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## Diversity of antibiotic-resistance genes in Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida*: dominance of pSN254b and discovery of pAsa8

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The bacterium *Aeromonas salmonicida* subsp. *salmonicida* is a common pathogen in fish farms worldwide. Since the antibiotic resistance of this bacterial species is on the increase, it is important to have a broader view on this issue. In the present study, we tested the presence of known plasmids conferring multi-drug resistance as well as antibiotic resistance genes by a PCR approach in 100 Canadian *A. salmonicida* subsp. *salmonicida* isolates. Our study highlighted the dominance of the conjugative pSN254b plasmid, which confers multi-drug resistance. We also identified a new multi-drug plasmid named pAsa8, which has been characterized by a combination of sequencing technologies (Illumina and Oxford nanopore). This new plasmid harbors a complex class 1 integron similar to the one of the *Salmonella genomic island 1* (SGI1) found in *Salmonella enterica* and *Proteus mirabilis*. Consequently, in addition to providing an update on the *A. salmonicida* subsp. *salmonicida* isolates that are resistant to antibiotics, our data suggest that this bacterium is potentially an important reservoir of drug resistance genes and should consequently be monitored more extensively. In addition, we describe a screening method that has the potential to become a diagnostic tool that is complementary to other methods currently in use.

The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is a fish pathogen that causes furunculosis worldwide<sup>1,2</sup>. In Quebec (Canada), furunculosis is the most common infection encountered in fish farms, especially brook trout farms, and causes between 30 and 60% of reported outbreaks every year<sup>3</sup>. Antibiotics are the most widely used treatment for furunculosis. In Canada, four antibiotics (oxytetracycline, florfenicol [a chloramphenicol analog], sulfadimethoxine/ormetoprim, and sulfadiazine/trimethoprim) are approved by the Veterinary Drugs Directorate (VDD) of Health Canada to treat infected fish. In Quebec, florfenicol is the antibiotic of choice because of its short withdrawal period (12 days) compared to antibiotics such as oxytetracycline and sulfadimethoxine/ormetoprim (42 days)<sup>3</sup>. However, intensive use of these four antibiotics has been correlated with growing number of antibiotic-resistant isolates<sup>3</sup>.

Antibiotic resistance genes in *A. salmonicida* subsp. *salmonicida* are mostly located on plasmids<sup>4</sup>. Among them, pSN254b and pAB5S9b plasmids, which bear multi-drug resistance genes, as well as variants of pRAS3 have been recently described in isolates from Canada<sup>5</sup>. To date, pSN254b has been identified in isolates from Quebec, New Brunswick (Canada) and Nova Scotia (Canada), while pAB5S9b and pRAS3 variants have been

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| Plasmid | Length (kb) | R-genes  | Found in Canada  | Reference |
|---------|-------------|--|------------------|-----------|
| pAsa7   | 5,276       | <i>cat</i>   | No               | 64        |
| pRAS3.2 | 11,823      | <i>tetA</i>  | No               | 65        |
| pRAS3.3 | 11,845      | <i>tetA</i>  | Yes              | 5         |
| pRAS3.1 | 11,851      | <i>tetA</i>  | No               | 65        |
| pAB5S9b | 25,540      | <i>tet(H), floR, sul2, strA, strB</i>                                  | Yes              | 5         |
| pASOT3  | ~39         | <i>aadA2, tetA</i>   | No               | 66,67     |
| pRAS1   | ~45         | <i>dfrA16, tetA, sul1</i>  | No               | 17        |
| pAr-32  | ~47         | <i>aadA2, sul1, catA2</i>  | No               | 17,21     |
| pASOT   | ~47         | <i>aadA2 or dfrIIc, tetA</i>   | No               | 66,67     |
| pASOT2  | ~47         | <i>aadA2, tetA</i>   | No               | 66,67     |
| pRAS2   | ~48         | <i>sul2, tet 31, strA, strB</i>  | No               | 17,68     |
| pSN254b | 152,216     | <i>tetA, floR, sul1, sul2, blaCMY, aadA, strA, strB, sugE2, qacEΔ1</i> | Yes              | 5         |
| pAsa4   | 166,749     | <i>tet(E), sul1, aadA, cat</i>   | Yes (pAsa4-like) | 5,43      |

**Table 1. R-plasmids found in *A. salmonicida*.**

identified only in New Brunswick. pAB5S9b and pSN254b bear genes coding for resistance to the three antibiotics used in veterinary medicine in Quebec. There are currently no government-approved antibiotics that are effective against bacterial isolates that host either one of these two plasmids. For their part, pRAS3 variants contain a gene coding for tetracycline resistance<sup>5</sup>. In addition to these plasmids conferring drug resistance (R-plasmids), many others were found in *A. salmonicida*, some of them having a large-panel of genes causing resistance to antibiotics (Table 1).

Even if the plasmidome (i.e., the total plasmid content) of *A. salmonicida* subsp. *salmonicida* harbor multiple R-plasmids, the dispersion of these plasmids and the gravity of the situation are presently unknown. In order to determine the fraction of isolates bearing antibiotic-resistance genes and to infer the geographic distribution of the R-plasmids, we investigated a collection of 100 *A. salmonicida* subsp. *salmonicida* Canadian isolates, using, among other methods, optimized multiplex PCR assays. This approach was also done to assess the presence of new R-plasmids.

This screening showed that pSN254b is the most common R-plasmid in tested Canadian isolates of *A. salmonicida* subsp. *salmonicida*. A new R-plasmid, pAsa8, was also found, which had genetic features that provided additional evidence that *A. salmonicida* subsp. *salmonicida* is an important reservoir for mobile genetic elements as well as antibiotic resistance genes.

## Results

**Plasmid content determined by PCR genotyping.** The presence of plasmids containing known antibiotic resistance genes (pAsa4, pAB5S9b, pRAS3.3, pSN254b) has been tested in 100 Canadian *A. salmonicida* subsp. *salmonicida* isolates<sup>5</sup> using the primers listed in Supplementary Table S1. The results of the genotyping for the 100 isolates are given in Supplementary Table S2. The pSN254b plasmid was the most prevalent (25/100), while the other plasmids were detected in two to four isolates each.

