ORIGINAL ARTICLE

Detection of the *floR* Gene in a Diversity of Florfenicol Resistant Gram-Negative Bacilli from Freshwater Salmon Farms in Chile

C. Fernández-Alarcón[1], C. D. Miranda[2], R. S. Singer[3,4], Y. López[1], R. Rojas[2], H. Bello[1], M. Domínguez[1] and G. González-Rocha[1]

[1] Laboratorio de Antibióticos, Departamento de Microbiología, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile
[2] Laboratorio de Patobiología Acuática, Departamento de Acuicultura, Universidad Católica del Norte, Coquimbo, Chile
[3] Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN, USA
[4] Instituto de Medicina Preventiva Veterinaria, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile

Impacts

- Antibiotic resistant bacteria exist in the environment, independent of antibiotic usage or other anthropogenic influences. This environmental reservoir, termed the resistome, can be augmented by the use of antibiotics.
- A diversity of florfenicol-resistant Gram-negative bacilli was isolated from freshwater salmon farms in Chile. Many of these isolates possessed the *floR* gene, but other isolates possessed a non-specific efflux pump that conferred florfenicol resistance.
- The florfenicol-resistant isolates that possessed the *floR* gene were multi-drug resistant. The diversity of antibiotics that are used in aquaculture could be selecting for a range of resistances through co-selection.
- Understanding the diversity of resistance within the bacteria as well as within the aquaculture environment will help improve our understanding of the animal and human health risks associated with antibiotic use in aquaculture.

Keywords:
Florfenicol; antibiotic resistance; *floR* gene; salmon; Chile

Correspondence:
R. S. Singer. Department of Veterinary and Biomedical Sciences, University of Minnesota, 1971 Commonwealth Ave., St. Paul, MN 55108, USA. Tel.: +1 612 625 6271; Fax: +1 612 625 5203; E-mail: singe024@umn.edu

Received for publication June 3, 2008

doi: 10.1111/j.1863-2378.2009.01243.x

Summary

Florfenicol is an important antibiotic in veterinary medicine that is used extensively in aquaculture, including salmon farming in Chile. We analysed a set of 119 florfenicol-resistant Gram-negative bacilli from seven freshwater Chilean salmon farms for the molecular determinants involved in the florfenicol resistance. Ninety-seven of these strains were glucose non-fermenting bacilli, mainly belonging to the *Pseudomonas* genus, whereas 22 strains were glucose-fermenters. The *floR* gene was detected in 26 strains (21.8%) that had been isolated from three of the seven salmon farms. Most of the *floR*-carrying strains were glucose fermenters (21 strains), and most of the *floR*-carrying strains were also resistant to streptomycin, chloramphenicol and oxytetracycline. The minimum inhibitory concentrations against florfenicol were assessed in the presence and absence of the efflux pump inhibitor Phe-Arg-β-naphthylamide (MC-207,110). There was evidence that in the majority of non-fermenting bacteria (82 strains), florfenicol resistance was at least partially mediated by non-specific efflux pump systems. Given the diversity of antibiotic resistance patterns observed in this study in the *floR*-positive isolates, a single antibiotic has the potential to co-select for a diversity of resistances. For this reason, human health as well as animal health can potentially be impacted by the use of antibiotics in aquaculture. To assess this potential risk, future studies should focus on the ability of different antibiotics used in aquatic environments to co-select for multiple resistances, the molecular basis of this diversity of resistance, and whether the genes conferring resistance can be transferred to other bacteria, including those of human health concern.

Introduction

Despite the fact that various authors have emphasized the potential negative impact derived from the use of antimicrobial agents in fish farms (Alderman and Hastings, 1998; Schwarz et al., 2001; Cabello, 2006), very few studies have been conducted on the diversity and evolution of antimicrobial resistance in aquaculture (Dang et al., 2007). Intensive aquaculture production in Chile has increased over the recent years, and antimicrobials are commonly used for the treatment and prevention of bacterial diseases (Cabello, 2004). Within the Chilean salmon industry, studies on antibiotic resistance have mostly been focused on phenotypic aspects of tetracycline resistance (Miranda and Zemelman, 2002a,b).

