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Pore worms: Using *Caenorhabditis elegans* to study how bacterial toxins interact with their target host

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Abstract

The interaction of pathogenic bacteria with a target host is regulated both by bacterial virulence factors and by host components that either protect the host or that promote pathogenesis. The soil nematode *Caenorhabditis elegans* is a host for a number of bacterial pathogens, as briefly reviewed here. *Bacillus thuringiensis* (*Bt*) is a pathogenic bacteria that *C. elegans* is likely to encounter naturally in the soil. The pore-forming Crystal (Cry) toxins made by *Bt* are recognized as the dominant virulence factor in this host-pathogen interaction. Forward genetic screens for *C. elegans* mutants resistant to the Cry toxin, Cry5B, have identified a host carbohydrate structure that promotes pathogenesis. Data suggest this structure is likely to be a Cry5B receptor expressed in the host intestine. This finding is discussed in light of other carbohydrate receptors for bacterial toxins. To investigate host-toxin interactions on a global level, the response of *C. elegans* to the pore-forming Cry5B is also being investigated by gene transcription profiling (microarrays). These data are beginning to reveal a diverse intracellular response to toxin exposure. To put these investigations in perspective, host responses to other pore-forming toxins are discussed. Investigations with Cry5B in *C. elegans* show a promising beginning in helping to elucidate host-toxin and host-pathogen interactions.

Key words: Pore-forming toxin – crystal toxin – *Caenorhabditis elegans* – *Bacillus thuringiensis* – carbohydrate receptor – transcription profiling – host-pathogen interactions

Introduction

The microbiological basis of infectious disease has fascinated scientists as far back as Koch's postulates in the late 1800s and continues to fascinate us today. How pathogenic bacteria interact with their hosts is complex, and modern biological techniques are delving deeper and deeper into this complexity. The nematode *Caenorhabditis elegans* is recently being developed as a model system to study host-pathogen interactions. *C. elegans* provides a facile and rapid system for studying this interaction as it naturally feeds on bacteria. The pathogenicity of specific strains of bacteria can be directly determined by feeding the nematodes the bacteria and looking for effects within hours or a few days. The ability to perform forward and reverse genetics in *C. elegans* also lends itself to understanding host pathways that respond to pathogens (for reviews see (Aballay and Ausubel, 2002; Alegado et al., 2003; Ewbank, 2002)).

Many Gram-negative and Gram-positive bacteria have been identified as *C. elegans* pathogens (Table 1). Virulence factors made by bacteria are

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Table 1. Examples of bacteria that are pathogenic towards *C. elegans*.

Bacteria	References
Gram-negative	
Aeromonas hydrophila	(Couillault and Ewbank, 2002)
Burkholderia pseudomallei	(Gan et al., 2002; O'Quinn et al., 2001)
Pseudomonas aeruginosa (PA01)	(Darby et al., 1999; Gallagher and Manoil, 2001)
Pseudomonas aeruginosa (PA14)	(Mahajan-Miklos et al., 1999; Tan et al., 1999)
Salmonella typhimurium	(Aballay et al., 2000; Labrousse et al., 2000)
Serratia marcescens	(Kurz et al., 2003; Mallo et al., 2002)
Yersinia pestis	(Darby et al., 2002)
Yersinia pseudotuberculosis	(Darby et al., 2002)
Gram-positive	
Bacillus thuringiensis	(Leyns et al., 1995; Marroquin et al., 2000)
Enterococcus faecalis	(Garsin et al., 2001; Sifri et al., 2002)
Microbacterium nematophilum	(Hodgkin et al., 2000)
Staphylococcus aureus	(Garsin et al., 2001; Sifri et al., 2003)
Streptococcus pneumoniae	(Garsin et al., 2001)
Streptococcus pyogenes	(Jansen et al., 2002)

