Antimicrobial resistance genes in marine bacteria and human uropathogenic *Escherichia coli* from a region of intensive aquaculture

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**Summary**

Antimicrobials are heavily used in Chilean salmon aquaculture. We previously found significant differences in antimicrobial-resistant bacteria between sediments from an aquaculture and a non-aquaculture site. We now show that levels of antimicrobial resistance genes (ARG) are significantly higher in antimicrobial-selected marine bacteria than in unselected bacteria from these sites. While ARG in tetracycline- and florfenicol-selected bacteria from aquaculture and non-aquaculture sites were equally frequent, there were significantly more plasmid-mediated quinolone resistance genes per bacterium and significantly higher numbers of *qnrB* genes in quinolone-selected bacteria from the aquaculture site. Quinolone-resistant urinary *Escherichia coli* from patients in the Chilean aquacultural region were significantly enriched for *qnrB* (including a novel *qnrB* gene), *qnrS*, *qnrA* and *aac(6')-Ib*, compared with isolates from New York City. Sequences of *qnrA1*, *qnrB1* and *qnrS1* in quinolone-resistant Chilean *E. coli* and Chilean marine bacteria were identical, suggesting horizontal gene transfer between antimicrobial-resistant marine bacteria and human pathogens.

**Introduction**

The concept that excessive use of antimicrobials in terrestrial animal husbandry could be detrimental to human and animal health was initially challenged but is now generally accepted (Prescott, 2006; Davies and Davies, 2010; Marshall and Levy, 2011). Even though aquaculture is an ancient human activity, industrial aquaculture (including that of salmon and shrimp) is relatively recent (Diana, 2009; Cabello *et al.*, 2013). This industrialization has been accompanied by increased use of prophylactic, metaphylactic and therapeutic antimicrobials against bacterial diseases arising from increased numbers of larger farms, higher densities of fish in pens and deficiencies in preventive and sanitary conditions (Asche *et al.*, 2010; Barton and Fløysand, 2010). Such use may have potential implications for human health.

Emerging antimicrobial resistance genes (ARG) in human pathogens such as *qnr*, *tetG* and *tetC*, *floR* and some β-lactamase genes have been identified as potentially of aquatic origin (Baquero *et al.*, 2008; Taylor *et al.*, 2011; Cabello *et al.*, 2013; Cantas *et al.*, 2013). ARG and mobile genetic elements such as plasmids, integrons and integrative and conjugative elements have also been shown to be shared between aquatic bacteria and terrestrial animal and human pathogens (Baquero *et al.*, 2008; Poirel *et al.*, 2012; Cabello *et al.*, 2013; Cantas *et al.*, 2013). These findings suggest a potential uni- or bidirectional flow of ARG and mobile genetic elements between bacteria of the aquatic and terrestrial environments linking the aquatic and human resistomes.

Antimicrobials heavily used in Chilean aquaculture (tetracycline, florfenicol, quinolones) were selected for antimicrobial resistance in bacteria cultured from marine sediments (Buschmann *et al.*, 2012). This selection was probably related to the presence of acquired ARG in these bacteria and to an intrinsic resistome for these antimicrobials (Buschmann *et al.*, 2012; Olivares *et al.*, 2013; Perry *et al.*, 2014; Shah *et al.*, 2014). Sediments both close to and far from aquacultural activities contained residual flumequine, a heavily used quinolone in this aquacultural
region of Chile. We have now studied acquired ARG in the presence of tetracycline, florfenicol and quinolones in antimicrobial-resistant bacteria (ARB) from water columns and sediments of these sites. To investigate genetic links between quinolone-resistant marine bacteria and human pathogens, plasmid-mediated quinolone resistance (PMQR) genes in quinolone-resistant marine bacteria in Chile and in clinical isolates of quinolone-resistant human urinary tract *Escherichia coli* from coastal areas near aquacultural sites were compared.

