



# *Bdellovibrio bacteriovorus* HD100, a predator of Gram-negative bacteria, benefits energetically from *Staphylococcus aureus* biofilms without predation

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## Abstract

*Bdellovibrio bacteriovorus* HD100 is a predatory bacterium which lives by invading the periplasm of Gram-negative bacteria and consuming them from within. Although *B. bacteriovorus* HD100 attacks only Gram-negative bacterial strains, our work here shows attack-phase predatory cells also benefit from interacting with Gram-positive biofilms. Using *Staphylococcus aureus* biofilms, we show this predator degrades the biofilm matrix, obtains nutrients and uses these to produce and secrete proteolytic enzymes to continue this process. When exposed to *S. aureus* biofilms, the transcriptome of *B. bacteriovorus* HD100 was analogous to that seen when present intraperiplasmically, suggesting it is responding similarly as when in a prey. Moreover, two of the induced proteases (Bd2269 and Bd2692) were purified and their activities against *S. aureus* biofilms verified. In addition, *B. bacteriovorus* HD100 gained several clear benefits from its interactions with *S. aureus* biofilms, including increased ATP pools and improved downstream predatory activities when provided prey.

*Bdellovibrio bacteriovorus* is a Gram-negative obligate predatory bacterium which lives by attacking other Gram-negative bacteria [1], including a very wide range of human pathogens [2, 3] but does not predate on Gram-positive bacterial strains [2, 4–7]. 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. Although a previous study showed the addition of glutamate or glutamate and other

amino acids prevented loss of viability [8], it has long been held that this predator is dependent on prey cells for both its replication and as a source of chemical energy. In addition to attacking planktonic prey, this predator is quite capable of reducing Gram-negative biofilm populations [2, 9, 10], even when two preys were present together [2]. In nature, however, *B. bacteriovorus* is thought to be mainly associated with biofilms [11, 12] and, as such, encounters non-prey Gram-positive strains within these biofilms.

To evaluate how *B. bacteriovorus* HD100 responds to Gram-positive biofilms, therefore, *Staphylococcus aureus* was selected since it is a non-prey bacterium [5] and a well-known human pathogen. Figure 1a illustrates a basic characteristic of predatory bacteria, namely that they only prey on Gram-negative bacteria, leading to an increase in their numbers. We found that when added to pre-formed biofilms, the predatory culture dispersed *S. aureus* biofilms (Fig. 1b) to the same degree as prey, i.e., *Salmonella enterica*, biofilms based upon crystal violet (CV) staining (Fig. 1c). Figure 1d shows that, while the CV results were similar, the *S. enterica* biofilm population was reduced by more than 1-log while that of *S. aureus* was reduced by only 59%. Though mild in comparison, this loss was significant, proving *B. bacteriovorus* HD100's ability to disperse *S. aureus* biofilms.

Given the susceptibility of *S. aureus* biofilms to proteases [5, 13–15], we hypothesized residual proteases from

