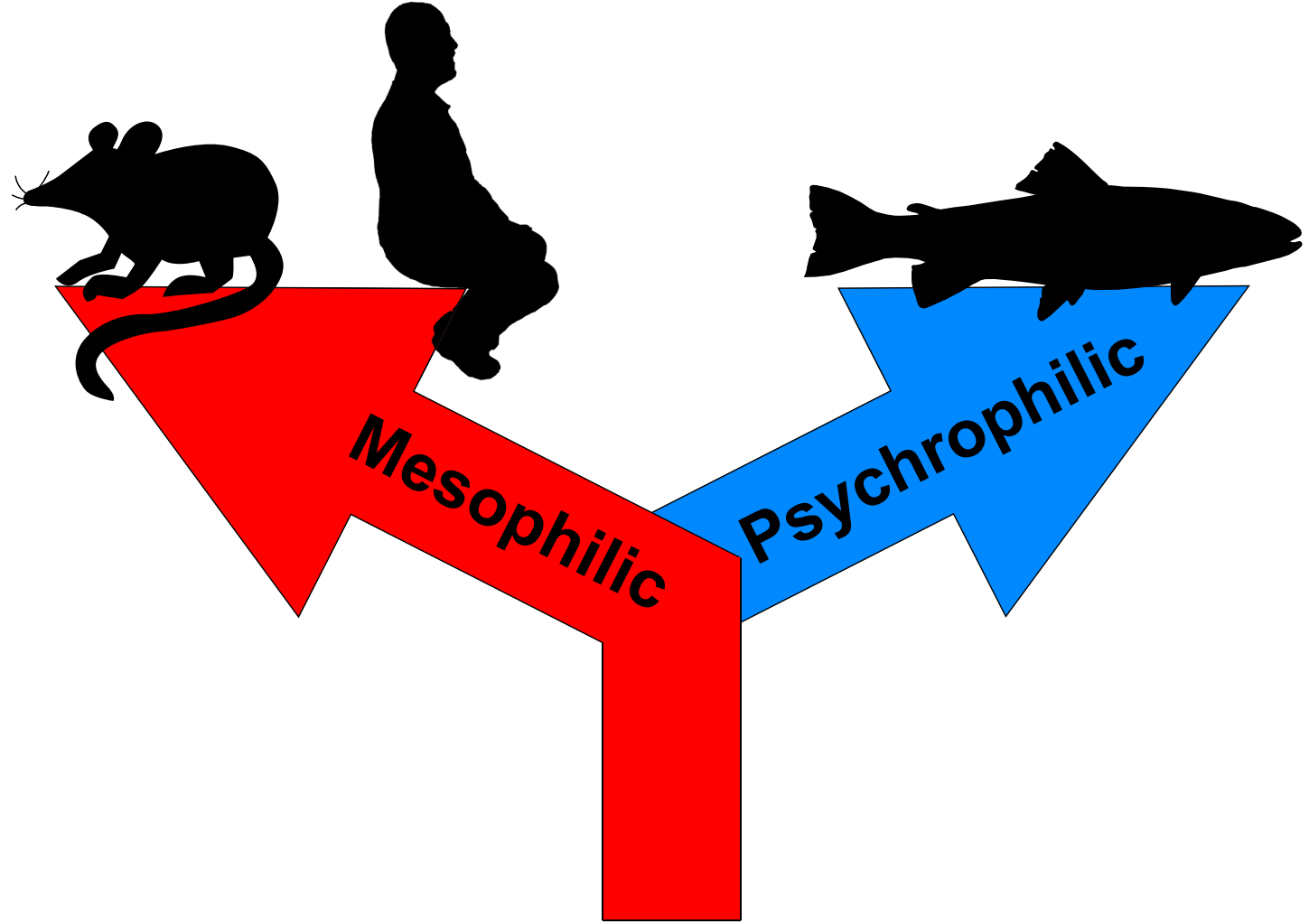

Aeromonas salmonicida

Research article

Investigation of the virulence and genomics of *Aeromonas salmonicida* strains isolated from human patients

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Running Title

Pathogenic *A. salmonicida* from human patients

Abstract

The bacterium *Aeromonas salmonicida* is known since long time as a major fish pathogen unable to grow at 37°C. However, some cases of human infection by putative mesophilic *A. salmonicida* have been reported. The goal of the present study is to examine two clinical cases of human infection by *A. salmonicida* in Spain and to investigate the pathogenicity in mammals of selected mesophilic *A. salmonicida* strains. An evaluation of the pathogenicity in a mouse model of clinical and environmental *A. salmonicida* strains was performed. The genomes of the strains were sequenced and analyzed in order to find the virulence determinants of these strains. The experimental infection in mice showed a gradient in the virulence of these strains and that some of them can cause necrotizing fasciitis and tissue damage in the liver. In addition to demonstrating significant genomic diversity among the strains studied, bioinformatics analyses permitted also to shed light on crucial elements for the virulence of the strains, like the presence of a type III secretion system in the one that caused the highest mortality in the experimental infection. Clinicians and microbiologists should consider these results for the inclusion of *A. salmonicida* in diagnosis tests since it is now clear that some mesophilic strains are also pathogens for humans.

Keywords

Aeromonas salmonicida; Infection; Necrotizing fasciitis; Pathogenicity; Type III Secretion Systems; Whole Genome Sequencing

1. Introduction

The Gram-negative bacterium *Aeromonas salmonicida* has been well known for decades to be a fish pathogen (Austin and Austin, 2016). Officially, *A. salmonicida* has five subspecies (Martin-Carnahan and Joseph, 2005): *salmonicida*, *smithia*, *achromogenes*, *masoucida* and *pectinolytica*. Although the taxonomy of *A. salmonicida* has always been subject to debate (Austin, 2011), it was only in 2000, with the publication of the discovery of the subspecies *pectinolytica* (Pavan et al., 2000) that the diversity of this bacterium was truly revealed. While the other defined *A. salmonicida* subspecies grow only at temperatures below 25°C, *pectinolytica* strains can grow efficiently at 37°C and are thus considered to be mesophilic (Pavan et al., 2000). This dichotomy in the maximum growth temperatures of *A. salmonicida* was reported before the official publication of the subspecies *pectinolytica* (Altwegg et al., 1990; Guérin-Faublée et al., 1997; Janda et al., 1996; Rouf and Rigney, 1971). However, at that time, the intra-species delineation of *A. salmonicida* into subspecies was not systematically used and genome sequences were not available, making conclusions difficult. Moreover, classification of *A. salmonicida* based on biochemical characteristics or 16S rRNA gene sequence has been extremely difficult and many times impossible (Beaz-Hidalgo et al., 2010).

Recently, four mesophilic *A. salmonicida* strains isolated from food in India were sequenced and characterized to shed light on genomic signatures that could explain why some evolutionarily close subspecies have such large differences in their maximum growth temperatures (Vincent et al., 2017, 2016). In accordance with previous experimental evidence based on the *salmonicida* subspecies (Tanaka et al., 2012), investigation of these genomes revealed that insertion sequences could be one of the major genomic determinants between the mesophilic and the psychrophilic strains (Vincent et al., 2017, 2016).

Although our knowledge about *A. salmonicida* has increased significantly during recent years, the infectious potential of mesophilic strains remained unknown. While psychrophilic *A. salmonicida* subspecies are known to infect various fish species (Austin and Austin, 2016), no host is certainly known for mesophilic strains. Early studies found that mesophilic *A. salmonicida* strains (known as hybridization group 3 [HG3]) could be isolated from human and animal hosts (Abbott et al., 1992; Altwegg et al., 1990; Aravena-Román et al., 2011; Janda et al., 1996; Janda and Abbott, 2010). Although rigorous, these studies were made before the democratization of DNA sequencing and the recent advances in the taxonomy of *A. salmonicida* based on core genome sequence analysis. In addition, no clinical background was available for the isolates mentioned above, letting difficult to draw conclusions on the medical importance of *A. salmonicida* for humans.

In 2008, a first case of human infection by *A. salmonicida* with clear clinical background was reported (Yang et al., 2008). More precisely, a 68-year-old diabetic woman on continuous ambulatory peritoneal dialysis was diagnosed as infected by *A. salmonicida* after having been admitted for abdominal pain and cloudy peritoneal fluid. Unfortunately, there is no indication on how the strain was identified as *A. salmonicida*. Recently, in India, *A. salmonicida* was reported to have been recovered from: (i) the blood of a 34-year-old female patient (Tewari et al., 2014), (ii) a skin infection of a 67-year-old immunocompetent male (Kamble, 2015) and (iii) the right eye of 55-year-old female who had recovered from a cataract surgery (Varshney et al., 2017). However, although interesting for clinical backgrounds, the taxonomic identification of these strains is putative given the inherent complexity of *A. salmonicida*.

In 2017, a study reported the isolation of a multidrug-resistant strain, ASG1, from a 15-year-old boy who had recovered from a finger surgery (Ruppé et al., 2017). This time, the strain

was clearly identified as belonging to *A. salmonicida* species. Although it demonstrated once for all that mesophilic *A. salmonicida* could infect humans, the pathogenicity of these isolates and specific mechanisms that allow such infections are still unknown.

Here, we investigate two mesophilic *A. salmonicida* strains isolated from human patients in Spain, one that suffered from an acute gastroenteritis and the other that had a cellulitis in a foot after a trauma. These two clinical strains, in addition to four environmental mesophilic *A. salmonicida* strains, were tested for pathogenicity in an immunosuppressed rodent model. The complete genomes of the strains were also investigated to figure out the putative determinants implicated in the virulence of the strains.

2. Materials and Methods

2.1. Isolation of the clinical strains

The strain AJ83 and 947C were isolated at a hospital in Guadalajara (Spain) (Table 1). The strain AJ83 was recovered from a cellulitis in the right foot of a 49-year-old man that also suffered of fasciitis due to trauma. The strain 947C came from the faeces of an 8-year-old girl that had an acute gastroenteritis. Both strains were first identified at the hospital as *Aeromonas hydrophila* using MicroScan W/A identification system (Dade MicroScan, Inc., Sacramento, Calif). Using the same equipment and based on the Clinical and Laboratory Standards Institute guidelines of 2015, the resistance to various antibiotics was assessed for each strain. Both strains were re-identified more thoroughly as *A. salmonicida* by sequencing the *rpoD* gene using primers and condition used in another study (Beaz-Hidalgo et al., 2010).

2.2. *In vivo* experiments

All *A. salmonicida* strains included in this study (Table 1) were grown on tryptic soy agar (TSA) plates and incubated at 30°C for 24 h. The colonies were then scraped off with a sterile loop and were suspended in sterile phosphate-buffered saline (PBS) solution. For each strain, the concentration of bacterial cells was determined by plating 10-fold dilutions onto TSA plates and then by counting the number of CFU after 24 h.

