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# Flagellar regulation mediated by the Rcs pathway is required for virulence in the fish pathogen *Yersinia ruckeri*

Anna K.S. Jozwick[a], Scott E. LaPatra[b], Joerg Graf[c], Timothy J. Welch[d,*]

[a] Center for Natural Science, Goucher College, Baltimore, MD, USA
[b] Clear Springs Foods, Inc., Research Division, Buhl, ID, USA
[c] Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut, USA
[d] National Center for Cool and Cold Water Aquaculture, Agricultural Research Service/U.S. Department of Agriculture, Kearneysville, West Virginia, USA

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ABSTRACT

The flagellum is a complex surface structure necessary for a number of activities including motility, chemotaxis, biofilm formation and host attachment. Flagellin, the primary structural protein making up the flagellum, is an abundant and potent activator of innate and adaptive immunity and therefore expression of flagellin during infection could be deleterious to the infection process due to flagellin-mediated host recognition. Here, we use quantitative RT-PCR to demonstrate that expression of the flagellin locus *fliC* is repressed during the course of infection and subsequently up-regulated upon host mortality in a motile strain of *Yersinia ruckeri*. The kinetics of *fliC* repression during the infection process is relatively slow as full repression occurs 7-days after the initiation of infection and after approximately 3-logs of bacterial growth *in vivo*. These results suggests that *Y. ruckeri* possesses a regulatory system capable of sensing host and modulating the expression of motility in response. Examination of the master flagellar operon (*flhDC*) promoter region for evidence of transcriptional regulation and regulatory binding sites revealed potential interaction with the Rcs pathway through an Rcs(A)B Box. Deletion of *rcsB* (Δ*rcsB*) by marker-exchange mutagenesis resulted in overproduction of flagellin and unregulated motility, showing that the Rcs pathway negatively regulates biosynthesis of the flagellar apparatus. Experimental challenge with Δ*rcsB* and Δ*rcsB*Δ*fliC1*Δ*fliC2* mutants revealed that mutation of the Rcs pathway results in virulence attenuation which is dependent on presence of the flagellin gene. These results suggest that the inappropriate expression of flagellin during infection triggers host recognition and thus immune stimulation resulting in attenuation of virulence. In addition, RNAseq analyses of the Δ*rcsB* mutant strain verified the role of this gene as a negative regulator of the flagellar motility system and identified several additional genes regulated by the Rcs pathway.

## 1. Introduction

*Yersinia ruckeri* is a gram-negative Enterobacterium that causes Enteric Red Mouth disease (ERM), a severe septicemia of farmed salmonid fish. The majority of outbreaks of ERM occur in rainbow trout and are most frequently caused by a genetically homogenous group of serotype 01 strains of the pathogen [1]. *Y. ruckeri* was first identified on a commercial rainbow trout farm in Idaho in the 1950s and was initially described as having a limited geographic distribution, however *Y. ruckeri* is now globally distributed, presumably through the transport of infected animals during the development and globalization of the trout aquaculture industry [2,3]. Vaccines for ERM were commercialized in the 1970s and their use successfully controlled this disease for many years. However, a recent increase in ERM outbreaks has been reported including outbreaks in previously vaccinated fish. Notably, many of the *Y. ruckeri* strains identified from these outbreaks have been non-motile, phospholipase secretion deficient and have been termed Biotype 2 (BT2) based on these phenotypes [4–6]. These BT2 strains are variants of motile serotype 01 strains that have emerged independently several times through mutation of essential Class II flagellar loci (e.g. *fliR, flgA, flgB*) [7–9]. BT2 strains appear to be rapidly displacing motile strains in areas where *Y. ruckeri* is endemic suggesting that this trait is somehow important for the success of these strains. Loss of flagellar motility is not unusual in the evolution of bacterial pathogens. For example, *Yersinia pestis* is thought to have lost motility relatively recently during its transition from a gastrointestinal pathogen to a vector-borne pathogen

* Corresponding author. National Center for Cool and Cold Water Aquaculture, Agricultural Research Service, US Department of Agriculture, 11861 Leetown Road, Kearneysville, WV, 25430, USA.
*E-mail address:* tim.welch@ars.usda.gov (T.J. Welch).

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**Table 1**
Bacterial strains and plasmids used in this study.

| Bacterial strain | Description | Source or reference |
|---|---|---|
| *Yersinia ruckeri* strains | | |
| CSF007-82 | Serovar 1, Biotype 1, Idaho 1982 | Evenhuis et al., 2009 |
| Δ *fliC*1 Δ*fliC*2 | CSF007-82-derived Δ *fliC*1 Δ*fliC*2 | This study |
| Δ*rcsB* | CSF007-82-derived Δ*rcsB* | This study |
| Δ *fliC*1 Δ*fliC*2 Δ*rcsB* | CSF007-82-derived Δ*fliC*1 Δ*fliC*2 Δ*rcsB* | This study |
| *Escherichia coli* strains | | |
| *E. coli* EC100D *pir* + | F- *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX*74 *recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L (StrR) *nupG pir* + (DHFR) | Epicentre |
| *E. coli* CC118λ*pir* pEVS104 | R6K, *tra*, *trb*, Km-R, Conjugative helper | Choi et al., 2005 |
| **Plasmid Name** | **Contents** | **Source** |
| pCR2.1 | cloning vector | Invitrogen |
| pAKJ4 | pTW-MEV with Δ*fliC*1 Δ*fliC*2 cloned into SacI and XbaI sites | This study |
| pAKJ5 | pTW-MEV with Δ*rcsB* cloned into SacI and XbaI sites | This study |

[10]. Similar mutational loss of motility has occurred in the evolution of *Shigella* and *Aeromonas salmonicida* subsp. *salmonicida* [11–13]. Motility in these cases has likely become obsolete or deleterious as these pathogens have evolved to accommodate a new host, mode of transmission, or other conditions critical to their environmental persistence or spread. Our previous work has shown that the non-motile phenotype of a BT2 strain, resulting from a naturally occurring mutation in *fliR*, does not confer a competitive advantage during infection of rainbow trout [14]. This study also provided evidence that the master flagellar locus, *flhDC*, was essential for the transcriptional activation of Class II and III flagellar loci and that this master regulator is important for not only flagellar biosynthesis, but also a number of other processes. While these results suggest that loss of motility is not important to the virulence of *Y. ruckeri,* the selective pressures driving the loss of motility in this pathogen remain uncertain.

