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Vulnerability of Pathogenic Biofilms to *Micavibrio aeruginosavorus*▽

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The host specificity of the gram-negative exoparasitic predatory bacterium *Micavibrio aeruginosavorus* was examined. *M. aeruginosavorus* preyed on *Pseudomonas aeruginosa*, as previously reported, as well as *Burkholderia cepacia*, *Klebsiella pneumoniae*, and numerous clinical isolates of these species. In a static assay, a reduction in biofilm biomass was observed as early as 3 hours after exposure to *M. aeruginosavorus*, and an ~100-fold reduction in biofilm cell viability was detected following a 24-h exposure to the predator. We observed that an initial titer of *Micavibrio* as low as 10 PFU/well or a time of exposure to the predator as short as 30 min was sufficient to reduce a *P. aeruginosa* biofilm. The ability of *Micavibrio* to reduce an existing biofilm was confirmed by scanning electron microscopy. In static and flow cell experiments, *M. aeruginosavorus* was able to modify the overall *P. aeruginosa* biofilm structure and markedly decreased the viability of *P. aeruginosa*. The altered biofilm structure was likely caused by an increase in cell-cell interactions brought about by the presence of the predator or active predation. We also conducted a screen to identify genes important for *P. aeruginosa-Micavibrio* interaction, but no candidates were isolated among the ~10,000 mutants tested.

Biofilms are dense aggregations of microbial cells attached to a surface (9, 11). These surface-attached communities are known to have a significant impact on human health when they form on medical and surgical implants (4, 13, 16, 18, 34, 36, 40). A major difficulty in controlling surface-attached bacteria is their enhanced resistance to antimicrobial agents. Biofilms can be 10 to 1,000 times more resistant to antimicrobial agents than their planktonic counterparts (5, 20, 26, 27, 35). The difficulty in controlling biofilms by conventional antibiotic therapy led researchers to examine other methods of biofilm control. Among these alternative techniques is the use of biological control agents, including invertebrates, protozoa, and bacteriophages (10, 14, 15, 19, 21, 28, 29, 33, 38, 43, 45, 46). Predatory prokaryotes from the genus *Bdellovibrio* have also been shown to have potential for biofilm control (17, 22).

In 1982, while searching for *Bdellovibrio* samples in wastewater, Lambina and colleagues isolated a new species of exoparasitic bdellovibrio-like bacteria that they called *Micavibrio* (24). Like *Bdellovibrio* spp., *Micavibrio* spp. are characterized by an obligatory parasitic life cycle. *Micavibrio* organisms are gram negative, small (~0.5 to 1.5 μm long), rod shaped, and curved and have a single polar flagellum. Phylogenetic analyses have placed *Micavibrio* spp. within the α subgroup of proteobacteria (12). The *Micavibrio* cycle of development includes the following stages: motile *Micavibrio* organisms attach to the cell surfaces of host bacteria, followed by growth of the exoparasite on the surface of the host and, finally, death of the infected cells (2, 25). Unlike *Bdellovibrio*, *Micavibrio* spp. were shown to have a high degree of host specificity; for example, *Micavibrio aeruginosavorus* strain ARL-13 was shown to prey only on *Pseudomonas aeruginosa* among 55 bacteria of different taxonomic groups that were tested (25).

In this study, we evaluated the ability of *M. aeruginosavorus* strain ARL-13 to infect pathogenic bacteria grown planktonically and in biofilms. Direct enumeration and microscopy of static and flow-cell-grown biofilms were used to quantify and visualize the extent and nature of damage inflicted on these communities after *M. aeruginosavorus* treatment. We also describe host cell-cell interactions brought about by predation, indicating that *M. aeruginosavorus* can promote biofilm formation under some conditions.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Unless otherwise noted, the strains used in this study were from the laboratory collection. *M. aeruginosavorus* strain ARL-13 was provided by E. Jurkevitch from the Hebrew University of Jerusalem (12). The following bacteria were obtained from Presque Isle cultures (Presque Isle, Erie, PA): *Acetobacter aceti* (ATCC 23747), *Bordetella bronchiseptica* (ATCC 10580), *Burkholderia cepacia* (ATCC 25416), *Enterococcus faecalis* (ATCC 19433), *Erwinia amylovora* (ATCC 7400), *Klebsiella pneumoniae* (ATCC 13883), *Mycobacterium smegmatis* (ATCC 14468), *Salmonella enterica* (ATCC 14028), *Serratia marcescens* strain D (ATCC 27117), *Shigella flexneri* (ATCC 12022), *Staphylococcus simulans* (ATCC 11631), and *Yersinia enterocolitica* (ATCC 23715). *Escherichia coli* strain ZK2686 was provided by R. Kolter (32, 37), *Vibrio cholerae* El Tor (3) was provided by R. Taylor, and *Pseudomonas syringae* BD301D was obtained from M. Klotz. Clinical isolates were provided by J. Schwartzman and R. Kowalski. *E. amylovora*, *Pseudomonas fluorescens*, *Pseudomonas putida*, and *P. syringae* were routinely grown in LB medium at 30°C. All other bacteria were grown at 37°C.

Cells were enumerated as CFU on LB agar plates. *M. aeruginosavorus* and *Bdellovibrio bacteriovorus* were maintained as plaques, as reported previously (41). *M. aeruginosavorus* populations were quantified as PFU developed on a lawn of prey cells. *M. aeruginosavorus* lysates were obtained by adding a plug of agar containing an *M. aeruginosavorus* plaque ($\sim 1 \times 10^6$ PFU/ml) to $\sim 1 \times 10^8$ CFU/ml washed prey, followed by a 24-h incubation in DDNB medium. DDNB medium is a 1:50 dilution of nutrient broth with 3 mM $MgCl_2 \cdot 6H_2O$ and 2 mM $CaCl_2 \cdot 2H_2O$. Mixtures of *M. aeruginosavorus* and host were incubated at 30°C on a rotary shaker at 200 rpm, and populations of the predator reached a final concentration of $\sim 1 \times 10^8$ PFU/ml. To harvest *M. aeruginosavorus*, the 24-h lysates were passed three times through a 0.45-μm-pore-size filter to remove residual prey and cell debris. These lysates are referred to hereafter as "*Micavibrio* lysates." As a control, a *Micavibrio* lysate was passed three times through

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a 0.22-μm-pore-size filter to remove all of the predator cells, yielding what will be referred to as "sterile lysate." The *Micavibrio* lysate was plated on LB agar medium to confirm that no viable host bacterial cells were present in the sample. No predator or host, as judged by CFU and PFU, respectively, could be detected in the sterile lysate (not shown). Dilutions were prepared in saline solution (150 mM NaCl) or 25 mM HEPES buffer containing 2 mM $CaCl_2 \cdot 2H_2O$ (pH 7.8).

Host range. The host specificity of *M. aeruginosavorus* was assessed by the ability of the predator to form a lytic halo on a lawn of prey cells, using a modification of the double-layer plaque assay (42). Host bacteria were grown for 18 h in LB medium, and 100 μl of washed cells was spread on DNB medium solidified with 1.5% agar. *Micavibrio* lysate (20 μl) was spotted on a lawn of host bacteria. DNB medium is 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). Lytic halo assay plates were incubated at 30°C for up to 4 weeks and examined for the formation of a zone of clearing where the lysates were spotted. Each lytic halo assay was performed at least four times in triplicate, with the *Micavibrio* lysate and the sterile lysate control spotted on each plate.