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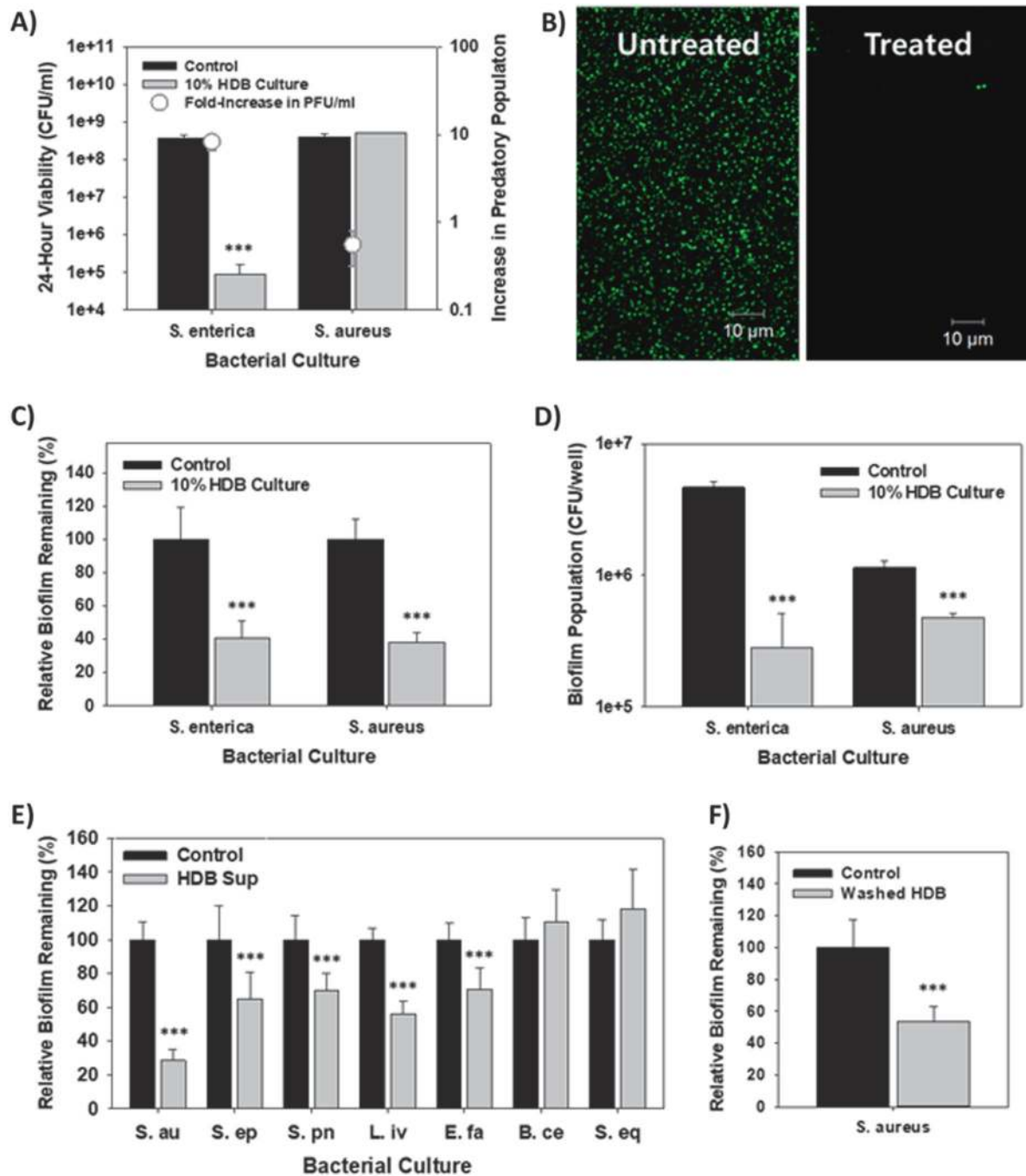
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the previous predation cycle, i.e., when culturing this predator for these tests, were responsible for the dispersions seen. The presence of proteases in the cell-free spent media (HDB Sup) was confirmed using the Azocoll assay, with average residual activities equivalent to  $3.2 \pm 1.3$  ng/ml proteinase K. This was sufficient, even when diluted tenfold, to significantly disperse several different Gram-positive biofilms (Fig. 1e). The use of proteinase K led to similar levels of dispersion while the addition of AEBSF, a potent inhibitor of serine proteases,

reduced the activity of the predatory supernatants (Figures S1 and S2). One interesting finding was the treatment of *S. enterica* biofilms with proteases actually increased the biomass (Figure S2). This increase is thought to be due to loss of the flagella, which causes the cells to adhere to the bottom of the wells as described by Teplitski et al [16]. Similarly, the differences seen between the individual Gram-positive biofilms is likely due to variations in their EPS content, a concept that should be studied in a future work.

