SPIROPLASMA ARP SEQUENCES: RELATIONSHIPS WITH EXTRACHROMOSOMAL ELEMENTS

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SPIROPLASMA ARP SEQUENCES: RELATIONSHIPS

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CHAPTER I

LITERATURE REVIEW

Background

Spiroplasmas are wall-less, helical prokaryotes that belong to the family *Spiroplasmataceae* of the order *Entomoplasmatales* in the class *Mollicutes*. Mollicutes, including spiroplasmas, mycoplasmas and acholeplasmas, evolved by genome reduction from ancestral Gram-positive bacteria, such as *Clostridium innocuum* and *C. ramosum*, that have low G+C contents (~ 24 to 26 %) [1, 2]. Their genome sizes range from 0.8 to 2.2 Mb. Mycoplasmas, mollicutes distantly related to spiroplasmas, have genomes of ~ 0.5 Mb. In some mollicutes, the UGA codon can be translated into tryptophan rather than being a translation termination codon [3], which makes the expression of spiroplasma genes in *E. coli* difficult. This problem may be overcome by, among other approaches, using spiroplasmas themselves as expression hosts [4].

Economic Importance

Spiroplasmas, initially termed mycoplasma-like organisms (MLOs), were originally isolated from citrus with stubborn disease and maize with corn stunt disease [5, 6]. To date, there are more than 50 spiroplasma species from various hosts including ticks, wasps, beetles, bees, flies, mammals and from plants [7-9]. Of the spiroplasmas

that are pathogenic, so far only three species are known to cause diseases on plants. *S. citri* [10-12] is the causative agent of stubborn disease of citrus and brittle root disease of horseradish; *S. kunkelii* [13-15] is the etiological agent of corn stunt; and *S. phoeniceum* [16] causes periwinkle yellows. The phytopathogenic spiroplasmas, and the distantly related phytopathogenic phytoplasmas, inflict millions of dollars of economic losses worldwide every year [17, 2].

Classification

Spiroplasmas are classified based on 16S rDNA sequences, DNA-DNA homology, G+C %, serological tests and biochemical properties into different groups [18-19]. *S. citri*, *S. kunkelii*, *S. phoeniceum* and *S. melliferum* belong to group I [20]. 16S rDNA analysis of several spiroplasmas and other representative mollicutes by various phylogenetic analysis methods revealed four main clusters within the Class *Mollicutes*. Within the *S. citri* cluster, there are three clades, including group I (containing the phytopathogenic spiroplasmas) and group II spiroplasmas, viz. the citri clade. The two others are the chrysopicola and mirum clades, containing pathogens distributed over very wide host and geographical ranges. Spiroplasmas from group I are further classified into eight subgroups based on serological data, with *S. citri* forming subgroup I-1, *S. kunkelii* being in subgroup I-3 and *S. phoeniceum* in subgroup I-8 [20]. Phylogenetically, *S. citri* is closer to *S. melliferum* than any other spiroplasma in the group, whereas *S. kunkelii* is more related to *S. phoeniceum* than any other member of the group.

Transmission in Nature

Phytopathogenic spiroplasmas are transmitted from plant to plant by insect vectors. Phloem-feeding insects, predominantly leafhoppers, transmit these pathogens in nature [21]. The beet leafhopper, *Circulifer tenellus*, transmits *S. citri* within the U.S. and the related species, *C. haematoceps*, transmits *S. citri* in the Eastern Hemisphere [22], whereas the leafhopper *Dalbulus maidis* transmits *S. kunkelii*. For *S. phoeniceum*, only experimental transmission by the leafhopper *Macrosteles fascifrons* is known so far [23]. *S. citri* is deposited into, and multiplies within, the phloem sieve tube cells of the plant host and is translocated to various parts of the plant through phloem. For successful transmission to the plant host, *S. citri* must cross the gut wall within the leafhopper after ingestion, enter the haemocoel and multiply in the haemolymph. It also must evade the insect's immune system, cross the salivary gland barrier and enter and multiply in the glands before exiting through the saliva during the feeding of the insect. There are three physical barriers in the leafhopper gut and salivary glands: the apical plasmalemma, the basal plasmalemma and the basal lamina [25]. The leafhopper basal lamina is devoid of pores and channels. How spiroplasmas cross this barrier is unclear.

Structures on the surface of intact *S. kunkelii* cells interpreted as similar to pili and fimbriae have been reported [26], and were speculated to have a role in conjugation [27]. Certain spiroplasma viruses, similar in size and shape to the surface structures reported, associate with the host plasma membrane and can appear similar to those structures [28].

The leafhopper haemolymph may serve as a supportive environment for spiroplasma maintenance and multiplication within the vector. *S. citri* and *S. kunkelii* have been shown to cross the gut wall of their natural vectors, *C. tenellus* and *D. maidis*,

respectively. Kwon *et al*. [29], in their electron micrographs, showed entry of *S. citri* into the cytoplasm of leafhopper midgut and salivary gland cells. Similarly, Ozbek *et al*. [26] reported *S. kunkelii* crossing the midgut and Malpighian tubule barriers in *D. maidis*. Further, *S. citri* is known to lose its helical morphology and become pleiomorphic a week after microinjection into its experimental vector *Euscelidius variegatus* [30]. It can be speculated that spiroplasma transmission by insect vectors is mediated by interactions between specific pathogen surface factors and insect cell surface receptors. Receptor-mediated endocytosis may be involved in the process [31].

Molecular Mollicute-host Interactions

Many animal- and human-infecting mycoplasmas initiate their infection cycle by the attachment of specific surface proteins (adhesins) to the host receptors [32]. The P1 adhesin of *Mycoplasma pneumoniae* and the adhesin MgPa of *M. genitalium* [33-35] are among the well characterized mollicute adhesins. The P1 protein forms the tip of a structure in *M. pneumoniae* called "the attachment organelle", which is necessary for the adherence of the pathogen to the host cells. In nonpathogenic *M. pneumoniae* strains, however, P1 is present uniformly over the pathogen surface [31].

Molecular Spiroplasma-host Interactions

The role of adhesins in *S. citri* transmission by leafhoppers is unclear, although the presence of proteins on its surface is known [36]. Unlike phytoplasmas, spiroplasmas can be cultured *in vitro* and, thus, have become models in studying the molecular mollicute–vector interactions. When *S. citri* BR3, isolated in Illinois [12], was experimentally maintained in different regimes [25], *S. citri* lines descending from it

developed genetic and phenotypic variations. BR3 was first triply cloned to get a homogeneous BR3 stock culture that was frozen. The derivative line was named BR3-3X. When BR3-3X was maintained, for many generations, in periwinkle plants by graft transmission rather than by insect transmission, the resulting line, BR3-G, lost its insect transmissibility. BR3-T, the line resulting after long-term maintenance of BR3-3X in turnip plants by leafhopper transmission, however retained its transmissibility. The chromosomal gene organization of BR3-G differed from that of BR3-3X, but those of BR3-T and BR3-3X remained similar to each other. BR3-G had sustained a large chromosomal inversion associated with a deletion of about 10 kb at each of the two borders of the inversion [37]. Within one of the deleted regions, an open reading frame (ORF) encoding a protein of ~58 kDa, P58, was observed. P58 (designated P58A) is distantly similar in sequence to two mycoplasma adhesins [38] and contains a putative transmembrane helix, suggesting that it is a surface protein. Recent work [J. Comer *et al*., *manuscript submitted*] showed that P58A is a member of a multigene family in *S. citri* BR3-3X. A homologous family of P58 genes was also found in *S. kunkelii* CR2-3X, whose genome sequence is nearly complete and is publicly available (www.genome.ou.edu/spiro.html). Moreover, recent data also suggest that, in both *S. citri* and *S. kunkelii*, the P58 multigene family arose by recombination between genes resembling ancestral adhesin and bacteriophage terminase genes [J. Comer *et al*., *manuscript submitted*]. However, the exact functions of P58 proteins in *S. citri* are not yet known.

Yu *et al*. [32] successfully developed enzyme-linked immunosorbent assay (ELISA)-based and radioisotope-based microtiter plate assays to investigate the binding

of *S. citri* cells to tissue-cultured *C. tenellus* cells. Using such an assay, they observed significant reduction in the adherence by prior treatment of the spiroplasmas with proteinase K or pronase. Electrophoresis and western blotting of spiroplasma membrane proteins, before and after exposure of intact spiroplasmas to proteases, revealed the concomitant disappearance of a major *S. citri* membrane protein (P89) and appearance of a new polypeptide of ~46-kDa (P46) in the protease-treated preparations. Labeling of P46 with anti-P89 serum suggested that it may be a breakdown product of P89. Moreover, regeneration of P89 after proteinase K treatment of spiroplasmas (after their transient incubation in broth medium) was directly associated with restoration of the pathogen's ability to attach to the vector cells. In addition, a protein of approximately the same size as P89 (92-kDa) was differentially expressed in transmissible and non-transmissible *S. citri* BR3-derived lines [39]. These results showed that P89 (designated SARP1 for spiroplasma adhesion related protein 1) is a surface protein and suggested that it has a role in *S. citri* adherence to *C. tenellus* cells [32]. It is therefore hypothesized that SARP1 is an adhesin involved in spiroplasma attachment to the leafhopper tissues. Further, recent work in *S. citri* GII-3 [40] revealed a surface protein highly similar to SARP1, P80, which is absent from the non-transmissible strains and present in transmissible ones and is one of eight Scarp (*S. citri* adhesion-related protein) proteins reported to be present in the pathogen.

The gene encoding SARP1 (*arp1*) was isolated, cloned and characterized (GenBank Accession number AJ297706) [41]. The mature SARP1 protein has a domain, named sarpin, at its N-terminus that is made of six repeats of 39-42 amino acids each, which belong to a novel family of amino acid repeats. The six repeats in the sarpin

domain were predicted to form a propeller-like structure rich in □-sheets followed by a long shaft. Near the C-terminus, SARP1 has a predicted transmembrane helix with a short C-terminal tail [41]. Anti-SARP1 antibodies were used to localize SARP1 on intact spiroplasma cells by immuno-gold labeling [Wayadande and Berg, *unpublished data*]. When tested with a polyclonal antibody, a dense decoration on the surface of intact *S. citri* cells and a moderate decoration on intact *S. kunkelii* cells were seen, substantiating that SARP1 is a surface protein and, therefore, a putative adhesin.

Recent work [40] in *S. citri* GII-3, a phytopathogen from the Eastern Hemisphere, showed that spiralin, an abundant surface protein, is necessary for transmission of the spiroplasmas by the insects, but is not involved in plant pathogenicity of the microbes. Moreover, another protein, P32, encoded by a large plasmid in *S. citri* GII-3 and expressed only in the transmissible strains, likely plays a role in the spiroplasma attachment to the leafhopper salivary gland cells [42]. However, since functional complementation of the gene encoding P32 in the non-transmissible strains did not restore insect transmissibility, P32 probably isn't the only protein involved in the transmission of the spiroplasmas.

Mollicute Extrachromosomal DNAs

There have been numerous reports of extrachromosomal DNAs in mollicutes [43-48]. Among the phytopathogenic spiroplasmas, only *S. citri* and *S. kunkelii* have been shown to harbor plasmids. Davis *et al*. [49] recently isolated and sequenced a plasmid, pSKU146, from *S. kunkelii* CR2-3X that encodes the *S. kunkelii* homolog of SARP1, Sk-ARP1. Moreover, from *S. citri* GII-3, five *arp*-containing plasmids (pSci1-5), ranging from 13–28 kb in size, have been sequenced and their sequences deposited in GenBank

(Foissac *et al*., *unpublished data*). A total of eight *arp*-related genes are scattered over these plasmids, with pSci5 containing three *arp* genes and pSci6 having none. Different *S. citri* strains contain extrachromosomal DNAs with different restriction patterns [43-45]. There also are replicative forms (RFs) of different viruses, such as SpV1 through SpV4 and SVTS2, and other uncharacterized extrachromosomal DNAs in *S. citri* [47]. Integrated virus-like sequences cause extensive genome rearrangements in spiroplasmas [47]. Among the phytopathogenic phytoplasmas, the beet leafhopper-transmitted virescence agent (BLTVA) and the onion yellows phytoplasma have been shown to contain plasmids [48 and 50]. Among mycoplasmas, *Mycoplasma mycoides* subsp. *mycoides* harbors a 1.7 kb plasmid, pADB201, encoding a staphylococcal repF homolog [51].

