Infection of the *Circulifer haematoceps* cell line Ciha-1 by *Spiroplasma citri*: the non-insect-transmissible strain 44 is impaired in invasion

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Successful transmission of *Spiroplasma citri* by its leafhopper vector requires a specific interaction between the spiroplasma surface and the insect cells. With the aim of studying these interactions at the cellular and molecular levels, a cell line, named Ciha-1, was established using embryonic tissues from the eggs of the *S. citri* natural vector *Circulifer haematoceps*. This is the first report, to our knowledge, of a cell line for this leafhopper species and of its successful infection by the insect-transmissible strain *S. citri* GII3. Adherence of the spiroplasmas to the cultured Ciha-1 cells was studied by c.f.u. counts and by electron microscopy. Entry of the spiroplasmas into the insect cells was analysed quantitatively by gentamicin protection assays and qualitatively by double immunofluorescence microscopy. Spiroplasmas were detected within the cell cytoplasm as early as 1 h after inoculation and survived at least 2 days inside the cells. Comparing the insect-transmissible GII3 and non-insect-transmissible 44 strains revealed that adherence to and entry into Ciha-1 cells of *S. citri* 44 were significantly less efficient than those of *S. citri* GII3.

INTRODUCTION

Spiroplasma citri is a phloem-limited, plant-pathogenic bacterium belonging to the class *Mollicutes*. It is the aetiological agent of the citrus stubborn and horseradish brittle root diseases (Fletcher *et al.*, 1981; Saglio *et al.*, 1971, 1973). The spiroplasmas inhabit the phloem sieve elements and are transmitted from plant to plant by phloem-sap-feeding insects, the leafhoppers *Circulifer haematoceps* in the Mediterranean area and Middle East (Fos *et al.*, 1986) and *Circulifer tenellus* in the USA (Liu *et al.*, 1983). The development of specific genetic tools and the availability of an experimental transmission system have made *S. citri* a unique model for studying plant mollicute–host interactions (Bové *et al.*, 2003; Foissac *et al.*, 1996; Gaurivaud *et al.*, 2000; Renaudin & Lartigue, 2005). Experimental transmission of *S. citri* to periwinkle (*Catharanthus roseus*) plants through injection into the leafhopper vector *C. haematoceps* has also revealed that some *S. citri* strains, such as wild-type GII3, were transmitted while others, such as

S. citri R8A2 and 44, were not, although their multiplication rates in the leafhopper were similar to that of GII3 (Berho *et al.*, 2006a, 2006b).

Various studies have addressed the movement of spiroplasmas into their leafhopper vectors (Ammar *et al.*, 2004; Ammar & Hogenhout, 2005; Kwon *et al.*, 1999; Ozbek *et al.*, 2003). For transmission to occur, the ingested spiroplasmas must pass through the intestinal barrier and spread via the haemolymph where they multiply and circulate to distant tissues, including the salivary glands, from which they are injected into the phloem through the salivary duct during feeding. To explain how spiroplasmas cross the two barriers, i.e. the gut epithelium and salivary gland membrane, a model based on electron microscopy observations has been proposed. Spiroplasmas probably adhere to receptors on the apical membrane of the gut epithelial cells and are then taken into the cells by endocytosis (Ammar *et al.*, 2004; Fletcher *et al.*, 1998). According to this model, spiroplasmas migrate within the cells and are released by exocytosis before reaching the haemolymph through the basal lamina. A similar inverse mechanism has been suggested for migration of the

Abbreviations: CLSM, confocal laser scanning microscopy; ScARP, *S. citri* adhesion-related protein.

spiroplasmas from the haemolymph into the salivary duct through the salivary gland cells (Kwon *et al.*, 1999). In the insect host, spiroplasmas also invade various other cell types, including muscle and epithelial cells of Malpighian tubules (Kwon *et al.*, 1999; Ozbek *et al.*, 2003). Thus, intimate interactions are likely to occur between spiroplasmas and cells of different insect organs. However, little is known about the bacterial and insect proteins involved in these interaction processes.

To efficiently establish infection, pathogenic bacteria express a variety of surface proteins, including adhesins. These molecules play a key role in the interactions with host cells, especially in the mollicutes that lack a cell wall (Rottem, 2003). In the plant mollicute *S. citri*, several proteins have been implicated in the transmission of the spiroplasma by its leafhopper vector. In *S. citri* BR3, the adhesion-related protein SARP1 was thought to be required for adhesion of spiroplasmas to insect cells *ex vivo*, suggesting that this protein is involved in the interaction of the spiroplasmas with insect cells *in vivo* (Berg *et al.*, 2001; Yu *et al.*, 2000). Using a reverse genetics approach, it has been shown that inactivation of the gene encoding the putative lipoprotein Sc76 resulted in a dramatic decrease of transmission efficiency and that transmission was restored through complementation with the wild-type gene, indicating that this protein plays a role in the transmission process (Boutareaud *et al.*, 2004). Similarly, an *S. citri* mutant devoid of spiralin, the major lipoprotein at the cell surface, was poorly transmitted compared with the wild-type strain GII3 (Duret *et al.*, 2003). Experiments *in vitro* revealed that spiralin acts as a lectin, binding to glycoproteins of the vector insect, and therefore may function as a ligand able to interact with uncharacterized insect surface protein receptors (Killiny *et al.*, 2005). More recently, sequencing of *S. citri* plasmids revealed that they encode proteins potentially involved in transmission (Saillard *et al.*, 2008). In *S. citri* GII3, plasmid pSci6 encodes the hydrophilic protein P32 associated with insect transmissibility (Killiny *et al.*, 2006) whereas pSci1–5 encode eight adhesion-related proteins (ScARPs) sharing strong similarities with SARP1 of pBJS-O of *S. citri* BR3 (Joshi *et al.*, 2005). Interestingly, the non-insect-transmissible strain *S. citri* 44 has no P32 and no ScARPs and even does not carry pSci1–6 (Berho *et al.*, 2006b). Moreover, successful transmission of *S. citri* 44 transformants containing pSci6 via injection into the leafhopper vector strongly suggests that genetic determinants required for insect transmission of *S. citri* are encoded by pSci6 (Berho *et al.*, 2006a). As a whole, these studies clearly indicate that various spiroplasmal proteins are involved in the transmission process. However, the precise role of these spiroplasma factors in the interactions with insect cells remains to be elucidated.

