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Expansion of the tetracycline resistome in *Aeromonas salmonicida* with a *tet*(D) gene found in plasmids pAsa-2900 and pAsa-2900b

Sarah B. Girard[1,2], Pierre-Étienne Marcoux[1,2],
Valérie E. Paquet[1,2], Sahra Zoubaï[1,2], Thuy Nhu Vy Can[1],
Sabrina A. Attéré[1,2], Antony T. Vincent[1,2,3]
and Steve J. Charette[1,2]*

[1]Institut de biologie intégrative et des systèmes (IBIS), Université Laval, Quebec, QC, Canada,
[2]Département de biochimie, de microbiologie et de bio-informatique, Faculté des sciences et de
génie, Université Laval, Quebec, QC, Canada, [3]Département des sciences animales, Faculté des
sciences de l'agriculture et de l'alimentation, Université Laval, Quebec, QC, Canada

Plasmids carrying antibiotic resistance genes occur frequently in *Aeromonas salmonicida* subsp. *salmonicida*, an aquatic pathogen. In this study, we describe three new plasmids found in strains of *A. salmonicida* subsp. *salmonicida* from Québec, Canada: pAsa-2358, pAsa-2900, and pAsa-2900b. The last two plasmids have a *tet*(D) resistance gene against tetracycline, which has not been previously found in *A. salmonicida*. The only difference between these two plasmids is the presence of a florfenicol resistance gene (*floR*) surrounded by two copies of the same insertion sequence, indicating that pAsa-2900 and pAsa-2900b are plasmid variants. All three plasmids represent an issue for aquaculture because they bear resistance genes against antibiotics used to treat infected fish: *floR* and *tet*(A) for pAsa-2358; *floR*, *sul2*, and *tet*(D) for pAsa-2900; and *sul2* and *tet*(D) for pAsa-2900b. Conjugation assays confirmed that the three plasmids are capable of conjugation with one *A. salmonicida* mesophilic strain, one *A. hydrophila* strain, and *Escherichia coli* DH5α; except for pAsa-2358 that is not able to conjugate into *E. coli* DH5α. Based on the results of antibiotic resistance assays, the *tet*(D) gene provides a greater resistance to tetracycline than the *tet*(A) gene found in many *A. salmonicida* subsp. *salmonicida* plasmids. This is the case whether the plasmids are in their parental *A. salmonicida* subsp. *salmonicida* strains or in *Aeromonas* sp. transconjugates. The presence of these three plasmids in *A. salmonicida* subsp. *salmonicida* strengthens the role of this bacterium as a reservoir for antibiotic resistance genes.

Introduction

The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is a major fish pathogen responsible for significant economic losses in the aquaculture industry worldwide (Austin and Austin, 2016). *A. salmonicida* subsp. *salmonicida* causes furunculosis, a disease with high morbidity and mortality rates, particularly affecting farmed salmonids (Toranzo et al., 2005; Austin and Austin, 2016). The disease manifests in two forms: chronic and acute. While both forms can be fatal, the acute form can kill its host within two to three days (Bernoth, 1997; Burr et al., 2005). The disease spreads rapidly in fish farms, especially under unfavorable conditions such as low oxygen levels or overcrowding in fishponds (Barton and Iwama, 1991; Ashley, 2007; Du et al., 2015). One of the bacterium's virulence factors is the Type Three Secretion System (TTSS), which translocates effector toxins directly into the host's cell cytoplasm (Reith et al., 2008; Vanden Bergh and Frey, 2014).

In Canada, and particularly in the province of Québec, antibiotic treatments for *A. salmonicida* subsp. *salmonicida* infections are widely used. In Québec's salmonid farms, florfenicol is the predominant antibiotic, constituting approximately 95% of prescribed treatments for combating furunculosis, followed by tetracycline. Occasionally, treatments incorporating sulfonamide and trimethoprim are also employed (Farley, pers. comm.). Veterinarians' statements and prescriptions are required to be able to purchase antibiotics for furunculosis treatment. By the time this process is completed, and the treatment is started, a significant number of fish could already have died from the disease.

A. salmonicida subsp. *salmonicida* contains a very diverse plasmidome (Vincent et al., 2021), including a large variety of plasmids that carry antibiotic resistance genes (ARGs), especially in Canadian isolates (McIntosh et al., 2008; Vincent et al., 2014; Tanaka et al., 2016; Trudel et al., 2016; Massicotte et al., 2019; Vaillancourt et al., 2021; Vincent et al., 2021; Fournier et al., 2022). Some strains even bear plasmids that make them resistant to all antibiotics authorized by Canadian authorities for aquaculture (Trudel et al., 2016). Tetracycline resistance is increasing in Québec (Fournier et al., 2022), and various genes have been observed in *A. salmonicida* subsp. *salmonicida* in a myriad of plasmids (Table 1). Since no other options available on the market are efficient enough to treat furunculosis at the moment—one example being auto-administered vaccines, which are expensive and complex to administer—antibiotics remain the main solution (Dallaire-Dufresne et al., 2014; Origgi et al., 2017; Vincent et al., 2019). If resistance occurs, it can be even harder for fish farms to recover from the losses. For instance, between 2009 and 2013 in Québec, about 30% of *A. salmonicida* subsp. *salmonicida* strains characterized were resistant to all approved antibiotics for aquaculture in Canada (Trudel et al., 2016).

Plasmid variants occur quite frequently in *A. salmonicida* subsp. *salmonicida*. The pRAS3 plasmid family is a good example (Table 1), where the number of small specific repeated sequences and the presence or absence of an insertion sequence (IS) can explain the diversity in this family (Fournier et al., 2022). Three variants of plasmid pAsa4 have also been described (Tanaka et al.,

2016). In this case, the variations between the plasmids are more complex and result, among other things, in a different ARG content between the plasmids. Moreover, variants of plasmids found in other bacterial species have been observed in *A. salmonicida* subsp. *salmonicida*. Examples include pSN254b, which is an IncA/C plasmid and a variant of a plasmid found in *Salmonella enterica* (McIntosh et al., 2008).

