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Minireview

MINIREVIEW

Arthropod–*Spiroplasma* relationship in the genomic era

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One Sentence Summary: MiniReview focused in Spiroplasma-Arthropod symbiosis in the context of the bacterial sequenced genomes and the elucidation of functional and evolutionary traits shaping these relationships.
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ABSTRACT

The genus *Spiroplasma* comprises wall-less, low-GC bacteria that establish pathogenic, mutualistic and commensal symbiotic associations with arthropods and plants. This review focuses on the symbiotic relationships between *Spiroplasma* bacteria and arthropod hosts in the context of the available genomic sequences. *Spiroplasma* genomes are reduced and some contain highly repetitive plectrovirus-related sequences. *Spiroplasma*'s diversity in viral invasion susceptibility, virulence factors, substrate utilization, genome dynamics and symbiotic associations with arthropods make this bacterial genus a biological model that provides insights about the evolutionary traits that shape bacterial symbiotic relationships with eukaryotes.

Key words: *Spiroplasma*; symbiosis; comparative genomics

INTRODUCTION

The *Spiroplasma* genus consists of cell-wall-less, helical, low-GC bacteria belonging to the class *Mollicutes*. Spiroplasmas are described as facultative anaerobes that exhibit a wide range of growth temperatures between 5 and 41°C (Konai *et al.*, 1996). These bacteria establish symbiotic associations mainly with arthropods. Associations with dipteran and coleopteran insect orders are frequent and have been largely reported (Wedincamp *et al.*, 1996). Other insect orders where spiroplasmas have been isolated are Hemiptera, Homoptera, Hymenoptera, Lepidoptera and Odonata (Hackett and Clark 1989; Hackett *et al.*, 1990; Watanabe *et al.*, 2014). Also, spiroplasmas have been isolated from non-insect arthropods and plants (Davis, Lee and Worley 1981; Saillard *et al.*, 1987; Wang *et al.*, 2004; Goodacre *et al.*, 2006).

The majority of *Spiroplasma* species described as insect symbionts have no effect on the hosts and are considered as commensal bacteria (Gasparich 2010). In some hosts like shrimp (Nunan *et al.*, 2004), honeybees (Clark 1977), and mosquitoes (Phillips and Humphery-Smith 1995), spiroplasmas have been characterized as pathogens. Pathogenicity is related to the capacity to cross the midgut lumen barrier, hemolymph invasion and therefore colonization of other host tissues, that lead in some cases to host death (Nunan *et al.*, 2005).

A significant effect on hosts like *Drosophila* (Williamson *et al.*, 1999) and other insects (Tabata *et al.*, 2011) is the male-killing phenotype, where maternally inherited *Spiroplasma* kill host male offspring in early stages of development.

Spiroplasma as mutualistic symbionts can be found in *Drosophila* and aphids providing host protection against

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Table 1. General features of the sequenced *Spiroplasma* genomes.

	S. chryso-picola DF-1	*S. syrphy-dicola* EA-1	*S. citri* Gll3-3X	*S. melli-ferum* IPMB4A	*S. melli-ferum* KC3	*S. dimi-nutum* CUAS-1^T	*S. taiwa-nense* CT-1^T	*S. apis* B31^T	*S. culici-cola* AES-1^T	*S. sabau-diense* Ar-1343^T
Arthropod host	*Chrysops sp.*	*E. arbustorum*	*C. haema-toceps*	*Apis-Mellifera*	*A. Mellifera*	*Culex-annulus*	*C. tritaeni-orhynchus*	*A. Mellifera*	*Aedes-sollicitans*	*stricticus/ A. vexans*
Symbiotic relationship	Commensal	Commensal	Commensal*	Pathogenic	Pathogenic	Commensal	Pathogenic	Pathogenic	Pathogenic	Commensal
Genome Size (bp)	1123 322	1107 344	1525 756	1098 846	1260 174	945 296	1075 140	1160 554	1175 131	1075 953
Chromo-somal contigs	1	1	39	24	4	1	1	1	1	1
G + C content (%)	28.8	29.2	25.9	27.5	27	25.5	23.9	28.3	26.4	30.2
rRNA operon	1	1	1	1	1	1	1	1	1	2
tRNA	29	29	29	29	29	29	29	29	29	30
Number of plasmids	0	0	7	4	0	0	1	0	0	0
Protein-coding genes	1009	1006	1170	920	1046	858	991	997	1071	924

*Commensal: insect is used only as a vector to infect plants.

parasitoid wasps (Xie, Vilchez and Mateos 2010), nematodes (Jaenike *et al.*, 2010; Cockburn *et al.*, 2013) and fungal pathogens (Lukasik *et al.*, 2013). *Spiroplasma kunkelii* increases the survival rate of the leafhooper *Dalbulus maidis* during cold and dry periods when the leafhopper's host plant is not accessible (Ebbert and Nault 1994).

