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Abstract

Bdellovibrio bacteriovorus is a predator bacterial species found in the environment and within the human gut, able to attack Gram-negative prey. Cystic fibrosis (CF) is a genetic disease which usually presents lung colonization by Pseudomonas aeruginosa or Staphylococcus aureus biofilms. Here, we investigated the predatory behaviour of B. bacteriovorus against these two pathogenic species with: i) broth culture; ii) ‘static’ biofilms; iii) field emission scanning electron microscope (FESEM); iv) ‘flow’ biofilms; v) zymographic technique. We had the first evidence of B. bacteriovorus survival with a Gram-positive prey, revealing a direct cell-to-cell contact with S. aureus and a new ‘epibiotic’ foraging strategy imaged with FESEM. Mean attaching time of HD100 to S. aureus cells was 185s, while ‘static’ and ‘flow’ S. aureus biofilms were reduced by 74% (at 24h) and 46% (at 20h), respectively. Furthermore, zymograms showed a differential bacteriolytic activity exerted by the B. bacteriovorus lysates on P. aeruginosa and S. aureus. The dual foraging system against Gram-negative (periplasmic) and Gram-positive (epibiotic) prey could suggest the use of B. bacteriovorus as a ‘living antibiotic’ in CF, even if further studies are required to simulate its in vivo predatory behaviour.

Keywords

Bdellovibrio bacteriovorus, Staphylococcus aureus, Pseudomonas aeruginosa, biofilm, predation, Cystic fibrosis, FESEM

Introduction

Cystic Fibrosis is a lethal genetic disease (Davis et al., 1996; Lyczak et al., 2002) in which mutations in the CF transmembrane Conductance Regulator (CFTR) gene result in defective function and/or processing of the mutant protein CFTR (Zielenski and Tsui, 1995; Gadsby et al., 2006). Patients are prone to chronic, persistent and recurrent respiratory tract infections, with an exaggerated inflammatory response, leading to progressive respiratory deficiency (Boucher, 2002; Dakin et al., 2002; Rajan and Saiman, 2002). Even if the lung is usually inhabited by various bacterial species (Goddard et al., 2012), in CF disease only one or two pathogenic species prevail (Moore et al., 2005; Harrison, 2007; Sibley and Surette, 2011), usually the Gram-negative P. aeruginosa and the Gram-positive S. aureus. These two species are able to establish chronic infections through biofilm formation and resistance, leading to clinical
exacerbations (Lyczak et al., 2002; Rajan and Saiman, 2002). Due to the polymicrobial nature of healthy lung microbiota and, conversely, to the predominance of only one/two pathogenic species in CF (Moore et al., 2005), it was suggested how manipulating lung communities would be effective against chronic infections (Harrison, 2007). From literature it was reported that a bacterial predator, *Bdellovibrio bacteriovorus*, is capable to attack different Gram-negative bacterial genera (*Escherichia*, *Salmonella*, *Legionella*, *Pseudomonas*) and their pre-formed biofilms (Socke and Lambert, 2004; Kadouri and O'Toole, 2005). *B. bacteriovorus* is ubiquitous in the environment, where it’s alleged to act as an ‘ecological balancer species’ (Varon, 1981; Yair et al., 2003; Dwidar et al., 2012), and it was recently found in the human gut of all healthy individuals examined (Iebba et al., 2013). *B. bacteriovorus* has the natural ability to predate Gram-negative bacteria by invading their periplasmic space, where it undergoes a complex replication cycle culminating in prey killing and release of progeny (Rendulic et al., 2004; Lambert et al., 2006). Lytic action exerted by *B. bacteriovorus* can rapidly reduce prey populations, making this predatory species, or its lytic enzymes (Dori-Bachash et al., 2008; Lambert et al., 2010; Lerner et al., 2012), potential therapeutic candidates (Socke and Lambert, 2004; Dwidar et al., 2012). Research is still necessary to give insights into the predatory spectrum of *Bdellovibrio*. The present study aimed at evaluating the predatory activity of *B. bacteriovorus* strain HD100 on bacterial strains commonly isolated from CF patients, such as the Gram-negative *P. aeruginosa* and the Gram-positive *S. aureus*.