Flagellin, the structural component of the flagellum, is an abundant (20,000 monomers/flagellum) and highly immunogenic protein with important immune signaling activities, including in rainbow trout and salmon [15–17]. In motile pathogens, repression of the flagellar apparatus during infection is common and is thought to be necessary for avoidance of flagellin-mediated host recognition which could result in attenuation of pathogenesis [18–20]. For example, in both enteropathogenic *Yersinia* species (*Y. enterocolitica* and *Y. pseudotuberculosis*) temperature is an important environmental cue for the coordinated inverse regulation of flagellar motility and virulence, as motility is repressed and virulence induced at mammalian body temperature [19,21] Regulation of flagellar synthesis is mediated through the FlhDC transcriptional activator which triggers an ordered cascade required for expression of all genes in the flagellar regulon. Environmental factors control this cascade through the action of transcriptional response regulators acting on the *flhDC* promoter [22]. Such regulators have been shown to either activate or repress *flhDC* in response to numerous environmental conditions including catabolite repression, quorum sensing, temperature, osmotic pressure, pH and membrane damage [22–24]. The Rcs system has been well studied in mammalian enteric pathogens and has been shown to regulate motility through its effect on the expression on *flhDC* master flagellar regulator [25–27]. RcsF is activated upon cell envelope perturbations, which consequently leads to the phosphorylation of RcsB and downstream activation or repression of genes [28]. The *E. coli* Rcs pathway is activated by challenge with lysozyme and responds by inducing lysozyme inhibitors [29]. Similarly, *S. enterica* senses exposure to cationic antimicrobial peptides (CAMPs) via the Rcs system, responding adaptively to CAMP-induced membrane damage [23].

We have previously documented a lack of measurable difference in virulence between biotype 1 (motile) and biotype 2 (non-motile) *Y. ruckeri* strains [14]. This was unexpected since mutational loss of flagellar motility presumably occurs to allow immune evasion. These observations led us to suspect that flagellar synthesis may be repressed during infection in motile strains to avoid flagellin-mediated host recognition. In this work we sought to investigate the regulation of flagellar motility in a motile (biotype 1) strain of *Y. ruckeri.* We demonstrate that the *Y. ruckeri* flagellin gene (*fliC*) is repressed during infection and subsequently de-repressed upon host death when tested in rainbow trout. We also show that the Rcs system is a negative regulator of motility in *Y. ruckeri* and that Rcs-mediated regulation of motility is critical for virulence in rainbow trout. Our results suggest that the *Y. ruckeri* Rcs regulatory system is responsible for sensing host signals during the infection process and regulating gene expression to adapt to the host environment, likely through the induction of virulence genes and the repression of genes deleterious to the infection process, including the flagellar system.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *Y. ruckeri* was routinely cultured at 28 °C with aeration in tryptic soy broth (TSB) or on tryptic soy agar (TSA). *Escherichia coli* strains were grown at 37 °C with aeration in Luria Broth (LB) or on Luria Agar (LA). When required, Kanamycin (Kan) at 50 μg/mL for *E. coli,* or Ampicillin (Amp) (Fisher Scientific, Fair Lawn, NJ, USA) at 100 μg/mL for *E. coli* and 400 μg/mL for *Y. ruckeri* was added to the media. To examine motility, an inoculating loop with approximately 2 μL of overnight culture was inserted half-way into semi-solid media (½ X TSB + 0.3% agar). The plates were incubated upright for ~18 h. Phospholipase activity was evaluated by spotting 2 μL of overnight culture onto phospholipase indicator media (T-media [30]: 10.0 g Difco Bacto-Peptone (Becton, Dickinson & Company, Sparks, MD, USA), 5.0 g NaCl (Sigma-Aldrich), 0.1 g $CaCl_2 \cdot 1$ H2O (Fisher Scientific), 1 L dH2O, pH 7.4, with 1.5% agar (Becton, Dickinson & Company), supplemented with 5 mL Tween 80 (OmniPur, Gibbstown, NJ, USA). The plates were incubated for 48 h and phospholipase production assessed by measured by calcium precipitation surrounding the colony.

### 2.2. Quantitative reverse transcriptional analysis of Y. ruckeri loci within infected tissue

To examine expression of *fliC* during infection, rainbow trout (~40 g) were challenged with wild type *Y. ruckeri* (CSF007-82) by immersion exposure to $2 \times 10^8$ viable cells/mL for 1 h in a static bath with dissolved oxygen applied during the exposure. Water was maintained at 16 °C throughout the experiment. Cells for the challenge were grown using conditions known to induce motility as follows, a single colony of *Y. ruckeri* was used to inoculate 1 L TSB in a 2.8 L Fernbach

flask and incubated at 28 °C with shaking (150 rpm) for ~24 h. Fish were sampled post challenge by random netting followed by euthanasia with 200 mg/L tricaine methanesulfonate (Tricaine-S, Western Chemical) and aseptic harvesting of spleens. Preliminary studies showed that the spleen harbored a homogenous bacterial load. Consequently, spleens were cut in half so that spleen load and *fliC* expression could be examined within each spleen. Half of the organ was homogenized in 10 µL PBS/mg tissue using a Biospec Mini-beadbeater (Biospec Products, Bartlesville, OK, USA) and dilutions were plated onto tryptic soy-blood agar to determine spleen load. The second portion of each spleen (50–100 mg tissue) was placed into 1 mL TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA), homogenized by bead beating and RNA isolated according to the manufacturer's protocol. DNA contamination was removed from each sample by treating 10 µg RNA with the TURBO DNA-free kit (Life Technologies) using the protocol provided by the manufacturer. The absence of DNA was verified by running a PCR using the RNA as template and the 260/280 ratio determined to assess RNA quality. First strand cDNA synthesis was then performed using 1500 ng total RNA with a 2 pmol reverse primer cocktail (gyrBqPCRrev, flhDqPCRrev, and fliCqPCRrev), 10 mM dNTP mix and Superscript III Reverse Transcriptase (Life Technologies).

*Y. ruckeri* flagellin loci *fliC* cDNA was amplified in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using 400 nM primer sets (each primer used in first strand synthesis was paired with its respective forward primer), SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA), and 2 µL cDNA template with the following cycle parameters: 50 °C for 2 min, 95 °C for 2 min, then 40 cycles of 95 °C for 15 s and 54.8 °C for 1 min, and a final increasing temperature gradient from 60 °C to 95 °C in 15 s increments. Internal standard curves were developed using the long primer sets (Table 2) as described previously [14]. All samples were run in triplicate and averaged for analysis. At least eight replicates were performed for each challenge group at each time point. Melt curves were included in all runs, with only one melting peak observed. Negative controls were included in each qPCR run. Absolute quantification of the samples, relative to standards was determined with Applied Biosystems SDS 2.2.2 software and reported as gene copies/µL. The efficiency for the *fliC* qPCR primer set was 96.5% (slope of standards = −3.41 and $R^2 = 0.993$) and the *gyrB* primer set was 100.9% (slope of standards = −3.30 and $R^2 = 0.987$). Comparison of *gyrB* expression across experimental groups, when normalized to CFU per spleen, was not significantly different (ANOVA $p = 0.0774$). Consequently, this housekeeping gene was used to normalize *fliC* expression. This finding corresponds with the use of *gyrB* for normalization during qPCR analysis in the closely related *Yersinia pestis* [31–33].

