Zebrafish Embryo Model of *Bartonella henselae* Infection

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Abstract

Bartonella henselae (*Bh*) is an emerging zoonotic pathogen that has been associated with a variety of human diseases, including bacillary angiomatosis that is characterized by vasoproliferative tumor-like lesions on the skin of some immunosuppressed individuals. The study of *Bh* pathogenesis has been limited to *in vitro* cell culture systems due to the lack of an animal model. Therefore, we wanted to investigate whether the zebrafish embryo could be used to model human infection with *Bh*. Our data showed that *Tg(fli1:egfp)^{y1}* zebrafish embryos supported a sustained *Bh* infection for 7 days with >10-fold bacterial replication when inoculated in the yolk sac. We showed that *Bh* recruited phagocytes to the site of infection in the *Tg(mpx:GFP)^{uwml}* embryos. Infected embryos showed evidence of a *Bh*-induced angiogenic phenotype and an increase in the expression of genes encoding pro-inflammatory factors and pro-angiogenic markers. However, infection of zebrafish embryos with a deletion mutant in the major adhesin (BadA) resulted in little or no bacterial replication and a diminished host response, providing the first evidence that BadA is critical for *in vivo* infection. Thus, the zebrafish embryo provides the first practical model of *Bh* infection that will facilitate efforts to identify virulence factors and define molecular mechanisms of *Bh* pathogenesis.

Introduction

THE GENUS *BARTONELLA* includes fastidious, Gram-negative, facultative intracellular bacteria that infect a wide range of hosts.^{1,2} While humans are the reservoir hosts for *Bartonella bacilliformis* and *Bartonella quintana*, cats are the natural host for *Bartonella henselae* (*Bh*) in which the bacteria cause an asymptomatic intraerythrocytic infection. The prevalence of *Bh* infection in cats ranges from 25% to as high as 54%, and infected cats can be bacteremic for several months to years.^{3,4} The organism can be transmitted between cats through cat fleas (*Ctenocephalides felis*), and from infected cats to humans by bites and scratches. Patients who suffer from cat scratch disease (CSD), which is a self-limiting infection, are typically immunocompetent. Centers for Disease Control and Prevention (CDC) estimated that 22,000–24,000 cases of CSD are diagnosed yearly, mainly in children, in the United States. Although CSD is considered a self-limiting infection, more than 10% of those cases require hospitalization and if not treated, the bacteria can disseminate to other organs such as the liver, the brain, and the heart, leading to more serious complications. In contrast, immunosuppressed individuals infected with *Bh* can develop a more systemic infection characterized by vasoproliferative tumor-like lesions called bacillary angiomatosis (BA) and bacillary peliosis (BP) on

the skin and liver, respectively.^{1,5} These lesions are a result of proliferation of vascular endothelial cells in close association with aggregates of *Bh* found at the site of infection.⁶ The exact incidence of BA and BP is not known; however, cases have been reported in all 50 states in the United States.

For the past decade, *Bartonella* research has been advancing at a fast pace, due, in part, to the availability of the complete genome sequence for *Bh*.⁷ This has enabled the establishment of gene function and the definition of important virulence factors associated with *Bh*-induced pathogenesis for a further *in vitro* study. Among these is the *Bartonella* adhesin A (BadA), which belongs to the class of trimeric autotransporter adhesins along with *Yersinia enterocolitica* (YadA) and *Neisseria meningitidis* (NadA). This group of proteins represents important virulence factors of Gram-negative bacteria in the form of an extracellular filament consisting of head and stalk domains assembled on a C-terminal membrane anchor domain.^{8,9} *In vitro* studies of *Bh* infection have shown that BadA is critical for adhesion to host cells and extracellular matrix proteins such as fibronectin and collagens, as well as for inhibition of phagocytosis and induction of angiogenesis.¹⁰ The expression of BadA has been shown to correlate with a pro-angiogenic cell response via activation of hypoxia-inducible factor-1 alpha (HIF-1 α) and the subsequent secretion of the pro-angiogenic vascular

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endothelial growth factor (VEGF).^{10–13} Substantial knowledge has been acquired about *Bh*-host interactions through the use of cell culture models. However, due to the lack of a practical animal model, the role of BadA and other virulence determinants in causing the unique angiogenic host response seen in humans has not been reproduced *in vivo*.

The efforts to establish a suitable animal model of *Bh* infection have met with limited success. For instance, cats infected with *Bh* do not exhibit symptoms such as lymphadenopathy and vasoproliferation as seen in humans infected with *Bh*.¹⁴ A primate rhesus macaque (*Macaca mulatta*) animal model was successfully used to study human infection with *B. quintana*,¹⁵ but use of this model system for *Bh* has not been reported. Moreover, efforts were made to infect C57BL/6 mice as well as BALB/c mice by inoculating 10⁷–10⁸ *Bh* colony-forming units (CFUs) intraperitoneally or subcutaneously. However, both strains of mice were shown to clear the bacteria rapidly after inoculation with no increase in bacterial burden, the mechanism by which the bacteria were cleared was not fully elucidated.^{16,17} For those reasons, we wanted to investigate whether the zebrafish embryo model could be used to study *Bh* pathogenesis and response to infection.

For decades, the zebrafish embryo (*Danio rerio*) model has been an invaluable tool for genetic and developmental studies.^{18,19} More recently, the use of zebrafish embryos was extended to study bacterial pathogenesis and tumor angiogenesis.^{20–22} Zebrafish embryos are translucent during the first weeks postfertilization, making them suitable for microscopy and enabling real-time analysis of bacterial infection.²⁰ Their *ex utero* development enables access to embryos at all stages of development, making postfertilization genetic manipulation of zebrafish embryos much easier. Importantly, the zebrafish immune system shares similarities with the human system, having both innate and adaptive immune systems featuring the myeloid and lymphoid cells such as macrophages, neutrophils, dendritic cells, T cells, and B cells.^{23–25} Moreover, since the adaptive immune system is not fully functional until 4 weeks postfertilization, it is possible to study the innate immune response to infection independent of the adaptive immune response.

In this study, we developed a zebrafish embryo model of *Bh* infection and host response. We investigated whether the zebrafish embryo *Tg(fli1:egfp)*^{y1} transgenic line could become infected with *Bh* Houston-1 by microinjection. In this transgenic line, green fluorescence protein is driven by the FLi1 promoter, which is constitutively expressed in the vasculature. Live imaging by confocal microscopy was used to monitor *Bh* infection of embryos in real time. The infection pattern was monitored by plating embryo homogenates to determine viable bacteria (CFUs) as well as quantitative polymerase chain reaction (qPCR) at different time points. The response to *Bh* infection in the zebrafish embryo was assessed phenotypically by microscopic imaging and confirmed by microarray and quantitative real-time PCR (qRT-PCR) of select genes. We found that *Bh* can mount a nonlethal infection in the zebrafish embryo, and the bacteria persist in the infected embryos through the duration of the experiment (1 week) with bacterial burden peaking between day 3 and 4 postinfection. We also found that *Bh* infection induces a pro-angiogenic response as well as a pro-inflammatory response involving the migration of phagocytic cells and the expression of pro-inflammatory and pro-angiogenic markers.