Materials and Methods

**Bacterial strains and cultivation.** The predator *Bdellovibrio bacteriovorus* strain HD100 (DSM No. 50701), was acquired by the German microorganisms collection DSMZ (Braunschweig, Germany), and arrived in our lab in a ‘double-layer agar plate’ containing diluted Nutrient-broth (NB) (1:10 dilution of NB amended with 3mM MgCl$_2$$\cdot$6H$_2$O and 2 mM CaCl$_2$$\cdot$2H$_2$O [pH 7.2]) and agar (0.6% upper layer) with enclosed prey cells of *P. aeruginosa* and *B. bacteriovorus* forming lysis plaque (Starr, 1975). Prey bacterial strains (*P. aeruginosa* and *S. aureus*) used in this study were recovered from glycerol stocks stored at -80°C, and were previously collected from sputa of two Cystic fibrosis patients with chronic mono-colonization attending the Cystic fibrosis center of Lazio Region, ‘Sapienza’ University of Rome. All strains were identified with the automated Vitek 2 system (Biomérieux, Marcy l'Etoile, France). Strains were first suspended in Tryptone Soya Broth (TSB), and, to verify the purity of the culture, 100 μL of overnight culture were
spread onto Tryptone Soya Agar (TSA) plates. Vitek2 assessed strain identification again. The predator strain *B. bacteriovorus* was grown as previously reported (Kadouri and O'Toole, 2005; Jurkevitch, 2006; Lambert and Sockett, 2008) in DNB minimal medium. Briefly, DNB growth medium contained: 0.8 g/L Bacto Nutrient Broth (NB) and 0.1 g/L yeast extract, with the separated addition of 0.45 µm-filtered 0.3 g/L of Casaminoacids, 0.5 g/L of CaCl$_2$·2H$_2$O and 0.6 g/L of MgCl$_2$·6H$_2$O. To obtain an enriched predator preparation to be used in predation assays, we modified the cultivation media doubling the concentration of NB from 0.8 g/L to 1.6 g/L, and the resulting medium was hereafter named ‘2X DNB’.

**Preparation of *B. bacteriovorus* suspension for predatory assays.** Two small pieces of agar were removed from a commercially available ‘double-layer agar plate’ of *B. bacteriovorus* preying on *P. aeruginosa*, as recommended by DSMZ instructions, and added to 60 mL of ‘2X DNB’. Incubation was performed at 30 °C under agitation (180 rpm) for 48h hours. Through microscopic observations at 100X magnification and the hanging drop technique, we were able to follow and measure every 2h the growth of *B. bacteriovorus*, discernible by a reduction in OD$_{600}$ turbidity (clear lysate). Upon reaching 48h, 30 mL of the clear lysate were 0.45 µm-filtered for three times in order to remove prey cells. One-hundred µL of the filtrate were plated on TSA agar plates, and incubated at 37 °C overnight to exclude carryover of the prey (*P. aeruginosa*). Finally, to have a three-times concentrated suspension of *B. bacteriovorus* and to remove the ‘2X DNB’ broth, which initial tests showed to interfere with prey biofilm (data not shown), the 0.45 µm filtrate was centrifuged at 10000g for 30 min and pellet was suspended in 10 mL of TSB. *B. bacteriovorus* suspension was prepared fresh each time for subsequent experiments.

**Predation assays on prey cultures.** One colony of prey (*P. aeruginosa* or *S. aureus*) was picked up from TSA plates and grown overnight in 20 mL of TSB at 37°C, 200 rpm. Bacterial culture was centrifuged (5000g, 15 min) and pellet suspended in pre-warmed TSB till reaching an OD$_{600}$=1. One-hundred µL of suspended culture were used to inoculate 60 mL of pre-warmed TSB, and incubated at 30°C with shaking (200 rpm). Bacterial growth was followed each hour with a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany), and at OD$_{600}$=1 the prey culture was split into three different 100 mL-flasks at equal volumes (20 mL): i) the first left as it is; ii) the second added with 2 mL of *B. bacteriovorus* suspension (see ‘*B. bacteriovorus* suspension for predatory assays’ paragraph); iii) the third added with 2 mL of *B. bacteriovorus* suspension 0.22 µm-filtered (for assessing the action of lytic enzymes eventually released into
the medium). Flasks were incubated at 30°C with shaking (200 rpm) into the same incubator/shaker GFL 3031 (MicroGlass Heim, Naples, Italy) and bacterial growth was measured every hour in two different ways: i) OD_{600} (BioPhotometer, Eppendorf, Hamburg, Germany); ii) bright field through the ‘hanging drop’ technique (optical microscope DM 5000-D, Leica Microsystems, Wetzlar, Germany). Prey levels (P. aeruginosa and S. aureus) were assessed plating every two hours, on TSA plates, 100 µL taken from the abovementioned first and second flasks, along with appropriate serial dilutions: colonies formed on plates after 17h at 37°C were automatically counted by means of the TotalLab TL120 software (Nonlinear Dynamics), ‘colony counting’ module. Predation assays in TSB broth were performed in triplicate: OD_{600} values and colony forming units (CFU) per mL were expressed as mean ± standard deviation of the mean. Graphs and statistical tests were done with Prism 5 software (GraphPad, La Jolla, California, USA).