Many plasmids found in various *A. salmonicida* subspecies that carry ARGs have been shown to be transferrable by conjugation to other bacteria from the same genus or to other genera (Sandaa and Enger, 1996; Vincent et al., 2014; Piotrowska and Popowska, 2015; Tanaka et al., 2016; Massicotte et al., 2019; Vincent et al., 2021; Marcoux et al., 2023a, b). The presence of *tra* genes suggests the ability of a plasmid to conjugate, but this is not always true; for example, pAsa4c is not conjugable in the same conditions tested for other members of the pAsa4 family, although it does bear several *tra* genes (Tanaka et al., 2016). Thus, experimental demonstration of conjugation activity is always necessary and relevant when characterizing a plasmid carrying *tra* genes.

Transposons and integrons were detected in plasmids found in *A. salmonicida* subsp. *salmonicida*. Typically, these genetic elements bear ARGs and other genes that provide diverse advantages to the bacteria (Piotrowska and Popowska, 2015). The pAsa4 plasmid variants contain transposon Tn*21* with an In*2* integron (Tanaka et al., 2016), and a similar Tn*21* is also found on pSN254b; this

TABLE 1 Tetracycline resistance genes found in resistance-bearing plasmids of *A. salmonicida* subsp. *salmonicida* known to date.

Plasmid	Tetracycline resistance gene	Reference
pAsa10	*tet*(A)	(Attéré et al., 2017)
pSN254b	*tet*(A)	(Vincent et al., 2014)
pRAS1b	*tet*(A)	(Vaillancourt et al., 2021)
pAsa8	*tet*(A) and *tet*(G)	(Trudel et al., 2016)
pAsa5–3432	*tet*(A) and *tet*(G)	(Massicotte et al., 2019)
pRAS3.1	*tet*(C)	(L'Abée-Lund and Sørum, 2002)
pRAS3.2	*tet*(C)	(L'Abée-Lund and Sørum, 2002)
pRAS3.3	*tet*(C)	(Vincent et al., 2014)
pRAS3.4	*tet*(C)	(Fournier et al., 2022)
pRAS3.5	*tet*(C)	(Fournier et al., 2022)
pRAS3–3432	*tet*(C)	(Massicotte et al., 2019)
pRAS3–3759	*tet*(C)	(Fournier et al., 2022)
pAsa4	*tet*(E) also known as *tetA*(E)	(Reith et al., 2008)
pAsa4b	*tet*(E) also known as *tetA*(E)	(Tanaka et al., 2016)
pS121–1a	*tet*(E) also known as *tetA*(E)	(Vincent et al., 2021)
pS121–1b	*tet*(E) also known as *tetA*(E)	(Vincent et al., 2021)
pAB5S9b	*tet*(H)	(Vincent et al., 2014)

transposon also carries mercury resistance genes (Vincent et al., 2014). Another frequent transposon in *A. salmonicida* subsp. *salmonicida* is Tn*1721*. Different complete and truncated forms of this transposon have been observed in plasmids such as pRAS1, pAsa8, or pAsa10 (Attéré et al., 2017; Vincent et al., 2021).

In this study, using high-throughput sequencing technologies, conjugation assays, PCR genotyping, and antibiotic susceptibility tests, we investigated three new plasmids—pAsa-2359, pAsa-2900, and pAsa-2900b—which bear ARGs and are found in *A. salmonicida* subsp. *salmonicida* strains isolated in 2021 and 2022 in Québec (Canada). The analyses of these new plasmids revealed, for the first time, the presence of the *tet*(D) gene in this bacterial subspecies; this gene is responsible for tetracycline resistance and provides a higher resistance than *tet*(A), which is present in the other plasmid described in this study.

Materials and methods

Bacterial strains

Twenty-eight strains were isolated from different types of fish in 2021 and seven strains were isolated in 2022; these were all included in our study. All the strains were provided by the Centre de diagnostic vétérinaire de l'Université de Montréal (CDVUM) and the Direction générale de l'expertise en santé et bien-être des animaux (DGESBEA, ministère de l'agriculture, des pêcheries et de l'alimentation du Québec (MAPAQ)). A detailed list of these strains is presented in Table 2. All strains were isolated from different tissues samples from infected fish, including kidney, liver, skin, or muscle.

Strains SHY21–2358, SHY21–2900, and SHY21–3098 were used in all experiments as representatives of strains bearing the plasmids characterized in this study. In PCR analyses, strain *A. salmonicida* subsp. *salmonicida* 01-B526 was used as a negative bacterial control. In conjugation assays, the three plasmid-bearing strains mentioned above were used as donor strains, while *A. hydrophila* strain HER1210, *A. salmonicida* subsp. *salmonicida* mesophilic strain 19-K308, and *Escherichia coli* strain DH5α were used as recipient bacteria. Minimum inhibitory concentration (MIC) assays were also conducted with the same donor and recipient strains, as well as with the transconjugants obtained from conjugation assays. To provide additional information on tetracycline resistance, an additional transconjugant from past experiments was tested (Vaillancourt et al., 2021). This strain bears pRAS1b, which possesses one tetracycline resistance gene and is found in a comparable host bacterium, *A. hydrophila* HER1210. Details on this additional strain are available in Table 3.

PCR analyses

DNA lysates of each strain were obtained using a previously described protocol (Attéré et al., 2017). PCR analyses were first performed to detect the presence of ARGs against chloramphenicol/florfenicol (*cat* and *floR*), sulfonamide (*sul1* and *sul2*), and tetracyclines (*tet*(A), *tetA*(C), *tetA*(E), *tet*(H), *tet*(G)) in the strains of interest using a previously described multiplex PCR approach (Trudel et al., 2016) before genomic sequencing (see the primer description in Supplementary Table S1). PCR analyses were also performed to detect plasmids previously detected in this region (pAsa1, pAsa2, pAsa3, pAsal1, pAsa5, pAsa8, pAsa9, pRAS3, and pSN254b) (Vincent et al., 2014; Tanaka et al., 2016; Trudel et al., 2016; Fournier et al., 2022). As a negative control, *A. salmonicida* subsp. *salmonicida* strain 01-B526 was used. To investigate the ARGs and plasmids, strains from our laboratory collection, in which each ARG or plasmid had already been characterized, were used as positive controls.

