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Genetic characterization of clinically relevant class 1 integrons carried by multidrug resistant bacteria (MDRB) isolated from the gut microbiota of highly antibiotic treated *Salmo salar*

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ABSTRACT

Objectives: The main objective of this study was the genetic characterization of clinically relevant class 1 integrons carried by multidrug resistant bacteria isolated from the intestinal microbiota of aquaculture salmon treated with high concentrations of antibiotics.
Methods: In 82 multidrug resistant bacterial isolates, the prevalence of both the conserved elements of the integrons, *qacEΔ1* and *sul1* genes, and the variable region (VR) was determined. Further, whole genome sequencing and complete genetic analysis was performed in VR-positive isolates.
Results: Despite the fact that 100% of the bacterial isolates presented the *intI1* gene, only 12.3% carried the *qacEΔ1* and *sul1* genes and only two (2.4%) presented a VR with gene cassettes. In the *Pseudomonas baetica* 25P2F9 isolate, a VR carrying *aac(6')31, qacH,* and bla_{OXA-2} gene cassettes was described, whereas the VR of *Aeromonas salmonicida* 30PB8 isolate showed a *dfrA14* gene cassette. The array of gene cassettes found in the *Pseudomonas* isolate appears with high frequency in clinically relevant pathogens such as *Pseudomonas aeruginosa* or *Escherichia coli*. Additionally, it was possible to determine that these integrons are contained in plasmids and coul be easily transferred. Resistome analysis demonstrated that both isolates carried a great diversity of antibiotic resistance genes, including many β-lactamases. Even in the *Aeromonas* isolate a new oxacillin-hydrolyzing beta-lactamase gene was described ($bla_{OXA-956}$).
Conclusion: The presence of multidrug resistant bacteria and clinically relevant genetic elements in the salmon intestinal microbiota make the aquaculture a hotspot in the phenomenon of antibiotic resistance; therefore, the control of antibiotics used in this activity is a key point to avoid its escalation.

1. Introduction

Aquaculture is a hotspot for the evolution, mobilization, and dissemination of antimicrobial resistance (AMR) [1], because large amounts of antibiotics are used to control bacterial pathogens [2]. Chile is the second largest producer of farmed salmonids and consumes the most antimicrobials [3]. The main route of antibiotic administration in salmon is through medicated food. This leads to recurrent exposure to antibiotics of their gut microbiota, which

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promotes the selection of multidrug resistant bacteria (MDRB) enriched in antibiotic resistant genes (ARGs) [4]. These bacteria are eliminated through the feces, whereby they pollute the environment and transform the salmon production in a focus of emergency and dispersion of MDRB and ARGs [4].

One of the most interesting mobile genetic elements (MGEs) that participates in the process of evolution and mobilization of ARGs is the integrons [5]. These elements are bacterial genetic platforms that facilitate the acquisition, storage, cleavage, rearrangement, and expression of genes located in mobilizable elements called *gene cassettes*, which correspond to the variable region (VR) of the integron [6]. Five classes of integrons based on the aminoacidic sequence of their integrase have been described [7]; however, only classes 1, 2, and 3 are highly associated with the dissemination and maintenance of ARGs [8]. Eventually, any gene cassette that is incorporated into an integron can be expressed independently of the genetic background of the host [9]. Thus, integrons have a high impact on human health because they can incorporate ARGs of clinical importance such as extended spectrum β-lactamases, carbapenemases, and *mcr*-like genes [10]. In Chilean salmon production, integrons carrying gene cassettes with *dfrA* and *addA* genes have been described in both freshwater [11] and seawater salmon farms [12]. However, there is no available information about integrons harbored in the intestinal microbiota of farmed salmon treated with antibiotics despite the fact that a large number of MDRB carrying ARGs have been isolated [4].

In a previous study published by our group, we characterized 91 bacteria-resistant isolates from fish microbiota collected in four salmon farms where high amounts of antibiotics were used [4]. These isolates presented a high prevalence of genetic elements involved in resistance to florfenicol (*floR* and *fexA* genes) and oxytetracycline (*tetA, tetE, tetH, tetL,* and *tetM* genes), the main antibiotics used in recent years in the salmon industry in Chile. Likewise, 89 of the 91 isolates were positive for the gene that encodes the integrase of class 1 integrons *intI1,* and 82 of them showed an MDR phenotype because they were resistant to at least one antibiotic from three different categories. However, the characterization of MDRB carrying integrons was not performed. It is for this reason that the main objective of the present manuscript is the characterization of the integrons present in these 82 MDR isolates.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Eighty-two MDRB isolated from the salmon gut microbiota from four fish farms treated with high concentrations of antibiotics and previously described by Higuera Llanten et al [4]. were selected for this study. The isolates had the following characteristics: they were positive for the *intI1* gene and they showed an MDR phenotype; they were resistant to at least one antibiotic from three different categories (antibiotic tested: oxytetracycline, tetracycline [tetracyclines]; florfenicol, chloramphenicol [phenicols]; erythromycin [macrolide]; kanamycin [aminoglycoside]; ciprofloxacin [quinolone]; and ampicillin [β-lactam]). Bacterial strains were routinely grown in LB (Lisogeny broth) medium and incubated at 15°C or 25°C for 24 h with shaking. For antibiotic susceptibility assays, bacterial cultures were grown overnight and then diluted in sterile 0.85% NaCl to a final OD_{600nm} of 0.01. Next, 100 µL of these dilutions were seeded over Mueller-Hinton agar plates, which were incubated for 24 h at 25°C or 15°C depending on the isolate.

