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Review article

The symbiotic relationship between filarial parasitic nematodes and their *Wolbachia* endosymbionts – A resource for a new generation of control measures

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Abstract

Filarial parasites are responsible for millions of human infections each year, mostly in developing parts of the world. International programs supported largely by the World Health Organization have worked to control the impact of onchocerciasis and lymphatic filariasis using mass administration of single or combination drugs for long periods of time in eligible populations. The success of these programs is now being hampered by the probability of programmatic failure in the event of emerging drug resistance. Additional research is critically needed to develop a new generation of tools for the control and treatment of these infections. These would include drugs that target adult worms and vaccines, with the goal to overcome potential resistance to the currently available drugs and complement present control measures. The majority of human filarial parasites carry intracellular symbiotic bacteria, *Wolbachia*, that appear to be essential for development and reproduction of the parasite. The recent availability of genomic data for both filaria and *Wolbachia* provides insight into essential aspects of the symbiotic relationship between the endosymbiont and its nematode host. We present an overview of how this knowledge opens up avenues in the identification of new targets for the control of these parasitic infections.

Keywords: *Wolbachia*, filaria, mutualism, symbiosis, *Brugia malayi*, *Onchocerca volvulus*, vaccines, drugs

1. Introduction

Filarial parasites represent important pathogens of humans infecting close to 150 million people worldwide, causing debilitating diseases including river blindness due to *Onchocerca volvulus*, and lymphatic filariasis (elephantiasis) due to *Brugia malayi* and *Wuchereria bancrofti*. Filarial nematodes are all obligate parasites of mammals, and therefore rely upon a successful interaction with the host to survive. Of 28 species of filaria, all except two harbor the intracellular symbiotic bacteria *Wolbachia*. *Wolbachia* are alphaproteobacteria related to Rickettsial pathogens (Fenn and Blaxter, 2004). Nematode *Wolbachia* are found only in vector transmitted filarial worms and the relationship between the endosymbiont and their filarial nematode hosts is one of mutualism (Fenn and Blaxter, 2004). The endosymbiont is thought to be necessary for

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several essential physiological functions of the parasite, including reproduction and molting (Bandi et al., 1999; Hoerauf et al., 1999; Casiraghi et al., 2002). In return, the bacteria need the parasite for their continued existence as they cannot replicate outside of their host cells. The *Wolbachia* endosymbiont apparently interacts with the mammalian host as well, as proteins expressed by the bacteria are known to be involved in some of the pathologic manifestations associated with infection with filarial parasites (Bandi et al., 2001; Gillette-Ferguson et al., 2006; Hise et al., 2007). Recently, the genome sequences of both *B. malayi* (Ghedin et al., 2007) and its *Wolbachia* endosymbiont (Foster et al., 2005a) have been determined, revealing interesting biology and complemented gaps, supporting the hypothesis of a mutualistic relationship. The *B. malayi Wolbachia* (*w*Bm) is similar to related *Rickettsia* and to *Wolbachia* found in *Drosophila* (*w*Mel). These organisms have reduced genomes typical of endosymbiotic bacteria. The *w*Bm genome is the most reduced in size yet it has retained more intact metabolic pathways than *Rickettsia* (Foster et al., 2005a).

Filarial infections have been treated for decades using a small group of drugs that include diethylcarbamazine (DEC), the benzimidazoles (e.g. albendazole), and the avermectins (ivermectin, moxidectin). Ivermectin is a microfilaricide which predominantly targets the larval stages of the parasite. It is highly effective in the short-term, but requires repeated administration over years due to the length of time of adult survival (up to 8 years for lymphatic filarial worms and more than 25 years for *O. volvulus* adult worms). The benzimidazoles and avermectins target, respectively, cytoskeletal components and ion channels that are conserved in mammals; the drugs therefore can be toxic to humans. Resistance to these compounds and larval recrudescence is also increasingly common (Cook et al., 2001; Osei-Atweneboana et al., 2007). There is no vaccine to prevent filariasis.

