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14. ABSTRACT

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burns and wound infections. *Bdellovibrio* and *Micavibrio* are Gram-negative obligatory predators that feed on other Gram-negative bacteria. The focus of the study was to evaluate the potential application of predatory bacteria (Pb) to control human pathogens most associated with war-related infections. Our findings demonstrated that Pb are able to attack many of the pathogens tested, including bacteria from the genus *Acinetobacter, Aeromonas, Aggregatibacter, Bordetella, Burkholderia, Citrobacter, Eikenella, Enterobacter, Escherichia, Klebsiella, Listonella, Morganella, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio,* and *Yersinia.* Pb were also able to attack *A. baumannii* drug resistant clinical isolates obtained from Wounded Warriors. Positive predation was measured in single and multi-species microbial cultures as well as on monolayer and multilayer pre-formed biofilms grown in static and flow cell systems. The predators were also able to inhibit biofilm formation and remove metabolically inactive biofilms. *Bdellovibrio* and *Micavibrio* were shown to prey at 37°C but were unable to prey under oxygen limiting conditions. Our study also established that applying biofilm EPS (extracellular polymeric substance) degrading enzymes, with the *Bdellovibrio,* could enhance the biofilm removal aptitude of the predators. In conclusion, the work presented here highlights the potential use of predatory bacteria as biological based agent for eradicating infection and will pave the way for future studies in animal and human subjects.

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Introduction.

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burns and wound infections, with infections becoming more difficult to treat with antibiotics. Drug resistance can be considered a natural response to the selective pressure of a drug and can develop in both free-floating bacteria, as well as in surface-attached bacteria or biofilms. Biofilms form when bacteria adhere to surfaces and begin to excrete a glue-like substance that protects and anchors them to materials and tissue. One of the major difficulties in controlling surface-attached bacteria is their enhanced resistance to drugs. The problem of enhanced drug resistant infections led researchers to examine other methods of controlling microorganisms. Among these alternative techniques is the use of biological control agents such as viruses and medical maggots. Recent studies have demonstrated the potential use of predatory bacteria to control and reduce some pathogenic bacteria grown in liquid suspension and as biofilms. These unique predators or parasites, which could be found in nature, exclusively feed on other bacteria. We propose that predatory bacteria might be able to serve as a novel therapeutic agent in controlling war-related burns and wound infections. The aim of this research is to evaluate the ability of predatory bacteria to attack microorganisms associated with burns and wounds.

Body.

Bdellovibrio and *Micavibrio* are both Gram-negative bacteria ubiquitous to many natural environments. Unlike most bacteria, these organisms are obligatory parasites that survive by feeding exclusively on other Gram-negative bacteria. Earlier studies have suggested that *Bdellovibrio* and *Micavibrio* have an ability to rapidly reduce and kill more than 99.9% of cells grown in liquid suspension and as biofilms [2, 3], this includes drug resistant pathogens typically associated with war burns and wound infections, such as *Pseudomonas aeruginosa, Burkholderia cepacia, Klebsiella pneumoniae* and *Escherichia coli*. With the increasing need to develop new and improved methods for controlling drug resistant bacteria and biofilms, there are potential advantages using predatory prokaryotes: (1) They are highly specific for infecting Gram-negative bacteria. (2) Laboratory experiments and DNA sequencing have shown no evidence of gene transfer between *B. bacteriovorus* and its prey, therefore it is unlikely that the organism would acquire genes that would make it pathogenic to humans or other mammals. (3) It is believed that the cell surface of *B. bacteriovorus* is only weakly immunogenic and will not provoke serious immune reactions. (4) *Bdellovibrio* and *Micavibrio* are capable of attacking human pathogens as well as drug resistant bacteria [2, 3]. (5) Since these organisms multiply rapidly on the microbial infection, the initial dose of the predator that needs to be applied could be low. (6) Predatory prokaryotes are thought to possess the capability to access extremely thick biofilms and are not restricted to the surface of the biofilm [2, 3]. (7) Finally, it is believed that no host cell resistance develops as a result of predation.

With the emergence of new multidrug resistant bacteria becoming an increasing problem in war- related injury infections and in hospital settings, the need for new and innovative approaches for controlling human pathogens is becoming essential.

We hypothesize that predatory prokaryotes might be able to serve as a novel therapeutic agent in controlling war-related burns and wound infections, and as a mean of enhancing the potency of existing drugs used to treat bacteria and biofilms.

Objectives.

The specific aims of this proposal are: **(i)** To evaluate the ability of *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* to reduce drug resistant bacteria associated with war burn and wound infections. **(ii)** To assess the ability of *B. bacteriovorus* and *M. aeruginosavorus* to penetrate and reduce single and multi-species biofilms. **(iii)** To enhance the potency of existing antimicrobial and antibiofilm drugs by incorporating their use in concert with microbial predators.

<u>Aim I.</u> Evaluating the ability of *Bdellovibrio* and *Micavibrio* to reduce drug resistant bacteria associated with burns and wounds.

The goal of this task was to evaluate the host range of *Bdellovibrio* spp. and *Micavibrio* spp. Although some initial work had been conducted in order to demonstrate the ability of predatory prokaryotes to attack human pathogens [3, 8-10], no work was carried out to verify that these predators are capable of attacking the new emerging multi-drug resistant bacteria encountered in wounded soldiers returning from the recent arm conflicts. To this end, emphasis was placed on examining human pathogens that are becoming predominant in recent war-related burns and wound infections. Other human and zoonotic pathogens were also examined for their susceptibility to predation.

Main methods used.

Growing the predators. Growing and maintaining the predators was done as described previously [2, 3]. In brief, the predators *Bdellovibrio bacteriovorus* strain 109J and *Micavibrio aeruginosavorus* strain ARL-13 were maintained as plaques in double-layered diluted nutrient broth (DNB) (0.8 gr/l 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) (Starr, 1975). *B. bacteriovorus* and *M. aeruginosavorus* were counted as plaque forming units (PFU) developing on a lawn of prey cells. Standard induced lysates were obtained by adding a plug of agar containing *B. bacteriovorus* or *M aeruginosavorus* plaque (about $1x10^6$ PFU/ml) to $1x10^8$ CFU/ml washed host cells, incubated for 18 hrs in DNB at 30°C on a rotary shaker set at 200 rpm, to reach a final concentration of $1x10^8$ PFU/ml predator. To harvest the predator cells, 18 hr lysates were passed three times through a

0.45 μm pore-size filter in order to remove residual prey and cell debris (filtered lysate). As a control, filtered sterilized lysate was prepared by sequentially passing the predator containing lysate through three 0.22 μm pore-size filters. After filtration, no predator, as judged by PFU, could be detected [2].

General host range predation assay. To evaluate the ability of *Bdellovibrio* and *Micavibrio* to prey on selected bacteria, lysates (cocultures) were prepared in which washed bacteria (about $1x10^8$ CFU/ml) were incubated with harvested *B. bacteriovorus* or *M. aeruginosavorus* ($1x10^6$ PFU/ml) in DNB. As a control, filtered sterilized lysate was used. The ability of predators to prey was confirmed by the reduction in host cell viability, measured by CFU enumeration, caused by the lysis of host cells during predation. Additional confirmation of active predation was done by microscopy evaluation (x1000 magnification). Each experiment was carried out at least three times.

<u>Subtask 1. 1.</u> Measuring host range specificity of *Bdellovibrio* spp. and its effectiveness in reducing cell viability of microbial pathogens.

In order to investigate the host range of *Bdellovibrio* and its ability to reduce and attack microbial cells, standard lysates were prepared in which *Bdellovibrio bacteriovorus* strain 109J (ATCC 43826) was incubated with the selected pathogen. In this study, 105 host bacteria were examined representing 26 different genera.

Results.

The results for subtask 1. 1. are presented in Table-1. In brief, *B. bacteriovorus* was able to prey, attack and reduce 87 of the 105 examined bacteria. Among the bacteria positively reduced were bacteria from the genus *Acinetobacter, Aeromonas, Aggregatibacter, Bordetella, Burkholderia, Citrobacter, Eikenella, Enterobacter, Escherichia, Klebsiella, Listonella, Morganella, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio,* and *Yersinia.* No predation was detected on bacteria from the genus *Campylobacter, Porphyromonas, Prevotella, Stenotrophomonas,* and the non-Gram-negative bacteria *Mycobacterium, Enterococcus and Staphylococcus.*

Table 1. Host range specificity of *B. bacteriovorus.*

Bacteria tested	Predation on planktonic cells*	CFU log reduction following predation¥	Predation on surface attached lawn cells◊
Acinetobacter			
A. species ATCC 49466	+	4-5	+
A. species ATCC 10153	+	3	+
A. baumannii ATCC 19606	+	3-4	+
A. baumannii NCIMB 12457	+	3-5	+
A. baumannii ATCC BAA-747	+	5	+
A. calcoaceticus PIC 346	+	0.5-1	-
A. haemolyticus ATCC 19002	+	5	+
A. lwoffii ATCC 15309	+	2-3	+
A. lwoffii ATCC 17925	+	3-4	+
Aeromonas			
A. hydrophila PIC 191	+	4	+
A. salmonicida ATCC 33658	+	5-6	+
Aggregatibacter actinomycetemcomitans 10 clinical isolates	+	2	n.e
Bordetella bronchiseptica PIC 402	+	3	+
Burkholderia cepacia 2 clinical isolates	+	3	n.e
Campylobacter			

C. jejuni ATCC 29428	-	0	n.e
C. jejuni ATCC BAA-1153	-	0	n.e
Citrobacter			
C. freundii NCTC 9750	+	2-3	+
C. freundii ATCC 43864	+	0.5	+
C. freundii ATCC 8090	+	3	+
Eikenella corrodens 9 clinical isolates	+	2	n.e
Enterobacter			
E. aerogenes ATCC 13048	+	2	+
E. aerogenes ATCC 35029	+	2	+
E. aerogenes ATCC 51697	+	5	+
E. aerogenes NCIMB	+	3	+
E. amnigenus ATCC 51816	+	4	+
E. cloacae ATCC 700323	+	2-3	+
E. cloacae ATCC 35030	+	2-3	+
E. cloacae ATCC 49141	+	4	+
E. gergoviae ATCC 33028	+	3-4	+
Enterococcus faecalis PIC 522B	-	0	n.e
Escherichia coli 3 lab strains	+	6-8	+
Klebsiella			
K. pneumoniae ATCC 33495	+	2	+
K. pneumoniae ATCC BAA-1706	+	4	+
K. pneumoniae ATCC BAA-1705	+	2	+
K. pneumoniae 6 clinical isolates	+	4-6	+

Listonella anguillarum ATCC 14181	+	5	n.e
Morganella			
M. morganii ATCC 25829	+	3	+
M. morganii ATCC 25830	+	3	+
M. morganii PIC 329	+	1	+
Mycobacterium			
M. smegmatis PIC 6972	-	0	n.e
M. lacticola PIC 697	-	0	n.e
Porphyromonas gingivalis 2 clinical isolates	-	0	n.e
Prevotella intermedia	-	0	n.e
Proteus			
P. mirabilis ATCC 35659	+	1-2	+
P. mirablis ATCC 43071	+	4	+
P. mirabilis ATCC 25933	+	3-4	+
P. mirabilis NCIMB 13283	+	4	+
P. mirabilis ATCC 7002	+	4	+
P. mirabilis PIC 366	+	3	+
P. morganii PIC 3661	+	1	+
P. rettgeri ATCC 9250	+	4-5	+
P. vulgaris ATCC 33420	+	4-5	+
P. vulgaris ATCC 49132	+	5	+
P. vulgaris ATCC 8427	+	4	+
P. vulgaris NCTC 4636	+	4-5	+

P. vulgaris PIC 365	+	8	+
Pseudomonas			
P. aeruginosa PA14	-	0	-
P. aeruginosa PA01	-	0	-
P. aeruginosa ATCC BAA-427	+	1	n.e
P. aeruginosa ATCC 10145	-	0	-
P. fluorescens	+	2	+
P. syringae	+	n.e	+
P. putida	+	n.e	+
Salmonella enterica PIC 371	+	4	n.e
Serratia marcescens PIC 361	+	3-4	n.e
Shigella			
S. flexneri PIC 387	+	5	n.e
S. sonnei PIC 388	+	6	n.e
Staphylococcus aureus	-	n.e	-
Stenotrophomonas maltophilia 6 clinical isolates	-	0	-
Vibrio			
V. angulara PIC 232	+	2	+
V. cholerae EL Tor	+	4	n.e
V. parahaemolyticus PIC 234	+	0.5-1	+

Yersinia			
Y. enterocolitica PIC 330	+	2	n.e
Y. pseudotuberculosys PIC 399	+	3	n.e

* Lysates were prepared by adding $1x10^8$ CFU/ml host cells to harvested *B. bacteriovorus* predator cells. Predation was evaluated after 24 and 48 hrs of incubation by light microscopy, (+) positive predation by *Bdellovibrio*; (-) no predation by *Bdellovibrio*; (n.e) not evaluated.

ψ Values represent maximum log reduction in host cell viability counts (CFU/ml) compared to the *Bdellovibrio* minus control.

Φ Twenty microliters of *Bdellovibrio* (~1 x 10^8 cfu/ml) was spotted on a lawn of the indicated bacteria. Predation was scored as the formation of lytic zone at the point of *Bdellovibrio* inoculation, (+) positive predation by *Bdellovibrio*; (-) no predation by *Bdellovibrio*; (n.e) not evaluated.

PIC-Presque Isle Culture Collection

ATCC- American Type Culture Collection

Each experiment was carried out three times yielding similar results.

<u>Subtask 1. 1. A.</u> Measuring the ability of *B. bacteriovorus* to attack drug resistant bacteria isolated from Wounded Warriors.

In order to investigate the ability of *Bdellovibrio* to attack drug-resistant clinical isolates of *A. baumannii*, standard lysates were prepared in which *B. bacteriovorus* 109J was incubated with 31 different clinical isolates collected from infected warriors. Predation was measured by CFU counts of the remaining host following predation.

Results.

The results are presented in Table-1.A. *B. bacteriovorus* was able to prey, attack and reduce all of the examined drug-resistant clinical samples. The majority of the samples (93%) were reduced by more than 2.5logs within 24 hrs of incubation.

** Samples were provided by Dr. Daniel V. Zurawski, Senior Scientist, Department of Wound Infections, Walter Reed Army Institute of Research. Samples were sent after filling the appropriate material transfer agreements.

Table 1. A. Reduction of drug- resistant clinical isolates of *A. baumannii* by *B. bacteriovorus.*

Strain #	With *Bdellovibrio*	Control no *Bdellovibrio*	Source
AB967	$1.3x10^4$	$5x10^8$	Blood/Sepsis
AB2828	$6x10^4$	$2.5x10^8$	Blood/Sepsis
AB3340	$6x10^3$	$5x10^8$	Blood/Sepsis
AB3560	$4x10^4$	$4.5x10^8$	Blood/Sepsis
AB3638	$5x10^4$	$5x10^8$	Posterior Wound
AB3785	$5x10^4$	$5x10^8$	Blood/Sepsis
AB3806	$9x10^3$	$2x10^8$	Leg Wound
AB3917	$3x10^7$	$5x10^8$	Blood/Sepsis
AB3927	$2x10^5$	$5x10^8$	Tibia/Osteomyelitis
AB4025	$3x10^4$	$5x10^8$	Femur/Osteomyelitis
AB4026	$1x10^4$	$3x10^8$	Fibula/Osteomyelitis
AB4027	$1.5x10^5$	$5x10^8$	Femur/Osteomyelitis
AB4052	$8x10^4$	$5x10^8$	War wound
AB4269	$3.5x10^4$	$4.5x10^8$	War wound
AB4448	$4.5x10^5$	$5.5x10^8$	War wound
AB4456	$2x10^4$	$3x10^8$	Tracheal Aspirate
AB4490	$2.5x10^4$	$2.5x10^8$	War wound
AB4498	$2.5x10^4$	$5x10^8$	Blood
AB4795	$9x10^4$	$2.5x10^8$	Bone/Osteomyelitis
AB4857	$9x10^4$	$8x10^8$	Ischial/Osteomyelitis
AB4878	$7x10^5$	$5x10^8$	War wound
AB4932	$5.5x10^4$	$9x10^8$	Sputum
AB4957	$5x10^3$	$6x10^8$	Sacral/Osteomyelitis
AB4991	$4x10^5$	$5x10^8$	War wound
AB5001	$2x10^5$	$5x10^8$	Blood/Sepsis
AB5075	$2x10^5$	$2x10^8$	Tibia/Osteomyelitis
AB5197	$2x10^3$	$4x10^8$	STS/Tissue

AB5256	$2.5x10^7$	$5x10^8$	Blood/Sepsis
AB5674	$4.5x10^5$	$5x10^8$	Blood/Sepsis
AB5711	$1.5x10^3$	$5x10^8$	Blood/Sepsis
AB 4700	$4x10^5$	$3x10^8$	Blood/Sepsis

*Data represents total numbers (CFU/ml) of host remaining after incubation.

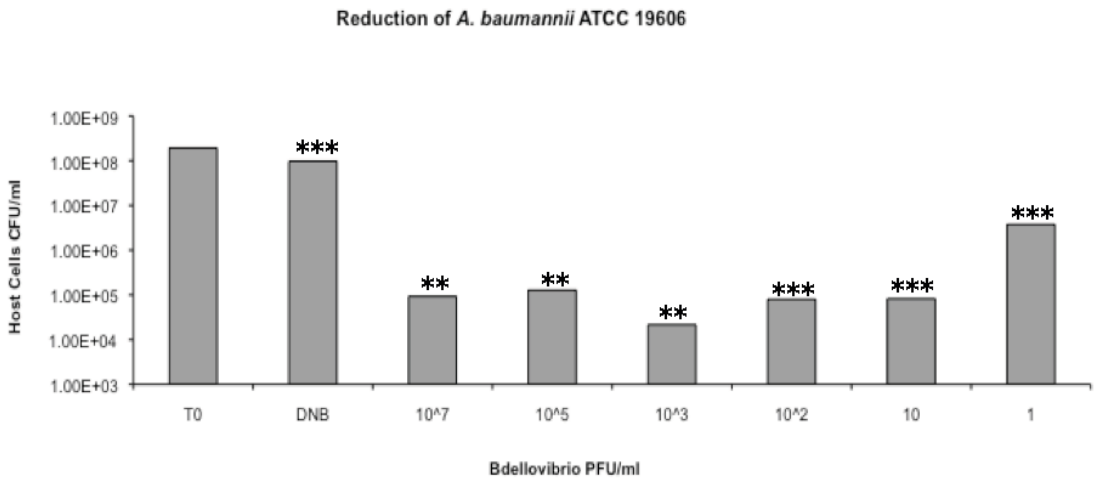
<u>Subtask 1. 1. B.</u> Threshold amount needed for active predation and host reduction.

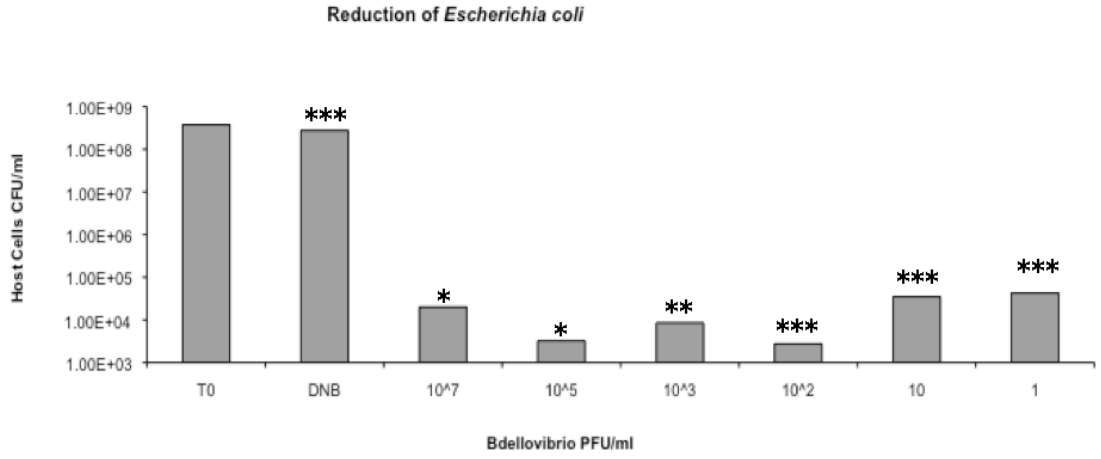
To evaluate the minimum concentration of *Bdellovibrio* required for predation, standard lysates were prepared in which serial diluted *B. bacteriovorus* 109J was added to host bacteria (*A. baumannii* ATCC 19606; *Escherichia coli; K. pneumoniae* ATCC 33495; *K. pneumoniae* clinical isolate). Predation was measured by CFU counts following 24, 48 and 72 hrs of incubation.

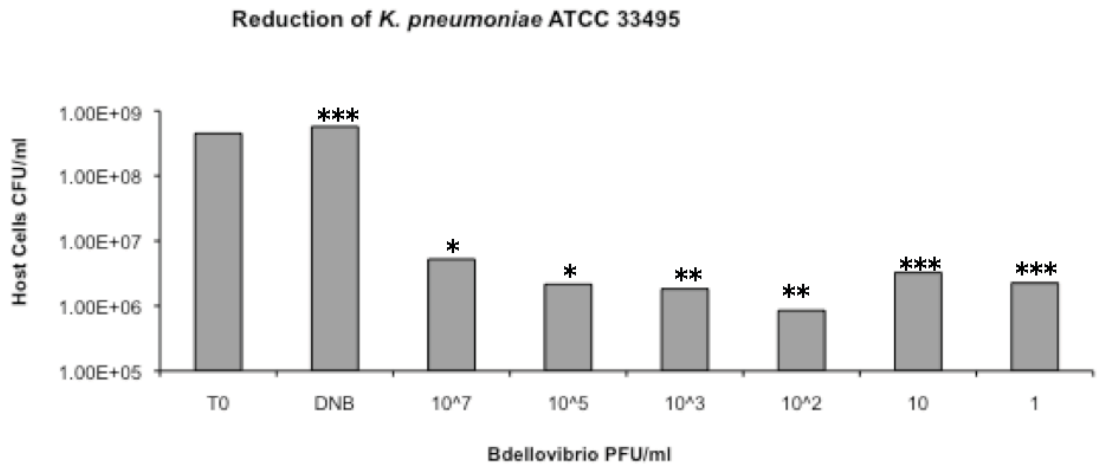
Results.

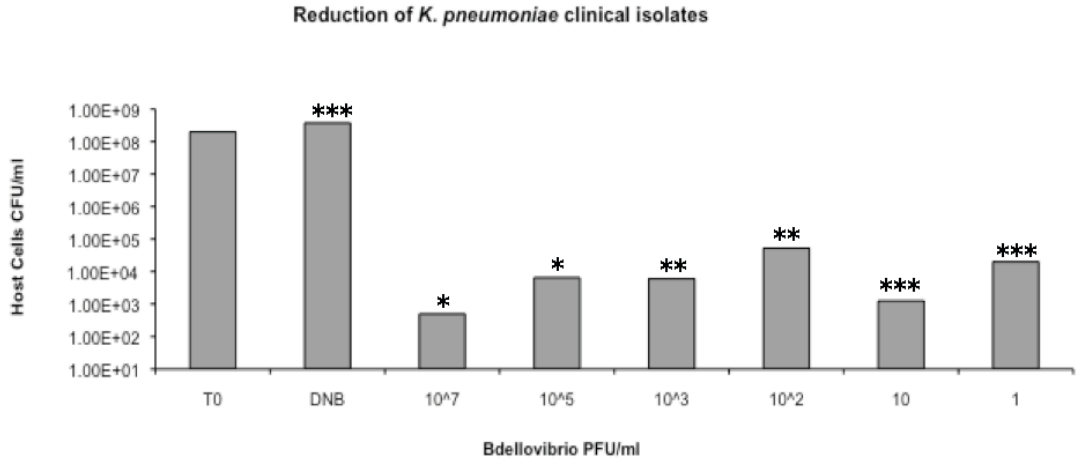
The addition of only 1 PFU/ml predator was sufficient to reduce the numbers of the examined bacteria. Extended incubation periods were required when a low number of *Bdellovibrio* was used (Fig-1).

Figure 1. Reduction of host cells by different concentrations of *B. bacteriovorus* 109J.









T_0- Initial host concentration.

DNB- *Bdellovibrio* free control.

* CFU of host remaining after 24 hrs of incubation.

** CFU of host remaining after 48 hrs of incubation.

*** CFU of host remaining after 72 hrs of incubation.

<u>Subtask 1. 2.</u> Measuring host range specificity of *Micavibrio* and its effectiveness in reducing cell viability of human pathogens.

In these experiments, 177 host bacteria representing 18 different genera were examined. Standard lysates were prepared in which *Micavibrio aeruginosavorus* ARL-13 was incubated with the selected pathogens.

Results.

The results for subtask 1. 2. are presented in Table-2. In brief, *Micavibrio* was able to prey, attack and reduce 145 of the 177 examined bacteria. The most profound reduction in host cell viability was measured for bacteria from the genus *Burkholderia, Escherichia, Klebsiella, Pseudomonas* and *Shigella.* A more moderate reduction was measured in lysates that included bacteria from the genus *Acinetobacter, Enterobacter Proteus* and *Yersinia.* No predation was seen when the host used was from the genus *Bordetella, Citrobacter, Enterococcus, Erwinia, Morganella, Mycobacterium, Serratia, Stenotrophomona, Vibrio.* The ability of *Micavibrio* to reduce *Burkholderia, Klebsiella,* and *Pseudomonas* are in line with a previous study reported by us [3]. However, our current study shows an increase in *Micavibrio* host range specificity, when compared to our previous study.

Table 2. Host range specificity of *M. aeruginosavorus.*

Bacteria tested	Predation on planktonic cells*	CFU log reduction following predationψ	Predation on surface attached lawn cellsΦ
Acinetobacter			
A. species ATCC 49466	-	0	-
A. species ATCC 10153	-	0	-
A. baumannii ATCC 19606	-	0	-
A. baumannii NCIMB 12457	-	0	-
A. baumannii ATCC BAA-747	-	0	-
A. calcoaceticus PIC 346	+	1	+
A. haemolyticus ATCC 19002	+/-	0-0.5	-
A. lwoffii ATCC 15309	+	0.5-1	+
A. lwoffii ATCC 17925	+	0.5-1	+
Bordetella bronchiseptica PIC 402	-	0	-
Burkholderia cepacia 2 clinical isolates	+	3	+
Citrobacter			
C. freundii NCTC 9750	-	0	-
C. freundii ATCC 43864	+	0.5	-
C. freundii ATCC 8090	-	0	-
Enterobacter			
E. aerogenes ATCC 13048	-	0	-
E. aerogenes ATCC 35029	+	1	+

E. aerogenes ATCC 51697	+	2	+
E. aerogenes NCIMB	+	0.5	+
E. amnigenus ATCC 51816	+	2	+
E. cloacae ATCC 700323	+	1-2	+
E. cloacae ATCC 35030	+	2	+
E. cloacae ATCC 49141	+	1	+
E. gergoviae ATCC 33028	+	1.5	+
Enterococcus faecalis PIC 522B	-	0	n.e
Erwinia amylovora PIC 351	-	0	-
Escherichia coli 3 lab strains	+	3	+
Klebsiella			
K. pneumoniae ATCC 33495	+	2	+
K. pneumoniae ATCC BAA-1706	+	2-3	+
K. pneumoniae ATCC BAA-1705	+	1-2	+
K. pneumoniae 6 clinical isolates	+	2-3	+
Morganella			
M. morganii ATCC 25829	-	0	-
M. morganii ATCC 25830	-	0	-
M. morganii PIC 329	-	0	-
Mycobacterium smegmatis PIC 6972	-	0	n.e
Proteus			
P. mirabilis ATCC 35659	+	0.5-1	+
P. mirablis ATCC 43071	+	1	+
P. mirabilis ATCC 25933	+	0.5	-

P. mirabilis NCIMB 13283	+	0.5	-
P. mirabilis ATCC 7002	-	0	-
P. mirabilis PIC 366	+/-	0-0.5	-
P. morganii PIC 3661	-	0	-
P. rettgeri ATCC 9250	+	0.5	-
P. vulgaris ATCC 33420	+	0.5-1	n.e
P. vulgaris ATCC 49132	+	0.5-1	+
P. vulgaris ATCC 8427	-	0	-
P. vulgaris NCTC 4636	-	0	-
Pseudomonas			
P. aeruginosa PA14	+	3	+
***P. aeruginosa* PA01	+	n.e	+
P. aeruginosa ATCC BAA-427	+	3-4	+
P. aeruginosa ATCC 10145	+	0.5	-
***P. aeruginosa* 16 clinical isolates - urine	+	n.e	+
***P. aeruginosa* 21 clinical isolates - sputum from non CF patients	+	n.e	+
***P. aeruginosa* 7 clinical isolates - sputum from CF patients	+	n.e	+
** *P. aeruginosa* 38 clinical isolates - eye	+	n.e	+
***P. aeruginosa* 22 clinical isolates - miscellaneous organs	+	n.e	+
***P. fluorescens*	-	n.e	-
***P. syringae*	-	n.e	-
***P. putida*	-	n.e	-

Serratia marcescens PIC 361	-	0	-
Shigella			
S. sonnei PIC 388	+	2	+
Stenotrophomonas maltophilia 6 clinical isolates	-	0	-
Vibrio			
V. angulara PIC 232	-	0	-
***V. cholerae* EL Tor**	-	*n.e*	-
V. parahaemolyticus PIC 234	-	0	-
Yersinia			
Y. pseudotubercu PIC 399	+	1-2	n.e

* Lysates were prepared by adding $1x10^8$ CFU/ml host cells to harvested *M. aeruginosavorus* predator cells. Predation was evaluated after 24 and 48 hrs of incubation by light microscopy, (+) positive predation by *Micavibrio*; (-) no predation; (+/-) inconclusive; (n.e) not evaluated.

** Examined previously by our group [3].

ᵠ Values represent maximum log reduction in host cell viability counts (CFU/ml) compared to the *M. aeruginosavorus* minus control.

ᵠ Twenty microliters of *Micavibrio* (~1 x 10^8 cfu/ml) was spotted on a lawn of the indicated bacteria. Predation was scored as the formation of lytic zone at the point of *M.*

aeruginosavorus inoculation, (+) positive predation; (-) no predation; (+/-) inconclusive (n.e) not evaluated.

PIC-Presque Isle Culture Collection

ATCC- American Type Culture Collection

Each experiment was carried out three times yielding similar results.

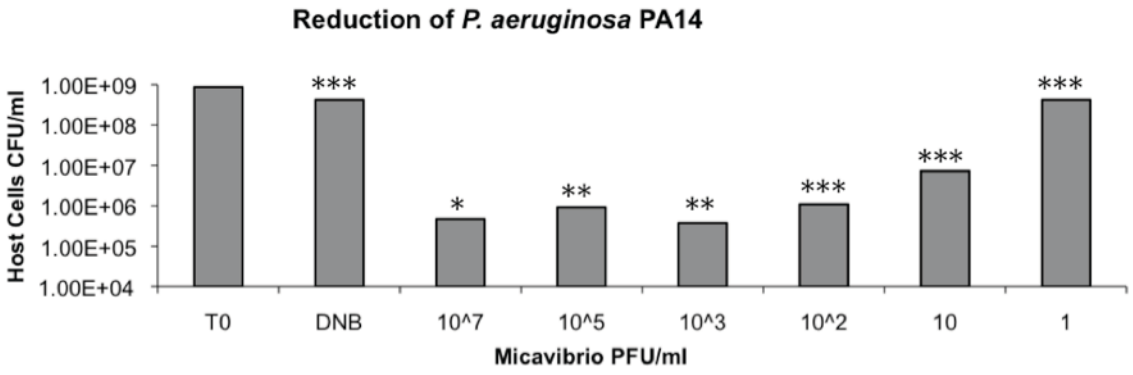
<u>Subtask 1. 2. A.</u> Threshold amount needed for active predation and host reduction.

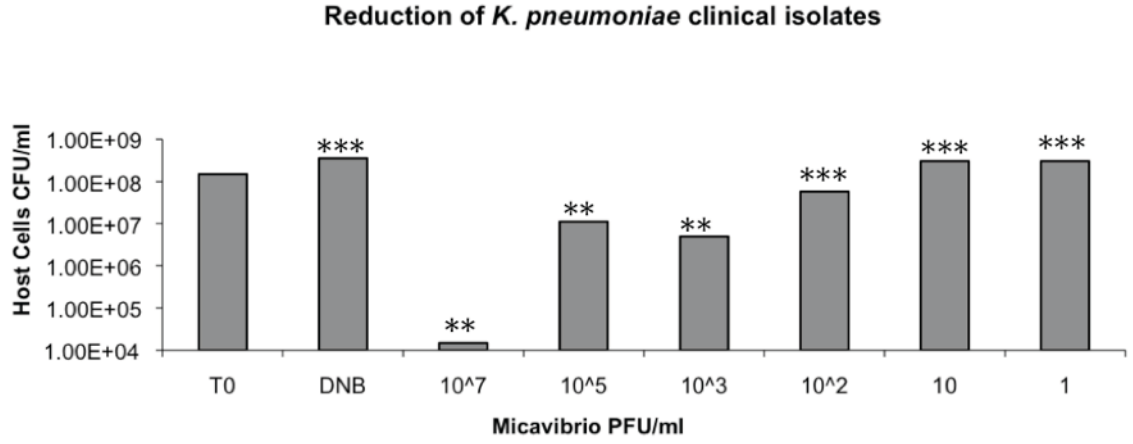
To evaluate the minimum concentration of *Micavibrio* required for predation, standard lysates were prepared in which serial diluted *M. aeruginosavorus* was added to *K. pneumoniae* clinical isolate and *P. aeruginosa* PA14. Predation was measured by CFU counts following 24, 48 and 72 hrs of incubation.

Results.

The addition of diluted predator was sufficient to reduce the numbers of the examined bacteria (10 PFU/ml and 100 PFU/ml for predation on *P. aeruginosa* and *K. pneumoniae* respectively). A positive correlation between the amount of *Micavibrio* used and the extent and pace of host reduction was seen (Fig. 2).

Figure 2. Reduction of host cells by different concentrations of *M. aeruginosavorus*.





T_0- Initial host concentration.

DNB- *Micavibrio* free control.

* CFU of host remaining after 24 hrs of incubation.

** CFU of host remaining after 48 hrs of incubation.

*** CFU of host remaining after 72 hrs of incubation.

<u>Subtask 1. 3.</u> Assessing the ability of predatory bacteria to reduce multi-species microbial cultures.

The following sets of experiments were aimed at evaluating the ability of the predator to attack and reduce pathogenic bacteria in a mixed culture. *B. bacteriovorus* was selected for this study since it exhibits a broader host range than *Micavibrio*, allowing us to be more flexible in selecting the bacteria to be examined. Standard lysates were prepared as described above, with each lysate containing a combination of host cells. Single host lysates were also incubated for comparison. The host cells co-culture included; *A. baumannii* NCIMB 12457 and *K. pneumoniae* ATCC 33495; *E. gergoviae* ATCC 33028 and *K. pneumoniae* ATCC BAA-1706; *A. baumannii* ATCC 19606 and *E. cloacae* ATCC 35030. CFU enumeration of the remaining host cells was done by plating the lysates on selective antibiotic agar plates.

Results.

As seen in Table-3 the ability of *B. bacteriovorus* to reduce host bacteria in multi-species microbial cultures was comparable to that of a single species culture. No reduction in *Bdellovibrio* predation efficacy was seen on any of the host combinations tested.

Table 3. Ability of *B. bacteriovorus* to prey on bacteria inoculated in a multi-species culture suspension.

Culture 1.

Bacteria tested	**CFU log reduction following predation in a <u>single</u> species lysate**	**CFU log reduction following predation in a <u>mixed</u> species lysate**
A. baumannii NCIMB 12457	3	4
K. pneumoniae ATCC 33495	2	2

Culture 2.

Bacteria tested	**CFU log reduction following predation in a <u>single</u> species lysate**	**CFU log reduction following predation in a <u>mixed</u> species lysate**
E. gergoviae ATCC 33028	4	3
K. pneumoniae ATCC BAA-1706	4	4

Culture 3.

Bacteria tested	**CFU log reduction following predation in a <u>single</u> species lysate**	**CFU log reduction following predation in a <u>mixed</u> species lysate**
A. baumannii ATCC 19606	3	3
E. cloacae ATCC 35030	3	3

Lysates were prepared by adding $1x10^8$ CFU/ml of single species or multi-species host cells to *B. bacteriovorus*. Predation was evaluated after 24 and 48 hrs of incubation. Values represent maximum log reduction in host cell viability counts (CFU/ml) compared to the *Bdellovibrio* minus control.

Additional experiments.

As the main goal of this proposal is to investigate the potential use of predatory bacteria as a bio-control agent, we conducted additional experiments aimed at examining the influence of different growth conditions on predation. We have selected conditions that might be encountered in "real-life" *in-vivo* settings such as elevated temperature and limited oxygen concentrations.

A. *B. bacteriovorus* predation capability in adverse culture conditions.

As some pathogens might reside within wounds where the oxygen concentration is limited and the environmental temperature is above 30^{o}C, we were interested in investigating the ability of *Bdellovibrio* to prey in anaerobic and microaerophilic conditions as well as elevated temperatures. To this end, standard *B. bacteriovorus* induced lysates were prepared using washed *E. coli* as prey. The cultures were placed in a BD GasPak Jar Systems with a disposable gas generating anaerobic or microaerophilic envelope (BD Diagnostic Systems, Franklin Lakes, NJ). The jars were incubated at 30^{o}C on a rotary shaker at 200 rpm. Additional lysates were placed at room oxygen levels at 30^{o}C and 37^{o}C.

Results.

CFU enumeration of the surviving host bacteria revealed that *B. bacteriovorus* was unable to prey under oxygen limiting conditions (Fig. 3). Other predation experiments in which the lysate was placed in an MACS MG 250 anaerobic chamber (10% CO_2, 10% H_2 and 80% N_2) also produced no reduction in host population (data not shown). Interestingly, when the lysates were removed from the oxygen limiting conditions (following 72 hrs) and placed in an aerobic environment, predation did occur, reducing host cell CFU numbers by 4 logs. Although *Bdellovibrio* was restricted in its ability to prey in anaerobic and microaerophilic conditions, it was not restricted to prey at higher temperatures of 37^{o}C (Fig. 3).

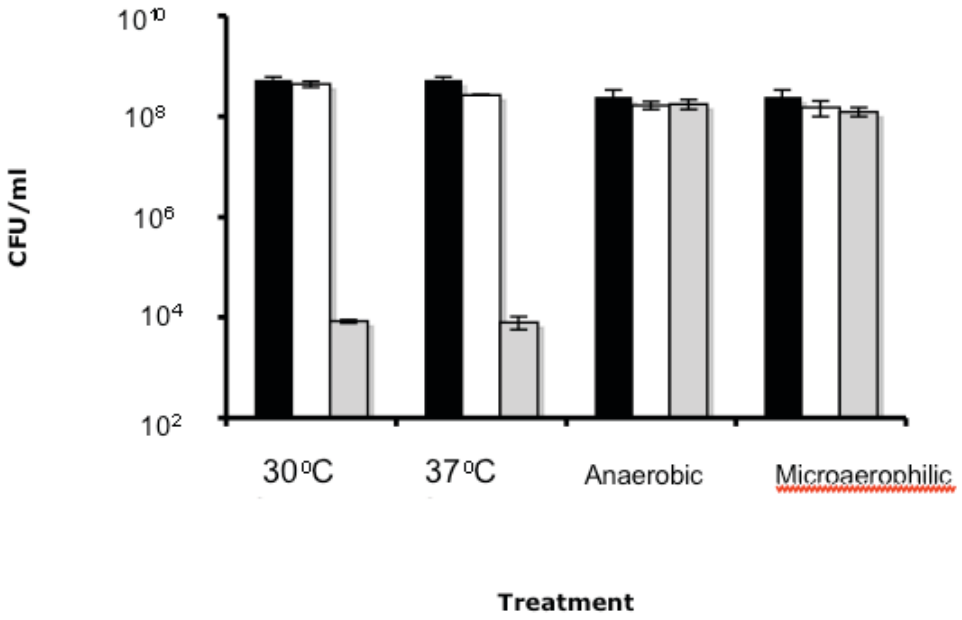


Fig. 3. *B. bacteriovorus* predation capability in adverse culture conditions. *E. coli* host cells were incubated for 48 hrs in the presence of *B. bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). The lysates were cultured at 30°C and 37°C (normal oxygen levels) or at 30°C under anaerobic or microaerophilic growth conditions. Black-bars represent host cell numbers at time-0. Each value represents the mean of 3 lysates. Error bars are shown as one-standard deviation.

B. Factors effecting predation by *M. aeruginosavorus.*

Due to the limited research conducted on *M. aeruginosavorus* biology, we were interested to determine which factors might influence predation. Two host bacteria *Klebsiella pneumonia* (ATCC 13883) and *Pseudemonas aeruginosa* PA14 were used in our experiments.

B. 1. Measuring the effect of oxygen levels on predation.

Micavibrio lysates were prepared using *P. aeruginosa* or *K. pneumonia* as hosts. The lysates were incubated at 30^{o}C in anaerobic and microaerophilic conditions using the BD BBL GasPak jar system. The efficiency of predation was measured by microscopic observation and CFU counts of the remaining host cells following 24 hrs of incubation

Results.

As seen in Fig-4A and 4B. *Micavibrio* was able to reduce *K. pneumonia* and *P. aeruginosa* population by 2-3 logs under room oxygen conditions but was not able to attack and reduce the host cell population when incubated under reduced oxygen levels (Fig 4).

B. 2. Temperature predation experiments. The following experiment was designed to assess the optimal temperature for growth and predation. *Micavibrio* lysates were prepared using *P. aeruginosa* or *K. pneumoniae* as hosts. The lysates were incubated at 25^{o}C, 30^{o}C and 37^{o}C. The efficiency of predation was measured by microscopic observation and CFU counts.

Results.

When *K. pneumoniae* was used as host, the most rapid reduction in host CFU counts was seen at 30^{o}C (Fig. 5A). By 48 hrs, comparable reduction in CFU numbers was registered at all three experimental temperatures.

In *P. aeruginosavorus* lysates, a similar predation pattern was seen at 25^{o}C and 30^{o}C, however, a reduction in *Micavibrio* predation capability was noted at 37^{o}C (Fig 5B).

These experiments confirm that like *Bdellovibrio*, *Micavibrio* also has the ability to prey and attack host cells at higher "medically relevant" incubation temperatures.

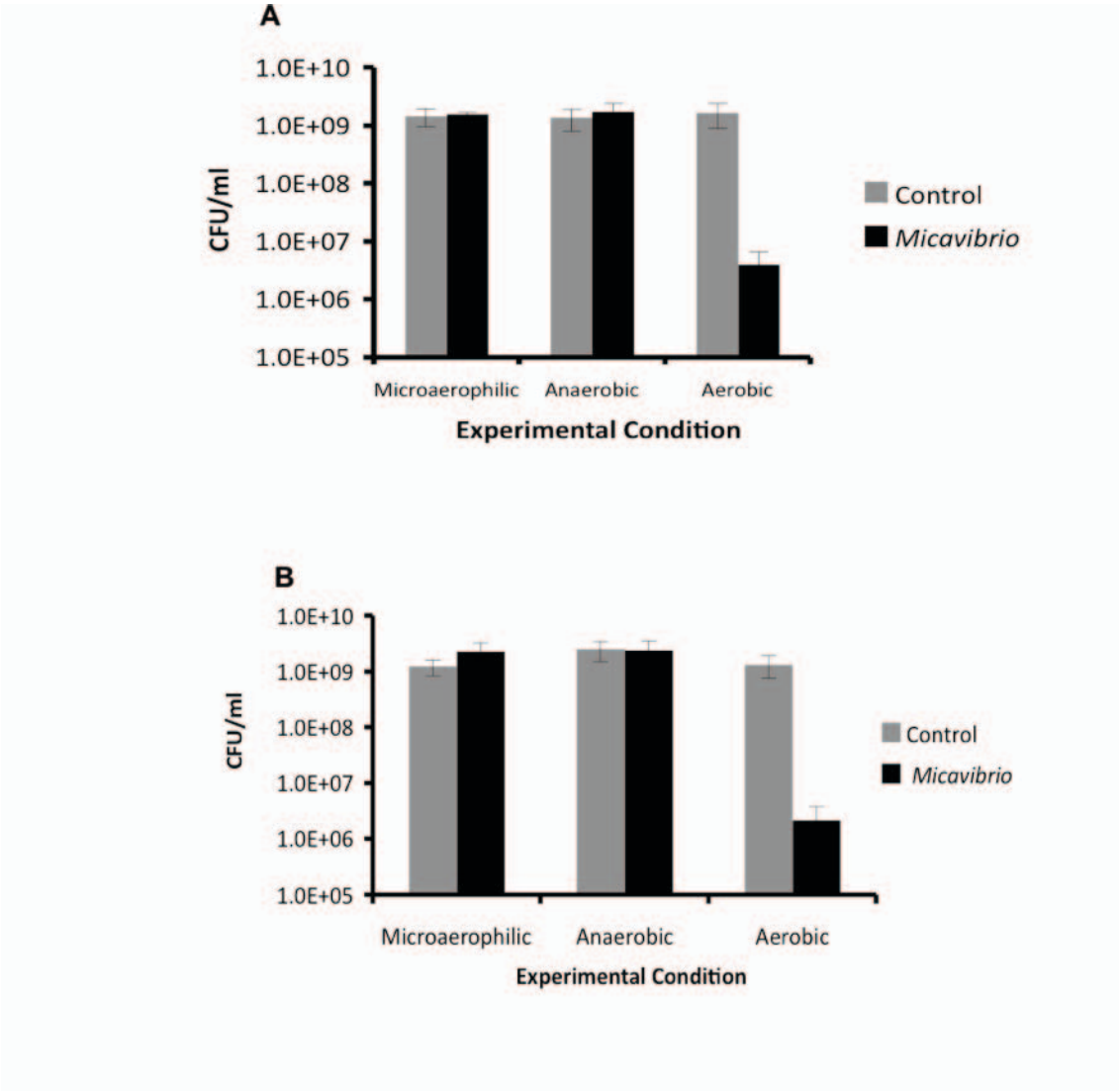


Fig 4. Effect of oxygen levels on predation by *M. aeruginosavorus*. *K. pneumonia* (A) and *P. aeruginosa* (B) lysates were incubated with *M. aeruginosavorus* for 24 hrs in anaerobic, microaerophilic and aerobic oxygen levels. Efficiency of predation was measured by CFU counts of the remaining host cells.

