

Determination of the Predatory Capability of *Bdellovibrio bacteriovorus* HD100

Cristina Herencias¹, M. Auxiliadora Prieto¹ and Virginia Martínez^{1, 2, *}

¹Environmental Biology Department, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain;

²Present address: Evolva Biotech A/S, Copenhagen, Denmark

*For correspondence: virginiaml83@gmail.com

[Abstract] *Bdellovibrio bacteriovorus* HD100 is an obligate predator that preys upon a wide variety of Gram negative bacteria. The biphasic growth cycle of *Bdellovibrio* includes a free-swimming attack phase and an intraperiplasmic growth phase, where the predator replicates its DNA and grows using the prey as a source of nutrients, finally dividing into individual cells (Sockett, 2009). Due to its obligatory predatory lifestyle, manipulation of *Bdellovibrio* requires two-member culturing techniques using selected prey microorganisms (Lambert *et al.*, 2003). In this protocol, we describe a detailed workflow to grow and quantify *B. bacteriovorus* HD100 and its predatory ability, to easily carry out these laborious and time-consuming techniques.

Keywords: *Bdellovibrio bacteriovorus*, Predatory bacteria, Predatory quantification

[Background] In the last years, *Bdellovibrio* has attracted the interest of the scientific community and several applications have been developed, such as evolution studies (Davidov and Jurkevitch, 2009), identification of new biocatalysts (Martínez *et al.*, 2012), therapeutic applications (Atterbury *et al.*, 2011), or biotechnological applications using *Bdellovibrio* as a lytic agent for the recovery of value added intracellular bioproducts (Martínez *et al.*, 2016). Due to the growing interest in *Bdellovibrio*, different indirect methods to quantify this predatory bacterium have been developed (Mahmoud *et al.*, 2007; Lambert and Sockett, 2008; Van Essche *et al.*, 2009). However, direct quantification of *Bdellovibrio* via double-layer method is still necessary to thoroughly characterize *Bdellovibrio* predatory capability. Here, we describe a well-established, reliable, and broadly used method that allows *Bdellovibrio* cell number quantification in predatory co-cultures.

Materials and Reagents

1. 0.45 µm sterilization filter (Sartorius, catalog number: 16555-K)
2. 0.22 µm sterilization filter (Sartorius, catalog number: 16532-K)
3. 10-ml glass test tubes (Fisher Scientific, catalog number: 15175134)
4. Glass microscope slides (76 x 26 mm) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 10143562BEF) (see Note 1)
5. Glass microscope coverslips (22 x 22 mm) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 3306)
6. 10 ml syringe (BD, catalog number: 307736)

7. *Bdellovibrio bacteriovorus* HD100 (ATCC, catalog number: 15356) (Stolp and Starr, 1963)
8. *Pseudomonas putida* KT2440 (ATCC, catalog number: 47054) (Nelson *et al.*, 2002)
9. Glycerol (EMD Millipore, catalog number: 104094)
10. Bacto tryptone (BD, Bacto™, catalog number: 211705)
11. Yeast extract (Conda, catalog number: 1702)
12. Sodium chloride (NaCl) (EMD Millipore, catalog number: 106404)
13. Sodium hydroxide (NaOH) pellets for analysis (EMD Millipore, catalog number: 106498)
14. Agar (BD, Bacto™, catalog number: 214010)
15. Nutrient broth (NB) (BD, Difco™, catalog number: 234000)
16. Calcium chloride dihydrate (CaCl₂·2H₂O) (EMD Millipore, catalog number: 102382)
17. Magnesium chloride hexahydrate (MgCl₂·6H₂O) (EMD Millipore, catalog number: 105833)
18. HEPES buffer (Sigma-Aldrich, catalog number: H3375)
19. Lysogeny broth (LB) medium (1 L, pH 7.5) (see Recipes)
20. LB, 1.5% (w/v) agar (see Recipes)
21. NB medium (1 L) (see Recipes)
22. CaCl₂ and MgCl₂ salts (see Recipes)
23. Diluted nutrient broth (DNB) medium (1 L, pH 7.4) (see Recipes)
24. DNB, 0.7% (w/v) agar (see Recipes)
25. DNB, 1.5% (w/v) agar (see Recipes)
26. HEPES buffer (see Recipes)

