For publication

**Identification and differential production of ubiquinone-8 in the bacterial
predator *Bdellovibrio bacteriovorus***

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Abstract

Bdellovibrio bacteriovorus 109J, a predatory bacterium with potential as a bacterial control agent, can exist in several lifestyles that differ both in predatory capacity and color. We determined that levels of ubiquinone-8 contribute to the distinctive but variable yellow color of different types of *Bdellovibrio* cells. Steady-state ubiquinone-8 concentrations did not differ markedly between conventional predatory and host-independent *B. bacteriovorus* despite upregulation of a suite of ubiquinone-8 synthesis genes in host-independent cells. In contrast, in spatially organized *B. bacteriovorus* films, the yellow inner regions contain significantly higher ubiquinone-8 concentrations than the off-white outer regions. Correspondingly, RT-PCR analysis reveals that the inner region, previously shown to consist primarily of active predators, clearly expresses two ubiquinone biosynthesis genes, while the outer region, composed mainly of quiescent or stalled bdelloplasts, expresses those genes weakly or not at all. Moreover, *B. bacteriovorus* cells in the inner region of week-old interfacial films, which are phenotypically attack-phase, have much higher UQ8 levels than regular attack-phase bdellovibrios, most likely because their “trapped” state prevents a high expenditure of energy to power flagellar motion.

Keywords: Bacterial metabolism; Electron transport; Gene expression; Phase variation; Predator-prey interactions

1. Introduction

The Gram-negative, predatory bacterium *Bdellovibrio bacteriovorus* has attracted interest in recent years due to its potential to control a broad range of bacterial populations without chemical antibiotics. *B. bacteriovorus* is a small (~1 μm in length) organism with a single polar flagellum that propels it at speeds of up to 100 cell lengths s^{-1} . Upon attaching to a Gram-negative prey cell, it enters the periplasm, sometimes dropping its flagellum [1]. The prey cell, which dies within 10 min after penetration, combined with the attacking *Bdellovibrio*, is termed a bdelloplast. The predator begins a growth phase in which it consumes the prey cytoplasm and elongates inside the bdelloplast. As the prey cell's cytoplasm becomes exhausted, *B. bacteriovorus* septates into multiple cell progeny, each of which develop a new flagellum and ultimately exit the depleted bdelloplast as new attack-phase *B. bacteriovorus* cells.

In addition to *Bdellovibrio*'s predatory life cycle, historically termed "host-dependent" (HD), saprophytically-growing variants or "host-independent" (HI) cells have been documented since *B. bacteriovorus* was first discovered [2]. These HI cells may arise out of mutation [3-5] or phenotypic variation [6,7]. Moreover, another *B. bacteriovorus* lifestyle was documented in which a differentiated film forms on a rich media agar surface [8]. Gene regulation in biofilms can differ markedly from that in planktonic cells, leading to distinct phenotypes [9]. Although the spatially organized films examined here are not consistent with biofilms as conventionally defined, understanding more about these different lifestyles may yield insights about *Bdellovibrio*'s adaptability with applications in human and animal health, agriculture, and industry.

HI *Bdellovibrio* cells show great variety in morphology, flagellation, and color [5,10]. Most colonies are some hue of yellow and it was previously asserted that this color was due to a

1 carotenoid [5,11]. Moreover, our previous study of *B. bacteriovorus* in an interfacial, high-
2 nutrient environment yielded a film with two spatially segregated regions of *B. bacteriovorus*
3 cells, only one of which was yellow. When a small drop of prey lysate containing mostly attack
4 phase *Bdellovibrios* and a few remaining *E. coli* prey was pipetted onto LB agar, the resulting
5 growth after 3-7 days consisted of a distinctive slightly-raised, yellow inner region surrounded
6 by an off-white outer region, appearing much like a fried egg. Experiments with FISH, AFM,
7 and PCR confirmed that *B. bacteriovorus* was the predominant species in both regions but that *E.*
8 *coli* was often present as well. The inner region contained mostly small, flagellated *Bdellovibrio*
9 predators that could readily clear new prey lysates but could not form new HI colonies on LB. In
10 contrast, the outer region was comprised largely of bdelloplasts based on FISH and microscopy
11 data, but the cells thrived neither on prey cells nor on rich media. Thus, these cells in the outer
12 region of the film appeared to be in an arrested bdelloplast state [8].

13 In this work we investigate the origin of the differences in color among HD and HI *B.*
14 *bacteriovorus* and the inner and outer regions of the previously described interfacial film. We
15 find that ubiquinone-8 (UQ8) contributes to a yellow bacterial hue and further explore how the
16 UQ8 concentrations of *B. bacteriovorus* in HD, HI, or interfacial film lifestyles relate to the
17 functions of the compound.

2. Materials and methods

2.1. Bacterial growth and extraction

2.1.1. Predatory *B. bacteriovorus*

Plaque-forming units of HD *B. bacteriovorus* (ATCC 109J) were cultivated according to the procedures given by Lambert and Sockett [12]; briefly, successive dilutions of HD *B. bacteriovorus* were added to double layer agar plates in which the top layer contained *E. coli* as well as a dilute nutrient medium. Appearance of plaques after 3-5 days indicated that single cells of *B. bacteriovorus* had propagated and cleared the surrounding agar of *E. coli*. A single such plaque was added to HEPES metals buffer (HM; 1 mM HEPES, 1 mM CaCl₂, 0.1 mM MgCl₂). *E. coli* was grown in Luria Broth (LB) overnight and this suspension (approximately 25% of the volume of HM used) was centrifuged, decanted, resuspended in a small volume of HM, and added to the HD *B. bacteriovorus* suspension. The resulting mixture was shaken at 30°C for 1-3 days until it was no longer cloudy. The suspension, termed a prey lysate, was centrifuged at 1500 × *g* for 5 min to remove residual prey cells, and the resulting supernatant was again centrifuged at 10,000 × *g* for 30 min to pellet HD cells. The cells were washed with distilled deionized H₂O and recentrifuged.

