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Bdellovibrio sp: An Important Bacterial Predator in Lake Geneva?

Ezzedine JA, Pavard G, Gardillon M and Jacquet S*

Universite Savoie Mont-Blanc, INRAE, France

***Corresponding author:** Jacquet S, INRAE-Universite Savoie Mont-Blanc, UMR CARRTEL, Thonon-Les-Bains, France, Tel: 33450267812; Email: stephan.jacquet@inrae.fr

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Abstract

We describe a new *Bdellovibrio* and like organism (BALO) isolated from Lake Geneva. The bacterial predator is a new member of the genus *Bdellovibrio (Bdellovibrionaceae)*, enable to reproduce mainly on *Pseudomonas* spp preys. To achieve this goal, we used a variety of preys so that we also tested in parallel the host range of other well-known and characterized strains of *Bdellovibrio* such as *B. exovorus* JSS. Among original results, we observed that these BALOs could also grow on a fish pathogen, i.e. *Aeromonas salmonicida salmonicida* which is responsible for salmonid furunculosis, providing thus a new potential therapeutic agent against this bacterial infection in freshwater fish farming. This work sheds light on a functional group of bacteria still largely unknown.

Keywords: *Bdellovibrio* sp; Bacterial Predator; Preys; Isolation; Lake Geneva

Abbreviations: BALO: Bdellovibrio and Like Organisms; SDS: Sodium Dodecyl Sulfate; BSA: Bovine Serum Albumin.

Introduction

Aquatic microorganisms, i.e. viruses, bacteria, Achaea and unicellular eukaryotes, are abundant, diversified, and play major roles in the functioning of ecosystems [1]. Microbiologists have long relied on the principle that microorganisms existed only if they were cultivable [2]. However, it is well known now that only a very small fraction (<1%) of these microorganisms are likely to grow on laboratory conditions [3]. This is not really surprising when one knows that unicellular organisms may live, grow and reproduce in a variety of environments where a multitude of parameters may intervene both in terms of quantity and quality (e.g. light, temperature, nutrients, pH, etc…) not to mention the biotic interactions (predation, parasitism, etc…). In fact, the huge diversity of microorganisms has been highlighted mainly by the advent and use of tools derived from molecular biology: PCR, cloning-sequencing, meta genomics, high-throughput sequencing. It remains, however, that the different interactions between these microorganisms and their environment, as well as their functional roles, often have to be explored, and, for this purpose, isolation based methods still represent a scientific interest. This is typically the case for many bacteria (comprising a large microbial community, consisting of species considered pathogenic to humans, animals and plants, as well as beneficial species that interact with other organisms, not to mention their various ecological roles), for instance the Bdellovibrio and like organisms (BALOs).

BALOs are composed of Gram-negative bacteria, and they are obligate predatory bacteria of other Gram-negative bacteria. Two life cycles exist among BALOs, the endo-biotic cycle where the prey is shaped as a bdelloplast and consumed from the inside, vs. a periplasmic cycle with an external consumption of the prey [4]. BALOs are composed of 5 families: the *Bdellovibrionaceae*, the *Peredibacteraceae*, the *Bacteriovoracaceae*, the *Halobacteriovoraceae* and the *Pseudobacteriovoracaceae,* all belonging to the Oligoflexia class [5]. A little bit apart, one genus, the *Micavibrio*, belongs to the Alpha-proteo bacteria class. In general, BALOs are

morphologically similar and look like a comma shape form with a flagellum, and measure between 0.2 to 0.5 µm in width and 0.5 to 2.5 µm in length [6]. BALOs have different predation strategies; some are generalists with a broad spectrum of preys, while others are specialized on a few preys [7]. The high diversity and ubiquitous nature of BALOs has implications on the structure and dynamics of microbial communities [8]. Indeed, it is assumed that BALOs may act as an ecological balancer". In other words, they could regulate bacterial biomass and diversity like bacteriophages and small protists do [9,10]. BALOs in freshwater ecosystems have been poorly studied [11-16], and this is particularly true for lakes.

Recently, we proved the existence of BALOs in large and deep peri-alpine lakes [17]. Using a cloning-sequencing (Sanger) approach and quantitative PCR, we found that, while the *Peredibacteraceae* family was represented mainly by a single species (*Peredibacter starrii*), it could represent up to 7% of the total bacterial cell abundances. Comparatively, the abundances of two other families (i.e. the *Bdellovibrionaceae* and *Bacteriovaracaceae*) were significantly lower. In addition, the distribution in the water column was very different between the three groups suggesting various life strategies/niches: *Peredibacteraceae* dominated near the surface while *Bdellovibrionaceae* and *Bacteriovaracaceae* were more abundant at greater depth. On the basis of these results and to go deeper in the knowledge about the possible importance of BALOs in the microbial functioning of lakes, we tried to isolate and characterize new BALOs as well as their potential preys from Lake Geneva. Our aims were thus multiple and consisted in: (I) isolating, growing and identifying potential BALOs and preys from different origins, and (ii) defining host-range for the predators.

