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Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254

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***Objectives*: The aim of this study was to examine the molecular basis for multiple antibiotic and mercury resistance in Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida*.**

***Methods*: Phenotypic and genotypic methods were employed to identify plasmid-associated antibiotic and mercury resistance genes and to determine the organization of those genes in multidrug-resistant (MDR) *A. salmonicida* isolates.**

***Results*: The MDR phenotype was transferable via conjugation using *Escherichia coli*, *Aeromonas hydrophila* and *Edwardseilla tarda* as recipients. Antibiotic and mercury resistance genes were carried by a conjugative IncA/C plasmid. Three distinct antibiotic resistance cassettes were characterized; first a class I integron containing an *aadA7* gene encoding for an aminoglycoside-3′-adenyltransferase, the second cassette showed 99.9% nucleotide sequence homology to a cassette previously identified in the *Salmonella enterica* IncA/C plasmid pSN254, containing *floR*, *tetA*, *sulII* and *strA/strB* sequences. The third cassette showed 100% nucleotide sequence similarity to a transposon-like element, containing a bla_{CMY-2} β-lactamase in association with *sugE* and *blc* sequences. This element is known to be widely distributed among clinical and food-borne *Salmonella* and other Enterobacteriaceae throughout Asia and the United States. Mercury resistance was linked to the presence of a *mer* operon that showed 100% nucleotide sequence homology to the *mer* operon carried by plasmid pSN254.**

***Conclusions*: Each MDR *A. salmonicida* isolate carried the same plasmid, which was related to plasmid pSN254. This is the first report of plasmid-mediated florfenicol-resistant *A. salmonicida* in North America. In addition, it is the first report of a plasmid-associated AmpC β-lactamase sequence in a member of the Aeromonadaceae.**

Keywords: AmpC β-lactamase, aquaculture, florfenicol, co-selection

Introduction

Aeromonas salmonicida subsp. *salmonicida* is the aetiological agent of furunculosis, an economically important disease of both wild and farmed salmonid fish.[1] Plasmid-mediated resistance to some, but not all, antibiotics utilized in the treatment of bacterial infections in aquaculture has been widely documented in clinical isolates of this pathogen.[2–7] Indeed, the emergence of

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multidrug-resistant (MDR) isolates towards the end of the 1980s represented a significant barrier to the successful development of the European salmonid aquaculture industry.[8] Fortunately, the successful development and subsequent large scale administration of oil-adjuvanted, injectable furunculosis vaccines in the early 1990s served to limit both the frequency and severity of *A. salmonicida* infections among populations of farmed salmonids. Consequently, the level of antibiotic chemotherapy employed in salmonid aquaculture has been reduced to only a fraction of that used previously.[9,10]

Excessive and imprudent antibiotic use is generally considered to be the principal driving force behind the growing prevalence of antibiotic resistance in both clinical and environmental bacteria.[11,12] Yet, it is pertinent to note that non-antibiotic agents, including mercury and other heavy metals, have been implicated as potential vehicles for the indirect co-selection of antibiotic resistance.[13,14] In this context, antibiotic and heavy metal resistance genes are frequently located on the same mobile genetic elements,[15,16] and a correlation between antibiotic and mercury resistance has been documented in the faecal and intestinal bacteria of primates with mercury amalgam fillings, where bacteria containing a mercury resistance operon were more likely to exhibit multiple antibiotic resistance.[17] In relation to fisheries, potential links between heavy metal and antibiotic resistance were reported by Pettibone *et al.*,[18] in *Aeromonas* spp. isolated from brown bullhead (*Ictalurus nebulosus*), in *Vibrio parahaemolyticus* isolated from shrimps[19] and for motile aeromonads and pseudomonads from rainbow trout (*Oncorhynchus mykiss*) farms in Australia.[20] In addition, nucleotide sequences comprising a mercury resistance operon and several antibiotic resistance genes all carried on a large plasmid recovered from a North American isolate of *A. salmonicida* subsp. *salmonicida* were recently deposited in the GenBank (accession number NC 009349) as part of the *A. salmonicida* genome sequencing project (accession number NC 009348).