Predatory activity of B. bacteriovorus on ‘static’ biofilms. Biofilms of P. aeruginosa and S. aureus were pre-formed on 48-well plates as already reported (Merritt et al., 2005). Briefly, 200 µL of prey overnight cultures diluted in TSB at OD_{600}=1 were used to inoculate the 48-well plate, followed by incubation at 37°C for 24h. Planktonic bacteria were removed by Phosphate Buffered Saline (PBS) washing. After washing, 200 µL of B. bacteriovorus preparation (see ‘Preparation of B. bacteriovorus for predatory assays’ paragraph) were added to ½ plate, while ¼ was added with 200 µL of 0.22 µm-filtered B. bacteriovorus preparation, and another ¼ was added with 200 µL of TSB (control). Plates were incubated at 37°C for additional 24h to allow B. bacteriovorus predation against prey biofilm. Then, all wells were washed with PBS for three times, then 100 µL of 1% crystal violet were added to each well and left in contact for 5 minutes. The dye in excess was eliminated by three washes with PBS, and the plate dried in a thermostat. 250 µL of 33% glacial acetic acid were added to each well and left in contact for 15 minutes. Subsequent OD_{570} readings were done with VMax® Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Wilcoxon Signed Rank test was employed to assess differences in biofilm amount, and a P value less than or equal to 0.05 was considered statistically significant. Graphs and statistical tests were done with Prism 5 software (GraphPad, La Jolla, California, USA).

Field-Emission Scanning Electron Microscopy (FESEM) of S. aureus biofilms. FESEM technique was employed to visualize S. aureus biofilm before and after a 24h-challenge of B. bacteriovorus. As a substrate for FESEM microscopy,
double-sided polished silicon wafers were reduced into many thin pieces by using a diamond cutter along natural crystallographic lines. Pieces of silicon wafers (hereafter mentioned as "wafers") were sterilized at 121°C for 15 minutes and then aseptically used as a substrate for bacterial biofilm growth. Wafers were separately immersed in 5 mL of $10^6$ CFU/mL of P. aeruginosa or S. aureus broth cultures respectively. After 24 hours of incubation at 37°C, 180 rpm, the supernatant and the planktonic preys were gently removed and replaced by B. bacteriovorus suspension (see ‘B. bacteriovorus suspension for predatory assays’ paragraph). The predator was left in presence of the different prey-biofilms for additional 24 hours. After 24 hours of contact between predator and preys, all wafers were gently rinsed by sterile saline solution (PBS) and immediately immersed in a fixative solution consisting of glutaraldehyde and PBS (2%-v/v). At this step, all samples were maintained in the dark at 25°C for 1 hour, then washed three times in PBS and immediately after immersed in osmium tetroxide 1% aqueous solution (cat# 75632-10ML, Sigma-Aldrich, St. Louis, MO, USA) and kept in the dark for 24 hours at 4°C. To obtain the necessary dehydration, after three PBS washes, wafers were immersed for 10 min each time in subsequent ethanol solutions increasing progressively in concentration from 30% to 99% (30%-50%-70%-80%-90%-99%). After drying at room temperature, samples were observed by FESEM microscopy. Identical preparation procedure has been adopted for all the control samples without predator or prey. FESEM images were captured using a Zeiss Auriga 405 (Carl Zeiss AG, Oberkochen, Germany). Different extraction voltages (5-10 keV) and a specific working distance (around 4 mm) were employed to find an affordable compromise among avoiding the radiation damage and enhancing the contrast between bacterial cells and siliceous substrate. FESEM images were captured without any additional surface coating of the sample, in order to evaluate the actual surface morphology of bacterial cells.

**Predatory activity of B. bacteriovorus on ‘flow’ biofilms.** BioFlux system (Fluxion Biosciences, South San Francisco, CA) was used to visualize and follow in a time-dependent manner the development and the subsequent predatory activity of B. bacteriovorus. Microfluidic plates used were BioFlux 200 WPM 24 well plates 0-20 Dynes (cat. #910-0009), and instrumental setup was set following manufacturer’s instructions and literature (Benoit et al., 2010). Two wells were inoculated with 800 $\mu$L of a suspension of the bacterial prey (P. aeruginosa or S. aureus) in TSB (OD$_{600}$=1), to allow biofilm formation within each microfluidic channel. An initial pressure of 2 dyne/cm$^2$ for 4s was applied in the upper side of the inlet allowing the initial flow of bacterial inoculum. Flow was then stopped for 30 min to allow the
initial stages of biofilm formation, then 2 mL of pre-warmed TSB medium were added, and flow started again at 0.5 dyne/cm$^2$, 37°C for 24h. Pre-formed biofilm was then added with 2 mL of *B. bacteriovorus* preparation (upper channel), while the control microfluidic well (lower channel) was added with 2 mL of TSB. When the *B. bacteriovorus* preparation and TSB were added, the flow was started again at 0.5 dyne/cm$^2$, 37°C for 24h. Still frames were taken every minute during the entire procedure (biofilm formation, predation) with a QICAM 12-bit camera (QImaging, Surrey, Canada) at a resolution of 1392 x 1040 pixels (4.65µm x 4.65µm pixel size) and 12-bit of image depth. By means of a specific software (ImageJ, National Institutes of Health, USA), measurements of grey intensity were made for each frame in a 0-255 scale, where 0 is black and 255 is white. Due to the different bacterial cell sizes of *P. aeruginosa* (rod-shaped, mean length and width, 3.0 µm and 0.8 µm, respectively), *S. aureus* (spherical, mean diameter 1.0 µm), and *B. bacteriovorus* (rod-shaped, mean length and width, 1.2 µm and 0.4 µm, respectively), the gray intensity value given by *B. bacteriovorus* alone was resulted to be 240.7±1.8 (with a component of around 4% on the total mean gray intensity), and such a value was subtracted from each frame. Experiments were performed in triplicate by the same operator, while gray intensity measures were done in triplicate on each still frame from three different operators.

