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Abstract

Aeromonas salmonicida AS03, a potential fish pathogen, was isolated from Atlantic salmon, *Salmo salar*, in 2003. This strain was found to be resistant to ≥1000 mM HgCl2 and ≥32 mM phenylmercuric acetate as well as multiple antimicrobials. Mercury (Hg) and antibiotic resistance genes are often located on the same mobile genetic elements, so the genetic determinants of both resistances and the possibility of horizontal gene transfer were examined. Specific PCR primers were used to amplify and sequence distinctive regions of the *mer* operon. *A. salmonicida* AS03 was found to have a pDU1358-like broad-spectrum *mer* operon, containing *merB* as well as *merA, merD, merP, merR* and *merT*, most similar to Klebsiella *pneumonaie* plasmid pRMH760. To our knowledge, the *mer* operon has never before been documented in Aeromonas spp. PCR and gene sequencing were used to identify class 1 integron associated antibiotic resistance determinants and the Tet A tetracycline resistance gene. The transposase and resolvase genes of Tn*1696* were identified through PCR and sequencing with Tn*21* specific PCR primers. We provide phenotypic and genotypic evidence that the *mer* operon, the aforementioned antibiotic resistances, and the Tn*1696* transposition module are located on a single plasmid or conjugative transposon that can be transferred to *E. coli* DH5α through conjugation in the presence of low level Hg and absence of any antibiotic selective pressure. Additionally, the presence of low-level Hg or chloramphenicol in the

mating media was found to stimulate conjugation, significantly increasing the transfer
30 frequency of conjugation above the transfer frequency measured with mating media lacking both antibiotics and Hg. This research demonstrates that mercury indirectly
32 selects for the dissemination of the antibiotic resistance genes of *A. salmonicida* AS03.

Introduction

34 *Aeromonas salmonicida*, a common fish pathogen, is the bacterium responsible for furunculosis in temperate and coldwater salmonid fish (1, 36). Outbreaks of this
36 pathogen can be controlled by vaccination or, more commonly, by antibiotic administration. However, excessive usage of antibiotics in fish feed has caused the
38 emergence of multiply antibiotic resistant fish pathogens (36). As a result of these multi-drug resistant fish pathogens, countries such as Denmark, Norway, Sweden, and the
40 United Kingdom have begun to monitor antibiotic usage in fish farms (36). The acquisition of antibiotic resistance genes by *A. salmonicida* confounds disease control
42 and is thus economically costly to the aquaculture industry(35). Mobile genetic elements such as Tn*1721* and class 1 integrons isolated from *A. salmonicida* strains are commonly
44 located on resistance plasmids (36). Aeromonad resistance plasmids are often IncU plasmids or genetically similar to IncU plasmids, suggesting that they share a common
46 origin and have since diverged due to different selective pressures(9, 37). IncU plasmids belong to the plasmid incompatibility group U, which consists of similar plasmids that
48 cannot co-exist in any bacterial strain and are thus said to be incompatible (37) Therefore, the resistance determinants associated with fish farm pathogens are of further
50 significance because they are very similar to those found in pathogenic enterobacteria in humans and hospital effluents (37). Antibiotic resistances associated with resistance

plasmids, such as oxytetracycline resistance, have been transferred from *Aeromonas sp.* to other bacterial species, including *E. coli* (11).

Mercury (Hg) resistance is prevalent among environmental bacterial isolates due to the selective pressure of elevated mercury contamination (3). The most common mechanism for bacterial mercury resistance involves the reduction of toxic mercury(II) to volatile Hg(0) by the mercuric reductase enzyme (3, 4). The gene encoding mercuric reductase, *merA*, is usually located in the *mer* operon, where it is associated with other genes coordinating mercury resistance. In addition to *merA*, the typical *mer* operon contains two genes responsible for mercury transport into the cell (*merT* and *merP*) and two regulatory genes (*merD* and *merR*). Some *mer* operons also contain the accessory genes *merB*, *merC* and *merF* (25). Since mercury resistance determinants are often located on mobile genetic elements such as plasmids and transposons, the *mer* operon can be transferred between species (3, 43). Several studies have demonstrated the transfer of mercury resistance via conjugation between bacterial species(3, 43). Although excessive antibiotic usage is largely responsible for the growing prevalence of antibiotic resistance, non-antibiotic selective agents, including mercury and other heavy metals, have been implicated as potential vehicles for the indirect selection of antibiotic resistance (34). Antibiotic and mercury resistance genes are frequently adjacent to each other on mobile genetic elements and furthermore located the same integrons (5, 28, 38, 43). This correlation between antibiotic and mercury resistance has been documented in the fecal and intestinal bacteria of primates with mercury amalgam fillings, where bacteria containing a *mer* operon are more likely to exhibit multiple antibiotic resistance(38). Furthermore, McArthur and Tuckfield observed increased antibiotic resistance in

bacterial strains exhibiting mercury resistance isolated from the sediment of mercury-
76 polluted streams (28). Although many plasmid-borne antibiotic resistant genes have been characterized in *Aeromonas sp.*, no aeromonad to our knowledge has been documented to
78 contain the *mer* operon conferring mercury resistance (1, 36).

