

***Bdellovibrio Bacteriovorus* : A NATURAL SOLUTION FOR CONTROLLING HARMFUL BACTERIA**

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ABSTRACT

Bdellovibrio bacteriovorus is a gram-negative predatory bacterium it is prey on harmful gram-negative bacteria. Their predatory nature is supported by various structural, biochemical, physiological and genetic adaptations. From their genetic studies it was found that their genes are specifically involved in prey recognition, chemotaxis, motility and to produce enzymes those are involved in nutrient acquisition and degradation of prey. Some important proteolytic enzymes and peptidoglycan hydrolases biochemically produced by them this can be used in biotechnological industries. Due to the presence of their distinct LPS various in vitro and in vivo studies shows that their use is safe and it can elicit minimal inflammatory responses in mammalian systems. They have some antibacterial properties including reduction of *Klebsiella pneumoniae*, *Salmonella enteritidis* like pathogens. Some significant characteristics of *B.bacteriovorus* makes them a non-toxic and novel alternative to the traditional antibiotics.

Keywords: *Antibacterial property, Bdellovibrio bacteriovorus, predatory bacteria*

Introduction

Bdellovibrio bacteriovorus is a small delta proteobacterium, it has a unique mode of their survival by invading the periplasm

of gram-negative bacteria, they consume the cytoplasmic materials of their respective prey and destroys them totally (Negus et al,2017). In terrestrial and aquatic environment, they are widely distributed (Varon et al,1980, Jurkevitch et al,2000). They are also present in mammalian feces, suggesting their presence in mammalian gut (3B). Field Emission Scanning Electron Microscopy (FESEM) demonstrated that this predatory bacterium can also prey on gram positive *Staphylococcus aureus* through an epibiotic mechanism (Lebba et al,2014). It is important to find alternatives to antibiotics and assess the vulnerability of pathogenic prey to *B.bacteriovorus* predation (Nrgus et al,2017). This predatory bacteria also prey on multidrug-resistance gram negative bacteria *A. baumannii* and *K.pneumoniae* studied under in vitro condition (Kadouri DE et al,2013). From the in vivo studies it was found that this bacteria can reduce *K.pneumoniae* load in rat lungs (Shatzkes K et al,2016). It helps to overcoming eye infections and burn wounds, lung infections in cystic fibrosis patients and periodontal infections (Shanks et al,2013; Lebba et al,2014; Bonfiglio et al,2020). This bacterium can be used as a veterinary medicine to target *Moraxella bovis* a causative agent of conjunctival and corneal infections in cattle, it also prevents *Salmonella enterica* infections in chicks (Atterbury et al,2011). In agricultural purpose this is effective to prevent bacterial soft rot in potatoes (Youdkes et al,2020) and bacterial blight caused by *Pseudomonad glycibea* (Scherf et al, 1972). *B.bacteriovorus* whole genome is sequenced to identify the metabolic products produced by this bacteria and to explore their potential use in biotechnological field (Bratanis et al ,2020). This review focused on the predation strategies and potential application of *Bdellovibrio* sp. in various fields. It also paving the way to use *Bdellovibrio* sp.in the field of cancer treatment, in food preservatives and as a conventional antibiotic thus reducing the use of chemicals.

***Bdellovibrio* sp.Characteristics:**

These bacteria have various important characteristics that enable it to act as a predator those are as follows -

Structural characteristics: -

1.Unique Cell Shape: - It has a spiral or vibroid (comma shaped) shape,which helps it to move rapidly and efficiently. (Lambert et al. 2006)

2.Cell Wall Modifications: - The activity of Penicillin-Binding Proteins (PBPS) in *B. bacteriovorus* which acts on prey cell wall is important.PBP4 proteins (DacBs, Bd0816 and Bd3459) act as endopeptidases, breaking D-amino acid containing peptide cross links in prey cell walls to round and relax them for predator entry (Lerner TR et al,2012), but it has a self-protection protein which inhibits the function of PBPS proteins when *Bdellovibrio* sp. Is insight the predator and in this way, they can protect their own cell wall (Lambert C et al,2015).

Physiological Characteristics:

1.High Motility:It has high motility rate;their swimming speeds range from 35µm/s for strain 109 J to 106 µm/s for the HD100 strain (Rendulic et al. 2004).Motility is essential which helps to generate forces required for attachment or penetration of prey cell,researchers proposed that active,motile 'drilling' of the *Bdellovibrio* sp.was responsible for prey entry (Burnham et al.1968).

2.Chemotaxis: *Bdellovibrio* sp. exhibits chemotaxis activity,due to the presence of this feature this bacterium moves towards chemical signals secreted by prey bacteria.

Biochemical Characteristics:

1.Proteolytic Enzymes: *Bdellovibrio* sp. produces some proteolytic enzymes that helps the bacteria to get entry in prey by penetrating and breaking down the prey's cell wall(Sockett et al.2009).

2.Peptidoglycan Hydrolases: *Bdellovibrio* sp.secretes peptidoglycan hydrolases that assist in degrading the prey's cell wall (Sockett et al.2009).

Genetic Characteristics :-

1.Predation Genes: *Bdellovibrio* sp. contains some genes which

predisposing this bacterium towards predatory nature, such genes actually codes for some proteolytic enzymes and toxins (Kuru et al. 2017).

2. Gene Regulation: *Bdellovibrio* sp. regulates their genes in this way which enhance their predatory nature, those genes are basically related to motility and prey recognition being up regulated during this process (Karun Ker et al. 2013).

