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Research Letter

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AsaGEI2b: a new variant of a genomic island identified in the *Aeromonas salmonicida* subsp. *salmonicida* JF3224 strain isolated from a wild fish in Switzerland

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One sentence summary: Identification of *AsaGEI2b*, a new genomic island as an additional indicator of the geographic origin of *Aeromonas salmonicida* subsp. *salmonicida* isolates.
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ABSTRACT

Aeromonas salmonicida subsp. *salmonicida* is the causal agent of furunculosis in salmonids. We recently identified a group of genomic islands (*AsaGEI*) in this bacterium. *AsaGEI2a*, one of these genomic islands, has almost exclusively been identified in isolates from North America. To date, *Aeromonas salmonicida* subsp. *salmonicida* JF3224, a strain isolated from a wild brown trout (*Salmo trutta*) caught in Switzerland, was the only European isolate that appeared to bear *AsaGEI2a*. We analyzed the genome of JF3224 and showed that the genomic island in JF3224 is a new variant of *AsaGEI*, which we have called *AsaGEI2b*. While *AsaGEI2b* shares the same integrase gene and insertion site as *AsaGEI2a*, it is very different in terms of many other features. Additional genomic investigations combined with PCR genotyping revealed that JF3224 is sensitive to growth at 25°C, leading to insertion sequence-dependent rearrangement of the locus on the pAsa5 plasmid that encodes a type three secretion system, which is essential for the virulence of the bacterium. The analysis of the JF3224 genome confirmed that *AsaGEI*s are accurate indicators of the geographic origins of *A. salmonicida* subsp. *salmonicida* isolates and is another example of the susceptibility of the pAsa5 plasmid to DNA rearrangements.

Keywords: *AsaGEI2b*; genomic island; *Aeromonas salmonicida* subsp. *salmonicida*; type three secretion system

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INTRODUCTION

The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is an opportunistic fish pathogen that is responsible for significant economic losses in salmonid fish farms (Bernoth *et al.* 1997). It is the causal agent of furunculosis, a predominantly salmonid disease with high morbidity and mortality (Janda and Abbott 2010).

The international fish trade may contribute to introducing pathogens into new environments (Hedrick 1996). Additional epidemiological tools are thus required to track the dissemination of *A. salmonicida* subsp. *salmonicida* isolates into new ecosystems.

We recently described a new genomic island (*Aeromonas salmonicida genomic island*, *AsaGEI*) in this bacterial species (Emond-Rheault *et al.* 2015). Genomic islands (GEIs) are genetic elements ranging in size from 10 to 200 kb that are inserted in the bacterial chromosome. They are not present in all strains of a species but can provide various functional advantages to those possessing them, including increased pathogenicity (Juhas *et al.* 2009; Bellanger *et al.* 2013; Darmon and Leach 2014). To date, we have identified three forms of *AsaGEI* (1a, 1b and 2a) that range in size from 50 to 53 kb. They all contain many phage genes. Their contribution to the host phenotype is unknown (Emond-Rheault *et al.* 2015).

Nevertheless, *AsaGEIs* are interesting from a molecular epidemiological point of view. Each *AsaGEI* appears to be strongly associated with a specific geographic region, suggesting that they can be used as indicators of geographic origin. *AsaGEI1a* has only been found in the Great Lakes-St. Lawrence River system, while *AsaGEI1b,* except for two isolates, was found in Europe. On its side, *AsaGEI2a* has been exclusively detected in North American isolates, with the exception of JF3224 (Emond-Rheault *et al.* 2015), which was isolated from a wild brown trout (*Salmo trutta*) captured in 2004 in a prealpine Swiss river (Burr *et al.* 2005). More precisely, of 139 isolates tested, 82 bore an *AsaGEI2a*, while only one of 25 isolates from Europe (JF3224) harbored this GEI. This exception prompted us to investigate this isolate using whole genome sequencing in order to determine whether it was an example of human-driven pathogen dissemination. In fact, our analyses revealed that this isolate contains a novel variant of *AsaGEI*.