Class 1 integrons, which are often found in close proximity to *mer* operons, are
80 found in many gram-negative clinical isolates and have been shown to carry a high diversity of antibiotic resistance cassettes Class 1 integrons have been documented to
82 associate with transposons encoding both mercury and tetracycline resistance, thus mobilizing the integron and the transposon's resistance determinants together (27, 30).
84 The genes encoding class 1 integrases associated with transposons carrying mercury resistance are identical to those encoding integrases not associated with transposons,
86 suggesting that the incorporation of integrons into transposons occurred recently and is an ongoing process (15). Integron-borne antimicrobial resistances could therefore be easily
88 spread to pathogenic bacteria via transposons, especially with the indirect selective pressure exerted by mercury (30).

90 The bacterial strain characterized in this study, *A. salmonicida* AS03, was isolated from hatchery-grown Atlantic salmon, *Salmo salar* in 2003. *A. salmonicida AS03* was
92 screened for resistance to $HgCl_2$, phenylmercuric acetate (PMA), and a total of 26 antimicrobial agents. Distinctive regions of the *mer* operon, class 1 integron genes, and
94 *Tet* A, a tetracycline resistance determinant typical of many multiple-drug resistant aeromonads isolated from aquaculture facilities, were partially characterized
96 genotypically. We provide evidence that the *mer* operon and multiple antibiotic resistance genes in *A. salmonicida* AS03 can be concomitantly transferred to *Escherichia*

coli DH5α by conjugation and are, therefore, associated with at least one mobile genetic element. Furthermore, low levels of either mercury or the antibiotic chloramphenicol stimulated higher transfer frequencies of conjugation.

Materials and Methods

Bacterial isolates and cultivation conditions. An isolate of *Aeromonas salmonicida*, designated strain AS03, was found to be resistant to both mercury and antimicrobials. Strain AS03 was isolated from 7 of 60 Atlantic salmon (*Salmo salar* L.) during a routine health inspection in 2003 of hatcheries in Northeastern North America. *Escherichia coli* DH5α (Invitrogen Life Technologies, Carlsbad, CA) was used as the recipient in the conjugation experiments. *A. salmonicida* AS03 and E. coli DH5α were grown on Brain Heart Infusion (BHI) agar (Difco, Detroit, MI) at 22°C and at 37°C, respectively. Both strains were stored in BHI broth with 50% (vol/vol) glycerol at -80°C.

Genomic DNA extraction and confirmation of strain identity. 16S rRNA gene sequencing was done to confirm the identity of the *A. salmonicida* AS03 strain. Total genomic DNA was extracted (E.N.Z.A. Bacterial DNA Kit, Omega Bio-tek, Doraville, GA) according to the manufacturer's protocol. A hypervariable, 174-200 bp region of the 16S rDNA gene was PCR amplified using primers and cycling conditions described by Muzyer *et al*(29) and sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Isolate identification was based on 16S rDNA sequence similarities (Blastn against GenBank at http://www.ncbi.nlm.nih.gov/BLAST).

Mercury minimum inhibitory concentrations (MICs). Hg MIC values for both $HgCl_2$ and phenylmercuric acetate (PMA; Sigma-Aldrich, Inc., St.Louis, MO) were determined for *A. salmonicida* AS03 and *E. coli* DH5α by the method of Wang et al.(42) using BHI

plating media solidified with Noble agar (Difco, Sparks, MD). Bacterial suspensions (4
122 .0 µl; 2.0 McFarland) of fresh (24-48 hr) solid plating media cultures in sterile, demineralized water were inoculated on BHI plating media containing 0, 50, 100, 250,
124 500, 750, and 1000 µM $HgCl_2$, and BHI plating media containing 0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 µM PMA and incubated for two days at 22°C. Hg MICs were recorded
126 as the lowest concentration of Hg that inhibited bacterial growth. Two control strains, mercury-susceptible *Bacillus subtilis* 168 and mercury-resistant *E. coli* SK1592, were run
128 in parallel with the experimental strains(18)

