Molecular Evolution of the Substrate Utilization Strategies and Putative Virulence Factors in Mosquito-Associated Spiroplasma Species

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Abstract

Comparative genomics provides a powerful tool to characterize the genetic differences among species that may be linked to their phenotypic variations. In the case of mosquito-associated Spiroplasma species, such approach is useful for the investigation of their differentiations in substrate utilization strategies and putative virulence factors. Among the four species that have been assessed for pathogenicity by artificial infection experiments, Spiroplasma culicicola and S. taiwanense were found to be pathogenic, whereas S. diminutum and S. sabaudiense were not. Intriguingly, based on the species phylogeny, the association with mosquito hosts and the gain or loss of pathogenicity in these species appears to have evolved independently. Through comparison of their complete genome sequences, we identified the genes and pathways that are shared by all or specific to one of these four species. Notably, we found that a glycerol-3-phosphate oxidase gene (glpO) is present in S. culicicola and S. taiwanense but not in S. diminutum or S. sabaudiense. Because this gene is involved in the production of reactive oxygen species and has been demonstrated as a major virulence factor in Mycoplasma, this distribution pattern suggests that it may be linked to the observed differences in pathogenicity among these species as well. Moreover, through comparative analysis with other Spiroplasma, Mycoplasma, and Mesoplasma species, we found that the absence of glpO in S. diminutum and S. sabaudiense is best explained by independent losses. Finally, our phylogenetic analyses revealed possible recombination of glpO between distantly related lineages and local rearrangements of adjacent genes.

Key words: Mollicutes, Spiroplasma, mosquito, virulence factor, glycerol-3-phosphate oxidase, glpO.

Introduction

Comparative analysis of gene content among related species with distinct phenotypes has provided a powerful tool to investigate the underlying genetic mechanisms. For example, examination of the presence and absence of genes between bacterial species that differ in pathogenicity can be used to identify putative virulence factors. This genome-scale screening is a high-throughput and cost-effective approach of narrowing down the list of candidate genes, which may greatly facilitate the downstream experimental verification and functional characterization. To demonstrate the utility of this comparative approach, the mosquito-associated Spiroplasma species provide a good study system.

The genus Spiroplasma contains a diverse group of wall-less bacteria that are mostly associated with various insect hosts (Whitcomb 1981; Gasparich et al. 2004; Regassa and Gasparich 2006; Gasparich 2010). To date five characterized Spiroplasma species have been found to be associated with mosquitoes, including Spiroplasma culicicola (Hung et al. 1987), S. sabaudiense (Abalain-Colloc et al. 1987),...
S. taiwanense (Abalain-Colloc et al. 1988), S. cantharicola (Whitcomb et al. 1993), and S. diminutum (Williamson et al. 1996). All of these five Spiroplasma species belong to the Apis clade within the genus. Interestingly, examination of their serotypes, phylogenetic placements, and the host associations of other related species suggest that the associations with mosquitoes have multiple independent origins (Gasparich et al. 2004; Lo, Ku, et al. 2013; the phylogeny and host associations are summarized in fig. 1). Because of the interests in developing these mosquito-associated bacteria for biological control of insect pests, a series of artificial infection experiments have been performed to examine the pathogenicity of these Spiroplasma species (Chastel and Humphery-Smith 1991; Humphery-Smith, Grulet, Chastel, et al. 1991; Humphery-Smith, Grulet, Le Goff, et al. 1991; Vorms-Le Morvan et al. 1991; Vazeille-Falcoz et al. 1994; Phillips and Humphery-Smith 1995). Based on these results, infection by S. taiwanense or S. culicicola increased the mortality of mosquitoes, no significant effect was found for the infection by S. diminutum or S. sabaudiense, while the effects of S. cantharicola infection remained to be tested.

To investigate the genetic mechanisms that may explain these observed differences in artificial infection experiments, we have determined the complete genome sequences of S. taiwanense and S. diminutum for comparative analysis (Lo, Ku, et al. 2013). One main finding from this pairwise genome comparison is that S. taiwanense has a copy of glpO encoding a glycerol-3-phosphate (G3P) oxidase, while S. diminutum does not. Because this gene is involved in reactive oxygen species (ROS) production, the presence of this gene in the S. taiwanense genome provides an explanation for the observation of tissue damage (Phillips and Humphery-Smith 1995) and increased mortality (Humphery-Smith, Grulet, Chastel, et al. 1991; Humphery-Smith, Grulet, Le Goff, et al. 1991; Vorms-Le Morvan et al. 1991; Vazeille-Falcoz et al. 1994) in infected hosts. Moreover, functional characterizations have provided experimental evidence that this gene is the main virulence factor in the closely related Mycoplasma mycoides (Pilo et al 2005, 2007) and the more distantly related M. pneumoniae (Hames et al. 2009).

