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Nematode-Bacterium Symbioses—Cooperation and Conflict Revealed in the "Omics" Age

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Abstract. Nematodes are ubiquitous organisms that have a significant global impact on ecosystems, economies, agriculture, and human health. The applied importance of nematodes and the experimental tractability of many species have promoted their use as models in various research areas, including developmental biology, evolutionary biology, ecology, and animal-bacterium interactions. Nematodes are particularly well suited for the investigation of host associations with bacteria because all nematodes have interacted with bacteria during their evolutionary history and engage in a variety of association types. Interactions between nematodes and bacteria can be positive (mutualistic) or negative (pathogenic/parasitic) and may be transient or stably maintained (symbiotic). Furthermore, since many mechanistic aspects of nematode-bacterium interactions are conserved, their study can provide broader insights into other types of associations, including those relevant to human diseases. Recently, genome-scale studies have been applied to diverse nematode-bacterial interactions and have helped reveal mechanisms of communication and exchange between the associated partners. In addition to providing specific information about the system under investigation, these studies also have helped inform our understanding of genome evolution, mutualism, and innate immunity. In this review we discuss the importance and diversity of nematodes, "omics"' studies in nematode-bacterial systems, and the wider implications of the findings.

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Introduction

Nematodes are among the most abundant and diverse organisms on the planet, comprising as many as 1 million species in 12 clades and numerically accounting for as much as 80% of all animals (Lambshead and Boucher, 2003; Holterman *et al.*, 2006). They have been found in all trophic levels within a wide range of environments, including 1 km beneath the Earth's surface (Ettema, 1998; De Ley, 2006; Borgonie *et al.*, 2011). As a consequence, they have a global impact on ecosystems, economies, and human health. Many nematodes are viewed as targets for eradication because of their devastating effects on agriculture and health (Perry and Randolph, 1999; Bird and Kaloshian, 2003; Chitwood, 2003). In particular, parasitic nematodes known as helminths cause a wide range of diseases in humans and animals, and it is estimated that greater than 10% of the world's population is at risk for helminthic infection every year (Crompton, 1999). Two severe forms of helminth-caused disease, lymphatic filariasis (elephantiasis) and onchocerciasis (river blindness), are due to infection by filarial nematodes (Taylor *et al.*, 2010). An estimated 150 million people suffer from these two diseases, with another billion at risk (Molyneux *et al.*, 2003; Taylor *et al.*, 2010). The devastating impact of parasitic nematodes on human productivity and health has spurred efforts to develop treatments and preventions by elucidating parasite biology using new technologies (Kumar *et al.*, 2007; Mitreva *et al.*, 2007; Taylor *et al.*, 2011).

Despite their sinister reputation, parasitic nematodes can also have many beneficial impacts on human interests and health. For example, entomopathogenic nematodes (EPNs),

such as steinernematids and heterorhabditids, are commercially used as biological control agents for crop pests (Grewal *et al.*, 2005). Also, human-parasitic nematodes are being tested for therapeutic use in many autoimmune diseases (Summers *et al.*, 2005; Schneider and Ayres, 2008; Liu *et al.*, 2009; Kuijk and van Die, 2010; Correale and Farez, 2011).

The simplicity, tractability, and conserved genes of many nematode species have supported their use as models for diverse biological processes, including human diseases, aging, immunity, development, ecology, evolution, and host-bacterial interactions (Aboobaker and Blaxter, 2000; Couillault and Ewbank, 2002; Goodrich-Blair, 2007; Mitreva *et al.*, 2009; Markaki and Tavernarakis, 2010; Neher, 2010; Xu and Kim, 2011). This last phenomenon—the intimate associations between two of the most speciose organisms on the planet—is the focus of the remainder of this review.

Nematode-bacterium associations can be beneficial (mutualistic) or harmful (pathogenic/parasitic) and can range from facultative, temporary interactions to stably maintained, long-term symbioses. Bacteria can be a potential food source for nematodes (Poinar and Hansen, 1986). Bacterivory occurs only in select nematode species and can be nonspecific (such as in *Caenorhabditis elegans* (Freyth *et al.*, 2010)) or specialized. In specialized interactions, the nematodes preferentially depend on select genera or species of bacteria, and these bacteria may be purposefully introduced or raised by the nematode (Ott *et al.*, 1991; Goodrich-Blair, 2007).

As well as being a food source, bacteria can be pathogens of nematodes. Many of these are the same or similar to pathogens of humans, which has spurred the use of *C. elegans* as a model host of human infectious diseases (Couillault and Ewbank, 2002; Waterfield *et al.*, 2008; Irazoqui *et al.*, 2010; Pukkila-Worley and Ausubel, 2012). In addition to trophic and pathogenic interactions, bacteria can serve as mutualists by aiding nematodes in development, defense, reproduction, and nutrient acquisition (Poinar and Hansen, 1986; Zhou *et al.*, 2002; Goodrich-Blair and Clarke, 2007; Musat *et al.*, 2007; Slatko *et al.*, 2010; Hansen *et al.*, 2011; Foster *et al.*, in press).

In recent years, "omics" studies, high-throughput analyses of whole cell, organism, and population-wide data sets, have begun to reveal the mechanistic underpinnings of many nematode-bacteria interactions. Genome sequencing has opened the door for transcriptomics to examine nematode and bacterium transcriptional profiles as well as for proteomics to identify and quantify proteins in complex mixtures (Malmstrom *et al.*, 2011). While these types of omics studies have been applied to only a few of the myriad nematode-bacterium associations, the findings have been integral to the understanding of other aspects of nematode biology and are paving the way for comparative analyses with non-nematode symbioses.

Model Systems of Nematode-Bacterium Symbiosis

Nematodes and their bacterial associates exist in marine, freshwater, soil, and plant or animal host environments. The most exhaustively studied of the nematodes, *Caenorhabditis elegans,* is a terrestrial nematode whose relationships with bacteria are predatory (Brenner, 1974), defensive (Tan and Shapira, 2011), and possibly commensal (Portal-Celhay and Blaser, 2012). The long experimental history of *C. elegans* has made it an unparalleled model of numerous biological processes (Blaxter, 2011; Xu and Kim, 2011), including bacterial pathogenesis and host immunity (Irazoqui *et al.*, 2010; Tan and Shapira, 2011; Pukkila-Worley and Ausubel, 2012). This body of work also has facilitated the advancement of studies of nematode-bacterium associations in which the nematode and bacteria engage in specific, persistent, mutualistic relationships. We emphasize three such associations here: terrestrial entomopathogenic nematodes associated with *Xenorhabdus* and *Photorhabdus* bacteria, *Laxus oneistus* marine nematodes with thiotrophic surface-colonizing bacteria, and parasitic filarial nematodes colonized by intracellular *Wolbachia* symbionts (Fig. 1) (Table 1).

Entomopathogenic nematodes (EPNs) and bacteria

At least two genera of nematodes, *Steinernema* and *Heterorhabditis,* have evolved symbiotic associations with Gammaproteobacteria, *Xenorhabdus* and *Photorhabdus* respectively, that allow them to kill insects and utilize the cadavers as food sources (Dillman *et al.*, 2012a). A specialized infective stage of EPNs carries the symbionts within the intestine and releases them upon invasion of an insect host. There, the bacteria contribute to killing the insect, help degrade the insect cadaver for nutrients, and protect the cadaver from opportunists. Once the insect resources are consumed, the EPN progeny nematodes develop into the colonized infective stage and emerge to hunt for a new insect host (Fig. 1) (Herbert and Goodrich-Blair, 2007; Clarke, 2008). There are three species recognized within the *Photorhabdus* genus: *P. temperata, P. luminescens,* and *P. asymbiotica*. The last was originally isolated from human wounds, but was recently discovered to colonize, like the other species, a heterorhabditid nematode host, of which there are 18 recognized species (Nguyen and Hunt, 2007; Nguyen, 2010; Stock and Goodrich-Blair, 2012). In contrast, there are 22 species of *Xenorhabdus* (Tailliez *et al.*, 2010, 2011) that colonize one or more of the more than 70 known species of *Steinernema* nematodes (Nguyen and Hunt, 2007; Nguyen, 2010; Stock and Goodrich-Blair, 2012). In both types of associations, the bacteria and nematodes can be cultivated independently or together, and molecular genetic techniques are available for the bacteria and, in some cases, for the nematodes (Ciche and Sternberg, 2007; Goodrich-Blair, 2007; Clarke, 2008). This technical tractability has enabled the use of EPNs and bacteria as