2.2. DNA extraction and manipulation

For PCR amplification, the total DNA of all bacterial strains were extracted using the AxyPrep^{TM} Bacterial Genomic DNA Miniprep Kit (Axygen Biosciencies, NY, USA) following the manufacturer's instructions. PCR assays were carried out using the GoTaq® ADN-polymerase Green Master Mix (Promega, USA) with the primers and conditions described in Supplementary Table S1. To determine the presence of class 2 and 3 integrons, partial sequences of *intI2* and *intI3* genes were amplified by PCR using paired primers, IntI2F-IntI2R and IntI3F-IntI3R [6]. To characterize the conserved region of the class 1 integrons, the *sul1* and *qacEΔ1* (3'-CS) genes were amplified using paired primers Sul1F-Sul1R [13] and qacEΔ1F-qacEΔ1R [14], respectively. Furthermore, in order to amplify the gene cassettes contained in the VR, the Hep58 and Hep59 primers were used according to White et al [15]. This study did not include a search for the *orf5* gene. All purified PCR products were cloned into pCR2.1 TOPO® TA plasmid (Invitrogen, USA) and sequenced by Sanger.

For whole genome sequencing (WGS) of the strains that carry gene cassettes, the total DNA of bacterial isolates were extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions. The DNA concentration and quality were measured using the multimodal reader machine Cytation 5 (BioTek), and the samples were shipped to the Microbial Genome Sequencing Center (Pittsburgh, PA) for sequencing (https://www.migscenter.com/).

2.3. Analysis of Sanger sequencing

All DNA sequences were analyzed by BLAST (Basic Local Alignment Search Tool) against the GenBank (April 2021) and INTEGRALL [16] databases, and integrons were assembled using the CLC Genomics Workbench 10 (Qiagen, USA). The sequences of the VR (flanked by Hep58 and Hep59 paired primers) were deposited in the GenBank database *(Aeromonas salmonicida* 30PB8 ID MG738684 and *Pseudomonas baetica* 25P2F9 ID MG738685).

2.4. WGS, assembly, and annotation

Genome sequencing for the strains carrying gene cassettes was performed at the Microbial Genome Sequencing Center on an Illumina NextSeq 2000 platform (2 × 151 bp reads) following the standard protocol. Quality and adapter trimming were performed using Trimmomatic 0.36 [17] followed by removal of possible reads matching the PhiX genome using BBduk [18]. Cleaned and trimmed reads were assembled using SPades [19] for genome assembly and plasmidSPades [20] to evaluate for possible plasmids in the genome sequences. SPAdes and plasmidSPades assemblies were compared to find contigs with the same length, and a BlastN search was performed to compare the nucleotide sequences. All assembled genomes and plasmid sequences were annotated using Prokka [21]. Evaluation of the presence of antibiotic resistance genes was done using ABRIcate [22] with the CARD (Comprehensive Antibiotic Resistance Database) database as a reference [23]. The WGS data of the *A. salmonicida* 30PB8 and *P. baetica* 25P2F9 strains were deposited in the NIH (National Institutes of Health) genomes database. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAGUQQ000000000 (*A. salmonicida* 30PB8) and JAIHLQ000000000 (*P. baetica* 25P2F9). The versions described in this paper are JAGUQQ000000000.1 and JAIHLQ000000000.1, respectively. To characterize the active site and conserved domains of OXA-956, the InterProScan server was used (https://www.ebi.ac.uk/interpro/search/sequence/)

2.5. Analysis of plasmid content in WGS data

WGS assemblies JAGUQQ000000000 (*A. salmonicida* 30PB8) and JAIHLQ000000000 (*P. baetica* 25P2F9) were analyzed to determine the presence of plasmids. Two bioinformatic tools Platon [24] and viralVerify [25] were used for this purpose. Platon detects plasmid contigs from WGS short-read assemblies. The main metric of this tool is denominated replicon distribution score (RDS), which shows the empirically measured frequency biases of protein sequence distributions between plasmids and chromosomes precomputed on complete NCBI (National Center for Biotechnology Information) RefSeq replicons, with an accuracy of 96.6%. For the interpretation of this parameter, contigs with RDS values < -7.7 depict chromosomal classification, whereas values < -7.7 represent plasmid classification. Moreover, viralVerify tool, which is based on PlasmidVerify [20], was used because it has more accuracy for plasmid analysis. It has the ability to predict genes' (Prodigal) proteins (Hidden Markov Models, HMMs) and classifies the contig as viral or nonviral by applying the naive Bayes classifier. For the set of predicted HMMs, viralVerify uses trained naive Bayes classifier to classify this set to be viral, plasmid, or chromosomal. Contig scores > 3 represent viral or plasmid prediction and contigs < 3 depict chromosomal classification.

