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Susceptibility of Biofilms to *Bdellovibrio bacteriovorus* Attack

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Biofilms are communities of microorganisms attached to a surface, and the growth of these surface attached communities is thought to provide microorganisms with protection against a range of biotic and abiotic agents. The capability of the gram-negative predatory bacterium *Bdellovibrio bacteriovorus* to control and reduce an existing *Escherichia coli* biofilm was evaluated in a static assay. A reduction in biofilm biomass was observed as early as 3 h after exposure to the predator, and an 87% reduction in crystal violet staining corresponding to a 4-log reduction in biofilm cell viability was seen after a 24-h exposure period. We observed that an initial titer of *Bdellovibrio* as low as 10^2 PFU/well or an exposure to the predator as short as 30 min is sufficient to reduce a preformed biofilm. The ability of *B. bacteriovorus* to reduce an existing biofilm was confirmed by scanning electron microscopy. The reduction in biofilm biomass obtained after the first 24 h of exposure to the predator remained unchanged even after longer exposure periods and reinoculation of the samples with fresh *Bdellovibrio*; however, no genetically stable resistant population of the host bacteria could be detected. Our data suggest that growth in a biofilm does not prevent predation by *Bdellovibrio* but allows a level of survival from attack greater than that observed for planktonic cells. In flow cell experiments *B. bacteriovorus* was able to decrease the biomass of both *E. coli* and *Pseudomonas fluorescens* biofilms as determined by phase-contrast and epifluorescence microscopy.

Biofilms are surface-attached microbial communities with phenotypic and biochemical properties distinct from free-swimming planktonic cells (4, 6). Biofilm formation is thought to begin when bacteria sense environmental conditions that trigger the transition to life on a surface, followed by a multistep process leading to the formation of a mature biofilm (7, 29, 46). Various techniques have been evaluated for their capability to manage and control biofilms, among them are the use of different materials and coatings to reduce initial cell adhesion to surfaces and a variety of treatments aimed at decreasing or destroying already existing biofilms, such as heat, cleaning regimens, low-power laser, sonication, chemical treatments, antibiotics, quorum-sensing analogs, and lectins (1, 2, 13, 26, 28, 30, 35, 38). Recently, there has been a renewed interest in the use of biological control agents against biofilms. These agents include the use of invertebrates and protozoa to reduce biofilms by means of grazing (21, 23, 24, 31, 37, 49) and the use of bacteriophages (8, 9, 14, 15, 43).

One organism that might have a potential use against biofilms is a bacterium from the genus *Bdellovibrio*. *Bdellovibrio* spp. are gram negative, motile, and uniflagellated bacteria. This genus is characterized by predatory behavior (or an obligatory parasitic life cycle). Bdellovibrios attack other gram-negative cells, penetrate their periplasm, multiply in the periplasmic space, and finally burst the cell envelope to start the cycle anew (16, 39, 41). Bdellovibrios are largely found in wet, aerobic environments and were first isolated from soil, where they are commonly encountered (41). However, they can also be found in fresh and brackish water, sewage, water reservoirs, and seawater (16, 17, 34). Another environmental niche with which bdellovibrios have been associated are biofilms (18, 48). It is believed that biofilms might offer good conditions for bdellovibrios' survival since these organisms have been found in natural marine biofilms but are not always recovered from the surrounding water (17, 18, 48). It is suggested that in a biofilm bdellovibrios can benefit from higher prey density, which has been shown to be necessary for *Bdellovibrio* survival (19, 44).

In the present study we have used a 96-well microtiter static assay to evaluate the capability and dynamics of *B. bacteriovorus* 109J predation on *Escherichia coli* biofilm communities. Staining of the biofilm, direct enumeration, and electron microscopy were used to estimate and visualize the extent and nature of damage inflicted on biofilms after *B. bacteriovorus* treatment. The relative resistance of biofilm and planktonic cells to attack by *B. bacteriovorus* was assessed. In additional experiments we also monitored the ability of *B. bacteriovorus* to impact *E. coli* and *Pseudomonas fluorescens* biofilms in a flow cell system.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions. *B. bacteriovorus* strain 109J was obtained from the American Culture Type Collection (ATCC 43826), and *E. coli* strain ZK2686 (a derivative of W3110) was obtained from R. Kolter (32). Biofilm-defective *E. coli* strains of ZK2686 (mutants *fimH*::*cm* and *wcaF*::*cm*) (5, 32, 42) were used to assess the difference in predation between biofilms and planktonic cells. *P. fluorescens* WCS365 (12) was used for flow-cell analysis. *E. coli* and *P. fluorescens* were grown routinely in LB medium at 37 and 30°C, respectively. Cells were enumerated as CFU on LB agar plates. *B. bacteriovorus* was maintained as plaques in double-layered diluted nutrient broth (DNB) (a 1:10 dilution of nutrient broth amended with 3 mM $MgCl_2 \cdot 6H_2O$ and 2 mM $CaCl_2 \cdot 2H_2O$ [pH 7.2]) agar (0.6% agar in the top layer) (40). *B. bacteriovorus* was counted as PFU developing on a lawn of prey cells. Standard *B. bacteriovorus*-induced lysates were obtained by adding a plug of agar containing a *B. bacteriovorus* plaque (ca. 10^6 PFU/ml) to 10^8 CFU of washed prey/ml and incubated 18 h in DDNB (a 1:50 dilution of nutrient broth with 3 mM $MgCl_2 \cdot 6H_2O$ and 2 mM $CaCl_2 \cdot 2H_2O$) at 30°C on a rotary shaker at 200 rpm to reach a final concentration of 10^9 PFU of predator/ml. To harvest *B. bacteriovorus*, the 18 h