important elements of pathogenic attack on the host. The interactions of several bacterial virulence factors with the *C. elegans* host have been investigated. For example, the death of *C. elegans* caused by *Pseudomonas aeruginosa* strain PA14 was separated into a fast killing mode (hours) and a slow killing mode (days) that depended on the medium used for the assay (Tan et al., 1999). Subsequent studies on the fast killing mode identified phenazines, pigments that are secreted from *P. aeruginosa*, as toxins that are associated with killing of the worms (Mahajan-Miklos et al., 1999). A different strain of *P. aeruginosa*, PA01, was found to lead to rapid paralysis and death of *C. elegans*, and the toxin responsible for this effect was demonstrated to be hydrogen cyanide (Darby et al., 1999; Gallagher and Manoil, 2001). A transposon-based mutagenic screen in *Serratia marcescens* identified a reduced virulence mutant with a transposon inserted in the shlBA operon that is required for production of the pore-forming toxin hemolysin (Kurz et al., 2003). An as yet unidentified toxin (or toxins) have also been associated with the pathogenicity of *Burkholderia pseudomallei* in *C. elegans* (Gan et al., 2002; O'Quinn et al., 2001). Toxins have also been associated with the killing of *C. elegans* by Gram-positive bacteria. *Streptococcus pyogenes* grown on Todd-Hewitt broth supplemented with yeast extract killed *C. elegans*, and the toxicity was mediated through hydrogen peroxide (Jansen et al., 2002). The pore-forming toxin α-hemolysin was identified as one of several virulent factors from *Staphylococcus aureus* (Sifri et al., 2003).

Our laboratory is studying the Crystal (Cry) toxins made by *Bacillus thuringiensis* (*Bt*), a Gram-positive soil bacterium. Cry proteins are toxic to insects and nematodes (de Maagd et al., 2001; Schnepf et al., 1998; Wei et al., 2003). These virulence factors are made by *Bt* upon sporulation and have been used extensively in transgenic plants for control of insect pests and as sprays for control of black flies and mosquitoes. Since both *Bt* and nematodes exist in the soil, it is likely that they naturally interact. Using *Bt* lysates, Cry5B and Cry6A were the first Cry proteins shown to be nematicidal towards *C. elegans* (Marroquin et al., 2000). Expression of Cry proteins in *Escherichia coli* as well as the use of purified Cry toxin verified directly that specific Cry proteins are responsible for the toxicity against nematodes (Griffitts et al., 2001; Marroquin et al., 2000; Wei et al., 2003). Cry toxicity is directed against intestinal cells of *C. elegans* and leads to vacuole formation, pitting, and eventual degradation of the intestine following Cry exposure (Marroquin et al., 2000).

The ability to intoxicate *C. elegans* with Cry toxins allows for identification of host factors that are required for the intoxication process as well as host factors that defend against the pore-forming toxin (Figure 1; more on its pore-forming activity below). For example, isolation of *C. elegans* mutants (and subsequent cloning of genes) resistant to a Cry toxin reveals host factors used by the toxin for the pathogenic process (Fig. 1A). Conversely, isolation of *C. elegans* mutants (and subsequent cloning of genes) hypersensitive to a Cry toxin reveals host factors that are responsible for defending against the toxin (Fig. 1B). These defense factors can be specific for a single toxin or part of a more general response of the innate immune system directed against multiple pathogens. The following sections illustrate how

