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Comparative analysis of novel *Pseudobdellovibrionaceae* genera and species yields insights into the genomics and evolution of bacterial predation mode

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Abstract

Bacteria of the family *Pseudobdellovibrionaceae* belong to a group of bacteria that kill and feed on other bacteria. The diversity of predation strategies, habitats, and genome characteristics of these bacteria are largely unexplored, despite their ecological and evolutionary importance in microbial communities. Therefore, we characterized new *Pseudobdellovibrionaceae* strains isolated from the direct environments of three animal hosts: the zebrafish (*Danio rerio*), the threespine stickleback fish (*Gasterosteus aculeatus*), and the nematode *Caenorhabditis elegans*. We used transmission electron microscopy (TEM) and genomic analyses to characterize the morphology and predation modes of our isolates. While most of our isolates exhibited periplasmic (i.e. endoparasitic) predation, one isolate clearly exhibited epibiotic (i.e. exoparasitic) predation and represents only the third confirmed epibiotic strain within its clade. Of our isolates, six are members of five new species in the genus *Bdellovibrio* and two strains likely represent new genera within the family *Pseudobdellovibrionaceae*. From metabarcoding data, we found indications that *Pseudobdellovibrionaceae* are widespread among our three animal hosts. Genomic analyses showed that epibiotic predators lack genes involved in host independence (i.e. prey-independent feeding) and peptidoglycan modification. However, genes unique to epibiotic predators may underlie this predation mode, particularly those involved in cell wall remodeling and recycling. With robust phylogenomic analyses, we show that our novel isolates cluster with previously described *Pseudobdellovibrionaceae* isolates according to predation mode. Further, by placing *Pseudobdellovibrionaceae* predators within a wider evolutionary history including other predatory and non-predatory bacteria, we postulate periplasmic predation as the ancestral mode, with more derived epibiotic predators exhibiting genome streamlining.

Introduction

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For decades, researchers have documented the mechanisms by which top-down control via predation drives structure and diversity in communities of plants and animals [1, 2]. More recent evidence suggests that predation by bacteria may similarly structure microbial communities, including those associated with animal hosts (e.g. in corals [3, 4]). Moreover, the presence, abundance, and richness of bacterial predators are positively correlated with overall microbiome diversity for numerous host-microbiome systems, most likely because predation on highly abundant species allows rare taxa to thrive [5]. Understanding the potential contribution of bacterial predators to host microbiome dynamics is important given the central role that microbiomes can play in host health and well-being.

One of the best-studied bacterial predators, *Bdellovibrio bacteriovorus*, was discovered over 60 years ago [6]. It is now considered a member of the "*Bdellovibrio* and like organisms" (BALOs), a group of obligate predators of Gram-negative bacteria. Since then, detailed information has been obtained on the distribution of BALOs across terrestrial and aquatic habitats and their complex strategies of predation, growth, and reproduction [7, 8]. BALOs play a role in microbial population control and nutrient cycling through substantial contributions to bacterial death, despite their relatively low abundance [9]. However, most BALO research has been restricted to a few type strains, and we are only beginning to uncover and comprehensively describe the taxonomic and genetic diversity of BALOs [10]. For instance, our understanding of bacterial predation modes largely reflects research done with two type strains from the *Pseudobdellovibrionaceae* family (formerly *Bdellovibrionaceae* [11, 12]): the periplasmic predator *B. bacteriovorus* HD100 and the epibiotic predator *Pseudobdellovibrio. exovorus* JSS. Both predators employ a biphasic lifestyle including an attack phase in which the small, motile predator cells actively search for prey cells. Once anchored to the prey cell, a periplasmic predator enters the prey periplasm, forms a *bdelloplast*, and grows by consuming the prey's cellular components. Then, the predator cell replicates, septates, and lyses the bdelloplast,

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76 releasing offspring cells. In contrast, epibiotic predators attach to the prey cell and consume the
77 prey's cytoplasmic content without intrusion. The epibiotic predator *P. exovorus* then undergoes
78 binary or non-binary fission [13] and produces two to three attack phase daughter cells.
79 Next-generation sequencing technologies have uncovered genomic signatures of
80 predation among the BALOs [14], and genetic elements that differentiate the periplasmic and
81 epibiotic predation modes [15]. In an initial evaluation, epibiotic predators were deficient in
82 numerous functions due to their smaller genomes compared to the periplasmic predators.
83 However, the recent genomic characterization of an epibiotic predator placed within the genus
84 *Bdellovibrio* with a larger genome suggests that the genomic diversity in epibiotic predators is
85 under sampled [16].
86 In order to expand our understanding of BALO taxonomic and genetic diversity,
87 especially in animal hosts, we isolated nine novel *Pseudobdellovibrionaceae* strains from the
88 immediate environments of three important model animal species: the zebrafish (*Danio rerio*),
89 the threespine stickleback fish (*Gasterosteus aculeatus*), and the nematode *Caenorhabditis*
90 *elegans*. We characterized these strains using microscopy across the life cycle to infer predation
91 mode. We surveyed metabarcoding data from all three animal hosts to infer the prevalence of
92 these novel strains. Finally, we sequenced and assembled the genomes of these
93 *Pseudobdellovibrionaceae* strains *de novo* and compared them to existing genomes of predators
94 of the genera *Bdellovibrio* and *Pseudobdellovibrio*. These analyses expand substantially on
95 previous comparative genomic analyses of predatory bacteria [14, 15, 17, 18], led to the formal
96 description of new genera and species within the family *Pseudobdellovibrionaceae*, and provide
97 the most comprehensive phylogenetic history to date of predation mode, revealing conserved
98 genomic features differentiating these two predation lifestyles.
99
100 **Methods**

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All materials and methods can be found in the supplemental material.

Results

Newly obtained *Pseudobdellovibrionaceae* isolates varied in size and predation mode

From the direct environments of three animal hosts, we isolated 9 novel BALO strains (Table 1). Transmission electron microscopy (TEM) after negative staining was employed to confirm predation strategies (Figure 1, Figure S1). The presence of a bdelloplast containing at least one BALO cell was considered an indication of periplasmic predation. Bacteria of all isolates had a curved-rod (vibrioid) shape and all periplasmic strains had thicker flagellar filaments, which likely represent ensheathed flagella [19] and may help periplasmic predators to retract their flagellum during prey invasion [20]. In contrast, the only epibiotic strain had a notably thinner and longer flagellar filament, which suggests that this strain does not have a sheathed flagellum. Moreover, flagellar waves differed between periplasmic predators and the epibiotic *Bdellovampiro gaculeatus* SBM16 strain (see below for justification and a formal description of all newly identified species). In detail, periplasmic predators showed the tapered waves as described for *Bdellovibrio bacterivorus* [21, 22]. In contrast, the flagellum of *Bdellovampiro gaculeatus* SBM16 consisted of homogenous waves with smaller amplitude.

The length of flagella and the size of predator cells varied between different strains (Table 1) and across independent experiments of single strains (Table S1). Cells sampled after the addition of fresh prey appeared larger and had a shorter flagellum than those from prey-cleared standard overnight cultures. Variability in cell size within strains and even within the same culture may be explained by an earlier observation that BALO flagella [23] and cells continue to elongate after exiting the bdelloplast [24]. The negative correlation between flagellum length and cell size is, however, puzzling. Notably, in the epibiotic strain, variation in

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cell size was larger than in periplasmic strains, as illustrated by two differently sized attack phase cells of SBM16 (Figure 1).

Between strains cell size and flagellum length showed no correlation (Table 1). For example, the epibiotic *Bdellovampiro gaculeatus* strain SBM16 cells exhibited both a large cell size (0.77 ± 0.24 μm^2) and the longest flagellum (7.13 ± 1.57 μm) among the analyzed strains (Figure 1). In contrast, *Bdellovibrio tomkyle* strain MYbb1, despite having a small cell size (0.19 ± 0.02 μm^2), had an average flagellum length (3.02 ± 0.22 μm), while *Bdellovibrio bagaluti* strain MYbb10 displayed a comparable flagellum length (2.9 ± 0.34 μm) but a larger cell size (0.29 ± 0.04 μm^2) (Table 1, Figure S1). Notably, potential damage to the flagella during sample preparation may have affected the measured flagellum lengths, possibly leading to underestimation.