Bacteria that showed sensitivity to *Micavibrio* attack in the lytic halo assay were further assessed for predation in a liquid lysate assay. In the liquid lysate assay, sensitivity of the host to *Micavibrio* was determined by a reduction in host CFU and/or the reduction of turbidity, using a Spectronic 20 spectrophotometer (Spectronic Instruments Inc., Westbury, NY) at 600 nm. Each liquid lysate test was carried out at least three times.

Biofilm and predation assays. Biofilm formation in 96-well polyvinyl chloride microtiter dishes (Becton Dickinson, Franklin Lakes, NJ) was measured by staining with 0.1% crystal violet (CV) in water as described previously (22, 30, 31), with the following modification. Microtiter wells were inoculated (100 μl per well) with an 18-h LB-grown host culture diluted 1:50 in the following media: for *P. aeruginosa* PA14 biofilms, diluted King's B medium was used (a 1:10 dilution of King's B medium containing 2% proteose-peptone, 1% glycerol, 8.6 mM K_2HPO_4, and 1 mM $MgSO_4 \cdot 7H_2O$ [pH 7.2]); *K. pneumoniae* biofilms were developed in M63 minimal salts (32) supplemented with 1 mM $MgSO_4 \cdot 7H_2O$, 14 mM $Na_3C_6H_5O_7 \cdot 2H_2O$, and 34 mM L-proline; and for *B. cepacia* biofilms, M63 minimal salts supplemented with 1 mM $MgSO_4 \cdot 7H_2O$, 5% glycerol, and 0.4% Casamino Acids was used. Quantification of biofilm bacteria by CFU was performed as described previously (22, 30). For statistical analyses, *P* values were determined using Student's *t* test performed with Microsoft Excel software. Error bars show 1 standard deviation.

Flow cell experiments. For biofilms grown under flow conditions, bacteria were cultivated in a four-channel flow cell, with square 2- by 2-mm glass capillaries (Friedrich and Dimmock Inc., Millville, NJ) serving as the channels. The flow system was assembled as described previously (8) and inoculated with 18-h LB-grown cultures diluted 10-fold in 30% King's B medium. The medium flow was stopped prior to inoculation and for 1 hour after inoculation. After the development of a mature, multilayered biofilm (24 h following inoculation with *P. aeruginosa*), the flow was stopped, and the chambers were inoculated with 1 ml ($\sim 1 \times 10^8$ PFU/ml) of *Micavibrio* lysate, prepared as described above, or 1 ml of sterile lysate as a control. After 1 h, the flow was resumed, and 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 pump (Watson-Marlow, Cornwall, England). Five experiments were performed for each strain, with two replicates for each treatment.

Imaging. Epifluorescence and phase-contrast microscopy and viability staining were performed as described previously (22). Quantification of macrocolony formation was performed by visually counting the numbers of macrocolonies in a field of view (magnification, ×100) using phase-contrast microscopy, and at least 50 fields were evaluated for each treatment. Cell clusters with a diameter of >50 μm were considered macrocolonies in this study.

SEM. Scanning electron microscopy (SEM) experiments were performed as described previously (22).

Quantitative measurement of aggregation. The extent of aggregation was measured during growth with the predator according to the method of Burdman et al. (6), modified as described below. The liquid culture containing aggregates was allowed to stand for 20 min to allow aggregates to settle to the bottom of the tube. Turbidity of the suspension (optical density of the suspension [OD_s]) was measured using a Molecular Devices VMax kinetic microplate reader (Sunnyvale, CA) at 600 nm. The culture was then dispersed by sonication using a VC505 sonicator (Sonics and Materials Inc., Newtown, CT) for 10 s, and the total turbidity was measured (OD_t). The percentage of aggregation was estimated as follows: % aggregation = $[(OD_t - OD_s) \times 100]/OD_t$.

Genetic approach for studying host-predator interaction. A collection of ~10,000 random transposon mutants of *P. aeruginosa* PA14 carrying the transposon Tn*5*-B30 (Tc^r) (31, 39) were grown individually in 96-well microtiter dishes for 18 h to allow biofilm formation and washed three times with DDNB medium, and 100 μl of *Micavibrio* lysate was added. In a parallel experiment, 100 μl of the sterilized lysate was added to the preformed biofilms. After 24 h of incubation, the wells were stained with crystal violet to assess predation. Biofilm-defective strains among the collection of Tn*5*-B30 (Tc^r) transposon mutants were screened in the lytic halo assay described above.

TABLE 1. Host range of *M. aeruginosavorus*

Organism(s) and origin	Predation (no. of attacked strains/no. of strains tested)	
	Planktonic cells[a]	Biofilm cells[b]
Acetobacter aceti	−	NA
Bordetella bronchiseptica	−	NA
Burkholderia cepacia	+	+
B. cepacia clinical isolate	1/1	1/1
Enterococcus faecalis	−	NA
Erwinia amylovora	−[c]	NA
Escherichia coli	−[c]	NA
Klebsiella pneumoniae	+[c]	+
K. pneumoniae clinical isolates	10/13	3/3
Mycobacterium smegmatis	−	NA
Pseudomonas aeruginosa PA14	+	+
P. aeruginosa PAO1	+	+
P. aeruginosa clinical isolates		
Urine	16/16	15/15
Sputa from non-cystic fibrosis patients	21/27	6/13
Sputa from cystic fibrosis patients	7/11	4/4
Eye	38/43	32/34
Miscellaneous organs	22/23	10/15
Pseudomonas fluorescens	−[c]	NA
Pseudomonas putida	−[c]	NA
Pseudomonas syringae	−	NA
Salmonella enterica	−[c]	NA
Serratia marcescens	−	NA
Shigella flexneri	−	NA
Staphylococcus aureus	−	NA
Staphylococcus simulans	−	NA
Yersinia enterocolitica	−	NA
Vibrio cholerae	−	NA

[a] Twenty microliters of a *Micavibrio* lysate ($\sim 1 \times 10^8$ CFU/ml) was spotted on a lawn of the indicated bacteria. Predation was scored as the formation of a lytic zone at the point of *Micavibrio* inoculation. +, predation by *M. aeruginosavorus* both in the lytic halo assay and in the liquid lysate assay; −, no predation by *M. aeruginosavorus*.

[b] Biofilms were formed in 96-well microtiter dishes for 18 h, and then the *Micavibrio* lysate was added to the preformed biofilm. Biofilm reduction was assessed by the reduction of CV staining and CFU counts. +, predation by *M. aeruginosavorus*; −, no predation by *M. aeruginosavorus*; NA, not assessed.

[c] Predation by *Bdellovibrio bacteriovorus* 109J.