Objectives of the Present Study

Davis and colleagues [49] reported the complete sequence of the *S. kunkelii* CR2-3X plasmid, pSKU146, which encodes a homolog of SARP1, SkARP1. Hence, in the first part of the work performed it was of interest to isolate and characterize extrachromosomal DNA from *S. citri* BR3-3X. the publication describing the isolation, distribution and characterization of pBJS-O, a novel, SARP1-encoding plasmid from *S. citri* BR3-3X [52], also reported the presence of *arp2*, a homolog of *arp1*, on the BR3-3X chromosome, but distribution of the gene in various mollicutes was not presented. Therefore, analysis of distribution of *arp2* was undertaken as a second study. Joshi and co-workers [52] also showed that the extrachromosomal DNAs of *S. citri* R8A2 and ASP-1 lack *arp1*, but share sequences with that of pBJS-O in other regions. Therefore, it

was of interest to determine the extent of sequences absent from their plasmids with respect to pBJS-O.

Thus, in Chapter II isolation, distribution and sequence characterization of a SARP1-encoding plasmid, pBJS-O, from *S. citri* BR3-3X are shown. Those findings were published in *BMC Genomics* in December 2005. Chapter III presents the distribution of *arp2* in various spiroplasmas and select mycoplasmas. Chapter IV highlights isolation and sequence characterization of plasmids from *S. citri* ASP-1 and R8A2. Finally, the overall understanding of *Spiroplasma arp* sequences, their relationships with extrachromosomal elements and with spiroplasma transmission by leafhoppers and some of the proposed directions of future work are presented in Chapter V.

CHAPTER II

SPIROPLASMA CITRI BR3-3X PLASMID pBJS-O: ISOLATION, DISTRIBUTION, SEQUENCING AND EVOLUTION

Abstract

Spiroplasma citri BR3-3X and *S. kunkelii* CR2-3X cause serious diseases worldwide on citrus and maize species, respectively. *S. citri* BR3-3X harbors a plasmid, pBJS-Original (pBJS-O), that encodes the spiroplasma adhesion related protein 1 (SARP1), a protein implicated in binding of the pathogen to cells of its leafhopper vector, *Circulifer tenellus*. The *S. kunkelii* CR2-3X plasmid, pSKU146, encodes a homolog of SARP1, SkARP1. Due to the close phylogenetic relationship of the two pathogens, we hypothesized that the two plasmids are closely related as well. The nucleotide sequence of pBJS-O was determined and compared to the sequences of a plasmid from BR3-T (pBJS-T), which is a multiply passaged leafhopper transmissible derivative of BR3-3X, and to known plasmid sequences including that of pSKU146. In addition to *arp1*, the 13,374 bp pBJS-O sequence putatively contains nine genes, recognized as open reading frames (ORFs). Several pBJS-O ORFs have homologs on pSKU146. However, the sequences flanking *soj*-like genes on both plasmids were found to be more distant from one another than sequences in any other region. Further, unlike pSKU146, pBJS-O lacks the conserved *oriT* region characteristic of the IncP group of bacterial plasmids. We were unable to identify a region in pBJS-O resembling a known plasmid origin of

transfer. In regions where sequence was available for pBJS from both BR3-3X and BR3-T, the pBJS-T sequence had a 0.4 kb deletion relative to its progenitor, pBJS-O. Southern blot hybridization of extrachromosomal DNA from various *S. citri* strains and spiroplasma species to an *arp*-specific probe and a probe made from the entire plasmid DNA of BR3-3X revealed limited conservation of both sequences in the genus *Spiroplasma*. Finally, we also report the presence on the BR3-3X chromosome of *arp2*, an *S. citri* homolog of *arp1* that encodes the predicted protein SARP2. The C-terminal domain of SARP2 is homologous to that of SARP1, but its N-terminal domain is distinct. Our data suggest that pBJS is a novel *S. citri* plasmid that does not belong to any known plasmid incompatibility group. The differences between pBJS-O and pSKU146 suggest that one or more events of recombination have contributed to the divergence of the plasmids of the two sister *Spiroplasma* species; pBJS itself has diverged slightly during the derivation of *S. citri* BR3-T from BR3-3X. Our data also show that pBJS encodes the putative adhesin SARP1. The presence of *traE* and *mob* on pBJS suggests a role for the plasmid in spiroplasmal conjugation.

Background

The phytopathogenic spiroplasmas and phytoplasmas, which cause serious diseases of economically important plant species worldwide [17 and 2], are wall-less prokaryotes phylogenetically related to Gram-positive eubacteria with low G+C content [1]. They are transmitted in nature by phloem-feeding insects, predominantly leafhoppers, in a propagative manner [21]. Even though there are close to forty recognized spiroplasma species, only three plant pathogenic spiroplasmas have been identified and characterized to date. *S. citri* [10, 11 and 12] is the causative agent of

stubborn disease of citrus and brittle root disease of horseradish; *S. kunkelii* [13, 14 and 15] is the etiological agent of corn stunt; and *S. phoeniceum* [16] causes periwinkle yellows. Unlike phytoplasmas, spiroplasmas can be cultured *in vitro*. Therefore, the relationships between *S. citri* and its insect vectors, the beet leafhopper, *Circulifer tenellus*, and the related species, *C. haematoceps* [22], have been investigated extensively, serving as models for investigating the molecular aspects of mollicute–vector interactions.

Spiroplasma binding to insect host and non-host cells, both in tissue-culture and within the intact insect, has been reported [24]. The loss and restoration of the ability of *S. citri* to adhere to tissue-cultured *C. tenellus* cells was associated with degradation and restoration of P89 (designated SARP1), a spiroplasma membrane protein [32]. Due to the possible direct involvement of SARP1 in the spiroplasma-leafhopper interaction, it was hypothesized that SARP1 is an adhesin. Later, Berg *et al* [41] reported cloning and characterization of *arp1*, the gene encoding SARP1, from *S. citri* BR3-T. They also reported that mature SARP1 protein contains a novel domain at the N-terminus, called "sarpin", made of six repeats of 39-42 amino acids each.

S. citri harbors several extrachromosomal DNAs with unique restriction patterns [44, 45, 46 and 43]. *S. citri* lines, derived from a clone, and sister clones of the same lines showed differences in their extrachromosomal DNAs [39]. In addition to known plasmids, there are replicative forms (RFs) of several viruses and other uncharacterized circular extrachromosomal DNAs in *S. citri* [47].

Plasmids have also been noted in strains of *S. kunkelii* [27]. Recently, Davis and colleagues [49] reported the complete sequence of the *S. kunkelii* CR2-3X plasmid

pSKU146, which encodes a homolog of SARP1, SkARP1. In the present study, we isolated and characterized a related indigenous plasmid, designated pBJS-Original (pBJS-O), from *S. citri* BR3-3X. This is a report of the discovery, distribution and characterization of that plasmid. Among other genes, pBJS-O contains *arp1*. The significance of the discovery of pBJS-O in relation to our current understanding of the *S. citri*-leafhopper interactions and potential genetic manipulations in mollicutes is discussed. Implications for the evolution of both pBJS-O and pSKU146 are also presented.

Results

Detection and Analysis of *arp2*

SARP1 has been characterized previously and the gene encoding it, *arp1*, has been cloned and sequenced [GenBank:AJ297706] from *S. citri* BR3-T [41]. In the process, an *Rsa*I restriction fragment was cloned and sequenced from BR3-T genomic DNA; the alignment of this fragment with AJ297706 revealed 92% similarity in the 3' 660 nucleotides of the former sequence. However in the 5' 55 bases of the total 715 bp, upstream from position 2370 in AJ297706, the new fragment was not similar to the known sequence. We designated this gene, which resembles but is not identical to *arp1*, as *arp2* and its putative protein product as SARP2. As also noted by Bai *et al*. [27], the *S. kunkelii* CR2-3X genome (URL: http://www.genome.ou.edu/spiro.html) contains two sequences similar to those of *S. citri* BR3-T *arp* genes. The predicted protein, Sk-ARP1 (for *S. kunkelii* adhesion related protein 1), encoded by the first sequence, *Sk-arp1*, contains seven rather than six sarpin repeats and has C-terminal domains resembling

those of SARP1 [41]. The second sequence encodes a putative protein whose C-terminus is homologous to that of SARP1, but has an unrelated N-terminus. This protein is designated Sk-ARP2 (*S. kunkelii* adhesion related protein 2) and the corresponding gene is named *Sk-arp2*. SARP1 has sequence similarity with known adhesins. Fleury *et al*. [53] have shown that the predicted amino acid sequence of P40, a *Mycoplasma agalactiae* cytadhesin, is similar not only to that of SARP1 but also to the one of P50, an adhesin of *M. hominis*.

Isolation and Distribution of *Spiroplasma* Extrachromosomal DNA

We isolated extrachromosomal DNA from *S. citri* BR3-3X to test the hypothesis that this DNA contains an *arp*-like gene as in *S. kunkelii*. Restriction of the DNA with single enzymes, including *Bgl*II and *Nde*I, converted a DNA migrating with 9 kb into a fragment migrating close to 7 kb (Figure 1). These results were consistent with the presence of a single major plasmid. We designated the plasmid pBJS-O. By nucleotide sequencing, we determined that the actual size of the plasmid was 13,374 bp and deposited the sequence in the EMBL Nucleotide Sequence Database [EMBL:AJ972409]

To test the conservation of pBJS-O in *S. citri* strains derived from *S. citri* BR3, plasmid preparations from *S. citri* BR3-3X and from BR3-G, BR3-T, BR3-M and BR3-P, lines derived from BR3-3X, were probed with a DNA fragment derived from *arp1* (Figure 2 and Table I). All hybridized with the probe, producing two or more bands. To

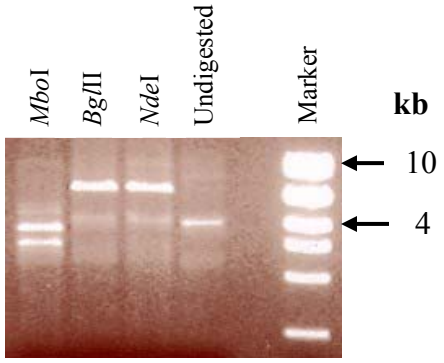

Figure 1. Restriction Digests of pBJS-O DNA with *Mbo*I, *Bgl*II and *Nde*I. The marker used was the High Mass Ladder (Invitrogen Corp., Carlsbad, CA, USA). Sizes of the fragments are denoted in kb.

