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Genomic and virulence insights of Western European *Aeromonas salmonicida* subsp. *salmonicida* and development of *Galleria mellonella* infection assay

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Abstract

Aims: *Aeromonas salmonicida* subsp. *salmonicida* is the etiological agent of furunculosis, a fish disease highly aggressive for salmonids and responsible for significant economic losses in aquaculture worldwide. This study aimed to explore genomic and antimicrobial resistance traits of Western European *A. salmonicida* subsp. *salmonicida* strains and to develop an adapted infection model using larvae of the greater wax moth *Galleria mellonella* to assess the pathogenic potential of this psychrophilic subspecies.

Methods and results: Three *A. salmonicida* subsp. *salmonicida* strains, isolated from salmonids displaying clinical signs of furunculosis, were tested against a panel of antibiotics and sequenced to characterize their genome. Virulence was evaluated in *G. mellonella* larvae using bacterial doses ranging from 10^1 to 10^6 CFU/larva. Two isolates exhibited multidrug resistance to antibiotics commonly used against furunculosis. Although closely related to the reference strain A449, genomic analyses revealed multiple plasmids known to encode antibiotic resistance genes. Virulence assays showed that this subspecies was lethal at doses as low as 10^1 CFU/larva, and that a fully functional Type III secretion system (T3SS) is not essential for the infection of *G. mellonella*, likely due to the presence of other virulence factors in T3SS-deficient strains.

Conclusions: These findings enhance the genomic characterization of European *A. salmonicida* subsp. *salmonicida* and validate the use of *G. mellonella* larvae as a relevant alternative infection model for studying this psychrophilic subspecies.

Impact Statement

The validation of *Galleria mellonella* as an alternative infection model with characterized *A. salmonicida* subsp. *salmonicida* strains offers a cost-effective, ethical, and scalable tool for studying virulence mechanisms and evaluating control strategies for this economically significant fish pathogen.

Keywords: European *Aeromonas salmonicida*; virulence; *Galleria mellonella*; antimicrobial resistance; mobile genetic elements

Introduction

Aeromonas salmonicida subsp. *salmonicida*, a psychrophilic Gram-negative bacterium, is a globally distributed opportunistic pathogen causing furunculosis in salmonids (Austin and Austin 2016). Initially observed in freshwater fish, this pathogen has since also been isolated from marine species (Roberts 2012). Furunculosis ranks among the main bacterial diseases responsible for significant economic losses in the aquaculture industry worldwide (Park et al. 2020). In a European country as highly involved in this sector as France, furunculosis has been reported to affect 20% of freshwater salmonid farms, making it the primary indication for using sulfonamides and flumequine in the field (Le Bouquin et al. 2021). In addition, the genus *Aeromonas*, including in particular the pathogenic species *A. salmonicida*, is one of the most studied genera for antimicrobial resistance (AMR). Resistances to (fluoro)quinolones, streptomycin, oxytetracycline, chloramphenicol, florfenicol, trimethoprim/sulfamethoxazole and beta-lactams are indeed commonly reported (Samanta and Bandyopadhyay 2020, Hayatgheib et al. 2021, Roh and Kannimuthu 2023).

The *A. salmonicida* species officially includes the four psychrophilic subspecies *salmonicida*, *achromogenes*, *masoucida*, and *smithia*, as well as the mesophilic subspecies *pectinolytica* (Pavan et al. 2000, Brenner et al. 2005, Vincent and Charette 2022). However, there has been an increasing number of unclassified mesophilic strains described in recent years, highlighting the greater genetic diversity of this group compared to the psychrophilic one (Vincent et al. 2019). The psychrophilic

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subspecies are further categorized as "typical" or "atypical" based on their isolation source and their composition in certain genomic elements, particularly insertion sequences (ISs) (Long et al. 2023). The *salmonicida* subspecies is the only member of the typical category as this subspecies is responsible for furunculosis classically observed in salmonids, especially in Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), and brown trout (*Salmo trutta*). Atypical strains have a broader fish host range, affecting both salmonids and non-salmonids (Wiklund and Dalsgaard 1998, Dallaire-Dufresne et al. 2014).

Genetic diversity in *A. salmonicida* is partly attributed to its abundance of mobile genetic elements (MGEs) (Charette 2021). In addition to being involved in the complex classification of *A. salmonicida*, MGEs in typical strains such as plasmids, particularly the pAsa5, play a crucial role in the virulence since they carry the majority of genes encoding the Type III secretion system (T3SS), an essential virulence factor in *A. salmonicida* subsp. *salmonicida* (Vanden Bergh and Frey 2014). Moreover, it is well known that the plasmid repertoire of this waterborne bacterium serves as a reservoir for antibiotic resistance, with at least 15 of its plasmids already identified as harboring antibiotic resistance genes (Vincent et al. 2021a). ISs also significantly contribute to the genomic plasticity of this species, and an increasing number of studies highlight their ability in modulating the virulence of typical psychrophilic strains when exposed to stressful conditions, such as temperatures of 25°C or higher.

Concrete examples include homologous recombination of the pAsa5 plasmid involving the IS*As11* or IS*As5* in the majority of *A. salmonicida* subsp. *salmonicida* strains tested (Marcoux et al. 2020). These IS-dependent rearrangements, obtained by culturing original strains for few days to 2 weeks at 25°C, result in the loss of the T3SS locus (Emond-Rheault et al. 2015a, Tanaka et al. 2012, 2017). Consequently, attenuated virulence has already been observed in a surrogate infection model using *Dictyostelium discoideum* when *A. salmonicida* subsp. *salmonicida* strains with an impaired T3SS region were tested (Daher et al. 2011). A reduced virulence, induced artificially by mutagenesis of key genes of this secretion system, has also been demonstrated *in vivo* in other studies (Burr et al.,2005 Dacanay et al. 2006, Froquet et al. 2007), highlighting the crucial role of T3SS integrity in this subspecies to be fully virulent.

Beyond its descriptive aspects, studying the diversity of *A. salmonicida* is important from an epidemiological perspective and can be useful for the development of effective control strategies. For instance, a comparative genomic analysis of a hundred Danish strains enabled Bartkova and colleagues, in 2017, to identify multiple bacterial introductions into the country and to suggest pathogen dispersal via carrier fish. Additionally, several genetic elements such as prophages (e.g. Prophage 3) and genomic islands (*AsaGEIs 1a*, *1b*, *2a*, *2b*, *2c*, and *2d*) have been associated with the geographical origins of *A. salmonicida* subsp. *salmonicida* strains, potentially aiding in isolate tracing during furunculosis outbreaks (Emond-Rheault et al. 2015b, Long et al., 2016Vincent et al. 2016, 2021b, Leduc et al. 2023). Given the predominance of non-European strains in genomic databases, the sequencing and characterization of new European isolates would help expand our understanding of *A. salmonicida* diversity. It may also lead to the discovery of new region-specific genetic elements, with a particular interest in countries from where no strains of *A. salmonicida* subsp. *salmonicida* have been sequenced yet, such as Belgium and Luxembourg.

The pathogenicity of *A. salmonicida* is associated with several virulence factors, including the T3SS, the A-layer protein, the AspA serine protease, and the GCAT lipase (Garduño et al. 2000, Dallaire-Dufresne et al. 2014, Vanden Bergh and Frey 2014). Various fish models have been used to characterize *A. salmonicida* virulence and assess the role of certain virulence factors, but ethical and practical concerns have led to the emergence of alternative models such as the unicellular amoebae *D. discoideum* (Froquet et al. 2007, Daher et al. 2011) and the greater wax moth larvae *Galleria mellonella* (Pintor-Cora et al. 2023). However, this last study was conducted with an *A. salmonicida* strain isolated from vegetables and cultured at 28°C, and even 37°C during the infection with *G. mellonella* larvae. Pintor-Cora and colleagues did not identify their *A. salmonicida* strain but given the bacterium's intense virulence against larvae at 37°C, it is likely a mesophilic strain rather than a psychrophilic strain, which are unable to grow at this temperature (Vincent et al. 2016). In this context, our work introduces an innovative adaptation of the *G. mellonella* larvae model to study the virulence of *A. salmonicida* subsp. *salmonicida*. *Galleria mellonella* is an invertebrate model, cost-effective, easy to implement, and ethically more acceptable than vertebrate models. Unlike the amoebae, this model also has the advantage of possessing an innate immune system (Desbois and Coote 2012, Cutuli et al. 2019, Ménard et al. 2021).

In this study, three European *A. salmonicida* subsp. *salmonicida* strains were isolated from salmonids displaying clinical signs of furunculosis. Their genomes were sequenced, analyzed and compared to existing *A. salmonicida* complete genomes. In addition, the *G. mellonella* larvae model was adapted to assess the virulence of *A. salmonicida* subsp. *salmonicida*. Overall, this study enriched the European collection of characterized *A. salmonicida* subsp. *salmonicida* genomes and contributed to the adaptation of a new infection model for this subspecies.

Materials and methods

Isolation and phenotypic identification

Aeromonas salmonicida strains were isolated at the Fish Diseases Laboratory (CER Groupe, Marche-en-Famenne, Belgium) from salmonids displaying hemorrhagic lesions in the context of routine diagnostics. Kidney tissues were sampled using a sterile Pasteur pipette and seeded on sheep blood tryptic soy agar. Plates were incubated for 48 h at room temperature (22°C–25°C). Typical brownish-producing-colonies were subcultured on the same medium to obtain a pure culture with uniform morphology. Isolates were initially identified according to their biochemical profile by API® galleries (API®20NE, BioMerieux, Marcy-l'Etoile, France) and their protein profile by MALDI-TOF® mass spectrometry (Maldi Biotyper MBT Smart, Bruker Daltonics, Bremen, Germany). The MALDI-TOF® mass spectrometry spectra were analyzed with the Maldi Biotyper 2.0 software package, MBT Compass RUO, and compared to a database containing 9607 spectral projections. Bacterial strains have been cryopreserved in tryptic soy broth with glycerol (40%*v/v*) at −20°C and −80°C.

