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2 Supplemental Information

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4 Resazurin to Rapidly Enumerate *Bdellovibrio*-and-Like 5 Organisms (BALOs) and Evaluate Their Activities

6 Running Title: Quick fluorescence-based estimation of BALO numbers

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18 **Table S1.** Bacterial strains used in this study

Bacterial Strain	Characteristics
Bdellovibrio bacteriovorus HD100	Bacterial predator – Type-strain
Bdellovibrio bacteriovorus 109J	Bacterial predator
Halobacteriovorax str. JA-1	Bacterial predator isolated from near Jangheung, South Korea
Halobacteriovorax str. JA-2	Bacterial predator isolated from near Jangheung, South Korea
Escherichia coli MG1655/pUCDK	Prey strain for the *Bdellovibrio* predatory strains
Klebsiella pneumoniae NCCP 15782	Clinical pathogen isolated from human blood samples

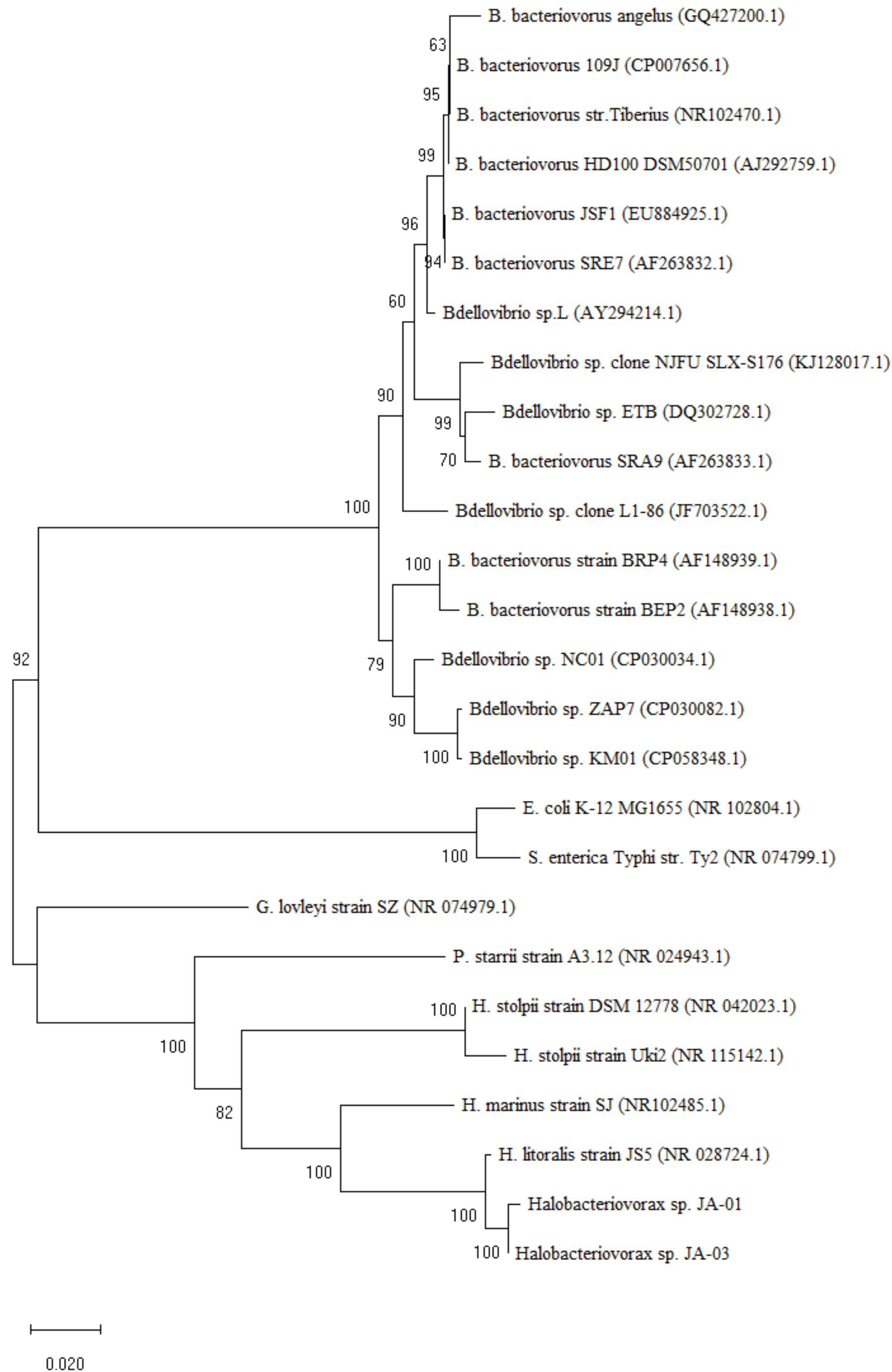
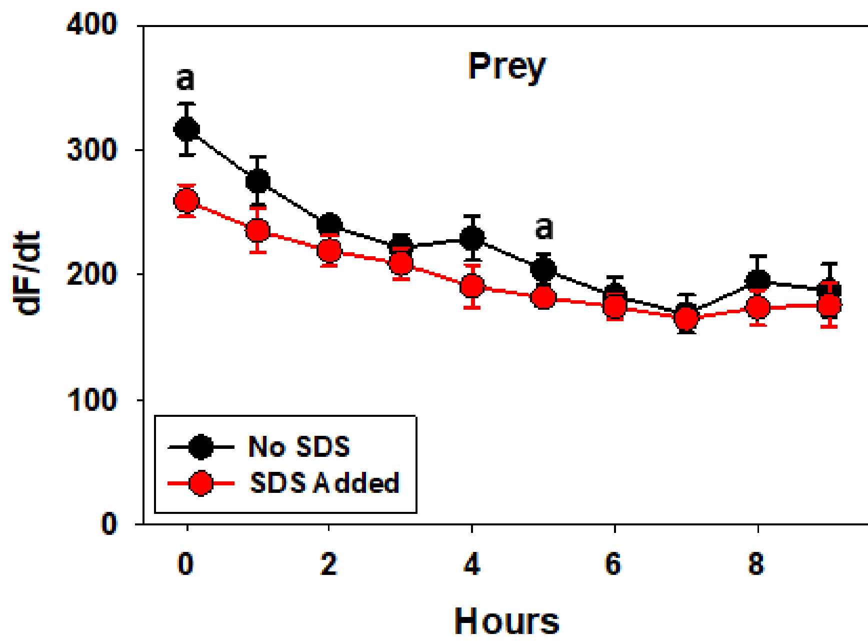