Wilcoxon Signed Rank test was employed to assess differences in mean grey intensity on each movie frame, and a $P$ value less than or equal to 0.05 was considered statistically significant. Graphs and statistical tests were done with Prism 5 software (GraphPad, La Jolla, California, USA).

**Zymographic technique.** The zymographic is an electrophoretic technique which includes a substrate copolymerized in a polyacrylamide gel useful for the detection of enzyme activity. Zymographic technique included the use of fresh lysates of *B. bacteriovorus* loaded on polyacrylamide gel copolymerized with prey cells, and was used as stated in literature (Audy et al., 1989; Lantz and Ciborowski, 1994) with minor modifications. Briefly, polyacrylamide mini-gels contained 10% polyacrylamide, Tris-HCl (pH 8.8), sodium-dodecyl-sulphate (SDS) 0.1 %, and 15% of prey cell suspension at OD$_{600}$=20. This huge bacterial density was necessary in order to hinder the action exerted by SDS, especially for *P. aeruginosa*. The *B. bacteriovorus* preparation was sonicated 10 times with a 300VT ultrasonic homogenizer (Biologics Inc., Manassas, Virginia, USA) to disrupt predator cells and bring enzymes in solution. Then, an equal volume of sample buffer (Bromophenol blue in 20 % glycerol in 2:1 ratio) was added to the sonicated *B. bacteriovorus*, and loaded. The run was set with a constant voltage (120 volts for 1 hour). Renaturation of proteins in
polyacrylamide gel was obtained by washing the gel for three hours, with changes of the solution every 30 minutes, in renaturing buffer (50 mM Na-phosphate buffer at pH 7 with 1% Triton X-100). After the three hours of washings, the gel was left in renaturing buffer for additional 24h at 37°C. Gel was stained with 0.25% (w/w) Coomassie Brilliant Blue R-250. Clear band on opalescent gel matrix indicated bacteriolytic activities. Zymograms were analysed by TotalLab TL120 software (Nonlinear Dynamics) and molecular weights of unknown lytic bands were inferred by a logarithmic interpolation of marker bands (unstained SDS-PAGE low range standard, Bio-Rad cat #161-0304).

Results

**B. bacteriovorus HD100 predation on P. aeruginosa and S. aureus in broth culture.** A first assessment of *B. bacteriovorus* predatory activity was done on prey species (*P. aeruginosa* and *S. aureus*) in TSB broth. Predation curve of *B. bacteriovorus* on *P. aeruginosa* reached stabilization at OD$_{600}$=0.30 (around 7.5×10$^7$ CFU/mL) after 10h, and this turbidity level was stable till the end of assay (Fig. 1, panel A). Interestingly, we observed predation of *B. bacteriovorus* against the Gram-positive *S. aureus* (Fig. 1, panel B), and in this case the predation curve stabilized itself at OD$_{600}$=0.38 (around 2.2×10$^8$ CFU/mL) after 7h. A slight reduction in OD$_{600}$ measurements was visible when 0.22 µm-filtered suspension of *B. bacteriovorus* was added to prey cultures, but this reduction was not significant in both prey species (data not shown). Due to the unexpected observation of *B. bacteriovorus* HD100 predation on *S. aureus* in broth culture, we decided to see its actual predatory behaviour utilizing the ‘hanging drop’ technique and making a movie with an optical microscope (bright field) at 100X magnification (Supplementary Video S1). In Fig. 2 are reported eight frames from Movie S1 depicting four distinct phases of *B. bacteriovorus* predation on *S. aureus*: sensing (a-c), attacking (d, e), breaching (f-h), and detaching (i, j). As shown in supplemental movie (Supplementary Video S1) and Fig. 2, while *B. bacteriovorus* swims at high speed from a lower depth level (a), it surpasses *S. aureus* cells (b), swiftly turns back (c, d), and hooks one of them (e), exhibiting a bending of its cellular body (e). After twisting for around 11s trying to breach its prey (f-h), *B. bacteriovorus* detaches from Gram-positive prey cell (i) and moves away (j). Time spent by *B. bacteriovorus* during the breaching phase on *S. aureus* (from attacking till detaching) was computed for different predator/prey couples, and resulted to be 185.5±25.7 seconds (mean ± standard deviation of the mean). Interestingly, predators who spent more time attached to *S. aureus* cells trying to breach them, in most cases were steady, with no
detectable twisting (Fig. 2 and Supplementary Video S1, bright blue prey; Supplementary Video S2). Such an
observation shows an unconventional predatory behaviour of *B. bacteriovorus* HD100, which usually holds on in
twisting upon attaching Gram-negative prey to breach and penetrate into periplasmic space (Medina et al., 2008). No
bdelloplast formation was observed throughout the entire period of observation (12h).