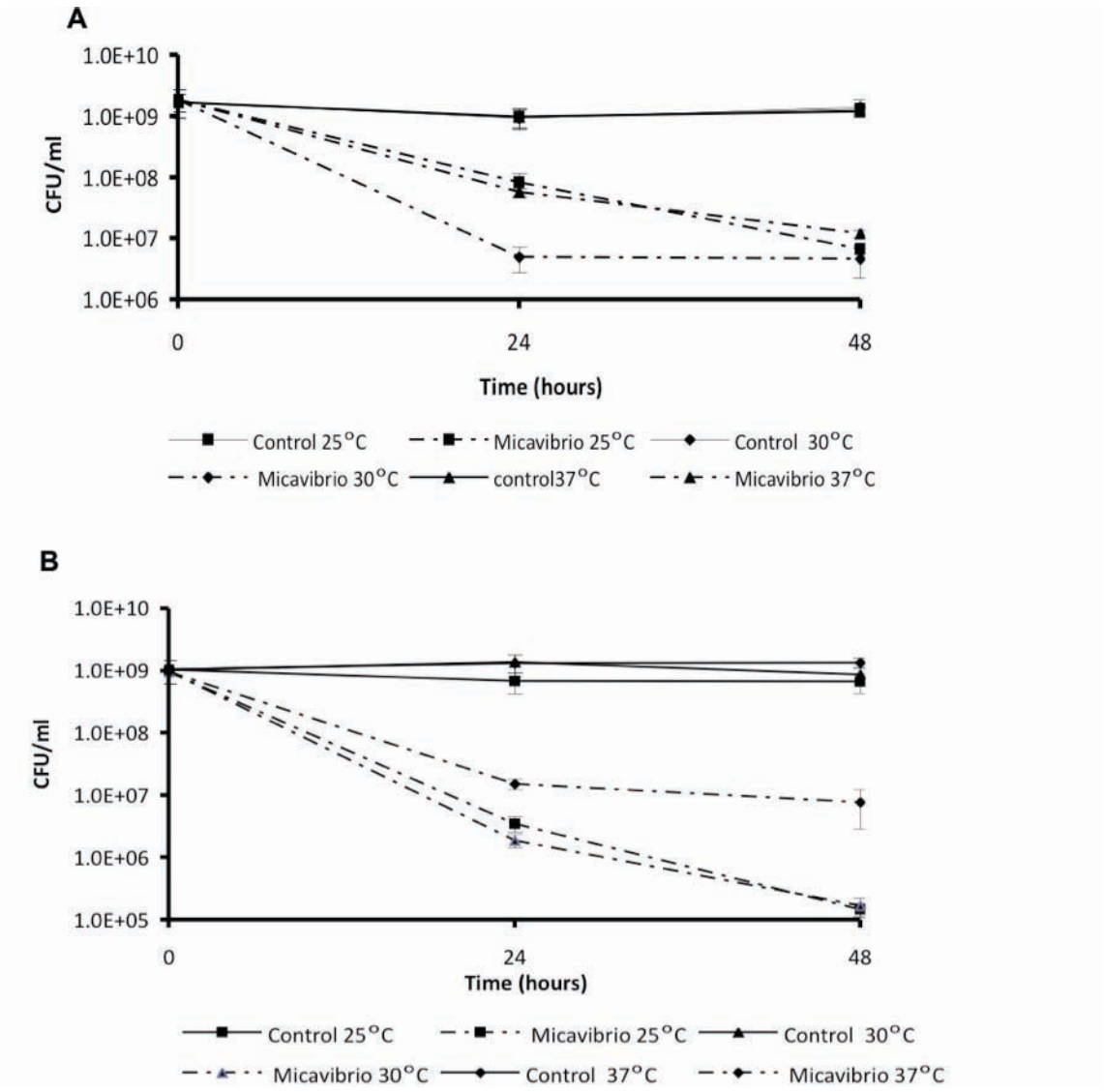


Fig 5. Effect of incubation temperature on *M. aeruginosavorus* predation. *K. pneumonia* (A) and *P. aeruginosa* (B) lysates were incubated with *M. aeruginosavorus* for 24-48 hrs at 25℃, 30℃ and 37℃. Efficiency of predation was measured by CFU counts of the remaining host cells.

B. 3. Predation of *Micavibrio* on metabolically inactive host cells. It was previously demonstrated that *Bdellovibrio* is capable of attacking non-viable host cells. In order to investigate whether *Micavbrio* is able to feed and proliferate on non-viable host cells, experiments were conducted in which *M. aeruginosavorus* was cocultured with three separate host bacteria: *E. coli*, *K. pneumoniae* ATCC 33495 and *P. aeruginosa* PA14. The cells were killed using high UV radiation, heating the cells for 18 hrs at 65^{o}C, or heating the cells for 30 min at 95^{o}C. CFU counts confirmed that the host cells were non-viable after the killing treatment. Viable host cells were used as a control. The ability of the predator to attack and proliferate on the host cells was measured by the change in predator numbers (PFU/ml) following incubation.

Results.

As seen in Table-4, *Micavibrio* was unable to proliferate on non-viable *E. coli* and *K. pneumoniae* cells. However, predation did occur when non-viable *P. aeruginosa* was used.

Table 4. Predation of *M. aeruginosavorus* on metabolically inactive host cells.

Host used	**T_0**	**Control**	**UV**	**65^{o}C**	**95^{o}C**
E. coli	$4x10^5$	$1x10^7$	$3x10^5$	$3x10^5$	$2.5x10^5$
K. pneumoniae	$3x10^5$	$2x10^7$	$2x10^5$	$1x10^5$	$1x10^5$
P. aeruginosa	$2x10^5$	$2x10^7$	$1x10^7$	$1x10^6$	$5x10^6$

T_0- *Micavibrio* initial concentration (PFU/ml).

Control- PFU/ml *Micavibrio* following predation on viable host cells.

UV- PFU/ml *Micavibrio* following predation on non-viable UV radiated host cells.

65^{o}C- PFU/ml *Micavibrio* following predation on non-viable host cells exposed to 65^{o}C.

95^{o}C- PFU/ml *Micavibrio* following predation on non-viable host cells exposed to 95^{o}C.

C. The effect of carbohydrates on host prey interactions. A key question in predatory bacteria biology is what are the mechanisms that govern host specificity and host-predator recognition. One factor that might be involved in predator-prey interactions and host specificity is protein-carbohydrate interactions. Lectins are sugar-binding proteins that play a role in many biological recognition phenomena, one of which is recognition of host cells by microorganisms. We hypothesize that if lectin receptors are involved in predation, we could block predation by adding exogeneous sugars to the co-cultures. *In-vivo* lectin receptors, glycoproteins and glycopeptides are present on numerous cells throughout the body as well as in intercellular matrixes and extracellular fluids, such as collagen, mucin and serum. Thus, the interaction of sugar molecules with predatory bacteria is significant.

C. 1. Effect of carbohydrates on predation by *B. bacteriovorus*. To examine the effect of sugars on predation, *B. bacteriovorus* 109J was cocultured with *E. coli* in the presence of several carbohydrates (Fig. 6A) and at different concentrations (Fig. 6B, 6C). The efficiency of predation was measured by host CFU enumeration.

Results.

As seen in Fig. 6A, the presence of 0.1 M dextrose or 5.4 M (50% v/v) glycerol completely inhibited predation. Dextrose and glycerol predation-inhibiting effect was found to be dose dependent, effectively inhibiting predation at 0.05 M dextrose (Fig. 6B) and 0.01 M (0.1% v/v) glycerol (Fig. 6C).

To examine if dextrose and glycerol effect on predation is host specific, predation experiments were done using four additional host cells. 0.1 M dextrose reduced *B. bacteriovorus* ability to prey on *K. pneumoniae, E. coli* DH5-α and S17-1. A more moderate inhibition effect was seen on *A. baumannii* (Fig. 6D). 0.54 M (5% v/v) glycerol also halted predation of *K. pneumoniae, E. coli* DH5-α and S17-1, with a reduced predation inhibiting effect measured on *A. baumannii* (Fig. 6E).

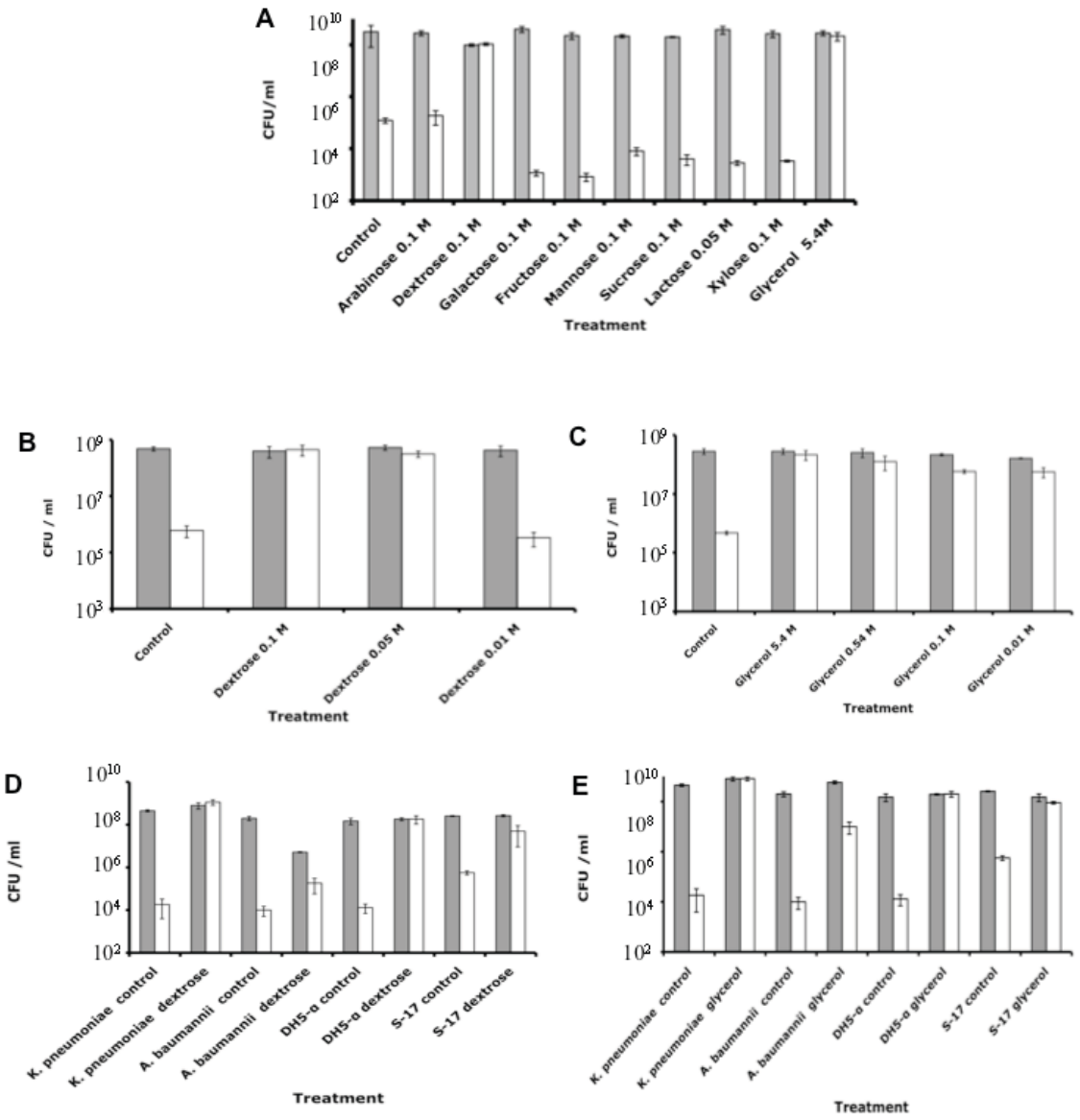


FIG. 6. *B. bacteriovorus* predation in the presence of carbohydrates. (A) *E. coli* was cultured in DNB (control) and DNB supplemented with carbohydrates. No *B. bacteriovorus* (gray bars), with *B. bacteriovorus* (empty bars). Predation in the presence of varying concentrations of dextrose **(B)** and glycerol **(C)**. Predation of *K. pneumoniae*, *E. coli* DH5-α, *E. coli* S17-1 and *A. baumannii* in the presence 0.1 M dextrose **(D)** and 0.54 M glycerol **(E)**. All cultures were incubated for 24 hrs. Predation was evaluated by CFU enumeration of the remaining host cells. Each value represents the mean of 3 cocultures. Error bars are shown as one-standard deviation.

C. 2. Effect of carbohydrates on predation by *M. aeruginosavorus*. To examine the effect of carbohydrates on predation by *Micavibrio*, *M. aeruginosavorus* was cocultured in the presence of carbohydrates with *K. pneumoniae* and *E. coli* used as host.

Results.

As seen in Fig. 7A, all of the selected carbohydrates were able to inhibit predation of *K. pneumoniae* by *M. aeruginosavorus*. Carbohydrate-inhibiting effect was also seen when *E. coli* was used as host, with only sucrose and lactose having no predation inhibiting capability (Fig. 7B).

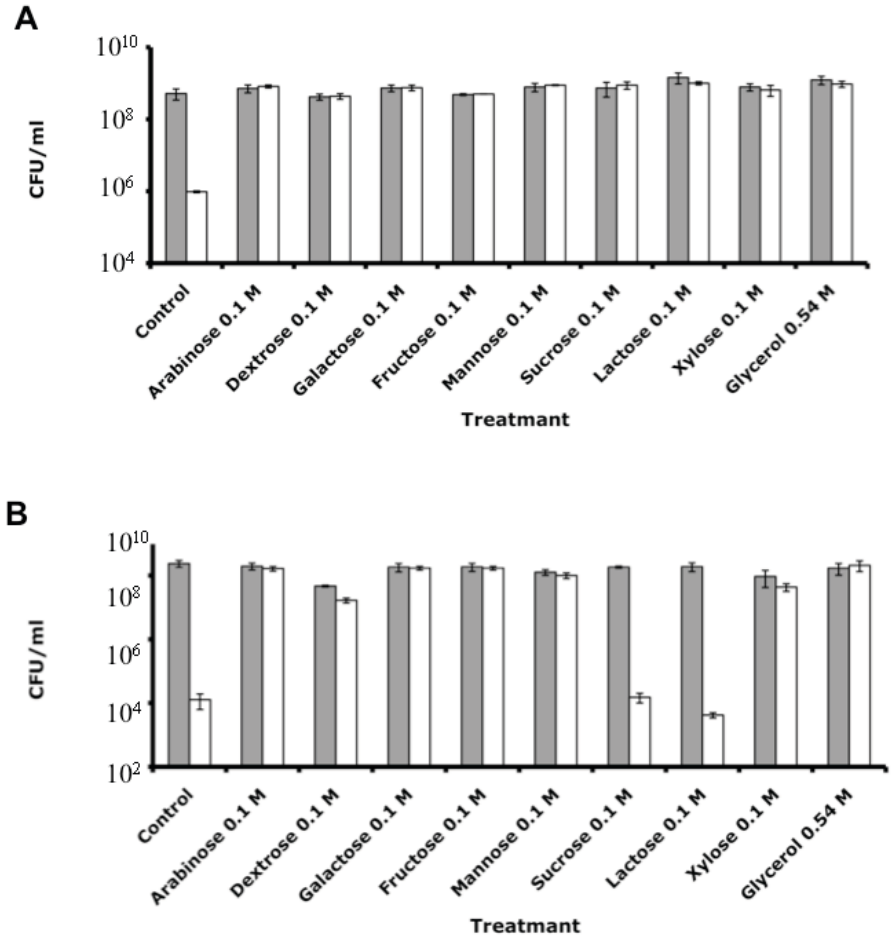


FIG. 7. *M. aeruginosavorus* predation in the presence of carbohydrates. *K.*

pneumoniae **(A)** and *E. coli* **(B)** were cultured in DNB (control) and DNB supplemented with carbohydrates. No *M. aeruginosavorus* (gray bars), with *M. aeruginosavorus* (empty bars). Cultures were incubated for 24 hrs. Predation was evaluated by CFU enumeration of the remaining host cells. Each value represents the mean of 3 cocultures. Error bars are shown as one-standard deviation.

******Since our initial data demonstrated that the presence of carbohydrates could block predation of both *Bdellovibrio* and *Micavibrio,* additional experiments aimed at better understanding the root of the sugar inhabitation effect were conducted. Further investigation established that inhibition of predation was due to media acidification by the metabolic activity of the host and not due to a blocking of a putative sugar-binding protein. Our findings were recently published in Applied and Environmental Microbiology (Dashiff, A., Keeling T, G. and Kadouri, E. D. 2011. Host Cell Metabolic Activity in the Presence of Carbohydrates Inhibits Predation by *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus*. Applied and Environmental Microbiology, 77:2224-2231).

We would like to note that the additional work was supported by funding from the Department of Oral Biology at UMDNJ.

The data presented in our work might be of value when storing, growing, and cultivating predatory bacteria, as well as when considering environmental conditions that might influence predation in the field.

D. Effect of proteases on predation. Many body fluids such as serum, saliva and tears contain protease, mainly serine and cysteine protease. As some of the predators' cell surface structures might be involved in predation (pili, flagellum and cell surface receptors), the presence of proteases in the environment might interfere with the predation process. Furthermore, since proteases might be used in conjunction with the predator as a way of enhancing its biofilm removal ability, understanding the effect of protease on predation is important.

In order to examine the effect of proteases on predation, *Bdellovibrio* and *Micavibrio* were cocultured with *E. coli* in the presence of trypsin and proteinase-K.

Results.

As seen in Table 5A, and 5B the presence of proteinase-K blocked predation by both *Bdellovibrio* and *Micavibrio*. However, no reduction in predation was measured when trypsin, a serine protease that exhibits different cleaving properties than proteinase-K, was used. Thus, the ability of the enzyme to inhibit predation seems to be specific.

Table 5. Ability of predatory bacteria to prey in the presence of trypsin and proteinase-K.

A. Predation by *B. bacteriovorus* in the presence of protease.

Host used	Host initial concentration (CFU/ml)	CFU/ml of host following predation in the presence of proteinase-K (100 µg/ml)	CFU/ml of host following predation in the presence of trypsin (50 µg/ml)
E. coli	$3.1x10^8$	$2.3x10^8$	$4.5x10^4$

B. Predation by *M. aeruginosavorus* in the presence of protease.

Host used	Host initial concentration (CFU/ml)	CFU/ml of host following predation in the presence of proteinase-K(100 µg/ml)	CFU/ml of host following predation in the presence of trypsin (50 µg/ml)
P. aeruginosa	$7x10^8$	$6.2x10^8$	$1x10^6$
K. pneumoniae	$9x10^8$	$1x10^9$	$4x10^6$

Aim II. Evaluate the ability of *B. bacteriovorus* and *M. aeruginosavorus* to penetrate and reduce single and multi-species biofilm.

Main methods used.

Microbial lawn predation assay. The capacity of *Bdellovibrio* and *Micavibrio* to attack a thin biofilm grown as a monolayer was assessed by their ability to form lytic halos on a lawn of prey cells using a modification of the double-layered plaque assay [3]. Host bacteria were grown for 18 hrs in LB and 100 μl of washed cells was spread on DNB medium solidified with 1.5% agar. Predator cell lysates were cultured as described above. Predators were purified by passing the lysate 3 times through a 0.45 μm pore-size filter. Twenty microliters of the predator lysate was spotted on a lawn of host bacteria. As a control, a predator lysate was passed three times through a 0.22 μm pore-size filter to remove all of the predator cells. No predator or host, as judged by CFU and PFU, respectively, could be detected in the 0.22 μm filtered sterile lysate (data not shown). Lytic halo assay plates were incubated at 30^{o}C for up to 3 weeks and examined for the formation of a zone of clearing where the lysates were spotted. Each lytic halo assay was performed at least three times.

General biofilm predation assays. The capacity of the predator to attack a multilayerbiofilm was evaluated by its ability to reduce a pre-formed biofilm grown in a 96-well static system as described previously [2, 3]. Microtiter wells were inoculated (100 μl per well) with 18 hr grown host culture diluted 1:50 in fresh medium. Cells were grown for 18 hrs at 30^{o}C (pre-formed biofilm) before they were stained with crystal violet (CV) and quantified as described [6] using a Molecular Devices Vmax kinetic microplate reader (Sunnyvale, CA). Absorbance of the CV solution was determined at 600 nm. To assess predation dynamics of predator on the host biofilms, the pre-formed biofilms were grown as described above, washed 3x with DDNB to remove planktonic cells and 100 μl of a lysate (~1 x 10^{7} PFU/well) was added to each well. Alternatively, as a control, 100 μl of 0.22 μm filtered sterile lysate was added to wells. The microtiter dish was incubated at 30^{o}C for the duration of the experiment.

Quantification of biofilm bacteria by CFU was performed as described [2, 6]. Briefly,

the wells of the microtiter plate were washed six times with saline to remove planktonic cells, 100 µl of saline was added to each well and the samples were sonicated individually for 8 sec using a VC505 sonicator (Sonics and Materials Inc., Newtown, CT) followed by dilution plating. The CFU of planktonic cells, before and after sonication, was determined to verify that the sonication procedure did not reduce host cell viability. 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 host cell viability yet was sufficient to remove all of the attached cells (data not shown). Each experiment was carried out at least three times with 4 to 8 wells for each treatment.

Scanning electron microscopy.

Experiments were performed as described previously [2, 3]. In brief, biofilms were developed on a 12x12 mm PVC plastic cover slip (Fisher Scientific, Pittsburgh, PA.). The cover slips were placed in a 24-well polystyrene cell culture plate (Corning Inc., Corning, NY). Pre-formed biofilms and predation assay were prepared as described above. The experiments were carried out in a 1.0 ml volume. Biofilms were rinsed to remove any planktonic cells before being fixed in 2% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1% ruthenium red. Images were collected from biofilms grown at the air-liquid interface. The imaging was done using a Zeiss Auriga cross-beam field emission scanning electron microscope (FE-SEM).

Subtask 2. 1. Measuring *Bdellovibrio* predation efficacy on monolayer-biofilms.

To investigate the ability of *Bdellovibrio* to prey on thin layers of surface-attached bacteria, predation assays were conducted. *B. bacteriovorus* strain 109J was purified and spotted on a lawn of host cells. In this study, 62 host bacteria were examined representing 13 different genera.

Results.

The results for subtask 2.1. are presented in Table-1 (Page-6, Predation on surface attached lawn cells). In general, the ability of the predator to attack and form lytic halos

on the host cells mirrored the host range specificity of the predator. *B. bacteriovorus* was able to prey and form halos on 51 of the 62 examined bacteria. Among the bacteria positively reduced were bacteria from the genus *Acinetobacter, Aeromonas, Bordetella, Citrobacter, Enterobacter, Escherichia, Klebsiella, Morganella, Proteus, Pseudomonas,* and *Vibrio.* No halos were detected on bacteria from the genus *Stenotrophomonas* and *Staphylococcus.*

Subtask 2.2. Measuring *Micavibrio* predation efficacy on a monolayer-biofilms.

To investigate the ability of *Micavibrio* to prey on a thin layer of surface attached bacteria, predation assays were conducted. *M. aeruginosavorus* ARL-13 was purified and spotted on a lawn of host cells. In this study, 168 host bacteria representing 15 different genera were examined.

Results.

The results for subtask 2.2. are presented in Table-2 (Page-16, Predation on surface-attached lawn cells). As before, the ability of the predator to attack and form lytic halos on the host cells mirrored the host range specificity of the predator. *Micavibrio* was able to prey and form halos on 131 of the 168 examined bacteria. Among the bacteria positively lysed were bacteria from the genus *Burkholderia, Escherichia, Enterobacter, Klebsiella, Pseudomonas* and *Shigella.* Halos formed on some examined species from the genus *Acinetobacter, Proteus.* No halos formed when the host used was from the genus *Bordetella, Citrobacter, Erwinia, Morganella, Serratia, Stenotrophomona,* and *Vibrio.* The ability of Micavibrio to reduce *Burkholderia, Klebsiella,* and *Pseudomonas* were reported in a previous study conducted by us [3]. As was discovered in the host specificity experiments, a slight increase in host range susceptibility of *Micavibrio* was also noted here.

Subtask 2.3. Assessing the ability of predatory bacteria to reduce multi-layer microbial biofilms.

The following sets of experiments were aimed at examining the ability of the predator to attack and reduce a multilayer biofilm that was developed in a 96-well static system. *B. bacteriovorus* was selected to serve as the predator based on its extensive predation characteristics which allowed us to be more flexible in selecting the host.

Experiment 1. Measuring biofilm redaction by CV staining. The host bacteria used to grow the biofilms were; *A. baumannii* ATCC 19606, *A. baumannii* NCIMB 12457, *A. species* ATCC 49466, *A. lwoffii* ATCC 17925, *C. freundii* NCTC 9750, *E. gergoviae* ATCC 33028, *K. pneumoniae* ATCC 33495, *K. pneumoniae* ATCC BAA-1706, and *M. morganii* ATCC 25829.

Results.

Incubating the pre-formed biofilm with the predator had resulted in a significant reduction of biofilm biomass (Fig. 8), with *A. baumannii* ATCC 19606 showing a 66% reduction in CV staining compared to the control; *A. baumannii* NCIMB 12457 75%; *A. species* ATCC 49466 77%; *A. lwoffii* ATCC 17925 58%; *C. freundii* NCTC 9750 68%; *E. gergoviae* ATCC 33028 61%; *M. morganii* ATCC 25829 26%; *K. pneumoniae* ATCC 33495 73% and *K. pneumoniae* ATCC BAA-1706 59% reduction in CV staining compared to the *Bdellovibrio*- free control.

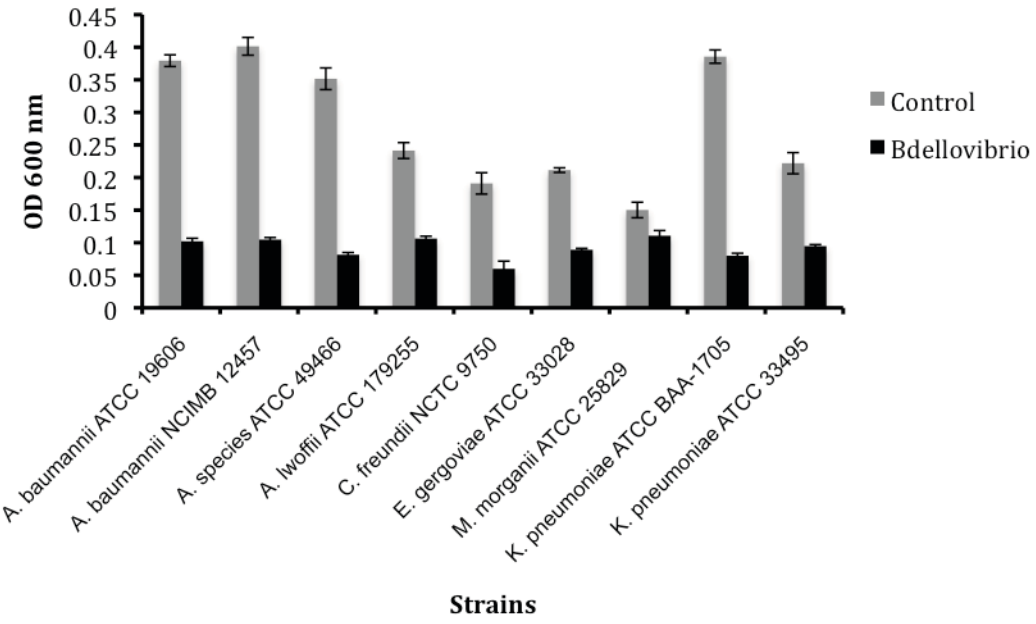


Fig. 8. Reduction of multi-layer microbial biofilms by *B. bacteriovorus*. Biofilms were formed in a 96-well static system. Thereafter, the pre-formed biofilm was rinsed and incubated with *B. bacteriovorus* or *Bdellovibrio*- free control. The impact of predation on the biofilm was assessed by CV staining following 24 hrs of incubation.

Experiment 2. Measuring biofilm reduction by CFU enumeration. The host bacteria used to grow the biofilm were; *A. baumannii* ATCC 19606, *A. baumannii* NCIMB 12457, *A. lwoffii* ATCC 17925, *E. gergoviae* ATCC 33028, *K. pneumoniae* clinical isolate, *K. pneumoniae* ATCC 33495, *K. pneumoniae* ATCC BAA-1706, and *M. morganii* ATCC 25829.

Results.

Incubating the pre-formed biofilm with the predator resulted in a 2-3 log reduction in CFU counts within the first 24 hrs of incubation. A 6-7 log reduction was measured by 48 hrs in biofilms composed of *A. baumannii* ATCC 19606, *A. baumannii* NCIMB 12457, *A. lwoffii* ATCC 17925, and the *K. pneumoniae* clinical isolate (Fig. 9; Table-6, CFU log reduction following predation in a single species biofilm). No reduction in CFU host cell viability was measured in the biofilms inoculated with the *Bdellovibrio*- free sample (Fig. 9).

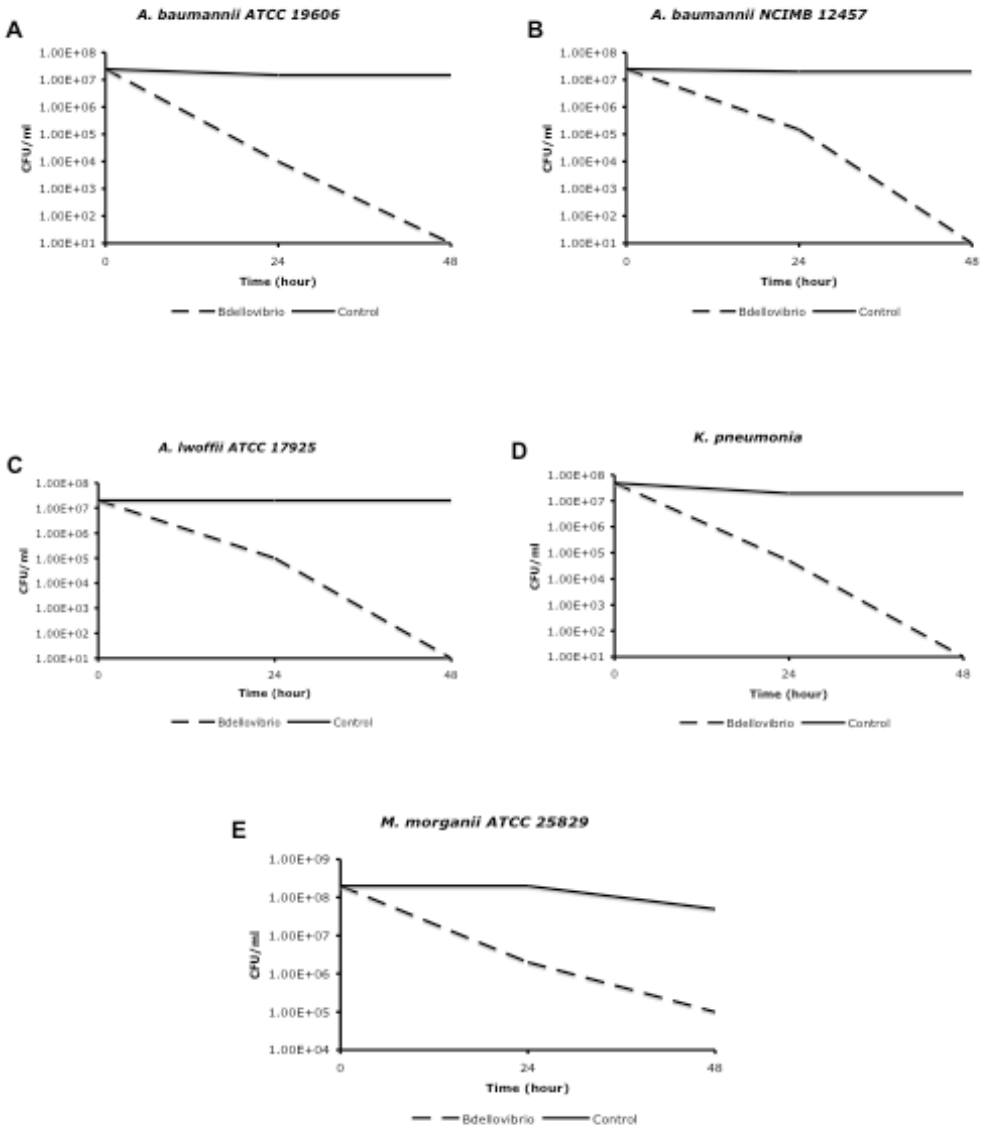


Fig. 9. Reduction of multi-layer microbial biofilms by predatory bacteria. Biofilms composed of microbial pathogens (A-E) were formed in a 96-well static system. Thereafter, the pre-formed biofilm was rinsed and incubated with *B. bacteriovorus* or *Bdellovibrio* free control media. The effect of the treatment on biofilm cell population was assessed by CFU enumeration of the remaining biofilm following incubation with the predator.

Experiment 3. Examining biofilm reduction by SEM. In order to obtain high resolution images of the impact of predation on biofilms, biofilms of selected pathogens were developed on PVC plastic cover slips. Thereafter, the biofilms were exposed for 24 hrs to the predator. The effect of predation was examined using a cross-beam field emission scanning electron microscope (FE-SEM). *Bdellovibrio*- free media was used as a control.

Result.

As seen in Fig-10, adding *Bdellovibrio* to the biofilm had a significant effect on the biofilm surface coverage. It is clear that the bulk of the biofilm was removed leaving behind what appears to be a few *Bdellovibrio* cells attached to the surface and biofilm debris.

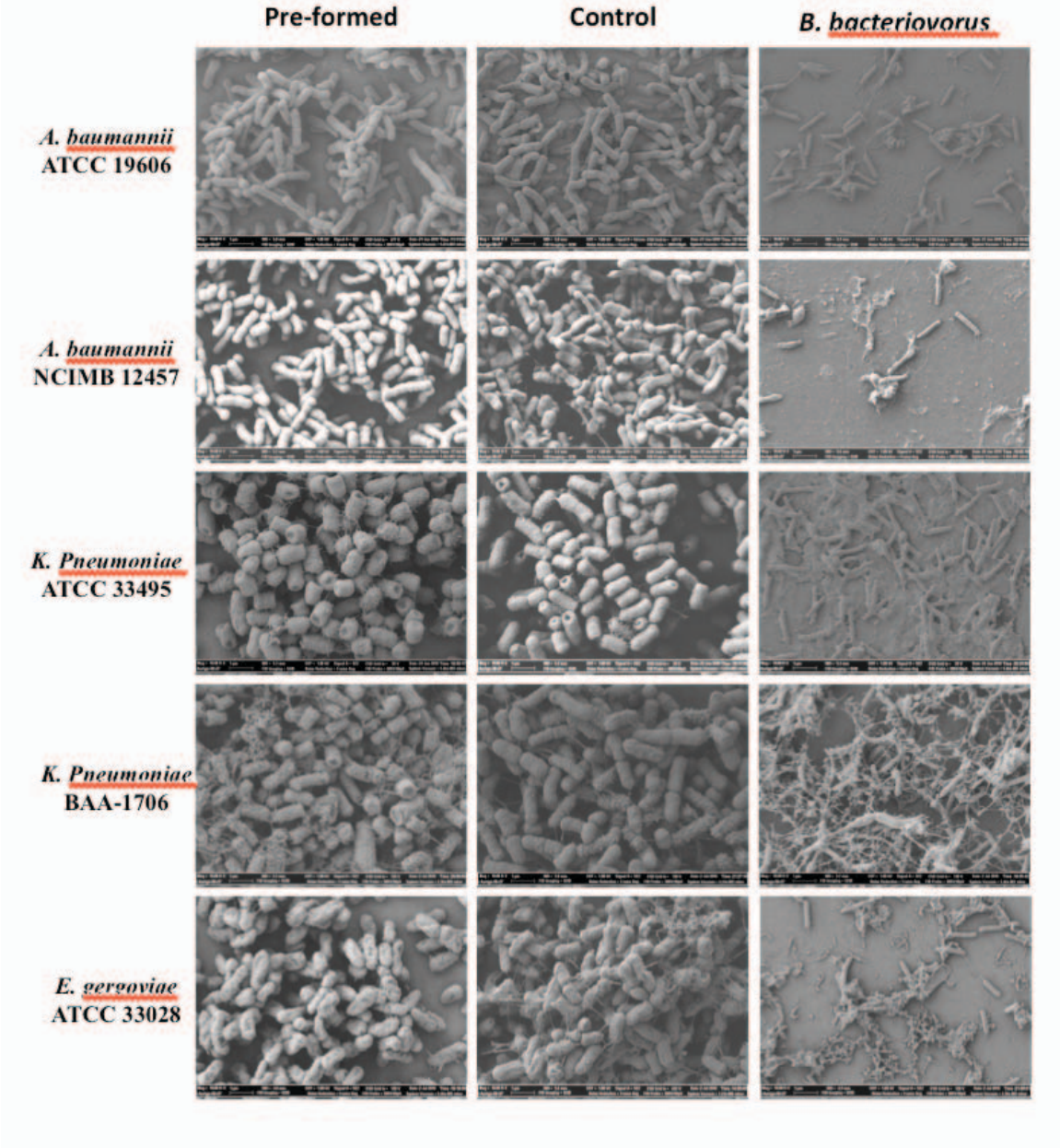


Fig. 10. SEM analysis of biofilm predation. Biofilms were developed on PVC plastic cover slips for 18 hrs (Pre-formed). Thereafter, the biofilms were washed and incubated for 24 hrs with *B. bacteriovorus* or filtered sterilized lysate (Control). Magnification, x10,000. Images were collected from biofilms grown at the air-liquid interface.

Subtask 2.4. Assessing the ability of predatory bacteria to inhibit biofilm formation.

In the following experiment, bacteria were allowed to form biofilms in the presence and absence of *B. bacteriovorus.* Biofilms were developed in 50% LB media in 96-well plates. Final biofilm buildup in the treated and non-treated samples was measured by CV staining following 24 hrs of incubation.

Results.

Incubating the bacteria in the presence of *Bdellovibrio* resulted in a significant reduction in biofilm buildup (Fig. 11). The biofilm buildup of *A. baumannii* ATCC 19606 was 40% less than that achieved in the *Bdellovibrio*- free sample; 53% less for *A. baumannii* NCIMB 12457; 39% less for *A. species* ATCC 49466; 73% less for *E. coli;* 30% less for *E. gergoviae* ATCC 33028; 57% less for *M. morganii* ATCC 25829, and76% and 34% less for *K. pneumoniae* clinical isolate and *K. pneumoniae* ATCC BAA-1706, respectively.

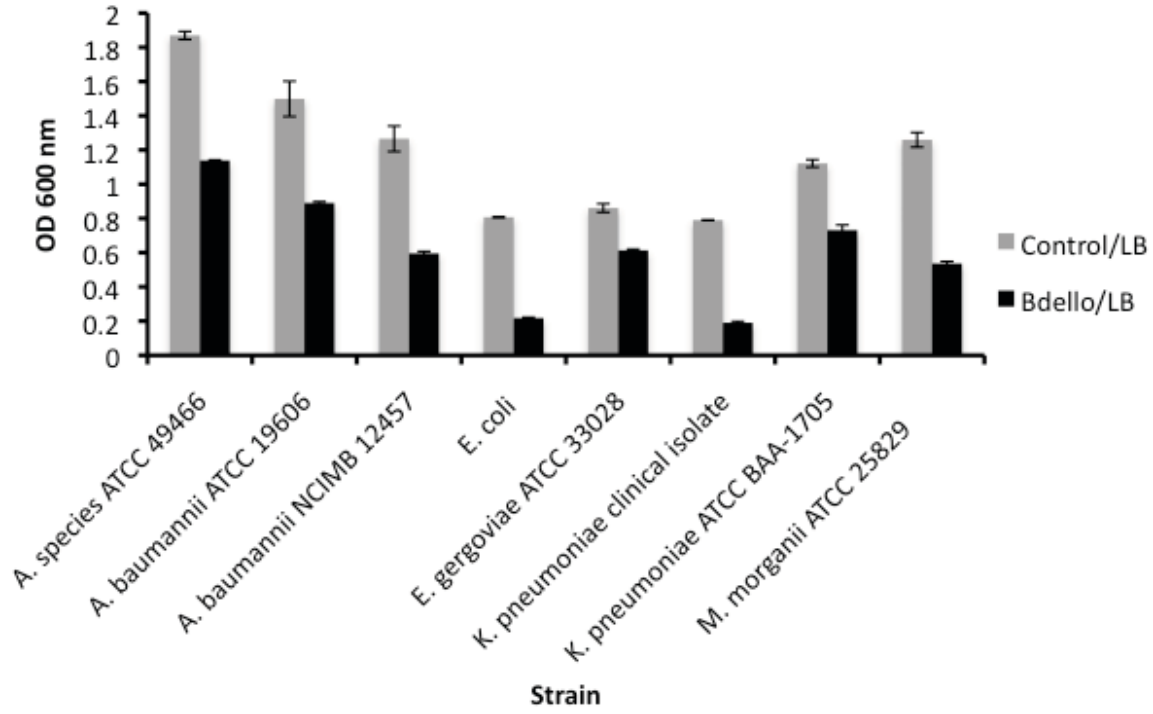


Fig. 11. Inhibition of microbial biofilms by *B. bacteriovorus*. Biofilms were developed with and without the addition of the predator. Thereafter, the biofilm was rinsed and stained with CV.

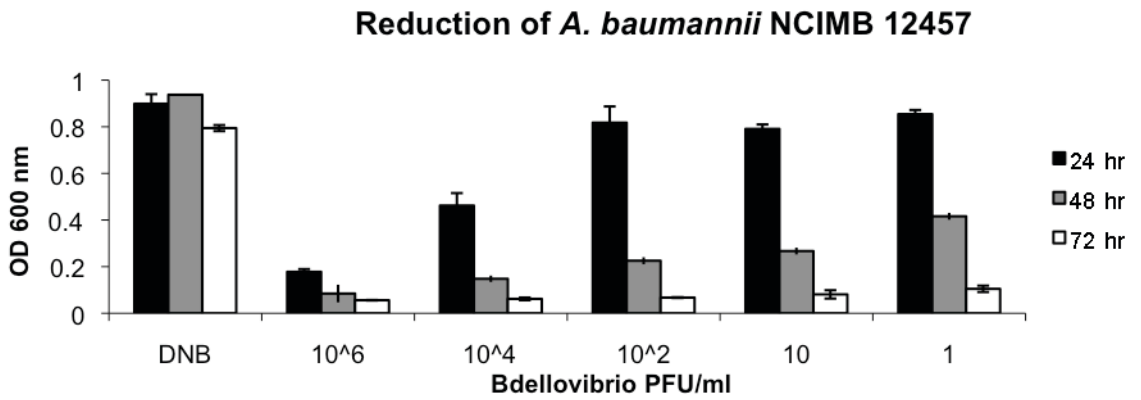
<u>Subtask 2.5.A.</u> Threshold amount of predator needed for active predation and biofilm host reduction.

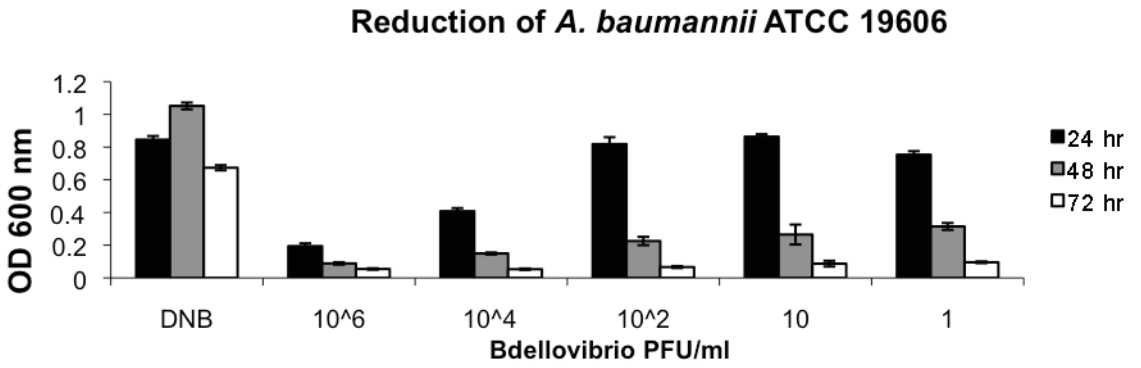
To evaluate the minimum concentration of *Bdellovibrio* required for biofilm removal, biofilms were developed in 96-well plates. Thereafter, serial diluted *B. bacteriovorus* was added to the biofilms. Biofilm reduction was measured by CV staining following 24-72 hrs of incubation. *Bdellovibrio*- free media was used as a control.

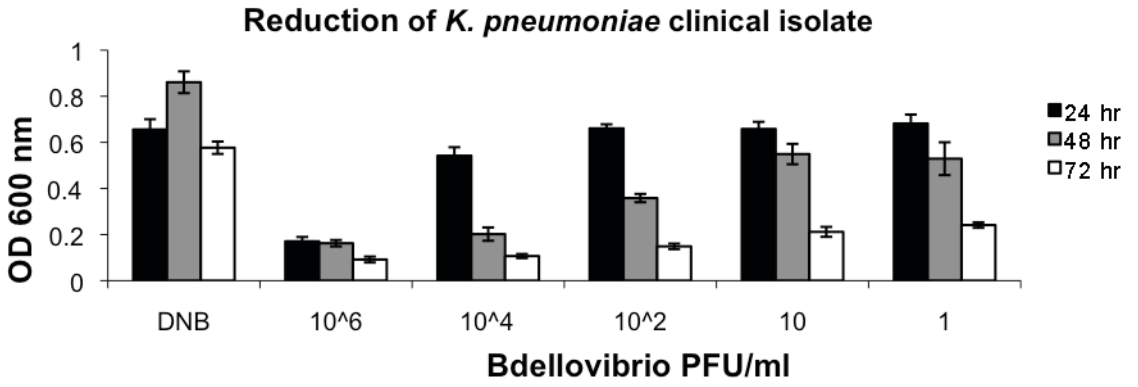
Results.

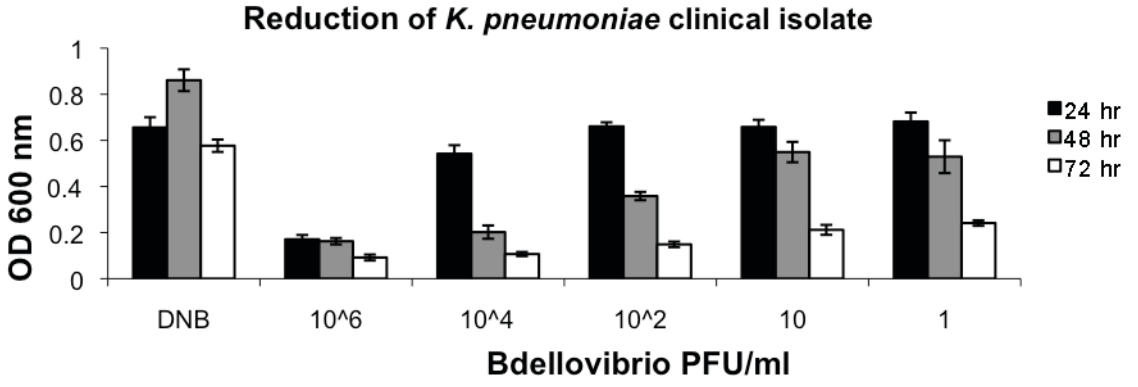
The addition of 1 PFU/ml predator was sufficient to significantly reduce the biofilm biomass of all of the selected pathogens. A positive correlation between the amount of *Bdellovibrio* used and the extent and pace of biofilm reduction was seen (Fig. 12).

Figure 12. Reduction of biofilms by different concentrations of *B. bacteriovorus* 109J.









DNB- *Bdellovibrio* free control.

* Biofilms were developed in 96-well plates. Thereafter, serial diluted *Bdellovibrio* was added to the biofilms. Data represents biofilm CV staining following 24, 48 and 72 hrs of incubation.

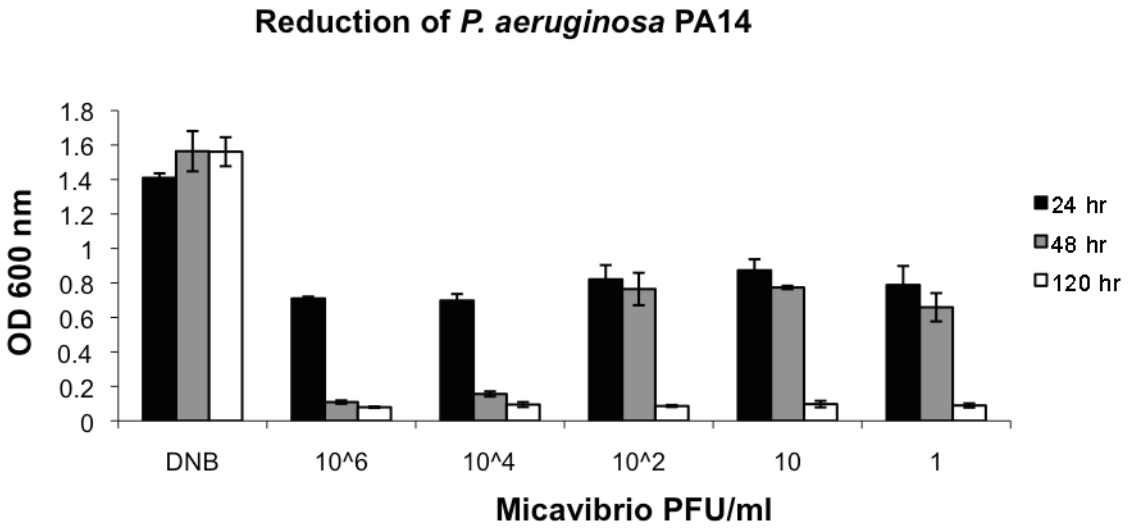
<u>Subtask 2.5.B.</u> Threshold amount needed for *Micavibrio* biofilm reduction.

