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MINIREVIEW

Bdellovibrio Host Dependence: the Search for Signal Molecules and Genes That Regulate the Intraperiplasmic Growth Cycle

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In 1962, while attempting to isolate bacteriophage from soil samples, Stolp and Petzhold (29) noted a number of odd plaques. Instead of taking hours to appear, these plaques took days to develop and continued to grow in size for more than a week. Intrigued, the investigators examined some of the plaque material under the light microscope. What they observed were small, highly motile, vibrio-shaped cells. Stolp and Petzhold had discovered bdellovibrios.

The discovery of bdellovibrios proved particularly exciting as it revealed a previously unrecognized type of prokaryotic cell-cell interaction: the intraperiplasmic (IP) development cycle (Fig. 1; see below). Over the years, much has been learned about the biochemistry and physiology of IP development and a number of unusual properties have been described for *Bdellovibrio* spp., including an exceptionally high Y_{ATP} (biomass yield per ATP molecule expended) (17), the ability to take up and utilize nucleoside monophosphates (18, 22), and the incorporation of host cell fatty acids (11) and lipopolysaccharides (13) into corresponding bdellovibrio macromolecules. These and related aspects of *Bdellovibrio* biology are covered in previous review articles (15, 21, 30), as are the topics of *Bdellovibrio* ecology (27, 32), cell envelope modifications (20) and *Bdellovibrio*-host cell signalling (7). This minireview focuses on the subject of *Bdellovibrio* host dependence.

NATURE OF *BDELLOVIBRIO* HOST DEPENDENCE

All wild-type *Bdellovibrio* isolates described to date display an obligate requirement for host cells in order to multiply (21). Determining the nature of this host dependence is fundamental to an understanding of the *Bdellovibrio*-host cell interaction. In addition, as the molecular basis for obligate host dependence is not well understood in any system, including the cell-cell interactions established by medically important obligate intracellular pathogens such as chlamydiae (12) and rickettsiae (33), such knowledge could have broad biological significance. Here we summarize what is known about the biochemistry and genetics of the *Bdellovibrio* host-dependent (H-D) phenotype. While our current understanding in these areas is rudimentary, important basic principles have been established and testable hypotheses regarding the nature of *Bdellovibrio* host dependence have been advanced.

Background: the IP development cycle. The IP development cycle is composed of two fundamental stages: an attack phase alternating with a growth phase (Fig. 1). During the attack phase, a highly motile bdellovibrio searches for a suitable gram-negative bacterium, generally referred to as either the host or prey cell, depending on whether the interaction is viewed as a host-parasite or predator-prey interaction; the cycle has characteristics in common with each (21). When a host is encountered, the bdellovibrio attaches to it and, after a period of 5 to 10 min, quickly penetrates into the host, becoming lodged in the periplasmic space. During the invasion process, the bdellovibrio drops its flagellum and initiates the transition from attack phase to growth phase.

The first 30 min of the growth phase is a period in which the bdellovibrio prepares for multiplication. Two dramatic events that occur early are the conversion of the host cell into a stable spherical structure termed the bdelloplast and the destruction of the host's ability to generate energy (16, 19). The bdellovibrio also directs a methodical degradation of the host cell's macromolecules to products that are generally too large to escape the bdelloplast but small enough to pass through the permeabilized plasma membrane; in essence, the bdellovibrio creates a niche, the bdelloplast, where it has sole access to a rich source of nutrients. At about 45 min into the growth phase, the bdellovibrio initiates DNA replication. During the subsequent 2 to 3 h, the bdellovibrio grows into a long filament, which, at the end of growth, fragments into individual motile cells. The remaining bdelloplast is then lysed, and the progeny are released into the environment.

Regulation of *Bdellovibrio* growth and development. Attack-phase bdellovibrios are capable of carrying out a wide range of cellular functions: they are motile, they can elongate, they respire a number of substrates, and they synthesize macromolecules, including RNA, protein, and peptidoglycan (16, 21, 30). They do not, however, initiate rounds of DNA replication. Indeed, all attempts to culture wild-type *Bdellovibrio* isolates axenically on commercial media have failed (5, 8, 14, 21). One possible explanation for this host dependency is that wild-type bdellovibrios might be auxotrophs that require a nutritional factor that is absent from standard complex media. Alternatively, attack-phase cells might be locked into a search mode, requiring a signal from the host to initiate IP growth. Such a negative regulatory model was initially suggested by Shilo (26). This model takes into account the fact that mutations are generally more likely to result in a loss of function than in a gain of function.

