Characterizing the replication and stability regions of *Spiroplasma citri* plasmids identifies a novel replication protein and expands the genetic toolbox for plant-pathogenic spiroplasmas

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Spiroplasma citri strain Gll3 contains seven plasmids, pSciA and pSci1–6, that share extensive regions of sequence homology and display a mosaic gene organization. Plasmid pSci2 comprises 12 coding sequences (CDS), three of which encode polypeptides homologous to proteins Soj/ParA, involved in chromosome partitioning, and TrsE and Mob/TraG, implicated in the type IV secretion pathway. One CDS encodes the adhesin-like protein ScARP3d whereas the other eight encode polypeptides with no homology to known proteins. The pSci2 CDS *pE* and *soj* have counterparts in all seven plasmids. Through successive deletions, various pSci2 derivatives were constructed and assessed for their ability to replicate by transformation of *S. citri* 44, a strain which has no plasmid. The smallest functional replicon was found to contain a single CDS (*pE*) and its flanking intergenic regions. Shuttle (*S. citri*/*Escherichia coli*) plasmids, in which CDS *pE* was disrupted, failed to replicate in *S. citri*, suggesting that *pE* is the replication protein of the *S. citri* plasmids. Successive propagations of pSci2-derived transformed spiroplasmas, in the absence of selection pressure, revealed that only pSci2 derivatives having an intact *soj* gene were stably maintained, indicating that the *soj*-encoded polypeptide is most likely involved in plasmid partitioning. Upon transformation, pSci2 derivatives, including shuttle (*S. citri*/*E. coli*) plasmids, were shown to replicate in all *S. citri* strains tested regardless of whether the strain possesses endogenous plasmids, such as strain Gll3, or not, such as strain R8A2. In addition, the pSci replicons were introduced efficiently into the plant-pathogenic spiroplasmas *Spiroplasma kunkelii* and *Spiroplasma phoeniceum*, the transformation of which had never, to our knowledge, been described before. These studies show that, besides their implications for the biology of *S. citri*, the pSci plasmids hold considerable promise as vectors of general use for genetic studies of plant-pathogenic spiroplasmas. As an example, a HA-tagged *S. citri* protein was expressed in *S. kunkelii*. Detection of *pE*-hybridizing sequences in various group I spiroplasma species indicated that *pE* replicating plasmids were not restricted to the three plant-pathogenic spiroplasmas.

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INTRODUCTION

Spiroplasmas are a group of helical, cell-wall-free bacteria belonging to the class *Mollicutes*. Three spiroplasma species, *Spiroplasma citri* (Saglio *et al.*, 1973), *Spiroplasma kunkelii* (Whitcomb *et al.*, 1986) and *Spiroplasma phoeniceum* (Saillard *et al.*, 1987), have been associated with plant diseases. *S. citri* is the aetiological agent of the citrus stubborn and horseradish brittle root diseases and was the

first plant mollicute to be cultured *in vitro* (Fletcher *et al.*, 1981; Saglio *et al.*, 1971). Since then, *S. citri* has been studied extensively (Bové *et al.*, 1989, 2003). The availability of both an experimental transmission assay via the leafhopper vector (Foissac *et al.*, 1996) and gene transfer systems (Foissac *et al.*, 1997; Renaudin, 2002; Renaudin & Lartigue, 2005) has made it a unique model for studying plant mollicute–host interactions (André *et al.*, 2005; Berho *et al.*, 2006a; Bové *et al.*, 2003; Killiny *et al.*, 2005).

In spiroplasmas, plasmids were first detected as extra-chromosomal, covalently-closed-circular DNAs (Ranhand

Abbreviation: CDS, coding sequence(s).

Five supplementary figures and a supplementary table are available with the online version of this paper.

et al., 1980) and some of them were mapped and/or cloned in *Escherichia coli* (Archer *et al.*, 1981; Mouchès *et al.*, 1983a). Extrachromosomal DNAs, including plasmids and viral DNAs, were most frequently detected in group I spiroplasmas (Gasparich *et al.*, 1993), especially in *S. citri* strains. However, these plasmids have for a long time been designated cryptic since no phenotypic character had been associated with their presence. In addition, due to the lack of sequence and functional data, the engineering of natural plasmids to produce cloning vectors has had very limited success (Salvado *et al.*, 1989). More recently sequencing of plasmids pBJS-O from *S. citri* strain BR3-3X (Joshi *et al.*, 2005), and pSciA and pSci1-6 from *S. citri* GII3 (Saillard *et al.*, 2008), revealed that they encode proteins, including adhesin-like molecules, which have been tentatively associated with insect transmission (Killiny *et al.*, 2006; Yu *et al.*, 2000). Furthermore, it was shown that plasmids pSci1-6 from the insect-transmissible strain GII3 were absent in the non-insect-transmissible strains of *S. citri* (Berho *et al.*, 2006b). However, the first clear-cut evidence of a phenotypic trait associated with spiroplasmal plasmids was the finding that pSci6 from *S. citri* GII3 conferred insect-transmissibility to the plasmid-free, non-insect-transmissible strain 44 (Berho *et al.*, 2006a). Besides assessing their role in insect transmission, the successful transfer of pSci plasmids between strains of *S. citri* raised their potential as genetic tools. In addition to adhesin-like proteins, pSci1-6 encode proteins with similarities to components of bacterial type IV secretion systems, suggesting the possibility of conjugative transfer of these plasmids. In turn, none of the plasmid-encoded proteins shared similarity with known replication proteins (Saillard *et al.*, 2008).

Previously, we showed that sequences essential for replication and maintenance of pSci2 in *S. citri* were located within an 8 kbp restriction fragment (Berho *et al.*, 2006a). In the present study, we describe the functional characterization of the replication and stability regions of *S. citri* pSci-derived plasmids, with the aim of further investigating their role in spiroplasmal biology. We also demonstrate the usefulness of pSci-derived plasmids as vectors for gene transfer and expression in plant-pathogenic spiroplasmas other than *S. citri*.

METHODS

Spiroplasma strains, growth conditions and transformation. *S. citri* GII3 wild-type strain was originally isolated from its leafhopper vector *Circulifer haematodeps*, captured in Morocco (Vignault *et al.*, 1980). *S. citri* 44 was isolated from a stubborn-diseased sweet orange tree in Iran (Hosseini Pour, 2000). Other spiroplasma strains used in this study are listed in Supplementary Table S1, available with the online version of this paper. Spiroplasmas were grown at 32 °C in SP4 medium (Whitcomb, 1983) from which fresh yeast extract was omitted. Spiroplasma cells were transformed by electroporation (Stamburski *et al.*, 1991) using 2–10 µg purified plasmid or various ligation mixtures. Spiroplasmal transformants were selected in the presence of 2 µg tetracycline ml⁻¹, and further propagated in broth

medium containing 5 µg tetracycline ml⁻¹. The Spiroplasma species of tetracycline-resistant transformants were confirmed by PCR-RFLP analysis of the spiralin genes, using amplification with primer pair SR28/SR29 (Table 1) and digestion with *Mbo*I and/or *Rsa*I.

