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Predatory bacteria as living antibiotics – where are we now?

Robert J. Atterbury[1] and Jess Tyson[2,*]

Abstract

Antimicrobial resistance (AMR) is a global health and economic crisis. With too few antibiotics in development to meet current and anticipated needs, there is a critical need for new therapies to treat Gram-negative infections. One potential approach is the use of living predatory bacteria, such as *Bdellovibrio bacteriovorus* (small Gram-negative bacteria that naturally invade and kill Gram-negative pathogens of humans, animals and plants). Moving toward the use of *Bdellovibrio* as a 'living antibiotic' demands the investigation and characterization of these bacterial predators in biologically relevant systems. We review the fundamental science supporting the feasibility of predatory bacteria as alternatives to antibiotics.

Antibiotics have revolutionized modern medicine, but their effectiveness is threatened by the spread of multi-drug-resistant bacteria for which there is no available treatment [1–3]. Antimicrobial resistance (AMR), often driven by unnecessary antibiotic use, is a serious, global health and economic threat [4–7]. Of particular concern is the declining antibiotic pipeline with limited antibiotics in development (in both number and diversity) to meet current and anticipated patient demands [8–11]. Moreover, scientific and economic challenges have contributed to many large global pharmaceutical companies discontinuing their antibiotic development programmes [12], making the need for new approaches to infection control ever more exigent [13].

Over the last few years, fundamental scientific research into alternatives to antibiotics has gathered momentum. These include 'living antibiotics'; live agents such as bacteriophage (hereafter referred to as phage) or indeed their components [14], predatory bacteria and probiotics, and the competitive exclusion of pathogens [15, 16]. Whilst evaluation of all potential novel non-traditional approaches currently under investigation is beyond the scope of this review, their development and associated challenges has been the subject of recent publications and symposia [8, 16–23]. Most new technologies, particularly those which are disruptive to an industry, are subject to many technical and regulatory challenges, which in the case of 'living antibiotics' include activity spectrum, pharmacokinetics, immune response, manufacturing issues (including production, stability, storage and delivery), regulatory and quality-control frameworks and market acceptance. Individually, these potential non-traditional antibiotics may never entirely fill the vacuum left by antibiotics, however, they may prove to be invaluable in complimenting conventional chemotherapy, either used separately or in combination with them. As such, it is imperative that the research community debates their use and builds scientific and clinical evidence prior to their requirement, and there are encouraging signs that this is happening [18].

One novel approach to treat infections is the use of living predatory bacteria, such as *Bdellovibrio bacteriovorus*, small Gram-negative bacteria found ubiquitously in soil and aquatic environments, that naturally invades and kills other Gram-negative bacteria. The feasibility of using *Bdellovibrio* therapeutically revolves around answering several important questions: Against which pathogens is *Bdellovibrio* effective? Is *Bdellovibrio* harmful to the patient either directly or indirectly via products of predation? and Do prey develop resistance to *Bdellovibrio*? Although predatory bacteria have been researched by the academic community since the 1960s, recent work supported by DARPA's visionary and multidisciplinary pathogen predators programme has driven fundamental scientific research towards experimentally addressing these questions [24].

While there are many different predatory bacteria [25], this review chiefly focuses on *B. bacteriovorus* and the fundamental science supporting its potential translation.

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Author affiliations: [1]School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Leicestershire, LE12 5RD, UK; [2]School of Life Sciences, University of Nottingham, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH, UK.

***Correspondence:** Jess Tyson, jess.tyson@nottingham.ac.uk

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PREDATORY LIFESTYLE OF *B. BACTERIOVORUS*

B. bacteriovorus consumes its Gram-negative bacterial prey in a process that typically lasts 3–4 h. This predatory lifecycle is a complex process that is beginning to be understood at the molecular level [26–33]. Initially, *B. bacteriovorus* recognizes, attaches to [34] and enters the prey cell, reinforcing, traversing and re-sealing its entry port [35]. Invasion is accompanied by prey cell-rounding, establishment of a stable bdelloplast and, at this point, death of the prey cell [36]. Through the sequential release of an arsenal of enzymes [37], *B. bacteriovorus* digest the prey and use the resulting nutrient pool to grow as a long filament. Synchronous division of this single filament produces an odd or even number of progeny cells, which each develop flagella or gliding engines (depending on conditions), then burst from the dead prey cell and begin to seek new prey. Depending of the size of the prey cell, and hence the nutrients available from inside the prey bacterium, an average of 4–6 *B. bacteriovorus* progeny are released per cell [38]. Although recognized as an obligate predatory bacterium, *B. bacteriovorus* can switch to a host-independent lifestyle, demonstrating axenic growth on complete media [39].

WHY PREDATORY BACTERIA?

Several features of the *B. bacteriovorus* lifecycle and genome make it attractive as a potential therapeutic agent against Gram-negative bacterial pathogens. During the predatory lifecycle the prey cell is killed in a short time (<30 min) [40] and therefore the prey would have to express means of defence quickly enough to resist predation, something not yet seen [41]. Unlike some antibiotics, which can cause a cascade of events that lead to bacterial autolysis and release of inflammatory molecules [42], *B. bacteriovorus* predation does not result in the initial lysis of the prey, as the prey contents are consumed from within a stable bdelloplast structure prior to lysis. In addition, there is no single receptor for prey recognition and attachment. After prey invasion there is upregulation, both in number and functional diversity, of prey-destructive enzymes [43] with potential genetic redundancy [44], suggesting that simple prey resistance to predation by *B. bacteriovorus* is unlikely to occur.

EFFECT OF ADMINISTRATION OF *BDELLOVIBRIO* ON THE HOST RESPONSE

In order for the therapeutic potential of *Bdellovibrio* to be realized, predation of Gram-negative pathogens must be fully characterized in biologically relevant systems. Such characterization must address issues such as the host response, toxicity, inflammation, tissue damage or the inhibition of wound healing.

