Avicenna J Environ Health Eng. 2015 December; 2(2):e4541. doi: 10.17795/ajehe-4541.

Published online 2015 December 26. **Research Article**

The Potential of *Bdellovibrio* For the Biocontrol of the Infectious Agent *Vibrio cholerae*

Natalia Olsson Markelova[1,2,*]

[1]Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Russia
[2]University of Maryland Biotechnology Institute, Baltimore, Maryland, USA

[*]*Corresponding author*: Natalia Olsson Markelova, Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Russia. E-mail: nmarkelova@mail.ru

Received 2015 October 29; **Revised** 2015 November 15; **Accepted** 2015 December 15.

Abstract

Members of the genus *Bdellovibrio* are small and highly motile Gram-negative predators of other Gram-negative bacteria. *Bdellovibrio* enters the prey cell, transforming it into a structure that is referred to as a bdelloplast. It then grows and divides inside the bdelloplast, ending in lysis and the release of the *Bdellovibrio* progeny. Because of this capability, *Bdellovibrio* is a potential antibacterial agent. In this article, we report the results of studies on the interactions of *Bdellovibrio* with actively growing and viable but nonculturable (VBNC) *Vibrio cholerae*. A significant observation was that *Bdellovibrio* attacked both VBNC and actively growing V. cholerae. These results indicate that *Bdellovibrio*, a "living antibiotic," has potential as an antibacterial agent in environmental and public health bioprotection.

Keywords: Microbial Contamination, Antibacterial Agents, Interaction With VBNC *Vibrio cholerae*

1. Introduction

The microbial contamination of ecosystems is associated with health risks in both humans and animals. The decontamination problem may be solved by understanding how nature avoids contamination and by intelligently applying this mechanism in our practices. From this point of view, the predatory bacteria genus of *Bdellovibrio*, which is widely spread in various aquatic and soil ecosystems, is of great interest. The control of bacterial abundance in nature is a complicated process because of the activities of protozoa, phages, competition among microorganisms, and the activities of higher organisms. However, *Bdellovibrio* is considered the main contributor to the control of Gram-negative bacterial abundance. Gram-negative bacteria of the *Bdellovibrio* genus, which are phylogenetically assigned to the subgroup of Deltaproteobacteria, are predators of other gram-negative bacteria, and they need prey cells to invade and utilize as a substrate for growth and reproduction. Their life cycle is biphasic. A free-living attack phase and an intra-periplasmic growth phase take place in the periplasm of their bacterial prey, which are referred to as bdelloplasts (1).

The ecological role of these bacteria is to maintain a balance between gram-negative bacteria in nature. Their ability to kill prey cells suggests that they play an important role as antibacterial and bioprotection agents. The use of Bdellovibrio's predatory activity against selected pathogenic microorganisms was reported (2-4) to have limited success. Previous studies were carried out using *Bdellovibrio* cells in suspension. There was a rapid loss of activity of *Bdellovibrio* cells when the concentration of prey cells was reduced below a level that was able to support *Bdellovibrio* development. Since its discovery in the 1960s (5), *Bdellovibrio* spp. were believed to be planktonic bacteria; however, in the 1990s, we developed a new concept of surface-associated predation in nature and immobilization in a two-component predator-prey system. Based on our results, we suggested an effective experimental model for analyzing the phenomenon of predation (6-8). Previous studies have demonstrated that *Bdellovibrio* cells are associated with surfaces (9-13).

The use of *Bdellovibrio* in the decontamination of polluted water has advantages because the approach is based on the use of natural regulators of bacterial abundance, and thus it is ecologically safe. A previous study examined the efficiency of the decontamination of polluted water by introducing immobilized *Bdellovibrio* cells (14). It was shown that the introduction of immobilized *Bdellovibrio* cells led to a ten-order increase in their density in the sewage bulk. It is remarkable that at the end of the experiment, the densities of Gram-negative bacteria were 26-fold less than those in the control. It was concluded that the introduction of immobilized *Bdellovibrio* into polluted water is effective for both accelerating and increasing the efficacy of the purification process. Immobilized systems have been shown to be more stable for longer periods because

of the renewed infection cycles on surfaces. This capability allows for several potential applications of *Bdellovibrio* in medicine, food protection, and ecotoxicological monitoring (monitoring microbial contamination of the environment) against the "bloom" in some aquatic ecosystems. Further potential applications include the decontamination of active sludge in water treatment plants against phytopathogenic bacteria, the decontamination of microbial industrial and domestic sewage, ecological crisis prevention, and bi-trrrism preparedness.

