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Predatory Prokaryotes: An Emerging Research Opportunity

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Abstract

Predatory prokaryotes have evolved a unique strategy of obtaining energy and biosynthetic materials from their surroundings: acquiring them from other living bacterial cells. These types of microbes have been found in a diverse variety of environments, and may play an important role in modulating microbial population structure and dynamics, as has been hypothesized for marine viruses and possibly protists. Only one genus of predatory bacterium, *Bdellovibrio*, has been extensively described and studied, though several other examples have been reported in the literature.

In this review, the four basic strategies used by currently described predatory prokaryotes will be discussed: ''wolfpack'' group predation, epibiotic attachment, direct cytoplasmic invasion, and periplasmic invasion. Special adaptations to each approach will be considered, and compared overall to the genetic and biochemical characteristics of symbiotic or pathogenic prokaryotes living within eukaryotic cells.

Two specific examples of predatory microbes, *Bdellovibrio* and *Ensifer*, will be described in terms of predation strategy, association with host cells, and host range. The prospects for bringing to bear the tools of molecular microbial genetics to the study of predatory prokaryotes will be explored, using current research with *Bdellovibrio* and *Ensifer* as examples.

Introduction

> ''So, naturalists observe, a flea
> Hath smaller fleas that on him prey;
> And these have smaller still to bite 'em
> And so proceed *ad infinitum*.''
> Jonathan Swift, *On Poetry, A Rhapsody* (1733)

While Swift's poem *was* referring to jealousy between authors (the segment above is followed by ''Thus every poet in his kind/Is bit by him that comes behind'') (Swift, 1733), this quote could just as well be applied to the microbial world. Current evidence suggests, in fact, that the microbial world is characterized by competition and predation (Casida, 1988; Crespi, 2001; Ruby, 1992), almost an ''eat or be eaten'' microcosmos involving bacteriophages, protists, and the subject of this short review, predatory prokaryotes. The role that these sorts of interactions play in microbial community structure, cycling of various elements, and even health or medical issues remains to be fully investigated.

This level of complex interaction is only now coming fully to light. It is hoped that this review will interest readers and investigators in exploring a new niche for microbiological research, by describing the modest amount of detail known concerning predatory prokaryotes, the possible significance of such interactions, and the types of tools and approaches amenable to the study of these organisms. It is indeed an area of molecular genetic research that is ripe with possibilities and opportunities to match the technical challenges.

Bacterial Predators can be Viral, Protistan, or Prokaryotic

Over the past fifteen years, it has become increasingly apparent that aquatic environments, and the oceans in particular, carry a large biological burden of viruses not due to human influences, instead relating to resident prokaryotic populations (Wommack and Colwell, 2000). These bacteriophages have been shown to change their overall number and diversity depending on season and geographical location, in essence reflecting the changing microbial populations that they require for proliferation (Fuhrman, 1999). Estimates have suggested that up to twenty percent of aquatic bacterial populations are lysed by bacteriophages on a daily basis (Suttle, 1994), directly impacting nutrient availability and cycling.

The dynamics of marine bacteriophages versus sensitive populations of bacteria are only now being explored. For example, it is possible that certain classes of bacteriophages are modulators of cyanobacterial planktonic populations (Wommack and Colwell, 2000), and thus may influence primary productivity of particular environments, as well as overall microbial community structure, dynamics, and nutrient cycling as suggested above (Fuhrman, 1999).

Eukaryotic protists are also grazers or predators of prokaryotic populations (Hahn and Hofle, 2001). Some researchers consider them to be major factors in controlling microbial community structure and food web cycling (Hahn and Hofle, 2001). Alternatively, attempts to investigate the impact of grazing protists on introduced populations of soil bacteria in agricultural settings have not been so clear cut (Jjemba, 2001). What is certain is that protistal grazers of prokaryotes are ubiquitous in terrestrial, marine, and freshwater

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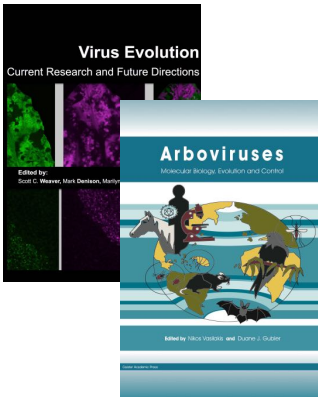

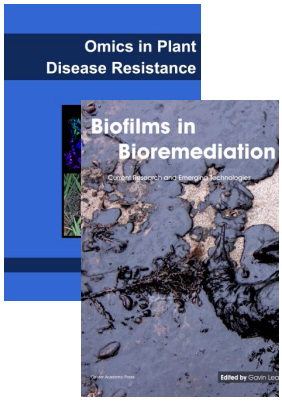

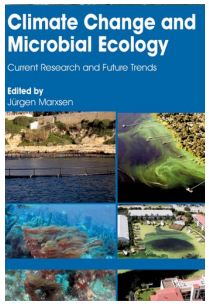

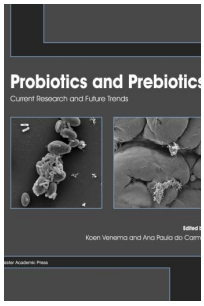

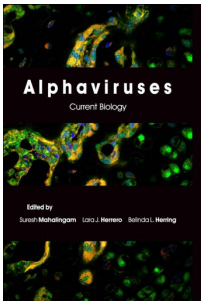

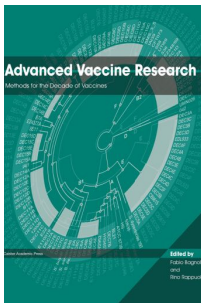

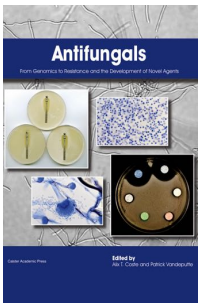

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environments, and clearly influence the population size and diversity of the local microbial ecology.

Interactions between prokaryotes in nature seem to be the rule rather than the exception (Casida, 1980; Casida, 1988; Fallon and Brock, 1979; Guerrero *et al*., 1986; Ruby, 1992); this differs from the pure cultures and single colony isolates with which microbiologists study most prokaryotes. Antagonistic interactions can range from antibiotic or bacteriocin like inhibition of growth of sensitive cells due to the presence of a specific organism (Burnham *et al*., 1981; Long and Azam, 2001) to the possibility of one bacterium literally living inside another in apparent symbiosis, as has recently been described for mealybug bacterial symbionts (von Dohlen *et al*., 2001). The focus of this short review is on antagonistic associations between bacteria, but it is important to recognize that no prokaryote appears to be truly ''alone'' in nature, as illustrated by the ubiquitous presence of biofilms inhabited by many bacterial species (Davey and O'Toole, 2000).

