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Specific gene targeting in *Spiroplasma citri*: improved vectors and production of unmarked mutations using site-specific recombination

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In *Spiroplasma citri*, where homologous recombination is inefficient, specific gene targeting could only be achieved by using replicative, *oriC* plasmids. To improve the probability of selecting rare recombination events without fastidious, extensive passaging of the transformants, a new targeting vector was constructed, which was used to inactivate the *crr* gene encoding the IIA component of the glucose phosphotransferase system (PTS) permease. Selection of recombinants was based on a two-step strategy using two distinct selection markers, one of which could only be expressed once recombination had occurred through one single crossover at the target gene. According to this strategy, spiroplasmal transformants were screened and multiplied in the presence of gentamicin before the *crr* recombinants were selected for their resistance to tetracycline. In contrast to the wild-type strain GII-3, the *crr*-disrupted mutant GII3-gt1 used neither glucose nor trehalose, indicating that in *S. citri* the glucose and trehalose PTS permeases function with a single IIA component. In addition, the feasibility of using the transposon $\gamma\delta$ TnpR/res recombination system to produce unmarked mutations in *S. citri* was demonstrated. In an arginine deiminase (*arcA*-disrupted) mutant, the *tetM* gene flanked by the *res* sequences was efficiently excised from the chromosome through expression of the TnpR resolvase from a replicative *oriC* plasmid. Due to *oriC* incompatibility, plasmid loss occurred spontaneously when selection pressure was removed. This approach will be helpful for constructing unmarked mutations and generating multiple mutants with the same selection marker in *S. citri*. It should also be relevant to other species of mollicutes.

INTRODUCTION

Spiroplasmas and phytoplasmas are plant-pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less micro-organisms phylogenetically related to low G + C content, Gram-positive bacteria (Weisburg *et al.*, 1989). Plant-pathogenic mollicutes are associated with many diseases affecting economically important crops such as ornamentals, vegetables, fruit trees and grapevine (Lee *et al.*, 2000; Seemüller *et al.*, 2002). Both phytoplasmas and spiroplasmas are restricted to the phloem sieve tubes of affected plants and are transmitted by phloem sap-feeding, leafhopper or psyllid vectors, in which they also multiply. Spiroplasmas are, in contrast to phytoplasmas, cultivable *in vitro*. *Spiroplasma citri*, the causal agent of citrus stubborn disease, has been cultured since 1970 (Saglio *et al.*, 1971, 1973). Over the years it has become a model organism for studying the relationships of plant mollicutes with their hosts, the plant and the insect vector (Bové *et al.*, 2003; Fletcher *et al.*, 1998). The ongoing determination of spiroplasma genome sequences (http://www.genome.ou.edu/spiro.html) promises to increase our knowledge of the spiroplasmal biology. However, an efficient methodology to generate defined mutations is essential for studying gene function. In *S. citri*, mutagenesis has been achieved in a random or targeted manner (Duret *et al.*, 1999; Foissac *et al.*, 1997; Gaurivaud *et al.*, 2000b; Jacob *et al.*, 1997; Renaudin, 2002), leading to the identification of genes involved in insect transmission and pathogenicity (André *et al.*, 2005; Boutareaud *et al.*, 2004; Duret *et al.*, 2003; Foissac *et al.*, 1997; Gaurivaud *et al.*, 2000a). Although double-crossover allelic exchange is easy to perform with many bacteria, it remains very difficult with others and in particular with mollicutes (Dybvig & Volker, 1996; Razin *et al.*, 1998; Renaudin, 2002). A 'suicide' vector approach using recombinant plasmids unable to

Abbreviations: ADI, arginine deiminase; CK, carbamate kinase; CDS, coding sequences; HNPP, 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate; OTC, ornithine carbamoyltransferase; PPLO, pleuropneumonia-like organisms; PTS, phosphotransferase system.

replicate has been successfully used in *Acholeplasma laidlawii* (Dybvig & Woodward, 1992), *Mycoplasma genitalium* (Dhandayuthapani *et al.*, 1999) and *Mycoplasma gallisepticum* (Markham *et al.*, 2003). In *S. citri*, where homologous recombination is inefficient, specific gene targeting could only be achieved by using replicative, *oriC* plasmids as disruption vectors (Duret *et al.*, 1999; Gaurivaud *et al.*, 2000b; Renaudin, 2002). However, plasmid recombination required extensive passaging of the transformants, and in most cases recombination occurred at *oriC* rather than at the target gene. To enhance the probability of recombination at the target gene, *oriC* vectors in which the *oriC* fragment was reduced to the minimal sequences required for replication were constructed (Lartigue *et al.*, 2002). These plasmids have been successfully used to inactivate genes *spi* and *ptsG*, encoding spiralin, the major membrane protein and the IICB component of the glucose phosphotransferase system (PTS) permease, respectively (Lartigue *et al.*, 2002; Duret *et al.*, 2003; André *et al.*, 2005). However, due to low recombination frequencies, a minimal length of homologous sequences was required for selecting recombination events. For example, attempts to inactivate the *crr* gene (encoding the glucose PTS permease IIA component) by recombination within a 220 bp fragment were unsuccessful (A. André & J. Renaudin, unpublished data).

This report describes a new gene targeting vector allowing selection of rare recombination events. The two-step strategy involves two distinct selection markers. First transformants are screened for their resistance to gentamicin, and then site-specific recombinants are selected for their resistance to tetracycline, which can only be expressed through recombination at the target gene. The usefulness of this strategy was established by the construction of a *crr*-disrupted mutant, the characterization of which demonstrated that glucose and trehalose PTS permeases of *S. citri* share a single IIA component. Further functional genetic studies will certainly require complementation and multiple mutation experiments. In *S. citri*, however, such experiments are limited by the very small number of suitable selectable markers. In fact, only genes *aacA-aphD* of Tn*4001* and *tetM* of Tn*916* conferring resistance to gentamicin and tetracycline are currently used. *S. citri* is also sensitive to chloramphenicol but o*riC* plasmids carrying the chloramphenicol acetyltransferase gene of Tn*9* transform spiroplasma cells at low frequencies (Renaudin, 2002). To avoid this limitation, the production of unmarked mutants was investigated. An unmarked, *arcA*-disrupted mutant was produced by using the transposon $\gamma\delta$ TnpR/res recombination system (Reed, 1981). In this system, the resolvase TnpR mediates the efficient resolution of the cointegrate intermediate generated during transposition by binding-specific recombination sites (res) (Grindley, 2002). In our study, the efficient elimination of the *tetM* resistance gene flanked with the *res* sequences was obtained through expression of the resolvase from an *oriC* plasmid.