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Antibiotic susceptibility testing

Antibiotic susceptibility profiles of the three isolated CER1, CER2, and CER3 *A. salmonicida* strains were assessed by a disk diffusion assay using Mueller–Hinton agar plates and antibiotic discs (Axonlab, Machelen, Belgium) at 22°C for 48 h. The assay was conducted with 23 antibiotics from 14 antibiotic classes (Table S1). The Gram-negative *Escherichia coli* ATCC 25922 was used as quality control (QC) strain. The EUCAST (European Committee on Antimicrobial Susceptibility Testing), CA-SFM (*Comité de l'Antibiogramme de la Société Française de Microbiologie*), and CA-SFM VET (2023 editions) clinical breakpoints and recommended inhibition diameter ranges for QC were used for result interpretation.

Extraction of genomic DNA, sequencing, assembly, and annotation

The total genomic DNA of the three CER strains was extracted using the NucleoSpin Microbial DNA Mini kit (Macherey-Nagel, Dueren, Germany) following manufacturer's instructions. Genomic libraries were prepared using Illumina DNA Prep, (M) Tagmentation (96 samples) and IDT for Illumina DNA/RNA UD Index Set A and were sequenced on an Illumina Miseq System (Illumina, San Diego, CA, USA) with a Reagent Kit v2 (500 cycles) by the platform of the *Unité génétique virale et Biosécurité* (ANSES, Ploufragan, France). The raw read sequences were cleaned using fastp v0.20.1 (CER1 and CER2) or v0.23.2 (CER3) (Chen et al. 2018), then assembled into contigs using shovill v0.9.0 (https://github.com/tseemann/shovill). Assembly quality and contamination were investigated using, respectively, QUAST v5.0.2 (https://github.com/ablab/quast, Gurevich et al. 2013) and ConFindr v0.7.4 (github.com/OLC-Bioinformatics/ConFindr, Low et al. 2019). Contigs were further reordered with Mauve Contig Mover v2.4.0.r4736 (Rissman et al. 2009) based on the A449 genome (CP000644.1), the closest reference *A. salmonicida* subsp. *salmonicida*, after removing contigs shorter than 200 bp or with a coverage depth lower than two. Assemblies were annotated using the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline v6.6.

Phylogenetic and average nucleotide identity analyses based on core genome

Comparative genomic analyses were conducted using the three CER draft genomes and the 32 *A. salmonicida* complete genomes available in the NCBI database (accessed in February 2024) (Table 1). Panaroo v1.3.0 (Tonkin-Hill et al. 2020) was used to analyze the core genome and obtain common genes between the 35 genomes (options "–clean-mode moderate –remove-invalid-genes –a core"). An alignment suitable for phylogenetic analysis was obtained using mafft (Katoh and Toh 2008) in Panaroo. The phylogenetic tree was then created using IQtree v2.2.0.3 (Minh et al. 2020) with core gene alignment (options "–safe -T AUTO -B 1000 -alrt 1000 -m MFP"). The genome of *A. bestiarum* strain YK48 (NZ_CP064746.1) was included as an outgroup to root the tree. The graphical representation of the phylogenetic trees was produced with iTOL (Letunic and Bork 2019). Average nucleotide identities (ANI) were computed using pyani (https://github.com/widdowquinn/pyani, Pritchard et al. 2015) following the MUMmer method (ANIm). An additional phylogenetic tree without branch length was created to clarify branch subdivisions. The latter was obtained with an in-house R script removing branch length from the phylogenetic tree exported from iToL (File S1).

Multi-locus sequence typing

A multi-locus sequence typing (MLST) was performed by considering the six housekeeping genes *gyrB*, *groL*, *gltA*, *metG*, *ppsA,* and *recA* (Martino et al. 2011) of the 35 *A. salmonicida* genomes listed in Table 1. Genomes were submitted to the MLST search tool v2.0.9 of the CGE platform (Center for Genomic Epidemiology, Technical University of Denmark) using MLST configuration for *Aeromonas* spp. to determine their identity against existing alleles. MLST allele sequences and profile data of the CGE platform were obtained from the PubMLST public databases (https://pubmlst.org/). Each strain was classified into its sequence type (ST) by the combination of alleles from the six genes.

Mobile genetic elements

All the MGEs were investigated based on sequencing reads in the three *A. salmonicida* CER genomes. ISs were identified using the 2020 version of ISfinder database (https://github.com/thanhleviet/ISfinder-sequences, Siguier et al. 2006) cleaned for redundant sequences. Reads were mapped on ISs using BWA-MEM2 v2.2.1 (Vasimuddin et al. 2019). Only matches with a breadth of coverage of at least 70% were retained. The approximative relative abundance of each IS was determined based on the average depth of genome coverage using SAMtools v1.14 (Li et al. 2009). *AsaGEIs* and plasmids were investigated using Bowtie2 v2.3.5.1 with the parameters "end-to-end" and "very-fast" (Langmead and Salzberg 2012), SAMtools, and Integrative Genomics Viewer v2.8.13 (Robinson et al. 2011). The reference A449 strain chromosome, *AsaGEI* variants 1a, 1b, 2a, 2b, 2c, and 2d (accession numbers KJ626178, KJ626179, KJ626180, KP861348, KU923576, and MW218448, respectively), and known plasmids (Vincent et al. 2021a) of *A. salmonicida* subsp. *salmonicida* were used as mapping reference sequences. Unmapped reads were used as query sequences in NCBI online Megablast to reveal any additional plasmid. All genomic elements with at least 50% of breadth of coverage and 50% of sequence identity were retained and used for final read mappings. The breadth of coverage and the sequence identity between each consensus mapping sequence and its corresponding reference sequence were computed. Read mappings were also used to assess the presence of prophage-derived sequences (Prophages 1, 2, and 3). As Prophages 1 and 2 were initially described in the A449 *A. salmonicida* subsp. *salmonicida* genome (Reith et al. 2008), their presence in the CER genomes was confirmed by read alignments on the two specific regions of this reference strain. As Prophage 3 is not found in all strains of this subspecies, its presence was investigated using the sequence of a lysogenic phage, the vB_AsaM_LPM4 (LPM4), which was demonstrated to be identical to Prophage 3 (accession number OL348188, Leduc et al. 2023).

Antibiotic resistance genes detection

Antibiotic resistance genes were investigated in the three CER genomes as well as in nine other *A. salmonicida* subsp. *salmonicida* complete genomes (YK, BG, A449, 01-B526, J223, J225, J228, 890 054, and SHY16-3432) using the online Resistance Gene Identifier v6.0.3 on the comprehensive antibi-

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Table 1. General information of the 35 *A. salmonicida* genomes used for phylogenetic and ANIs analyses.

Strain	Subspecies	Isolation year	Country	Source	Host	Accession number	Reference
CER1	*salmonicida*	2009	Luxembourg	Cold water fish	*Salmo trutta*	JBANEZ000000000	This study
CER2	*salmonicida*	2014	France	Cold water fish	*Salmo salar*	JBANEY000000000	This study
CER3	*salmonicida*	2004	Belgium	Cold water fish	*Salmo trutta*	JBANEX000000000	This study
BG	*salmonicida*	2013	China	Cold water fish	*Coregonus peled*	CP110650-51	Long et al. (2023)
YK	*salmonicida*	2012	China	Cold water fish	*Coreius guichenoti*	CP110648-49	Long et al. (2023)
A449	*salmonicida*	1975	France	Cold water fish	*Salmo trutta*	CP000644-46	Reith et al. (2008)
890 054	*salmonicida*	1989	Canada	Cold water fish	*Salvelinus fontinalis*	CP085533-41	Gauthier et al. (2021)
01-B526	*salmonicida*	2001	Canada	Cold water fish	*Salvelinus fontinalis*	CP027000	Vincent and Charette (2018)
SHY16-3432	*salmonicida*	2016	Canada	Cold water fish	*Salvelinus fontinalis*	CP038102-105	Massicotte et al. (2019)
J225	*salmonicida*	2017	Canada	Cold water fish	Salmon (unknown species)	CP116258-62	–
J223	*salmonicida*	1999	USA	Cold water fish	*Salmo salar*	CP048223-27	Vasquez et al. (2022)
J228	*salmonicida*	2017	Canada	Cold water fish	Salmon (unknown species)	CP116263-67	–
J411	*salmonicida*	2018	Canada	Cold water fish	*Anoplopoma fimbria*	CP052034-35	Vasquez et al. (2022)
J409	*salmonicida*	2018	Canada	Cold water fish	*Anoplopoma fimbria*	CP047374-75	Vasquez et al. (2022)
J410	*salmonicida*	2018	Canada	Cold water fish	*Anoplopoma fimbria*	CP047376-77	Vasquez et al. (2022)
JNG	–*	2018	China	Unknown fish	Unknown fish	CP122987-89	Xu et al. (2023)
RZ6S-1	*masoucida*	2016	China	Cold water fish	*Scophthalmus maximus*	CP049830-33	Wang et al. (2020)
AS1	–	2013	China	Cold water fish	*Kareius bicoloratus*	CP110654-56	Long et al. (2023)
BR19001YR	*masoucida*	2018	Korea	Cold water fish	*Sebastes schlegeli*	CP060030-33	Kang et al. (2021)
RFAS1	*masoucida*	2009	Korea	Cold water fish	*Sebastes schlegeli*	CP017143-45	Kim et al. (2018)
S68	–	2015	China	Cold water fish	*Salmo salar*	CP022182-86	–
S121	–	2015	China	Cold water fish	*Salmo salar*	CP022170-75	–
AS2	–	2016	China	Cold water fish	*Salmo salar*	CP110652-53	Long et al. (2023)
S44	–	2014	China	Cold water fish	*Salmo salar*	CP022176-81	–
FN1	–	2022	China	Environment	Poultry farm	CP101948	–
34mel	*pectinolytica*	1988	Argentina	Environment	River	CP022426	Pfeiffer et al. (2018)
A527	–	2007	India	Food	Food	CP022550	Vincent et al.2017
ZAS	–	2016	China	Mammal	Pig	CP110647	Long et al. (2023)
SRW-OG1	–	2018	China	Warm water fish	*Epinephelus coioiaes*	CP051883	Zhong et al. (2021)
wx3-1	*masoucida*	2022	China	Shellfish	*Procambarus clarkii*	CP102172	Yang et al. (2024)
AS3	–	2012	China	Environment	Soil	CP110645-46	Long et al. (2023)
57	–	2020	China	Food	Meat	CP124841-44	–
ASscau0313	–	2019	China	Unknown fish	Carp (unknown species)	CP091480	–
O23A	–	2010	Poland	Environment	Mine	CP021654-58	Uhrynowski et al. (2017)
29	–	2020	China	Food	Meat	CP124840	–

