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Wolbachia genomes: revealing the biology of parasitism and mutualism

Katelyn Fenn and Mark Blaxter

Institute of Evolutionary Biology and Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Ashworth Laboratories, King's Buildings, Edinburgh, UK, EH9 3JT

Wolbachia **bacteria are endosymbiotic partners of many animal species, in which they behave as either parasites (in arthropod hosts) or mutualists (in nematode hosts). What biochemistry and biology underpin these diverse lifestyles? The recent complete sequencing of genomes from *Wolbachia* that infect the arthropod *Drosophila melanogaster* and the nematode *Brugia malayi*, together with the partial genome sequencing of three *Wolbachia* strains found in drosophilids, enables this question to begin to be addressed. Parasitic arthropod *Wolbachia* are characterized by the presence of phages that carry ankyrin-repeat proteins; these proteins might be exported to the host cell to manipulate reproduction. In nematode *Wolbachia*, which lack these phages, several biochemical pathways can deliver essential metabolites to the nematode hosts. Nematode *Wolbachia* might also have a role in modulating the mammalian host immune system but the sequenced *Wolbachia* genomes lack the genes to synthesize lipopolysaccharide, raising questions about the nature of the inducing molecule. The *Wolbachia* surface protein might carry out this function.**

Why do filarial nematodes have *Wolbachia* endosymbionts?

Wolbachia are Alphaproteobacteria – members of the Anaplasmataceae that are related to rickettsial pathogens and that live intracellularly within arthropod and nematode hosts [1,2]. Arthropod *Wolbachia* are found in most orders of hexapods (insects), in crustaceans (woodlice) and in chelicerates (spiders). They cause several reproductive manipulations, including the induction of cytoplasmic incompatibility, the induction of parthenogenesis, and feminization or even killing of genetic males. Because arthropod *Wolbachia* are usually transmitted vertically from mother to daughter, all of these manipulations promote the fitness of infected females. Thus, antibiotic treatment of infected insects results in 'cure', with (usually) no adverse effects on the arthropod host. Arthropod *Wolbachia* are, therefore, parasites [1,2].

Nematode *Wolbachia* have a more restricted host distribution than that of their arthropod counterparts [3], being found only in vector-transmitted Onchocercidae such as *Onchocerca volvulus*, *Brugia malayi* and *Wuchereria bancrofti* – causative agents of human filariases [4]. The relationship between nematode *Wolbachia* and their hosts, although one of intracellular symbiosis, has features of mutualism [1]. Treatment of infected nematodes with antibacterial agents not only harms the *Wolbachia* but also adversely affects the host, resulting in delayed moulting, reduced growth rates, failure of embryogenesis (i.e. effective sterilization) and death [5–7]. In trials using infected human populations, tetracycline is a powerful adjunct to more-established pharmacological interventions [8,9].

Wolbachia are classified by their place in molecular phylogenies, which define six different 'supergroups' of *Wolbachia* [10] (Figure 1). Nematode *Wolbachia* belong to supergroups C (i.e. from *O. volvulus*, *Dirofilaria immitis* and relatives of both genera), D (i.e. from *B. malayi*, *W. bancrofti* and relatives of both genera) [4,11] and F (from *Mansonella ozzardi*) [12]. The majority of arthropod *Wolbachia* belongs to supergroups A and B. Supergroups E, F and G have been described from only a restricted set of crustacean, chelicerate, hexapod and nematode hosts.

Why do filarial nematodes carry *Wolbachia*? Are these bacteria parasites of parasites, affecting nematode biology in ways that are still not understood, or do they have an essential role in the parasitic lifestyle of nematodes? One way to answer these and other questions is to compare the genomes of *Wolbachia* to identify pathways and products that might have roles in the interface between bacterial and eukaryotic cells. In filarial nematode *Wolbachia*, for which there is evidence that *Wolbachia*-derived products affect the immune system of the hosts of the nematodes [13,14], genome sequencing might also identify *Wolbachia* genes that help the nematodes to survive.