Herein, we report the characterization of multiple drug and mercury-resistant isolates of *A. salmonicida* subsp. *salmonicida* recovered from Atlantic Canadian aquaculture facilities during 2002–04. Nucleotide sequence analysis revealed that the genes encoding the resistance phenotypes were organized into three separate resistance cassettes carried on a large transferable IncA/C plasmid backbone. The organization of the resistance cassettes showed a high degree of sequence homology to the recently described *Salmonella enterica* plasmid pSN254.[21] Our data support the hypothesis that environmental selective pressures, associated with the land-based use of antibiotics and possibly heavy metal pollution, may contribute to the development of resistance mechanisms that are subsequently transferred to other environmental compartments, in this case aquaculture facilities.[13,22]

Materials and methods

Bacterial isolates, determination of antibiotic resistance phenotypes and mercury MICs

A total of 13 MDR *A. salmonicida* subsp. *salmonicida* isolates, recovered from juvenile Atlantic salmon (*Salmo salar*) aquaculture facilities throughout New Brunswick and Nova Scotia during 2002, 2003 and 2004, were included in this study (Table 1). An additional seven isolates, from a variety of geographic locations and showing a range of resistance phenotypes, were included for comparative purposes (Table 1). Individual colonies of each isolate were used to perform antibiotic susceptibility testing (disc diffusion method) and to establish stock cultures. *Escherichia coli* DH5α (Invitrogen Life Technologies; Carlsbad, CA, USA) and antibiotic-susceptible field isolates of *Aeromonas hydrophila* and *Edwardsiella tarda* recovered from Canadian salmon aquaculture facilities, during 2004, were employed as recipients in conjugation experiments.

Antibiotic susceptibility assays employed a total of 13 antibiotics: ampicillin (10 μg), amoxicillin (10 μg), ceftiofur (30 μg), florfenicol (30 μg), enrofloxacin (5 μg), nalidixic acid (30 μg), sulfamethoxazole/trimethoprim (25 μg), sulfadimethoxine/ormetoprim (25 μg), trimethoprim (5 μg), oxytetracycline (30 μg), erythromycin (15 μg), gentamicin (10 μg) and streptomycin (10 μg). Disc diffusion antibiotic susceptibility testing was performed according to the criteria of Miller *et al.*[23] Commercial antibiotic discs were purchased from Oxoid, Canada, with the exception of the florfenicol discs that were provided by Schering Plough Veterinary Products (Kenilworth, NJ, USA).

MIC values for both $HgCl_2$ and phenylmercuric acetate (PMA; Sigma-Aldrich, Inc., St Louis, MO, USA) were determined for *A. salmonicida* and *E. coli* DH5α by the method of Wang *et al.*,[24] using brain heart infusion (BHI) plating media solidified with 1.5% (w/v) Noble agar (Difco-BBL, Sparks, MD, USA). Bacterial suspensions prepared as described above were used as inocula for plates containing 0, 50, 100, 250, 500, 750 and 1000 μM $HgCl_2$, or 0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 μM PMA and incubated for 2 days at 22°C. Mercury MICs were recorded as the lowest concentration of Hg that inhibited bacterial growth. Three control strains, *A. salmonicida* (ATCC 33658), mercury-susceptible *Bacillus subtilis* 168 and mercury-resistant *E. coli* SK1592, were run in parallel with the experimental strains.

Assessment of ability to transfer antibiotic and Hg resistance via conjugation

Broth conjugation experiments involved the production of overnight cultures of MDR isolates (donors) and *E. coli* DH5α (Invitrogen Life Technologies), *A. hydrophila* and *E. tarda* (recipients), in tryptone soya broth. Equal volumes (1 mL) of individual donor and recipient cultures were mixed and incubated at 22°C for 5 h. Thereafter, 100 μL aliquots of each mixture were used to inoculate plates of tryptone soya agar, supplemented with one of the following antibiotics: ampicillin (50 mg/L), florfenicol (20 mg/L), tetracycline (10 mg/L) or streptomycin (10 mg/L), or supplemented with $HgCl_2$ (75 μM) and the plates were incubated at 37°C overnight. It had been established previously that the recipients were (i) capable of growth at 37°C and (ii) susceptible to each of these antibiotics and $HgCl_2$ (with the exception of ampicillin in the case of *A. hydrophila*). In contrast, the donors were resistant to each of these drugs but were unable to grow at 37°C. Thus, the only bacteria that should have been able to grow in the presence of the antibiotics at 37°C would be transconjugants, recipients which had acquired the resistance phenotype via conjugative plasmid transfer. The frequency of transfer was determined according to the method of Sorum *et al.*[25] Three individual transconjugants were recovered from each of the different selective agar plates and their resistance phenotypes were evaluated using the disc diffusion assay described earlier. Genotypic analysis of transconjugants was performed by PCR as described below.