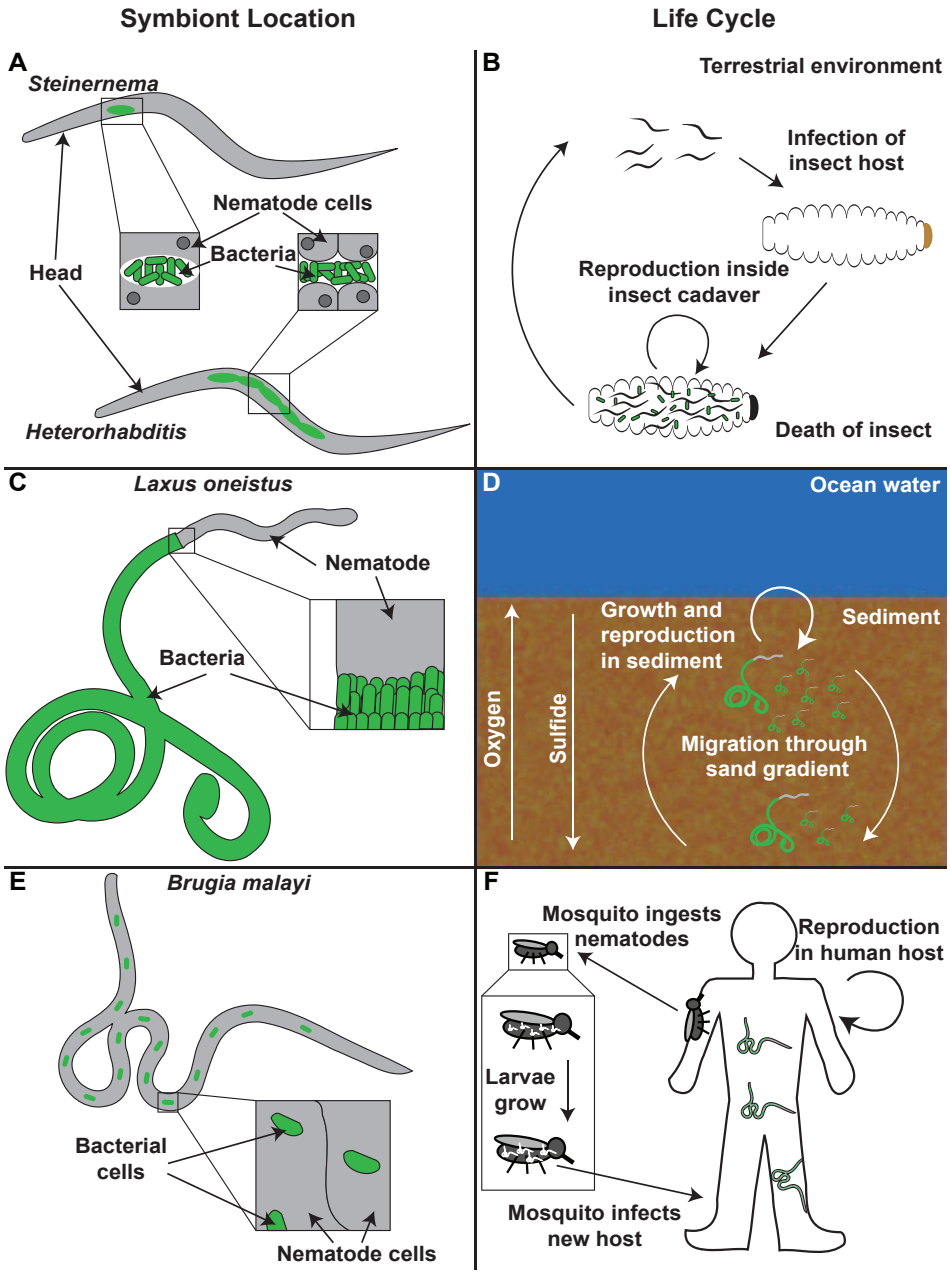

Figure 1. Figure 1. Schematic of model nematode-bacterium symbioses: symbiont location and life cycle. (A) *Xenorhabdus* and *Photorhabdus* (green) are located within infective juveniles (environmental stage) of *Steinernema* and *Heterorhabditis* nematodes respectively. The bacteria are located in the lumen between intestinal epithelial cells (gray with dark gray nuclei) (insets). (B) The infective juveniles of *Steinernema* and *Heterorhabditis* parasitize insect hosts. The nematodes and bacteria kill the insect and reproduce within the insect cadaver. The nematodes then re-associate with their bacterial symbiont and migrate away from the cadaver into the environment to seek new hosts. (C) The ectosymbiont (green) of *Laxus oneistus* (gray) is located on the outside of all nematode life stages. The bacteria are arranged perpendicularly to the exterior of the nematode (inset). (D) *L. oneistus* nematodes grow and reproduce in the sediment of the sea floor. Their thiotrophic ectosymbiont profits from nematode migrations in oxygen and sulfide gradients (see text for more details). (E) The *Wolbachia* symbionts (green) of *Brugia malayi* nematodes (gray) are localized to the hypodermal cells of the lateral chords in all nematode life stages and in the reproductive tissues of females. These bacteria are intracellular (inset). (F) *B. malayi* is transmitted to a human host through a mosquito vector. The nematodes reproduce within the human host and produce a larval stage that can be taken up by new mosquitoes. Larval stages grow within the mosquito and can then infect new human hosts when the mosquito takes a blood meal.

Table 1

*"Omics" studies applied to nematode-bacterium symbioses**

Symbiosis†	Nematode omics	References	Bacterium omics	References
Parasites of invertebrates				
Steinernema (Clade 10)-*Xenorhabdus*	Genomes and transcriptomes of *S. carpocapsae*, *S. scapterisci*, *S. monticolum*, *S. feltiae*, and *S. glaseri*	Dillman *et al.*, 2012b	Genomes of *X. nematophila* and *X. bovienii*	Latreille *et al.*, 2007 Chaston *et al.*, 2011
Heterorhabditis (Clade 9)-*Photorhabdus*	Genome and transcriptome of *H. bacteriophora*	Ciche, 2007 Harris *et al.*, 2010 Bai *et al.*, unpubl. Bai *et al.*, 2009 Hao *et al.*, 2012	Genomes of *P. luminescens* and *P. asymbiotica* Proteomes of *P. luminescens* TT01 variants Transcriptome of *P. luminescens* TT01 variants	Duchaud *et al.*, 2003 Gaudriault *et al.*, 2006 Gaudriault *et al.*, 2008 Ogier *et al.*, 2010 Wilkinson *et al.*, 2009 Derzelle *et al.*, 2004 Turlin *et al.*, 2006 Lanois *et al.*, 2011
Parasites of vertebrates				
Brugia malayi (Clade 8)-*Wolbachia*	Genome sequence Transcriptomes Proteomes	Bennuru *et al.*, 2011 Bennuru *et al.*, 2009 Choi *et al.*, 2011 Ghedin *et al.*, 2009 Ghedin *et al.*, 2007	Genome Proteome	Foster *et al.*, 2005 Bennuru *et al.*, 2009 Bennuru *et al.*, 2011
Free-living nematodes				
Laxus oneistus (Stilbonematinae, Clade 4)	Transcriptomes	Bulgheresi, 2012a Bulgheresi, 2012b	Draft genome of *L. oneistus* ectosymbiont	Available upon request at http://rast.nmpdr.org/rast.cgi
Plant-parasitic nematodes				
Meloidogyne incognita (Clade 12)	Genome	Abad *et al.*, 2008	NA	NA
Meloidogyne hapla (Clade 12)	Genome	Opperman *et al.*, 2008	NA	NA

* Only those nematode-bacterium associations discussed in this review for which there are available omics data are listed.
† Clades refer to those defined by Holterman *et al.* (2006).

models of mutualism, virulence, evolution, behavior, ecology, and drug discovery (Clarke, 2008; Ram *et al.*, 2008; Adhikari *et al.*, 2009; Bode, 2009; Richards and Goodrich-Blair, 2009; Eleftherianos *et al.*, 2010; Hallem *et al.*, 2011; Bashey *et al.*, 2012). Furthermore, since these nematode-bacterium complexes are pathogenic toward a wide but varying range of insects, an additional goal in studying EPNs is improving their use in biological control of insect pests (Stock, 2004). In particular, investigators have focused on identifying nematode traits associated with host range and successful parasitism to help improve the field efficacy of EPNs, and on identifying products of the entomopathogenic bacteria with insecticidal properties, efforts facilitated by sequencing of both bacterial and nematode genomes (Duchaud *et al.*, 2003; Ciche, 2007; Wilkinson *et al.*, 2009; Chaston *et al.*, 2011; Dillman *et al.*, 2012b; Bai [The Ohio State University] *et al.*, unpub.).