**Table 2**
PCR primers used in this study.

| Primer Name | 5' - 3′ sequence | Ta (°C) |
|---|---|---|
| sighookF | CTTGCAGAGCATCGTAGCG | 55 |
| sighookR | GATACTTGCCATGTCTAATCCTTAAT | |
| 5′rcsBFor_SacI | *CTAGGT*<u>*GAGCTC*</u>TGCAGATGACCATCCAATCGT | 65 |
| 5′rcsBRev_XhoI | *TGCTAG*<u>*CTCGAG*</u>AAGCGTAATACCGTCACCGT | |
| 3′rcsBFor_XhoI | *CTAGCA*<u>*CTCGAG*</u>TCAGTGCTAACGGCTATGGC | 65 |
| 3′rcsBRev_XbaI | *TGCTAG*<u>*TCTAGA*</u>GAAGACGGTGAGTGATGGCA | |
| gyrBqPCRfor | CAACCGGTATGCATGATGAG | 54.8 |
| gyrBqPCRrev | TTACCGCCAGCATGTAACAC | |
| fliCqPCRfor | CAGCGCTAAAGATGATGCAG | 54.8 |
| fliCqPCRrev | AATACCGTCGTTGGCGTTAC | |
| gyrBlongFor | CGCTGGCCACTGTAAAGAAA | 54 |
| gyrBlongRev | GGCACTTTAACCGATACCACG | |
| fliClongFor | ACAGCCTGTCTTTGCTGACC | 55 |
| fliClongRev | TGATTTTCTGGGCATCTTGC | |
| rcsBF | GGTTCTCGGCAAGCAACTGT | 55 |
| rcsBR | TTTATTGAATGGTACAGCTAGGC | |
| filC compF | TTAATATTCAAGTGTCGATACCGGG | 55 |

The italicized bases were added for restriction digestion and the underlined bases are the restriction cut sites.

### 2.3. Construction of ΔfliC1 ΔfliC2, ΔrcsB and ΔfliC1 ΔfliC2 ΔrcsB strains

Null mutants were created in *Y. ruckeri* CSF007-82 using marker exchange-eviction mutagenesis, based on a method by Ried and Collmer [34]. To knockout both of the *fliC* loci (two tandem copies *fliC*1 and *fliC*2), an in-frame 702 bp and 714 bp region was deleted from the middle of each locus (55% of each gene deleted), respectively, as described below. First, a 3673 bp region, encoding *fliC*1 and *fliC*2 was amplified by PCR using 10 pmol primers sighookF and sighookR, with Hot Star Taq Plus Master Mix (Qiagen, Valencia, CA, USA), 30 cycles and a $T_a$ of 55 °C. The PCR product was purified using the PCR Purification Kit (Qiagen) and digested with EcoRV (New England Biolabs, Ipswich, MA, USA). This restriction enzyme has 2 cut sites within each *fliC* locus, creating 5 DNA segments, which included a 629 bp region encoding the sighookF sequence and 321 bp of the 5′ end of the *fliC*1, along with an 854 bp region harboring 255 bp of the 3' end of *fliC*2 and sighookR sequence. These segments were PCR purified, ligated together the using the restriction digest as template for the Quick Ligation kit (New England Biolabs) and amplified with the sighookF and sighookR primers. The Δ*fliC1* Δ*fliC2* fragment (1483 bp) was purified with the Gel Extraction Kit (Qiagen) and inserted into pCR2.1 with the TOPO TA Cloning Kit (Invitrogen). The marker exchange vector pAKJ4, constructed by inserting Δ*fliC*1 Δ*fliC*2 into pTW-MEV using SacI and XbaI, was mobilized into *Y. ruckeri* CSF007-82 and allelic exchange was performed as previously described [9].

The Δ*rcsB* mutant was constructed by creating a 211 bp deletion (WT gene: 651 bp) that resulted in a frame shift starting at the 70$^{th}$ codon and introduced a premature stop codon at 99th codon. This mutation removes the C-terminal DNA binding domain and should only impact *rcsB* transcription since this gene is at the end of an operon. First, the 5′ and 3′ ends of *rcsB* were amplified by PCR using 5'rcsBFor_SacI and 5'rcsBRev_XhoI or 3'rcsBFor_XhoI and 3'rcsBRev_XbaI, respectively. Both PCR products were digested with *Xho*I, ligated, and the ligation used as template in a PCR with 5'rcsBFor_SacI and 3'rcsBRev_XbaI. The resulting 441 bp Δ*rcsB* amplicon was cloned into pTW-MEV using the *Sac*I and *Xba*I cut sites, creating pAKJ5, and mobilized into *Y. ruckeri* CSF007-82 wild-type (WT) and allelic exchange was performed as described above. The Δ*fliC1* Δ*fliC2* Δ*rcsB* double mutant strain was constructed by introducing the Δ*rcsB* mutation into the Δ*fliC1* Δ*fliC2* strain using the method described above. Mutants were verified by PCR with genomic DNA. The *rcsB* mutant was complemented *in trans* by first PCR amplifying the gene using primers rcsBF and rcsBR followed by cloning into pCR2.1 with the TOPO TA Cloning Kit (Invitrogen). The *rcsB* gene was then cloned into pBR322 as an *Eco*RV-*Bam*HI fragment oriented appropriately for expression from the Tet promoter within pBR322. The Δ*fliC1* Δ*fliC2* strain was complemented using a similar approach by amplification using fliC compF and fliCqPCRrev1 and cloning into pCR2.1 followed by cloning the fliC gene into pBR322 as an *Ssp*1-*Bam*HI fragment into the *Eco*RV-*Bam*HI sites in pBR322. Plasmids were moved into mutant strains by electroporation as described previously [9] and complementation was assessed using motility assays described above.

### 2.4. In silico analyses

*Y. ruckeri flhD* and 1000 bp upstream (GenBank Accession # LN681231) were examined for transcriptional start sites and transcription factor binding sites manually and using the freely available analysis tools BPROM [35] and Virtual Footprint Promoter Analysis Version 3.0 [36].

