Attack-Phase Bdellovibrio bacteriovorus Responses to Extracellular Nutrients Are Analogous to Those Seen During Late Intraperiplasmic Growth

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Attack-Phase *Bdellovibrio bacteriovorus* Responses to Extracellular Nutrients Are Analogous to Those Seen During Late Intraperiplasmic Growth

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Abstract

Bdellovibrio bacteriovorus is a predatory bacterium which lives by invading the periplasm of gram-negative bacteria and consuming them from within. This predator was thought to be dependent upon prey for nutrients since it lacks genes encoding for critical enzymes involved in amino acid biosynthesis. This study, however, found that planktonic attack-phase predators are not just dependent

upon prey for nutrients, but rather, they respond to nutrients in the surrounding medium and, subsequently, synthesize and secrete proteases in a nutrient-dependent manner. The major secreted proteases were identified through mass spectrometry analyses. Subsequent RT-qPCR analyses found that the nutrient-induced proteases are similar to those expressed within the prey periplasm during the late intraperiplasmic growth phase. Furthermore, RNA sequencing found that incubating the planktonic attack-phase cells in a nutritious environment for a short period of time (4 h) changes its gene expression pattern to a status that is akin to the late intraperiplasmic phase, with more than 94% of the genes previously identified as being late intraperiplasmic-specific also being induced by nutrient broth in this study. This strong correlation between the gene expression patterns hints that the availability of hydrolyzed prey cell components to the predator is likely the stimulus controlling the expression of late intraperiplasmic *B. bacteriovorus* genes during predation.

Keywords

Bdellovibrio bacteriovorus
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Serine proteases
Transcriptomics
Extracellular

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Electronic supplementary material

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Introduction

Bdellovibrio bacteriovorus is a gram-negative predatory bacterium which lives by attacking other gram-negative bacteria [1]. This predator enters the periplasm of its prey, where it hydrolyzes and consumes the prey cell components, grows, and septates before lysing the prey and proceeding to attack another. As such, the life cycle of *B. bacteriovorus* is very complex for a bacterium and involves several different stages [2, 3].

Although the genome for *B. bacteriovorus* HD100 and other predatory strains has been sequenced [4, 5, 6, 7], much remains to be learned about their predatory lifestyle. In particular, the environmental stimuli and the regulatory networks involved in controlling gene expression during the different stages of their life cycle have not yet been fully elucidated. Recent studies by different groups have shed light on some aspects of this genetic regulation such as the diverse roles c-di-GMP plays in regulating gene expression [8]. Another report also demonstrated the presence of an attack-phase (AP) massively expressed and presumably c-di-GMP-responsive riboswitch RNA (merRNA) that is significantly downregulated once the predator enters its prey [9]. A more recent study found that interactions with both the prey cell membrane and the prey soluble fraction were needed for *B. bacteriovorus* to start its intraperiplasmic growth phase [10]. In that study, the authors demonstrated that planktonic AP predatory cells could not grow when incubated with prey extract alone or with a rich medium such as peptone yeast extract (PYE) even when this rich medium was supplemented with prey envelopes.

Although that study also mapped the expression patterns of several key genes related with *B. bacteriovorus*' life cycle, it was limited to only a selection, and the effects a rich medium has on the genome-wide transcriptomics of this predator have not been pursued. Planktonic AP *B. bacteriovorus* cells are generally regarded as hungry cells which swim very fast in the medium [11] in search for a prey where they can find the necessary nutrients needed for their growth and development [12]. However, much is still not known about how AP predatory cells behave when they encounter nutrients outside of their prey.

Although, historically, *B. bacteriovorus* was regarded as an obligate predator that is dependent upon its prey for survival, variants of this predator that grow axenically within complex media can be isolated [13, 14, 15, 16]. These host-independent *B. bacteriovorus* (HIB) cells were found to secrete extracellular proteases when growing in PYE media [13]. This suggested that the secretion of these proteases was either inherent to the HIB lifestyle or that it is a general phenotype that can be induced by the presence of extracellular nutrients.

In this study, we evaluated this hypothesis and found that planktonic AP *B. bacteriovorus* HD100 cells also secrete proteases when provided with a source of amino acids. Aside from increasing the extracellular protease activities, the presence of nutrients also induced a genome-wide transcriptional response that is comparable to that seen when *B. bacteriovorus* is inside its prey, a result that

implies that the induced expression of these genes during predation results partially from the hydrolysis of prey macromolecules.

Materials and Methods

Microorganisms and Culture Conditions

The predatory bacterium *B. bacteriovorus* was maintained using *Escherichia coli* MG1655 as the prey in HEPES buffer with $CaCl_2$ and $MgCl_2$ salts added (4 and 2 mM, respectively) as described in previous reports [17, 18]. We selected this *E. coli* strain as the prey since it is nonpathogenic and a common strain in many labs. The same buffer was used as a control for all experiments. To count the predatory *B. bacteriovorus* cells, double-layer DNB agar plates with *E. coli* prey were employed. For the HIB, PYE (peptone 10 g/L and yeast extract 3 g/L) agar plates supplemented with $CaCl_2$ and $MgCl_2$ were used as described previously [13].