Figure S1. Phylogenetic analyses for *Halobacteriovorax* str. JA-1 and JA-3. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.67038433 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches.

24 The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary
25 distances used to infer the phylogenetic tree. The evolutionary distances were computed using
26 the Jukes-Cantor method and are in the units of the number of base substitutions per site. The
27 analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were
28 eliminated. There were a total of 956 positions in the final dataset. Evolutionary analyses were
29 conducted in MEGA7.



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31 **Figure S2**. Addition of SDS (0.02% (w:v) final) has only a mild impact of the resorufin

32 fluorescence from *E. coli*, confirming this prey is not negatively impacted by this surfactant.

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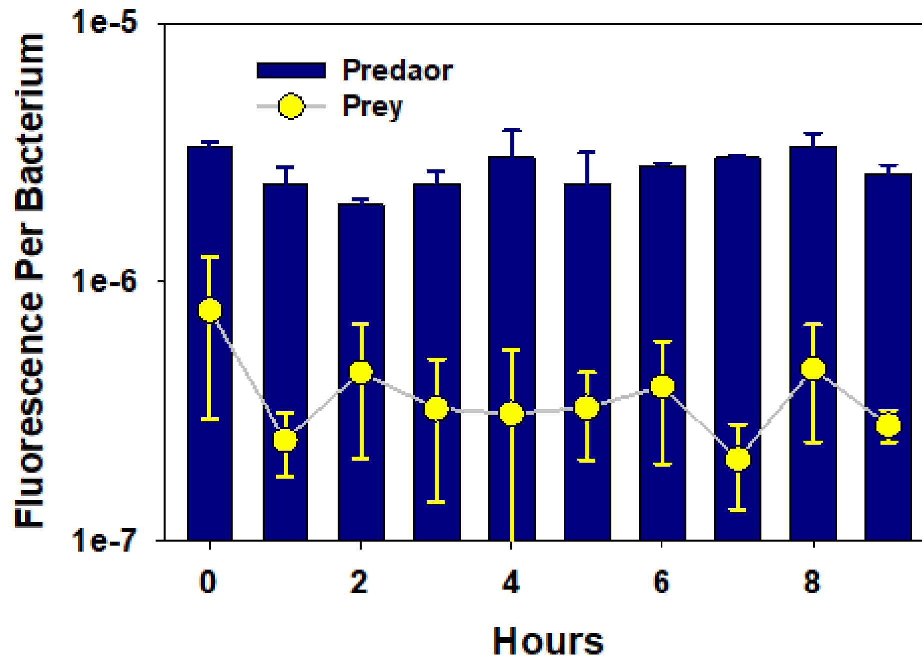