Cell lines of animal origin have often been used to study interactions between pathogenic organisms and host cells *ex vivo* (Cherry, 2008; Veiga & Cossart, 2006). These cellular models are currently used to study the mechanisms

of cell infection by a wide variety of animal pathogens, including mycoplasmas (Burnett *et al.*, 2006; Drasbek *et al.*, 2007; Fleury *et al.*, 2002; Giron *et al.*, 1996; Svenstrup *et al.*, 2002; Winner *et al.*, 2000). However, leafhopper cell lines that have been established to study the interactions between plant pathogenic mollicutes and their specific insect vectors are still very few (Omura & Kimura, 1994; Steiner *et al.*, 1984; Wayadande & Fletcher, 1998). Among them, the CT1 cell line was derived from *C. tenellus* embryos, but this cell line consists of a heterogeneous population of cells, made up primarily of four different cell types (Wayadande & Fletcher, 1998). Hitherto, studying the interactions between *S. citri* and cells of its leafhopper vector *C. haematoceps*, and in particular those during the entry process, has been limited by the lack of a relevant cell culture system. This prompted us to develop a cell line from *C. haematoceps* as a complementary approach to compare *S. citri* strains that differ in their insect transmissibility. In this study, we have used embryos of *C. haematoceps* to initiate primary tissue cultures. From three initial primary cultures, one led to the development of a continuous cell line, named Ciha-1, the first, to our knowledge, reported for this leafhopper. We also compared the uptake of two *S. citri* strains, the insect-transmissible GII3 and the non-insect-transmissible 44, by these cultured cells.

METHODS

***C. haematoceps* eggs treatment.** Healthy *C. haematoceps* were reared on stock plants, in cages housed in a room with a 16 h:8 h light:dark photoperiod at 27 °C during the day and 24 °C during the night. Eggs in which eyespots had migrated approximately two-thirds of the length of the embryo were removed from midribs and veins with fine needles. Approximately 100 intact eggs were collected in a microcentrifuge tube containing 1 ml Schneider's *Drosophila* medium (Invitrogen). After medium removal, the eggs were surface sterilized with a 20% bleach solution for 3 min, followed by two sterile water rinses. They were further treated with 70% ethanol for 3 min, and rinsed three times with sterile water. The eggs were gently ground in 100 µl culture medium made of 200 ml Schneider's *Drosophila* medium, 25 ml Grace's insect cell culture medium (Invitrogen), 2.5 ml histidine buffer [0.057 M histidine monohydrate (Sigma), pH 6.2], 25 ml heat-inactivated fetal bovine serum (Lonza), 1.2 ml G-5 supplement (Invitrogen) supplemented with 1.25 µg fungizone ml⁻¹ (Invitrogen) and 50 µg penicillin/streptomycin ml⁻¹ (Invitrogen). After centrifugation at 1000 g for 3 min, the pellet was suspended in 400 µl cell dissociation buffer (Invitrogen) and incubated at 28 °C for 10 min. The mixture was then centrifuged at 1000 g for 3 min. Fresh medium was added to the pellet to a final volume of 0.5 ml. The entire volume, including chorions, was transferred to a sterile 12.5 cm² culture flask and incubated at 28 °C for 1 h, before 2.5 ml fresh medium was added to the flasks. The culture medium was replaced by fresh medium the following day and then two-thirds of the medium was changed every week until the first colonies grew.

Cell line initiation and culture. When the surface of a colony reached a size of about 4 mm² (approximately 4 months), the cells were trypsinized with TrypLE (Invitrogen) and placed into a 24 mm diameter well; two-thirds of the culture medium was changed twice a week for 3 months, after which the cells were passed every 7 days.

Aliquots (1 ml) of cultured cells were frozen in liquid nitrogen for culture reference. *C. haematoceps* cells were designated Ciha-1 (*C. haematoceps*-1). After the cell line was established the cells were cultivated at 32 °C and passed every 7 days with an additional change of medium during the week.

S. citri strains. The *S. citri* GII3 wild-type strain was originally isolated from its leafhopper vector *C. haematoceps*, captured in Morocco (Vignault *et al.*, 1980), and was experimentally transmitted to periwinkle (*Catharanthus roseus*) through injection to the leafhopper *C. haematoceps* (Foissac *et al.*, 1996). *S. citri* 44 was isolated from a stubborn-diseased sweet orange tree in Iran (Hosseini Pour, 2000). In contrast with *S. citri* GII3, *S. citri* 44 cannot be transmitted to periwinkle under these conditions. Spiroplasmas were grown at 32 °C in SP4 medium (Whitcomb, 1983) from which yeast extract was omitted.

PCR amplification and partial sequencing of 16S rDNA from C. haematoceps insect and Ciha-1 cells. Primers LR-J-12887 (5'-CCGGTYTGAACCTCARATCA-3') and LR-N-13398 (5'-CRMCTGT-TTAWCAAAAACAT-3') were used to amplify and partially sequence the mitochondrial 16S rDNA from leafhopper and Ciha-1 cell DNA (Takiya *et al.*, 2006). *C. haematoceps* DNA extraction was performed as described by Maixner *et al.* (1995). DNA from the Ciha-1 cell line was extracted from 10⁵ cells using a Wizard Genomic DNA purification kit (Promega), following the supplier's instructions. PCR was performed on isolated DNA using primers LR-J-12887 and LR-N-13398 under standard reaction conditions with about 10 ng template DNA. The PCR products were analysed by agarose gel electrophoresis and sequenced. A search for homologies in general databases was carried out using the BLAST program.

