Identification of a *Spiroplasma citri* hydrophilic protein associated with insect transmissibility

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With the aim of identifying *Spiroplasma citri* proteins involved in transmission by the leafhopper *Circulifer haematocaps*, protein maps of four transmissible and four non-transmissible strains were compared. Total cell lysates of strains were analysed by two-dimensional gel electrophoresis using commercially available immobilized pH gradients (IPGs) covering a pH range of 4–7. Approximately 530 protein spots were visualized by silver staining and the resulting protein spot patterns for the eight strains were found to be highly similar. However, comparison using PDQuest 2-D analysis software revealed two trains of protein spots that were present only in the four transmissible strains. Using MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry and a nearly complete *S. citri* protein database, established during the still-ongoing *S. citri* GII-3-3X genome project, the sequences of both proteins were deduced. One of these proteins was identified in the general databases as adhesion-related protein (P89) involved in the attachment of *S. citri* to gut cells of the insect vector. The second protein, with an apparent molecular mass of 32 kDa deduced from the electrophoretic mobility, could not be assigned to a known protein and was named P32. The P32-encoding gene (714 bp) was carried by a large plasmid of 35.3 kbp present in transmissible strains and missing in non-transmissible strains. PCR products with primers designed from the *p32* gene were obtained only with genomic DNA isolated from transmissible strains. Therefore, P32 has a putative role in the transmission process and it could be considered as a marker for *S. citri* leafhopper transmissibility. Functional complementation of a non-transmissible strain with the *p32* gene did not restore the transmissible phenotype, despite the expression of P32 in the complemented strain. Electron microscopic observations of salivary glands of leafhoppers infected with the complemented strain revealed a close contact between spiroplasmas and the plasmalemma of the insect cells. This further suggests that P32 protein contributes to the association of *S. citri* with host membranes.

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INTRODUCTION

The first-cultured and most-studied spiroplasma is *Spiroplasma citri*, the causal agent of citrus stubborn disease, one of the three plant-pathogenic, sieve-tube-restricted, and leafhopper-vector-transmitted mollicutes (Bové *et al.*, 1989; Bové & Garnier, 2003). The main vector of *S. citri* in the Mediterranean area and the Near East is the leafhopper *Circulifer haematocaps* (Fos *et al.*, 1986), and *Circulifer tenellus* is thought to be the most important natural vector in California (Oldfield *et al.*, 1976; Kaloostian *et al.*, 1979). Spiroplasmas ingested via phloem-sap feeding traverse the insect gut wall and move into the haemolymph, where they multiply and circulate. They eventually invade the salivary glands, where they multiply further (Liu *et al.*, 1983; Kwon

et al., 1999). Probably delivered by exocytosis into the salivary duct, they are introduced with saliva into the phloem of a new host plant (Fletcher *et al.*, 1998). Thus, *S. citri* cells undergo a series of molecular and cellular interactions with the insect vector that are required for transmission to a plant. The detailed mechanisms by which these events take place remain to be elucidated (Fletcher *et al.*, 1998; Kwon *et al.*, 1999). Although many *S. citri* strains multiply within the haemocoel, the ability to cross insect gut and salivary gland barriers is lost by some strains maintained for a long time *in vitro* or *in planta* without passage through an insect host (Bové *et al.*, 1989; Wayadande & Fletcher, 1995).

Attachment of bacteria to host cells is thought to be a critical step leading to colonization of a particular tissue, and bacterial pathogens typically express adhesins, i.e. bacterial surface proteins that promote host cell attachment. In the case of human and animal mycoplasmas, adhesins play an important role in invasion and pathogenicity (Rottem,

Abbreviations: IPG, immobilized pH gradient; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; ScARP, *S. citri* adhesion-related protein.

2003). Transmission of *S. citri* by its leafhopper vector also involves adherence and invasion of insect host cells (Liu *et al.*, 1983).