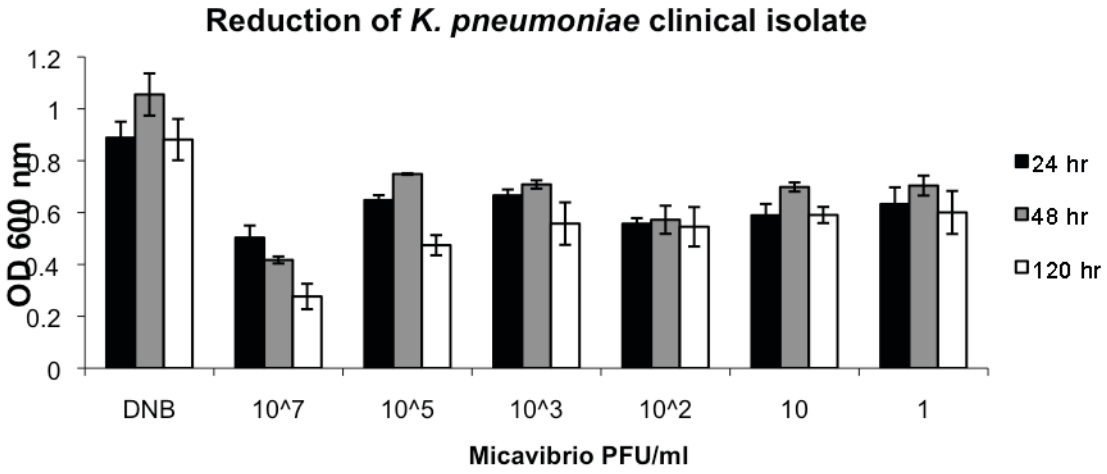
To assess the minimum concentration of *Micavibrio* required for biofilm removal, biofilms were pre-developed in 96-well plates. Thereafter, serial diluted *M. aeruginosavorus* was added to the biofilms. Biofilm reduction was measured by CV staining following 24-120 hrs of incubation. Predator- free media was used as a control.

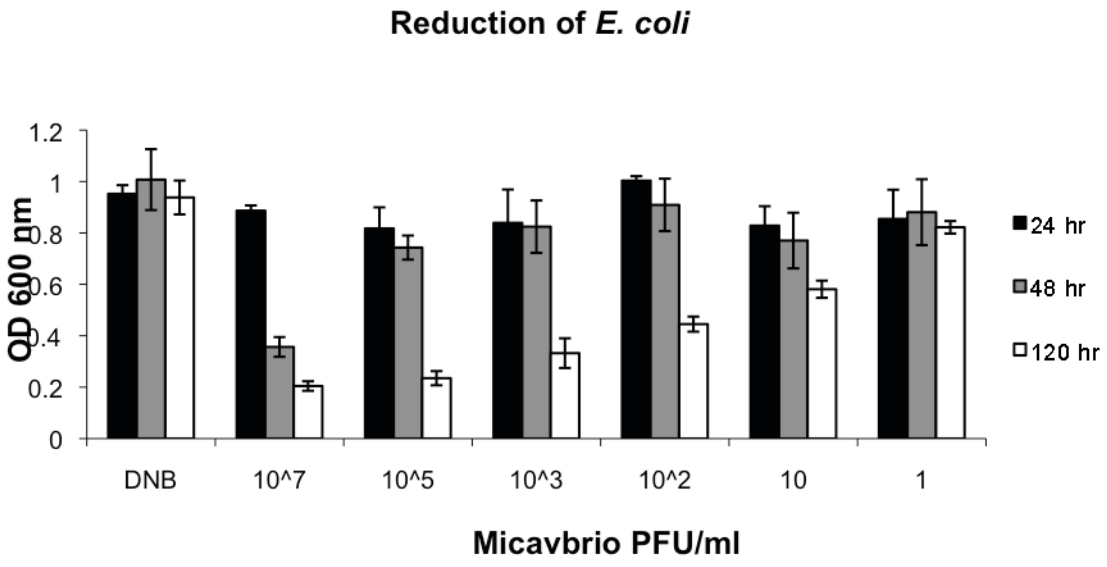
Results.

The addition of 1 PFU/ml predator was sufficient to reduce the biofilm biomass of *P. aeruginosa.* Higher concentrations of *Micavibrio* (above 10 PFU/ml) was required to remove *E. coli* biofilms and *K. pneumoniae* biofilms. As before, the extent and rate of reduction was correlated to the amount of predator used (Fig. 13).

Figure 13. Reduction of biofilms by different concentrations of *Micavibrio*.







DNB- *Bdellovibrio* free control.

* Biofilms were developed in 96-well plates. Thereafter, serial diluted *Micavibrio* was added to the biofilms. Data represents biofilm CV staining following 24, 48 and 120 hrs of incubation.

Subtask 2.6. Assessing the ability of *B. bacteriovorus* to reduce multi-species biofilms.

Experiment 1. To measure the influence of *B. bacteriovorus* 109J predation on biofilms composed of more than one microbial species, mixed cultures were used to form a biofilm in a 96-well static system. The one day old pre-formed biofilm was exposed to the predator or to a *Bdellovibrio*- free control. The effect on the biofilm was evaluated by CFU enumeration of the remaining biofilm cells following a 24 hr incubation period. Single host biofilms were also incubated for comparison. The host cells co-culture included; *A. baumannii* NCIMB 12457 and *K. pneumoniae* ATCC 33495; *E. gergoviae* ATCC 33028 and *K. pneumoniae* ATCC BAA-1706. CFU enumeration was done by plating the lysates on selective antibiotic agar plates.

Results.

As seen in Table-6, the ability of *B. bacteriovorus* to reduce host bacteria in multi-species microbial cultures was mostly compatible to that of a single species culture. A slight reduction in *Bdellovibrio* predation efficacy was seen on biofilms composed of *A. baumannii* NCIMB 12457 and *K. pneumoniae* ATCC 33495.

Table 6. Reduction of multi-species microbial biofilms by predatory bacteria.

Experiment A.

Bacteria tested	**CFU log reduction following predation in a <u>single</u> species biofilm**	**CFU log reduction following predation in a <u>mixed</u> species biofilm**
A. baumannii NCIMB 12457	2-3	2
K. pneumoniae ATCC 33495	2	1

Experiment B.

Bacteria tested	**CFU log reduction following predation in a <u>single</u> species lysate**	**CFU log reduction following predation in a <u>mixed</u> species lysate**
E. gergoviae ATCC 33028	2	2
K. pneumoniae ATCC BAA-1706	3	3

Multi-species biofilms were formed for 24 hrs in a 96-well static system. Thereafter, the pre-formed biofilm was rinsed and incubated with *B. bacteriovorus* or *Bdellovibrio*- free control media for 24 hrs. The effect of the treatment on biofilm cell population was

assessed by CFU enumeration of the remaining biofilm. Single host biofilms experiments were also done for comparison.

Experiment 2. Measuring multi-species biofilm reduction by SEM.

In order to obtain high resolution images of the impact of predatory bacteria on biofilms composed of more than one microbial species, multi-species biofilms were developed on PVC plastic cover slip. Thereafter, the biofilms were exposed to the predators and impact of predation was examined using a FE-SEM. *Bdellovibrio*- free media was used as a control.

Results.

As seen in Fig.14, adding *Bdellovibrio* to the biofilm had a significant effect on the multi-species biofilm surface coverage. As before, the bulk of the biofilm was removed leaving behind what appears to be *Bdellovibrio* cells attached to the surface and biofilm debris.

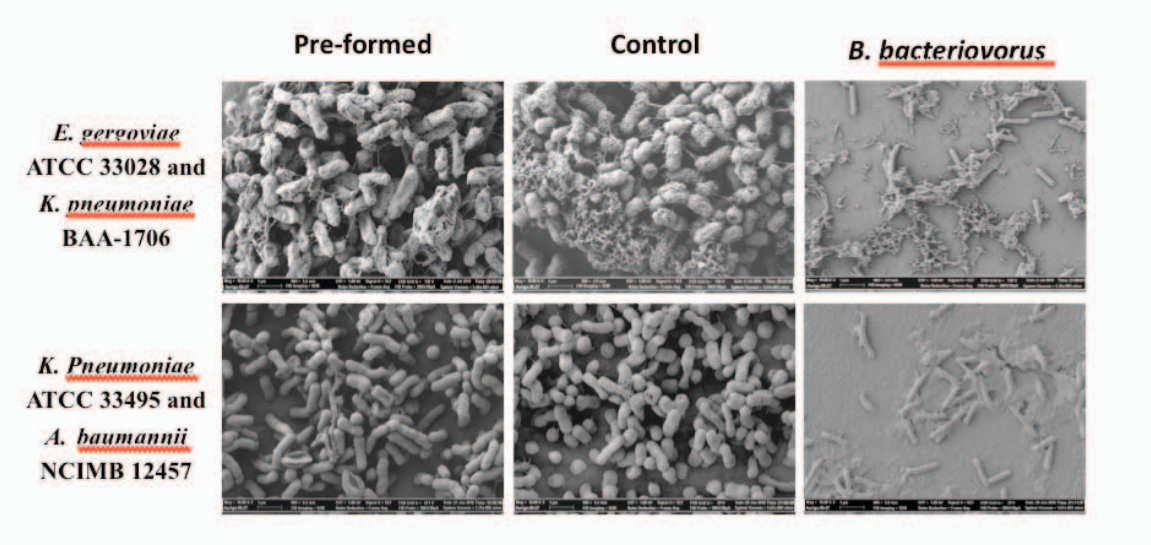


Fig. 14. SEM analysis of multi-species biofilm predation. Multi-species biofilms were developed on PVC plastic cover slips for 18 hrs (Pre-formed). Thereafter, the biofilms were washed and incubated for 24 hrs with *B. bacteriovorus* or filtered sterilized lysate (Control). Magnification, x10,000. Images were collected from biofilms grown at the air-liquid interface.

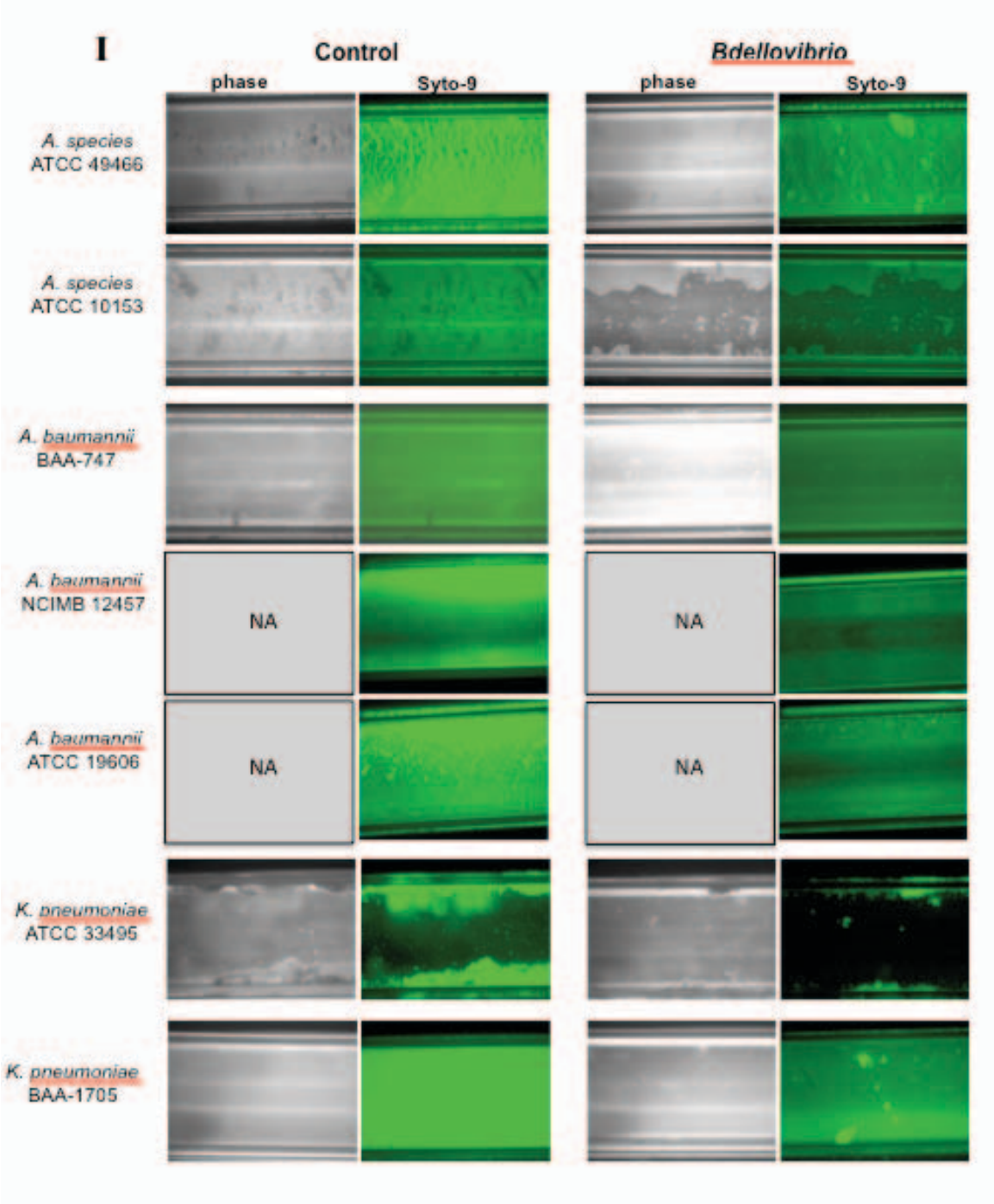
Subtask 2.7. Reduction of microbial biofilms in a flow cell system. In order to measure the ability of *Micavibrio* and *Bdellovibrio* to clear a robust biofilm grown under flowing conditions, a flow cell system was used. In this system, biofilms were cultivated in a four-channel flow cell with a square glass capillary (channel dimensions of 2 by 2 mm, Friedrich and Dimmock, Inc., Millville, NJ). The glass capillaries were placed on a 30^{o}C-heating block (Fig. 15). The flow system was assembled as described previously [2,3]. Cells were inoculated from 18 hr LB-grown cultures. The medium flow was turned off prior to inoculation and 1 hr after inoculation. After the development of a mature multilayered biofilm (24 hrs), the flow was turned off, and the chambers were inoculated once with 1 ml (10^{8} PFU/ml) of harvested *B. bacteriovorus* or *M. aeruginosavorus*. 1 ml of filtered sterilized predator- free lysate was used as a control. Thereafter, DNB medium was pumped through the flow cell at a constant rate of 4.8 ml/h for the duration of the experiment (48 hrs). To analyze the effect of predation on the overall biofilm biomass, the glass capillary channels were placed on a phase-contrast inverted microscope and viewed under bright light at 40x magnification. Images were also taken with florescent light after staining the biofilm with Syto-9. Syto-9 is a green nucleic acid stain that can penetrate both intact and damaged membranes (Molecular Probes, Eugene, OR). Since the biofilms were developed within closed glass capillaries, we were unable to access the biofilm to perform direct cell enumeration or SEM imaging.

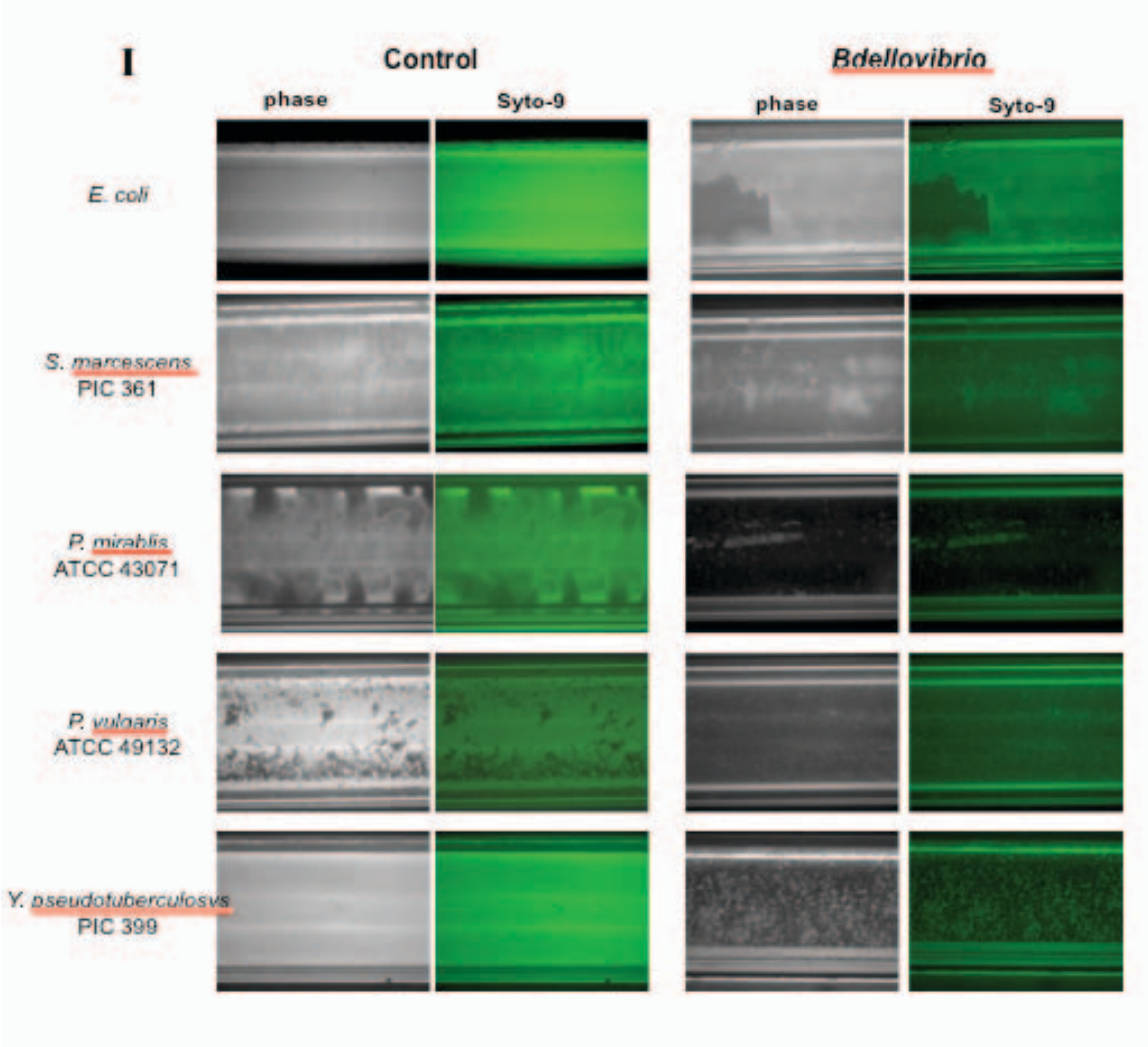
Fig 15. Flow cell system used.

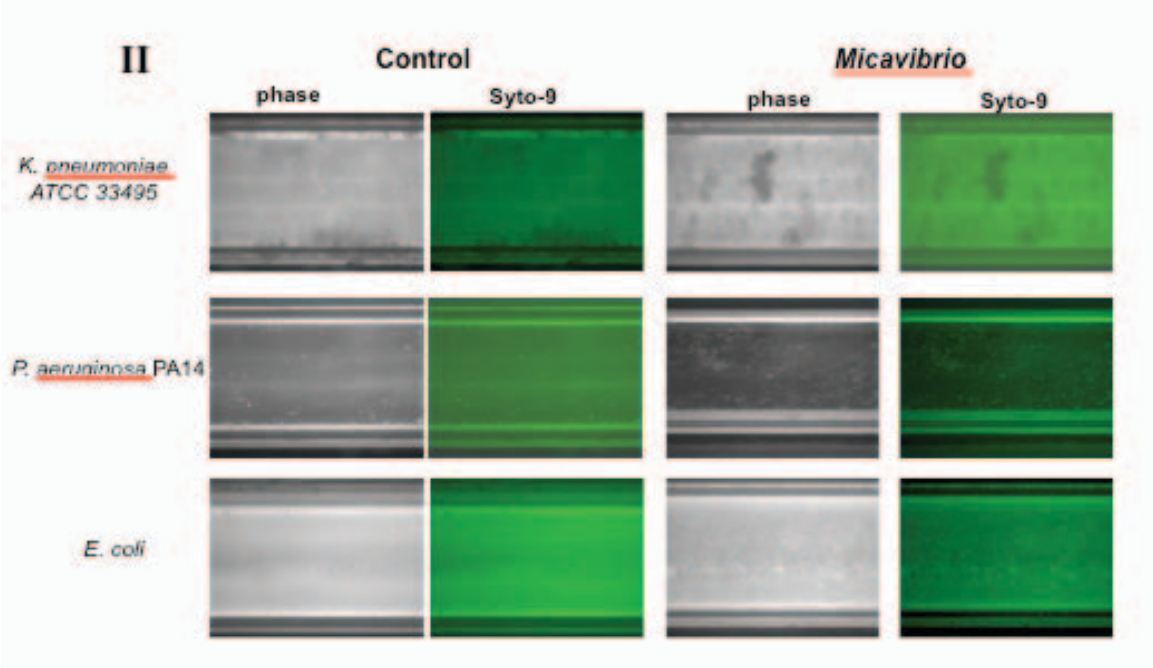
Results.

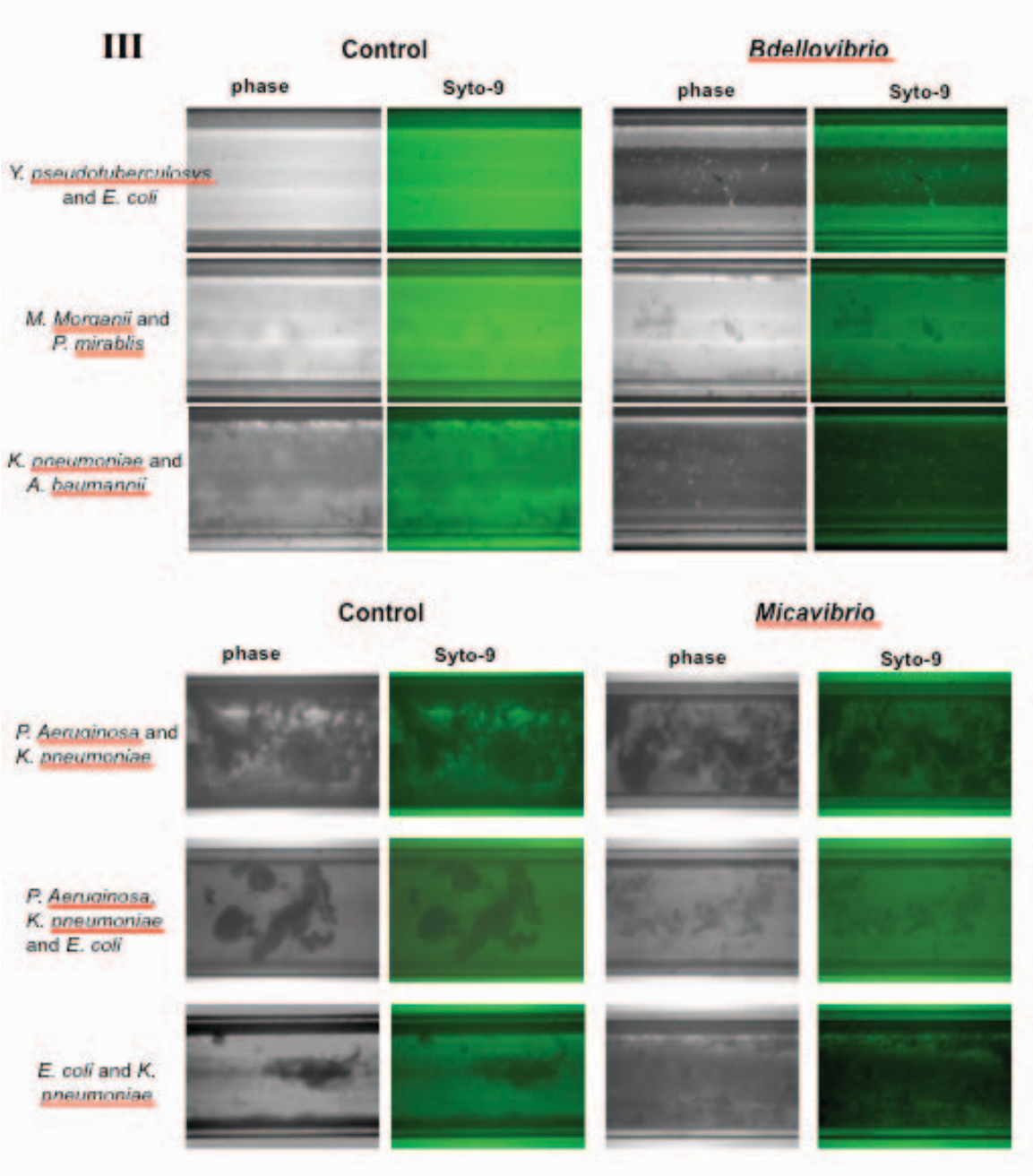
The results are shown in Fig-16. Treating the biofilms, which were composed of single-species (I, II) or multi-species bacteria (III), with *B. bacteriovorus* (I, III) or *M. aeruginosavorus* (II, III), caused a reduction in overall biofilm surface coverage. Reduction of biofilm biomass within the glass capillary was seen under bright light (phase) and fluorescence (Syto-9). In general, the biofilm reduction caused by *B. bacteriovorus* was more pronounced than that caused by *M. aeruginosavorus*.

Fig. 16. Monitoring predation in flow cell systems. Single-species (I and II) and multi-species biofilms (III) were developed in a flow cell system. Thereafter, the biofilms were injected once with *B. bacteriovorus* (I and III), *M. aeruginosavorus* (II, III) or predator-free media (control). After 48 hrs, the chambers were analyzed by phase-contrast microscopy (phase) (dark areas are adherent bacteria) or stained with Syto-9. Magnification, x40.


I
Control
Bdellovibrio
phase
Syto-9
phase
Syto-9
A. species
ATCC 49466
A. species
ATCC 10153
A. baumannii
BAA-747
A. baumannii
NCIMB 12457
NA
NA
A. baumannii
ATCC 19606
NA
NA
K. pneumoniae
ATCC 33495
K. pneumoniae
BAA-1705


I
Control
Bdellovibrio
phase
Syto-9
phase
Syto-9
E. coli
S. marcescens
PIC 361
P. mirahlis
ATCC 43071
P. vulgaris
ATCC 49132
Y. pseudotuberculosys
PIC 399


II
Control
Micavibrio
phase
Syto-9
phase
Syto-9
K. pneumoniae ATCC 33495
P. aeruginosa PA14
E. coli

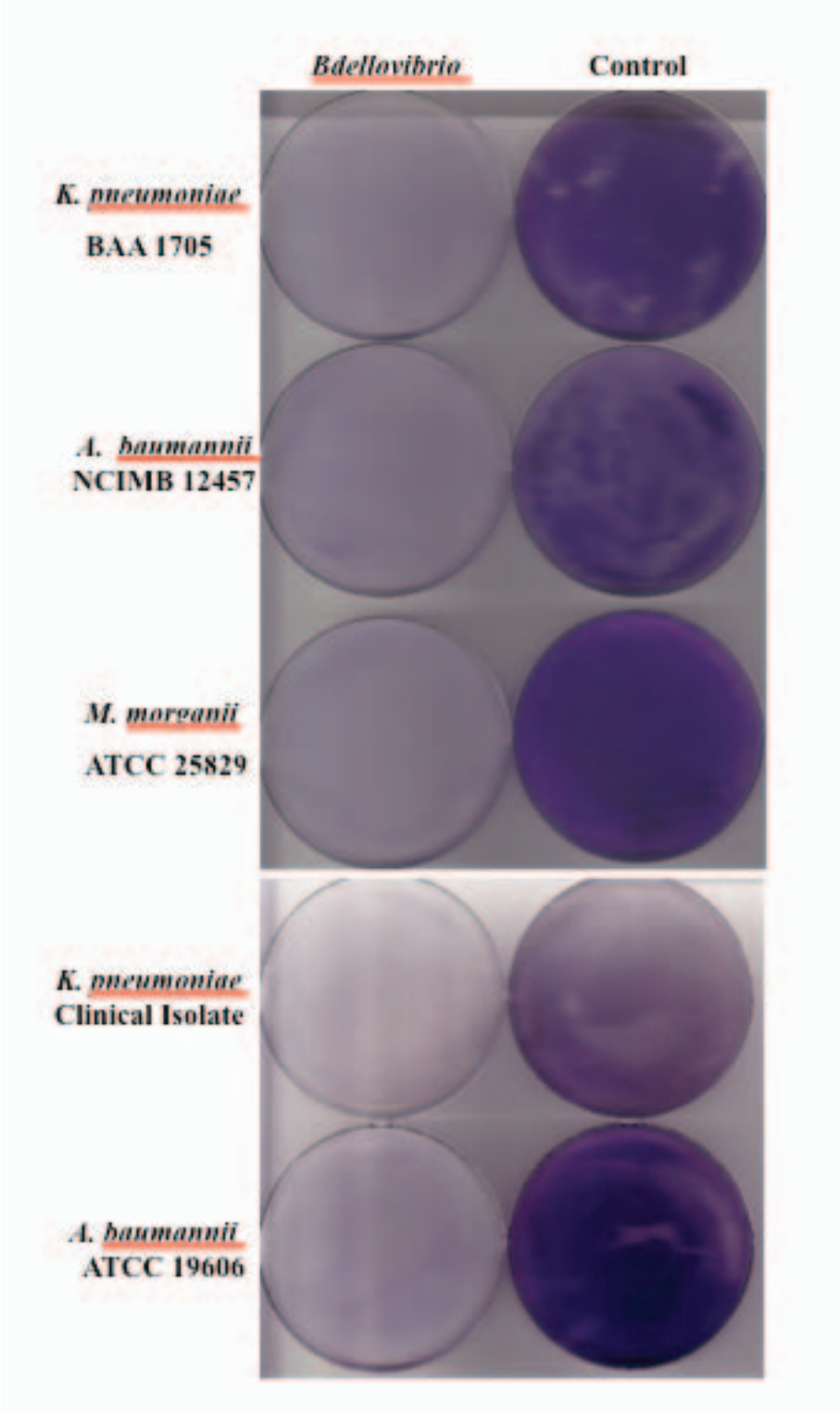

III
Control
Bdellovibrio
phase
Syto-9
phase
Syto-9
Y. pseudotuberculosys and E. coli
M. Morganii and P. mirablis
K. pneumoniae and A. baumannii
Control
Micavibrio
phase
Syto-9
phase
Syto-9
P. Aeruginosa and K. pneumoniae
P. Aeruginosa, K. pneumoniae and E. coli
E. coli and K. pneumoniae

Subtask 2.7.1. Reduction of robust microbial biofilms under shaking conditions. To further demonstrate the unique ability of *Bdellovibrio* to reduced robust biofilms on a large surface area, biofilms were developed in a tissue culture treated 94 mm dish. The plate was placed on an orbital shaker for 48 hrs under sheer forces. The constant sheer forces allowed the development of a robust and tightly attached biofilm on an extremely large surface area. Thereafter, the biofilm was treated with *Bdellovibrio* or *Bdellovibrio* free control.

Results.

A significant removal and clearing of the biofilm was seen following a 24 hr incubation period with the predator. Our findings demonstrate that *Bdellovibrio* is capable of detaching large biofilms formed on large surface areas. Our data also show that the sheer forces produce by the shaking of the plate did not reduce the ability of the predator to attack (Fig 16A).

FIG. 16A. Predation of robust biofilms covering a large surface. Biofilms were developed for 48 hrs in 94 mm dishes which were placed on an orbital shaker. The biofilms were than incubated for 24 hrs with *B. bacteriovorus* or predator- free media (control). CV staining was used to examine the biofilm bio-mass.



Additional experiments.

A. Examining early predator-biofilm attachment by SEM.

In the following experiments, single and multi-species biofilms were developed on PVC plastic cover slips. Thereafter, the biofilms were exposed to the predators for 2 hrs, rinsed to remove non-attached cells and examined using FE-SEM.

Results.

A 2 hr exposure period to the predators was sufficient for *Bdellovibrio* and *Micavibrio* to firmly bind to the biofilm and initiate an attack (Fig. 17). As both predators feed on the biofilm, the single predatory cells attached to the biofilm could proliferate and eventually lead to biofilm destruction.

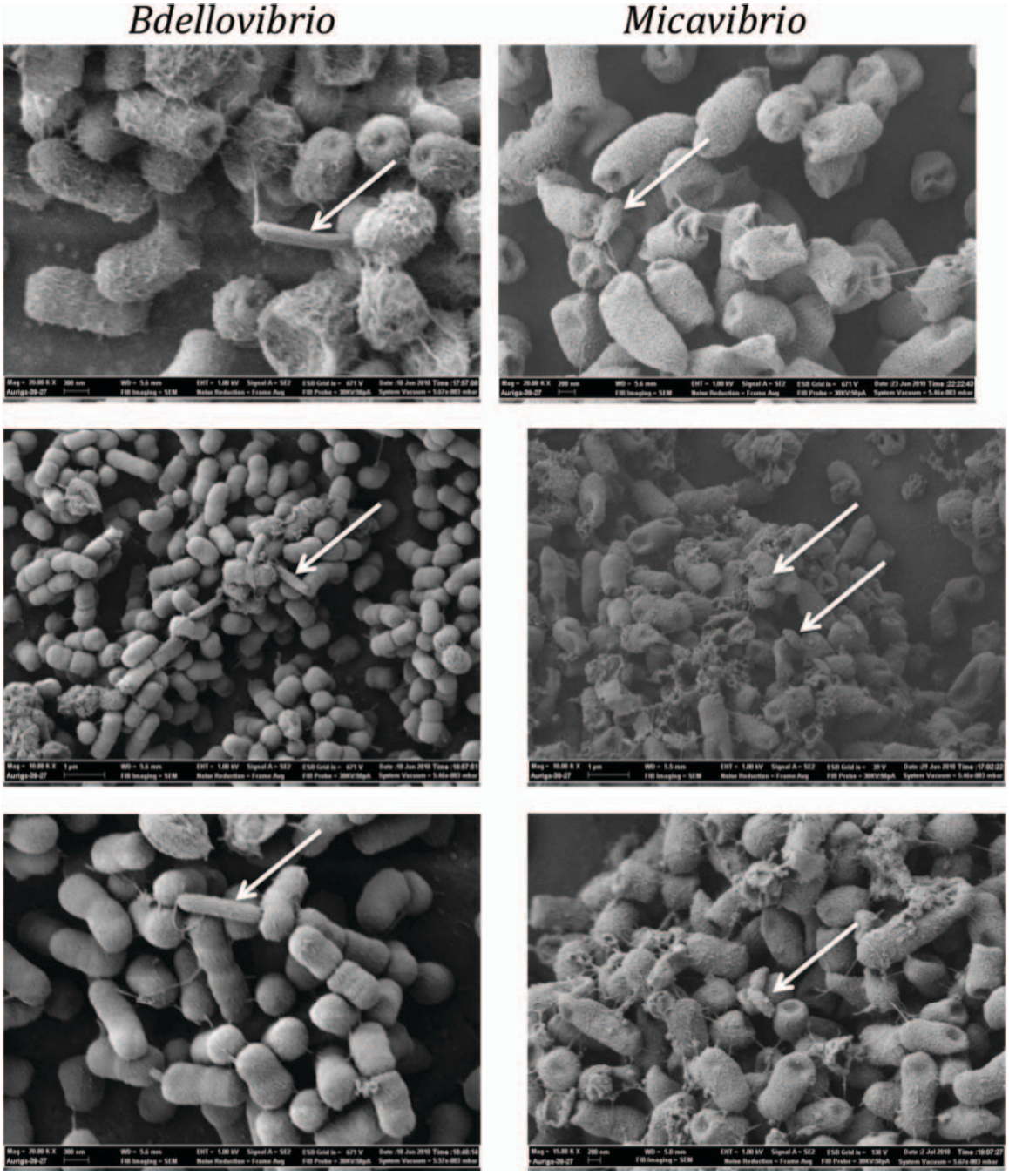


Fig. 17. High magnification images of early predatory biofilm attachment.

Single and multi-species biofilms were developed on PVC plastic cover slips for 18 hrs. Thereafter, the biofilms were incubated for 2 hrs with *B. bacteriovorus* or *M. aeruginosavorus*. The biofilms were rinsed to remove loosely attached cells and

examined by SEM. Magnification, x10,000-x20,000. Images were collected from biofilms grown at the air-liquid interface. Arrow indicates predatory bacteria.

B. Reduction of metabolically inactive biofilms.

It was previously suggested that cells within biofilms might exhibit limited growth, which in turn increases their resistance to antimicrobial challenges [5, 11, 12]. Thus, the ability of an antimicrobial agent to reduce and remove metabolically inactive surface-associated bacteria could be beneficial.

When working with biofilms of the oral pathogen *Aggregatibacter actinomycetemcomitans*, we noticed that although the pre-formed biofilm is extremely stable, CFU enumeration and viability assays revealed that the biofilm contained no viable cells following a 96 hr incubation period in DNB. At this point, an assay was carried out in which 24 hr pre-formed biofilms were washed, resuspended in DNB, and incubated for 120 hrs. The stable but nonviable biofilm was incubated with *Bdellovibrio* or filter-sterilized lysate.

The rationale for using *A. actinomycetemcomitans* in the experiments was its ability to form stable biofilms which showed little detachment with time. In one experiment, a reduction of 1.25% in biofilm biomass staining was measured during a 100-day incubation period in DNB (from A_{600}=0.83 at day one to A_{600}=0.79 at day 100), demonstrating the stability of the biofilms.

Results.

As seen in Fig.18, a reduction of 75% in CV staining was measured following incubation with *Bdellovibrio*, confirming removal of the metabolically inactive biofilm.

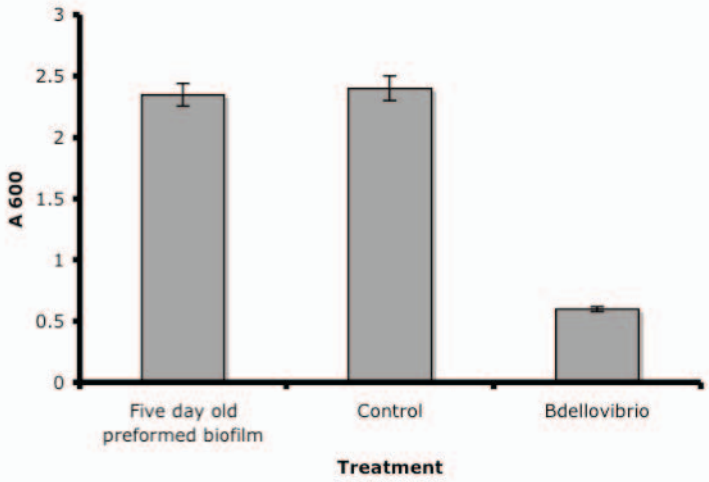


Fig. 18. Ability of *B. bacteriovorus* to remove metabolically inactive biofilms. Preformed overnight *A. actinomycetemcomitans* biofilms were developed in 96 well plates for 24 hrs. Thereafter, DNB was added to the biofilm and incubated for 96 hrs (Five day old preformed biofilm). Filtered sterilized lysate (Control) or *Bdellovibrio* was then added to the metabolically inactive biofilms. Biofilms were stained with CV and quantified. Each value represents the mean of 18 wells. Error bars are shown as one-standard deviation.

Aim III. Enhancing biofilm removal and potency of existing antimicrobial drugs.

Subtasks 3.1. Using biofillm-degrading enzymes to enhance biofilm removal. The aim of this task was to enhance the ability of the predator to penetrate and remove the biofilm by treating the biofilm with biofilm-degrading enzymes in concert or directly after the predator treatment.

A. Enhancing the ability of *Bdellovibrio* to remove biofilms of *A. actinomycetemcomitans*

It was previously reported that *A. actinomycetemcomitans* biofilms are composed of cells that are embedded in a self synthesized extracellular polymeric substance (EPS) which contains DNA, protein, and poly-N-acetylglucosamine (PGA) [1]. We hypothesized that by applying EPS degrading enzymes with or following the application of *Bdellovibrio*, greater biofilm removal could be obtained. To this end, biofilms of *A. actinomycetemcomitans* were formed and treated with *Bdellovibrio*, DNase-I, proteinase-K, and DspB (a PGA-hydrolyzing enzyme) [4, 7]. *A. actinomycetemcomitans* was selected for its enhanced ability to form extremely stable biofilms.

Results.

Treating the biofilm with *Bdellovibrio* for 48 hrs followed by a 2 hr incubation period with DNase-I (65μg/ml) resulted in a 12% increase in biofilm removal when compared to *Bdellovibrio* alone (71% reduction), and 45% more than DNase-I alone (which resulted in 38% reduction). Incubating the biofilm simultaneously with the enzyme and *Bdellovibrio* did not prove to be more effective in reducing the biofilm (66% reduction) when compared to the *Bdellovibrio* alone (Fig. 19A).

Incubating the biofilm with proteinase-K did not cause a measurable reduction in biofilm CV staining (Fig. 19B, PK treatment). Comparable biofilm removal was measured in both the *Bdellovibrio* treated sample and the sample treated with proteinase-K (100μg/ml) after the *Bdellovibrio* treatment (80% and 81% reduction, respectively). When incubating the pre-formed biofilm simultaneously with proteinase-K and *Bdellovibrio,* a loss of biofilm removal was seen (Fig. 19B, *Bdello* with PK). A reduction

in the *Bdellovibrio* biofilm-reducing ability was also detected in the samples that were first incubated with proteinase-K, washed, and then treated with the *Bdellovibrio* (Fig. 19B, PK followed by Bdello).

To further examine the effects of protease on predation, proteinase-K (100μg/ml) and trypsin (500μg/ml) were added to a standard lysate containing $2.5x10^8$ CFU/ml *E. coli* DH5α host cells. A 4-log reduction in CFU counts was measured in the *Bdellovibrio*-trypsin lysates with no host cell decrease occurring in the *Bdellovibrio*- free control ($3x10^4$ and $2.5x10^8$ CFU/ml respectively). No reduction in host cell population was measured in the *Bdellovibrio* proteinase-K lysates or the *Bdellovibrio*- free control ($2.5x10^8$ CFU/ml), concurring that proteinase-K could effectively inhibit predation.

Treating the pre-formed biofilm with a PGA-hydrolyzing enzyme (20μg/ml DspB) resulted in a 16% reduction in biofilm CV staining (Fig 19C, DspB). DspB was also capable of enhancing (by 14%) the ability of the *Bdellovibrio* to remove the biofilm when incubated in concert or before the application of the predator (Fig. 19C). Applying DspB after the *Bdellovibrio* treatment also proved to be more efficient in removing the biofilm when compared to the *Bdellovibrio* treatment alone (84% and 77% respectively).

In addition to the enhanced biofilm removal capability observed in the combined *Bdellovibrio* - DspB treatment, the time by which the *A. actinomycetemcomitans* biofilm was removed by the predator was considerably reduced in the presence of the enzyme. As seen in Fig. 20, treating the biofilm with a combination of *Bdellovibrio* and enzyme removed 88% of the biofilm within the first 24 hrs, whereas *Bdellovibrio* alone reduced the biofilm by 64% during the same time period.

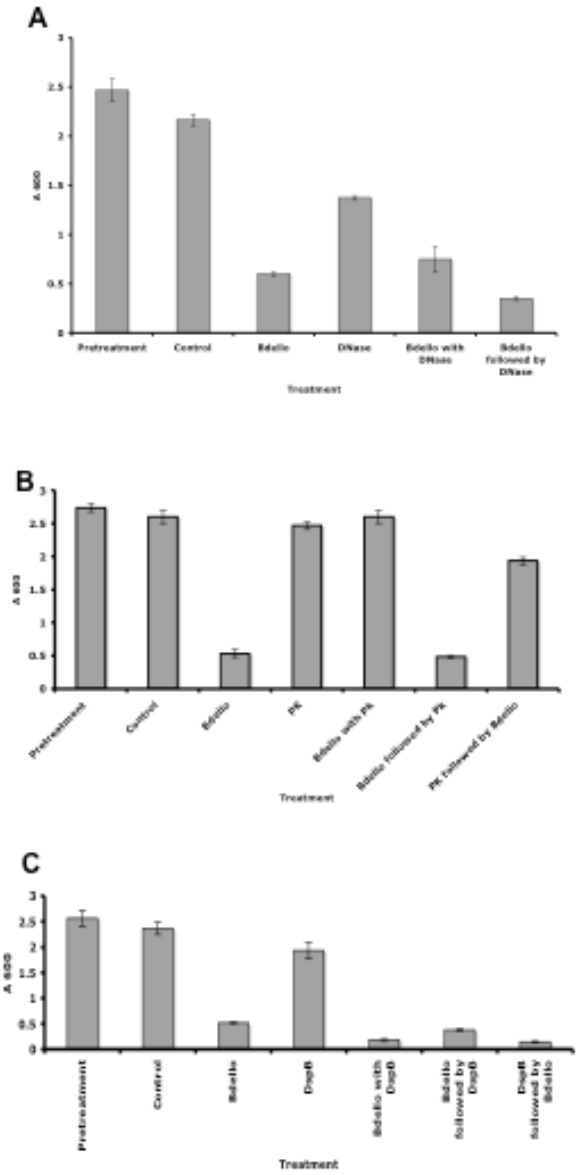


FIG. 19. Quantification of *A. actinomycetemcomitans* biofilm removal by *Bdellovibrio* and enzyme treatments. Preformed overnight *A. actinomycetemcomitans* biofilms (Pretreatment) were incubated for 48 hrs with filtered sterilized lysate (Control), *Bdellovibrio* (Bdello), DNase-I (A), proteinase-K (B), and DspB (C). Treatments also included incubating the biofilm simultaneously with *Bdellovibrio* and the selected enzymes (Bdello with enzyme), treating the biofilm for 2 hrs with the enzyme followed by a 48 hr *Bdellovibrio* treatment (enzyme followed by Bdello), or a 48 hr *Bdellovibrio* treatment followed by a 2 hr incubation period with the enzyme (Bdello followed by enzyme). Biofilms were stained and quantified. Each value represents the mean of 12 wells. Error bars are shown as one-standard deviation.

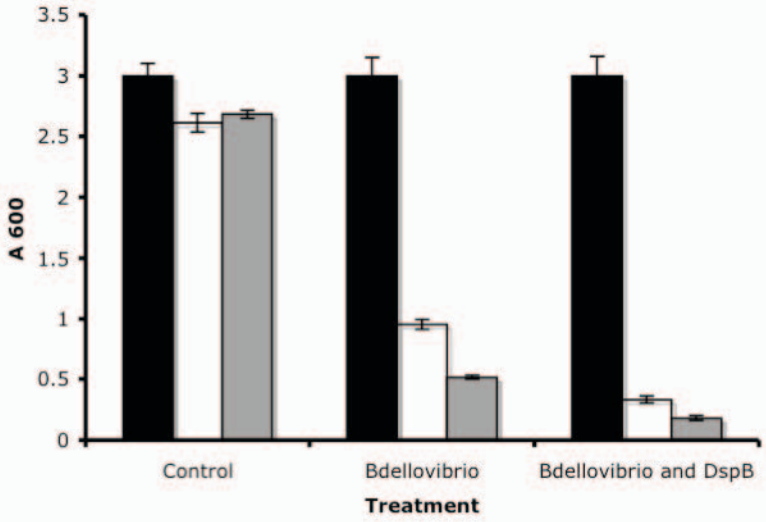


FIG. 20. Enhancing *Bdellovibrio* biofilm removal by the use of a PGA degrading enzyme. Preformed overnight *A. actinomycetemcomitans* biofilms (black bars) were incubated with filtered sterilized lysate (Control), *Bdellovibrio* or a combination of *Bdellovibrio* and DspB. Biofilms were stained after 24 (white bars) and 48 hrs (gray bars). Each value represents the mean of 12 wells. Error bars are shown as one-standard deviation.