Spiroplasma symbiotic associations with arthropods can be considered as biological model systems to study molecular mechanisms and evolutionary traits that shape contrasting bacterial-host interactions.

In this review, we will focus on arthropod-associated *Spiroplasma* species with complete genome sequences, the biological implications of harboring this bacteria and the information that genomic sequences provide towards understanding of symbiotic relationships between arthropods and spiroplasmas.

GENERAL FEATURES OF *Spiroplasma* GENOMES

It was not until 2010 that the first draft genome sequence of *S. citri* became available (Carle *et al.*, 2010). Currently, there are 10 genomes deposited in either draft or complete assembly in the NCBI genome database. Four sequenced strains are associated with mosquitoes. *Spiroplasma culicicola* AES-1 and *S. taiwanense* CT-1^T are known pathogens that produce tissue damage and increased mortality in their respective mosquito host (Humphery-Smith *et al.*, 1991). Infections by the sequenced strains of *S. sabaudiense* Ar-1343 and *S. diminutum* CUAS-1^T show no significant effects on mosquitoes and are considered commensal bacteria (Abalain-Colloc *et al.*, 1987; Williamson *et al.*, 1996). Three other *Spiroplasma* genomes correspond to honeybee pathogens, including two *S. melliferum* strains and one strain of *S. apis* (Bové *et al.*, 1983). Further, *S. chrysopicola* DF-1 and *S. syrphydicola* EA-1 strains are considered commensals of the deerfly *Chrysops* sp. (Whitcomb *et al.*, 1997) and the syrphyd fly *Eristalis arbustorum* (Whitcomb *et al.*, 1996), respectively. Finally, the sequenced strain of *S. citri* Gll3-3X is the causal agent of citrus stubborn disease in plants and is transmitted by leafhoppers that feed from phloem nutrients. In this case, the insect host act as a vector of the plant pathogen (Bové *et al.*, 2003). It is important to highlight that no genome sequences for mutalistic *Spiroplasma* genomes have been published or released to public repositories.

Spiroplasma genome features are summarized in Table 1. Their genome size ranges from 780 to 2220 kb. This range of genome sizes is wider than those of other mollicutes such as *Mycoplasma* (Carle *et al.*, 1995). G + C content of the sequenced genomes ranges from 23.9% of *S. taiwanense* CT-1^T to 30.2% of *S. sabaudiense* Ar-1343. All the sequenced genomes have one rRNA operon and 29 tRNA genes, except *S. sabaudiense*, which has two complete and identical rRNA operons and 30 tRNA genes with an extra copy of tRNA-Ser gene. Only few spiroplasmas have plasmids. *Spiroplasma citri* Gll3-3X has seven plasmids, the largest being pSci6 (35.3 kb) and the shortest pSciA (7.8 kb) (Saillard *et al.*, 2008). *Spiroplasma melliferum* IPMB4A has four plasmids of 4.7, 5.6, 9.86 and 14.45 kb (Alexeev *et al.*, 2011). *Spiroplasma taiwanense* CT-1^T has only one plasmid of approximately 11 kb (Gasparich and Hackett 1994).

Spiroplasmas have small genomes with large variations in gene content. For example, in the Citri–Chrysopicola–Mirum, the two strains of *S. melliferum* share 864 genes representing 78.4% of the total genes. If this comparison is made with more phylogenetically distant species like *S. citri*, the amount of common genes decreases to 51.7% of the total genes (Lo *et al.*, 2013a). In the Apis clade, *S. diminitum* and *S. taiwanense* share 59% of their genes. Comparisons between genomes of different clades resulted in lower values of common genes. *Spiroplasma melliferum* shares 38.84% of its genes with *S. diminitum* and only 34.5% with *S. taiwanense* (Lo *et al.*, 2013b). Recently, it has been proposed that 'a prokaryotic genus can be defined as a group of species with all pairwise percentage of conserved proteins values higher than 50%' (Qin *et al.*, 2014). In this context, the above-mentioned set of common genes between the *S. melliferum* strains and *S. citri* is on the borderline limit, even when they belong to the Citri–Chrysopicola–Mirum clade. Furthermore, the shared genes between spiroplasmas of different clades are lower than the proposed threshold which belong to the same genus. Reduced genome size is a common feature in spiroplasmas, but gene conservation seems to depend on host selective pressures.

