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Invited Mini Review

The dual probiotic and antibiotic nature of *Bdellovibrio bacteriovorus*

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Bdellovibrio bacteriovorus **is a predatory bacterium which attacks and consumes other bacterial strains, including the well known pathogens** ***E. coli*** **O157 : H7,** ***Salmonella typhimurium*** **and** ***Helicobacter pylori*****. This remarkable activity has been the focus of research for nearly five decades, with exciting practical applications to medical, agriculture and farming practices recently being published. This article reviews many of the exciting steps research into this bacterium, and similar bacteria, has taken, focusing primarily on their use as both an antibiotic to remove harmful and pathogenic bacteria and as a probiotic to help curb and control the bacterial populations within the intestinal tract. Owing to the unique and dual nature of this bacterium, this review proposes the use of "amphibiotic" to describe these bacteria and their activities. [BMB reports 2012; 45(2): 71-78]**

OVERVIEW OF BACTERIAL PREDATION OF BACTERIA

Currently, it is well known that predation is common among prokaryotes. Bacteria in their natural environments are subjected to predation from bacteriophage, protists and predatory prokaryotes (1). This last group of predators is unique in the fact that the predator is a bacterium that is clearly a living organism, as opposed to viruses and phages, and is smaller than the prey, in contrast with protists. Furthermore, of the three bacterial predators, this group has received the least amount of attention and investigation.

Predatory bacteria have different strategies for attacking their hosts (1) and can be categorized in part by the location of the predator with respect to the prey cell. They may attack in groups, referred to as a wolfpack, as observed with *Myxococci* (1, 2). Wolfpacks are located around the prey but do not appear to require physical attachment of the predator with the prey for the predation to occur. The predators in this scenario excrete a variety of hydrolytic enzymes that "chew up" nearby bacteria, thereby allowing the predators to consume the prey-derived nutrients. Another strategy is epibiotic predation, which is exemplified by *Vampirococcus* (3). Epibiotic predation requires the predator to attach to the outer surface of the prey cell, where it will begin hydrolyzying the components of the prey and consuming them. Invasion of the periplasmic space is accomplished by *Bdellovibrio*-and -like-organisms (BALOs). BALOs are perhaps the best studied and characterized predators within the bacterial predation of bacteria field and, as such, will be the focus of this review. They also represent one of the more complex predation schemes, with seven stages of predation (Fig. 1). The life cycle of *B. bacteriovorus* involves two distinct phases; an attack phase in which the cells are seeking the prey, during which the cells have a vibrio shape with a single polar flagellum, and an intra-periplasmic phase inside the prey. During this phase, the predator develops into a non-flagellated coiled non-septate filament which, just prior to exiting the exhausted prey, undergoes sep-

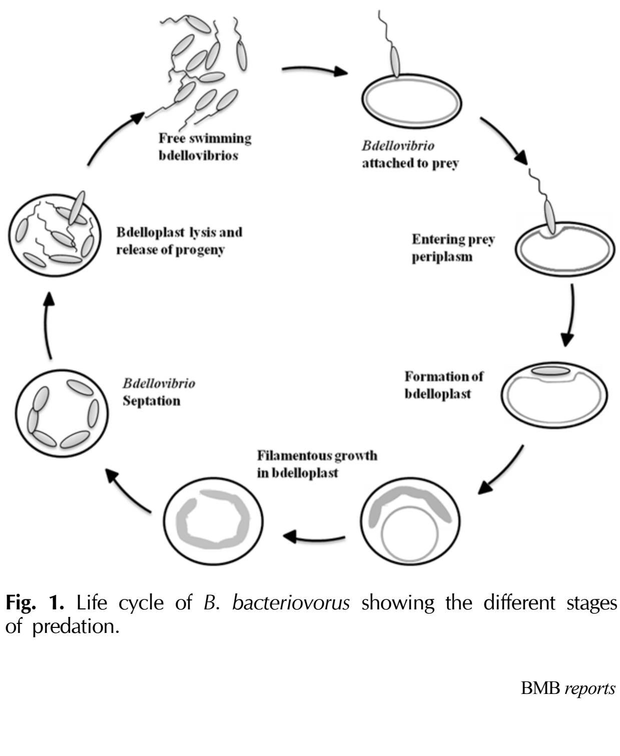

Fig. 1. Life cycle of *B. bacteriovorus* showing the different stages of predation.

