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Salmonella Typhimurium induces cloacitis-like symptoms in zebrafish larvae

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ABSTRACT

Pathogenic *Salmonella* strains have a set of virulence factors allowing them to generate systemic infections and damage in a variety of hosts. Among these factors, bacterial proteins secreted by specialized systems are used to penetrate the host's intestinal mucosa, through the invasion and destruction of specialized epithelial M cells in the intestine. On the other hand, numerous studies have demonstrated that humans, as well as experimental animal hosts, respond to *Salmonella* infection by activating both innate and adaptive immune responses. Here, through live cell imaging of *S.* Typhimurium infection of zebrafish larvae, we showed that besides the intestinal colonization, a deformed cloacae region and a concomitant accumulation of *S.* Typhimurium cells was observed upon bacterial infection. The swelling led to a persistent inflammation of infected larvae, although the infection was non-lethal. The *in vivo* inflammation process was confirmed by the co-localization of GFP-tagged *S.* Typhimurium with mCherry-tagged neutrophils at 72 h post exposition. Our live-cell analyses suggest that *Salmonella* Typhimurium induce cloacitis-like symptoms in zebrafish larvae.

1. Introduction

Salmonella serotypes associated with gastroenteritis coordinate intestinal inflammatory and secretory responses, while serotypes that cause enteric fever establish a systemic infection through their ability to survive and replicate in mononuclear phagocytes [1]. Particularly, *S.* Typhimurium infection of mice offers a well-characterized model for the pathogenesis of human typhoid fever [2], and a wide variety of classical and molecular genetic tools are available for the identification and characterization of potential *Salmonella* virulence factors [3]. Orally ingested bacteria infiltrate the intestinal mucosa and migrate via the lymph nodes to the spleen and liver to cause systemic disease [4]. During bacterial infection, macrophages operate as professional phagocytes and key effectors of the innate and adaptive immune responses [5]. *S.* Typhimurium exploits the phagocytic nature of the macrophages to reside intracellularly where it replicates within specialized vacuoles.

The penetration of the intestinal mucosal barrier is a crucial step in *Salmonella* pathogenesis [6]. Invasive *S.* Typhimurium, introduced into the stomachs of mice, became associated primarily with the lymphoid follicles or with the Peyer's patches of the small intestine rather than with the intestinal wall. After crossing the epithelium of the Peyer's patches, virulent *Salmonella* strains reach an environment populated with host lymphocytes and macrophages. To move into deeper tissues, these bacteria must be able to avoid and/or survive the killing by professional phagocytes following internalization [7,8].

The use of surrogate host models such as the amoeba *Dictyostelium discoideum*, the nematode *Caenorhabditis elegans*, the insect *Drosophila melanogaster*, and the fish *Danio rerio* for studying bacterial pathogenesis in human diseases is growing [9,10]. In particular, zebrafish (*D. rerio*) is a powerful and advantageous vertebrate model for developmental studies due to its small size, transparency, external fertilization and great regeneration capabilities, but now it has been used with success to study human pathogens [11,12]. Importantly, zebrafish possesses both innate and adaptive immune systems, making it a particularly suitable model organism for

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investigating host-pathogen interactions. *In vivo* cell imaging of host-pathogen interactions is fundamental to the study microbial pathogenesis and transgenic zebrafish larvae are increasingly used to study the function of the vertebrate innate immune system during this interaction [11,12]. Furthermore, this model allows to study the innate response separately from adaptive immune functions, since the innate immune system of the zebrafish embryo is capable to resist microbial infections as early as 1 day post-fertilization (dpf), while maturation of the adaptive immune system is completed only after 3 weeks of zebrafish development [13,14]. Our main goal is to established zebrafish as a model for study *Salmonella* infection to discover novel virulence factor and host responses.

Here, using static immersion of 6 dpf zebrafish larvae in a suspension of *S.* Typhimurium cells as a model of infection [15], we found that approximately 20% of the animals showed a deformed cloacae region. By using a transgenic zebrafish line harboring mCherry-labeled neutrophils, we confirmed the occurrence of an *in vivo* inflammation process. To our knowledge this is the first report of *Salmonella* causing a deformed anal region phenotype in animals.

2. Materials and methods

2.1. Bacterial strains and cell conditions

Escherichia coli DH5α, *Salmonella* Typhimurium 14028 strain (wild type) and the Δ*aroA* mutant derivative [16] were grown in 20 mL of Luria-Bertani broth (Triptone 10 g/L, Yeast extract, 5 g/L, NaCl 5 g/L) until stationary growth phase, under aerobic conditions, 37 °C and shaking at 180 rpm. The cells were harvested by centrifugation for 15 min at 6000 rpm at 25 °C. GFP-tagged *Salmonella* strains were obtained by transformation with pDiGc plasmid (Amp^r) [17]. *Escherichia coli* DH5α transformed with pDiGc [18] was used as negative control in live cell imaging experiments.

