Bdellovibrio Predation in the Presence of Decoys: Three-Way Bacterial Interactions Revealed by Mathematical and Experimental Analyses

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Bdellovibrio bacteriovorus **is a small, gram-negative, motile bacterium that preys upon other gram-negative bacteria, including several known human pathogens. Its predation efficiency is usually studied in pure cultures containing solely** *B. bacteriovorus* **and a suitable prey. However, in natural environments, as well as in any possible biomedical uses as an antimicrobial,** *Bdellovibrio* **is predatory in the presence of diverse decoys, including live nonsusceptible bacteria, eukaryotic cells, and cell debris. Here we gathered and mathematically modeled data from three-member cultures containing predator, prey, and nonsusceptible bacterial decoys. Specifically, we studied the rate of predation of planktonic late-log-phase** *Escherichia coli* **S17-1 prey by** *B. bacteriovorus* **HD100, both in the presence and in the absence of** *Bacillus subtilis* **nonsporulating strain 671, which acted as a live bacterial decoy. Interestingly, we found that although addition of the live** *Bacillus* **decoy did decrease the rate of** *Bdellovibrio* **predation in liquid cultures, this addition also resulted in a partially compensatory enhancement of the availability of prey for predation. This effect resulted in a higher final yield of** *Bdellovibrio* **than would be predicted for a simple inert decoy. Our mathematical model accounts for both negative and positive effects of predator-prey-decoy interactions in the closed batch environment. In addition, it informs considerations for predator dosing in any future therapeutic applications and sheds some light on considerations for modeling the massively complex interactions of real mixed bacterial populations in nature.**

Bdellovibrio bacteriovorus (20) is a small motile bacterium with an unusual biphasic predatory lifestyle. It preys upon, and replicates within, other gram-negative bacteria, including several human pathogens, such as *Pseudomonas* and *Salmonella*. The *Bdellovibrio* life cycle (Fig. 1a) includes a free-swimming attack phase (stage i) and an intraperiplasmic replication phase (stages iii to v). Upon collision with a suitable prey cell, *Bdellovibrio* enters the periplasm, seals up the entry pore once it is inside, and establishes itself within the periplasm, resulting in a rounding of the host cell and the formation of a bdelloplast. Inside the periplasm the *Bdellovibrio* cell grows by hydrolysis and uptake of prey cell cytoplasm components, forming a filamentous cell, which finally septates to give several progeny. The progeny become flagellate, and the *Bdellovibrio* cells lyse the bdelloplast (stage vi) and become free-swimming attack-phase cells. It is this biphasic life cycle and the apparent inability to invade mammalian cells (8) that may allow *Bdellovibrio* to be used as a potential alternative to topical antibiotics for treating bacterial infections in eukaryotes (18).

In natural environments or, if used in clinical applications, in infected wounds or on mucosal surfaces, *Bdellovibrio* is predatory in areas that contain potential prey and nonsusceptible cells, the latter of which include both gram-positive bacteria and eukaryotic cells. In the future *Bdellovibrio* may have antimicrobial therapeutic applications because it does not suffer from many of the problems associated with methods such as phage therapy. *Bdellovibrio* is not known to be prey specific,

infects a variety of gram-negative hosts, and has no known specific host recognition sites. In contrast, phage attach to specific molecular sites and hence are effective only against a narrow range of bacteria, which can in turn become resistant by simple mutations at the specific molecular attachment sites. In addition, some phage are unable to invade cells with capsules (16), whereas bacterial capsules have been shown to be an ineffective barrier to predation by *Bdellovibrio* (7). There have also been studies which have shown that some phage are inactivated by the presence of subtilisin (2), which, as we show here, was present in our decoy cultures but did not inhibit *Bdellovibrio* predation. In typical laboratory cultures *Bdellovibrio* is grown solely in the presence of its gram-negative prey, usually either *Escherichia coli* or *Pseudomonas* sp., or with a combination of potential prey species (15, 21). As such, the effectiveness of *Bdellovibrio* predation in environments containing mixed bacteria (both prey and nonsusceptible bacteria) is unknown, but it must be studied to understand dosage if *Bdellovibrio* is ever to be used as a therapeutic antimicrobial agent. Our work on predation in a "simple" three-member culture consisting of predator, prey, and decoy is the beginning of such an investigation.

Here we studied the predation of *E. coli* S17-1 (17) by *B. bacteriovorus* HD100 (20) in the presence of a nonsporulating (SpoIIID⁻) *Bacillus subtilis* 671 decoy (19). In our system the prey and decoy were viable and motile in late-log to stationary phase. We experimentally determined and modeled the inhibition of predation by the presence of decoy bacteria, and we compared our data to those of Wilkinson (23), who performed a solely theoretical study of predation in the presence of inert decoys. Our study showed that there was an unexpected enhancement of prey growth (Fig. 2C), probably as a result of the

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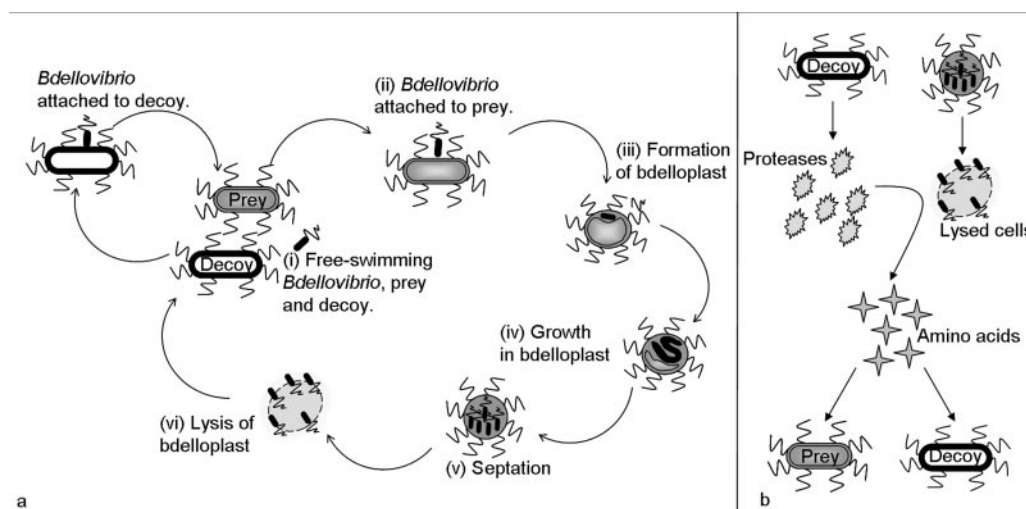
FIG. 1. *Bdellovibrio* life cycle and interactions occurring during the predation experiments. (a) Biphasic life cycle of *B. bacteriovorus*, showing the direct effect on predation caused by the *Bacillus* decoy occupying *B. bacteriovorus* in nonproductive attachments which reduce the availability of attack-phase predator cells to attach productively to prey cells. (b) Interactions between the *B. subtilis* decoy and the *E. coli* prey, including production of proteases, which degrade lysed bdelloplasts into their constituent amino acids, generating nutrients for both prey and decoy.