DNA extraction

Total genomic DNA of strains SHY21–2358, SHY21–2900, and SHY21–3098 was extracted using DNeasy Blood and Tissue kits (Qiagen, Montreal, QC, Canada) with the addition of an RNase A treatment step (20 μg/mL, Ambion, ThermoFisher, Mississauga, ON, Canada) according to the manufacturer's protocol.

Sequencing, sequence assembly and analyses

Sequencing libraries were prepared from purified bacterial DNA using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and the sequencing was performed using a MiSeq instrument system (Illumina, San Diego, CA, USA) at the Plateforme d'Analyse Génomique of the Institut de Biologie Intégrative et des Systèmes (Université Laval, Quebec City, QC, Canada). The DNA of strain SHY21–2900 was also sequenced by Plasmidsaurus (Eugene, OR) using Nanopore PromethION.

Prior to the assembly, reads were trimmed with Trimmomatic version 0.39 (Bolger et al., 2014). FastQC version 0.11.5 was used to assess the quality of the reads before assembly (Andrews, 2010). The sequencing reads were *de novo* assembled using Shovill version 1.1.0, a SPAdes-based assembler (Prjibelski et al., 2020). CONTIGuator version 2.7.5 was used with default parameters for mapping the contigs on the reference genome of strain A449 (NC_009348.1) (Galardini et al., 2011). The unmapped contigs were screened using BLAST (Altschul et al., 1990) against the nr/nt database of the NCBI. MOB-suite version 3.1.4 was used to determine which unmapped contigs were capable of circularization, establishing the contigs of interest for each strain after excluding contigs that mapped with *A. salmonicida* subsp. *salmonicida* pAsa5 plasmids and small cryptic plasmids of this subspecies (Attéré et al., 2017).

ABRicate version 1.0.1 (github.com/tseemann/ABRicate) with the ARG NCBI database (Feldgarden et al., 2019) was used to determine which resistance genes were present on the plasmid sequences. All the contigs of interest were annotated using Prokka version 1.14.6 (Seemann, 2014). The plasmid sequences were then further characterized, annotated, and checked manually using

TABLE 2 A. salmonicida subsp. salmonicida strains isolated and analyzed in this study.

Strain name	Antibiogram results[a,b]			Resistance gene detected	Plasmids detected by PCR											
	Tetracycline	Florfenicol	Sulfo-namide		pAsa1	pAsa2	pAsa3	pAsaI1	pAsa5	pAsa8	pAsa9	pRAS3[c]	pSN254b	pAsa-2358	pAsa-2900	pAsa-2900b
SHY21–2358	L	L	Information non-provided	floR, tet(A)	●	●		●	●					●		
SHY21–2900	R	R		sul2, floR, tet(D)	●	●	●		●						●	
SHY21–3098	R	S		sul2, tet(D)	●	●	●		●							●
SHY21–2476	R	S		tet(C)	●	●	●	●	●		●	●				
SHY21–2359	L	L		sul1, floR, tet(A), tet(G)	●	●	●	●	●	●						
SHY21–3064	S	S		tet(C)	●	●	●	●	●		●	●				
SHY21–3120	L	L		sul1, sul2, floR, tet(A)	●	●	●		●		●		●			
SHY21–3158	S	S		sul1, floR, tet(A), tet(G)	●	●	●	●	●	●						
SHY21–3372	S	S		tet(C)	●	●	●	●	●		●	●				
SHY21–3653	S	R		floR, tet(A)	●	●	●	●	●					●		
SHY21–3763	R	S		tet(C)	●	●	●	●	●		●	●				
SHY21–5243	S	L		sul1, sul2, floR, tet(A)	●	●	●		●		●		●			
SHY21–2425	R	L		sul2, floR, tet(D)	●	●	●		●						●	
SHY21–2450	R	R		sul2, floR, tet(D)	●	●	●	●	●						●	
SHY21–2998	R	S		sul2, tet(D)	●	●	●		●							●
SHY21–3290	R	R		sul2, floR, tet(D)	●	●	●		●						●	
SHY21–4310	R	S		sul2, floR, tet(D)	●	●	●		●						●	
SHY21–1627	S	S		-	●	●	●	●	●							
SHY21–2026	S	S		–	●	●	●	●	●							
SHY21–2780	S	S		–	●	●	●	●	●							
SHY21–3083	S	S		–	●	●	●	●	●							

(Continued)

TABLE 2 Continued

Strain name	Antibiogram results[a,b]			Resistance gene detected	Plasmids detected by PCR											
	Tetracycline	Florfenicol	Sulfo-namide		pAsa1	pAsa2	pAsa3	pAsaI1	pAsa5	pAsa8	pAsa9	pRAS3[c]	pSN254b	pAsa-2358	pAsa-2900	pAsa-2900b
SHY21–2759	S	S		–	■	■	■	■	■							
SHY21–3490	S	S		–	■	■	■		■							
SHY21–3526	S	S		–	■	■	■		■							
SHY21–3909	S	S		–	■	■	■		■							
SHY21–4417	S	S		–	■	■	■		■							
SHY21–4462	S	S		–	■	■	■	■	■							
SHY21–4557	S	S		–	■	■	■	■	■							
SHY22–5948	R	S	S	tet(C)	■	■	■	■	■		■	■				
STF22–2474	R	R	S	sul2, floR, tet(D)	■	■	■		■						■	
SHY22–1380	S	S	S	–	■	■	■	■	■							
SHY22–3656	S	S	S	–	■	■	■	■	■							
STF22–3084	S	S	S	-	■	■	■	■	■							
STF22–3310	S	S	S	-	■	■	■	■	■							

*: Black indicates the presence of the plasmid in the strain, white indicates the absence of the plasmid in the strain.

a: Provided by the diagnostic service.

b: R is used for Resistant, S is used for Sensitive and L is used for Limit.

c: The variants of pRAS3 were not determined.

TABLE 3 Other strains used in this study.

