Injections of Predatory Bacteria Work Alongside Host Immune Cells to Treat *Shigella* Infection in Zebrafish Larvae

**Highlights**

- Injected predatory *Bdellovibrio* bacteria persist non-pathogenically in zebrafish
- *Bdellovibrio* injection promotes *Shigella* killing and increases zebrafish survival
- *Bdellovibrio* are eventually cleared by the zebrafish immune system
- Antibacterial therapy is achieved via the host immune system working with *Bdellovibrio*

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**In Brief**

In an era of global antibiotic resistance, Willis et al. characterize the “living-antibiotic” action of predatory *Bdellovibrio* bacteria in zebrafish larvae versus the human pathogen *Shigella flexneri*. Results are proof of principle that predators assist the immune system to promote animal survival upon infection by Gram-negative pathogens.
Injections of Predatory Bacteria Work Alongside Host Immune Cells to Treat Shigella Infection in Zebrafish Larvae

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SUMMARY

Bdellovibrio bacteriovorus are predatory bacteria that invade and kill a range of Gram-negative bacterial pathogens in natural environments and in vitro [1, 2]. In this study, we investigated Bdellovibrio as an injected, antibacterial treatment in vivo, using zebrafish (Danio rerio) larvae infected with an antibiotic-resistant strain of the human pathogen Shigella flexneri. When injected alone, Bdellovibrio can persist for more than 24 hr in vivo yet exert no pathogenic effects on zebrafish larvae. Bdellovibrio injection of a zebrafish containing a lethal dose of Shigella promotes pathogen killing, leading to increased zebrafish survival. Live-cell imaging of infected zebrafish reveals that Shigella undergo rounding induced by the invasive predation from Bdellovibrio in vivo. Furthermore, Shigella-dependent replication of Bdellovibrio was captured inside the zebrafish larvae, indicating active predation in vivo. Bdellovibrio can be engulfed and ultimately eliminated by host neutrophils and macrophages, yet have a sufficient dwell time to prey on pathogens. Experiments in immune-compromised zebrafish reveal that maximal therapeutic benefits of Bdellovibrio result from the synergy of both bacterial predation and host immunity, but that in vivo predation contributes significantly to the survival outcome. Our results demonstrate that successful antibacterial therapy can be achieved via the host immune system working together with bacterial predation by Bdellovibrio. Such cooperation may be important to consider in the fight against antibiotic-resistant infections in vivo.

RESULTS AND DISCUSSION

Injected Predatory Bdellovibrio Persist in Zebrafish Larvae without Ill Effects and Treat Shigella Infection In Vivo

The rise in antimicrobial-resistant (AMR) Gram-negative bacterial infections in hospital patients has prompted an urgent search for novel antibacterial agents [3]. One candidate group is the naturally predatory bacteria Bdellovibrio bacteriovorus, which invade and kill a wide range of Gram-negative bacterial pathogens [1]. Invasion is followed by pathogen rounding, after which the prey pathogen dies [2]. Bdellovibrio replicate within the dead pathogen, which persists as a stable “bdelloplast” structure until being lysed 3–4 hr after invasion [4]. Prey lysis releases mature Bdellovibrio progeny that can seek further bacterial victims (Figure 1A).

Given that Bdellovibrio are ubiquitous in nature, it is likely that these bacteria are already being harmlessly ingested in food or water. Indeed, low levels of Bdellovibrio 16S rRNA have been detected in gut samples from healthy children [5]. Although no equivalent studies have been conducted in adults, there is no known association of Bdellovibrio with disease. Previously, non-injected administrations of Bdellovibrio have been shown to reduce pathogen numbers by oral administration versus Salmonella enteritidis in the gut of chickens [6] and by external application against eye infection in cattle [7–9]. Emerging global antibiotic resistance calls for new injected therapies to target infected wounds and body compartments. The capability of injected Bdellovibrio to treat bacterial infections has not been tested, nor has the interaction of predatory bacteria with leukocytes, and successes here could dramatically expand the therapeutic scope of predatory bacteria against life-threatening infections. It is, therefore, important to consider the extent to which injected Bdellovibrio survive in the presence of a vertebrate immune system and whether leukocyte interactions aid or prevent pathogen killing in vivo. Measuring these parameters is crucial to develop the therapeutic potential of Bdellovibrio.

