High prevalence of class 1 integrase and characterization of class 1 integron gene cassettes in multiresistant bacteria isolated from the gut microbiota of extended antibiotic treated *Salmo salar* fish farms

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Abstract

The use of antimicrobials in aquaculture is a common practice. Chile is second larger producer of salmon worldwide, but unfortunately is the first consumer of antibiotics. Tonnes of florfenicol and oxytetracycline yearly are used in the Chilean salmoniculture to control the pathogens that threaten the sustainability of the industry. This excessive use of antibiotics have selected populations of resistant bacteria from the sediments and the water column that sorround the fish farms. In a recent work, our lab described the high prevalence of multiresistant bacteria and Antibiotic Resistance Genes (ARGs) in the gut microbiota of Antlactic salmon (Salmo salar) treated with high doses of antibiotics. In this work, we revisited the analysis of the previously described gut multiresistant bacteria grouped in banks of florfenicol resistant isolates (FB) and oxytetracycline resistant isolates (OB) looking for the presence of integron-integrase elements. These elements have been described as an important players in the Antimicrobial Resistance (AMR) phenomenon and they are considered a good markers of the anthopogenic activities pollution. The results showed that the 100% of the multiresistant isolates present the class 1 intagrase. Despite this result, no isolate from FB showed the typical structure of class 1 integrons: the presence in 3'-CS of *qacEΔ1/sul1* genes. While in OB, only 23% of the isolates showed this characteristic structure. Additionally, only four isolates of OB and none of FB showed recognisable gene cassettes and no genes of resistance to florfenicol and oxytetacycline appeared in them. Of these four isolates, three of them showed a single gene cassette containing the *dfrA-14* gene, which confers resistance to trimethoprim. Whilst the other isolate showed the *aac(6')31-qacH-bla*$_{oxa2}$ genes, which confers resistance to aminoglycosides, quaternary ammonium compounds and beta-lactams, respectively. Finally, it was possible to demonstrate that the described integrons probably come from anthropogenic

activities like clinical settings and/or industrial animal husbandry, since they show integrases proteins identical to those carried by human pathogens.

Introduction

According to the FAO report, Chile is the second largest producer of farmed salmon after Norway, but at the same time, it is the country that consumes the greatest amount of antimicrobials in this activity [1]. This is because the constant infectious diseases that lead to the death of millions of fish. The main disease affecting the three salmonid species farmed in Chile, Atlantic salmon (*Salmo salar*); Coho salmon (*Oncorhynchus kisutch*); and Rainbow trout (*Oncorhynchus mykiss*), is the Salmon Rickettsial Syndrome (SRS), caused by the facultative intracellular bacterium *Piscirickettsia salmonis* [2]. Although this pathogen has been described in all major salmon producing countries, such as Norway, Canada, Scotland, Ireland and Australia [3–5], both the genetic conditions of the Chilean isolates and the particular environmental conditions of the fjords of southern Chile make it much more aggressive than those described in other countries [2]. In addition to the extreme aggressiveness shown by this bacterium in Chile, none of the 40 vaccines available provide sufficient protection to prevent the development of the disease [6–8]. All of the above have led to the use of antibiotics as the main way to control this bacterium.

Between 2007 and 2017, almost 5500 tonnes of antibiotics have been used as an active substance, reaching an average of 500 g of antibiotic per tonne of salmon produced [9]. According to the latest report on the use of antimicrobials by the Servicio Nacional de Pesca y Acuicultura (*National Fisheries and Aquaculture Service*) (Spanish acronym: Sernapesca), 393 tonnes of antibiotics were used in 2017, of which 92.2% corresponded to florfenicol, 6.7% to oxytetracycline and 1% to flumequine [9]. The main route of administration of these antibiotics is through medicated food [10,11]. The administration of medicated food fundamentally affects the gut microbiota of the fish [12], since the constant exposure to antimicrobials leads to the selection of resistant

bacteria and the increase of horizontal gene transfer (HGT) of those elements containing Antibiotic Resistance Genes (ARGs) [13]. In a recent article published by our laboratory, we have described that subsequent treatments with antibiotics select multiresistant bacteria with a high prevalence in ARGs both to florfenicol and oxytetracycline in the gut microbiota of fish [14].

One of the most important elements in the dispersal capacity of the ARGs are the Integron-integrases systems [15]. These elements are bacterial genetic platforms that allow the acquisition, storage, cleavage and rearrangement of genes located in mobilizable elements called gene cassettes [16]. The integron structure is formed by; i) an integrase, whose function is recombine circularized DNA known as gene cassettes; ii) a recombination site called *attI* and; iii) a promoter, PC, that control the genetic expression of the captured genes [17,18]. Integrons participate actively in the bacterial evolution and they are vehicles of gene exchange between the environmental resistome and commensal and pathogenic bacteria [19]. According to the amino acid sequence of integrase proteins, the integrons have been classified into 5 classes [15], however, only the class 1, 2 and 3 integrons are highly associated with the successful dispersion of the ARGs [20]. Even more the class 1 integrons are, precisely, the most described in pathogenic bacteria from humans and animals and, in turn, they are the most abundant integrases in the clinical environment since most of them show ARGs giving resistance to a wide variety of antimicrobials [19]. Thanks to these characteristics, class 1 integron-integrases elements have recently been proposed as indicators of pollution by Antibiotic Resistance Bacteria (ARB), ARGs, and other anthropogenic pollutants [21]

Taking into account the above mentioned facts, the main objective of this work is to determine the prevalence of class 1, 2 and 3 integron-intagrases in banks of bacteria resistant to florfenicol and oxytetracycline, which were obtained from the gut

