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MOLECULAR CHARACTERIZATION OF *Bdellovibrio* sp. AND PREY RANGE OF THE ESKAPE GROUP

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Abstract: In 2017, the World Health Organization (WHO) generated a statement of the highest priority pathogens based on their resistance to antibiotics (W.H.O., 2017), due to the increase in resistance to antimicrobials, included in the list of urgent global health problems (WHO, 2020). Due to the above and the great increase in infections caused by both Gram-negative and Gram-positive bacteria and given that these infections are difficult to control, due to the resistance that they have created to antimicrobials, the search for others and new alternatives to control them. *Bdellovibrio* is a Gram-negative bacterium that predators or consumes other Gram-negative bacteria and some Gram-positive bacteria, so it is believed that this is a viable alternative (Iebba *et al.*, 2014; Im *et al.*, 2014). The objective of this work was to molecularly characterize isolates of predatory bacteria with the capacity to attack bacteria of the ESKAPE group. Pathogenic bacteria of clinical interest were used as prey, and samples of water, soil, and animal feces, to isolate predatory bacteria, were confronted in order to observe lytic activity. 16S rRNA gene sequences were used to amplify by PCR for the *Bdellovibrio genus,* sequences from the Bdellovibrionaceae family, and prey range.
Keywords: Bacteria, prey, pathogens, *Bdellovibrio,* PCR.

INTRODUCTION

In the environment there is a great diversity of Gram-negative and Gram-positive bacteria, some are considered important for their investigation, because they are pathogenic for humans. In addition, the increase in resistance to antibiotics has caused infections that are difficult to control, which is why new alternatives to antimicrobials are being sought. In 2017, the World Health Organization generated a statement of the highest priority pathogens based on their resistance to antibiotics, said priority was called by categories as high, critical and medium (World Health Organization, 2017), and given The increase in resistance to antimicrobials, in 2020 the World Health Organization classified it as a public health problem, within the list of urgent health problems of a global dimension (WHO, 2020). Therefore, it is important to carry out research on new agents that support the reduction and control of infections caused by pathogens of interest to the World Health Organization, such as those of the ESKAPE group (Enterococcus faecium, Staphylococcus **aureus**, Klebsiella *pneumoniae***,** *Acinetobacter* baumannii, Pseudomonas *aeruginosa* and *Enterobacter* spp.). *Bdellovibrio* spp. is a ubiquitous, Gram-negative, uniflagellate, highly motile Deltaproteobacteria, preys on or consumes other Gram-negative bacteria, as well as reduces biofilms formed by Gram-positive bacteria, so it is believed that this is a viable alternative (Iebba *et al.*, 2014; Im *et al.*, 2014). Among the main mechanisms of predation of predatory bacteria are: motility, its polar flagellum, type IV pili, and the use of proteolytic enzymes with which they attack their prey causing cell lysis, death, taking advantage of the nutrients of the prey bacteria. Therefore, it is important to carry out the phenotypic and molecular characterization of new isolates of *Bdellovibrio* spp. with the capacity to attack bacteria of the ESKAPE group from organic and environmental samples from different states of the Mexican Republic through PCR amplification of the 16S rRNA gene using specific primers for the species, as well as determining the range of prey, which provides information to determine its possible applications.