As will be described below, predatory interactions between microbes fall into several general classes. Regardless of mechanism, predation of bacteria by other bacteria could have the same impact on sensitive populations as that of viruses or protists, and is even less well investigated.

Predatory interactions in general are plentiful, diverse, complex, and quite easy to demonstrate. Consider the simple ''baiting'' technique for identifying predators as described by Casida (Casida, 1980; Casida, 1982; Sillman and Casida, 1986). A sample of moistened soil is placed in a petri dish, covered with a piece of sterile 0.65 micron filter paper, followed by the addition of the host or prey organism to the upper surface of the filter paper. After a few days, a dizzying array of protist, bacteriophage, and prokaryotic predators appear on the upper surface of the filter, consuming the prey organism. Most of these predatory organisms have never been examined closely by microbiologists or microbial geneticists.

Discovery of *Bdellovibrio* and Other Prokaryotic Predators

In the early 1960s, during a search for bacteriophages of plant pathogenic bacteria, Stolp and coworkers found a most unusual organism (Stolp and Starr, 1963). The double layer plates that revealed the expected bacteriophage plaques after overnight growth showed additional ''late'' or tardy plaques that continued to grow in size, unlike genuine phage plaques. Observation of the material within these tardy plaques revealed small, highly motile curved rods now called *Bdellovibrio* (Latin for ''leech'' and ''curved rod,'' describing both form and habit), which could be purified through several plaque cycles, much like bacteriophage. This small organism could not normally be grown axenically on nutrient medium, and appeared to live by invading host cells and growing within the periplasm (the region between inner and outer membrane), described in more detail below. Thus, this organism appeared to alternate between a free swimming ''hunt'' phase and a host-dependent ''intracellular'' growth phase. The life cycle of *Bdellovibrio* is illustrated in Figure 1.

With the same techniques used to search for and purify strains of bacteriophage, it has been possible to isolate *Bdellovibrio* strains and species from a wide variety of environments, capable of attacking most Gram negative organisms (Ruby, 1992). In fact, many strains or species of *Bdellovibrio* are not particularly specific in terms of host cell preference; for example, strain 109J of *Bdellovibrio bacteriovorus* can attack and grow on bacterial genera as diverse as *Escherichia*, *Chromatium*, *Rhizobium*, *Pseudomonas*, and *Spirillum*. (Ruby, 1992; Stolp and Starr, 1963).

Bdellovibrio strains are ubiquitous in most environments, ranging from marine to plant associated to the purely terrestrial (Cotter, 1992; Jurkevitch and Ramati, 2000; Ruby, 1992), while alternating between free living and intracellular growth phases. The definition of the species name ''*Bdellovibrio*'' was originally based on this observable phenotype: the intraperiplasmic growth habit of the organism and its biphasic life history. It can be argued, in fact, that most ''species'' of *Bdellovibrio* are not closely related, based on G+C content (Donze *et al*., 1991; Ruby, 1992) and 16s rRNA phylogenetic analysis (Baer *et al*., 2000; Donze *et al*., 1991). This latter technique of classifying organisms using molecular chronometers and phylogenetic tree building has led one research group to suggest that some ''species'' of *Bdellovibrio* be redefined as a new genus named *Bacteriovorax* (Baer *et al*., 2000). In any event, based on 16s rRNA analysis, *Bdellovibrio* appears to be a δ-proteobacter (Ruby, 1992), most closely related to *Myxococcus* and sulfate reducing microbes such as *Geobacter*.

The role of *Bdellovibrio* in nature remains enigmatic. *Bdellovibrio* has been associated with sensitive *Legionella* populations in nature (Richardson, 1990), providing partial support for a role in controlling various populations of bacteria in specific environments.

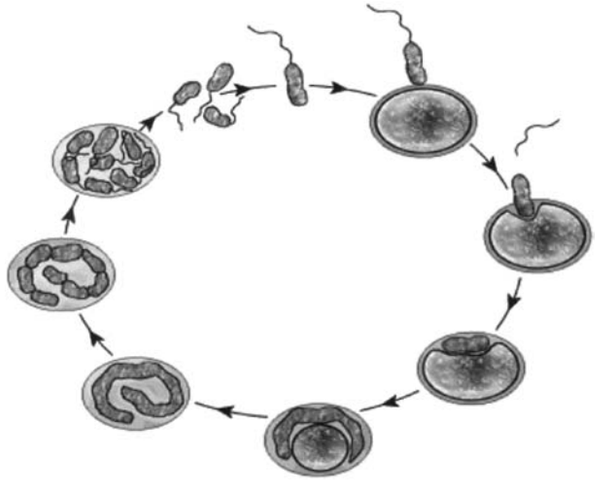

Figure 1. Diagram of the general lifecycle *of Bdellovibrio bacteriovorus*. Illustration courtesy of J.J. Quinn.

The numbers of *Bdellovibrio* and prey necessary to maintain viable populations in nature—particularly considering the ubiquitous occurrence of this organism—have been investigated by several groups (Varon *et al.*, 1984; Wilkinson, 2001; Williams, 1988). However, it may be that non-sensitive prey cells or ''decoys'' (Wilkinson, 2001) can modify these calculations, allowing more complex relationships between predator and prey, and preventing population crashes. Williams and his coworkers, interestingly, have shown a relationship between biofilms and *Bdellovibrio* in estuarine environments (Rice *et al.*, 1997; Williams, 1988), which leads to the question of how efficiently or directionally *Bdellovibrio* ''hunts'' prey cells.

On a more practical note, *Bdellovibrio* has been employed to reduce bacterial contamination in food preparation machinery (Fratamico and Cooke, 1996) and even to reduce the incidence of bacterial pathogens in agriculture (Scherff, 1972). However, until the population dynamics between predator and prey are better understood (Varon *et al.*, 1984; Wilkinson, 2001), it is difficult to see how *Bdellovibrio* could easily be used in health or agriculture related roles, despite recent progress in using bacteriophages as an antimicrobial tool, even in the treatment of disease (Biswas *et al.*, 2002).

Casida and coworkers (Casida, 1980; Casida, 1988) found many non-obligate predators of soil bacteria through a ''baiting'' technique much like the one described above, but none are as well characterized as *Bdellovibrio*. One such search resulted in *Ensifer*, which (unlike *Bdellovibrio*) has a strong specificity for attacking and consuming members of the genus *Micrococcus*, yet can also be grown on most standard laboratory media (Casida, 1982). This makes *Ensifer* a possible candidate for relatively straightforward genetic analysis. Surprisingly, 16s rRNA analysis of *Ensifer* shows a close relationship to the fast growing rhizobia in the α proteobacter group, with a marked similarity (97% identity across a short segment of rRNA) to *Sinorhizobium fredii* and *Sinorhizobium meliloti* strain 1021 (Rogel *et al.*, 2001, unpublished obser vations from this laboratory). This suggests that various strategies of predation may have evolved many times over the evolutionary history of the prokaryotes.