Table 1. *A. salmonicida* isolates employed in this study

Isolate code	Provided by	Location	Resistances	Year of isolation
FFA16	NBDAA	FW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2003
FFA17	NBDAA	SW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2003
FFA18	NBDAA	SW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2003
FFA19	NBDAA	FW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2003
FFA72	NBDAA	FW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2003
FFA74	NBDAA	SW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2003
AS03	Microtechnologies	FW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2003
FFA106	NBDAA	FW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2004
FFA108	NSDAF	FW (NS)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2004
FFA109	NSDAF	FW (NS)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2004
FFA111	NSDAF	FW (NS)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2004
FFA150	NBDAA	FW (NB)	AMP, FFC, OXT, NAL, STR, SUL, Hg	2004
FFA160	DFO	FW (NS)	AMP, FFC, OXT STR, SUL, Hg	2002
FFA20	NBDAA	FW(NB)	OXT	2003
ATCC 33658	ATCC	FW (USA)	none	1970
NVI 718	NVI	SW (Nor)	OXT, STR, SUL, TMP	1989
NVI 7076	NVI	SW (Nor)	OXT	1991
FFA110	NSDAF	NS	NAL	2004
FFA112	NSDAF	NS	OXT	2004
FFA113	NSDAF	NS	NAL, OXT	2004

FFA, Food Fisheries and Aquaculture; NBDAA, New Brunswick Department of Agriculture and Aquaculture; AS, *Aeromonas salmonicida*; NSDAF, Nova Scotia Department of Agriculture and Fisheries; DFO, Department of Fisheries and Oceans Canada (Moncton); ATCC, American Type Culture Collection; NVI, Norwegian School of Veterinary Science; FW, fresh water site; SW, sea water site; NB, New Brunswick; NS, Nova Scotia; Nor, Norway; AMP, ampicillin; FFC, florfenicol; OXT, oxytetracycline; NAL, nalidixic acid; STR, streptomycin; SUL, sulphonamide; TMP, trimethoprim; Hg, mercury.

Plasmid isolation

Plasmid isolation from *A. salmonicida* and transconjugants was performed using the E.N.Z.A. plasmid miniprep kit (Omega Bio-tek, Doraville, GA, USA) or the S.N.A.P. plasmid miniprep kit (Invitrogen Life Technologies). Plasmid profiles were determined on 0.8% agarose gels prepared in Tris–Borate–EDTA buffer, pH 8.0, stained with ethidium bromide and documented with an Imagemaster digital camera and associated annotation software (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

PCR-based detection of antibiotic and mercury resistance genes and sequences from IncA/C plasmids

Plasmid DNA preparations from MDR *A. salmonicida* and transconjugants were examined by PCR. Table S1 lists the primer sequences employed and is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/). Initially, primers for antibiotic resistance and integron gene detection were designed using sequences encoding products associated with resistance to tetracyclines, sulphonamides and streptomycin which had been previously identified in *A. salmonicida*. In contrast, the molecular basis for resistance to ampicillin, florfenicol and Hg had not been reported previously for *A. salmonicida*. Therefore, primers were designed based on sequences associated with resistance to those antibiotics and Hg in a variety of other animal and human pathogens. In order to determine if the resistance genes were present on an IncA/C plasmid, PCR-based analysis of MDR *A. salmonicida* and transconjugants was undertaken using a subset of previously described primers. Those assays employed a positive control, in the form of the IncA/C plasmid pYR1 from *Yersinia ruckeri* (kindly provided by Dr Timothy J. Welch, National Centre for Cool and Cold Water Aquaculture, USDA, Kearneysville, WV, USA).

