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Visualizing and quantifying *Pseudomonas aeruginosa* infection in the hindbrain ventricle of zebrafish using confocal laser scanning microscopy

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ABSTRACT

Pseudomonas aeruginosa colonizes surfaces using a stepwise process that involves several phases, including attachment, production of exopolysaccharides, formation of microcolonies and the eventual development of biofilms. This process has been extensively characterized in vitro using both light and electron microscopic techniques. However, our ability to visualize this process in situ at the site of infection has been limited by the nature of the vertebrate models available. The optically clear zebrafish (*Danio rerio*) is an emerging model well suited for imaging bacterial infections. In this study, we infected the hindbrain ventricle of 54 h post-fertilization zebrafish with *P. aeruginosa* PAO1 and visualized and quantified microcolony formation using confocal laser scanning microscopy and image analyses. In comparison to wildtype PAO1, infection with a *P. aeruginosa* mutant deficient in the ability to produce the exopolysaccharide Psl caused less zebrafish mortality and fewer, smaller microcolonies per zebrafish at both 18 h and 29 h post-infection. The work presented here demonstrates reproducible in situ visualization and quantification methods for determining the extent of *P. aeruginosa* infection in a vertebrate model. We demonstrate how this model system can be manipulated to understand the effect of virulence factors on pathogenicity. Furthermore, this model can be adapted to study biofilm formation in situ, thereby extending our understanding of how bacterial persistence leads to chronic infections.

1. Introduction

Pseudomonas aeruginosa is one of the most important opportunistic human pathogens that can cause life-threatening infections in individuals with compromised immune systems or cystic fibrosis (Høiby, 2011; Hogardt and Heesemann, 2010; Gellatly and Hancock, 2013). While a weakened host defense would be expected to favor infection by a variety of opportunistic pathogens, the dominance and persistence of *P. aeruginosa* in nosocomial settings have led to extensive studies of its pathogenicity (Høiby, 2011; Folkesson et al., 2012). *P. aeruginosa* expresses multiple virulence factors, including adhesins, lipopolysaccharide (LPS), toxins, and hydrolytic proteases, which enhance bacterial attachment to cellular surfaces, induce immune responses and cause tissue damage (Fothergill et al., 2007; Lam et al., 2011; Hahn, 1997). The adaptability of this species to the host environment and its easy transition from acute to chronic infections, characterized by forming biofilms, further enhance its pathogenicity (Folkesson et al., 2012; Gellatly and Hancock, 2013). These biofilms are difficult to eradicate and play a role in *P. aeruginosa* becoming highly resistant to antibiotics (Parsek and Singh, 2003; Folkesson et al., 2012).

In *P. aeruginosa* PAO1, the colonization of cells and formation of biofilms is coupled with the synthesis and secretion of many extracellular polymeric substances (EPS) that are required for attachment of the bacteria to both biotic and abiotic surfaces (Flemming and Wingender, 2010; Folkesson et al., 2012). Among these polymeric substances is the exopolysaccharide Psl (polysaccharide synthesis locus), which has been implicated in initial cell-to-surface and cell-to-cell attachment (Ma et al., 2006, 2007; Byrd et al., 2010; Zhao et al., 2013). A recent study by Byrd et al. found that Psl, in conjunction with flagellin, is required for surface adherence to A549 adenocarcinoma human alveolar basal epithelial cells in vitro (Byrd et al., 2010). The same authors also hypothesized that Psl plays an important role in binding to host cells in a chinchilla middle ear infection model (Byrd et al., 2011), thus highlighting the potential importance of this polysaccharide in promoting *P. aeruginosa* infections.

