Interactions with microbial pathogens*

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Abstract

A wide variety of bacterial pathogens, as well as several fungi, kill *C. elegans* or produce non-lethal disease symptoms. This allows the nematode to be used as a simple, tractable model host for infectious disease. Human pathogens that affect *C. elegans* include Gram-negative bacteria of genera *Burkholderia, Pseudomonas, Salmonella, Serratia* and *Yersinia;* Gram-positive bacteria *Enterococcus, Staphylococcus* and *Streptococcus;* and the fungus *Cryptococcus neoformans.* Microbes that are not pathogenic to mammals, such as the insect pathogen *Bacillus thuringiensis* and the nematode-specific *Microbacterium nematophilum,* are also studied with *C. elegans.* Many of the pathogens investigated colonize the *C. elegans* intestine, and pathology is usually quantified as decreased lifespan of the nematode. A few microbes adhere to the nematode cuticle, while others produce toxins that kill *C. elegans* without a requirement for whole, live pathogen cells to contact the worm. The rapid growth and short generation time of *C. elegans* permit extensive screens for mutant pathogens with diminished killing, and some of the factors identified in these screens have been shown to play roles in mammalian infections. Genetic screens for toxin-resistant *C. elegans* mutants have identified host pathways exploited by bacterial toxins.

1. Introduction

Nematodes began feeding on microbes long before the emergence of vertebrates. Microbial mechanisms to fend off nematodes - and perhaps even turn the animals into the microbe's food - must be of equally ancient origin. It is likely, then, that many of the microbial processes at work in human infectious diseases are adaptations of pathways that first evolved as defenses against nematodes. To the extent that this is the case, *C. elegans* can provide information about these virulence mechanisms that can be difficult to obtain with traditional animal models.

To fully understand a pathogen, researchers ideally would screen the microbe's genome comprehensively to identify all virulence genes. When mice or other mammals are the experimental host, such screens are impractical and prohibitively expensive, inasmuch as they require many thousands of animals. With *C. elegans*, the requirement for animals is not limiting because they grow rapidly in a simple and inexpensive culture system: production of 10,000 animals is trivial. As a result, a number of screens have been performed in which thousands of pathogen mutants have been tested individually against worms.

Of course, *C. elegans* offers far more than mere fecundity. Three decades of research has provided an extensive collection of mutants and clones that are available as off-the-shelf reagents for analysis of the host side of pathogen-host interactions. *De novo* genetic analysis of the host is feasible, e.g. by screening *C. elegans* for mutants that are either resistant or hypersensitive to a pathogen. Screens for hypersensitive mutants have been especially productive in elucidating the *C. elegans* innate immune system, as described in Signaling in the immune response by Jonathan J. Ewbank.

For convenience, pathogenic effects on *C. elegans* can be divided into two broad categories. In one, the nematode becomes infected, and presence of whole, live microbes within the animal or attached to the cuticle is correlated with death or disease. In the other, secreted microbial products, i.e. toxins, are responsible for symptoms, and *C. elegans* need not be exposed to live organisms. These categories are not mutually exclusive: one well-studied pathogen, *Pseudomonas aeruginosa*, has a "slow killing" mode involving colonization of *C. elegans* as well as two toxin-mediated pathways. Furthermore, it is not trivial to discern the mode of killing, and often pathogenesis appears to be multifactoral.

Table 1 lists microbial species known to be pathogenic to *C. elegans*, most of which are also human pathogens. Two of the pathogens (*Drechmeria coniospora* and *Cryptococcus neoformans*) are fungi, and the remainder are bacteria. Some of the bacteria, e.g. *Yersinia* sp. and *Salmonella* sp., are known to invade host cells during mammalian infections, but invasion of *C. elegans* cells has not been reported. No viruses or non-fungal eukaryotic parasites have been shown to infect or kill *C. elegans*.

Following are brief descriptions of the pathogens and their effects on *C. elegans*. In this chapter, "virulence factor" means any pathogen molecule involved in infection, including those that directly affect the host as well as those that function in the pathogen's own physiology, metabolism, and regulation. "Effector" and "toxin" refer to the subset of virulence factors that act directly on the host organism. For the sake of brevity, references have been omitted for general background on the pathogens.

Table 1.

Organism	Human pathogen?	*C. elegans* pathology	References
Aeromonas hydrophila	Yes	Diminished lifespan[a]	Couillault and Ewbank, 2002
Agrobacterium tumefaciens	Yes[b]	Diminished lifespan[a]	Couillault and Ewbank, 2002
Bacillus thuringiensis	No	Pore-forming toxin	Griffitts et al., 2003; Griffitts et al., 2001; Marroquin et al., 2000
Burkholderia cenocepacia	Yes	Gut infection; toxin	Coenye et al., 2001; Kothe et al., 2003
Burkholderia pseudomallei	Yes	Diminished lifespan[a]; toxin	Gan et al., 2002; O'Quinn et al., 2001
Cryptococcus neoformans	Yes	Gut infection; toxin	Mylonakis et al., 2002; Mylonakis et al., 2004
Drechmeria coniospora	No	Whole body parasitized	Jannson, 1994
Enterococcus faecaelis	Yes	Gut infection	Garsin et al., 2001; Kim et al., 2002; Sifri et al., 2002
Erwinia carotovora	No[c]	Diminished lifespan[a]	Couillault and Ewbank, 2002
Erwinia chrysanthemi	No[c]	Diminished lifespan[a]	Couillault and Ewbank, 2002
Escherichia coli	Yes	Gut infection	Garsin et al., 2001
Microbacterium nematophilum	No	Rectal & posterior cuticle infection	Hodgkin et al., 2000; Nicholas and Hodgkin, 2004
Photorhabdus luminescens	Yes[b]	Diminished lifespan[a]	Couillault and Ewbank, 2002
Pseudomonas aeruginosa	Yes	Gut infection; multiple toxins	Darby et al., 1999; Gallagher and Manoil, 2001; Kim et al., 2002; Mahajan-Miklos et al., 1999; Tan et al., 1999; Tan et al., 1999; Wareham et al., 2005
Salmonella enterica	Yes	Gut infection; germline cell death	Aballay and Ausubel, 2001; Aballay et al., 2000; Labrousse et al., 2000; Tenor et al., 2004
Serratia marcescens	Yes	Gut infection; tissue degradation	Kurz et al., 2003; Mallo et al., 2002
Shewanella frigidimarina	No[c]	Diminished lifespan[a]	Couillault and Ewbank, 2002
Shewanella massilia	No[c]	Diminished lifespan[a]	Couillault and Ewbank, 2002
Staphylococcus aureus	Yes	Gut infection	Bae et al., 2004; Begun et al., 2005; Garsin et al., 2001; Sifri et al., 2003
Streptococcus agalactiae (Group B)	Yes	Toxin (hydrogen peroxide)	Bolm et al., 2004
Streptococcus dysgalactiae	Yes	Toxin (hydrogen peroxide)	Bolm et al., 2004
Streptococcus mitis (Viridans group)	Yes	Toxin (hydrogen peroxide)	Bolm et al., 2004
Streptococcus oralis	Yes	Toxin (hydrogen peroxide)	Bolm et al., 2004
Streptococcus pneumoniae	Yes	Toxin (hydrogen peroxide)	Jansen et al., 2002
Streptococcus pyogenes (Group A)	Yes	Toxin (hydrogen peroxide)	Jansen et al., 2002
Xenorhabdus nematophila	No	Biofilm on cuticle; toxin	Couillault and Ewbank, 2002