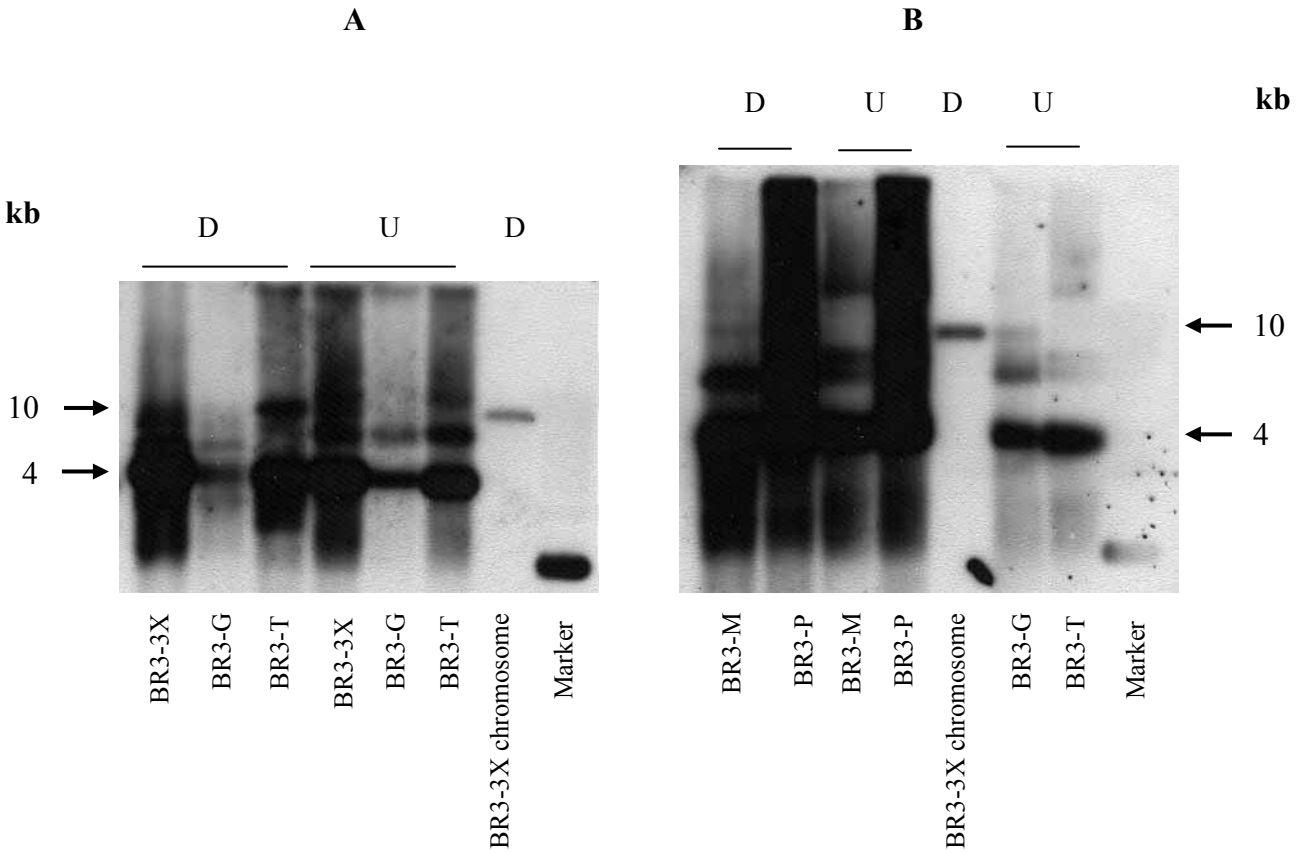

Figure 2. Southern Blotting Hybridization of (A) *S. citri* BR3-3X, BR3-G, BR3–T, and (B) *S. citri* BR3-M and BR3-P Plasmid Preparations to an *arp1*-derived Probe. *Eco*RI-digested *S. citri* BR3-3X chromosomal DNA and *Eco*RI-digested and undigested plasmid preparations from BR3-3X, BR3-G, BR3-T, BR3-M and BR3-P are shown. D, digested with *Eco*RI; U, undigested. Hybridization in the marker lane is due to presence of short pBluescript vector sequences in the probe.

TABLE I

RESULTS OF THE SOUTHERN HYBRIDIZATIONS OF UNDIGESTED PLASMID PREPARATIONS FROM VARIOUS SPIROPLASMA SPECIES AND *S. CITRI* STRAINS TO EITHER AN *ARP1*-DERIVED PROBE OR WHOLE pBJS-O PROBE

Spiroplasma		**Probe**		**Biological features**	
Species*	Strain	*arp1*	pBJS-O	Transmissibility	Pathogenicity
S. citri	BR3-3X	+	+	+	+
	BR3-G	+	+	-	-
	BR3-T	+	+	+	+
	BR3-M	+	+	+	+
	BR3-P	+	+	Very low	-
	ASP-1	-	+	Unknown	Unknown
	R8A2	-	+	-	Unknown
	Beni Mellal	-	-	-	Unknown
S. kunkelii	CR2-3X	+	+	+	+
S. phoeniceum	P40	-	-	+**	+
S. melliferum	TS2	+	+	Unknown	-
S. floricola	23-6	-	-	Unknown	-

**S. citri*, *S. kunkelii*, *S. phoeniceum* and *S. melliferum* belong to serogroup I, whereas *S. floricola* belongs to serogroup III. The biological features of the spiroplasmas, except for *S. phoeniceum*, are taken from references 20 and 25.

**Only experimental transmission to the plant host is known for this spiroplasma. It is unclear whether it can be naturally transmitted by leafhoppers.

+ and – denote positive and negative hybridizations, respectively.

test the conservation of pBJS-O in other *S. citri* strains, other plant-associated spiroplasmas and the closest relative of *S. citri*, *S. melliferum* [20], the plasmids of *S. kunkelii* CR2-3X, *S. melliferum*, *S. citri* strains R8A2, ASP-1 and Beni Mellal, *S. floricola* and *S. phoeniceum* also were probed with the *arp1*-derived probe (Figure 3A). Only *S. kunkelii* CR2-3X and *S. melliferum* reacted in the hybridization. However, when the same plasmids were probed with the whole pBJS-O plasmid as a probe (Figure 3B), all the sample preparations, except those from *S. citri* Beni Mellal, *S. floricola* and *S. phoeniceum*, hybridized with the probe. All of the above Southern hybridization experiments revealed multiple reactive species in the plasmid preparations and the hybridization patterns of *Eco*RI-digested and undigested plasmid samples were very similar to each other. For comparison, the blots included *Eco*RI-digested chromosomal DNA of *S. citri* BR3-3X. A single hybridization signal distinct from those of plasmid preparations was observed (Figure 2).

arp1 and *arp2* Locations in *S. citri* BR3-3X

The Southern blot hybridization results suggest that *arp*-related sequences are present on both a plasmid and the chromosome. *arp1* and *arp2* from BR3-T are nearly identical over a considerable portion of their nucleotide sequence. Hence, using a probe containing this conserved region should detect both genes. Nevertheless, *arp1* and *arp2* differ at several positions in those regions. To determine whether the BR3-3X plasmid and chromosomal sequences represented *arp1* or *arp2* genes, we determined parts of the sequences of BR3-3X plasmid and chromosomal DNAs by direct sequencing and by sequencing amplified PCR products. Comparison of the BR3-3X *arp* sequences with

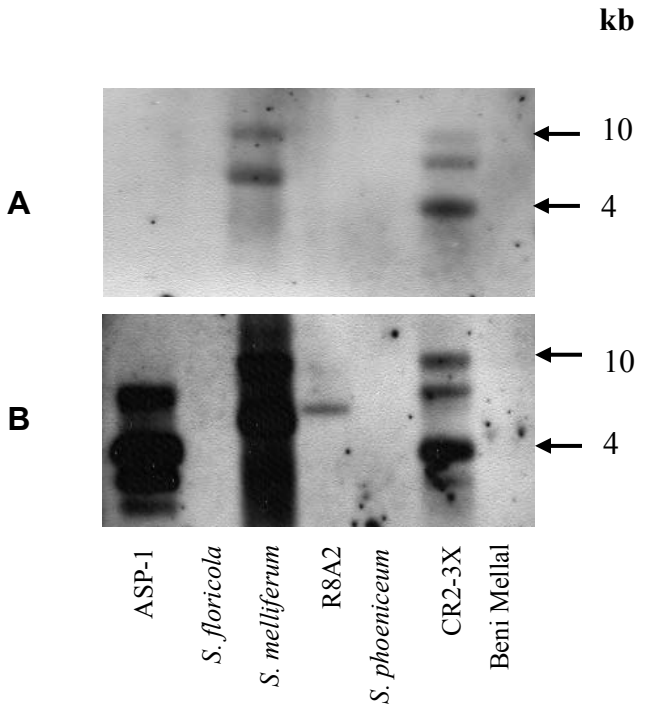

Figure 3. Southern blotting hybridization of undigested plasmid preparations from different *S. citri* strains and spiroplasma species to (A) an *arp1*-derived probe and (B) the whole pBJS-O probe. Plasmids from *S. citri* ASP-1, R8A2 and Beni Mellal, and from *S. floricola*, *S. melliferum*, *S. phoeniceum* and *S. kunkelii* CR2-3X were used. The blot shown in panel A was stripped and rehybridized using the whole pBJS-O probe, shown in panel B.

those of BR3-T revealed that the BR3-3X *arp2* sequence had diverged more from the other three sequences than the latter had from each other (Figures 4 and 5). At positions where the two BR3-T genes differed from one another, the chromosomal BR3-3X sequence had *arp2* residues in 21 positions and *arp1* residues in only 3 positions (Figures 4A and 4B). Conversely, at *arp1*- and *arp2*-specific positions, the BR3-3X plasmid DNA had no *arp2* residues and 28 *arp1* residues. Further, at all 57 positions at which chromosomal and plasmid sequences differed, the BR3-3X plasmid and *arp1* nucleotides were identical. Hence, we conclude that, in *S. citri* BR3-3X, the *arp1* gene resides on a plasmid and that the *arp2* gene most likely resides on the chromosome. The newly determined *arp2* sequences from BR3-3X and BR3-T were deposited in the EMBL Nucleotide Sequence Database [EMBL:AM040506 and EMBL:AM040505, respectively].

Complete pBJS-O Sequencing and Analysis

The 4273 bp sequence [GenBank:AJ297706] originally cloned and characterized from *S. citri* BR3-T [41] contains a partial ORF *soj*, followed by ORF2, P89 (*arp1*) and another partial ORF, ORF4. AJ297706 was used to design primers and initiate primer walking to determine the complete pBJS-O plasmid sequence and allow its characterization. During sequencing, a segment (from nucleotide 1-80) of the assembled sequence proved particularly difficult to sequence. It contained three of the six oligopurine/oligopyrimidine tracts of 12 or more bp in the entire plasmid sequence. That the sequence of the tracts was consistent with triple-helix formation suggests that this region of the plasmid may readily form triple-helical structures interfering with sequencing.

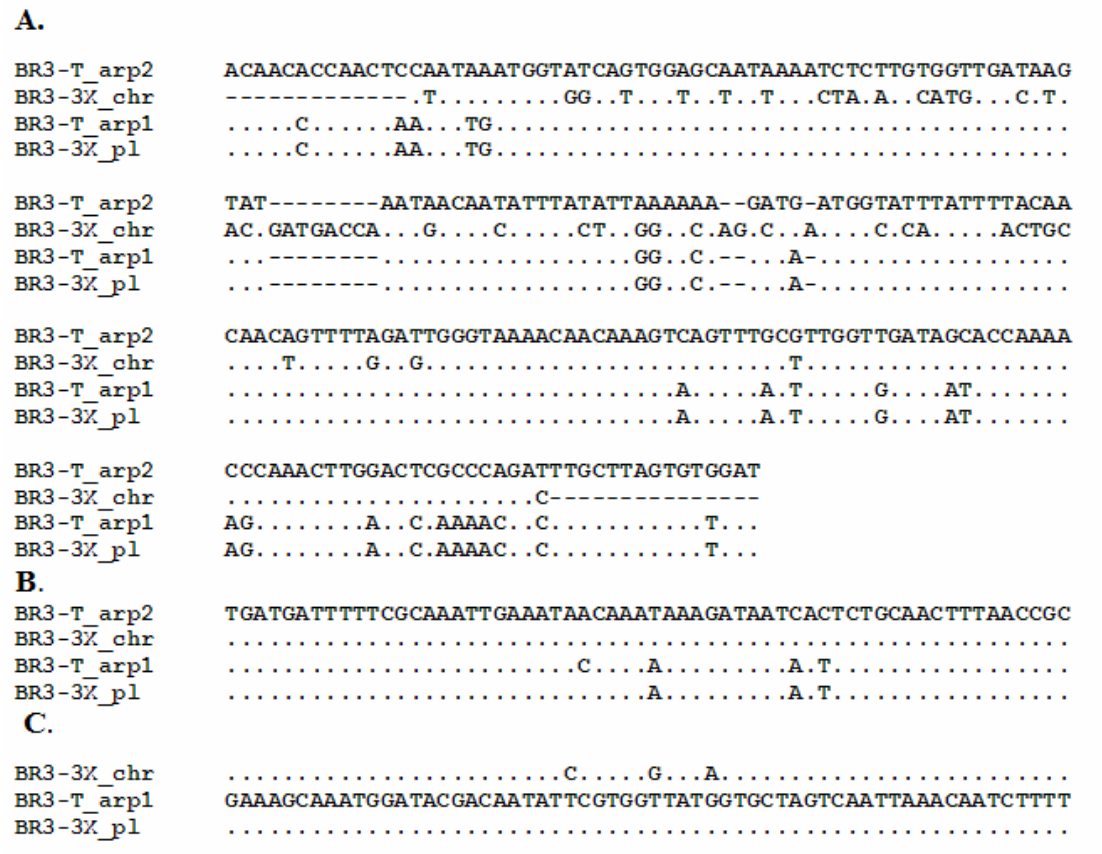

Figure 4. Comparison of Partial Nucleotide Sequences of *S. citri* BR3-3X Chromosomal (BR3-3X_chr) and Plasmid (BR3-3X_pl) Sequences to Those of Available BR3-T *arp1* and *arp2* Genes (BR3-T_*arp1* and BR3-T_*arp2*, Respectively). Only the regions containing polymorphic positions are shown. (A) region of *arp1* positions 3572 to 3800 (AJ297706). (B) region of *arp1* positions 4118 to 4177 and (C) region of *arp1* positions 4658 to 4717). *S. citri* BR3-T *arp2* is not available for the last sequence alignment. Gaps are denoted by dashed lines, whereas dots denote identical bases.

