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Research Article

RESEARCH ARTICLE

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

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One sentence summary: The florfenicol resistance gene *floR* was found in a *Chryseobacterium* isolate recovered from a rainbow trout; analyses suggested horizontal acquisition and that this was a rare finding in this important group of aquatic bacteria.

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ABSTRACT

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

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

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INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Bacterial isolates

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

Antimicrobial susceptibility testing

Susceptibility to 19 antimicrobial agents commonly used in veterinary medicine was assessed by broth microdilution for all isolates using Trek Sensititre Avian susceptibility plates for veterinary use (Trek Diagnostic Systems, Cleveland, OH). The antibiotics tested (and ranges) were as follows: enrofloxacin (0.12–2 μg/ml), gentamicin (0.5–8 μg/ml), ceftiofur (0.25–4 μg/ml), neomycin (2–32 μg/ml), erythromycin (0.12–4 μg/ml), oxytetracycline (0.25–8 μg/ml), tetracycline (0.25–8 μg/ml), amoxicillin (0.25–16 μg/ml), spectinomycin (8–64 μg/ml), sulfadimethoxine (32–256 μg/ml), trimethoprim (0.5–2 μg/ml)-sulfamethoxazole (9.5–38 μg/ml), florfenicol (1–8 μg/ml), sulphathiazole (32–256 μg/ml), penicillin (0.06–8 μg/ml), streptomycin (8–1024 μg/ml), novobiocin (0.5–4 μg/ml), tylosin tartrate (2.5–20 μg/ml) and clindamycin (0.5–4 μg/ml).

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

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

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

Susceptibility of *Chryseobacterium* spp. isolates MOF25P and BGARF1 to 16 antimicrobials in the presence of the drug efflux inhibitor phenylalanine-arginine β-naphthylamide (PAβN) was tested via a disc diffusion assay as previously described (Michel

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

For reporting of antimicrobial susceptibility, as neither clinical breakpoints or, indeed, robust ecological cutoffs have been established for the diversity of Flavobacteriaceae species tested in this study, MIC_{10}, MIC_{50} and MIC_{90} values were instead calculated for each antibiotic tested, as recommended by Schwarz *et al.* (2010).

DNA microarray testing

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

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

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

Twenty bases at the 5′ end of each read, which had low quality scores FastQC (Andrews 2010), were removed using the FASTX Toolkit (Gordon and Hannon 2010). Paired reads were assembled using Velvet (Zerbino and Birney 2008). A kmer value of 89 was used for assembly, together with a minimum contig length of 200 bp and an expected insert length of 200 bp. Automatic annotation was carried out using RAST (Aziz *et al.* 2008) and Prokka (Seemann 2014). Manual confirmation of a group of ORFs identified as putative virulence and AMR genes (Supplementary Table S4, Supporting Information) was undertaken using individual BLAST searches (Altschul *et al.* 1997). After assembling isolate MOF25P and BGARF1 using Velvet, contig sequences were compared against the *floR* gene. Any contig with a region strongly matching the *floR* gene was read into Artemis (Rutherford *et al.* 2000) and the GC content around the *floR* gene was examined. Contigs were also searched for putative AMR genes using the online Comprehensive Antibiotic Resistance Database (CARD) maintained by McMaster University (McArthur *et al.* 2013). The two draft genome sequences have been submitted to Genbank, with BioSample Accessions SAMN03764887 and SAMN03764910, corresponding to isolates MOF25P and BGARF1, respectively.

Analysis of publically available Flavobacteriaceae genomes for AMR genes

A list of draft and complete genomes from the Flavobacteriaceae family was retrieved from the NCIMB taxonomic database (accessed 27 November 2015), through the Entrez API (Entrez Programming Utilities Help, 2010) (Supplementary Datasheet 1, Supporting Information). Assembly and nucleotide datasets were downloaded from NCBI. All of the genomes were converted into Fasta files, with the DNA sequence of any contiguous sequence, or contig, assembled from the genome. All of the genomes were then added into a single blast database. The nucleotide sequences from the ARG-Annot database (Gupta *et al.* 2014) were aligned to the blast database using the Nucleotide Blast algorithm (Camacho *et al.* 2009). Potential AMR genes were excluded if the alignment length was <90% of the query sequence length, and the aligned sequences shared <90% sequence identity. Genomes were re-annotated with Prokka (Seemann 2014), using default settings. Annotated AMR genes were examined for proximity to mobile genetic elements, using custom Python scripts, and the Artemis genome viewer (Rutherford *et al.* 2000). In addition, genomes were analysed for the presence of genomic islands using the online tool IslandViewer 3 (Dhillon *et al.* 2015) and for plasmid sequences using PlasmidFinder (Carattoli *et al.* 2014) and custom Python scripts.