Florfenicol [d-threo-3-fluoro-2-dichloroacetamido-1-(4-methylsulfonylphenyl)-1-propanol] is a synthetic, primarily bacteriostatic, broad-spectrum fluorinated analogue of chloramphenicol that is active against Gram-negative and Gram-positive bacteria. The primary antimicrobial activity of this compound is through its binding with the 50S ribosome, thus preventing the transfer of amino acids from tRNA to the growing peptide chains during protein synthesis (Schwarz et al., 2004). Florfenicol has a variety of applications in veterinary medicine and has been used in aquaculture in Asia since the early 1990s. It was approved in the US by the Food and Drug Administration and Center for Veterinary Medicine in 1996 for the treatment of bovine respiratory disease pathogens and has since been approved for use in aquaculture and swine. In the European Union, it was approved for the treatment of respiratory tract infections in cattle in 1995 and in swine in 2000. Florfenicol was licensed in Chile in 1994 for the treatment of diverse bacterial infections occurring in Chilean salmon farming and is currently one of the most frequently used anti-bacterials in Chilean freshwater salmon farming (Miranda and Rojas, 2007).

Resistance to florfenicol in Gram-negative bacteria is mediated primarily by the *floR* gene, which is a specific drug exporter that confers resistance to florfenicol and chloramphenicol (Schwarz et al., 2004). The gene is widely disseminated among Gram-negative bacteria in diverse geographic locations (Schwarz et al., 2004), particularly from Gram-negative organisms from animal agricultural sources (Keyes et al., 2000; White et al., 2000; Bischoff et al., 2002; Singer et al., 2004; Gordon et al., 2008). Because of the importance of florfenicol in treating bacterial infections in animal populations, it is critical to monitor the emergence and spread of resistance to this compound in agriculture.

In our previous study of florfenicol resistance in bacteria from Chilean salmon farms, we found a diversity of bacteria with phenotypic florfenicol resistance (Miranda and Rojas, 2007). The resistance existed in areas that had not been directly exposed to florfenicol from aquaculture. What is not known is the mechanism by which these bacteria gained resistance to florfenicol. It is plausible that some of these isolates were intrinsically resistant to florfenicol rather than that they had acquired the resistance through mutation or horizontal gene transfer. Therefore, the objective of this study was to conduct a broader cross-sectional survey of seven freshwater salmon farms, investigate the diversity of bacteria with florfenicol resistance, and then identify the major resistance determinants responsible for this resistance. In particular, we wanted to determine the diversity of bacteria possessing the *floR* gene. We also wanted to determine if any of these isolates carried a broad-spectrum efflux pump that conferred resistance to florfenicol.

Materials and Methods

Bacterial strains

One hundred and nineteen Gram-negative bacilli strains resistant to florfenicol were isolated from seven different freshwater salmon farms located in the South of Chile (Table 1). The salmon farms were land-based (CC1-CC3) (Miranda and Zemelman, 2002b) or were located in the lakes Lago Rupanco (F1 and F2) and Lago Llanquihue (F3 and F4). Florfenicol was administered at the farms at Lago Rupanco (F1 and F2) approximately 2 weeks before sampling, whereas in the other sampled farms florfenicol had not been used for more than a year prior to sampling.

Samples were processed as previously described (Miranda and Zemelman, 2002b; Miranda and Rojas, 2007), and florfenicol-resistant strains were recovered by a spread plate method using Tryptic soy agar (TSA, Difco, Franklin Lakes, NJ, USA) containing 30 μg ml^{-1} of florfenicol (Schering-Plough, Kenilworth, NJ, USA). This level of florfenicol is higher than the established breakpoint for florfenicol resistance, but based on our previous work, bacterial isolates possessing the florfenicol resistance gene *floR* had MICs >32 μg ml^{-1} (Singer et al., 2004). The use of this concentration means that only those isolates with a high level of florfenicol resistance would have been detected; isolates with decreased susceptibility to florfenicol would be missed by this screening approach. Purified strains were stored at −85°C in Tryptic soy broth (Difco) supplemented with 20% glycerol and florfenicol (30 μg ml^{-1}) until use.