Results and discussion

Aquacultural activities in Chile take place in close proximity to the coast. Water, sediment and fish in the Chilean littoral are thus contaminated with ARB of animal and human origin (Silva *et al.*, 1987; Miranda and Zemelman, 2001). For these reasons, contamination of marine samples and bacterial isolates with non-aquatic bacteria was a real possibility. Bacterial species harbouring ARG were identified by 16S RNA gene sequence analysis (see Supporting information for details and other experimental procedures) in 26 randomly chosen tetracycline-resistant bacteria of 48 isolates, in 46 oxolinic acid-resistant bacterial isolates and in all 16 florfenicol-resistant bacterial isolates. Of 88 bacteria whose species was identified (Table 1), all have been previously found in the aquatic environment and were therefore not considered terrestrial contaminants. These findings suggest that indigenous marine bacteria contain ARG also found in animal and human pathogens (Taylor *et al.*, 2011; Cabello *et al.*, 2013).

*tetA*, *tetB*, *tetK* and *tetM* have been frequently reported in bacteria from aquatic environments (Chopra and Roberts, 2001; Roberts and Schwarz, 2009). These genes were found 39 times in 24 tetracycline-resistant strains from the aquaculture site and 39 times in 24 tetracycline-resistant strains from the non-aquaculture site (Table 2). There were no differences in their frequencies between isolates from sediments and water. Twenty-one isolates contained more than one tetracycline-resistance gene (Table S1). There were more isolates with *tetA/B* than *tetK/M* genes (47 vs. 31), probably reflecting the fact that most of the studied bacteria were Gram-negative (data not shown) and *tetA/B* genes are more frequent in Gram-negative than in Gram-positive bacteria (Chopra and Roberts, 2001). The number of *tetA/tetB/tetK* genes per bacterial cell in tetracycline-resistant selected bacteria in sediments from the aquaculture site was significantly greater than that previously found in unselected marine bacteria from these same sediments (Buschmann *et al.*, 2012): 1.16 and 0.58 genes per bacterium respectively (*P* < 0.05).

*flor* was present in seven of 16 florfenicol-resistant marine bacterial isolates from the aquaculture site and in nine of 16 florfenicol-resistant isolates from the non-aquaculture site (Table 2). The number of florfenicol resistance genes per bacterial cell in sediments from the aquaculture site in florfenicol-resistant selected bacteria was again significantly greater than that previously found (Buschmann *et al.*, 2012) in unselected marine bacteria from these same sediments, 0.71 vs. 0.17 (*P* < 0.05).

Thirty-one PMQR genes (*qnrA*, *qnrB74*, *qnrS*, *aac(6′)-1b*) were detected in 23 quinolone-resistant bacteria from the aquaculture site while 20 were detected in 23 quinolone-resistant bacteria from the non-aquaculture site (Table 2). None of the quinolone-resistant isolates contained *qnrC/qnrD/qepA/oqxA*. Thirty-four of 46 quinolone-resistant isolates carried PMQR genes, 17 with more than one of them (Table 2, Table S1). Importantly, PMQR genes were not detected in five quinolone-resistant strains from the aquaculture site and in seven from the non-aquaculture site. There were no significant differences between the total number of PMQR genes in isolates from aquaculture and non-aquaculture sites or in isolates from sediments and water. The frequency of isolates from the aquaculture site with multiple PMQR genes was significantly greater than those from

