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Bacterial invasion and killing by predatory *Bdellovibrio* primed by predator prey cell recognition and self protection

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Bacterial predation, as exemplified by the periplasm-invading model predator Bdellovibrio bacteriovorus, is a fascinating multistage process facilitated by several adaptations to 'regular' bacterial lifestyles. We are beginning to understand more about such adaptations at the molecular level, particularly those concerning processes near the beginning of the predatory lifecycle (recognition, invasion, prey cell wall manipulation). In this review we highlight recent advances in investigating predation and tie these into some of the classical observations and phenotypes that make this two-cell system such an interesting case study in evolution.

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The process of predation

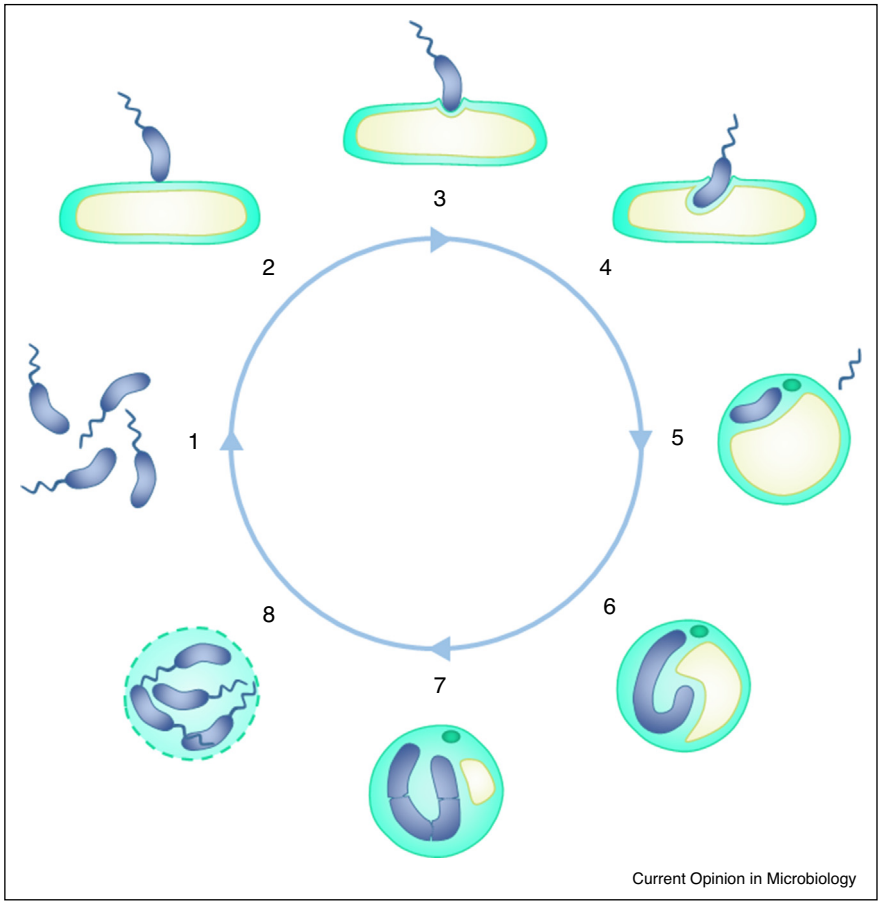
Bacterial predators of bacteria are natural solutions to the growing concern of antimicrobial resistance, offering the possibility of both isolated antibacterial enzymes and whole cell usage [1]. The model predator *Bdellovibrio bacteriovorus* kills prey via periplasmic invasion, entering through the outer membrane and consuming the prey from within; consequent growth and replication occur, with progeny exiting upon exhaustion of prey cell nutrients (Figure 1). The prey is effectively a 'substrate organism' wherein predator progeny production is dependent on prey cell biomass conversion. In decades of research, no acquired resistance to predation has been discovered, and *Bdellovibrio* can kill Gram-negative pathogens regardless of drug-resistance status [2]. Recent advances have demonstrated that *Bdellovibrio* can work in tandem with the immune system to eradicate pathogens in a zebrafish infection model [3]. Thus, therapeutic usage of predators holds promise (e.g. against the agent of plague [4•,5•], also working collectively with phage [6•]), and will benefit from an increased understanding of the molecular features governing successful predation.