A few *S. citri* proteins have been identified as possibly involved in spiroplasma–insect cell interactions. Two surface proteins, P58 and P89, are candidates for adherence to and invasion of insect host cells (Ye *et al.*, 1997; Yu *et al.*, 2000). Boutareaud *et al.* (2004) found that the ability of *S. citri* to be transmitted by *C. haematoceps* is clearly lost by disruption of a gene encoding a putative solute-binding protein of an ABC transporter, and restored by the addition of this gene. An *S. citri* spiralin-less mutant was transmitted by leafhopper to periwinkle plants less efficiently than the wild-type strain GII-3. This impaired transmissibility phenotype was observed despite the ability of the mutant to multiply to a high titre in the insect (Duret *et al.*, 2003). These data suggested that the absence of spiralin, the most abundant *S. citri* membrane protein, reduces the ability of the spiroplasma to invade the salivary glands or its ability to survive in the insect saliva. Recently, the GII-3 spiralin was shown to act *in vitro* as a lectin binding to glycoproteins of *C. haematoceps* and therefore might function as a ligand able to interact with uncharacterized insect surface protein receptors (Killiny *et al.*, 2005). However, spiralin is equally present in transmissible and non-transmissible *S. citri* strains, confirming that the ability to adhere to the host cell does not rely on only a single spiroplasmal protein. A combination of the effects of several proteins or complexes is probably involved (Razin & Jacobs, 1992).

Here we report the comparison of 2-D cell-lysate protein maps from transmissible and non-transmissible *S. citri* strains in order to identify proteins that differentiate strains according to their transmissibility. A 32 kDa protein specifically present in the transmissible strains enables unambiguous discrimination of transmissible and non-transmissible strains of different origins. This protein was further identified and characterized. We also demonstrated that the absence of P32 was correlated with the absence of the corresponding gene.

METHODS

Bacterial strains and experimental transmission assays. *Escherichia coli* strain DH10B [F'-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*Δ*M15*Δ*lacX74* *deoR* *recA1* *araD139* Δ (*ara*, *leu*)7697 *galU* *galK/L* *rpsL* *nupG*] (Stratagene) served as the host strain for cloning procedures and plasmid propagation.

Spiroplasma citri strains were isolated from stubborn-affected citrus trees, or leafhoppers (*C. haematoceps*). Their respective site of isolation and host are shown in Table 1. The Iranian strains 44 and 26 were kindly provided by Dr A. Hosseini Pour, Tarbiat Modares University, Iran. Spiroplasmas were grown at 32 °C in SP4 medium (Tully *et al.*, 1977) from which fresh yeast extract was omitted, until the colour of the phenol red indicator changed to yellow. From an early passage of *S. citri* strain GII-3 (Vignault *et al.*, 1980), a triply cloned strain was obtained (Duret *et al.*, 1999) and used in this study.

Transmission to periwinkle plants (*Catharanthus roseus*) via injection into the leafhopper vector (*Circulifer haematoceps*) was performed as described previously (Markham *et al.*, 1974; Foissac *et al.*, 1996b). Multiplication of spiroplasmas in the leafhoppers and in plants exposed to infection by injected leafhoppers was determined by culture assay (Foissac *et al.*, 1996b). Spiroplasmas that multiplied in the leafhopper at the same rate as the wild-type strain GII-3 and could not be recovered in plants were identified as non-transmissible strains.

Protein sample preparation. Spiroplasmas were harvested from 50 ml cultures by centrifugation at 20 000 *g* for 20 min at 4 °C. The pellet was washed four times by suspension in 50 ml washing buffer (8 mM HEPES, 280 mM sucrose, pH 7·4) and finally dissolved in 5 ml 0·3 % (w/v) SDS, 0·07 % β-mercaptoethanol, 1 mM PMSF, 1 mM PBS (0·1 M phosphate buffer pH 7·4 with 0·1 M NaCl). The lysate was boiled for 15 min. Protein concentration was determined with the Bio-Rad protein assay kit, according to the Bradford dye binding procedure with ovalbumin as the standard (Bradford, 1976). Generally, 1 mg of proteins was obtained from 50 ml *S. citri* culture. Proteins were precipitated by the addition of 4 vols ice-cold acetone containing 0·07 % β-mercaptoethanol, incubated overnight at –20 °C, and then collected by centrifugation at 4 °C. Finally, the proteins were solubilized in 1·5 ml of a rehydration solution containing 7 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 2 % (w/v) DTT and 2 % (v/v) Ampholine pH 3–10 for 2-D gel electrophoresis. Aliquots of 300 μl (200 μg protein) were stored at –80 °C until use.