The transparent zebrafish larva provides a unique opportunity to quantify and visualize in vivo both the spread or restriction of bacterial infection as well as bacterial interactions with immune cells [10]. In particular, the zebrafish hindbrain ventricle is highly amenable to imaging, enabling us to follow injected Bdellovibrio ± pathogenic bacteria over time (Figure S1A). To first test for any pathogenic effects of Bdellovibrio inside a vertebrate host, we injected the hindbrain ventricle of zebrafish larvae at 3 days post-fertilization (dpf) with 1–10 × 10^3 plaque-forming units (PFUs) of mCherry B. bacteriovorus HD100 alone. This assessment was also essential to determine whether injected Bdellovibrio alone would survive in the zebrafish long enough to successfully prey on pathogen infections. Live-cell imaging showed a gradual reduction in fluorescence from...
Figure 1. Injected Predatory *Bdellovibrio* Persist in Zebrafish Larvae without Ill Effects and Protect against *Shigella* Infection In Vivo

(A) Cartoon of *Bdellovibrio* life cycle. I–III Motile predatory *Bdellovibrio* attach to and invade the periplasm of Gram-negative bacteria such as *Shigella*. III Prey bacteria are rounded by DD-endopeptidase action on the cell wall. IV Prey bacteria are killed in ~30 min and kept intact as *Bdellovibrio* consume their contents and grow. V and VI Following replication, *Bdellovibrio* lyse prey 180–240 min after invasion, releasing further predators. These *Bdellovibrio* progeny can repeat the predatory cycle. CM, cytoplasmic membrane; CW, cell wall peptidoglycan; OM, outer membrane.

(B) Wild-type (WT) AB larvae were injected at 3 dpf in the hindbrain ventricle with 1–10^3^ PFUs of mCherry-*Bdellovibrio* (red). The same larvae were imaged over time to observe distribution. Representative images from a single larva are shown here. Scale bar, 100 μm.

(C) Enumeration of live *Bdellovibrio* in PBS-homogenates from larvae injected with mCherry-*Bdellovibrio* as in (B) over time. Each circle represents a count from an individual larva. Data are pooled from two independent experiments (n = 8 larvae per experiment). Mean ± SEM (horizontal bars) is shown. The p values (versus the 0 hpi time point) were determined by multiple t test. Significance with Bonferroni correction was defined as p < 0.0125. See also Figures S1 B–S1D for comparative evaluations of *Bdellovibrio* persistence from different doses in larvae at different developmental stages.

(D) Survival curve of WT AB larvae injected with mCherry-*Bdellovibrio* as in (B) and incubated at 28 °C for 48 hpi. Data are pooled from three independent experiments (n = 22–37 larvae per experiment).

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mCherry-Bdellovibrio following injection, although some bacteria were clearly observed in vivo after 24 hr post-infection (hpi) (Figure 1B). For quantification of the survival of Bdellovibrio in vivo, injected larvae were homogenized and predatory bacteria enumerated over time post-infection (Figure 1C). Consistent with observations from live-cell imaging, we detected a steady decline in Bdellovibrio numbers from 2 hpi, and by 48 hpi the bacteria were largely eliminated. Similar results were obtained using a 10-fold higher dose of Bdellovibrio or in larvae injected 2 dpf when the immune system is less developed (Figures S1B–S1D). These results reflect the obligatory prey-dependent lifestyle of Bdellovibrio. They are able to survive within zebrafish for extended periods of time but are unable to replicate in the absence of prey bacteria, giving an opportunity for Bdellovibrio to act therapeutically before ultimately being cleared. Moreover, Bdellovibrio-injected zebrafish larvae displayed normal morphology and locomotive activity, with no signs of developmental toxicity or reduced viability (Figures 1D and S1E). These results raise no concern for the use of Bdellovibrio as a therapeutic agent in vivo, and its natural clearance could be viewed as a beneficial property of a limited treatment.

