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Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

AsaGEI2d: a new variant of a genomic island identified in a group of *Aeromonas salmonicida* subsp. *salmonicida* isolated from France, which bears the pAsa7 plasmid

Antony T. Vincent[1], Laurent Intertaglia[2], Victor Loyer[3,4,5], Valérie E. Paquet[3,4,5], Émilie Adouane[6], Patrick Martin[7], Céline Bérard[7], Raphaël Lami[6] and Steve J. Charette[3,4,5,*,†]

[1]Département des Sciences Animales, Faculté des Sciences de l'agriculture et de l'alimentation, Université Laval, 2425, rue de l'Agriculture, Quebec City, QC G1V 0A6, Canada, [2]Sorbonne Université, CNRS, Bio2mar, Observatoire Océanologique de Banyuls sur Mer, Avenue Pierre Fabre, 66650, Banyuls-sur-Mer, France, [3]Institut de Biologie Intégrative et des Systèmes, Pavillon Charles-Eugène-Marchand, Université Laval, 1030 avenue de la Médecine, Quebec City, QC G1V 0A6, Canada, [4]Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec, 2725 Chemin Sainte-Foy, Quebec City, QC G1V 4G5, Canada, [5]Département de Biochimie, de Microbiologie et de Bio-informatique, Faculté des Sciences et de Génie, Université Laval, 1045 avenue de la Médecine, Quebec City, QC G1V 0A6, Canada, [6]Sorbonne Université, CNRS, Laboratoire de Biodiversité et Biotechnologies Microbiennes, Observatoire Océanologique de Banyuls sur Mer, Avenue Pierre Fabre, 66650, Banyuls-sur-Mer, France and [7]Conservatoire national du Saumon sauvage, Larma, 43 300 Chanteuges, France

*Corresponding author: Institut de Biologie Intégrative et des Systèmes (IBIS), Pavillon Charles-Eugène-Marchand, 1030 avenue de la Médecine, Université Laval, Quebec City, QC G1V 0A6, Canada. Tel: +418-656-2131, ext. 406914; Fax: +418-656-7176; E-mail: steve.charette@bcm.ulaval.ca

One sentence summary: *AsaGEI2d* is the sixth genomic island discovered in the bacterium *Aeromonas salmonicida* subspecies *salmonicida* further demonstrating the genomic diversity of this bacterium.

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†Steve J. Charette, http://orcid.org/0000-0002-0199-2852

ABSTRACT

Genomic islands (*Aeromonas salmonicida genomic islands*, *AsaGEIs*) are found worldwide in many isolates of *Aeromonas salmonicida* subsp. *salmonicida*, a fish pathogen. To date, five variants of *AsaGEI* (*1a, 1b, 2a, 2b* and *2c*) have been described. Here, we investigate a sixth *AsaGEI,* which was identified in France between 2016 and 2019 in 20 *A. salmonicida* subsp. *salmonicida* isolates recovered from sick salmon all at the same location. This new *AsaGEI* shares the same insertion site in the chromosome as the other *AsaGEI2*s as they all have a homologous integrase gene. This new *AsaGEI* was thus named *AsaGEI2d*, and has five unique genes compared to the other *AsaGEI*s. The isolates carrying *AsaGEI2d* also bear the plasmid pAsa7, which was initially found in an isolate from Switzerland. This plasmid provides resistance to chloramphenicol thanks to a *cat* gene. This study reveals more about the diversity of the *AsaGEI*s.

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INTRODUCTION

Aeromonas salmonicida subsp. *salmonicida* is an opportunistic worldwide fish pathogen (Austin and Austin 2016). This psychrophilic Gram-negative bacterium is the etiological agent of furunculosis, a major salmonid disease (Dallaire-Dufresne *et al.* 2014).