The severe social and economic impact of filariasis on some of the poorest populations in the world (e.g. sub-Saharan Africa and the Indian subcontinent) has attracted the attention of the international community, which is currently sponsoring onchocerciasis control programs in Africa and the Americas, along with filariasis control programs in 34 countries worldwide (Molyneux et al., 2003). These programs are presently only based upon a single strategy: the mass distribution of microfilaricidal drugs (Molyneux et al., 2003). The success thus rests upon achieving high coverage rates in the eligible populations for a long period of time, something that is expensive and logistically difficult. Furthermore, the reliance upon a single drug or drug combination as a control strategy increases the probability of programmatic failure in the event that drug resistance emerges. For these reasons, additional research is critically needed to develop a new generation of tools for control and treatment for these infections, including macrofilaricides (drugs targeting adult worms) and vaccines, to overcome potential resistance to the currently available drugs and to complement present control measures.

2. *Wolbachia* as a Drug Target in Filariasis Treatment

Recently, DNA sequencing of the genome of *Wolbachia* from *B. malayi* (*w*Bm) was completed (Foster et al., 2005a). The annotation identifies several potential foci that help understand the co-dependency of the host-endosymbiont relationship. Such information will aid in the identification of potential drug targets against filarial diseases. The absence of lactate dehydrogenase in *w*Bm and the lack of sugar transporters or sugar kinases implies that the abundant excretory metabolites, lactate and succinate, which are the major products of glucose utilization in filarial nematodes do not serve as growth substrates for *w*Bm. Instead it appears that pyruvate and Krebs cycle intermediates derived from amino acids are utilized in gluconeogenesis (the reverse of glycolysis). Enzymes for amino acid degradation are present in *w*Bm, as is a pyruvate dehydrogenase complex, a complete Krebs cycle and respiratory chain elements typical of alphaproteobacteria. The *w*Bm genome encodes many more proteases and peptidases than *Rickettsia* which likely degrade host proteins in the extracellular environment. Two Na+/alanine symporters not present in *Rickettsia* are encoded by *w*Bm, in addition to amino acid symporters of the PolE family (Foster et al., 2005a).

The ability to provide riboflavin, flavin adenine dinucleotide (FAD), heme and nucleotides are likely to be among the contributions of *w*Bm to the symbiotic relationship with its host. Unlike *Rickettsia*, *w*Bm contains all enzymes for biosynthesis of riboflavin and FAD and has complete pathways for *de novo* synthesis of purines and pyrimidines. This last feature is in contrast not only to *Rickettsia* but also many other endosymbionts and parasites such as *Buchnera*, *Blochmannia*, *Mycoplasma* and *Chlamydia*. A likely major role of the nematode host in this symbiotic relationship is to provide amino acids required for bacterial growth, since *w*Bm has very limited amino acid biosynthetic capacity. The cell wall biosynthesis pathways are devoid of genes required for the biosynthesis of lipopolysaccharide (LPS), similar to *w*Mel (Wu et al., 2004), *Ehrlicha* and *Anaplasma sp.* (Lin and Rikihisa, 2003). In addition, an unusual peptidoglycan structure is suggested with some possible similarities to peptidoglycan-derived bacterial cytotoxins. *w*Bm likely makes unmodified peptidoglycan while *w*Mel has retained genes that can modify peptidoglycan with oligosaccharide. Differences in peptidoglycan structure between *w*Bm and *w*Mel further suggest adaptations to their respective mutualistic or parasitic lifestyles. Both *w*Bm and *w*Mel lack many genes

involved in membrane biogenesis, rendering them unable to produce lipid A, the usual component of proteobacterial membranes. Possibly both organisms incorporate cholesterol into the cell wall as observed in *Ehrlichia* and *Anaplasma* (Lin and Rikihisa, 2003).

Other features include a common type IV secretion system and an abundance of ankyrin domain-containing proteins, which could regulate host gene expression as suggested for *Ehrlichia phagocytophilia* AnkA (Park et al., 2004), as well as several proteins predicted to localize to the cell surface. Ankyrin proteins are of interest because of their roles involving protein-protein interactions in a variety of cellular processes. Of the 12 ankyrin genes in *w*Bm, 7 are pseudogenes and of the remaining 5, at least 4 are expressed as evidenced by RT-PCR and preliminary microarray experiments (Ware, Foster and Slatko, unpublished; Scott and Slatko, unpublished).