Cell shape was further assessed using the Feret diameter ratio, which indicates the degree to which a particle is stretched or how similar its projected contour is to a circle, with higher values suggesting elongated shape (Table 1). Notably, the highest values were recorded for *Bdellovibrio krueschi* strain MYbb4, *Bdellovibrio kumpostii* strain MYbb5, *Bdellovibrio bagaluti* strain MYbb10, and *Bdellovampiro gaculeatus* strain SBM16, indicating these cells exhibited elongated or narrow morphologies compared to other BALO cells (Table 1).

Comparative genomic analysis reveals novel *Pseudobdellovibrionaceae* genera and species

To assign species and genus level taxonomic classifications to the novel strains, we used average nucleotide identity (ANI) (>95% [25]) and average amino acid identity (AAI) (>65% [26]). These comparative genomic analyses suggest novel genus and species relationships within the *Pseudobdellovibrionaceae* family (Figure S2; Table S2). Building on previous work [12, 18], AAI values support the existence of four genera within *Pseudobdellovibrionaceae* including existing (*Bdellovibrio*, *Pseudobdellovibrio*) and novel genera (*Bdellovenatio*, *Bdellovampiro*).

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Genus *Bdellovenatio* includes the novel strain *B. daniorerio* ZFWA1 which shares <62% AAI with all other genomes. Genus *Bdellovampiro* includes novel and existing strains *B. gaculeatus* SBM16 and *B. qaytius*, respectively, which share an AAI of 79.59%, and each have an AAI <62% with *Pseudobdellovibrio exovorus* JSS. *Bdellovibrio* species were confirmed for strains MYbb10 and MYbb7 (now *B. bagaluti* strains MYbb10 and MYbb7), strains MYbb2 and *B. bacteriovorus Tiberius* (now *B. tiberii* strains MYbb2 and Tiberius), and strains MYbb11 (now *B. bacteriovorus* strain MYbb11), *B. bacteriovorus* 109J, and *B. bacteriovorus* HD100. Further, the strains MYbb1 (now *B. tomkyle* strain MYbb1), MYbb4 (now *B. krueschi* strain MYbb4), and MYbb5 (now *B. kumpostii* strain MYbb5) were identified as separate species. The results also suggest that *B. bacteriovorus* strains SSB218315, kdesi, and W represent separate new species. Notably, ANI values between 85%-95% are comparatively rare and represent a discontinuity in the distribution of ANI values among closely related genomes [27]. A number of genomes had ANI values within this range, including strains of *B. bacteriovorus*, *B. tiberii*, *B. kumpostii*, and *B.* sp SSB218315, strains *B.* sp ZAP7 and *B.* sp KM01, and strains *B.* sp kdesi and *B. tomkyle* MYbb1. While considered "species-like", these sequence-discrete populations are generally ecologically differentiated and still considered distinct species [27].

Phylogenomic analysis identifies two distinct clades for periplasmic and epibiotic *Pseudobdellovibrionaceae*

To uncover the ancestral predation mode in predatory bacteria, a tree with representative outgroups including predatory and non-predatory bacteria, as well as one archaeon (Table S3), was generated. The phylogenetic analysis led to the identification of two distinct clades, with one clade containing all predatory bacteria with a periplasmic lifestyle (Figure 2a). *P. exovorus* and the non-predator *Oligoflexus tunisiensis* clustered within this clade as well. The other predatory bacteria with a cooperative or epibiotic predation strategy (*Myxococcus xanthus*, *Micavibrio*

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aeruginosavorus, and *Vampirovibrio chlorellavorus*) clustered within the second clade. It should be noted that the genus *Micavibrio* is also part of the family *Pseudobdellovibrionaceae*. Our phylogenomic analysis and others [12, 18] indicates that *Micavibrio* is not in close relation to the known isolates of the genera *Bdellovibrio* and *Pseudobdellovibrio* nor to our newly isolated strains and should be therefore re-classified. We are subsequently omitting *Micavibrio* from the following focused analysis and whenever we discuss members of the family *Pseudobdellovibrionaceae*.

We used core-genome sequence phylogenetic analysis to examine the evolutionary relationships between 21 *Pseudobdellovibrionaceae* strains including our nine new isolates. The maximum-likelihood phylogenetic tree was constructed based on the amino acid sequences of 1099 single-copy core genes (Figure 2b). *Pseudobdellovibrionaceae* strains fall into two distinct clades that differentiate based on predation strategy. The novel epibiotic predator isolated from the stickleback fish aquaculture water, here named *Bdellovampiro gaculeatus* strain SBM16, clusters with known epibiotic predators *B. qaytius* and *P. exovorus*. The novel isolate from zebrafish aquaculture water (*Bdellovenatio daniorerio* strain ZFWA1) was the most diverged periplasmic *Pseudobdellovibrionaceae strain*.

Epibiotic *Pseudobdellovibrionaceae* have distinct characteristics indicative of genome reduction

The predators included in Figure 2a were further explored by comparing genome characteristics (Figure 3). Epibiotic *Pseudobdellovibrionaceae* genomes are smaller, have lower GC content, and exhibit a higher coding density than most periplasmic *Pseudobdellovibrionaceae* genomes (Figure 3a). Within the family, *P. exovorus* has the smallest genome, while *B. qaytius* has the lowest GC content and highest coding density. Additionally, all periplasmic predator genomes have less than 350 COGs categorized as M or I functional

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categories (Figure 3b), indicative of a loss in genes associated with the production of fatty acids, phospholipids, and peptidoglycan [28]. Within this group, epibiotic predators have even lower numbers of M and I COGs and some of the smallest genomes. With high GC content and a larger genome, the cooperative predator *Myxococcus xanthus* differs from other predatory bacteria (Figure 3).

Periplasmic and epibiotic *Pseudobdellovibrionaceae* vary in gene content

In the comparative genomic analysis of all 21 *Pseudobdellovibrionaceae* strains, between 2616 and 3754 genes were annotated per strain, and 95.7% of genes were identified as belonging to 5897 orthogroups (Table S4, Figure 4a). Genome characteristics of novel strains and type strains are shown in Table 2. A total of 142 of these orthogroups (made up of 354 genes) were exclusive to a single strain. A total of 1360 core orthogroups were found in all genomes. Excluding these core orthogroups, *B. qaytius* and *B. gaculeatus* strain SBM16 had the greatest number of unique, shared orthogroups (271). *B. krueschi* strain MYbb4 had the greatest number of strain-specific orthogroups (63) comprising 3.9% of total genes, followed by *B. daniorerio* strain ZFWA1 with 17 strain-specific orthogroups (Table 2). *P. exovorus* JSS had the greatest number of exclusively absent orthogroups (i.e. found in all genomes but *P. exovorus* JSS) (64), followed by *B. daniorerio* strain ZFWA1 with 31 exclusively absent orthogroups.

Epibiotic strains shared 102 unique orthogroups that had a higher relative frequency of genes involved in the transport and metabolism of amino acids, nucleotides, coenzymes, and lipids as well as in secondary metabolite biosynthesis, intracellular trafficking, secretion, and vesicular transport (Figure S3). Orthogroup eggNOG-mapper annotations are listed in Table S5. Epibiotic predators also exclusively encoded some of the genes involved in folate biosynthesis (including *folB*, *folP*, and *folC*), as well as ABC transporters of substrates across the cytoplasmic membrane (including *dppB*), synthesis of glutamate (*glnN*) and purine (*purK*), and disposal of

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toxic ammonia (*rocF*). Orthogroups unique to periplasmic predators (168) had a higher relative frequency of genes involved in numerous COG categories including transcription, replication, recombination and repair, cell wall biogenesis, cell motility, and signal transduction mechanisms (Figure S3). Here, unique genes included genes encoding histidine kinases and genes involved in chemotaxis.

The distribution of several peptidoglycan-modifying enzymes differs between predation strategies. While all *Pseudobdellovibrionaceae* encode a housekeeping penicillin binding protein (*dacB*) involved in cell wall integrity, periplasmic predators exclusively share an *mltA*-like lytic transglycosylase (Bd3285) and a *slt* lytic transglycosylase (Bd1124) (Figure 4b). Epibiotic strains encode two peptidoglycan modifying enzymes that were absent from periplasmic strains (Figure 4b). These include anhydro-N-acetylmuramic acid kinase (anmK) and a polysaccharide deacetylase involved in cell wall recycling and peptidoglycan remodeling, respectively.

Many *Pseudobdellovibrionaceae* lack genes involved in host independence

Genomes of the epibiotic predators and *Bdellovibrio bagaluti* strains MYbb7 and MYbb10, as well as *Bdellovibrio* sp. ZAP7 and KM01, lack the *hit* locus gene Bd0108 (Figure 4b). A similar pattern was observed for the LysR family transcriptional regulator gene Bd3229. However, this gene was also absent in *B. bacteriovorus* W.