Genomic islands (GEIs) are genetic elements acquired via horizontal gene transfers, that are inserted in bacterial chromosomes. *AsaGEIs* (*A. salmonicida genomic islands*) are found in many *A. salmonicida* subsp. *salmonicida* isolates (Emond-Rheault *et al.* 2015a). Typically, GEIs encode proteins that may be beneficial to the bacteria that contain them, including increased pathogenicity (Hacker and Kaper 2000; Juhas *et al.* 2009, Bellanger *et al.* 2014). In the case of the *AsaGEIs*, no such role is known so far. To date, five variants of *AsaGEI* (*1a, 1b, 2a, 2b* and *2c*) have been described. The size of *AsaGEIs* varies from 50 to 53 kb and many phage genes are found in these genetic elements (Emond-Rheault *et al.* 2015a, b; Long *et al.* 2016).

Despite the as-yet-unknown role of *AsaGEIs* in the metabolism or virulence of *A. salmonicida* subsp. *salmonicida*, it has been shown that the various *AsaGEI* variants have a specific geographic distribution (Emond-Rheault *et al.* 2015a). In this study, *AsaGEI1a* and *2a* have been observed in several dozen strains from the Canadian Maritimes and the Great Lakes—St. Lawrence River System in North America. *AsaGEI1b* was found in 10 isolates spread across Europe and in two isolates in North America. In subsequent studies, *AsaGEI2b* was found in a single strain (JF3224) isolated from a wild fish in Switzerland and *AsaGEI2c* was described in a strain from China (Emond-Rheault *et al.* 2015b; Long *et al.* 2016). *AsaGEI1b* and putative uncharacterized *AsaGEIs* were also detected in a large number of Danish *A. salmonicida* subsp. *salmonicida* isolates (Bartkova *et al.* 2017). Isolates from both Europe and Canada are also known to have no *AsaGEI* (Emond-Rheault *et al.* 2015a; Bartkova *et al.* 2017; Marcoux *et al.* 2020).

Here we present the description of a new GEI, *AsaGEI2d*, found in a group of *A. salmonicida* subsp. *salmonicida* isolated from France. While it has the same insertion site in the chromosome as the other *AsaGEI2s*, this 52 648 bp genetic element has five unique open reading frames. Interestingly, these isolates also bear the pAsa7 plasmid, which confers resistance to chloramphenicol (Vincent *et al.* 2016).

MATERIALS AND METHODS

Isolation of *A. salmonicida* subsp. *salmonicida* strains

Aeromonas salmonicida subsp. *salmonicida* strains were isolated following the usual protocols (Austin and Austin 2012) at the *Conservatoire National du Saumon Sauvage* (Chanteuges, France). Adult Atlantic salmon (*Salmo salar*) raised at the hatchery in fresh water, in natural temperatures for this region and with a simulated natural photoperiod, were euthanized by anesthetic overdose (Eugenol), then dissected. All the fish collected for this study were displaying sign of illnesses (furuncles, multiple tissue damages [internal and external], multiple bleedings and rapid death in a few days). Kidney extracts, mucus and blood from these fish were diluted in physiological saline, and typical brownish pigment-producing colonies were detected on Tryptic Soy Agar after incubation at 25°C or 18°C (TSA, Biokar Diagnostics, Allonne, France) for two successive subculturing steps on the same medium. Each isolate was then grown in tryptic soy broth (TSB, Biokar Diagnostics) for 48 h at 25°C or 18°C while being agitated (100 rpm). Bacteria were then cryopreserved as previously described (Quere *et al.* 2019) and included in the Banyuls Bacterial Culture Collection (BBCC; Table 1). Isolates are issued from 15 different specimen collected over four different years.

Table 1. *Aeromonas salmonicida* subsp. *salmonicida* isolates analyzed in this study were sampled from sick fish specimens from the *Conservatoire National du Saumon Sauvage* and are shown above the line in this table. Strains used as control for genotyping are shown below the line. The whole genome was sequenced for the strain that is shown in bold text.