Classification of *Bdellovibrio* sp. based on their predation nature: On the basis of predation *Bdellovibrio* sp. fall into three main groups those are as follows –

- I. Endobiotic, in this case predatory organism invades prey and multiplies there.
- II. Epiobiotic, here predatory organism attaches and kill prey from outside.
- III. Transbiotic where lytic proteins secreted by the predator and they feed cellular contents released from the prey (Seccareccia et al, 2015).

Endobiotic Predation of *B. bacteriovorus* :-

The predatory activity involves an attack phase (AP), during which free-living Organisms locates its prey, where it detects a specific signal in the prey's cell wall, and it becomes transfer from the attack phase to the growth phase (GP). Using type IV pilus predator attaches to form the pre cell wall, and predator releases some hydrolytic enzymes in response to another cellular signal that create a channel in the cell wall. Via this channel predator get entry in the periplasm of prey (Kuru E, et al 2017). After entering into the periplasm, it triggers the development of a spherical structure known as a 'bdelloplast', utilise the prey cell's contents to grow into multicellular filament, undergoes septation, and finally lyses the prey cells.

Epiobiotic Predation: *B. bacteriovorus* and *B. exovorus*:

B. exovorus is an epiobiotic predator and surprisingly, *B. bacteriovorus* epiobiotically attack *S. aureus* in both planktonic suspensions (Pantanella F et al, 2018; Iebba V et al, 2014). Electron microscopy reveals *B. bacteriovorus* can

externally attached to the partially disrupted *S. aureus*, indicates that it can destroy the cell externally and extract all the contents in a characteristic epibiotic fashion (Novick et al. 2021).

Transbiotic Predation *Bdellovibrio* Sp.: This type of predator is not of clinical interest but some of their products play a major important role but further research is necessary to get the information on transbiotic predation.

The Predatory Life Cycle Of *Bdellovibrio* bacteriovorus :-

Bdellovibrio bacteriovorus attack gram negative bacteria *Escherichia coli*, a typical prey cell. The life cycle of similar in both *Bdellovibrio* bacteriovorus strain the smaller ($0.35 \times 1.1 \mu\text{m}$) vibroid shaped *B. bacteriovorus* HD100 (complete genome sequence identified already) and the larger ($0.5 \times 1.5 \mu\text{m}$) rod shaped *B. bacteriovorus* 109 J (most biochemical studies happen on it). *B. bacteriovorus* HD100 has slightly greater than the larger *B. bacteriovorus* 109J.

The life cycle of a typical *Bdellovibrio* bacteriovorus consists of eight phases, those are as follows-

1. **Attack-Phase:** *Bdellovibrio* sp. use active flagellar motility to move towards the *E. coli*, their chemotaxis in nature helps them to find bacteria-rich areas where they randomly collided with prey.
2. **Attachment (5-15 min.):** -After colliding with a prey cell *Bdellovibrio* sp. either detached within few seconds (abortive attachments) or permanently attached with prey *E. coli* cells.
3. **Invasion (12-25 min.):** -*Bdellovibrio* sp. secrete some cell wall degrading enzymes to invade prey cells. The flagellar motility ceases as *Bdellovibrio* sp. penetrates the prey's (*E. coli*) periplasm.
4. **Establishment:** *Bdellovibrio* sp. settles down within the periplasm and also get attached to the cytoplasmic membrane of a prey (*E. coli*) cell.
5. **Growth In Bdelloplast (25-180 min.):** -Cell wall remodeling happens in this phase thus the prey's cell appears rounded, and it starts growing and simultaneously replicates its DNA co-

encytically within the periplasm, thus a significant structure Bdelloplast is formed.

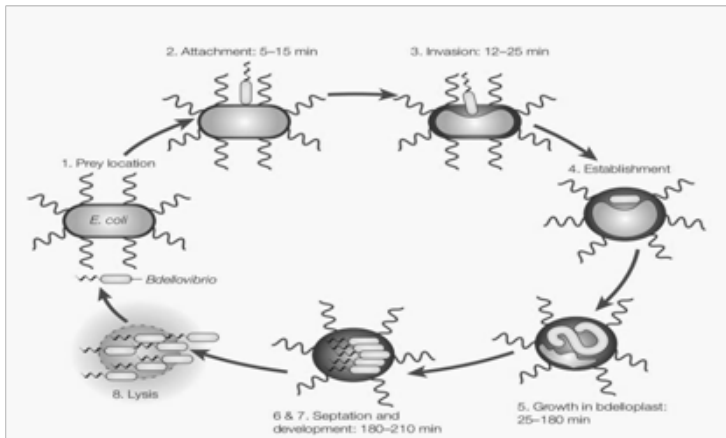


Fig 1. The predatory life cycle of *Bdellovibrio bacteriovorus* HD100 and *Bdellovibrio bacteriovorus* 109 J (Reference:- Sockett et al .2004)

6.&7. Septation And Development (180-210 min):-In septation phase *Bdellovibrio* sp. recognize that the cytoplasmic resources of prey are exhausted this time they divide into individual cells. At the development phase *Bdellovibrio* sp. Produce flagella and swims within the depleted Bdelloplast.

8.Lysis :-At last prey's outer membrane get ruptured and releasing *Bdellovibrio* sp.in the environment that they can back to the attack phase to hunt new prey (Sockett et al,2004).

In this way an *Bdellovibrio* sp. complete their predatory life cycle in their corresponding prey bacteria.

- **PREY RANGE:-** *Bdellovibrio* sp.have broad spectrum of pray range *Pseudomonas* sp. was resistance against *Bdellovibrio* sp. White *Vibrio* sp.was found to be most susceptible against *Bdellovibrio* sp.(Jurkevitch et al,2000).It is reported that *Bdellovibrio bacteriovorus* was prey on the strains those are belonging to the genera *Pseudomonas* sp. , *Chromatium* sp. ,*Spirillum* sp. and *Escherichia* sp.(Martin et

al,2002).

