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3 **Detection of the florfenicol resistance gene *floR* in *Chryseobacterium***
4 **isolates from rainbow trout. Exception to the general rule?**

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21

22 **Abstract**

23 Bacteria from the genus Flavobacteriaceae often show low susceptibility to antibiotics. With the
24 exception of two *Chryseobacterium* spp. isolates that were positive for the florfenicol resistance
25 gene *floR*, no clinical resistance genes were identified by microarray in 36 Flavobacteriaceae isolates
26 from salmonid fish that could grow in ≥ 4 mg/L florfenicol. Whole genome sequence analysis of the
27 *floR* positive isolates revealed the presence of a region that contained the antimicrobial resistance
28 (AMR) genes *floR*, a *tet(X)* tetracycline resistance gene, a streptothricin resistance gene and a
29 chloramphenicol acetyltransferase gene. *In silico* analysis of 377 published genomes for
30 Flavobacteriaceae isolates from a range of sources, confirmed that well-characterized resistance
31 gene cassettes were not widely distributed in bacteria from this group. Efflux pump-mediated
32 decreased susceptibility to a range of antimicrobials was confirmed in both *floR* positive isolates
33 using an efflux pump inhibitor (phenylalanine-arginine β -naphthylamide) assay. The *floR* isolates
34 possessed putative virulence factors, including production of siderophores and, haemolysins, and
35 were mildly pathogenic in rainbow trout. Results support the suggestion that, despite the detection
36 of *floR*, susceptibility to antimicrobials in Flavobacteriaceae is mostly mediated via intrinsic
37 mechanisms rather than the horizontally acquired resistance genes more normally associated with
38 Gram-negative bacterial pathogens such as Enterobacteriaceae.

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40 **Keywords: Flavobacteriaceae; *Chryseobacterium*; fish pathogen;**
41 **antimicrobial resistance; virulence; horizontal gene transfer;**

42 **Introduction**

43 Bacteria from the family Flavobacteriaceae are important components of freshwater aquatic
44 ecosystems (Bernardet *et al.* 1996). They include a number of recognised fish pathogens, in
45 particular *Flavobacterium psychrophilum* (Madsen, Møller, and Dalsgaard, 2005) and *Flavobacterium*
46 *columnare* (Starliper and Schill 2011). The group also includes human pathogens, particularly
47 *Elizabethkingia meningoseptica* and *Chryseobacterium indologenes* (Woodford *et al.* 2000; González
48 and Vila 2012).

49 As well as recognised pathogens, there are a number of other organisms that share phenotypic and
50 genotypic characteristics of the main *Flavobacterium* spp., and these are regularly isolated from
51 freshwater fish species ([Michel et al. 2005](#)). Some of these organisms may cause disease in fish.
52 Antibiotics are routinely applied to control disease in fish suspected of being infected with bacterial
53 diseases, including Flavobacteriaceae. However there is only limited knowledge of the relative
54 virulence, their antibiotic susceptibility and their resistance mechanisms of some of these
55 organisms. It has previously been shown that Flavobacteriaceae from fish can grow in high
56 concentrations of a range of antimicrobials ([Chang et al. 1997](#); Rangdale, Richards and Alderman
57 1997; [Bruun et al. 2000](#); [Michel et al. 2005](#); [Clark et al. 2009](#); [Hesami et al. 2010](#); [Henríquez-Núñez et](#)
58 [al. 2012](#)). Studies have shown that clinical strains of *E. meningoseptica* and *C. indologenes* can
59 express extended-spectrum beta-lactamases (ESBLs) ([Bellais et al., 2000, 2002a, 2002b](#); [González](#)
60 [and Vila, 2012](#); [Matsumoto et al., 2012](#); [Woodford et al., 2000](#)). Other studies have demonstrated
61 the presence of multidrug efflux pumps in this group ([Michel et al. 2005](#); [Clark et al. 2009](#)) and there
62 is evidence that quinolone resistance can be mediated via changes in DNA gyrase and topoisomerase
63 genes ([Shah et al. 2012](#)). However, the presence of other well-characterized clinically important
64 resistance genes, particularly those commonly transferred between species of Gram-negative
65 bacteria via mobile elements, such as transposons and plasmids, has not typically been observed in
66 this major group of bacteria, although exceptions include the recording of *dfrA1*, *sul1* and *sul2* genes
67 in a collection of clinical *E. meningoseptica* isolates from China ([Jiang et al. 2012](#)). Multidrug resistant
68 isolates of the duck pathogen *Riemerella anatipestifer* carrying a range of clinical resistance genes
69 have also been identified ([Chen et al. 2012](#); [Sun et al. 2012](#)).

70 Previous work has shown that a range of clinical resistance genes have been identified in other Gram
71 negative pathogens and commensals of fish ([McIntosh et al. 2008](#); [Verner-jeffreys et al. 2009](#); [Welch](#)
72 [et al. 2009](#)). However only limited work has been done to date to identify such genes in
73 Flavobacteriaceae from fish.

74 In this study, a collection of 86 Flavobacteriaceae spp. isolates from fish was analysed for *in vitro*
75 susceptibility to antibiotics using a combination of phenotypic and molecular methods. We also
76 undertook a wider survey of published Flavobacteriaceae genomes from a variety of sources to
77 determine whether the types of antimicrobial resistance mechanisms more commonly associated
78 with Gram-negative clinical and animal pathogens are widely distributed in this important group of
79 bacteria.

80 **Materials and Methods**

81 **Bacterial isolates**

82 A total of 86 *Flavobacterium* spp. isolates were used in this study (Supplementary Table S1). Isolates
83 were recovered from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum), ayu (*Plecoglossus*
84 *altivelis*, Temminck and Schlegel) and Atlantic salmon (*Salmo salar*, Linnaeus) from different origins
85 over a 20-year period (Supplementary Table S1). The isolates were stored at 4°C on tryptic yeast
86 extract salts agar (Holt, Hovec and Fryer 1993) and were then grown at 15°C in Anacker and Ordal
87 broth (Anacker and Ordal 1959). The isolates were all characterized as members of the
88 Flavobacteriaceae, based on morphological and biochemical criteria. Isolates were also tested by a
89 16S rRNA nested PCR method (Wiklund *et al.* 2000) to determine whether they were *Flavobacterium*
90 *psychrophilum* or other species of Flavobacteriaceae. A subset of these other Flavobacteriaceae
91 were further identified on the basis of partial 16S rRNA gene sequencing and analysis using the
92 online tool RDP SEQMATCH (Cole *et al.* 2005).

93 **Antimicrobial susceptibility testing**

94 Susceptibility to 19 antimicrobial agents commonly used in veterinary medicine was assessed by
95 broth microdilution for all isolates using Trek Sensititre Avian susceptibility plates for veterinary use
96 (Trek Diagnostic Systems, Cleveland, Ohio). The antibiotics tested (and ranges) were: enrofloxacin
97 (0.12-2 µg/ml), gentamicin (0.5-8 µg/ml), ceftiofur (0.25-4 µg/ml), neomycin (2-32 µg/ml),
98 erythromycin (0.12-4 µg/ml), oxytetracycline (0.25-8 µg/ml), tetracycline (0.25-8 µg/ml), amoxicillin
99 (0.25-16 µg/ml), spectinomycin (8-64 µg/ml), sulfadimethoxine (32-256 µg/ml), trimethoprim (0.5-2

100 µg/ml)-sulfamethoxazole (9.5-38 µg/ml), florfenicol (1-8 µg/ml), sulphathiazole (32-256 µg/ml),
101 penicillin (0.06-8 µg/ml), streptomycin (8-1024 µg/ml), novobiocin (0.5-4 µg/ml), tylosin tartrate
102 (2.5-20 µg/ml) and clindamycin (0.5-4 µg/ml).