Adhesion assays. The binding assay was performed at 4 °C, a temperature that is likely to prevent bacterial internalization. Ciha-1 cells (about 2 × 10⁵ cells per well of 24-well plates) were inoculated with 30–300 spiroplasmas per cell in 1 ml culture medium with or without serum, and without antibiotics. To calculate the m.o.i., the trypsinized Ciha-1 cells were counted using a Malassez chamber. The spiroplasma titre was determined as c.f.u. counts. Cells were pre-incubated at 4 °C for 1 h before incubation with spiroplasmas at 4 °C for 4 h. The unbound spiroplasmas were removed by washing the cells three times with 1 ml Schneider's Drosophila medium and three times with 1 ml 1 × PBS, pH 7.2, before trypsinization with TrypLE (Invitrogen). Serial dilutions of the mixture were plated onto SP4 agar and incubated at 32 °C for counting c.f.u. The percentage of cells with adherent spiroplasmas was estimated by dividing the total c.f.u. counts per well by the number of cells in one well × 100. In this evaluation, 1 c.f.u. is expected to represent one Ciha-1 cell associated with spiroplasma. To estimate the release of spiroplasmas from the cells during trypsinization, cells with associated spiroplasmas were trypsinized and then pelleted by centrifugation at 1000 g for 3 min before the spiroplasma titre in the supernatant was determined by c.f.u. counting. In the control experiment, cells were omitted to evaluate non-specific adherence of spiroplasmas to the plastic plate. The percentage of adherent spiroplasmas was estimated by dividing the total c.f.u. counts per well by the number of spiroplasmas in one well × 100. Each experiment was carried out four times. Student's *t*-test was used for statistical analyses.

Invasion assays. Ciha-1 cells (about 2 × 10⁵ cells per well of 24-well plates) were inoculated at an m.o.i. of 15–40 spiroplasmas per cell and incubated for 4 h at 28 or 32 °C, which is the optimal growth temperature of *S. citri*, in culture medium with or without serum, and without antibiotics. Entry of the spiroplasmas into the Ciha-1 cells was quantified using the gentamicin protection assay and visualized using confocal laser scanning microscopy (CLSM) of immunofluorescent preparations (see below). Cells were washed three times in Schneider's Drosophila medium to remove extracellular bacteria. To

determine the total number of cells with associated spiroplasmas, the cells were trypsinized immediately after washing. For invasion assays, fresh culture medium supplemented with 400 µg gentamicin ml⁻¹ was added and after incubation at 32 °C for 3 h to kill extracellular bacteria, the cells were washed three times with 1 ml Schneider's Drosophila medium and three times with 1 ml PBS, trypsinized and plated for c.f.u. counting. In a control experiment, it was shown that gentamicin treatment of a spiroplasma culture (10⁸ c.f.u. ml⁻¹) repeatedly killed more than 99.8% of spiroplasmas within 3 h (i.e. 0.2% spiroplasmas escaped this gentamicin treatment). The percentage of cells with associated spiroplasmas (determined in the absence of gentamicin) and the percentage of infected cells (i.e. cells containing internalized spiroplasmas, determined in the presence of gentamicin) were estimated by dividing the total c.f.u. per well by the number of cells in one well × 100. In this evaluation, 1 c.f.u. is expected to represent one Ciha-1 cell with associated spiroplasma or containing internalized spiroplasmas. The percentage of cells with adherent spiroplasmas was calculated as the difference between the percentage of cells with associated spiroplasmas and that of infected cells. The number of false-positives of infected cells due to adherent spiroplasmas that were resistant to the gentamicin treatment depended on the number of spiroplasmas attached to insect cells. The percentage of false-positives was estimated as follows: [(c.f.u. in the absence of gentamicin × 0.2%)/c.f.u. in the presence of gentamicin] × 100. Trypan blue vital staining was used to study Ciha-1 viability. Each experiment was carried out three times. For statistical analysis Student's *t*-test was used when appropriate.

S. citri survival in Ciha-1 cells. The insect cells were infected by *S. citri* GII3 at an m.o.i. of 30 and incubated at 32 °C for 18 h in 24-well plates. After three washes in Schneider's Drosophila medium, fresh culture medium containing 100 µg gentamicin ml⁻¹ was added in order to kill extracellular spiroplasmas, and the cells were grown for 1 or 2 days at 32 °C. At the end of the incubation period, cells were washed and trypsinized and the number of infected cells was determined by plating dilutions onto SP4 agar plates. The release and intercellular dissemination of spiroplasmas was evaluated using the same protocol except for the following modifications. After the incubation period and three washes in Schneider's Drosophila medium, extracellular bacteria were killed by a treatment with 400 µg gentamicin ml⁻¹ for 3 h. After removing the supernatant, fresh culture medium without antibiotic was added to the monolayer and infected cells were further incubated for 2 h. The c.f.u. in the supernatant (spiroplasmas released from cells in the medium) and in the cell fraction (spiroplasmas released from cells but still adherent) were determined by plating successive dilutions on agar plates and comparing with those obtained in the presence of gentamicin, as determined in parallel control experiments. The infected cells were further incubated 1 and 2 days after gentamicin treatment. Each experiment was performed in triplicate.