Organism	Human pathogen?	*C. elegans* pathology	References
Yersinia pestis	Yes	Biofilm on cuticle, feeding blocked	Darby et al., 2002
Yersinia pseudotuberculosis	Yes	Biofilm on cuticle, feeding blocked	Darby et al., 2002

[a]"Diminished lifespan" indicates that the organism's killing mode has not been determined in more detail.
[b]Rare reports of human infection by this species.
[c]Rare reports of human infection by some species in this genus.

2. Bacterial infections of the intestine

Numerous bacteria infect *C. elegans*. Experimenters take advantage of the fact that *C. elegans* eats microbes and simply place L4 or young adult animals on pathogen lawns to initiate infection. The transparency of *C. elegans* allows direct observation of infectious organisms within the worm by differential interference contrast microscopy (DIC); this is facilitated by using pathogens transformed to express green fluorescent protein (GFP; Figure 1). In many cases, the intestine becomes distended; it is not apparent whether this is due simply to physical pressure exerted by the growing pathogen or to some physiological response of the nematode. Colonization can be quantified by grinding worms into a homogenate, plating on solid media, and counting colonies. Mortality is usually reported as time for 50% of animals to die (LT_{50}).

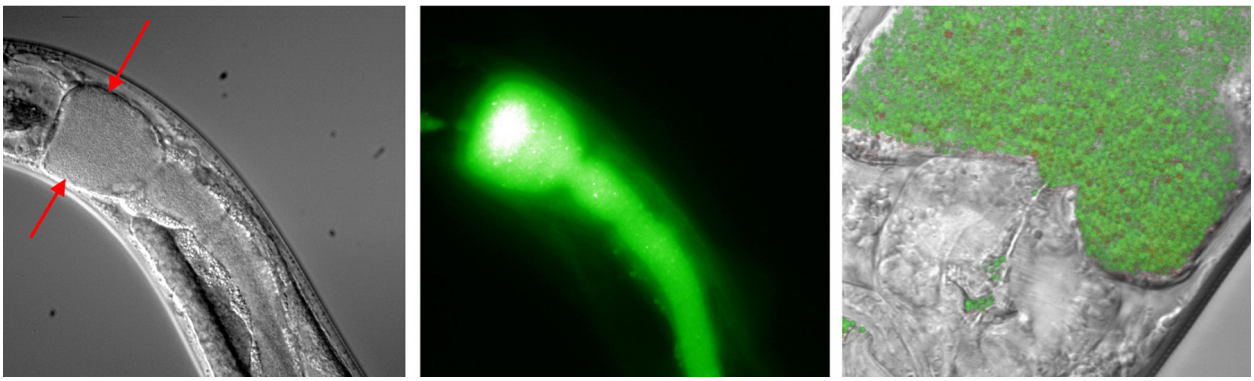

Figure 1. *C. elegans* colonized by *Staphylococcus aureus* expressing GFP. The anterior intestine is distended to the full width of the animal (arrows, left panel). Epifluorescence image of the same field (middle) shows that *S. aureus* colonizes the entire intestine. Individual bacteria can be resolved at high-magnification, as shown in merged bright-field and epifluorescence images (right). Photo: Jakob Begun and Frederick M. Ausubel.

E. coli OP50, the standard laboratory food, does not colonize wild-type *C. elegans*, but many pathogens do. Is intestinal colonization deleterious *per se*? It appears that the answer is no. *Enterococcus faecium* heavily colonizes the *C. elegans* gut, growing to a titer of more than 10^4 bacteria per nematode, without causing significant mortality (Garsin et al., 2001). This implies that when microbial killing occurs, there are specific pathogenic mechanisms at work.

2.1. *Enterococcus faecalis*

E. faecalis is a Gram-positive organism that is part of the normal gut flora of many persons, but is also a serious pathogen that can cause meningitis, endocarditis and urinary tract infections. *E. faecalis* colonizes *C. elegans* and kills fairly rapidly, with an LT_{50} of about 4 days (d) (Garsin et al., 2001). Continuous feeding on *E. faecalis* is not necessary for heavy colonization; a small inoculum proliferates in the animal. Death was more rapid with *E. faecalis* expressing cytolysin, an effector that can lyse host cells. *E. faecalis* with a deletion in *fsrB*, part of a quorum-sensing system that responds to high culture density, was attenuated for killing, but colonized as efficiently as its wild-type parent.