RESULTS

Host range. *M. aeruginosavorus* strain ARL-13 was first isolated some 20 years ago on *P. aeruginosa* as a host. To reassess the host specificity of *M. aeruginosavorus*, we analyzed the ability of this predator to attack different bacterial species. *M. aeruginosavorus* had the ability to attack and form lytic halos on 3 of 19 bacterial species tested (Table 1). The bacteria that were preyed upon by *M. aeruginosavorus* ARL-13 were *B. cepacia*, *K. pneumoniae*, and *P. aeruginosa*, including both strains PA14 and PAO1 and numerous clinical isolates. Liquid lysate assays were performed to confirm predation observed on plates. The predator reduced the host population 100- to 1,000-fold from a starting population of $\sim 10^8$ CFU/ml by 48 h.

No significant reduction in CFU ($P > 0.1$) was measured in control treatments.

To further investigate the specificity of the predator, we conducted lytic halo experiments, using clinical isolates as hosts (Table 1). *M. aeruginosavorus* had the ability to attack 104 of 120 *P. aeruginosa* clinical isolates tested as well as 10 of 13 *K. pneumoniae* strains and the one *B. cepacia* clinical isolate tested. These data show that the predator has some flexibility in its ability to attack strains within a given species. No obvious conserved characteristics, such as colony morphology or excessive exopolysaccharide production, were observed among the resistant strains.

***P. aeruginosa* biofilm predation assay.** Because *M. aeruginosavorus* strain ARL-13 was first isolated as a predator of *P. aeruginosa*, a major opportunistic pathogen and a key model for the study of biofilm formation, we assessed the ability of *M. aeruginosavorus* to attack biofilms of this microbe. To measure the effect of *M. aeruginosavorus* on *P. aeruginosa* biofilms over time, we developed conditions that yielded stable *P. aeruginosa* biofilms in a 96-well dish. *P. aeruginosa* biofilms were grown in DNB medium for 18 h. Thereafter, the medium was replaced by DDNB, yielding biofilms comprising $\sim 1 \times 10^8$ CFU/ml that could be maintained stably for up to 144 h.

The *P. aeruginosa* biofilm formed after ~18 h in a 96-well microtiter plate was exposed to a *Micavibrio* lysate or a sterile lysate as a control. As shown in Fig. 1A (pretreatment), the untreated 18-h-old biofilm produced was easily visualized by CV staining. Treatment with the *Micavibrio* lysate (Fig. 1A, *+M.a.*) markedly reduced the CV staining compared to that of the sterile lysate control (Fig. 1A, *−M.a.*). Quantification of the effect of *M. aeruginosavorus* on *P. aeruginosa* biofilms over time revealed a 69% reduction in CV staining after 12 h and an 87% reduction after 24 h (Fig. 1B, filled squares) relative to that of a biofilm treated with the sterile lysate control. At 48 h, the reduction in CV staining was 85% compared to the initial time point ($t = 0$), and no further reduction occurred with 96 h of incubation. In contrast, a 13% increase in CV staining in the control was measured after 24 h (Fig. 1B, empty squares).

We also assessed the degree of biofilm decrease by direct enumeration of adherent, viable bacteria. By 24 h, a 16-fold reduction in biofilm cell count, from 1.1×10^8 to 6.6×10^6 CFU/ml, was detected after treatment with *M. aeruginosavorus* (Fig. 1C, filled squares). The reduction in viable counts of biofilm cells obtained after the first 24 h remained largely unchanged even after an additional 48 h of incubation. In comparison, no reduction in viable biofilm cells was observed in the control wells after 72 h (Fig. 1C, empty squares).

To study the threshold amount of predator needed to reduce biofilm biomass, we varied the total amount of *M. aeruginosavorus* added to the wells (from 1×10^8 to 1 PFU/well). An initial titer as low as 10 PFU/well was sufficient to reduce a preformed biofilm by 74% after 96 h, as measured by CV staining (from OD_{550} of 0.6 ± 0.08 to OD_{550} of 0.18 ± 0.04). To determine if continuous exposure to *Micavibrio* is necessary for the large decrease in the biofilm population, we monitored the biofilm after a brief exposure (30 min) to $\sim 1 \times 10^8$ PFU of the predator, followed by six washes with saline to remove planktonic *Micavibrio*. After 24 h, the biomasses of biofilms that were exposed to *Micavibrio* for 30 min showed nearly the same reduction as that resulting from a continuous 24-h exposure to the predator (78% reduction versus 81% reduction in CV staining, respectively). SEM images taken 30 min after the introduction of *Micavibrio*, followed by extensive washes to remove unattached cells, confirmed that a 30-min exposure time is sufficient for the predator to attach to cells in the biofilm (data not shown).

Microscopy studies. To visualize the effect of *Micavibrio* predation on biofilms, biofilms that were formed on a plastic coverslip were exposed to either a *Micavibrio* lysate or a sterile lysate control and then analyzed by SEM. A clear difference in the biofilm was observed 24 h after inoculation with the predator compared to inoculation with the control (Fig. 1D). The *P. aeruginosa* cells remaining in the *Micavibrio*-treated sample were 74% smaller than the biofilm cells in the control (0.29 ± 0.08 μm and 1.13 ± 0.23 μm in length, respectively; $P < 0.001$). Furthermore, the amount of cell debris and matrix was much more abundant in the treated sample than in the control. No discernible changes were observed in the control biofilms.

Predation on *P. aeruginosa* clinical isolate biofilms. We assessed the ability of the predator to attack biofilms formed by *P. aeruginosa* clinical isolates. Only 67.5% (81 of 120) of the *P. aeruginosa* isolates had the ability to form a stable biofilm in a 96-well dish under the conditions tested; *Micavibrio* had the ability to reduce the biofilms of 67 of these 81 clinical isolates by ≥80% (Table 1).

Biofilm versus planktonic cell susceptibility to *Micavibrio* attack. We reported that *E. coli* biofilms have increased resistance or tolerance to predation by *B. bacteriovorus* compared to planktonic *E. coli* cells (22). Therefore, we were interested in investigating whether biofilm-grown *P. aeruginosa* cells were more resistant to *Micavibrio* attack than their planktonic counterparts. The survival of planktonic cells was determined by simultaneously inoculating the predator and planktonic *P. aeruginosa* into DDNB medium in the wells of a microtiter dish. Under these conditions, the planktonic cells are not allowed to form a biofilm before they encounter the predator, and control experiments confirmed that *P. aeruginosa* does not form biofilms under the conditions tested (data not shown). A small but statistically significant increase ($P = 0.04$) was noted in the ability of *Micavibrio* to reduce the number of planktonic cells versus surface-attached cells (from $5.1 \times 10^8 \pm 0.2 \times 10^8$ to $1.3 \times 10^7 \pm 0.5 \times 10^7$ CFU/ml for planktonic cells and from $2.1 \times 10^8 \pm 0.8 \times 10^8$ to $1.3 \times 10^7 \pm 0.3 \times 10^7$ for biofilm cells).