Four-week-old male OF1 mice weighing approximately 30 g each (Charles River, Criffa S.A., Barcelona, Spain) were used to perform the experiments. All animals were maintained under standard conditions. The designed experiments and care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were immunosuppressed 2 days prior to infection by intraperitoneal injection of 200 mg/kg body

119 weight of cyclophosphamide (Genoxal®; Laboratories Funk S.A., Barcelona, Spain) and
120 thereafter the same procedure was performed once every 5 days (Sanchis et al., 2016).

121 Groups of 8 animals were infected intravenously at the tail with 0.2 mL of sterile PBS
122 containing 1×10^7 or 1×10^9 CFU/mouse of the respective *A. salmonicida* strains. Parameters
123 were selected based on previous experiments of mouse infections with *Aeromonas* (Romero et
124 al., 2016). In all experiments, a control group of 8 mice injected with only 0.2 mL of PBS was
125 used. At the end of the experiment, mice were euthanized by anoxia in a CO_2 chamber, followed
126 by cervical dislocation.

127 The Kaplan-Meier function was used through the R package survival to verify if the
128 survival curves were significantly different from each other. The p-values from the log-rank test
129 were adjusted with the Bonferroni method ($\alpha = 0.05$).

130 **2.3. Bacteria quantification from the different organs and histopathological analysis.**

131 The liver and kidney from the mice infected at both concentrations were directly aseptically
132 collected when the animal died on day 10 post-infection. Each organ was divided in two parts: one
133 part was directly frozen at –80°C and was used for bacterial DNA quantification by real time PCR
134 (qPCR), and the other half was directly fixed in 10% buffered formalin for histopathological
135 studies.

136 The DNA was extracted using the Easy-DNA™ Kit (Invitrogen, CA), according to the
137 manufacturer's instructions. Real-time PCR was performed on the purified DNA using the kit DNA
138 TargetSpecies dtec-qPCR Test for *Aeromonas* sp. (Genetic PCR solutions, SP) and the
139 StepOnePlus™ Real-Time PCR System (Applied Biosystems) equipment. The number of copies
140 was calculated on the basis of the standard curve and the corresponding amplification cycle

141 threshold (Ct). At the time of collection of the liver and kidney, the organs were examined to detect
142 any macroscopic lesions. After fixation, the tissues were embedded in paraffin and sectioned before
143 staining with hematoxylin, eosin, and Giemsa. The sections were evaluated with microscopy (CX
144 33, Olympus).

145 **2.4. DNA extraction, sequencing and analysis**

146 The strains AJ83, 947C and A308 were grown on TSA at 30ºC for 24 h and the genomic
147 DNA extracted using Easy-DNA™ Kit (Invitrogen, Carlsbad, CA), according to the
148 manufacturer's instructions. The DNA of strain A308 (=Popoff C316), was also sequenced since
149 this environmental strain is considered to be a reference of the mesophilic *A. salmonicida* (known
150 as hybridization group 3 [HG3]) by several studies (Abbott et al., 1992; Altwegg et al., 1990;
151 Martínez-Murcia et al., 2005).

152 The purified DNA was used to prepare sequencing libraries using a KAPA Hyper Prep
153 kit. The resulting libraries were sequenced using Illumina MiSeq technology (IBIS, Université
154 Laval). The final reads were *de novo* assembled using A5-miseq version 20160825 (Coil et al.,
155 2015). The resulting sequences were annotated using the Prokaryotic Genome Annotation
156 Pipeline (PGAP) of the NCBI and were deposited in GenBank (Table 1).

157 All the genome sequences of mesophilic *A. salmonicida* strains (including AJ83, 947C
158 and A308 that are from the present study), the ones of selected psychrophilic *A. salmonicida*
159 strains and finally the ones of 30 other *Aeromonas* were annotated using Prokka version 1.12
160 (Seemann, 2014) (see Supplementary Table S1). Homologous links between the translated
161 coding sequences were defined using GET_HOMOLOGUES version 20180103 (Contreras-
162 Moreira and Vinuesa, 2013) with two algorithms: COG and OMCL (see Supplementary Fig. S1).
163 The 2026 gene sequences (excluding paralogs) corresponding to the softcore, defined as the

sequences present in more than 95% of the genomes, were recovered and aligned by codons using TranslatorX version 1.1 (Abascal et al., 2010). The resulting alignments were filtered using BMGE version 1.12 (Criscuolo and Gribaldo, 2010) and concatenated in a partitioned supermatrix using AMAS (Borowiec, 2016). The best-fit model of each partition was determined using ModelFinder (Kalyaanamoorthy et al., 2017) through IQ-TREE version 1.6.1 (Nguyen et al., 2015). The maximum-likelihood phylogeny was itself done using IQ-TREE by performing 10,000 ultrafast bootstraps (Hoang et al., 2017). The Average Nucleotide Identity (ANI) values were computed for genome sequences of *A. salmonicida* using pyani (https://github.com/widdowquinn/pyani).

The antibiotic resistance genes were predicted using ABRicate version 0.8.7 (https://github.com/tseemann/abricate) and the CARD database (Jia et al., 2016). A gene sequence had to have a minimum of 80% identity on at least 70% of the length in order to annotate it as an antibiotic resistance gene. Annotation of the genes was then manually curated.

178 **3. Results**

179 **3.1. Clinical pictures**

180 In 2007, a 49-year-old man was hospitalized at the Guadalajara University Hospital for
181 cellulitis and fasciitis in the right foot after trauma. The clinical background of the patient
182 includes diabetes mellitus and Reiter syndrome, being treated with prednisone. The patient was
183 treated by piperacillin/tazobactam and surgical debridement. The patient healed without
184 complications. A microbial investigation at the wound exudate revealed a polymicrobial infection
185 of *Aeromonas hydrophila*, *Staphylococcus aureus and Klebsiella oxytoca*, after MicroScan
186 identification. No stool or blood culture was performed, since the patient did not have a fever.
187 The *rpoD* sequence of the *Aeromonas* strain, named AJ83, revealed that this strain does not
188 belong to the *hydrophila* species, but surprisingly to the *salmonicida* species (data not shown).
189 This strain is resistant to three antibiotics: cefazolin, ampicillin and ticarcillin, while sensitive to
190 piperacillin/tazobactam (see Supplementary Table S2).

191 One year later, at the same hospital, an 8-year-old girl without a clinical background was
192 hospitalized for an acute gastroenteritis. The patient had bloody stools with mucus. The stool
193 culture revealed the presence of *Campylobacter jejuni* in addition to *Aeromonas hydrophila.* A
194 blood culture was not performed since the young girl had no fever. She had a treatment with
195 hydration and no antibiotic was administered. Like strain AJ83, the *rpoD* sequence of the
196 *Aeromonas* strain (named here 947C) revealed that it belongs to the species *salmonicida*. Strain
197 947C was shown to be resistant to cefazolin, ampicillin and cotrimoxazole (see Supplementary
198 Table S2).

199

3.2. Taxonomic validation of the strains

The genome of the clinical strains AJ83 and 947C was sequenced, *de novo* assembled and used to perform a robust molecular phylogeny based on 2026 gene sequences (Fig. 1). Without any doubt, the clinical strains AJ83 and 947C are belonging to the *salmonicida* species as they clustered with the type strain of the subspecies *pectinolytica* (34mel^T) and with the other already known *A. salmonicida* mesophilic strains. Moreover, they cluster along other mesophilic strains, as strain A308. Interestingly, strain 947C cluster with strain A308, which is environmental. On its side, strain AJ83 form a group with Y567 and Y47, two strains isolated from food in Mumbai (India) and for which no host is known (Fig. 1). The ANI values revealed that available genomes of mesophilic strains are distant in terms of nucleotide sequences, although they come from strains of the same species (ANI $\geq$ 0.96) (Fig. 1). Only strains AJ83 and Y567 were more closely related comparatively to other strains (ANI value of 99%).

3.3. Pathogenicity of strains

The pathogenicity of six mesophilic *A. salmonicida* strains was evaluated by infecting mice (Fig. 2). Two doses were tested, 1×10^7 and 1×10^9 CFU/mouse. At 1×10^7, a clear dichotomy in the survival rate of mice can be observed between strains (Fig. 2-A). The most virulent strain is the clinical one 947C followed by strain A308, which has been isolated from fresh water. There is no statistical significant difference in the mortality caused by both strains (see Supplementary Table S3), which are in the same phylogenetic cluster (Fig. 1). The less virulent strains include A527, Y47, AJ83 and 34mel^T (subspecies *pectinolytica*). Here again, there is no significant statistical difference in the mortality caused by those strains.

Three groups of strains based on virulence can be observed at the dose of 1×10^9 CFU/mouse (Fig. 2-B). The pathogenicity of 947C is striking, with all mice being death after

223 only three days post-infection. As seen in the test at the dose of 1×10^7 CFU/mouse, strains
224 A527, Y47 and 34mel^T are the less virulent and without significant difference in the mortality
225 caused by them (see Supplementary Table S4). At this dose, the environmental strain A308
226 showed to have an intermediate virulence, along with strain AJ83. Although they are less virulent
227 than strain 947C, both strains A308 and AJ83 killed all mice before the end of the experiment.

228 Interestingly, both clinical strains 947C and AJ83 and the Indian strain Y47 produced
229 lesions on the mouse tails, at the injection site (Fig. 2-C). This cutaneous infection that is typical
230 of necrotizing fasciitis was only seen at the lowest dose (1×10^7 CFU/mouse) for 947C, likely
231 because mice died too quickly at the dose of 1×10^9 CFU/mouse. Strains A308, A527 and
232 34mel^T did not produce visible cutaneous infections.

233 **3.4. Bacteria quantification from the different organs and histopathological studies**

234 The presence of bacterial DNA in the liver and kidney of mice was quantified by qPCR
235 (Fig 3). Significantly higher amounts of DNA ($p<0.05$) were found in both organs for the clinical
236 strains (947C and AJ83), than for the environmental strains. A higher amount of *Aeromonas* DNA
237 was detected in liver than in kidney. It is interesting to note that the DNA of the environmental
238 strain A308 was present in larger quantities than other environmental strains, in both organs. In
239 liver and at a higher dose, more DNA of strain A308 was detected than clinical strain AJ83 (Fig.
240 3A). The results obtained with the clinical strains showed a significantly greater amount of DNA
241 of strain 947C, the most pathogenic one, at a lower dose (1×10^7) than for strain AJ83 at both
242 doses.

243 Histopathological examination with hematoxylin and eosin or Giemsa staining showed no
244 damage in the kidney (see Supplementary Fig. S2). However, the liver revealed various levels of
245 multifocal and diffuse necrotic changes and infiltration of polymorphonuclear cells (PMNs), with

246 inflammatory response as shown in Fig. 4. Specifically, tissues collected from animals infected
247 with strain 947C at dose 1 x 10^7 showed more PMN infiltration and necrotic cells (Fig. 4A), than
248 for strain AJ83 at dose 1 x 10^9 (Fig 4B). In addition, the Giemsa staining confrimed the observation
249 of PMN cells and the inflammatory response (Fig 4C).