126 microbiota of fish coming from four fish farms subjected to treatments with high doses
127 of antibiotics. Moreover, we have been able to demonstrate that these class 1 integron-
128 integrases come from the other anthropogenic activities like clinical settings or lan
129 industrial animal husbandry different to the aquaculture and that the constant exposure
130 to antibiotics allows them to remain in the salmon farming system in Chile. The
131 presence of these elements, indicators of contamination by human activities, in the gut
132 microbiota of fish, make this system a perfect environment for the exchange of ARGs
133 between environmental bacterial and fish commensal bacteria. Finally, these genetic
134 elements could be easily released to the environment through the faeces of the fish.
135

136 # Materials and Methods

137 ## Banks of resistant bacteria to florfenicol and oxytetracycline

138 For this study, characterized banks of resistant bacteria to florfenicol and
139 oxytetracycline isolated in our lab were used [14]. Shortly, four Atlantic salmon (*Salmo*
140 *salar*) fish farms were chosen, located in the Aysén Region, North Patagonia, Chile.
141 The farms were selected because at the time of the sample all of them had applied more
142 than one medicated food treatment with antibiotics. Bacteria were isolated from the
143 faeces and the intestine of the fish and they were plated in TSA medium and incubated
144 at 15, 25 and 37 ºC. Minimal Inhibitory Concentration (MIC) to florfenicol and
145 oxytetracycline for all isolates were estimated. Those isolated showing a MIC ≥128
146 µg/mL for florfenicol and ≥32 µg/mL for oxytetracycline were considered to be
147 resistant bacteria, according to EUCAST clinical standards for enterobacteria group.
148 Both banks were taxonomically characterized by amplification and sequencing of the
149 16S gene. The bank of bacteria resistant to florfenicol (FB) consists of 47 isolates, while
150 the bank of bacteria resistant to oxytetracycline (OB) consists of 44 isolates [14]. This

151 study was carried out in accordance with law 20,380 regarding animal welfare, as set
152 out by the Chilean Health Ministry in the use of wild or protected animal species in
153 biomedical research and approved by the National Fisheries Service (SERNAPESCA)
154 and the Pontificia Universidad Católica de Valparaíso Bioethical Committee.
155

156 ## DNA extraction and manipulation

157 Total DNA of all isolates was extracted using the AxyPrep™ Bacterial Genomic DNA
158 Miniprep Kit (Axygen Biosciencies, USA) following the manufacturer's instructions.
159 All PCR products were purified from the gel using the UltraClean® GelSpin® DNA
160 extraction kit (MoBio, USA). All PCR products were cloned using the pCR2.1 TOPO™
161 TA plasmid (Invitrogen, USA). All plasmids were extracted with the E.Z.N.A.™
162 Pasmid mini kit Omega Bio-Tek, USA).
163

164 ## Detection of class 1, 2 and 3 integrases in resistant banks of bacteria

165 In order to determine and characterize the presence of integron-integrase
166 elements in the banks of bacteria resistant to florfenicol and oxytetracycline, partial
167 sequences of the genes which encode for integrase proteins *int1, int2 and int3* were
168 amplified. With this purpose, the primers Int1F-Int1R [22], Int2F-Int2R and Int3F-Int3R
169 [23]were used. Primer used in this study are collected in table 1.
170

171 ## Characterization of class 1 integrons

172 In order to characterize the complete structure of the class 1 integrons, the *sul1*
173 and *qacEΔ1* (3'-CS) genes were amplified, using specific primers in the class 1
174 integrase positive isolates. Hence, the primers Sul1F-Sul1R [24] and qacEΔ1F-

175 qacEΔ1R [25] were used. Furthermore, and in order to amplify gene cassettes contained
176 in the variable region, the primers Hep58 and Hep59 were used according to White et
177 al 2000 [26]. The sequence of the primers and the PCR conditions are collected in Table
178 1. All PCR products were cloned in the pCR2.1 TOPO™ TA (Invitrogen, USA) plasmid
179 and sequenced. All sequences were analysed using the BLAST program [27] and the
180 variable regions were assembled using the CLC Genomics Workbench 10 (QIAGEN).
181 The complete sequence of the integrons were deposited in the GenBank database.
182 Accession numbers of the complete class 1 integrons described are collected in table 4

Primer name	Primer sequence	Product	Annealing temp (°C)	Fragment size (pb)	Reference
int1F int1R	5'-CAGTGGACATAAGCCTGTTC-3' 5'-CCCGAGGCATAGACTGTA-3'	Class 1 integrase gene	59	160	[22]
int2F int2R	5'-GTAGCAAACGAGTGACGAAATG-3' 5'-CACGGATATGCGACAAAAAGGT-3'	Class 2 integrase gene	59	788	[23]
int3F int3R	5'-GCCTCCGGCAGCGACTTTCAG-3'. 5'-ACGGATCTGCCAAACCTGACT-3'	Class 3 integrase gene	59	979	[23]
sul1F sul1R	5'-CTTCGATGAGACCCGGCGGC-3' 5'-GCAAGGCGGAAACCCGCGCC-3'	Sulfonamide resistance gene	65	436	[24]
qacE1F qacE1R	5'-ATCGCAATAGTTGGCGAAGT-3' 5'-CAAGCTTTTGCCCATGAAGC-3'	Quaternary ammonium compounds resistance gene	68	250	[25]
hep58 hep59	5'-TCATGGCTTGTTATGACTGT-3' 5'-GTAGGGCTTATTATGCACGC-3'	Variable region of class 1 integrons	59	Variable	[26]
int01-F int01-R	5'-CTACCTCTCACTAGTGAGG 3' 5'-ACAGTCATAACAAGCCATGA 3'	Complete class 1 integrase gene	58	1014	This study