Although *Bdellovibrio* was discovered almost 50 years ago, only its bacteriolytic properties for active growth prey bacteria have been studied. However, many bacterial species have been shown to enter a state where they do not grow in conventional bacteriological media but maintain both viability and virulence (15). Such bacteria are known as viable but not culturable cells (VBNC), and they exist in the environment in large numbers for extended periods, even years. They are detectable only by employing appropriate methods (16). However, in the last six years, we have progressed in studying the interaction of *Bdellovibrio* with VBNC bacteria (17).

The current study analyses the relationships between pathogens and predators under different conditions, in liquid media, and in conditions that simulating the environment, such as biofilms on solid surfaces. Both actively growing and VBNC of V. cholerae were analyzed for their potential utilization of *Bdellovibrio* as an antibacterial agent.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

Bdellovibrio bacteriovorus 100 NCIB 9529 (Dr. Stolp, Germany), *Escherichia coli* IBPM B 102 (All Russian Collection of Microorganisms), and *Vibrio cholerae* 14035, both as VBNC and actively growing cells (Center of Marine Biotechnology, University of Maryland Biotechnology Institute, USA), were analyzed.

E. coli and *V. cholerae* prey were grown on trypticase soy agar (TSA) (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD, USA) for 24 – 48 hour at 30°C and then harvested by washing with 10 mL deionized distilled water (DDW) containing a 0.001 M salt solution (0.1 M $CaCl_2$ and 0.1 M $MgCl_2$ 6 H_2O) that was sterilized by filtration through 0.2 μm membrane filters (Nalgene Co., Rochester, NY, USA). VBNC cells of *Vibrio cholerae* were maintained in a 0.1% solution of Instant Ocean (18).

Bdellovibrio bacteriovorus (103 cells/mL) were grown in suspensions of prey (109 cells/mL) for 24 - 48 hour at 30°C with gentle shaking until the ratio (determined by enumeration) of predator and prey reached approximately 109:103. The suspensions were used as inoculae for cultures that had been through at least three passages of prey bacterial cells in DDW. *B. bacteriovorus* concentrations were enumerated by determining the number of plaques (PFU) on a lawn of susceptible prey bacteria, using the double-layer agar method with bacteriological agar (Difco Laboratories, Detroit, MI, USA). A lysis test was conducted, in which bacterial suspensions were incubated directly for 1 - 3 days were evaluated by employing both the clarity of the suspension and direct microscopy of the cells as means of enumeration (19). Colony-forming units of V. cholerae prey cells (CFU) on TSA were also enumerated.

2.2. Prey and Predator Experiments

2.2.1. Free-Living Cells

Predator-prey interactions in suspension were analyzed by counting plaques (PFU) and colonies (CFU) at four-hour intervals over 72 hour. TSA plates were used to determine CFU by employing the method previously described in (19). Predator-prey interactions on solid media were analyzed by counting the number of plaques on double layer of agar.

VBNC of V. cholerae were prepared using the method in Xu et al. (18). Briefly, bacterial cells of *V. cholerae* in logarithmical growth phase were suspended in a sterile salts solution and incubated at 4°C as described above (19). Under these conditions, the cells entered a stage in which they were no longer able to be cultured in standard microbiological media while maintaining their viability.