Virulence factor identification

Haemolysin production

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

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

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

Iron acquisition and growth in iron-deprived media

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

Challenge experiments

Experiments were undertaken to assess the potential virulence of MOF25P and BGARF1 when injected intramuscularly into rainbow trout. For challenge experiments, cultures of MOF25P and BGARF1 were grown in MVA broth at 15°C for 72 h, washed twice in sterile phosphate-buffered saline (PBS) and resuspended in sterile PBS. For the first experiment, three groups of six rainbow trout (~3 g) were injected with of MOF25P at 5×10^4, 5×10^3 and 5×10^2 cfu/fish. For the second experiment, two groups of 16 (~4 g) fish were each injected with either MOF25P or BGARF1 at 1.5×10^5 cfu/fish. Fish were maintained at 10°C –12°C in 10-L flow-through tanks for up to 11 days. Moribund fish were removed

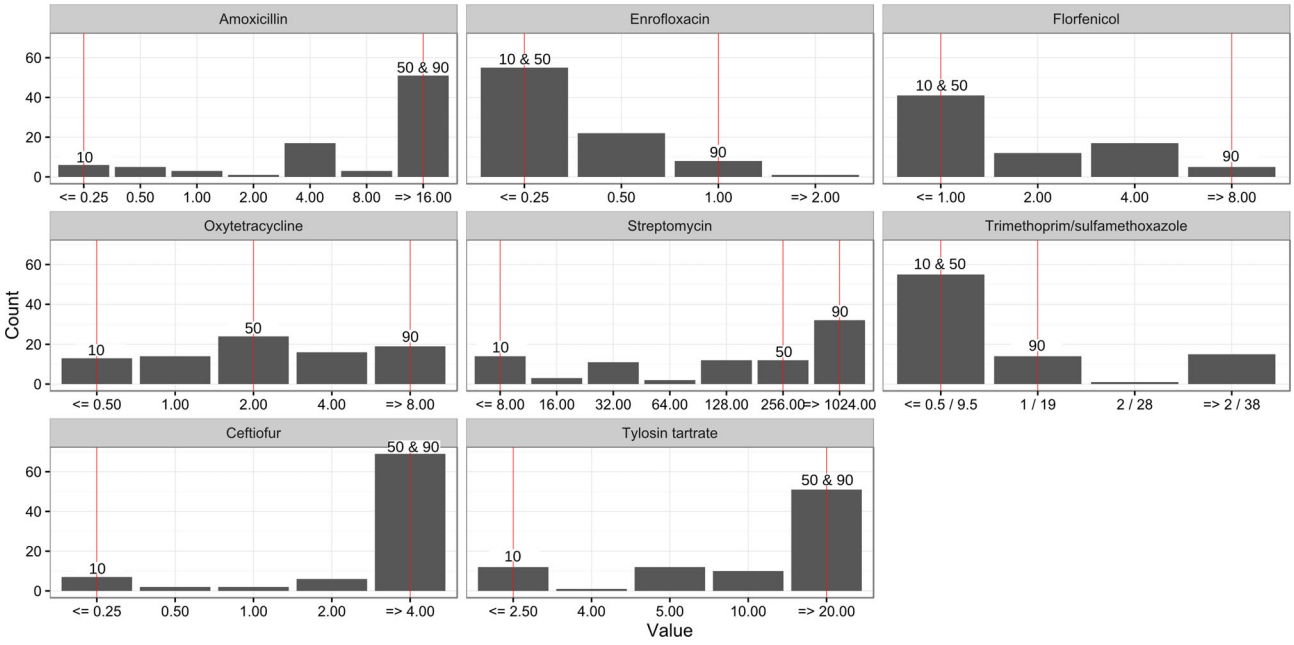

Figure 1. Histogram distributions of MICs for 86 Flavobacteriaceae isolates against eight antimicrobials. MIC_{10}, MIC_{50} and MIC_{90} values are indicated.

