




2006

A broad-spectrum mer operon in a multi-drug resistant strain of the fish pathogen, *Aeromonas salmonicida*

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Recommended Citation

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2 **A broad-spectrum *mer* operon in a multi-drug resistant strain of the fish pathogen,**
3 ***Aeromonas salmonicida*.**

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5 **Colby College Biology Department**
6 **Honors Thesis**
7 **Submitted May 17, 2006**

8 **Abstract**

10 *Aeromonas salmonicida* AS03, a potential fish pathogen, was isolated from
12 Atlantic salmon, *Salmo salar*, in 2003. This strain was found to be resistant to ≥ 1000 mM
14 HgCl₂ and ≥ 32 mM phenylmercuric acetate as well as multiple antimicrobials. Mercury
16 (Hg) and antibiotic resistance genes are often located on the same mobile genetic
18 elements, so the genetic determinants of both resistances and the possibility of horizontal
20 gene transfer were examined. Specific PCR primers were used to amplify and sequence
22 distinctive regions of the *mer* operon. *A. salmonicida* AS03 was found to have a
24 pDU1358-like broad-spectrum *mer* operon, containing *merB* as well as *merA*, *merD*,
26 *merP*, *merR* and *merT*, most similar to *Klebsiella pneumoniae* plasmid pRMH760. To our
28 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 Tn1696 were identified through PCR and sequencing with Tn21
specific PCR primers. We provide phenotypic and genotypic evidence that the *mer*
operon, the aforementioned antibiotic resistances, and the Tn1696 transposition module
are located on a single plasmid or conjugative transposon that can be transferred to *E. coli*
DH5 α 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 Tn1721 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

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

54 Mercury (Hg) resistance is prevalent among environmental bacterial isolates due
to the selective pressure of elevated mercury contamination (3). The most common
56 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
58 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
60 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
62 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
64 be transferred between species (3, 43). Several studies have demonstrated the transfer of
mercury resistance via conjugation between bacterial species(3, 43). Although excessive
66 antibiotic usage is largely responsible for the growing prevalence of antibiotic resistance,
non-antibiotic selective agents, including mercury and other heavy metals, have been
68 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
70 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
72 and intestinal bacteria of primates with mercury amalgam fillings, where bacteria
containing a *mer* operon are more likely to exhibit multiple antibiotic resistance(38).
74 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 HgCl₂, 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*

98 *coli* DH5 α by conjugation and are, therefore, associated with at least one mobile genetic
element. Furthermore, low levels of either mercury or the antibiotic chloramphenicol
100 stimulated higher transfer frequencies of conjugation.

Materials and Methods

102 **Bacterial isolates and cultivation conditions.** An isolate of *Aeromonas salmonicida*,
designated strain AS03, was found to be resistant to both mercury and antimicrobials.
104 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*
106 DH5 α (Invitrogen Life Technologies, Carlsbad, CA) was used as the recipient in the
conjugation experiments. *A. salmonicida* AS03 and *E. coli* DH5 α were grown on Brain
108 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.

110 **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
112 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
114 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
116 Biosystems, Foster City, CA). Isolate identification was based on 16S rDNA sequence
similarities (Blastn against GenBank at <http://www.ncbi.nlm.nih.gov/BLAST>).

118 **Mercury minimum inhibitory concentrations (MICs).** Hg MIC values for both HgCl₂
and phenylmercuric acetate (PMA; Sigma-Aldrich, Inc., St.Louis, MO) were determined
120 for *A. salmonicida* AS03 and *E. coli* DH5 α 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 HgCl₂ , 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 Tn21 genes and antibiotic resistance genes.

156 Primers (Table 1) were used to amplify Tn21, 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 °C for 1 min, 53-57°C for 1 min and
72°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 HgCl₂ following incubation at 37°C.
These concentrations of chloramphenicol and HgCl₂ 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 $\mu\text{g ml}^{-1}$ chloramphenicol.
190 Donor and recipient strains were mixed as described above on BHI agar plates containing
1 $\mu\text{g ml}^{-1}$ chloramphenicol, a concentration determined to be sub-lethal to recipient *E.*
192 *coli* DH5 α . 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 $\mu\text{M HgCl}_2$.
196 Donor and recipient strains were mixed as described above on BHI agar plates containing
25 $\mu\text{M HgCl}_2$, and the mating mixtures were incubated as described above. Transfer
198 frequencies were done as described above except that selection for *E. coli* DH5 α
transconjugants was done on BHI plating media with 250 $\mu\text{M HgCl}_2$.