Moreover, by using a deletion mutant, BadA was shown to play a role in *Bh* replication and host response in the embryo. The application of this model should prove valuable in studies aimed at broadening our understanding of the mechanisms of *Bartonella* pathogenesis in an *in vivo* environment.

Materials and Methods

Bacterial strains and growth conditions

An in-frame deletion mutant of the full-length *badA* (BH01510) was constructed in *Bh* Houston-1 using the two-step mutagenesis strategy described by Mackichan *et al.* with some modifications.²⁶ Briefly, *Bh* genomic DNA was used as a template for PCR to generate two fragments of the gene. The first fragment contained an upstream noncoding region and included a small segment of the 5' part of the gene, and the second fragment contained the 3' coding region of the gene and the downstream noncoding region. The two purified PCR products were used as templates for mega prime PCR using only the forward primer from fragment 1 and the reverse primer from fragment 2. The resulting product was ligated into the "suicide" plasmid pJM05 at the *Bam*HI restriction site, propagated in *Escherichia coli* DH12S, and then incorporated into *Bh* Houston-1 by transconjugation.²⁶ Transconjugates were selected by plating on Heart Infusion agar with 5% rabbit blood supplemented with kanamycin (30 µg/mL) and then counter-selected on agar containing 10% sucrose to select for a second cross-over event, resulting in replacement of the full-length gene with the truncated version. The deletion mutant was verified by sequencing the amplicon from the deleted region and by RT-PCR to ensure the absence of the *badA* mRNA.

The plasmid vector pDsRed2 (Clontech) was used as a template to amplify the *Discosoma* sp. *dsred2* gene for cloning into pNS2T5 vector containing a kanamycin resistance gene.²⁷ The resulting plasmid pNS2T5-DsRed2 was electroporated into *Bh* Houston-1 wild-type (WT) and the Δ *badA* mutant making red fluorescent protein (RFP)-expressing strains *Bh*/pNS2T5DsRed2 and *Bh* Δ *badA*/pNS2T5DsRed2, respectively.

A chromosome knock-in strain of *Bh* Houston-1 expressing DsRed2 (*Bh*::DsRed2) was constructed by cloning the T5 promoter-*dsred2* into EZ-Tn5 pMOD-6<Kan-2/MCS transposon construction vector (Epicentre). The KanT5 DsRed transposon DNA was amplified by PCR, and the stable transposome was prepared with the transposon DNA and the transposase enzyme according to the manufacturer's protocol and electroporated into *Bh* Houston-1. After kanamycin selection, transposon knock-in strains were identified by confocal microscopy as those expressing RFP and confirmed by inverse PCR and sequencing out from the DsRed2 transposon. It was determined that the knock-in strain selected for further use had the transposon introduced in an intergenic region upstream of BH14030 and downstream of BH14040. No difference was observed in the in-growth curves between the chromosomally inserted DsRed2 or the plasmid encoded DsRed2 *Bh* Houston-1 strains compared with WT *Bh* (data not shown).

The DsRed2 labeled kanamycin-resistant *Bh* strains were cultured on heart infusion agar supplemented with 1% bovine hemoglobin (chocolate agar) and 50 µg/mL of kanamycin in Schneider's liquid media (Sigma) supplemented with 10%

fetal bovine serum and 50 $\mu\text{g/mL}$ of kanamycin for 3–4 days at 37°C in the presence of 5% CO_2 .²⁸ *E. coli* carrying the pNS2T5DsRed2 plasmid was grown in Luria-Bertani (LB) broth supplemented with 50 $\mu\text{g/mL}$ of kanamycin overnight at 37°C. All manipulations of *Bh* have been approved by the USF Institutional Biosafety Committee.

Animal care and strains

WT (AB) adult zebrafish and *Tg(mpx:GFP)uwm1* embryos were purchased from Zebrafish International Resource Center (Eugene Oregon). In this strain, GFP is highly expressed in the neutrophils, but at a lower level of expression in a subpopulation of the monocyte/macrophage lineage, under the control of the myeloid-specific peroxidase (mpx) gene promoter.^{29–31} Adult breeders of the transgenic line *Tg(fli1:egfp)*³¹ were a kind gift from Dr. Brandt Weinstein (NCI, Rockville, MD). Zebrafish breeders were maintained at 28°C $\pm$ 0.5°C in a 14 h:10 h light/dark cycle in our breeding system designed by Aquatic Habitats in accordance with standards established in the “Zebrafish Book”.³² Adult breeders were mated and eggs were collected and maintained at 30°C in embryo water, supplemented with 50 $\mu\text{g/mL}$ of kanamycin to help prevent contamination, for approximately 1 week.

Ethics statement

This study was performed in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Zebrafish embryos, and adults were maintained in accordance with standards established in the “Zebrafish Book,”³² and by the guidelines outlined by the Institutional Animal Care and Use Committee (IACUC) at the University of South Florida.

Zebrafish embryo staging and microinjection

Before microinjection, embryos were staged at 24–28 hours postfertilization (hpf) and manually dechorionated using two pulled-glass needles under a dissecting scope (Nikon). Embryos were anesthetized in 0.02% tricaine methanesulfonate (Sigma) in embryo water.³² Microinjection needles were pulled from 1.0-mm borosilicate glass micropipette using the PC-10 vertical puller (Narishige).

Bh and *E. coli* cultures were pelleted by centrifugation at 3000 *g* for 5 min and resuspended in 0.05% phenol red in phosphate-buffered saline (PBS, pH 7.2) to help monitor the microinjected solution. The suspension was diluted and plated to determine the number of CFUs to quantify the number of viable bacteria. All *Bh* plates were incubated at 37°C with 5% CO_2 for 10 days; *E. coli* were plated on LB agar and incubated overnight at 37°C. For microinjection, anesthetized embryos were placed into a holding groove in 3% agarose to immobilize them for microinjection. Embryos were microinjected in the yolk sac or the blood circulation using thin pulled-glass needles attached to a Kite micromanipulator (WPI), a PV830 pneumatic pump microinjector (WPI), and an SMZ 1500 dissecting microscope (Nikon). The inoculum was collected during and after microinjection and plated for CFU enumeration or saved for qPCR experiments. For angiogenic response experiments, positive control embryos were injected with 10 ng of recombinant zebrafish VEGF (rzfVEGF) (R&D Systems).

