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I-*Sce*I-mediated plasmid deletion and intra-molecular recombination in *Spiroplasma citri*

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ABSTRACT

S. citri wild-type strain GII3 carries six plasmids (pSci1 to -6) that are thought to encode determinants involved in the transmission of the spiroplasma by its leafhopper vector. In this study we report the use of meganuclease I-*Sce*I for plasmid deletion in *S. citri*. Plasmids pSci1NT-I and pSci6PT-I, pSci1 and pSci6 derivatives that contain the *tetM* selection marker and a unique I-*Sce*I recognition site were first introduced into *S. citri* strains 44 (having no plasmid) and GII3 (carrying pSci1–6), respectively. Due to incompatibility of homologous replication regions, propagation of the *S. citri* GII3 transformant in selective medium resulted in the replacement of the natural pSci6 by pSci6PT-I. The spiroplasmal transformants were further transformed by an *oriC* plasmid carrying the I-*Sce*I gene under the control of the spiralin gene promoter. In the *S. citri* 44 transformant, expression of I-*Sce*I resulted in rapid loss of pSciNT-I showing that expression of I-*Sce*I can be used as a counter-selection tool in spiroplasmas. In the case of the *S. citri* GII3 transformant carrying pSci6PT-I, expression of I-*Sce*I resulted in the deletion of plasmid fragments comprising the I-*Sce*I site and the *tetM* marker. Delineating the I-*Sce*I generated deletions proved they had occurred though recombination between homologous sequences. To our knowledge this is the first report of I-*Sce*I mediated intra-molecular recombination in mollicutes.

1. Introduction

Spiroplasma citri is the etiological agent of citrus stubborn disease (Saglio et al., 1973). The spiroplasmas inhabit the phloem sieve tubes and are transmitted by phloem sap-feeding leafhopper vectors in a persistent and propagative manner (Liu et al., 1983; Fos et al., 1986). Many *S. citri* strains have been shown to carry plasmids (Ranhand et al., 1980; Archer et al., 1981; Mouchès et al., 1983; Gasparich et al., 1993). For years, spiroplasma plasmids were designated as cryptic, since no phenotypic trait had been associated with their presence until recently. Sequencing of pBJS-O from *S. citri* BR3 (Joshi et al., 2005), and pSciA and pSci1 to -6 from *S. citri* GII3 (Saillard et al., 2008) revealed that they encode proteins, which have been tentatively associated with insect transmission (Yu et al., 2000; Berho et al., 2006b; Killiny et al., 2006). In addition, the recent finding that transfer of pSci6 from *S. citri* GII3 conferred insect transmissibility to the non-transmissible strain *S. citri* 44 (Berho et al., 2006a) strongly suggested that genetic determinants required for insect transmission were encoded by pSci6. In this respect, plasmid curing of *S. citri* GII3 would be of primary importance to assess the role of plasmid-encoded genes in a given chromosomal background. However, in *S. citri* GII3, conventional curing methods such as growth at sub lethal temperature and/or in the presence of curing agent such as acridine orange, acriflavine and novobiocin, were inefficient in that only spiroplasma strains lacking one, two or three plasmids out of 7 were obtained (Berho et al., 2006b). Furthermore, these procedures are stressful to the host and suffer the problem that part of the bacterial stress response is to increase the mutation rate, altering the host phenotype irrespective of whether the plasmid has been displaced.

An alternative approach for curing a host cell is to use plasmid incompatibility to displace the resident plasmid by a selectable derivative that can be further counter-selected. We recently reported plasmid replacement based on incompatibility in *S. citri* (Breton et al., 2010a). In these studies, transformation of *S. citri* GII3 by a selectable plasmid derivative resulted in the nearly specific replacement of the related natural plasmid. However, subsequent loss of the introduced plasmid required extensive propagation of the spiroplasmal transformant in the absence of selection. In addition, in some cases, plasmid curing through incompatibility may not be straightforward due to plasmid complexity such as the presence of multiple copies of the replication region in pSci6 (Breton et al., 2008; Saillard et al., 2008) and the lack of counter-selection method in this organism.

Here we report the use of the homing endonuclease I-*Sce*I as a counter-selection tool as well as the ability of the meganuclease to stimulate intra-molecular recombination in *S. citri* plasmids.