Bacterial identification

Phenotypic characteristics, Gram stain, oxidase production and oxidative/fermentative utilization of glucose were

Table 1. Identification of florfenicol-resistant strains used in this study

	Fish farm							
Species	CC1	CC2	CC3	F1	F2	F3	F4	Total
Acinetobacter johnsonii					1			1
Acinetobacter lwoffii			2					2
Aeromonas hydrophila	1							1
Alcaligenes faecalis							1	1
Burkholderia cepacia	2							2
Cedecea davisae							1	1
Citrobacter amalonaticus						1		1
Citrobacter freundii						1		1
Citrobacter youngae						1		1
Enterobacter aerogenes				2				2
Enterobacter amnigenus						6		6
Enterobacter cloacae							1	1
Hafnia alvei				2				2
Kluyvera ascorbata				3			1	4
Pantoea sp.							2	2
Pseudomonas aeruginosa			1					1
Pseudomonas alcaligenes							1	1
Pseudomonas chlororaphis				1				1
Pseudomonas fluorescens	16	4	6	4	2			32
Pseudomonas ludensis					2			2
Pseudomonas maculicola					2			2
Pseudomonas nitroreducens					1		1	2
Pseudomonas putida	3		1		8	2	4	18
Pseudomonas sp.	4		1	2	4	1	1	13
Pseudomonas synxantha				1				1
Pseudomonas viridilivida					4		1	5
Ralstonia pickettii	1						1	2
Raoultella terrígena				1				1
Sphingobacterium multivorum							1	1
Stenotrophomonas maltophilia		3						3
Not identified					3		3	6
Total	27	7	11	16	27	12	19	119

determined for every selected isolate as previously described (Miranda and Rojas, 2007). Strains possessing the *floR* gene (described below) were further characterized by using the GN Microplate system (Biolog Inc., Hayward, CA, USA) to confirm their bacterial identification (Miranda and Rojas, 2007). Bacterial identification was performed with the MICROLOG SYSTEM 4.2 identification software (Biolog Inc.). Approximately 20% of the isolates were tested in duplicate to check the reproducibility of the assay.

Antimicrobial susceptibility testing and efflux pump inhibition

Minimum inhibitory concentrations (MICs) of florfenicol against each strain were determined by an agar plate dilution method and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) MIC Interpretative Standards (National Committee for Clinical Laboratory Standards, 2002). Briefly, serial 2-fold dilutions of the antibiotic were added to Mueller-Hinton agar (Oxoid Ltd., Basingstoke, UK) to obtain final concentrations ranging from 8 to 256 μg ml^{-1}. Triplicate plates were inoculated and incubated for 24 h at 30°C. MIC was defined as the lowest concentration of antibiotic that inhibited growth on at least two of the three plates. Reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were included as controls (National Committee for Clinical Laboratory Standards, 2002).

To test for the presence of a broad-spectrum efflux pump that could potentially be conferring the observed florfenicol resistance, the agar dilution procedure was repeated in the presence (20 μg ml^{-1}) and absence of the efflux pump inhibitor (EPI) Phe-Arg-β-naphthylamide (MC-207,110) (Sigma, St. Louis, MO, USA). A decrease of at least 4-fold in the MIC value (two dilutions) in the presence of EPI was considered indicative of the presence

of an efflux mechanism (Kadlec et al., 2007). *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and two *floR* gene-positive *Acinetobacter baumannii* strains from our collection were used as controls.

Detection of the *floR* gene

The *floR* gene was detected by PCR. Genomic template was prepared by mixing 200 μl of overnight bacterial culture with 800 μl of sterile distilled water (SDW), boiling for 15 min, and then centrifuging at 16 000 ***g*** for 5 min. The supernatant was used directly as the source of template. Each 25 μl reaction mixture contained 2.5 μl 10× PCR buffer (Invitrogen, Carlsbad, CA, USA); 2.5 μl each dNTP (1.25 mM); 1.25 μl $MgCl_2$ (50 mM, Invitrogen); 1.5 μl each primer (25 pmol μl^{-1}); 0.125 μl Taq DNA polymerase (5 U μl) and SDW to 25 μl of reaction volume. Amplification of the *floR* gene was performed in a Gene Amp PCR System 2400 thermal cycler (Perkin-Elmer, Foster City, CA, USA) using the primers: F: 5′-AATCACGGGCCACGCTGTATC-3′ and R: 5′-CGCCG-TCATTCTTCACCTTC-3′ (Bolton et al., 1999). DNA was amplified by PCR using the following cycle conditions: 96°C for 30 s, followed by 30 cycles of 96°C for 15 s, 52°C for 30 s, and 70°C for 1 min. There was a final extension at 70°C for 5 min. PCR products were detected by electrophoresis in 1.5% agarose gels and visualized by ultraviolet illumination after staining the gels with ethidium bromide (0.5 mg l^{-1}). The expected size of PCR products was 215 bp. A *floR*-positive *E. coli* and *E. coli* K-12 ATCC 19215 served as positive and negative controls, respectively, and were included in each PCR run.