Antibiotic MICs. The *in vitro* activity of antimicrobial agents against *A. salmonicida*
130 AS03 and *E. coli* DH5α isolates was assessed by the MIC method following the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. MICs were
132 determined using the Sensititre® (MJ Panel; Trek Diagnostic Systems, Westlake, OH) dried susceptibility panels. Manufacturer's instructions were followed for *E. coli* DH5α,
134 however, modifications for *A. salmonicida* AS03 included incubation at 22°C for 48h. Antibiotic MIC assays were done in triplicate for each strain, and MIC was determined as
136 the lowest concentration of the antimicrobial agent able to inhibit growth. As recommended by NCCLS and Sensititre® guidelines, the following reference strains were
138 included as internal standards: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Enterococcus faecalis* (ATCC 29212). For specific
140 antimicrobials that were not included in the Sensititre MJ panel, additional susceptibility testing was performed on BHI plating media with antimicrobial discs containing
142 streptomycin (10 µg), tobramycin (10 µg), kanamycin (30 µg) and chloramphenicol (30 µg) (Sensi-Disc; Becton, Dickinson and Co., Sparks, MD). *A. salmonicida* cultures were

144 incubated at 22°C while *E. coli* cultures were incubated at 37°C before zones of inhibition were measured.

146 **PCR amplification and sequencing of *mer* genes**. Primers (Table 1) were used to probe for and amplify regions of the *mer* operon using genomic DNA as a template (25). The 148 short (288 bp) *merA* PCR product was cloned according to the manufacturer's protocol (TA Cloning Kit ; Invitrogen Life Technologies, Carlsbad, Calif.) and sequenced. All 150 other PCR products were cleaned (E.N.Z.A. CyclepureKit or E.Z.N.A Gel Extraction Kit; Omega Bio-tek, Doraville, GA) according to manufacturer's protocol and sequenced. All 152 *mer* sequences were collected and aligned in Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI). *mer* gene sequences were entered in the blastn database to confirm gene 154 identity and identify similar sequences.

PCR amplification and sequencing of Tn*21* genes and antibiotic resistance genes. 156 Primers (Table 1) were used to amplify Tn*21*, class 1 integron genes, and the tetracycline resistance gene Tet A. These reactions were carried out on a gradient thermocycler 158 (iCycler, BioRad Hercules, CA) for 35 cycles of 94 ^{o}C for 1 min, 53-57^{o}C for 1 min and 72^{o}C for 2.5 min. The 1 kb PCR product of *in-F* and *in-B*, the 1 kb Tet A amplicon, a 411 160 bp *tnpA* amplicon, and a 240 bp *tnpR* amplicon were sequenced

Conjugation experiments and plasmid profiling. The ability of *A. salmonicida* AS03 162 to transfer mercury- and antibiotic resistance genes to *E.coli* DH5α (Invitrogen Life Technologies; Carlsbad, CA) was examined by conjugation experiments using the solid 164 plating medium method (21). Growing colonies of the *A. salmonicida* AS03 were mixed with approximately equal amounts of growing recipient strain *E. coli* DH5α on BHI

166 plates, with mercury and antibiotic amendments as specified below, and incubated at 22°C from 1 to 2 days

168 The frequency of transfer was determined according to the method of Sorum, et al. (37) with the following modifications. Transfer frequency was measured by dividing 170 the number of transconjugants per ml by the number of recipients per ml in the mating mixture. The total number of recipients was estimated after overnight incubation on BHI 172 solid plating media without selection agents at 37°C, at which the growth of the donor strain was suppressed, while selection of transconjugants was done on BHI agar plates 174 with either 8 μg ml^{-1} of chloramphenicol or 250 μM $HgCl_2$ following incubation at 37°C. These concentrations of chloramphenicol and $HgCl_2$ were found to inhibit growth of the 176 *E. coli* DH5α recipient. All *E. coli* DH5α transconjugants were subjected to mercury and antibiotic MIC assays at a temperature of 37°C to determine their phenotypic resistance 178 characteristics. The transfer frequencies were calculated on the basis of the average number of colonies counted from duplicate plates after appropriate serial dilution (2.0 180 McFarland) of the mating mixture.

Several conjugation experiments were performed to determine if the transfer of 182 resistance genes is stimulated by exposure of the donor *A. salmonicida* AS03 to mercury and chloramphenicol. Three experiments were conducted under each condition of either 184 chloramphenicol or mercury exposure of the donor strain and mating mixture. To assess the stability of the resistance determinants in the *E. coli* DH5α transconjugants, both 186 antibiotic and mercury MICs were done on the transconjugants after 10 subculture passages on BHI plating media at 37°C.

188 **(i) Conjugation in the presence of chloramphenicol.** *A. salmonicida* AS03 was grown overnight on BHI solid plating media amended with 8 μg ml^{-1} chloramphenicol. 190 Donor and recipient strains were mixed as described above on BHI agar plates containing 1 μg ml^{-1} chloramphenicol, a concentration determined to be sub-lethal to recipient *E. coli* DH5α. 192 The mating mixtures were incubated and the frequencies of transfer were determined as described above.

194 **(ii) Conjugation in the presence of mercury.** The donor strain, *A salmonicida* AS03, was grown overnight on BHI solid plating media with added 25 μM $HgCl_2$. 196 Donor and recipient strains were mixed as described above on BHI agar plates containing 25 μM $HgCl_2$, and the mating mixtures were incubated as described above. Transfer 198 frequencies were done as described above except that selection for *E. coli* DH5α transconjugants was done on BHI plating media with 250 μM $HgCl_2$.