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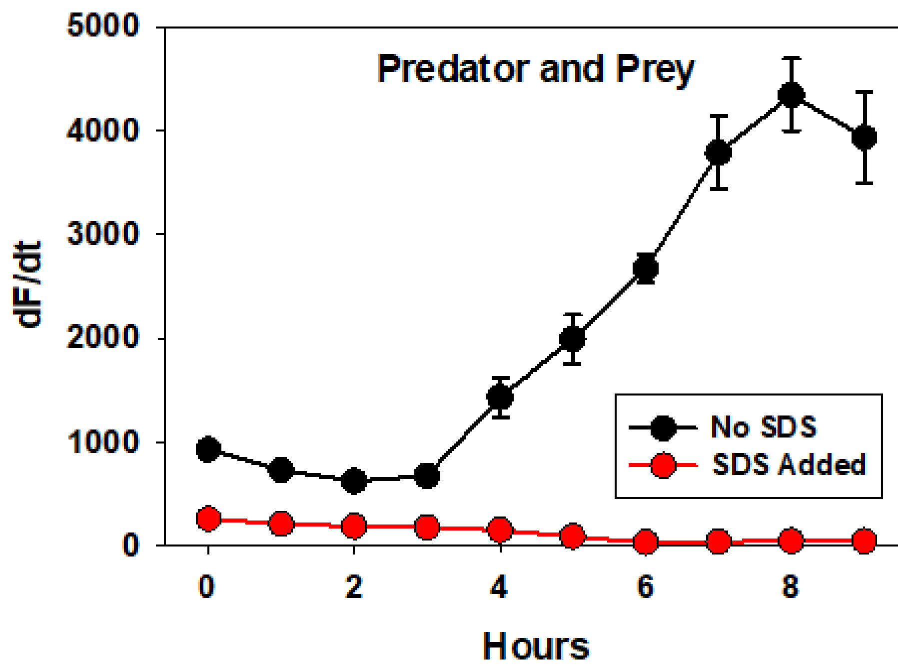

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36 **Figure S3**. Resorufin fluorescence generated per bacterium, showing the significantly higher
37 (7-fold) fluorescence signal from *B. bacteriovorus* HD100 than *E. coli*. The samples were taken
38 at each hour during the predation tests. For the prey, these samples had no predator or SDS
39 addition. For the predator, the values were calculated based on the difference after SDS
40 addition (Lower). Each was divided by the measured viable counts at each time point. (n = 3)

41 **Materials and Methods**

42 **Isolation and Identification of the *Halobacteriovorax* Strains**

43 Samples of the tidal flats were taken south of Jangheung near Sumoon, South Korea. These
44 samples (soil and water) were incubated with shaking at 250 rpm and 30ºC for two hours. After
45 centrifugation (500xg, 5 min) to remove the soil, the supernatant was filtered (0.45 µm syringe
46 filter, Millipore, USA) to remove any large bacterial cells. To the filtrate, *Klebsiella*
47 *pneumoniae* NCCP 15782, a septicemia-related pathogen, was added to an optical density (600
48 nm) of 1.0. The cultures were grown under shaking (250 rpm) at 30ºC for several days until
49 the optical density (OD 600 nm) cleared (below 0.2). The cultures were then filtered (0.45 µm
50 syringe filter, Millipore, USA) to remove any remaining prey and bdelloplasts. The filtrate was
51 diluted in 35% artificial seawater (ASW) and added to top agar made with the same media and
52 prey bacterial strain added. The composition of ASW (100%) was described previously
53 (Artificial Seawater (2012)). These plates were incubated at 30ºC for up to a week to allow
54 plaques to form.