200 Plasmid DNA was isolated from *A. salmonicida* AS03, *E. coli* DH5 α recipient,
and the *E. coli* DH5 α 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°C. Genomic DNA was extracted from *A.*
204 *salmonicida* AS03, *E. coli* DH5 α recipient, and the *E. coli* DH5 α transconjugant as
described above in order to serve as template DNA in the following PCR amplifications.
206 PCR primer sets A1-s.F and A5-n.R, *In-F* and *In-B*, *tnpR-F* and *tnpR-B*, Tet A FW and
Tet A RV, and p5-II and p2-II (Table 1) were used to probe the *E. coli* DH5 α recipient
208 and transconjugant for the presence of the *merA*, *aadA7*, *tnpR*, Tet A, and *tnpA* genes that
were present in *A. salmonicida* AS03.

210 **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
 212 XXXXXXXXXX. 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
 214 numbers XXXXXXX through XXXXXXX

216

Table 1: PCR primers used in this study

Primer	Gene	Sequence	Source or Reference
A1-s.F	<i>merA</i>	TCCGCAAGTNGCVACBGTNGG	(41)
A5-n.R	<i>merA</i>	ACCATCGTCAGRTARGGRAAVA	(41)
R1-F	<i>merR-merT</i>	GCGGATTTGCCTCCACGTTGA	(25)
T1-R	<i>merR-merT</i>	CCAGGCAGCAGGTTCGATGCAAG	(25)
P1-F	<i>merP-merA</i>	GGCTATCCGTCCAGCGTCAA	(25)
A0-R	<i>merP-merA</i>	GTCGCAGGTCATGCCGGTGATTTT	(25)
B1-F	<i>merB</i>	TCGCCCATATATTTTAGAAC	(25)
B2-R	<i>merB</i>	GTCGGGACAGATGCAAAGAAA	(25)
D2-F	<i>merD</i>	CCAGGCGGCTACGGCTTGTT	(25)
D3-R	<i>merD</i>	GGTGGCCAACTGCACTTCCAG	(25)
A6-F	<i>merA-merD</i>	GCCGACCAGTTGTTCCCCTACCTGACG	(25)
D1-R	<i>merA-merD</i>	CGCACGATATGCACGCTCACCC	(25)
<i>In-F</i>	Integron	GGCATCCAAGCAGCAAGC	(40)
<i>In-B</i>	Integron	AAGCAGACTTGACCTGAT	(40)
p5-II	Tn21 <i>tnpA</i>	TACTGCCGCGCATCAAGATC	(10)
p2-II	Tn21 <i>tnpA</i>	AGAAAGTTCGTCCTGGGCTG	(10)
tnpR-F	Tn21 <i>tnpR</i>	GGCGACACCGTGGTGGTGCATAGC	(6)
tnpR-B	Tn21 <i>tnpR</i>	CGGTAAGCCCCGCGTTGCTTGGC	(6)
qacF	<i>qacEA1-sul1</i>	ATCGCAATAGTTGGCGAAGT	(35)
Sul R	<i>qacEA1-sul1</i>	GCAAGGCGGAAACCCGCGCC	(35)
tetA FW	Tet A	GTAATTCTGAGCACTGTTCGC	(17)
tetA RV	Tet A	CTGCCTGGACAACATTGCTT	(17)

218

Results

220 **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).

224

226

228

230 **Table 2.** Antibiotic minimum inhibitory concentrations ($\mu\text{g/ml}$) as determined by Sensititre panels for *A. salmonicida* AS03, *E. coli* DH5 α , and the transconjugant.

Antimicrobials by class (<i>a</i>)	<i>Aeromonas salmonicida</i> AS03 Donor	<i>Escherichia coli</i> DH5 α recipient	<i>Escherichia coli</i> DH5 α 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			
Ciprofloxacin (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 $\mu\text{g/mL}$

232

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

234 *Aeromonas salmonicida* AS03 demonstrated resistance to $\geq 1000 \mu\text{M}$ HgCl_2 and $\geq 32 \mu\text{M}$
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 *pneumoniae* 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' adenylyltransferase enzyme conferring
resistance to streptomycin and spectinomycin. This gene exhibited the most similarity to
254 *aadA7* in *Escherichia coli* (AF234167.1), *Vibrio cholerae* (DQ196322.1) and *Salmonella*
enterica (AY463737) and explains the streptomycin resistant phenotype of *A.*