Digital imaging and microscopy of zebrafish embryos

To monitor bacterial infection and host interaction in real time, embryos from the experimental and control groups were anesthetized and suspended in a depression slide in 0.05% agarose/embryo water. Live embryos were examined with a 20 $\times$ LUC Plan FLN 0.45 N.A. objective using an Olympus FV1000 confocal laser scanning microscope (Olympus America). EGFP vasculature of the embryo and DsRed2-expressing bacteria were imaged by 488 nm laser with 475–519 nm spectral emission setting and 543 nm laser with 525–615 nm emission, respectively. For time-lapse imaging, live embryos were viewed with a 20 $\times$ UPLSAPO 0.75 NA, WD 0.65 mm objective using an Olympus IX81 inverted microscope equipped with the 3i Yokogawa spinning disk scanner and CDD cameras. Some pictures were viewed using the FluorView 10-ASW 1.7 and analyzed by using the NIH ImageJ software. Others were analyzed using the SlideBook 5.5 software and the SMRecorder software to make three-dimensional (3D) surface view videos showing the interaction of the RFP-expressing bacteria and the GFP-expressing endothelial cells. Adobe Photoshop CS5.1 software was subsequently used to arrange and format the images, and all micrographs are reported as projected z-series.

Bacterial enumeration from zebrafish embryos

Bh infection and viability in the zebrafish embryo host were determined at different time points after infection. Three embryos from each of the infected and control groups were washed individually thrice with 1 mL 1 $\times$ sterile PBS and transferred to 1.5 mL tubes. Whole embryo tissues were disrupted in 1 mL of 0.1% saponin/PBS solution using sterile disposable plastic pestles. The suspension was serially diluted and plated on chocolate agar plates containing 50 $\mu\text{g/mL}$ of kanamycin. The plates were incubated at 37°C with 5% CO_2 for 10 days to determine the number of CFUs. The means of triplicate samples were used for a comparison between experimental and control groups at different time points.

Genomic DNA extraction and qPCR

Infected and control zebrafish embryos were individually stored at -80°C at different time points for genomic DNA extraction. Embryos were homogenized, and total DNA was extracted using the Wizard Genomic DNA Purification Kit according to the manufacturer's instructions (Promega). qPCRs were run in 25 μL volume reactions containing 12.5 μL of 2 $\times$ iQSYBR Green Supermix, 300 nmol of each primer designed from the *Bh* NADH dehydrogenase gamma subunit G gene (*nuoG*) (BH08890) (Table 1), and 5 μL of DNA (total DNA including zebrafish and bacteria DNA) extracted from embryo homogenate. Cycling parameters were as follows: 95°C for 3 min, followed by 40 cycles of 60°C for 30 s and 55°C for 1 min. A melting curve analysis was performed to confirm that no primer dimers were generated. All samples were run in triplicate, and the average genome equivalent (GE) for 5 embryos was calculated by comparison to a standard curve constructed by using known amounts of *Bh* genomic DNA as a template for qPCR. The GE per microliter/template was calculated by dividing the concentration ($\text{g}/\mu\text{L}$) of the bacterial DNA by the molecular weight of *Bh* genome (2.08×10^{-15} g/copy), enabling the conversion of RT-PCR cycle threshold values to *Bh* genome copies per embryo.³³

TABLE 1. PRIMERS USED IN THIS STUDY

Genes	Sequence (5'-3')	Source
<i>nuoG</i>	For GCGGTGATTGTTCTCGTTA Rev CACGACCACGGCTATCAAT	33
<i>il-1b</i>	For ATCAAACCCCAATCCACAGAGT Rev GGCACCTGAAGACACCACGTT	68
<i>il-8</i>	For TGTTCCTCTGGCATTCTGACC Rev TTTACAGTGTGGGCTTGGAGGG	68
<i>Vegf</i>	For TGCTCCTGCAAATTCACACAA Rev ATCTTGGCTTTTCACATCTGCAA	69
<i>flk1</i>	For CACAAGAAGTCCAGCGATCA Rev CAGGGGACCACAAAATATGG	70
<i>angptl2</i>	For GGTCATGGATGTTCTTCAC Rev GTCCTGCTCAGTGGAAAGGTG	71
<i>eflα</i>	For AGAAGGAAGCCGCTGAGATG Rev TGTCCAGGGGCATCAATAAT	72
<i>rpl13a</i>	For TCTGGAGGACTGTAAGAGGTATGC Rev AGACGCACAATCTTGAGAGCAG	73

RNA isolation

Pools of 10 embryos were stored at -80°C for RNA extraction and purification as described by Leung *et al.*³⁴ Briefly, frozen embryos were homogenized with Trizol Reagent (Invitrogen) using an RNase-free Kontes pellet pestle (Fisher Scientific) and a QIAshredder column (Qiagen). Subsequently, total RNA was extracted twice with chloroform in Heavy Phage Lock Gel tubes (Fisher Scientific) and further purified using the RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer's instructions. The RNA was eluted using diethylpyrocarbonate-treated water and subsequently treated with Turbo DNase (Ambion) according to the manufacturer's protocol. The yield and the integrity of the extracted total RNA was evaluated by measuring the ultraviolet absorbance using the ND-1000 nanodrop spectrophotometer (Thermo Scientific) and by agarose gel electrophoresis.

Microarray

The microarray experiments and data analysis were performed in the Moffitt Cancer Center and Research Institute Molecular Genomics Core Facility. RNA from pools of *Bh*-infected and control embryos was isolated as described earlier. The resulting RNA (100 ng) was used as a template to generate amplified complementary DNA (cDNA) according to the manufacturer's instructions (NuGEN Technologies). The cDNA was labeled using the FL-Ovation™ cDNA Biotin Module V2 (NuGEN). The hybridization mixture was prepared to accommodate 5 μg of cDNA target. The Zebrafish Gene 1.0 ST Arrays were hybridized, washed, and stained according to the Affymetrix protocol. GeneChips were scanned using a GCS3000 7G scanner (Affymetrix), and images (DAT files) were converted to CEL files using AGCC software (Affymetrix). Robust multi-array average (RMA) data analysis was performed using expression console V1.2 (Affymetrix). Differential gene expression analysis was performed using Excel.

cDNA synthesis and qRT-PCR

The DNase-treated RNA (1 μg) was reverse transcribed into cDNA in a 20 μL reaction using the iScript cDNA Synthesis Kit (Bio-RAD). RT-PCR was performed using the

iCycler IQ real-time PCR detection system (Bio-Rad). Each reaction was performed in a 25 μL volume containing 100 ng of cDNA, 12.5 μL of 2 $\times$ iQSYBR Green Supermix (Bio-Rad), and 300 nmol of each primer. All reactions were performed in triplicate, and zebrafish elongation factor-1 (*efl1 α*) or ribosomal protein L13 (*rpl13*) was used as an endogenous gene for normalization (Table 1). Cycling parameters were 95°C for 3 min, followed by 40 cycles of 60°C for 30 s and 55°C for 1 min. Melting curve analysis was performed to confirm that no primer dimers were generated. Results were analyzed using the comparative C_T method ($2^{-\Delta\Delta C_T}$).³⁵

Statistical analysis

The SigmaPlot 11.0 software was used to graph and statistically analyze the data. One-way repeated measures of analysis of variance test was used to assess the significance in the increase in bacterial burden at different time points post-infection. Student's *t*-test was used to determine the angiogenic phenotype differences in the mean values among the infected and the control groups to show statistically significant differences at the intersection points (IPs) and the number and length of the subintestinal vessel (SIV). The Mann-Whitney Rank-Sum Test was used to analyze the difference in the infectivity of *Bh* WT compared to the $\Delta badA$ mutant. The error bars represent the standard error of the mean.