Recent studies found that *Bdellovibrio bacteriovorus* is able to attack some species those are belonging to the genera *Bardetella* sp., *Citrobacter* sp., *Acinetobacter* sp., *Burkholderia* sp., *Escherichia* sp., *Listonella* sp., *Enterobacter* sp., *Klebsiella* sp., *Proteus* sp., *Salmonella* sp., *Shigella* sp., *Yersinia* sp., *Aeromonas* sp., *Morganella* sp., *Pseudomonas* sp., *Serratia* sp., and *Vibrio* sp. (Dashiff et al,2011). *Bdellovibrio bacteriovorus* actually prey on several gram negative bacteria but it does not show any type of predatory effect on itself, even if they are grown axenically as prey and co-cultured with another predator strain, because their outer membrane is different from the other gram-negative bacteria (Hobley L et al,2012). Schwudke et al. found that in the lipid A of *Bdellovibrio bacteriovorus*, shows mannose substitution in place of phosphates. Due to this alternation negative charge is typically reduced in polar head group which enabling closer interaction with the other membrane of a typical prey organisms (Schwudke D et al,2003). This modification distinguishes *Bdellovibrio* sp. as non-prey thus reducing their susceptibility to cationic antibiotics. It also exhibits greater sensitivity to Triton X-100 compared to the other gram-negative bacteria (Tudor J.J et al,2008; Negus D et al,2017). Recently we found an information that *Bdellovibrio bacteriovorus* also interacts with gram-positive bacteria, including *Staphylococcus aureus* (Iebba V et al,2014; Pontanella F et al,2018)

- Isolation Of *Bdellovibrio bacteriovorus* From Environmental Samples:- Soil samples and waste water samples are collected to isolate *Bdellovibrio bacteriovorus* from the environment.

- Isolation Process From soil sample :-

Bdellovibrio bacteriovorus Isolation Process from The Soil Samples Described Here -

1. Initial Culture Preparation: *E. coli* MG1655 culture was inoculated in 50 ml LB broth and incubated overnight aerobically at 37°C. After that this culture was centrifuged at 4000 rpm for 5 minutes and the pellet was resuspended in 50

ml HM buffer.

2. Enrichment Culture: This resuspended sample was added to a 250 ml Erlenmeyer flask containing 100 mg of soil sample to make enrichment culture. This enrichment cultures was incubated aerobically for 48 hours at 30°C with shaking condition at 150 rpm. Turbidity depletion checked periodically to identify predation happen or not.

3. Filtration And Dilution: After 48 hours, the culture was centrifuged at 300 xg for 10 minutes to remove sediment and debris. This supernatant was filtered by 0.45 µm PES syringe filter (Gene Bio Systems, Burlington, ON, Canada) to exclude any *E. colicells*. Upto 105 dilutions were prepared using HM buffer.

4. Double-Layer Agar (DLA): Assay: In 15 ml glass tubes 100 ml of filtered dilution, 250 ml of *E. coli* MG1655, and 4 ml molten YPSC top agar were combined, and vortexed it into a petridish solidified YPSC bottom agar in petridishes. Those plates were incubated 3-10 days aerobically or anaerobically at 30°C to observe Plaques (Indicative Of Predation)

5. Plaque Purification: Individual plaques were picked by using inoculation loop and transferred it to a 15 ml conical containing 5 ml of HM buffer and 500 ml of a overnight *E. coli* MG1655 culture resuspended in HM buffer (Approx OD600 of 1.0). This co-culture were subjected to another round of DLA plating for purification.

This process was repeated until pure isolates of *Bdellovibrio bacteriovorus* were obtained.

6. Cryopreservation: Once turbidity reduction was observed , 200 ml aliquotes were mixed with 2 ml of skim milk freezing media in a 2 ml cryovial, gently mixing it and preserve it at -80°C for further use.

7. Microscopic Verification: The presence of *Bdellovibrio bacteriovorus* cells were verified using phase contrast microscopy. 10 ml aliquotes of enrichment culture or filtered samples were plated onto the microscopic slides under 100X magnification on a Leica DM200 (Leica Microsystem, Wetzlar, Germany).

If Dark, small, vibrio-shaped cells were observed it indicates successful isolation of *Bdellovibrio bacteriovorus*.

From Wastewater Sample:

Raw sewage samples (36 samples) were collected from December 2017 to March 2019 from North municipal wastewater treatment plant at Isfahan in Iran.

1. Sample Collection: Raw sewage samples were collected with a PH of 7.4, BOD =55 mg per liter, and COD=274 mg per liter. These samples were collected in clean bottles and transported immediately to the laboratory after collection (Oyedara OO et al,2016).

2. Centrifugation: These samples were centrifuged at 500 Xg for 5 minutes at 4°C. After centrifugation pellet was discarded and the supernatant was further centrifuged at 20,000 Xg at 4°C for 10 minutes. Then the final pellet was resuspended in 4 millilitres of cold HEPSE buffer (Oyedara OO et al,2016).

3. Filtration: This suspension was filtered through a 1.2 µm micropore filter to remove larger bacteria and debris (Oyedara OO et al,2016).

4. Dilution And Cultivation: Serial dilution of the filtrate were done using HEPES buffer. These dilutions were mixed with 200 ml of *E. coli* host suspension (1×10^9 CFU/ml) and finally it is spreading over broth DNB bottom agar (1.5%) plates (Oyedara OO et al,2016; Feng S et al,2016)

5. Inoculation: Those plates were incubated at 30°C for 3-6 days (12,13 E) with the addition of 3 mM $MgCl_2 \cdot 6H_2O$ and 2 mM $CaCl_2 \cdot 2H_2O$. After incubation plaques were observed over the DNB agar plates (Oyedara OO et al,2016; Feng S et al,2016).

