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Worm-stars and half-worms

Novel dangers and novel defense

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In a recent paper, we reported the isolation and surprising effects of two new bacterial pathogens for *Caenorhabditis* and related nematodes. These two pathogens belong to the genus *Leucobacter* and were discovered co-infecting a wild isolate of *Caenorhabditis* that had been collected in Cape Verde. The interactions of these bacteria with *C. elegans* revealed both unusual mechanisms of pathogenic attack, and an unexpected defense mechanism on the part of the worm. One pathogen, known as Verde1, is able to trap swimming nematodes by sticking their tails together, resulting in the formation of "worm-star" aggregates, within which worms are killed and degraded. Trapped larval worms, but not adults, can sometimes escape by undergoing whole-body autotomy into half-worms. The other pathogen, Verde2, kills worms by a different mechanism associated with rectal infection. Many *C. elegans* mutants with alterations in surface glycosylation are resistant to Verde2 infection, but hypersensitive to Verde1, being rapidly killed without worm-star formation. Conversely, surface infection of wild-type worms with Verde1 is mildly protective against Verde2. Thus, there are trade-offs in susceptibility to the two bacteria. The Leucobacter pathogens reveal novel nematode biology and provide powerful tools for exploring nematode surface properties and bacterial susceptibility.

Keywords: bacterial infection, coryneform, Leucobacter, nematode trapping, autotomy, glycosylation, trade-off

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Introduction

Most of our knowledge of *C. elegans* comes from studies performed under laboratory conditions, usually by growing worms on the surface of agar plates in benign conditions. However, an increasing amount of research has explored the natural environment and natural enemies of the worm, with particular reference to microbial pathogens. The Leucobacter pathogens were discovered as a part of a long-term program aimed at the better understanding of the ecology, distribution, and diversity of Caenorhabditis species.[1] Isolation of wild *C. elegans* and related nematodes has been greatly aided by the realization that rotting fruit provides excellent material for isolating microbivorous nematodes. Sampling of such material has yielded both the first nematode microsporidians[2] and the first nematode viruses.[3] On a collecting trip to Cape Verde, Marie-Anne Félix examined decaying banana trunks and other rotting vegetable matter for nematode populations, and obtained several samples both of *Caenorhabditis briggsae* and another hermaphroditic species,[4] *Caenorhabditis n. sp. 11*. Cape Verde is probably too warm to support *C. elegans* populations, so its absence was not surprising. One of the *Caenorhabditis n. sp. 11* samples was noticeably infected with a dense coating of bacteria on the surface of the worms, and the worms also exhibited a swollen

tail or Dar (Deformed Anal Region) phenotype. This phenotype was reminiscent of the defensive Dar response that occurs as a consequence of the infection of *C. elegans* by a coryneform pathogen known as *Microbacterium nematophilum*.[5,6] *M. nematophilum* has been isolated as a laboratory contaminant of *C. elegans* on several independent occasions in different research institutions,[7] because the tail swelling is very noticeable. However, *M. nematophilum* has never been isolated from the wild and its real ecological niche is unknown. Indeed, the bacteria responsible for the tail swelling both in the Cape Verde isolate and in a Dar isolate of *C. elegans* from Kakegawa, Japan, proved to belong to a different coryneform genus, *Leucobacter*. Nevertheless, the isolation of these diseased wild isolates demonstrates that the Dar response occurs naturally and can be elicited by a variety of pathogens.

Double Infection

Surprisingly, the bacteria causing the tail swelling in the Cape Verde isolate were distinct from the bacteria densely coating the worms, although both belong to genus *Leucobacter*. Our further characterization of these two strains (in preparation) indicates that they represent distinct species: the bacteria causing the dense surface coating define a subspecies of a previously described bacterium,[8] *L. celer*, while the Dar-inducing bacteria define a new species. For convenience we refer to them as Verde1 and Verde2, respectively.

Wild-type *C. elegans* growing on a bacterial lawn containing Verde1 acquired a coating of adherent bacteria, like the original strain of *Caenorhabditis n. sp. 11*. This resulted in slower growth and impaired movement, but the animals were able to grow and reproduce indefinitely in the presence of the surface infection, and never exhibited swollen tails. However, Verde1 did elicit a defense response in the epidermis, which was revealed by the induction of the antimicrobial peptide NLP-29. This peptide is expressed at higher levels in response to infection by the fungus *Drechmeria coniospora*, as well as surface damage or hyperosmotic conditions.[9] Substantial upregulation of the *nlp-29* gene in the epidermis of *C. elegans* was seen within a few hours of exposure to bacterial lawns containing Verde1.