Further, epibiotic predators and *Bdellovenatio daniorerio* strain ZFWA1 lack the genes *motAB1* (Bd0144, Bd0145) and *motAB2* (Bd3021, Bd3020 except for SBM16), and a histidine kinase (Bd3126), which are genes previously reported to be involved in host independence (HI; i.e., prey-independent feeding, see discussion). In contrast, two other putative HI genes, *rhlB* (Bd3461) and *pcnB* (Bd3464), as well as *motAB3* (Bd3254 and Bd3253) were present in all strains. Additionally, the described HI-related histidine kinase Bd3650/Bd1535/Bd2843 was absent in all these strains, and also in MYbb1 and *B. bacteriovorus* W. Two other histidine

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kinase genes (Bd3393 and Bd2335) and Bd2152 (a glycerol-3-phosphate transporter [29]) were exclusively absent in epibiotic predators. Additionally, epibiotic predators and ZFWA1 exclusively share 12 orthologous genes with annotated functions involved in initial binding of peptides in periplasmic space (*dppA*) for ABC-type transport, methionine salvage (*mtnA* and *mtnP*), cell wall formation (*murB*), and tyrosine metabolism (*hgo*).

***Pseudobdellovibrionaceae* strains show indications of habitat specificity**

We found ASVs matching the 16S rDNA of our newly isolated strains in host-microbiome datasets of zebrafish, stickleback, and worms (Table 3). Interestingly, zebrafish microbiomes only contained *Pseudobdellovibrionaceae* ASVs matching strains that had been isolated from aquatic habitats. In contrast, stickleback microbiomes contained a more diverse set of *Pseudobdellovibrionaceae*, including strains that had been isolated from the direct environment of zebrafish (ZFW), stickleback (SBM), and worms (MYbbs), and were present in up to 80% of samples. Lastly, worm microbiomes rarely contained *Pseudobdellovibrionaceae* ASVs (3% of samples) and did not harbor *Pseudobdellovibrionaceae* ASVs associated with zebrafish or stickleback. However, they did contain *Pseudobdellovibrionaceae* ASVs that had been previously associated with aquatic habitats, although most of the *Pseudobdellovibrionaceae* ASVs belonged to strains isolated from worm-associated environments or other terrestrial habitats.

Discussion

Bdellovibrio and like organisms (BALOs) play an important role in microbial communities [30–32] and there is growing interest in using BALOs to manipulate microbial communities, especially within host organisms [33–35]. To broaden our understanding of BALO diversity, especially those associated with animal hosts, we isolated nine novel

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Pseudobdellovibrionaceae strains from the environments of three important model animals: the zebrafish, threespine stickleback fish, and the nematode *C. elegans*. We characterized these strains using TEM, comparative genomics and phylogenetic analysis. Two of these nine strains were sufficiently diverged genetically to be considered new genera, including only the third *Pseudobdellovibrionaceae* strain known to use an epibiotic predation strategy, while six strains can be grouped into five new species within the genus *Bdellovibrio.* Our phylogenetic analysis indicates that there are two clades within the family *Pseudobdellovibrionaceae*, separating periplasmic from epibiotic genera (Figure 2b). These novel isolates significantly expand our understanding of BALO biology, especially the genetics and evolution of predation strategies.

Epibiotic predation is a derived trait among BALOs

Phylogenetic analysis of our novel isolates allowed us to better understand the evolution of predation modes among BALO species. Bacterial predation is not a monophyletic trait, and predatory bacteria are found in other bacterial phyla. When including other bacterial phyla in the broader phylogeny (Figure 2a), we identified a single clade that contained all periplasmic BALO genera, suggesting that periplasmic predation may be the ancestral phenotype. In contrast, epibiotic BALO genera are dispersed amongst non-predators and periplasmic predators alike, suggesting that epibiotic predation arose in multiple independent lineages. This evolutionary hypothesis differs from that of Deeg et al. [16], who proposed that epibiotic predation is ancestral and periplasmic predation the derived state within the *Pseudobdellovibrionaceae*. However, Deeg et al. [16] used a single gene (16S rRNA) to infer their phylogeny, while we used a set of over 250 single-copy core genes.

Our comparative genomic analyses provided additional support for our hypothesis that periplasmic predation is ancestral. Pasternak et al. [14] noted genome reduction in periplasmic predators driven by the dependence on prey amino acids and vitamins as is observed in host-

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dependent bacteria [36]. If periplasmic predation is ancestral, derived epibiotic predators therefore would exhibit a secondary phase of genome streamlining as first hypothesized by Pasternak et al. [15]. In the present study, epibiotic *Pseudobdellovibrionaceae* genomes show signs of genome degradation, similar to what has been shown for host-associated symbionts [28]. This includes higher AT content and coding density, smaller genome size, including fewer DNA repair genes and fewer genes involved in cell envelope biosynthesis and morphology when compared to non-predators (Figure 3 and Figure S3). However, we can base this interpretation only on observations of the three epibiotic *Pseudobdellovibrionaceae* strains described so far. However, this streamlining of epibiotic predators seems to be restricted to *Pseudobdellovibrionaceae* species as periplasmic BALOs in the family *Bacteriovoracaceae* including *Halobacteriovorax marinus*, *Bacteriovorax stolpii*, and *Peredibactor starii* had low GC content and high coding density comparable to epibiotic *Pseudobdellovibrionaceae*. Likewise, epibiotic BALOs *Micavibrio aeruginosavorus* and *Vampirovibrio chlorellavorus* had higher GC content and lower coding density than all *Pseudobdellovibrionaceae* predators (Figure 3b).

The evolution of epibiotic predation may have involved the loss of "host independence" genes

Our comparative genomic analysis identified multiple genes that may have been lost in epibiotic predators during their evolution from a periplasmic ancestor. Many of these are genes involved in "host independence" in *Bdellovibrio bacteriovorus* (Figure 4b), a prey-independent feeding strategy that may be induced under limited prey conditions in nature and on protein-rich media in the laboratory [6, 37] and is caused by a mutation in any of a number of genes. We identified multiple genes associated with host independence (specifically, but not exclusively Type I host independence; [38–43]), including Bd3126 (a histidine kinase), and one of the three

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motAB gene clusters, Bd0144/Bd0145 (*motAB1*), that were absent in the epibiotic *Pseudobdellovibrionaceae* species and their closest relative that used periplasmic predation (*Bdellovenatio daniorerio* ZFWA1), but were present in other periplasmic predators (Figure 4b).

Functional differences in peptidoglycan modification differentiate predation strategies

Our comparative analyses provided novel information regarding the biochemistry underlying different predation strategies and builds upon previous studies [15, 18]. Epibiotic genomes lack some peptidoglycan modifying enzymes that have been shown to be important in periplasmic predation (Figure 4B; [16]). Assuming periplasmic predation as the ancestral state, this likely represents a gene loss for epibiotic predators. All *Pseudobdellovibrionaceae* strains encode *dacB*, a housekeeping PBP4 (penicillin binding protein) likely involved in the maintenance of the predator's own cell wall [44]. Periplasmic *Bdellovibrio* predators in the clade that contains both strains HD100 and MYbb1 (Table 2a) all possess a suite of enzymes involved in the modification of the prey cell wall. For example, two *dacB*-like genes, Bd0816 and Bd3459, involved in prey cell rounding to form an osmotically stable bdelloplast and reducing the rate of secondary invasions [44], were absent from some more diverged periplasmic strains and all epibiotic strains. The rate of wasteful secondary invasions may be higher in strains that lack these genes, but this is currently unknown. Similarly, L,D-transpeptidases, Bd0886 and Bd1176, strengthen the prey bdelloplast wall to resist bursting during predator growth within the prey cell [45]. While absent from various strains, an ortholog of these genes is encoded in *B. gaculeatus* strain SBM16. Since bdelloplast formation is not necessary for epibiotic predators, these genes may perform a different role in SBM16 or may be fated for removal in further genome streamlining. Interestingly, all four genes involved in bdelloplast integrity were absent from *B. krueschi* strain MYbb4. This, together with the abundance of exclusively present orthogroups (Table 2) may reflect MYbb4's evolved specificity to predate *Ochrobactrum*,

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although host range was not exhaustively examined here. Further, the diversity, distribution, and function of predatory peptidoglycan-modifying genes is likely influenced by structural and biochemical properties of the prey cell wall such as cross-link chemistry [45], bacterial capsules, surface layers, or acetylation state [46].