Organization of the antibiotic resistance genes

Plasmid minipreps from either *A. salmonicida* or MDR transconjugants did not yield sufficient quantities of plasmid to perform direct sequence analysis. To overcome this obstacle, the individual antibiotic resistance genes and flanking sequences were cloned and analysed as follows. Plasmid preparations were partially digested with the restriction endonuclease *Sau3*A, and the resulting fragments were ligated with *Bam*HI digested vectors; either pUC19 or pUS972.[26] The ligation mixes were used to transform electrocompetent cells of *E. coli* DH5α with transformants selected on plates containing ampicillin (50 mg/L), florfenicol (20 mg/L), streptomycin (20 mg/L) or tetracycline (20 mg/L). Plasmid mini-preparations of individual transformants were produced and a minimum of 10 clones, containing inserts of different sizes, were selected to sequence and map the regions surrounding the resistance genes and to establish the organization of possible resistance cassettes. Sequencing of the inserts was achieved by primer walking on both strands, starting with M13 universal primers in the case of pUC19 or the primers 972F and 972R for pUS972 (Table S1). Cycle sequencing was performed using a Big Dye Terminator Kit and the associated protocol (PE Applied Biosystems, Foster City, CA, USA). All nucleotide sequence determinations were performed on an ABI 310 Genetic Analyzer (PE Applied Biosystems). Sequence alignments and the generation of resistance cassette contigs were performed using Sequencher (Version 4.6, Gene Codes Corporation, Ann Arbor, MI, USA). All sequences were entered into the BLAST search algorithm and the NCBI nucleotide database to confirm gene identity.

Results and discussion

As with land-based, food animal agriculture, the intensive culture of aquatic species requires the periodic use of antimicrobial compounds to control bacterial disease outbreaks. In the specific case of furunculosis, the introduction of highly efficacious vaccines and improved husbandry practices in the early 1990s resulted in a dramatic reduction in the use of antibiotics to treat this condition.[8] However, breakdowns in vaccine performance can and do occur, and in such situations, it is necessary to resort to treatment with a limited selection of antibiotics licensed for use in aquaculture.[10]

Resistance phenotypes of MDR isolates

The *A. salmonicida* isolates examined in the current study were recovered from both freshwater and marine salmon farming sites throughout Atlantic Canada during 2002, 2003 and 2004 and were responsible for significant (~$18 million CAD) economic losses. Initial analysis of four isolates (FFA16, 17, 18 and 19), performed at the NBDAA laboratory in the late summer of 2003, indicated that they were resistant to three antibiotics (oxytetracycline, florfenicol and potentiated sulphonamides). However, using an extended panel of antibiotics, including many never employed in Canadian aquaculture, it was demonstrated that the isolates were in fact hexa-resistant (ampicillin/florfenicol/oxytetracycline/nalidixic acid/streptomycin/sulphonamides). Clear resistance was recorded, using the breakpoint zone sizes reported by Miller *et al.*,[23] towards ampicillin and amoxicillin, chloramphenicol and florfenicol, nalidixic acid, oxytetracycline, sulphonamides and streptomycin. In addition, it was noted that in comparison to the ATCC 33658 control strain, the hexa-resistant isolates had developed a degree of cross-resistance towards some of the other drugs tested including the cephalosporin ceftiofur and the quinolone enrofloxacin (Table 2). The same hexa-resistant profile noted for FFA16, 17, 18 and 19 was subsequently recorded for the isolates AS03, FFA72, FFA74, FFA106 and FFA150 recovered from New Brunswick sites and for FFA108, FFA109 and FFA111 provided by the Nova Scotia Department of Agriculture, Fisheries and Aquaculture (NSDAFA). The isolate FFA160, recovered from a site in Nova Scotia during 2002, showed a penta-resistant phenotype (susceptibility was shown towards nalidixic acid). All of the MDR *A. salmonicida* isolates demonstrated resistance to $\geq$1000 μM $HgCl_2$ and $\geq$32 μM PMA.

Plasmid analysis and assessment of ability to transfer antibiotic and Hg resistance

As shown in Figure 1, all strains, including ATCC 33658, carried the characteristic triplet of cryptic plasmids (pAsa1, pAsa2 and pAsa3) common to the majority of strains of *A. salmonicida* subsp. *salmonicida*.[27] None of the MDR strains carried a plasmid of molecular weight similar to those of the R-plasmid pRAS1.[6] However, strains FFA17, FFA18 and FFA20 each carried a plasmid with the same molecular mass as pRAS3; 11.8 kb[7] (Figure 1). This finding explained the tetracycline resistance phenotype (encoded by a *tetC* sequence) of isolate FFA20. The pRAS3 plasmid was subsequently detected in FFA74, FF112, FFA113 and FFA160.