Spiroplasma GENOME SEQUENCES AND THEIR PHYLOGENOMIC RELATIONSHIPS

The Entomoplasmatales order is composed of four clades: Mycoides–Entomoplasmataceae, Apis, Citri–Chrysopicola–Mirum and Ixodetis. *Mycoplasma*, *Mesoplasma* and *Entomoplasma* genera are restricted to Mycoides–Entomoplasmataceae clade. *Spiroplasma* species are distributed in the other three clades.

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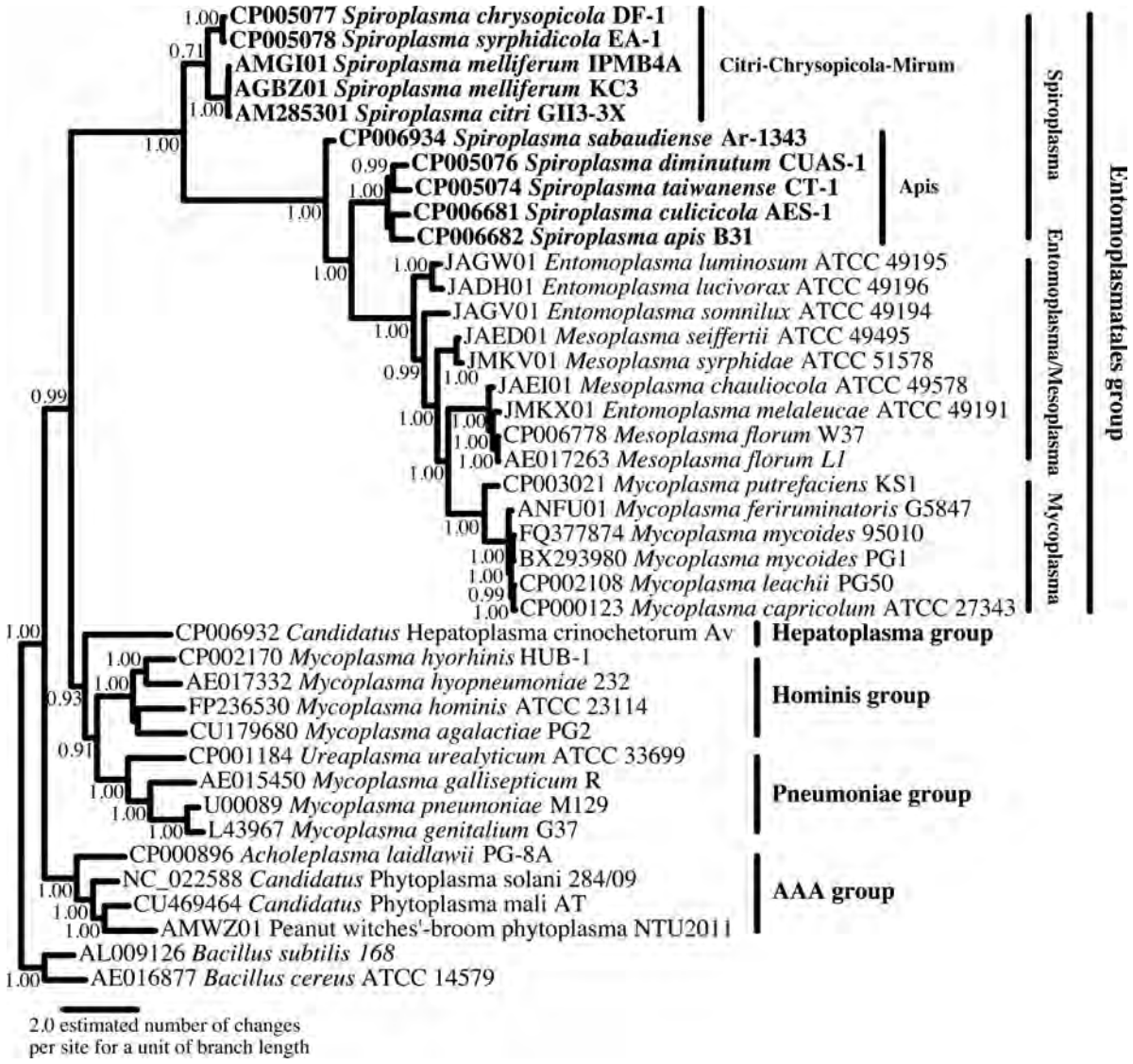

