, Psychrobacter, Shewanella*, and *Sulfitobacter* (Table S.2). The genus *Pseudoalteromonas* and *Vibrio* are ubiquitous in the marine environment. Moreover, some species of genus *Vibrio* (V. alginolyticus and *V. harveyi)* are important opportunistic pathogens of marine organisms and have been reported as rare opportunistic human pathogens (Thompson et al. 2004). The resistance rates of the 58 isolates to the tested antibiotics were as follows: tetracycline, 94.8%; florfenicol, 82.8%; sulfamethoxazole, 81.0%; trimethoprim, 72.4%; enrofloxacin, 69%, streptomycin, 94.8%; erythromycin, 74.1% (Table 1). All resistant isolates were screened for the transposase gene of IS*CR2* and the gene was identified in 31 strains (53.4%). The resistance rates of isolates carrying IS*CR2* were much higher than those of isolates without IS*CR2*, suggesting that IS*CR2* plays a role in the dissemination ARGs in fish farms.

IS*CR2* is associated with sulfamethoxazole, florfenicol, tetracycline, and streptomycin resistance genes present in *Vibrio sp*. strain L-85

To determine the ARGs associated with IS*CR2*, we sequenced the genome of a *Vibrio* sp. We isolated strain L-85 harbouring IS*CR2*. Amplification and sequencing of 16 S ribosomal DNA from the isolate indicated that it shared 99.0% identity with *Vibrio harveyi* strain ATCC 33843 (CP009467). The draft genome of *Vibrio* sp. strain

Table 1 The proportion of antibiotic resistant strains among 58 isolates

Sample group (no. of isolates)[a]	% Resistant[b]						
	ERY	FFC	ENR	STR	SMZ	TMP	TC
Total isolates (58)	74.1	82.8	69	94.8	81	72.4	94.8
IS*CR2*+ isolates (31)	71	96.8	93.5	100	100	100	100
IS*CR2*− isolates (27)	77.8	66.6	40.7	88.9	59.2	40.7	88.9

[a]Total isolates: 58 isolates from sick fish; IS*CR2*+ isolates: 31 isolates contained IS*CR2* among the 58 isolates; IS*CR2*− isolates: 27 isolates lacked IS*CR2* among the 58 isolates

[b]*ERY* erythromycin, *FFC* florfenicol, *ENR* enrofloxacin, *STR* streptomycin, *SMZ* sulfamethoxazole, *TMP* trimethoprim, *TC* tetracycline

L-85 was 6.4 Mb in length with an average G+C content of 46 mol%. A total of 5873 protein-coding genes were predicted based on Uniprot, KEGG, Swissprot, and RefsEq. We found eight ORFs that shared high identity with known resistance genes. The contigs carrying resistance genes were subjected to a BLAST search against the NCBI Genome Database and identified by PCR amplification. Two mobile elements were identified, integron 1 and IS*CR*2. Integron 1 harbours four resistance determinants: *arr2* (ADP-ribosyl transferase) conferring rifampicin resistance, *dfrA27* (dihydrofolate reductase) conferring trimethoprim resistance, *aadA16* (aminoglycoside 3′-adenylyltransferase) conferring streptomycin resistance and *sul1* (dihydropteroate synthase 1) conferring sulfonamide resistance. IS*CR*2 is adjacent to a hypothetical protein, a *floR* gene encoding a florfenicol efflux, a putative Lys regulator, a truncated IS*CR*2, a *strB* gene encoding streptomycin phosphotransferases, *tetA* and *tetR* genes encoding a tetracycline efflux pump and its cognate regulator, and *strA, strB*, and *sul*2 genes encoding dihydropteroate synthase conferring sulfonamide resistance (Fig. 1, type I). The arrangement of IS*CR*2 and *sul*2 exhibited significant similarity (99%) to the fragment from *V. cholera* (AB114188), one of the most threatening pathogens of the present day causing the disease cholera.