6. Identification Of *Bdellovibrio Bacteriovorus* :- After incubation period *Bdellovibrio* sp. plaques were separate out from the DNB plate using scalpel and resuspended it in HEPES buffer, after that this plaque containing buffer solution passed through a 0.45 µm syringe filter and were further used for molecular analysis and microscopic examination to identify *Bdellovibrio* sp. strains (Oyedara OO et al,2016).

Whole genome sequencing of *Bdellovibrio bacteriovorus* isolates:-

After isolation of this predatory bacteria we have to know the gene sequence of them to get information of their phylogenetic position, pathogenicity, antibiotic resistance tracking and to develop any diagnostic tools from them. The process of whole genome sequencing are described below-

1. Illumina whole genome sequencing of *B.bacteriovorus* isolates:- Minimum 5ng/μl concentration of genomic DNA (gDNA) was extracted from *B.bacteriovorus*. For gDNA extraction and concentration analysis Qiagen QIAamp powerFecal pro kit (modified) and Qubit dsDNA HS assay kit were used respectively. Based on their availability at the time of experiment isolates were selected and sent them to the Microbial genome sequencing center for Illumina-based whole genome sequencing. Illumina DNA prep kit and IDT 10bp UDI indices were used to prepare DNA libraries. After that sequencing was performed on an Illumina Next seq200 platform using 2×151bp paired-end reads. Demultiplexing, quality control and trimming was performed with bcl-convert software (v3.9.3). Trimmomatic, MEGHIT and proKKA was used for trim reading, assemble contigs and genome annotation respectively (Greg Higgins, 2024).

2. Nanopore whole genome sequencing of *B.bacteriovorus* isolates:- At first genomic DNA was prepared using the Oxford nanopore SQK-LSK109 native ligation sequencing kit after that multiplexing was achieved using EXP-NBD104 kit. The sequencing library was run on a MIN106D flow cell using a MinION MK1C device. Fast guppy model was used to perform live basecalling via Minknow software. Porechop was used for adapter removal. NanoFilt was used to remove low-quality reads (<200bp) and apply a minimum quality score threshold of 8 (Greg Higgins, 2024).

3. Hybrid genome assembly and analysis of *B.bacteriovorus* isolates genome sequences:- Hybrid assembly and Illumina-only assembly were used to process each isolate. ProKKA was used for genome annotation. Genome quality was evaluated using checkM, it ensures completeness and accuracy. After

that potential exogenous microbial contamination was removed using JGI RQC filter pipeline. Then RAST tool was used to identify biological subsystem distributions across *B.bacteriovorus* and *P.exovorus* JSS genomes. Further ANI analysis were conducted using FastANI and ANI cluster map to compare isolate genomes and reference strains (Greg Higgins, 2024).

Genetic mechanisms that govern *Bdellovibrio* sp predation strategies:

- Gene expression during life cycle phases: Following gene oligonucleotide microarrays, gene expression analysis and reverse transcription qPCR it was found that approximately 40% of genes among the whole genome potentially involved in the predatory life cycle of *B.bacteriovorus* (Lambert et al .2010). *B.bacteriovorus* co-cultured with *E.coli* to further characterise the Attack phase and Growth phase genes by using Illumina sequencing (Karunker et al. 2013). From this studies authors found that 67% of genes expressed by *B.bacteriovorus* during growth phase (1557 genes) were active, 15% were active during the attack phase (353 genes) and 18% were expressed in both growth phase and attack phase.
- Attack phase genes: In *Bdellovibrio bacteriovorus* 59% genes encode hypothetical proteins and 41% genes were involved in their motility, chemotaxis and their cell surface composition.
- Growth phase genes: Growth phase genes mostly coded for cell division, ribosome biogenesis, DNA polymerase, chromosome partitioning and energy metabolism.
- Attack phase: In attack phase non-replicating bacterial cells use a single sheathed flagellum for high-speed prey hunting (Lambert et al. 2006). In this phase upregulation of *bd108* gene happen which control the extrusion or retraction of type IV a pilus (Rotem et al. 2015). Also upregulation of *merRNA* was happen in this phase.
- Transition to Growth phase: When *B.bacteriovorus* irreversibly binds to a prey cell its gene expression become

shifts to the growth phase. As a result this predatory bacteria creates a pore to the prey cell wall to enter in the periplasmic space and this time they secrete peptidoglycan-modifying enzymes to seal the pores that forms earlier than bdelloplast formation takes place (Lambert et al. 2010; Lerner et al. 2012). These are some peptidoglycan-modifying enzymes are present those are D-ala-D-ala carboxypeptidase, lytic murein transglycosylase, a polysaccharide de-acetylase which helps in the modification of peptidoglycan of prey cell wall during bdelloplast formation and those enzyme also helps to lyse prey cell when progeny get matured.

- Growth phase: When *Bdellovibrio* sp. enter into the growth phase this time upregulation of some genes happened those encoding some hydrolytic enzymes, regulatory factors and energy metabolism proteins (Rotem et al. 2015). But this time σ^{28} regulon, *mer* RNA and bdelloplast forming genes remains silenced.

Study of host immune responses to predatory bacteria:

If we want to choose this predatory bacterium as an alternative to antibiotics to treat multidrug-resistance bacterial infections, we have to understand their interaction with mammalian cells and the immune responses stimulated by them.