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lysates were passed three times through a 0.45-μm-pore-size filter in order to remove residual prey and cell debris. Dilutions were made in saline solution (150 mM NaCl) or 25 mM HEPES buffer containing 2 mM $CaCl_2 \cdot 2H_2O$ (pH 7.8).

Biofilm assays and predation assay. Biofilm formation in microtiter dishes was measured as described previously (27). Microtiter wells were inoculated (100 μl per well) from 18 h *E. coli* LB-grown cultures diluted 1:100 in LB. Cells were grown for 18 h at 30°C (preformed biofilm) before they were stained with crystal violet (CV) and quantified as described previously (27) by using a Molecular Devices Vmax kinetic microplate reader (Sunnyvale, CA) at 550 nm. To assess predation and dynamics of *B. bacteriovorus* on *E. coli* biofilms, the preformed biofilms were grown as described above, washed three times with DDNB in order to remove any planktonic cells, and 100 μl of *B. bacteriovorus* from 18 h lysate was added (10^8 PFU/well). As a control, 100 μl of a filtered sterilized lysate was prepared by passing the *B. bacteriovorus*-containing lysate through a 0.22-μm-pore-size filter three times. After filtering through a 0.22-μm-pore-size filter, no predator, as judged by PFU, could be detected. The microtiter dish was incubated at 30°C for the duration of the experiments. Preliminary studies confirmed that the use of DDNB maintained the predeveloped biofilm on the surface of microtiter wells for as long as 120 h (data not shown).

Quantification of biofilm bacteria before and after treatment was performed as follows. The wells were washed six times with saline in order to remove any planktonic cells, 100 μl of saline was added to each well, and the samples were independently sonicated for 8 s by using a VC505 sonicator (Sonics and Materials, Inc., Newtown, CT), followed by dilution plating. The CFU and PFU counts were performed on planktonic cells before and after sonication to verify that the sonication procedure did not reduce cell viability, and CV staining and microscopy were used to determine the efficacy of sonication to remove surface-attached cells. These control experiments demonstrated that the sonication regimen did not reduce cell viability and yet was sufficient to remove all of the attached cells (data not shown). For planktonic cell count, total liquid volume (100 μl) was taken for dilution plating prior to the sonication step. Each experiment was carried out at least three times with 24 to 48 wells for each treatment. For statistical analyses, *P* values were determined by using a Student *t* test performed with Microsoft Excel software. Error bars are shown as one standard deviation.

Flow cell experiments. For biofilms grown under flow conditions, bacteria were cultivated in a four-channel flow cell with a square glass capillary channel dimensions of 2 by 2 mm (Friedrich and Dimmock, Inc., Millville, NJ). The flow system was assembled as described previously (3). For *E. coli* and *P. fluorescens* flow cell studies, cells were inoculated from 18 h LB-grown cultures diluted 10-fold in 20 or 4% LB, respectively. The medium flow was turned off prior to inoculation and for 1 h after inoculation. After the development of a mature multilayered biofilm with "mushroom-like" structures ~30 μm in depth (62 h after inoculation for *E. coli* and 36 h after inoculation for *P. fluorescens*), the flow was turned off, and the chambers were inoculated with 1 ml (10^9 PFU/ml) of harvested *B. bacteriovorus* lysates, prepared as described above, or 1 ml of filtered sterilized lysate as control. Thereafter, DDNB medium was pumped through the flow cell at a constant rate of 4.8 ml/h for the duration of the experiment. The flow cells were incubated at room temperature. The flow was controlled with a PumpPro MPL (Watson-Marlow, Cornwall, England). Three experiments were carried out for each strain, with two replicates for each treatment.

Imaging. Epifluorescence and phase-contrast microscopy were performed with a model DM IRBE microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Orca model C4742-5 charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan). Images were acquired and processed on a Macintosh G4 loaded with OpenLab software (Improvision, Coventry, England). Fluorescence intensity of the images was quantified by using OpenLab software. To determine viability of the cells, the samples were stained with BacLight live/dead bacterial viability kit (Molecular Probes, Eugene, OR). BacLight is comprised of two dyes, Syto-9 and propidium iodide (PI). Syto-9 is a green nucleic acid stain that can penetrate both intact and damaged membranes. PI is a red florescent dye that only penetrates damaged membranes. Cells staining red are generally considered "dead." Cells staining green have intact membranes and are considered "alive" in this assay.