Triton X-114 fractionation. Triton X-114 fractionation was used to enrich membrane and membrane-associated proteins (Bordier, 1981). Spiroplasma cells from 150 ml of culture were washed twice

Table 1. Origins of *S. citri* strains used in this study

Strain	Origin	Isolated from	Reference*	Transmission†
GII-3	Morocco	Leafhopper	Vignault <i>et al.</i> (1980)	+
Corsica	France	Leafhopper	Fos <i>et al.</i> (1986)	+
Cyprus	Cyprus	Citrus leaves	Bové (1995)	+
Palmyra	Syria	Leafhopper	Fos <i>et al.</i> (1986)	+
R ₈ A ₂	Morocco	Citrus leaves	Saglio <i>et al.</i> (1971)	–
44	Iran	Citrus leaves	Hosseini Pour (2000)	–
26	Iran	Citrus leaves	Hosseini Pour (2000)	–
ASP-1‡	Israel	Citrus leaves	Townsend <i>et al.</i> (1977)	–

*Reference of the first report for isolation of the strain.
†Experimentally transmitted strains are indicated by (+) and non-transmitted strains by (–).
‡Non-helical and motile strain.

in washing buffer and extracted in a total volume of 1 ml, with 10% Triton X-114 in Tris/NaCl buffer (10 mM Tris/HCl, 154 mM NaCl, pH 7.5) containing 1 mM PMSF for 40 min on ice. The suspension was centrifuged for 5 min at 12 000 g and the supernatant was incubated at 37 °C for 5 min. The detergent phase was separated from the aqueous phase by centrifugation for 3 min at 25 °C (20 000 g). Proteins from the insoluble fraction were precipitated with 10 vols ice-cold methanol containing 0.07% β -mercaptoethanol; those present in the soluble fraction were precipitated in 4 vols ice-cold acetone containing 0.07% β -mercaptoethanol. Pellets from both fractions were suspended in 200 μ l 0.1 M PBS, pH 7.4. Protein concentration was determined with the Bio-Rad protein assay kit. Then, proteins were precipitated with ice-cold acetone and redissolved in 1.5 ml rehydration solution. Aliquots of 300 μ l (200 μ g protein) were frozen at -80 °C until use.

2-D electrophoresis. For the first dimension, proteins (200 μ g) were solubilized in a rehydration solution according to the manufacturer's instructions (Bio-Rad). Immobilized pH gradient strips (17 cm, Bio-Rad) covering a pH range of 4–7 were rehydrated in 300 μ l of this protein solution for about 13 h under mineral oil. Then they were subjected to IEF in a Protean II xi cell. After IEF, strips were equilibrated for 10 min in 6 M urea, 2% (w/v) SDS, 0.375 M Tris/HCl pH 8.8, 20% (v/v) glycerol with 130 mM DTT and then for 10 min in the same buffer without DTT but containing 135 mM iodoacetamide. Equilibrated strips were transferred onto a 12.5% polyacrylamide gel. Strips were bonded to the gels using 1% low-melting-point agarose in 1 M Tris/HCl pH 6.8. Gels were run in the Protean II xi gel tank at 20 mA per gel at room temperature until the dye front ran off the gels. For routine use proteins were visualized by silver staining as previously described (Blum *et al.*, 1987). Gels intended for MALDI-TOF analysis were stained by Coomassie brilliant blue (Fairbanks *et al.*, 1971).

The digitized gel images were imported into PDQuest (version 7.0; Bio-Rad) and were used for detection of spots and gel matching analysis among the strains.

MALDI-TOF mass spectrometry and protein identification. Proteins of interest were excised from stained 2-D gels and digested with trypsin. The resulting peptides were analysed directly by MALDI-TOF (Applied Biosystems, Voyager DE Super STR). The incomplete genome of strain GII-3-3X (100% of extrachromosomal elements and 93% of the chromosomal information) was translated into protein sequence (unpublished data) for matching the resulting peptides obtained by MALDI-TOF. *S. citri* GII-3-3X extrachromosomal sequences and annotation data are available under EMBL accession numbers AJ969069, AJ969070, AJ969071, AJ969072, AJ969073 and AJ969074. Peptide matches allowed us to determine the sequence of each protein spot. Then, the function of the proteins was predicted by similarity with other proteins in the non-redundant protein database (NCBI) and the MolliGen database (<http://cbi.labri.fr/outils/mollogen>), in which all the complete mollicute genome sequences are available (Barré *et al.*, 2004). In addition, TBLASTN algorithms were used to search for homologies between *S. citri* protein sequences and proteins deduced from the partially sequenced genome of one other phytopathogenic spiroplasma: *Spiroplasma kunkelii* (<http://www.genome.ou.edu/spiro.html>).