*: not known.

otic resistance database (CARD) v3.3.0 (Alcock et al. 2023) and ABRicate v1.0.1 (https://github.com/tseemann/abricate) on the ResFinder database v4.1 (Bortolaia et al.). Only genes with a minimum length coverage of 80% and identity of 95% were retained. Additionally, the *cat* gene conferring chloramphenicol resistance was investigated through TBLASTN alignments (*e*-value $\leq$1e-20) and the presence of reads at the best matching loci according to *e*-values was confirmed by read mapping with Bowtie2 v2.3.5.1.

Virulence genes detection and pAsa5 plasmid genotyping

Virulence-related genes in the three CER genomes were predicted using the *Aeromonas* virulence genes set of the virulence factors database (VFDB, https://www.mgc.ac.cn/VFs/, Liu et al. 2022) along with nine other *A. salmonicida* subsp. *salmonicida* complete genomes (YK, BG, A449, 01-B526, J223, J225, J228, 890 054, and SHY16-3432). This analysis was complemented by the search for additional genes encoding some species-specific important virulence factors not included in the *Aeromonas* set of VFDB, such as the A-layer protein (*vapA*), AspA protease (*aspA*), GCAT lipase (*satA*), A1 phospholipase (*pla*), and AopP T3SS-associated effector (*aopP*) in CER genomes (Garduño et al. 2000, Dallaire-Dufresne et al. 2014, Vanden Bergh and Frey 2014), using the MyDdFinder v2.0 search tool of the CGE platform.

As mentioned above, *A. salmonicida* grown at 25°C or higher can undergo IS-dependent recombinations of its pAsa5 plasmid leading to the loss of the T3SS locus. Consequently, the pAsa5 plasmid integrity was investigated by Polymerase Chain Reaction (PCR) targeting four genes covering the pAsa5 length: *P5G011*, *ati2*, *ascC,* and *resD*, with the *ati2* and *ascC* genes being part of the T3SS locus (Daher et al. 2011). The three pAsa5 plasmid recombinations described to date (11B/C, 11A/C, and 5Z/A) in A449 or 01-B526 derived strains were also tested by PCR using IS-associated primer sequences designed by Tanaka and colleagues (Tanaka et al. 2012, 2017). A schematic representation of the targeted genes and recombinations on the pAsa5 plasmid is shown in Fig. 1.

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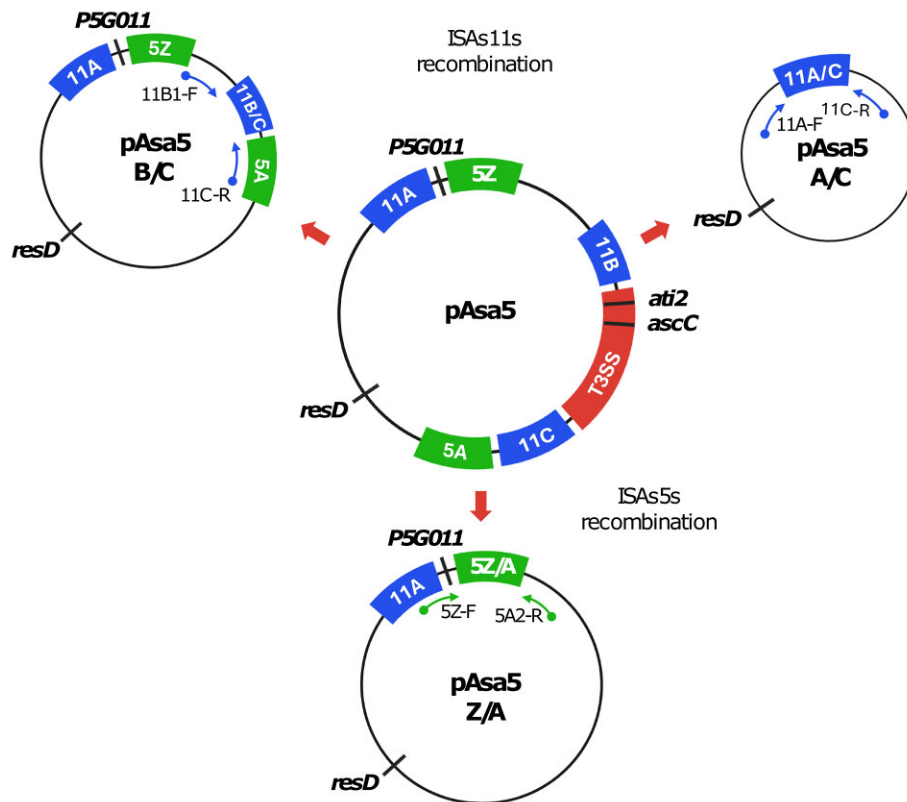

Figure 1. Schematic representation of an intact 01-B526 pAsa5 plasmid with IS*As11* and IS*As5* copies, targeted genes locations, and its known IS-dependent homologous recombination events. The 01-B526 pAsa5 plasmid carries the T3SS locus (*red box*), and several copies of IS*As11* (11A, 11B, and 11C; *blue boxes*) and IS*As5* (5A and 5Z; *green boxes*). The four annotated genes *P5G011*, *ati2*, *ascC*, and *resD* are targeted by PCR to control plasmid integrity (Daher et al. 2011). The pAsa5 B/C results from the IS*As11B*/IS*As11C* recombination (*top left circle*), the pAsa5 A/C from the IS*As11A*/IS*As11C* (*top right circle*), and pAsaZ/A from the IS*As5Z*/IS*As5A* (*bottom circle*). These recombinations can be revealed using primer pairs 11B1-F/11C-R, 11A-F/11C-R (*blue arrows*), and 5Z-F/5A2-R (*green arrows*), respectively (Tanaka et al. 2012, 2017). These primer pairs would not produce any amplicon on an intact pAsa5 plasmid as they are too far apart.

The primer pairs used are described in Table 2. DNA templates were obtained as described above and PCR assays were performed in duplicate with a Vapo.Protect Mastercycler® Pro (Eppendorf, Hambourg, Germany) according to manufacturer's recommendations in 50 µl reaction volume. The enzyme used was the FastGene® Taq ReadyMix 2X (Nippon genetics, Tokyo, Japan). The primer pairs (Integrated DNA Technologies, Leuven, Belgium) were used at a final concentration of 0.5 µM. For short amplicons (≤550 bp), 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 30 s at 72°C, followed by a final extension for 10 min at 72°C. For longer amplicons, the PCR program was similar but with an annealing temperature of 55°C and an extension time of 3 min for each cycle. The samples were separated on 1.5% agarose gels with Midori Green DNA stain.

Virulence assessment in the *G. mellonella* larvae model

Bacterial strains and culture conditions

In addition to the CER1, CER2, and CER3 strains, the two 01-B526 and 01-B516 *A. salmonicida* subsp. *salmonicida* strains containing an intact pAsa5 plasmid were used as controls. Moreover, a 01-B526-derived strain, the R35, no longer possessing a functional T3SS due to an 11B/C recombination (Tanaka et al. 2012, 2017) was also used to assess the role of this virulence factor in the infection of *G. mellonella* by *A. salmonicida* subsp. *salmonicida*. These three additional strains have been obtained from the *Institut de Biologie Intégrative et des Systèmes* (IBIS, Laval University, Quebec, Canada). The pAsa5 plasmid integrity in the 01-B526 and 01-B516 strains and its rearrangement in the R35 strain were checked by PCR in duplicate using the same primer pairs described above (Table 2). Liquid bacterial cultures corresponding to a concentration of 10^8 CFU·ml^{-1} were obtained by incubating 50 µl of an overnight liquid culture of each of these six strains in fresh Luria–Bertani medium with shaking until reaching an optical density at 600 nm (OD_{600}) of 0.3. To preserve the pAsa5 plasmid integrity, this experiment was carried out at 18°C.