Laxus oneistus *symbiosis*

Stilbonematids occur in marine sand and establish ectosymbioses with thiotrophic Gammaproteobacteria (Ott *et al.*, 2004a, b; Bulgheresi, 2011). 18S rRNA-gene-based phylogeny indicates that stilbonematids form a monophyletic, distinct group of closely related genera within the *Desmodoridae* (Kampfer *et al.*, 1998; Bayer *et al.*, 2009). Stilbonematids are hypothesized to trophically depend on their ectosymbionts, and these in turn are assumed to profit from nematode migrations through the sulfide gradient in the marine sediment (Fig. 1) (Ott *et al.*, 1991). Stilbonematid sexual reproductive biology and development are poorly known. Two distinctive morphological characters unifying all stilbonematids are a poorly muscularized pharynx and the presence of unique epidermal organs called glandular sense organs (GSOs) (Bauer-Nebelsick *et al.*, 1995). GSOs appear to play a key role in the ectosymbiosis because they express a Ca^{2+}-dependent lectin (C-type lectin) that mediates ectosymbiont aggregation and host attachment (Bulgheresi *et al.*, 2006, 2011). Each GSO is composed of at least two gland cells and a sensory neuron (Bauer-Nebelsick *et al.*, 1995). Secretory products from the gland cells accumulate into a canal that crosses the epidermis and cuticle and terminates in a hollow bristle (seta). Therefore, with the

cuticle being like a sieve, a continuum exists between each GSO and the nematode surface.

L. oneistus ectosymbiont cells are rod-shaped and aligned perpendicularly to the nematode surface, forming an epithelium-like monolayer (Fig. 1). Notably, the cuticle thins at the bacterial coat onset (Urbancik *et al.*, 1996). The bacteria belong to the marine oligochaete and nematode thiotrophic symbiont (MONTS) cluster, which comprises 16S rRNA-gene sequences retrieved from Gammaproteobacterial sulfur-oxidizers associated with these invertebrates, as well as sequences of environmental origin (Heindl *et al.*, 2011). The closest cultivable relatives of MONTS members are free-living purple sulfur bacteria (*Chromatiaceae*). Beside their 16S rRNA-gene-based phylogenetic placement, uptake of ^{14}C bicarbonate (Schiemer *et al.*, 1990) and the presence of RuBisCo enzymatic activity indicate *Laxus* ectosymbiont autotrophy (Polz *et al.*, 1992). Enzymatic activity of ATP sulfurylase and sulfite oxidase, the presence of elemental sulfur in symbiotic but not in aposymbiotic *L. oneistus* (Polz *et al.*, 1992), and the cloning of the symbiont's *apr*A gene, encoding the alpha subunit of adenosine-5-phosphosulfate (APS) reductase (Bayer *et al.*, 2009), indicate sulfur-oxidation capability. Moreover, metabolic studies suggest denitrification capability (Hentschel *et al.*, 1999). The available genome draft (Table 1) confirms nitrate respiration and suggests additional capabilities for nitrite respiration and ammonia assimilation.

A distinguishing quality of stilbonematids is their ability to form monospecific *ecto*symbioses. The fact that host and ectosymbiont can be easily separated from one another makes stilbonematids an excellent system for dissecting the molecular base of symbiosis-specificity. Indeed, both host-secreted and microbe-associated molecular patterns (MAMPs) identified through omics can be expressed *in vitro* and directly tested on these nematode-bacteria consortia. In addition, *L. oneistus* represents an example of how the study of nematode-bacterial associations can have direct impacts in solving societal problems: the C-type lectin mentioned above is structurally and functionally similar to a human HIV-1 receptor, and could also block viral infection of human immune cells (Nabatov *et al.*, 2008).

Filaria nematode-Wolbachia *symbiosis*

Wolbachia are Alphaproteobacteria belonging to the order Rickettsiales and closely related to *Anaplasma*, *Ehrlichia,* and *Rickettsia. Wolbachia* are perhaps the most abundant of all intracellular bacteria, being found in filarial nematodes and arthropods, with around 70% of insect species colonized (Hilgenboecker *et al.*, 2008; Werren *et al.*, 2008). It remains unresolved if the *Wolbachia* bacteria present in different hosts or different invertebrate phyla represent distinct bacterial species or strains (Pfarr *et al.*, 2007). These maternally inherited, intracellular bacteria are generally considered reproductive parasites of arthropods due to the various reproductive manipulations they induce (cytoplasmic incompatibility, parthenogenesis induction, feminization, male killing) that serve to promote the reproductive success of infected females and the spread of *Wolbachia* through populations (Werren *et al.*, 2008). However, there is recent evidence that *Wolbachia* may confer fitness advantages to arthropods in certain situations. For example, *Wolbachia* increases resistance to viral pathogens in both fruit flies and mosquitoes and may be involved in nutritional provisioning in times of metabolic stress (Schneider and Chambers, 2008; Teixeira *et al.*, 2008; Brownlie *et al.*, 2009; Moreira *et al.*, 2009; Osborne *et al.*, 2009; Bian *et al.*, 2010; Glaser and Meola, 2010). The *Wolbachia* found in most filarial nematode species is believed to be an obligate mutualist and has shared a long, stable co-existence with its worm hosts (Foster *et al.*, in press). Clearance of *Wolbachia* with antibiotics has dire consequences for the nematode host with disrupted development, blockage of embryogenesis, and eventual death of the worm (Taylor *et al.*, 2005; Foster *et al.*, in press). Consequently, *Wolbachia* represents a major new drug target for control of filarial diseases, and doxycycline has been used in several clinical trials in Africa and Asia (Taylor *et al.*, 2010).

Within filarial nematodes, *Wolbachi*a is found in the hypodermal cells of the lateral chords of both sexes and in the ovaries, oocytes, and developing intrauterine embryonic stages of females. *Wolbachia* is present in all developmental stages of the worm but undergoes extensive multiplication within a week of the nematode transitioning from its insect vector to the mammalian host (Fig. 1). *Wolbachia* titer increases further as the larvae develop to adulthood and as the oocytes and embryonic stages become infected (Fenn and Blaxter, 2004; McGarry *et al.*, 2004). These observations suggest a molecular crosstalk that serves to regulate *Wolbachia* titer. Complete genome sequences of both *Brugia malayi* (causes lymphatic filariasis) and its *Wolbachia* endosymbiont are available (Foster *et al.*, 2005; Ghedin *et al.*, 2007) and have facilitated subsequent microarray, transcriptomic, and proteomic studies (Table 1) that are beginning to tease apart aspects of the filarial nematode–*Wolbachia* symbiosis.

Omics Insights into Nematode-Bacterial Mutualism

Experimental systems of nematode-bacterium mutualism provide an opportunity to test existing symbiosis theory (Douglas, 2008) including how benefits and costs within each association vary depending on environment and partner, how specific partners are transmitted between generations, how the development of cheating is prevented or maintained at acceptable levels, how tolerance or avoidance of host immunity is achieved, and how symbiosis impacts

the evolution of an organism. Through numerous approaches, including omics, these questions are beginning to be answered in several nematode-bacterium symbioses.

Nutrient provisioning between filarial nematodes and Wolbachia *symbionts*

Filarial nematodes depend on their *Wolbachia* symbiont for normal development, embryogenesis, and viability, raising the hypothesis that the bacteria may provide essential nutrients to their nematode host (Taylor *et al.*, 2005; Foster *et al.*, in press). In turn, *Wolbachia* bacteria are unable to be cultured outside host cells, indicating it too receives some nutritive benefit from its host. The genome sequences of *B. malayi* (Ghedin *et al.*, 2007) and its *Wolbachia* endosymbiont (Foster *et al.*, 2005) together with transcriptomic approaches (see below) have revealed several candidate examples of metabolic provisioning between the bacterium and its nematode host. *Wolbachia* is very limited in its production of amino acids but encodes several proteases and importers, which presumably enable the bacterium to grow on host-derived amino acids. Surprisingly, *B. malayi* lacks genes required for *de novo* synthesis of purines and pyrimidines but maintains salvage pathways; conversely *Wolbachia* has retained *de novo* synthesis but lacks nucleotide salvage pathways. Similarly, *B. malayi* is deficient in genes required for biosynthesis of heme, riboflavin, and FAD, while *Wolbachia,* despite having a streamlined genome typical of intracellular bacteria, has retained these biosynthetic capabilities.

