Microbial Ecology (2021) 81:347–356
https://doi.org/10.1007/s00248-020-01585-8

ENVIRONMENTAL MICROBIOLOGY

Diffusible Signaling Factor, a Quorum-Sensing Molecule, Interferes with and Is Toxic Towards *Bdellovibrio bacteriovorus* 109J

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Received: 7 June 2020 / Accepted: 24 August 2020 / Published online: 6 September 2020

Abstract

Bdellovibrio bacteriovorus 109J is a predatory bacterium which lives by predating on other Gram-negative bacteria to obtain the nutrients it needs for replication and survival. Here, we evaluated the effects two classes of bacterial signaling molecules (acyl homoserine lactones (AHLs) and diffusible signaling factor (DSF)) have on *B. bacteriovorus* 109J behavior and viability. While AHLs had a non-significant impact on predation rates, DSF considerably delayed predation and bdelloplast lysis. Subsequent experiments showed that 50 μM DSF also reduced the motility of attack-phase *B. bacteriovorus* 109J cells by 50% (38.2 ± 14.9 vs. 17 ± 8.9 μm/s). Transcriptomic analyses found that DSF caused genome-wide changes in *B. bacteriovorus* 109J gene expression patterns during both the attack and intraperiplasmic phases, including the significant downregulation of the flagellum assembly genes and numerous serine protease genes. While the former accounts for the reduced speeds observed, the latter was confirmed experimentally with 50 μM DSF completely blocking protease secretion from attack-phase cells. Additional experiments found that 30% of the total cellular ATP was released into the supernatant when *B. bacteriovorus* 109J was exposed to 200 μM DSF, implying that this QS molecule negatively impacts membrane integrity.

Keywords *Bdellovibrio bacteriovorus* · Quorum sensing · Diffusible signaling factor · Acyl homoserine lactone · Predation

Introduction

Bdellovibrio bacteriovorus is a Gram-negative, highly motile bacterium which lives by predating on other Gram-negative bacteria to get the nutrients needed for its growth and survival. Due to its unique lifestyle in the prokaryotic world, and its high potential as a biocontrol agent against harmful bacteria [1, 2], *B. bacteriovorus* has received a lot of attention during the last two decades, especially considering the current strive to identify novel biological tools and their use as alternatives to current chemical antibiotics [2–5]. Interest was enhanced further due in part to recent studies showing that *B. bacteriovorus* can attack a wide range of Gram-negative pathogens responsible for various human and animal infections [6–8].

Despite intensive research, much remains unknown about how predatory bacteria interact with other bacterial communities in the environment and how they respond to signaling molecules produced by those communities, including quorum sensing (QS) molecules. In the prokaryotic world, researchers have identified several different classes of signaling molecules, including acyl homoserine lactones (AHLs) [9, 10],

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-020-01585-8) contains supplementary material, which is available to authorized users.

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the diffusible signaling factor (DSF) [11], and indole [12, 13], among others. Within this group, AHLs represent the best studied quorum sensing molecules and likely are the most widely distributed group in nature, with bacteria from a range of genera capable of producing, detecting and responding to AHLs, often by homologues of the LuxR protein [9, 14]. Some of the Gram-negative bacterial genera that produce AHLs include *Vibrio*, *Pseudomonas*, *Yersinia*, and *Burkholderia* [10, 15, 16] where these molecules regulate various intra- and inter-specific interactions, including biofilm formation, bioluminescence, and virulence.

DSF is a group of α, β-unsaturated fatty acids which serve as extracellular signals for cell-to-cell communication among bacteria and also between bacteria and fungi [17]. The prototype DSF (*cis*-11-methyl-2-dodecenoic acid) was originally discovered in the plant pathogen *Xanthomonas campestris* [18], and, soon thereafter, DSF molecules were found to be widely distributed among Gram-negative bacteria where they regulate a variety of individual and community behaviors, including motility, biofilm formation, antibiotic resistance, in vivo persistence, and virulence of plant and animal pathogenic bacteria [19, 20]. *X. campestris* inoculated into the model host plant *Nicotiana benthamiana* produced a considerable amount of DSF (40~100 μM) in the first 2 days of inoculation, which induced a hypersensitivity reaction (HR)–like response and programmed cell death in the diseased plant [21]. Furthermore, in a study by Twomey et al. [20], DSF was found at a concentration of about 0.25 μM in the sputum of cystic fibrosis (CF) patients infected with *Burkholderia cenocepacia* or *Stenotrophomonas maltophilia*. The same study also showed that synthetic DSF increased the antibiotic resistance of 50 clinical isolates of *Pseudomonas aeruginosa*.

Owing to the highly diverse functions of both DSF and AHL, and their widespread impact on bacterial communities, we were curious if they also affect the behavior of *B. bacteriovorus* 109J. This is especially true as both QS molecules are produced by Gram-negative bacterial strains, i.e., the prey for *B. bacteriovorus*. Moreover, aside from indole [22], little is known about how other bacterial signaling molecules impact bacterial predation.