Immunofluorescence. Detection of *S. citri* GII3 within insect cells was performed by using the double immunofluorescence assay described by Heesemann & Laufs (1985) with minor modifications. Ciha-1 cells were grown on coverslips in 24-well plates and infected with *S. citri* as described above. All further steps were carried out at room temperature. After three washes in Schneider's Drosophila medium, cells and bacteria were fixed for 15 min with 4% paraformaldehyde in PBS. Spiroplasmas were stained in a double-step procedure using two different fluorescent dyes. The cells were incubated with an anti-*S. citri* rabbit serum diluted 1:500 in PBS–BSA solution (PBS containing 1% BSA) for 30 min. After three washes with PBS, the cells were incubated for 30 min with Alexa 633-conjugated goat anti-rabbit antibodies (Invitrogen) diluted 1:200 in PBS–BSA buffer. As a result, only extracellular bacteria were stained with Alexa 633. After three washes in PBS, the cells were permeabilized by treatment with 0.2% Triton X-100 in PBS–BSA

solution for 15 min, washed twice with PBS and overlaid with anti-*S. citri* rabbit serum diluted 1:500 in PBS-BSA solution for 30 min. After three washes with PBS, the cells were then incubated for 30 min with both Alexa 488-conjugated goat anti-rabbit antibodies (Invitrogen) diluted 1:200 in PBS-BSA buffer and Alexa 568-phalloidin (Invitrogen) to stain the actin filaments. The specificity of immunostaining was evaluated by omitting the anti-spiroplasma antibodies. The coverslips were mounted in anti-fading ProLong Gold Reagent (Invitrogen). Immunofluorescent samples were imaged using a TCS SP2 upright Leica confocal laser scanning microscope, with a $\times 63$ oil immersion objective lens at 1024×1024 pixel resolution. Fluorochromes were detected sequentially frame by frame. The images were coded blue (Alexa 633) and green (Alexa 488), giving cyan in merged images. Actin (Alexa 568) was coded red.

Transmission electron microscopy. Ciha-1 cells were grown on coverslips in 24-well plates and infected with *S. citri* at an m.o.i. of 30 for 18 h in culture medium. Cells and bacteria were fixed for 45 min with 2.5 % glutaraldehyde in Schneider's medium at room temperature. They were washed three times in PBS and post-fixed with 1 % buffered osmium tetroxide for 45 min. After three washes in PBS, they were maintained in 1 % aqueous tannic acid for 15 min. They were dehydrated in a graded ethanol series. Then ethanol was progressively replaced by Epon resin. The samples were embedded by turning gelatin capsules upside down, directly on the coverslips coated with the cell monolayer. Polymerization was carried out at 60 °C for 24 h. Micrographs were taken at 80 kV on a FEI CM10 transmission electron microscope equipped with an AMT $\times 60$ digital camera (Elexience).

RESULTS

Ciha-1 cell line

Continuous culturing of cells isolated from fragmented embryos of leafhopper (*C. haematoceps*) for 7 months resulted in three cell lines, the tissue origin of which was unknown. Based on its homogeneous cell population consisting of 90 % of cells with epithelial-type morphology, the first cell line was selected and designated Ciha-1. Similar cells represented approximately one-third of the cell population in the second cell line, which also contained two other cell types. The third cell line was quite distinct from the other two, as the majority of cells had a smaller size and it was proved to be much more resistant to trypsinization. The Ciha-1 cells were passed once a week with a 1:3 dilution over 50 times. The doubling time, as estimated from the growth curves, was approximately 2 days. After freezing in liquid nitrogen, successful recovery of Ciha-1 cells could be obtained for over 1 year.

Cell morphology was examined by light microscopy. Fig. 1 shows a monolayer of Ciha-1 cells, some of which tend to form aggregates as indicated by the arrow (Fig. 1a). A large majority of cells were probably epithelial cells, as suggested by their morphology (Fig. 1b). Whereas the isolated cells looked elongated and granulated, they sprawled when at confluence (Fig. 1).

The leafhopper origin of the Ciha-1 cell line was checked by partial sequencing of the 16S rDNA sequences from both the cultured Ciha-1 cells and the whole insect.

Sequences from the 511 bp fragments amplified from Ciha-1 cell DNA and from the *C. haematoceps* insect DNA were found to be identical (data not shown). A BLAST search in the GenBank database revealed that this sequence shares 83 % identity with the 16S rDNA of the leafhopper *Nakahararus maculosus* as the closest relative.

Adhesion to Ciha-1 cells by *S. citri*

Depending on the pathogenic bacteria, adhesion assays are carried out in the presence (Boyle *et al.*, 2007) or absence (Felek *et al.*, 2008) of serum. In the case of *S. citri*, the ability to adhere to Ciha-1 cells was determined both in the presence and absence of serum. Each c.f.u. count was expected to represent one Ciha-1 cell with adherent spiroplasmas rather than one isolated spiroplasma. The treatment of spiroplasmas with trypsin in the absence of Ciha-1 cells had no effect on spiroplasmal viability and hence the number of c.f.u. It should be noticed that trypsin treatment of Ciha-1 cells resulted in the release of a few cell-adherent spiroplasmas. The amount of these released spiroplasmas accounted for one-tenth of the c.f.u. counts for both strains GII3 and 44. As a result, the number of cells with adherent spiroplasmas may be slightly overestimated. Since this bias occurs for both strains GII3 and 44, this has no effect on the determination of the relative adherence/entry of *S. citri* 44 compared with GII3.

S. citri GII3 did adhere to the Ciha-1 cells in culture, regardless of the presence or absence of serum in the medium (Table 1). The number of cells with adherent spiroplasmas ranged from 2 % at low m.o.i. (40) to 30 % at high m.o.i. (300). In the case of the non-insect-transmissible strain *S. citri* 44, the adhesion rate was significantly lower than that of *S. citri* GII3 (Table 1; $P < 0.01$, using Student's *t*-test). Similar to *S. citri* GII3, the presence of serum in the medium had no effect on *S. citri* 44 adhesion. The proportion of adherent spiroplasmas was estimated to be 0.05–0.09 % and 0.01 % for *S. citri* GII3 and *S. citri* 44, respectively. However, these percentages could be slightly underestimated because under these experimental conditions, the number of spiroplasmas adherent to the cells varied from one to two per cell (data not shown). In the absence of cells, these percentages were 0.005 and 0.01 % for *S. citri* GII3 and *S. citri* 44, respectively.