2.1.2. HI *B. bacteriovorus*

HI cells were isolated from a filtered prey lysate of ATCC strain 109J as described previously; cells were confirmed to not be mutated in the Bd0108 gene [7]. Liquid-grown cells were shaken in LB for 2 days at 30°C before centrifuging. For agar-grown cells, HI cells were shaken overnight in liquid LB, then spread in 100 µL aliquots onto LB agar plates. After 2 days, the cell mass was scraped into microcentrifuge tubes using a sterilized spatula. Both liquid- and agar-

grown cells were washed once with HM and once with distilled deionized water. *E. coli* was grown under liquid and agar conditions identical to HI *B. bacteriovorus*.

2.1.3.Spatially organized films

Six- μ L aliquots of cleared HD prey lysates, as described above, were pipetted either onto a polycarbonate filtration membrane (0.2 μ m pore size) supported by an LB plate (for RT-PCR) or directly pipetted onto LB agar (for extraction). After 3-7 days the resulting film developed a yellow center and off-white outer region. A sterilized spatula was used to collect the different regions; several films (~20) were collected to gather sufficient cell material for extraction. Cells undergoing collection and extraction were washed once with HM and once with distilled deionized water.

2.2. Extraction of the yellow compound

2.2.1.Extraction method 1

To gather sufficient material from HI cells to identify the yellow compound, a simple extraction described by Friedberg [11] was used. HPLC-grade acetone was added to the cell pellet and the suspension was agitated, allowed to sit for 10-15 min, and centrifuged. The acetone layer was collected and dried down. If the cell pellet was still yellow, more acetone was added and the extraction was repeated.

2.2.2.Extraction method 2

This method was used for quantification of UQ8 in HD and HI cells as well as in spatially organized films. The microcentrifuge tubes containing the washed cells were dried in a centrifugal vacuum evaporator, added to new, pre-weighed microcentrifuge tubes, and extracted using a procedure based on that of Block [13]. Samples were crushed with a glass stir rod before

1 recording the new mass (typically 50-150 mg dried cell material). Next, 0.25 g of 0.5 mm glass
2 beads and 1 mL of 50:50 chloroform:hexanes were added to each tube. The resulting mixture
3 was vortexed at 3000 rpm for 80 min. The crude extract was passed through microcentrifuge
4 filter devices (0.22 μm), washing once each with hexanes and chloroform. The filtrate was dried
5 with a centrifugal evaporator and resuspended in chloroform prior to analysis by HPLC or DEP-
6 MS.

7 8 *2.3 Identification of yellow compound*

9 *2.3.1.Semi-prep HPLC*

10 An Agilent 1100 Series HPLC with an Agilent eclipse XDB 5 μm column (9.4 $\times$ 250 mm) was
11 used in gradient mode using isopropyl alcohol:acetonitrile in a ratio ranging from 30:70 to 70:30
12 (see Table S1 for details) at a flow rate of 4 mL min⁻¹. The peak of interest eluted at 22.5 min.

13 *2.3.2.Mass spectrometry*

14 Tandem MS was performed using an Agilent LC/MSD Trap in APCI negative ion mode. High
15 resolution MS was also conducted using a Waters LCT Premier in APCI mode.

16 *2.3.3.NMR*

17 A 300 MHz Jeol NMR was used to collect ¹H-NMR, ¹³C-NMR, DEPT, COSY, and NOESY
18 spectra.

19 20 21 *2.4. Quantification of UQ8*

22 A Waters 600 HPLC with Waters 996 photodiode array detector was used to flow 30:70
23 isopropyl alcohol:methanol at 1 mL min⁻¹ through a Nova-Pak C18 column (3.9x150 mm, 4 μm).

When developing the method and confirming that the peak at 5.2 min was UQ8, eluate from multiple injections was collected and concentrated, then run on EI-MS and CI-MS using a direct exposure probe. Both the molecular ion peak and the fragments at 197 and 235 [14,15] confirmed the peak identity.

2.5. Ubiquinone biosynthesis gene expression

Total RNA was obtained from prey lysates from HM liquid media, inner and outer regions of spatially organized films, and whole films using a TRIzol® Max™ Bacterial RNA Isolation Kit (Invitrogen). Primers used for cDNA amplification for RT-PCR are detailed in Table 1. The RT-PCR kit used was SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen). Next, PCR-amplified products (30 cycles) were analyzed by gel electrophoresis. For additional verification of the amplified sequence, restriction enzyme HindIII was used to digest the PCR-amplified sample.