103 Minimum inhibitory concentration (MIC) testing followed guidelines from the Clinical and Laboratory
104 Standards Institute (CLSI 2004), with the minor modifications recommended for testing *F.*
105 *psychrophilum* applied for all the isolates (Hesami *et al.* 2010). Two control strains, *Escherichia coli*
106 ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* NCMB 1102 were also included in
107 parallel in all testing.

108 Resistance to florfenicol is a particular concern as it is the main antimicrobial used to treat bacterial
109 disease caused by *Flavobacterium* spp. in the UK (Verner-Jeffreys and Taylor 2015). Isolates that
110 grew in at least 4 mg/L florfenicol were also tested using Trek Sensititre custom-made florfenicol
111 plates to determine MIC with concentrations from 0.12 mg/L to 128 mg/L. These florfenicol isolates
112 with low susceptibility to florfenicol were also tested for susceptibility to a further 22 antimicrobials
113 typically used to treat clinical Gram-negative infections using Trek Sensititre GN2F plates
114 (Supplementary Table S3).

115 ESBL production in two *Chryseobacterium* spp. isolates (MOF25P and BGARF1; see below) able to
116 grow in high concentrations of florfenicol was tested via a disc diffusion assay (Carter *et al.* 2000).
117 Four different antibiotic discs (Abtek Biologicals, UK) containing cefotaxime (30 µg), cefotaxime +
118 clavulanate (30 µg/10 µg), cefpodoxime (10 µg) and cefpodoxime + clavulanate (10µg/1 µg) were
119 used to assess clavulanate synergy. The inhibition zone sizes were recorded after 24 and 48 h for
120 *Klebsiella pneumoniae* (positive control) and the two *Chryseobacterium* spp. isolates respectively.

121 Susceptibility of *Chryseobacterium* spp. isolates MOF25P and BGARF1 to 16 antimicrobials in the
122 presence of the drug efflux inhibitor phenylalanine-arginine β-naphthylamide (PAβN) was tested via
123 a disc diffusion assay as previously described ([Michel *et al.* 2005](#)) using *Pseudomonas aeruginosa*

124 (NCTCC 12903) as a positive control. The plates were incubated at 22°C or 30°C for *Chryseobacterium*
125 *spp.* and *P. aeruginosa* respectively. The inhibition zone sizes were recorded after 24 and 48 hours
126 for *P. aeruginosa* and *Chryseobacterium spp.* respectively (Michel *et al.* 2005).

127 For reporting of antimicrobial susceptibility, as neither clinical breakpoints or, indeed, robust
128 ecological cutoffs have been established for the diversity of *Flavobacteriaceae* species tested in this
129 study, MIC₁₀, MIC₅₀ and MIC₉₀ values were instead calculated for each antibiotic tested, as
130 recommended by (Schwarz *et al.* 2010).

131 **DNA microarray testing**

132 Isolates were screened for the presence of 75 antimicrobial resistance (AMR) genes using a
133 previously described DNA microarray (Card *et al.* 2013). Approximately 1 µg of genomic DNA was
134 linearly amplified using antisense primers and simultaneously labelled with biotin. The single-
135 stranded labelled amplified products were hybridized to the microarray using the HybPlus Kit (Alere
136 Technologies, Jena, Germany), as described previously (Card *et al.* 2015). Microarray signals were
137 detected with the ArrayMate device (Alere Technologies) using IconoClust software (Standard
138 version; Alere Technologies). Mean signal intensities of two replicate spots per probe were used for
139 analysis and values ≥ 0.5 were considered positive, a signal intensity < 0.5 as negative. Positive
140 results for the florfenicol resistance gene *floR* were confirmed by PCR (Welch *et al.* 2007).

141 **Whole genome sequencing of *Chryseobacterium* isolates MOF25P and BGARF1.**

142 Two of the isolates, MOF25P and BGARF1, that had low susceptibility to florfenicol and were positive
143 for the resistance gene *floR* by microarray and PCR (see Results) were whole genome sequenced and
144 examined for the presence of antibiotic resistance genes. For whole genome sequencing, isolates
145 were sequenced using 150 bp paired end reads on an Illumina MiSeq platform.

146 Twenty bases at the 5' end of each read, which had low quality scores FastQC (Andrews 2010), were
147 removed using the FASTX Toolkit (Gordon and Hannon 2010). Paired reads were assembled using
148 Velvet (Zerbino and Birney 2008). A kmer value of 89 was used for assembly, together with a

149 minimum contig length of 200 bp and an expected insert length of 200 bp. Automatic annotation
150 was carried out using RAST ([Aziz *et al.* 2008](#)) and Prokka (Seemann 2014). Manual confirmation of a
151 group of ORFs identified as putative virulence and antimicrobial resistance genes (Supplementary
152 Table S4) was undertaken using individual BLAST searches ([Altschul *et al.* 1997](#)). After assembling
153 isolate MOF25P and BGARF1 using Velvet, contig sequences were compared against the *floR* gene.
154 Any contig with a region strongly matching the *floR* gene was read into Artemis (Rutherford *et al.*
155 2000) and the GC content around the *floR* gene was examined. Contigs were also searched for
156 putative AMR genes using the online Comprehensive Antibiotic Resistance Database (CARD)
157 maintained by McMaster University ([McArthur *et al.* 2013](#)). The two draft genome sequences have
158 been submitted to Genbank, with BioSample Accessions SAMN03764887 and SAMN03764910,
159 corresponding to isolates MOF25P and BGARF1, respectively.

160 **Analysis of publically available Flavobacteriaceae genomes for AMR genes**

161 A list of draft and complete genomes from the Flavobacteriaceae family was retrieved from the
162 NCIMB taxonomic database (accessed 27 Nov 2015), through the Entrez API (Entrez Programming
163 Utilities Help, 2010), (Supplementary Datasheet 1). Assembly and nucleotide datasets were
164 downloaded from NCBI. All of the genomes were converted into Fasta files, with the DNA sequence
165 of any contiguous sequence, or contig, assembled from the genome. All of the genomes were then
166 added into a single blast database. The nucleotide sequences from the ARG-Annot database (Gupta
167 *et al.* 2014) were aligned to the blast database using the Nucleotide Blast algorithm (Camacho *et al.*
168 2009). Potential AMR genes were excluded if the alignment length was less than 90% of the query
169 sequence length, and the aligned sequences shared less than 90% sequence identity. Genomes were
170 re-annotated with Prokka (Seemann 2014), using default settings. Annotated AMR genes were
171 examined for proximity to Mobile Genetic Elements (MGEs), using custom Python scripts, and the
172 Artemis genome viewer ([Rutherford *et al.* 2000](#)). In addition, genomes were analysed for the
173 presence of genomic islands using the online tool IslandViewer 3 ([Dhillon *et al.* 2015](#)) and for
174 plasmid sequences using PlasmidFinder (Carattoli *et al.* 2014) and custom Python scripts.

175 **Virulence factor identification**

176 **Haemolysin production**

177 Isolates were streaked onto sheep blood agar and rainbow trout blood agar. Plates were incubated
178 at 22°C for 7 days and observed daily for growth and zones of clearing. This experiment was carried
179 out at 10, 15, 22, 30 and 37°C for isolates MOF25P and BGARF1.

180 A microplate hemolysis assay was carried out as described previously (Hogfors-Ronnholm and
181 Wiklund 2010) with sheep blood in Alsever's solution (Oxoid), instead of rainbow trout blood.
182 Isolates were classed as hemolytic if they caused 20% or more hemolysis using the following

183 formula: $\frac{\text{Experimental value} - \text{negative control}}{\text{Positive control} - \text{negative control}} \times 100$.