SEM. For scanning electron microscopy (SEM) experiments *E. coli* biofilms were developed on a 12-by-22-mm PVC plastic coverslip (Fisher Scientific, Pittsburgh, PA). The coverslips were placed with a 55° angle in a 24-well polystyrene cell culture plate (Corning, Inc., Corning, NY). Preformed biofilms and *B. bacteriovorus* were prepared as described above. The experiments were carried out in a 1.5-ml volume. The coverslips were rinsed six times in DDNB to remove any planktonic cells and fixed for 2 h in a solution containing 2% glutaraldehyde, 0.1 M sodium cacodylate (NaCac), and 0.1% ruthenium red (RR) at pH 7.4, followed by three washes in 0.1 M NaCac buffer with 0.1% RR and a 1-h secondary osmium fixation (1% osmium tetroxide, 0.1 M NaCac, 0.1% RR). The samples were washed in sterile water, dehydrated in ethanol to absolute ethanol, and placed in 100% hexamethyl disilizane (HMDS). The HMDS was dehydrated in a vacuum overnight, and the samples were gold palladium coated (Au/Pd 60/40) for 1.5 min. All of the chemicals were obtained from Electron Microscopy Sciences, Inc. (Hatfield, PA). An FEI XL-30 field emission gun/environmental scanning electron microscope, operated in high vacuum mode, was used to examine the samples. Each experiment was carried out three times with triplicate samples. Images were viewed at the air-liquid interface.

RESULTS

Biofilm predation assay and dynamics. In order to measure the effects of *B. bacteriovorus* on *E. coli* biofilms over time, we developed conditions that yield stable *E. coli* biofilms in a 96-well dish. The *E. coli* biofilm was formed in LB medium for ~18 h. After 18 h, the medium was removed and replaced with DDNB medium as described in the Materials and Methods. Using this method, an *E. coli* biofilm comprised of ~10^8 CFU/well could be stably maintained for up to 120 h.

The *E. coli* biofilm formed after ~18 h in a 96-well microtiter plate was exposed to *B. bacteriovorus* lysate or a filter-sterilized lysate as control. As shown in Fig. 1A (pretreatment), the untreated 18-h-old biofilm produced was easily visualized with CV staining. Treatment with 10^8 PFU of *B. bacteriovorus* (Fig. 1A, +*B.b.*) markedly reduced the CV staining compared to the control (Fig. 1A, −*B.b.*). Quantification of the effect of *B. bacteriovorus* on *E. coli* biofilms over time revealed a 15% reduction in CV staining at 3 h posttreatment and an 87% reduction after 24 h (Fig. 1B). At 48 h, the reduction in CV staining was 89% compared to the initial time point ($t = 0$), and no further reduction occurred with 72 h of incubation (data not shown). In contrast, only an 18% reduction in CV staining was measured after 48 h in the control sample (Fig. 1B).

In a parallel experiment, fresh *B. bacteriovorus* was added to a preformed *E. coli* biofilm every 24 h over a period of 3 days. After 72 h there was no statistically significant change in biofilm biomass as judged by CV staining compared to the 24-h time point (optical densities at 550 nm [OD_{550}] of 0.29 ± 0.02 and 0.28 ± 0.04, respectively; $P > 0.1$).

To study the threshold amount of predator needed for reducing biofilm biomass, we varied the total *Bdellovibrio* added to the wells (from 10^8 to 10 PFU/well). An initial titer as low as 10^2 PFU/well was sufficient to reduce a preformed biofilm by ~90% after 48 h as measured by CV staining. To determine whether continuous exposure to *Bdellovibrio* is necessary for the large decrease in the biofilm population, we monitored the biofilm after a brief exposure (30 min) to 10^8 PFU of *B. bacteriovorus*, followed by six washes with saline in order to remove planktonic *Bdellovibrio*. No decrease in CV staining was measured after this 30-min exposure to *Bdellovibrio* (OD_{550} = 1.3 ± 0.08); however, 24 h later the reduction in biofilm biomass achieved by the 30-min exposure treatment (OD_{550} = 0.31 ± 0.02, a 77% reduction) was less than the reduction resulting from a continuous 24-h exposure to the predator (OD_{550} = 0.23 ± 0.01, an 82% reduction; $P < 0.03$). SEM images taken 30 min after the introduction of *Bdellovibrio*, followed by extensive washes to remove unattached cells, confirmed that a 30-min exposure time is sufficient for *Bdellovibrio* to attach to the biofilm (data not shown).

