

NOTE

Construction of a virulent, green fluorescent protein-tagged *Yersinia ruckeri* and detection in trout tissues after intraperitoneal and immersion challenge

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ABSTRACT: A green fluorescent protein (GFP) expressing strain of *Yersinia ruckeri* was created by the transposition of a Tn10-GFP-kan cassette into the genome of *Y. ruckeri* Strain YRNC10. The derivative, YRNC10-gfp, was highly GFP fluorescent, retained the *gfp-kan* marker in the absence of kanamycin selection, and exhibited *in vitro* growth kinetics similar to those of the wild type strain. YRNC10-gfp colonized and caused mortality in immersion and intraperitoneally challenged rainbow trout *Oncorhynchus mykiss*, although it was modestly attenuated compared to the wild type strain. The distribution and location of YRNC10-gfp in infected fish was visualized by epifluorescence microscopy. Abundant extracellular bacteria and a small number of intracellular bacteria were observed in the kidney, spleen and peripheral blood. To determine the percentage of trout cells containing intracellular bacteria, GFP fluorescence was measured by flow cytometry. A small population of GFP positive leukocytes was detected in peripheral blood (1.6%), spleen (1.1%) and anterior kidney (0.4%) tissues. In summary, this is the first report of the construction of a virulent, GFP-tagged *Y. ruckeri*, which may be a useful model for detecting and imaging the interactions between an aquatic pathogen and the natural salmonid host.

KEY WORDS: *Yersinia ruckeri* · Green fluorescent protein · Flow cytometry · Epifluorescence microscopy

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INTRODUCTION

Yersinia ruckeri was first recognized as a pathogen of rainbow trout in the Northwestern United States in the 1950s and has since appeared throughout North America and Europe, primarily in areas where trout are intensively cultured (Stevenson 1997). This Gram-negative bacterium is the causative agent of Enteric Redmouth Disease (ERM), an acute hemorrhagic septicemia. ERM outbreaks are often associated with poor water quality, excessive stocking densities, handling of fish and the occurrence of environmental stressors. *Y. ruckeri* can also persist in an asymptomatic carrier state in which bacteria are shed in the

faeces, with carrier fish serving as a reservoir for recurrent infections, often occurring at times of stress (Busch 1978, Stevenson 1997). Despite the early recognition of *Y. ruckeri* as an economically important pathogen of cultured salmonids, little work has been done to characterize the virulence mechanisms of this pathogen, and as a result, a model for its pathogenesis has not yet been developed.

Genetic tagging of pathogenic microorganisms with the gene encoding the green fluorescent protein (GFP) from *Aequorea victoria* has become an important tool for elucidating the interactions between bacterial pathogens and their hosts (Valdivia & Falkow 1996, Valdivia et al. 1996, Southward & Surette 2002). GFP-

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tagging allows *in situ* detection of bacteria–host interactions in real time. GFP fluorescence associated with tagged cells can easily be visualized and quantified by epifluorescence microscopy, flow cytometry, spectrofluorometry or fluorescence colony counting (Chalfie et al. 1994, Valdivia & Falkow 1996, Valdivia et al. 1996). More recently, artificial plasmids carrying the gene encoding the green fluorescent protein have been used to tag several fish pathogens (Ling et al. 2000, 2001, O'Toole et al. 2004), while these tagged strains have been useful for determining the host–pathogen interactions during the early stages of infection, the inherent instability of plasmid-mediated tags may limit their use in studies involving the long-term persistence of pathogens. Herein, we describe the use of a specialized transposon to construct a highly stable *gfp*-tagged derivative of *Yersinia ruckeri* and the use of this strain to visualize and quantify the interaction of this pathogen with trout immune cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Yersinia ruckeri* Strain YRNC10 is a Serotype 1 strain that was isolated from a moribund rainbow trout collected at a fish farm in North Carolina. YRNC10 and its derivatives were cultured at 24°C in trypticase soy broth (TSB), or trypticase soy agar (TSA). *Escherichia coli* strains were grown at 37°C in Luria broth or Luria agar. When required, antibiotics (Sigma) were added at the following concentrations: ampicillin (100 µg ml⁻¹); kanamycin (100 µg ml⁻¹ for *Y. ruckeri* and 50 µg ml⁻¹ for *E. coli*).