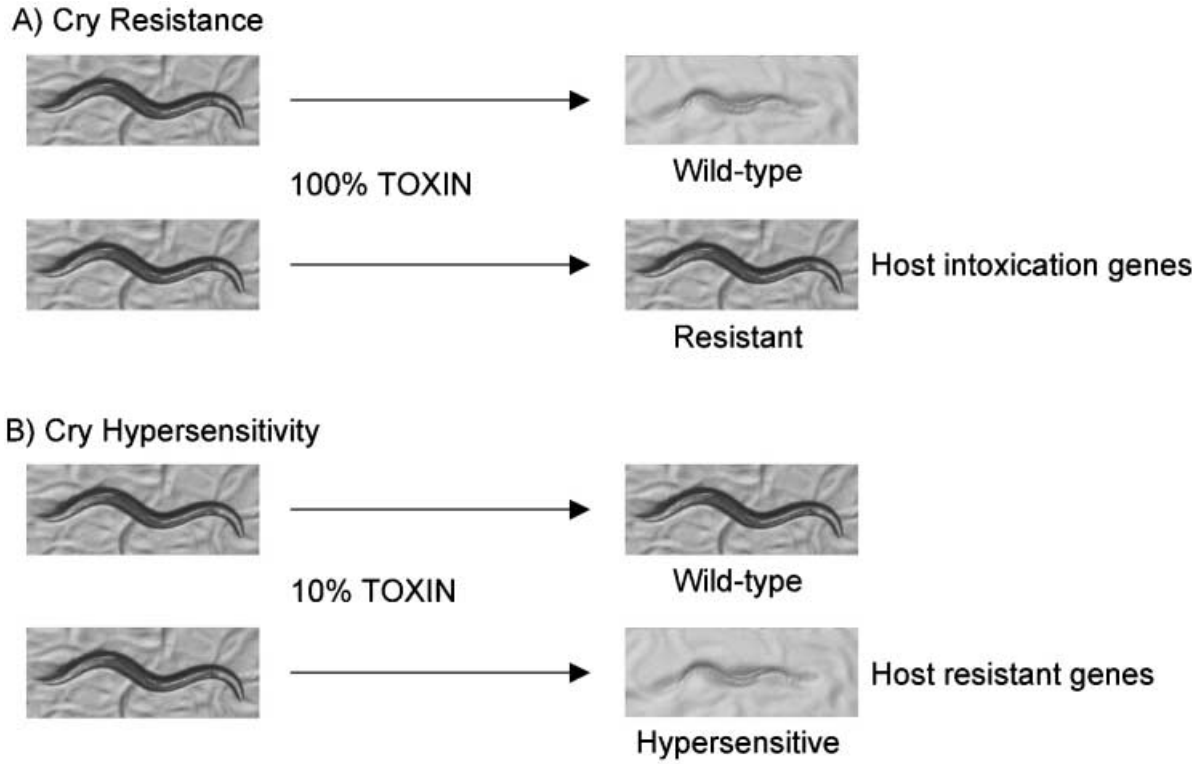

Fig. 1. Paradigm for understanding interactions between Cry toxins and *C. elegans*. A) Host resistance. Wild-type worms fed *E. coli* in which 100% of the bacteria express Cry protein become intoxicated as indicated by their small size and pale color while worms with a resistant mutation do not. Identification of the mutant alleles in the resistant worms will demonstrate host components that are required for Cry toxicity. B) Host hypersensitivity. Wild-type worms fed a mixture of *E. coli* in which only 10% of the bacteria produce the Cry protein do not become intoxicated. Hypersensitive worms have increased susceptibility to the Cry protein and become intoxicated on this lower dose. Identification of alleles that confer a hypersensitive phenotype will illustrate host components that are required for defense against the toxin.

this paradigm is being used to identify genes and pathways in *C. elegans* responsible for both the intoxication and the defense of the host when exposed to a pore-forming Cry toxin.

Toxin-host interaction via carbohydrates is a major intoxication process revealed by genetics

To determine host factors that cause resistance against Cry5B, an ethylmethanesulfonate mutagenesis screen was performed in which *C. elegans* resistant to the pore-forming toxin were selected (see Fig. 1A). This screen identified five genes named *bre-1* to *bre-5*, for *Bt*-toxin resistant (Marroquin et al., 2000). *bre-5* encodes a glycosyltransferase (Griffitts et al., 2001, 2003). Recently, *bre-2*, *bre-3*, and *bre-4* have also been shown to be glycosyltransferase genes (Griffitts et al., 2003). *bre-2*, *bre-3*, *bre-4*, and *bre-5* function in the intestine in a single genetic pathway required for intoxication (Griffitts et al., 2003). Unlike wild-type nematodes, *bre* mutants are not able to internalize Cry5B into intestinal cells (Fig. 2; (Griffitts et al., 2001, 2003)).