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tation to give numerous motile flagellated progeny that start a new round of predation(4). Finally, direct invasion of the prey cytoplasm is also known, ex. *Daptobacter* (3). In such cases, the bacteria can readily degrade the host components, consume them and then lyse the cell to continue the process.

HISTORICAL PERSPECTIVE

The early years (1962-1992)

Bdellovibrio bacteriovorus is a tiny (0.2-0.5 um × 0.5-2.5 um) Gram negative predatory bacteria discovered by chance in 1962 by Stolp and Petzold while they were trying to isolate bacteriophages for plant pathogenic bacteria from soil (5). After their initial discovery, *Bdellovibrio*s were isolated from various habitats and found to be widely distributed in aquatic and terrestrial environments (6-9). Since its discovery and up to the time of writing this review, more than 390 articles have been published concerning this bacterium and similar organisms. Compared to other bacterial predators, *Bdellovibrio* received more attention during the last decades, owing to its interesting and mysterious life style and also because of its great potential to be applied as an antibacterial agent in industry, agriculture or medicine (Fig. 2).

During the early periods of predatory bacteria research, the efforts were concentrated mainly on identifying the life cycle of this organism, its strategies for penetrating the host cells, periplasmic growth and other aspects related to its life cycle. Starting about a decade later, researchers began to think about the possible application of this unique bacteria in different applications, such as in fighting plant pathogens (10) and in reducing bacterial numbers/contamination in food industries (11). However, researchers were limited in their potential applications since genetic tools and knowledge were both limited, particularly for this bacterium. As a result, for the first 30 years of *B. bacteriovorus* research, very little genetic work was accomplished.

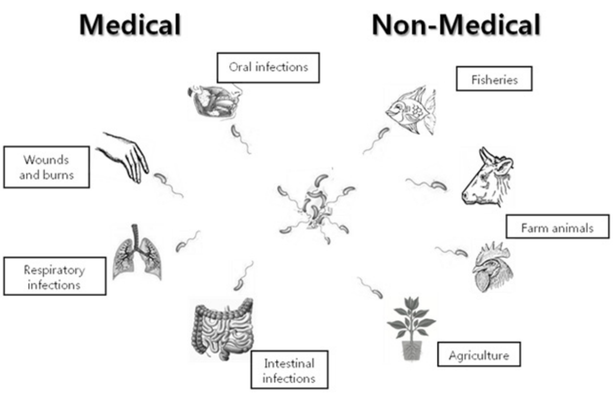

Fig. 2. Beneficial applications of BALOs.

The genetic era of predatory bacteria (1992-2011)

This changed in 1992 when Cotter and Tomashow tried to determine the genetic locus (hit locus) associated with the shifting to host independent mode of life (12, 13), opening up a new chapter in *B. bacteriovorus* research. Consequently, the last decade has seen progressively more attention given to the genetics of *B. bacteriovorus*. The complete genome sequence for the type strain (*Bdellovibrio bacteriovorus* HD100) was determined by Schuster *et al.* and published in 2004 (14), while Pan *et al.* expanded on this and earlier this year analyzed the genome and proteome composition of *B. bacteriovorus* (15). One remarkable finding from the sequencing results is that *B. bacteriovorus* showed little recent horizontal gene transfer with other bacteria (14). It is also not surprising for an obligate predator like *B. bacteriovorus* to find a large number of potential hydrolyzing enzymes in its genome (150 proteases/peptidases, 20 DNases, 9 RNases, 10 glycanases, etc). Besides, it is capable of synthesizing only 11 of the amino acids needed for protein synthesis while also lacks the degradation pathways for 10 amino acids, suggesting strongly that this bacterium synthesize its proteins almost exclusively during the intraperiplasmic stage.