184 **Iron acquisition and growth in iron deprived media**

185 Growth of *Chryseobacterium* spp. isolates MOF25P and BGARF1 in iron deprived (100 µM 2,2'-
186 bipyridyl amended) tryptone soy broth was tested as previously described (Hirst, Hastings and Ellis
187 1991). *A. salmonicida* isolate 96001 from the Cefas culture collection was included as a positive
188 control. Siderophore indicator chrome azurol S (CAS) agar (Schwyn and Neilands 1987) was
189 inoculated with MOF25P or BGARF1 and monitored for growth and agar colouration for 10 days. *A.*
190 *salmonicida* isolates Cefas 96001 and Cefas 01190 were used as positive and negative controls
191 respectively (Holzberg and Artis 1983; Schwyn and Neilands 1987).

192 **Challenge experiments**

193 Experiments were undertaken to assess the potential virulence of MOF25P and BGARF1 when
194 injected intramuscularly into rainbow trout. For challenge experiments, cultures of MOF25P and
195 BGARF1 were grown in MVA broth at 15 °C for 72 h, washed twice in sterile phosphate buffered
196 saline (PBS) and re-suspended in sterile PBS. For the first experiment, three groups of six rainbow
197 trout (approx. 3 g) were injected with of MOF25P at 5×10^4 , 5×10^3 and 5×10^2 cfu/fish. For the
198 second experiment two groups of sixteen (approx. 4g) fish were each injected with either MOF25P
199 or BGARF1 at 1.5×10^5 cfu/fish. Fish were maintained at 10-12°C in 10L flow-through tanks for up to

200 11 days. Moribund fish were removed from the tank and killed by terminal anaesthesia and
201 destruction of the brain. Spleen and head kidney swabs were taken from moribund fish, mortalities
202 and fish killed at the end of the study. In addition, samples of gill, kidney, spleen, intestine and liver
203 were fixed in neutral buffered formalin, processed to paraffin blocks, sectioned and stained with
204 haematoxylin and eosin for assessment of pathological changes. Specific mortalities were confirmed
205 from the growth of swabs inoculated onto MVA. Inoculated plates were monitored daily for growth.
206 All experiments involving fish were performed at the University of Stirling under the authority of the
207 relevant UK Home Office Animals (Scientific Procedures) Act 1986 Project Licenses and approved by
208 the University of Stirling Animal Welfare Ethical Review Body. All regulated procedures were
209 undertaken by staff holding A(SP)A Personal Licenses for the particular techniques.

210 **Results**

211 **Antimicrobial susceptibility**

212 Isolates tested covered a range of Flavobacteriaceae species (Supplementary Table S1). In general, it
213 can be stated that many of the isolates had low susceptibility to the antimicrobials tested (Figure 1
214 and Supplementary Figure S1). There were also no obvious temporal or geographic variations in
215 observed patterns of susceptibility observed. Many isolates were able to grow in the presence of
216 relatively high concentrations of the main antimicrobials that are used to control bacterial diseases
217 in farmed fish in the UK and elsewhere in the world. For example, the MIC₅₀ value for amoxicillin
218 was ≥ 16 mg/L. MIC₅₀ values for both oxytetracycline and florfenicol were 2mg and their MIC₁₀
219 values were ≥ 8 and 32 mg/L respectively. Although most isolates were highly susceptible to
220 sulfamethoxazole/trimethoprim (MIC₅₀ <0.5/9.5 mg/L), the MIC₁₀ value was $\geq 2/3$ mg/L.

221 We also tested susceptibility to a range of other antimicrobials commonly used to treat bacterial
222 diseases in humans and other animals (Figure 1, Supplementary Figure S1 and Supplementary Tables
223 S2-3). Many of the isolates could grow in high concentrations of 3rd generation cephalosporins (e.g.
224 the MIC₅₀ for ceftiofur was ≥ 4 mg/L) and aminoglycosides (the MIC₅₀ for streptomycin was ≥ 32
225 mg/L). Most isolates were inhibited by low concentrations of the fluoroquinolone enrofloxacin,

226 although the (MIC₁₀ was still 1 mg/L). A total of 56 % of isolates were able to grow in high
227 concentrations of four or more representatives of different structural classes of antimicrobials
228 (Figure 2), suggesting intrinsic resistance or acquired reduction in susceptibility to multiple
229 antibiotics.

230 Out of the 86 isolates, 36 isolates that grew in the highest florfenicol test concentration (4 mg/L) in
231 the Trek Sensititre Avian susceptibility plates were chosen for further analysis (Supplementary
232 Tables S2-3). As shown in Supplementary Table S2, susceptibility to florfenicol in these 36 isolates
233 varied from 2 to 128 mg/L when re-tested using the custom florfenicol testing panels. Susceptibility
234 to a range of antimicrobials used to treat Gram-negative clinical infections was also assessed
235 (Supplementary Table S3). Many of the isolates able to grow in ≥ 4 mg/L florfenicol also displayed
236 low susceptibility to 1st, 2nd and 3rd generation cephalosporins, and other classes of antimicrobials
237 (Supplementary Table S3). Many grew in the highest concentrations of antimicrobials tested in the
238 panels used. For instance 32/36 isolates grew in ≥ 32 mg/L ceftriaxone (a 3rd generation
239 cephalosporin), 28/36 in ≥ 32 mg/L cefepime (a 4th generation cephalosporin) and 27/36 in ≥ 64 mg/L
240 nitrofurantoin ; Supplementary Table S3). Isolates showed most sensitivity to the two carbapenems
241 tested, imipenem and meropenem, although even here, 12 and 4 out of the 36 tested isolates were
242 able to grow in >4 mg/L of meropenem and imipenem respectively.

243

244 **Resistance gene arrays**

245 The 36 isolates that could grow in ≥ 4 mg/L florfenicol were examined by microarray for the presence
246 of clinically important resistance genes. No clinical resistance genes were identified by microarray in
247 any of the isolates, with exception of two of the isolates, MOF25P and BGARF1. Both these isolates
248 could grow in high concentrations of florfenicol *in vitro* (MIC of 128 and 64 mg/L respectively). These
249 were strongly positive for the resistance gene *floR* by microarray (values of 0.84 and 0.89
250 respectively). The positive results by microarray were confirmed as *floR* positive by PCR and whole
251 genome sequencing (see below).

252 **Draft whole genome sequencing of *Chryseobacterium* isolates MOF25P and BGARF1**

253 The total length of draft genomes of MOF25P and BGARF1 were estimated at 4,661,207 bp and
254 4,675,015 bp respectively. A total of 310 contigs were obtained from strain MOF25P and 260 contigs
255 from BGARF1, with N50 values of 44,054 for MOF25P and 71,571 bp for BGARF1. Minimum coverage
256 was estimated at more than 30 fold for both isolates. Using the RAST annotation pipeline, a total of
257 4336 coding regions were found in the genome of BGARF1, of which 1275 (29%) could be annotated
258 functionally. The functional comparison of genome sequences available on the RAST server revealed
259 the closest neighbours of both MOF25P and BGARF1 to be *Chryseobacterium gleum* ATCC 35910
260 (score of 546). Similarity to a sequenced strain of *F. psychrophilum* JIP 02/86 (Taxonomy ID: 402612)
261 was 188. Comparison of the (identical) near full length 16S rRNA genes recovered from both BGARF1
262 and MOF25P with sequences derived from other *Chryseobacterium* spp. showed the closest
263 neighbours to be *Chryseobacterium piscium* and *Chryseobacterium bastulinum*, but distinct from
264 both (Figure 3).

