Received: 27 May 2024 | Revised: 29 July 2024 | Accepted: 5 August 2024

DOI: 10.1111/jfd.14007

RESEARCH ARTICLE

Journal of Fish Diseases WILEY

Differential expression of the type III secretion system genes in *Yersinia ruckeri*: Preliminary investigations in different environmental conditions

Hadis Rahmatelahi[1] | Simon Menanteau-Ledouble[2] | Astrid S. Holzer[1] | Mansour El-Matbouli[1] | Mona Saleh[1]

[1]Division of Fish Health, Department for Farm Animals & Veterinary Public Health, University of Veterinary Medicine, Vienna, Austria

[2]Norce, Nygårdsgaten, Bergen, Norway

Correspondence
Mona Saleh, Division of Fish Health, Department for Farm Animals & Veterinary Public Health, University of Veterinary Medicine, Vienna, Austria.
Email: mona.saleh@vetmeduni.ac.at

Abstract

Type III secretion system (T3SS) is an important virulence system in Gram-negative bacteria. In this investigation, different environmental conditions that regulate the expression of T3SS genes in *Yersinia ruckeri* were investigated aimed at obtaining a better understanding about its modulation after various environmental challenges. Four isolates of *Y. ruckeri* CSF007-82, ATCC29473, A7959-11 and YRNC10 were cultivated under the diverse in vitro challenges iron depletion, high salt, low pH and in the presence of fish serum or in the fish cell culture (Chinook Salmon Embryo – CHSE). The transcriptional modulation of the chromosomal genes *ysaV*, *ysaC*, *ysaJ* and *prgH* of *ysa* were investigated using quantitative real-time PCR. The expression of *prgH*, *ysaV*, *ysaC* and *ysaJ* was differentially expressed in all four strains under evaluation. The highest gene expression levels were observed for *Y. ruckeri* YRNC10 AN after addition of 0.3 M NaCl in Luria Bertani broth. The results obtained from this study provide initial insights into T3SS responses in *Y. ruckeri*, which pave the way for further studies aimed at expanding our knowledge on the functional roles of the T3SS genes in *Y. ruckeri*.

KEYWORDS
environmental challenges, fish pathogen, gene modulation, Q-PCR, type III secretion system, *Yersinia ruckeri*

1 | INTRODUCTION

Yersinia ruckeri is recognized as a significant fish pathogen, primarily identified as the causative agent of enteric red mouth disease in salmonid fish species belonging to the *JKL gammaproteobacterium* (Calvez et al., 2014; Kumar et al., 2015; Nelson et al., 2015). Two biotypes have been described for this bacterium, motile and non-motile biotypes, mostly differentiated based on the silencing of flagellar genes in the non-motile biotype. The flagellum is often a major antigenic determinant of bacteria, and therefore, silencing of flagellar genes is a recurring occurrence in the evolution of infectious bacteria. In *Y. ruckeri*, this silencing might have been facilitated by the widespread adoption of a vaccine strategy to control ERM.

While different families of T3SS have been described, their general structure tends to be conserved between families. It includes a basal body anchoring the T3SS into the cell membranes

and harbouring a sorting platform within the bacterial cytoplasm aimed at directing the effector proteins into the needle. The needle extends outside of the cell, and the needle tip inserts effectors through the membrane to the target cells (Moest & Méresse, 2013; Troisfontaines & Cornelis, 2005).

This secretion system is found among *Enterobacteriaceae*; however, its effectors and functions in *Y. ruckeri* remain elusive. T3SS has virulence activity by interfering with cell signalling pathways to overcome target cells. Moreover, T3SS suppresses the immune system through the activity of its effectors (Pinaud et al., 2018; Rahmatelahi et al., 2021). Thus far, two groups of effectors have been associated with this virulence factor, known as *ysa* and *ysp* (Venecia & Young, 2005).

The entire genetic sequence of the ysa locus was discovered within the chromosome of the virulent *Y. ruckeri* SC09 strain. The *ysa* locus has pathogenic features in *Y. enterocolitica* Pathogenicity Island I (Venecia & Young, 2005). It demonstrates homology with *Salmonella enterica* Pathogenicity Island 1, and the chromosome encoded T3SS of *Y. enterocolitica* biotype 1B (Liu et al., 2016).

Several genes from the T3SS have been identified based on this homology, including *prgH*, which plays a role as an early protein, cooperating with switch proteases like *spaS* in the needle compartmental completion process (Monjarás Feria et al., 2015). Similarly, other studies showed *ysaC* is a chromosomal-encoded effector located in the outer membrane. It plays a role in forming a channel between bacterial cytoplasm and that of the host cells during pathogenesis (Haller et al., 2000). *ysaJ* is a further gene-related T3SS that resides in the outer membrane. Its function is defined as its structural role in forming lipoprotein (Haller et al., 2000). *YsaV* has homology to the inner membrane transporter component of the type III secretion system.

YsaC, *ysaV* and *ysaJ* as chromosomal T3SS genes are homologous to *Shigella flexneri* effectors, mxiD, mxiA and mxiJ, and to *Y. enterocolitica* (PYV) effectors, yscC, yscV and yscJ, respectively (Haller et al., 2000).

The evaluation of T3SS-related genes and monitoring their expression under various in vitro challenges could provide valuable insights into the factors regulating the expression of T3SS in *Y. ruckeri*. In the present study, we focused on two isolates of biotype I and two isolates of biotype II to gain a deeper understanding of how environmental factors influence this virulence and invasion-related T3SS system.