Given that Bdellovibrio have no adverse effects on zebrafish, we tested their efficacy as an antibacterial agent against a streptomycin- and carbenicillin-resistant strain of Shigella flexneri M90T. Shigella is a Gram-negative enteroinvasive pathogen that is responsible for 163 million illness episodes and over 1 million deaths annually [11]. Similar to many other Gram-negative pathogens in hospital patients, cases of antibiotic-resistant Shigella are rising [12]. Bdellovibrio invade Gram-negative pathogens without a simple receptor-based recognition system, making Bdellovibrio resistance genetically complex for prey to acquire. Previous studies have used the zebrabream as a model organism to study the infection biology of S. flexneri M90T infection in vivo [13, 14]. We developed this model of Shigella infection to incorporate the additional injection of B. bacteriovorus and test its therapeutic potential. Here, 30–90 min after an otherwise lethal (at 48–72 hpi) hindbrain inoculation of GFP-Shigella M90T (>5 × 10⁵ colony-forming units [CFUs]), we injected 1–2 × 10⁵ PFUs of mCherry-Bdellovibrio into the hindbrain ventricle of zebrafish larvae. In the absence of Bdellovibrio injection, zebrfish larvae showed increasing fluorescence of GFP-Shigella (Figure 1E; Movie S1). Strikingly, larvae injected with Bdellovibrio showed diminishing fluorescence of GFP-Shigella in regions contacting mCherry-Bdellovibrio (Figure 1E; Movie S1). Consistent with this, Shigella enumeration demonstrated that zebrafish larvae injected with Bdellovibrio were able to control Shigella replication significantly better than those infected with Shigella alone (Figure 1F). Moreover, Bdellovibrio could rescue zebrafish from lethal Shigella infection, increasing survival by ~35% at 72 hpi (Figure 1G).

**Bdellovibrio Prey on Shigella In Vitro and In Vivo inside Living Zebrafish**

To confirm susceptibility of Shigella to Bdellovibrio predation, we performed co-incubation assays in vitro, and we observed mCherry-Bdellovibrio invade GFP-Shigella and, subsequently, induce prey rounding to form bdelloplasts (Figure 2A). In live-cell counting assays of viability in buffer, Shigella numbers were reduced >4,000-fold by Bdellovibrio treatment (Figure 2B). Plate reader assays measuring optical density of Shigella and fluorescence of mCherry-Bdellovibrio confirmed that reduction in Shigella numbers was followed by a rise in Bdellovibrio numbers after an ~3 hr predatory cycle, during which they invade and grow within Shigella and then emerge (Figures 2C and 2D).

To investigate Bdellovibrio predation of Shigella in vivo, we imaged the zebrfish tail muscle after sequential GFP-Shigella and mCherry-Bdellovibrio co-injections. Shigella are rod-shaped bacteria (~0.5 × 2.0 μm); however, Shigella in the presence of Bdellovibrio were mostly rounded (~1.0 × 1.0 μm), suggesting their invasion by Bdellovibrio inside zebrafish (Figure 2E). To investigate this further, we followed in vivo predator-prey interactions at the level of single cells (Figure 2F). Remarkably, confocal microscopy inside live zebrafish confirmed that mCherry-Bdellovibrio invade individual GFP-Shigella to form green rounded bdelloplasts containing red Bdellovibrio over a similar time frame to that seen in vitro (Movie S2). To test for Bdellovibrio predation of Shigella at the whole-animal level, we quantified Bdellovibrio over time after sequential GFP-Shigella and mCherry-Bdellovibrio co-injections (Figure 2G). As expected from experiments performed both in vitro (Figure 2D) and in vivo (Figure 1C), in the absence of Shigella, prey zebrafish larvae showed decreasing numbers of Bdellovibrio over the 24 hr following infection (Figure 2G). In contrast, zebrafish larvae infected with Shigella and Bdellovibrio showed significantly increased numbers of Bdellovibrio at 5 hpi, indicating predatory replication inside Shigella in vivo. Thus, live bacterial predation is occurring within zebrafish. To dissect the efficacy of Bdellovibrio therapy in the context of innate immunity, we next tested how leukocytes can affect the efficacy of Bdellovibrio predation in our zebrafish infection model.