The *Wolbachia* surface protein (WSP) contains transmembrane domains and a standard signal peptide for secretion. The protein shows similarity to the major outer membrane protein of *Ehrlichia*. WSP is one of several *Wolbachia* proteins to which antibody responses have been observed (Taylor et al., 2005) and WSP immunoreactivity has been shown to correlate with the onset of chronic filarial morbidity (Punkosdy et al., 2003; Taylor et al., 2005; Turner et al., 2006). WSP, as well as additional candidate surface proteins may be useful for phylogenetic and immunological studies and as potential therapeutic targets. Eighteen putative membrane surface proteins, in addition to WSP, have been identified in the *w*Bm genome. A number of other molecules are of interest as potential drug targets. For example, glutathione biosynthesis genes may provide a source of glutathione for the protection of the host nematode from oxidative stress or immunological effector molecules. Heme produced from *w*Bm (6 of 7 synthesis genes are present) could be vital to worm embryogenesis, as there is evidence that molting and reproduction are controlled by ecdysteroid-like hormones (Warbrick et al., 1993), whose synthesis requires heme. Depletion of *Wolbachia* might therefore halt production of these hormones and block molting and/or embryogenesis. In this context, it is interesting to note that most, if not all, nematodes, including *B. malayi*, appear to be unable to synthesize heme, but must obtain it from extraneous sources, such as the media, the food supply, or perhaps, via endosymbionts.

Of further interest is the observation that *w*Bm may be an essential source of nucleotides for the host, especially during embryogenesis where the nucleotide requirement may be high. Further, in the host *B. malayi* genome sequence, the purine metabolism genetic pathway appears to be absent (Ghedin et al., 2007). This may provide yet another area of potential drug targeting.

3. Host Immune Responses to *Wolbachia* in Filarial Infections

Pathogenesis in onchocerciasis is largely due to the presence of microfilaria. During the infection cycle, both adults and microfilaria become surrounded by many immune modulators, including macrophages, eosinophils, neutrophils, NK cells, mast cells, B and T cells, indicating that both arms of the immune response are involved (Brattig, 2004). However, the major cause of pathogenesis is due to the death of microfilariae causing dermatitis reactions and edema. More importantly, inflammation in the eye causes opacification and, following recurrent inflammatory responses, scarring of the cornea leading to the river blindness phenotype.

In contrast, the pathogenesis in lymphatic filariasis is largely due to the death of the adult worms in the lymphatic vessels. Secondary infections due to lymphatic system damage can occur, in addition to recurrent inflammation responses which can lead to elephantiasis (Turner et al., 2006). However, secondary infections are not the major cause of lymphedema and elephantiasis.

In both lymphatic filariasis and onchocerciasis, *Wolbachia* have been implicated as having a major involvement in the pathogenesis, as many of the immune responses were not seen to occur in experiments with filarial parasites not harboring *Wolbachia* or by using extracts depleted of *Wolbachia* immune stimulatory molecules (Saint Andre et al., 2002; Taylor et al., 2005; Turner et al., 2006).

It has been proposed that the development of lymphedema in patients infected with *Wuchereria bancrofti* is associated with a switch from predominantly Th2 responses to Th1 responses (Lammie et al., 2002). Th2 responses are associated with larval development whereas Th1 responses are characterized as anti-filarial responses associated with lymphedema (Lammie et al., 2002). The transition from a Th2 to a Th1 response is potentially associated with worm death and release of *Wolbachia*. The increase of anti-WSP IgG following the onset of lymphedema development in human serum samples argues in favor of this theory (Punkosdy et al., 2003).

Following treatment with albendazole and ivermectin, which are standard anti-filarial treatments, adverse reactions are frequently observed, mostly fever, rash and itching. These reactions are associated with microfilaremia and increased *Wolbachia* levels in the serum (Turner et al., 2006). Treatment with tetracycline, an antibiotic that will affect *Wolbachia*, reduces severe and moderate post-treatment reactions (Turner et al., 2006). This implies that *Wolbachia* release following macro- and microfilaricidal treatment is a major factor in causing post-treatment reactions.