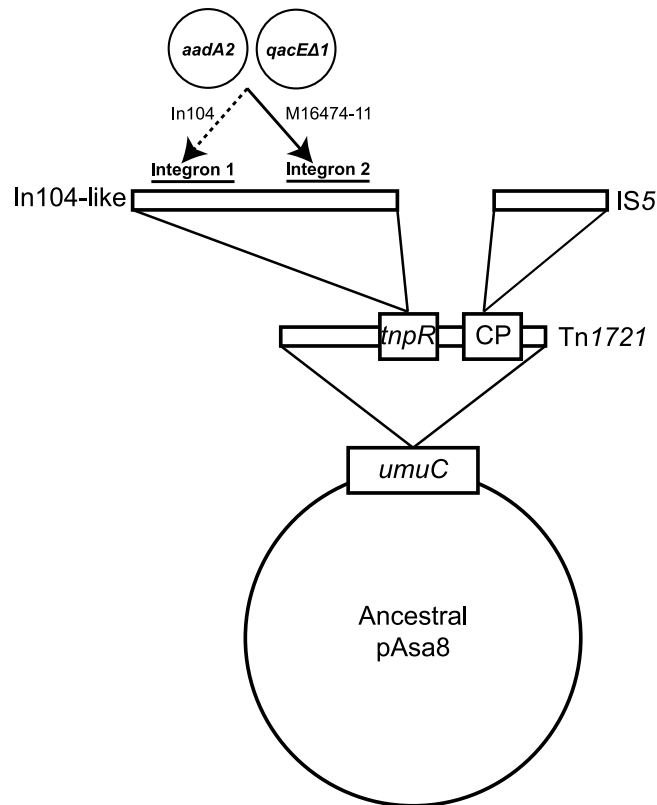
**Multiplex PCR to detect antibiotic resistance genes.** We subsequently developed four multiplex PCR assays (see the Methods section) to detect the antibiotic resistance genes known to be present in *A. salmonicida* subsp. *salmonicida* isolates: *floR*, *cat*, *sul1*, *sul2*, *tetA*, *tet(C)*, *tet(E)*, and *tet(H)*. The results of these tests for the 100 *A. salmonicida* subsp. *salmonicida* isolates are presented in Supplementary Table S2.

The *floR* gene was detected in 29 of the 100 isolates, while the *cat* gene was not detected in any of the isolates excepted in A449, which was the positive control. The *floR* gene, which has been detected in isolates with the pSN254b and pAB5S9b plasmids<sup>5</sup>, was also detected in the M15448-11 and M16474-11 isolates, despite the fact that they do not host either of the two plasmids. This result suggests that an unknown genomic entity might be responsible for the presence of the *floR* gene in these two isolates.

The *sul1* and *sul2* genes were detected in 30 and 28 isolates, respectively. Both genes were detected in isolates that contained pSN254b, while only *sul2* was detected in isolates carrying pAB5S9b, as reported previously<sup>5</sup>. The *sul1* gene was detected in four isolates (three from Quebec and one from Ontario (Canada)) with a pAsa4-like plasmid. This multiplex PCR experiment also revealed that the *sul1* gene was present in M15448-11 and M16474-11.

The *tet(C)* gene was detected in four isolates with the pRAS3 plasmid while *tet(H)* was detected in two isolates harboring the pAB5S9b plasmid<sup>5</sup>. The *tet(E)* gene was present in four isolates with a pAsa4-like plasmid. The *tetA* gene was detected in isolates with the pSN254b plasmid<sup>5</sup> as well as in the M15448-11 and M16474-11 isolates.

**Characterization of pAsa8.** The presence of multiple closely related antibiotic resistance genes in M15448-11 and M16474-11 isolates without the detection of any recognized plasmids in these isolates prompted us to sequence the complete DNA of M16474-11 by Illumina and Oxford nanopore (MinION) technologies. Only the



**Figure 1. Putative evolutionary scenario of the multidrug region of pAsa8.** Only the elements involved are shown: the gene *umuC* of ancestral pAsa8, the genes *tnpR* and the one encoding for the chemotaxis protein (CP) located on the transposon Tn1721, the insertion sequence IS5 and finally the integron In104-like. Cassettes *aadA2* and *qacEΔ1* are located within integron 1 of In104 (dashed arrow), and also within integron 2 of In104-like found on pAsa8 (solid line arrow) (isolate M16474-11).

DNA of the M16474-11 isolate was sequenced since both isolates (i.e., M15448-11 and M16474-11) exhibited the same profile.

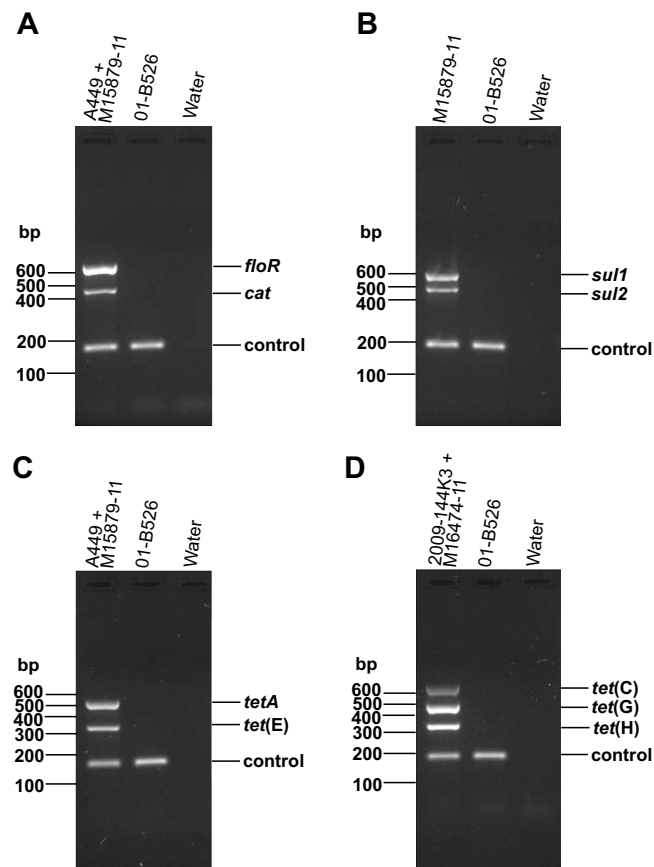
The sequencing revealed the presence of a new plasmid, that we named pAsa8, of 110 577 bp and which has a region containing many drug resistance genes. This region is the product of multiple integration events involving mobile genetic elements (Fig. 1). One of them is a transposon Tn1721<sup>6</sup>, which is integrated in the gene *umuC*, encoding for a UV mutagenesis and repair protein, of the ancestral pAsa8. This transposon brings a *tetA* gene, causing resistance to tetracycline, which explains the positive result of the multiplex PCR.