(E) WT AB zebrafish larvae were injected in the hindbrain ventricle at 3 dpf with >5 × 10⁵ CFUs of GFP-S. flexneri (green), followed by a hindbrain injection of either PBS or 1–2 × 10⁵ PFUs of mCherry-Bdellovibrio (red), 30–90 min after the initial Shigella infection. Representative images of the hindbrain ventricle in PBS- or Bdellovibrio-treated larvae are shown with Shigella in green and Bdellovibrio in red. For each treatment, the same larvae was imaged over time. Scale bar, 100 μm. See also Movie S1.

(F) Enumeration of live Shigella in homogenates of larvae injected with S. flexneri and treated with injections of either PBS or Bdellovibrio as in (E) over time. Each circle represents a count from an individual larva. Half-filled circles represent enumerations from larvae at time 0 and are representative of inocula for both conditions. Only viable larvae were included in the analysis. Data are pooled from four independent experiments (up to n = 3 larvae per time point per experiment). Mean ± SEM (horizontal bar) is shown. The p values (between conditions at cognate time points) were determined by unpaired one-tailed Student’s t test. Significance was defined as p < 0.05.

(G) Survival curve of larvae injected with S. flexneri and treated with either PBS or Bdellovibrio as in (E). Larvae were incubated at 28°C for 72 hpi. Data are pooled from three independent experiments (n = 22–48 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 2, 24, 48, and 72 hr time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance was defined as p < 0.05. See also Figure S1.
Figure 2. *Bdellovibrio* Prey on *Shigella* In Vitro and In Vivo inside Living Zebrafish

(A) GFP-*S. flexneri* (green) were incubated in vitro, in the presence or absence of mCherry-*Bdellovibrio* (red), and visualized by wide-field fluorescent microscopy. Representative images, including rod-shaped *Shigella* and rounded *Shigella* invaded by smaller comma-shaped *Bdellovibrio*, were taken at 1 hr post-mixing. Scale bar, 1 μm.

(B) 5–12 × 10^8 CFUs of GFP-*S. flexneri* were incubated, in vitro, in 10 mL CaHEPES buffer for 21 hr in the presence or absence of ~6.2 × 10^10 PFUs of mCherry-*Bdellovibrio*. Live *Shigella* were enumerated over time. Data are pooled from three independent experiments. Mean ± SEM (horizontal bars) is shown. The p value between conditions was determined by paired one-tailed Student’s t test. Significance was defined as p < 0.05.

(C and D) 2–7 × 10^7 CFUs of GFP-*S. flexneri*, 8.4–10.4 × 10^9 PFUs of mCherry-*Bdellovibrio*, or both GFP-*S. flexneri* and mCherry-*Bdellovibrio* were incubated in vitro in CaHEPES buffer at 37°C. (C) Optical density 600 (OD_600) representing *Shigella* numbers (*Bdellovibrio* are too small to contribute to OD_600) or (D) mCherry fluorescence intensity representing *Bdellovibrio* numbers was measured every 30 min for 6 hr using a microplate reader (results plotted every 1 hr). Mean ± SEM (legend continued on next page)
**Bdellovibrio Is Recognized and Engulfed by Zebrafish Leukocytes In Vivo**