A Novel Worm-Trapping Mechanism

An entirely different response was seen when nematodes were exposed to Verde1 bacteria while swimming in liquid. Under these conditions, Verde1 caused worms to stick to each other by their tail spikes, leading to the formation of aggregates containing dozens or hundreds of worms, all radiating outward from their entangled tail regions. We call these aggregates "worm-stars." Star formation was rapid (beginning within a few minutes of exposure to bacteria), efficient (requiring only a few hundred bacteria per worm), largely irreversible, and ultimately lethal to the worms. If the worm-stars were picked from liquid onto an agar surface, most worms remained trapped in the stars and died within 24–48 h. Dying worms exhibited loss of cuticular integrity in posterior regions, as revealed by dye penetration. The continuing attempts of worms to escape from the worm-stars led to increased entanglement and probably contributed to damage and bacterial invasion in their tail regions. Verde1 bacterial counts within worm-stars were found to increase over time, indicating that the worms were being converted into bacterial biomass. The formation of worm-stars therefore represents a new pathogenesis mechanism: Verde1 bacteria are able to immobilize groups of worms, kill them, and use them as a source of nutrients.

This trap-and-degrade strategy is reminiscent of the trapping mechanisms used by various soil fungi, which form specialized hyphae that can immobilize nematodes by adhesion or noose formation, followed by hyphal invasion, and death of the trapped worms.[10-12] The Verde1 trapping strategy represents an ingenious variation on this mechanism, and is also an impressive example of asymmetric warfare, in that a small number of bacteria are able to immobilize and then kill the vastly larger host worms, simply by tying their tails together.

Escape by Autotomy

Worms that managed to escape from stars within the first hour after star formation survived with little damage. A remarkable additional escape mechanism was observed when stars were allowed to form from populations of late larval (L4) worms and left on an agar surface. Over the next 24 h, 5–10% of the worms in the L4 worm-stars underwent a form of autotomy, splitting their bodies into a trapped posterior portion and an anterior "half-worm" that was able to crawl away. Scission was seen to occur at any point from just behind the pharynx (30% of worm length) to the pre-anal region (90% of worm length). The half-worms could remain viable for several days, as indicated by continued movement and pharyngeal pumping, even though their intestinal tracts were truncated. As an escape strategy, whole-body autotomy works for *C. elegans* because it is self-fertile, and the gonads in most half-worms had developed far enough to allow the generation of a few self-progeny.

Autotomy has not previously been reported for any nematode species, although comparable escape strategies occur in other animal groups,[13] for example, in the well-known tail-shedding exhibited by lizards in response to predators. The basic nematode body plan, which depends on a hydrostatic skeleton and a high internal pressure, might seem to preclude autotomy, but evidently it occurs. Substantial tissue remodeling must be required in order to complete the formation of a viable half-worm: ectopic fusion of intestinal cells is needed to form a blind gut ending, as well as fusion of epidermal cells and cuticle to create an intact but truncated posterior. It is possible that a special developmental program needs to be invoked in order to achieve autotomy, but how this might be triggered is not obvious. Half-worms were never seen to arise from adult worm-stars, only

from stars formed from L4 larvae, which leads us to speculate that the necessary tissue remodeling occurs during the last larval molt, when other kinds of reorganization such as final vulval morphogenesis are normally occurring. It remains to be determined whether other hermaphroditic nematodes, or even male/female nematode species, can undergo similar autotomy, if they become trapped by Verde1 or other hazards.

Host Range

As is usual with any newly discovered pathogen, the preferred host for Verde1 is unknown. We explored its host-range by exposing various other rhabditid nematodes to Verde1 in the same liquid swimming conditions that resulted in star formation for *C. elegans.* All tested species of Caenorhabditis formed worm-stars, though with varying efficiency; so we also did some representatives of related genera such as *Oscheius*. Nematodes that are unable to swim vigorously in liquid did not form worm-stars, perhaps because they rarely make contact with each other. Some species, such as the extensively studied *Pristionchus pacificus*,[14] formed loosely adherent tangles or stars, which were unstable over time and did not result in worm death. However, *P. pacificus* could be permanently trapped and killed by incorporation into mixed-species stars with *C. elegans* (**Fig. 1**). The ability of Verde1 to create mixed-species stars therefore effectively expands its host-range. The formation of mixed-species stars also suggests that the surface properties that allow adhesion by Verde1 are conserved between different rhabditids.

