Predatory mechanisms of *Bdellovibrio* and like organisms

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Bdellovibrio and like organisms (BALOs) are predatory, Gram-negative delta-proteobacteria with a complex developmental lifecycle. In the free-living attack phase they are highly motile and seek out prey bacteria that they invade. The ensuing intracellular growth and replication is characterized by the development of a long filament that septates into individual cells that differentiate further into the flagellated attack-phase bacterium. The prey bacterium is lysed and motile predators are released. BALOs have recently been considered to have potential as living antibiotics. The idea of using predatory bacteria as therapeutic agents to combat pathogenic Gram-negative bacteria is intriguing, as they can prey upon human pathogenic bacteria including *Salmonella*, *Pseudomonas* and *Escherichia coli*. However, our current knowledge of the amazing biology of these prokaryotes that cause the systematic destruction of Gram-negative bacteria is still rather limited. More has to be learned about their predatory lifestyle before their application as therapeutic agents will become feasible.

Predatory bacteria were discovered more than 40 years ago in experiments designed for isolating phages by using the common double-layer plate technique. Plaques developed on lawns of susceptible bacteria 2–3 days after the onset of the experiment and increased slowly in size for up to 1 week [1,2]. These plaques were found to contain small, highly motile, Gram-negative bacteria. Further analysis revealed that these bacteria were equipped with a single polar flagellum and lysed the prey bacteria. *Bdellovibrio* and like organisms (BALOs) were found to prey on Gram-negative but not on Gram-positive bacteria. Since these studies, a wide variety of Gram-negative bacteria has been found to be susceptible, including members of the *Aeromonadales*, *Enterobacteriales*, *Vibrionales* and *Pseudomonadales* [2–4]. Predators have been isolated from a number of environments, including the plant rhizosphere, salt and fresh water, and even the animal gut [5–11].

Originally, all isolates were grouped into the genus *Bdellovibrio*, but more detailed genetic analyses revealed distinct taxonomical divergences among the isolates. Presently, all BALOs are grouped into two families belonging to the order *Bdellovibrionales*: *Bdellovibrionaceae* include, for example, *Bdellovibrio bacteriovorus* and *Bdellovibrio* spp.W, while the two new taxonomic genera, *Bacteriovorax* and *Peredibacter* form the family *Bacteriovaoraceae* [12–14].

B. bacteriovorus is the best-characterized obligate predatory species. Genome sequencing of the type strain *B. bacteriovorus* HD100 revealed a single circular chromosome consisting of approximately 3.8 million base pairs (bp) [15]. The genome is predicted to comprise 3584 genes, but only 55% of all putative open reading frames (ORFs) show homology to known proteins. The GC content differs in only four genomic regions from the average, indicating that recent uptake of foreign genetic information by horizontal gene transfer has probably been a rare event. Another study using a variety of phylogenetic and comparative genomics analyses postulated that ancient lateral gene transfer has shaped the genome of *B. bacteriovourus* to a great extent [16]. The genomic sequence of *B. bacteriovorus* HD100 was interpreted using the physiological data obtained from *B. bacteriovorus* 109J, another strain, which had been used in the majority of the earlier studies. Although the two strains show identity in 16S rRNA sequences, there may be considerable diversity elsewhere, as shown by the analysis of the outer membrane protein genes. The major outer membrane proteins (Omps) of the two strains show only a conservation of 82%, which suggests a high degree of strain specificity for the function of these proteins, particularly as the major Omp of another strain (HD114) is even more diverse [17,18].

Currently, further genome sequences on *Bacteriovorax marinus* and *Bdellovibrio* spp. W are in progress and will increase knowledge regarding the predatory mechanisms of BALOs. Genetic manipulation of *B. bacteriovorus* has been achieved by using a conjugation procedure based on suicide plasmids [19] and was successfully used to inactivate a putative chemotaxis gene and flagellin genes (Figure 1) [20,21].

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cells by *B. bacteriovorus* 109J [20]. Strain HD100 has 20 MCP genes and a chemotaxis machinery for signaling environmental changes to the flagellar motor [15]. Aerotaxis of BALOs was clearly demonstrated earlier [30], but whether these findings are connected to a mechanism of prey location has yet to be tested experimentally.

Most of the early research addressing prey location in liquid cultures concluded that the encounter between prey and predator resulted from random collisions [31]. This means that the chances for collision between predator and prey are directly dependent on the cell density of both and can be described with mathematical models [32]. High motility is important for efficient encounters with prey bacteria.

