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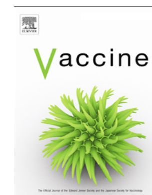

# *Francisella noatunensis* subspecies *noatunensis clpB* deletion mutant impairs development of francisellosis in a zebrafish model

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ABSTRACT

*Background:* *Francisella noatunensis* ssp. *noatunensis* (*F.n.n.*) is the causative agent of francisellosis in Atlantic cod and constitutes one of the main challenges for future aquaculture on this species. A facultative intracellular bacterium like *F.n.n.* exert an immunologic challenge against which live attenuated vaccines in general are most effective. Thus, we constructed a deletion in the *F.n.n. clpB* gene as Δ*clpB* mutants are among the most promising vaccine candidates in human pathogenic *Francisella*.
*Purpose:* Characterization of *F.n.n.* Δ*clpB* using primary Atlantic cod head kidney leukocytes, the zebrafish embryo and adult zebrafish model with focus on potential attenuation, relevant immune responses and immunogenic potential.
*Main results:* Interleukin 1 beta transcription in Atlantic cod leukocytes was significantly elevated from 24 to 96 h post infection with *F.n.n.* Δ*clpB* compared to *F.n.n.* wild-type (wt). Growth attenuation of the deletion mutant in zebrafish embryos was observed by fluorescence microscopy and confirmed by genome quantification by qPCR. In the immunization experiment, adult zebrafish were immunized with $7 \times 10^6$ CFU of *F.n.n.* Δ*clpB* before challenge four weeks later with $6 \times 10^8$ CFU of *F.n.n.* wt. One day after challenge, immunized zebrafish responded with significantly lower interleukin 8 levels compared to the non-immunized control. Immunized fish were protected against the acute mortality observed in non-immunized zebrafish after challenge and bacterial genomes quantified by qPCR were reduced to a minimum 28 days post challenge, indicating protective immunity stimulated by *F.n.n.* Δ*clpB*.
*Conclusion:* Deletion mutation of *clpB* in *F.n.n.* causes *in vitro* and *in vivo* attenuation and elicits a protective immune response in adult zebrafish against a lethal dose of *F.n.n.* wt. Taken together, the results presented increases the knowledge on protective immune responses against *F.n.n.*
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## 1. Introduction

Caseinolytic protease B (ClpB) is a chaperone and part of a conserved system of ATP dependent proteases [1] participating in stress responses in several bacterial species including *Francisella* sp. [2]. The chaperone disaggregates and reactivates aggregated proteins assisted by the DnaK/DnaJ chaperone system [3]. ClpB contributes to virulence in several bacterial species; *Listeria monocytogenes* [4], *Leptospira interrogans* [5], *Porphyromonas gingivalis* [6], *Salmonella typhimurium* [7] and *Francisella* spp. [2,8–10]. In *Francisella*, *clpB* deletion mutants are attenuated in several aspects e.g. *in vitro* intracellular growth [11], mouse macrophage cytopathogenicity and stress resistance against heat, ethanol and pH [2]. Attenuation *in vivo* is described by decreased virulence [9,10] and replication failure in target organs in mice [2,10], but the degree of attenuation is affected by dose, mouse strain, parental *Francisella* strain and administration. Mice are protected against

*Abbreviations:* ClpB, Caseinolytic protease B; cDNA, complementary DNA; CFU, colony forming unit; dpi, days post injection; dpc, days post challenge; *F.n.n.*, *Francisella noatunensis* ssp. *noatunensis*; gDNA, genomic DNA; GFP, green fluorescent protein; HE, Hematoxylin and eosin; hpi, hours post infection; ORF, open reading frame; PAS, Periodic acid Schiff; wt, wild-type.

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**Table 1**
Strains and plasmids used in this study.

| Strains | Subspecies/genotype | Reference |
|---|---|---|
| *E. coli* | | |
| DH5α λpir | λpir/φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA*1 *hsdR*17 *deoR thi*-1 *supE*44 *gyrA*96 *relA*1 | [20] |
| DH5α λpir | pCR4-TOPO::*clpB* | |
| S17-1 | *thi thr leu tonA lacY supE recA*:: RP4-2-Tc:: Mu, Kn:: Tn7 | [21] |
| S17-1 | pDMK::OFOR *clpB* | |
| *F. noatunensis* | subsp. *noatunensis* | |
| *F.n.n.* wt | NCIMB14265$^T$ | [22] |
| *F.n.n. gfp* | pKK289Km::*gfp* | [23] |
| *F.n.n.* Δ*clpB* | *clpB* deletion mutant constructed by allelic exchange without antibiotic resistance marker gene | This study |
| *F.n.n.* Δ*clpB gfp* | pKK289Km::*gfp* | This study |
| *F.n.n. clpB* + . | pKK289Km::*clpB* | This study |
| *Plasmids* | | |
| pDMK2 | Suicide plasmid. *sacB*, mobRP4, oriR6K, Km$^r$ | [24] |
| pCR4-TOPO::*clpB* | pCR4-TOPO cloning vector containing *clpB* PCR product, Km$^R$, Amp$^R$ | ThermoFischer Scientific |
| pKK289Km::*gfp* | pKK289 *Francisella* replicating plasmid expressing *gfp* gene from groEL promoter, Km$^R$ | [19] |
| pKK289::*clpB* | pKK289 expressing *clpB* from groEL promoter, Km$^R$ | This study |

lethal aerosol challenge after immunization with live Δ*clpB* mutants derived from *F. tularensis* ssp. *tularensis* strain SCHU S4 and two *F. tularensis* ssp. *holarctica* strains, LVS and FSC200 [2,8,9]. Thus, the attenuated, but still immunogenic *F. tularensis clpB* deletion mutants are potential human vaccine candidates.

Atlantic cod was a promising new aquaculture species in the late 20st century, but along with farming came disease challenges including francisellosis. The disease is caused by *F.n.n.* and is often associated with increased mortality especially during periods of high or fluctuating water temperature. Externally, sick fish do not display specific clinical signs, and typical pathologic findings in Atlantic cod with francisellosis are granulomas mainly in internal organs like kidney, spleen and liver [12,13]. *F.n.n.* and *Francisella* sp. are in general highly infectious [13], which combined with high density of susceptible hosts in an aquatic environment in fish farming, a chronic disease pattern and largely internal pathology results in high prevalence at the time of diagnosis. As a result, francisellosis has caused great economic losses in the currently collapsed Norwegian Atlantic cod aquaculture and the benefit of a vaccine is apparent.

As *F.n.n.* is facultative intracellular, infects multiple cell types [12,14] and grows within macrophages [15], the vaccination strategy is different from many common bacterial diseases in fish aquaculture caused by extracellular bacteria where antigens and adjuvant provide a protective antibody response. A cellular immune response is central in protective immunity against many intracellular bacteria including human pathogenic *Francisella* sp. and is most efficiently stimulated by live attenuated vaccines [16,17].