Bdellovibrio bacteriovorus, although the best studied and characterized strain, is not the only intraperiplasmic predatory strain or genus within nature. Phylogenetic studies of *Bdellovibrio*-and-like-organisms (BALOs) based on 16S rDNA sequence analysis were done (16, 17), with numerous strains of predatory bacteria identified. Edouard Jurkevitch's group at the Hebrew University of Jerusalem is taking advantage of this fact and has also recently sequenced the genomes of several different BALOs with the goal being to identify genes common to all of the strains [personal communication]. Furthermore, Roschanski and Strauch (18) recently developed the pSUP series of conjugative shuttle plasmids that stably replicate inside *B. bacteriovorus*, thereby providing an important tool that will facilitate downstream genetic approaches.

Ushering in the therapeutic BALO period (2011-)

The lack of these tools and plasmids did not hinder early researchers from beginning research into therapeutic applications of BALO (11, 19), with one group even applying for a patent in France highlighting the use of *Bdellovibrio*s in the treatment of gastrointestinal diseases (referenced in (20)). A search of the literature shows that different research groups around the world are concentrating more on this area nowadays (20-25), with all of these studies being published within the last two years. They include research characterizing *B. bacteriovorus* in different animal hosts, including chicks (21) and cows, particularly their tears (22), and evaluated its effects on various pathogens, such as *Helicobacter pylori* (20), *Salmonella* sp. (23), and oral pathogens of humans, *i.e., Porphyromonas gingivalis* (24).

Consequently, the main aims of this review are to present the progress towards applying *B. bacteriovorus* as an antibacterial agent to address real environmental, industrial and medical issues and to define both the advantages and challenges that face *B. bacteriovorus* as both a promising probiotic and an alternative for antibiotics in the future.

BENEFITS OFFERED BY BALOs

BALOs are non pathogenic to higher organisms

For *B. bacteriovorus* to be used medically for humans, as well as animals, their safety should first be confirmed. Various studies and techniques have shown that BALOs are not harmful to humans or animals (Table 1). In 1973 a study done by Verluka showed that *Bdellovibrio* was not pathogenic upon injecting it into mice, rabbits and guinea pigs (26). Another study also on animal models was conducted few years later by Kramer's group (27). In their study, *Bdellovibrio* strain MS7 was fed to fishes, mice and frogs and, upon examining those animals, they found that *Bdellovibrio* was able to multiply in the intestinal contents of these animals *in vitro*. However, it was unable to establish itself in the intestinal flora of these animals *in vivo*. They studied this further in the same report and found that the number of viable *Bdellovibrio* strain MS7 cells gradually decreased over 24 hrs when inoculated into rabbit ileal loops.

In 1978, Lenz and Hespell co-cultivated different strains of BALOs (*B. bacteriovorus* 109 J, *Bacteriovorax stolpii* UKi2 and *Peredibacter starrii* A3.12) with animal cells isolated from mouse livers, hamster kidneys and bovine mammary glands (28). In their experiments, the BALOs didn't grow or attach to the animal cells. Furthermore, when they cultured the bacterial strains with erythrocyte suspensions, they found only a very low level of penetration, but no growth was observed. Finally, they also micurgically injected these three strains into either the perivitelline space or the cytoplasm of rabbit ova to determine if any of these BALOs can grow within animal cells and found no significant increase in the BALO numbers after 18 hrs.

Studies about the immunogenicity of *Bdellovibrio* were also conducted and the results appear highly promising for human health issues. Firstly, the lipopolysaccharide layers of BALOs were found to be unique in that their Lipid A contains α-D-mannose residues instead of the phosphate groups and, hence, their Lipid A is devoid of any negatively charged groups (29). As a consequence, they have a significantly lower binding affinity to the LPS receptors in human cells as well as a much lower endotoxic activity when compared to that of *E. coli*. The latter was confirmed by the very low induction levels of TNF-α and interleukin-6 from human macrophages when exposed to the bacteria (29).