Results

Conditions for zebrafish embryo infection with *Bh*

In most zebrafish infectious disease models, infection is achieved by microinjecting the microbe into the embryo. However, zebrafish infection by immersion has been achieved by some natural fish pathogens as is the case for *Edwardsiella tarda*.³⁶ We attempted to infect dechorionated zebrafish embryos at 24 hpf by static immersion with 10^8 CFU/mL of *Bh* Houston-1 (WT) carrying a plasmid expressing RFP (*Bh/pNS2T5DsRed2*) for 16 h. Results from confocal microscopy and CFU counts from homogenate plating of embryos were negative for *Bh* (data not shown). Therefore, we used a microinjection technique as the means of introducing the bacteria into the embryos. Since zebrafish optimal temperature is 28.5°C and *Bh* are exposed to 37°C in the human host, we assessed the infection pattern in infected embryos maintained at constant temperatures of 28.5°C versus those that are maintained at 30°C or 33°C . No significant difference in infection pattern was observed, and the proper development of the embryos did not seem to be compromised (data not shown). Growth of *Bh* in liquid culture medium showed that the bacteria grow faster at 37°C compared with 30°C , although the bacteria seem to reach the same density at day 7 (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/zeb). For all subsequent experiments, embryos were maintained at 30°C to enable sufficient bacterial growth and maintain the health of the embryos.

For most bacteria studied using the zebrafish embryo model, infection was achieved by a microinjection either into the blood circulation (using the posterior blood island, the caudal vein, and the yolk sac circulating valley as injection sites) or into the yolk sac (Fig. 1A).^{37–39} In an effort to determine the optimal infection route, *Tg(fli1:egfp)^{y1}* transgenic embryos were staged at 28 hpf and microinjected into either the yolk sac or the blood

FIG. 1. Infection of *Tg(fli1:egfp)^{y1}* zebrafish embryos with *Bh*. (A) Schematic representation of the zebrafish embryo highlighting the possible sites of injection (not drawn to scale). (B) Representative micrographs of embryos inoculated in the yolk sac circulation valley (row 1) or the yolk sac (row 2) at 28 hours postfertilization (hpf) with an average of 3×10^3 colony-forming unit (CFU) *Bh*::DsRed2. The same embryos were photographed by confocal microscopy at the indicated time points. Scale bar = 100 μ m. (C) Confocal imaging of embryos inoculated with 3×10^3 genome equivalents (GEs) of *Bh*::DsRed2 (row 1) or phosphate-buffered saline (PBS) phenol red control (row 2) by a microinjection into the yolk sac. All images shown are representatives of the pool of embryos photographed. Scale bar = 100 μ m. (D) Average CFUs ($\pm$ SEM) from homogenate plating of embryos inoculated with $\sim 5 \times 10^3$ CFUs of *Bh*. Three embryos from infected and control groups were homogenized individually at the indicated time points and plated in triplicate. (E) Embryos microinjected with $\sim 3 \times 10^3$ GEs of *Bh* at 28 hpf were homogenized; the bacterial nucleic acid was extracted, and the GE/embryo was determined by quantitative polymerase chain reaction (qPCR). Values are the means ($\pm$ SEM) of triplicate wells for five different embryos at the indicated time points. A significant increase in bacteria burden was observed from day 0 to 2 with $**p=0.013$, from day 0 to 3 with $*p=0.02$, and from day 0 to 4 with $***p=0.002$. As expected, the *nuoG* sequence was not amplified from uninfected control embryos. Color images available online at www.liebertpub.com/zeb

circulation valley with $\sim 3 \times 10^3$ CFU of *Bh* Houston-1 (WT) expressing RFP from a single transposome inserted into the chromosome (*Bh*::DsRed2). Bacteria injected in the blood circulation were cleared by day 2 postinjection (dpi) as shown by the DsRed2 signal, whereas those inoculated in the yolk sac near the developing SIVs replicated and persisted throughout the duration of the experiment (3 dpi) (Fig. 1B). For subsequent experiments, all embryos were inoculated in the yolk sac.

Bh survival in the zebrafish embryo host

In order to assess *Bh* infection and viability in the zebrafish embryo host, *Tg(fli1:egfp)^{y1}* transgenic embryos were staged at 28 hpf and microinjected in the yolk sac near the developing SIVs with 3×10^3 GEs of *Bh*::DsRed2. Microscopy results showed an increase in bacterial burden with peak fluorescence observed on 3 dpi and persistence through the entire duration of the experiment (6 dpi) (Fig. 1C). To determine the viability of the bacteria in the host, embryos inoculated with $\sim 5 \times 10^3$ CFUs or PBS phenol red control were homogenized individually and plated on selective kanamycin chocolate agar plates (50 μ g/mL) at different time points postinfection for bacterial enumeration. Results

showed an increase in CFU count, which peaked at 3 dpi and started decreasing at 4 dpi (Fig. 1D). An alternative qPCR method using primers specific to the *Bh* NADH dehydrogenase subunit G gene (*nuoG*, BH08890) was used to determine GE/embryo. Embryos inoculated with 3×10^3 *nuoG* GEs mirrored the infection pattern observed by confocal microscopy, and homogenate plating with bacterial burden increased by almost 10-fold by day 3 ($*p=0.02$) and persisted in the embryos for 8 days (Fig. 1E). The limit of detection of the assay was ~ 10 copies of *nuoG* per reaction, and the sequence was not amplified from the uninfected control embryos (data not shown).

Bh interaction with zebrafish embryo vascular endothelium

The location of the bacteria in relationship to the vascular endothelial cells (EC) in infected *Tg(fli1:egfp)^{y1}* embryos was determined by confocal microscopy (Fig. 2). Microscopy evidence showed that some of the red fluorescent *Bh* are colocalized with the green fluorescent angioblasts, the precursor of vascular endothelial cells, at 1 dpi (Fig. 2B), and with the mature vascular endothelium at 2 dpi (Fig. 2C) as

FIG. 2. Interaction of *Bh* with GFP-labeled endothelial cells in *Tg(fli1:egfp)^{y1}* zebrafish embryo microinjected at 28 hpf. (A) Noninfected control embryo at day 1 postinoculation with PBS/phenol red. (B and C) Micrographs of the same infected embryo at day 1 and 2 postinoculation, respectively. Arrows show areas of colocalization of the red fluorescent protein (RFP)-labeled bacteria with GFP-labeled EC of the vessels. Supplementary Videos S1 and S2 are higher-magnification images of the selected area of *Bh* and EC interaction. These areas are also depicted in video files showing adherence of the bacteria to EC as well as the intracellular location of the bacteria in the infected zebrafish embryos (Supplementary Videos S1 and S2). Images are representatives of pools of embryos micrographed. Scale bar = 50 μ m. Color images available online at www.liebertpub.com/zeb

shown by the arrows at both time points. However, subsequent analysis was necessary to ascertain whether the bacteria were located either inside or merely interacting with the host cells. The SlideBook 5.5 software was used for 3D analysis of the micrographs, and videos were made using the SMRecorder software (Supplementary Videos S1 and S2). Analysis showed that aggregates of bacteria were interacting with the EC; some were inside of the cells, while most were located outside or away from the endothelium.