Some recent reports have commented on the susceptibility of selected Gram-positive strains (to predator exoenzymes, not invasion [7,8] — given that the equivalent space to the Gram-negative periplasm is apparently incapable of accommodating another cell). This review will focus on classical Gram-negative intraperiplasmic predation, where the staged ~4 hour lifecycle is synchronized thus: (i) freely moving predators locate prey, with the precise role of directed chemotaxis yet to be fully explored; (ii) recognition commences, progressing from an initially reversible association to a 'committed' predator:prey attachment [9]; (iii) the outer membrane of prey is breached, and the predator pulls itself through a dedicated invasion pore; (iv) this pore is then resealed, ensuring minimal loss of prey cell metabolites; (v) metabolism/assimilation of prey macromolecules then drives filamentous growth of the predator; (vi) the filamentous form generates progeny via multiple septa, this adaptation allowing a variable number to be produced thus maximizing economy; (vii) the progeny then develop, lyse the prey and start the predation cycle anew. This unique bacterial lifestyle, phenotypically-rich, does not benefit 'easily' from comparative genomics, given that many of the genes encoded (~3600 in commonly studied strain HD100) are unique to *Bdellovibrio* and related predators [10]. Interestingly, epibiotic *Bdellovibrio* that attach to but do not enter prey, were originally believed to have a relatively smaller genome [11], which has changed with the recent discovery of an epibiotic strain with a genome size similar to the endoperiplasmic HD100 [12].

Observation of predation phenomena

Bdellovibrio is small (~0.2–0.5 μm wide by 0.5–2.5 μm long, exact size depends on lifecycle and nutritive state), with a fully-sheathed flagellum, and is thus extremely fast, swimming at ~100 cell lengths per second [13]. Observations of high-speed impact with prey historically led to the idea of a 'drilling' mechanical component to prey cell invasion, which was later disproven via the predatory capability of motility-compromised mutants [13]. Given that predation has been shown to be effective in high-viscosity environments [14,15] and that penetration was inhibited

Figure 1



Lifecycle of *Bdellovibrio bacteriovorous*.
1. Free swimming attack phase *Bdellovibrio bacteriovorus* 2. Attachment 3–4. Invasion 5. Flagellum loss, pore sealing and bdelloplast formation, 6. Filamentous growth 7. Septation 8. Lysis.

when protein synthesis was blocked [16], one can imagine dedicated adhesins (possibly with a secondary signalling function) initiating predation. The adhesion 'toolkit' will presumably be multifactorial given the lack of prey resistance development, unlike phage, and should correlate with prey-range, which varies between *Bdellovibrio* strains [17]. Adhesins have remained somewhat cryptic, although predator attachment times have been shown to differ between rough and smooth variants of *Salmonella* prey, indicating that the inner core of lipopolysaccharide could represent one example ligand [18]; the absence of lipopolysachharide has also been observed to expedite predation [19]. Predators preferentially enter at the sidewall/long axis of prey, sampling the site of entry using smooth gliding motility [20]. Elegant use of an '*ex-vivo*' culturing method has demonstrated that predators utilize two signals to initiate growth, one from the prey envelope and one from the prey cytoplasm [21]. One interesting recent finding was that when prey nutrient content is low, *Bdellovibrio* is capable of stalling and completing DNA replication via multiple, sequential kills [22•].

The study of attachment and entry have benefitted from a continuum of microscopic studies, including conventional phase-contrast microscopy [23], electron microscopy [24] and cryo-electron tomography [25]; it has also been possible to 'see-inside' the invaded cell using fluorescent-backlighting [26] and helium-ion microscopy milling [27•]. Electron microscopy and tomography reveal that the predator enters through a pore thinner than itself — the comparatively soft *Bdellovibrio* becoming deformed as it squeezes through. The considerable pushback from osmotic forces of the prey cell is partly mitigated through the use of type IV pili to pull the predator inward [28]. The use of fluorescent D-amino acids to monitor peptidoglycan deposition and tailoring revealed that the invasion pore is stabilized by a collar of peptidoglycan, which is then resealed post-invasion by another wave of synthesis [29•]. Restricting the invasion machinery to a collar-localized zone of entry prevents the spread of damage and premature lysis of the prey, akin to being able to pierce a balloon with a pin by placing tape over it!

