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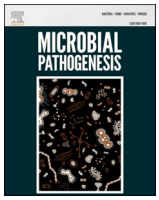

Detection of *Aeromonas salmonicida* subsp. *salmonicida* infection in zebrafish by labelling bacteria with GFP and a fluorescent probe based on the siderophore amonabactin

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ABSTRACT

Zebrafish (*Danio rerio*) is an excellent model to study bacterial infections in fish and their treatment. We used zebrafish as a model of infection for *Aeromonas salmonicida* subsp. *salmonicida* (hereinafter *A. salmonicida*), the causative agent of fish furunculosis. The infection process of *A. salmonicida* was studied by immersion of zebrafish larvae in 2 different doses of the bacteria and the fish mortality was monitored for three days. The bacterium caused a high mortality (65 %) in zebrafish larvae only when they were exposed to a high bacterial concentration (10^7 bacterial cells/mL). To evaluate the use of fluorescence microscopy to follow *A. salmonicida* infection *in vivo*, two different fluorescent strains generated by labeling an *A. salmonicida* strain with either, the green fluorescent protein (GFP), or with a previously reported siderophore amonabactin-sulforhodamine B conjugate (AMB-SRB), were used. The distribution of both labeled bacterial strains in the larvae tissues was evaluated by conventional and confocal fluorescence microscopy. The fluorescent signal showed a greater intensity with the GFP-labeled bacteria, so it could be observed using conventional fluorescence microscopy. Since the AMB-SRB labeled bacteria showed a weaker signal, the larvae were imaged using a laser scanning confocal microscope after 48 h of exposure to the bacteria. Both fluorescent signals were mainly observed in the larvae digestive tract, suggesting that this is the main colonization route of zebrafish for waterborne *A. salmonicida*. This is the first report of the use of a siderophore-fluorophore conjugate to study a bacterial infection in fish. The use of a siderophore-fluorophore conjugate has the advantage that it is a specific marker and that does not require genetic manipulation of the bacteria.

1. Introduction

The species of the genus *Aeromonas* are Gram-negative γ-proteobacteria, bacillus-shaped, and facultative anaerobic [1]. They are responsible for numerous diseases in fish, amphibians, and reptiles, as they are found mainly in aquatic environments [1,2]. Moreover, they can also cause human infections, such as gastroenteritis, conjunctivitis, septicemia, and endocarditis [3]. *Aeromonas* species are especially known because of the great economic losses they cause in aquaculture, due to their negative impact in fish health and their high resistance to common antibiotics, which makes their study relevant [4].

A. salmonicida is a non-motile psychrophilic species composed of five subspecies (*salmonicida*, *achromogenes*, *masoucida*, *pectinolytica*, and *smithia*) [5]. *A. salmonicida* subsp. *salmonicida* (hereinafter simply referred to as *A. salmonicida*) causes furunculosis in a wide variety of fish (e.g. salmonids, turbot, sea bream, sole) [3,6]. The clinical signs of the

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disease include septicemia along with hemorrhages in the fins, muscles, and internal organs, and typical skin ulcerations [7]. The optimal growth temperature for *A. salmonicida* is within the range 22–25 °C [6].

The development of methodologies to study the invasion routes and host colonization mechanisms of *Aeromonas* is important not only to increase our knowledge about their virulence mechanisms but also for investigating the fish immunological responses. All this will be useful to develop novel tools to control bacterial infections caused by *Aeromonas* spp. In recent decades, the use of zebrafish (*Danio rerio*) as a model for studying diseases in aquaculture fish species has grown. Its advantages over other animal models and its exceptional *in vivo* imaging capabilities have enabled new insights in this field [8]. Previous studies indicates that zebrafish may be a suitable model for *A. salmonicida* infections [9].

Green Fluorescent Protein (GFP) has revolutionized fluorescent microscopy in recent years. This protein was originally isolated from the jellyfish *Aequorea*, but exhibits intrinsic fluorescence in heterologous hosts without the need for any other cofactor [10]. By introducing mutations into the *gfp* gene, it was possible to generate new proteins with higher fluorescence intensities or even new emission spectra, which resulted in a new palette of hues and colors [11]. All of the above, together with the possibility of noninvasive detection, has led GFP and its derivatives to be widely used as biomarkers for studying gene expression, protein localization (of GFP-tagged proteins) and interactions [7]. Thus, GFP is widely used in a number of fields, including cell biology, developmental biology, neurobiology, and ecology [12]. The use of GFP for labelling bacteria has been of great importance for many microbial cell biology studies [13]. Virulent and avirulent *A. salmonicida* strains tagged with GFP were used to track the colonization of turbot [14].