Material and Methods

Prey isolation, selection, and culturing from different origins Preys used in this study were from four different origins. Firstly, five standard reference (Gram-) bacteria were ordered from the Centre International de Ressources Microbiennes (CIRM) (https://www6.inra.fr/cirm_eng/)": *Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 10536, *Hafnia alvei* ATCC 13337, *Pseudomonas fluorescens* ATCC 13525 and *P. putida* ATCC 12633. These bacteria were cultured on liquid LB medium (Trypton 10 g, Yeast extract 5 g, NaCl 10 g) and incubated at 25°C under low shaking at 200 rpm. The second source of potential preys was obtained from a mixed sample of water issued from Lakes Geneva, Bourget and Annecy and of the Dranse River (ALBD). From each source, 100 ml of water was collected and mixed. The third source of bacteria was obtained from 25 non-axenic cultures of freshwater microalgae, all isolated from Lake Geneva and stored in the Thonon Culture Collection (TCC)" (https://www6.inra.fr/carrtel-collection_eng/) hold in our laboratory. Briefly, 3 ml of culture were collected and mixed together. For the two latter sources, samples were filtered through microfiber filters (Whatman, GF/F, 47 mm), and polycarbonate filters of 5 and 2 µm (ipPoRE). Then filtrates were distributed in Falcon tube of 50 ml and centrifuged at 12,000 g for 20 min. The pellet was dissolved with 3 ml of PBS 1X (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g). Finally, 1 ml were distributed into 3 flasks containing 30 ml of LB medium and incubated for 48 h at 25°C under low shaking at 200 rpm. After incubation, the cell concentration was estimated by flow cytometry (not shown). The cultures were then diluted 10 fold up to 10^{-7} in PBS 1X, and 100 µl of each dilution was spread on LB agar plate (15 g/l) and incubated for 24 h at 25°C. Isolated colonies so obtained of different shapes and colors were selected and cultured in liquid LB medium for 24 h at 25°C under low shaking at 200 rpm. Then, the culture was centrifuged at 5,000 g at 4°C for 10 min and the supernatant was discarded. The pellet was dissolved in a few milliliter of HM buffer (HEPES 6 g, 15g agar, 6 ml $CaCl_2$ (0.5 M), 3.33 ml $MgCl_2$ (0.6 M)) to obtain a concentration of about 10^{10} cells per ml in order to be used for BALOs enrichment. The cell concentration was obtained by reading optical density (OD) at 600 nm. Each culture was colored using a Gram stain kit (Sigma-Aldrich) according to the manufacturer's instructions. Finally, the species *Aeromonas salmonicida salmonicida* SA28 recently characterized and isolated from farmed Arctic chars (*Salvelinus alpinus*) infected with furunculosis (Jacquet unpublished) was also tested as a potential prey. Specific Fur medium (Tryptone 10 g, yeast extract 5 g, NaCl 2.5 g, Tyrosine 1.0 g) was used to grow the species.

Sampling and Enrichment of BALOs

A few litters of water mixed with biofilms on rocks and sediment were sampled on April 2, 2019 in a coastal area of Lake Geneva next to our research institute. Immediately, the sample was filtered as above and 100 ml was used for the enrichment step (see below), with 5 ml of each concentrated prey sources (CIRM, ALBD, TCC). A negative control was prepared with filtered sterile lake water to which preys were added. All flasks were placed in a hot chamber at 25°C and gently shacked at 200 rpm. Each flask was examined daily for a decrease in turbidity, a marker of bacterial prey concentration decrease. Also, a drop of each sample was observed under phase-contrast microscopy for fast moving cells, a marker of BALO presence.

Predator Isolation and Growth on Double-Layered Agar Plates

We applied the protocol proposed by Jurkevitch [18] to isolate BALOs. 50 ml of each enriched culture were

centrifuged at 500 g for 5 minutes at 4˚C. The supernatant was then centrifuged at 27,000 g for 20 minutes at 4˚C. The pellet was taken up with 3 mL of HM buffer and filtered through 1.2 µm. This filtrate containing the possible predators was used to achieve a dilution range of 10 to 10^{-4} in a final volume of 5 mL of HM buffer. 100 µL of each dilution were added to 4 mL of molten HM top agar (0.5%), supplemented with 300 µL of prey at 10^{10} cells/ml. For each dilution of predator, one type of prey was added. After mixing it rapidly, the HM top was poured on a Petri dish containing 20 mL of solid HM (15%). Once the top HM was solidified, the dishes were sealed with Parafilm and incubated at 25˚C. After 5 to 8 days of incubation, the presence of transparent halos of a few millimeters (resembling lysis plaques) testified of the consumption of the tested prey. These predation plaques were then removed by means of a 1 ml pipettor tips having been previously cut by a sterile scalpel. The agar piece was then resuspended in 500 µL of HM buffer. The presence of BALOs in the suspension was confirmed by microscopy observation. When fast moving cells were detected, the suspension was filtered through 0.45 µm filter. This isolation step was repeated three times to obtain a "purified" strain of BALO. Finally, half of the suspension was stored in glycerol (20%) at -80˚C and half was processed for DNA extraction.