Quantitative Protease Assay

The protease activity of the *B. bacteriovorus* supernatants and other solutions was tested using the Azocoll reagent (Sigma-Aldrich Co., USA). The assay was performed using proteinase K as a standard. The detailed procedure used for the assay has been described previously [13]. One modification, however, was that the 37 °C incubation was done for 24 h before the samples were centrifuged, and the absorbance of the supernatant was measured.

Survival Assay of *B. bacteriovorus*

Survival of *B. bacteriovorus* HD100 in nutrient broth (NB) or HEPES was measured over 5 days. For this, predatory bacteria cultured overnight as described above were centrifuged and washed with HEPES. The washed cells were inoculated into HEPES or NB media at a concentration of 1×10^9 ~~PFU~~plaque-forming units (PFU)/mL. They were then incubated at 30 °C for 5 days without shaking. Every 24 h, the number of HDB and HIB ~~is~~cells were enumerated and SEM imaging was performed as described previously [19].

Mass Spectrometric Analysis of Attack-Phase *B. bacteriovorus*-Secreted Proteases

AP *B. bacteriovorus* HD100 from overnight predatory cultures were filtered twice through 0.45-μm filters to remove the remaining *E. coli* cells. The *B. bacteriovorus* cells were then centrifuged and washed three times before being re-suspended in 1×

NB media at a concentration of ~1 × 10^8 PFU/mL. After 14 h of incubation at 30 °C in a shaking incubator, the cells were counted through plaque-forming unit enumeration to make sure that no significant lysis or change in the number happened during this incubation. The cultures were then filtered twice through 0.22-µm filters to remove the *B. bacteriovorus* cells, and 5 mL of the supernatant was concentrated to 80 µL using 3 kDa molecular weight cutoff centrifugal filters (Amicon Ultra, Millipore). The samples were then boiled with 5× reducing sample buffer and run on a 12% Tris-glycine SDS-PAGE gel (Bio-Rad, USA). As a negative control, a freshly prepared nutrient broth was concentrated, treated similarly, and analyzed in parallel. After staining with colloidal Coomassie blue, the proteins in the range of approximately 30~80 kDa were sliced in six consecutive portions followed by in-gel tryptic digestion, as described by Shevchenko and co-workers [20]. The resulting tryptic peptides were analyzed by LC-MS/MS.

All mass analyses were performed on a LTQ-Orbitrap (Thermo, Bremen, Germany) equipped with a nanoelectrospray ion source. To separate the peptide mixture, we used a C18 reverse-phase HPLC column (150 mm × 75 ~~m~~µm ID) using an acetonitrile/0.1% formic acid gradient from 13 to 30% for 90 min at a flow rate of 300 nL/min. For MS/MS analysis, the precursor ion scan MS spectra (*m*/*z* 400~2000) were acquired in the Orbitrap at a resolution of 60,000 at *m*/*z* 400 with an internal lock mass. The 20 most intensive ions were isolated and fragmented in the linear ion trap by collisionally induced dissociation (CID). All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; Version 1.4.1.14) and X! Tandem (GPM; Version Cyclone (2010.12.01.1)). Both programs were set up to search the *B. bacteriovorus* protein sequence database (3763 entries, UniProt (http://www.uniprot.org /)) assuming the digestion enzyme trypsin. Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 ppm. Carbamidomethylation of cysteine was specified as fixed modification. Deamidation of asparagine and glutamine; methylation of glutamic acid, lysine, glutamine, and arginine; oxidation of methionine; acetylation of lysine, serine, and threonine; and the *N*-terminus phosphorylation of serine, threonine, and tyrosine were specified in Sequest and X! Tandem as variable modifications. Additionally, Glu->pyro-Glu at the *N*-terminus, ammonia loss of the *N*-terminus, and gln->pyro-Glu at the *N*-terminus were also specified in X! Tandem as variable modifications. Scaffold (Version 4.4.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 81.0% probability to achieve a false discovery rate

(FDR) less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve an FDR less than 1.0% and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [21]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

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Time-Dependent RNA Extraction Throughout the Predation Cycle and RT-qPCR Analysis

Three independent overnight cultures of *B. bacteriovorus* HD100 in HEPES buffer were filtered through 0.45-μm filters to remove bdelloplasts and un-predated *E. coli* cells. These filtrates were then concentrated 10 times and mixed with *E. coli* suspensions in HEPES buffer which were previously adjusted to an OD_{600} of 4.3 and incubated at 30 °C for 1 h in a shaking incubator. After mixing the two suspensions, the final multiplicity of infection (MOI) of these cultures was about 3.7. Samples were taken from the *B. bacteriovorus* cultures immediately before mixing (attack-phase samples) and then at different time points after mixing (30, 60, 120, and 180 min).

Each sample was centrifuged at 2000 rcf for 2 min; then, the supernatant was decanted. This was followed by a brief washing with the HEPES buffer and 1 min more of centrifugation at 1000×*g* before decanting the supernatant again. This was done to get rid of the majority of the free attack-phase *B. bacteriovorus* cells that did not invade *E. coli*. RNA was then extracted using the TRIzol/chloroform extraction method [22]. RNA processing and RT-qPCR analyses were performed as described previously [23, 24]. The expression of each gene was calculated relative to 16S ribosomal RNA (rRNA) gene expression and is shown relative to the expression level of the AP cells. Table S3 lists all primers used in this study.