183 **Table1:** Primers used in this study

184

185 ## Phylogenetic relationship of the class 1 integrase proteins

186 In order to identify the origin of class 1 integrase proteins, the full sequence was
187 amplified using the primers int01-F and int01-R (Table 1). Furthermore, an extensive
188 search for class 1 integrase proteins from different environments such as soil, fresh
189 water, sea water and human pathogens were performed using the INTEGRALL
190 database [28], with the purpose of identifying the phylogenetic origin of the integrase
191 proteins from the banks of oxytetracycline and florfenicol. For the construction of the

192 phylogenetic tree, the amino acid sequences from integrase proteins were used. The
193 analysis of these sequences was performed using MEGA7 applying the maximum
194 likelihood methods with 1,000 bootstraps. Accession number for the complete sequence
195 of the integrases are collected in the supplementary table 1 (Table S1)
196

197 Results

198 Prevalence of integron-integrase elements in florfenicol and
199 oxytetracicline isolate-banks

200 In order to determine the prevalence of the three types of integron-integrases
201 elements, the search was carried out by PCR in both banks of resistant bacteria. We
202 were able to identify that 100% of the isolates from both banks was positive for class 1
203 integrases (Tables 2 and 3). However, we were not able to find class 2 and 3 integrases.
204 Furthermore, a search for the conserved elements of class 1 integrons of genes *sulI* and
205 *qacE*Δ1 was made. One of the most important findings is that, despite all isolates of FB
206 were positive for the class 1 intagrase, none of them was a carrier for the genes *sulI* and
207 *qacE*Δ1 (Table 2). Likewise, 57% of the isolates showed the gene *sulI* in OB, while
208 only 27% showed the gene *qacE*Δ1. Similarly, 23% showed both genes, which is the
209 typical structure of a class 1 integron. Without a doubt, the most important feature of an
210 integron is its variable region. Out of the 44 isolates of OB, only four were positive for
211 the variable region (Table 3) and two isolates were found in different isolates of the
212 same species *Serratia proteomaculans*. In the same way, another variable region was
213 found in one of the important pathogens of salmon farming: *Aeromonas salmonicida*.

Source	Florfenicol Bank	Strain	*int1*	*sul1*	*qacEΔ1*	Variable region	Resistance phenotype	Origin
Farm 1	*Pseudomonas fragi*	13H4	+	-	-	-	AMP, CHL, ERY FLO, OTC	F
	Pseudomonas sp.	13G1	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas fragi	7C5	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F

	Pseudomonas fragi	5B1	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas fluorescens	7A3	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas sp.	4B2	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Serratia sp.	4D1	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas sp.	9A1	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas azotoformans	8C2	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Serratia sp.	9A2	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
Farm II	*Serratia sp.*	1B4	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas fragi	1E2	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas fluorescens	2B4	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas sp.	1D2	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas fragi	6A4	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas sp.	6C5	+	-	-	-	AMP, CHL, ERY, FLO, KM, OTC, TET	F
	Pseudomonas sp.	5B8	+	-	-	-	AMP, CHL, ERY, FLO, OTC	I
	Pseudomonas fluorescens	6H4	+	-	-	-	AMP, CHL, ERY, FLO, KM, OTC, TET	I
	Pseudomonas sp.	5C8	+	-	-	-	AMP, CHL, ERY, FLO, KM	I
	Pseudomonas fluorescens	7B8	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas fluorescens	6D3	+	-	-	-	FLO, OTC, TET	F
	Pseudomonas psychrophila	6A5	+	-	-	-	FLO, KM, OTC, TET	I
	Pseudomonas psychrophila	4G7	+	-	-	-	AMP, CHL, ERY, FLO, OTC	I
	Pseudomonas sp.	6H3	+	-	-	-	AMP, CHL, ERY, FLO, KM, TET	F
	Pseudomonas sp.	4D7	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
Farm III	*Pseudomonas sp.*	1A8	+	-	-	-	AMP, CHL, ERY, FLO, KM	I
	Pseudomonas sp.	1C7	+	-	-	-	AMP, CHL, ERY, FLO, KM	F
	Pseudomonas migulae	3C4	+	-	-	-	AMP, CHL, ERY, FLO	F
	Pseudomonas fluorescens	4A11	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas migulae	7F11	+	-	-	-	AMP, CHL, ERY, FLO, TET	I
	Hafnia sp.	6B1	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas fragi	8B12	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas fluorescens	8F12	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas fluorescens	11H8	+	-	-	-	AMP, CHL, ERY, FLO, KM	F
	Pseudomonas sp.	9F10	+	-	-	-	AMP, CHL, ERY, FLO, KM	I
	Aeromonas molluscorum	9E11	+	-	-	-	AMP, CHL, ERY, FLO	I
Farm IV	*Pseudomonas jessenii*	1G10	+	-	-	-	AMP, CHL, ERY, FLO	I
	Pseudomonas sp.	1H11	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas sp.	1F11	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Pseudomonas sp.	3A8	+	-	-	-	AMP, CHL, ERY, FLO	F
	Pseudomonas fragi	5B4	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas sp.	4C3	+	-	-	-	AMP, CHL, ERY, FLO, KM, OTC, TET	F
	Pseudomonas sp.	10G1	+	-	-	-	AMP, CHL, ERY, FLO	F
	Pseudomonas sp.	8A7	+	-	-	-	AMP, CHL, ERY, FLO, OTC	I
	Pseudomonas sp.	9B4	+	-	-	-	AMP, CHL, ERY, FLO	I
	Pseudomonas sp.	9C6	+	-	-	-	AMP, CHL, ERY, FLO	F