DNA Extraction

DNA extraction was performed on both preys and predators. For BALOs, liquid medium were filtered with 0.45 µm pore size filters in order to remove larger microorganisms such as prey cells. All samples were then centrifuged for 3 min at 6,000 g and at 4˚C. The supernatant was discarded and the pellet was used for DNA extraction. We used a homemade protocol combined with GenEluteTM-LPA (Sigma-Aldrich) solution. Briefly, the protocol began with a lysis step by adding 300 µL of TE buffer (TRIS: 1M - pH8, EDTA: 0.5M - pH8) and 200 µL of lysis solution (TRIS: 1M - pH8, EDTA: 0.5M - pH8 and sucrose: 0.7 M). After a thermic shock at -80˚C for 15 min and at 55˚C for 2 min, 50 µL 10% sodium dodecyl sulfate (SDS) and 10 µL of proteinase K (20 mg/mL) were added. The sample was incubated at 37˚C for 1 h with gentle stirring and placed again in the block heater at 55˚C for 20 min. After a quick centrifugation step (13,000 rpm at 4˚C for 3 min), the supernatant was collected, and 50 µL of sodium acetate (3M – pH 5.2) plus 1 µL of GenEluteTM-LPA (Sigma-Aldrich, 25µg/µL) were added. One volume of isopropanol was then added and the tubes were centrifuged for 10 min at 12,000 g and 4°C. Two rounds of ethanol (80%) washing were carried to purify the pellet of DNA. The remaining ethanol was evaporated using the SpeedVac for 20 min. Finally, 30 µL of TE were added and tubes were left for 1 h at 37°C. DNA concentration was measured using Nano Drop 1000 spectrophotometer. When DNA concentration was superior to 25 ng/µL, a dilution was carried. All tubes were stored at -20˚C until analysis.

PCR *Amplification*

PCR amplification of the 16S rRNA gene were performed using specific primers (Bd529F and Bd1007R for *Bdellovibrio*, Bac676F and Bac1442R for *Bacteriovorax* and Per676F and Per1443R for *Peredibacter*, [18], as well as universal primers targeting bacteria (515F and 909R, [19]). Adopted from Davidov Y, et al. [4] the specific amplification PCR mixture volume was at 25 µL and consisted of (final concentration): 1x buffer, 0.2 mM dNTP, 3 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin (BSA) and 0.625 U Biotaq DNA polymerase (Bioline). In a second step, primers (Forward and Reverse) for each BALO genus were added to the different mixtures. Finally, 1 µL of template DNA (BALOs extracted DNA) concentrated at 25 ng/µL was added. A negative control was included and the PCR program was as follows: 94˚C – 5 min, 30 x (94˚C – 1 min, 58˚C – 1 min, 72˚C – 3 min), [4] and with a final extension step at 72˚C for 5 min. For the universal amplification, the mixture volume was at 25 µL and consisted of (final concentration): 1x buffer, 0.4 mM dNTP, 2 mM $MgCl_2$, 0.4 mg/mL bovine serum albumin (BSA), 0.2 µM of forward primer 515F, 0.2 µM of reverse primer 909R and 0.5 U Biotaq DNA polymerase (Bioline). Finally, 1 µL of template DNA concentrated at 25 ng/µL were added. A negative control was included and the PCR program was as follows: 95˚C – 2 min, 30 x (94˚C – 30 sec, 58˚C – 30 sec, 72˚C-30 sec), and with a final extension step at 72˚C for 5 min. Agarose gel analysis was performed for verification of PCR products.

Cloning, Sanger Sequencing and Taxonomic Assignment

When plaques were detected, both the predator and the prey were extracted, amplified and cloned for sequencing. Prior to cloning, PCR products were purified using GE healthcare illustra GFX kit according to the manufacturer's instructions. The purified products were then measured using a Nano drop 1000 spectrophotometer. Cloning was conducted using TOPO TA Cloning kit (Thermo Fisher Scientific) following manufacturer's recommendation. Briefly, ligation step was carried in 6 µL volumes consisting of 1 µL of salt solution, 1 µL of topo vector and 4 µL of purified PCR product. The mix was incubated for 20 min at ambient temperature. After the incubation time, the mix was put in icy water to stop the ligation process. Next, the transformation process was performed by adding 2 µL of the ligation product in 50 µL of competent *E. coli*. A thermic shock was executed in icy water for 10 min, immediately thawed into a block heater at 42˚C for 40 sec, and then again in ice for 3 min. After, 250 µL of SOC medium were added. The sample was incubated at 37˚C for 1h30 with gentle stirring. Then,