Anti-microbial resistance patterns

Isolates positive for the *floR* gene (26 strains) were tested for susceptibility to 15 anti-bacterials by an agar disk diffusion method according to the CLSI MIC Interpretative Standards (National Committee for Clinical Laboratory Standards, 2002) using Müeller–Hinton agar plates (Oxoid Ltd.). Disks containing the following anti-bacterial agents were used: ampicillin (AM, 10 μg), cefotaxime (CTX, 30 μg), chloramphenicol (CM, 30 μg), florfenicol (FFC, 30 μg), streptomycin (S, 10 μg), gentamicin (G, 10 μg), kanamycin (K, 30 μg), oxytetracycline (OT, 30 μg), nalidixic acid (NA, 30 μg), oxolinic acid (OA, 2 μg), flumequine (UB, 30 μg), enrofloxacin (ENR, 5 μg), furazolidone (FX, 100 μg) and sulphamethoxazole/trimethoprim (SXT, 1.25 and 23.75 μg). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were included as quality control strains (National Committee for Clinical Laboratory Standards, 2002). All disks except florfenicol (Schering-Plough) were supplied by Oxoid Ltd.

Results

Identification of florfenicol-resistant strains

Out of the 119 florfenicol-resistant Gram-negative bacilli isolates analysed in this study, 97 (81.5%) were glucose non-fermenting bacteria and 22 (18.5%) were glucose fermenters. Most isolates were in the *Pseudomonas* genus (80 strains), with a high frequency of *Pseudomonas fluorescens* and *Pseudomonas putida* (32 and 18 strains respectively) (Table 1). Other species of this genus isolated at low frequency included *Pseudomonas aeruginosa* (1), *Pseudomonas alcaligenes* (1), *Pseudomonas chlororaphis* (1), *Pseudomonas ludensis* (2), *Pseudomonas maculicola* (2), *Pseudomonas nitroreducens* (2), *Pseudomonas synxantha* (1) and *Pseudomonas viridilivida* (5). With the exception of one *Aeromonas hydrophila* strain recovered from the CC1 farm, all resistant strains isolated from the land-based farms (CC1-CC3) were non-fermenters. The remaining glucose-fermenting strains were all enteric species, mainly belonging to the species *Enterobacter amnigenus* (six strains) and *Kluyvera ascorbata* (four strains) (Table 1). As multiple florfenicol-resistant colonies were selected from some samples, it is possible that they represent clones of the same isolate. For example, it is plausible that the six colonies of *E. amnigenus* from Farm F3 are all the same isolate (Tables 1 and 2).

Florfenicol resistance levels and efflux pump inhibition

The distribution of florfenicol MIC values in the presence and the absence of the EPI Phe-Arg-β-naphthylamide (MC-207,110) indicated that the EPI had an effect on florfenicol resistance in some of the studied strains and therefore demonstrated active participation of non-specific efflux pump systems in florfenicol resistance in these strains. Florfenicol MIC values without EPI ranged from 32 to >256 μg ml^{-1} (MIC_{50} and MIC_{90} values of 256 and >256 μg ml^{-1} respectively), while MIC values in the presence of EPI ranged from 8 to 256 μg ml^{-1} (MIC_{50} and MIC_{90} values of 32 and 256 μg ml^{-1} respectively). The effect of EPI on florfenicol resistance was observed for three glucose-fermenter and 80 non-fermenter strains. Twenty glucose-fermenter strains and 16 non-fermenter strain maintained high levels of resistance to florfenicol in the presence of EPI, suggesting that the resistance to florfenicol in these strains was not controlled by the targeted multi-drug transporters.