Iron is an essential element for most organisms since it participates in numerous metabolic processes [15]. Due to the low bioavailability of free iron, most pathogenic bacteria secrete siderophores, low molecular weight (500–1500 Da) compounds, to scavenge iron from their surroundings [16]. They have very high affinities for Fe(III) with affinity constants up to 10^{49} [17]. During our studies about siderophores secreted by fish pathogenic bacteria [18], and particularly by *A. salmonicida* [19–21], we have developed a new fluorescent probe called AMB-SRB (Fig. 1) [22]. This probe was generated by conjugation of a synthetic analogue of the siderophore amonabactin, produced by *A. salmonicida*, and the fluorophore sulforhodamine B. We have demonstrated that AMB-SRB is successfully internalized by *A. salmonicida* through the amonabactin outer membrane transporter FstC [22]. AMB-SRB, like GFP, absorbs radiation at specific UV wavelengths (absorption bands at 575 and 535 nm) and emits light as a result (bands between 550 and 750 nm) [23]. This makes siderophore-fluorophore conjugates valuable tools for studying Fe(III) uptake mechanisms in organisms that secrete siderophores or for detecting the presence of Fe(III) [24,25].

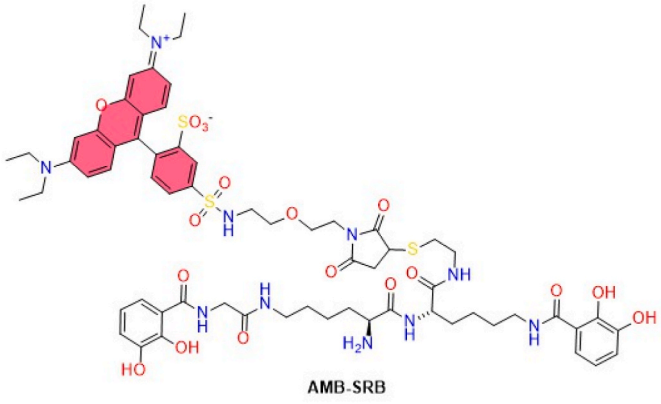

Fig. 1. Structure of the fluorescent probe AMB-SRB.

The main aim of this study was to evaluate the use of alternative fluorescent markers based on siderophore conjugates to analyze the colonization route of waterborne *A. salmonicida* in zebrafish. For this purpose, we compared GFP and AMB-SRB labeled *A. salmonicida* strains. Our results show that this bacterium does compromise the zebrafish viability and seems to colonize them through the digestive tract. Although the visualization of bacterial infections in zebrafish using GFP-labeled bacteria has been already reported [13], siderophore-based fluorescent probes to label bacteria has not been used so far to study host-pathogen interactions in zebrafish.

2. Materials and methods

2.1. Zebrafish husbandry

Zebrafish (*Danio rerio,* Fam. Cyprinidae) individuals used in this study belong to the wild-type line Tüpfel long fin (TL). Adults were kept in a commercial housing system following standard conditions [26], including feeding twice a day with dry food and *Artemia* sp., maintained at 28 ± 1 °C and under a light cycle of 14 h light and 10 h dark. To obtain embryos, adults were transferred to breeding tanks and placed in shelves exposed to the light source. Eggs were collected the next morning by using a sieve and placed in Petri dishes with sterile dechlorinated tap water (SDTW). Non fertilized embryos were detected under a stereomicroscope and removed from the Petri dishes. Embryos were maintained in an incubator at 28 ± 1 °C until exposed to the bacteria by 4 days post-fertilization (dpf). Developmental stage was determined following the method described by Kimmel et al. [27].