200 Plasmid DNA was isolated from *A. salmonicida* AS03, *E. coli* DH5α recipient, and the *E. coli* DH5α transconjugant using the E.N.Z.A. Plasmid miniprep kit (Omega 202 Bio-tek, Doraville, GA). Plasmids were visualized using pulsed field gel electrophoresis (PFGE) in a 0.8% agarose gel at 175 V at 4^{o}C. Genomic DNA was extracted from *A. salmonicida* AS03, *E. coli* DH5α recipient, and the *E. coli* DH5α transconjugant as 204 described above in order to serve as template DNA in the following PCR amplifications. PCR primer sets A1-s.F and A5-n.R, *In-F* and *In-B*, tnpR-F and tnpR-B, Tet A FW and 206 Tet A RV, and p5-II and p2-II (Table 1) were used to probe the *E. coli* DH5α recipient and transconjugant for the presence of the *merA*, *aadA7*, *tnpR*, Tet A, and *tnpA* genes that 208 were present in *A. salmonicida* ASO3.

Nucleotide sequence accession numbers. The sequence of the 16S rRNA gene has been deposited in the GenBank database under the following accession number: strain AS03 XXXXXXXXX. Partial sequences of the *merA*, *merD*, *merR-T*, *merB*, Tet A, and *aadA7* genes of strain AS03 have been deposited in the GenBank database under accession numbers XXXXXX through XXXXXXX

Table 1: PCR primers used in this study

Primer	Gene	Sequence	Source or Reference
A1-s.F	*merA*	TCCGCAAGTNGCVACBGTNGG	(41)
A5-n.R	*merA*	ACCATCGTCAGRTARGGRAAVA	(41)
R1-F	*merR-merT*	GCGGATTTGCCTCCACGTTGA	(25)
T1-R	*merR-merT*	CCAGGCAGCAGGTCGATGCAAG	(25)
P1-F	*merP-merA*	GGCTATCCGTCCAGCGTCAA	(25)
A0-R	*merP-merA*	GTCGCAGGTCATGCCGGTGATTTT	(25)
B1-F	*merB*	TCGCCCCATATATTTTAGAAC	(25)
B2-R	*merB*	GTCGGGACAGATGCAAAGAAA	(25)
D2-F	*merD*	CCAGGCGGCTACGGCTTGTT	(25)
D3-R	*merD*	GGTGGCCAACTGCACTTCCAG	(25)
A6-F	*merA-merD*	GCCGACCAGTTGTTCCCCTACCTGACG	(25)
D1-R	*merA-merD*	CGCACGATATGCACGCTCACCC	(25)
In-F	Integron	GGCATCCAAGCAGCAAGC	(40)
In-B	Integron	AAGCAGACTTGACCTGAT	(40)
p5-II	Tn21 *tnpA*	TACTGCCGCGCATCAAGATC	(10)
p2-II	Tn21 *tnpA*	AGAAAGTTCGTCCTGGGCTG	(10)
tnpR-F	Tn21 *tnpR*	GGCGACACCGTGGTGGTGCATAGC	(6)
tnpR-B	Tn21 *tnpR*	CGGTAAGCCCCGCGTTGCTTGGC	(6)
qacF	*qacEΔ1-sul1*	ATCGCAATAGTTGGCGAAGT	(35)
Sul R	*qacEΔ1-sul1*	GCAAGGCGGAAACCCGCGCC	(35)
tetA FW	Tet A	GTAATTCTGAGCACTGTCGC	(17)
tetA RV	Tet A	CTGCCTGGACAACATTGCTT	(17)

Results

16S rDNA gene sequencing

The 16S rDNA sequence of *A. salmonicida* AS03 was identical (100%) to that of *A.*

222 *salmonicida* (AY297782.1), *A. salmonicida* subspecies subspecies Achromogenes

(AY910844.1) and *A. salmonicida* isolate 3-St 2-6 (DQ133187.1).

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Table 2. Antibiotic minimum inhibitory concentrations (μg/ml) as determined by Sensititre panels for *A.*

230 *salmonicida* AS03, *E. coli* DH5α, and the transconjugant.