Plasmid constructs. Plasmids pSRT1 and pSRT2 are pBS⁺ (Stratagene)-derived, ColE1 replicons carrying the tetracycline resistance gene *tetM* under the control of the spiralin gene promoter (Lartigue *et al.*, 2002). Plasmid pSRT1 differs from pSRT2 in that the *tetM* cassette is in the opposite orientation. Plasmids pSci1NT and pSci6PT were obtained by inserting the *tetM* cassette (as a 2.3 kbp *Pst*I fragment) into the *Nsi*I and *Pst*I sites of *S. citri* pSci1 and pSci6, respectively, whereas pSci21NT resulted from the combination of the *tetM* cassette with the 8034 bp *Nsi*I fragment of pSci2 (Berho *et al.*, 2006a). In pSci1NTΔS, the *scarp3d* gene was disrupted by inserting the 20 bp oligonucleotide duplex SBamF1/SBamR1 (Table 1) into the *Nhe*I site of pSci1NT. Similarly, in pSci21NTΔsoj, the *soj* gene was disrupted by insertion of the 19 bp SojBamF1/SojBamR1 duplex into the *Sml*I site of pSci21NT. As a result, the *Soj* coding sequence (CDS) encodes a truncated polypeptide of 67 amino acids as compared with 260 in the wild-type. Plasmid pFL3 was obtained by self-ligation of the 6675 bp *Eco*RI–*Bgl*II fragment of pSci21NT. Plasmid pFL4 was constructed by deleting the 891 bp *Hinc*II–*Hae*II fragment within the *mob* gene of pFL3, and pFL5 by deletion of the 504 bp *Acl*I–*Eco*RV fragment of pFL4. Plasmid pBS1 was derived from pBS⁺ by inserting the EPBg1/EPBg2 duplex at the *Eco*RI site to introduce a *Bgl*II site. Plasmid pBST1 was then obtained by combining the *Bgl*II-linearized pBS1 with the *tetM* cassette (as a 2311 bp *Bam*HI–*Bgl*II fragment) of pSRT2. Plasmid pFL6, containing the *pE* gene and the flanking, intergenic regions, resulted from the ligation of the 2265 bp *Pac*I fragment from pFL4 to the 2329 bp *Pac*I fragment of pBST1 containing the *tetM* cassette. Plasmid pFL7 (4259 bp) was obtained by deleting the 341 bp *Acl*I–*Bgl*II fragment of pFL6. The shuttle (*S. citri*/*E. coli*) plasmid pBSFL6 resulted from the combination of *Pst*I-linearized pBS⁺ and pFL6. To construct pSDE1, the amplification product of pSci2 with primer pair PEF1/IEFR2 was inserted into pSRT1 after they were both digested with *Bam*HI + *Eco*RI. Similarly, the Rep1-F1_*Bgl*II/Rep1-R1_*Bgl*II amplification product, comprising the *pE* gene and the flanking intergenic regions of pSci1, was digested with *Bgl*II, and the 1963 bp fragment was inserted into *Bgl*II-linearized pSRT1 to yield pMBRE. While pBSFL6 comprised the *pE* region of pSci2, pMBRE contained the comparable *pE* region of pSci1 having a *Mlu*I restriction site within the *pE* gene. To construct pMBRAE1, pMBRE was linearized with *Mlu*I, and recircularized by ligation after the 4 bp cohesive ends were made blunt-ended. In this plasmid, disruption of *pE* led to a truncated polypeptide of 97 amino acids instead of 217 in the wild-type. Similarly, in pMBRAE2 inserting the 19 bp oligonucleotide duplex EBamF1/EBamR1 into the *Mlu*I site led to a truncated PE of 95 amino acids. Plasmid pNAB32H resulted from insertion of the *p32-HA* tagged gene inserted into pBSFL6. Each plasmid construct was confirmed by restriction mapping and sequencing the appropriate regions.

DNA isolation, Southern blot hybridization and Western immunoblotting. Spiroplasma genomic DNA was prepared from 10 ml cultures using the Wizard genomic DNA purification kit (Promega), whereas plasmid DNA was purified from 25 ml cultures with the Wizard SV minipreps DNA purification kit (Promega). Southern blot hybridization of spiroplasmal DNA with appropriate digoxigenin-dUTP-labelled probes has been described elsewhere (André *et al.*, 2003). Hybridization signals were detected with anti-digoxigenin-alkaline phosphatase conjugate and 2-hydroxy-3-naphthoic acid-2'-phenylamide phosphate (HNPP) as the substrate, following the supplier's instructions (Roche Diagnostics). Fluorescent signals were detected using a Fluor-S Multimager Phosphorimager (Bio-Rad). Probes specific to SCARs, P32 and PE were generated by PCR amplification with primer pairs S235F/S235R, P32F/P32R and PEF1/PER1, respectively (Table 1).

Table 1. Oligonucleotides and primers used in this study

Name	Nucleotide sequence (5'–3')	Position	Accession no.
S235F	TAAACATTGATATTGCCAACCCC	3617–3639	AJ969069
S235R	GGTTAAAGTTGCAGAATTATTATC	3973–3996	AJ969069
SR28	ACTTTTATCGATTTTAGCAG	114268–114287	AM285305
SR29	ATCTTTTGCTTTAACTGTTACT	114886–114908	AM285305
P32F	TAACGAATTAAATCATTCTAATAGC	24852–24876	AJ969074
P32R	TAGTTCCGGCTTGCTCACCA	24332–24351	AJ969074
PEF1	CCCACG gaATTCTT CTATACACCTATTAAG	11022–11051	AJ969070
PER1	AGTATT ggATc CATTGTCTTGCTACGCTGT	12046–12075	AJ969070
Rep1-F1_BglII	ACAGTT AgAtc TGCTTGCTCTGAATAAC	10208–10235	AJ969069
Rep1-R1_BglII	TTAAT CaGaTCT GTGCATAATTTCAACTCCT	12159–12188	AJ969069
IEFR2	CTAAATTTAA GgATCCT CATTTATTAATCATATC	12620–12653	AJ969070
EPBg1	AATTCCTTAATTAAC AGATCT CCTTAATTAACCG	–	–
EPBg2	AATTCGGTTAATTAAG AGATCT GGTTAATTAAGGG	–	–
EBamF1	CGCGAT AGGAT CCAACTAT	–	–
EBamR1	CGCGATAGTT GGATC CTAT	–	–
SBamF1	CTAGATT AGGAT CCACTAAT	–	–
SBamR1	CTAGATTAGT GGATC CTAAT	–	–
SojBamF1	TCAAT GGAT CCATAGCTAC	–	–
SojBamR1	TTGAGTAGCTAT GGATC CA	–	–

*Bold type indicates restriction enzyme sites. Lower-case type indicates mismatched nucleotides.

Spiroplasma proteins were separated by SDS-PAGE and further analysed by immunoblotting as described previously (Duret *et al.*, 2003) except that the proteins reacting with the primary antibodies were visualized by using a goat anti-rabbit immunoglobulin G-peroxidase conjugate and the SuperSignal West Pico chemiluminescent substrate (Pierce).

In silico analyses. The BLAST program (Altschul *et al.*, 1997) was used to search for homologies in general databases (<http://www.ncbi.nlm.nih.gov/blast/>), the mollicute dedicated database MolliGen (Barré *et al.*, 2004), and the *S. kunkelii* partially sequenced genome (http://www.genome.ou.edu/blast/spiro_blastall.html). Conserved domains were detected by CD-Search against the NCBI's conserved domain database (Marchler-Bauer & Bryant, 2004). Global sequence alignments were accomplished using a Needleman–Wunsch algorithm implemented in the Needle program (Needleman & Wunsch, 1970). Multiple alignments were done with T-Coffee (Notredame *et al.*, 2000) and/or CLUSTAL W (Thompson *et al.*, 1994). For subsequent phylogenetic analyses, poorly aligned positions and divergent regions were eliminated using the alignment curation program Gblocks (Castresana, 2000). Neighbour-joining trees based on these alignments were constructed using the MEGA 3.1 program (Kumar *et al.*, 2004) and maximum-likelihood trees were computed with the WAG substitution model using the PhyML program (Guindon & Gascuel, 2003). All trees were bootstrapped 100 times.