250 **3.5. Genomic investigation**

251 When checking the genome of strain 947C, the most virulent one, several genes involved
252 in a type III secretion system (T3SS) were found (see Supplementary Table S5). However, it is
253 unclear what make strains AJ83 and A308 virulent. A high number of genes that encode for
254 hypothetical proteins were predicted to be encoded in their genomes and we cannot rule out that
255 some of them are implied in virulence.

256 When looking for the presence of CDSs that encode known virulence factors (Rasmussen-
257 Ivey et al., 2016), the gene *ast* (cytotonic enterotoxin) was found exclusively in the genomes of
258 clinical strains 947C and AJ83 (see Supplementary Table S6). Finally, the mouse infections
259 clearly demonstrated that strains 947C, AJ83 and Y47 can cause necrotizing fasciitis (Fig. 2).
260 Only five orthologous genes, not yet associated with virulence in *Aeromonas salmonicida*, were
261 found to be present in the genomes of these three strains and absent from those of strains 34mel^T,
262 A308 and A527 (Table 2). Interestingly, four of these five genes were already listed in the
263 literature as virulence factors in human pathogens such as *A. hydrophila*, *Helicobacter pylori*,
264 *Leptospira* sp. and *Salmonella enterica* (Table 2).

265 It was also interesting to investigate the genes that could be involved in antibiotic
266 resistance for the mesophilic strains of *A. salmonicida* (see Supplementary Table S7). All strains
267 have genes predicted to be involved in antibiotic resistance (from 2 to 12 genes). Two genes were
268 predicted to be encoded in the genome of all strains: *OXA-12* (resistance to cephalosporin and

269 penam) and *cphA5* (resistance to carbapenem) genes. The two strains with the most antibiotic
270 resistance genes are ASG1 (12 genes), isolated from a human patient, and ECFood+05 (10 genes)
271 for which little information is available. The most virulent strain in the mouse model (Fig. 2),
272 947C, is predicted to have genes involved in resistance to several compounds: aminoglycoside,
273 cephalosporin, penam and carbapenem. The second strain isolated from a human patient for the
274 present study, AJ83, presents almost the same antibiotic resistance pattern as 947C, only differing
275 by the absence of the gene involved in resistance to aminoglycoside compounds.

276 **4. Discussion**

277 Earlier studies on human cases of *A. salmonicida* infections lack clinical metadata or are
278 taxonomically uncertain (Abbott et al., 1992; Altwegg et al., 1990; Aravena-Román et al., 2011;
279 Janda et al., 1996; Kamble, 2015; Ruppé et al., 2017; Tewari et al., 2014; Varshney et al., 2017)
280 compared to what can be done now with core genome phylogeny (Vincent et al., 2016). Recently,
281 the strain ASG1, clearly identified as *A. salmonicida*, was isolated from a 15-year-old boy that
282 recovered from a finger surgery (Ruppé et al., 2017). Unfortunately, another pathogen,
283 *Stenotrophomonas maltophilia,* was co-isolated with strain ASG1, making it impossible to draw
284 firm conclusions on clinical aspects of the ASG1 strain. The present study clearly demonstrated
285 for the first time by combining experimental infection essays and whole genome analyses that
286 some mesophilic *A. salmonicida* strains are able to infect mammals.

287 It is not surprising that T3SS seems to be a major virulence factor, as shown by the
288 striking mortality caused by strain 947C. T3SS is known to be an important virulence factor in
289 several Gram-negative bacteria, including the human pathogens *A. hydrophila* and *Aeromonas*
290 *veronii* (Chacón et al., 2004; Vilches et al., 2004) and the fish pathogen *A. salmonicida* subsp.
291 *salmonicida* (Frey and Origgi, 2016).

292 Interestingly, some *A. salmonicida* strains have the ability to cause cutaneous infections
293 that look like necrotizing fasciitis. In addition to the pathogenicity tests done in the present study,
294 ASG1 was isolated from a finger that recovered from surgery (Ruppé et al., 2017) and AJ83
295 isolated from the right foot of 49-year-old man that suffered of fasciitis due to trauma.
296 Investigation of the genomes revealed five genes that are candidates to explain why only three
297 strains cause necrotizing fasciitis (Table 2). In addition to these genes, which may help explain
298 the ability of some strains to cause necrotizing fasciitis, it was observed that even a low level of
299 virulence can cause this type of infection. Strain 947C, which is the most virulent, causes a
300 necrotizing fasciitis only at the lowest dose (Fig. 2). The other two strains that can cause this skin
301 infection, AJ83 and Y47, cause a low or intermediate mortality level. It is possible to postulate
302 some similarity with the subspecies *salmonicida*, which causes two forms of furunculosis in
303 salmonids (Austin and Austin, 2016). The chronic form of the disease causes a low mortality rate
304 and is often characterized by a cutaneous appearance known as furuncles, hence the name of the
305 disease. The acute form of the disease causes a high mortality rate (2 to 3 days) due to
306 septicaemia and does not manifest cutaneously.

307 A significant amount of *Aeromonas* DNA was found in the livers of fish (more than in
308 their kidneys) by qPCR (Fig. 3). Similar results were described with *A. hydrophila* in channel
309 catfish, where the bacterium was detected only in the liver more than 48 hours post-infection and
310 was eliminated from the other organs, including the kidney, of the fish (Zhang et al., 2016). The
311 quantification obtained from the clinical strains correlated with the results of the
312 histopathological examination, which showed important pathological changes in the liver while
313 no damage was observed in the kidney (Fig .4 and Supplementary Fig. S2). The fact that bacterial

DNA was detected in the kidney at relatively low levels could be related to the process of their elimination with the urine.

Although preliminary, the degree of pathogenicity does not seem to be associated with strains of a specific phylogenetic group. However, the study of the pathogenicity of mesophilic *A. salmonicida* is still in its infancy and strains from various hosts will be needed to clarify the evolutionary links between these strains. The fact that the genomes of only two out of ten mesophilic *A. salmonicida* strains are similar at the nucleotide level demonstrates a great diversity in the mesophilic strains of this bacterium (Fig. 1). One of these two strains, AJ83, has a clinical origin while the second, Y567, was isolated from food.

The psychrophilic strains of *A. salmonicida* are officially divided into different subspecies: *salmonicida, smithia, achromogenes* and *masoucida,* whereas there is only one official mesophilic subspecies, *pectinolytica.* However, according to the molecular phylogeny and ANI values, the mesophilic strains of *A. salmonicida characterized* so far have greater genetic diversity than the psychrophilic strains of the same species. This fact rises, as mentioned before (Vincent et al., 2017), a certain taxonomic dilemma. It is obvious that it will be necessary to review the taxonomy of *A. salmonicida* in order to unify in a cohesive manner the mesophilic and psychrophilic strains of this species. A first scenario could be to classify mesophilic strains into different subspecies. A second scenario would be to make two subspecies, one comprising all the mesophilic strains and the other all the psychrophilic strains. In any case, before considering one of these scenarios, it will be necessary to continue to isolate new mesophilic and psychrophilic strains of *A. salmonicida* in order to obtain a broader view of the different genetic and phenotypic characteristics, thus making it possible to establish a robust and representative taxonomy of this bacterium. Also, it is crucial to take into account that the mesophilic

A. salmonicida strains can be easily misidentified as *A. hydrophila* and that the use of molecular methods such as the sequence of the *rpoD* gene are required to correctly assign the taxonomy of these strains (Beaz-Hidalgo et al., 2010).

The two clinical strains investigated in the present study were shown to be resistant to some antibiotics (see Supplementary Table S2) and also to harbor genes known to be involved in resistance (see Supplementary Table S7). In fact, the resistance gene repertoires of strains 947C and AJ83 differ only in that 947C has a gene that causes resistance to aminoglycoside antibiotics. It is surprising that strain AJ83 is resistant to ticarcillin, belonging to the penem drug class, while strain 947C is sensitive to this antibiotic (see Supplementary Table S2). Similarly, strain 947C is resistant to cotrimoxazole, belonging to sulphonamide/diaminopyrimidinedrug class, while strain AJ83 is sensitive. It is still unclear why strains 947C and AJ83 differ in their resistance to these antibiotics. Other species of the genus *Aeromonas* are known to harbor genes involved in antibiotic resistance (Piotrowska and Popowska, 2015). This is the case, for example, of the fish pathogen *A. salmonicida* subsp. *salmonicida*, for which several strains are multi-resistant to all antibiotic approved in aquaculture in Canada (Trudel et al., 2016; Vincent et al., 2014). A similar pattern of multiple resistance seems to be apparent in mesophilic strains of the *salmonicida* species where some strains, such as ASG1 and ECFood+05, were predicted to harbor more than 10 genes involved in resistance to antibiotic compounds. This is even more interesting given the context that these two strains cluster together in the phylogenetic tree (Fig. 1), suggesting that some mesophilic *A. salmonicida* strains that arise from a particular common ancestor could be more prone to having antibiotic resistance genes. Given that both ASG1 and ECFood+05 only share four genes (*OXA-12*, *cphA5*, *aadA* and *tet*(E)), it is reasonable to believe that the other genes could have been acquired by horizontal gene transfers. Moreover, the multiple resistance to

antibiotics of ASG1 strain was confirmed experimentally (Ruppé et al., 2017). Closely monitoring mesophilic *A. salmonicida* will be essential to effectively treat cases of infection by strains of this bacterium.

4.1. Concluding remarks

In this study, it was possible to demonstrate robustly that the mesophilic strains of *A. salmonicida* can infect mammals, with varying levels of pathogenicity between strains. It will be essential in the future to isolate new mesophilic *A. salmonicida* strains and to verify their geographical distribution. The clinical strains AJ83 and 947C investigated in the present study come from Spain. However, several clinical studies have documented cases in India of infections in humans from putatively mesophilic *A. salmonicida* strains (Kamble, 2015; Tewari et al., 2014; Varshney et al., 2017). Moreover, environmental strains from India have clearly been identified as mesophilic *A. salmonicida* (Vincent et al., 2017, 2016).

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Conflicts of interest

The authors have no conflicts of interest to declare.

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384

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Table 1. Mesophilic strains of *A. salmonicida* used in the present study.

Strain	Source	Country	Year	Accession number	Reference
34mel^T	River	Argentina	1988	NZ_CP022426.1	(Pavan et al., 2000)
Y47	Chicken[a]	India	2006	JZTF00000000	(Nagar et al., 2011)
A527	Giant river prawn[a]	India	2007	CP022550	(Nagar et al., 2011; Vincent et al., 2017)
A308[b]	Fresh water	France	1962	PSZJ00000000	Present study
AJ83	Human	Spain	2007	PSZI00000000	Present study
947C	Human	Spain	2008	PSZK00000000	Present study

a: Isolated in food markets in India (Nagar et al., 2011). The real hosts are considered unknown.
b: Strain A308 = Popoff C316 = CDC 0434-84 = CECT 5171 = LMG 13451. This strain is considered as a reference for mesophilic *A. salmonicida* (Abbott et al., 1992; Altwegg et al., 1990; Martínez-Murcia et al., 2005).