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

RESULTS

Antimicrobial susceptibility

Isolates tested covered a range of Flavobacteriaceae species (Supplementary Table S1). In general, it can be stated that many of the isolates had low susceptibility to the antimicrobials tested (Fig. 1 and Supplementary Fig. S1, Supporting Information). There were also no obvious temporal or geographical variations in observed patterns of susceptibility observed. Many isolates were able to grow in the presence of relatively high concentrations of the main antimicrobials that are used to control bacterial diseases in farmed fish in the UK and elsewhere in the world. For example, the MIC50 value for amoxicillin was ≥16 mg/L. MIC50 values for both oxytetracycline and florfenicol were 2 mg and their MIC10 values were ≥8 and 32 mg/L, respectively. Although most isolates were highly susceptible to sulfamethoxazole/trimethoprim (MIC_{50} <0.5/9.5 mg/L), the MIC10 value was ≥2/3 mg/L.

We also tested susceptibility to a range of other antimicrobials commonly used to treat bacterial diseases in humans and other animals (Fig. 1, Supplementary Fig. S1 and

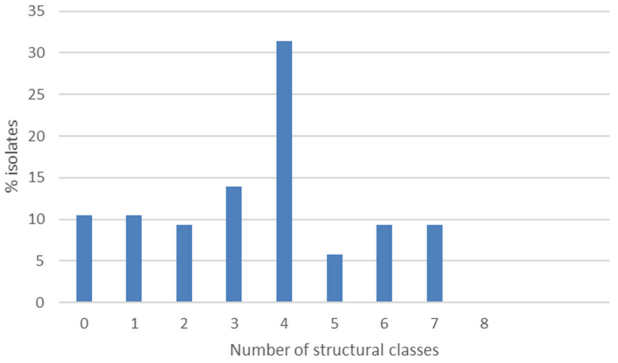

Figure 2. Proportions (%) of isolates showing low susceptibility to numbers of different structural classes of antimicrobial. Isolates were defined as of low susecptibility if they could grow in high concentrations of a representative aminoglycocide (≥64 mg/L streptomycin), fluoroquinolone (≥4 mg/L enrofloxacin),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

Supplementary Tables S2, S3, Supporting Information). Many of the isolates could grow in high concentrations of third-generation cephalosporins (e.g. the MIC_{50} for ceftiofur was ≥4 mg/L) and aminoglycosides (the MIC_{50} for streptomycin was ≥32 mg/L). Most isolates were inhibited by low concentrations of the fluoroquinolone enrofloxacin, although the MIC_{10} was still 1 mg/L. A total of 56 % of isolates were able to grow in high concentrations of four or more representatives of different structural classes of antimicrobials (Fig. 2), suggesting intrinsic resistance or acquired reduction in susceptibility to multiple antibiotics.

Out of the 86 isolates, 36 isolates that grew in the highest florfenicol test concentration (4 mg/L) in the Trek Sensititre Avian susceptibility plates were chosen for further analysis (Supplementary Tables S2 and S3). As shown in Supplementary Table S2, susceptibility to florfenicol in these 36 isolates varied from 2 to 128 mg/L when re-tested using the custom

florfenocol testing panels. Susceptibility to a range of antimicrobials used to treat Gram-negative clinical infections was also assessed (Supplementary Table S3). Many of the isolates able to grow in ≥4 mg/L florfenicol also displayed low susceptibility to first-, second- and third-generation cephalosporins, and other classes of antimicrobials (Supplementary Table S3). Many grew in the highest concentrations of antimicrobials tested in the panels used. For instance, 32/36 isolates grew in ≥32 mg/L ceftriaxone (a third-generation cephalosporin), 28/36 in ≥ 32 mg/L cefepime (a fourth-generation cephalosporin) and 27/36 in ≥64 mg/L nitrofurantoin (Supplementary Table S3). Isolates showed most sensitivity to the two carbapenems tested, imipenem and meropenem, although even here, 12 and 4 out of the 36 tested isolates were able to grow in >4 mg/L of meropenem and imipenem, respectively.