Table 2. Antibiotic susceptibilities of five test strains of *A. salmonicida*

	Diameter (mm) of zone of inhibition after 48 h for strain					
Antibiotic	FFA16	FFA17	FFA18	FFA19	FFA20	ATCC 33658
AMP (10)	11 (R)	10 (R)	12 (R)	11 (R)	32	42 (34–42)[a]
AMX (10)	12 (R)	12 (R)	12 (R)	12 (R)	36	42
XNL (30)	20 (R)	20 (R)	20 (R)	20 (R)	38	48
FFC (30)	10 (R)	8 (R)	8 (R)	10(R)	30	42 (32–44)[a]
ENR (5)	28 (IR)	28 (IR)	28 (IR)	28 (IR)	45	45 (36–46)[a]
NAL (30)	0 (R)	0 (R)	0 (R)	0 (R)	45	48
SXT (25)[b]	26	26	25	26	32	35 (27–40)[a]
SOR (25)[b]	22	22	23	22	24	32 (24–38)[a]
TMP (5)[b]	36	36	36	36	37	40
OXT (30)	10 (R)	8 (R)	8 (R)	8 (R)	0 (R)	36 (30–39)[a]
ERY (15)	28	28	28	28	28	26 (17–28)[a]
GEN (10)	28	28	28	28	28	28 (23–29)[a]
STR (10)	0 (R)	0 (R)	0 (R)	0 (R)	18	23

R, complete resistance; IR, intermediate resistance; AMP, ampicillin; AMX, amoxicillin; XNL, ceftiofur; FFC, florfenicol; ENR, enrofloxacin; NAL, nalidixic acid; SXT, sulfamethoxazole/trimethoprim; SOR, sulfadimethoxine/ormetoprim; TMP, trimethoprim; OXT, oxytetracycline; ERY, erythromycin; GEN, gentamicin; STR, streptomycin.

[a]The values in parentheses represent the approved quality control range established for these drugs by the CLSI (formerly NCCLS), USA. As determined by the Subcommittee on Veterinary Antimicrobial Susceptibility Testing-Aquaculture Working Group (VAST-AWG), see Miller *et al.*[23]

[b]'Fuzzy' zones were noted for these antibiotics. These extended 3–5 mm towards the disc from the point at which normal growth ceased. Details of the antibiotics used are provided in the Materials and methods section.

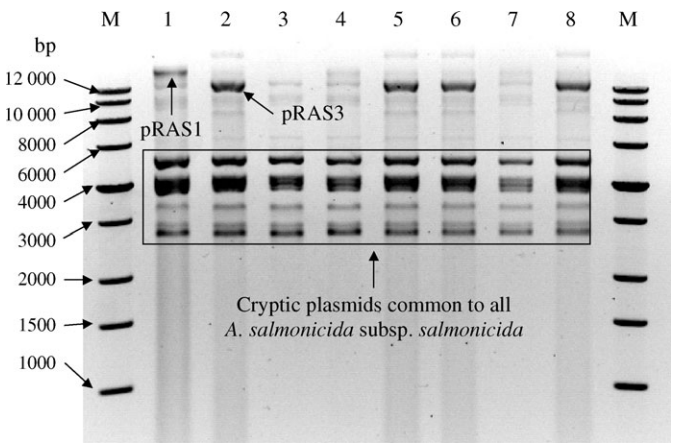

Figure 1. Plasmid profiles generated from reference strains and a sample of Atlantic Canadian isolates. M, 0.5–12 kb DNA ladder; lane 1, 718 (pRAS1); lane 2, 7067 (pRAS3); lane 3, ATCC 33658; lane 4, FFA16; lane 5, FFA17; lane 6, FFA18; lane 7, FFA19; and lane 8, FFA20.

Based on these data, it was considered possible that the resistance genes were associated with a very large novel R-plasmid, which because of its size, failed to migrate into the agarose gel during electrophoresis. Alternatively, this plasmid could have been present at very low copy numbers in