Much of our understanding about how *P. aeruginosa* colonizes surfaces has been gained through optical microscopy techniques (Watnick and Kolter, 2000; Hall-Stoodley et al., 2004). Using biofilm-cultivation devices with optically clear flow cells, researchers have been able to develop a visual framework for how *P. aeruginosa* PAO1 colonizes abiotic surfaces in real-time. Colonization of abiotic surfaces has been defined as a stepwise process, beginning with surface sampling and reversible attachment by individual bacterial cells. This initial attachment is followed by irreversible attachment aided by the secretion of exopolysaccharides such as Psl (Ma et al., 2007; Colvin et al., 2012; Flemming and Wingender,

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2010). Through cell division and co-aggregation events, these irreversibly attached cells proliferate to form microcolonies and eventually larger macrocolonies (Caiazza and O'Toole, 2004; Watnick and Kolter, 2000; Sauer et al., 2002). While insights into *P. aeruginosa* colonization and biofilm development has been primarily acquired using in vitro methods, the relevance of these results to the complex host environment remains unclear. Numerous studies have investigated this colonization process using more clinically relevant animal models (Dohar et al., 2005; Byrd et al., 2011; Komor et al., 2012; Schaber et al., 2007; Mulcahy et al., 2011; Seth et al., 2012). For example, Dohar et al. used scanning electron microscopy to define *P. aeruginosa* PAO1 aggregates surrounded by exopolysaccharide material in a nonhuman primate model of chronic suppurative otitis media (Dohar et al., 2005). However, these types of in vivo studies require dissection procedures in order to observe the site of infection, which presents obvious obstacles if one wants to use live-cell imaging techniques to monitor the infection process in real-time. Direct and high-resolution visualization of the infection process within a host has therefore been hindered by the lack of a vertebrate infection model with "*flow cell-like*" amenability to detailed microscopic analyses. Detailed in vivo observations at both the light and electron microscopic levels are critical for elucidating the mechanisms of bacterial pathogenesis and for directing research into the next generation of antimicrobials.

Over the past decade, zebrafish (*Danio rerio*) has emerged as a powerful tool to investigate host–microbe interactions (Davis et al., 2002; Deng et al., 2013; van der Sar et al., 2003; Li and Hu, 2012; Toh et al., 2013; van Soest et al., 2011; Phennicie et al., 2010; Clatworthy et al., 2009; Brannon et al., 2009). A zebrafish infection model has many advantages, including abundant egg production yielding large experimental populations, small and optically transparent embryos amenable to whole organism light microscopic and high-throughput analyses, and the presence of a vertebral immune system with well-defined temporal segregation of innate and adaptive immunity development (Clatworthy et al., 2009; Milligan-Myhre et al., 2011). Two major approaches, static immersion and microinjection, have been used to assess zebrafish-microbe interactions (Toh et al., 2013; Li and Hu, 2012; van Soest et al., 2011). Static immersion attempts to mimic the acquisition of a bacterial infection from an aquatic environment by briefly incubating zebrafish embryos in bacterial culture-containing egg water. While static immersion is appealing in its biological relevance, a close examination of host expression responses revealed high variability between individual embryos (van Soest et al., 2011). Furthermore, it is unlikely that *P. aeruginosa* causes infection via static immersion (Milligan-Myhre et al., 2011; Clatworthy et al., 2009), limiting the applicability of this approach for the study of *P. aeruginosa* pathogenicity. *P. aeruginosa* infection pathology by direct injection has been assessed at multiple anatomic sites within zebrafish (Brannon et al., 2009; Clatworthy et al., 2009; Phennicie et al., 2010; Deng et al., 2013). These studies demonstrate that dissemination and lethality of infection in larval zebrafish depends on the site of injection (Fig. 1A). For example, injection into the axial vein (Brannon et al., 2009) or the duct of Cuvier (Phennicie et al., 2010) results in a lethal systemic infection. In contrast, injection of bacteria into compartments such as the hindbrain ventricle (Li and Hu, 2012; Phennicie et al., 2010) (Fig. 1B) or pericardial cavity (Li and Hu, 2012) results in a more localized infection with reduced mortality.

In this study we develop a reproducible protocol for studying *P. aeruginosa* infection and microcolony formation within the hindbrain ventricle of zebrafish and use confocal laser scanning microscopy to monitor the processes. We demonstrate that this combination of the zebrafish model and confocal microscopy approach is an excellent system for obtaining visual information on microcolony formation within a live vertebrate host. In addition, using a *P. aeruginosa* mutant strain incapable of producing the exopolysaccharide Psl, we demonstrate the utility of this system to explore the role of virulence factors in colonization and immune evasion. This model is quantitative, and can be used to determine if decreased zebrafish survival correlates with increased bacterial proliferation within the host, or may be caused by other factors, such as increased bacterial persistence due to the success of the bacteria in evading the host's immune response. This system also sets the stage for studying chronic biofilm-mediated infections and can be adapted for live-cell imaging applications.