214 **Table 2: Florfenicol bank, antibiotic resistance profile and detection of class 1**
215 **integron elements** Antibiotics used, AMP (ampiciline), CHL (chloramphenicol), CIP
216 (ciprofloxacin), ERY (erythromycin), FLO (florfenicol), KAN (kanamycin), TET
217 (tetracycline). Resistance levels was defined by the EUCAST values for any antibiotic
218 for enterobacteria group. AMP ≥64 µg/ml, CHL ≥32 µg/ml, CIP ≥4, ERY ≥16 µg/ml,
219 FLO ≥128 µg/ml, TET ≥32 µg/ml. Origin: F= Fecal matter; I= Intestine.
220

221

Source	Oxitetracycline Bank	Strain	*int1*	*sul1*	*qacEΔ1*	Variable region	Resistance phenotype	Origin
Farm I	*Serratia proteamaculans*	P151C9	+	+	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Serratia proteamaculans	P151E2	+	+	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Serratia proteamaculans	25P1E12	+	+	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Serratia proteamaculans	30P1XC9	+	+	-	-	AMP, CHL, ERY, FLO,KM, OTC, TET	I
	Serratia proteamaculans	30P1XC8	+	+	-	+	CHL, ERY, FLO, OTC, TET	I
	Serratia proteamaculans	30P1XD3	+	+	-	+	AMP, CHL, ERY, FLO, OTC, TET	I
	Serratia proteamaculans	30PF8	+	+	+	-	CHL, ERY, FLO, OTC, TET	F
	Serratia proteamaculans	30PF10	+	+	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Serratia proteamaculans	30PF11	+	+	-	-	CHL, ERY, FLO, OTC, TET	I
	Serratia proteamaculans	30P1XE10	+	+	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Pseudomonas fragi	30P1XB10	+	+	+	-	CHL, FLO, OTC, TET	F
	Pseudomonas fragi	25P1H2	+	+	+	-	CHL, ERY, FLO,KM, OTC, TET	I
	Pseudomonas fragi	P30F12	+	-	-	-	CHL, ERY, FLO, OTC	I
	Pseudomonas fragi	25P1D4	+	+	+	-	AMP, CHL, ERY, FLO,KM, OTC, TET	I
	Pseudomonas fragi	30P1XE12	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Shewanella baltica	30P1XB8	+	+	+	-	CHL, FLO, OTC, TET	F
	Hafnia alvei	25P3D1	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Rouxiella chamberiensis	30PXG6	+	+	-	-	AMP, CHL, FLO, OTC, TET	I
Farm II	*Rouxiella chamberiensis*	154F5	+	-	-	-	AMP, CHL, FLO, OTC, TET	F
	Carnobacterium maltaromaticum	25P2A9	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET,	F
	Brochothrix thermosphacta	P30XA10	+	+	-	-	AMP, CHL, ERY, FLO,KM, OTC, TET	F
	Brochothrix thermosphacta	P30XB10	+	-	-	-	AMP, CHL, FLO, OTC, TET	I
	Rouxiella chamberiensis	30PXB9	+	+	+	-	AMP, CHL, FLO, OTC, TET	F
	Rahnella sp.	30PXF7	+	-	-	-	AMP, CHL, FLO, OTC, TET	I
	Rouxiella chamberiensis	P30XB3	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Carnobacterium maltaromaticum	P30XD4	+	-	+	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Carnobacterium maltaromaticum	P30XD5	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Carnobacterium maltaromaticum	P30XG8	+	-	-	-	AMP, CHL, FLO, KM, OTC, TET	F
Farm III	*Serratia proteamaculans*	25P1F12	+	+	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Brochothrix thermosphacta	25P3C5	+	-	-	-	FLO, OTC, TET	I
	Brochothrix thermosphacta	30P1XG3	+	+	-	-	CHL, ERY, FLO, OTC, TET	F
	Brochothrix thermosphacta	P30C3	+	-	-	-	CHL, FLO, OTC, TET	I
	Brochothrix thermosphacta	P30C4	+	+	-	-	AMP, CHL, ERY, FLO, OTC, TET	I

	Brochothrix thermosphacta	P30C5	+	-	-	-	CHL, FLO, OTC, TET	I
	Shewanella hafniensis	30PF3	+	-	+	-	CHL, FLO, OTC, TET	F
Farm IV	*Shewnella vesiculosa*	15P2G11	+	+	+	-	CHL, ERY, FLO, OTC	F
	Shewanella putrefaciens	25P3F1	+	-	-	-	ERY, FLO, OTC,TET, STR	I
	Shewanella putrefaciens	15P3A4	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Kluyvera intermedia	15P2D11	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	F
	Kluyvera intermedia	15P2G8	+	-	-	-	AMP, CHL, ERY, FLO, OTC, TET	I
	Psychrobacter nivimaris	15P2H7	+	+	+	-	CHL, ERY, FLO, OTC	F
	Pseudomonas baetica	25P2F9	+	+	+	+	AMP,ERY, FLO, OTC	I
	Pseudomonas fragi	25P2F4	+	+	-	-	CHL,ERY, FLO, OTC, TET	F
	Aeromonas salmonicida	30PB8	+	+	+	+	ERY, FLO, OTC, TET	F

Table 3: Oxytetracycline bank, antibiotic resistance profile and detection of class 1 integron elements Antibiotics used, AMP (ampiciline), CHL (chloramphenicol), CIP (ciprofloxacin), ERY (erythromycin), FLO (florfenicol), KAN (kanamycin), TET (tetracycline). Resistance levels was defined by the EUCAST values for any antibiotic for enterobacteria group. AMP ≥64 µg/ml, CHL ≥32 µg/ml, CIP ≥4, ERY ≥16 µg/ml, FLO ≥128 µg/ml, TET ≥32 µg/ml. Origin: F= Fecal matter; I= Intestine.