Materials and Methods

Bacterial isolates and growth conditions

The *A. salmonicida* subsp. *salmonicida* isolates used in the present study were JF3224, which was isolated from a wild brown trout (*Salmo trutta*) captured in 2004 in a prealpine Swiss river (Burr *et al.* 2005), and 01-B526, which was used as a reference strain with an intact pAsa5 plasmid (Daher *et al.* 2011; Charette *et al.* 2012). They were grown for 2 or 3 days at 18°C on furunculosis agar (10 g of Bacto-Tryptone, 5 g of yeast extract, 1 g of L-tyrosine, 2.5 g of NaCl and 15 g of agar per liter of distilled water) (Hanninen and Hirveri-koski 1997).

DNA extraction and genomic sequencing

The total genomic DNA of JF3224 was extracted using DNeasy blood and tissue kits (Qiagen, Canada). The TruSeq shotgun library was generated using purified genomic DNA and was sequenced by the Plateforme d'Analyse Génomique of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval) using Illumina technology on a MiSeq instrument.

Table 1. Primers used in this study.

Primer	Sequence (5′–3′)	Reference
	Control primers for pAsa5	
traC F	5′-TGCACTATCCCCAGCTATCC-3′	(Daher *et al.* 2011)
traC R	5′-TCGGTAATCGCGGTCTTGTC-3′	(Daher *et al.* 2011)
exsD F	5′-AGAAGTGATCCTGACCCAAGGCAA-3′	(Daher *et al.* 2011)
exsD R	5′-TTGCAACGACTGTTGCCAAGAACC-3′	(Daher *et al.* 2011)
resD F	5′-TCAGAAACTTGGCCATCGCTCACA-3′	(Daher *et al.* 2011)
resD R	5′-TGATGTGCAGATTTCCCTGGAGCA-3′	(Daher *et al.* 2011)
	B–C rearrangement primers	
11B1F	5′-GCGCACCACCACCATTTAATGTCA-3′	(Tanaka *et al.* 2012)
11CR	5′-AACTGGCAAGGATAGAGCTGCTGA-3′	(Tanaka *et al.* 2012)

Sequence analyses

A total of 1800 692 raw reads (1702 181 after quality control and error correction) with an average length of 300 bp were generated (mean coverage: 86.53×) and were *de novo* assembled using A5-miseq pipeline version 20140401 (Coil, Jospin and Darling 2015). The complete structures of the chromosome of the JF3224 isolate and the pAsa5 plasmid were determined by genomic alignment using the CONTIGuator webserver (Galardini *et al.* 2011) with the chromosome (GenBank accession number: NC_009348.1) and pAsa5 plasmid (GenBank accession number: NC_009350) from the A449 strain as reference sequences (Reith *et al.* 2008). The Whole Genome Shotgun project of *A. salmonicida* subsp. *salmonicida* JF3224 has been deposited in the DDBJ/EMBL/GenBank database under accession number JXTA00000000. The draft genome sequences were annotated using the NCBI Prokaryotic Genome Annotation Pipeline.

AsaGEI2b was annotated as previously described (Emond-Rheault *et al.* 2015). Briefly, the open reading frames (ORFs) were predicted by Prodigal (version 1.20) (Hyatt *et al.* 2010), confirmed by GeneMark (version 2.5) (Besemer, Lomsadze and Borodovsky 2001) and visualized in Artemis (version 16.0.0) (Carver *et al.* 2012). Each ORF was manually verified. BLASTn and BLASTp searches (NCBI) were performed to determine the putative functions of the ORFs (Altschul *et al.* 1997). The non-redundant (nr/nt) and whole-genome shotgun nucleotide databases were used for the BLASTn search and the non-redundant protein sequences (nr) database was used for the BLASTp search.