Equipment

1. Centrifuge (Eppendorf, model: 5810 R)
2. 100 ml flasks
3. 30 °C chamber (JP Select, catalog number: 001257)
4. 30 °C shaking incubator (250 rpm) (Eppendorf, New Brunswick™, model: Innova® 44)
5. Water bath (JP Select, catalog number: 6000138)
6. Phase contrast microscopy (Nikon Instruments, model: OPTIFHOT-2) (Note 1)
7. Spectrophotometer (Shimadzu, model: UV-260)
8. Leica DFC345 FX camera (Leica Microsystems, model: DFC345 FX)
9. Autoclave

Procedure

- A. Culture of *Bdellovibrio* preying upon a prey (Figures 1A and 1B)
 1. Preparation of prey cell suspension (Figure 1A)

- a. In this example, *P. putida* KT2440 is used as prey. To obtain cell suspensions, grow the prey in NB medium at 30 °C and 250 rpm for 16 h (see Note 2), centrifuge (30 min, 4000 x g, 4 °C) and resuspend to OD₆₀₀ = 10 in HEPES buffer (see Note 3).
 - b. Prey cells can be stored at 4 °C for up to 2 weeks. Immediately prior to use, dilute the cells to OD₆₀₀ = 1.
2. Preparation of the preinocule of *Bdellovibrio* (two-step cultivation) (Figure 1B)
 - a. Recover *Bdellovibrio* from glycerol stocks stored at -80 °C (see Note 4) by adding 50 µl directly to 10 ml of prey cell suspension prepared in DNB medium at OD₆₀₀ = 1. Incubate at 30 °C and 250 rpm.
 - b. After 24 h of predation, transfer 100-300 µl of the co-culture to 10 ml of prey suspension prepared in HEPES buffer at OD₆₀₀ = 1 (predator-prey ratio of 1:10). Incubate at 30 °C and 250 rpm for 24 h.

Note: Set up the co-cultures by adding 10 ml of suspension to 100 ml flasks.
 3. Isolation of *Bdellovibrio* cells (Figure 1B). After predation, filter co-cultures twice through a 0.45 µm filter to recover *B. bacteriovorus*.

Note: Use a new filter for the second filtration step.
 4. Set up the co-cultures of interest: transfer 100-300 µl of *Bdellovibrio* cells to 10 ml of prey suspension prepared in HEPES buffer at OD₆₀₀ = 1 (predator-prey ratio of 1:10). Incubate at 30 °C and 250 rpm for 24 h.

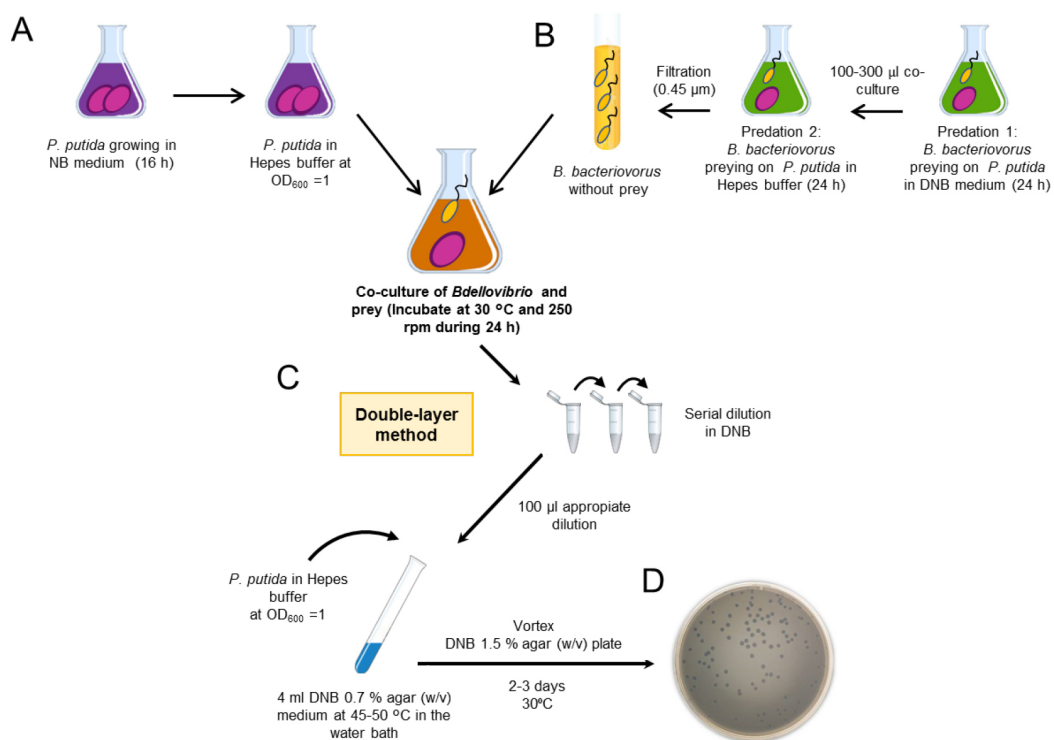


Figure 1. Workflow to set up *Bdellovibrio* co-cultures. A. Preparation of the prey cell suspension (here, *P. putida* KT2440). B. Preparation of *Bdellovibrio* cells. Two-step cultivation

