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Original Article

Predation Efficacy of *Bdellovibrio bacteriovorus* on Multidrug-Resistant Clinical Pathogens and Their Corresponding Biofilms

Yao Sun[1], Jianzhong Ye[2,3], Yuanbo Hou[4], Huale Chen[1], Jianming Cao[4], and Tieli Zhou[1]*

[1]*Department of Clinical Laboratory, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou;* [2]*State Key Laboratory for Diagnosis and Treatment of Infectious Disease, The First Affiliated Hospital, College of Medicine,* [3]*Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou; and* [4]*School of Laboratory Medicine and Life Science, Wenzhou Medical University, Wenzhou, China*

SUMMARY: The aim of the present study was to evaluate the predation efficacy of *Bdellovibrio bacteriovorus* on multidrug-resistant (MDR) or extensive drug resistant (XDR) gram-negative pathogens and their corresponding biofilms. In this study, we examined the ability of *B. bacteriovorus* to prey on MDR and XDR gram-negative clinical bacteria, including *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Results showed that *B. bacteriovorus* was able to prey on all planktonic cultures, among which the most efficient predation was observed for drug-resistant *E. coli*, with a 3.11 log10 reduction in viability. Furthermore, *B. bacteriovorus* demonstrated promising efficacy in preventing biofilm formation and dispersing the established biofilm. Reductions in biofilm formation of *E. coli, K. pneumoniae, P. aeruginosa*, and *A. baumannii* co-cultured with *B. bacteriovorus* were 65.2%, 37.1%, 44.7%, and 36.8%, respectively. Meanwhile, the established biofilms of *E. coli, K. pneumoniae, P. aeruginosa*, and *A. baumannii* were significantly reduced by 83.4%, 81.8%, 83.1%, and 79.9%, respectively. A visual analysis supported by scanning electron microscopy demonstrated the role of *B. bacteriovorus* in removing the established biofilms. This study highlights the potential use of *B. bacteriovorus* as a biological control agent with the capability to prey on MDR/XDR gram-negative pathogens and eradicate biofilms.

INTRODUCTION

The emergence and evolution of bacterial antibiotic resistance is one of the most significant threats to current public health and the economy (1). Increases in morbidity, length of hospitalization, healthcare cost, and mortality have been attributed to infections caused by multidrug-resistant (MDR) or extensive drug resistant (XDR) superbugs, which respond poorly to antimicrobial agents (2). Unfortunately, this situation has been further exacerbated since many chronic human diseases are biofilm-associated, and bacteria in biofilms are naturally more resistant to antibiotic treatments (3). Therefore, there is a pressing need to develop innovative strategies to control antibiotic-resistant organisms in hospitals.

Bdellovibrio bacteriovorus is a small, anaerobic/microaerobic, highly motile delta-proteobacterium that preys on a wide range of other gram-negative bacteria (4). The typical predatory life cycle of *Bdellovibrio* involves an attack phase and growth phase. During the attack phase, *Bdellovibrio* seeks, attaches to, and penetrates a bacterial host. Then, it occupies the periplasmic space and undergoes growth phase which culminates in prey killing and release of progeny (4). *B. bacteriovorus* is ubiquitous both in aquatic and terrestrial habitats and has recently been discovered in the human gut, where it is alleged to act as a "probiotic" (5,6). Previous research has demonstrated that *B. bacteriovorus* has the potential to prey upon several human pathogens which grow in planktonic cultures or in biofilm (7). The antimicrobial susceptibility of the bacterial hosts utilized in most studies has not been investigated. A pioneering work using 14 MDR gram-negative clinical strains as host cells for *B. bacteriovorus* has preliminarily demonstrated that *B. bacteriovorus* maintains its ability to prey on MDR bacteria regardless of its antimicrobial resistance (8). However, the capability of *B. bacteriovorus* to prey on MDR or even XDR clinical pathogens that grow in planktonic cultures or in biofilm has not been elaborately elucidated.

Here, we evaluated the ability of *B. bacteriovorus* 109J to prey on MDR and XDR gram-negative clinical bacteria in planktonic cultures, including *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Acinetobacter baumannii* from clinical settings. Moreover, prevention of biofilm formation and removal of established biofilm by *B. bacteriovorus* 109J were examined. The role of *B. bacteriovorus* in removing established biofilm was further evaluated using scanning electron microscopy (SEM).

