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1              **Endosymbiotic bacteria associated with nematodes, ticks and amoebae**

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27

1    **Abstract**

2    Endosymbiosis is a mutualistic, parasitic or commensal symbiosis in which one symbiont is

3    living within the body of another organism. Such symbiotic relationship with free-living

4    amoebae and arthropods has been reported with a large biodiversity of micro-organisms,

5    encompassing various bacterial clades and to a lesser extent some fungi and viruses. By

6    contrast, current knowledge on symbionts of nematodes is still mainly restricted to *Wolbachia*

7    and its interaction with filarial worms that lead to increased pathogenicity of the infected

8    nematode. In this review article, we aim to highlight the main characteristics of symbionts in

9    term of their ecology, host-cell interactions, parasitism, and co-evolution, in order to stimulate

10   future research in a field that remains largely unexplored despite the availability of modern

11   tools.

12

1

1                                    **Introduction**

2           Endosymbiosis is a symbiosis in which one symbiont dwells within the body of the

3    other. Usually, when talking about endosymbionts, we refer to bacteria, or, less frequently to

4    fungi living inside the eukaryotic cell or simply inside the body. Interestingly, the

5    endosymbiotic theory first articulated by the Russian botanist Konstantin Mereschkowski in

6    1905 (1) describes chloroplasts, mitochondria and other organelles as originating from

7    bacterial endosymbionts. Nearly 90 years ago, Paul Buchner, the father of symbiosis research,

8    documented a remarkable array of both endosymbiotic fungal and bacterial associates of

9    arthropods (2). More recently, evidence has also emerged that bacterial symbionts are present

10   in a large variety of additional eucaryotes, including nematodes, amoebae and plants (see

11   Table 1 for a summary of selected discoveries illuminating research on symbionts). In the

12   present review, we will focus on bacterial symbionts associated with nematodes, arthropods

13   and free-living amoebae.

14

15                              **Symbionts of nematodes**

16           Nematodes or 'roundworms' form a highly successful and abundant group of organisms

17   found in every ecosystem on Earth. Considering their ubiquity and enormous diversity, it is

18   surprising that only relatively few examples of bacterial endosymbioses have been described

19   in nematodes compared with amoebae and arthropods. Of these few examples only three have

20   been investigated in sufficient detail to unravel some of the biological features of the

21   symbiotic relationship.

22

23           The most extensively studied systems are the closely related gamma-proteobacteria,

24   *Photorhabdus* and *Xenorhabdus*, which colonise the guts of *Heterorhabditis* and *Steinemema*

25   nematodes respectively (see 3-5, for in depth reviews). The bacteria have intricate and distinct

1  roles in the nematode's life-cycle. On entering its insect prey, the infective juvenile stage of

2  the nematode regurgitates its bacteria into the haemolymph, which rapidly grows and kills the

3  insect, releasing nutrients to support the growth and development of the nematode. After the

4  adults reproduce, a process that is dependent upon symbionts, environmental cues stimulate

5  the progeny to enter the infective juvenile stage, which becomes re-colonised with one or two

6  bacteria through maternal inoculation. Therefore, throughout the nematode's life-cycle, the

7  bacteria exhibit both pathogenicity to the insect prey and mutualism for the growth and

8  development of the nematode. Although these symbiotic relationships share many common

9  features at the whole organism level, the molecular regulation of each phase of the

10  pathogenic/mutualistic interaction is dependent on both distinct and common pathways and

11  effector molecules (5). The amenability of these systems to experimental and genetic

12  manipulation coupled with post-genomic approaches will undoubtedly reveal further insight

13  into the regulation of pathogenesis and mutualism in these symbiotic associations (3-5).

14

15      The other example of a bacterial-nematode mutualism occurs between the

16  endosymbiont, *Wolbachia* and members of the Onchocercidae family of filarial nematodes

17  (Table 2), including medically important parasites of humans and animals (6). Members of

18  the genus *Wolbachia*, an alpha-proteobacterial group most closely related to *Ehrlichia,*

19  *Anaplasma* and *Rickettsia* species, are diverse and abundant endosymbionts of insects and

20  other arthropods, where they mainly display a parasitic association. Yet in nematodes the

21  bacterium appears to have become a mutualist, restricted to a sub-group of the family

22  *Onchocercidae* (7). Surveys of non-filarial nematodes have failed to detect *Wolbachia* outside

23  of this group (8), although some evidence for divergent *Wolbachia*-like sequences and

24  structurally distinct bacteria have been reported in the plant parasitic Tylenchid nematode,

25  *Radopholus similis* (9). Reports of PCR amplification of *Wolbachia* sequence from the

1    metastrongylid nematode, *Angiostrongylus cantonensis* (10) have not been reproduced and

2    appear to be due to laboratory contamination (11). A more in depth survey of sub-families of

3    the *Onchocercidae*, supports the view that *Wolbachia* arose late in the divergence of filarial

4    nematodes. It is absent from all ancestral groups and there are examples of the presence or

5    absence of *Wolbachia* both within nematode genera and species (12). Further evidence of a

6    different tissue tropism and distribution in the more recently acquired Clade F group in

7    *Mansonella* spp., also suggests a more complex evolutionary history and potentially more

8    diverse symbiotic relationships than previously thought (12).