2.2. Aeromonas salmonicida strains, media, and growth conditions

The strains of *A. salmonicida* subsp. *salmonicida* used were routinely grown at 25 °C in Trypticase Soy Agar (TSA) (Condalab, Madrid, Spain). The strains were also cultured in Miller's minimal medium [28] with 0.2 % casamino acids (CM9) and in iron deficiency by the addition of 2, 2′-dipyridyl 30 μM. All bacterial strains were preserved in cryovials at −80 °C.

Two strains of *A. salmonicida* subsp *salmonicida* were used in the infection experiments. As infection control strain we used *A. salmonicida ΔentB,* a mutant (derived from *A. salmonicida* VT45.1) which is unable to produce any siderophore, but keeps intact FstC protein, the outer membrane transporter for amonabactins [14,19]. We used this mutant instead a wild type strain to avoid interferences with the production of endogenous siderophores. This strain was first labeled with green fluorescent protein (GFP). The labeled strain was obtained by conjugating plasmid pRhokHi-2-GFP from *E. coli* Sm10 pRhokHi-2-GFP, following the method described by Dubert et al. [29]. This GFP-labeled strain was cultured into 3 mL of CM9 minimal medium containing 2,2′-dipyridyl 30 μM. It was incubated at 25 °C and 200 rpm continuous shaking overnight. The bacterial growth was followed by measuring the OD_{600} using a mySPEC spectrometer (Mode = Cuvette; Path Length = 10 mm), up to values of 0.6–0.8, which means a viable cell concentration of 4.8 x 10^8–6.4 x 10^8 cells/mL.

The other strain used was *A. salmonicida ΔentB* labeled with the fluorescent probe AMB-SRB as previously described by Cisneros-Sureda et al. [22]. The culture was done in the same way as that of *A. salmonicida* labeled with GFP, but adding 54 μL of the AMB-SRB probe solution at 721 μM (final concentration 13 μM) in methanol: milliQ-water (1:1) and stored at −20 °C.

2.3. Analysis of zebrafish mortality after exposure to A. salmonicida ΔentB

Zebrafish embryos were exposed to bacterial suspensions by static immersion. After 4 days post-fertilization (dpf), fish were transferred to twelve-well plates, with 10 embryos per well. All embryos had

spontaneously hatched from their chorion. Next, a suspension of *A. salmonicida ΔentB* was added to each well (500 μl per well). Two different concentrations were tested: 10^6 bacteria/mL and 10^7 bacteria/mL (four wells per concentration). Bacterial counts were performed by turbidimetry or optical density. Two wells were kept as negative control (fish maintained in SDTW). Embryos were kept at 28 ± 1 °C in the dark and monitored under a stereomicroscope every 24 h for the next 72 h. Fish showing any of the following endpoints were counted as dead [30–32]: a) coagulation; b) absence of heartbeat. Abnormal swimming behavior and lack of movement were also annotated. Three independent replicates of the experiment were carried out. After 72 h, all surviving larvae were euthanized.

2.4. Live-cell fluorecesce microscopy analysis

Zebrafish embryos were exposed to labeled strains of *A. salmonicida* (GFP and AMB-SRB) by static immersion. In brief, 4 dpf zebrafish were transferred to four-well plates, placing 6 larvae per well, and bacterial suspensions were added (10^6 bacteria/mL and 10^7 bacteria/mL; two wells per concentration). Two wells were kept as negative controls (fish kept in SDTW). Plates were maintained at 28 ± 1 °C in the dark until imaged 24 and 48 h later. Three independent replicates of the experiment were carried out.

Before observation, larvae were washed 3 times with SDTW, transferred into an imaging Petri dish (Ibidi) and anesthetized using tricaine methanesulfate. Imaging was performed using a fluorescence microscope (Nikon Eclipse E600FN) equipped with an eGFP dichroic filter (excitation 488 nm, emission 500–550 nm) and an TRITC dichroic filter (excitation 561 nm, emission 570–620 nm), that were used for imaging GFP and AMB-SRB- labeled *A. salmonicida*, respectively.

In addition, a laser scanning confocal microscope (Nikon A1R) was used for the analysis of the fluorescence and distribution of the bacteria labeled with AMB-SRB. The same imaging parameters were used for control larvae and larvae exposed to the bacteria (λ_{ex} = 561.4 nm, λ_{em} = 570–620 nm, HV = 185, Offset = −1). Bright field images were also collected using the transmitted light detector. Single images (x, y) and confocal stacks (x, y, z) were taken. Fiji software was used for image processing and analysis [33].