From the *Wolbachia* Genome Consortium (held in 1999) [15] came a plan to sequence representative genomes that covered the diversity of *Wolbachia*. The first *Wolbachia* genome – from *w*Mel, a supergroup A endosymbiont of *Drosophila melanogaster* that causes cytoplasmic incompatibility (Figure 1) – was published in late 2004 [16]. More recently, additional *Wolbachia* genome sequences have been published: two additional *Drosophila* host species, targets of nuclear genome sequencing, have yielded incomplete but informative clade A *Wolbachia* genomes [17] (Table 1), and a five-year effort has produced the complete genome sequence of *w*Bm, a supergroup D *Wolbachia* from *B. malayi* [18]. Informatively, genome sequences are also now available for related pathogens: *Rickettsia* (several species) [19–21], *Anaplasma marginale* [22] and *Ehrlichia ruminantium* [23] (Table 1). What

Corresponding author: Blaxter, M. (mark.blaxter@ed.ac.uk).

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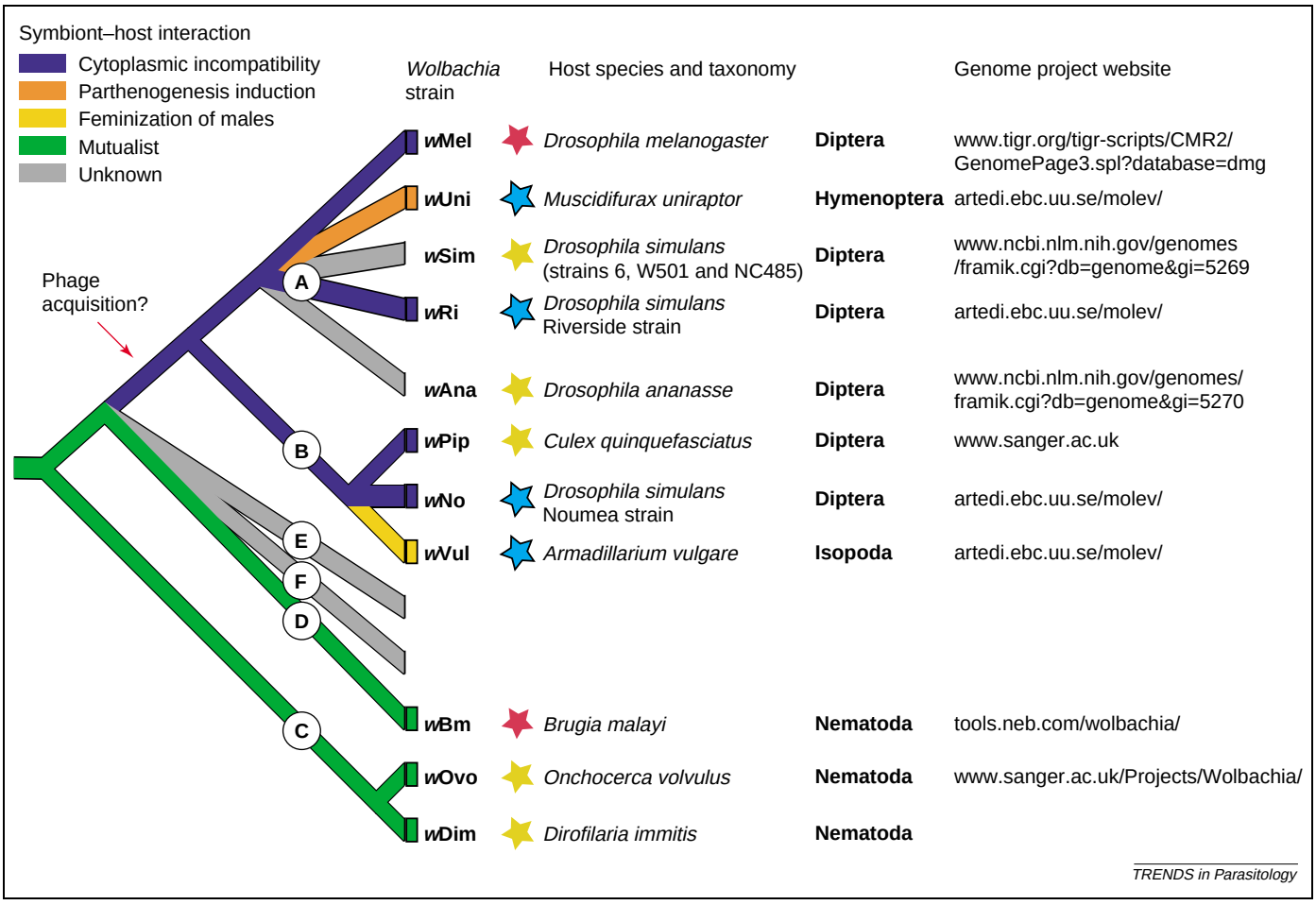