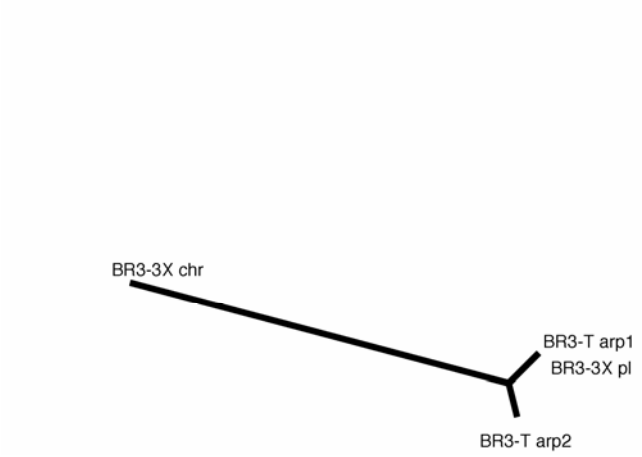

Figure 5. An Unrooted Phylogenetic Tree Representing the Nucleotide Sequence Alignment Shown in Figure 4A. The tree was generated using algorithms ClustalW and PHYLIP from the Biology Workbench using the neighbour-joining method.

The total plasmid sequence is 13,374 bp in length and contains ten predicted ORFs (Figure 6 and Table II), of which *orf2* (*S. citri* ORF2) has no homologs, and *orf9* and *orf10* appear to have distant relatives (E values 0.34 and 0.024, respectively). Of the ten, six putative pBJS-O-ORFs have homologs in pSKU146, the recently characterized *S. kunkelii* CR2-3X plasmid [49]: *arp1* (adhesin protein; E value 0.0), *orf4* (hypothetical protein pSKU146_11; E value 0.0), *traE* (conjugation ATPase; E value 0.0), *orf6* (hypothetical protein pSKU146_13; E value 9×10^{-45}), *mob* (mobilization protein; E value 0.0) and *orf8* (hypothetical protein pSKU146_17; E value 1×10^{-103}). Predicted products of *traE* and *mob* are similar to proteins involved in conjugative DNA transfer in other bacterial genera. In the regions where the plasmid sequence was available from both BR3-3X and BR3-T, pBJS-T (the plasmid from *S. citri* BR3-T) sequence had a 0.4 kb deletion relative to pBJS-O, bringing the *orf4* gene close to *arp1* and *traE*. In BR3-3X, however, *arp1* and *orf4* are separated by 281 bp. The nucleotide sequence variations between pBJS-O and pBJS-T were found to be clustered. Two regions of enhanced variation were observed over a 200 bp stretch in the ORF2-*arp1* intergenic region (positions 2700 to 2900 in pBJS-O). In a comparable stretch from position 5262 to 5544 in the *arp1*-ORF4 intergenic region, a single stretch of dissimilarity was found.

Algorithm TMHMM v. 2.0 was used to predict the locations of transmembrane helices and intervening loops in the putative products of *traE* (**Figure 7**), *mob* and *orf4*. Although the TraE polypeptide was predicted to contain three transmembrane helices, the third helix was predicted at a lower probability than were the other two. Assuming the presence of three transmembrane helices, the protein was predicted to have the N-terminal region (about 10% of the length of the polypeptide) in the cytosol and almost all

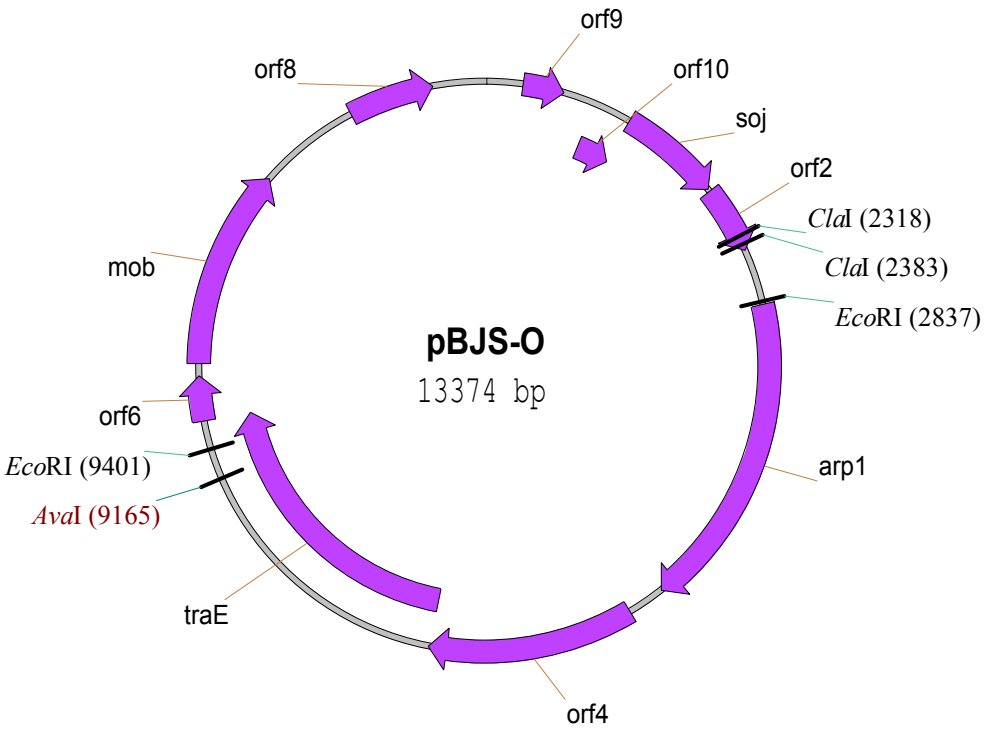

Figure 6. The ORF and Restriction Map of pBJS-O.

TABLE II

DESCRIPTIONS OF ORFS PRESENT ON pBJS-O

ORF #	Map Position	Length (bp)	Closest homolog (from BLASTP search)	E value
1	1114-1896	783	Soj-like protein [*S. citri*]*	1×10^{-116}
2	1916-2434	519	hypothetical protein [*S. citri*]*	1×10^{-100}
3	2859-5255	2397	putative adhesin P89 [*S. citri*]*	0
4	5536-7101	1566	hypothetical protein [*S. kunkelii*]	0
5	7091-9613	2523	conjugation ATPase [*S. kunkelii*]	0
6	9617-9955	339	hypothetical protein [*S. kunkelii*]	9×10^{-45}
7	10047-11558	1512	mobilization protein [*S. kunkelii*]	0
8	12338-12988	651	hypothetical protein [*S. kunkelii*]	1×10^{-103}
9	270-581	312	Unknown	0.34
10	831-1127	297	Unknown	0.024

* AJ297706, the sequence originally characterized from *S. citri* BR3-T, is the source of these hits.

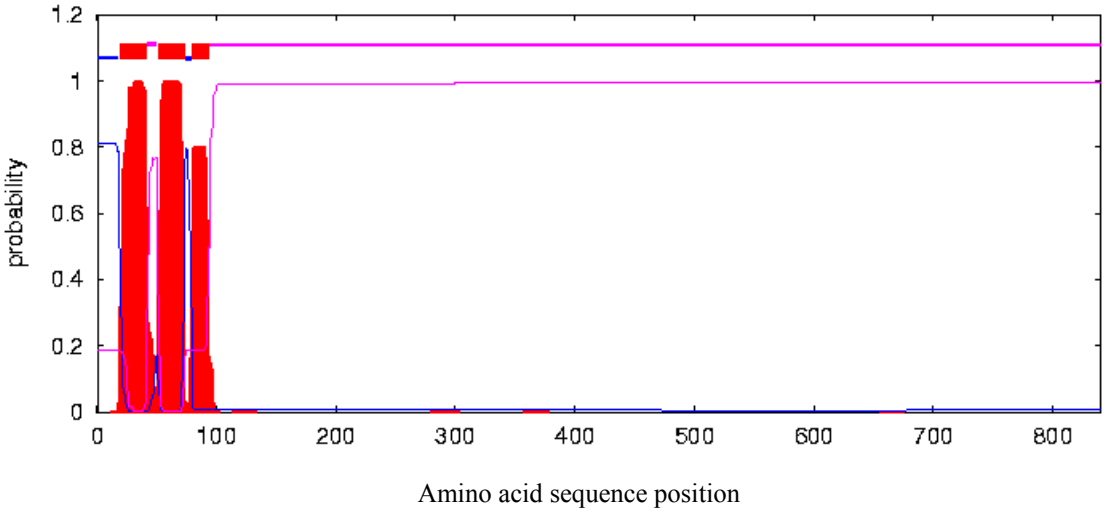

Figure 7. Predicted Locations of Transmembrane Helices and Intervening Loops in the Putative Protein Encoded by ORF5 (*traE*) of pBJS-O. The sequential amino acid positions in the primary sequence of the polypeptide are on the X-axis, while the probability score of each residue for being in a transmembrane helix is on the Y-axis in red. The blue and pink curves denote the probability of each amino acid in the sequence to be cytosolic or extracellular, respectively. In the schematic representation of the protein domains at the top, blue lines show the cytosolic portions, purple ones denote the extracellular portions and the thick horizontal bars denote the predicted transmembrane portions of the polypeptide, respectively.

of the rest of the protein extracellular.

Plasmid pSKU146 from *S. kunkelii* CR2-3X encodes the *S. kunkelii* homolog of SARP1, SkARP1. In addition to *skarp1*, pSKU146 contains 17 ORFs. The pSKU146-ORFs having counterparts on pBJS-O were listed above. However, although both plasmids contain genes encoding the ParA-like protein, Soj, sequences surrounding those genes are more distant from one another than are sequences in any other regions. Further, unlike pSKU146, pBJS-O lacks the conserved *oriT* region characteristic of the IncP group of bacterial plasmids. Also, we were unable to identify a region in pBJS-O resembling a known plasmid origin of transfer.

Discussion

In the present study we report isolation, distribution and structural characterization of pBJS-O, an indigenous *S. citri* BR3-3X plasmid. We also present evidence that pBJS-O harbors *arp1*, the gene encoding SARP1, and describe the presence on the BR3-3X chromosome of *arp2*, an *S. citri* homolog of *arp1*. Finally, the sequences of pBJS-O, pBJS-T and the *S. kunkelii* CR2-3X plasmid, pSKU146, in relation to plasmid evolution are discussed.

Conservation of *arp* and pBJS-O Sequences in *Spiroplasma*

In Southern hybridizations, the similarity in the hybridization patterns of *Eco*RI digested versus undigested pBJS-O preparations, despite the presence of two GAATTC recognition sequences, may be due to an adenine methylation system in *S. citri*. Restriction site modification in *S. citri* has been reported elsewhere. Rascoe *et al*. [54]

detected multiple bands of *S. citri* extrachromosomal DNA by Southern blotting, which they attributed to incomplete restriction due to variable restriction site modification in the DNA, and Ye *et al*. [55] reported protection of an *Eco*RI site in the *S. citri* 16S rDNA. Moreover, differential methylation of restriction sites in the RF of the spiroplasma virus, SVTS2, allowed Sha *et al*. [56] to clone the full-length DNA.