METHODS

Bacterial strains, transformation and growth conditions. *Escherichia coli* TG1 (*supE hsd*Δ5 *thi* Δ(*lac–proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] or DH10B [F$^-$ *mcr*A Δ(*mrr–hsdRMS–mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara–leu*)*7697 galK rpsL*(Str^R) *endA1 nupG*] were used as the host strains for cloning experiments and plasmid propagation. *E. coli* C600 [F$^-$ e14$^-$ (*mcrA*$^-$) *supE44 thi-1 thr-1 leuB6 lacY1 tonA21*] was used for construction and propagation of plasmids carrying *res* sequences. *E. coli* TG1 competent cells were transformed by heat shock at 42 °C, whereas C600 and DH10B were transformed by electroporation. Transformants were selected by plating on LB agar medium containing 50 μg ampicillin or chloramphenicol ml^{-1}. *S. citri* GII-3 wild-type strain was originally isolated from its leafhopper vector *Circulifer haematoceps* captured in Morocco (Vignault *et al.*, 1980). Spiroplasmas were grown at 32 °C in SP4 medium from which fresh yeast extract was omitted or in HSI medium in which the horse serum was replaced by 1 % pleuropneumonia-like organism (PPLO) serum fraction (Whitcomb, 1983). Electrotransformation of *S. citri* has been described previously by Stamburski *et al.* (1991). Transformants were selected in SP4 medium containing 2 μg tetracycline ml^{-1} or 100 μg gentamicin ml^{-1}. Selection of the *crr*-disrupted mutant, unable to use glucose, was performed in SP4 medium in which glucose had been replaced by fructose (SP4^{Fru}).

Construction of plasmids. The *S. citri oriC* plasmids pBOG, pC1/2, pC55 and pSR2 have been described elsewhere (Lartigue *et al.*, 2002; Renaudin, 2002). Plasmid pSD1 is identical to pSD2 (Lartigue *et al.*, 2002), except that the *oriC* fragment is in the opposite orientation. Plasmid pSD6 was obtained by inserting *res* sequences of the $\gamma\delta$ transposon at the *Bam*HI and *Bgl*II sites, respectively, upstream and downstream of the tetracycline resistance cassette of pSD1. The *res* sequences were retrieved from plasmid pCG118 (Malaga *et al.*, 2003) by digestion with *Bam*HI and *Bgl*II. Plasmid pSRCAT was obtained by inserting the *cat* gene (as the 765 bp fragment resulting from digestion of Cat1/Cat2 PCR product with *Bam*HI and *Bgl*II) at the *Bgl*II site of pSR2, downstream of the spiralin gene promoter. Plasmid pSD12 was obtained through insertion of the 163 bp *oriC* fragment of pC1/2 at the *Bam*HI site of pSRCAT. To construct pSD25, the resolvase gene (*tnpR*) was amplified from pCG123 (Malaga *et al.*, 2003) with primer pair TNP5/TNP6 and inserted at the *Bgl*II site of pSR2. Then, the 923 bp *Bam*HI–*Bgl*II fragment containing *tnpR* downstream of the spiralin gene promoter was retrieved from pSD25 and inserted into the *Bam*HI site of pSD12 to yield pSD261/262 (depending on *tnpR* orientation). To construct pSRG, the *aacA-aphD* gene encoding the gentamicin and kanamycin resistance determinant of Tn*4001* was amplified from pBOG with primers Gmr1 and Gmr2. After restriction with *Bam*HI plus *Bgl*II, the 1969 bp DNA fragment was inserted into the *Bgl*II site of pSR2 to yield pSRG. The 2303 bp *Pst*I cassette of pSRG containing the *aacA-aphD* coding sequence immediately downstream of the spiralin gene promoter was then inserted into the *Pst*I site of pC55 to yield pGOT1/2, depending on the insert orientation. Plasmids pGOT1/2, pSD61 and pGOT18 are described in Results.

DNA analyses. Genomic DNA from spiroplasma cells was isolated with the Wizard genomic DNA purification kit (Promega). Restricted DNA was fractionated by agarose gel electrophoresis, transferred to positively charged nylon membranes by the alkali procedure, and hybridized with appropriate [digoxigenin]dUTP-labelled probes using standard procedures (Sambrook *et al.*, 1989). Hybridization signals were detected with anti-digoxigenin–alkaline phosphatase conjugate and HNPP (2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate) as the substrate, following the supplier's instructions (Roche Diagnostics). Fluorescent signals were detected using a Fluor-S Multimager phosphoimager (Bio-Rad). The *arcA*,

Table 1. Primers used in this study

Primer	Nucleotide sequence (5′–3′)*	Position	Accession no.
ArcA1	TTATTTA**GGatcc**TTAGATAATC	554–576	DQ004462
ArcA2	ATTcTCAATT**AgaTcT**TTTGTTAC	933–956	DQ004462
ArcA3	GATAGGTATTCTGTGTAT	110–127	DQ004462
ArcA4	CCTTTTAAATTTACAGCC	1469–1486	DQ004462
Cat1	CTTCGACG**gGaTcc**TCAGGAG	32–52	U13846
Cat2	GACGA**AgaTCT**GCCATTCATC	794–814	U13846
Gmr1	CAATCGCTTAATT**GGAtCC**GTTCTTATGGACCTACATG	1190–1227	M18086
Gmr2	GCGTTTC**AgATcT**TATATAATtAATCTTTATAAGTCC	3148–3184	M18086
GPA1	GACAAT**GGaTcc**AAACACGGCATAATTC	2809–2836	AY230006
GPA3	AATTC**GGaTcc**AATTGCTCCTGTTGAAG	3731–3758	AY230006
GPA7	AGGTGA**GGaTcc**AGCAATTAAACCAAGTGC	3186–4015	AY230006
GPA8	AAATCA**AgATct**CATAATAAATCTCCTTG	3391–3419	AY230006
EV13	GGAA**GATc**GCACATTATTTTCCACG	1581–1605	Z19108
EV14	TTTT**gaTC**CTTACTTTAGTATATTCTG	1728–1754	Z19108
Res1	GCAACCGTCCGAAATATTATAAA	6–28	X03526
Res2	CATAAAAATGTATCCTAAATCAAATATC	105–132	X03526
Tet7	CCTAATTCTGTAATCGCTCCAC	302–323	X56353
TNP5	TTGTC**gGATcc**gaGAaAGGAGACATTTTTATGCGACTT TTTGGTTACGCACGGGTATCAACC	145–208	J01844
TNP6	tCggg**agAtCT**TTTATGTTAGTTGCTTTCATTTATTAC	707–744	J01844

*Bold letters indicate restriction enzyme sites. Lower-case letters indicate mismatched nucleotides.

crr, *oriC* and *res* probes were generated by PCR amplification with primer pairs ArcA3/ArcA4, GPA1/GPA3, EV13/EV14 and Res1/Res2, respectively. Primers used in this study are listed in Table 1.