Resistance gene arrays

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

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

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

Analysis of the assembled contigs for genes that likely code for AMR genes demonstrated the presence, in single contigs from both isolates, of a region that contained the florfenicol resistance gene *floR*, a *tet*(X) tetracycline resistance gene, a streptothricin resistance gene and a chloramphenicol resistance gene of the chloramphenicol acetyltransferase (cat) family (Fig. 4). In this structure, there is a putative relaxase/mobilisation protein upstream of the *floR* and *cat* genes and a putative transposase downstream of both genes (Fig. 4). In addition, an ~3-kb region containing the *floR* element and the putative transposase in both strains had a markedly higher GC content (58%) compared to the rest of the genomes (34%). When analysed by BLASTn, the high GC content region closely aligned (a single mismatch and no gaps or insertions over the same 3395 compared bases in each case) to parts of sequences derived from different Gram-negative bacteria and their associated plasmids (e.g. accession numbers CP014775, KU302354 and KF250428).

The area was identified as part of a possibly horizontally acquired genomic region in both isolates by two different genomic island prediction methods (SIGI-HMM (Waack *et al.* 2006) and IslandPath-DIMOB (Langille, Hsiao and Brinkman 2008)) when the draft genomes were analysed using IslandViewer3. Although IslandViewer3 identified seven other regions that may have been acquired by HGT, this was the only region identified by both prediction methods (Supplementary Fig. S2, Supporting Information). Furthermore, it was the only region with a markedly different (higher) GC content to the rest of the genome (Supplementary Fig. S2). In terms of other potential mobile elements, no sequences of putative plasmid origin were identified *in silico* in either draft genome. Putative AMR genes were also identified in other regions of the two genomes by both CARD (McArthur *et al.* 2013) and RAST (Aziz *et al.* 2008) (Supplementary Table S4). Beta-lactamase genes and, in particular one ORF, were present in both isolates (AB670_04238 in MOF-25P and AB671_02057 in BGARF1; Supplementary Table S4) coded for a protein containing 292 amino acids with greater than 70% identity to previously characterised Ambler Class A VEB-like ESBL recovered from other Flavobacteriaceae (Bellais, Naas and Nordmann 2002b). The presence of a number of ORFs with high identity to genes encoding multidrug efflux pumps was also noted (Supplementary Table S3). Confirming the results of the microarray analysis, with the exception of *floR*, no clinically important resistance gene sequences, which would hybridise with the probes represented on the array, were detected in the draft genome sequences of either isolate. It is likely that the region containing *floR* and *catB* in the two isolates was chromosomally located as no DNA sequences with known plasmid-related functions were detected on the two large contigs (70.5 and 144 kb, respectively) that the region was present on. Furthermore, examination of all the contigs containing putative AMR genes did not reveal any with much greater than average read depth, which would have been indicative of location on a multicopy plasmid.

