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POTENTIAL USE OF PREDATORY BACTERIA TO ATTACK SPECIES OF *AEROMONAS*

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Abstract: Antimicrobial resistance is a growing problem in recent years, due to its indiscriminate use in clinical and agricultural areas. *Aeromonas* mainly affect fish and shellfish, causing diseases such as furunculosis, impacting the economy of the aquaculture industry. In humans it causes diseases such as gastroenteritis and skin and soft tissue infections. The WHO has classified *Aeromonas* bacteria as a priority for the development of new alternatives as antimicrobial agents to control them. Predatory bacteria such as *Bdellovibrio*, termed live antibiotics, prey on a broader range of Gram-negative bacteria than phages, allowing them the potential to control pathogenic bacteria. In this study, ten predatory bacteria were isolated from various samples of environmental origin (soil, water, and feces from mammals), all belonging to the genus *Bdellovibrio*, and were identified with partial sequences of the 16S rRNA gene. Their phenotype and genotype were characterized, and their predation efficiency against twelve *Aeromonas* strains of clinical and environmental origin was determined. All isolates of *Bdellovibrio spp*. preyed on *Aeromonas* species.

According to the results obtained from predation efficiency, the *Bdellovibrio* isolates reduced the prey population 5 to 16.5 h after inoculation with different *Aeromonas* strains.

Keywords: Predatory bacteria, BALO, *Aeromonas*, phenotypic characterization, molecular characterization.

INTRODUCTION

Aeromonas are bacteria that cause various infections in humans, they have been found to be associated with clinical cases of gastroenteritis and mild infections in various organs and tissues, or serious ones such as septicemia (Parker and Shaw, 2011; Ku and Yu, 2015; Awan et al., 2018a; Awan et al., 2018b). These bacteria have long been thought to be opportunistic, however there is evidence of severe sepsis in immunocompetent patients caused by virulent strains of this genus (Ku and Yu, 2015). One of the characteristics that most favor these bacteria is their ecological adaptability, since they have a diversified metabolism, which allows *Aeromonas* to be present 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 most frequently associated with human disease are A. hydrophila (14.5%), A. caviae (37.6%), A. veronii bv. sober (27.2%) and *A. dhakensis* (16.5 %), which represent around 96% of gastroenteritis cases (Janda and Abbott, 2010; Teunis and Figueras, 2016). Due to the growing problem of antimicrobial resistance (AMR), the World Health Organization (WHO) has classified the bacteria on a priority list to develop and investigate new antibiotics or alternatives to mitigate them, according to this classification *Aeromonas* spp. it is in priority 1 or critical (WHO, 2017; WHO, 2020). Therefore, the study of new alternatives for the biological control of *Aeromonas* is essential. Currently, predatory bacteria have attracted attention due to their predatory ability and wide prey range, 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). The search for predatory bacteria that are potentially effective in fighting these highly virulent microorganisms is paramount. The phenotypic and molecular characterization of predatory bacteria allowed to know the characteristics of the isolates with the greatest potential to attack *Aeromonas* species of clinical and environmental origin.

METHODS

SAMPLING AND ISOLATION OF PREDATORY BACTERIA

Bacteria of clinical and environmental origin of the genus *Aeromonas* were used from the collection of pathogenic bacteria of the Laboratory of Genomic Biotechnology and the Laboratory of Molecular Biomedicine of the Center for Genomic Biotechnology of the National Polytechnic Institute, located at Blvd. del Maestro S/N, Esq. Elías Piña, Col. Narciso Mendoza, CP 88710, Cd. Reynosa, Tamaulipas, Mexico. The bacteria used as prey (hosts) were: *Salmonella enterica* and *Klebsiella pneumoniae*. The dams were cultured in Petri dishes containing LB agar, incubated at 37 °C for 18-24 h, later, a colony was taken and inoculated in 20 ml of LB broth contained in 50 ml conical "Falcon" tubes, incubated at 37 °C for 18-24 h at 150 rpm. Once growth was observed in the LB broth, they were kept refrigerated at 4 °C, being the culture base for the following experiments. Predatory bacteria were isolated from fecal samples of mammals, soil, and water from different areas of the Mexican Republic and placed in cocultures with bacteria of clinical origin., *Salmonella enterica* and *Klebsiella pneumoniae*, following the specifications described by Jurkevitch (Jurkevitch, 2012). The supernatants of the samples (soil, water and feces from mammals) previously obtained were added to a sterile 250 ml Erlenmeyer flask containing two prey pellets. Starting from the crop feet (*S. enterica* y *K. pneumoniae*) 100 µl were taken and inoculated into 20 ml of LB broth and incubated at 37 °C for 18-24 h. 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. prey pellets, *S. enterica* y *K. pneumoniae*, were placed for different samples. Finally, the 250 mL Erlenmeyer flask was shaken vigorously to homogenize the pellets and the supernatant of the samples in the flask, leaving a final volume of approximately 100 mL. The flasks were incubated at 28 °C for 7-10 days with constant shaking at 150 rpm, until predation was observed (visualization of cellular debris at the bottom of the flask).