METHODOLOGY

Soil, water, and animal feces samples were obtained in falcon-type tubes and transported to the Genomic Biotechnology Laboratory of the National Polytechnic Institute, Reynosa, Tamaulipas, Mexico. *Klebsiella pneumoniae* (dams) were inoculated individually in 20 ml of Luria Bertani (LB) broth, incubated at 37 °C/18 hours at 180 rpm, centrifuged at 3500 rpm/20 min, 4 °C, discarding the supernatant and obtaining pellets. of cells. On the other hand, 10 grams of soil or feces were incubated in 100 ml of milli-Q water at 180 rpm at 30 °C/1 hour, then centrifuged at 3500 rpm/20 min, 4 °C, keeping the liquid part. and discarding the solids. The dams were immediately resuspended with the liquid samples of soil and feces (co-culture), and they were incubated from 24 hours to 7 days at 180 rpm at 30 °C, until cell lysis was observed. For liquid samples, organic matter was removed with filter paper and mixed directly with the prey. The co-culture was repeated at least twice in order to obtain a larger population of the predatory bacteria. The double layer method was performed, in order to separate the predatory bacteria in case there was a consortium in the liquid co-culture. For each co-culture, 6 double layers were made with predator dilutions of -1 to -6 respectively, for a total of 360 double layers. Those samples that presented "early" lysis were chosen, that is, those that formed lysis halos between 24 and 48 h; to these, the double layer technique was redone up to 3 times to ensure that the predatory bacteria that would be used come from the same colony and are completely purified, and later, once the third double layer was finished, 3 halos were selected per plate considering different shape and size of the halo, in order to ensure that we take different predators. The DNA extraction was carried out by heat lysis and from this the presumptive PCR to determine the presence of possible *Bdellovibrio* spp., or BALOs, upon confirming positive by means of PCR, these were selected to obtain the DNA using the kit Wizard® Genomic DNA Purification Kit, Ref. A1120, Promega. PCR identification of Bdellovibrio *spp.* It was carried out according to (Van Essche *et al.*, 2009). The resulting products were analyzed by 2% agarose gel electrophoresis in 1X TAE buffer solution, for 1 hour at 80 volts. Finally, the gel was visualized in a Kodak® photo documenter with a Gel Logic 112 camera using the Kodak® ds 1D bioinformatics program. In the sequencing of the specific gene for *Bdellovibrio* spp. ExoSAP-IT™ was used to purify the PCR products, the sequencing reaction was performed using the commercial kit BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, #4337455). The resulting products were subjected to purification with the commercial BigDye® X-Terminator™ kit from Applied Biosystems. To confirm the amplified products from the isolates of predatory bacteria, the sequencing reaction was carried out using the capillary electrophoresis sequencing technique, in the Services Laboratory of the Genomic Biotechnology Center of the National Polytechnic Institute, using the ABI® 3130 equipment. Genetic Analyzer from Applied Biosystems. Sequencing analysis was performed using computer files in.ab1 format was used to create a consensus sequence from the sequences from isolates from predatory bacteria with the direct primer. The sequences were cleaned using FinchTV 1.4. 0 and the search for homologous sequences was carried out using NCBI BLAST. Finally, they were aligned using reference sequences from the Deltaproteobacteria class. properly the genera *Bdellovibrio* sp., *Peredibacter* sp. *and Bacteriovorax* sp., from the Bdellovibrionaceae and Bacteriovoracaceae families, with the Gblocks 0.91b programs,

the MEGA X program was used, the *find best DNA model* tool was used, it was determined to use the construction of a phylogenetic tree using the Neighbor-Joining and UPGMA method for the 16S rRNA sequence, with Kimura's two-parameter model at 1000 replicates using a Gamma distribution. For prey range determination. *Klebsiella pneumoniae* was inoculated for 18 hours at 37 °C/180 rpm, the pellets were washed 3 times with sterile milli-Q water, homogenizing and centrifuging the sample, the pellet was resuspended with 3.5 ml of 25 mM HEPES buffer, pH 7.4, mixing until dissolution. On the other hand, the isolate of *Bdellovibrio* spp. it was grown 4 days before in co-culture with *Klebsiella pneumoniae* in 4 ml of HEPES buffer, washing the co-culture, it was centrifuged to use the supernatant, filtering 3 times with 0.45 µm, the predator was concentrated by centrifuging at 15,750 rpm for 40 min at 4 °C and the pellet was resuspended with 8 ml of HEPES. 25 mM HEPES buffer, pH 7.4. 500 µl of predator per prey were added to obtain a nutrient clean co-culture and 7:1 ratio. Determining the decrease in the optical density of the co-cultures, confronting 3 BALO isolates, a reference strain and the negative control, which were confronted against 12 prey bacteria, of which 4 were Gram-positive.