Carbohydrate and arginine catabolism. In *S. citri*, carbohydrate fermentation results in the production of lactic and acetic acids that are released into the medium causing acidification (Miles, 1992). In this study, sugar fermentation was monitored by following the pH decrease during spiroplasma growth as described previously (André *et al.*, 2005). *S. citri* cells grown to the mid-exponential phase in SP4 medium were harvested by centrifugation (15 min, 12 000 ***g***, 18 °C), washed twice and resuspended in HEPES/sorbitol buffer [8 mM HEPES, pH 7·4, 10 % (w/v) sorbitol]. Two millilitres of sugar-free HSI medium supplemented with 0·5 % glucose, fructose, trehalose or sorbitol was inoculated with 5×10^8 washed cells. Sugar fermentation was followed by pH measurements every 24 h for 7 days. To determine the ability to hydrolyse arginine, spiroplasmas grown in SP4 were harvested as described above except that they were washed three times in HEPES/sorbitol buffer. Ten millilitres of HSI medium supplemented with 0·1 % fructose and/or 1 % arginine was inoculated with 5×10^8 washed cells. Sugar fermentation and/or arginine hydrolysis were followed by pH measurements for 14 days.

Experimental transmission assay. Microinjection of *S. citri* into the leafhopper (*C. haematoceps*) vector and transmission to the periwinkle (*Catharanthus roseus*) host plant have been described previously (Foissac *et al.*, 1996, 1997). Briefly, the insects were injected with 10^5–10^6 spiroplasma cells and then caged on young periwinkle plants (with 12 insects per plant and 5–10 plants per spiroplasma strain) for a 2 week transmission period. Plant symptoms were recorded for 8 weeks after transmission. Culture of *S. citri* from plants and insects were as described previously (Foissac *et al.*, 1996, 1997; Duret *et al.*, 2003). Titres of *S. citri* in the insects were determined at the end of the transmission period. In the plants, titres were determined 4 weeks after the transmission period.

RESULTS

Construction of the disruption vector pGOT18 and isolation of the *crr*-disrupted mutant

The pGOT1 vector was designed in order to facilitate the selection of recombinants in specific gene disruption experiments. This plasmid was constructed by inserting the 2303 bp *Pst*I cassette of pSRG, containing the gentamicin resistance gene *aacA-aphD* downstream of the spiralin gene promoter, into the *S. citri oriC* plasmid pC55. The resulting plasmid pGOT1 comprises two distinct selection markers, one of which can only be expressed once recombination has occurred at the target gene (Fig. 1). While the gentamicin resistance gene is constitutively transcribed from the spiralin gene promoter, the promoterless *tetM* gene cannot be transcribed from the free plasmid. The possible read-through transcription of *tetM* from the *lacZ* promoter is further prevented by two copies of the transcription terminator of the fibril protein gene. The *crr* gene encodes the IIA component of the glucose PTS permease, the organization of which has been described previously (André *et al.*, 2003). For inactivation, the internal *crr* fragment was obtained by PCR amplification of *S. citri* genomic DNA with primer pair GPA7/GPA8. After restriction with *Bam*HI plus *Bgl*II, the 220 bp fragment was inserted into the *Bam*HI site of pGOT1 to yield the disruption plasmid pGOT18 (Fig. 1). *S. citri* GII-3 was electroporated with 2 μg pGOT18 and the transformation mixture was plated onto $SP4^{Fru}$ plates containing either 100 μg gentamicin ml^{-1} or 2 μg tetracycline ml^{-1}. Gentamicin-resistant colonies were

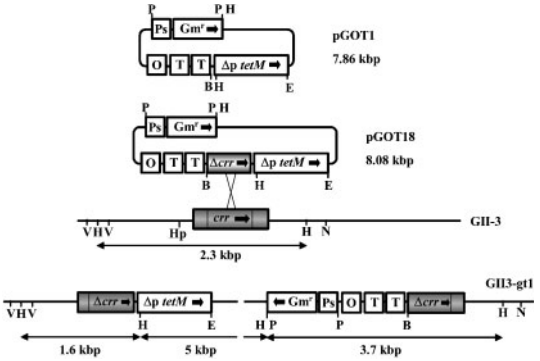

Fig. 1. Partial restriction maps of plasmids pGOT1 and pGOT18, and schematic representation of pGOT18 integration by recombination at the chromosomal *crr* gene. O, *S. citri oriC*; Δp *tetM*, *tetM* gene of Tn*916* without promoter; Δ*crr*, truncated *crr*; T, transcription terminator of the *S. citri* fibril protein gene. B, *Bam*HI; E, *Eco*RI; V, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; N, *Nsi*I; P, *Pst*I. Block arrows indicate direction of transcription.

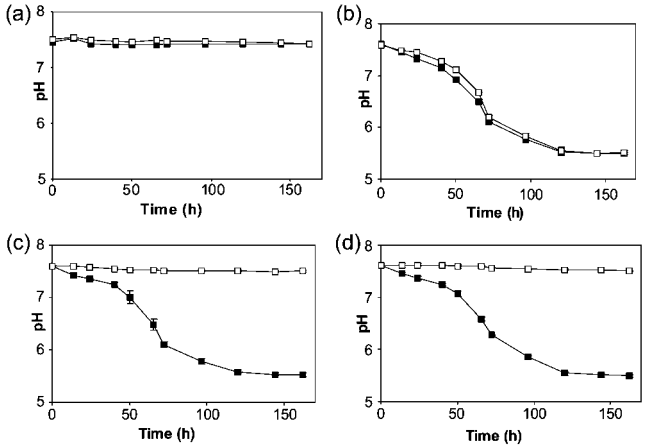

Fig. 3. Carbohydrate utilization by *S. citri* GII-3 wild-type (■) and the *crr*-disrupted mutant GII3-gt1 (□) as indicated by acidification of HSI medium supplemented with sorbitol (a), fructose (b), glucose (c) and trehalose (d).