Galleria mellonella *assay*

A total of 740 *G. mellonella* larvae (Nusect, Deerlijk, Belgium) in their final larval stage were divided into 37 groups of 20 larvae placed in 9 cm Petri dishes. The first 36 groups (6 x 6 groups) were inoculated with 10 µl of bacteria at six different concentrations per bacteria (from 10^1 to 10^6 CFU/larva). The last group was inoculated with 10 µl of Phosphate Buffered Saline (PBS) as a control group. Each larva was inoculated into the hemocoel via the last left proleg with a BD Plastipak™ 1 ml sterile syringe (Becton-Dickinson, Franklin Lakes, NJ, USA) and a sterile 30-gauge needle (Terumo Corporation, Tokyo, Japan) mounted on an automatic injector

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Table 2. Primer sequences used for pAsa5 plasmid and recombination genotyping.

Target	Primer	Sequence (5′ to 3′)	Amplified fragment length (bp)	Reference
pAsa5 genotyping				
resD	*resD*-F	TCAGAAACTTGGCCATCGCTCACA	504	Daher et al. (2011)
	resD-R	TCAACGTCCCAGTAACAGCGGATT		
P5G011	*P5G011*-F	TTGTCTGACTCTGCATCCAGCGAA	295	
	P5G011-R	AGGTGCCTGAATTACCACCAGTGA		
ati2	*ati2*-F	TTGACCTGTGGTCAGGTTAGCAGT	550	
	ati2-R	ACACGATGATACGCACCTAGCCAA		
ascC	*ascC*-F	GCATTGGAGCAACAGTCCCA	476	
	ascC-R	CCTTCAATCCCCTTGCGAT		
pAsa5 recombinations				
11B/C	11B1-F	GCGCACCACCACCATTTAATGTCA	∼2000	Tanaka et al. (2012)
	11C-R	AACTGGCAAGGATAGAGCTGCTGA		
11A/C	11A-F	AATAGGTGTCGCAAGCTGGGTTGA		
	11C-R	AACTGGCAAGGATAGAGCTGCTGA		
5Z/A	5Z-F	TCAGTGCCAATCAAATCAAACTCC	2888	Tanaka et al. (2017)
	5A2-R	TCCACGACAAACTGAATAAACTGG		
Chromosomal control				
tapA	*tapA*-F	ACATGAAGAAGCAATCAGGC	∼500	Ebanks et al. (2006)
	tapA-R	AGAGGTCATGCGTTAGCAG		

(Cole Parmer, Vernon Hills, IL, USA). Groups were maintained at 18°C and the time of death of each larva was recorded every 12 h over 4 days. Larvae were considered dead when they displayed no movement in response to physical stimulation. Dead larvae were systematically removed to avoid potential re-contamination during the experiment. In parallel, back-titrations were performed by serial 10-fold dilutions to confirm inoculum concentrations. Kaplan–Meier survival curves and log-rank tests were performed using GraphPad Prism v8.0.2. to highlight any significant difference in the survival rates between groups over time ($P \leq 0.05$). Adjusted *P*-values were generated using R v4.2.2. The lethal time 50% (LT_{50}) values were estimated for each group using nonlinear regressions in GraphPad Prism v8.0.2 to compare virulence at identical bacterial concentrations.

Results

Isolation and phenotypic identification

Two *A. salmonicida* strains, named CER1 and CER3, were isolated in 2009 and 2004 from brown trout (*Salmo trutta*) coming from fish farms in Luxembourg and Belgium, respectively. A third strain, named CER2, was isolated in 2014 from a parr (*Salmo salar*) coming from the *Conservatoire National du Saumon Sauvage* (CNSS, Chanteuges, France) and arrived on ice in the laboratory. Fish displayed hemorrhages in the eyes, at the base of the pectoral fins, and around the anus. The three CER1, CER2, and CER3 isolates have been identified as *A. salmonicida* subsp. *salmonicida* by API®20NE galleries (identities of 99.8%, 99.6%, and 99.8%, respectively). Phenotypic characteristics of the three isolates are shown in Table S2. The MALDI-TOF® identity scores for *A. salmonicida* subsp. *salmonicida* were between 1.76 and 1.84.

General features of the three *A. salmonicida* sequenced genomes

The *de novo* assembly of the CER1 and CER3 genomes resulted in lengths of 4.66 Mb in 174 contigs and 4.85 Mb in 193 contigs, the largest contigs being 251 533 and 240 863 bp, respectively. The CER2 strain presented a slightly longer assembly with 5.00 Mb in 177 contigs, the largest being 382 412 bp. Total assemblies had N50 values ranging from 65 348 to 85 304 bp and close GC content ranging from 58.30% to 58.54%. No contamination was reported for the three strains. The genome fractions reported by QUAST are 97.074%, 97.132%, and 97.150%, respectively, for CER1, CER2, and CER3 (while coverage on assembly is 100% for the three strains). These three assemblies were deposited on NCBI database under the BioProject PRJNA1075456 and the BioSamples SAMN40034175, SAMN40034220, and SAMN40034310 for CER1, CER2, and CER3 strains, respectively. Whole genome overviews are shown in Table S3.

Antibiotic resistance phenotypes and gene compositions

Antimicrobial susceptibility testing by disk diffusion assay (Table S4) showed that all three CER strains presented resistance to vancomycin, clindamycin, and nalidixic acid, and a surprising susceptibility to all the beta-lactams tested, except against cephalothin. The CER2 and CER3 strains showed a more resistant profile with additional resistance to tetracycline, sulfonamide, and a combination of trimethoprim and sulfamethoxazole. Moreover, the CER2 strain was the only one to present florfenicol and chloramphenicol resistance. Resistance phenotypes of the three CER strains against frequently used antibiotics to control furunculosis in fish are represented in Fig. 2a. While the *cphA5* gene, conferring resistance to cephalosporins and carbapenems, was detected in each *A. salmonicida* genome considered in the antibiotic resistance gene analysis, all the remaining resistance genes clustered in five distinct strains: CER2, CER3, A449, 890 054, and SHY16-3432. These genes confer resistance to various classes of antibiotics, including phenicol (*floR*, *cat*), tetracycline [*tet*(A), *tet*(C), *tet*(E), *tet*(G)], sulfonamide (*sul1*, *sul2*), diaminopyrimidine (*dfrA1*, *dfrB3*), aminoglycoside (*aadA*, *aadA2*), and carbapenem (*CARB*-2). Additionally, a resistance gene against quaternary ammonium compounds and certain cationic detergents (*qacEdelta1*) was also identified in these five strains (Fig. 2b).

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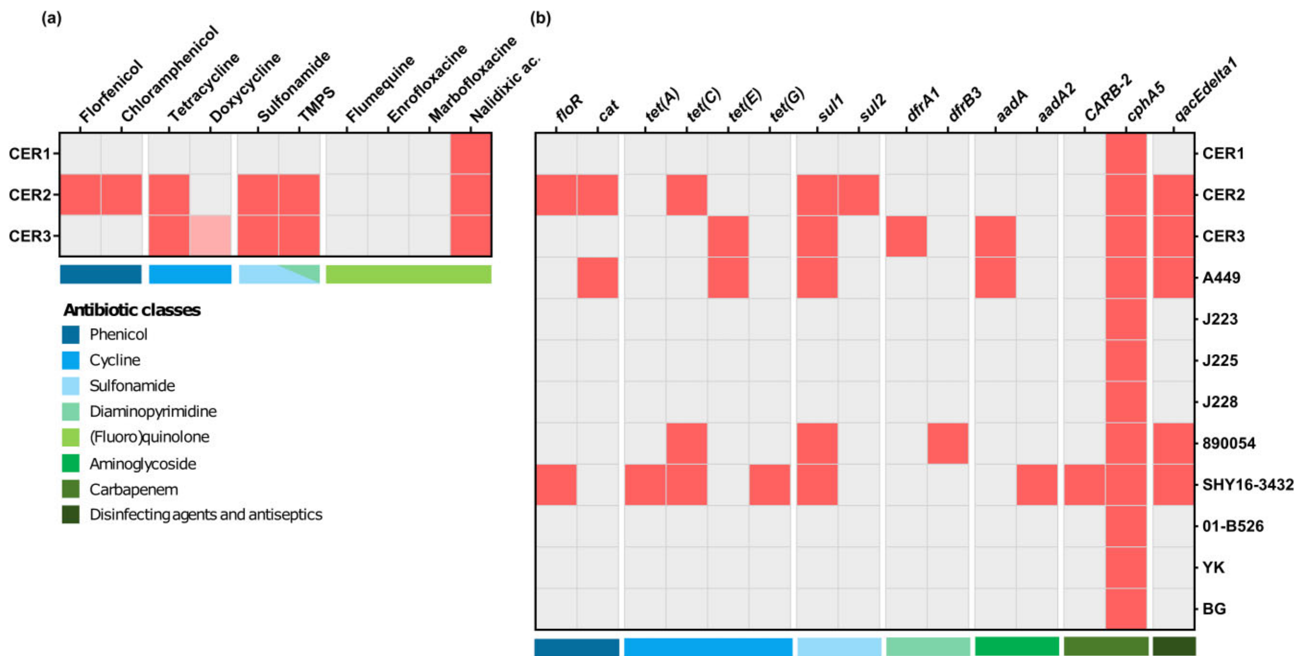

Figure 2. Antibiotic resistance phenotypes observed in the *A. salmonicida* CER1, CER2, and CER3 strains against frequently used antibiotics to control furunculosis in fish along with antibiotic resistance gene compositions of the CER genomes and the nine other *A. salmonicida* subsp. *salmonicida* complete genomes. (a) The red, light red, and grey colors represent, respectively, the resistant, intermediate, and susceptible phenotypes using EUCAST, CA-SFM, and CA-SFM VET (2023 editions) clinical breakpoints. (b) The red and grey colors represent, respectively, the presence and absence of genes sought through the CARD and ResFinder databases with ≥80% of coverage and ≥95% of identity. TMPS, trimethoprim/sulfamethoxazole; Ac., acid.