Time-lapse microscopy in the hindbrain showed that, by 6 hr following injection, the initially dispersed mass of mCherry-Bdellovibrio clustered and formed dynamic punctae of ~10 μm. Similar observations were made after caudal vein injection of Bdellovibrio into the bloodstream of zebrafish larvae (Figure S2A). These observations suggest that Bdellovibrio can reside within leukocytes. Although little studied, some features of Bdellovibrio have been predicted to allow a degree of “silent running” in the immune system of a vertebrate. Bdellovibrio are characteristically small, 0.25 × 1.0 μm, comprise a single-membrane-sheathed flagellum, and have a modified mannosylated lipopolysaccharide (LPS) outer membrane [15]. Furthermore, Bdellovibrio gene expression and surface protein production are significantly lower outside of bacterial prey hosts than inside [16, 17]. To test for innate immune detection of Bdellovibrio in vivo, we injected larvae with mCherry-Bdellovibrio and, measured the expression of the pro-inflammatory cytokines interleukin 1β (IL1β) and tumor necrosis factor α (TNF-α) by qRT-PCR. Increased expression of both IL1β and TNF-α was detected 4 hr after larva inoculation of Shigella, Bdellovibrio, or Shigella + Bdellovibrio combined (Figure S2B). Importantly, the cytokine response from Shigella + Bdellovibrio together is not additive beyond Shigella alone, demonstrating that Bdellovibrio is not solely stimulating a further immune response to help clear pathogenic bacteria. Cytokine signaling during zebrafish infection is typically accompanied by an active immune cell response [18, 19]. The innate immune system of zebrafish is highly homologous to that of humans, and responses are mediated by neutrophils and macrophages [19]. Leukocytes do not typically reside in the hindbrain during steady-state conditions, making this site ideal to study directed leukocyte migration in response to injected bacteria. To test Bdellovibrio interactions with leukocytes, we used Tg(mp5x:GFP)174 transgenic larvae with GFP-neutrophils and Tg(impeG1:Gal4-FF)205/Tg(UAS-E1b:nfsB:mCherry)205 transgenic larvae with mCherry-macrophages. Consistent with a stimulated cytokine response (Figure S2B), imaging of Bdellovibrio hindbrain injections in zebrafish with these fluorescent leukocytes revealed that both neutrophils and macrophages localize to the site of injection (Figures 3A and 3B; Movie S3). Despite detection of Bdellovibrio by innate immune cells in vivo, quantification of leukocyte recruitment to the larval head, via image analysis, revealed only a slight increase over PBS controls in neutrophils (1.5-fold ± 0.1) and macrophages (1.2-fold ± 0.1) in the hindbrain at 6 hpi (Figures 3C and 3D).

Analysis by confocal microscopy of Bdellovibrio-leukocyte interactions within live zebrafish confirmed that these bacteria are engulfed by both neutrophils and macrophages (Figures S2C and S2D; Movie S4). To assess the role of leukocytes in Bdellovibrio clearance, we performed experiments in immunocompromised zebrafish larvae using an antisense morpholino oligonucleotide targeting Pu.1, a zebrafish transcription factor driving myeloid gene expression [20]. Larvae depleted of leukocytes, via prior injection of Pu.1-targeting morpholino into the one- to eight-cell-stage embryo, were injected at 3 dpf in the hindbrain with 1–2 × 10^5 PFUs of Bdellovibrio. In agreement with a role for leukocytes in the clearance of Bdellovibrio in vivo, significantly more predatory bacteria were recovered from larval homogenates of Pu.1 morphants as compared to controls (Figures 3E and S2E). Survival of control or Pu.1 morphants injected with Bdellovibrio was not significantly different from each other (Figure S2F), highlighting that prolonged exposure to Bdellovibrio is not detrimental to the health of an immunocompromised animal.

**Bdellovibrio Work alongside Innate Immune Cells to Protect Against Shigella Infection In Vivo**

To understand better the relative contributions of Bdellovibrio and the host immune system to Shigella clearance, we performed infection studies in zebrafish larvae in which leukocytes were depleted by Pu.1 morpholino. Control and Pu.1 morphants were injected with lethal hindbrain doses of Shigella as before and treated with PBS or Bdellovibrio (Figure S3A). Enumerations of Shigella from larval homogenates showed that treatment with Bdellovibrio reduced Shigella numbers in both immune-compromised and immune-competent zebrafish larvae (Figure 4A), but survival was significantly greater in immune-competent larvae (Figure 4B). Remarkably, these results show that maximal therapeutic benefit of Bdellovibrio against a Gram-negative bacterial infection is ultimately the product of eukaryotic leukocytes working cooperatively with prokaryotic predators.