Ensifer attaches to host cells under low nutrient conditions, picket fence fashion, but does not appear to enter the periplasm or cytoplasm of host cells. Instead, *Ensifer* generates an uncharacterized diffusible lytic factor (Casida, 1980; Casida, 1982), much as described for the myxobacters below. In addition, a dark ''bar'' observable by electron microscopy can in some cases be seen stretching from *Ensifer* to prey cells, perhaps acting as some kind of nutritional conduit (though Casida is careful to state that this ''bar'' is not always seen, which is confirmed by work in this laboratory). This ''bar,'' incidentally, is the source of *Ensifer*'s name (Latin for ''swordbearer'') (Burnham *et al.*, 1981).

Also of interest is *Ensifer*'s habit of ''tracking'' prey cells (Burnham *et al.*, 1981). Recalling how *Ensifer* was originally isolated, it is not a surprise to learn that the predator microbe appears to ''pursue'' its host, *Micrococcus*. Thus, a dried line of *Micrococcus*, when crossed with a line of *Ensifer* on medium with a low concentration of nutrients, leads to *Ensifer* rapidly ''spreading'' along the length of the *Micrococcus* cells. This behavior does not occur when non-prey cells such as *E. coli* are used (see Figure 2). Close microscopic analysis (J. Leadbetter and M.O. Martin, unpublished results) show *Ensifer* moving rapidly throughout the exopolysaccharide—like substance overlaying the prey or host cells. It is not yet clear if tracking is dependent upon this viscous material.

Predatory bacteria have also been discovered solely by observation in specialized environments, particularly ones that display a ''bloom'' of one prokaryotic species. Such blooms perhaps allow a natural enrichment for specific predatory prokaryotes. In a study of a holomictic anaerobic lake in Spain, two new types of predatory microbes (*Vampirococcus* and *Daptobacter*) were found by electron microscopy. The two putative predators were shown to be in association with a common prokaryotic inhabitant of that environment, a member of the genus *Chromatiaceae* (Esteve *et al.*, 1983; Esteve *et al.*, 1992; Guerrero *et al.*, 1986). *Vampirococcus* has not been successfully grown axenically in pure culture, while *Daptobacter* has been

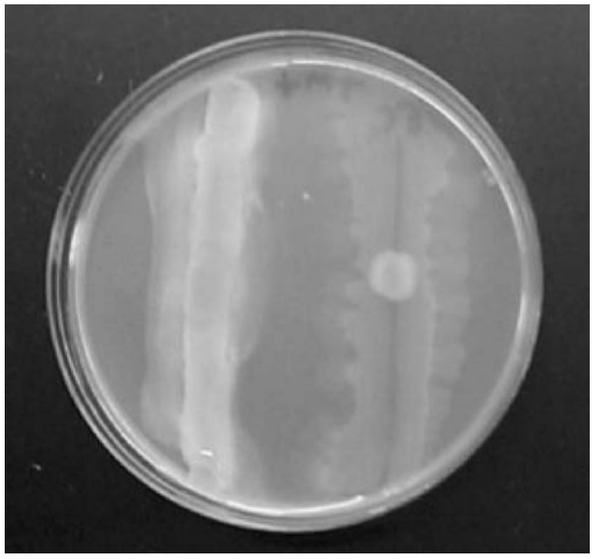

Figure 2. Photograph of ''tracking'' behavior by *Ensifer adhaerens. Ensifer adhaerens*, its prey organism *Micrococcus luteus*, and *E. coli* strain DH5α were grown to saturation in Nutrient Broth (Difco). A one milliliter aliquot of each organism was centrifuged and resuspended in 0.1X Heart Infusion Broth (Difco) supplemented with 0.1% glucose. A 100 microliter aliquot of washed *Micrococcus* (above, left side of petri dish) was evenly spread in a thin line across a 0.1X Heart Infusion Broth plus 0.1% glucose plate and allowed to dry. The procedure was repeated with the *E. coli* strain (right side of petri dish). The cells were allowed to dry for 30 minutes at room temperature. 10 microliters of the washed *Ensifer* culture were placed in the middle of each dried line of bacteria. The plates were allowed to incubate for four days at 30 degrees C. Note that *Ensifer* has spread all along the *Micrococcus* streak with copious lysis and exopolysaccharide production, but exhibits no such ''tracking'' behavior toward *E. coli*.

reported to be capable of growing in isolation (E.G. Ruby, personal communication).

It is also important to note that several species of prokaryotes have been found that non-specifically degrade nearby cells of other bacterial species, through the excretion of hydrolytic enzymes and other factors as described below (Burnham *et al.*, 1981; Crespi, 2001; Fallon and Brock, 1979; Lin and McBride, 1996). These prokaryotes include various species of *Myxococcus* (Burnham *et al.*, 1981; Ruby, 1992), as well as *Lysobacter* (Lin and McBride, 1996). Recent work with marine bacteria (Long and Azam, 2001) has shown that roughly half of the isolates studied had antagonistic activity of some form toward other prokaryotes in the same large group of microbes, especially for organisms associated with or attached to particles (Fallon and Brock, 1979; Long and Azam, 2001). The latter research, however, did not discriminate between antibiotic action and more direct and ''aggressive'' antagonistic interactions.

Relationships to Symbiotic and Pathogenic Microbes

Clearly, there are sets of genes expressed in the ''search'' phase of the predatory life cycle, while another set of genes is expressed during the process of predation and host cell assimilation. In *Bdellovibrio*, for example, ''attack phase'' cells do not replicate DNA, nor do they divide, but they do synthesize flagella (Cotter, 1992; Gray and Ruby, 1990; Ruby, 1992; Thomashow and Cotter, 1992). Intraperiplasmic phase *Bdellovibrio* cells, on the other hand, replicate DNA and divide, yet do not synthesize flagella (Cotter, 1992; Ruby, 1992; Thomashow and Cotter, 1992). In support of this concept, it is estimated from two dimensional gel electrophoresis that at least 30 *Bdellovibrio* proteins are specific to the intraperiplasmic state (McCann *et al.*, 1998). This principle of differential gene expression is certainly true of all predatory microbes on a simplistic level.