Figure 1. *Wolbachia* genomes currently being sequenced. There are six supergroups of *Wolbachia* (A–F), which are defined by phylogenetic analysis of 16S rRNA, *Wolbachia* surface protein and *ftsZ* genes [10]. Complete sequences have been determined for two *Wolbachia* genomes (red stars): *w*Mel of *Drosophila melanogaster* in clade A [16] and *w*Bm of *Brugia malayi* in clade D [18]. Partial sequences of three *Wolbachia* genomes were assembled from whole-genome shotgun-sequencing projects that are underway for two *Drosophila* species [17], and sequencing is underway for several other *Wolbachia* strains (yellow stars denote partial sequences that are available publicly; blue stars denote strains for which information has not yet been released). These additional genomes include two from clade D *Wolbachia* (*w*Ovo from *Onchocerca volvulus* and *w*Dim from *Dirofilaria immitis*), three from clade B and four from clade A. No clade E or F genomes are currently being sequenced, and additional *Wolbachia* supergroups might exist. To the left of the tree, symbiont–host interaction biology is mapped onto a *Wolbachia* phylogeny based on 16S rRNA genes [4]. The root of the *Wolbachia* tree might lie between clade D and the remainder of the supergroups (implying that *Wolbachia* have evolved from mutualistic symbiosis with nematode hosts to parasitic relationships with arthropod hosts) or between the nematode (C and D) and arthropod supergroups. Red arrow indicates where during evolution the *Wolbachia* WO phage could have invaded a pre-A and pre-B supergroup ancestor. Interestingly, acquisition of the phage correlates with evolution of the parasitic phenotype.

information have these genome sequences provided about the relationships of *Wolbachia* with their hosts and do they suggest new routes of drug treatment for filarial symbioses [22,24]? Because sequences are available for both parasitic and putative mutualist *Wolbachia*, are there obvious genomic features associated with parasitism? As with all genome-sequencing projects, the data answer some old questions and raise exciting new ones.

Life inside a eukaryotic cell

A common feature of intracellular symbionts such as *Wolbachia* is the loss of genetic material following adaptation to the host. Intracellular bacteria jettison 'exotic' metabolic capabilities and evolve a reliance on the basic metabolic pathways of their host [25]. This is evident in the completed *Wolbachia* genomes, which encode limited metabolic capacity [16,18]. For example, endogenous amino acid synthesis is extremely limited and *Wolbachia* import host amino acids for protein synthesis and for energy production through the tricarboxylic acid cycle and gluconeogenesis. In *Rickettsia* species, an ADP–ATP exchange protein enables the bacteria to scavenge ATP energy equivalents directly from the host cell [21] but this system is absent from *Wolbachia*, which can synthesize the full range of purine and pyrimidine nucleotide triphosphates. Interestingly, there is only a small number of regulatory genes in these genomes and, thus, differential mRNA expression in response to different host lifecycle stages or stress is likely to be minimal. The 'complete parts list' of *Wolbachia* indicates why these bacteria cannot survive outside their host cells, but perhaps this information could be used to devise culture conditions under which these essential nutrients are supplied. Such an extracellular culture system would be of great use in analyses of the genome and phenotype of other *Wolbachia* strains.

Surprisingly, given the drive towards miniaturization, both of the sequenced *Wolbachia* genomes contain a large proportion of repeated sequences, which is unusual in bacteria [16,18]. In the *w*Mel genome, these repeats are associated with a large number of insertion elements [16]. Although the *w*Bm genome has fewer

Table 1. The sequenced genomes of some rickettsial intracellular pathogens and symbionts

Species	*Wolbachia pipientis*				*Rickettsia conorii*	*Rickettsia prowazekii*	*Rickettsia typhi*	*Ehrlichia ruminantium*	*Anaplasma marginale*
Strain	*w*Mel	*w*Bm	*w*Ana[a]	*w*Sim[a]	Malish 7	Madrid E	Wilmington	Welgevonden	St Maries
Host species	*Drosophila melanogaster*	*Brugia malayi*	*Drosophila ananasse*	*Drosophila simulans*	Vertebrate pathogens with arthropod vectors	Vertebrate pathogens with arthropod vectors	Vertebrate pathogens with arthropod vectors	Vertebrate pathogens with arthropod vectors	Vertebrate pathogens with arthropod vectors
Genome size (Mb)	1.268	1.080	(1.441)	(1.063)	1.269	1.112	1.111	1.516	1.198
Number of protein-coding genes	1271	806	(1837)	(790)	1374	835	838	888–958[b]	949
Coding proportion of genome (%)	85	67	NE[c]	NE	81	76	76	86	62
Number of proteins with match to InterPro IPR002110 ankyrin domain	23 (+6)[d]	9	(34)	(25)	7	3[e]	2	5	4
Number of pseudogenes	94	98	NE	NE	41	11	101	32	14
GC content (%)	35	34	(35)	(35)	32	29	28	27	49
Refs	[16]	[18]	[17]	[17]	[20]	[21]	[19]	[23]	[22]