- Immune response in human: *B. bacteriovorus* is a gram-negative bacteria but due to the presence of unique lipopolysaccharide (LPS) it does not elicit strong inflammatory response like other pathogenic bacteria *E. coli* (Schwudke et al., 2003). Their LPS contains α -D-mannose instead of phosphate groups, which results in a lower binding affinity for LPS receptors on human cells and inflammatory responses is also reduced. From the studies it was found that *B. bacteriovorus* HD100 which contains neutrally charged LPS triggers 100 times less tumor necrosis factor α (TNF- α) production in human mononuclear cells compared to an *E. coli* K12 control.

- In vitro studies: Two experiments were done in laboratory to study human responses to the presence of *Bdellovibrio bacteriovorus*, those are as follows :-

1st Experiment: Monappa Ak uses four human epithelial cell lines (Intestinal HT29, Caco-2, T84 cells and Lung NuLi-1 cells) and murine microphage cell line (RAW 264.7) to *Bdellovibrio* strains HD100 and BY1 for in vitro studies. After 24 hours of incubation (without *B.bacteriovorus*) epithelial cell line viability is reduced slightly, while the murine macrophages were not affected at all. (Monappa et al,2016). But in the presence of *B. bacteriovorus* an increase in the production of TNF- α and IL-12 inflammatory cytokines observed from the RAW 264.7 macrophages after 6 hours exposure. However, the levels of these produced in response to *E. coli*, which reveals that the membrane components of *B. bacteriovorus* are relatively harmless.

2nd Experiment:(Gupta et al,2016)

The findings of Gupta et al. further confirmed the first experiment result. In their study, they tested three types of human epithelial cell lines (HaCaT, HepG2 and HK-2) and two human immune cell lines (Blood-derived monocytes and splenic monocytes, THP-1) by exposing them to *Bdellovibrio bacteriovorus* 109J and HD100. Their study basically measures cell line viability and cytokine responses.

After 24 hours of exposure, no deleterious effect was observed in any of the cell lines which is determined by cell imaging and cytotoxicity assays. Additionally, there was no significant elevation of several key cytokines [GM-CSF (Granulocyte Macrophage Colony-Stimulated Factor) interferon gamma (IFN- γ), IL-10, IL-12p70, IL-1 β , IL-2, IL-6, IL-8 and TNF- α] in four of the five human cell lines (HaCaT, HepG2, HK-2 and Spleen monocytes) over the same time period.

But, upon exposure of THP-1 macrophages to the predatory bacteria show increased level of two cytokines (IL-1 β and IL-6) at 4 hours duration and additional increase in GM-CSF, IL-10, IL-12p70 and TNF- α at 24 hours duration. But still, the levels of IL-1 β and TNF- α were lower than those seen in control cells when exposed to harmful *E. coli* 0157:H7. However, an anti-inflammatory role played by IL-10 which may help to balance the immune response.

- **In Vivo Studies:** Two experiments were conducted in laboratory to study mouse immune responses in the presence of *B. bacteriovorus*, those are as follows-

1st Experiment: In a mouse infection model, after administering predatory bacteria (*B. bacteriovorus* 109J) intranasally or intravenously an mild inflammatory responses was observed.

Intranasal Administration: Within 1 hour administration of *B. bacteriovorus* 109J, proinflammatory cytokines (IL-1 β , IL-23 and TNF- α) increased by fivefold or more in the lungs of mouse. But this cytokine levels returned to normal within 24 hours (58).

Intravenous Administration: At 3 hours post-inoculation significant level TNF- α and IL-6 were observed in the kidneys, liver and spleen, but it become dropped to baseline by 18 hours. Similarly in the blood IL-1 β , IL-6, IL-10, IL-12, CXCL-1, INF- γ and TNF- α cytokines was observed (Shatzkes K et al,2015).

2nd Experiment: A high dose of *B. bacteriovorus* strains HD100 and 109J was administrated intranasally as a result threefold or higher induction of CXCL-1, IL-6 and TNF- α was detected in the lungs of rat within 1 hour. But it returned to baseline levels within 24 hours. Similarly, IL-1 β and IL-13 was detected at 24 hours of post-inoculation which returned to baseline by 48 hours (Shatzkes K et al,2016).

3rd Experiment: Shank et al. showed that no cytotoxicity was found in human corneal-limbal epithelial cells upon exposure of predatory bacteria and no significant increase in pro inflammatory cytokines IL-8 and TNF- α was observed compared to *P. aeruginosa* positive control at 4 and 24h post-exposure (Romanowski EG et al,2016).

Antibacterial Activity Of *Bdellovibrio bacteriovorus*:

From the In vitro and In vivo studies, we have found that *Bdellovibrio bacteriovorus* also shows significant antibacterial activity.

In-Vitro Antibacterial Activity:

Several In vitro studies have found that *B. bacteriovorus* has a significant antibacterial activity.

In a study it was found that *B. bacteriovorus* reducing *Klebsiella pneumonia* populations by about 106 fold. But some of them get survival by developing phenotypic resistance strategies and regrow (Shatzkes K et al,2016). Interestingly, it was also found that a lytic bacteriophage associated with *B.bacteriovorus* has been found to enhance its efficacy, it leads to this complete eradication of prey bacteria in some cases (Hobley L et al,2020).

In another study, *B. bacteriovorus* showing antibacterial activity against biofilms farming oral Pathogens by penetrating and disrupting biofilms (Kadouri D et al,2005; Dashiff A et al,2011). Nevertheless some residual prey population can also persist within biofilms due to the presence of substance like cyanide or indole produced by prey organisms (Mu W et al,2017).

In Vivo Antibacterial Activity :-

In vivo studies demonstrate that *B.bacteriovorus* also exhibits some notable antibacterial activity.