Mutations in two protease genes under *fsrB* regulation, *gelE* and *sprE*, produced milder defects, suggesting that both *fsrB*-regulated effectors are involved in killing (Sifri et al., 2002). The *fsrB* mutant was also attenuated in a mouse model for peritonitis, establishing a connection between the *C. elegans* model and mammalian infection.

2.2. *Escherichia coli*

E. coli is known to *C. elegans* researchers primarily as worm food. The natural environment of *E. coli* is the digestive tract of warm-blooded animals, and in nature *C. elegans* is likely to encounter it only sporadically, e.g., in animal feces. In humans, *E. coli* can be either a harmless component of the natural gut flora or a serious pathogen.

C. elegans lived longer when grown on a common soil organism, *Bacillus subtilis*, than on the laboratory food strain of *E. coli*, OP50 (Garsin et al., 2003). Furthermore, when worms were fed OP50 grown on brain-heart infusion, a rich medium, their lifespan was shorter than with OP50 grown on standard NGM medium, a relatively poor source of bacterial nutrition (Garsin et al., 2001). These results suggest that *E. coli* is mildly pathogenic to *C. elegans* and that virulence increases on rich media. OP50 is a domesticated laboratory strain that lacks the O-antigen component of its outer membrane (S.L. Ananth and C.D., unpublished data), and is therefore not typical of pathogenic *E. coli*.

In mammalian infections, enteropathogenic *E. coli* (EPEC) produces attaching and effacing (AE) lesions in which microvilli of the small intestine are degraded and the bacteria intimately attach to the host epithelium. EPEC infection causes watery diarrhea in infants, which can lead to death by dehydration. *C. elegans* exposed to EPEC on NGM medium died prematurely, with an LT_{50} of 6.5 d, compared to 9.5 d for animals fed an *E. coli* K-12 control strain (J. Mellies, personal communication). After 24 h of exposure, EPEC titer was about 10^4 per nematode, versus about 10^2 for the controls. Microcolonies of EPEC expressing GFP were found primarily in the posterior intestine, and colonization was dependent on *ler*, a regulator required for AE lesions. However, *C. elegans* colonization does not require a type-three secretion system that injects bacterial proteins into host cytoplasm and is necessary for mammalian AE lesions. Bundle-forming pili, which adhere to mammalian host cells, also were not required in the worm. Furthermore, one EPEC clinical isolate killed *C. elegans* without colonizing, suggesting a toxin-mediated lethality.

2.3. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a common soil and water bacterium that is pathogenic to both animals and plants. It rarely affects healthy persons, but can cause devastating infections in patients compromised by inborn genetic defects, acquired diseases, or injuries such as burns. *P. aeruginosa* lung infections are a major cause of death in patients with the genetic disease cystic fibrosis.

Depending on the strain and culture conditions, *P. aeruginosa* can kill *C. elegans* rapidly by toxin-mediated mechanisms (see below) or slowly in an infectious process. In the "slow killing" model, bacteria colonize the intestine, but during the first 24 h of exposure no strong disease symptoms are observed and mortality is negligible (Tan et al., 1999). With continued exposure, the worms gradually cease pharyngeal pumping, become immobile, then die. L4 larvae are more susceptible than adults, with LT_{50} of 38 h and 48 h, respectively. Direct observation using *P. aeruginosa* expressing GFP showed that bacteria began accumulating 36 h after feeding on the pathogen commenced. The lethal outcome could be prevented by removing the animals from *P. aeruginosa* after 12 h of exposure; longer periods before removal resulted in progressively poorer survival. Heat killed bacteria caused no mortality.

To identify bacterial factors involved in *C. elegans* killing, Tan et al. screened 2,400 transposon insertions in *P. aeruginosa* PA14 and recovered eight mutants (Tan et al., 1999). Four were severely attenuated, with LT_{50} greater than 90 h, and each contained an insertion in a regulatory gene. Two of these genes encode transcriptional activators, *lasR* and *ptsP*; the others, *gacA* and *lemA*, function together in a two-component signal transduction system. The requirement for LasR, the transcriptional activator of a "quorum sensing" system that responds to high bacterial cell density, is consistent with the observation that at least 12 h of colonization was required to produce a lethal infection. The factors under LasR control involved in slow killing have not been determined.

The eight mutations reducing virulence against *C. elegans* were tested in a mouse burn model under conditions that kill 100% of mice infected with wild-type *P. aeruginosa* (Tan et al., 1999). One mutant failed to kill any mice, another killed 100%, and the remaining six had intermediate levels of killing. Among the genes with

reduced mouse killing was one which had no matches to known genes in databases at the time it was isolated. This suggests that the *C. elegans* slow killing model will be useful in identifying additional *P. aeruginosa* genes that are important in human infections.

Slow killing is likely to be multifactoral. A mutation eliminating exotoxin A, an enzyme that kills eukaryotic cells, was only moderately attenuated (LT_{50} 55-70 h), suggesting that other effectors contribute to pathogenesis (Tan et al., 1999). Four effectors that in other systems attack host cytoplasmic functions (exoenzymes S, T, U and Y) are not required for slow killing (Wareham et al., 2005).

2.4. *Salmonella enterica*

Salmonella is a genus of Gram-negative enteric bacteria that cause disease in a variety of vertebrates. In humans, *S. enterica* serovar Typhi causes the serious systemic infection known as typhoid fever; however, this organism is not highly pathogenic to mice. Conversely, *S. enterica* serovar Typhimurium is lethal to mice, causing a typhoid-like disease, but in humans causes nonfatal infection limited to the gastrointestinal tract. *S. enterica* serovar Typhimurium therefore has long been studied in the mouse as a model of systemic infection.