In the current study, we constructed an *F.n.n. clpB* deletion mutant to characterize its attenuation *in vitro* by infecting leukocytes extracted from Atlantic cod head kidney before investigating the immunogenic potential of the mutant *in vivo* in a zebrafish embryo and adult zebrafish model.

## 2. Material and methods

### 2.1. Bacterial strains and growth conditions

*F.n.n.* NCIMB 14265$^T$, originally isolated from Atlantic cod *Gadhus morhua* L. in Norway, and the Δ*clpB* mutant was grown as previously described [18] unless stated otherwise. Green Fluorescent Protein (GFP) fluorescent *F.n.n.* bacteria and the complemented *clpB* mutant were grown in presence of 15 μg ml$^{-1}$ kanamycin as *gfp* and *clpB* were expressed from the kanamycin resistant pKK289Km plasmid [19]. All bacterial strains used are listen in Table 1. Primers are listed in Supplementary Table S1.

#### 2.1.1. Construction of the F.n.n. ΔclpB mutant and the F.n.n. clpB + complementation strain

Construction of the *clpB* deletion mutant was performed as previously described in detail [25] and is based upon the method of allelic replacement in *Francisella tularensis* [26]. In short, the two ~1100 base pair (bp) flanking regions of *clpB* (primer 1 + 2 and 3 + 4) were amplified by PCR and the products fused by splicing by overhand extension (SOEing) PCR (primer 1 and 4). The end product was inserted into the pDMK2 vector (kanR), transformed into *Escherichia coli* and transferred to *F.n.n.* by conjugation [25]. 2487 bp was removed from the *clpB* locus, from nucleotide 61 to 2547. Nucleotide and amino acid sequence comparison between ClpB in *F.n.n.* and a selection of *Francisella* spp. was performed by BLAST [27] and alignment in CLC Main Workbench version 6.6.2 (https://www.qiagenbioinformatics.com) (Fig. 1A). Putative genes surrounding *clpB* in *F.n.n.* were identified by open reading frame (ORF) detection in CLC Main Workbench and RT-qPCR was run with primers 18 + 19 and 20 + 21 (Supplementary Table S1) amplifying the putative genes to discover potential polar effects of the deletion.

The pKK289Km::*clpB* construct was made essentially as described previously for *F.n.n. iglC* [25] except that the initial PCR product was first ligated into the cloning vector TOPO pCR4 before digestion with the restriction enzymes NdeI and SacI (New England Biolabs) and insertion into the correspondingly digested expression plasmid pKK289Km. PCR amplification of *clpB* was performed with primers 14 and 15 (Supplementary Table 1) with *F.n.n.* genomic DNA as template. The complemented mutant and GFP expressing mutant was made by transformation of *F.n.n.* Δ*clpB* with the expression plasmid pKK289Km::*clpB* or pKK289Km::*gfp* essentially as described [23].

### 2.2. Atlantic cod head kidney leukocyte extraction, infection and immunolabeling

Wild caught Atlantic cod were reared at 9 °C water temperature before euthanization and cell extraction of leukocytes from the head kidney as described previously [28] as this organ is a central hematopoietic site in bony fish with a majority of macrophages [29–31]. Fish were of both sexes ranging from 43.5 to 57.5 cm in length. Weight estimate was 0.85–1.98 kg, calculated according to Ulrich et al. [32]. Incubation medium (L-15+) and cell extraction