265 Analysis of the assembled contigs for genes that likely code for AMR genes demonstrated the
266 presence, in single contigs from both isolates, of a region that contained the florfenicol resistance
267 gene *floR*, a *tet(X)* tetracycline resistance gene, a streptothricin resistance gene and a
268 chloramphenicol resistance gene of the chloramphenicol acetyltransferase (*cat*) family (Figure 4). In
269 this structure, there is a putative relaxase/mobilisation protein upstream of the *floR* and *cat* genes

270 and a putative transposase downstream of both genes (Figure 4). In addition, an approximately 3kb
271 region containing the *floR* element and the putative transposase in both strains had a markedly
272 higher GC content (58%) compared to the rest of the genomes (34%). When analysed by BLASTn, the
273 high GC content region closely aligned (a single mismatch and no gaps or insertions over the same
274 3395 compared bases in each case) to parts of sequences derived from different Gram negative
275 bacteria and their associated plasmids (e.g. Accession numbers CP014775, KU302354 and
276 KF250428).

277 The area was identified as part of a possibly horizontally acquired genomic region in both isolates by
278 two different genomic island prediction methods (SIGI-HMM ([Waack et al. 2006](#)) and IslandPath-
279 DIMOB (Langille, Hsiao and Brinkman 2008)) when the draft genomes were analysed using
280 IslandViewer3. Although IslandViewer3 identified seven other regions that may have been acquired
281 by HGT, this was the only region identified by both prediction methods (Supplementary Figure 2).
282 Furthermore, it was the only region with a markedly different (higher) GC content to the rest of the
283 genome (Supplementary Figure 2). In terms of other potential mobile elements, no sequences of
284 putative plasmid origin were identified in silico in either draft genome. Putative AMR genes were also
285 identified in other regions of the two genomes by both CARD ([McArthur et al. 2013](#)) and RAST (Aziz
286 et al. 2008) (Supplementary Table S4). Beta-lactamase genes and, in particular one ORF, were
287 present in both isolates (AB670_04238 in MOF-25P and AB671_02057 in BGARF1; Supplementary
288 Table S4) coded for a protein containing 292 amino acids with greater than 70% identity to
289 previously characterized Ambler Class A VEB-like ESBL recovered from other Flavobacteriaceae
290 (Bellais, Naas and Nordmann 2002a). The presence of a number of ORFs with high identity to genes
291 encoding multidrug efflux pumps was also noted (Supplementary Table S3). Confirming the results of
292 the microarray analysis, with the exception of *floR*, no clinically important resistance gene
293 sequences, which would hybridise with the probes represented on the array, were detected in the
294 draft genome sequences of either isolate. It is likely that the region containing *floR* and *catB* in the
295 two isolates was chromosomally located as no DNA sequences with known plasmid-related functions

296 were detected on the two large contigs (70.5 and 144kb respectively) that the region was present
297 on. Furthermore, examination of all the contigs containing putative AMR genes, did not reveal any
298 with much greater than average read depth, which would have been indicative of location on a multi
299 copy plasmid.

300 *PA β N and ESBL assays*

301 For isolates MOF25P and BGARF1, moxalactam, ciprofloxacin, enrofloxacin, ofloxacin, oxolinic acid,
302 florfenicol, chloramphenicol, erythromycin and cephalothin all gave increased inhibition zones in the
303 presence of the efflux pump inhibitor PA β N (Figure 5). No change in inhibition zone size was seen for
304 ampicillin, amoxicillin or oxytetracycline and a decreased inhibition zone size was seen for
305 gentamicin and streptomycin (Figure 5). Isolate BGARF1 produced the same results, except for
306 flumequine which had a decreased inhibition zone size. The average inhibition zone differences
307 ranged from 4 to 17.5 mm and from 2 to 9.7 mm for isolates BGARF1 and MOF25P respectively. For
308 the positive control, *P. aeruginosa*, there was no inhibition zone difference for ampicillin or
309 amoxicillin, but there was an increase for all other antimicrobial compounds tested (Figure 5). The
310 difference in inhibition zone sizes in the presence and absence of the inhibitor ranged from 0 to 32.5
311 mm. The mean average inhibition zone difference was 15.2 mm.

312 The presence of the ESBL inhibitor clavulanate caused an inhibition zone increase for both
313 sequenced *Chryseobacterium spp.* isolates when using cefpodoxime and cefotaxime. The average
314 inhibition zone increase (across three experiments) for cefpodoxime was 3.5 and 4.7 mm for isolates
315 MOF25P and BGARF1, respectively. The average increase for cefotaxime was 10.1 and 12.4 mm for
316 MOF25P and BGARF1, respectively.

317 *In silico analysis of published Flavobacteriaceae genomes for AMR genes and mobile* 318 *elements*

319 To further test the hypothesis that AMR genes and mobile elements more commonly associated
320 with Gram-negative bacteria (particularly Proteobacteria) are not typically found within
321 Flavobacteriaceae, available genomes from this family of bacteria were examined *in silico*. A total of

322 377 genomes were accessed from publically available databases and examined for the presence of
323 both AMR genes and mobile elements. The sequenced genomes represented examples of a range of
324 genera and species from clinical and environmental sources (Supplementary Datasheet 1). A total of
325 81/377 isolates had one or more matches to resistance genes in the ARG-Annot database. The most
326 common resistance determinants identified (Supplementary Datasheets 2-3) were genes coding for
327 putative resistance to beta-lactams (65/377, tetracyclines (16/377). Genes coding for putative
328 resistance to macrolide/lincosamide/streptomycin G group antibiotics (4/377) and phenicols (3/377)
329 were also identified . The beta lactamase genes identified (Supplementary Data Sheet 3) were
330 typically those known as naturally occurring in *Chryseobacteria* and other Flavobacteriaceae that
331 code for Amber Class B and other enzymes, such as *bla_B*, *bla_{MUS}*, *bla_{TUS}* , *bla_{IND}*, *bla_{CFX}*, *bla_{JOHN}* and
332 *bla_{GOB}* (Bellais *et al.* 2000; Woodford *et al.* 2000; Bellais, Naas and Nordmann 2002a, 2002c;
333 Mammeri, Bellais and Nordmann 2002; Naas, Bellais and Nordmann 2003; Gonzalez and Vila 2012;
334 González and Vila 2012).

335 In terms of well characterized AMR genes typically associated with Gram-negative clinical and
336 veterinary pathogens, only *floR* and *catB* were identified. These were associated with plasmid
337 elements found in isolates of the duck pathogen *Riemerella anatipestifer* (Chen *et al.* 2012). The two
338 tetracycline resistance genes identified, *tet(X)* and *tet(Q)*, are primarily associated with
339 Bacteroidetes group organisms (Shoemaker *et al.* 2001; Yang *et al.* 2004).

340 Search of the integral database (Moura *et al.* 2009) also recovered a limited number of matches to
341 Flavobacteriaceae. For example, searches using the terms 'Flavobacterium' and 'Elizabethkingia'
342 returned no matches while 'Chryseobacterium' returned three accession numbers with sequences
343 containing putative class 1 integrons (Accession numbers: KM278188, KJ561906, KM278184). All
344 three deposited sequences were reportedly recovered from *Chryseobacterium* spp. isolated from
345 Chinese river water samples. The sequences all contained well-characterized resistance gene
346 cassettes (including dihydrofolate reductase (*drfA12*), aminoglycoside adenylyltransferase (*aadA2*,

347 *aacA4*) and rifampin ADP-ribosylating transferase (*arr-3*) genes. *R. anatispestifer* was different with a
348 number of matches in the integral database to well-characterized resistance gene cassettes and class
349 1 integrons. No matches to plasmid sequences on PlasmidFinder database were found.

350 **Additional phenotypic characterization of *Chryseobacterium spp.* isolates**

351 **Identification of other virulence factors**

352 Both isolates were able to grow in iron restricted conditions. They also produced siderophores when
353 grown on CAS agar. The isolates also produced haemolysins when grown on both sheep and rainbow
354 trout blood agar and in a micro plate assay with sheep erythrocytes. Genome analysis identified a
355 putative haemolysin-encoding gene in both isolates (Locus Tags AB670_00278 and AB671_02880,
356 Supplementary Table S4) and a putative aerobactin-like siderophore (Locus Tags AB670_03217 and
357 AB671_00904, Supplementary Table S4) in isolates MOF25P and BGARF1.