2 | MATERIALS AND METHODS

2.1 | Media and bacterial strains and growth conditions

Four strains from biotype I and biotype II collected from our fish clinic biobank were used in this study, and their identities were confirmed by PCR. These strains included Biotype I, *Y. ruckeri* CSF007-82 and ATCC29473, as well as Biotype II, YRNC10 and A7959-11. All strains have been isolated from rainbow trout and stored at −80°C until the start of the experiment. The bacteria were thawed, streaked out on Columbia agar (Sigma-Aldrich) and incubated overnight at 28°C. Single colonies were then inoculated into 7.5 mL starter cultures of Luria Bertani (LB; Sigma-Aldrich, Vienna, Austria). Bacteria were incubated until achieving appropriate OD600. Afterwards, 1 mL was taken out and further inoculated in 15 mL of modified broth overnight (OD600). The broth was modified to provide various environmental triggers to the expression of the T3SS, as described below. Furthermore, bacteria were exposed to fish sera and fish cells. All experiments were performed in biological triplicates.

The log phase was specifically selected for subsequent transcriptional analysis. During the log phase, the bacterial population experiences rapid growth, prompting the initiation of further procedures. This phase is particularly advantageous as it signifies a prominent increase in both gene expression and bacterial growth. The collected samples were used for the next steps including extraction of RNA, preparation of cDNA and quantitative polymerase chain reaction (Q-PCR), aiming to analyse the T3SS genes.

2.2 | Iron-starvation challenge

LB broth was supplemented with 1 mg per 60 mL of 2,2′-dipyridyl N-oxide (MW; 172.18 g/nMol; Sigma-Aldrich, Vienna, Austria). Bacterial single colonies collected from Columbia agar from overnight cultivated isolates were inoculated in supplemented and non-supplemented broth, then incubated for 5–6 h to get appropriate OD 600 (Figure 1). Afterwards, 1 mL taken out of the same broth was added to the 14 mL fresh supplemented and non-supplemented and incubated overnight with the same procedure explained above, then centrifuged. Finally, RNAs were extracted by following the manufacturer's protocol with the RNeasy Kit (Qiagen, Hilden, Germany).

2.3 | High-salt challenge

In this challenge, LB broth was supplemented with 0.3 M NaCl. Then, from the overnight cultivation of the *Y. ruckeri* strains CSF007-82, A7959-11, attenuated laboratory strain ATCC29473, and YRNC10, a single colony was isolated and inoculated in supplemented broth and non-supplemented broth. First, in 7.5 mL of high-salt LB and LB to appropriate OD600. Subsequently, for the second time, 1 mL of ideal OD600 broth was added to 14 mL of freshly supplemented LB and LB. After that, the broth was incubated overnight while shaking at 28°C. Lastly, the incubated broth was centrifuged at 16,000 *g*, pelleted and the RNA was extracted.

2.4 | Chinook salmon embryo cells (CHSE-214)

CHSE-214 cells were incubated with the overnight cultivated bacteria in non-supplemented LB broth for 4 h at room temperature. Overnight cultivated broth was pelleted by centrifugation at 16,000 *g* for 10 min at 4°C and the flow through discarded. The pellets were

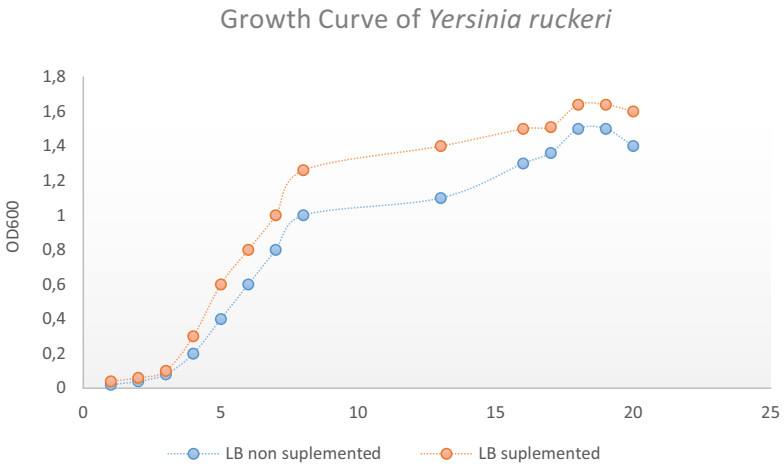

FIGURE 1 The growth curves of *Yersinia ruckeri* YRNC10, comparing non-supplemented and high-salt-supplemented conditions, revealed subtle differences at various time points.

TABLE 1 Type III secretion system genes and housekeeping genes used in this study.

Primer	Forward primer sequence	Revers primer sequence	Annealing temperatures (°C)	Amplicon size (bp)
YRA1	5′- TCTGGACATCGCTCTGG-3′	5′-AGTTTTTTTGCGTAGATAGGA-3′	58	183
Yruck16S1	5′- TAACCTCGCAAGAGCAAAGTG-3	5′- GACCGTGTCTCAGTTCCAGT-3′	59	155
PrgH	5′-TTGCCGGTCTCTTTGCCTTTA-3′	5′-CCAATCCATATCCCGCTGTGT-3′	53.5	164
YsaC	5′-GACCGCATCAAATACCTTGGC-3′	5′-CTGGTTACGTTTCCTCTGCCT-3′	55	119
YsaJ	5′- GTTGTAGCGTAGCCAGGACTT-3	5′-GCACTGTTATTTGCCGGTTCA-3′	53.6	109
YsaV	5′- AATCACTGGTAGCGACGGAG-3′	5′- TCTTCGGGCGGGACATTTTT-3′	54	89

resuspended in 10 mL of Gibco™ 1xGlutaMax Minimum Essential Eagle (MEM; Thermo Fisher scientific, Vienna, Austria) and mixed well to detach the pellet. Afterwards, 1 mL of the mixture of media and pellet was transferred into a smaller falcon and 4 mL from the fresh MEM medium was added to mixture. We retrieved half of the media from three wells of cell cultures. Subsequently, the remaining half of the media from these three wells was carefully removed before introducing the mixture of bacterial Gibco medium. This procedure was promptly executed to ensure the cells remained in a fresh state. Then, immediately, 1 mL of bacterial mixture was added to each well and incubated at room temperature for 4 h.