Prey bacteria attached to solid surfaces as part of biofilms [33,34] or fixed to filters [35] were also susceptible to predation by *B. bacteriovorus*. Even a short exposure time of 30 min was sufficient for a successful attack [34]. As in the experiments, *B. bacteriovorus* was directly applied to the prey bacteria, the mechanism of prey location in solid interfaces has not been addressed, although it was assumed that the initial encounter occurs by random collision [33].

Attachment

Microscopic observation has demonstrated that the predator exclusively attaches to prey via the pole opposite the flagellum. The initial attachment is reversible and does not involve specific structures or receptors. This was demonstrated by the fact that predatory strains can attach to Gram-positive, non-prey bacteria and even abiotic surfaces (e.g., glass) [26]. An irreversible, productive attachment was estimated to occur in only three per 100 collisions with prey bacteria [31]. However, the nature of the irreversible attachment between prey and predator remains unclear, as the existence of specific receptors or sites could not be unequivocally demonstrated [26]. In one study it was concluded that the prey receptor sites involved in attachment of *B. bacteriovorus* were partially located in the lipopolysaccharide (LPS) core, as both rough and smooth strains of *Salmonella* were susceptible to predation [36]. By contrast, in the same study the facultative predatory strain *B. stolpii* recognized outer membrane porins to a certain degree. While a thick polysaccharide capsule did not prevent the attachment and successive invasion of *E. coli* by *B. bacteriovorus* [37], paracrystalline protein surface layers (S-layers) protected Gram-negative bacteria from predation [38].

In a recent paper, *B. bacteriovorus* 109J was incubated in a mixture of two prey cells present in equal numbers [39]. In multiple prey pairings, *B. bacteriovorus* preferentially lysed on one prey over the other. When prey bacteria were individually incubated with *B. bacteriovorus*, they were preyed on with different efficiencies. Timing of attachment of *B. bacteriovorus* to prey cells also varied, with *Bdellovibrio* attachment to more preferred prey occurring the fastest. These results suggest that *B. bacteriovorus* 109J does not randomly infect prey cells but infects and kills some prey more readily than others, although the underlying mechanism remains unclear.

On the predator side, LPS is – due to its unique structure – an attractive candidate for recognition, since the LPS of *B. bacteriovorus* includes an uncharged lipid A lacking phosphate residues [40]. This LPS represents a novel bacterial structure and it is tempting to assume a correlation between this cell wall compound and the predatory lifestyle, as some other Gram-negative bacteria, living in specialized environments, also possess unusual lipid A structures [40]. After analyzing another predatory bacterium, Steiner and colleagues suggested that sphingolipids are involved in the recognition process. *B. stolpii* synthesizes a sphingophosphonolipid as well as the most common lipid species, phosphatidylethanolamine and phosphatidylglycerol [41,42]. Sphingolipids are rarely found in bacteria but are prevalent in eukaryotic cells. In mammalian cells they play a key role in invasion by pathogens and an important role in transmembrane signaling [43,44].

In 1991, Gray and Ruby wrote [26]:

"Bdellovibrios are capable of responding to a variety of cell surface characteristics as a means of identifying suitable prey. This reinforces the idea that bdellovibrios possess only very general, though highly adaptable, abilities of prey recognition that might more accurately be described as simple environmental sensing mechanisms"

This statement is still valid.

Penetration

In this stage of the lifecycle, *B. bacteriovorus* progress from the free-living attack phase to the intracellular growth phase. After irreversible attachment, the predator generates a pore in the envelope of the prey bacteria through which it can smoothly enter (within about 10 min) [45]. A prerequisite for penetration seems to be that the prey

BALOs were exposed to various prey cell extracts, both autoclaved Gram-negative and Gram-positive bacteria, as well as uncharacterized prey factors, were found to stimulate extracellular growth. The contradictory results of various studies (summarized in [26]) were probably a result of different prey–predator systems, leaving the nature of the prey-derived factors unclear. In a later study, axenic growth of *B. bacteriovorus* was stimulated by heat shock and it was concluded that the heat shock had altered the transcription of one or more genes and had generated a signal normally derived from prey [58]. These observations have not been investigated further.

Additionally, the continuous presence of prey-derived factors are necessary for elongation of *B. bacteriovorus*. By treatment with lytic enzymes normally produced by the predator at the end of the lifecycle, it was possible to induce the release of predator cells from the bdelloplast at various stages of intracellular growth. This premature release prompted the released bacteria to differentiate into motile attack-phase cells upon completion of their previously initiated rounds of DNA replication [59,60]. As the prey-derived factors were not limited to a single class of compounds, their identity remains unclear [61] and it is likely that more than one prey signal – perhaps a regulatory cascade – is required to commit predators to filamentous growth [57].