Some of these issues have been addressed using *in vitro* cell culture and *in vivo* animal models, evaluating the effect of predatory bacteria on both individual components of the immune system as well as the whole host. A number of human cell lines, including corneal-limbal epithelial cells, blood monocytes, macrophages, kidney epithelial cells, liver epithelial cells and spleen monocytes, have been exposed to different predatory bacteria, at a range of m.o.i. and duration (between 2 h and 24 h), and levels of pro- and anti-inflammatory cytokines measured [45–49]. Common to these studies were the pro- and anti-inflammatory cytokines IL1B, TNFa, IL6, IL8 and IL10; cytokines known to be stimulated in response to bacterial outer membrane lipopolysaccharide (LPS) [50], and essential for host defence against pathogens. These studies show that while *B. bacteriovorus* are not silent in terms of their immune response, the levels of inflammatory cytokines produced following exposure were negligible or low; *B. bacteriovorus* is not as immune-stimulatory as Gram-negative pathogens tested alongside [45, 46, 49], perhaps in part due to a unique lipid A structure [45], and its possession of sheathed flagellum [51]. Additional cell-viability imaging, cytotoxicity measurements and assessment of morphological changes to animal and human cultured cells on exposure to predatory bacteria preliminary indicate that *B. bacteriovorus* is non-toxic to human cells [46–48], although more studies are needed. Extending this for *B. bacteriorvorus*, Raghunathan and colleagues investigated the uptake, persistence and clearance of live predatory bacteria using a human macrophage cell line (U937 cells) [49]. By exposing U937 cells to fluorescently labelled *B. bacteriovorus*, Raghunathan and colleagues were able to microscopically visualize and count live intracellular bacteria. In parallel experiments, predatory bacteria engulfed by the U937 cells were recovered and enumerated following experimental lysis of the U937 cells. Assessing *Bdellovibrio* numbers post-administration is challenging, but these experimental approaches demonstrated that high numbers of predatory bacteria were able to survive up to 24 h inside cells (typically 14 bacteria per U937 cell (microscopy)), thereby defining a period of persistence and potential ability to prey on intracellular pathogens. *B. bacteriorvorus* had no measurable effect on the viability of the eukaryotic cell, supporting and amplifying findings from previous work [46–48]. In addition, by performing uptake experiments in the presence of pharmacological inhibitors, a role of the host actin cytoskeleton and its rearrangement in the uptake of *B. bacteriorvorus* was demonstrated. *B. bacteriorvorus* is ultimately trafficked via the phagolysosomal pathway, as evidenced by their targeting to acidic vacuoles [49]. This is supported by similar observations in a murine macrophage cell line [52], and both are important observations to consider for administration of *B. bacteriovorus* as a therapeutic. These studies illustrate the potential for *B. bacteriovorus* to target intracellular pathogens, which may be inaccessible to many antibiotics and other biological control agents such as phage; although this latter point is under active investigation [53–55]. These *in vitro* cellular experiments, along with animal models described below, have been important steps to investigate efficacy, lack of toxicity and potential predator bioavailability.

Early *in vivo* host response focussed on the viability of *Bdellovibrio* sp. in the intestines of endothermic and exothermic vertebrates. Westergaard and Kramer [56] investigated the viability and persistence of *B. bacteriovorus* strain MS7

experimentally administered in the intestines of catfish (*Ictalurus punctatus*), leopard frogs (*Rana pipiens*), mice and rabbits showing little or no recovery of *B. bacteriovorus* 24–48 h post-inoculation. *B. bacteriovorus* offered to mice via drinking water for 3 days could not be recovered at all from the intestinal tract. Likewise, either none or very few *B. bacteriovorus* were recovered from rabbit ileal loops approximately 24 h post-injection, regardless of whether axenic cultures were used, or *Escherichia coli* prey were co-injected. Importantly, *B. bacteriovorus* was shown to be non-pathogenic to the animals.

This early work of Westergaard and Kramer has been extended by several groups using experimental animal models. Atterbury *et al*. found that *B. bacteriovorus* did not produce any noticeable signs of disease when chickens were dosed with axenic cultures of *B. bacteriovorus* HD100 [57]. Administration of *B. bacteriovorus* was shown to be non-toxic and non-immunogenic to the ocular surface of rabbit eyes [58], and did not interfere with wound healing in a *Galleria* (wax moth larvae) model [46]. These studies have been extended to include assessments of host morbidity, histopathology, levels of pro- and anti-inflammatory cytokines, predatory bacterial dissemination in the body and long-term assessments of general health in rat [59–61], mouse [52, 62] and zebrafish larval models [63]. Overall, *in vivo* administration of *B. bacteriovorus* in a range of animal models and via a number of routes of administration has demonstrated that they are not detrimental to the health of these animals.

EXAMINING THE PREY RANGE OF *B. BACTERIOVORUS*: *IN VITRO* AND *IN VIVO* PREDATION

In vitro efficacy of *B. bacteriovorus* has been demonstrated against a wide range of prey including bacteria associated with gut, oral, wound and ocular infections [46, 64–66] and bioterrorism agents [67]. *B. bacteriovorus* successfully reduce pathogen numbers in both laboratory buffer and human serum [68] and against prey in biofilms [69–71], which are often recalcitrant to antibiotic treatment [72]. Importantly, a number of multi-drug-resistant human clinical isolates have been shown to be susceptible to predation by *B. bacteriovorus* [46, 65, 68, 70, 73, 74]; this list encompasses a number of the E(S)KAPE pathogens for which new treatments are needed [6] including Gram-negative colistin-resistant isolates expressing mcr-1 [75]. Work *in vitro* has also investigated the ratio of *B. bacteriovorus* to prey required for successful predation [68]; an important consideration for future *in vivo* experiments and clinical translation for use as a therapeutic if antibiotics fail to treat the infection.