Comparative genomics and MLST analysis

A total of 10 256 genes were predicted across the 35 genomes of *A. salmonicida* considered in this study, which constituted the pan-genome, and 3204 genes were conserved among them, which represented the core genome. Phylogenetic analysis based on the core genome clustered the 35 strains into two distinct groups (Fig. 3). The first group (orange branch group) comprises 11 strains isolated from highly diversified sources which are quite distant from each other. The second group comprises 24 strains isolated from cold-water fish for which genetic distances are smaller (Fig. S1). Within this second group, the distribution of phyla revealed two subgroups (blue and green branch groups) of 12 strains each. Based on previous studies (Long et al. 2023, Godoy et al. 2024), these three branch groups have been referred to as the mesophilic, the typical psychrophilic, and the atypical psychrophilic groups, respectively (Fig. 3). The three CER strains were distributed in the typical psychrophilic group, around the French *A. salmonicida* subsp. *salmonicida* A449 reference strain.

ANI analysis showed a high genomic identity between these 35 strains with values ranging from 97.016% (SRW-OG1 vs. YK) to 99.999% (J228 vs. J223). This analysis also distributed the strains into mesophilic, typical psychrophilic, and atypical psychrophilic groups. The mesophilic group contained the lowest ANI values among the three groups, i.e. from 97.104% (57 vs. FN1) to 97.838% (FN1 vs. 34mel). The typical psychrophilic group included the highest ANI values of the three groups, i.e. from 99.922% (YK vs. J225) to 99.999% (J228 vs. J223). The atypical psychrophilic group showed an intermediate range, i.e. from 99.797 (RZ6S-1 vs. J411) to 99.994 (S68 vs. S64). Concerning the three CER strains, genome identities were 99.979% between CER1 and CER3, 99.967% between CER1 and CER2, and 99.964% between CER2 and CER3. Compared to the 32 already sequenced *A. salmonicida* genomes, CER1, CER2, and CER3 showed the highest identity with the French strain A449 (99.966%, 99.965%, and 99.964% identity, respectively) (Fig. 3).

For the MLST profiling based on six housekeeping genes (*gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA*), the 35 *A. salmonicida* strains can be divided according to the same three subdivisions. Mesophilic strains showed a mosaic of different alleles for each of the six genes (Table S5). Except for the 34mel strain (ST 1952), strains in this group were assigned to a single or several nearest MLST profiles. No dominant profile could be identified. Typical psychrophilic strains exclusively displayed type 2 alleles for each of the six genes considered, which assigned them to the ST 2. Type 2 alleles were also observed for the four genes *gyrB*, *groL*, *metG*, and *recA* in atypical psychrophilic strains. However, the atypical subgroup differs from the typical one in the *gltA* gene for which allelic type 872 was exclusively observed, and in the *ppsA* gene for which two allelic types (2 and 966) were observed. The ST 1799 was assigned to atypical psychrophilic strains, except for strains J409, J410, and J411 for which the profile remained poorly defined (1799, 2, or 1973) (Fig. 3).

Mobile genetic elements

The ISs identification based on sequencing reads within the three CER strains of *A. salmonicida* revealed a repertoire of 17 different ISs from 11 IS families (Fig. 4). The three strains shared a common repertoire of 12 ISs from 8 IS families (Table S6). These ISs were comparably represented within the three CER strains, except for IS*As29* which was present in lower abundance in CER2. The strains CER2 and CER3 dif-

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Figure 3. Phylogenetic relationship among the CER1, CER2, CER3, and 32 *A. salmonicida* complete genomes based on their core genes (with branch length, *left tree* ; without branch length, *top tree*) and heatmap representing the ANI values. Beside the heatmap, the first two columns represent the isolation source and the associated country of each strain. The third column represents the simplified MLST profile of each strain (if one nearest profile has been proposed, this profile has been assigned; if several nearest profiles have been proposed, "Unknown" has been assigned for the clarity of the figure). The outgroup is not represented on this figure. Branch support was assessed using 1000 bootstrap replicates and 1000 approximate likelihood ratio tests (aLRT). G.D.L., Grand Duchy of Luxembourg.

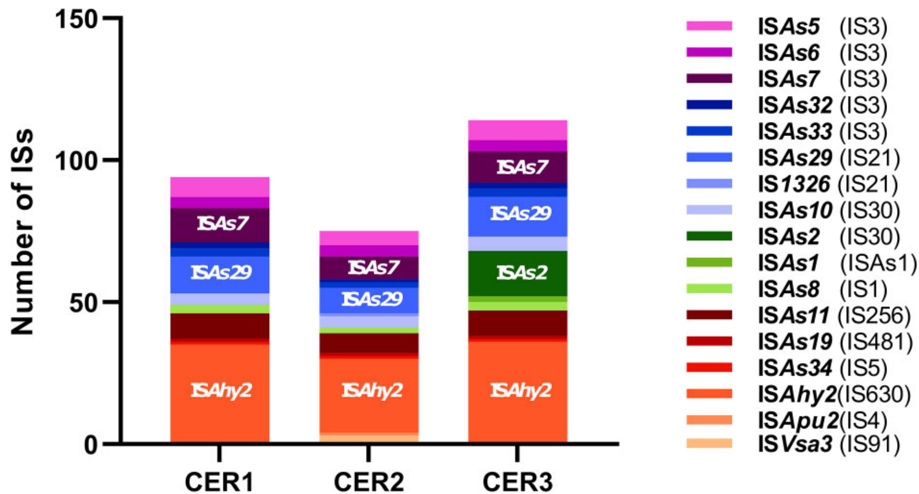

Figure 4. Distribution of relative ISs abundance in the *A. salmonicida* CER1, CER2, and CER3 genomes. The relative copy numbers per IS in each genome are described in Table S6.

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fered from the CER1 in the presence of additional ISs poorly represented, except for IS*As2* which was present in large numbers in CER3.

Read mappings of the three CER genomes on the closest reference *A. salmonicida*-specific plasmid sequences revealed the presence of several plasmids with different degrees of completeness. In each CER strain, the three small plasmids pAsa1, pAsa2, and pAsal1 were completely or nearly completely represented (≥99.70% coverage, ≥99.70% identity). The CER2 strain harbored three additional complete or nearly complete plasmids (100% coverage, ≥99.84% identity): the two small pAsa3 and pAsa7, and a variant of the pRAS3 family. The CER3 strain also matched nearly completely with an additional variant of the long pAsa4 plasmid, the pAsa4b (100% coverage, 99.96% identity). The use of *AsaGEI* sequences as mapping references revealed the presence of the nearly complete *AsaGEI2d* variant (52 648 bp; 99.98% coverage, 99.97% identity) in the French CER2 genome while none of them were found in the CER1 and CER3. Breadths of coverage and sequence identities of all detected plasmids, including pAsa5, pAsa6, and pAB5S9b for which only partial sequences were found, and the *AsaGEI2d* are shown in Table S7. No additional plasmids with ≥50% coverage and ≥50% sequence identity were found using unmapped reads as query sequences in NCBI online Megablast.

Finally, regarding prophage-derived sequences, read mappings on the reference European A449 strain chromosome confirmed the presence of the two prophage-derived regions typically associated with Prophages 1 and 2 in the three CER chromosomes. No match was found for Prophage 3 in any of the three strains using the lysogenic LPM4 phage sequence as a mapping reference (identical to Prophage 3, Leduc et al. 2023).

Virulence genes and pAsa5 plasmid genotyping

A total of 252 virulence genes associated with adherence, secretion systems, and toxin classes were investigated using the VFDB in the genomes of the three CER strains, along with nine other *A. salmonicida* subsp. *salmonicida* complete genomes (YK, BG, A449, 01-B526, J223, J225, J228, 890 054, and SHY16-3432) (Table S8). The distribution of predicted genes for each virulence factor across these 12 strains is summarized in Fig. 5. This analysis revealed that five strains were distinguished by the absence of most genes related to one or more secretion systems: the T3SS and T6SS for YK, the T6SS for BG, and the T3SS for CER2 and CER3. The absence of most T3SS genes in both CER2 and CER3 strains explains why they harbor a reduced total number of virulence genes (181 and 183 predicted genes, respectively) compared to the majority of other strains (between 223 and 225 predicted genes). The CER1 strain, with only 100 predicted genes, was unique in its absence of most T2SS and T3SS genes, as well as possessing a reduced number of genes associated with the other virulence factor classes investigated in this study. Furthermore, the search for additional genes coding for other important virulence factors in *A. salmonicida* but not included in the *Aeromonas* set of VFDB indicated that all three CER strains possess the *vapA* (A-layer protein), *aspA* (AspA protease), *satA* (GCAT lipase), *pla* (A1 phospholipase), and *aopP* (AopP T3SS-associated effector) genes.

The pAsa5 plasmid genotyping through the four genes *P5G011*, *ati2*, *ascC*, and *resD* revealed an incomplete plasmid sequence for the three CER strains. The plasmid is indeed present in each isolate but lacks the *P5G011*, *ati2*, and *ascC* genes. An 11A/C recombination was subsequently demonstrated in the three CER strains by PCR genotyping (Fig. 1 and Table 3).

Virulence assessment in the *G. mellonella* larvae model

The CER strains lacking T3SS, along with three other strains (01-B526, 01-B516, and R35), were tested using the larval infection model. The 01-B526 and 01-B516 strains possess a T3SS, as confirmed by PCR genotyping, while the R35 strain, derived from 01-B526, no longer carries it due to a B/C recombination of the pAsa5 plasmid (Table 3) (Tanaka et al. 2012, 2017).