In an extensive review by Moulder (Moulder, 1985), *Bdellovibrio* is compared to a wide variety of other intracellular parasites (generally ones that cause disease, often using unique and intricate biochemical and genetic mechanisms). In Moulder's view, *Bdellovibrio* is a greatly simplified example of such intracellular associations, and may shed light on the very basic strategies used by intracellular prokaryotes living within eukaryotic cells, and more specifically on the evolution of predation as a trait. It is true that a predator like *Bdellovibrio* invading another prokaryotic cell does not involve co-opting eukaryotic cytoskeletal machinery or phagocytosis (Bavoil and Ojcius, 2000; Gort and Miller, 2000; LaVier *et al.*, 2000). However, the basic principles—finding prey, entering or accessing some compartment of the prey cell, and degrading and utilizing prey macromolecules—are similar, and could provide a basic and simplified model with relevance to intracellular associations in general (LaVier *et al.*, 2000; Moulder, 1985).

A similar argument could be made for prokaryotes that enter into symbiotic associations with eukaryotic cells, such as the relationship between *Rhizobium* and legumes. Clearly, different suites of genes are active (or inactive) in the symbiotic, intracellular form of *Rhizobium* compared with the organism free living in the rhizosphere (Oke and Long, 1999). Thus, there must be specific biochemical and genetic accommodations made to the cytoplasmic environment, just as *Bdellovibrio* must make accommodations to the most unusual periplasmic environment (Ferguson, 1992). Strategies used for the study of genes specific to symbiotic and pathogenic microbes should be of equal utility to the study of predatory prokaryotes.

One such strategy is called *in vivo* expression technology (IVET) (Merrell and Camilli, 2000), which is based on an integrative plasmid vector with a promoterless drug resistance gene, in front of which small segments of bacterial genomic DNA are cloned. A collection of such plasmids is introduced into the pathogen or symbiont, and the recombinant organisms allowed to infect their eukaryotic host in the presence of the appropriate antibiotic. If a cloned segment contains a promoter to a bacterial gene only expressed within the host cell, then the drug resistance phenotype will only be expressed under those conditions. IVET can thus be used to enrich and eventually identify bacterial genes expressed only within the cytoplasm of host eukaryotic cells, both for pathogens and symbionts.

This approach has resulted in the isolation of a wide variety of ''intracellular-associated'' genes, including nutrient acquisition, DNA replication, and outer surface protein synthesis (Gort and Miller, 2000; Merrell and Camilli, 2000; Oke and Long, 1999). However, this approach has also yielded a number of unknown and unidentified genes (Bavoil and Ojcius, 2000; Gort and Miller, 2000; Merrell and Camilli, 2000), as well as unexpected ones, such as the role adenine methylation plays in *Salmonella* virulence and pathogenicity (Heithoff *et al.*, 1999). A similar approach to the study of predatory prokaryotes would no doubt also result in the expected and unexpected classes of genes and their products, and suggest many possible new areas of investigation in the genetic analysis of such microbes.

Predation Strategies

The goal of a predatory microbe is simple: to utilize energy and biosynthetic monomers and polymers collected by and contained within other organisms.

The first question that arises is nomenclatural. Are these bacteria truly predatory, by the ecological definition of the word, or are they parasites? In the case of *Bdellovibrio*, the correct term may in fact be *parasitoid* (*Bdellovibrio* cells are roughly 1/3 of the size of *E. coli* host cells, and only one *Bdellovibrio* cell seems to be successful in ''parasitizing'' a host cell). There has been much spirited debate on this subject in the literature (Guerrero *et al.*, 1986; Ruby, 1992; Stolp and Starr, 1963), yet most researchers are equally comfortable with terms like ''prey'' or ''host'' cell, ''predator'' or ''parasite.''

Regardless of the nomenclature, in order for one prokaryotic cell to attack and consume another, several steps must be accomplished.

(1) The predator must find the prey cell, either by chemotaxis or because of sufficient population sizes and resultant random collisions between cells.
(2) The predator cell must then associate irreversibly with the prey cell (except in strategy ''A,'' below).
(3) The predator cell must degrade or otherwise make available host specific macromolecules.
(4) Finally, the predator cell must assimilate the released nutrients, hopefully in a manner that is both specific and efficient.

Clearly, each step in the pathway above is ripe with possibilities for the enterprising microbial geneticist, given a tractable or developing genetic system.

Keeping the basic parameters of predation described above in mind, there are four basic strategies thus far observed among prokaryotes.

Wolfpack (Figure 3A)

The first approach is essentially ''group predation,'' in which a number of predator cells produce in common a variety of hydrolytic enzymes that degrade nearby bacteria. This degradation leads to the localized availability of host cell derived nutrients, though strategies to reduce diffusion of the released nutrients must be taken into account. Martin Dworkin has called such a predatory approach a ''wolfpack'' strategy (personal communication). *Myxococcus* (Burnham *et al.*, 1981; Fallon and Brock, 1979) and *Lysobacter* (Lin and McBride, 1996) are examples of these kinds of prokaryotes, which appear to be common in the soil. It is not yet clear if *Ensifer* uses epibiotic or wolfpack strategies to attack and degrade host cells (Casida, 1982).

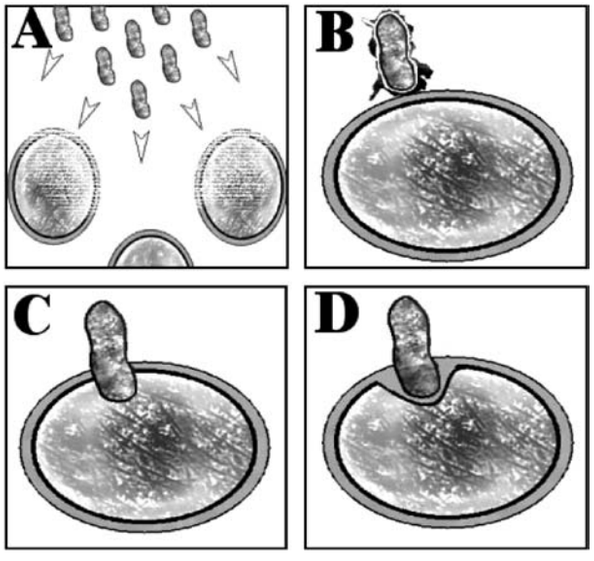

Figure 3. Different general strategies of predation by microorganisms, as described in the text. (A) Group or ''wolfpack'' predation (as observed with *Myxococcus*), (B) Epibiontic attachment (*Vampirococcus*), (C) Direct invasion of the cytoplasm (*Daptobacter*), and (D) Periplasmic invasion (*Bdellovibrio*). Illustrations courtesy of J.J. Quinn.

Epibiotic (Figure 3B)

The second approach is much more ''cell to cell'' than the previous strategy, and requires cell contact. In essence, the predator cell attaches to the outer surface of the host cell, and begins to degrade and assimilate host molecules through specialized structures. Guerrero and his coworkers describe *Vampirococcus* and its attacks on various species of *Chromatium* as a typical epibiotic predatory strategy (Esteve *et al.*, 1983; Esteve *et al.*, 1992; Guerrero *et al.*, 1986).