While experimental *in vitro* systems, especially laboratory buffer or growth medium-rich environments may demonstrate predatory efficacy, they are an inadequate substitute for the complexities of *in vivo* therapy, which must take into account the host immune response and pathogen survival and subversion tactics [76–78]. Animal infection models have been instrumental in assessing both the ability of *B. bacteriovorus* to prey upon Gram-negative bacteria *in vivo*, as well as addressing any host response (and therefore safety) to such administration (see Effect of administration of *Bdellovibrio* on the host response), adding to the growing evidence of the feasibility of using predatory bacteria for therapeutic applications, at least in animals and in the future extending this to humans.

Atterbury *et al*. [57] administered *B. bacteriovorus* HD100 orally to groups of chickens, which had been experimentally infected with *Salmonella* Enteritidis p125109. In the 3 days following *B. bacteriovorus* treatment, *Salmonella* numbers in the caeca of *B. bacteriovorus*-treated birds were significantly reduced compared with control animals. Moreover, the appearance of the caeca in *B. bacteriovorus*-treated birds was normal, compared with many of the caeca from control animals, which showed clear evidence of typhilitis consistent with *Salmonella* infection. Interestingly, *B. bacteriovorus* treatment in these birds was associated with a higher mean weight (although not statistically significant) than buffer-treated groups and also resulted in changes to native bacterial populations in the caeca. *B. bacteriovorus* were not isolated from the caeca of treated birds at the end of the trial, suggesting that *B. bacteriovorus* was a relatively short-lived population in the environment of the chicken gut. This study was also crucial in priming future studies considering any potential change in bacterial composition of the gut microbiome that would be associated with an administration of such a therapeutic.

Further important studies have demonstrated successful *in vivo* predation of pathogens by *B. bacteriovorus*. Using a rat model, Shatzkes and colleagues administered *B. bacteriovorus* 109J in a series of four intranasal doses to treat rats experimentally infected with *Klebsiella pneumoniae* in the lungs, showing that pathogen burden was significantly reduced in *B. bacteriovorus*-treated animals compared to control groups [59]. Shatzkes *et al*. [60] further investigated the use of *B. bacteriovorus* 109J to treat *K. pneumoniae* infections in rats, but this time with a septicaemic model of disease initiated by injection into the tail veins of rats. *B. bacteriovorus* were unable to significantly reduce the infection. This was the first administration of *B. bacteriovorus* directly into the bloodstream, and an important step in assessing the ability of predatory bacteria to clear bloodstream infections.

The Mostowy and Sockett laboratories employed a zebrafish larval localized (hindbrain) infection and predator treatment model [63, 79, 80]. Along with extensive genomic homology to humans [81], the physical characteristics of zebrafish larvaeinclude a well-understood, fully developed immune system and optically transparent nature which lends itself well to innovative live fluorescent microscopy. This means the larval model has great translational potential especially with regard to studying infection and treatment kinetics [80, 82]. Using live-cell imaging and bacterial enumeration, Willis *et al*. demonstrated successful *in vivo* predation of *Shigella flexneri* by *B. bacteriovorus* [63]. Using fluorescently labelled (GFP) *Shigella flexneri* and mCherry-labelled *B. bacteriovorus*, the authors were able to observe the *in vivo*

behaviours of both bacteria over time. In the absence of treatment, zebrafish larvae showed increasing GFP fluorescence over time. Upon treatment with *B. bacteriovorus*, this pathogen-associated GFP fluorescence was diminished. This was supported by enumeration of both bacterial species and confocal microscopy evidence of live bacterial predation inside zebrafish larvae, demonstrating that injection of *B. bacteriovorus* reduces otherwise lethal antibiotic-resistant Gram-negative pathogen *Shigella flexneri* numbers by active predation. This work has now been extended to include two additional antibiotic-resistant E(S)KAPE pathogens and expanded to simultaneously quantify *in vivo* predation, zebrafish host response and development of a mathematical model of infection and cure (Jess Tyson and Liz Sockett personal communication; manuscript submitted).

In a recent study, Russo and colleagues [83] demonstrated that *B. bacteriovorus* 109J could be used to significantly reduce *Yersinia pestis* numbers in the lungs of experimentally infected mice. In a separate study, groups of mice (SKH-1) were pre-treated with *B. bacteriovorus* via intraperitoneal injection, then infected with a lethal challenge of *Y. pestis* [84]. Following this, mice were treated daily with *B. bacteriovorus* and the spread of *Y. pestis* was tracked with *in vitro* imaging. *B. bacteriovorus* treatment was associated with significantly lower levels of *Y. pestis*, measured both by luciferase signal and by viable counts of the bacteria from the spleen at the end of the experiment. Interestingly, no protection was recorded for Balb/c mice infected with *Y. pestis* then treated with *B. bacteriovorus*, suggesting that the protective effect may be dependent on the genetic or immune background of the animal.

Not all *in vivo* administration of *B. bacteriovorus* has been successful in reducing pathogen load. Despite promising results *in vitro* using a tissue culture model in a prior investigation [85], administration of *B. bacteriovorus* to treat calves experimentally infected with *Moraxella bovis*, a causative agent of infectious bovine keratoconjunctivitis, failed to result in significant improvement in corneal ulcer formation compared with untreated control animals [86]. Although an important investigation, the authors themselves state that there are limitations to the study, and perhaps treatment failure could be explained by the observation that they were unable to record persistence of *B. bacteriovorus* in the tears of calves in this experimental model, despite having previously shown *B. bacteriovorus* persistence on the corneal surface.

As seen from the extensive research above, there is mounting evidence that *Bdellovibrio* sp. persist non-pathogenically and long enough to be therapeutically active, are not highly immune-stimulatory, have minimal adverse effects on the bacterial microbiota, and are not known to become part of normal host microbiota [56, 57, 61, 63]. Both successful and unsuccessful *in vivo* assays such as these reviewed here are vital for assessing what indications, routes of administration (topical versus intravenous) and sites of infection, a potential predatory bacterial administration would be most effective against.