Recently, in a study performed by Sockett's group (21), BALOs were administrated orally with antacids to young chicks. Their study found that the presence of the BALOs changed the overall natural microbiota of the gut but there were no adverse effects on the growth and well being of the animals. Meanwhile, another study found *Bdellovibrio* could be also isolated from animal faeces (16). In conclusion, based upon all of these reports, it can be concluded that BALOs to a great extent can be considered nonpathogenic and nontoxic to animals and humans.

BALOs attack pathogenic bacteria

In general, when compared to other biological tools, such as bacteriophages, *B. bacteriovorus* and other BALOs show a very non-specific predation of their host cells. Consequently, *B. bacteriovorus* can attack gram negative bacteria from very distinct and different genera. For example, it was reported that the *Bdellovibrio bacteriovorus* 109J host range includes strains from *Escherichia, Pseudomons, Chromatium* and *Spirillum* (1), while, more recently, Kadouri's group reported that the same strain can attack pathogenic bacteria from numerous genera, including *Acinetobacter, Aeromonas, Bordetella, Burkholderia, Citrobacter, Enterobacter, Klebsiella, Listonella, Morganella, Proteus, Serratia, Salmonella, Shigella, Vibrio* and *Yersinia* (23). Although according to their results, *B. bacteriovorus* 109 J could not attack *Campylobacter*, a study done by Markelova showed that another *B. bacteriovorus* strain, 100NCJB, could attack *Campylobacter jejuni* as well as *Helicobacter pylori* (20).

From the above results, it is clear that many pathogenic gram-negative bacterial strains which infect humans, in addition to animal and plant pathogens, can be predated upon by one or more strains of *Bdellovibrio*. It is also worthy to mention that in their study, Dashiff *et al*. (23) showed *Pseudo-*

Table 1. Highlights of *B. bacteriovorus* and its amphibiotic nature

Progress or Discovery	Year	Reference
Discovery of *B. bacteriovorus*	1962	(5)
B. bacteriovorus was not pathogenic upon injecting it into lab animals	1973	(26)
B. bacteriovorus was not toxic to lab animals when fed through gastrointestinal route	1977	(27)
B. bacteriovorus doesn't grow or attach to animal cells of different tissues	1978	(28)
B. bacteriovorus has low immunogenicity	2003	(29)
B. bacteriovorus can attack *H. pylori* and *C. jejuni*	2010	(20)
B. bacteriovorus attacks numerous periodontal pathogens	2011	(24, 25)
B. bacteriovorus predates various drug resistant human pathogens	2011	(23)
B. bacteriovorus controls *Salmonella* infections in young chicks	2011	(21)

monas aeruginosa, which infects patients with cystic fibrosis (30) and showed low susceptibility to attack by *B. bacteriovorus* 109 J, could be attacked by another predatory bacterium, Micavibrio. This bacterium, which doesn't belong to the *Bdellovibrio* genus, attacks by epibiotic processes and also replicates by binary fission instead of septation, in contrast to *Bdellovibrio*. These findings suggest that it may be possible to use a combination of different predators to concurrently attack multi-strain infections.

In recent studies also done by Teughel's group (31), *Bdellovibrio bacteriovorus* HD100 could attack *Aggregatibacter actinomycetemcomitans* (one of the major species which contribute to periodontitis). In a subsequent study by the same group (25), they showed that different bacterial species involved in periodontitis can be attacked by one or more of *Bdellovibrio* strains, even if the prey was strict anaerobe. This is remarkable since, on the hand, BALOs are strict aerobes and require oxygen to complete the predation cycle (7, 32) while, on the other hand, their prey will die if exposed to oxygen for too long.