A number of attempts have been made to identify a host signal molecule(s) that is capable of triggering bdellovibrio growth and development in the absence of host cells (5, 8, 14). While these studies have met with only limited success, they have established the important fact that wild-type bdellovibrios can be cultured on commercial complex media

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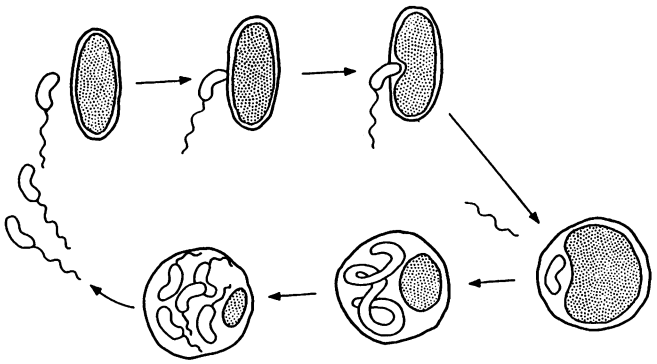

FIG. 1. Schematic representation of the *Bdellovibrio* life cycle. Redrawn from reference 30 with permission of Blackwell Scientific Publications, Ltd.

supplemented with extracts prepared from host cells. Only a fraction of the total population of bdellovibrios initiate growth in the presence of such extracts (up to about 50% of the cells), but those that do grow and multiply appear to go through the same developmental cycle that occurs intraperiplasmically: the cells drop their flagella, and after a lag phase, they initiate DNA synthesis, elongate into filaments, and fragment into individual motile cells (5, 8, 14).

Unfortunately, little progress has been made towards determining the nature of the factor(s) responsible for triggering axenic growth of wild-type bdellovibrios. Each of the described extracts has been a crude protein preparation required at relatively high concentration (milligrams per milliliter) (5, 8, 14). Moreover, there have been inconsistencies regarding the basic properties of the active component(s). For instance, one extract was inactivated by RNase and inhibited by 10 mM Mg^{2+} (8), while another was RNase insensitive and its activity was enhanced by 10 mM Mg^{2+} (5). There is a consensus, however, that the active component(s) is nondialyzable, heat stable, and resistant to digestion with DNase.

One possible explanation for the difficulty in purifying a signal molecule that triggers axenic growth is that there may be more than one host signal required during the IP growth cycle. Indeed, Gray and Ruby (7), in an extensive, thoughtful review on *Bdellovibrio*-host cell signalling, proposed that at least two distinct signals are required to complete the IP development cycle: one to trigger the differentiation of attack-phase bdellovibrios into growth-phase cells and another to initiate DNA replication. This hypothesis was based largely on the results of two studies. In the first, Ruby and Rittenberg (23) showed that premature release of wild-type bdellovibrios from bdelloplasts resulted in the bdellovibrios differentiating into attack-phase cells. This occurred regardless of whether the bdellovibrios were suspended in buffer or rich medium. If the bdellovibrios were released before the onset of DNA replication, they differentiated into attack-phase cells without initiating DNA replication. If they were released after the onset of DNA replication, the rounds of DNA synthesis that had been initiated were completed, but the bdellovibrios then differentiated into attack-phase cells. Thus, prematurely released bdellovibrios, although capable of synthesizing DNA, could not initiate new rounds of DNA replication even if suspended in rich medium.