**B. bacteriovorus HD100 reduces ‘static’ prey biofilms.** Twenty-four hours-old biofilms of *P. aeruginosa* and *S.
aureus* strains were challenged for 24h with *B. bacteriovorus* predation, and quantified by crystal violet staining
followed by OD$_{570}$ readings. *P. aeruginosa* biofilm was significantly reduced of 76%, while *S. aureus* biofilm was
reduced of 74% (*P*<0.0001, Fig. 3). A prey preference of *B. bacteriovorus* for *P. aeruginosa* was visible, whose biofilm
was reduced 9.3% more than *S. aureus* (*P*=0.0437) (Fig. 3). Even if significantly different, the similar biofilm amount
of the two challenged species could indicate a similar predatory level exerted by *B. bacteriovorus*, irrespective from
presence or absence of periplasmic replication. Biofilm amount in both prey species was not significantly affected by
0.22 µm-filtered suspension of *B. bacteriovorus* HD100, in which lytic enzymes would be eventually released (*P.
aeruginosa*, *P*=0.8667; *S. aureus*, *P*=0.8596), signifying that a specific predator-prey interaction is needed to challenge
pre-formed biofilms.

**B. bacteriovorus HD100 shows an epibiotic predation on *S. aureus*.** Results obtained with ‘hanging drop’ technique
(Fig. 2, Supplementary Video S1, Supplementary Video S2) allowed us to see the predatory behaviour of HD100
towards *S. aureus*, thus, the subsequent step was to find out a better visualization of *B. bacteriovorus* attacking its
Gram-positive prey. To this end, we employed FESEM technique on pre-formed biofilm of *S. aureus* before and after a
challenge with *B. bacteriovorus*. Biofilm of *S. aureus* was grown on a siliceous slice for 24h in TSB, then the medium
was carefully removed and replaced with 5 mL of HD100 suspension (see ‘Preparation of *B. bacteriovorus* suspension
for predatory assays’) allowing biofilm challenge for another 24h. In Figure 4 are shown the results. A 24h-old *S.
aureus* biofilm was established (Fig. 4, panel A), but after 24h the biofilm was thoroughly removed, leaving few prey
cells alive surrounded by many debris (Fig. 4, panel B) with a single *B. bacteriovorus* approaching through the long (4
µm) flagellum (Fig. 4, panel B, inset). A higher FESEM magnification showed two *B. bacteriovorus* predators attacking
two different *S. aureus* cells in an epibiotic manner, in which a direct contact (Fig. 4, panel C, white arrows) with prey
cell is maintained throughout the entire predation process, from the initial attack phase (Fig. 4, panel C, left) till the late
attack phase (Fig. 4, panel C, right). Interestingly, *B. bacteriovorus* HD100 was able to attack its prey with the anterior part, leaving the flagellum free to move, maybe aiding in pushing the predator body (Fig. 4, panel C, and Fig. 2). This new behaviour (epibiotic predation) against *S. aureus* is significantly different from what usually *B. bacteriovorus* HD100 does against Gram-negative prey (periplasmic predation).