S. citri BR3-3X showed probe-reactive sequences in both the chromosomal and extrachromosomal DNA fractions. However, that the patterns of hybridization of the two fractions differed significantly from each other demonstrates that the two fractions of BR3-3X DNA were not appreciably cross-contaminated. Sequence analyses of DNA from the two fractions showed that, in BR3-3X, *arp1* resides on pBJS-O and *arp2* on the chromosome. Hybridization of *S. citri* ASP-1 and R8A2 plasmid preparations with the pBJS-O probe (Figure 3B), but not with the *arp1* probe (Figure 3A), indicates that each of these two strains contained a plasmid related to pBJS-O, which differed from pBJS-O in lacking *arp1*. Although *S. citri* ASP-1 and R8A2 were originally derived from the same parent strain, both have undergone extensive cultivation *in vitro* since their first isolation, which may have contributed to the differences between their plasmids and pBJS-O. Moreover, the differences in the maintenance regimes of the various spiroplasmas tested may have contributed to the evolution of their plasmids. In this paper we could not correlate pBJS-O and pBJS-O like sequences with either transmissibility or phytopathogenicity of the spiroplasmas tested. However, it is still hypothesized that SARP1 is involved in *S. citri* transmission by the insect vector.

Frequent chromosomal rearrangements such as inversions and deletions, leading to genome instability, have been reported in spiroplasmas, such as in the lines derived

from *S. citri* strain BR3 [57 and 37]. In the present study, we detected a 0.4 kbp deletion in pBJS-T relative to pBJS-O. Unlike BR3-3X, which was stored frozen, *S. citri* BR3-T was maintained for several years in turnip plants via transmission by the natural insect vector *C. tenellus*, possibly leading to the sequence differences between pBJS-T and pBJS-O. A recombinational chromosomal rearrangement is indicated by the 5'-sequence differences between *arp1* and *arp2* reported above.

Recombination likely also played a role in the generation of pBJS-O like plasmids. The gene organization on pBJS-O is similar to that of the recently characterized IncP-like *S. kunkelii* CR2-3X plasmid, pSKU146. Yet, the two plasmids have substantially different sequences in the region including the *soj*-like gene in both plasmids and the IncP *oriT* sequence in pSKU146. Highly similar sequences in the remainder of the two plasmids suggest that recombination events have occurred during the generation of one or both plasmids.

Phage sequences have been implicated in many recombination events in spiroplasmas. Only a short region with similarity to a phage gene was found in pBJS-O. However, the observation of strong stops to sequencing reactions in the region of nucleotides 1 to 80 is reminiscent of a strong stop encountered during the sequencing of the SVTS2 phage [60]. This strong stop was attributed to potential secondary structure putatively involved in phage packaging. It is, thus, possible that pBJS-O has some phage-like properties.

pBJS-O Genes

As mentioned above, ORF3, encoding SARP1, and adjacent ORFs [GenBank:AJ297706], had been cloned and characterized from *S. citri* BR3-T [41]. ORF3 was flanked downstream by a partial ORF (ORF4) having no known homologs. Upstream, ORF3 was flanked by ORF2, encoding a hypothetical protein with no similarity to any known protein, and ORF1, a partial ORF encoding a putative homolog of a ParA-like protein, Soj, which oscillates from pole to pole [58] and is important for chromosome partitioning in *Bacillus subtilis* [59]. In this study, the putative protein product of *orf4* was predicted to contain eight transmembrane helices. Due to a 0.4 kb deletion in the derivation of pBJS-T, *orf4* is possibly a part of the same transcription unit as *arp1* and *traE* in this strain. In BR3-3X, *arp1* and *orf4* are separated by 281 bp, suggesting that they are transcribed separately. Consistent with different translational constraints on this region in BR3-T and BR3-3X, this region contains a large proportion of the differences between the lines. The translation start site of *traE* was predicted to be ten nucleotides upstream of the *orf4* translation stop site.

Consistent with the observation of Bai *et al*. [27], putative products of the other pBJS-O ORFs, *traE* [61] and *mob* [62], are homologous to proteins that are components of the bacterial type IV secretion system involved in conjugative DNA transfer. Members of the TraE family of proteins are thought to form pili that, in addition to conjugation, are involved in processes like virus infection and biofilm formation. Bai *et al*. [27] reported

the presence of three conserved transmembrane helices in four TraE homologs that they characterized from *S. kunkelii* M2. Ozbek *et al*. [26], in their transmission electron micrographs, reported the presence of structures resembling fimbriae and pili in *S. kunkelii* and Bai *et al*. [27] considered whether the structures may be involved in conjugation. Bové [28] reported that rod-shaped spiroplasma viruses, approximately 230-280 by 10-15 μm in size, can also be surface-associated. Because they can attach perpendicularly to the host membrane at their tips [30 and 63], they might resemble the structures reported as pili/fimbriae. In the putative TraE homolog reported here, unlike its *S. kunkelii* counterpart, two transmembrane helices were predicted at high probability and a third one at moderate probability. Should the third not actually be a transmembrane helix, the ATP binding site would be located intracellularly rather than extracellularly.

pBJS-O Gene Organization and Evolution

Unlike pSKU146, pBJS-O was found to lack the conserved *oriT* region characteristic of the IncP group of plasmids. We were also unable to identify a region in pBJS-O resembling any other known plasmid origins of transfer, suggesting that pBJS-O belongs to a hitherto unidentified group of plasmids. Horizontal transfer of a promiscuous plasmid, such as an IncP plasmid, between phylogenetically related and unrelated bacteria would help the hosts quickly adapt to different niches [64]. It is possible that an IncP-like plasmid was acquired by the ancestor of *S. citri* and *S. kunkelii*. The plasmid may have co-evolved with the host chromosomes after the divergence of the two species, leading to the emergence of pBJS-O and pSKU146, respectively, and to the adaptation of the pathogens to phylogenetically distinct leafhopper vectors and plant hosts.

Future Directions

Molecular genetic tools such as cloning and transposon-mediated mutagenesis are available for the study of mollicutes [65]. Cloned genes were expressed in *S. citri* GII-3 using artificial plasmids based on the *S. citri* chromosomal *oriC* [66-68]. However, those plasmids tend to integrate into the *S. citri* chromosome. When pCJ32, a derivative of the *oriC* plasmid pBOT1, containing an internal fragment of the gene *scm1* (a motility-related *S. citri* gene), was transformed into *S. citri* GII-3 cells it successfully integrated into the host chromosome by homologous recombination and disrupted *scm1*, resulting in non-motile *S. citri* GII-3 mutants [69]. However, attempts to use pBOT1 in *S. citri* BR3-3X have been unsuccessful (F. Ye, *unpublished data*), possibly due to the incompatibility of the plasmid with the host. The indigenous *S. citri* BR-3X plasmid, pBJS-O, will help us develop a better vector for genetic manipulation not only in *S. citri* BR3-3X but also in other spiroplasmas.

Conclusions

We have shown that the *S. citri* BR3-3X plasmid, pBJS-O, encodes the putative adhesin SARP1. This is the first report of an *S. citri* plasmid encoding a putative adhesin. We have further shown that the *arp1*-like gene, *arp2*, resides on the BR3-3X chromosome. The indigenous *S. citri* BR3-3X plasmid, pBJS-O, will be useful for the development of a better vector for genetic manipulation not only in *S. citri* BR3-3X but also in other spiroplasmas. Our data also suggest that pBJS-O is a novel *S. citri* plasmid that does not belong to any known plasmid incompatibility group. The differences

between pBJS-O and pSKU146 suggest that recombination has contributed to the divergence of the two plasmids.

Methods

Spiroplasmas

S. citri BR3 was isolated from horseradish plants with brittle root disease [12]. *S. citri* BR3-T, derived from the triply cloned parental isolate (BR3-3X) by repeated transmission in turnips via its insect vector C. tenellus, is insect-transmissible. BR3-M, derived by passage in liquid medium 43 times, is also a transmissible line. The lines BR3-G (maintained in periwinkle plants by graft transmission) and BR3-P (passed in liquid medium more than 130 times) are insect non-transmissible [25]. *S. citri* R8A2, isolated from citrus in Morocco [70], and its non-helical derivative ASP-1 (both obtained from R.E. Davis, USDA/ARS, Beltsville, MD), are non-transmissible. Also provided by R.E. Davis were *S. citri* Beni Mellal, originally isolated from *C. haematoceps* collected in Morocco; *S. melliferum* TS2, isolated from honeybees and *S. floricola* 23-6, isolated from a flower surface [71]. *S. phoeniceum* P40, a gift from G. Gasparich (Towson University, Towson, MD), was originally isolated from periwinkle in Syria [16]. *S. kunkelii* CR2-3X was isolated by one of us (J. Fletcher) from stunt-diseased corn collected in Costa Rica [72]. All spiroplasmas, except *S. kunkelii* CR2-3X, were grown in LD8 broth medium [73] at 31^0C. The latter was grown in LD8A3 broth medium [74] at 28^0C.

Purification of Chromosomal and Extrachromosomal

ds DNAs from Spiroplasmas

For Southern blot hybridization and PCR, extrachromosomal double-stranded (ds) DNA of spiroplasma strains was isolated using the QIAprep Spin Miniprep and the QIAGEN Plasmid Mini Kits (Qiagen, Santa Clarita, CA), following the manufacturer's protocols. For primer walking, *S. citri* BR3-3X extrachromosomal DNA was isolated using a previously published procedure [56]. The isolation of chromosomal DNA from *S. citri* BR3-3X cells was performed according to Murray and Thompson [75] and using 1.4 M NaCl, 2.5% cetyltrimethylammonium bromide [CTAB], 100 mM Tris-HCl, pH 8.0, and 20 mM EDTA in the extraction buffer.

PCR and Sequencing Using *S. citri* BR3-3X Plasmid

and Chromosomal DNAs

To amplify the 3'- and flanking regions of *arp* genes from *S. citri* BR3-3X plasmid and chromosomal DNAs, two oligonucleotides were designed, forward (#7686) 5'-AACACTATTTTCACTGCGG-3', from the *S. citri* BR3-T *arp1* sequence (GenBank accession number AJ297706), and reverse (#7960) 5'-TTTTCCATTGTTTTTGTCTCC-3', from the sequence homologous to ORF4 from the plasmid pSKU146 (pSKU146_11; accession number NC_006400). The PCR was carried out in a DNA thermal cycler (MJ Research, Waltham, MA) performing 35 cycles, each of 30 sec at 94°C, 1 min at 42°C and 3 min at 72°C. Reactions were performed separately in a volume of 50 µl containing 2.5 Units Taq polymerase (Promega), 0.20 µM primers, 200 µM of each dNTP, 1.5 mM $MgCl_2$, and 100-150 ng BR3-3X plasmid and ~3.5 µg chromosomal DNA. The

amplicons were sequenced using ~100 ng of each of the PCR products, 10 μM of the same primers used in the PCR in separate reactions by the ABI PRISM BigDye Terminator Cycle Sequencing method (version 1.0, Applied Biosystems, Foster City, CA) with an ABI PRISM 3700 Automated DNA Analyzer (Perkin Elmer Biosystems, Foster City, CA).

Southern Blotting

Extrachromosomal DNA of each spiroplasma strain was digested with *Eco*RI (Life Technologies, Inc.) for 4 h at 37ºC. The fragments were separated by electrophoresis on a 0.75% (w/v) agarose gel in 1X TAE running buffer and transferred to Hybond-N^+ nylon membranes (Amersham Biosciences, Uppsala, Sweden) according to standard procedures. The blots were subsequently hybridized to Dig-11-UTP-labeled *arp1*-derived and whole-plasmid probes, labeled using a DIG DNA Labeling Kit (Roche Molecular Biochemicals, Indianapolis, IN), following the manufacturer's instructions. The *arp1*-derived probe was obtained by PCR, using clone pP89B (an *Rsa*I fragment of *S. citri* BR3-T genomic DNA; [41]) as template, and primer pair T7 and #7483 (5'-TTTAACATCAACCGAACCC–3'). The probe comprised 657 bp of a DNA segment from *S. citri* BR3-T (AJ297706; positions 2315-2989) and 72 bp derived from the cloning vector (pBluescript). PCR was carried out in a DNA thermal cycler performing 34 cycles, each of 30 sec at 94°C, 30 sec at 54°C and 1 min at 72°C. Reactions were performed in a volume of 50 μl containing 1 Unit Taq polymerase, 0.25 μM primers, 250 μM of each dNTP, 50 - 100 ng template DNA, and 2.5 mM $MgCl_2$. Hybridizations were performed at 55°C in Church buffer (0.5 M sodium phosphate buffer, pH 7.2, 7% SDS, and 1 mM

EDTA) overnight followed by four washes, each of 20 min, at 55°C in washing buffer (40 mM sodium phosphate buffer, pH 7.2, containing 0.1% SDS). Detection of the DIG-labeled probes was performed using a DIG Luminescent Detection Kit (Roche) following the manufacturer's protocol.