Experimental Procedures

Microorganisms and Culturing Conditions

The strains used in this study were *Escherichia coli* MG1655 (ATCC 700926) and *Bdellovibrio bacteriovorus* 109J (ATCC 43826). All strains were kept as frozen glycerol stock at − 80 °C. Upon need, *E. coli* MG1655 was streaked on LB (Lysogeny Broth, Difco, USA) agar plates and one colony was inoculated in LB broth overnight at 37 °C. *B. bacteriovorus* was always cultured and maintained in HEPES buffer with $CaCl_2$, and $MgCl_2$ salts added to it as described previously [23, 24] using *E. coli* MG1655 as the prey.

Signaling Molecule Stock Solutions

DSF (*cis*-11-methyl-2-dodecenoic acid), 3-Oxo-C6-HSL (*N*-(β-ketocaproyl)-L-homoserine lactone), and 3-Oxo-C12-HSL (*N*-(3-Oxododecanoyl)-L-homoserine lactone) were purchased from Sigma-Aldrich (USA). Stock solutions of DSF were prepared in ethanol while the stocks for the AHLs were in DMSO. Each was stored at − 20 °C. Upon need, the stock solutions were diluted in HEPES buffer and added at the indicated concentrations to the predation cultures. For the control cultures, an equivalent quantity of DMSO or ethanol was added instead. The final concentration of ethanol in the culture was equal to or less than 0.4%.

Effect of DSF on Predation, Prey Attack, and Bdelloplast Lysis

These experiments were performed using the same protocols as described previously for indole [22].

Microscopic Analyses of *B. bacteriovorus* Motility

The microscope setting, time-lapse recording, and analyses were done as previously described [22]. Briefly, a fresh, active overnight predation culture was mixed with different concentrations of DSF (0, 50, or 100 μM) and incubated at 30 °C for 30 min. The predator motility in each tube was then examined under microscope (IX81, Olympus, USA) using time-lapse imaging. The image stacks were then analyzed using the tracking tool in Metamorph software to determine the average swimming velocity for the predator cells in each condition. A minimum of 30 predators were chosen randomly and tracked for each case, and their average swimming velocities were determined.

Protease Assay

Overnight cultures of *B. bacteriovorus* 109J were filtered through a 0.45 μm syringe filter (Millipore, USA) to remove any remaining *E. coli*. The predatory bacteria were then pelleted (5000×*g*, 30 min) and resuspended in 5× nutrient broth (NB) media (Difco, USA) at a concentration of 4×10^9 PFU/mL. The composition of the 5× NB is 25 g/L peptone and 15 g/L beef extract. The cultures were then mixed with an equal volume of 5× NB alone or 5× NB containing DSF. After a 24-h incubation at 30 °C in a shaking incubator (250 rpm), the cultures were centrifuged (16,000×*g*, 10 min) and the protease activities in the supernatants were determined. For this, a Protease Activity Assay Kit (Abcam, USA) was used

according to the manufacturer's suggested protocol with proteinase K as a standard.

ATP Assay

The protocol used was the same as used previously [25], but with slight modification. Briefly, overnight cultures of *B. bacteriovorus* 109J were filtered through a 0.45-μm syringe filter (Millipore, USA) to remove any remaining *E. coli* prey cells and bdelloplasts. The predatory bacteria were pelleted (16,000×*g*, 10 min) and resuspended in HEPES buffer at a concentration of 1×10^9 PFU/mL. DSF was then added to obtain a final concentration of 0 μM, 50 μM, or 200 μM. The cultures were incubated with shaking (250 rpm) at 30 °C for 2 h. To measure the total ATP of the culture, trichloroacetic acid (TCA) was added to the cells at final concentration of 1% to lyse them. Likewise, to measure the ATP content of the supernatants only, the cultures were filtered through 0.22-μm syringe filters (Millipore, USA) to remove the predatory bacterial cells and the filtrates were also treated with 1% TCA. In both cases, the TCA treatment proceeded on ice for 1 h, after which each sample was neutralized using 5 M NaOH solution. The total ATP present in each sample was then determined using the ENLITEN® ATP Assay System (Promega, USA) according to the manufacturer's suggested protocol.

RNA Extraction and Transcriptomic Analyses

Independent 150 mL overnight cultures of *B. bacteriovorus* were filtered (0.45 μm) to remove the remaining prey cells, centrifuged (16,000×*g*, 10 min), and concentrated 10-fold. For the attack-phase (AP) tests, portions of these cultures were incubated at 30 °C with or without 50 μM DSF for 30 min before the RNA was extracted. For the intraperiplasmic exposure tests, *E. coli* MG1655 cells from an overnight LB cultures were centrifuged (16,000×g, 5 min), resuspended in HEPES buffer to an OD of 4.3 ± 0.2, and incubated at 30 °C for 1 h. The concentrated *B. bacteriovorus* cultures were then mixed 1:1 (v:v) with the *E. coli* prey so that the MOI (multiplicity of infection) was approximately five. After incubating the mixed cultures for 2 h at 30 °C, either DSF (50 μM) or sterile HEPES buffer was added and they were incubated 30 min longer, after which the bdelloplasts were pelleted (16,000×*g*, 2 min) and the RNA extracted. The RNA extraction and RT-qPCR procedures were all performed as described previously [22, 25]. *Bdellovibrio bacteriovorus* extracted genomic DNA was serially diluted and used to prepare the standard curve. The expression of the genes was calculated using the 16 s rRNA gene as an internal standard. The RT-qPCR primers used are listed in Table S1. RNA sequencing was done by ChunLab (South Korea).