◀ **Fig. 1** *B. bacteriovorus* disperses Gram-positive biofilms. **a** *S. aureus* is not predated upon by *B. bacteriovorus* HD100. Cultures of *S. enterica* or *S. aureus* were incubated in HEPES buffer alone (Control) or in HEPES containing *B. bacteriovorus* HD100 (10% HDB Culture). The 24-h viability of each bacterium was then determined. For *B. bacteriovorus* HD100, the results are presented as the fold-increase in the predatory population, illustrating that *S. enterica* is a prey but *S. aureus* is not. \*\*\* $P < 0.001$ . ( $n = 3$ ). **b** Confocal images of the *S. aureus* biofilm showing its removal when treated with *B. bacteriovorus* HD100 in HEPES buffer. The untreated sample was incubated in sterile HEPES buffer. After treatment, the cells were stained with Syto-9 to visualize them. **c** *B. bacteriovorus* HD100 removes both *S. enterica* and *S. aureus* biofilms. Biofilms of each strain alone in 96-well plates were treated for 24 h with HEPES buffer alone (Control) or with HEPES containing *B. bacteriovorus* HD100 (10% HDB Culture). The results are presented as the relative biofilm biomass based upon crystal violet staining. \*\*\* $P < 0.001$ . ( $n = 27$ ). **d** *B. bacteriovorus* HD100 reduced *S. aureus* biofilm populations. The bacterial populations remaining after treatment with *B. bacteriovorus* HD100 as in **c** were determined, proving *S. aureus* is being removed. \*\*\* $P < 0.001$ . ( $n = 3$ ). **e** Dispersal of various Gram-positive biofilms by the cell-free predatory supernatant (HDB Sup). HDBsup was added at 10% (v:v) to preformed biofilms of each bacterium. The strains used in this figure were *S. au* (*Staphylococcus aureus* KACC 10768), *S. ep* (*Staphylococcus epidermidis* KACC 13234), *S. pn* (*Streptococcus pneumoniae* KACC 15313), *L. iv* (*Listeria ivanovii* subsp. *ivanovii* KACC 10767), *E. fa* (*Enterococcus faecalis* KACC 11304), *B. ce* (*Bacillus cereus* KACC 1000), and *S. eq* (*Streptococcus equinus* KACC 13808). The biomass remaining was determined after 24 h. \*\*\* $P < 0.001$ . ( $n = 10$ ). **f** *B. bacteriovorus* HD100 cells that were pelleted and washed to remove any extracellular proteases still dispersed the *S. aureus* biofilms. \*\*\* $P < 0.001$ . ( $n = 10$ ). The protocols used for these experiments are provided within the Supplemental Information and Data