Detection of the *floR* gene

Only 26 (21.85%) of the PCR-assayed strains were positive for the *floR* gene. Twenty-one of these strains were glucose-fermenters isolated from F1, F3 and F4 farms

Table 2. Source, identification, florfenicol Minimum Inhibitory Concentration (in μg ml^{-1}), and anti-bacterial resistance pattern of the *floR*-carrying strains recovered from Chilean salmon farms

Species	Source	Farm	MIC FFC	Resistance phenotype
Enterobacter aerogenes	Cage sediment	F1	256	AM-CTX-S-CM-FFC-OT-FX
Hafnia alvei	Mucus	F1	256	AM-CTX-S-CM-FFC-OT-FX
Kluyvera ascorbata	Mucus	F1	256	AM-S-K-CM-FFC-OT-NA
Hafnia alvei	Intestinal content	F1	256	AM-CTX-S-CM-FFC-OT-NA-FX
Kluyvera ascorbata	Intestinal content	F1	>256	AM-S-CM-FFC-OT-FX
Kluyvera ascorbata	Intestinal content	F1	>256	AM-S-K-CM-FFC-OT-FX
Raoultella terrigena	Intestinal content	F1	>256	AM-S-K-CM-FFC-OT-FX
Citrobacter amalonaticus	Mucus	F3	>256	AM-S-K-CM-FFC-OT
Citrobacter freundii	Mucus	F3	256	S-K-CM-FFC-OT
Citrobacter youngae	Mucus	F3	256	S-K-CM-FFC-OT-SXT
Enterobacter amnigenus	Mucus	F3	256	S-CM-FFC-OT
Enterobacter amnigenus	Mucus	F3	256	S-CM-FFC-OT
Enterobacter amnigenus	Mucus	F3	256	S-CM-FFC-OT
Enterobacter amnigenus	Mucus	F3	256	S-CM-FFC-OT
Enterobacter amnigenus	Mucus	F3	256	S-CM-FFC-OT
Enterobacter amnigenus	Mucus	F3	256	S-CM-FFC-OT
Cedecea davisae	Cage water	F4	256	AM-S-K-CN-CM-FFC-OT-FX
Sphingobacterium multivorum	Cage water	F4	256	AM-S-K-CN-CM-FFC-OT-OA-FX-SXT
Pseudomonas alcaligenes	Cage water	F4	>256	AM-S-CM-FFC-OT-SXT
Pseudomonas putida	Cage water	F4	>256	AM-CTX-S-FFC-CM-OT-NA-OA-UB-FX-SXT
Pantoea sp.	Cage water	F4	>256	AM-S-CM-FFC-OT-NA-OA-UB-FX
Pantoea sp.	Mucus	F4	256	AM-S-CN-CM-FFC-OT-FX
Alcaligenes faecalis	Mucus	F4	>256	AM-S-CN-CM-FFC-OT-NA-OA-UB-ENR-FX-SXT
Enterobacter cloacae	Mucus	F4	256	AM-S-CN-CM-FFC-OT-NA-FX
Ralstonia pickettii	Mucus	F4	>256	AM-CTX-S-K-CN-CM-FFC-OT-NA-OA-UB-FX-SXT
Kluyvera ascorbata	Mucus	F4	>256	AM-S-K-CM-FFC-OT-FX

AM, ampicillin; CTX, cefotaxime; S, streptomycin; K, kanamycin; CN, gentamicin; CM, chloramphenicol; FFC, florfenicol; OT, oxytetracycline; NA, nalidixic acid; OA, oxolinic acid; UB, flumequine; FX, furazolidone; SXT, trimethoprim-sulphametoxazole.

(Table 2). These were identified as *E. amnigenus* (6), *K. ascorbata* (4), *Hafnia alvei* (2), *Pantoea* sp. (2), *Enterobacter aerogenes* (2), *Enterobacter cloacae* (1), *Citrobacter youngae* (1), *Citrobacter freundii* (1), *Cedecea davisae* (1) and *Raoultella terrigena* (1). The *floR* positive non-fermenter strains were only recovered from the F4 farm and were identified as *Sphingobacterium multivorum*, *P. alcaligenes*, *P. putida*, *Ralstonia pickettii* and *Alcaligenes faecalis*. No strains recovered from the land-based farms (CC1-CC3) possessed the *floR* gene. It is important to note that the most *floR*-carrying strains were recovered from fingerling samples (20 strains), mainly salmon mucus. Three of the *floR*-carrying strains (*R. terrigena*, *Citrobacter amalonaticus* and *S. multivorum*) also were positive for the presence of a non-specific drug efflux pump.