PAβN and ESBL assays

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

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

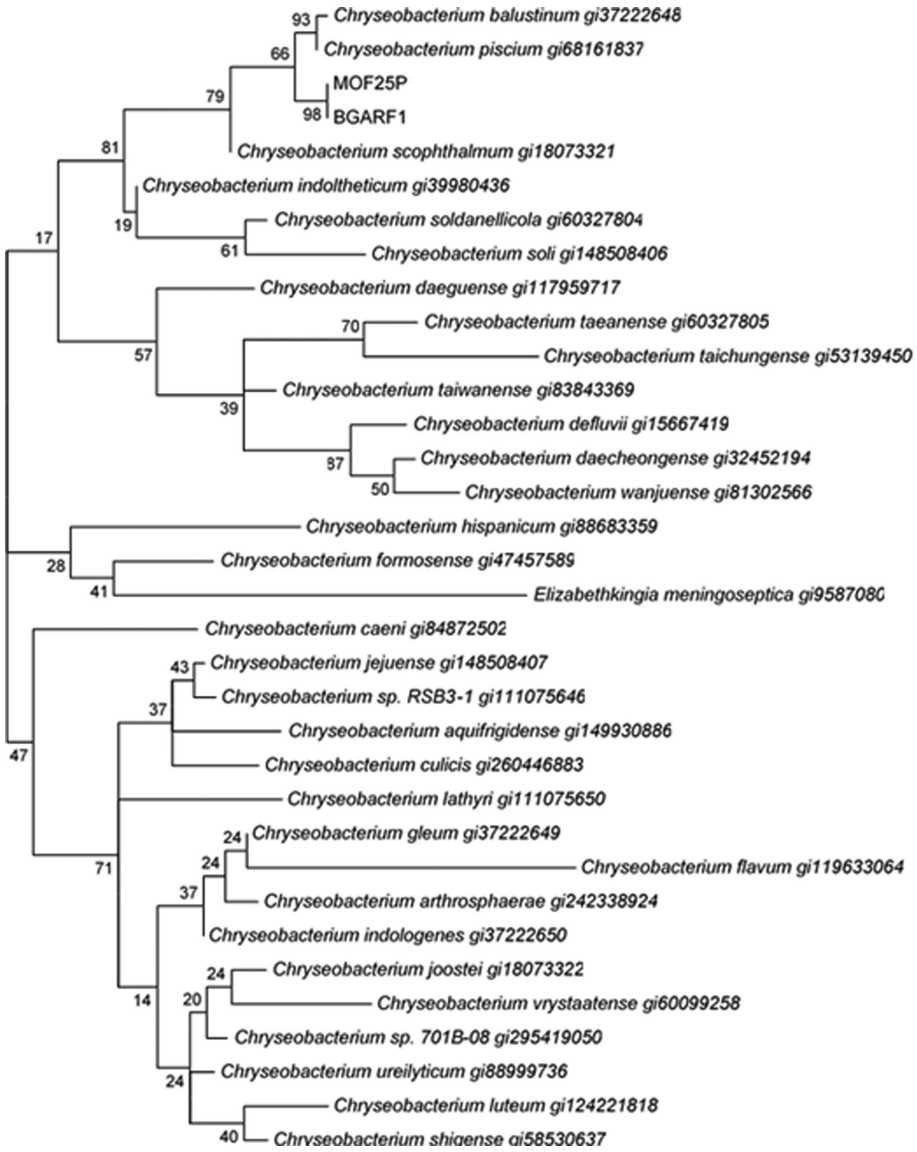

Figure 3. Phylogenetic relationships of partial 16S rRNA gene sequences derived from Chryseobacteria isolates to isolates BGARF1 and MOF25P. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.* 2011). Evolutionary analyses were conducted in MEGA5.

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

To further test the hypothesis that AMR genes and mobile elements more commonly associated with Gram-negative bacteria (particularly Proteobacteria) are not typically found within Flavobacteriaceae, available genomes from this family of bacteria were examined *in silico*. A total of 377 genomes were accessed from publically available databases and examined for the presence of both AMR genes and mobile elements. The sequenced genomes represented examples of a range of genera and species from clinical and environmental sources (Supplementary Datasheet 1). A total of 81/377 isolates had one or more matches to resistance genes in the ARG-Annot database. The most common resistance determinants identified (Supplementary Datasheets 2-3, Supporting Information) were genes coding for putative resistance to beta-lactams (65/377) and tetracyclines (16/377). Genes coding for putative resistance to macrolide/lincosamide/streptomycin G group antibiotics (4/377) and phenicols (3/377) were also identified. The beta-lactamase genes identified (Supplementary Data Sheet 3) were typically those known as naturally occurring in *Chryseobacteria* and other Flavobacteriaceae that code for Amber Class B and other enzymes, such as bla_{B}, bla_{MUS}, bla_{TUS}, bla_{IND}, bla_{CFX}, bla_{JOHN} and bla_{GOB} (Bellais *et al.* 2000; Woodford *et al.* 2000; Mammeri, Bellais and Nordmann 2002; Bellais, Naas and Nordmann 2002a,b; Naas, Bellais and Nordmann 2003; Gonzalez and Vila 2012.

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

Search of the integral database (Moura *et al.* 2009) also recovered a limited number of matches to Flavobacteriaceae. For

Figure 4. Schematic illustrating a region of the genome from strain MOF25P containing multiple AMR genes. The region bounded by broken red lines shows the high GC content region containing the resistance genes *floR* and a putative transposase. Adjacent to both sides of this region are other putative resistance genes encoding for resistance to chloramphenicol, tetracycline and streptomycin. Other genes potentially associated with mobilisation and recombination are also adjacent to this region (a putative DNA invertase of possible prophage origin and a relaxase/mobilisation nuclease domain protein). Green = possible insertion elements, dark red = antibiotic resistance, black = other, orange = unknown.