A second integration event involved a complex class 1 integron In104-like (In4-group) previously found in a 43-kb integrated element known as the *Salmonella genomic island 1* (SGI1) found in *Salmonella enterica*<sup>7</sup> and *Proteus mirabilis*<sup>8–11</sup> and for which numerous variants were found<sup>12</sup>. The In104-like integron allows resistance to florfenicol/chloramphenicol (*floR*), tetracyclines (*tet(G)*), sulfonamides (*sul1*), ampicillin/carbenicillin (*bla<sub>PSE-1</sub>*) and streptomycin/spectinomycin (*aadA2*) (Fig. 2) in the previously described M16474-11 *A. salmonicida* subsp. *salmonicida* isolate. The complex In104 integron contains two functional *IntI1* integron regions<sup>12,13</sup> with many variants reported<sup>14</sup>. The integron regions found in the In104-like of *A. salmonicida* differs from those of the original In104 found in *S. enterica* serovar *Typhimurium* DT104 in the sense that the cassettes *aadA2* and *qacEΔ1* found in the first integron (*IntI1*) in *S. enterica* are in the second (*groEL/IntI1*) for *A. salmonicida* subsp. *salmonicida* (Fig. 2). The evolutionary scenario that would account for the cassette swap is not clear, but a BLASTn revealed that the second integron region (*groEL/IntI1*) is identical to one of the transposon Tn2610, which is formed by parts of Tn1721, Tn21 and SGI1<sup>15</sup>. The third and last integration event involves the integration of an IS5 into the gene that encodes the chemotaxis protein of Tn1721.

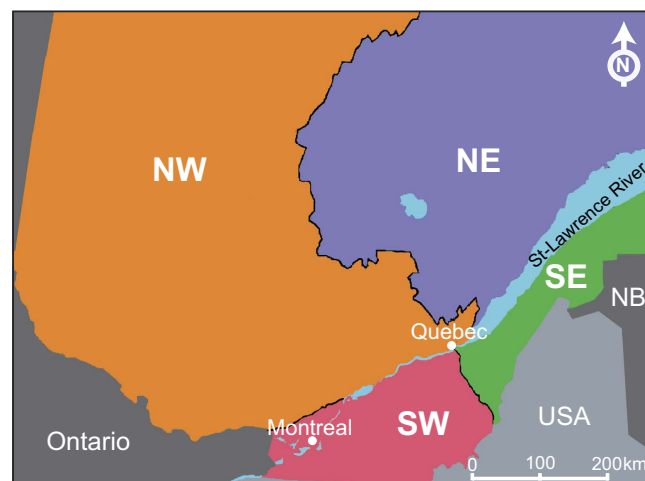
Interestingly, the IncU plasmid pRAS1, found in both typical and atypical *A. salmonicida*, was also reported to harbour a Tn1721 and an integron of the In4 family<sup>16,17</sup>. Unfortunately, this plasmid is not sequenced and consequently it would be perilous to postulate any evolutionary links between pRAS1 and pAsa8. As recently reviewed elsewhere, Tn1721 is present in several strains of the genus *Aeromonas*<sup>4</sup>.

The pAsa8 backbone contains several genes encoding for hypothetical proteins and also has a region containing genes promoting conjugative transfers. However, many of these ORFs are likely pseudogenes or are highly derived relative to sequences in the GenBank nr/nt database (based on BLASTp). No type 2 toxin-antitoxin system was found by TAFinder. A BLASTn query of the nr/nt database with the pAsa8 sequence only gave partial alignments (the multidrug region). However, a BLASTn analysis against the Whole Genome Shotgun (WGS) database (Gammaproteobacteria, taxid:1236) resulted in significant homology to the draft genome of *Aeromonas*





**Figure 3.** Multiplex PCR targeting genes coding for resistance to chloramphenicol/lorfenicol (A), sulfonamides (B), and tetracyclines (C,D). Electrophoresis gels with amplicons generated using DNA isolated from positive controls (A449 + M15879-11 in (A,C), M15879-11 in B, 2009-144K3 + M16474-11 in D) and from a negative control (01-B526). A specific control for *A. salmonicida* subsp. *salmonicida* using a primer pair that amplified an element of the prophage 1 was included in each multiplex PCR reaction. The amplicon of this product can be seen at the bottom of each gel (control). (A) The target *cat* (448 bp) and *floR* genes (632 bp) and (B) the *sul2* (449 bp) and *sul1* (550 bp) genes were detected when present. (C) Amplicons for *tet(E)* (351 bp) and *tetA* (526 bp). (D) Amplicons for *tet(H)* (326 bp), *tet(G)* (460 bp), and *tet(C)* (629 bp). Water was used as a negative control.