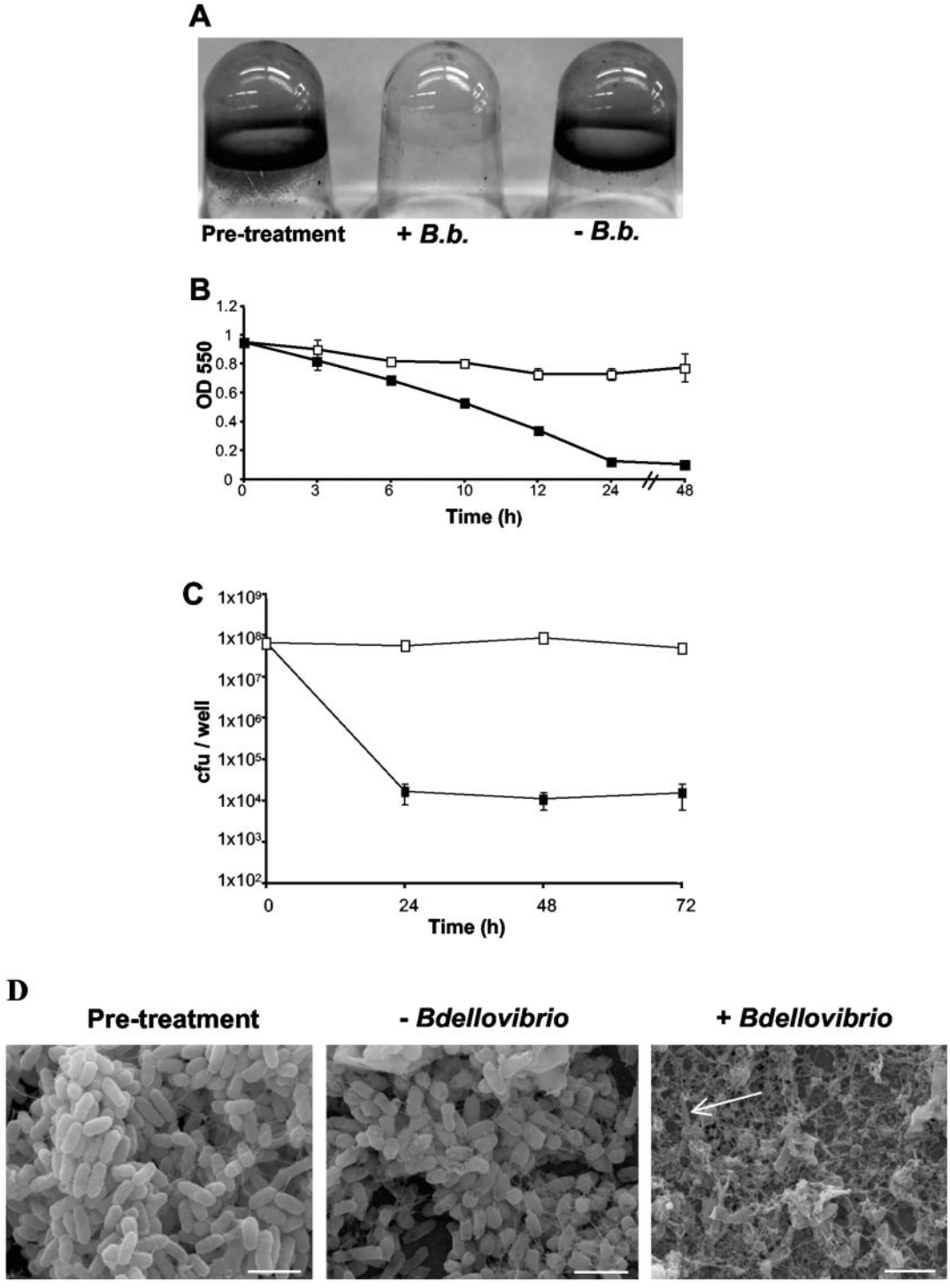

FIG. 1. Predation on *E. coli* biofilms by *B. bacteriovorus*. (A) *E. coli* biofilms were developed for 18 h in 96-well microtiter plates (pretreatment), followed by a 24-h exposure to *B. bacteriovorus* lysate (+*B.b.*) or a sterile lysate solution (−*B.b.*), and then rinsed and stained with CV. (B) Quantification of biofilm biomass over time. *B. bacteriovorus* lysate (■) or a sterile lysate solution control (□) was added to a developed *E. coli* biofilm, the dishes were rinsed and stained with CV, and the amount of CV staining was quantified at OD_{550} for each time point. Each value represents the mean of 24 wells from one representative experiment; error bars indicate the standard errors. Each experiment was carried out three times, yielding similar results each time. The difference in OD_{550} at each time point from 6 h to 48 h was statistically significant ($P < 0.001$) (C) Quantification of biofilm cell viability. *E. coli* biofilms were developed ~18 h in a 96-well microtiter plate, followed by exposure to *B. bacteriovorus* or a sterile lysate. Biofilm cell viability assays of *B. bacteriovorus*-treated (■) and control, sterile lysate-treated (□) samples were performed after the wells were rinsed and sonicated. Each value represents the mean of four wells from one representative experiment; error bars indicate the standard errors. Each experiment was carried out three times yielding similar results. The difference in viability between these treatments at each time point was statistically significant ($P < 0.001$). (D) SEM images taken after *E. coli* biofilms were developed for 18 h on a polyvinyl chloride plastic coverslip (pretreatment) and exposed for 24 h to a sterile lysate solution (−*Bdellovibrio*) or a *B. bacteriovorus* lysate (+*Bdellovibrio*). The arrow indicates attached *B. bacteriovorus.* Scale bar, 2 μm. Magnification, ×10,000. Each experiment was carried out three times, yielding similar results. Images were viewed at the air-liquid interface.