BALOs and biofilms

It was estimated that 80% of bacterial chronic inflammatory and infectious diseases in humans involve biofilms (33), which include the biofilms made by uropathogenic *E. coli* in urinary tract infections, enterohemorragic *E. coli* in gastrointestinal infections, and wound, burn and respiratory infections caused by *P. aeruginosa*. In general, the main complication resulting from biofilm formation is that bacteria inside these biofilms are protected well and become much more resistant to antibiotic treatment as compared to planktonic cells (34-37) and this, in turn, contributes to the further progression of the infection. One special character about *Bdellovibrio* that distinguishes it from other biological-based antibacterial tools, such as bacteriophages (38), is their ability to invade biofilms, penetrating them deeply and effectively destroying them, as demonstrated in the studies done by Kadouri's group (23, 39). Furthermore, they reported that *Bdellovibrio* was even more efficient in attacking biofilms than the planktonic cells, leading to a greater loss in bacterial counts, purportedly due to the higher prey density in biofilms and hence faster and easier access to the prey. *B. bacteriovorus* was also able to attack biofilms made by single species and mixed species (23).

Also in a study done by the same group about *B. bacteriovorus* predation of biofilms made by oral pathogens (24), i.e., *Eikenella corrodens* and *A. actinomycetemcomitans*, they found that *B. bacteriovorus* could remove biofilms developed on hydroxyapatite surfaces and it could also attack metabolically inactive biofilms. Another important finding in that study was its ability to attack in presence of saliva. This will be of great significance for the use of BALO in the future as an oral antibacterial agent in mouthwashes, gargles, etc. as saliva is well known for its antibacterial effects it exerts on broad spectrum of microorganisms such as *Streptococcus mutans, Legionella pneumophila, Salomnella typhimurium, Pseudomonas aeruginusa* (40) owing to its many protective and antimicrobial proteins (41, 42), including peroxidase, mucins, proline-rich glycoprotein, immunoglobulins, agglutinin, lactoferrin, cystatins and lysozyme. Besides, small cationic antimicrobial peptides, e.g. defensins, cathelicidin and the histatins, have been found in human saliva and are currently being studied (42). These findings, along with those listed above in this section, strongly support the concept of using *B. bacteriovorus* for treatment of disease-related biofilms and, particularly, oral infections (23).

NON-MEDICAL APPLICATIONS OF BALOs

Although the focus of this review thus far has been primarily on human health and pathogens, numerous other studies with BALOs illustrate their potential within a wide range of areas. As with human health, they have shown promise in reducing the number of pathogenic and potentially harmful bacteria in the areas of agriculture and animal health.

Agriculture

BALOs are naturally found within soils and aquatic environments. In fact, it was found that *B. bacteriovorus* can survive in dried soils at undetectable levels but will revive and respond quickly to the presence of a large number of prey cells (43). It should come, then, as no surprise that BALOs are predominantly found within the rhizosphere (44). Upon analysis of *Capsicum annum L* rhizosphere soil, BALOs were detected in all tested samples in higher numbers compared to non rhizospher soil while having no apparent effect on the beneficial rhizosphere bacterial community (44).

The first attempt to apply *Bdellovibrio* in plant diseases control was peformed by Scherff in 1973 (10). In their study, *B. bacteriovorus* (strain Bd-17) was isolated from the rhizosphere of soybean roots and tested for the ability to control bacterial blight induced by *Pseudomonas glycine*a infection. The results show that Bd-17 could effectively inhibit the development of bacterial blight. In another study done by Song (45), six *B. bacteriovorus* strains were tested against bacteria associated with antibiosis or nitrogen fixation, including *Azospirillum brasilense, Paenibacillus polymyxa, Pseudomonas fluorescens, Pseudomonas putida,* and *Serratia marcescens*, and also pathogenic bacterial strains including *Burkholderia glumae*, No predation occurred for the nitrogen fixers or antibiosis- associated bacteria while *B. glumae* was predated upon, suggesting once more that *Bdellovibrio* is not harmful for beneficial plant symbionts.