2.2. Zebrafish husbandry

Zebrafish (*Danio rerio*) embryos were obtained by natural spawning of Tab5 and *Tg*(*BACmpo:mCherry*) lines [19]. Fertilized eggs were raised in petri dishes containing E3 medium (5 mM NaCl,

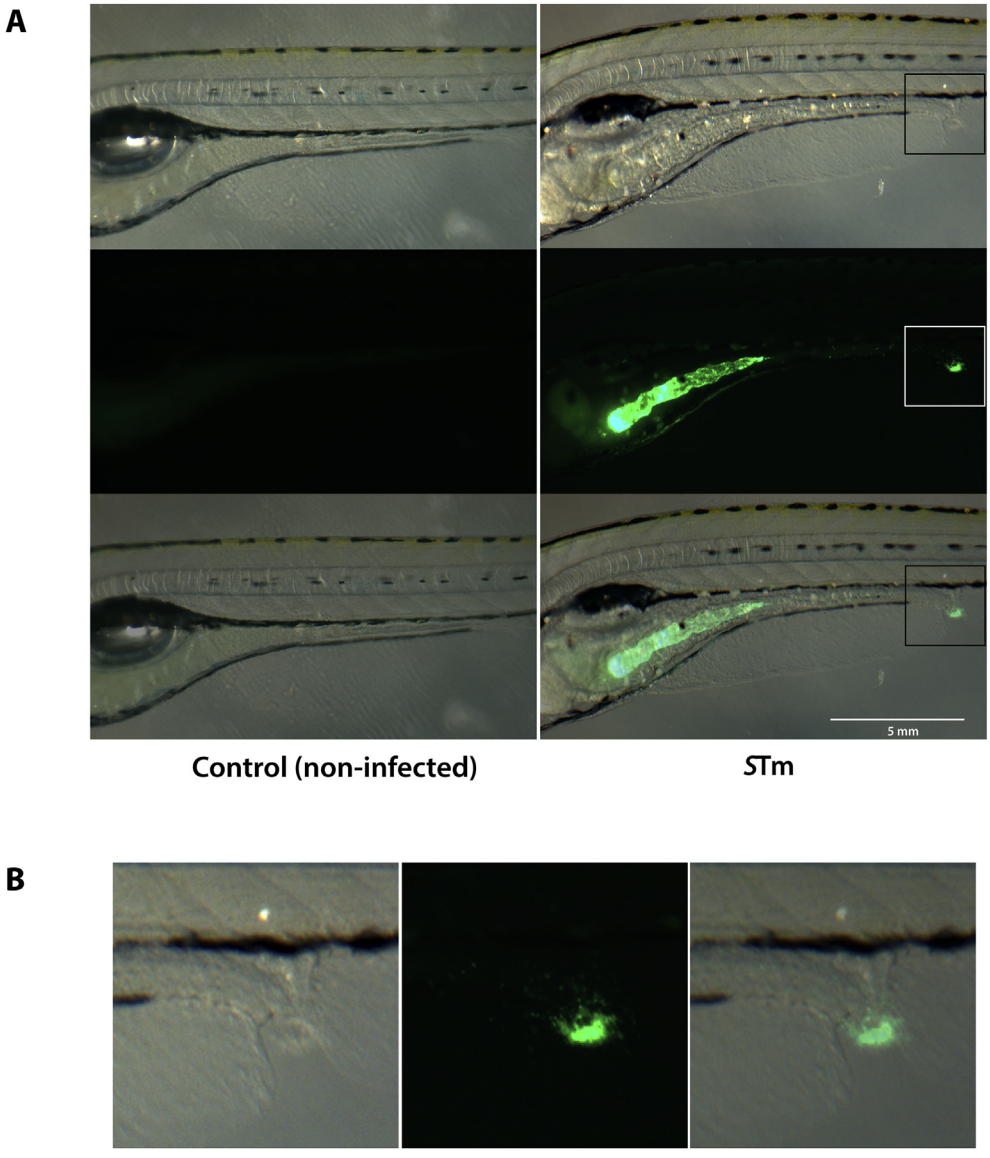

Fig. 1. Live cell imaging of *S.* Typhimurium infection in 9 dpf wild type (Tab5) zebrafish larvae. **(A)** Intestinal and cloacal colonization of GFP-tagged *S.* Typhimurium using the immersion protocol. (B) Zoom of the deformed cloacal region colonized by *S.* Typhimurium.

