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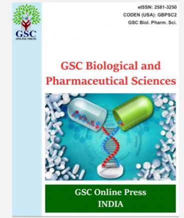

(Research Article)

Bdellovibrio: A possible application for the control of *Aeromonas* species

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Abstract

In the last decade there has been a growing increase in antimicrobial resistance, this has been due to bad practices and excessive use in the clinical and agricultural area. *Aeromonas* species cause infections in fish and shellfish, causing furunculosis, causing large economic losses in fish farms. In humans *Aeromonas* causes gastroenteritis and infections in wounds and soft tissues. According to the WHO *Aeromonas* is classified as a priority pathogen for the development of new strategies for its control. *Bdellovibrio* is a predatory bacterium that has a wide range to attack Gram-negative bacteria even greater than some phages, which represents a great potential for the control of pathogenic bacteria. In this study, ten bacteria of the genus *Bdellovibrio* were isolated from environmental samples (soil, water and feces of mammalian animals), all were identified within the genus *Bdellovibrio*, through partial sequences of the 16S rRNA gene. Their predation efficiency against *Aeromonas* strains of clinical and environmental origin was evaluated. All *Bdellovibrio* spp. isolates showed efficiency in preying on *Aeromonas* species. In the predation efficiency results, *Bdellovibrio* isolates showed a reduction in the population of *Aeromonas* strains after 5 to 16.5 h of their inoculation.

Keywords: *Aeromonas*; *Bdellovibrio*; Predatory bacteria; Antimicrobials; Infections; Predation

1. Introduction

Aeromonas bacteria are ubiquitous, however, they are mainly found in aquatic environments. This type of bacteria causes various infections in humans. They have been found associated with clinical pictures of gastroenteritis and mild infections in various organs and tissues, or severe infections such as septicemia (Parker and Shaw, 2011; Ku and Yu, 2015; Awan *et al*., 2018a; Awan *et al*., 2018b). Over time, it has been thought that these bacteria were opportunistic, however, there is evidence of severe septicemia in immunocompromised patients caused by virulent strains of this genus (Ku and Yu, 2015). An important factor that favors this type of bacteria is its adaptation, diverse metabolic capacity, among others; allowing *Aeromonas* to persist in almost any environment and to be transmitted by various routes and vectors (Figueras *et al*., 2017; Hoel *et al*., 2017; Ruppé *et al*., 2018; Li *et al*., 2015). The *Aeromonas* species of clinical importance and most frequently associated with human diseases are *A. hydrophila* (14.5%), *A. caviae* (37.6%), *A. veronii* bv. sobria (27.2%) and *A. dhakensis* (16.5%), which represent around 96% of cases of gastroenteritis (Janda and Abbott, 2010; Teunis and Figueras, 2016). There is an increase in antibiotic resistance, which is why the World Health Organization (WHO) has classified bacteria such as *Aeromonas* on a priority list with the purpose of developing and researching new antibiotics or alternatives to control it, according to this classification *Aeromonas* are in priority 1 or critical, (WHO, 2017; WHO, 2020). Therefore, it is important to study new alternatives for the control of the *Aeromonas* genus. An alternative for its control is the potential use of predatory bacteria, which has attracted attention due to its ability to prey on a wide range of prey, making them a viable alternative (Atterbury *et al*., 2011; Cao *et al*., 2012; Loozen *et al*., 2015; Raghunathan *et al*., 2019; Li *et al*., 2018). It is important to continue isolating and characterizing predatory bacteria with the potential to control these highly virulent microorganisms. In this study, the phenotypic and molecular characterization of predatory bacteria (*Bdellovibrio* and similar organisms, BALOs) allowed

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us to understand the capabilities of the isolates with the greatest potential to attack *Aeromonas* species of clinical and environmental origin.