9

10    In filarial nematodes that host *Wolbachia*, most studies have naturally focused on the

11    endosymbiont's relationship with pathogenic nematode species; *Brugia malayi*, a lymphatic

12    filarial parasite of humans, *Onchocerca volvulus*, the cause of human onchocerciasis or 'river

13    blindness' and *Dirofilaria immitis*, the cause of dog heartworm disease (6, 13). These,

14    together with the majority of related species and genera host either clade C or D *Wolbachia*,

15    which are ubiquitous in all specimens and stages of the nematode and share a close congruent

16    co-evolution. In these species, *Wolbachia* is an essential requirement for larval and embryonic

17    growth and development, fertility and viability of the nematode host (7).

18

19    In species that display an obligate mutualistic association, the bacteria are mostly

20    distributed throughout the syncytial hypodermal chord cells in large numbers (Figure 1) and

21    contained within host-derived vacuoles (7). This tissue tropism develops early in embryonic

22    development, where *Wolbachia* localizes to the posterior of the egg and upon fertilization

23    segregates asymmetrically in a cell-lineage specific pattern (14). Although it was previously

24    assumed that *Wolbachia* enters oocytes through the female germline, a recent observation

25    suggests that the genital primordia remain free of bacteria, which instead appear to translocate

1  from the hypodermis through the pseudocoelomic cavity and across the ovarial epithelium to

2  infect oocytes at the onset of oocyte development (15). Embryonic development is entirely

3  dependent on *Wolbachia*, with about 70 bacteria being transmitted in each embryo (16).

4  These numbers remain static throughout embryonic development and in the microfilariae and

5  the L2 and L3 larval stages, which develop in the insect vector (17). Only after the L3 larvae

6  have infected the mammalian host does the population of *Wolbachia* rapidly expand to

7  populate the hypodermal tissues with further expansion in reproductively active adult females

8  (17).

9

10  The variation in population density between developmental stages and the sensitivity of

11  larval and embryonic development to antibiotic treatment, suggest that *Wolbachia* bacteria are

12  most important during periods of high metabolic activity, presumably through the provision

13  of key nutrients or metabolites to support the rapid growth, organogenesis and development of

14  L4 larvae and embryos. Further evidence in support of this hypothesis comes from

15  observations made on the nematode cellular and nuclear structure following antibiotic

16  depletion of *Wolbachia*. Loss of *Wolbachia* results in extensive and profound apoptosis

17  throughout reproductive cells, embryos and microfilaria, which correlate closely with the

18  tissues and processes initially perturbed following antibiotic therapy. The induction of

19  apoptosis occurs in a non-cell autonomous pattern extending to numerous cells not previously

20  infected with the endosymbiont, implying that a factor derived from *Wolbachia* hypodermal

21  populations is essential for the avoidance of nematode cell apoptosis (16).

22

23  Although L4 and embryonic growth and development are the biological processes most

24  sensitive to *Wolbachia* depletion, other phases of the nematode life-cycle including early

25  larval development and transmission through the vector (18) and the viability and longevity of

1     adult worms (19, 20) are also either partially dependent upon the bacterial endosymbionts or

2     alternatively, may occur through indirect mechanisms associated with *Wolbachia* infection.

3     These include protection from oxidative stress, contribution to the nematodes' evasion, and

4     subversion of host immunity.

5

6     The molecular basis of the mutualistic role of *Wolbachia* remains unresolved.

7     Comparative genomic analysis of *B. malayi Wolbachia* (wBm), with other *Wolbachia*

8     'strains' and related rickettsial species together with that of the host nematode, has revealed

9     that although much of the wBm genome appears degenerate, certain key metabolic pathways

10     remain intact. These pathways include the biosynthesis of haem, nucleotides, riboflavin and

11     FAD, which are absent from the host nematode genome and related bacteria (21, 22). How

12     and when these factors contribute to the mutualistic association is the subject of ongoing

13     research.