RESULTS

Twenty-five environmental samples (soil, water and animal feces) were obtained, placed in co-culture with the human pathogen *Klebsiella pneumoniae as prey, obtaining a total of 75 Bdellovibrio* spp. isolates. presumably by cell lysis Figure 1.

Figure 1. Co-cultures representative samples of environmental samples against human pathogen *Klebsiella pneumoniae* as prey, cell lysis was observed.

From the 75 isolates of *Bdellovibrio* spp. presumably due to cell lysis, 30 co-cultures were selected with *Klebsiella pneumoniae,* a human pathogen as prey, in 9 different samples lysis halos were observed between 24 and 48 hours, in 17 samples it was observed that there were lysis halos after 8 days and in 4 samples there was no presence of lysis even after 15 days Figure 2.

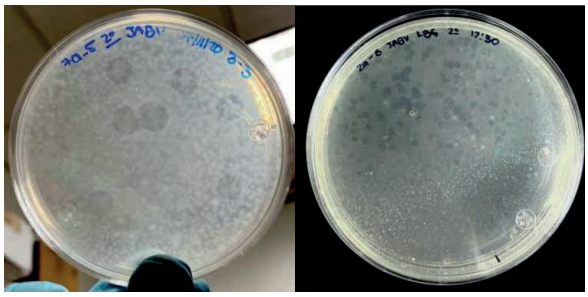

Figure 2. **Double layer.** representative samples, Left, double layer of water sample from captive hippo pond from Taxco de Alarcón, Guerrero, México, with *Klebsiella Pneumoniae* strain C16KP0077, used as prey; right, double layer of fecal sample from captive hippopotamus from Chilpancingo de Bravo, Guerrero, Mexico; using *Klebsiella oxytoca* strain as prey NCTC11355.

From the 9 samples where lysis halos were observed between 24 and 48 h, the genomic DNA was obtained, Table 1 and Figure 3.

sample no.	dna	Sample Origin
M1	Yeah	hippo feces
m2	Yeah	hippo feces
M3	Yeah	hippo feces
M4	Yeah	hippo feces
M5	Yeah	hippo feces
M6	Yeah	hippo feces
M7	Yeah	PSV floor
M8	Yeah	PSV floor
M9	Yeah	PSV floor

Table 1. Extraction of DNA from predatory bacteria.

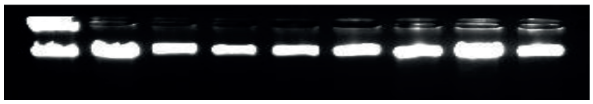

Figure 3. **Extraction of gDNA from predatory bacteria.** 1% agarose gel run at 80 V for 80 min showing DNA extraction by the Wizard® Genomic DNA Purification Kit method of samples 1 to 9.

PCR amplification with primer BdsF and BdsR, specific for *Bdellovibrio,* shows the 900 bp fragments for predatory bacteria, samples from M1 to M9 with consecutive number are observed in Figure 4.

It was possible to carry out the partial sequencing of 9 *Bdellovibrio* sp. isolates, including M7, a predatory bacterium that had greater efficiency in the prey range, once the sequences were obtained and analyzed in the NCBI Blast, Table 2 shows the identification part of the isolates.

In the prey range of the total of the 9 predatory bacteria, the one that showed the highest efficiency was M7. The measurements of reduction of the optical density in the predation efficiency of one of the predatory bacteria against a collection of pathogenic bacteria as prey were obtained, among them some of the ESKAPE group, both Gram - negative as Gram-positive Table 3.