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions: A total of 200 clinical strains, including *E. coli, K.*

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*Corresponding author: Mailing address: Department of Clinical Laboratory, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. Tel: +86-0577-8669-9370, E-mail: wyztli@163.com

pneumoniae, P. aeruginosa, and *A. baumannii*, were collected from the First Affiliated Hospital of Wenzhou Medical University from 2013 to 2014 and were tested as prey cells. Each species was divided into 2 groups according to the drug resistance characteristics, which consisted of 25 MDR or XDR strains (5 MDR strains and 20 XDR strains in the *K. pneumoniae* group, 25 XDR strains in the *A. baumannii* group, and 25 MDR strains in the *E. coli* and *P. aeruginosa* group) and 25 sensitive strains. MDR was defined as non-susceptibility to more than one agent from at least 3 antimicrobial categories, while XDR was defined as non-susceptibility to more than one agent in all but 2 or fewer antimicrobial categories (9). All prey cells were maintained in frozen glycerol stocks at −80°C. Upon need, they were streaked on trypticase soy agar (TSA, Difco, Detroit, MI, USA) plates and grown overnight at 37°C. Then, one colony was picked up and inoculated into lysogeny broth (LB, Difco) overnight at 37°C with agitation (200 rpm). Harvested by centrifugation (8,500 $\times g$ for 10 min), the cells were washed and diluted in dilute nutrient broth (DNB), a 1:10 dilution of nutrient broth (Difco) amended with 3 mmol/L $MgCl_2$ and 2 mmol/L $CaCl_2$.

The predator *B. bacteriovorus* 109J (ATCC 43826) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cultivation of *B. bacteriovorus* 109J on *E. coli* was performed as described previously (7). As a control, filtered sterilized lysate was prepared by sequentially passing the *B. bacteriovorus* culture through three 0.22 μm track-etched nuclepore membranes (Millipore, Billerica, MA, USA). As judged by plaque-forming units (PFU), no predator could be detected after filtration.

Predation assays on planktonic cultures: Predation assays on planktonic cultures were conducted as previously described (10) with little modification. Briefly, 0.5 mL of washed prey cells (approximately 1×10^8 CFU mL^{-1}) and 0.5 mL of freshly harvested *B. bacteriovorus* 109J (approximately 1×10^8 PFU mL^{-1}) or a predator-free control were added into DNB media to construct a 5 mL co-culture system. The cultures were incubated at 30°C on a rotary shaker set at 200 rpm for 48 h. The ability of *B. bacteriovorus* to prey was confirmed by the reduction in host cell viability compared to the predatory free control, which was measured by CFU enumeration. Each co-culture was performed in triplicate.

Screening of biofilm production in drug-resistant strains: Biofilm-forming MDR and XDR strains which simultaneously carried genes involved in biofilm formation were selected as the experimental cells for further experiments. Phenotypically, quantification of biofilm formation under static conditions was determined by microtiter assay as already reported with little modification (11). Briefly, 18 h LB-grown cultures diluted 1:100 in fresh LB containing 0.5% glucose were inoculated (200 μL per well) into tissue culture-treated 96-well polystyrene microtiter plates (Costar, Corning, NY, USA). After 24 h incubation at 37°C, the plates were washed twice with 0.1 mol/L (pH 7.2) sterile phosphate-buffered saline (PBS). Subsequently, each well was stained with 200 μL of 0.1% crystal violet and incubated for 20 min at room temperature before it was washed and dried. Finally, 250 μL of ethanol supplemented with 2% acetic acid was added to each well for 30 min. The OD_{570} reading of each well was performed with a VMax® Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Sterile LB supplemented with 0.5% glucose served as a control. All experiments were conducted 3 times independently. The strain was considered to be phenotypically biofilm-forming positive at OD_{570} values greater than or equal to 0.12 and was otherwise adherence-negative (11).

Genotypically, drug-resistant strains were screened for genes involved in biofilm formation by PCR using primers as described previously (11–15). The primer sequences and corresponding product length used in this study are presented in Table 1. The positive PCR products were screened by electrophoresis on 1.0% agarose gel and sequenced by Shanghai Majorbio Bio-Pharm Technology Co. (Shanghai, China).