### 2.5. Flagellin western analysis

To examine flagellin production by the Δ*rcsB* strain, *Y. ruckeri* parent and mutant strains were grown in TSB O/N at 16 °C, with 3 replicates per strain. The cultures were then serially diluted from 1: 10,000 to 1: 320,000 in 10 mL TSB and incubated at 16 °C and 170 rpm. Cultures with an $OD_{600}$ ~ 1.0 (~30 h incubation) were harvested by pelleting 1 mL of culture at 8000 × *g* for 5 min at 16 °C. The cell pellets were resuspended in 100 μL Laemmli sample buffer and incubated at 100 °C for 10 min. The samples were then stored at −80 °C until use. Rabbit anti-flagellin anti-serum production and immunoblot experiments were performed as described in Ref. [9].

### 2.6. Experimental fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained as certified disease-free eyed eggs (Trout Lodge, USA) and maintained at the National Center for Cool and Cold Water Aquaculture (NCCCWA; Kearneysville, WV) in 12.5 °C fresh water under flow-through conditions using an automated light control system that simulates seasonal photoperiod. Oxygen concentration was maintained at 10 mg/L. Fish were fed 6 days per week using a commercially available diet (Zeigler Finfish G 3.0 mm). During infection experiments fish were fed once daily by hand to avoid overfeeding due to infection-caused loss of appetite. The NCCCWA Institutional Animal Care and Use Committee approved all experimental procedures.

### 2.7. In vivo and in vitro competition assays

To compare colonization and virulence capabilities of the different *Y. ruckeri* strains we used a competition infection method to measure the ability of each mutant to compete with its wild-type parent *in vivo* during infection. For the competition experiments WT (CSF007- 82) and mutant strains were mixed at a 1:1 ratio and used either to expose naïve trout by immersion, to assess their competitive performance *in vivo*, or diluted into fresh culture media to assess their performance *in vitro*. A 2 h time point was used to assess invasive virulence (colonization) and the 96 h time point was used to assess *in vivo* replication or persistence. A competitive index of 1 indicates equal capabilities of the strains, while a value greater than or less than 1 signifies that the mutant or WT strain, respectively, has a competitive advantage. Cells for these studies were grown on TSA at 20 °C for 48 h and then resuspended in 1X phosphate buffered saline (PBS) in preparation for both *in vitro* and infection experiments. To examine *in vitro* growth, a 1:1 mixture of mutant to parent cultures was used to inoculate 200 mL TSB at an $OD_{600}$ of 0.2 per strain. The cultures were incubated at 16 °C and 170 rpm for 1 h and then sub-cultured at a 1:1000 dilution into fresh TSB and incubated for 96 h. At 1 and 96 h, the input and output ratios of the mutant: parent were determined by plating serial dilutions onto tryptic soy-blood agar (Remel) and incubating plates at 28 °C for 48 h. Strain ratios were determined by replicate picking colonies (n = 150 per sample) onto motility (Δ*fliC1* Δ*fliC2* or Δ*fliC1* Δ*fliC2* Δ*rcsB* vs WT) or phospholipase indicator (Δ*rcsB* vs WT) media and incubating at 24 °C or 32 °C, respectively, for 18 h and phenotypes were recorded. Known controls were used on every plate and strains were verified by PCR for the genotype throughout the experiment.

To investigate *in vivo* competition, rainbow trout (~33 g) were challenged by immersion exposure to $2 \times 10^8$ cell/mL (1:1 mixture of mutant to parent cultures) in tank water. Input ratios were determined by measuring the CFU in the challenge tanks and strain ratios were determined by replica plating on indicator media as described above. At 2 and 96 h post challenge, fish (10 per competition) were euthanized by immersion in 200 mg/L tricaine methanesulfonate (Tricaine-S, Western Chemical) and whole spleens were aseptically removed, homogenized in PBS (10 μL/mg spleen) and CFU estimated by plating onto tryptic soy-blood agar. Colonies were replica picked onto indicator media, as described in the *in vitro* competition experiment to determine output mutant: parent ratio. The 2 h time point gives an indication of colonization while the 96 h time point is used to describe the or *in vivo* growth or persistence of the strains. Competitive index (CI) was calculated as the output ratio (mutant: WT) divided by the input ratio (mutant: WT).

### 2.8. Experimental infection

For each of the bacterial strains analyzed in Fig. 6, two replicate groups of 25 fish (mean weight, 9.5 g) were exposed to a dilution of a culture containing $5 \times 10^8$ viable bacteria/mL for 1 h in a static bath with supplemental oxygen. Cells for the challenge were grown as described for the *in vivo* and *in vitro* competition assays above. After experimental exposure fish were placed in 19 L tanks receiving 15 °C ultra-violet treated spring water. Fish were monitored for 28 days and mortalities removed and recorded daily. In all cases data from replicate tanks were pooled as there was no significant difference between the survival curves at a 95% confidence level using the Log-rank (Mantel-Cox) test. Mortality due to *Y. ruckeri* infection was confirmed by plating kidney tissue onto TSA from at least 20% of daily mortalities in each tank and confirmed as *Y. ruckeri* by serotyping. When necessary, fish were euthanized by MS-222 (200 mg/L) overdose. Stock and test fish were confirmed free of bacterial infection just prior to experimentation using standard microbiological methods. These experiments and general animal care were carried out at Clear Springs Foods Inc. using protocols approved by the Clear Springs Foods Animal Care and Use Committee.

### 2.9. RNAseq analysis of ΔrcsB

To examine the transcriptional profile of the Δ*rcsB*, *Y. ruckeri* was grown as described in the flagellin western analysis methods and cultures with an $OD_{600}$ ~ 1.0 were harvested by pelleting 1 mL of culture at 8000 × *g* for 5 min at 16 °C. Most of the supernatant was removed, and the remaining 20 μL and cell pellet were flash frozen in liquid nitrogen and stored at −80 °C until further processing. Total RNA was isolated and analyzed as described previously [14]. After RNA isolation, rRNA was removed using the RiboZero rRNA removal kit for bacteria (Illumina, San Diego, CA). ScriptSeq (Illumina, San Diego, CA) was used to generate strand-specific libraries. A Bioanalyzer (Agilent, Santa Clara, CA) was to assess the quality and Qubit (ThermoFisher, Waltham, MA) was used to assess the quantity of the RNA and library. Barcoded libraries were prepared and sequenced on an Illumina NextSeq (1 × 75 bp). Three replicates from each strain were performed. The raw data from this experiment can be found in GenBank (BioProject PRJNA513071). The GenBank Accession numbers are SAMN10695552, SAMN10695558 and SAMN10695559 for the *Y. ruckeri* CSF007-82 replicates and SAMN10695561, SAMN10695560, and SAMN10695567 for the Δ*rcsB* replicates.