Use of BALOs with animals

Recently, the restrictions for application of antibiotics in poultry industry are increasing and therefore, similar to humans, there is also a great need to find effective prebiotics and probiotics as alternatives (46). In the study mentioned earlier done by Atterbury *et al*. (21), oral administration of BALOs to young chicks pre-dosed with a gut colonizing *S. enterica* found that these predatory strains could effectively attack *Salmonella*, thereby reducing its number in the cecum and significantly mitigating cecal inflammation caused by the infection. *B. bacteriovorus* was also found to decrease the severity of keratoconjuctivitis caused by *Shigella flexneri* in rabbit eyes provided that they were administrated in the first 48 hr of infection (19). Another study evaluating the effects of BALOs on eye infections (22) focused on the control of *Moraxella bovis*, which causes serious ocular infections in cows. Once more, *B. bacteriovorus* was found to reduce the number of *M. bovis* attached to bovine epithelial cells in an *in vitro* model and the authors also showed *B. bacteriovorus*' ability to remain viable in tears for up to 24 hours in the absence of prey bacteria. These studies show the potential of BALOs to combat disease causing bacteria but more importantly that they may be effective when applied topically to the eye.

Owing to their proclivity to swim fast, it would seem that BALOs would also be effective within aqueous environments. This was demonstrated within a recent study done by Chu and Zhu (2010), where a strain by *B. bacteriovorus* (BdC-1) effectively protected cultured fish in ponds against an experimental infection of *A. hydrophila J-1* (47). The mortality of groups immersed with BdC-1 was lower than the groups without BdC-1 addition, which in turn strongly illustrates the possible use of BALOs to control infectious bacteria within lakes and ponds.

In conclusion, it seems that the use of *Bdellovibrio* as an antibacterial agent has various applications not only in medicine but also in many industrial and environmental issues and it is going to expand more in the future provided it proves itself in real industrial, environmental and medical problems.

Food industry

In one study done by Fratamico and Whiting (48), *B. bacteriovorus* 109 J was tested for its ability to lyse 32 bacterial strains comprising six genera of food-borne pathogens. The study demonstrated the potential usefulness of BALOs in the biological control of pathogenic and food spoilage-related organisms, even when used at low temperatures (12 and 19^{o}C). However, the incubation period required was extended compared to tests performed at higher temperatures (30 and 37^{o}C). In another study by the same group, the authors wanted to test the ability of *B. bacteriovorus* to remove the gram negative bacteria from the surfaces of food processing equipment (11). Their isolated strains of *B. bacteriovorus* were capable of lysing *E. coli* O157 : H7 and *Salmonella* sp. present on stainless steel surfaces.

The benefits of *B. bacteriovorus* are not limited to only steel surfaces. As noted above, there does not seem to be any clear negative impact when *B. bacteriovorus* is consumed. Consequently, in another study (49), three isolated *B. bacteriovorus* strains and one *Peredibacter* sp. (BD2GS) were tested for their ability to attack various potentially pathogenic *S. typhimurium* strains. Being more effective, *Peredibacter* sp. (BD2GS) was tested further for its ability to control the growth of *S. typhimurium* on tilapia fillets and the results show that it has a great potential and thus the authors concluded by suggesting that BD2GS can be used for this purpose within food industries.

Potential limitations

As demonstrated in the above references, BALOs, and particularly *B. bacteriovorus*, clearly is effective at reducing the population of harmful bacteria, support for its potential use as a probiotic. Moreover, in contrast with traditional probiotic bacteria, BALO strains achieve this reduction through predation, thereby killing the harmful bacteria, not merely displacing them. Based upon this dual nature as an antibiotic and a probiotic, we suggest calling BALOs "amphibiotic" to include both traits. These two characteristics are very attractive, particularly with regard to the continuously evolving resistance of bacteria to various classes of antibiotics. However, there are still many challenges BALOs need to overcome to improve their potential acceptance as amphibiotic agents.