549 **Table 2. CDSs present only in strains 947C, AJ83 and Y47**

Protein	Virulence trait	Ref
Two pore domain potassium channel family protein	N/A[a]	N/A
Hemerythrin	*A. hydrophila* survival in host macrophages	(Zeng et al., 2016)
Pseudaminic acid cytidylyltransferase	Colonisation of *H. pylori*	(Wahid, 2017)
Catalase KatE[b]	Virulence of *Leptospira* spp. in animal models	(Eshghi et al., 2012)
UDP-N-acetylglucosamine-1-phosphate transferase[c]	Production of enterobacterial antigen in *S. enterica*	(Gilbreath et al., 2012)

550 a: N/A, none-applicable
551 b: The catalase was annotated as KatE by PATRIC (Wattam et al., 2017)
552 c: The CDS in strain Y47 appears to be divergent compared to those of strains 947C and AJ83
553

554 **FIGURES LEGENDS**

555 **Figure 1. Phylogenetic tree of 51 strains of *Aeromonas*.** The tree is based on 2026 gene
556 sequences using the methodology described in the Materials and Methods section. For the sake of
557 clarity, the focus is on mesophilic (red) and psychrophilic (blue) strains of the species
558 *salmonicida*. Bootstrap values are only shown if they are less than 100. The heatmap represents
559 the ANI values.

560

561 **Figure 2. Virulence tests in a mouse model.** Survival rate of mice at doses of (A) 1×10^7 and
562 (B) 1×10^9 CFU/mouse of six mesophilic *A. salmonicida* strains. (C) Pictures showing the
563 lesions at the infection site caused by strains 947C, AJ83 and Y47. The pictures of the mouse tail
564 infected with A308 provides a negative control for the lesions observed with the other strains.

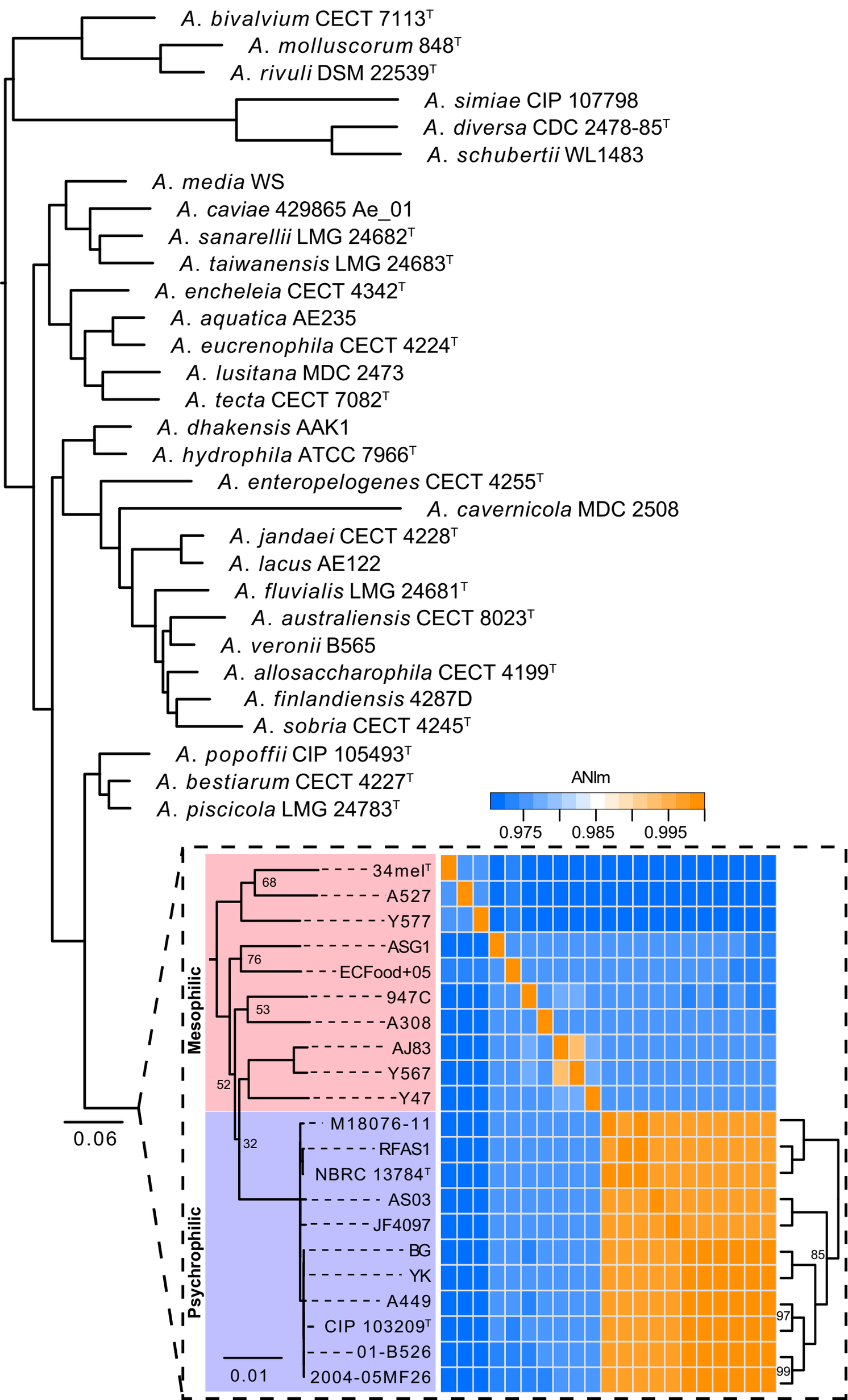
565

566 **Figure 3.** Concentration of *Aeromonas* DNA determined by qPCR in mice liver (A) and kidney
567 (B) tissues 10 days after intravenous infection at doses 1×10^7 and 1×10^9. *Statistical
568 significance ($p < 0.05$). Tissue = non-infected tissue, Water = only water without tissue; both
569 used as negative controls.

570

571 **Figure 4.** Histopathological examination of mouse liver tissue 10 days after intravenous infection
572 with two *Aeromonas* strains (AJ83 and 947C) of clinical origin. (A) Strain AJ83 at dose 1×10^9
573 CFU with hematoxylin/eosin staining. (B) Strain 947C at dose 1×10^7 CFU with