Recruitment and accumulation of phagocytes to the site of infection with Bh

To investigate whether *Bh* could recruit phagocytes to the site of infection, we injected *Bh::DsRed2* in the yolk sac of *Tg(mpx:GFP)uwml* embryos at 50 hpf, a transgenic zebrafish line that expresses GFP under the neutrophil-specific myeloperoxidase promoter.³⁰ On microscopic examination of live infected embryos using the Olympus IX81 inverted microscope equipped with the 3i Yokogawa spinning disk scanner and CDD cameras, we observed that the infection induced the accumulation and colocalization of neutrophils (bright green) with the bacteria (red) at the site of infection within 2 hours postinfection (hpi) (Fig. 3; top panel). The number of phagocytic cells seemed to increase during the course of infection as shown by the increase in neutrophils (bright green) (Fig. 3; arrow; top panel) and what we presumed to be macrophages (larger, light green) (Fig. 3,

FIG. 3. Infiltration and colocalization of GFP-labeled phagocytes with *Bh*. Representative micrographs from pools of *Tg(mpx:EGFP)uwml* embryos inoculated at 50 hpf with 300 CFU of *Bh* wild type (WT) (top panel) or PBS/phenol red control (bottom panel) at the indicated time points postinoculation. White arrows are DsRed2 *Bh* colocalized with GFP-labeled neutrophils (bright green; 2 hours post-infection [hpi] and day 4 postinjection [dpi]). Arrowheads are DsRed2 *Bh* interacting with macrophages (larger phagocytes) (4 dpi only). Compared with the infected embryos, minimal numbers of GFP-labeled phagocytes are observed in the noninfected embryos (2 hpi; 4 dpi). Scale bar = 50 μ m. Color images available online at www.liebertpub.com/zeb

arrowhead; top panel) at 4 dpi in the infected embryos. Time-lapse imaging further confirmed our observation with the slow moving macrophages (Supplementary Video S3). Macrophages have previously been observed by 24 hpf in zebrafish embryos based on morphology and phagocytic capacity.^{29,40,41} As shown in the videos, some bacteria were engulfed by the neutrophils and macrophages; however, most of the bacteria were seen outside of the phagocytic cells. Compared with the infected embryos, the sham-injected control embryos did not show as many phagocytes in the yolk sac (Fig. 3; 2 hpi and 4 dpi; bottom panels).

Angiogenic phenotype and inflammatory and angiogenic responses in *Bh*-infected embryos

In order to evaluate the angiogenic effect induced by *Bh* infection, *Tg(fli1:egfp)^{y1}* transgenic embryos were inoculated with an average of 3×10^3 GEs of *Bh::DsRed2*. Confocal laser scanning microscopy was used to examine the embryos and to evaluate blood vessel formation and morphology near the site of infection. Infected embryos were analyzed for vessel sprouting at 48 hpi (Fig. 4A). Note that the red fluorescent overlay showing the RFP bacteria in the infected panel (Fig. 4A, ii) was omitted to help better visualize the subintestinal basket (branching network of blood vessels within the yolk sac of the embryos), so the inset image represents the same embryo showing the bacteria at the site of infection. Compared with the uninfected control group (Fig. 4A, i) and the rzfVEGF-injected control group (Fig. 4A, iii), blood vessel sprouting, shown by the white arrows, could be seen at the subintestinal basket of the infected embryos (Fig. 4A, ii). Moreover, the phenotypic changes induced by *Bh* infection in the transgenic embryos infected with *Bh/pNST5DsRed2* from an independent experiment were assessed at 4 dpi (Fig. 4B); the inset image showed the presence of the red fluorescent bacteria at the site of infection. A larger subintestinal basket was observed in the infected embryos (Fig. 4B, ii, white oval) compared with the noninfected control embryos (Fig. 4B, i) and similar to the positive control injected with rzfVEGF (Fig. 4B, iii), suggesting that the bacteria triggered an angiogenic response that affected the pattern of the SIVs (vessels located in the oval) at the site of infection. This response was quantitatively analyzed using the ImageJ software measuring the length, the number, and the IPs (are the intersections where the SIVs branch out) of SIVs from six different embryos from the infected group and five different embryos from the control group. Compared with the negative control embryos, the infected and the positive control embryos had significantly more SIV (** $p < 0.001$) and IPs (* $p = 0.003$) (Fig. 4C), as well as significant lengthening of the SIVs (** $p < 0.001$ and * $p = 0.002$ for WT and positive control, respectively) (Fig. 4D).

To determine whether the observed angiogenesis was accompanied by a pro-angiogenic response, the host response to *Bh* infection was assessed by qRT-PCR measuring the mRNA level of pro-inflammatory cytokines and pro-angiogenic markers in pools of 10 zebrafish embryos per group at 3 dpi. Those markers were selected due to their roles in tumor angiogenesis,^{42–44} and because they have been shown to be upregulated in *Bh* infection in *in vitro* models.^{11,45–50} The data showed that the expression of IL-1b, IL-8 (Cxc18), zVEGF₁₆₅ (one of the dominant spliced isoforms of VEGF

FIG. 4. Subintestinal vessel (SIV) remodeling and angiogenic marker expression in response to infection with *Bh*. (A) SIV morphology of embryos at 48 hpi: (i) uninfected PBS/phenol red control; (ii) embryos infected with $\sim 3 \times 10^3$ GEs of *Bh::DsRed2* (the overlay showing the RFP bacteria was omitted to help better visualize the subintestinal basket); *inset* image is the same infected embryos showing the presence of the red fluorescent bacteria at the site of infection; *white arrows* show SIV sprouting in the infected embryo; (iii) Embryos injected with 10 ng recombinant zebrafish vascular endothelial growth factor (rzfVEGF) in the yolk sac near the developing SIV. Images are representative of the pool of infected and control embryos imaged. Scale bar = 100 μ m. (B) Blood vessel morphology of *Tg(fli1:egfp)^{y1}* zebrafish embryos analyzed at 4 days postinoculation: (i) PBS/phenol red control at 4 dpi; (ii) embryos infected with *Bh/pNST5DsRed2* visualized at 4 dpi (the overlay showing the RFP bacteria was omitted to help better visualize the subintestinal basket); *white oval* indicates the area of SIVs remodeling in infected embryos; *inset* image is the same infected embryos showing the presence of the red fluorescent bacteria at the site of infection; (iii) embryos injected with 10 ng of rzfVEGF (positive control) in the yolk sac near the developing SIV. Images shown are representative of the pool of six embryos photographed from the infected group and five embryos photographed from the control groups. Scale bar = 100 μ m. (C) Number of SIV and intersection points (IP) in the yolk sac from six embryos (4 dpi) from each group (mean $\pm$ SEM), (** $p < 0.001$; * $p = 0.003$). (D) Length (in relative units) of SIVs (measured from 100 $\times$ images) in the yolk sac from six embryos (4 dpi) from infected group and 5 embryos from the control groups (mean $\pm$ SEM) versus negative control (** $p < 0.001$; * $p = 0.002$). (E) Quantitative real-time PCR (qRT-PCR) of pro-angiogenic markers in zebrafish embryos infected with *Bh*. Results are expressed as the mean fold change in transcript levels of *Bh*-infected compared with uninfected control embryos at 3 dpi ($n = 2$). Color images available online at www.liebertpub.com/zeb