First of all, it seems that BALOs don't completely kill their prey, *i.e.*, there are always some prey bacteria that are not attacked even at high predator to prey ratios. It should be noted, however, that this resistance is due to a plastic phenotype rather than permanently genetically encoded one (50) and, to date, no group has isolated a completely resistant mutant prey. Although a limitation, BALO resistance does not face the same problem as chemical antibiotics where, owing to the extensive use of the antibiotics since the discovery of penicillin, bacteria of different genera have become resistant to many of the commonly used antibiotics. Furthermore, this widespread resistance is expected to worsen in the future as the discovery of new classes of antibiotics continues to decrease (51, 52). The fact that the resistance to BALOs is lost quickly (50) suggests that the mechanism for attack may utilize a cellular component necessary for survival of the prey and, consequently, the prey will always be prone to predation.

Another potential limitation of BALOs is their broad host range. At first glance it would seem to be a beneficial trait, such as when mixed bacterial species are present in the infections, as happens in cystic fibrosis (30, 53). However, since their effects on the natural flora of different body cavities has not been studied thoroughly and should be taken into consideration, the use of BALOs in this case would need to be monitored.

According to early studies of BALOs (7, 32), these bacteria were found to be strict aerobes, and this of course can be a drawback that limits their potential use in oxygen-limited environments within the human body, such as gut or urinary tract. However, it should be noted that according to a study done by Schoffield *et al*. (54), BALO strains could survive for up to nine days under anoxic environments and some BALO strains could grow and attack under microaerobic conditions, albeit at a reduced rate compared to fully aerobic tests. Also, a genome analysis of *B. bacteriovorus* HD100 shows that this strain may have the potential to live under microaerophilic conditions since, aside from existence of the usual cytochrome oxidase CYTaa3 gene, there is also a gene for CYTbb3 with a higher affinity for oxygen (for microaerophilic conditions) (55). In addition, genes for nitrate reductase and nitric oxide reductase both are present, indicating the possible use of other electron acceptors when under oxygen limiting conditions (55). Even if BALOs are not so effective under oxygen-limited environments, like gastrointestinal or urinary tracts, they still present a very useful treatment regime for superficial infections, such as in wounds and burns, or in the form of aerosols for respiratory tract infections.

Another challenge for BALOs is their inability to attack Gram negative bacteria with S-layer on their surfaces (56) and that their activity is affected by the physiological status of their prey and by the presence of other bacteria, which may interfere and decrease or even increase their activity (57). Inside the human body, BALOs will be required to attack their prey in presence of various decoys like Gram-positive bacteria, epithelial cells and cell debris. In a study performed by Sockett's group (58), predation was studied in presence of a *Bacillus* strain as a live bacterial decoy. The results showed that although the presence of decoy did significantly reduce the rate of predation from 3 to 7 h, the predation still occurred, and eventually, the presence of decoy didn't significantly reduce the time at which all prey cells were almost predated. It should be noted that in that study, the decoy cells produced proteases which promoted the growth of the prey and hence the predator resulting in a higher final yield of the predator.

BALO activity is also reduced significantly in presence of some chemicals, such as high concentrations of glucose or glycerol, and also at low pH (59). However, a study done by Fratamico and Whiting (1995) showed that *B. bacteriovorus* was effective against *E. coli* in the pH range of 5.6 to 8.6 (48). Meanwhile, one class of chemicals that was found to have no effect on BALOs was B-lactam antibiotics (55, 60). Consequently, BALOs can be used in conjunction with these antibiotics since the predatory strains will still be active and attack the prey species. This attribute is clearly beneficial when a patient is struggling with an antibiotic resistant strain. These kinds of positive and negative interactions with the surrounding environment would definitely affect the efficiency of predation in real application.

CONCLUSIONS

This review article was undertaken to present the recent progress made in applying *Bdellovibrio bacteriovorus* and other BALOs as a tool for human, animal and plant health and to evaluate its potential as both a probiotic and an antibiotic. The studies presented above and their data demonstrate that *Bdellovibrio* strains, as well as other BALOs, are generally not harmful to humans, animals and plants but offer many benefits. This would need to be clarified further, however, before this bacterium can be used in a wide spread manner as both an antibiotic and probiotic.

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