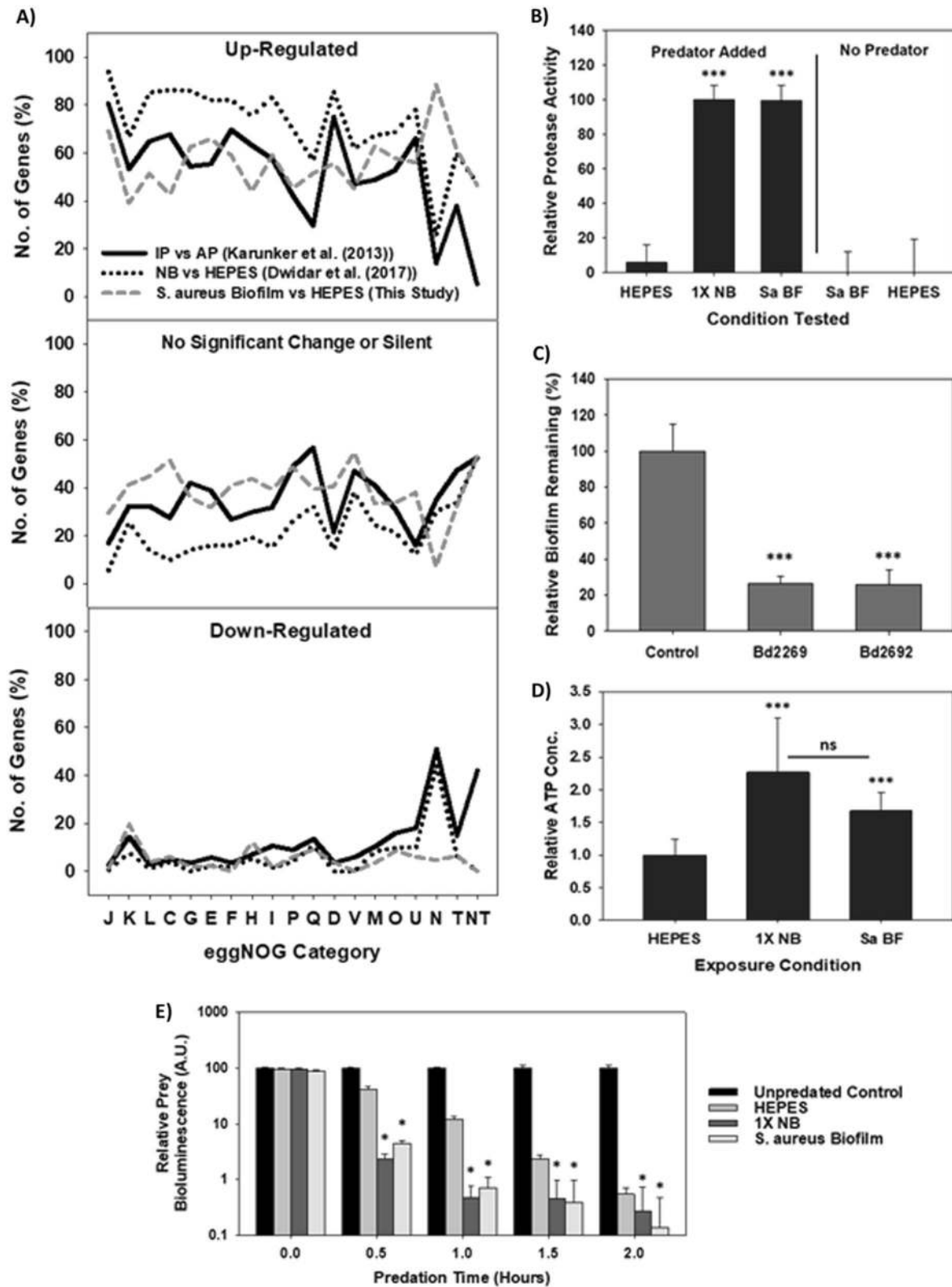
In subsequent experiments, we found *S. aureus* biofilms were still dispersed even after the *B. bacteriovorus* HD100 cells were pelleted and washed to remove the residual secreted proteases (Fig. 1f and S3), implying de novo protease expression also occurred. As shown in Figure S3, this was also true for other *S. aureus* biofilms, demonstrating this activity is not limited to a single strain of *S. aureus*. Figure S4 demonstrates the proteolytic activities were from the predatory strain, and not the *S. aureus* biofilms, as the resulting proteolytic activity levels were nearly identical and the level of dispersion was comparable regardless if the biofilm was viable or UV-killed.

We next studied how *B. bacteriovorus* HD100 responds to *S. aureus* biofilms by sequencing the total cellular RNA. As shown in Figure 2a, the transcriptomic responses during the exposure are highly reminiscent of those seen both during intraperiplasmic (IP) growth [17] and when *B. bacteriovorus* HD100 was incubated in nutrient broth (NB) [18]. One striking difference between the *S. aureus* biofilm-induced transcriptomic responses and the other conditions was EggNOG (Evolutionary genealogy of genes: Non-supervised Orthologous Groups) category “N” (Fig. 2a), which includes the genes related with cell motility. Whereas most of the genes included in this category were down-regulated in the NB-treated and IP predatory cultures, the