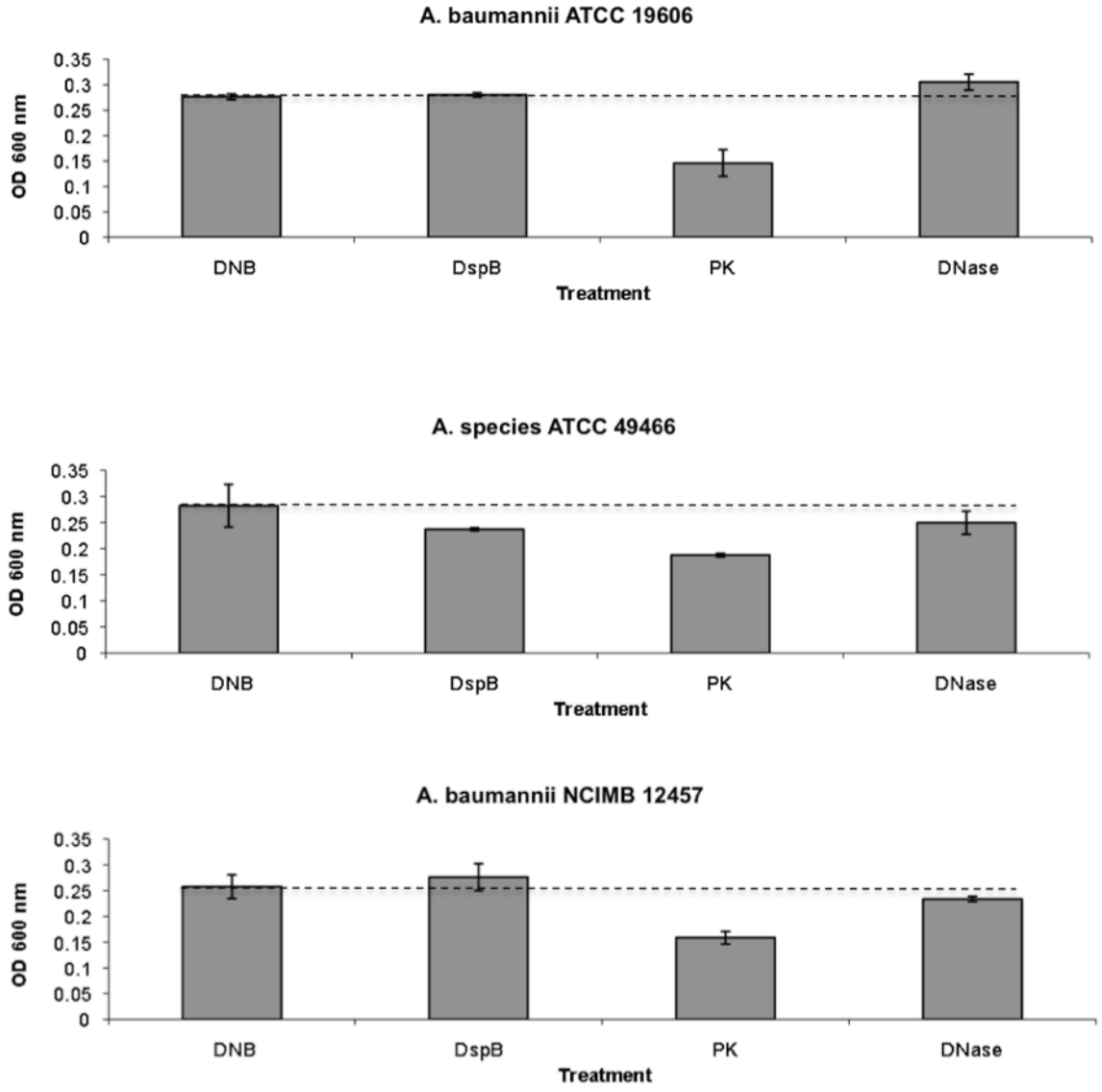
B. The use of biofilm degrading enzymes to increase biofilm removal post-predation.

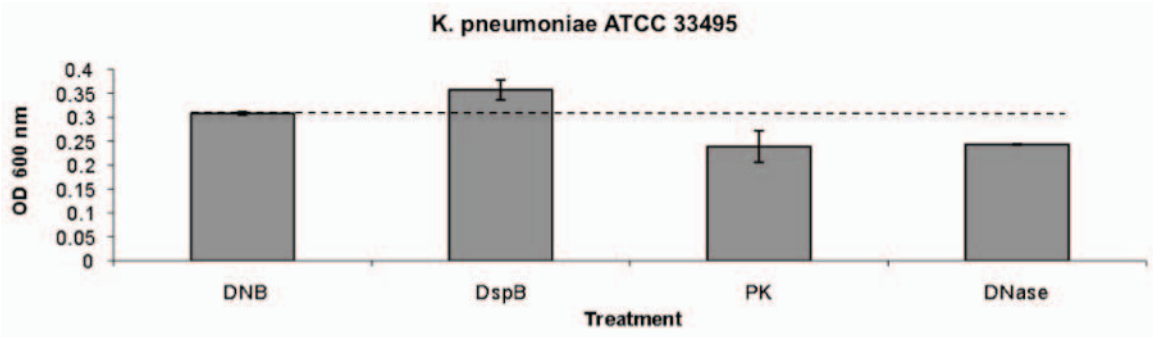
In the following experiments, pathogenic bacteria were allowed to form biofilms in 96-well plates. The biofilms were inoculated with *Bdellovibrio* for 18 hrs. Thereafter, enzymes were added to the *Bdellovibrio*-reduced biofilms and incubated for 3 hrs at 37^{o}C. The aim was to examine if enzyme treatment could cause further clearing of the biofilm. The change in biofilm reduction was evaluated by CV staining. Enzyme free media was used as a control (DNB). The enzymes used in this experiment were: DNase-I (100 μg/ml), proteinase-K (100 μg/ml) and DspB (20 μg/ml).

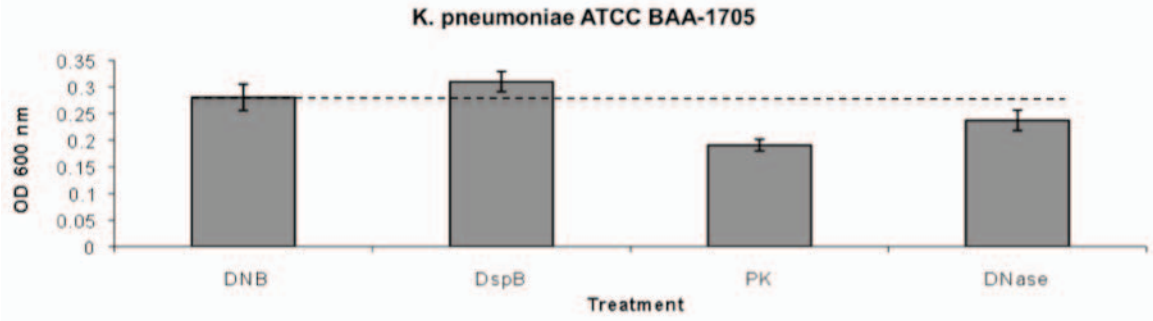
Results.

As seen in Fig. 21, the majority of the enzymes had little or no effect on the residual biofilm left after predation. Proteinase-K had the best biofilm reducing effect, followed by DNase-I. DspB had little effect on the biofilms. The inability of DspB to remove the biofilm is probably due to the fact that PGA is most likely not a major component in the biofilm matrix of the tested bacteria. Some biofilms were increased when DspB was used. This increase could be explained by the fact that the DspB enzyme is suspended in glycerol. Our preliminary experiments showed that adding trace amounts of glycerol to some bacteria, such as *K. pneumoniae,* will cause rapid biofilm buildup, as the bacteria could utilize the glycerol as a sole carbon source.

Fig. 21. Biofilm reduction by predation followed by enzyme treatment.




K. pneumoniae ATCC 33495
OD 600 nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
DNB
DspB
PK
DNase
Treatment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0.15
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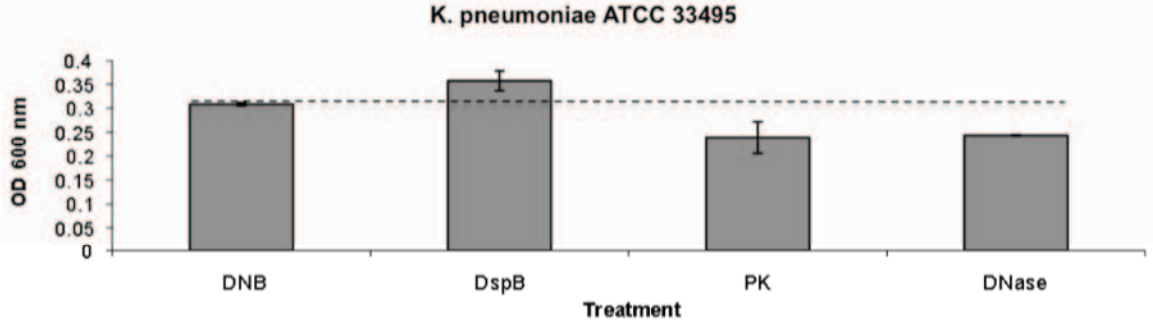
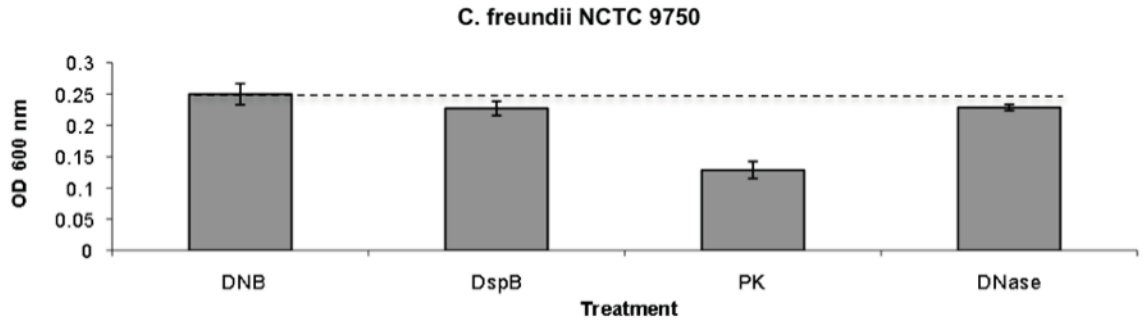
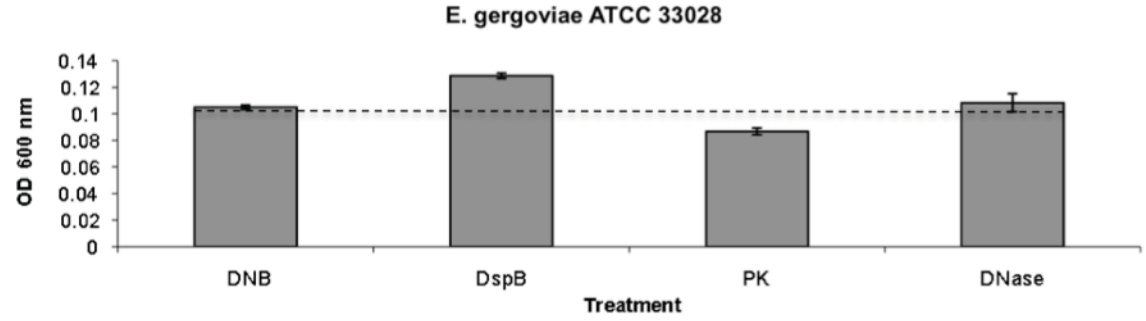
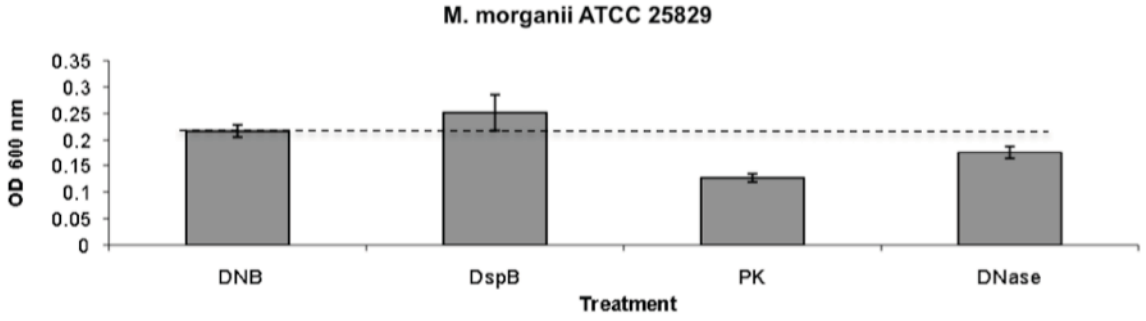
DspB
PK
DNase
Treatment


K. pneumoniae ATCC 33495
OD 600 nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
DNB
DspB
PK
DNase
Treatment







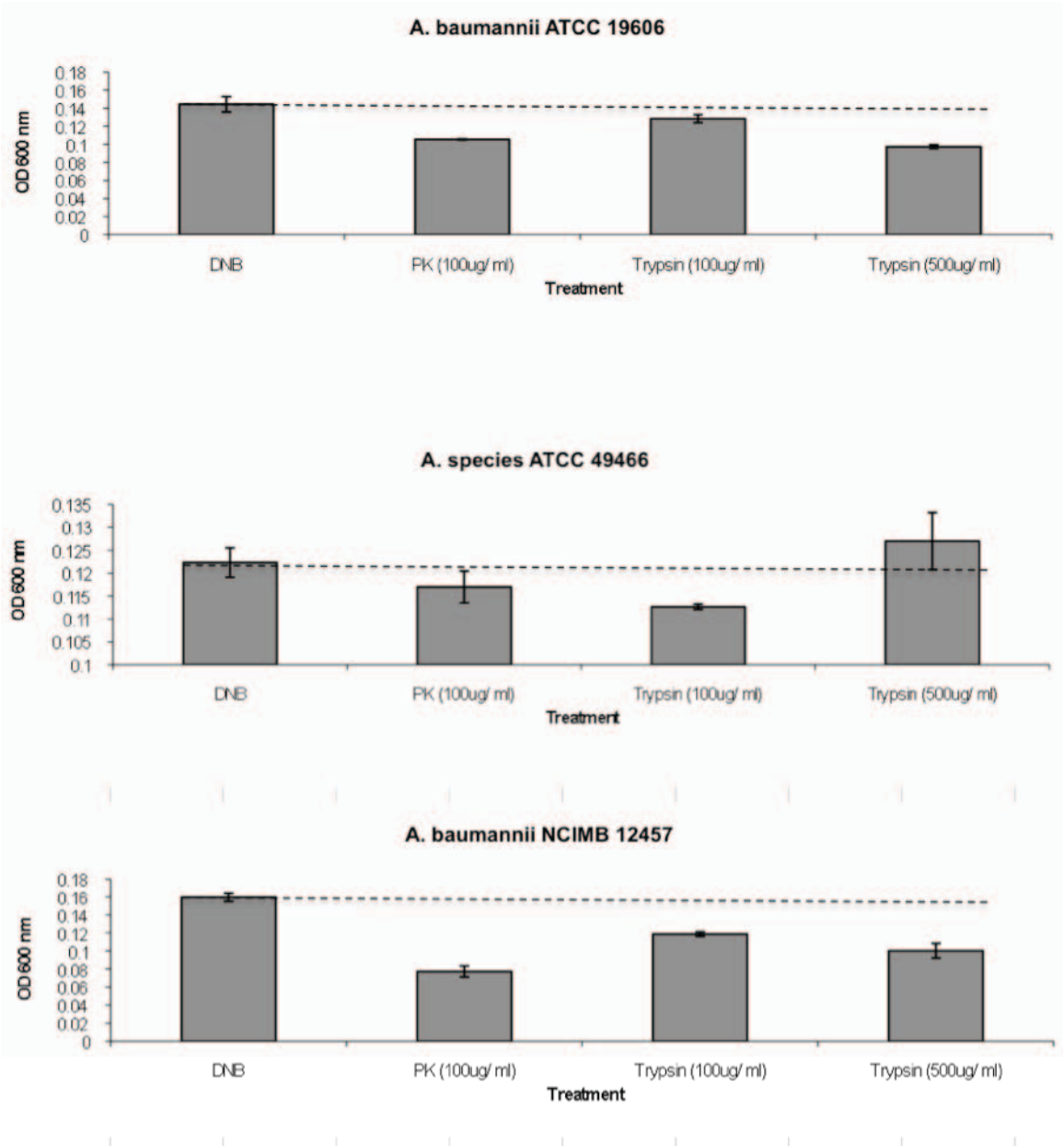
* Biofilms were developed for 24 hrs in 96-well plates followed by an 18 hr incubation period with *Bdellovibrio*. Thereafter, the remaining biofilm was inoculated for 3 hrs at 37^{o}C with the selected enzyme. Enzyme free media was used as a control (DNB). Dotted line indicates the change in biofilm biomass compared to the non-treated enzyme control.

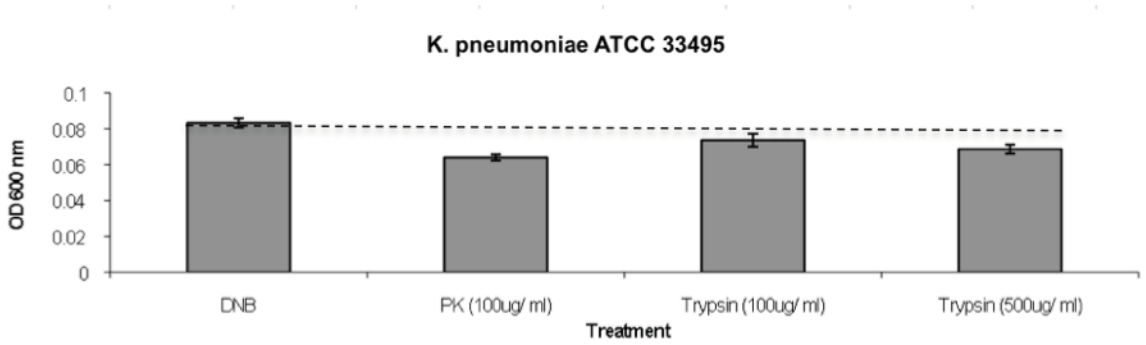
C. The use of protease to increase biofilm removal post-predation. Based on the data provided in Fig. 21, the biofilm removing ability of proteinase-K seemed to be the most effective among the enzymes used. In order to examine if other proteases would have a similar effect, biofilms were treated with an additional protease. The biofilms were formed in 96-well plates and treated for 18 hrs with the predator. The predator-reduced biofilm was incubated for 3 hrs with proteinase-K (100 μg/ml), trypsin (100 μg/ml) or 500 μg/ml trypsin. As before, enzyme free media was used as a control (DNB).

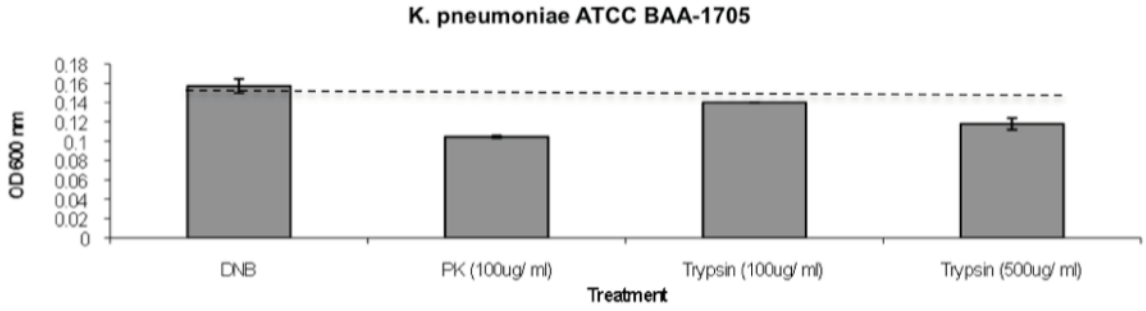
Results.

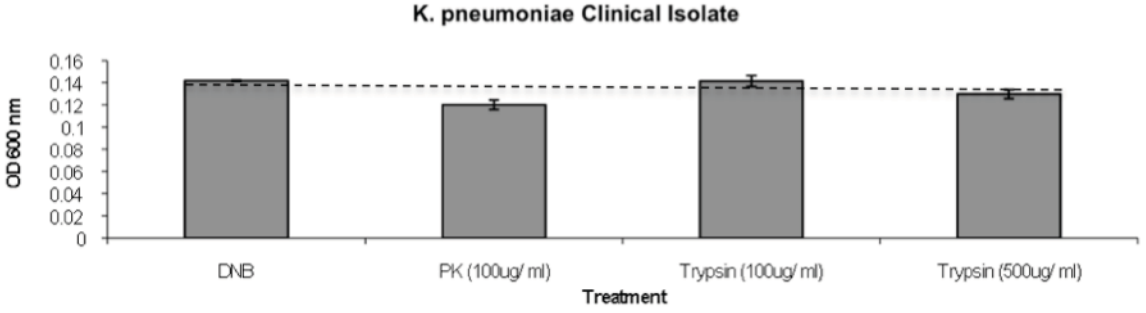
In general, incubating the biofilms with proteinase-K or trypsin increased removal of the residual biofilm after predation.

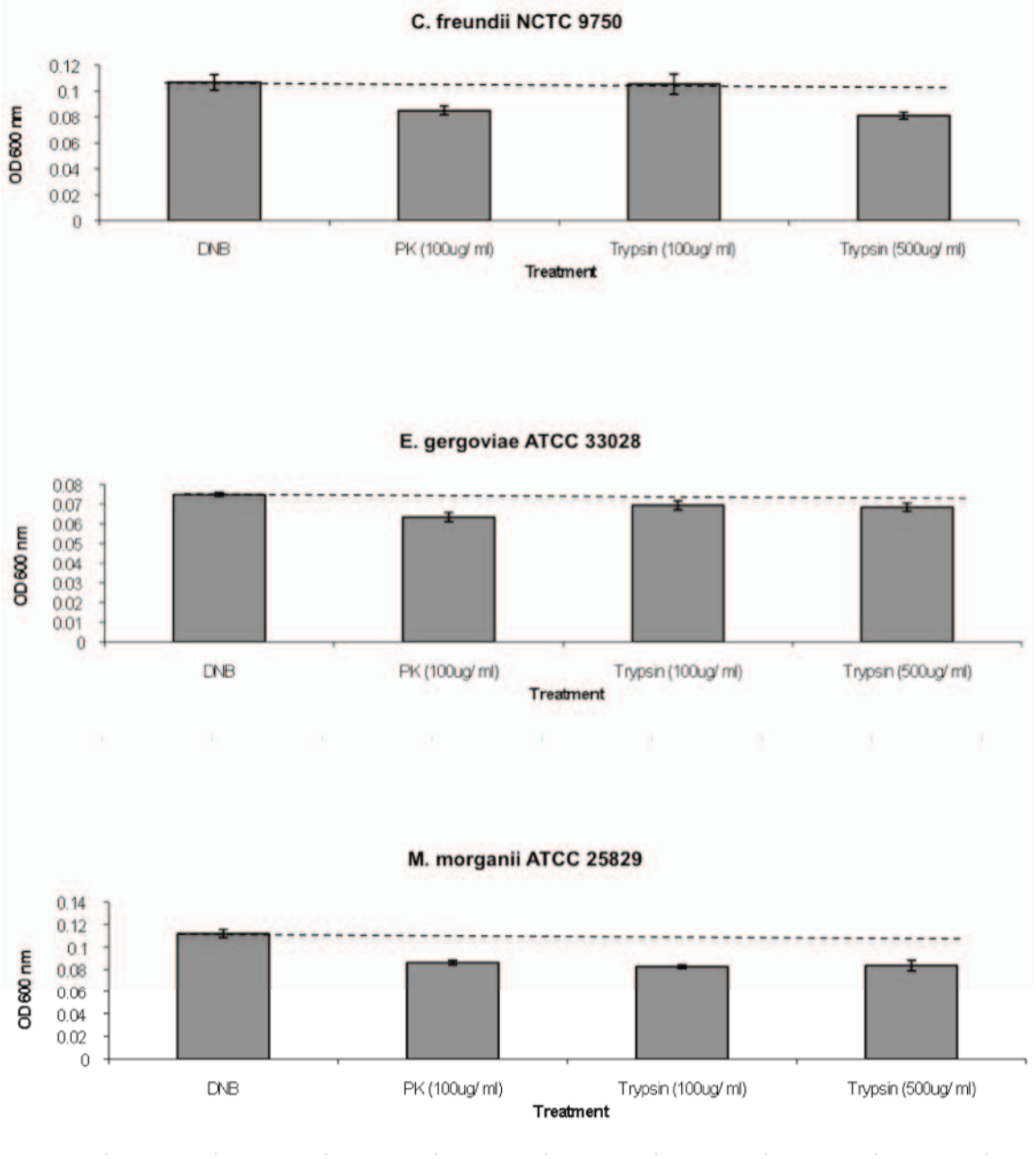
Fig. 22. Biofilm reduction by predation followed by protease treatment.




K. pneumoniae ATCC 33495
OD600 nm
0.1
0.08
0.06
0.04
0.02
0
DNB
PK (100ug/ ml)
Trypsin (100ug/ ml)
Trypsin (500ug/ ml)
Treatment


K. pneumoniae ATCC BAA-1705
OD600 nm
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
DNB
PK (100ug/ ml)
Trypsin (100ug/ ml)
Trypsin (500ug/ ml)
Treatment


K. pneumoniae Clinical Isolate
OD600 nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
DNB
PK (100ug/ ml)
Trypsin (100ug/ ml)
Trypsin (500ug/ ml)
Treatment



* Biofilms were developed in 96-well plates for 24 hrs followed by an 18 hr incubation period with *Bdellovibrio*. Thereafter, the remaining biofilm was inoculated with protease for 3 hrs at 37^{o}C. Enzyme free media was used as a control (DNB). Dotted line indicates the change in biofilm biomass compared to the non-treated enzyme control.

Subtasks 3.2. Enhancing the potency of antimicrobial drugs.

The aim of this task was to enhance the biofilm reducing aptitude of known antimicrobials by first exposing the biofilm to the predator for a relatively short period. The rationale behind these experiments is that the relatively brief exposure to the predator will be sufficient for "damaging" the biofilm and allowing the increase of antimicrobial biofilm penetration and a decrease in biofilm cell resistance.

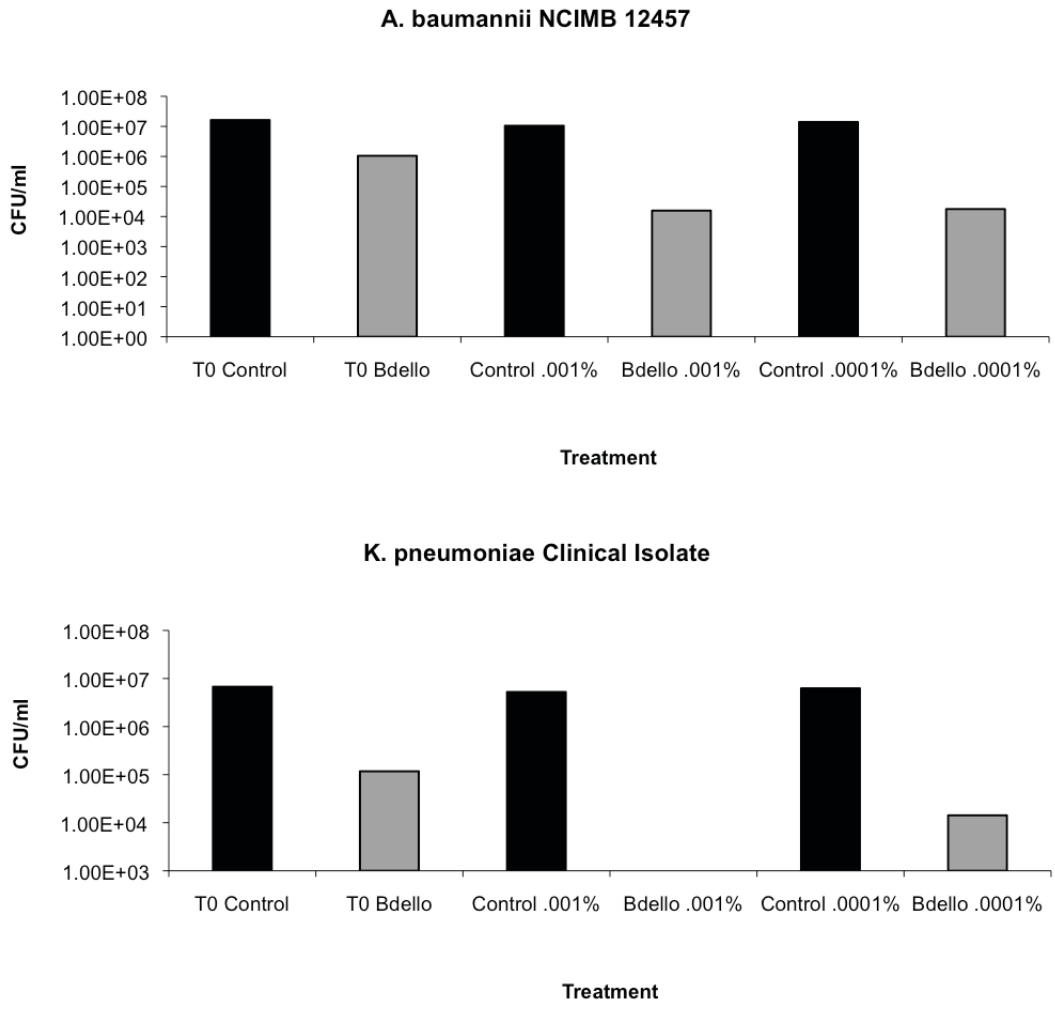
A. Increasing the antimicrobial activity of iodine.

In the following experiments, biofilms were developed in 96 well plates. The biofilms were exposed to *Bdellovibrio* for 6 hrs. Thereafter, different concentrations of iodine (Povidone-Iodine solution, Ricca chemical company, Arlington, TX) were placed on the biofilm for 5 min. As a control, *Bdellovibrio*- free media was used. The amount of viable cells remaining after the treatment was evaluated by CFU enumeration of the remaining biofilm.

Results.

Pre-treating biofilms of *K. pneumoniae* and *A. baumannii* with *Bdellovibrio* resulted in reduction the of biofilm iodine resistance. Exposing the naive, non-*Bdellovibrio* treated biofilm (control) to 0.01-0.001% Iodine did not affect cell viability. However, when the *Bdellovibrio* 'assaulted' biofilm was exposed to similar iodine concentrations a reduction in cell viability was measured (Fig. 23).

Fig. 23. The use of predatory bacteria to reduce biofilm resistance to iodine.



Biofilms were developed in 96-well plates for 18 hrs followed by a 6 hr incubation period with *Bdellovibrio* or predator-free control (control). Thereafter, the control treated (T_0 control) and *Bdellovibrio* exposed biofilm (T_0 Bdello) were incubated for 5 min with 0.01 and 0.001 of iodine. The biofilms were removed and the amount of viable cells was measured.

B. Increasing the bactericidal activity of iodine.

The aim of the experiment was to reduce the iodine bactericidal concentration needed for total biofilm cell killing by first exposing the biofilm to *Bdellovibrio*. To this end, *A. baumannii* biofilms were developed in 96-well plates for 18 hrs, followed by a 6 hr incubation period with *Bdellovibrio* or predator- free control (control). Thereafter, the biofilms were treated for 5 min with varying concentrations of iodine. The biofilm cells were washed, removed by sonication, plated on LB agar plates and incubated for 24 hrs at 37^{o}C. The Minimum Bactericidal Concentration (MBC) was determined as the lowest concentration of iodine needed to kill the cells.

Results.

Treating the biofilm for 6 hrs with *Bdellovibrio* resulted in a reduction in biofilm resistance to iodine. The iodine Minimum Bactericidal Boncentration (MBC) needed to kill the naive biofilm (MBC_{100}) was found to be 0.007%. However, after a relatively short exposure to the predator, the MBC_{100} was reduced to 0.005% (Fig. 24A)

C. Increasing the bactericidal activity of tetracycline.

The aim of the experiment was to reduce antibiotic MBC by first exposing the biofilm to *Bdellovibrio*. *A. baumannii* biofilms were developed in 96-well plates for 18 hrs, followed by a 6 hr incubation period with *Bdellovibrio* or predator- free control (control). Thereafter, the biofilms were inoculated overnight with varying concentrations of tetracycline. The biofilms were washed, removed, plated on LB agar plates and incubated at 37^{o}C. The MBC_{100} was determined as the lowest concentration of tetracycline needed to kill the cells.

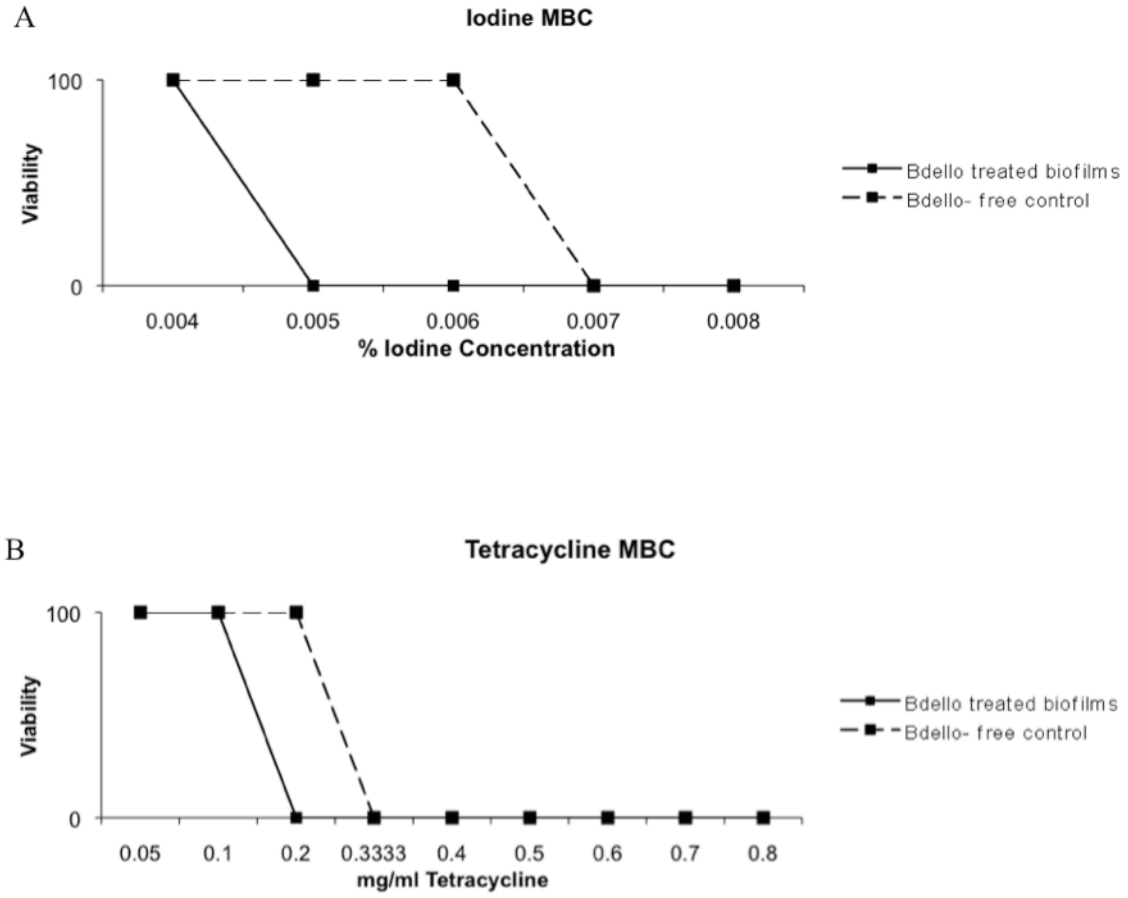
Results.

Treating the biofilm for 6 hrs with *Bdellovibrio* resulted in a reduction in biofilm resistance to tetracycline. The tetracycline MBC needed to kill the non-*Bdellovibrio*

treated biofilm was found to be 0.33 mg/ml. However, after exposing the biofilm to the predator, the MBC_{100} was reduced to 0.2 mg/ml (Fig. 24B).

We have attempted to conduct similar experiments using cetylpyridinium chloride (CPC), chlorhexidine digluconate (CHX) and benzalkonium chloride (BKC) as well as two additional antibiotics kanamycin and nalidixic acid. However, we were unable to obtain consistent results. The main problem was that the MBC measured in the control varied from one experiment to another so the differences seen in the *Bdellovibrio* treated sample was difficult to interpret.

Fig. 24. Increasing the bactericidal activity of iodine and tetracycline.



A. baumannii NCIMB 12457 biofilms were developed in 96-well plates for 18 hrs followed by a 6 hr incubation period with *Bdellovibrio* (Bdello treated biofilm) or predator- free control (Bdello- free control). Thereafter, the control treated and the *Bdellovibrio* exposed biofilm were incubated for 5 min with iodine (A) or overnight with tetracycline (B). The biofilms were removed, plated and the MBC_{100} (Viability 0) was determined.

Key research accomplishments and findings:

Aim I main findings:

- *Bdellovibrio* is able to attack and reduce bacteria from the genus *Acinetobacter, Aeromonas, Aggregatibacter, Bordetella, Burkholderia, Citrobacter, Eikenella, Enterobacter, Escherichia, Klebsiella, Listonella, Morganella, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio,* and *Yersinia.*

- *B. bacteriovorus* is capable of reducing the microbial loads of bacteria most associated with burns and wound infections by 99% to 99.9999%. For example; *A. baumannii* (3-5 log reduction), *E. aerogenes* (2-5 log reduction), *E. coli* (6-8 log reduction), *K. pneumoniae* (2-6 log reduction) and *P. mirabilis* (2-5 log reduction).

- *B. bacteriovorus* is capable of reducing the microbial loads of freshly isolated drug resistant *A. baumannii* samples collected from wound sites.

- The addition of 1 PFU/ml *B. bacteriovorus* is sufficient to initiate predation and lead to host reduction.

- *Micavibrio aeruginosavorus* is able to attack and reduce bacteria from the genus *Burkholderia, Escherichia, Klebsiella, Pseudomonas,* and *Shigella* by 99-99.99%.

- *M. aeruginosavorus* has a moderate ability (10%-99%) to reduce bacteria from the genus *Acinetobacter, Enterobacter Proteus* and *Yersinia.*

- Sequential passing of *M. aeruginosavorus* could alter the predator's host range characteristics, broadening its host range and ability to attack and reduce host.

- The addition of 10-100 PFU/ml *M. aeruginosavorus* is sufficient to initiate predation and lead to host reduction.

- *B. bacteriovorus* ability to reduce multi-species microbial cultures is comparable to its ability to reduce similar host bacteria in a mono-species culture.

- *B. bacteriovorus* could prey on host cells at temperatures of up to 37^{o}C.

- *B. bacteriovorus* is unable to affect host cell populations in microaerophilic and anaerobic conditions.

- Although unable to prey, *B. bacteriovorus* is able to survive and withstand periods of microaerophilic and anaerobic conditions and resume predation after oxygen levels become adequate.

- *M. aeruginosavorus* could prey on host cells at temperatures of 25^{o}C – 37^{o}C.

- *M. aeruginosavorus* is unable to affect host cell populations in microaerophilic and anaerobic growth conditions.

- Depending on the host, *Micavibrio* is able to attack and proliferate on nonviable host cells.

- The presence of carbohydrates in the coculture could block predation by *B. bacteriovorus.*

- The presence of carbohydrates in the coculture could block predation by *M. aeruginosavorus.*

- It was concluded that inhibition of predation was due to media acidification by the metabolic activity of the host and not to a blocking of a putative sugar-binding protein.

- Proteinase-K could inhibit predation by both *Bdellovibrio* and *Micavibrio*.

- Trypsin did not inhibit predation of either *Bdellovibrio* or *Micavibrio.*

- Since not all serine proteases were able to block predation, it is tempting to speculate that predation could occur *in-vivo* even in the presence of some proteases.

Aim II main findings:

- *B. bacteriovorus* is able to prey on human pathogens grown in monolayer-biofilms. Among the bacteria positively reduced were bacteria from the genus *Acinetobacter, Aeromonas, Bordetella, Citrobacter, Enterobacter, Escherichia, Klebsiella, Morganella, Proteus, Pseudomonas,* and *Vibrio.*

- The ability of *B. bacteriovorus* to attack and form lytic halos on host cells grown in monolayer-biofilms mirrors the host range specificity of the predator.

- *M. aeruginosavorus* is able to prey on human pathogens grown in monolayer-biofilms. Among the bacteria positively reduced were bacteria from the genus *Burkholderia, Escherichia, Enterobacter, Klebsiella, Pseudomonas* and *Shigella.*

- The ability of *M. aeruginosavorus* to attack and form lytic halos on host cells grown in monolayer-biofilms mirrors the host range specificity of the predator.

- *B. bacteriovorus* is capable of <u>reducing</u> pre-formed multi-layer microbial biofilm of bacteria most associated with wound infections.

- *B. bacteriovorus* is capable of <u>inhibiting</u> the formation of microbial biofilms.

- The addition of 1 PFU/ml *B. bacteriovorus* is sufficient to initiate predation and lead to biofilm reduction.

- *B. bacteriovorus* could reduce multi-layer biofilms of *A. baumannii*, *A. lwoffii* and *K. pneumoniae* by 3 and 7 logs within 24 and 48 hrs, respectively.

- The addition of 1 PFU/ml *M. aeruginosavorus* is sufficient to initiate predation and lead to *P. aeruginosa* biofilm reduction.

- *B. bacteriovorus* could reduce multi-species multi-layer biofilms associated with wound infections.

- As seen by high resolution SEM imaging, *B. bacteriovorus* is able to remove and clean an established biofilm from the surface, leaving behind what appears to be biofilm debris. The reducing effect was seen in both single and multi-species biofilms.

- In flow cell systems, introducing predatory bacteria could effectively reduce biofilm biomass. The effect was seen on numerous pathogens in both single-species and multi-species biofilms. In general, the biofilm reduction caused by *B. bacteriovorus* was more pronounced than that caused by *M. aeruginosavorus*.

- Attachment of *B. bacteriovorus* and *M. aeruginosavorus* cells to the biofilm occurs within a few minutes of inoculation. The initial attached predator cells are sufficient to initiate an attack and lead to biofilm destruction.

- *B. bacteriovorus* is able to attack and remove metabolically inactive biofilms.

Aim III main findings:

- Applying biofilm EPS (extracellular polymeric substance) degrading enzymes could enhance the biofilm removal aptitude of *B. bacteriovorus*.

- Treating *A. actinomycetemcomitans* biofilms with DNase-I before or after the application of *Bdellovibrio* enhances the biofilm removal capacity of the predator.

- The addition of Proteinase-K could inhibit predation of *Bdellovibrio* (similar findings were found in aim-I).

- Although Proteinase-K disrupts predation, other proteases, such as trypsin, do not seem to inhibit predation (similar findings were found in aim-I).

- Treating *A. actinomycetemcomitans* biofilms with a poly-N-acetylglucosamine hydrolyzing enzyme (DspB) markedly enhances the ability of *Bdellovibrio* to remove the pre-formed biofilm.

- The addition of some enzymes, such as protease, after predation might increase the removal and cleaning of the residual biofilm.

- A short-term exposure to predation could enhance the anti-biofilm activity of antimicrobial drugs.

- Exposing the biofilm for 6 hrs to *Bdellovibrio* resulted in an increase in iodine biofilm antimicrobial activity and a decrease in the amount of iodine and tetracycline needed for full biofilm cell killing.

Reportable outcomes:

Manuscripts.

The data collected throughout this project was incorporated into three manuscripts which were recently published in peer review journals. We would like to point out that although the majority of the work was done using funds from this award, some of the published data was generated by funding from UMDNJ. PDF reprints of the manuscripts are provided in the appendix.

- Dashiff, A. and **Kadouri, E. D.** 2011. Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J. Molecular Oral Microbiology, PMID: 21214870.

- Dashiff, A., Junka, R. A., Libera, M. and **Kadouri, E. D.** 2011. Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus.* Journal of Applied Microbiology, 110:431-444.

- Dashiff, A., Keeling T, G. and **Kadouri, E. D.** 2011. Host Cell Metabolic Activity in the Presence of Carbohydrates Inhibits Predation by *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus*. Applied and Environmental Microbiology, 77:2224-2231

Oral Presentations.

Several of the findings supported by this grant were presented at the following institutes and conferences:

- **Kadouri, D**. Predatory Bacteria- The use of biological agents to control biofilms Department of Biology. The University of Virginia. Charlottesville, VA. December. 2009. Copy of the abstract is provided in the appendix.

- **Kadouri, D**. Biofilm: Everything you wanted to know but were afraid of asking. Undergraduate Summer Research Program. New Jersey Medical School. Newark, NJ. June. 2010. No Abstract submitted.

- **Kadouri, D**. Predatory Prokaryotes- from basic research to application. Department of wound infections. Walter Reed Army Institute of Research. Silver Spring, MD. August. 2010. Copy of the abstract is provided in the appendix.

- **Kadouri, D.** Predatory Prokaryotes. Department of Biology. Bard College. Annandale-on-Hudson, NY. October 2010. No Abstract submitted.

- **Kadouri, D**. Biofilm: The potential use of predatory bacteria to control human pathogens. Department of Oral Biology seminar series. New Jersey Medical School. Newark, NJ. December. 2010. No Abstract submitted.

- **Kadouri, D.** The Use of Predatory Prokaryotes to Control Drug Resistant Bacteria and Microbial Biofilms Associated with Burn and Wound Infections. US Army Medical Research and Material Command Wound Symposium. San Antonio, TX, May 2011. Copy of the abstract is provided in the appendix.

- **Kadouri, D**. and A. Dashiff. Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. 4th Congress of European Microbiologists. Geneva, Switzerland, June, 2011. Copy of the abstract is provided in the appendix.

* We would like to note that although we presented data generated during this project, funding to attend these meetings were not obtained through this proposal.

General non-peer reviewed publications.

In addition to the peer review publications, our work was also highlighted and picked up by several popular science magazines, news agencies and web sites.

- BBC Focus magazine.
- UMDNJ Press Release.
- About .com
- Newswise
- Bloomberg Businessweek.
- ICT-Infection Control Today
- CDMRP website.

As required, the grant manager and Gail Whitehead, CDMRP Public Affairs person, were informed before any interviews took place. A copy of the articles are provided in the appendix.

Student research opportunities.

This project had given a number of students the opportunity to conduct research and gather hands-on scientific experience.

The following students participated in this study:

- Bradford Craigen- M. S. student (GSBS-UMDNJ).
- Lydia Yoo - Rotation M. S. student (GSBS-UMDNJ).
- Anukriti Gupta - Rotation Ph. D. candidate (GSBS-UMDNJ).
- Joseph Sedlock- Undergraduate student (Rutgers- Newark).
- Aliza Dashiff- Research assistant (UMDNJ).
- Tai Ha- Rotation M. S. student (GSBS-UMDNJ).
- Thomas Gregory Keeling- Rotation M. S. student (GSBS-UMDNJ).
- Radoslaw Junka (GSBS Undergraduate Summer Research Program, Stevens Institute of Technology, NJ).
- Dipti Godboley- M. S. student (GSBS-UMDNJ).
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- Chris Russo- Rotation M. S. student (GSBS-UMDNJ).

Conclusion:

The purpose of this research was to examine the potential use of predatory bacteria in controlling and eradicating microbial pathogens associated with burn and wound infections. Two Gram-negative predatory bacteria were used in this study, the endoparasite *Bdellovibrio bacteriovorus*, and the exoparasite *Micavibrio aeruginosavorus.* In the first part of the study (Aim-I), we examined the host range specificity of *Bdellovibrio* and *Micavibrio.* Our findings demonstrate that *Bdellovibrio* is capable of attacking the majority of pathogens tested, including bacteria from the genus *Acinetobacter, Aeromonas, Aggregatibacter, Bordetella, Burkholderia, Citrobacter, Eikenella, Enterobacter, Escherichia, Klebsiella, Listonella, Morganella, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio,* and *Yersinia.* We have also demonstrated that *B. bacteriovorus* is capable of attacking and reducing the microbial loads of freshly isolated drug resistant *A. baumannii* collected from Wounded Warriors. Thus, the predation capacity of *Bdellovibrio* is well suited to deal with infections found in military settings. In dilution experiments, the introduction of extremely low numbers (1 PFU/ml) of *Bdellovibrio* was sufficient to initiate predation and lead to host reduction. This is probably due to the ability of the predator to rapidly proliferate within its host and reach high cell numbers without the need of re-introducing additional *Bdellovibrio* to the system.