[a]The genomes of the *Wolbachia* endosymbionts of *D. ananasse* and *D. simulans* are incomplete [17] and, thus, the estimates of gene content are subject to error. The values in brackets are based on the length of assembled sequence obtained. The predicted genome size of *w*Ana is ~20–30% greater than of other *Wolbachia* strains, and the number of genes is similarly higher. Our analysis of this sequence indicates that the assembly contains many duplicated regions, possibly because of misassembly (indeed, 34 of the genes from *w*Ana seem to be of insect origin) or the presence of more than one *Wolbachia* strain in the sequenced *D. ananasse* stock (C. Whitton and M. Blaxter, unpublished). Infections of arthropods with multiple *Wolbachia* strains are relatively common. Initial analysis of *Drosophila mojavensis* whole-genome shotgun data suggested that a third partial *Wolbachia* genome was present [17] but this has subsequently been shown to be because of the mislabelling of sequence reads from other drosophilid projects [58,59].

[b]The number of protein-coding genes in *E. ruminantium* strain Welgevonden is under debate because two independent groups have predicted 888 (GenBank accession number CR767821) and 958 (GenBank accession number CR925678) proteins; *E. ruminantium* strain Gardei has a genome of 1.500 Mb and 950 predicted proteins (GenBank accession number CR925677).

[c]Not estimated (NE) because the genome sequences are partial.

[d]Although only 23 ANK genes were predicted in the genome publication, 29 different *w*Mel genes have matches to the ankyrin domain model in InterPro IPR002110.

[e]One of the *R. prowazekii* ANK genes is a RecJ homologue that is also present in the other *Rickettsia* genomes. However, the RecJ of other *Rickettsia* species does not have a significant match to the ankyrin domain model and, thus, the presence of the ankyrin domain model in *R. prowazekii* RecJ is likely to be an overprediction.

repeats than does the *w*Mel genome (5.4% compared with >14% of the genome, respectively), they are associated with breakpoints in synteny between the two genomes. Other members of the order Rickettsiales also have a relatively large proportion of repeats [19–23], suggesting that it is a feature of this family of bacteria that might be linked to the generation of novelty by gene duplication.

What do *Wolbachia* do for, or to, their hosts?

Because *w*Mel and *w*Bm differ in their phenotypic effects, comparison of their genomes has focused on functions hypothesized to be directly involved in parasitism or mutualism. Three major groups of genes have been investigated: (i) those involved in the type IV secretion machinery and the proteins that it might secrete; (ii) those encoding an unexpected diversity of proteins containing ankyrin-repeat motifs; (iii) and those present in apparently mobile bacteriophages.

Bacterial type IV secretion systems are leader-peptide-independent mechanisms for exporting effector proteins, or 'virulence factors', and are implicated in the pathogenesis of many bacterial species. Complete type IV systems, as two operons, are present in both of the sequenced *Wolbachia* genomes [16,18]. Intriguingly, one operon is closely associated with a homologue of the *Wolbachia* surface protein (WSP), a molecule that might be exported to the vacuole in which the bacterium resides [16]. Is wspB a virulence factor of *Wolbachia*? What other proteins are exported by this mechanism? The genome sequences suggest many candidates, including some of the ankyrin-repeat proteins.