recycling of protein from prey cell debris by the *B. subtilis* decoy (Fig. 1b). We believe that the combination of modeling and live decoy-prey predation experiments provides reliable insight into the variables that must be considered if researchers wish to use *Bdellovibrio* to control pathogens in dynamically interacting, mixed bacterial populations.

MATERIALS AND METHODS

Bacterial strains. *B. bacteriovorus* genome-sequenced strain HD100 (12, 20) was the predator in all experiments. The prey used was *E. coli* S17-1 (17), and the decoy bacterium was *B. subtilis* 671 (19), a *spoIIID* mutant which is unable to sporulate yet otherwise grows vegetatively as a wild type. We examined *B. subtilis* 671 by phase-contrast microscopy using a Nikon Eclipse 600 to establish its cell size and motility and to determine that *Bdellovibrio* attaches to it and subsequently detaches upon recognition that the *Bacillus* cell is not suitable prey (as it is gram positive).

Establishing decoy-prey ratios in experimental cultures. The aim of the experimental procedure was to establish a 1:150 predator/prey ratio along with a decoy/prey ratio of 1:2.5 in experimental cultures alongside matched control cultures (Table 1). Because *Bdellovibrio* cells are too small to be enumerated by measuring optical density, we could determine the actual numbers of viable cells only many days after the experiment by viable-cell counting, so the procedure used for preculturing (outlined below) was followed exactly to match the numbers and viabilities of predator, prey, and decoy in each replicate.

Establishment of predatory experimental cultures. For all cultures the numbers of cells per ml are shown in Table 1. Predatory experimental cultures in 250-ml conical flasks contained 50 ml Ca/HEPES buffer (25 mM HEPES supplemented with 2 mM $CaCl_2$; pH 7.6) and were incubated for the times indicated below in an orbital shaker at 29°C at 200 rpm. A 1-ml aliquot of a *B. bacteriovorus* preculture containing attack-phase cells (as determined by phase-contrast microscopy to ensure complete prey lysis) was inoculated into the Ca/HEPES buffer along with 3 ml of a late-log-phase *E. coli* S17-1 prey preculture when appropriate. At the time of inoculation of prey and predators, 3 ml of *B. subtilis* 671 cells was added to the appropriate cultures as a decoy, and controls containing single bacterial cultures were also enumerated (Table 1).

Precultures of *B. bacteriovorus* attack-phase cells were grown on the same *E. coli* S17-1 prey for 24 h until complete prey lysis was achieved before use as inocula in the experiments. *E. coli* and *B. subtilis* starter cultures were grown from single colonies in 250-ml conical flasks containing 40 ml YT broth (0.8% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl; pH 7.5) and incubated at 37°C with shaking at 200 rpm overnight to obtain late-log-phase cultures. Portions (1 ml) of these starter cultures were inoculated into 40 ml YT broth and incubated at 37°C with shaking at 200 rpm overnight to obtain the prey and decoy precul-

tures used in the predation experiments. Optical densities at 600 nm (OD_{600}) were determined, and the prey and decoy precultures were diluted using supernatants from the appropriate cultures (to avoid perturbing growth with fresh medium) to obtain OD_{600} matching those of the other experimental replicates immediately prior to addition to the predation experiment flasks.

Although there were slight variations in OD_{600}, this procedure ensured that for each repetition we had a 2:1 ratio of S17-1 to 671. *B. bacteriovorus* was cultured every 24 h for a minimum of 3 days before the experiment, ensuring that each *Bdellovibrio* population was in the same state of motility and that the population densities were similar.

Seven cultures were studied, and their contents are shown in Table 1. Each culture was set up in a 250-ml flask and incubated at 29°C with shaking at 200 rpm. Cells were enumerated every 2 h using appropriate serial dilutions, as outlined below. Flasks were removed from the incubator for the minimal amount of time to avoid drastic changes in culture temperature. Due to the need to track the populations over 24 h, the experiments were run for 0 to 14 h three times and then for 12 to 24 h three times (again with initial enumeration). The data presented below are means for all experiments. Variations in experimental values for replicates were estimated by expressing the killing or growth of prey decoy and predator cultures as a percentage of the starting inoculated cell number in each replicate experiment. For example, the average standard deviation for all times for the number of prey cells (for a comparison of replicates of each type of culture containing *E. coli*) was 11% when each result was expressed as a percentage of the starting value, showing that the level of variation between replicate experiments was not significant.

Enumeration of predators, prey, and decoys. *E. coli* and *B. subtilis* in cultures containing either the prey or the decoy were enumerated using serial dilutions spread onto YT agar plates. The organisms in cultures containing both the prey and the decoy were enumerated simultaneously on chromogenic agar (Oxoid chromogenic *E. coli* coliform medium CM956), on which the *E. coli* strain formed purple colonies and the *B. subtilis* strain formed cream colonies. All plates were incubated at 29°C overnight.

B. bacteriovorus was enumerated using the double-layer agar technique on YPSC agar plates (0.1% yeast extract, 0.1% peptone, 0.05% CH_3CO_2Na, 0.025% $MgSO_4 \cdot 7H_2O$ [pH 7.6], with $CaCl_2$ added after autoclaving to give a concentration of 0.25 g liter^{-1}; the bottom layer was solidified with 1% agar, and the top layer was solidified with 0.6% agar). Plaques (representing single *Bdellovibrio* cells or bdelloplasts) formed within 3 to 7 days with incubation at 29°C. The presence of any carried-over *B. subtilis* cells in the culture did not impede the production of plaques as the *Bacillus* cells formed small colonies within the *E. coli* lawn, around which the *Bdellovibrio* could form plaques.