2.6. Bioinformatics

We scrutinized the supplemental information from two transcriptomics studies [16,17] comparing attack-phase *Bdellovibrio* to HI *Bdellovibrio* or to HD *Bdellovibrio* that had been in a bdelloplast for 30 or 180 min to determine the relative up- and downregulation of genes related to ubiquinone biosynthesis, the electron transport pathway, flagella, or gliding motility. To assemble a full complement of genes related to ubiquinone biosynthesis and the electron transport pathway, the KEGG Pathway database was used to identify the terpenoid backbone biosynthesis (map 00900), ubiquinone and other terpenoid biosynthesis (map 00130), and oxidative phosphorylation (map 00190) pathways for genes with homologs in *B. bacteriovorus*

1 HD100. Lambert et al. [16] and Karunker et al. [17] assigned two additional genes to
2 ubiquinone biosynthesis and one other to ubiquinone oxidoreductase; BLAST confirmed strong
3 homology between these *B. bacteriovorus* proteins in a host of other proteobacteria and green
4 sulfur bacteria species ($E < 10^{-56}$). *B. bacteriovorus* and many other bacteria do not have the
5 cytochrome *bc₁* complex (Complex III) in the KEGG oxidative phosphorylation pathway, but
6 Yanyushin et al. [18] described a novel class of bacterial membrane oxidoreductases and
7 identified amino acid sequences contributing to Complex III function that were conserved among
8 many species of bacteria including *B. bacteriovorus*. BLAST was used to identify the *B.*
9 *bacteriovorus* genes with which they were associated.

10 Genes involved in gliding motility were also compiled based on published assignments in the
11 *B. bacteriovorus* HD100 genome [16], and HD100 genes with homology to genes known to
12 regulate flagellar and motor synthesis were obtained from Lambert et al. [19].

3. Results

3.1. Identity of a yellow chromophore in *Bdellovibrio*

When extracted membrane-associated material from HI *B. bacteriovorus* cells (extraction method 1) was separated by HPLC, two peaks were detected at 406 nm. We focused our analysis on the larger and more quickly-eluting peak. A second, smaller, more non-polar peak that subsequently decomposed upon addition of CDCl_3 was presumed to be a carotenoid, potentially that observed by Friedberg [11] and Barel and Jurkevitch [5].

The purified eluate from the main HPLC peak yielded a UV-visible spectrum with a large peak at 272 nm, a shoulder at 326 nm, and a small peak at 406 nm. In MS-MS analysis, the striking pattern of removal of a 69 atomic mass unit (amu) fragment and 6 other visible 68 amu fragments is clearly indicative of an isoprenoid tail (Fig. 1a). Given that isoprene is used to synthesize a wide variety of lipids, the identity of the head group remained to be determined. NMR analysis revealed that the molecule was a ubiquinone (Fig. 1b; Fig. S1-3), and this was confirmed by comparison to commercially available UQ10. Moreover, the initial losses of two 15 amu fragments in MS analysis are consistent with the two methoxy groups on the quinone. High-resolution mass spectrometry verified that the compound was $\text{C}_{49}\text{H}_{74}\text{O}_4$ (sodiated mass 749.5446, measured compound mass 726.5548; calculated mass 726.5587), or ubiquinone-8 (UQ8).

Although ubiquinone is an expected membrane component of Gram-negative bacteria, it is striking that it is the *primary* contributor to the yellow appearance of HI *B. bacteriovorus* cell extract. When using extraction method 2 to process smaller batches of cell material, a handful of peaks were visible on the HPLC chromatogram at 275 nm, but only the UQ8 peak remained at 406 nm (Fig. S4). Measured UQ8 levels correlated with cell color, but the contribution of

1 carotenoids cannot be fully discounted, particularly after observing a likely signal during HPLC
2 analysis of the first extraction. The lack of an observed carotenoid signal in the second
3 extraction could stem from the centrifugal evaporation of the cells, subsequent exposure to air, or
4 addition of different solvents. The high reactivity of many carotenoids, which is often perceived
5 as exacerbated at small concentrations due to reaction with trace compounds, likely renders them
6 unstable in the second extraction method.

7 8 3.2. UQ8 production by *B. bacteriovorus* grown under different conditions

9 After identifying UQ8 as a yellow chromophore in HI cells, we considered whether different
10 lifestyles of *B. bacteriovorus* might show distinct trends in UQ8 production reflecting their
11 different metabolic needs. In Fig. 2, UQ8 levels are compared for predatory and HI
12 bdellovibrios, with the latter grown both in LB broth and on LB agar. Notably, predatory and
13 HI cells produce roughly similar amounts of UQ8. Neither does motility seem to play a major
14 role in UQ8 concentration, since HI cells in both liquid and semi-solid agar media (where gliding
15 is critical) produce comparable amounts of UQ8. Nevertheless, HI cells grown either in broth or
16 on agar show substantially higher *variability* than predatory cells or *E. coli* grown on both media
17 types. This variation in overall normalized UQ8 concentrations among different HI samples does
18 not appear to be due to instrumental variability or culturing conditions but instead to genuine
19 inconsistency of UQ8 concentrations in individual cell populations: the replicate isolations and
20 measurements of parallel HD and *E. coli* samples are quite similar. Such variability is not
21 surprising given the inherent genotypic and phenotypic variability in HI isolates [20], but it does
22 make it difficult to draw fine comparisons between UQ8 production in HI and HD bdellovibrios.

1 Since *E. coli* has been well-studied with respect to UQ8 production and this prey strain is a
2 potential contributor of UQ8 to the interfacial film, we considered its UQ8 concentration as well.
3 In general, UQ8 content in *E. coli* appears slightly smaller than that in predatory *B.*
4 *bacteriovorus* but this difference is not significant at the $p = 0.05$ level.