S. enterica serovar Typhimurium colonizes the *C. elegans* intestine (Aballay et al., 2000; Labrousse et al., 2000). For adults transferred to *S. enterica* and incubated at 25° C, the TD_{50} was 5.1 d, compared to 9.9 d for control animals on *E. coli* OP50 (Aballay et al., 2000). When worms were exposed to *S. enterica* for only 3 h, then removed to OP50, there was significant early death. Invasion of host cells is an essential aspect of *Salmonella* sp. pathogenesis in mammalian systems, but *S. enterica* does not appear to invade *C. elegans* cells.

S. enterica infection is accompanied by an increase in programmed cell death (PCD) in the germline (Aballay and Ausubel, 2001). This requires *ced-3* and *ced-4*, indicating that the germline death involves the canonical PCD pathway that has been studied extensively in somatic cells (see Programmed cell death). However, there was no evidence that *S. enterica* induced somatic cell death. *ced-3* and *ced-4* mutants are more sensitive to *S. enterica* infection, suggesting that some PCD-related process is protective. Because the bacterial infection is limited to the intestine, it is not obvious how germline cell death itself could provide this protection. A pleiotropic *S. enterica* mutant, which lacks the *phoP/phoQ* virulence signaling pathway, failed to elicit germline cell death (Aballay and Ausubel, 2001), as did mutants for effector secretion (*invH*) and a transcriptional regulator (*hilD*; Tenor et al., 2004). Two other mutants, which are defective in lipopolysaccharide synthesis, exhibited reduced levels of killing and failed to trigger PCD, despite accumulating in the intestinal lumen (Aballay et al., 2003). In mammalian cell-culture systems, *S. enterica* SipB has been shown to be sufficient for apoptosis of host cells (Hersh et al., 1999); it has not been reported whether SipB is required for *C. elegans* germline cell death.

A screen of 960 transposon insertions in *S. enterica* produced 15 mutations with reduced killing of *C. elegans*, only some of which were in known virulence genes (Tenor et al., 2004). Some of the mutants were defective in assays for inducing neutrophil migration and invading epithelial cells, suggesting roles in mammalian pathogenesis. The genes represent a wide range of known or putative functions, including transcriptional activation, secretion, adhesion, outer-membrane synthesis and metabolism.

2.5. *Serratia marcescens*

Serratia marcescens is a broad-host-range, opportunistic pathogen. This Gram-negative organism colonizes the *C. elegans* intestine, with an LT_{50} of approximately 4 d and complete killing in 6-7 d (Kurz et al., 2003). Early larvae are resistant to infection, but L4 larvae and adults are highly susceptible. *S. marcescens* infection of L4s increases the ability of *E. coli* cells to pass through the pharynx unlysed, suggesting that the pathogen disrupts the pharyngeal grinder. The infection also causes some intestinal cells to lyse and autofluorescent vacuoles to appear in the lumen. Neither heat-killed *S. marcescens* nor bacterial culture supernatants caused death, suggesting that killing requires live infection and that a diffusible toxin is not sufficient.

Kurz et al. (2003) screened for attenuated bacteria, identifying 19 loci out of 2,300 transposon insertions tested. Fewer than half of the mutants were attenuated in a *Drosophila melanogaster* model for *S. marcescens*, and only three were defective in a cytotoxicity assay against human epithelial cells. When the three were tested in a mouse lung infection model, only one was defective, and the mutated gene encodes a hemolysin that has been previously characterized.

2.6. *Staphylococcus aureus*

Staphylococcus aureus causes a wide variety of diseases, including skin infections, food poisoning, toxic shock syndrome and septic shock. *S. aureus* kills all developmental stages of *C. elegans* (Sifri et al., 2003). Several strains have an LT_{50} of only about 1 d, making *S. aureus* among the swiftest of killers that infect the worm. Exposure of at least 8 h to the pathogen was required for killing.

Conflicting results were obtained by different laboratories regarding two well-studied *S. aureus* virulence regulators. Sifri et al. (2003) found strong attenuation with mutation of the *agr* locus and slight attenuation from a mutation in *sarA*. Working with a different *S. aureus* strain, Bae et al. (2004) reported that transposon insertions in either locus did not significantly reduce *C. elegans* killing. Both studies found that α-hemolysin, a secreted protein that lyses mammalian host cells, is important in *C. elegans* pathogenesis.

One group that screened for virulence mutations found 10 that were attenuated in more than one *S. aureus* strain background (Begun et al., 2005). Five of the genes identified had been found previously in mammalian models. The other five mutants were tested in a mouse model, and one was significantly attenuated. Because the transposon used for mutagenesis had a strong bias for one chromosomal region, the screen was not comprehensive.

Another group tested about 1,700 *S. aureus* transposon mutants and identified 71 genes, of which 41 could be assigned functions in metabolism, regulation, or production of extracellular factors (Bae et al., 2004). Only two extracellular factors, α-hemolysin and a secreted nuclease, were required for *C. elegans* killing. There was no attenuation in *C. elegans* killing for a strain defective in displaying about 20 surface proteins used in adhesion to mammalian hosts.

2.7. *Staphylococcus epidermidis*

S. epidermidis is a Gram-positive pathogen that frequently grows as a biofilm, a population of microbes that adheres to a surface by means of an extracellular matrix the microbes secrete. *S. epidermidis* biofilms can grow on the surfaces of medical devices such as catheters and synthetic heart valves, sometimes leading to lethal infections. *S. epidermidis* infects and kills *C. elegans*, and killing requires the *ica* genes that code for synthesis of an extracellular polysaccharide biofilm component (J. Begun, C. Sifri and F. Ausubel, personal communication).

3. Bacterial infections of the cuticle

3.1. *Microbacterium nematophilum*

Microbacterium nematophilum is a Gram-positive organism whose pathogenic effect on *C. elegans* was discovered when the bacteria contaminated a laboratory culture (Hodgkin et al., 2000). Unlike most of the organism studied with *C. elegans* models, *M. nematophilum* is not known to be a pathogen of mammals.