Figure 1. Topology of a phylogenomic analysis showing the predicted evolutionary relationships of sequenced *Spiroplasma* strains within the *Mollicutes*. The tree was reconstructed with PhyloPhlAn using a multisequence alignment of 388 conserved proteins. PhyloPhlAn performs individual alignments from each protein set recovered from the *Mollicutes* input genomes. PhyloPhlAn then concatenates the most discriminative positions in each protein alignment into a single long sequence to reconstruct a phylogenetic tree using FastTree. *Spiroplasma* strains are shown in bold in the tree. Accession numbers are indicated for all sequenced genomes. *Mollicutes* groups are indicated in the tree. Two *Bacillus* strains were used as outgroup. Numbers at the branch points represent SH-like local support values (based on 1000 resamples). The scale bar represents the estimated number of amino acid changes per site for a unit of branch length.

Based on the 16S rRNA gene phylogeny, the genus *Spiroplasma* is not monophyletic (Gasparich 2002).

Spiroplasma strains with available genome sequences correspond to representative members from the Citri–Chrysopicola–Mirum and Apis clades. No *Spiroplasma* genome from the Ixodetis clade is yet available. A phylogenomic approach using multiple amino acid markers (Segata *et al.*, 2013) clearly distinguishes the Citri–Chrysopicola–Mirum clade from the Apis clade, which seems to share a common ancestor with the Entomoplasma/Mesoplasma and Mycoplasma groups (Fig. 1).

Average Nucleotide Identity (ANI) and DNA–DNA Hybridization (DDH) pairwise comparisons between *Spiroplasma* genomes are congruent with the phylogenomic tree. ANI and DDH values highlight the genomic divergence between the Citri–Chrysopicola–Mirum clade from the Apis clade. Species with highest ANI and DDH values are *S. syrphidicola* and *S. chrysopicola* in the Apis clade and *S. citri* with both strains of *S. melliferum* in the Citri–Chrysopicola–Mirum clade. *Spiroplasma sabaudiense* has relatively low values compared with all other sequenced spiroplasmas, but is more closely related to the Apis clade (Tables S1 and S2, Supporting Information). 16S rRNA gene pairwise alignments also revealed striking differences between both clades as they share approximately 90% sequence identity over the entire marker gene (Table S3, Supporting Information). It has been suggested that a cutoff of 94.9% ± 0.4 should define genus boundaries based on 16S rRNA gene sequence identities (Yarza *et al.*, 2008, 2010). The wide range of *Spiroplasma* identities has a minimum of 90.08% and a maximum of 99.67%, which also supports the great divergence between the different species in the genus. Even more revealing is the difference between the highest identities of some Apis clade species against those of the Citri–Chrysopicola–Mirum clade, similar to the observed

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ANI and DDH values. For example, both strains of *S. melliferum* are 97.15% identical, and different Apis clade species such as *S. chrysopicola* and *S. syrphidicola*, which are 99.67% identical.

PLECTROVIRAL SEQUENCES

One of the most striking features of some *Spiroplasma* genomes is the presence of a great amount of plectroviral sequences. Plectroviruses are bacteriophages that infect exclusively cell-wall-less bacteria. Genomes of plectroviruses are present in multiple regions of bacterial genomes (Rakonjac 2012). The presence of plectroviral sequences was detected since the first report of the *S. kunkelii* 85 kb genomic sequence (Zhao *et al.*, 2003). Plectroviral sequences have been found in other *Spiroplasma* genomes. In *S. citri*, *S. melliferum* IPMB4A and *S. melliferum* KC3, repetitive plectroviral sequences and proteins of viral origin were found distributed all over their chromosome. The absence of plectroviral sequences in the genomes of the Chrysopicola and Apis clades seems to indicate that susceptibility of viral invasions may be restricted only to the Citri clade. *Spiroplasma chrysopicola* and *S. syrphidicola* genomes do not have any trace of plectroviral sequences (protein-coding or non-coding) unlike the Citri clade genomes. This may be in relation to the presence of antiviral systems in these strains, such as clustered regularly interspaced short palindromic repeats and type 1 and 2 restriction/modification (R/M), which were found in *S. syrphidicola* and *S. chrysopilica*, respectively (Ku *et al.*, 2013). However, the type 1 R/M system is truncated in *S. citri*.