Based on these and other findings, we hypothesize that the proteins encoded by the *bre* genes synthesize a carbohydrate receptor for Cry5B (Griffitts et al., 2001, 2003). *bre* mutants fail to internalize toxin because they lack this receptor. An alternative interpretation is that the *bre* genes do not synthesize a receptor but rather influence the membrane or membrane proteins in a way that promotes toxin binding. Interestingly, carbohydrates have been previously implicated as playing a role in insecticidal Cry toxin-receptor interactions since the sugar N-acetyl-D-galactosamine inhibits binding of Cry1Ac to brush border membrane vesicles (Knowles et al., 1991). Based on the homology of two BRE proteins with two glycosyltransferases characterized in *Drosophila*, the structure modified by the BRE enzymes may be a glycolipid (Griffitts et al., 2003). Thus, screening for host mutants that strongly resist attack by the pore-forming Cry toxin invariably leads to

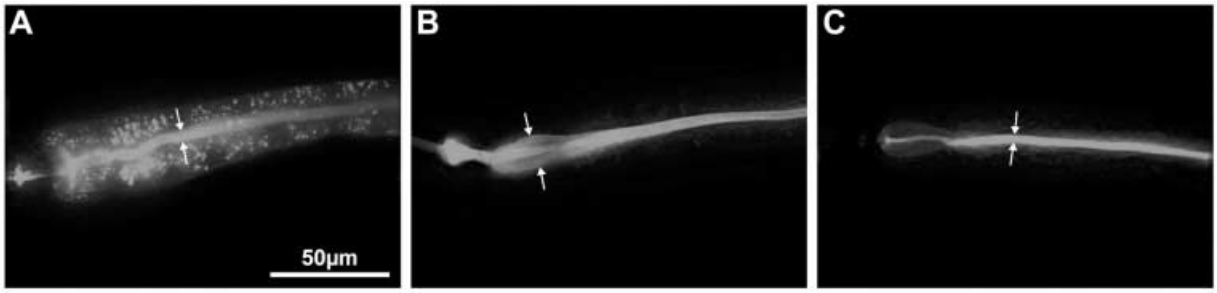

Fig. 2. Internalization of Cry5B is impaired in the *bre-5* mutant. Rhodamine labeled Cry5B was fed to A) a wild-type worm, B) a wild-type worm after the toxin was boiled to denature, and C) a *bre-5* mutant worm. The white arrows demarcate the edges of the intestinal lumen for each worm. Unlike the wild-type worm, the *bre-5* mutant is not able to uptake the toxin into intestinal cells, suggesting the mutant lacks a toxin receptor. The failure of the wild-type worm to internalize denatured toxin indicates a specific toxin structure is required for the endocytosis.

Table 2. Known carbohydrate receptors for bacterial toxins.

Toxin (Bacteria)	Receptor	References
Cholera toxin (*Vibrio cholerae*)	GM1 glycolipid	(Holmgren et al., 1973)
Heat-labile enterotoxin LT (*E. coli*)	GM1 glycolipid	(Moss et al., 1981)
Heat-labile enterotoxin LT-IIa (*E. coli*)	GD1b glycolipid	(Fukuta et al., 1988)
Heat-labile enterotoxin LT-IIb (*E. coli*)	GD1a glycolipid	(Fukuta et al., 1988)
Shiga toxin (*Shigella dysenteriae*)	Gb3 glycolipid	(Jacewicz et al., 1986)
Verotoxin (*E. coli*)	Gb3 glycolipid	(Lingwood et al., 1987)
Aerolysin (*Aeromonas hydrophila*)	GPI anchored proteins	(Abrami et al., 1998; Nelson et al., 1997)

loss of host carbohydrate structures required for interaction of Cry toxin with intestinal cells. These data suggest that toxin-receptor interactions are of central importance in the intoxication process.