Utilization of prey compounds by *B. bacteriovorus* is astonishingly efficient. A mass balance shows that 50–55% of the substrate cell carbon is assimilated, 15% is respired and the remainder is discarded [45]. Several ideas have been put forward to explain how BALOs take up nutrients from the cytoplasm [62]:

- The expression of new channel proteins encoded by prey genes is induced by the invading predator;
- The predator synthesizes and inserts its own pore-forming proteins;
- The predator reutilizes existing prey channel proteins to achieve access.

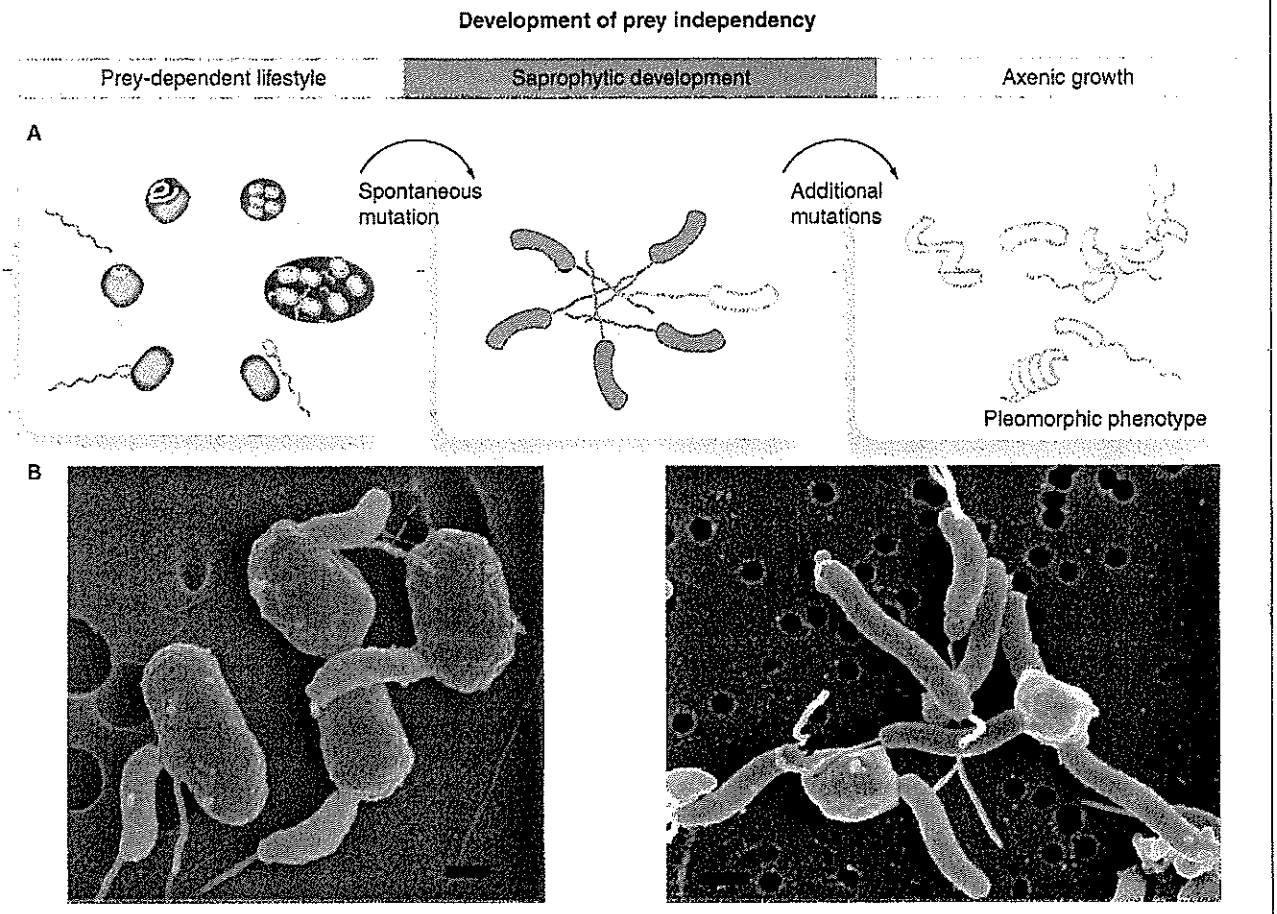
The last concept was supported by a number of studies, mainly from one group, showing that *B. bacteriovorus* has the ability to reutilize prey porins. Relocation of *E. coli* OmpF into the predator outer membrane was described and used to explain the metabolic efficiency of the predator ([45], and the literature cited therein) [63]. However, another controversial study refuted the transfer of OmpF to the outer membrane of the predator and showed that intraperiplasmic *B. bacteriovorus* synthesized it's own OmpF-like protein [64]. In later studies, translocation of the *B. bacteriovorus* OmpF-like protein into the prey's cytoplasmic membrane was reported [65,66], providing evidence that the predator gains access to the cytoplasm by forming a new channel in the cytoplasmic membrane of the prey, killing it in the process. Two recent studies using mass spectrometry and reverse genetics clearly demonstrated that different *B. bacteriovorus* strains produce a highly abundant innate Omp, whereas no evidence for an Omp relocation was found [17,18]. Protein data from these studies clearly suggested that the major Omp of *B. bacteriovorus* is the OmpF-like protein described earlier [64–66]. The polypeptide was also found to be associated with membranes in prey ghosts [18]. The *B. bacteriovorus* Omps form a new family of Omps that lack similarity to known proteins and for which differences in the primary structures indicate a high degree of strain specificity, although a porin function has yet to be proven. Analyses of outer membrane fractions of more predatory strains indicate that related Omps are widely distributed in BALOs [56].