To confirm that the decrease in planktonic cell population was due to killing by *Micavibrio*, not to initiation of biofilm formation, we performed the same study described above with a nonmotile flagellar stator *P. aeruginosa* PA14 mutant (Δ*motABCD*) which is incapable of biofilm formation (44). There was no difference in the planktonic growth rate between the wild type and the mutant strain (data not shown) and no significant difference ($P > 0.1$) in the ability of the predator to reduce the planktonic cell population of the Δ*motABCD* mutant (from $2.1 \times 10^8 \pm 0.8 \times 10^8$ to $1.3 \times 10^7 \pm 0.3 \times 10^7$) compared to its ability to reduce the wild-type biofilm after 48 h (from $2.5 \times 10^8 \pm 0.7 \times 10^8$ to $1.1 \times 10^7 \pm 0.9 \times 10^7$). Additional experiments performed in tubes incubated with agitation also showed no difference in predation between the wild-type and mutant strains (data not shown).

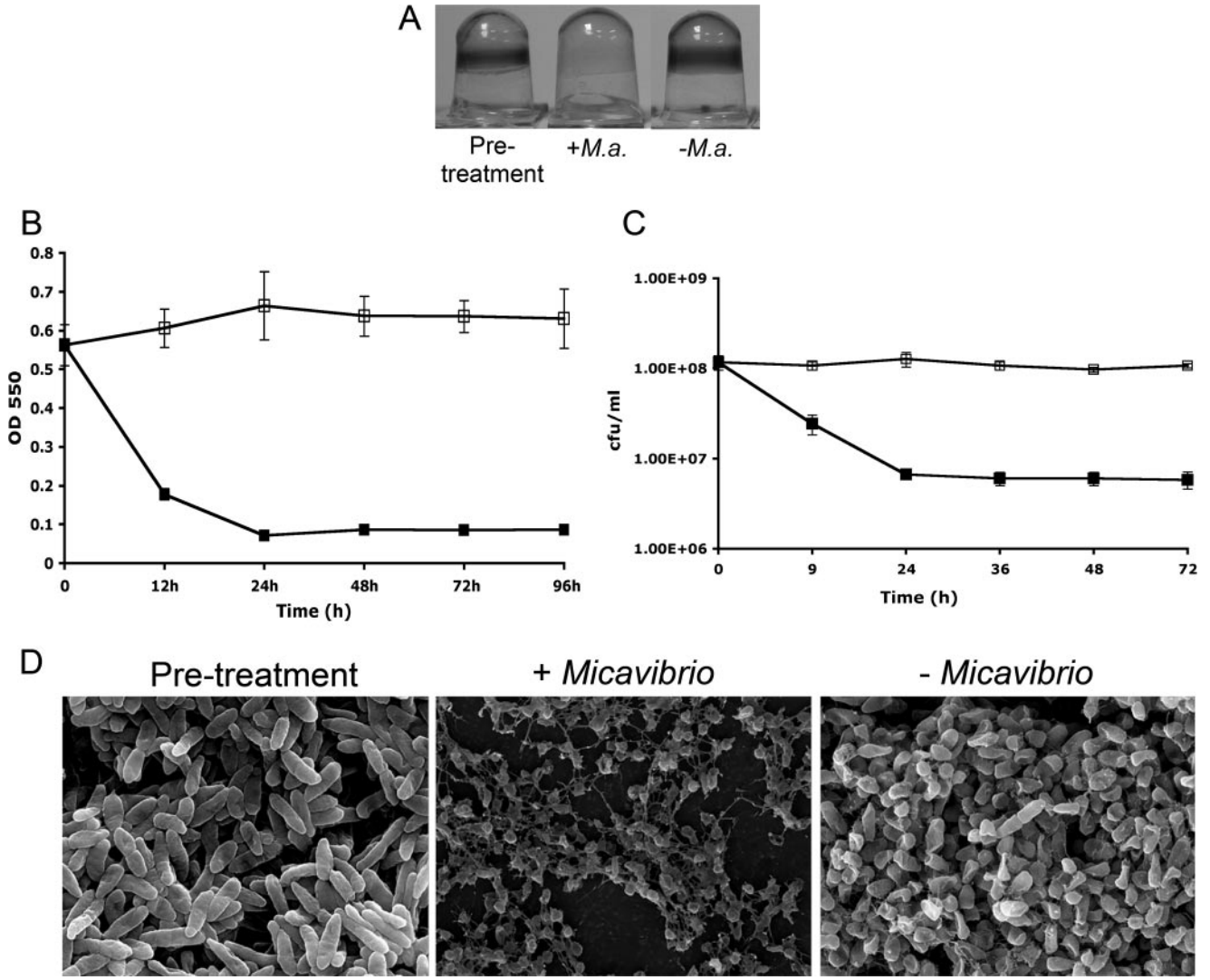

FIG. 1. Predation of *P. aeruginosa* PA14 biofilms by *M. aeruginosavorus* ARL-13. (A) *P. aeruginosa* biofilms were developed for 18 h in 96-well microtiter plates (pretreatment), followed by 24 h of exposure to a *Micavibrio* lysate (+*M.a.*) or a sterile lysate (−*M.a.*), and then rinsed and stained with CV. (B) Quantification of biofilm biomass over time. A *Micavibrio* (■) or sterile (□) lysate was added to a preformed *P. aeruginosa* biofilm, the dishes were rinsed and stained with CV, and the amount of CV staining was quantified as the OD_{550} for each time point. Each value represents the mean for 24 wells from one representative experiment. Error bars show 1 standard deviation. Each experiment was carried out five times, yielding similar results. The difference in OD_{550} at each time point from 12 h to 96 h was statistically significant ($P < 0.001$). (C) Quantification of biofilm cell viability. *P. aeruginosa* biofilms were developed for ~18 h in a 96-well microtiter plate, followed by exposure to a *Micavibrio* (■) or sterile (□) lysate. Samples were obtained after the wells were rinsed and sonicated. Each value represents the mean for three wells from one representative experiment, and error bars indicate standard errors. Each experiment was carried out three times, yielding similar results. The difference in viability between the treatments at each time point was statistically significant ($P < 0.001$). (D) Scanning electron micrographs after *P. aeruginosa* biofilms were developed for 18 h on polyvinyl chloride plastic coverslips (pretreatment) and exposed for 24 h to a *Micavibrio* lysate (+*Micavibrio*) or a sterile lysate (−*Micavibrio*). Magnification, ×10,000. Each experiment was performed three times, yielding similar results. Images were viewed at the air-liquid interface.

Predation experiments in flow cells. To assess the resistance of mature biofilms to attack by *Micavibrio*, we utilized a flow cell system to examine the predation of *P. aeruginosa* PA14 biofilms. Biofilms were grown in flow cells for 24 h, resulting in a uniform lawn of cells (depth, 12 ± 3 μm). The flow-cell-grown biofilms were inoculated with a single pulse of 1 ml (~1×10^8 PFU/ml) of *Micavibrio* lysate, or sterile lysate as a control, and the viability of the cells was assessed 72 h later.

In the control samples, a uniform biofilm lawn was detected by phase-contrast microscopy, with a relatively small number of "mushroom-like" macrocolony structures (Fig. 2, left panels). In contrast, many more macrocolonies were observed for the biofilm treated with *Micavibrio*. Quantification of these structures revealed a 6.5-fold increase in the number of macrocolonies in the predator-treated sample relative to that in the control (18.2 ± 4.7 and 2.8 ± 1.6 macrocolonies/field, respectively; $P < 0.001$).