256 *salmonicida* AS03 determined by antimicrobial susceptibility disks. PCR primers *qacF*
and *sulR* yielded an approximately 800 bp PCR product which was identified by blastn
258 analysis on the reverse sequence as *sull*. This *sull* gene resembled *sull* genes of *E. coli*
plasmid PQR-1 (AY655485) and *K. pneumoniae* pRMH760 (AY123253). The forward
260 sequence confirmed the presence of *qacEΔI* and was most similar to *qacEΔI* in *E. coli*
pSa (L06822.4) and pRMH760 (AY123253) in the blastn database. PCR and gene
262 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,
264 including *E. coli* pAPEC-OR-R (AY214164), *S. S. typhimurium* pU302L (AY333434),
and *A. salmonicida* pRAS1 (AJ131405).

266 **Transposon genes.** Because this strain exhibited simultaneous antibiotic and Hg
resistance phenotypes, the presence of Tn21, which carries both resistance determinants,
268 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
270 similar to the *tnpA* from Tn1696 than that of *tnpA* of Tn21, and was almost identical to
the Tn1696 *tnpA* of *P. arguinosus* R1033 (U12338.2) and *K. pneumoniae* pRMH760
272 (AY123253). Similarly, the 240 bp amplicon obtained from PCR with primers *tnpR-R*
and *tnpR-F* was identified as *tnpR* from Tn1696 and was near identical to the Tn1696
274 *tnpR* of *P. arguinosus* p1033 and *K. pneumoniae* pRMH760.

Conjugation. *A. salmonicida* AS03 was able to transfer both mercury and antibiotic
276 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
278 μM HgCl₂, 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,
280 especially among the tetracycline, penicillin, and cephem classes of antibiotics (Table 2).
The folate pathway inhibitor sulfisoxazole was also expressed maximally in the
282 transconjugant. Notably, antibiotics of the fluoroquinolone class were not transferred,
even though the donor, *A. salmonicida* AS03, exhibited low levels of resistance to some
284 of the antimicrobials in both the fluoroquinolone classes.

Similarly, mercury MICs in the transconjugant were identical under conditions of
286 either chloramphenicol or HgCl₂ selection. The donor *A. salmonicida* AS03 exhibited
high resistance to mercury with MICs of ≥ 1 mM HgCl₂ and ≥ 32 μ M PMA, whereas the
288 *E. coli* DH5 α recipient showed low levels of mercury resistance with MICs of 100 μ M
HgCl₂ and 16 μ M PMA. After mating with *A. salmonicida* AS03, the *E. coli* DH5 α
290 transconjugant had mercury MICs of ≥ 1 mM HgCl₂ and ≥ 32 μ M PMA. No plasmids
were isolated from the *E. coli* DH5 α recipient nor the transconjugant, while at least
292 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.
294 PCR amplification from the genomic DNA of the *E. coli* DH5 α transconjugant showed
the presence of the *merA*, *aadA7*, *tnpR*, *Tet A*, and *tnpA* genes that were previously
296 sequenced in the *A. salmonicida* AS03 donor. These genes were not amplified in the *E.*
coli DH5 α recipient and, therefore, must have been acquired by the *E. coli* DH5 α
298 transconjugant via conjugative transfer from the *A. salmonicida* AS03 donor (Figure 1).