55

56 Individual plaques were inoculated into sterile 35% artificial seawater (ASW), *i.e.*, with an
57 osmolality between 300 and 350 mOsm/kg, with the same prey to initiate predation once more.
58 Once the culture OD cleared, stocks were prepared and the predator was stored as a bdelloplast
59 formulation in 20% glycerol and -80ºC using the same pathogen as the prey. As needed, fresh
60 plaques of these strains were grown. Subcultures of these isolates were always grown using
61 30% artificial seawater (ASW; 350 mOsm/kg) with *K. pneumoniae* NCCP 15782 as the prey.
62 After obtaining pure cultures as described above, the newly isolated predators were then
63 identified via 16S rDNA sequencing using the universal primer 27F/1492R set as described
64 previously (Wen et al., 2009; Monnappa et al., 2016) followed by phylogenetic analysis with
65 MEGAversion 6 software (Tamura et al., 2013) (Figure S1).

66

67 Growth of the Bacterial Strains

68 All of the bacterial strains used in this study are listed in Table S1. The prey bacteria used to
69 culture the *B. bacteriovorus* strains was *E. coli* MG1655 as described previously (Monnappa
70 et al., 2014; Im et al., 2018) while *K. pneumoniae* NCCP 15782 was used for the
71 *Halobacteriovorax* isolates. Briefly, each prey was cultivated overnight in Lysogeny Broth (LB)
72 (Difco BD, USA) at 30°C and 250 rpm in a shaking incubator. The prey cells were then pelleted
73 (2,200 x g, 10 min), washed with HEPES buffer (25 mM, pH 7.4) with $CaCl_2$ and $MgCl_2$ added
74 (3 and 2 mM, respectively) and resuspended to an optical density (OD) at 600 nm of 1.0. To
75 these cultures, the predatory bacteria were added (1:100 dilution) and grown in flask cultures.
76 After 24 hours, when the optical density was below 0.2, the predated culture was filtered using
77 0.45 μM syringe filters (Millipore, USA) to remove any surviving prey and bdelloplasts from
78 the attack-phase predatory cells.

79

80 Resazurin Enumeration of the Predatory Bacterial Strains

81 The resazurin stock (0.2% (w/v)) was prepared using distilled water and filter-sterilized (0.22
82 μm syringe filter, Millipore, USA). For the experiments, this stock was diluted 1:3 (v:v) into
83 HEPES buffer (25 mM, pH 7.4) with $CaCl_2$ and $MgCl_2$ added (3 and 2 mM, respectively).
84 After growth overnight as described above, the predatory cultures were filtered (0.45 μm
85 syringe filter, Millipore, USA) and serially diluted in either HEPES buffer (*Bdellovibrio*) or
86 30% ASW (*Halobacteriovorax*). From these dilutions, 200 μl aliquots were transferred into the
87 wells of a black 96-well plate (Greiner, USA) and 20 μl of the diluted resazurin stock were
88 added, giving a final concentration of 0.005% resazurin (w/v). The fluorescence from each well
89 was then immediately read using a plate reader (TECAN, USA; Ex 530nm, Em 580nm). The
90 plate reader was set to mix the samples, maintain the temperature at 30ºC and read the

91 fluorescence signal in each well every ten minutes. Using the fluorescence values obtained, the
92 change in fluorescence per minute (dF/dt) was calculated manually using the zero- and ten-
93 minute data as follows:

94
$$\frac{FL_{t=10\ min} - FL_{t=0\ min}}{dt\ (10\ minutes)}$$

95

96 **SDS Tests**

97 To perform the SDS sensitivity experiments with *B. bacteriovorus* HD100, these experiments
98 were performed as described above and previously (Cho et al., 2019) except the predatory
99 cultures, after 0.45 µm filtration, were diluted ten-fold, *i.e.*, into 180 µl of sterile media
100 (HEPES buffer alone or HEPES buffer with SDS added) 20 µl of the filtered predatory culture
101 were added and mixed within the wells of a black 96-well plate (SPL, Korea). For
102 *Halobacteriovorax* str. JA-1. the same protocol was used except the media used was 25 mM
103 HEPES in 35% ASW. The plates were then incubated at 30 ºC with shaking in a TECAN Spark
104 plate reader. After one hour, 20 µl of the diluted resazurin were added to each well and the
105 fluorescence was measured as described above.