14

15     One puzzle, which has confounded the broad acceptance of *Wolbachia* as an obligate

16     mutualist, is the apparent secondary loss of the endosymbiont from some of the more

17     evolutionarily 'advanced' species, including the human filaria, *Loa loa*, the rodent parasite,

18     *Acanthocheilonema viteae* and the deer parasite, *Onchocerca flexuosa* (7). Support for the

19     secondary loss of the symbiont comes from genomic sequencing, which showed evidence of

20     *Wolbachia* gene fragments having been integrated into the host nematode genome through

21     lateral gene transfer (LGT), facilitated by the close association of the bacteria and germline

22     cells (23). The process of LGT appears to be common among *Wolbachia* insect and nematode

23     hosts, with almost an entire *Wolbachia* genome inserted into the nuclear genome of

24     *Drosophila ananassae* (24). Although evidence for gene transcription has been reported for

25     some of these LGT events, further work is needed to determine whether they represent a

1 mechanism by which the nematodes have been able to dispense with the endosymbionts by

2 acquiring the key genes required for obligate mutualism, or if they simply represent a genetic

3 'ghosts' from previous encounters in their evolutionary history.

4

5 Another area in which *Wolbachia* has been shown to play an important role is in driving

6 inflammatory disease pathogenesis and inflammatory adverse reactions to anti-nematode

7 drugs in lymphatic filariasis, onchocerciasis and heartworm disease (7, 25). The release of

8 *Wolbachia* bacteria and their products from the nematode has been shown to stimulate innate

9 and adaptive inflammatory immunity through recognition of lipoproteins via Toll-like

10 receptors TLR-2 & TLR-6 (26). This drives the recruitment of inflammatory cells, leading to

11 damage of parasitized tissues, including the cornea and lymphatics (7, 25, 26). Recent work

12 has shown that this TLR-2- and *Wolbachia*-dependent stimulation of inflammation can impart

13 a selective advantage to the parasite, through activating mast cells in the skin, which enhances

14 the establishment of the parasite by increasing vascular permeability (26). Some have even

15 suggested that *Wolbachia*-mediated inflammatory responses may act to block anti-nematode

16 immunity (27) and so contribute indirectly to the unusual longevity of filarial nematodes.

17

18 Probably the most important outcome from the discovery of *Wolbachia* mutualism in

19 filarial nematodes has been to create the opportunity to use antibiotics as a novel treatment of

20 filarial diseases (6, 22). Treatment with tetracycline or rifamycin antibiotics results in the

21 clearance of the endosymbiont from the nematode, leading to blockage of embryogenesis,

22 sterilization of adult worms and the eventual death of the adult parasites, an outcome that has

23 remained elusive with existing anti-nematode drugs. Existing treatment regimes require a

24 four-week course of doxycycline to deplete the bacteria. Although this produces a superior

25 therapeutic efficacy compared to existing anti-nematode drugs with benefits to individual

1   point-of-care treatment, the prolonged course of therapy together with contraindications in

2   children and pregnant women, restrict its use in widespread community mass drug

3   administration (MDA) programs. This stimulated the formation of the anti-*Wolbachia* (A-

4   WOL) consortium 2007, which was funded by the Bill and Melinda Gates Foundation to

5   discover and develop new anti-wolbachial drugs suitable for MDA control programs.

6   Currently, the A-WOL consortium has developed a portfolio of drug discovery projects with

7   the potential to generate at least one new anti-wolbachial chemotype for eventual deployment

8   as a macrofilaricide and is evaluating more than 200 'hits' from registered or re-purposed

9   drugs to improve on existing regimes (http://www.a-wol.com/). The goal of this research is to

10   deliver anti-wolbachial therapy that can be used in endemic communities, to sustain the

11   achievements of existing control programmes and provide the means to deliver the

12   elimination of filarial diseases.

13

## Symbionts of ticks

15     Ticks are small arachnids in the order *Ixodida*, subclass *Acarina*. They are ectoparasites,

16   living by hematophagy on the blood of mammals, birds, reptiles and amphibians. The lifestyle

17   of many Ixodid (hard) ticks, that are the important vectors and reservoirs of many human and

18   veterinary pathogens, encompasses three primary stages of development: larval, nymphal, and

19   adult. Most ticks take a blood meal only three times in their life (lasting up to 10 years for

20   some species). This type of feeding (almost always a sterile meal), slows down metabolism

21   and a very hard chitin covering make ticks the walking "cans" with very limited exchange

22   with the environment. So, any bacterium found inside the tick should (i) either (directly or

23   indirectly) kill it in order to exit and pursue its life cycle or, (ii) become endosymbiotic in

24   order to facilitate coexistence. Specifically integrated in the « can » system, bacteria may be

25   beneficial or neutral to the host. Symbionts of ticks represent sophisticated systems with an

1   intimate host/endosymbiont relationship and a specific type of transmission from one

2   generation to another. Transovarial transmission enables bacterial colonization very early in

3   the tick life cycle; copulation and egg fertilization could also favour bacterium-tick

4   associations through possibly infected sperm or the microbiota associated with the female

5   genital tract (29).

6       However, surprisingly, no "classical" primary or secondary endosymbionts have been

7   described for ticks up to date. Moreover, the microbiome of ticks remains largely unexplored.