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2. Materials and methods

2.1. Bacterial strains, transformation, and growth conditions

S. citri GII3 wild-type strain was originally isolated from *Circulifer haematoceps* leafhoppers captured in Morocco (Vignault et al., 1980). Through injection into the leafhopper vector, *S. citri* GII3 can be experimentally transmitted to periwinkle (*Catharanthus roseus*) plants, in which it multiplies and induces severe symptoms (Foissac et al., 1997). *S. citri* 44 was isolated from stubborn-diseased sweet orange in Iran (Hosseini Pour, 2000). In contrast to GII3, *S. citri* 44 is described as a non-insect-transmissible strain, as attempts to infect periwinkle plants through injection of spiroplasmas into the leafhopper repeatedly failed.

Spiroplasmas were grown at 32 °C in SP4 medium (Whitcomb, 1983), from which fresh yeast extract was omitted. Spiroplasma cells were electrotransformed as previously described (Stamburski et al., 1991), using 1–2 μg of purified plasmid. Spiroplasmal transformants were selected in the presence of 100 μg/ml gentamicin or 2 μg/ml tetracycline.

2.2. DNA isolation, Southern blot hybridization, and western immunoblotting

Spiroplasma genomic DNA was prepared from 2 ml cultures using the Wizard genomic DNA purification kit (Promega), and plasmids were 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′-phenylanilide phosphate (HNPP) as the substrate, following the supplier's instructions (Roche Diagnostics). Fluorescent signals were detected using a Fluor-S Multimager Phosphoimager (Bio-Rad). Probes S235, P32, and TetM, respectively specific to the *scarp*, *p32*, and *tetM* genes have been described elsewhere (Berho et al., 2006a). Probe GmR specific to the gentamicin resistance gene *aac-aphD* was obtained by PCR amplification of pBOG with primer pair GR1/MG3 (Table 1). Spiroplasmal proteins were separated by SDS-PAGE and further analyzed by immunoblotting as described previously (Breton et al., 2008).

2.3. Plasmid constructions

S. citri GII3 contains 7 plasmids, pSciA and pSci1 to -6, most of which can be linearized by *Nsi*I (pSci1, 3, 4, and 5) or *Pst*I (pSci6) (Saillard et al., 2008). Plasmids pSci1NT and pSci6PT have been described elsewhere (Berho et al., 2006a). Incorporation of a I-*Sce*I restriction site into the pSci plasmids was carried out as follows: A phosphorylated oligonuleotide duplex containing the I-*Sce*I recognition site (ISceIF: 5′-*GATC*GTAGGGATAACAGGGTAAT-3′; ISceIR: 5′-*GATC*ATTACCCTGTTATCCCTAC-3′) was first inserted into the *Bgl*II site immediately downstream of the *tetM* gene of pSRT2 (Lartigue et al., 2002), yielding pSRT-I. Then, the 2.3 kbp tetracycline-resistance cassette containing the I-*Sce*I site was rescued from pSRT-I by digestion with *Pst*I and ligated to *Nsi*I or *Pst*I-digested extrachromosomal DNA from *S. citri* GII3 before the ligation mixtures were used to transform *S. citri* 44, a strain that has no plasmid. Tetracycline resistant transformants were screened and their plasmid contents characterized by Southern blot hybridization. One *S. citri* 44 transformant carrying the recombinant plasmid pSci1NT-I (pSci1 containing the *tetM* cassette and the I-*Sce*I site) was selected for further experiments. Similarly, pSci6PT-I was obtained by inserting the tetracycline resistance cassette of pSRT-I into the unique *Pst*I site of pSci6. *S. citri* strains 44 transformed by pSci1NT-I and GII3 transformed by pSci6PT and pSci6PT-I were named 44/1-I, GII3/6, and GII3/6-I, respectively.

Table 1
Primers used in this study.