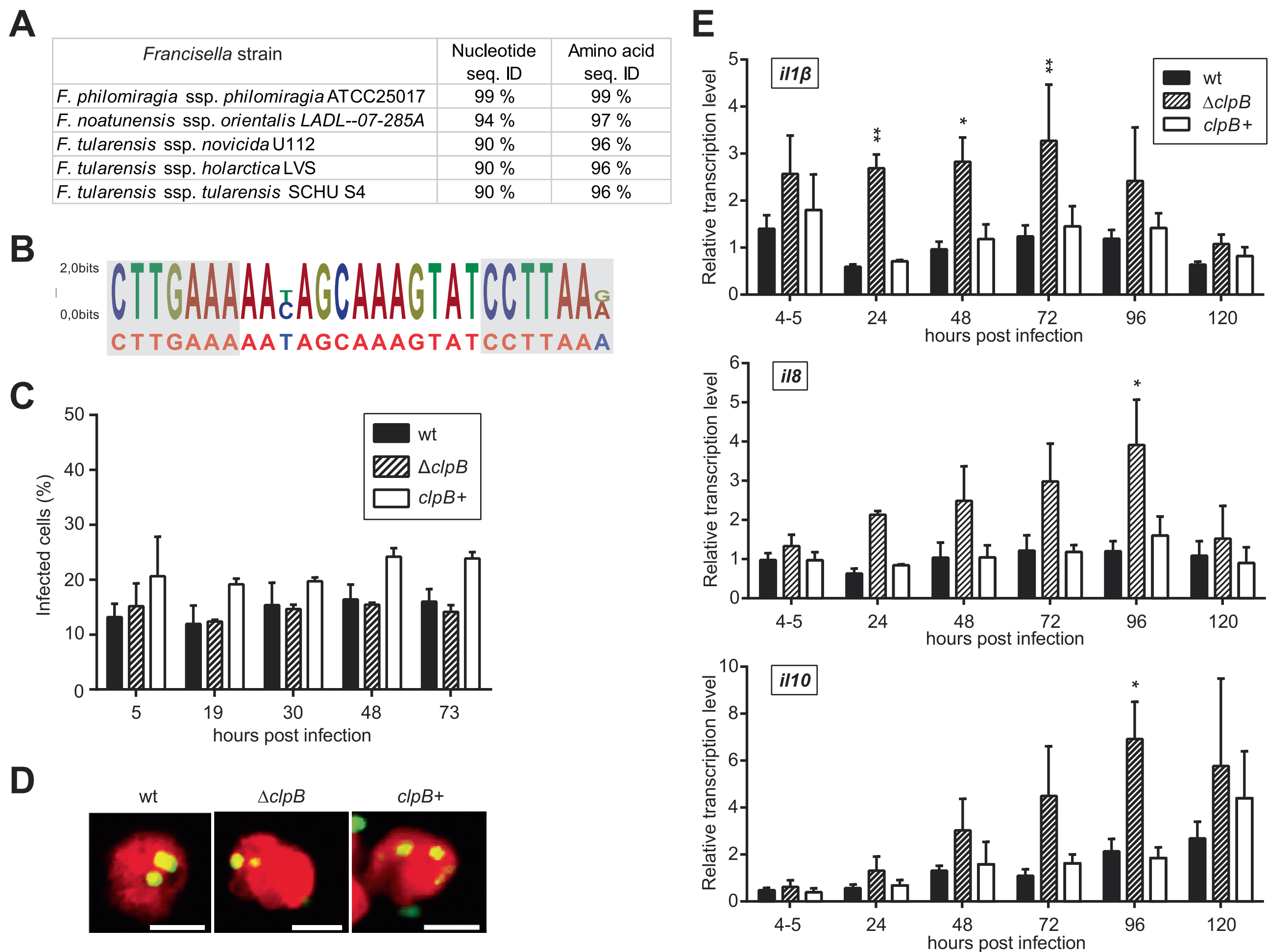


**Fig. 1.** *In silico* analysis of the *clpB* locus and the σ[32]-like promoter sequence in *Francisella* spp. and immune response of Atlantic cod head kidney-derived leukocytes after infection with *Francisella noatunensis* ssp. *noatunensis* Δ*clpB*. (A) Nucleotide and translated amino acid sequence comparison between *clpB* in *F.n.n.* and a selection of *Francisella* spp. (B) Sequence logo of the σ[32]-like promoter sequence after comparison of *Francisella noatunensis* subsp *orientalis, Francisella philomiragia, Francisella tularensis* subsp *holarctica* LVS and *Francisella tularensis* subsp *tularensis* SCHU S4. The lower *F.n.n.* sequence is blue in the positions where it differs from some of the *Francisella* spp. The grey background marks the promoter binding sequence. (C) Manual counting of immunolabeled Atlantic cod head kidney leukocytes after infection experiments (N = 3). Results are presented as mean ± SEM. (D) Representative micrographs of Atlantic cod head kidney-derived leukocytes 5 hpi in the infection experiments labeled with Alexa Fluor 594 conjugated Wheat Germ Agglutinin (red) infected with *F.n.n. gfp* (wt), *F.n.n.* Δ*clpB gfp* (Δ*clpB*) or *F.n.n. clpB* + labeled with rabbit anti-*F.n.n.* antibodies and goat anti-rabbit Alexa Fluor 488 conjugated antibodies (green). Scale bars = 5 μm. (E) The relative transcription level of *il1β*, *il8* and *il10* of mutant-infected Atlantic cod leukocytes measured by RT-qPCR (N = 3). Results are presented as mean ± SEM. Asterisks represent a statistically significant difference between *F.n.n.* wt and *F.n.n.* Δ*clpB* at each time point (* p value <.05, ** p value from 0.001 to 0.01; 2-tailed unpaired Student's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

medium (L-15+ with 100 μg ml$^{-1}$ gentamicin and 10 U ml$^{-1}$ heparin) were prepared as described by Furevik et al. [33]. After extraction, cells were incubated at 13 °C for 4 h before being washed twice in L-15+ and kept at 13 °C overnight in L-15+ with 100 μg ml$^{-1}$ gentamicin.

The primary cod leukocyte infections including subsequent RNA extraction or fixation for immunolabeling in triplicate followed the same protocol as described in Lampe et al. [28]. In one RNA replicate cells were infected, washed and incubated in the same well throughout infection without reseeding. RNA extraction samples were made at 5, 24, 48, 72, 96 and 120 h post infection (hpi) while cells for immunolabeling and counting were PFA-fixed at: 5, 19, 30, 48 and 73 hpi.

Immunolabeling was performed as previously described [28] except from using polyvalent rabbit antiserum against *F.n.n.* [34] as primary antibodies diluted 1:20 000 for all samples in order to also label the complemented mutant.

We could not test the fish for *F.n.n.* exposure before the experiments, but all control cells from all fish used were negative for *F.n.n.* in qPCR analysis with *F.n.n.*-specific primers and/or in immunofluorescence experiments using *F.n.n.*-specific antibodies.

### 2.3. Zebrafish embryo rearing and microinjection

Zebrafish (*Danio rerio*) AB wt embryos were obtained and reared as described [25]. Phenyl-2-thiourea (Sigma-Aldrich, St. Louis, MO, USA) was added to 0.006% w/v concentration to reduce pigmentation for imaging purposes.

*F.n.n.* wt and Δ*clpB* both expressing GFP *in trans* were used for zebrafish embryo microinjections into the Duct of Cuvier (arrow, Fig. 2A) and subsequent monitoring, handling and euthanization was performed as described previously [14,25]. The injected dose per embryo was estimated by plating decadic dilutions as described [14] and found to be between $10^2$ and $10^3$ colony forming units (CFU). A control group was injected with sterile phosphate buffered saline solution (PBS, pH 7.4). Groups consisted of at least 24 embryos. The tail region (rectangle, Fig. 2A) of two representative embryos per group was imaged as described [25] on 1,