S. citri GII3 adhesion to the Ciha-1 cells was confirmed by transmission electron microscopy. Spiroplasmas were observed in close proximity of the Ciha-1 cells (Fig. 2c, infected cells and Fig. 2a, control cells). Ciha-1 cells showed small extensions of the cell body that could be easily distinguished from the spiroplasmas according to the density and nature of the content (Fig. 2b and d). Close contacts (gap size 7–13 nm) between the spiroplasma surface and the Ciha-1 cell membranes were observed (Fig. 2d and e). The invaginations of the eukaryotic membrane, as illustrated in Fig. 2(e), strongly suggested that the spiroplasmas adhered to the Ciha-1 cells.

Fig. 1. Morphology of Ciha-1 cells cultured from embryos of *C. haematoceps*. (a) Low magnification ($\times 20$) of the Ciha-1 monolayer by phase-contrast microscopy. A cellular aggregate is indicated by the arrow. (b) Enlarged view by differential interference contrast microscopy (scale bar, 10 μm).

Invasion of Ciha-1 cells by *S. citri*

To compare the ability of *S. citri* GII3 and 44 to enter the leafhopper cells, gentamicin protection assays were carried out. Due to limited accumulation of gentamicin into the eukaryotic cells, the intracellular spiroplasmas are thought to be protected from the bactericidal effect, whereas the extracellular spiroplasmas are killed. The spiroplasmas were grown in the cell culture medium, in which they maintained their helical morphology and grew at the same rate as in the SP4 medium (data not shown). In these experiments, gentamicin was used at $400 \mu\text{g ml}^{-1}$, i.e. 10 times the MIC for both strains GII3 and 44. Such a treatment had no effect on Ciha-1 cell viability.

Gentamicin protection assays proved that *S. citri* GII3 was able to invade Ciha-1 cells (Table 2). In these experiments, the percentage of the false-positives due to spiroplasmas resistant to the gentamicin treatment was estimated to be only 1–4 % of total infected cells and proved the gentamicin protection assay reliable for estimating the number of cells with internalized spiroplasmas. The percentage of infected cells was significantly higher at 32°C (2.2 ± 0.4) than at 28°C (1.0 ± 0.05) ($P < 0.01$). *S. citri* GII3 was significantly more invasive than *S. citri* 44, regardless of the conditions used (Table 2, $P < 0.01$ or $P < 0.001$). The entry of *S. citri* 44 into insect cells did not vary with the temperature but in response to the presence or absence of serum in the medium.

The percentage of infected cells was six times lower when serum was present in the medium ($P < 0.01$). At a low m.o.i., the proportion of cells with adherent spiroplasmas in the cell culture medium was significantly higher at 32°C than at 4°C for both bacterial strains ($P < 0.001$). In the presence of serum, the number of cells with adherent spiroplasmas increased in the case of *S. citri* GII3 ($P < 0.01$) and decreased in the case of *S. citri* 44 ($P < 0.01$). For strain 44, the effect of temperature on adhesion was evenly observed in the presence or absence of serum in the incubation medium ($P < 0.01$).

CLSM observations

In the double immunofluorescence assay (Fig. 3), each picture is a representative image from one of three independent experiments. Fig. 3(a–d) shows three CLSM micrographs of the same area of the Ciha-1 monolayer infected for 4 h with *S. citri* GII3 as well as the overlay of these images. Through the double staining method using fluorescent antibodies, intracellular spiroplasmas (green) could clearly be distinguished from those remaining outside the cells for both strains GII3 (Fig. 3d) and 44 (Fig. 3e) (cyan). However, it should be noted that internalization of *S. citri* 44 was a rare event, as the large majority of spiroplasmas associated with Ciha-1 cells remained outside the cells. No fluorescence was detected in the non-infected monolayer

Table 1. Adhesion to Ciha-1 cells by insect-transmissible (GII3) and non-insect-transmissible (44) strains of *S. citri*

Cells were incubated for 4 h at 4°C in culture medium containing serum (Serum) or in the absence of serum (No serum).

Strain	m.o.i.	Incubation medium	c.f.u. ($\pm$ SD)	% cells with adherent spiroplasmas ($\pm$ SD)
GII3	40	Serum	5.4×10^3 ($\pm 1.7 \times 10^3$)	2.0 (± 0.7)
	40	No serum	8.7×10^3 ($\pm 3.1 \times 10^3$)	3.2 (± 1.1)
	300	Serum	5.4×10^4 ($\pm 8.8 \times 10^3$)	20.0 (± 3.3)
	300	No serum	8.1×10^4 ($\pm 2.6 \times 10^4$)	29.8 (± 9.5)
44	30	Serum	1×10^3 ($\pm 1.9 \times 10^2$)	0.4 (± 0.07)*
	30	No serum	7.8×10^2 ($\pm 3.4 \times 10^1$)	0.3 (± 0.01)*
	200	Serum	7×10^3 ($\pm 6 \times 10^2$)	2.6 (± 0.2)*
	200	No serum	7.5×10^3 ($\pm 9.5 \times 10^2$)	2.7 (± 0.3)*

* $P < 0.01$, Student's *t*-test *S. citri* GII3 versus *S. citri* 44 under identical experimental conditions at a similar m.o.i.

Fig. 2. Transmission electron micrograph of Ciha-1 cells infected with *S. citri* GII3. (a, b) Non-infected Ciha-1 cells; note small expansions of the cell body (b). (c–e) Different Ciha-1 cells with adherent *S. citri* GII3 at low magnification (c and d) and at high magnification (e). (f) Detail of (c) showing an extracellular spiroplasma with helical morphology. External spiroplasmas are indicated by arrows (c) or by asterisks (d–f) and zones of contact between spiroplasmas and insect cells are indicated by arrowheads (d and e). m, Mitochondrion; n, nucleus. Bars, 100 nm (f) and (e); 500 nm (b) and (d); 2 µm (a) and (c).