of *Bdellovibrio* is needed to obtain the predatory cells for the experiment. C. Double layer method to quantify the cell number of *B. bacteriovorus* HD100. D. Development of *B. bacteriovorus* HD100 on a lawn of prey on DNB agar plates after 2-3 days of incubation at 30 °C. *Bdellovibrio* cell number can be quantified as plaque-forming-units (pfu/ml).

B. Calculation of the viability of *Bdellovibrio* and prey cells (Figure 1C)

Make serial dilutions of the co-cultures from 10^{-1} to 10^{-7} (see Note 5) in DNB medium.

1. Determine *Bdellovibrio* viability using the double layer method (see Figure 1C) (Lambert *et al.*, 2003):
 - a. Add 4 ml of DNB 0.7% (w/v) agar into a glass tube and keep at 45-50 °C in a water bath (see Note 6).
 - b. Add 0.5 ml of prey cell suspension (*P. putida* KT2440 prepared at $OD_{600} = 10$ in HEPES buffer; see Figure 1A).
 - c. Add 0.1 ml of the appropriate dilution from the co-cultures.
 - d. Vortex the tube gently.
 - e. Rapidly, pour the mixture onto a DNB 1.5 % (w/v) agar plate.
 - f. Incubate the plates for 2-3 days at 30 °C. *Bdellovibrio* growth is monitored as plaque-forming units per milliliter (pfu/ml) developing on a lawn of the prey (Figure 1D).
2. Calculation of prey cell viability
Place 10 µl of each dilution on LB agar plates (see Note 5) and incubate them at 30 °C. Prey cells are counted as colony-forming units per milliliter (cfu/ml).

Data analysis

A representative graph of the predatory activity of *Bdellovibrio* is shown below (Figure 2). Predator and prey cell number at the beginning of the experiment (0 h) and after 24 h of predation upon *P. putida* KT2440 are represented.

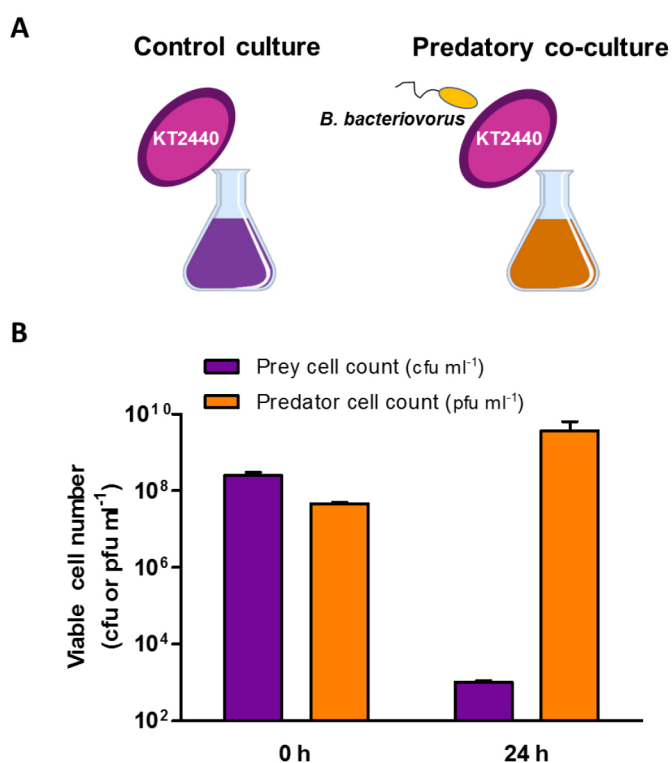


Figure 2. Predation profile to study the predatory capability of *B. bacteriovorus* HD100 upon *P. putida* KT2440. A. Schematic representation of the co-culture involving *B. bacteriovorus* HD100 and the control culture of *P. putida* without predator. B. Viability of the prey and predator cells at the beginning of the experiment (0 h) and after 24 h of incubation. The purple bars represent the viability of the prey cells and the orange bars correspond to the predator viable cell number. Error bars indicate the standard deviation of the mean (n ≥ 3).