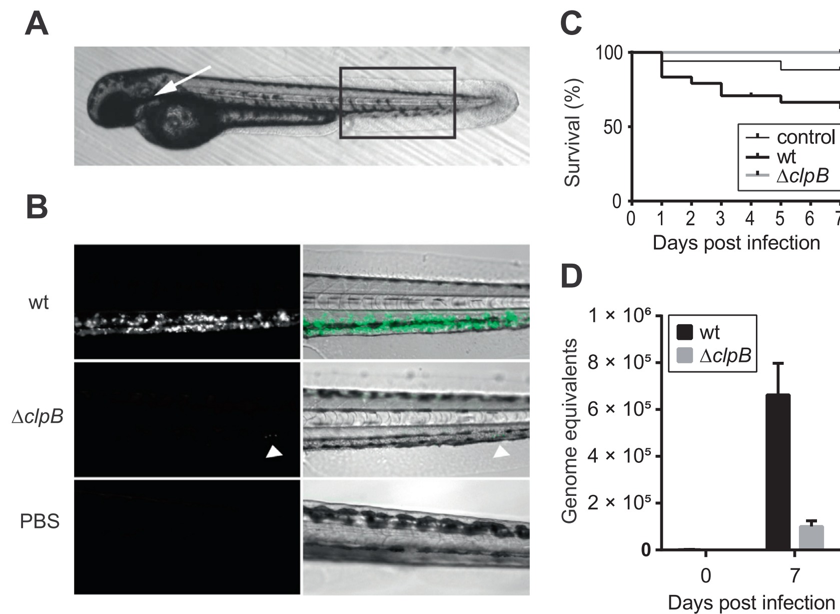


**Fig. 2.** Intravascular injection experiment of zebrafish embryos with green fluorescent *F.n.n.* wt, *F.n.n.* Δ*clpB* and PBS suggests growth attenuation of the deletion mutant. (A) Injections of $10^2$ –$10^3$ CFU were administered into the Duct of Cuvier (arrow) of zebrafish embryos (AB wt) and micrographs were made from the tail region (rectangle). (B) Micrographs from the tail region of zebrafish embryos 7 days post injection with green fluorescent *F.n.n. gfp* (wt), *F.n.n. ΔclpB gfp* (Δ*clpB*) and PBS. Only few weakly green fluorescent foci were visible in mutant infected embryos (arrowhead), 10x magnification. (C) Kaplan – Meier curve of cumulative survival of zebrafish embryos injected with *F.n.n. gfp*, *F.n.n.* Δ*clpB gfp* or PBS (control). The difference in cumulative survival between the mutant-injected and the wt-injected embryos was statistically significant (p = .0087). (D) Quantification of *F.n.n.* genome equivalents by qPCR on gDNA zebrafish embryos injected with *F.n.n. gfp* or *F.n.n.* Δ*clpB gfp* at 0 or 7 days post injection (dpi). Results are presented as mean ± SEM. Bacterial load was significantly higher in wt injected embryos 7 dpi (wt day 0 versus day 7, p = .0089 and ΔclpB day 0 versus day 7, p = .0175 and wt versus ΔclpB day 7, p = .0063). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4 and 7 days post injection (dpi). At 0 dpi three samples consisting of three embryos each were sampled per group while four samples were sampled 7 dpi.

### 2.4. Adult zebrafish rearing and F.n.n. infections

Adult zebrafish, strain AB wt, from the zebrafish facility at the Norwegian University of Life Sciences (NMBU, Oslo, Norway) were acclimatized to 22 °C for at least 1 week prior to the experiments. Fish weight was 400–600 mg, fish age was 11.7–14.7 months with equal proportion of each gender. All zebrafish experiments were approved by the Norwegian Animal Research Authority, approval No. 2014/306, FOTS ID 6001 and treated according to institutional guidelines. The experimental details on adult zebrafish rearing, infection protocol, euthanization, sampling, sample processing and analyzing methods are described in the previously published study on *F.n.n.* Δ*iglC* [28] as both mutants were tested concomitantly. The injection site was cranially to the pelvic fin base, close to the midline and with the needle tip right within the peritoneal cavity. The experimental set up is described in Table 2.

### 2.5. Dose-response assessment zebrafish

*F.n.n.* wt and Δ*clpB* grown to exponential phase [33] were administered by intraperitoneal injections at doses of $3 \times 10^7$ (lower dose) or $3 \times 10^9$ (high dose) CFU per fish with an injection volume of 15 µl. Group sizes are described in Table 2. Five fish were sampled at 2 and 14 dpi whereupon heart, spleen and kidney were transferred separately into RNA*later*® (Ambion) and stored at 4 °C.

### 2.6. Immunization and challenge of zebrafish

Adult zebrafish were immunized by intraperitoneal injections with *F.n.n* Δ*clpB* $7 \times 10^6$ CFU while the control group was injected with PBS. Four weeks after the *F.n.n.* Δ*clpB* immunization, both groups were challenged with *F.n.n.* wt of $6 \times 10^8$ CFU by intraperitoneal injections. Four fish per tank were sampled for RNA extraction the day before challenge (dbc) and 1, 3 and 7 days post challenge (dpc) except the hearts from 1 dbc from which DNA was extracted. Five fish per group were sampled for DNA extractions at day 7, 14, 21 and 28 dpc. Heart, spleen and kidney samples were stored in RNA*later*® at 4 °C.

**Table 2**
Experimental setup for infection and immunization in adult zebrafish AB wt.

| Group | Immunization dose/fish | Challenge dose/fish | Number of fish |
|---|---|---|---|
| *Dose-response experiment* | | | |
| PBS control | – | – | 24 |
| *F.n.n.* wt lower dose | – | $3 \times 10^7$ | 15 |
| *F.n.n.* wt high dose | – | $3 \times 10^9$ | 25 |
| *F.n.n.* Δ*clpB* lower dose | – | $3 \times 10^7$ | 15 |
| *F.n.n.* Δ*clpB* high dose | – | $3 \times 10^9$ | 24 |
| *Immunization experiment* | | | |
| PBS + infected | – | $6 \times 10^8$ | 59 |
| Immunized + infected | $7 \times 10^6$ | $6 \times 10^8$ | 58 |

### 2.7. RNA and genomic DNA extraction

RNA extraction from the Atlantic cod leukocytes and RNA and gDNA extraction from zebrafish tissues was performed as described in detail previously for adult zebrafish [28] and zebrafish embryos [25,35].

### 2.8. Quantitative real-time PCR

Five µl of diluted cDNA samples were used as template in RT-qPCR reactions with the same setup, machine and calculation method to determine the relative transcription levels for each gene

and time point as previously described [28]. Primers are listed in Table S1.

Transcription of Atlantic cod genes associated with immune response was investigated by RT-qPCR on 1:6 diluted cDNA from cod cell experiments using primers amplifying transcripts of interleukin 1 beta (*il1β*), interleukin 6 (*il6*), interleukin 8 (*il8)* and interleukin 10 (*il10*) and interleukin 12, subunit p40 (*il12p40*) with Elongation factor 1 alpha in cod (*ef1α*) as reference gene.

The immune response of infected adult zebrafish kidney and spleen cells was examined by RT-qPCR on 1:10 diluted cDNA synthesized from RNA extracted from dissected tissue. Transcription of tissue necrosis factor alpha (*tnfα*), *il1β*, *il6, il8, il10, il12a* (IL12, subunit p35), interferon-gamma isoform 1_1 (*ifng1_1*) and 1_2 (*ifng1_2*) was investigated. *ef1α* (zf) and beta actin in zebrafish (*β-actin*) were used as reference genes for the dose experiment and 18S ribosomal RNA in zebrafish (*18S rRNA*) was added as a third reference gene for the immunization experiment.

### 2.9. Francisella-specific quantitative PCR

Extracted genomic DNA was used as template for qPCR with validated *Francisella noatunensis* ssp. *noatunensis*-specific primers [36] as described previously for zebrafish embryos [25] and adult zebrafish tissue [28] using 1 ng gDNA from *F.n.n.* wt as equilibrator.

### 2.10. Histologic sample preparation

Histology samples were fixed, prepared, mounted and stained with Hematoxylin and Eosin (HE), and Periodic Acid Schiff (PAS) as previously described [28].

### 2.11. Statistical analysis

Statistical analysis of the data sets was performed using Graphpad Prism 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Kaplan-Meier survival curves and differences between groups were deemed statistically significant if p-value <0.05 using Log-rank (Mantel-Cox) test. Differences in bacterial quantification and transcription between groups were deemed statistically significant if $p < .05$ after using unpaired two-tailed Student's *t*-test assuming unequal variance.