2.2.2. Attached Cells

Suspensions of prey cells were placed on cover slips and allowed to stand for 10 minutes to enable them to attach to the surface of the cover slip, followed by the transfer of one drop of a 24 - 48 hour suspension of predator cells. The mixture was allowed to stand for 30 minutes before washing in distilled water as described above. Glass cover slips with immobilized cells of both prey and predator were allowed to float on the surface of DDW in 3 mL wells of sterile tissue culture plates (Corning, USA). At four-hour intervals over 72 hour, the cover glasses were removed and acridine orange stained. Predators, infected prey (bdelloplasts), and uninfected prey (i.e., a three-member population) were enumerated.

All experiments were performed in replicates of four, and the results are presented as mean values.

3. Results and Discussion

Bdellovibrio grows and divides inside the prey cell, which then lyses, releasing the *Bdellovibrio* to prey upon

more bacteria. Because of this capability, *Bdellovibrio* is a potential antibacterial agent. However, since its discovery in the early 1960s (5), it still has not been applied as an antibacterial agent in real practice. Subsequently, we discovered the role of surface structures in predation (6, 8), which was supported by the work of other scientists with using genetic methodologies (20-23). The objective of this study was to expand the understanding of the ability of *Bdellovibrio* to infect VBNC bacterial cells in suspension as well as when they are attached to surfaces. They are more effective under surface-associated conditions.

The interactions of predator and prey in two-component cultures of *B. bacteriovorus* 100 and *V. cholerae* 14035 are shown in Figure 1. *V. cholerae* without *B. bacteriovorus* served as the control in these experiments. Oscillations in the numbers of prey and predator cells were observed, and it was found that the number of *Bdellovibrio* cells was directly proportional to the number of unlysed prey cells.

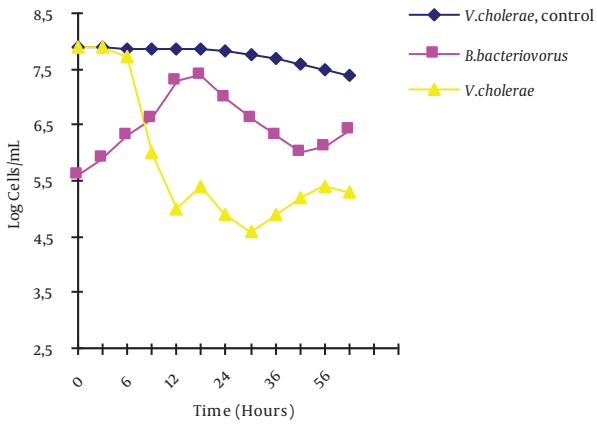

Figure 1. Dynamics of the Interaction of *B. bacteriovorus* With *V. cholerae* in Cell Suspensions

Figure 2 illustrates interactions of a two-component bacterial system in which attached cells were employed, i.e., immobilized *B. bacteriovorus* and *V. cholerae*. The components of the system included free *Bdellovibrio* cells, bdelloplasts, and intact cells of *V. cholerae*, a constant cycle of prey-predator. The *V. cholerae* population was replaced by *B. bacteriovorus*. Peaks of free *Bdellovibrio* cells occurred when both the bdelloplasts and *V. cholerae* decreased. The results clearly showed that the reduction of *V.cholerae* was more effective under surface-associated conditions than in suspension. This result also confirms the positive role of surfaces in the survival of *Bdellovibrio*.

Our early experiments showed that the immobilization of *Bdellovibrio* and prey on glass cover slips provided

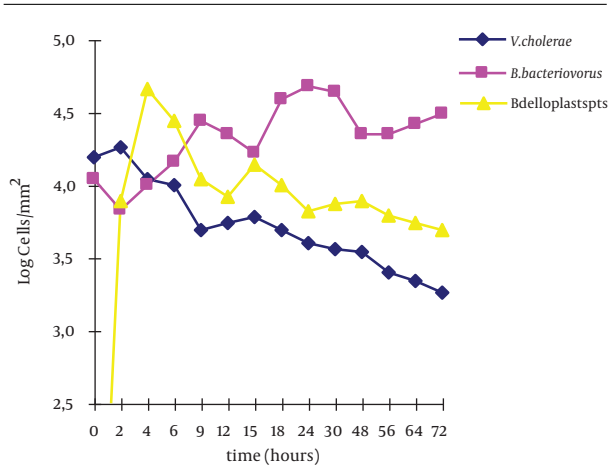

Figure 2. Dynamics of the Interaction *B. bacteriovorus* with *V. cholerae* as Immobilized, Surface Attached Cells

a convenient visualization of the process and the interaction between cells, which could be monitored by direct observation using epifluorescent microscopy (8).