Experimental studies based on these genomic insights suggest that the *Wolbachia* heme pathway may indeed be critical for the *B. malayi* host (Wu *et al.*, 2009). Furthermore, a microarray study that compared gene expression in tetracycline-treated *Litomosoides sigmodontis* (a closely related filarial worm) to untreated worms that retained their *Wolbachia* showed higher expression in treated worms of a nematode heme-binding globin as well as several heme- and riboflavin-containing respiratory chain components encoded by the mitochondrion (Strubing *et al.*, 2010). These transcriptional changes were not observed in a filarial nematode that naturally lacks *Wolbachia,* suggesting that the responses observed in *L. sigmodontis* were a true consequence of *Wolbachia* clearance. These results highlight the power of genomics to focus experimentation on key specific, testable hypotheses.

In *L. sigmodontis,* expression of genes involved in translation, transcription, protein folding/sorting, structure, motility, metabolism, signaling, and immunomodulation was also affected by *Wolbachia* clearance (Strubing *et al.*, 2010). Broadly similar changes were observed in a comparable microarray experiment conducted in *B. malayi* (Ghedin *et al.*, 2009). In this study, genes in certain classes (*e.g.,* signaling) showed a bimodal pattern of regulation: they were upregulated soon after antibiotic treatment started, then quickly downregulated, before becoming upregulated again after the end of treatment (Ghedin *et al.*, 2009). Since antibiotics affect embryogenesis in advance of worm viability, the authors postulated that early changes in gene transcript levels reflect disruption of the embryo program, while later transcriptional changes are the result of reduction of the *Wolbachia* load in the hypodermis (Ghedin *et al.*, 2009). Although the cDNA preparation selected against *Wolbachia* transcripts, some were detectable. As might be expected, after antibiotic treatment *Wolbachia* probes that hybridized showed downregulation almost exclusively. However, three *Wolbachia* genes (hypothetical, short-chain alcohol dehydrogenase and stress-induced morphogen) were upregulated after treatment (Strubing *et al.*, 2010), although the significance of this observation is not understood.

The relative costs and benefits of bacterial association can be influenced by the developmental stage of the organisms, and therefore key insights can be gained by using transcriptomics to monitor aspects of mutualism throughout the life cycles of the associates. Expressed sequence tag (EST) sequencing from 25 cDNA libraries made from different life-cycle stages of *B. malayi* has produced over 25,000 sequences that cluster to nearly 10,000 genes. Similar data sets are available for other filarial nematode species (Elsworth *et al.*, 2011; Blaxter, 2012). In addition, a recent comprehensive RNASeq transcriptomic profiling of seven life-cycle stages of *B. malayi* (Choi *et al.*, 2011) will be invaluable for tracking the temporal transcription of nematode genes predicted to be involved in the symbiotic relationship with *Wolbachia*. Stage-specific proteomic studies on *B. malayi* (Bennuru *et al.*, 2009) and its excreted or secreted proteins (Bennuru *et al.*, 2011) have confirmed production of about two-thirds of the predicted proteome and validated about half of the genes annotated as hypothetical. Of note, *Wolbachia* proteins were also found among the excretory or secretory products, suggesting integration of nematode and bacterial physiology. A recent genome-wide computational prediction of protein-protein interactors in six species of parasitic nematodes, including *B. malayi* as well as the free-living *C. elegans,* was undertaken to highlight interactors as candidate drug targets (Taylor *et al.*, 2011). This study did not include the *Wolbachia* proteome with the *Brugia* data set, but prediction of the *Wolbachia-Brugia* interactome is highly warranted given their likely physiological integration. On the basis of the hypothesis that outer membrane proteins such as *Wolbachia* surface protein (WSP) might interact with nematode proteins, WSP was used to bind *B. malayi* protein extracts, for panning a *Brugia* cDNA library and for ELISA and pull-down assays (Melnikow *et al.*, 2011). One *Brugia* protein annotated as hypothetical was identified by all approaches and provides the first example of a *Brugia-Wolbachia* in-

teracting protein pair. Thus, the combination of transcriptomic and proteomic data from the host nematode and its symbiont allows detailed investigation of the presence and abundance of nematode and *Wolbachia* gene products throughout the life cycle and will lead to enhanced understanding of the host-bacterial interactome and the symbiosis in general.

Specificity in the EPN-bacteria symbiosis

Photorhabdus and *Xenorhabdus* bacteria are closely related to each other phylogenetically, and both infect a similar range of insect hosts, but each associates with an EPN from a different clade (Table 1). Both bacteria make similar symbiotic contributions to the fitness of their nematode hosts: helping establish infection in insects, defending the insect host from predators and competitors, and promoting normal nematode development (Goodrich-Blair and Clarke, 2007). However, comparative analyses of the four sequenced bacterial genomes (*P. luminescens*, *P. asymbiotica, X. nematophila*, and *X. bovienii*) (Duchaud *et al.*, 2003; Wilkinson *et al.*, 2009) revealed that these similar fitness traits are the product of convergent evolution (Chaston *et al.*, 2011). For example, each symbiont limits the growth of competitor microbes, but does so through the production of different types of antimicrobial compounds (Chaston *et al.*, 2011). In contrast, the genes involved in entomopathogenicity, such as those encoding insecticidal toxins, appear to be conserved among the four bacterial species. On the basis of the apparent convergent evolution of genes involved in nematode-association and conservation of those involved in insect virulence, this study also predicted which bacterial genes may be involved in either of these symbiotic behaviors (Chaston *et al.*, 2011). The analysis was based on the idea that genes present in both *Xenorhabdus* and *Photorhabdus* but absent in non-insect pathogens may be enriched for those that encode activities necessary for killing and digesting insects. Similarly, genes that are unique to either *Xenorhabdus* or *Photorhabdus* should be enriched for those that are necessary for interactions with the nematode host. The study found 243 *X. nematophila* genes common to *Xenorhabdus* and *Photorhabdus* but absent in non-insect pathogens, including many with predicted roles in pathogenesis, and 290 genes specific to *Xenorhabdus*. Perhaps not surprisingly, genes of unknown function predominate in the latter "nematode host interaction" category, suggesting that bacterial genes involved in nematode interactions remain to be functionally characterized (Chaston *et al.*, 2011). Further application of proteomic and panning approaches, such as those described above for *Wolbachia*-filaria interactions, would be useful for exploring this set of potential host-interaction genes.

In addition to comparative genome approaches, genome sequencing of EPN symbionts facilitated genetic screens that lent insights into the biology involved in host-microbe interactions. As with all mutualistic symbiotic associations, a key component of the EPN-bacterium symbiosis is transmission of the bacterial symbiont to the next generation. In EPNs this occurs by bacterial colonization of the intestines of progeny-infective juveniles and carriage to the next insect host. Bacterial colonization of the infective juvenile stage can be highly selective, such that in some EPN-bacterium associations only one species of bacterium is capable of colonizing a particular species of nematode (Goodrich-Blair, 2007; Clarke, 2008). Transposon mutagenesis screens in both *X. nematophila* and *P. luminescens* have revealed novel genes involved in this specificity (Heungens *et al.*, 2002; Easom *et al.*, 2010; Somvanshi *et al.*, 2010). In one study, nine *X. nematophila* genes essential for normal colonization of the infective stage of *Steinernema carpocapsae* nematodes were identified. Three of these genes, *nilA, B,* and *C*, are encoded together on a 3.5-kb locus (Heungens *et al.*, 2002). Further study revealed that this locus is not present in other *Xenorhabdus* bacterial symbionts and is sufficient to confer colonization of *S. carpocapsae* on naturally non-colonizing bacteria, establishing for the first time a genetic element conferring host range expansion in an animal-bacterial association (Cowles and Goodrich-Blair, 2008). *nilB* is similar to genes found in animal-associated microbes, including mucosal pathogens (Heungens *et al.*, 2002; Bhasin *et al.*, 2012), supporting the idea that common molecules or mechanisms maintain many host-bacterial interactions regardless of whether the outcome of the interaction is mutualistic or pathogenic (McFall-Ngai *et al.*, 2010). The function of NilB, a surface-exposed outer membrane protein (Bhasin *et al.*, 2012), remains unclear, but analysis of the EPN symbiont genome sequences has provided some clues. Relaxed search parameters revealed that each of the four sequenced genomes of EPN symbionts, including *X. nematophila* itself, encodes a NilB-like protein in a conserved genomic context. Adjacent genes are predicted to encode TonB-like transporters and TonB-dependent receptors involved in metabolite transport across the membrane. This finding leads to the hypothesis that NilB and NilB-like proteins may be involved in transport of a class of molecules that varies among different nematode hosts, allowing their function to dictate host range specificity (Bhasin *et al.*, 2012). Alternatively, the NilB-like orthologs may play a role in other aspects of the EPN symbiont biology, such as insect virulence.