## 3. Results and discussion

### 3.1. High sequence identity between clpB in F.n.n. and a selection of Francisella sp. and identification of the $\sigma^{32}$-like promoter sequence upstream of F.n.n. clpB

ClpB is a known virulence determinant in other *Francisella* sp. and *F.n.n clpB* displays the highest similarity to those of *F. philomiragia* (99% identity) and *F. noatunensis* subsp. *orientalis* (94% nucleotide and 97% amino acid sequence identity) (Fig. 1A and Supplementary Fig. S1). The *clpB* similarity between *F.n.n.* and the *F. tularensis* lineage ranges from 90% at nucleotide to 96% at amino acid similarity (Fig. 1A). The nucleotide sequence upstream of *clpB* in *F.n.n.* reveals a striking homology with the previously described $\sigma^{32}$-like promoter sequence associated with regulation of heat shock response induced by misfolded proteins in the cytoplasm [2,37] (Fig. 1B and Supplementary Fig. S2). This suggests a similar regulation of *clpB* transcription also in *F.n.n.* which remains to be studied. The generation of the *clpB* deletion mutant from *F.n. n.* facilitates studies of gene regulation and virulence contribution also in a fish pathogenic species, which so far has not been published.

### 3.2. In vitro infection with F.n.n. wt, the ΔclpB mutant and complemented clpB mutant in cod head kidney-derived leukocytes show a steady infection level in all three strains

In head kidney-derived Atlantic cod leukocytes, the *clpB* deletion mutant displayed a steady infection level of ~14.4% from 5 hpi until 73 hpi, similar to the average *F.n.n.* wt infection level of 14.8% (see Fig. 1C). The complemented mutant, *clpB*+, appeared to reach a higher initial infection level of approximately 20.6%, but also this strain remained at a stable level throughout the experiment. Representative images from the infections are presented in Fig. 1D. *F.n.n.* growth is slow in broth culture and *in vitro* in Atlantic cod leukocytes [15,33], but the lack of growth observed from immunolabeling experiments in the current study was unexpected. However, a discrepancy has been reported between immunolabeling results and parallel genomic quantification [28] or flow cytometry analysis [15] from Atlantic cod head kidney leukocytes infections with *F.n.n.* and could also be associated with loss of highly infected cells through the immunolabeling protocol.

The immune response of *in vitro* infected cod leukocytes was measured by qPCR for *il1β, il6, il8, il10* and *il12 p40.* While no significant difference was detected for *il6* or *il12p40,* the immune response was significantly different between the wt and *clpB* mutant for *il1β, il8 and il10* (Fig. 1E). The *F.n.n.* Δ*clpB* mutant triggered significantly elevated transcription of the proinflammatory cytokine *il1β* compared to *F.n.n.* wt at 24, 48 and 72 hpi. Transcription of *il8* and *il10* in Δ*clpB* infected leukocytes was significantly elevated compared to *F.n.n.* wt infected cells at 96 hpi. While IL8 is a chemokine attracting leukocytes, the combination with the inhibitory role of IL10 during immune responses [38,39] suggests a higher level of control in the response of the *clpB* mutant infected cod leukocytes. The immune response triggered by the complemented mutant resembled the response of wt infected cells for *il1β*, *il8* and *il10*. All strains triggered *il6* transcription to a similarly higher level than non-infected cells (Supplementary Fig. 3). *F.n.n.* wt infection caused a minute, but significantly higher transcription of *il12p40* at the earliest timepoint compared to Δ*clpB* infected cells, but this minor transcription difference was not considered a functional difference (Supplementary Fig. S3).

### 3.3. F.n.n. ΔclpB is growth attenuated and displays reduced virulence in zebrafish embryos

When a previously established *F.n.n.* - zebrafish embryo model was infected [14,25], it is apparent that the *F.n.n.* wt infected embryos are highly infected with multiple green fluorescent nodules in the tail region (representative micrographs from 7 dpi are presented in Fig. 2 B). Only a few green foci are detectable in the Δ*clpB* infected embryo, suggesting growth attenuation. All embryos injected with *F.n.n.* Δ*clpB* survived the experiment while the Kaplan-Meier cumulative survival was 88% in the PBS group and 62% in the *F.n.n.* wt group 7dpi (Fig. 2C). The difference in cumulative survival between the mutant-injected and the wt-injected embryos was statistically significant ($p = .0087$), indicating attenuation also regarding mortality in zebrafish embryos. The two mortalities (1 and 5 dpi) in the PBS group were considered injection-related or due to low natural fitness as circulatory abnormalities were observed in the second embryo. Quantification of genomic equivalents performed as previously described [25] shows a significantly lower bacterial load 7 dpi in embryos injected with *F.n.n.* Δ*clpB* compared to *F.n.n.* wt. Together with the fluorescence micrographs (Fig. 2B) and survival analysis in Fig. 2C, results suggest attenuation regarding intracellular replication and mortality. However, replication of both bacterial strains occurred from 0 to 7 dpi and bacterial load was significantly higher in wt injected

embryos 7 dpi (Fig. 2 D, wt day 0 versus day 7, p = .0089 and Δ*clpB* day 0 versus day 7, p = .0175 and wt versus Δ*clpB* day 7, p = .0063).

### 3.4. F.n.n. ΔclpB is growth attenuated in adult zebrafish despite inducing acute mortality after high dosage

When challenging an adult zebrafish infection model, zebrafish infected with a lower dose of the *clpB* mutant experienced 100% survival (Fig. 3A) while lower dose wt infected fish displayed 77% survival (not statistically significant, p = .1132). Splenomegaly previously described in lower dose wt infected fish [28] was absent in the corresponding Δ*clpB* infected fish. Lower dose Δ*clpB* infected fish displayed significantly decreased bacterial load in spleen from 2 to 14 dpi (Fig. 3B, heart: p = .0639, spleen: p = .0027). Also, significantly lower *tnfα* transcription was observed 2 dpi (p = .0079) compared with the wt group and only the wt group triggered *tnfα* at this timepoint compared with the PBS group (p = .0069) (Fig. 3C). Transcriptional differences between lower dose wt and mutant infected zebrafish were not significant for *il1β, il6, il8, il10, il12a, ifng 1_1 or ifng 1_2*. The high dose mutant group displayed acute mortality like the high dose wt group (Fig. 3A), both reaching the preset humane endpoint of 60% mortality after 9 and 7 days, respectively. Despite this, the bacterial load in the high dose mutant group appeared to decrease from 2 to 9 dpi although not to significant levels (Fig. 3B, heart: p = .2267, spleen: p = .2928). Both high dose groups responded similarly 2 dpi with a significantly higher transcription of *il1β* (wt: p=<.0001, Δ*clpB*: p = .002), *il6* (wt: p = .0003, Δ*clpB*: p = .0007), *il8* (wt: p = .0277, Δ*clpB*: p = .0023), *il10* (wt: p = .0057, Δ*clpB*: p = .0008), *il12a* (wt: p = .0006, Δ*clpB*: p = .035) *and ifng 1_2* (wt: p = .0046, Δ*clpB*: p = .0398) compared to the PBS group, but no clear *tnfα* and *ifng1_1* transcription was triggered at 2dpi. At the endpoint of both high dose groups, transcription levels of *il1β* (p = .0071), *il6* (p = .0035) and *ifng1_2* (p = .0113) were significantly higher in the mutant infected group 9 dpi compared with the wild-type group 7 dpi, but this is interpreted with caution as the sampling day is different. For the other immune genes studied, no significant difference was detected between wt and mutant infected zebrafish (Supp. Fig. S4).

