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THE BACTERIA *WOLBACHIA* IN FILARIAE, A BIOLOGICAL RUSSIAN DOLLS' SYSTEM: NEW TRENDS IN ANTIFILARIAL TREATMENTS

MARTIN C.* & GAVOTTE L.**

Summary :

Filarial nematode species can host *Wolbachia* bacterial endosymbionts. To understand the symbiosis, a higher level of complexity should be considered, taking in account the tripartite association between *Wolbachia*, filariae and mammals. This overview article discusses the biology of *Wolbachia* in filariae, including their distribution and phylogeny, mechanisms of action, inflammatory consequences on mammal host and biological control implications for filariases. Potential directions for future research are also discussed.

KEY WORDS : *Wolbachia*, Onchocercidae, filaires, symbiosis, antibiotherapy

Résumé : *WOLBACHIA* ET FILAIRES, UN SYSTÈME EN POUPÉE RUSSE: NOUVELLES TENDANCES DANS LES TRAITEMENTS ANTIFILARIENS

Les filaires sont des nématodes qui peuvent héberger des endosymbiontes bactériens Wolbachia. *Pour comprendre la symbiose, un niveau plus haut de complexité doit être considéré, qui prend en compte l'association tripartite entre* Wolbachia, *les filaires et leurs hôtes mammifères. Cette revue discute la biologie de* Wolbachia *dans les filaires, leur distribution et phylogénie, leurs mécanismes d'action, les conséquences inflammatoires sur l'hôte mammifère et les implications biologiques dans le contrôle des filarioses. Des directions potentielles pour la recherche future sont également discutées.*

MOTS CLÉS : Wolbachia, *Onchocercidae, filariae, symbiose, antibiothérapie.*

INTRODUCTION

Human filariases, *e.g.* lymphatic filariasis, onchocerciasis and loiasis, are parasitic diseases from subtropical areas affecting one person out of six in endemic areas (Hotez *et al.*, 2009; Molyneux *et al.*, 2009). They are caused by filarial nematodes. The life cycle of these parasites is complex, and includes a haematophagous vector. In this vector they develop from larval stage 1 (L1) to infective larval stage 3 (L3). They are inoculated in a vertebrate host where the L3 moults into larval stage 4 and into male or female adult worms producing L1 (microfilaria). The control of the haematophagous vectors and the anti-filarial chemotherapies (e.g. ivermectin) have been shown to reduce the disease and the transmission (Molyneux *et al.*, 2003; Specht & Wanji, 2009). However, the transmission continues even after 10-12 years of treatment in some situations (Borsboom *et al.*, 2003). The main problem with the current treatment strategy is that ivermectin kills the L1 but not the adults, thereby preventing complete interruption of transmission. The search for new strategies is justified by the following features: the re-emergence of onchocerciasis in areas so far controlled, the development of resistances to existing treatments (Bourguinat *et al.*, 2008, Bourguinat *et al.*, 2007), the co-endemicity with loiasis in which ivermectin treatment may induce serious side effects (Boussinesq, 2008; Mackenzie *et al.*, 2007), and the appearance of zoonoses due to animal *Onchocerca* species (Takaoka *et al.*, 2004; Takaoka *et al.*, 2005). Some of the filarial nematodes have a unique feature, as they harbour the mutualistic *Wolbachia* bacterial endosymbiont which is essential for worm development, fertility and survival (Hoerauf *et al.*, 2003b) and is also a component of inflammatory disease pathogenesis (Turner *et al.*, 2009). These mutualistic endosymbionts have been a novel target for antibiotic based therapy in filariasis. Identifying key molecules and pathways is critical to the understanding of this mutualistic bacterial-nematode symbiosis. It remains an area of active research and makes use of structural, functional genomics and proteomic analysis.

WOLBACHIA IN FILARIAE

DESCRIPTION, ABUNDANCE AND TISSULAR LOCALISATION

Wolbachia Hertig, 1936 are common and widespread symbiotic bacteria (order Rickettsiales) in arthropods. It is estimated that

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these Gram-negative alphaproteobacteria infect 20-80 % of all insect species (Hilgenboecker *et al.*, 2008) but are also present in many chelicerates (Baldo *et al.*, 2007; Goodacre *et al.*, 2006) and terrestrial crustaceans (Bouchon *et al.*, 1998).