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The text continues with detailed descriptions of experimental methods and results, including enumeration of any replicated predators, quantification of leukocyte response from infected larvae, and analysis of immune cell response to Bdellovibrio. The conclusions highlight the collaborative role of Bdellovibrio with innate immune cells for protection against Shigella infection.
Figure 3. *Bdellovibrio* Is Recognized and Engulfed by Zebrafish Leukocytes In Vivo

(A) 1–2 × 10^5 PFUs of mCherry-*Bdellovibrio* were injected into the hindbrain ventricle of Tg(*mpx*:GFP)*i114* zebrafish larvae at 3 dpf, and interactions between neutrophils (green) and *Bdellovibrio* (red) were visualized by fluorescent stereomicroscopy. Representative images from a single larva over time are shown. Scale bar, 100 μm. See also Movie S3.

(B) 1–2 × 10^5 PFUs of mTeal-*Bdellovibrio* were injected into the hindbrain ventricle of Tg(*mpeg1*:Gal4-FF)/Tg(UAS-E1b:nfsB.mCherry)*c264* zebrafish larvae at 3 dpf, and interactions between macrophages (green) and *Bdellovibrio* (red) were visualized by fluorescent stereomicroscopy. Representative images from a single larva over time are shown. Scale bar, 100 μm. See also Movie S3.

(C) Tg(*mpx*:GFP)*i114* zebrafish larvae were injected with PBS or *Bdellovibrio* as in (A), and GFP-expressing neutrophils present in the head region were quantified at 6 hpi. Each circle represents a count from an individual larva. Data are pooled from two independent experiments. The p value between conditions was determined by unpaired one-tailed Student’s t test. Significance was defined as p < 0.05.

(D) Tg(*mpeg1*:Gal4-FF)/Tg(UAS-E1b:nfsB.mCherry)*c264* zebrafish larvae were injected with PBS or *Bdellovibrio* as in (B), and mCherry-expressing macrophages present in the head region were quantified at 6 hpi. Each circle represents a count from an individual larva. Data are pooled from two independent experiments. The p value between conditions was determined by unpaired one-tailed Student’s t test. Significance was defined as p < 0.05.

(E) Tg(*mpx*:GFP)*i114* zebrafish larvae were pre-treated using control (CTRL) or Pu.1-targeting morpholino (MO) to deplete leukocytes. Morphants were injected in the hindbrain ventricle at 3 dpf with either PBS or 3–5 × 10^5 PFU mCherry-*Bdellovibrio*. Live *Bdellovibrio* were enumerated from PBS homogenates of larvae. Each circle represents a count from an individual larva. Half-filled circles represent enumerations from larvae at time 0 and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. The p value (between conditions at cognate time points) was determined by unpaired one-tailed Student’s t test. Significance was defined as p < 0.05. As inoculums from independent experiments were variable up to a log-fold, a representative of three independent experiments performed is shown. See also Figure S2E.

See also Figure S2.
Figure 4. Bdellovibrio Work alongside Innate Immune Cells to Protect against Shigella Infection In Vivo
(A) Tg/mpx:GFP114 zebrafish larvae were pre-treated using control (CTRL) or Pu.1-targeting morpholinos (MO) to deplete leukocytes. Morphants were injected in the hindbrain ventricle at 3 dpf with >5 × 10^3 CFUs of GFP-S. flexneri followed by a hindbrain injection of PBS or 1–2 × 10^5 PFUs of mCherry-Bdellovibrio 30–90 min after the initial Shigella infection. Live Shigella were enumerated from larval homogenates. Each circle represents a count from an individual larva. Half-filled circles represent enumerations from larvae at time 0 and are representative of inocula for both conditions. Only viable larvae were included in the analysis. Data are pooled from three independent experiments (up to n = 3 larvae per time point per experiment). Mean ± SEM (horizontal bars) is shown. Top graph represents collated data. Bottom graph represents only Bdellovibrio-treated larvae, a subset of the above data. The p value (between conditions at cognate time points) was determined by unpaired one-tailed Student’s t test. Significance was defined as p < 0.05. ND, not determined at 72 hpi due to high morphant mortality reducing the samples available.