The important role of *Wolbachia* in the pathogenesis of river blindness is emphasized by the observation that a strain of *O. volvulus* that causes severe eye disease carries significantly more *Wolbachia* bacteria per worm than a mild strain (Higazi et al., 2005). In that study, large variations in *Wolbachia*/nematode DNA ratios were observed among individual parasites within one location, but severe strains of *O. volvulus* had a *Wolbachia*/nematode DNA ratio 12-fold higher than that of the mild strain. The reason behind the immunopathology is at least in part due to the major surface protein of *Wolbachia* (WSP) which on its own is capable of inducing immune responses (Brattig et al.. 2004). Earlier studies described this component as lipopolysaccharide-like (Brattig et al., 2000), however no lipopolysaccharide-like molecules from *Wolbachia* could be identified and genome annotation indicated lipopolysaccharide biosynthesis is lacking (Foster et al., 2005a). It has been demonstrated that the immune response in the form of neutrophil migration and corneal disease is dependant on the presence of *Wolbachia* (Saint Andre et al., 2002). In this study, worms were excised from patients treated with doxycycline and from untreated patients. Protein extracts from these worms were injected into mouse corneas and neutrophil infiltration appeared to be reduced in protein extracts that lacked *Wolbachia*. Along the same line of reasoning, it was demonstrated that nodules from the *Wolbachia* positive strain *Onchocerca jakutensis* contained neutrophil infiltrates whereas no neutrophils were found surrounding *Onchocerca flexuosa* – a *Wolbachia* negative strain (Brattig et al., 2001).

Toll-like Receptors (TLRs) are a family of 11 germline encoded receptors related to the insect 'Toll', and which recognize microbial products. Studies have sought to identify which TLRs are activated by *Wolbachia*, and to determine the role in a mouse model of river blindness. Assays with TLR reporter cells and macrophages from TLR gene knockout mice demonstrated that *Wolbachia* utilized TLR2 and TLR6 (Hise et al., 2007). *In vivo* murine models of river blindness identified a role for the common TLR adaptor molecule myeloid differentiation factor 88 (MyD88) in the recruitment of neutrophils to the corneal stroma (Gillette-Ferguson et al., 2006). The requirement of TLR2 remains even in the presence of adaptive immune responses (Daehnel et al., 2007). Most interestingly, only Th1-associated responses, such as neutrophil infiltration to the cornea and splenocyte IFNγ-production, depend on the presence of TLR2. Th2-associated responses such as IL-5 production by splenocytes and eosinophil infiltration into the cornea are, on the other hand, independent of TLR2 (Daehnel et al., 2007). Current studies are investigating the role of *Wolbachia* in the induction of Th1-associated responses. Post-treatment reactions are significantly decreased in the absence of *Wolbachia* (Keiser et al., 2002). Patients were treated with either diethylcarbamazine or ivermectin and serum samples were analyzed for the presence of *Wolbachia* DNA. Levels of *Wolbachia* DNA were correlated to the severity of post-treatment reactions and found to be positively linked (Keiser et al., 2002). All the evidence gained over the last few years points to *Wolbachia* as a main culprit for onchocercal disease and post-treatment reactions, likely due to *Wolbachia* release upon death of the worms and/or during worm development and reproduction.

4. Vaccine Development

As mentioned above, the *Wolbachia* endosymbiont is necessary for several essential physiological functions of the parasite, including molting and reproduction, processes that were also identified as potential targets for vaccines and/or macrofilaricides (drugs which kill or permanently sterilize the adult worms). One of the attempts to develop new means to control infection with the filarial nematode *O. volvulus* has mostly focused on the development of a prophylactic vaccine. Larval *O. volvulus,* including L3, molting L3 (mL3) and fourth-stage larvae (L4), were selected as the targets for the vaccine (Cook et al., 2001). It was anticipated that immune responses which kill any of these stages would prevent the development of adult worms and thereby production of their microfilarial offspring, which are responsible for the manifestation of disease. Moreover, assuming that there are common protective mechanisms against all filarial nematodes and that the target antigens may be similar, it was postulated that antigens, which appear to be protective against the *O. volvulus* parasites are potentially also relevant to protective immunity against the parasites *B. malayi* and *W. bancrofti.* Fifteen *O. volvulus* proteins were identified that are able to induce partial protection; 5 of which were protective in multiple experiments and were, therefore, the top candidates for development of a future vaccine (Lustigman et al., 2002; 2003). Comparative analysis of the *O. volvulus* vaccine candidates and the *B. malayi* genome confirmed that all 15 proteins are in fact also encoded by *B. malayi* genes and could, therefore, potentially serve as vaccine candidates against the lymphatic filariae.

