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Introducing two isolates of *Bdellovibrio* from Iran-Rafsanjan

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ABSTRACT

Bdellovibrio bacteriovorus is an obligate predator of other Gram-negative bacteria. It grows in the periplasm of the prey cell and finally lyses them, so it has high potentials to be used in biocontrol of plant pathogenic bacteria. The objective of the present study was to isolate and characterize *Bdellovibrio*s bacteria from soil and water samples of Rafsanjan, Kerman province, Iran. To do this, after sampling, two *Bdellovibrio*s isolates (Sar and Pj) were isolated by using *Escherichia coli* strain K12 as prey on the two layer agar. The isolates were identified by phenotypic and 16S rDNA sequencing. The sequencing and phylogenetic studies showed that the strains have more than 99% similarity to *B. bacteriovorus*. Then, the host range and specificity of the strain were evaluated on 9 different Gram negative bacterial species. The results showed that the strains have predatory effects only on *E. coli* as prey and do not have any effect on *Pseudomonas fluorescence*.

Key words: Predatory bacteria, *Bdellovibrio bacteriovorus*, prey range, biological control and 16S rDNA.

INTRODUCTION

Gram-negative bacteria *Bdellovibrio bacteriovorus* is a predator species that can use other gram-negative bacteria and has received considerable attention in recent years. This bacterium will be used as an antimicrobial agent in industry, agriculture or medicine (Dwidar *et al*. 2012). The size of this bacterium is very small (0.25-2.5 micrometers). This predator has been isolated from soil, water, sewage, compost, and gastrointestinal tract of humans and horses (Rendulic *et al*. 2004, Jurkevitch, 2006).

The species belongs to delta-Protobacteria and attacks other gram-negative bacteria parasites. After the establishment and metabolization of the prey, the prey disintegrates and eventually drops out (Scherff, 1973, Lambert *et al*. 2015). After binding to the host, the bacterium penetrates into the outer membrane and remains in the periplasmic space to the internal membrane of the prey and forms a bdeloplast. In the bdeloplast stage, the size of the bacteria reaches its maximum longitudinal growth and has a spiral shape, and ultimately creates new cells through fragmentation simultaneously, this synchronization also occurs even if the prey attacked by two *Bdellovibrios*. The signal or mechanisms that make this coincidence still are unknown (Wolf, 2010). These bacteria introduced more as a predator than a parasite, as it generally lasts for a total of 210 minutes from the onset of the attack to the complete lyses of the prey. They cease the vital activities of the prey cell about 15 minutes after entering the periplasm. Therefore, in general, it does not establish a long-term relationship with its prey (Ahmadzadeh, 2012). Due to the inherent ability of bdellovibrios to parasitize and leach the prey, they can