Protease production assay. Protease production by *B. subtilis* strain 671 was initially determined by culturing bacteria at room temperature on gelatin-solidified PY plates (1% peptone, 0.3% yeast extract; solidified with 5% gelatin). Liquefaction around a colony indicated protease production. For
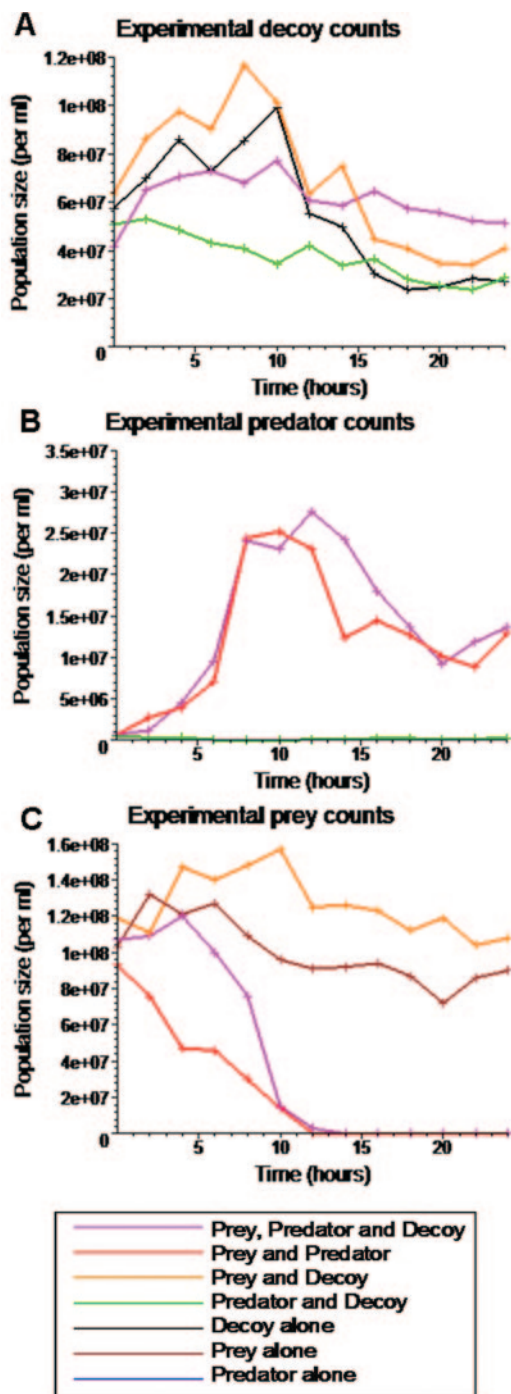
FIG. 2. Experimental results. The data are means from three repeats of the experiments. The population size is expressed as the number of cells per ml and is plotted against the time from the start of the experiment.

quantification of subtilisin production by *B. subtilis* 671 we used milk agar plates (0.1% peptone, 0.5% NaCl, 2% agar, 1% skim milk powder). Three milliliters of an overnight *Bacillus* culture was harvested and resuspended in 100 μl Ca/HEPES. Ten microliters of this preparation was stabbed into a plate. Similarly, 10-μl portions of various concentrations of subtilisin (Sigma subtilisin A dissolved in Ca/HEPES and filter sterilized) were stabbed into plates for comparison. The plates were incubated at 29°C for 3 days, and zones of clearing were measured.

RESULTS AND DISCUSSION

Ratios of cells used in experimental cultures. In our experiments we aimed to use a mean initial predator/prey ratio of 1:150, and the decoy was added to obtain an initial decoy/prey ratio of 1:2.5. There were slight variations between replicates (e.g., a 1:150 predator/prey ratio was actually 1:142 or 1:148, and the actual decoy/prey ratio was 1:2.6) due to the nature of an experiment, in which actual cell viability could be determined only after the experiment (see Materials and Methods).

***B. subtilis* was a true *Bdellovibrio* decoy.** In contrast to Stolp and Starr's observation that *Bdellovibrio* did not attach to *Bacillus megaterium* (20), our phase-contrast microscopy observations showed that attachment did occur between *Bdellovibrio* and *B. subtilis*, but the attachment was found to be reversible, with the time of attachment varying from less than 1 min to up to 5 min. We also established microscopically that the cell size and flagellar swimming motility of *B. subtilis* 671 were similar to the cell size and flagellar swimming motility of the *E. coli* S17-1 prey. Thus, *B. subtilis* 671 did act as a true living decoy in our experimental system.

Predation was effective in our cultures, and the decoy reduced predation at some time points. We found that predation by *Bdellovibrio* on *E. coli* reduced the *E. coli* population from a mean value of 1×10^8 cells ml^{-1} at time zero to less than 1×10^5 cells ml^{-1} after 14 h, both in the case of incubation with the *Bacillus* decoy and in the case of incubation without the *Bacillus* decoy (Fig. 2C). As expected, there was a significant lag time in the predation of *E. coli* by *Bdellovibrio* between 3 and 7 h when predation occurred in the presence of *B. subtilis* 671 as a live bacterial decoy (Fig. 2C). This was due to the collisions with and attachment of the *Bdellovibrio* cells to the *B. subtilis* decoy cells and due to the time between attachment and recognition as a nonsusceptible cell and subsequent detachment (which ranged from less than 1 min to 5 min). These nonproductive interactions caused a lag by preventing productive attachment to prey, when the ratio of decoy to prey was high at the start of the experiment. After 10 h of incubation and the resulting rounds of *Bdellovibrio* replication, the ratio in the three-member culture (Fig. 2C) had changed from an starting value of 148 prey cells per predator cell to 0.66 prey cell per predator cell. At this time there was not a significant difference between the predation in the presence of the decoys and the predation in the absence of the decoys, even though the ratio of decoy to prey had risen from a starting value of 1 decoy cell to 2.6 prey cells to 5 decoy cells to 1 prey cell. At the same time in the cultures without the decoy (Fig. 2C), the prey/predator ratio had dropped from 142 prey cells per predator cell to just 0.56 prey cell per predator cell. Thus, the chances of a random collision between predator and prey were similar in the two types of cultures, and the curves converge at 10 h (Fig. 2C).