RESULTS

Plasmids from *S. citri* GII3 all contain CDS *pE* and *soj*

S. citri strain GII3 carries seven plasmids, pSciA (7.8 kbp) and pSci1–6 (13–35 kbp) (Saillard *et al.*, 2008), which share a large number of highly conserved regions and

display a mosaic gene organization (Fig. 1). Most of the CDS are present on several plasmids and, reciprocally, very few of them are specific to one given plasmid. Such an exception is *p32*, encoding a hydrophilic protein specific to pSci6 (Killiny *et al.*, 2006). Surprisingly, most of the plasmid CDS have no orthologues in other bacterial genera, with the exceptions of *soj*, *trsE*, *traG* and *mob*, the naming of which indicates that they encode proteins closely related to proteins involved in partitioning (Soj/ParA family COG1192) and transfer (TrsE, TraG and Mob) of DNA molecules. The *scarp* genes of pSci1–5 encode proteins sharing strong similarities with the adhesin-like protein SARP1 from *S. citri* BR3 (Berg *et al.*, 2001). Plasmid pSci2 (14.4 kbp) seems to represent the 'core plasmid' as it displays a gene organization (*pE*, *pF*, *pA*, *soj*, *pB*, *scarp*, *pC*, *pD*, *trsE*, *p4*, *mob*) which is conserved in pSci1 (13 kbp) and larger plasmids pSci3 (19.3 kbp) and pSci4 (20.2 kbp) (Fig. 1). Plasmid pSci3 possesses an additional CDS (*pG*), whereas pSci4 carries two *scarp* genes, two copies of *soj*, and the additional CDS *pH*, which is absent in the other three plasmids pSci1–3. In pSci1, pSci5 and pSci6, the *mob*-like gene is disrupted. The large plasmids pSci5 and pSci6 probably result from recombination between plasmids and/or from gene duplication. Whereas pSci1–3 contain a single copy of *pE* and *soj*, pSci4–6 have two copies of *soj*, and multiple copies of *pE* are present in pSci5 (2 copies) and pSci6 (4 copies). Interestingly, *pE* and *soj* are the only two CDS that are conserved, uninterrupted, in all seven plasmids, pSciA and pSci1–6, suggesting that these genes may be essential for replication and maintenance of the plasmids.

Fig. 1. Gene organization of plasmids pSciA and pSci1–6 from *S. citri* GII3. CDS *pE* and *soj* are indicated by black and white arrows, respectively. Numbers indicate the percentages of identical amino acids shared by the *pE* and *soj* protein products with their counterparts from pSci2. Conserved CDS are named (A to N, corresponding to pA–N in the databases) whereas orphan CDS are not. Positions of *Bst*BI restriction sites (B) of pSci6 are indicated. *scarp*, genes encoding *S. citri* adhesion-related proteins. * indicates truncated CDS. For alignment, pSciA and pSci1–6 were linearized arbitrarily regardless of the sequence numbering (GenBank accession numbers AJ966734 and AJ969069–74).

Replication region of pSci2

Preliminary data strongly suggested that the replication region of pSci2 was located within the *Nsi*I fragment of 8034 bp (Berho *et al.*, 2006a). This fragment encodes seven putative polypeptides, including a Mob-like ATPase and a Soj-like protein (Fig. 2). Polypeptides PA and PE, as well as the truncated PB and PF, are hypothetical proteins that have counterparts in other pSci plasmids, whereas the hypothetical polypeptide HP shares no homology with other proteins. None of these polypeptides share homology with known replication proteins. To identify the replication region of pSci2, various pSci2 derivatives were constructed by successive deletions of pSci21NT, and tested for their ability to replicate through transformation of *S. citri* 44, a strain which contains no plasmid. Plasmid constructs are described in Methods. The finding that pFL3, pFL4, pFL5, pFL6 and pFL7 all replicated in *S. citri* showed that the replication region was located within the 1876 bp *Hae*III–*Acl*I fragment of pFL3 (Fig. 2). Interestingly, this DNA fragment comprised a single CDS, suggesting that the *pE*-encoded polypeptide might be the replication protein and that the flanking intergenic regions contained the *cis*-acting binding sites. *In silico* analyses revealed the region upstream of *pE* to contain specific features including the imperfect repeats ATTTAATCCCC and ATTTAACCCCC (at positions 10963 and 11084 of pSci2) and four sequences (GTTTCCATA, ATTTCCACA, TCTACCACA and TTTTCCAAA, at positions 10833, 10899, 10977 and 11268, respectively) resembling the DnaA boxes

(TTTTCACACA) of the *S. citri* chromosomal replication origin (*oriC*) (Ye *et al.*, 1994). However, in contrast to the situation at *oriC*, these sequences did not cluster within a short region. Immediately downstream of *pE*, two tandem repeated sequences of 11 nucleotides (TAACCTCCCTA), as well as an A-T rich region, were detected (Fig. 2). However, none of the six inverted repeat sequences identified in the intergenic region downstream of *pE* (Saillard *et al.*, 2008) was located within the 1876 bp minimal replicon, indicating that these sequences, which putatively lead to hairpin structures, are not essential for plasmid replication. To further delineate the replication region, DNA fragments were retrieved from pFL6 by digestion or PCR amplification and inserted into ColE1 replicons by cloning in *E. coli*. Whereas the recombinant plasmid pBSFL6, which contained the *pE* gene and the flanking intergenic regions, readily replicated in *S. citri*, plasmid pSDE1 did not, indicating that replication probably requires sequences upstream of primer PEF1.

CDS E encodes the replication protein

To further demonstrate the role of PE in plasmid replication, shuttle plasmid pMBRE and its *pE*-disrupted derivatives pMBRAE1 and pMBRAE2 were used to transform *S. citri* 44. As expected, transformation by pMBRE yielded tetracycline-resistant transformants at relatively high frequency ($\sim 10^{-4}$ transformants c.f.u. $^{-1}\mu\text{g}^{-1}$), indicating that the plasmid replicated in these cells. The presence of the relevant plasmid in the

Fig. 2. Structural organization of pSci2 deletion derivatives, and ColE1 replicons carrying the pSci1 or pSci2 replication region. Plasmid constructs are described in Methods. Putative DnaA boxes (□), repeated sequences (▶), iteron-like sequences (small white arrows), and an AT-rich region (|||||) are indicated in the minimum replicon pFL7 only. A, *AcI*; B, *Bgl*; E, *EcoRI*; EV, *EcoRV*; H, *HincII*; Ha, *HaeIII*; M, *MluI*; N, *NsiI*; P, *PacI*; Ps, *PstI*; S, *SmaI*; nd, not done. + and – indicate the ability or inability to replicate or to be stably maintained in the absence of selection pressure. * indicates truncated CDS of pSci2.

spiropalasmal transformants was confirmed by PCR and Southern blot hybridization (data not shown). In contrast, no transformants were obtained through transformation with pMBRAE1 and pMBRAE2, in which the *pE* gene was disrupted (see Methods and Fig. 2). As a control, disruption of the *scarp* gene in pSci1NTΔS had no effect on the ability of the plasmid to replicate.

Considering PE as the replication protein, one would expect pMBRAE1 and pMBRAE2 to replicate in *S. citri* GII3, a strain with plasmids, from which wild-type PE was known to be expressed, as indicated by proteome analyses (unpublished data). However, transformation of *S. citri* GII3 by these plasmids failed repeatedly, indicating that replication of pMBRAE1 and pMBRAE2 cannot be driven by the PE protein when provided *in trans* from endogenous plasmids.

Alignments of the PE proteins from pSci1–6 showed that they share strong similarities to each other, with the conserved regions being distributed all along the protein sequence. Identities ranged from 76.1 % between pSci1-PE and pSci6-PE4 to 97.7 % between pSci3-PE and pSci5-PE1

(Fig. 1 and Supplementary Fig. S1). The pSciA-PE was much less conserved with identities ranging from 25.2 % with pSci1-PE to 28.4 % with pSci6-PE1. Unexpectedly, despite the PE protein being essential for plasmid replication, no putative DNA-binding motif such as helix-turn-helix, coiled coil, zinc finger, or leucine zipper was predicted in the PE sequence. It is noteworthy that most of the PE polypeptides end with an identical C-terminus (ELDNLD), except that from pSciA and three of the four PE from pSci6, which have distinct C-termini (Supplementary Fig. S1). The autonomous replication of the 16195 bp circularized *Bst*BI fragment of pSci6PT (unpublished data) indicated that at least one PE of pSci6 (pSci6 E2 in Fig. 1) functioned as the replication protein. Whether the other three are functional or not has not yet been investigated. Studying the genetic relatedness of the *S. citri* GII3 proteins PEs showed that they clustered within three distinct groups (Fig. 3). The largest group consisted of PEs from pSci1–5 and the PE counterparts of pBJS-O from *S. citri* BR3 (Joshi *et al.*, 2005) and pSKU146 from *S. kunkelii* CR2-3X (Davis *et al.*, 2005). Unexpectedly, whereas one PE (PE2) from pSci6 also fell in this group, the

Fig. 3. Phylogenetic tree of the PE proteins from *S. citri* (pSciA, pSci1–6, and pBJS-O) and *S. kunkelii* (pSKU146) plasmids. Sequences were aligned with the T-Coffee program and analysed as described in Methods.

other three (PE1, 3 and 4) formed a distinct, heterogeneous group. Such variability is consistent with the hypothesis that, in contrast to PE2, PE1, 3 and 4 might not be functional. Whether the occurrence of multiple copies of the *pE* gene in pSci6 resulted from gene duplication or recombination between plasmids is unclear. Intriguingly, the pSciA PE did not cluster with any other PE proteins, suggesting that pSciA had a distinct origin. This is in agreement with the fact that, except for PE and Soj, the pSciA CDS have no counterparts in any other *S. citri* plasmids.