3. Results

3.1. Mortality of zebrafish larvae exposed to A. salmonicida ΔentB

To test the uptake of waterborne *A. salmonicida* by zebrafish and consequent impact in survival, we immersed zebrafish embryos [4 days post-fertilization (dpf)] in two bacterial suspensions (10^6 and 10^7 bacteria/mL) of *A. salmonicida* mutant *ΔentB* (a mutant, derived from *A. salmonicida* wild-type strain VT45.1, that is unable to produce any siderophore, and hence has a low degree of virulence). Fish were then examined at 24, 48, and 72 h.

For the highest concentration of *A. salmonicida ΔentB*, we observed that fish exhibited clear symptoms of disease, including erratic or abnormal swimming behavior after 24 h exposure (Fig. 2). In the next two days, there was a significant increase in larvae mortality compared to controls, with 10 % mortality by 48 h and 65 % by 72 h (Fig. 2).

Absence of swimming in some of the live larvae was also observed. For the zebrafish larvae exposed to the lowest concentration of the bacteria, no significant increase in mortality was observed. However, compared to controls, fish did exhibit erratic or abnormal swimming behavior.

3.2. Visualization of the distribution of GFP-labeled A. salmonicida in zebrafish larvae

The general morphology of larvae was not altered compared to controls (skin, musculature, fins, eyes). All zebrafish larvae (control and exposed) showed yolk autofluorescence (Fig. 3A–C), which persisted throughout the experiment. Larvae showed fluorescent signal along the digestive tract and in the cloaca (Fig. 3B, E), which was not present in control individuals (Fig. 3A, D). Fluorescent punctae could be distinguished along the digestive tract after 24 h immersion (Fig. 3E). The fluorescent signal along the digestive tract was much more intense after 48 h (Fig. 3C, F). Although the fluorescence in the larvae exposed to a concentration of 10^6 bacteria/mL also increased from 24 to 48 h compared to the control (not shown), the fluorescence observed was much weaker than in larvae exposed to a concentration of 10^7 bacteria/mL.

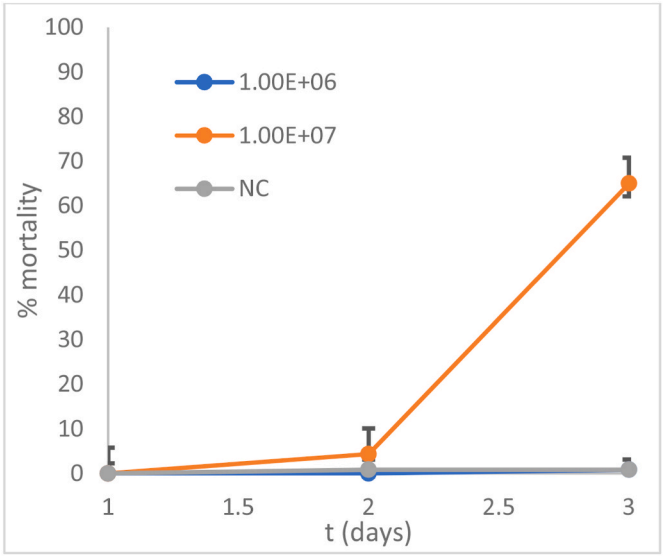

Fig. 2. Evolution of the mortality rate of 4 dpf zebrafish embryos exposed to two concentrations of *A. salmonicida* (10^6 and 10^7 bacteria/mL) and the negative control (NC).

3.3. Visualization of the distribution of AMB-SRB-labeled A. salmonicida in zebrafish larvae

As before, the general morphology of the exposed larvae was not altered. Very weak fluorescent signal was observed (not shown), so larvae were imaged using a laser scanning confocal microscope after 48 h exposure. Although fluorescence was observed along the digestive tract of both control and exposed larvae, the intensity of the fluorescence was much greater in those exposed to the bacteria (Fig. 4).