The ankyrin-repeat domain is a motif of ~33 amino acids that is found in many eukaryotic proteins, often in tandem arrays, in which it mediates protein–protein interactions [26]. There are >110 different ankyrin-repeat-containing (ANK) proteins in *D. melanogaster* [27] – including cell-cycle regulators that are active in the early zygote, such as PLUTONIUM [28,29]. ANK proteins have been found in bacteria, in which they are implicated in host–pathogen interactions [30], but usually only a small number is found per genome (Table 1). The *w*Mel genome contains a surprisingly large number (23) of ANK genes, some of which are secreted [16] (Table 1). The *Wolbachia* of *Drosophila ananasse* (*w*Ana) has 34 putative ANK proteins but this number could be large because of unassembled duplications (Table 1). *w*Bm has only nine ANK genes but several of them seem to be pseudogenes [18], and of those that seem to be functional only two are orthologues of *w*Mel ANK proteins. *Wolbachia* ANK genes might be involved in regulating the host cell cycle or in interacting

with the host cytoskeleton, possibly through mechanisms related to *plutonium* function [29,31].

Eight of the *w*Mel ANK genes are located within integrated prophage segments. These *Wolbachia*-specific (WO) bacteriophages were discovered in the flour moth *Ephestia kuehniella* and have been identified in many arthropod *Wolbachia* strains [32,33]. There is evidence that WO phages can move both within a bacterial genome and between bacteria in multiply infected hosts. Because bacteriophages can drive the evolution of bacterial genomes by transducing genes of exotic function across species barriers, they are often a source of innovation in terms of introducing genes, in novel combinations, into novel genomic environments. As would be expected from their sequestered habitat, the *Wolbachia* genomes contain few, if any, gene segments that have the signature of lateral gene transfer from other bacteria [16]. Thus, WO phages might have an important role in generating diversity within arthropod *Wolbachia* [34,35]. Closely related *Wolbachia* strains can induce different phenotypes in their arthropod hosts, whereas distantly related strains can induce the same phenotype; are these differences mediated by genes that are transduced by WO phages [34,35]? It is striking that the *w*Bm genome does not contain WO prophages or any obvious remnants thereof [18]. The small number of ANK genes in *w*Bm is linked to this lack of WO-phage-encoded members. More filarial *Wolbachia* strains must be analysed to determine whether prophages are absent from supergroups C and D, and whether the association between parasitism and WO phages holds up. ANK and other genes in the WO phage are now prime targets for functional genomic analysis of putative 'genes for parasitism'. Excitingly, genetic analysis of the cytoplasmic incompatibility induced by *Wolbachia* parasites of the malaria-transmitting mosquito *Culex quinquefasciatus* suggests a core role for highly variable WO-phage-associated ANK genes in the incompatibility reaction [36]. Are ANK genes and WO phages involved in all of the phenotypes induced by parasitic *Wolbachia*?

Metabolic mutualism?

Does the *w*Bm genome assist the identification of candidate metabolic pathways that could account for its mutualism with *B. malayi* [18]? Tetracycline treatment has multiple effects on nematodes that could be used to identify the physiological processes in which *w*Bm has a major role (Table 2). *w*Bm has complete sets of riboflavin and haem biosynthesis genes [18] and, based on data from the ongoing *B. malayi* nuclear genome project [37], it is thought that *B. malayi* cannot synthesize riboflavins or haem endogenously. Haem is essential for cytochrome functions: for example, the modification of hormones such as ecdysteroids. Although there is a lack of compelling evidence of the role of ecdysteroids in *Caenorhabditis elegans* physiology, ecdysteroids are involved in moulting and reproduction in filarial nematodes [38–40]. However, some nematodes – including *C. elegans* – also lack the ability to synthesize haem [41], yet do not seem to be dependent on an endosymbiont as a source of this metabolite. Filarial nematodes also have access to other sources of haem. The *Wolbachia*-positive rodent filarial parasite *Litomosoides sigmodontis* takes up host red blood cells (and, thus, the encapsulated haem) into its gut during growth [42]. The existence of filarial nematodes lacking *Wolbachia* [1,4,43–45] also indicates that there is not an exclusive role for *Wolbachia* in the provision of vital nutrients, although these bacteria might supplement other sources in a restrictive environment. The *w*Mel genome also encodes these pathways; do arthropod *Wolbachia* also have a mutualist characteristic?

These postulated dependencies between *w*Bm and *B. malayi* indicate the movement of metabolites between the two organisms. It remains unclear how this traffic can cross both the host vacuolar membrane and the bacterial cell walls. Further investigation of such transfers must be initiated because they could be excellent drug targets.