Ku *et al.* (2013) proposed a model for *Spiroplasma* evolution in relation to viral susceptibility. Allegedly, the common ancestor of Chrysipicola and Citri clades had an active antiviral system or systems and therefore was resistant to the virus, similar to *S. chrysipicola* and *S. syrphiciola*. The Chrysipicola clade diverged and the ancestor of the Citri clade lost its antiviral system(s) and began to accumulate viral fragments. The consequences of these viral infections were the increase in genome sizes and higher homologous recombination rates due to the copies of viral fragments, and even non-viral DNA acquisition. Concomitantly, there should be a counterbalance of genomic acquisitions with loss of old fragments as new viral sequences were inserted. The rearrangement effect of the old fragments seems to be untraceable from the present distribution of the viral fragments.

Viruses can help to horizontally transfer virulence related genes among bacteria (Moore and Lindsay 2001). The genomes susceptible to viral infections are those from *Spiroplasma* pathogenic to bees and plants from the Citri clade. Pathogenicity genes were probably acquired by lateral transfer mediated by virus; thus, a correlation of viral infections with pathogenic lifestyle of spiroplasmas has been proposed (Ku *et al.*, 2013). Genome plasticity emerges from constant sequence acquisitions and losses under strong selective pressures. This plasticity may have led *Spiroplasma* to develop mechanisms that allow it to be undetected by the host immune system (Anbutsu and Fukatsu 2010; Herren and Lemaitre 2011).

The *recA* gene is truncated in strains of *S. citri* and *S. melliferum* MC3 (Marais, Bove and Renaudin 1996). RecA mediates homologous recombination, essential for maintaining genomic integrity and generating genetic diversity (Chen, Yang and Pavletich 2008). *Spiroplasma melliferum* IPMB4A genome lacks the machineries for mismatch repair and homologous recombination, *recA* included (Lo *et al.*, 2013a). The loss of a functional RecA in these species seems to be a relatively recent event. It is suggested that genome instability occurred before *recA* loss (Ku *et al.*, 2013).

PATHOGENICITY FACTORS

Spiroplasma pathogenicity in arthropods has been correlated with the ability of the bacteria to cross the epithelial gut lumen barrier. After trespassing the gut tissue, bacteria infect other host tissues via the hemolymph (Kwon, Wayadande and Fletcher 1999). To accomplish this task, pathogens need a set of molecular tools with different specialized functions. Genomic sequencing has provided insights into possible genes associated with the gradual steps of host tissue invasion.

Chitin degradation genes including chitinase A (*chiA*) and a putative chitin deacetylase were proposed to be used for the first step of invasion, which is the permeabilization of bacterial load through the epithelial barrier. The discovery of the protein product of chitin deacetylase gene in the *S. melliferum* KC3 proteome provides evidence that this gene is being expressed (Alexeev *et al.*, 2011). Chitin is a structural biopolymer of various insect cuticles including the gut lumen (Merzendorfer and Zimoch 2003). Chitin degradation causes permeation of structural components of the peritrophic matrix of the gut epithelium. However, *chiA* and putative chitin deacetylase homologues are found in commensal *S. chrysopicola* and *S. syrphidicola*, and not in pathogenic *S. taiwanense*. Chitin is also present in the cell walls of fungi; it remains to be elucidated if *Spiroplasma* chitinases could have an effect on fungal gut microbiota, and consequently on hosts.