Antimicrobials by class *(a)*	*Aeromonas salmonicida* AS03 Donor	*Escherichia coli* DH5α recipient	*Escherichia coli* DH5α transconjugant
Tetracyclines			
Tetracycline (0.25-8)	>8	1	>8
Penicillins			
Ampicillin (0.5-16)	>16	2	>16
Ampicillin/Sulbactam (8/4-16/8)	>16/8	0	>16/8
Mezlocillin (4-64)	0	0	16
Ticarcillin/Clauvulanic Acid (4/2-64/2)	8/2	0	64/2
Amoxicillin/Clauvulanic Acid (0.5/0.25-16/8)	>16/8	4/2	>16/8
Piperacillin (4-64)	0	0	16
Cephems			
Cefazolin (1-16)	>16	2	>16
Cephalothin (8-16)	>16	0	>16
Cefoxitin (2-16)	>16	0	>16
Ceftazidime (1-16)	16	0	>16
Ceftriaxone (4-32)	8	0	16
Cefuroxime (2-16)	8	0	>16
Aminoglycosides			
Amikacin (4-16)	0	0	0
Gentamicin (0.25-8)	0.5	0	0
Fluoroquinolones			
Ciprofoxacin (0.06-2)	0.25	0	0
Norfloxacin (4-8)	0	0	0
Ofloxacin (0.25-4)	0.5	0	0
Lomefloxacin (0.5-2)	2	0	0
Folate Pathway Inhibitors			
Trimethoprim/Sulfamethoxazole (0.5/9.5-2/38)	>2/38	0	0
Sulfisoxazole (256)	>256	0	>256

a Ranges tested in MIC in μg/mL

232

Hg and Antibiotic minimum inhibitory concentrations of *A. salmonicida* AS03.

234 *Aeromonas salmonicida* AS03 demonstrated resistance to ≥1000 μM $HgCl_2$ and ≥32 μM PMA as well as maximal resistance to 11 antimicrobials. In addition, some low-level

236 resistance to ceftriaxone, ciprofloxacin, ofloxacin, and trimethoprim/sulfamethoxazole was observed (Table 2). Antimicrobial susceptibility disc assays showed that *A.*

238 *salmonicida* AS03 was also resistant to 10 μg streptomycin and 30 μg chloramphenicol, and susceptible to 10 μg tobramycin and 30 μg kanamycin

240 **Genes encoding the *mer* operon.** Regions of the *merA*, *merB*, *merD*, *merR*, *merP*, and *merT* genes were amplified and sequenced in *A. salmonicida* AS03. All *mer* gene PCR

242 amplifications of *A. salmonicida* AS03 produced single PCR bands of the following expected sizes: 288 bp for *merA*, 134 bp for *merA-P*, 225 bp for *merR-T*, 502 bp for

244 *merB*, 219 bp for *merD*, and 181 bp for *merA-D*. Blastn searches confirmed the identities of the amplicons. All genes demonstrated the most sequence similarity to *Klebsiella*

246 *pneumonaie* pRMH760 (>95% sequence similarity), which contains a pDU1358-like *mer* locus (accession no. AY123253). PCR mapping of the *merD-merA* region verified

248 the presence of *merB* between *merA* and *merD* (3, 24, 25).

Antibiotic resistance genes. PCR amplification with class 1 integron primers *in-F* and

250 *in-B* yielded several amplicons in *A. salmonicida* AS03. A 1 kb amplicon from this amplification was successfully sequenced and identified through blastn analysis as

252 *aadA7*, which encodes an aminoglycoside 3' adenyltransferase enzyme conferring resistance to streptomycin and spectinomycin. This gene exhibited the most similarity to

254 *aadA7* in *Esherichia coli (*AF234167.1 *), Vibrio cholerae* (DQ196322.1) and *Salmonella enterica* (AY463737) and explains the streptomycin resistant phenotype of *A.*

salmonicida AS03 determined by antimicrobial susceptibility disks. PCR primers *qacF* and *sulR* yielded an approximately 800 bp PCR product which was identified by blastn analysis on the reverse sequence as *sul1* This *sul1* gene resembled *sul1* genes of *E. coli* plasmid PQR-1 (AY655485) and *K. pneumonaie* pRMH760 (AY123253). The forward sequence confirmed the presence of *qacEΔ1* and was most similar to *qacEΔ1* in E. coli pSa (L06822.4) and pRMH760 (AY123253) in the blastn database. PCR and gene sequencing with primers *tetA FW* and *tetA RV* amplified an approximately 1 kb amplicon, which demonstrated the most sequence similarity with *tetA* from multiple genera, including *E. coli* pAPEC-OR-R (AY214164), *S. S. typhimurium* pU302L (AY333434), and *A. salmonicida* pRAS1 (AJ131405).

Transposon genes. Because this strain exhibited simultaneous antibiotic and Hg resistance phenotypes, the presence of Tn*21*, which carries both resistance determinants, was assayed using primers described by Dahlberg et al. and Guerra et al.(6, 10). A 411 bp fragment was amplified and sequenced using primers *p5-II* and *p2-II,* which was more similar to the *tnpA* from Tn*1696* than that of *tnpA* of Tn*21,* and was almost identical to the Tn*1696 tnpA* of *P. arguinosa* R1033 (U12338.2) and *K. pneumonaie* pRMH760 (AY123253). Similarly, the 240 bp amplicon obtained from PCR with primers *tnpR-R* and *tnpR-F* was identified as *tnpR* from Tn*1696* and was near identical to the Tn*1696* *tnpR* of *P. arguinosa* p1033 and *K. pneumonaie* pRMH760.