The 01-B526, 01-B516, CER1, CER2, CER3, and R35 strains were all responsible for a significantly lower larval survival rate across the six bacterial concentrations tested (from 10^1 to 10^6 CFU/larva) compared to the control group injected with PBS at 18°C. Specifically, the 01-B526 and 01-B516 virulent control strains exhibited a strong dose-dependent virulence in this model, with survival rates reaching 0% at 96 h post-inoculation (HPI) for nearly all groups. At the highest dose tested (10^6 CFU/larva), first mortalities were recorded at 24 HPI for 01-B526 and 36 HPI for 01-B516, with survival rates dropping to 15% and 5% at 36 HPI, respectively. Kaplan–Meier survival curves at all six concentrations for these two control strains are shown in Figs 6a and b. For groups infected with the CER1, CER2, and CER3 strains, the same trend as for control strains was observed, although the log-rank tests revealed a significantly delayed mortality in CER1-inoculated groups at lower doses compared to those inoculated with 01-B516 (10^1 CFU/larva, $P = 0.0085$) and CER3 (10^2 CFU/larva, $P = 0.0045$; 10^1 CFU/larva, $P = 0.019$). A comparison of survival curves for the 01-B526, 01-B516, CER1, CER2, and CER3 strains at the highest (10^6 CFU/larva) and lowest (10^1 CFU/larva) concentrations is shown in Fig. 6c. Finally, when groups inoculated with the rearranged R35 strain were compared to those inoculated with the wild-type strain (01-B526), a similar dynamic was observed except at the lowest dose (10^1 CFU/larva) where a significantly delayed mortality was demonstrated ($P = 0.0414$). A comparison of survival curves for the R35 and 01-B526 strains at the highest (10^6 CFU/larva), an intermediate (10^4 CFU/larva) and the lowest (10^1 CFU/larva) concentrations is shown in Fig. 6d. Complete survival results for the CER1, CER2, CER3, and R35 strains are presented in Fig. S2.

In addition to survival rate comparisons, the lethal time 50% (LT_{50}) values were estimated to refine the evaluation of each strain's virulence (Table 4). As expected, the LT_{50} mean values increased progressively with decreasing bacterial doses, reflecting a dose-dependent effect. Notably, standard deviation values also tended to increase in the same way, illustrating differences in virulence at lower bacterial concentrations.

Discussion

It is currently well known that *A. salmonicida* strains can be classified as typical or atypical depending on their isolation

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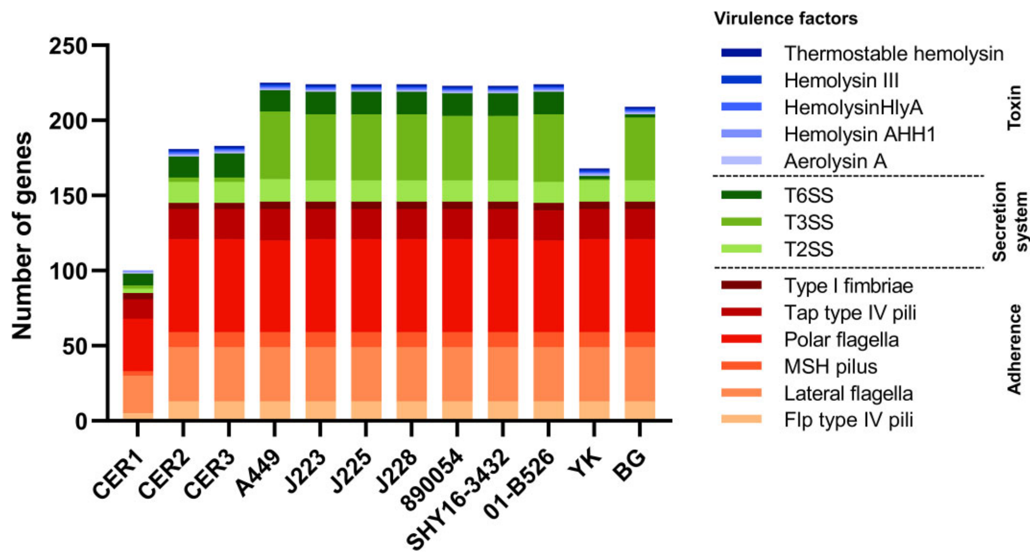

Figure 5. Distribution of virulence genes per virulence factor in the CER1, CER2, CER3, and nine other *A. salmonicida* subsp. *salmonicida* genomes. The number of virulence genes in each genome was predicted based on the VFDB.

Table 3. pAsa5 profile of 01-B526 and 01-B516 *A. salmonicida* subsp. *salmonicida* control strains, R35, CER1, CER2, and CER3 strains.

	01-B526	01-B516	R35	CER1	CER2	CER3
pAsa5 genotyping						
resD	+	+	+	+	+	+
P5G011	+	+	+	−	−	−
ati2	+	+	−	−	−	−
ascC	+	+	−	−	−	−
Recombinations						
11B1/C	−	−	+	−	−	−
11A/C	−	−	−	+	+	+
5Z/A2	−	−	−	−	−	−
Chromosomal control						
tapA	+	+	+	+	+	+

source (Wiklund and Dalsgaard 1998, Dallaire-Dufresne et al. 2014), and as mesophilic or psychrophilic according to their optimal growth temperature (Pavan et al. 2000, Boone et al. , Vincent and Charette 2022). In 2023, Long and colleagues proposed a combination of these two classifications with the clustering of *A. salmonicida* strains into three distinct groups: (i) typical psychrophilic, (ii) atypical psychrophilic, and (iii) mesophilic groups. In their view, these three groups could correspond respectively to (i) strains of *A. salmonicida* subsp. *salmonicida* isolated from cold-water fish living in rivers or lakes, (ii) strains of the other subspecies isolated from marine benthic fish living in temperate water, and (iii) strains isolated from warm hosts, the environment or warm-water fish. They also demonstrated that the phylogenetic relationships of the strains were consistent with their isolation source, but not with the originating country. Notably, mesophilic strains were recently isolated from brook trouts in Quebec (Canada), highlighting the fact that this group can still originate from cold-water fish (Attéré et al. 2023). Following our phylogenetic analysis, the CER1, CER2, and CER3 *A. salmonicida* strains were assigned to the typical psychrophilic group containing exclusively the *salmonicida* subspecies (Fig. 3). More specifically, it has been shown that the three CER strains and the typical *A. salmonicida* subsp. *salmonicida* A449 strain share a common ancestor and cluster together with 99.96% nucleotide identity. This comparative analysis effectively distinguished the three aforementioned groups, further validating previous observations (Vasquez et al. 2022, Long et al. 2023, Godoy et al. 2024), although the inclusion of a larger number of genomes would have broadened the impact of current results. Interestingly, our analysis links to associate three poorly described Chinese strains (29, 57, and ASscau0313 strains) to the mesophilic group.

Despite the outstanding interspecies and intraspecies genetic variability in *Aeromonas* spp., several MLST profiles were proposed for this genus (Martinez-Murcia et al. 2011, Martino et al. 2011, Roger et al. 2012). Based on a set of six genes (gyrB, groL, gltA, metG, ppsA, and recA), Martino et al. (2014) showed a specific distribution of *Aeromonas* species depending on their isolation source, highlighting the adaptation toward specific habitats. In our study, we inves-

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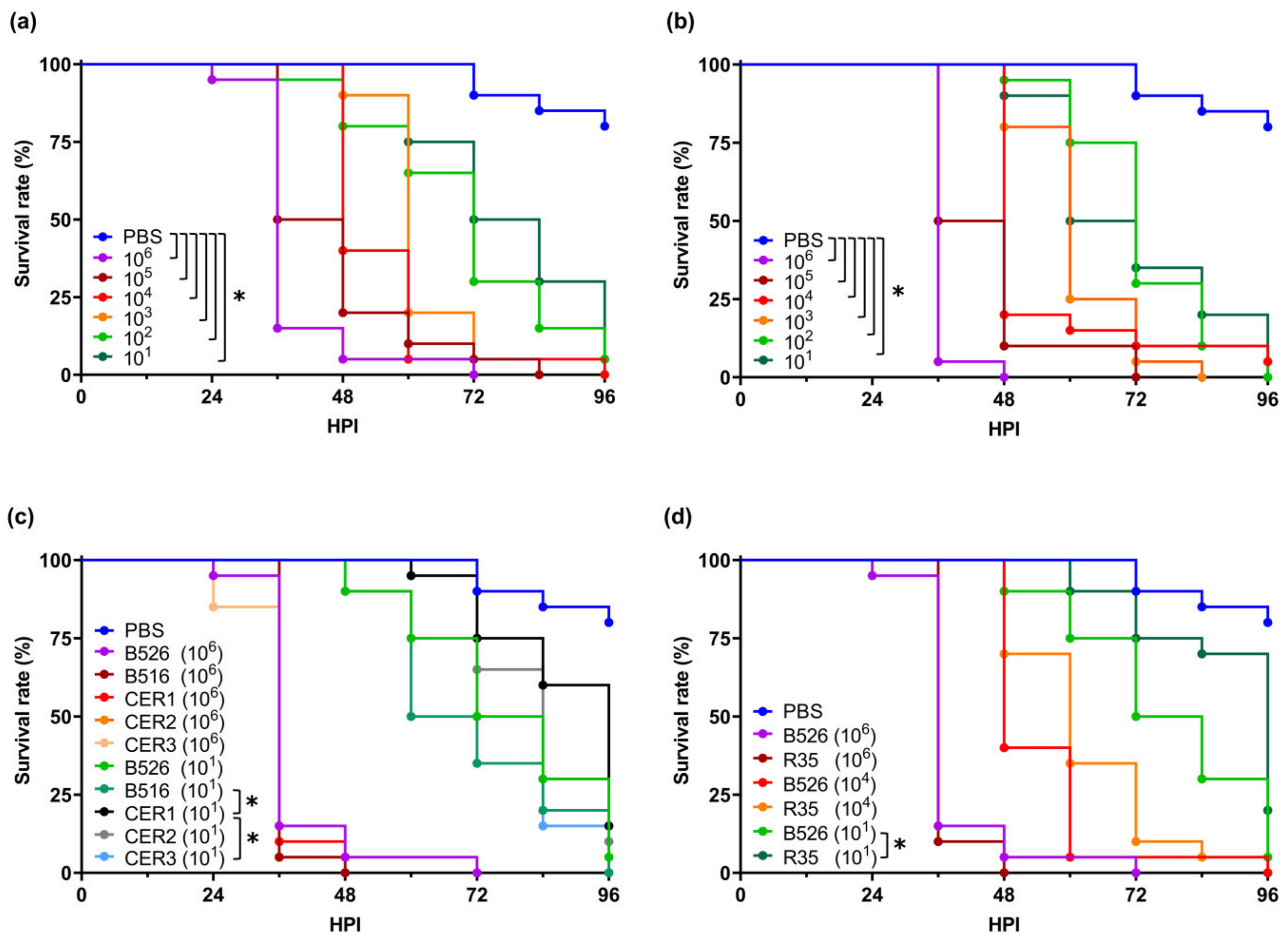