Notably, an *mltA*-like lytic transglycosylase is exclusive to periplasmic predators. In *Bdellovibrio bacteriovorus* strain HD100, this protein is secreted into the prey periplasm and cleaves the peptidoglycan septum of dividing prey, facilitating the conversion of actively dividing prey into a single spherical bdelloplast [47]. All strains contain an LD carboxypeptidase involved in predator cell wall curvature which causes the formation of a vibrioid and facilitates efficient intracellular growth of HD100 in a spherical prey niche [48]. What role this plays in epibiotic strains is yet to be determined. Most periplasmic predators and *P. exovorus* strain JSS encode peptidoglycan deacetylases (Bd0468 and Bd 3279 or orthologs) that deacetylate the peptidoglycan of the prey [49]. This allows the predator to differentiate between the cell wall of the prey and that of the predator itself with the enzyme dslA (Bd0314) which cuts peptidoglycan depending on acetylation state and functions as an exit enzyme by destroying the prey cell wall [50]. Strains MYbb4, ZFWA1, and JSS all lack *dslA*, suggesting that MYbb4 and ZFWA1 possess an alternative exit strategy.

Epibiotic *Pseudobdellovibrionaceae* strains exclusively share *anmK*, which encodes a bifunctional glycosidase/kinase involved in recycling of cell-wall derived anhydroMurNAc [51]. Interestingly, all non-*Pseudobdellovibrionaceae* epibiotic predators also encode AnmK as do non-predator relatives (*A. capsulatum*, *V. chlorellavorus*, *M. aeruginosavorus*, *E. adhaerens*, and *S. maltophilia*). While not present in periplasmic predators, this widely conserved gene may be integral to epibiotic predation. Cell-wall recycling through MurNAc dissimilation is a pathway by which bacteria can utilize peptidoglycan fragments from the environment or from the endogenous cell wall [51]. This could be advantageous for predatory bacteria to scavenge prey

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cell wall material [52]. However, *murQ* is also necessary for anhydroMurNAc recycling, but is absent in *Pseudobdellovibrionaceae* strains. Both *anmK* and *murQ* are present in epibiotic predators *M. aeruginosavorus* and *V. chlorellavorus* as well as *E. adhaerens*. While some epibiotic predators may be able to utilize prey cell wall material through this process, the role and exclusivity of *anmK* in epibiotic *Pseudobdellovibrionaceae* is yet to be uncovered.

The family *Pseudobdellovibrionaceae* is more genus and species rich than previously thought

Using ANI, we identified new *Pseudobdellovibrionaceae* strains belonging to five new species and two new genera. Further, existing strains proposed to belong to *B. bacteriovorus* likely represent separate species. Renaming these strains as distinct species would enhance our understanding of *Pseudobdellovibrionaceae* diversity and evolution.

Targeting few environments in relation to host organisms of interest was enough to isolate novel BALOs. Thus, true BALO species diversity is largely underexplored. This may be particularly true for difficult to culture species such as epibiotic predators. In general, epibiotic predators have a narrower prey range than periplasmic predators and produce fewer offspring per prey cell, resulting in lower population growth rates [13, 16, 17], a pattern we also observed for our novel epibiotic isolate B. gaculeatus strain SBM16.

***Pseudobdellovibrionaceae* are likely widespread among host microbiomes**

Although there is growing interest in using BALOs to manipulate host-associated microbiomes, there have been very few studies that have attempted to isolate host-associated BALO species [53–57]. By sampling the immediate environment of three model animal hosts (zebrafish, threespine stickleback, and *C. elegans*) we successfully isolated new *Pseudobdellovibrionaceae* and detected ASVs (highly) similar to the 16S rDNA sequences of

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those strains in microbiomes of the hosts: *Bdellovenatio daniorerio* strain ZFWA1 ASVs were found in zebrafish eggs and larvae, as well as in samples of threespine stickleback guts (Table 3). Zebrafish microbiomes exclusively contained *Pseudobdellovibrionaceae* ASVs of strains originally isolated from aquatic environments. In contrast, stickleback microbiomes contained *Pseudobdellovibrionaceae* ASVs of species originally isolated from many different environments (Table 3), including two *Bdellovibrio* species that we isolated from compost (*B. tomkyle* strain MYbb1 and *B. krueschi* strain MYbb4). Interestingly, ASVs of *B. krueschi* strain MYbb4 were highly prevalent across stickleback gut samples. MYbb4 specifically preys on *Ochrobactrum*, and we indeed detected *Ochrobactrum* ASVs in these same stickleback samples. We found ASVs of aquatic *Pseudobdellovibrionaceae* in worm microbiomes (Table 3). One example is the Tiberius strain, isolated from the nutrient-rich Tiber River in Italy. This strain belongs to the same species as *B. tiberii* MYbb2, which we isolated from compost material in Kiel, Germany. This indicates that the same *Bdellovibrio* strain can be found in multiple habitats and suggests that habitat preferences may depend more on the presence of suitable prey than on the habitat itself.

The prey preferences of different *Pseudobdellovibrionaceae* species likely influences their distributions. For example, *B. daniorerio* strain ZFWA1 was isolated using *Aeromonas veronii*, and this genus is commonly found associated with fish as well as in free-living aquatic microbiomes. We observed ASVs of *B. daniorerio* in zebrafish and stickleback microbiomes, which also contained *Aeromonas* species. Similarly, most of our compost (MYbb) isolates were isolated using *E. coli* as prey. *E. coli* strains are widespread across different environments, and indeed we find many *Pseudobdellovibrionaceae* ASVs in microbiomes from both worms and stickleback fish. In contrast, ASVs of *B. gaculeatus* strain SBM16 were exclusively present in stickleback microbiomes. This species was isolated from a stickleback aquaculture facility, a moderately saline environment. We observed that this species had higher population growth rates

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in the laboratory when a saline medium was used. However, further research is needed to confirm whether this strain truly exhibits strong habitat specificity toward saltwater. Interestingly, this strain had a distinct flagellum morphology compared to the other isolates (i.e., smaller wave amplitudes), which may have functional implications in saline environments. In *Shewanella*, a smaller wave amplitude led to decreased spreading in soft agar (consistent with our observation that SBM16 does not grow well on double-layer agar plates) but increased velocity at higher viscosity (as in salt water) [58], a phenomenon observed in other bacteria as well [59].

Isolating several new *Pseudobdellovibrionaceae* strains, including an epibiotic BALO, revealed that the diversity within the family is highly underestimated and helped improve genome-based predictions regarding predation mode and its evolutionary origin. Efforts in isolating and sequencing new predatory bacteria will further enhance our knowledge and allow for the testing and application of these strains as alternative probiotics or antibiotics in the future.

Formal description of five new species and two new genera within the *Pseudobdellovibrionaceae*

Bdellovibrio tiberii sp. nov. (/tiˈbeːri.iː/, derived from the name of the river Tiber, from which the first strain was isolated) with type strain MYbb2^{T}

Highly motile cells of vibrioid shape (0.25 ± 0.04 µm^2 in size) with a single flagellum (3.34 ± 0.36 µm in length) and a periplasmic predation strategy. The type strain was isolated from *C. elegans*-associated compost material, collected from the Kiel Botanical Garden using *Escherichia coli* ML35 as prey. Cells grow on prey lawns as plaques at 28°C.