Biofilm prevention assay: Overnight culture (100 μL, 10^8 CFU ml^{-1}) and *B. bacteriovorus* preparation or the predator-free control were added to 96-well polystyrene microtiter plates. The plates were incubated at 30°C for 48 h before they were washed and stained with

Table 1. The primer sequences and product length for genes involved in biofilm formation

Strain	Target gene	Sequence	Product length (bp)
E. coli	*pgaA*	F: AGGGACTGCGCATTGATTAC R: GTTCACGTTCGACAACATCG	195
	csgD	F: ACAGCTCTCTTGCAGCACCT R: ACGGGTAATCTTCAGGCGTA	214
K. pneumoniae	*mrkA*	F: CGGTAAAGTTACCGACGTATCTTGT ACTG R: GCTGTTAACCACACCGGTGGTAAC	498
	fimH	F: GCTCTGGCCGATAC(C/T)AC(C/G)AC GG R: GC(G/A)(A/T)A(G/A)TAACG(T/C)GCC TGGAACGG	423
P. aeruginosa	*pslA*	F: CACTGGACGTCTACTCCGACGATAT R: GTTTCTTGATCTTGTGCAGGGTGTC	1,119
A. baumannii	*abaI*	F: GTACAGTCGACGTATTTGTTGAATAT TTGGG R: CGTACGTCTAGAGTAATGAGTTGTT TTGCGCC	382

crystal violet. All experiments were conducted 3 times independently. The absorbance at 570 nm was measured as described above.

Biofilm removal assays: As mentioned above, the pre-formed biofilms were prepared. Eighteen-hour LB-grown cultures diluted 1:100 in fresh LB containing 0.5% glucose were inoculated into microtiter plates and incubated for 24 h at 37°C. The plates were washed with PBS to remove planktonic cells and then inoculated with 200 µL of *B. bacteriovorus* preparation or predator-free control at 30°C for 48 h. Quantification of the biofilm was conducted as described above. Experiments were repeated 3 times.

Scanning electron microscopy analysis: To visualize biofilms before and after 48 h challenge of *B. bacteriovorus*, SEM analysis was employed. Briefly, overnight-grown strains were diluted 100 fold in fresh LB with 0.5% glucose, 2 mL of which were inoculated into 6-well cell culture plates (Costar). Then, a cover slip on which biofilms developed was placed on the bottom of each well. After 24 h incubation at 37°C, the inoculum was gently discarded by aspiration and the plates were washed 3 times with 0.1 mol/L sterile PBS to remove the planktonic preys. Then, an equal volume of harvested predator was added to the plates. After an additional 48 h of interaction between the predator and prey-biofilms at 30°C, all cover slips were gently rinsed with 0.1 mol/L sterile PBS, followed by critical-point drying. Finally, they were gold palladium-coated (sputter coater 5150A; Edwards High Vacuum International, Crawley, England) and examined with a scanning electron microscope (Hitachi S-3000N, Tokyo, Japan).

Statistical analysis: For the analysis of the data acquired from predation assays on planktonic cultures, the Mann-Whitney test was performed, while for biofilm prevention and removal assays, the Student's t-tests and chi-square test were used. Probability values of $p < 0.05$ were considered to be significant. All the statistical analyses were performed by SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

***B. bacteriovorus* predation on planktonic cultures:** *B. bacteriovorus* was able to prey on all planktonic cultures, regardless of drug susceptibility patterns. Greater than 1.49 log10 reductions of attacked strains were measured following 48-h incubation (Fig. 1). No obvious changes were observed in the *B. bacteriovorus*-free control group. The most efficient predation was found for *E. coli* in the MDR group, with a 3.11 log10 reduction in viability. The differential capacity of *B. bacteriovorus* to prey upon the 4 species investigated in this study was observed ($p < 0.05$). However, predation efficiencies of the predator to attack strains within the same species were similar whether the isolates were sensitive to antimicrobials or not (Fig. 1), with no statistical differences observed ($p > 0.05$).

Biofilm prevention and removal assay: Sixteen out of 25 (64%) *E. coli* strains as well as 23 out of 25 (92%) strains for each of *K. pneumoniae, P. aeruginosa*, and *A. baumannii* were confirmed as biofilm-forming positive strains both phenotypically and genotypically and were utilized for further experiments. Biofilm-forming positive strains in 25 MDR or XDR strains of 4 bacteria species in our study exhibited high biofilm formation rates and were strong biofilm producers.