Locomotion and Pathogenesis

Verde1 was tolerated by *C. elegans* when the worms are growing on the surface of an agar plate, and worm-stars were never seen to form under these conditions, which are physically different from growth in bulk liquid. *C. elegans* grows well in liquid suspension, as long as bacterial food is present, but the behavior of worms under these conditions has been relatively little investigated, partly because it is so much easier to examine worms when they are crawling on a two-dimensional surface, rather than swimming in liquid. In fact, Sydney Brenner's original choice of nematodes as ideal lab organisms for investigating animal development and neurobiology was influenced by his avoidance of simple animals that fly (like *Drosophila*) or swim (like rotifers or water-fleas).[15] It is not known how frequently *C. elegans* goes swimming in its natural habitats, but liquid swimming conditions must often be encountered, for example, after rainfall or flooding. We simulated such a scenario by allowing fragments of banana stem or mushroom to decay in the presence of water and Verde1, and found that worm-stars could form readily in the resulting suspensions.

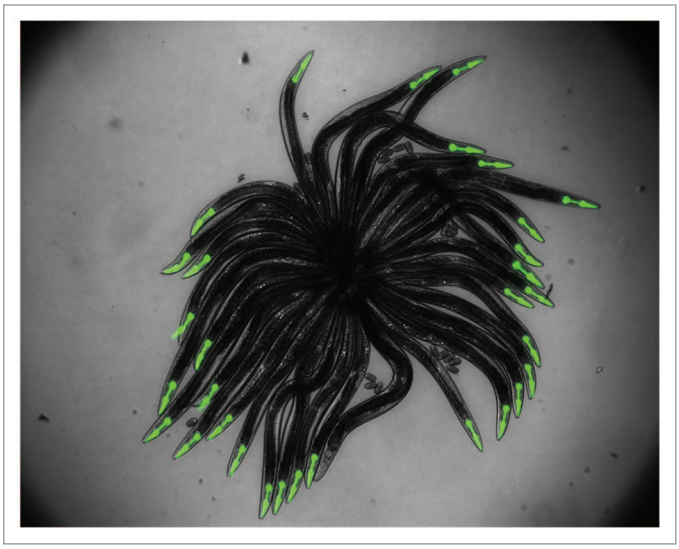

Figure 1. This shows a worm-star formed from 31 *C. elegans* that had been tagged with a pharyngeal GFP transgene, plus one *P. pacificus*.

Comparable star-like aggregates of nematodes have occasionally been reported by nematologists, both for the human parasite *Wuchereria bancrofti*[16] (described as "Medusa heads") and for free-living soil nematodes[17,18] (described as "rosettes"). No convincing explanation has previously been given for these assemblages, and it seems possible that at least some of them may have resulted from an undetected bacterial attack. Nematode populations are not always examined under free-swimming conditions, so it may be that worm-star formation occurs in nature more frequently than the rarity of these publications suggest.

One lesson that arises from our observation of worm-stars is that more attention could be paid to how *C. elegans* develops and behaves under liquid growth conditions, and in three-dimensional soil culture. Another lesson is that host movement can have a strong influence on the response to pathogens. For nematodes, host mobility should therefore be regarded as another important variable like temperature or nutrient conditions, which can determine whether an infection is benign or virulent in its consequences.

Complementary Pathogenesis

The second Leucobacter strain, Verde2, was also found to have lethal effects on *C. elegans* and related species, but of a different kind. When worms were grown on bacterial lawns containing Verde2 and incubated at 25 °C, most died within 24–48 h. Dying adult worms were seen to be distended with large internal vacuoles filling the pseudocoelom. How the Verde2 bacteria caused the formation of these vacuoles is not known, and comparable vacuolation has not been seen in worms exposed to *M. nematophilum,* even in immunocompromised hosts.[6] At

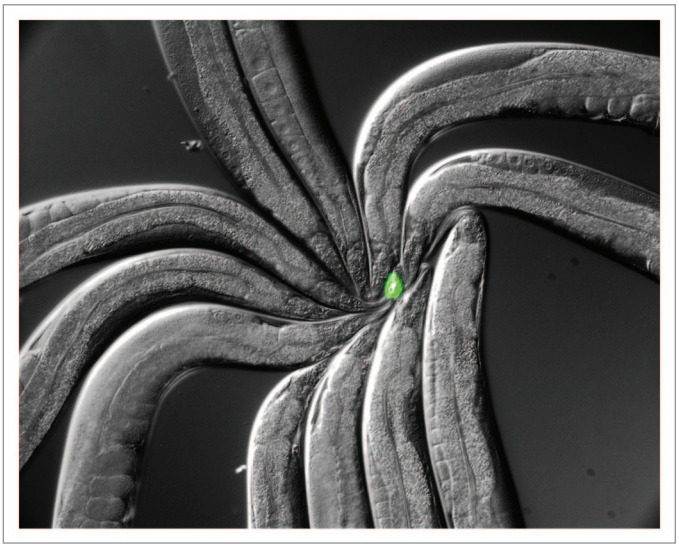

Figure 2. This shows a worm-star formed from Verde1 bacteria that had been fluorescently labeled with the dye SYTO13; green fluorescence from adherent bacteria is only visible in the region of the entangled tail-spikes.