Strain name	Species	Lifestyle	Commentary	Reference
01-B526	*A. salmonicida* subsp. *salmonicida*	Psychrophilic	Negative control for minimum inhibitory concentration experiments	(Charette et al., 2012)
HER1210	*A. hydrophila*	Mesophilic	Recipient strain for conjugation experiments	ATCC 7966
19-K308	*Aeromonas* sp.	Mesophilic	Recipient strain for conjugation experiments	(Attéré et al., 2023)
DH5α	*Escherichia coli*	Mesophilic	Recipient strain for conjugation experiments	ATCC 29552
HER1210 + pRAS1b	*A. hydrophila*	Mesophilic	Strain used to compare the efficiency of tetracycline resistance	(Vaillancourt et al., 2021)

BLAST protein database version 2.14.0 (Altschul et al., 1990), and sequences were visualized on Artemis version 18.2.0 (Carver et al., 2011). PCR analyses were conducted to confirm what was visualized on Artemis. When confirming the identity of the plasmids by PCR, strains SHY21–2358, SHY21–2900 and SHY21–3098 were used for pAsa-2358, pAsa-2900 and pAsa-2900b, respectively as positive controls. *A. salmonicida* subsp. *salmonicida* strain 01-B526 was used as a negative control, as this strain does not carry the new plasmids. Finally, circularization of the plasmid maps was done using SnapGene version 7.1 (https://www.snapgene.com).

For strain SHY21–2900, Illumina reads were filtered using Fastp version 0.23.4 (Chen et al., 2018) with default parameters, while Nanopore reads were filtered using Filtlong version 0.2.1 (https://github.com/rrwick/Filtlong), retaining the best 90% of reads above 1000 bp or until only 500 Mbp remained. A hybrid assembly was then performed using Unicycler version 0.5.0 (Wick et al., 2017).

Sequence comparison analyses were conducted using the National Center for Biotechnology Information (NCBI) BLAST tools. These analyses utilized the Nucleotide collection (nr/nt) and Whole Genome Shotgun (wgs) databases. We only included results with a coverage of over 80% and an identity greater than 95%. Plasmid comparison visualizations were done with EasyFig version 2.2.2 (Sullivan et al., 2011), and Inkscape version 1.3.2 was used for the finalization of the plasmid maps (Inkscape, 2020). All three plasmid sequences were deposited in DDBJ/ENA/Genbank under accession number PP592929 (pAsa-2358), PP592930 (pAsa-2900), and PP592931 (pAsa-2900b).

Bacterial conjugation assays

Bacterial conjugation assays were performed as previously described (Tanaka et al., 2016) to transfer all three plasmids from the donor strains (SHY21–2358, SHY21–2900, and SHY21–3098) to *A. hydrophila* strain HER1210 (ATCC 7966), *A. salmonicida* mesophilic strain 19-K308 (Attéré et al., 2023), and *E. coli* strain DH5α (ATCC 29552). All three donor strains were pre-cultivated in 10 mL of Tryptic Soy Broth (TSB) (Wisent, St-Bruno, QC, Canada) at 18°C overnight with shaking at 200 revolutions per minute (rpm). All three recipient strains were pre-cultivated in 10 mL of TSB also but were incubated at 37°C overnight with shaking at 200 rpm. For each conjugation experiment, cultures of donor and recipient cells (1 mL of each) were harvested by centrifugation at

17,200 x g for 1 minute, suspended in 20 µL of TSB, mixed together, and spotted on tryptic soy agar (TSA) (Wisent, St-Bruno, QC, Canada) without selection for 24h at 18°C. Afterwards, the culture was suspended in TSB, diluted up to 10^{-3} and each dilution was plated on TSA with 10 µg/mL of tetracycline. Preliminary tests indicated that 10 µg/mL tetracycline was sufficient as a selection marker with the recipient strains used. Plates were incubated overnight at 37°C to select against *A. salmonicida* subsp. *salmonicida* which is psychrophilic (Vincent et al., 2016). Recipient strains are not capable of growing in the presence of tetracycline, unless they acquire the plasmid of interest. Large colonies were picked and plated on new TSA plates with selection. PCR analyses were performed on the lysates of colonies plated to detect the presence of the three plasmids in transconjugants using the primers described in Supplementary Table S1. The absence of *A. salmonicida* subsp. *salmonicida* among the transconjugants was confirmed by PCR with primers that targeted the *tapA* gene (Ebanks et al., 2006). If the *tapA* gene was detected in the lysates, it indicated that the *A. salmonicida* subsp. *salmonicida* donor strain was still present, suggesting that the conjugation was unsuccessful and did not select only the recipient strain (Ebanks et al., 2006; Tanaka et al., 2016). Primers targeting the backbone of each plasmid of interest were used to confirm that the recipient strains possessed the new plasmid. Primers for 16S rRNA gene were used to confirm that each lysate contained bacterial cell material (Marchesi et al., 1998). In each PCR trial, *A. salmonicida* subsp. *salmonicida* strain 01-B526 was used as a positive control for 16S rRNA gene and as a negative control for the presence of the conjugated plasmid. Strains SHY21–2358, SHY21–2900, and SHY21–3098 were used as positive controls for the plasmids pAsa-2358, pAsa-2900, and pAsa-2900b, respectively. Conjugation assays were performed twice.

Assessment of the MIC

The bacteria were inoculated on TSA directly from frozen stocks and were grown at 18°C for 72 h or 37°C for 24 h, depending on the nature of the strains used. Selective medium (TSA containing 10 µg/mL tetracycline) was inoculated, from frozen stocks, with the potential transconjugants obtained in the conjugation assays. The assay was performed at least twice. MIC was assessed on solid media (TSA). Nine concentrations were

tested: 256, 128, 64, 32, 16, 8, 4, and 2 μg/mL, as well as a control without tetracycline but with ethanol (1.6%) to assure that the compound present in the tetracycline stock did not inhibit bacterial growth. Each bacterium of interest was streaked on the selective media with sterile toothpicks in a single line. Petri dishes were incubated at 37°C for 24 h, except for the parental donor strains that were incubated at 18°C for 24 h. After the incubation time, Petri dishes were analyzed to determine at which concentration bacterial growth was inhibited. Parental donor strains and recipient strains were used as controls to ensure that they could not grow with tetracycline without the presence of acquired plasmids of interest in this study. *A. salmonicida* subsp. *salmonicida* strain 01-B526 was also used as a negative control for the antibiotic selection. The analyses were performed in duplicate to ensure reproducibility.