in zebrafish corresponding to human VEGF₁₆₅⁵¹), Flk1, and angiopoietin-2 was upregulated by 4- to 23-fold in embryos inoculated with *Bh* WT compared with those inoculated with PBS phenol red (Fig. 4E). To determine whether this angiogenic response was *Bh*-specific, embryos were microinjected at 28 hpf in the yolk sac with an average of 4×10^3 CFU of *Bh* pNS2T5DsRed2 or *E. coli* DH12S/pNS2T5DsRed2. At that particular dose, *E. coli* DH12S, a nonpathogenic strain, leads to a lethal infection in >90% of the embryos by 20 hpi versus zero death for *Bh*-injected and sham-injected embryos (data not shown). Our confocal microscopy showed evidence of dying embryos infected with *E. coli* compared with sham-injected and *Bh*-infected embryos (Supplementary Fig. S2). Thus, the lethal infection caused by *E. coli* precludes any assessment of an angiogenic response in these embryos. Our data showed that zebrafish embryos inoculated with heat-killed *Bh* exhibited a reduced inflammatory and angiogenic responses compared with those inoculated with live *Bh*; angiopoietin-2 is upregulated by 3-fold, IL-1b by 3.5-fold, VEGF by 4-fold, FLK-1 by 5-fold, and IL-8 by 5.5-fold (Supplementary Fig. S3).

The overall response of the zebrafish embryo to *Bh* was characterized by analyzing the total mRNA levels in infected embryos compared with controls by microarray using the GeneChip Gene 1.0 ST arrays designed by Affymetrix. The gene chips were scanned and data were background adjusted, normalized, and summarized by an RMA algorithm. In total, more than 883 genes were upregulated by twofold or more, and 886 genes were downregulated by at least twofold (Supplementary Table S1). While several of those genes are

uncharacterized, many of them are involved in metabolic pathways, immune response, and tissue remodeling. The microarray result was validated by qRT-PCR using the remaining RNA from the microarray experiment. Fold change in gene expression was calculated with a cut-off value of 1.9, and a select list of candidate genes involved in inflammatory response (IL-1b, complement component c3c) and angiogenesis response (VEGF, IL-8) was assembled (Table 2).

The *Bh* Δ badA mutant exhibits attenuation in vivo

To further characterize *Bh* infection and the importance of BadA in *Bh* pathogenesis and survival in the zebrafish embryo host, embryos were staged at 28 hpf and microinjected with *Bh* Houston-1 containing an in-frame deletion for full-length *badA* (Δ badA/pNS2T5DsRed2). Microscopy results indicated that the Δ badA mutant could survive in the embryos with peak fluorescence observed at 3 dpi, although the levels of red fluorescence did not appear as strong as the WT (Fig. 5A). In a subsequent experiment, zebrafish embryos were randomly grouped and inoculated with an average of 7.4×10^3 *Bh* pNS2T5DsRed2 and 6.7×10^3 Δ badA pNS2T5DsRed2. Bacterial infection was evaluated by qPCR at different time points. WT bacteria were able to replicate, with GE increasing to more than 2×10^5 in 4 days, and remained detectable throughout the duration of the experiment. In contrast, the Δ badA deletion mutant did not appear to replicate as compared with the WT in the infected embryos (Fig. 5B). Although there was some variability in the number of bacteria in the initial inocula, significantly higher numbers of

TABLE 2. SELECTED GENES INVOLVED IN RESPONSE TO INFECTION WITH *BARTONELLA HENSELAE*

Genes	Accession number	Microarray fold change	qRT-PCR fold change	Description/ functions ^a
mapre3b	NM_001002170	+7.07		Regulate dynamics of microtubule cytoskeleton
Rho family GTPase 3a	NM_199522	+2.29		Regulate cytoskeletal dynamic
Cdc42se1	NM_200060	+2.67		Cytoskeletal assembly
IL-8	XM_001342570	+5.54	+2.6	Facilitate migration of immune cells to inflammation site; angiogenesis
IL-1b	NM_212844	+1.93	+21.3	Inflammatory response, cell proliferation, and differentiation
VEGF	NM_001044855	+1.92	+6	Cell proliferation; angiogenesis
Apoptosis inhibitor 5	NM_199540	+4.23	+9.22	Prevent apoptosis after growth factor deprivation
FGF13a	NM_001007399	+6.22		Cell growth; tissue repair
E-cadherin	NM_131820	+2.01		Calcium-dependent adhesion molecule
FGF receptor 1b	NM_001161732	+5.20		Regulation of cell growth, formation of blood vessels
Complement component c3c	NM_001037236	-2.46		Promote phagocytosis during an inflammatory response against pathogens
FGF2	NM_212823	-3.78		Cell survival; angiogenesis
Interferon regulatory factor 2a	NM_207054	-2.18		Regulate transcription of interferon through the JAK-STAT signaling pathway

^aThe description and function of those genes are in humans. Some genes have the same function in zebrafish as in humans; however, the functions of some of those genes have not yet been fully investigated in the zebrafish model.

qRT-PCR, quantitative real-time polymerase chain reaction.

FIG. 5. Zebrafish embryo infected with *Bh* $\Delta badA$ mutant. (A) Confocal micrographs of *Tg(fli1:egfp)*^{y1} transgenic embryos infected with 3×10^3 CFU of *badA* mutant expressing DsRed2 compared with the WT expressing DsRed2. Both strains persist in the embryos at least 5 dpi with a peak fluorescence observed at 3 dpi with the fluorescence intensity is at least twice as much in the WT-infected compared with the *badA* mutant-infected embryos. All images shown are representative of the pool of embryos imaged. Scale bar = 100 μ m. (B) Infection pattern in zebrafish embryos inoculated at 28 hpf with 6.7×10^3 CFU of *Bh* $\Delta badA$ /pNS2T5DsRed2 or 7.4×10^3 CFU of *Bh*/pNS2T5DsRed2. The means of GEs between the two infected groups were calculated by qPCR at different time points (mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.016$ and *** $p < 0.008$). (C) Differential expression in pro-inflammatory genes and pro-angiogenic factors by qRT-PCR in pools of embryos infected with $\Delta badA$ compared with WT. Results are expressed as the mean fold change of WT-infected or $\Delta badA$ mutant-infected compared with uninfected control embryos at 3 dpi ($n = 2$). Color images available online at www.liebertpub.com/zeb

GEs (** $p < 0.016$, ** $p < 0.016$, * $p < 0.05$, and *** $p < 0.008$ at days 2, 3, 4, and 5, respectively) were detected in embryos infected with the WT than observed in those inoculated with $\Delta badA$. Growth curves constructed for WT and the $\Delta badA$ mutant exhibited no differences in overall growth rates and patterns (Supplementary Fig. S4).