**Figure 4.** Map of the Quebec showing the regions to which the various isolates were assigned. Northwest (NW, orange), northeast (NE, purple), southwest (SW, pink), and southeast (SE, green). Most of the fish farms analyzed were less than 200 km from the St. Lawrence River, which crosses the province from west to east. The map has been drawn using Adobe Photoshop CS4 version 11.0.2 ([www.adobe.com](http://www.adobe.com)).



| Region <sup>a</sup>                     |            | NE             | SE              | SW              | NW              | Quebec as a whole |
|---|------------|----------------|-----------------|-----------------|-----------------|-------------------|
| <b>Number of isolates</b>               |            | <b>16</b>      | <b>11</b>       | <b>14</b>       | <b>45</b>       | <b>86</b>         |
| Plasmid found in the isolates           | pSN254b    | 0              | 5               | 6               | 12              | 23                |
|   | pAsa4-like | 0              | 0               | 0               | 3               | 3                 |
|   | pAsa4      | 0              | 0               | 0               | 0               | 0                 |
|   | pAB5S9     | 0              | 0               | 0               | 0               | 0                 |
|   | pRAS3      | 0              | 0               | 0               | 0               | 0                 |
|   | pAsa8      | 0              | 0               | 0               | 2               | 2                 |
| Number of antibiotic-resistant isolates |            | 0              | 5               | 6               | 17              | 28                |
| % of resistant isolates <sup>b</sup>    |            | 0 [0.00, 0.11] | 45 [0.20, 0.73] | 43 [0.20, 0.68] | 38 [0.25, 0.52] | 33 [0.23, 0.43]   |

**Table 2. Geographical distribution of the antibiotic resistant isolates in Quebec.** <sup>a</sup>see Fig. 4. <sup>b</sup>The binomial 95% confidence interval (Jeffreys method) is indicated between brackets.

major problem when it comes to the treatment of infected fish with antibiotics. The high prevalence of pSN254b can be mostly explained by (1) its capacity to be transferred by conjugation<sup>22</sup> and (2) the presence of genes conferring resistance to various antibiotics, thus enhancing the selection pressure for isolates having this plasmid. In fact, up to 40% of the *A. salmonicida* subsp. *salmonicida* bacteria isolated from diseased fish in Quebec, except in the NE region, harbor antibiotic resistance genes (Table 2). pAsa4 and pAsa8 were found in the NW region while the pSN254b plasmid was present in the SW, SE, and NW regions.

The veterinarians who collected the *A. salmonicida* subsp. *salmonicida* isolates from diseased fish proposed three reasons explaining the geographic distribution of the antibiotic resistant isolates<sup>23</sup>. First, there is more infection outbreak in regions with higher aquaculture activity (mainly NW, SW and SE). Second, it is known that fish farmers exchange fish between regions which may contribute in part to the spread of the antibiotic resistant isolates. Finally, there is an increased likelihood of diagnosis for the fish farms in the surrounding regions of the veterinary service (e.g. NW and SW).

As mentioned in Vincent *et al.* (2014), plasmids conferring resistance to antibiotics may be transferable between *A. salmonicida* subsp. *salmonicida* and human pathogens such as *Salmonella enterica*<sup>5</sup>. This is exemplified by the IncA/C pSN254b plasmid, a variant of pSN254 found in *S. enterica*<sup>5</sup>. The plasmids of the IncA/C group are known to be conjugative and found, in addition to *A. salmonicida* and *S. enterica*, in a broad range of bacterium<sup>24</sup> such as *Yersinia pestis*<sup>25</sup>, *Klebsiella pneumoniae*<sup>26</sup>, *Aeromonas hydrophila*<sup>27,28</sup>, *Photobacterium damsela* subsp. *piscicida*<sup>29</sup> and *Escherichia coli*<sup>30,31</sup>. The IncA/C plasmids are also known to regulate the excision of SGI1<sup>32</sup> and to serve as helpers for its mobilization *in trans*<sup>33</sup>. Interestingly, pAsa8 (a new R-plasmid described here) of the M16474-11 and M15448-11 *A. salmonicida* subsp. *salmonicida* isolates appeared to have a Tn1721 and a complex class 1 integron similar to the one found in SGI1. Even if we have a putative mechanism explaining the multiple integration events in the ancestral pAsa8 plasmid (Fig. 1), it is unclear if they occurred in *A. salmonicida*, in other bacteria such as *S. enterica*, or in both.

In summary, we analyzed 100 *A. salmonicida* subsp. *salmonicida* Canadian isolates for their antibiotic resistance genes and R-plasmids repertoire to shed light on their occurrence and distribution. We found that the conjugative R-plasmid pSN254b is dominant with 25% of the isolates having it. In fact, 37% of the isolates have at least one R-plasmid. This situation is worrying considering that no government-approved antibiotics can be used against these isolates to prevent furunculosis. We suggest that *A. salmonicida* subsp. *salmonicida* should be monitored worldwide to verify the trend found in Canada revealed in this study. We also discovered and characterized a new R-plasmid named here pAsa8. This plasmid hosts multiple antibiotic resistance genes from mobile elements such as Tn1721 and a complex class 1 integron In104-like. Since In104 is usually found within the genomic island SGI1 of the human pathogen *S. enterica*, this study reinforces the idea that *A. salmonicida* subsp. *salmonicida* could be an important reservoir of mobile DNA conferring drug resistance and raises the possibility of exchange of this genetic material with other waterborne bacteria. If indeed this is the case, it is even more worrying from a One Health perspective (i.e. the idea that human, animal and environmental health are all interconnected)<sup>34</sup>. Considering antimicrobial resistance in a One Health context is a part of the fourth intervention plan of the last report produced by the *Review on Antimicrobial Resistance*<sup>35</sup>. It is now well known that environmental bacteria, such as *Aeromonas*<sup>36</sup>, may act as a reservoir of antibiotic resistance genes<sup>37–39</sup>. Therefore, our results add substantial evidence that there is an urgent need to develop alternative strategies to efficiently mitigate furunculosis prevalence without selecting for other resistance genes. Pre and probiotic strategies, phage therapy and vegetable extracts such as essential oils might be promising research avenues against *A. salmonicida* subsp. *salmonicida* as it is the case for other bacteria<sup>40–42</sup>.

## Methods

**Bacterial isolates and growth conditions.** All 100 *A. salmonicida* subsp. *salmonicida* isolates used in this study were from Canada (Supplementary Table S2). The A449 reference strain from France<sup>43</sup>, for which the annotated chromosome and plasmid sequences are publicly available, was also included. All the isolates were grown on furunculosis agar for two or three days at 18 °C as previously described<sup>44</sup>.