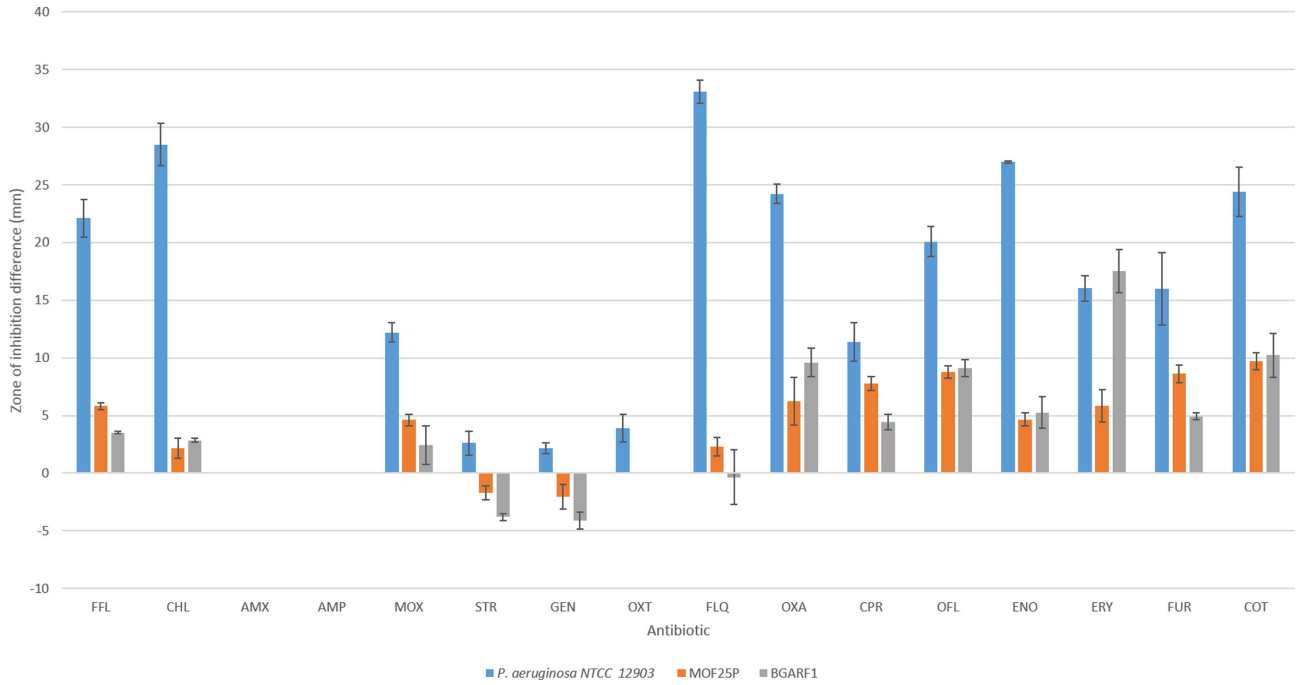

Figure 5. Average difference in inhibition zone size (across three experiments) for isolates MOF25P, BGARF and the control strain (*P. aeruginosa* NCTCC no 12903) in the presence and absence of the efflux pump inhibitor PAβN, when using the disc diffusion method to assess antimicrobial susceptibility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

example, searches using the terms 'Flavobacterium' and 'Elizabethkingia' returned no matches while 'Chryseobacterium' returned three accession numbers with sequences containing putative class 1 integrons (accession numbers: KM278188, KJ561906, KM278184). All three deposited sequences were reportedly recovered from *Chryseobacterium* spp. isolated from Chinese river water samples. The sequences all contained well-characterised resistance gene cassettes (including dihydrofolate reductase (*drfA12*), aminoglycoside adenyltransferase (*aadA2*, *aacA4*) and rifampin ADP-ribosylating transferase (*arr-3*) genes. *Riemerella anatipestifer* was different with a number of matches in the integral database to well-characterised resistance gene cassettes and class 1 integrons. No matches to plasmid sequences on PlasmidFinder database were found.