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be used as biological control agents against the gram-negative bacteria (Epton, 1989). *B. bacteriovorus* can attacked to *Escherichia coli* and species of *Pseudomonas*, *Burkholderia*, *Salmonella*, *Proteus*, *Serratia*, *and Helicobacter pylori* and some other bacteria in the human, animal or plant group. Unlike bacteriophages, *Bdellovibrios* can attack non-specific gram-negative bacteria belonging to different genus and far away. These bacteria can be used as a biological control agent and, on the other hand, can be an important barrier to the establishment of many beneficial bacteria from the group of gram-negative bacteria, hence these bacteria called amphibiotic (Dwidar *et al*. 2012). The first attempt to control of plant diseases by using *B. bacteriovorus* happened in 1972. In that research, Bd-17 isolate was isolated from soybean root rhizosphere and its effect on population and survival of *Pseudomonas glycinea* in the soybean rhizosphere was proved (Sherff, 1972). In a study, 30 strains of *Bdellovibrio* from rhizosphere and non-rhizosphere of beans and tomato fields were isolated by using of *Pseudomonas corrugate*, *Erwinia carotovora* subsp. *carotovora* and *Agrobacterium tumefaciens* as prey (Jurkevitch *et al*. 2000). Biocontrol effect of *Bdellovibrio*s on a number of pathogenic bacteria such as *Burkhulderia cepacia* has been proven (McNeely *et al*. 2017). In recent years, numerous investigations have been carried out to isolate and identify BALOs (Bdellovibrio and like organisms) that affect plants and livestock bacteria and have achieved very good results (Kadouri & O'Toole, 2005, Atterbury *et al*. 2011). In 2011 a group of researchers for the first time was used *B. bacteriovorus* as a live antibiotic in young chickens and observed that the *salmonella* population significantly decreased in the digestive tract of chickens, also the predator did not have any adverse effects on the growth and health of chickens (Atterbury *et al*. 2011). *Bdellovibrio* also reduced liver necrotic disease in shrimp (Kongrueng *et al*. 2017). *B. bacteriovorus* had a significant impact on human pathogens that are resistant to all types of drugs, such as *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and their biofilms (Sun *et al*. 2017). Although some Gram-negative species are reported to be *B. bacteriovorus* prey, it seems that in case of nutrient deficiency this predatory bacterium can also take advantage of some Gram-positive species (Pantanella *et al*. 2018). Considering the positive results, it seems that the use of these bacteria in the future will be expanded as an antibacterial agent not only in medicine, but also in resolving agricultural, industrial and environmental problems (Shatzkes *et al*. 2011). In this regard for the first time, in the present study, we tried to isolate, identify, and find prey range of *Bdellovibrio* bacteria from soil and water samples of Rafsanjan.

Materials and Methods

1- Collect samples

For soil sampling, a half kg of soil from a pistachio garden and near the shade of trees in Jafarabad was taken and surrounding soils were removed. For water sampling, 300 ml liquate of irrigation water from Aliabad area (irrigation water related to Sarcheshme Dam) in Rafsanjan-Kerman province was taken. Finally samples were transferred to the laboratory quickly.

2- Isolation of *Bdellovibrio*

In this study we used the culture dependent technique for isolation of

Bdellovibrio. For this purpose, the combination of Jorkevitch (2012) and Koval (2013) methods has been used with some changes.

2-1- Enrichment of *Bdellovibrio*s in samples

For soil samples, first 10 to 50 grams of soil sample was mixed with 1: 2 to 1:10 of sterilized water depending on the soil texture (for heavy clay soils, the minimum amount was used and for sandy soils maximum amount was used), and then the potential prey with the concentration of 10^{10} CFU/ml was added to the soil slurry. Then samples were stirred at 28 ° C for 24 to 48 hours with rotary shakers at 200 rpm. After 2 to 4 days, the optical density of the specimens was measured, and the presence of small cells with high motility was detected by contrast microscopy. If such cells were not found, incubation continued for two weeks to see the cells. After observing the cells, the remaining steps were performed according to the instructions for isolation.

2-2- Isolation from soil and water samples

After enriching the soil and ensuring the presence of the *Bdellovibrio* bacteria, the soil slurry was centrifuged at 5000 g for 5 minutes at 4 ° C. Then the supernatant was passed through Nucleopore filters in two stages. At first, a filter with a pore size of 0.6 or 0.8μm, and then a filter with a pore size of 0.4μm were used. In the next step, a 10-fold serial dilutions of the filtered sample was prepared in a HM buffer [HEPES buffer contains three ml of HEPES plus 2 ml $CaCl_2.2H_2O$ (1M) and also 0.17 $MgCl_2.7H_2O$ (0.1M)] with a final volume of 5 ml at room temperature. Then top-agar infusions were prepared in the following manner. First 250 to 300μl (5×10^9 to 10^{10} CFU/ml) suspension of potential prey (*E. coli* was used here) were added into 10-ml test tubes containing 4 ml molten HM top agar (HM buffer + 0.7% agar). Then the tubes were rapidly vortexed and 100μl of filtrate dilution added. Finally HM top agar vortexed and quickly poured onto an HM plate (HM buffer + 1.5% agar). These steps repeat for each dilution until all dilutions have been plated (Two to three replicate plates made per dilution). The top agar was then allowed to solidify at room temperature (about 60 minutes). Afterwards, plates were covered with Parafilm and the sealed plates were incubated upside-down for 3 to 8 days at 28°C. After the plaques were formed, each plaque was taken separately and placed inside a 500μl HM buffer. After a few minutes a drop of suspension was placed on a clean glass slide and it covered with a cover slip and the cells were observed by contrast microscopy.