UNDERSTANDING THE RELATIONSHIP OF *BDELLOVIBRIO* PREDATION AND HOST RESPONSE

The clinical outcome of an infection in animals and humans following therapy depends on the interaction of factors such as the patient's immune system and general health, the nature of the pathogen and the type and delivery of treatment. In considering *in vitro* and *in vivo* bacterial predation as separate entities to host response, the interaction and cooperation between bacterial predation of a Gram-negative pathogen and response of the host is not captured. The zebrafish hindbrain infection model has been instrumental in exploring this [63]. This model demonstrated that *B. bacteriovorus* persisted non-pathogenically for a sufficient duration to effectively prey on *Shigella* during an experimental infection [63]. It was in this work that interactions with cells of the host immune system were observed microscopically. Moreover, through the use of Pu.1 morpholinos to knockdown host macrophages and neutrophils, this study found that the maximal therapeutic benefits of *B. bacteriovorus* resulted from the synergy of bacterial predation and the host immune system. Post-administration, the predatory bacteria population is ultimately cleared by interaction with the host's immune system [49, 63] and indeed this clearance may be of benefit in (self-) limiting the treatment. By employing RNAseq analysis in a zebrafish model of infection and predatory cure, we are beginning to understand this complex relationship (Jess Tyson and Liz Sockett personal communication; manuscript submitted).

COMPARISON TO OTHER MICROBIOLOGICAL ANTIBACTERIAL APPROACHES

In thinking about treatment of infections with predatory bacteria, researchers can look to the phage research and clinical community for motivation in addressing challenges from *in vitro* to clinical setting. Although in use for years in some countries [87, 88] recent high-profile cases have demonstrated the successful administration of phage cocktails in treating drug-resistant infections in a clinical setting [89, 90].

Superficially, *B. bacteriovorus* and phage share many characteristics as potential biological control agents. They both exhibit predatory or parasitic lifecycles, they are self-replicating and self-limiting, persisting only while susceptible hosts or prey are present and, in the case of lytic phage, both have a broadly similar lifecycle. They also appear to have little or no adverse effect when introduced into the bodies of animals and humans. However, there are some notable differences between the two, which may restrict their use in certain indications.

Firstly, *B. bacteriovorus* are unequivocally 'living' and metabolically active, compared with phage, which are inert particles until they establish contact with a host via a specific receptor. This may be both an advantage and disadvantage for *B. bacteriovorus* in that it may use energy-requiring processes such as the active 'locating' of prey that are unavailable to phages. However, if no prey is found, *B. bacteriovorus* cannot

enter a dormant state and will die. Conversely, phages can remain 'viable' for many years without expending any energy, but are unable to actively pursue their hosts. They must rely on stochastic probability to encounter their hosts, and compensate for this comparative inefficiency by replicating more quickly than *B. bacteriovorus* and with a much greater burst size. Previous studies with *Salmonella* typhi and *E. coli* have shown that phage do not always eliminate the whole population of their hosts [91]. Often in these cases, the titres of phage and host increase and decline with characteristic out-of-phase population oscillations [92]. This phenomenon has also been described in predatory protozoa [92] and *Bdellovibrio* [91, 93]. As such, the replication of both *B. bacteriovorus* and phage may be subject to a minimum host/prey threshold, below which they cannot sustain their populations indefinitely. However, this may be therapeutically beneficial as elimination of a pathogen is often not necessary to achieve significant reductions or complete resolution of disease symptoms [63, 94]. The host range of an individual phage is usually restricted to a number of strains within a species or one or two closely related species of bacteria, which makes the maintenance of their populations more challenging than *B. bacteriovorus*. The host specificity and rapid acquisition of bacterial resistance to phage needs to be considered; the broad bacterial prey range and the lack of simple resistance mechanisms of predatory bacteria give it an advantage in comparison. Interestingly, recent work by Hobley and colleagues has highlighted the combinatorial power of using predatory bacteria alongside phage [95]; the separate kinetics of phage susceptibility and *Bdellovibrio* predation allowed greater bacterial cell killing of *E. coli* in certain conditions.

Both *B. bacteriovorus* and phages will encounter challenging conditions when introduced into the body of endothermic animals. Phage are more likely to be resilient when encountering harsh physical and chemical conditions, such as higher temperatures and extremes of pH, compared with *B. bacteriovorus*. Phage can cross the blood-brain barrier but may not be able to persist intracellularly without deliberate modification. Indeed, previous studies suggest that when they enter the bloodstream they can be quickly sequestered by the reticuloendothelial system [96]. This may be countered, at least in some cases, with the serial passage of phage in mammals to re-isolate phage capable of circulating in mammals for long periods of time [96]. Phages are known to be capable of transferring DNA between bacteria through generalized and specialized transduction, which is a risk that does not apply to *B. bacteriovorus* [37]. This risk can be mitigated by careful screening of phage genomes to remove particular phage possessing integrases for example, and others which may harbour genes encoding bacterial toxins or other virulence factors.

The therapeutic application of *Bdellovibrio* and phages require the industrial production of both. This presents different challenges for each, as phages can be produced in much greater numbers and more quickly, but their production may require the co-culturing of large quantities of pathogenic host bacteria. Conversely, non-pathogenic hosts of *Bdellovibrio* such as *E. coli* and *P. putida* can be used, but these would need to be efficiently separated from the final therapeutic preparation. In addition, maintaining the viability of a *Bdellovibrio* preparation is likely to be more challenging than for phage. It will be interesting to see what lessons can be learnt from the technical challenges of producing therapeutic preparations of these biocontrol agents [97], and also determining whether any synergy can be achieved when using them together as treatments.