- In a rat lung infection model which is infected by *Klebsiella pneumonia* upon treating with *B.bacteriovorus* it seems to reduce intrapulmonary bacterial titers by 103 fold(Shatzkes K et al,2016).But after intravenous infection it does not decrease harmful bacterial loads in organs ,instead it improving the health of treated rats compared to the untreated rats (Shatzkes K et al,2017).
- In other studies showed *Salmonella* sp. infection in chicks are attenuated by *B.bacteriovorus* (Atterbury RJ et al,2011).This predatory bacteria can also cooperating with immune cells to care *K.pneumoniae* infections in Zebra fish larvae,and reducing mice infected with *Yersinia pestis* (Findlay JS et al,2019).

Surface Antigen profiling: A key to enhancing bacterial recognition and vaccine efficacy:

Researchers studied the outer membrane proteins (oMPs) of *B.bacteriovorus* and other related *Bdellovibrio* sp. like organisms (BALOs) to identify suitable targets for antibody development. They focus to find conserved surface proteins to develop antibodies applicable across various BALOs. A candidate of BALOs (primarily *B.bacteriovorus*HD100) antigens from homologs of known OMPs in *E.coli* were identified using uniprot, emphasizing topology, localization and expression and completeness of genomic details. BALOs genomic data were analysed via ExPASy and BLASTp for similarity evaluation and sequence alignment. Two BALO OMPs were chosen for antibody development those are slc39 and TolC (Fig 2.). Where slc39 is a fall under solute carrier family and functions as an iron-regulated transporter (Uniprot C et al,2023). Also Tolc act as an outer membrane export factor that involved in efflux and it has the potential to act as an multidrug resistance factor (Uniprot C et al,2023).



Fig 2. Predictive structures of Slc39 (A) and TolC (B) *B.bacteriovorus* proteins generated by Pacific Immunology. Transmembrane domains are highlighted in yellow. Regions that would be most suitable for antigen development are highlighted in red (Reference: Greg Higgins, 2024).

- Developing antigens and antibodies based on selected OMPs:

The selected protein sequences of Slc39 and TolC were forwarded to design antigen and antibody synthesis purposes. Peptides from outside regions of transmembrane domains were selected and those two peptides were predicted as suitable antigens for each of Slc39 and TolC (TABLE). These peptides were attached to a keyhole Limpet Hemocyanin carrier and it was injected into rabbits, thus generating four antibodies with two biological replicates for each protein. For isolation purposes final production bleeds were purified by affinity column chromatography (Greg Higgins, 2024).

Verification of *B.bacteriovorus* OMP recognition:-

- **Preparation of coverslips:** Circular coverslips were sterilized by autoclaving and treated them with 70% ethanol for 15 minutes then dried them under UV light. Then these coverslips were coated with 200µl of 0.1% poly-L-lysine at room temperature. After that excess poly-L-lysine was removed and 3× with 1× PBS is used to wash coverslips. Further coated coverslips were dried under UV.
- **Application and fixation of cells:** 50µl of prepared *B.bacteriovorus* HD100 cells were applied to the coverslips and incubated them at room temperature for 1 hour. Then fixation was performed by using 200µl of 4% paraformaldehyde for 15 minutes at room temperature. Further coverslips were washed using 1× PBS.
- **Blocking and antibody staining:** 500µl of a 10% normal goat serum is used as a blocking solution and coverslips were coated with this solution followed by overnight incubation at 4°C. Then blocking solution was removed and 50µl of the primary antibody were added to a triplicate wells followed by 1 hour of incubation. Washing step repeats again and further 50µl of secondary antibody followed by incubating them 1 hour in dark condition.
- **Counterstaining:** After washing a DAPI counterstain was added to the coverslips by applying 300µl of diluted DAPI followed by 5 minutes incubation at room temperature

in dark condition.

- **Mounting and Imaging:** After final washing coverslips were mounted on slides using Mowiol 4-88 and stored them at 4°C until imaging them using a Leica DM 500B epifluorescence microscope at 100× magnification. Velocity software used to analyze the images.

Safety studies to show that *Bdellovibrio bacteriovorus* does not affect animal health:-

- **Ocular safety :-** For the potential treatment of eye infections *B.bacteriovorus* applied on the ocular surface of live rabbits demonstrating its non-toxicity nature. Any clinical signs of inflammation or interference with corneal wound healing were not observed upon applying this predatory bacterium (Romanowski et al,2016).
- **Respiratory and intravenous Administration:** Upon administering high dose of *B.bacteriovorus* (4×10^9 PFU intranasally, 1×10^8 PFU intravenously) were well-tolerated in mice and all mice were survived during 20 day observation period without any signs of distress. Their histological analysis revealed no adverse pathology in examined organs (Shatzke et al,2015).
- **Oral administration in poultry:** Oral dosing of *Bdellovibrio bacteriovorus* reduced salmonella sp. number in the ceca of birds it also preventing gut inflammation. It was observed that gut microbiota diversity was altered in non-infected chicks those were treated with *B.bacteriovorus*, but interestingly no adverse effect were observed in them (Atterbury et al ,2011).
- **Lung infection in rats:** Upon administration of *B.bacteriovorus* intranasally sublethal lung infection of *Klebsiella pneumonia* in rat reduced upto $3.0 \log_{10}$ amount than the previous bacterial load (Shatzkes et al,2016).
- **Zebra fish Embryo model:** Lethal injection of *Shigella flexneri* into the hindbrain of zebra fish embryos were rescued by *B.bacteriovorus* injections into the same compartment. Here *B.bacteriovorus* predation nature is supported by the host immune cells but this predatory bacteria was ultimately

cleared by macrophages and neutrophils of the fish (Willis et al,2016).