8   Only few studies are available that describe the diversity of the microbiota associated with

9   hard ticks. Most attempts aimed at identifying the bacterial species associated with ticks used

10  standard culture methods on various solid media (30,31). In almost all studies, only

11  environmental free-living bacteria were isolated. Most probably, these represent occasional

12  members of the bacterial microbiota, either ingested or covering the chitin coat of the tick.

13      Almost all endosymbiotic bacteria are quite difficult to isolate; typical primary

14  endosymbionts of arthropods were never isolated in pure culture (32-34). In order to identify

15  bacteria ecologically and evolutionarily associated with ticks, other methods should be used,

16  such as special cell culture system (tick cell lines), enriched broth, and/or 16S rRNA-based

17  analysis. The most comprehensive method to characterize bacterial diversity is the bar-coded

18  16S rRNA pyrosequencing technique. A recent study using this method (35) reports the

19  presence of bacteria of 121 genera in different tissues and stages of *Rhipicephalus microplus*,

20  an important vector of veterinary pathogens. Most of these were free-living environmental γ-

21  proteobacteria, Gram-positive cocci and anaerobes without strict association with ticks. These

22  data confirmed previous culture-based studies (30, 31).

23      However, several groups of bacteria isolated or identified in ticks are of high interest as

24  possible endosymbionts or, at least, as closely-associated bacteria (Table 3). Some examples

25  are highlighted below.

1    ***Coxiella*-like bacteria.** The *Coxiella*-like microorganisms comprise a group of

2    genetically similar bacteria that have not yet been isolated in pure culture. These γ-

3    proteobacteria are phylogenetically close to the obligate intracellular *Coxiella burnetii*, the

4    agent of Q fever and the only recognized species of the genus. These bacteria were detected

5    by PCR in many tick species, mostly from the genera *Rhipicephalus, Haemaphysalis*,

6    *Amblyomma* and *Dermacentor* (35-38). In most studies, these bacteria are present in almost

7    100% of ticks of both sexes. In a recent study, Andreotti *et al.* showed the presence of

8    *Coxiella*-like bacteria in ovaries, eggs and adult males of *Rh. microplus* ticks. In ovaries, this

9    constitutes more than 98% of all identified bacterial species. This may indicate that some

10   bacteria of the *Coxiella* genus are tick-associated primary endosymbionts that can be

11   transmitted vertically (35). Interestingly, the reproductive fitness of *Amblyomma americanum*

12   infected with a *Coxiella* spp. endosymbiont was reduced by an antibiotic treatment (39).

13   Moreover, as expected for a tick symbiont, the genome of the *Coxiella*-like bacteria was

14   reduced in size as compared to *C. burnetii* genome, with a lack of several hypothetical

15   proteins of *C. burnetii* including the *recN* gene productinvolved in DNA repair (38).

16   ***Arsenophonus nasoniae.*** Bacteria of the genus *Arsenophonus* are considered as

17   endosymbionts of many insects (hymenoptera, whiteflies, triatomine bugs, hyppoboscids and

18   lice) (40). *A. nasoniae* induces the male killing phenomenon in the wasp *Nasonia vitripennis,*

19   a parasite of several fly species (41). Interestingly, the strain of *A. nasoniae* was identified in

20   hard ticks of the genera *Amblyomma* and *Dermacentor* in the USA (42-43). Recently, a strain

21   almost identical to *A. nasoniae* from wasps was isolated from the nymph of a *Ixodes ricinus*

22   tick collected in Slovakia. Molecular screening of the ticks from the same location showed

23   that 37% of the nymphs contain this bacterium, while only 3.6% of adults do. This suggests

24   that the bacterium is pathogenic toward early developmental stages of the tick, or that its

25   presence in ticks' bodies depends on the developmental stage. *A. nasoniae* may play a role in

1    tick fitness and/or development, but data on the precise nature of the bacteria/tick relationship

2    are still lacking. The pathogenicity of *Arsenophonus* spp. for vertebrates is also yet unknown.

3        ***Diplorickettsia massiliensis.*** The recently described bacterium *Diplorickettsia*

4    *massiliensis* was isolated from the hard tick *Ixodes ricinius* (44). It is an obligate intracellular

5    Gram-negative bacterium phylogenetically close to the genus *Rickettsiella*, a clade of

6    intracellular bacteria that infect a wide range of arthropods including insects, crustaceans and

7    arachnids (46). Further, it can be grouped into the Family *Coxiellaceae* and the Order

8    *Legionellales* (γ-proteobacteria). The *Coxiellaceae* Family currently includes three genera:

9    *Diplorickettsia, Coxiella* and *Rickettsiella* (45). *Coxiella*-like bacteria, as described above,

10   should be placed in the same family, when isolated and fully characterized.

11       *D. massiliensis* bacteria are localized within an intracellular compartment (vacuole) in

12   pairs; the internal structure includes crystal-like and multi-layer sheath-like structures of

13   unknown composition and function. The role of *D. massiliensis* in tick natural history and its

14   influence on tick's fitness is unknown. However, a recent study suggests that this bacterium is

15   pathogenic towards humans (47).