When examining the host range specificity of *M. aeruginosavorus,* we found that as previously demonstrated [3], *Micavibrio* is able to attack bacteria from the genus *Burkholderia, Klebsiella,* and *Pseudomonas.* However, active and significant predation on *Escherichia* and *Shigella* was also achieved. One possibility for the increase in *Micavibrio* host range might be the routine and sequential re-culturing of the bacteria in the lab.

The capacity of *Bdellovibrio* to attack pathogenic bacteria was also demonstrated in mixed species cultures, highlighting its possible use as a bio-control agent in real-life wound infections, which frequently involve more than one microbial pathogen.

As the main goal of this proposal was to investigate the potential use of predatory bacteria as a bio-control agent in “real-life” settings, we conducted additional

experiments aimed at examining the influence of different growth conditions on predation. Our results demonstrate that both *Bdellovibrio* and *Micavibrio* are capable of preying at elevated temperatures of 37^{o}C, however, microaerophilic and anaerobic conditions impaired the ability of *Bdellovibrio* and *Micavibrio* to prey. Predation by *Bdellovibrio* did resume when the lysates were placed at room oxygen levels, demonstrating that *Bdellovibrio* could survive periods of limited oxygen concentrations. Therefore, the incapacity of the predators to prey in microaerophilic and anaerobic conditions should be considered when applying the predators against bacteria located deep within wounds, where the oxygen concentrations might be limited.

Depending on the host, *Micavibrio* was able to attack and proliferate on nonviable host cells. At this point we could only speculate as to why *Micavibrio* is only able to attack certain metabolically inactive cells. One explanation is that the nutrient requirements needed for *Micavibrio* growth is degraded in some non-viable host cells, which in turn eliminates *Micavibrio* proliferation.

Our study also established that the presence of carbohydrates in the coculture could block predation. However, this effect was found to be the result of media acidification by the metabolic activity of the host and not due to a blocking of a putative sugar-binding protein. Our findings also demonstrated that the presence of proteinase-K could inhibit predation by both *Bdellovibrio* and *Micavibrio*. This effect was not seen when trypsin was used. It was previously demonstrated that *Bdellovibrio* cell surface structures, such as pilli, is required for predation. It could be speculated that proteinase-K might cleave the cell surface structures needed for predation. Since not all serine proteases were able to block predation, it is tempting to speculate that predation could occur *in-vivo* even in the presence of some proteases.

As the majority of wound infections are caused by bacteria which attach to tissue and surfaces and form tightly packed microbial communities known as biofilms, we were interested in measuring the effect of the predators on microbial biofilm communities (Aim-II). To this end, three biofilm modules were examined; a thin biofilm lawn which produces a monolayer-biofilm that is fed from beneath, a multilayer biofilm grown in a 96 well static system, and a flow cell system, which allows the development of extremely robust biofilms under media, flow conditions. Our study shows that the ability of the

predators to attack thin biofilm lawns and form a zone of clearance on host cells mirrored their host specificity and ability to prey on bacteria grown in liquid suspension.

Seeing that thin biofilms did not inhibit the ability of the predators to attack, a second biofilm module system was used. In this system, multilayer biofilms were formed in 96 well plates. After establishing the biofilm, *Bdellovibrio* was added to the system and the degree of cell-biofilm killing was measured. CV staining, high-resolution SEM imaging and CFU enumeration had all confirmed that *B. bacteriovorus* could significantly reduce multi-layer biofilms of *A. baumannii*, *A. lwoffii* and *K. pneumoniae* by 7 logs within 48 hrs. Thus, the biofilm structure, which usually enhances the antibiotic resistance of microbial pathogens and their ability to evade host immune response, does not pose a significant barrier to predation by *Bdellovibrio*. In addition to the ability of the predator to reduce pre-formed biofilms, culturing the bacteria with the predator resulted in the reduction in biofilm formation. As was seen for host cells grown in culture suspension, the addition of 1 PFU/ml of predator was sufficient to initiate predation and eventually lead to biofilm reduction. Moreover, SEM imaging confirmed that the attachment of *B. bacteriovorus* and *M. aeruginosavorus* cells to the biofilm occurs within a few minutes of inoculation.

The ability of the predator to reduce both single-species and multi-species biofilms was also evaluated in flow cell systems. In these systems biofilms are developed under media flowing condition. Injecting the biofilms with a single dose of predators was found to be effective in reducing the biofilm biomass.

It was previously suggested that cells within biofilms might exhibit limited growth. When placed on a five-day-old metabolically inactive *A. actinomycetemcomitans* biofilm, *B. bacteriovorus* was able to measurably reduce the biofilm. The ability to attack and remove metabolically inactive biofilms could be of great significance when considering that the limited cell growth rate, usually exhibited within biofilm communities, plays a vital role in enhancing biofilm antibiotic resistance [5, 11, 12]. Thus, a biofilm-control agent, such as *Bdellovibrio*, which is not influenced by the metabolic activity of its target cell, could be of great value.

Previous studies showed that the EPS of *A. actinomycetemcomitans* biofilms contains extracellular DNA, protein, and poly-N-acetylglucosamine (PGA) and that degradation of the EPS compounds could lead to biofilm dispersal [1]. In an attempt to enhance biofilm removal by *Bdellovibrio* (Aim-III), DNA, protein, and PGA degrading enzymes were incorporated in the biofilm predation experiments. The use of DNase-I following the predation period caused an increase in biofilm removal. However, the addition of proteinase-K during predation prevented the removal of the biofilm by *Bdellovibrio*. These results suggest that proteinase-K might affect specific surface proteins on the host cell or the *Bdellovibrio* that are required for predation. As before, although proteinase-K was able to inhibit predation, trypsin, a serine protease that exhibits different cleaving properties than proteinase-K, did not reduce predation. Treating the biofilm with DspB, a known PGA-hydrolyzing enzyme, prior or simultaneously with *Bdellovibrio*, resulted in an increase in biofilm removal. The DspB-*Bdellovibrio* treatment also significantly shortened the time required for *A. actinomycetemcomitans* biofilm removal.

The addition of enzymes, such as protease, after predation, was also found to increase the removal and cleaning of the residual biofilm. These results demonstrate that applying EPS degrading enzymes with or following the application of *Bdellovibrio* could enhance the biofilm removal aptitude of the predator. Thus, our data emphasize that treating the biofilms with *Bdellovibrio* followed by a brief enzyme exposure might be an efficient method to remove the residual biofilm and biofilm debris left after predation.

Finally, we have established that a relatively short exposure to the predator might be used to enhance the efficacy of antimicrobials and disinfectants. We believe that exposing the biofilm to the predator for a few hours could breach the biofilm defensive EPS structure and allow better antimicrobial penetration. This increase penetration could reduce biofilm resistance and increase drug efficacy.

In conclusion, our data demonstrate the potential use of predatory bacteria in controlling human pathogens associated with wound infections. Their broad host range, ability to significant reduce microbial loads of multidrug resistant pathogens, and capacity to penetrate and remove surface attached biofilms, regardless of the metabolic state of the

biofilms, make these organisms ideal candidates to be used as biological control agents. Based on the results presented here, one could consider a few potential applications which involve the use of predatory bacteria. For example; as a preventative measure, applied immediately after an injury occurs or during medical treatment to prevent the establishment of resistant biofilms; as a biocontol method used to eradicate an already existing infection or biofilm; as a means to reduce biofilm accumulation on medical devices such as catheters and implants; and as a way to treat hospital acquired drug resistant bacteria. Other potential military relevant applications in which predatory bacteria might be used is in the treatment and decontamination of surfaces after exposure to a biological weapon, including decontamination of military hardware, military personnel, water reservoirs, and large contaminated land areas. The use of predatory bacteria could also be applied in homeland security for decontamination of large areas after an exposure to Gram-negative bacteria that might be used in bio-terrorism.

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Appendix

Peer review manuscripts

molecular oral microbiology

molecular oral microbiology

Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J

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SUMMARY

Periodontal diseases are multifactorial infections elicited by a complex of primarily gram-negative bacteria that interact with host tissues and lead to the destruction of the periodontal structures. *Bdellovibrio bacteriovorus* is a gram-negative bacterium that preys upon other gram-negative bacteria. It was previously shown that *B. bacteriovorus* has an ability to attack and remove surface-attached bacteria or biofilms. In this study, we examined the host specificity of *B. bacteriovorus* strain 109J and its ability to prey on oral pathogens associated with periodontitis, including; *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Tannerella forsythia.* We further demonstrated that *B. bacteriovorus* 109J has an ability to remove biofilms of *Ei. corrodens* as well as biofilms composed of *A. actinomycetemcomitans*. *Bdellovibrio bacteriovorus* was able to remove *A. actinomycetemcomitans* biofilms developed on hydroxyapatite surfaces and in the presence of saliva, as well as to detach metabolically inactive biofilms. Experiments aimed at enhancing the biofilm removal aptitude of *B. bacteriovorus* with the aid of extracellular-polymeric-substance-degrading enzymes demonstrated that proteinase-K inhibits predation. However, treating *A. actinomycetemcomitans* biofilms with DspB, a poly-*N*-acetylglucosamine (PGA) -hydrolysing enzyme, increased biofilm removal. Increased biofilm removal was also recorded when *A. actinomycetemcomitans* PGA-defective mutants were used as host cells, suggesting that PGA degradation could enhance the removal of *A. actinomycetemcomitans* biofilm by *B. bacteriovorus*.

INTRODUCTION

Periodontal diseases are multifactorial infections elicited by a complex of bacterial species that interact with host tissues and lead to the destruction of the periodontal structures, including the tooth-supporting tissues, alveolar bone and periodontal ligament. Periodontal disease is a significant global public-health concern and is probably the most common chronic infectious disease in humans. In the United States alone, more than 70% of the population is afflicted with the disease with approximately 54% of all US adults aged 30 years or more suffering from some form of periodontal disease (Albandar & Kingman, 1999). The importance of bacteria in dental plaque and the key role of plaque in the causation of periodontal disease is well established. Whereas oral bacteria colonize and produce disease primarily in the oral cavity, they can also produce systemic disease (Scannapieco, 1998; Garcia *et al.*, 2001). Therefore, the mitigation of oral infection is of broad clinical importance beyond the boundaries of the oral cavity. Among the bacteria frequently isolated from periodontal pockets are the

gram-negative bacteria *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Tannerella forsythia*, all of which are strongly associated with various forms of periodontitis including localized aggressive periodontitis, generalized early onset periodontitis and chronic periodontitis (Dzink *et al.*, 1985; Wilson *et al.*, 1991; Bolstad *et al.*, 1996; Tanner & Izard, 2006; Fine *et al.*, 2007). In addition to their role in periodontal disease, the presence of these bacteria was also implicated as a causative agent of several non-oral infections (Kaplan *et al.*, 1989; Beck *et al.*, 1996; Offenbacher *et al.*, 1996; Dibart *et al.*, 1998; Takamatsu *et al.*, 1999; Roberts, 2000; Chang *et al.*, 2004; Hombach *et al.*, 2007; Miller *et al.*, 2007; Kajiya *et al.*, 2008).

The difficulty in removing oral plaque or biofilms by conventional therapies led researchers to examine other alternative methods for biofilm control, such as biological control agents. One biological agent that might be used to control pathogenic bacteria is the predatory prokaryotes from the genus *Bdellovibrio*. *Bdellovibrio* are gram-negative bacteria that feed on other gram-negative bacteria (Stolp & Starr, 1963; Sockett, 2009). Recently, it was demonstrated that *B. bacteriovorus* 109J can significantly reduce biofilms developed in a microtiter dish-based static assay as well as in a flow cell system (Kadouri & O'Toole, 2005; Nunez *et al.*, 2005; Medina *et al.*, 2008). *Bdellovibrio bacteriovorus* strain HD100 was also shown to be able to attack and kill four smooth strains and one rough biofilm-forming strain of *A. actinomycetemcomitans* (Van Essche *et al.*, 2009).

In this study, the susceptibility of oral pathogens to predation by *B. bacteriovorus* 109J was examined in liquid suspension and on biofilms. The predatory ability of *B. bacteriovorus* was also assessed under different clinically relevant growth conditions. Finally, an attempt was made to enhance the biofilm removal aptitude of *B. bacteriovorus* with the use of other biofilm-degrading enzymes.

METHODS

Bacteria strains, media and growth conditions

The strains used in this study are listed in Table 1. *Aggregatibacter actinomycetemcomitans* was routinely grown on brain–heart infusion (BHI) and *Ei. corrodens* was grown on tryptic soy broth (TSB) blood agar plates (5% defibrinated sheep blood and 1.5% agar) or in TSB containing 2 mg ml^{-1} KNO_3 and 5 μg ml^{-1} hemin. The *F. nucleatum* was cultured on TSB blood agar plates; *Pr. intermedia*, *Po. gingivalis* and *T. forsythia* were cultured on TSB blood agar plates supplemented with 5 μg ml^{-1} hemin and 1 μg ml^{-1} menadione. *N*-Acetylmuramic acid (0.001%) was added to the *T. forsythia* plates. Both *A. actinomycetemcomitans* and *Ei. corrodens* were cultured at 37°C in 10% CO_2, whereas *F. nucleatum*, *Pr. intermedia*, *Po. gingivalis* and *T. forsythia* were cultured anaerobically (10% CO_2, 10% H_2 and 80% N_2) in a MACS MG 250 anaerobic chamber (Microbiology International, Frederic, MD) at 37°C. The *B. bacteriovorus* was maintained as plaques in double-layered diluted nutrient broth [DNB; 0.8 g l^{-1} 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) (Starr, 1975). To initiate a lysate, *B. bacteriovorus* co-cultures were obtained by adding a plug of agar containing *B. bacteriovorus* plaque to 1×10^8 colony-forming units (CFU) ml^{-1} washed *Escherichia coli* prey in DNB, and incubated at 30°C on a rotary shaker set at 200 r.p.m. until the co-culture became clear (stock lysate). To harvest *B. bacteriovorus*, co-cultures were prepared in which 2 ml overnight-grown washed *E. coli* host cells (1×10^9 CFU ml^{-1}) were incubated with 2 ml stock lysate in 20 ml DNB. The co-cultures were incubated for 18 h to reach a final concentration of approximately 1×10^8 plaque-forming units ml^{-1} of predator. At this point the lysate was passed three times through a 0.45-μm Millex pore-size filter (Millipore, Billerica, MA) to remove residual prey and cell debris (filtered lysate). As a control, filtered sterilized lysate was prepared by sequentially passing the *B. bacteriovorus* culture through three 0.22-μm pore-size filters. After filtration, no predator, as judged by plaque-forming units, could be detected (Kadouri & O'Toole, 2005).

Biofilm assays

Biofilms of *A. actinomycetemcomitans* were developed as described previously (Izano *et al.*, 2007, 2008) with some modifications. *Aggregatibacter actinomycetemcomitans* rough-colony strains were grown on plates for 48 h. The colonies were scraped into fresh BHI medium and homogenized to reach a final concentration of 1×10^7 CFU ml^{-1} absorbance

Table 1 Bacteria used in the study

Name	Serotype	Source
Aggregatibacter actinomycetemcomitans		
Rough-colony strains		
NJ5000	a	UMDNJ (Kaplan *et al.*, 2002)
DL 1159	a	UMDNJ
NJ3500	b	UMDNJ (Kaplan *et al.*, 2002)
DL865	b	UMDNJ
DL 1067-1	b	UMDNJ
DL 1171	b	UMDNJ
DL 639-2	b	UMDNJ
DL 772	b	UMDNJ
DF2300	c	UMDNJ (Kaplan *et al.*, 2002)
DL 1093-2	c	UMDNJ
DL 1108-2	c	UMDNJ
DL 1148	c	UMDNJ
IDH781	d	UMDNJ (Kaplan *et al.*, 2002)
NJ9500	e	UMDNJ (Kaplan *et al.*, 2002)
CU1000	f	UMDNJ (Kaplan *et al.*, 2002)
HW1018 (CU1000 *pgaC*::IS903ϕ*kan*)	f	UMDNJ (Izano *et al.*, 2007)
Smooth-colony strains		
ATCC 29523	a	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
SUNYab75	a	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
ATCC 29524	b	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
JP2	b	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
NK1651	b	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
Y4	b	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
Aa307	c	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
IDH781S	d	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
IDH1705	e	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
CU1060	f	UMDNJ (Rupani *et al.*, 2008)
Eikenella corrodens		
ATCC 23834	–	USC (Henriksen, 1969)
H2S-1	–	USC
D4P-1	–	USC
D11P-1	–	USC
RMA 12256	–	RMARL
RMA 12259	–	RMARL
RMA 12794	–	RMARL
RMA 15501	–	RMARL
Ec-f2	–	Forsyth Institute
Fusobacterium nucleatum		
PK1594	–	UMDNJ (Rupani *et al.*, 2008)
ATCC 10953	–	UMDNJ (Rupani *et al.*, 2008)
Prevotella intermedia		
ATCC 25611	–	ATCC (Shah & Collins, 1990)
Porphyromonas gingivalis		
ATCC 33277	–	ATCC (Tran & Rudney, 1996)
W83/ATCC BAA-308	–	Forsyth Institute
Tannerella forsythia		
ATCC 43037	–	Forsyth Institute (Sakamoto et al., 2002; Tanner & Izard, 2006)
Bdellovibrio bacteriovorus		
109J/ATCC 43826	–	ATCC (Kadouri & O'Toole, 2005)

UMDNJ, Department of Oral Biology strain collection, University of Medicine and Dentistry of New Jersey; RMARL, R.M. Alden Research Laboratory, Santa Monica CA; USC, Division of Periodontology, University of Southern California School of Dentistry; Forsyth Institute, Department of Molecular Genetics, The Forsyth Institute; and ATCC, American Type Culture Collection.

at 595 nm (A_{595}) = 0.07. Cells (100 μl) were transferred to the wells of a 96-well, flat-bottom, tissue culture-treated, polystyrene microtiter plate and incubated for 24 h at 37°C in 10% CO_2. Non-adherent cells were removed by washing, and adherent cells were stained with 0.1% crystal violet (CV) as described previously (Merritt *et al.*, 2005). Photographs of the plate were taken using a Canon-scan 4400F digital scanner. The CV was solubilized using 50% acetic acid for 10 min. Relative biofilm formation was assayed by measuring the absorbance of the CV solution at 600 nm (A_{600}). Biofilms of *Ei. corrodens* were developed for 36 h in six-well, tissue culture-treated, polystyrene plates, which contained harvested *Ei. corrodens* cells resuspended in fresh TSB broth containing KNO_3 and hemin.

For biofilm formation on hydroxyapatite, 3 × 3 mm hydroxyapatite squares, prepared from sintered food-grade hydroxyapatite (NEI Industries, Sesser, IL; Sreenivasan *et al.*, 2009), were inserted into a 12-well polystyrene plate. The wells were filled with 0.5 ml BHI medium containing *A. actinomycetemcomitans* cells and incubated for 24 h to allow biofilm development.

Prey range assay

To evaluate the ability of *B. bacteriovorus* to prey on the selected oral bacteria, co-cultures were prepared in which washed host cells were incubated in 20 ml DNB with 2 ml harvested *B. bacteriovorus*. As a control, filtered sterilized lysate was used. The cultures were incubated at 30°C on a rotary shaker set at 200 r.p.m. The ability of *B. bacteriovorus* to prey was confirmed by the reduction in culture turbidity caused by the lysis of host cells during predation. Culture turbidity was examined by removing 100-μl aliquots and reading the absorbance in a BioRad 680 microplate reader (A_{595}). Additional confirmation of active predation was provided by microscopy evaluation (×1000 magnification).

Biofilm removal assays

To assess the ability of *B. bacteriovorus* 109J to remove *A. actinomycetemcomitans* and *Ei. corrodens* biofilms, the biofilms were grown as described above, washed twice with DNB to remove planktonic cells, and 100 μl filtered *B. bacteriovorus* from an 18-h lysate was added. As a control, 100 μl filtered sterilized lysate was used. The dishes were incubated at 30°C for the duration of the experiments.

Removal of *A. actinomycetemcomitans* biofilms in the presence of saliva

To investigate whether biofilm removal could occur in the presence of saliva, preformed *A. actinomycetemcomitans* biofilms were developed, and washed, and fresh DNB media containing varying amounts of filter-sterilized unstimulated saliva (from 0 to 100%), and *B. bacteriovorus*, were added. Unstimulated saliva was collected, on ice, in a 50-ml tube, centrifuged for 2 min at 5000 ***g*** to remove cell debris, and filter sterilized using 0.22-μm pore-size filter.

Predation by *B. bacteriovorus* under oxygen-limiting conditions

To measure the ability of *B. bacteriovorus* to prey in anaerobic and microaerophilic conditions, *B. bacteriovorus* host co-cultures were prepared and placed in a BD GasPak Jar Systems with a disposable gas-generating anaerobic or microaerophilic envelope (BD Diagnostic Systems, Franklin Lakes, NJ). The jars were incubated at 30°C on a rotary shaker at 200 r.p.m. Predation was measured using CFU of the surviving host cells.

Obtaining metabolically inactive biofilms

To obtain metabolically inactive biofilms of *A. actinomycetemcomitans*, the *A. actinomycetemcomitans* CU1000 biofilms were developed as described above, washed and incubated for 96 h in DNB. To confirm loss of biofilm viability, the biofilm cells were removed with the aid of a tissue culture scraper, and plated on BHI plates for CFU enumeration. Additional verification of cell metabolic activity loss was measured by adding Alamar-Blue cell viability reagent (Invitrogen, Carlsbad, CA) to the biofilm and measuring the change in fluorescence (Pettit *et al.*, 2005). AlamarBlue® works as a cell viability and proliferation indicator through the conversion of resazurin to resorufin. Resazurin, a non-fluorescent indicator dye, is converted to highly red fluorescent resorufin via reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells (product literature; Trek Diagnostic Systems,

Cleveland, OH). No fluorescence signal or colony growth was measured in the 4-day-old, DNB-suspended biofilm, confirming loss of biofilm cell viability.

Scanning electron microscopy

Experiments were performed as described previously (Kadouri & O'Toole, 2005). In brief, biofilms were developed on a 12 × 12-mm PVC plastic cover slip (Fisher Scientific, Pittsburgh, PA). The cover slips were placed in a 24-well polystyrene cell culture plate (Corning Inc., Corning, NY). Preformed biofilms and predation assay were prepared as described above. The experiments were carried out in a 1.0 ml volume. Biofilms were rinsed to remove any planktonic cells before being fixed in 2% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1% ruthenium red. Images were viewed at the air–liquid interface using a Zeiss Auriga field emission scanning electron microscope.

RESULTS

Host range specificity

To examine the ability of *B. bacteriovorus* 109J to prey on bacteria commonly associated with periodontal disease, bacteria were cultured, then incubated in the presence of *B. bacteriovorus.* As seen in Fig. 1A, *B. bacteriovorus* was able to prey on all *A. actinomycetemcomitans* serotypes tested. Microscopy evaluation of the co-cultures had further confirmed predation, showing a reduction in *A. actinomycetemcomitans* cells (fewer than 10% of total cells in a field of view) and an increase in *B. bacteriovorus* cells (90% of the cell population in each field of view), following a 48-h incubation period. In this assay, only *A. actinomycetemcomitans* strains that exhibited smooth-colony morphology were used. These strains were selected for their inability to attach to the surface of the tube and form biofilms or aggregates (Fine *et al.*, 1999; Rupani *et al.*, 2008), thus reducing the likelihood of culture turbidity decrease as a result of biofilm formation or cell aggregation. In addition to the ability of *B. bacteriovorus* to prey on *A. actinomycetemcomitans*, predation was also observed when *Ei. corrodens* was used as host (Fig. 1B). The *B. bacteriovorus* was unable to prey on *Po. gingivalis*, *Pr. intermedia*, *T. forsythia* and *F. nucleatum* ATCC 10953 (data not shown); however, a positive reduction in turbidity was measured when *F. nucleatum* PK1594 was used as host. A reduction in culture turbidity, from A_{595} = 0.26 to A_{595} = 0.091 was

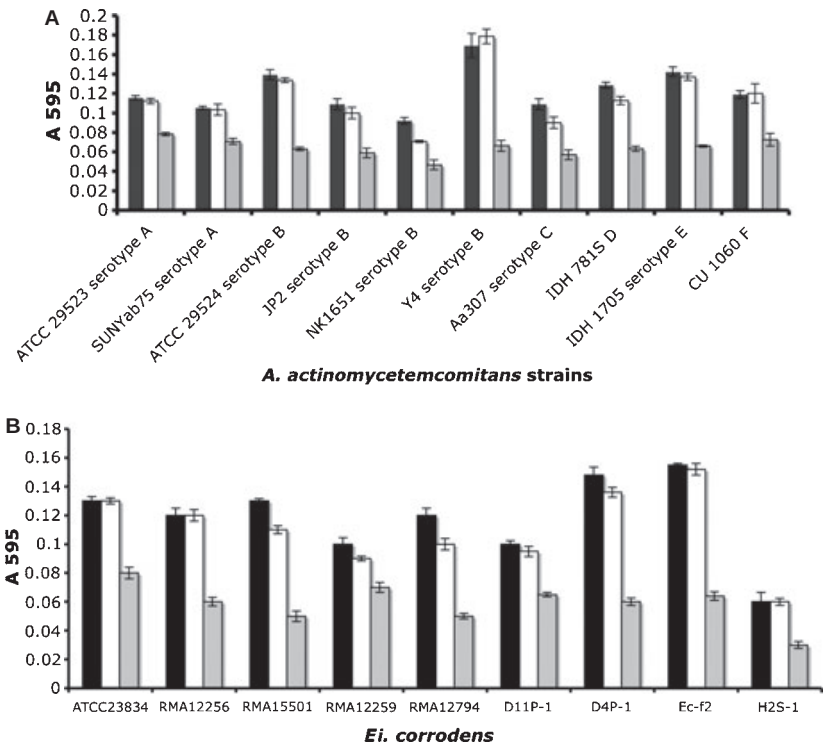

Figure 1 Host range predation assay. Overnight cultures of *Aggregatibacter actinomycetemcomitans* (A) and *Eikenella corrodens* (B) were incubated with harvested *Bdellovibrio bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). Cocultures were incubated for 48 h. Predation was confirmed by the reduction in culture turbidity measured at 595 nm (A_{595}). Black bars represent culture turbidity at time-0. Each value represents the mean of 3 cocultures. Error bars are shown as one-standard deviation.

measured following 48 h incubation with the predator, with limited turbidity reduction measured in the *B. bacteriovorus* – free control (from A_{595} = 0.26 to A_{595} = 0.24). Active predation on the *F. nucleatum* PK1594 was also verified by light microscopy.

A. actinomycetemcomitans biofilm removal assay

To measure the effect of *B. bacteriovorus* 109J on *A. actinomycetemcomitans* biofilms, we developed conditions that yield stable *A. actinomycetemcomitans* biofilms in a 96-well dish. Biofilms were formed in BHI medium for 24 h. Thereafter, the medium was removed and replaced with DNB as described in the Methods. Using this method, stable biofilms were developed from 15 *A. actinomycetemcomitans* isolates, all exhibiting rough-colony morphology phenotype, which is typical of fresh clinical isolates (Fine *et al.*, 1999). The preformed biofilms were incubated with *B. bacteriovorus* or a control filtered sterilized lysate. By 48 h, a measurable reduction (>81%) in biofilm biomass was recorded for all of the strains tested (Fig. 2A,B). In another experiment, *A. actinomycetemcomitans* CU1000 biofilms were developed on hydroxyapatite squares (Fig. 2C, preformed biofilm). When incubated in the presence of *B. bacteriovorus*, a substantial reduction in biofilm CV staining was seen by 72 h, with no reduction in the control inoculated sample (Fig. 2C, *B. bacteriovorus* and control respectively).

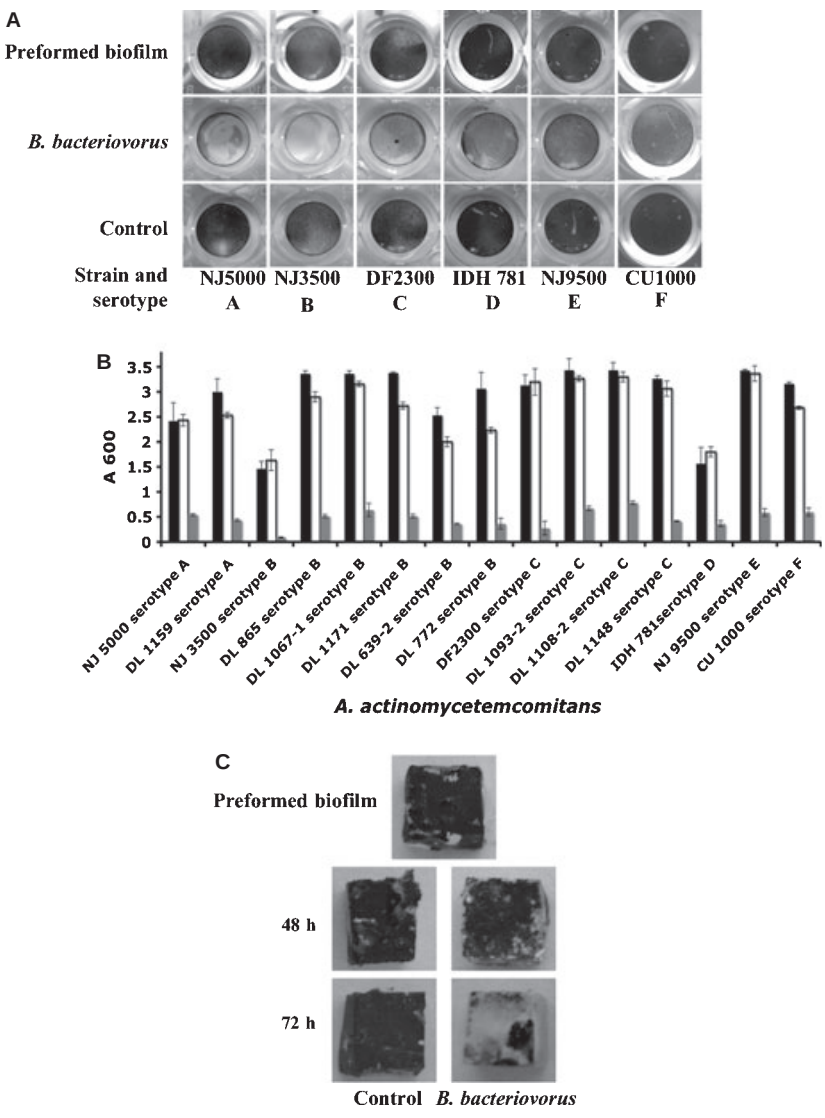

Figure 2 Removal of *Aggregatibacter actinomycetemcomitans* biofilms by *Bdellovibrio bacteriovorus*. (A) Biofilms of *A. actinomycetemcomitans* were developed in 96-well plates for 24 h (Preformed biofilm) following a 48-h incubation period with *B. bacteriovorus* 109J (*B. bacteriovorus*) or filtered sterilized lysate (Control). Biofilms were rinsed and stained with CV. (B) Quantification of biofilm reduction. Preformed overnight biofilms (black bars) were incubated for 48 h with *B. bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). The wells were rinsed, stained with CV, and the amount of CV staining was quantified at 600 nm (A_{600}). Each value represents the mean of 12 wells. Error bars are shown as one-standard deviation. (C) Biofilms of *A. actinomycetemcomitans* CU1000 were developed on hydroxyapatite squares then treated with the predator (*B. bacteriovorus*) or filtered sterilized lysate (Control).

Ei. corrodens biofilm removal assay

The ability of *B. bacteriovorus* to remove biofilms composed of *Ei. corrodens* was also assessed (Fig. 3 A–C). *Eikenella corrodens* preformed biofilms were developed in six-well plates, washed and inoculated with the predator. By 48 h, a measurable reduction in biofilm CV staining was obtained on all of the examined strains. However, the degree of reduction varied from 81 to 45%, as several of the tested isolates did not form stable biofilms resulting in some degree of biofilm detachment within the control sample. Removal of *Ei. corrodens* biofilms by *B. bacteriovorus* was further visualized and confirmed by SEM imaging, showing a reduction in surface cell coverage in the *B. bacteriovorus* treated sample.

In the following experiments, *A. actinomycetemcomitans* CU1000 was used. This isolate was selected for its ability to form stable biofilms, which showed little detachment with time. In one experiment, a reduction of 1.25% in biofilm biomass staining was measured during a 100-day incubation period in DNB (from $A_{600} = 0.83$ at day one to $A_{600} = 0.79$ at day 100), demonstrating the stability of the biofilms.

Removal of *A. actinomycetemcomitans* biofilms in the presence of saliva

To investigate whether biofilm removal could occur in the presence of saliva, preformed *A. actinomycetemcomitans* biofilms were incubated with *B. bacteriovorus* and varying amounts of filter-sterilized saliva. As seen in Fig. 4A, a substantial reduction (77%) in biofilm was measured in the saliva-free *B. bacteriovorus*-treated sample. Incubating *B. bacteriovorus* in 100% saliva did not hinder the ability of the predator to remove the biofilm. A reduction of 43% was registered in the saliva plus *B. bacteriovorus*-treated samples when compared with the control biofilm incubated with saliva alone (Fig. 4A, 100% saliva).

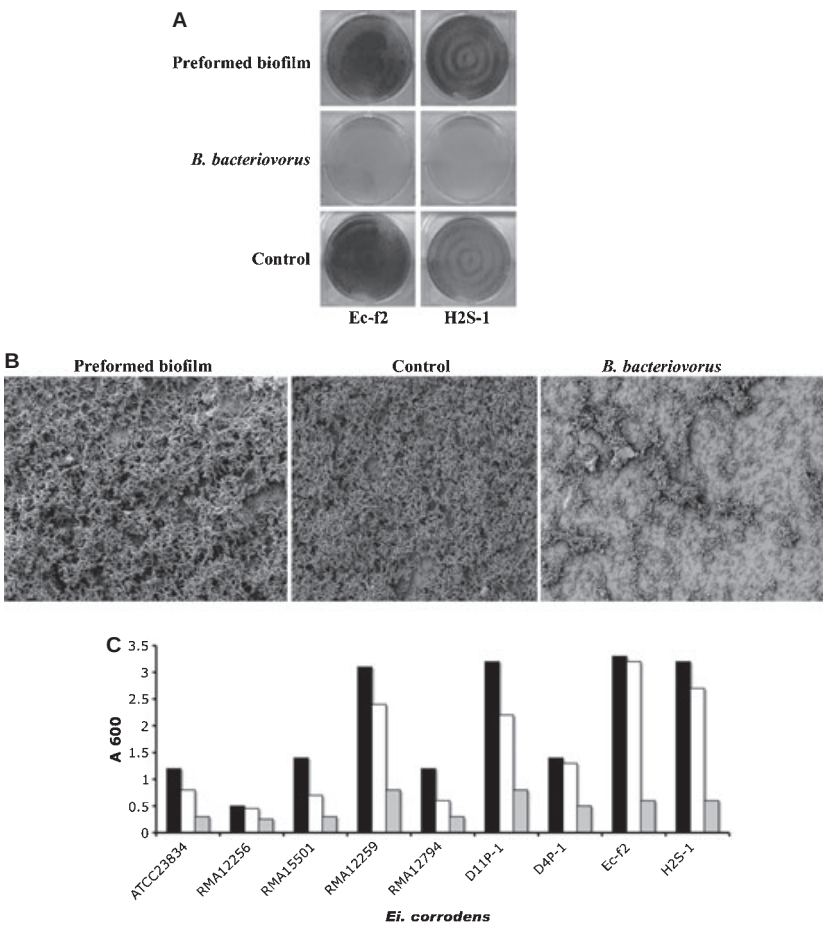

Figure 3 Predation of *Eikenella corrodens* biofilms by *Bdellovibrio bacteriovorus*. (A) Biofilms of *Ei. corrodens* were developed in six-well plates for 36 h (Preformed biofilm) followed by a 48-h incubation period with *B. bacteriovorus* 109J (*B. bacteriovorus*) or filtered sterilized lysate (Control). Biofilms were rinsed and stained with CV. For SEM imaging, *Ei. corrodens* H2S-1 biofilms were developed on PVC plastic cover slips. Scanning electron micrographs were viewed at 1000× magnification (B). (C) Quantification of biofilm reduction. Preformed overnight biofilms (black bars) were incubated for 48 h with *B. bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). The wells were rinsed, stained with CV, and the amount of CV staining was quantified. Each value represents the mean of two wells from one representative experiment.

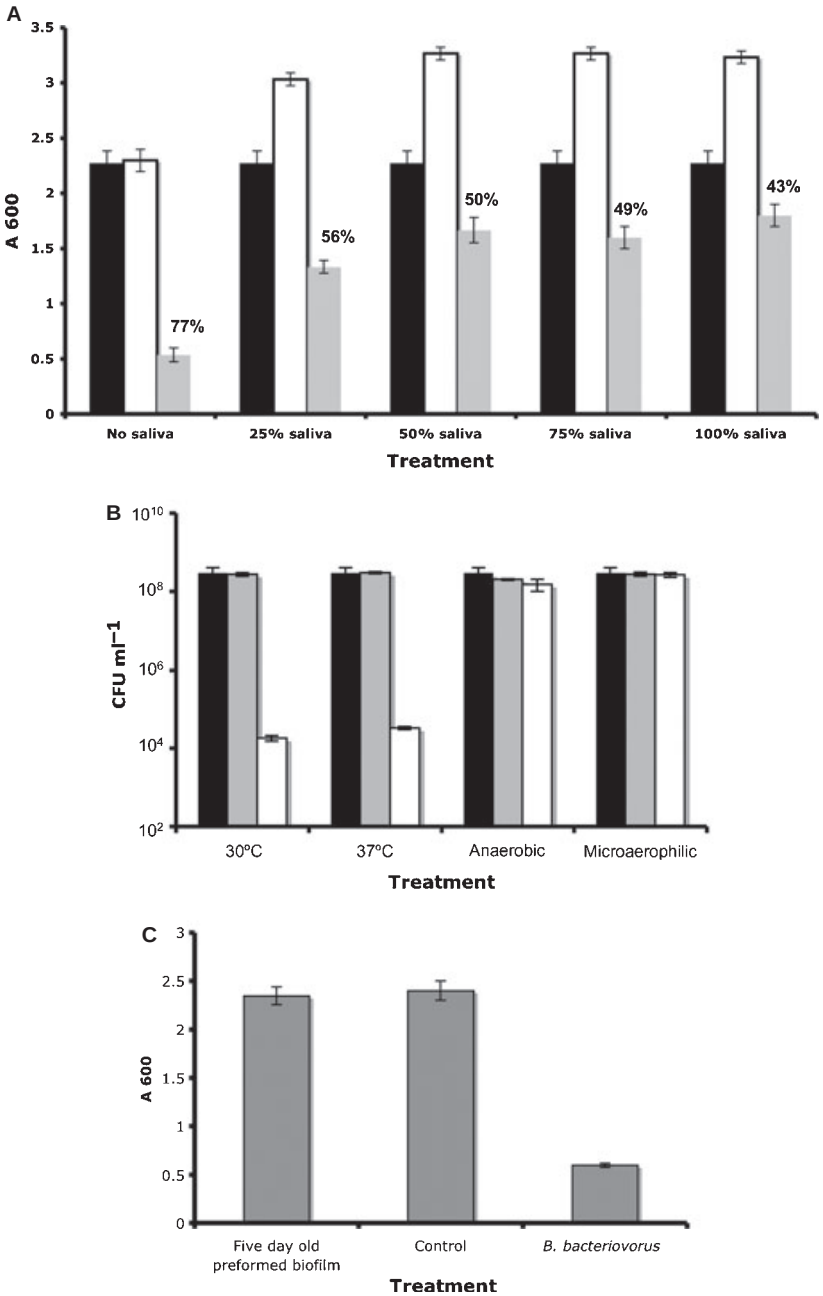

Figure 4 Reduction of host cells under various conditions. (A) Quantification of biofilm reduction in the presence of saliva. Preformed overnight *Aggregatibacter actinomycetemcomitans* CU1000 biofilms (black bars) were incubated with varying concentrations of filtered sterilized unstimulated saliva (from 0 to 100%), containing *Bdellovibrio bacteriovorus* 109J (gray bars) or filtered sterilized lysate (white bars). After 48 h, the wells were rinsed, stained with CV, and the amount of CV staining was quantified. Each value represents the mean of 12 wells. Error bars are shown as one-standard deviation. Numbers above the bars represent the average percent reduction in biofilm biomass compared with the control. (B) *B. bacteriovorus* predation capability in adverse culture conditions. *Escherichia coli* ZK2686 host cells were incubated for 48 h in the presence of *B. bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). The co-cultures were incubated at 30 and 37°C (normal oxygen levels) or at 30°C under anaerobic or microaerophilic growth conditions. Black bars represent host cell numbers at time 0. Each value represents the mean of three cultures. Error bars are shown as one-standard deviation. (C) Ability of *B. bacteriovorus* to remove metabolically inactive biofilms. Preformed overnight *A. actinomycetemcomitans* CU1000 biofilms were developed in 96-well plates for 24 h. Thereafter, DNB was added to the biofilm and incubated for 96 h (5-day-old preformed biofilm). Filtered sterilized lysate (Control) or *B. bacteriovorus* was then added to the metabolically inactive biofilms. Biofilms were stained and quantified. Each value represents the mean of 18 wells. Error bars are shown as one standard deviation.

B. bacteriovorus predation in adverse culture conditions

As some oral pathogens reside within the subgingival area, where the oxygen concentration is limited, we were interested in investigating the ability of *B. bacteriovorus* to prey in anaerobic and microaerophilic conditions. To this end, standard *B. bacteriovorus* host co-cultures were prepared using washed *E. coli* strain ZK2686, a derivative of W3110, (Pratt & Kolter, 1998) as prey. The cultures were placed in a BD GasPak Jar systems with a disposable gas generating anaerobic or microaerophilic envelope. Counting the CFU of the surviving host revealed that *B. bacteriovorus* was unable to prey under oxygen-limiting conditions (Fig. 4B). Other predation experiments in which the co-cultures were placed in a MACS MG 250 anaerobic chamber (10% CO_2, 10% H_2 and

80% N_2) also produced no reduction in host population (data not shown). Interestingly, when the cultures were removed from the oxygen-limiting conditions (after 72 h) and placed in an aerobic environment, predation did occur, reducing host cell CFU numbers by 3 logs (from 2×10^8 to 1×10^5 CFU ml^{-1}). The inability of *B. bacteriovorus* to prey under anaerobic and microaerophilic conditions was also seen when *E. coli* strain DH5α was used as prey (data not shown) or when *A. actinomycetemcomitans* strain JP2 was used (from an initial optical density of $A_{595} = 0.112$ to $A_{595} = 0.109$ and $A_{595} = 0.102$ for anaerobic and microaerophilic conditions, following 48 h of incubation, respectively). As was seen for *E. coli*, reduction in turbidity did occur when the anaerobic co-cultures were replaced in aerobic conditions ($A_{595} = 0.075$). Although *B. bacteriovorus* was restricted by its ability to prey in anaerobic and microaerophilic conditions, it was not restricted to prey at higher temperatures of 37°C (Fig. 4B).

Reduction of metabolically inactive biofilms

It was previously suggested that cells within biofilms might exhibit limited growth, which in turn increases their resistance to antimicrobial challenges (Keren *et al.*, 2004; Werner *et al.*, 2004; Yang *et al.*, 2008; Hoiby *et al.*, 2010). Hence, the ability of an antimicrobial agent to reduce and remove metabolically inactive surface-associated bacteria could be beneficial.

When growing biofilms of *A. actinomycetemcomitans* CU1000, we noticed that although the preformed biofilm is extremely stable, CFU enumeration and Alamar-Blue cell viability reagent assays revealed that the biofilm contained no viable cells, following a 96-h incubation period in DNB. At this point, an assay was carried out in which 24-h-preformed biofilms were washed, resuspended in DNB, and incubated for 120 h. The stable, but unviable biofilm was incubated with *B. bacteriovorus* or filter-sterilized lysate. As seen in Fig. 4C, a reduction of 75% in CV staining was measured following incubation with *B. bacteriovorus*, confirming removal of the metabolically inactive biofilm.

Enhancing the ability of *B. bacteriovorus* to remove biofilms of *A. actinomycetemcomitans*

It was previously reported that *A. actinomycetemcomitans* biofilms are composed of cells that are embedded in a self-synthesized extracellular polymeric substance (EPS) which contains DNA, protein and poly-*N*-acetylglucosamine (PGA) (Izano *et al.*, 2008). We hypothesized that by applying EPS-degrading enzymes with or following the application of *B. bacteriovorus*, greater biofilm removal could be obtained. To this end, biofilms of *A. actinomycetemcomitans* CU1000 were formed and treated with *B. bacteriovorus*, DNase-I, proteinase-K, and DspB (a PGA-hydrolysing enzyme) (Kaplan *et al.*, 2004; Ramasubbu *et al.*, 2005).

DNase treatment

Treating the biofilm with *B. bacteriovorus* for 48 h, followed by a 2-h incubation period with DNase-I (65 μg ml^{-1}) had resulted in a 12% increase in biofilm removal when compared with *B. bacteriovorus* alone (71% reduction), and 45% more than DNase-I alone (which resulted in 38% reduction). Incubating the biofilm simultaneously with the enzyme and *B. bacteriovorus* did not prove to be more effective in reducing the biofilm (66% reduction) when compared with the *B. bacteriovorus* alone (Fig. 5A).

Protease treatment

Incubating the biofilm with proteinase-K did not cause a measurable reduction in biofilm CV staining (Fig. 4 B, PK treatment). Comparable biofilm removal was measured in both the *B. bacteriovorus*-treated sample and the sample treated with proteinase-K (100 μg ml^{-1}) after the *B. bacteriovorus* treatment (80 and 81% reduction, respectively). When incubating the preformed biofilm simultaneously with proteinase-K and *B. bacteriovorus*, a loss of biofilm removal was seen (Fig. 5B, *Bdello* with PK). A reduction in the *B. bacteriovorus* biofilm-reducing ability was also detected in the samples that were first incubated with proteinase-K, washed, and then treated with the *B. bacteriovorus* (Fig. 5B, PK followed by *Bdello*).

To further examine the effects of protease on predation, proteinase-K (100 μg ml^{-1}) and trypsin (100 μg ml^{-1}) were added to a *B. bacteriovorus* host co-cultures containing 3.1×10^8 CFU ml^{-1} *E. coli* strain ZK2686 host cells. A 4-log reduction in CFU counts was measured in the *B. bacteriovorus* plus trypsin cultures with no host cell decrease occurring in the *B. bacteriovorus*-free control (4.5×10^4 and

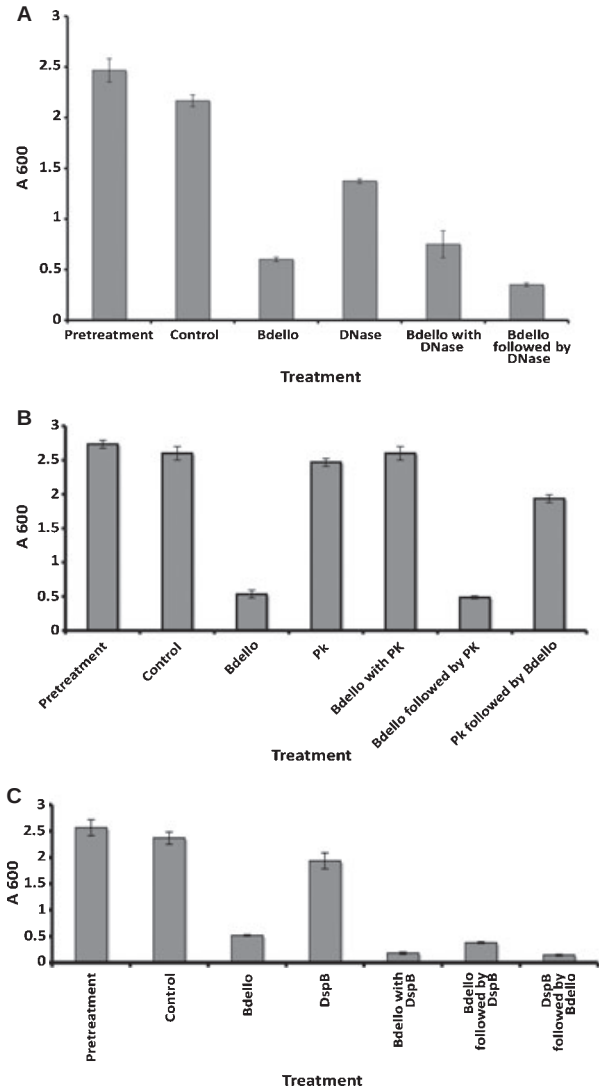

Figure 5 Quantification of *Aggregatibacter actinomycetemcomitans* biofilm removal by *Bdellovibrio bacteriovorus* and enzyme treatments. Preformed overnight *A. actinomycetemcomitans* CU1000 biofilms (Pretreatment) were incubated for 48 h with filtered sterilized lysate (Control), *B. bacteriovorus* (Bdello), DNase-I (A), proteinase-K (B), and DspB (C). Treatments also included incubating the biofilm simultaneously with *B. bacteriovorus* and the selected enzymes (Bdello with enzyme), treating the biofilm for 2 h with the enzyme followed by a 48-h *B. bacteriovorus* treatment (enzyme followed by Bdello), or a 48-h *B. bacteriovorus* treatment followed by a 2-h incubation period with the enzyme (Bdello followed by enzyme). Biofilms were stained and quantified. Each value represents the mean of 12 wells. Error bars are shown as one standard deviation.