2. Materials and methods

2.1. Bacterial strains, growth conditions and inoculum preparation

P. aeruginosa PAO1 (Hancock and Carey, 1979) and WFPA800 (PAO1 with an in-frame *psl* promoter deletion, referred to hereafter as PAO1-Δ*psl*) (Ma et al., 2006) were grown in liquid cultures in lysogeny broth (LB, also called Luria Bertani broth; Becton Dickinson, Mississauga, ON). All liquid cultures were incubated at 37 °C in a rotary shaker at 200 rpm, unless stated otherwise. A single colony was first inoculated into 5 mL of LB and incubated for 16 h. To obtain log-phase bacteria for injection into zebrafish, 50 μL of overnight culture was inoculated into 25 mL of fresh LB, incubated for 3 h, and then placed at room temperature for 1 h. Up to 12 mL of culture was pelleted by centrifugation at 1500 ×*g* for 5 min at room temperature, and the pellet was resuspended with a 26-gauge needle (five up-and-down sequences) in 1 mL of standard phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, and a pH of 7.4). The suspension of cells was then further diluted in PBS to achieve the desired bacterial density (OD_{600} of 0.2 and 1.0 for 100 CFU of Δ*psl* and wt PAO1 strains, respectively). The resuspended inoculum was visualized using light microscopy to ensure the inoculum conditioning method limited injection of pre-formed bacterial aggregates. Approximately 2–3 μL of inoculum was spotted onto a glass microscope slide, a coverslip was added, and the suspension was imaged using a Leica DM2000 LED upright light microscope with a 40× phase objective (Leica Microsystems Inc., Concord, Ontario). No large bacterial aggregates (>5 cells) and only a few small bacterial aggregates (<3–4 cells) were observed. To heat-kill bacteria, 100 μL of diluted culture was placed in an Eppendorf tube and submerged in a boiling-water bath for 45 min. The final inoculum was prepared by adding 8 μL of diluted culture to 2 μL of 0.5% phenol red in PBS, of which ~0.5 nL was injected into the hindbrain ventricle of the zebrafish (see below for control experiments).

2.2. Zebrafish maintenance and microinjections

All zebrafish experiments were performed in accordance to the Canadian Animal Care Committee and the University of Guelph Animal Care Committee (AUP# 1530). Zebrafish eggs from different females were collected and pooled in Falcon 3004 Petri dishes (Thermo Fisher Scientific Inc., Waltham, Massachusetts) containing egg water (0.6 g/L aquarium salt) with 0.01% methylene blue anti-fungal agent (Sigma-Aldrich, St. Louis, Missouri) and incubated at 28 °C. At 23 h post-fertilization (hpf) (28 somite stage), the embryos were placed in egg water containing 100 μM 1-phenyl 2-thiourea (PTU; Acros Organics, Geel, Belgium) to inhibit pigmentation (Karlsson et al., 2001). Dechorionation was performed manually at 32 hpf as previously described (Knox et al., 2014).

Micropipette needles were prepared by pulling 100 mm thin-wall glass capillaries (World Precision Instruments, Sarasota, Florida) using a Model P-1000 Flaming/Brown micropipette puller (Stutter Instrument, Novato, California) with the following settings: Heat 525, pull 65, velocity 25, time 50, and pressure 500 (all arbitrary units, based on the manufacturer's instructions). The inoculum was agitated by Vortex for 10 s prior to loading 3 μL into a micropipette needle. Needles were clipped approximately 1.0 mm from the closed end with forceps positioned perpendicular to the needle so as to produce a beveled end. The inoculum-filled needle was then fixed to a micro-injection PV820 Pneumatic PicoPump (World Precision Instruments, Sarasota, Florida), maneuvered into a 10 μL droplet of Halocarbon oil 27 (Sigma-Aldrich, St. Louis, Missouri) atop a 1.0 mm/0.01 mm scale micrometer slide.