Figure 3A and B illustrates the interactions of immobilized *B. bacteriovorus* and *V. cholerae*. It shows VBNC cells and the time-dependent transformation of *V. cholerae* cell into bdelloplasts and then the release of *Bdellovibrio* cells with a uniform distribution of the three components. The occurrence of intact prey cells, intraperiplasmatic bdellovibrios (bdelloplasts), and free *Bdellovibrio* cells demonstrated the continuous interaction in the two-component bacterial system.

Importantly, the *Bdellovibrio* progeny were morphologically variable. In addition to normal cells were curved oblong cells that were significantly larger than usual and derived from actively growing cells (index 1 in Figure 3A and B). Figure 3B illustrates the two morphological types of *B. bacteriovorus* cells that were released when *B. bacteriovorus* interacted with VBNC *V. cholerae*. This phenomenon was described in an earlier work on VBNC *H. pylori* cells (17). It could be explained by the presence of vegetative cells in *Bdellovibrio*. *Bdellovibrio* has been regarded as consisting of obligate parasites, but these findings suggest that axenic growth is also possible. However, additional molecular genetic investigations are required. Similar research is done at the university of Nottingham, UK (24).

Interestingly, the finding that the VBNC of *V. cholerae* were also attacked supports the potential application of Bdellovibrios in environmental biocontrol. holera is a contagious disease that is induced by *V. cholerae* cells, which recently were shown to be mostly VBNC. The disease is endemic in tropical regions, where it is exacerbated by

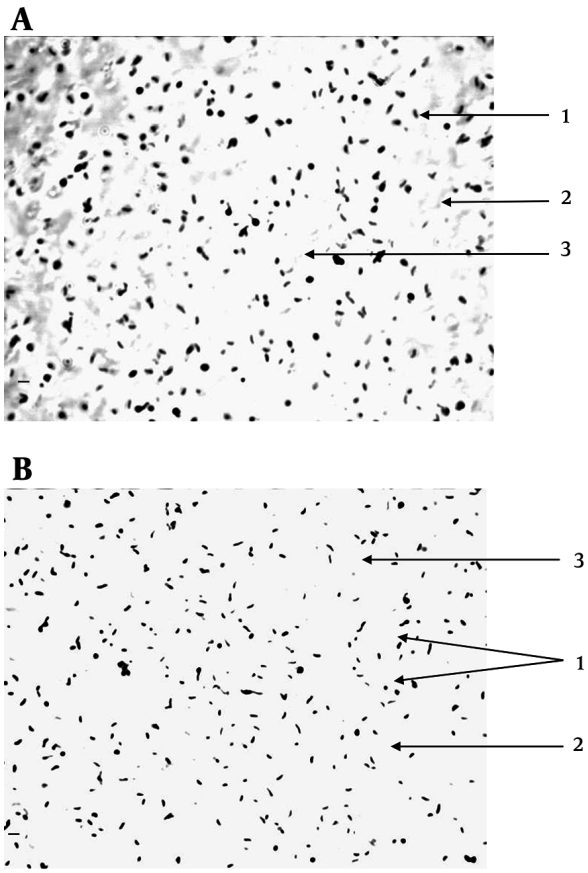

Figure 3. Interactions of *B. bacteriovorus* with Surface Attached VBNC *V. cholerae*, A, 6 hours; B, 24 hours; 1, free cells *B. bacteriovorus*; 2, intraperiplasmatic *B. bacteriovorus* (bdelloplasts); 3, intact cells *V. cholera*; Bar, 10 μm.