2.5×10^8 CFU ml^{-1} respectively). No reduction in host cell population was measured in the *B. bacteriovorus* proteinase-K lysates (2.3×10^8 CFU ml^{-1}), indicating that proteinase-K could effectively inhibit predation. Similar results were obtained when using *E. coli* DH5α as host (data not shown) or when co-culturing *B. bacteriovorus* with JP2, a smooth-colony variant of *A. actinomycetemcomitans,* in the presence of proteinase-K (from an initial optical density of $A_{595} = 0.108$ to $A_{595} = 0.103$, following 48 h of incubation).

Treating the preformed biofilm with a PGA-hydrolysing enzyme (20 μg ml^{-1} DspB) resulted in a 16% reduction in biofilm CV staining (Fig. 5C, DspB). DspB was also capable of enhancing (by 14%) the ability of the *B. bacteriovorus* to remove the biofilm when incubated in concert or before the application

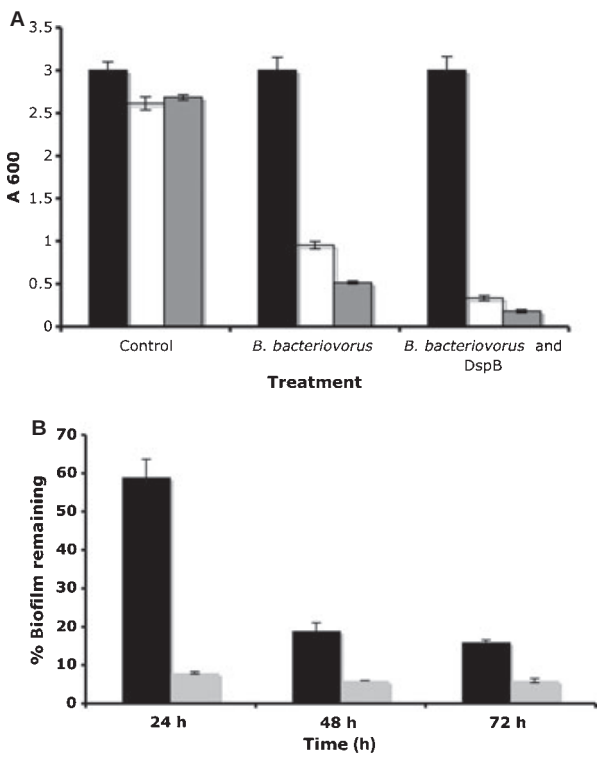

Figure 6 The role of poly-*N*-acetylglucosamine (PGA) in biofilm removal. (A) Preformed overnight *Aggregatibacter actinomycetemcomitans* CU1000 biofilms (black bars) were incubated with filtered sterilized lysate (Control), *Bdellovibrio bacteriovorus* or a combination of *B. bacteriovorus* and DspB. Biofilms were stained after 24 h (white bars) and 48 h (gray bars). Each value represents the mean of 12 wells. Error bars are shown as one standard deviation. (B) *A. actinomycetemcomitans* CU1000 biofilms (black bars) or *A. actinomycetemcomitans* HW1018 biofilms (gray bars) were treated with *B. bacteriovorus* for 24, 48 and 72 h, stained, and the percentage of biofilm remaining was calculated. Each value represents the mean of 18 wells. Error bars are shown as one standard deviation.

of the predator (Fig. 5C). Applying DspB after the *B. bacteriovorus* treatment also proved to be more efficient in removing the biofilm when compared with the *B. bacteriovorus* treatment alone (84 and 77%, respectively).

DspB treatment

In addition to the enhanced biofilm removal capability observed in the combined *B. bacteriovorus* plus DspB treatment, the time by which the *A. actinomycetemcomitans* biofilm was removed by the predator was considerably reduced in the presence of the enzyme. As seen in Fig. 6A, treating the biofilm with a combination of *B. bacteriovorus* and enzyme removed 88% of the biofilm within the first 24 h, whereas *B. bacteriovorus* alone reduced the biofilm by 64% during the same time period.

To further attribute the enhanced biofilm removal ability of the combined treatment to the degradation of the biofilm PGA matrix, *A. actinomycetemcomitans* CU1000 PGA mutants were used. Although HW1018 is unable to synthesize PGA, it is still capable of forming a robust biofilm (Izano *et al.*, 2007, 2008). When incubating the preformed biofilm with *B. bacteriovorus* for 24 h, a reduction of 41% was seen in the WT CU1000 biofilm, whereas, *B. bacteriovorus* was able to remove 92% of the HW1018-PGA mutant within the same incubation period (Fig. 6B).

DISCUSSION

In the work presented here, the ability of the predatory bacterium *B. bacteriovorus* 109J to prey on bacteria associated with periodontal diseases was examined. When cultured in liquid suspension, *B. bacteriovorus* 109J had the ability to prey on all the *A. actinomycetemcomitans* serotypes tested. Predation was observed on both smooth-colony, biofilm-negative variants (Fig. 1A) and on biofilms composed of rough-colony variants (Fig. 2). The ability of *B. bacteriovorus* strain 109J to prey on *A. actinomycetemcomitans* is in agreement with a previous study, in which predation of *A. actinomycetemcomitans* by *B. bacteriovorus* strain HD100 was documented (Van Essche *et al.*, 2009). Predation was also observed when *Ei. corrodens* was used as host. The ability of *B. bacteriovorus* to attack *Ei. corrodens* was observed on all of the isolates tested, and occurred on host cells that were cultured both planktonically or as a biofilm (Figs 1B and 3). Predation was also observed on *F. nucleatum* ATCC 10953; but no predation was detected when *Po. gingivalis*, *Pr. intermedia*, *T. forsythia* and *F. nucleatum* ATCC 10953 were used as host. As the mechanisms governing host specificity of *B. bacteriovorus* are far from being fully understood, we could only speculate on the reasons leading to *B. bacteriovorus* ability or inability to use some gram-negative bacteria and not others. One possibility that could be presented is the inability of the strict anaerobes to survive in the aerobic conditions in which the predation experiments were conducted. This might also explain why *B. bacteriovorus* was able to prey on *F. nucleatum*, as some isolates of *F. nucleatum* have been shown to tolerate higher oxygen levels (Diaz *et al.*, 2000, 2002). However, the ability of *B. bacteriovorus* to prey on metabolically inactive host cells (Varon & Shil, 1968) might suggest that additional factors are involved.

To further investigate the potential use of *B. bacteriovorus* to remove surface-attached bacteria, the predominant state in which oral bacteria reside within the oral cavity, additional experiments were conducted. We focused our work on the ability of the predator to remove *A. actinomycetemcomitans* biofilms CU1000, which formed extremely stable biofilms. The *B. bacteriovorus* was capable of detaching and removing *A. actinomycetemcomitans* grown on hydroxyapatite squares, as well as removing *A. actinomycetemcomitans* biofilms submerged in saliva (Figs 2C and 4A). The ability of a bio-control agent to withstand the antimicrobial activity associated with human saliva (Tenovuo, 2002; De Smet & Contreras, 2005; Abiko & Saitoh, 2007; Weinberg, 2007; Gorr, 2009) is key when considering the *in vivo* use of the agent within the oral cavity. Other factors that might be encountered in the oral cavity and that might influence the ability of the predator to attack and remove the biofilms are temperature, oxygen concentrations and slow or low metabolic activity of the host cell. To this end, experiments were conducted in which lysates were placed in an elevated temperature and in a limited oxygen environment. As documented by Seidler & Starr (1969), a temperature of 37°C did not inhibit predation, however microaerophilic and anaerobic conditions did halt the ability of *B. bacteriovorus* to prey. Predation did resume when the cultures were re-placed at room oxygen levels,

demonstrating that *B. bacteriovorus* could survive periods of limited oxygen concentrations. The inability to prey in an oxygen-limited environment was previously reported (Schoeffield *et al.*, 1996). Although the incapacity of *B. bacteriovorus* to prey in microaerophilic and anaerobic conditions might prove to be a disadvantage when applied against bacteria located deep within the low oxygen environment of the periodontal pocket, it may still be an efficient strategy to reduce periodontopathogens, such as *A. actinomycetemcomitans*, *Ei. corrodens* and *F. nucleatum* (Muller *et al.*, 1997, 2001; Mager *et al.*, 2003), which could be detected throughout the oral cavity and in saliva, or as a means to prevent recolonization of the periodontal pocket following periodontal therapy (De Soete *et al.*, 2001; Quirynen *et al.*, 2001; Van Essche *et al.*, 2009). The *B. bacteriovorus* might also be effective in penetrating oral plaque, removing the oxygen tolerant bacteria and exposing the anaerobic pathogens harbored deep within the biofilm (Diaz *et al.*, 2002). Although *B. bacteriovorus* 109J was unable to prey in an oxygen-limited environment, other *Bdellovibrio*-like organisms might be more adapted to prey under these conditions (Schoeffield *et al.*, 1996; Van Essche *et al.*, 2009). Although viable *Bdellovibrio* were never cultured from the oral cavity, 16S ribosomal RNA analysis did identify the presence of a *Bdellovibrio* genomic sequence in an oral sample (Paster *et al.*, 2002; Chen *et al.*, 2010). The ability of *B. bacteriovorus* to survive at elevated temperatures, during periods of oxygen limitation and in the presence of saliva suggests that the oral cavity could be an adequate environment to support *B. bacteriovorus* growth.

When placed on a 5-day-old, metabolically inactive, *A. actinomycetemcomitans* biofilm, *B. bacteriovorus* was able to reduce the biofilm by 75% (Fig. 4C). The ability to attack and remove metabolically inactive biofilms could be of great significance when considering that the limited cell growth rate, usually exhibited within biofilm communities, plays a vital role in enhancing biofilm antibiotic resistance (Keren *et al.*, 2004; Werner *et al.*, 2004; Yang *et al.*, 2008; Hoiby *et al.*, 2010). Hence, a biofilm-controlling agent that is not influenced by the metabolic activity of its target cell could be of value.

Previous studies showed that the EPS of *A. actinomycetemcomitans* biofilms contains extracellular DNA, protein, and PGA and that degradation of the EPS compounds could lead to biofilm dispersal (Izano *et al.*, 2008). In an attempt to enhance biofilm removal by *B. bacteriovorus*, DNA-, protein- and PGA-degrading enzymes were incorporated in the biofilm predation experiments. The use of DNase-I simultaneously with the predator did not increase *A. actinomycetemcomitans* biofilm removal. A slight increase in biofilm removal was measured when DNase-I was added to the biofilm following the predation period (Fig. 5A). The limited biofilm removal effect seen with DNase-I and *B. bacteriovorus* could be explained by the finding that, in the presence of PGA, DNA may not be a major compound of *A. actinomycetemcomitans* (Izano *et al.*, 2008). The addition of proteinase-K during predation prevented the removal of the biofilm by *B. bacteriovorus*. The predation-inhibiting effect of proteinase-K was further confirmed in a standard lysate containing *E. coli* as host cells or a smooth-colony variant of *A. actinomycetemcomitans*. These results suggest that proteinase-K might affect specific surface proteins on the host cell or the *B. bacteriovorus* that are required for predation. No reduction in host CFU or host-independent *B. bacteriovorus* CFU were measured after incubation with proteinase-K (Medina & Kadouri, 2009). Therefore, it is less likely that the loss of predation in the presence of proteinase-K is caused by a reduction in cell viability. Although proteinase-K was able to inhibit predation, trypsin, a serine protease that exhibits cleaving properties different from those of proteinase-K, did not reduce predation. Hence, the ability of the enzyme to inhibit predation is specific and might explain the ability of *B. bacteriovorus* to prey in the presence of saliva, which is known to harbor trypsin-like proteases (Ingman *et al.*, 1993; Sun *et al.*, 2009).

Treating the biofilm with DspB, a known PGA-hydrolysing enzyme, before or simultaneously with *B. bacteriovorus*, resulted in an increase in biofilm removal (Fig. 5C). The DspB plus *B. bacteriovorus* treatment also significantly shortened the time required for *A. actinomycetemcomitans* biofilm removal (Fig. 6A). Additional confirmation of the enhanced biofilm removal by *B. bacteriovorus* in the absence of PGA was seen when HW1018, a PGA-deficient mutant, was used (Fig. 6B).

We propose that the improved biofilm removal, measured in the presence of DspB and in the PGA mutant, could result from an increased ability of

B. bacteriovorus to penetrate and remove a biofilm that has a low or no PGA content in its EPS. Another possibility is that following predation; PGA and EPS residues still remain on the surface. Hence, removal of the PGA by the enzyme or by using a PGA mutant reduces the surface-attached debris remaining after predation, leading to a reduction in CV staining. Further experiments are currently underway to fully understand the role played by PGA in biofilm maintenance during predation. In conclusion, our data demonstrate the potential use of biofilm EPS degrading enzymes as a means to enhance the ability of *B. bacteriovorus* to remove surface-attached bacteria.

In this study we have demonstrated the potential use of *B. bacteriovorus* 109J in controlling oral pathogens *in vitro* and the conditions that might influence and enhance the bio-control aptitude of this unique predator. It was previously shown that, like other predators in nature, *B. bacteriovorus* does not consume all of its prey (Shemesh & Jurkevitch, 2004; Kadouri & O'Toole, 2005) making it less adequate to be used as a bio-control agent; however, its ability to infiltrate biofilms could still render it effective in partially reducing the biofilm and allowing host immune defenses or additional antimicrobial agents better access to the surface-attached cells. Another question that needs to be addressed is what will be the impact of adding a relatively non-specific predator like *B. bacteriovorus* to the gram-negative microbial commensal population. Though we are incapable of answering this question at this point, one should remember that the majority, if not all, of the antimicrobial agents used to date are also non-specific and target the commensal flora. Therefore, while the potential ability of *B. bacteriovorus* to be used to control human pathogens has been discussed (Richardson, 1990; Fratamico & Cooke, 1996; Martin, 2002; Sockett & Lambert, 2004) the full impact of using *Bdellovibrio* as a live antibiotic *in vivo* should be the focus of future studies.

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ORIGINAL ARTICLE

Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*

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Abstract

Aims: The focus of this study was to evaluate the potential use of the predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* to control the pathogens associated with human infection.
Methods and Results: By coculturing *B. bacteriovorus* 109J and *M. aeruginosavorus* ARL-13 with selected pathogens, we have demonstrated that predatory bacteria are able to attack bacteria from the genus *Acinetobacter*, *Aeromonas*, *Bordetella*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Listonella*, *Morganella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio* and *Yersinia*. Predation was measured in single and multispecies microbial cultures as well as on monolayer and multilayer preformed biofilms. Additional experiments aimed at assessing the optimal predation characteristics of *M. aeruginosavorus* demonstrated that the predator is able to prey at temperatures of 25–37°C but is unable to prey under oxygen-limiting conditions. In addition, an increase in *M. aeruginosavorus* ARL-13 prey range was also observed.
Conclusions: *Bdellovibrio bacteriovorus* and *M. aeruginosavorus* have an ability to prey and reduce many of the multidrug-resistant pathogens associated with human infection.
Significance and Impact of the Study: Infectious complications caused by micro-organisms that have become resistant to drug therapy are an increasing problem in medicine, with more infections becoming difficult to treat using traditional antimicrobial agents. The work presented here highlights the potential use of predatory bacteria as a biological-based agent for eradicating multidrug-resistant bacteria, with the hope of paving the way for future studies in animal models.

Introduction

Infectious complications caused by micro-organisms that have become resistant to drug therapy are an increasing problem in medicine, with more infections becoming difficult to treat using traditional antimicrobial agents. Many of the infections are caused by Gram-negative bacteria such as *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Davis *et al.* 2005; Joseph *et al.* 2010; Jung *et al.* 2010; Smith *et al.* 2010). Recently, new Carbapenem-resistant NDM-1 (New Delhi metallo-beta-lactamase-1) Gram-negative bacteria have been identified with the potential of becoming an endemic worldwide in the next few years (Nordmann *et al.* 2009; Yong *et al.* 2009; Karthikeyan *et al.* 2010). Drug resistance can be considered a natural response to the selective pressure of a drug and can develop in both single-cell free-floating bacteria and surface-attached bacteria or biofilms. Biofilms form when bacteria adhere to surfaces and begin to excrete a glue-like extracellular polymeric substance that protects and anchors them to materials and tissue. One of the major difficulties in controlling surface-attached bacteria is their enhanced resistance to antimicrobial agents – biofilms can be up to 1000 times more resistant to

antimicrobial agents than their planktonic counterparts (Cos *et al.* 2010; Hoiby *et al.* 2010; Nucleo *et al.* 2010).

Two organisms that are capable of specifically targeting and preying on Gram-negative micro-organisms are the bacteria from the genus *Bdellovibrio* spp. and *Micavibrio* spp. *Bdellovibrio* and *Micavibrio* are Gram-negative, motile and uniflagellated bacteria characterized by predatory behaviour (or an obligatory parasitic life cycle). *Bdellovibrio* life cycle and biology are relatively well studied and consists of an attack phase cell that attaches to other Gram-negative bacteria, penetrates their periplasm, multiplies in the periplasmic space and finally bursts the cell envelope to start the cycle anew (Stolp and Starr 1963; Rendulic *et al.* 2004; Lambert *et al.* 2006; Sockett 2009). *Micavibrio*, a less studied predator, exhibits a 'vampire-like' lifestyle leeching externally to its host as it feeds (Lambina *et al.* 1982, 1983; Afinogenova *et al.* 1987; Davidov *et al.* 2006). Unlike *Bdellovibrio* spp., *Micavibrio* spp. were shown to have a high degree of host specificity, with *Micavibrio aeruginosavorus* strain ARL-13 preying only on *Ps. aeruginosa*, *Burkholderia cepacia* and *Kl. pneumoniae* (Lambina *et al.* 1983; Kadouri *et al.* 2007).

The aim of this study was to conduct a comprehensive investigation into the host range specificity of *Bdellovibrio bacteriovorus* 109J and *M. aeruginosavorus* ARL-13, as well as measure the impact of predation on host cells grown in a single or multispecies culture and biofilm. Special emphasis was put on examining predation on pathogens most associated with wounds, burns and multidrug-resistant infection. However, other medically relevant bacteria were examined as well. *M. aeruginosavorus* temperature and aeration optimal predation requirements were also examined.

Materials and methods

Bacteria strains, media and growth conditions

The host strains used in this study are listed in Tables 1 and 2. The predatory bacteria used were *B. bacteriovorus* 109J (ATCC 43826) and *M. aeruginosavorus* strain ARL-13 (Lambina *et al.* 1983; Kadouri *et al.* 2007). *Micavibrio aeruginosavorus* and *B. bacteriovorus* were maintained as plaques in double-layered diluted nutrient broth (DNB) agar, a 1 : 10 dilution of nutrient broth amended with 3 mmol l^{-1} $MgCl_2 \cdot 6H_2O$ and 2 mmol l^{-1} $CaCl_2 \cdot 2H_2O$ [pH 7·2] and agar (0·6% agar in the top layer) (Starr 1975). To initiate a lysate, cocultures were obtained by adding a plug of agar containing *B. bacteriovorus* or *M. aeruginosavorus* plaque to washed prey cells in DNB and incubated at 30°C on a rotary shaker set at 200 rev min^{-1} until the coculture became clear (stock lysate). To harvest the predators, cocultures were prepared in which 2 ml of washed host cells (*c.* 1×10^9 CFU ml^{-1}) was incubated with 2 ml of stock lysate in 20 ml of DNB. The cocultures were incubated for 18 and 48 h (for *B. bacteriovorus* and *M. aeruginosavorus*, respectively) to reach a final concentration of *c.* 1×10^8 PFU ml^{-1} predator. At this point, the lysate was passed three times through a 0·45-μm Millex pore-size filter (Millipore, Billerica, MA, USA) to remove residual prey and cell debris (filtered lysate). As a control, filtered sterilized lysate was prepared by sequentially passing the lysates through three 0·22-μm pore-size filters. After filtration, no predator, as judged by PFU, could be detected (Kadouri and O'Toole 2005; Kadouri *et al.* 2007).

Prey range assay

To evaluate the ability of *B. bacteriovorus* and *M. aeruginosavorus* to prey on the selected pathogens, cocultures were prepared in which washed prey cells were incubated in 5 ml of DNB with harvested *B. bacteriovorus* or *M. aeruginosavorus*. As a control, filtered, sterilized lysate was used. The cultures were incubated for up to 48 h, at 30°C on a rotary shaker set at 200 rev min^{-1}. The ability of predators to prey was confirmed by the reduction in host cell viability, measured by CFU enumeration, compared to the predator-free control. Each coculture was performed three times.

Plaque predation assays

The ability of the predator to form a lytic halo on a relatively thin lawn of surface-attached prey cells was determined using a modification of the double-layered plaque assay (Stolp and Starr 1963; Kadouri *et al.* 2007). Prey cells were grown for 18 h in LB (Luria–Bertani). One hundred microlitres of ten times concentrated washed cells were spread on DNB medium solidified with 1·5% agar. Harvested *B. bacteriovorus* or *M. aeruginosavorus* were prepared as described above. Three 20-μl drops of the filtered predator were spotted on the lawn of the examined host bacteria. Lytic halo assay plates were incubated at 30°C for up to 3 weeks and examined for the formation of a zone of clearing where the predator was spotted. Each halo assay was performed three times with filtered sterilized lysate used as a negative control.

Biofilm and predation assays

Biofilms were formed in a nontissue culture treated, 96-well polyvinyl chloride microtiter dishes (Becton Dickinson, Franklin Lakes, NJ, USA) as previously described (Kadouri and O'Toole 2005; Merritt *et al.* 2005). Briefly, microtiter wells were inoculated (100 μl per well) with 18-h LB-grown host culture diluted 1 : 100 in fresh LB media. The plates were incubated for 18 h at 30°C (preformed biofilm) before they were stained with crystal

Table 1 Host range specificity of *Bdellovibrio bacteriovorus*

Bacteria tested	Predation on planktonic cells*	CFU log reduction following predation†	Predation on surface-attached lawn cells‡
Acinetobacter			
Acinetobacter species ATCC 49466	+	4·5 ± 0·5	+
Acinetobacter species ATCC 10153	+	3	+
Acinetobacter baumannii ATCC 19606	+	3·5 ± 0·5	+
Ac. baumannii NCIMB 12457	+	4 ± 1	+
Ac. baumannii ATCC BAA-747	+	5	+
Acinetobacter calcoaceticus PIC 346	+	0·75 ± 0·25	–
Acinetobacter haemolyticus ATCC 19002	+	5	+
Acinetobacter lwoffii ATCC 15309	+	2·5 ± 0·5	+
Ac. lwoffii ATCC 17925	+	3·5 ± 0·5	+
Aeromonas			
Aeromonas hydrophila PIC 191	+	4	+
Aeromonas salmonicida ATCC 33658	+	5·5 ± 0·5	+
Bordetella bronchiseptica PIC 402	+	3	+
Burkholderia cepacia 2 clinical isolate§	+	3	n.a
Campylobacter			
Campylobacter jejuni ATCC 29428	–	0	n.a
Camp. jejuni ATCC BAA-1153	–	0	n.a
Citrobacter			
Citrobacter freundii NCTC 9750	+	2·5 ± 0·5	+
Cit. freundii ATCC 43864	+	0·5	+
Cit. freundii ATCC 8090	+	3	+
Enterobacter			
Enterobacter aerogenes ATCC 13048	+	2	+
Ent. aerogenes ATCC 35029	+	2	+
Ent. aerogenes ATCC 51697	+	5	+
Ent. aerogenes NCIMB	+	3	+
Enterobacter amnigenus ATCC 51816	+	4	+
Enterobacter cloacae ATCC 700323	+	2·5 ± 0·5	+
Ent. cloacae ATCC 35030	+	2·5 ± 0·5	+
Ent. cloacae ATCC 49141	+	4	+
Enterobacter gergoviae ATCC 33028	+	3·5 ± 0·5	+
Enterococcus faecalis PIC 522B	–	0	n.a
Escherichia			
Escherichia coli ZK2686/W3110	+	6·5 ± 0·5	+
E. coli PIC 336	+	7 ± 1	+
E. coli DH5α	+	7 ± 1	+
Klebsiella			
Klebsiella pneumoniae ATCC 33495	+	2	+
Kl. pneumoniae ATCC BAA-1706	+	4	+
Kl. pneumoniae ATCC BAA-1705	+	2	+
Kl. pneumoniae 6 clinical isolates§	+	5 ± 1	+
Listonella anguillarum ATCC 14181	+	5	n.a
Morganella			
Morganella morganii ATCC 25829	+	3	+
Mo. morganii ATCC 25830	+	3	+
Mo. morganii PIC 329	+	1	+
Mycobacterium			
Mycobacterium smegmatis PIC 6972	–	0	n.a
Mycobacterium lacticola PIC 697	–	0	n.a
Proteus			
Proteus mirabilis ATCC 35659	+	1·5 ± 0·5	+
Pr. mirabilis ATCC 43071	+	4	+
Pr. mirabilis ATCC 25933	+	3·5 ± 0·5	+
Pr. mirabilis NCIMB 13283	+	4	+

Table 1 (*Continued*)

Bacteria tested	Predation on planktonic cells*	CFU log reduction following predation†	Predation on surface-attached lawn cells‡
Pr. mirabilis ATCC 7002	+	4	+
Pr. mirabilis PIC 366	+	3	+
Proteus morganii PIC 3661	+	1	+
Proteus rettgeri ATCC 9250	+	4·5 ± 0·5	+
Proteus vulgaris ATCC 33420	+	4·5 ± 0·5	+
Pr. vulgaris ATCC 49132	+	5	+
Pr. vulgaris ATCC 8427	+	4	+
Pr. vulgaris NCTC 4636	+	4·5 ± 0·5	+
Pr. vulgaris PIC 365	+	8	+
Pseudomonas			
Pseudomonas aeruginosa PA14	–	0	–
Ps. aeruginosa PA01	–	0	–
Ps. aeruginosa ATCC BAA-427	+	1	n.a
Ps. aeruginosa ATCC 10145	–	0	–
Pseudomonas fluorescens PIC 105	+	2	+
Pseudomonas syringae	+	n.a	+
Pseudomonas putida PIC 107	+	n.a	+
Salmonella enterica PIC 371	+	4	n.a
Serratia marcescens PIC 361	+	3·5 ± 0·5	n.a
Shigella			
Shigella flexneri PIC 387	+	5	n.a
Shigella sonnei PIC 388	+	6	n.a
Staphylococcus aureus	–	n.a	–
Stenotrophomonas maltophilia§ 6 clinical isolates	–	0	–
Vibrio			
Vibrio angulara PIC 232	+	2	+
Vibrio cholerae EL Tor	+	4	n.a
Vibrio parahaemolyticus PIC 234	+	0·75 ± 0·25	+
Yersinia			
Yersinia enterocolitica PIC 330	+	2	n.a
Yersinia pseudotuberculosys PIC 399	+	3	n.a

PIC, Presque Isle Culture Collection; ATCC, American Type Culture Collection.

*Cocultures were prepared by adding host cells to harvested *B. bacteriovorus* predator cells. Predation was evaluated after 48 h of incubation by light microscopy, (+) predation by *B. bacteriovorus*; (–) no predation by *B. bacteriovorus*; (n.a) not evaluated.

†Values represent the average reduction in host cell viability (CFU ml^{-1}) compared to their respective predator-free filtered sterilized lysate control.

‡Twenty microlitres of *B. bacteriovorus* was spotted on a lawn of the indicated bacteria. Predation was scored as the formation of lytic zone at the point of *B. bacteriovorus* inoculation, (+) predation by *B. bacteriovorus*; (–) no predation by *B. bacteriovorus*; (n.a) not evaluated.

§Similar reduction was seen on each clinical isolate.

violet. To assess the biofilm predation, the preformed biofilms were washed 2× with DNB to remove planktonic cells and 100 μl of harvested predator was added to each well. Alternatively, as a control, 100 μl of sterile lysate was added to the wells. The microtiter dishes were incubated at 30°C for the duration of the experiment. Quantification of biofilm bacteria before and following predation was performed by washing the microtiter plates with DNB, to remove nonadhering cells, 100 μl of fresh DNB was added to each well and the samples were sonicated for 8 s using a VC505 sonicator (Sonics and Materials Inc., Newtown, CT, USA) followed by dilution plating and CFU enumeration (Kadouri and O'Toole 2005; Merritt *et al.* 2005; Kadouri *et al.* 2007).

Micavibrio aeruginosavorus predation under oxygen-limiting conditions

To measure the ability of *M. aeruginosavorus* to prey in anaerobic and microaerophilic conditions, *M. aeruginosavorus*

Table 2 Host range specificity of *Micavibrio aeruginosavorus*

Bacteria tested	Predation on planktonic cells*	CFU log reduction following predation†	Predation on surface-attached lawn cells‡
Acinetobacter			
Acinetobacter species ATCC 49466	–	0	–
Acinetobacter species ATCC 10153	–	0	–
Ac. baumannii ATCC 19606	–	0	–
Ac. baumannii NCIMB 12457	–	0	–
Ac. baumannii ATCC BAA-747	–	0	–
Acinetobacter calcoaceticus PIC 346	+	1	+
Acinetobacter haemolyticus ATCC 19002	+/–	0·25 ± 0·25	–
Acinetobacter lwoffii ATCC 15309	+	0·75 ± 0·25	+
Ac. lwoffii ATCC 17925	+	0·75 ± 0·25	+
Bordetella bronchiseptica PIC 402	–	0	–
Burkholderia cepacia 2 clinical isolate§	+	3	+
Citrobacter			
Citrobacter freundii NCTC 9750	–	0	–
Cit. freundii ATCC 43864	+	0·5	–
Cit. freundii ATCC 8090	–	0	–
Enterobacter			
Enterobacter aerogenes ATCC 13048	–	0	–
Ent. aerogenes ATCC 35029	+	1	+
Ent. aerogenes ATCC 51697	+	2	+
Ent. aerogenes NCIMB	+	0·5	+
Enterobacter amnigenus ATCC 51816	+	2	+
Enterobacter cloacae ATCC 700323	+	1·5 ± 0·5	+
E. cloacae ATCC 35030	+	2	+
E. cloacae ATCC 49141	+	1	+
Enterobacter gergoviae ATCC 33028	+	1·5	+
Enterococcus faecalis PIC 522B	–	0	n.a
Erwinia amylovora PIC 351	–	0	–
Escherichia			
Escherichia coli ZK2686/W3110	+	2·5 ± 0·5	+
E. coli PIC 336	+	2·5 ± 0·5	+
E. coli DH5α	+	3	+
Klebsiella			
Klebsiella pneumoniae ATCC 33495	+	2	+
Kl. pneumoniae ATCC BAA-1706	+	2·5 ± 0·5	+
Kl. pneumoniae ATCC BAA-1705	+	1·5 ± 0·5	+
Kl. pneumoniae 6 clinical isolates§	+	2·5 ± 0·5	+
Morganella			
Morganella morganii ATCC 25829	–	0	–
Mo. morganii ATCC 25830	–	0	–
Mo. morganii PIC 329	–	0	–
Mycobacterium smegmatis PIC 6972	–	0	n.a
Proteus			
Proteus mirabilis ATCC 35659	+	0·75 ± 0·25	+
Pr. mirabilis ATCC 43071	+	1	+
Pr. mirabilis ATCC 25933	+	0·5	–
Pr. mirabilis NCIMB 13283	+	0·5	–
Pr. mirabilis ATCC 7002	–	0	–
Pr. mirabilis PIC 366	+/–	0·25 ± 0·25	–
Proteus morganii PIC 3661	–	0	–
Proteus rettgeri ATCC 9250	+	0·5	–
Proteus vulgaris ATCC 33420	+	0·75 ± 0·25	n.a
Pr. vulgaris ATCC 49132	+	0·75 ± 0·25	+
Pr. vulgaris ATCC 8427	–	0	–
Pr. vulgaris NCTC 4636	–	0	–

Table 2 (*Continued*)

Bacteria tested	Predation on planktonic cells*	CFU log reduction following predation†	Predation on surface-attached lawn cells‡
Pseudomonas			
Pseudomonas aeruginosa PA14	+	3	+
Ps. aeruginosa PA01	+	n.a	+
Ps. aeruginosa ATCC BAA-427	+	3·5 ± 0·5	+
Ps. aeruginosa ATCC 10145	+	0·5	–
Ps. aeruginosa 16 clinical isolates§	+	n.a	+
Pseudomonas fluorescens PIC 105	–	n.a	–
Pseudomonas syringae	–	n.a	–
Pseudomonas putida	–	n.a	–
Serratia marcescens PIC 361	–	0	–
Shigella			
Shigella sonnei PIC 388	+	2	+
Stenotrophomonas maltophilia 6 clinical isolates§	–	0	–
Vibrio			
Vibrio angulara PIC 232	–	0	–
Vibrio cholerae EL Tor	–	n.a	–
Vibrio parahaemolyticus PIC 234	–	0	–
Yersinia			
Yersinia pseudotuberculosys PIC 399	+	1·5 ± 0·5	n.a

PIC, Presque Isle Culture Collection; ATCC American Type Culture Collection.
*Cocultures were prepared by adding host cells to harvested *M. aeruginosavorus* predator cells. Predation was evaluated after 48 h of incubation by light microscopy, (+) predation by *M. aeruginosavorus*; (–) no predation; (+/–) inconclusive; (n.a) not evaluated.
†Values represent the average reduction in host cell viability counts (CFU ml^{-1}) compared to their respective predator-free filtered sterilized lysate control.
‡Twenty microlitres of *M. aeruginosavorus* was spotted on a lawn of the indicated bacteria. Predation was scored as the formation of lytic zone at the point of *M. aeruginosavorus* inoculation, (+) positive predation; (–) no predation; (+/–) inconclusive (n.a) not evaluated.
§Similar reduction was seen on each clinical isolate.

host cocultures were prepared and placed in a BD GasPak Jar Systems with a disposable gas generating anaerobic or microaerophilic envelope (BD Diagnostic Systems). The jars were incubated at 30°C on a rotary shaker at 200 rev min^{-1}. Predation was measured by CFU enumeration of the surviving host cells.

Scanning electron microscopy

Experiments were performed as described previously (Kadouri and O'Toole 2005; Kadouri *et al.* 2007). In brief, biofilms were developed on a 12 × 12 mm PVC plastic cover slip (Fisher Scientific, Pittsburgh, PA, USA). The cover slips were placed in a 24-well polystyrene cell culture plate (Corning Inc., Corning, NY, USA). Preformed biofilms and predation assay were prepared as described above. The experiments were carried out in a 1·0-ml volume. Biofilms were rinsed to remove any planktonic cells before being fixed in 2% glutaraldehyde, 0·1 mol l^{-1} sodium cacodylate and 0·1% ruthenium red. Images were collected from biofilms grown at the air–liquid interface. The imaging was performed using a Zeiss Auriga crossbeam field emission scanning electron microscope (FE-SEM; Carl Zeiss NTS, LLC, Peabody, MA).

Results

Bdellovibrio bacteriovorus 109J host specificity

To examine the host range specificity of *B. bacteriovorus* and its effectiveness in reducing cell viability of microbial pathogens, bacteria were cultured, then incubated in the presence of *B. bacteriovorus* for 48 h. To measure predation, 100-μl aliquots were removed and CFU enumeration of the viable remaining prey cells was performed. As seen in Table 1, *B. bacteriovorus* was able to prey, attack and reduce 68 of the 83 examined bacteria. Among the bacteria reduced were those from the genus *Acinetobacter*, *Aeromonas*, *Bordetella*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Listonella*, *Morganella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio* and *Yersinia*. No predation was detected on bacteria from

the genus *Campylobacter*, *Stenotrophomonas*, and the non-Gram-negative bacteria *Mycobacterium*, *Enterococcus* and *Staphylococcus*.

Micavibrio aeruginosavorus host range specificity

The ability of *M. aeruginosavorus* to prey on microbial pathogens is shown in Table 2. *Micavibrio aeruginosavorus* was able to prey and reduce 57 of the 89 examined bacteria. The most profound reduction in host cell viability was measured for bacteria from the genus *Burkholderia*, *Escherichia*, *Klebsiella*, *Pseudomonas* and *Shigella*. A more moderate reduction was measured in cocultures that included bacteria from the genus *Acinetobacter*, *Enterobacter Proteus* and *Yersinia*. No predation was seen when the host used was from the genus *Bordetella*, *Citrobacter*, *Enterococcus*, *Erwinia*, *Morganella*, *Mycobacterium*, *Serratia*, *Stenotrophomonas*, *Vibrio*. The ability of *M. aeruginosavorus* to reduce *Burkholderia*, *Klebsiella* and *Pseudomonas* is in line with a previous study (Kadouri *et al.* 2007). However, our current study shows an increase in *M. aeruginosavorus* host range specificity. Further verification of the ability of *M. aeruginosavorus* to attach and prey on *E. coli* was performed by SEM evaluation in which the predator was added to a preformed *E. coli* ZK2686 biofilm (Pratt and Kolter 1998). SEM micrographs (Fig. 1) clearly show *M. aeruginosavorus* prey cells attaching to the *E. coli* host. No *B. bacteriovorus* cell contamination was seen in the samples.

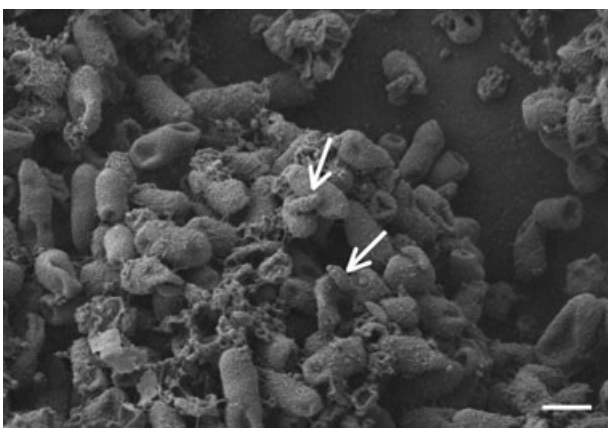

Figure 1 Predation of *Micavibrio aeruginosavorus* on *Escherichia coli* host cells. *Escherichia coli* preformed biofilm was incubated in the presence of *M. aeruginosavorus* for 24 h. The biofilm was washed to remove nonadhered cells, and SEM micrographs were taken. Arrows indicate attached *M. aeruginosavorus* prey cells to *E. coli* host. Scale bar, 1 μm. Magnification, ×10 000.

Assessing the ability of *Bdellovibrio bacteriovorus* to reduce multispecies microbial cultures

The following sets of experiments were aimed at evaluating the ability of the predator to attack and reduce pathogenic bacteria in a mixed culture. *Bdellovibrio bacteriovorus* was selected for this study as it exhibits a broader host range, which allowed for more flexibility in selecting the bacteria to be examined. Standard cocultures were prepared as described above, with each culture containing a combination of host cells. Single host cocultures were also incubated for comparison. The host cells coculture included the following: *Ac. baumannii* NCIMB 12457 and *Kl. pneumoniae* ATCC 33495; *Enterobacter gergoviae* ATCC 33028 and *Kl. pneumoniae* ATCC BAA-1706; *Ac. baumannii* ATCC 19606 and *Enterobacter cloacae* ATCC 35030. CFU enumerations of the remaining host cells were performed by plating the cocultures on selective antibiotic agar plates. The results of the experiments demonstrate that the ability of *B. bacteriovorus* to reduce host bacteria in multispecies cocultures is compatible to that of a single-species culture (Table 3).

Table 3 Ability of *Bdellovibrio bacteriovorus* to prey on bacteria inoculated in a multi-species culture suspension

Bacteria tested	CFU log reduction following predation in a single-species lysate	CFU log reduction following predation in a mix species lysate
Culture 1		
Acinetobacter baumannii NCIMB 12457	3	3·5 ± 0·5
Klebsiella pneumoniae ATCC 33495	2	2
Culture 2		
Enterobacter gergoviae ATCC 33028	4	3·5 ± 0·5
Kl. pneumoniae ATCC BAA-1706	4	4
Culture 3		
Ac. baumannii ATCC 19606	3	3
Enterobacter cloacae ATCC 35030	3	3

Cocultures were prepared by adding multispecies host cells to *B. bacteriovorus*. Predation was evaluated after 48 h of incubation. Values represent the average reduction in host cell viability counts (CFU ml^{-1}) compared to *B. bacteriovorus* minus control. Single-species host cocultures were prepared for comparison.

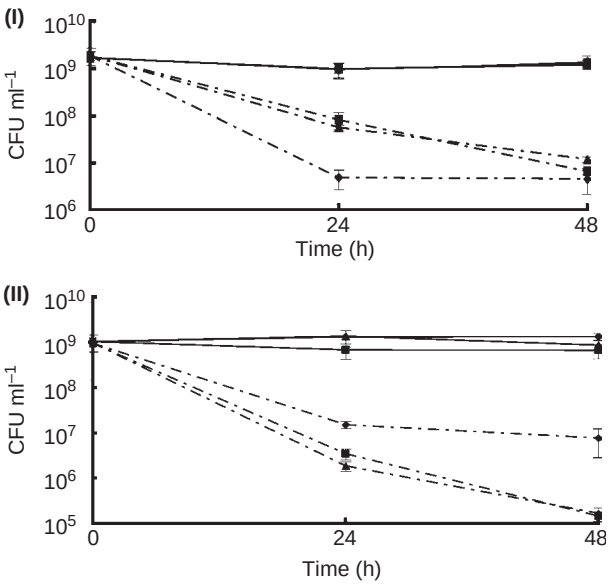

Figure 2 Effect of incubation temperature on *Micavibrio aeruginosavorus* predation. *Klebsiella pneumoniae* (I) and *Pseudomonas aeruginosa* (II) cocultures were incubated with *M. aeruginosavorus* (broken line) or predator-free control (full line) for 24–48 h. Cocultures ware placed at 25°C (square), 30°C (diamond) and 37°C (triangle). Efficiency of predation was measured by CFU counts of the remaining host cells. Each value represents the mean of three coculture. Error bars are shown as one-standard deviation.

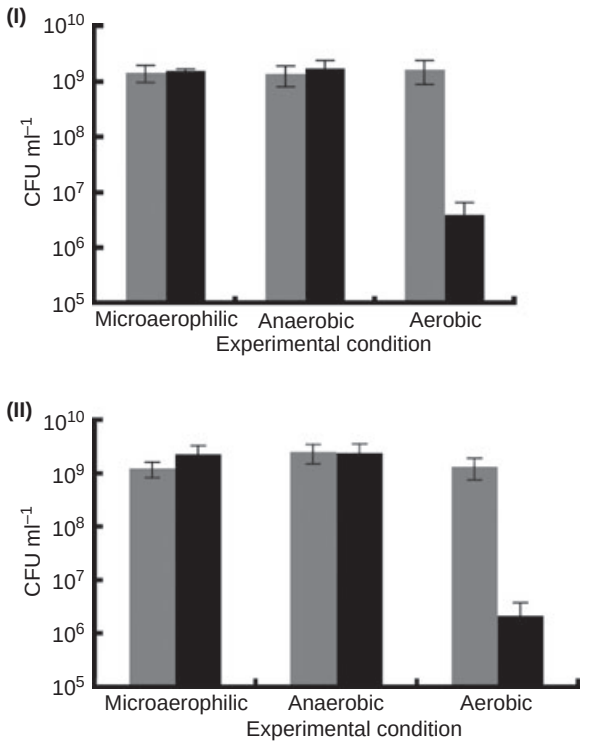

Figure 3 Effect of oxygen levels on predation by *Micavibrio aeruginosavorus*. *Klebsiella pneumoniae* (I) and *Pseudomonas aeruginosa* (II) cocultures were incubated for 48 h with *M. aeruginosavorus* (black bars) or predator-free control (grey bars) in anaerobic, microaerophilic and aerobic conditions. Efficiency of predation was measured by CFU counts of the remaining host cells. Each value represents the mean of three cocultures. Error bars are shown as one-standard deviation.

The effect of temperature and aeration on *Micavibrio aeruginosavorus* predation

Because of the limited research conducted on *M. aeruginosavorus* biology, we were interested in determining the optimal temperature as well as the effect of oxygen levels on predation. Two host bacteria, *Kl. pneumoniae* and *Ps. Aeruginosa*, were used in experiments in which the cocultures were placed at 25, 30 and 37°C. When *Kl. pneumoniae* was used as host, the most rapid reduction in host CFU counts was seen at 30°C (Fig. 2a). By 48 h, comparable reduction in host CFU numbers was measured at all three experimental temperatures. In *Ps. aeruginosa* cocultures, similar predation patterns were seen at 25 and 30°C; however, a reduction in *M. aeruginosavorus* predation capability was noted at 37°C (Fig. 2b). When placed in anaerobic and microaerophilic conditions, a loss of predation was seen, regardless of the host cell used (Fig. 3).

Evaluate the ability of *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* to form lytic halos on lawn grown bacteria

When spotted on a lawn of host bacteria, which was used to mimic a thin monolayer biofilm, *B. bacteriovorus* was able to attack and form lytic halos on 56 of the 67 examined bacteria. In general, the ability of the predator to attack and form lytic halos on the host cells mirrored the host range specificity of the predator. Among the bacteria reduced were those from the genus *Acinetobacter*, *Aeromonas*, *Bordetella*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Proteus*, *Pseudomonas* and *Vibrio*. No halos were detected on bacteria from the genus *Stenotrophomonas* and *Staphylococcus* (Table 1, predation on surface-attached lawn cells). The ability of *M. aeruginosavorus* to prey on lawns of host cells was also parallel to the host range specificity of the predator that was measured in liquid suspension. *Micavibrio aeruginosavorus* was able to prey and form halos on 48 of the 85 bacteria examined. Among the bacteria positively lysed were bacteria from the genus *Burkholderia*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Pseudomonas* and *Shigella*. Halos formed on a few of the examined species from the genus *Acinetobacter* and *Proteus*. No halos were formed when the host used was from the genus *Bordetella*, *Citrobacter*, *Erwinia*, *Morganella*, *Serratia*, *Stenotrophomonas* and *Vibrio* (Table 2, predation on surface-attached lawn cells).

Assessing the ability of predatory bacteria to reduce multilayer microbial biofilms

The following sets of experiments were aimed at examining the ability of the predator to attack and reduce a multilayer biofilm that was predeveloped in a 96-well static system. The host bacteria used to grow the biofilm were as following: *Ac. baumannii* ATCC 19606, *Ac. baumannii* NCIMB 12457, *Acinetobacter lwoffii* ATCC 17925, *Kl. pneumoniae* clinical isolate and *Morganella morganii* ATCC 25829. As before, *B. bacteriovorus* was selected to serve as the predator based on its extensive predation characteristics, which allowed for more flexibility in selecting the prey to be used. Incubating the preformed biofilm with the predator resulted in a 2–3 log reduction in CFU counts within the first 24 h of incubation. A 6–7 log reduction was measured by 48 h in biofilms composed of *Ac. baumannii* ATCC 19606, *Ac. baumannii* NCIMB 12457, *Ac. lwoffii* ATCC 17925 and the *Kl. pneumoniae* clinical isolate. No reduction in CFU host cell viability was measured in the biofilms inoculated with the *B. bacteriovorus*-free sample (Fig. 4).

Assessing the ability of *Bdellovibrio bacteriovorus* to reduce multispecies biofilms

To measure the effect of *B. bacteriovorus* predation on biofilms composed of a multispecies biofilm, mix cultures were used to form a biofilm in a 96-well static system.