Stability of pSci plasmids

S. citri strain GII3, originally isolated in 1980, has been triply cloned and further propagated for hundreds of generations. Plasmids pSci1–6 from this strain, GII3-3X, displayed characteristic hybridization patterns when probed with a mixture of probes *scarp* and *p32* (Berho *et al.*, 2006b). To further confirm the presence of the six plasmids in every cell, the spiroplasma strain was further subcloned twice, and the plasmid contents of 10 isolated colonies were characterized by Southern blot hybridization of total DNA. The hybridization patterns of all 10 subclones tested were identical to each other and to that of the original culture (Supplementary Fig. S2), indicating that each spiroplasma cell contained the whole set of plasmids, pSci1–6. Identical hybridization patterns were obtained with spiroplasma cultures isolated from the leafhopper vector and from the host plant after experimental transmission of GII3-3X, by injection, to its leafhopper vector (data not shown).

As indicated above, each *S. citri* plasmid contains at least one *soj*-like gene, encoding a polypeptide with significant

similarities to proteins of the Soj/ParA, ATPase family involved in chromosome partitioning. Similarities to the chromosomal ParA of *S. citri* and the Soj protein of *Bacillus subtilis* ranged from 41.3 to 46.3% and from 41.6 to 50.8%, respectively. In the plasmid-encoded Soj, COG1192 (Soj) and COG0455 (ATPases involved in chromosome partitioning) were predicted with significant probabilities (e-values ranging from 3×10^{-10} to 1×10^{-28}). Like the chromosomal ParA, the pSci-encoded Soj all possessed the deviant Walker-type ATPase consensus motif with the typical A, A', and B boxes (Koonin, 1993) (Supplementary Fig. S3).

To investigate the role of Soj in *S. citri* plasmid partitioning, we assessed the stability of various pSci2 derivatives in *S. citri* 44 transformants. In the experiment shown in Fig. 4, *S. citri* 44 cells transformed with pSci21NT (containing *soj*) or pFL3 (lacking *soj*) were propagated in the absence of selection pressure. After 5, 15 and 30 passages (30 passages correspond to 100 generations), the plasmid contents of 10 individual clones were analysed by Southern blot hybridization. The results showed that pSci21NT was detected in each of the 10 clones tested, regardless of the passage number (5, 15 or 30). In contrast, pFL3 was lost in three clones out of ten, as early as the fifth passage. After 15 passages, pFL3 was detected in only two clones, and after 30 passages, none of the ten clones carried pFL3. The finding that, in the absence of selection pressure, pSci21NT was stably maintained for many generations whereas pFL3 was rapidly lost strongly suggested that the *soj*-encoded protein was indeed involved in partitioning of pSci2. In accordance with these results, plasmid loss was also noticed in spiroplasmas transformed with pSci2 derivatives pFL4, pFL5 and pFL6, all of which lack *soj*. However, when compared with pSci21NT these plasmids

Fig. 4. Segregational stability of pSci21NT and pFL3. Southern blot hybridization of *Hinc*II-restricted DNAs from *S. citri* GII3 (lane G) and *S. citri* 44 transformants (lanes 1–10) with probe *pE*. Total DNAs were extracted after 5, 15 and 30 passages in the absence of selection pressure. (a) pSci21NT; (b) pFL3.

lack not only *soj* but also three additional CDS, the two conserved hypothetical proteins PA and PB as well as the orphan CDS HP (Fig. 2). The role of *Soj* was further demonstrated by evaluating the stability of pSci21NTΔ*soj*, a plasmid in which the *soj* gene was disrupted, in *S. citri* 44 transformants. After propagation for up to 30 passages in the absence of selection pressure, the presence of the plasmid was checked by monitoring the percentage of spiroplasma cells growing on selective (tetracycline) agar medium. As expected from the above experiment, nearly 100 % (285 / 294) of cells from pSci21NT transformants were still tetracycline-resistant after 30 passages. In contrast, in pSci21NTΔ*soj* transformants, the ratio of tetracycline-resistant to total cells progressively decreased to 81 % (515/638) at the fifth passage, 27 % (132/496) at the 15th passage, and less than 0.2 % (less than 1/417) after 30 passages. These results clearly indicated that the *soj* gene product is involved in the stability of pSci plasmids.

Phylogeny of plasmid *Soj* proteins

Sequence alignments of predicted *Soj* proteins (Supplementary Fig. S3) revealed that those from plasmids pSci1–5 were more closely related to each other (identities ranging from 50.4 to 82.9 %) than to *Soj* from pSci6 (22.4 to 28.3 % identity), and that those from pSci1–6 shared a low percentage (24 to 29.2 %) of identical amino acids with pSciA. The bootstrapped phylogenetic tree (Fig. 5) indicates that plasmid-encoded *Soj* were distinct from the chromosomal *Soj*/ParA and clustered in two separate groups: *Soj* from pSci1–5 (*S. citri* GII3) and pBJS-O (*S. citri* BR3) in one group, and *Soj* from pSciA and pSci6 (*S. citri* GII3), and pSKU146 (*S. kunkelii* E275) in the other, suggesting that these groups had evolved separately and

disjointedly from the chromosomal ParA. In an effort to understand the origin of these plasmids, phylogenetic analyses of spiroplasma *Soj* proteins were extended to a set of representative ParA homologues (Gerdes *et al.*, 2000) including the 23 annotated ParA homologues from mollicute genomes (<http://cbi.labri.fr/outils/molligen/>). The phylogenetic tree showed that, as in many bacteria (McLeod *et al.*, 2006), the chromosomally encoded ParA proteins of spiroplasmas had phylogenies congruent with those of their hosts. In contrast, the plasmid-encoded *Soj*/ParA proteins were more diverse and their clustering did not follow the organismal phylogeny (Supplementary Fig. S4). When restricted to the mollicutes and several other firmicutes, these analyses identified four major clusters supported by strong bootstrap values (Supplementary Fig. S5). One coherent cluster was made from the *S. citri* chromosomal ParA and other chromosomal ParA proteins of firmicutes (except those of mycoplasmas). A second cluster consisted of the mycoplasmal chromosome-encoded ParA/*Soj* proteins, whose genes are located in the vicinity of the chromosome replication origin. These proteins are clearly distinct from ParA/*Soj* proteins of other firmicutes and the spiroplasmas in particular. The third cluster comprised plasmid-encoded *Soj* proteins from *S. citri* pSci1–5 and pBJS-O; the fourth cluster consisted of *Soj* proteins from *S. citri* pSciA and pSci6, *S. kunkelii* pSKU146, and chromosomal *Soj* proteins that are encoded by putative or demonstrated mobile elements of mycoplasmas (Marenda *et al.*, 2006; Sirand-Pugnet *et al.*, 2007).