Bacteria have been detected in filariae (Kozek, 1977; Kozek & Marroquin, 1977; McLaren *et al.*, 1975) and were later identified as *Wolbachia* (Bandi *et al.*, 1998; Casiraghi *et al.*, 2004; Sironi *et al.*, 1995). The presence of the bacteria is limited to the family Onchocercidae. They were not identified in other nematode groups infecting arthropods, during the larval (spirurids) or adult (mermithids, steinernematids) phase (Duron & Gavotte, 2007). The presence of a *Wolbachia*-like endosymbiont in a plant-parasitic nematode has also recently been described (Haegeman *et al.*, 2009).

In filarioid nematodes, *Wolbachia* have only been identified in two subfamilies of Onchocercidae: Onchocercinae and Dirofilariinae (Bain *et al.*, 2008; Casiraghi *et al.*, 2004). For example, *Wolbachia* is present in *Onchocerca volvulus*, the filaria responsible for human onchocerciasis, in *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*, agents of lymphatic filariases, and in *Dirofilaria immitis*, known as heart worm in dogs. However, in both subfamilies, some filarial species do not contain *Wolbachia* (Bain *et al.*, 2008; Casiraghi *et al.*, 2004). Indeed, on a broader number of analysed filarioid species, 10 out of 18 genera are positive for *Wolbachia*. The complexity of the presence/absence of *Wolbachia* in these genera is increased when the species level is considered. For example, among *Litomosoides* or *Onchocerca*, a large majority of analysed species are infected by *Wolbachia*; in the genus *Cercopithifilaria*, none of the analysed species was positive for *Wolbachia* except *C. japonica* (Uni, 1983; Bain *et al.*, 2008; Casiraghi *et al.*, 2004).

In filariae, *Wolbachia* infection appears to be fixed in natural populations of many species (Bandi *et al.*, 1998) as illustrated by the analysis of a large number of *Litomosoides sigmodontis* and *Onchocerca volvulus* specimens. A mutualistic relationship between bacteria and filariae is probably the source of this observation: advantages conferred to nematode by symbiont are so important that symbiotic nematodes exhibit a significantly higher fitness than aposymbiotics. However the picture may not be so clear regarding *Mansonella perstans,* as some geographic isolates of *M. perstans* may exist in which filariae contain or not *Wolbachia* (Keiser *et al.*, 2008).

Wolbachia from arthropods were mainly studied because they modify the reproduction of their host. In addition to being present in germ-line tissues, *Wolbachia* infections have been found in somatic tissues of several arthropods (Cheng *et al.*, 2000; Clark *et al.*, 2003). Many interactions have been reported, including cytoplasmic incompatibility (Dobson, 2004), genetic male feminisation (Bouchon *et al.*, 1998), killing of male embryos (Hurst *et al.*, 2000) or parthenogenesis (Huigens *et al.*, 2000). All these phenotypes allow bacteria to take advantage of the reproduction of infected females compared to those that are uninfected. These manipulations seem to have evolved toward a facilitation of invasion of host populations by *Wolbachia*.

In filariae, *Wolbachia* are located in the female reproductive apparatus (Fig. 1) but not in that of males (Bain *et al.*, 2008; Casiraghi *et al.*, 2004; Hoerauf *et al.*, 2003b). However, among the infected filarioid species, the tissue distribution appears to be more complex: the majority of filarial species harbour *Wolbachia* in the hypodermis of the lateral chords, such as many *Onchocerca*, *Brugia*, *Dirofilaria*, whilst other species are negative in the hypodermis, such as *Loxodontofilaria caprini* (Uni & Bain, 2006) and *O. dewittei japonica* (Uni, Bain & Takoka, 2001; Bain *et al.*, 2008). *Wolbachia* are transmitted vertically from female worms to progeny, through egg cytoplasm. *Wolbachia* are present in all larval stages, in the haematophagous vector as well as in the vertebrate host (Bandi *et al.*, 2001; Fenn & Blaxter, 2004). In microfilariae from *Onchocerca*, *Brugia* and *Dirofilaria*, *Wolbachia* are present in the hypodermal lateral chords (Egyed *et al.*, 2002; Kozek, 1977; McLaren *et al.*, 1975).