4. Discussion

Understanding the pathologies that affect fish in aquaculture is crucial for reducing economic losses and improving animal welfare. While zebrafish (*Danio rerio*) is not an aquaculture fish, it could serve as an ideal model organism for studying the development and progression of fish diseases. However, few studies have been conducted on *A. salmonicida* subsp. *salmonicida* in zebrafish [8] and they were mainly focused on immunology studies [9,34].

In this study, we examined the effects of *A. salmonicida* subsp. *salmonicida* infection in zebrafish larvae. We also took advantage of the transparency of the larvae to visually monitor the disease and analyze the distribution of two labeled strains of the bacterium in the larvae.

Our mortality study results suggest that *A. salmonicida* is pathogenic to zebrafish larvae. Although intraperitoneal injection is a common method for experimentally infecting fish, it does not accurately represent natural infection conditions in aquaculture facilities. In this work, we infected zebrafish larvae by immersing them in suspensions of the bacteria [13,35]. We observed that both concentrations tested (10^6 and

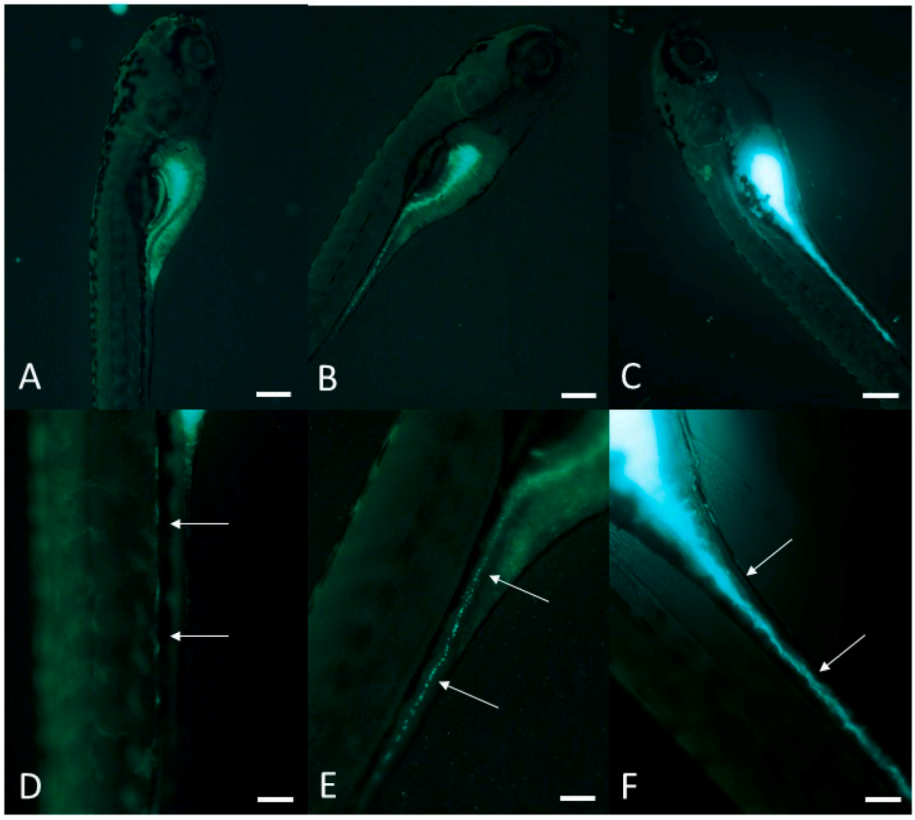

Fig. 3. Visualization of zebrafish larvae (*Danio rerio*) by fluorescence microscopy. A) Control. B) After 24 h of exposure to GFP-labeled *A. salmonicida*. C) After 48 h of exposure. D) Detail of A at the level of the digestive tract. E) Detail of B at the level of the digestive tract. F) Detail of C at the level of the digestive tract. Lateral views, anterior to the top. Arrows point to the digestive tract. Scale bars: 200 μm (A–C), 100 μm (D–F).

10^7 bacteria/mL) cause changes in the larvae behavior, and the highest concentration caused a high percentage of death (65 %) in the time range tested (72 h). In a previous study, adult zebrafish were infected with *A. salmonicida*, also causing death between 24 and 30 h after infection [9].