IS*CR*2 is associated with multiple resistance genes in diverse strains

The association between IS*CR*2 and *sul2* presence in *Vibrio* sp. strain L-85 and the association frequent reported in the literature prompted us to examine the presence of *sul2* in isolates carrying IS*CR*2, and found 22 of the 31 isolates contained *sul2*. Primers targeting IS*CR*2-*sul2* were designed according to the sequence found in *Vibrio* sp. strain L-85. Other five sequences arrangement types of IS*CR*2 plus ARGs were obtained from 17 isolates (Fig. 1). The type II sequence lost *strB* downstream of the truncated IS*CR*2 gene when compared to *Vibrio* type I, and was found in one isolate. Amplification and sequencing of 16 S rRNA gene from the isolate indicated that it shared 100% similarity with *Vibrio alginolyticus* NSTH35 (KF886646.1) (Table 2). The type III sequence was missing *tetA* and *tetR* when compared to type II, and was found in four isolates belonging to *Pseudoalteromonas, Vibrio*, and *Shewanella*. The *tet31* gene conferring tetracycline resistance and its cognate regulator *tetR* were located between IS*CR*2 and *sul2* in the type IV sequence found in *Pseudoalteromonas espejiana*. A hypothetical protein that shared low identity with macrolide phosphotransferases and a macrolide efflux pump was located between IS*CR*2 and *sul2* in the type V sequence, which was found in seven strains of *Pseudoalteromonas*. Type VI was the simplest arrangement in which IS*CR*2 was adjacent to Δ*glmM*, followed by *sul2*, and was distributed in strains of *Pseudoalteromonas* and *Psychrobacter*.

Furthermore, different types of IS*CR*s and ARGs have been found in pathogens. The type I sequence is present on the fragment from *V. cholera* (AB114188). The type II sequence is present on plasmids of *E. coli* (HQ023863), *Klebsiella pneumoniae* (JX442976), *S. enterica* (CP009409), and *V. cholera* (CP007636). The type III sequence is present on the SXT conjugative element from *V. cholera* KN14 (AB535680.1). The type VI

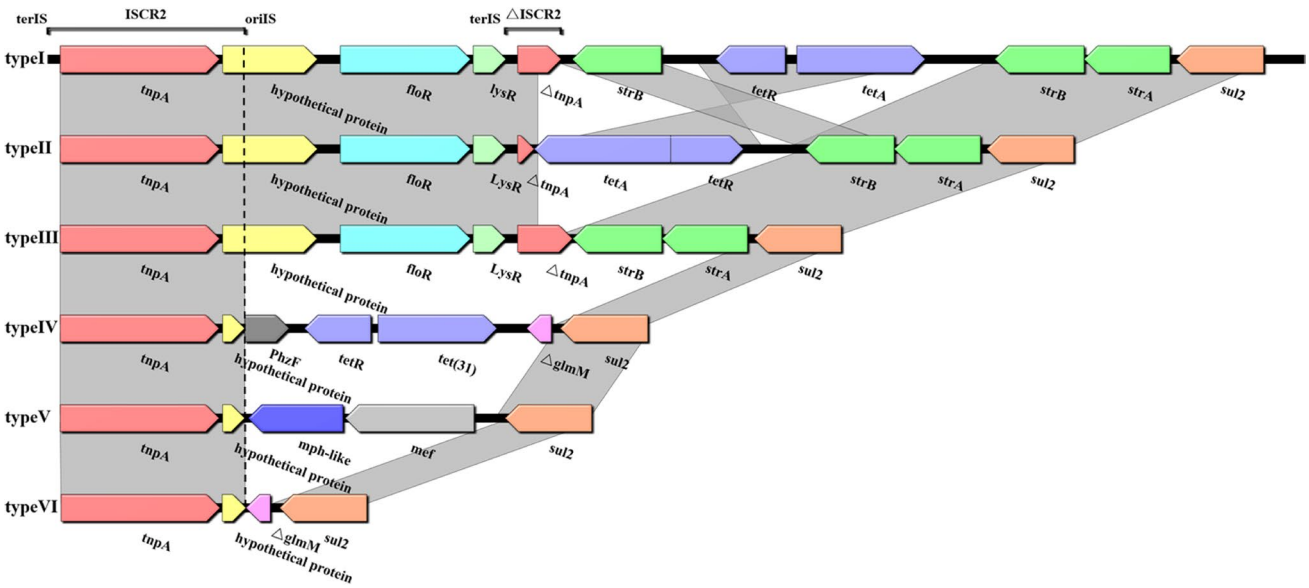

Fig. 1 Various arrangements of IS*CR*2 and *sul*2 in diverse strains

Table 2 Distribution of arrangements of IS*CR*2 and *sul*2 among strains

Best hit	IS*CR*2 Type					
	I	II	III	IV	V	VI
Pseudoalteromonas espejiana				1	2	
Pseudoalteromonas issachenkonii			1		4	
Pseudoalteromonas mariniglutinosa					1	
Pseudoalteromonas shioyasakiensis						1
Psychrobacter celer						1
Shewanella algae			1			
Vibrio alginolyticus		1				
Vibrio azureus			1			
Vibrio harveyi	1		1			
SUM	1	1	4	1	7	2