During growth, *B. bacteriovorus* incorporates prey cell components that have been predigested and the incorporation of prey DNA- and RNA-derived nucleotides as well as fatty acids has been described ([25], and the literature cited therein) [45,67]. Interestingly, *B. bacteriovorus* HD100 has biosynthetic pathways for only eleven of the amino acids and ten amino acid degradation pathways are missing [15].

Septation & development

Septation into daughter cells begins when the filament has reached a size several times that of the free-living predator. The final length of the filament and the number of progeny cells seems to be determined by the size of the prey cell. This was shown using *E. coli* K-12 Hfr strains that grow to variable lengths of up to 100 µm. The number of daughter cells obtained varied, from as few as three to four in small prey cells up to as many as 90 in filamentous, multinucleate *E. coli* [45,68]. The dependence of growth duration on the size of the prey cell suggests that the filament extends until nutrient depletion and that differentiation into attack phase is initiated in response to nutrient-limited conditions. Finally, the filament is multinucleate and cross-walls appear simultaneously and equidistantly.

Figure 2. Development of wild-type *Bdellovibrio bacteriovorus* towards prey independency.



(A) From wild-type strains (left), prey-independent (host-independent) mutants can be isolated that are able to grow saprophytically on heat-killed bacteria (middle). The mutants are initially facultative predators and show morphological variations. Further cultivation in the absence of prey generates axenic mutants (right) that only grow on rich media and have lost the ability to invade prey cells. These axenic mutants show major pleomorphism. **(B)** Electron micrographs of wild-type *Bdellovibrio bacteriovorus* HD100 (orange) invading *Escherichia coli* K-12 cells (left) and saprophytic mutants of *B. bacteriovorus* HD100 (blue) growing on heat-killed *E. coli* K-12 (right). Bars represent 300 nm.

survival? In laboratory experiments the prey-dependent lifestyle was superior to the prey-independent lifestyle, as cocultivation of a predatory strain and a prey-independent strain on the same prey always led to elimination of the axenic mutants. In addition, cultivation of axenic mutants alone in the presence of living prey, soon led to the development of prey-dependent revertants [31].

Future perspective: predatory prokaryotes as therapeutic agents?

The use of predatory bacteria to combat pathogenic bacteria is obviously an intriguing possibility [15,67]. The development of new antimicrobial strategies is imperative given the global increase of antibiotic-resistant microorganisms.

Indeed, a successful reduction in the number of food-borne pathogenic and spoilage bacteria was reported using *B. bacteriovorus* 109J [76]. The ecological role of BALOs has been mostly studied in aquatic environments and BALOs were shown to decrease the number of viable Gram-negative bacteria in polluted waste water sewage plants [77]. Investigations in natural environments revealed that BALOs may survive best in biofilms [78]. Recent studies confirmed the ability of *B. bacteriovorus* to prey successfully on bacteria in biofilms, although it was found that the level of surviving prey in biofilms was higher than that observed for free cells [34] and the efficiency of predation on a biofilm depends on nutrient conditions of the environment [33]. In a recent study,

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