***Bdellovibrio* yield in the batch cultures.** The yield of *Bdellovibrio* in all cultures containing both predators and prey was low, given the starting ratio of ca. 150 prey cells per predator cell, and the maximum increase in the *Bdellovibrio* level was 40 times the starting population upon complete (measurable) death of all prey in the cultures. The batch cultures were set up in such a way that both decoy and prey could be enumerated on the same plate, that the ratio of decoys to predators present at the outset would cause significant attachment to occur between

TABLE 1. Mean starting number of cells in each experimental culture[a]

Culture	No. of cells/ml		
	B. bacteriovorus	E. coli	B. subtilis
S17-1 prey only		$1.03 \times 10^8 \pm 3.27 \times 10^7$	
S17-1 prey and 671 decoy		$1.19 \times 10^8 \pm 1.47 \times 10^7$	$6.36 \times 10^7 \pm 2.38 \times 10^7$
671 decoy only			$5.75 \times 10^7 \pm 1.61 \times 10^7$
S17-1 and HD100	$6.59 \times 10^5 \pm 2.50 \times 10^5$	$9.35 \times 10^7 \pm 2.62 \times 10^7$	
S17-1, 671, and HD100	$7.21 \times 10^5 \pm 3.24 \times 10^5$	$1.07 \times 10^8 \pm 3.31 \times 10^7$	$4.15 \times 10^7 \pm 0.95 \times 10^7$
671 and HD100	$4.88 \times 10^5 \pm 2.27 \times 10^5$		$5.07 \times 10^7 \pm 0.97 \times 10^7$
HD100 predator only	$7.14 \times 10^5 \pm 4.6 \times 10^5$		

[a] One milliliter of inoculum was added to each culture containing *Bdellovibrio*. For each culture, 3 ml of *E. coli* or *B. subtilis* inoculum (from a preculture) was added after the cultures were matched by optical density to give the final cell concentrations. The data are means ± standard deviations for three replicates. The total amount of liquid in each culture was adjusted with Ca/HEPES buffer to obtain a final volume of 57 ml in a 250-ml conical flask. The aim of the experimental setup was to standardize the cell numbers added each time at a predator/prey ratio of 1:150, although viable counting could give actual cell numbers only several days after the experiment.

them, and that predation could be monitored over 24 h, not a shorter period. All of these experimental requirements necessarily restricted the nature of the cultures in which predation was studied, so the modeling took account of issues of cellular crowding within the batch cultures. Thus, cultures could have been experimentally established so that there was a much higher yield of *Bdellovibrio* and less cell death due to nonpredatory factors, but such cultures would not have been suitable for experimental monitoring or for modeling over a time course. Nonetheless, we are satisfied that these conditions were biologically relevant, and in modeling the experimental data, it was found that the overall yield was 3.8 *Bdellovibrio* cells per prey cell.

This suggests that not every bdelloplast formed actually yielded viable progeny cells (factored into the model) and that significant death due to starvation (5; also see below) of free-swimming *Bdellovibrio* occurred during the 24-h incubation period. These are parameters that are relevant to high-cell-density conditions, such as wounds, in which *Bdellovibrio* may be used as an antibacterial agent in the future.

Bdellovibrio yield after 10 to 12 h of incubation was not reduced by the presence of the decoy, although there was a slight lag in the peak yield. Interestingly, although there was a significant difference between early (3- to 7-h) predation rates depending on the presence of the decoys, Fig. 2B shows that the yield of *Bdellovibrio* peaked at 10 h for predator-prey cultures and at 12 h for predator-prey-decoy cultures (the reasons for a decrease after the peak later in the experiments are discussed below).

Slightly surprisingly, the yield from the predator-prey-decoy culture was similar to, or even slightly greater than, the yield from the predator-prey culture. As discussed below, this may have been due to decoy recycling of prey debris that supported more prey and decoy growth in the otherwise nonnutritive Ca/HEPES medium. The *Bdellovibrio* cells grown in the presence of decoys maintained their population size longer (Fig. 2B), probably due to the increased prey availability.

The prey counts were indeed similar at 12 h, but the enhancement of prey growth and the resulting extra predator replication occurred earlier in the experiment; thus, by 12 h significant exposure to the extra predators had already partially taken place. Some evidence that supports this is shown in Fig. 2B, which shows that the numbers of predators

at 8 h were similar for the predator-prey and predator-prey decoy cultures, despite the initial lag in predation. The model (see below) predicts that a predation cycle takes 2 h 25 min, so that, as shown in Fig. 2B, the last excess prey contributes to the late peak in the numbers of *Bdellovibrio* cells at 12 h when they had attached to, entered, and killed the prey before the 10-h point.

Bdellovibrio cells are not as robust as prey and decoy cells, and some Bdellovibrio cells died in the closed buffer culture. In both cultures in which *Bdellovibrio* was actively predatory, the *Bdellovibrio* population increased to a maximum level of 40 times the starting population by 12 h (Fig. 2B), before the previously documented (5) loss of viability (14 to 24 h) (Fig. 2B) due to starvation (due to very low prey numbers in the culture), which resulted in an overall endpoint *Bdellovibrio* population increase that resulted in a population size that was 20 times the initial population size after 24 h of incubation (Fig. 2B). This result correlates with the 50% decrease in viability over 10 h of starvation documented for *Bdellovibrio* strain 109J by Hespell et al. (5). The death of *Bdellovibrio* may also have been partially due to crowding of the culture. Laboratory-style batch buffer cultures, such as the cultures used in this work, contain far denser populations of bacteria than would be found in any terrestrial or aquatic environment from which *Bdellovibrio* is isolated. As such, the amounts of toxic metabolic by-products from prey and decoys may be significantly higher, and the local dissolved oxygen concentrations may be lower than those normally encountered and adapted to by *Bdellovibrio*; this possible effect has been included in the mathematical model (see below).