358 **Challenge experiments**

359 Injection of groups of six 4g rainbow trout with doses of 5×10^5 cfu /fish of both MOF25P and
360 BGARF1 resulted in high mortalities in both groups by day 11 post-infection (5/6 and 4/6
361 respectively), when the experiment was terminated for welfare reasons (Figure 6). There were no
362 mortalities in the groups of 3 g rainbow trout injected with doses of MOF25P or BGARF1 at 5×10^4 , 5
363 $\times 10^3$ or 5×10^2 cfu/fish. No consistent pathology was seen, although renal haematopoietic tissue
364 necrosis was present in one and two fish infected with MOF25P or BGARF1 respectively. Bacteria
365 were not recovered by culture from affected tissues.

366 **Discussion**

367 This study confirms the results of earlier reports that many of the Flavobacteriaceae isolated from
368 fish, the environment and clinical samples, have low susceptibility to a range of antimicrobials (Kirby
369 *et al.* 2004; [Michel *et al.* 2005](#)). Despite high MICs to a wide range of antimicrobials of different
370 structural classes, analysis of 36 isolates using a microarray revealed that carriage of clinically
371 relevant AMR genes commonly found in other Gram-negative bacteria was rare. Indeed, only two
372 isolates were microarray positive for a single AMR gene, in both cases the florfenicol resistance gene

373 *floR*. Additional *in silico* analysis of 377 publically available genomes further demonstrated that such
374 clinical resistance genes were not widely distributed in this family. [Michel et al. \(Michel et al. 2005\)](#)
375 analyzed multidrug resistant *Chryseobacterium* isolates recovered from fish species and did not
376 detect any clinically relevant AMR genes, including *floR*. This is in contrast to previous studies other
377 Gram negative bacterial pathogens of fish (McIntosh et al. 2008; Verner-jeffreys et al. 2009; Welch
378 et al. 2009)

379 The results from the efflux pump inhibitor assays showed that susceptibility to a range of antibiotics
380 (including moxalactam, flumequine, ciprofloxacin, chloramphenicol, florfenicol, erythromycin,
381 furazolidone, enrofloxacin, oflaxofacin and oxolinic acid) increased in the presence of the inhibitor
382 PA β N for both MOF25P and BGARF1 (Figure 5). Genome analysis identified a number of putative
383 multidrug efflux pumps in the *Chryseobacterium spp.* genomes (Supplementary Table S4). Results in
384 general therefore agree with [Michel et al. 2005](#) who concluded that low susceptibility to
385 antimicrobials in these organisms is largely intrinsic, particularly through expression of multidrug
386 efflux pumps. Other studies have also identified that multidrug efflux pumps are widely distributed
387 in this as in other groups of bacteria, suggesting they have an ecological role related to survival in
388 the aquatic environment ([Pidcock 2006a](#); [Martinez et al. 2009](#)), as well as potentially conferring
389 resistance to antimicrobials in clinical and aquaculture settings (Van Bambeke, Balzi and Tulkens
390 2000; Pidcock 2006b; Poole 2007; Nikaido and Pagès 2012). As well as efflux mechanisms, it is
391 possible that other mechanisms may also be important, including decreased permeability via porins
392 (Pages, James and Winterhalter 2008).

393 Conversely, oxytetracycline, amoxicillin and ampicillin susceptibility was unaffected by PA β N, which
394 suggests the presence of different mechanisms are responsible for susceptibility to these
395 antimicrobials in the *Chryseobacterium spp.* isolates MOF25P and BGARF1. It is possible that point
396 mutations to chromosomal genes may have a role in lowered susceptibility, as has been
397 demonstrated for other Gram negative bacteria to a number of antibiotics. These include

398 quinolones ([Shah et al. 2012](#)), sulphonamides ([Vedantam et al. 1998](#)) and beta lactams (Zapun,
399 Contreras-Martel and Vernet 2008).

400 Beta-lactamase genes were detected in the organisms. The isolates also appeared to demonstrate a
401 degree of clavulanate synergy for the two cephalosporins tested, as inferred via double disc diffusion
402 testing with ESBL inhibitors. Beta-lactamases, including ESBLs, have been detected in a range of
403 other Flavobacteriaceae, including *Chryseobacterium* spp. ([Bellais et al. 2000](#); Bellais, Naas and
404 Nordmann 2002b, 2002c; [Yum et al. 2010](#)). Further work is needed to validate that the observed
405 clavulanate synergy observed was related to production of ESBLs by the two organisms. In this
406 regard, the potential role of a putative VEB-like ESBL (Locus tag AB670_04238 ; Supplementary Table
407 S4) also needs to be determined. Genes encoding tetracycline inactivating enzymes (*tet(X)*) were
408 also found, likely explaining the observed resistance to oxytetracycline, even in the presence of the
409 efflux pump inhibitor. It was noteworthy that susceptibility to florfenicol *in vitro* was reduced in both
410 isolates in the presence of the efflux pump inhibitor, suggesting that the high MICs may result from a
411 combination of the effects of *floR* gene expression and efflux pumps.

412 An identical genomic region containing four AMR genes, including *floR*, was found in the genomes of
413 both *Chryseobacterium* spp. isolates we sequenced: MOF25P and BGARF1 (Figure 4). There is
414 evidence that a small part of this region, around 3kb in size, was introduced through horizontal
415 transfer, given the close match against plasmids (*e.g.* accession number KF787110.1) in other
416 bacterial species, such as *Proteus mirabilis*, *Klebsiella pneumoniae* and *Vibrio cholerae*, with very few
417 mismatches, or indels in the alignment and the higher GC percentage across the same region. The
418 remaining genes coding for antibiotic resistance, and gene transfer, which fall outside of this 3kb
419 region, aligned less well against published sequences, and had a lower GC percentage more typical
420 for the genome as a whole. However, the extent to which the genes in this area are actively
421 expressed and functional in these organisms at high level is uncertain. Further functional-genetic
422 work will be necessary to determine if these genes contribute to the antimicrobial susceptibility

423 phenotypes observed in these strains. Further work is also required to more accurately assign
424 isolates MOF25P and BGARF1 phylogenetically. They may represent examples of a new
425 *Chryseobacterium* species (Figure 3).

426 Additional *in silico* analysis of published Flavobacteriaceae genomes was undertaken, to determine
427 whether such elements are commonly associated with this group. Results suggest they are indeed
428 relatively rare. The majority of the AMR genes identified coded for beta-lactamase and tetracycline
429 resistance genes, known to be constitutively expressed in this group of bacteria. There were also
430 only limited records in the integral database of integrase class 1 genes and resistance gene cassettes.
431 An apparent exception to this general trend appears to be the duck pathogen *R. anatipestifer*,
432 particularly isolates that are circulating in farmed ducks in China ([Chen *et al.* 2012](#); [Sun *et al.* 2012](#)).
433 As well as a range of important clinical resistance gene cassettes, *floR*, has also been isolated from
434 this pathogen. It is noteworthy that *floR* has only been identified to date in Flavobacteriaceae from
435 fish and livestock farming environments, where it is likely that florfenicol and other veterinary
436 antibiotics are widely applied.

437 It is possible that transfer of clinically relevant resistance genes between the Proteobacteria spp.
438 that typically host such elements and Flavobacteriaceae, and their subsequent active expression and
439 maintenance, may be restricted due to genetic incompatibility between these evolutionary distant
440 groups of bacteria. It has long been known that genetic manipulation of bacteria of the phylum
441 *Bacteroidetes* is problematical as the selectable markers, cloning vectors, transposons, and
442 convenient methods of gene transfer used for manipulation of Proteobacteria and other organisms
443 do not tend to function in this group (McBride and Baker 1996; Staroscik *et al.* 2008).