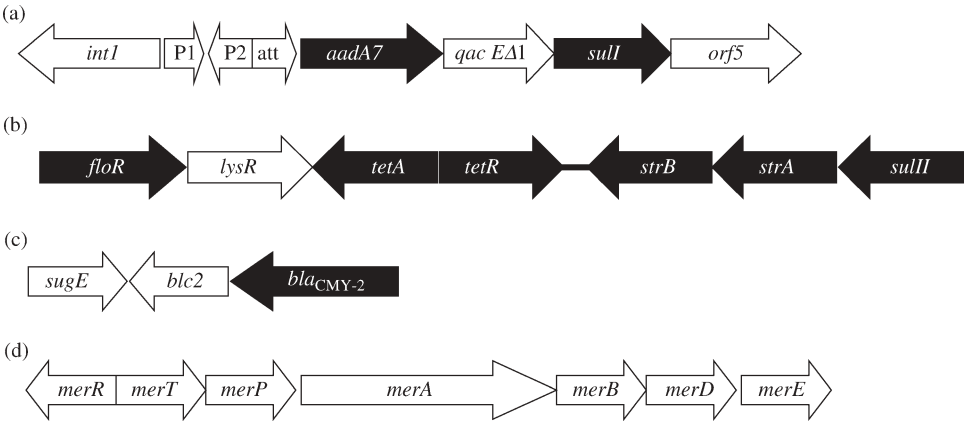

Figure 2. Schematic representation of the antibiotic and mercury resistance cassettes identified in MDR *A. salmonicida* subsp. *salmonicida*. Sequences encoding antibiotic resistance determinants are shaded black. (a) Class 1 integron (3812 bp) carrying an *aadA7* sequence. (b) A 7483 bp region with the predicted genes *floR*, *tetA/R*, *strA/B* and *sulII*—this region showed 99.9% sequence homology to nucleotides 25 530–33 012 of the plasmid pSN254 (GenBank accession no. CP000604). (c) A 3431 bp region predicted to include bla_{CMY-2}, *blc and sugE* genes and showing 100% nucleotide sequence homology to nucleotides 415–3845 of a transposon-like element (GenBank accession no. AY253913). (d) Gene organization of a 4266 bp *mer* operon (*merRTPABDE*) showing 100% sequence homology to nucleotides 143 996–148 261 of the plasmid pSN254.

A. salmonicida and failed to produce a detectable band on ethidium bromide stained gels.

Data from conjugation experiments suggested that the different resistance genes were associated with a plasmid. Thus, following incubation with *A. salmonicida* donors, each of the recipient strains (*A. hydrophila, E. coli* and *E. tarda*) gave rise to similar levels of growth (transfer frequencies were in the range of 2×10^{-4}–4×10^{-4}), on each antibiotic (ampicillin, florfenicol, streptomycin and oxytetracycline) or $HgCl_2$ containing medium. Three individual transconjugants were taken from each of the different antibiotic containing media and their resistance phenotypes were evaluated using the disc diffusion assay. It was found that irrespective of the antibiotic used for initial isolation, all transconjugants demonstrated resistance to ampicillin, florfenicol, streptomycin, oxytetracycline and $HgCl_2$. Taken as a whole, these data strongly suggested that all of the resistance genes were associated with the same mobile genetic element. As expected, the transfer of resistance to nalidixic acid was not detected in any of the transconjugants examined. Definitive evidence for the presence of an IncA/C plasmid in the MDR isolates was provided by probing both those isolates and transconjugants by PCR using four sets of primers (Table S1) designed to amplify the IncA/C replicon and three regions of the IncA/C plasmid backbone.[21] It was shown that amplicons of the expected size were generated from control DNA from *Y. ruckeri* (pYR1) and from each of the MDR test samples. In contrast, the other *A. salmonicida* isolates and unconjugated recipient strains were universally negative.

Detection and characterization of antibiotic and Hg resistance genes

The antibiotic and mercury resistance cassettes identified in MDR *A. salmonicida* subsp. *salmonicida* are represented schematically in Figure 2. Given the difficulty in producing a good quality plasmid preparation, our initial analysis involved screening by PCR for known antibiotic resistance genes and for sequences associated with integrons. This approach identified the presence of a class 1 integron, which was shown to contain an *aadA7* gene encoding for an aminoglycoside-3′-adenyltransferase, conferring resistance to streptomycin and spectinomycin. Resistance to spectinomycin was subsequently confirmed phenotypically on agar plates containing this antibiotic at a concentration of 10 mg/L (data not shown). The ability of *A. salmonicida* to develop multiple antibiotic resistance phenotypes via the acquisition of R-plasmids and the association of resistance genes with mobile elements including integrons and transposons has been well documented.[2,3,25] Against this background, the detection of a class 1 integron carrying a sequence encoding resistance to streptomycin was not entirely unexpected. Certainly, integrons have been shown to be widely distributed throughout microbial populations, including *A. salmonicida*, found in aquatic environments[28,29] and streptomycin resistance genes are frequently associated with them.[30,31] However, the *aadA7* sequence detected in all MDR isolates of *A. salmonicida* has not previously been reported in aquatic environments. The *aadA7* gene was first detected in *E. coli*,[32] but has subsequently been reported to be carried by class 1 integrons present in *S. enterica*.[33] As highlighted by other authors, the ample distribution of sequences conferring resistance to streptomycin among drug-resistant *A. salmonicida* is difficult to explain, given that neither this compound nor any other aminoglycoside is licensed for use in aquaculture. In contrast to the situation reported for streptomycin-resistant *A. salmonicida* isolated in Europe,[5,25] where different single sequences were responsible for conferring resistance, all MDR isolates examined in the current study possessed two distinct mechanisms for resistance to streptomycin, i.e. the *aadA7* and *strA/strB* gene products, which were located within distinct resistance cassettes (see below).