PCR amplification. Primers 32F1 (5'-TAACGAATTAATCATTC-TAATAGC-3') and 32R (5'-TAGTTCCGGCTTGCTACCA-3') were designed from the *p32* gene sequence (accession no. CAI93836), located from nucleotide 24219 to nucleotide 24935 on plasmid pSci6 (AJ969074). The use of these primers in PCR amplification with *S. citri* genomic DNA as template leads to a 544 bp amplicon. The PCR reaction was carried out in 30 μ l of reaction mixture containing 1 μ M of each primer, 200 μ M of each of the four dNTPs, 2 mM MgCl₂, 20 mM Tris/HCl pH 8.4, 50 mM KCl, 1.5 U *Taq* polymerase

(Promega), and 100 ng DNA template. The reaction was performed in a thermal cycler (Perkin-Elmer Cetus) with the following programme: 40 cycles each at 94 °C for 30 s, 66 °C for 45 s, and 72 °C for 45 s. Amplifications with primers designed on the spiralin gene were performed as described before (Najar *et al.*, 1998) and constituted the positive control of our PCR experiments. Primers Tet1 (5'-CTGCAAAAGATGGCGTAC-3') and Tet2 (5'-CGTAAATGTAG-TACTCCAC-3') correspond, respectively, to nucleotides 521 to 538 and 1037 to 1055 of the *tetM* gene (Burdett *et al.*, 1982). Amplification was carried out as previously described (Duret *et al.*, 1999). Following amplification, 10 μ l aliquots of reaction mixture were analysed by electrophoresis on 1% agarose gels. PCR products used as probes were labelled by the addition of 1 nmol digoxigenin 11-dUTP to 40 μ l PCR reaction mixture.

DNA manipulations. *Spiroplasma* genomic DNAs were prepared from 10 ml cultures using the Wizard genomic DNA purification Kit (Promega). Small-scale preparations of plasmid DNA amplified in *E. coli* were carried out according to standard procedures (Sambrook *et al.*, 1989). Recombinant DNA manipulations were conducted according to standard techniques and by following the manufacturer's recommendations.

DNA was blotted onto positively charged membranes by the alkali transfer procedure (Sambrook *et al.*, 1989). Hybridizations with appropriate digoxigenin-labelled DNA probes were carried out by using the standard method described by the supplier (Roche Applied Science). Detection of hybridized probes was achieved using anti-digoxigenin antibodies coupled to alkaline phosphatase and the fluorescent substrate HNPP (2-hydroxy-3-naphthoic acid-2'-phenylanilide phosphate) (Roche Molecular Biochemicals). Chemifluorescence was detected by using a high-resolution camera (Fluor-S, Bio-Rad) and Quantity One, a dedicated software for image acquisition (Bio-Rad).

Complementation of *S. citri* Iranian strain 44. Gene *p32* of strain GII-3 with its promoter and terminator regions was recovered by PCR amplification using primers 32F Eco (5'-CAGACCGC-GAATTCACAAAC-3') and 32R Eco (5'-TCGCCGATATGAATTC-GGTGC-3'), which include an artificially introduced *EcoRI* site (underlined). After *EcoRI* digestion of the amplified DNA, obtained with the Platinum *pf*x DNA Polymerase (Invitrogen), the 1176 bp *EcoRI*-restricted DNA fragment was inserted into *EcoRI*-linearized plasmid pSD4. The *oriC* plasmid pSD4 (Renaudin, 2002), containing the *tetM* gene, replicates in *S. citri* as a free plasmid before its integration into the spiroplasmal chromosome by recombination at the *oriC* region. The resulting complementing plasmid, named pSD4-32, was used to transform *S. citri* strain 44. Transformation was achieved by electroporation, as described previously (Stamburski *et al.*, 1991). Transformants were selected by plating on SP4 medium containing 2 μ g tetracycline ml⁻¹. Individual colonies were picked to inoculate broth medium containing tetracycline (2 μ g ml⁻¹). During propagation, the tetracycline concentration was progressively increased to 15 μ g ml⁻¹. To determine whether pSD4-32 was maintained extrachromosomally as a free plasmid or was integrated into the spiroplasmal genome, total DNA of transformants was hybridized with probes *tetM* and 32F1/32R. To provide a spiroplasma control, *S. citri* strain 44 was also transformed with pSD4, and a randomly picked and propagated clone was used in transmission experiments.