Distribution and host-range of *S. citri* pSci plasmids

Previous studies showed that, in contrast to GII3, various *S. citri* strains did not contain plasmids pSci1–6, as revealed by the failure of genomic DNAs to hybridize with *scarp* and *p32* probes (Berho *et al.*, 2006b). To investigate the occurrence of PE replicating plasmids in spiroplasmas further, genomic DNAs from various strains of *S. citri* and other spiroplasma species were hybridized with probes *scarp* and *pE* (Fig. 6, Table 2). Both *scarp* and *pE* hybridization signals were detected in strains GII3, Corse, Palmyre, Hinckley, IP85 and Israel. In contrast, no hybridization signal was detected in ASP-1, R8A2 and 44. Detection of *pE* but not *scarp* sequences in strain Alcanar 254 is consistent with the fact that this strain carries a single plasmid nearly identical to pSci6 (unpublished data), which contains no *scarp* genes. Interestingly, *pE*-hybridizing signals were also detected in the plant-pathogenic spiroplasmas *S. kunkelii* E275 (subgroup I-3) and *S. phoeniceum* P40 (subgroup I-8), indicating that PE replicons were not restricted to the species *S. citri*, i.e. subgroup I-1 of the spiroplasmal classification (Williamson *et al.*, 1998). In addition, the *pE* probe was found to hybridize with genomic DNAs from the bee pathogen *Spiroplasma melliferum* (subgroup I-2) strains BC3, G1 and AS576, the rabbit tick spiroplasma 277F (I-4), the plant bug spiroplasma sp. LB-12 (I-5), and the plant surface

Fig. 5. Phylogenetic relationships of plasmid *Soj* proteins from *S. citri* (pSciA, pSci1–6 and pBJS-O) and *S. kunkelii* (pSKU146), and chromosomal *Soj* proteins from *S. citri* and *B. subtilis*. Sequences were aligned with the T-Coffee program and analysed as described in Methods.

Fig. 6. Southern blot hybridization of *HincII*-restricted DNAs from various spiroplasmas with the *pE* probe. DNAs were from *S. citri* GII3, Palmyre, Israel, Hinckley, IP85 and R8A2 (lanes 1–6), ASP-1 and 44 (lanes 13 and 14), M4 and MHcl2 (lanes 15 and 16), Corse (lane 21), *S. melliferum* BC3 and G1 (lanes 19 and 20), *S. kunkelii* E275 (lane 7), *Spiroplasma* sp. 277F and LB12 (lanes 8 and 12), *S. phoeniceum* P40 (lane 9), *S. floricola* 23.6 (lane 10), *S. apis* B31 (lane 11), *S. mirum* SMCA (lane 17) and *S. ixodetis* Y32 (lane 18).

Spiroplasma sp. N525 (I-7) but not with DNAs from strains B13 and B31 of the bee pathogen *Spiroplasma apis* (group IV) and strain 23.6 of the plant surface *Spiroplasma floricola* (group IV). From these data, the PE replicons appear to be restricted to species of group I spiroplasmas. Unlike pSci1–5 from *S. citri* GII3, these replicons do not necessarily contain conserved *scarp* genes. Similarly to *S. citri* Alcanar 254, PE replicons from *S. melliferum* did not hybridize with the *scarp* probe.

The occurrence of PE replicons in many spiroplasma species suggested that these plasmids might have a broad host-range. Therefore we explored the possibility of transforming various strains of *S. citri* as well as other spiroplasma species with pSci21NT and pFL6, two derivatives of pSci2 from *S. citri* GII3. Both plasmids were introduced successfully and replicated in all *S. citri* strains tested (GII3, ASP-1, Alcanar 254, R8A2 and 44), regardless of the strains' natural plasmid contents. Transformation frequencies ranged from 10^{-5} to 10^{-3} transformants c.f.u.⁻¹ ($\mu\text{g plasmid}$)⁻¹. In particular, it is noteworthy that strain R8A2, which could only be transformed at extremely low efficiency (less than 10^{-8} transformants c.f.u.⁻¹ μg^{-1}) by *oriC* plasmids (Renaudin *et al.*, 1995) was repeatedly transformed by pSci21NT and pFL6 at frequencies close to 10^{-5} transformants c.f.u.⁻¹ μg^{-1} . Interestingly, transformation of *S. kunkelii* E275 and *S. phoeniceum* P40 by pSci21NT, pFL6, and its shuttle derivative pBSFL6 also yielded tetracycline-resistant transformants at frequencies of 10^{-5} – 10^{-4} transformants c.f.u.⁻¹ μg^{-1} . The possibility that these tetracycline-resistant cells could be spontaneous mutants or contaminants was excluded by showing that they did contain the relevant plasmid, by PCR and Southern blot hybridization, and by confirming the spiroplasma

species through PCR-RFLP analysis of the spiralin gene (Methods).

pSci-derived plasmids as gene vectors

To demonstrate the usefulness of pSci-derived plasmids as gene vectors, the plasmid construct pNAB32H, in which the *p32* gene fused to a HA tag was inserted into the shuttle plasmid pBSFL6, was introduced into *S. kunkelii* E275 by electrotransformation. The presence of pNAB32H in the spiroplasmal transformants was confirmed by PCR and Southern blot hybridizations (data not shown). In the experiment shown in Fig. 7, total proteins were probed by Western immunoblotting with P32- and HA-specific, polyclonal antibodies (Fig. 7a and b, respectively). Cells from *S. citri* GII3 and *S. kunkelii* E275 transformed by pSci1NT were used as controls. As expected, P32 was detected in *S. citri* GII3 (Fig. 7a, lane 1) but not in untransformed *S. kunkelii* (lane 2) or in *S. kunkelii* transformed with pSci1NT (lane 6). In contrast, P32-related proteins were detected in *S. kunkelii* cells transformed by pNAB32H, carrying the *p32*–HA fusion gene (lanes 3 and 4), or pSci6PT containing the wild-type *p32* gene (lane 5). As expected, the HA-tagged protein was clearly detected in pNAB32H-transformed cells, indicating that the fusion protein was expressed from the recombinant plasmid in *S. kunkelii* E275 (Fig. 7b, lanes 3 and 4). In addition, the relative intensities of the P32 detection signals were consistent with an expression level of the P32–HA fusion protein from pNAB32H similar to that of the wild-type P32 encoded by pSci6 in *S. citri* GII3.

DISCUSSION

Replication protein PE

The occurrence of highly conserved regions in the plasmids from *S. citri* GII3 suggests that they have a common origin. Except for *soj*, *traG*, *trsE* and *mob*, most of the plasmid genes encode hypothetical proteins with no counterparts in bacteria other than spiroplasmas. Moreover, homology searches failed to identify a putative replication protein, indicating that these plasmids probably represent a new plasmid family. In particular, they do not share any homology with the phytoplasma plasmids that encode putative replication proteins (Rep) having the conserved motifs associated with the rolling circle mechanism of replication (Oshima *et al.*, 2001; Tran-Nguyen & Gibb, 2006). *S. citri* plasmids pSciA and pSci1–6 all possess genes *soj* and *pE*, which are also conserved in plasmids pBJS-O from *S. citri* BR3 (Joshi *et al.*, 2005) and pSKU146 from *S. kunkelii* CR2-3X (Davis *et al.*, 2005). In this study, we showed that *pE* is essential and the only plasmid-encoded protein required for plasmid replication. The finding that inactivation of *pE* by a single 4 bp insertion prevented replication clearly indicates that the polypeptide, rather than the nucleotide sequence, is required. The fact that the

Table 2. Distribution of *scarp* and *pE* sequences among various spiroplasma strains

Group	Species	Strains*	Habitat	Probe†	
				<i>scarp</i>	<i>pE</i>
I-1	<i>S. citri</i>	ASP-1	Phloem/leafhopper	—	—
		C189		ND	—
		Corse		+	+
		Alcanar 254		—	+
		GII3		+	+
		Hinckley		+	+
		Israel		+	+
		IP85		+	+
		MHcl2		ND	+
		M4		ND	+
		Palmyre		+	+
		R8A2		—	—
		44		—	—
I-2	<i>S. melliferum</i>	AS576	Honey bee	—	+
		BC3		—	+
		G1		—	+
I-3	<i>S. kunkelii</i>	E275	Phloem/leafhopper	+	+
I-4	<i>Spiroplasma</i> sp.	277F	Rabbit tick	+	+
I-5	<i>Spiroplasma</i> sp.	LB-12	Plant bug	—	+
I-6	<i>S. insolitum</i>	M55	Flower surface	—	—
I-7	<i>Spiroplasma</i> sp.	N525	Plant surface	—	+
I-8	<i>S. phoeniceum</i>	P40	Phloem/leafhopper	+	+
III	<i>S. floricola</i>	23.6	Plant surface	—	—
IV	<i>S. apis</i>	B13	Honey bee	—	—
		B31		—	—
V	<i>S. mirum</i>	SMCA	Rabbit tick	ND	—
VI	<i>S. ixodetis</i>	Y32	Ixodid tick	ND	—
XV	<i>Spiroplasma</i> sp.	I-25	Leafhopper	—	—
XXIII	<i>S. gladiatoris</i>	TG-1	Horse fly	—	—

*The references for spiroplasma strains are given in Supplementary Table S1.