DISCUSSION

It was possible to isolate predatory bacteria from fecal samples of mammals such as hippopotamus, bat, and horse, which showed the ability to predate on the different human pathogens of the ESKAPE group used as prey. In 2017, the effect of predatory bacteria on the gastrointestinal tract in rats was evaluated, infecting the mice with *Klebsiella pneumoniae,* no evidence of

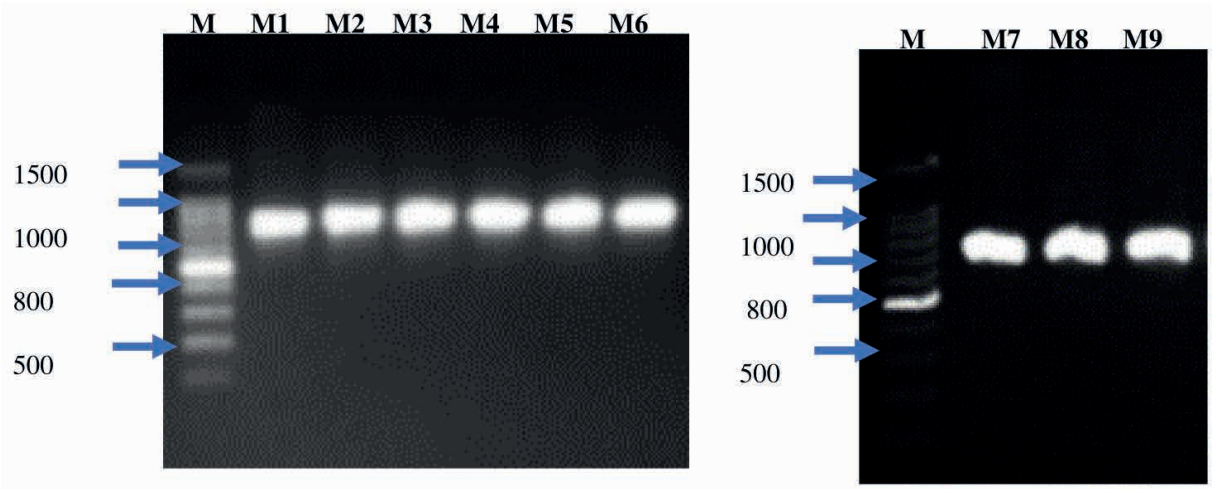

Figure 4. **PCR with primer specific for *Bdellovibrio.*** 2% agarose gel run at 80 V for 70 min showing amplification of predatory bacteria with BdsF and BdsR primers.

Sample No.	Origin	Identified predatory bacteria	% of identity	BLAST reference
M3	hippo feces	*Bdellovibrio bacteriovorus* spp.	99.60%	MK779947.1
M7	Soil Platón Sánchez	*Bdellovibrio bacteriovorus* spp.	96.50%	AF148938.1
M12	hippo pond	*Bdellovibrio* sp.	97.14%	CP058348.1
M16	Atezca Lagoon	*Bdellovibrio bacteriovorus* spp.	99.60%	CP007656.1
M20	Papagayo river soil	*Bdellovibrio bacteriovorus* spp.	100%	BX842648.2
M21	Mezcala River	*Bdellovibrio* sp.	98.50%	FM956319.1
M25	Sea Pie de la Cuesta	*Bdellovibrio* sp.	99.73%	AF148938.1
M27	equine feces	*Flavobacterium* sp.	96.43%	KP875419.1
M30	bat feces	*Peredibacter* sp.	98.14%	KR153976.1

Table 2. Sequencing of predatory bacteria.

Prey	Predation by *Bdellovibrio bacteriovorus* (M7)	Approximate time of predation
* *Enterococcus faecalis*	+	11 a.m.
enterococcus faecium	+	7 a.m.
* *Staphylococcus aureus*	+	7 p.m.
Staphylococcus epidermidis	+	12 noon
Streptococcus anginosus	+	9 p.m.
* *Klebsiella pneumoniae*	+	6 a.m.
Klebsiella oxytoca	+	7 a.m.
Klebsiella aerogenes	+	3 p.m.
proteus mirabilis	+	7 a.m.
Proteus vulgaris	+	12 noon
enteric salmonella	+	12 noon
Stenotrophomonas maltophilia	+	7 a.m.
Serratia marcesens	+	12 noon
* *Acinetobacter baumannii*	+	3 p.m.
Citrobacter diverse	+	12 noon
Vibrio cholerae	+	2 p.m.
Vibrio parahaemolyticus	+	3 p.m.
* *Pseudomonas aeruginosa*	+	11 a.m.
Aeromonas hydrophila	+	4 p.m.
Escherichia coli	+	5 p.m.