50, 80 and 100 µL of *E. coli* were streaked on agar plate. The next day, white clones were selected for DNA amplification. White clones were heated at 95°C for 10 min in order to burst the DNA from *E. coli* cell. 1 µL of DNA was pipetted and added to a PCR mix of 29 µL composed of: 1x buffer, 2 mM dNTP, 2 mM $MgCl_2$, 0.1 µM of forward primer M13F (-20) (5'-GTAAAACGACGGCCAG-3'), 0.1 µM of reverse primer M13R (5'-CAGGAAACAGCTATGAC-3') and 0.6 U Biotaq DNA polymerase (Bioline). A negative control was included and the PCR program was as follows: 94°C-10 min, 30 x (94°C-60 sec, 55°C-60 sec, 72°C – 60 sec), and with a final extension step at 72°C for 10 min. Agarose gel analysis was performed for PCR products verification. With these PCR products, 2 plates of 96 wells were prepared and sent to GATC / Euro fins Genomics for single end Sanger sequencing. 10 clones for each prey and 16 clones for each predator with universal and specific primer were sent. Taxonomic assignment of sequences obtained from Sanger sequencing was performed online with NCBI Blastn [20].

Phylogeny

On one hand, 8 *Bdellovibrio* reference sequences (including type species) of the 16S rRNA gene were downloaded from NCBI [21], supp. Table 2) and *Vampirovibrio chlorellavorus* was used here as an out group. On the other hand, 30 assigned sequences of *Bdellovibrio* (Cloning-sequencing) were used to construct the phylogenetic tree. To begin, all sequences were aligned visually using the program MEGA7 [22] via MUSCLE alignment [23]. The alignment was trimmed at both ends in order for all the sequences to fully overlap with each other (255 positions with gaps). The generated file was then formatted to Phylip and Nexus to be respectively used in PhyML 3.1 2010 [24] and MrBayes 3.2.7a [25]. Model Generator v.85 [26] was used to select the best nucleotide substitution model under corrected Akaike information criterion (AICc) [27] with Number of discrete gamma categories" set to 4. The program returned TIM + G as the best substitution model. The tree was built under Maximum likelihood (ML) method using the PhyML 3.1 program with Tree topology search operation" set to SPR and with 100 bootstraps. Then the Bayesian tree was constructed using MrBayes 3.2.7a [24] program with 500,000 generations and a burn-in value of 25%. In the final ML tree, Posterior" probability (PP) and Bootstraps" (BS) values (PP/BS) were added respectively at each node when possible.

Data Accession Numbers

The *Bdellovibrio* sequences are available in the Gen Bank database with the following accession numbers: MN556920 to MN556941.

Predatory Spectrum Experiment

For each possible predator, a predatory spectrum (host-range) experiment was carried by presenting one prey at a time for the predator in the double-layered agar plate. Two replicates were set for each experiment. The *Bdellovibrio exovorus* JSS strain was also tested with a variety of preys.

Results

Characterization of the Preys and Predators

All colonies isolated from the ALBD sample and selected for the isolation were Gram-negative bacteria. For the TCC samples, 4 colonies were identified as Gram-negative and one colony was Gram-positive (Table 1).

Table 1: Gram Coloration of Isolated Prey Colonies from ALBD and TCC Samples.

ALBD colonies		TCC colonies	
A1	*Gram-negative*	T1	*Gram-negative*
A2	*Gram-negative*	T2	*Gram-negative*
A3	*Gram-negative*	T3	*Gram-positive*
A4	*Gram-negative*	T4	*Gram-negative*
A5	*Gram-negative*	T5	*Gram-negative*

Following the isolation procedure to obtain one or several predators, samples for which turbidity was reduced were then sub-sampled and observed using microscopy. For some of them, we could detect fast moving cells, likely to be BALOs, so that a more in-depth analysis was performed. In fine, predators referred to as T1 and A4 displayed distinct plaques. After PCR amplification, cloning and sequencing the taxonomic assignation confirmed that they were BALOs, more particularly *Bdellovibrio sp*. Indeed, following the molecular approach using the specific primers, 13 out of 16 sequences for both A4 and T1 could be associated to *Bdellovibrio sp*. while only 4 for T1 and 0 for A4 when using the bacterial

universal primer. The phylogenetic analysis revealed that A4 and T1 was in fact the same microorganism (Figure 1).

On the other hand, all clones associated to the preys and also referred to as A4 and T1 were *Pseudomonas sp.* Preys referred to as A5 and A3 were assigned to *Acinetobacter sp*. and *Pseudomonas sp*, respectively, while A5 and A3 predators could not be isolated and identified despite the observation of discrete plaques.