16       ***Spiroplasma ixodetis.*** Spiroplasmas (class *Mollicutes*) are helical, motile, wall-less

17   prokaryotes associated with a variety of insects, other arthropods, and some plant hosts (48).

18   They are usually considered as commensal organisms in their arthropod hosts, but several are

19   pathogenic for insects and plants. Several species were associated with a male-killing

20   phenomenon (49). Spiroplasmas have been identified in ticks (*Haemaphysalis*

21   *leporispalustris*, *Ixodes pacificus*), blood-sucking members of the *Diptera*, including

22   horseflies (*Tabanus* spp.), deerflies (*Chrysops* spp.), and mosquitoes (*Aedes* spp., *Culex* spp.).

23       *Spiroplasma ixodetis* was first isolated from *Ixodes pacificus*, a principal vector of Lyme

24   disease on the west coast of the United States of America (USA) (48). Later, a nearly identical

25   bacterium was isolated from a pool of *Ixodes* ticks in North Rhine-Westphalia (Germany)

1  (50), and another strain of *Spiroplasma* spp. (genetically very close to *S. ixodetis*) was

2  recently isolated on the XTC cell line from a *Ixodes ricinus* tick sampled in Slovakia, where

3  prevalence of tick infection by *Spiroplasma* was 2.5% (Raoult *et al.*, unpublished).

4      Virtually nothing is known about the relationship between *Spiroplasma* and ticks.

5  However, several publications support the pathogenic role of this bacterium towards humans.

6  Thus, Lorenz *et al.* (51) found a *Spiroplasma* sp. infection causing a unilateral cataract in a

7  premature human baby. Spiroplasmas have been reported to be involved in neurodegenerative

8  diseases such as scrapie or Creutzfeldt-Jakob disease (52, 53).

9

10     In addition to the 4 potential tick endosymbionts discussed above and to established

11  human pathogens known to be transmitted by ticks (Table 3), several other fastidious

12  intracellular bacteria have been shown to be closely associated with ticks, including

13  *Candidatus* Midichloria mitochondrii (54), *Francisella*-like bacteria (55), *Wolbachia* spp.,

14  and different *Rickettsiales*.  More studies are needed in this emerging field, whose results may

15  have many applications, including the control of vector-borne diseases of humans and

16  animals. Indeed, the concept of targeting endosymbionts as a mean to control ticks and tick-

17  borne diseases has been tested using a chemotherapeutic approach (56). Novel methods for

18  isolation and characterization of tick-associated bacteria will likely promote new approaches

19  to control ticks by targeting their endosymbionts.

20

21                          **Symbionts of amoebae**

22      Amoebae are widespread in the environment, mainly at water-air and water-soil

23  interfaces and these protists are especially prevalent where procaryotes are growing, since

24  they graze on bacteria (57). However, some bacteria are resistant to the microbicidal effectors

25  of amoebae (i) by being either true symbionts, i.e. living in close association during a specific

1   period of their time-life with amoebae, or (ii) by being true amoebal pathogens able to lyse

2   the amoebae before or after completing an intra-amoebal replication cycle (58, 59). Amoebae

3   may thus be considered as a replicative niche for both amoebal symbionts and amoebal

4   pathogens. However, amoebae are not a neutral replicative site, but a potent evolutionary crib

5   that promotes selection of virulence traits leading to survival against phagocytic cells (60-63).

6   This supports the use of amoebae as a model to assess the bacterial virulence of amoebae-

7   resisting micro-organisms (67). Amoebae also represent protective armour for the internalized

8   bacteria when encysted, and at least for some symbionts, a source of energy and nutrients.

9      The evidence of the importance of amoebae as a reservoir of *Legionella* spp. led T.

10   Rowbotham to use amoebae as cells in a cell culture system to culture *Legionella* species

11   (71). Since that time, this amoebal co-culture method (see reference 70 for an up-to-date

12   protocol) has proven successful for the recovery by culture of a large biodiversity of

13   amoebae-resisting bacteria (57, 61, 68, 70).

14      Amoebae are also increasingly considered as an Agora where gene exchanges take place

15   (63-65). This intra-amoebal cross-talk has been corroborated by a recent analysis of gene

16   exchanges occurring between amoebae-resisting microorganisms, whereby as many as 9

17   horizontal gene transfer events between *Legionella* species, *Chlamydia*-related bacteria and

18   members of the Order *Rickettsiales* (66) were identified. Moreover, the genome of amoebae-

19   resisting bacteria are commonly encoding proteins sharing a domain conserved in eukaryotic

20   proteins (66, 75), suggesting that horizontal transfer may also be at play between the bacterial

21   symbiont and the amoebal host.