The initiation of predation and role of cyclic-di-GMP

One clue as to what commits *Bdellovibrio* to the initial stages of the predatory lifecycle was obtained by study of the predation-specific role of the generalized bacterial second messenger cyclic-di-GMP (cdg). In 'regular' non-predatory bacteria, cdg is known to govern switching between behaviours, notably motile:sessile transitions, planktonic:biofilm growth and virulence initiation of pathogens [30]. The enzymes responsible for cdg production (GGDEF motif-containing domains) and hydrolysis (EAL- or HDGYP-containing domains) are gated by often cryptic appended sensory domains that respond to select stimuli [30]. By monitoring the phenotypes of GGDEF-knockout strains, Hobley *et al.* revealed that both entry and exit from the predatory lifecycle was licensed by cdg production [31]. Strains lacking *dgcB* (diguanylate cyclase B, a GGDEF enzyme) were unable to initiate predation, and could only be grown outside prey as HI (host-independent) cultures. Those lacking *dgcA* were rendered non-predatory in a different manner, able to complete prey killing/metabolism, but unable to propagate due to being trapped in the prey debris by a gliding motility deficit at the end of the lifecycle. Interestingly, loss of one GGDEF gene was not complemented by presence of the others, indicating that temporal and spatial factors were likely to be important.

A recent study by Meek *et al.* has provided the molecular details behind the role of DgcB as gatekeeper to the initiation of predation [32•]. This work was directed by the hypothesis that the predator:prey interaction would ultimately result in cyclic-di-GMP production by DgcB. A high resolution X-ray crystallographic structure of DgcB revealed that the C-terminal enzymatic GGDEF domain is appended to an N-terminal FHA (Forkhead-associated) domain, via a flexible linker region (Figure 2). This full-length structure was representative of a post-activation state of DgcB wherein the produced cdg has bound into an inhibitory I-site, locking the enzyme in a feedback-inhibited dimeric form (DgcB is predicted to be a cytoplasmic, soluble protein in all states). Despite this, there were clues to the nature of DgcB activation, given that the appended FHA sensory domain retained the classical motifs/binding cleft known from its usage as a phosphopeptide sensor in other systems [33]. From a pattern of predator sequence conservation and structural similarity to a metabolic checkpoint protein of mycobacteria [33], the authors surmised that the (stimulus) peptide sensed was derived from the N-terminus of DgcB itself, which was disordered in the original structure [32•].

Predation would putatively be initiated by a protein kinase acting on the disordered N-terminal tail of DgcB, which would conformationally switch the appended GGDEF domain into a productive dimeric state, able to convert GTP into cdg. This hypothesis was subsequently proven by firstly obtaining a co-crystal complex between the DgcB FHA domain (residues 32–132) and exogenously added tail phosphopeptide (residues 7–17), and secondly using this structure to design and assay a disulphide mutant that locked the tail in place (without a requirement for phosphorylation). The cdg produced by DgcB then activates a number of downstream targets to license predation: these remain less well-defined, but include CdgA whose deletion exhibits slower invasion [31], and additionally a subset of the 84 *Bdellovibrio* proteins identified by cdg-resin capture methods [34].

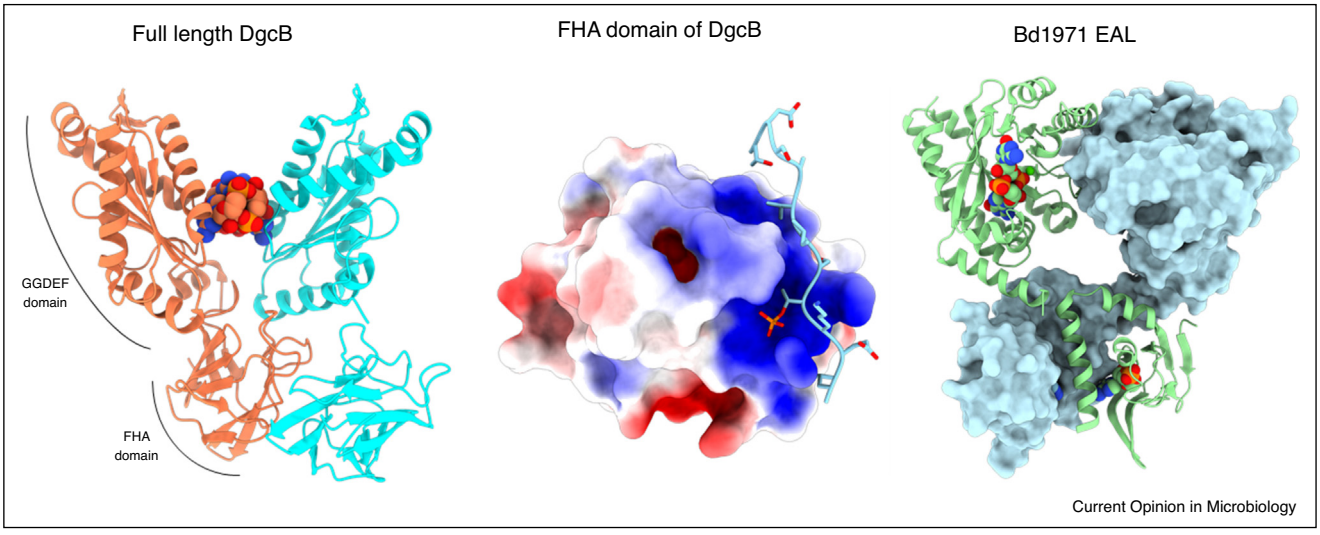
Placement of DgcB as an intermediary between prey sensing and downstream effector production raised the question as to the nature of the activating kinase specific for the DgcB tail peptide. Meek and coworkers found that a related predator encoded a fused kinase-tail-FHA-GGDEF gene, and homology to this thus implicated HD100 gene *bd3148* as responsible for DgcB phosphorylation. Consistent with this, *bd3148* was identified as an essential invasion gene in a recent high throughput study of *Bdellovibrio* [35•]. Any activated kinase is likely to have a host of substrate proteins beyond DgcB, and analysis of the HD100 genome [10] reveals FHA domain proteins encoded from predation-relevant gene loci (type two secretion proteins, gliding motility apparatus components).