Bioinformatics Analysis

RNA Seq data (Paired end reads) was quality trimmed using Nesoni pipeline (https://github.com/Victorian-Bioinformatics-Consortium/nesoni). Quality trimmed reads were mapped on the reference genome using the Salmon pipeline [26]. The relative abundances for each transcript, in units of transcripts per million (TPM), were used for the downstream analysis. Pairwise two group comparisons were performed using Welch's *t* test with Benjamini-Hochberg false discovery rate (FDR) correction. The raw transcriptome data is available in the GEO Database in the National Centers for Biotechnology Information (NCBI) under Accession No. GSE150985.

Reproducibility and Statistical Analysis

Unless mentioned otherwise, all experiments were conducted using three independent samples. The error bars on the graphs represent the standard deviations of the samples. Student's *t* test was applied to compare the data and significant differences are noted (*, **, and ***) in the graphs to indicate for *p* values of less than 0.05, 0.01, and 0.001, respectively. Analyses of variance test (ANOVA) together with the Tukey post hoc test was used to compare three or more sets of data. In this case, the letters a, b, c, d, and e were used to identify statistically different groups with *p* values of less than 0.05.

Results

Predation Is Not Affected by AHLs but Is Delayed with DSF

In a previous study, we showed that indole delays *B. bacteriovorus* HD100 predation on both *E. coli* MG1655 and *Salmonella enterica* KACC 11595 [22] and that it accomplishes this by both slowing down the attack-phase predator and delaying its development within the bdelloplast. Since indole is a bacterial signaling molecule [12, 13], we were curious if other signaling molecules had similar impacts on bacterial predation. To test this, two different classes of molecules were selected for evaluation, namely DSF (i.e., *cis*-11-dodecenoic acid), the prototype molecule for the DSF family, and two AHLs (i.e., 3-Oxo-C6-HSL and 3-Oxo-C12-HSL). These two AHL molecules were chosen because they have different chain lengths and are produced by a wide spectrum of proteobacteria strains. 3-Oxo-C6-HSL is naturally produced by strains such as *Vibrio fischeri* [27], *Pantoea stewartii* [28], *Yersinia pseudotuberculosis* [29], and *Pseudomonas putida* [30] while 3-Oxo-C12-HSL is produced by *Pseudomonas aeruginosa* [31] and *Pseudomonas putida* [30]. Moreover, the concentrations selected are based on the

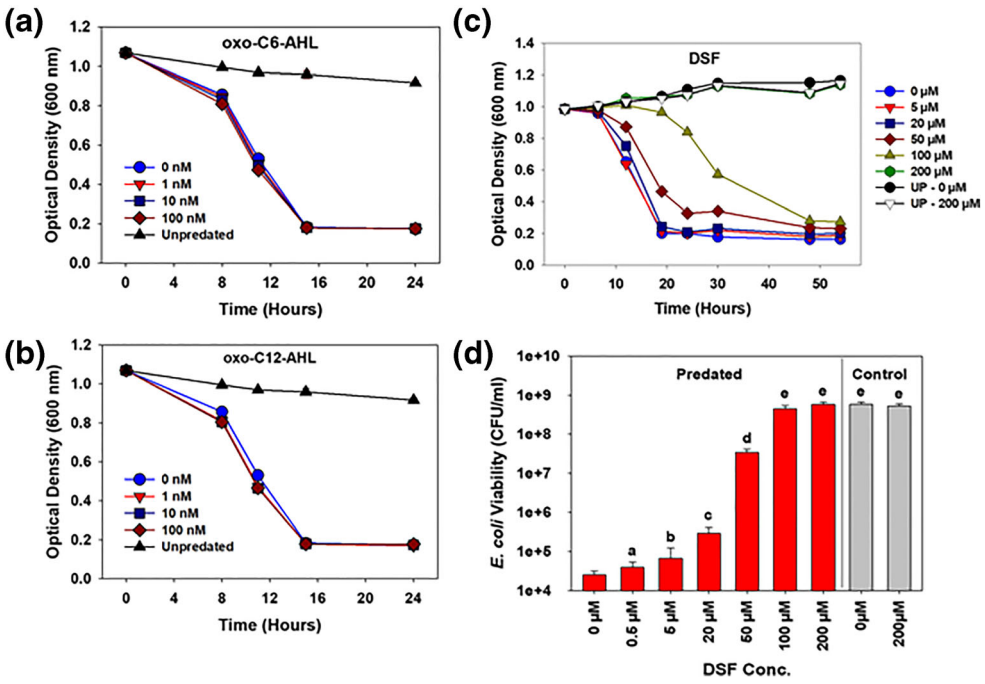


Fig. 1 Effects of the quorum sensing molecules on predation. **A**, **B** Two AHL molecules, 3-Oxo-C6-HSL and 3-Oxo-C12-HSL, respectively, had no obvious impacts on the predatory activities of *B. bacteriovorus* 109J. **C** Predation rates were apparently slower when the DSF concentration was 50 μM or higher, with 200 μM completely inhibiting predation for more than 2 days. UP unpredated ($n = 3$). **D** Plot showing the prey (*E. coli*) viability at 19 h post-predation, showing the dose-dependent results based on the DSF concentration. Control—unpredated. a, b, c, d, and e = $p < 0.05$. ($n = 3$)