444 The two *Chryseobacterium* spp. isolates MOF25P and BGARF1 produced positive results in the CAS
445 agar assay for production of siderophores and all three hemolysis assays. This complements the
446 genome analysis, which identified a putative haemolysin and an aerobactin-like siderophore
447 encoding genes in each genome (Supplementary Table S4). Siderophore production has also been

448 detected in *F. psychrophilum* isolates (Møller *et al.* 2005). It is possible that the haemolysins
449 produced by the *Chryseobacterium spp.* isolates are involved in disease progression, as they are
450 likely to be in *F. psychrophilum* (Secades, Alvarez and Guijarro 2003; Pérez-Pascual *et al.* 2011).

451 MOF25P and BGARF1 were recovered from rainbow trout with disease symptoms. However the
452 challenge results were equivocal, with high mortalities observed in groups of rainbow trout injected
453 with high doses of the two bacteria, but not in a group of rainbow trout injected with lower doses of
454 the organism, or in groups of Atlantic salmon challenged separately at Cefas Weymouth Laboratory
455 (unpublished data). *Chryseobacterium* species, including *C. piscium*, that isolates MOF25P and
456 BGARF1 share a degree of genetic identity with, have been strongly associated with disease in
457 Atlantic salmon and rainbow trout (Ilardi *et al.* 2010; Zamora *et al.* 2012). However, as with this
458 study, other workers report that reliable induction of disease symptoms following direct exposure to
459 these organisms is difficult to achieve (Ilardi *et al.* 2010). This suggests that these organisms may
460 have a role in disease only under certain circumstances (e.g. when animals are stressed or otherwise
461 compromised). Further work is required to determine, at a mechanistic level, potential barriers to
462 gene transfer and functional expression of antibiotic resistance genes, such as *floR*, typically
463 associated with other groups of bacteria, e.g. Proteobacteria (Guglielmini *et al.* 2011) into
464 Flavobacteriaceae .

465 There is considerable interest in the role that aquatic and other environmental organisms may have
466 in the development, acquisition, maintenance and spread of AMR (Taylor, Verner-Jeffreys and Baker-
467 Austin 2011). Particularly how this may relate to development of drug resistant infections in humans
468 and animals, including fish. Thus a deeper understanding of the mechanisms that may restrict, as
469 well as promote, the acquisition and spread of AMR genes between environmental, clinical and
470 veterinary pathogens is required to inform robust risk assessments in this highly important area.

471 In summary, low susceptibility to antibiotics is common in Flavobacteriaceae but this appears to be
472 largely intrinsic and conferred by genes/mechanisms different to those present in many clinically

473 relevant Gram-negative bacteria (e.g. Enterobacteriaceae). In some respects it is reassuring that
474 these bacteria do not appear to be significant reservoirs of many of the AMR genes that are causing
475 problems in humans and terrestrial animals. However the demonstration that *floR* and *tet(X)* were
476 likely acquired in bacteria from this group via a horizontal transfer event from an unrelated organism
477 may represent a new risk for the dissemination of resistance into the aquatic environment.

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662 **Figures**

663 **Figure 3** Phylogenetic relationships of partial 16S rRNA gene sequences derived from
664 Chryseobacteria isolates to isolates BGARF1 and MOF25P. The evolutionary distances were
665 computed using the Maximum Composite Likelihood method (Tamura et al., 2011). Evolutionary
666 analyses were conducted in MEGA5

667 **Figure 4.** Schematic illustrating a region of the genome from strain MOF25P containing multiple AMR
668 genes. The region bounded by broken red lines shows the high GC content region containing the
669 resistance genes *floR* and a putative transposase. Adjacent to both sides of this region are other
670 putative resistance genes encoding for resistance to chloramphenicol, tetracycline and
671 streptomycin. Other genes potentially associated with mobilisation and recombination are also
672 adjacent to this region (a putative DNA invertase of possible prophage origin and a

673 relaxase/mobilisation nuclease domain protein). Green = Possible insertion elements. Dark Red =
674 antibiotic resistance, Black = other, Orange = Unknown.

675 **Figure 5** Average difference in inhibition zone size (across 3 experiments) for isolates MOF25P,
676 BGARF and the control strain (*P. aeruginosa* NCTCC No 12903) in the presence and absence of the
677 efflux pump inhibitor PA β N, when using the disc diffusion method to assess antimicrobial
678 susceptibility (error bars given are \pm the standard error). AMP=ampicillin, AMX=amoxicillin,
679 MOX=moxolactam, FLQ=flumequine, CPR=ciprofloxacin, ENO=enrofloxacin, OFL=ofloxacin,
680 OXA=oxolinic acid, FFL=florfenicol, CHL=chloramphenicol, OTC=oxytetracycline, GEN=gentomicin,
681 STR=streptomycin, ERY=erythromycin, FUR=furazole, COT=cotrimoxazole.

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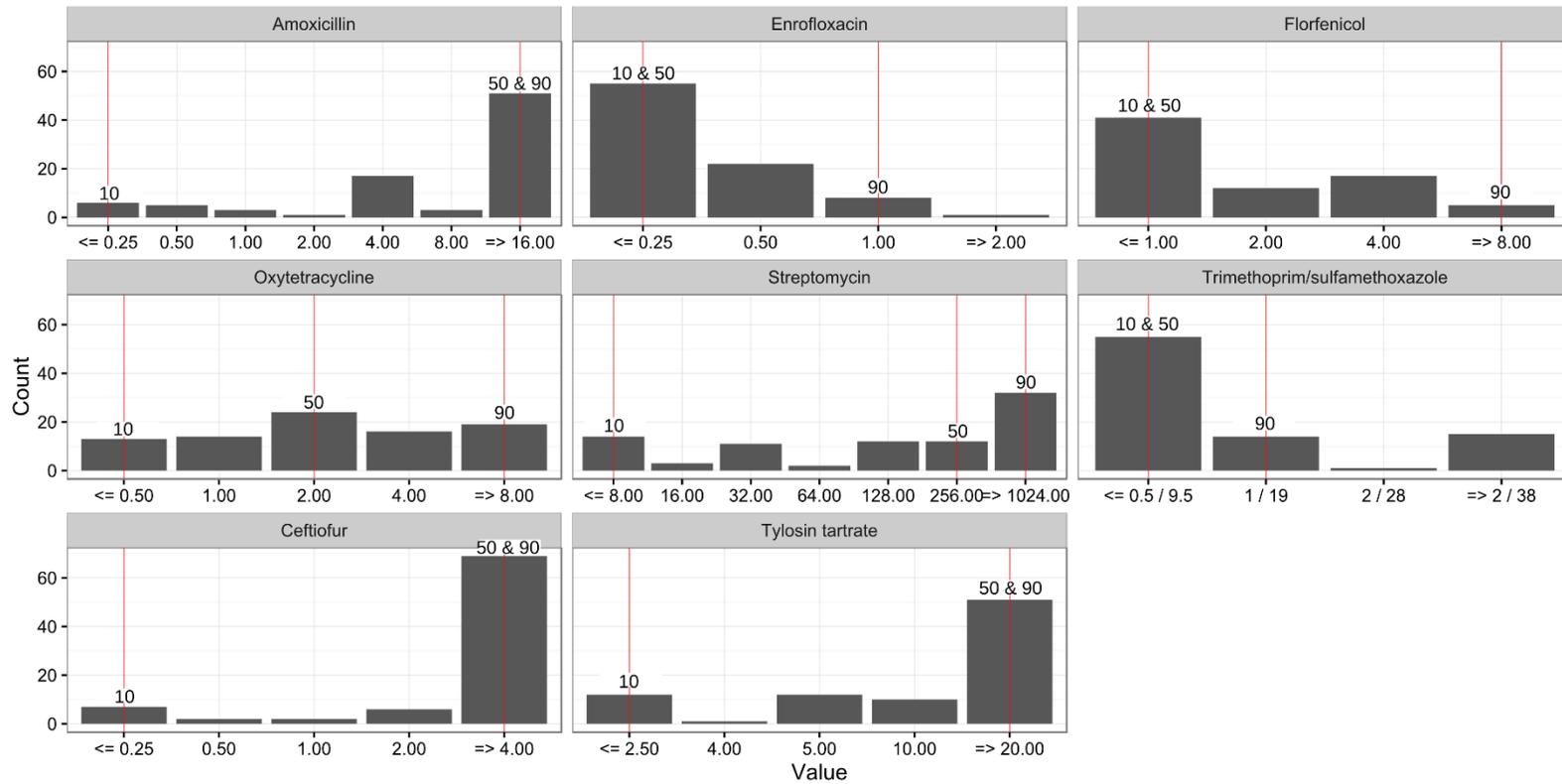
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691 **Figure 1** Histogram distributions of Minimum Inhibitory Concentrations for 86 Flavobacteriaceae isolates against 8 antimicrobials. MIC₁₀, MIC₅₀ and MIC₁₀₀
 692 values are indicated.