majority were up-regulated when *B. bacteriovorus* HD100 was exposed to *S. aureus* biofilms. Of the 38 EggNOG category “N” genes up-regulated in this study, 35 are annotated as being involved in flagellar biosynthesis and were located within eight clusters or operons throughout the chromosome. The other three are annotated as a transporter related with gliding motility (*Bd1025*), a chemotaxis protein (*CheX*, *Bd1823*) and a hypothetical protein located within a flagellar operon (*Bd3396*). The differences between the three studies are thought to be due to the nature of the predator and its environments; the release of amino acids from the *S. aureus* biofilm through the induced proteolytic activities may act as a chemoattractant for swimming predator cells, as demonstrated by LaMarre et al. [19] in their study with *Bacteriovorax stolpii* UKi2. In contrast, IP growth requires little or no movement while the media used by Dwidar et al. [18] was homogeneous, precluding the need for the flagella or chemotactic responses. Likewise, the predators exposed to the *S. aureus* biofilms tended to have fewer EggNOG category “D” genes upregulated than the other published conditions. The reason for this is the variability in the gene expression levels; although many genes had average expression levels that were upregulated several-fold, their values were not significant based upon the Student's *t*-test. Consequently, these were categorized as being “No Significant Change or Silent”.

When viewed alongside the findings of Dwidar et al. [18], where *B. bacteriovorus* HD100 altered its transcriptome and secreted proteases in response to nutrients in the form of nutrient broth, the above results imply *B. bacteriovorus* HD100 also responds to *S. aureus* biofilms as if they were a source of amino acids and secretes proteases in reply. This was confirmed by both the transcriptomic data, where the expression levels for several different serine protease genes were elevated when *B. bacteriovorus* HD100 was exposed to the *S. aureus* biofilms for 4 h (Figure S5), and the increased extracellular proteolytic activities, which paralleled those seen within tests with NB (Fig. 2b). Two of the proteases (*Bd2269* and *Bd2692*) induced in *B. bacteriovorus* HD100 by the *S. aureus* biofilms (Figure S5) were cloned, then expressed and purified (Figures S6 and S7). When tested, we found *S. aureus* biofilms were susceptible to both (Fig. 2c). Their clear activities against *S. aureus* biofilms, alongside their expression patterns, imply these two proteases are likely involved in the dispersals seen above and that they provide this predatory bacterium with a source of amino acids.

These amino acids not only provided *B. bacteriovorus* HD100 with the necessary components to express and secrete proteases but also provided it with a source of energy. This is illustrated in Figure 2d where *B. bacteriovorus* HD100's ATP pool was significantly higher (1.7-fold) after an exposure to the *S. aureus* biofilms when



compared to predatory cells incubated for the same amount of time in HEPES. The higher ATP levels were slightly less than but statistically comparable to those seen when *B. bacteriovorus* HD100 was provided nutrients in the form of

NB medium. The higher energy content in both predatory cultures correlated with better predatory activities downstream, as illustrated in Figure 2e. In this assay, the prey was a bioluminescent strain of *E. coli* and the relative loss in