0.17 mM KCl, 0.33 mM $CaCl_2$, 0.3 mM $MgSO_4$) and 0.1% methylene blue until 6 dpf. All procedures complied with national guidelines of the Animal Use Ethics Committee of the University of Chile and the Bioethics Advisory Committee of Fondecyt-Conicyt (1120209) (the funding agency for this work). Larvae were feed using UV-steriled Vipan staple food (Sera, Denmark).

2.3. Bacterial immersion experiments

Once harvested, bacterial cells were washed and subsequently suspended in filtered and sterile system water (60 mg/L Instant Ocean, Aquarium Systems, Mentor, OH, USA, pH 7.0), adjusting the suspension to an optical density at 600 nm (OD_{600}) of 1.4, equivalent to 0.5-1x10^9 CFU/mL. To determine the CFU/mL, the bacterial suspension was plated on LB-agar supplemented with 100 μg/ml ampicillin. 6-dpf zebrafish were washed with system water and 10 larvae per well were placed in a 6-well plate. Each well was filled with the bacterial suspension to obtain an OD_{600} of 0,7 in a final volume of 8 ml. Zebrafish larvae were immersed in the bacterial suspension simultaneously with the first feeding, and incubated during 24 h. Three replicates in three independent experiments for a total of 90 zebrafish larvae where analyzed in each condition. Then, the larvae were washed with system water and fed every 24 h, and observed after 72 h post-exposition (hpe).