5 We next considered UQ8 production in a spatially organized film consisting mostly of *B.*
6 *bacteriovorus* with a minor presence of prey *E. coli*. (Fig. 3a, inset). Given the striking
7 difference in color between the yellow inner regions and off-white outer regions, we compared
8 UQ8 levels in the inner versus the outer regions of these films. Indeed, despite large variability
9 among samples, the yellow inner region consistently has much higher UQ8 levels than the off-
10 white outer region. If a single batch of films is cultivated and sampled over several weeks, it is
11 evident that the UQ8 content of the inner region decreases as the film ages (Fig. 3b). This time
12 dependence is also evident across the different sample sets, with the 6- and 7-day films having
13 higher UQ8 than the 10-day films (Fig. 3a). Moreover, all three of the younger films have
14 exorbitantly high UQ8 levels in the inner region compared to HD *B. bacteriovorus* (or *E. coli*);
15 one set of inner region cells collected from 10-day films also has UQ8 levels higher than the
16 range exhibited by HD *B. bacteriovorus*. The UQ8 concentrations measured in the outer film
17 region cells range from significantly lower than the lower range of *E. coli* to slightly above the
18 upper range of HD. Note that when collecting the outer and inner regions, some degree of cross-
19 contamination is likely despite removal of the boundary region, which may explain why samples
20 with the highest UQ8 content in the inner region also have relatively high UQ8 levels in their
21 outer regions.

22 The striking difference between ubiquinone content in the inner and outer regions of
23 phenotypically differentiated films grown on LB agar warranted further study. Since this

spatially organized film has not been previously examined for gene expression beyond those experiments needed to identify cells as *B. bacteriovorus* or *E. coli*, we conducted RT-PCR to probe how the expression of two genes involved in ubiquinone biosynthesis (Bd0422 and Bd2350) compared in the two film regions. Bd0422 was chosen to represent UQ biosynthesis genes that are upregulated in both early bdelloplasts and HI cells, whereas the Bd2350 gene is unique in that it is the only UQ biosynthesis gene to show no up- or downregulation among the different HD and HI cultures examined in previous studies [16,17]. Positive and negative controls performed as expected (Fig. S5): RNA from HD *B. bacteriovorus* prey lysate hybridized to primers from Bd0422 and Bd2350 as well as from *B. bacteriovorus* 16S rRNA. In contrast, the *E. coli* ML35 negative control exhibited no signal for any of the three *Bdellovibrio*-derived RNA sequences. Although the *E. coli* genome contains UQ8 biosynthesis genes that have some homology to those in *B. bacteriovorus*, the two primers used here match the *E. coli* genome for only 15 and 13 out of 20 nucleotides, resulting in poor amplification. Thus, the observed gene expression is not due to contamination by prey bacterial RNA, and even if a *B. bacteriovorus* sample is slightly “diluted” by prey nucleic acids, sufficient PCR cycles were conducted to ensure that even a small amount of hybridizing RNA would generate a strong signal.

Fig. 4 extends examination of the expression of these two ubiquinone-related genes to spatially organized films of *B. bacteriovorus*. The inner yellow region, which contains primarily small (<1 μ m), highly motile cells that readily clear a lawn of *E. coli* [8], clearly expresses both Bd0422 and Bd2350. In contrast, the band for the Bd2350 amplicon in the outer white region is ambiguous and Bd0422 expression is not detected at all, indicating that UQ8 production via these gene pathways is extremely low or non-existent in the outer region. The whole film, as expected, shows features of both the inner and outer regions. Note that a control with *B.*

1 *bacteriovorus* 16S rRNA primers was positive for both the inner and outer regions as well as the
2 whole film (Fig. S6), confirming that *Bdellovibrio* cells are found in both regions of this film.
3 For additional verification, restriction enzyme HindIII was used to digest the PCR-amplified
4 sample (Fig. S7). These results further corroborate the higher measurements of UQ8 in the inner
5 region compared to the outer region.

6 Additional bands were observed in the lanes featuring the outer region for both Fig. 4 and Fig.
7 S7. Since this outer region is highly pleiomorphic and includes many bdelloplasts, yet does not
8 induce clearing [8], it was suggested that the observed bdelloplasts were similar to the “stable
9 bdelloplasts” as originally described by Sanchez-Amat and Torella for *Bacteriovorax* spp. [21],
10 perhaps formed as a way for these predators to persist in environments with low prey density.
11 Stalled bdelloplasts that lyse after several days, if at all, have also been reported for *B.*
12 *bacteriovorus* under genomic and environmental conditions affecting cytoskeletal protein levels
13 [22]. Thus, the additional bands in the PCR gels may stem from cross-hybridization of smaller
14 RNA fragments created as the *B. bacteriovorus* in the bdelloplasts slowly break down prey
15 components.

16 Bioinformatic mining of publically available transcriptomic data allows us to place our
17 observations of UQ8 upregulation into a broader organismal perspective. Lambert et al. [16]
18 used microarrays to compare gene expression in attack phase *B. bacteriovorus*, 30 min
19 bdelloplasts, and HI cells, whereas Karunker et al. [17] used RNA-seq to compare attack-phase
20 cells to 3 h bdelloplasts. Although data from microarrays and RNA-seq cannot be combined
21 quantitatively, we can compare trends in the transcriptomics studies for attack phase, bdelloplast,
22 and HI cells to the HD and HI growth conditions in our study. Unfortunately, there are no direct
23 parallels to the inner and outer regions of the spatially organized film. Though Lambert et al.

