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ABSTRACT

Defensive microbes are of great interest for their roles in arthropod health, disease transmission, and biocontrol efforts. Obligate bacterial passengers of arthropods, such as *Spiroplasma*, confer protection against the natural enemies of their hosts to improve their own fitness. Although known for less than a decade, *Spiroplasma*'s defensive reach extends to diverse parasites, both microbial and multicellular. We provide an overview of known defensive phenotypes against nematodes, parasitoid wasps, and fungi, and highlight recent studies supporting the role of *Spiroplasma*-encoded ribosome-inactivating proteins in protection. With cellular features well-suited for life in the hemolymph, broad distribution among invertebrate hosts, and the capacity to repeatedly evolve vertical transmission, *Spiroplasma* may be uniquely equipped to form intimate, defensive associations to combat extracellular parasites. Along with insights into defensive mechanisms, recent significant advances have been made in male-killing – a phenotype with interesting evolutionary ties to defense. Finally, we look forward to an exciting decade using the genetic tools of *Drosophila*, and the rapidly-advancing tractability of *Spiroplasma* itself, to better understand mechanisms and evolution in defensive symbiosis.

INTRODUCTION

Symbiotic microbes have been increasingly recognized as influential players in animal health, ecology, and evolution. One of the most important ways symbionts can affect their hosts is through protection[1-3]. These relationships have been especially well documented and studied in inherited insect symbioses. Protection may be accomplished in different ways, but symbiont toxins are emerging as a common mechanism[1]. For example, philanthine wasps, commonly known as beewolves, harbor *Streptomyces* symbionts within specialized crypts in their antennae. These symbionts produce a cocktail of antimicrobials to protect their hosts from pathogenic fungi and bacteria as they pupate in underground burrows[4]. Symbionts also protect hosts from much larger enemies, as in the case of *Pseudomonas* symbionts of *Paederus* rove beetles, that synthesize a highly reactive polyketide toxin called pederin, used by the insect host to dissuade predators, such as spiders[5]. In this review, we highlight a proficient symbiotic defender, *Spiroplasma*, that has demonstrated protection against both microbes and multicellular eukaryotes.

Spiroplasma are helical, cell wall-less bacteria belonging to an ancient lineage of host-associated Mollicutes that also includes the vertebrate- and plant-associated *Mycoplasma*, and the insect-vectored plant pathogenic *Phytoplasma*. *Spiroplasma* are broadly distributed among invertebrate hosts, often crustaceans, spiders, and insects; they are estimated to occur in about 7% of all terrestrial arthropods[6]. Interestingly, highly divergent *Spiroplasma* strains have recently been found associated with marine and deep-sea invertebrates, including jellyfish and sea cucumbers, and the biology of these lineages is essentially unknown[7,8]. *Spiroplasma* exhibits great variation in

transmission mode, tissue tropism, and fitness effects, ranging from gut commensals, to insect-vectored plant pathogens, to symbionts that are highly efficiently maternally transmitted. The biology and infection dynamics of symbiotic *Spiroplasma* have been thoroughly reviewed elsewhere[9]; however, its defensive capabilities have only come to light within the last decade. Within this brief period, defensive roles against highly divergent natural enemies – entomopathogenic fungi, nematodes, and parasitoid wasps – have been described. We discuss how recent discoveries and methodological advances have *Spiroplasma* poised for development into an ideal model to study defensive symbiosis.