M. nematophilum colonizes the *C. elegans* rectum, which is cuticle-lined, and a small peri-anal region of the exterior cuticle (Figure 2). The area around the anus becomes distended and swollen, a phenotype known as Dar (deformed anal region). The infection is not lethal to wild-type *C. elegans*, and the animals can grow and reproduce on a pure culture of these bacteria, although more slowly than on *E. coli* (Hodgkin et al., 2000). However, larvae of several other *Caenorhabditis* sp., including *C. plicata* and *C. drosophilae*, are highly susceptible and die within 24 h of exposure (J. Hodgkin, personal communication). Genetic methods have not been reported for *M. nematophilum*, and the mechanisms by which it induces Dar are unknown.

Because *M. nematophilum* attaches to the cuticle, mutants with altered surface properties were examined. *srf-2*, *srf-3* and *srf-5* animals were not colonized on the peri-anal cuticle and were not Dar (Hodgkin et al., 2000). Absence of Dar is known as Bus (bacterially unswollen).

The Dar response requires components of the ERK MAP kinase cascade (Nicholas and Hodgkin, 2004), which is also involved in cell fate specification. Infection without anal swelling is observed for worms with mutations encoding ERK pathway kinases (*lin-45*, *mek-2*, *mpk-1*), a scaffolding protein (*ksr-1*), and transcriptional activators (*sur-2*, *lin-25*). When infected, these mutants become severely constipated and their fertility is sharply reduced; this suggests that Dar is a defensive response of the nematode. Mutations in *let-60*, which activates the ERK pathway in development, did not produce Bus, suggesting a different means of activating ERK in response to *M. nematophilum*.

Screens for Bus mutants have identified 20 loci, and the mutants include alleles of *sur-2* (Nicholas and Hodgkin, 2004) and *srf-3* (Hoflich et al., 2004).

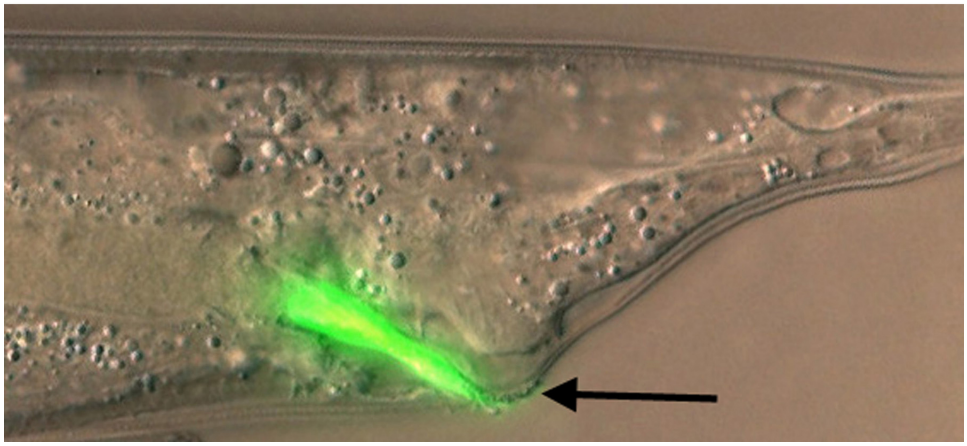

Figure 2. Rectal and peri-anal colonization by *Microbacterium nematophilum*. Bacteria are stained with Syto13 dye. Arrow, *M. nematophilum* adhering to cuticle immediately posterior to anus. Photo: Hannah Nicholas, Delia O'Rourke and Jonathan Hodgkin.

3.2. *Yersinia* sp.

Y. pestis, the etiological agent of bubonic plague, and *Y. pseudotuberculosis*, a closely related pathogen that causes less serious infections, produce biofilms that attach to the cuticle of *C. elegans*, particularly on the head (Darby et al., 2002; Joshua et al., 2003). The biofilm matrix, which is polysaccharide rich (Tan and Darby, 2004), covers the mouth and blocks feeding (Figure 3), thereby inhibiting growth. This is similar to the manner in which *Y. pestis* colonizes and blocks feeding of its vector, the flea, to promote its transmission to mammals. Nematode biofilms and flea blockage both require *Y. pestis hms* genes that are involved in polysaccharide synthesis (Darby et al., 2002).

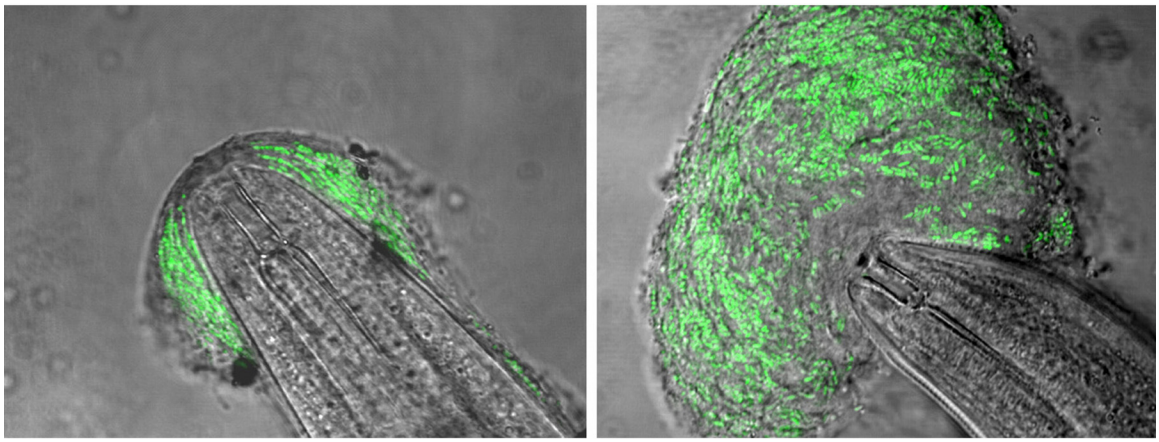

Figure 3. Biofilms on head formed by *Yersinia pseudotuberculosis* after one hour (left) or overnight (right) incubation on bacterial lawns. Nematodes were washed in M9 buffer before mounting for confocal epifluorescence microscopy. *Yersinia* cells express GFP; non-fluorescent material adhering to the head is bacterially-synthesized extracellular polysaccharide. Note that polysaccharide covers the mouth at one hour. Photo: Creg Darby.