The response to infection in pools of embryos inoculated with the $\Delta badA$ mutant was investigated by qRT-PCR at 3 dpi. Compared with embryos inoculated with WT, those inoculated with $\Delta badA$ mutant showed a reduced induction of IL-1 by 7-fold and pro-angiogenic markers IL-8, zVEGF₁₆₅, Flk1, and angiopoietin-2 by 2.5-fold, 2-fold, 3-fold, and 2-fold, respectively (Fig. 5C). Thus, the $\Delta badA$ mutant not only has a diminished ability to replicate *in vivo* but also has a dampened host response.

Discussion

Bartonella are facultative intracellular pathogens with more than thirty species described that infect a range of mammalian hosts.^{2,52} *Bh*, *B. quintana*, and *B. bacilliformis* are the species most commonly known to cause human disease. Depending on the immune status of the infected individual, *Bh* infection can span the range of self-limiting CSD to the more serious and life-threatening BA.¹ A unique aspect of some *Bartonella* species is their ability to induce blood vessel proliferation in humans characterized by blood-filled capillaries resulting from systemic infection.⁵ The angiogenic response induced by *Bh* in patients with BA is believed to be a multi-step process that involves the proliferation of endothelial cells, inhibition of endothelial cell apoptosis, and angiogenic reprogramming of infected host cells. The BadA protein of *Bh* is considered as playing a leading role in eliciting an angiogenic host response during infection,^{10,12,45,53}

but the VirB/VirD4 type IV secretion system is also involved in promoting *Bh*-induced angiogenesis.^{10,12,45,53}

Although much is known about *Bh* and those virulence factors from studies using *in vitro* systems, their molecular mechanisms and their overall contribution to *Bh* pathogenesis remains elusive and can only be fully understood by using an *in vivo* model. Therefore, there has been a great need for a practical animal model to study *Bh* pathogenesis and host response. To date, efforts to establish an *in vivo* model to study *Bh* pathogenesis have, for the most part, been unsuccessful. The fact that zebrafish have a well-developed immune system with many similarities to mammalian systems makes them an attractive model of human diseases. The zebrafish embryo is a proven model for bacterial pathogenesis^{20,36,38,39,41}; moreover, their transparency and the availability of the *Tg(fli1:egfp)*^{y1} transgenic strain with GFP-labeled vasculature has provided a unique opportunity to study *Bh* infection and its distinctive ability to induce vessel proliferation.

We have determined the conditions necessary to maintain a sustained infection with *Bh*, including the appropriate route of infection, dose and site of inoculation, the proper stage of development of the embryos, and the means of enumerating bacteria after infection. We have found that inoculation of *Bh* into the yolk sac as compared with the blood circulation leads to a more persistent infection. It has been reported that yolk sac infections with fast-growing bacterial species have resulted in massive bacterial growth and early death of embryos.^{20,39} The yolk sac is perhaps preferred by *Bh*, because it is a fastidious and slow growing bacterium; the yolk sac may provide the nutrients needed and enough time to adapt to the new environment before facing a strong antimicrobial response. Furthermore, we have determined that zebrafish embryos *Tg(fli1:egfp)*^{y1} sustained a consistent infection

pattern when microinjected at 24–28 hpf in the yolk sac with an inoculum of $\sim 3 \times 10^3$ GEs of *Bh*.

One area, which proved problematic, is bacterial enumeration of *Bh* via plating of embryo homogenates. This assay provides direct counts of the number of viable bacteria in the embryos. However, the autoagglutination of the bacteria as well as contamination seen even on kanamycin selective media plates caused some variability in enumeration as evidenced by the error bars (Fig. 1D). This contamination is likely, in part, due to the normal flora present in the embryos. In addition, rapidly growing water-borne bacteria and fungi that are resistant to kanamycin also proved problematic, as *Bh* requires at least 5 days for colonies to appear on plates, and the contaminants usually grew much faster and often prevented accurate plate counting. Therefore, we alternatively used both confocal microscopy to qualitatively visualize *Bh* in embryos and a qPCR to assess the GE/infected embryo. This method is more sensitive, as it uses primers specific to the *Bh* NADH dehydrogenase subunit G gene to quantify the GEs of bacteria per embryo. It is likely that these primers amplify DNA from nonviable bacteria; however, there is a notable reduction in GEs from day 4 to 6, suggesting that gDNA from dead bacteria may degrade and is likely less suitable as a PCR template (Fig. 1E). This reduction pattern in GEs was similar to that observed by microscopy (Fig. 1C) and by CFU count in previous experiments (Fig. 1D). Despite the possible detection of nonviable bacteria, the qPCR method proved to be far more consistent and reliable than the plating method.

Much of the understanding of the mechanisms required for angiogenesis has been derived from cancer models. Similar to cancer, *Bh* infection of endothelial cells induces the activation of HIF-1 α and subsequent secretion of angiogenic factors such as VEGF, leading to cell proliferation and angiogenesis.^{10,13} An *in vivo* model is critical, as multiple cell types, including endothelial cells, macrophages, and epithelial cells, are considered as contributing to *Bh*-mediated angiogenesis via the paracrine loop model.^{11,46} We have shown that aggregates of *Bh* interact with the EC of the embryo with some intracellular bacteria observed. However, many are distant from the EC in the yolk sac, and it is difficult to accurately quantify the number of intracellular *Bh* in infected embryos. It should be noted that in humans, histological studies of samples from BA patients have shown very few intracellular *Bh* and most of the bacteria are seen as epicellular.⁵⁴

A histological examination of BA lesions from patients with *Bh* infection has shown the infiltration of polymorphonuclear cells and macrophages located in close proximity of the proliferating endothelial.^{54,55} Our evidence shows that *Bh* infection induces migration and accumulation of neutrophils and macrophages at the site of infection. This result is in agreement with what was observed in tissue samples as well as with what has been shown in *in vitro* studies, revealing the role of phagocytic cells in *Bh* pathogenesis.⁴⁶ There should be a balance between *Bh* causing phagocytic cells to produce proangiogenic factors and simultaneously suppressing bacterial killing. Although the yolk sac may be considered a site of relative immune privilege in the zebrafish embryo, a robust innate response against infection has been described in the yolk sac, and macrophages and neutrophils have been observed as ingesting bacteria in both the blood circulation and

the yolk sac.^{20,39,56,57} We hypothesize that the resistance to a lethal infection by *Bh* from infected embryos is very likely due to the activation of an innate immune response. While our real-time observation of *Bh*-infected zebrafish embryos showed an accumulation of phagocytic cells at the site of infection, the role of these cells in killing *Bh* was not clearly demonstrated. It remains to be investigated whether or not this is due to the ability of this bacterium to inhibit phagocytosis.