Name	Source	Date of sampling
BBCC2492	Mucus	2016
BBCC2493	Mucus	2016
BBCC2495	Mucus	2017
BBCC2727	Kidneys	2017
BBCC2728	Furuncle	2017
BBCC2729	Spleen	2017
BBCC2730	Blood	May 2017
BBCC2731	Mucus	2017
BBCC2732	Furuncle	2017
BBCC2871	Furuncle	July 2017
BBCC2873	Furuncle	2017
BBCC2874	Furuncle	2017
BBCC2886	Mucus	April 2018
BBCC2887	Mucus	April 2018
BBCC2984	Mucus	2019
BBCC3005	Mucus	2019
BBCC3006	Mucus	2019
BBCC3007	Mucus	2019
BBCC3050	Mucus	2019
BBCC3051	Mucus	2019
Name	***AsaGEI***	**Reference**
A449	None	Reith *et al.* (2008)
01-B526	*AsaGEI1a*	Charette *et al.* (2012)
HER1108	*AsaGEI1b*	Daher *et al.* (2011)
01-B516	*AsaGEI2a*	Daher *et al.* (2011)
JF3224	*AsaGEI2b*	Emond-Rheault *et al.* (2015b)

Strain identification by 16S rRNA gene sequencing

After genomic DNA extraction with the Wizard Genomic DNA purification kit (Promega, Charbonnières-les-Bains, France), the 16S rRNA gene was analyzed after PCR and sequencing, as described previously (Fagervold *et al.* 2013), to confirm the genus and species of all the new isolates. These analyses were performed thanks to the Bio2Mar technical facilities at the Observatoire Océanologique de Banyuls (France).

Antibiogram analysis

Antibiotic susceptibility tests were performed using a standard disk diffusion test after having grown *A. salmonicida* subsp. *salmonicida* strains on TSA media plates at 18°C or 25°C (Austin and Austin 2012). Zone sizes were measured and interpreted according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM/EUCAST). For each strain, the susceptibility of 14 different antibiotics were tested.

DNA extraction and whole genome sequencing

To further analyze the isolates at the genomic level, the total genomic DNA of one of them (isolate BBCC2887) was extracted as previously described (Vincent *et al.* 2019a). Sequencing was performed using a MiSeq (Illumina, San Diego, CA) system at the Plateforme d'Analyse Génomique of the Institut de Biologie Intégrative et des Systèmes (Université Laval, Quebec City, QC, Canada).

Sequence assembly and analyses

The sequencing reads of the isolate BBCC2887 were *de novo* assembled and annotated as previously described (Vincent *et al.* 2019b). The sequence was deposited in DDBJ/ENA/GenBank under the accession number JADKRF000000000. The sequencing reads were deposited in SRA under the accession number PRJNA264317. The new genetic element was annotated using the RAST webserver (Aziz *et al.* 2008) and then manually curated. The annotated sequence was deposited under the accession number MW218448.

Molecular phylogeny was used to determine the phylogenetic position of strain BBCC2887 among the other *A. salmonicida* (Table S1, Supporting Information). This was done as previously described (Vincent *et al.* 2019a). The Average Nucleotide Identity (ANI) values were computed for genome sequences of *A. salmonicida* using pyani (https://github.com/widdowquinn/pyani). Genes unique to BBCC2887 were found using PATRIC (Wattam *et al.* 2017).

Antibiotic resistance genes were found with the help of ABRicate, using a % identity of 80% and a minimum coverage of 70%, as suggested in the literature (Vincent *et al.* 2019b). The NCBI Antibiotic Resistance Database was used. Finally, a TBLASTN search of the *AsaGEI2d* integrase sequence against the NCBI database identified genomes with similar genes (e-value of at least $1e^{-10}$ and a percentage similarity greater than 50%). The identified integrase sequences were aligned using MAFFT (Katoh and Standley 2013), and the phylogeny was performed using IQ-TREE (Nguyen *et al.* 2015; Hoang *et al.* 2018). The POCP analysis between genomes that have integrases similar to that of *AsaGEI2d* was performed using GET_HOMOLOGUES (Contreras-Moreira and Vinuesa 2013).