[16] described the overarching ways in which gene expression in attack-phase HD and HI *B. bacteriovorus* related to lifestyle differences, we focus here on genes related to UQ synthesis or function.

Both of the *B. bacteriovorus* transcriptomics studies cited above employed strain HD100 rather than HD109J as used in this work, but comparisons here are not likely to be affected significantly. First, Wurtzel et al. [23] determined that the nucleotide sequence for 109J differs from that of HD100 [24] by only 0.75%, and the total percentage of non-synonymous changes is 0.11%. Moreover, since ubiquinone is such a fundamentally important molecule, genes associated with ubiquinone are much more likely to be conserved among *B. bacteriovorus* strains.

The HD100 genome contains 11 genes known to be associated with UQ biosynthesis and the prerequisite terpenoid backbone biosynthesis. These, along with the genes identified as part of the oxidative phosphorylation pathway, are detailed in Table 2. Although there is little significant difference in expression of the selected genes for 30 min bdelloplasts compared to attack phase cells, both 3 h bdelloplasts and HI cells demonstrate *overwhelming upregulation* of these genes relative to attack phase cells. When the average upregulation of the upregulated ubiquinone-related genes is normalized by the average upregulation of all upregulated genes in the genome (5.65 for the microarray study; 79 for the RNA-seq study), it is evident that the terpenoid and ubiquinone biosynthesis processes are moderately upregulated in HI and 3 h bdelloplasts, and genes involved in all steps of oxidative phosphorylation are upregulated strongly. Cells are actively growing in both HI and 3 h bdelloplast samples, whereas they do not grow in the attack phase, so this uptick in electron transport is to be expected. Given the degree

1 to which proteins encoded by the oxidative phosphorylation genes are upregulated, it is not
2 surprising that more UQ8 must be synthesized.

3

4

4. Discussion

We determined that the distinctive yellow color of *B. bacteriovorus* correlates with UQ8 content, although at least one type of carotenoid is likely also present. Ubiquinone of varying isoprenoid chain lengths is found in most Gram-negative aerobic bacteria. Some facultative anaerobes produce both ubiquinone and menaquinone, whereas anaerobes and Gram-positive bacteria generally produce menaquinone only [15,25]. Due to its ability to undergo two reversible 1-electron transfers accompanied by proton transfers, ubiquinone plays a critical role in the electron transport chain and generation of proton-motive force (PMF). It and its reduced form, ubiquinol, have also been documented to participate in redox sensing [26,27], enhance membrane fluidity [28,29], serve as a membrane-based antioxidant [27], and promote antibacterial activity in animals [13,30]. Different types of tissue have been shown to contain varying amounts of ubiquinone in rats, mice and humans [31], most likely reflecting differential needs depending on the tissue function. In an effort to understand the suite of functions that this molecule plays in *B. bacteriovorus*, we quantified the UQ8 in *B. bacteriovorus* cells growing in different lifestyles.

The most striking observation is the large difference between UQ8 levels in the inner and outer regions of the prey lysate films grown on LB agar. This distinction is corroborated by RT-PCR experiments with two ubiquinone biosynthesis genes. These trends in ubiquinone are consistent with the aforementioned description of cellular segregation because cells in an attack phase phenotype (inner region) need to employ the electron transfer pathway and generate PMF considerably more than cells in a quiescent state (outer region). FISH, RT-PCR, and AFM analyses have shown that *E. coli* are sometimes detected and sometimes not found in both the inner and outer regions of the spatially organized film. When *E. coli* cells are detected, they are

generally found in modest numbers [8]. Thus, although we cannot completely discount the potential contribution of UQ8 derived from *E. coli* rather than *B. bacteriovorus*, this concern is of only minor importance for two reasons. First, *E. coli* cells produce similar or slightly smaller quantities of UQ8 per dry cell mass than *B. bacteriovorus* (Fig. 1), so a minority population of *E. coli* would be expected to produce only a fraction of the UQ8. Moreover, the inner region has a significantly greater UQ8 concentration than either typical *B. bacteriovorus* or *E. coli* (Fig. 2a).

Note that of Bd0422 and Bd2350, the two genes found in this study to be expressed differently between the inner and outer regions, only Bd0422 was shown to be upregulated for HI or bdelloplasts. In contrast, Bd2350 changes expression relative to attack-phase neither in HI nor in bdelloplasts, perhaps reflecting a base level of UQ8 production. Its ambiguous expression in Fig. 4 is consistent with the outer region population being composed primarily of senescent cells.

Remarkably, despite the gene upregulation for HI cells as well as 3 h bdelloplasts in Table 2, there is no significant difference in the cell mass-normalized UQ8 levels measured in predatory *B. bacteriovorus* compared to HI cells grown either in liquid or on agar media. Clearly, the high genotypic and phenotypic variability in HI cells contributes to this conservative statement; certain HI samples have extremely high UQ8 concentrations that dwarf those in the typical HD sample. But on average, the lack of distinction of UQ8 levels between HI and HD *B. bacteriovorus* is likely due to the experimental focus on the *steady-state* concentration of UQ8 in this work compared to the two transcriptomics studies. In the attack phase, locating and penetrating prey are the key priorities, so ATP is consumed to run the flagellar motor, but no synthesis of DNA is occurring. In contrast, after entering the prey cell periplasm and forming a bdelloplast, *B. bacteriovorus* uses prey-cell derived molecules to grow and divide. HI cells are also in growth phase but use nutrient media. UQ8 upregulation due purely to cell growth and

1 production of more biomass would not stand out in this study, despite potentially prodigious
2 UQ8 production, due to the normalization of UQ8 signal by dry cell mass. In addition, an
3 increase in UQ8 production concomitant with a proportional increase in cellular consumption of
4 UQ8 would show no effect here.