C. elegans mutations in *srf-2*, *srf-3* and *srf-5*, which change the surface characteristics of the worm, prevent biofilm attachment (Joshua et al., 2003), a phenotype known as Bah (biofilm absent on head). In a *de novo* screen for Bah mutants, an allele of *srf-3* was obtained (Hoflich et al., 2004), as were alleles of *srf-2*, three *bus* genes, and three apparently novel genes, *bah-1*, *bah-2* and *bah-3* (author's unpublished results).

4. Bacteria with multiple or undetermined killing modes

This section describes pathogen models in which the mode of killing, i.e. by infection or intoxication or both, has not been definitively determined, or in which both modes appear to be functional.

4.1. *Burkholderia cenocepacia*

Burkholderia cepacia complex (BCC) is a group of closely related bacteria, including several that can infect the lungs of cystic fibrosis patients. BCC is taxonomically complex, with many ambiguities and changes in nomenclature (Coenye et al., 2001). In the two studies described in this subsection, different species names were used for the same strain; it is referred to here by the most recently used name, *B. cenocepacia*.

B. cenocepacia kills *C. elegans* slowly when the bacteria and worms are grown on standard media. GFP labeling of the bacteria showed extensive colonization of the intestine in this slow-killing assay, and death occurred in 1-4 d (Kothe et al., 2003). On high-osmolarity medium, *B. cenocepacia* killed completely within 24 h, and there was no detectable colonization in this fast killing. Conditioned media containing *B. cenocepacia* diffusible products caused some killing under high-osmolarity conditions; curiously, some worms were immobile and appeared dead at 4 h, but had recovered by 24 h. The toxin has not been identified, but cyanide, one of several *P. aeruginosa* toxins, was not detected in *B. cenocepacia* cultures.

Both modes of killing require the *B. cenocepacia cep* quorum-sensing regulatory system (Kothe et al., 2003). Mutation of a gene under quorum-sensing control, *aidA*, rendered the bacteria incapable of colonization and slow killing, but did not significantly affect fast killing.

4.2. *Burkholderia pseudomallei*

Melioidosis, an infection that can be fatal, is caused by *B. pseudomallei*, a soil organism endemic in Southeast Asia and northern Australia. *B. pseudomallei* exposure causes *C. elegans* pharyngeal pumping to slow or cease, followed by a general decline in locomotion and eventual death (O'Quinn et al., 2001). The most virulent strain assayed had an LT_{50} of about 10 h. No assay for nematode colonization was reported. Curiously, *B. pseudomallei* cells inactivated by ultraviolet irradiation did not kill nematodes, but bacteria treated with high doses of gamma rays retained killing. The speed of the killing and the lethality of gamma-irradiated cultures suggests the action of a cell-free toxin. However, culture supernatants did not kill, nor did conditioned solid media, which contained diffusible products from bacterial growth but not live bacteria.

Another study, though, found killing from *B. pseudomallei*-conditioned media, although at reduced levels from that seen with whole cells (Gan et al., 2002). The decrease could mean that killing is multifactoral and includes processes that require live bacteria. Alternatively, toxin may be sufficient for killing, but less is available in conditioned media than when *C. elegans* is incubated continuously with live bacteria. As with the previous study, colonization of nematodes was not described. In this study, heat-killed bacteria did not kill nematodes, suggesting that the toxin is a protein or other heat labile substance.

Gan et al. screened 3,400 transposon insertion mutants of *B. pseudomallei* and obtained five with reduced killing of *C. elegans*. The five mutants were all attenuated to various degrees when inoculated intranasally into mice (Gan et al., 2002).

4.3. Plant, fish and insect pathogens

Couillault and Ewbank (2002) examined a variety of bacteria, including plant, fish and insect pathogens, assaying survival over time. Diminished lifespan resulted from exposure to a wide range, including plant pathogens *Agrobacterium tumefaciens*, *Erwinia carotovora* and *Erwinia chrysanthemi*; a marine bacterium that spoils stored fish, *Shewanella frigidimarinia*; and a freshwater fish pathogen that sometimes infects humans, *Aeromonas hydrophila*.

Photorhabdus luminescens and *Xenorhabdus nematophila* are related bacteria that live symbiotically in the intestines of *Heterorhabditis* sp. and *Steinernema* sp. nematodes, respectively. These entomopathogenic nematodes are parasites of insects, and it is their symbiotic bacteria that kill the insect host. Both *Photorhabdus luminescens* and *Xenorhabdus nematophila* were pathogenic to *C. elegans* (Couillault and Ewbank, 2002). Heat-killed *X.*

nematophila retained significant killing ability, suggesting production of a heat-stable diffusible toxin. *P. luminescens* killing, while particularly rapid, was lost when bacteria were heat killed. *X. nematophila* colonized the head of *C. elegans* in a biofilm-like structure that resembles the one formed by *Yersinia* sp.

5. Fungal infections

5.1. *Cryptococcus neoformans*

C. neoformans is a yeast-form fungus that can infect immunocompromised persons. When fed to *C. elegans*, the animals are colonized with a distended intestine and die with an LT_{50} of 5 - 7 d (Mylonakis et al., 2002). Two nonpathogenic cryptococci, *C. laurentii* and *C. keutzingii*, did not diminish the worm lifespan; in fact, animals lived longer on *C. laurentii* than on *E. coli* OP50. Several mutants known to be attenuated in mouse infections were also less pathogenic to *C. elegans*, and a mutation conferring hypervirulence in the mouse model killed *C. elegans* more rapidly than wild-type yeasts. These results suggest overlap between disease mechanisms in mammals and *C. elegans*.