22      Three major groups of amoebae-resisting bacteria have been extensively investigated,

23   the *Legionella*, mycobacteria and *Chlamydia*-related organisms (Figure 2), and several

24   relatively recent reviews are already available (63, 67-69). Here, we thus focus on rickettsial

25   symbionts, and on two other *Candidatus* species for which recently available genomic data

1    illuminate the biology and their interactions with amoebae: *Odysella thessalonicensis* and

2    *Amoebaphilus asiaticus*.

3    **Rickettsial symbionts.** Symbionts related to *Rickettsia* have been so far only poorly

4    studied, although *Caedibacter* was already observed in an *Acanthamoeba* amoeba in 1985

5    (71). The taxonomic position of these rickettsial symbionts was confirmed by coupled 16S

6    rRNA sequencing & FISH approaches (72), *Caedibacter acanthamoebae, Paracedibacter*

7    *acanthamoebae* and *Paraceadibacter symbiosus* sharing (i) only 93.3%, 87.5% and 86.5%

8    16S rRNA sequence similarity, respectively, with  *Caedibacter caryophilus*, their closest

9    neighbour (a symbiont of paramecium) and (ii) 84 to 86% with *Holospora obtusa*. Due to the

10   limited available research reports on rickettsial symbionts, it is likely that a much larger

11   biodiversity of *Rickettsia*-like bacteria remains to be discovered, as suggested by the

12   observation in *Acanthamoeba* of a small rod exhibiting 85.4% 16S rRNA sequence similarity

13   with *Rickettsia sibirica* (74). Future work should thus aim at better defining the distribution,

14   prevalence, host range and pathogenicity towards animals and humans of these amoebal

15   endosymbionts.

16   *Odysella thessalonicensis.* Like *Rickettsia* spp.*, Odysella thessalonicensis* is an alpha-

17   proteobacterium, exhibiting a strict dependency to cells. It has been isolated by amoebal co-

18   culture from an air conditioning system of a Greek hospital in the city of Thessalonika (58).

19   This bacterium could only be grown in *Acanthamoeba* spp. and induced amoebal lysis after 7

20   and 4 days at 30 and 37°C, respectively. This contrasted with the stability of its symbiotic

21   relationship with the same amoebal strain at 22°C for at least 3 weeks (58). Its biology and

22   potential pathogenicity remain largely unknown.

23   *Amoebophilus asiaticus*. *Amoebophilus asiaticus* is a strict intracellular symbiont

24   related to *Cardinium hertigii* and both belong to the *Bacteroidetes* group (76). *A. asiaticus*

25   was discovered within an amoeba isolated from sediments of an Austrian lake (75).  The

1     analysis of its genome revealed a circular chromosome of 1884 kb, encoding 1557

2     hypothetical proteins (76). Thus, contrarily to symbionts of arthropods that exhibit small

3     genomes (< 0.8 kb), this amoebal symbiont does not present a highly compact genome,

4     despite the absence of extrachromosomal elements. This suggests that, as observed for

5     *Legionella*, *Chlamydia*-related bacteria and giant viruses (63-65), the sympatric intra-amoebal

6     life of *Amoebophilus asiaticus* has prevented a significant reduction of the genome size.

7     Indeed, mobile elements represent 24% of the whole genome coding capacity of this

8     endosymbiont (76). Moreover, *A. asiaticus* exhibits a reduced number of genes encoding

9     metabolic functions (17% of the coding capacity) and encodes as many as 82 proteins

10     involved in the transmembrane transport of metabolites, a feature expected for an amoebal

11     symbiont (76). Like *Legionella* spp. and *Chlamydia*-related bacteria (see above)*, A. asiaticus*

12     encodes different proteins exhibiting eukaryotic domains suggesting that amoebae-resisting

13     bacteria widely use such eukaryotic motifs to manipulate the host cell. These eukaryotic

14     domains include U-box and F-box, leucine-rich repeats (LRRs) and ankyrin repeats, among

15     others. U–box and F-box motifs are likely interfering with the ubiquitin system involved in

16     the degradation of proteins by the proteasome whereas ankyrin proteins are likely controlling

17     the interactions of the intracellular bacteria in its host cell. Finally, the LRRs domain, also

18     largely present in the genome of *Protochlamydia amoebophila* (77), may be involved in

19     decreasing recognition of the bacteria by the innate immune system.