5 In addition, because HI cells are substantially larger than HD cells, equal cell masses of HD
6 and HI yield a greater number of HD cells, meaning that the UQ8 amount *per cell* is much
7 greater for HI cells. However, due to UQ8's role in basic cellular maintenance, larger cells are
8 expected to contain more UQ8 molecules. The added advantage of quantifying UQ8 per dry cell
9 mass is that enumeration of cells by plaque-forming units (for HD *B. bacteriovorus*) and colony
10 counts (for *E. coli* and HI *B. bacteriovorus*) are not critical. Although it would ideally be
11 desirable to determine the exact numerical breakdown of *B. bacteriovorus* and *E. coli* in the
12 inner and outer regions of the spatially organized film, this is not possible due to film
13 heterogeneity.

14 Comparing steady-state, cell-mass-normalized UQ8 concentrations to time-dependent up- and
15 downregulation of UQ8-related genes also sheds light on a seeming disparity: the attack-phase
16 cells in the inner region of the 6- to 7-day film have extraordinarily high UQ8 concentrations
17 compared to all HD and most HI samples. These attack phase cells in the inner region of the
18 film may have similar UQ8 production rates compared to typical HD cells, but their confinement
19 in a small, relatively dry area prohibits significant flagellar activity, thus conserving both energy
20 and UQ8. Although UQ8 gets recycled during the electron transport pathway, each cycle
21 provides a new opportunity for wasted energy and UQ8 degradation. Moreover, Pasternak et al.
22 [32] demonstrated that *B. bacteriovorus*, along with all 10 other predatory bacteria species with
23 published genomes, use the mevalonate pathway to synthesize ubiquinone, as do animals,

whereas other bacteria and plants use the non-mevalonate pathway. In the non-mevalonate pathway, pyruvate and glyceraldehyde-3-phosphate are used to ultimately make isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which are building blocks for a suite of biologically relevant isoprenoid molecules including ubiquinone. In contrast, the mevalonate pathway uses acetyl CoA or acetoacetyl CoA as a precursor. Although pyruvate is required to make acetyl CoA, and therefore the mevalonate pathway would be expected to be less energetically favorable, using acetyl CoA previously synthesized by prey species is clearly more advantageous. Both prey cells and LB media contain acetyl CoA that could be co-opted for this purpose, so the predatory *B. bacteriovorus* cells growing as a film on LB agar would not be limited by available precursor molecules. Thus, they could produce more UQ8 while not consuming it as quickly, resulting in a greater steady-state concentration.

Pasternak et al. [32] also noted that homologues of the TT1927b protein from *Thermus thermophilus* were more common in *Bdellovibrio* and like organisms than in non-predatory bacteria. The crystal structure of this protein suggests that this protein family (the YceI-like family) is involved in the metabolism, transport or storage of isoprenoid quinones such as UQ8 [33]. A PSI-BLAST search on the *B. bacteriovorus* HD100 genome yielded 4 close homologs of the TT1927b protein. All four of these gene homologs demonstrated similar expression between attack phase HD and 30-minute bdelloplasts, whereas two of the four genes were comparatively downregulated for HI growth, one was upregulated, and one showed no significant difference [16]. It is perhaps not surprising that regulatory patterns differ among the four genes given that UQ8 transport, metabolism or storage would be expected to yield different patterns.

4.1. Functions of UQ8 in *B. bacteriovorus*

The primary function of ubiquinone in *B. bacteriovorus*, as with the ubiquinones, menaquinones or phyloquinones produced by nearly all life forms, is most likely its role in electron transport. Attack-phase bdellovibrios do not grow, although other cell maintenance is, of course, necessary. Rather, growth occurs during the bdelloplast part of the life cycle, which was not examined in this study. HI cells, on the other hand, actively grow in rich media. The differentiated film resulting from prey lysate pipetted onto LB agar is particularly intriguing, since prey lysates filtered to remove residual prey cells do not grow without new prey except as HI isolates. Yet, both the inner and outer regions of the film undergo macroscopic growth. That of the outer region may be due to growth of *E. coli* that are subsequently attacked by *B. bacteriovorus*, whereas the expansion of the inner region could stem from an increase in attack-phase cells after the first several cycles of predatory growth during the first few days when the film is developing.

As UQ8 is reduced first to a semiquinone and then to ubiquinol-8, the 2-electron transfer is accompanied by the acceptance of 2 protons. This generates a proton motive force (PMF) across the cytoplasmic membrane, which, while best known for facilitating ATP generation, also directly powers *Bdellovibrio*'s polar flagellum [34,35] as well as adventurous gliding [36-38]. As a response to being grown on agar, one might expect UQ8 levels to be scaled up to better power gliding motility, but that was not observed in this study, either for HI or *E. coli*.