Experimental fish. Rainbow trout were obtained from a commercial supplier (Trout Lodge, Washington). Stock and test fish were maintained at 16°C in freshwater under flow-through conditions and a fed a standard diet 5 d per week. Fish were anaesthetized with MS-222 (200 mg l⁻¹) and, when necessary, anaesthetized fish were euthanized by cervical dislocation.

Transposition of Tn10-*gfp-kan* cassette. Transposition was carried out using the mini-Tn10-*gfp-kan* transposon (Stretton et al. 1998), according to the method of de Lorenzo et al. (1990), with the following modifications. Conjugation mixes were incubated at 24°C for 12 to 18 h on TSA media, after which the cells were resuspended in TSB, diluted, and plated on TSA containing 100 µg ml⁻¹ kanamycin to select for the transposon. Counter selection for the donor *Escherichia coli* strain was accomplished by incubation at 14°C.

Immersion infection and estimation of bacterial loads in exposed fish. Rainbow trout were challenged by immersion exposure with approximately 10⁸ viable bacteria ml⁻¹ tank water for 1 h in a static bath. Dissolved oxygen levels were maintained throughout the

exposure procedure. Test fish were then maintained at 16°C in fresh water under flow-through conditions for 1 mo, and dead fish were removed and recorded daily. Necropsies were performed on each fish and mortality due to infection by *Yersinia ruckeri* was confirmed by microbiological analysis of spleen tissue. Bacterial loads in the spleens of exposed fish were quantified by direct plate count assay as follows. Whole organs were removed aseptically, weighed and homogenized in phosphate-buffered saline (PBS, 10 µl mg⁻¹) by repeated passage through a 0.5 ml syringe. Homogenates were then serially diluted and 100 µl from each dilution plated on TSA. Colonies were enumerated after 24 h of incubation at 24°C. Based on the amount of tissue homogenate plated, the detection limit of this assay was 1 bacterium per 10 mg spleen tissue.

Statistical analysis. Bacterial load data was analyzed by 1-way ANOVA, and paired comparisons between wild-type (WT)- and GFP-infected fish were analyzed by Tukey's test. All statistical analysis was carried out using the GraphPad Prism 4 software package (GraphPad Software).

Cell samples and flow cytometry. For flow cytometry experiments, fish were intraperitoneally injected with 10⁶ bacteria in 0.1 ml PBS. Tissues and blood were collected at 48 h post infection. Fish were anaesthetized with MS-222 (200 mg l⁻¹) and peripheral blood was collected from the caudal vein using a 20 gauge needle and a heparinized blood collection tube (BD Vacutainer). After blood removal, fish were euthanized by cervical dislocation and spleen and anterior kidney tissues removed aseptically. The samples were placed in 0.5 ml of L15 medium (Sigma) containing 2% fetal bovine serum, FBS (Sigma) and homogenized using a 0.5 ml syringe. Large tissue fragments were allowed to settle by incubation for 10 min; the remaining suspended cells were transferred to a new tube and pelleted by centrifugation at 500 × *g* for 10 min at 4°C. Peripheral blood (0.5 ml) was diluted 10-fold in L15-2% FBS prior to centrifugation. Pelleted cells were washed and resuspended in L15-2% FBS medium containing 0.1% sodium azide. Erythrocytes were removed by discontinuous gradient centrifugation; 5 ml of cell suspension was layered on 6 ml histopaque 1077 (Sigma) in a 15 ml tube and centrifuged at 500 × *g* for 40 min at 4°C. After centrifugation, white blood cells located at the interface were collected and washed twice with 10 ml Dulbeccos PBS containing 2% FBS and 0.1% sodium azide. The light scattering and fluorescent properties of the cells were analyzed using a 3-color FACSCalibur (Becton Dickinson Biosciences). Data were analyzed using CellQuest Pro (Version 5.1.1). We recorded 20 000 events for each sample, and gating was used to remove autofluorescent cells (FL1 and FL2 double positive) from the analysis.