obtained at a frequency of approximately 10^{-6} transformants μg^{-1} c.f.u.$^{-1}$. In contrast, no (less than 10^{-9}) tetracycline-resistant colonies were obtained, indicating that the *tetM* gene was not or was very poorly expressed from the free pGOT18. Gentamicin-resistant colonies were grown in liquid medium and then plated onto tetracycline agar medium. Tetracycline-resistant colonies were obtained at a frequency of $1–2 \times 10^{-8}$. Six tetracycline-resistant transformants were grown in the presence of the antibiotic for three passages and their DNAs were analysed by Southern blot hybridization with a *crr* probe (Fig. 2). In clones 2, 5, 6 and 7 (lanes 3–6) the probe hybridized with two *Hin*dIII fragments of 2·3 and 3 kbp corresponding to the wild-type *crr* and the free pGOT18 fragment, respectively. In contrast, these fragments were not detected in clones 8 and 9 (lanes 7 and 8), suggesting that pGOT18 had integrated into the *crr* region of the chromosome as early as the third passage. In these clones, the sizes (1·6 and 3·7 kbp) of the hybridizing fragments were consistent with plasmid recombination at *crr*, as illustrated in Fig. 1. The *crr*-disrupted mutant was triply cloned and named GII3-gt1.

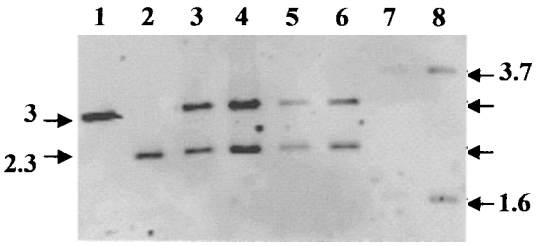

Fig. 2. Southern blot hybridization between restricted DNA of *S. citri* and the *crr* probe. Purified pGOT18 (lane 1), and genomic DNAs from *S. citri* GII-3 (lane 2) and six transformants (lanes 3–8) were restricted by *Hin*dIII. The *crr* probe consisted of a DNA fragment amplified with primer pair GPA1/GPA3. Sizes are indicated in kbp.

Sugar catabolism in GII3-gt1

Sugar fermentation in *S. citri* results in lactic acid production, and therefore can be monitored by measuring the pH decrease of the culture medium during spiroplasma growth. We have compared the spiroplasma growth of the *crr*-disrupted mutant GII3-gt1 to that of the wild-type GII-3 in HSI medium supplemented with sorbitol, fructose, glucose or trehalose (Fig. 3a, b, c and d, respectively). As expected, the wild-type strain GII-3 was found to acidify the medium supplemented with fructose, glucose and trehalose but not sorbitol (used as the control). In the case of mutant GII3-gt1, pH decrease was observed in the presence of fructose but not in the presence of glucose or trehalose. In the presence of fructose the pH curves of GII-3 and GII3-gt1 were indistinguishable. These results clearly indicated that GII3-gt1 was unable to use glucose and trehalose as well, indicating that the *crr*-encoded, glucose-PTS permease IIA^{Glc} polypeptide is required for glucose and trehalose import.

Insect transmission and pathogenicity of GII3-gt1

To determine whether the inability to import glucose and trehalose affected spiroplasmal pathogenicity, the *S. citri* mutant GII3-gt1 was experimentally transmitted to periwinkle plants via injection into the leafhopper vector *C. haematoceps*. Insects were microinjected with cultures of *S. citri* GII-3 (used as the control) or GII3-gt1, and caged on young periwinkle plants for 2 weeks. After the transmission period, the spiroplasma titres in the insects were determined, and plant symptoms were recorded for 6 weeks (Table 2). The results showed that, similarly to the wild-type

Table 2. Transmission of *S. citri* GII-3 (wild-type) and GII3-gt1 (*crr*-disrupted mutant) to periwinkle (*Catharanthus roseus*) plants via injection into the leafhopper vector (*Circulifer haematoceps*)

S. citri strain	Spiroplasma titre in insects*	Symptomatic plants†	Spiroplasma titre in plants‡		
			Weeks post-transmission:		
			2	3	4
GII-3	$1{\cdot}2\times10^{6}\pm1{\cdot}7\times10^{5}$	5/5	$3{\cdot}8\times10^{6}$	$6{\cdot}2\times10^{5}$	$1{\cdot}2\times10^{7}$
GII3-gt1	$6{\cdot}3\times10^{5}\pm2{\cdot}3\times10^{4}$	4/10	$3{\cdot}6\times10^{6}$§	$4{\cdot}3\times10^{6}$§	$5{\cdot}7\times10^{6}$§

*Spiroplasma titres in the insects were determined at the end of the transmission period. Each value, indicated as c.f.u. per insect, represents the mean of two (GII-3) or four (GII3-gt1) independent determinations.

†Symptoms were recorded 6 weeks post-transmission.

‡Spiroplasma titres in plants (c.f.u. per g midrids).

§Mean of four determinations.

strain GII-3, the *S. citri* mutant GII3-gt1 multiplied in the injected leafhoppers, and was transmitted to periwinkle plants, in which it multiplied and induced severe symptoms. Characterization of spiroplasmas isolated from these symptomatic plants proved that symptom expression was due to multiplication of GII3-gt1, and not to the presence of contaminants or spiroplasmas having reverted to the wild-type phenotype. However, while all five plants (of five) were infected in the case of *S. citri* GII-3, only four plants of ten developed symptoms in the case of mutant GII3-gt1. Furthermore, symptoms appeared with a delay (1–2 weeks) suggesting that, in these plants, a smaller amount of spiroplasmas was injected by the insects. Nevertheless, the low transmission efficiency of the mutant (as compared with the wild-type) was not due to the failure to multiply in the leafhopper. In spite of its inability to use trehalose, which is the major sugar in the insect haemolymph (Wyatt, 1967), GII3-gt1 was found to multiply at approximately the same rate as the wild-type strain, reaching a plateau within 4 days after injection (Fig. 4). After 2 weeks (i.e. at the end of the transmission period), the GII3-gt1 titre in the insects ($6{\cdot}3\times10^{5}$ c.f.u. per insect) was slightly lower than that ($1{\cdot}2\times10^{6}$ c.f.u. per insect) of GII-3, but higher than the minimal spiroplasma titre (10^{4}–10^{5} c.f.u. per insect) required for efficient transmission. In the light of previous studies (André *et al.*, 2005; Boutareaud *et al.*, 2004, Duret *et al.*, 2003), the delay in symptom production and the fact that only some of the plants were infected strongly suggested that GII3-gt1 was affected in its transmission rather than in its ability to multiply in the insect haemolymph. These data indicate that, in the insect, the ability of the spiroplasma to multiply does not simply rely on its ability to import glucose or trehalose. However, the lower transmission efficiency of the GII3-gt1 mutant suggests that glucose and/or trehalose might play a role in the insect transmission process. In this respect, it has been hypothesized that the failure to use glucose might hinder spiroplasmal multiplication in the salivary gland cells, resulting in decreased transmission efficiency (André *et al.*, 2005).