Conjugation. *A. salmonicida* AS03 was able to transfer both mercury and antibiotic resistance to *E. coli* DH5α in conjugation experiments. Initial conjugation experiments showed that, regardless of the selective agent, either 8 μg ml^{-1}chloramphenicol or 250 μM $HgCl_2$, the *E. coli* DH5α transconjugant had multiple antibiotic resistances with MIC

levels either identical to or exceeding that of the donor *A. salmonicida* AS03 strain, especially among the tetracycline, penicillin, and cephem classes of antibiotics (Table 2). The folate pathway inhibitor sulfisoxizole was also expressed maximally in the transconjugant. Notably, antibiotics of the fluoroquinolone class were not transferred, even though the donor, *A. salmonicida* AS03, exhibited low levels of resistance to some of the antimicrobials in both the fluoroquinolone classes.

Similarly, mercury MICs in the transconjugant were identical under conditions of either chloramphenicol or $HgCl_2$ selection. The donor *A. salmonicida* AS03 exhibited high resistance to mercury with MICs of ≥1 mM $HgCl_2$ and ≥32 μM PMA, whereas the *E. coli* DH5α recipient showed low levels of mercury resistance with MICs of 100 μM $HgCl_2$ and 16 μM PMA. After mating with *A. salmonicida* AS03, the *E. coli* DH5α transconjugant had mercury MICs of ≥1 mM $HgCl_2$ and ≥32 μM PMA. No plasmids were isolated from the *E. coli* DH5α recipient nor the transconjugant, while at least three plasmids, estimated to be between 4-10 kB using the BAC-Tracker Supercoiled DNA Ladder (Epicentre, Madison, WI), were visualized in the *A. salmonicida* donor. PCR amplification from the genomic DNA of the *E. coli* DH5α transconjugant showed the presence of the *merA*, *aadA7*, *tnpR*, *Tet* A, and *tnpA* genes that were previously sequenced in the *A. salmonicida* AS03 donor. These genes were not amplified in the *E. coli* DH5α recipient and, therefore, must have been acquired by the *E. coli* DH5α transconjugant via conjugative transfer from the *A. salmonicida* AS03 donor (Figure 1).

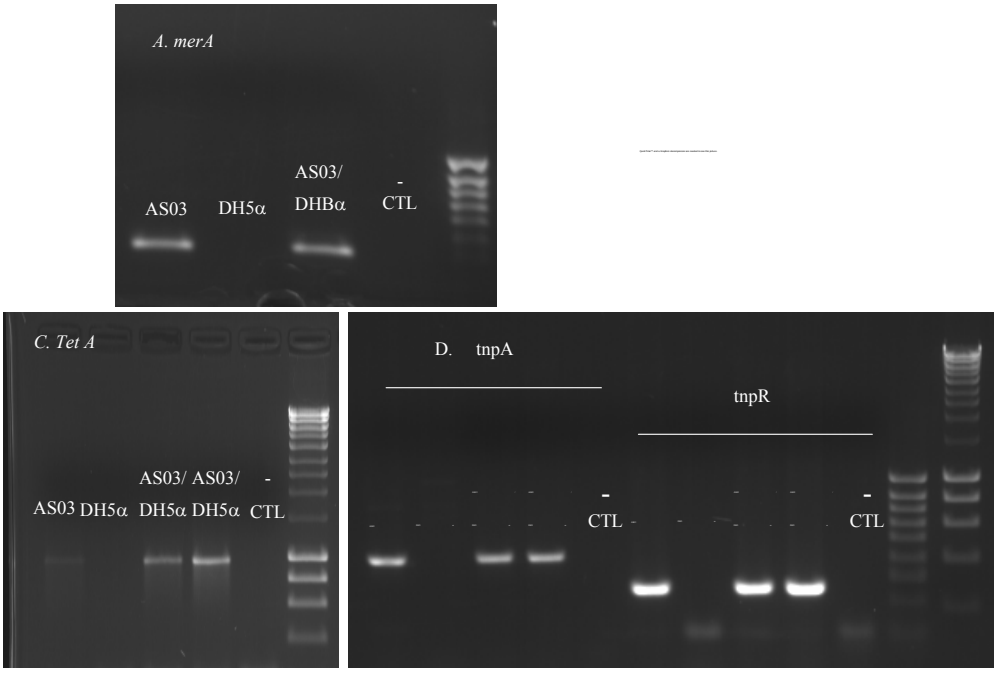

Figure 1. The genes *merA* (A.), *aadA7* (B.), Tet A (C.), and *tnpA* and *tnpR* (D.) are present in the *A. salmonicida* AS03 donor and the *E. coli* DH5α transconjugant but absent in the *E. coli* DH5α recipient, showing that these genes were transferred to the *E coli* DH5α transconjugant from *A. salmonicida* AS03 via conjugation. The notation AS03/DH5α is used to denote the transconjugant on the gel pictures.