Slight overall decreases in *E. coli* numbers were seen when this organism was incubated in the absence of predators; the values corresponded to a 12% decrease by the 24-h endpoint when *E. coli* was incubated alone (Fig. 2C) and a 9% decrease when *E. coli* was incubated with the *Bacillus* decoy (Fig. 2C). These decreases were most likely due to natural cell death over 24 h caused by lack of nutrients in the buffer, reduced oxygen concentrations, and the possible release of potentially damaging metabolic by-products. In all cultures except those containing *Bdellovibrio* preying on *E. coli*, there were significant decreases of between 37 and 53% in the *B. subtilis* decoy population (Fig. 2A). However, when the *Bacillus* strain was

incubated in the presence of both *Bdellovibrio* and *E. coli*, this trend was reversed, and the *Bacillus* population grew by about 23% (Fig. 2A) by the 24-h endpoint.

Growth of the decoy and inferred growth of the prey due to proteolytic recycling of cell debris remaining after predation. As mentioned above, the endpoint numbers of *B. subtilis* cells increased only in three-member predator-prey-decoy cultures. Also, the number of *Bdellovibrio* cells produced by predation in the presence of decoys was as high as the number without decoys, and *Bdellovibrio* viability was sustained for a longer time in the presence of the decoy. We postulate that this may have been due to proteolytic degradation causing recycling of prey debris that provided extra nutrients for decoy and prey replication (as inferred from the higher numbers of *Bdellovibrio* cells), as shown in Fig. 1b. As all the cultures were incubated in Ca/HEPES buffer, there was no source of nutrition for heterotrophic bacteria such as the prey and decoy bacteria, but proteolysis of the ghosts of bdelloplasts lysed by *Bdellovibrio* might have provided such a source. *B. subtilis* 671 did produce proteases that liquefy gelatin, but *E. coli* S17-1 did not produce such proteases. These *B. subtilis* decoy proteases include subtilisin (3), which aids degradation of proteins into their composite amino acids.

Decoy proteases do not adversely affect prey and predator. As we hypothesized that proteases could be active in our three-member cultures, we tested whether they might negatively affect predator or prey viability. The levels of proteases from the *B. subtilis* decoy were determined on milk agar plates to be equivalent to 100 μg/ml subtilisin. This concentration was used (as an approximation for the concentration of decoy proteases) to measure effects on both prey and predators, incubated separately and during predation. Cultures were set up as described above for experiments containing the *Bacillus* decoys, except that the *Bacillus* strain was replaced with 2.9 ml of Ca/HEPES and 100 μl of 100 μg/ml subtilisin. Population viability measurements were obtained at 0, 12, 16, 20, and 24 h for each culture (data not shown). The resulting numbers of each organism were not significantly altered in the presence of the subtilisin, although we could not test other *Bacillus* exoenzymes in this way.

Initial unplanned growth of prey and decoy was seen in cultures. Late-log-phase inocula of prey and decoy were used throughout this study, and no heterotrophic medium was supplied; only Ca/HEPES was supplied, yet increases in cell numbers were seen early in the experiments. The numbers of *E. coli* prey cells increased by up to 20% compared to the starting values when *E. coli* was incubated in the presence of the *B. subtilis* decoy over the first 10 h of incubation, whereas when *E. coli* was incubated alone, a slight decrease in the number of cells was seen over the same time span. When *B. subtilis* was incubated alone, the numbers of cells also increased in the first 12 h (Fig. 2A), and the viability of the decoy was sustained for the longest time (to the end of the experiment) when the decoy was incubated with both prey and predator. The fact that in the culture with just *Bdellovibrio* and *B. subtilis* there was no such increase in numbers of cells may indicate that starving *Bdellovibrio*, not cycling through prey, has negative effects even on gram-positive bacteria, possibly due to secretion of active compounds. The increases in numbers of prey and decoy

cells for such a length of time could not have been due to division of already replicated cells in the inoculum. It was likely due to proteolytic recycling of protein from dead prey or decoy cells that were killed either by osmotic shock upon inoculation into the buffer or by predation.

Mathematical models. While there have been several theoretical attempts to model *Bdellovibrio* predation, only the attempt of Wilkinson (23) included the effects of decoys (latex spheres representing unsusceptible bacteria) on predation efficiency, and he focused on the role of the decoy in reducing the rate of attachment of predator to prey. Other modelers considered only predators and prey. Varon and Zeigler (22) used traditional Lotka-Volterra equations to model their data for *Bdellovibrio* predation on luminous *Photobacterium*, which gave reasonable agreement. Marchand and Gabignon (9, 10) used a time delay term in their model to represent the time between the formation of the bdelloplast and the release of the live progeny. We considered including such delay effects in our modeling approach, but the fit with the experiments achieved using a differential equation formulation implied that such a refinement was not warranted. Crowley et al. (1) used differential equations to model *Bdellovibrio* predation in nutrient chemostats; however, they did not include any experimental data and focused on a system involving prey, *Bdellovibrio*, and bdellophage.

Our modeling approach. Our model considers more of the biological and environmental effects on each species than is accounted for by the Lotka-Volterra equations used by Varon and Zeigler (22). Instead of using a time delay term to represent the bdelloplast stage, we consider the bdelloplasts members of a separate, transient "population," which allows the effects of the culture environment on the bdelloplasts to be investigated more readily.

Populations in the model. We consider the predators to exist in four possible states: free swimming [population $B_f(t)$], attached to prey [$B_{aE}(t)$], attached to a decoy [$B_{aD}(t)$], or inside a bdelloplast [$P(t)$]. Thus, to compare the results with the experimental viable count we use predator viable count at time t as follows: $\sim B_f(t) + B_{aE}(t) + B_{aD}(t) + P(t)$.

The prey cells are considered to be in one of two states: free swimming [$E(t)$] or attached to a predator [$B_{aE}(t)$]. Thus, the prey viable count at time t is $\sim E(t) + B_{aE}(t)$.

Similarly, the decoy cells are in one of two states: free swimming [$D(t)$] or attached to a predator [$B_{aD}(t)$]. Thus, the decoy viable count at time t is $\sim D(t) + B_{aD}(t)$.