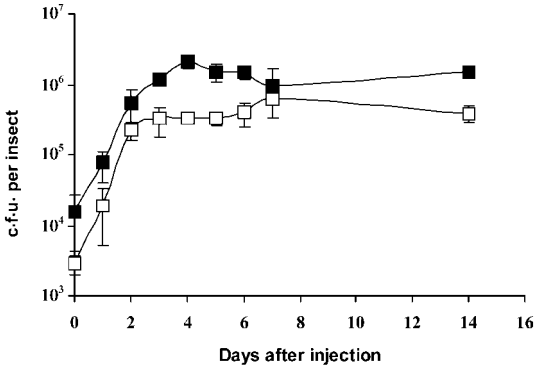

Fig. 4. Multiplication of *S. citri* GII-3 (■) and GII3-gt1 (□) in the leafhopper vector *C. haematoceps*.

Gene organization of the *S. citri* arginine deiminase (ADI) operon

With the aim to create an insertional mutant unable to hydrolyse arginine, we first characterized the gene cluster encoding the ADI pathway of *S. citri*. Briefly, from the data of the ongoing *S. citri* genome sequencing project, sequences encoding putative polypeptides with high similarities to enzymes of the arginine catabolism pathway were identified. The 5·2 kbp region of the chromosome (GenBank accession no. DQ004462) was found to contain four coding sequences (CDS), which we named *arcA*, *arcB*, *arcC* and *arcD*, as they encode proteins homologous to ADI, ornithine carbamoyltransferase (OTC), carbamate kinase (CK) and arginine–ornithine antiporter (ArcD), respectively (Fig. 5). The best BLAST hits were with ADI from *Clostridium perfringens* (50 % identity, 70 % similarity), OTC from *Mycoplasma capricolum* (63 % identity, 77 % similarity), CK from *Mycoplasma mycoides* (59 % identity, 73 % similarity) and ArcD from *Mesoplasma florum* (40 % identity, 61 % similarity). All four CDS start with an ATG initiation codon preceded by a typical ribosome-binding sequence and end with a TAA

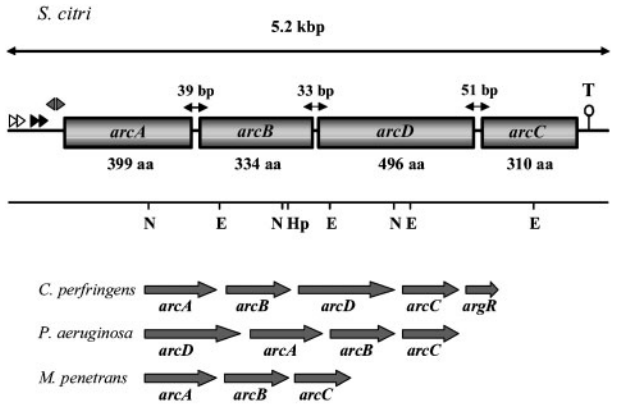

Fig. 5. Partial restriction map and gene organization of the *S. citri* ADI operon. Comparison with the ADI gene clusters of *Clostridium perfringens*, *Pseudomonas aeruginosa* and *Mycoplasma penetrans*. Arrowheads indicate direct and inverted repeat sequences. E, *Eco*RI; Hp, *Hpa*I; N, *Nsi*I; T, transcription terminator structure.

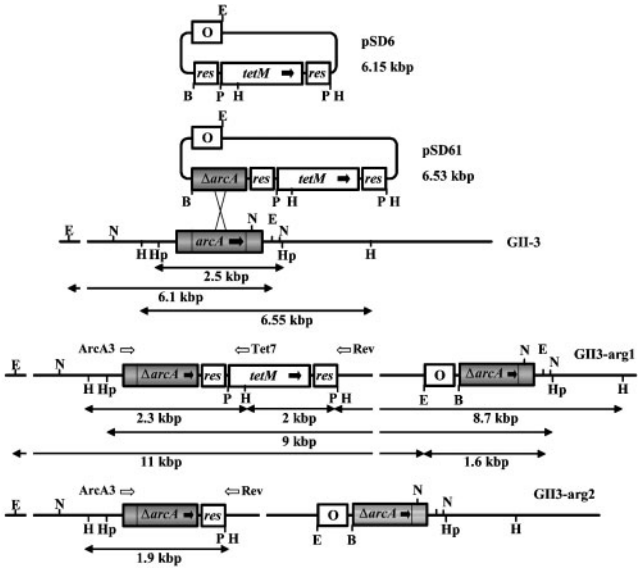

Fig. 6. Schematic representation of pSD61 recombination at the *arcA* gene. Partial restriction maps and gene organization of the *arcA* regions of *S. citri* mutants GII3-arg1 and GII3-arg2. Positions of primers ArcA3, Tet7 and Rev (universal reverse) are indicated by open arrows. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nsi*I; P, *Pst*I.

(*arcA*, *arcC*, *arcD*) or TAG (*arcB*) stop codon. The occurrence of very short (less than 50 nt) intergenic regions, as well as the absence of transcription terminator-like structures in between the *arc* genes, strongly suggests that they are transcribed as a single transcription unit. An imperfect inverted repeat located immediately downstream of *arcC* might represent the transcription terminator. The gene order, *arcABDC*, of the *S. citri* ADI operon is similar to those of *Bacillus licheniformis* (Maghnouj *et al.*, 1998) and *C. perfringens* (Ohtani *et al.*, 1997), except that no gene homologous to *arcR* (encoding the arginine regulator) could be identified, either in the vicinity of the ADI operon or somewhere else in the chromosome. The presence of direct and inverted repeat sequences in the intergenic region upstream of *arcA* suggests that this operon might be regulated. However, no palindromic sequences resembling *arcR* binding sites were identified.