Another proposed mechanism of transmissibility and invasion of insect cells is receptor-mediated endocytosis (Özbek *et al.*, 2003; Ammar *et al.*, 2004). Spiralin has been proposed as a protein for intestinal epithelium receptor recognition, along with P89, P58, sc76 and P32 (Ye, Melcher and Fletcher 1997; Yu, Wayadande and Fletcher 2000; Boutareaud *et al.*, 2004; Killiny *et al.*, 2006). Spiralin genes are present among the sequenced Citri–Chrysopicola–Mirum *Spiroplasma* genomes and the gene products account for up to 30% of the total protein mass of spiroplasmas (Wroblewski *et al.*, 1977). Protein sequence pairwise comparisons between spiralins revealed low identities between species. The highest protein identity is 99% between both strains of *S. melliferum*. *Spiroplasma citri* strain identities range from 92.7 to 99% (Khanchezar *et al.*, 2014) . The lowest identity among the five sequenced species is 38% between *S. chrysopicola* and both strains of *S. melliferum*. The highest interspecies identity is 71% between *S. citri* and *S. melliferum* KC3. These values support that spiralin is a highly divergent protein (Foissac *et al.*, 1996; Meng *et al.*, 2010). *In vitro*, spiralin binds glycoproteins from its insect vector (Killiny, Castroviejo and Saillard 2005); although it is not important for pathogenicity in plants, it is essential for *S. citri* infection (Duret *et al.*, 2003). Regularly, spiralin is distributed along the cell. During adhesion of *S. citri* with *Circulifer haematoceps* cells, spiralin relocates to the space of contact and acts as an adhesin, which allow further internalization of bacterial cells into the insect cell (Duret *et al.*, 2014).

Once the intestinal lumen has been crossed, spiroplasmas reach hemolymph, where they start to proliferate. It has been suggested that proliferation is limited by the availability of nutrients, specifically hemolymph lipids (Herren *et al.*, 2014).

Spiroplasma citri (Gaurivaud *et al.*, 2000) and *S. melliferum* (Chang and Chen 1983) can ferment trehalose, the main sugar and carbon source in insect hemolymph. TreB is a transporter involved in the uptake of trehalose and TreA is the enzyme that converts trehalose-6P to glucose-6P. Both genes are present in *S. melliferum*, *S. citri* and *S. diminutum* (non-pathogenic). *Spiroplasma taiwanense* lacks *treA* and *treB*, so it may have alternative metabolic capacities to survive in its host hemolymph.

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Genomic comparisons of pathogenic and commensal spiroplasmas of mosquitoes indicated that *L*-α-glycerophosphate oxidase (GlpO) might be a virulence factor. GlpO converts *sn*-glycerol 3-phosphate + O_2 to glycerone phosphate + H_2O_2 (Chang *et al.*, 2014). Previously, it was found that GlpO plays a central role in virulence of *Mycoplasma mycoides* due to the production and translocation of H_2O_2 into the host cell (Bischof, Vilei and Frey 2009). In *Spiroplasma*, the two pathogenic species *S. culicicola* and *S. taiwanense* share a copy of *glpO*, along with the transporter genes *ugpA*, *ugpC* and *ugpE*, which allow the *sn*-glycerol 3-phosphate uptake and a glycerol kinase for glycerol phosphorylation (*glpK*) (Chang *et al.*, 2014). In contrast, the mosquito commensals *S. diminutum* and *S. sabaudiense* lack these genes. Interestingly, *glpO* is conserved in the commensal species *S. chrysopicola* and *S. syrphidicola* from deer flies and syrphid flies. These two species also have an *ugpA* ortholog annotated as a hypothetical protein, but no tissue damage in their hosts has been reported.

COMPARATIVE GENOMICS AND METABOLISM

Spiroplasmas have some common biochemical characteristics such as glucose fermentation, arginine hydrolysis and inability to hydrolyze urea, and the majority require an external source of sterols (Regassa and Gasparich 2006). Like other Mollicutes, spiroplasmas have very limited biosynthetic capabilities (Petzel and Hartman 1990) and are considered fastidious organisms due to the complex nutritional requirements needed to grow in culture. They lack almost all of the genes required for amino acid synthesis. In contrast, they conserve a set of genes that encode transporters like the arginine/ornithine antiporter present in *S. sabaudiense*, *S. citri* and *S. syrphidicola* among other amino acid permeases. But the putative main system to acquire amino acids from the media is the oligopeptide transport system, which is conserved among all the *Spiroplasma* genomes (*oppA, oppB, oppC, oppD, oppF*).