Identification of gene cassettes in class 1 integrons

In order to identify possible resistance genes contained in the positive variable regions found in the bank of oxytetracycline resistant bacteria, the potential gene cassettes, amplified from the variable region, were sequenced and identified (Table 4). It was possible to identify gene cassettes in the strains *Pseudomonas baetica* 25P2F9 and *Aeromonas salmonicida* 30PB8, both isolated from fish farm IV. The gene cassette found in the isolate of *Pseudomonas baetica* contained the genes *aac(6')31-qacH-bla*$_{oxa-2}$ which encodes for both aminoglycoside adenylyltransferase - a protein resistant to quaternary ammoniums-, and for oxacillin-hydrolyzing class D beta-lactamase, respectively. With regard to *Aeromonas salmonicida* 30PB8, it was possible to identify the gene *dfrA14* which encodes for a trimethoprim resistance determinant, a dihydrofolate reductase enzyme. Finally, both isolates from the species *Serratia proteomaculans* showed the same gene cassette with the gene *dfrA14* (Figure 1). The other strains containing the genes *sulI* and *qac*Δ*E*, in the typical structure of a class 1

integron, showed no gene cassettes in the variable region, which implies that the system has a great potential to incorporate and express new antibiotic resistance genes. Nonetheless, it is important to note that none of the antibiotic resistance genes to florfenicol and oxytetracycline described in both banks by Higuera-Llanten et al 2018 [14] are found in the integrons of the isolated bacteria. Currently, a complete metagenomic analysis is being carried out to find the relationship between these resistance determinants and these genetic elements that could probably be established in non-culturable bacteria.

Oxitetracycline Bank	Strain	Source	*int1*	*attI* site	Gene cassettes[a]	Size (bp)	Integron Genbank Accesion number
Serratia proteamaculans	30P1XC8	Farm I	+	+	*dfrA14*	483	MG738686
Serratia proteamaculans	30P1XD3	Farm I	+	+	*dfrA14*	483	MG738687
Pseudomonas baetica	25P2F9	Farm IV	+	+	*aac(6')-31, qacH, oxa-2*	547/333/828	MG738684
Aeromonas salmonicida	30PB8	Farm IV	+	+	*dfrA14*	483	MG738685

Table 3: Gene cassette composition in positive Class 1 integrase variable region strains. Genes found: i) *dfrA14* (Trimethoprim-resistant dihydrofolate reductase), resistance to trimethoprim; ii) *aac(6')-31* (Aminoglycoside adenylyltransferase), resistance to aminoglycosides; iii) *qacH* (Quaternary ammonium protein), resistance to quaternary ammonium compounds and; iv) *oxa-2* (Oxacillin-hydrolyzing class D beta-lactamase), resistance to beta-lactam antibiotics.

Figure 1. Scheme of Class 1 integrons. A) Basic class 1 integrons platform.5’ CS: 5’- conserved segment consists of *intI* (gene which encodes for integrase), Pc (promotor), *attI* (recombination site). Structured gene cassettes formed by the recombination site *attC*. 3’ CS: 3’-conserved segment consists of the genes: i) *qacEΔ1* (resistance to quaternary ammonium); ii) *sul1* (resistance to sulfonamides) and; iii) *orf5*,

whose function is completely unknown. **B) Integrones found in the microbiota of *Salmo salar*.**

Class 1 integrases isolated from gut microbiota of *Salmo salar* show a close phylogenetic relationship with clinical important bacteria

In order to evaluate the phylogenetic relationship and, therefore, the possible origin of class 1 integrases identified in the gut microbiota of *Salmo salar*, the complete ORF of 23 genes of the class 1 integrase from both banks was obtained, which were translated into their amino acid sequence. It is worth noting that it was not possible to obtain the gene *intI1* in all the isolates with the primers used. Despite this, a phylogenetic analysis was carried out with the 23 integrases of the isolates from both banks of resistant bacteria, along with various integrases obtained from different environments such as sea water, soil, fresh water and human pathogens. In the obtained phylogenetic tree (Figure 2), 3 differential clades are clearly shown, where integrases from marine environment, soil, fresh water, and from human pathogens are identified. The 23 integrases from the isolates of gut microbiota of *Salmo salar* are grouped in the clade corresponding to human pathogens of clinical importance, such as *Acinetobacter baumannii*, *Salmonella enterica*, *Enterobacter cloacae, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. These data suggest that there is an important contribution component from land anthropogenic activities like clinical settings and/or animal husbandry to the gut microbiota of salmon, and the antibiotics used in this productive activity select indirectly these elements keeping them and dispersing them in the marine environment.