RESULTS AND DISCUSSION

Generation of GFP tagged *Yersinia ruckeri* strain

Transposition was used to deliver the mini Tn10-*gfp-kan* cassette to the genome of *Yersinia ruckeri*, yielding a bank of approximately 6000 kanamycin resistant clones, each presumably containing a random mini Tn10-*gfp-kan* insertion. This transposon contains a promoterless *gfp* gene and therefore *gfp* expression occurs only if transposition results in transcriptional fusion to an active promoter within the recipient genome (Stretton et al. 1998). Of the 6000 transconjugants, 50 (0.8%) were strongly GFP fluorescent under blue light-emitting diode (LED) light, indicating fusion to a strong *Y. ruckeri* promoter. Tn10-*gfp-kan* insertion in some transconjugants could compromise the ability of the resulting strain to colonize or cause disease; this may occur if the transposon insertion has altered the expression of a gene involved in either of these processes. Therefore, an *in vivo* selection strategy was used to enrich a tagged clone that had retained its ability to colonize rainbow trout. For this, the 50 GFP positive clones were grown individually to stationary phase, pooled, and the resulting mixture used to challenge rainbow trout by immersion. Moribund fish were observed 6 d after exposure, and fluorescence microscopy revealed high levels of GFP positive bacteria in the spleen and anterior kidney of these individuals. Standard microbiological methods were used to isolate bacteria from these tissues into pure culture, and a single strongly GFP-fluorescent clone (YRNC10-*gfp*) was selected for further study. YRNC10-*gfp* displayed strong GFP fluorescence in all phases of growth in liquid as well as on solid culture media, and had no discernible abnormalities in growth on these media (data not shown).

YRNC10-*gfp* retention of pathogenicity for rainbow trout

To validate the utility of YRNC10-*gfp* as a model for *Yersinia ruckeri* infection, it was important to verify that this modified strain had retained its ability to colonize and cause disease in exposed rainbow trout. Fish were immersion challenged with the YRNC10-*gfp* or the isogenic WT strain, and splenic bacterial loads were assessed as an indication of colonization and disease progression. Spleen colony counts were determined on Days 1, 3, 6, 9, 12, and 15 post-infection and a scatter plot of these data is presented in Fig. 1A. With the exception of Day 6, there were no significant differences in cfu mg⁻¹ spleen tissue between WT- and YRNC10-*gfp* immersion exposed fish. Between 8 and 16 d after infection, a low level of mortality was ob-