As shown in Figs 2 and 3, *B. bacteriovorus* 109J possessed a robust predation activity that significantly inhibited biofilm formation or dispersed established biofilms of MDR and XDR strains in all 4 species. In comparison to the predator-free control, the absorbance at 570 nm of *E. coli, K. pneumoniae, P. aeruginosa*, and *A. baumannii* declined by 65.2%, 37.1%, 44.7%, and 36.8%, respectively, indicating that *B. bacteriovorus* was able to prevent biofilm formation (Fig. 2). Among the 4 species, the most efficient predation of *B. bacteriovorus* was observed in *E. coli* ($p < 0.05$). After challenge for 48 h with *B. bacteriovorus*, established biofilms of

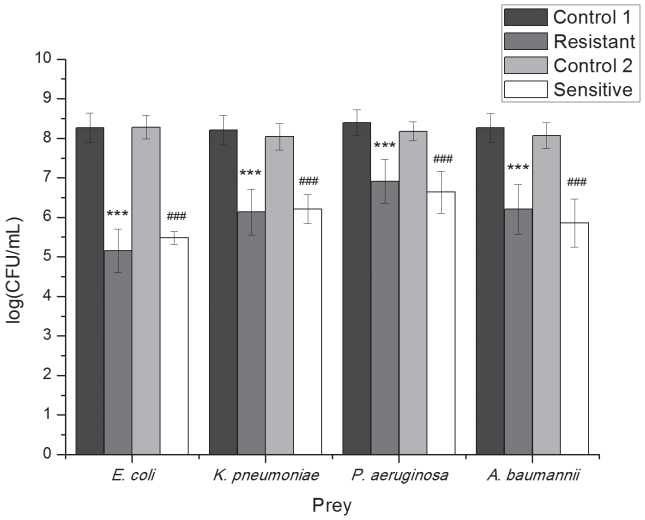

Fig. 1. Efficiency of *B. bacteriovorus* predation on planktonic cultures. Drug-resistant and drug-sensitive preys were incubated for 48 h with *B. bacteriovorus* (resistant bars and sensitive bars) or corresponding predator-free control (control 1 bars and control 2 bars). Each value represents the mean of 3 cocultures for all the tested strains of that bacterium. Error bars are shown as one-standard deviation. Control 1 compared to resistant, *** $p < 0.001$. Control 2 compared to sensitive, ### $p < 0.001$.

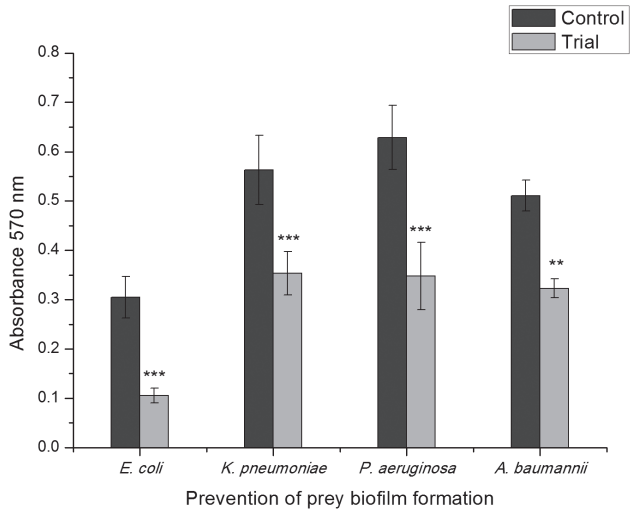

Fig. 2. Prevention of prey biofilms formation by *B. bacteriovorus*. 100 µL of overnight culture were incubated for 48 h with *B. bacteriovorus* (black bars) or predator-free control (white bars) in 96-well plates. Each value represents the mean of triplicate for all the biofilm-forming positive strains of that bacterium. Error bars are shown as standard errors. ** $p < 0.01$, *** $p < 0.001$.