Care must be taken, however, to ensure that predation is actually taking place, rather than solely epibiotic attachment. In natural populations, large prokaryotes are often covered with other prokaryotes, which appear to be straightforward surface colonizers (J.R. Leadbetter, H. Overmann, personal communications). Guerrero's group reports promising electron microscopic evidence of cytoplasmic ''bridges'' between *Vampirococcus* and *Chromatium*, and associations in which the prey cells are clearly degrading (Esteve *et al.*, 1983). The best test of this association is to use radioactively or fluorescently labeled host cell components and determine if the labeled material is transferred to the predator cell. Unfortunately, this is a quite difficult experiment in the case of an obligate predator like *Vampirococcus*, which cannot be cultivated easily in the laboratory.

Direct Invasion (Figure 3C)

A third approach would be for a predator cell to directly enter the host cytoplasm, in a process that Moulder calls *diacytosis* (Moulder, 1985). This strategy is attractive, since it mimics predation outside the microbial world. Guererro and his coworkers have described an intriguing organism, found in the same sulfurous lake as *Vampirococcus*, called *Daptobacter* (Guerrero *et al.*, 1986). *Daptobacter* also appears to prey on members of genus *Chromatiaceae*, specifically *Chromatium minus*. *Daptobacter* is reportedly capable of being cultivated axenically (Guerrero *et al.*, 1986, E.G. Ruby, personal communication), but no other researchers have reported or published investigations of this fascinating microorganism.

Periplasmic (Figure 3D)

The last category of predation is, ironically, the one that is best understood. The predator cell invades and grows within a specific compartment found in Gram negative cells, the periplasm (Ferguson, 1990; Ferguson, 1992; Hobot *et al.*, 1984). Long called the ''periplasmic space,'' this compartment is crowded with solutes, enzymes, and oligosaccharides of various kinds, and is estimated to possess the same osmolarity as the cytoplasm (Hobot *et al.*, 1984). The predatory organism that is best known to invade the periplasm of host cells as part of its life cycle is *Bdellovibrio* (Cotter, 1992; Ruby, 1992; Stolp and

Starr, 1963). Figure 1, mentioned earlier, illustrates the basic life cycle of this organism, and a description follows below.

The genus *Bdellovibrio* consists of small (0.3 μm by 2 μm), highly motile bacterial cells that prey upon other Gram negative prokaryotes (for an extensive review, see Ruby, 1992). This bacterium possesses two distinct ''developmental'' stages as discussed earlier: an attack or search phase independent of the host bacterium, and an intraperiplasmic growth phase within the host or prey cell (Thomashow and Cotter, 1992).

Attack phase bdellovibrios are free swimming and metabolically highly active, but are not normally capable of DNA replication or cell division (Ruby, 1992; Thomashow and Cotter, 1992). When an attack phase bdellovibrio cell contacts an appropriate prey bacterium, it swiftly attaches irreversibly to the outer cell wall of the host cell, detaches its flagellum, and enters the periplasmic compartment by one of several proposed mechanisms involving either glycanases or peptidases (Schelling and Conti, 1986; Tudor *et al*., 1990).

The host bacterium is then rapidly modified by the invading predator in several ways; the outer cell wall is extensively altered (Ruby, 1992), and the hydrophobicity of the host membrane changed (Cover and Rittenberg, 1984), apparently to prevent further infection by other *Bdellovibrio* cells. The inner membrane, on the other hand, becomes ''leaky'' to low molecular weight compounds (Romo *et al*., 1992). The parasitized host bacterium ''rounds up'' into a structure termed a ''bdelloplast'' (Thomashow and Cotter, 1992), within which the growth phase *Bdellovibrio* cell rapidly utilizes host macromolecules for energy and biosynthesis. Within minutes of infection, the *Bdellovibrio* cell introduces proteases and nucleases into the host cytoplasm by an unknown mechanism (Gray and Ruby, 1990; Romo *et al*., 1992; Rosson and Rittenberg, 1979; Thomashow and Cotter, 1992), and rapidly destroys the host cell's ability to generate ATP by oxidative phosphorylation (Hespell, *et al*., 1973; Romo *et al*., 1992; Ruby, 1992).

The intraperiplasmic growth form of the invading microbe does not appear to use carbohydrates as a major source of energy and carbon (Hespell, 1976; Hespell, *et al*., 1973; Romo *et al*., 1992; Ruby and McCabe, 1988), instead relying on the degradation and utilization of host nucleic acids, lipids, and proteins (Hespell, *et al*., 1973; Rosson and Rittenberg, 1979; Ruby, 1992; Ruby *et al*., 1985). *Bdellovibrio* remains one of the most energetically efficient prokaryotes known, presumably due to its ability to utilize several high energy host macromolecules (such as ATP) directly (Hespell, *et al*., 1973; Ruby *et al*., 1985). There is also evidence that *Bdellovibrio* incorporates membrane proteins and segments of host membrane into itself (Diedrich, 1988; Tudor and Karp, 1994). The growing *Bdellovibrio* cell elongates into a coiled nonseptate structure, which after several hours divides into individual flagellated motile progeny cells (Ruby, 1992). The bdelloplast lyses at this point, releasing 3–5 progeny attack phase *Bdellovibrio* cells to complete the life cycle.

This unusual life strategy is thought to be used by *Bdellovibrio* to obtain an exclusive and rich source of nutrients under conditions of oligotrophic nutrient availability, as well as a method for avoiding ultraviolet light and bacteriophages (Gray and Ruby, 1990; Ruby, 1992; Thomashow and Cotter, 1992). The entire life cycle of *Bdellovibrio* is generally complete within 3–4 h of initiation (Cotter, 1992; Thomashow and Cotter, 1992). Figure 4 depicts the general order of events, based on biochemistry or microscopic observation (and is by no means complete).

Very little is known regarding the transition from search to growth phase programs of gene expression in *Bdellovibrio* (Gray and Ruby, 1990; McCann *et al*.,1998; Thomashow and Cotter, 1992). Clearly, motility (and the genes encoding this function) is a phenotype only observed in search phase bdellovibrios, as the flagellum is detached after attachment but just prior to invasion of the host cells (Stolp and Starr, 1963). Several phenomena are characteristic and indicative of a transition to the growth phase: an early ''rounding up'' of the invaded host cell into a bdelloplast, the later initiation of DNA replication in the *Bdellovibrio* cell, and the still later septation of the long filament into progeny cells prior to release from the depleted bdelloplast (Thomashow and Cotter, 1992).