In this model it is assumed that each bdelloplast forms a single plaque on an overlay plate (1 PFU), although there may be more than one viable predator inside, and that a prey cell attached to (but not yet invaded by) a predator is still a viable CFU. For the decoy, it was assumed that any attached *Bdellovibrio* had no effect on decoy viability.

In addition to modeling the six "subspecies" described above, we also considered the quantity of protein produced from the dead cells [$U(t)$], the useable amino acids produced from these proteins [$F(t)$] (used in cryptic growth as described by Kolter et al. [6]), and the protease levels in the culture [$T(t)$]. Although protein parameters have not been directly measured experimentally, their effects are seen in the viable counts.

Model equations. The equations used in the model are:

TABLE 2. Parameters used in the model[a]

Parameter	Symbol(s)	Simulation value(s)
Cell generation time	μ_E, μ_D	2.23×10^5, 9×10^5
Rate of attachment of predator	k_E, k_D	4×10^{-7}
Rate of lysis of bdelloplasts	κ	3/7
Rate of early bdelloplast lysis	η	17/21
No. of progeny per bdelloplast	N	3.8
Detachment of *Bdellovibrio* from *B. subtilis*	K	30
Formation of bdelloplast	ε	12
Effect of crowding	γ_E, γ_B, γ_D	4×10^{-5}, 5×10^{-4}, 1×10^{-4}
Contribution to crowding effect	θ_E, θ_B, θ_D	1×10^{-6}, 1×10^{-5}, 1×10^{-6}
Amt of dead cell protein	β_E, β_B, β_D	1.54×10^{-13}, $0.1475\beta_E$, β_E
Amt of protein from lysed and unlysed bdelloplasts	δ_L, δ_U	$\beta_E - (N-1)\beta_B$, $\beta_E + \beta_B$
Protein degradation	λ	1.905×10^{-7}
Amt (g) of usable amino acids per g of degraded protein	ϕ	0.95
Amt of amino acids required per cell	α_E, α_D	β_E, β_D
Protease production/decay	ρ_+, ρ_-	4.2×10^{-17}, 0

[a] Subscripts indicate the species that the parameters refer to, as follows: E, *E. coli* (prey); B, *Bdellovibrio* (predator); D, *B. subtilis* (decoy); U, unproductive bdelloplast; L, productive bdelloplast. The parameters described previously include k_E, κ, ε, β_E, β_B, β_D, α_E, α_D, δ_L, δ_U, and λ. The parameters found in experiments include k_D, K, and ρ_+. The remaining parameter values were obtained by methodically evaluating the mathematical model, with consideration of the biological relevance of the parameter values.

$$\frac{dE(t)}{dt} = \mu_E F(t)[E(t) + B_{aE}(t)] - k_E E(t)B_f(t) - \gamma_E E(t)O_d(t)$$
$$+ \gamma_B B_{aE}(t)O_d(t) \quad (1)$$

$$\frac{dB_f(t)}{dt} = -k_E E(t)B_f(t) - k_D D(t)B_f(t) + \kappa N P(t) + K B_{aD}(t)$$
$$- \gamma_B B_f(t)O_d(t) + \gamma_E B_{aE}(t)O_d(t) + \gamma_D B_{aD}(t)O_d(t) \quad (2)$$

$$\frac{dB_{aE}(t)}{dt} = k_E E(t)B_f(t) - \varepsilon B_{aE}(t) - \gamma_E B_{aE}(t)O_d(t) - \gamma_B B_{aE}(t)O_d(t) \quad (3)$$

$$\frac{dB_{aD}(t)}{dt} = k_D D(t)B_f(t) - K B_{aD}(t)\gamma_D B_{aD}(t)O_d(t) - \gamma_B B_{aD}(t)O_d(t) \quad (4)$$

$$\frac{dP(t)}{dt} = \varepsilon B_{aE}(t) - \kappa P(t) - \eta P(t) \quad (5)$$

$$\frac{dD(t)}{dt} = \mu_D F(t)[D(t) + B_{aD}(t)] - k_D D(t)B_f(t) + K B_{aD}(t)$$
$$- \gamma_D D(t)O_d(t) + \gamma_B B_{aD}(t)O_d(t) \quad (6)$$

$$\frac{dU(t)}{dt} = +\delta_L \kappa P(t) + \delta_U \eta P(t) - \lambda U(t)T(t) + \beta_E \gamma_E[E(t)$$
$$+ B_{aE}(t)]O_d(t) + \beta_B \gamma_B[B_f(t) + B_{aE}(t) + B_{aD}(t)]O_d(t)$$
$$+ \beta_D \gamma_D[D(t) + B_{aD}(t)]O_d(t) \quad (7)$$

$$\frac{dF(t)}{dt} = \lambda \phi U(t)T(t) - \alpha_E \mu_E F(t)[E(t) + B_{aE}(t)]$$
$$- \alpha_D \mu_D F(t)[D(t) + B_{aD}(t)] \quad (8)$$

$$\frac{dT(t)}{dt} = \rho_+[D(t) + B_{aD}(t)] - \rho_- T(t) \quad (9)$$

where $O_d(t) = \theta_E[E(t) + B_{aE}(t)] + \theta_B[B_f(t) + B_{aE}(t) + B_{aD}(t)] + \theta_D[D(t) + B_{aD}(t)]$. $O_d(t)$ represents the effect of crowding on the culture due to toxic metabolic by-products and/or reduction in the local dissolved oxygen concentrations. The parameters in the model are summarized in Table 2.

Initial conditions. The initial values, $E(0)$, $B_f(0)$, and $D(0)$, correspond to the values obtained in the experiments (Table 1), with $B_{aE}(0)$, $B_{aD}(0)$, and $P(0)$ assumed to be zero. The final three initial conditions, $U(0)$, $F(0)$, and $T(0)$, were unknown, and hence values were obtained by using a method similar to that used for the parameter values described below, which enabled the model to reproduce the experimental data best. These initial values correspond to the carryover of proteins, amino acids, and proteases from the starter cultures and hence vary for different experimental cultures depending on the populations added. Specifically, this led to the following values: for cases involving decoys the $T(0)$ value used was 4.4×10^{-9} g proteases per ml, and otherwise the value used was zero as we assumed that the proteases were produced only by the decoys. The presence of prey or decoys resulted in 2.84×10^{-7} g of protein per ml being added to $U(0)$ and 1.3×10^{-7} g of amino acids per ml being added to $F(0)$ for each of the species, while the presence of the predators did not add anything to either $U(0)$ or $F(0)$.