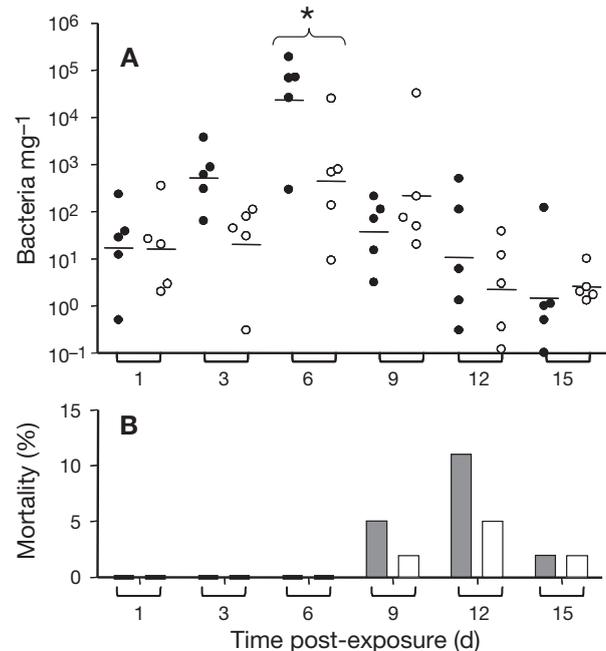


Fig. 1. *Oncorhynchus mykiss*. Splenic bacterial loads and mortality after immersion exposure to wild-type and *gfp*-expressing *Yersinia ruckeri*; 75 fish (50 to 100 g) were immersion exposed to wild-type *Y. ruckeri* (○) or *Y. ruckeri* YRNC10-*gfp* (●) for 1 h at a concentration of 10⁸ bacterial ml⁻¹. (A) Bacterial load in spleen of individual fish quantified at indicated times after exposure; *: significant difference ($p < 0.05$). Mean denoted by horizontal line. (B) Percent fish that died in intervals between sampling events. Shaded bars: wild-type *Y. ruckeri*; open bars: YRNC10-*gfp*

served in both WT- and YRNC10-*gfp* infected fish (Fig. 1B). Affected individuals showed gross pathology features typical of enteric redmouth disease, including hypertrophied spleen, exophthalmia, haemorrhaging in the lower intestine and frequently reddening around the lower jaw. The trends observed in bacterial splenic loads and mortality in the above experiment suggest that *in vivo* replication of the YRNC10-*gfp* strain is slower than that of its wild-type parent; as a result, this strain may be somewhat compromised in its ability to cause disease. To investigate whether there was a subtle difference in virulence, an immersion challenge was carried out using quadruplicate tanks per strain (Fig. 2). The total percent mortality observed for the YRNC10-*gfp* strain averaged 36% and ranged from 28 to 43%. In comparison, the wild-type strain yielded an average of 65% mortality that ranged from 61 to 67%. These data show a statistically significant reduction in mortality caused by the YRNC10-*gfp* strain and verify that this modified strain is impaired in its ability to cause disease compared to the wild type. Since YRNC10-*gfp* does not show any apparent growth defects on plate culture, the reduced virulence may be either due to GFP expression *in vivo*, or the GFP cas-

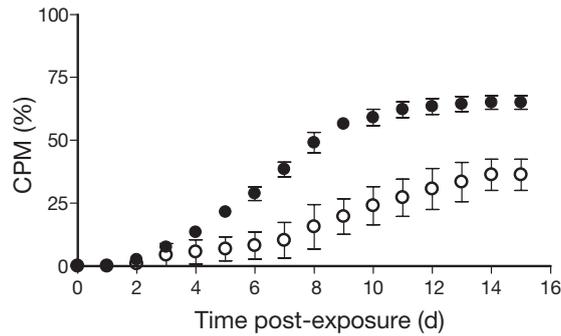


Fig. 2. *Oncorhynchus mykiss*. Mortality induced by immersion exposure to YRNC10-*gfp* or wild-type *Yersinia ruckeri*; 4 groups of 80 fish (average weight 5 g) were immersion exposed to wild-type *Y. ruckeri* (●) or *Y. ruckeri* YRNC10-*gfp* (○) for 1 h at a concentration of 10^8 bacterial ml^{-1} . Cumulative percent mortality (CPM) was recorded daily for 15 d after exposure. Error bars represent standard deviation (n = 4 replicates per strain)

sette insertion into a genomic region that contributes to virulence. Regardless, this finding does not invalidate the utility of YRNC10-*gfp* as a model for studying bacterial–host interactions, as the YRNC10-*gfp* strain

readily colonizes trout, causes pathology indicative of ERM and induces significant mortality.

It should be noted that the media used for all plating assays, including those for the YRNC10-*gfp* strain, did not contain kanamycin and therefore were not selective for the *gfp-Km* marker. In addition, all bacterial clones isolated from YRNC10-*gfp*-infected fish were found to be GFP fluorescent, indicating a high degree of stability of the *gfp-Km* marker in this strain during infection. Mortality was also induced by intraperitoneal challenge with YRNC10-*gfp* (data not shown).