PURIFICATION OF PREDATORY BACTERIA USING THE DOUBLE-LAYER TECHNIQUE IN A PETRI DISH

DNB media (for its acronym in English "Diluted Nutrient Broth") were prepared with the following specifications, for one liter they were added: 0.8 g of nutrient broth, 2 ml of MgCl2 • 7 H2O 1 M (2 mM final), 3 ml of CaCl2 • 2 H2O 1 M (3 mM final), 15 g of agar-agar for DNB agar bottom and 7 g for DNB agar top; each medium was divided into 15 ml conical "Falcon" tubes, placing 10 ml in each and subsequently sterilized. The DNB bottom agar was placed in Petri dishes and allowed to solidify. 900 µl of 25 mM HEPES buffer pH 7.4 were placed in 1.5 ml microtubes and dilutions from 1x10-1 to 1x10-6 were made for each coculture. 100 µl of the corresponding dilution and 250 µl of prey were added to each tube with DNB top agar, then emptied onto each Petri dish with the solidified DNB bottom agar. They were left to incubate at 30 °C for 5 to 10 days, until halos of cell lysis were observed (indication of predation).

MOLECULAR IDENTIFICATION USING SPECIFIC OLIGONUCLEOTIDES OF THE 16S RRNA GENE FOR BALOS

To detect the presence of BALOs 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, 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 (not purified) was used for the PCR reactions. After obtaining gDNA by the lysis method, a PCR mix was prepared for amplification with specific oligonucleotides of the 16S rRNA gene for the presumptive detection of BALOs-specific genes (Jurkevitch, 2012), which contains: 13.25 µl of milli-Q water, 5 µl of MyTaq® 5X buffer (1X final), 0.25 µL 50 mM MgCl2 (1.5 mM final), 0.25 µL 10 mM dNTPs (0.2 mM final), 0.5 µL 5 µM Forward oligonucleotide (0.1 µM final), 0.5 µL 5 µM Reverse oligonucleotide (0.1 µM final), 0. 25 µl MyTaq® Taq Polymerase 5 U/µl (final 0.05 U/µl) and 5 µl gDNA (extracted by cell lysis method). The conditions for the thermocycler were the following: initial 94 °C for 4 min; 35 cycles of 94 °C for 1 min, Tm °C (Tm of the Forward and Reverse pair of oligonucleotides of the 16S rRNA gene specific for BALOs) (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 carried out, the resulting products were analyzed by 1% agarose gel electrophoresis, run for 60 min at 80 V, using 0.5X TAE as buffer. The loading buffer mixture with the PCR product was placed in each well of the gel; corresponds to 2 µl of loading buffer (1X SYBR® Gold Nucleic Acid Gel Stain #S11494, INVITROGEN, USA; with 0.25% bromophenol blue and 0.25% Xylene cyanol dyes) with 5 µl of the PCR product. The agarose gel was visualized on the Kodak® photodocumenter with a Gel Logic 112 camera using the Kodak® dS 1D v. 3.0.2.

SEQUENCING REACTION USING SPECIFIC OLIGONUCLEOTIDES OF THE 16S RRNA GENE FOR BALOS

The PCR product was purified following the methodology of the manufacturer ExoSAP-IT (#78200, USB, USA). The sequencing reaction was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit. The sequencing reactions were sent to the Services Laboratory of the Genomic Biotechnology Center of the National Polytechnic Institute for sequencing on the Applied Biosystems ABI® 3130 Genetic Analyzer equipment. Once the sequencing was done on the computer, the files were downloaded in .ab1 format. Sequences were cleaned using MEGA11 v. 11.0.10 and a BLAST (Basic Local Alignment Search Tool) search for homologous sequences was performed in the NCBI (National Center for Biotechnology Information) database to determine the identity of each predatory bacterium used for predation efficiency.