Consistent with the latter hypothesis, screens for *P. luminescens* mutants defective in colonizing their nematode host *H. bacteriophora* did not reveal the NilB-like ortholog, nor any of the other colonization genes identified in *X. nematophila* (Heungens *et al.*, 2002; Easom *et al.*, 2010; Somvanshi *et al.*, 2010). This finding further supports the convergent abilities of *Xenorhabdus* and *Photorhabdus* to mutualistically associate with their respective nematodes

(Chaston *et al.*, 2011). Putative *P. luminescens* nematode colonization genes revealed by mutant screens include those involved in lipopolysaccharide metabolism, fimbriae biosynthesis, and regulation (Easom *et al.*, 2010; Somvanshi *et al.*, 2010). Subsequent microarray work established that the colonization gene *hdfR* encodes a transcription factor that regulates more than 100 genes, including many involved in metabolic processes. Nematodes co-cultivated with the *hdfR* mutant display a developmental lag, suggesting that *hdfR* is required for normal nematode development (Easom and Clarke, 2012). As the roles of bacteria in EPN development are elucidated, it will be particularly interesting to compare these findings to those in the filarial nematode-*Wolbachia* associations to determine if common themes are revealed.

Another avenue toward elucidating the molecular dynamics of nematode-bacterium mutualism is identification of genes that are expressed specifically during association. Such an approach has been applied to *P. luminescens* and *P. temperata*. Selective capture of transcribed sequences (SCOTS) identified 106 *P. temperata* transcripts that had altered levels when cells were grown in liquid culture rather than colonizing the nematode host (An and Grewal, 2010). The authors identified genes involved in cell surface structure, regulation, stress response, nucleic acid modification, transport, and metabolism, and found that half of the transcriptional changes overlap with that of the bacterial starvation response (An and Grewal, 2010). This overlap and the metabolic shifts that occur in sugar metabolism and amino acid biosynthesis indicate the likelihood that the nematode is a nutrient-poor environment. The authors hypothesized that this could be a mechanism by which the nematode controls the bacterial population (An and Grewal, 2010), which again echoes the potential of filarial nematodes to control their *Wolbachia* symbiont titer (Fenn and Blaxter, 2004; McGarry *et al.*, 2004).

Comparative-omics to elucidate the molecular dialog between host and symbiont

Nematodes likely interact with their symbiont partners through immune pathways. For example, nematode immunity may be downregulated by the symbiont, which may in turn produce antimicrobials to protect the immuno-depressed host from pathogens. Alternatively, the symbiont may induce, but be resistant to, nematode immunity. Also, the nematode may immunologically tolerate the symbiont (Schneider and Ayres, 2008). In each of these scenarios the nematode resistance, response, or tolerance to microbes and the relevant immune pathways must be identified to fully unravel the molecular dialog between host and symbiont.

The C. elegans *immune system.* Our knowledge of nematode innate immune defense derives primarily from *C. elegans* and its interactions with pathogens (Alper *et al.*, 2007; Schulenburg *et al.*, 2008; Irazoqui *et al.*, 2010; Ewbank and Zugasti, 2011; Tan and Shapira, 2011). *C. elegans* does not have circulating immune cells. Therefore, if behavioral avoidance cannot spare it from deleterious microorganisms (Pradel *et al.*, 2007), it relies on epithelial immunity to respond to pathogens. Three principal pathways activate distinct but overlapping sets of immune effectors: the p38 mitogen-activated protein kinase (MAPK) pathway, the insulin/IGF-1 signaling (IIS) pathway, and a transforming growth factor-beta (TGF-beta) pathway. Despite the undisputed role of Toll-like receptors in mammalian immunity, *C. elegans* epithelial immunity does not rely on them (Pujol *et al.*, 2001). Moreover, many genes encoding Toll-NF-kB pathway components are absent from all the available nematode genomes (Irazoqui *et al.*, 2010). *C. elegans* can distinguish between nonpathogenic and pathogenic, but also between different classes of microbes. The specificity of this customized immune response may arise at the recognition level or at the effector level. It may also be achieved through differential immune regulation (*e.g.,* different microbes cause a different degree of activation of one or more signaling pathways or a different integration among pathways; Schulenburg *et al.*, 2008).

In *C. elegans* p38 MAPK-mediated epidermal immunity, the binding of an unknown ligand to an unknown receptor results in successive activation of heterotrimeric G protein, protein kinase(s) C, and the p38 MAPK module. Activation of the module results in the expression of antimicrobial peptide-encoding genes such as *nlp-29*. Additionally, neuronally secreted DBL-1 may also ignite epidermal immunity, though the identity of the DBL-1-secreting neurons is unknown. In this case, DBL-1 receptor-regulated Smad proteins would activate an unknown transcription factor or factors, which in turn would switch on transcription of antimicrobials such as caenacins in the epidermal cell.

C. elegans intestinal immunity differs from epithelial immunity; in the latter the p38 MAPK pathway (Kim *et al.*, 2002) is integrated with the neuronally activated TGF-beta pathway, whereas in the former, it is integrated with the insulin/IGF-1 signaling (IIS) pathway (Garsin *et al.*, 2003). The IIS pathway is also neuronally activated, and it is a conserved regulator of metabolism, stress resistance, and immune homeostasis (Becker *et al.*, 2010; Peng, 2010). Activation of the insulin/IGF-1 receptor DAF-2 by insulin-like ligands triggers a phosphorylation cascade involving lipid and serine/threonine kinases. These phosphorylation events lead to the cytoplasmic retention of the transcription factor homolog DAF-16. If DAF-2 is not activated, or if its function is reduced, DAF-16 is translocated into the nucleus, and this triggers the expression of antimicrobial genes, such as those encoding lysozymes and saposin-like proteins. DAF-16 was long hypothesized to be the only transcription factor capable of conferring pathogen resistance, and it is probably the most crucial stress-protective transcription factor (Tan and Shapira, 2011). In the recently

described *C. elegans* model for persistent intestinal colonization, *daf-2* mutants exhibited reduced colonization by *E. coli*, while *daf-16* mutants showed increased colonization; but neither mutation appeared to influence the competitive advantage of *Salmonella* relative to *E. coli* for colonization (Portal-Celhay and Blaser, 2012), indicating that these factors generally influence colonization, but do not necessarily contribute to specificity.

Since no nematode has been as extensively tested as *C. elegans*, it is unclear how different nematodes respond to microbial challenge. A comparison of current genome or transcriptome nematode databases reveals many regulatory components of the epithelial pathway described above, and other immunity pathways seem to be conserved across nematodes (Table 2, Appendix, and Supplemental Table 1, http://www.biolbull.org/content/supplemental). Indeed, both DAF-2 and DAF-16 appear to have orthologs in every nematode species examined, highlighting their critical roles in nematode biology. The increasing availability and decreasing costs of omics techniques promises that nematode immunity will slowly but surely be revealed, answering such questions as how nematodes respond to the physical presence of bacterial cells on their cuticle, how they recognize one type of bacterium from another (and therefore select for beneficial associates while defending against pathogens), and how they control symbiont populations.

L. oneistus *immunity pathways putatively involved in symbiosis.* Transcriptomics has revealed potential immune pathways functioning in the *L. oneistus*-bacterium symbiosis. A manual search of adult *L. oneistus* transcriptomic data (Bulgheresi, 2012a) for immunity genes based on the *C. elegans* annotation (Harris *et al.*, 2010) indicates that *L. oneistus* expresses the p38 MAPK module (Table 2). Putative p38 MAPK module activators expressed in *L. oneistus* include heterotrimeric G protein component beta RACK-1 and protein kinase C PLC-3, as well as a Tribbles homolog 1 (*C. elegans* NIPI-3). The presence of DBL-1 transcripts in the *L. oneistus* transcriptome may indicate that neuronally secreted DBL-1 triggers the epidermal TGF-beta pathway, the basic components of which are also expressed by *L. oneistus*. At present, it is not known whether the p38 MAPK and the TGF-beta pathways are triggered in *L. oneistus* epidermal cells by bacteria contacting the worm's surface. Although epidermal cells underlying an intact cuticle may be insensitive to microbes attached to it, there is a continuum between each GSO lumen and the nematode surface (see background on *L. oneistus* above). Moreover, the gland and neuronal cells making up each GSO are in direct contact with one another. Therefore, it is very tempting to speculate that the GSO gland cells may mount an immune response instead of—or in addition to—the epidermal cells, and that the GSO neuronal cells may locally modulate their response.