Carbohydrates that function as bacterial toxin receptors

The suggestion that the *bre* genes synthesize a carbohydrate receptor for Cry5B is consistent with a recurring theme in bacterial pathogenesis – host carbohydrates as receptors for virulence factors (Table 2). The GM1 ganglioside was one of the first carbohydrates to be identified as a bacterial toxin receptor. Cholera toxin was initially demonstrated to interact specifically with the GM1 ganglioside (Holmgren et al., 1973). The related *E. coli* heat-labile enterotoxin LT was also found to preferentially bind GM1 compared to other gangliosides (Moss et al., 1981). Both cholera toxin and LT led to an increase in cAMP in mouse fibroblasts after GM1 was incorporated into the cells, indicating this ganglioside is a functional receptor (Moss et al., 1979). A crystal structure of the cholera toxin B subunit complexed with the GM1 pentasaccharide has revealed the molecular interactions of the toxin with the sugar residues, thereby demonstrating the carbohydrate portion of GM1 directly binds the toxin (Merritt et al., 1994). Distinct glycolipids are used as receptors for other bacterial toxins. *E. coli* enterotoxin LT-IIa binds GD1b while enterotoxin LT-IIb binds GD1a (Fukuta et al., 1988). The shiga toxin from *Shigella dysenteriae* and shiga-like toxins from *E. coli* use the globotriosylceramide (Gb3) glycolipid as receptors (Jacewicz et al., 1986; Lingwood et al., 1987). In addition to the carbohydrate moiety of glycolipids, carbohydrates within glycoproteins can also serve as receptors for bacterial toxins. The toxin aerolysin from *Aeromonas hydrophila* was demonstrated to bind glycosylphosphatidylinositol (GPI)-anchored proteins (Abrami et al., 1998; Nelson et al., 1997). The carbohydrate portion of the GPI is required for this interaction (Diep et al., 1998), and specific sugar residues within the carbohydrate structure were found to affect toxin affinity (Abrami et al., 2002). The specific protein anchored by the GPI may also affect the ability of the toxin to bind (Abrami et al., 2002). Similarly, current evidence indicates the tetanus and botulinum neurotoxins, produced from *Clostridium* bacteria, may interact with carbohydrate as well as protein receptors on cell surfaces (Pellizzari et al., 1999).

Delving into the global response of a host to a pore-forming toxin

Although the binding of pore-forming toxins to their receptors is a major event, intracellular events that follow are undoubtedly important as well. These intracellular events can include modifications in protein activity, alterations in cytosol composition, morphological changes to the cell or intracellular organelles (e.g., Cry5B induces vacuole formation in *C. elegans* intestinal cells), and modified gene expression. Some of these events are induced by the toxin in the host cell to promote pathogenesis while other events are the efforts of the host cell to protect itself. Without further information, we sometimes cannot be sure whether a cellular response is one or the other (see below). The toxin molecule itself might give us clues, but what sort of intoxicating or protecting responses might we expect to see with a pore-forming toxin? *Bt* crystal proteins make pores ~ 2 nm in diameter that show little ionic specificity (Peyronnet et al., 2002). This is comparable to some other bacterial pore-forming toxins, such as the hemolysin made by *Vibrio cholerae* (Zitzer et al., 1997) (Table 3). We will review what is known about responses induced by pore-forming toxins below. But first we present our current directions in investigating this intriguing area.

Because intracellular events of intoxication include both toxin-inflicted injuries and host-mounted defenses, a molecular examination of host response can unearth strategies of both the toxin and the host. As a means of uncovering the host response, we are using Affymetrix gene chips to determine how gene expression profiles change in *C. elegans* whole animals upon exposure to the crystal toxin Cry5B. We exposed synchronous populations of larvae to Cry5B toxin by feeding them a non-toxic attenuated *E. coli* strain transformed with the Cry5B gene and allowing them to feed for increasing lengths of time before harvesting their RNA. Given that Cry5B uptake is observed after 1–3 hours of toxin exposure (Griffitts et al., 2001, 2003), we chose to focus on the first eight hours of the intoxication process in an effort to establish the primary host response. Since *C. elegans* typically die on *E. coli* expressing Cry5B after 4–5 days, the transcriptional changes seen at eight hours do not represent the transcriptional profiles of dead or nearly dead animals. The results of repeated chip experiments surprisingly show that $>5\%$ of the genome is transcriptionally regulated in response to Cry5B and implicate a variety of host cellular processes. This result is different from that found by Mallo et al. (2002) who used partial cDNA arrays and detected seven genes induced by *C. elegans* upon infection with *Serratia marcescens*.