Figure 6. Kaplan–Meier survival curves of *G. mellonella* larvae groups inoculated at 18°C with PBS or with *A. salmonicida* subsp. *salmonicida* (a) 01-B526 control strain at six different concentrations, (b) 01-B516 control strain at six different concentrations, (c) 01-B526, 01-B516, CER1, CER2, and CER3 strains at the highest and lowest concentrations, (d) 01-B526 and R35 strains at the highest, an intermediate, and the lowest concentrations. Each group contained 20 larvae. HPI, hours post-inoculation. *P*-value (∗) ≤0.05.

Table 4. Lethal time 50% (LT_{50})-based comparison of *A. salmonicida* subsp. *salmonicida* virulence across six inoculation doses in *G. mellonella.*

Strain	LT_{50} (hours post-inoculation) at each dose (CFU/larva)					
	10^6	10^5	10^4	10^3	10^2	10^1
01-B526	30.37	37.09	46.79	56.31	64.67	73.22
01-B516	29.98	36.13	45.45	54.67	66.88	64.01
CER1	30.17	37.86	50.73	54.96	78.68	86.67
CER2	29.98	37.47	49.77	54.10	66.80	76.97
CER3	29.98	34.78	44.20	49.10	59.09	72.36
R35	30.17	37.66	55.06	62.55	63.80	89.17
Mean (± SD)	30.11 (0.16)	36.83 (1.18)	48.67 (4.00)	55.28 (4.34)	66.65 (6.54)	77.07 (9.45)

tigated the MLST profile of the three CER genomes as well as 32 *A. salmonicida* complete genomes based on this proposed set of six genes to determine if a similar trend within the *A. salmonicida* species could be observed. Psychrophilic strains presented two distinct MLST sequence types, ST 2 or ST 1799, while mesophilic strains presented a more diversified panel of MLST profiles (Table S5). Based on this observation, the MLST profile could represent an additional argument to distinguish mesophilic and psychrophilic strains of *A. salmonicida*. Furthermore, among psychrophilic strains, the possibility of classifying typical and atypical strains according to the MLST profile could be considered knowing that typical strains were exclusively assigned to the ST 2 and atypical strains were mainly assigned to the ST 1799. However, this may be imprecise as intermediate results were observed for the three atypical strains J409, J410, and J411. Analysis of a larger number of strains would be required to confirm this hypothesis.

The genomic plasticity of *A. salmonicida* is partly due to its ISs, particularly the IS*As1*, IS*As2*, IS*As5*, and IS*As11* (also known as IS*As3*), given their implication in gene inactivation and plasmid recombination. More precisely, these endogenous events involve the loss of the outer A-layer protein after the transposition of IS*As1* and IS*As2* in the *vapA* or *abcA* genes,

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which are necessary for the assembly and display of this surface protein (Gustafson et al. 1994, Chu et al. 1995). For its part, the loss of the T3SS locus on the pAsa5 plasmid by homologous recombinations is associated with IS*As11* or IS*As5* (Tanaka et al. 2012, Trudel et al. 2013, 2017). Moreover, the activity of some of these ISs has already been shown to be increased during growth at 25°C or higher (Gustafson et al. 1994, Tanaka et al. 2012), which was the case for all three CER strains. In this study, the ISs repertoire of CER strains is consistent with previous studies for typical psychrophilic strains in which IS*Ahy2*, IS*As7*, and IS*As29* were the most represented; IS*As19*, IS*As32*, IS*As33*, and IS*As34* the least represented, and IS*As4* absent (Fig. 4 and Table S6) (Vasquez et al. 2022, Long et al. 2023). More interestingly, ISs known to be involved in mutagenesis events, such as IS*As11* and IS*As5*, were found in multiple copies in all three strains, and CER3 was the only one to carry IS*As1* and IS*As2*. The presence of these MGEs, particularly IS*As11* here, could therefore explain the recombination events observed within the three CER pAsa5 plasmids. Indeed, the European strain A449, from which the CER strains are the closest, would be less prone to IS*As5*-recombination because its pAsa5 contains fewer copies of this IS than the 01-B526, the strain which led to the discovery of the 5Z/A recombination (Tanaka et al. 2017).

Three different prophages (Prophages 1, 2, and 3) and six *AsaGEIs* (*1a*, *1b*, *2a*, *2b*, *2c*, and *2d*) have already been identified in the genome of *A. salmonicida* subsp. *salmonicida* (Emond-Rheault et al. 2015a, Vincent et al. 2016, Leduc et al. 2023). The study of the composition in prophages and genomic islands in this *Aeromonas* species is interesting from an epidemiological perspective because some of these viral sequences seem to be linked to the geographical origin of the strains. These elements include the Prophage 3, *AsaGEI1a*, and *AsaGEI2a* that are found almost exclusively in North American strains. European strains show either no *AsaGEI*, or variants *1b*, *2b* or the recently identified *AsaGEI2d* (Emond-Rheault et al. 2015b, Vincent et al. 2021b, Reith et al. 2008). As expected, the presence of the Prophages 1 and 2 together with the absence of the Prophage 3 have been confirmed in the three CER strains from Europe. Regarding *AsaGEIs*, none of them was found in strains from Luxembourg (CER1) and Belgium (CER3), while the recently identified *AsaGEI2d* variant was detected in the strain from France (CER2). The presence of this variant in the French strain coming from the *Conservatoire National du Saumon Sauvage* (CNSS, Chanteuges, France) is consistent with previous results since *AsaGEI2d* was initially discovered in French strains isolated in the same structure (Vincent et al. 2021b).

In Europe, only tetracycline, (fluoro)quinolone, sulfonamide, and phenicol classes are approved to treat furunculosis in fish (European Medicines Agency 2022). However, it has already been demonstrated that intensive use of these antibiotics is correlated with a growing number of antibiotic-resistant *A. salmonicida* (Morin 2010). The French CER2 and Belgian CER3 strains expressed resistance against frequently used antibiotics, e.g. tetracycline, florfenicol, sulfonamide, and trimethoprim/sulfamethoxazole (Fig. 2), which is in line with this trend. From a genomic perspective, the CER2 strain carries three plasmids or close variants (pAsa7, pRAS3.5-like, and pAB5S9b-like) known to encode *cat*, *tet*(C), *tet*(H), *sul2*, *strA/B*, and *floR* genes (Vincent et al. 2014, Vincent et al. 2021b, Fournier et al. 2022). The CER3 harbors a sequence similar to the large pAsa4b plasmid, which includes *tet*(E) and *sul1* (Tanaka et al. 2016). The location of these genes on plasmids is of major clinical concern as it facilitates horizontal gene transfer between aquatic bacteria. For instance, Girard et al. (2024) described novel plasmids in *A. salmonicida* carrying *tet*(D*)* and *floR*, conferring a high-level resistance to tetracyclines and phenicols, and they demonstrated their transfer to other *Aeromonas* spp. and *E. coli*. Additionally, *Aeromonas* and its class 1 integrons, another well-known vector for resistance gene, have recently been proposed as biomarkers for environmental AMR monitoring (Barraud et al. 2023). Altogether, these findings reinforce the role of *Aeromonas* as a reservoir of antibiotic resistance genes and highlight their contribution to treatment failures in aquaculture. Compared to the CER1 strain for which no resistance-associated plasmid was detected, the multi-resistant phenotype shown by the CER2 and CER3 strains could logically be attributed in part to the presence of these antibiotic resistance vectors or their variants. Although this study provided valuable insights into the genomic characterization of these *A. salmonicida* strains, the use of Illumina sequencing technology remains a limitation. Short-read sequencing complicated the analysis of MGEs such as plasmids and ISs, making it difficult to accurately resolve their composition in the three CER strains. Incorporating long-read sequencing techniques would therefore be necessary to clearly identify these genetic elements, particularly to distinguish known plasmids from potential new variants.