2.5. Determination of antibiotic susceptibility

Minimal inhibitory concentrations (MICs) of β-lactam antibiotics in the bacterial isolates that carry recognizable gene cassettes were determined using ETEST® strips (BioMérieux, France). The antibiotics used were piperacillin, ceftazidime, ceftriaxone (CRO), cefotaxime (CTX), cefepime, cefoxitin (FOX), aztreonam, imipenem, and meropenem. Strips were placed on the surface of Mueller-Hinton agar plates (one strip per plate), and the experiments were repeated three times. The susceptible/resistant phenotype in *P. baetica* 25P2F9 was defined according to the MIC breakpoints of *Pseudomonas* spp. established in Performance Standards for Antimicrobial Susceptibility Testing (M100) [26] by CLSI-2021 (Clinical & Laboratory Standards Institute). For *A. salmonicida* 30PB8, MIC breakpoints stablished in Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria (M45) for *Aeromonas* spp. were considered [27].

3. Results

3.1. Prevalence of class 1 conserved genetic elements in MDRB

To search for the presence of class 2 and 3 integrons, the respective genes coding for the integrases *intI2* and *intI3* were amplified by PCR. None of these genes were found in the isolates evaluated. *sul1* and *qacE*Δ1 genes were also screened to analyze the 3' conserved sequence (3' CS) associated with class 1 integrons, and 15 out of 82 (18.3%) resistant isolates carried *sul1* or *qacEΔ1* genes while 10 out of 82 (12.3%) presented both genes. However, only two isolates (2.4%), *P. baetica* 25P2F9 and *A. salmonicida* 30PB8, presented a VR with gene cassettes as well as *intI1, sul1,* and *qacE*Δ1. Although *orf5* was not searched by PCR, this gene was detected by WGS analysis in both *P. baetica* 25P2F9 and *A. salmonicida* 30PB8, showing the complete class 1 integron array (Fig. 1A).

3.2. Identification of gene cassettes in class 1 integrons

To identify ARGs contained in the VR of class 1 integrons, gene cassettes were amplified using the paired primers Hep58-Hep59 and sequenced, which showed that only two out of 82 isolates presented recognizable gene cassette structures (Fig. 1B). A cassette carrying the *dfrA14* gene that encodes a dihydrofolate-reductase and confers resistance to both sulphonamides and trimethoprim was found in the *A. salmonicida* 30PB8 isolate. Remarkably, the integron found in the *P. baetica* 25P2F9 strain contains a VR with three ARGs that could be relevant in terms of food safety and human/fish health because they confer resistance to clinical- and aquaculture-related antimicrobial drugs: aminoglycosides (*aac[6′]-31*), β-lactams ($bla_{\text{OXA-2}}$), and quaternary ammonium compounds (*qacH*). The last one is commonly used as an industrial disinfectant.

To compare the structure of the VR of this last integron, a comparative nucleotide sequence alignment by BLAST was performed by modifying the algorithm parameters for the alignment of a maximum of 2000 matches. Only the integrons that presented 85% of coverage and carried at least two gene cassettes of the integron described in this manuscript were considered to perform the structural analysis. The result showed that this integron presents a high similarity to other integrons found in MDR *Pseudomonas aeruginosa* and *Escherichia coli* (Fig. 2A,B); both classify as clinically relevant Gram-negative pathogens. Furthermore, this class 1 integron also showed a high similarity to a previously described integron from *Achromobacter denitrificans* (Fig. 2C)—an environmental β-proteobacteria recently classified as an infectious agent with human health relevance that can cause bacteremia and lung infection in immunocompromised patients [28]. In all closely related matches, the presence of $bla_{\text{OXA-2}}$ and different alleles for at least one quaternary ammonium resistance gene (*qacH* and *qacL*) and one aminoglycoside resistance gene (*aacA [6], aacA [6′]* and *aacA4*) were found. However, none of the gene cassettes found in this analysis showed the presence of exactly the same gene alleles or the same organization as the integron found in *P. baetica* 25P2F9, suggesting that this is a integron that has not been previously described.