<table>
<thead>
<tr>
<th>Gene</th>
<th>Bacteria</th>
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<tbody>
<tr>
<td><em>tetA</em></td>
<td><em>Pseudoalteromonas</em> sp., <em>Shewanella</em> sp., <em>Psychrobacter</em> sp., <em>Cobetia</em> sp., <em>North Sea bacterium</em> H7</td>
</tr>
<tr>
<td><em>tetB</em></td>
<td><em>Pseudoalteromonas</em> sp., <em>Shewanella</em> sp., <em>Psychrobacter</em> sp., <em>Cobetia</em> sp., <em>Vibrio</em> sp.</td>
</tr>
<tr>
<td><em>tetK</em></td>
<td><em>Pseudoalteromonas</em> sp., <em>Shewanella</em> sp., <em>Cobetia</em> sp., <em>Vibrio</em> sp.</td>
</tr>
<tr>
<td><em>tetM</em></td>
<td><em>Pseudoalteromonas</em> sp., <em>Shewanella</em> sp., <em>Cobetia</em> sp., <em>Vibrio</em> sp., <em>Pseudomonas</em> sp.</td>
</tr>
<tr>
<td><em>qnrA</em></td>
<td><em>Alcanivorax</em> sp., <em>Acrobacter</em> sp., <em>Arthrobacter</em> sp., <em>Kytococcus</em> sp., <em>Marinobacter</em> sp., <em>Microbacterium</em> sp., <em>Pseudomonas</em> sp., <em>Rhodococcus</em> sp., uncultured bacterium</td>
</tr>
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<td><em>qnrB</em></td>
<td><em>Actinobacterium</em> sp., <em>Cellulophaga</em> sp., <em>Flavobacteriaceae</em>, <em>Erythrobacter</em> sp., <em>Kytococcus</em> sp., <em>Marinobacter</em> sp., <em>Rhodococcus</em> sp., <em>Tsukamurella</em> sp., uncultured bacterium</td>
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<td><em>qnrS</em></td>
<td><em>Acrobacter</em> sp., <em>Arthrobacter</em> sp., <em>Cellulophaga</em> sp., <em>Dietzia</em> sp., <em>Erythrobacter</em> sp., <em>Marinobacter</em> sp., <em>Microbacterium</em> sp., <em>Pseudomonas</em> sp., <em>Rhodococcus</em> sp., uncultured bacterium</td>
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<tr>
<td><em>aac(6′)-1b-cr</em></td>
<td><em>Arthrobacter</em> sp., <em>Cellulophaga</em> sp., <em>Flavobacteriaceae</em>, <em>Erythrobacter</em> sp., <em>Kytococcus</em> sp., <em>Rhodococcus</em> sp., uncultured bacterium</td>
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<tr>
<td><em>floR</em></td>
<td><em>Pseudoalteromonas</em> sp., <em>Marinobacter</em> sp., <em>Shewanella</em> sp., <em>Cobetia</em> sp., <em>Halomonas</em> sp.</td>
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the non-aquaculture site (13 of 18 vs. 4 of 16) \((P<0.05)\). For example, *Rhodococcus* sp. and *Kytococcus* sp. isolated from the aquaculture site harboured *qnrA-qnrB-qnrC* and *qnrB-aac(6')-Ib-cr*, respectively. In contrast, *Microbacterium* sp. and *Kytococcus sedentarius* isolated from the non-aquaculture site harboured only *qnrS* and *qnrA*, respectively (data not shown).

The number of PMQR genes per bacterium at the aquaculture site was significantly greater than at the non-aquaculture site, 1.35 vs. 0.87 \((P=0.05)\). Furthermore, the number of PMQR genes per bacterial cell in sediments from the aquaculture site in quinolone-resistant selected bacteria was significantly greater than that previously found in unselected marine bacteria from these same sediments (Buschmann *et al.*, 2012), 1.58 vs. 0.58 \((P<0.05)\). There were also differences in distribution of specific genes among the quinolone-resistant isolates (Table S1). In 23 quinolone-resistant isolates, *qnrA* was present in six quinolone-resistant isolates from the non-aquaculture site and in four from the aquaculture site \((P>0.05)\); *qnrB* was present in 13 quinolone-resistant isolates from the aquaculture site and in two from the non-aquaculture site \((P<0.05)\); and *aac(6')-Ib* was present in six isolates from the aquaculture site and three from the non-aquaculture site \((P>0.05)\). Three of these nine *aac(6')-Ib* genes were sequenced; all corresponded to the *aac(6')-Ib-cr* allele, suggesting that this allele is highly represented among these bacteria (data not shown). The *aac(6')-Ib-cr* has been previously found in aquatic *Laribacter hongkongensis* and *Aeromonas* (Chen *et al.*, 2013; Deng *et al.*, 2014).