300

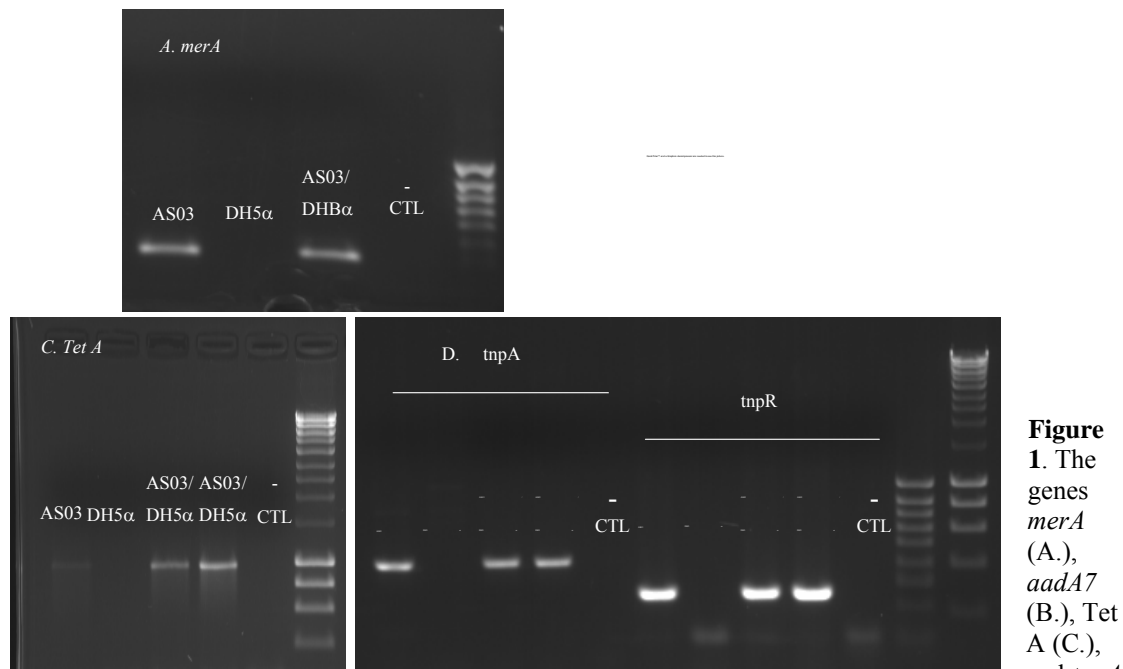


Figure 1. The genes *merA* (A.), *aadA7* (B.), Tet A (C.), and *tnpA*

326 and *tnpR* (D.) are present in the *A. salmonicida* AS03 donor and the *E. coli* DH5α transconjugant but absent
 328 in the *E. coli* DH5α recipient, showing that these genes were transferred to the *E. coli* DH5α transconjugant
 330 from *A. salmonicida* AS03 via conjugation. The notation AS03/DH5α is used to denote the transconjugant
 on the gel pictures.

332 Conjugative transfer of resistance determinants to the *E. coli* DH5α recipient
 varied under different conditions of the donor and mating mixture. The highest transfer
 334 frequencies were observed when the donor was grown on BHI plating media amended
 with either 8 μg ml⁻¹ chloramphenicol or 25 μM HgCl₂ prior to conjugation and the
 336 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 HgCl₂). Statistically, there
 338 was no significant difference between the transfer frequencies of these two exposure
 conditions. However, exposure to either chloramphenicol or mercury stimulated
 340 significantly greater transfer frequencies than observed in the control, which contained
 neither mercury nor chloramphenicol (Table 3). Regardless of the exposure condition,
 342 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* DH5 α recipient and an *A. salmonicida*
AS03 donor on solid BHI media under different conditions

Conjugation Condition	Transfer Frequency ^a \pm SE
BHI agar	$(2.20 \pm .48) \times 10^{-2}$
25 μ M Hg in BHI agar	$(4.85 \pm .94) \times 10^{-2}$
8 μ M 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 μ g ml⁻¹ chloramphenicol
divided by the number of CFU on non-selective BHI media

352

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 Tn21 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 Tn1696 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 Tn1696 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 adenylyltransferase,
382 *aadA7*, was first found in an *E. coli* strain in which the *mer* operon was located on the
mobile genetic element Tn21(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
390 a higher degree of similarity to these genes found on Tn1696 than those carried on Tn21.
This transposition module has not previously been reported in *A. salmonicida*. However,
392 the integron In4, which is typically associated with Tn1696, has been detected in *A.*
salmonicida (36). It is possible that the *mer* operon and associated antibiotic resistance
394 genes in *A. salmonicida* AS03 are under the control of the Tn1696 transposition module
as part of a composite transposon.

396 The conjugation data strongly indicate that the antibiotic resistance determinants
in *A. salmonicida* AS03, with the exception of those encoding resistance to
398 fluoroquinolones, the *mer* operon and Tn1696 transposition module were located together
on a mobile genetic element capable of conjugative gene transfer. Supporting this
400 observation is the fact that, with the exception of fluoroquinolone resistance, the
antibiotic resistance determinants previously reported in *A. salmonicida* are encoded on
402 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
404 resistance determinants in addition to the *mer* operon and Tn1696 transposition module
and these genes were similarly transferred to *coli* DH5 α (32). Multiple conjugation
406 experiments were conducted under different mating mixture conditions of
chloramphenicol and mercury exposure. However, all transconjugants exhibited identical
408 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
410 elements.

Because these resistance genes are transferable via conjugation, it is likely that the mobile genetic element containing the *mer* operon, the Tn1696 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* DH5 α 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 Tn1696, 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* DH5 α 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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