RNA Extraction from AP *B. bacteriovorus* HD100 Cells for RT-qPCR and RNA Sequencing

AP *B. bacteriovorus* HD100 from 24- h predatory cultures prepared in HEPES buffer were filtered (0.45 μM), concentrated, and then re-suspended in HEPES buffer, 0.2× NB, 1× NB, or 5× NB (20 mL each). All the media were supplemented with $CaCl_2$ and $MgCl_2$ as usual. After a 4- h incubation in a 30 °C shaking incubator, *B. bacteriovorus* cells from all the tubes were collected by

centrifugation, the RNA was extracted, and RT-qPCR was performed as described previously [24].

RNA sequencing and alignment was done by ChunLab (Seoul, South Korea, http://chunlab.com). For this, the total RNA quality was assessed based on RNA integrity number (RIN) using Agilent 2100 Bioanalyzer (Agilent, USA). Ribosomal RNA depletion was performed using the Ribo-Zero rRNA Removal Kit (Epicentre, USA). Libraries for sequencing were made using the TruSeq Stranded Messenger RNA (mRNA) Sample Preparation Kit (Illumina, USA). The sequencing was done using single-end 50 bp sequencing on the Illumina HiSeq 2500 platform. *B. bacteriovorus* reference genome was downloaded from NCBI database, and the quality filtered reads were aligned to the genome using Bowtie 2. Analyses and visualization of the RNA sequencing reads were performed using CLRNASeq™ program (ChunLab, South Korea). The RNA sequencing reads in each sample were normalized using three different methods: ~~reads per kilobase million~~Reads Per Kilobase per Million mapped reads (RPKM) [25], ~~RLE~~Relative Log Expression (RLE) [26], and ~~TMM~~Trimmed Mean of M values (TMM) [27]. The results of these three normalization methods were then compared to the RT-qPCR results. The RPKM method, however, showed the best correlation (Table S2, worksheet 2), and consequently, it was chosen for further analyses. The differential expression of the AP-specific small RNAs was analyzed using the Artemis program [28]. The RNA sequencing data together with the raw extracted files were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) website under accession number GSE82035.

Reproducibility and Statistical Analyses

All experiments were done at least in triplicate, and the standard deviations between the samples are shown as error bars on the graphs. Statistical analysis was done using Student's *t* test to compare two sets of data, and the statistical significance was shown on the graphs using the marks *, **, and *** for *p* values of less than 0.05, 0.01, and 0.001, respectively. To compare three or more sets of data, analysis of variance test (ANOVA) was used followed by Tukey's post hoc test. Groups with significant statistical difference were assigned different letters on the graphs (a, b, c, d, and e).