**PCR analyses.** The DNA templates, PCR mixtures, and program cycles used in this study have been previously described<sup>45</sup>. The PCR assays were performed at least twice. Suitable positive and negative controls were included in each assay.

| Primer  | Sequence 5'–3'           | T <sub>m</sub> (°C) | Amplicon size (bp) | Target               |
|---|--------------------------|---------------------|--------------------|----------------------|
| Multiplex PCR targeting genes coding for chloramphenicol/florfenicol resistance |                          |                     |                    |                      |
| MT-ctrl-chloram-F1  | GCTTACCTCAGATAATGAGTCGTC | 54,8                | 172                | Prophage 1 (Control) |
| MT-ctrl-chloram-R1  | GCCAATAAGAGCCCTACTCTTC   | 55                  |                    |                      |
| MT-cat-F1   | CTATTTTGACAATACGCCCTGC   | 54,3                | 448                | <i>cat</i>           |
| MT-cat-R1   | CTCCCAAACGTAAATATCGGC    | 54                  |                    |                      |
| MT-floR-F1  | TTGAGCCTCTATATGGTGATGC   | 54,4                | 632                | <i>floR</i>          |
| MT-floR-R1  | GTTGTCACGATCATTACAAGCG   | 54,3                |                    |                      |
| Multiplex PCR targeting genes coding for sulfonamide resistance                 |                          |                     |                    |                      |
| MT-ctrl_sul-F1  | TTCATTTTCGTCTTGGGTCTAGC  | 54,8                | 175                | Prophage 1 (Control) |
| MT-ctrl_sul-R1  | GGACTACAGATCTACCATAATCCG | 54                  |                    |                      |
| MT-sul1-F1  | GGGCTACCTGAACGATATCC     | 54,7                | 550                | <i>sul1</i>          |
| MT-sul1-R1  | CTAGGCATGATCTAACCCCTCG   | 54,4                |                    |                      |
| MT-sul2-F1  | ATCATCTGCCAAACTCGTCG     | 55,2                | 449                | <i>sul2</i>          |
| MT-sul2-R1  | TTCTTGCGGTTTCTTTCAGC     | 53,9                |                    |                      |
| Multiplex PCR targeting genes coding for tetracycline resistance                |                          |                     |                    |                      |
| First multiplex PCR targeting genes coding for tetracycline resistance          |                          |                     |                    |                      |
| MT-ctrltet1-F1  | CCAGAATGACGAATGAATGTCG   | 54,3                | 175                | Prophage 1 (Control) |
| MT-ctrltet1-R1  | GGACCTCTTTACTCCAGTCG     | 54,4                |                    |                      |
| MT-tetA(E)pAsa4-F1  | GATGTCACACCTGAGGAATCC    | 55,1                | 351                | <i>tetA(E)</i>       |
| MT-tetA(E)pAsa4-R1  | TCCGAATAAAACCCATAATGTTGC | 53,9                |                    |                      |
| MT-tetApSN54b-F1  | CAAGCAGGATGTAGCCTGTG     | 55,9                | 526                | <i>tetA</i>          |
| MT-tetApSN54b-R1  | ATTGCCGATATCACTGATGG     | 52,4                |                    |                      |
| Second multiplex PCR targeting genes coding for tetracycline resistance         |                          |                     |                    |                      |
| MT-ctrltet2-F1  | ATTCATTTTCGTCTTGGGTCTAGC | 55                  | 176                | Prophage 1 (Control) |
| MT-ctrltet2-R1  | GGACTACAGATCTACCATAATCCG | 54                  |                    |                      |
| MT-tetHpAB5S9b-F1   | ACGACTGTCTGATAAATACGGC   | 54,6                | 326                | <i>tetH</i>          |
| MT-tetHpAB5S9b-R1   | ATATCGAGTGTGAAATAGCGGC   | 54,9                |                    |                      |
| MT-tetA(C)pRAS3-F1  | CTGTAGGCATAGGCTTGGTTAT   | 54,4                | 629                | <i>tetA(C)</i>       |
| MT-tetA(C)pRAS3-R1  | CTGTCCTACGAGTTGCATGATA   | 54,1                |                    |                      |
| MT-tetGs62-F1   | GGTTCGCATCAAACCATTTCG    | 54,8                | 460                | <i>tetA(G)</i>       |
| MT-tetGs62-R1   | GCTTAGATTGGTGAGGCTCG     | 55,6                |                    |                      |

**Table 3. Primers used for the multiplex PCR.**

The PCR primers used to identify the isolates harboring antibiotic-resistance plasmids (pAsa4, pAB5S9, pSN254, pRAS3) are listed in Supplementary Table S1, as are the primers used to genotype pAsa8 of the M16474-11 isolate.

**Multiplex PCR.** The sequences of orthologous genes causing resistance to chloramphenicol/florfenicol (*cat* and *floR*), tetracycline (*tetA*, *tet(C)*, *tet(E)*, *tet(H)*, *tet(G)*) and sulfamethoxazole (*sul1* and *sul2*) in *A. salmonicida* subsp. *salmonicida* were aligned to identify the conserved regions. The chloramphenicol resistance gene *catA2* found on the pAr-32 plasmid identified in a Japanese isolate<sup>46</sup> was not included in the analysis due to the lack of an appropriate positive control. The alignment for each resistance gene sequence was performed using MUSCLE<sup>47</sup> and BLASTn<sup>48</sup> through Geneious version 6.1.8<sup>49</sup>. Primers were designed based on the conserved sequences using Primer3<sup>50</sup> also through Geneious version 6.1.8.

The DNA templates were prepared by resuspending an inoculum of bacterial culture in 1 mL of SWL buffer (50 mM KCl, 10 mM Tris, pH 8.3, 2.5 mM MgCl<sub>2</sub>, 0.45% NP-40, and 0.45% Tween 20). The lysates were heated for 15 min at 95 °C, and the DNA concentrations in the solutions were adjusted to 100 ng/μL. The PCR mixture contained 1X Go-Taq buffer (Promega, USA), 2 mM dNTP, 0.2 μM forward and reverse primers, 1 U of GoTaq (5 U; Promega), and 100 ng of DNA templates. Depending on the number of primers used for the multiplex PCR, various volumes of filtered water were added to adjust the final reaction volume to 20 μL. The PCR program was as follows: 2 min 30 s at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1 min 30 s at 68 °C, followed by a final 5-min extension step at 68 °C. The PCR products were separated on 1.3% agarose gels and were stained with 0.5 μg/mL of ethidium bromide. For all the multiplex PCR, water and 01-B526, an antibiotic-sensitive isolate<sup>51</sup>, were used as negative controls. The primers used for each multiplex PCR are given in Table 3.