hematoxylin/eosin staining (C) Strain 947C at dose 1×10^7 CFU with Giemsa staining. Bars represent 100 μm.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A. diversa CDC 2478-85T
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A. media WS
A. caviae 429865 Ae_01
A. sanarellii LMG 24682T
A. taiwanensis LMG 24683T
A. encheleia CECT 4342T
A. aquatica AE235
A. eucrenophila CECT 4224T
A. lusitana MDC 2473
A. tecta CECT 7082T
A. dhakensis AAK1
A. hydrophila ATCC 7966T
A. enteropelogenes CECT 4255T
A. cavernicola MDC 2508
A. jandaei CECT 4228T
A. lacus AE122
A. fluvialis LMG 24681T
A. australiensis CECT 8023T
A. veronii B565
A. allosaccharophila CECT 4199T
A. finlandiensis 4287D
A. sobria CECT 4245T
A. popoffii CIP 105493T
A. bestiarum CECT 4227T
A. piscicola LMG 24783T
0.06
ANIm
0.975
0.985
0.995
Mesophilic
Psychrophilic
34melT
A527
Y577
ASG1
ECFood+05
947C
A308
AJ83
Y567
Y47
M18076-11
RFAS1
NBRC 13784T
AS03
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85
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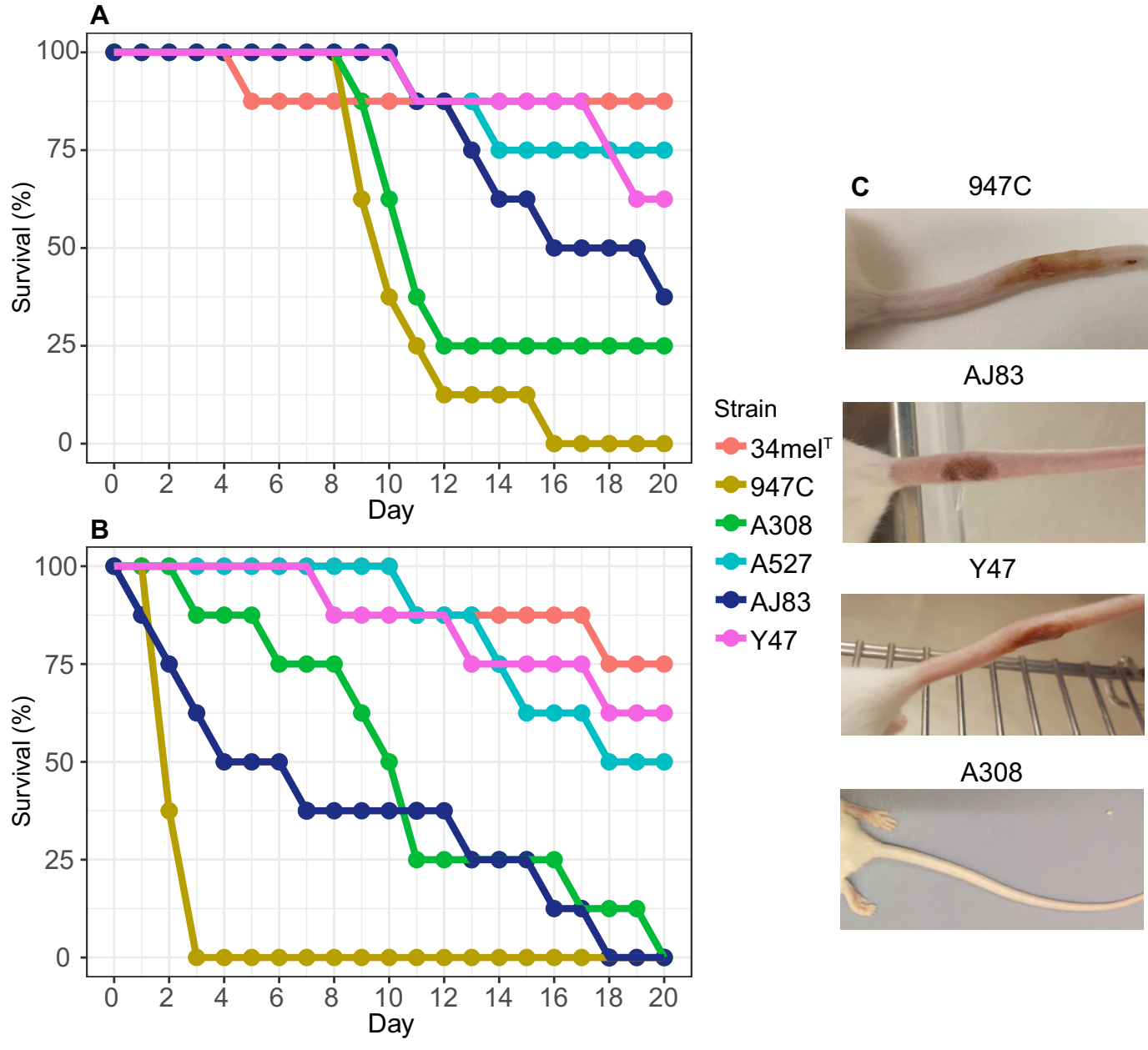
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Survival (%)
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34mel^{T}
947C
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AJ83
Y47
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AJ83
Y47
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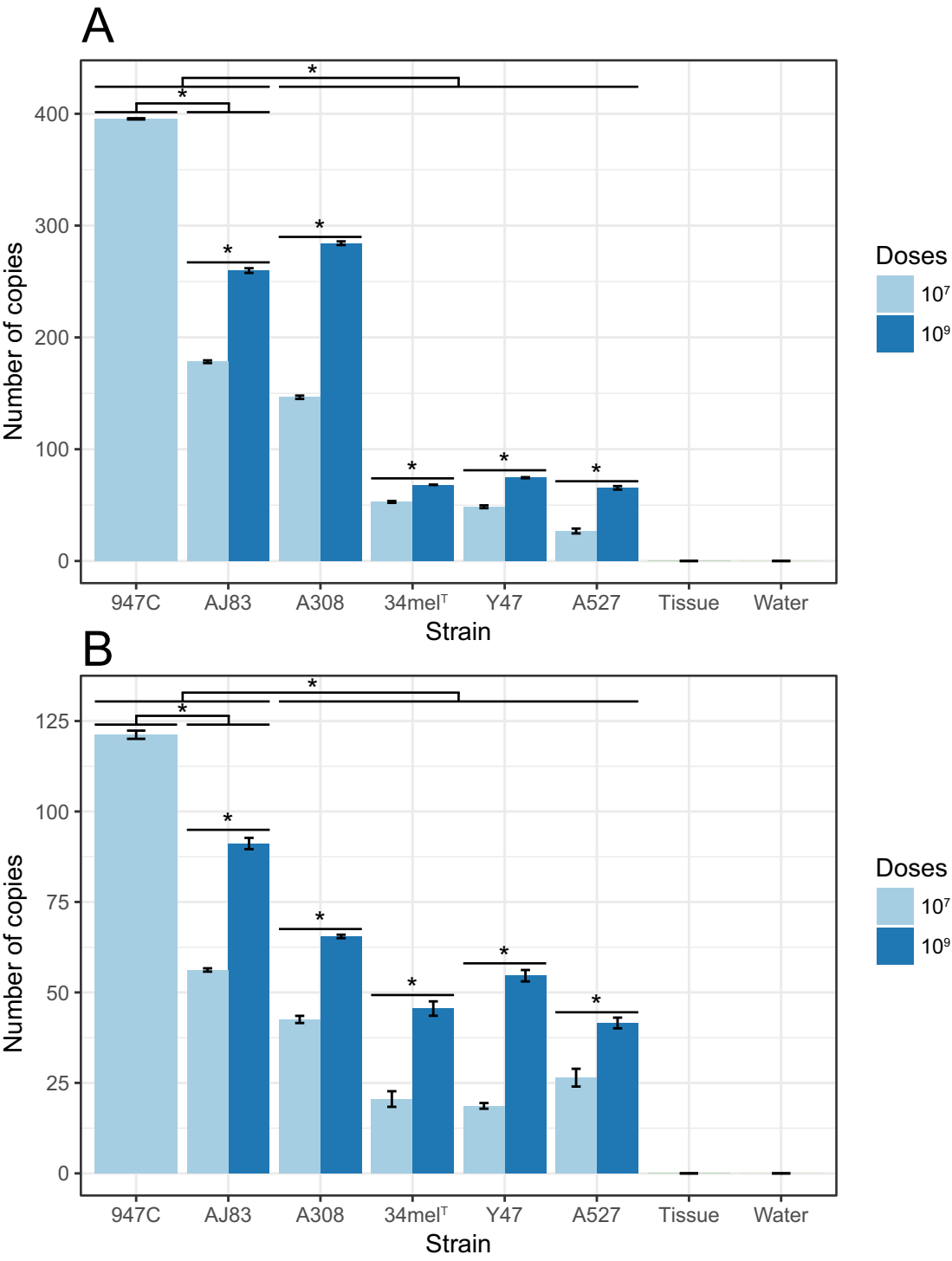
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Number of copies
Strain
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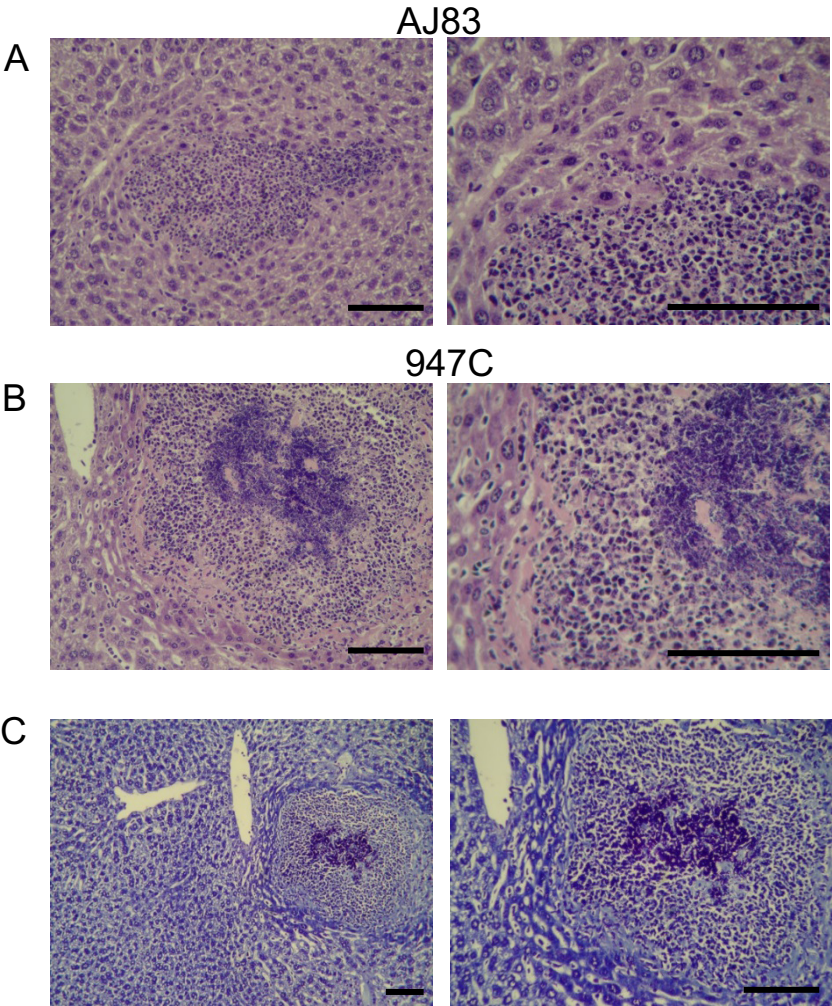
34mel^{T}
Y47
A527
Tissue
Water
Doses
10^{7}
10^{9}
*


AJ83
A
947C
B
C

Supplementary Materials

Investigation of the virulence and genomics of *Aeromonas salmonicida* strains isolated from human patients

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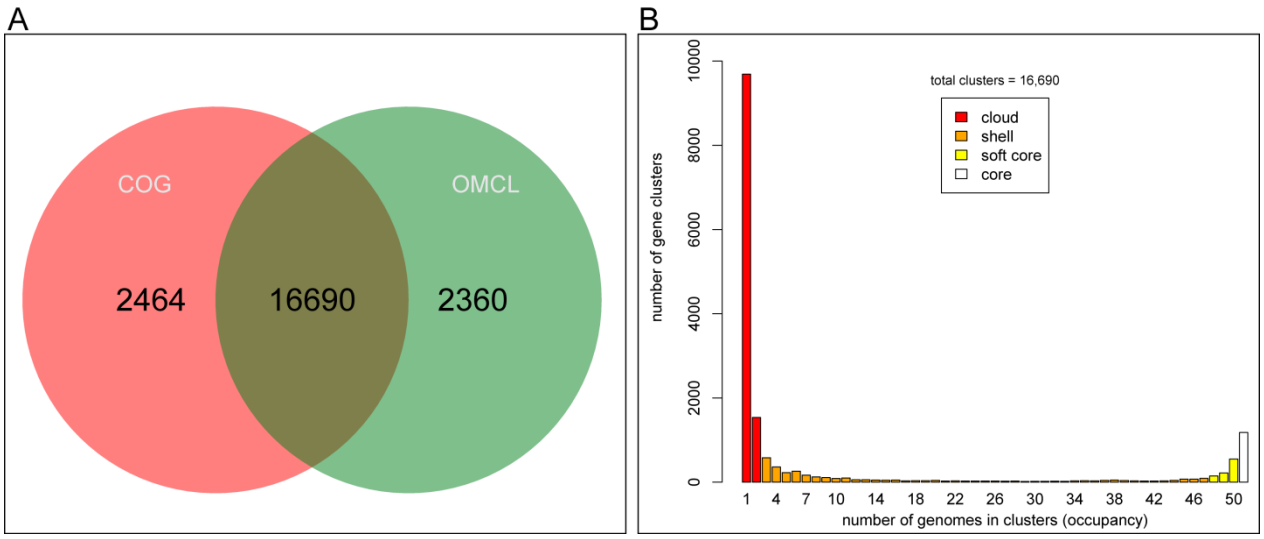

Figure S1. Pan-genome analysis for the 51 *Aeromonas* genome sequences used in the present study. (A) Gene clusters found by the COG (Kristensen et al., 2010) and OMCL (Li et al., 2003) algorithms that were used by the tool GET_HOMOLOGUES (Contreras-Moreira and Vinuesa, 2013) to find homologous links between the coding sequences encoded by the set of genomes (Table S1). (B) Distribution of the pan-genome in four categories.