2.5 | Serum challenge

Bacterial pellets were harvested as previously described then resuspended in the freshly 2 mL serum of rainbow trout (*Oncorhynchus mykiss*), pipetting well and incubated for 4 h at room temperature.

2.6 | RNA extraction and cDNA preparation

Bacterial RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Prior to the extraction, the overnight-cultivated (OD 600) *Y. ruckeri* samples from all four challenges were centrifuged at 16,000*g* for 10 min at 4°C. The pellets were washed with PBS and then lysed by adding RLT buffer containing β-mercaptoethanol. The final amount of RNA was measured using a NanoDrop 2000 spectrophotometer with a 4200 TapeStation (Agilent, Santa Clara, CA, USA).

For reverse transcription, extracted RNA was converted in cDNAs using 20 ng of RNA per reaction according to the manufacturer's protocol for the Qiagen iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

2.7 | Primer design

Primers were designed (Table 1) using the NCBI primer design tool Primer-BLAST after alignment of all available *Y. ruckeri* genomes. The specificity of the primers was confirmed by analysing them in conventional PCR and running them on an agarose gel. Obtained bands were purified from the agarose gel, purified amplicons were prepared for sequencing (LGC Genomics, Berlin, Germany), and the sequences were confirmed using the Basic Local Alignment Sequence Tool (NCBI). YRA1 and Yruck16S were used as housekeeping genes according to Bastardo et al. (2012).

2.8 | Reverse transcription-quantitative real-time PCR (RT-qPCR) analysis

The synthesized cDNA was tested in quantitative real-time PCR to analyse the expression levels of the targeted T3SS genes using the CFX96 Touch Real-Time PCR detection system (Bio-Rad,

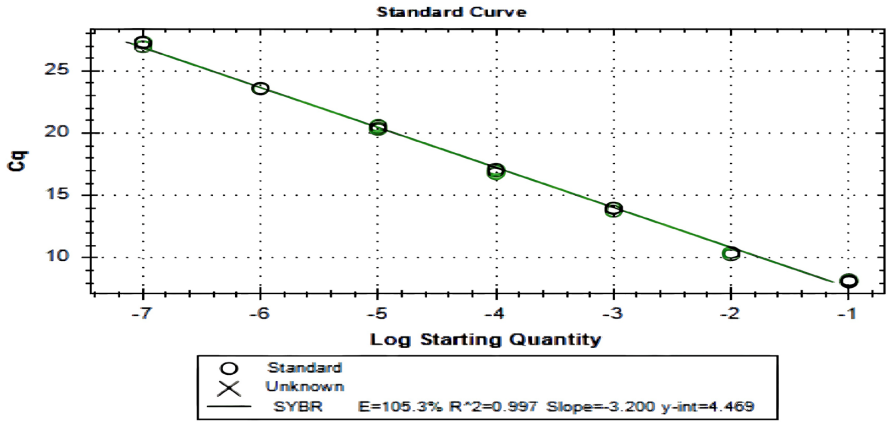

FIGURE 2 Standard curves for each gene and for housekeeping genes were obtained using bacterial DNA dilution (10^{-1}–10^{-7} ng).

München, Germany). Each of these RTqPCRs was performed in technical triplicates. Standard curves for each gene and for housekeeping genes were obtained using bacterial DNA dilution (10^{-1}–10^{-7} ng; Figure 2). The real-time PCR assay for every gene was performed in a total volume of 25 µL containing 12.5 µL of 2× SsoAdvancedTM Universal SYBR Green Supermix (Bio-Rad); 1 µL of forward and reverse primer; 5.5 µL of nuclease-free water; and 3 µL of 1:20 dilution of cDNA samples. The real-time qPCR cycling conditions included an initial denaturation step at 95°C for 5 min, followed by 39 cycles of denaturation at 95°C for 30 s. Annealing temperatures ranged from 54°C to 59°C, and annealing lasted 30 s with an elongation at 72°C. The final elongation step has been run at 95°C for 30s. Melting curve analysis was performed at 60–90°C with an increment of 0.5°C per 10 s.

2.9 | Statistical analysis

Gene expression was normalized to the mean of both reference genes. The relative gene expression of genes between exposed and control groups was calculated using the $2^{-\Delta\Delta Ct}$ method. A student's *t*-test was employed for analysing the variance between the normal and challenged groups according to a rate of *p* value less than .05 ($p < .05$).

3 | RESULTS

The modulation of the T3SS investigated genes *ysaJ*, *ysaC*, *ysaV* and *prgH* is shown in fold changes in the charts 3–6. The levels of gene expression are shown compared to a normal condition.

As shown in charts 3–6, genes related to T3SS were differentially modulated in all four *Y. ruckeri* strains. Specifically, the genes are highly expressed in the strains CSF007-82 and YRNC10. The lowest levels of stimulation were observed in the two isolates, ATCC29473 and A7959-11. Under these challenges, *ysaJ* in isolate CSF007-82 is highly stimulated. *ysaC* expression is highly stimulated by fish cell lines, rainbow trout serum and high salt. *ysaV* expression is stimulated in all performed challenges in the two isolates CSF007-82 and YRNC10. *PrgH* expression is upregulated in YRNC10 in all performed challenges. Under all challenges performed, gene modulation is considered significant when the p value is greater than 0.05 ($p < .05$).

3.1 | Iron starvation challenge

The data demonstrate (Figure 3) the modulation of T3SS genes in the isolates of *Y. ruckeri* CSF007-82, A7959-11, YRNC10 and ATCC29473. Iron starvation challenge showed differential gene expression of the tested T3SS genes in each isolate. The expression of ysaJ in CSF007-82 was increased (>247-fold), while the gene expression of ysaC, ysaV and prgH showed <8-fold induction. The isolate A7959-11 showed increased gene expression in the range of 5- to >41-fold. The isolate YRNC10 showed highest expression for ysaV (>521-fold) and prgH (>120-fold), and ysaJ and ysaC showed increased expression rates (>22-fold) and (>32-fold), respectively. The isolate ATCC29473 showed lower stimulation among the four tested isolates in the range of >3- to >11-fold for ysaJ and prgH respectively.