More than two-thirds of isolated ARB contained ARG potentially responsible for their resistance to tetracycline, florfenicol and quinolones. This suggests that the presence of antimicrobials used in aquaculture exerted important selective pressure in this environment (Buschmann *et al.*, 2012; Shah *et al.*, 2014). It also suggests that marine bacteria in this environment contain other as yet undescribed and uncharacterized tetracycline, quinolone and florfenicol resistance genes and mechanisms of resistance in their acquired and intrinsic resistome (Miranda *et al.*, 2003; van Hoek *et al.*, 2011; Cabello *et al.*, 2013; Olivares *et al.*, 2013; Perry and Wright, 2014). While 81% of tetracycline-resistant bacteria contained the tested tetracycline resistance genes and 74% quinolone-resistant bacteria contained the tested PMQR genes, only 50% of florfenicol-resistant bacteria contained the *floR* gene (Table 2). Tetracycline- and quinolone-resistant bacteria usually contained more than one ARG per isolate, implying high levels of resistance in these bacteria by gene dosage effect. The high and multiple numbers of ARG detected in this study are in contrast to the lower numbers previously found in bacteria from the same area (Shah *et al.*, 2014). This difference might be due to the

<table>
<thead>
<tr>
<th>Site</th>
<th>Tetracycline</th>
<th>Quinolones</th>
<th>Florfenicol</th>
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<tbody>
<tr>
<td>Aquaculture Water</td>
<td>Tested 12</td>
<td>Tested 12</td>
<td>Tested 4</td>
</tr>
<tr>
<td>Aquaculture Sediment</td>
<td>Tested 12</td>
<td>Tested 12</td>
<td>Tested 4</td>
</tr>
<tr>
<td>Non-aquaculture Water</td>
<td>Tested 12</td>
<td>Tested 12</td>
<td>Tested 4</td>
</tr>
<tr>
<td>Non-aquaculture Sediment</td>
<td>Tested 12</td>
<td>Tested 12</td>
<td>Tested 4</td>
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use of high concentrations of antimicrobials in agar plate selection. Such a methodological difference could preferentially select for highly resistant bacteria because of gene dosage effects stemming from multiple ARG for the same antimicrobial in bacterial cells and from potential location of ARG in high-copy plasmids (Aedo et al., 2014).

Although frequencies of antimicrobial genes in bacterial isolates at both sites were significantly greater than those observed in unselected bacteria from the same sediments, there was no significant difference in frequencies of tetracycline and florfenicol antimicrobial-resistant isolates between the two sites. This absence of differences between aquaculture and non-aquaculture sites could plausibly be ascribed to the previously demonstrated contamination of the control site by flumequine residues as a result of the heavy and continuous application of antimicrobials in connection with aquacultural activities in the area for many years (Buschmann et al., 2012). ARB present in water could also be carried from aquaculture sites to non-aquaculture sites by local water currents. Potential differences between aquaculture and non-aquaculture sites may have been underestimated since only culturable bacteria and a limited number of acquired ARG responsible for these resistances were studied, and the mechanisms of resistance of the intrinsic resistome were not explored (Cabello et al., 2013; Olivares et al., 2013).