By using the BacLight viability stain, it was apparent that for the control samples, the majority of the cells could be considered live (staining green), but for the *Micavibrio*-treated sam-

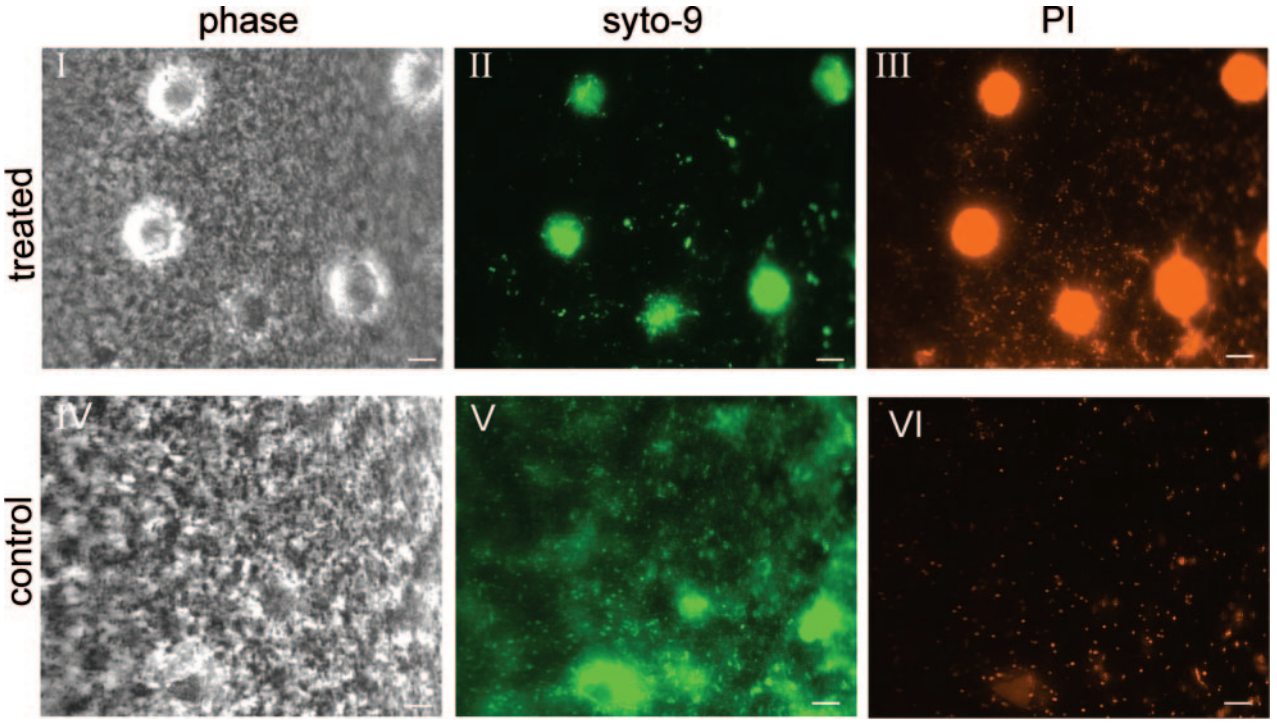

FIG. 2. Monitoring *Micavibrio* attack in flow cells. *P. aeruginosa* PA14 biofilms were developed in a flow cell system for 24 h following inoculation with a *Micavibrio* (I to III) or sterile (IV to VI) lysate. Seventy-two hours after treatment, the chambers were analyzed by phase-contrast microscopy (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). Bar, 20 μm; magnification, ×650. Each experiment was performed three times, with two replicates for each treatment, yielding similar results. At least 10 different areas of each sample were examined, and representative images are shown.

ples, the vast majority of the cells on the surface and in the macrocolonies were likely dead or had compromised membranes (staining red). OpenLab computer analysis of propidium iodide (PI)-dependent fluorescence measured $3.4 \times 10^4 \pm 0.3 \times 10^4$ arbitrary fluorescence units after 72 h for the *P. aeruginosa* biofilm treated with *Micavibrio*, in contrast to the control, measuring $3.6 \times 10^3 \pm 1.6 \times 10^3$ arbitrary fluorescence units, which is an ~10-fold difference ($P < 0.001$).

Cell-cell interactions are induced by predation. The flow cell studies showed an increase in macrocolony formation in the predator-treated sample compared to that in the control, indicating that *M. aeruginosavorus* impacted the multicellular behavior of *P. aeruginosa*. We found that in rich medium (such as King's B medium), rather than the nutrient-limited conditions used in the planktonic cell susceptibility assay described above, the addition of *Micavibrio* induced *P. aeruginosa* biofilm formation at early time points. Microtiter wells were inoculated (100 μl per well) with 18-h LB-grown *P. aeruginosa* cells diluted 1:50 in 10% King's B medium and mixed at a 1:1 ratio with a *Micavibrio* lysate or a sterile lysate as the control. There was a 50% increase in CV staining in the treated sample (Fig. 3A, *+M.a.*) compared to that in the control (Fig. 3A, *−M.a.*) at 24 h of incubation. This was followed by a reduction in CV staining of 78% in the predator-treated sample (Fig. 3A, 48h, *+M.a.*) and a 53% increase in CV staining in the control (Fig. 3A, 48 h, *−M.a.*).

Additional verification of the dynamics of biofilm development in the presence of the predator came from direct enumeration of adherent, viable bacteria (Fig. 3B). By 24 h, the number of biofilm CFU in the *Micavibrio*-treated sample was 3.4-fold higher than that in the control ($3.4 \times 10^8 \pm 0.7 \times 10^8$ and $1.0 \times 10^8 \pm 0.1 \times 10^8$ CFU/ml, respectively; $P < 0.01$), followed by a reduction in the treated sample ($9.5 \times 10^7 \pm 0.1 \times 10^7$ CFU/ml) and an increase in the control sample ($1.0 \times 10^9 \pm 2.0 \times 10^9$ CFU/ml) after 48 h. Therefore, the results of assays in 96-well dishes mirror those of assays in flow cells, with an initial boost in biofilm formation followed by a reduction of the viable population for the *Micavibrio*-treated sample.