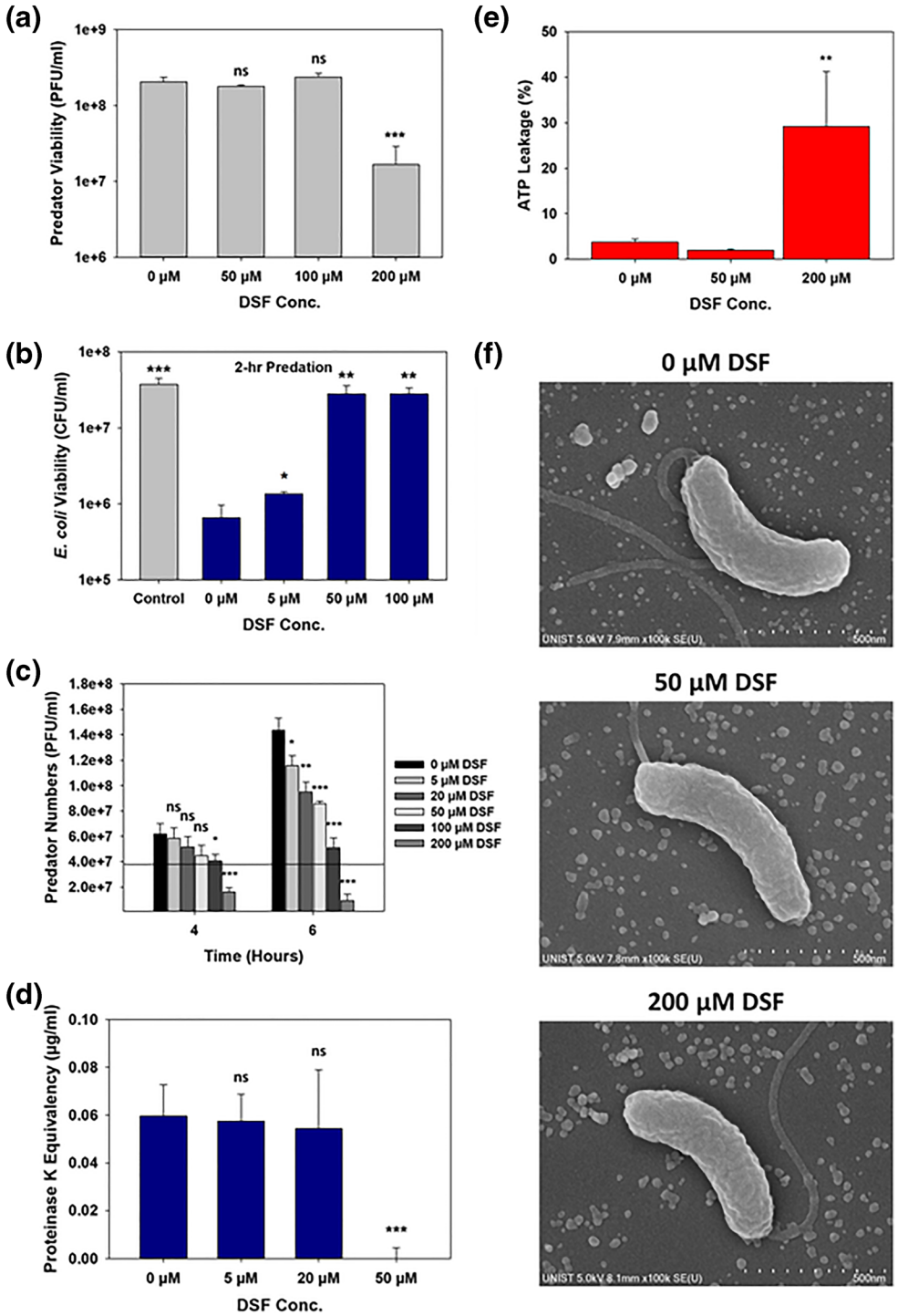
known activities of these molecules, i.e., DSF usually exerts biological responses when present in the low to medium micromolar range [20, 21, 32, 33], while AHL molecules usually have an impact when their concentration is low to medium nanomolar [34–37].

The two AHL molecules were evaluated first (in the range of 1~100 nM) and found to lead to a slight, yet insignificant, improvement in the predation kinetics, especially with the longer 3-Oxo-C12-HSL (Fig. 1A and B). Repeating the experiment with higher concentrations of the AHL molecules (up to 10 μM) found again only slight improvement in predation kinetics with the longer 3-Oxo-C12-HSL (Fig. S1). When experiments were performed using DSF, however, predation was significantly delayed (Fig. 1C). As illustrated here, the activity of DSF was dose-dependent, with concentrations less than 20 μM having no obvious effects based on the OD measurement, while higher concentrations led to a marked delay in predation. When a concentration of 200 μm was tested, predation was completely inhibited. The dose-dependent impact of DSF was also verified using the prey viability counts at 19 h. As shown in Fig. 1D, the viable *E. coli* counts mirrored the OD_{600} measurements for the same time point in Fig. 1C, with 50 μM DSF giving midline results while the higher concentrations tested significantly inhibited predation. Furthermore, lower DSF concentrations (0.5~5 μM) showed also slight but statistically significant effect based on prey cell counting. As neither of the AHL molecules had a significant impact on predation, all subsequent experiments focused on DSF.