To confirm the decrease in the CV staining of the biofilm in the static assay upon addition of *B. bacteriovorus*, we also assessed the degree of biofilm decrease by direct enumeration of adhered, viable bacteria. The biofilm bacteria were removed from the walls of the 96-well plate by sonication (as described in Materials and Methods), and viable bacteria were counted by plating the cells on LB agar medium (Fig. 1C). By 24 h, a 1,000-fold reduction in biofilm cell count from 6.7×10^7 to 1.7×10^4 CFU/well was detected after treatment with *B. bacteriovorus*. However, as observed for the CV staining assay above (Fig. 1B), the reduction in viable count of biofilm cells obtained after the first 24 h remained unchanged even after an additional 48 h of incubation. At 72 h when the biofilm population was 1.6×10^4 CFU/well, a large population of the predator (2.3×10^5 PFU/well) could still be found associated with the biofilm cells. No planktonic *E. coli* cells were detected prior to sonication in the *Bdellovibrio* treated wells (data not shown), verifying that detachment of the biofilm and movement of the cells to the planktonic fraction did not cause the reduction in biofilm cell counts. In comparison, a <1.5-fold reduction in biofilm cell viability from 6.7×10^7 to 5×10^7 CFU/well was seen after 72 h in the control wells (Fig. 1C).

Microscopy studies. To visualize the *E. coli* biofilm after treatment with *Bdellovibrio*, biofilms that were predeveloped on a plastic coverslip were exposed to either a *B. bacteriovorus* lysate or a filtered sterilized lysate control and then analyzed by SEM. As observed for CV staining and direct enumeration, *Bdellovibrio* was able to significantly reduce the biofilm after 24 h (Fig. 1D). In this figure, the small rod-shaped *Bdellovibrio* (indicated by the arrow) can be distinguished from the larger rod-shaped *E. coli* cells. No discernible changes were observed for the biofilm in the control treatment. Similar results were observed by using phase-contrast microscopy or epifluorescence microscopy of Syto-9-stained samples (data not shown).

Biofilm versus planktonic cell susceptibility to *Bdellovibrio* attack. Because biofilm cells have increased resistance to or tolerance of a wide range of chemical, physical, and biological insults (2, 22, 38), we investigated whether biofilm cells are more resistant to attack from *Bdellovibrio* than their planktonic counterparts. The survival of planktonic cells was determined by simultaneous addition of *Bdellovibrio* and planktonic *E. coli* to the wells of a 96-well dish. Under these conditions, the planktonic cells are not allowed to form a biofilm before they encounter the predator. The survival of the preformed biofilm to *Bdellovibrio* attack was assessed as described above.

As shown in Fig. 2, although *Bdellovibrio* did reduce the numbers of biofilm bacteria by ~1,000-fold, $\sim 10^4$ CFU/well could be recovered after 48 h of incubation. In contrast, the planktonic population was reduced to ~10 CFU/well at 24 h and fell below detection at the 48-h time point. This ~10,000-fold difference in viability of biofilm versus planktonic cells at the 48-h time period strongly suggests that the biofilm bacteria were better able to withstand attack by *Bdellovibrio*.

To confirm that the decrease in planktonic cell population was due to killing by *Bdellovibrio* and not the initiation of biofilm formation, we performed the same assays described above with two biofilm-defective *E. coli* mutants: a type I pili mutant (*fimH*::*cm*) and a colanic acid production mutant (*wcaF*::*cm*). The isolation and characterization of these biofilm-defective mutants has been described elsewhere and, as

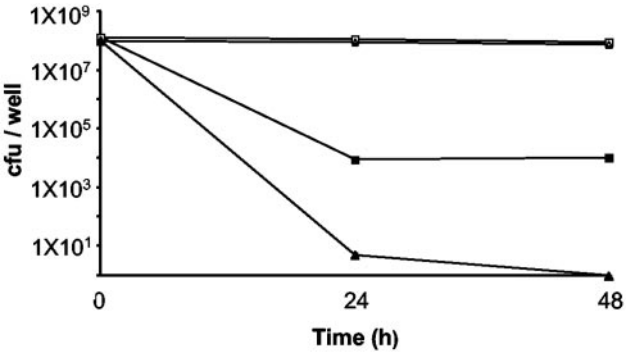

FIG. 2. Cell viability counts of biofilm and planktonic *E. coli*. Wild-type *E. coli* biofilms were formed for 18 h in a 96-well microtiter plate, followed by 48 h of exposure to *B. bacteriovorus* (■) or sterile lysate (□), and viability counts were determined after the wells were rinsed and sonicated. Planktonic *E. coli* were mixed with *B. bacteriovorus* (▲) or with sterile lysate (△), and the bacterial viability counts were determined. Each value represents the mean of four wells from one representative experiment, and error bars indicate the standard errors. The difference in viability at 24 and 48 h between the biofilm versus planktonic cells was statistically significant ($P < 0.001$). Each experiment was carried out three times, yielding similar results.

previously reported (5, 32, 42), no difference in planktonic growth was seen among the wild-type and mutant strains when grown in LB (data not shown). There was no significant difference in residual planktonic cell populations after 24 h of predation for the wild-type strain (~10 CFU/well) compared to the biofilm mutants (~18 CFU/well for the *fimH*::*cm* mutant and ~17 CFU/well for the *wcaF*::*cm* mutant). At 48 h, the planktonic population of the wild type and the two biofilm mutant strains fell below detection. Additional experiments performed in tubes incubated with agitation also showed no difference in predation among the wild-type and mutant strains (data not shown). These data confirm that the decreases in planktonic cell counts were a result of predation by *B. bacteriovorus* and not a consequence of the planktonic cells attaching to the surface and forming a biofilm.