Name[a]	Nucleotide sequence (5′–3′)[b]	Position	Accession number
1F	AAATCGGAAAAGTTAGCAGA	831–850	AJ969074
2R	CTAACAATGCCGAAACCGAC	1031–1050	AJ969074
3F	TGTTGAGGGTGGTATTCTTGC	4728–4748	AJ969074
4R	AGTTGCTAATTCTTCGGCACTC	5776–5797	AJ969074
5F	TGCCAAAAATGTAACAACCA	6037–6056	AJ969074
6F/R	CGCCAGGAATGAAAACGTAACT	7459–7480	AJ969074
7F/R	TGCTCCAGCAATACCTGAACT	8903–8923	AJ969074
8R	CCAGCAATGTTGGTTAGTGTGT	10000–10021	AJ969074
9F	TGTTGAGGGTGGTATTCTTGC	10545–10565	AJ969074
10R	AGTTGCTAATTCTTCGGCACTC	11593–11614	AJ969074
11F	TGCCCAAAATGTAACAACCA	11854–11873	AJ969074
12R	TGAAAATAAATTGCGTTGAA	6375–6394	AJ969074
13F	CGAAATCTGAAGCAACAAGG	14669–14688	AJ969074
14R	AGGGTTTTAAGTAGGGGGGTAT	15429–15440	AJ969074
15F	AGAAGCATTGGAACTCGAACA	807–827	X56353
16R	TCCGTCACATTCCAACCATA	1690–1709	X56353
17F	TTAACACTAGTACAACCACC	15677–15696	AJ969074
18R	TTTCTGCAAGATCGTTGCTA	16756–16775	AJ969074
19F	CCACACCTATAAATTAGATGTTGTACAA	16920–16947	AJ969074
20R	TATGAGGTTATGTTTTGCTCTAAACTTG	18224–18251	AJ969074
21F	GTTGACTCAATCAGATAAACAAG	18570–18592	AJ969074
22R	GCTCATCAAAAGCGTTGACAG	19264–19284	AJ969074
23F	GGTCAACTTTTGATAATG	21664–21681	AJ969074
24R	CTTCCGATAAAAGACC	22317–22332	AJ969074
25F	TAGTTCCGGCTTGCTCACCA	24332–24351	AJ969074
26R	GTAGTCAAACCGATATCGAGC	24962–24982	AJ969074
27F/R	CGAATTAAATAAAGCTTCTGAAC	28429–28451	AJ969074
28R	CATAAAAATgGatCCTAAATTAATGC	29655–29680	AJ969074
ISceI–BglII–F	TAGAG**AgAtcT**GAAATGCATCAAAAAAACC	499–528	AF170481
ISceI–BamHI–R	ACACA**GGAtcC**AGCTATGACCATGATTAC	1244–1272	AF170481
GR1	AAGATATAGTTGAAGAATATTATCC	3046–3070	M18086
MG3	AAGCTTGCGCATCATTGGATG	3521–3541	M18086
P32F	TAACGAATTAAATCATTCTAATAGC	24852–24876	AJ969074
P32R	TAGTTCCGGCTTGCTCACCA	24332–24351	AJ969074
PEF1	CCCACGgaATTCTTCTATACACCTATTAAG	11022–11051	AJ969070
PER1	AGTATTggATcCATTGTCTTGCTACGCTGT	12046–12075	AJ969070
PstIF	TTAAGTTTAATTGGAGCAGCTA	15533–15554	AJ969074
PstIR	CCACTAGAACCGAGCGAAAC	15974–15993	AJ969074
S235F	TAAACATTGATATTGCCAACCCC	3617–3639	AJ969069
S235R	GGTTAAAGTTGCAGAATTATTATC	3973–3996	AJ969069
Tet1	CTGCAAAAGATGGCGTAC	521–538	X56353
Tet2	CGTAAATGTAGTACTCCAC	1037–1055	X56353

[a] For oligonucleotides indicated nF/R, both forward and reverse complement sequences have been used as primers.
[b] Bold letters indicate restriction enzyme sites. Lower-case letters indicate mismatched nucleotides.

To construct pSRIS, the I-*Sce*I gene was PCR-amplified from pUC19RP12 (kindly provided by Dr G. Posfai) using primers ISceI-BglII-F and ISceI-BamHI-R (Table 1), digested by *Bam*HI + *Bgl*II, and inserted into the *Bgl*II site, downstream of the spiralin gene promoter of pSR2 (Lartigue et al., 2002). For expressing the I-*Sce*I endonuclease in *S. citri*, the 1044-kbp *Pst*I fragment containing the I-*Sce*I gene under the control of the spiralin gene promoter was rescued from pSRIS and inserted into the *Pst*I site of the *S. citri oriC* plasmid pBOG (Renaudin, 2002). The resulting plasmid was named pBIS.

2.4. Experimental transmission assay

Injection of *S. citri* cultures into *C. haematoceps* leafhoppers and transmission to periwinkle plants were adapted from a previously described method (Foissac et al., 1996) as further outlined in Breton et al. (2010a). The leafhoppers were micro-injected with approximately 10^5 spiroplasma cells and after a 2-week latency period on stock (*Matthiola incana*) plants the infected insects were transferred to young periwinkle (*Catharanthus roseus*) plants (5 insects per plant, 5 to 10 plants per spiroplasma strain) for a 2-week transmission