DSF Impacts Several Key Characteristics of Predation

When AP *B. bacteriovorus* 109J were exposed to DSF in absence of prey, we found 200 μM DSF led to a 91.9% loss

Fig. 2 Characterizing DSF effects on *B. bacteriovorus* 109J. **a** DSF at a ▶ concentration of 200 μM is toxic for *B. bacteriovorus* 109J, but not at the lower concentrations tested. DSF was added to the *B. bacteriovorus* suspension in absence of prey, and the predator viability was assessed after 2 h of incubation. Statistical analyses were performed against the untreated controls. ns non-significant; $***p < 0.001$ ($n = 3$). **b** DSF inhibits prey attack at sub-lethal concentrations. The predator was incubated with DSF for 30 min prior to introducing the prey to the culture. The prey viability was then assessed 2 h post-infection, showing even 5 μM has an effect while 50 and 100 μM completely inhibited prey attack during the first 2 h. The predator-to-prey ratio used for these tests was five. Statistical analyses were performed against the untreated, i.e., no DSF, predated cultures. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ ($n = 3$). **c** DSF delays bdelloplast lysis and the release of *B. bacteriovorus* progeny. DSF was added 1 h after mixing the predator with the prey. The predator viability was assessed immediately after adding DSF (1 h post-predation (shown as a line within the graph)) and after 3 and 5 additional hours (i.e., 4 and 6 h post-predation). Statistical analyses were performed against the untreated, i.e., no DSF, predated cultures at each time point. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ ($n = 3$). **d** AP *B. bacteriovorus* extracellular protease activities were abrogated by 50 μM DSF. The protease values were compared against the untreated cultures for the statistical analyses. $***p < 0.001$ ($n = 3$). **e** Exposure to high doses of DSF leads to membrane leakage. Using an ATP assay, we determined the amount of cellular ATP released when AP *B. bacteriovorus* 109J was exposed to 50 or 200 μM DSF. Statistical analyses were performed against the untreated cultures. $**p < 0.01$ ($n = 3$). **f** Electron microscopic images of *B. bacteriovorus* 109J cells exposed to DSF. Although the results in **e** suggest that 200 μM DSF reduces the membrane integrity of the predator; no obvious changes were observed in *B. bacteriovorus* morphology



in viability within just 2 h (Fig. 2a), proving that this quorum sensing molecule is toxic towards this predator. Although neither 50 nor 100 µM DSF had any discernible impact on the predator's viability, both concentrations still effectively inhibited prey attack, as shown in Fig. 2b, a finding that is similar to what was reported previously with indole [22]. Based on published results with indole [22], we hypothesized this may be due to DSF reducing the motility of AP *B. bacteriovorus* 109J. Tests performed using either 50 or 100 µM DSF proved that this was the case, with 50 µM DSF reducing their speed by 53.4%, i.e., from 38.2 ± 14.9 to 17 ± 8.9 µm/s, while 100 µM DSF decreased it further, i.e., to 11.7 ± 5.1 µm/s (a 69.4% loss). More than just impacting AP predators, DSF also affected intraperiplasmic (IP) *B. bacteriovorus* 109J, impeded the development and release of progeny from bdelloplasts in a dose-dependent manner

(Fig. 2c), and inhibited the secretion of extracellular proteases from the AP cells (Fig. 2d).

B. bacteriovorus 109J's Membrane Integrity Is Disrupted by DSF

As DSF is a very hydrophobic molecule (Log P_{OW} 5.11), we hypothesized that it is attacking and destabilizing the predator's membrane. To evaluate this, the amount of ATP that leaked from AP *B. bacteriovorus* 109J cells when exposed to either 50 μM or 200 μM DSF was measured. Figure 2e shows that treatment with 50 μM DSF led to no obvious impact, while 200 μM DSF led to nearly 30% of the total ATP pool being leaked into the supernatant. When the cells were imaged, however, no damage was readily obvious (Fig. 2f), implying that 200 μM DSF decreased the membrane integrity but did not completely rupture it.

DSF Induces Oxidative and Osmotic Stress Responses in *B. bacteriovorus* 109J

While the ATP assay proves that higher concentrations of DSF can disrupt the cellular membrane of *B. bacteriovorus* 109J, it did not explain why 50 μM DSF delayed predation (Fig. 1C and D). To evaluate this further, the transcriptomic responses from both AP and intraperiplasmic (IP) *B. bacteriovorus* 109J to 50 μM DSF were mapped. While approximately 40% of the predator's genes (1458 genes) were significantly downregulated (> 1.5-fold, Corr. $p < 0.05$) in 50 μM DSF-exposed AP cells, this number was much lower in the DSF-exposed IP predators, i.e., only 261 (Table S2), with 107 genes present in both groupings. Similarly, of the 93 and 574 genes that were significantly upregulated (> 1.5-fold, Corr. $p < 0.05$) in AP and IP predators, respectively, 53 were shared by both populations.

The transcriptional data supported the protease results in Fig. 2d, with the seven AP-specific serine proteases experiencing significantly lower expression levels after an exposure to 50 μM DSF (Table S3). This was proven further using reverse transcription quantitative PCR (RT-qPCR), as shown in Fig. S2. Similarly, the expression of most flagellum assembly genes (33 out of 44) were significantly downregulated (> 1.5-fold, Corr. $p < 0.05$) (Table S3) during the AP. In fact, based on SEED database analyses [38], 11.3% of all significantly downregulated AP genes were categorized as either motility or chemotaxis (Table S4), a result that agrees with the reduced swimming speeds mentioned above.