2. Material and methods

Sampling and isolation of *Bdellovibrio*: Bacteria of the *Aeromonas* genus of clinical and environmental importance from the collection of pathogenic bacteria of the Genomic Biotechnology Laboratory of the Genomic Biotechnology Center of the National Polytechnic Institute, located at Blvd. del Maestro S/N, Esq. Elías Piña, Col. Narciso Mendoza, CP 88710, Cd. Reynosa, Tamaulipas, Mexico, were used. *Salmonella enterica* was used as prey to isolate *Bdellovibrio*. Each prey was inoculated in Petri dishes containing Luria Bertani agar and incubated at 37 °C for 18-24 h, then a colony was inoculated in 20 ml of LB broth in 50 ml conical Falcon tubes, incubated at 37 °C for 18-24 h at 200 rpm (culture stand). Several isolates of *Bdellovibrio* were obtained from soil, water and feces samples from mammalian animals from different locations in Mexico, when confronted in co-culture with the prey, *Salmonella enterica*. 20 g of soil and feces samples were weighed, and 20 ml for water, each separately in 250 ml Elrenmeyer flasks, and placed in 50 ml of 25 mM HEPES buffer pH 7.4 in the case of solid samples, and for water samples 30 ml of 25 mM HEPES buffer pH 7.4 were added, they were left to incubate at 29 °C for 1 h at 200 rpm, subsequently, the samples with organic particles were filtered with coarse mesh filters, then starting from the culture feet (*S. enterica*) 100 µl were taken and inoculated in 20 ml of LB broth and incubated at 37 °C for 18-24 h at 200 rpm. After this time, they were centrifuged at 5 °C for 20 min at 3,500 rpm, the supernatant was discarded and 25 ml of 25 mM HEPES pH 7.4 were added to each pellet. Two pellets were placed for each sample. The prey pellets, *S. enterica*, were placed for different samples. Finally, each 250 ml Erlenmeyer flask was shaken vigorously to homogenize the prey pellets and the sample in the flask, leaving a final volume of approximately 100 ml. The flasks were incubated at 29 °C for 7-10 days with constant shaking at 200 rpm until cell lysis of the prey was observed (visualization of cellular debris at the bottom of the flask) following the protocol described by Jurkevitch (Jurkevitch, 2012).

2.1. PCR identification using the 16S rRNA gene specific for *Bdellovibrio* and BALOs

For identification of *Bdellovibrio* in the samples, 1 ml of coculture was placed in a sterile 1.5 ml microtube. It was incubated in a thermomixer (Eppendorf, Germany) at 95 °C for 10 min (heat lysis), then placed on ice for 5 min and centrifuged at 5 °C for 5 min at 14,000 rpm; the supernatant was transferred to a new sterile microtube and the gDNA was used for PCR reactions. Next, a PCR mixture was prepared for amplification with *Bdellovibrio*-specific oligonucleotides of the 16S rRNA gene, which contains: 13.25 µl of milli-Q water, 5 µl of 5X MyTaq® buffer (final 1X), 0.25 µl of 50 mM MgCl2 (final 1.5 mM), 0.25 µl of 10 mM dNTPs (final 0.2 mM), 0.5 µl of 5 µM Forward oligonucleotide (final 0.1 µM), 0.5 µl of 5 µM Reverse oligonucleotide (final 0.1 µM), 0.25 µl of 5 U/µl MyTaq® Taq Polymerase (final 0.05 U/µl) and 5 µl of gDNA. The conditions for the thermocycler were as follows: initial 94 °C for 4 min; 35 cycles of 94 °C for 1 min, Tm °C (Tm of the Forward and Reverse oligonucleotide pair of the 16S rRNA gene specific for *Bdellovibrio*) (Jurkevitch, 2012) for 1 min and 72 °C for 1 min; 72 °C for 10 min and final 8 °C for 5 min. Once the PCR was performed, the products were analyzed by electrophoresis in a 1% agarose gel, run for 60 min at 80 V, using 0.5X TAE as buffer. The loading buffer mixture was placed in each well of the gel with 5 µl of the PCR product. The agarose gel was visualized in the Kodak® photodocumenter with a Gel Logic 112 camera using the Kodak® dS 1D v. 1 bioinformatics program. 3.0.2.

2.2. Sequencing reaction using the 16S rRNA gene specific for *Bdellovibrio*

The PCR product was purified according to the manufacturer's instructions ExoSAP-IT (#78200, USB, USA). The sequencing reaction was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit. The sequencing reactions were performed on the ABI® 3130 Genetic Analyzer from Applied Biosystems. The files in .ab1 format were cleaned using MEGA11 v11.0.13. The search for homologous sequences was performed in BLAST of the NCBI database to determine the identity of each predatory bacterium that was used for predation efficiency. The phylogenetic tree was generated in MEGA11 v11.0.13 using the Maximum Likelihood method.