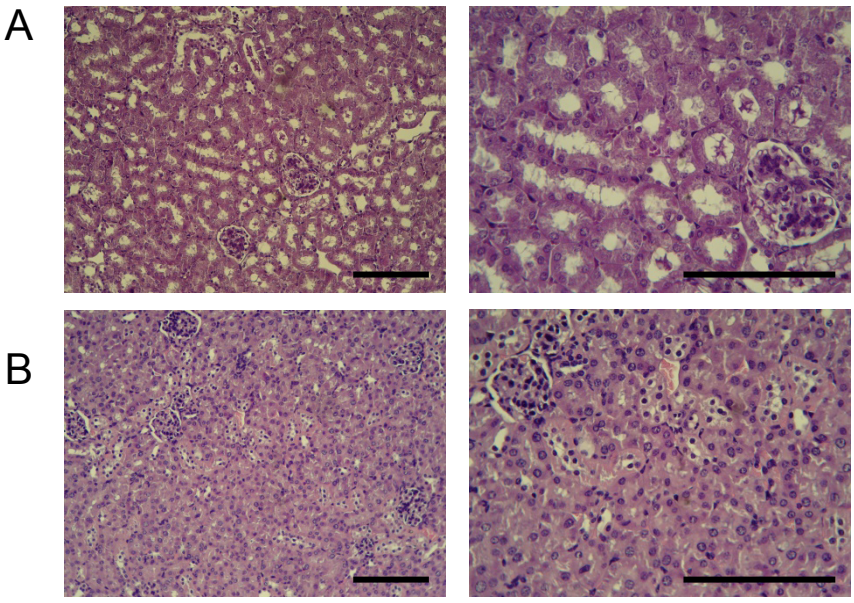

Figure S2. Histopathological examination of kidney mice tissues 10 days after intravenous infection with two *Aeromonas* strains (AJ83 and 947C) of clinical origin. Neither strain seems to have caused any apparent damage in the glomeruli nor in the tubules of the kidney. (A) Strain AJ83 at dose 1×10^9 CFU with hematoxylin/eosin staining. (B) Strain 947C at dose 1×10^7 CFU. Bars represent 100 μm.

Table S1. Genome sequences of *Aeromonas* used for the phylogenetic analysis.

Species	Strain	GenBank	Reference
A. allosaccharophila	CECT 4199^T	NZ_CDBR00000000	(Colston et al., 2014)
A. aquatica	AE235	NZ_JRGL00000000	(Hossain et al., 2014)
A. australiensis	CECT 8023^T	NZ_CDDH00000000	(Colston et al., 2014)
A. bestiarum	CECT 4227^T	NZ_CDDA00000000	(Colston et al., 2014)
A. bivalvium	CECT 7113^T	NZ_CDBT00000000	(Colston et al., 2014)
A. cavernicola	MDC 2508	NZ_PGGC01000000	(Martínez-Murcia et al., 2013)
A. caviae	429865 Ae_01	NZ_LIIX01000001	(Padilla et al., 2015)
A. dhakensis	AAK1	NZ_BAFL00000000	(Wu et al., 2012)
A. diversa	CDC 2478-85^T	NZ_APVG00000000	(Farfán et al., 2013)
A. encheleia	CECT 4342^T	NZ_CDDI00000000	(Colston et al., 2014)
A. enteropelogenes	CECT 4255^T	NZ_CDDE00000000	(Colston et al., 2014)
A. eucrenophila	CECT 4224^T	NZ_CDDF00000000	(Colston et al., 2014)
A. finlandiensis	4287D	NZ_JRGK00000000	(Beaz-Hidalgo et al., 2015)
A. fluvialis	LMG 24681^T	NZ_CDBO00000000	(Colston et al., 2014)
A. hydrophila	ATCC 7966^T	NC_008570.1	(Seshadri et al., 2006)
A. jandaei	CECT 4228^T	NZ_CDBV00000000	(Colston et al., 2014)
A. lacus	AE122	NZ_JRGM00000000	(Beaz-Hidalgo et al., 2015)
A. lusitana	MDC 2473	PGCP01000000	(Martínez-Murcia et al., 2016)
A. media	WS	NZ_CP007567.1, NZ_CP007568.1	(Chai et al., 2012)
A. molluscorum	848^T	NZ_AQGQ00000000	(Spataro et al., 2013)
A. piscicola	LMG 24783^T	NZ_CDBL00000000	(Colston et al., 2014)
A. popoffii	CIP 105493^T	NZ_CDBI00000000	(Colston et al., 2014)
A. rivuli	DSM 22539^T	NZ_CDBJ01000000	(Colston et al., 2014)
A. sanarellii	LMG 24682^T	NZ_CDBN00000000	(Colston et al., 2014)
A. schubertii	WL1483	NZ_CP013067.1	(Liu et al., 2016)
A. simiae	CIP 107798	NZ_CDBY00000000	(Colston et al., 2014)
A. sobria	CECT 4245^T	NZ_CDBW01000000	(Colston et al., 2014)
A. taiwanensis	LMG 24683^T	NZ_BAWK00000000	(Wang et al., 2014)
A. tecta	CECT 7082^T	NZ_CDCA00000000	(Colston et al., 2014)
A. veronii	B565	NC_015424.1	(Li et al., 2011)
A. salmonicida subsp. *salmonicida*	01-B526	AGVO00000000	(Charette et al., 2012)
A. salmonicida subsp. *salmonicida*	2004-05MF26	JRYW00000000	(Vincent et al., 2015)
A. salmonicida subsp. *salmonicida*	A449	CP000644.1	(Reith et al., 2008)
A. salmonicida subsp. *salmonicida*	CIP 103209^T	CDDW00000000	(Colston et al., 2014)
A. salmonicida subsp. *salmonicida*	BG	LUHO00000000	(Long et al., 2016)
A. salmonicida subsp. *salmonicida*	YK	LUHP00000000	(Long et al., 2016)
A. salmonicida subsp. *achromogenes*	AS03	AMQG00000000	(Han et al., 2013)

A. salmonicida subsp. *smithia*	JF4097	JZTI00000000	(Vincent et al., 2016)
A. salmonicida subsp. *masoucida*	NBRC 13784^{T}	BAWQ01000000	N/A
A. salmonicida subsp. *masoucida*	RFAS1	NZ_CP017143.1	(Han et al., 2011)
A. salmonicida	M18076-11	NQMJ00000000.1	(Rouleau et al., 2018)
A. salmonicida	Y47	JZTF00000000	(Vincent et al., 2016)
A. salmonicida	Y567	JZTG00000000	(Vincent et al., 2016)
A. salmonicida	Y577	JZTH00000000	(Vincent et al., 2016)
A. salmonicida	A527	CP022550	(Vincent et al., 2017)
A. salmonicida	ECFood+05	NZ_NVQH01000000	N/A
A. salmonicida	ASG1	PRJNA377399	(Ruppé et al., 2017)
A. salmonicida	A308[a]	PSZJ00000000	Present study
A. salmonicida	947C	PSZK00000000	Present study
A. salmonicida	AJ83	PSZI00000000	Present study
A. salmonicida subsp. *pectinolytica*	34mel^{T}	NZ_CP022426.1	(Gulla et al., 2016; Pavan et al., 2015)

a: Strain A308 = Popoff C316 = CDC 0434-84 = CECT 5171 = LMG 13451. This strain is considered to be a reference for mesophilic *A. salmonicida* (Abbott et al., 1992; Altwegg et al., 1990; Martínez-Murcia et al., 2005).

Table S2. Sensitivity and resistance to various antibiotics

			Minimum inhibitory concentration mg/L	
Antibiotic	**Class**	**Accession**	**947C**	**AJ83**
Gentamicin	aminoglycoside	ARO[a]	< 4 S[b]	< 4 S
Tobramycin	aminoglycoside	ARO:3000052	< 4 S	< 4 S
Amikacin	aminoglycoside	ARO:3000013	< 8 S	< 8 S
Meropenem	carbapenem	ARO:3000073	< 4 S	< 4 S
Imipenem	carbapenem	ARO:3000170	2 S	< 1 S
Cefazolin	cephalosporin	ARO:3000058	> 16 R	> 16 R
Cefuroxime	cephalosporin	ARO:3000063	< 8 S	< 8 S
Cefotaxime	cephalosporin	ARO:3000645	< 0.5 S	< 0.5 S
Ceftazidime	cephalosporin	ARO:3000060	< 1 S	< 1 S
Cefepime	cephalosporin	ARO:3000059	< 1 S	< 1 S
Cefoxitin	cephamycin	ARO:3000008	< 8 S	< 8 S
Ciprofloxacin	fluoroquinolone	ARO:3000036	< 0.12 S	< 0.12 S
Ofloxacin	fluoroquinolone	ARO:3000663	< 0.5 S	< 0.5 S
Aztreonam	monobactam	ARO:3000550	< 1 S	< 1 S
Piperacillin	penam	ARO:3000078	< 16 S	< 16 S
Piperacillin/tazobactam	penam	ARO:3004021	< 16 S	< 16 S
Amoxicillin/clavulanate	penam	ARO:3003997	< 4 S	< 4 S
Ampicillin	penam	ARO:3000637	> 16 R	> 16 R
Ticarcillin	penem	ARO:3003832	< 16 S	64 R
Cotrimoxazole	sulphonamide/diaminopyrimidine	ARO:3004024	> 2 R	<2 S

a: gentamicin A = ARO:3004015; gentamicin B = ARO:3000655; gentamicin C = ARO:0000014.
b: S = sensitive; R = resistant.

Table S3. p-values for the pairwise comparisons of mice mortality caused by the strains at 1×10^7 CFU/mouse

Strain	34mel^T	947C	A308	A527	AJ83
947C	0.0160	-	-	-	-
A308	0.3849	1.0000	-	-	-
A527	1.0000	0.0082	0.3906	-	-
AJ83	1.0000	0.0363	1.0000	1.0000	-
Y47	1.0000	0.0034	0.8064	1.0000	1.0000

Table S4. p-values for the pairwise comparisons of mice mortality caused by the strains at 1×10^9 CFU/mouse

Strains	34mel^T	947C	A308	A527	AJ83
947C	0.0011	-	-	-	-
A308	0.0100	0.0042	-	-	-
A527	1.0000	0.0011	0.1209	-	-
AJ83	0.0085	0.3835	1.0000	0.0958	-
Y47	1.0000	0.0011	0.0774	1.0000	0.0351