In the first multiplex PCR, targeting *floR* and *cat*, a mix of the sequenced isolates M15879-11 and A449<sup>43,52</sup> was used as a positive control. The *floR* gene gives resistance to both chloramphenicol and florfenicol while the *cat* gene provides resistance only to chloramphenicol<sup>53</sup>. Bioinformatics analyses confirmed that the M15879-11 isolate bears a pSN254b plasmid similar to that of the 2004-05MF26 isolate and had the same set of antibiotic resistance genes<sup>5,54</sup>. In the second multiplex PCR, developed to detect the *sul1* and *sul2* genes, the M15879-11 isolate

was used as positive control. A subset of tetracycline resistance genes (*tetA* and *tet(E)*) was targeted by a third multiplex PCR. A mix of M15879-11 and A449 was used as a positive control for these genes. The last multiplex PCR assay aimed at detecting *tet(C)*, *tet(G)* and *tet(H)*. Two isolates were used as positive controls: 2009-144K3 (pAB5S9b and pRAS3.3) and M16474-11 (pAsa8).

**DNA extraction and genomic sequencing.** The total DNA of the M15879-11 and M16474-11 *A. salmonicida* subsp. *salmonicida* isolates was extracted using DNeasy Blood and Tissue kits (Qiagen, Canada) and was sequenced by next-generation sequencing (NGS) at the Plateforme d'Analyse Génomique of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval). The DNA of the M15879-11 isolate was fragmented at a 5-kb size and sequenced by pyrosequencing using a GS-FLX+ apparatus as previously described<sup>52</sup>. The resulting sequencing reads were *de novo* assembled with Newbler version 2.5.3<sup>55</sup>. The total draft assembly of M15879-11 was annotated by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and deposited in GenBank under accession number LAIS00000000.

The M16474-11 library was prepared using a KAPA Hyper Prep kit and was sequenced by a MiSeq (Illumina) sequencing system. The resulting reads were filtered with Trimmomatic version 0.32<sup>56</sup> using the manual-recommended parameters for paired-end reads<sup>57</sup>. The DNA of M16474-11 was also extracted by phenol/chloroform by following the protocol *Extracting DNA Using Phenol-Chloroform* provided by Pacific Biosciences (<http://www.pacb.com>). The DNA was sheared at 8-kb using a standard Covaris g-TUBE protocol and was used to prepare an Oxford nanopore technologies sequencing library using the protocol GDE\_1002\_v1\_revF\_17Nov2015. The library was sequenced with a MinION sequencer. The basecalling was done by the Oxford nanopore technologies's Metrichor cloud service with the *2D Basecalling for SQK-MAP006* version 1.69 application. All the reads (1D and 2D)  $\geq 750$  bp were converted from the native HDF5/FAST5 format to FASTQ by poretools version 0.5.1<sup>58</sup>. Finally, a hybrid assembly of both Illumina and Oxford nanopore technologies reads was performed using SPAdes version 3.7.1<sup>59</sup> with kmer lengths of 21, 33, 55, 77, 99 and 127. The pAsa8 plasmid was recovered in a single contig. To assess the quality of the sequence, the Illumina reads were mapped on the pAsa8 sequence using BWA-MEM<sup>60</sup> and evaluated with Pilon version 1.17<sup>61</sup>. No corrections occurred during step, reinforcing our confidence in the quality of the pAsa8 sequence. This sequence was annotated with the webserver RAST<sup>62</sup> and manually curated. The sequence was analyzed using TAFinder (TADB) to find putative type 2 toxin-antitoxin systems<sup>63</sup>. The complete annotated sequence of pAsa8 was deposited in GenBank under the accession number KX364409.

## References

- Hiney, M. & Olivier, G. In *Fish diseases and disorders, vol III: viral, bacterial and fungal infections* (eds P. T. K. Woo & D. W. Bruno) 341–425 (CAB publishing Oxford, 1999).
- Dallaire-Dufresne, S., Tanaka, K. H., Trudel, M. V., Lafaille, A. & Charette, S. J. Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol* **169**, 1–7, doi: 10.1016/j.vetmic.2013.06.025 (2014).
- Morin, R. Utilisation des antibiotiques pour combattre la furunculose chez l'omble de fontaine gène de l'antibiorésistance chez *Aeromonas salmonicida*. *Laquicole* **15**, 2–6 (2010).
- Piotrowska, M. & Popowska, M. Insight into the mobilome of *Aeromonas* strains. *Front Microbiol* **6**, 494, doi: 10.3389/fmicb.2015.00494 (2015).
- Vincent, A. T. *et al.* Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug resistance, interspecies exchanges, and plasmid reshaping. *Antimicrob Agents Chemother* **58**, 7367–7374, doi: 10.1128/AAC.03730-14 (2014).
- Allmeier, H., Cresnar, B., Greck, M. & Schmitt, R. Complete nucleotide sequence of Tn1721: gene organization and a novel gene product with features of a chemotaxis protein. *Gene* **111**, 11–20 (1992).
- Boyd, D. A., Peters, G. A., Ng, L. & Mulvey, M. R. Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol Lett* **189**, 285–291 (2000).
- Bi, S. *et al.* New variant *Salmonella* genomic island 1-U in *Proteus mirabilis* clinical and food isolates from South China. *J Antimicrob Chemother* **66**, 1178–1179, doi: 10.1093/jac/dkr030 (2011).
- Lei, C. W. *et al.* Molecular characteristics of *Salmonella* genomic island 1 in *Proteus mirabilis* isolates from poultry farms in China. *Antimicrob Agents Chemother* **58**, 7570–7572, doi: 10.1128/AAC.03992-14 (2014).
- Siebor, E. & Neuwirth, C. Emergence of *Salmonella* genomic island 1 (SGI1) among *Proteus mirabilis* clinical isolates in Dijon, France. *J Antimicrob Chemother* **68**, 1750–1756, doi: 10.1093/jac/dkt100 (2013).
- Qin, S. *et al.* Emergence of Extensively Drug-Resistant *Proteus mirabilis* Harboring a Conjugative NDM-1 Plasmid and a Novel *Salmonella* Genomic Island 1 Variant, SGI1-Z. *Antimicrob Agents Chemother* **59**, 6601–6604, doi: 10.1128/AAC.00292-15 (2015).
- Kiss, J., Nagy, B. & Olasz, F. Stability, entrapment and variant formation of *Salmonella* genomic island 1. *PLoS One* **7**, e32497, doi: 10.1371/journal.pone.0032497 (2012).
- Michael, G. B., Hall, R., Fanning, S. & Schwarz, S. In *Salmonella in domestic animals* (eds P. A. Barrow & U. Methner) 120–135 (2013).
- Mulvey, M. R., Boyd, D. A., Olson, A. B., Doublet, B. & Cloeckert, A. The genetics of *Salmonella* genomic island 1. *Microbes Infect* **8**, 1915–1922, doi: 10.1016/j.micinf.2005.12.028 (2006).
- Takaya, A., Watanabe, M. & Yamamoto, T. Organization of tn2610 containing two transposition modules. *Antimicrob Agents Chemother* **50**, 1143–1147, doi: 10.1128/AAC.50.4.1143-1147.2006 (2006).
- Sandaa, R. A. & Enger, O. Transfer in Marine Sediments of the Naturally Occurring Plasmid pRAS1 Encoding Multiple Antibiotic Resistance. *Appl Environ Microbiol* **60**, 4234–4238 (1994).
- Sorum, H., LÅbee-Lund, T. M., Solberg, A. & Wold, A. Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* **47**, 1285–1290 (2003).
- Colston, S. M. *et al.* Bioinformatic genome comparisons for taxonomic and phylogenetic assignments using *Aeromonas* as a test case. *MBio* **5**, e02136, doi: 10.1128/mBio.02136-14 (2014).
- Galardini, M., Biondi, E. G., Bazzicalupo, M. & Mengoni, A. CONTIGuator: a bacterial genomes finishing tool for structural insights on draft genomes. *Source Code Biol Med* **6**, 11, doi: 10.1186/1751-0473-6-11 (2011).
- Dixon, B. A. The biology of antibiotic resistance. *World Aquac* **32**, 63–65 (2001).
- Aoki, T., Egusa, S., Kimura, T. & Watanabe, T. Detection of R factors in naturally occurring *Aeromonas salmonicida* strains. *Appl Microbiol* **22**, 716–717 (1971).