(Fig. 3f) or in infected cells when the anti-spiroplasma primary antibodies were omitted (Fig. 3g).

The presence of spiroplasmas within the insect cells infected *ex vivo* was detected as early as 1 h after inoculation (data not shown). The CLSM observations also revealed that spiroplasmas were able to form micro-colonies when adhering to Ciha-1 cells, even though the vast majority of them adhered as single cells (data not shown). In the cell culture medium, spiroplasmas showed typical helical

morphology (Fig. 2f and Fig. 3d, arrow). In contrast, once inside the cells, they lost their helical morphology and appeared pleomorphic or round (Fig. 3d, arrowhead).

S. citri survival in Ciha-1 cells

Localization of spiroplasmas in insect cells has previously been studied by using electron microscopy (Ozbek *et al.*, 2003). Nevertheless, microscopy analyses did not provide

Table 2. Infection of Ciha-1 cells by insect-transmissible (GII3) and non-insect-transmissible (44) strains of *S. citri*, in gentamicin protection assays

Cells were infected for 4 h at 28 and 32 °C in culture medium containing serum (Serum) and at 32 °C in the absence of serum (No serum).

Strain	m.o.i.	Culture conditions	c.f.u. in wells without gentamicin ($\pm$ SD)	% cells with associated spiroplasmas ($\pm$ SD)	c.f.u. in wells with gentamicin ($\pm$ SD)	% of invaded cells ($\pm$ SD) (% false positives)
GII3	40	Serum, 28 °C	6.9×10^4 ($\pm 1.1 \times 10^4$)	18.9 (± 3.1)	3.7×10^3 ($\pm 1.7 \times 10^2$)	1.0 (± 0.05) (4%)
	30	Serum, 32 °C	7.4×10^4 ($\pm 4.2 \times 10^3$)	14.1 (± 1.3)	9.9×10^3 ($\pm 2 \times 10^3$)	2.2 (± 0.4) (1%)*
	15	No serum, 32 °C	1.4×10^4 ($\pm 5.5 \times 10^3$)	5.8 (± 2.3)*	3×10^3 ($\pm 7.8 \times 10^2$)	1.3 (± 0.3) (1%)
44	30	Serum, 28 °C	2.9×10^3 ($\pm 8 \times 10^2$)	0.8 (± 0.2)†	2.6×10^2 ($\pm 1.2 \times 10^2$)	0.07 (± 0.03) (2%)†
	25	Serum, 32 °C	1.4×10^4 ($\pm 2.1 \times 10^3$)	2.5 (± 0.4)†‡	6×10^2 ($\pm 3.6 \times 10^2$)	0.1 (± 0.08) (4%)†
	20	No serum, 32 °C	1.1×10^4 ($\pm 4.2 \times 10^2$)	4.3 (± 0.2)*	1.4×10^3 ($\pm 2.5 \times 10^2$)	0.6 (± 0.1) (2%)*§

*Significant difference between serum at 28 °C versus serum at 32 °C, or serum at 32 °C versus no serum at 32 °C (by Student's *t*-test), $P < 0.01$.

†Significant difference between *S. citri* GII3 versus *S. citri* 44 under identical experimental conditions (by Student's *t*-test), $P < 0.001$.

‡Significant difference between serum at 28 °C versus serum at 32 °C, or serum at 32 °C versus no serum at 32 °C (by Student's *t*-test), $P < 0.001$.

§Significant difference between *S. citri* GII3 versus *S. citri* 44 under identical experimental conditions (by Student's *t*-test), $P < 0.01$.

Fig. 3. Confocal observation of Ciha-1 cells infected by *S. citri* in the presence of serum at 32 °C. (a) Alexa 633 fluorescence (blue) of extracellular *S. citri* GII3. (b) Endogenous actin, detected using Alexa 568-phalloidin, is shown in red. (c) Alexa 488 fluorescence (green) indicating both extracellular and intracellular *S. citri* GII3. (d) Superimposed image of (a), (b) and (c) indicate extracellular (cyan) and intracellular (green) spiroplasmas, which are circled. The arrow indicates an extracellular helical spiroplasma and the arrowhead shows a round intracellular spiroplasma. (e) Overlay of images corresponding to *S. citri* 44-infected cells stained with the double immunofluorescent assay. (f) Overlay of images corresponding to uninfected cells stained with the double immunofluorescence assay. (g) Overlay of images corresponding to infected cells stained with the double immunofluorescence assay where the primary anti-GII3 antibodies were omitted. Bars, 8 μm (a), (b), (c), (d) and (e); 20 μm (f) and (g).

any clues regarding the survival of spiroplasmas in the intracellular environment. To assess the ability of *S. citri* GII3 to survive within the leafhopper cells, Ciha-1 cells were infected for 18 h in order to increase the number of initially infected cells. The cells were washed and further incubated for 24–48 h in the presence of 100 μg gentamicin ml⁻¹ to kill extracellular spiroplasmas. As a control, it was shown that addition of 100 μg gentamicin ml⁻¹ to a *S. citri* culture (10⁷ c.f.u. ml⁻¹) killed 100 % of the spiroplasmas within 8 h. Though the percentage of infected cells decreased after 24 and 48 h incubations in the presence of gentamicin, it remained high enough (4 %), suggesting that *S. citri* GII3 was able to survive inside the Ciha-1 cells (Fig. 4a). This finding is in agreement with images obtained by confocal microscopy which show spiroplasmas inside the cells after 2 days of infection (Fig. 5a and c). Unexpectedly, some spiroplasmas (or spiroplasmal antigens) were detected outside the cells by immunofluorescence after a 24 and 48 h treatment with 100 μg gentamicin ml⁻¹. These spiroplasmas could either be resistant to the gentamicin treatment or be released from infected Ciha-1 cells.