We observed the location of both GFP and AMB-SRB labeled strains of *A. salmonicida* along the digestive tract of zebrafish larvae, but not in other body parts. Although it is not clear if the digestive tract is an infection route for *A. salmonicida*, the presence of the bacteria has previously been observed in the digestive tracts of various fish species [36]. Further studies are needed to determine if the bacteria adhere and colonize the gastrointestinal tract, as well as to characterize the local response in the larvae (e.g. production of mucus [36,37] or neutrophil recruitment [35]).

Zebrafish larvae had not been previously used to study infection routes and distribution of *A. salmonicida*, but they were used to study other labeled pathogenic bacteria, such as *Vibrio anguillarum* (exposure by immersion) [13,37] or *Salmonella typhimurium* (exposure by immersion and microinjection) [35]. For the present work, the study published by O'Toole et al. was used as an example of infection of zebrafish larvae [13]. In the case of GFP-labeled *V. anguillarum*, the fluorescent signal was observed in the larvae after 2 and 6 h of exposure, with a strong signal in the digestive tract and on the skin surface [13]. However, with our GFP-labeled *A. salmonicida*, it was necessary to wait between 24 and 48 h for the fluorescence to be seen clearly, and it was limited to the digestive tract. These results suggest that infection with *V. anguillarum* is faster than with *A. salmonicida* and may involve different pathogenic mechanisms. In the case of exposure by immersion to a wild type strain of *Salmonella typhimurium*, it was observed that the bacteria colonize the gut and there is neutrophil recruitment, but the bacteria are unable to cause mortality after 72 h exposure [35]. This differs with our results, as we do observe high mortality after 72 h exposure to *A. salmonicida*.

There are no previous reports of zebrafish larvae infection studies using siderophore-fluorophore conjugates, such as the AMB-SRB probe. The present work is the first example of using siderophore-based fluorescent probes for this type of studies. The images of larvae exposed to AMB-SRB-labeled *A. salmonicida* showed fluorescence in the digestive tract, although less intense than with the GFP-labeled strain. This is due to the different emission wavelength of the AMB-SRB probe's SRB fluorophore compared to that of GFP. Both probes can detect areas of zebrafish larvae most affected by *A. salmonicida*. The use of siderophore-based fluorescent probes for bacteria labeling has the advantage that it does not require the introduction of reporter genes, such as GFP, in the bacteria. Thus, these probes could be used as well to study other *Aeromonas* spp. strains, like atypical *A. salmonicida*, or other pathogenic bacteria refractory to genetic manipulation. Since *A. salmonicida* is an intracellular pathogen [6], these probes could be also interesting to study the fate of *A. salmonicida* cells inside host cells.

Based on the results obtained here, zebrafish larvae seem to be a promising model for the study of furunculosis and this type of study could be extended to other species of fish. In addition, it should be noted that siderophore-fluorophore type conjugates are also of special interest for the development of new antimicrobials based on the conjugation of siderophore analogs with antibiotics, known as the Trojan Horse strategy [38].

In conclusion, the exposition of zebrafish (*Danio rerio*) with two strains of *A salmonicida* labeled with green fluorescent protein (GFP) or the siderophore amonabactin-sulforhodamine B conjugate (AMB-SRB) display fluorescent signals in the digestive tract. This work represents the first report of using pathogenic bacteria tagged with a siderophore fluorophore conjugate to study the infection in zebrafish.