PCR analyses

The PCR primers used for genotyping are listed in Table S2 (Supporting Information). DNA templates were prepared as previously described (Trudel *et al.* 2013). The PCR mixture and conditions were the same as previously described (Attere *et al.* 2015).

Ethics statement

The study was carried out indoors at the Conservatoire National du Saumon Sauvage (CNSS), Chanteuges, France (Agreement N° B43056005; according to the 'ARRETE N° DDCSPP/CS/2016/40'). The research project and experiments were performed in accordance with the guidelines and regulations approved by the 'Ethics Committee for Animal Experiments of Languedoc-Roussillon (C2EA-LR/C2EA-36)' N° A6601601, and following the European Union regulations (European directive 2010/63/EU).

RESULTS AND DISCUSSION

A total of 20 bacterial isolates were recovered from sick salmon at the *Conservatoire National du Saumon Sauvage* (CNSS, Larma, Chanteuges, France) from 2016 to 2019. These isolates were identified as *A. salmonicida* subsp. *salmonicida* by sequencing their 16S rRNA gene. Identical sequences were obtained for all isolates. They were also 100% identical to the A449 reference strain (Reith *et al.* 2008).

To form a clearer picture of the identity of these isolates, the entire genome of BBCC2887 was sequenced. Based on the soft-core genome, a robust phylogenomic three was produced (Fig. 1 and Figure S1, Supporting Information). The phylogenetic position of strain BBCC2887 and the ANI values confirm that it is an *A. salmonicida* subsp. *salmonicida* strain, related to other European strains, as expected.

European strains have either no *AsaGEI*, or they have variants *1b* or *2b*. Sequence comparisons have shown the presence of a new *AsaGEI* in strain BBCC2887 (Fig. 2). This *AsaGEI* is at the same integration site as the *AsaGEI2s*, in the $ARNt^{LEU}$ adjacent to prophage 1 (Emond-Rheault *et al.* 2015a). Since *AsaGEI2a*, *2b* and *2c* were already described, this new *AsaGEI* was therefore named *AsaGEI2d*.

Analysis of the sequence of *AsaGEI2d* revealed a gene organization very similar to that of other *AsaGEI2s* (Figure S2, Supporting Information and Fig. 2). In fact, only five ORFs are predicted to be unique to *AsaGEI2d* (ORF4, ORF5, ORF9, ORF10 and ORF30), and to encode for hypothetical proteins (Figure S2, Supporting Information). *AsaGEI2d* also presented specific sequence divergence in other ORFs including, among others, ORF38 and ORF45. *AsaGEI2d* has a gene that encodes for an integrase homologous to those of other *AsaGEI2s*. This observation may explain why all *AsaGEI2s*, including *AsaGEI2d*, share the same insertion site. A TBLASTN search identified seven genomes, in addition to other *AsaGEI2s*, with genes similar to the integrase found in *AsaGEI2d* (Fig. 3). All of these genomes come from phages. Coupled with the presence of several viral genes in *AsaGEI2s* (Figure S2, Supporting Information), this result corroborates the notion that *AsaGEI2s* originate from phages. Despite a high level of similarity between the integrase sequences of the *AsaGEI2s* and those of the seven identified phages, a POCP analysis reveals a different gene repertoire between the *AsaGEI2s* and the phages. It is possible that the integrase gene had a high conservation pressure in order to preserve its function, whereas the other phage genes found in the GEI (Emond-Rheault *et al.* 2015a) could have evolved following the integration of the ancestor of *AsaGEI2* into the genome of *A. salmonicida* subsp. *salmonicida*.

Some of the regions that are unique to this new *AsaGEI* (ORF4, ORF5, ORF9, ORF38 and ORF45) were used as targets to develop a PCR genotyping method to detect this genomic island (Table S2, Supporting Information and Fig. 4). This approach also demonstrated that this new *AsaGEI* was also present in all the other isolates from the CNSS included in this study (Table 1).