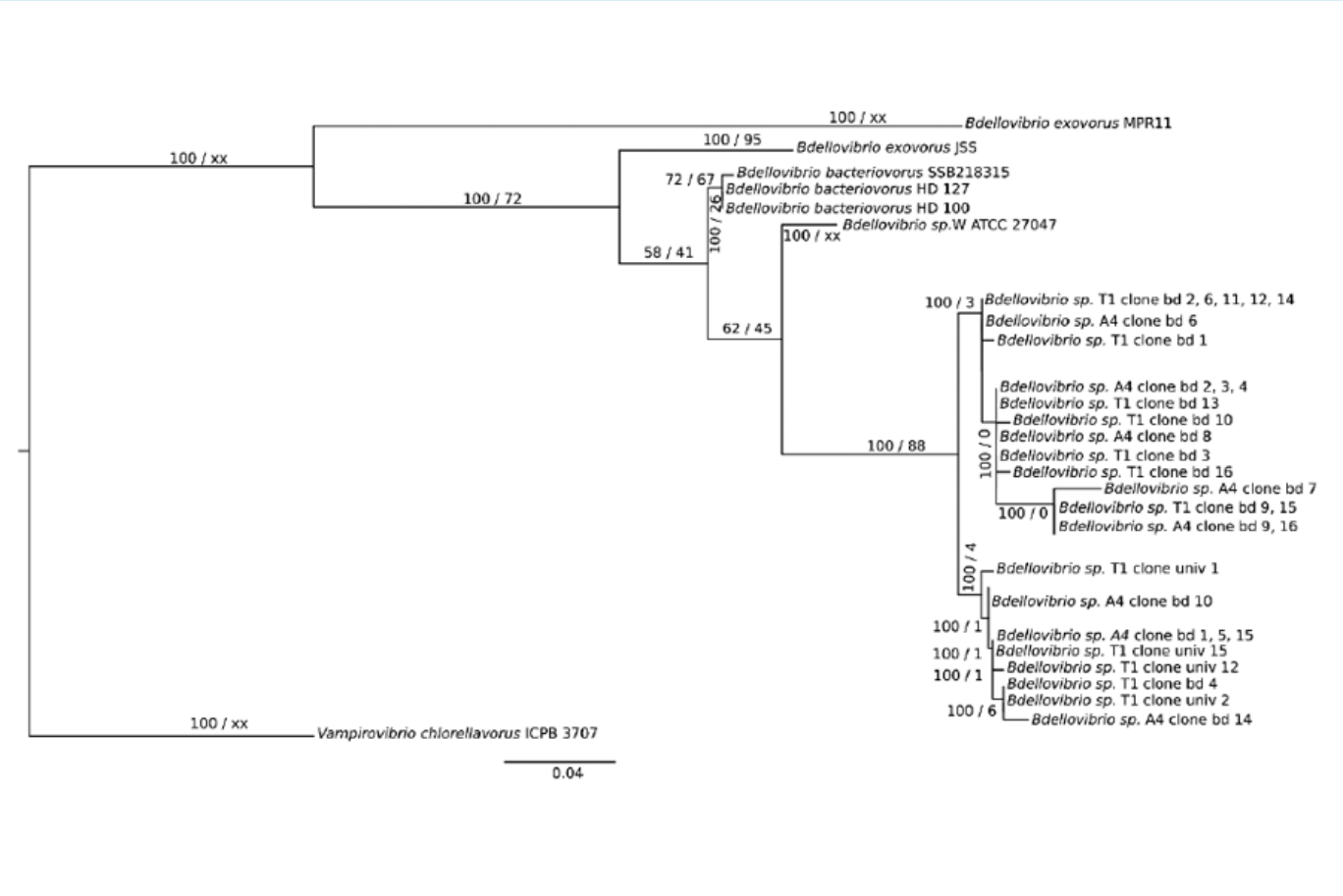


Figure 1: Phylogenetic analysis of 16S rRNA of *Bdellovibrio* clones obtained after Sanger sequencing. Phylogenetic tree was constructed by the maximum likelihood method using PhyML-3.1 and Bayesian inference was conducted with MrBayes 3.2.7.a. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). *Vampirovibrio chlorellavorus* was used as an out group to root the tree. On the other hand, all clones associated to the preys and also referred to as A4 and T1 were *Pseudomonas sp*. Preys referred to as A5 and A3 were assigned to *Acinetobacter sp*. and *Pseudomonas sp*, respectively, while A5 and A3 predators could not be isolated and identified despite the observation of discrete plaques.

Predation Spectrum (Host-Range Experiment)

The experiment was carried with *Bdellovibrio sp*. T1, A4 and *B. exovorus*. Typical plaques were observed with T1 (*Pseudomonas sp*.), A4 (P. sp.; Figure 2), *P. fluorescens* and A3 (P. sp.). However, no plaques were detected when using the T4, *C. freundii, E. coli*, *H. alvei*, T3 and A5 (Table 2) and other preys. In addition, plaques were observed for all the *Bdellovibrio* tested with *A. salmonicida salmonicida* (Supplementary Tables 1 & 2; Supplementary Figure 1).