TRANSMISSION OF DEFENSIVE *SPIROPLASMA*

Many strains of *Spiroplasma* maintain infection through vertical transmission. In *Drosophila*, *Spiroplasma* have evolved vertical transmission on at least four separate occasions[10]. In recent years, studies have shown that some of these vertically-transmitted *Spiroplasma* are protective[11-14]. In fact, all of the known protective strains of *Spiroplasma* (and those of many other defensive microbes) are vertically-transmitted. This is perhaps unsurprising, given that this mode of transmission links symbiont fitness to that of the host. Unlike many inherited insect symbionts, such as the well-known *Wolbachia*, *Spiroplasma* is primarily extracellular and can replicate to high titers in host hemolymph[15,16]. Evasion, and in some cases, suppression[17,18] of the host immune system by *Spiroplasma* facilitates its existence here. Hemolymph localization may have a significant influence on host shifts within *Spiroplasma*. Despite its vertical transmission, phylogenetic discordance between *Spiroplasma* and hosts suggests horizontal transmission among unrelated hosts occurs frequently[10]. Parasite-mediated

transfer via mites[19] has been observed in the lab and host-matched strains detected in mites collected from *Drosophila* in the field[20]. The ease with which strains can be horizontally transferred has benefits for studying *Spiroplasma* as well, because it allows symbionts to be easily swapped between hosts by moving infected hemolymph from one organism to another via intrathoracic microinjection.

DEFENSIVE PHENOTYPES

Protection against a parasitic nematode

The first documented case of host protection by *Spiroplasma* involved defense against parasitic nematodes. The mushroom-feeding North American woodland fly, *Drosophila neotestacea* is commonly infected by a virulent generalist nematode, *Howardula aoronymphium*; infection prevalence can reach 30% in the wild[12,21]. Parasitism is crippling; until only recently, virtually all infected females were rendered sterile. However, flies that harbor a strain of *Spiroplasma poulsonii* are resistant to *Howardula* infection - female flies are no longer sterilized and mature nematodes are severely reduced in size and produce virtually no infective juveniles[12]. The benefit conferred by *Spiroplasma* is so great that symbiont-infected flies are rapidly replacing their uninfected counterparts and spreading across N. America[12,21]. As far as we are aware, this is still the only known case of endosymbiont-mediated protection against nematodes in nature. An interesting recent study successfully established four new stable *Spiroplasma* symbioses in *D. neotestacea* via hemolymph transfer. Two of the transferred symbionts were different strains of *S. poulsonii* while the two others were *Spiroplasma* from other clades (citri and ixodetis). None of these *Spiroplasma* were able to protect *D.*

neotestacea from *Howardula*, suggesting that nematode protection requires special features that are present in *D. neotestacea's* native strain[22].

Protection against parasitoid wasps

Three strains of *S. poulsonii*, from *D. hydei*, *D. melanogaster*, and *D. neotestacea*, have been found to protect their hosts from two distantly-related lineages of parasitic wasp (braconids in the genus *Asobara*, and figitids in the genera *Leptopilina* and *Ganaspis*)[11,23,24]. At present, all susceptible parasitoids are larval endoparasites. One pupal ectoparasite, a pteromalid in the genus *Pachycrepoideus*, has also been tested, and it is not susceptible[25]. Although protection always results in wasp death, there is variation in the outcome for the fly host. Two recent studies showed that *Spiroplasma*-infected flies survive attack by the specialist wasp *L. boulardi*, but not the generalist wasp *L. heterotoma*[23,25], possibly because the venoms of some wasps are lethal on their own[26], even while those of sister species have more mild effects such as immune suppression. Interestingly, early work demonstrating wasp defense by *S. poulsonii* of *D. melanogaster* used isolines established from recently wild-caught flies and found they did not survive protection even when matched against *L. boulardi*[13]; likewise, *Spiroplasma*-protected *D. hydei* strongly recovered following attack by *L. heterotoma*[11]. Thus, host-parasite coevolution likely shapes venom resistance such that this pairing, and not *Spiroplasma*'*s* activity, determines whether flies survive attack. Wasps themselves show evidence of delayed development very early after hatching within *Spiroplasma*-infected hosts, and die during the host pupal stage[11,13,23,27].