One possibility for the increase in biofilm formation at earlier time points is that cell debris produced by predation became available to the host, thereby stimulating host growth and biofilm formation. We predicted that if this were the case, we would also expect to observe an increase in the planktonic *P. aeruginosa* population at early time points after addition of the predator. To evaluate this hypothesis, we conducted experiments in which host cells and *Micavibrio* were simultaneously mixed in 5-ml tubes and grown under shaking conditions. There was no growth increase in planktonic *P. aeruginosa* measured in the predator-treated sample during the first 18 h of predation (from $1.3 \times 10^8 \pm 0.2 \times 10^8$ to $5.5 \times 10^5 \pm 1.2 \times 10^5$ CFU/ml), but an increase in viable *P. aeruginosa* cells was detected in the sterile lysate control treatment (from $5.6 \times 10^7 \pm 3.1 \times 10^7$ to $3.5 \times 10^9 \pm 1.2 \times 10^9$ CFU/ml). These data are not consistent with the hypothesis that the increased biofilm formation in the presence of *Micavibrio* is due to increased growth of *P. aeruginosa*. We also observed no difference in the

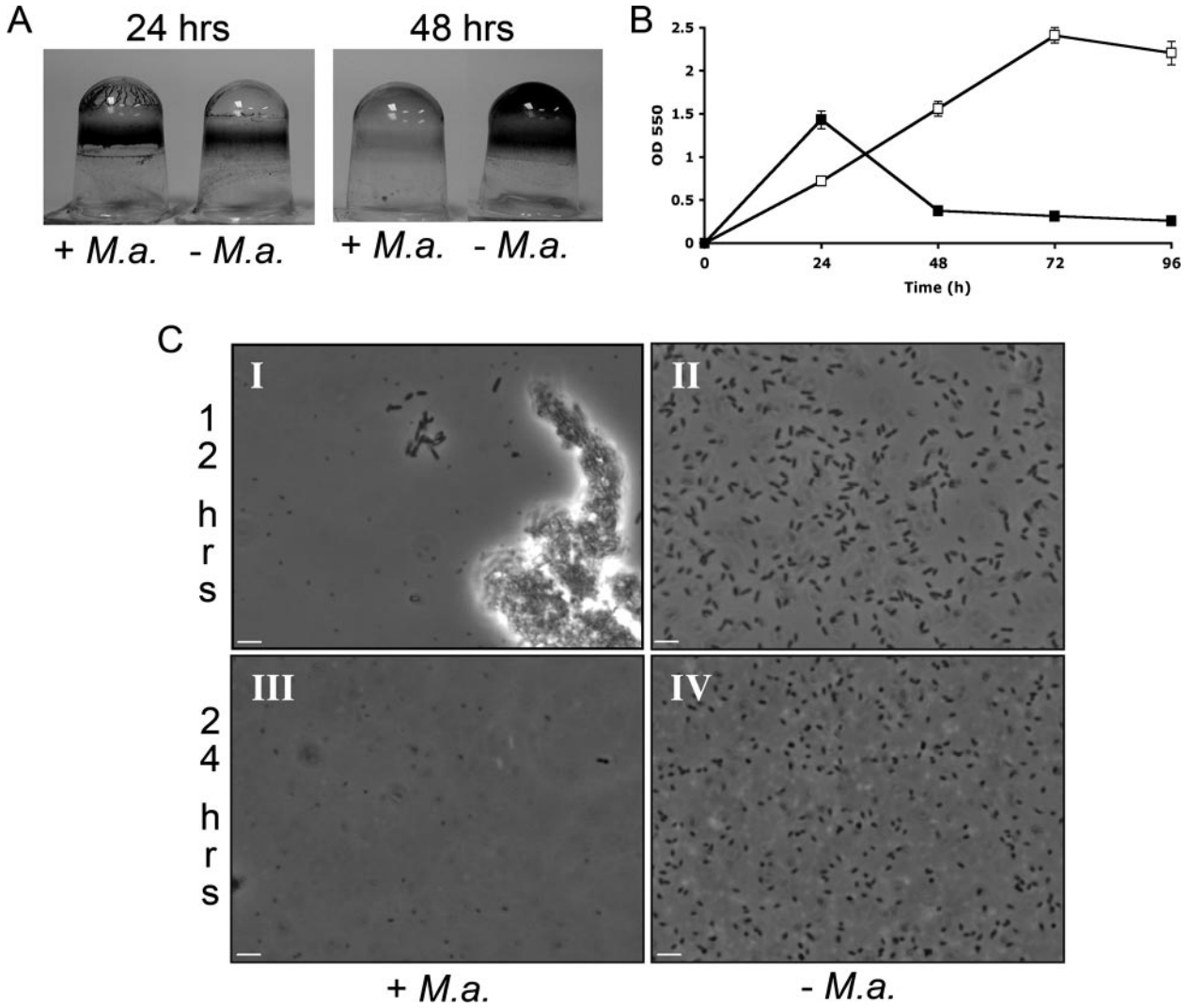

FIG. 3. Predation promotes early biofilm formation and cell aggregation. (A) *P. aeruginosa* biofilms were simultaneously mixed with a *Micavibrio* (+*M.a.*) or sterile (−*M.a.*) lysate, and the wells were rinsed and stained with CV after 24 and 48 h of incubation. (B) Quantification of biofilm biomass over time. A *Micavibrio* (■) or sterile (□) lysate was simultaneously mixed with *P. aeruginosa* cells, the dishes were rinsed and stained with CV, and the amount of CV staining was quantified as the OD_{550} for each time point. Each value represents the mean for 24 wells from one representative experiment, and error bars indicate 1 standard deviation. Each experiment was carried out five times, yielding similar results. The difference in OD_{550} at each time point from 24 h to 96 h was statistically significant ($P < 0.001$). (C) Phase-contrast microscopy images taken 12 (I and II) and 24 (III and IV) h after *P. aeruginosa* was simultaneously mixed with a *Micavibrio* lysate (+*M.a.*) or sterile lysate (−*M.a.*). Bar, 4 μm; magnification, ×650. This experiment was performed three times, yielding similar results.

growth of *P. aeruginosa* when the *Micavibrio* lysate versus a filter-sterilized supernatant of a *P. aeruginosa* culture was used as growth medium.

In analyzing predation by phase-contrast microscopy, it was quite evident that most of the host cells in the *Micavibrio*-treated sample formed aggregates within the first few hours, whereas no cell aggregation was observed in the control sample or in a sample that was mixed with heat-killed *Micavibrio* cells (incubated for 45 min at 65°C). By 12 h, a decrease in host cell number and an increase in predator cell population were clearly noted in the *Micavibrio*-treated sample, with most of the host cells being aggregated (Fig. 3C, +*M.a.*, panel I). In contrast, many more individual *P. aeruginosa* cells were observed in the control sample, with no cell aggregation observed by phase-contrast microscopy (Fig. 3C, −*M.a.*, panel II). Measuring the extent of aggregation revealed a significant difference ($P < 0.01$) in aggregation between the samples, with 4.6% ± 1.7% aggregation measured for the control and 18.2% ± 3.3% aggregation measured for the *Micavibrio*-treated sample. Only a very few *P. aeruginosa* cells were detected by microscopy after 24 h of incubation with the predator, in contrast to the control, where a large number of cells were clearly visible (Fig. 3C, +*M.a.*, panel III, and −*M.a.*, panel IV, respectively). These micrographs confirmed the quantitative analysis shown above. Taken together, these results suggest that the increase in biofilm formation observed at early time points in the *Micavibrio*-treated sample is not likely to be caused by stimulation of cell growth but is due instead to an increase in cell-cell interactions