Notes

1. If possible, predation events should be checked using a phase contrast microscope to ensure the success of the following steps (see Figure 3).
2. Select the culture medium based on the prey cells used to grow *Bdellovibrio*.
3. To measure OD₆₀₀, prepare a 1/10 dilution of the culture of interest. *E.g.*, Add 100 µl of the culture to 900 µl of saline solution and mix.
4. To store *Bdellovibrio* at -80 °C, add 0.3 ml of 85 % (w/v) glycerol and 0.7 ml of the co-culture of *Bdellovibrio* and the prey bacteria to a cryogenic vial, and place the tube directly in the -80 °C freezer. *Bdellovibrio* cells can be revived by simply scratching the ice of the cryogenic vial with a sterile loop and adding it to the prey suspension. There is no need to de-freeze the cryogenic vial for the inoculation.
5. Dilute samples further if a high concentration of cells is expected. *E.g.*, Prepare 1:10 serial dilutions by adding 100 µl of the co-culture to 900 µl of DNB medium in Eppendorf tubes and repeat until the desired concentration is obtained.

6. DNB 0.7 % (w/v) agar tubes can be previously prepared and stored at room temperature. Prior to use, melt the tubes in a water bath at 150-200 °C and keep them at 45-50 °C.
7. Prepare DNB medium (or HEPES buffer) and the CaCl₂ and MgCl₂ salts separately. Add the salts immediately prior to use.

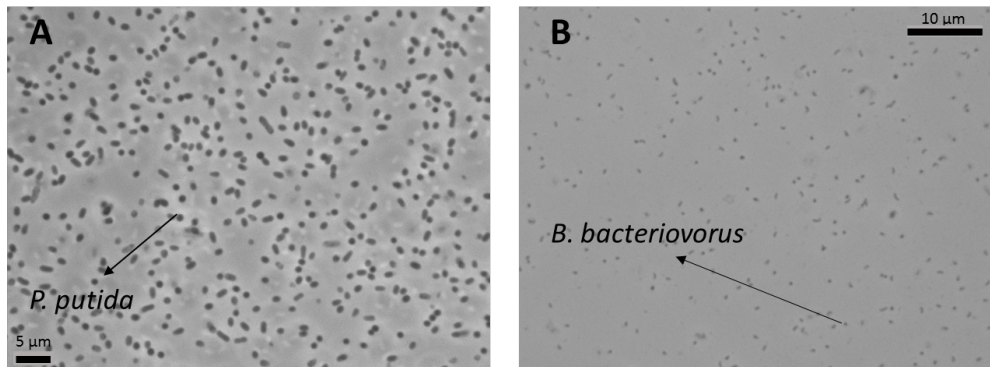


Figure 3. Co-culture of *B. bacteriovorus* HD100 preying on *P. putida* KT2440 under the microscope. A. Co-culture at the onset of predation (time zero); B. After 24 h of incubation, only predatory cells can be observed. Cultures are routinely visualized using a 100x phase-contrast objective and images taken with a Leica DFC345 FX camera.

Recipes

1. Lysogeny broth (LB) medium (1 L)
10 g Bacto tryptone
5 g yeast extract
10 g NaCl
Adjust to pH 7.5 using 1 N NaOH
Autoclave at 121 °C for 20 min
2. LB, 1.5% (w/v) agar (1 L)
10 g Bacto tryptone
5 g yeast extract
10 g NaCl
Adjust to pH 7.5 using 1 N NaOH
15 g agar
Autoclave at 121 °C for 21 min
3. NB medium (1 L)
8 g NB
Autoclave at 121 °C for 20 min
4. CaCl₂ and MgCl₂ salts
2 mM CaCl₂·2H₂O

- 3 mM MgCl₂·3H₂O
Sterilize by filtration through a 0.22 µm filter into a sterile container
5. Diluted nutrient broth (DNB) medium (1 L) (see Note 7)
0.8 g NB
Adjust to pH 7.4 using 1 N NaOH
Autoclave
Add CaCl₂ and MgCl₂ salts (see Recipe 4)
 6. DNB, 0.7% (w/v) agar (1 L) (see Note 7)
0.8 g NB
Adjust to pH 7.4 using 1 N NaOH
7 g agar
Autoclave at 121 °C for 20 min
Add CaCl₂ and MgCl₂ salts (see Recipe 4)
 7. DNB, 1.5% (w/v) agar (1 L) (see Note 7)
0.8 g NB
Adjust to pH 7.4 using 1 N NaOH
15 g agar
Autoclave at 121 °C for 20 min
Add CaCl₂ and MgCl₂ salts (see Recipe 4)
 8. HEPES buffer (see Note 7)
25 mM HEPES buffer
Adjust to pH 7.8 using NaOH pellets
Autoclave at 121 °C for 20 min
Add CaCl₂ and MgCl₂ salts (see Recipe 4)