Results and discussion

A total of 35 strains were isolated in 2021 and 2022 and were screened for various genetic elements, such as the presence of resistance genes and plasmids. Among these new strains, unusual profiles of resistance genes were found in comparison to what is normally observed in *A. salmonicida* subsp. *salmonicida* strains from the province of Québec (Table 2). In particular, strains displaying the presence of *floR* and *tet*(A), or *floR* and *sul2*, or *sul2* alone have never been previously observed. In addition, an antibiogram analysis, carried out on eight strains by the diagnostic service providing the strains, suggested resistance to tetracycline. However, the usual PCR assay for *tet* genes did not reveal the responsible determinants (Table 2). Consequently, these results suggest that some of the strains isolated in 2021 and 2022 potentially carry new plasmids or new ARGs.

To investigate the presence of these new plasmids, we selected strains SHY21–2358, SHY21–2900, and SHY21–3098, all isolated in 2021 from the kidney of a dead furunculosis-infected juvenile brook char (*Salvelinus fontinalis*) in the province of Québec, as representatives of the strains displaying these unusual resistance gene profiles (Table 2). The genomic DNA of these strains was sequenced using a short-read approach. For strain SHY21–2900, long-read sequencing data was also required to successfully assemble the sequence of the new plasmid present in this strain. The plasmids pAsa-2358 (155,767 bp), pAsa-2900 (153,415 bp), and pAsa-2900b (149,430 bp) were identified in strains SHY21–2358, SHY21–2900, and SHY21–3098, respectively.

Antibiotics tested by the diagnostic service were coherent with ARGs identified in the newly discovered plasmids. Tetracycline resistance detected by the diagnostic service could be explained by the presence of the gene *tet*(D) found on the pAsa-2900 and pAsa-2900b plasmids (Table 2). After identification of the gene involved in tetracycline resistance by bioinformatics using ABRicate and designing new primers (Supplementary Table S1), the presence of this tetracycline resistance gene was confirmed in strains SHY21–2900 and SHY21–3098. It was also confirmed in all the other 2021 and 2022 strains displaying tetracycline resistance that could not be detected using primers detecting *tet* genes previously reported in *A. salmonicida* subsp. *salmonicida* (Table 2). After screening different

strains from our laboratory's collection that were isolated before 2021, we confirmed that pAsa-2900, pAsa-2900b, and pAsa-2358 were all absent from previous strains; therefore, these represent new plasmids in the *salmonicida* subspecies. By possessing resistance genes to florfenicol and tetracycline, the pAsa-2358 and pAsa-2900 plasmids join the group of the most problematic plasmids for fish farming in Quebec with the pAsa8 plasmids and more particularly pSN254b (Vincent et al., 2014; Trudel et al., 2016) since these antibiotics are the most used in this agri-food sector (Fournier et al., 2022). It will be necessary for veterinarians to be even more attentive to the risks of resistance to these antibiotics and to therapeutic failures.

By comparing *tet*(D) resistance gene sequences with the NCBI BLAST tool using the nr/nt (nucleotide collection) and wgs (whole-genome shotgun contigs) databases, there is no similarity found with any documented *A. salmonicida* subsp. *salmonicida* sequence registered in the databases. Also, no significant similarity is found when comparing the *tet*(D) resistance gene sequence with all *A. salmonicida* subspecies. When searching for *Aeromonas* taxa in the nr/nt database, the *tet*(D) resistance gene was found in *A. veronii, A. hydrophila*, and *A. liquefaciens*. When searching only for *Aeromonas* taxa in the wgs database, the *tet*(D) sequence was also previously identified in two strains of *A. veronii*: L924 and L975. The *tet*(D) gene was also reported in *A. hydrophila* and *A. caviae* isolates from Turkey (Capkin et al., 2017). Our analysis suggests that this is the first report of a *tet*(D) resistance gene in *A. salmonicida* species.

Plasmid maps of pAsa-2358, pAsa-2900, and pAsa-2900b are shown in Figures 1–3, respectively. The plasmid pAsa-2358 is a large plasmid with many backbone genes, as well as *tra* genes that are of interest in conjugation assays. What is most interesting is that this plasmid contains a putative composite transposon that includes the *floR* resistance gene (highlighted by the yellow line in Figure 1). This putative composite transposon is bordered by an identical IS on both sides. The plasmid pAsa-2900 also contains the same putative composite transposon bearing the *floR* resistance gene (highlighted by the yellow line in Figure 2). Plasmid pAsa-2358 shares no other high-similarity characteristics with pAsa-2900 and pAsa-2900b (Figure 4).

This putative *floR*-containing composite transposon is the sole difference observed between plasmids pAsa-2900 and pAsa-2900b; the latter does not contain this hypothetical transposon and only contains one copy of IS*91* (highlighted by the yellow line Figures 3 and 4). Therefore, these two plasmids can be considered as variants since the loss of the composite transposon of pAsa-2900 could directly generate the plasmid pAsa-2900b. Interestingly, transposon instability has been previously observed for plasmid pAsa-2939, an unrelated plasmid to those described in this study that was also found in *A. salmonicida* subsp. *salmonicida* (Marcoux et al., 2023a). In subsequent analyses, it will be interesting to verify whether the same phenomenon occurs with pAsa-2900. Another putative composite transposon is observed in pAsa-2900 and pAsa-2900b; two ISs (IS*6*) surround the *tet*(D) resistance gene (highlighted by the pink line in Figures 2 and 3).