lower temperatures, exposure to Verde2 elicited a strong Dar (swollen tail) phenotype, but did not always cause lethality. In both circumstances, Verde2 bacteria were seen to adhere preferentially to the rectal/anal region, but not elsewhere, as with *M. nematophilum*. Verde2 is therefore similar to *M. nematophilum* in its pathogenicity, but more virulent. Numerous *C. elegans* mutants have been isolated on the basis of resistance to *M. nematophilum* ("Bus," or bacterially un-swollen mutants), many of which have altered cuticle properties and abnormal glycosylation.[19-22] Most of these Bus mutants were found to be fully resistant to Verde2, even at 25 °C, extending the similarity between the Verde2 and *M. nematophilum* infections. Presumably, the cuticle alterations prevented initial adhesion by either Verde2 or *M. nematophilum* bacteria, thereby conferring resistance.

Exposure of these Verde2-resistant mutants to Verde1 revealed a conspicuous trade-off in susceptibility. As described above, Verde1 is not lethal to wild-type worms when they are growing on a surface. However, almost all the Verde2-resistant Bus mutants were rapidly killed by Verde1 when growing on a surface, with an apparent loss of integrity over the whole surface of the worm. After 24 h exposure, most worms were dead, with a collapsed appearance. Verde1 can therefore kill *C. elegans* both by worm-star formation and by direct surface attack if the cuticle is abnormal. Possibly, some other nematode species naturally have surface properties that confer the same kind of resistance to Verde2 and hypersensitivity to Verde1. A further interaction between the two pathogens was seen in that pre-exposure of wild-type *C. elegans* to Verde1 was partly protective against the lethal effects of subsequent exposure to Verde2. This effect may explain why the original *Caenorhabditis n. sp. 11* isolate was able to tolerate a double infection of both Verde1 and Verde2, although Verde2 alone is lethal to it.

Tail-Spike Function

A notable feature of the worm-star mode of attack is that it depends on rapid attachment of the Verde1 bacteria to the tail-spikes of worms, followed by the entanglement and knotting together of their tails. Fluorescently labeled Verde1 bacteria in worm-stars could be seen to adhere preferentially to tail-spikes (**Fig. 2**), even though more prolonged exposure resulted in general surface attachment. Possessing a tail-spike is therefore disadvantageous, when worms are swimming in the presence of Verde1. It is not obvious what compensatory advantage might come to worms from the possession of a tail-spike, and there is considerable variation between nematode species in this part of the anatomy. One possibility that might be considered is that tail-spikes could assist in nictation, the distinctive pattern of nematode behavior in which worms stand on their tails and wave their heads in the air, thereby increasing their chances of dispersal.[23,24] The correlation between nictating species and those with a tail-spike is not perfect, but this does not disprove the hypothesis, since some nictating species may have lost their tail spikes as a result of selective pressure by Verde1 or equivalent pathogens. The preferential attachment of Verde1 to tail-spikes also suggests that this part of the nematode surface must have different biochemical characteristics from the rest of the cuticle.

A Possible Rodent Parallel

The formation of worm-stars is an unexpected phenomenon in studies of *C. elegans*, but we can point to an intriguing precedent in mammalian cryptozoology. There is a strange tradition in European folklore, dating back many centuries, of finding "Rattenkönig" or "rat kings," which are collections of rat corpses tied together by their tails. Examples can still be found in German and Estonian museums.[25] It has usually been assumed that the rat kings have formed only by accident or human intervention, but our discovery of worm-star formation as a pathogenic mechanism leads us to wonder whether something similar might sometimes happen to rats.

Concluding Remarks

Irrespective of such speculation, the biological effects of Verde1 and Verde2, demonstrate how much remains to be discovered about the interactions of *C. elegans* with pathogens, as well as the existence and activation of latent defenses such as autotomy. Moreover, the 100% lethality exerted by each of these pathogens under appropriate conditions (Verde2 on wild-type worms at 25 °C, and Verde1 on Bus mutant worms)

makes them powerful experimental tools, allowing efficient selection of resistance mutants and enabling new modifier screens. Lastly, identification of the bacterial virulence factors underlying these unexpected modes of pathogenesis may lead to the development of novel biological control agents that would be effective against parasitic nematodes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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