Despite the dramatic impact of *Spiroplasma* on parasitoid wasps in the laboratory, protection in the wild has not been shown. Parasitoid wasps are a major

contributor to host mortality in the wild[28]. Because of this and given *Spiroplasma's* propensity to transfer among species of *Drosophila*, one might expect to find *Spiroplasma* at much higher frequency in the wild. Yet, most wild flies are free of *Spiroplasma*. This apparent disparity remains to be investigated, but it is likely due to the fact that parasitoid resistance to *Spiroplasma* is common. Although in some regard, it appears defense is broad-reaching, i.e. protection has been demonstrated against distantly-related parasitoids of the super families Ichneumonoidea and Cynipoidea[9,23], Mateos and colleagues recently reported the discovery of resistant wasps, and that resistance has evolved independently in at least two lineages[24].

Surprisingly, one wasp-defensive *Spiroplasma*, *S. poulsonii* of *D. melanogaster*, also known as the melanogaster sex-ratio organism, or strain MSRO, is also a reproductive parasite that kills male offspring of infected mothers. Studies on male-killing by several *Drosophila*-infecting *S. poulsonii* strains predate the discovery of its defensive properties by half a century, but at present strain MSRO is the only one known to be both a parasite and a defensive symbiont.

Protection against fungal infection

Symbiont-mediated protection against parasitoid wasps is also seen in aphids, where it is mediated by *Hamiltonella defensa* phages and several other facultative symbionts, though notably not *Spiroplasma* (although an interesting recent study[29] found that parasitic wasps prefer volatiles from plants that were fed on by uninfected aphids over ones that carried facultative symbionts, including *Spiroplasma*). However, some, but not all, aphid-infecting *Spiroplasma* strains confer protection against a virulent fungal pathogen, *Pandora neoaphidis*, enhancing aphid survival and reducing the frequency of

sporulation[14]. Aphid *Spiroplasma* belongs to the ixodetis clade, far removed from the poulsonii clade, and one of the most widespread groups of arthropod *Spiroplasma* in general. A similar and in most cases more complete protection is also produced by four other facultative symbionts, *Rickettsia*, *Rickettsiella*, *Regiella,* and *Fukatsuia*[14, 30]. While virtually nothing is known of the mechanism behind protection, it is notable that the phenotype is fully transferrable to the grain aphid, *Sitobion avenae*, following transfer of *Spiroplasma* by microinjection[31]. Many open questions about this strain remain, and indeed about the genetic basis of ixodetis clade phentotypes in general because, like poulsonii clade strains, they are not only defensive but also proficient male-killers.

MECHANISMS OF DEFENSE

A major goal in the study of defensive symbiosis is identification of the mechanism. This is critical to help understand and predict costs for the host and responses by natural enemies[2,3]. There are three general, non-mutually exclusive mechanisms of protection: production of toxins, immune system recruitment, and resource competition. Hamilton and colleagues recently identified a *Spiroplasma*-encoded toxin, a ribosome-inactivating protein (RIP) and implicated it in defense by demonstrating the toxic activity of purified protein *in vitro* and of *Spiroplasma*-produced toxin on nematodes *in vivo* in *D. neotestacea*[32]. Subsequently, the same hallmark of RIP activity was reported alongside wasp mortality in protected *D. neotestacea* and *D. melanogaster*[25]. RIPs are N-glycosidases of ribosomal RNA (rRNA). They bind to 28S rRNA and cleave an essential adenine base from a highly-conserved loop structure required for translation initiation, irreversibly inhibiting protein synthesis and triggering apoptosis and eventually necrosis[33].

Concurrently with the discovery of RIPs came the first genome assemblies of defensive *Spiroplasma,* those of strain MSRO[34,35] and the symbiont of *D. neotestacea*[25, 36]. These are reduced genomes typical of the genus, yet each encodes a diverse family of RIP genes. The forces driving RIP diversity in *Spiroplasm*a are unknown and remain a focus of ongoing research. One hypothesis is that diverse RIPs exhibit specialized functions as a consequence of target cell specificity. For example, the *S. poulsonii* strain of *D. neotestacea* encodes two RIPs very similar to those of strain MSRO, and two others lacking MSRO orthologs, which is one possible explanation for the additional nematode protection displayed by this symbiont.