2.3. Determination of predation efficiency of *Bdellovibrio* species

The determination of predation efficiency was measured by spectrophotometry by reading the optical density of the ten predators isolated in co-culture with each *Aeromonas* of clinical and environmental origin. The co-cultures were maintained at 29 oC, 1 ml of each co-culture was deposited in plastic cells (1.5 ml semi-micro PS cell, #KART1938, KARTELL, USA), the OD reading was measured in the visible light spectrum at 600 nm (Spectrometer, Cintra 10e, GBC). The first reading was taken at 0 h, and the following readings at 5, 8.5, 12.5, 16.5, 20.5, 24.5, 28.5, 32.5, 36.5, 41, 46, 48.5, 52.5, 57, 60.5, 64.5 and 68.5 h

3. Results

Forty-one *Bdellovibrio* isolates were obtained from water, soil and feces samples from mammals in the states of Tamaulipas, Durango, Puebla and Tlaxcala (Mexico), and the formation of cellular debris was observed (Figure. 1).

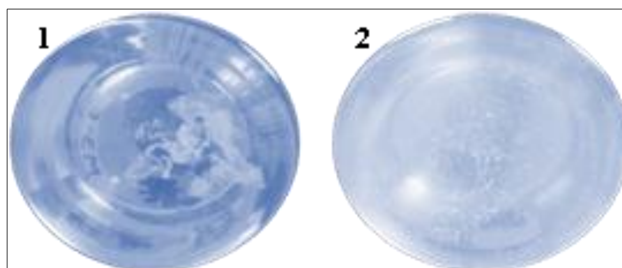

Figure 1 Coculture flasks with formation of cellular debris (cell lysis). 1) M3 (soil sample). 2) M5 (soil sample). Isolates with *Salmonella enterica* as prey

Some isolates were identified by PCR using *Bdellovibrio*-specific oligonucleotides (Figure 2).

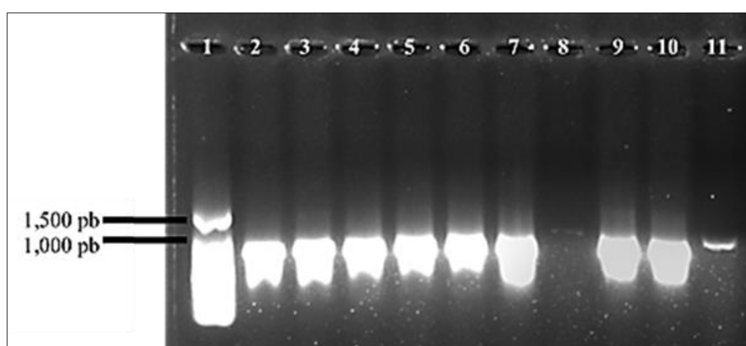

Figure 2 Amplification of 16S rRNA gene fragments specific for *Bdellovibrio* BdsF:BdsR (800 bp). Extraction by Wizard® Genomic DNA Purification Kit method. (1) 100 bp DNA Ladder (#G210A, Promega, USA), (2) B1, (3) B3, (4) B5, (5) B7, (6) B19, (7) B34, (8) B37, (9) B40, (10) B41, and (11) B4J

From the sequencing reactions run on the Applied Biosystems ABI® 3130 Genetic Analyzer with BigDye® XTerminatorTM Purification Kit (#4376486, Applied Biosystems, USA), files were obtained in .ab1 format and analyzed using the MEGA11 v11.0.13 program. A search for homologous sequences was performed with the files in FASTA format using BLAST in the NCBI database to determine the identity of each predatory bacterium. The evolutionary relationships between the different *Bdellovibrio* isolates are observed in the phylogenetic tree constructed with the sequences that showed the highest percentage of identity in the NCBI database (Figure 3).