Tissue processing for transmission electron microscopy. Heads separated (by gentle pulling) from insect bodies, with undamaged salivary glands, were fixed under vacuum with 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 3 h at room temperature. They were postfixed with 2% osmium tetroxide in the same buffer for 2 h at 20 °C. Salivary glands were then dissected in phosphate buffer. After dehydration in a graded ethanol series, samples were embedded in Epon resin. Ultrathin sections (60–80 nm) were stained with 5% aqueous uranyl acetate

for 40 min then with 0.5% aqueous lead citrate for 5 min. Micrographs were taken at 80 kV with a Philips CM 10 transmission electron microscope equipped with a side-port digital camera (AMT XR-60)

RESULTS

Comparison of 2-D protein patterns of transmissible and non-transmissible *S. citri* strains

The aim of this protein pattern comparison was to detect the conserved differences between four transmissible and four non-transmissible strains. Care was taken to ensure that all gels were stained to the same extent. From time to time, variability was however seen amongst very faint spots just at the silver-stain detection limit. Nevertheless, comparisons carried out by PDQuest showed that the eight resulting protein spot patterns were highly similar to each other. For all strains, the resulting patterns within a pI range from 4 to 7 consisted of approximately 530 well-resolved protein spots of varying intensities with molecular mass ranging from 10 to 100 kDa. Only two consistent differences obvious by visual examination were noticed between the protein patterns of transmissible strains and non-transmissible strains: two trains of spots at positions 85 kDa and 32 kDa were present only on the protein patterns of the transmissible strains. Gel regions displaying spot differences between the transmissible strain GII-3 and the non-transmissible strain 44 are shown in Fig. 1. Gel sections around 32 kDa, showing differences in protein spots detected between the transmissible strains Cyprus,

Corsica and Palmyra, and the non-transmissible strains R₈A₂, ASP-1 and 26, are also shown in more detail in Fig. 1.

Protein identification

In order to identify these proteins, the corresponding spots were excised from the gels after Coomassie blue staining and digested with trypsin, and a peptide spectrum was generated by MALDI-TOF. The identification of the protein was performed by peptide mass fingerprinting comparison to the *S. citri* theoretical and nearly complete proteome deduced from its sequenced genome. The protein with an apparent molecular mass of 85 kDa was identified as protein P89, an *S. citri* adhesion-related protein (ScARP) implicated in adhesion to cells of the leafhopper vector *C. tenellus* (Yu *et al.*, 2000). The protein present in the second spot surrounded by a box in Fig. 1 displays an electrophoretic mobility yielding an apparent molecular mass of about 32 kDa. This protein was termed P32. Identification by MALDI-TOF MS analysis revealed a protein with a theoretical molecular mass of about 27.6 kDa. No significant hits were found with proteins present in general and MolliGen databases and those deduced from the partially sequenced genome of one other phytopathogenic spiroplasma (*S. kunkelii*).

Characterization of P32 as a cytoplasmic protein

Hydrophilic properties of P32 protein were tested by Triton X-114 phase partitioning as detailed in Methods. In this method, integral hydrophobic membrane proteins are incorporated into the Triton X-114 micelles while hydrophilic

Fig. 1. Silver-stained 2-D gels of proteins from *S. citri* transmissible strain GII-3 and non-transmissible 44. Proteins (200 µg) were resolved by SDS electrophoresis in a 12.5% polyacrylamide gel after isoelectric focusing in a non-linear immobilized pH gradient from pH 4 to 7 (IPG strip 17 cm). The region where protein spots differ between the two strains is outlined. The gel sections corresponding to the P32 area of three additional transmissible and non-transmissible strains are displayed in the small panels on the right. Relative molecular masses are indicated on the left side and strain designations are indicated under each panel.

Fig. 2. 2-D protein profiles of Triton X-114 insoluble fraction (a) and soluble fraction (b) from *S. citri* GII-3. Proteins (200 µg) were separated on a 17 cm IPG strip (5–8) and a vertical 12.5% polyacrylamide SDS gel. Gels were silver stained. The spiralin and P32 protein spots are circled and indicated.

proteins are sequestered in the aqueous phase. Fig. 2 shows the 2-D protein patterns of soluble and insoluble GII-3 proteins after Triton X-114 fractionation. Spiralin, a well-documented *S. citri* membrane protein, was found in the hydrophobic phase (Fig. 2a). In contrast P32 protein was almost completely partitioned in the soluble fraction (Fig. 2b). Ten washings of the insoluble fraction failed to remove the small amount of P32 in the hydrophobic detergent phase, suggesting an association between P32 and membrane proteins.

In silico analysis of P32 protein

Analysis of the predicted amino acid sequence of the 27.2 kDa protein revealed that it had a hydrophilic nature, which is in accordance with the results of Triton X-114 partitioning. No signal peptide or transmembrane domains were predicted along the 238 amino acids of P32. Eight potential sites of phosphorylation were found.