BLAST analyses performed against the NCBI nr/nt database in February 2024 using the entire pAsa-2358 plasmid sequence
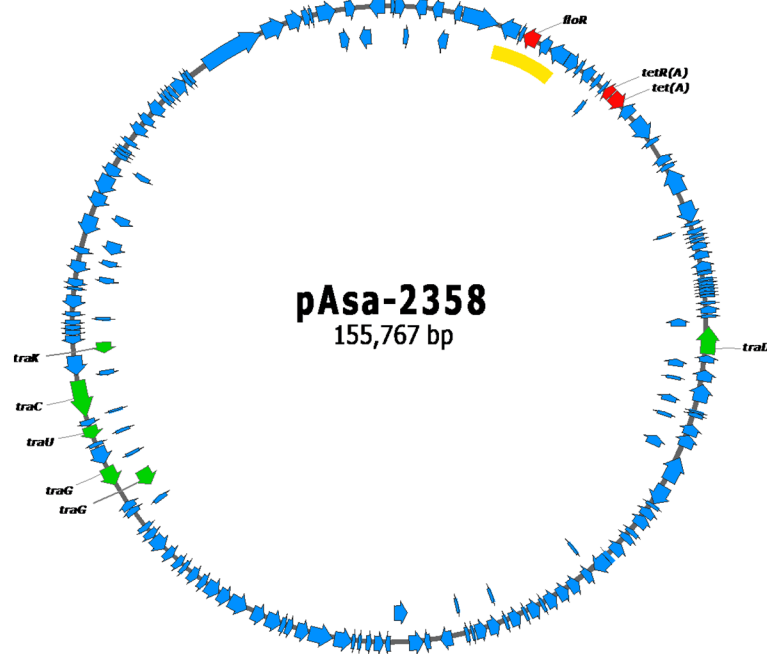
FIGURE 1
Gene map of the pAsa-2358 plasmid. The red genes code for antibiotic resistance proteins, the green genes for transfer proteins, and the blue genes for other proteins. The yellow line highlights the putative composite transposon containing the *floR* resistance gene.

revealed that two plasmids, pAER-F909 (NZ_CP026221.1) and pQZ124 (CP121101.1), shared a high sequence identity with pAsa-2358. No hits with more than 80% query coverage were identified in the analyses on the WGS database. Plasmids pAER-F909 (NZ_CP026221.1) and pQZ124 (CP121101.1) are carried by *Aeromonas* sp. strain ASNIH4 and *A. hydrophila* strain QZ124, respectively. The similar regions between pAsa-2358 and these two plasmids correspond to the plasmid backbone and the transfer
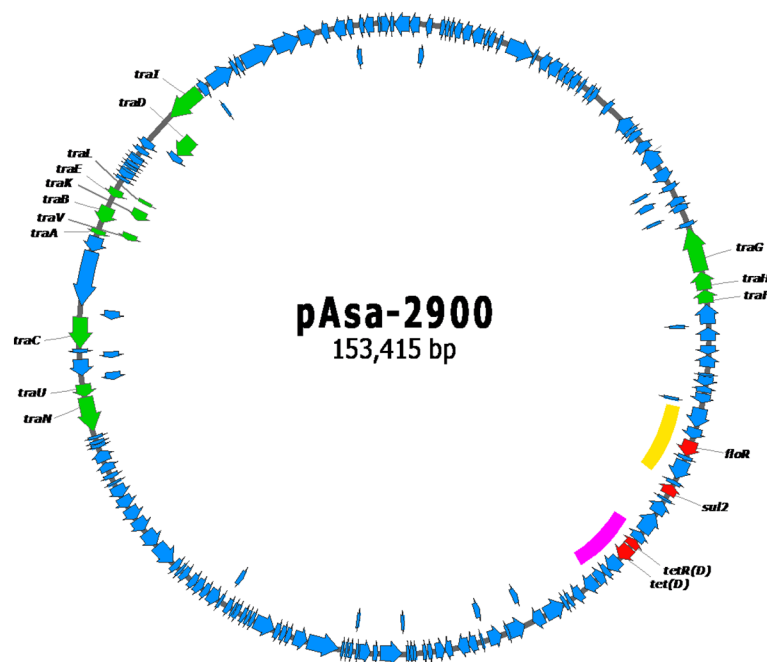

FIGURE 2
Gene map of the pAsa-2900 plasmid. The red genes code for antibiotic resistance proteins, the green genes for transfer proteins, and the blue genes for other proteins. The yellow line highlights the putative composite transposon containing the *floR* resistance gene. The pink line highlights the putative composite transposon containing the *tet*(D) resistance gene.
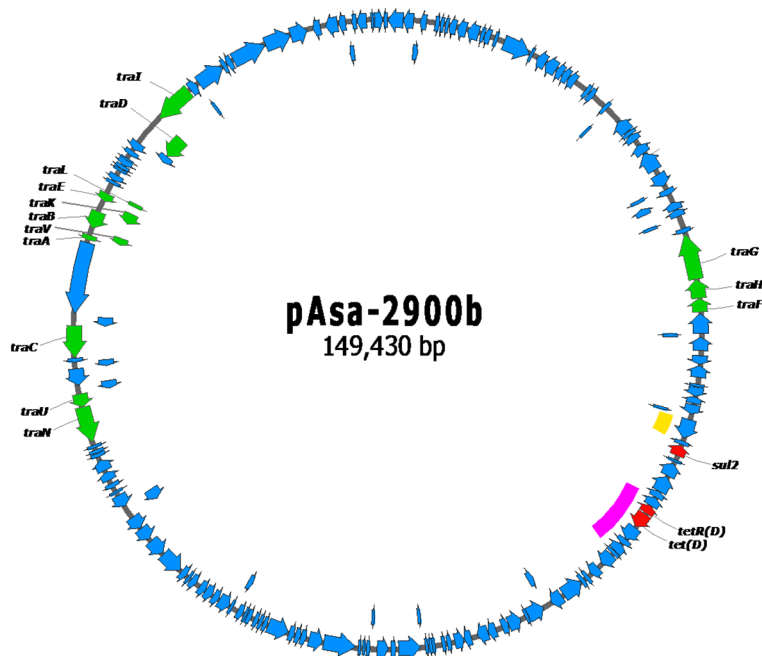
FIGURE 3
Gene map of the pAsa-2900b plasmid. The red genes code for antibiotic resistance proteins, the green genes for transfer proteins, and the blue genes for other proteins. The yellow line highlights the only copy of IS*91*, part of the putative composite transposon in the variant pAsa-2900, here lacking all other genes included. The pink line highlights the putative composite transposon containing the *tet*(D) resistance gene.

genes, while the regions carrying the resistance genes are highly divergent (Supplementary Figures S1, S2). Furthermore, plasmids pAER-F909 and pQZ124 are significantly larger in size than pAsa-2358, as clearly illustrated in Supplementary Figures S1, S2. It cannot be excluded that these three plasmids have a common ancestor which would have diverged over time depending on the host bacteria through the acquisition, among other things, of antibiotic resistance genes by distinct mechanisms.