Anti-microbial resistance patterns of the *floR*-carrying strains

Among the florfenicol-resistant strains carrying the *floR* gene isolated from the F1, F3 and F4 farms, a high incidence of multi-drug resistance (MDR) was observed, with all strains resistant to at least 5 anti-bacterials (Table 2). All of the *floR*-carrying strains were resistant to chloramphenicol, streptomycin and oxytetracycline in addition to florfenicol, and most of the strains recovered from the F1 and F4 farms also exhibited resistance to ampicillin and furazolidone. Resistance to gentamicin, nalidixic acid, oxolinic acid and flumequine was only detected in some strains isolated from the F4 farm, mainly belonging to non-fermenting species, and it is possible that resistance to one or more of these antibiotics could be intrinsic.

Discussion

There are few reports of the *floR* gene in aquatic environments. In a recent study of tetracycline resistance in bacteria from aquaculture in China, some of the isolates also possessed the *floR* gene (Dang et al., 2007). Another recent study of an *Aeromonas bestiarum* strain from a freshwater stream assessed the molecular structure of the *floR* gene in this isolate and found the gene linked to a tetracycline resistance gene (Gordon et al., 2008). In both of these cases, the *floR* gene was found in potential pathogens in aquaculture. In our study, there was a diversity of strains that possessed the *floR* gene, but most commonly

the gene was found in the glucose-fermenting strains. The diversity of bacteria possessing the *floR* gene suggests considerable horizontal gene transfer, although a more detailed sequence-based analysis of the *floR* genes would be needed to understand the evolutionary history of these transfer events.

There were considerable differences in the microbiota composition and in the prevalence of the *floR* gene among the farms in this study, even within the same company. For example, for the farms in Lago Rupanco, the gene was only detected in the F1 farm microbiota, despite the fact that both farms belong to the same company and have a similar history of florfenicol usage. This difference could be explained by the lack of glucose-fermenting strains recovered from the F2 farm, given that the presence of the *floR* gene was highly associated with the glucose fermenters. There was a high concordance in the resistance phenotypes of the *floR*-carrying strains isolated from the F1 and F4 farms, but anti-bacterial resistance patterns of the *floR*-carrying strains recovered from salmon farms located at Lago Llanquihue were remarkably different, where the F4 farm strains exhibited higher multi-drug resistance levels than those isolated from the F3 farm. In addition, among the *floR*-carrying bacteria, sulfonamide resistance was observed in the strains recovered from F4 but not in the strains from F3. This variability is probably due largely to the cross-sectional design of the study.

Previous studies demonstrated that the presence of the *floR* gene is usually associated with high florfenicol MIC values (White et al., 2000; Bischoff et al., 2002). In this study, strains carrying the *floR* gene showed MIC values of ≥ 256 μg ml^{-1}, and are in accordance with Singer et al. (2004), who found that the isolates harbouring the *floR* gene exhibited MIC values of ≥ 256 μg ml^{-1}. It should be emphasized, though, that samples were screened in the presence of 30 μg ml^{-1} of florfenicol and therefore *floR*-positive isolates with low florfenicol MICs would not have been detected.

Significant reductions in the MIC values of most of the non-fermenter strains following EPI suggest direct involvement of broadly non-specific multi-drug efflux pumps. These mechanisms have been frequently reported for *Pseudomonas* species (Li et al., 1994; Poole, 2002). These results agree with Michel et al. (2005), who found a high frequency of constitutive multi-drug resistance mediated by efflux mechanisms in *Chryseobacterium* strains isolated from aquatic environments. These strains had a high resistance to florfenicol but were negative for the *floR* gene. Efflux pump systems are extensively observed in environmental bacteria, and it has been argued that this is a result of non-specific exposure of the micro-organism to a wide variety of substances that may have nothing to do with anti-bacterials (Paulsen et al., 1996).