The breakdown of cdg, ensuring levels remain low at crucial timepoints, was shown to be catalysed by a cyclic-AMP-activated EAL enzyme (Figure 2), thus linking cyclic mononucleotide and cyclic dinucleotide pathways directly and potentially introducing a metabolic sensor into the process [36•]. Levels of cyclic-AMP may alter as the predatory lifecycle progresses. *Bdellovibrio* is also likely to use 3',3'-cGAMP signalling given that it possesses genes for the metabolism of this additional second messenger [37•,38].

Peptidoglycan metabolism, niche formation and predator self-protection

Following prey recognition, predator invasion into the periplasm commences, and involves a range of interactions with the prey cell wall. It is clear that prey cell manipulation and reshaping is important to the process of predation: the prey cell is more than just a husk to be whittled away, it must provide support and protection for the majority of the timecourse of the intraperiplasmic stage. A pioneering triple publication by Thomashow and Rittenberg discovered that various modifications of the prey cell wall were central to niche formation – (i) endopeptidase activity breaking some of the wall cross-links [39], (ii) deacetylase activity generating a chemical difference between predator and prey [40], and (iii) a remarkable molecular sleight-of-hand in which the Braun lipoprotein link between wall and outer membrane was cut and replaced (topology unknown) by acylation of

Figure 2



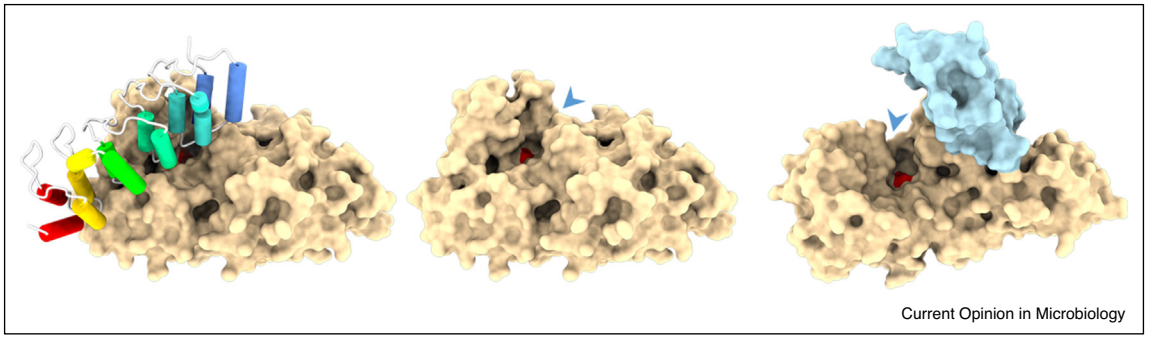
Sensory mechanisms behind cyclic-di-GMP production and degradation.
Left: Full length DgcB in an asymmetric dimer configuration, one chain in coral, one in cyan. Cyclic-di-GMP is shown as spheres, bound by the I-sites of both GGDEF domains. (PDB code 6hbz) **Centre:** The FHA domain of DgcB in complex with a peptide comprising the phosphorylated DgcB N-terminus. A positively charged pocket is present where the phosphothreonine is bound, forming multiple electrostatic interactions and hydrogen bonds (PDB code 6hc1). **Right:** Bd1971 dimer with one chain in cartoon form and one as a surface. The upper lobe binds cyclic-di-GMP and the lower lobe binds cyclic-AMP (both shown as spheres (PDB code 6hq5).

peptidoglycan using long chain fatty acids [41]. The lipidation modification currently remains obscure, but recent progress has characterized the enzymes and physiological function behind both the endopeptidase and deacetylase activities.