Conjugative transfer of resistance determinants to the *E. coli* DH5α recipient varied under different conditions of the donor and mating mixture. The highest transfer frequencies were observed when the donor was grown on BHI plating media amended with either 8 μg ml^{-1} chloramphenicol or 25 μM $HgCl_2$ prior to conjugation and the mating mixtures contained either chloramphenicol or mercury at levels sub-lethal to the *E. coli* DH5α recipient (1 μg ml^{-1} chloramphenicol or 25 μM $HgCl_2$). Statistically, there was no significant difference between the transfer frequencies of these two exposure conditions. However, exposure to either chloramphenicol or mercury stimulated significantly greater transfer frequencies than observed in the control, which contained neither mercury nor chloramphenicol (Table 3). Regardless of the exposure condition, transconjugants maintained the same mercury and antibiotic resistance phenotypes they had demonstrated immediately after conjugation following ten sequential transfers on

344 BHI plating media, indicating that all of the transferred resistance determinants were stably inherited.

346

348 **Table 3**. Transfer frequencies of a conjugation between an *E. coli* DH5α recipient and an *A. salmonicida* AS03 donor on solid BHI media under different conditions

Conjugation Condition	Transfer Frequency [a] ± SE
BHI agar	$(2.20 \pm .48) \times 10^{-2}$
25 μM Hg in BHI agar	$(4.85 \pm .94) \times 10^{-2}$
8 μM Chloramphenicol in BHI agar	$(6.60 \pm 1.0) \times 10^{-2}$

350 [a] Transfer frequency is the number of CFU on selective BHI media containing 8 μg ml^{-1} chloramphenicol divided by the number of CFU on non-selective BHI media

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Discussion

354 Hg resistance in *A. salmonicida* AS03 was found to be encoded by a broad-spectrum pDU1358-like *mer* operon. Plasmid pDU1358 is a multiple-antibiotic resistance 356 IncC incompatibility group plasmid, specifying broad-spectrum mercurial resistance, that was originally isolated from a hospital strain of *Serratia marcescens* (16) *A. salmonicida* 358 AS03 exhibited a similar amplicon pattern to that of pDU1358 locus 8 and a dissimilar pattern to that of Tn*21* based upon the results reported by Liebert et al.(25). Additionally, 360 the *mer* sequence in blastn with the highest degree of similarity to all *A. salmonicida mer* sequences, pRMH760, is a pDU1358-like *mer* locus. pRMH760 is a conjugative plasmid 362 that carries resistances to chloramphenicol, ampicillin, gentamicin, kanamycin, neomycin, streptomycin, spectinomycin, sulfamethoxazole, tobramycin, and 364 trimethoprim as well as a Tn*1696* transposition module and a broad-spectrum *mer* locus(32). The mobile genetic element in *A. salmonicida* AS03 was also shown to carry 366 ampicillin, streptomycin, spectinomycin, sulfamethoxazole, and trimethopim resistances

in addition to the broad-spectrum *mer* locus and Tn*1696* transposition module. Hence,
368 similar integron-containing composite transposons could be present in *A. salmonicida* AS03 and pRMH760. Several gram-negative isolates have been reported to possess more
370 than one type of *mer* locus (25, 33). Data from this study does not support the existence of more than one type of *mer* locus in *A. salmonicida* AS03. Whereas Liebert et al. (20)
372 found multiple mer loci in their bacterial isolates, as determined by the presence of multiple PCR bands, using the same PCR primer pairs (B1 and B2, D2 and D3, P1 and
374 AO and R1 and A5), we observed only a single amplicon for the same PCR amplifications in *A. salmonicida* AS03.

376 Class I integrons are prevalent in antibiotic-resistant clinical isolates of gram-negative bacteria (32). Each class 1 integron may contain several gene cassettes
378 encoding antibiotic resistance, and the global reservoir for these cassettes is large (22, 27). Our data support the presence of at least one class 1 integron in *A. salmonicida*
380 AS03. The class 1 integron genes *sul1, qacEΔ1* and *aadA7* were sequenced from this strain. The aminoglycoside resistance gene that encodes for adenyltransferase,
382 *aadA7*,was first found in an *E. coli* strain in which the *mer* operon was located on the mobile genetic element Tn*21*(27). *aadA7* has never been reported in *A. salmonicida* (2,
384 12, 23, 27, 31). The tetracycline resistance gene, Tet A, has previously been found in *A. salmonicida*, purportedly as a result of the widespread use of tetracycline in veterinary
386 medicine (22). This tetracycline resistance determinant was probed for in this study as this gene has been found in many *A. salmonicida* strains and, along with Tet E, is the
388 most common tetracycline resistance determinant isolated from aquatic bacteria(21, 22)

The transposase genes *tnpA* and *tnpR* amplified and sequenced in this study show a higher degree of similarity to these genes found on Tn*1696* than those carried on Tn*21*. This transposition module has not previously been reported in *A. salmonicida*. However, the integron In4, which is typically associated with Tn*1696*, has been detected in *A. salmonicida* (36). It is possible that the *mer* operon and associated antibiotic resistance genes in *A. salmonicida* AS03 are under the control of the Tn*1696* transposition module as part of a composite transposon.