†+ and — indicate positive and negative hybridization under standard stringent conditions. ND, Not done.

Fig. 7. Western immunoblotting of total proteins from *S. citri*, *S. kunkelii* and *S. kunkelii* transformants with P32- (a) and HA- (b) specific polyclonal antibodies. Lanes: 1, *S. citri* GII3; 2–6, *S. kunkelii* E275 wild-type strain (lane 2), transformed with pNAB32H (lanes 3 and 4), pSci6PT (lane 5) and pSci1NT (lane 6).

pE-disrupted plasmids failed to replicate in *S. citri* GII3 (a strain that produces PE from endogenous plasmids) suggests that PE, unlike other replication initiator proteins (Emond *et al.*, 2001) might be a *cis*-acting protein, unable to support replication when provided *in trans*. In this experiment, however, the hypotheses of a retro-control system specific for *S. citri* GII3, or incompatibility between plasmids having identical replication regions, cannot be excluded (Bouet *et al.*, 2007). Therefore, complementation of the mutation carried by the *pE*-disrupted plasmids in *S. citri* 44 will be required to definitely establish whether or not PE is a *cis*-acting protein.

Although the PE protein was found to be essential for replication, it lacks the leucine zipper and helix-turn-helix motifs found in many Rep proteins (Del Solar *et al.*, 1998). The lack of sequence and/or structural similarity between PE and known replication proteins suggests that replication of spiroplasma plasmids pSci involves a distinctive mechanism. Circular bacterial plasmids use two modes of

DNA replication known as rolling circle (RCR) and theta. RCR plasmids are generally recognized by their small size (less than 12 kbp), their high copy number, the generation of single-stranded intermediates, and the presence of specific functional features comprising a *rep* gene, encoding the replication initiator protein, and the replication origin sequences (Khan, 2005). None of these typical features of RCR plasmids apply to pSci plasmids from *S. citri* GII3 (Saillard *et al.*, 2008), suggesting that pSci plasmids replicate through a theta mechanism. The presence of four putative DnaA boxes and an AT-rich region upstream of *pE* is consistent with this hypothesis. Also, the 11 bp tandem repeated sequences separated by 9 bp and located immediately downstream of *pE* might represent iterons, i.e. binding sites for the replication initiator protein. Interaction of the replication protein with its cognate iteron repeat is an important determinant of plasmid incompatibility (Bouet *et al.*, 2007). Obviously more work is needed to determine whether or not these sequences that are strictly conserved in plasmids pSci1–6 and pBJS-O of *S. citri* and pSKU146 of *S. kunkelii* are the binding site of the replication protein PE.

Partitioning protein Soj

In bacteria, active segregation of low-copy-number plasmids into daughter cells relies on plasmid-encoded active partitioning (*par*) systems. In *S. citri*, the fact that pSci1–6 were stably maintained both *in vitro* and *in vivo* indicates that, in spite of highly conserved sequences, they exhibit no incompatibility with each other and hence suggests that each plasmid has its own partitioning system. The finding that, provided they carry a functional *soj* gene, the pSci2-derived plasmids are stably maintained in the absence of selection pressure indicates that *S. citri* plasmids possess an active partition system involving the Soj protein. In general, the *par* locus encodes two *trans*-acting proteins expressed from an operon and one or more *cis*-acting centromere-like sites (Bouet *et al.*, 2007; Ebersbach & Gerdes, 2005). The *par* loci are divided into two main groups depending on the type of ParA ATPase encoded by the first gene of the *parA/B* operon. Type I loci encode Walker box ATPases and type II loci encode actin-like ATPases. The type I systems can be further divided into type Ia and type Ib based on the similarities and sizes of the Par proteins, the localization of the partition site, and the mechanism of the transcriptional regulation (Gerdes *et al.*, 2000). *S. citri* pSci plasmids have *soj* genes predicted to encode ParA superfamily ATPases. These ParA homologues possess the deviant Walker-type ATPase motif of the type I plasmid partitioning system (Koonin, 1993) and the genetic organization of *soj* loci is more reminiscent of type Ib. The pSci-encoded Soj proteins have reduced sizes (255–260 residues) and lack the N-terminal extension containing a DNA-binding domain, usually present in type Ia. Considering that mollicutes are phylogenetically related to Gram-positive bacteria (Weisburg *et al.*, 1989), this is in agreement with the fact that type Ia loci have only been

reported in plasmids from Gram-negative bacteria, whereas loci of type Ib have been described in both Gram-negative and Gram-positive bacteria (Gerdes *et al.*, 2000). The hypothesis that CDS such as *pB* located immediately downstream of *soj* in pSci2 could encode a ParB-like component cannot be excluded as these components frequently exhibit no conserved sequences (Ebersbach & Gerdes, 2005). Phylogenetic analyses revealed that Soj proteins from spiroplasma plasmids clustered within two distinct groups that are clearly unrelated to spiroplasma chromosomal ParA/Soj (see Supplementary Fig. S4). Clustering of Soj from *S. citri* pSciA and pSci6 and *S. kunkelii* pSKU146 with those of the mycoplasma mobile elements is consistent with previous observations that the arrangement of three CDS of pSKU146 paralleled that of their counterparts in the integrated conjugative element (ICE) of *Mycoplasma fermentans* (Calcutt *et al.*, 2002; Davis *et al.*, 2005), and suggests that spiroplasma plasmids and mycoplasma ICEs might have a common ancestor. It is noteworthy that, in contrast to spiroplasmas, most mycoplasmas do not possess *parA/soj* genes (Livny *et al.*, 2007), and that mycoplasmas having *parA/soj*-like sequences all belong to the ‘pneumoniae’ phylogenetic group. However, the absence of *parB* and *parS* equivalents in these mycoplasmas raises the question of whether the mycoplasma ParA function as chromosome partitioning proteins.

Distribution of PE replicons

The PE replicons were found to be widely distributed in group I spiroplasmas, including the three plant-pathogenic spiroplasmas *S. citri*, *S. kunkelii* and *S. phoeniceum*. In *S. citri*, however, strains R8A2 and 44 do not contain plasmids pSciA and pSci1–6 (Berho *et al.*, 2006b). In contrast, ASP-1 is known to carry plasmid pIJ2000 (Archer *et al.*, 1981), highly similar if not identical to pSciA. In this case, the lack of hybridization signal probably resulted from the fact that *pE* sequences are poorly conserved in pSciA (Supplementary Fig. S1). Therefore, in our experiments, the failure to hybridize with the *pE* probe does not exclude the presence of pSciA-related plasmids. In *S. phoeniceum*, both *scarp* and *pE* probes were found to hybridize with total DNA from *S. phoeniceum* P40, indicating the presence of PE replicons in this strain. For unknown reasons, these data do not fit with those of Joshi *et al.* (2005) showing that undigested plasmid preparations from *S. phoeniceum* did not hybridize with the whole pBJS-O probe.

Expanding the genetic toolbox of spiroplasmas

Limited cloning tools are available for the genetic modification of *S. citri*. Artificial *oriC* plasmids have been used as vectors to complement mutants (Gaurivaud *et al.*, 2000; Jacob *et al.*, 1997). However, due to incompatibility with the chromosomal *oriC*, the spiroplasma transformants have a reduced growth rate and the plasmids are unstable, as they tend to integrate into the host chromosome. In addition, transformation frequencies of

S. citri by *oriC* plasmids are relatively low, and some *S. citri* strains cannot be transformed consistently (Renaudin, 2002; Renaudin & Lartigue, 2005). Characterizing the replication and stability regions of pSci2 proved the *S. citri* plasmids and their shuttle derivatives to hold considerable advantages for the development of new vectors: (i) they transform the various *S. citri* strains tested at relatively high frequencies (10^{-4} – 10^{-3} transformants c.f.u. $^{-1}$ μg^{-1}), (ii) the doubling times of the transformants are not significantly affected as compared with the wild-type, (iii) according to the demand they are either stably maintained or not, depending on the presence of *soj*, and never integrate into the chromosome, and (iv) their host range is not restricted to *S. citri*. Indeed, they were shown to replicate in *S. phoeniceum* P40 and *S. kunkelii* E275, in which expression of the foreign protein P32-HA was demonstrated. To our knowledge this is the first report of transformation and expression of foreign genes in plant-pathogenic mollicutes other than *S. citri*.