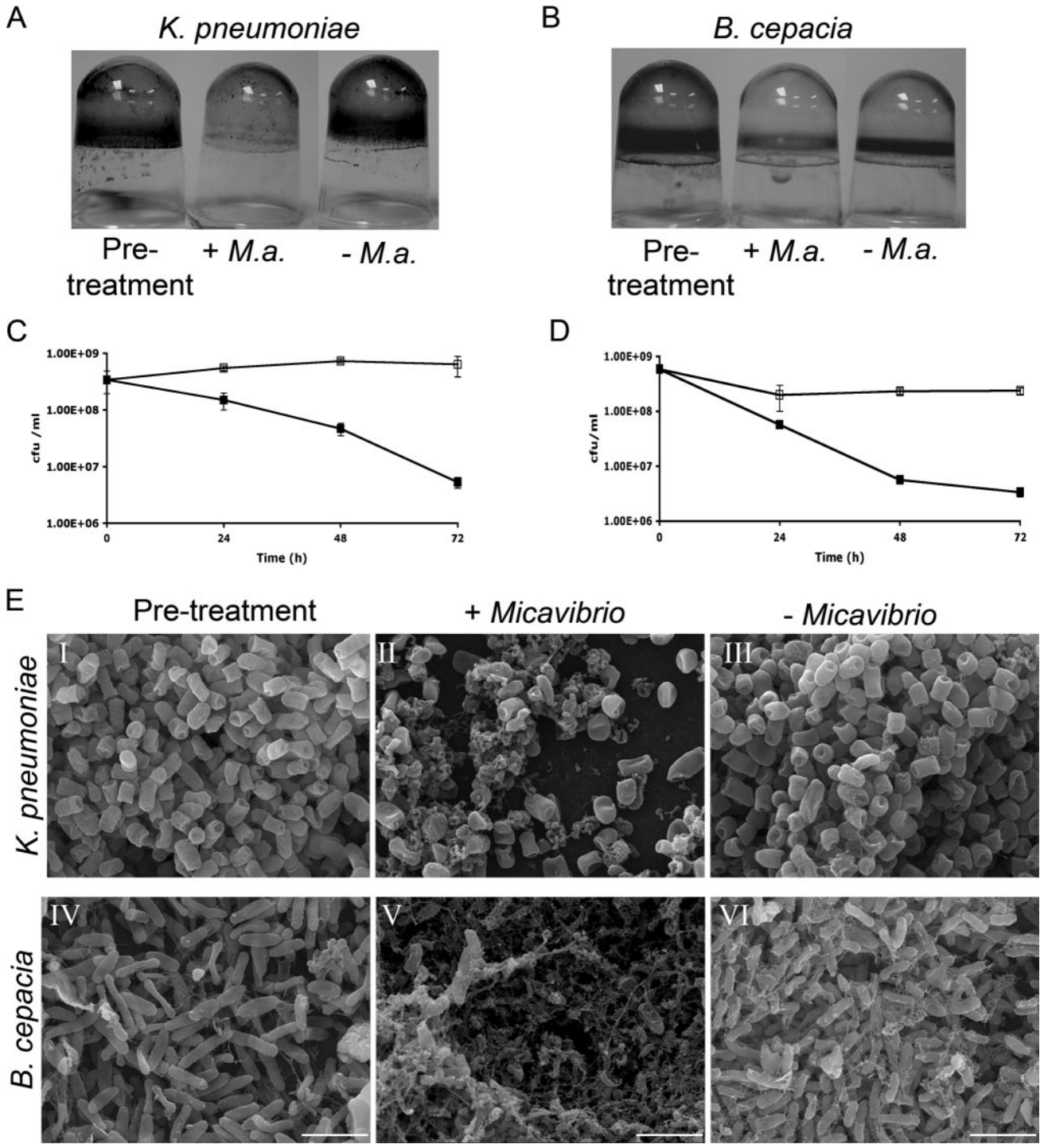

FIG. 4. Predation on *K. pneumoniae* and *B. cepacia* biofilms by *M. aeruginosavorus* ARL-13. (A) *K. pneumoniae* and (B) *B. cepacia* biofilms were developed for 18 h in 96-well microtiter plates (pretreatment), followed by 24 h of exposure to a *Micavibrio* lysate (+*M.a.*) or a sterile lysate (−*M.a.*), and then rinsed and stained with CV. (C and D) Quantification of biofilm cell viability. *K. pneumoniae* (C) and *B. cepacia* (D) biofilms were developed for ~18 h in 96-well microtiter plates, followed by exposure to a *Micavibrio* lysate (■) or a sterile lysate (□), and biofilm cell viability was assessed as described in the legend for Fig. 1. The difference in viability between the treatments at each time point was statistically significant ($P < 0.01$). (E) Scanning electron micrographs taken after *K. pneumoniae* (I to III) and *B. cepacia* (IV to VI) biofilms were developed for 18 h on polyvinyl chloride plastic coverslips (I and IV, pretreatment) and exposed for 24 h to a *Micavibrio* lysate (II and V, + *Micavibrio*) or a sterile lysate (III and VI, − *Micavibrio*). Bar, 4 μm; magnification, ×10,000. Each experiment was performed three times, yielding similar results. Images were viewed at the air-liquid interface.

brought about by the presence of the live predator or active predation.

Predation of *Micavibrio* on *K. pneumoniae* and *B. cepacia* biofilms. Our experiments showed that *M. aeruginosavorus* has the ability to attack both *K. pneumoniae* and *B. cepacia* in liquid culture (Table 1). To determine the ability of the predator to attack biofilms composed of these bacteria, we identified strains that had the ability to form stable and robust biofilms in a 96-well dish for extended time periods, including three clinical isolates of *K. pneumoniae* (1840, 1867, and 1868) and two isolates of *B. cepacia* (a clinical isolate and ATCC 25416). Because initial testing confirmed that *M. aeruginosavorus* could attack 18-h biofilms of these selected isolates equally well (data not shown), one isolate of each species was chosen for the subsequent experiments (*K. pneumoniae* isolate 1840 and *B. cepacia* ATCC 25416).

K. pneumoniae and *B. cepacia* biofilms were formed for 18 h, and then the medium was removed and replaced with DDNB medium containing a *Micavibrio* lysate or a sterile lysate as a control. The untreated 18-h-old biofilm produced by each strain was visualized by CV staining (Fig. 4A and B, pretreatment). Treatment with *M. aeruginosavorus* (Fig. 4A and B, *+M.a.*) markedly reduced the CV staining for each strain compared to that of the control (Fig. 4A and B, *−M.a.*). These data were confirmed by direct enumeration of adherent, viable bacteria (Fig. 4C and D). We also assessed the ability of *M. aeruginosavorus* to attack preformed biofilms of *K. pneumoniae* and *B. cepacia* clinical isolates. *Micavibrio* had the ability to reduce the biofilms of all *K. pneumoniae* isolates tested (three of three) as well as those of the single clinical isolate of *B. cepacia* examined (Table 1).

The effect of *Micavibrio* predation on *K. pneumoniae* and *B. cepacia* biofilms was visualized by SEM imaging. Again, a clear difference in biofilms was observed 24 h after inoculation with the predator compared to inoculation with the control (Fig. 4E). In the *Micavibrio*-treated sample, the number of intact cells was noticeably reduced, with much more cell debris and matrix (Fig. 4E, panels II and V, + *Micavibrio*), with no discernible changes observed for the biofilm in the control treatment (Fig. 4E, panels III and VI, − *Micavibrio*).