PREDATION EFFICIENCY OF ISOLATED PREDATORY BACTERIA

The cocultures were kept at room temperature (29 oC), and 1,000 µl of each coculture was taken, which were deposited in plastic cells (1.5 ml semi-micro PS cell, #KART1938, KARTELL, USA) to measure the optical density (OD) in a spectrophotometer (UV-Visible Spectrometer, Cintra 10e, GBC). The first reading was performed 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.

RESULTS

A total of 41 cocultures were obtained from soil, water, and feces samples of mammals from the states of Durango, Puebla, Tamaulipas, and Tlaxcala, in which the formation of cellular debris was observed (Figure 1).

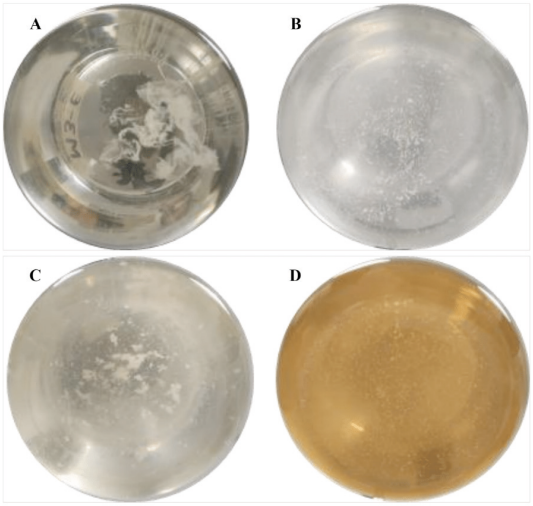

Figure 1. Cocultures with formation of cellular debris.Cellular debris in cocultures indicates predation activity. Soil samples: (A) M3 with Salmonella enterica prey, (B) M5 with Salmonella enterica prey, and (C) M34 with Salmonella enterica prey. Sheep feces sample: (D) M40 with Klebsiellapneumoniae prey.

Cell lysis halos were isolated from the cocultures in a Petri dish for the purification of predatory bacteria, Figure 2. With the double-layer method in a Petri dish, those samples that did not form cell lysis halos, that is, absence of predation, were discarded.

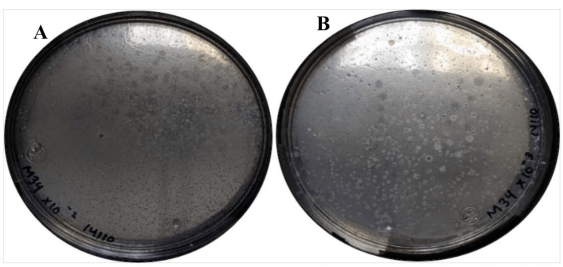

Figure 2. Double layer in Petri dish with formation of cell lysis halos. It can be observed the predation of the sample (A) M34 1x10-2 with Salmonella enterica prey, (B) M341x10-3 with Salmonella enterica prey.

Different isolates were identified by molecular method with specific BALO oligonucleotides, shown in Figure 3 and Table 1.

From the sequencing reactions run on the ABI® 3130 Genetic Analyzer from Applied Biosystems purified with the BigDye® XTerminatorTM Purification Kit (#4376486, Applied Biosystems, USA), the files were obtained in.ab1 format and analyzed using the MEGA11 v. 11.0.10, and with the sequences in FASTA format, a search for homologous sequences was performed using BLAST in the NCBI database to determine the identity of each predatory bacterium (Table 2).