Factors present in host cell extracts have been shown to induce some developmental stage specific activity (Gray and Ruby, 1990), but the factor or factors have not been isolated nor characterized reproducibly. Part of the confusion is the possibility that more than

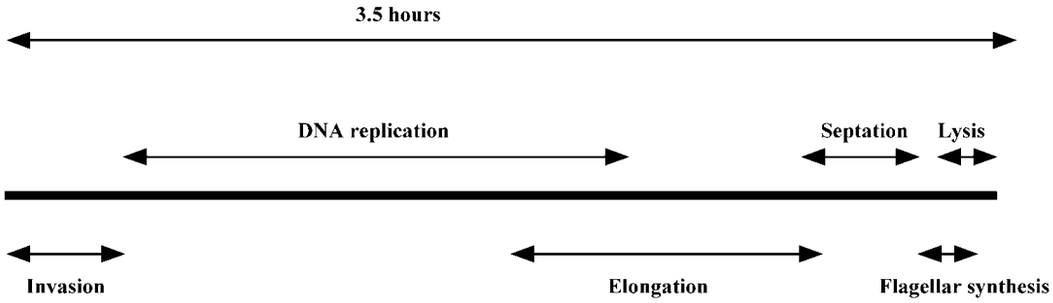

Figure 4. Schematic of the probable order of events in the life cycle of *Bdellovibrio bacteriovorus*. Total time, from initiation of invasion to release of progeny attack phase cells, consists of about 3.5 hours. This time line was assembled from references 9, 50, and 60.

one host signal—or a regulatory ''cascade''—may be necessary to ''commit'' the predator cell to a growth phase cell genetic program (Ruby, 1992; Thomashow and Cotter, 1992).

Wild type *Bdellovibrio* can only grow on host cells, but it is possible to isolate mutants of *Bdellovibrio* capable of growing on various forms of nutrient medium. These mutants are called ''host independent'' (HI) strains (Cotter, 1992; Diedrich *et al.*, 1970; Ruby, 1992). Unfortunately, the more quickly HI strains of *Bdellovibrio* grow (allowing molecular genetic techniques to be employed), the less effective the mutants are at carrying out the intraperiplasmic stage of their life cycle (Barel and Jurkevitch, 2001; Diedrich *et al.*, 1970, E.G. Ruby, personal communication, unpublished observations from this laboratory).

One of the only published molecular genetic studies of *Bdellovibrio*, by Cotter and Thomashow (Cotter, 1992; Cotter and Thomashow, 1992), demonstrated that a plasmid from a genomic library could partially ''correct'' the HI phenotype to a more typical plaque morphology, but remained intermediate to wild type (Cotter and Thomashow, 1992). This research resulted in the incomplete sequence of a genetic locus, *hit*, that appeared to be associated with the host independent/dependent phenotype (Barel and Jurkevitch, 2001; Cotter and Thomashow, 1992). The open reading frame altered in HI mutants had the characteristics of a membrane bound protein, suggesting a signaling role for the *hit* locus product (Cotter, 1992). Unfortunately, the intact *hit* locus has not been subcloned nor characterized. Jurkewitz and his colleagues (Barel and Jurkevitch, 2001) have done extensive research regarding the nature of the HI mutation in general, and it has become clear that the HI phenotype is due to at least two independent mutations. Thus, it is reasonable to conclude that the HI phenotype is complex, and does not reflect the free living conditions of *Bdellovibrio* during attack or hunt phase.

Adaptations to the Predatory State

Most information regarding this subject is observational, and focuses on *Bdellovibrio*. Regardless, there remains a great deal of uncertainty in published reports. On a simplistic level, *Bdellovibrio* moves from a nutrient poor (outside the host cell) to a nutrient rich (inside the host cell periplasm) environment. Thus, *Bdellovibrio* may be seen as existing in a ''starvation state'' while in search of prey cells (Gray and Ruby, 1990; Thomashow and Cotter, 1992), then moving into a rich nutrient source within minutes of predation. Ability to rapidly adapt to changing metabolic states would be advantageous to this organism, presumably.

Although *Bdellovibrio* has been shown to be weakly chemotactic toward amino acids (Straley and Conti. 1977), no evidence has been shown that the predator ''hunts'' host cells *via* chemotaxis (Ruby, 1992; Straley and Conti. 1977). This suggests a random ''collision model'' of prey acquisition. However, this does not agree with the finding of some marine *Bdellovibrio* species associating with biofilms (Williams, 1988, H. Williams, personal communication) in estuarine environments; such *Bdellovibrio* cells must be ''pursuing'' prey to remain near a submerged biofilm community (Rice *et al.*, 1997; Williams, 1988; H. Williams, personal communication). Entry of *Bdellovibrio* into a biofilm community would require synthesis of appropriate hydrolytic enzymes to break down the tough matrix of these organized collections of surface colonizing cells (Davey and O'Toole, 2000) to find fresh prey.

Bdellovibrio cells tend to adhere to many objects when observed microscopically, but those associations are reversible. The nature of irreversible attachment of *Bdellovibrio* to prey cells involves certain aspects of the lipopolysaccharide of the host envelope, but in a complex fashion (Schelling and Conti, 1986). Glycanase and related activities have been shown to be present in the search or attack phase cells, and are thought to be responsible for initial entry activity (Schelling and Conti, 1986; Stolp and Starr, 1963; unpublished observations). Additionally, the presence of a host cell capsule was shown not to be an impediment to attack by *Bdellovibrio* (Koval and Bayer, 1997). The goal of host membrane modification following entry by *Bdellovibrio* is presumably to prevent superinfection by *Bdellovibrio*, and several enzymatic activities have been reported which alter the host membranes and peptidoglycan network to this end (Thomashow and Cotter, 1992), as previously stated.

Once within the host periplasm, *Bdellovibrio* elaborates a number of hydrolytic enzymes that degrade host cell macromolecules, including proteases, DNAses, and RNAses (Cotter, 1992; Diedrich *et al.*, 1970; Rosson and Rittenberg, 1979; Stolp and Starr, 1963). There is strong evidence for the introduction of such enzymes into the cytoplasm of host cells shortly after attack by *Bdellovibrio* (Romo *et al.*, 1992). Monomers and other substances (including ATP) from the host are quickly taken up by the growing *Bdellovibrio* cell, which appears to have a preference for oxidizing amino acids and components of nucleic acids (Hespell, 1976; Hespell, *et al.*, 1973). After the *Bdellovibrio* cell elongates at the expense of the host cell, septates, and synthesizes flagella, a lytic factor is produced which lyses what is left of the host bdelloplast (Ruby, 1992), releasing the progeny *Bdellovibrio*.

Other predatory prokaryotes, though not so well described as *Bdellovibrio*, surely have specific mechanisms that aid their activities while locating, attacking, and consuming host cells. The four basic mechanisms described above should apply to most instances of predation. It is interesting to note that *Bdellovibrio* and *Ensifer* both nonspecifically bind to objects in their microenvironment (Casida, 1980; Cotter, 1992; Schelling and Conti, 1986; Stolp and Starr, 1963), yet only some associations (with the appropriate host) lead to nonreversible binding and the next stage in predation.