When the top ten differentially expressed genes in either phase of growth (AP or IP) were identified (Fig. 3), the majority were hypothetical proteins, but several encoded for stress-related proteins. One of these was EP01_RS18125, which was strongly repressed by DSF in AP *B. bacteriovorus* 109J (Fig. S3). This gene encodes for an pY-homologue, i.e., ribosome-associated protein Y, which inhibits translation by blocking the aminoacyl-tRNA from binding to the ribosomal A site [39].

Several stress-related genes were also very strongly induced, including EP01_RS00585 (Fig. S4), which encodes for a MarR family transcriptional regulator, and EP01_RS03185 (Fig. S5), which encodes for a BON (bacterial OsmY and nodulation) domain-containing protein and is typically induced by osmotic shock [40]. A third stress-related gene that was highly upregulated was EP01_RS09060 (Fig. S6), which encodes for a DPS homologue, i.e., a protein that protects the cellular DNA from reactive oxygen species (ROS) and other stresses [41, 42]. Although not in the top ten genes, several other oxidative stress genes were also strongly induced, including two superoxide dismutase genes and one catalase gene (Fig. S6).

Discussion

In this study, we report that *B. bacteriovorus* 109J has disparate responses to different quorum-sensing molecules. For instance, the two AHL molecules tested did not have a significant effect on the predation kinetics. There is still a possibility, however, that *B. bacteriovorus* may be responding to other types of AHLs. In fact, the EP01_RS05130 gene in *B. bacteriovorus* 109J (equivalent to Bd1826 in *B. bacteriovorus* HD100) is annotated as a *luxR* homologue, i.e., the transcriptional regulatory protein used to detect and respond to AHL molecules. Likewise, several other genes are predicted to have *luxR* domains including EP01_RS10135, EP01_RS06875, EP01_RS06945, EP01_RS15980, and EP01_RS12280 (Equivalent to Bd2837, Bd2139, Bd2154, Bd1267, and Bd0093, respectively). DSF, on the other hand, delayed predation in a dose-dependent manner similar to that observed previously with indole [22].

In addition to delaying predation, several similarities in the effects these two signaling molecules had on bacterial predators were noted. For instance, both impeded the development and release of progeny from bdelloplasts in a dose-dependent manner. One interesting finding was that at the physiologically relevant concentration of 5 μM [33, 43], DSF had a statistically significant impact on predation (Fig. 1d), causing inhibition of prey attack (Fig. 2b), and leading to a 19% reduction in progeny numbers at 6 h (Fig. 2c), indicating that *B. bacteriovorus* 109J is highly sensitive to this compound. Another similarity between the activities of DSF and indole was that both compounds reduce the expression of flagellar assembly and chemotaxis genes in the AP predators, leading to slower motilities. This is reminiscent of the effect indole has on *E. coli* [44] and DSF has on *Xanthomonas oryzae* pv. *oryzae*, a DSF-producing plant pathogen [45], where the motility of both strains and the expression of sixteen chemotaxis