* Bacteria of the ESKAPE group; Predation: Where: + indicates that there was bacterial predation, – indicates that there was no bacterial predation.

Table 3. Prey range of a soil isolate of *Bdellovibrio bacteriovorus* (M7).

damage was shown in the mouse by intranasal inoculation of the predatory bacteria and after 48 hours, predatory bacteria were found to be viable in the feces of the mice (Shatzkes *et al.*, 2015). The predation capacity of the predatory bacteria isolated against Gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus anginosus*, *Enterococcus faecium* and *Enterococcus faecalis* was evaluated and it was confirmed that *Bdellovibrio* sp. showed the ability to control and eradicate populations of Gram bacteria -positive possible human pathogens. In 2018, the predation capacity of *Bdellovibrio bacteriovorus* HD100 was evaluated with a strain of *Staphylococcus aureus* with the capacity to produce biofilms and it was observed that the rapprochement of the predatory bacterium with the prey was rare, but it did have the capacity to control the bacterial population. (Pantanella *et al.*, 2018). *Peredibacter* sp., was isolated from bat feces (guano), in a cave in the city of Cuetzalan, Puebla, under conditions of high humidity (75%) and at an ambient temperature of 12 °C, for which it is suggested which, like various predatory bacteria *Peredibacter* sp., is a ubiquitous bacterium; however, our isolate did not show the ability to prey on 19 of 20 bacterial hosts used in the prey range. *Peredibacter starri* has only been isolated from soil samples at a temperature of 35 °C and it is suggested that, having a lifestyle similar to that of *Bdellovibrio* sp., it has a wide prey range (Jurkevitch, 2020). It was confirmed that the *Bdellovibrio sp.* isolates showed the ability to prey on Gram-positive and Gram-negative bacteria; however, all our *Bdellovibrio* sp. isolates had the ability to control the host bacteria from 6, 8 or 12 hours, that is, before the established average time. Several authors affirmed that *Bdellovibrio* sp. has a wide range of prey, both Gram-negative and Gram-positive bacteria and that it has the ability to prey on Gram-negative bacteria in an average of 18 to 24 hours (Pérez et al.., 2016; Jurkevitch 2020*)* (Rumbaugh, and Sauer, 2020) (Vestby, *et al.*, 2020).

CONCLUSIONS

Predatory bacteria were isolated from soil, water and fecal samples of mammals at temperatures between 12 °C and 41 °C, in conditions of high humidity (maximum 87%) and in dry soils. The presence of predatory bacteria was confirmed in fecal samples from mammals. *Bdellovibrio* sp., had a wide range of prey, including Gram-negative and Gram-positive human pathogens. *Bdellovibrio* sp., isolated from the hippopotamus pond in the city of Taxco de Alarcón, Guerrero, Mexico, presented an effective predation mean of 12 hours to control and eradicate both Gram-negative and Gram-positive bacteria. The presence of predatory bacteria in saline environments was confirmed, since predatory bacteria were isolated from seawater samples in Pie de la Cuesta, Acapulco, Guerrero, Mexico.

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INTEREST CONFLICT

The authors declare that there is no conflict of interest

PERSPECTIVES

The Genomic Biotechnology Laboratory of the National Polytechnic Institute has carried out the complete sequencing and analysis of the genomes of some isolates of predatory bacteria such as *Bdellovibrio* sp., in order to know the functions of the genes that code for enzymes that are involved in the predation process and with this knowledge look for future applications.

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