Additional phenotypic characterisation of *Chryseobacterium* spp. isolates

Identification of other virulence factors

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

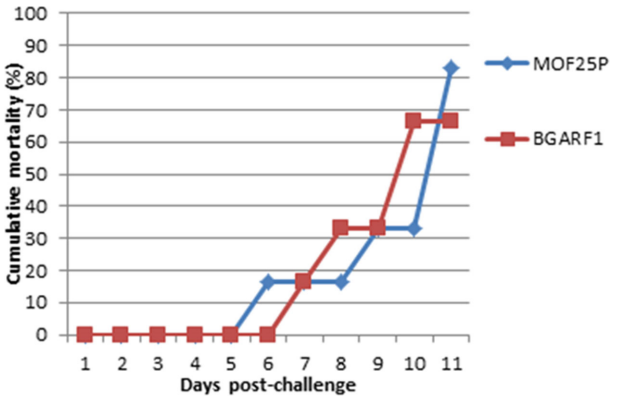

Figure 6. Cumulative % mortality in two groups of ten 4-g rainbow trout injected intramuscularly with isolates MOF25P and BGARF1 (5×10^5 cfu/fish).

Challenge experiments

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

DISCUSSION

This study confirms the results of earlier reports that many of the Flavobacteriaceae isolated from fish, the environment and clinical samples have low susceptibility to a range of antimicrobials (Kirby *et al.* 2004; Michel *et al.* 2005). Despite high MICs to a wide range of antimicrobials of different structural classes, analysis of 36 isolates using a microarray revealed that carriage of clinically relevant AMR genes commonly found in other Gram-negative bacteria was rare. Indeed, only two isolates were microarray positive for a single AMR gene, in both cases the florfenicol resistance gene *floR*. Additional *in silico* analysis of 377 publically available genomes further demonstrated that such clinical resistance genes were not widely distributed in this family. Michel *et al.* (2005) analysed multidrug-resistant *Chryseobacterium* isolates recovered from fish species and did not detect any clinically relevant AMR genes, including *floR*. This is in contrast to previous studies of other Gram-negative bacterial pathogens of fish (McIntosh *et al.* 2008; Verner-Jeffreys *et al.* 2009; Welch *et al.* 2009)

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

Conversely, oxytetracycline, amoxicillin and ampicillin susceptibility was unaffected by PAβN, which suggests that the presence of different mechanisms is responsible for susceptibility to these antimicrobials in the *Chryseobacterium* spp. isolates MOF25P and BGARF1. It is possible that point mutations to chromosomal genes may have a role in lowered susceptibility, as has been demonstrated for other Gram-negative bacteria to a number of antibiotics. These include quinolones (Shah *et al.* 2012), sulphonamides (Vedantam *et al.* 1998) and beta-lactams (Zapun, Contreras-Martel and Vernet 2008).

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

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

Additional *in silico* analysis of published Flavobacteriaceae genomes was undertaken to determine whether such elements are commonly associated with this group. Results suggest that they are indeed relatively rare. The majority of the AMR genes identified coded for beta-lactamase and tetracycline resistance genes, known to be constitutively expressed in this group of bacteria. There were also only limited records in the integral

database of integrase class 1 genes and resistance gene cassettes. An apparent exception to this general trend appears to be the duck pathogen *Riemerella anatipestifer*, particularly isolates that are circulating in farmed ducks in China (Chen *et al.* 2012; Sun *et al.* 2012). As well as a range of important clinical resistance gene cassettes, *floR*, has also been isolated from this pathogen. It is noteworthy that *floR* has only been identified to date in Flavobacteriaceae from fish and livestock farming environments, where it is likely that florfenicol and other veterinary antibiotics are widely applied.

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

The two *Chryseobacterium* spp. isolates MOF25P and BGARF1 produced positive results in the CAS agar assay for production of siderophores and all three haemolysis assays. This complements the genome analysis, which identified a putative haemolysin and an aerobactin-like siderophore encoding genes in each genome (Supplementary Table S4). Siderophore production has also been detected in *Flavobacterium psychrophilum* isolates (Møller *et al.* 2005). It is possible that the haemolysins produced by the *Chryseobacterium* spp. isolates are involved in disease progression, as they are likely to be in *F. psychrophilum* (Secades, Alvarez and Guijarro 2003; Pérez-Pascual *et al.* 2011).

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

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

In summary, low susceptibility to antibiotics is common in Flavobactericeae but this appears to be largely intrinsic and conferred by genes/mechanisms different to those present in many clinically relevant Gram-negative bacteria (e.g. Enterobacteriaceae). It is some respects it is reassuring that these bacteria do not appear to be significant reservoirs of many of the AMR genes that are causing problems in humans and terrestrial animals. However, the demonstration that floR and *tet*(X) were likely acquired in bacteria from this group via a horizontal transfer event from an unrelated organism may represent a new risk for the dissemination of resistance into the aquatic environment.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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