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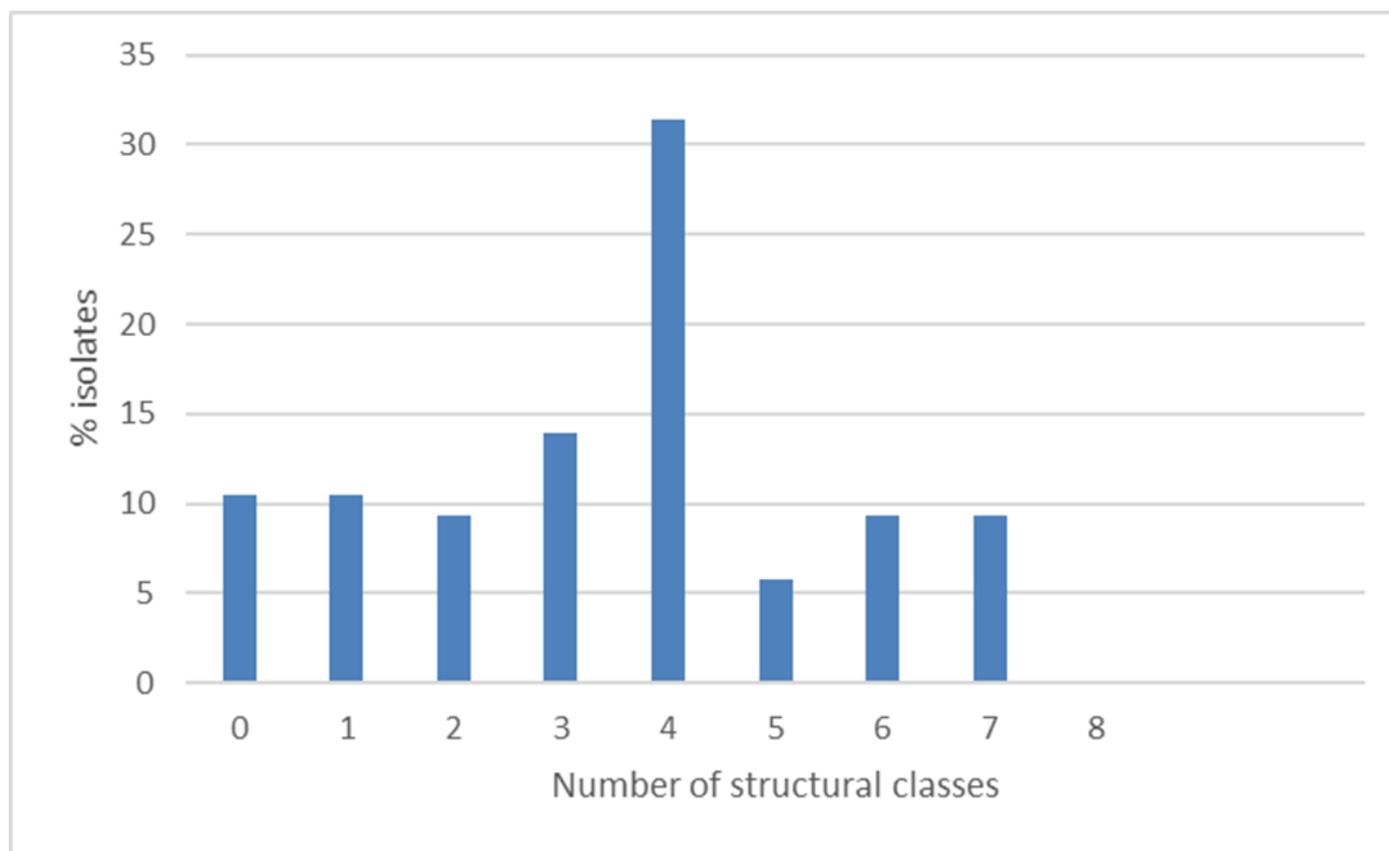


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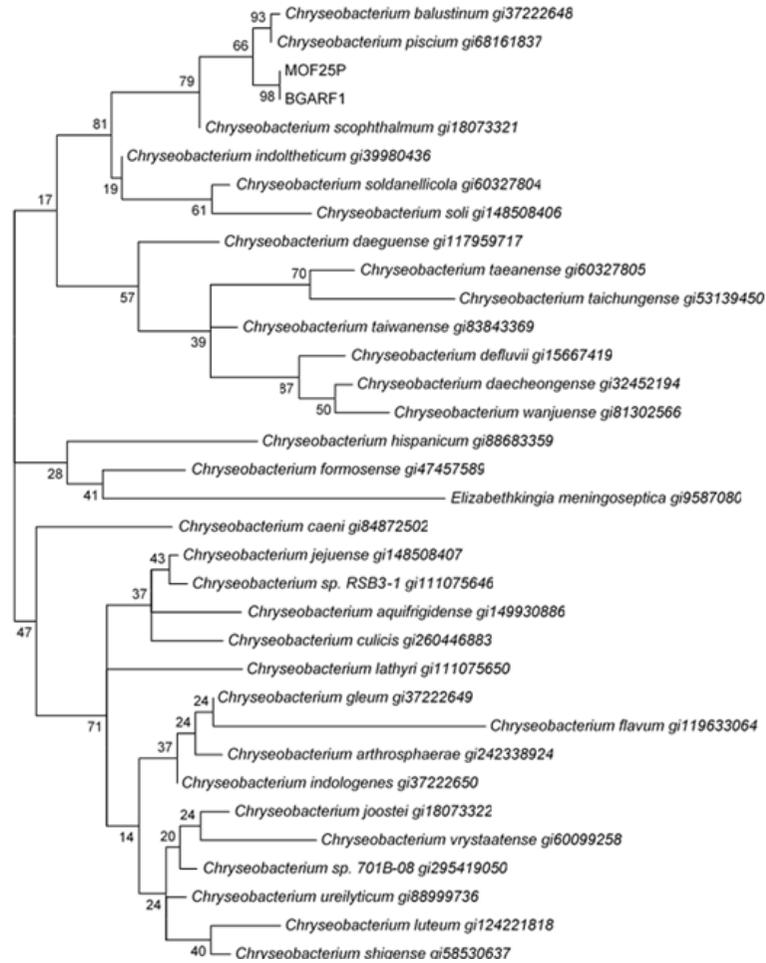
698 **Figure 2** Proportions (%) of isolates showing low susceptibility to numbers of different structural classes of antimicrobial. Isolates were defined as of low
699 susceptibility if they could grow in high concentrations of a representative aminoglycoside (≥ 64 mg/L streptomycin), fluoroquinolone (≥ 4 mg/L
700 enrofloxacin), macrolide (≥ 20 mg/L tylosin tartrate), penicillin (≥ 4 mg/L amoxicillin), sulphonamide (≥ 152 mg/L sulphadiazine), tetracycline (≥ 8 mg/L
701 oxytetracyclin), phenicol (≥ 8 mg/L florfenicol) and third generation cephalosporin (≥ 4 mg/L ceftiofur).