The decrease in the number of internalized spiroplasmas measured by gentamicin protection assays raised the question of whether the spiroplasmas died within the insect cells or whether they were killed by gentamicin when leaving the cells. To further assess the capability of the internalized spiroplasmas to leave the cells, their presence outside of the infected cells was determined as described in Methods. Assuming that one infected Ciha-1 cell was associated with at least one spiroplasma, 19 % or more (number estimated minus the 7 % of false-positives due to resistance to gentamicin treatment) of the internalized spiroplasmas escaped from the cells within 2 h of the gentamicin treatment. Among these escaped spiroplasmas, 80 % were found to be adherent to the cells. During the 2 days following the gentamicin treatment (400 μg ml⁻¹) the percentage of infected cells increased from 11 to 17, suggesting that in spite of spiroplasma release, the extracellular bacteria multiply in the medium and keep infecting insect cells (Fig. 4b). The finding that the number of both intracellular and extracellular spiroplasmas detected by immunofluorescence increased between 24

Fig. 4. Survival of *S. citri* GII3 in Ciha-1 cells. Cells were infected with *S. citri* GII3 at an m.o.i. of 30 for 18 h (0). (a) After 18 h of infection, 100 μg gentamicin ml⁻¹ was added to the medium for 1 or 2 days. (b) After 18 h of infection, 400 μg gentamicin ml⁻¹ was added for 3 h with further incubation in antibiotic-free culture medium for 1 or 2 days. Error bars represent SEM.

Fig. 5. Survival of *S. citri* GII3 in Ciha-1 cells examined by confocal microscopy. Cells were infected with *S. citri* GII3 at an m.o.i. of 130 for 4 h at 32 °C in the presence of serum. Gentamicin treatment was at 100 µg ml⁻¹ for 1 day (a) or 2 days (c) or at 400 µg ml⁻¹ for 3 h with further incubation in antibiotic-free culture medium for 1 day (b) or 2 days (d). Panels represent superimposed immunofluorescence images as described in Fig. 3 plus DAPI staining. The locations of extracellular spiroplasmas (cyan) are circled; green spots represent intracellular spiroplasmas. Bars, 20 µm.

and 48 h is in good agreement with these results (Fig. 5b and d).

DISCUSSION

Cell culture systems have been extensively used for studying the interactions of pathogenic bacteria with their eukaryotic host at the molecular and cellular levels. With the aim of providing new tools for deciphering the molecular mechanisms that govern the interactions of *S. citri* with its leafhopper vector *C. haematops*, we have developed a new insect cell culture system. The Ciha-1 cell line is derived from primary tissue cultures of unknown origin, muscle, nerve or epithelial cells, issued from leafhopper egg embryos. Like in other insect tissue cultures (Hunter & Hsu, 1996), contracting tissues were observed in the first developing explants. The Ciha-1 cell line resulted from epithelial-type cells attaching to the plate. Cells with such a morphology had been previously observed in cultured, embryonic tissues of leafhoppers (Maramorosch, 1979) and in the CT1 cell line of *C. tenellus* (Wayadande & Fletcher, 1998). However, in addition to epithelial cells, the CT1 cell population comprises several other cell types. In contrast, the Ciha-1 cell line consists of a large majority (more than 90 %) of cells with a single epithelial-type morphology and hence is more suitable for obtaining consistent results.

The ability of *S. citri* to invade the Ciha-1 cells was demonstrated by two independent methods, the gentamicin protection assay and immunofluorescence microscopy. In mollicutes, the gentamicin protection assay has been

previously used to study the invasion of human and animal mycoplasmas into eukaryotic cells. Because *Mycoplasma penetrans* was relatively insensitive to gentamicin, monitoring the invasion of HeLa cells required addition of Triton X-100 at low concentration to kill the external mycoplasmas (Andreev *et al.*, 1995). In *Mycoplasma pneumoniae*, *Mycoplasma gallisepticum* and *Mycoplasma hominis*, the killing effect was obtained with 400, 100 and 50 µg gentamicin ml⁻¹, respectively, in the absence of Triton X-100 (Dessi *et al.*, 2005; Winner *et al.*, 2000; Yavlovich *et al.*, 2004). In *S. citri*, the most appropriate treatment was found to be 400 µg gentamicin ml⁻¹ for 3 h, despite the fact that the MIC was only 40 µg gentamicin ml⁻¹. Although high concentrations of gentamicin may be toxic to eukaryotic cells in some cases (Martinez-Salgado *et al.*, 2007), the gentamicin treatment had no lethal effect on Ciha-1 cells. The intracellular location of spiroplasmas was further confirmed by a double immunofluorescence labelling technique combined with CLSM. Differential labelling of extracellular and intracellular bacteria has been used to prove that mycoplasmas invade eukaryotic host cells (Dessi *et al.*, 2005; Winner *et al.*, 2000). Nevertheless, this is the first time, to our knowledge, that this technique has been applied to monitor the infection of leafhopper insect cells by *S. citri* *ex vivo*.

For transmission to occur, spiroplasmas must invade cells of the leafhopper vector. Adhesion is therefore a prerequisite to the invasion process. Temperature variations are known to influence the adhesion of bacteria to cells cultured *in vitro* (Amorena *et al.*, 1990). In agreement with this, the adhesion

rate of *S. citri* to Ciha-1 cells was found to be 5–10 times higher at 32 °C than at 4 °C. The low temperature (4 °C) might affect the interactions between spiroplasmas and the eukaryotic cells by inhibiting the cellular metabolism. Whether or not the expression and/or accessibility of spiroplasma adhesins could be induced by the insect cells for successful adhesion remains to be explored. Also, dissimilarities between the Ciha-1 cells and leafhopper cells encountered by spiroplasmas *in vivo* are likely to exist and could explain the low percentage of Ciha-1 cells with adherent spiroplasmas. It can be hypothesized that the cell receptor(s) recognized by the spiroplasma *in vivo* is under produced *in vitro* in Ciha-1 cells.