Distinction of flagellar and gliding motility becomes murkier when comparing HD and HI *B. bacteriovorus*. Flagellar motion is only advantageous in aqueous environments, including the prey lysate conditions in which HD *B. bacteriovorus* are typically cultured. Both published studies on gene expression in *B. bacteriovorus* have demonstrated overwhelming downregulation of flagella-related genes in bdelloplasts and HI cells compared to attack-phase HD cells (Table

S2), even though the HI cells examined had flagella [16,17]. Gliding motility, on the other hand, is crucial for HD cells in low-moisture environments [10,39] such as agar, soil, and human and animal intestines, but genes related to gliding motility are mostly upregulated in HI cells compared to attack-phase HD cells (Table S2). Consistent with exiting the bdelloplast, 3 h bdelloplasts also showed partial upregulation of gliding motility genes compared to attack-phase cells. This offers a possible reason for the lack of significant difference in the steady-state UQ8 levels between HI and HD found in this study. In predatory cells, UQ8 would be useful for its role in generating PMF for flagellar propulsion, whereas in HI cells, that PMF could be directed toward powering gliding motility.

To assess the biological effect of externally applied UQ8 on cell growth, crude extract from extraction method 2 was spotted and streaked onto LB agar both before and directly after spotting and streaking both *E. coli* and HI *B. bacteriovorus* cells. No impact on cell growth was observed.

Ubiquinone has also been well-characterized in *E. coli* as part of a redox-sensing pathway [26,27,40-42]. Under anaerobic conditions, ArcB, a two-component sensor kinase, autophosphorylates and transfers a phosphate group to ArcA, a transcriptional regulator that stimulates anaerobic respiration. Under aerobic conditions, however, when the ubiquinone/ubiquinol pool is primarily ubiquinone, ubiquinone serves as the oxidant to form two disulfide bonds in a thiol-based redox switch, thus inhibiting ArcB's activity and maintaining aerobic metabolism [27]. If quinones are not present, the reducing conditions in the cytosol prevent disulfide bond formation and the cell undergoes anaerobic metabolism even if grown in aerobic conditions [26]. Although *B. bacteriovorus* has historically been termed an obligate aerobe in the laboratory, the environment inside the bdelloplast is likely microaerobic.

Moreover, *Bdellovibrio*'s common existence in the human gut [43] indicates that *B. bacteriovorus* is indeed capable of thriving in an anaerobic environment. Like *E. coli* and other facultative anaerobes, *B. bacteriovorus* has a suite of menaquinone synthesis genes in addition to those for ubiquinone synthesis. Although production of UQ8 for the purpose of maintaining a UQ8 concentration sufficient to sense the quinone/quinol ratio may be an important factor in *B. bacteriovorus* persistence in a range of redox conditions, the experimental conditions employed here were all aerobic. Future studies examining gene expression and steady-state concentrations of both ubiquinone and menaquinone in microaerobic and anaerobic environments would provide interesting fodder for examining this role.

In conclusion, UQ8 has been identified as a contributor to the yellow color of *B. bacteriovorus* cells. Steady-state quantities of UQ8 do not differ significantly for HD and HI bdellovibrios, indicating that the upregulation in UQ biosynthesis genes for HI shown in previous studies is due to cell growth and the resulting production of all components related to the electron transport pathway. Thus, although more UQ8 synthesis is needed, the amount per mass dried cells remains approximately constant. UQ8 is required to help generate PMF to power both flagellar motion (important for HD) and adventurous gliding (important for HI), but the upregulation of these two sets of cellular machineries and the concomitant UQ8 requirement appear to balance out. When unfiltered HD prey lysate is pipetted onto LB agar, spatially organized films with a yellow inner region and off-white outer region, both of which contain *B. bacteriovorus*, develop within days. UQ8 levels are dramatically higher for the yellow inner region compared to the off-white outer region, and RT-PCR experiments confirm greater expression of *B. bacteriovorus* UQ8 biosynthesis genes in the inner region. These findings support active metabolism with limited opportunity for flagellar activity in the inner region and the existence of primarily

1 quiescent stalled bdelloplasts in the outer region. *Bdellovibrio*'s adaptation to a surface-
2 associated lifestyle and its differential production of UQ8 is a novel example of phase variation,
3 providing clues about how *B. bacteriovorus* may persist in diverse environments including the
4 human body.

5

6

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1   **Legends to figures**

2   **Fig. 1.** (a) Tandem MS spectrum of purified extract from HI *B. bacteriovorus* using APCI
 3   ionization in negative ion mode. m/z values 726.6, 711.7 and 696.7 were sequentially selected
 4   prior to this mass spectrum collection. (b) ^1H -NMR spectra of the purified yellow compound in
 5   CDCl_3 with UQ8 structure (inset). In some cases non-equivalent protons are labeled with
 6   identical letters due to the similarity of their spin environments and resulting NMR signals.

7   **Fig. 2.** UQ8 signal based on HPLC analysis normalized to dry cell mass for predatory (HD) *B.*
 8   *bacteriovorus*, HI *B. bacteriovorus* (grown both on agar and in liquid), and *E. coli* (grown both
 9   on agar and in liquid). Each column represents a replicate culture. Error bars denote one
 10   standard deviation based on three injection replicates.