In pathogenic bacteria, plasmids are usually considered as being non-essential genetic elements that confer a selective advantage under specific conditions (Stewart *et al.*, 2005; Vivian *et al.*, 2001). Whether plasmids are required for *S. citri* survival in nature is unknown. Nevertheless, plasmid-encoded determinants have been tentatively associated with insect transmission (Berg *et al.*, 2001; Berho *et al.*, 2006a; Killiny *et al.*, 2006). In *S. citri* GII3, ~10 copies of each plasmid co-exist within the same cell. Transformation of *S. citri* 44 by extrachromosomal DNA from *S. citri* GII3 also revealed that many transformants contained more than one plasmid (Berho *et al.*, 2006a). Therefore, the compatibility of the *S. citri* plasmids would permit the design of experiments in which two distinct plasmids must be stably maintained in the same cell. Conversely, from the preliminary observation that transformation of *S. citri* GII3 by selectable pSci2 derivatives led to rapid loss of the wild-type pSci2, incompatibility of identical replication regions would provide a means to displace natural plasmids by their mutated/deleted derivatives and hence to investigate the biological function of plasmid-encoded determinants.

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REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

André, A., Maccheroni, W., Doignon, F., Garnier, M. & Renaudin, J. (2003). Glucose and trehalose PTS permeases of *Spiroplasma citri* probably share a single IIA domain, enabling the spiroplasma to adapt quickly to carbohydrate changes in its environment. *Microbiology* **149**, 2687–2696.

André, A., Maucourt, M., Moing, A., Rolin, D. & Renaudin, J. (2005). Sugar import and phytopathogenicity of *Spiroplasma citri*: glucose and fructose play distinct roles. *Mol Plant Microbe Interact* **18**, 33–42.

Archer, D. B., Best, J. & Barber, C. (1981). Isolation and restriction mapping of a spiroplasma plasmid. *J Gen Microbiol* **126**, 511–514.

Barré, A., de Daruvar, A. & Blanchard, A. (2004). MolliGen, a database dedicated to the comparative genomics of Mollicutes. *Nucleic Acids Res* **32**, D307–D310.

Berg, M., Melcher, U. & Fletcher, J. (2001). Characterization of *Spiroplasma citri* adhesion related protein SARP1, which contains a domain of a novel family designated sarpin. *Gene* **275**, 57–64.

Berho, N., Duret, S., Danet, J. L. & Renaudin, J. (2006a). Plasmid pSci6 from *Spiroplasma citri* GII-3 confers insect transmissibility to the non-transmissible *S. citri* strain 44. *Microbiology* **152**, 2703–2716.

Berho, N., Duret, S. & Renaudin, J. (2006b). Absence of plasmids encoding adhesion-related proteins in non-insect-transmissible strains of *Spiroplasma citri*. *Microbiology* **152**, 873–886.

Bouet, J. Y., Nordstrom, K. & Lane, D. (2007). Plasmid partition and incompatibility – the focus shifts. *Mol Microbiol* **65**, 1405–1414.

Bové, J. M., Carle, P., Garnier, M., Laigret, F., Renaudin, J. & Saillard, C. (1989). Molecular and cellular biology of spiroplasmas. In *The Mycoplasma*, vol. 5, pp. 243–364. Edited by R. F. Whitcomb & J. G. Tully. New York: Academic Press.

Bové, J. M., Renaudin, J., Saillard, C., Foissac, X. & Garnier, M. (2003). *Spiroplasma citri*, a plant pathogenic mollicute: relationships with its two hosts, the plant and the leafhopper vector. *Annu Rev Phytopathol* **41**, 483–500.

Calcutt, M. J., Lewis, M. S. & Wise, K. S. (2002). Molecular and genetic analysis of IECF, an integrative conjugative element that is present in the chromosome of *Mycoplasma fermentans* PG18. *J Bacteriol* **184**, 6929–6941.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* **17**, 540–552.

Davis, R. E., Dally, E. L., Jomantiene, R., Zhao, Y., Roe, B., Lin, S. & Shao, J. (2005). Cryptic plasmid pSKU146 from the wall-less plant pathogen *Spiroplasma kunkelii* encodes an adhesion and components of a type IV translocation-related conjugation system. *Plasmid* **53**, 179–190.

Del Solar, G., Giraldo, R., Ruiz-Echevarria, M. J., Espinoza, M. & Diaz-Orejas, R. (1998). Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev* **62**, 434–464.

Duret, S., Berho, N., Danet, J. L., Garnier, M. & Renaudin, J. (2003). Spiraline is not essential for helicity, motility, or pathogenicity but is required for efficient transmission of *Spiroplasma citri* by its leafhopper vector *Circulifer haematocaps*. *Appl Environ Microbiol* **69**, 6225–6234.

Ebersbach, G. & Gerdes, K. (2005). Plasmid segregation mechanisms. *Annu Rev Genet* **39**, 453–479.

Emond, E., Lavallée, R., Drolet, G., Moineau, S. & Lapointe, G. (2001). Molecular characterization of a theta replication plasmid and its use for development of a two-component food-grade cloning system for *Lactococcus lactis*. *Appl Environ Microbiol* **67**, 1700–1709.

Fletcher, J., Schultz, G. A., Davis, R. E., Eastman, C. E. & Goodman, R. M. (1981). Brittle root disease of horseradish: evidence for an etiological role of *Spiroplasma citri*. *Phytopathology* **71**, 1073–1080.

Foissac, X., Danet, J. L., Saillard, C., Whitcomb, R. F. & Bové, J. M. (1996). Experimental infections of plants by spiroplasmas. In