Construction of the disruption vector pSD61 and isolation of the *arcA*-disrupted mutant

Plasmid pSD61 was constructed from pSD6, in which the *tetM* gene was flanked by the *res* sequences. The *arcA* internal fragment was obtained by PCR amplification of *S. citri* genomic DNA with primer pair ArcA1/ArcA2. After restriction by *Bam*HI and *Bgl*II, the 380 bp fragment was inserted into the *Bam*HI site of pSD6 to yield pSD61 (Fig. 6), which was used to transform *S. citri* GII-3. Tetracycline-resistant colonies were obtained at a frequency of 5×10^{-6} transformants μg^{-1} c.f.u.$^{-1}$. Ten transformants were propagated in liquid medium containing tetracycline for 10 passages. To check for plasmid recombination at *arcA*, genomic DNAs from five transformants were analysed by Southern blot hybridization with the *arcA* probe. The results indicated that one transformant still carried pSD61 as free extrachromosomal DNA, whereas the other four carried pSD61 sequences integrated into the chromosome. Plasmid recombination was found to occur either at the spiralin gene promoter (one transformant) or at *oriC* (two transformants). The hybridization pattern of the fifth transformant indicated a mixed population of cells, some in which plasmid recombination had occurred at *oriC* and others in which recombination had occurred at *arcA* (data not shown). Indeed, subcloning this transformant yielded two distinct profiles, one of which was consistent with plasmid recombination at *arcA* (Fig. 6) and the other with recombination at *oriC*. The relevant clones were named GII3-arg1 and GII3-ori1, respectively. As shown in Fig. 7, the wild-type *arcA* fragments (3·5 kbp *Nsi*I, 2·5 kbp *Hpa*I, 6·1 kbp *Eco*RI and 6·55 kbp *Hin*dIII), which were detected in *S. citri* GII-3 (lanes 1, 7, 10 and 14) and GII3-ori1 (lanes 3, 9, 12 and 16), were not detected in GII3-arg1 (lanes 2, 8, 11 and 15), indicating that, in this mutant, recombination had occurred within the *arcA* region. In mutant GII3-arg1, the sizes of the restriction fragments hybridizing with the *arcA* probe, such as the 9 kbp *Hpa*I and the two *Eco*RI fragments of 1·6 and 11 kbp (lanes 8 and 11), were consistent with integration of pSD61 into the chromosome by one crossover recombination at *arcA*, as illustrated in Fig. 6.

Isolation of the unmarked ADI mutant GII3-arg2

In order to produce an unmarked mutant, the ADI mutant GII3-arg1 was transformed with pSD262 carrying the resolvase gene *tnpR* under the control of the spiralin gene

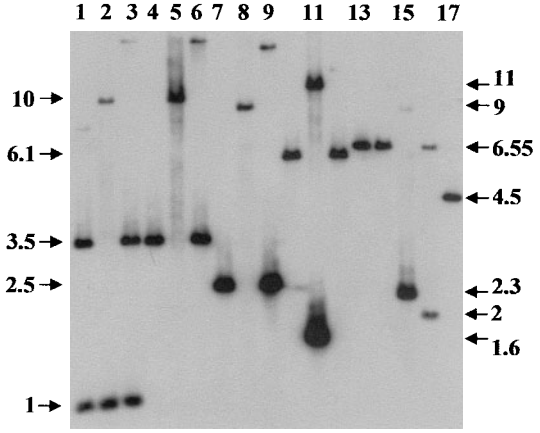

Fig. 7. Southern blot hybridization between restricted DNAs from *S. citri* GII-3 (wild-type), GII3-arg1 and GII3-ori1, and the *arcA* probe. Purified pSD61 (lanes 13 and 17) and genomic DNAs from GII-3 (lanes 1, 4, 7, 10 and 14), GII3-arg1 (2, 5, 8, 11 and 15) and GII3-ori1 (lanes 3, 6, 9, 12 and 16) were restricted by *Nsi*I (lanes 1–3), *Eco*RV (lanes 4–6), *Hpa*I (lanes 7–9), *Eco*RI (lanes 10–13) and *Hin*dIII (lanes 14–17). Sizes of DNA fragments are indicated in kbp.

promoter. Following expression of *tnpR*, the resolvase was expected to excise the DNA fragment in between the *res* sequences, i.e. the *tetM* gene, leading to a loss of tetracycline resistance. When *S. citri* GII3-arg1 was electrotransformed with pSD262, chloramphenicol-resistant colonies were obtained at a frequency of $2–5 \times 10^{-8}$ transformants μg^{-1} c.f.u.$^{-1}$. Twelve transformants (1–12) were grown in the presence of chloramphenicol for two passages, and screened for the presence of *cat* and *tetM* genes by PCR amplification with primer pairs Cat1/Cat2, ArcA3/Tet7 and ArcA3/Rev (Fig. 8a, b and c, respectively). As expected, all 12 transformants but one (which probably represents a spontaneous chloramphenicol-resistant mutant, in lane 6) yielded positive amplification with primer pair Cat1/Cat2, indicating that they all contained pSD262 (Fig. 8a, lanes 3–5 and 7–14). Interestingly, while most of the tranformants (9 of 12) yielded positive amplification with primer pair ArcA3/Tet7 (Fig. 8b, lanes 4 and 7–14), two of them seemed not to contain the *tetM* gene, as indicated by the absence of PCR product (Fig. 8b, lanes 3 and 5). In addition, detection of the 1 kbp ArcA3/Rev PCR product suggested that in all 11 transformants a number of cells had lost the *tetM* gene (Fig. 8c, lanes 3–5 and 7–14). To further confirm excision of the *tetM* gene, genomic DNAs were restricted by *Hin*dIII and hybridized with the *res* probe. In mutant GII3-arg1, the two *res* copies were detected as two *Hin*dIII hybridizing fragments of 2 and 2·3 kbp (Fig. 9, lane 2), whereas in pSD262 transformants 1 and 3, the probe hybridized with one single DNA fragment of 1·9 kbp (Fig. 9, lanes 3 and 5), a size that was in perfect agreement with excision of the *tetM* gene through recombination between the two *res* sequences (see Fig. 6). In all other transformants (Fig. 9, lanes 4 and

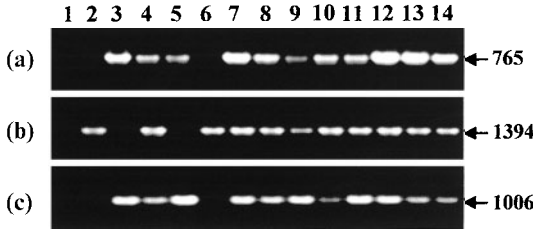

Fig. 8. PCR amplification of genomic DNAs from *S. citri* GII-3, GII3-arg1 and pSD262 transformants with primer pairs Cat1/Cat2 (a), ArcA3/Tet7 (b) and ArcA3/Rev (pUC/M13 reverse) (c). Lane 1, control without DNA; lane 2, DNA from GII3-arg1; lanes 3–14, DNAs from pSD262 transformants 1–12.