2-2-1- Preparing the prey cells

First, prey bacteria were cultured in NB (Nutrient Broth) medium. Grown cells were collected at the end of exponential to early stationary phase and centrifuged them at 5000 g for 10 minutes at 4°C. Then they were washed with HM buffer and finally re-suspended in HM buffer to a final concentration of 5×10^9 to 10^{10} CFU/ml. For routine growth, prey stock can be used 10 to 14 days if kept at 4°C. In this study, nine gram-negative bacteria were used as prey, as shown in Table 2.

2-2-2- Storage

For maintenance, each individual plaque was cultured in DNB (Diluted Nutrient Broth) medium with prey. For

long term storage, a 24–48 h culture of cell suspension was centrifuged and concentrated at 10,000 g for 10 minutes, and the pellet was re-suspended by fresh DNB and finally was frozen in the presence of 25 % (w/v) glycerol at -80°C. For storage in a refrigerator, after the plaque were cultured in DNB medium with prey, cultures were transferred to sterile screw-capped tubes contain HM buffer and kept at 4°C for up to 1 month.

3- Primers and PCR conditions

At first, a fresh predator suspension was passed through a 0.45µm filter syringe, and then centrifuged at 10,000 g for 10 minutes at 4°C, and re-suspended in sterile water at 1/10 the original volume. For DNA extraction, the bacteria were immersed in liquid nitrogen then in hot water (80°C) for 3 minutes, and it was repeated twice. The samples were finally cooled on ice and added dimethyl sulfoxide (DMSO) at a final concentration of 10% (v / v) (Jurkevitch, 2012). Each reaction mixture contained 4µl of DNA, 1µl of each primer, 1.5µl of BSA, 5.6µl of master mix (prepared from the Pishgam company), and finally DDW (Double-distilled Water) was added to a final volume of 25µL.

Specific oligonucleotides for *Bdellovibrio*s: 1007R: 5’-TCTTCCAGTACATGTCAAG-3’ and 529F: 5’-GGTAAGACGAGGGATCCT-3' were used in PCR (Jurkevitch, 2012).

The program used for amplifying was: 2 minutes 95°C, 35 cycles (30 seconds 95 °C, 45 seconds at 52 °C for annealing and 2 minutes at 72 °C for extension) and 10 minutes at a temperature of 72 °C for final extension. Verification of the presence of a PCR product was performed by running 5µl of PCR product on a 1% agarose gel in TAE (Voytas, 2000). Finally the gel stained by ethidium bromide and stains visualized by using of shortwave UV light.

4- DNA sequencing

Products obtained from PCR performed with primers 1007R and 529F sent to Takapouzist Company for sequencing.

5- Phylogenetic analysis

Nucleotide sequences were compared by using the online Blast search tool (NCBI). And sequences were aligned separately by Mesqite 3.01 software (Maddision and Maddision, 2011). To find phylogenetic tree, the RAxML-HPC2 program on XSEDE was used, as implemented on the CIPRES Portal V.1.0. (Stamatakis, 2006) and phylogenetic tree were constructed based on maximum similarity. The consistency of the phylogenic tree was also verified by bootstrapping.

6- Determination of prey range

Bdellovibrio isolates from this study (Pj and Sar) were separately cultured in NB culture media and centrifuged after 24 hours (4400 g for 10 minutes at 4 °C). The pellet was then suspended with HEPES buffer. Then the fresh lysates of the *Bdellovibrio*s were passed through a 0.45 µm-pore-size filter in order to remove residual prey and cell debris, centrifuged at 10,000 *g* for 15 min at 4°C, and re-suspended in HEPES to a final concentration of 10^6 to 5×10^6 cells ml^{-1}. The supernatant was filtered through a 0.22µm-pore-size membrane filter and used as a control. Prey suspensions (150µl) and predator suspensions (60µl) were added to 96-well microtiter plates. The controls

included *Bdellovibrio*-free prey suspensions amended with buffer and prey suspensions amended with filtered lysates. The plates were incubated overnight at 30°C on a rotary shaker at 200 rpm. Turbidity (cell density) was determined at 570 nm (Jurkevitch, 2000). Three replicates of each predator-prey combination were prepared, and each experiment was carried out twice.