Analysis of the BBCC2887 genome revealed that the type three secretion system (TTSS) genes usually found on the pAsa5 plasmid were absent. The loss of the TTSS locus on pAsa5 is the

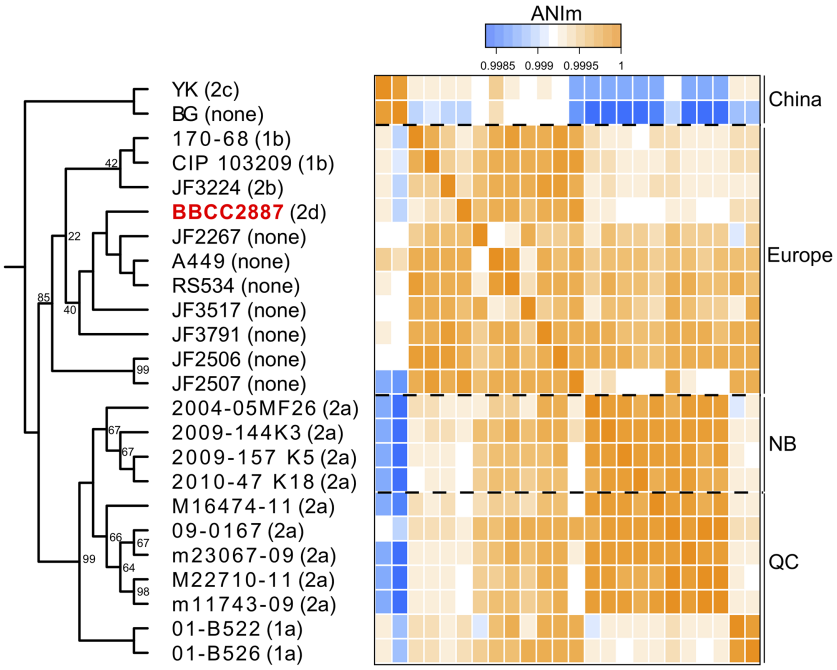

Figure 1. Phylogenetic positioning of BBCC2887 among other *A. salmonicida* psychrophilic strains. This molecular phylogeny is based on the sequences of 2012 orthologous genes (1 845 956 phylogenetically informative sites). The matrix represents the values of ANI. The *AsaGEI* variant is indicated in parentheses for each of the strains. The strain described in the present study is in bold red. Only bootstrap values less than 100 are shown.

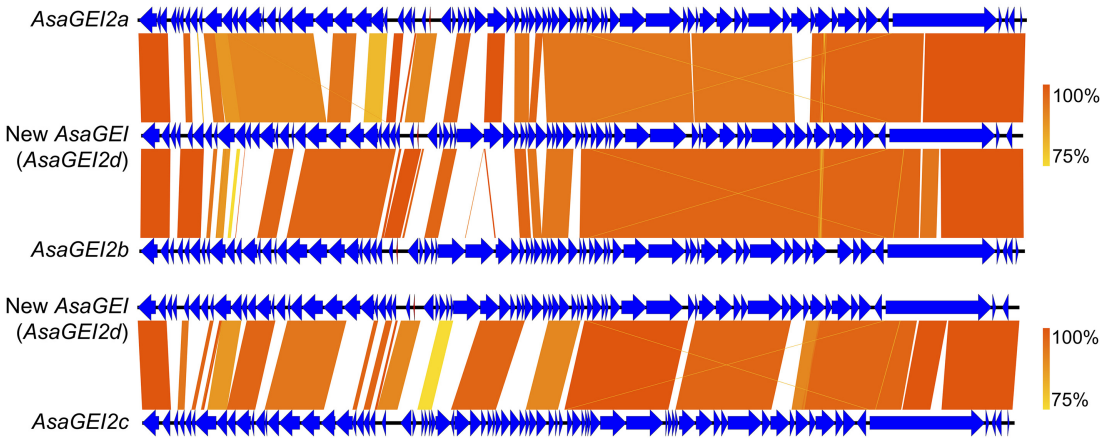

Figure 2. A representation of the genomic alignments of *AsaGEI2d* found in BBCC2887 with other *AsaGEI2s*. High identity regions are shown in orange and genes in blue.