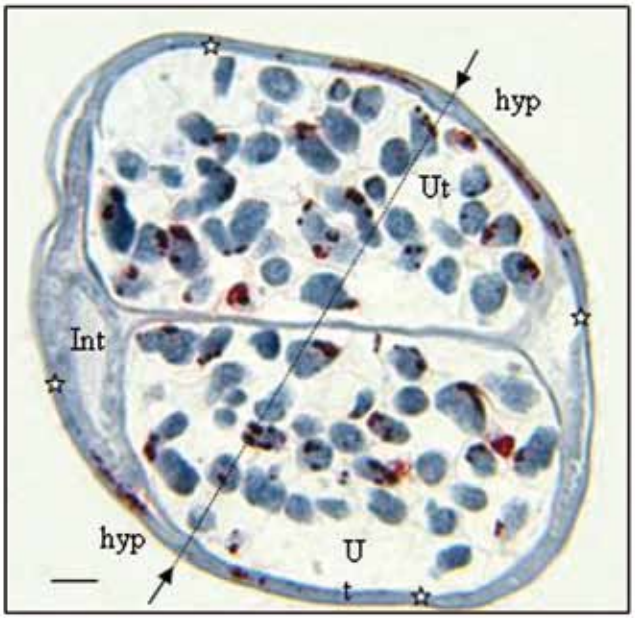

Fig. 1. – Visualization of *Wolbachia* in filaria by immunostaining. Transverse distal section of female *Litomosoides sigmodontis*, laboratory strain, showing two uteri with divided eggs, stained with a rabbit polyclonal antiserum against *Wolbachia* Surface Protein (WSP) of *B. pahangi Wolbachia* (Wol-Bp-WSP, dilution 1:2000, Streptavidin-HRP, AEC; hematoxylin counterstaining). *Wolbachia* (red spots) are observed in uterine contents (eggs) and in hypodermis (Int, Intestine; Ut, Uterus; black arrows and dot line represent the lateral plan; hyp, hypodermis delineated by white stars; Scale: bar = 25 µm.

Taxonomy, phylogenic position and co-evolution

Due to the specificity of *Wolbachia*, each strain is normally characterised according to three parameters: i) Host species; ii) Induced phenotype; iii) Genetic sequence (typically using *wsp* gene or MLST method). Strains are generally named following a specific nomenclature: w (for *Wolbachia*), the first letter of host gender, the three first letters of host species and a number or a letter to distinguish different strains in the host (characterized by induced phenotype or DNA sequences). Thus, a strain infecting *Drosophila melanogaster* is named wDmel. However, in some cases a precise identification is difficult to make. For example, the *Wolbachia* strains infecting the mosquito *Culex pipiens* cannot be discriminated using DNA sequences because they display dozens of different phenotypic strains. Rather, they are discriminated by complex crossing experiments based on the cytoplasmic incompatibility induced by the bacteria in this species (Guillemaud *et al.*, 1997; Laven, 1967; Sinkins *et al.*, 2005; Walker *et al.*, 2009). More recently, the multi-locus sequencing typing (MLST) method revealed multiple ambiguous lineages (Baldo & Werren, 2007). These studies further showed that *Wolbachia* is a highly diversified bacterium and that the exact taxonomic level of *Wolbachia* (species, gender or family) remains in question.

Wolbachia strains are distributed into 8 supergroups or types (Lo *et al.*, 2002; Lo *et al.*, 2007) (Fig. 2) named from A to H. The supergroup G has been discarded due to recombination issues (Baldo & Werren, 2007). However, the diversity may be higher because a new *Wolbachia* supergroup (type K) has been reported in the mite *Bryobia* species V (Ros *et al.*, 2009). Moreover, these authors defined new supergroups for the *Wolbachia* strains recovered from fleas (type I) and from a filarial species (type J). Although controversial, all of the supergroups are currently thought to represent one single taxon, namely *Wolbachia pipientis* Hertig, 1936 (Lo *et al.*, 2007; Pfarr *et al.*, 2007).

Three main types of *Wolbachia* have been associated with filariae (Bain *et al.*, 2008; Casiraghi *et al.*, 2004). Types C and D are exclusively hosted by filariae whereas type F is harboured by a few filarial species as well as by arthropods (Casiraghi *et al.*, 2005). However, additional molecular diversity is appearing among Onchocercidae. For example, *Wolbachia* from *Dipetalonema gracile* belongs to the supergroup J (Ros *et al.*, 2009) (work in progress).

Phylogenetic studies show contrasting results between arthropod endosymbionts, types A and B, and onchocercid endosymbionts, types C and D. The other types are not sufficiently documented. Arthropod *Wolbachia* phylogenies are not congruent with host phylogenetic trees (Werren *et al.*, 1995), even within closely related species groups (Schilthuizen & Stouthamer, 1997; Shoemaker *et al.*, 2002). This indicates that *Wolbachia* 'jump' between various host species (i.e. horizontal transfers) at an evolutionary scale. Since multiple infections are less abundant than would be expected by such a high level of horizontal transfer, it appears that the arthropod *Wolbachia* evolutionary dynamic is a large movement of acquisition and loss of hosts at an evolutionary scale (Vavre *et al.*, 1999).