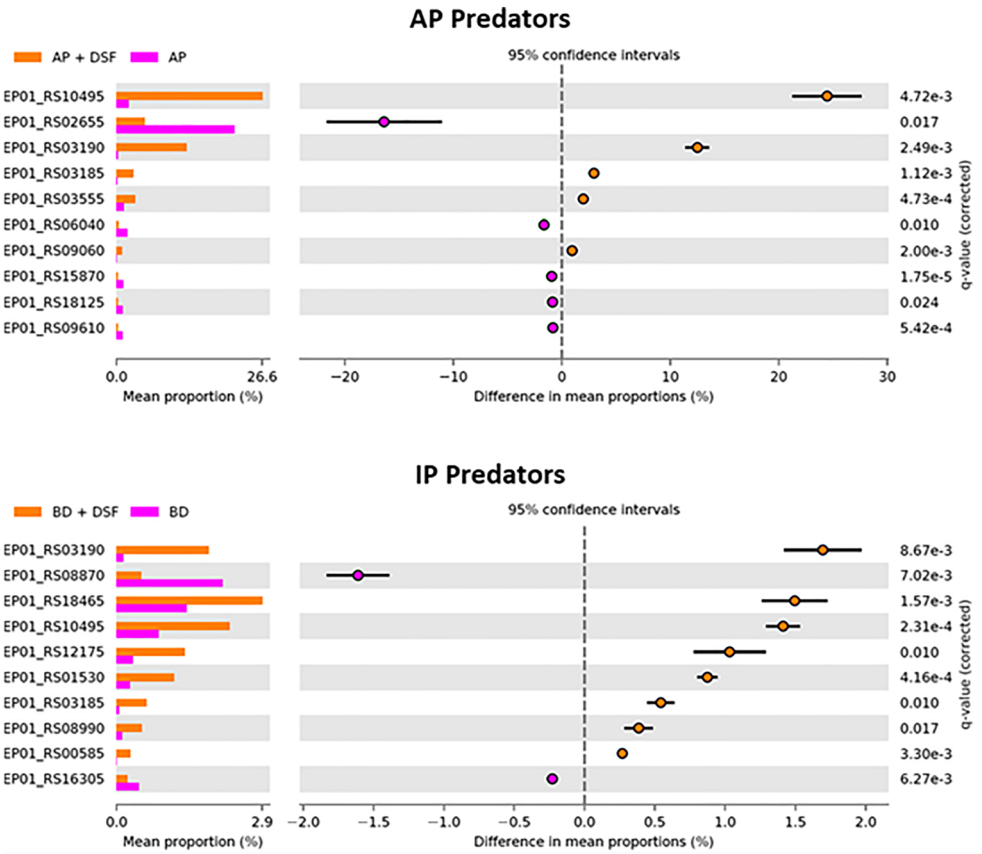


Fig. 3 Top ten *B. bacteriovorus* 109J genes differentially expressed during exposure to 50 μM DSF. Welch *t* test (multiple test correction = Benjamini-Hochberg FDR, minimum difference between proportions = 0.35 and minimum ratio of proportions = 2) based top 10 differentially expressed genes upon DSF treatment for attack phase (AP) cells and bdelloplast (BD) predators

genes in the latter are reduced by these signaling molecules, respectively. However, in *B. bacteriovorus* 109J, these altered activities likely stem from the fact that DSF was harmful towards this predator.

In fact, this was the one major noteworthy difference observed between indole and DSF, namely that DSF was toxic while indole was not. This is evident when focusing on the reported inhibitory concentrations for each, i.e., 2 mM for indole [22] and 200 μM for DSF, as no loss in viability was reported with 2 mM indole while the latter caused a 91.9% loss in viability in the current study. As DSF (212.33 Da) is a very hydrophobic molecule (Log P_{OW} 5.11), we hypothesized it acts by attacking the predatory membrane, particularly at a concentration of 42.5 mg/L (200 μM). However, we noted that both indole (117.15 Da) and violacein (343.34 Da) are also hydrophobic chemicals, with Log P_{OW} values of 2.14 and 3.34 [46, 47], respectively, yet neither harmed *B. bacteriovorus* when added at similar or higher concentrations, i.e., 234 and 20 mg/L, respectively [5, 22].

A recent study reported that chemical surfactants, particularly sodium dodecyl sulfate (SDS), were highly toxic towards different predatory strains [48]. When the toxicity of DSF was contrasted against that of SDS, their toxicities towards *B. bacteriovorus* 109J were quite comparable, e.g., 200 μM (or 42.5 mg/L) DSF and 347 μM SDS (or 100 mg/L) led to 91.9% and 73.5% losses in viability, respectively, even though SDS has a much higher water solubility (Log P_{OW} 1.6). Moreover, both compounds were also harmful towards IP predators and reduced their viabilities. As illustrated in Fig. 2c, the treatment of the bdelloplasts with 200 μM DSF led to a 74.1% loss in the *B. bacteriovorus* 109J viability, while 100 mg/L SDS caused a comparable loss (76.0%) with *B. bacteriovorus* HD100 bdelloplasts [48]. These results not only show the similar activities of SDS and DSF towards *B. bacteriovorus*, but they also imply the hydrophobicity of the compound matters less than its structure. With both containing long-chain aliphatic hydrocarbon chains, and SDS's established activity as a membrane disrupting compound, it led us to consider if DSF was likewise acting like a surfactant. This was proven further through the ATP assays, where nearly 30% of the total cellular ATP was leaked when AP predators were treated with 200 μM DSF. The more evident general downregulation of the genes during the AP compared with the IP phase (1458 genes vs. 261 genes; Table S2) suggests that DSF is more toxic for the AP cells. This could be in part due to the protection the predator possesses inside the bdelloplast as DSF needs to first diffuse through the prey outer membrane before reaching the predator.

Although this is the first clear demonstration that DSF acts like a surfactant towards *B. bacteriovorus* 109J and disrupts its cellular membranes, activities that help explain its toxic nature at the higher concentrations tested, it also led us to

question why lower, non-toxic concentrations (i.e., 50 μM) had pleiotropic impacts on the predator, such as reducing its speed, its secretion of proteases, and its development in the bdelloplast. It is possible, therefore, that some of the two-component signal transduction systems in *B. bacteriovorus* may be responding to DSF. In *X. campestris*, for instance, DSF is sensed through the RpfC/RpfG two component system, where RfpC serves as the sensing histidine kinase while the HD-GYP phosphodiesterase RfpG acts as the response regulator and works by hydrolyzing the second messenger cyclic di-GMP [17]. *B. bacteriovorus* HD100 genome codes for 6 HD-GYP proteins, one of which, Bd1817 (Equivalent to EP01_RS05090), shares 48% similarity and 24% identity with RpfG. However, as the function of Bd1817 has yet to be determined [49], its potential role in regulating these responses to DSF should be studied further.

Within AP predatory cells, the expression of flagellar and chemotaxis genes is known to be of special importance as they function in locating prey [50, 51]. Focusing on these genes, we found that the expression of most flagellar assembly genes (33 out of 44) were significantly downregulated (> 1.5-fold, Corr. $p < 0.05$) (Table S3), providing transcriptional support for the reduced motilities observed. Although the effect of DSF on the expression of flagellar genes during the IP was variable, the modest downregulation of some flagellar genes in this phase may be one factor accounting for the delayed progeny release from the bdelloplast, as was shown previously [52].