TRANSLATING FUNDAMENTAL SCIENCE INTO APPLICATION TO MEET THE CHALLENGE OF AMR

Academic researchers, including most recently some working on the DARPA pathogen predators programme, have transformed the research field and demonstrated the potential of using whole live predatory *B. bacteriovorus* to kill a broad range of antimicrobial-resistant (AMR) clinical pathogens *in vitro* and *in vivo*; supporting further its promise as a therapeutic. To be considered as a credible therapeutic option, we need compelling evidence from future human trials that this treatment is effective, has no (or minimal) negative effect on the patient and has some advantage over existing treatments, at least in specific circumstances. We need to demonstrate clinical value and measure treatment effect to illustrate the benefit to patients. Future economic and regulatory framework discussions for non-traditional approaches need to be fulfilled, and whilst beyond the scope of this review, momentum is gathering in this area [9, 12, 22] (follow http://amr.solutions/blog-index.html for updates, comprehensive expert analysis and opinion). But do we have all the information we need? and if not, what still needs to be addressed?

Informed by *in vivo* animal models, we need to move towards both safety and efficacy trials in humans. Animal models are crucial, but have limitations, with the protocols to date administering predatory bacteria prior to, or soon after, the pathogen of interest. Investigation of more established infections is needed, with support for defining predatory bacteria dose range (and possibly pathogen numbers), dose number and schedule required to treat the infection. Perhaps surprisingly, knowledge on the actual numbers of pathogens at different human bodily sites during infections and recovery is still very much a developing field. Development of large-scale predator growth and purification methods are currently limited [98] and need expanding for larger-scale safety and efficacy trials. Infection treatment is time critical therefore an assessment of stability, long-term storage and delivery of a live, predatorily-active therapeutic needs further work.

What is an appropriate clinical model? What does the treatment endpoint look like? We need to define the indications in which an advantage of using predatory bacteria can be seen. Not every infection may be accessible to predatory bacteria – evidence from animal models to date suggests topical or localized rather that systemic applications would

be more amenable. We need to define what constitutes a successful treatment outcome and for this we may need to measure pathogen levels and debate whether it is sufficient to reduce pathogen numbers to a level at which a healthy human immune system will clear the infection itself [99, 100].

Further work is needed to evaluate the dissemination of predatory bacteria from the administration site, along with determining any long-term effects of exposure on the host or their resident microbiota. Animal models and the ability to recover and count live predators in cellular models are important milestones in assessing the effect of predatory bacterial treatment on the host, its bioavailability and dissemination. It would be interesting to expand this work to visualize the detailed subcellular location of *B. bacteriovorus* in the presence of a pathogen [101] to inform the availability to prey on intracellular pathogens.

In conclusion, it may seem counter intuitive to treat a bacterial infection with administration of another bacterium, and this requires a paradigm shift. The potential effects of treating a Gram-negative bacterial infection with administration of a live predatory bacterium must be considered in context with the burden of an infection on the host. Although non-replicating in the absence of prey, an immune response, albeit a low one, to the presence of *B. bacteriovorus* carrying out predation, would be expected as part of the treatment, but this may be actually beneficial. However, faced with an otherwise untreatable Gram-negative infection, could this 'cost' be considered acceptable? One final note once the scientific community has addressed the scientific challenges ahead of their requirement, we are faced with a dilemma to use them as a therapeutic or keep them in reserve. Thinking about the striking analogy to fire prevention, and of antibiotics being the fire extinguishers of modern medicine [102] that are developed and hopefully never used, should we preserve and have *B. bacteriovorus* ready and available should a fire start?

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. WHO. 2020. Antimicrobial resistance. https://www.who.int/health-topics/antimicrobial-resistance
2. Livermore DM. Has the era of untreatable infections arrived? *J Antimicrob Chemother* 2009;64:i29–36.
3. CDC. Antibiotic resistance threats in the United States. http://www.cdc.gov/DrugResistance/Biggest-Threats.html 2019.
4. O'Neill J. The review on antimicrobial resistance. tackling drug-resistant infections globally: final report and recommendations. https://amr-review.org/Publications.html 2016.
5. Morehead MS, Scarbrough C. Emergence of global antibiotic resistance. *Prim Care* 2018;45:467–484.
6. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M *et al*. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis* 2018;18:318–327.
7. Laxminarayan R, Van Boeckel T, Frost I, Kariuki S, Khan EA *et al*. The Lancet infectious diseases Commission on antimicrobial resistance: 6 years later. *Lancet Infect Dis* 2020;20:e51–e60.
8. The Pew Charitable Trusts A Scientific Roadmap for Antibiotic Discovery. https://www.pewtrusts.org/en/research-and-analysis/reports/2016/05/a-scientific-roadmap-for-antibiotic-discovery 2016.
9. Luepke KH, Suda KJ, Boucher H, Russo RL, Bonney MW *et al*. Iii past, present, and future of antibacterial economics: increasing bacterial resistance, limited antibiotic pipeline, and societal implications. *Pharmacotherapy* 2017;37:71–84.
10. Hesterkamp T. *Antibiotics Clinical Development and Pipeline. How to Overcome the Antibiotic Crisis Current Topics in Microbiology and Immunology*, 398. Cham: Springer; 2015. pp. 447–474.
11. Livermore DM. On behalf of the British Society for antimicrobial chemotherapy Working Party on the urgent need: regenerating antibacterial drug discovery and development B, Martin, Carrs O, Cassell G, fishman N, a Guidos R, et al. discovery research: the scientific challenge of finding new antibiotics. *J Antimicrob Chemother* 2011;66:1941–1944.
12. Ardal C, Balasegaram M, Laxminarayan R, McAdams D, Outterson K *et al*. Antibiotic development - economic, regulatory and societal challenges. *Nat Rev Microbiol.*2019.
13. Sharland MGS, Huttner B, Moja L, Pulcini C, Zeng M *et al*. Eml expert Committee and antibiotic Working Group. encouraging AWaRe-ness and discouraging inappropriate antibiotic use-the new 2019 essential medicines list becomes a global antibiotic stewardship tool. *Lancet Infect Dis* 2019;19:1278–1280.
14. Lukacik P, Barnard TJ, Keller PW, Chaturvedi KS, Seddiki N *et al*. Structural engineering of a phage lysin that targets gram-negative pathogens. *Proc Natl Acad Sci U S A* 2012;109:9857–9862.
15. Allen HK, Trachsel J, Looft T, Casey TA. Finding alternatives to antibiotics. In: Bush K (editor). *Antimicrobial Therapeutics Reviews: Infectious Diseases of Current and Emerging Concern*, 13232014. Annals of the New York Academy of Sciences. pp. 91–100.
16. Ghosh C, Sarkar P, Issa R, Haldar J. Alternatives to conventional antibiotics in the era of antimicrobial resistance. *Trends in Microbiology* 2019;27:323–338.
17. Duke-Margolis Center for Health Policy. Understanding development challenges associated emerging non-traditional-antibiotics. https://healthpolicy.duke.edu/events/understandingdevelopment-challenges-associated-emerging-non-traditional-antibiotics 2018.
18. Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H *et al*. Alternatives to antibiotics-a pipeline Portfolio review. *Lancet Infect Dis* 2016;16:239–251.
19. Tse BN, Adalja AA, Houchens C, Larsen J, Inglesby TV *et al*. Challenges and opportunities of nontraditional approaches to treating bacterial infections. *Cli Infect Dis* 2017;65:495–500.
20. Rello J, Parisella FR, Perez A. Alternatives to antibiotics in an era of difficult-to-treat resistance: new insights. *Expert Rev Clin Pharmacol* 2019;12:635–642.
21. Theuretzbacher U, Piddock LJV. Non-traditional antibacterial therapeutic options and challenges. *Cell Host Microbe* 2019;26:61–72.
22. Rex JH, Fernandez Lynch H, Cohen IG, Darrow JJ, Outterson K. Designing development programs for non-traditional antibacterial agents. *Nat Commun* 2019;10:3416.