poor sanitary conditions and the lack of clean water. The finding that *Bdellovibrio* attacked and killed both actively growing and VBNC *V. cholerae* supports the application of these bacteria as antibacterial agents. This finding also confirms our previous findings of differences in the responses of surface-associated and suspended two-member predator-prey systems to selected environmental factors (7, 8), which supports the hypothesis that bdelloplasts not only allow *Bdellovibrio* to survive but they also play an important role in the surface attachment for their growth and reproduction. The results indicate that immobilized *Bdellovibrio* has potential as an antibacterial agent against both the actively growing and VBNC cells of bacteria.

4. Conclusions

Because antibiotic resistance in Gram-negative pathogens recently has reached alarming levels, it is important to study the ability of *Bdellovibrio* to infect pathogenic bacteria. The results of the present study showed for the first time that *Bdellovibrio* could kill both actively growing and VBNC cells of *V. cholerae*. This finding is especially important in regions where cholera is endemic, which have difficulties in predicting the occurrence of infection because of the conversion of environmental pathogens to the VBNC form. Based on the finding that the VBNC of *V. cholerae* were also attacked, *Bdellovibio* could be applied in the ecotoxicological monitoring of contaminated environments. Thus, Bdellovibrios, the "living antibiotic" has great potential as an agent in environmental bioprotection, water cleanup, and crop and food protection. It could also be applied as an agent in medical therapy, public health protection, and bioterrorism preparedness.

Acknowledgments

This research was supported in part by the Fulbright scholarship program. The author gratefully acknowledges Drs. R. R. Colwell and A. Huq for providing the opportunity to work at the center of biotechnology, university of Maryland, USA.

References

1. Starr MP, Baigent NL. Parasitic interaction of Bdellovibrio bacteriovorus with other bacteria. *J Bacteriol.* 1966;**91**(5):2006–17. [PubMed: 5327913].
2. Plissier M, inventor. Medicament, notamment pour le treatment des maladies infectieuses, et applications de sa substance active [in France]. 1976.
3. Scherff RH. Control of Bacterial Blight of Soybean by Bdellovibrio bacteriovorus. *Phytopath.* 1973;**63**(3):400–2. doi: 10.1094/Phyto-63-400.
4. Fratamico PM, Cooke PH. Isolation of Bdellovibrios That Prey on Escherichia Coli O157:H7 and Salmonella Species and Application for Removal of Prey from Stainless Steel Surfaces. *J Food Safety.* 1996;**16**(2):161–73. doi: 10.1111/j.1745-4565.1996.tb00157.x.
5. Stolp H, Petzold H. Untersuchungen über einen obligat parasitischen Mikroorganismus mit lytischer Activität fur Pseudomonas-Bacterien. *Phytopath Z.* 1962;**45**:364–90. doi: 10.1111/j.1439-0434.1962.tb02050.x.
6. Markelova NY, Colwell RR. Two-component predator-prey bacterial system immobilized on the surface of transparent cover glasses is a promising model for investigation of the role of bacterial predators in ecosystems. *Mikrobiol.* 1999;**68**(3):387—90.
7. Markelova NY. Effect of toxic pollutants on Bdellovibrio. *Proc Biochem.* 2002;**37**:1177—81.
8. Markelova NY, Gariev IA. Predatory bacteria Bdellovibrio: survival strategy. *Process Biochem.* 2005;**40**(3-4):1089–94. doi: 10.1016/j.procbio.2004.03.017.
9. Williams HN, Schoeffield AJ, Guether D, Kelley J, Shah D, Falkler WJ. Recovery of bdellovibrios from submerged surfaces and other aquatic habitats. *Microb Ecol.* 1995;**29**(1):39–48. doi: 10.1007/BF00217421. [PubMed: 24186637].
10. Kadouri D, O'Toole GA. Susceptibility of biofilms to Bdellovibrio bacteriovorus attack. *Appl Environ Microbiol.* 2005;**71**(7):4044–51. doi: 10.1128/AEM.71.7.4044-4051.2005. [PubMed: 16000819].