**B. bacteriovorus HD100 reduces ‘flow’ prey biofilms.** CF patients’ lungs show a somewhat impaired mucociliary clearance (MCC), even if precise *in vivo* measurements lead to controversial results depending on upper/lower lobes involvement (Tarran et al., 2005): such a diminished MCC allows bacterial establishment and permanence in biofilms (Mall et al., 2004;Donaldson et al., 2007). We then hypothesized that a physiologic shear-stress of 0.5 dyne/cm², as found in periciliary liquid (PCL) of healthy and CF individuals (Regnis et al., 1994;McShane et al., 2004;Tarran et al., 2005), could affect *B. bacteriovorus* predation of prey biofilms. To test such hypothesis, evaluation of *B. bacteriovorus* predatory activity on preformed biofilms was done in dynamic settings, by means of BioFlux microfluidics apparatus. The first significant reduction of *P. aeruginosa* biofilm (-31%) was visible after 9h upon *B. bacteriovorus* challenge (*P*=0.0175), while after 20h it was reduced by 38% (Fig. 5, panel A). Interestingly, after a fixed decreasing rate (from 6h to 11h) of *P. aeruginosa* biofilm amount (-6.3% / h), an oscillation with a period $T_p = 4h$ was visible starting around at 12h, reminiscent the predator/prey Lotka-Volterra model (Varon and Zeigler, 1978). Such an oscillation was not due to the BioFlux peristaltic pump, which showed instead a period $T_{pump} = 0.7h$. A significant reduction of *S. aureus* biofilm (-33%) was visible after 14 hours of contact with the predator (*P*=0.0380), and after 20h it was reduced by 46% (Fig. 5, panel B). From 6h to 8.5h a fixed decreasing rate of *S. aureus* biofilm amount (-17.5% / h) was observed, but no oscillations were visible thereafter, maybe owing to the absence of a host-dependent replicative cycle of *B. bacteriovorus*. Strikingly, as observed in static conditions, after 20h of predation in flow settings both biofilm amounts reached a similar level of grey density (169.1±18.0 for *P. aeruginosa*, 170.2±7.4 for *S. aureus*, Fig. 5), with no significant difference (*P*=0.9269).

**B. bacteriovorus HD100 uses different non-released degradative enzymes to lyse *P. aeruginosa* and *S. aureus.** Results obtained with the ‘hanging drop’ technique (Fig. 2, Supplementary Video S1, Supplementary Video S2), ‘static’ biofilms (Fig. 3), and FESEM images (Fig. 4), suggested that a direct interaction of *B. bacteriovorus* HD100 is needed to ensure predation, and that its degradative enzymes eventually released into the medium do not exert a role in lysing
neither free prey nor their pre-formed biofilms. Zymographic technique was used to find non-released bacteriolytic enzymes of *B. bacteriovorus* against cellular substrates of *S. aureus* or *P. aeruginosa*. Zymograms densitometry profiles were divided in 312 pixels and molecular weights of lytic bands (expressed as kDa) were inferred by a logarithmic interpolation of the marker (Log curve, $y = 111.6e^{-0.009371x} + 14.66$, $R^2=1$). A differential bacteriolytic activity against *P. aeruginosa* and *S. aureus* was observed (Fig. 6), with a unique clear band at around 22 kDa for the Gram-negative prey, and three different lytic bands at 22 kDa, 44 kDa, and 67 kDa for the Gram-positive prey (Fig. 6). SDS added to polyacrylamide gels allowed us to be quite confident in assessing the molecular weights of lytic bands (Lantz and Ciborowski, 1994), especially for *S. aureus*, even if a proper characterization of enzymes involved will be done in forthcoming experiments.

**Discussion**

Since its discovery in 1963 (Stolp and Starr, 1963), *Bdellovibrio* was recognized as a predator species capable to prey Gram-negative bacteria, maybe exerting a role in regulating microbial communities (Varon, 1981;Yair et al., 2003;Dwidar et al., 2012). Previous reports suggested to use *B. bacteriovorus*, or similar predatory species, as a biological agent against bacterial pathogens, and found it to be actually useful in challenging such infections (Chu and Zhu, 2010;Atterbury et al., 2011;Dashiff et al., 2011;Dashiff and Kadouri, 2011). CF is usually accompanied to an exaggerated bacterial colonization of the lower respiratory tract (Moore et al., 2005;Harrison, 2007;Sibley and Surette, 2011), in which *P. aeruginosa* and *S. aureus* trigger an inflammatory response that leads to progressive clinical exacerbation (Lyczak et al., 2002;Rajan and Saiman, 2002). The objective of the present study was to evaluate the predatory activity of *Bdellovibrio bacteriovorus* strain HD100 against two bacterial pathogens usually isolated from CF patients’ sputa, *P. aeruginosa* and *S. aureus*. To this end, the activity of *B. bacteriovorus* was evaluated versus cells prey in culture and on preformed biofilms, both in ‘static’ and ‘flow’ conditions. Bioflux results on *P. aeruginosa* revealed the instauration of a Lotka-Volterra predator/prey oscillation after 12h of *B. bacteriovorus* attack: further experiments with fluorescent dyes will be addressed to ascertain the predator/prey ratio at the origin of this oscillation. Interestingly, the reduction rate of both Gram-negative and Gram-positive biofilms started at 6h upon *B. bacteriovorus* challenge, and the final biofilm amount settled at around -38% in both prey species (Fig. 5). We cannot exclude that *S.
... biofilm was still growing while *P. aeruginosa* biofilm was at its plateau (Fig. 5), but we found that such a biofilm development and subsequent predation was reproducible under simulated physiologic shear-stress (‘flow’ condition) of 0.5 dyne/cm² for both preys. ‘Flow’ conditions diminished predatory activity of *B. bacteriovorus* by 38% (*P. aeruginosa*) and 28% (*S. aureus*) compared to ‘static’ conditions, and this observation could be useful if an *in vivo* treatment would be employed for *B. bacteriovorus* in CF disease. In this view, further studies are needed to validate the effect of shear-stress in PCL on *B. bacteriovorus* predation by using CF animal models.