Complete Nucleotide Sequencing of pBJS-O

The sequence AJ297706 was used to design primers to initiate primer walking to completely sequence and characterize the unknown portion of pBJS-O. The sequencing reactions were performed using ~1.2 μg of pBJS-O DNA and 40 μM of primers with the ABI PRISM BigDye Terminator Cycle Sequencing method and the ABI PRISM 3700 Automated DNA Analyzer, as mentioned above. The total 134 sequence reads with an average length of 600 bases gave us about 6X coverage of the entire plasmid sequence. The fragments were assembled from the trace files using the software package PipeOnline 2.0 [76]. Physical gaps in the sequence were closed by PCR and cloning of the products into vector pGEM-T (Promega). The clones were sequenced using primers T7 and SP6. The consensus sequence of the final assembly was annotated using the BLASTX search program [77] and the ORF Finder tool at NCBI (URL: http://www.ncbi.nlm.nih.gov/gorf/gorf.html), in which a minimum length of 100 bases was used for the nucleotide sequence of a putative ORF. The nucleotide and amino acid sequence analysis tools offered by the Biology Workbench at the San Diego Supercomputer Center (URL: http://workbench.sdsc.edu), such as ClustalW and PHYLIP for generating the unrooted phylogenetic tree of the *S. citri arp* sequences, were used to further analyze the plasmid and the polypeptide sequences. BLASTN and BLASTP

searches were carried out to find out relationships with the closest homologs. *S. kunkelii* CR2-3X genome sequence data were accessed and BLAST searches were performed at the Spiroplasma Genome Sequencing Project Web site mentioned above.

Authors' Contributions

BDJ performed isolation, distribution and sequence characterization of pBJS-O. JR carried out pBJS-O sequencing and assisted BDJ in primer design and sequence assembly. MB performed *S. citri* BR3-T *arp2* gene cloning and sequencing, and also assisted BDJ in pBJS-O distribution experiments. BDJ, MB, UM and JF planned the research, BDJ and UM wrote the manuscript and MB and JF reviewed it.

Acknowledgements

We are indebted to Dr. Robert E. Davis of USDA/ARS, Beltsville, MD for personally communicating his work on the plasmid related to pBJS-O from *S. kunkelii* CR2-3X and sharing with us some of the unpublished data. Staff members of the Recombinant DNA/Protein Resource Facility at OSU are thanked for oligonucleotide synthesis, DNA sequencing, and valuable technical assistance and advice. Dr. Samir Gunjan is thanked for providing technical help in cloning pBJS-O PCR products while filling the gaps in the sequence. The people involved in the *S. kunkelii* CR2-3X genome sequencing project [B.A. Roe, S.P. Lin, H.G. Jia, H.M. Wu, D. Kupfer, and R.E. Davis] are thanked for making the sequence data publicly available.

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CHAPTER III

THE GENE ENCODING THE SPIROPLASMA *ADHESION RELATED PROTEIN 2 (SARP2): DISTRIBUTION IN THE MEMBERS OF CLASS* MOLLICUTES

Introduction

Yu *et al*. [32] found that loss and restoration of the ability of *S. citri* to adhere to a monolayer of cultured *C. tenellus* cells is clearly associated with degradation and restoration of a specific spiroplasma membrane protein, P89. Their results suggested that P89 (designated SARP1, for *s*piroplasma *a*dhesion *r*elated *p*rotein 1) is directly involved in spiroplasma-insect cell interaction. Hence, the gene encoding SARP1 (*arp1*) from *S. citri* BR3-T, was isolated and characterized [Gen-Bank:AJ297706] [41]. During characterization, a BR3-T genomic DNA fragment was sequenced, whose 3' 660 nucleotides were 92% similar to AJ297706. However, upstream from position 2370 in AJ297706 the new fragment was not similar to the known sequence. This gene, resembling *arp1*, was named *arp2*. The corresponding protein was called SARP2. The *S. kunkelii* CR2-3X genome revealed two sequences like the *S. citri* BR3-T *arp* sequences, as was also noted by Bai and colleagues [27]. Sk-ARP1 (for *S. kunkelii* adhesion related protein 1), the predicted product of the first sequence (*Sk-arp1*), has seven sarpin repeats as opposed to six in *S. citri* and its C-terminal domains are similar to those of SARP1 [41]. The putative protein product of the second sequence has its C-terminal portion like that of SARP1, with a different N-terminal region. This second protein was named Sk-ARP2 (*S. kunkelii* adhesion related protein 2) and its gene was called *Sk-arp2*.

Recently, Joshi and colleagues [52] reported the presence of *arp1* on a plasmid, pBJS-O, in *S. citri* BR3-3X. They also reported the presence of *arp2* on the BR3-3X chromosome but did not present distribution of the gene in mollicutes. It was important to determine whether the *arp1*-containing spiroplasmas possess other *arp*-related sequences on their chromosomal DNAs. Further, the possible evolutionary relationships of all the *arp* sequences with leafhopper transmission of the spiroplasmas were explored. Thus, here we report the distribution of *arp2*, which encodes the SARP1-homolog, SARP2, among spiroplasmas and select mycoplasmas.

Methods

Spiroplasmas and Mycoplasmas

The mollicute isolates used in this study are shown in Table III. *S. phoeniceum* P40 was grown in LD8 broth medium [73] at 31^0C. *S. kunkelii* CR2-3X was grown in LD8A3 broth medium [74] at 28^0C. All the other spiroplasmas and the mycoplasmas were grown by A. Chandrasekaran [78]. All the *S. citri* strains, *S. floricola* and *S. melliferum* were grown in LD8. Initially, 5 ml cultures were maintained at 32^0C and grown to the titer of 10^8 cells/ml. After checking for contamination by dark-field microscopy the entire cultures were inoculated

TABLE III

SPIROPLASMA AND MYCOPLASMA ISOLATES

Organism	Host	Comment(s)
S. citri		
BR3-3X	Horseradish, IL	Derived from *S. citri* BR3
BR3-T		Derived from BR3-3X
BR3-G		Derived from BR3-3X
Beni Mellal	*C. haematoceps*, Morocco	Obtained from R.E. Davis, MD
SPA-T		Obtained by JF and AW
MDHR3	Horseradish, MD	Obtained from R.E. Davis, MD
GO4	Orange, CA	Obtained from George Oldfield, CA
Aceratagallia	Aceratagallia leafhoppers	Obtained from R. Whitcomb
R8A2	Citrus, Morocco	Obtained from R.E. Davis, MD
M200H		Derived from R8A2
ASP-1		Derived from R8A2
S. kunkelii (CR2-3X)	Corn	Cultured by JF; is from Costa Rica
S. kunkelii (CSS-Mex)	Corn	Obtained from R.E. Davis, MD; is from Mexico
S. melliferum (TS2)	Honeybees	Obtained from R.E. Davis, MD
S. phoeniceum (P40)	Periwinkle	Obtained from G. Gasparich, MD
S. floricola (23-6)	Flower surfaces	Obtained from R.E. Davis, MD
S. apis	Honeybees	Obtained from G. Gasparich, MD
S. syrphidicola	Syrphid flies	Obtained from G. Gasparich, MD
Mycoplasma bovis	Cattle	Obtained from R. Welsh, OK
Mycoplasma felis	Cats	Obtained from R. Welsh, OK

into 100 ml broth medium and grown as mentioned above for 5 ml cultures. Pellets of *S. apis* and *S. syrphidicola* cells were provided by G. Gasparich (Towson University, MD). The mycoplasmas were grown in Friis liquid medium [79] at 37ºC under reduced oxygen or oxygen-free environments until the pH indicator in the medium turned pink to pale

orange and were used for further analyses before the medium turned yellow to avoid cell death.

DNA Extraction

DNA of spiroplasma strains *S. kunkelii* CR2-3X and *S. phoeniceum* P40, cultured in broth, was isolated using a DNeasyTM Tissue Kit (Qiagen, Santa Clarita, CA, USA) following the manufacturer's protocol. The isolation of DNA from all other spiroplasmas was performed according to Murray and Thompson [75] with slight modifications of the extraction buffer (1.4 M NaCl, 2.5% cetryltrimethylammoniumbromide (CTAB), 100 mM Tris-HCl, pH 8, and 20 mM EDTA). The mycoplasma DNAs were isolated by A. Chandrasekaran [78].

Southern Blotting

About 1.0 µg spiroplasma DNA of each strain was digested with restriction enzyme *Eco*RI (20 units) following the manufacturer's (Life Technologies Inc., Rockville, MD, USA) recommendations. The DNA fragments were separated by electrophoresis in a 0.75% agarose gel and transferred to nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Uppsala, Sweden) according to Sambrook *et al*. [80]. The blot was subsequently hybridized with a Dig-11-UTP DNA probe labeled with a DIG DNA Labeling Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) following the manufacturer's protocol. The probe used for hybridization was obtained by PCR amplification, using clone cP89b [41] as template and primer pair T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and 7483 (5'-TTTAACATCAACCGAACCC–3'). The probe comprises 657 bp of a DNA sequence from *S. citri* BR3-T conserved between *arp1* and

arp2 (pos. 2315-2989, accession number AJ297706) and 72 bp derived from the cloning vector (pBluescript™). PCR was carried out in a DNA thermal cycler (MJ Research Inc., Waltham, MA) performing 34 cycles, each of 30 sec at 94°C, 30 sec at 54°C and 1 min at 72°C. Reactions were performed in a volume of 50 µl containing 1 Unit Taq polymerase (Promega, Madison, WI), 0.25µM primers, 250 µM of each dNTP, 50 - 100 ng template DNA, and 2.5 mM $MgCl_2$.

Hybridizations were at 55°C in Church buffer (0.5 M sodium phosphate buffer, pH 7.2, 7% SDS, and 1 mM EDTA) overnight followed by four washes, each 20 min, at 55°C in washing buffer (40 mM sodium phosphate buffer, pH 7.2, containing 0.1% SDS). Detection of the DIG-labeled probe was performed using a DIG Luminescent Detection Kit following the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN).

Results

Occurrence of arp2 Among Selected Mollicutes

Chromosomal DNAs of *S. citri* strains, other spiroplasma species and selected mycoplasmas were tested for the occurrence of *arp*-related sequences by Southern blot hybridization (Figure 8 and Table IV) using a probe derived from BR3-T *arp1*. DNA fragments sharing similarities with the *arp1*-probe were detected in all *S. citri* lines derived from strain BR3 and in most other *S. citri* strains tested. However, their patterns of hybridization differed considerably. *S. citri* strains BR3-3X, BR3-T and Beni Mellal showed one major band each, but the one from BR3-3X was larger in size than the other

two, which were similar in size to each other. BR3-G showed two bands, the larger one was similar in size to the one from BR3-X and the other one was smaller than the ones from the rest of the three strains. The bands from the two corn stunt spiroplasma (CSS) strains were larger than the ones from all the *S. citri* strains and the one from *S. citri* SPA-T was the largest of all. *S. citri* strain R8A2 and its derivative lines, ASP-1 and M200H, on the other hand, did not react with the probe (data not shown). Of the DNA from other tested spiroplasmas, only that from *S. kunkelii* and *S. melliferum*, which belong to the same serogroup as *S. citri*, hybridized with the probe, whereas DNAs of *Mycoplasma bovis* and *M. felis* did not react.

Discussion

The probe used in the Southern blot hybridizations in this study was designed from a region in *S. citri* BR3-T DNA that is conserved between *arp1* and *arp2*, as mentioned above. Hence, if any plasmid DNA contamination existed in the genomic DNA preparations used here, then low molecular weight bands would have been detected with the probe. Since we did not see any such bands, we conclude that our genomic DNA preparations were adequately pure for this study.