5. Macrofilaricide Development

In the definitive host, filarial nematodes molt, grow and mature for a period following infection, after which they devote their energy almost entirely to microfilaria production. Therefore, another promising approach to discovery of new targets for anti-nematode vaccines and drugs is the identification of nematode specific genes essential for viability of larvae. The completion of the *C. elegans* genome and the development of high throughput RNAi screens in *C. elegans* combined with comparative

genomics have greatly bolstered such an effort. Recently, through a *C. elegans* genome-wide RNA-interference screen, novel *C. elegans* endrocrine and enzymatic regulators of molting were identified (Frand et al., 2005). Inactivation of 159 genes interfered with molting (molting defects in 10 to 100% of the treated worms were observed). Products of the 159 genes discovered include annotated transcription factors, secreted peptides, transmembrane proteins, and extracellular matrix enzymes essential for molting.

Molting is a conserved process in nematodes that involves three major steps: 1) separation of the old cuticle from the hypodermis (apolysis); 2) formation of new cuticle arising from the outermost surface of the hypodermis; and 3) the shedding of the old cuticle (ecdysis) (Singh and Sulston, 1978). The L3 to L4 molt of filarial nematodes occurs in the human host within 3–10 days after infection.

Using comparative analysis of the *C. elegans* molting genes identified through the genome-wide RNA-interference screen (Frand et al., 2005) and the peptide database for the *B. malayi* genome project, established that the majority of *C. elegans* essential proteins for molting are also expressed in *B. malayi*; some of which encode potentially attractive drug targets. For example, 46 out of the 47 essential *C. elegans* molting genes (>90% reduction in molting due to gene specific RNAi) belonging to the categories: novel, proteases, protease inhibitors, peroxidases, ECM, Sterol-sensing domain, DNA binding, Nucleic acid interacting, Signaling, WD domains and others, are also potentially encoded by *B. malayi* transcripts.

Identifying genes essential for the molting of *O. volvulus* and *B. malayi* could enable the development of safe and effective nematicides that target gene products conserved only in nematodes or ecdysozoans. For example, molting genes such as the extracellular matrix proteins NOAH-1 and NOAH-2 are conserved only in insects and nematodes. On the other hand, other molting genes such as proteases, like NAS-37 and NAS-36, may also represent attractive targets for the development of small-molecule antagonists, although they have homologues in mammals, given the success of drug development on protease targets for HIV (Cvetkovic and Goa, 2003). In a recent review (Craig et al., 2007), the authors propose to focus on anti-nematode drugs that are based on inhibition of key targets in the molting degradome. It appears that the importance of peptidases is becoming increasingly apparent, not only for controlled synthesis of the new cuticle and release of the old but also as mediators of signaling events.

The development of macrofilaricides for filarial infections has been refractory to traditional approaches. However, the recent advances in molecular biology and genomics have provided also outstanding new opportunities for basic research on filarial parasites as they have provided the tools required to identify and validate the functions of the filarial genes and the biochemical pathways in which they participate. Criteria desirable for an ideal macrofilaricide have been developed (Behm et al., 2005). One factor making identification of a macrofilaricide less daunting is that it would likely be co-administered with ivermectin, a drug that temporarily paralyzes and sterilizes the adult worm presumably making it more vulnerable to killing by a second agent. Moreover, a rapid comparative genomic filtering approach has been devised to support the identification and prioritization of molecular targets in nematodes for drug discovery (Kumar et al., 2007; Foster et al., 2005b; McCarter, 2004) that together with functional genomics filters, such as RNAi, can allow the selection of gene products essential for survival and reproduction of the filarial parasites within the human host.