23. Theuretzbacher U, Outterson K, Engel A, Karlén A. The global preclinical antibacterial pipeline. *Nat Rev Microbiol* 2020;18:275–285.
24. DARPA. Pathogen predators program. https://www.darpa.mil/program/pathogenpredators 2015.
25. Pérez J, Moraleda-Muñoz A, Marcos-Torres FJ, Muñoz-Dorado J. Bacterial predation: 75 years and counting! *Environ Microbiol* 2016;18:766–779.
26. Negus D, Moore C, Baker M, Raghunathan D, Tyson J *et al*. Predator versus pathogen: how does predatory Bdellovibrio bacteriovorus interface with the challenges of killing gram-negative pathogens in a host setting? *Annu Rev Microbiol* 2017;71:441–457.
27. Laloux G. Shedding light on the cell biology of the predatory bacterium *Bdellovibrio bacteriovorus*. *Front Microbiol* 2020;10:3136.
28. Sockett RE. Predatory lifestyle of Bdellovibrio bacteriovorus. *Annu Rev Microbiol* 2009;63:523–539.
29. Milner DS, Ray LJ, Saxon EB, Lambert C, Till R *et al*. DivIVA controls progeny morphology and diverse para proteins regulate cell division or gliding motility in Bdellovibrio bacteriovorus. *Front Microbiol* 2020;11:542.
30. Meek RW, Cadby IT, Moynihan PJ, Lovering AL. Structural basis for activation of a diguanylate cyclase required for bacterial predation in Bdellovibrio. *Nat Commun.* 2019;10:4086.
31. Bratanis E, Andersson T, Lood R, Bukowska-Faniband E. Biotechnological potential of Bdellovibrio and like organisms and their secreted enzymes. *Front Microbiol* 2020;11:662.
32. Harding CJ, Huwiler SG, Somers H, Lambert C, Ray LJ *et al*. A lysozyme with altered substrate specificity facilitates prey cell exit by the periplasmic predator Bdellovibrio bacteriovorus. *Nat Commun* 2020;11:4817.
33. Caulton SG, Lovering AL. Bacterial invasion and killing by predatory Bdellovibrio primed by predator prey cell recognition and self protection. *Curr Opin Microbiol* 2020;56:74–80.
34. Evans KJ, Lambert C, Sockett RE. Predation by *Bdellovibrio bacteriovorus* HD100 requires type IV pili. *J Bacteriol Res* 2007;189:4850–4859.
35. Kuru E, Lambert C, Rittichier J, Till R, Ducret A *et al*. Fluorescent D-amino-acids reveal bi-cellular cell wall modifications important for Bdellovibrio bacteriovorus predation. *Nat Microbiol* 2017;2:1648–1657.
36. Im H, Kim D, Ghim C-M, Mitchell RJ. Shedding light on microbial Predator–Prey population dynamics using a quantitative bioluminescence assay. *Microbial Ecology* 2014;67:167–176.
37. Rendulic S, Jagtap P, Rosinus A, Eppinger M, Baar C *et al*. A predator unmasked: life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. *Science* 2004;303:689–692.
38. Fenton AK, Kanna M, Woods RD, Aizawa SI, Sockett RE. Shadowing the actions of a predator: backlit fluorescent microscopy reveals synchronous nonbinary septation of predatory Bdellovibrio inside prey and exit through discrete bdelloplast pores. *J Bacteriol* 2010;192:6329–6335.
39. Roschanski N, Klages S, Reinhardt R, Linscheid M, Strauch E. Identification of genes essential for prey-independent growth of Bdellovibrio bacteriovorus HD100. *J Bacteriol* 2011;193:1745–1756.
40. Rittenberg SC, Shilo M. Early host damage in the infection cycle of *Bdellovibrio bacteriovorus*. *J Bacteriol* 1970;102:149–160.
41. Lambert C, Ivanov P, Sockett RE. A transcriptional "Scream" early response of *E. coli* prey to predatory invasion by Bdellovibrio. *Curr Microbiol* 2010;60:419–427.
42. Wolf AJ, Liu GY, Underhill DM. Inflammatory properties of antibiotic-treated bacteria. *J Leukoc Biol* 2017;101:127–134.
43. Lambert C, Chang CY, Capeness MJ, Sockett RE. The first bite-profiling the predatosome in the bacterial pathogen Bdellovibrio. *PLoS One* 2010;5:e8599.
44. Duncan MC, Gillette RK, Maglasang MA, Corn EA, Tai AK *et al*. High-Throughput analysis of gene function in the bacterial predator Bdellovibrio bacteriovorus. *mBio* 2019;10.