◀ **Fig. 2** Extracellular nutrients and biofilms induce the secretion of proteases by attack-phase host-dependent *B. bacteriovorus*. **a** EggNOG-based analysis of *B. bacteriovorus* HD100 gene expression patterns when exposed to *S. aureus* biofilms or HEPES buffer alone. This figure shows the results of this study juxtaposed against previously published data, i.e., an NB/HEPES transition [18] and a GP3/AP transition [17]. Excluding the NT group, any genes located within more than one category were excluded from the analyses. In addition, only those genes showing more than 1.5-fold change in their expression (with  $P < 0.05$ ) were considered. For the GP3/AP transition [17], the authors provided Bonferroni values instead of  $p$ -values. J: Translation, ribosomal structure and biogenesis, K: Transcription, L: Replication, recombination and repair, C: Energy production and conversion, G: Carbohydrate transport and metabolism, E: Amino acid transport and metabolism, F: Nucleotide transport and metabolism, H: Coenzyme transport and metabolism, I: Lipid transport and metabolism, P: Inorganic ion transport and metabolism, Q: Secondary metabolites biosynthesis, transport and catabolism, D: Cell cycle control, cell division, chromosome partitioning, V: Defense mechanisms, M: Cell wall/membrane/envelope biogenesis, O: Posttranslational modification, protein turnover, chaperones, U: Intracellular trafficking, secretion, and vesicular transport, N: Cell motility, T: Signal transduction mechanisms, NT: Both cell motility and signal transduction mechanisms. Portions of this figure were reprinted with permission from ref. [18]. **b** *S. aureus* biofilms and NB media induce similar levels of protease secretion from *B. bacteriovorus*. Washed attack-phase *B. bacteriovorus* were exposed for 24 h to HEPES buffer alone (HEPES), NB media (1 × NB) or *S. aureus* biofilms in HEPES buffer (Sa BF) within 96-well plates. The control wells had only HEPES alone (HEPES) or *S. aureus* biofilms alone (Sa BF), i.e., without the predator added. The relative protease activity (based on 1 × NB) of each sample was measured after 24 h and compared with that of the predator in HEPES. \*\*\* $P < 0.001$ . ( $n = 6$ ). **c** Purified *B. bacteriovorus* proteases are capable of removing *S. aureus* biofilms. The *S. aureus* biofilms were treated for 24 h with HEPES buffer alone or supplemented with the individual proteases, Bd2269 or Bd2692, which were added at an activity equivalent of 1 ng-Proteinase K /ml based upon the Azocoll protease assay. \*\*\* $P < 0.001$ . ( $n = 10$ ). **d** Incubation of *B. bacteriovorus* HD100 with NB media or *S. aureus* biofilms increases the predatory cellular ATP pools. Washed attack-phase *B. bacteriovorus* were exposed for 6 h to HEPES buffer alone, 1 × NB or *S. aureus* biofilms in HEPES buffer. The cellular ATP content was determined afterwards, showing the significantly higher ATP pools in the predatory cells incubated in NB media or with the non-prey biofilms. \*\*\* $P < 0.001$ ; ns = not significant. ( $n = 3$ ). **e** Incubation with *S. aureus* biofilms improves the predatory activity against *E. coli*. The activity of the predators in **d** were measured using a bioluminescent *E. coli* prey as described previously [20]. The results show that the predatory activity of *B. bacteriovorus* cells exposed to the *S. aureus* biofilms are significantly better than those exposed to HEPES alone. \* $P < 0.001$ . ( $n = 28$ ). The protocols used for these experiments are provided within the Supplemental Information and Data

bioluminescence was used to compare the predatory activities [20]. The faster and more significant loss in bioluminescence seen with the NB- and *S. aureus* biofilm-incubated *B. bacteriovorus* HD100 cells show these cultures were more active than those incubated in HEPES buffer.

To date, this predator was thought to be dependent upon Gram-negative prey cells as both a source of chemical energy and for its replication, restricting many of the studies published to only those interactions. The findings presented

here implicate that bdellovibrios may have an even greater impact on bacterial communities than previously thought and that their interactions within nature are not limited to only Gram-negative strains and biofilms. Although *B. bacteriovorus* does not predate on Gram-positive bacterial strains, despite one study allegedly claiming otherwise [21], at present very little is known about how this predator responds to non-prey strains and, in particular, their biofilms. Here, we demonstrate *B. bacteriovorus* HD100 benefits from such interactions, obtaining amino acids from non-prey biofilms and using them to produce and secrete proteases as well as augment their ATP pools and predatory activities. These activities in no manner whatsoever suggest predation is occurring, as illustrated by the fact that the predatory numbers do not increase when provided *S. aureus* (Fig. 1a), but suggest a hitherto unknown aspect on how predatory bacteria inherently survive and thrive within natural environments, particularly in niches where Gram-positive, non-prey cells are present.

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**Author contributions** MD and HI designed and carried out the experiments. RJM supervised the experimental work. MD, HI, and RJM evaluated the data. MD, HI, and RJM wrote the manuscript.

## Compliance with ethical standards

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

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