Acknowledgments

This protocol was modified and adapted from a predatory assay previously described (Martínez *et al.*, 2016; Lambert *et al.*, 2003). This work was funded by the EU Seventh Framework Programme under grant agreement No. 311815 (SYNPOL), and by grants from the Comunidad de Madrid (P2013/MIT2807) and the Spanish Ministerio de Economía y Competitividad, (BIO2010-21049, BIO2013-44878-R).

References

1. Atterbury, R. J., Hopley, L., Till, R., Lambert, C., Capeness, M. J., Lerner, T. R., Fenton, A. K., Barrow, P. and Sockett, R. E. (2011). [Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and *Salmonella* colonization of young chicks](#). *Appl Environ Microbiol* 77(16): 5794-5803.

2. Davidov, Y. and Jurkevitch, E. (2009). [Predation between prokaryotes and the origin of eukaryotes](#). *Bioessays* 31(7): 748-757.
3. Gophna, U., Charlebois, R. L. and Doolittle, W. F. (2006). [Ancient lateral gene transfer in the evolution of *Bdellovibrio bacteriovorus*](#). *Trends Microbiol* 14(2): 64-69.
4. Jurkevitch, E. and Davidov, Y. (2007). [Phylogenetic diversity and evolution of predatory prokaryotes](#). In: Jurkevitch, E. (Ed.). *Microbiol Monogr Predatory Prokaryotes Heidelberg. Springer*, pp: 11-56.
5. Jurkevitch, E., Minz, D., Ramati, B. and Barel, G. (2000). [Prey range characterization, ribotyping, and diversity of soil and rhizosphere *Bdellovibrio* spp. isolated on phytopathogenic bacteria](#). *Appl Environ Microbiol* 66(6): 2365-2371.
6. Lambert, C., Smith, M. C. and Sockett, R. E. (2003). [A novel assay to monitor predator-prey interactions for *Bdellovibrio bacteriovorus* 109 J reveals a role for methyl-accepting chemotaxis proteins in predation](#). *Environ Microbiol* 5(2): 127-132.
7. Lambert, C. and Sockett, R. E. (2008). [Laboratory maintenance of *Bdellovibrio*](#). *Curr Protoc Microbiol* 9:7B.2.1-7B.2.13.
8. Mahmoud, K. K., McNeely, D., Elwood, C. and Koval, S. F. (2007). [Design and performance of a 16S rRNA-targeted oligonucleotide probe for detection of members of the genus *Bdellovibrio* by fluorescence *in situ* hybridization](#). *Appl Environ Microbiol* 73(22):7488.
9. Martínez, V., de la Peña, F., García-Hidalgo, J., Mata, I., García, J. L., and Prieto, M. A. (2012). [Identification and biochemical evidence of a medium-chain-length polyhydroxyalkanoate depolymerase in the *Bdellovibrio bacteriovorus* predatory hydrolytic arsenal](#). *Appl Environ Microbiol* 78: 6017-6026.
10. Martínez, V., Herencias, C., Jurkevitch, E. and Prieto, M. A. (2016). [Engineering a predatory bacterium as a proficient killer agent for intracellular bio-products recovery: the case of the polyhydroxyalkanoates](#). *Sci Rep* 6: 24381.
11. Nelson, K. E., Weinl, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A., Fouts, D. E., Gill, S. R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R. T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J. A., Timmis, K. N., Dusterhoft, A., Tumbler, B. and Fraser, C. M. (2002). [Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440](#). *Environ Microbiol* 4(12): 799-808.
12. Sockett, R. E. (2009). [Predatory lifestyle of *Bdellovibrio bacteriovorus*](#). *Annu Rev Microbiol* 63: 523-539.
13. Stolp, H. and Starr, M. P. (1963). [Bdellovibrio Bacteriovorus gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism](#). *Antonie Van Leeuwenhoek* 29: 217-248.

14. Van Essche, M., Sliepen, I., Loozen, G., Van Eldere, J., Quirynen, M., Davidov, Y., Jurkevitch, E., Boon, N and Teughels, T. (2009). [Development and performance of a quantitative PCR for the enumeration of *Bdellovibrionaceae*](#). *Environ Microbiol Rep* 1(4):228-33.