It has long been hypothesized that adult stilbonematids feed on their symbionts; while this has not yet been observed (Ott *et al.*, 1991), it remains possible that at some developmental stages the ectosymbiont is present, undigested, in the *L. oneistus* gut. This is even likelier in light of the fact that in contrast to *C. elegans*, stilbonematids do not possess a grinder that can efficiently crush ingested bacteria (Hoschitz *et al.*, 2001). How might adult *L. oneistus* intestinal cells react to and limit bacterial proliferation? They express a DFK-2 ortholog, and this kinase could activate the p38 MAPK cascade. Additionally, a neuronally activated IIS pathway might play a role in mediating microbial recognition in the gut (Table 2).

L. oneistus appears to constitutively express signaling pathway components necessary to react to the presence of its ectosymbiont. In particular, transcripts encoding all the members of the TGFβ pathway, which is central in *C. elegans* epidermal immunity, are present. Secondly, more conservation seems to exist among signaling pathways working in *C. elegans* and *L. oneistus* than among the downstream effectors that they regulate (Table 2). These are notoriously poorly conserved, and it is therefore likely that investigating diverse systems will provide greater insights into host responses to and selectivity for bacteria and enable the discovery of novel antimicrobials.

Contrasting immunity in free-living and host-associated nematodes. While many nematodes, like *L. oneistus* and the EPNs, are either free-living or have free-living stages, there are also nematodes such as *B. malayi*, *Ascaris suum,* and *Trichinella spiralis* that complete most of their life cycles within animal hosts and have less exposure to bacterial diversity. For example, *Wolbachia* is intracellular, mostly restricted to *B. malayi* reproductive tissue and hypodermal chords, and likely to have existed in a long-term evolutionarily stable relationship with its nematode host. A recent report documented low numbers of *Wolbachia* in the excretory-secretory canal of *B. malayi,* raising a potential mechanism for release of *Wolbachia* to the nematode surface or surrounding tissue (Landmann *et al.*, 2010). *Wolbachia* has also been observed in the intestinal wall of a related filarial nematode (Ferri *et al.*, 2011). Any effects the bacteria in these locations might have on epidermal or intestinal immunity are unknown. There is extensive transcriptomic data for seven life-cycle stages of *B. malayi* (Choi *et al.*, 2011) which reveals that all the predicted immunity-related genes indicated in Table 2 are transcribed with the exception of the MAP kinase kinase, MEK-2.

The lifestyle features of nematodes such as *B. malayi, A. suum,* and *T. spiralis* might be expected to result in a very reduced spectrum of nematode immune defense mechanisms. However, the repertoire of immune regulators seems to be broadly conserved across the phylum (Table 2). When looking more specifically at the abundance of immune effectors such as lysozymes, defensin-like ABF proteins, thaumatins, and C-type lectin domain-containing proteins (CTLDs) (Table 3), there seems to be more of a pattern.

Table 2

Orthology analysis of selected proteins known to play a significant role in the immunity of Caenorhabditis elegans

		C. elegans protein	Protein description	*C. elegans*	*Laxus oneistus*	*Steinernema carpocapsae*	*Brugia malayi**	*Ascaris suum**	*Trichinella spiralis**
	TGF-beta	DBL-1	TGF-beta ligand	1	1	1	1	1	0
		SMA-6	Type I TGF-beta receptor	1	1	1	2	1	1
		SMA-2 & SMA-3	Smad protein	3	2	3	3	3	2
		SMA-4	Smad protein	1	1	1	3	2	1
	Insulin/ IGF-1	GOA-1	G protein alfa subunit	1	1	1	0	1	1
		DGK-1	Diacylglycerol kinase beta	1	2	1	4	1	1
		INS-7	Insulin/IGF-1-like peptide	8	0	0	0	0	0
	Epithelial cell	DAF-2	Insulin/IGF-1 receptor	1	1	3	4	2	2
		AGE-1	Phosphotidylinositol 3-kinase	1	3	1	1	1	0
		AKT-1	Rac Ser/Thr protein kinase	1	3	0	0	0	0
		AKT-2	Rac Ser/Thr protein kinase	1		1	1	1	1
		SGK-1	Serum/glucocorticoid regulated kinase 1	1	2	1	0	1	0
		DAF-16	FOXO family transcription factor	1	2	2	2	1	1
p38 MAPK pathway	Epidermal immunity	RACK-1	G protein beta subunit	1	1	2	1	1	1
		PLC-3	Phospholipase C gamma	1	1	2	1	1	1
		PKC-3	Protein kinase C iota type	1	1	1	2	1	1
		GPA-12	G protein alpha subunit	1	0	1	1	1	1
		NIPI-3	Tribbles homolog 1 (TRB-1)	1	1	1	1	1	0
	Intestinal immunity	EGL-30†	G protein G(q) alpha subunit	1	2	1	2	2	1
		EGL-8†	Phospholipase C beta homolog	1	0	2	2	1	2
		DKF-2	Ser/Thr protein kinase D	1	2	1	2	1	1
		RAB-1	Ras-related GTPase Rab-1A	1	5	1	1	1	2
		NSY-1	ASK1 MAPKKK	1	2‡	2	2	1	1
		SEK-1	MKK3, MKK6, MAPKK	1	4	1	1	1	1
		PMK-1	p38 MAPK	1	3	1	1	1	1
	Other immune effectors	SPP-10	Saposin-like protein	1	3	2	1	1	0
		LYS-8	Lysozyme	5	1	2	1	0	0
		LYS-4,5,6, & 10	Lysozyme	4	5	2	0	1	0
		CLEC-48 & 50	C type domain-containing proteins (CTLD)	3	23	2	1	1	0
		CLEC-178	CTLD	1	3	0	0	1	0
		CLEC-56	CTLD	5	1	2	0	0	0
		CLEC-3,10, & 11	CTLD	43	3	0	0	0	0
		CLEC-150	CTLD	1	1	0	0	0	0
		FIP-1-like	FIDR protein	1	1	0	0	0	0

The protein names for *C. elegans* are given in the leftmost column with protein descriptions given in the second column from the left. The number of proteins found in orthology clusters with the proteins in the leftmost column are labeled under each species examined. The orthology analysis was run using the species listed across the top and also included *B. xylophilus* and *P. pacificus* as nonparasitic nematodes as well as *Nasonia vitripennis*, the parasitoid wasp, as an arthropod outgroup (see Appendix 1 for details). All protein data were taken from whole-genome releases except for *L. oneistus*, for which protein data from transcriptomics were used. All individual orthology results and the protein identifiers for the clustered orthologs can be found in Supplemental Table 1 (http://www.biolbull.org/content/supplemental).

* Nematodes with limited or no free-living stages.

† EGL-30 and EGL-8 are known to be involved in both the Insulin/IGF-1 pathway and intestinal immunity in *C. elegans*.

‡ No NSY ortholog was identified in *L. oneistus*; *L. oneistus* proteins identified in this cluster are orthologs of human TGF-beta activated kinase MAPKKK7.

Nematodes that are either free-living or have free-living stages seem to possess a greater abundance and diversity of both general and adaptively specific immune regulators than those with limited or no free-living stage (Tables 2 and 3). The orthology analysis seems to suggest that the evolution of immune effectors has been sculpted to the lifestyle of each nematode, with those lineages encountering a potentially broader array of microbes having experienced expansions in these protein families. Notably, *T. spiralis*, an intracellular mammalian parasite with no free-living stage, shows a high level of conservation of immune regulators but a contraction of immune effector protein families (Tables 2 and 3). *B. malayi* and *A. suum,* though closely related phylogenetically, differ in the presence and abundance of immune effector orthologs. *A. suum*, which lives in the intestine and likely experiences more bacterial interactions,

Table 3

A broad protein orthology analysis of all known Caenorhabditis elegans *proteins in the listed immune effector categories**

Immune effector	# of clusters	*C. elegans*	*Pristionchus pacificus*	*Bursaphelechus xylophilus*	*Steinernema carpocapsae*	*Brugia malayi*†	*Ascaris suum*†	*Trichinella spiralis*†
Lysozymes	3	15	14	14	8	2	2	0
Antimicrobial caenacins	0	11	0	0	0	0	0	0
Caenopores or saposin-like	9	23	6	2	7	1	4	1
Neuropeptide-like proteins (NLPs)	18	47	9	10	15	7	11	1
Thaumatins (THNs)	1	8	1	1	1	0	0	0
Defensin-like ABF proteins	2	6	3	0	0	0	5	0
C type lectin domain-containing proteins (CTLD)	34	265	66	4	15	3	19	0

* For example, there are 265 *C. elegans* proteins labeled CLEC (1-266 with no protein assigned as CLEC-200), but not all of these have been functionally shown to play a role in immunity. This table shows the total number of clusters generated by an orthology analysis including the species listed across the top as well as the parasitoid wasp, *Nasonia vitripennis* as an arthropod outgroup.