Under stress due to iron-starvation, significant upregulation of *ysaV* expression in YRNC10 (>527-fold) and *prgH* (>120-fold). In addition, *ysaJ* was significantly increased (>247-fold) in isolate CSF007-82.

3.2 | High-salt challenge

High-salt challenge revealed varied gene expression values in the four tested isolates (Figure 4). In this challenge, gene expression levels of all the four genes were high in CSF007-82. In YRNC10, high gene expression levels of *prgH* (>768-fold) and *ysaV* (>224-fold) were observed.

In isolate CSF007-82, gene expression increased for *ysaV*, *ysaC* and ysaJ (>209-fold), (>178-fold), (>161-fold) and for *prgH* (>64-fold), respectively, as compared with the control group. As observed in *Y. ruckeri* CSF007-82, gene expression revealed stimulation with moderate diversity. In isolate A7959-11 highest gene expression upregulation was observed for *ysaV* (>108-fold), the rest of genes have shown lower values compared to *ysaC*. Herein, as it is demonstrated for *ysaJ* (>9-fold), *ysaV* (>3-fold) and *prgH* (>2-fold). Isolate YRNC10 showed highest gene expression levels for *prgH* (>768-fold) and *ysaV* (>224-fold) compared to the rest of the genes and other under tested strains. In the YRNC10 strain, high-salt induced gene expression of *ysaC* (>57-fold)

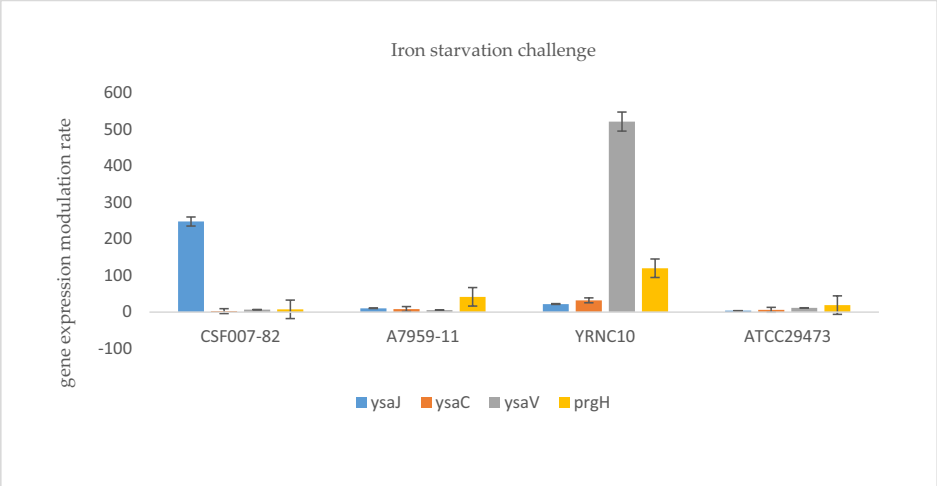

FIGURE 3 In this chart, data were normalized with housekeeping genes, compared with control groups and demonstrated as fold change.

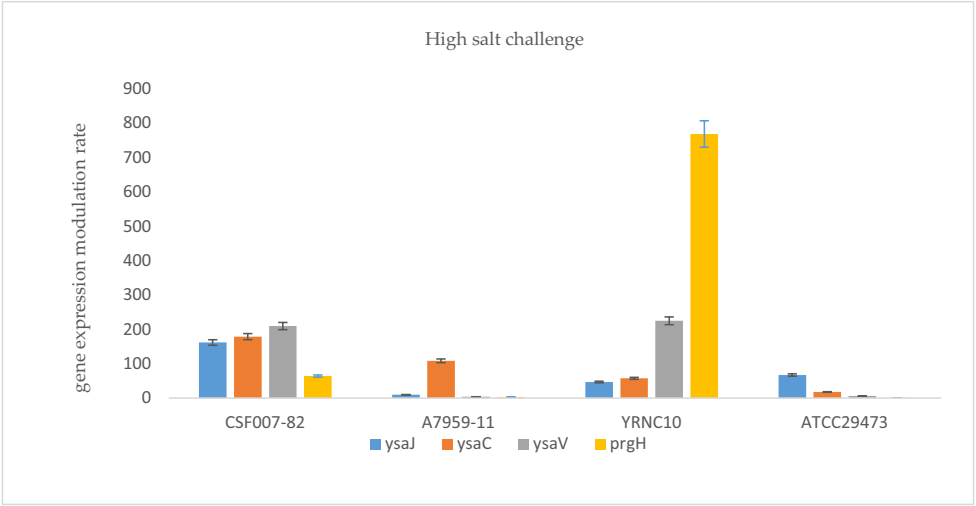

FIGURE 4 In this chart, data were normalized with housekeeping genes, compared with control groups and demonstrated as fold change.

and *ysaJ* (>46-fold). The isolate ATCC 29473 demonstrated lower gene expression values for all genes, *ysaJ* (>67-fold), *ysaC* (>17-fold), *ysaV* (>5-fold), *prgH* (>1-fold) compared to the rest of the tested strains.