289 **Figure 2. Phylogenetic relationship of class 1 integrases from the salmon industry**
290 **in Chile.** The phylogenetic tree shows class 1 integrases from diverse environments
291 such as seawater, soil, fresh water and human pathogens. It is clearly observed that the
292 integrases from the salmon farming in Chile are grouped together with integrases of
293 pathogens of clinical importance and marked with the Δ symbol. XerC proteins from
294 *Escherichia coli* and *Thiobacillus denitrificans* were used as outgroup. The results were
295 obtained using the maximum likelihood methods after 1,000 bootstraps.
296

297 # Discussion

298 In this work, the high prevalence of class 1 integron-integrase elements in banks
299 of bacteria resistant to florfenicol and oxytetracycline, isolated from the gut microbiota
300 of the Atlantic salmon (*Salmo salar*) treated with high doses of antibiotics in salmon
301 farms has been reported. In a previous laboratory work, it was demonstrated that the
302 high use of antimicrobials brings with it a high abundance of multiresistant bacteria
303 with a high presence of ARGs to both florfenicol and oxitetrecycline. More than 50% of
304 the isolates, counting both banks, showed resistance against at least 4 of the seven
305 antibiotics tested. Despite the fact that an extensive work was performed, in this last
306 work, the presence of integron-like elements was not measured. These genetic elements
307 have been considered of great importance in the antimicrobial resistance phenomenon,
308 since they could play a fundamental role in the transfer of genes between the clinical
309 and the natural environment [16,19]; hence, it has even been estimated that its role in
310 the marine environment is much more important than in the terrestrial environment
311 [29].

312 The occurrence of the three classes of integrons-integrases in the environment
313 has been studied in different works, especially the abundance of class 1 integrons-

314 integrases and the composition of their respective gene cassettes. In this study, the
315 presence of the three classes of integrons-integrases so far described Classes 1, 2 and 3
316 was measured, and it was found that 100% of the isolates from both banks showed class
317 1 integrons; however, no isolate showed class 2 and class 3 integrons. This type of
318 result had already been described in salmonid production systems. In salmon farms of
319 rainbow trout in Australia, 31% of the isolates of *Aeromonas* spp. showed class 1
320 integrons. Nevertheless, class 2 and class 3 integrons were not detected [30]. The same
321 result was obtained with isolates from *Pseudomonas* spp., where 23% was positive for
322 class 1 integrons, while class 2 and class 3 integrons were not detected either [31]. The
323 same was found in catfish farming , where 33% of the isolates from *Pseudomonas* and
324 28% of the isolates from *Aeromonas* showed class 1 integrases, but all of them were
325 negative for class 2 and class 3 intregrases [32]. Only in confined eel farming, in China,
326 the presence of the three classes of integrons in systems related to aquaculture has been
327 demonstrated [33]. Although class 2 integrons-integrases have been detected frequently
328 in different types of environments, class 3 integrons-integrases have rarely been
329 detected outside the clinical setting [16,33]. The high frequency of the appearance of
330 class 1 integrons lacking the typical structure of the 3'-CS is another interesting result
331 of this work, since only 23% of OC showed the genes *qacE*Δ1/*sulI*, while none of the
332 isolates of FB, showed these genes in its structure.

333 But without a doubt, the most important result of this work is that despite the
334 fact that all bacteria showed a high prevalence of antibiotic resistance genes to
335 florfenicol and oxytetracycline, none of these genes were part of these genetic elements.
336 Although tetracycline resistance genes are found with a high frequency in all
337 environments impacted by human activity [34], their presence in class 1 integrons is
338 very scarce. Only sometimes the genes *tetC* and *tetE* have been found to be associated

339 with class 1 integrons [35]. However, due to the high amount of antibiotics used in this
340 industry and the high volume of resistance genes present in the obtained resistant
341 isolates, there is a tendency to think that the incorporation of these genes in this type of
342 gene elements could be favoured, but it seems that the dynamics of the integron-
343 integrases elements in this system is not so evident. A similar case occurs with the
344 genes resistant to florfenicol, *floR* and *fexA*. Up to now, the gene *fexA* has never been
345 described in integrons, while the gene *floR* has rarely been found in class 1 integrons.
346 This is mainly because they are more directly related to horizontal transfer elements
347 such as plasmids and transposons [33]. This fully confirms that despite a high incidence
348 of the genes *floR* and *fexA* in the resistant bacteria isolated from the fish's microbiota,
349 none of these genes could be found as a part of the integrons.

350 Although the 91 resistant isolates present in both banks were positive for the
351 presence of class 1 integrase, only four of them showed recognisable gene cassette
352 structures. In all isolates, it was possible to find genes that appear with high frequency
353 in class 1 integrons. Such is the case of the cassette array found in the species
354 *Pseudomonas baetica*, where the genes *aac(6')-31, qacH* and bla_{oxa-2} are found. The
355 first encodes for aminoglycoside adenylyltransferase; these genes appear with a very
356 high frequency in class 1 integrons [16] since it has been described that more than 80%
357 of these elements contain these types of enzymes in their structures [36]. Thus, it has
358 even been described that genes resistant to aminoglycosides are permanent elements in
359 integrons described in the marine environment [29,37] which gives even more
360 dynamism to the antibiotic resistance phenomenon in aquatic environments [38].
361 Regarding the gene *qacH*, which encodes for a quaternary ammonium resistance
362 mechanism, it was first described in members of the genus *Staphylococcus* [39] but,
363 nowadays, it is frequently found in Gram-negative bacteria, giving them resistance to a

364 broad spectrum of quaternary ammonium compounds, unlike for other genes *qac*
365 [40,41]. Another characteristic of this gene is that it appears relatively often in the food
366 contaminating species *Listeria monocytogenes*, which is associated with resistance to
367 disinfectants derived from benzalkonium chloride [42,43]. This element of resistance
368 has also been found in other bacteria that are frequent food contaminants, such as the
369 members of the genus *Salmonella* [44] and *Staphylococcus aureus*, which contaminates
370 milk [45]. Considering that salmon meat is intended for human consumption, the
371 presence of this element of resistance could represent a risk for eliminating this type of
372 bacteria from the processing plant.