45. Schwudke D, Linscheid M, Strauch E, Appel B, Zahringer U *et al*. The obligate predatory Bdellovibrio bacteriovorus possesses a neutral lipid A containing alpha-D-Mannoses that replace phosphate residues: similarities and differences between the lipid as and the lipopolysaccharides of the wild type strain B. bacteriovorus HD100 and its host-independent derivative HI100. *J Biol Chem* 2003;278:27502–27512.
46. Shanks RMQ, Davra VR, Romanowski EG, Brothers KM, Stella NA *et al*. An eye to a kill: using predatory bacteria to control gram-negative pathogens associated with ocular infections. *PLoS ONE* 2013;8:e66723.
47. Monnappa AK, Bari W, Choi SY, Mitchell RJ. Investigating the responses of human epithelial cells to predatory bacteria. *Scientific Reports* 2016;6.
48. Gupta S, Tang C, Tran M, Kadouri DE. Effect of predatory bacteria on human cell lines. *Plos One* 2016;11.
49. Raghunathan D, Radford PM, Gell C, Negus D, Moore C *et al*. Engulfment, persistence and fate of Bdellovibrio bacteriovorus predators inside human phagocytic cells informs their future therapeutic potential. *Scientific Reports* 2019;9.
50. Rossol M, Heine H, Meusch U, Quandt D, Klein C *et al*. LPS-induced cytokine production in human monocytes and macrophages. *Crit Rev Immunol* 2011;31:379–446.
51. Seidler RJ, Starr MP. Structure of the flagellum of Bdellovibrio bacteriovorus. *J Bacteriol* 1968;95:1952–1955.
52. Findlay JS, Flick-Smith HC, Keyser E, Cooper IA, Williamson ED *et al*. Predatory bacteria can protect SKH-1 mice from a lethal plague challenge. *Scientific Reports* 2019;9.
53. Huh H, Wong S, St Jean J, Slavcev R. Bacteriophage interactions with mammalian tissue: therapeutic applications. *Adv Drug Deliv Rev* 2019;145:4–17.
54. Moller-Olsen C, SFS H, Shukla RD, Feher T, Sagona AP. Engineered K1F bacteriophages kill intracellular Escherichia coli K1 in human epithelial cells. *Sci Rep* 2018;8:17559.
55. Zhang L, Sun L, Wei R, Gao Q, He T *et al*. Intracellular *Staphylococcus aureus* control by virulent bacteriophages within MAC-T bovine mammary epithelial cells. *Antimicrob Agents Chemother* 2017;61.
56. Westergaard JM, Kramer TT. Bdellovibrio and the intestinal flora of vertebrates. *Appl Environ Microbiol* 1977;34:506–511.
57. Atterbury RJ, Hobley L, Till R, Lambert C, Capeness MJ *et al*. Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and *Salmonella* colonization of young chicks. *Appl Environ Microbiol* 2011;77:5794–5803.
58. Romanowski EG, Stella NA, Brothers KM, Yates KA, Funderburgh ML *et al*. Predatory bacteria are nontoxic to the rabbit ocular surface. *Scientific Reports* 2016;6.
59. Shatzkes K, Singleton E, Tang C, Zuena M, Shukla S *et al*. Predatory bacteria attenuate Klebsiella pneumoniae burden in rat lungs. *mBio* 2016;7.
60. Shatzkes K, Singleton E, Tang C, Zuena M, Shukla S *et al*. Examining the efficacy of intravenous administration of predatory bacteria in rats. *Scientific Reports* 2017;7.
61. Shatzkes K, Tang C, Singleton E, Shukla S, Zuena M *et al*. Effect of predatory bacteria on the gut bacterial microbiota in rats. *Scientific Reports*2017;7.
62. Shatzkes K, Chae R, Tang C, Ramirez GC, Mukherjee S *et al*. Examining the safety of respiratory and intravenous inoculation of Bdellovibrio bacteriovorus and Micavibrio aeruginosavorus in a mouse model. *Scientific Reports* 2015;5.
63. Willis AR, Moore C, Mazon-Moya M, Krokowski S, Lambert C *et al*. Injections of predatory bacteria work alongside host immune cells to treat Shigella infection in zebrafish larvae. *Curr Biol* 2016;26:3343–3351.
64. Dashiff A, Junka RA, Libera M, Kadouri DE. Predation of human pathogens by the predatory bacteria Micavibrio aeruginosavorus and Bdellovibrio bacteriovorus. *J Appl Microbiol* 2011;110:431–444.