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448 The species belongs to the genus *Bdellovibrio* (within the family *Pseudobdellovibrionaceae*).
449 The genome has a G+C content of 49.8% and is approximately 4.05 Mb in size. The strain is
450 accessible at the DSMZ () and the ATCC ().
451
452 *Bdellovibrio kumpostii* sp. nov. (/kumˈpɔs.ti.iː/, latinized from the German word Kompost
453 describing the isolation source, a compost heap in Kiel, Germany) with type strain MYbb5^T
454 Highly motile cells of vibrioid shape (0.21 ± 0.03 µm^2 in size) with a single flagellum (3.02 ±
455 0.55 µm in length) and a periplasmic predation strategy. The type strain was isolated from *C.*
456 *elegans*-associated compost material, collected from the Kiel Botanical Garden using
457 *Escherichia coli* ML35 as prey. Cells grow on prey lawns as plaques at 28°C.
458 The species belongs to the genus *Bdellovibrio* (within the family *Pseudobdellovibrionaceae*).
459 The genome has a G+C content of 49.9% and is approximately 3.89 Mb in size. The strain is
460 accessible at the DSMZ () and the ATCC ().
461
462 *Bdellovibrio tomkyle* sp. nov. (/tɔmˈki.le/, derived from the Low German binomial tom kyle
463 referring to the old name for the city of Kiel in Germany, meaning tom “at the” kyle “fjord”, as
464 the strain was isolated in Kiel) with type strain MYbb1^T
465 Highly motile cells of vibrioid shape (0.19 ± 0.02 µm^2 in size) with a single flagellum (3.02 ±
466 0.22 µm in length) and a periplasmic predation strategy. The type strain was isolated from *C.*
467 *elegans*-associated compost material, collected from the Kiel Botanical Garden using
468 *Escherichia coli* ML35 as prey. Cells grow on prey lawns as plaques at 28°C.
469 The species belongs to the genus *Bdellovibrio* (within the family *Pseudobdellovibrionaceae*).
470 The genome has a G+C content of 48.8% and is approximately 3.88 Mb in size. The strain is
471 accessible at the DSMZ () and the ATCC ().
472

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Bdellovibrio bagaluti sp. nov. (/bagaˈluːti/, derived from the Low German word Bagalut, referring to someone who is mischievous or unruly to reflect the predatory nature of the strain) with type strain MYbb7^{T}

Highly motile cells of vibrioid shape (0.21 ± 0.03 µm2 in size) with a single flagellum (2.99 ± 0.44 µm in length) and a periplasmic predation strategy. The type strain was isolated from *C. elegans*-associated compost material, collected from the Kiel Botanical Garden using *Escherichia coli* ML35 as prey. Cells grow on prey lawns as plaques at 28°C.

The species belongs to the genus *Bdellovibrio* (within the family *Pseudobdellovibrionaceae*). The genome has a G+C content of 45% and is approximately 3.8 Mb in size. The strain is accessible at the DSMZ () and the ATCC ().

Bdellovibrio krueschi sp. nov. (/ˈkʁʏʃi/, derived from the Low German word krüsch which describes someone who is picky reflecting the small prey range of the species) with type strain MYbb4^{T}

Highly motile cells of vibrioid shape (0.25 ± 0.03 µm2 in size) with a single flagellum (2.58 ± 0.33 µm in length) and a periplasmic predation strategy. The type strain was isolated from *C. elegans*-associated compost material, collected from the Kiel Botanical Garden using *Ochrobactrum* MYb71 as prey. Cells grow on prey lawns as plaques at 28°C. So far, only members of the genus *Ochrobactrum* have been identified as prey.

The species belongs to the genus *Bdellovibrio* (within the family *Pseudobdellovibrionaceae*). The genome has a G+C content of 45.2% and is approximately 3.52 Mb in size. The strain is accessible at the DSMZ () and the ATCC ().

Bdellovenatio gen nov. (/bdɛloʊvəˈneɪʃiˌoʊ/, derived from Latin *bdella* meaning “leech” and *venatio* meaning “hunting”, referring to the rapid movement of the cells) with type species

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daniorerio sp. nov. (/dæniɔːˈrɛrioʊ/, derived from the Latin binomial *Danio rerio,* the scientific name of the zebrafish in which this bacterium has been found) and type strain ZFWA1^T

Highly motile cells of vibrioid shape (0.25 ± 0.05 µm^2) with a single flagellum (2.77 ± 0.25 µm in length) and a periplasmic predation strategy. The type strain was isolated from water collected at the University of Oregon zebrafish aquaculture facility on the campus in Eugene, Oregon, USA, using *Aeromonas veronii* ZOR0001as prey. Cells grow on prey lawns as plaques at 28°C. The species belongs to a unique genus in the family *Pseudobdellovibrionaceae*. The genome of has a G+C content of 42.9% and is approximately 3.21 Mb in size. The strain is accessible at the DSMZ () and the ATCC ().

Bdellovampiro gen nov. (/bdɛloʊˈvæmpɪroʊ/, derived from Latin *bdella* meaning “leech” and *vampiro* meaning “vampire” referring to its epibiotic predation strategy) with type species *gaculeatus* sp. nov. (/gɑːkjuːˈliːətəs/, a contraction of the Latin binomial *Gasterosteus aculeatus,* the scientific name of the threespine stickleback fish in which this bacterium has been found) and type strain SBM16^T

Highly motile cells of elongated vibrioid shape (0.77 ± 0.24 µm^2) with a thin and very long (7.13 ± 1.57 µm) single flagellum and an epibiotic predation strategy. The type strain was isolated from water collected at the University of Oregon threespine stickleback aquaculture facility on the campus in Eugene, Oregon, USA, using *Escherichia coli* ML35 as prey. Cells grow together with prey at 20°C. This strain is mesohalophilic, growing optimally in media amended with 2 g/L sea salt. The species belongs to a unique genus in the family *Pseudobdellovibrionaceae*. The genome has a G+C content of 43.5% and is approximately 3.08 Mb in size. The strain is accessible at the DSMZ () and the ATCC ().

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Competing Interests

The authors declare no competing interests.

Data Availability Statement

All raw sequences are available in the SRA under PRJNA1185762 and assembled *Pseudobdellovibrionaceae* genomes are available in the NCBI under PRJNA1185297.

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702

703 **Tables**

704 **Table 1. Phenotypic characterization of novel *Pseudobdellovibrionaceae* isolates.** Values are
705 expressed as mean ± standard error (MYbb1, MYbb2, MYbb4, ZFWA1) or as mean ± standard
706 deviation (MYbb5, MYbb7, MYbb10, MYbb11, SBM16). Respective highest/lowest values are
707 highlighted in bold. Measurements for all independent experiments and grids are given in Table
708 S1.

709 [a]Species named according to results of comparative genome analysis, see section below for
710 details.

711 [b] The Feret diameter ratio was used as a proxy for cell size and shape, with higher values
712 indicating elongated cells.

713 [c]Phenotype confirmed by TEM imaging after negative staining.

714 [d]Description for strains with outstanding phenotype.

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Table 2. Genome comparison of novel *Pseudobdellovibrionaceae* isolates with *Bdellovibrio* and *Pseudobdellovibrio* type strains. Genome quality estimates of % completion (% comp.) and % contamination (% cont.) were obtained from Checkm2. The number of coding genes (CDS) and different types of RNA genes were predicted using Prokka. Exclusively present orthogroups (OG) defined by Orthofinder were only found in a single genome, and exclusively absent orthogroups were found in all genomes but one. Novel isolates are bolded, and type strains are indicated with an asterisk.

[a]Other programs reported deviating levels of contamination, including checkm1 (5.41%), BUSCO (3.2%), and NCBI fcs-gx (0%).

Table 3: *Pseudobdellovibrionaceae* strains can be found in 16S rDNA data sets of relevant hosts. Number of the different *Pseudobdellovibrionaceae* ASVs with up to 4 mismatches (i.e. 99% identity) and their prevalence are shown for each microbiome study and all *Pseudobdellovibrionaceae* strains used in this study.

$^{0\text{-}4}$: number of mismatches to reference sequences

[a]Skin and gut samples collected in 2018 from wildtype adult zebrafish (238 days post fertilization or dpf) reared at the University of Oregon Zebrafish Facility under standard husbandry conditions. Unpublished. Sequenced 16S rRNA region: V4.

[b]Egg samples from 0, 1, and 2 dpf wildtype zebrafish collected in 2022 at the University of Oregon Zebrafish Facility under standard husbandry conditions. Unpublished. Sequenced 16S rRNA region: V4.

[c]Egg (0 and 2 dpf) and larval gut (5 and 7 dpf) samples from wildtype zebrafish collected in 2023 at the University of Oregon Zebrafish Facility under standard husbandry conditions. Unpublished. Sequenced 16S rRNA region: V4.

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[d]Gut samples collected in 2016 from juvenile threespine stickleback (60dpf) reared at the Cresko Stickleback facility at the University of Oregon under standard husbandry conditions. Published in [60]. Sequenced 16S rRNA region: V4.

[e]Gut samples collected in 2015 from adult male threespine stickleback (12-16 months) reared at the Cresko Stickleback facility at the University of Oregon under standard husbandry conditions. Published in [61]. Sequenced 16S rRNA region: V4.

[f]Natural worm samples collected from a compost heap in 2016-2017 and published in [62]. Sequenced 16S rRNA region: V3-V4.

[g]Natural worm samples collected from apples on a compost heap in 2019-2020 and published in [63]. Sequenced 16S rRNA region: V3-V4.