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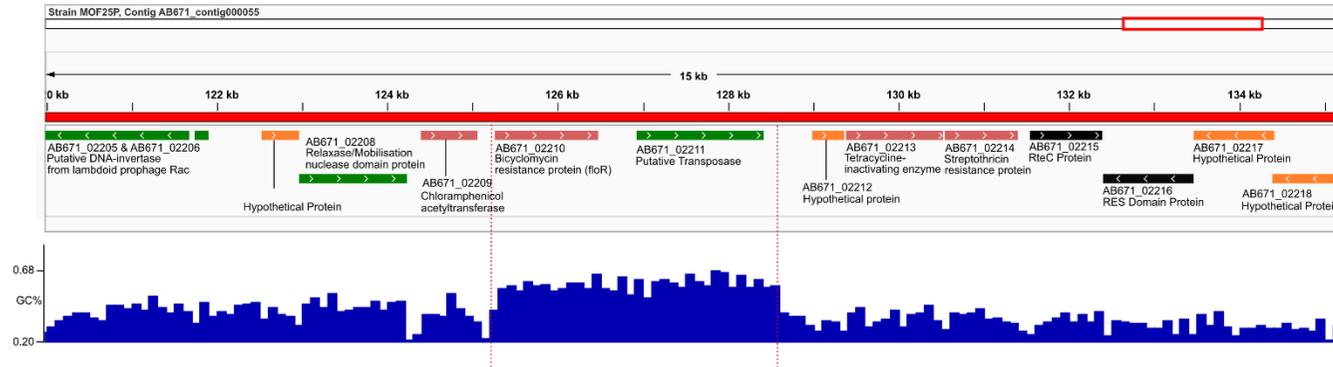
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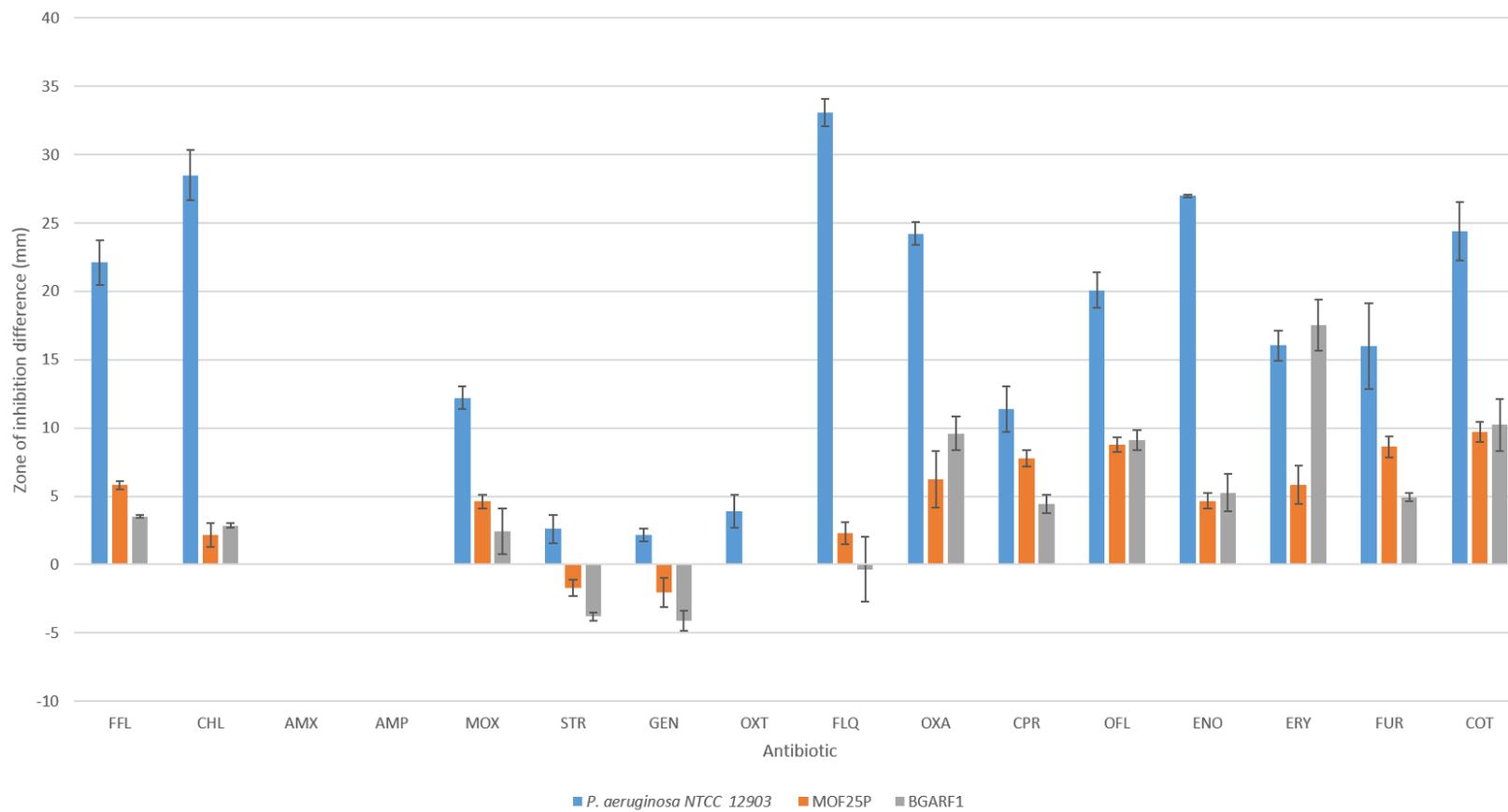
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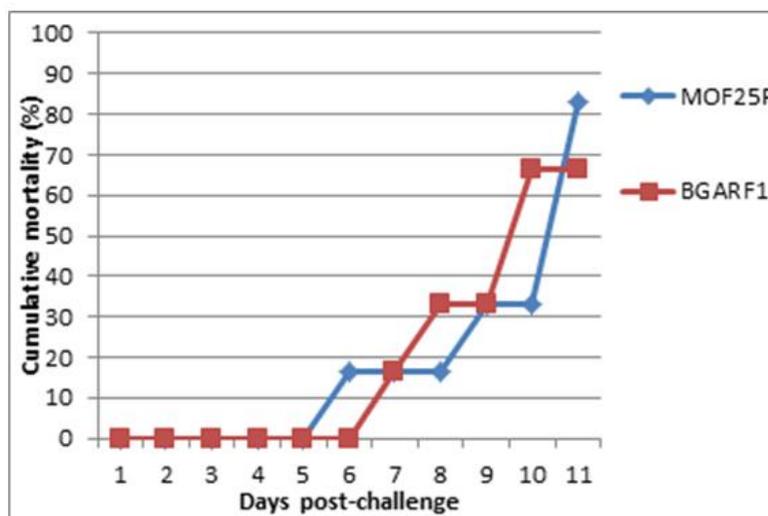
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720 **Figure 6** Cumulative % mortality in two groups of ten 4 g rainbow trout injected intramuscularly with isolates MOF25P & BGARF1 (5×10^5 cfu/fish) .

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