### 2.10. Statistical analysis

All data analyses, with the exception of the RNAseq experiment, were performed with GraphPad Prism ver. 5.01 for Windows (GraphPad Software, San Diego, California, www.graphpad.com). Quantitative RT-PCR and competition experiments were analyzed using one-way ANOVAs and Tukey's post hoc tests. Survival curves were created using the Kaplan-Meier method and compared using the log-rank test. RNAseq data was analyzed using the DESeq2 version 1.12.3 package [37] in RStudio version 0.99.902. Significant differences in transcript abundance were determined by calculating the adjusted p-value, which corresponds to p-value adjusted for multiple testing using Benjamini-Hochberg method, which controls for the false discovery rate [38].

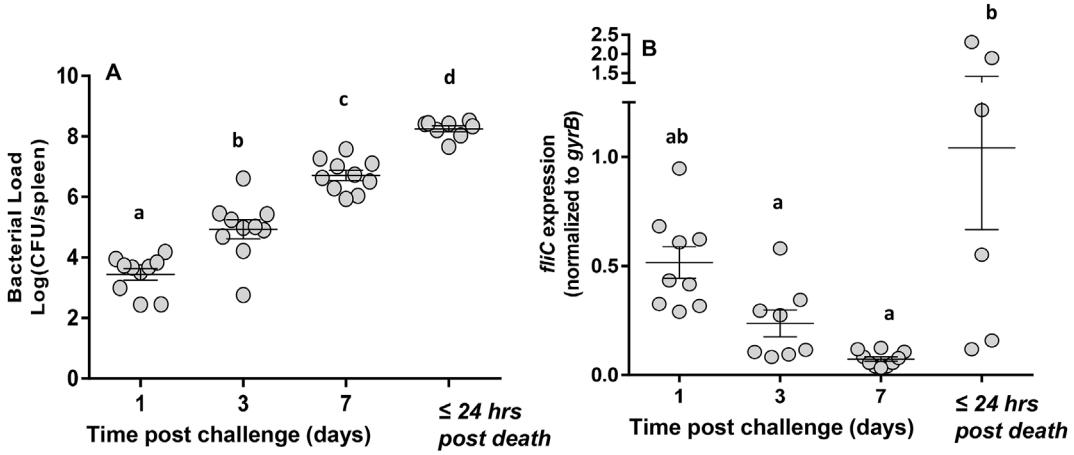


**Fig. 1.** Transcriptional repression of *Y. ruckeri* flagellin (*fliC*) during infection. A. Bacterial load within infected spleens throughout an experimental immersion challenge (ANOVA $p < 0.0001$). B. Expression of *Y. ruckeri fliC* from infected trout spleens (ANOVA $p = 0.0006$). Each dot represents an individual sample. Error bars represent 1 standard error of the mean (S.E.M.). Letters above each group indicate significant differences between experimental groups.

## 3. Results

### 3.1. Flagellin gene expression is repressed during infection

Expression of the flagellin gene (*fliC*) during infection was examined using qRT-PCR. To accomplish this, rainbow trout were immersion exposed to motile *Y. ruckeri* and both bacterial load and *fliC* expression were measured from the spleens of individual fish as a function of time after exposure. Splenic bacterial load increased throughout the course of infection significantly (ANOVA, $p < 0.0001$), with the highest bacterial counts occurring in fish sampled ≤ 24 h postmortem (Fig. 1A). Note that the postmortem samples were taken from animals that had died sometime in the 24 h interval between daily mortality removal time points and therefore the exact time of sampling postmortem is unknown. The corresponding *fliC* qRT-PCR analysis indicated an active repression of *fliC* expression whereby expression decreased as the infective load increased over time (Fig. 1B). Interestingly, *fliC* was dramatically upregulated in fish that had died due to infection when compared to expression 3 and 7 days post challenge (ANOVA $p = 0.0006$). These results suggest that *Y. ruckeri* responds to host signals by triggering the repression of *fliC* expression. Remarkably, the reversal of *fliC* repression upon death of the host shows that these regulatory signal(s) are specific to a living host. We suggest that *Y. ruckeri* possesses an environmental response regulatory system with which it senses host signals and regulates gene expression to adapt to the host environment, likely through the induction of virulence genes and the repression of genes deleterious to the infection process including the flagellar system.

### 3.2. Characterization of flhDC promoter region suggests complex transcriptional regulation

Regulation of flagellar motility is complex and typically occurs through the action of response regulators that modulate the expression of the flagellar master regulator *flhDC* [22]. In order to identify putative host sensing systems responsible for the observed *in vivo* regulation of flagellar biosynthesis we examined the *flhDC* promoter region for evidence of transcriptional regulation and regulatory binding sites using Virtual Footprint version 3.0 (http://prodoric.tu-bs.de/vfp/vfp_promoter.php). This analysis resulted in putative interactions with cAMP-CRP, H-NS, RcsB, OmpR, Fis, Fur, and quorum sensing regulators, which have all been shown to influence *flhDC* in related bacteria [22,39–41]. Preliminary studies *in vitro* did not reveal a correlation with carbon sources or quorum sensing molecules and the motility phenotype or flagellin production (Authors, unpublished). Consequently, we concluded that catabolite repression and quorum sensing are likely not major factors regulating flagellar synthesis. We chose to target the Rcs pathway, specifically the transcriptional regulator *rcsB,* for the following reasons. First, the *Y. ruckeri flhDC* promoter region contains a Rcs(A)B box sequence that is almost identical (13/14 bp; TAgGAATATTCCTA) to the described consensus [42] (Fig. 2). Secondly, Rcs pathway activation occurs upon perturbations of the cell membrane which may occur during infection through exposure to host-produced defense factors [23,29]. Lastly, binding of RcsB to the *flhDC* promoter has been shown to result in repression of flagellar biosynthesis in other bacteria [25,27].