In predation efficiency, the initial concentrations of prey and BALOs were 0.301 and 0.142 A, respectively. B1 started predation at 5 h in 16.66 % of the prey (A8, A12); B3 in 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) at 50%; B34 in 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). The BALO B19 showed the highest efficiency when starting predation time at 5 h in 83.33 % of the prey items (A1, A2, A3, A5, A6, A7, A8, A9, A10 and A11), while B1 had the lowest efficiency when starting predation at 5 h in only 16.66 % of the prey items. *Aeromonas* (A8, A12) and starting predation at 12.5 h in 41.66 % of the prey (A1, A2, A5, A7, A9). These results indicate that the predatory bacteria displayed very different predation characteristics, depending on the specific strains of the prey, even though the prey belong to the same genus.

DISCUSSION

Predation was found in the cocultures made from the three types of samples available for the study, which correspond to soil, water, and feces of mammals, confirming that predatory bacteria are ubiquitous and can be found in various ecological niches as described by other

Figure 3. Amplification of 16S rRNA gene fragments specific for BALOs, BdsF:BbsR, PerF:PerR, 21BdsF:1260BdsR, McvF:McvR. (1)100 pb DNA Ladder(#G210A, Promega, USA), Bdellovibrio: 800 pb(BdsF:BbsR707):(2)M3 x10-1, (3) M40 1x10-2[1], Peredibacter: 1,200 pb(PerF:PerR):(4) M7 1x10-2[2], Bdellovibrio: 1,200-1,500 pb(21BdsF:1260BdsR):(5) M5 1x10-2[3], (6) M5 1x10-2[1], (7) M5 1x10-3[2], (8) M5 1x10-3[1], (9) M5 1x10-4[7], (10) M5 1x10-4[6], (11) M5 1x10-4[5], (12) M5 1x10-4[1], (13) M5 1x10-4[8], (14) M5 1x10-4[3], (15) M5 1x10-4[4], (16) M5 1x10-4[2], (17) M5 1x10-6[2], (18) M5 1x10-6[1], Micavibrio: 800 pb(McvF:McvR): (19) M34 1x10-2[6].

Species	Size (pb)	Oligonucleotides	Specific amplification for BALOs					
Bdellovibrio	800	BdsF	M2	M3	M7	M18	M40	M41
		BdsR						
Peredibacter	1,200	PerF	M1	M3	M18	M40	M41	
		PerR						
Bdellovibrio	490	BbsF216	M19	M21	M34			
		BbsR707						
Micavibrio	800	McvF	M4	M6	M19	M34	M39	
		McvR						
Bacteriovorax	1,000	Bac676F	M5					
		Bac1442R						

Table 1. Cocultures that amplified for predatory bacteria with specific BALO oligonucleotides.

ID	Oligonucleotides	Scientific name	% of identification	Access
B1	BdsF:BdsR	*B. bacteriovorus* CW6	97.03	KC480584.1
		B. bacteriovorus MK1	94.64	MZ934718.1
		B. bacteriovorus MX3	94.64	MZ934717.1
B3	BdsF:BdsR	*B. bacteriovorus* HD100	97.21	NR_027553.1
		B. bacteriovorus FSBD5	97.03	OQ553821.1
		B. bacteriovorus SSB218315	96.75	CP020946.1
B5	BdsF:BdsR	*B. bacteriovorus* HD100	93.82	NR_027553.1
		B. bacteriovorus 109J	93.60	CP007656.1
		B. bacteriovorus SSB218315	93.44	CP020946.1
B7	BdsF:BdsR	*B. bacteriovorus* HD100	95.33	NR_027553.1
		B. bacteriovorus FSBD5	95.14	OQ553821.1
		B. bacteriovorus SRE7	95.14	AF263832.1
B19	BdsF:BdsR	*B. bacteriovorus* MK1	96.31	MZ934718.1
		B. bacteriovorus MX3	96.31	MZ934717.1
		B. bacteriovorus Y38	96.18	OM846611.1
B34	BdsF:BdsR	*B. bacteriovorus* SSB218315	99.98	CP020946.1
		B. bacteriovorus N322	99.98	KC836746.1
		B. bacteriovorus FSBD5	99.37	OQ553821.1
B37	BdsF:BdsR	*B. bacteriovorus* HD100	95.80	NR_027553.1
		B. bacteriovorus 109J	95.80	CP007656.1
		B. bacteriovorus 100	95.80	AF084850.1
B40	BdsF:BdsR	*B. bacteriovorus* SDWB-DG2	94.31	MK779946.1
		B. bacteriovorus GF2	94.31	MK415060.1
		B. bacteriovorus Kdesi	94.31	MG957118.1
B41	BdsF:BdsR	*B. bacteriovorus* HD100	98.72	NR_027553.1
		B. bacteriovorus FSBD5	98.71	OQ553821.1
		B. bacteriovorus 109J	98.53	CP007656.1
B4J	BdsF:BdsR	*B. bacteriovorus* 109J	98.24	CP007656.1
		B. bacteriovorus HD100	98.24	NR_027553.1
		B. bacteriovorus HD100 cg	98.24	BX842648.2