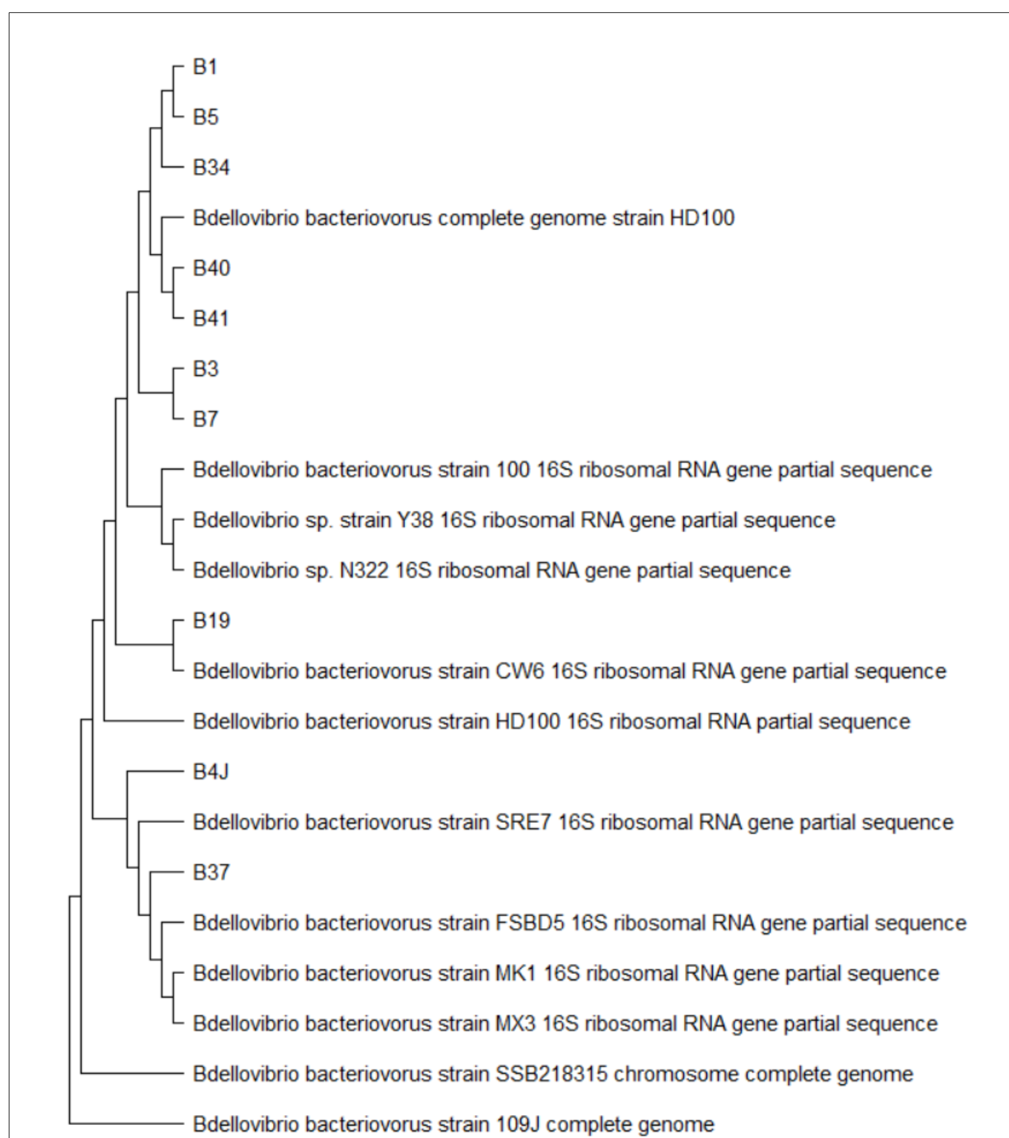

Figure 3 Phylogenetic tree of *Bdellovibrio* isolates. Evolutionary relationships between the different *Bdellovibrio* isolates with the sequences that showed the highest percentage of identity in the NCBI database

In predation efficiency, initial concentrations of prey and BALOs were 0.301 and 0.142 A, respectively. B1 initiated predation at 5 h on 16.66% of prey (A8, A12); B3 on 41.66% (A5, A9, A10, A11, A12); B5 (A2, A5, A6, A9, A10, A12), B37 (A2, A4, A5, A8, A11, A12), B41 (A1, A4, A6, A7, A9, A10), and B4J (A2, A4, A5, A7, A10, A12) on 50%; B34 on 58.33% (A2, A4, A5, A6, A8, A10, A11); B7 (A2, A3, A5, A6, A7, A9, A10, A11, A12) and B40 (A2, A3, A5, A6, A7, A8, A9, A11, A12) in 75%; and B19 in 83.33% (A1, A2, A3, A5, A6, A7, A8, A9, A10 and A11). BALO B19 showed the highest efficacy by initiating predation time at 5 h in 83.33% of the prey (A1, A2, A3, A5, A6, A7, A8, A9, A10 and A11), whereas, B1 had the lowest predation efficacy by initiating predation at 5 h in only 16.66% of *Aeromonas* (A8, A12) and initiating predation at 12.5 h in 41.66% of the prey (A1, A2, A5, A7, A9). These results reveal that predatory bacteria have very different predation characteristics depending on the specific prey strains (*Aeromonas* species), although the prey belong to the same genus.

4. Discussion

Predation was found in cocultures made from the three types of samples available for the study, which correspond to soil, water and feces of mammalian animals, confirming that predatory bacteria are ubiquitous and can be found in various ecological niches as described by other authors (Oyedara *et al.*, 2016; El-Shanshoury *et al.*, 2016; Taylor *et al.*, 1974).