However, several questions remained regarding the molecular evolution of glpO in mosquito-associated Spiroplasma species. For example, was the gene gained in the lineage leading to S. taiwanense or lost in S. diminutum? Do other Spiroplasma species possess this gene as well? To address these questions, we determined the complete genome sequences of the other two mosquito-associated Spiroplasma species that have been tested in artificial infection experiments, S. culicicola and S. sabaudiense, for more comprehensive comparative analyses in this study. Because the two species that have been found to be pathogenic (i.e., S. taiwanense and S. culicicola) do not form a monophyletic clade when other mosquito-associated species are considered (Lo, Ku, et al. 2013), this expansion in taxon sampling provides us with the opportunity to investigate the possibility of multiple independent gains or losses of putative virulence factors in these bacteria. Additionally, the recent increased availability of complete genome sequences from other Spiroplasma species (Ku et al. 2013, 2014) has improved our ability to establish the ancestral states of gene content and to perform molecular phylogenetic inference. Taken together, we aim to improve our understanding of the substrate utilization strategy and putative virulence factors in mosquito-associated Spiroplasma species.

**Materials and Methods**

**Genome Sequencing**

The two bacterial strains sequenced in this study, S. culicicola AES-1 and S. sabaudiense Ar-1343, were acquired from the American Type Culture Collection (ATCC catalog numbers 35112 and 43303, respectively). For the whole genome shotgun sequencing of S. culicicola, one paired-end (∼160-bp insert; 151-bp reads; ∼0.81-Gb raw reads) and one mate-pair....
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results and discussion

Genome Sequences of Mosquito-Associated Spiroplasma Species

The genome assembly statistics and chromosomal organization of the four mosquito-associated Spiroplasma species are provided in table 1 and figure 2. Both of the two newly sequenced species contain a circular chromosome that is approximately 1.1 Mb in size (S. culicicola: 1,175,131 bp; S. sabaudiense: 1,075,953 bp). These genome sizes are slightly smaller than those reported previously based on pulsed-field gel electrophoresis (Carle et al. 1995). The values for GC content are similar to those estimates based on the buoyant density method (Abalain-Colloc et al. 1987; Hung et al. 1987).

The availability of these two additional Spiroplasma genomes, together with the previously established species phylogeny (Lo, Ku, et al. 2013), provided several insights into the phylogeny of the Apis clade. The second set expands the taxon sampling to include S. apis in the Apis clade, four Mycoplasma/Mesoplasma species in the sister Mycoides–Entomoplasmataceae clade (M. mycoides [BX293980], M. leachii [CP002108], M. putrefaciens [CP004357], and Me. florum [AE017263]) and two Spiroplasma species in the Chrysopicola clade as the outgroup (S. chrysopicola and S. syphidica). In these two sets of comparative analyses, the homologous gene clusters among the genomes being compared were identified by OrthoMCL (Li et al. 2003). The lists of homologous gene clusters were examined to investigate the patterns of gene presence and absence.

For the four mosquito-associated Spiroplasma species, we utilized MUMmer v3.23 (Kurtz et al. 2004) for pairwise genome alignments. We increased the minimum match length (option “–l”) to 24 from the default setting of 20 to reduce spurious hits. The chromosome of S. taiwanense was chosen as the reference to be compared with the other three species. To estimate the genome-wide sequence divergence levels, the single-copy orthologous genes shared by these four species were used for sequence alignment by MUSCLE v3.8 (Edgar 2004). The alignments of individual genes were concatenated for calculation of sequence similarities by the DNADIST and PROTDIST programs of PHYLIP v3.69 (Felsenstein 1989).