### 3.3. Rcs pathway negatively regulates motility and phospholipase secretion

To examine the role of *rcsB* in flagellar regulation we used marker-exchange mutagenesis to produce an *rcsB* deletion mutant (Δ*rcsB*). The Δ*rcsB* strain overexpressed flagellin (FliC) when assessed by SDS-PAGE and Western blot analysis (Fig. 3). In addition, Δ*rcsB* was hypermotile and displayed increased phospholipase secretion when assessed by plating on motility or lipase media (Fig. 4). Flagellar motility and therefore secretion is repressed at 32 °C in the wild type strain and the Δ*rcsB* strain expressed both motility and phospholipase activity at this normally non-permissive temperature. These observations are consistent with a role for RcsB as a negative regulator of flagellar biosynthesis. To further investigate the role of *rcsB* on flagellar biosynthesis and virulence, a Δ*fliC1* Δ*fliC2* mutant was constructed in wild type *Y. ruckeri*, in addition to an Δ*fliC1* Δ*fliC2* Δ*rcsB* double mutant. Mutations within the flagellin loci, which are class III flagellar genes, should eliminate motility, but leave the flagellar secretion apparatus intact. As expected, absence of intact *fliC* loci resulted in loss of motility (Fig. 4A), but maintained an intact flagellar motor complex, as evidenced by the secretion of phospholipase (Fig. 4B). The Δ*fliC1* Δ*fliC2* Δ*rcsB* strain was


**Fig. 2.** *Y. ruckeri* encodes a putative Rcs(A)B box 247–260 bp upstream of the *flhD* start codon. A web logo depiction of the RcsAB box created by combining the *Y. ruckeri* putative RcsAB box (5′ – taggaatattccta – 3′) with those defined in *E coli* [25] and *S. enterica* and conseneus sequences from Refs. [42,50] http://weblogo.berkeley.edu/.

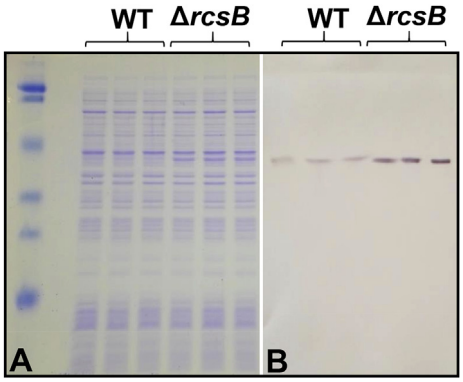


**Fig. 3.** SDS-PAGE (A) and western blotting for detection of flagellin (B) from whole-cell extracts.

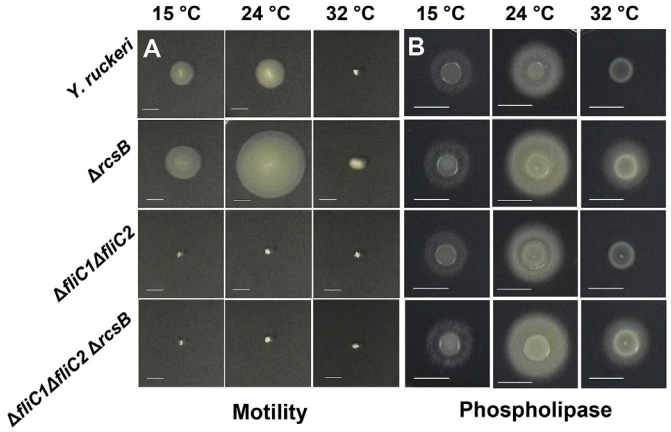


**Fig. 4.** Motility (A) and phospholipase activity (B) phenotypes of *Y. ruckeri* strains incubated at indicated temperatures. Scale bar represents 1 cm.

also non-motile (Fig. 4A), but maintained unregulated phospholipase secretion. These mutants were constructed to enable the differentiation between the effects of the Δ*rcsB* mutation on flagellin production versus its effect on flagellar secreted products (i.e. phospholipase) on the virulence phenotype in the experiments described in the next section.

### 3.4. Rcs pathway is required for full virulence

To examine the effect of the Δ*rcsB* mutation on virulence we first used a co-infection competition assay to determine the ability of each mutant to compete with its wild type parent during infection. For these experiments wild type and mutant strains were mixed at a ratio of 1:1 and then used either to immersion expose rainbow trout to assess competitive performance *in vivo* or diluted into culture media to assess competitive performance *in vitro*. For *in vivo* assays splenic bacterial loads were measured to determine competitive index (CI) at 2 and 96 h after exposure. The CI is defined as the observed output (mutant/parent) ratio divided by the input ratio (1/1). Here, a CI greater or less than 1 indicates that the mutant or parent, respectively, has a competitive advantage. Competition of Δ*rcsB* to its wild type parent demonstrated similar colonization capabilities (measured 2 h after infection) by each strain (Fig. 5A), indicating that loss of *rcsB* does not significantly alter the ability of *Y. ruckeri* to colonize fish when exposed by immersion. However, at 96 h post challenge, the wild type parent strain had significantly outcompeted the Δ*rcsB* strain *in vivo*, while there was only a slight competitive advantage for the parent when allowed to compete *in vitro* (rich culture media). When the Δ*fliC1* Δ*fliC2* Δ*rcsB* mutant was tested in competition with its wild type parent, colonization was comparable to the parent strain. However, at 96h post-challenge the Δ*fliC1* Δ*fliC2* Δ*rcsB* mutant competed better against wild type then that of the Δ*rcsB* strain, indicating the that the attenuating defect caused by the Δ*rcsB* mutation was partially dependent on the flagellin gene (Fig. 5B). There was no competitive advantage for wild type or Δ*fliC1* Δ*fliC2* when these strains were tested (Fig. 5C) which is consistent with our previous findings showing that there is no virulence defect in strains lacking flagellar production [8,14]. The virulence phenotype of these strains was further examined using a standard individual strain challenge model with mortality as the endpoint. Survival analysis from this experiment is presented in Fig. 6 and is in agreement with the competition assays described above. Challenge with the Δ*rcsB* mutant resulted in 77% survival under conditions that resulted in only 22% survival when challenged with the wild type parent strain. Mutation of *fliC* in the wild type background had no effect on virulence but partially restored virulence in the Δ*rcsB* mutant background. These results demonstrate that mutational loss of *rcsB* causes virulence attenuation and that this attenuation is dependent, in part, on flagellin production. These results suggests RcsB is responsible for the *in vivo* repression of flagellin production and that this repression is necessary for *Y. ruckeri* virulence. In addition, the observation that loss of the *fliC* loci only partially recovered the virulence phenotype within Δ*fliC1* Δ*fliC2* Δ*rcsB* suggests that ectopic expression of flagellin in Δ*rcsB* is not the only factor contributing to the attenuation seen in this strain and other virulence factors may also be regulated by the Rcs pathway.