[*]multiple ASVs in worm samples matched similarly well to multiple *Pseudobdellovibrionaceae* strains

Figure Legends

Figure 1: Cell shape and predation mode of *Pseudobdellovibrionaceae* strains MYbb2, MYbb4, ZFWA1 and SBM16. Life cycles of periplasmic (purple) and epibiotic (yellow) *Pseudobdellovibrionaceae* isolates are schematically represented on the left. Numbers in image corners correspond to the proposed life cycle stage. The upper panels illustrate periplasmic *Pseudobdellovibrionaceae* isolates. Stage 1 images represent *Pseudobdellovibrionaceae* cells in attack phase. In stage 2, predators are attached to prey cells (p). Stages 4-7 demonstrate intra-periplasmic growth in bdelloplasts (bd), with one or more *Pseudobdellovibrionaceae* cells confined within the outer membrane of a prey cell. Stage 8 depicts *Pseudobdellovibrionaceae* cells leaving the prey ghost cell (gh). The lower panels display the epibiotic *Bdellovampiro gaculeatus* strain SBM16. This strain has a long flagellum, as illustrated in stage 1 images with

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765 white arrows at the start and end point of the flagellum. After attachment to and penetration of
766 the outer membrane, epibiotic cells grow and divide outside prey cells (stages 4-5) as indicated
767 by different sizes of cells and by septation in elongated bacteria. Samples were imaged by TEM
768 after negative staining. Scale bar is 500 nm. Life cycle images created in BioRender. Wülbern, J.
769 (2024) BioRender.com/t31h353.
770
771 **Figure 2. Phylogenetic relationship between various predatory and non-predatory**
772 **prokaryotes.** IQ-TREE was used to infer phylogenetic trees by maximum likelihood with
773 bootstrap approximation. a) Phylogenetic relationships between selected prokaryotic species
774 based on phylogenomic analysis using 265 single-copy genes found in 82.4% of species
775 predicted by Orthofinder. All branch support values are reported in blue. b) Relationships among
776 *Pseudobdellovibrionaceae* strains based on phylogenomic analysis using 1099 single-copy core
777 genes predicted by Orthofinder. All branch support values from bootstrapping approximation
778 were 100. Novel isolates are bolded and listed by the newly proposed names based on 65% AAI
779 genus and 95% ANI species designations.
780
781 **Figure 3. Relationship between different genome metrics of predatory and non-predatory**
782 **bacteria.** a) Relationship between coding density and GC content. b) Relationship between the
783 total combined number of genes in the M (cell envelope biogenesis, outer membrane) and I (lipid
784 metabolism) clusters of orthologous groups (COG) categories and genome size. All bacterial
785 species in Figure 2b are included.
786
787 **Figure 4. Variation in gene content by predation strategy.** a) The number of shared
788 orthologous genes in *Pseudobdellovibrionaceae* strains identified using Orthofinder. The top 22
789 shared orthologous gene sets are shown. b) Presence of genes involved in peptidoglycan

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790 modification and host independence in *Pseudobdellovibrionaceae* isolates. Dark gray cells
791 indicate the presence of the gene with the best protein match listed under seed ortholog. Light
792 gray cells indicate genes with a different seed ortholog that belong in the same orthogroup. The
793 asterisk indicates that only gene Bd3021 is present in SBM16, not Bd3020. All novel isolate
794 names are bolded.
795
796 **Supplementary Tables**
797 **Table S1. Size measurements for *Pseudobdellovibrionaceae* strains described in this study.**
798 Measurements of bacteria were performed on images taken at 11,000x (TIA camera) or 16,500x
799 (MegaView III camera) magnification using FIJI software (Schindelin et al., 2012). A segmented
800 line tool was used to measure the length of flagella, and a polygon tool was used to measure the
801 cell body area, perimeter and Feret diameter. Culture column indicates whether fresh prey was
802 added before imaging or whether standard overnight cultures were used. The mean, standard
803 deviation (SD) and count numbers are displayed for flagellum and cell body measurements.
804 Units for each measurement are indicated in square brackets. Count column refers to the number
805 of images analyzed for mean values. Run column indicates how many independent experiments
806 (the first number) and how many independent grids (the second number) were analyzed for each
807 sample.
808
809 **Table S2. Average Nucleotide Identity (a) and Average Amino Acid Identity (b) between**
810 **strains.** Pairwise ANI was calculated with fastANI v1.34 and pairwise AAI was calculated with
811 CompareM v0.1.2.
812
813 **Table S3. Genbank accession numbers of genome assemblies included in phylogenomic**
814 **analyses.** Prokaryotic genomes were used to infer phylogenetic relationships between

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periplasmic and epibiotic BALOs. *Pseudobdellovibrionaceae* genomes were used to infer phylogenetic relationships between periplasmic and epibiotic existing and novel *Pseudobdellovibrionaceae* strains.

Table S4. Orthofinder output of gene counts by *Pseudobdellovibrionaceae* isolate in each orthogroup. Rows are predicted orthogroups and columns are genome assemblies. Counts represent the number of gene calls from a genome that belong to a specific orthogroup.

Table S5. EggNOG-mapper annotations of each Prodigal-predicted gene in Orthofinder-predicted orthogroups. Each row represents a unique gene identified by Query. Each gene is annotated from a specific *Pseudobdellovibrionaceae* genome and belongs to an orthogroup. Orthogroups may have multiple genes from the same and/or different genomes. Each query has a Seed ortholog that is the best matching sequence in the eggNOG protein space. Genome id is the Prodigal-assigned id to the genome assembly. Input into eggNOG-mapper was the Prodigal faa file output of each genome assembly. Genome names reflect current database names.

Supplementary Figures

Figure S1: Cell shape and predation mode of periplasmic *Pseudobdellovibrionaceae* strains MYbb1, MYbb5, MYbb7, MYbb10 and MYbb11. Stages in the life cycle of periplasmic *Pseudobdellovibrionaceae* isolates are shown on top. Numbers in image corners correspond to the proposed life cycle stage. Stage 1 images represent *Pseudobdellovibrionaceae* cells in attack phase. In stage 2, predators are attached to prey cells (p). After penetration of the prey's outer membrane, *Pseudobdellovibrionaceae* cells enter stages 4-7 with intraperiplasmic growth in bdelloplasts (bd), with one or more predator cells confined within a prey cell (p). Samples were

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839 imaged by TEM after negative staining. Scale bar is 500 nm. Life cycle images created in
840 BioRender. Wülbern, J. (2024) BioRender.com/t31h353.
841
842 **Figure S2. Average Nucleotide Identity (a) and Average Amino Acid Identity (b) between**
843 **strains.** Pairwise ANI was calculated with fastANI v1.34 and pairwise AAI was calculated with
844 CompareM v0.1.2. Both were visualized in R with ComplexHeatmap v2.20.0. Novel isolates are
845 listed in bold.
846
847 **Figure S3. Relative frequency of COG categories in orthogroups unique to the core,**
848 **epibiotic, and periplasmic genomes.** Orthogroups were annotated with a COG category using
849 eggNOG-mapper and classified as core (found in all genomes), epibiotic, or periplasmic. Total
850 orthogroups per classification are listed in the figure legend. Relative frequency is calculated by
851 summing COG annotations per category within a classification and divided by the total number
852 of orthogroups per classification.
853