On the contrary, while less information are available for filarial symbionts than for arthropods, a congruency between filarial hosts and bacteria phylogenies has been observed in supergroups C and D (Bandi *et al.*, 1998). For example, nine out of ten analysed *Onchocerca* species harbour the type C, and five out of six

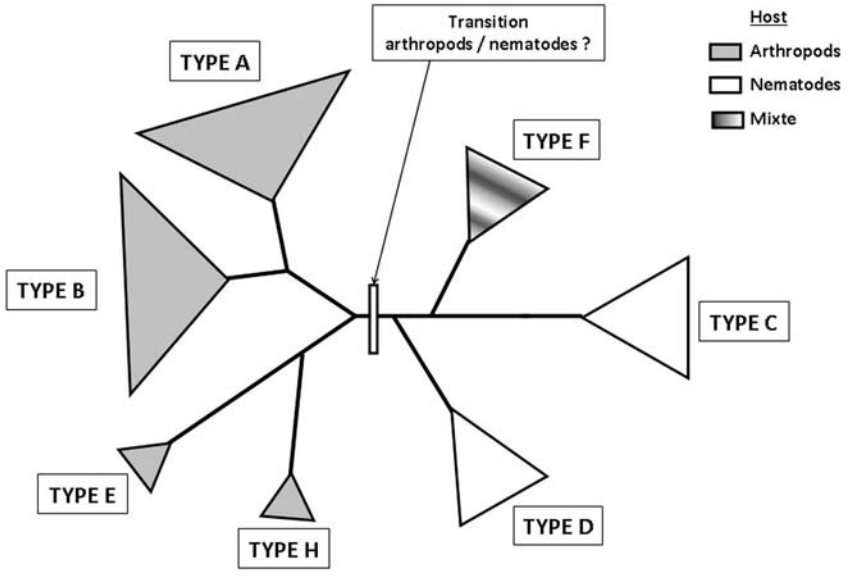

Fig. 2. – *Wolbachia* supergroup phylogeny, adapted from (Werren *et al.*, 2008). The eight established supergroups are represented. Type C and D are specific of filariae; type F is shared by filariae and arthropods.

analysed *Litomosoides* species harbour the type D; the four studied *Mansonella* species are positive for type F (Casiraghi *et al.*, 2004)(unpublished data). This evolutionary pathway is probably related to the mutualistic relationships in the symbiotic Onchocercidae – *Wolbachia* couples.

The association between Onchocercidae and the bacterial types C and D appears to be one of long-term (> 100 million years) (Bandi *et al.*, 1998; Fenn *et al.*, 2006) but the relationship between filariae and the type F might have occurred more recently (work in progress).

What do genomes teach on *Wolbachia* life style?

Today, very few fully sequenced genomes *of Wolbachia* strains' are available: three symbionts of arthropods (bacteria infecting *Drosophila melanogaster* (Wu *et al.*, 2004), *D. simulans* (Klasson *et al.*, 2009b) and *C. pipiens* (Salzberg *et al.*, 2009)) and only one of filaria (bacteria of *Brugia malayi* (Foster *et al.*, 2005; Ghedin *et al.*, 2007)). At least five other strains are in the process of being sequenced, symbionts of two drosophila species (*D. ananassae* and *D. willistoni*), one hymenopteran parasitoid (*Muscidifurax uniraptor*), one mosquito (*Culex pipiens quinquefasciatus*) and one crustacean isopod (*Armadillidium vulgare*).

One of the difficulties in obtaining pure bacterial DNA (essential for sequencing under optimal conditions) is due to the inability to cultivate *Wolbachia*. Moreover, *Wolbachia* genomes contain a large range of mobile and repeated DNA sequences, thereby making the concatenation of sequences highly problematic (Wu *et al.*, 2004). In filarial models, another difficulty arises with the low number of available host individuals, resulting in insufficient material for molecular studies. To overcome this problem, an alternative approach has been used, which involves realising the symbiont genome sequencing along with that of the nematode host (Foster *et al.*, 2005).