In this study, nine bacteria were used as prey that listed in table 1.

Results

1- Isolation

After three to eight days from the time of adding the soil and water extract to the HM plates containing *E. coli* K12, some plaques were observed on the plates (Fig. 1). The size of these plaques was different. Usually bigger ones were observed in plates related to soil samples with more organic matter, as well as soil samples near the root plants. Also depending on the soil organic matter and soil type, the number of plaques that were formed was different. Generally it was possible to identify distinct plaques in the serial dilutions of four and five. After observing these plaques by using the phase contrast microscopy, *Bdellovibrio*s presence in the specimens was confirmed due to the very active, swimming, small cells of these bacteria. Finally, one isolate from water and one isolate from the soil was isolated and kept in both - 80 freezer and refrigerator.

2- Molecular identification of species

The final product that was amplified by oligonucleotides (1007R, 529F) was about 480 bp and is a part of *16SrRNA* gene. These two oligonucleotides are specific for *Bdellovibrio*s so the obtained PCR product from these two strains is an evidence for being *Bdellovibrio* (fig. 2). After sequencing of PCR products, BLAST results showed that the two strains have more than 99% similarity to *B. bacteriovorus*. Our phylogenetic analysis confirmed that these two isolates are belonging to *Bdellovibrio* genus. In phylogenetic tree, isolate Sar kx451004.1 and straines angelus, DM8C and HD100 were found to be identical. Although isolate Pj belonged to the *B. bacteriovorus* group, it was more distantly related, exhibiting 54% identity with strain BDH (Fig. 3).

3- Prey range

The effects of these two isolates (Sar and Pj) on predation of nine gram-negative bacteria were carried out. In some cases, increases in optical density of control wells was observed that were not inoculated with bdellovibrios or in prey suspensions that were not attacked by the predator; these increases may have been due to utilization of capsule or storage materials. Controls, which were prepared by using cell-free lysate of each of the *Bdellovibrio* strains tested and each type of prey, were always negative in increasing of optical density. Strains Sar and Pj exhibited the same prey range. Both isolates used *E. coli* as prey, which was expected because both *Bdellovibrio* isolates were isolated by this bacterium. In general, different isolates show different predatory behavior, but in this case the two isolates Sar and Pj had the same predatory behavior, and both attacked only *E. coli* and did not attack other preys (Table 4). Also both isolates did not attack *Pseudomonas fluorescent* that is an important biological agent.

Discussion

The higher soil organic matter and the higher amount of oxygen in the soil, the

greater the number of *Bdellovibrio*s in the soil, which can be due to a higher population of prey bacteria in these soils than in other soils. Also, due to root extracts and more nutrients around the root, rhizosphere is a richer part of soil with the various types of prey bacteria that are attractive for *Bdellovibrio*s. In this regard, the results of the present study were consistent with the results of Jurkevitch, which confirmed that there were different populations of *Bdellovibrio* at bulk and rizosphere soil (Jurkevitch *et al*. 2000). These bacteria generally lives in minimum population and, as the prey population increases, it responds quickly and increases its population (Ahmadzadeh, 2012). In this research, the population of *Bdellovibrio* increased after enrichment of specimens. *Bdellovibrio* is also seen in places where there are biofilms of bacteria or any accumulation of bacteria from different genus. Therefore, the formation of biofilms is not an obstacle to being safe from the attack of this predator (Ahmadzadeh, 2012). Even the climatic conditions prevailing in different regions affect the populations of this predatory bacterium. According to a research, geologically, there are sometimes different strains of *Bdellovibrio* in different areas. Factors that affect its dispersal include salinity, food source, temperature, and oxygen (Jurkevitch *et al*. 2000).