For molecular phylogenetic inference, homologous genes from selected genomes were aligned using MUSCLE v3.8 (Edgar 2004). The maximum likelihood phylogenies were inferred using PhyML v3.0 (Guindon and Gascuel 2003). The proportion of invariable sites and the gamma distribution parameter were estimated from the data set, and the number of substituting rate categories was set to four. Bootstrap supports were estimated based on 1,000 replicates generated by the SEQBOOT program of PHYLIP v3.69 (Felsenstein 1989).
genome evolution in the Apis clade. For example, the low GC content, low coding density, and high frequency of pseudogenes observed in the *S. taiwanense* genome (Lo, Ku, et al. 2013) appear to be derived states specific to this lineage. Additionally, *S. sabaudiense* belongs to the basal group and has several distinct genomic features. First, *S. sabaudiense* has the highest GC content (30.2%) among the *Spiroplasma* genomes sequenced to date. For comparison, the other three mosquito-associated *Spiroplasma* species have a GC content of 23.9–26.4%. Considering that the *S. apis* genome has a GC content of 28.3% (Ku et al. 2014) and the more distantly related *Spiroplasma* species in the Chrysopicola clade have a GC content of 28.8–29.2%, it is possible that a relatively high GC content of ~28–30% (Ku et al. 2014) and the more distantly mosquito-associated genomes sequenced to date. For comparison, the other three mosquito-associated *Spiroplasma* species have a GC content of ~28–30% (Ku et al. 2014) and the more distantly mosquito-associated genomes sequenced to date. For comparison, the other three mosquito-associated *Spiroplasma* species have a GC content of ~28–30% (Ku et al. 2014) and the more distantly mosquito-associated genomes sequenced to date.

### Table 1

**Genome Assembly Statistics**

| Genome     | *S. diminutum* CUAS-1 | *S. taiwanense* CT-1 | *S. culicicola* AES-1 | *S. sabaudiense* Ar-1343 |
|------------|-----------------------|----------------------|-----------------------|--------------------------|
| GenBank accession | CP005076 | CP005074 | CP006681 | CP006934 |
| Chromosome size (bp) | 945,296 | 1,075,140 | 1,175,131 | 1,075,953 |
| GC content (%) | 25.5 | 23.9 | 26.4 | 30.2 |
| Coding density (%) | 92.7 | 82.5 | 92.2 | 90.0 |
| Protein-coding genes | 858 | 991 | 1,071 | 924 |
| Length distribution (Q1/Q2/Q3) (aa) | 177/283/443 | 137/247/397 | 176/283/437 | 189/296/455 |
| Hypothetical proteins | 310 | 452 | 460 | 368 |
| Annotated pseudogenes | 0 | 54 | 0 | 7 |
| rRNA genes/operons | 3/1 | 3/1 | 3/1 | 6/2 |
| tRNA genes | 29 | 29 | 29 | 30 |
| Plasmid (GenBank accession) | 0 | 1 (CP005075) | 0 | 0 |

The chromosome of *S. taiwanense* is used as the reference for pairwise genome alignments, the patterns are consistent with the expectation based on the species phylogeny (Lo, Ku, et al. 2013) and the levels of sequence similarity (fig. 3). Compared with the most closely related *S. diminutum* (fig. 3A), the chromosomal organizations are largely conserved, except for the ~0.5–0.7 Mb region that contains the putative replication terminus at ~0.58 Mb and possibly involves an inversion. In contrast, the highly divergent *S. sabaudiense* exhibits low levels of sequence similarity and synteny conservation (fig. 3C).

### Comparison of Gene Content and Substrate Utilization Strategies

A total of 1,634 homologous gene clusters were found among the four mosquito-associated *Spiroplasma* species (fig. 4 and supplementary table S1, Supplementary Material online). Among these, 552 are shared by all four species (>50% of all the protein-coding gene in each species). These core genes include those involved in essential cellular processes conserved among bacteria (Koonin 2003; Lapière and Gogarten 2009; Chen et al. 2012), such as DNA replication, transcription, and translation. Furthermore, genes that have been suggested as shared between the Apis and the Citrus clade within *Spiroplasma* (Lo, Ku, et al. 2013), such as those involved in glucose uptake and utilization (ptsG and crn), fructose uptake and utilization (fruA and fruK), N-acetyl-glucosamine (GlcNAc) uptake and utilization (nagE, nagA, and nagB), glycolysis (pgi, pfkA, fbaA, gap, gapN, pgk, pgm, eno, and ppy; the dotted line in fig. 5), nucleotide biosynthesis (e.g., adk, apt, gmk, hprT, purA, purB, pyrG, pyrH, rdgB, tdk, thyA, tmk, upp, etc), the nonmevalonate pathway for isopentenyl pyrophosphate synthesis (dxr, dks, ispD, ispE, ispF, ispG, and ispH), protection from oxidative stress (sufB, sufC, sufD, sufS, sufU, and tpx), and putative secreted proteins containing GH18 chitinase and SGNH hydrolase domains are found to be conserved in these four species. Intriguingly, among these conserved genes, we observed several cases of lineage-specific gene family expansions. For example, in a previous analysis of the *S. taiwanense* genome (Lo, Ku, et al. 2013), three oligopeptide ABC transporter genes (*oppC, oppD*, and *oppF*) were found to have three copies each. Because these genes are single copy in the other three *Spiroplasma* genomes compared to the *S. taiwanense* genome (Lo, Ku, et al. 2013), three oligopeptide ABC transporter genes (*oppC, oppD*, and *oppF*) were found to have three copies each. Because these genes are single copy in the other three *Spiroplasma* genomes compared...
In this study, this observation is best explained by *S. taiwanense*-specific tandem duplications. In addition, the 6-phospho-beta-glucosidase gene (*bgl*) was found to exhibit a high level of copy number variation, ranging from single copy in *S. taiwanense* to eight copies in *S. culicicola*. Most of the species-specific genes are annotated as hypothetical proteins, such that we are unable to infer their function.