3.3 | CHSE line challenge

Chinook salmon embryo cells (CHSE-214) have been used as a model for studying various isolates of *Y. ruckeri* to investigate the levels of infection and protein release in vitro. As shown in Figure 5, the expression in YRNC10 modulated *ysaC* increased significantly (>673-fold), *ysaV* (>185-fold) and *prgH* (>606-fold) compared to control unstimulated bacteria respectively. Isolate CSF007-82 showed increased gene expression values for *ysaJ* (ca. 164-fold), *ysaC* (>56-fold) and *ysaV* (>47-fold), and for *prgH* (>24-fold). Isolate A7959-11 showed gene expression upregulation for *ysaJ* (ca. 7-fold), *ysaC* (>158-fold), *ysaV* (>3-fold) and for *prgH* (<1-fold). YRNC10 demonstrated highest gene expression levels for *ysaC* (>673-fold), *prgH* (>606-fold) compared to the other tested isolates, but *ysaJ* showed lowest gene expression upregulation (>2-fold) compared to other isolates. In isolate ATCC29473, the gene expression was upregulated for *ysaJ* (ca. 44-fold), *ysaC* (>123-fold) and *ysaV* (>57-fold), and for *prgH* (>2-fold).

3.4 | Rainbow trout (RBT) serum challenge

As shown in Figure 6, isolate CSF007-82 showed increased gene expression values for *ysaJ* (>40-fold), *ysaC* (>272-fold) and *ysaV* (>82-fold), and for *prgH* (>26-fold). A7959-11 also showed increased gene expression values for *ysaJ* (>15-fold), *ysaC* (ca. 88-fold) and *ysaV* (>42-fold), and for *prgH* (>20-fold). YRNC10 demonstrated the highest gene expression levels for *prgH* (ca. 869-fold), *ysaC* (>472-fold) and *ysaV* (ca. 309-fold). Isolate ATCC29473 exhibited increased gene expression in the range of (>83- to ca. 3-fold) for *ysaJ* to *prgH*, respectively.

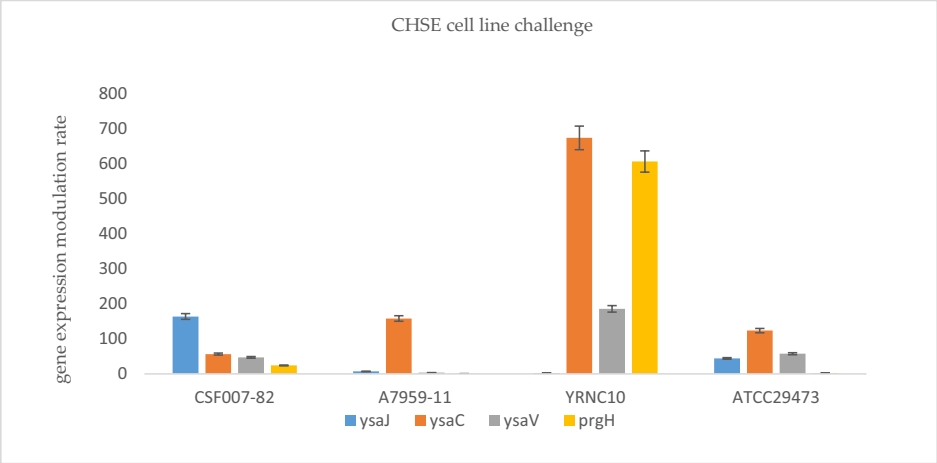

FIGURE 5 In this chart, data were normalized with housekeeping genes, compared with control groups and demonstrated as fold change.

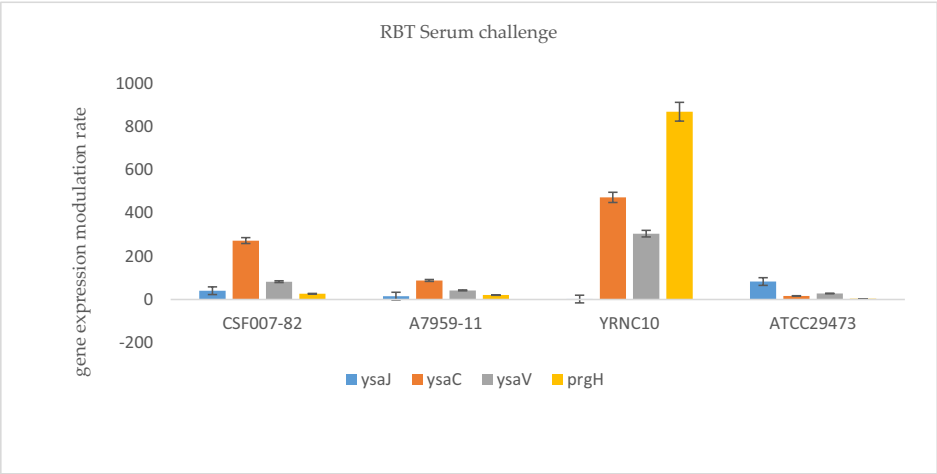

FIGURE 6 In this chart, data were normalized with housekeeping genes, compared with control groups and demonstrated as fold change.

As expected, significant increase of *ysaC* (>472-fold), *ysaV* (>304-fold) and *prgH* (ca. 869-fold) was observed in YRNC10. In addition, the expression of *ysaC* was increased (>274-fold) in CSF007-82.

4 | DISCUSSION

The rationale behind studying these *Y. ruckeri* strains (Biotype I: CSF007-82 and ATCC29473; Biotype II: YRNC10 and A7959-11) and their T3SS responses lies in understanding how different biotypes and strains adapt to different environments. The selection of different environmental conditions, such as the inclusion of 0.3 M NaCl in LB broth, was intended to mimic various stresses that *Y. ruckeri* encounter in its natural habitat and during infection. High-salt concentrations can simulate osmotic stress, which is prevalent in aquatic environments where this pathogen typically resides. Such conditions help to understand how T3SS and other virulence mechanisms are regulated in response to environmental changes. Comparing T3SS gene expression to environmental stimuli across biotypes and strains can uncover key regulatory mechanisms that govern virulence and adaptation. Such knowledge is crucial for developing targeted strategies for disease prevention and control, as well as for understanding the evolutionary dynamics of bacterial pathogens in aquatic ecosystems.