The results of Ruby and Rittenberg (23) suggested that bdellovibrio growth and multiplication required the continued presence of a host cell factor. In accord, Gray and Ruby (6) found that prematurely released bdellovibrios would initiate DNA replication in a rich medium if it was supplemented with microbial cell extracts. The factor(s) responsible for stimulating DNA replication was apparently different from that responsible for the switch between attack phase and growth phase, since extracts that could stimulate DNA replication in prematurely released wild-type bdellovibrios had no effect on attack-phase cells. Interestingly, extracts that could stimulate bdellovibrio DNA replication could be prepared from both host (*Escherichia coli*) and nonhost (*Bacillus subtilis*) bacteria, but not from *Bdellovibrio bacteriovorus*. Initial characterization of the extracts indicated that the activity was soluble, heat stable, resistant to treatment with RNase or DNase, and composed of protein-like compounds that fractionated over a wide range of molecular masses (10 to >200 kDa).

The results of bdellovibrio protein synthesis studies (1) are of particular interest given the Gray and Ruby two-signal model. Specifically, it was found that two distinct waves of protein synthesis occur early in the interaction of bdellovibrios with host cells (Fig. 2). The first is initiated during the invasion process, beginning with attachment at 5 min and ending at about 20 min with the bdellovibrios lodged in the periplasmic space of the bdelloplasts (e.g., the protein labelled A in Fig. 2). The second is initiated at about 20 min, continues throughout the bdellovibrio multiplication phase, and ends around the time of bdelloplast lysis, about 2 to 3 h after invasion (e.g., protein C). At that time, the synthesis of a major new protein begins (protein B). It is tempting to speculate that the two early waves of protein synthesis are triggered by the two hypothetical host signals proposed by Gray and Ruby. Whether or not this is the case, the data indicate that the IP growth cycle involves multiple cascades of differential gene expression.

Analysis of host-independent mutants. Early in the study of bdellovibrios, it was established that mutants able to grow on rich media in the absence of host cells spontaneously arise (4, 25, 28). Such host-independent (H-I) mutants have been reported to occur at a frequency of about 10^{-6} to 10^{-7} (25, 31), suggesting that the H-I phenotype can result from single mutations. Gray and Ruby (7) noted that this posed a problem for their two-signal model, as one might think that H-I mutants would be double mutants; putatively, they would have lost the requirement for two distinct signalling events. Of course, there is the plausible explanation put forward by the authors that certain single-site mutations might have pleiotropic effects obviating the need for both proposed signals. However, a reexamination of the literature, summarized below, offers another possible explanation: H-I mutants that can grow in the absence of cell extracts do not arise from single mutations at a frequency of 10^{-6} to 10^{-7}.

Seidler and Starr (25) were the first to report a procedure that reliably yielded *Bdellovibrio* H-I mutants and found that wild-type bdellovibrios produce colonies on peptone-yeast extract agar plates at a frequency of 10^{-6} to 10^{-7} (compared with PFUs); we have made similar observations, using the same mutant isolation procedure (2, 3). However, the colonies obtained do not have a single phenotype. They vary widely in size, ranging from barely perceptible to relatively large (about 3 mm), and they differ dramatically in their subsequent culturing characteristics. Our experience with *B. bacteriovorus* 109J (2, 3) is that the small H-I colonies generally do not give growth upon restreaking unless they are pooled with other small colonies, and even then they grow only in close proximity with each other in the heavily