Gut colonization is not essential for *C. neoformans* killing. Two *C. neoformans* strains that lack a polysaccharide capsule failed to accumulate in the intestine, but nevertheless killed *C. elegans* (Mylonakis et al., 2002). Heat-killed capsule-positive yeasts also killed, although more slowly than live fungi, but heat-killed capsule-negative strains were highly attenuated. These results suggest that a component of the *C. neoformans* capsule is toxic.

A screen of 350 *C. neoformans* insertion mutants yielded seven with attenuated virulence (Mylonakis et al., 2004). A mutation in *kin1*, encoding a serine/threonine kinase, did not affect *C. elegans* colonization but produced substantially less killing. The mutation did not alter previously studied *C. neoformans* virulence phenotypes, including capsule formation. When tested in mice by intranasal or intravenous injection, the *kin1* mutant had a diminished ability to colonize several tissues, and killing was substantially slowed.

5.2. *Drechmeria coniospora*

D. coniospora is an endoparasitic fungus that infects and consumes a narrow range of nematodes (Jannson, 1994). Conidia, which are spores, attach to the *C. elegans* head at the labial and amphid sensilla, and also attach to the vulva. The conidia produce threadlike hyphae that grow throughout the animal and consume it. When infection begins at the head, it is not apparent whether the fungus penetrates the cuticle or simply bypasses it by way of the sensilla openings. Protease treatment of worms before exposure decreased conidia attachment, suggesting involvement of nematode surface or secreted proteins. *D. coniospora* were able to bind *che-12* and *che-14* mutants, which have defective amphids; conidia also bound *mec-1* animals, which have displaced amphid neurons, and *vab-1* mutants, which have malformed heads.

Couillault et al. (2004) reported specific induction of antimicrobial peptide genes in C. elegans after infection by *D. coniospora*. One of these, *nlp-31*, encodes a peptide with demonstrable antifungal activity against *D. coniospora*.

6. Toxin-mediated killing

6.1. *Bacillus thuringiensis*

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium used in insect control because it makes toxins that do not affect vertebrates. *Bt* expressing a single pore-forming toxin, Cry5B, causes intestinal damage to *C. elegans* in a manner that resembles the pathology observed in toxin-treated insects (Marroquin et al., 2000). The animals turn pale, the intestine becomes pitted, vacuolated and constricted, and brood size is reduced. There are no obvious effects on the neuromuscular systems of the worm. Expression of Cry5B in *E. coli* recapitulated these results, indicating that the damage is toxin-specific, not a general response to *Bt*.

Screening for *C. elegans bre* (*Bacillus* toxin resistant) mutants identified five genes (Marroquin et al., 2000). *bre-2*, *bre-3*, *bre-4*, *bre-5* encode glycosyltransferases that appear to function in a single pathway and are required for intestinal cells to take up Cry5B (Griffitts et al., 2003; Griffitts et al., 2001). Binding of Cry5B to glycolipids that are absent in *bre-3*, *bre-4* and *bre-5* mutants has been demonstrated (Griffitts et al., 2005).

6.2. *Pseudomonas aeruginosa*

P. aeruginosa kills *C. elegans* in two distinct toxin-mediated modes. In the first, known as "fast killing," bacterial strain PA14 is used, and a high osmolarity medium is required (Mahajan-Miklos et al., 1999). At least 50%, and in some assays about 90%, of L4 animals died within 4 h of exposure. Killing is mediated by diffusible toxins and does not require contact with live bacteria.

A screen of 3,300 *P. aeruginosa* transposon insertions produced seven mutants with reduced killing (Mahajan-Miklos et al., 1999). Four mutants had reduced levels of pyocyanin, a pigmented secondary metabolite belonging to the phenazine family of tricyclic compounds. However, three killing-defective mutants made normal amounts of pyocyanin. Together, these results suggest that toxicity is multifactoral, involving pyocyanin (or other phenazines) and unidentified other molecules.

Pyocyanin is a redox-active compound toxic to eukaryotic cells, suggesting that fast killing is due at least in part to oxidative stress. Analysis of *C. elegans* mutants lent support to this hypothesis. *age-1* mutants, which are resistant to other forms of oxidative stress, were significantly resistant to fast killing (Mahajan-Miklos et al., 1999). Conversely, *mev-1* and *rad-8* mutants, which are hypersensitive to oxidative stress, were more sensitive in fast-killing assays.

The *C. elegans* genes *pgp-1* and *pgp-3* encode P-glycoprotein membrane transporters. A *pgp-1*;*pgp-3* double mutant was sensitive to fast killing without the need for high osmolarity (Mahajan-Miklos et al., 1999). Furthermore, the double mutant was hypersensitive to *P. aeruginosa* mutants that retained phenazine production, but was as resistant as wild type to phenazine-deficient mutants.

The second toxin-mediated killing model uses a different *P. aeruginosa* strain, PAO1. When PAO1 is grown to high density on an especially rich medium, animals placed on the lawn cease pharyngeal pumping almost instantaneously, become progressively paralyzed, and die within two hours (Darby et al., 1999). These effects are mediated by bacterially produced cyanide, a toxin that blocks respiratory electron transport (Gallagher and Manoil, 2001).

In a screen for *C. elegans* resistant to cyanide-mediated killing, two alleles of *egl-9* were recovered (Darby et al., 1999). Although *egl-9* worms were completely resistant to killing by live bacteria, dose-response experiments *in vitro* showed that the mutants can be killed by sufficiently high cyanide concentrations (Gallagher and Manoil, 2001). *egl-9* encodes a dioxygenase that functions as a negative regulator of a hypoxia-response pathway (Epstein et al., 2001). This suggests that in *egl-9* mutants, cyanide resistance is mediated by constitutive activation of the pathway. The role of cyanide in human *P. aeruginosa* infections, if any, is not known.