Predation is one of the causes of bacterial mortality due to the impact on the structure and composition of the community, which regulates the size of the population of bacteria (Kandel *et al*. 2014), so that predatory pressure leads to the selection of strains that are not attacked by predators or less favored predators. This is also true about BALOs and can be an inhibitor to the formation of suppressive soils, because one of the factors contributing to the creation of suppressive soils is the presence of fluorescent pseudomonads that can be threatened by this predator. Therefore, further research is needed on these bacteria. In a study, adding plant pathogens *Pseudomonas corrugate*, *Erwinia carotovora* subsp. *carotovora* and *Agrobacterium tumefaciens*, and others in the soil increased the population of predator bacteria, which was higher in the rhizosphere (Jurkevitch *et al*. 2000). This predator was able to reduce the biofilm biomass in two bacteria, *Pseudomonas fluorescens* and *E. coli* (Kadouri and O'Toole, 2005). In another study, the effect of six strains of *Bdellovibrio* was investigated on several beneficial bacteria that are involved in antibioses and nitrogen fixation, such as *P. fluorescens*, *P. putida*, *Serratia marcescens* and the pathogenic bacterium *Burkholderia glumae* (Song, 2004). In this research, the phenomenon of predation was reported only for *B. glumae* bacteria and did not show a negative effect on beneficial bacteria.

In present study, it seems that two introduced isolates are not able to hunt beneficial bacteria such as *P. fluorescence* and their preferred host is *E. coli*. On the other hand, because both bacteria are isolated from a region with the same climate, this behavior may be equally affected by the same conditions. In other words, due to the use of *E. coli* as a prey in the steps of enrichment and isolation of these isolates, it is therefore natural that these bacteria tend to be more sensitive to *E. coli*. Due to the limited prey range of these two bacterial predators, it seems that these two isolates can be used to clear *E. coli* bacteria from sewage water. But that does not mean that *E. coli* is the only prey for these two strains, but more research is needed with more number of

prey bacteria. Koval introduced a new species of *Bdellovibrio*, named *B. exovorus*, previously named as *B. bacteriovorus*, due to a similar resemblance of 93%, but was named a new species due to differences in predation behavior (Koval *et al*. 2013). Here, the two isolates obtained are similar to *B. bacteriovorus*, but in the phylogenetic study Pj isolate were placed in a separate clade with a strain from China, *B. bacteriovorus* strain BDH (HM219229.1) and it seems that this isolate also appear in a new species.

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Table1- The name and location of the bacteria used in this research as a prey

Prey strains	Source
Escherichia coli K12	Laboratory of medical diagnostics of Mehr
Pseudomonas fluoresencs UTPF5	Biological control collection in University of Tehran
Pseudomonas syringae pv. *syringae*	Bacterial collection of Vali-e-Asr university of Rafsanjan
Ralstonia solanacearum two species: kobI and PsI	Seed and plant improvement institute of Karaj
Pectobacterium atrosepticum	Seed and plant improvement institute of Karaj
Pectobacterium carotovorum subsp. *carotovorum*	Seed and plant improvement institute of Karaj
Xanthomonas axenopodis pv. *vesicotor*	Seed and plant improvement institute of Karaj
Erwinia amylovora	Seed and plant improvement institute of Karaj

Table2- Prey range of two isolates of *Bdellovibrio* (Sar and Pj) by optical density measurement of prey and predator combination after one night; - reducing optical density below 0.04, + reducing optical density between 0.04-0.07, ++ reducing optical density above 0.07 to 0.1, +++ reducing optical density more than 0.1

Prey bacteria	Prey range of: Sar	Pj
Escherichia coli K12	++	++
Pseudomonas fluoresencs UTPF5	_	_
Pseudomonas syringae pv. *syringae*	_	_
Ralstonia solanacearum kobI	_	_
Ralstonia solanacearum PsI	_	_
Pectobacterium atrosepticum	_	_
Pectobacterium carotovorum subsp. *carotovorum*	_	_
Xanthomonas axenopodis pv. *vesicotor*	_	_
Erwinia amylovora	_	_