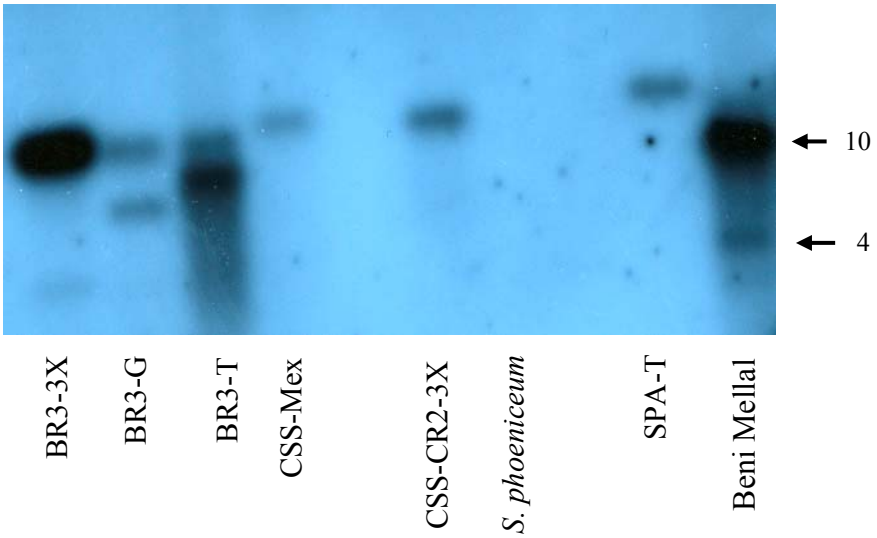

Figure 8. Southern Blot Hybridizations of the Genomic DNAs of BR3-derived *S. citri* Strains, CSS-Mex and CSS-CR2-3X, *S. phoeniceum*, and *S. citri* SPA-T and Beni Mellal with an *arp* Probe. The band sizes are in Kb.

TABLE IV

RESULTS OF SOUTHERN BLOT ANALYSIS OF DNA FROM SPIROPLASMAS AND MYCOPLASMAS DIGESTED WITH RESTRICTION ENZYME *Eco*RI AND HYBRIDIZED WITH A PROBE DERIVED FROM *arp1*

Organism	**Group**	**Southern blot**
S. citri		
BR3-3X	I	+[a]
BR3-T		+
BR3-G		+
Beni Mellal		+
SPA-T		+
MDHR3		+
GO4		+
Aceratagallia		+
R8A2		-
M200H		-
ASP-1		-
S. kunkelii (CR2-3X)	I	+
S. kunkelii (CSS-Mex)	I	+
S. melliferum (TS2)	I	+
S. phoeniceum (P40)	I	-
S. floricola (23-6)	III	-
S. apis	IV	-
S. syrphidicola	VIII	-
Mycoplasma bovis	-	-
Mycoplasma felis	-	-

[a] "+" indicates that the *arp1*-probe reacted with the DNA of the tested organism. The symbol "-" means no reaction in Southern blot hybridization.

The Southern hybridization data showed an absence of *arp2* in *S. citri* R8A2 and its derivative lines ASP-1 and M200H. Since M200H was not used in the study of

distribution of *arp1*, we do not know whether a plasmid with an *arp*-like sequence is absent from that strain also. Chromosomal DNA of other *S. citri* strains and of other Group I spiroplasmas, *S. kunkelii* and *S. melliferum*, did react with the probe. Out of the several non-BR3-derived *S. citri* strains tested in this study, only Beni Mellal was tested for the presence of both a plasmid and a chromosomal *arp* sequence. Previously [52] it was shown that *arp1* as well as any plasmid related to pBJS-O is absent from its extrachromosomal DNA. In contrast, here we find that it has *arp*-related sequences in its genome. *S. phoeniceum*, one of the three plant pathogenic spiroplasmas, did not react with the probe in this study. *S. apis* and *S. syrphidicola*, two other insect-associated spiroplasmas that belong to groups other than *S. citri*, also did not react with the probe in this study and neither did the two mycoplasma isolates used here.

The non-transmissible *S. citri* strain, BR3-G, not only possesses *arp2* in its genome, but carries pBJS-O, the plasmid containing *arp1* [52]. In contrast, *S. phoeniceum* neither possesses *arp1*, *arp2* nor any of the other regions related to the plasmid pBJS-O. These findings suggest that genes other than *arp1* and *arp2* may be involved in the transmission of these phytopathogens. Interestingly, Berho and colleagues [40] found a putative adhesin highly similar in sequence with SARP1, P80 (designated, Scarp4a), from *S. citri* GII-3 differentially expressed between the wild type and non-transmissible strains. They had made the comparison, however, using only three non-transmissible strains. The group also reported the presence of eight *Scarp* genes on six different plasmids in *S. citri* GII-3. Gene duplication and recombination may have played roles in giving rise to those genes. Furthermore, in this study *S. citri* Beni Mellal was found to lack *arp1*-like sequences, but was found to possess *arp*-like sequences on its

chromosome, suggesting that it probably lost the extrachromosomal *arp*-related sequences during its adaptation to the insect vector *C. haematoceps*. The differences in the long-term maintenance regimes of the *S. citri* BR3-derived strains may have led to the differences among their hybridization patterns, like the differences in protein profiles between transmissible and non-transmissible *S. citri* BR3-derived lines caused by the differences in the way *S. citri* BR3 was maintained [39]. Similarly, the differences in the hybridization patterns among the *S. citri* and the CSS strains may reflect adaptation to different vector and plant environments. As far as the absence of *arp*-related sequences from mycoplasmas is concerned, more conclusive results may be obtained if a different *arp* probe is used. Taken together, these results suggest that *arp2* may be sufficient but may not be necessary for the leafhopper transmissibility of the spiroplasmas.

CHAPTER IV

SPIROPLASMA CITRI ASP-1 AND R8A2 PLASMIDS: ISOLATION, CHARACTERIZATION AND EVOLUTION

Background

There have been several reports of extrachromosomal DNAs from *S. citri* as well as other spiroplasmas from ticks, insects and plants and from phytoplasmas [43; 44; 45; 46; 39; 47, 48 and 40]. Recently Joshi and coworkers [52] reported sequencing and structural characterization of pBJS-O, an indigenous, SARP1-encoding plasmid from *S. citri* BR3-3X. Plasmids have also been noted in *S. kunkelii* M2 [27] and Davis and colleagues [49] completely sequenced and characterized the *S. kunkelii* CR2-3X plasmid, pSKU146, which encodes an *S. kunkelii* homolog of SARP1, Sk-ARP1.

As mentioned in CHAPTER II, the extrachromosomal DNAs of *S. citri* R8A2, an *S. citri* strain isolated from citrus from Morocco, and *S. citri* ASP-1, a non-helical derivative of R8A2, lack *arp1*, the gene encoding SARP1, but possibly share sequences with that of pBJS-O in other regions [52]. SARP1 is hypothesized to be involved in the attachment of *S. citri* to the cells of its leafhopper vector *C. tenellus*. We hypothesized that *arp1*-related sequences were deleted from a pBJS-O-like ancestral plasmid present in *S. citri* R8A2 and ASP-1 during the evolution of the strains. To test this hypothesis and to determine the extent of sequences absent from ASP-1 and R8A2 plasmids with respect to pBJS-O, we sequenced and characterized extrachromosomal DNAs of the two strains.

Materials and Methods

Spiroplasmas

S. citri BR3-3X was originally isolated from brittle root diseased horseradish plants in Illinois [12], triply cloned [25], and frozen. Leafhopper-transmissibility and plant pathogenicity of BR3-3X was confirmed [25]. *S. citri* R8A2, isolated from citrus from Morocco [70], and its non-helical derivative, ASP-1, both obtained from R.E. Davis, USDA/ARS, Beltsville, MD, are non-transmissible. All the three *S. citri* strains were grown in LD8 broth medium [73] at 31^0C.

Purification of Extrachromosomal DNAs

S. citri ASP-1 and R8A2 extrachromosomal double-stranded (ds) DNAs were isolated (Figure 9) using the QIAprep Spin Miniprep Kit (QIAGEN Sciences, MD), following the manufacturer's protocol.

Cloning of *S. citri* ASP-1 and R8A2 Plasmids

Approximately 1 µg each of *S. citri* ASP-1 and R8A2 extrachromosomal DNAs were digested with 20 units of *Taq*I (Invitrogen Corp., Carlsbad, CA) at 65^0C for 4 hrs

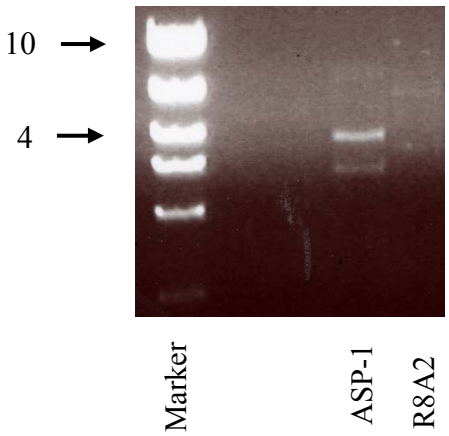

Figure 9. Undigested *S. citri* ASP-1 and R8A2 Plasmid Preparations. The marker used was High Mass Ladder (Invitrogen) and the band sizes are in Kb.

(Figure 10) and the reactions were stopped by heating at 80^0C for 20 min. About 15 µg of pBluescript™ (Stratagene, Cedar Creek, TX) vector DNA was digested with 20 units of *Cla*I (GIBCOBRL, Bethesda, MD) at 37ºC for 2h and the enzyme was inactivated by heating at 65^0C for 10 min. About 10 ng of the vector DNA was mixed with ~ 200 ng of the insert DNAs and the ligations were carried out with 0.1 units of T4 DNA Ligase

(Promega) at 14^0C for about 16 hrs. The reactions were stopped by heating at 70^0C for 10 min. Two microliters of each reaction was mixed with 50 µl of XL2-Blue Ultracompetent *E. coli* cells (Stratagene). Transformations were carried out by first keeping the tubes on ice for 30 min, followed by a heat shock of 45 seconds at 42^0C and keeping the tubes again on ice for 2 min [81]. Fresh LB broth (950 µl) was added to each tube. The transformants were grown at 37ºC for 90 min and 50 µl of each culture was plated onto separate LB-agar plates supplemented with 50 µg/ml ampicillin, 33 µg/ml X-gal and 8.1 µg/ml IPTG. The plates were incubated at 37ºC for 17 hrs. Individual white colonies were streaked onto separate LB-agar plates containing 50 µg/ml ampicillin, 33 µg/ml X-gal and 8.1 µg/ml IPTG as above, incubated at 37ºC for 17 hrs and 5 ml mid log phase LB broth cultures, containing 50 µg/ml ampicillin, from each of the colonies were grown at 37ºC for 13 hrs.

Sequencing of the Recombinant Clones

Cells from 3 ml of each of the LB broth cultures were harvested by centrifugation at 15, 800 x *g* for 5 min at 4^0C and the pellets were used to prepare plasmids using the QIAprep Spin Miniprep Kit (QIAGEN). Approximately 1 µg of each of the plasmids were digested with 20 units of *Pvu*II (Promega) at 37ºC for 4 hrs and electrophoresed on

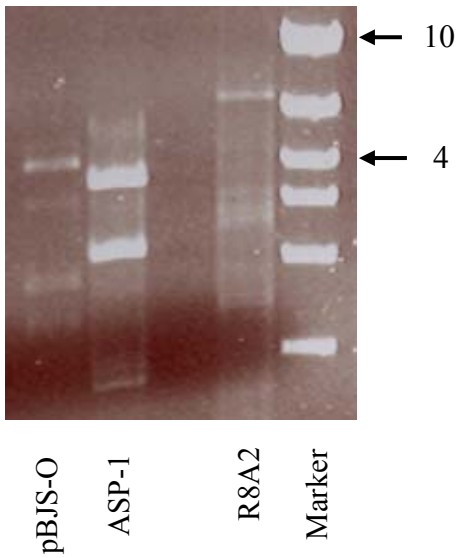

Figure 10. *Taq*I restriction patterns of pBJS-O, *S. citri* ASP-1 extrachromosomal DNA and *S. citri* R8A2 extrachromosomal DNA. The marker used was High Mass Ladder (Invitrogen) and the band sizes are in Kb.

a 0.75% (w/v) agarose gel with 1X TAE as running buffer to confirm the presence of inserts. ~ 1 μg of the recombinant plasmids containing inserts of different sizes were end-sequenced in both directions using 5 pmoles each of T3 (5'-ATT AAC CCT CAC TAA AGG GA-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') primers by the ABI PRISM BigDye Terminator Cycle Sequencing method (version 1.0, Applied Biosystems, Foster City, CA) and an ABI PRISM 3700 Automated DNA Analyzer (Perkin Elmer Biosystems, Foster City, CA). The raw sequences were screened for vector contamination using the software tool VecScreen from NCBI and the vector-free sequences were aligned with known sequences by BLAST searches [77] at the NCBI server and by using software tool AlignX provided by Vector NTI Suite 6.0 (Invitrogen).