PCR analyses

The DNA templates, PCR mixtures and program cycles were as previously described (Trudel *et al.* 2013). The PCR assays were performed in duplicate with the appropriate positive and negative controls for each condition. The PCR primers used are listed in Table 1.

To compare the pAsa5 plasmid from the JF3224 isolate in Switzerland to the pAsa5 plasmid in the isolate shipped to Canada as well as its susceptibility to incubation at 25°C, the same PCR assays were performed in Switzerland on the original isolate grown on tryptic soy agar for 3 days at 18°C or 25°C. A pool of approximately 20 colonies grown at 18°C or 25°C were suspended in 450 μl of lysis buffer (100 mM Tris-HCl, pH 8.5, 0.05% Tween 20 and 240 μg ml^{-1} of proteinase K) and were incubated for 1 h at 60°C and then for 15 min at 97°C. The cell lysates were used as DNA templates for the PCR assays described above.

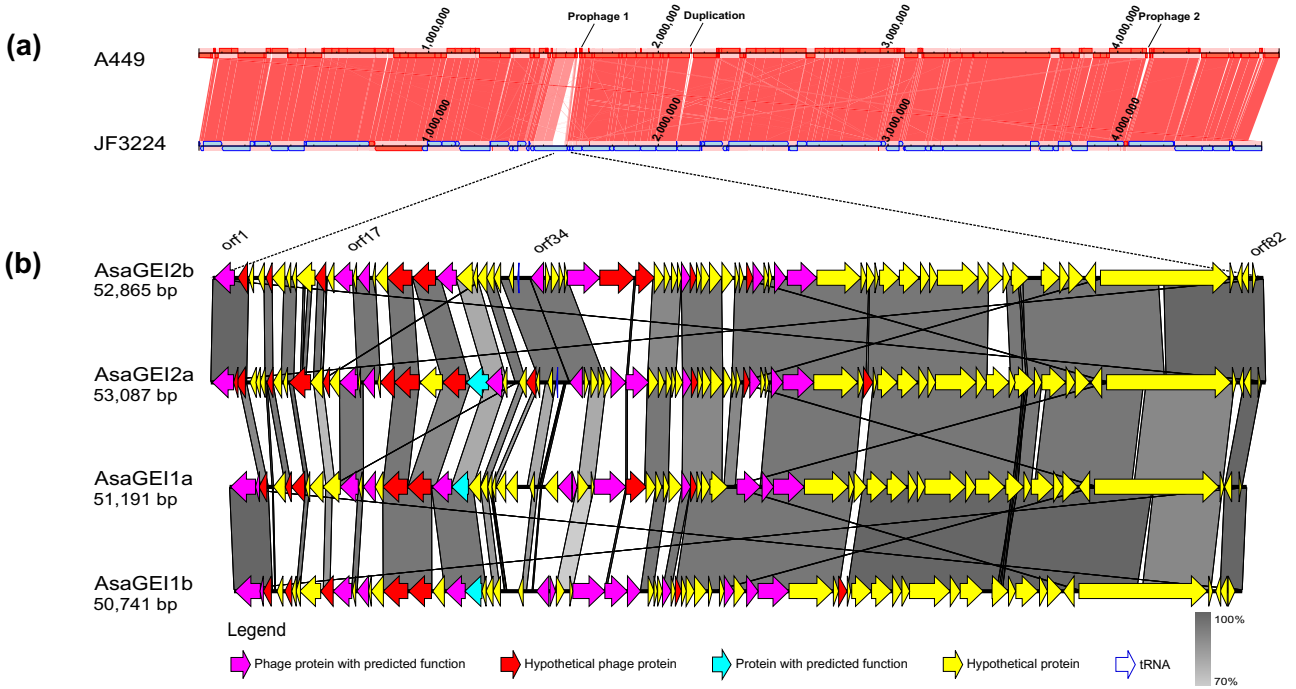

Figure 1. Identification of *AsaGEI2b* in the genome of the JF3224 *A. salmonicida* subsp. *salmonicida* isolate. (**a**) Chromosomal alignment of the JF3224 contigs with the A449 reference strain (Reith *et al.* 2008) using the CONTIGuator webserver (Galardini *et al.* 2011). (**b**) Representation of the genomic alignments of the new *AsaGEI* element in the genome of JF3224 (*AsaGEI2b*) with the other three *AsaGEIs* identified in a previous study (Emond-Rheault *et al.* 2015) using Easyfig (Sullivan, Petty and Beatson 2011).