A salient feature of *Wolbachia* genomes is the dramatically high level of mobile DNA elements compared to other obligatory endosymbionts and other bacteria (Fig. 3). Indeed, although bacteria, such as *E. coli*, have an average of 1.7 % of their genome occupied by repeated DNA (Foster *et al.*, 2005; Frank *et al.*, 2002), *Wolbachia* infecting *Drosophila simulans* (wDsim strain) display up to 22 % of repeated DNA (Klasson *et al.*, 2009b) and *B. malayi* symbiont (wBmal) 6 % (Foster *et al.*, 2005). In addition, the presence of active bacteriophages (Masui *et al.*, 2000a) and numerous intensively active transposons in arthropod bacterial strains (Cordaux *et al.*, 2008) represents a distinct evolutionary pattern.

Large host acquisition/lost process for wDmel and more strict co-evolution for wBmal were related to *Wolbachia*'s evolutionary process. This indicates the potential involvement of the mobile DNA in *Wolbachia* horizontal transfer, which is a major factor in wDmel, but is absent

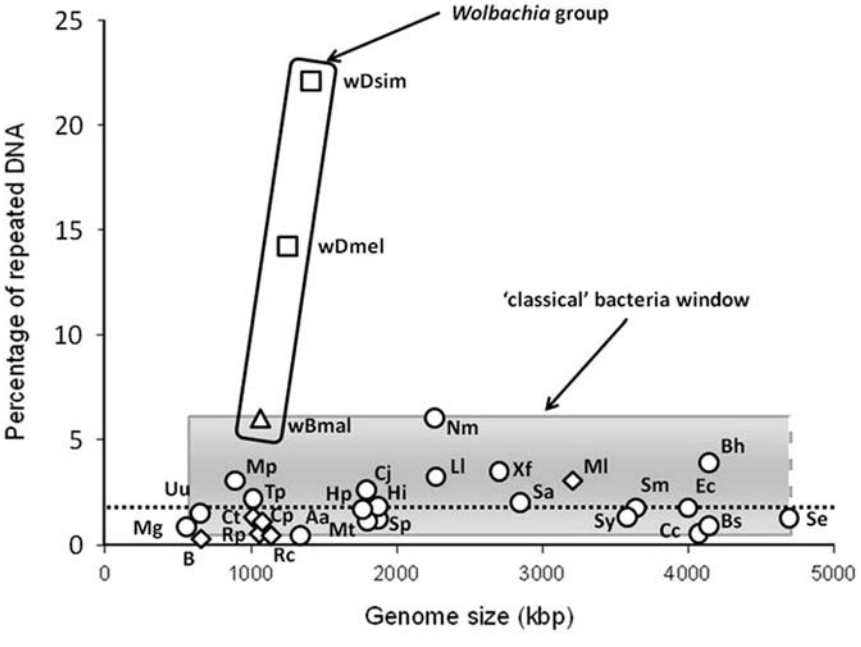

Fig. 3. – Repeated DNA percentage in function of genome size on different bacteria. Wolbachia are indicated with squares, other endosymbionts by diamonds and free living bacteria by circles. Strict endosymbiont are surrounded by a light blue oval. Abbreviations of species: Aa (*Aquifex aeolicus*), B (*Buchnera sp.*), Bh (*Bacillus halodurans*), Bs (*Bacillus subtilis*), Cc (*Caulobacter crescentus*), Cj (*Campylobacter jejuni*), Cp (*Chlamydia pneumoniae*), Ct (*Chlamydia trichomalis*), Ec (*Escherichia coli*), Hi (*Haemophilus influenza*), Hp (*Helicobacter pylori*), Ll (*Lactobacillus lacti*), Mg (*Mycoplasma genitalium*), Ml (*Mycobacterium leprae*), Mp (*Mycobacterium pneumoniae*), Mt (*Mycobacterium tuberculosis*), Nm (*Nesseiria meningitidis*), Ph (*Pyrococcus horikoshii*), Rc (*Rickettsia conorii*), Rp (*Rickettsia prowaseki*), Sa (*Staphylococcus aureus*), Se (*Salmonella enterica*), Sm (*Sinorhizobium meliloti*), Sp (*Streptococcus pneumoniae*), Sy (*Synechocystis sp.*), Tp (*Treponema pallidum*), Uu (*Ureaplasma urealytica*), wBmal (*Wolbachia infecting Brugia malayi*), wDmel (*Wolbachia infecting Drosophila melanogaster*), wDsim (*Wolbachia infecting Drosophila simulans*), Xf (*Xylella fastidiosa*). The red line shows the average of repeated DNA for prokaryotes (1.7 %). Adapted from (Frank *et al.*, 2002; Klasson *et al.*, 2009a; Wu *et al.*, 2004).

in wBmal (Foster *et al.*, 2005). Indeed, genome rearrangements facilitated by mobile and repeated DNA could be fundamental to immediate adaptations during horizontal transfer between host species which are not closely related (very frequent among arthropods' *Wolbachia*). Finally, *Wolbachia* genome specificities seems to indicate that genomic fluidity (maintained by a high percentage of mobile DNA) is a very important feature of this bacterium and could be a powerful tool for *Wolbachia* dispersion and evolution.