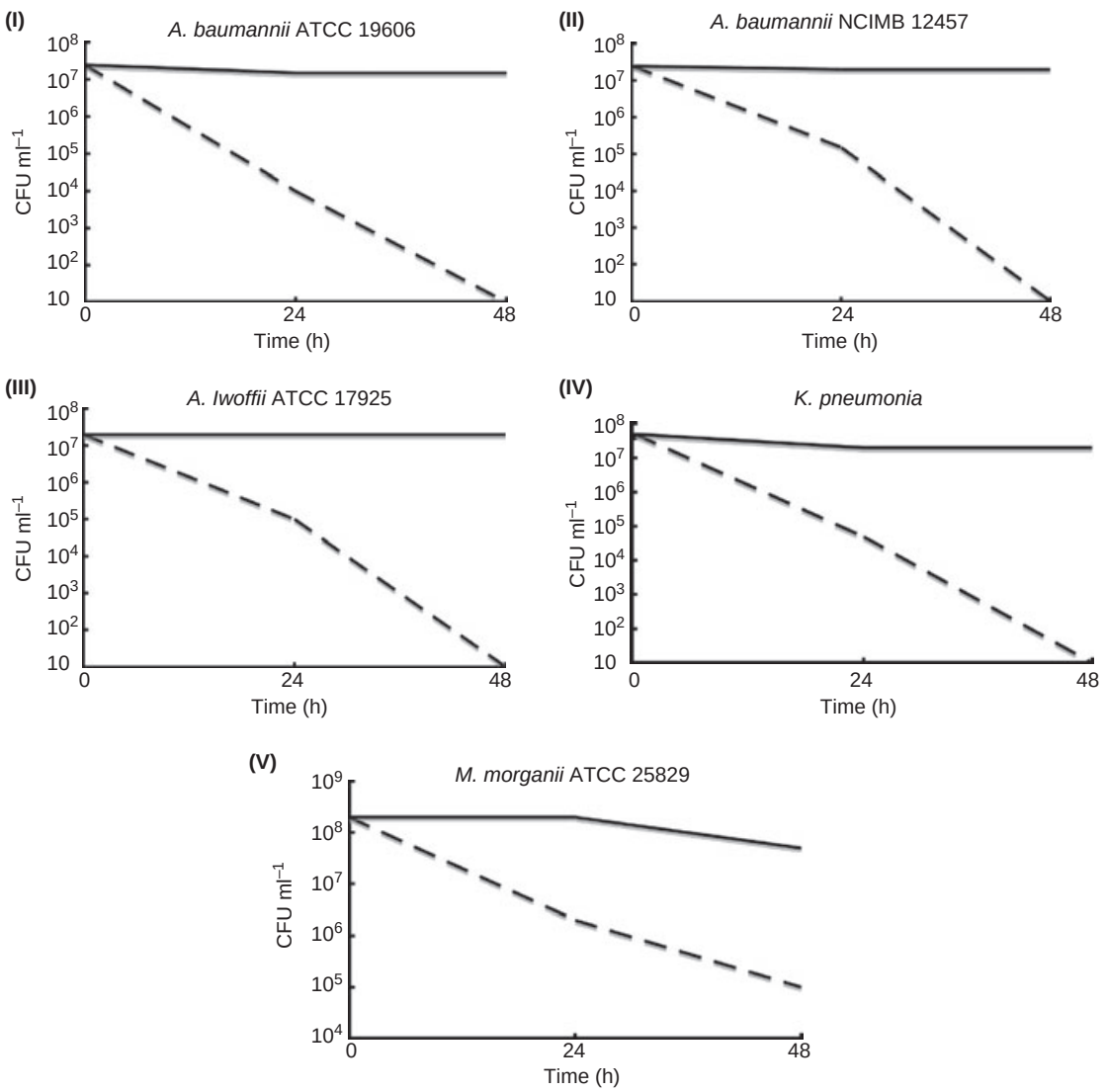

Figure 4 Reduction in multilayer microbial biofilms. Biofilms composed of microbial pathogens (I–V) were formed in a 96-well static plate. Thereafter, the preformed biofilm was rinsed and incubated with *Bdellovibrio bacteriovorus* (broken line) or predator-free control (full line). The effect of predation on the biofilm cell population was assessed by CFU enumeration of the remaining biofilm following 24 and 48 h of incubation.

Table 4 Reduction in multispecies microbial biofilms by *Bdellovibrio bacteriovorus*

Bacteria tested	CFU log reduction following predation in a single-species biofilm	CFU log reduction following predation in a mix species biofilm
Experiment 1		
Acinetobacter baumannii NCIMB 12457	2·5 ± 0·5	2
Klebsiella pneumoniae ATCC 33495	2	1
Experiment 2		
Enterobacter gergoviae ATCC 33028	2	2
Kl. pneumoniae ATCC BAA-1706	3	3

Multispecies biofilms were formed for 18 h in a 96-well static system. Thereafter, the preformed biofilm was rinsed and incubated for 24 h with *B. bacteriovorus* or *B. bacteriovorus*-free control. Values represent the average reduction in host cell viability counts (CFU ml^{-1}) compared to *B. bacteriovorus* minus control. Single host biofilms experiments were also prepared for comparison.

The 1-day-old preformed biofilm was exposed to the predator or to a predator-free control. The effect on the biofilm was measured by CFU enumeration of the remaining biofilm cells following a 24-h incubation period. Single host biofilms were also incubated for comparison. The host cells coculture included *Ac. baumannii* NCIMB 12457 and *Kl. pneumoniae* ATCC 33495 and *Ent. gergoviae* ATCC 33028 and *Kl. pneumoniae* ATCC BAA-1706. CFU enumeration was performed by sonicating the remaining biofilm and plating the cells on selective antibiotic agar plates. As seen in Table 4, the ability of *B. bacteriovorus* to reduce host bacteria in multispecies microbial cultures was similar to that of a single-species culture. A slight reduction in *B. bacteriovorus* predation efficacy was seen on biofilms composed of *Ac. baumannii* NCIMB 12457 and *Kl. pneumoniae* ATCC 33495.

SEM analysis of biofilm predation

To further visualize the impact of predation by *B. bacteriovorus* on single and multispecies biofilms, Biofilms were developed on PVC plastic cover slip (Fig. 5, preformed biofilms). Thereafter, the biofilms were incubated, for 24 h, in the presence of *B. bacteriovorus* (Fig. 5, *B. bacteriovorus*) or predator-free control (Fig. 5, control). A clear reduction in biofilm biomass was seen on all the examined biofilms, with the majority of the biofilm host cells being cleared, leaving *B. bacteriovorus* cells attached to the remaining biofilm debris.

Discussion

In the work presented here, the host specificity of *B. bacteriovorus* 109J and *M. aeruginosavorus* ARL-13 was examined. Although the host range susceptibility of both predators was previously examined (Nakamura 1972; Lambina *et al.* 1983; Richardson 1990; Fratamico and Cooke 1996; Kadouri *et al.* 2007), this is the first study, to our knowledge, aimed at specifically assessing the capacity of these predators to prey on micro-organisms which are becoming predominant in medical settings and untreatable multidrug-resistant infections.

In general, *B. bacteriovorus* demonstrated an extremely broad host range when compared to *M. aeruginosavorus*, attacking a wide array of medically relevant Gram-negative pathogens. Furthermore, the prey-reducing ability of *B. bacteriovorus* was greater than that of *M. aeruginosavorus*, as measured by CFU enumeration. This could be explained by the fact that whereas *M. aeruginosavorus* replicates by binary fission, producing one daughter cell at a time (Lambina *et al.* 1982), *B. bacteriovorus* produces numerous progeny, which escape from the bdelloplast after consumption of the host (Sockett 2009). Although the host range of *M. aeruginosavorus* is relatively narrow, it might still be of significance as it could attack host cells, such as *Ps. aeruginosa*, which are less susceptible to predation by *B. bacteriovorus*. An interesting observation was that the host range specificity of *M. aeruginosavorus* ARL-13 seemed to increase, when compared to a previous study (Kadouri *et al.* 2007). *Micavibrio aeruginosavorus* ARL-13 was found to prey on bacteria from the genus *Burkholderia*, *Escherichia*, *Klebsiella*, *Pseudomonas* and *Shigella* as well as a limited ability to attack bacteria from the genus *Acinetobacter*, *Enterobacter*, *Proteus* and *Yersinia*. SEM micrographs have provided additional evidence supporting the finding that *M. aeruginosavorus* is able to attach to *E. coli* cells (Fig. 1). As no predation was seen in the control samples, which were inoculated with the 0·22-μm filtered *M. aeruginosavorus* lysates, nor did we find any *B. bacteriovorus* cells in the *M. aeruginosavorus* cocultures, we could conclude that the redaction in host cells resulted from a breach in *M. aeruginosavorus* host specificity and not by a phage or *Bdellovibrio* contamination. Early studies performed on *M. aeruginosavorus* demonstrated that the ARL-13 strain exhibits a narrow host range, preying only on *Ps. aeruginosa* (Lambina *et al.*

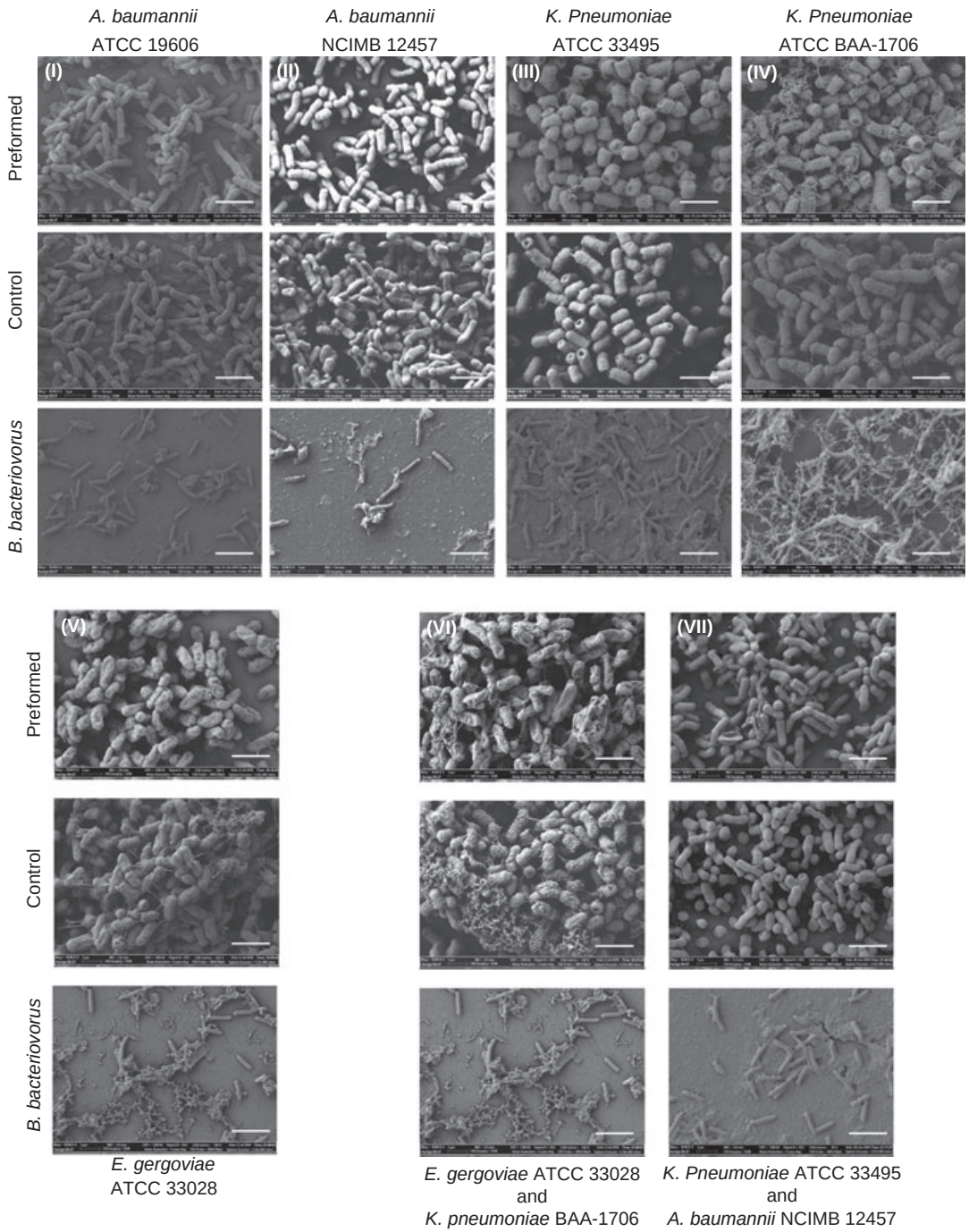

Figure 5 SEM analysis of biofilm predation. Single (I–V) and multispecies (VI–VII) biofilms were developed on PVC plastic cover slips for 18 h (preformed). Thereafter, the biofilms were washed and incubated for 24 h with *Bdellovibrio bacteriovorus* or filtered sterilized lysate (control). Scale bar, 2 μm. Magnification, ×10 000. Images were collected from biofilms grown at the air–liquid interface.

1983). The first breach in host specificity was reported in 2007 (Kadouri *et al.* 2007) in which a stored sample of ARL-13, provided by Dr Jurkevitch from the Hebrew University of Jerusalem, demonstrated an ability to prey on *B. cepacia* and *Kl. pneumoniae*. No predation was observed at the time on *Acinetobacter*, *E. coli*, *Enterobacter*, *Shigella* and *Yersinia*; thus, it seems that a change in host specificity does occur with time. The loss of host specificity, as a result of extended storage periods, was reported for *Micavibrio admirandus* ARL-14. It was shown that the

ARL-14 strain lost its host specificity following a 3-year storage period in liquid culture, in which it was reseeded numerous times (Afinogenova et al. 1986). As the mechanisms that govern predation are far from being fully understood, it is difficult to speculate why a breach in host specificity occurs. However, the fact that other *Micavibrio* species, such as *M. admirandus* ARL-14, do possess an ability to prey on Enterobacteriaceae suggests that other *Micavibrio* spp. have the potential to attack Enterobacteriaceae and broaden their host range specificity if needed. Although the breach of host specificity could be considered an advantage, as it might allow the use of *Micavibrio* spp. against additional pathogens, it should be carefully investigated to assure the inability of the predator to attack eukaryotic cells. In our study, we focused on the host range specificity of *B. bacteriovorus* 109J and *M. aeruginosavorus* ARL-13; however, other representatives from the genus *Bdellovibrio* and *Micavibrio* might exhibit different host range characteristics and should be examined in the future.

As infections could arise from single or multispecies pathogens, the ability of predatory bacteria to reduce host cells in a mix culture suspension was evaluated. Compatible reduction in cell viability was seen in host cells cultured separately or in multispecies suspension (Table 3). It was previously shown, in mix culture predation studies, that *B. bacteriovorus* differentially preys on one host over another. However, cell viability was measured only 3 h after the onset of introducing the predator (Rogosky *et al.* 2006). Thus, it seems that when left for extended time periods, *B. bacteriovorus* will ultimately consume both hosts indistinctively.

When considering the use of predatory bacteria as a 'live antibiotic', one needs to deliberate on the environment in which predation needs to occur. Infection mitigation could require predation in fully aerated sites at 30–35°C, such as superficial burns and wounds, or in predominantly anaerobic settings with temperatures of 37°C. Such conditions may exist in indwelling medical device infections. To this end, the optimal prey condition of *M. aeruginosavorus* was examined. As reported for *B. bacteriovorus* (Seidler and Starr 1969), *M. aeruginosavorus* was able to prey on both *Kl. pneumoniae* and *Ps. aeruginosa*, at temperatures ranging from 25 to 37°C, with 25 and 30°C being the optimal temperature for predation on *Ps. aeruginosa* and 25°C for *Kl. pneumoniae*. As *M. aeruginosavorus* does have a capacity to attack at 37°C, an attempt could be made to enrich for *M. aeruginosavorus* variants that are much more suitable to prey at 37°C and above by sequentially reculturing the predator at higher temperatures. Although able to prey at elevated temperatures, *M. aeruginosavorus* predation did halt under microaerophilic and anaerobic conditions. The incapacity to prey in oxygen-limiting conditions was also reported for *B. bacteriovorus* (Schoeffield *et al.* 1996). Therefore, the aeration conditions of the infection site should be taken into account when considering the application of the predators.

It is widely believed that the majority of infections, particularly chronic infections, are caused by communities of micro-organisms which exist as biofilms (Burmolle *et al.* 2010; Ehrlich *et al.* 2010; Ferreira *et al.* 2010; Percival *et al.* 2010). Therefore, the ability of the predators to prey on surface-associated bacteria was examined. When spotted on a thin lawn of host cells, the ability of the predators to prey and form a lytic zone of clearance mirrored the predator's host range seen in liquid suspension. The biofilm lawn assay was used to represent a thin biofilm that is nourished from beneath. Such a biofilm could be encountered in superficial wound and burn infections. *Bdellovibrio bacteriovorus* was also able to attack and significantly reduce more robust biofilms developed in 96-well plates. Biofilm reduction was seen in both single and multispecies biofilms. The reduction in CFU numbers, following predation by *B. bacteriovorus*, was consistently higher in biofilm-associated host cells than in planktonic cocultures. This is probably because of the high cell density in which biofilm bacteria exist and the relative ease in which the predator can encounter its prey. Further confirmation of the biofilm degrading ability of *B. bacteriovorus* was obtained by SEM micrographs, which showed a profound reduction in biofilm biomass within 24 h of predation. The images also emphasize that the action of predation is not restricted to the exterior of the biofilm, which is frequently observed with invertebrates protozoan and bacteriophage (Doolittle *et al.* 1996; Lawrence *et al.* 2002; Matz *et al.* 2004), but could influence thicker and more robust biofilms, such as those developed at the air–liquid interface. The ability of predatory bacteria to attach, penetrate, proliferate and reduce a preformed biofilm is in agreement with previous reports (Fratamico and Cooke 1996; Kadouri and O'Toole 2005; Nunez *et al.* 2005; Kadouri *et al.* 2007).

With the emergence of new multidrug-resistant bacteria such as *Ac. baumannii*, *E. coli* and *Kl. pneumoniae* bearing NDM-1 (Nordmann *et al.* 2009; Yong *et al.* 2009; Karthikeyan *et al.* 2010) and biofilm related infections, the need to invest in new antimicrobial strategies is becoming evident. One such strategy might be incorporating predatory bacteria, or antimicrobials derived from these organisms, as a biologically based treatment. The advantages of using predatory bacteria are numerous. For example, they could prey on several of the most medically relevant pathogens and yet appear to be harmless to eukaryotic cells (Westergaard and Kramer 1977; Sockett and Lambert 2004); they are effective against bacteria regardless of their ability to resist antibiotic treatment;

they could attack biofilms as well as planktonically grown bacteria (Fratamico and Cooke 1996; Koval and Bayer 1997; Kadouri and O'Toole 2005; Nunez *et al.* 2005; Kadouri *et al.* 2007); genetically stable host resistance does not seem to develop as a response to predation (Shemesh and Jurkevitch 2004). However, many questions regarding the full potential of predatory bacteria as a biocontrol agent still remain unanswered. For example, what will be the efficiency of predation in the presence of a host immune system? Will predatory bacteria provoke an aggressive immune response? Will predation be sufficient to clear the site from the infection or at least reduce the microbial load to levels which will allow the host immune system to rid itself from the infection? What will be the fate of the commensal Gram-negative microbial population. All these questions will need to be thoroughly investigated in *in-vivo* model systems before the use of predatory bacteria could be considered. This will probably be the focus of future investigations.

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Inhibition of Predation by *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* via Host Cell Metabolic Activity in the Presence of Carbohydrates▽

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***Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are highly motile Gram-negative predatory bacteria with the potential of being used as biocontrol agents or living antibiotics. It was suggested previously that sugar-binding proteins play a role in *M. aeruginosavorus* and *B. bacteriovorus* host specificity and predator-prey interactions. The effect of carbohydrates on predation was reexamined in this study. It was demonstrated that the presence of carbohydrates could indeed block predation. However, further investigation demonstrated that inhibition of predation was due to medium acidification by the metabolic activity of the host and not to a blocking of a putative sugar-binding protein. The data presented here might be of value when storing, growing, and cultivating predatory bacteria, as well as when considering environmental conditions that might influence predation in the field.**

Bdellovibrio and *Micavibrio* species are Gram-negative, motile, and uniflagellate bacteria characterized by predatory behavior or an obligatory parasitic life cycle. Recently, these bacteria have drawn new interest for their potential use as "live antibiotics" (9, 30). The *Bdellovibrio* life cycle, with *Bdellovibrio bacteriovorus* being the most studied representative of the genus, consists of an attack-phase cell that attaches to other Gram-negative bacteria, penetrates their periplasm, multiplies in the periplasmic space, and finally bursts the cell envelope to start the cycle anew (24, 29, 31). Unlike that of *Bdellovibrio* spp., our knowledge of *Micavibrio* biology is somewhat modest. *Micavibrio* belongs to the alpha subgroup of proteobacteria (11); they are small (0.5 to 1.5 μm long), rod shaped, and curved and have a single polar flagellum. The *Micavibrio* life cycle includes the attachment, or leeching, of a motile attack-phase cell to its prey, followed by growth on the surface of the host and, finally, the death of the infected cells (2, 18, 19). Unlike *B. bacteriovorus*, which is considered to have a broad host range (9, 31), *Micavibrio* spp. such as *M. aeruginosavorus* are host specific (1, 9, 16, 19). To date, one of the key questions puzzling researchers is what governs host specificity and host-predator recognition.

One factor that might be involved in predator-prey interactions and host specificity is protein-carbohydrate interactions. Lectins are sugar-binding proteins that play a role in many biological recognition phenomena, one of which is recognition of host cells by microorganisms. For example, the adherence of bacteria to host cells is in many cases mediated by lectin-like adhesins on the bacterial surface that bind to carbohydrate receptors present on the host cell surface as part of the membrane glycoproteins and glycolipids (5, 15, 23). Lectins are also involved in recognition and attachment of fungi, protozoa, and viruses to their host cells during infection (3, 13, 20, 25, 32, 34).

In 1984, Chemeris and colleagues demonstrated that the predation of *B. bacteriovorus* could be inhibited by the addition of sugars to coculture media and by modification of the host cell surface polysaccharide (8). Similar findings were reported for *Micavibrio*, in which a range of exogenous sugars prevented predation of *Pseudomonas aeruginosa* by *Micavibrio admirandus* ARL-14 (7). Based on these data, the authors concluded that carbohydrate-protein receptors play a role in host-predator interaction.

In this study, the putative role of carbohydrates in predator-prey interactions was reexamined. Initial experiments concurred that carbohydrates block predation by both predators. However, further examination revealed that the sugar inhibition effect resulted from medium acidification caused by host metabolic activity and not from the blocking of a sugar-binding protein.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The host strains used in this study were *Acinetobacter baumannii* NCIMB 12457, *Klebsiella pneumoniae* ATCC 13883 (16), *Escherichia coli* strains DH5α, S17-1, and WM3064, a diaminopimelic acid auxotroph (26). The predatory bacteria used were *B. bacteriovorus* 109J (ATCC 43826), HI-A, a facultative host-independent (HI) variant of *B. bacteriovorus* 109J (22), and *M. aeruginosavorus* strain ARL-13 (16, 19). Host bacteria were grown routinely in LB media at 37°C. Strain WM3064 medium was supplemented with 0.3 mM diaminopimelic acid (DAP). HI-A was grown in peptone-yeast extract (PYE) (22). *M. aeruginosavorus* and *B. bacteriovorus* were maintained as plaques in double-layered, diluted nutrient broth (DNB) agar, a 1:10 dilution of nutrient broth amended with 3 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$ [pH 7.2], and agar (0.6% agar in the top layer). In order to initiate a lysate, cocultures were obtained by adding a plug of agar containing *B. bacteriovorus* or *M. aeruginosavorus* plaques to washed prey cells in DNB, which were incubated at 30°C on a rotary shaker set at 200 rpm until the coculture became clear (stock lysate). To harvest the predators, cocultures were prepared by adding 2 ml of washed host cells ($\sim 1 \times 10^9$ CFU/ml) and 2 ml of stock lysate to 20 ml DNB. The cocultures were incubated for 18 and 48 h (for *B. bacteriovorus* and *M. aeruginosavorus*, respectively) to reach a final concentration of $\sim 1 \times 10^8$ PFU/ml predator. Thereafter, the lysate was passed through a 0.45-μm-pore-size Millex filter (Millipore, Billerica, MA) in order to remove residual prey and cell debris (filtered lysate). As a *Bdellovibrio*-free control, filtered sterilized lysate was prepared by passing the lysates through a 0.22-μm-pore-size filter (9).

Chemicals. The following compounds were used in the study: arabinose, dextrose (D-glucose), galactose, fructose, mannose, sucrose, lactose, xylose, L-glu-

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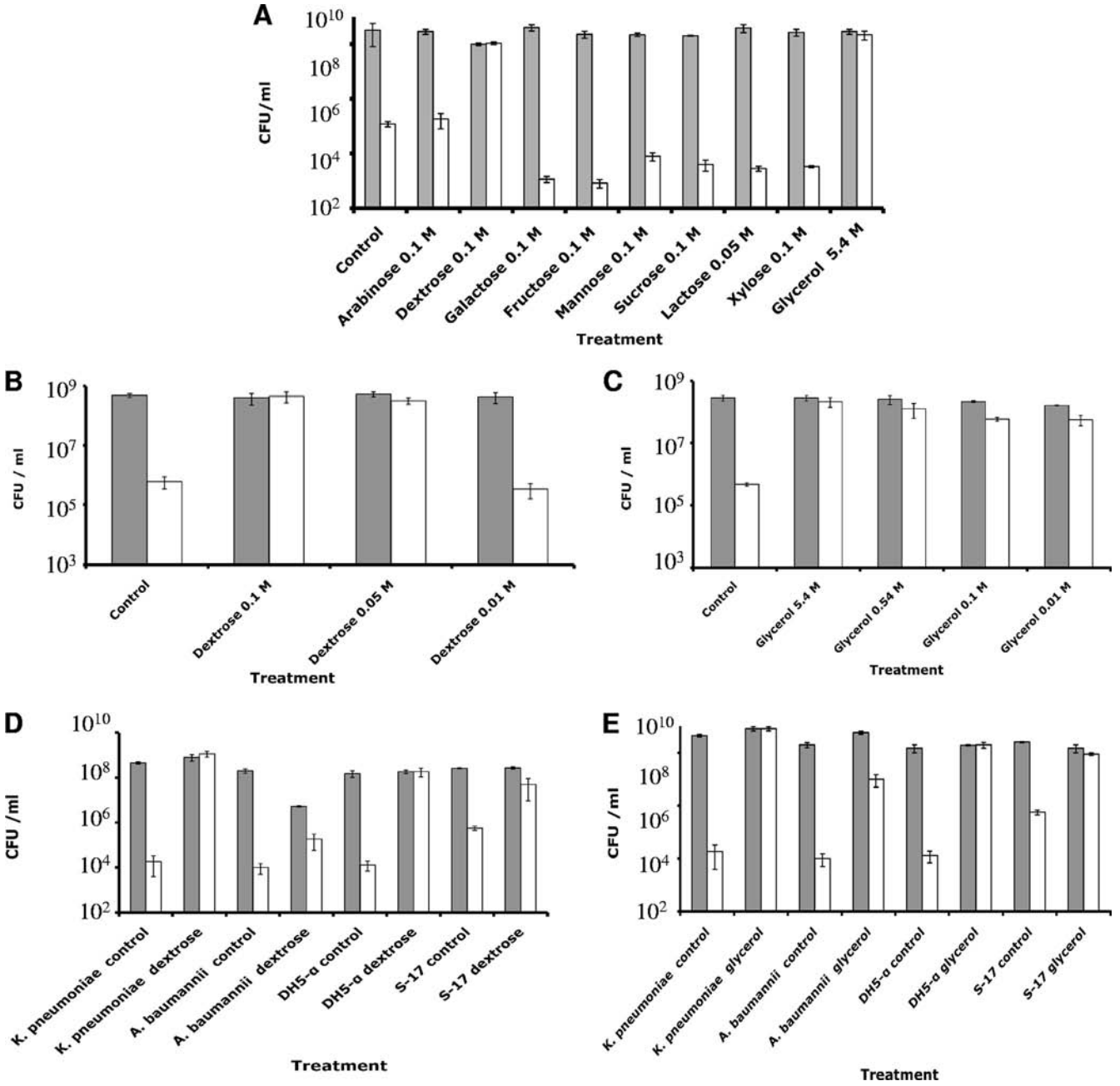

FIG. 1. *B. bacteriovorus* predation in the presence of carbohydrates. (A) *E. coli* WM3064 was cultured in DNB (control) and DNB supplemented with carbohydrates. Gray bars, without *B. bacteriovorus*; white bars, with *B. bacteriovorus*. (B and C) Predation in the presence of various concentrations of dextrose (B) and glycerol (C). (D and E) Predation of *K. pneumoniae*, *E. coli* DH5α, *E. coli* S17-1, and *A. baumannii* in the presence 0.1 M dextrose (D) and 0.54 M glycerol (E). All cultures were incubated for 24 h. Predation was evaluated by CFU enumeration of the remaining host cells. Each value represents the mean of results from three cocultures. Error bars are shown as 1 standard deviation.

cose, methyl-α-D-glucopyranoside, and glycerol. All compounds were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO).

Predation experiments. To evaluate the ability of *B. bacteriovorus* and *M. aeruginosavorus* to prey in the presence of carbohydrates, cocultures were prepared as described before (9, 10). In brief, washed prey cells were incubated in 5 ml DNB media with harvested *B. bacteriovorus* or *M. aeruginosavorus*. Filtered, sterilized lysate was used as a control. Selected compounds were added to the cocultures at a final concentration of 0.1 M, unless stated differently below. The cocultures were incubated at 30°C on a rotary shaker set at 200 rpm. The ability of predators to predate was confirmed by the reduction in host cell viability, measured by CFU enumeration. Each experiment was performed three times. Predation on metabolically inactive host cells was done by heating the host cells to 65°C for 50 min. No viable host cells remained after the heat treatment, as confirmed by CFU enumeration. The metabolically inactive cells were cocultured with the predator. Predation was evaluated by the change in culture turbidity, measured at 595 nm.

RESULTS

Carbohydrates inhibit the predation of *B. bacteriovorus*. To examine the effect of sugars on predation, *B. bacteriovorus* 109J was cocultured with *E. coli* strain WM3064. As seen in Fig. 1,

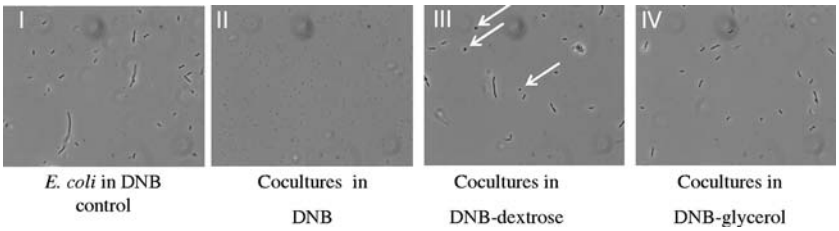

FIG. 2. Predation in the presence of dextrose and glycerol. Microscopic evaluation of overnight cocultures. (I) *E. coli* alone; (II) *B. bacteriovorus-E. coli* cocultures incubated in DNB; (III) *B. bacteriovorus-E. coli* cocultures incubated in DNB and 0.1 M dextrose; (IV) *B. bacteriovorus-E. coli* cocultures incubated in DNB and 0.54 M glycerol. Arrows indicate bdelloplasts. Magnification, ×1,000. Each experiment was carried out three times, with 10 to 20 fields viewed each time, yielding similar results; representative images are shown here.

the presence of 0.1 M dextrose or 5.4 M (50%, vol/vol) glycerol completely inhibited predation. The dextrose and glycerol predation-inhibiting effect was found to be dose dependent, effectively inhibiting predation at 0.05 M dextrose (Fig. 1B) and 0.01 M (0.1%, vol/vol) glycerol (Fig. 1C).

Dextrose's and glycerol's effect on predation is not host specific. Predation experiments were done using four additional host cells. Dextrose (0.1 M) reduced *B. bacteriovorus*'s ability to prey on *K. pneumoniae* and *E. coli* DH5α and S17-1. A more moderate inhibition effect was seen on *A. baumannii* (Fig. 1D). Glycerol (0.54 M; 5%, vol/vol) also halted the predation of *K. pneumoniae*, *E. coli* DH5α, and S17-1, with a reduced predation-inhibiting effect measured for *A. baumannii* (Fig. 1E).

Dextrose and glycerol do not affect *B. bacteriovorus* viability. To measure the effect of dextrose and glycerol on *B. bacteriovorus*'s viability, pure *B. bacteriovorus* prey cells (4.7×10^7 PFU/ml) were resuspended in DNB and DNB supplemented with 1 M dextrose or 0.54 M glycerol. Cell viability was measured by PFU enumeration after 24 h. Similar reductions in *B. bacteriovorus* cell viability were measured in all of the tested samples (2.1×10^7, 1.9×10^7, and 2.5×10^7 PFU/ml with DNB, dextrose, and glycerol, respectively).

The predation-inhibiting effect of dextrose is transient. To assess if dextrose's and glycerol's effects on predation are reversible, *B. bacteriovorus* was cocultured with *E. coli* (2.1×10^8 CFU/ml) in DNB supplemented with 0.1 M dextrose or 0.01 M glycerol. As before, no predation occurred in the overnight cocultures grown in DNB containing dextrose or glycerol, with 2×10^8 CFU/ml host remaining. Thereafter, the cells were collected by centrifugation and washed to remove residual carbohydrates. The cells were divided into two flasks, one resuspended in DNB alone and one in DNB supplemented with 0.1 M dextrose or 0.01 M glycerol. Removing the dextrose and resuspending the cells in dextrose-free medium restored predation (with 8.8×10^2 CFU/ml of the host remaining). However, no predation occurred in the culture that was resuspended in new dextrose solutions (2×10^8 CFU/ml host). Although predation inhibition by dextrose was transient, the inhibiting effect of glycerol was found to be irreversible. No reduction in host cells was seen after incubating the glycerol-grown cocultures in fresh glycerol-free medium (2×10^8 CFU/ml host).

Microscopic examination of the overnight cocultures revealed that the host was consumed by the *B. bacteriovorus* in the DNB-incubated control, with only *B. bacteriovorus* attack-phase cells seen in the field of view (Fig. 2, panel II). In contrast, no *B. bacteriovorus* cells were viewed in the *B. bacteriovorus*-free control or in the DNB-glycerol cocultures, with only intact *E. coli* cells present (Fig. 2, panels I and IV, respectively). Although no evidence of predation was seen in the DNB-dextrose cocultures, host cells containing *B. bacteriovorus* (bdelloplasts) were present. After 20 fields of view were inspected, it was estimated that 15% ± 5% of the host cells appear to be bdelloplasts (Fig. 2, panel III).

Glucose analogs do not inhibit predation. To examine the effect of nonmetabolized sugars on predation, L-glucose and methyl-α-D-glucopyranoside, two glucose analogs not utilizable by *E. coli* (4), were used. As seen in Fig. 3A, adding a 0.1 M concentration of the glucose analogs to the *B. bacteriovorus-E. coli* cocultures did not inhibit predation. Similar results were obtained when *K. pneumoniae* was used as the host (Fig. 3B).

Host cell viability is required for the dextrose and glycerol predation-inhibiting effect. When cocultured in the presence of nonviable heat-killed *E. coli* host cells, dextrose and glycerol were unable to affect predation. A similar reduction in culture turbidity was measured in cocultures suspended in DNB alone (positive control), DNB-dextrose, and DNB-glycerol (Fig. 3C).

Carbohydrate utilization and medium acidification plays a role in inhibiting predation. Although dextrose was able to inhibit predation in DNB media, no predation-inhibiting effect was seen in *E. coli* or *K. pneumoniae* cocultures that were prepared in 25 mM HEPES buffer (pH 7.5) supplemented with 0.1 M dextrose (Table 1). Similar results were observed when glycerol was added to the HEPES buffer (data not shown).

To determine if medium acidification occurred in the presence of carbohydrates, *E. coli* cells were incubated in DNB media supplemented with 0.1 M dextrose or 0.54 M glycerol. A drop in medium pH from 6.5 to below 4 was measured in both cultures within the first 5 h of incubation. No reduction in pH was seen when cells were grown with L-glucose and methyl-α-D-glucopyranoside (reaching a pH of 6.5), or when *E. coli* cells were grown with dextrose or glycerol which was suspended in HEPES buffer (reaching a pH of 7.5). Heat-deactivated *E. coli* cells were also unable to acidify DNB media supplemented with dextrose and glycerol (reaching a pH of 6.5 to 7). It was also noted that medium acidification by *A. baumannii* took longer to develop and was not as extreme as seen in *E. coli* and *K. pneumoniae* (reaching a pH of 4.5 after 18 h).

A correlation between medium acidification and predation was also seen in *B. bacteriovorus-K. pneumoniae* cocultures. When these organisms were incubated in the presence of car-

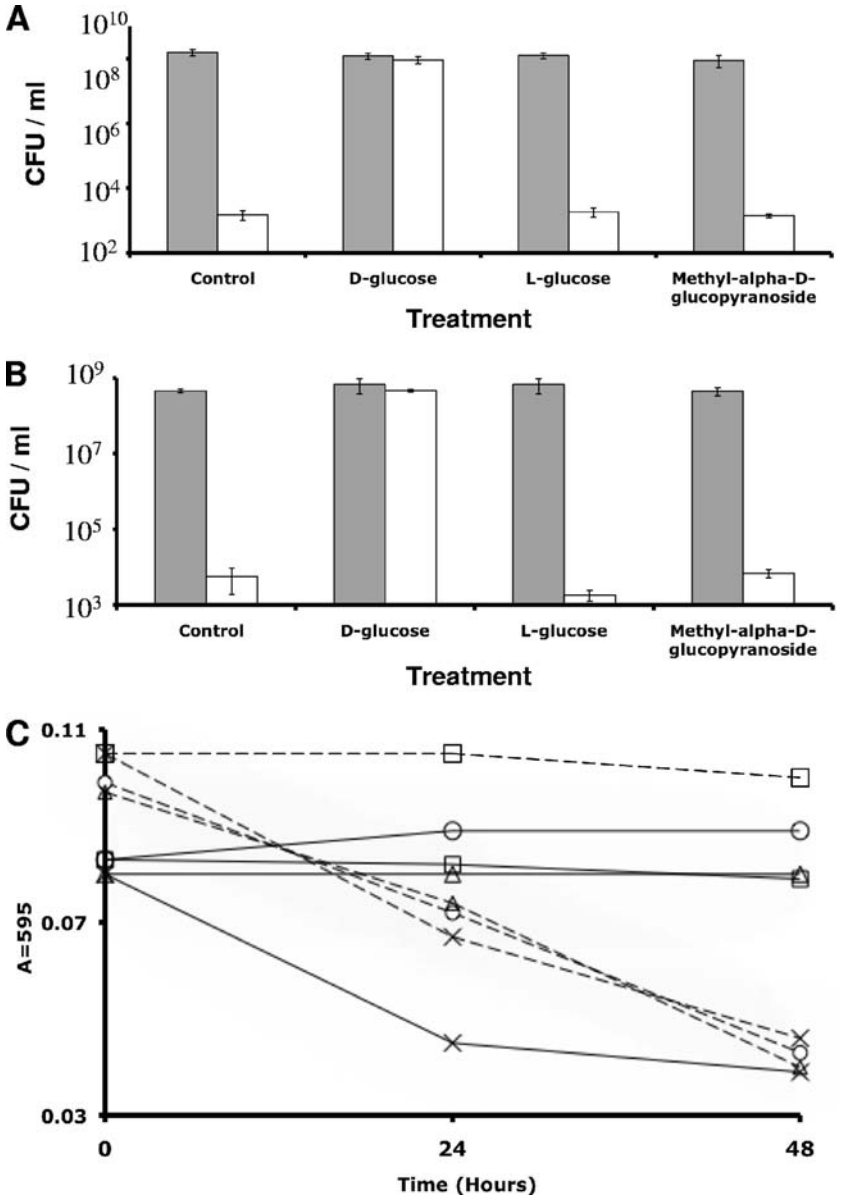

FIG. 3. Inhibition of predation by dextrose requires active utilization of the sugar by the host. Predation in the presence of glucose analogs. *E. coli* (A) and *K. pneumoniae* (B) were cultured alone (gray bars) or with *B. bacteriovorus* (white bars). Cultures were prepared in DNB or DNB supplemented with 0.1 M each compound. Cultures were incubated for 24 h. Predation was evaluated by CFU enumeration of the remaining host cells. Each value represents the mean of results from 3 cocultures. Error bars are shown as 1 standard deviation. (C) Predation on nonviable host cells. Cocultures were prepared using viable (unbroken lines) or heat-deactivated (broken lines) host cells. *E. coli* was cultured in DNB alone (□) or with *B. bacteriovorus* in DNB (×), DNB-dextrose (○), and DNB-glycerol (△). Predation was evaluated by the change in culture turbidity measured at 595 nm.

bohydrates, a drop in pH levels from 6.5 to below 4 and a loss of predation were measured in all of the examined carbohydrates (Table 2). Active predation was measured only in the sugar-free DNB control, in which no pH drop was recorded.

Medium acidification causes rapid *B. bacteriovorus* cell death. In order to investigate whether acidification of the media caused *B. bacteriovorus* cell death and consequently predation inhibition, three experiments were done. First, *B. bacteriovorus* wild-type (WT) and HI-A cells were resuspended in DNB medium that had been acidified by pyruvic acid to pH 4. As seen in Fig. 4A, a rapid reduction of *B. bacteriovorus* and HI-A cell viability was seen in the acidified medium but not in the nonacidified control. In a second experiment, *B. bacteriovorus-E. coli* cocultures were prepared and incubated in acidified DNB (pH 4) and in regular DNB (pH 6.5). As expected, predation occurred only in the nonacidified control (Fig. 4B). In the third experiment, *E. coli* was grown in DNB, DNB with 0.1 M dextrose, or DNB supplemented with 0.54 M glycerol. Following 18 h of incubation, the cells were removed by centrifugation and the supernatant was filter sterilized. The pH of each medium was measured. The cells grown in DNB did not reduce the pH of the medium (pH 6.5). However, medium

TABLE 1. Predation in HEPES buffer[a]

Medium	Host	*E. coli*		*K. pneumoniae*	
		CFU/ml	pH	CFU/ml	pH
DNB	Control	1.1×10^8	6.5	4.5×10^8	6.5
	Bdellovibrio	2.1×10^3	7	2.5×10^3	7
	Bdellovibrio and dextrose	1.3×10^8	4	4.6×10^8	3.5
HEPES	Control	1.2×10^8	7.5	3.4×10^8	7.5
	Bdellovibrio	1.3×10^3	7.5	2.8×10^3	7.5
	Bdellovibrio and dextrose	1.3×10^3	7.5	4.6×10^3	7.5

[a] *E. coli* (1.3×10^8 CFU/ml) and *K. pneumoniae* (4.4×10^8 CFU/ml) were cultured in DNB (pH 6.5) and HEPES buffer (pH 7.5) with no *B. bacteriovorus* (control), with *B. bacteriovorus* (*Bdellovibrio*), or with *B. bacteriovorus* and 0.1 M dextrose (*Bdellovibrio* and dextrose). Medium pH and host CFU numbers were measured following 24 h of incubation.

TABLE 2. Predation of *K. pneumoniae* in the presence of carbohydrates

Carbohydrate	No. of host CFU/ml	pH of medium
Control	5×10^4	7
Ara	8.1×10^8	2.5
Dex	5×10^8	3
Gal	8×10^8	2
Fru	8.5×10^8	3
Man	9×10^8	2.5
Suc	9.2×10^8	4.2
Lac	8×10^8	2.5
Xyl	9.8×10^8	2.5
Gly	1.1×10^8	4.25

K. pneumoniae (5.5×10^8 CFU/ml) was cocultured in DNB medium (pH 6.5) with *B. bacteriovorus* (control) or *B. bacteriovorus* supplemented with a 0.1 M concentration (exceptions noted) of the following carbohydrates: arabinose (Ara), dextrose (Dex), galactose (Gal), fructose (Fru), mannose (Man), sucrose (Suc), lactose (Lac), xylose (Xyl), and glycerol (Gly; 0.54 M). Medium pH and host CFU numbers were measured following 24 h of incubation.

acidification did occur in supernatants derived from cultures that were grown in DNB-dextrose and DNB-glycerol (pHs 4 and 3.5, respectively). Thereafter, *B. bacteriovorus-E. coli* cocultures were prepared in each one of the sterile supernatants and incubated for 18 h. Numbers of CFU of the remaining host revealed that predation occurred only in cocultures which were cultivated in DNB supernatants. A reduction in *B. bacteriovorus* cell viability was also measured in cocultures incubated in spent sterilized supernatants that originated from DNB-dextrose- and DNB-glycerol-grown cells (Fig. 4C).

Carbohydrates inhibit predation by *M. aeruginosavorus*. To examine the effect of carbohydrates on predation by *Micavibrio*, *M. aeruginosavorus* was cocultured in the presence of carbohydrates, with *K. pneumoniae* or *E. coli* strain WM3064 used as a host (Fig. 5). As seen in Fig. 5A, all of the selected carbohydrates were able to inhibit the predation of *K. pneumoniae* by *M. aeruginosavorus*. The carbohydrate inhibiting effect was also seen when *E. coli* strain WM3064 was used as the host, with only sucrose and lactose having no predation-inhibiting capability (Fig. 5B). Additionally, a correlation between the ability of the host cells to acidify the media and inhibition of predation was observed in all of the cocultures (Fig. 5A and B).

Although DNB media supplemented with 0.1 M dextrose inhibited predation by *M. aeruginosavorus*, no inhibitory effect was seen when DNB cocultures were supplemented with 0.1 M glucose analogs (L-glucose and methyl-α-D-glucopyranoside) or when the dextrose was added to cocultures suspended in HEPES buffer (pH 7.5) (Fig. 5C). As before, a positive correlation between the ability of the host to acidify the media and inhibition of predation was observed (Fig. 5C).

***M. aeruginosavorus* viability is lost at low pHs.** To establish that the inhibition of predation resulted from the inability of the predator to survive at low pHs, 6.5×10^5 PFU/ml *M. aeruginosavorus* was suspended for 24 h in DNB, DNB supplemented with 0.1 M dextrose or fructose (pH 6), and DNB acidified by pyruvic acid to pHs of 5, 4, and 3. Suspending the cells in DNB, DNB at pH 5, DNB-dextrose, and DNB-fructose did not result in a substantial reduction in *M. aeruginosavorus* viability (5.5×10^5, 2.5×10^5, 4.5×10^5, and 5.5×10^5 PFU/ml, respectively). However, medium acidification to below pH 5 caused a significant reduction in cell numbers (<1 PFU/ml).

To validate that *M. aeruginosavorus* cell death occurred in the presence of dextrose, *K. pneumoniae* and *M. aeruginosavorus* were cocultured in DNB and DNB-dextrose. After 24 h of incubation, a reduction in host viability (from 2.8×10^9 to 8×10^5 CFU/ml) and an increase in *M. aeruginosavorus* cell numbers (from 1.5×10^5 to 5.6×10^7 PFU/ml) was seen in the DNB coculture control, whereas in the dextrose cocultures, no surviving *M. aeruginosavorus* organisms were detected (<1 PFU/ml) and no reduction in host cell viability was measured (2.7×10^9 CFU/ml).

DISCUSSION

With the renewed interest in using predatory bacteria as "live antibiotics" (9, 30), the necessity of understanding the mechanisms which govern host specificity and early predator-prey interactions are evident. One possible factor suggested to play a role in *M. aeruginosavorus* and *B. bacteriovorus* predator-host interactions is lectin sugar-binding proteins. The first evidence that host cell surface composition might be involved in *Bdellovibrio* host specificity was reported by Varon and Shilo (36) and Houston and colleagues (14), who demonstrated that cell wall host peptidoglycan R antigens are involved in *B. bacteriovorus*'s interaction with *E. coli* and *Salmonella enterica*. Other studies suggested that covering the surface of the host with paracrystalline protein surface layers (S layers) might provide resistance to predation by *Bdellovibrio* (17). In a later study, a putative role of surface sugar-binding proteins was proposed. Chemeris et al. (8) demonstrated that the addition of a variety of sugars, such as mannose, dextrose, and galactose, inhibited predation of *E. coli* by *B. bacteriovorus* 109D. Other sugars, including arabinose, sucrose, lactose, and xylose, inhibited the predation of *Erwinia carotovora* by *B. bacteriovorus* strain B-608. A putative role of sugar-binding proteins in *Micavibrio* host interactions was also demonstrated. When added to a *P. aeruginosa-M. admirandus* coculture, dextrose, mannose, sucrose, lactose, and xylose at a final concentration of 0.01 M were able to inhibit predation (7).