373 Unquestionably, beta-lactamases are the most studied elements in the antibiotics
374 resistance phenomenon, since they represent the biggest problem for human health.
375 OXA-type beta-lactamases have been widely characterised and more than 350 different
376 alleles have been described worldwide [46]. OXA-type beta-lactamases confer
377 resistance to any type of penicillin and some of them can extend their spectrum of
378 activity to cephalosporins [47]. Most of them are part of plasmids in Gram-negative
379 bacteria and they appear relatively often in class 1 integrons, exclusively in human
380 pathogens such as *Pseudomonas aeruginosa* [48], *Salmonella tiphymurium* [49],
381 *Escherichia coli* [50], and *Acinetobacter baumanii* [51]. Those, that have been detected
382 outside the clinical environment, have always been detected in pathogenic bacteria or
383 human commensals [52,53]. Therefore, this is the first time that an OXA-type beta-
384 lactamases betalactamasa, taking part of a class 1 integron associated with aquaculture
385 bacteria, is described.

386 Regarding the integrons found in *Aeromonas salmonicida* and *Serratia*
387 *proteomaculans*, in both cases, the resistance gene to trimethoprim *dfrA14* has been
388 found, which encodes for one of the most distributed alleles of a dihydrofolate

389 reductase. The association of integrons with genes which encode for dihydrofolate-
390 reductases has been widely documented in aquaculture [30,33,54], it has even been
391 possible to demonstrate the presence of these elements in marine sediments associated
392 with the production of salmon in Chile [55,56].

393 Integrons are widely distributed elements in all environments; however, based
394 on the in the IntI Integrase protein sequence, a classification that has allowed them to be
395 classified into different types has been established, and it has been demonstrated that
396 some of them are much more frequent depending on the environment in which they are
397 found [16]. This is how integrases typical of soil bacteria and fresh water, marine
398 bacteria and pathogenic bacteria, both veterinary and human, have been classified [17].
399 It was expected that the bacteria present in the fish's microbiota would be rich in
400 integrons, either from freshwater, from its initial breeding, or from seawater bacteria.
401 Nevertheless, all sequenced integrases are those normally found in human pathogens.
402 This suggests that these elements have come from the land anthropogenic activities like
403 clinical settings and/or animal husbandry and have been highly successful in the salmon
404 farming environment, which could be dangerous due to the probability of exchange of
405 ARGs between land and marine systems.

406 The high presence of integron-integrases elements in aquaculture has been
407 described in several papers [30,33,57,58], even further, the presence of integrons in
408 resistant bacteria isolated from the sediment of salmon farms in Chile has been
409 described in a recently published paper by [56] which has even reached clinical isolates
410 of the bacteria *E. coli*. Among the gene cassettes described in this work, the high
411 abundance of genes *dfrA12* and *dfrA17,* which confer resistance to trimethoprim, as
412 well as genes resistant to aminoglycosides *aadA2* and *aadA5*, can be observed. These
413 genes appear with high frequency in the integron-integrases elements; however, none of

414 them contained genes for resistance to florfenicol or oxytetracycline either, which are
415 the most used antimicrobials in salmon farming in Chile. These results are fully
416 validated in this work.

417 With the data obtained in the present study, we can conclude that the presence of
418 integron-integrase elements is highly abundant in the gut microbiota in farmed Atlantic
419 salmon subjected to treatments with high doses of antimicrobials florfenicol and
420 oxytetracycline. In addition to this, we can say that these elements apparently come
421 from land human activities like clinical settings and/or animal husbandry, since the class
422 1 integrase gene is identical to that found in human pathogenic bacteria of clinical
423 importance such as *P. aeruginosa* or *A. baumanii*. Thus, the contribution of the human
424 activity is could be the main cause of dispersion and dissemination of ARGs in natural
425 environments; also, the large amount of antimicrobials used in aquaculture only favours
426 the maintenance and perpetuation of these elements in the environment. This becomes
427 even more delicate since, if we consider the gut microbiota of fish as a "semi-isolated"
428 system from the environment and due to its direct contact ability with the used
429 antibiotics, it could become the perfect place for genetic exchange to occur between
430 bacteria from different environments. Hence, the gut microbiota of fish treated with
431 high doses of antibiotics could become an ideal reservoir for ARGs, which have a high
432 probability of being dispersed through the faeces, loading the marine environment with
433 these types of genetic elements. The high use of antimicrobials requires a quick
434 solution, consequently, the different production companies are already beginning to take
435 measures to reduce the use of antimicrobials, in the hope that by the year 2020, the use
436 of these drugs is reduced by at least 50%.