### 3.5. F.n.n. ΔclpB reduced mortality in adult zebrafish exposed to lethal challenge of parental wild-type strain

One fish in the immunized group died the first day and one PBS injected fish died 2 dpi, after which no mortalities were observed for the *F.n.n.* Δ*clpB* immunized fish during the 28 days prior of challenge. Survival 28 dpc was 70% in the group immunized with the *clpB* mutant compared with 45% in the non-immunized control group (Fig. 4A) which was terminated 10 dpc at 45% survival, as mortality rapidly approached the humane endpoint mentioned previously. Upon termination of the non-immunized group, survival in the immunized group 10 dpc was significantly different at 87% (p = .0005). The Relative Percent Survival (RPS) calculated

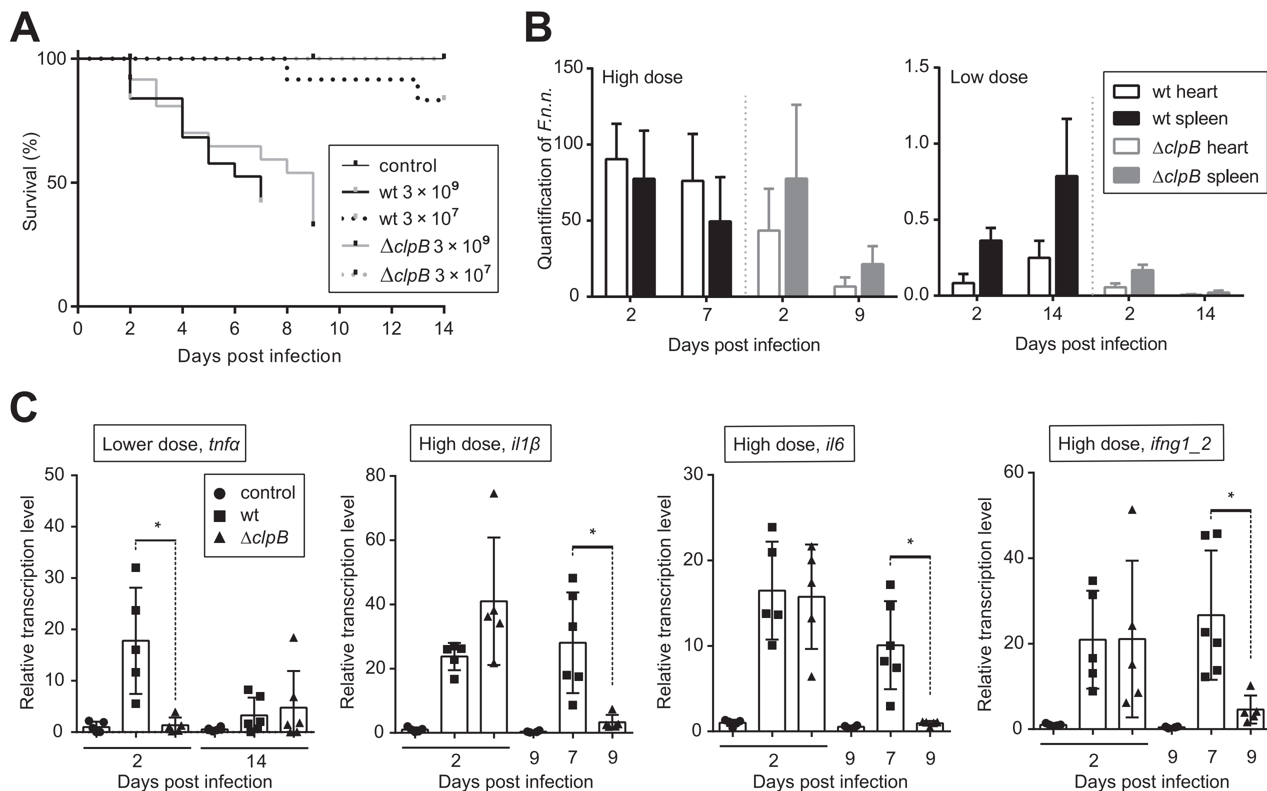


**Fig. 3.** Infection experiment comparing different doses of *F.n.n.* Δ*clpB* and *F.n.n.* wt in adult zebrafish AB wt. (A) Kaplan – Meier curve of cumulative survival of adult zebrafish infected with high dose ($3 \times 10^9$) or lower dose ($3 \times 10^7$) of *F.n.n.* wt and Δ*clpB*. Cumulative survival of lower dose wt versus Δ*clpB* was not statistically significant, p = .1132. (B) Bacterial burden of heart and spleen infected with lower and high dose of *F.n.n.* wt and Δ*clpB* quantified by qPCR on genomic DNA. Significantly decreased bacterial load in spleen from 2 to 14 dpi was found in lower dose Δ*clpB* infected fish (heart: p = .0639, spleen: p = .0027). Bacterial load in the high dose Δ*clpB* group appeared to decrease from 2 to 9 dpi (not significant; heart: p = .2267, spleen: p = .2928). (C) Transcription of *tnfα, il1β, il6* and *ifng1_2* in kidney tissue infected with lower or high dose *F.n.n.* wt and *F.n.n* Δ*clpB* measured by RT-qPCR. Results are presented as means ± SEM. Asterisks indicate significant upregulation (* p < .05; 2-tailed unpaired Student's *t*-test). Significantly lower *tnfα* transcription was observed in lower dose mutant infected fish 2 dpi (p = .0079) compared with the wt group and only the wt group triggered *tnfα* at this timepoint compared with the PBS group (p = 0.0069). Both high dose groups responded 2 dpi with a significantly higher transcription of *il1β* (wt: p=<.0001, Δ*clpB*: p = .002), *il6* (wt: p = .0003, Δ*clpB*: p = .0007), *il8* (wt: p = .0277, Δ*clpB*: p = .0023), *il10* (wt: p = .0057, Δ*clpB*: p = .0008), *il12a* (wt: p = .0006, Δ*clpB*: p = .035) *and ifng 1_2* (wt: p = .0046, Δ*clpB*: p = .0398) compared to the PBS group, but no clear *tnfα* and *ifng1_1* transcription was triggered at 2dpi. At the endpoint of both high dose groups, transcription levels of *il1β* (p = .0071), *il6* (p = .0035) and *ifng1_2* (p = .0113) were significantly higher in the mutant infected group 9 dpi compared with the wild-type group 7 dpi, but this is interpreted with caution as the sampling day is different.