Visualization of *gfp*-expressing *Yersinia ruckeri* in infected tissue and host cells by epifluorescence microscopy

YRNC10-*gfp* cells can be distinguished from WT cells by plating (Fig. 3A) and are easily visualized as motile, non-cell associated bacteria within rainbow trout tissue wet-mounted slides (Fig. 3 and authors' unpubl. data). In addition to the freely motile bacteria, a small number of intracellular bacteria were observed

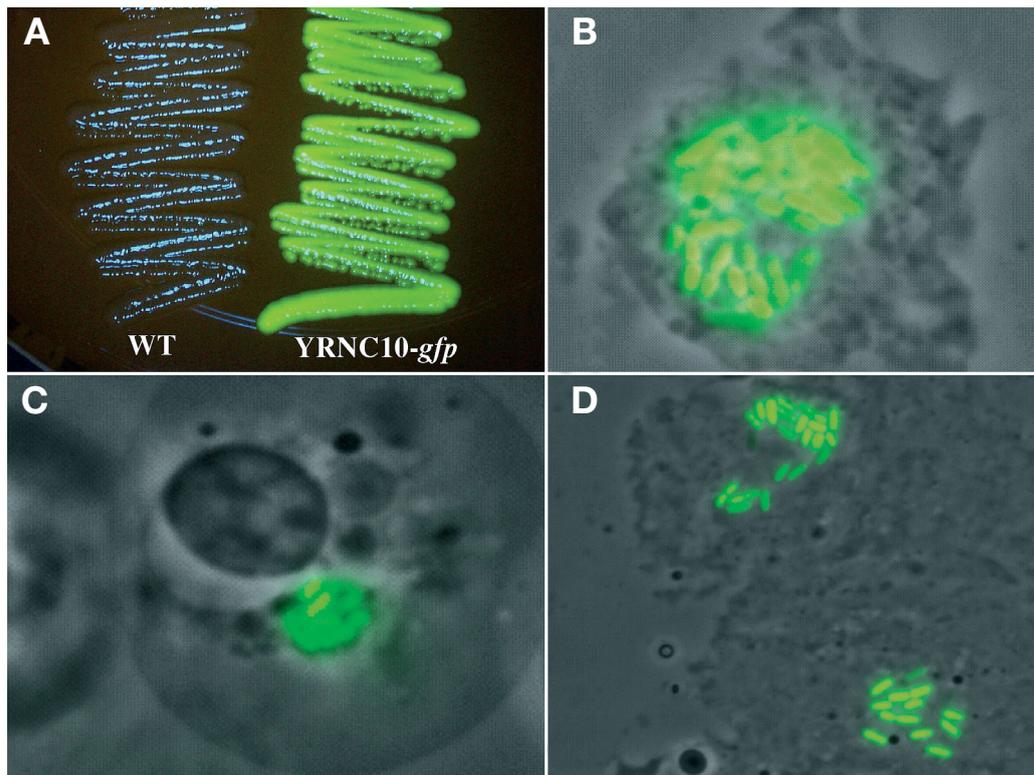


Fig. 3. Detection of green fluorescent protein (GFP)-producing *Yersinia ruckeri* in *Oncorhynchus mykiss*. Visualization of *Y. ruckeri* strain YRNC10-*gfp*. (A) Blue light-emitting diode (LED) illumination of cells grown on TSA. YRNC10-*gfp* cells were also observed in purified leukocytes enriched from (B) anterior kidney and (C) peripheral blood 48 h after intraperitoneal injection with approximately 10^6 cells. (D) Homogenized anterior kidney tissue from a moribund fish from the immersion infection experiment in Fig. 1 (micrographs are merged phase contrast, epifluorescence images acquired with Nikon Eclipse E600 microscope at 55× magnification)

in purified peripheral blood, anterior kidney and spleen leukocytes 48 h after intraperitoneal injection (Fig. 3B,C and authors' unpubl. data). Bacteria in these cells appeared to be contained within vacuole-like compartments. Formalin-killed YRNC10-*gfp* cells retained their GFP fluorescence, but were not detected 24 or 48 h after injection at a similar concentration. Large numbers of GFP-positive bacteria were also detected associated with infected spleen and kidney from several of the fish killed during the immersion challenge experiment described in the previous subsection (data not shown). The observation of GFP-positive YRNC10-*gfp* cells at several time points after infection and at all times when grown in culture suggests that the transcriptional fusion to the *gfp* gene in this strain resulted in constitutive production of GFP