Table 2. Partial sequences of purified bacterial predators: Homologous sequences obtained in BLAST in the NCBI database.

authors (Oyedara et al., 2016; El-Shanshoury et al., 2016; Taylor et al., 1974).

Isolates B40 and B41 showed the ability to prey on *Klebsiella pneumoniae*, bacteria used as prey for its isolation and purification. Shatzkes et al. in 2017, they evaluated the effect of predatory bacteria on the gastrointestinal tract in mice, infecting the mice with *Klebsiella pneumoniae*, no evidence of mouse damage was shown by intranasal inoculation of predatory bacteria, and at 48 hours predatory bacteria were viable in the feces of mice (Shatzkes et al., 2017a; Shatzkes et al., 2017b). Several studies have affirmed that species of *Bdellovibrio* they have a wide range of prey for Gram-negative bacteria, and 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 species of *Aeromonas* predation was observed after 5 h. The gender: *Peredibacter* was found in the soil sample M7 from the Tepetitla River, Tlaxcala, Tlaxcala, Mexico, but it was not possible to purify it, so it can be determined that, like various predatory bacteria, Peredibacter is an ubiquitous bacterium. However, the phenotypic characteristics of the isolate have only been determined with the prey used for its isolation.: *S. enterica*. *Peredibacter starri*, has been isolated only from soil samples at a temperature of 35 °C, it is suggested that by having a lifestyle similar to that of *Bdellovibrio* sp., has a wide prey range (Jurkevitch and Jacquet, 2017). The gender *Micavibrio* was found in sample M34 of garden soil from Cd. Victoria, Tamaulipas, Mexico, but it was not possible to purify it, this isolate showed predation with the prey used for its isolation:*S. enterica*. The capacity of *Micavibrio aeruginosavorus* to prey on pathogens of clinical interest such as *P. aeruginosa* and *K. pneumoniae*, has been shown in different studies, in the same way 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 kill and reduce 57 of the 89 bacteria examined (Kadouri et al., 2007; Dashiff et al., 2011).

CONCLUSIONS

Of a total of 41 samples, 36 were from soil, 3 from water and 2 from feces from mammals, from which 9 BALOs were obtained: 6 from soil (4 from the Tepetitla River, Tlaxcala, Mexico; 1 from the textile zone of Tlaxcala, Tlaxcala, Mexico and 1 from garden soil from Cd. Victoria, Tamaulipas, México), 1 from water (from the beach in Cd. Madero, Tamaulipas, México). and 2 from fecal feces of mammals (both from 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:1260B dsR, PerF:PerR and McvF:McvR, respectively. The balos present in the samples M3, M5, M19, M34, M37 (which correspond to the balos, B3, B5, B19, B34 and B37, respectively) were purified with a salmonella enterica prey and, for the samples M40 and M41 (which correspond to the balos, B40 and B41, respectively) with Klebsiella Pneumonia Dellovibrio. Sample M7 amplified for oligonucleotides specific to the genera Peredibacter and B*dellovibrio* (corresponding to BALO B7) with Salmonella enterica prey. Sample M34 amplified for oligonucleotides specific to the genera Micavibrio and *Bdellovibrio* (corresponding to BALO B34) with Salmonella enterica prey. The BALOs

Peredibacter and Micavibrio could not be purified by double layering in a Petri dish. In M40 and M41 (which correspond to BALOs, B40 and B41, respectively) the presence of predatory bacteria was confirmed in fecal samples from mammals, with which it can be concluded that BALOs do not represent a danger to 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 and B4J -LBG collection-) demonstrated 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 after 5 h and until 16.5 h of coculture. The 10 isolates of predatory bacteria represent a viable alternative to attack *Aeromonas* species of clinical and environmental origin.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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