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, 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
parasitoid wasps (Xie, Vilchez and Mateos 2010), nematodes (Jea- nieke et al., 2010; Cockburn et al., 2013) and fungal pathogens (Lukasik et al., 2013). Spiroplasma kunkelii increases the survival rate of the leafhopper 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<sup>7</sup> are known pathogens that produce tissue damage and increased mortality in their respective mosquito host (Humphrey-Smith et al., 1991). Infections by the sequenced strains of S. sabaudiense Ar-1343 and S. diminutum CUAS-1<sup>7</sup> 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. chrysop- dacola DF-1 and S. syr- phydica EA-1 strains are considered commensals of the deerfly Chrysops sp. (Whitcomb et al., 1997) and the syrphid fly Eristalis arbutorum (Whitcomb et al., 1996), respectively. Finally, the sequenced strain of S. citri GI3-3X 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 mutualistic Spiroplasma genomes have been published or released to public repositories. Spiroplasma genome features are summarized in Table 1. Their genome sizes range from 7,880 to 22,200 kb. This range of genome sizes is wider than those of other mollicutes such as Mycoplasma (Carle et al., 1995). C + G content of the sequenced genomes ranges from 23.9% of S. taiwanense CT-1<sup>7</sup> to 33.2% of S. sabaudiense Ar-1343. All the sequenced genomes have one mRNA operon and 29 tRNA genes, except S. sabaudiense, which has two complete and identical mRNA operons and 30 tRNA genes with an extra copy of tRNA-Ser gene. Only few spiroplasmas have plasmids. Spiroplasma citri GI3-3X 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, 8.66 and 14.45 kb (Alexeev et al., 2011). Spiroplasma taiwanense CT-1<sup>7</sup> 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–Chrysopodica–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. diminutum 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. diminutum 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–Chrysopodica–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 Genomes and Their Phylogenomic Relationships**

The Entomoplasmatales order is composed of four clades: Mycoideal–Entomoplasmataceae, Apis, Citri–Chrysopodica–Mirum and Ixodetes. Mycoplasma, Mesoplasma and Entomoplasm genera are restricted to Mycoideal–Entomoplasmataceae clade. Spiroplasma species are distributed in the other three clades.

<table>
<thead>
<tr>
<th>Arthropod host</th>
<th>S. chrysopodica DF-1</th>
<th>S. syrphydica EA-1</th>
<th>S. citri GI3-3X</th>
<th>S. melliferum IPMB4A</th>
<th>S. melliferum KC3</th>
<th>S. diminutum CUAS-1&lt;sup&gt;7&lt;/sup&gt;</th>
<th>S. taiwanense CT-1&lt;sup&gt;7&lt;/sup&gt;</th>
<th>S. apis B31&lt;sup&gt;7&lt;/sup&gt;</th>
<th>S. culicicola AES-1&lt;sup&gt;7&lt;/sup&gt;</th>
<th>S. sabaudiense Ar-1343&lt;sup&gt;7&lt;/sup&gt;</th>
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<tr>
<td>Commensal</td>
<td>1123 322</td>
<td>1107 344</td>
<td>1526 756</td>
<td>1098 846</td>
<td>1260 174</td>
<td>945 296</td>
<td>1075 140</td>
<td>1160 554</td>
<td>1175 131</td>
<td>1075 953</td>
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<td>28.8</td>
<td>29.2</td>
<td>25.9</td>
<td>27.5</td>
<td>27</td>
<td>25.5</td>
<td>23.9</td>
<td>28.3</td>
<td>26.4</td>
<td>30.2</td>
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<td>Protein-coding genes</td>
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<td>1006</td>
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<td>920</td>
<td>1046</td>
<td>858</td>
<td>991</td>
<td>997</td>
<td>1071</td>
<td>924</td>
</tr>
</tbody>
</table>

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

Table 1. General features of the sequenced Spiroplasma genomes.
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 Ixodetes 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 phylogenetic 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 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
ANI and DDH values. For example, both strains of *S. melliferum* are 97.15% identical, and different Apis clade species such as *S. chrysopica* and *S. syrphidicola*, which are 99.67% identical.

**PECTROVIRAL 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. kunkeli* 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 Chrysopica and Apis clades seems to indicate that susceptibility of viral invasions may be restricted only to the Citri clade. *Spiroplasma chrysopica* 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 clusted regularly interspaced short palindromic repeats and type 1 and 2 restriction/modification (R/M), which were found in *S. syrphidicola* and *S. chrysopica*, 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 Chrysipica and Citri clades had an active antiviral system or systems and therefore was resistant to the virus, similar to *S. chrysopica* and *S. syrphidicola*. The Chrysipica 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 (Ambutsu and Fukatsu 2010; Herren and Lemaître 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. chrysopica* 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-Chrysopica-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 spiralin 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. chrysopica* 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 *C. haematocoept* 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.
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 + O2 to glycerone phosphate + H2O2 (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 H2O2 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 1pgA, 1pgC and 1pgE, 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. sabaudienese 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 1pgA 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. sabaudienese, 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 (fuaA). 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 pyrophosphate synthesis and the pathway for nucleotide biosynthesis. The non-mevalonate pathway for isopentenyl pyrophosphate (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 mutabilistic 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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