- Molecular and Diagnostic Procedures in Mycoplasma*, vol. 2, pp. 385–389. Edited by S. Razin & J. G. Tully. New York: Academic Press.
- Foissac, X., Saillard, C. & Bové, J. M. (1997). Random insertion of transposon Tn4001 in the genome of *Spiroplasma citri* strain GII3. *Plasmid* 37, 80–86.
- Gasparich, G. E., Hackett, K. J., Clark, E. A., Renaudin, J. & Whitcomb, R. F. (1993). Occurrence of extrachromosomal deoxyribonucleic acids in spiroplasmas associated with plants, insects, and ticks. *Plasmid* 29, 81–93.
- Gaurivaud, P., Danet, J. L., Laigret, F., Garnier, M. & Bové, J. M. (2000). Fructose utilization and phytopathogenicity of *Spiroplasma citri*. *Mol Plant Microbe Interact* 13, 1145–1155.
- Gerdes, K., Moller-Jensen, J. & Jensen, R. B. (2000). Plasmid and chromosome partitioning: surprises from phylogeny. *Mol Microbiol* 37, 455–466.
- Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52, 696–704.
- Hosseini Pour, A. (2000). *Determination of some molecular and cellular characteristics of Spiroplasma citri, the causal agent of citrus stubborn disease in Kerman, Fars and Mazadran provinces*. PhD thesis, Tarbiat Modares University, Tehran.
- Jacob, C., Nouzieres, F., Duret, S., Bové, J. M. & Renaudin, J. (1997). Isolation, characterization, and complementation of a motility mutant of *Spiroplasma citri*. *J Bacteriol* 179, 4802–4810.
- Joshi, B. D., Berg, M., Rogers, J., Fletcher, J. & Melcher, U. (2005). Sequence comparisons of plasmids pBJS-O of *Spiroplasma citri* and pSKU146 of *S. kunkelii*: implications for plasmid evolution. *BMC Genomics* 6, 175–185.
- Khan, S. A. (2005). Plasmid rolling-circle replication: highlights of two decades of research. *Plasmid* 53, 126–136.
- Killiny, N., Castroviejo, M. & Saillard, C. (2005). *Spiroplasma citri* spiralin acts in vitro as a lectin binding to glycoproteins from its insect vector *Circulifer haematocaps*. *Phytopathology* 95, 541–548.
- Killiny, N., Batailler, B., Foissac, X. & Saillard, C. (2006). Identification of a *Spiroplasma citri* hydrophilic protein associated with insect transmissibility. *Microbiology* 152, 1221–1230.
- Koonin, E. V. (1993). A superfamily of ATPases with diverse functions containing either classical or deviant ATP-binding motif. *J Mol Biol* 229, 1165–1174.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 5, 150–163.
- Lartigue, C., Duret, S., Garnier, M. & Renaudin, J. (2002). New plasmid vectors for specific gene targeting in *Spiroplasma citri*. *Plasmid* 48, 149–159.
- Livny, J., Yamaichi, Y. & Waldor, M. K. (2007). Distribution of centromere-like *parS* sites in bacteria: insights from comparative genomics. *J Bacteriol* 189, 8693–8703.
- Marchler-Bauer, A. & Bryant, S. H. (2004). CD-Search: protein domain annotations on the fly. *Nucleic Acids Res* 32, W327–W331.
- Marenda, M., Barbe, V., Gourgues, G., Manganot, S., Sagne, E. & Citti, C. (2006). A new integrative conjugative element occurs in *Mycoplasma agalactiae* as chromosomal and free circular forms. *J Bacteriol* 188, 4137–4141.
- McLeod, M. P., Warren, R. L., Hsiao, W. W., Araki, N., Myhre, M., Fernandes, C., Miyazawa, D., Wong, W., Lillquist, A. L. & other authors (2006). The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. *Proc Natl Acad Sci U S A* 103, 15582–15587.
- Mouchès, C., Barroso, G. & Bové, J. M. (1983a). Characterization and molecular cloning in *Escherichia coli* of a plasmid from the mollicute *Spiroplasma citri*. *J Bacteriol* 156, 952–955.
- Needleman, S. B. & Wunsch, C. D. (1970). A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J Mol Biol* 48, 443–453.
- Notredame, C., Higgins, D. G. & Heringa, J. (2000). T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J Mol Biol* 302, 205–217.
- Oshima, K., Kakizawa, S., Nishigawa, H., Kuboyama, T., Miyata, S., Ugaki, M. & Namba, S. (2001). A plasmid of phytoplasma encodes a unique replication protein having both plasmid- and virus-like domains: clue to viral ancestry or result of virus/plasmid recombination? *Virology* 285, 270–277.
- Ranhand, J. M., Mitchell, W. O., Popkin, T. J. & Cole, R. M. (1980). Covalently closed circular deoxyribonucleic acids in spiroplasmas. *J Bacteriol* 143, 1194–1199.
- Renaudin, J. (2002). Extrachromosomal elements and gene transfer. In *Molecular Biology and Pathogenicity of Mycoplasmas*, pp. 347–370. Edited by S. Razin & R. Herrmann. New York: Kluwer Academic/Plenum.
- Renaudin, J. & Lartigue, C. (2005). *OriC* plasmids as gene vectors for mollicutes. In *Mycoplasmas: Pathogenesis, Molecular Biology, and Emerging Strategies for Control*, pp. 3–30. Edited by A. Blanchard & G. Browning. Norwich, UK: Horizon Scientific Press.
- Renaudin, J., Marais, A., Verdin, E., Duret, S., Foissac, X., Laigret, F. & Bové, J. M. (1995). Integrative and free *Spiroplasma citri* *oriC* plasmids: expression of the *Spiroplasma phoeniceum* spiralin in *Spiroplasma citri*. *J Bacteriol* 177, 2870–2877.
- Saglio, P., Lafèche, D., Bonisol, C. & Bové, J. M. (1971). Culture *in vitro* des mycoplasmes associés au stubborn des agrumes et leur observation au microscope électronique. *C R Acad Sci Paris* 272, 1387–1390.
- Saglio, P., Lhospital, M., Lafèche, D., Dupont, G., Bové, J. M., Tully, J. G. & Freundt, E. A. (1973). *Spiroplasma citri* gen. and sp. nov.: a mycoplasma-like organism associated with stubborn disease of citrus. *Int J Syst Bacteriol* 23, 191–204.
- Saillard, C., Vignault, J. C., Bové, J. M., Raie, A., Tully, J. G., Williamson, D. L., Fos, A., Garnier, M., Gadeau, A., Carle, P. & Whitcomb, R. F. (1987). *Spiroplasma phoeniceum* sp. nov., a new plant pathogenic species from Syria. *Int J Syst Bacteriol* 37, 106–115.
- Saillard, C., Carle, P., Duret-Nurbel, S., Henri, R., Killiny, N., Carrère, S., Gouzy, J., Bové, J. M., Renaudin, J. & Foissac, X. (2008). The abundant extrachromosomal content of *Spiroplasma citri* strain GII3–3X. *BMC Genomics* 9, 195–207.
- Salvado, J. C., Barroso, G. & Labarere, J. (1989). Involvement of a *Spiroplasma citri* plasmid in the erythromycin-resistance transfer. *Plasmid* 22, 151–159.
- Sirand-Pugnet, P., Lartigue, C., Marenda, M., Jacob, D., Barré, A., Barbe, V., Schenowitz, C., Manganot, S., Couloux, A. & other authors (2007). Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. *PLoS Genet* 3, e75.
- Stamburski, C., Renaudin, J. & Bové, J. M. (1991). First step toward a virus-derived vector for gene cloning and expression in spiroplasmas, organisms which read UGA as a tryptophan codon – synthesis of chloramphenicol acetyltransferase in *Spiroplasma citri*. *J Bacteriol* 173, 2225–2230.
- Stewart, P. E., Byram, R., Grimm, D., Tilly, K. & Rosa, P. A. (2005). The plasmids of *Borrelia burgdorferi*: essential genetic elements of a pathogen. *Plasmid* 53, 1–13.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22, 4673–4680.

Tran-Nguyen, L. T. & Gibb, K. S. (2006). Extrachromosomal DNA isolated from tomato big bud and *Candidatus* Phytoplasma australiense phytoplasma strains. *Plasmid* **56**, 153–166.

Vignault, J. C., Bové, J. M., Saillard, C., Vogel, R., Faro, A., Venegas, L., Stemmer, W., Aoki, S., McCoy, R. E. & other authors (1980). Mise en culture de spiroplasmes à partir de matériel végétal et d'insectes provenant de pays circum méditerranéens et du Proche Orient. *C R Acad Sci Paris* **290**, 775–780.

Vivian, A., Murillo, J. & Jackson, R. W. (2001). The roles of plasmids in phytopathogenic bacteria: mobile arsenals? *Microbiology* **147**, 763–780.

Weisburg, W. G., Tully, J. G., Rose, D. L., Petzel, J. P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T. G. & other authors (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* **171**, 6455–6467.

Whitcomb, R. F. (1983). Culture media for spiroplasma. In *Methods in Mycoplasmaology*, vol. I, pp. 147–158. Edited by S. Razin & J. G. Tully. New York: Academic Press.

Whitcomb, R. F., Chen, T. A., Williamson, D. L., Liao, C., Tully, J. G., Bové, J. M., Mouchès, C., Rose, D. L., Coan, M. E. & Clark, T. B. (1986). *Spiroplasma kunkelii* sp. nov.: characterization of the etiologic agent of corn stunt disease. *Int J Syst Bacteriol* **36**, 170–178.

Williamson, D. L., Whitcomb, R. F., Tully, J. G., Gasparich, G. E., Rose, D. L., Carle, P., Bové, J. M., Hackett, K. J., Adams, J. R. & other authors (1998). Revised group classification of the genus *Spiroplasma*. *Int J Syst Bacteriol* **48**, 1–12.

Ye, F., Renaudin, J., Bove, J. M. & Laigret, F. (1994). Cloning and sequencing of the replication origin (*oriC*) of the *Spiroplasma citri* chromosome and construction of autonomously replicating artificial plasmids. *Curr Microbiol* **29**, 23–29.

Yu, J., Wayadande, A. C. & Fletcher, J. (2000). *Spiroplasma citri* surface protein P89 implicated in adhesion to cells of the vector *Circulifer tenellus*. *Phytopathology* **90**, 716–722.

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