Parameter values used for simulations. The parameter values used for simulations were obtained from a combination of data from previously published studies and our experimentation (e.g., protease production), and some (such as those for the crowding effects) were obtained by fitting the resulting graphs to the experimental values. The resulting parameter values are shown in Table 2.

Parameter values related to timing in the *Bdellovibrio* life cycle were obtained from previous work performed in our laboratory and other laboratories, as reviewed by Sockett and Lambert (18). In the model, attachment of *Bdellovibrio* to both

prey and decoy was assumed to occur due to random collision; hence, as the size and motility of the prey are similar to the size and motility of the decoy, the rate of attachment of *Bdellovibrio* to the decoy was set to the rate of attachment to the prey.

For the protein degradation rate (λ) we used the standard values for subtilisin (3), assuming that all *Bacillus* proteases have similar levels of enzymatic activity. The protease production rate (ρ_+) was obtained from the milk agar plate experiment. The protease decay constant (ρ_-) was set at zero, as it was insignificant for the conditions and time studied here.

The mass of protein per *E. coli* cell (α_E, β_E) was obtained from the work of Neidhardt (11), and the comparative protein mass for *B. bacteriovorus* 109J (β_B) obtained from the work of Rittenberg and Hespell (13) was 14.75% of that of a typical *E. coli* cell. We assumed that the protein mass of the *Bacillus* decoy (α_D, β_D) is approximately the same as that of the *E. coli* prey due to the similarity of the cell sizes. We assumed that the amount of protein released from the early unproductive lysis of a bdelloplast (δ_U) is equal to the amount of protein in its component parts, one *E. coli* cell (β_E) and one *Bdellovibrio* cell (β_B). The amount of protein released during productive lysis (δ_L) is considered to be the sum of the amount of protein in a prey cell (β_E) and the amount of protein in a *Bdellovibrio* cell (β_B) minus the amount of protein used to make nitrogen-containing biopolymers during the formation of the *Bdellovibrio* progeny ($N\beta_B$).

Crowding parameters were estimated from fits to the experimental population curves. The contributions made by prey and decoy were considered equal, due to the similarities in cell sizes and respiration rates, whereas the contribution made by the *Bdellovibrio* was defined as 10 times the contribution made by the prey due to the increased respiration rate, which has been shown for strain 109J to be six to seven times that of a typical *E. coli* strain (4). We assumed, due to higher rates of predator replication and flagellar rotation, that the respiration rate of *Bdellovibrio* strain HD100 used in this study may be slightly higher than that of the 109J strain.

Simulations and parameter sensitivity. The set of differential equations were solved numerically (by the Runge-Kutta Fehlberg method) using the MAPLE package (Maple 7.0; Waterloo Maple Inc., Waterloo, Ontario, Canada), and the resulting graphs are shown in Fig. 3. Parameter sensitivity studies have confirmed that good qualitative agreement arises robustly, providing support for the modeling framework adopted.

Impact on predation. The decoys had similar effects on predation in the model, as shown by the experimental results. Equivalent prey killing occurred slightly later in the simulations with a decoy than in the simulations without a decoy (Fig. 3C). The *Bdellovibrio* grew to a maximum population size of 2.7×10^7 cells per ml in the model of the decoy culture (Fig. 3B), approximately matching the size of the experimental decoy culture (maximum population size, 2.8×10^7 cells per ml) (Fig. 2B). The growth of the predators also occurred slightly later in the model, with a slightly higher peak yield, when decoys were added to the simulation (Fig. 3B), which correlates with the experimental results (Fig. 2B).

Incubation effects on cell viability. The model includes limited growth of both prey and decoy populations constrained by the availability of amino acids and the death of all three species caused by the crowding effects in the culture. An overall slight
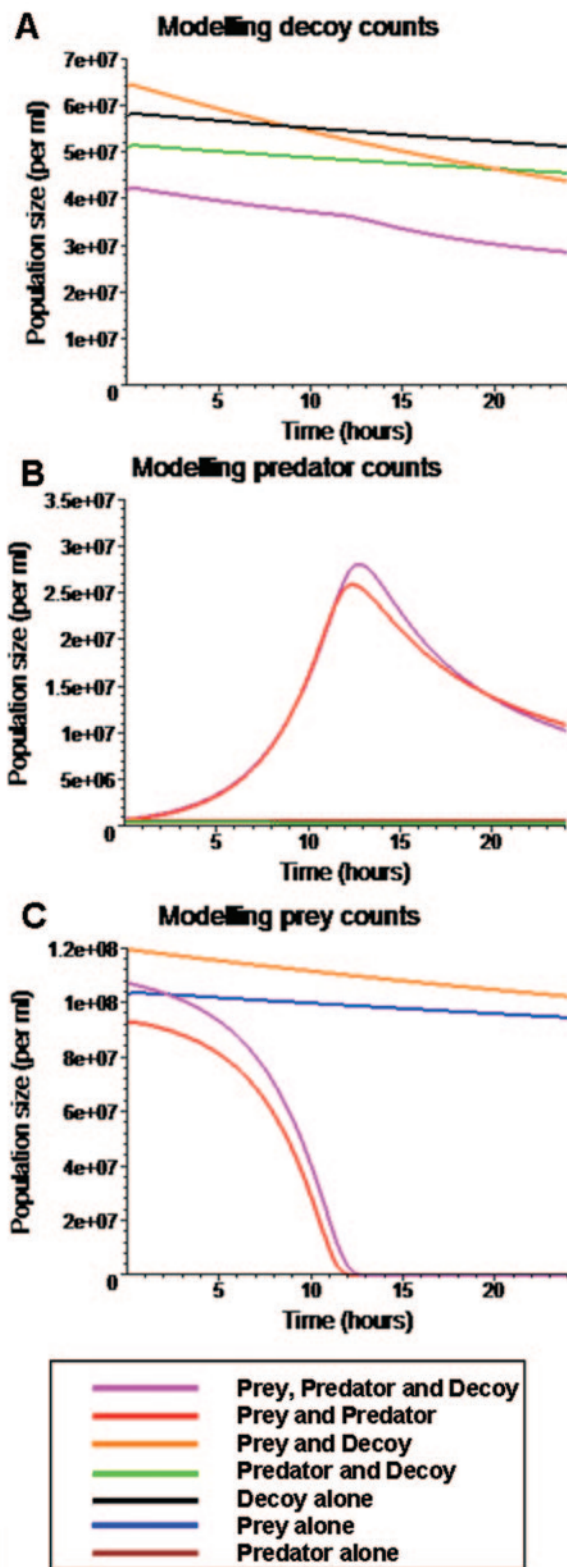
FIG. 3. Modeling results. The graphs correspond to the model, with the parameter values shown in Table 2. The colors of the lines correspond to those in Fig. 2. The curves have been tested and shown to fit within 1 standard deviation of the mean the majority of the time. The estimated population size is plotted against the time.