The function of these Cry5B-responsive genes in host/toxin relations can be explored using *C. elegans* genetics. If a particular gene plays a role that favors intoxication, reducing the function of that gene should make the animals more resistant to the toxin (Fig. 1A). Conversely, if the gene product serves to protect the animal, reducing the function of the gene should make the animals more susceptible to the toxin (Fig. 1B). For example, MAP kinase and TGF-β pathways have been implicated in the defense of *C. elegans* against bacterial pathogens (not virulence factors per se) since loss of genes in these pathways makes the animals more susceptible to the pathogenic bacteria (Kim et al., 2002; Mallo et al., 2002). For several of the host genes regulated (induced/repressed) by toxin exposure, we ordered *C. elegans* strains available from the stock center with single mutations in these genes. These strains are being visually examined for either an increase or decrease in Cry5B susceptibility. The initial results of this primary screen indicate that while many of the genes appear to have no functional role in the toxin-host interaction, some of these genes do play a functional role in either helping the toxin or helping the host. Given the lifestyle of *C. elegans* as a soil nematode that regularly encounters and ingests a variety of soil bacteria, including most likely *Bt*, we speculate that the nematode has evolved to respond robustly to this type of bacterial virulence factor and attack.

Table 3. Comparison of pore sizes made by bacterial toxins.

Pore-forming toxin	Pore diameter	Reference
Vibrio cholerae hemolysin	1–2 nm	(Zitzer et al., 1997)
Aerolysin	3.8–4.6 nm	(Tschodrich-Rotter et al., 1996)
Cry toxin	1.5–2.6 nm	(Peyronnet et al., 2002; Vie et al., 2001)
Streptolysin O	30 nm	(Bhakdi et al., 1985)

Delving into mammalian responses to pore-forming toxins

What is known of responses to bacterial pore-forming toxins in mammalian host cells? There are some recurring themes – induction of a calcium response, initiation of cell death, and formation of vacuoles. Increases in intracellular calcium levels or induction of calcium oscillations caused by a pore-forming toxin have commonly been reported. By monitoring the first several minutes of toxin exposure, Krause et al. (1998) found that purified aerolysin at concentrations of 100 ng/ml causes an increase in calcium levels in human granulocytes that is di-phasic, with the early phase occurring almost immediately upon toxin exposure. This dose-dependent response was found to be the result of calcium release from intracellular stores mediated by G-protein activation. Staphylococcal α-toxin and streptolysin O were also found to have effects on calcium levels in this same study. Interestingly, studies examining lower concentrations of aerolysin in T lymphoma cells found that even at subnanomolar concentrations, cytosolic calcium levels increase (Nelson et al., 1999). These increases, however, were contributed to influx from extracellular sources rather than release from stores. *Listeria monocytogenes* is also capable of inducing rapid, multi-phasic calcium elevations in a macrophage-like cell line that are dependent on the pore-forming toxin listeriolysin O (LLO) (Goldfine and Wadsworth, 2002; Wadsworth and Goldfine, 1999). Further work in epithelial cells and kidney cells indicates the source of calcium in these LLO-induced influxes is the external medium (Dramsi and Cossart, 2003; Repp et al., 2002). Influx through the cell membrane is also the cause of the calcium oscillations seen in neuroblastoma cells treated with the pneumococcal toxin pneumolysin (Stringaris et al., 2002). Calcium increase in these cells was followed by cell death, with 40% of the cells dying within three hours of treatment. Here, calcium chelators inhibited this cell death response, suggesting that increased calcium levels caused by pneumolysin may serve to promote cell death. Similar observations have been made in models of aerolysin intoxication where its been proposed that the toxin-induced calcium increases act as an apoptotic signal (Nelson et al., 1999). Although the association between poisonous levels of calcium and cell death has been well characterized, the function of calcium flux as a host response and the downstream consequences to these fluxes remain unclear. Because calcium plays a role in so many cellular processes, there is potential for these calcium elevations to have widespread impact.