Table S5. Specific genes found in a group of mesophilic *A. salmonicida* strains.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain 947C			
PGF_00557086	17 kDa surface antigen precursor	1	113	0
PGF_00426666	5-methylcytosine-specific restriction enzyme A (EC 3.1.21.-)	1	225	0
PGF_00035135	Adenine-specific methyltransferase (EC 2.1.1.72)	1	223	0
PGF_06393555	Aminoglycoside 3''-phosphotransferase (EC 2.7.1.87) => APH(3'')-I	1	267	0
PGF_01082199	Aminoglycoside 6''-phosphotransferase (EC 2.7.1.72) => APH(6")-Ic/APH(6)-Id	1	247	0
PGF_00416326	Capsular polysaccharide synthesis enzyme Cap5L	1	346	0
PGF_00417450	Chaperone protein YscY (Yop proteins translocation protein Y)	1	114	0
PGF_05997426	ClpB-like protein	1	880	0
PGF_00418902	Conjugative transfer protein TrbB	1	355	0
PGF_00418905	Conjugative transfer protein TrbC	1	127	0
PGF_00418908	Conjugative transfer protein TrbD	1	90	0
PGF_00418912	Conjugative transfer protein TrbE	1	816	0
PGF_00418915	Conjugative transfer protein TrbF	1	234	0
PGF_00418918	Conjugative transfer protein TrbG	1	330	0
PGF_00418922	Conjugative transfer protein TrbI	1	426	0
PGF_00418926	Conjugative transfer protein TrbJ	1	245	0
PGF_00418931	Conjugative transfer protein TrbK	1	108	0
PGF_00418937	Conjugative transfer protein TrbK	1	91	0
PGF_03070810	Conjugative transfer protein TrbL	1	454	0
PGF_00419496	CopG domain-containing protein	1	154	0
PGF_00419652	Coupling protein VirD4, ATPase required for T-DNA transfer	1	659	0
PGF_00421978	DNA-binding protein	1	381	0
PGF_00422437	DUF1176 domain-containing protein	1	332	0
PGF_00423998	EF hand domain protein	4	318.7	230.2
PGF_00425042	Exodeoxyribonuclease VIII (EC 3.1.11.-)	1	463	0
PGF_00000945	FIG00904992: hypothetical protein	1	135	0
PGF_06889881	Flagellar motor rotation protein MotA	1	285	0
PGF_04572835	Flagellar regulatory protein FleQ	1	419	0
PGF_00008924	Glycosidases	1	181	0
PGF_00896896	Homology to phage-tail assembly proteins	1	183	0
PGF_01196079	IcmF-related protein	1	1165	0
PGF_00557082	LPXTG-motif cell wall anchor domain protein	1	472	0
PGF_00815743	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	1	453	0
PGF_00018067	Lysozyme (EC 3.2.1.17)	1	165	0
PGF_00022405	Modification methylase EcoRI (EC 2.1.1.72)	1	339	0
PGF_03821650	Outer membrane protein ImpK/VasF, OmpA/MotB domain	1	259	0
PGF_00032526	Phage protein	1	418	0
PGF_02742028	Phage terminase, large subunit	1	618	0
PGF_02599114	Plasmid replication initiator protein	1	284	0
PGF_00036213	Predicted purine nucleoside transporter, MFS superfamily	1	387	0

PGF_00037022	Probable dTDP-4-dehydrorhamnose reductase (EC 1.1.1.133)	1	295	0
PGF_01929932	Protein ImpG/VasA	1	588	0
PGF_00037960	Protein StbA	1	347	0
PGF_02923769	Putative large exoprotein involved in heme utilization or adhesion of ShlA/HecA/FhaA family	1	621	0
PGF_00047100	RecT protein	1	350	0
PGF_01022609	Resolvase	1	204	0
PGF_00056927	Tir chaperone	1	128	0
PGF_04402936	Transcriptional regulator, Xre family	1	110	0
PGF_00698550	Transposase	1	775	0
PGF_04646018	Type III secretion bridge between inner and outer membrane lipoprotein (YscJ,HrcJ,EscJ, PscJ)	1	240	0
PGF_00063481	Type III secretion chaperone protein for YopD (SycD)	1	167	0
PGF_02808153	Type III secretion chaperone protein for YopE (SycE)	1	131	0
PGF_00063488	Type III secretion cytoplasmic ATP synthase (EC 3.6.3.14, YscN,SpaL,MxiB,HrcN,EscN)	1	440	0
PGF_00063489	Type III secretion cytoplasmic LcrG inhibitor (LcrV,secretion and targeting control protein, V antigen)	1	272	0
PGF_00063492	Type III secretion cytoplasmic protein (YscF)	1	85	0
PGF_00063494	Type III secretion cytoplasmic protein (YscI)	1	112	0
PGF_00063498	Type III secretion cytoplasmic protein (YscL)	1	212	0
PGF_00063508	Type III secretion host injection and negative regulator protein (YopD)	1	298	0
PGF_00063511	Type III secretion host injection protein (YopB)	1	394	0
PGF_00063525	Type III secretion inner membrane channel protein (LcrD,HrcV,EscV,SsaV)	1	705	0
PGF_00063540	Type III secretion inner membrane protein (YscQ,homologous to flagellar export components)	1	308	0
PGF_00063543	Type III secretion inner membrane protein (YscR,SpaR,HrcR,EscR,homologous to flagellar export components)	1	217	0
PGF_00063548	Type III secretion inner membrane protein (YscS,homologous to flagellar export components)	1	88	0
PGF_00063553	Type III secretion inner membrane protein (YscT,HrcT,SpaR,EscT,EpaR1,homologous to flagellar export components)	1	262	0
PGF_03097682	Type III secretion inner membrane protein (YscU,SpaS,EscU,HrcU,SsaU, homologous to flagellar export components)	1	352	0
PGF_00063566	Type III secretion outer membrane contact sensing protein (YopN,Yop4b,LcrE)	1	293	0
PGF_00063575	Type III secretion outer membrane pore forming protein (YscC,MxiD,HrcC, InvG)	1	609	0
PGF_00063603	Type III secretion spans bacterial envelope protein (YscG)	1	117	0
PGF_00063613	Type III secretion thermoregulatory protein (LcrF,VirF,transcription regulation of virulence plasmid)	1	95	0
PGF_05125858	Type IV secretory pathway, VirD2 components (relaxase)	1	660	0
PGF_03935134	Type IV secretory pathway, protease TraF	1	199	0
PGF_00063807	Type VI secretion lipoprotein/VasD	1	171	0
PGF_00063810	Type VI secretion protein VasI	1	201	0
PGF_00063969	UDP-N-acetyl-L-fucosamine synthase (EC 5.1.3.28)	1	377	0
PGF_00064344	UPF0380 proteins YafZ and homologs	1	276	0
PGF_06066221	Uncharacterized protein ImpA	1	481	0
PGF_00064962	Uncharacterized protein ImpC	1	492	0
PGF_02979767	Uncharacterized protein ImpH/VasB	1	332	0
PGF_00064987	Uncharacterized protein ImpI/VasC	1	411	0
PGF_00064990	Uncharacterized protein ImpJ/VasE	1	444	0
PGF_00065739	Uncharacterized protein similar to VCA0109	1	143	0
PGF_00066079	Unknown, probably involved in type III secretion	1	147	0

PGF_00071340	flagellin modification protein FlmH	1	174	0
PGF_00407860	putative lipoprotein	1	183	0
PGF_00408050	putative lipoprotein	1	119	0
PGF_02896985	putative lipoprotein	2	219.5	3.5
PGF_00410187	putative plasmid stabilization protein	1	688	0
PGF_00177460	sigma-54-dependent transcriptional regulator	1	512	0
PGF_00414781	y4eB gene in pNGR234a homolog	1	104	0
	122 Hypothetical proteins			
	Present only in the genome of strain A308			
PGF_00417140	ABC transporter, ATP-binding protein	1	236	0
PGF_01027157	ABC transporter, permease protein	1	401	0
PGF_01027719	ATP-binding protein	1	457	0
PGF_00046216	Alpha-L-Rha alpha-1,3-L-rhamnosyltransferase (EC 2.4.1.-)	1	298	0
PGF_00058720	Anticodon nuclease	1	380	0
PGF_00399672	Baseplate assembly protein J	1	293	0
PGF_00415078	CMP-binding factor	1	289	0
PGF_00416106	CRISPR-associated protein, Csy3 family	1	356	0
PGF_00418464	Coenzyme F420-dependent oxidoreductase	1	440	0
PGF_00420893	D-arabinitol 4-dehydrogenase (EC 1.1.1.11)	1	455	0
PGF_00927728	D-arabinitol operon repressor	1	313	0
PGF_00421005	D-mannonate oxidoreductase (EC 1.1.1.57)	1	486	0
PGF_02272288	DNA helicase IV (EC 3.6.4.12)	1	925	0
PGF_00002325	FIG01223779: hypothetical protein	1	350	0
PGF_03479854	Flavodoxin	1	195	0
PGF_03207284	Lipid carrier : UDP-N-acetylgalactosaminyltransferase (EC 2.4.1.-)	1	185	0
PGF_00019272	Mannonate dehydratase (EC 4.2.1.8)	1	393	0
PGF_01767866	Metal-dependent hydrolases of the beta-lactamase superfamily II	1	292	0
PGF_00667779	Mobile element protein	1	267	0
PGF_01482614	Modulator of drug activity B	1	217	0
PGF_02718770	Mu-like prophage FluMu I protein	1	390	0
PGF_02779037	NADH-dependent butanol dehydrogenase A (EC 1.1.1.-)	1	382	0
PGF_03059183	Outer membrane protein romA	1	367	0
PGF_00031706	Phage baseplate assembly protein	1	123	0
PGF_00031742	Phage capsid scaffolding protein	1	273	0
PGF_00032032	Phage major capsid protein	1	350	0
PGF_00032231	Phage protein	1	524	0
PGF_03336619	Phage replication protein	1	795	0
PGF_00032787	Phage terminase, ATPase subunit	1	570	0
PGF_00032790	Phage terminase, endonuclease subunit	1	227	0
PGF_03122294	Phage-related capsid packaging protein	1	358	0
PGF_00036218	Predicted pyrophosphatase	1	378	0
PGF_00040787	Putative exported protein precursor	1	353	0
PGF_04274853	Putative phage-encoded peptidoglycan binding protein	1	274	0