Of particular interest is the possibility of targeting cysteine proteases that have been identified as potential targets for drug or vaccine development in many parasite systems (McKerrow et al., 1999; Newton and Meeusen, 2003). Based on indirect evidence, cathepsin L- and Z-like proteases of the filarial nematodes as well as their endogenous inhibitor, cystatin, were postulated to be involved in a variety of important biological processes: molting, cuticle remodeling, and embryogenesis (Lustigman et al., 1992; 1996; 2004; Guiliano et al., 2004). Functional analyses of *C. elegans* cathepsin L (Ce-cpl-1), cathepsin Z (Ce-cpz-1) and cystatin (Ce-cpi-2) have revealed that these genes are required for oogenesis, embryogenesis, and/or molting (Hashmi et al., 2002; 2004; 2006). To also establish the functions of homologous filarial genes, RNAi assays were performed on *O. volvulus* L3 and *B. malayi* female adult worms, resulting in successful transcript reduction and observed phenotypes; inhibition of molting and disruption in the process of embryogenesis, respectively (Lustigman et al., 2004) (S. Lustigman, unpublished results). These studies demonstrated that these genes are not only conserved among nematodes but that they are also likely to be functional orthologs.

As there are remarkable parallels between the roles of cysteine proteases and *Wolbachia* during filarial development, molting and embryogenesis, in our more recent studies we have begun investigating whether cysteine proteases also participate in the filariae/*Wolbachia* endosymbiotic relationship. We used double-strand RNA of *Bm-cpl-5* to knock out by RNAi the gene expression of this cysteine protease (S. Lustigman, unpublished results) and analyzed the presence of *Wolbachia* in the RNAi treated worms. Preliminary results show that the number of *Wolbachia* in the hypodermis and microfilariae was reduced considerably in the RNAi treated worms. In comparison, the number of *Wolbachia* in the oocytes and embryos was similar to those of normal worms (Fig. 1). The explanation behind this dramatic reduction is presently being investigated.