Resistance of biofilm cells to predation. Because a population of $\sim 10^4$ biofilm cells remained even after prolonged exposure to *Bdellovibrio* (Fig. 2), we considered that there may be an "adaptation" of the biofilm cells to a more resistant phenotype. To further examine this proposition, we sought to enrich for biofilm cells that were more resistant to attack by *Bdellovibrio*. A preformed *E. coli* biofilm was exposed to *Bdellovibrio* attack for 24 h, followed by extensive washing to remove the bulk of the predator. LB medium was then added to the *Bdellovibrio*-treated biofilms to allow the outgrowth of any putative resistant *E. coli* cells in the population. After 24 h of outgrowth, the wells were washed, and then fresh predator was added. Under these conditions, we predicted that any resistant subpopulation of *E. coli* present should become enriched leading to a progressive increase in the biofilm (as judged by increased CV staining) that survived *Bdellovibrio* treatment. This enrichment regimen was performed up to five times, successively, and the CV staining was measured. No statistically significant difference ($P > 0.6$) in the decrease of the biofilm was seen with each subsequent exposure to *Bdellovibrio*; an 81.2% ± 1% reduction in CV staining was brought about by the first

encounter with the predator, and an 83.6% ± 2.6% decrease of the biofilm was measured after five rounds of enriching for any resistant subpopulation. These data suggest that no genetically stable population of resistant *E. coli* cells was produced.

Predation experiments in flow cells. To assess the resistance of thicker biofilms to attack by *Bdellovibrio* and to extend our findings to an important environmental organism, we utilized a once-through, continuous flow cell system to examine the predation of *E. coli* and *P. fluorescens* biofilms. Biofilms were grown as described in Materials and Methods resulting in macrocolonies with a maximum thickness measuring 37 ± 8 μm for *E. coli* and 25 ± 5 μm for *P. fluorescens*. The flow-cell-grown biofilms were inoculated with a single pulse of 1 ml (10^9 PFU/ml) of *B. bacteriovorus* or filtered sterilized lysate as control. At 48 h after inoculation with *Bdellovibrio* flow cells were stained with BacLight live/dead stain to assess bacterial cell viability.

In the control samples, large "mushroom-like" structures were detected by phase-contrast microscopy (Fig. 3, left-hand panels), as would be expected for the *E. coli* and *P. fluorescens* biofilms growing under these conditions. In contrast, the *Bdellovibrio*-treated samples were a relatively uniform monolayer. By using BacLight viability stain it was apparent that for the control samples the majority of the cells could be considered live (e.g., staining green) and mushroom structures were readily visualized; however, in the *Bdellovibrio*-inoculated samples the vast majority of the cells were stained red (e.g., considered dead or membrane compromised) for both *E. coli* (Fig. 3A) and *P. fluorescens* (Fig. 3B) biofilms. OpenLab computer analysis of PI-dependent fluorescence measured 2.3 ± 0.8 × 10^5 arbitrary fluorescence units (AFU) after 48 h for the *E. coli* biofilm treated by *Bdellovibrio* compared to the control, measuring 7 ± 2 AFU, an ~5-log difference ($P < 0.001$). A similar difference in PI-mediated fluorescence was observed for *P. fluorescens* (Fig. 3B), wherein the fluorescence values measured for *Bdellovibrio*-treated and control treatment were 8.7 ± 1.8 × 10^5 AFU and 1 ± 0.4 AFU ($P < 0.001$), respectively.

We also monitored the *Bdellovibrio* population in these experiments. At 48 h after inoculation, 1 ml of outflow medium was harvested to determine the *B. bacteriovorus* population. *B. bacteriovorus* in the outflow was measured at 1.6 ± 0.02 × 10^6 PFU/min for *E. coli* and 1.8 ± 0.04 × 10^5 PFU/min for *P. fluorescens*. These data are consistent with the observation that a high population of *B. bacteriovorus* can be recovered from biofilms in the static assay even after 72 h and, furthermore, suggest that the predator can maintain a relatively high population under flow conditions over the course of several days.

We also obtained a rough assessment of the stability of the *Bdellovibrio*-treated and untreated biofilms. At the completion of the flow cell studies, we rinsed the flow cells with 0.5% sodium hypochlorite at a high flow rate of 250 ml/h. We observed that, although the *Bdellovibrio*-treated chambers of the flow cell were immediately cleaned of biomass and became transparent, the control chambers needed to be washed for at least 6 min in order to attain a similar outcome (data not shown).