**Fig. 2.** Chromosomal organization of the four mosquito-associated *Spiroplasma* species. Rings from the outside in: (1) scale marks (unit: Mb), (2 and 3), protein-coding genes on the forward and reverse strand, respectively (color coded by the functional categories), (4) GC skew (positive: green; negative: yellow), and (5) GC content (above average: orange; below average: blue; rRNA operons are labeled by black triangles).
Among these four species, *S. diminutum* has the lowest number of species-specific gene clusters (fig. 4), possibly due to the fact that it has the smallest chromosome and the fewest protein-coding genes. Intriguingly, *S. sabaudiense* has a large family of species-specific hypothetical proteins with 27 copies. These genes are often found as clusters of two to four adjacent copies on the chromosome in regions with unexpected GC skew patterns (e.g., ~0.2, ~0.4, and ~1.0 Mb in fig. 2; the assembly in these regions have been verified by PCR), suggesting that these DNA have been integrated recently. However, because database searches provided no identifiable homolog or conserved protein domain, the function and the origin of these hypothetical proteins remained unknown.

In the few cases that the functional roles of species-specific genes can be inferred, they reveal interesting information about the metabolism differences among these species. For example, *S. sabaudiense* is the only species that has the complete set of genes for arginine utilization (*arcA*, *arcB*, and *arcC*), which is consistent with previous biochemical tests (Abalain-Colloc et al. 1987; Hung et al. 1987; Abalain-Colloc et al. 1988; Williamson et al. 1996). Additionally, sucrose utilization (*scrB* and *scrK*) appears to be limited to *S. diminutum*, while glycerophosphocholine (GPC; substrate of *glpU* and *glpQ*) utilization appears to be limited to *S. culicicola*. Intriguingly, one putative secreted protein specific to *S. culicicola* (SCULL_v1c06250) was found to contain a partial Pfam03318 domain (*Clostridium epsilon* toxin ETX/*Bacillus* mosquitocidal toxin MTX2), which may contribute to its pathogenicity toward mosquitoes.

Other than the genes that are shared by all four species or specific to one of the species, genes with more variable phylogenetic distribution patterns are important in promoting our understanding of these mosquito-associated bacteria (fig. 5 and supplementary table S1, Supplementary Material online). Two sets of genes are of particular interest because of the differences in pathogenicity toward mosquitoes observed in
**Fig. 5.**—Patterns of gene presence and absence in selected metabolic pathways and transporters.

**Fig. 6.**—Chromosomal locations of the glpF-glpO-glpK genes. The circular chromosomes are presented as linear for visualization; the first base of dnaA is used as the leftmost position for each chromosome. The sizes of individual genes are not drawn to scale.
previous artificial infection experiments (Chastel and Humphery-Smith 1991; Humphery-Smith, Grulet, Chastel, et al. 1991; Humphery-Smith, Grulet, Le Goff, et al. 1991; Vorms-Le Morvan et al. 1991; Vazeille-Falcoz et al. 1994; Phillips and Humphery-Smith 1995). For the two species without apparent pathogenicity (i.e., *S. diminutum* and *S. sabaudiense*), they were found to share *murP* and *murQ* for the uptake and utilization of *N*-acetylmuramic acid (MurNAc) and *nrdD* for the conversion of CTP to dCTP (Fontecave et al. 1989). For the two species that exhibit pathogenicity (i.e., *S. culicicola* and *S. taiwanense*), they were found to share a copy of *glpO* for ROS production. This finding provides further support for our previous inference that *glpO* is likely a virulence factor in these mosquito-associated *Spiroplasma* species (Lo, Ku, et al. 2013). To provide the substrate for *glpO*, these two species both contain the genes coding for *sn*-glycerol-3-phosphate ABC transporter (*ugpA*, *ugpC*, and *ugpE*) for direct import of G3P and glycerol kinase (*glpK*) for glycerol phosphorylation. Furthermore, a pseudogenized copy of glycerophosphoryl diester phosphodiesterase (*glpQ*) was found in the *S. taiwanense* genome (Lo, Ku, et al. 2013), suggesting that the metabolic capacity to utilize GPC was ancestral as well.