Applications of *Bdellovibrio* sp.:*Bdellovibrio* sp. is a predatory bacterium, it has gained considerable interest in recent years due to its distinctive predatory behaviour and it has the potential to be used in multiple fields. As a natural predator of gram-negative bacteria, *Bdellovibrio* sp. presents a promising approach for managing bacterial populations across different settings. These bacteria have various applications in fields such as antibacterial development, environmental cleanup, biomedical research and agricultural, aquaculture enhancement. Those are as follows-

- Therapeutic potential: From the various studies we have found that *B.bacteriovorus* can be used as a therapeutic or biological control agents in various purposes those are as follows-
- It can reduce the *Klebsiella pneumonia* bacterial load within the lungs of rats. The *B.bacteriovorus* also did not affect negatively on host after their inoculation (Shatzkes K et al,2016).
- It also plays an important role to provide protection and increase life span in mice that were infected with lethal injections of *Yersinia pestis* which is responsible for the plague (Sutherland et al,2004).
- These bacteria reduce the infection of *Salmonella enterica* in chick ceca of a chicken (Atterbury et al,2011).
- Low toxicity: The LPS of a typical *Bdellovibrio* sp. has reduced toxicity compared to the other gram-negative bacteria, thus lowering the risks of inflammatory responses in human cells (Kessel M & Shilo et al,1976; Thomashow et al,1978).
- *B.bacteriovorus* act as a prey on gram-negative oncomicrobes those are found in human gut, and this property helps to be novel approach to be used in therapies for CRC treatment (Greg Higgins,2024).

- **Potential in combination therapies:** It can be used as antiseptics or β -lactam antibiotics because it shows limited resistance development and it works effectively in synergy (Thomashow et al,1978; Cotter et al,1992).

■ **Probiotic approaches:**

■ **Presence in mammals:** From the recent studies we have found that *Bdellovibrio* sp. is present in mammalian guts, and it has the potential to act as a probiotic to combat pathogens in human microbiota (Lambert et al,2002).

■ **Selective targeting:** This bacterium specifically targets gram-negative bacteria without harming human cells that making it a promising candidate for safe probiotic applications (Ruby et al,1992).

■ **Role in microbial ecology:** It has the ability to influence the microbial balance in ecosystems suggesting that it could positively impact in human gut health by reducing pathogenic bacteria (Fackrell et al,1973).

■ **Food Industry:**

■ **Improvement of food safety in animals:** BALOs were tested in various animals to demonstrate significant reductions of *Salmonella* sp. and other pathogenic bacterial colonization to improve food safety (Atterbury et al,2011).

■ **Food preservation:** *Bdellovibrio* sp. has the ability to reduce food borne pathogens such as *E.coli* on chicken and canned meat, it minimizing the use of chemical preservative while improving food safety (Ottavina et al, 2020) .

■ **Water treatment :-**

■ **Potable water pre-treatment:** This is used in the pre-treatment step to reduce microbial load in waste water, thus limiting biofilm formation and enhancing membrane filter efficiency (Kim et al 2013) C.

■ **Rainwater purification:** Their predatory capability was successfully paired with solar-based methods to enhance the disinfection of harvested rain water (Waso et al , 2020).

■ In biotechnological field :-

■ **Enzyme source:** *Bdellovibrio* sp. produces enzymes like proteases, DNases and RNases, which could serve as an antimicrobial agents or tools for antibody analysis and protein research (Bratanis et al , 2020) .

■ **Biofuel production:** This bacteria promotes microalgae growth by controlling bacterial contamination, aiding in eco-friendly biofuel production (Li et al ,2018).

■ **Recovery of Intracellular products:-** This predatory bacteria used as a biological agent to recovering bioplastics like polyhydroxyalkanoates by lysing gram-negative bacteria, thus in this way they reducing the need for chemical solvents (Martinez et al ,2016).

■ In Agricultural field:-

■ **Prevention of spoilage:** It was found *Bdellovibrio* sp. is used to fight against *Pseudomonas tolaasii* a harmful bacteria that infect post-harvested mushrooms (Saxon et al, 2014).

■ **Prevention of soft rot in potatoes:** BALOs prevent bacterial soft rot in potato slices, but their protective effect become reduced if applied after the pathogen had already infected the crop. So we have to apply this as a pesticide before contamination (Youdeowei et al ,2020).

■ In Aquaculture field:

■ **Enhancement of Marine Aquaculture species:** *Bdellovibrio* sp. improved growth , survival rates and overall health of species like black tiger shrimp (*Penaeus monodon*) and turbot fish (*Scophthalmus maximus*) by targeting harmful bacteria in rearing waters (Wen et al ,2010; Guo et al,2017).

■ **Curative effects against pathogens in fish:** *Bdellovibrio* sp. has the curative effects against fish pathogens thus improving channel catfish and turbot farming (Chen & cai 2011; Zeng et al,2017).

■ **Environmental benefits in Aquaculture:** *Bdellovibrio*

sp. reduces heterotrophic bacterial populations and biofilm formation in aquaculture systems, thus promoting healthier environment to the aquatic organisms (Wen et al ,2010; Li et al ,2014).