22. McIntosh, D. *et al.* 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. *J Antimicrob Chemother* **61**, 1221–1228, doi: 10.1093/jac/dkn123 (2008).
23. Uhland, C. F. Rapport des activités du Laboratoire d'Ichtyopathologie (1 avril 2004 – 31 mars 2005). 16 pages (Saint-Hyacinthe (Québec), 2005).
24. Johnson, T. J. & Lang, K. S. IncA/C plasmids: An emerging threat to human and animal health? *Mob Genet Elements* **2**, 55–58, doi: 10.4161/mge.19626 (2012).
25. Welch, T. J. *et al.* Multiple antimicrobial resistance in plague: an emerging public health risk. *PLoS One* **2**, e309, doi: 10.1371/journal.pone.0000309 (2007).
26. Doublet, B. *et al.* Complete nucleotide sequence of the multidrug resistance IncA/C plasmid pR55 from *Klebsiella pneumoniae* isolated in 1969. *J Antimicrob Chemother* **67**, 2354–2360, doi: 10.1093/jac/dks251 (2012).
27. Aoki, T., Egusa, S., Ogata, Y. & Watanabe, T. Detection of resistance factors in fish pathogen *Aeromonas liquefaciens*. *J Gen Microbiol* **65**, 343–349, doi: 10.1099/00221287-65-3-343 (1971).
28. Fricke, W. F. *et al.* Comparative genomics of the IncA/C multidrug resistance plasmid family. *J Bacteriol* **191**, 4750–4757, doi: 10.1128/JB.00189-09 (2009).
29. Kim, M. J. *et al.* Complete DNA sequence and analysis of the transferable multiple-drug resistance plasmids (R Plasmids) from *Photobacterium damsela* subsp. *piscicida* isolates collected in Japan and the United States. *Antimicrob Agents Chemother* **52**, 606–611, doi: 10.1128/AAC.01216-07 (2008).
30. Call, D. R. *et al.* blaCMY-2-positive IncA/C plasmids from *Escherichia coli* and *Salmonella enterica* are a distinct component of a larger lineage of plasmids. *Antimicrob Agents Chemother* **54**, 590–596, doi: 10.1128/AAC.00055-09 (2010).
31. Fernandez-Alarcon, C., Singer, R. S. & Johnson, T. J. Comparative genomics of multidrug resistance-encoding IncA/C plasmids from commensal and pathogenic *Escherichia coli* from multiple animal sources. *PLoS One* **6**, e23415, doi: 10.1371/journal.pone.0023415 (2011).
32. Kiss, J. *et al.* The master regulator of IncA/C plasmids is recognized by the *Salmonella* Genomic island SGII as a signal for excision and conjugal transfer. *Nucleic Acids Res* **43**, 8735–8745, doi: 10.1093/nar/gkv758 (2015).
33. Douard, G., Praud, K., Cloeckaert, A. & Doublet, B. The *Salmonella* genomic island 1 is specifically mobilized in trans by the IncA/C multidrug resistance plasmid family. *PLoS One* **5**, e15302, doi: 10.1371/journal.pone.0015302 (2010).
34. Robinson, T. P. *et al.* Antibiotic resistance is the quintessential One Health issue. *Trans R Soc Trop Med Hyg* **110**, 377–380, doi: 10.1093/trstmh/trw048 (2016).
35. O'Neill, J. Tackling Drug-Resistant Infections Globally: final report and recommendations. (2016).
36. Gordon, L. *et al.* Complete sequence of the floR-carrying multiresistance plasmid pAB559 from freshwater *Aeromonas bestiarum*. *J Antimicrob Chemother* **62**, 65–71, doi: 10.1093/jac/dkn166 (2008).
37. Biyela, P. T., Lin, J. & Bezuidenhout, C. C. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci Technol* **50**, 45–50 (2004).
38. Hatosy, S. M. & Martiny, A. C. The ocean as a global reservoir of antibiotic resistance genes. *Appl Environ Microbiol* **81**, 7593–7599, doi: 10.1128/AEM.00736-15 (2015).
39. Wellington, E. M. *et al.* The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. *Lancet Infect Dis* **13**, 155–165, doi: 10.1016/S1473-3099(12)70317-1 (2013).
40. Haba, E. *et al.* Rhamnolipids as emulsifying agents for essential oil formulations: antimicrobial effect against *Candida albicans* and methicillin-resistant *Staphylococcus aureus*. *Int J Pharm* **476**, 134–141, doi: 10.1016/j.ijpharm.2014.09.039 (2014).
41. Krylov, V. N. Bacteriophages of *Pseudomonas aeruginosa*: long-term prospects for use in phage therapy. *Adv Virus Res* **88**, 227–278, doi: 10.1016/B978-0-12-800098-4.00005-2 (2014).
42. Llewellyn, M. S., Boutin, S., Hoseinifar, S. H. & Derome, N. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Front Microbiol* **5**, 207, doi: 10.3389/fmicb.2014.00207 (2014).
43. Reith, M. E. *et al.* The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* **9**, 427, doi: 10.1186/1471-2164-9-427 (2008).
44. Daher, R. K. *et al.* Alteration of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas salmonicida* in stressful conditions. *Vet Microbiol* **152**, 353–360, doi: 10.1016/j.vetmic.2011.04.034 (2011).
45. Trudel, M. V. *et al.* Insertion sequence AS5 (ISAS5) is involved in the genomic plasticity of *Aeromonas salmonicida*. *Mob Genet Elements* **3**, e25640, doi: 10.4161/mge.25640 (2013).
46. Aoki, T., Mitoma, Y. & Crosa, J. H. The characterization of a conjugative R-plasmid isolated from *Aeromonas salmonicida*. *Plasmid* **16**, 213–218 (1986).
47. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792–1797, doi: 10.1093/nar/gkh340 (2004).
48. Altschul, S. F. *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402 (1997).
49. Kears, M. *et al.* Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649, doi: 10.1093/bioinformatics/bts199 (2012).
50. Untergasser, A. *et al.* Primer3—new capabilities and interfaces. *Nucleic Acids Res* **40**, e115, doi: 10.1093/nar/gks596 (2012).
51. Charette, S. J. *et al.* Draft genome sequence of the virulent strain 01-B526 of the fish pathogen *Aeromonas salmonicida*. *J Bacteriol* **194**, 722–723, doi: 10.1128/JB.06276-11 (2012).
52. Vincent, A. T., Boyle, B., Derome, N. & Charette, S. J. Improvement in the DNA sequencing of genomes bearing long repeated elements. *J Microbiol Methods* **107**, 186–188, doi: 10.1016/j.mimet.2014.10.016 (2014).
53. Mascaretti, O. A. *Bacteria versus antibacterial agents: an integrated approach*. (ASM Press, 2003).
54. Vincent, A. T. *et al.* Draft genome sequences of two *Aeromonas salmonicida* subsp. *salmonicida* isolates harboring plasmids conferring antibiotic resistance. *FEMS Microbiol Lett* **362**, doi: 10.1093/femsle/fnv002 (2015).
55. Margulies, M. *et al.* Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376–380, doi: 10.1038/nature03959 (2005).
56. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120, doi: 10.1093/bioinformatics/btu170 (2014).
57. Coil, D., Jospin, G. & Darling, A. E. A5-misec: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics* **31**, 587–589, doi: 10.1093/bioinformatics/btu661 (2015).
58. Loman, N. J. & Quinlan, A. R. Poretools: a toolkit for analyzing nanopore sequence data. *Bioinformatics* **30**, 3399–3401, doi: 10.1093/bioinformatics/btu555 (2014).
59. Bankevich, A. *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* **19**, 455–477, doi: 10.1089/cmb.2012.0021 (2012).
60. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754–1760, doi: 10.1093/bioinformatics/btp324 (2009).
61. Walker, B. J. *et al.* Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* **9**, e112963, doi: 10.1371/journal.pone.0112963 (2014).