Table 3 Functional analysis of putative macrolide phosphotransferases expressed in *E. coli*

Strain/plasmid	MIC (mg L^{-1})[a]				
	ERY	CLR	AZM	MDM	SPM
E. coli K-12/pUC18::*mph-like*	128	128	16	256	128
E. coli K-12/pUC18	4	4	2	16	16

[a] *ERY* erythromycin, *CLR* clarithromycin, *AZM* azithromycin, *MDM* midecamycin, *SPM* spiramycin

sequence is present on plasmids of *Acinetobacter baumannii* (AP014650, CP001921), *E. coli* (KF787110), *K. pneumoniae* (KF250428), and *S. enterica* (KM670336). Altogether, the *Pseudoalteromonas* spp. and *Vibrio* spp. studied here share a mobile resistome frequently found in human pathogens, strongly suggesting that bacteria isolated from fish may have an active role in the fish farm-to-clinic transfer of ARGs.

A novel macrolide phosphotransferase is associated with IS*CR*2

A hypothetical protein found in the type V sequence shared homology with known macrolide phosphotransferases: 39, 40, and 35.8–36.5% amino acid identities with MphA, MphB, and MphC proteins, respectively, and 34.5–41.9 and 36.8–40.3% with Mphs of Cluster A and Cluster B, respectively, which we identified in a *Bacillus cereus* group recently (Wang et al. 2014). We cloned the *mph*-like protein into *E. coli* and found that recombinant plasmids carrying functional protein increased the resistance of their *E. coli* host to 14-, 15-, and 16-membered macrolides (clarithromycin, azithromycin, and midecamycin) by 8–32-folds (Table 3). Seven *Pseudoalteromonas* strains contained the novel macrolide phosphotransferase gene and all seven were resistant to clarithromycin, azithromycin, and midecamycin. Three *Pseudoalteromonas* strains that did not contain the phosphotransferase gene were more susceptible to the three antibiotics than the seven strains carrying the gene, suggesting that the novel macrolide

Table 4 Macrolide MICs for *Pseudoalteromonas* spp.

No. of strains	Best hit	Presence of *mph-like*	MICs (mg L^{-1})[a]		
			CLR	AZM	MDM
51	*P. mariniglutinosa* strain J19	+	256	32	128
52	*P. issachenkonii strain* K-W24	+	512	64	128
54	*P. espejiana* strain XH142	+	128	64	256
71	*P. espejiana* strain XH142	+	512	64	256
72	*P. issachenkonii* strain 5-4-4	+	256	32	256
73	*P. issachenkonii* strain K-W24	+	512	64	256
74	*P. issachenkonii* strain 5-4-4	+	256	32	256
58	*Pseudoalteromonas* sp. A-5-1	–	4	0.5	128
81	*Pseudoalteromonas* sp. SMW6	–	16	4	128
104	*Pseudoalteromonas* sp. A-5-1	–	16	4	256

CLR clarithromycin, *AZM* azithromycin, *MDM* midecamycin

phosphotransferase confers resistance in *Pseudoalteromonas* spp. (Table 4).

Conclusion

Sulfonamides, chloramphenicol, and tetracycline are used in aquaculture and their resistance genes have been extensively documented (Muziasari et al. 2014; Shah et al. 2012); our studies confirm these ARGs in fish farms. Our results reveal that IS*CR*2 is associated with multiple resistance genes among *Vibrio* spp., *Pseudoalteromonas* spp., and other aquatic bacteria. Florfenicol was used to treat the infection of fish in fish farm and florfenicol resistance gene, *floR* was found in three type of IS*CR* arrangement, suggesting the correlation between antibiotic pressure and prevalence of mobile elements. The different arrangements of IS*CR*2 and ARGs found in aquatic bacteria are also present in human pathogens, suggesting that the fish farm is a critical environmental reservoir for bacterial ARGs and the association of ARGs and mobile elements enhance their likelihood of transfer among pathogens.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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