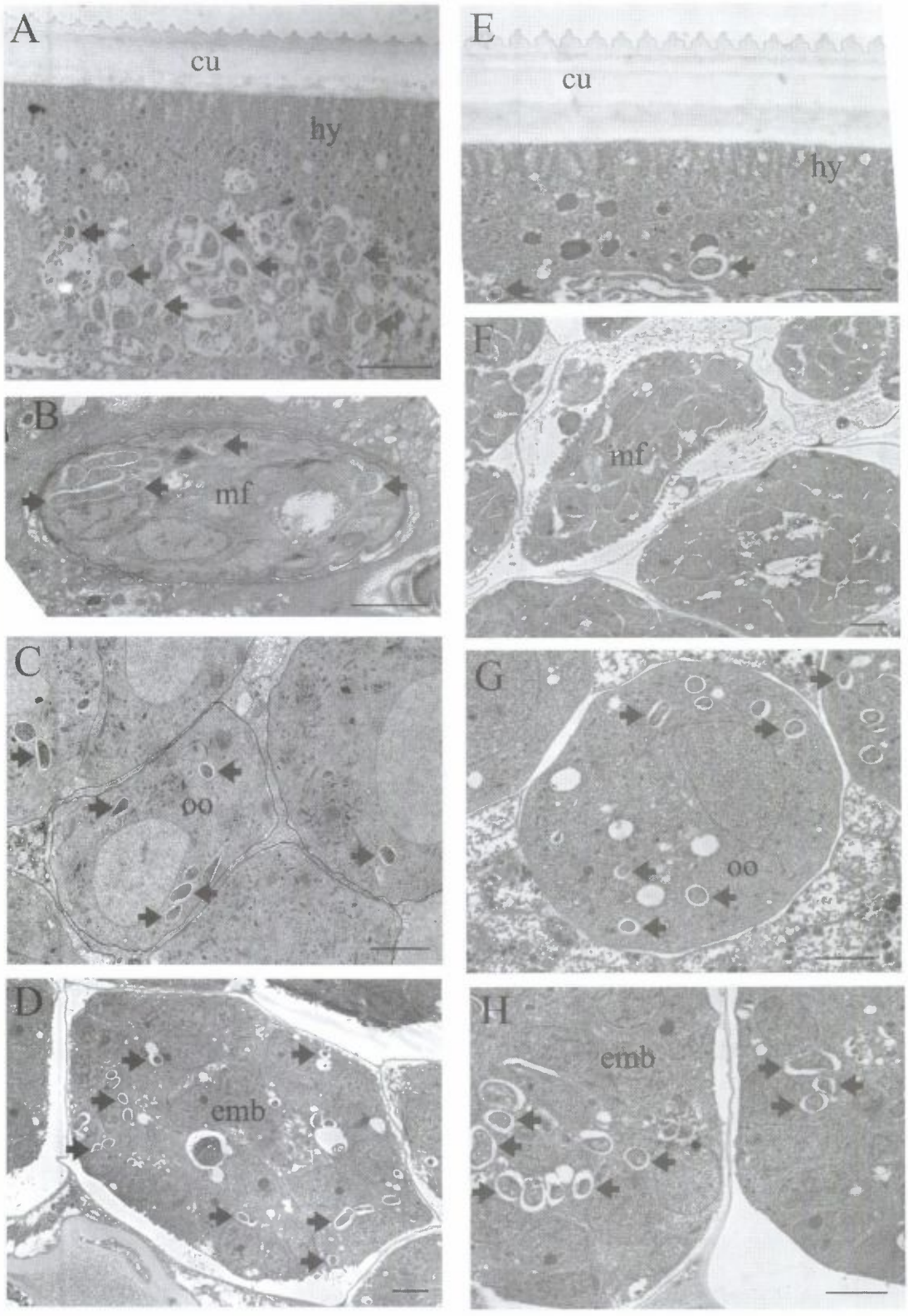

Figure 1. *Wolbachia* in normal female *B. malayi* worms and in *Bm-cpl-5* dsRNA treated worms. In normal female *B. malayi* worms *Wolbachia* (see arrows) is found in the hypodermis (A) and in all stages of the embryo's development (B, C, D). In *Bm-cpl-5* dsRNA treated worms, very few *Wolbachia* are found in the hypodermis (E), none were found in microfilaria (F), however, similar number of *Wolbachia* is found in the early development stages; oocytes and embryos (G, H). Abbreviations: cu - cuticle, hy - hypodermis, mf - microfilaria, oo - oocyte, emb - embryo. Bar = 2 microns.

6. Future Directions

The completion of the *B. malayi* and *w*Bm genomes offers a wealth of information, which may help to understand the molecular basis for the endosymbiosis between filarial nematodes and *Wolbachia*. We now know which metabolites may be potentially provided by *w*Bm to the nematode and which are presumably required by the endobacteria (and provided by the nematode). This may open up the exciting possibility to find and test drugs already registered for use in humans, which may inhibit key biochemical pathways in the *Wolbachia* that could lead to sterility or killing of the adult worms. Genome projects are also underway for the *Wolbachia* endosymbionts from *O. volvulus* and *Dirofilaria immitis*. The latter genome is presently the smallest sized *Wolbachia* genome at approximately 0.95 Mbp (Sun et al., 2001) compared to 1.1 Mbp for the *Wolbachia* from *B. malayi* (Foster et al., 2005a), and 1.27 to 1.66 Mbp for *Wolbachia* from different strains or species of *Drosophila* (Wu et al., 2004; Sun et al., 2001). Comparisons of these genomes may shed light on different endosymbiont-nematode symbioses and, in the case of the *Dirofilaria Wolbachia*, reveal even further areas of genome reduction.

The multiple effects of antibiotic depletion on filarial nematodes harboring *Wolbachia* and the dynamics of bacterial populations in different developmental stages suggest the worms have become dependent on the bacteria for a diverse range of biological processes that may have distinct stage-specific functions. Further studies incorporating biochemical and functional genomic approaches should help unravel the role of these different metabolic pathways throughout the nematode life cycle and identify those suitable as targets for novel anti-symbiotic and/or anti-filarial therapies that might indirectly affect the survival of the endosymbiont within the host and thus ultimately also kill the parasite or prevent its reproduction.

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