Induction of cell death is also a property that has been reported for many pore-forming toxins and is a good example of how intoxication is the work of both the toxin and the host cell machinery. For the vacuolating cytotoxin from *Helicobacter pylori*, VacA, promoting cell death appears to involve the direct activity of the toxin in the cytosol. When expressed in HeLa cells as a GFP fusion, VacA-GFP localized to the mitochondria where it activated caspase-3 and caused apoptotic-like cell death (Galmiche et al., 2000). In the case of pneumolysin, recent work suggests that the toxin promotes cell death in neuroblastoma cells by activating the mitogen-activated kinase, p38 (Stringaris et al., 2002). p38 MAP kinase is part of a conserved signal transduction cascade that regulates inflammation, immune response and apoptosis and responds to a variety of stress stimuli. This result not only demonstrates that pore-forming toxins can act as a signal to the host to trigger immune pathways, but it also shows that the action of pneumolysin at the cell membrane can be translated into activation of the highly regulated, intracellular program of apoptosis. Currently, many questions remain about whether cell death is largely a protective response that limits the spread of the bacteria, or whether it's a mechanism used by the toxin to kill off host immune cells. Ultimately, the function of cell death may be found to vary depending on the site and stage of infection.

Some bacteria make pore-forming toxins that cause vacuolization of cellular organelles. The VacA protein from *Helicobacter pylori* is a classic example. The vacuoles caused by this toxin are thought to be the result of fusion of late endosomal and lysosomal compartments (Molinari et al., 1997; Papini et al., 1997). The hemolysin produced by *Vibrio cholerae* also causes vacuolization in Vero cells within 30 minutes (Moschioni et al., 2002). These vacuoles contain membranes from the trans-Golgi network as well as from endosomes and lysosomes. The hemolysin from *Serratia marcescens* also causes vacuolization in epithelial cells (Hertle et al., 1999). Abrami et al (1998) found that aerolysin is capable of inducing vacuolization of the endoplasmic reticulum in a variety of cell types. These vacuoles were detectable within an hour of toxin exposure. In this study, vacuoles were not observed in BHK cells treated with Streptolysin O, indicating that any permeabilization of the plasma membrane is not sufficient for vacuolization. Furthermore, calcium ionophores failed to induce vacuole formation, ruling out the possibility that vacuoles are the consequence of calcium influx.

These findings showing vacuolization of mammalian cells by certain bacterial pore-forming toxins are of particular interest to us because Cry5B has been shown to cause vacuole formation in *C. elegans* intestinal cells. It remains to be seen how these vacuoles contribute to animal intoxication.

Given the complexity of host responses to pore-forming bacterial proteins in these mammalian systems, perhaps it is not surprising that Cry5B invokes such an enormous transcriptional response in *C. elegans*. Even though this response involves many genes, the power of a model organism that can be grown in a Petri dish is the ability to assess the importance of each toxin-responsive gene individually. Gene knockouts are easily generated in *C. elegans* by feeding animals double stranded RNA corresponding to the gene of interest, a technique known as RNA interference (RNAi; (Bargmann, 2001; Barstead, 2001)). Those responses that are important can be distinguished from those that do not significantly alter the intoxication outcome. We have already seen that the ability of the *Bt* pore-forming toxin Cry5B to cause harm to *C. elegans* is dependent on host genes, specifically, the glycosyltransferase genes the products of which help build the toxin-binding epitope of the receptor. Which *C. elegans* genes influence intracellular events of this intoxication remains to be discovered. Because pore-forming toxins play such critical roles in the virulence of many bacterial pathogens in both vertebrate and invertebrate hosts, describing host-pathogen interactions in a simplified, in vivo system will continue to bring insight to the field of bacterial pathogenesis.

Conclusions

C. elegans is becoming a model animal system for studying host-pathogen interactions, including studying the interaction of bacterial virulence factors with a host. One such interaction involves the crystal pore-forming toxins made by *Bt* that attack nematodes and insects. As is the case with some bacterial toxins that attack mammals, the nematicidal *Bt* toxin Cry5B appears to use a carbohydrate receptor. The transcriptional response of *C. elegans* to the pore-forming toxin is being studied with microarrays, the goal being to eventually characterize the function of responding host genes involved in intoxication and defense.

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