There were significant differences between the aquaculture and non-aquaculture sites by Fisher’s exact test as regards higher frequencies of bacteria with multiple PMQR genes. Unfortunately, the number of isolates may not be sufficient to distinguish differences between sites by Fisher’s exact test. However, the high frequency and variety of PMQR genes in these bacteria may be the result of selection by residual amounts of flumequine (Buschmann et al., 2012), which is not easily degraded in the marine environment (Björklund et al., 1991; Samuelsen et al., 1994).

tet, floR and PMQR genes were present in previously unreported bacteria: tetA was present in Shewanella and in North Sea bacterium H7; tetB was present in Shewanella and Psychrobacter; tetK in Shewanella and Cobetia; tetM in Cobetia; and floR in Marinobacter, Cobetia and Shewanella. Among the PMQR genes, qnrA was detected in Alcanivorax, Kytococcus and Rhodococcus; qnrB in Marinobacter, Kytococcus and Flavobacteria; qnrS in Dietzia sp. and Rhodococcus and aac(6′)-1b-cr in Arthrobacterium, Flavobacterium, Erythrobacter and Cellulophaga sp. In the present case, detection of these PMQR genes in species (or genera) of bacteria that have not been previously identified as carrying them greatly expands the host range of marine bacteria harbouring these genes and indicates that geography may influence their distribution. The high frequency with which qnrB, aac(6′)-1b and its variant, aac(6′)-1b-cr were detected in marine bacterial species in which they previously had not been found provides increasing support to the hypothesis that at least some PMQR genes have an aquatic origin (Poirel et al., 2005; Saga et al., 2005; Cattoir et al., 2007; Jacoby et al., 2011; Fonseca and Vicente, 2013; Jacoby and Hooper, 2013).

To investigate genetic links between quinolone-resistant marine bacteria and human pathogens, 25 quinolone-resistant E. coli isolates (determined by disc diffusion) from urinary tract infections in patients from a coastal region in Chile adjacent to industrial aquaculture sites and 23 E. coli quinolone-resistant isolates from urinary tract infections in patients from New York were examined for the presence of PMQR genes. None of these isolates harboured qnrC/qnrD/qepA/oqxA (Table 3). The total number of PMQR genes harboured by the Chilean uropathogenic isolates was significantly higher than those of the New York isolates (41 vs. 20) (P < 0.05). Twenty of the 25 E. coli quinolone-resistant isolates from Chile harboured qnrA/qnrB/qnrS/aac(6′)-1b, with 15 having more than one PMQR gene (Table S3). Sequencing of aac(6′)-1b genes from two randomly chosen Chilean E. coli quinolone-resistant clinical isolates indicated that both corresponded to the aac(6′)-1b-cr allele. Significantly fewer of the quinolone-resistant E. coli isolates from the United States harboured PMQR genes (15 of 23) and only five of these harboured more than one PMQR gene (P < 0.05). The distribution of PMQR genes between these two groups of isolates also differed. Although more qnrB, qnrS and aac(6′)-1b genes were detected in the Chilean isolates than in the New York isolates, this difference was only significant for qnrB and aac(6′)-1b (P < 0.05). In contrast, the quinolone-resistant North American clinical isolates harboured more qnrA genes than clinical isolates from Chile.
*qnr* genes obtained from three randomly chosen marine bacterial isolates and three randomly chosen clinical isolates of *E. coli* were sequenced. Sequences of one *qnrA1* gene from a putative uncultured marine bacteria species, one *qnrB1* gene from *Rhodococcus* sp. and one *qnrS1* gene from *Microbacterium* sp. were identical to sequences of *qnrA1*, *qnrB1* and *qnrS1* genes found in three randomly chosen, quinolone-resistant Chilean urinary tract *E. coli* isolates from the aquaculture area. A different Chilean *E. coli* clinical isolate harboured a *qnrB* gene whose sequence was not present in databases. Phylogenetic analysis confirmed that this novel *qnrB* gene (*qnrB74*) was indeed a member of the *qnrB* family (Fig. S1).