One of the main virulence factors responsible for the acute virulence of typical *A. salmonicida* strains in fish and other models is the T3SS (Burr et al. 2005, Frey and Origgi 2016) which, like other secretion systems, exports toxins from the bacterial cytoplasm to the extracellular space or directly into a target cell using a complex protein machinery (Costa et al. 2015). The key T3SS effectors of *A. salmonicida* subsp. *salmonicida* are AexT, AopH, Ati2, AopP, AopO, and AopN, and their main role is to enable the bacteria to evade phagocytosis (Dallaire-Dufresne et al. 2014, Vanden Bergh and Frey 2014, Frey and Origgi 2016). In the *salmonicida* subspecies, most of the genes involved in this secretion system (structural, regulator, and effector genes) are carried by the large pAsa5 plasmid, while the *aopP* and *aexT* genes are located, respectively, on the small plasmid pAsal1 and the chromosome (Stuber et al. 2003, Fehr et al. 2006, Attéré et al. 2017). In the three CER genomes, none of the genes related to structural and regulatory elements of this system could be found. However, genes encoding important T3SS effectors such as the plasmidic *aopO* and *aopP*, and the chromosomic *aexT* were detected (Table S8). It is currently well-established that the pAsa5 plasmid can undergo significant temperature-dependent rearrangements, particularly in the region carrying the T3SS genes (Stuber et al. 2003, Daher et al. 2011). As previously mentioned, this phenomenon could be partly attributed to the IS*As11* and IS*As5* which encircle the altered regions (Tanaka et al. 2012, 2013, 2017) and are both present in multiple copies in the three CER strains (Table S6). Here, we ultimately revealed that an 11A/C recombination (Fig. 1) occurred in all three CER strains. Since this event can lead to a non-functional T3SS, it can have a considerable impact on the virulence of these *A. salmonicida* subsp. *salmonicida* strains. The rearrangement of the pAsa5 plasmid in CER strains likely occurred during their isolation in the laboratory, due to the culture temperature used, as previously observed (Emond-Rheault et al. 2015a, Vincent et al. 2021b).

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Various fish models using *A. salmonicida* have already been tested, including zebrafish (*Danio rerio*) in both their adult (Lin et al. 2007) and larval stages (Missawi et al. 2024), rainbow trout (*Oncorhynchus mykiss*) (Burr and Frey 2007, Kim et al. 2015), Atlantic salmon (*Salmo salar*) (Verner-Jeffreys et al. 2007) or carp (*Cyprinus carpio*) (Hussain Bhat et al. 2021). The use of the bacteria's natural host is undoubtedly the most relevant model. However, housing fish to inoculate them with infectious agents requires specific facilities as well as the management of contaminated water. The murine model is not particularly adapted for studying pathogens like *A. salmonicida* which typically infects cold-blooded vertebrates living at low temperatures (Froquet et al. 2007). In this context, alternative models are gradually emerging, such as the use of the unicellular amoebae *D. discoideum* or the greater wax moth larvae *G. mellonella*. Invertebrate models have the advantage of being low-cost, easy to implement, and ethically more acceptable. The thermal flexibility is also a well-known benefit of the *G. mellonella* model (Pereira et al. 2020, Giammarino et al. 2024), allowing the incubation temperature to be adjusted to match the optimal temperature at which full pathogen virulence is expressed. To our knowledge, only one virulence study has been conducted in *G. mellonella* with *Aeromonas* species, including one *A. salmonicida* strain (Pintor-Cora et al. 2023). However, the subspecies of the strain used by Pintor-Cora and collaborators is unknown, and since it was isolated from vegetables and cultured at 28°C, it is unlikely to represent a typical strain of the *salmonicida* subspecies responsible for furunculosis in fish. The first objective of *G. mellonella* larvae infection experiments was therefore to assess the virulence of typical *A. salmonicida* subsp. *salmonicida* in the model. For this purpose, the 01-B526 and 01-B516 Canadian strains of the *salmonicida* subspecies isolated from diseased fish, harboring an intact pAsa5 plasmid and known for being virulent in *D. discoideum* (Daher et al. 2011) and *S. fontinalis* (only 01-B526 in Dautremepuits et al. 2006), were inoculated to *Galleria* larvae from 10^1 to 10^6 CFU/larva at 18°C. This experiment demonstrated that the *salmonicida* subspecies is highly virulent to *G. mellonella* at 18°C, with nearly 100% mortality at the lowest dose tested (10^1 CFU/larva) at 96 h post-inoculation, and first mortalities occurring as early as 24 HPI for the 01-B526 strain at 10^6 CFU/larva (Fig. 6a and b). In this context, given the high pathogenicity of this subspecies in the *G. mellonella* model, the LT_{50} approach can be considered as a more sensitive tool than the LD_{50} for virulence comparisons (Axline et al. 2025). Although this model is frequently highlighted for its thermal tolerance, it is important to note that temperature has a great influence on *G. mellonella* immune response (Wojda 2017). For instance, while the exposure of larvae to mild thermal shock conditions such as 4 or 37 °C for 24 h induces immune responses (Mowlds and Kavanagh 2008), incubation at 15°C results in a weaker immune response (Browne et al. 2015). The incubation temperature of 18 °C, used to maintain the full bacterial virulence in this study, likely induces an altered immune response in infected *G. mellonella* larvae. The incubation temperature used in this model to compare virulence among strains of this psychrophilic subspecies is therefore a key parameter for minimizing inter-experimental variability.

Based on these first findings, the second objective of the *in vivo* experiments was to characterize the virulence of our three European isolates, CER1, CER2, and CER3. Surprisingly, the three CER strains (without an intact T3SS locus) proved to be as lethal as the Canadian control strains (with an intact T3SS locus) in *G. mellonella* at nearly all tested doses. An exception was observed for CER1-infected larvae at the lowest dose tested (10^1 CFU/larva) where a significantly delayed mortality was noticed compared to 01-B516 and CER3 strains at the same concentration (Fig. 6c and Fig. S2), consistent with a higher LT_{50} value for this concentration (Table 4). This result may be partly attributed to the reduced total number of virulence genes found in the CER1 genome (Fig. 5). Additionally, the 01-B526-derived R35 (without an intact T3SS locus) was used in parallel to specifically investigate the implication of this secretion system in the virulence of *A. salmonicida* subsp. *salmonicida* in the *G. mellonella* model. Once again, a similar dynamic was observed between the rearranged and the wild-type strains, except at the lowest R35 dose (10^1 CFU/larva), where a virulence attenuation was noticed (delayed mortality, Fig. 6d and Fig. S2d; increased LT_{50}, Table 4). A hypothesis would be that *G. mellonella* may be sensitive to other *A. salmonicida* virulence factors at 18°C. A logical follow-up to the present study would be to test in this model *A. salmonicida* strains deleted in (i) the T3SS as well as the entirety of its effectors, (ii) their other secretion systems, or other virulence factors such as (iii) the A-layer protein, AerA aerolysin, AspA serine protease, or GCAT lipase.

While the virulence test in the amoeba *D. discoideum* relies on varying the number of hosts while maintaining a constant bacterial load, the *G. mellonella* model, in contrast, allows for a significant reduction in the number of bacteria inoculated into the host. This opens the door to analyses that the amoebic model cannot provide. Specifically, in a recent study by St-Laurent and colleagues, an approach was introduced to study highly virulent mesophilic *A. salmonicida* strains by using a *D. discoideum* strain with greater voracity and reducing the richness of the culture medium (St-Laurent et al. 2024). However, the authors noted that their model was unable to distinguish psychrophilic strains with low resistance to predation. In this regard, our *G. mellonella* model offers a valuable opportunity to analyze these strains, as demonstrated by the results of the CER1 strain compared to CER3 and 01-B516. Thus, these two surrogate models provide complementary advantages for studying the virulence of *A. salmonicida*, and our study highlights the relevance of the *G. mellonella* model for investigating the typical psychrophilic strains of this bacterial species.

In conclusion, this study provides a comprehensive characterization of three European *A. salmonicida* subsp. *salmonicida* strains and presents an innovative application of the *G. mellonella* larvae model to investigate the virulence of this psychrophilic subspecies. Among these strains, which are closely related to the European reference strain A449, two were found to exhibit resistance to commonly used antibiotics for furunculosis treatment in Europe, while also harboring multiple plasmids known to carry antibiotic resistance genes. Virulence assays in *G. mellonella* larvae showed that *A. salmonicida* subsp. *salmonicida* can be lethal at doses as low as 10^1 CFU/larva even without a functional T3SS, indicating that *G. mellonella* larvae are sensitive to other virulence factors of this subspecies. Moreover, the reduced number of virulence genes detected in the CER1 strain was associated with a virulence attenuation in this model, which supports the use of *G. mellonella* for a finer distinction of typical psychrophilic strains. These results contribute to a deeper understanding of *A. salmonicida* diversity and highlight the value of

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using both *G. mellonella* larvae and *D. discoideum* amoeba as complementary surrogate models for the detailed analysis of *A. salmonicida* virulence. The main perspective of this work would be to further investigate more thoroughly the ability of the *Galleria* model to differentiate strains deleted in specific virulence factors such as the A-layer protein, AerA aerolysin, AspA serine protease, GCAT lipase, or other secretion systems.

Author contributions

Salomé Desmecht (Conceptualization, Formal Analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing), Fabrice Touzain (Data curation, Formal Analysis, Visualization, Writing – review & editing), Thibaut Olivier (Data curation, Formal Analysis, Visualization, Writing – review & editing), Céline Antoine (Conceptualization, Writing – review & editing), Véronique Beven (Investigation), Cécile Meex (Investigation), François Lieffrig (Resources, Writing – review & editing), Steve J. Charette (Conceptualization, Resources, Writing – review & editing), Damien Thiry (Conceptualization, Funding acquisition, Supervision, Writing – review & editing)

Supplementary data

Supplementary data is available at *JAMBIO* Journal online.

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Data availability

Sequencing was submitted as NCBI BioProject PRJNA1075456. This BioProject includes the BioSamples SAMN40034175 (CER1), SAMN40034220 (CER2), and SAMN40034310 (CER3).

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