Amplification and genomic location of the gene encoding P32 protein

With primers 32F1 and 32R designed from the *p32* gene of 718 bp, a PCR product of 544 bp was obtained with DNAs extracted from transmissible strains (Fig. 3a, lanes 1–5). No amplification occurred with DNAs of non-transmissible strains (Fig. 3a, lanes 6–9). Amplification with spiralin gene primers was carried out on the same DNA preparations as a control (Fig. 3b). As expected, a PCR product of 330 bp was obtained for all strains.

In order to locate the *p32* gene in the *S. citri* genome, Southern blot analysis was performed using the 32F1/32R amplified fragment as probe. Fig. 4(a) illustrates the migration in 0.5% agar gel of the undigested DNA from transmissible strain GII-3 and non-transmissible strain 44. As shown in Fig. 4(b), hybridization with probe 32F1/32R, corresponding to a part of the *p32* gene, revealed only one fragment in undigested DNA from strain GII-3 whereas no

Fig. 3. Agarose gel electrophoresis of PCR amplifications with primer pair 32F1/32R (a) and primers designed from the spiralin gene (b) on DNA from *S. citri* transmissible (lanes 2–5) and non-transmissible (lanes 6–9) strains. Lane 1, no DNA. Transmissible strains: lane 2, GII-3; lane 3, Corsica; lane 4, Cyprus; lane 5, Palmyra. Non-transmissible strains: lane 6, 44; lane 7, 26; lane 8, R₈A₂; lane 9, ASP-1. M, molecular size markers (1 kb ladder).

Fig. 4. (a) Agarose gel electrophoresis of total DNA from *S. citri* transmissible strain GII-3 and non-transmissible strain 44. Concentration of the agarose gel was 0.5%, and 2 µg of DNAs migrated under 20 V for 60 h. (b) Southern blot hybridizations of genomic DNAs of strains GII-3 and 44 with 32F1/32R probe. Lanes M₁ and M₂ contain the molecular size markers, the yeast chromosome marker and 1 kb ladder respectively.

DNA fragment was observed in that from strain 44. For both strains, no hybridization occurred between the chromosomal DNA and the probe. No additional faster-migrating fragments were observed in strain GII-3. These results indicate that the *p32* gene is present in the transmissible strain on only one extrachromosomal DNA, not present in the non-transmissible strain 44.

Transmissibility of strain 44 after complementation with the *p32* gene

To determine whether the P32 protein played a role in the transmission of *S. citri*, we attempted to restore the transmission of the non-transmissible strain 44, by transformation of it with the plasmid pSD4-P32. In insects or plants, spiroplasmas are not subjected to antibiotic selection pressure. For this reason it was important to obtain a stable complemented strain in which the complementing plasmid pSD4-32 had integrated into the chromosome. From hybridizations between total DNA of complemented strain 44 and probes specific to the *tetM*, *oriC* and *p32* genes, integration of the plasmid was shown to be at the *oriC* region (data not shown). Expression of the *p32* gene in the complemented strain 44 (named 44-P32) was revealed by protein analysis on a 2-D gel. Comparison of the protein patterns of strains 44 and 44-P32 confirmed the heterologous expression of

P32 as revealed by the presence of the protein in the 2-D pattern of strain 44-P32 (Fig. 5).

To further study the ability of the complemented strain to be transmitted by the insect vector, experimental transmission assays were carried out. Spiroplasmas were transmitted to periwinkle plants via injection of the leafhopper vector *C. haematoceps* as indicated in Methods. In the insect, the wild-type GII-3 and the complemented strain 44 multiplied to the same titre of 10^7 spiroplasmas per insect whereas the multiplication of strain 44 was slightly lower (10^6 per insect). After the 2 week transmission period, the insects were removed and symptom production was monitored for at least 12 weeks. As expected, with strain GII-3, plants developed severe symptoms within 2 weeks after the transmission period. In the case of the control strain 44, in which plasmid pSD4 had integrated at the *oriC* region, plants did not develop symptoms, showing that the presence of pSD4

Fig. 5. 2-D analyses of proteins from *S. citri* strain 44 and strain 44-P32 obtained after complementation of strain 44 with the *p32* gene. Proteins (200 µg) were submitted to analysis as described in Methods. Gels were silver stained. P32 protein is present only in the complemented strain 44-P32.

sequences in the chromosome of strain 44 did not affect its non-transmissibility. Despite the expression of P32 in strain 44-P32, no transmission to periwinkle plants occurred up to 12 weeks after the transmission period.