In *Spiroplasma kunkelii*, electron microscopy observations showed that spiroplasmas use the cytoplasm of the intestinal epithelial cells to move from the gut lumen to the haemolymph (Ozbek *et al.*, 2003). Spiroplasmas were located in vesicles as single particles, suggesting no or low replication of the bacteria within the epithelial cells. In *S. citri*, the gentamicin assays also suggested that spiroplasmas entered the Ciha-1 cells, were released, and possibly reinfected insect cells. However, the turnover of the endocytosis–exocytosis phenomenon remains to be investigated. Because of the difficulty of lysing the host cells without killing the cell-wall-less spiroplasmas, direct counting of the intracellular spiroplasmas cannot be carried out. Alternatively, 5-BrdU incorporation has been used to assess *M. hominis* multiplication in *Trichomonas vaginalis* cells (Dessi *et al.*, 2005). By using the gentamicin protection assays combined with double immunofluorescence microscopy, we have shown the ability of the internalized spiroplasmas to survive within the insect cells for at least 2 days. Similar rates of survival have been reported for other mollicutes including *M. hominis*, *M. penetrans*, *M. pneumoniae* and *M. gallisepticum* (Baseman *et al.*, 1995; Dessi *et al.*, 2005; Winner *et al.*, 2000; Yavlovich *et al.*, 2004). *In vivo*, entry of spiroplasmas into the insect cells may or may not cause cytopathologic changes depending on whether cells are muscle cells or Malpighian epithelial cells, respectively (Ozbek *et al.*, 2003). In Ciha-1 cells, no cytopathologic effect was observed up to 2 days after infection when gentamicin was maintained in the medium. In addition, confocal microscopy did not reveal any clusters of internalized spiroplasmas. Therefore, it is likely that in the Ciha-1 cell, spiroplasmas multiply at a low rate or not at all.

When compared with the insect-transmissible strain GII3, the non-insect-transmissible strain 44 was affected in adhesion and entry into the Ciha-1 cells during the first 4 h of infection. However, a small proportion of *S. citri* strain 44 entered the Ciha-1 cells. This is reminiscent of the finding that the non-insect-transmissible strain BR3-G also had the ability to invade the CT1 leafhopper cells *ex vivo* (Wayadande & Fletcher, 1998). Nevertheless, no quantitative data supporting differences between the insect-transmissible and non-insect-transmissible strains BR3-T and BR3-G were obtained in this study. In our work, the

finding that *S. citri* 44 is less adherent and invasive than GII3 strongly suggests a link between the ability to invade Ciha-1 cells and the interactions that occur *in vivo* in the insect vector. However, the ability to invade cells *ex vivo* may not fully reflect the ability to be transmitted by the leafhopper vector.

In the presence of serum, the adhesion and invasion abilities of *S. citri* 44 at 32 °C significantly decreased, whereas adhesion of *S. citri* GII3 significantly increased. While it is known that components from the serum are adsorbed at the spiroplasma surface, how the presence of serum interferes with the invasion process is not. The present data could be correlated with the differences in the membrane protein content in strains 44 and GII3, as reported previously in the literature (Killiny *et al.*, 2006). It can be hypothesized that the non-insect-transmissible strain 44 is deprived of components that are required for the spiroplasmas to efficiently enter the Ciha-1 insect cells. Indeed, it has been shown that, in contrast with GII3, *S. citri* 44 lacks plasmids pSci1–6, which encode the adhesion-related proteins ScARPs (Berho *et al.*, 2006b). These proteins share extensive homology with protein SARP1 of *S. citri* BR3 (Berg *et al.*, 2001), which has also been shown to be involved in adherence to insect cells *ex vivo* (Yu *et al.*, 2000). *S. citri* 44 also lacks the pSci6-encoded protein P32, which has been associated with insect transmission (Killiny *et al.*, 2006). In particular, it has been suggested that P32 contributes to the association of spiroplasmas with membranes of the insect host.

It is known that spiroplasma cells may have distinct morphologies depending on their environment. While helical forms are observed in the phloem sieve elements of the host plant (Bové, 1997), in the gut lumen of the leafhopper host (Ammar *et al.*, 2004) and in cell-free culture medium (Bové, 1997), spiroplasmas within the insect cells display a non-helical, ovoid or flask-shaped morphology. These non-helical forms have been observed in cells of the intestinal epithelium, salivary glands and Malpighian tubule epithelium (Ammar *et al.*, 2004; Kwon *et al.*, 1999; Ozbek *et al.*, 2003). Similar round-shaped morphology was also observed in Ciha-1 cells infected by *S. citri* GII3, suggesting that the spiroplasma morphology shifts from helical to round-shaped during endocytosis. This strongly suggests that the same molecular events are involved in the morphological changes that were observed both *in vivo* and *ex vivo* during insect cell invasion. Therefore, the Ciha-1 cells provide a suitable model for studying morphological changes that occur during the invasion of insect cells by *S. citri*. These specific mechanisms might be induced by spiroplasma–insect cell interactions and might reflect metabolic changes that are crucial for the spiroplasmas to invade and/or survive in the vacuolar environment of the leafhopper cells.

To summarize, the Ciha-1 cell line described in this work is one of the very few established from *Cicadellidae* insects and the first, to our knowledge, from the *S. citri* natural

vector *C. haematoceps*. These cells were infected *ex vivo* by *S. citri* and differences in adhesion/entry were observed between transmissible and non-insect-transmissible strains. In addition, the morphological changes of spiroplasmas that were observed *in vivo* in *S. citri*-infected leafhoppers were also found to occur *ex vivo*, upon infection of Ciha-1 cells.

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