We found that *B. bacteriovorus* strain HD100 could survive for a prolonged time (at least 20h) exclusively preying a Gram-positive species, here a *S. aureus* CF isolate. Previous papers reported how some bacterial predators genera, namely *Cupriavidus* (Casida, 1988), *Lysobacter* (Bonner et al., 1988), and *Myxococcus* (Shimkets, 1990), could prey Gram-positive preys in an epibiotic manner, but such an evidence was never reported for *B. bacteriovorus* species, nor *Bdellovibrio* genus, which instead requires an intra-periplasmic replication for its survival. Only a single study reported a similar epibiotic predation for a different *Bdellovibrio* strain, JSS, which is able to predate only the Gram-negative *Caulobacter crescentus* (Chanyi et al., 2013;Koval et al., 2013;Pasternak et al., 2014), and only another study used Gram-positive species as decoys for *B. bacteriovorus* HD100 in a triple-planktonic predation system (Hobley et al., 2006). Unlike our results (Fig. 3), a recent study reported how supernatant of a host-independent mutant of *B. bacteriovorus* HD100 is able to reduce *S. aureus* biofilm by 75%, but predator itself is not able to do that (Monnappa et al., 2014). Monnappa and colleagues had this evidence on three different *S. aureus* strains, but no direct attack of *B. bacteriovorus* on *S. aureus* was visible. In our study, through FE-SEM, we had the first evidence of predation and survival of strain HD100 preying exclusively a Gram-positive species, with an attack-phase and an epibiotic predation (Fig. 2 and Fig. 4), leading to a diminution of *S. aureus* biofilm of 74% (Fig. 3). Morphological changes in the predator/prey ‘region of contact’ are currently under investigation, by means of transmission electron microscopy (TEM) and scanning-transmission electron microscopy (STEM) in our lab. Once depicted by TEM and STEM the intimate contact among *B. bacteriovorus* and *S. aureus*, the forthcoming step will be to study the underlying molecular mechanisms of *S. aureus* killing: due to the short mean predator/prey contact of 185s, it will be interesting to test if cell wall hydrolysis or protein synthesis interference, as previously observed for *E. coli* (Varon et al., 1969), could be responsible. One could also consider that *B. bacteriovorus* predation on *S. aureus* would be prey strain-dependent, or it...
would be different if an axenic host-independent (HI) \textit{B. bacteriovorus} is used (Monnappa et al., 2014). In our study we decided to see the actual predatory behaviour of \textit{B. bacteriovorus} strain HD100 against \textit{S. aureus}, thus, an axenic host-independent (HI) variant was not used. We thus evidenced a specific interaction of HD100 with \textit{S. aureus} cellular surface (Fig. 4, panel C, white arrows), in which the destruction of \textit{S. aureus} cell seemed to occur through the rupture of its membrane and exudation of the cell content (Fig. 4, panel C). We also found that \textit{B. bacteriovorus} HD100 was always attached epibiotically to \textit{S. aureus} from the initial to the latter phase of prey destruction. It is conceivable that strain HD100 could use a specific repertoire of bacteriolytic enzymes to rupture \textit{S. aureus} membrane to start exudation.

Taking into account the \textit{B. bacteriovorus} HD100 annotated genome (Rendulic et al., 2004), it is noteworthy how this strain owns a huge repertoire of hydrolytic enzymes, such as proteases, glycanases, and DNases. Interestingly, \textit{S. aureus} zymogram showed three lytic bands, at 22 kDa, 44 kDa and 67 kDa, among which the last two may represent an additional bacteriolytic activity compared to \textit{P. aeruginosa} zymogram. Further studies are required to understand the mechanism of interaction among \textit{B. bacteriovorus} and Gram-positive species, such as \textit{S. aureus}, along with characterization of lytic enzymes involved and their mode of delivery.