In the present study, we investigated the triggers of the *Y. ruckeri* chromosomal type III secretion system. Given the differences among the selected isolates, we utilized various biotypes of *Y. ruckeri* to explore the impact of environmental challenges on the expression of T3SS genes, which serve as crucial virulence factors.

Principally, upon contact and interaction with the host cell, the T3SS genes act as an invasive apparatus that becomes stimulated. This stimulation influences cell signalling cascades between host cells and bacterial invasive procedures in fish, allowing bacteria to overcome the protective response and defensive barriers of the host (Klein et al., 2000). Previous investigations on invaded mammalian cells have reported that Yop effectors, which are encoded by plasmids, are translocated or secreted to the C-terminal residues and released via chaperones and flagella (Haller et al., 2000). Herein, challenges were carried out to induce stress on the bacterial isolates in vitro, thus circumventing the use of live fish.

Y. ruckeri is recognized as a major microbial pathogen, associated with severe and economically costly outbreaks in aquaculture settings, notably in rainbow trout (Ma & Guijarro, 2004; Wrobel et al., 2019). Due to the recognized susceptibility of rainbow trout to *Y. ruckeri*, an assessment was conducted to examine the modulation of the T3SS genes. After incubation of *Y. ruckeri* in LB broth overnight, the isolates were cultivated with CHSE-214 cells according to the protocol previously described by Menanteau-Ledouble et al. (2018) as the cytotoxic effect was investigated as part of another experiment involving CHSE and the same strains of *Yersinia ruckeri*. Significant stimulation was observed for all investigated genes, but at varying levels. Furthermore, ysaC, recognized as one of the key T3SS genes responsible for host cell membrane penetration, was significantly induced in all evaluated strains during this challenge.

PrgH is located adjacent to other conserved EscJ/PrgK/YscJ family members in the inner membrane and outer membrane. As previously reported in Spreter et al. (2009), the *prgH* gene belongs to a group of genes involved in the invasion of host cells. Significant stimulation was observed in all under evaluation genes but with differential levels of modulation.

The highest level of *prgH* expression was observed in high salt, CHSE cell line and rainbow trout serum challenges in biotype II YRNC10 as one of the virulent isolates responsible for symptomatic disease in fish. Furthermore, in iron starvation, YRNC10 showed high expression values of *prgH*. PrgH is one of the structural components of the T3SS apparatus (Monjarás Feria et al., 2015). The crystal structure of T3SS in *S. typhimurium prgH* has been described as an inner membrane-conserved protein associated with the secretome (Spreter et al., 2009).

In the current study, the modulation of the *prgH* gene in response to environmental challenges potentiates its pivotal role as one of the fundamental T3SS elements in *Y. ruckeri*.

Iron starvation represents an external stress factor that significantly impacts bacterial activity. For instance, the identification of yhIBA through In vivo expression technology showcased elevated expression levels under conditions of iron deprivation. Within this cluster, two genes, yhIA and yhIB, exhibit cytolytic activity, which is accentuated under iron stress and low-temperature conditions (18°C; Guijarro et al., 2018). Furthermore, under conditions of iron limitation and a temperature of 18°C, the genes encoding ruckerobactin are stimulated to facilitate iron acquisition for bacterial growth. Ruckerobactin enhances iron levels by transporting iron molecules from the environment or from host cells across the membrane of infected cells (Ma & Guijarro, 2004; Wrobel et al., 2019). Our observations indicate that inducing iron starvation challenges also have diverse effects on gene modulation, albeit without following a regular pattern.

Overall, ysaV, ysaC and ysaJ are contact-dependent and directed towards overcoming host cells, playing a crucial role in the invasion process (Haller et al., 2000; Matsumoto & Young, 2006). The expression values of *ysaV* and *ysaC* do not follow the regular pattern in biotype I and II strains in this study. Regardless of whether strains being biotype I or biotype II, ysaC as a component of the T3SS syringe-like needle complex structure has a key role in host cell invasion process considering its increased gene expression under various challenges (Dean, 2011; Rahmatelahi et al., 2021). *YopJ* e has an inhibitory role, inhibiting MAPK and NF-$_k$B by affecting phosphorylation in this signalling cascade (Russo et al., 2019).

The regulatory proteins ysrS and rcsG encoded by the ysa locus in T3SS were stimulated under high-salt conditions (Venecia & Young, 2005). High concentration of salt as a stress factor is able to trigger expression of genes related to T3SS for invasion or secretion process (Haller et al., 2000). Hence, high-salt challenge was performed as stress inducing the expression of T3SS genes. The expression of the T3SS gene of *S. enterica* SPI2 was induced in response to environmental challenges, which in turn triggers the expression of other related genes and protein (Rappl et al., 2003). Similarly, throughout all the environmental challenges conducted in this study, the modulation of *Y. ruckeri* T3SS virulence-associated genes varied among one or more strains. This variation depended on the specific challenge conditions and the strain being examined.