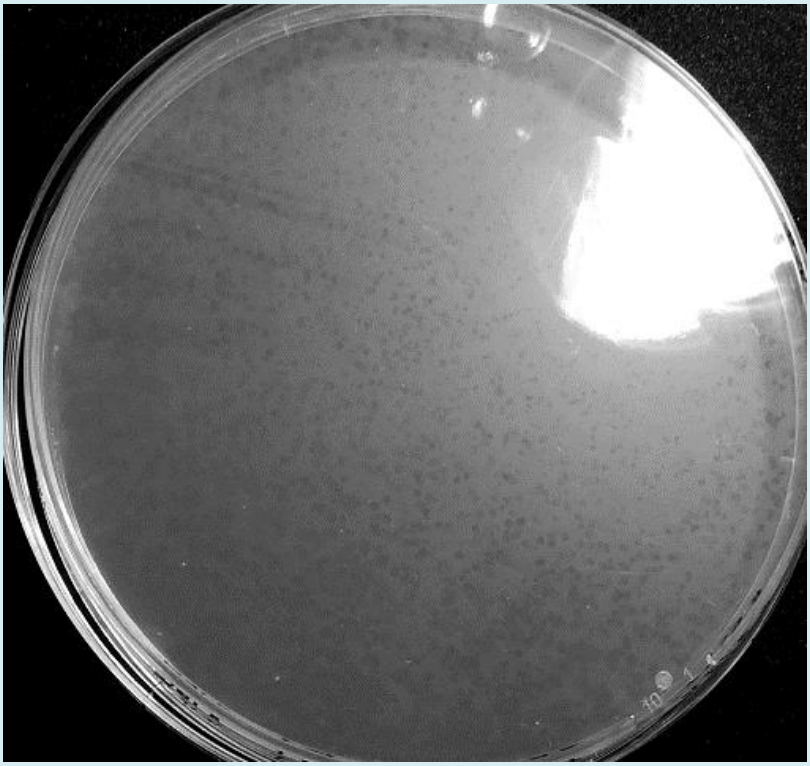


Figure 2: Predation Experiment between *Bdellovibrio Sp*. T1 and A4 Prey (*Pseudomonas sp*.) showing distinct small plaque typical of a predation process.

Table 2: Predation experiment with Bdellovibrio sp. T1 and B. *exovorus*.

Predator	Prey	Predation
Bdellovibrio sp. T1	T1 (*Pseudomonas sp.*)	+++
	A4 (*Pseudomonas sp.*)	+++
	Pseudomonas fluorescens	++
	A3 (*Pseudomonas sp.*)	+
	T4 (Gram-negative)	-
	Aeromonas salmonicida salmonicida SA28	+
	Citrobacter freundii	-
	Escherichia coli	-
	Hafnia alvei	-
	T3 (Gram-positive)	-
	A5 (*Acinetobacter sp.*)	-
Bdellovibrio exovorus	*Aeromonas* salmonicida *salmonicida* SA28	+

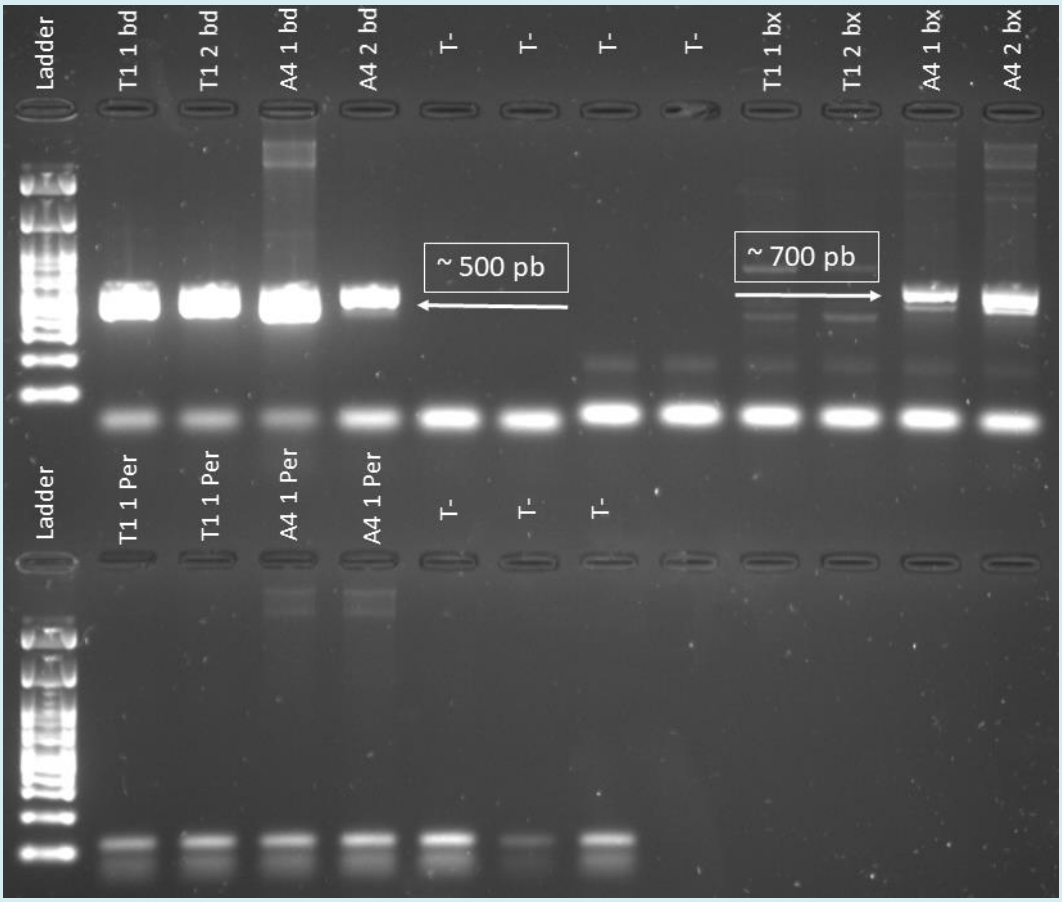
(-) No predation. (+++) multiple and distinct predation plaques. (++) moderate and distinct predation plaques. (+) few predation plaques