(B) Survival curve of control (CTRL) or Pu.1 morphant larvae, injected with S. flexneri and treated with Bdellovibrio as in (A). Larvae were incubated at 28°C for 72 hpi. Data are pooled from three independent experiments (n = 12–40 larvae per condition per experiment). Up to three larvae per condition were taken for CFU at 6, 24, and 48 hr time points. Top graph represents collated data. Bottom graph represents only Bdellovibrio-treated larvae, a subset of the above data. The p value between conditions was determined by log-rank Mantel-Cox test. Significance was defined as p < 0.05.

(legend continued on next page)
The *Bdellovibrio* predatory process, which we have shown to be effective in vivo, could possibly allow treatment of infection sites in humans [21]. We propose that, following *Bdellovibrio* application, an early predatory-killing phase can reduce pathogen burden to a level manageable for clearance by the vertebrate innate immune system (Figure 4C). Predation will release small pathogen-derived fragments into the vertebrate, albeit in a digested state, after predator enzyme action. The limited immune stimulation by the injected *Bdellovibrio* (and possibly these pathogen fragments) is not detrimental to the well-being of zebrafish larvae. This limited immune stimulation may contribute to the clearance of *Shigella*, but active predation with predator-prey encounters in vivo occurs as early as 30 min post-injection (Figure 2F; Movie S1), time points before extensive leukocyte infiltration. Moreover, *Bdellovibrio* have replicated extensively in vivo at 5 hr, in a process dependent on *Shigella* killing (Figure 2G).

Predator-prey interactions in our experiments occurred early, when a significant percentage of pathogens would be outside of leukocytes. *Bdellovibrio* engulfed by leukocytes inside zebrafish were detectable by their mCherry fluorescence within the vacuoles of those leukocytes for up to 6 hr (Movie S4). This suggests that predatory bacteria may persist intracellularly within immune cells. Future studies beyond this work will be testing whether intracellular *Bdellovibrio* are able to access, invade, and kill Gram-negative pathogens, which themselves invade leukocytes, such as *Shigella* [22].

In conclusion, these results highlight the first successful use of *Bdellovibrio* in vivo as an injected antibacterial therapy, improving survival in live infected animals. The zebrafish infection model reveals host recognition and clearance of *Bdellovibrio* within days following treatment, a feature that provides a useful limitation to an applied therapy. Most importantly, we show that injected *Bdellovibrio* persist in vivo sufficiently long enough with predatory capacity to reduce numbers of pathogenic bacteria, before themselves being removed by immune action of the host.

In our study, the prokaryotic predator *Bdellovibrio* works together with the host immune system, which would otherwise be overwhelmed by a Gram-negative infection. These biological experiments suggest that when tackling pathogenic AMR bacterial infections in a human medical setting, active predation and any associated/limited immune-stimulatory side effects can be beneficial as long as patient physiology and well-being can be supported. Future experiments will allow us to characterize the host immune response in more detail, determine how predators can be prepared with modified immune-stimulatory properties, and examine how multiple doses of predators can be applied in more long-lived infections. The data in this study represent key milestones in future use of *Bdellovibrio* as a “living antibiotic” in vivo, and they warrant further research into the development of predatory bacteria as an antibacterial agent for infected sites or wounds in higher vertebrates and, ultimately, humans. The strength of such prokaryotic-predator: eukaryotic-leukocyte combinations is an important therapeutic consideration as we move forward in responding to new Gram-negative bacterial threats.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, three figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.09.067.

**AUTHOR CONTRIBUTIONS**

S.M. and R.E.S. acted jointly as senior authors of this collaboration; in line with journal policy, S.M. has been designated as lead contact as the majority of the work by all authors was performed at his lab. A.R.W., S.M., and R.E.S. designed the research and carried out analysis along with C.M. A.R.W., R.E.S., M.M.-M., C.M., C.L., and S.K. performed experiments with supervision and assistance from S.M. R.T. constructed the fluorescent *Bdellovibrio* strains, and A.R.W., C.M., M.M.-M., and S.M. carried out zebrafish husbandry. A.R.W., S.M., and R.E.S. wrote the manuscript with assistance from C.M. and input from all authors.

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