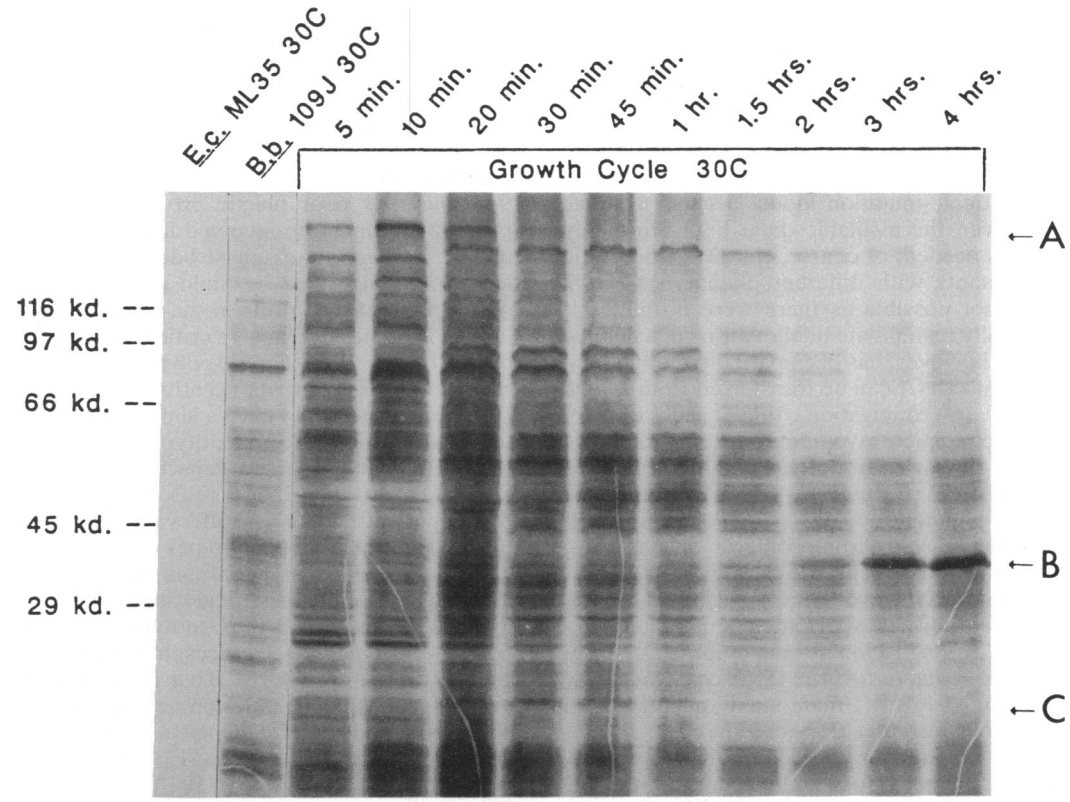

FIG. 2. Changes in protein synthesis during IP growth of *B. bacteriovorus*. A synchronous culture of *B. bacteriovorus* 109J.1 (streptomycin resistant) growing on *E. coli* ML35 (streptomycin sensitive) was pulse-labelled (1.5 min) with [^{35}S]methionine at various times during the IP growth cycle. Protein synthesis by *E. coli* was inhibited by addition of 50 μg of streptomycin per ml to the culture. The leftmost lane shows that little host protein was labelled with ^{35}S under these conditions. The next lane shows the profile of bdellovibrio proteins labelled with ^{35}S in the absence of host cells. The remaining lanes show the profile of bdellovibrio proteins labelled with ^{35}S at various times (indicated above lanes) during the synchronous culture. Proteins labeled A, B, and C are examples of bdellovibrio proteins synthesized during the early, late, and middle stages of IP growth, respectively.

streaked portion of the plate. In contrast, the large H-I colonies, which make up only a small proportion of the CFUs (on the order of 1%), can usually be picked and restreaked without difficulty to give well-isolated colonies.

These observations have interesting parallels with the findings of both Shilo and Bruff (28) and Ishiguro (9). Shilo and Bruff (28) reported that H-I derivatives of strain A3.12 [also designated *B. starrii* (24)] would form colonies on rich media only if they were plated at high cell density, about 10^4 organisms per plate. If, however, the mutants were plated on a rich medium supplemented with autoclaved supernatants (1% vol/vol) from axenic cultures of H-I bdellovibrios, they did not display the density-dependent phenotype; they would form well-isolated colonies. Similarly, Ishiguro (9) could detect H-I mutants of *B. bacteriovorus* 109D only if the plating medium was supplemented with cell extracts prepared from various hosts or nonhosts or cell lysates of intraperiplasmically grown bdellovibrios. However, continued culture of the GIF (growth initiation factor)-dependent H-I mutants in the absence of hosts gave rise to GIF-independent mutants. Such mutants could be cultured on standard commercial media without addition of cell extracts or lysates.

Taken together, the genetic data suggest that wild-type bdellovibrios spontaneously give rise to at least two classes of H-I mutants. Type I mutants display a density-dependent growth phenotype, forming isolated colonies on nutrient agar plates only if the medium is supplemented with various cell extracts. Type II mutants display a density-independent growth phenotype, forming isolated colonies on commercial media without addition of cell extracts. The H-I mutants described by Shilo and Bruff (28) and the GIF-requiring mutants described by Ishiguro (9) would fall into the type I class, while the GIF-independent mutants described by Ishiguro (9) would be type II mutants. The work of Ishiguro (9) further suggests that type II mutants are double mutants that arise from type I single mutants.