Genomic and phylogenetic analysis reveals the evolutionary history of *Spiroplasma* RIPs as one shaped by dynamic gains and losses through gene duplication and death, as well as horizontal transfer among strains throughout the genus[37]. Despite the prevailing pattern of evolutionary mobility of RIPs and clear precedent for genetic transfer among facultative symbionts such as *Wolbachia*, *Rickettsia*, and *Cardinium*, e.g. ref [38], this diversity of RIP genes appears not to have leaked to other symbiont lineages. The alternative genetic code of *Spiroplasma* encodes the amino acid tryptophan using the standard stop codon, UGA, making *Spiroplasma* genes dead-on-arrival in most recipient genomes and effectively hoarding special host phenotypes produced by RIPs within the genus[39].

Toxin-based defense is one of many features shared between *Spiroplasma* and a symbiont that confers parasitoid protection in aphids, *Hamiltonella defensa* harboring toxin-encoding phage. Other similarities include maternal transmission, hemolymph localization, toxin gene localization on accessory genomes (e.g. phage, plasmids), and

the evolution of resistance among wasps[40–42]. The rarity of wasp protective symbionts may be attributed to the difficulty of surviving freely in host hemolymph as a bacterial cell. Avoiding this problem is yet another common thread between the two symbionts. However, there are also notable differences. For example, *Spiroplasma*'s immune evasion is inherent, as it lacks a peptidoglycan-rich cell wall, while *Hamiltonella's* is due to a peculiarity of aphid immunity, the inability to sense peptidoglycan[43]. For these reasons, *Spiroplasma* may be particularly well-suited to protect diverse insect hosts from extracellular parasites.

Defensive strains are not the only *Spiroplasma* that encode RIPs – there is striking RIP diversity distributed throughout the genus, at least 11 of the currently sequenced strains encode RIPs, including pathogens (*S. eriocheiris*), commensals (*S. sabaudiense*, *S. atrichopogonis*) and vertically-transmitted symbionts with unknown host effects (e.g. diverse citri clade strains widespread in the ant genus *Myrmica*)[25,32,39,44]. The function and specificity of these RIPs is completely unknown, but effects on pathogenesis and microbiome composition are promising candidates for investigation. There are also numerous intriguing reports of *Spiroplasma* influencing host ecology in ways that are not yet understood. The corn stunt agent, *S. kunkelii*, has a temperature-dependent effect of prolonging the lifespan of its insect host, the corn leafhopper[45]. Interestingly, not only does it not appear to protect its host against parasitism by dryinid wasps, but wasps instead reduce the presence of *Spiroplasma*[46]. Additionally, *Spiroplasma* negatively impact occurrence or titer of defensive and other facultative symbionts in pea aphids[47] and *Drosophila*[48]. In the case of aphids, *Spiroplasma* may not

directly exclude other symbionts, rather the infrequency of co-occurrence in sampled individuals could be a reflection of the higher cost co-infections[47,49].

Also unexplored are the evolutionary relationships and transitions between parasitic and defensive strains of *Spiroplasma* in the poulsonii and ixodetis clades. Both defense and male-killing have been tied to single effector genes. In the case of defense, these are the RIPs, and in male-killing, an orphan toxin called Spaid has been implicated[50]. Spaid, like some RIPs, is encoded on an extrachromosomal plasmid, suggesting potential for a highly dynamic ebb and flow in the frequency and rate at which these phenotypes emerge and are lost. The degree to which these few genetic effectors are capable of recapitulating male-killing and defense on their own and in distantly related hosts remains to be studied. In the case of defense, it is of interest to identify determinants of specificity toward different parasites, for example, whether *Spiroplasma* RIPs encode domains that facilitate cell entry, or if additional factors are required. Other mechanisms could contribute to wasp mortality as well. For example, Paredes and colleagues also implicate competition between *Spiroplasma* and wasps over lipids[23], as each are unable to synthesize lipids and must scavenge them from host hemolymph. They found that although wasp presence does not hinder the growth of *Spiroplasma*, wasps develop poorly in *Spiroplasma*-free host larvae when lipids are artificially depleted. With regard to male-killing, Spaid is sufficient for the phenotype in *Drosophila*, but we expect the first genome of an ixodetis clade male-killing strain will help clarify its mobility, or conversely, reveal evidence of convergent evolution of male-killing in *Spiroplasma*.