RESULTS AND DISCUSSION

JF3224 isolate was initially thought to bear *AsaGEI2a* (Emond-Rheault *et al.* 2015). However, PCR assays to determine the presence of *orf21*, which is usually found in *AsaGEI2a* (GenBank accession number: KJ626180), indicated that JF3224 does not possess *orf21* (data not shown), suggesting that this isolate may harbor an *AsaGEI2a* variant. This possibility was verified by genome sequencing.

The *de novo* genome assembly of the JF3224 isolate produced 133 contigs, with an N_{50} length of 120 108 bp. The smallest and largest contigs were 599 and 382 412 bp long, respectively. The G+C content was 58.44%. The genomic alignment determined using CONTIGuator (Galardini *et al.* 2011) of the contigs from JF3224 with either the chromosome or the pAsa5 plasmid from the reference strain A449 (Reith *et al.* 2008) showed that the 75 and 12 contigs mapped on both. The BLASTn analyses (Altschul *et al.* 1997) of the unmapped contigs showed that contigs number 81, 76 and 73 (GenBank accession numbers: JXTA01000081, JXTA01000076 and JXTA01000073, respectively) corresponded to the high-copy-number plasmids pAsa1, pAsa2 and pAsa3, respectively (Boyd *et al.* 2003). Another high-copy-number plasmid (pAsal1) (Fehr *et al.* 2006) was also mapped on several contigs of the JF3224 genome. The remaining unmapped contigs were associated with repeated elements in the *A. salmonicida* subsp. *salmonicida* genome and corresponded to insertion sequences (IS), rRNA clusters, conserved sequences shared by prophages 1 and 2 and a 6000-bp chromosomal duplication.

The results of the genomic alignment comparison of the reference strain (A449) and the contigs of JF3224 using CONTIGuator (Galardini *et al.* 2011) were visualized with ACT (Carver *et al.* 2005) to determine the reasons for the genome assembly breaks. The majority of the assembly breaks could be explained by repeated elements (data not shown), as reported in another study (Vincent *et al.* 2014). The chromosome alignment suggested that there is a 52.8-kb sequence inserted in the JF3224 chromosome (Fig. 1a).

The detailed genome sequencing analyses indicated that JF3224 possesses a new variant of *AsaGEI* since it is markedly different from *AsaGEI2a* and other *AsaGEIs* (Fig. 1b). However, the novel genetic variant contained an integrase gene (*orf1*) identical to the *orf1* in *AsaGEI2a* (Fig. 1b). In addition, *AsaGEI2a* and the new genetic variant shared the same insertion site in the chromosome (data not shown). The new *AsaGEI* was thus called *AsaGEI2b*. The query cover and identities between *AsaGEI2a* and *AsaGEI2b*, which were determined using megablast (word size of 28) (Altschul *et al.* 1997) on the NCBI server, were 76 and 94%, respectively, indicating that approximately 25% of the genes in these *AsaGEIs* (i.e. *AsaGEI2a* and *AsaGEI2b*) are completely different. This level of identity is similar to that observed with *AsaGEI1a* and *AsaGEI1b* (Emond-Rheault *et al.* 2015).

AsaGEI2b was annotated, and the sequence is available in GenBank (accession number: KP861348). Seventy-four percent of the genes of *AsaGEI2b* code for hypothetical proteins. Like the other three *AsaGEIs*, no function has so far been attributed to this genomic element (Emond-Rheault *et al.* 2015). BLASTn searches (Altschul *et al.* 1997) using the NCBI webserver showed that *AsaGEI2b* is not present in any other bacterial strains.