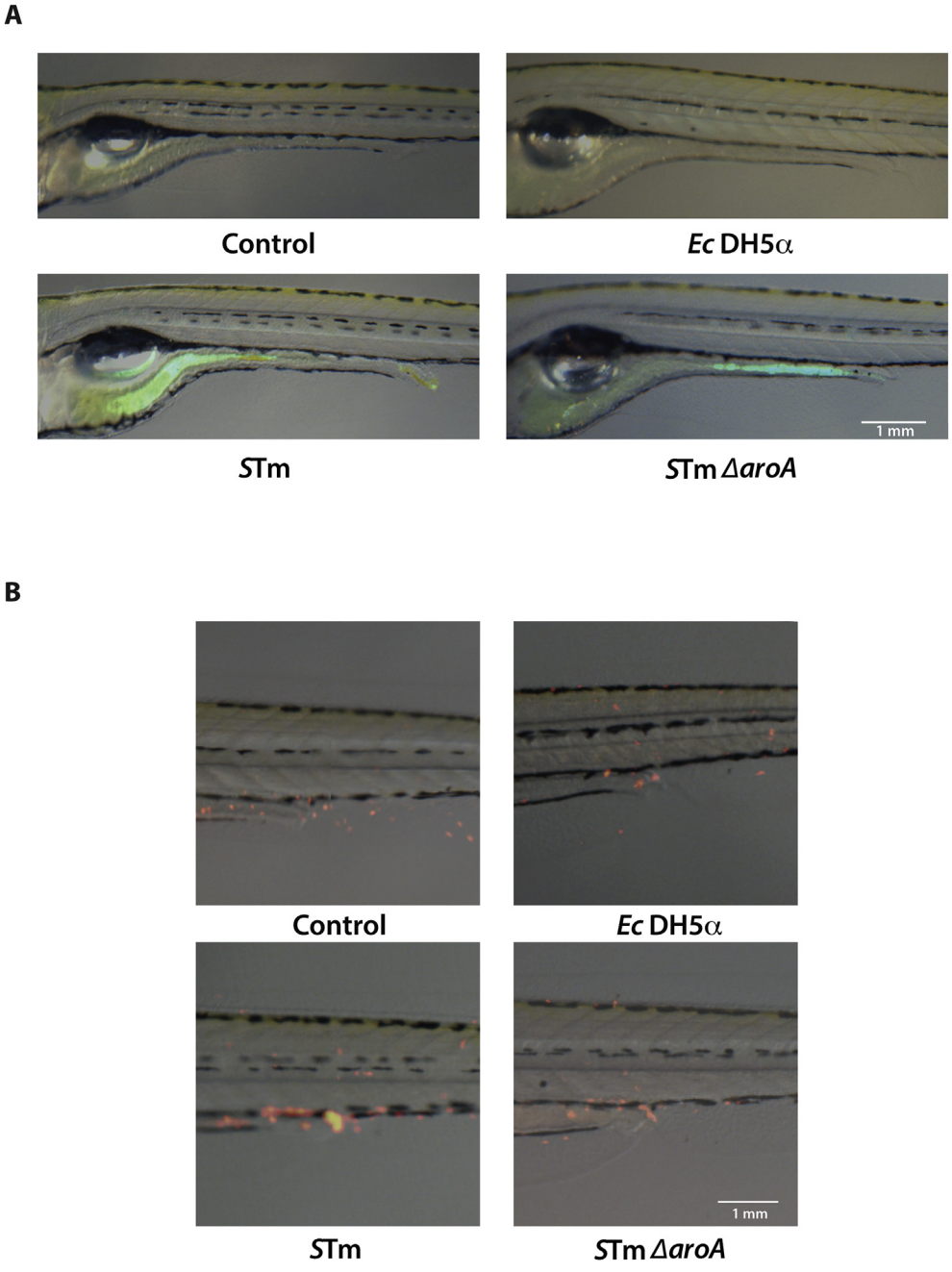

Fig. 2. Live cell imaging of the inflammatory response in 9 dpf zebrafish larvae caused by *S.* Typhimurium infection. (A) Intestinal localization of GFP-tagged *S.* Typhimurium (wild type and Δ*aroA*) or *E. coli* DH5∝ (control) cells in 9 dpf Tab5 zebrafish larvae. **(B)** Co-localization of recruited neutrophils and GFP-tagged *S.* Typhimurium cells (wild type or Δ*aroA*) in 9 dpf transgenic zebrafish larvae (BACmpo:mCherry).

2.4. Live cell imaging of host-pathogen interaction

Infected 9 dpf larvae were anesthetized with 0.01% tricaine for 2 min and mounted into a bed of low melting point agarose (1%). GFP-tagged *Salmonella* localization and i*n vivo* recruitment of neutrophils were observed using an Olympus MVX10 (Japan) fluorescence microscopy at 72 hpe. *Escherichia coli* DH5α was used as negative control for live cell experiments.

3. Results and discussion

Zebrafish larvae are a powerful host model of the innate immune response to infection. The small size and optical transparency of larval zebrafish allow live-cell imaging of host-pathogen interaction experiments [12,20]. Numerous protocols are available adapted to image and manipulate host interactions with several pathogens. However, most of them rely in the microinjection of the bacterial pathogen and few static immersion methods has been described [15,21]. In the present study, using an static immersion protocol with zebrafish larvae as a host model for *S.* Typhimurium infection, we observed that in addition to the expected gut colonization, in 20% of individuals a small patch of bacteria was seen sticking around the cloaca region immediately behind the anus with extensive swelling (Fig. 1). The presence of the deformed anal region (Dar) phenotype in some animals suggests that some host susceptibility would be required for the phenotype. Similar results were observed in *C. elegans* where *Microbacterium nematophilum,* induces a similar morphological change in the nematode [22].

In some animals, the ends of the digestive, urinary and/or reproductive tract combine to form a common chamber and a single opening to the external environment. This structure, called the cloaca or vent, can become infected and inflamed in a condition known as cloacitis. In order to explore signs of bacterial infection and host inflammation in Dar larvae, a transgenic zebrafish line harboring mCherry-labeled neutrophils was used. As shown in Fig. 2, 72 hpe, GFP-tagged *Salmonella* and a zone displaying an intense neutrophil recruitment co-localized in the cloaca region of infected larvae, suggesting the occurrence of a cloacitis-like syndrome.

To further explore the nature of the zebrafish Dar phenotype, a well-characterized attenuated mutant of *S.* Typhimurium (Δ*aroA*) was used [16]. Salmonella Δ*aroA* mutants present strong defects in colonization and intracellular survival [16,23], therefore, we aimed to determine if a Δ*aroA* mutant presents the same Dar phenotype in zebrafish. As shown in Fig. 2 Δ*aroA* mutant strain displayed a different colonization pattern than the wild type strain and was unable to induce the Dar phenotype in zebrafish larvae. Attenuation of Δ*aro* mutants in *Salmonella* is caused not only by their impairment to synthesize aromatic metabolites but also by their deficiency in cell wall and outer membrane functions [16]. This suggest that some of the virulence factors absent in attenuated strain are required to cause this kind of infection, although further experiments will be required to know which bacterial or host factor are important for originating the Dar phenotype. To our knowledge; this is the first report of cloacitis-like symptoms triggered by *Salmonella* infection. Our finding in zebrafish opens the door for exploring the occurrence of *Salmonella* anal infection in other murine or avian host models.

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