20

21     **General conclusions**

22       We hope that this review on symbionts of nematodes, ticks and amoebae will help the

23     reader to understand the importance of the symbiont in determining the virulence of its host,

24     as exemplified with *Wolbachia* in nematodes; similarly, an amoebal endosymbiont may also

25     be implicated in the pathogenesis of *Acanthamoeba* keratitis, by potentially exacerbating local

1   inflammation. This review also recaps the importance of the host in the ecology of its

2   endosymbiont, by directly impacting its survival in the environment, its dissemination, and its

3   mode of transmission to humans and animals. This is of paramount importance, since ecology

4   strongly controls the gene content of the symbionts. Sympatric amoebal symbionts exhibit

5   much larger genomes and much more frequent genes exchange events than those living in an

6   allopatric environment in nematodes and ticks. Symbionts have also clearly played an

7   important role by "feeding" eucaryotes with significant amounts of genetic information

8   during evolution, (i) as previously exemplified by the identification of the role of an ancestral

9   member of the *Rickettsiales* in the biogenesis of current mitochondria (78), and (ii) as recently

10  exemplified by the acquisition by a fruit fly of a nearly complete wolbachial genome content

11  (24). The fact that at least one member of the Order *Rickettsiales* has been identified in all

12  three eucaryote lineages discussed in this review further supports the hypothesis that an

13  ancestral rickettsia was already intracellular more than one billion years ago, when it

14  exchanged genes encoding an ADP/ATP transporter with an ancestral *Chlamydiales* (79).

15  Moreover, this explains why rickettsiologists are in the fore-front of research on

16  endosymbiont-host interactions. Other important lessons provided by studying symbionts are

17  that (i) their diverse nature (large biodiversity encompassing several clades) as well as (ii)

18  their intimate relationship with their specific host, provides no guaranty of their

19  innocuousness towards other eukaryotes encountered by chance, for instance in a modified

20  ecosystem such as man-made water networks.

21

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1    **Table 1.** Selected discoveries or hypotheses illuminating research on symbionts*

2

| Year | Discovery – hypothesis | References |
|------|------------------------|------------|
| 1879, De Bary | Concept of symbiosis | (61) |
| 1905, Merezhkowsky | Endosymbiotic bacteria may be at the origin of mitochondria and other eucaryotic organelles | (1) |
| 1912, Büchner | Description of bacterial and fungal endosymbionts of arthropods | (2) |
| 1967, Jeon | Accidental infection of *Amoeba proteus*, by a symbiont initially reffered as the "x-bacterium" before being renamed *Legionella jeonii* | (80) |
| 1998, Sullivan | Concept of "symbiosis island" to refer to a genomic island, which is important in mutualistic relationship of a symbiont with its host | (61) |
| 1998, Andersson | An ancestral rickettsial endosymbiont identified to be at the origin of mitochondria | (78) |
| 2001, Moran | First evidence of strong genome reduction (demonstrated for an aphid symbiont) | (81) |
| 2003, Greub | Long history of co-evolution of *Rickettsiales* and *Chlamydiales* with their hosts (already energy parasites of their hosts > 1 billion year ago) | (79) |
| 2003, La Scola | Discovery of a giant virus of amoebae | (82) |
| 2004, Greub | Conjugative DNA transfer may occur between strict intracellular bacteria (between amoebal endosymbionts) | (83) |
| 2007, Kikuchi | First report of horizontal gene transfer in an insect symbiont | (84) |
| 2010, Moliner | Reductive evolution among allopatric symbionts (of ticks & nematodes) and large genomes for sympatric symbionts present in amoebae | (65) |

3    * adapted from reference 61.

4

1    **Table 2.** Bacteria-nematode mutualism between the *Wolbachia* endosymbiont and filarial

2         nematodes.

3

| Effect of *Wolbachia* endosymbiont | References |
|---|---|
| 1. Provide key nutrients: haem, nucleotides, riboflavine and FAD (especially important during embryogenesis up to L4 larval stages) | (17, 21, 22) |
| 2. Prevent apoptosis of the reproductive cells | (16) |
| 3. Prevent oxidative stress | (25) |
| 4. Pro-inflammatory effect: | |
| - TLR2/TLR6 recognition of wolbachial lipoproteins | (26) |
| - Facilitates nematode entry, i.e. represents a mechanism of immune subversion | (27-28) |

4

5

1    **Table 3.** Bacterial species known to be associated with ticks

| Species | Ticks | Comments |
|---|---|---|
| *Arsenophonus nasoniae* | *Amblyomma, Dermacentor, Ixodes ricinus* | Endosymbionts of many insects<br><br>Unknown pathogenicity |
| *Borellia* spp. | Ixodes spp. | Agent of Lyme disease |
| *Bartonella* spp. | Uncommonly transmitted by ticks[1] | Agent of cat-scratch disease, trench fever, bacillary angiomatosis and other bartonella infections |
| *Coxiella burnetii* | Uncommonly transmitted by ticks | Agent of Q fever |
| *Coxiella*-like bacteria | *Rhipicephalus, Haemaphysalis, Amblyomma, Dermacentor* | Antibiotic treatment reduces the reproductive fitness of infected ticks |
| *Diplorickettsia massiliensis* | *Ixodes ricinus* | *Diplorickettsia* genus is part of the *Coxiellaceae* family as *Coxiella* and *Ricketsiella* genera<br>Possible new pathogen of humans |
| *Ehrlichia/Anaplasma* spp. | Various species | Agents of ehrlichiois |
| *Francisella tularensis* | | Agent of tularemia |
| *Francisella*-like bacteria | Various species | Unknown human pathogenicity; yet uncultured |
| *Rickettsia* spp. | Various species | Agents of various spotted fevers<br><br>Reductive evolution of the genome associated with increased pathogenicity<br><br>Ticks determine the geographical repartition and the clinical presentation[2] |
| *Spiroplasma ixodetis* | *Ixodes* spp. | Belong to the *Molliculites* class (like *Mycoplasma*) |