TARGETING *WOLBACHIA* AGAINST FILARIASES

Inflammatory response of the vertebrate host activated by *Wolbachia*

Many studies have highlighted the key role of *Wolbachia* in filarial-induced inflammation. Filarial extracts containing *Wolbachia* activate human and rodent neutrophils (Brattig *et al.*, 2001; Gillette-Ferguson *et al.*, 2004) as well as human monocytes (Brattig *et al.*, 2000). They also induce activation and tolerance of murine macrophages (Taylor *et al.*, 2000; Turner *et al.*, 2006). Evidence from onchocerciasis in cattle suggests that worm killing in onchocercomas is associated with a shift in the dominant granulocyte type surrounding the parasites, from neutrophils to eosinophils (Nfon *et al.*, 2006). Neutrophil recruitment is caused primarily by the presence of *Wolbachia*; following *Wolbachia* antibiotic treatment, few neutrophils are found at sites where most bacteria were killed, whereas eosinophils (cells adapted to kill worms) were present in increased numbers around worms and were observed degranulating on the filarial cuticle (Nfon *et al.*, 2006).

Wolbachia seems to be an especially important stimulus of the inflammatory response to *O. volvulus* microfilariae in the eye, which results in ocular impairment. *Wolbachia*-positive *O. volvulus* extracts induce neutrophil recruitment to the cornea and corneal opacity in a mouse model of ocular onchocerciasis (Gillette-Ferguson *et al.*, 2007; Saint Andre *et al.*, 2002); if the extracts were depleted of *Wolbachia*, none of these effects were observed (Saint Andre *et al.*, 2002).

Similar observations were made in onchocercian patients (Brattig *et al.*, 2004) and in *Dirofilaria immitis* infected dogs (Bazzocchi *et al.*, 2003) on the effects of *Wolbachia* Surface Protein (WSP), a major surface protein of *Wolbachia*. WSP induces neutrophil chemotactism probably by promoting interleukin (IL)-8 production, a strong chemoattractant for neutrophils (Bazzocchi *et al.*, 2003; Brattig *et al.*, 2004). IL-8 also inhibits neutrophil apoptosis (Bazzocchi *et al.*, 2007). WSP also promotes production of TNF-alpha, IL-12, and interferon (IFN)–gamma production (Brattig *et al.*, 2004).

The activation of macrophages and their subsequent desensitisation by *Wolbachia* molecules is dependent both on Toll-like receptor (TLR)2 and on the adapter protein MyD88 (Gillette-Ferguson *et al.*, 2006; Turner *et al.*, 2006). It was established that *Wolbachia*-induced inflammation is dependent on TLR2 and TLR6, and partially on TLR1, and that the MyD88/Mal signal transduction pathway is mediated by TRIF and TRAM (Hise *et al.*, 2007). Recently *Wolbachia* diacyl lipoproteins were identified as being responsible for TLR2 and TLR6 activation and being involved in proinflammatory cytokine production (Turner *et al.*, 2009).

Current therapeutic approach with antibiotic treatments targeting *Wolbachia*

In the last ten years, investigators have emphasized the association between *Wolbachia* and filariae and have suggested that the *Wolbachia* bacteria could provide a novel target for antibiotic-based therapy or a novel antisymbiotic chemotherapy.

Tetracyclines, such as doxycycline, were known to be efficient against Rickettsiae. They act by inhibiting bacterial protein synthesis. Interestingly, targeting *Wolbachia* with tetracyclines has biological consequences on filariae: the bacterial depletion blocks female worm development as well as early embryogenesis (Hoerauf *et al.*, 2000); it also interferes with the last moulting process from larval stage 4 to adults (Casiraghi *et al.*, 2002) and it impairs microfilariae development into L3 (Arumugam *et al.*, 2008). Furthermore, antibiotic treatment inhibits microfilarial production in *O. volvulus* and in *W. bancrofti* and has some macrofilaricidal effects (Hoerauf *et al.*, 2001; Hoerauf *et al.*, 2003a; Hoerauf *et al.*, 2008; Mand *et al.*, 2008; Mand *et al.*, 2009; Specht *et al.*, 2008). In summary, antibiotic treatment of filarial nematodes results in sterility of worms and inhibits larval development and adult worm viability. Treatment has no consequences on filariae which do not contain *Wolbachia* (Brouqui *et al.*, 2001). This observation underlines the importance of symbiosis on filarial survival.