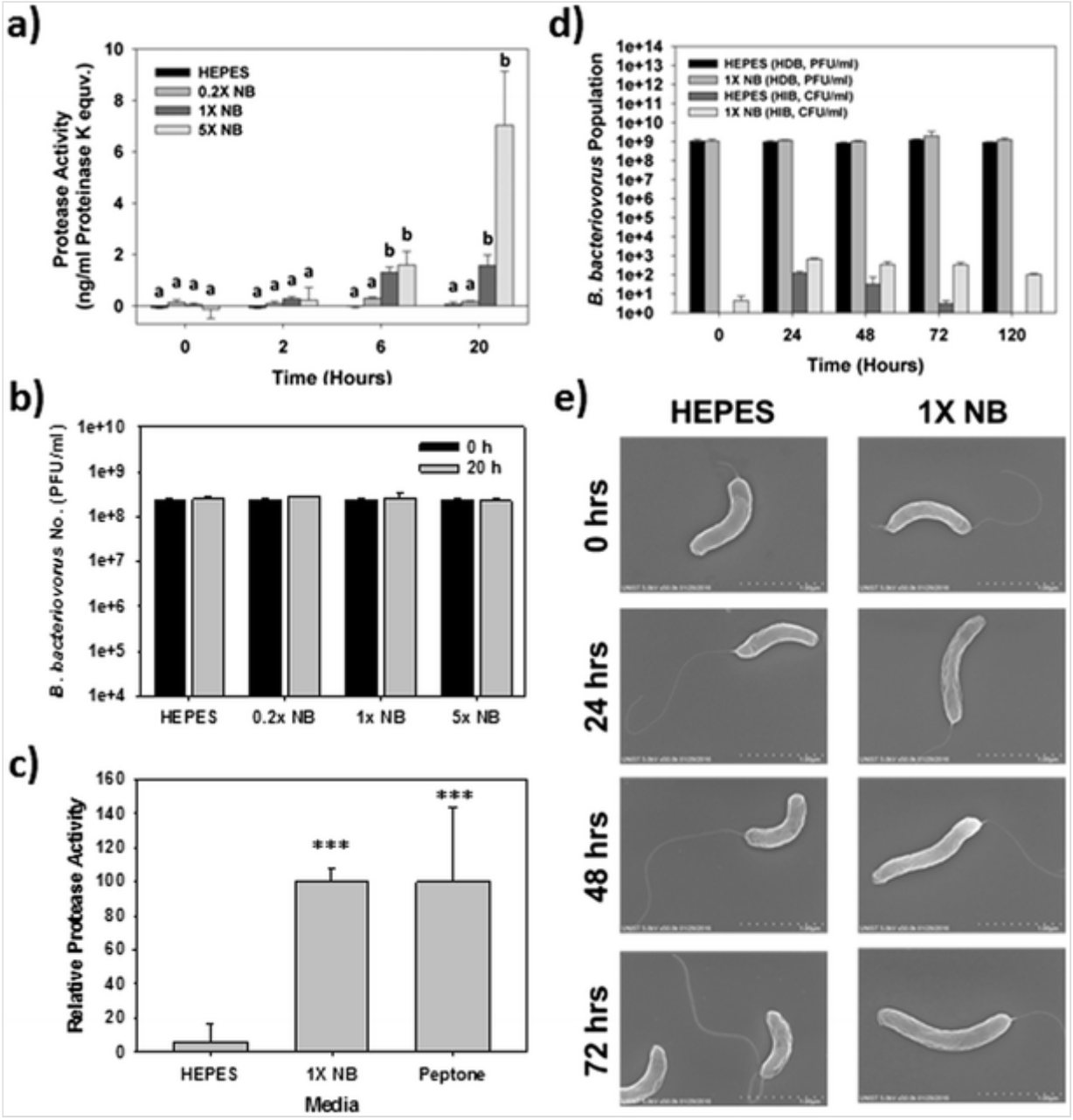
Results

Extracellular Nutrients Induce the Secretion of Proteases by Attack-Phase *B. bacteriovorus* HD100

When AP *B. bacteriovorus* HD100 were incubated in nutrient media for several hours in the absence of prey, we found that proteases were secreted (Fig. 1a). The extracellular proteolytic activities increased in a time-dependent fashion. Although similar activities were seen initially with the different media preparations, the richer media led to stronger activities as the incubation was extended. The *B. bacteriovorus* population in each of these media was stable over the 20- h experiment (Fig. 1b), while experiments in 1% peptone gave basically identical protease activities (Fig. 1c), illustrating that the predator is utilizing amino acids to produce these proteases.

Fig. 1

Extracellular nutrients induce the secretion of proteases by attack-phase (AP) *B. bacteriovorus* HD100. **a** *B. bacteriovorus* protease activity within various media over time. Washed AP *B. bacteriovorus* were re-suspended in HEPES buffer, 0.2× nutrient broth (NB), 1× NB, or 5× NB at a concentration of approximately 8×10^8 PFU/mL. Samples were taken over time and filter-sterilized before determining the protease activity present. *a* and *b* = $P < 0.05$ ($n = 3$). **b** *B. bacteriovorus* HD100 does not grow in the nutrient media. The predatory viability initially and after 20 h was measured in each of the nutrient media preparations, showing that the *B. bacteriovorus* population does not increase ($n = 3$). **c** Amino acids induce the secretion of proteases from AP *B. bacteriovorus* HD100. Washed AP *B. bacteriovorus* were exposed for 24 h to HEPES buffer, 1× NB, or 1% peptone. The protease activity relative to that found in 1× NB was then measured ($n = 6$). **d** Host-independent *B. bacteriovorus* variants appear but remain only a very minor population. As in **b**, the AP *B. bacteriovorus* HD100 populations remained steady for 120 h (5 days) in both HEPES and NB. Although HIB variants were also observed in each media, their populations were 6-log lower than AP *B. bacteriovorus* HD100 ($n = 3$). **e** Scanning electron microscopic (SEM) images of predatory cells according to the media and time. Cells were sampled from the cultures in **d** and imaged. The sizes of the cells are listed in Table 1



Mass spectrometric analyses of the extracellular proteins produced in 1× NB identified 68 proteins common among three independent preparations (Table S1). This list included three serine proteases, i.e., *Bd2269*, *Bd2321*, and *Bd2692*, all of which are also secreted by host-independent variants of *B. bacteriovorus* (HIB), as demonstrated previously [13]. Consequently, we tested if HIB appeared during long-term incubations in HEPES buffer or the nutrient media (Fig. 1d). Although HIB were found, their population was very low, i.e., less than 680 bacteria per mL. The rate of spontaneous development was approximately 10^{-6} to 10^{-7}, which is a frequency that is similar with that reported previously [15, 16]. Figure 1e shows some images of the predators during this extended incubation, and Table 1 lists the average lengths measured. Although the predatory cells elongate when incubated in

the nutrient media, they do not form filamentous cells like those described for HIB [11] but have a morphology typical of planktonic *B. bacteriovorus* cells [29].

Table 1

Sizes of attack-phase *B. bacteriovorus* cells in HEPES and nutrient broth

	Size (μm)			
Time (h)	0	24	48[a]	72[a]
HEPES	1.03 ± 0.12	0.95 ± 0.04	0.84 ± 0.07	0.86 ± 0.1
NB	1.02 ± 0.09	1.34 ± 0.4	1.58 ± 0.16	1.40 ± 0.32
Length was measured as the linear distance between the two poles of the cells (n = 16)				
[a]The sizes at 48 and 72 h were significantly different between the NB and HEPES samples (n = 16; $P < 0.01$)				

Extracellular Nutrients Induce the Transcription of Several *B. bacteriovorus* HD100 Protease Genes

Since the serine proteases encoded by the *Bd2269*, *Bd2321*, and *Bd2692* genes were secreted by AP *B. bacteriovorus*, the transcriptional levels for each of these genes were subsequently determined using the same media preparations (Fig. 2a). Alongside these, the transcription patterns for five other serine proteases were also evaluated. Three of these additional proteases (encoded by the *Bd0449*, *Bd2545*, and *Bd2627* genes) were seen in two of the three mass spectrometry results performed, while the remaining two (encoded by the *Bd1283* and *Bd1432* genes) were not found in any of the samples but are annotated as being potential extracellular serine proteases.