106

107 **Osmolality tests**

108 The osmolality of the various samples was measured as described previously (Im et al., 2017)
109 using an Advanced 3220 Freezing-Point Osmometer (Advanced Instruments, USA). Briefly,
110 to adjust the osmolality of the samples, artificial seawater (ASW) solutions were mixed with
111 the HEPES preparations to give 0, 25, 50, 75 and 100% ASW.

112

113 The short-term effects of the osmolality on the predators were conducted as described above
114 for the resazurin tests. After filtration, the predator was diluted into the different media (1:10)
115 and 200 µl of the culture were incubated in a black 96-well plate (SPL, Korea) at 30 ºC with
116 shaking in a TECAN Spark plate reader. After one hour, 20 µl of the diluted resazurin were
117 added to each well and the fluorescence was measured as described above.
118
119 To test the impact of the osmolality on 24-h predation, the prey bacteria were grown as
120 described above, pelleted by centrifugation (13,000 x g, 5 min) and resuspended in the different
121 ASW media at an OD of 1.0. To this, the predatory filtrate was then added (1:100 dilution).
122 These samples were then incubated for 24 hours at 30 ºC with agitation (250 rpm), after which
123 the prey and predator viabilities in each media were determined.
124
125 **Predation tests**

126 Predation tests were performed using *B. bacteriovorus* HD100 with *E. coli* MG1655 as the
127 prey in 25 mM HEPES buffer (pH 7.2) with 3 mM $MgCl_2$ and 2 mM of $CaCl_2$ added. The prey
128 OD was set to 1.0 and the filtered predator was added so that the initial predator-to-prey ratio
129 was approximately 0.3 (*i.e.*, one predator for three prey). The cultures were incubated at 30 ºC
130 with shaking (250 rpm). Every hour, a sample was taken and used to measure the viabilities
131 using agar plates for the prey and top agar plates for the predator, as described previously (Cho
132 et al., 2019). Briefly, for *B. bacteriovorus* HD100, *E. coli* MG1655 was used as the prey and
133 the top agar consisted of 0.7 % agar prepared using dilute nutrient broth (DNB), *i.e.*, 1/10 NB,
134 with 3 mM $MgCl_2$ and 2 mM $CaCl_2$ added. Similarly, for *Halobacteriovorax* str. JA-1, *K.*
135 *pneumoniae* NCCP 15782 was used as the prey and the 0.7 % top agar was prepared using
136 DNB with 35 % ASW added. Further additions of $MgCl_2$ and $CaCl_2$ were not needed as they
137 were already present in the ASW.

These hourly aliquots were also tested using resazurin, which we performed without treatment or after a ten-minute treatment with 0.02% SDS. Briefly, 180 µl of the culture were mixed with either 20 µl of HEPES buffer or 20 µl of HEPES with SDS added. After a ten-minute incubation, 20 µl of the diluted resazurin was added and the fluorescence was measured as described above in black 96-well plates (SPL, Korea).

Reproducibility and statistical analysis

Unless specified, each experiment was performed in triplicate and the standard deviations are presented on the graphs as error bars. Normal distribution of each dataset was verified using the Shapiro-Wilk test. For those samples that did not show a substantial departure from the normality ($p > 0.05$), the student t-test was used, with significance indicated within the graphs using: a - $p < 0.05$; b - $p < 0.01$; c - $p < 0.001$. For the resorufin fluorescence per predatory density, Shapiro-Wilk found them to be significant ($p < 0.05$) and, thus, the correlations (R^2 values) between the predatory density and the resorufin fluorescence were calculated using the Spearman Correlation Test.

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