In our study, we examined how various in vitro challenges affect the transcriptional regulation of Y. ruckeri T3SS virulence-associated genes. Unexpectedly, differences in the response of the T3SS were observed between even closely related strains. The differences in T3SS gene expression among the four *Y. ruckeri* strains and the varying responses of strains within the same biotype to environmental challenges can be attributed to several factors, including genetic variation, regulatory mechanisms and adaptation to specific environmental conditions. Variance in upstream regulatory sequences or the presence of additional regulators can modulate T3SS gene expression differently across strains (Beaumont et al., 2009; Lee & Marx, 2012). Strains within the same biotype may exhibit different responses to environmental challenges due to several reasons. Despite belonging to the same biotype, individual strains can carry unique genetic variations that influence their regulatory networks and responses to environmental stimuli. For example, differences in epigenetic modifications, such as DNA methylation or histone modifications, can alter gene expression patterns in response to environmental cues. Strains may have been adapted to different ecological niches or environmental conditions over time, resulting in distinct phenotypic responses to stimuli like osmotic stress (0.3 M NaCl). In fact, research on bacterial adaptation has highlighted that even subtle genetic differences or variations in regulatory elements can lead to significant phenotypic diversity in response to environmental changes (Beaumont et al., 2009; Lee & Marx, 2012). It is well known that different strains of *Y. ruckeri* can display vast difference in the expression of their virulence factors (Wrobel et al., 2019), a fact that would be consistent with the 'epidemic population structure' described by Bastardo et al. and Barnes et al. (Barnes et al., 2016; Bastardo et al., 2012). In this model, recombinatory changes primarily drive the genetic diversity of *Y. ruckeri*. Afterwards, this diversity is expanded through disease outbreaks, highlighting the importance of virulence factors to drive the diversity of *Y. ruckeri*. The best known

example of phenomenon in *Y. ruckeri* is likely the flagellar apparatus for which silencing is known to have occurred multiple times independently and which has become an important discriminant markers between strains and biotypes of *Y. ruckeri* (Barnes et al., 2016; Riborg et al., 2022). The variation in T3SS gene expression among *Y. ruckeri* strains and the differential responses of strains within the same biotype to environmental challenges reflect the complex interplay between genetic diversity, regulatory mechanisms and environmental adaptation in bacterial pathogens.

The observed modulation suggests that each environmental challenge could serve as a stimulator of T3SS genes. The in vitro method utilized in this research stands out as a practical and less invasive approach for monitoring virulence-related genes, providing an alternative to more aggressive and invasive in vivo experiments. In future studies, the investigation of further environmental conditions and combined in vitro stresses may provide options for evaluating gene behaviour, secreted proteins and isolate manipulation.

5 | CONCLUSION

The T3SS genes were stimulated by exposing *Y. ruckeri* to various environmental conditions in vitro. The modulation of gene expression showed that our tested environmental conditions could individually induce the T3SS virulence elements. Although there is a differential modulation under each type of in vitro stress, we could not observe a regular pattern among modulated genes in different isolates under applied stress conditions. These unexpected findings could suggest that the T3SS of *Y. ruckeri* is under the control of a complex regulatory system, allowing input from multiple environmental clues.

Investigating factors that influence and trigger the activation of *Y. ruckeri* T3SS-associated genes is pivotal for obtaining a deeper understanding of virulence enhancers and inhibitors. This exploration not only contributes valuable insights into the nature of these factors but also holds the potential and paves the way for innovative strategies in *Y. ruckeri* management. Furthermore, such research efforts may play a crucial role in the development of advanced drugs targeting *Y. ruckeri* virulence factors, with the primary goal of inhibiting and preventing disorders in fish.

AUTHOR CONTRIBUTIONS

Hadis Rahmatelahi: Methodology; investigation; writing – original draft; validation. **Simon Menanteau-Ledouble:** Conceptualization; writing – review and editing; validation. **Astrid S. Holzer:** Writing – review and editing; resources. **Mansour El-Matbouli:** Conceptualization; writing – review and editing; supervision. **Mona Saleh:** Writing – review and editing; resources; supervision.

FUNDING INFORMATION

This research did not recieve any specific grant.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

ORCID

Mansour El-Matbouli https://orcid.org/0000-0001-8148-0218
Mona Saleh https://orcid.org/0000-0001-9273-1502

REFERENCES

Barnes, A. C., Delamare-Deboutteville, J., Gudkovs, N., Brosnahan, C., Morrison, R., & Carson, J. (2016). Whole genome analysis of *Yersinia ruckeri* isolated over 27 years in Australia and New Zealand reveals geographical endemism over multiple lineages and recent evolution under host selection. *Microbial Genomics*, *2*, e000095. https://doi.org/10.1099/mgen.0.000095

Bastardo, A., Ravelo, C., & Romalde, J. L. (2012). Highly sensitive detection and quantification of the pathogen *Yersinia ruckeri* in fish tissues by using real-time PCR. *Applied Microbiology and Biotechnology*, *96*(2), 511–520. https://doi.org/10.1007/s00253-012-4328-1

Beaumont, H., Gallie, J., Kost, C., et al. (2009). Experimental evolution of bet hedging. *Nature*, *462*, 90–93. https://doi.org/10.1038/nature08504

Calvez, S., Gantelet, H., Blanc, G., Douet, D. G., & Daniel, P. (2014). Yersinia ruckeri biotypes 1 and 2 in France: Presence and antibiotic susceptibility. *Diseases of Aquatic Organisms*, *109*(2), 117–126. https://doi.org/10.3354/dao02725

Dean, P. (2011). Functional domains and motifs of bacterial type III effector proteins and their roles in infection. *FEMS Microbiology Reviews*, *35*(6), 1100–1125. https://doi.org/10.1111/j.1574-6976.2011.00271.x

Guijarro, J. A., García-torrico, A. I., Cascales, D., & Méndez, J. (2018). The infection process of *Yersinia ruckeri*: Reviewing the pieces of the jigsaw puzzle. *Frontiers in Cellular and Infection Microbiology*, *8*, 1–10. https://doi.org/10.3389/fcimb.2018.00218

Haller, J. C., Carlson, S., Pederson, K. J., & Pierson, D. E. (2000). A chromosomally encoded type III secretion pathway in *Yersinia enterocolitica* is important in virulence. *Molecular Microbiology*, *36*(6), 1436–1446. https://doi.org/10.1046/j.1365-2958.2000.01964.x

Klein, J. R., Fahlen, T. F., & Jones, B. D. (2000). Transcriptional organization and function of invasion genes within *Salmonella enterica Serovar Typhimurium Pathogenicity orgA*, *orgB*. *And orgC Genes*, *68*(6), 3368–3376.