Firstly, profiling of the genes upregulated during invasion, the 'predatosome', revealed that (predicted) peptidoglycan-modifying functions feature predominantly during this timepoint [42]. Two of these genes, bd0816 and bd3459 had a moderate similarity to characterized peptidoglycan DD-endopeptidases (e.g. 24% identity to *Escherichia coli* PBP4) that break the 3,4-crosslink of bacterial cell walls. Lerner *et al.* made a double knockout of *bd0816|bd3459*, confirming that these activities were responsible for the characteristic rounding of the prey cell as *B. bacteriovorus* invades [43]. Conversion of the initially rod-shaped prey into a rounded form (termed the bdelloplast) was historically presumed to be a mechanism for ensuring correct progeny development, but was revealed to not affect this. Instead, non-rounded cells invaded by the Δ*bd0816|bd3459* strain were prone to superinfection (additional entry by 'tailgating' predators), demonstrating rounding to be an adaptation that signalled occupancy to kin and drove avoidance of wasteful secondary predation events. High resolution structures were informative as to the high activity of the Bd0816 and Bd3459 rounding DD-endopeptidases – these swap out the inhibitory domain of PBP4 [44] for an entirely different non-obstructed fold, increasing the availability of the active site (Figure 3). Given that the predator retains its shape during invasion, and that *Bdellovibrio* possesses a similar peptidoglycan composition to prey strains, it was a mystery as to how the endopeptidases avoided deleterious action against self. This was later explained by discovery of a co-transcribed immunity protein, Bd3460 (Figure 3), which was able to complex both endopeptidases [45]. Retention of the protective Bd3460 in the *Bdellovibrio* periplasm, and secretion of Bd0816/Bd3459, thus allowed the selective targeting of prey cell walls. Deletion of the *bd3460* gene confirmed this hypothesis, wherein immunity-deficient strains attached to prey, induced endopeptidase production and spectacularly rounded and lysed themselves. Interestingly, the ankyrin repeat-based nature of Bd3460 is a fold often used by intracellular parasites/pathogens of eukaryotes [46] evocative of the intraperiplasmic *Bdellovibrio*.

A different means of predator self protection arises from the deployment of peptidoglycan deacetylases first observed by Thomashow and Rittenberg [40]. Peptidoglycan deacetylases are best-characterized in Gram-positive pathogens where they confer protection from mucosal lysozyme [47]. Lambert, Lerner and coworkers identified the *Bdellovibrio* deacetylases as Bd0468 and Bd3279, the latter possessing an unusual active site plug, both active on *N*-Acetylglucosamine [48]. Strains lacking both genes were predation-competent, but left behind

Figure 3



Self-protection during prey cell wall modification.
Left: Structure of DD-endopeptidase Bd0816 (surface – khaki, homologous to Bd3459) and its cognate immunity protein Bd3460 (Cartoon with rainbow coloured helices; PDB code 5cer). **Centre:** The structure of Bd0816 with the immunity protein removed. The Bd3460 ankyrin fold blocks the active site cleft (blue arrowhead), the catalytic serine is shown in red. **Right:** Structure of PBP4 from *E. coli* (PDB code 2exb). When viewing the carboxypeptidase domain in the same orientation as Bd0816, a large inhibitory subdomain can be observed (blue surface) obscuring the active site cleft (arrowhead).

prey cell ghost envelopes, indicating that full, efficient predation must use specialized enzymes that recognize the deacetylation modification. Self-protection would ensue given that the predator does not modify its own wall in this manner, and so is resistant to these late-acting enzymes. Combination of this phenotype with the rounding deficit (a Δ*bd0468|bd0816|bd3279|bd3459* quadruple mutant) resulted in the creation of rod-shaped ghosts.

These peptidoglycan modifications, and their catalysis by multiple enzymes, highlight the importance of redundancy in *Bdellovibrio*. Indeed, the duplication of endopeptidases and deacetylases is dwarfed by the 19^{+} LD-transpeptidases in strain HD100 [10], which group into several families at least in part responsible for the collar/trapdoor prey modifications observed in fluorescent D-amino acid experiments [49]. Broader enzyme specificity, achieved via gene duplication, may even contribute to increasing prey range by the ability to work on biologically diverse substrates. Duplication and diversification also extends to other invasion phenomena, given that *Bdellovibrio* has multiple pilin genes and gliding motility operons [10]. We await further revelations on the fascinating biology of this complex system, whose rich function and potential utility continues to excite.

Conflict of interest statement

We, Andrew Lovering and Simon Caulton state that we have no conflict of interest.

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