**Molecular Evolution of the Glycerol Metabolism Genes**

Based on the comparison of their substrate utilization strategies (fig. 5), glycerol metabolism and the associated production of ROS are likely to be linked to the observed pathogenicity of *S. culicicola* and *S. taiwanense* in artificial infection experiments (Chastel and Humphery-Smith 1991; Humphery-Smith, Grulet, Chastel et al. 1991; Humphery-Smith, Grulet, Le Goff, et al. 1991; Vazeille-Falcoz et al. 1994; Phillips and Humphery-Smith 1995). Our examination of the chromosomal locations of these glycerol metabolism genes revealed that the gene order of *glpF-glpO-glpk* is largely conserved among the *Spiroplasma* species with complete genome sequences available (fig. 6); *S. apis* represents the only exception due to the insertion of *glpQ* and *glpU*.

![Figure 7](image-url)
between glpO and glpK. For comparison, the gene order is glpO-glpK-glpF in the three Mycoplasma species belonging to the Mycoides–Entomoplasmataceae clade, which seems to be a derived state due to one or more rearrangements. Based on the phylogenetic distribution pattern of this gene cluster, its absence in S. diminutum, S. sabaudiense, and Me. florum is best explained by independent losses.

For more detailed investigation of the molecular evolution of these genes, we compared the individual gene trees to the species phylogeny (fig. 7). Surprisingly, despite the conservation in gene order among the Spiroplasma species, all three gene trees support the clustering of S. taiwanense homologs with those from the Mycoides–Entomoplasmataceae clade. This unexpected conflict between gene order and gene phylogenies is difficult to explain. Future investigation that incorporates additional sequence data from more diverse lineages in the Apis and the Mycoides–Entomoplasmataceae clade is essential to confirm the gene phylogenies.

In the examination of gene order and gene phylogenies, we found several interesting points regarding the glycerol uptake facilitator protein gene (glpF). In addition to the copy adjacent to glpO and glpK, several Spiroplasma genomes contain a second copy of glpF in other regions of the chromosomes (fig. 6). In all cases, these isolated copies exhibit high levels of sequence divergence from other homologs (fig. 7B; the locus tags are assigned sequentially starting from dnaA and reflect their relative positions on the chromosome). The second copy from S. apis (SAPIS_v1c05780; located at ~0.69 Mb in fig. 6) was not included in the gene phylogeny because it was not grouped in the same homologous gene cluster with all other copies of glpF. This pattern of sequence divergence may be explained by the release from selective constraint for these redundant copies. For S. diminutum and perhaps also S. sabaudiense, the glpF gene may be in the process of nonfunctionalization because the downstream glpK has been lost (fig. 5). Such gradual degradation of gene content is common among host-associated bacteria (Ochman and Davalos 2006; McCutcheon and Moran 2012) and is likely to be driven by a combination of mutational biases toward deletions and high levels of genetic drift (Mira et al. 2001; Kuo et al. 2009; Kuo and Ochman 2009; Kuo and Ochman 2010). Eventually, these Spiroplasma lineages may lose their glpF just as what has occurred for Me. florum.

Conclusions

In summary, the gene content comparison presented in this study provides an overview on the substrate utilization strategies across diverse mosquito-associated Spiroplasma species. Moreover, our result demonstrates that glpO is conserved across diverse Spiroplasma lineage. The absence of glpO in S. diminutum and S. sabaudiense is best explained by independent losses and may be linked to the lack of pathogenicity in these two species. The clustering of the S. taiwanense glpF(glpO)(glpK) with those from the Mycoides–Entomoplasmataceae clade is an intriguing point that requires further investigation. Finally, future tool development for the genetic manipulation of these bacteria is necessary for the functional characterization of their putative virulence factors.

Supplementary Material

Supplementary table S1 is available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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