When considering pAsa-2900 and pAsa-2900b variants, BLAST analyses (February 2024) on the nr/nt and WGS databases revealed that these two plasmids belong to a group of plasmids found in the *Aeromonas* genus, particularly the *salmonicida* species, which share a very similar backbone (over 95% identity) while presenting divergent regions bearing distinct ARGs. pAsa-2900 and pAsa-2900b are the only plasmids in this group to carry the *tet*(D) gene. Within this group, notable members include the variants of the pAsa4 plasmid family, as well as the pAsmA (NZ_CP050188.1) and pA (NZ_CP049831.1) plasmids found in *A. salmonicida* subsp. *masoucida* strain RZ6S-1 which show the highest identity with pAsa-2900 and pAsa-2900b. Here too, the shared regions between pAsa-2900 and these two plasmids correspond to the plasmid backbone and the transfer genes only (Supplementary Figures S3, S4). It is interesting to note that among the plasmids in this group sharing the same backbone, pAsa-2900 and pAsa-2900b are the smallest with other plasmids often bigger than 200 kb, as illustrated in Supplementary Figures S3, S4. Here again, the scenario of a common ancestor having diverged cannot be excluded. However, as mentioned above pAsmA and pA do not have the *tet*(D) gene.

A previous study suggested that *tet*(D) was present in aquaculture facilities in Turkey, where the plasmids bearing this gene in other species were capable of conjugation, such as in *Aeromonas caviae, Bulkholderia cepacian*, and *Plesimonas shigelloides* (Capkin et al., 2017). The current study found transfer genes (*tra*) in pAsa-2358, pAsa-2900, and pAsa-2900b (Figures 1–3), which indicate the possible exchange of these plasmids with other bacteria in nature through conjugation. This hypothesis was confirmed by conducting conjugation assays with different receptor strains. The presence of plasmids in transconjugants was confirmed by PCR using specific primers (Supplementary Table S1). More than 20 transconjugants were tested after each conjugation experiment (n = 2). Of all the plasmids tested, pAsa-2358 (SHY21–2358), pAsa-2900 (SHY21–2900), and pAsa-2900b (SHY21–3098) could be transferred by conjugation into *A. hydrophila* HER1210 (ATCC 7966)*, A. salmonicida* sp. mesophilic strain 19-K308, and *E. coli* DH5α, except for pAsa-2358 which could not be conjugated into *E. coli* DH5α.

Different hypotheses could explain the incapacity of pAsa-2358 to effectively be transferred into *E. coli* DH5α. Conjugation is a very specific mechanism of exchange between bacterial cells, and the donor and recipient cells require a minimum compatibility of receptors (Virolle et al., 2020). Considering the phylogenetic distance between the two species, the SHY21–2358–*E. coli* DH5α pair are likely less efficient at conjugation than the other pairs studied here. Also, analysis of the plasmid structures indicated that pAsa-2358 has fewer *tra* genes (Figures 1–3), which could suggest a more stringent capacity for conjugation. Although conjugation was
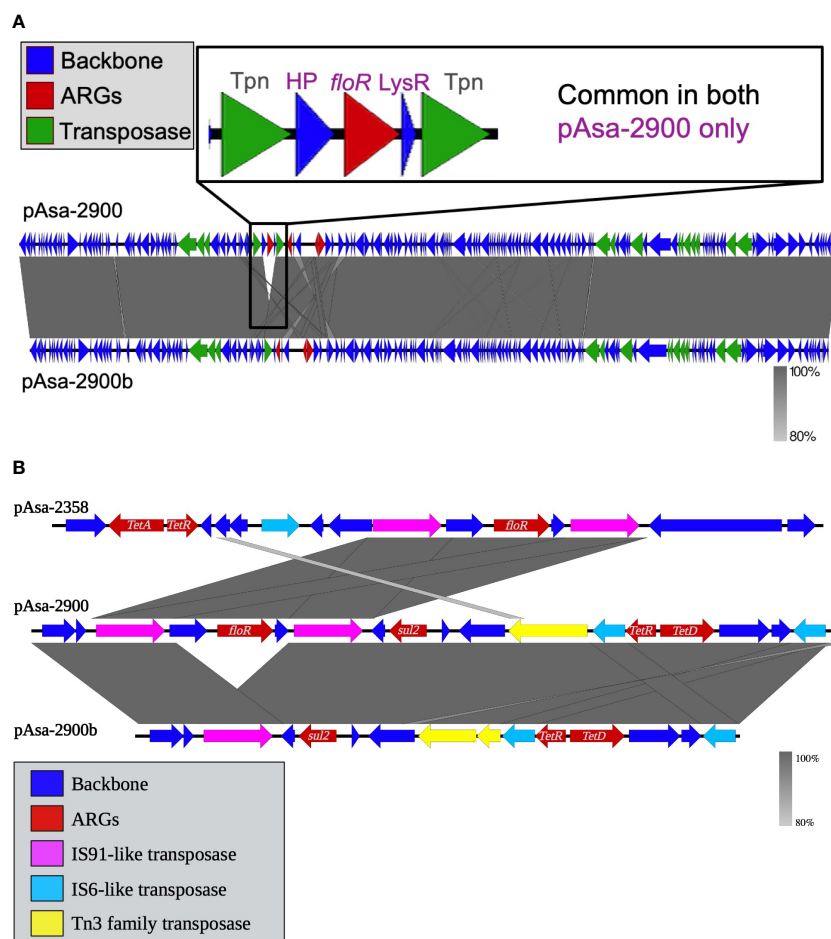
FIGURE 4
Nucleotide alignment of the sequence of pAsa-2900 and its variant, pAsa-2900b, is shown in **(A)**. The red genes code for antibiotic resistance proteins, the green genes for transfer proteins, and the blue genes for other proteins. A zoomed-in view of the *floR* resistance gene presence on pAsa-2900, inside the putative composite transposon but lacking in pAsa-2900b, is shown. **(B)** Easyfig map for comparison of the *floR*- and *tet*(D)-containing putative composite transposons among the three plasmid sequences of this study.

not efficient with *E. coli* DH5α, pAsa-2358 was still transferred when an *Aeromonas* strain was the recipient. The limited conjugation could also be related to the presence of other conjugation genes that are not in the *tra* family, such as the relaxase *virD2* in the version of the putative composite transposon found in pAsa-2358 (Vogel and Das, 1992; Cascales and Christie, 2004). The most diluted bacterial culture resulting from the conjugation assay was not diluted enough to obtain isolated colonies after conjugation with donor strains SHY21–2900 and SHY21–3098, demonstrating high conjugation efficiency. Assays with SHY21–2358 showed that most of the time, bacterial colonies grew faintly after selection and could be observed without obtaining actual transconjugants.