The EPI caused at least a 4-fold reduction in the MIC values of a high number of assayed strains, but MIC values still remained over the breakpoint value stated for florfenicol resistance (8 μg ml^{-1}) (White et al., 2000; Singer et al., 2004), suggesting that other resistance factors are also involved in florfenicol resistance in these strains. It has been reported that many *Pseudomonas* species may express different drug transporters, and the inhibition of an efflux pump can potentially be compensated by the overexpression of other pumps with overlapping spectra (Lee et al., 2000). Simultaneous overexpression of two efflux mechanisms was demonstrated for a clinical isolate of *P. aeruginosa* (Pumbwe and Piddock, 2000), providing evidence that two (or even more) efflux systems can be overexpressed and function together in one bacterium. In addition, it has been reported that several types of multi-drug transporters may coexist together in the same bacterium with specific transporters (Lee et al., 2000; White et al., 2000). In this study, there were three strains that demonstrated simultaneous expression of a florfenicol specific exporter, such as FloR and non-specific multi-drug efflux pumps.

Recently, studies have begun to elucidate the diversity of antibiotic resistance genes that exist in the environment (Riesenfeld et al., 2004), particularly in areas with little to no antibiotic use or other anthropogenic influence (Allen et al., 2008). This diversity of antibiotic resistance that exists naturally in the environment has been termed the resistome (D'Costa et al., 2006), but unfortunately the resistome of aquaculture environments is understudied. To understand the impacts that antibiotic usage in aquaculture can have on human and animal health, it is imperative to take a more ecological view of resistance (Singer et al., 2006). We must explore the diversity of antibiotic resistant bacteria and antibiotic resistance genes that exist in an environment, even when no antibiotics are used, and then evaluate how antibiotics affect these microbial populations.

The isolates in this study that possessed the *floR* gene were commonly multi-drug resistant, and these bacterial strains could potentially be serving as a reservoir for a diversity of antibiotic resistance genes. Any antibiotic that is used in aquaculture has the potential to select for antibiotic resistant bacteria. Given the diversity of antibiotic resistance patterns observed in this study in the *floR*-positive isolates, a single antibiotic has the potential to co-select for a diversity of resistances. For this reason, human health as well as animal health can potentially be impacted by the use of antibiotics in aquaculture. To assess this potential risk, future studies should focus on the ability of different antibiotics used in aquatic

environments to co-select for multiple resistances, the molecular basis of this diversity of resistance, and whether the genes conferring resistance can be transferred to other bacteria, including those of human health concern. To put these data in context, though, much more work must be carried out on the resistome in aquatic environments, particularly in farms that do not use antibiotics.

Acknowledgements

This study was supported by the Science and Technology National Council (CONICYT) of Chile by the Project Grant No. 1040924.

References

Alderman, D. J., and T. S. Hastings, 1998: Antibiotic use in aquaculture: development of antibiotic resistance – potential for consumer health risks. *Int. J. Food Sci. Technol.* 33, 139–155.

Allen, H. K., L. A. Moe, J. Rodbumrer, A. Gaarder, and J. Handelsman, 2008: Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *ISME J.* 3, 243–251.

Bischoff, K. M., D. G. White, P. F. McDermott, S. Zhao, S. Gaines, J. J. Maurer, and D. J. Nisbet, 2002: Characterization of chloramphenicol resistance in beta-hemolytic *Escherichia coli* associated with diarrhea in neonatal swine. *J. Clin. Microbiol.* 40, 389–394.

Bolton, L. F., L. C. Kelley, M. D. Lee, P. J. Fedorka-Cray, and J. J. Maurer, 1999: Detection of multidrug-resistant *Salmonella enterica* serotype typhimurium DT104 based on a gene which confers cross-resistance to florfenicol and chloramphenicol. *J. Clin. Microbiol.* 37, 1348–1351.

Cabello, F. C., 2004: Antibiotics and aquaculture in Chile: implications for human and animal health. *Rev. Med. Chil.* 132, 1001–1006.

Cabello, F. C., 2006: Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ. Microbiol.* 8, 1137–1144.

D'Costa, V. M., K. M. McGrann, D. W. Hughes, and G. D. Wright, 2006: Sampling the antibiotic resistome. *Science* 311, 374–377.

Dang, H., X. Zhang, L. Song, Y. Chang, and G. Yang, 2007: Molecular determination of oxytetracycline-resistant bacteria and their resistance genes from mariculture environments of China. *J. Appl. Microbiol.* 103, 2580–2592.