Other important conserved permeases among all the genomes are those for the transport of glucose (*ptsG*) and fructose (*fruA*). Besides the transporters, spiroplasmas have the complete set of genes involved in glycolysis. Furthermore, *S. diminutum* has genes for sucrose uptake (*scrA*), and its conversion to glucose-6P (*scrB*) or fructose-6P (*scrK*). It is important to note that the presence of genes does not necessarily mean that they are expressed. Future transcriptomic analyses are required to confirm the functionality of these genes.

Among the few common biosynthetic capabilities of spiroplasmas are the non-mevalonate pathway for isopentenyl pyrophospate synthesis and the pathway for nucleotide biosynthesis. The non-mevalonate pathway for isopentenyl pyrophospate (I-PP) is composed of seven genes (*dxr, dxs, ispD, ispE, ispF, ispG* and *ispH*). This pathway takes as input pyruvate to produce I-PP, a precursor for the biosynthesis of terpenes. For numerous microbial pathogens, the non-mevalonate pathway is the only source of terpenoids (Rohdich *et al.*, 2002). However, spiroplasmas have no annotated genes involved in the next steps of terpenoid biosynthesis, but only the intermediate *uppS* gene. This gene transforms farnesyl pyrophosphate into undecaprenyl pyrophosphate. Spiroplasmas apparently lack the enzyme that converts I-PP into farnesyl-PP: this missing gene should link both pathways and challenges whether the spiroplasmas can produce undecaprenyl pyrophosphate terpene.

SEX-RATIO DISTORTION MECHANISM

Spiroplasma is widely recognized because of the male-killing phenotype induction in *Drosophila* flies. These bacteria are vertically transmitted maternally and kill male eggs before gastrulation (Counce and Poulson 1962). Several strains of male-killing *Spiroplasma* have been isolated from different species of *Drosophila* (Williamson and Poulson 1979; Pool, Wong and Aquadro 2006), butterflies (Jiggins *et al.*, 2000) and ladybird beetles (Tinsley and Majerus 2006), in addition to other strains that do not express male-killing phenotype in their hosts (Kageyama *et al.*, 2006). The molecular mechanisms underlying this phenotype have begun to be elucidated. Recently, two mechanisms have been described: apoptosis-dependent epidermal cell death and apoptosis-independent neural malformation. *Drosophila* embryos infected with male-killing spiroplasmas develop a remarkable neural malformation. Additionally, *Drosophila* embryos with *Spiroplasma* show an up-regulated, male-specific apoptotic pathway mainly targeted to embryonic epithelial cells (Martin, Chong and Ferree 2013). The two mechanisms seem to be independent because even if the host apoptotic pathway is disrupted, the male-specific neural malformation occurs (Harumoto, Anbutsu and Fukatsu 2014).

An important observation in the study of the male-apoptosis-dependent epidermal cell death mechanism is that *Spiroplasma* abundance is not the factor responsible for the phenotype. The signal that triggers these effects on male embryos should be *Spiroplasma*-derived factor(s) that act(s) selectively. Unfortunately, currently there are no genomic sequences of *Spiroplasma* isolated from *Drosophila* hosts. Transcriptomic studies with male-killing and non-male-killing strains may unveil *Spiroplasma* factors produced in the presence of *Drosophila* embryos.

CONCLUSIONS

The presence of repetitive phage sequences hampered the complete assembly of *Spiroplasma* genomes. Despite the difficulties, *Spiroplasma* genome sequencing projects have elucidated important information on metabolism, pathogenicity and genome dynamics. Other biological aspects such as the molecular male-killing mechanisms or the possible genes involved in mutualistic symbiosis functions have not been revealed by comparative genomics. Hypothetical proteins account for approximately 40% of the total protein coding genes and the majority of the species-specific genes across genome comparisons are annotated as hypothetical. Species-specific genes could be the most important elements to understand the intimate and unique associations that *Spiroplasma* establishes with their hosts. Ongoing *Spiroplasma* genome sequencing projects will enrich phylogenomic and comparative genome analyses. Parallel studies of *in vivo* transcriptomic or proteomic analysis should be done to understand the gene expression dynamics of *Spiroplasma* genes and proteins in the presence of the host. Furthermore, the creation of mutant banks from these strains could help elucidate functions for novel, hypothetical or unknown annotated *Spiroplasma* genes.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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