11. Nunez ME, Martin MO, Chan PH, Spain EM. Predation, death, and survival in a biofilm: Bdellovibrio investigated by atomic force microscopy. *Colloids Surf B Biointerfaces.* 2005;**42**(3-4):263–71. doi: 10.1016/j.colsurfb.2005.03.003. [PubMed: 15893228].
12. Pineiro SA, Stine OC, Chauhan A, Steyert SR, Smith R, Williams HN. Global survey of diversity among environmental saltwater Bacteriovoracaceae. *Environ Microbiol.* 2007;**9**(10):2441–50. doi: 10.1111/j.1462-2920.2007.01362.x. [PubMed: 17803770].
13. Lambert C, Fenton AK, Hobley L, Sockett RE. Predatory Bdellovibrio bacteria use gliding motility to scout for prey on surfaces. *J Bacteriol.* 2011;**12**(3):139–41.
14. Afinogenova AV, Markelova NY. Decontamination of water by means of introduction of bdellovibrios, immobilized on a fibrous carrier. *Chemie and Technologiya Wodi.* ;**19**(2):203–7.
15. Roszak DB, Colwell RR. Survival strategies of bacteria in the natural environment. *Microbiol Rev.* 1987;**51**(3):365–79. [PubMed: 3312987].
16. Hasan JA, Huq A, Tamplin ML, Siebeling RJ, Colwell RR. A novel kit for rapid detection of Vibrio cholerae O1. *J Clin Microbiol.* 1994;**32**(1):249–52. [PubMed: 8126193].
17. Markelova NY. Predacious bacteria, Bdellovibrio with potential for biocontrol. *Int J Hyg Environ Health.* 2010;**213**(6):428–31. doi: 10.1016/j.ijheh.2010.08.004. [PubMed: 20850380].
18. Xu HS, Roberts NC, Adams LB, West PA, Siebeling RJ, Huq A, et al. An indirect fluoresent antibody staining procedure for detection of Vibrio cholerae serovar 01 cells in aquatic environmental samples. *J Microbiol Methods.* 1984;**2**(4):221–31. doi: 10.1016/0167-7012(84)90017-4.
19. Shilo M. Predatory bacteria. *Science.* 1966;**2**:33–7.
20. Medina AA, Shanks RM, Kadouri DE. Development of a novel system for isolating genes involved in predator-prey interactions using host independent derivatives of Bdellovibrio bacteriovorus 109J. *BMC Microbiol.* 2008;**8**:33. doi: 10.1186/1471-2180-8-33. [PubMed: 18284687].
21. Lambert C, Smith MCM, Sockett RE. A novel assay to monitor predator-prey interactions for Bdellovibrio bacteriovorus 109 J reveals a role for methyl-accepting chemotaxis proteins in predation. *Environm Microbiol.* 2003;**5**(2):127–32. doi: 10.1046/j.1462-2920.2003.00385.x.
22. Tudor JJ, Davis JJ, Panichella M, Zwolak A. Isolation of predation-deficient mutants of Bdellovibrio bacteriovorus by using transposon mutagenesis. *Appl Environ Microbiol.* 2008;**74**(17):5436–43. doi: 10.1128/AEM.00256-08. [PubMed: 18621871].
23. Kadouri DE, To K, Shanks RM, Doi Y. Predatory bacteria: a potential ally against multidrug-resistant Gram-negative pathogens. *PLoS One.* 2013;**8**(5):ee63397. doi: 10.1371/journal.pone.0063397. [PubMed: 23650563].
24. Hobley L, Fung RK, Lambert C, Harris MA, Dabhi JM, King SS, et al. Discrete cyclic di-GMP-dependent control of bacterial predation versus axenic growth in Bdellovibrio bacteriovorus. *PLoS Pathog.* 2012;**8**(2):ee1002493. doi: 10.1371/journal.ppat.1002493. [PubMed: 22319440].