PGF_03078435	RND efflux system, membrane fusion protein	1	383	0
PGF_00047450	RelB/StbD replicon stabilization protein (antitoxin to RelE/StbE)	1	74	0
PGF_02853126	Repair of Iron Centers di-iron protein	1	221	0
PGF_00048740	Ribose ABC transporter, periplasmic ribose-binding protein RbsB (TC 3.A.1.2.1)	1	319	0
PGF_00050723	Sensor protein copS (EC 2.7.3.-)	1	482	0
PGF_00055454	TPR domain protein	1	246	0
PGF_04111601	TRAP-type C4-dicarboxylate transport system, large permease component	1	433	0
PGF_06137558	TRAP-type C4-dicarboxylate transport system, periplasmic component	1	326	0
PGF_00055872	TRAP-type C4-dicarboxylate transport system, small permease component	1	168	0
PGF_05620245	Thiosulfate:cyanide sulfurtransferase PspE (EC 2.8.1.1)	1	107	0
PGF_01626418	TnpA transposase	2	500.5	3.5
PGF_01121714	Transcriptional regulator	1	254	0
PGF_00061764	Transposon Tn21 resolvase	1	208	0
PGF_00063471	Type III restriction-modification system methylation subunit (EC 2.1.1.72)	1	706	0
PGF_00064885	Uncharacterized protein AF_1681	1	214	0
PGF_00066263	Uronate isomerase (EC 5.3.1.12)	1	474	0
PGF_00066310	Usg protein	1	477	0
PGF_00066371	Uxu operon transcriptional regulator	1	256	0
PGF_01667392	Xylulose kinase (EC 2.7.1.17)	1	487	0
PGF_03294113	alkylhydroperoxidase like protein, AhpD family	1	113	0
PGF_03854940	putative flippase	1	485	0
PGF_00409842	putative nuclease	1	233	0
	128 Hypothetical proteins			
	Present only in the genome of strain AJ83			
PGF_02282781	Acetyltransferase (isoleucine patch superfamily)	1	216	0
PGF_00050752	Amidohydrolase family protein	1	465	0
PGF_00066715	Aspartate aminotransferase (EC 2.6.1.1)	1	396	0
PGF_01771319	Bacteriocin/lantibiotic efflux ABC transporter, permease/ATP-binding protein	1	685	0
PGF_04991091	Bipolar DNA helicase HerA	1	587	0
PGF_00417608	Chitinase (EC 3.2.1.14)	1	783	0
PGF_00418006	Chromosome segregation ATPase	2	425	19
PGF_00418879	Conjugative transfer ATP-dependent DNA helicase	1	667	0
PGF_00419582	Copper tolerance protein	1	177	0
PGF_06243817	Cytochrome c551/c552	1	105	0
PGF_01370898	D-glycero-D-manno-heptose 1-phosphate guanosyltransferase	1	350	0
PGF_06102630	DNA primase, phage associated	1	754	0
PGF_04276033	Fimbriae usher protein StfC	1	834	0
PGF_00552044	Helix-turn-helix domain protein	1	113	0
PGF_00014121	Inner membrane protein YbcI	1	180	0
PGF_03033306	Integral membrane protein	1	157	0
PGF_00016826	Legionaminic acid biosynthesis protein PtmG	1	368	0
PGF_08041143	MaoC family protein	1	138	0
PGF_03672087	Nitrous oxide reductase maturation protein, outer membrane lipoprotein NosL	1	181	0

PGF_00026086	Nitrous oxide reductase maturation transmembrane protein NosY	1	276		0
PGF_06331579	O-antigen export system permease protein RfbD	1	269		0
PGF_00027601	OsmC/Ohr family protein	1	162		0
PGF_00036518	Predicted transporter component	1	148		0
PGF_00036619	Prevent host death protein, Phd antitoxin	2	70.5		24.5
PGF_00037709	Prophage Lp2 protein 6	1	363		0
PGF_03098502	Putative DNA processing chain A	1	444		0
PGF_00043825	Putative ribosomal-protein-serine acetyltransferase	1	181		0
PGF_00401204	Subclass B3 beta-lactamase (EC 3.5.2.6)	1	337		0
PGF_00056192	Tellurite resistance protein-related protein	1	222		0
PGF_06487367	UDP-N,N'-diacetylbacillosamine 2-epimerase (hydrolyzing) (EC 3.2.1.184)	1	380		0
PGF_06750368	beta-glycosyl hydrolase	1	892		0
PGF_07397007	gluconolactonase family protein	1	293		0
PGF_00404750	prophage CP4-like integrase	1	402		0
	126 Hypothetical proteins				
	Present only in the genome of strains 947C and A308				
PGF_04400245	GDP-4-amino-4,6-dideoxy-alpha-D-acetylglucosamine N-acetyltransferase	2	254		0
PGF_00557102	Lactoylglutathione lyase, YQJC B.subtilis ortholog	2	133		1
PGF_00032298	Phage protein	2	504.5		0.5
PGF_02516485	Protein containing aminopeptidase domain	2	451.5		1.5
	4 Hypothetical proteins				
	Present only in the genome of strains A308 and AJ83				
PGF_03244749	Bis-ABC ATPase SPy1206	2	519		0
PGF_01949586	Chloramphenicol O-acetyltransferase (EC 2.3.1.28) => CatB family	2	211		0
PGF_03438544	DNA or RNA helicase of superfamily II	2	1047		0
PGF_02955071	Mu-like prophage FluMu protein gp29	2	532		0
PGF_00025736	Nitrate/nitrite transporter NarK	2	470		0
PGF_08117723	Phage integrase	2	481		5
PGF_00032042	Phage major capsid protein	2	302		0
PGF_00032839	Phage terminase, small subunit	2	191		0
PGF_00045452	Putative transposase	2	737		0
PGF_02967959	Putative transposase	2	626		3
PGF_00047563	Replication initiation ATPase; bacteriophage DNA transposition B protein	2	240		0
PGF_00047728	Respiratory nitrate reductase alpha chain (EC 1.7.99.4)	2	1256		0
PGF_00047730	Respiratory nitrate reductase beta chain (EC 1.7.99.4)	2	512		0
PGF_00047732	Respiratory nitrate reductase delta chain (EC 1.7.99.4)	2	238		0
PGF_00047734	Respiratory nitrate reductase gamma chain (EC 1.7.99.4)	2	225		0
PGF_00557133	Ribonuclease Z (EC 3.1.26.11) (RNase Z) (tRNase Z) (tRNA 3 endonuclease)	2	342		0
PGF_00748608	Transcriptional regulator, LysR family	2	299		0
PGF_03752321	Transposase, mutator type	3	302	97.8	
PGF_00404789	prophage MuSo1, transcriptional regulator, Cro/CI family	2	248		0
PGF_00404790	prophage MuSo2, portal protein, putative	2	523		0

PGF_00557065	putative lipoprotein	2	59.5	17.5
PGF_00411276	putative transposition protein	2	335	0
	43 Hypothetical proteins			
	Present only in the genome of strains 947C, A308 and AJ83			
PGF_03033259	Serine/threonine protein phosphatase (EC 3.1.3.16)	3	261	0
PGF_01493148	TPR repeat	3	394	0
	4 Hypothetical proteins			

Table S6. Presence and absence of known virulence genes in six mesophilic *A. salmonicida* strains

		Strain					
Gene	Function	947C	AJ83	A308	34mel	A527	Y47
aerA	Aerolysin	+	+	+	+	+	+
ahh1	Extracellular hemolysin	+	+	+	+	+	+
ahpB	Serine Protease	+	+	+	+	+	+
alt	Cytotonic enterotoxin (lipase)	+	+	+	+	+	+
ast	Cytotonic enterotoxin	+	+	-	-	-	-
dam	DNA adenine methyltransferase	+	+	+	+	+	+
eno	Enolase (surface-expressed)	+	+	+	+	+	+
eprA1	EprA1 (extracellular protease)	+	+	+	+	+	+
gidA	Glucose-inhibited division protein	+	+	+	+	+	+
rtxA	RTX toxin (repeat in toxin A)	-	+	Truncated	+	Truncated	+
ser	Serine protease	+	+	+	+	+	+
tagA	ToxR-regulated lipoprotein (TagA)	+	+	+	+	+	+
vacB	Exoribonuclease R	+	+	+	+	+	+
AHA_1741	Collagenase	+	+	+	+	+	+
AHA_3147	Invasin	+	+	+	+	+	+
AHA_3217	Thermostable hemolysin	+	+	+	+	+	+
AHA_3493	Hemolysin III	+	+	+	+	+	+

Table S7. Antibiotic resistance genes predicted from the genome sequences of mesophilic *A. salmonicida* strains

				Strain									
Gene	**AMR Gene Family**	**Drug Class**	**Accession #**	**34mel^T**	**A527**	**Y577**	**ASG1**	**ECFood+05**	**947C**	**A308**	**AJ83**	**Y567**	**Y47**
AAC(3)-IIc	AAC(3)	aminoglycoside antibiotic	ARO:3002535	-	-	-	+	-	-	-	-	-	-
AAC(6')-Ia	AAC(6')	aminoglycoside antibiotic	ARO:3002545	-	-	-	-	+	-	-	-	-	-
ANT(2'')-Ia	ANT(2'')	aminoglycoside antibiotic	ARO:3000230	-	-	-	-	+	-	-	-	-	-
APH(3'')-Ib	APH(3'')	aminoglycoside antibiotic	ARO:3002639	-	-	-	-	-	+	-	-	-	-
APH(3')-Ia	APH(3')	aminoglycoside antibiotic	ARO:3002641	-	-	-	+	-	-	-	-	-	-
APH(6)-Id	APH(6)	aminoglycoside antibiotic	ARO:3002660	-	-	-	-	-	+	-	-	-	-
CTX-M-3	CTX-M beta-lactamase	cephalosporin	ARO:3001866	-	-	-	+	-	-	-	-	-	-
FOX[a]	FOX beta-lactamase	cephamycin, cephalosporin	ARO:3002156	-	+	-	-	-	-	+	+	+	-
OXA-12	OXA beta-lactamase	cephalosporin, penam	ARO:3001407	+	+	+	+	+	+	+	+	+	+
TEM-1	TEM beta-lactamase	penem, cephalosporin, monobactam, penam	ARO:3000873	-	-	-	+	-	-	-	-	-	-
VEB-1	VEB beta-lactamase	cephalosporin, monobactam	ARO:3002370	-	-	-	-	+	-	-	-	-	-
aadA	ANT(3'')	aminoglycoside antibiotic	ARO:3002601	-	-	-	+	+	-	-	-	-	-
catII	chloramphenicol acetyltransferase (CAT)	phenicol antibiotic	ARO:3002684	-	-	-	+	-	-	-	-	-	-
cmlA5	major facilitator superfamily (MFS) antibiotic efflux pump	phenicol antibiotic	ARO:3002695	-	-	-	-	+	-	-	-	-	-
cphA5	CphA beta-lactamase	carbapenem	ARO:3003101	+	+	+	+	+	+	+	+	+	+
dfrA12	trimethoprim resistant dihydrofolate reductase dfr	diaminopyrimidine antibiotic	ARO:3002858	-	-	-	+	-	-	-	-	-	-
dfrA14	trimethoprim resistant dihydrofolate reductase dfr	diaminopyrimidine antibiotic	ARO:3002859	-	-	-	-	+	-	-	-	-	-
dfrA15	trimethoprim resistant dihydrofolate reductase dfr	diaminopyrimidine antibiotic	ARO:3003013	-	-	-	+	-	-	-	-	-	-
qacH	small multidrug resistance (SMR) antibiotic efflux pump	fluoroquinolone antibiotic	ARO:3003836	-	-	-	-	+	-	-	-	-	-
sul1	sulfonamide resistant sul	sulfonamide antibiotic, sulfone antibiotic	ARO:3000410	-	-	-	+	-	-	-	-	-	-
tet(E)	major facilitator superfamily (MFS) antibiotic efflux pump	tetracycline antibiotic	ARO:3000173	-	-	-	+	+	-	-	-	-	+
			Total	**2**	**3**	**2**	**12**	**10**	**4**	**3**	**3**	**3**	**3**

a. May be FOX-2, FOX-3, FOX-4 or FOX-5

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