consequence of the growth of *A. salmonicida* subsp. *salmonicida*, a psychrophilic bacterium, at high temperatures (25°C and higher; Stuber *et al.* 2003; Daher *et al.* 2011; Tanaka *et al.* 2012). Typically, the loss of TTSS occurs by homologous recombination of the insertion sequences (ISs) found on either side of the TTSS locus on the plasmid pAsa5. For the A449 strain, which is phylogenetically very close to the BBCC2887 strain, the ISAS11s are those involved in these rearrangements. The ISAS11B and ISAS11C are directly on either side of the TTSS locus, and cause a BC rearrangement (also known as a Type 1 loss profile). ISAS11A, which is located further upstream on the plasmid, can also combine with ISAS11C to give an AC rearrangement called a Type 2 loss profile (Tanaka *et al.* 2012). Bacterial growth during the isolation process from the sick fish at the CNSS was performed at 25°C except for some isolates in 2019. This may explain the TTSS loss for strain BBCC2887.

PCR assays with appropriate primers were performed to determine whether the TTSS loss seen in BBCC2887 was also observed in the other isolates (Emond-Rheault *et al.* 2015b) (Table S2, Supporting Information). As shown in Table 2, among the 20 isolates analyzed, only six have an intact pAsa5 plasmid based on PCR analysis, including two isolates grown at 18°C in 2019 instead of 25°C. All the other strains lost their TTSS locus. It was shown in a recent study that for strains likely to lose their TTSS, it is rarely all the cells that lose the locus encoding the TTSS. This rate varies from less than 5% to more than 80% depending on the strain (Marcoux *et al.* 2020). Thus, this explains why some strains recovered from the CNSS and cultivated at 25°C can despite still possess their TTSS locus.

Antibiogram analyses revealed that all isolates were resistant to chloramphenicol, vancomycin penicillin and streptomycin (except one intermediate strain), while all isolates were sensitive