Results and Discussion

Since the plasmids of *S. citri* ASP-1 and R8A2 do not contain *arp1*, we wanted to determine the precise extent of pBJS-O sequences missing from ASP-1 and R8A2 extrachromosomal DNA. As seen before in Southern blot hybridizations of *S. citri* ASP-1 and R8A2 extrachromosomal DNA with pBJS-O probe [52], multiple forms of native plasmids from the two strains were observed in this study (**Figure 9**). When the extrachromosomal DNAs were treated with *Taq*I, their restriction patterns differed completely from that of pBJS-O. Out of the seven restriction fragments observable from pBJS-O, none were conserved in ASP-1 and R8A2 (**Figure 10**).

Sequence Analysis of *S. citri* ASP-1 and R8A2 Clones

Alignment of the preliminary nucleotide sequences of *S. citri* ASP-1 plasmid clones by BLAST searches at the NCBI server revealed strong similarity with different regions of plasmid pSciA, an approximately 8.0 kb plasmid from *S. citri* GII-3 [EMBL:AJ966734]. BLASTX search using one of the ASP-1 sequences also revealed limited similarity with ORF 10 of pBJS-O and ORF 17 of pSKU146.

A dot plot analysis revealed no significant similarity between pBJS-O and pSciA nucleotide sequences. We recently reported [52] the detection of pBJS-O-related sequences in *S. citri* ASP-1 extrachromosomal DNA by Southern blot hybridization using pBJS-O itself as a probe, but not with an *arp*-related probe. It is possible that some pSciA-related sequences were also present along with pBJS-O in the probe. Furthermore, it appears that *soj*-related nucleotide sequences on pBJS-O and pSciA are not similar enough to be detected by a dot plot, although their protein products are 25% identical to each other in sequence (E value: 2e-14). The SOJ-like protein encoded by pSciA is more similar to its counterparts encoded by pSKU146 and by the five other pSci plasmids of *S. citri* GII-3 than to the one encoded by pBJS-O. Moreover, a 35 bp GC-rich sequence was found in pSciA upstream of the *soj* gene. A BLASTN search, using that sequence as a query, detected almost all of the sequence, with more than 95% identity, in all of the five *arp*-containing plasmids of *S. citri* GII-3 [40] and in pSKU146, in which the sequence was found about 30 bases upstream of the palindromic region, palSK1 [49]. Interestingly, this sequence was not detected in pBJS-O at all.

Analysis of the preliminary sequence data of *S. citri* R8A2 clones revealed strong matches with a spiroplasmavirus, SVTS2, from *S. citri* GII-3 and *S. melliferum* [60 and

X. Foissac, *unpublished data*] and with plasmids pBJS-O and pSKU146 of *S. citri* BR3-3X and *S. kunkelii* CR2-3X, indicating the presence of multiple plasmids in the extrachromosomal DNA. The sequence from primer T3 was similar to SVTS2 of *S. melliferum*, whereas the one from primer T7 was similar to the *S. citri* GII-3 version of the virus. In both versions of the virus sequence, however, the R8A2 sequence was similar to that of an open reading frame (ORF) C. In this study, multiple plasmids were also detected in the extrachromosomal DNA of *S. citri* BR3-3X; the major one being a related to plasmid pSciA of *S. citri* GII-3 [X. Foissac, *unpublished data*] and pBJS-O being in the minority.

Spiroplasmas contain numerous virus-related sequences integrated within their genomes, leading to extensive genome rearrangements [47]. Such events have significant impacts on the evolution of genomes of this important group of pathogens. Four types of spiroplasmaviruses, SpV1-4, have been identified. One genus of viruses, plectroviruses, that are rod-shaped and have circular single-stranded DNA genomes, has its members scattered in the *S. citri* genome [60]. One such virus, SpV1-C74, for instance, was found to be a member of the insertion sequence 3 (IS3) family whose insertion had inactivated a DNA adenine methylase gene in the host chromosome. Another one, SpV1-R8A2 B, contained several deletions due to multiple homologous recombination events with related sequences within the *S. citri* chromosome [47]. Further, we detected an SpV1-R8A2 B-like sequence in pBJS-O [52], which may have hybridized with a related sequence on the *S. citri* R8A2 extrachromosomal DNA in the Southern blot hybridization. Thus, virus-like sequences in *S. citri* R8A2 extrachromosomal DNA may have arisen by recombination with a similar sequence of the chromosomal DNA.

CHAPTER V

DISCUSSION AND FUTURE WORK

The relationships of chromosomal and extrachromosomal *arp* sequences of the genus *Spiroplasma* with insect transmissibility of phytopathogenic members of the genus are presented in this dissertation. The overall research comprises three parts that are reported here in three separate chapters, viz. Chapter II, Chapter III and Chapter IV.

The first study was of the isolation, distribution and sequence characterization of a novel SARP1-encoding plasmid, pBJS-O, from *S. citri* BR3-3X, which is reported in CHAPTER 2. To date, pBJS-O does not belong to any known incompatibility group of bacterial plasmids. Those findings were published in *BMC Genomics* in December 2005.

The second was of the distribution of an *arp* sequence from the chromosome of *S. citri* BR3-3X, *arp2*, a homolog of the plasmid-borne gene, *arp1*, in various mollicutes. It revealed that *Spiroplasma arp* sequences can be located on the chromosomal DNA and may not be present on the extrachromosomal DNA alone. Since the *arp* sequences can be present on plasmids, it would make a genetic exchange and/or a loss of the sequences possible through horizontal DNA transfer, depending upon the selection pressures encountered by the pathogens within the leafhopper and in the plant host. This might explain the absence of *arp*-like sequences from certain phytopathogens such as *S. phoeniceum*. It must be noted here that certain spiroplasma isolates used in these studies, such as *S. citri* ASP-1, R8A2 and Beni Mellal, were passaged in artificial media to a high degree here at OSU, which may have led to the loss of *arp* sequences from their DNAs and to the differences in their insect transmissibilities and phytopathogenicities, as

compared to the original isolates. Furthermore, two separate plasmids, with sequences similar to SVTS2 and to pBJS-O and pSKU146, were recently found in the extrachromosomal DNA of *S. citri* R8A2 [Joshi *et al*., *unpublished data*], which may have also arisen by horizontal DNA transfer and recombination. Such a mixture of plasmids was also recently found in the extrachromosomal DNA of *S. citri* BR3-3X. Contrary to the published report [52], majority of the extrachromosomal DNA is represented by a 7 Kb plasmid related to pSciA of *S. citri* GII-3 and only a small, but considerable, portion contains pBJS-O. This fact explains the observed disparities between the *in silico* and *in vitro* restriction digestion patterns of the extrachromosomal DNA obtained with *Bgl*II, *Nde*I, *Eco*RI and *Eco*RV. The exact extent of sequence conservation among pSciA, pBJS-O, pSKU146 and the related plasmids from *S. citri* BR3-3X and R8A2 is not yet known.

Finally, isolation and sequence characterization of plasmids from *S. citri* ASP-1 and R8A2, which lack *arp1*, is presented. Our data suggest that the extrachromosomal DNAs of both ASP-1 and R8A2 have little similarity with pBJS-O. Previous data suggest that ASP-1 does not bind the tissue-cultured cells of the leafhopper *C. tenellus* [F. Ye, *unpublished data*]. Hence, it could be used as a host for the functional complementation of adherence-related genes using its indigenous plasmid, pSciA, as a molecular vector. This vector might be better than the one made from pBJS-O and the vectors currently available for gene manipulation in *S. citri*. Random transposon mutagenesis of pBJS-O was carried out to develop it into a molecular vector for molecular genetic applications in *S. citri*, first using pMOD-5 (Epicentre Biotechnologies, Madison, WI), a cloning vector containing a *Tn*5-derived transposon having an *ori*C and a multiple cloning site (MCS),

for amplification in *Escherichia coli* and then using R6K*γori*/KAN-2 (Epicentre), another *Tn*5-derived transposon, but containing a kanamycin-resistance marker gene, used for *in vitro* insertion into target DNA. With the first approach, insertion of the transposon into pBJS-O failed, possibly because of damage to the transposase recognition sequences due to overexposure to UV during gel-extraction of the free transposon, whereas unusual topologies of pBJS-O molecules inhibited transposon insertion with the other approach.

Thus, overall the data presented in this dissertation suggest that *Spiroplasma arp* genes can be distributed over chromosomal as well as extrachromosomal DNAs and SARP1 may be sufficient, but not necessary, for *S. citri* transmission by *C. tenellus*. None of the genes, however, is solely necessary for the transmission of the pathogens by leafhoppers. Genes other than *arp1* and *arp2* may also be involved in the process and more work needs to be done to utilize spiroplasma plasmids as vectors for gene manipulations in mollicutes. Finally, it can be said that differences in hosts, geographical niches and maintenance regimes of spiroplasmas can lead to differences in their plasmid content.

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***S. kunkelii* Genome Project** [http://www.genome.ou.edu/spiro.html]

ORF Finder [http://www.ncbi.nlm.nih.gov/gorf/gorf.html]

Biology Workbench [http://workbench.sdsc.edu/]

VITA

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Thesis: *SPIROPLASMA ARP* SEQUENCES: RELATIONSHIPS WITH EXTRACHROMOSOMAL ELEMENTS

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Title of Study: *SPIROPLASMA ARP* SEQUENCES: RELATIONSHIPS WITH EXTRACHROMOSOMAL ELEMENTS

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Major Field: Biochemistry and Molecular Biology

Scope and Method of Study: Spiroplasmas, members of Class *Mollicutes*, are scattered over wide host and geographic niches. Those which are plant pathogenic, such as *Spiroplasma citri*, are transmitted from plant to plant by homopteran insect vectors, leafhoppers, and cause devastating damages to several crops world-wide. A variety of model systems are available to study the transmission of phytopathogenic spiroplasmas by leafhoppers, their predominant vectors. *Spiroplasma citri*-*Circulifer tenellus* is one such system. Because spiroplasmas can be cultured in artificial media, they offer an excellent means to investigate the complex molecular interactions underlying the transmission of *S. citri* by the insect vector *C. tenellus*. Evidence suggests that an *S. citri* membrane protein, *s*piroplasma *a*dhesion *r*elated *p*rotein 1 (SARP1), is involved in the adhesion of *S. citri* cells to tissue-cultured *C. tenellus* cells and, thus, is thought to be a putative adhesin. The gene encoding SARP1 (*arp1*) has been isolated, cloned and characterized. In this study, Southern blot hybridizations, PCR, cloning, sequencing and sequence comparisons of *S. citri* chromosomal and extrachromosomal DNAs were performed to investigate the relationships of the *arp* genes present on them with insect transmissibility and plant pathogenicity of various members of Class *Mollicutes*.

Findings and Conclusions: In this dissertation, *arp1* is shown to be present on a novel, indigenous plasmid, pBJS-O, in *S. citri* BR3-3X. Furthermore, evidence of *arp1*-related sequences on the chromosomal DNA in certain spiroplasmas and the partial characterization of plasmids from two *S. citri* strains that lack *arp1*, *S. citri* ASP-1 and R8A2, is presented. The presence of multiple plasmids in the extrachromosomal DNAs of *S. citri* BR3-3X and R8A2 is also shown. Taken together, the data presented suggest that SARP1 may be sufficient, but not necessary, for *S. citri* transmission by *C. tenellus* and the differences in host species, geographical niches and maintenance regimes of spiroplasmas can lead to differences in their plasmid content.

ADVISER'S APPROVAL: Dr. Ulrich Melcher