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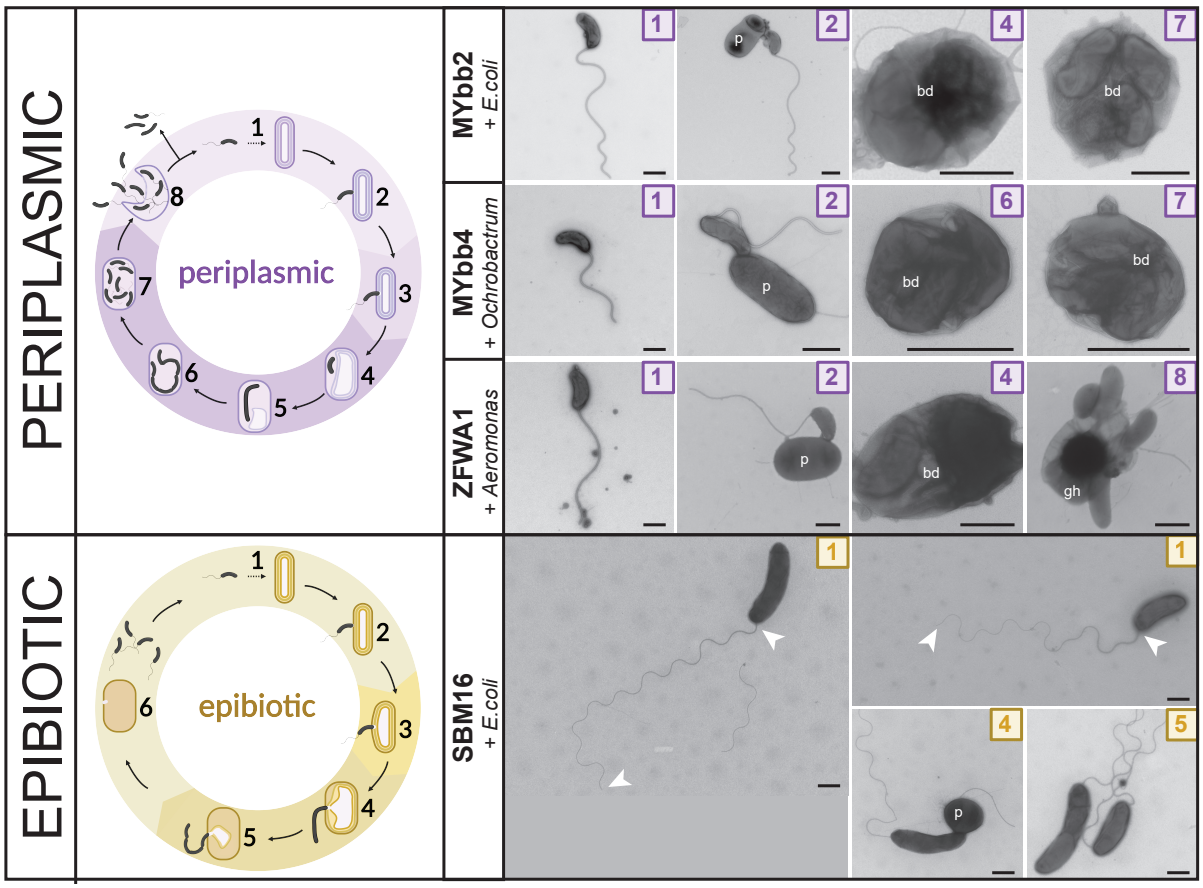

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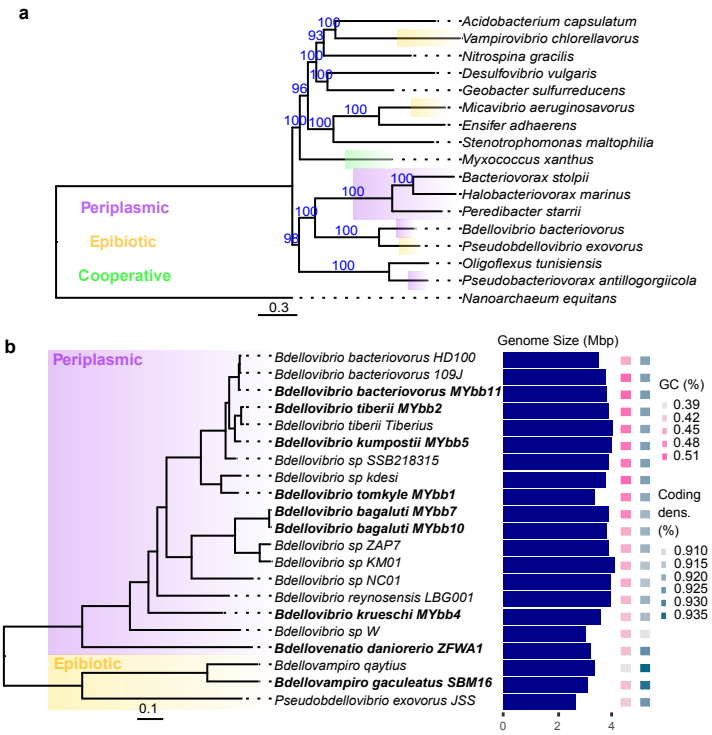

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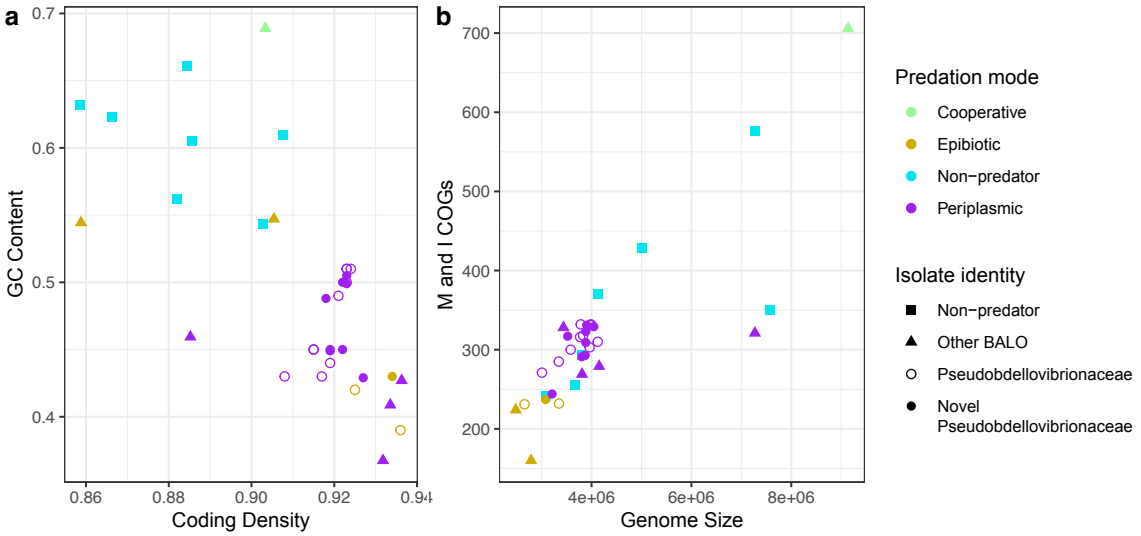

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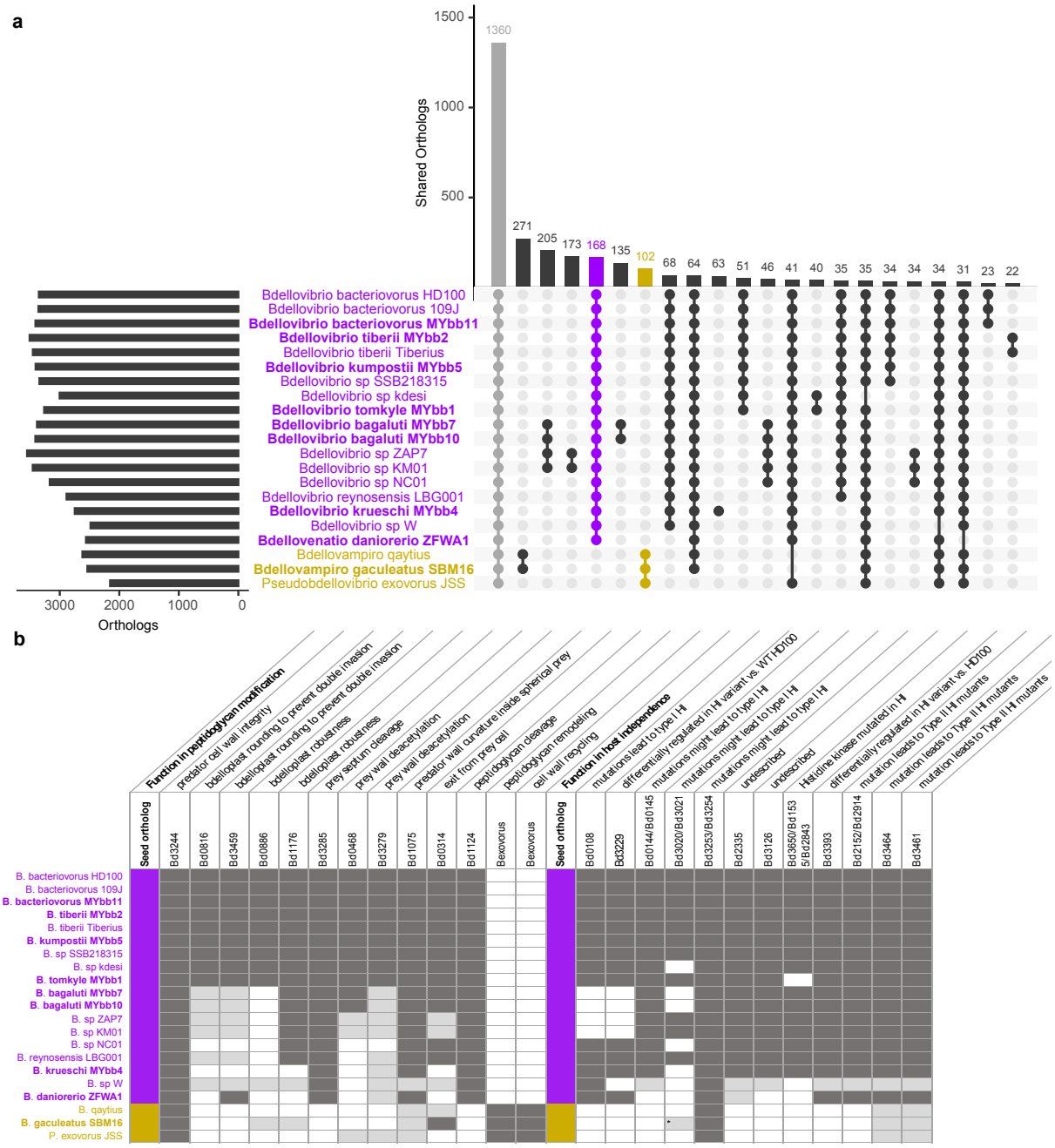