Due to the spread of resistance among clinical pathogens, it was suggested how \textit{B. bacteriovorus} could be used as a ‘living-antibiotic’ (Rendulic et al., 2004; Sockett and Lambert, 2004). It is noteworthy that the dual foraging strategy exhibited by \textit{B. bacteriovorus} on \textit{S. aureus} (epibiotic) and \textit{P. aeruginosa} (periplasmic), would be a favourable condition to actually significantly reduce bacterial loads and established biofilms both in mono- and bi-colonized CF patients, rather than disperse them. Dispersal of alive pathogen bacterial cells, as seen by using specific \textit{B. bacteriovorus}-derived proteases (Monnappa et al., 2014), could be detrimental within an inflamed lung, leaving planktonic cells free to colonize other niches, thus exacerbating CF symptoms. \textit{B. bacteriovorus} was found to be unable to infect mammalian cells (Sockett and Lambert, 2004), and was found in the healthy human gut (Iebba et al., 2013), making it a good candidate to treat infections \textit{in vivo}, and results obtained in this study point towards its use in the treatment of CF pulmonary infections.


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Author Contributions

Additional Information

Authors declare no competing financial interests.

Legends to illustrations

Fig. 1. *B. bacteriovorus* predation on broth cultures of *P. aeruginosa* and *S. aureus*. Prey species (*P. aeruginosa*, red, panel A; *S. aureus*, blue, panel B) were grown in TSB till OD$_{600}$=1, then 2 mL of *B. bacteriovorus* suspension were added to 20 mL of culture. Treated and control flasks were in the same incubator at 37°C and 200 rpm. OD$_{600}$ measurements were done in triplicate every hour, while CFU/mL counts were done plating 100 µL of culture (along with serial dilutions) onto TSA plates, leaving colonies to grow overnight at 37°C.

Fig. 2. *B. bacteriovorus* predation on two *S. aureus* prey cells. Frames (a-j) from Supplementary Video S1 (bright field, 100X magnification) depicting predation phases of *B. bacteriovorus* on two *S. aureus* cells. Time (t) is expressed as ‘seconds:frames’, where each second is made of 25 frames. A single *B. bacteriovorus* enters from the left, attacks its prey (right, dark blue) and after around 12s moves away. A second *B. bacteriovorus* is already attached to its prey (left, bright blue), perpendicularly to the frame level during the entire Supplementary Video S1.

Fig. 3. Predation of *B. bacteriovorus* on ‘static’ prey biofilm. Biofilm of *P. aeruginosa* (Ps, red) and *S. aureus* (St, blue) were grown on 48-well plates for 24h, and challenged with *B. bacteriovorus* for additional 24h. After crystal-violet staining, OD$_{570nm}$ was used to measure biofilm amount, and Wilcoxon Signed Rank test was used to assess statistical differences.

Fig. 4. Predation of *B. bacteriovorus* on ‘static’ biofilm of *S. aureus*. SEM images of *S. aureus* biofilm (panel A, 30000X) grown for 24h on a silicon plate, and after 24h of *B. bacteriovorus* HD100 predation (panel B, 20000X). A free *Bdellovibrio* is visible with its long polar flagellum (estimated length, 4 µm) (arrow, panel B, inset, 50000X).
higher SEM magnification (88830X) shows the initial attack phase of HD100 on a prey cell (panel C, left) and the late attack phase, with a destroyed \textit{S. aureus} cell (panel C, right). White arrows in panel C show the direct interaction of \textit{B. bacteriovorus} HD100 with \textit{S. aureus}.

**Fig. 5. Predation of \textit{B. bacteriovorus} on ‘flow’ prey biofilm.** Biofilms of \textit{P. aeruginosa} (panel A, red) and \textit{S. aureus} (panel B, blue) were grown in 24-well microfluidic plates (BioFlux), then challenged with \textit{B. bacteriovorus} suspension for 24h. Biofilm amount was expressed as mean grey intensity (from 255-white to 0-black) of each movie frame, and statistical differences were assessed by Wilcoxon Signed Rank test.

**Fig. 6. Zymograms.** \textit{P. aeruginosa} e \textit{S. aureus} were embedded into different polyacrylamide gels and wells were loaded with sonicated \textit{B. bacteriovorus}. Species-specific lytic bands (white) were visible in correspondence of \textit{B. bacteriovorus}-derived lytic enzymes, after blue coomassie staining. Densitometry profiles (green) were added to appreciate the differential bacteriolytic activity exerted by \textit{B. bacteriovorus} on prey cells. M, marker (unstained SDS-PAGE low range standard, Bio-Rad cat #161-0304).
Figure 3.

Biofilm amount (OD$_{570}$)

- **Ps**
- **Ps + Bd 0.22 μm**
- **Ps + Bd 0.45 μm**
- **St**
- **St + Bd 0.22 μm**
- **St + Bd 0.45 μm**

- **P < 0.0001** for **Ps + Bd 0.22 μm** compared to **Ps**
- **P < 0.0001** for **St + Bd 0.22 μm** compared to **St**
Figure 5. TIF

A

Biofilm amount (inverted grey intensity)

Time (h)

Flow start

P = 0.0175

Ps

Ps + Bd

B

Biofilm amount (inverted grey intensity)

Time (h)

Flow start

P = 0.0380

St

St + Bd
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