† indicates nematodes with limited or no free-living stages. See Appendix 1 for analysis methods. All the individual protein names from individual species, identified as orthologs, can be found in Supplemental Table 2 (http://www.biolbull.org/content/supplemental).

is armed with more immune effectors than *B. malayi*, which resides in the lymphatic system. It is possible that this difference in the presence and abundance of immune effectors could result from incomplete sequencing, significant sequence divergence of orthologs such that they are no longer detectable by sequence similarity, the evolution of different immune effectors not orthologous to the *C. elegans* ones, or the expansion of these gene families in *C. elegans.* Nevertheless, it is tempting to speculate that the diversity of effectors present in the genome positively correlates with the nematode's exposure to microbes and the consequent need for immunity.

Orthology analysis across several genomes suggests that some immune effectors are lineage-specific. For example, there is no evidence for orthologs of any of the 11 antimicrobial caenacins of *C. elegans* (Table 3). Similarly, orthologs of *C. elegans* genes encoding potentially antimicrobial neuropeptide-like proteins *nlp-29*, *-31* or *-33* or other candidate antimicrobial *nlp* genes encoding a YGGYG motif (*nlp-24* through *-33*) (Gravato-Nobre and Hodgkin, 2005; McVeigh *et al.*, 2008) were not identified (Supplemental Table 2, http://www.biolbull.org/content/supplemental). Interestingly, genes encoding antimicrobial proteins also appear absent in the necromenic nematode *Pristionchus pacificus* and the migratory endo-plant-parasitic nematode *Bursaphelechus xylophilus* despite their both having free-living stages (Table 3). In fact, in a survey of 33 nematode EST data sets, orthologs of the three *C. elegans nlp* genes encoding antimicrobials were not found. Sequences with YGGYG motifs were identified, albeit sporadically and predominantly only in representatives of nematode clades 9–12 (Gravato-Nobre and Hodgkin, 2005; McVeigh *et al.*, 2008). Although the bulk of diversity within Nematoda remains to be explored, *B. malayi*, *A. suum*, *L. oneistus,* and *T. spiralis* belong to clades 8, 8, 4, and 2 respectively, indicating that, although preliminary, analyses including these species span a considerable segment of the phylum (Holterman *et al.*, 2006). Therefore, the absence of known antimicrobial-encoding *nlp* genes in *B. malayi*, *A. suum*, *L. oneistus,* and *T. spiralis* suggests that they are an immune adaptation that is unique to *C. elegans.*

Although our orthology analysis described above relies on knowledge of the *C. elegans* immune system, it does suggest that omics-acquired data can provide provocative hypotheses including how transient bacterial exposure, symbiosis, and environmental adaptation affect the evolution of nematode immune effectors and other immune pathways.

Exploring Parasitism, Pathogenesis, and Competition Through Omics

To date almost 700,000 nematode ESTs have been generated, representing about 230,000 genes from 62 nematode species (Elsworth *et al.*, 2011). Sequencing of ESTs from diverse nematodes offers a powerful approach toward uncovering candidate drug targets, lineage-specific parasitic traits, and conserved features of parasitism. For example, transcriptomics have been used to identify *S. carpocapsae* and *H. bacteriophora* nematode genes that may be involved in parasitism. In one study, subtractive hybridization was used to enrich for ESTs expressed by a virulent wild isolate of *H. bacteriophora* relative to a less virulent wild isolate. This approach revealed 87 ESTs differentially regulated between the strains that may contribute to pathogenesis, almost half of which lacked similarity to sequences in the public database (Hao *et al.*, 2012). In the *S. carpocapsae* study, investigators sequenced ESTs from the infective stage exposed to insect hemolymph. Of the 1592 unique transcripts, 37% lacked similarity to database sequences (Hao *et al.*, 2010). In both the *H. bacteriophora* and *S. carpocasae* studies, among those that do have significant

similarity to database sequences are those predicted to be involved in signaling (*e.g.,* G protein), metabolism (*e.g.,* fatty acid catabolism), stress response (*e.g.,* heat shock and oxidative stress-response proteins), and host-parasite interactions (*e.g.,* protease inhibitors, chitinases, and lectins). To identify proteins specific to parasitism, Bai *et al.* (2009) sequenced a library of 31,485 ESTs of the EPN *H. bacteriophora* TT01 (Bai *et al.*, 2009), and classified these ESTs on the basis of their presence in parasitic nematodes and absence in free-living nematodes. This approach yielded 554 genes as candidates for being involved in the parasitic lifestyle of the heterorhabditid nematodes. Again, the majority of these (412) have no matches to known proteins in the public sequence database.

In another study, transcriptome comparison of inbred, laboratory-cultured lines with deteriorated parasitism traits relative to those of parental lines was used to identify potential parasitism genes in *H. bacteriophora* using microarrays against 15,220 EST probes (Bilgrami *et al.*, 2006; Adhikari *et al.*, 2009). Genes that showed differential expression in the two nematode lines were enriched in metabolism, signal transduction, virulence, and longevity, with the ratio of primary to secondary metabolism being lower in the inbred strain. One of the genes present in higher levels in the inbred line relative to the parent line was nitric oxide synthase interacting protein, predicted to be a negative regulator of NO production (Adhikari *et al.*, 2009). Since NO may be involved in nematode virulence (*e.g.,* it is present in filarial nematode excretory products that inhibit immune cell proliferation (Pfarr *et al.*, 2001)) downregulation of NO might be one contributor to decreased virulence in insects of the inbred line relative to the parent line. Similarly, a microarray study comparing mosquito-vectored third-stage larvae of the filarial nematode *B. malayi* to those maintained in culture found numerous differentially expressed genes (Li *et al.*, 2009). Transcripts from mosquito-derived nematodes were enriched for those encoding stress resistance and immune modulation (such as cysteine proteinase inhibitors, which were also identified in *H. bacteriophora* ESTs), while genes differentially expressed by cultured nematodes were enriched for cell growth and molting (Li *et al.*, 2009). In a recurring theme, of the *B. malayi* mosquito-derived nematode-specific transcripts, 28% were of unknown function and may represent novel virulence determinants (Li *et al.*, 2009).

The studies described above highlight that while omics can focus the attention of researchers toward likely genes of interest, comprehensive understanding of molecular and cellular processes can only come from in-depth genetic and biochemical analyses. Since many candidate parasitism genes lacking significant homologs in the database are therefore absent from the genetically tractable model organism *C. elegans*, investigations into their function must necessarily be conducted in nematode parasites. Therefore, it is critical to continue developing tools such as transformation and RNA interference that are necessary to investigate gene function in a broader array of nematode genera. Furthermore, there is a need for in-depth comparative analyses of transcriptome data sets from diverse nematode systems to facilitate the identification of conserved and diverged mechanisms by which parasitic nematodes overcome their hosts' immune defenses.

The bacterial symbiont partners can also contribute to parasitism. For example, EPNs rely on their bacterial symbionts to help kill the insect host and to support reproduction in the cadaver. These bacterial symbionts can themselves be *bona fide* insect pathogens, capable of killing insects within several hours after injection into the insect blood cavity (Eleftherianos *et al.*, 2006; Richards and Goodrich-Blair, 2009). Comparative transcriptomics have been applied to identify *P. luminescens* TT01 genes potentially involved in insect pathogenesis. Genes differentially regulated between a virulent strain (TT01a) and an attenuated phenotypic variant included those encoding toxins, secreted enzymes, and proteins involved in oxidative stress (Lanois *et al.*, 2011). An *et al. (*2009) used Selective Capture of Transcribed Sequences (SCOTS) to identify *X. koppenhoeferi* and *P. temperata* genes expressed more highly during infection of insects than during laboratory growth, in an effort to identify virulence factors commonly and distinctly used by these bacteria. Both bacteria displayed *in vivo* upregulation of genes involved in stress response, toxin production (*tcaC*), hemolysins, fatty acid biosynthesis (reminiscent of the *H. bacteriophora* ESTs identified in more virulent strains described above), and metal transport. These authors further analyzed their data using a pathway-building program (PathwayStudio, Ariadne, Rockville, MD) to reveal patterns and pathways involved in virulence of the two EPN bacteria. Continued mapping of both nematode and bacterial metabolic pathways induced during infection has the potential to reveal metabolic integration in the symbiosis.