A combination of PCR and primer walking of cloned resistance sequences identified the presence of a second resistance cassette 7483 bp in size, containing *floR* (encoding resistance to florfenicol), *tetA/tetR* (tetracycline resistance), *sulII* (sulphonamide resistance) and *strA/strB* (streptomycin resistance) sequences. This cassette showed 99.9% sequence homology to nucleotides 25 530–33 012 of the plasmid pSN254 (GenBank acession no. CP000604), which was isolated from the

food-borne pathogen *S. enterica* serotype Newport SL254.[21] Interestingly, a similarly organized cassette, albeit lacking the *tetA* and *tetR* sequences, had previously been identified as part of the SXT integrative and conjugative element (ICE) carried by South East Asian isolates of drug-resistant *Vibrio cholerae*[34] (GenBank accession no. AY034138).

It is pertinent to note that the absence of any previous reports of plasmid-associated resistance to florfenicol in *A. salmonicida* had elevated this antibiotic to the status of treatment of choice for furunculosis outbreaks in Canadian aquaculture facilities. Hence, our finding that *A. salmonicida* had acquired a transferable mechanism to resist treatment with florfenicol represented a significant development, which will undoubtedly have implications for the management of furunculosis in Atlantic Canada and beyond. A further point for consideration is the fact that the resistance cassette carrying the *floR* gene was in itself able to be mobilized, as demonstrated by its previous isolation in association with IncA/C plasmids in *Salmonella* spp. and the SXT element of *V. cholera*. Nevertheless, given the paucity of reported associations between those species and cold water aquaculture, we consider it unlikely that either could have served as the direct donor of the resistance cassette or the plasmid. Instead, we would suggest that both the IncA/C plasmid and resistance cassette are maintained in circulation via a series of non-pathogenic, intermediate hosts, which are widely distributed throughout aquatic environments, including aquaculture facilities.[11]

Initial attempts to detect the gene-encoding resistance to ampicillin using primer pairs designed to amplify bla_{TEM}, bla_{SHV}, bla_{PSE} and bla_{OXA} β-lactamase sequences were unsuccessful. The subsequent use of primers specific for the AmpC class of β-lactamase[35] generated an amplicon which, upon sequencing, was found to represent a $bla_{\mathrm{CMY\text{-}2}}$ gene. Once again, this gene was not contained within a class 1 integron; rather it formed part of a 3439 bp cassette, in association with the sequences *sugE* and *blc* which encoded for a multidrug efflux pump and outer membrane lipoprotein, respectively.[36] This cassette was identical, 100% nucleotide sequence homology to nucleotides 415–3845, to a transposon-like element (GenBank accession no. AY253913) known to be widely distributed among clinical and food-borne *Salmonella* and other Enterobacteriaceae throughout Asia and the United States.[21,36,37]