To analyze the clinical relevance of each one of the gene cassettes found in the *P. baetica* 25P2F9 integron, we investigated in which species they were more frequently described using the resistance gene identifier tool available on the CARD (Comprehensive Antibiotic Resistance Database) platform [23]. In the case of the *aac(6')-31,* 80% were found in *P. aeruginosa*, 10% in *Acinetobacter baumannii*, and 8% in *Klebsiella pneumoniae*; all of these have been declared critical pathogens by the World Health Organization. In contrast, the *qacH* gene seemed to be highly associated with *Enterobacteriaceae* because it had a 58% prevalence in *E. coli*, 25% in *K. pneumoniae*, and 8% in *Salmonella enterica*, showing only 4% in *P. aeruginosa*. Finally, $bla_{\text{OXA-2}}$ was the gene with the greatest host diversity in the database, where 38% has been described in *P. aeruginosa*, 29% in *K. pneumoniae*, and 8% in *E. coli*, 8% *Enterobacter* Spp., 5% in *Serratia marcescens*, and 4% in other bacterial species.

3.3. WGS prediction of integron location in P. baetica 25P2F9 and A. salmonicida 30PB8

After the characterization of VR-harboring integrons, we determined the genetic location of the integron. For that purpose, the total DNA from the two strains harboring complete integrons, *A. salmonicida* 30PB8 and *P. baetica* 25P2F9, were fully sequenced by Illumina technology. When the corresponding trimming and quality analysis were completed, the genomes were de novo assembled with SPades [19] software, and the presence of the respective integrons were confirmed by BLAST. To complement the genome assembly, we used plasmidSPades [20] to identify putative plasmid contigs within the genome assembly. This approach can facilitate the identification of specific contigs based on the properties of the assembly graph. These contigs were then used to search the corresponding integrons by BLAST, and the results demonstrated that both integrons have a high probability of being localized in plas-

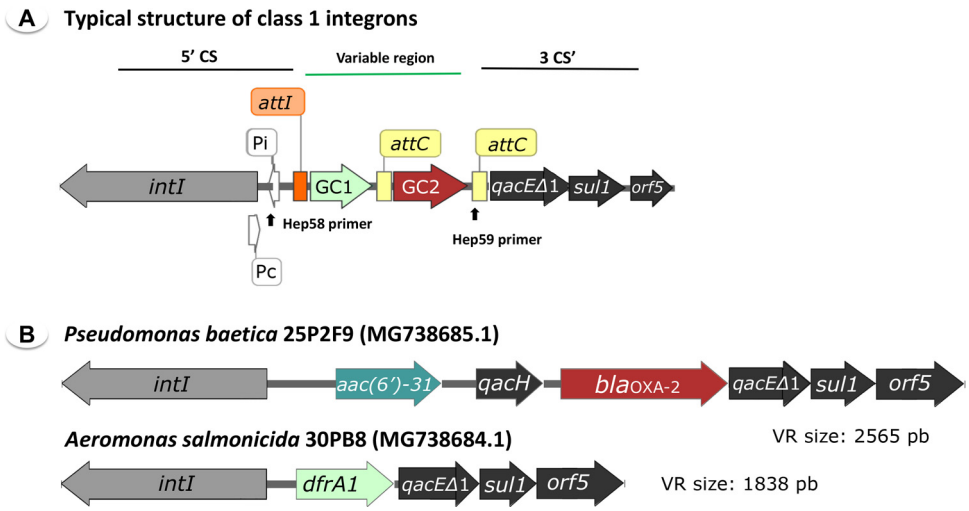

Fig. 1. Structure of class 1 integrons. (A) Canonical structure of class 1 integrons showing the 5′ conserved sequence (CS), 3′ CS, and different gene cassettes (GC) in the variable region. The location and the direction of transcription of genes are indicated by the direction of the horizontal arrows. The GC is inserted into the variable region by IntI integrase (left grey arrow) through a site-specific recombination mechanism between *attI* (orange) and *attC* (yellow) sites. Pc and Pi (both in white) are the two promoters in the integron system and control the expression of the entire GCs and integrase, respectively. The little vertical black arrow shows Hep58-Hep59 primers used to amplify the variable region. The *qacE*Δ1 and the *sul1* resistance genes as well as the open reading frame *orf5* (less common than the other features) are located in the 3′ CS (right black arrows). (B) Structure of class 1 integrons containing an identifiable variable region in this work (size scaled). The same features mentioned above for the typical structure of class 1 are shown, and the GC arrays detected were *aac(6′)-31, qacH, dfrA14*, and bla_{OXA-2}.

mids. In addition, Platon and viralVerify tools were used to detect integron location. Platon analysis in *A. salmonicida* 30PB8 (WGS JAGUQQ000000000) showed seven contigs with a high probability of being classified as plasmids (Supplementary Table S2). The class 1 integron characterized in this strain is located in the contig JAGUQQ010000067.1, with a length of 22 087 pb and RDS = 20.4, and a value that classified this contig as a plasmid. Moreover, the same analysis was made in *P. baetica* 25P2F9, which shows nine contigs with plasmid attributes (Supplementary Table S3). A class 1 integron is located in the contig JAIHLQ010000089.1, with a length of 6133 and RDS =13.1, also classified as a plasmid. Furthermore, viralVerify tool analysis provided similar results. Contig JAGUQQ010000067.1 has a score value of 45.43 (Supplementary Table S4), and this contig is predicted to be a plasmid. The same situation occurs in contig JAIHLQ010000089.1 with a score of 8.50, also classified as a plasmid (Supplementary Table S5). In summary, these results provided by both tools show strong evidence that the class 1 integrons characterized in both strains are localized in plasmids.