Ensifer produces the uncharacterized lytic factor mentioned earlier, possesses the chemotaxis like ''tracking'' effect toward host cells, and a possible

''conduit'' for obtaining host resources (the mysterious ''bar'' described by Casida in Casida, 1982). *Vampirococcus* appears, via electron microscopy, to have a specialized ''holdfast'' structure that may act as a conduit of host cell molecules (Esteve *et al.*, 1983; Guerrero *et al.*, 1986). *Daptobacter* must have some specific mechanism to degrade and take up host nutrients while lodged in the cytoplasm, as well. The ''wolfpack'' bacteria described earlier appear to ''hunt'' in groups, thereby taking advantage of the released nutrients from the lysed cells before diffusion dilutes them (Burnham *et al.*, 1981; Ruby, 1992).

Genetic Analysis of Predatory Prokaryotes

As the tools of modern molecular genetics are extended to more and more unusual bacterial genera, it is only a matter of time until such approaches are brought to the study of predatory prokaryotes. Currently, several laboratories are investigating *Bdellovibrio* and *Ensifer*, including this one.

Certain tools are essential to genetic analysis of ''undomesticated'' prokaryotes. These include transposon mutagenesis, ''shuttle plasmids'' between the organism of interest and *E. coli*, and transductional systems (though it is often possible to ''work around'' the lack of one of these tools) (Salyers *et al.*, 2000). Toward that end, genetic tools have been brought to bear on the study of *Bdellovibrio* by Cotter and Thomashow (Cotter, 1992; Cotter and Thomashow, 1992) and Jurkevitz (Jurkevitch and Ramati, 2000). Cotter and Thomashow, though not reporting success with transposon mutagenesis, did in fact develop a cosmid ''shuttle'' vector based on pVK101 (Cotter and Thomashow, 1992; Knauf and Nester, 1982).

Currently, some genetic analysis is being carried out with *Ensifer*, specifically in the area of introducing various vectors in that organism (Rogel *et al.*, 2001; unpublished observations). However, this work appears to focus more on the relationship between *Ensifer* and other *Rhizobiaceae* that are capable of nodulating leguminous plants (Rogel *et al.*, 2001).

The eventual goal of these studies is to identify and characterize predation specific genes, just as many researchers are currently investigating pathogenicity specific or intracellular specific genes of pathogens (Merrell and Camilli, 2000; Moulder, 1985) and symbionts (Oke and Long, 1999) of eukaryotic cells. However, predation is such an unusual phenotype that it may be necessary to very carefully define subdivisions of the predation process (reversible versus irreversible attachment, for example) for genetic dissection and biochemical analysis. Temporal analysis may be particularly important, and most feasible with *Bdellovibrio*, given the ability to prepare synchronized cultures of the predator microbe (Ruby, 1992).

Work in this laboratory has made significant progress in the study of the genetics of *Bdellovibrio*. Various transposable elements were conjugated into host independent *Bdellovibrio* according to standard procedures (Ausubel *et al.*, 1987; deLorenzo *et al.*, 1990), and useful frequencies of transposition events per recipient cell were observed (see Figure 5).

One of the transposons used, TnphoA-1' (Wilmes-Riesenberg and Wanner, 1992) carries a *lac*Z gene (despite its name). As *Bdellovibrio* lacks β-galactosidase activity (Hespell, 1976; Romo *et al.*, 1992; unpublished observations), such a transposon would be an excellent reporter gene of utility in the study of *Bdellovibrio*. Figure 6-A illustrates that some insertions of this transposon into the chromosome were into active transcriptional units of *Bdellovibrio*, and others into transcriptionally quiescent regions (based on hydrolysis of the chromogen Xgal as a measure of transcription). Figure 6-B is a Southern blot (Ausubel *et al.*, 1987) confirming the observation that the transposition of TnphoA-1' is random into the genome of *Bdellovibrio*. Similar results were found for mini-Tn5 elements carrying a promoterless *lux* gene cassette (deLorenzo *et al.*, 1990) and displayed differing levels of bioluminescence from exconjugant to exconjugant (data not

Transposon	Relevant phenotypes	Transposition frequency (per recipient cell)	Reference
MiniTn5*lux*AB	Lux, Tc^R	2.1×10^{-5}	deLorenzo, et al, 1990
Tn*pho*A	Pho, Kan^R	2.3×10^{-5}	Wilmes-Riesenberg and Wanner, 1992
Tn*pho*A'-1	LacZ, Tc^R	3.4×10^{-6}	Wilmes-Riesenberg and Wanner, 1992
MiniTn5*Cm*	Cm^R	$<1 \times 10^{-9}$	deLorenzo, et al, 1990
MiniTn5*lac*Z1	LacZ, Kan^R	7.3×10^{-6}	deLorenzo, et al, 1990
MiniTn5*Km*	Kan^R	8.0×10^{-5}	deLorenzo, et al, 1990
Tn5*gfp*1	GFP, Tc^R	4.5×10^{-6}	Burlage, et al, 1996

Figure 5. Transpositional efficiency of selected transposons into host independent *Bdellovibrio bacteriovorus* strain 109J-KAI. Transposon mutagenesis was carried out by plate matings, dilutions, and plating on selective medium as described in deLorenzo, et al, 1990, using a spontaneous rifamycin resistant mutant of HI *Bdellovibrio* as recipient.