The significantly higher number of uropathogenic *E. coli* harbouring PMQR genes isolated from the aquaculture coastal region in Chile strongly suggests that the population of uropathogenic *E. coli* in the aquacultural regions is under more intense selective pressure than those in New York. This is not unexpected since veterinary use of quinolones, especially in aquaculture, exceeds their use in human medicine in Chile by several-fold, and wild-caught fish for human consumption in this region may contain residual quinolones (Fortt *et al.*, 2007; Cabello *et al.*, 2013). Distribution and nucleotide sequences of these genes in uropathogenic *E. coli* mimicked those among marine bacteria isolated from the aquaculture site in this region. This suggests that either excessive use of quinolones and other antimicrobials in the region selects for the same PMQR genes in marine bacteria and uropathogenic *E. coli* or that genes selected in the marine or terrestrial environment find their way into bacteria from both environments by uni- or bidirectional horizontal gene transfer (Forsberg *et al.*, 2012).

This latter hypothesis is supported by the DNA sequence identity of *qnrA1*, *qnrB1* and *qnrS1* in three marine bacteria and three uropathogenic *E. coli* in the present study and from previous reports of DNA sequence identity of *aac(6’)-1b-cr* between marine bacteria and *E. coli* in this same geographical area (Aedo *et al.*, 2014). Identity of the DNA sequences of these PMQR genes in these different bacterial species from these separate environments is certainly consistent with exchanges by horizontal gene transfer (Forsberg *et al.*, 2012; Aedo *et al.*, 2014). A limitation of this study is that only some of the *aac(6’)-1b* genes were sequenced. We cannot therefore be certain that all of them correspond to the cr variant able to inactivate piperazinyl quinolones (Robicsek *et al.*, 2006). Additional limitations of the study are the small numbers of bacterial isolates studied, the small number of PMQR genes in quinolone-resistant marine bacteria and uropathogenic *E. coli* isolates that were sequenced and a lack of direct assessment of their genetic location and transmissibility. We plan to address these limitations with appropriate molecular and genetic experiments in future studies. Despite these limitations, our results strongly suggest that increased characterization of the bacterial resistome in places with increased aquatic antimicrobial use similar to the one sampled in this study may yield more examples of genes in the resistome of human pathogens of likely aquatic origin (Cabello *et al.*, 2013).

In sum, we have identified ARG in marine bacteria isolated from Chilean aquaculture and non-aquaculture sites endowing them with resistance to tetracycline, florfenicol and quinolones. PMQR genes were more frequently present in uropathogenic *E. coli* isolates from a Chilean aquaculture-bordering coastal site than in uropathogenic *E. coli* isolates from a non-aquaculture urban site in the United States. Some Chilean uropathogenic *E. coli* contained PRQR genes whose sequences were identical to those in local Chilean marine bacteria, suggesting linkage of these marine and terrestrial bacterial populations by uni- or bidirectional gene flow mediated by horizontal gene transfer. Aquacultural areas may thus constitute hotspots of evolution towards antimicrobial resistance (Baquero *et al.*, 2008; Taylor *et al.*, 2011; Cabello *et al.*, 2013; Cantas *et al.*, 2013).

**Nucleotide accession number**

The GenBank accession number of the new *qnrB* sequence, *qnrB74*, reported in the present study is KJ415247.

**Acknowledgements**

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**References**


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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Phylogenetic bootstrap analysis of a novel *qnrB1* gene (*qnrB74*) and other *qnrB* genes using the maximum likelihood method based on the Kimura two-parameter model. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories; $570 + G$, parameter $= 0.3371$). The tree with the highest log likelihood ($-3773$) is drawn to scale with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is indicated next to the branches; values $< 75\%$ have been omitted. The analysis involved 63 nucleotide sequences with 642 positions in the final dataset after all positions containing gaps and missing data had been eliminated.

**Table S1.** Marine bacterial isolates with single or multiple ARG

**Table S2.** Primers for PCR and positive control bacteria used in this study

**Appendix S1.** Experimental procedures