437

438

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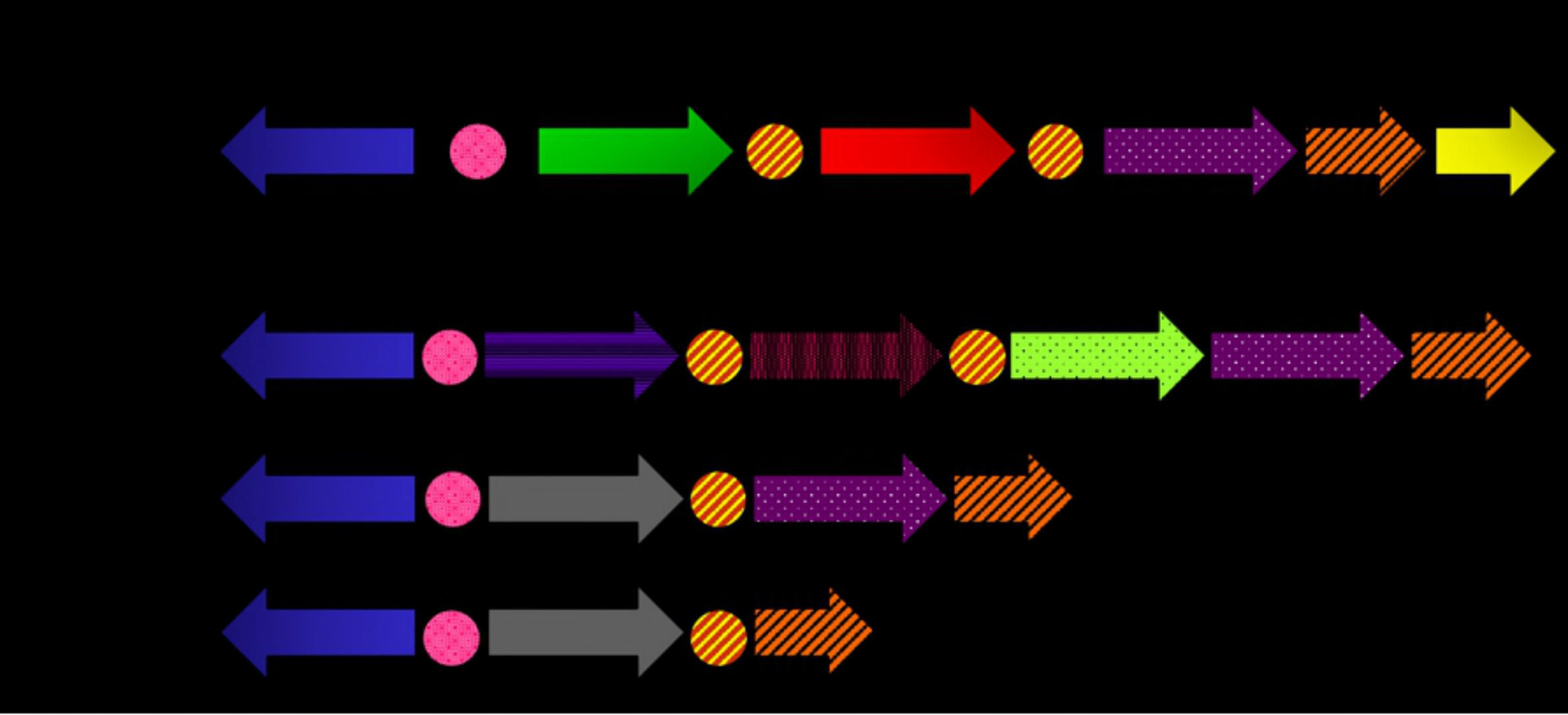

Figure 1

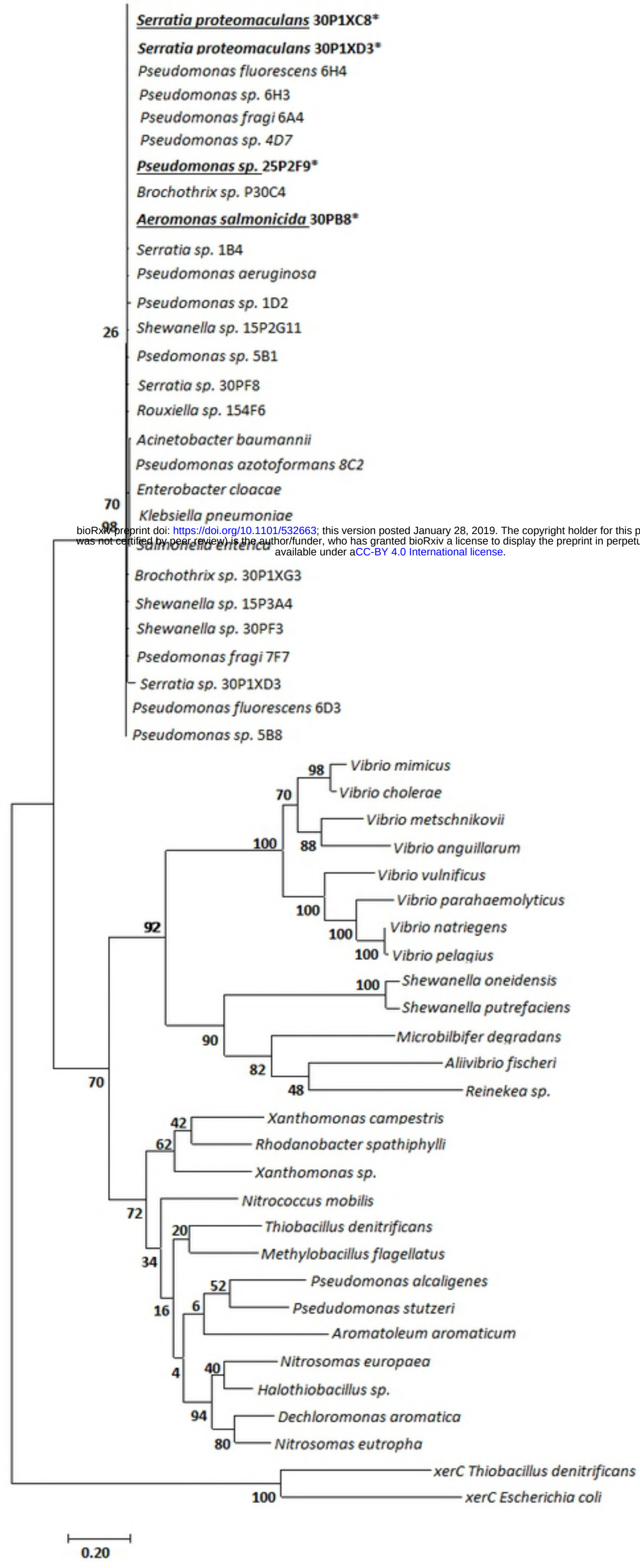

Figure 2