To further compare the level of tetracycline resistance conferred by the *tet*(D) gene in plasmids pAsa-2900 and pAsa-2900b, and the *tet*(A) gene in plasmid pAsa-2358, MIC assays were conducted with both *A. salmonicida* subsp. *salmonicida* strains carrying these plasmids and with *Aeromonas* sp. transconjugants to test the plasmids in the same genetic background. It was confirmed that the *tet*(A) gene from pAsa-2358 results in a lower level of resistance than the *tet*(D) gene from pAsa-2900 and pAsa-2900b (MIC value of 64 μg/mL for pAsa-2358, 128 μg/mL for pAsa-2900 and pAsa-2900b) (Table 4) when testing their respective *A. salmonicida* subsp. *salmonicida* host strains. When testing the transconjugants, the resistance conferred by the acquired plasmids seemed to be similar or even higher than in the host strain (Table 4).

The *tet*(A) and *tet*(D) genes both encode tetracycline-efflux pumps that are part of the same group of the major facilitator superfamily (MFS). These genes are specifically reported in Gram-negative bacteria and are similar in function (Chopra and Roberts, 2001; Grossman, 2016). The *tet*(A) resistance gene has been extensively studied because it is one of the most well-known and commonly found tetracycline resistance genes. In contrast, *tet*(D) is known but less frequently identified or investigated in bacterial strains exhibiting tetracycline resistance. Only a few articles have focused on *tet*(D), and these did not reveal any significant differences that would explain our MIC results. One study indicated that among a panel of tested tetracycline resistance genes, including *tet*(A) and *tet*(D), the latter was the most effective in conferring resistance in a MIC assay. These results,

TABLE 4   Minimum inhibitory concentration of tetracycline at 18°C for plasmids pAsa-2358, pAsa-2900, and pAsa-2900b host strains, as well as transconjugants in *Aeromonas hydrophila* HER1210 and *A. salmonicida* 19-K308.

Donor strain	Transconjugant name	Species of the recipient strain	Plasmid of interest	Combined replicates (2) MIC results
01-B526	Not Applicable	Not Applicable	None, sensitive control	< 2 µg/mL
SHY21–2358	Not Applicable	Not Applicable	pAsa-2358 (host)	64 µg/mL
	pAsa-2358-C1	*A. hydrophila* HER1210	pAsa-2358	64 µg/mL
	pAsa-2358-C2	*A. salmonicida* 19-K308		64 µg/mL
SHY21–2900	Not Applicable	Not Applicable	pAsa-2900 (host)	128 µg/mL
	pAsa-2900-C1	*A. hydrophila* HER1210	pAsa-2900	256 µg/mL
	pAsa-2900-C2	*A. salmonicida* 19-K308		> 256 µg/mL
SHY21–3098	Not Applicable	Not Applicable	pAsa-2900b (host)	128 µg/mL
	pAsa-2900b-C1	*A. hydrophila* HER1210	pAsa-2900b	256 µg/mL
	pAsa-2900b-C2	*A. salmonicida* 19-K308		256 µg/mL
2004–072	pRAS1b-clone1	*A. hydrophila* HER1210	pRAS1b	128 µg/mL

although obtained with *Enterobacter*, align with our findings (Banihashem et al., 2020).

In conclusion, the discovery of three new plasmids, pAsa-2358, pAsa-2900, and pAsa-2900b, with the latter two also featuring a novel *tet*(D) resistance gene against tetracycline, underscores the evolving landscape of antibiotic resistance in *A. salmonicida* subsp. *salmonicida*. The appearance of these plasmids in a region where continuous monitoring over the last decade has been carried out demonstrates the dynamics of antibiotic resistance in salmonids in this region. After a few years where tetracycline monoresistant plasmids were mainly detected (Fournier et al., 2022), new plasmids carrying a *floR* gene represent a real problem given the significant use of florfenicol for the treatment of furunculosis in this region. The presence of these plasmids in the genus *Aeromonas* not only poses challenges for aquaculture because of their repertoire of ARGs, but also highlights the role of this bacterium as a reservoir for ARGs. Conjugation assays further confirm the potential transferability of these plasmids, emphasizing the urgency of continued surveillance and intervention strategies to mitigate the spread of antibiotic resistance in aquatic environments. More specifically, fish farmers must continue their efforts to reduce their use of antibiotics, but this reduction in the use of antibiotics must be global since we have described the presence of new plasmids conferring resistance to tetracycline in an industry where this antibiotic is used very little, suggesting the effects on fish farming in this region of other activities using this antibiotic heavily. In future analyses, it will be relevant to monitor the presence of these plasmids in fish farms and their surrounding environments. Continuous characterization of the genomic characteristics of *A. salmonicida* subsp. *salmonicida* isolates presenting resistance to antibiotics is also necessary, especially in a context where bacterial genome sequencing is increasingly accessible and affordable. Moreover, the observed tetracycline resistance conferred by the *tet*(D) gene *A. salmonicida* subsp. *salmonicida* underscores the importance of understanding the

genetic mechanisms underlying antibiotic resistance for effective management strategies in fish farms in this region.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI Nucleotide, PP592929, PP592930, PP592931.

Author contributions

SG: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. PM: Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. VP: Writing – review & editing, Validation, Resources, Methodology, Investigation. SZ: Writing – review & editing, Investigation. TC: Writing – review & editing, Investigation. SA: Writing – review & editing, Visualization, Validation, Resources, Methodology. AV: Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Formal analysis, Data curation. SC: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbrio.2024.1418706/full#supplementary-material

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