Detection of trout cells associated with *gfp*-expressing *Yersinia ruckeri* by flow cytometry

We examined whether flow cytometry could be used to measure the number, size and granularity of leukocytes that contained either intracellular or tightly-associated extracellular *gfp*-expressing *Yersinia ruckeri*. Leukocytes isolated by gradient centrifugation from the peripheral blood, spleen and anterior kidney of sham-, *Y. ruckeri* WT- and YRNC10-*gfp*-injected fish were analyzed by flow cytometry. Dot plots of the cellular forward scatter (FSC) and side scatter (SSC) are shown in Fig. 4. Cells falling within the known lymphocyte, monocyte and neutrophil regions (Kollner et al. 2001, Stafford et al. 2001, Moritomo et al. 2003) were included in the R1 gate. Spleen and anterior kid-

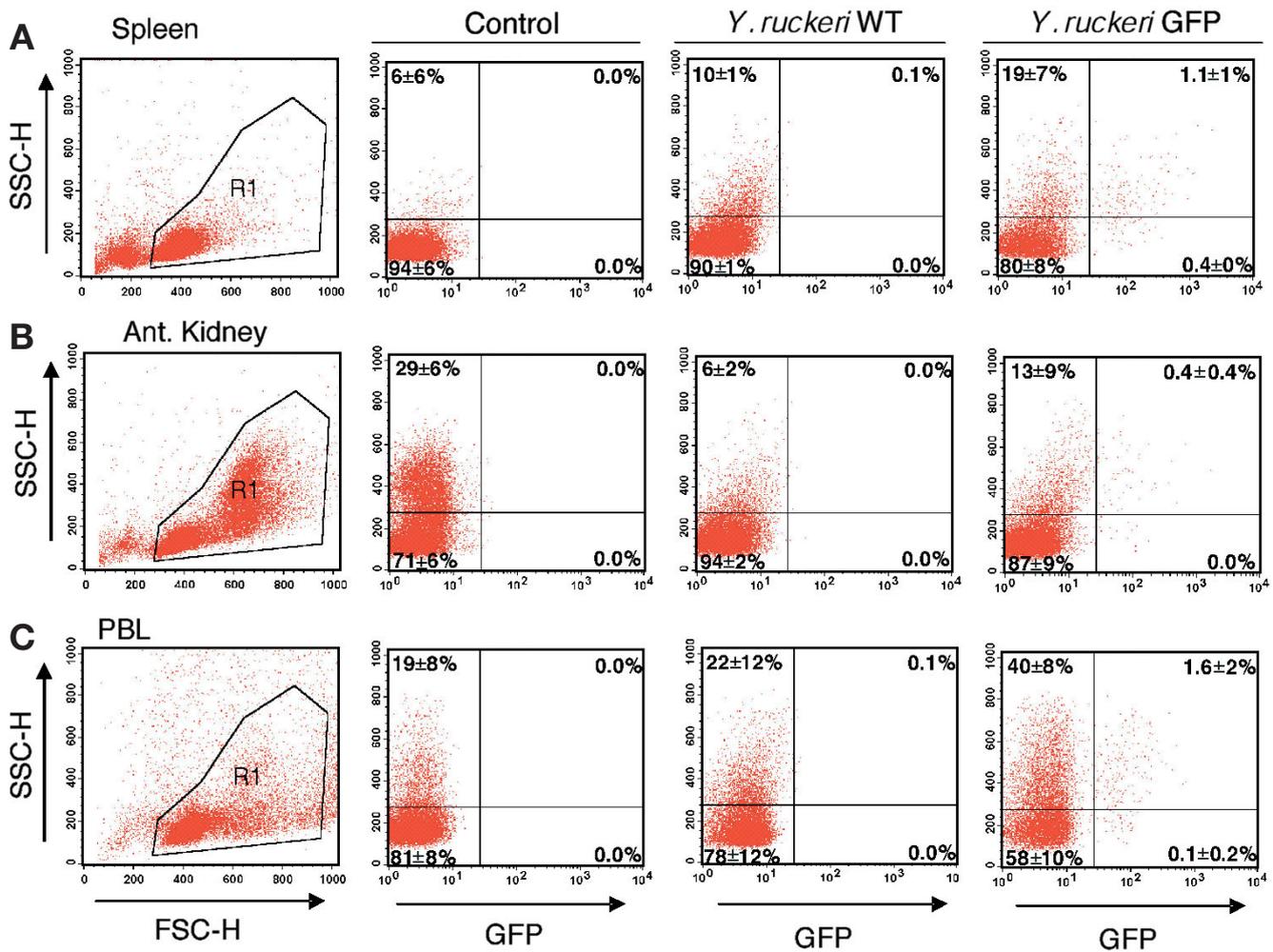


Fig. 4. Detection of green fluorescent protein (GFP)-producing *Yersinia ruckeri* in *Oncorhynchus mykiss* by flow cytometry. Leukocytes were isolated from the (A) spleen, (B) anterior kidney and (C) peripheral blood of (PBL) sham-, *Y. ruckeri* WT (wild-type)- and YRNC10-*gfp* (GFP+)-injected fish; size and granularity of cells determined by forward (FSC) and side scatter (SSC) plots ('control' = uninfected fish). Cells falling within the leukocyte R1 gate were analyzed for GFP fluorescence in the FL1 channel (abscissa) and for granularity (ordinate). Percentages in each quadrant represent average and standard deviation of 3 (sham-injected and *Y. ruckeri* WT-injected) or 4 (*Y. ruckeri* GFP+)-injected fish

ney tissues from both *Y. ruckeri* WT and YRNC10-*gfp* infections contained increased numbers of autofluorescent cells compared to sham-injected controls (data not shown). The nature of these cells is unknown and they were removed from the analyses by a second, R2 gate (data not shown). Small populations of trout cells were clearly identified in YRNC10-*gfp*-injected fish spleen, peripheral blood lymphocytes and anterior kidney samples which were not present in either the sham-injected or *Y. ruckeri* WT-injected