Genetic screen to identify loci important for host-predator interactions. In an attempt to identify genes required for the host-predator interaction, we screened a transposon mutant library of *P. aeruginosa* organisms grown as biofilms for mutants resistant to *Micavibrio* attack. *Micavibrio* had the ability to attack and reduce all mutant strains tested, as assessed by CV staining. The biofilm-negative strains among the ~10,000 mutants were also tested in the lytic halo assay and shown to be susceptible to attack by *Micavibrio*. No reduction in CV staining was observed in the sterile lysate control (data not shown), and an 85% decrease was observed for the wild-type *P. aeruginosa* biofilm treated with a *Micavibrio* lysate used as a positive control.

DISCUSSION

In a previous study, we showed that the predatory bacterium *B. bacteriovorus* could attack and reduce existing *E. coli* and *P. fluorescens* biofilms (22). For this work, we were interested in determining whether we could employ predatory prokaryotes against *P. aeruginosa* biofilms. Preliminary testing had shown that *B. bacteriovorus* 109J is limited in its ability to decrease existing *P. aeruginosa* biofilms (unpublished data). Reevaluating the host specificity of *M. aeruginosavorus* ARL-13 revealed that, as previously described (25), this exoparasite has the ability to attack lab strains as well as numerous clinical isolates of *P. aeruginosa*. Furthermore, *M. aeruginosavorus* ARL-13 also attacked two other opportunistic human pathogens, namely, *B. cepacia* and *K. pneumoniae* (Table 1 and Fig. 4).

While *Micavibrio* species typically exhibit relatively narrow host ranges (2, 25), under suboptimal storage conditions these predators may lose species specificity and become parasites with a broad host spectrum. For example, *Micavibrio admirandus* ARL-14 lost its host specificity after a 3-year storage period in liquid culture, in which it was reseeded numerous times (1). In our experiments, *M. aeruginosavorus* ARL-13 was grown under suitable conditions and still maintained a restricted host range. Thus, we believe that *B. cepacia* and *K. pneumoniae* can naturally be preyed upon by ARL-13 and that predation was not brought about by a breach in host specificity. However, this point needs to be investigated further using additional *M. aeruginosavorus* strains when they become available.

A microtiter dish-based static assay was used to monitor the ability of *Micavibrio* to attack *P. aeruginosa* PA14, *B. cepacia*, and *K. pneumoniae* biofilms as well as biofilms derived from several clinical isolates. Both CV staining and viable counts showed that *Micavibrio* was capable of markedly reducing biofilm biomass (Fig. 1 and 4). The extent of damage brought about by *M. aeruginosavorus* on 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 PFU/well of *Micavibrio* was sufficient to reduce *P. aeruginosa* biofilms by 75% after 96 h, and furthermore, biofilm-attached *Micavibrio* visualized by SEM imaging 30 min after initial inoculation confirmed that this brief exposure period was sufficient to initiate infection.

We reported that *E. coli* biofilms exhibit an increase in resistance towards *Bdellovibrio* attack compared to a planktonic population of the same bacterium (22). In this work, we did not observe a marked difference between the abilities of *Micavibrio* to attack *P. aeruginosa* cells as biofilms and as free-floating cells. This outcome could be explained by the inability of the predator to completely eradicate its planktonic prey, as previously demonstrated for bdellovibrios (23). Another explanation may be that under the conditions tested, biofilm formation does not enhance the ability of *P. aeruginosa* to withstand predation compared to that of planktonic cells. This observation holds promise that *Micavibrio* treatment may be effective for reducing *P. aeruginosa* biofilms, at least under some conditions.

P. aeruginosa has the ability to adapt to environmental predators, such as grazing protozoa, by developing grazing-resistant macrocolonies (29). By concurrently incubating *P. aeruginosa* and *M. aeruginosavorus* in a rich medium, we were able to study *P. aeruginosa* biofilm formation and its response to *Micavibrio* attack. A more robust biofilm was formed in the presence of the predator than in the control lacking *Micavibrio*, followed by a decrease in biofilm biomass in the *Micavibrio*-treated sample and a biofilm increase in the control, as measured by both CV

staining and viable counts (Fig. 3 and related text). By growing *P. aeruginosa* with and without *Micavibrio* in liquid cultures, we showed that there is no increase in the host planktonic population in response to predation, and thus the biofilm increase is not likely to be a consequence of an increased number of planktonic cells in the system. However, we did note an increase in cell-cell interactions in the *Micavibrio*-treated sample (Fig. 3C). This aggregation phenomenon was detected only when the host was mixed with live predator and did not occur when heat-killed *Micavibrio* or filtered sterilized lysate was added. At this point, we cannot determine if the increase in aggregation is an active process or is merely an indirect occurrence caused by an increase in cell debris, extracellular DNA, etc. Our results also suggest that under certain conditions, perhaps in which *P. aeruginosa* is provided with sufficient nutrients, this microbe can adapt to attack by increasing biofilm formation, as was also recently shown for protozoan grazers of *P. aeruginosa* biofilms (29). Weitere et al. (45) demonstrated that *P. aeruginosa* PAO1 macrocolonies confer only partial protection against protozoan grazers. This work is consistent with our finding that while *Micavibrio* predation did bring about an increase in the formation of macrocolonies by *P. aeruginosa* PA14, these biofilms were still susceptible to *Micavibrio* attack (Fig. 2).

An early study of *M. admirandus* demonstrated that certain carbohydrates inhibited the initial interactions between the host and predator, thus preventing predation. These data indicated that the host-predator interaction might be mediated by the availability of sugar receptors on one of the partners (7). In an attempt to identify genes required for host-predator interaction in our system, we screened ~10,000 *P. aeruginosa* PA14 transposon mutants grown as biofilms to identify strains resistant to attack by *Micavibrio*. No predation-resistant mutants were isolated from this initial screen. At this point, we can only speculate about the reason that no resistant mutants were identified. For example, a putative receptor responsible for host-predator interaction may be essential, or genes or pathways required for these interactions are redundant. Finally, we are aware that our screen is not yet fully saturated.

With the increasing interest in developing improved methods for controlling biofilms, there are many potential advantages of using *M. aeruginosavorus* for the biological control of *P. aeruginosa* biofilms, including the following: (i) it could be assumed that the narrow host range and specificity for infecting bacteria cells demonstrated so far might indicate that *Micavibrio* is harmless to commensal and nonbacterial organisms, (ii) *Micavibrio*'s ability to feed on the host allows the use of low initial doses to carry out an attack, and (iii) the toxins secreted by *P. aeruginosa* do not seem to inhibit *Micavibrio*'s ability to prey on this host, as is the case with other predators (29, 45). Furthermore, our data indicate that growth in a biofilm does not confer any additional protection to *P. aeruginosa* compared to growth as a planktonic population, suggesting that *M. aeruginosavorus* may be able to overcome some aspects of biofilm-mediated resistance. Future work using the methods developed in this work should allow us to broaden our understanding of factors important for host-predator interactions and the host-specific response to *Micavibrio* attack, as well as to perform a more rigorous assessment of the potential use of *Micavibrio* as a biocontrol agent for biofilms.

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