Kumar, G., Menanteau-Ledouble, S., Saleh, M., & El-Matbouli, M. (2015). *Yersinia ruckeri*, the causative agent of enteric redmouth disease in fish. *Veterinary Research*, *46*(1), 1–10. https://doi.org/10.1186/s13567-015-0238-4

Lee, M. C., & Marx, C. J. (2012). Repeated, selection-driven genome reduction of accessory genes in experimental populations. *PLoS Genetics*, *8*(5), e1002651. https://doi.org/10.1371/journal.pgen.1002651

Liu, T., Wang, K. Y., Wang, J., Chen, D. F., Huang, X. L., Ouyang, P., Geng, Y., He, Y., Zhou, Y., & Min, J. (2016). Genome sequence of the fish pathogen *yersinia ruckeri* SC09 provides insights into niche adaptation and pathogenic mechanism. *International Journal of Molecular Sciences*, *17*(4), 1–21. https://doi.org/10.3390/ijms17040557

Ma, I., & Guijarro, J. A. (2004). Identification of specific in vivo-induced (ivi) genes in yersinia ruckeri and analysis of ruckerbactin, a catecholate siderophore iron acquisition. *System*, *70*(9), 5199–5207. https://doi.org/10.1128/AEM.70.9.5199

Matsumoto, H., & Young, G. M. (2006). Proteomic and Functional Analysis of the Suite of Ysp Proteins Exported by the Ysa Type III Secretion System of *Yersinia Enterocolitica* Biovar 1B. *Molecular Microbiology*, *59*, 689–706. https://doi.org/10.1111/j.1365-2958.2005.04973.x

Menanteau-Ledouble, S., Lawrence, M. L., & El-Matbouli, M. (2018). Invasion and replication of *Yersinia ruckeri* in fish cell cultures. *BMC Veterinary Research*, *14*(1), 1–11. https://doi.org/10.1186/s12917-018-1408-1

Moest, T. P., & Méresse, S. (2013). Salmonella T3SSs: Successful mission of the secret(ion) agents. *Current Opinion in Microbiology*, *16*(1), 38–44. https://doi.org/10.1016/j.mib.2012.11.006

Monjarás Feria, J. V., Lefebre, M. D., Stierhof, Y. D., Galán, J. E., & Wagner, S. (2015). Role of autocleavage in the function of a type iii secretion specificity switch protein in salmonella enterica serovar typhimurium. *MBio*, *6*(5), 1–8. https://doi.org/10.1128/mBio.01459-15

Nelson, M. C., LaPatra, S. E., Welch, T. J., & Graf, J. (2015). Complete genome sequence of *Yersinia ruckeri* strain CSF007-82, etiologic agent of red mouth disease in salmonid fish. *Genome Announcements*, *3*(1), 4–5. https://doi.org/10.1128/genomeA.01491-14

Pinaud, L., Sansonetti, P. J., & Phalipon, A. (2018). Host cell targeting by enteropathogenic bacteria T3SS effectors. *Trends in Microbiology*, *26*(4), 266–283. https://doi.org/10.1016/j.tim.2018.01.010

Rahmatelahi, H., Matbouli, M. E., & Ledouble, S. M. (2021). Delivering the pain: An overview of the type III secretion system with special consideration for aquatic pathogens. *Veterinary Research*, *1–18*, 146. https://doi.org/10.1186/s13567-021-01015-8

Rappl, C., Deiwick, J., & Hensel, M. (2003). Acidic pH is required for the functional assembly of the type III secretion system encoded by salmonella pathogenicity Island 2. *FEMS Microbiology Letters*, *226*(2), 363–372. https://doi.org/10.1016/S0378-1097(03)00638-4

Riborg, A., Colquhoun, D. J., & Gulla, S. (2022). Biotyping reveals loss of motility in two distinct *Yersinia ruckeri* lineages exclusive to Norwegian aquaculture. *Journal of Fish Diseases*, *45*, 641–653. https://doi.org/10.1111/jfd.13590

Russo, B. C., Duncan, J. K., Wiscovitch, A. L., Hachey, C., & Id, M. B. G. (2019). Activation of shigella flexneri type 3 secretion requires a host-induced conformational change to the translocon pore. *PLoS Pathogens*, *15*, 1–20. https://doi.org/10.1371/journal.ppat.1007928

Spreter, T., Yip, C. K., Sanowar, S., André, I., Kimbrough, T. G., Vuckovic, M., Pfuetzner, R. A., Deng, W., Yu, A. C., Finlay, B. B., Baker, D., Miller, S. I., & Strynadka, N. C. J. (2009). A conserved structural motif mediates formation of the periplasmic rings in the type III secretion system. *Nature Structural and Molecular Biology*, *16*(5), 468–476. https://doi.org/10.1038/nsmb.1603

Troisfontaines, P., & Cornelis, G. R. (2005). Type III secretion: More systems than you think. *Physiology*, *20*(5), 326–339. https://doi.org/10.1152/physiol.00011.2005

Venecia, K., & Young, G. M. (2005). Environmental regulation and virulence attributes of the Ysa type III secretion system of *Yersinia enterocolitica* biovar 1B. *Infection and Immunity*, *73*(9), 5961–5977. https://doi.org/10.1128/IAI.73.9.5961-5977.2005

Wrobel, A., Leo, J. C., & Linke, D. (2019). Overcoming fish defences: The virulence factors of *Yersinia ruckeri*. *Genes*, *10*(9), 700. https://doi.org/10.3390/genes10090700

How to cite this article: Rahmatelahi, H., Menanteau-Ledouble, S., Holzer, A. S., El-Matbouli, M., & Saleh, M. (2024). Differential expression of the type III secretion system genes in *Yersinia ruckeri*: Preliminary investigations in different environmental conditions. *Journal of Fish Diseases*, *47*, e14007. https://doi.org/10.1111/jfd.14007