The main difficulties associated with antibiotic treatment are treatment duration (daily drug administration for 4-6 weeks) and the precautions necessary for their use: these drugs are not recommended for use during pregnancy and they should not be used by children under eight years of age. Caution is also advised for use in older children. Such precautions make doxycycline unsuitable for mass drug administration (MDA). However, its use is of particular interest in individual treatment (IDA) for onchocerciasis where loiasis is also endemic (Hoerauf, 2008), because the MDA of antifilarial drugs such as ivermectin for onchocerciasis is

associated with serious adverse events in co-endemic area with loiasis (Who, 2009, Boussinesq, 2008).

Communication between *Wolbachia* and filariae: potential therapeutic targets?

Ongoing studies on *Wolbachia* will investigate mechanisms of interaction with its hosts (Fig. 4) and the potential for filariasis-specific pharmacological intervention. Among *Wolbachia* genome, four components may be promising as therapeutic targets: i) the proteins with ankyrin repeat domains are studied as mediators between the symbiont and its environment, the host cell; ii) the bacteriophage WO, a potential powerful tool for *Wolbachia* genetic transformation; iii) the Type IV Secretion System involved in bacterial excretion and subsequent interactions with the host and iv) the haem biosynthetic pathways involved in metal (iron) regulation and supposedly in mutualistic relationships with hosts.

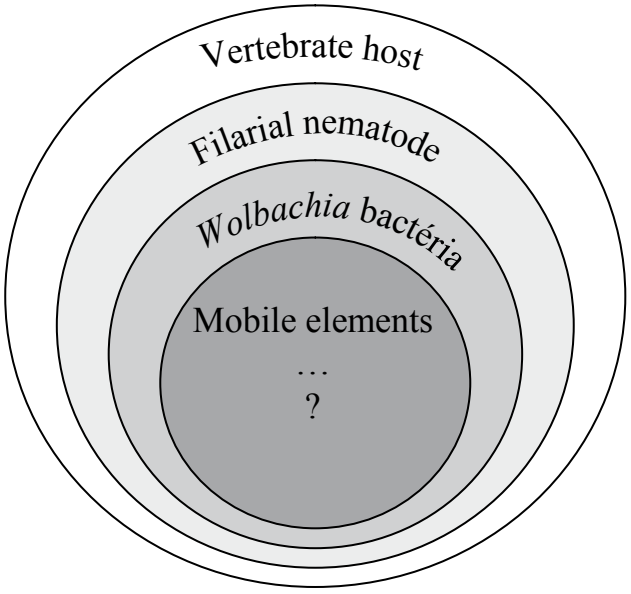

Fig. 4. – The multipartite association between *Wolbachia*, filarial nematode and the vertebrate host. Taking in account all the partners involved in the relationship between the bacteria and filariae increased the complexity of the symbiosis. The bacteria can host mobile genetic elements and the filariae are parasites of vertebrates.

• Ankyrin protein domains

Arthropod *Wolbachia* genomes display genes that include ankyrine repeat domains (Ank genes – Ank proteins) (Duron *et al.*, 2007, Foster *et al.*, 2005, Iturbe-Ormaetxe *et al.*, 2005, Wu *et al.*, 2004). These protein domains are present in nearly all organisms from viruses to mammals. Ank proteins have a large range of functions including cyclin-dependent kinase (CDK) inhibitors, transcriptional regulators, cytoskeleton organisers, developmental regulators and toxins (Sedgwick and Smerdon, 1999).

Interestingly, *Wolbachia* Ank genes seem to be closely related to various eukaryote Ank genes (Wu *et al.*, 2004), suggesting gene transfer or mimetic convergence, and allowing the bacteria to interfere with host's cellular signals. Arthropod *Wolbachia* strains contain numerous Ank genes, 23 in *D. melanogaster* (Wu *et al.*, 2004), 35 in *D. simulans* (Klasson *et al.*, 2009b) or 60 in *C. pipiens* (Klasson *et al.*, 2008), which is very unusual compared to related α-proteobacteria such as *Rickettsia* whose genomes typically contain only one to three genes encoding ankyrin repeats (Andersson *et al.*, 1998). In contrast, *B. malayi* displays five Ank genes and seven related degenerated non-coding sequences (Foster *et al.*, 2005). This purge suggests a particular importance of Ank gene diversity for parasitism in arthropods. The maintenance of five Ank genes in *B. malayi* could be related to i) a primary function for symbiont preservation, ii) a role for some, or all of these genes in host mutualism, or iii) an unachieved purge. To discriminate between these hypotheses, the characterization of Ank genes in other filarial symbionts will be necessary to provide some new clues.