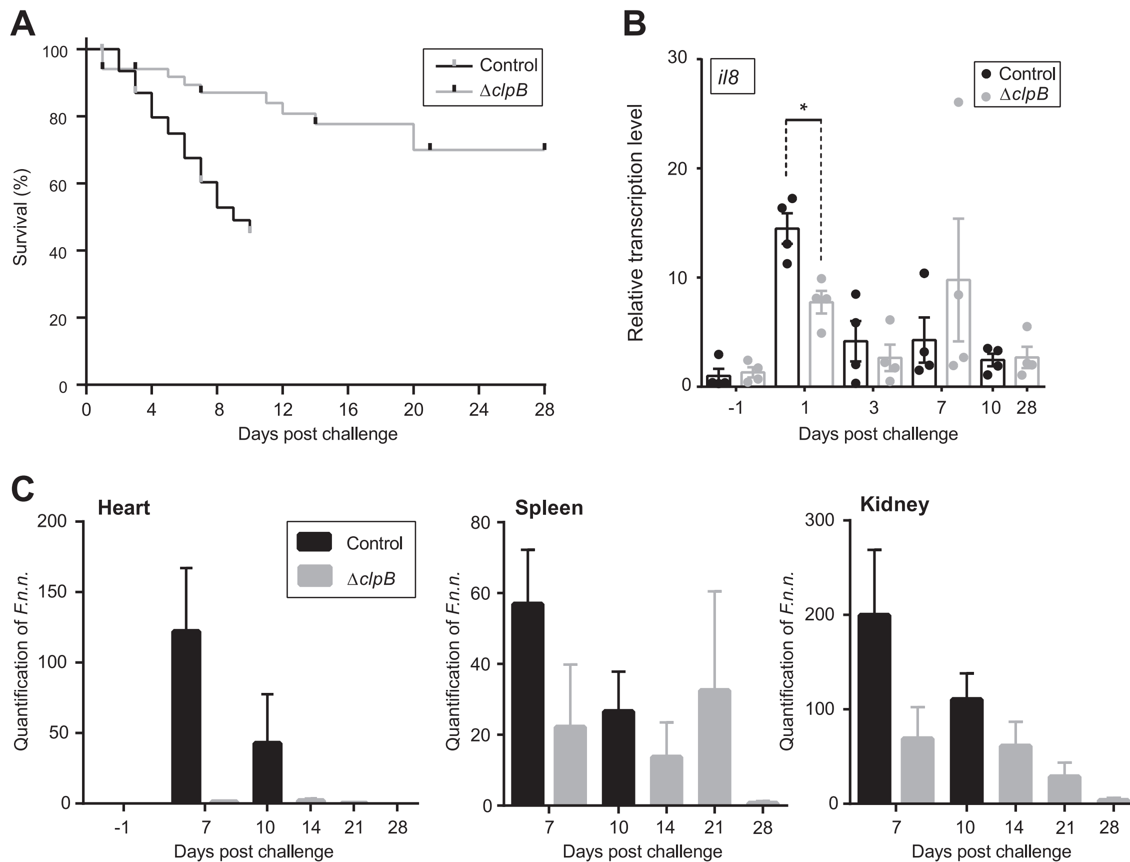


**Fig. 4.** Immunization with *F.n.n.* Δ*clpB* protected adult zebrafish from acute mortality after challenge with a lethal dose ($6 \times 10^8$ CFU) of *F.n.n.* wt. (A) Kaplan – Meier curve of cumulative survival of adult zebrafish that were challenged with $6 \times 10^8$ CFU of *F.n.n.* wt four weeks after immunization with $7 \times 10^6$ *F.n.n.* Δ*clpB* or PBS-injection. Upon termination of the non-immunized group, survival in the immunized group 10 dpc was significantly different at 87% (p = .0005). (B) Kidney transcription was measured by RT-qPCR on extracted RNA and revealed a significantly lower *il8* transcription level in mutant immunized zebrafish after *F.n.n.* wt challenge compared with non-immunized zebrafish. Results are presented as means ± SEM. Asterisks indicate significant upregulation (* p < .05; 2-tailed unpaired Student's *t*-test). Kidney *il8* transcription at 1 dpc (p = .0097) was significantly lower in immunized fish compared with the non-immunized control. (C) Quantification of *F.n.n.* by qPCR on gDNA from zebrafish heart, spleen and kidney relative to zebrafish genomes. Results are presented as means ± SEM.

at termination of the non-immunized control group (10 dpc) was 92.6%, which had dropped to 54.8% at endpoint (28 dpc). RPS at endpoint would likely have been higher if the non-immunized control group had not been terminated at 10 dpc. The fish were immunized by intraperitoneal injections and subsequently challenged by injections into the same anatomical compartment. Transcription of *il1β*, *il6*, *il8*, *il10* and *ifng1_2* was significantly increased 1 dpc in kidneys and spleens of immunized and non-immunized fish (Fig. 4B and Supplementary Fig. S5). Interestingly, the only significant difference in transcription was found in kidney *il8* at 1 dpc (p = .0097) which was significantly lower in immunized fish compared with the non-immunized control among the immunorelevant transcripts studied (Fig. 4B and Supplementary Fig. S4). As IL8 is a chemokine recruiting leukocytes like neutrophils to sites of inflammation [38], the triggered, but significantly lower transcription in kidneys from immunized fish compared to non-immunized fish 1 dpc suggests that a more modest recruitment of leukocytes contributes to protection against francisellosis. The relative quantification of *F.n.n.* genomic DNA 7 dpc was generally lower in tissues of immunized fish compared to non-immunized fish (Fig. 4C), suggesting immunogenic interference with bacterial growth, infection kinetics, nutrient acquisition or bactericidal processes. Few *F.n.n.* genomes were detected in hearts of immunized fish 7 dpc compared to the non-immunized fish hearts and compared to the other tissues of both groups. After 28 dpc, bacterial load in hearts of immunized fish was diminished considerably to almost undetectable levels. Bacterial load in non-immunized fish tissues upon termination of the group at 10 dpc was similar to bacterial load 7 and 14 dpc in the immunized group, but with concomitant acute disease in the non-immunized group.