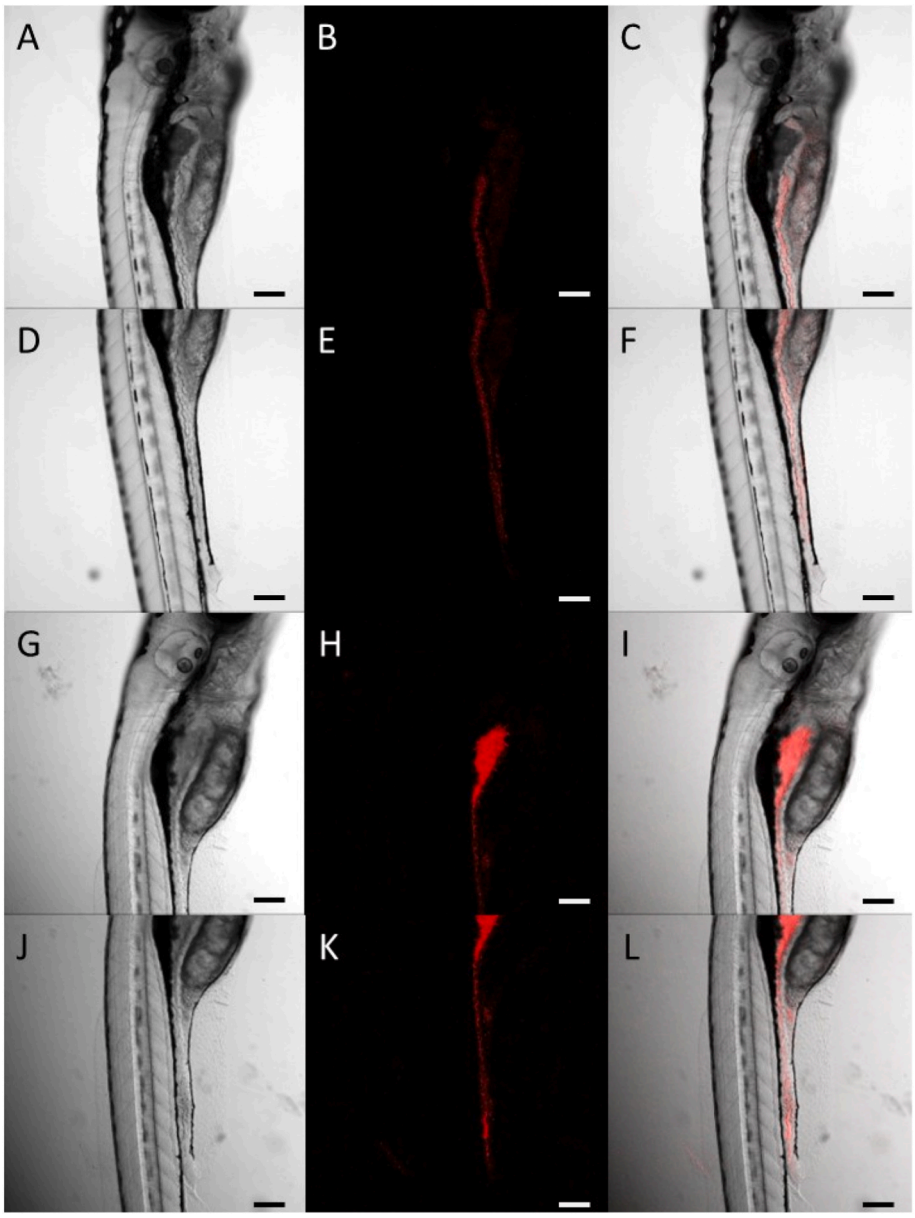

Fig. 4. Visualization of zebrafish larvae (Danio rerio) by confocal microscopy. A–F: Controls. G–L: After 48 h of exposure to AMB-SRB-labeled A. salmonicida. A/D/G/J: bright field. B/E/H/K: Fluorescence. C/F/I/L: Fluorescence and bright field overlay. Lateral views, anterior to the top. Scale bar: 100 μm.

Ethics approval and consent to participate

All experiments performed using zebrafish were in accordance with animal experimentation regulations included in Spanish RD 53/2013 and EU Directive 10/63/EU. The project had been positively evaluated by the Institutional Animal Care and Use Committee of ICARDCFR (Ref. No. DCFR/IACUC/25/01/2021/7) for sampling, maintenance, handling and sacrificing of fishes during experiments.

Consent for publication

All authors have consented to publish this manuscript.

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

A. Rodríguez-Pedrouzo: Writing – original draft, Investigation, Data curation. **J. Cisneros-Sureda:** Resources. **D. Martínez-Matamoros:** Investigation. **D. Rey-Varela:** Investigation. **M. Balado:** Investigation. **J. Rodríguez:** Writing – review & editing, Conceptualization. **M.L. Lemos:** Writing – review & editing, Data curation. **M. Folgueira:** Writing – review & editing, Supervision, Data curation. **C. Jiménez:**

Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing, Supervision, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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