fish (Fig. 4). The percentage of this population was highest in the peripheral blood (1.6 + 2%; n = 4) and lowest in the anterior kidney (0.4 + 0.4%; n = 4). The majority of the GFP-positive cells were in the non-lymphocyte quadrant (Fig. 4, compare percentage in upper right quadrant to lower right quadrant), consistent with known properties of rainbow trout phagocytic cells (Kollner et al. 2001, Moritomo et al. 2003). Interestingly, there was an increase in the percentage of larger, granular cells in the spleen and peripheral blood of infected fish compared to sham injected controls, while there was a decrease in these types of cells in the anterior kidney. These data are consistent with migration of leukocytes from the anterior kidney to the periphery during infection. In summary, a small percentage of rainbow trout cells could be identified by flow cytometry that either contained intracellular or tightly associated extracellular GFP-positive *Y. ruckeri* cells.

In this report we have described the construction and characterization of YRNC10-*gfp*, a virulent strain of *Yersinia ruckeri* that stably and constitutively expresses high levels of the green fluorescent protein. In addition, we used this strain to identify a small population of phagocytic cells containing intracellular bacteria. The small number of intracellular bacteria observed in these studies suggests that *Y. ruckeri* is predominantly an extracellular pathogen; however, a high proportion of bacteria could reside inside cells earlier in infection or in the chronic form of the disease. The detection of GFP-expressing fish pathogens by flow cytometry will allow the characterization of physiologically relevant trout cells that interact with pathogenic bacteria. This strain can also be easily identified in complex environmental samples containing multiple bacterial species (data not shown), making it especially

useful for characterizing factors that allow its persistence and spread outside its host.

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