Fig. 2

Differential transcription of several proteases in attack-phase (AP) and intraperiplasmic (IP) *B. bacteriovorus* HD100. **a** Relative transcriptional levels of the selected proteases in AP *B. bacteriovorus* in the different nutrient media preparations. AP *B. bacteriovorus* were incubated for 4 h in HEPES buffer, 0.2× nutrient buffer (NB), 1× NB, or 5× NB. The RNA was then extracted, and the expression levels of the selected protease genes were determined using RT-qPCR. Each is shown relative to the expression levels obtained in HEPES. *a*, *b*, and *c* = $P < 0.05$ (n = 3). **b** Transcriptional levels of the protease genes during IP growth of *B. bacteriovorus*. RNA was collected from AP *B. bacteriovorus* cells and from *E. coli* bdelloplasts at different time points during predation. The expression levels of each protease gene

were measured using RT-qPCR and are shown relative to the expression levels seen during the AP ($n = 3$)

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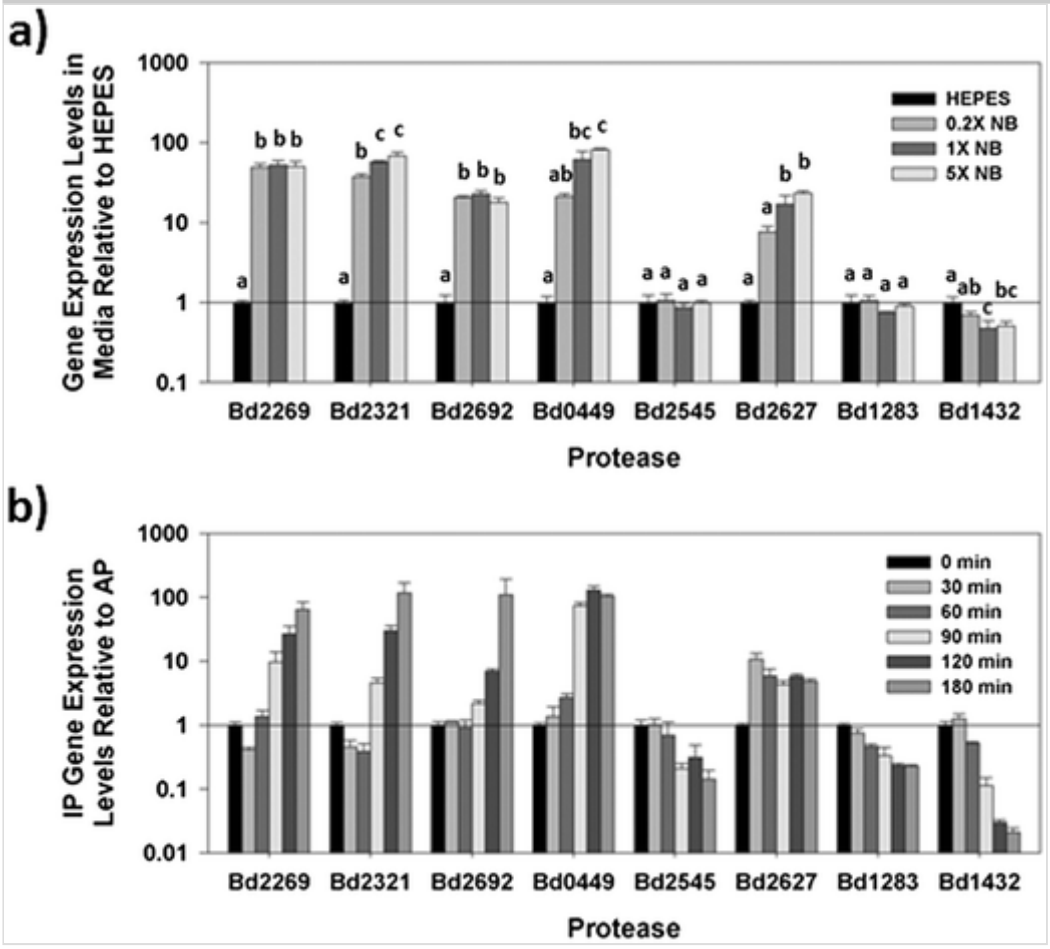


As shown in Fig. 2a, of the six proteases found in the media, the transcriptional levels for five were significantly higher (greater than 10-fold) when *B. bacteriovorus* HD100 was incubated in nutrient media. In contrast, the transcriptional levels for *Bd2545* and *Bd1283* were not affected, while that of Bd1432 actually decreased as the concentration of nutrients in the media increased.