Plasmid-encoded AmpC β-lactamases have been found in isolates from livestock, including swine and cattle, and from companion animals such as dogs.[38] Yet, to our knowledge, our data represent the first such isolation from a pathogen of aquatic animals and the first documented cases of ampicillin-resistant *A. salmonicida* subsp. *salmonicida* in North America. A limited incidence of resistance to ampicillin was previously reported among Scottish isolates of *A. salmonicida*, but no details were provided as to the molecular basis for this phenomenon.[39] Furthermore, in contrast to the Canadian isolates, the ampicillin resistance seen in Scotland could not be transferred to other bacteria in laboratory-based conjugation experiments. β-Lactam antibiotics have rarely been used as an antibiotic treatment in aquaculture; therefore, the acquisition and maintenance of resistance to this class of drug is intriguing. Plasmid-mediated AmpC β-lactamases represent a threat to the successful treatment of a number of human and veterinary infections since they confer resistance to cephalosporins, are not affected by commercially available β-lactamase inhibitors (e.g. clavulanic acid) and can, in strains with loss of outer membrane porins, provide resistance to carbapenems. Resistance mediated by plasmid-associated *ampC* genes has been found around the world, may cause nosocomial outbreaks and appears to be increasing in prevalence.[36,40] Clearly, the detection of plasmid-encoded AmpC-mediated resistance, located on a transposable element, in a member of the Aeromonadaceae is a cause for concern and suggests that such sequences are more widely distributed than was thought previously.

To fully understand antibiotic resistance, we must enhance our knowledge of gene flow and the factors that influence these dynamics.[12] The results of the present work confirm the hypothesis that antibiotic use in aquaculture can result in the acquisition of antibiotic resistance determinants by fish pathogens. Yet, contrary to the assertions of some workers that antibiotic resistance determinants associated with aquaculture are being transferred to bacteria of land animals and humans,[41,42] our data provide support to the conclusions of Smith *et al.*,[43] that the flow of these resistance determinants is predominantly in the opposite direction. Differences in the organization of the resistance cassettes seen in the MDR *A. salmonicida* and those reported in IncA/C plasmids in terrestrial bacteria, particularly those in plasmid pSN254, have most likely arisen from 'genetic slippage'[12] as the plasmid passed through a variety of non-pathogenic hosts prior to being acquired by *A. salmonicida*. Such plasmids most likely persist due to a steady input by means of both human and animal pathogens and more importantly faecal-associated commensal bacteria, which enter the aquatic environmental compartment via run off from land used for the production of farm animals[44,45] or to a lesser extent via hospital effluent.[4,12]

It is intriguing that despite the metabolic burden associated with the carriage of large resistance plasmids, such molecules persist and circulate between microbial populations in distinct environmental niches in the absence of significant antibiotic selective pressure.[11,13,14] In the case of the present study, it is worth considering that other selective pressures, namely mercury pollution, may have contributed to maintenance of the IncA/C plasmid by *A. salmonicida*. Phenotypic data had revealed the presence of a mechanism for mercury resistance, and in the process of sequencing a streptomycin-resistant clone containing a 10 kb insert, we detected the presence of sequences (*merD* and *merB*) associated with a mercury resistance operon. Additional PCR and sequence analysis revealed the presence of *merA*, *merE*, *merP*, *merR* and *merT* genes organized in an identical manner to the same sequences present in the *mer* operon, i.e. *merRTPABDE* (nucleotides 143 996–148 261) of the plasmid pSN254.[21]

The relationship between mercury and antibiotic resistance in the indigenous microflora of humans, other primates and pigs is well documented,[14] but there have been relatively few studies regarding this relationship in fish, and those have examined only point source contamination sites. However, fish microbiologists have largely ignored this correlation in non-point source, low-level contamination areas. Yet, the continued input of mercury via atmospheric deposition in water systems or when present in feed may be acting as a selective agent to facilitate the spread of antibiotic resistance genes in the total genomic reservoir of a population.[14] In this context, McArthur and Tuckfield[46] found a strong positive correlation between stream sediment mercury concentrations and antibiotic resistance in resident sediment bacteria and concluded that mercury pollution may help to promote antibiotic resistance through indirect selection.

In addition to representing a fascinating example of horizontal gene transfer between terrestrial and aquatic environments, the emergence of the MDR *A. salmonicida* was a significant health problem for the Atlantic Canadian salmon industry during 2003 and 2004. Fortunately, the application of molecular methods to the detailed characterization of the resistance mechanisms present in the 2003 isolates allowed the rapid identification of the same genes in the 2004 isolates resulting in the efficient implementation of control measures, namely extermination of the entire smolt population, followed by complete disinfection of the single infected hatchery. The emergence of a variant of this pathogen that was essentially untreatable, using the limited range of antimicrobials licensed for use in Canadian aquaculture, served to highlight the need for continued vigilance and rigorous adherence to the vaccination, biosecurity and good husbandry practices required to control this pathogen. In this context, no new isolations of the MDR isolate have occurred since 2004.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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