11   **Fig. 3.** (a) UQ8 signal based on HPLC analysis normalized to dry cell mass is shown for the
 12   inner (dark) and outer (light) regions of spatially organized film from different samples. Error
 13   bars show one standard deviation based on three injection replicates. The colored horizontal
 14   regions show the overlapping ranges of UQ8 levels found in HD *B. bacteriovorus* (green and
 15   yellow-green) and *E. coli* (yellow and yellow-green). Inset: The spatially organized film that
 16   develops within days of pipetting an unfiltered *B. bacteriovorus* prey lysate on LB agar. (b)
 17   Normalized UQ8 levels decrease as a function of time in both the inner (black diamonds) and
 18   outer (white circles) regions of the differentiated film.

19   **Fig. 4.** Gel electrophoresis of RT-PCR products prepared with spatially organized films of *B.*
 20   *bacteriovorus* 109J. Lane 1 contains a 100 bp ladder (asterisk at 600 bp). Lanes 2-4 show
 21   amplicons produced with the Bd0422 primer hybridized to cDNA from the inner region, outer
 22   region, and whole film, respectively. Likewise, lanes 5-7 contain the Bd2350 primer hybridized
 23   to cDNA from the inner region, outer region and whole film, respectively.

Figure 1

Figure 2

Figure 3

Figure 4

Table 1. Primers used for RT-PCR.

Gene	Primers	Amplicon size (bp)
Bd0422	F 5' – GGCGATACCGGAAGAGCGCA – 3'	758
	R 5' – GCATTGCCCAGGTCACCGC – 3'	
Bd2350	F 5' – GGCATCCTGGAACGGCAGGT – 3'	608
	R 5' – ACAAGGCCCTGGAGCCACAC – 3'	
16S rRNA	F 5' – GATCCCTCGTCTTACCGC – 3'	765
	R 5' – GCGGTAAGACGAGGGATC – 3'	

F: forward (complementary to template strand)

R: reverse (complementary to coding strand)

Table 2. Relative expression of UQ8-related genes in HI, 30-min bdelloplasts, and 3-hour bdelloplasts compared to attack-phase bdellovibrios. Values in red and blue correspond to up- and down-regulated genes, respectively. Dashes indicate no significant change in gene expression. Black values in the title subsection bars represent the average upregulation for the upregulated genes in the corresponding subsection normalized by the average upregulation of all upregulated genes in the entire genome.

Gene Number	Likely gene homolog	X-fold change in HI ^a	X-fold change for 30 min bdelloplast ^a	X-fold change for 3-hr bdelloplast ^b	Gene Number	Likely gene homolog	X-fold change in HI ^a	X-fold change for 30 min bdelloplast ^a	X-fold change for 3-hr bdelloplast ^b
Terpenoid backbone biosynthesis		0.9		0.8	Complex II - succinate dehydrogenase		3		3
Bd0404	atoB	7.1	-	4.6	Bd0945	etf-QO	6.3	-	18
Bd1627		6.0	-	93	Bd0024	sdhC	28	-	540
Bd1630	mvk	1.8	-	20	Bd0025	sdhA	15	-	110
Bd1629		1.9	-	33	Bd0026	sdhB	6.1	-	110
					Complex III – quinol oxidase		5		4
Bd0199	yqiD	7.4	-	56	Bd1607		6.6	-	150
Bd0557	ispB	4.9	-	150	Bd1608	hmoA	22	-	170
Ubiquinone biosynthesis		1		0.4	Bd1609		34	-	400
* Bd0422	aarF	3.1	-	44	Bd1610		34	-	270
* Bd2350		-	-	-	Bd1611		34	-	210
Bd3543		5.4	1.5	54	Bd1612		9.6	-	230
Bd3487	ubiE	7.3	1.4	32	Complex IV - Cytochrome c oxidase		5		3
Bd3625	ubiG	0.25	-	9.0	Bd0286	ctaC	21	-	160
Oxidative phosphorylation					Bd0290	ctaB	0.65	-	260
Complex I - NADH hydrogenase		3		2	Bd0288	ctaE	32	-	190
Bd0332	ndh	-	-	32	Bd0287	ctaD	25	-	240
Bd3080	nuoI	40	-	88	Bd2217	ctaA	-	-	-
Bd3892	nuoA	9.6	-	320	Bd2611	fixN	51	-	-
Bd3086	nuoB	27	-	140	Bd2613	fixP	9.8	-	-
Bd3085	nuoD	9.8	-	49	Complex V - ATP synthase		3		6
Bd3084	nuoE	12	-	40	Bd1010	ppk	9.7	-	7.1
Bd3083	nuoF	19	-	77	Bd0571		8.7	-	4.8
Bd3081	nuoG	22	-	93	Bd0009	atpB	8.9	-	660
Bd3891	nuoH	17	-	520	Bd3899	atpA	42	2.1	540
Bd3890	nuoJ	21	-	200	Bd3897	atpB	47	2.0	850
Bd3889	nuoK	10	-	130	Bd3898	atpG	15	2.0	600
Bd3888	nuoL	15	-	190	Bd3900	atpD	11	1.5	170
Bd3887	nuoM	30	-	240					

Bd3886	nuoN	13	-	360	Bd3896	atpC	4.1	1.7	940
					Bd3902	atpF	11	1.4	310
					Bd0010	atpE	4.0	1.7	970

*Examined by RT-PCR in this study.

^aFrom Lambert *et al.* [14], based on DNA microarrays

^bFrom Karunker *et al.* [15], based on RNA-seq