7–14) detection of the three fragments of 1·9, 2 and 2·3 kbp suggested the presence of a mixed population of cells, some of which still carried *tetM* and others not. Indeed, plating these transformants onto SP4 and tetracycline SP4 plates revealed that 20–70 % of spiroplasmal cells had lost the *tetM* gene, as indicated by their sensitivity to the antibiotic. Interestingly enough, due to incompatibility of *oriC* plasmids, pSD262 carrying *tnpR* was spontaneously lost during propagation of the transformants in the absence of chloramphenicol. After only four passages, no plasmid could be detected by PCR with primer pair Cat1/Cat2 (data not shown).

Arginine catabolism in *S. citri* GII-3 and GII3-arg1

To determine the ability of the *arcA*-disrupted mutant to metabolize arginine, *S. citri* GII-3 (wild-type) and GII3-arg1 (mutant) were grown in HSI medium supplemented with various sugars and/or arginine, and the pH curves were compared (Fig. 10). Carbohydrate fermentation in *S. citri* essentially results in lactic acid production and, consequently, leads to a pH decrease in the culture medium. When *S. citri* GII-3 (wild-type) and GII3-arg1 (*arcA*-disrupted mutant) were grown in the presence of fructose, the pH progressively decreased from 7·6 to 5·5 within 3 days. In contrast, no such pH decrease was observed in the presence of sorbitol, which is not metabolized by *S. citri*. Interestingly, when *S. citri* GII-3 was grown in the presence of fructose and arginine, the pH first dropped

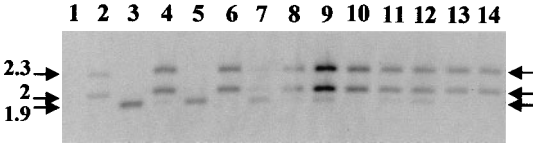

Fig. 9. Southern blot hybridization between genomic DNAs from *S. citri* GII-3, GII3-arg1 and pSD262 transformants and the *res* probe. Lane 1, GII-3; lane 2, GII3-arg1; lanes 3–14, pSD262 transformants 1–12.

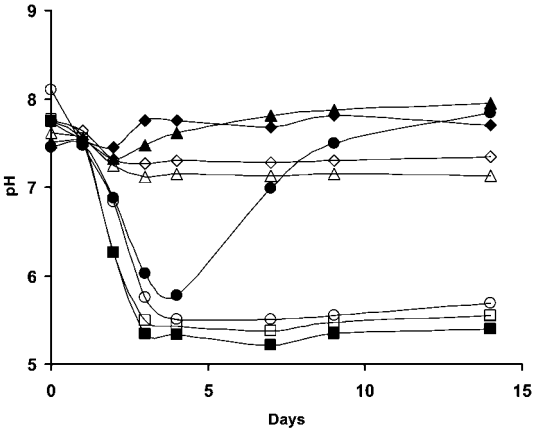

Fig. 10. Arginine utilization by *S. citri* GII-3 (filled symbols) and the *arcA*-disrupted mutant GII3-arg1 (open symbols) grown in HSI medium supplemented with sorbitol (◆, ◇), fructose (■, □), arginine (▲, △) and fructose plus arginine (●, ○).

from 7·5 to 5·75 as a result of fructose fermentation. Then, due to the arginine catabolism and subsequent ammoniac release, the pH progressively increased to reach 7·8 after 14 days (Fig. 10). In the case of the *arcA* mutant GII3-arg1, no pH increase was observed, indicating that this mutant was unable to metabolize arginine. It has been shown previously that complete utilization of arginine by *S. citri* only occurred when an alternative energy source, glucose or fructose, was present to encourage growth (Townsend, 1976). Accordingly, a minor pH increase was observed when *S. citri* GII-3 was grown in the absence of fructose. As expected, no such pH increase was observed with mutant GII3-arg1 (Fig. 10). The unmarked mutant GII3-arg2 displayed an identical phenotype.

Insect transmission and pathogenicity of GII3-arg1

Insect transmission and pathogenicity of the *arcA*-disrupted mutant was determined through experimental transmission to periwinkle plants as described above for mutant GII3-gt1. Four weeks after transmission, all 10 plants displayed symptoms undistinguishable from those produced by the wild-type strain GII-3. These results indicate that the GII3-arg1 mutant was efficiently transmitted to plants by the leafhopper vector. Determination of spiroplasma titres revealed that GII3-arg1 multiplied in the insects and in the plants similarly to *S. citri* GII-3 (data not shown). These data indicate that arginine degradation through the ADI pathway is not critical for the spiroplasma to complete its life cycle in the vector insect and in the host plant.

DISCUSSION

In the present study, disruption of *crr* was achieved with a pGOT1-based disruption vector. This plasmid vector carries two selection markers, one of which (the gentamicin resistance gene Gm^r) is constitutively expressed, whereas the other (*tetM*), devoid of promoter, can only be expressed when recombination has occurred at the target gene. In this case, transcription of *tetM* is driven by the target gene promoter. Due to low recombination frequency, direct selection of recombinants by plating the transformed cells on tetracycline medium was unsuccessful in agreement with the fact that, in the presence of tetracycline, pGOT1 behaves as a suicide plasmid. Selection of recombinants required two steps. First, transformants carrying the disruption plasmid were selected for their resistance to gentamicin, and following propagation, spiroplasma cells in which recombination had occurred at the target gene were selected by plating onto tetracycline plates. The advantage of such a two-step selection procedure lies in the almost unlimited number of cells carrying the disruption vector, increasing the probability for selection of rare recombination events. Unexpectedly, some tetracycline-resistant transformants still carried free plasmid, indicating that in spite of the absence of promoter the *tetM* gene was somehow transcribed. These data suggest that transcription of *tetM* could proceed from a DNA sequence carried on the *crr* DNA fragment. Considering the high A + T content (70 mol%) of the *crr* DNA fragment, the occurrence of sequences resembling the −35 and −10 regions of eubacterial promoters recognized by the RNA polymerase cannot be excluded.