62. Overbeek, R. *et al.* The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* **42**, D206–D214, doi: 10.1093/nar/gkt1226 (2014).
63. Shao, Y. *et al.* TADB: a web-based resource for Type 2 toxin-antitoxin loci in bacteria and archaea. *Nucleic Acids Res* **39**, D606–D611, doi: 10.1093/nar/gkq908 (2011).
64. Vincent, A. T. *et al.* Antibiotic resistance due to an unusual ColE1-type replicon plasmid in *Aeromonas salmonicida*. *Microbiology*, doi: 10.1099/mic.0.000286 (2016).
65. LAbee-Lund, T. M. & Sorum, H. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. *Plasmid* **47**, 172–181 (2002).
66. Adams, C. A., Austin, B., Meaden, P. G. & McIntosh, D. Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. *Appl Environ Microbiol* **64**, 4194–4201 (1998).
67. LAbee-Lund, T. M. & Sorum, H. Class 1 integrons mediate antibiotic resistance in the fish pathogen *Aeromonas salmonicida* worldwide. *Microb Drug Resist* **7**, 263–272, doi: 10.1089/10766290152652819 (2001).
68. LAbee-Lund, T. M. & Sorum, H. Functional Tn5393-like transposon in the R plasmid pRAS2 from the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida* isolated in Norway. *Appl Environ Microbiol* **66**, 5533–5535 (2000).

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## Author Contributions

Conceived and designed the experiments: M.V.T., A.T.V., N.D. and S.J.C. Performed the experiments: M.V.T., A.T.V., S.A.A. and M.L. Analyzed the data: M.V.T., A.T.V., S.A.A. and S.J.C. Contributed reagents/materials/analysis tools: N.D., A.I.C. and S.J.C. Wrote the paper: M.V.T., A.T.V., N.D., A.I.C. and S.J.C. All authors reviewed the manuscript.

## Additional Information

**Accessions code:** The complete annotated sequences of M15879-11 and pAsa8 were deposited in GenBank under the accession numbers LAIS00000000 and KX364409, respectively.

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