Shatzkes *et al*. In 2017a, they evaluated the effect of predatory bacteria on the gastrointestinal tract in mice, infecting the mice with *Klebsiella pneumoniae*, no signs of damage were shown in the mice by intranasal inoculation of the predatory bacteria and after 48 hours, the predatory bacteria were viable in the mouse feces (Shatzkes *et al*., 2017a; Shatzkes *et al*., 2017b). Several studies have stated that *Bdellovibrio* species have a wide prey range for Gram-negative bacteria, and that they have the ability to prey on them in an average of 18 to 24 hours (Chu and Zhu, 2010; Dashiff *et al*., 2011; Atterbury *et al*., 2011; Dwidar *et al*., 2012; Pérez *et al*., 2016; Jurkevitch and Jacquet, 2017).

In this study, predatory bacteria were isolated that showed the ability to prey on bacteria of clinical interest used for their isolation and purification, *Salmonella enterica* and *Klebsiella pneumoniae*. In addition, when confronted with *Aeromonas* species, predation was observed after 5 h. The *Peredibacter* genus was found in soil sample M7 from the Tepetitla River, Tlaxcala, Mexico, but it was not purified, so it can be determined that, like several predatory bacteria, *Peredibacter* is a ubiquitous bacterium. However, only the phenotypic characteristics of the isolate have been determined with the prey used for its isolation and purification: *S. enterica*. *Peredibacter starrii*, which has only been isolated from soil samples at a temperature of 35 °C, is suggested to have a similar lifestyle to *Bdellovibrio* and a wide prey range (Jurkevitch and Jacquet, 2017).

The genus *Micavibrio* was found in soil sample M34 from a garden in Cd. Victoria, Tamaulipas, Mexico, but it could not be purified. This isolate showed predation with the prey used for its isolation and purification: *S. enterica*. The ability of *Micavibrio aeruginosavorus* to prey on pathogens of clinical interest such as *P. aeruginosa* and *K. pneumoniae* has been shown in different studies. Likewise, an increase in the prey range of this predator has been seen, since Dashiff *et al*. In 2011, they showed that it was able to hunt and reduce 57 of the 89 bacteria examined (Dashiff *et al*., 2011; Kadouri *et al*., 2007).

5. Conclusion

Of a total of 41 samples, 36 were soil, 3 water and 2 mammalian animal feces, from which 9 BALOs were obtained: 6 soil (4 from the Tepetitla River, Tlaxcala, Mexico; 1 from the textile zone of Tlaxcala, Tlaxcala, Mexico and 1 garden soil in Cd. Victoria, Tamaulipas, Mexico), 1 water (beach in Cd. Madero, Tamaulipas, Mexico) and 2 mammalian animal feces (Gómez Palacio, Durango, Mexico), managing to isolate the genera *Bdellovibrio*, *Peredibacter* and *Micavibrio* identified by amplification with oligonucleotides of the 16S rRNA gene specific for BALOs, being BbsF216: BbsR707, BdsF:BbsR, 21BdsF:1260BdsR, PerF:PerR and McvF:McvR, respectively. The BALOs present in samples M3, M5, M19, M34, M37 (corresponding to BALOs, B3, B5, B19, B34 and B37, respectively) were purified with *Salmonella enterica* prey and, for samples M40 and M41 (corresponding to BALOs, B40 and B41, respectively) with *Klebsiella pneumoniae* prey, and correspond to the *Bdellovibrio* genus. Sample M7 amplified for the specific oligonucleotides of the *Peredibacter* and *Bdellovibrio* genera (corresponding to BALO B7) with *S. enterica* prey. Sample M34 amplified for the specific oligonucleotides of the *Micavibrio* and *Bdellovibrio* genera (corresponding to BALO B34) with *S. enterica* prey. The *Peredibacter* and *Micavibrio* BALOs could not be purified by double layer purification on a Petri dish. In M40 and M41 (corresponding to BALOs, B40 and B41, respectively) the presence of predatory bacteria was confirmed in fecal samples of mammalian animals, thus, it can be concluded that BALOs do not represent a danger for animals, and their resistance to stomach acids allows them to persist in the intestine. The isolated BALOs (B1, B3, B5, B7, B19, B34, B37, B40, B41, B4J) demonstrated to have a wide prey range on *Aeromonas* species (A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12), since the presence of predation was observed from 5 h to 16.5 h of co-culture. The ten predatory bacteria isolates represent a viable alternative to attack *Aeromonas* species of clinical and environmental origin.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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