### 3.5. RNAseq analysis of ΔrcsB demonstrates global regulation

The Rcs pathway has been described as a global regulator in other bacteria and we sought to define the extent of genes regulated by this pathway in *Y. ruckeri* using RNAseq (Table 3). Since the loss of motility only partially recovered virulence of the Δ*rcsB* mutant, we hypothesized that RNAseq analysis would uncover additional potential virulence factors regulated by this pathway. Using the DESeq2 package in R Studio, the RNAseq comparison of Δ*rcsB* to its wild type parent strain showed that out of the 3631 genes analyzed, 3544 loci had at least 1 count (non-zero total read counts). Of these loci, 2.3% (n = 83) were up-regulated and 2.8% (n = 99) were down-regulated at an adjusted p-value < 0.1 (Supp. Table 1). There were no outliers in the dataset estimated prior to the statistical analysis. The Wald test Padj were not calculated for 1030 loci (29%) due to low counts.

In *Y. ruckeri* Δ*rcsB* there were 47 significantly (adjusted p-value < 0.05) upregulated genes (21 loci associated with motility [from Classes I, II and III] and chemotaxis; 5 transporters, 6 membrane proteins, 4 hypothetical) and 56 significantly downregulated genes, (including *rcsB,* 4 transporters, 4 hypothetical). This information is consistent with the hypermotile phenotype of this mutant strain, suggesting that *rcsB* is involved in transcriptional downregulation of class I flagella biosynthesis loci. The analysis also identified two genes whose expression is altered in the *rcsB* mutant and may have potential roles in virulence. Putative membrane protein 4, is upregulated 2.08-fold and a NCBI BLAST search identified this protein as the diguanylate cyclase HmsT. Hypothetical protein 121, which is downregulated 15.12-fold, shares significant homology within *Yersinia* and is a putative histidine kinase, but the protein has yet to be characterized in the literature. A NCBI BlastP search identified a conserved helix-turn-helix domain and the sequence showed closest relation to a histidine kinase in *Yersinia mollaretii* (74% identity, NCBI Reference Sequence: WP_050539145.1) and ~70% identity to hypothetical proteins within the *Yersinia* genus. These data provide further evidence for the role of the Rcs pathway as a global regulator in *Y. ruckeri* and identify several non-motility genes that may be either induced or repressed *in vivo* during infection.

## 4. Discussion

In this study we show that *Y. ruckeri* possesses a regulatory system capable of sensing its host and responding by repressing the expression

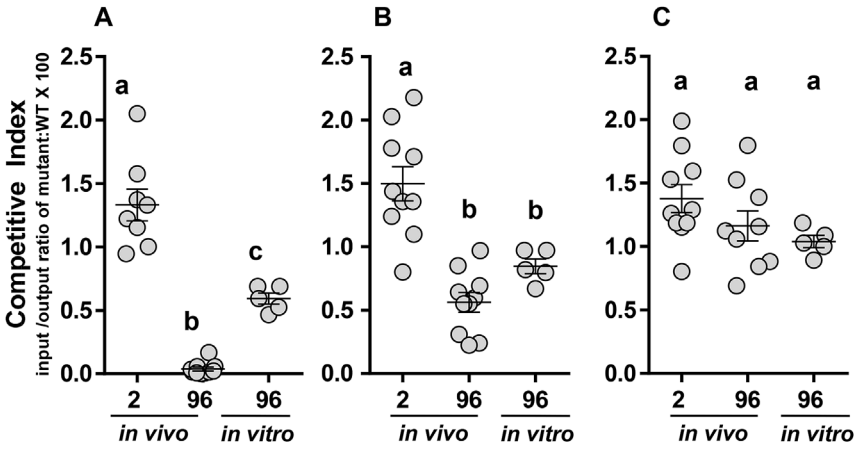


**Fig. 5.** Colonization and virulence examined by competition experiments, comparing the following mutants with the parent WT *Y. ruckeri* strain. Strains were competed in rainbow trout (*in vivo*) and in culture (*in vitro*): **A.** Δ*rcsB* (ANOVA $p < 0.0001$) **B.** Δ*fliC1* Δ*fliC2* Δ*rcsB* (ANOVA $p < 0.0001$) **C.**. Δ*fliC1* Δ*fliC2* (ANOVA $p = 0.1383$) Competitive index is calculated as the input ratio of mutant:WT divided by the output ratio of mutant:WT. Letters about groups indicate significant differences. Error bars signify 1 S E M.

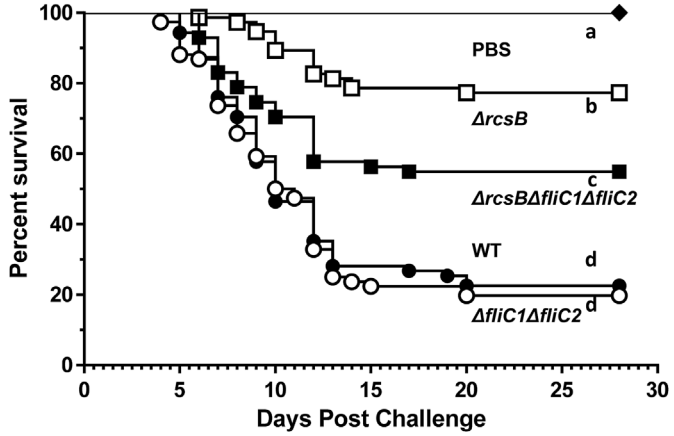


**Fig. 6.** Survival curves for trout challenged with indicated strains. Three replicate tanks housing 25 rainbow trout each were challenged and data pooled for each strain since there were no significant differences among replicate survival curves. Letters indicate significant differences (Log rank test $p < 0.0001$).

of the flagellin gene. Remarkably, our results also show that when fish die from infection, flagellin gene repression is reversed. Flagellar repression during infection is not surprising since the flagellin protein, the major structural component of the flagellum, is a very abundant and potent stimulator of both innate and adaptive immunity and thus expression of this protein during infection could be deleterious to the infection process [17,43]. Similar *in vivo* flagellar regulation has been documented in mammalian pathogens where coordinated regulation of virulence, including repression of flagellar motility, is dependent on growth temperature. For example, in both enteropathogenic *Yersinia* species (*Y. enterocolitica* and *Y. pseudotuberculosis*) temperature is an important environmental cue for the regulation of flagellar motility as motility is repressed at mammalian body temperature [19,21]. Clearly in an aquatic animal pathogen, whose host is an aquatic poikilotherm, temperature is not a useful surrogate cue for host. While motility is regulated by temperature in *Y. ruckeri*, it is clearly not a host signal for this pathogen since motility is expressed at the full range of temperatures suitable for trout and flagellin gene synthesis is de-repressed in an animal that has succumbed to disease at the same temperature that *in vivo* repression occurred (Fig. 1).