Our initial experiments, in which carbohydrates were added to the cocultures, supported earlier findings that sugars could block predation. Inhibition of predation by carbohydrates was

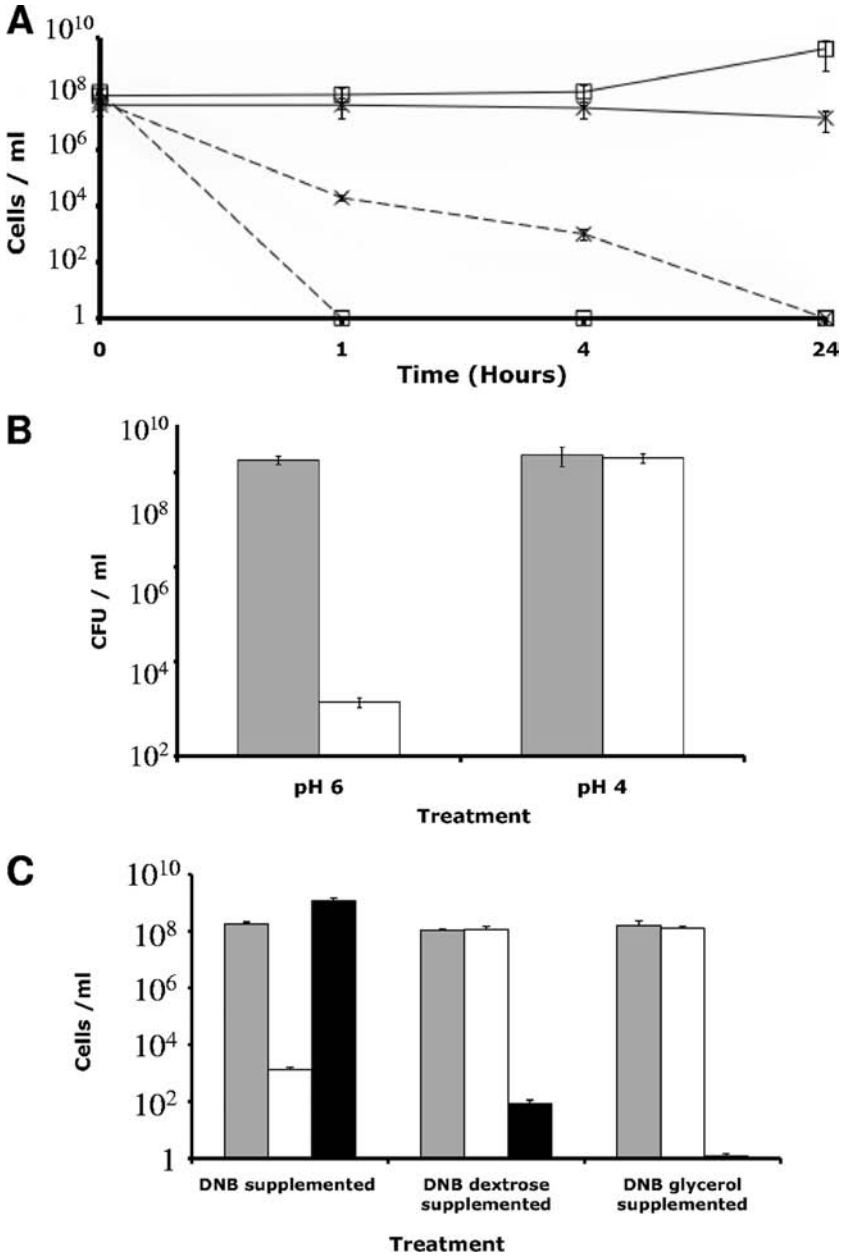

FIG. 4. Survival and predation of *B. bacteriovorus* in the presence of acidified media. (A) *B. bacteriovorus* survival at low pHs. *B. bacteriovorus* WT (×) and HI-A (□) were incubated in DNB media at pH 6.5 (unbroken lines) and pH 4 (broken lines). Cell viability was measured at time zero and following incubation. Each value represents the mean of results from 3 cocultures. (B) Predation in acidified media. *E. coli* was cultured with (empty bars) or without (gray bars) *B. bacteriovorus*. Cultures were prepared in DNB (pH 6.5) or acidified DNB (pH 4) and incubated for 24 h. Predation was evaluated by CFU enumeration of the remaining host cells. (C) Predation and *B. bacteriovorus* cell viability in the presence of a pregrown *E. coli* supernatant. *E. coli* was cultured for 18 h in DNB (DNB), DNB supplemented with 0.1 M dextrose, or DNB supplemented with 0.54 M glycerol. Thereafter, filter-sterilized supernatants were collected and used as media for culturing *E. coli* with (white bars) or without (gray bars) *B. bacteriovorus.* Cultures were incubated for an additional 24 h. Predation was evaluated by CFU enumeration of the remaining host cells and PFU enumeration of *B. bacteriovorus* cells (black bars). Each value represents the mean of results from 3 cocultures. Error bars are shown as 1 standard deviation.

seen in both *B. bacteriovorus* and *M. aeruginosavorus*. Although dextrose inhibited predation, L-glucose, which is not metabolically utilized by *E. coli* but might still possess the ability to bind to a putative receptor, did not have any effect on predation by both predators. Similar findings were seen when methyl-α-D-glucopyranoside was used. Like L-glucose, *E. coli* is unable to utilize methyl-α-D-glucopyranoside (4). To further validate the idea that utilization of sugar by the host is required for inhibition, dextrose was added to a coculture containing nonviable host cells. The ability of *B. bacteriovorus* to infect heat-killed and metabolically inactive prey was previously reported (37). The addition of dextrose to the coculture did not reduce the ability of *B. bacteriovorus* to infect the metabolically inactive cells, confirming that host viability is required for the sugar

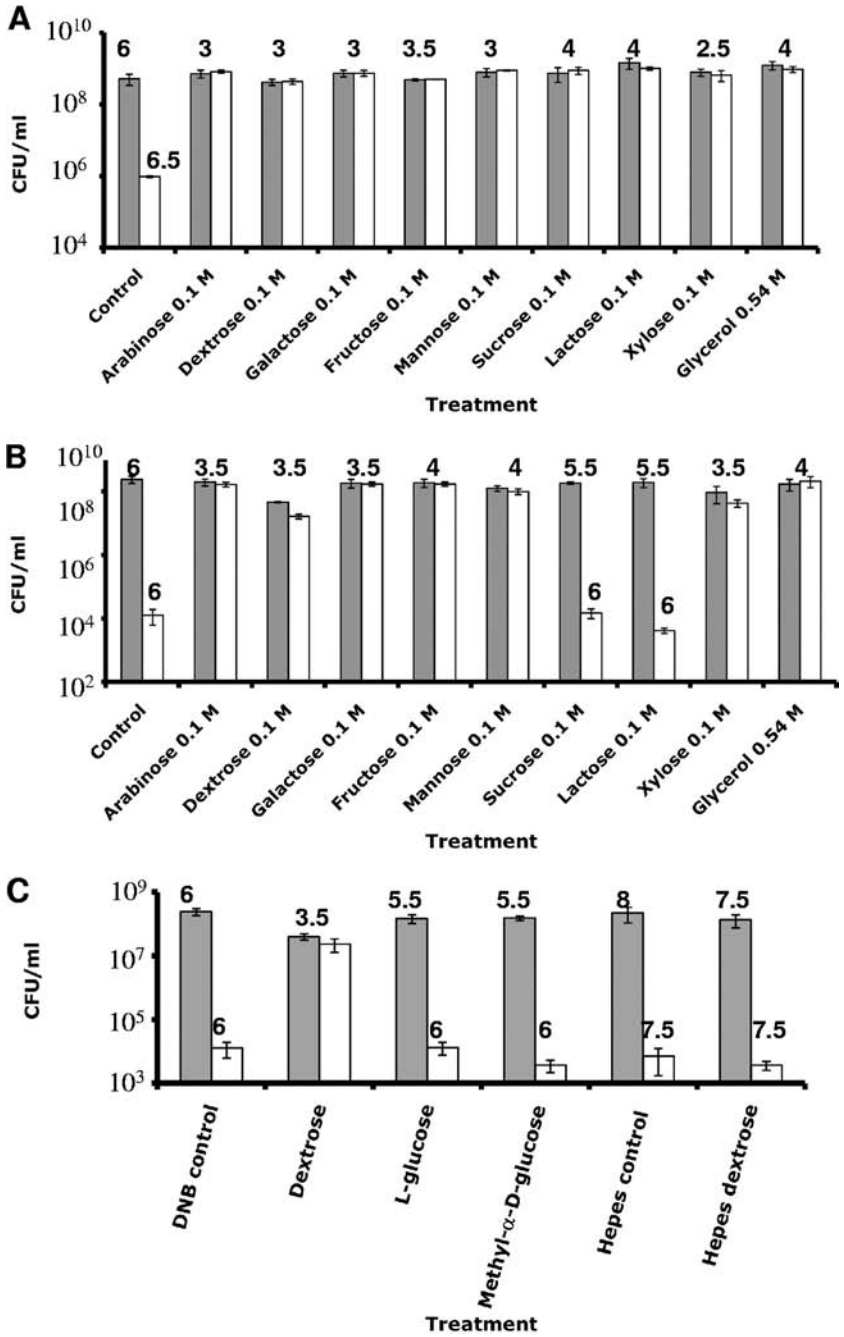

FIG. 5. *M. aeruginosavorus* predation in the presence of carbohydrates. *K. pneumoniae* (A) and *E. coli* (B) were cultured in DNB (control) and DNB supplemented with carbohydrates. Gray bars, without *M. aeruginosavorus*; white bars, with *M. aeruginosavorus*. (C) Predation in HEPES buffer and glucose analogues. *E. coli* cells were cultured alone (gray bars) or with *M. aeruginosavorus* (white bars). Cultures were prepared in sugar-free DNB and HEPES buffer (no-sugar control), medium supplemented with 0.1 M glucose, and DNB supplemented with 0.01 M glucose analogs. Cultures were incubated for 24 h. Predation was evaluated by CFU enumeration of the remaining host cells. Each value represents the mean of results from 3 cocultures. Error bars are shown as 1 standard deviation. Values above the bars represent the pHs of the cultures following incubation.

inhibiting effect (Fig. 3C). Since fermentation and catabolism of carbohydrates by many microorganisms, including *Enterobacteriaceae*, could lead to medium acidification (6), it was hypothesized that utilization of the carbohydrates by the host cells would acidify the DNB medium, kill the predator, and halt predation. As anticipated, a strong correlation was seen between acidification of the media and predation. No inhibition of predation or medium acidification was seen when nonfermentable sugar analogs were used. Additionally, the predation-inhibiting effect was lost when cocultures were incubated in HEPES-buffered medium (Fig. 5C and Table 1). It is important to note that earlier studies aimed at assessing the effect of sugar on predation were all done using water (7, 8). Finally, it was demonstrated that cultivating *B. bacteriovorus* and *M.*

aeruginosavorus in medium which was acidified to below pH 5 led to a rapid loss in predator viability and consequently to a loss of predation. The optimal pH range of above 5 and the inability of *B. bacteriovorus* WT and HI variants to survive at low pHs are in line with previous findings (12, 28, 35). The ability of *B. bacteriovorus* to renew predation after removal of the dextrose from acidified media could be explained by the ability of some attack cells to penetrate into the host and form bdelloplasts prior to medium acidification. The conditions within the bdelloplasts might provide refuge from the surrounding environment, allowing the *Bdellovibrio* cells to escape after the external conditions become more suitable. The protective characteristics of bdelloplasts and their ability to provide a degree of resistance against a variety of stresses, such as starvation, elevated temperatures, sonication, and desiccation, were previously reported (21, 27, 33). It could be speculated that when glycerol was used, *B. bacteriovorus* organisms were unable to form stable bdelloplasts, as a result of either rapid medium acidification or intracellular acidosis caused by the utilization and internalization of the compound, making the glycerol inhibiting effect permanent. However, we could not rule out the possibility that glycerol influences host-predator interactions by other means, such as altering cell membrane properties.

In conclusion, the inability of the predator to prey on a specific host is usually attributed to a structural feature that inhibits attachment, penetration, replication, or escape from the host. Our study demonstrated that the host could also influence its surrounding environment in a way that will make it less suitable for predator survival and proliferation. The data presented in this study could be of significance when considering the medium composition used to store, grow, and maintain the predators as well as when evaluating *Bdellovibrio* and *Micavibrio* host range specificities. Environmental factors, such as pH and the presence of carbohydrates, which might alter host metabolic activity and predator viability, should also be taken into account when considering the application of predatory bacteria *in vivo*.

ACKNOWLEDGMENTS

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Presentations abstracts

Department of Biology. The University of Virginia. Charlottesville, VA. December. 2009.

The title of the talk: Predatory Bacteria- The use of biological agents to control biofilms

Presentation summary: Most bacteria found in natural medical and industrial settings persist in complex microbial communities attached to surfaces known as biofilms. One of the major difficulties in controlling biofilms is their enhanced resistance to antimicrobial agents - biofilms can be up to 1000 times more resistant to antimicrobial agents than their planktonic counterparts. Recently, there has been a renewed interest in the use of biological control agents against biofilms. Among these agents is the use of invertebrates, protozoa and bacteriophages. In our lab we are using bacteria from the genus *Bdellovibrio* spp. and *Micavibrio* spp. in order to reduce and control surface attached bacteria. In addition to the antimicrobial application of those predators, we are also focusing our research on the biology of these unique organisms, specifically we are interested in the mechanisms involved in predation and predator prey interaction. The long-term goal of our study is to harness the therapeutic potential of these predators to control pathogenic bacteria and biofilms both in medical and industrial settings.

US Army Medical Research and Materiel Command Wound Symposium. San Antonio, TX, May 2011.

The Use of Predatory Prokaryotes to Control Drug – Resistant Bacteria and Microbial Biofilms Associated with Burn and Wound Infections

DR080241

Daniel E Kadouri

July 2009 - May 2011

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of wound infections. *Bdellovibrio* and *Micavibrio* are Gram-negative obligatory parasites that feed on other Gram-negative bacteria. The focus of the study was to evaluate the potential use of predatory bacteria to control human pathogens. Our findings demonstrated that predatory bacteria are able to attack many of the pathogens associated with war-related wound infections. Positive predation was measured in single and multi-species microbial cultures as well as on monolayer and multilayer pre-formed biofilms. The predators were also able to prey and remove metabolically inactive biofilms. *Bdellovibrio* and *Micavibrio* were shown to prey at 37^{o}C. Positive predation was also measured on biofilms grown in flow-cell systems. In conclusion, the work presented here highlights the potential use of predatory bacteria as biological based agent for eradicating infection and will pave the way for future studies in animal and human subjects.

4th Congress of European Microbiologists. Geneva, Switzerland, June 26-30, 2011.

Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*

Kadouri, D. and A. Dashiff

Background: Infectious complications caused by microorganisms that have become resistant to drug therapy are an increasing problem in medicine, with more infections becoming difficult to treat using traditional antimicrobial agents.

Objectives: The focus of our study was to evaluate the potential use of predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* to control pathogens associated with human infection, as well as measure the impact of predation on host cells grown in a single or multispecies culture and biofilm.

Methods: By coculturing *B. bacteriovorus* 109J and *M. aeruginosavorus* ARL-13 with selected pathogens, we have demonstrated that predatory bacteria are able to attack bacteria from a variety of genera including: *Acinetobacter, Aeromonas, Aggregatibacter, Bordetella, Burkholderia, Citrobacter, Eikenella, Enterobacter, Escherichia, Fusobacterium, Klebsiella, Listonella, Morganella, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio* and *Yersinia*. Predation was measured in single and multispecies microbial cultures as well as on monolayer and multilayer preformed biofilms.

Conclusions: *B. bacteriovorus* and *M. aeruginosavorus* have an ability to prey and reduce many of the multidrug-resistant pathogens associated with human infection. The work presented here highlights the potential use of predatory bacteria as a biological-based agent for eradicating multi-drug-resistant bacteria and biofilms.

Abstract A-291-0067-00707

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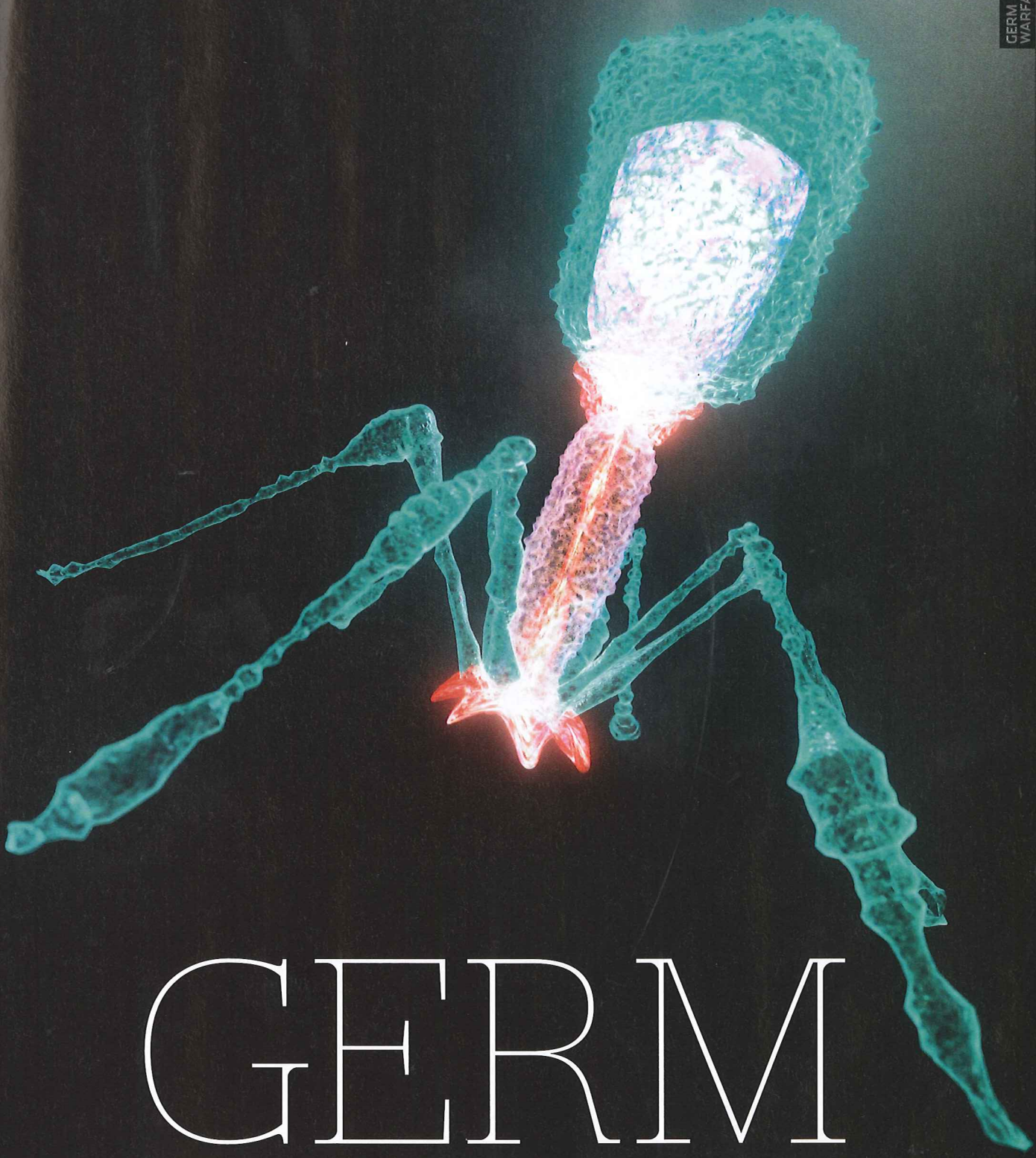


GERM WARFARE

New superbugs could render traditional antibiotics redundant. But, as **Daniel Bennett** discovers, viruses and friendly bacteria could be recruited to fight infections

SCIENCE PHOTO LIBRARY

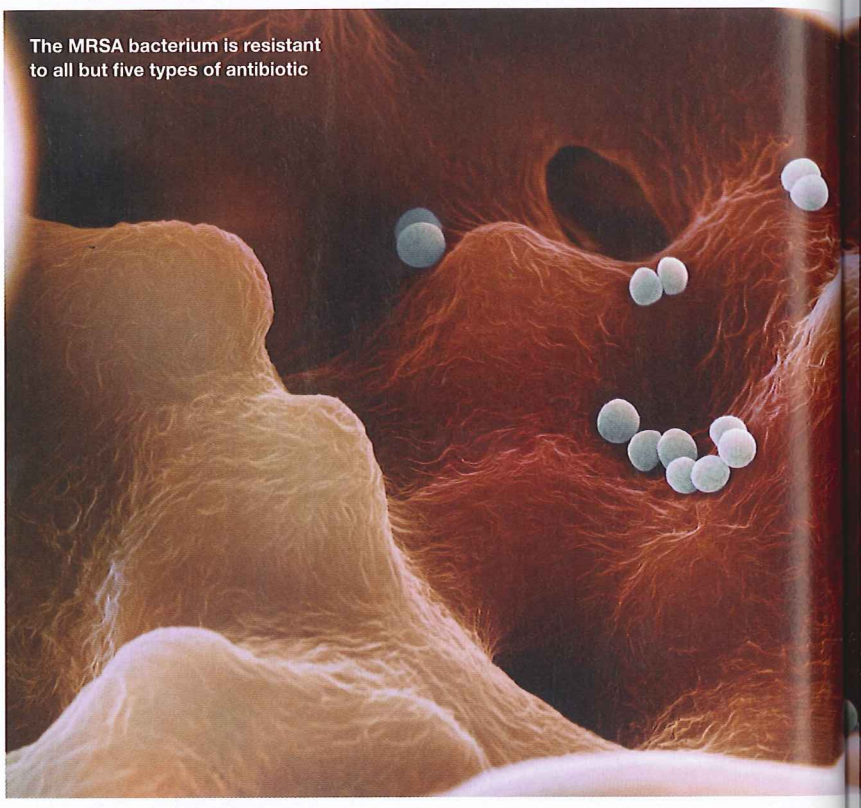
Bacteria are constantly laying siege to our bodies' defences. Until recently, our most potent protection against microscopic invaders has been antibiotics – wonder drugs that have kept most infections and diseases at bay for the last 70 years.

But now a new breed of superbug has evolved, and it's resistant to almost all current treatments. NDM-1, named after the antibiotic-destroying enzyme the superbug produces, is immune to all but two types of antibiotic, one of which has nasty side-effects and the other is only partially effective. MRSA, by comparison, is resistant to all but five.

But it isn't the bug itself that poses the biggest threat to our health: the real problem is that it can spread its resistance to any bacteria it encounters by allowing them to pick up a piece of DNA containing the gene for resistance. In fact, when the NDM-1 bug was first identified in 2008, other bacterial 'flora' living in the patient's gut, such as *E. coli*, were already resistant to most antibiotics.

Follow-up tests by epidemiologists led by Prof Tim Walsh at Cardiff University revealed that cholera also became untreatable after contact with bacteria carrying the NDM-1 gene. When cases appeared in the UK earlier this year, they published a paper in *The Lancet* entitled 'Is this the end of antibiotics?'

"Without five to 10 drugs that NDM-1 isn't resistant to, modern medicine, with infection-free surgery, could be history in 20 years"



The MRSA bacterium is resistant to all but five types of antibiotic

Drug-resistant bacteria pose a serious threat to surgical procedures

SCIENCE PHOTO LIBRARY X5, PETER BYRON/UMDNJ, PHOTOLIBRARY.COM

According to Walsh, the answer is 'yes'. "There'll come a point where bacteria becomes resistant to all current treatments. Right now, there's barely anything to treat NDM-1 with, and there's nothing on the horizon either."

Even if new drugs are discovered, it'll only be a temporary solution. "If a wonder-antibiotic appears, we'll all start using it and resistance will invariably ensue," warns Walsh. "We want five or 10 drugs on the horizon so that NDM-1 bacteria won't be a threat. Without them, modern medicine with infection-free surgery could be history in 20 years."

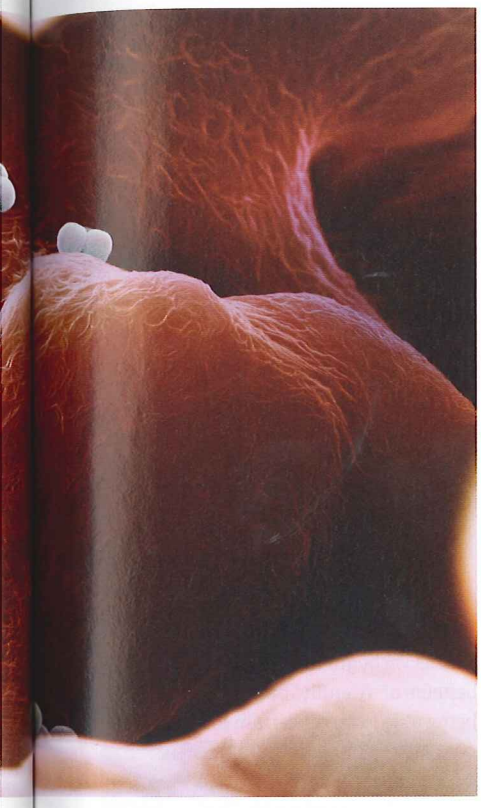
Friendly bacteria

Microbiologists are already aiming to stockpile an arsenal of treatments to prepare for this eventuality. At the University of Medicine of New Jersey, Dr Daniel Kadouri has found that the most promising weapon against superbugs may be bacteria itself.

"Until now, traditional research organisations have been squeamish about the idea of using bacteria to kill bacteria," says Kadouri. His lab has been 'training' bacterial predators to eat infectious species. "If there's bacteria out there, then there's probably something that eats them. We sort out what's eating what so we can use them to attack specific infections."

These predators are more like parasites than hunters: they find a species they like the taste of, then burrow inside them. Once they've fed on the insides of their host, they divide and burst out, before moving on to nearby cells. And once they've gorged themselves, they die off without touching any human cells.

Dr Kadouri (left) fighting bacteria with bacteria



This treatment strategy has another advantage, too. "Antibiotics encourage the creation of multi-drug resistant bacteria through natural selection," Kadouri explains. "Over time, the mutant bacteria that survive a course of antibiotics create generations of increasingly resistant bacteria – this doesn't happen with the predatory bacteria." It's not yet known why a resistance to these 'friendly bacteria' doesn't develop, but Kadouri hopes more research will reveal what's going on.

But the vital question is whether the strategy can stop the likes of NDM-1. "Integrating this with other systems will work. So, you could use predatory cells to kill 99 per cent of the initial infection and then finish it off with a potent antibiotic. As long as we don't apply too much pressure from one particular angle, we won't generate resistance."

Bio-weapons

Another approach is to turn a bacterium's own systems against itself. Bioengineers led by Prof Jim Collins at Boston ▸

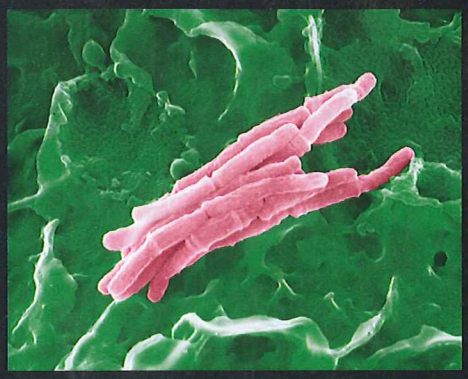
PLAN B

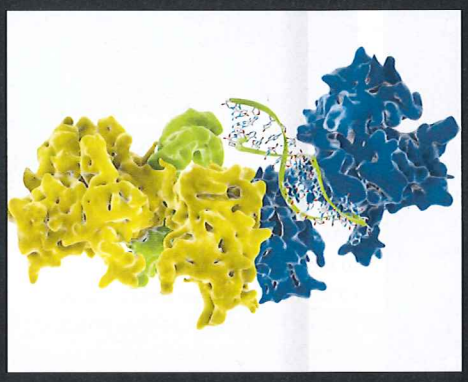
Future treatments being developed to combat bacterial disease

COMMUNICATION DISRUPTION

When bacteria infect a host, they rely on chemical signals to measure how many of its kind are nearby. While few in number, the bacteria lie in wait without causing any harm. Once there's a sufficient number, a chemical signal is sent out telling them to attack.

This ability to determine when they have adequate forces is known as 'quorum sensing'. Dr Bonnie Bassler at the University of Princeton was the first to identify the molecule that bacteria use to achieve this. Since then, Bassler and other researchers have been searching for a way to interrupt this process. Disrupting a bacterium's quorum-sensing ability would render it impotent without the risk of it becoming immune to treatment.





SUPER-ANTIBIOTICS

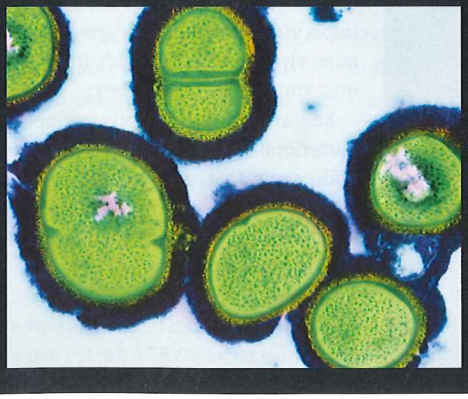
A bacteria becomes resistant to antibiotics by developing enzymes that can metabolise the drug. Finding new antibiotics is a huge economic drain, but researchers from Texas Tech University have been working on a chemical coating that could make old antibiotics effective once again.

Known as aptamers, these chemicals bind to the enzymes produced by superbugs, stopping them from disabling the antibiotics. In this way, defunct drugs, used in conjunction with the right aptamers, should destroy bacteria that were previously resistant to antibiotics. It's possible that this technique could extend the lifespan of current antibiotic treatments.

NATURE'S DEFENCES

Peptides are part of the innate immune response to bacterial infections – molecules that act as natural antibiotics. MRSA has already developed resistance to the peptides the human body produces. As most animals produce these molecules, microbiologists have been hunting for the peptides that could be synthesised to combat superbugs.

Last year, German researchers from the Christian-Albrechts-University of Kiel found that *Hydra*, simple organisms related to jellyfish, produce peptides that can kill MRSA. This only took place in the Petri dish, however, and the treatment is a long way from becoming a pill. The next step is to hunt for other oganisms that produce MRSA-killing chemicals.



STERILE ENVIRONMENTS

Superbugs like MRSA will happily live on surfaces like hospital walls and surgical tools while waiting to infect a human host. So far, sterilisation and quarantine procedures in UK hospitals have kept levels of infection at bay. At the Rensselaer's Centre for Biotechnology, Prof Jonathan Dordick has come up with a nano-coating that kills any MRSA bacterium that lands on it. It's made from lysostaphin, a naturally occurring enzyme, and carbon nanotubes, which act as microscopic packages for the chemical. In tests, this 'conjugate' was mixed with ordinary latex house paint and killed 100 per cent of MRSA within 20 minutes of contact with the coating.

JOHN MCAFEE

The anti-virus software creator on fighting biological infections

What made you want to switch to biological research?
It became apparent to me quite a while ago that the current methodology of developing antibiotics was going to hit a brick wall eventually. Killing bacteria and stopping it reproducing is guaranteed to produce a new strain as the resistant mutations survive and reproduce. This approach is not going to work in the long run – over 50 to 100 years. We're seeing it right now in the advent of superbugs and some of the older antibiotics like azithromycin are virtually useless now. It was a massively effective antibiotic and therefore was prescribed heavily and as a result it became useless very rapidly.

You're now bio-prospecting for anti-quorum-sensing drugs (see 'Plan B, Communication disruption', p47) in the Belize rainforest. Why?
What I wanted to do is study plants, because plants are susceptible to bacteria just like animals. Plants however have been around much longer than animals and they have one unique disadvantage when it comes to bacteria in that they can't move. So they have to have a far more effective defence against them. So I came at it from the perspective that plants must use some kind of anti-quorum-sensing and we've discovered that is the case. And Belize is one of the most bio-diverse places on the planet.

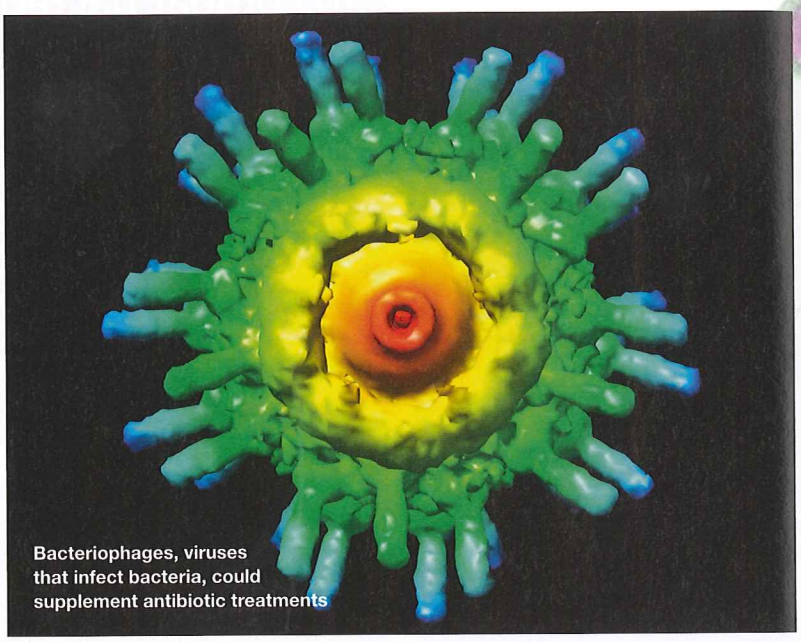
It's also said you've been doing work based on leads from Mayan elders, is that right?
Absolutely, if we didn't, we'd still be at ground zero. There are some 4000-odd unique plants within 32km of where we live. It's a time-consuming process to test each one for anti-quroum-sensing activity. So we asked the local healers, what treatments they were using. We found most of them didn't have any effect, but we found that a few did.

What's it like trying to develop a drug in the depths of the Belize rainforest?
We live in the middle of the jungle, by a crocodile-infested river. Between the hours of four in the afternoon and seven in the evening, the clouds of mosquitoes are so thick that you can barely see through them. We have leeches, several varieties of deadly, poisonous snakes not to mention the scorpions and spiders.

Has your career fighting computer viruses helped you battle biological bugs?
Computer viruses are basically programs that 'infect' computers, much like bacteria that infect organisms. They're transmitted from computer to computer through sharing of data, contact with or proximity to a crowded space (the internet). This is the digital equivalent of transmission mediums for organic infections.

A computer virus mimics, in great detail, the infection process of bacteria. For example: when a pathogenic bacterium first enters a host, it doesn't cause any harm to the host. It sits quietly and multiplies until there are sufficient numbers to mount an effective attack against the host's immune system. It then becomes pathogenic. Computer viruses, by and large, do the same thing. They don't immediately destroy data or cause harm. They generally wait for a given date, a signal from the author of the virus, or some other event, to begin causing damage. This gives the virus time to propagate undetected and infect large numbers of computers.

So, epidemiologically, computer viruses and bacteria follow very similar infection strategies. And this is just one of hundreds of other similarities between computer viruses and biological infections, where computer viruses mimic living infections.



Bacteriophages, viruses that infect bacteria, could supplement antibiotic treatments

▶ University have found that it's possible to kick-start a series of events that can cause infectious bacteria to 'commit suicide' – exposure to the chemical hydroxyurea induces bacteria to produce molecules that they are toxic to.

According to Collins, this could be the future of antibiotic treatment. "The next step will be to deliver this in the form of a molecule that's small enough to act further down the chain of events that stimulate this suicide."

Viruses that infect bacteria – bacteriophages – could also be used as a weapon in the war against disease-causing bacterial species. Bacteriophages clamp themselves to a cell and inject genetic material inside using their tails as a hypodermic needle. The virus's DNA turns a bacterium's cellular machinery into a virus-making factory that produces more viruses, which explode from the host and attack other bacteria.

Researchers are currently engineering bacteriophages to infect specific species. Collins says that, until recently, this was thought to be a limitation because it required complicated production processes for a treatment that would affect only a small range of afflictions. "Bacteriophages can be used as part of an anti-bacterial SWAT team to clear up whatever other methods leave behind." And as we begin to understand the benefits of 'friendly' bacteria in the body, being able to target certain foes is now seen as a valuable asset. "Ten years ago we were looking for a drug that could handle a broad spectrum of infections," says Collins. "But there is now increased call for specific antibacterial therapies that will wipe out the pathogen, but leave natural flora behind."

Evolution has provided us with the tools needed to fend off the latest strain of superbugs, but can we put them into production in time to prevent the 'end of antibiotics'? Collins believes that scientists are capable of finding a solution, it's just a matter of money, time and commitment. "We can deal with it in time, but we'll need additional resources to address the problem. If there isn't a large international focus on solving this soon, we will all be in trouble." ■

***Daniel Bennett is the editorial assistant at* Focus**

FIND OUT MORE

The Antibiotic Paradox
Stuart B Levy, (Perseus, 2001)

Allies and Enemies, How the World Depends on Bacteria
Anne Maczulak, (FT Press, 2010)

Can Predatory Bacteria Succeed Where Antibiotics Fail?

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There are predators in the bacterial world that consume other bacteria, much as predators attack prey in the animal world. A team led by researchers at the University of Medicine and Dentistry of New Jersey-New Jersey Dental School suggests that some of these predator microbes might be put to work against disease-causing bacteria that have become resistant to antibiotics. Their findings have been published online by the Journal of Applied Microbiology.

Lead author Daniel Kadouri, PhD, an assistant professor of oral biology at New Jersey Dental School, and his team focused on two bacteria: Micavibrio aeruginosavorus and Bdellovibrio bacteriovorus. Kadouri says the two microorganisms were chosen because they are true predators. “They actually have to consume other bacteria in order to complete their life cycles,” notes Kadouri. “They have a great ability to seek out other bacteria, invade them, grow in or on them, and kill them.”

The researchers found that in a laboratory environment, M. aeruginosavorus was able to reduce populations of 57 of 89 bacteria examined. B. bacteriovorus reduced 68 forms of bacteria out of 83 tested. The bacteria effectively attacked include Klebsiella pneumoniae, a cause of lung infection; Pseudomonas aeruginosa, which can be fatal for patients suffering from the lung disorder cystic fibrosis; and Acinetobacter, which in its drug-resistant form can produce extremely hard-to-treat infections in wounds.

Kadouri hopes that one day medical practitioners can use these predator bacteria to supplement antibiotic drugs in treating life-threatening infections. “We have been living with bacteria all our lives,” he reminds us. “There are bacteria in and on us, and they are a part of our ecology. When we eat yogurt and cheese, for example, we are eating bacteria.” Kadouri adds that the predator bacteria he is examining are among the many bacteria in our environment that are considered harmless to humans.

A big unknown at the moment is whether predator bacteria can have the same effect on harmful microbes inside the human body as they do in the lab. It is possible that the human immune system would neutralize these bacteria before they could do their beneficial work. But if that problem can be avoided, or solved, Kadouri is confident that a new disease-fighting tool may one day be put into use.

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ENT Blog

By Kristin Hayes, About.com Guide

Could Predatory Bacteria Be the Answer to Antibiotic Resistance?

Saturday January 29, 2011

The University of Medicine and Dentistry of New Jersey is studying predatory bacteria. What, you may be wondering, is predatory bacteria? Predatory bacteria is bacteria that eats other bacteria. The research team is focusing on two specific strains of bacteria that are truly ultimate bacterial predators, *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. "They actually have to consume other bacteria in order to complete their life cycles," Daniel Kadouri, Ph.D., an assistant professor of oral biology says. "They have a great ability to seek out other bacteria, invade them, grow in or on them, and kill them." By studying these predators the research team hopes that one day these types of bacteria might be used to kill harmful bacteria inside of the body, curing illnesses that are now treated with antibiotics.

There are still many hurdles standing in the way. No one knows for sure if *Micavibrio aeruginosavorus* and *Bdellovibrio bacterivorus* will behave the same way in the human body as it does in the lab. It's possible that the human immune system might immediately kill these bacterial predators, before they get a chance to devour their intended target. However, with antibiotic resistance becoming a huge problem, I, for one, am comforted by the thought of new solutions just over the horizon.

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Can Predatory Bacteria Succeed Where Antibiotics Fail?

Released: 1/10/2011 4:30 PM EST
Source: University of Medicine and Dentistry of New Jersey (UMDNJ)

Newswise — There are predators in the bacterial world that consume other bacteria, much as predators attack prey in the animal world. A team led by researchers at the University of Medicine and Dentistry of New Jersey-New Jersey Dental School suggests that some of these predator microbes might be put to work against disease-causing bacteria that have become resistant to antibiotics. Their findings have been published online by the *Journal of Applied Microbiology*.

Keywords:

Daniel Kadouri, New Jersey Dental School, predatory bacteria, Antibiotic Resistant Bacteria, Antibiotic Resistant Infections, Antibiotic Resistance, MRSA, UMDNJ, University of Medicine and Dentistry of New Jersey

Contact Information

Available for logged-in reporters only

Description

Now that increasing numbers of disease-causing bacteria are resistant to antibiotics, a new approach to fighting serious infections might be needed. Microbiologist Daniel Kadouri of UMDNJ-New Jersey Dental School has made progress toward finding one.

Lead author Daniel Kadouri, Ph.D., an assistant professor of oral biology at New Jersey Dental School, and his team focused on two bacteria: Micavibrio aeruginosavorus and Bdellovibrio bacteriovorus. Dr. Kadouri says the two microorganisms were chosen because they are true predators. “They actually have to consume other bacteria in order to complete their life cycles,” notes Kadouri. “They have a great ability to seek out other bacteria, invade them, grow in or on them, and kill them.”

The researchers found that in a laboratory environment, M. aeruginosavorus was able to reduce populations of 57 of 89 bacteria examined. B. bacteriovorus reduced 68 forms of bacteria out of 83 tested. The bacteria effectively attacked include Klebsiella pneumoniae, a cause of lung infection; *Pseudomonas aeruginosa*, which can be fatal for patients suffering from the lung disorder cystic fibrosis; and Acinetobacter, which in its drug-resistant form can produce extremely hard-to-treat infections in wounds.

Kadouri hopes that one day medical practitioners can use these predator bacteria to supplement antibiotic drugs in treating life-threatening infections. “We have been living with bacteria all our lives,” he reminds us. “There are bacteria in and on us, and they are a part of our ecology. When we eat yogurt and cheese, for example, we are eating bacteria.” Kadouri adds that the predator bacteria he is examining are among the many bacteria in our environment that are considered harmless to humans.

A big unknown at the moment is whether predator bacteria can have the same effect on harmful microbes inside the human body as they do in the lab. It is possible that the human immune system would neutralize these bacteria before they could do their beneficial work. But if that problem can be avoided, or solved, Kadouri is confident that a new disease-fighting tool may one day be put into use.

The University of Medicine and Dentistry of New Jersey (UMDNJ) is the nation's largest free-standing public health sciences university with more than 6,000 students attending the state's three medical schools, its only dental school, a graduate school of biomedical sciences, a school of health related professions, a school of nursing and its only school of public health on five campuses. Annually, there are more than two million

patient visits at UMDNJ facilities and faculty practices at campuses in Newark, New Brunswick/Piscataway, Scotch Plains, Camden and Stratford. UMDNJ operates University Hospital, a Level I Trauma Center in Newark, and University Behavioral HealthCare, which provides a continuum of healthcare services with multiple locations throughout the state.

Bloomberg Businessweek
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EXECUTIVE HEALTH January 14, 2011, 16:00 EST

Predatory Bacteria May Help Control Antibiotic-Resistant Germs

In lab, tiny hunters invaded, destroyed majority of dangerous bacteria tested on them

FRIDAY, Jan. 14 (HealthDay News) -- Bacteria that prey on other bacteria may prove useful in controlling antibiotic-resistant germs, say U.S. researchers.

They tested two types of predatory bacteria -- *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus* -- on dozens of other bacteria.

"They actually have to consume other bacteria in order to complete their life cycles. They have a great ability to seek out other bacteria, invade them, grow in or on them, and kill them," lead author Daniel Kadouri, an assistant professor of oral biology at the University of Medicine and Dentistry of New Jersey-New Jersey Dental School, said in a university news release.

He and his colleagues found that *M. aeruginosavorus* reduced populations of 57 of 89 bacteria and *B. bacteriovorus* reduced populations of 68 of 83 bacteria. The bacteria effectively attacked by the predatory bacteria included lung infection-causing *Klebsiella pneumoniae*; *Pseudomonas aeruginosa*, which can be deadly for cystic fibrosis patients; and *Acinetobacter*, which in its drug-resistant form can cause difficult-to-treat infections in wounds.

The study was recently published online in advance of print publication in the February issue of the *Journal of Applied Microbiology*.

The predatory bacteria being examined by Kadouri and his colleagues are considered harmless to humans. But it's not clear whether the human immune system would destroy these bacteria before they could attack harmful bacteria in the body.

More information

The U.S. Food and Drug Administration has more about antibiotic resistance.

-- Robert Preidt

SOURCE: University of Medicine and Dentistry of New Jersey, news release, Jan. 11, 2011

Press Release

Date: 01-10-11
Name: Rob Forman
Phone: 973 972 7276
Email: formanra@umdnj.edu

Can Predatory Bacteria Succeed Where Antibiotics Fail?

NEWARK, N.J. - There are predators in the bacterial world that consume other bacteria, much as predators attack prey in the animal world. A team led by researchers at the University of Medicine and Dentistry of New Jersey-New Jersey Dental School suggests that some of these predator microbes might be put to work against disease-causing bacteria that have become resistant to antibiotics. Their findings have been published online by the *Journal of Applied Microbiology*.

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CDMRP website.

Using Predatory Bacteria to Combat Drug-Resistant Bacteria

Daniel E. Kadouri, Ph.D., University of Medicine and Dentistry of New Jersey, Newark, New Jersey

Complex traumatic injuries are often complicated by bacterial infections. These infections are associated with increased morbidity and mortality in patients. Many of these infections are caused by drug resistant bacteria such as *Staphylococcus aureus*, *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae* , and *Pseudomonas aeruginosa*. The numbers of drug-resistant bacteria are increasing as a natural consequence of broad spectrum antimicrobials being inappropriately prescribed and patients not following antibiotic regimes to completion. To combat the expanding number of threats many scientists are working to identify new antimicrobials, vaccines, and drug delivery systems to combat this emerging threat. Dr. Daniel Kadouri of the University of Medicine and Dentistry of New Jersey decided to take a different approach. In his Fiscal Year 2008 Hypothesis Development Award from the Deployment Related Medical Research Program, Dr. Kadouri and his team are using the Gram-negative bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* to control wound infections and to enhance the potency of existing drugs to treat bacteria and biofilms. The advantage of using the predatory bacteria is that they are unlikely to be pathogenic or strongly immunogenic to humans, their prey is not known to develop resistance, and they replicate rapidly as they feed on their prey.

In a recent Annual Report and paper published in the Journal of Applied Microbiology, Dr. Kadouri and his team report the host range and robustness of predation. Of the nearly 200 prey bacteria tested, the predatory bacteria were able to attack and lyse approximately 80% bacteria species tested. Predation was observed in free-floating planktonic cells as wells as within single and multispecies bacterial biofilms. The observation that the predatory bacteria can penetrate biofilms is particularly promising. Bacterial biofilms are a collection of bacterial and extracellular secretions composed of DNA, protein and polysaccharides. These secretions function to secure the bacteria to the wound and also protect the bacteria from antibiotics; in fact bacteria within biofilms can be up to 1000 times more resistant to antibiotics than their free floating counterparts. In the next reporting period Dr. Kadouri will perform experiments that will assess if the predatory bacteria biofilm penetrating capability can work in concert with existing antimicrobials and anti-biofilm agents to further enhance bacterial pathogen reduction.

Publication:

A. Dashiff, R.A. Junka, M. Libera and D.E. Kadouri. 2010. Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. *Journal of Applied Microbiology* 110:443-444.