S. citri infection of salivary glands

The distribution of the three *S. citri* strains GII3, 44 and 44-P32 was investigated in the salivary glands of *C. haematoceps*, 12 days after injection. Whatever the strain injected into the insects, non-helicoidal forms of *S. citri* were encountered in two types of salivary gland cells, containing secretion granules or not. For GII-3, individual or clustered spiroplasmas were found in cytoplasmic membrane-bound vesicles, located at the periphery of the salivary cells (Fig. 6a). The basal lamina and plasmalemma remained intact. In the salivary glands of insects infected with strain 44 (Fig. 6b), numerous round-shaped wall-free bacteria accumulated between the basal lamina and the plasmalemma. No degradation of the membranes was seen. No spiroplasmas appeared to be attached to the plasmalemma and none was seen inside the cytoplasm. However, in the salivary glands of insects infected by the complemented strain 44-P32, spiroplasmas were extensively found between the basal lamina and the plasmalemma, in cells containing secretion granules. Few spiroplasmas were observed in close contact with the plasmalemma (Fig. 6c).

DISCUSSION

Comparison of a GII-3 reference map with maps obtained from transmissible and non-transmissible strains revealed a remarkable conservation of overall protein profiles. Such conservation, in addition to the reproducibility of the *in vitro* culture and protein extraction conditions we used, is in agreement with published and unpublished data on the variation in electrophoretic mobility of spiralin (Foissac *et al.*, 1996a; C. Saillard & A. Hosseini Pour, unpublished

data). According to spiralin gene variability, all the strains used in this study belong to the *S. citri* group originating from the Mediterranean and Middle East countries. Despite this feature, two obvious differences were observed: two trains of spots present in the protein patterns of the transmissible strains were missing in the non-transmissible strains. The first train of protein spots, having a molecular mass of approximately 85 kDa, comprised a set of eight proteins homologous to the previously described P89 (Berg *et al.*, 2001), a spiroplasma membrane protein directly involved in spiroplasma–insect cell interaction (Yu *et al.*, 2000). In strain GII-3, these eight proteins belonging to the ScARP protein family were encoded by five plasmids ranging from 12.9 to 27.8 kbp (accession nos AJ969069, AJ969070, AJ969071, AJ969072, AJ969073).

The abundant protein in the second train of spots with an apparent molecular mass of 32 kDa but a theoretical mass of 27.5 kDa had no significant hits with sequences in the GenBank or MolliGen databases, or those deduced from the partially genome sequence of *S. kunkelii* translated on the six possible frames. This protein, not found in the phytopathogenic strain of *S. kunkelii*, was probably specific to *S. citri* and is a putative candidate to play a role in the transmission process.

Our Southern blot hybridizations of genomic DNA from transmissible and non-transmissible strains with a *p32* probe, as well as PCR amplification of the *p32* gene, showed that this gene was present only in the transmissible strains. In the genome of strain GII-3, the *p32* gene is carried by an extrachromosomal DNA of high molecular mass corresponding to the larger plasmid of 35.5 kbp predicted by the *in silico* analysis of the *S. citri* plasmid content (accession no. AJ969074). The non-transmissible strain 44 has no plasmid encoding the P32 protein. Further Southern blot experiments have shown that all non-transmissible strains from different origins have lost the plasmids carrying the ScARP

Fig. 6. Transmission electron micrographs of *S. citri* in *C. haematoceps* salivary glands. (a) Strain GII-3; individual bacteria were located in cytoplasmic vesicles presumably derived from the plasmalemma. (b) Strain 44; numerous bacteria accumulated within the basal lamina and the plasmalemma. (c) Strain 44-P32; note the close contact between some *S. citri* cells and the plasmalemma (arrows with *). Arrows indicate basal lamina (BL) and plasmalemma (PL). Bars, 0.5 nm.

and *p32* genes ranging from 12.5 to 35.5 kbp (data not shown). Taken together, these results demonstrate that strains lacking all plasmids and devoid of P32 and ScARP proteins are non-transmissible by insects. A correlation between the loss of high-molecular-mass plasmids and the non-transmissible phenotype was observed for all *S. citri* strains used in our study.