Gordon, L., A. Cloeckaert, B. Doublet, S. Schwarz, A. Bouju-Albert, J. P. Ganiere, B. H. Le, A. Le Fleche-Mateos, and E. Giraud, 2008: Complete sequence of the *floR*-carrying multiresistance plasmid pAB5S9 from freshwater *Aeromonas bestiarum*. *J. Antimicrob. Chemother.* 62, 65–71.

Kadlec, K., C. Kehrenberg, and S. Schwarz, 2007: Efflux-mediated resistance to florfenicol and/or chloramphenicol in *Bordetella bronchiseptica*: identification of a novel chloramphenicol exporter. *J. Antimicrob. Chemother.* 59, 191–196.

Keyes, K., C. Hudson, J. J. Maurer, S. Thayer, D. G. White, and M. D. Lee, 2000: Detection of florfenicol resistance genes in *Escherichia coli* isolated from sick chickens. *Antimicrob. Agents Chemother.* 44, 421–424.

Lee, A., W. Mao, M. S. Warren, A. Mistry, K. Hoshino, R. Okumura, H. Ishida, and O. Lomovskaya, 2000: Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance. *J. Bacteriol.* 182, 3142–3150.

Li, X. Z., D. M. Livermore, and H. Nikaido, 1994: Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. *Antimicrob. Agents Chemother.* 38, 1732–1741.

Michel, C., O. Matte-Tailliez, B. Kerouault, and J. F. Bernardet, 2005: Resistance pattern and assessment of phenicol agents' minimum inhibitory concentration in multiple drug resistant *Chryseobacterium* isolates from fish and aquatic habitats. *J. Appl. Microbiol.* 99, 323–332.

Miranda, C. D., and R. Rojas, 2007: Occurrence of florfenicol resistance in bacteria associated with two Chilean salmon farms with different history of antibacterial usage. *Aquaculture* 266, 39–46.

Miranda, C. D., and R. Zemelman, 2002a: Antimicrobial multiresistance in bacteria isolated from freshwater Chilean salmon farms. *Sci. Total Environ.* 293, 207–218.

Miranda, C. D., and R. Zemelman, 2002b: Bacterial resistance to oxytetracycline in Chilean salmon farming. *Aquaculture* 212, 31–47.

National Committee for Clinical Laboratory Standards, 2002: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard, 2nd edn. National Committee for Clinical Laboratory Standards, Wayne, PA.

Paulsen, I. T., M. H. Brown, and R. Skurray, 1996: Proton-dependent multidrug efflux systems. *Microbiol. Rev.* 60, 575–608.

Poole, K., 2002: Multidrug Efflux Pumps and Antimicrobial Resistance in Pseudomonas aeruginosa and Related Organisms in Microbial Multidrug Efflux. Horizon Scientific Press, Norfolk, pp. 273–298.

Pumbwe, L., and L. J. Piddock, 2000: Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 44, 2861–2864.

Riesenfeld, C. S., R. M. Goodman, and J. Handelsman, 2004: Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ. Microbiol.* 6, 981–989.

Schwarz, S., C. Kehrenberg, and T. R. Walsh, 2001: Use of antimicrobial agents in veterinary medicine and food animal production. *Int. J. Antimicrob. Agents* 17, 431–437.

Schwarz, S., C. Kehrenberg, B. Doublet, and A. Cloeckaert, 2004: Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol. Rev.* 28, 519–542.

Singer, R. S., S. K. Patterson, A. E. Meier, J. K. Gibson, H. L. Lee, and C. W. Maddox, 2004: Relationship between phenotypic and genotypic florfenicol resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* 48, 4047–4049.

Singer, R. S., M. P. Ward, and G. Maldonado, 2006: Can landscape ecology untangle the complexity of antibiotic resistance? *Nat. Rev. Microbiol.* 4, 943–952.

White, D. G., C. Hudson, J. J. Maurer, S. Ayers, S. Zhao, M. D. Lee, L. Bolton, T. Foley, and J. Sherwood, 2000: Characterization of chloramphenicol and florfenicol resistance in *Escherichia coli* associated with bovine diarrhea. *J. Clin. Microbiol.* 38, 4593–4598.