DISCUSSION

In the work presented here, a microtiter dish-based static assay was developed to monitor the ability of *Bdellovibrio* to attack an *E. coli* biofilm. Both CV staining and viable counts showed that *Bdellovibrio* was capable of markedly reducing biofilm biomass (Fig. 1). The extent of damage brought about by *Bdellovibrio* on *E. coli* biofilms was further visualized by SEM imaging, wherein the bulk of the biofilm cells were shown to be destroyed, leaving behind what appears to be cell residue and matrix. An initial titer of as low as 10^2 PFU/well of *Bdellovibrio* was sufficient to reduce biofilm by ~90% and, furthermore, biofilm-attached *Bdellovibrio* visualized by SEM imaging 30 min after initial inoculation confirmed that this brief exposure period was sufficient to initiate infection. In contrast to the biofilm bacteria, the planktonic population was completely eliminated in the static assay (Fig. 2). Interestingly, under the experimental conditions typically used to assess bdellovibrio predation, it is widely reported that bdellovibrios never completely eradicate their planktonic prey (19, 45). The low volume and/or the high host-predator ratio in our static system may explain why no planktonic cells were detected after predation. It is possible that these culture conditions could be exploited as the basis of a selection to identify strains resistant to *Bdellovibrio* attack.

B. bacteriovorus was also able to successfully attack the thicker biofilms grown in flow cell experiments, suggesting that the action of this predator is not restricted to the surface of the biofilm, as is frequently observed with invertebrates and protozoan biofilm grazing experiments, and with studies using bacteriophage (9, 21, 24). Furthermore, by collecting and quantifying *B. bacteriovorus* in the outflow 48 h after inoculation, it was apparent that the predator not only survived in the biofilm but also could feed, proliferate, and escape in order to start a new cycle of predation. These results correspond to the observation in the static microtiter assay, in which a large population of the predator could still be found associated with the biofilm cells 72 h after infection. This ability to survive and proliferate in the biofilm emphasizes the possible significance of biofilms as a natural reservoir of bdellovibrios in the environment.

From the results obtained here it was apparent that upon exposure to a high titer of *Bdellovibrio* the majority of biofilm reduction occurred within the first 24 h of attack (Fig. 2). No additional reduction of the biofilm was achieved by longer incubation times with the predator or by reinoculation of the biofilm with fresh *Bdellovibrio*. Attempts to select for a population of biofilm cells recalcitrant to *Bdellovibrio* attack did not yield a genetically stable resistant population. Similarly, attempts to select for planktonic bacterial populations resistant to *Bdellovibrio* in batch culture have been unsuccessful (39); therefore, resistance to *Bdellovibrio* predation of planktonic cells was concluded to be a plastic phenotypic response rather than a mutational event (36); perhaps biofilm-grown organisms also develop such a phenotypic resistance. Alternatively, as reported in other systems (22), a subpopulation of bacteria in the biofilm may be growing more slowly, be nutritionally deprived, or induce a stress response, thus decreasing their susceptibility to *Bdellovibrio* attack. Although we do not understand the nature of this resistance, previous work has shown successful adaptation of other biofilm bacteria to attack by predators. For example, *P. aeruginosa* biofilms that were exposed to the surface-feeding flagellate *Rhynchomonas nasuta*