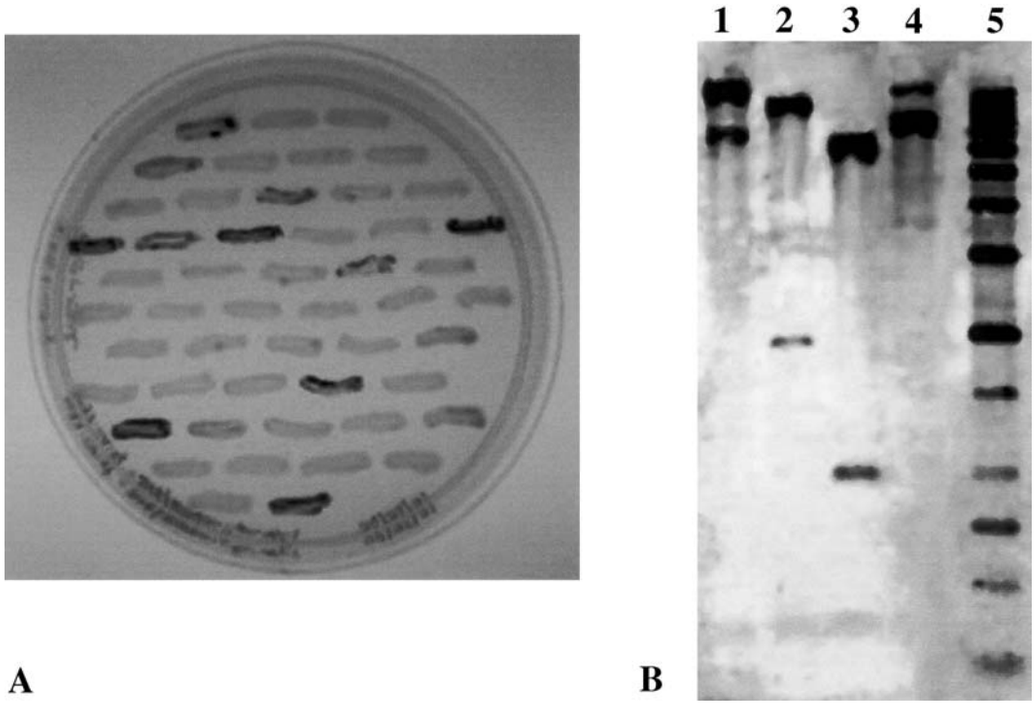

Figure 6. Demonstration of random nature of TnphoA-1' insertion into *Bdellovibrio* genome, on both phenotypic and molecular levels. (A). Different levels of β-galactosidase activity in different Tn*pho*A-1' mutants of *Bdellovibrio bacteriovorus*. 50 randomly selected colonies from a Tn*pho* A-1' transposon mutagenesis experiment were patched onto fresh agar with added kanamycin and the chromogen Xgal, allowed to grow for four days at 30 degrees C., then photographed. Differences in blue pigment indicate that Tn*pho*A-1' transposes into many different sites in the *Bdellovibrio* genome. (B). Four randomly selected Tn*pho*A-1' mutants were selected, genomic DNA isolated, digested with *Eco*RI, run on agarose gels, blotted to a nylon filter, hybridized to fluorescently labeled Tn*pho*A-1' specific DNA, and non-radioactively visualized (Illuminator System, Stratagene, Inc.) by autoradiography and standard molecular biological techniques (1). Different sizes of hybridizing bands reflect different sites of Tn*pho*A-1' insertion. Lanes 1- 4: Tn*pho*A-1' mutants. Lane 5: 1 kb pre-labeled DNA ladder.

shown). Recently, our laboratory has identified several screenable phenotypes, including α-amylase activity and a casein degrading protease; these should make excellent targets for transposon mutagenesis in a species that otherwise has few.

Of course, this work has focused on HI *Bdellovibrios* that are clearly regulated in a different fashion than the wild type strain (Diedrich *et al.*, 1970; Ruby, 1992; Thomashow and Cotter, 1992). It is possible to transfer plasmids and transposons into wild type *Bdellovibrio* (Cotter, 1992, unpublished observations), but it is difficult to manipulate and analyze such relatively rare events among a large group of free swimming bacteria. It should be possible, however, to screen among transposon mutants for colonies that respond to host cell extract, which is known to elicit ''partial wild type'' responses from HI *Bdellovibrios* (Gray and Ruby, 1990; Thomashow and Cotter, 1992), and this laboratory has embarked on such a search. It should also be possible to apply random transposon mutagenesis approaches to search for essential predation genes directly (Judson and Mekalanos, 2000), provided a truly facultative HI mutant of *Bdellovibrio* can be isolated.

Currently, this laboratory has used functional complementation (Ausubel *et al.*, 1987), various *E. coli* deletion mutants, and a genomic library of wild type *Bdellovibrio* DNA in a cosmid vector (Cotter, 1992) to clone a variety of genes of interest—adenylate cyclase, amylase, *gro*EL, and *rec*A—which are currently under analysis. This laboratory is also experimenting with a number of *Bdellovibrio* specific bacteriophages (in collaboration with B. Fane of the University of Arizona), and is attempting to demonstrate generalized transduction in that organism. A simplified method for transferring genetic markers from HI to wild type strains of *Bdellovibrio* would be very useful as the genetics of this prokaryote becomes increasingly tractable.

The recent use of IVET (*in vivo* expression technology, mentioned above) approaches to study bacterial genes that are transcriptionally active within the cytoplasm of eukaryotes (as with pathogens and symbionts) presents another opportunity to study predation. It should be possible to adapt IVET technology to the *Bdellovibrio* system, and begin to identify *Bdellovibrio* genes that are active only in the periplasm of host cells. This work is greatly assisted by the ability to set up synchronized cultures of *Bdellovibrio* and host cells (Ruby, 1992). To be sure, just as in the study of pathogens, the IVET approach will yield obvious candidates: DNA replication genes, cell division genes, etc (see Figure 4). Yet the IVET approach to the study of pathogens and symbionts has routinely yielded unusual genes as well (Gort and Miller, 2000; Merrell and Camilli, 2000); similar results will surely be found in the study of *Bdellovibrio*.

Work in this laboratory with *Ensifer* is much less advanced. As have other investigators (Rogel *et al.*, 2001), we have shown that incP and incQ plasmids are capable of replicating in *Ensifer*. Attempts have been made to carry out transposon mutagenesis in this organism, but natural resistance to several antibiotics has complicated this.

It would be extremely interesting to carry out transposon mutagenesis on *Ensifer* and search for nonmotile mutants, as well as mutants greatly reduced in exopolysaccharide production. Perhaps these are necessary factors for tracking and predation by *Ensifer*. Investigations with *Ensifer* should be simplified by its nonobligately predatory nature, and its ability to grow on a number of defined types of nutrient media.

If predatory bacteria like *Vampirococcus* and *Daptobacter* can be reliably grown axenically, the above approaches could be used in a similar fashion to begin to genetically dissect the biochemical and genetic strategies that are part of the complex phenotype called predation in those species. There will be differences among the predation strategies and similarities, as well as fascinating genetic and biochemical adaptations. By comparing the different ways that prokaryotic predators carry out the four basic stages of predation, it should be possible to synthesize basic principles and compare them in a useful fashion to the strategies used by intracellular pathogens and symbionts of eukaryotic organisms (LaVier *et al.*, 2000; Moulder, 1985).

Future Prospects

As researchers begin to study how prokaryotic organisms interact with each other in complex systems and environments, it is probable that many more predatory microbes will be found and analyzed, and added to the relatively small amount of information uncovered thus far.

It is almost certain that even a modest effort by several investigators would result in the isolation of new predatory prokaryote isolates, each falling within one of the predation ''types'' listed above (or perhaps in new categories). As with the study of marine viruses, increased attention to this fascinating subject could well create a new and potentially fertile field of investigation with relevance to medicine, ecology, and the study of basic microbiology.

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