The notion of two classes of H-I mutants is consistent with the two-signal model of Gray and Ruby (7). The single mutation putatively suffered by type I mutants would nullify the requirement for one of the two host signal molecules, but robust growth on rich media (ability to form isolated colonies) would still be dependent on the second signal being supplied by cell lysates or extracts. Type II mutants, on the other hand, would be double mutants that no longer require host signals and thus grow on rich media in the absence of added lysates or extracts. The frequency of obtaining type I mutants is in the range of 10^{-6} to 10^{-7} (9, 28), which is reasonable for single mutations. The frequency at which type II mutants occur, however, is less clear. The large-colony type II H-I mutants of *B. bacteriovorus* 109J that we isolated (2, 3) arose at a frequency of about 10^{-8} to 10^{-9}. This would seem somewhat high for a double mutation. However, it is not clear whether the mutants suffered both

putative mutations before being plated. That is, it is possible that the type II mutants arose from type I mutants after they had grown to some degree on the initial selection plate (type I mutants will grow in close proximity with other type I mutants).

Identification of a genetic locus associated with *Bdellovibrio* host independence. While the two-signal model of Gray and Ruby (7) and the double-mutation model discussed above seem reasonable given the available data, both are highly speculative. What is needed, of course, is a detailed genetic analysis of H-I mutants with different phenotypes. Until recently, this was not possible as there were no methods available to genetically manipulate bdellovibrios in a defined way. This situation, however, has been partially rectified (2). It has been shown that DNA can be transferred from *E. coli* to *B. bacteriovorus* by conjugation. While such transfer might seem odd—the recipients would be expected to invade and kill the donors!—it works. Both IncQ and IncP plasmids can be transferred from *E. coli* to either wild-type *B. bacteriovorus* or H-I mutants by using IncP *tra* functions carried on either the *E. coli* chromosome or on plasmids. IncQ plasmids autonomously replicate in *B. bacteriovorus*; IncP plasmids do not. However, IncP plasmids can be maintained in *B. bacteriovorus* via homologous recombination and integration through cloned bdellovibrio DNA sequences. Libraries of *B. bacteriovorus* DNA constructed by using the IncP vector pVK101 (10) are stably maintained in *E. coli* and can be transferred to *B. bacteriovorus*.

The *Bdellovibrio* conjugation procedure has been exploited to identify a genetic locus affected in three H-I mutants derived from *B. bacteriovorus* 109J (3). The mutants—*B. bacteriovorus* BB3, *B. bacteriovorus* BB4, and *B. bacteriovorus* BB5—are type II: they form large isolated colonies on peptone-yeast extract plates without supplementation with cell extracts. Like most H-I mutants, they are facultative and form small turbid plaques on lawns of host cells. Transfer of wild-type *B. bacteriovorus* 109J cosmid libraries into H-I mutant *B. bacteriovorus* BB5 resulted in the isolation of recombinants that produced large clear plaques that were similar, if not identical, to those formed by wild-type cells. Analysis of 27 of the enhanced-plaque recombinants indicated that each had one of only two overlapping cosmids integrated into the genome.

The results of the conjugation-recombination experiments were consistent with the enhanced-plaque phenotype being brought about by specific wild-type DNA sequences. The question raised was whether this region of the *Bdellovibrio* genome corresponded to a site that had suffered a mutation in *B. bacteriovorus* BB5. Additional genetic and DNA sequence analyses indicated that it did (3). Specifically, the region of the wild-type genome that gave the enhanced-plaque phenotype was narrowed down to a 1-kb *Xba*I-*Eco*RI fragment, and DNA sequence analysis indicated that *B. bacteriovorus* BB5 had suffered a single base pair deletion within this region of the genome. Moreover, it was found that both *B. bacteriovorus* BB3 and *B. bacteriovorus* BB4 had also suffered mutations within the 1-kb *Xba*I-*Eco*RI fragment: *B. bacteriovorus* BB3 had a single base pair deletion, and *B. bacteriovorus* BB4 had a 34-bp deletion. As this locus had a clear effect on the plaque-forming ability of the bdellovibrio, it was designated *hit* for host-interaction.