Increased Transcription of the Protease Genes During Intraperiplasmic Growth

When the mRNA expression levels for the same eight proteases were monitored during intraperiplasmic (IP) growth of *B. bacteriovorus* HD100, the same five genes, i.e., *Bd2269*, *Bd2692*, *Bd0449*, *Bd2321*, and *Bd2627*, were induced (Fig. 2b). One of these genes (*Bd2627*) was rapidly induced (30 min) and constitutively transcribed during the rest of the IP growth stage, while the expression levels of the other four increased after 60 min and displayed time-dependent patterns. In

contrast, transcription of *Bd1283*, *Bd1432*, and *Bd2545* decreased as predation ensued. Much like what was seen with the different media preparations in Fig. 2b, the expression of *Bd1432* was inhibited most significantly during IP growth.

Genome-Wide Transcriptional Patterns Induced by Media in AP Predators Are Comparable to Those Seen in IP Predators

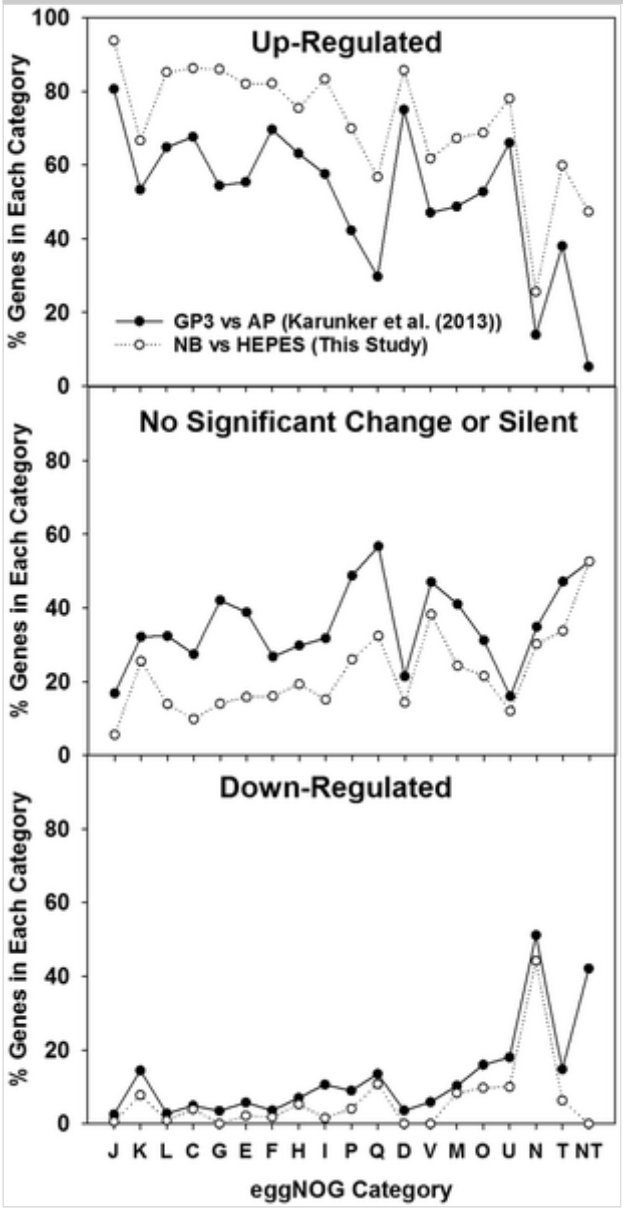
The transcriptional levels for many of the protease genes in Fig. 2a were similar to those seen during IP growth, particularly during the latter stages of predation (Fig. 2b). The similar expression levels from both conditions suggested that an exposure of planktonic AP predatory cells to extracellular nutrients can induce gene expression patterns that are comparable to those generated inside the prey. To evaluate this further, we compared the genome-wide gene expression levels of *B. bacteriovorus* HD100 when incubated in 1× NB with those found during the late IP stage of growth (GP3), as published previously by Karunker et al. [9].

Comparative sequencing of the RNA extracted from AP *B. bacteriovorus* incubated in either HEPES buffer or 1× NB found that, among the 3587 genes encoded by this predator, the transcriptional levels for 2242 (62.5%) were upregulated 1.5-fold or greater ($P < 0.05$) in the nutrient media (Table S2). When the genes were grouped into their respective evolutionary genealogy of genes: Nonsupervised Orthologous Groups (eggNOG) categories and compared with the results from Karunker et al. [9], the similarities between the two treatments were readily apparent (Fig. 3). A more detailed comparison between the two studies is provided in the Supplemental Note.