Challenges for *Bdellovibrio* sp. survival: *Bdellovibrio bacteriovorus* a typical predatory bacteria facing various difficulty for their survival those are discussed below-

- **Discovery of Bdellophages:-** A tail less icosahedral single-stranded DNA bacteriophage was found that infecting *B.bacteriovorus* they known as “bdellophages”. This viruses infect both host-dependent and host-independent variants of *B.bacteriovorus* (Hashimoto et al 1970; Althausen et al, 1972).
- Upon using *B.bacteriovorus* to treat gut related infections and disease require anaerobic environment but *B.bacteriovorus* is an obligatory aerobic microorganisms.
- **Threats from protists:-***B.bacteriovorus* faces threats not only from bacteriophages but also from phagotropic protists that influence this bacterial community composition .Some ciliated protists varieties can also consume both living and dead *B.bacteriovorus* cells (Johnke et al, 2017).
- *Bdellovibrio bacteriovorus* have an unusual large genome (3.8 Mbp) were several genes support anaerobic respiration condition of this bacteria (Rendulic S et al,2004).
- **Impact of secondary metabolites:***Chromobacterium piscinae* produce some secondary metabolites, such as violacein and cyanide which inhibit predation of *B.bacteriovorus*. This inhibition happen in a calcium/HEPES buffer but not in diluted nutrient broth, indicating medium dependent effects (Mun et al, 2017).
- It is difficult use this predatory bacteria to treat gut related diseases, but some studies mentioned that infection of *Salmonella enterica* in chick ceca is prevented by *B.bacteriovorus* supporting their possible anaerobic growth (Atterbury et al,2011).

Potential mechanisms by which pathogens avoid predation:

- **Outer membrane as a barriers to predation:**

When *B.bacteriovorus* get survived in the extracellular environment they next faces the challenge to overcome prey's outer membrane. A wide range of gram-negative bacteria potentially prey to this predatory bacteria but still variations in susceptibility to predation was observed even within the same species (Dashiff et al,2011). But variations in the outer membrane does not completely avoid predation by *B.bacteriovorus* (Varon M et al,1969). Further testing requires to predict their role in averting predation due to new outer membrane variants (fig 3a).

- **Role of capsules:** Bacterial capsule can prevent bacteriophage infections (Sutherland IW,1965). From the studies we have found that *E.coli* k29 capsule does not inhibit predatory attachment or penetration, because predator usually penetrates the capsule by flagellar motility (Koval SF et al,1997).(fig 3b)

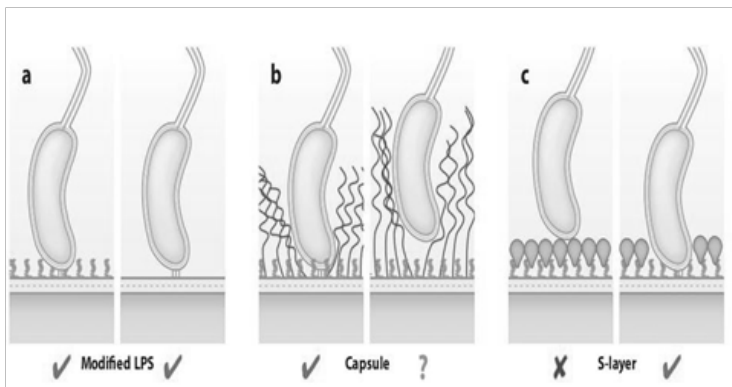


Fig .3 (a) *Bdellovibrio bacteriovorus* (yellow) attaches to a gram-negative bacterium with LPS outer membrane O antigen (left) or no antigen (right) (Varon M et al,1969). Other LPS modifications have yet to be studied. (b) *B.bacteriovorus* attaches to a gram-negative bacterium with a capsule. *E.coli* polysaccharide capsules are not a barrier of predation(left) (Koval SF et al,1991), but the effects of other biochemically

distinct prey capsules on predation have yet to be studied (right). (c) *B.bacteriovorus* attaches to a gram-negative bacterium with a surface layer (S-layer) only at point where the S-layer is incomplete (right), so predation is less efficient on S-layer-expressing prey (Koval SF et al,1991)

- **Role of S-layer in predation:** The S-layer is a common bacterial structure which is made up of crystalline protein subunits that may inhibit *B.bacteriovorus* attachment. *B.bacteriovorus* predation is protected by the S-layer of *Aquaspirillum serpens*, *A.sinuosum* and *Aeromonas salmonicida* bacteria (Koval SF et al,1991). partial expression of the S-layer can expose the outer membrane, making it susceptible to predation and highlighting how even small vulnerabilities in this defences can be exploited (fig 3c). Further investigation is needed to understand the role of the S-layer in reducing predation by medically significant pathogens.

- **LPS and O-antigen as barriers:** The LPS of gram-negative bacteria act as a potential barrier to the predator so they cannot access the outer membrane and periplasm. It was identified that O-antigen a capsular component can also hinder predation. From the studies we have found that those *Vibrio cholerae* isolates lacking O-antigen were more susceptible to *Bdellovibrio* sp . than their wild type isolates (Seed et al,2012).

Conclusion: *Bdellovibrio bacteriovorus* is a predatory bacteria that holds a great promise to act as a biocontrol agent against various pathogenic bacteria and colorectal cancer (CRC) associated oncomicrobes including *Fusobacterium nucleatum*, enterotoxigenic *Bacteroides Fragilis* and pKs+ *E.coli*. It's specialized features such as high motility, chemotaxis and production of proteolytic enzymes enables them to efficiently target pathogenic bacteria thus paving the way towards living antibiotic. Various research demonstrates its safety in vivo and in vitro condition, minimal inflammatory response and its ability to reduce bacterial populations.

However, some challenges remain such as reduced effectiveness in anaerobic environment, development of

bacterial resistance, presence of Bdellophage and presence of some predatory protists against them. Even *B. bacteriovorus* a innovative alternative to conventional antibiotics ,along with their potential applications in infection control, agriculture and biotechnology. Further research is necessary to enhancing its anaerobic efficiency, solve the resistance issue to totally use its potential as a therapeutic agent for harmful pathogens and CRC associated bacteria.

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