Furthermore, in the plant pathogen *Xanthomonas campestris*, DSF signaling positively regulates the secretion of hydrolytic enzymes, including proteases and glycanases [11, 53]. In contrast, several papers have reported AP *B. bacteriovorus* increase their expression and secretion of proteases when provided extracellular amino acids [25, 54, 55]. In this study, we employed this response to study the impacts of DSF on AP *B. bacteriovorus* 109J and found that 50 μM DSF completely abrogated the secretion of the proteases, even when the predator was provided sufficient amino acids (Fig. 2d). This response was supported by transcriptome analyses where the expression of all seven AP-specific serine proteases were significantly lower after AP predatory cells were exposed to 50 μM DSF (Table S3).

The stress brought by DSF on *B. bacteriovorus* is further supported by the transcriptional responses of several stress-related genes. For instance, one of the genes that was very strongly induced, i.e., 165- and 294-fold during the AP and IP, respectively (Fig. S4), was EP01_RS00585, which encodes for a MarR family transcriptional regulator. Although not studied previously within *B. bacteriovorus* 109J, in other bacterial strains, MarR acts as a repressor of many genes (including efflux pumps [56, 57]) and is derepressed when bound by hydrophobic compounds, such as phenolics, antibiotics, and xenobiotic chemicals [58–60], making it a central stress response node for harmful compounds. As such, the massive expression of this gene in *B. bacteriovorus* 109J is a clear toxicity "fingerprint" and proves DSF is injurious to both AP and IP predators. Similarly, EP01_RS03185 encodes for a BON domain-containing protein and was particularly upregulated in the AP predators (Fig. 3; Fig. S5). As the BON domain is a membrane-binding domain typically involved in osmotic shock protection and other cell membrane–localized processes [40], the increased expression of EP01_RS03185 is transcriptional proof that DSF disrupts membrane integrity.

One gene that was strongly repressed by DSF in AP *B. bacteriovorus* 109J was a pY-homologue (EP01_RS18125). Considering its function is to prevent translation, that it is strongly expressed in *B. bacteriovorus* 109J during the AP stage and nearly "shut off" during the IP stage (Fig. S3), this protein likely regulates the production of unwanted proteins during the AP, i.e., when nutrients are low. This fits the profile for pY since it is also a stress-related protein [39, 61] and is typically produced during cold shock or the stationary phase, during which it inhibits elongation during protein translation. As such, its 4.5-fold repressed expression in DSF-treated AP *B. bacteriovorus* 109J may be viewed as a shift in this predator, permitting other stress-related proteins to be translated in response to, and to protect against the activities of, DSF.

In addition to the genes listed above, one of the stresses that DSF clearly induces in this predator is oxidative radicals and damage. This can be seen in the strongly induced expression of multiple oxidative stress genes, including DPS (EP01_RS09060), two superoxide dismutase genes (EP01_RS08140 and EP01_RS13195), and one of the catalase genes (EP01_RS17880). Therefore, it seems quite certain that ROS are being produced in *B. bacteriovorus* 109J. Although the production of ROS by DSF has not been reported previously, it falls in line with what is known about several antibiotics, including ampicillin and kanamycin. In their study, Kohanski et al. [62] reported both antibiotics indirectly lead to the generation of reactive oxygen species by hyperactivating the electron transport chain and depleting the bacterial NADH pools. They proposed this led to the generation of superoxide radicals, which attacked Fe–S clusters, releasing Fe^{2+}, and that this free Fe^{2+} produced hydroxyl radicals via the Fenton reaction.

In conclusion, this study shows that *B. bacteriovorus* 109J has disparate responses to different bacterial signaling molecules. While the two AHLs tested did not significantly alter predation rates, the effects of DSF were dose-dependent, with the highest concentration leading to a significant loss in viability. Both experimentation and the transcriptome confirmed that DSF is harmful towards this predator, leading to a significant loss in membrane permeability and inducing a variety of stress responses, including osmotic shock, ROS, and induction of the MarR regulon. Taken together, *B. bacteriovorus*

109J is quite sensitive to this molecule, a finding that raises the question if the role of DSF in nature is 2-fold, both as a signaling molecule and as a deterrent against predatory bacteria, an idea that we are currently pursuing. Given that DSF and AHL molecules are widely produced by Gram-negative bacteria, future work is also needed to determine if *B. bacteriovorus* is producing its own type of DSF- or AHL-like molecules.

Author Contributions MD and RJM conceived the idea; MD, HJ, WM, HI, and SC conducted the experiments; MD and RJM analyzed the data; NS, SY, and DN analyzed the transcriptome; MD, NS, and RJM prepared the manuscript.

Funding Funding for this project was sponsored by the National Research Foundation of Korea under the Mid-Career Project (Grant No. 2020R1A2C2012158) and the Space Core Technology Development Project (Grant No. 2017M1A3A3A02016642). We appreciate the support.

Data Availability The strains used in this study were *Escherichia coli* MG1655 (ATCC 700926) and *Bdellovibrio bacteriovorus* 109J (ATCC 43826). The raw transcriptome data is available in the GEO Database in the National Centers for Biotechnology Information (NCBI) under Accession No. GSE150985.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Statement This article does not contain any studies with human participants or animals performed by any of the authors.

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