In addition to the presence of a new *AsaGEI* in the JF3224 chromosome, we also observed another particularity related to the pAsa5 plasmid in the genome of this isolate. The alignment of pAsa5 from A449 (Reith *et al.* 2008) with the JF3224 contigs visualized with ACT (Carver *et al.* 2005) indicated that assembly breaks were mainly caused by repeated elements such as ISs (Fig. 2a). The assembly break sites were at the same locations as those in two other pAsa5 plasmids studied in another study (Vincent *et al.* 2015). The JF3224 isolate was previously

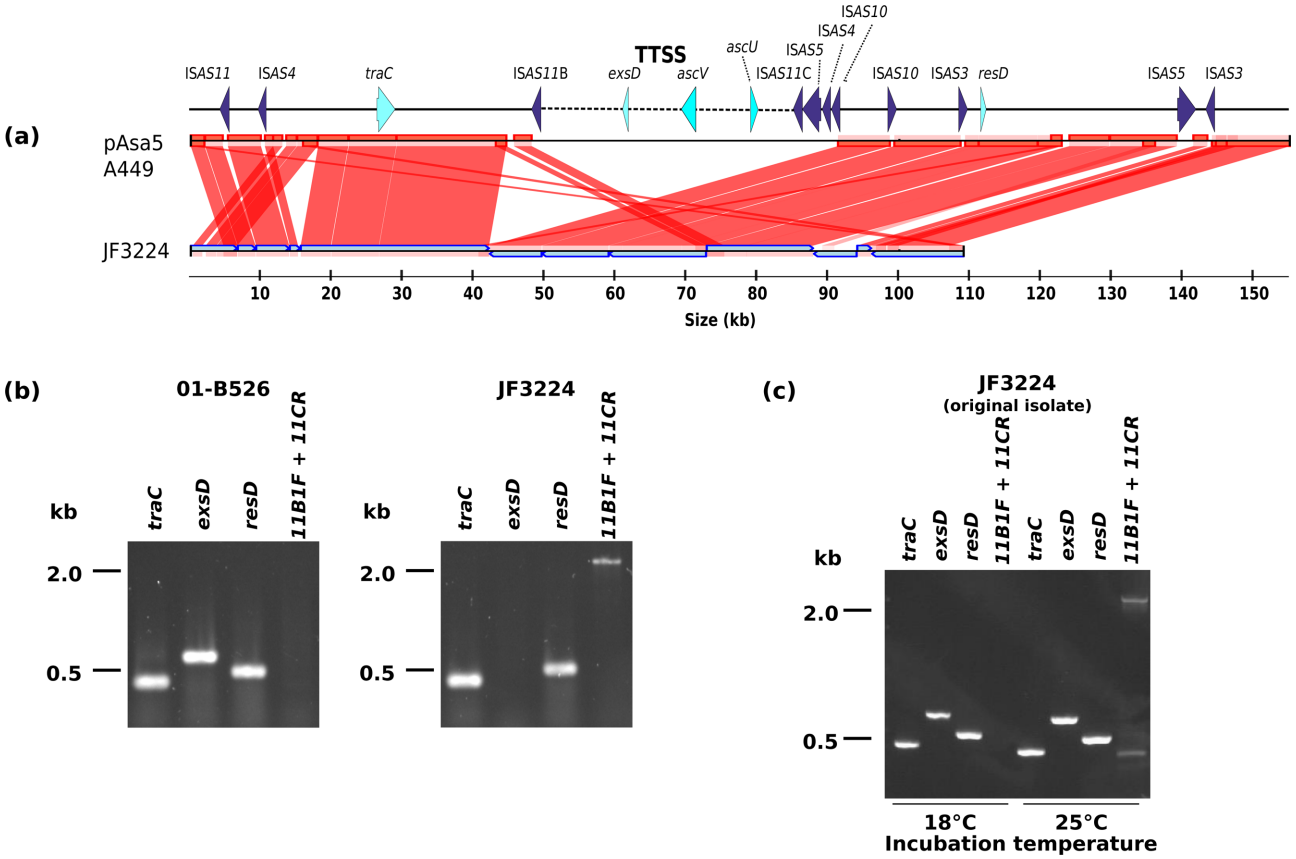

Figure 2. Loss of the TTSS locus from the pAsa5 plasmid of the JF3224 isolate. (**a**) Alignment of plasmid pAsa5 from A449 with the JF3224 contigs using CONTIGuator (Galardini *et al.* 2011). The locations of the 11 ISs and the *traC*, *exsD*, *ascV*, *ascU* and *resD* genes on pAsa5 were labeled using Easyfig (Sullivan *et al.* 2011). (**b**) Confirmation by PCR genotyping of the pAsa5 rearrangement in JF3224. Primers 11B1F and 11CR were used to assess B–C rearrangements while primers targeting *traC*, *exsD* and *resD* were used as controls for the presence of the various sections of pAsa5. The 01-B526 isolate was used as a control for an intact pAsa5 plasmid (Daher *et al.* 2011; Charette *et al.* 2012). (**c**) The original JF3224 isolate in Switzerland has an intact pAsa5 plasmid that is sensitive to insertion sequence-dependent rearrangements when incubated at 25°C. The bacteria were grown for 3 days at 18 or 25°C. Pools of approximately 20 colonies were tested in each PCR assay using the same primers as in (b).

reported to possess the *ascV* and *ascU* genes (Studer, Frey and Vanden Bergh 2013), which are located in the type three secretion system (TTSS) locus (Fig. 2a). The results of the PCR analyses (Fig. 2b) and genome sequencing performed during the present study indicated that JF3224 does not harbor the TTSS locus. We suspect that the time it took (5 days) to transport the isolate from the Swiss laboratory to the Canadian laboratory exposed the bacteria to