• WO phage

Many *Wolbachia* are infected by an active bacteriophage named WO (Masui *et al.*, 2000a), but a strict endosymbiotic lifestyle is usually related to an absence of phage infection. Bacteriophage WO has only been detected in arthropod symbionts (Gavotte *et al.*, 2007); it was not reported in filarial nematode bacteria (Foster *et al.*, 2005; Gavotte *et al.*, 2007). Bacteriophage WO is not directly involved in *Wolbachia* phenotypes nor in variations of these phenotypes (Gavotte *et al.*, 2007).

Bacteria and phage phylogenies are not congruent, indicating a general dynamic of bacteriophage horizontal transfer among *Wolbachia* strains, except for filariae symbionts (Braquart-Varnier *et al.*, 2005; Gavotte *et al.*, 2007; Masui *et al.*, 2000a).

Besides the phage ecology and evolution, utilisation of a virus able to genetically transform *Wolbachia* was envisaged early on and is still being investigated (Tanaka *et al.*, 2009). The possibility of transforming filarial mutualists would open a large range of therapeutic procedures to limit the parasites' pathogenicity.

• Type IV Secretion System (T4SS)

The Type IV Secretion System (T4SS) is an efficient structure mediating the transfer of DNA and/or proteins from bacteria to eukaryotic cells. T4SS are predominantly found in various pathogenic gram-negative bacteria, such as *Agrobacterium* sp., *Bordetella* sp., *Helicobacter* sp., *Brucella* sp., *Legionella* sp., *Rickettsia* sp. etc. Protein secretion plays an important role in the virulence of these bacteria (Burns, 1999). In *Wolbachia*,

genes encoding T4SS proteins have been identified and characterised at two separate operons (Rances *et al.*, 2008, Wu *et al.*, 2004). The sequences and organisation of these operons are strictly conserved among numerous arthropod *Wolbachia* strains (Masui *et al.*, 2000b; Pichon *et al.*, 2009). This strict evolutionary conservation of the T4SS suggests the importance of this system in *Wolbachia* biology and survival in host cells. However molecules translocated by this secretion system remain unknown.

The two operons of T4SS genes are also present and largely conserved in wBmal genome (Foster *et al.*, 2005), reinforcing the hypothesis of T4SS importance for *Wolbachia* but also suggesting that the function of T4SS is fundamental to symbiont maintenance in filarial hosts.

• Iron metabolism – Heme containing proteins

The *Wolbachia* genome encodes a large number of genes involved in metal metabolism (Wu *et al.*, 2004). The *Wolbachia* interaction with the host's iron metabolism has been suspected for few years (Brownlie *et al.*, 2009) and was recently demonstrated in the parasitoid wasp (Kremer *et al.*, 2009). Iron is tightly controlled by organisms; it is stored in a complex form with proteins and is very important for the efficiency of various enzymes and other proteins. Consequently, the host milieu lacks a freely available form of iron, thereby obliging parasites such as filarial nematodes to develop specific compensatory mechanisms. *Wolbachia* could help to acquire and keep iron for filarial metabolism as observed in arthropods (Kremer *et al.*, 2009). Some groups are investigating the possibility of using haem biosynthetic pathways as a potential anti-filarial drug target (Wu *et al.*, 2009).

CONCLUSION

Despite intensive research on filarial nematodes, *Wolbachia* has been largely ignored for decades. The symbiotic relationship between bacteria and filariae represents a new and potentially effective therapeutic approach. Antibiotherapies eliminating *Wolbachia* are now being widely investigated and available evidence suggests they could are efficient. Such drugs could provide valuable control of symptoms (e.g. to reduce filarial-induced inflammation), effective prevention strategy by breaking the parasitic cycle (e.g. by microfilaricidal effect on *W. bancrofti*) or effective support of parasite elimination (e.g. on onchocerciasis). Recent genomic advances will enable identification of a range of new therapeutic targets and/or tools, including key molecules involved in bacteria-filariae symbiosis.

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