decrease in the decoy population size was seen in all simulations (Fig. 3A), and a slight decrease in the prey population size was seen in the simulations without predators (Fig. 3C), which correlates with the experimental data in Fig. 2. The model does not show the unexpected, initial, experimentally measured increases in the levels of prey and decoy, but it does give similar overall population sizes at 24 h.

Bdellovibrio viability during replication. The model considers the bdelloplasts to be a separate, transient population, which allows the environmental effects on the bdelloplasts to be investigated. The model shows that a significant proportion of bdelloplasts do not lyse and release viable progeny. Earlier models have made the assumption that every bdelloplast lyses, yielding viable progeny, whereas here we show that this is not true. Experimental investigation into early bdelloplast death or aborted replication has not been reported previously but is worthy of future work.

Proteolytic recycling of cell debris. The model includes the effects of decoy proteases on the recycling of cell debris into usable amino acids, using the $U(t)$, $F(t)$, and $T(t)$ equations. Interestingly, single-parameter sensitivity studies have shown that individual changes to parameters within the protein-amino acid-protease cycle do not have a major effect on the overall cell populations. However, this is likely to be because the presence of protein for recycling into amino acids depends upon more than one parameter; i.e., protein is made available when predator kills prey and decoy proteases are required to obtain amino acids from it. Therefore, the cycle is not dependent on the value of a single parameter but depends on a set of parameters.

The protease cycle terms allow the model to be applied to situations involving larger quantities of available proteins and amino acids, such as the quantities present in protein-rich serum encountered in potential therapeutic applications but not in a nonnutritional buffer like the Ca/HEPES buffer used here.

Wilkinson (23) modeled chemostat biomass yields during *Bdellovibrio* predation in the presence of inert theoretical decoys. He predicted that the steady-state prey population should vary linearly with the decoy population, because he did not anticipate positive effects of live decoys. Our experiments were conducted with batch cultures of late-log-phase cells, not with chemostats, but both the viability counts and the continued flagellar motility of prey and decoy cultures, throughout the infection, showed that they were in an approximately steady state. We found that at high prey concentrations, the *Bdellovibrio* cells dwelled predominantly within prey bdelloplasts; thus, in assessing Wilkinson's model we must pay keen attention to predator/prey ratios and bear in mind the nonsynchronous 2- to 3-h infection periods between releases of *Bdellovibrio*.

Utility of the model. The model predicts that in the conditions used, the predation cycle for *B. bacteriovorus* HD100 takes 2 h 25 min. It also allows us to model the times at which prey numbers are reduced below a certain threshold either in the presence or in the absence of decoy bacteria. These are important parameters to establish before factoring in the possible effects of neutrophil engulfment of predator, prey, and/or decoy when considering therapeutic applications in an immunologically active environment, such as a wound. In such cases the predator, prey, and decoy could be actively removed at different rates by the action of the host immune system. This would affect the therapeutic dose of predator required and is where our experimentation and modeling are taking us next.

Summary. We showed that *Bdellovibrio* predator-prey interactions are complex but still productive in the presence of live bacterial decoys. Live decoys at an initial ratio of 1 decoy cell to every 2.5 prey cells did significantly reduce the efficiency and rate of predation at a lower predator multiplicity of infection for culture times from 3 to 7 h. However, this decoy level did not significantly reduce the time at which all prey cells were measurably extinguished. Thus, any topical therapeutic application of *Bdellovibrio* should be at high multiplicity of infection with respect to prey. Live decoys can produce extracellular factors, such as proteases, which can promote the growth of prey and hence predators. Culture density can have a significant influence on the viability of all three of the species involved, but particularly on the viability of *Bdellovibrio*. Our model accounts for these negative and positive influences on population numbers, which must be considered if *Bdellovibrio* is used to treat infected wounds.

Robson and coworkers have shown that a wound with a bacterial population larger than 10^5 cells g^{-1} of tissue can be considered infected (14). If treatment reduces the bacterial load to less than 10^5 cells g^{-1} without removing all the bacteria, the wound is less open to new colonization, but spontaneous healing can take place. Therefore, to consider the use of *Bdellovibrio* as a possible treatment for infected wounds, we need to show that *Bdellovibrio* can reduce the pathogenic bacterial load to less than 10^5 cells ml^{-1} ($\sim 10^5$ cells g of tissue^{-1}). Here we show that a prey level of 10^8 cells ml^{-1} is reduced to less than 10^5 cells ml^{-1} in less than 10 h in the presence of 7×10^5 decoy cells ml^{-1}. Thus, even if a wound contains both species at concentrations above the wound infection threshold, *Bdellovibrio* is, in theory, able to eliminate the gram-negative infection and then die (as predicted from the fragility of *Bdellovibrio* in our experimental culture system), allowing spontaneous healing. Obviously, in an infected wound the immune response of the patient affects not only the species infecting the wound but also the *Bdellovibrio*, and serum may provide a continuous source of nutrients for the prey. These effects can be incorporated into the model, easily expanding it to apply to these therapeutic settings, along with a range of other environmental factors whose importance may differ for different prey or decoy species. The model presented here is a framework with which to analyze these factors and to predict the effectiveness of *Bdellovibrio* as a therapeutic antimicrobial agent in infected wounds.

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