Supplementary Table 1: List of cultured phytoplankton from which prey bacteria were isolated

Class	Family	Genus Species	Source	ID in TCC
Chlorophyceae	Ulotrichaceae	*Ulothrix sp.*	Lake Geneva	TCC1a
Cyanophyceae	Phormidiaceae	*Planktothrix rubescens*	Lake Geneva	TCC13
Cyanophyceae	Synechococcaceae	*Synechococcus rhodobaktron*	Lake Geneva	TCC33
Zygophyceae	Desmidiaceae	*Staurastrum sebaldii*	Lake Geneva	TCC106
Chlorophyceae	Oocystaceae	*Mychonastes homosphaera*	Lake Geneva	TCC108b
Chlorophyceae	Scenedesmaceae	*Scenedesmus serratus*	Lake Geneva	TCC110
Chlorophyceae	Chlamydomonadaceae	*Chlamydomonas intermedia*	Lake Geneva	TCC113
Chlorophyceae	Scenedesmaceae	*Scenedesmus acutus*	Lake Geneva	TCC116
Chlorophyceae	Hydrodictyaceae	*Pediastrum duplex*	Lake Geneva	TCC120
Chlorophyceae	Dictyosphaerioideae	*Botryococcus protuberans*	Lake Geneva	TCC123
Chlorophyceae	Volvocaceae	*Eudorina elegans*	Lake Geneva	TCC125
Chlorophyceae	Coelastraceae	*Coelastrum reticulatum*	Lake Geneva	TCC129
Chlorophyceae	Oocystaceae	*Mychonastes sp.*	Lake Geneva	TCC136-1
Diatomophyceae	Bacillariaceae	*Nitzschia palea*	Lake Geneva	TCC139-3
Chlorophyceae	Prasiolaceae	*Stichococcus bacillaris*	Lake Geneva	TCC145-7
Chlorophyceae	Chlorellaceae	*Chlorella vulgaris*	Lake Geneva	TCC213
Chlorophyceae	Chlorellaceae	*Monoraphidium contortum*	Lake Geneva	TCC223
Diatomophyceae	Fragilariaceae	*Fragilaria crotonensis*	Lake Geneva	TCC365
Diatomophyceae	Fragilariaceae	*Fragilaria perminuta*	Lake Geneva	TCC743
Chlorophyceae	Scenedesmaceae	*Scenedesmus costatus*	Lake Geneva	TCC744
Diatomophyceae	Achnanthidiaceae	*Achnanthidium minutissimum*	Lake Geneva	TCC746
Diatomophyceae	Fragilariaceae	*Fragilaria perminuta*	Lake Geneva	TCC747
Diatomophyceae	Achnanthidiaceae	*Achnanthidium minutissimum*	Lake Geneva	TCC748
Diatomophyceae	Fragilariaceae	*Fragilaria perminuta*	Lake Geneva	TCC749
Diatomophyceae	Achnanthidiaceae	*Achnanthidium straubianum*	Lake Geneva	TCC833

Supplementary Table 2: Accession numbers of the sequence downloaded from NCBI to construct the phylogenetic tree.

Sequence name	Accession number
Bdellovibrio bacteriovorus strain HD 127	AJ292760.1
Bdellovibrio sp. W strain ATCC 27047	AJ292518.1
Bdellovibrio bacteriovorus strain 109J	M61234.1
Bdellovibrio bacteriovorus strain HD100	NR_027553.1
Bdellovibrio bacteriovorus strain SSB218315	KT807464.1
Bdellovibrio exovorus strain JSS	EF687743.1
Bdellovibrio exovorus strain MPR11	MH230062.1
Bdellovibrio exovorus strain KL8	NR_115142.1
Vampirovibrio chlorellavorus strain ICPB 3707	NR_104911.1



Supplementary Figure 1: Gel photo showing amplification at ~500 pb when using the specific primer of Bdellovibrionaceae on T1 and A4 predators DNA extract. In addition amplification was observed at ~700 pb when using Bacteriovoracaceae specific primer on A4 predator DNA extract. However, no amplification is detected when using *Peredibacter* specific primer on T1 and A4 predators DNA extract.

Discussion

BALOs have been mainly studied and isolated in marine and terrestrial environments [4,28,29]. Hence, knowledge gaps for fresh waters are important, especially for (peri-alpine) lakes. In a previous work, we found that *Peredibacteracaceae* seem to be the most abundant BALO family in Lakes Geneva, Annecy and Bourget and these bacteria were mostly localized in surface [17]. In another study, we revealed that *Bdellovibrionaceae* reads were the highest in Lake Geneva by Ezzedine JA, et al.[45]. The different results clearly suggested that BALOs can be numerically and functionally important in Lake Geneva. Thus, isolating, characterizing and maintaining one or several BALOs in culture may help to determine their importance in this lake and beyond.