2    [1]*Bartonella quintana* is generally transmitted by body lice whereas *Bartonella henseale* is mainly
3    transmitted by cat or dog scratch and to a lesser extend by cat fleas.

4    [2]due to the attack strategy of *Amblyomma* ticks, several inoculation eschars are common in the same
5    patient; due to the hair tropism of *Dermacentor* ticks, the inoculation eschar is often missed and first hint
6    for diagnosis of *R. slovacae* infection is the presence of a loco-regional adenopathy, according to which
7    the disease has been named: tick-borne lymphadenitis (TIBOLA);

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3      1    **Figure legends.**
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5      2    Figure 1. Electron micrograph of *Wolbachia* (arrows) in the hypodermal chord cell of *Brugia*
6      3    *malayi* (a filarial nematode). Bar represents about 0.5 μm.
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8      4    Figure 2. A *Chlamydia*-related bacteria (*Criblamydia sequanensis*) recently isolated by
9      5    amoebal co-culture from the Seine river water. The bacteria (here within *Acanthamoeba*
10     6    *castellanii* amoebae) exhibits typical star-shaped elementary bodies. Electron microscopy,
11     7    magnification 20,000x; bar represents 0.2 μm.
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Figure 1. Electron micrograph of Wolbachia (arrows) in the hypodermal chord cell of Brugia malayi (a filarial nematode). Bar represents about 500 nm.
130x149mm (300 x 300 DPI)

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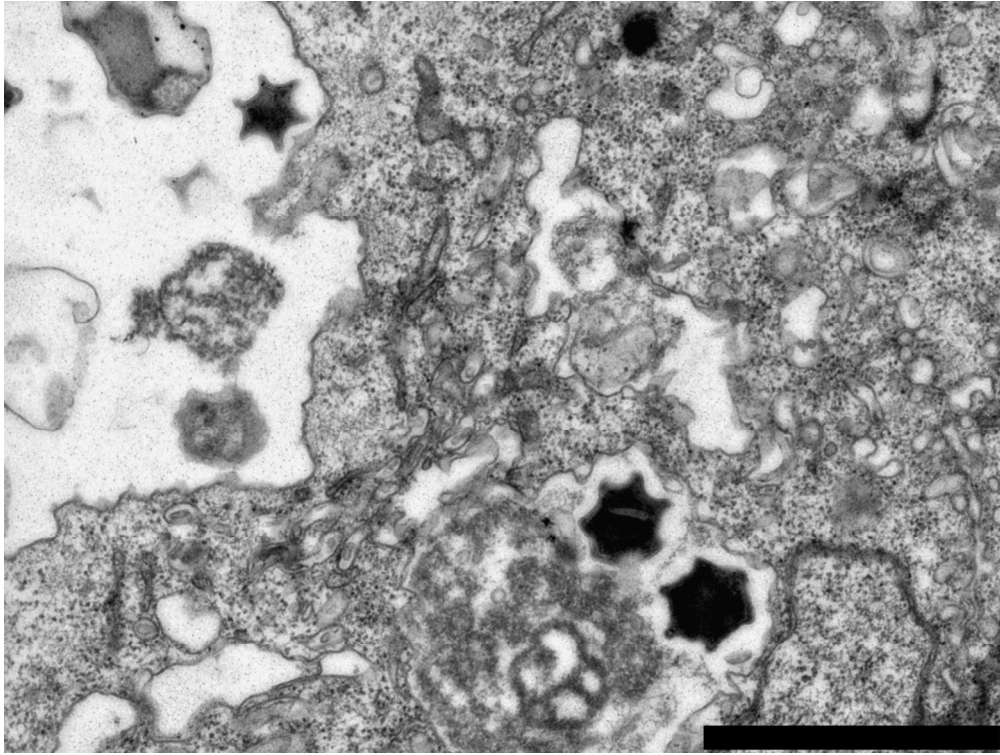


Figure 2. A Chlamydia-related bacteria (Criblamydia sequanensis) recently isolated by amoebal co-culture from the Seine river water. The bacteria (here within Acanthamoeba castellanii amoebae) exhibits typical star-shaped elementary bodies. Electron microscopy, magnification 20,000x; bar represents 200 nm.
130x97mm (300 x 300 DPI)