3.4. Analysis by WGS of the resistome and determination of β-lactam susceptibility profile in P. baetica 25P2F9 and A. salmonicida 30PB8

The resistomes of *P. baetica* 25P2F9 and *A. salmonicida* 30PB8 were analyzed using de novo assemblies by WGS and the software ABRIcate [22]. The results (Table 1) showed that both strains, in addition to integron-associated ARGs, presented a *tetA* gene that is responsible for their tetracycline resistance phenotype that was previously reported [4]. Further, *A. salmonicida* 30PB8 presented other ARGs related to the resistance to β-lactams (FOX-4; *cphA5;* $bla_{OXA-427}$-like), aminoglycosides (APH[3′′]-Ib; APH[6]-Id), phenicols (*floR*), and sulfonamides (*sul2*). From a salmon aquaculture perspective, the most interesting finding is *floR* because it confers resistance to florfenicol, which is a very dangerous concern if this bacterium is a salmon pathogen. Upon further analysis we were able to find that in *A. salmonicida* 30PB8, eight of the 11 ARGs found are present in plasmids, and in the same way, six of the seven ARGs found in *P. baetica* 25P2F9 are carried by plasmids.

To determine whether or not the presence of β-lactam resistance genes could influence the susceptibility of *P. baetica* 25P2F9 and *A. salmonicida* 30PB8 to these antibiotics, the MICs of a representative group of β-lactams were determined. The results (Table 2) showed that *A. salmonicida* 30PB8 — the strains with wider diversity of β-lactam resistance genes (FOX-4; *cphA5;* $bla_{OXA-427}$-like) — had a narrower spectrum of β-lactam resistance (FOX) than *P. baetica* 25P2F9 (CRO, CTX, FOX, and aztreonam), which only presented the bla_{OXA-2} gene. These resistance profiles are in accordance with the ARGs found because FOX is a substrate of FOX-4, and most OXA-2 type of β-lactamases presented a similar substrate profile to that described here in *P. baetica* 25P2F9.

3.6. New OXA-type β-lactamase in A. salmonicida 30PB8

The data obtained from WGS showed that *A. salmonicida* 30PB8 presented a $bla_{OXA-427}$-like gene that shared 85.91% of identity according to the analysis in CARD (Table 1). Due to the low percentage of homology, we submitted the entire genome data to the NCBI database in order to determine whether this gene corresponded to a new OXA variant. After the analysis from the database curators, the new OXA allele was designated as OXA-956. Interestingly, despite OXA-427 being considered a carbapenemase enzyme [29], the susceptibility profile observed in the host strain *A. salmonicida* 30PB8 suggests that the substrate profile in OXA-956 is narrower than that in OXA-427. Even when an aminoacidic sequence analysis was performed, no differences were found (Supplementary Fig. S1), corroborating that the host is essential in defining the range of action of the enzyme.

4. Discussion

Class 1 integrons are highly successful MGEs that promote the dissemination of ARGs. In aquaculture, the presence of class 1 integrons carrying ARGs has been described in various production systems and MDRB harboring class 1 integrons carrying ARGs have also been documented in salmon production in Chile. In all of these cases, the integron-harboring bacteria were isolated from sediment, but none of them were isolated from the intestinal mi-

Table 1
Determinants of resistance and phenotype involved in AMR in bacterial isolates carrying integrons with variable regions