Immunological enigmas

The presence of a bacterial symbiont within a metazoan parasite challenges simple models of how an intact immune system should respond. Filarial disease is characterized by specific immunosuppression and extreme longevity of adult nematodes in otherwise immunocompetent hosts [46]. Do *Wolbachia* products have a role in the induction and maintenance of this state? An early part of the mouse immune response to *Wolbachia*-infected filarial nematodes is characterized by an innate response involving Toll-like receptor (TLR)-4 – a mode of response that indicates a bacterial component of induction [13,14,47]. The inducing component has properties similar to those of lipopolysaccharide (LPS), in that it is blocked by specific LPS inhibitors [12]. Other Anaplasmataceae do not express LPS [48], and the relevant biosynthetic machinery is absent from the genomes of *Wolbachia*, *Anaplasma* and *Ehrlichia* [19–23]. None of the analysed genomes encodes the pathways for synthesizing lipid A, the murein sacculus synthetic machinery of these bacteria is incomplete and genes involved in maintenance

Table 2. *w*Bm metabolic pathways that might supply essential products to the host *Brugia malayi*

*w*Bm metabolic pathway	Physiological importance	Possible tetracycline-induced phenotypes
Riboflavin and flavin adenine dinucleotide biosynthesis	Essential coenzymes; biosynthesis genes are lacking from *B. malayi*	Failure to grow to maturity [6]
Haem biosynthesis	Prosthetic group of cytochromes that catalyses the biosynthesis of steroid hormones	Delayed or abortive moulting, delayed reproductive maturation [6]
Nucleotide biosynthesis	Might supplement host nucleotide pool	Disruption of oogenesis and embryogenesis [6]
Glutathione biosynthesis	An essential metabolite for protection against oxidative stress; might supplement host defences	Failure to grow to maturity [6]

of the outer membrane are absent. The composition of the cell wall of these bacteria remains unknown, despite the availability of their genome sequences. It is predicted that the *w*Bm peptidoglycan is not extensively crosslinked through amino sugars but it might have novel peptidic composition [16].

A major response to *Wolbachia* products, notably the WSP, can be detected in infected humans and in animal models; this response is biased towards *Wolbachia* in the context of the nematode third-stage infective larvae [49,50]. The much lower relative concentration of *Wolbachia* in third-stage larvae compared with that in adult nematodes [51,52] suggests that there is a stage-specific expression of, or heightened exposure to, bacterial antigens. WSP is recognized in a TLR-2-dependent and TLR-4-dependent manner and induces a T helper (Th)1-type response, suggesting that it is an important bacterial stimulant of this specific arm of the immune system [50].

Other Alphaproteobacteria also have unusual cell walls, and these species also activate mammalian immune systems in novel ways. Natural killer (NK) cells are activated by glycolipids bound to the cell-surface molecule CD1d. In infections with several LPS-negative *Sphingomonas* species and *Ehrlichia muris*, CD1d on, for example, dendritic cells presents glycosphingolipids to NK cells, thus activating the NK cells in an antigen-specific manner [53–56]. In the model systems tested, this activation was independent of TLR signalling. We suggest that, given the relationships of these bacteria, *Wolbachia* induces an immune response, through glycolipid antigens other than LPS, that has a role in nematode survival. The bacterial pathways of glycosphingolipid biosynthesis have not yet been elucidated [57], although analogues have been studied in yeast and mammals (in which their involvement in inherited disease and the nervous system has spurred much work). It is believed that the pathways are at least similar to those in animal cells but the genomes of Alphaproteobacteria do not encode clear orthologues of the glycosyl transferases involved (M. Blaxter, unpublished). Are these pathways potential new drug targets that will both disable the bacteria and, simultaneously, incapacitate the immune-evasion strategies of the nematodes?

Concluding remarks

The discovery of *Wolbachia* in nematodes is one of the most exciting recent developments in filarial research, and the *Wolbachia* genome sequences, in conjunction with the forthcoming completion of the nuclear genome sequence of *B. malayi*, will spur additional discoveries. The predictions made from the *Wolbachia* genome sequences must now be tested. Do the ANK genes interact with host-cell products? Are WO phages mediators of parasitic behaviour [36]? Which metabolites are transported and how is this achieved? Which components of the bacteria interact with the early innate immune system of the mammalian host and how does this affect nematode survival? Most important is the use of this information to devise effective and lasting intervention strategies for filarial disease [24], in which drugs eliminate *Wolbachia* and nematodes from communities while avoiding, as much as possible, the risk of selecting for genetic resistance or for *Wolbachia*-cured but healthy nematodes.

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