We were moderately successful since we only obtained one *Bdellovibrio sp*. representative despite an important effort to try to obtain several bacterial predators. We obtained it from a mixture of water sediment and biofilms from the shore. Thus, we do not know if we selected a benthic or a pelagic species, a free-living population or an attached group. It is noteworthy, however, that it has been reported that BALOs can be more present in surface biofilms and sediments than in the water column [30,31]. We also chose a location with natural clean water and far from any wastewater treatment pipes, and this may have reduced our success to isolate different populations. Indeed, some authors reported that BALOs are more concentrated in the waters at the outlet of wastewater treatment plants [32]. However, polluted waters have also been reported by Markelova NY [33,34] to drive BALOs into entering a bdelloplast state in order to survive. At last, while we tried to be not too limiting in terms of prey range, our selection remained relatively low (with only 10 different species or strains) and this may also have contributed to a low isolation success.

We obtained however very interesting results. Firstly, the only predators we could isolate were *Bdellovibrio*. The reason behind this result, specifically why this genus and not another BALO is difficult to explain since we used only one sample, from one location, and one date. On one hand, maybe the coastal environment of Lake Geneva at this period of time is richer with *Bdellovibrio* than in other BALOs. In another hand, *Bdellovibrio spp* could be the easiest BALOs to isolate since they dominate existing isolates. As optimal temperature for growing *Peredibacter* is between 20 and 30˚C [8], *Bacteriovorax* between 15 and 35˚C [35], and *Bdellovibrio* between 28 and 30˚C [4,15,36], our temperature incubation about 25˚C was unlikely to explain our result. The isolated *Bdellovibrio sp*. was characterized by small, numerous and distinct predation plaques, smaller but globally similar to those of *Bdellovibrio* cells growing on *Pseudomonas corrugata* [37]. In another hand, plaques were different in size and shape from *Bdellovibrio* growing on *E. coli*. Isolated [15] from Nigerian freshwater bodies. Such results are not surprising since [37] stated that both the shape and size of predation plaques depend on the nature of the predator which produces them. Since these predators belong to different environments, each one may have a different predation strategy or simply behave differently in different laboratory conditions.

Secondly, we found that the predator had a narrow host range with a clear preference for *Pseudomonas spp*. Indeed, the isolated *Bdellovibrio* seemed to be a specialist since predation plaques were mainly observed with *Pseudomonas*, i.e. P. *fluorescens* ATCC 13525, T1, A4 and A3 preys that were also *Pseudomonas sp*. species. This predation activity against *Pseudomonas sp* is interesting when one knows that different species or strains of this genus can carry enteric diseases for humans. Unlike Iebba V, et al. [10] and Pantanella F [38] who reported that *B. bacteriovorus* may predate on Gram-positive bacteria (in case of nutrient deficiency), such a situation was not observed here. In any case, further experiment should be carried to evaluate the impact of BALOs predation on a largest variety of prey. *Bdellovibrio* could also predate on *A. salmonicida salmonicida* (here a strain recently isolated in our lab from Arctic charr). *Bdellovibrio exovorus*, which is an *epibiotic* predator with no bdelloplast formation, was shown to grow on a few variety of preys including *Caulobacter crescentus, Acinetobacter johnsonii* RS3-17, A. *junii* RS3-41, *Delftia acidovorans* RS3-16, and *Aeromonas hydrophila* AS12a [39,40]. The appearance of predation plaques on *A. salmonicida salmonicida* SA28 reminds thus the result obtained with *A. hydrophila*. Such a result is promising since bacterial predators of bacteria could be used as bio agents controlling specifically some toxigenic bacterial strains, among which the ones that are multi-resistant to antibiotics, for instance in fish farming [11,41,42] reported that *B. bacteriovorus* H16 and *Halobacteriovorax* HBXCO1 predated *Vibrio* species that infect and kill shrimps (*Penaeus vannamei*) and bivalves. In addition [43] showed a correlation between the health status of domestic animals and the abundance of BALOs. The more BALOs are present, the less these animals were exposed to pathogens. Here, a new potential application is highlighted here for salmonids infected by Furunculosis that can cause a significant mortality in a variety of species, including salmons, and in the peri-alpine lakes the Arctic charr [44].

Conclusion

The isolation and characterization of a new obligate bacterial predator of bacteria highlights the necessity to interest to this functional group of bacteria and probably to other (non-obligate) predators to better understand the functioning of microbial ecosystems. As also proposed in a parallel study for Lake Geneva, we believe that BALOs constitute an important group of bacteria likely to play key roles in the structure, dynamics and diversity of the heterotrophic bacterial community, constituting thus an unseen elephant in the room [45]. In the future, new attempts of isolation will be required as well as the conduction of experiments to precisely define mortality rates induced by such predators.

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