Fig. 3

Comparative eggNOG-based analyses of the attack-phase *B. bacteriovorus* gene expression patterns in nutrient broth (NB) vs HEPES. This figure shows the comparable gene transcription patterns according to the eggNOG categorization resulting from NB/HEPES transition (this study) and GP3/AP transition (Karunker's study, 2013). Except for the *NT* group, genes located within more than one category were excluded from the analyses and only those showing more than 1.5-fold change in their expression (with $P < 0.05$) were considered significant. For GP3/AP transition (Karunker et al. 2013), the authors provided Bonferroni values instead of *p* values. Translation, ribosomal structure, and biogenesis (*J*); transcription (*K*); replication, recombination, and repair (*L*); energy production and conversion (*C*); carbohydrate transport and metabolism (*G*); amino acid transport and metabolism (*E*); nucleotide transport and metabolism (*F*); coenzyme transport and metabolism (*H*); lipid transport and metabolism (*I*); inorganic ion transport and metabolism (*P*);

secondary metabolite biosynthesis, transport, and catabolism (*Q*); cell cycle control, cell division, and chromosome partitioning (*D*); defense mechanisms (*V*); cell wall/membrane/envelope biogenesis (*M*); post-translational modification, protein turnover, and chaperones (*O*); intracellular trafficking, secretion, and vesicular transport (*U*); cell motility (*N*); signal transduction mechanisms (*T*); both cell motility and signal transduction mechanisms (*NT*)



Discussion

During its IP growth stage, *B. bacteriovorus* hydrolyzes the prey cell components, including the proteins, RNA, and DNA, to provide the requisite building blocks it needs for growth and replication [23]. This process is particularly critical for *B. bacteriovorus*, as it lacks the ability to produce and degrade many amino acids, a

characteristic that makes it dependent upon other organisms [7]. Although often viewed as being strictly a predator that is dependent upon its prey, *B. bacteriovorus* can also enter an axenic lifestyle [13], commonly referred to as a HIB. In a previous study, we reported that HIB cells secrete proteases when growing axenically in PYE media [13]. This suggested that the secretion of proteases was either inherent to the HIB lifestyle or that it is a general phenotype that can be induced by the presence of extracellular nutrients. We show here that it is the latter, namely, that planktonic AP *B. bacteriovorus* HD100 also secretes proteases when proteins are available in the surrounding media.

When AP predatory cells were incubated in the different nutrient media preparations, we found that the extracellular protease activity increased significantly. The maximum protease activities detected increased dose-dependently and in a manner that correlated well with the concentration of the nutrients in the media. This is shown in Fig. 1a, where the maximum protease activity seen in 1× NB was 5-fold higher than with 0.2× NB (1.5 vs 0.29 ng proteinase K equivalent/mL). Likewise, the maximum seen in 5× NB was 4.7-fold higher than the maximum seen in 1× NB (7.0 vs 1.5 ng proteinase K equivalent/mL).

As nutrient broth is a complex medium, experiments were also performed using 1% peptone to provide only amino acids and small peptides. The resulting protease activity levels in 1% peptone were very similar to those seen in 1× NB (Fig. 1b), a result that is consistent with the conclusion that planktonic predators utilize extracellular amino acids to produce their proteases. Given that *B. bacteriovorus* HD100 lacks the anabolic and catabolic pathways for many amino acids [7], its need for amino acids is not surprising. These results demonstrate that, much like HIB cells [13], planktonic *B. bacteriovorus* HD100 also secrete proteins when extracellular amino acids are available. Not only does it secrete these proteins, but the expression data in Fig. 2a shows that it also synthesizes them. The data in this figure clearly shows that these genes are not expressed constitutively and simply not translated due to a lack of amino acids. Rather, planktonic *B. bacteriovorus* HD100 actively responds to the presence of extracellular nutrients by changing its transcriptome and increasing the production of proteases. Secretion of these proteases will, in turn, hydrolyze extracellular proteins and generate more amino acids, which can serve as building blocks and an energy source for the further production of proteases and other proteins in an iterative process.

Changes in its transcriptome extended far beyond these few protease genes, however, as genome-wide RNA sequencing found 1× NB induced more than 60%

of *B. bacteriovorus*' genes by 1.5-fold or more. Focusing on genes annotated as putative serine proteases (either extracellular or of unknown location), we found that their expression patterns resembled those reported by Karunker et al. [9] when the AP and the late IP growth phase (GP3) were compared (Fig. S1). We also looked at the putative protease genes showing upregulation in Lambert et al. study [12] at 30 min post-predation. However, only 4 of the 10 genes were significantly upregulated ($P > 0.05$) in our study (Table S2, worksheet 4). This suggests that the other six genes are likely induced by interactions between the predator and the prey, or its components, rather than nutrients.

When we looked deeper into the whole transcriptomic profile provided by the RNA sequencing data, we noticed that the overall patterns were similar with those reported previously during the late IP growth [9], as illustrated when each gene was classified according to its gene ontology (Fig. 3). The similarity went deeper, however, as among the 1557 genes identified as being specific for late IP growth by Karunker et al. [9], 94% (1464 genes) were also significantly upregulated by 1× NB media in this study. Given that the reference samples in both studies were basically identical, i.e., planktonic AP *B. bacteriovorus* within HEPES buffer, the similarities between the gene expression patterns and the eggNOG results suggest that the conditions experienced by late IP predators within their prey and planktonic AP predators in 1× NB are analogous to one another. This similarity also implies that the gene expression patterns seen during the late IP growth may result from amino acids becoming available to the predator as prey proteins are hydrolyzed. This will be studied further in a subsequent study.