temperatures exceeding 18°C, and that this may have caused the TTSS deletion. The instability of the TTSS locus on pAsa5, a thermolabile plasmid, has already been attributed to the incubation 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). PCR assays with the appropriate primers were used to determine whether the TTSS deletion was caused by a recombination of ISAS11B and ISAS11C (Fig. 2a), as previously reported by Tanaka *et al.* (2012). The results indicated that ISAS11B/C were involved in the loss of the TTSS locus (Fig. 2b). This was confirmed by sequencing the amplicons (data not shown).

To verify whether pAsa5 was intact in the original isolate in Switzerland, the same PCR assays were performed on this isolate. The original isolate in Switzerland was also grown for 3 days at 25°C to determine its sensitivity to this stressful condition. As shown in Fig. 2c, the original isolate gave PCR products for the TTSS *exsD* gene, confirming the integrity of the pAsa5 plasmid. The PCR amplification of DNA from a pool of 20 colonies grown at 25°C amplified both the *exsD* gene and the ISAS11B/C rearrangement, suggesting that some of the colonies contained a pAsa5 plasmid with an IS-dependent rearrangement while the others contained an intact plasmid. The present study provided additional proof that *A. salmonicida* subsp. *salmonicida* is sensitive to stressful conditions that can affect the genes involved in a major virulence mechanism.

The discovery of *AsaGEI2b* revealed, until proven otherwise, that *AsaGEI2a* is only found in North American isolates. Rather than illustrating a case of trade-driven intercontinental dissemination of *A. salmonicida* subsp. *salmonicida*, the draft genome of JF3224 revealed that *AsaGEIs* are more diverse than previously thought and further confirmed the usefulness of these genomic islands as indicators of the geographic origins of *A. salmonicida* subsp. *salmonicida* isolates.

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Conflict of interest. None declared.

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