Strain	Gene	Product description	% coverage	% identity	Database	Accession number	Plasmid prediction by PLATON/Viralverify	Predictive resistance phenotype	Resistance phenotype observed[a]
A. salmonicida 30PB8	FOX-4	FOX-4 is a β-lactamase found in *Escherichia coli*.	96.52	86.92	CARD	AJ277535:0-1149	-	Cephalosporin; cephamycin	Cefoxitin Chloramphenicol
	sul2	Sul2 is a sulfonamide-resistant dihydropteroate synthase of Gram-negative bacteria usually found on small plasmids.	100	100	CARD	AY055428.1:21084-20268	+	Sulfonamide	Tetracycline
	floR	FloR is a plasmid or chromosome-encoded chloramphenicol exporter.	100	99.75	CARD	AF231986:3307-4522	+	Phenicol	
	APH(3′′)-Ib	APH(3′′)-Ib is an aminoglycoside phosphotransferase encoded by plasmids, transposons, integrative conjugative elements, and chromosomes.	100	99.88	CARD	AF313472:15593-16397	+	Aminoglycoside	
	APH(6)-Id	APH(6)-Id is an aminoglycoside phosphotransferase encoded by plasmids, integrative conjugative elements, and chromosomal genomic islands	100	99.88	CARD	AF024602:3155-3992	+	Aminoglycoside	
	dfrA14	DfrA14 is an integron-encoded dihydrofolate reductase.	100	100	CARD	EU780012:2162-2645	+	Diaminopyrimidine	
	qacEΔ1	QacEdelta1 is a resistance gene conferring resistance to antiseptics. It is different from QacE only at the 3′-terminus.	100	100	CARD	U49101.1:1490-1838	+	Acridine dye; disinfecting agents and intercalating dyes	
	sul1	Sul1 is a sulfonamide-resistant dihydropteroate synthase of Gram-negative bacteria. It is linked to other resistance genes of class 1 integrons.	100	100	CARD	JF969163:1053-1893	+	Sulfonamide	
	tet(A)	TetA is a tetracycline efflux pump found in many species of Gram-negative bacteria.	97.8	100	CARD	AF534183.1:2970-4245	+	Tetracycline	
	cphA5	CphA5 is an Ambler Class B MBL; subclass B2 originally isolated from *Aeromonas salmonicida*	99.87	94.65	CARD	AY227051:0-765	-	Carbapenem	
	$bla_{\text{OXA-956}}$	From the Lahey list of β-lactamases. Not yet released.	100	85.91	CARD	KX827604.1:0-795	-	Carbapenem; cephalosporin; penam	
P. baetica 25P2F9	*mexF*	MexF is the multidrug inner membrane transporter of the MexEF-OprN complex.	98.81	83.25	CARD	AE004091.2:2810008-2813197	-	Diaminopyrimidine; fluoroquinolone; phenicol	Ceftriaxone Cefotaxime Cefoxitin
	AAC(6′)-31	AAC(6′)-31 is an integron-encoded aminoglycoside acetyltransferase.	100	98.65	CARD	AM283489:2655-3174	+	Aminoglycoside	Aztreonam Chloramphenicol Tetracycline
	qacH	QacH is a subunit of the qac multidrug efflux pump.	100	90.99	CARD	DQ149925.1:188-521	+	Disinfecting agents and intercalating dyes	Ciprofloxacin
	$bla_{\text{OXA-2}}$	OXA-2 is a β-lactamase mainly found in the *Enterobacteriaceae* family.	100	100	CARD	M95287.4:2455-3283	+	Carbapenem; cephalosporin; penam	
	qacEΔ1	QacEdelta1 is a resistance gene conferring resistance to antiseptics. It is different from QacE only at the 3′-terminus.	100	100	CARD	U49101.1:1490-1838	+	Acridine dye; disinfecting agents and intercalating dyes	
	sul1	Sul1 is a sulfonamide-resistant dihydropteroate synthase of Gram-negative bacteria. It is linked to other resistance genes of class 1 integrons.	100	100	CARD	JF969163:1053-1893	+	Sulfonamide	
	tet(A)	TetA is a tetracycline efflux pump found in many species of Gram-negative bacteria.	97.8	100	CARD	AF534183.1:2970-4245	+	Tetracycline	

AMR, antimicrobial resistance. CARD, Comprehensive Antibiotic Resistance Database.