Results of Triton X-114 partitioning of *S. citri* GII-3 total cell lysate revealed that P32 was mostly found in the hydrophilic fraction. Attempts to extract the small amount of P32 protein from the detergent fraction were unsuccessful, supporting the prediction that P32 is a cytoplasmic protein that could be associated with membrane proteins. Analysis of the amino acid sequence of the 27.5 kDa protein was in agreement with the experimental results and revealed the absence of transmembrane domains. *S. citri* was able to grow and survive in two different hosts (plant and leafhopper), and its survival required adaptation to these different environments. During transmission, a range of factors such as tissue environment (gut and salivary glands) and physiological state of the vector could induce interaction of the P32 cytoplasmic protein with membrane proteins. In addition, *in silico* analysis predicted several putative phosphorylation sites in P32, suggesting the presence of functional kinases and phosphatases in *S. citri*. To adapt to environmental changes, *S. citri* might have developed a complex network of regulatory systems acting at different levels including post-translational modification. In prokaryotes, protein phosphorylation and dephosphorylation have been shown to be involved in survival and virulence of pathogens within the host (Wang *et al.*, 1998; Cowley *et al.*, 2004). In a variety of conditions inside the hosts, reversible P32 phosphorylation could lead to a conformational change of the protein and unexpected functions. Thus, P32 could participate in different processes important for adaptation to physiological events encountered in the hosts during transmission and be closely associated with surface membrane proteins mediating attachment of *S. citri* to insect cells. One reported example concerns the elongation factor EF-Tu, mostly found in the cytoplasm, that was also associated with the membrane in *E. coli* (Jacobson & Rosenbusch, 1976) and in *Mycoplasma pneumoniae* (Dallo *et al.*, 2002). In this latter mollicute, EF-Tu exhibited a novel function of binding to fibronectin, which may aid adhesion to host cells and colonization of tissues (Dallo *et al.*, 2002). The possible involvement of EF-Tu phosphorylation in the regulation of protein synthesis for adaptation of *Listeria monocytogenes* to the stressful environments in the host has also been reported (Archambaud *et al.*, 2005). Two known *S. citri* proteins are possible candidates to interact with P32 in a protein complex. These are ScARPs, associated with spiroplasma adhesion to insect cells (Yu *et al.*, 2000; Berg *et al.*, 2001), and spiralin, acting as a lectin binding to insect glycoproteins (Killiny *et al.*, 2005).

Functional complementation of the non-transmissible strain 44 with the *p32* gene did not restore the transmissible

phenotype despite the expression of P32 in the complemented strain 44 (44-P32). As strains 44, 44-P32 and GII-3 reached titres usually considered more than enough for an efficient transmission (10^5 spiroplasmas per insect), the failure to restore the transmission suggested that strain 44-P32 was probably affected, like strain 44, in its ability to move from the haemolymph into the salivary glands. In the non-transmissible strain 44-P32 the group of ScARP proteins is also missing. These results support the idea that P32 may be necessary but not sufficient for spiroplasma adhesion and invasion of insect cells.

Our electron microscopic observations of the transmissible strain GII-3 within membranous pockets, apparently formed by invagination of the plasmalemma, also suggest that a receptor-mediated endocytotic mechanism was probably involved in the spiroplasma's crossing of salivary gland barriers, as postulated by others (Fletcher *et al.*, 1998). Such a mechanism necessarily implies a specific recognition between the spiroplasma and a receptor on the plasmalemma outer surface. In *C. haematocaps* salivary glands infected by strain 44, spiroplasmas accumulated within the space between the basal lamina and the plasmalemma. No spiroplasmas appeared to be attached to the plasmalemma and no cytoplasmic vesicles were observed, suggesting no specific recognition between *S. citri* and the plasmalemma. The basal lamina crossed by the spiroplasmas was intact, as described previously for *S. citri*-infected *C. tenellus* salivary glands (Kwon *et al.*, 1999). In contrast, a physical degradation of the lamina by the tip structure of *S. kunkelii* in the midgut epithelium of *Dalbulus maidis* was observed (Özbek *et al.*, 2003). In the salivary glands of *C. haematocaps* infected with the *p32*-complemented strain 44, which remained non-transmissible, we frequently noticed a close contact between round-shaped sections of spiroplasmas and the plasmalemma of the insect cells. A unique difference between the two non-transmissible strains is the presence of P32 protein in the complemented strain 44-P32. This suggested that P32 allowed the spiroplasma to recover a part of its affinity for a membranous factor. Does P32 act as a recruiting protein necessary for adhesion but insufficient for invasion of insect vector salivary gland cells? Further experiments will be necessary to explore such a possibility.

Taken together our results show clearly that there was more than one protein involved in the adhesion, revealing a complex dialogue between *S. citri* and insect cells during transmission. Even though P32, ScARPs and spiralin were shown to participate in adhesion to host cells, their precise role during the process of transmission remains to be determined. The P32 protein present only in the transmissible strains is a useful marker for insect transmission.

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