The last decade of research has yielded transformative insight into the diversity and mechanisms of *Spiroplasma*-host interactions. Undoubtedly, identifying candidate mechanisms of defense, and likewise the male-killing toxin, have been important steps forward in *Spiroplasma* research. Equally exciting is the very recent success in establishing a stable, host-free culture of *S. poulsonii* strain MSRO[35]. We expect these discoveries and resources to open avenues for innovative research and attract new expertise that will drive forward understanding of *Spiroplasma's* roles in the health, ecology, and evolution of their invertebrate hosts in the coming decade.

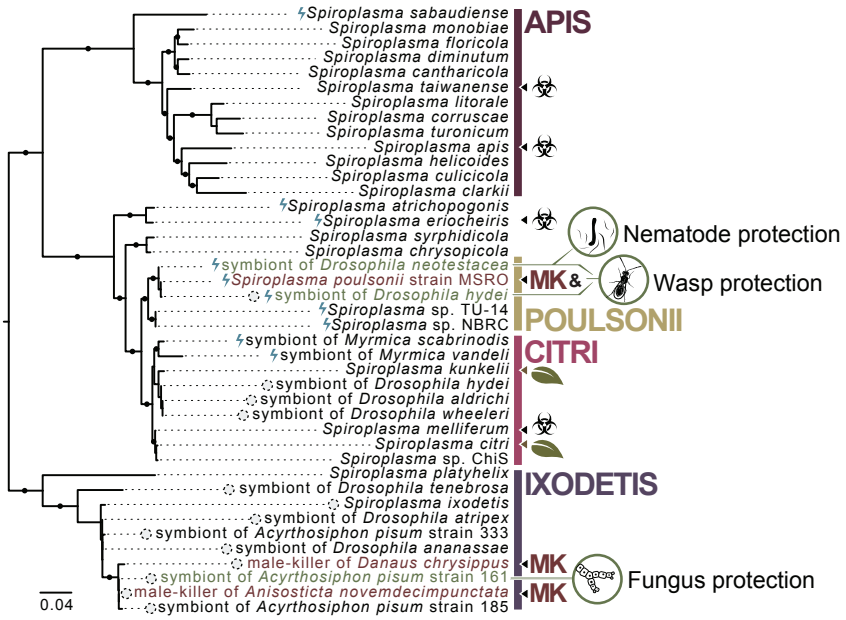

Fig 1. Evolution and diversity of host interactions in the genus *Spiroplasma*

Evolutionary relationships between strains of bacteria in the genus *Spiroplasma*, inferred by maximum-likelihood phylogenetic analysis of the nucleotide loci rpoB, 16S rRNA, ITS, and 23S rRNA gene nucleotide sequences. Branches supported with approximate likelihood ratio test scores of 1.0 are indicated with a filled circle. The conventional clades within the genus, apis, poulsonii, citri, and ixodetis, are labeled to the right of taxon labels. Defensive taxa are colored with green text and marked with a green circle and type of natural enemy against which protection is effective. Male-killing taxa are colored with red text and an MK label. Invertebrate pathogens are marked with a biohazard symbol. Plant pathogens are marked with a leaf symbol. To the left of taxon labels, a small lightning icon designates strains that encode one or more ribosome-inactivating protein (RIP) and a small dashed circle designates strains for which genome-scale sequencing data has not yet been collected.

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