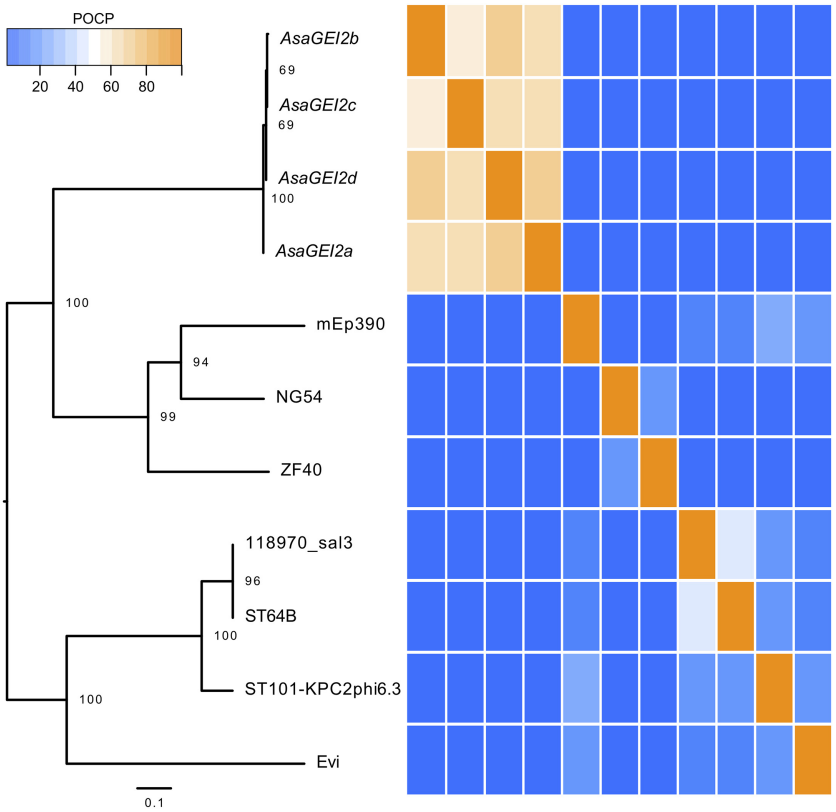

Figure 3. Phylogenetic analysis of integrase genes. The matrix corresponds to the POCP values between the complete sequences while the phylogeny is based on the integrase genes. The bootstrap values for each of the nodes are shown. The accession numbers are: *AsaGEI2a* = KJ626180, *AsaGEI2b* = KP861348, *AsaGEI2c* = KU923576, *AsaGEI2d* = MW218448, mEp390 = JQ182729, NG54 = MK047638, ZF40 = JQ177065, 118970_sal3 = KU927493, ST64B = AY055382, ST101-KPC2phi = MK416017 and Evi = LR597642.

Table 2. PCR genotyping results for the presence of TTSS and pAsa7 in the isolates bearing an *AsaGEI2d*.

Strains (BBCC)	Year	Growth temperature (°C)	TTSS[a, b]	pAsa7[a]
2492	2016	25	–	+
2493	2016	25	+	+
2495	2016	25	+	+
2727	2017	25	–	+
2728	2017	25	–	+
2729	2017	25	–	+
2730	2017	25	–	+
2731	2017	25	–	+
2732	2017	25	–	+
2871	2017	25	–	+
2873	2017	25	–	+
2874	2017	25	–	+
2886	2018	25	–	+
2887	2018	25	–	+
2984	2019	25	+	+
3005	2019	18	+	+
3006	2019	18	+	+
3007	2019	25	–	+
3050	2019	25	+	+
3051	2019	25	–	+

[a]: Presence indicated by a plus (+) symbol and absence by a minus symbol (-).
[b]: TTSS absent when no PCR product obtained for the *exsD* gene found in the TTSS locus (Emond-Rheault *et al.* 2015b).

to rifampicin, cefotaxime and tetracycline (except one resistant strain; Table S3, Supporting Information). For the other antibiotics tested (ciprofloxacin, polymyxin, ofloxacin, kanamycin and erythromycin), the resistance was variable from one isolate to another and may be explained by resistance acquired individually by the isolates through point mutations on chromosomal genes (Woodford and Ellington 2007; Li *et al.* 2019). It is interesting to note that the majority of the 2019 isolates were resistant to ciprofloxacin and ofloxacin; this is not true of isolates from previous years (Table S3, Supporting Information), which suggests that the bacteria are evolving.

Several antibiotic resistance genes were found in the BBCC2887 genome sequence (Table S4, Supporting Information). Our bioinformatics analyses did not allow us to identify the mutations in chromosomal genes that might explain the differences in the resistance profile observed with the antibiogram analyses. The antibiotic resistance gene found in BBCC2887 of particular interest was the gene *cat*, which confers resistance to chloramphenicol. This gene was found so far on plasmids such as pAsa4, pAsa4c and pAsa7 (Reith *et al.* 2008; Tanaka *et al.* 2016; Vincent *et al.* 2016).