It is not known why mutations at the *hit* locus result in a small-turbid-plaque phenotype or how this relates to the ability of H-I mutants to grow axenically on rich culture media. It is interesting, however, that one of the *hit* open reading frames is deduced to encode a 10.6-kDa protein that is probably targeted to the periplasm (3); the polypeptide has a putative signal peptide, and the mature polypeptide is hydrophilic. Such a location is intriguing as it suggests that the polypeptide might have a direct role in the *Bdellovibrio*-host interaction. One possibility is that the polypeptide has a structural function or enzymatic activity required for host invasion. Mutations at *hit* might then result in poor penetration and thus poor plaque production. Alternatively, the polypeptide might be involved in *Bdellovibrio*-host cell signalling. Perhaps attack-phase bdellovibrios are repressed for growth, as suggested by Shilo (26), with the 10.6-kDa *hit* polypeptide having a role in the negative regulatory circuit. Upon encountering a host, a critical signal molecule might be bound by the 10.6-kDa *hit* polypeptide, thus modifying its activity and relieving its negative effect on axenic growth; mutations at *hit* could have a similar effect. In this scenario, the small-turbid-plaque phenotype of the H-I mutants would result from an increased capacity of the bdellovibrios to grow in the absence of host cells. Such a regulatory model is particularly attractive as it offers a potential link between the plaquing and plating phenotypes of the H-I mutants.

The occurrence of mutations at the *hit* locus in three individual H-I mutants strongly suggests that this locus has a role in the axenic-growth phenotype. However, recombination of wild-type *hit* sequences into the H-I mutants did not abolish axenic growth (3). That is, *hit* recombinants could form large isolated colonies on peptone-yeast extract plates, albeit at a reduced frequency as compared with the H-I mutants: whereas the CFU-to-PFU ratio in the H-I mutants was about 1, it was about 10^{-4} in the *hit* recombinants. There are a number of possible explanations for this intermediate axenic-growth phenotype. As discussed above, *B. bacteriovorus* BB3, *B. bacteriovorus* BB4, and *B. bacteriovorus* BB5 are type II H-I mutants, and thus they may be double mutants. If so, the *hit* recombinants might retain a second site mutation that is responsible for the intermediate axenic-growth phenotype. The mutations at *hit* might not, in fact, have a direct role in axenic growth at all; they might be secondary mutations that enhance the ability of axenic mutants to grow without hosts. Finally, there is the possibility that *hit* mutations fully account for the H-I phenotype—both the plaquing phenotype (small, turbid) and the plating phenotype (axenic growth)—but that recombination of the wild-type *hit* sequences into the H-I mutants was unable to restore the wild-type phenotype because of allelic interactions between the wild-type and mutant copies of the *hit* locus present in the merodiploid *hit* recombinants. Distinguishing between these and additional possibilities is now a major challenge, but it should be possible by using the available conjugation system.

CONCLUDING REMARKS

Determining the molecular basis for the H-D phenotype of wild-type bdellovibrios is fundamental to our understanding of *Bdellovibrio* biology. At present, our knowledge in this area is elementary. However, significant progress has been made. Particularly important have been the biochemical studies suggesting that *Bdellovibrio* IP development is regulated by more than one host signal molecule and the genetic studies indicating that the *Bdellovibrio* requirement for host cells can be obviated by mutation. Indeed, ever since the isolation of the first H-I mutant, it has been anticipated that genetic studies would add greatly to our understanding of *Bdellovibrio* host dependence. This promise has not been realized, in large part because of a lack of genetic systems to

manipulate the *Bdellovibrio* genome. This deficiency, however, has now been partially rectified, and the first genetic locus associated with the H-I phenotype has been identified. We are optimistic that the ability to genetically manipulate bdellovibrios will stimulate a burst of research that will soon provide significant new insight into the nature of host dependence in this remarkable prokaryote.

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