An angiogenic phenotype was observed in our transgenic zebrafish embryo model when infected with *Bh*. It was characterized by an increase in length and number of the SIVs and IPs at the site of infection in the infected compared with controls. Moreover, disruption of the smooth and normal pattern of those vessels could also be observed. Although the extent of the phenotypic response observed is not as dramatic as what is seen in tumor angiogenesis, a similar response was observed in the zebrafish yolk membrane angiogenesis assay developed by Nicoli *et al.*^{22,58} The angiogenic phenotype observed in the infected embryos was further substantiated by qRT-PCR analysis showing the induction of proangiogenic factors such as IL-8 (Cxcl8), zVEGF-165, zVEGFR2, and angiopoietin-2 in the infected compared with control embryos. These potent angiogenic factors have been shown to stimulate endothelial cell proliferation and angiogenic phenotypes in response to *Bh* infection *in vitro*.^{11,47,49} Our efforts to use *E. coli* DH12S strain as a negative control for an angiogenic response in the zebrafish embryo was unsuccessful, as embryos inoculated with *E. coli* in the yolk sac died within 20 h postinoculation. The death of the embryos may be a result of septic shock due to the high endotoxicity of bacterial lipopolysaccharide (LPS) of *E. coli* compared with the remarkably low endotoxicity of LPS of *Bh*.⁵⁹ We have been unable to identify a suitable control bacterium which exhibits the same sub-lethal infection pattern as that seen with *Bh*. However, when heat-killed *Bh* was injected into zebrafish embryos, the pro-angiogenic pattern of gene expression was greatly diminished compared with the embryos infected with live *Bh* (Supplementary Fig. S3), indicating that live *Bh* is required for this unique host response.

Our microarray data provide evidence that there are many genes which are involved not only in inflammatory and angiogenic responses, but also in cytoskeletal rearrangement and metabolic processes. This suggests that the bacteria have a widespread impact on host gene expression patterns. *Bh* entry into EC and epithelial cells can happen by two alternative routes: single bacterium uptake via a zipper-like mechanism or in the form of large bacterial aggregates in a structure called invasome.^{2,60} The zipper-like mechanism has been documented in several bacteria, including *L. monocytogenes*. It has been shown that the entry of *L. monocytogenes* into epithelial cells requires the interaction of the internalin protein with E-cadherin on the host cell.⁶¹ In addition to its role in cell–cell adhesion and involvement in early tissue formation in zebrafish embryo,⁶² E-cadherin may also play a role in *Bh* entry into the host cell as *Bh* infection induces an upregulation of the gene encoding for E-cadherin protein. Formation of the invasome structure leading to the uptake of *Bh* aggregates was shown to be dependent, in part, on the expression of small Rho family GTPases and CDC42.⁶³ Rho family GTPase 3a and CDC42 small effector 1 (Cdc42se1) were upregulated in the infected embryos, suggesting that they may play a role in *Bh*-host interactions.

The *Bh*-induced angiogenesis is a multi-step process that partly depends on the expression of BadA protein which induces proangiogenic cell response via activation of HIF-1 and NF- κ B, and the subsequent secretion of VEGF and IL-8, respectively.^{10,11,13} *Bh*-induced angiogenesis in the zebrafish embryo may occur through the same pathway as previously shown in cell culture studies, as those genes including HIF- α (NM_200223) and NF- κ B (NM_203184) were seen to be upregulated in the infected embryos. Some notable genes that were downregulated in the infected embryos include FGF2, which plays an important role in cell survival and angiogenesis, and complement component c3c, which is involved in promoting phagocytosis during an inflammatory response against pathogen.^{64–66} Although FGF2 expression in *Bh*-induced angiogenesis has not been fully investigated *in vitro*, it is somewhat surprising that the infection with *Bh* would suppress its expression. On the other hand, *Bh* has been shown to inhibit phagocytosis,¹² so it is not surprising that the evidence shows that expression of complement component c3c is suppressed by *Bh* infection in the zebrafish embryos.

The genome of *Bh* contains at least two forms of BadA-encoding genes: the full-length version (BH01510), which was the gene deleted in this study, and a truncated version (BH01490). It has been reported that some *Bh* isolates do not express a functional BadA, and due to passaging histories, defined *Bh* strains may exhibit distinct characteristics and infection phenotypes.⁶⁷ However, our *Bh* Houston-1 strain was early passage and displayed a rough phenotype which correlates with the presence of full-length BadA protein which was further confirmed by reactivity with an antibody that was specific for BadA (data not shown). While a deletion mutant for the major trimeric autotransporter adhesin BadA appears to increase in fluorescence intensity within the embryos over time, qPCR data indicated that this mutant was unable to replicate and remained at a relatively constant bacterial burden as determined by the GE/embryo. It has been reported that full-length BadA is essential for *Bh* adhesion to host cells and extracellular matrix proteins, leading to a direct angiogenic response by stimulating HIF-1 α production and VEGF secretion.^{10,11,13} Therefore, we hypothesized that, in absence of full-length BadA, *Bh*'s ability to adhere to target cells, establish infection, and initiate the induction of an angiogenic response would be hampered. Our data showed that the *Bh* mutant for full-length BadA exhibited an abrogated angiogenic response in the zebrafish embryo compared with WT, although it may be possible that the diminished ability of this mutant to replicate in the zebrafish embryos precluded the establishment of a pro-angiogenic host response. It is important to note that no difference was observed for the *in vitro* growth rate of the *Bh badA* mutant as compared with the parental WT control (Supplementary Fig. S4). Thus, our *in vivo* data support a major role of BadA in establishing infection and also possibly inducing a pro-angiogenic host response. Although the function of the truncated version of BadA (BH01490) in *Bh* has not been fully investigated, a compensatory role in inducing an angiogenic phenotype after the loss of full-length BadA cannot be eliminated in the $\Delta badA$ mutant.

In conclusion, we have developed an animal model to study *Bh* infection and pathogenesis. We demonstrated that zebrafish embryos microinjected in the yolk sac became infected with *Bh* and exhibited evidence of an angiogenic response as well as an inflammatory response involving re-

cruitment and accumulation of neutrophils and macrophages at the site of infection. We also found that the *Bh* mutant for full-length BadA showed a limited ability to replicate when compared with the WT, suggesting that this *in vivo* model is a useful system in which to assess virulence. The infection pattern with *Bh* was not assessed beyond day 8 postinoculation, as embryos are capable of obtaining nutrition from the yolk sac during this time period and extension of the period of infection would require supplementary food sources—possibly introducing microbial contaminants. Regardless of this, we believe that the use of zebrafish embryos as a model of *Bh* infection proved highly successful and provided valuable insights which can only be gathered from an *in vivo* model system.

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Disclosure Statement

No competing financial interests exist.

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