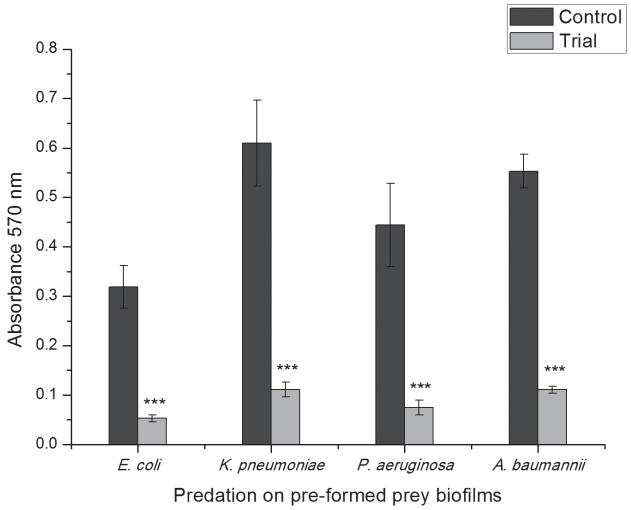

Fig. 3. Predation of *B. bacteriovorus* on pre-formed prey biofilms. Biofilms of prey were grown on 96-well plates for 24 h, and challenged with *B. bacteriovorus* (black bars) or predator-free control (white bars) for additional 48 h. Biofilm amount was measured by crystal violet staining followed by OD_{570} readings. Each value represents the mean of triplicate for all the biofilm-forming positive strains of that bacterium. Error bars are shown as standard errors. *** $p < 0.001$.

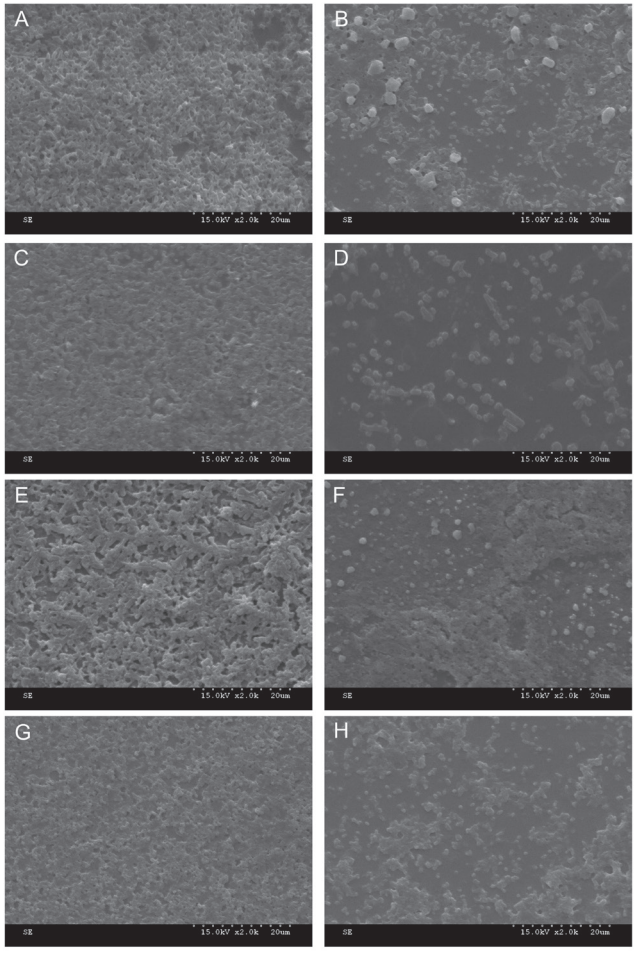

Fig. 4. SEM analysis of biofilm predation by *B. bacteriovorus*. *E. coli, K. pneumoniae, P. aeruginosa*, and *A. baumannii* biofilms were developed on cover slips for 24 h (pre-formed). Thereafter, the biofilms were washed and incubated for 24 h with *B. bacteriovorus* (B, D, F, H) or predator-free control (A, C, E, G). Magnification, × 2,000.

E. coli were significantly reduced by 83.4%, while *K. pneumoniae, P. aeruginosa*, and *A. baumannii* biofilms showed reduction by 81.8%, 83.1%, and 79.9%, respectively (Fig. 3). No statistically significant difference was observed between the 4 species. The similar biofilm amount loss for all challenged species indicates a similar predatory efficacy exerted by *B. bacteriovorus*. Biofilm amounts of 4 prey species treated with the predator-free control were not significantly changed.

SEM analysis of biofilm predation: Further confirmation of the biofilm-devouring capacity of *B. bacteriovorus* was visualized by SEM imaging. The results showed that the *B. bacteriovorus* penetrated the biofilm extracellular matrix. The bulk of all the examined biofilms was destroyed within 48 h of predation, leaving behind what appeared to be cell debris and matrix (Fig. 4).