65. Kadouri DE, To K, Shanks RMQ, Doi Y. Predatory bacteria: a potential ally against multidrug-resistant gram-negative pathogens. *PLoS ONE* 2013;8:e63397.
66. Patini R, Cattani P, Marchetti S, Isola G, Quaranta G *et al*. Evaluation of predation capability of Periodontopathogens bacteria by Bdellovibrio bacteriovorus HD100. An in vitro study. *Materials* 2019;12:E2008.
67. Russo R, Chae R, Mukherjee S, Singleton EJ, Occi JL *et al*. Susceptibility of select agents to predation by predatory bacteria. *Microorganisms* 2015;3:903–912.
68. Baker M, Negus D, Raghunathan D, Radford P, Moore C *et al*. Measuring and modelling the response of Klebsiella pneumoniae KPC prey to Bdellovibrio bacteriovorus predation, in human serum and defined buffer. *Scientific Reports* 2017;7.
69. Dashiff A, Kadouri DE. Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J. *Mol Oral Microbiol* 2011;26:19–34.
70. Sun Y, JZ Y, Hou YB, Chen HL, Cao JM *et al*. Predation efficacy of Bdellovibrio bacteriovorus on multidrug-resistant clinical pathogens and their corresponding biofilms. *Jpn J Infect Dis* 2017;70:485–489.
71. Kadouri D, O'Toole GA. Susceptibility of biofilms to Bdellovibrio bacteriovorus attack. *Appl Environ Microbiol* 2005;71:4044–4051.
72. Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW. The physiology and collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol* 2002;46:202–256.
73. Im H, Choi SY, Son S, Mitchell RJ. Combined application of bacterial predation and violacein to kill polymicrobial pathogenic communities. *Scientific Reports* 2017;7:14415.
74. Im H, Son S, Mitchell RJ, Ghim C-M. Serum albumin and osmolality inhibit Bdellovibrio bacteriovorus predation in human serum. *Scientific Reports* 2017;7:5896.
75. Dharani S, Kim DH, Shanks RMQ, Doi Y, Kadouri DE. Susceptibility of colistin-resistant pathogens to predatory bacteria. *Res Microbiol* 2018;169:52–55.
76. Bengoechea JA, Sa Pessoa J. *Klebsiella pneumoniae* infection biology: living to counteract host defences. *FEMS Microbiology Review* 2019;43:123–144.
77. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA *et al*. Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. *Science* 2014;343:204–208.
78. Donnenberg MS. Pathogenic strategies of enteric bacteria. *Nature* 2000;406:768–774.
79. Torraca V, Mostowy S. Zebrafish infection: from pathogenesis to cell biology. *Trends Cell Biol* 2018;28:143–156.
80. Gomes MC, Mostowy S. The case for modeling human infection in zebrafish. *Trends Microbiol* 2020;28:10–18.
81. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C *et al*. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 2013;496:498–503.
82. Meijer AH, Spaink HP. Host-Pathogen interactions made transparent with the zebrafish model. *Curr Drug Targets* 2011;12:1000–1017.
83. Russo R, Kolesnikova I, Kim T, Gupta S, Pericleous A *et al*. Susceptibility of virulent Yersinia pestis bacteria to predator bacteria in the lungs of mice. *Microorganisms* 2019;7.
84. Findlay JS, Flick-Smith HC, Keyser E, Cooper IA, Williamson ED *et al*. Predatory bacteria can protect SKH-1 mice from a lethal plague challenge. *Sci Rep* 2019;9:7225.
85. Boileau MJ, Clinkenbeard KD, Iandolo JJ. Assessment of Bdellovibrio bacteriovorus 109J killing of Moraxella bovis in an in vitro model of infectious bovine keratoconjunctivitis. *Can J Vet Res* 2011;75:285–291.
86. Boileau MJ, Mani R, Breshears MA, Gilmour M, Taylor JD *et al*. Efficacy of Bdellovibrio bacteriovorus 109J for the treatment of dairy calves with experimentally induced infectious bovine keratoconjunctivitis. *Am J Vet Res* 2016;77:1017–1028.
87. Kutateladze M, Adamia R. Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends Biotechnol* 2010;28:591–595.
88. Aslam S, Schooley RT. What's old is new again: bacteriophage therapy in the 21st century. *Antimicrob Agents Chemother* 2019;64.
89. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J *et al*. Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant Acinetobacter baumannii infection. *Antimicrobial Agents and Chemotherapy* 2017;61.
90. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K *et al*. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant Mycobacterium abscessus. *Nat Med* 2019;25:730–733.
91. Alexander M. Why microbial predators and parasites do not eliminate their prey and hosts. *Annu Rev Microbiol* 1981;35:113–133.
92. van den Ende P. Predator-prey interactions in continuous culture. *Science* 1973;181:562–564.
93. Varon M. Selection of predation-resistant bacteria in continuous culture. *Nature* 1979;277:386–388.
94. Bhandare S, Colom J, Baig A, Ritchie JM, Bukhari H *et al*. Reviving phage therapy for the treatment of cholera. *J Infect Dis* 2019;219:786–794.
95. Hobley L, Summers JK, Till R, Milner DS, Atterbury RJ *et al*. Dual predation by bacteriophage and *Bdellovibrio bacteriovorus* can eradicate *Escherichia coli* prey in situations where single predation cannot. *J Bacteriol* 2020;202.
96. Merril CR, Biswas B, Carlton R, Jensen NC, Creed GJ *et al*. Long-circulating bacteriophage as antibacterial agents. *Proc Natl Acad Sci U S A* 1996;93:3188–3192.
97. Garcia R, Latz S, Romero J, Higuera G, Garcia K *et al*. Bacteriophage production models: an overview. *Front Microbiol* 2019;10:1187.
98. Boileau MJ, Mani R, Clinkenbeard KD. Lyophilization of Bdellovibrio bacteriovorus 109J for long-term storage. *Curr Protoc Microbiol* 2017;45:7B 3 1-7B 3 15.
99. Robson MC, Mannari RJ, Smith PD, Payne WG. Maintenance of wound bacterial balance. *Am J Surg* 1999;178:399–402.
100. Robson MC. Wound infection. A failure of wound healing caused by an imbalance of bacteria. *Surg Clin North Am* 1997;77:637–650.
101. Greenwood DJ, Dos Santos MS, Huang S, Russell MRG, Collinson LM *et al*. Subcellular antibiotic visualization reveals a dynamic drug reservoir in infected macrophages. *Science* 2019;364:1279–1282.
102. Rex JH, Outterson K. Antibiotic reimbursement in a model delinked from sales: a benchmark-based worldwide approach. *Lancet Infect Dis* 2016;16:500–505.