It should be noted that although there is a significant correlation between our study and that of Karunker et al. [9], the differential gene expression patterns in Karunker's study tended to be sharper in general for both upregulated and downregulated genes for both serine proteases and across the whole genome (Figs. S1 and S2, respectively). One intriguing idea is that these difference result from regulatory networks within the predator. For instance, in Karunker et al. [9], eight small RNAs (sRNAs) were identified that are specific to the attack phase and highly downregulated in the late IP growth phase. The range of downregulation for these sRNAs was between 16- and 5295-fold. In this study, with the exception of APsRNA5 which was actually expressed more strongly, these AP-specific sRNAs were downregulated only slightly in 1× NB when compared to HEPES (1.3- to 5.1-fold; Table S2, worksheet 4). This included the merRNA, which was repressed only 1.3-fold when *B. bacteriovorus* HD100 was incubated in 1× NB. By comparison, the merRNA expression was downregulated 5006-fold when the predator shifted

from AP to late IP growth [9]. As the merRNA was found to harbor a c-di-GMP riboswitch, Karunker et al. speculated that it may act in a "sponge-like action," absorbing the cellular c-di-GMP during the AP and, upon entering the prey, releasing this signaling molecule [9]. If true, this would help to explain some of the differences seen between the two studies as the merRNA population remains largely unaffected in nutrient media. However, one may gather from these results that the merRNA does not strongly regulate many of the late IP genes as their expression is still induced during the AP by extracellular nutrients.

It is also worthy to mention that the *hit* locus and its nearby pilus genes (*Bd0108~Bd0119*) were downregulated by approximately 2~3-fold (Table S3, worksheet 4). Given the importance of this locus for predation [30, 31] and its high expression level in the attack phase compared to the intraperiplasmic phase [9, 32], this might partially explain why *B. bacteriovorus* predation is less efficient in rich media as previously found by other groups [33, 34]. In Rotem et al., it was found that incubating planktonic AP predatory cells with ghost prey cells together with either PYE medium or prey extract led to the silencing of the hit locus (*Bd0108*) and that was attributed mainly to the presence of the ghost prey cells [10]. However, downregulation of *Bd0108* in this study suggests that the availability of nutrients inside the prey may also be an additional stimulus.

Looking at the eggNOG categories shown in Fig. 3 found that the *N* group, which contains mainly the flagellar assembly genes, had more genes downregulated than upregulated upon shifting from HEPES to NB (this study) or from AP to GP3 [9]. However, for the GP3/AP shift, another group was also downregulated, the *NT* group. This group mainly contains genes involved in chemotaxis (Table S3). Given this observation, our study suggests that incubating the predator in a nutritious environment does not shut the chemotactic system down. This in turn suggests that, although *B. bacteriovorus* is provided the nutrients necessary for its metabolism and maintenance, this predator is still searching for prey.

Images of the predators incubated for up to 3 days in HEPES show that their sizes were reduced, a result that has been seen with other bacteria [35, 36] and presumably occurs from the ~~bacterium~~bacteria consuming their own cellular components to maintain their viability [37, 38, 39]. The predators in nutrient media, however, were elongated, reaching average lengths that were nearly twice as long as the predators in the HEPES buffer (Fig. 1e and Table 1). Taken together with the transcriptome results, where most of the genes are induced in the nutrient media, it is clear that planktonic AP *B. bacteriovorus* use the nutrients in the media for more

than simply producing proteases. For *B. bacteriovorus*, it was reported that prey cell extracts alone are not sufficient to induce growth and division but that interactions with the prey cell membrane are also required [10]. In their study, Rotem et al. saw the predator number increasing by more than 10-fold in 2 days when prey extracts and ghost envelopes were used together [10]. In contrast, when the cell extracts or ghost envelopes were used alone, or ghost envelopes with nutrient media (PYE), the predator populations did not increase. The results here agree with their findings, as well as several other studies [30, 40], and support the idea that some specific conditions or signals provided by the prey are required for *B. bacteriovorus* replication to occur. Although our predatory cells were elongated after 48 h of incubation in nutrient media, their population did not increase.

AQ6

In conclusion, although *B. bacteriovorus* lacks the genes necessary for the biosynthesis and degradation of numerous amino acids and, thus, it was suggested that planktonic *B. bacteriovorus* cells ~~exist in a pseudo-starvation mode and~~ may be dependent on their amino acid storage pool for protein synthesis during the AP [7], this study demonstrates that this is not completely true. We show here that when planktonic AP *B. bacteriovorus* HD100 cells encounter amino acids in the surrounding media, they utilize them to synthesize and secrete proteases. Moreover, the presence of extracellular nutrients also induces significant changes in the transcriptome of this predator. These transcriptome changes were found to be analogous to those seen during late IP growth of the predator, a finding that implies that the induced expression of these genes during predation results partially from the hydrolysis of prey proteins.

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Author Contributions

M.D. and H.I. designed and carried out the experiments. R.J.M. supervised the experimental work. M.D., H.I., and R.J.M. evaluated the data. M.D. and J.S.K. performed the mass spectrometry experiments. J.S.K. performed the mass spectrometry analyses. M.D., H.I., J.S.K., and R.J.M. wrote the manuscript.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Electronic Supplementary Material

ESM 1 A replacement supplementary text is attached in which the annotations in the references were slightly corrected to keep consistency

(DOCX 780 kb)

ESM 2

(XLSX 16 kb)

ESM 3

(XLSX 1127 kb)

References The "DOI" was removed from all references and the annotations were slightly corrected to keep consistency

AQ7

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