6.3. *Streptococcus* sp.

S. pneumoniae is a Gram-positive organism whose only natural reservoir is humans. Many persons are colonized asymptomatically, but the bacterium can also cause pneumonia and meningitis. *S. pneumoniae* kills *C. elegans* rapidly (Garsin et al., 2001), but does not colonize the worm, and bacterially synthesized hydrogen peroxide is sufficient for killing (Jansen et al., 2002). Other Streptococci that kill *C. elegans* with hydrogen peroxide include *S. pyogenes* (also known as group A Streptococcus) and *S. agalactiae* (group B; Bolm et al., 2004).

6.4. Expression of bacterial effectors in transgenic *C. elegans*

Pathogenic bacteria produce a wide variety of effectors that act on the host, and often the targets have conserved eukaryotic functions. Organisms that produce these effectors nevertheless may not be pathogenic to *C. elegans* because disease requires a variety of other functions, e.g. sensing of the host environment, gene induction, binding to host receptors, and evasion of host defenses. A strategy to overcome these limitations is to express bacterial effectors directly in transgenic *C. elegans*. This approach was demonstrated using pertussis toxin (PTX), a well-characterized effector known to enzymatically inactivate a class of heterotrimeric G proteins. Expression of PTX produced a phenotype indistinguishable from that of loss-of-function mutations in *goa-1*, which encodes the only PTX target in *C. elegans* (Darby and Falkow, 2001). PTX expression also suppressed a gain-of-function *goa-1* mutation.

A mutation in the *Salmonella enterica* gene *sptP* was recovered in a screen for mutants with defects in killing *C. elegans* (Tenor et al., 2004). In mammals, SptP is injected into host cells, where it reverses cytoskeletal

disruption produced earlier by another *S. enterica* effector (Fu and Galan, 1999). Transgenic animals were generated with *sptP* under the promoter of the intestinal gene *mtl-2*. The animals were then exposed to either *S. enterica* or *Pseudomonas aeruginosa*infection to determine whether ectopic expression of SptP enhances pathogenicity. In both cases a slight increase was observed in relative mortality (ratio of LT_{50} on pathogen to LT_{50} on *E. coli*) compared to untransformed controls.

7. Conclusions

The first paper describing *C. elegans* as a model host for a microbial pathogen was published in 1999. The field has grown rapidly since, and now investigates a diverse range of human pathogens that includes numerous bacteria and one fungus. Several microbes that are pathogenic to *C. elegans* but not to humans also are being examined.

As a rapidly growing, inexpensive host, *C. elegans* permits large-scale screens of pathogen genomes, and thousands of bacterial mutants have been tested in screens of *P. aeruginosa* (Gallagher and Manoil, 2001; Mahajan-Miklos et al., 1999; Tan et al., 1999), *B. pseudomallei* (Gan et al., 2002), *S. marcescens* (Kurz et al., 2003) and *S. aureus* (Bae et al., 2004). Some of the mutations recovered have been in virulence genes already known from mammalian studies, which validates the screening strategy (Bae et al., 2004; Kurz et al., 2003; Sifri et al., 2003; Tan et al., 1999; Tenor et al., 2004).

The ultimate goal is to discover new virulence factors for mammalian pathogenesis, and here results have been mixed. Some of the *P. aeruginosa* (Tan et al., 1999) and *B. pseudomallei* (Gan et al., 2002) mutants defective against *C. elegans* were also attenuated in a mouse models. On the other hand, no new mammalian virulence mutants were found in a screen of *S. marcescens* (Kurz et al., 2003), and investigators who screened *S. aureus* concluded that for this pathogen, there is limited overlap between *C. elegans* and mammalian pathogenesis (Bae et al., 2004).

With infection models, some screens for bacterial mutant appear biased toward identifying regulators. For instance, five of eight *P. aeruginosa* mutations in the slow killing model were in genes known or inferred to encode regulators (Tan et al., 1999). This suggests that slow killing is multifactoral, and that regulators are identified because mutations disrupt expression of several downstream factors. Screens in a multifactoral system may not have sufficient resolution to identify individual effectors. However, this drawback may be pathogen-specific, because a screen of *S. enterica* produced a mutation in *sptP*, which is known from mammalian studies to encode an effector that acts in the cytoplasm of host cells (Tenor et al., 2004).

In toxin-mediated killing models, screens of *P. aeruginosa* have proven useful in characterizing the toxins. A mutation in the bacterial *hcnC* gene, encoding hydrogen cyanide synthase, prompted experiments showing that cyanide is necessary and sufficient for lethal paralysis (Gallagher and Manoil, 2001). In the fast-killing model, mutations in *phzB* prompted further investigation of phenazines as candidate toxins (Mahajan-Miklos et al., 1999).

On the *C. elegans* side of the interaction, genetics can provide information about the host pathways that pathogens exploit. A notable success is the work of Aroian and colleagues on *C. elegans* mutants resistant to a *Bt* toxin. The screen identified a set of glycosyltransferases that function together in *C. elegans* (Griffitts et al., 2003; Griffitts et al., 2001; Marroquin et al., 2000), and this in turn facilitated identification of a glycolipid toxin receptor (Griffitts et al., 2005). However, screens to find resistant organisms do not always identify the pathways that interact directly with the pathogen. A screen for worms resistant to *P. aeruginosa* paralysis produced alleles of *egl-9* (Darby et al., 1999); later characterization suggested that the resistance phenotype results from activation of a bypass pathway (Epstein et al., 2001).

Because *C. elegans* models for microbial pathogenesis are still new, the extent to which they are relevant to human disease remains to be determined. Obviously the overlap will not be complete, but this is true for any animal model. It is encouraging that some virulence gene discovered using *C. elegans* have been found to be required for full pathogenesis in mouse infections. These genes have not yet been characterized thoroughly, however. The task ahead is to take novel results from *C. elegans* systems and determine their importance in human infectious disease.

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