### *3.6. Immunized fish develop focal granulomas after wild-type challenge*

Histology sections of immunized fish sampled one day before challenge revealed no granulomas, as shown in histographs from liver (Fig. 5A) and kidney (Fig. 5B) indicating that the immunization did not produce the granulomas observed later in the experiment. However, at 28 dpc, immunized fish revealed multifocal granulomas (Fig. 5C - G2) with signs of encapsulation (arrows, Fig. 5C, D, E2 and F) surrounded by normal tissue. Granulomas were found adjacent to the liver (Fig. 5C and D), in the kidney (Fig. 5E1, E2 and F) and the pancreas (Fig. 5G1 and G2). The pancreatic granulomatous processes appeared less defined than the other lesions with little sign of encapsulation, but the lesions appeared to remain within the pancreatic tissue. Fig. 5D and Fig. F - G2 are PAS stained sections in which the PAS positive character of granular cells within the granulomas is evident, as also observed in previous *F.n.n.* infections [28].

Although *clpB* mutant immunized zebrafish developed granulomatous lesions after *F.n.n* wt challenge, the surrounding normal tissue and decreased bacterial load in kidney, spleen and heart indicates a more successful containment compared to

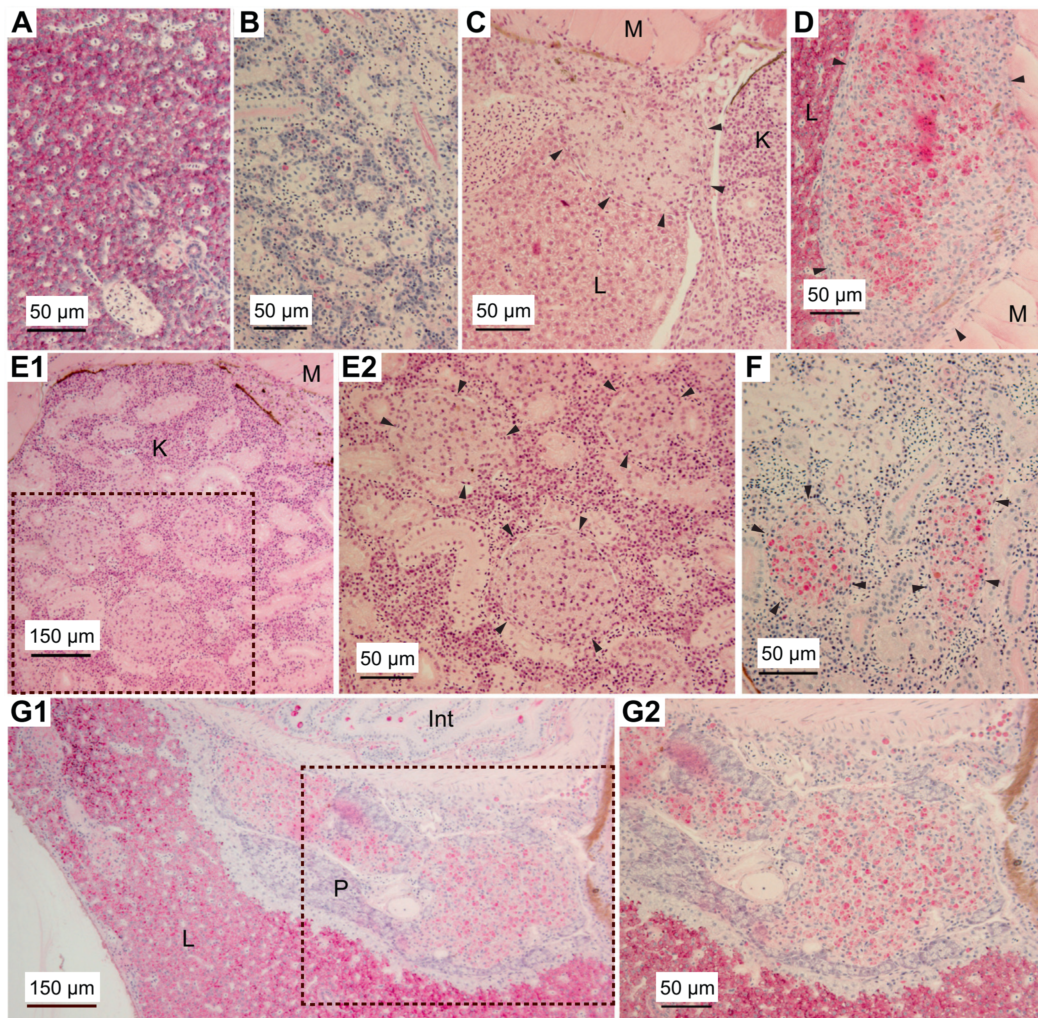


**Fig. 5.** Histology sections of *F.n.n.* Δ*clpB*-immunized adult zebrafish before and after challenge with a lethal dose of *F.n.n.* wt. Sections are stained with Hematoxylin and eosin (HE) or Periodic acid Schiff's reagent (PAS). PAS stained liver (A) and kidney (B) tissue of zebrafish 27 days after immunization with *F.n.n.* Δ*clpB* (one day before challenge). (C-G2) Histology sections from mutant-immunized zebrafish 28 days post challenge (dpc) with *F.n.n.* wt. (C) A granulomatous process delineated by arrowheads is located in the liver, L next to kidney, K and muscle tissue, M. H&E staining. (D) A large liver granuloma adjacent to muscle tissue, PAS staining. (E1) Overview of HE stained kidney tissue with rectangle showing area magnified in (E2), presenting three localized granulomatous processes (arrowheads). (F) PAS stained section of kidney revealing two granulomas. (G1) Overview of PAS stained tissue with a large granulomatous process localized within pancreatic tissue, P, next to the intestines, Int, with rectangle showing magnified area in (G2).

unvaccinated fish, which likely contributes to reduction of the wt bacterial load.

## 4. Conclusion

In the present study, we found that *clpB* deletion in *F.n.n.* generates an immunogenic strain that after intraperitoneal injection in adult zebrafish induced protective immunity against an acute lethal dose of the parental strain delivered by the same route. The *F.n.n* Δ*clpB* mutant is deficient for replication in both adult zebrafish and in an embryo zebrafish model. The immune response of the Atlantic cod head kidney leukocytes and adult zebrafish reveals cytokines associated with *F.n.n.* infection and protection. Further studies should be performed to investigate if the immune response induced by the *clpB* mutant can protect against other routes of infection, such as water-borne exposure or injection into a different anatomical compartment. It is important to note that the zebrafish is only a model used for initial assessment of the mutant in this study, and such studies on *F.n.n.* Δ*clpB* should preferably be performed in the natural host, Atlantic cod.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2017.11.009.

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