DISCUSSION

Ever since its accidental discovery in 1963 (16), *Bdellovibrio* has been found to be ubiquitous in nature, occurring in marine sediments, polluted waste-water sewage, cultured fish ponds, and human intestines (5). Its promising ability to reduce bacterial populations is linked to its application as a biocontrol agent to clear different habitats of plant, fish, and food-borne pathogens (17,18). Moreover, it has been proposed that *B. bacteriovorus* 109J can be used as a probiotic bacterium because of its ability to prey on gram-negative bacteria and their biofilms (19). With the increased occurrence of MDR and XDR clinical pathogens, many of which have brought about therapeutic challenges, the concept of utilizing predatory bacteria as live antimicrobials is gaining momentum. However, the majority of previous research has failed to investigate whether predation of *B. bacteriovorus* against bacteria is affected by antibiotic susceptibility (10,20). Hence, further research on the predation efficacy of *B. bacteriovorus* against MDR or XDR clinical pathogens and their biofilms is encouraged.

A significant reduction in viability of all prey species indicated that *B. bacteriovorus* possessed the ability to prey on planktonic cultures. The most efficient predation was found for *E. coli* in the MDR group, with a 3.11 $\log_{10}$ reduction in viability. The prey cells were not completely eradicated, but similar findings have been widely reported (7,21). It was noteworthy that the predator maintained its ability to prey on the clinical strains regardless of MDR or XDR status (Fig. 1). This study largely confirmed the research performed by the Kadouri group showing that *B. bacteriovorus* can prey on MDR strains grown planktonically (8), highlighting the potential therapeutic use of *B. bacteriovorus* strain 109J as an alternative approach to combat antimicrobial-resistant bacterial infections. The results showed no statistical difference between drug-sensitive and resistant groups in each species. However, mechanisms governing host specificity of *B. bacteriovorus* are not fully understood, and efforts should be made to explore the reason for the diversity. A limitation of this predator is its inability to prey on gram-positive bacteria, such as the important nosocomial pathogen *Staphylococcus aureus*.

Notably, MDR and XDR strains in this study exhib-

ited high biofilm formation rates (64–92%) and were strong biofilm producers. Encouragingly, the results presented here demonstrated that *B. bacteriovorus* strain 109J possesses a robust predation activity to significantly inhibit biofilm formation or disperse established biofilms of MDR and XDR strains (Figs. 2, 3). Statistical analysis showed no significant difference in the ability of *B. bacteriovorus* 109J to prey on MDR and XDR strains both in planktonic cultures and in biofilms. The reduction of pre-formed biofilms shown by OD_{570} readings, following 48 h predation by *B. bacteriovorus*, was surprisingly higher than the data shown in the biofilm prevention assay. This might be explained by the high cell density of bacteria existing in the biofilm, which makes it easier for the predator to encounter its prey. Further, a previous study reported that relatively metabolically inactive biofilms, which are considered to play a vital role in enhancing antibiotic resistance, can be significantly reduced by *B. bacteriovorus*, indicating that the predator is not influenced by the metabolic activity of its target cells (7). Bacterial biofilms, within which the cells exhibit increased antibiotic resistance, are the basis of many chronic infections. Multiple mechanisms, including the biofilm matrix, which prevents antimicrobials from reaching the cells and altering the pattern of gene expression in the biofilm population, contribute to the antimicrobial resistance of biofilms (22). Nevertheless, previous research showed that the thick polysaccharide capsule is not difficult for Bdellovibrio to penetrate (23), making it easier for *B. bacteriovorus* to access and efficiently disperse biofilms. It is reported that *B. bacteriovorus* is able to access biofilms as thick as 30 μm (24). To further visualize biofilm predation after treatment with *B. bacteriovorus*, biofilms predeveloped on coverslips were exposed to *B. bacteriovorus* or a predator-free control and then analyzed by SEM (Fig. 4). The results showed that *B. bacteriovorus* devoured biofilms of all the examined species, with the majority of the biofilms being cleared.

Collectively, all data obtained in this study demonstrated that *B. bacteriovorus* 109J maintained its ability to reduce bacterial loads and established biofilms and provided further insight into its role in inhibiting biofilm formation of clinical pathogens, regardless of the antimicrobial resistance profile of the prey cells. Given the fact that *B. bacteriovorus* is nonpathogenic and nontoxic to higher organisms (25,26) and its presence in the healthy human gut (6), it seems that *B. bacteriovorus* is a good candidate for treating infections and biofilms removal.

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Conflict of interest None to declare.

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