The conjugation data strongly indicate that the antibiotic resistance determinants in *A. salmonicida* AS03, with the exception of those encoding resistance to fluoroquinolones, the *mer* operon and Tn*1696* transposition module were located together on a mobile genetic element capable of conjugative gene transfer. Supporting this observation is the fact that, with the exception of fluoroquinolone resistance, the antibiotic resistance determinants previously reported in *A. salmonicida* are encoded on plasmids, in which resistance genes are often closely linked on mobile genetic elements(22, 24, 27, 30, 43). The plasmid pRMH760 carries many of the same antibiotic resistance determinants in addition to the *mer* operon and Tn*1696* transposition module and these genes were similarly transferred to. *coli* DH5α (32). Multiple conjugation experiments were conducted under different mating mixture conditions of chloramphenicol and mercury exposure. However, all transconjugants exhibited identical antibiotic and mercury resistance phenotypes (Table 2). These data strongly suggest that these genes are located on a single mobile genetic element as opposed to multiple genetic elements.

Because these resistance genes are transferable via conjugation, it is likely that the mobile genetic element containing the *mer* operon, the Tn*1696* transposition module, and the genes encoding the transferred antibiotic resistances is either a conjugative R plasmid or conjugative transposon. Plasmids were not detected in either the *E. coli* DH5α recipient or the transconjugant that had phenotypically similar mercury and antibiotic resistance capabilities as the donor *A. salmonicida* AS03. The resistance genes could either be present on a low-copy number plasmid that was not detectable by standard experimental protocols or that they were located on a conjugative transposon that, after conjugative transfer, integrated in the recipient's chromosome. Previous studies involving conjugative transposons in various conjugation systems have reported the inability to detect plasmids in the transconjugants (19) Due to the high levels of antimicrobial and mercury resistance in the transconjugant, it would be expected that plasmids containing these resistance determinants to be in high copy number. Therefore, our data suggest that the mercury and antibiotic resistance genes, as well as the transposition module of Tn*1696*, were encoded on a conjugative transposon. Conjugative transposons are known to carry both mercury and antibiotic resistance genes. The conjugative transposon SXT, characterized in *Vibrio cholerae*, encodes resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (20). In this study, all of antibiotic genes encoded on SXT were observed to be transferable in our conjugation system (Table 2).

Furthermore, it was demonstrated that low-level Hg and chloramphenicol can stimulate conjugation as evidenced by increased the transfer frequencies (Table 3). Due to atmospheric deposition, mercury levels in the environment are rising and its presence

434 in the landscape is a threat to wildlife as well as human health in the Northeastern United States and Atlantic Canada (4, 14, 26, 39). High mercury levels have been documented in
436 freshwater and marine fish in Atlantic Canada and the United States and fish consumption advisories have been issued in all five Canadian Provinces as well as thirty-
438 nine states in the U.S (7, 8, 13, 14). Our study provides evidence that environmental mercury might have provided the direct selection pressure necessary to facilitate the
440 acquisition of broad-spectrum Hg resistance, as well as the indirect selection pressure that facilitated multiple-drug resistance by *A. salmonicida* AS03. Due to Hg pollution, bodies
442 of water contain low-levels of Hg. Not only can this mercury continually select for the presence of plasmids or transposons with mercury resistance genes but this mercury can
444 stimulate the transfer of these resistance genes between bacterial species. In addition, antibiotic resistance genes were transferred to *E. coli* DH5α along with mercury
446 resistance genes in the complete absence of antibiotics and in the presence of low-level mercury in the mating mixtures. This demonstrates that mercury can in fact directly select
448 for the transfer of mercury resistance genes and indirectly select for the horizontal transfer of antibiotic resistance genes in a system in which the antibiotic and mercury
450 resistance genes are both located on mobile genetic elements. This potentially pathogenic aeromonad is significant not only because it is resistant to many antibiotics but because
452 its antibiotic and mercury resistances are located on mobile genetic elements, which may include conjugative R-plasmids or conjugative transposons, and can disseminate these
454 genes to other organisms by horizontal gene transfer. This genetic element can easily be transferred to other bacterial strains, such as salmonid commensal bacteria or other
456 pathogenic hatchery bacteria when low-level Hg acts as a selection agent.

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