The *A. salmonicida* subsp. *salmonicida* isolates generally have a repertoire of small plasmids that include pAsa1, pAsa2, pAsa3 and pAsal1, the latter having a gene coding for an effector of TTSS (AopP; Fehr *et al.* 2006; Attere *et al.* 2015). The plasmids pAsa1, pAsa2, pAsa3 are present in BBCC2887, but pAsal1 is absent. This is common in European isolates (Attere *et al.* 2015). A more precise search revealed that strain BBCC2887 has an identical plasmid (100% identity match) to pAsa7 of the JF3791

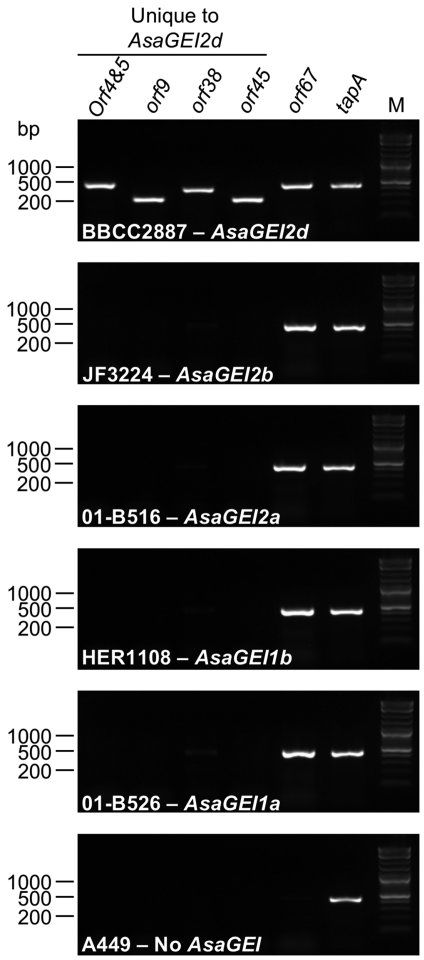

Figure 4. PCR genotyping. Specific primer pairs have been designed (*orf4–5*, *orf9*, *orf38* and *orf45*) to target genes unique to *AsaGEI2d*, and these primer pairs do not amplify strains that bear another *AsaGEI* or that have no *AsaGEI*. A primer pair targeting a gene common to all *AsaGEIs* (*orf67*) was used as a control. A primer pair that amplifies the *tapA* gene found on the chromosome of all *A. salmonicida* subsp. *salmonicida* isolates (Emond-Rheault *et al.* 2015a) was also used as a control.

strain isolated in Switzerland in 2006 (Vincent *et al.* 2016). The presence of this plasmid explains the *cat* gene found in the BBCC2887 genome. By PCR genotyping, the presence of pAsa7 was confirmed in all the other isolates from the CNSS included in this study. Having found this rare plasmid in strains other than JF3791 is interesting, and makes it possible that pAsa7 may eventually spread.

Considering the presence of *AsaGEI2d* and pAsa7 in all of the isolates from the CNSS that were analyzed in this study, it is interesting to propose that all these isolates have likely a clonal origin, which suggests a persistent antibiotic resistance problem at the CNSS. The result regarding different profiles for pAsa5 and its TTSS genes among the isolates is not contradictory with a clonal origin, because the loss of the TTSS locus and the rearrangement of pAsa5 in some isolates most likely occurred after the bacteria were sampled from the sick fish; these discrepancies were likely caused by the culture conditions.

This study demonstrates that the diversity of the *AsaGEIs* is even greater than suspected and suggests that other genomic islands will probably be discovered in the future. It will also be interesting to see if *AsaGEI2d* is detected in other strains in the future, particularly in Europe. Compared to *AsaGEI1a*, *1b* and *2a*, which have been found in numerous strains that have shown their role as markers of the geographical origin of the strains bearing them, *AsaGEI2b*, *2c* and *2d* have only been identified in one isolate or group of related isolates. They must therefore be found in other unrelated isolates of *A. salmonicida* subsp. *salmonicida* before it will be possible to confirm their role as geographic markers as for the other *AsaGEIs*. The analysis of the CNSS isolates is further proof that the plasmid pAsa5 is sensitive to stressful culture conditions. Finally, it is interesting to have found the antibiotic-resistance plasmid pAsa7 in additional *A. salmonicida* subsp. *salmonicida*, reinforcing the idea that this plasmid must be monitored during routine analyses by veterinary diagnostic services, especially in Europe.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

Conflicts of interest. None declared.

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