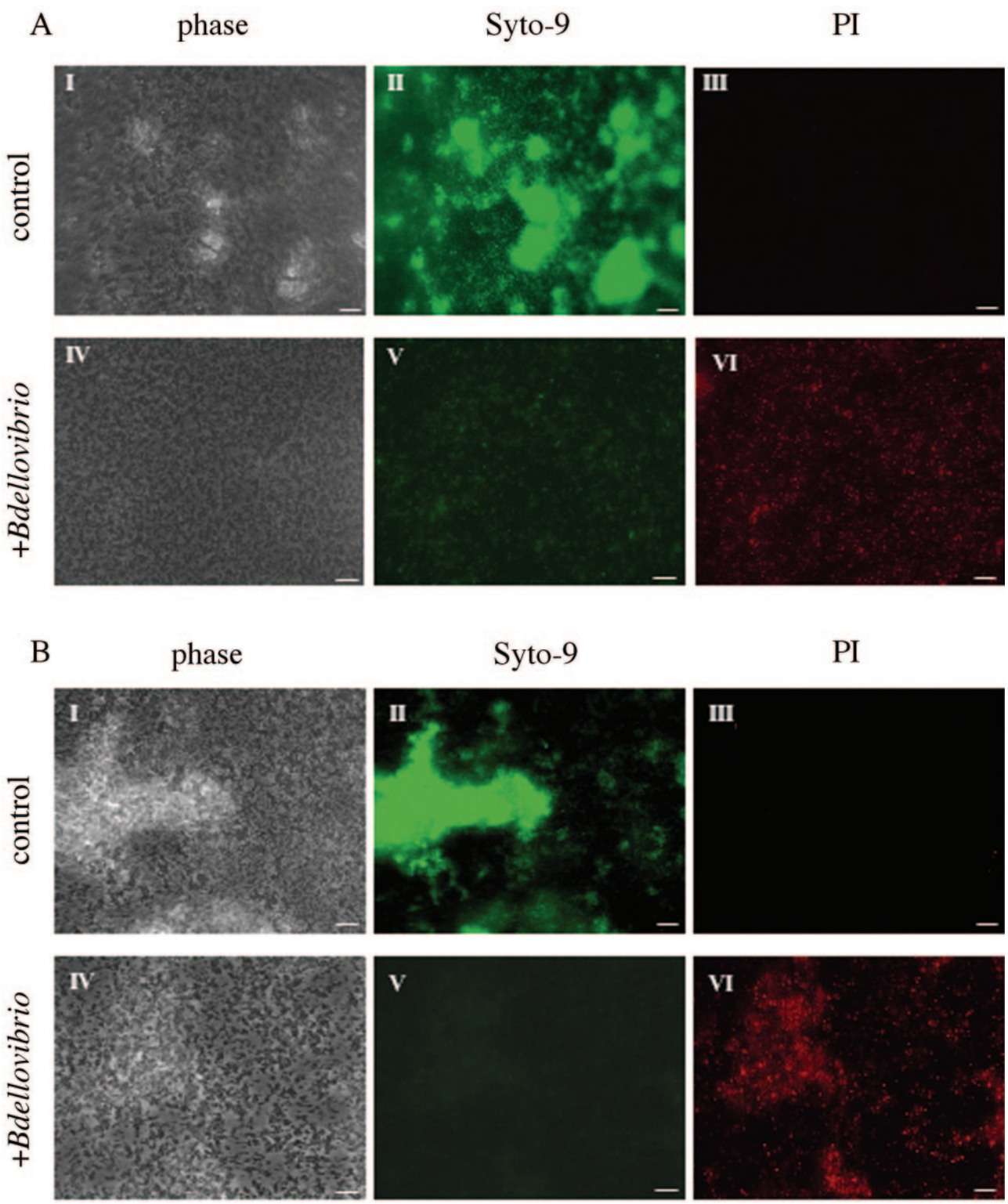

FIG. 3. Monitoring *Bdellovibrio* attack in flow cells. *E. coli* (A) and *P. fluorescens* (B) biofilms were developed in a flow cell system after inoculation with sterile lysate solution (I to III) or *B. bacteriovorus* lysate (IV to VI). After 48 h the chambers were analyzed by phase-contrast microscopy (I and IV) (dark areas are adherent bacteria) or stained with the BacLight viability stain for 45 min and then rinsed for 45 min to remove excess dye. Syto-9 panels (II and V) indicate viable cells (green, intact membranes), and PI panels (III and VI) indicate dead or compromised cells (red, damaged membranes). Scale bar, 20 μm. Magnification, ×650. Each experiment was carried out three times, with two replicates for each treatment, yielding similar results. At least 10 different areas of each sample were examined; representative images are shown here.

developed microcolonies in the presence of the flagellate, whereas biofilms without predator were undifferentiated (24).

With the increasing interest in developing improved methods for controlling biofilms, there are number of potential advantages of using *Bdellovibrio* for the biological control of biofilms. For example, although bdellovibrios have a broad host range (39), they are highly specific for infecting bacteria and thus are harmless to nonbacterial organisms. Furthermore, the initial dose of the *Bdellovibrio* can be low since this predator multiplies in the host cells, releasing new bdellovibrios upon lysis. As we have observed here, a *Bdellovibrio* population is maintained in the biofilm even though a majority of the host bacteria have been destroyed. *Bdellovibrio* is also likely to be effective even against bacteria that have multiple resistances to antibiotics, which is the situation in many biofilm settings (22). Finally, a key difficulty encountered in the use of biological control agents in reducing biofilm population is the inability of biological control agents to access the cells within the biofilm. Working with capsulated and noncapsulated planktonic *E. coli*, Koval and Bayer (20) have shown that the thick polysaccharide capsule does not impose a barrier against *Bdellovibrio*; thus, the penetration difficulty encountered in other biological systems does not appear to be the case for *Bdellovibrio*. The results obtained in the present study (Fig. 3) demonstrated that *B. bacteriovorus* does have the capability to access biofilms as thick as 30 μm and is not restricted to the surface of the biofilm.

The use of *Bdellovibrio* for reducing bacterial population has been attempted previously by other investigators with some success. Among these efforts was the use of *Bdellovibrio* in controlling *Xanthomonas oryzae* from rice paddy fields, controlling bacterial blight caused by *Pseudomonas glycinea*, and the reduction of soft rot and black leg in potato (10, 33, 44). In animal models, *Bdellovibrio* was used to treat *Shigella flexneri*-induced keratoconjunctivitis in rabbits and as a predator against pathogens in the intestinal tract (25, 47). From these studies it is difficult to determine whether the *Bdellovibrio* was attacking planktonic or biofilm bacterial populations; however, the ability of soil-isolated *Bdellovibrio* to reduce *E. coli* O157:H7 attached to a stainless steel surface has been reported (11). The methods developed here should allow a more rigorous assessment of the potential use of *Bdellovibrio* as a biocontrol agent versus biofilms. For example, the static biofilm assay allows the potential for rapid and large-scale screening of conditions promoting biofilm killing by *Bdellovibrio*. Furthermore, it was apparent from our studies that when flow cells were rinsed with sodium hypochlorite at high velocity, the biofilm in the chambers treated with the predator was removed faster than in the control treated chambers. These data suggest that *Bdellovibrio* may be an effective tool to improve classical biofilm control strategies.

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