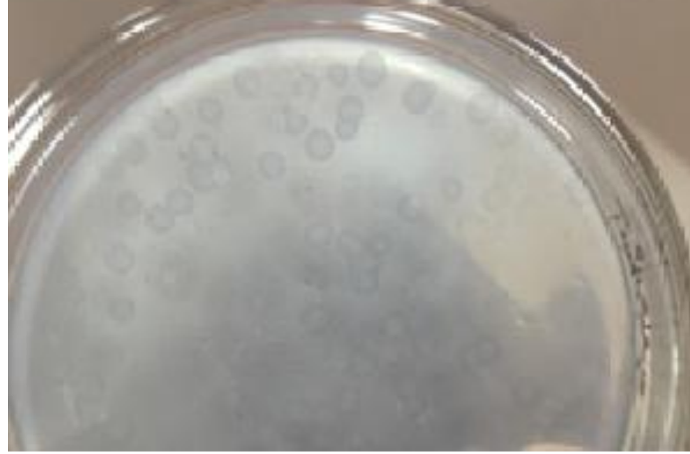

Figure1- *Bdellovibrio*’s plaques on double layer plate

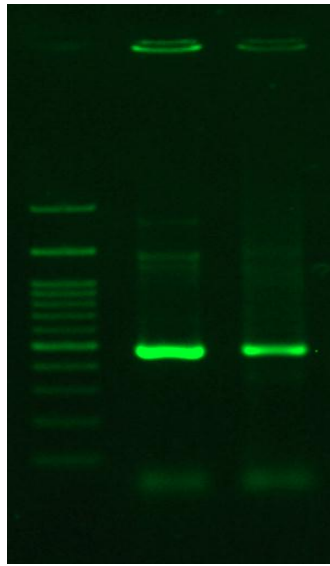

Figure2- Amplification of a fragment of the 16S rDNA related to *Bdellovibrio* isolates

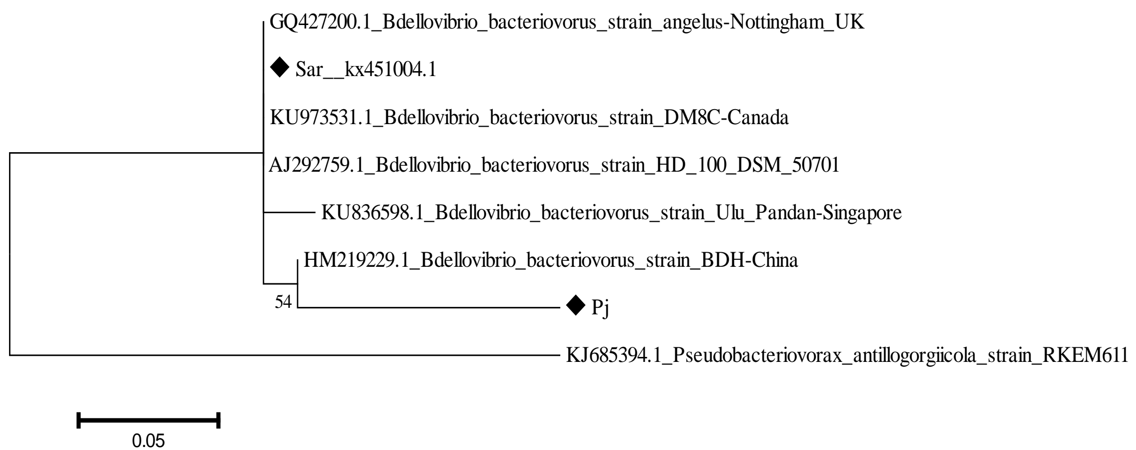

Figure 3. 16S rDNA-based phylogenetic tree for *Bdellovibrio* isolates, Sar and Pj (♦) and previously published sequences, using RAxML-HPC2 software. The scale of 0.05 shows the expected change in each location. The bootstrap values at the nodes are percentages based on 100 iterations; only values greater than 50% are shown.

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