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Strain	Species[a]	Flagellum length [µm]	Cell body area [µm^2]	Perimeter [µm]	Feret diameter ratio[b]	Cultivation prey	Predation strategy[c]	Morphology[d]
MYbb11	***Bdellovibrio bacteriovorus***	2.87 ± 0.57	0.22 ± 0.03	1.85 ± 0.17	1.81	*E.coli*	periplasmic	
MYbb2	***Bdellovibrio tiberii***	3.34 ± 0.36	0.25 ± 0.04	2.01 ± 0.2	1.92	*E.coli*	periplasmic	
MYbb5	***Bdellovibrio kumpostii***	3.02 ± 0.55	0.21 ± 0.03	1.9 ± 0.16	2.08	*E.coli*	periplasmic	narrow cell
MYbb1	***Bdellovibrio tomkyle***	3.02 ± 0.22	**0.19** ± 0.02	**1.67** ± 0.1	**1.78**	*E.coli*	periplasmic	
MYbb7	***Bdellovibrio bagaluti***	2.99 ± 0.44	0.21 ± 0.03	1.82 ± 0.19	2.01	*E.coli*	periplasmic	
MYbb10	***Bdellovibrio bagaluti***	2.9 ± 0.34	0.29 ± 0.04	2.36 ± 0.21	2.29	*E.coli*	*periplasmic* (no confirmation)	narrow cell
MYbb4	***Bdellovibrio krueschi***	**2.58** ± 0.33	0.25 ± 0.03	2.15 ± 0.2	2.16	*Ochrobactrum sp.*	periplasmic	short flagellum, narrow cell
ZFWA1	***Bdellovenatio daniorerio***	2.77 ± 0.25	0.25 ± 0.05	1.99 ± 0.2	1.9	*Aeromonas sp.*	periplasmic	
SBM16	***Bdellovampiro gaculeatus***	**7.13** ± 1.57	**0.77 ± 0.24**	**4.33 ± 0.97**	**3.24**	*E.coli*	epibiotic	long flagellum, narrow cell

[a]Species named according to results of comparative genome analysis, see section below for details.
[b] The Feret diameter ratio was used as a proxy for cell size and shape, with higher values indicating elongated cells.
[c]Phenotype confirmed by TEM imaging after negative staining.
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	Genome size	**GC content (%)**	**# of contigs**	**% comp.**	**% cont.**	**CDS**	**tRNA**	**rRNA**	**Other RNA**	**Exclusively present OG**	**Exclusively absent OG**
*Bdellovibrio bacteriovorus HD100**	3,782,950	50.6	1	99.99	0.15	3,563	36	6	1	0	1
Bdellovibrio bacteriovorus strain MYbb11	3,901,330	50.5	1	100	0.34	3,647	36	6	1	0	0
Bdellovibrio tiberii strain MYbb2*	4,045,310	49.8	19	100	0.34	3,821	35	3	1	3	1
Bdellovibrio kumpostii strain MYbb5*	3,886,825	49.9	1	100	0.28	3,672	35	6	1	1	0
Bdellovibrio tomkyle strain MYbb1*	3,884,186	48.8	1	100	0.25	3,702	37	6	1	2	1
Bdellovibrio bagaluti strain MYbb7*	3,803,878	45	1	99.99	0.88	3,689	34	6	1	1	0
Bdellovibrio bagaluti strain MYbb10	3,873,366	44.9	1	99.99	0.84	3,754	34	6	1	0	0
Bdellovibrio krueschi strain MYbb4*	3,522,424	45.2	5	99.95	11.38[a]	3,479	38	6	2	63	7
Bdellovenatio daniorerio strain ZFWA1*	3,209,714	42.9	4	99.98	0.79	3,082	38	6	1	17	31
Bdellovampiro gaculeatus strain SBM16*	3,080,354	43.5	16	97.12	0.76	2,885	31	4	1	3	2
Pseudobdellovibrio exovorus JSS*	2,657,893	41.9	1	99.99	0.4	2,616	33	3	0	14	64

[a]Other programs reported deviating levels of contamination, including checkm1 (5.41%), BUSCO (3.2%), and NCBI fcs-gx (0%)

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Table 3: *Pseudobdellovibrionaceae* strains can be found in 16S rDNA data sets of relevant hosts. Number of the different *Pseudobdellovibrionaceae* ASVs with up to 4 mismatches (i.e. 99% identity) and their prevalence are shown for each microbiome study and all *Pseudobdellovibrionaceae* strains used in this study.

Host microbiome	ZFW strain	SBM strain	MYbb strains	Other, aquatic strains	Other, terrestrial strains	Total prevalence in organisms
Zebrafish[a]				JSS in 1/29 samples[0]		1 ASVs in 1/29 samples (3.5%)
Zebrafish[b]	1 ASV in 78/210 samples[0]			2 ASVs: ZAP[2], JSS [3] each in 1/210 samples		4 ASVs in 79/210 samples (37.6%)
Zebrafish[c]	2 ASVs in 4/384 samples[0,1]					2 ASVs in 3/384 samples (0.8%)
Stickleback[d]		2 ASVs: in 2/296 samples[0] & in 7/296 samples[2]				2 ASVs in 9/296 samples (3%)
Stickleback[e]	1 ASV in 104/145 samples[4]	1 ASV in 11/145 samples[0]	2 ASVs: MYbb4 in 42/145 samples[2], MYbb1 in 2/145 samples[1]	JSS in 9/145 samples[4]		5 ASVs in 116/145 samples (80%)
Worm[f,*]			✓	✓	✓	4 ASVs in 7/361 samples (2%)
Worm[g,*]			✓	✓	✓	7 ASVs in 8/257 samples (3.1%)

[0-4]: number of mismatches to reference sequences
[a]Skin and gut samples collected in 2018 from wildtype adult zebrafish (238 days post fertilization or dpf) reared at the University of Oregon Zebrafish Facility under standard husbandry conditions. Unpublished. Sequenced 16S rRNA region: V4.
[b]Egg samples from 0, 1, and 2 dpf wildtype zebrafish collected in 2022 at the University of Oregon Zebrafish Facility under standard husbandry conditions. Unpublished. Sequenced 16S rRNA region: V4.
[c]Egg (0 and 2 dpf) and larval gut (5 and 7 dpf) samples from wildtype zebrafish collected in 2023 at the University of Oregon Zebrafish Facility under standard husbandry conditions. Unpublished. Sequenced 16S rRNA region: V4.
[d]Gut samples collected in 2016 from juvenile threespine stickleback (60dpf) reared at the Cresko Stickleback facility at the University of Oregon under standard husbandry conditions. Published in [60]. Sequenced 16S rRNA region: V4.
[e]Gut samples collected in 2015 from adult male threespine stickleback (12-16 months) reared at the Cresko Stickleback facility at the University of Oregon under standard husbandry conditions. Published in [61]. Sequenced 16S rRNA region: V4.
[f]Natural worm samples collected from a compost heap in 2016-2017 and published in [62]. Sequenced 16S rRNA region: V3-V4.
[g]Natural worm samples collected from apples on a compost heap in 2019-2020 and published in [63]. Sequenced 16S rRNA region: V3-V4.
[*]multiple ASVs in worm samples matched similarly well to multiple *Pseudobdellovibrionaceae* strains