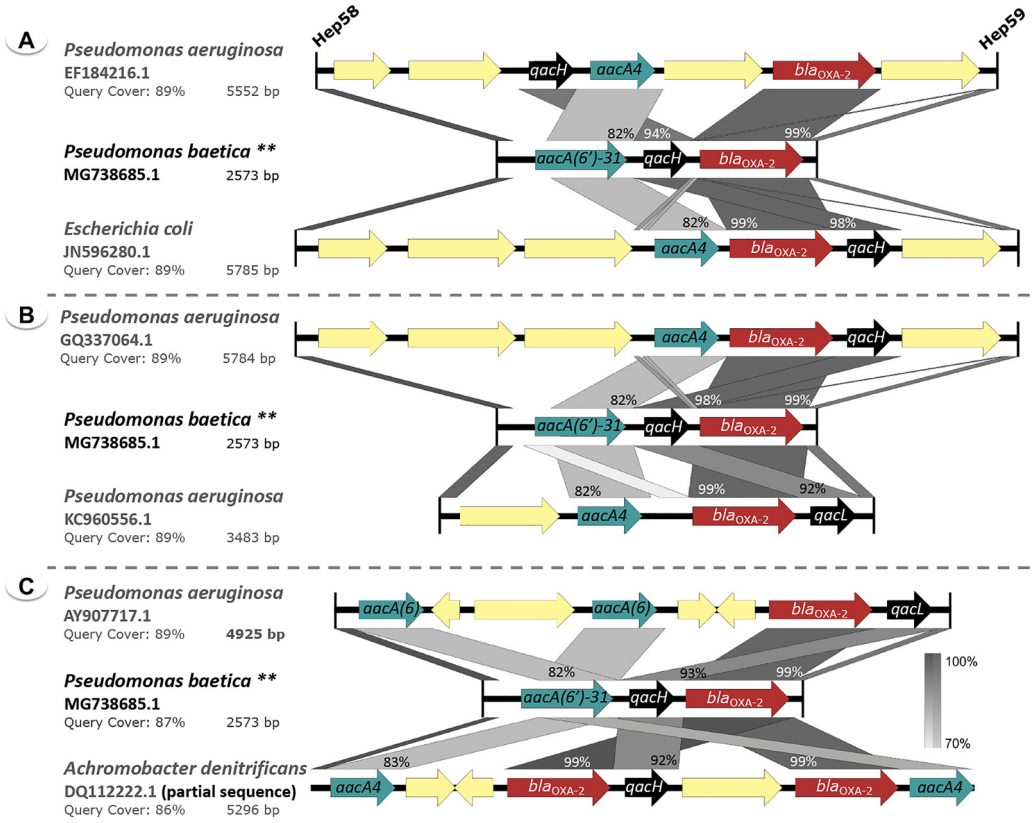

Fig. 2. Sequence analysis of the class 1 integron described in the *Pseudomonas baetica* 25P2F9 strain. BLAST (Basic Local Alignment Search Tool) analysis was performed by modifying the algorithm to obtain a maximum of 2000 matches. Only those integrons that had a coverage greater than 85% and that also carried all three gene cassettes harbored in the new integron were selected to make the structural comparison. This figure shows the independent alignment of the new class 1 integron described here (ID: MG738685.1) with the integrons ((A) EF184216.1 and JN596280.1; (B) GQ337064.1 and KC960556.1; (C) AY907717.1 and DQ112222.1. These were previously described in human pathogens *Pseudomonas aeruginosa, Escherichia coli,* and *Achromobacter denitrificans*.

Table 2
β-lactams MIC estimation using ETEST® strips in bacterial isolates with integrons variable regions

Antibiotics		MIC (µg/mL) *Aeromonas salmonicida* 30PB8	*Pseudomonas baetica* 25P2F9
Penicillins	PIP	8	12
Cephems	CAZ	0.094	0.75
	CRO	0.19	**24**
	CTX	0.64	**>32**
	FEP	0.094	0.5
	FOX	**16**	**>256**
Monobactam	ATM	0.016	**32**
Carbapenems	IPM	0.19	0.5
	MEM	0.125	0.19
	DOR	0.19	0.19
	ETP	0.38	0.38
Phenicols	CHL	**32**	**32**
	FLO	**512**	**256**
Tetracyclines	TET	**32**	**32**
	OXY	**>64**	**>64**
Aminoglycosides	KAN	8	8
Macrólides	ERY	64	1024
Fluoroquinolones	CIP	**2**	**2**

Bold characters indicate resistance values for MIC breakpoints established by CLSI in M100 and M45 manuals. For *Aeromonas salmonicida* 30PB8, MIC breakpoints for *Aeromonas* Spp. established in Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria (M45) [27] were considered. For *Pseudomonas baetica* 25P2F9, criteria for *Pseudomonas* Spp. in Performance Standards for Antimicrobial Susceptibility Testing (M100) by CLSI-2021 [26] were used.
ATM, aztreonam; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CLSI,Clinical & Laboratory Standards Institute; CRO, ceftriaxone; CTX, cefotaxime; DOR, doripenem; ERY, erythromycin; ETP, ertapenem; FEP, cefepime; FLO, florphenicol; FOX, cefoxitin; IPM, imipenem; KAN, kanamycin; MIC, minimal inhibitory concentration; MEM, meropenem; MIC, minimal inhibitory concentrations; OXY, oxytetracycline;PIP, piperacillin; TET, tetracycline.

crobiota of farmed fish. This is probably the most affected system because the main route of antibiotic administration in fish is through medicated food. Taking this into account, we address the hypothesis that the high use of antibiotics in salmon farms can select and maintain integrons carrying ARGs in the fish microbiota, leaving some of them with a high relevance from a human health perspective.

In the present study, it was possible to demonstrate that despite the fact that all bacterial isolates analyzed presented the *intI1* gene, only 12.3% also presented 3'CS, conformed by *qacED1* and *sul1* genes. Of these, only two isolates (2.4%) presented ARGs in the VR, which were fully sequenced by WGS. The integron found in *A. salmonicida* 30PB8 presented a VR with a *dfrA14* gene, which contributes to sulfonamides/trimethoprim resistance. The *dfr* genes have been strongly associated with MGEs such as plasmids and integrons. This impacts the emergence, evolution, and dissemination of sulfonamide resistance in aquatic environments. This is extremely interesting because many of the genes involved in resistance to oxytetracycline (i.e., *tetA*) and florfenicol (i.e., *floR*) (the most widely used antibiotics in salmon production in Chile) are carried by highly disseminable plasmids [30,31]. This study determined that the integron harbored by *A. salmonicida* 30PB8 has a high probability of being found in a plasmid, and, given the occurrence of the *floR* and *tetA* genes, this integron has likely been maintained in this isolate due to a coselection phenomenon.