We have shown previously that in *S. citri*, the glucose PTS permease enzyme II was split into two distinct polypeptides IIA^{Glc} and $IICB^{Glc}$ encoded by two separate genes *crr* and *ptsG*, and that the trehalose PTS permease did not possess its own IIA component (André *et al.*, 2003). By using a yeast two-hybrid system, we also showed that the IIA^{Glc} domain bound not only the IIB^{Glc} but also the IIB^{Tre} domain, suggesting that glucose and trehalose permeases shared a single IIA domain (André *et al.*, 2003). The finding that the *crr*-disrupted mutant GII3-gt1 used neither glucose nor trehalose definitely demonstrates that, in *S. citri*, glucose and trehalose PTS permeases function with a single IIA domain. In spite of its inability to import these two sugars, the *crr*-disrupted mutant GII3-gt1 multiplied in the leafhopper vector and was transmitted to periwinkle plants, in which it induced symptoms. In insects, trehalose is the main sugar in the haemolymph but glucose and fructose also are present (Florkin & Jeuniaux, 1974). Therefore, it is likely that multiplication of GII3-gt1 in the leafhopper vector mainly relies on the use of fructose. However, fructose import is not an absolute requirement for *S. citri* multiplication in the insect, as the fructose operon mutant GMT553 multiplies to high titre in the leafhopper (Gaurivaud *et al.*, 2000a). These data attest the capability of *S. citri* to adapt to carbohydrate changes in its environment. We have shown previously that insect transmission of the *ptsG*-disrupted mutant GII3-glc1, which is unable to import glucose, was less efficient than that of the wild-type strain GII-3 (André *et al.*, 2005). As expected, transmission of GII3-gt1 also was found to be poorly efficient. *In vitro*, *S.*

citri metabolizes glucose, fructose and trehalose equally well (Chang *et al.*, 1994). However, when both fructose and glucose are present, *S. citri* uses fructose preferentially (André *et al.*, 2005). As a result, while the *S. citri* mutant GMT553, which is unable to use fructose, is non-pathogenic to plants (Foissac *et al.*, 1997; Gaurivaud *et al.*, 2000a), GII3-glc1 induces symptoms identical to those produced by the wild-type strain, indicating that glucose import is not essential for pathogenicity (André *et al.*, 2005). Similarly, the *crr*-disrupted mutant GII3-gt1 proved to be highly pathogenic to periwinkle plants, showing that trehalose import, like glucose import, is not an absolute requirement for pathogenicity.

Arginine metabolism leading to the synthesis of ATP through the ADI pathway is considered to be the primary energy conserving route in non-glycolytic mollicutes (Pollack *et al.*, 1997). However, carbohydrates and arginine can be metabolized concomitantly by glycolytic mollicutes, including the plant pathogen *S. citri* (Townsend, 1976; Igwegbe & Thomas, 1978; Stevens *et al.*, 1984; Pollack *et al.*, 1997). The ADI pathway comprises three reactions catalysed by ADI, OTC and CK, and converts arginine to ornithine, ammonia and CO_2, with concomitant generation of ATP. In mollicutes, the ADI gene clusters display very diverse gene organizations depending on the mollicute species. A rapid survey using the Molligen software (Barré *et al.*, 2004) revealed that *M. penetrans* lacks *arcD* with the organization *arcABC*, *M. mycoides* lacks *arcA*, with *arcBD* and *arcC* being located on two distinct transcription units, and *M. gallisepticum* has two separated copies of *arcA* and no *arcB*, *arcC* and *arcD*. In *S. citri*, we found the relevant genes *arcA*, *arcB* and *arcC* to be clustered in a single operon, together with a fourth gene (*arcD*) encoding an arginine–ornithine antiporter, and located in between *arcB* and *arcC*. As reported previously (Townsend, 1976), we found that complete utilization of arginine only occurred when an alternative energy source in the form of glucose or fructose was present to encourage growth. Inactivation of the *arcA* gene through homologous recombination completely abolished the use of arginine. However, the *arcA*-disrupted mutant GII3-arg1 was found to multiply *in vitro*, in its leafhopper vector, and in its host plant to approximately the same rate as the wild-type strain GII-3. These results confirm that, in *S. citri*, the ADI pathway is not the major energy-generating system and might not be essential for the spiroplasma to complete its life cycle. In most bacteria, energy depletion is an essential signal for inducing the ADI pathway (Cunin *et al.*, 1986). In *S. citri* also, the ADI pathway is inducible (Igwegbe & Thomas, 1978) and therefore might play a role in nutrient stress response. From the *arcA*-disrupted mutant GII3-arg1, we have produced an unmarked *arcA* mutant, free of the *tetM* selection marker, by using the TnpR/res recombination system of the *E. coli* $\gamma\delta$ transposon. The site-specific recombinase TnpR has been used as a reporter of gene expression in *Vibrio cholerae* (Camilli *et al.*, 1994) and for producing unmarked mutations in mycobacteria (Bardarov *et al.*, 2002; Malaga *et al.*, 2003). In our experiments, expression of *tnpR* driven by the spiralin gene promoter resulted in efficient excision of the *tetM* marker flanked with the *res* sequences. Indeed, *tetM* excision was detected in all pSD262 transformants after only two successive propagations. Moreover, plating onto tetracycline agar plates revealed that one of these transformants yielded no (less than 10^{-4}) tetracycline-resistant colonies, indicating that *tetM* had been excised in a large majority of cells. Production of unmarked mutations usually requires the use of counterselectable markers (Reyrat *et al.*, 1998) for curing the plasmid from which the resolvase is expressed (Malaga *et al.*, 2003). However, in mycobacteria, plasmid loss could also be achieved using a delivery system made of a pair of replicating plasmids, which are incompatible (Pashley *et al.*, 2003). In our study, we have used the incompatibility of *S. citri oriC* plasmids as the selection pressure for plasmid loss. During propagation of transformants in the absence of chloramphenicol, the spontaneous loss of the *oriC* plasmid carrying the resolvase gene avoided the use of a counterselectable marker. Our results demonstrate that the TnpR resolvase was functional in spiroplasmas and catalyses site-specific recombination between two *res* sequences in direct orientation on the spiroplasmal chromosome.

In summary, we have developed new genetic tools for reverse genetic studies in *S. citri*. For specific gene targeting through homologous recombination, the use of pGOT1-based disruption vectors was shown to improve the probability to select rare recombination events through a two-step procedure. As an example, we produced the *crr*-disrupted mutant GII3-gt1, the characterization of which proved the glucose and trehalose PTS permeases to share a unique IIA component. Also, we have shown that the transposon $\gamma\delta$ site-specific recombination system functions in *S. citri*. An unmarked ADI mutant was produced through excision of the *tetM* gene, allowing the successive use of the same antibiotic to produce multiple mutations. These new tools, like the formerly described *oriC* plasmids (Renaudin, 2002; Renaudin & Lartigue, 2005), should be applicable to a wide variety of mollicute species.

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