One of the mutualistic services provided by the bacteria to their nematode partners is protection of the cadaver from scavengers and opportunistic organisms that may compete for nutrients. The *Xenorhabdus* and *Photorhabdus* genera therefore offer tremendous potential as a source of anti-insecticidal, antimicrobial, and other bioactive molecules, and genomics has opened numerous doors to the discovery of novel metabolites. To date, the genomes of four EPN bacterial symbionts have been sequenced and analyzed from a comparative perspective (Duchaud *et al.*, 2003; Latreille *et al.*, 2007; Wilkinson *et al.*, 2009; Ogier *et al.*, 2010; Chaston *et al.,* 2011). These sequences revealed numerous loci predicted to encode secondary metabolites with potential pharmaceutical and agricultural uses (Bode, 2009). There are at least 23 biosynthetic gene clusters in *P. luminescens* TT01 (Duchaud *et al.*, 2003; Bode, 2009), primarily

non-ribosomal peptide synthetases (NRPS). Similarly *P. asymbiotica* encodes a rich diversity of NRPS or polyketide synthetase loci (Wilkinson *et al.*, 2009). This potential for secondary metabolite production was revealed through genome sequencing and belies the few compounds that were known from experimental approaches. Also, while some molecules had been biochemically characterized, the genes encoding them had not been identified, precluding detailed analysis of their synthesis and efforts to engineer high-output production. Genome sequences have provided an invaluable resource for identification of genes responsible for secondary metabolite production. For example, the genes responsible for synthesis of xenematide, a molecule with antimicrobial activity, were bioinformatically predicted (Crawford *et al.*, 2011).

Genomic Analyses Reveal Bacterial Contributions to Nematode Genome Evolution

Lateral gene transfer between nematodes and bacteria

Nematode-bacterium symbioses have contributed to our understanding of genome evolution, including genome plasticity and microbial-eukaryotic lateral gene transfer (LGT). LGT between eukaryotes and prokaryotes has been documented through transcriptome and genome analysis of plant parasitic nematodes (Scholl and Bird, 2011) with genes encoding glucanases and pectate lyases that are absent in other animals but are similar to those of rhizosphere bacteria. These genes are fully integrated into the genomes, with introns and mRNA processing typical of eukaryotes. They are prevalent among plant-parasitic nematodes such as *Meloidogyne incognita* and *M. hapla*, suggesting ancient acquisition (Scholl *et al.*, 2003; Scholl and Bird, 2011). Investigations on the genomes of *Pristionchus pacificus* (Dieterich *et al.*, 2008) and *Bursaphelenchus xylophilus* (Kikuchi *et al.*, 2011) provide additional compelling evidence that LGT of microbial genes is a component of nematode evolution (Mayer *et al.*, 2011).

Bacterial symbionts that are closely associated with the germ-line of their hosts are most likely to contribute to LGT events, and therefore it might not be surprising to find evidence of LGT in nematodes symbiotically associated with such bacteria. Indeed, fragments of *Wolbachia* DNA appear to be present in noncoding regions of the filarial nematodes *Onchocerca volvulus, O. ochengi* (Fenn *et al.*, 2006), *B. malayi,* and *Dirofilaria immitis* (Dunning Hotopp *et al.*, 2007). Further evidence comes from 454 pyrosequencing that identified *Wolbachia* genes in two naturally *Wolbachia*-free filarial nematodes: *Acanthocheilonema viteae* and *Onchocerca flexuosa* (McNulty *et al.*, 2010). Based on the hypothesis that the ancestor of extant filarids in the Onchocercinae and Dirofilariinae was in a symbiosis with *Wolbachia* (Casiraghi *et al.*, 2004), the authors posited that the presence of *Wolbachia* DNA in these uncolonized symbionts is evidence of former infection and ancient LGT. That these genes might play some functional role in the nematode is supported by evidence that some of the *Wolbachia* sequences are expressed in specific tissues (McNulty *et al.*, 2010). The presence of *Wolbachia* DNA in the genomes of many filarial nematodes raises intriguing possibilities about the role of symbiont DNA in shaping nematode evolution. In addition to *Wolbachia*-derived fragments, filarial nematodes also contain a functional ferrochelatase gene (last step in heme biosynthesis) that includes introns and a mitochondrial targeting signal but appears to be the result of horizontal transfer from a Rhizobiales bacterium (Slatko *et al.*, 2010).

Since cross-kingdom LGT horizontal gene transfer from bacteria to nematodes has been revealed in worms from several different clades, this method of gene acquisition may be commonplace among nematodes, or at least in chromadorean nematodes, and may represent an additional route by which nematodes may gain essential functions—a symbiosis of sorts.

Insights into bacterial genome evolution revealed by comparative genomics

Aspects of genome evolution have been explored through comparative analysis of noncore regions of the genomes of the EPN symbionts *Photorhabdus* spp. and *Xenorhabdus* spp. Flexible genome regions, or regions of genome plasticity (RGP), are defined as DNA sequences that are absent from one or more genomes being analyzed. In *Photorhabdus* and *Xenorhabdus* comparisons, as much as 60% of the genomic content falls into this class (Ogier *et al.*, 2010). Analysis of these regions revealed that RGP are made up of modules that can be shuffled by recombination and are proposed to be the actual units of genome plasticity. Indeed, the authors show that a *P. luminescens* TTO1-derived strain that had been associated with laboratory-reared nematodes had several deletions within RGP compared to the reference strain. These deletions encompassed modules, rather than entire RGP, and appeared to result from a single block deletion event.

Another observation of this study was that *P. asymbiotica*, the species isolated from human wounds, has a higher proportion, relative to the other *Photorhabdus* and *Xenorhabdus* genomes, of RGP that do not have canonical markers of mobile genetic elements (*e.g.,* they lack transposases, insertion elements, or genes encoding DNA modification enzymes). The authors suggest that understanding the functions of genes encoded on these regions might give insights into the evolution of *P. asymbiotica* as a human pathogen (Ogier *et al.*, 2010).

Conclusions

The biology of nematode-bacterial symbiotic associations is far-reaching and fundamental. Although in its infancy, the broad knowledge gained by "omics" studies in diverse biological disciplines—including symbiosis, evolution, immunology, infectious disease, and secondary metabolism—is already remarkable. These studies have revealed several key interactions that are common within nematode-bacterial interactions, such as bacterial contribution to nematode development and genome content. However, they also highlight that while the themes are common, the molecular mechanisms underlying them are likely specific to the system, as in the immune pathways involved in direct communication. While large-scale data-generating omics-style experiments have been critical for identifying important themes and mechanisms, they are only a starting place, producing many provocative hypotheses that remain to be functionally tested. As a result, it is important that the necessary tools for subsequent mechanistic explorations (*e.g.,* transformation and RNAi) continue to be developed in diverse nematode systems. As more data sets from previously unexplored clades of the phylum are produced, continued conversation between systems will be critical to further our understanding of conserved and unique patterns in the evolving relationships between nematodes and the bacteria they encounter.

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Appendix

Orthology Analysis

OrthoMCL ver. 1.4 (Chen *et al.,* 2006) was used to predict orthologous groups of proteins among species to facilitate analysis of protein evolution that could have an impact on symbiosis or other nematode-bacteria interactions. Proteins were grouped using the Markov Cluster algorithm to predict orthologs and paralogs. Complete proteomes were analyzed where possible, and EST data were analyzed in the case of *Laxus oneistus*. Nematode proteomes were downloaded from WormBase (www.wormbase.org, access date 1/7/12) from this site: "ftp://ftp.sanger.ac.uk/pub2/wormbase/releases/WS228/species/".

All proteomes used were from the WS228 release, except for *Bursaphelechus xylophilus* and *Ascaris suum*, which are from the WS229 release. The parasitoid wasp proteome from *Nasonia vitripennis* was used as an outgroup. The 1.2 version from NasoniaBase (www.hymenopteragenome.org, accessed 1/15/12) was downloaded and used in this analysis. The resulting clusters were analyzed for proteins known to play a role in *Caenorhabditis elegans* immunity.