The most interesting integron found in our study was identified in the *P. baetica* 25P2F9 isolate. The composition of this integron contains the gene cassettes *aac(6′)-31, qacH,* and $bla_{\text{OXA-2}}$. The first encodes for aminoglycoside adenylyltransferase, which confers resistance to aminoglycosides (streptomycin and spectinomycin), with a high prevalence in class 1 integrons. This gene has been predominantly identified in *P. aeruginosa, A. baumannii*, and *K. pneumoniae* [32]. The *qacH* gene encodes for a small multidrug resistant efflux pump and is associated with resistance to benzalkonium chloride [33]—a type of quaternary ammonium compound commonly used in industrial disinfectants, and it is frequently found in Gram-negative bacteria, mainly in *Enterobacteriaceae* but also in food-contaminating bacteria such as *Listeria monocytogenes* [34]. In contrast, this resistance element has been reported only once in aquaculture — in an *Aeromonas hydrophila* isolated from tilapia [35]. The results reported here suggest that the association between *qacH* genes and integrons in the salmon gut microbiota represents a potential risk for salmon farms because some of the disinfectants commonly used in this food industry are quaternary ammonium compounds.

The third gene cassette corresponds to the $bla_{\text{OXA-2}}$ gene, which encodes an OXA-type β-lactamase. This type of enzyme has been widely characterized, and more than 350 different alleles have been described worldwide [36]. They are class D β-lactamases frequently disseminated in Gram-negative bacteria. In particular, OXA-2 mediates resistance to penicillins and some cephalosporins, but some aminoacidic modifications confer resistance to expanded spectrum cephalosporins such ceftazidime, thus turning it into an extended spectrum β-lactamase [37]. Nevertheless, diverse studies have shown that OXA-2 can even hydrolyze carbapenems in specific isolates of *A. baumannii* and *P. aeruginosa* [38]. However, this last is not the case of the OXA-2 isolated in the present study because although it is associated with resistance to several cephalosporins (CRO, CTX, and FOX), the host strain *P. baetica* 25P2F9 remains susceptible to carbapenems (imipenem and meropenem) and to expanded spectrum cephalosporins (ceftazidime and cefepime). Similarly to *aac(6′)-31* and *qacH* genes, this enzyme has been frequently found in hospital environments and has been isolated from clinically important bacteria such as *A. baumannii* and *P. aeruginosa*. However, other papers have reported the enzyme outside clinical environments, including in sewage water as free DNA, associated with plasmids of the IncP1-β family, in *E. coli* isolates from rivers and lakes, and in different water bodies, including seawater [39,40]. In all of these environments, this β-lactamase is considered to be a pollutant. Although this gene is considered a pollutant derived from human waste, it has also been described in *Aeromonas* sp. strains isolated from aquaculture systems, demonstrating that they can easily reach these systems and can even be maintained depending on the antibiotic selection pressure.

All genes carried by the integron described in *P. baetica* 25P2F9 presented a high prevalence in clinically relevant human pathogens; therefore, the possibility that all of these gene cassettes come from contamination of human clinic activity should not be discarded, even more so if in this study we have determined that this integron has a high probability of being part of a plasmid. However, when an integron with this structure and configuration is acquired by a florfenicol/oxytetracycline-resistant bacteria of the fish microbiota, the identification may be collaterally selected via the use of antibiotics during the fish treatment. Here is where the concept of coselection becomes more relevant, because although the integron does not contain any florfenicol- or oxytetracycline-resistance determinant, they can be maintained in such an environment. Likewise, in both isolates, the ARGs (not only described in the integrons) are mostly in plasmids, which facilitates their dispersal and transmission to fish and human pathogenic bacteria. These reasons explain aquaculture as a hotspot for AMR phenomenon. There are highly successful MGEs such as integrons that accelerate the evolution and dissemination of ARGs; likewise, the combination of fish feces carrying ARGs and food medicated with antibiotics facilitate AMR in the environment.

The results explain why the use of antibiotics in animal production must be controlled —even those considered as nonclinic antibiotics: MDRB carry a complete arsenal of resistance determinants and may be selected and disseminated in many environments, constituting a risk to both human and veterinary health.

5. Conclusion

The constant use of antibiotics in salmon production in Chile selects MDRB. Although 100% of the MDR bacteria analyzed in this study carried the *intI1* gene, only 2.3% carried a variable region. However, the integron characterized in the isolate of *P. baetica* 25P2F9 presents clinically relevant genetic elements with a high prevalence in pathogens classified as critical by the World Health Organization, like *P. aeruginosa, A. baumannii,* and *E. coli*. Likewise, it was determined that the integrons described in this study have a high probability of being found in plasmids, which greatly facilitates the mobilization of clinically relevant ARGs between different microbiomes, making them not only a public health problem but a real One Health problem.

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Competing interests

The authors declare no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.02.003.

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