The Rcs pathway is a negative regulator of flagellar motility in *Y. ruckeri*. *In vitro* the Δ*rcsB* mutant overexpresses flagellin protein production and displays a hyper-motile phenotype, both characteristics consistent with the absence of a negative regulator. The identification of a putative RcsB Box upstream of *flhDC* suggests that *Y. ruckeri* RcsB acts by modulating the expression of this master flagellar regulator. Indeed, the Rcs pathway has been shown to regulate motility by repressing *flhDC* transcription in *E. coli* and *Salmonella* [25–27]. When tested in experimental challenge models the Δ*rcsB* mutant displayed no defect in host colonization but was clearly defective in growth and/or persistence *in vivo*. In the absence of the flagellin gene (Δ*fliC1*Δ*fliC2* Δ*rcsB* mutant) virulence was partially restored thus showing that the attenuating phenotype expressed by the Δ*rcsB* strain is dependent, in part, on a functional flagellin gene. This result provides indirect evidence that a lack of Rcs regulation causes inappropriate expression of flagellin during infection and thus flagellin-mediated host recognition. Note that the Δ*fliC1* Δ*fliC2* mutation by itself had no effect on virulence in the wild type background. Ectopic expression of flagellin during infection has been shown to attenuate virulence in other motile bacterial pathogens. In *Y. enterocolitica* artificial expression of flagellin in a mouse oral inoculation model caused complete attenuation of virulence under conditions resulting in 100% mortality in mice infected with the wild-type control strain [19]. Similar virulence effects have been observed when flagellin is expressed inappropriately during infection in *Salmonella enterocolitica* [20] and *Bordetella pertussis* [18]. To our knowledge, this is the first study to show that *in vivo* flagellar repression is critical to the success of a fish pathogen.

The Rcs pathway is activated by cell surface stress or damage caused by various perturbations or drugs that act at the cell wall or cell membrane [28]. This includes lysozyme in *E. coli* [29] and CAMPs in *S. enterica* [23]. The kinetics of *fliC* repression was remarkably slow as full repression occurs 7-days after the initiation of infection and after

**Table 3**
Summary of RNAseq datasets.

| Sample | No. reads | Total no. mapped reads | No. uniquely mapped reads | Source |
|---|---|---|---|---|
| CSF007-82 sample 1 | 53,891,051 | 6,144,946 | 5,523,134 | [14] |
| CSF007-82 sample 2 | 31,930,693 | 5,101,627 | 4,858,967 | [14] |
| CSF007-82 sample 3 | 34,969,804 | 4,050,308 | 3,764,434 | [14] |
| CSF007-82 Δ*rcsB* sample 1 | 63,757,069 | 5,865,739 | 5,184,671 | this study |
| CSF007-82 Δ*rcsB* sample 2 | 45,606,407 | 5,902,145 | 5,543,047 | this study |
| CSF007-82 Δ*rcsB* sample 3 | 46,379,630 | 6,227,709 | 5,881,993 | this study |

approximately 3-logs of bacterial growth *in vivo* (Fig. 1). This suggests that the factors involved in host sensing by *Y. ruckeri* are likely induced by the fish host during the infection process which is consistent with membrane-damaging host-produced innate immune mechanisms such as antimicrobial peptides, lysozyme and complement. The reversal of *fliC* repression post-mortem could then because by the depletion of membrane damaging host factors causing the release from immune surveillance. This is supported by the observation that pure recombinant *Y. ruckeri* flagellin strongly induces expression of several anti-microbial peptides in rainbow trout [17]. It's possible then that the *Y. ruckeri* Rcs pathway represses flagellin synthesis in response to host immune factors induced by exposure to flagellin. Further work will be necessary to examine this possibility. Note that while this proposed mechanism of signaling is consistent with the known function of the Rcs system in other bacterial species, we cannot discount the possibility that changes in nutrient composition or availability occurring after host death influence *fliC* repression.

RcsB is a global regulator in *Y. ruckeri*. RNAseq analysis revealed that almost half of the upregulated loci (21/47 genes) in the Δ*rcsB* mutant were associated with motility and chemotaxis, including *flhC*, supporting the hypothesis that RcsB influences *flhDC* expression. While the loss of *rcsB* also led to an attenuation of virulence, the deletion of *fliC* loci in the *rcsB* mutant background showed only a partial recovery of virulence (Figs. 5C and 6). This suggests that additional virulence factors are regulated by RcsB. The RNAseq study identified two potential loci of interest that require further investigation. Within the *Y. ruckeri* Δ*rcsB* strain, there was also derepression of the diguanylate cyclase gene *hmsT* and a 15-fold decrease in a putative histidine kinase (hypothetical protein 121) transcripts. The Rcs pathway has been shown to repress transcription of *hmsT* in *Y. pestis* and *Y. pseudotuberculosis* [44] and play a role in haemin storage [45] as well as biofilm formation [46]. Histidine kinases, typically a part of two-component signaling, can contribute to virulence [47] and are currently being targeted by novel antimicrobials [48].

We, and others, have shown that non-motile BT2 strains of *Y. ruckeri* are recent mutant derivatives of previously motile strains and that these strains have emerged and displaced motile strains in areas where *Y. ruckeri* is endemic [6,7,9]. Initially we hypothesized that these mutations may confer a selective advantage during infection because mutational loss of motility would safeguard against flagellin-mediated host recognition which may be deleterious to the infection process. However, we have recently showed that moving a naturally occurring biotype 2-causing mutation into a motile *Y. ruckeri* strain ablated motility but had no effect on virulence. This showed that the biotype 2-causing mutation on its own did not confer a selective advantage during infection [14]. The *in vivo* repression of flagellar synthesis observed herein helps to explain this lack of effect on virulence between motile and emerging non-motile strains. The repression of flagellar motility during infection should serve to protect *Y. ruckeri* from host recognition of flagellin, as this protein has previously been shown to stimulate the rainbow trout immune system [17,49]. Further research is necessary to investigate the role of TLR5 signaling in the attenuation phenotype seen in the *rcsB* mutant strain.

In summary, our current hypothesis is that upon entering the fish host, the Rcs pathway is activated leading to the production of RcsB which binds to the RcsB box in the promoter of the master flagellar operon (*flhDC*). This results in the repression of flagellin production, preventing the induction of flagellin-induced innate immune responses and enabling infection progression. Upon death of the host, the Rcs pathway is no longer activated and flagella production is reactivated.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.036.

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