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Perspective

Immune response and survival of *Circulifer haematoceps* to *Spiroplasma citri* infection requires expression of the gene hexamerin

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ABSTRACT

Spiroplasma citri is a cell wall-less bacterium that infects plants. It is transmitted by the leafhopper *Circulifer haematoceps*, which hosts this bacterium in the haemocel and insect tissues. Bacterial factors involved in spiroplasma colonization of the insect host have been identified, but the immune response of the leafhopper to *S. citri* infection remains unknown. In this study, we showed that *C. haematoceps* activates both humoral and cellular immune responses when challenged with bacteria. When infected by *S. citri*, *C. haematoceps* displayed a specific immune response, evidenced by activation of phagocytosis and upregulation of a gene encoding the protein hexamerin. *S. citri* infection also resulted in decreased phenoloxidase-like activity. Inhibition of hexamerin by RNA interference resulted in a significant reduction in phenoloxidase-like activity and increased mortality of infected leafhoppers. Therefore, the gene hexamerin is involved in *S. citri* control by interfering with insect phenoloxidase activity.

1. Introduction

Spiroplasmas are helical, motile bacteria of the class Mollicutes (Cole et al., 1973; Davis and Worley, 1973), a group of cell wall-less microorganisms phylogenetically related to Gram-positive bacteria (Weisburg et al., 1989; Woese et al., 1980). Spiroplasmas are commonly found in arthropods, mostly insects and crustaceans. Some are commensal in the gut of leafhoppers and beetles (Konai et al., 1996; Ammar et al., 2011), while others are mutualists in some *Drosophila* and aphid (Łukasik et al., 2013; Mateos et al., 2006), or pathogenic for honeybee, *Drosophila*, mosquito, moth, crab and shrimp (Anbutsu and Fukatsu, 2003; Clark et al., 1985; Humphery-Smith et al., 1991; Mouches et al., 1984; Nunan et al., 2005; Phillips and Humphery-Smith, 1995; Tabata et al., 2011; Wang et al., 2011; Williamson et al., 1999). Of the 52 spiroplasma species described to date, 3 (i.e., *Spiroplasma citri*, *Spiroplasma kunkelii*, and *Spiroplasma phoeniceum*) are plant pathogens transmitted by insects. These spiroplasmas have the ability to colonize two separate hosts, the leafhopper vector and the host plant, in which they induce disease symptoms (Gasparich, 2010).

S. citri is the causative agent of citrus stubborn and horseradish brittle root diseases (Fletcher et al., 1981; Saglio et al., 1971). In nature, transmission of spiroplasmas to plants is mainly mediated by the two sap feeding leafhoppers (hemipterans): *Circulifer haematoceps* (Fos et al., 1986) and *Circulifer tenellus* (Liu, 1983b) in a persistent propagative manner. Successful transmission of *S. citri*

Abbreviations: AMP, antimicrobial peptide; CFU, colony-forming unit; dsRNA, double-stranded RNA; EST, expressed sequence tag; GFP, green fluorescent protein; GNBP, gram negative binding protein; HLS, haemocyte lysate supernatant; IgG and IgM, immunoglobulin G and M; IL-1, interleukin 1; IMD, immune deficiency; LPS, lipopolysaccharide; MAMP, microbial-associated molecular patterns; NSRO, *Drosophila nebulosa* sex ratio organism; PG, peptidoglycans; PGRPs, peptidoglycan recognition proteins; PO, phenoloxidase; PTU, phenylthiourea; RNAi, RNA interference; RNA-seq, RNA sequencing; ScARPs, *S. citri* adhesion-related proteins; SSH, suppression substractive hybridization; TNF, tumour necrosis factor.

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requires that spiroplasmas cross two physical barriers, the gut epithelium and the salivary gland cells, and multiply in the insect haemocel (Kwon et al., 1999; Liu, 1983a; Russo et al., 1976). Crossing these barriers occurs by mechanisms of endocytosis/exocytosis (Fletcher et al., 1998). To date, two main virulence factors have been described in *S. citri*: the adhesins spiralin and *S. citri* adhesion-related proteins (ScARPs), both of which are involved in the invasion of insect cells by spiroplasmas (Béven et al., 2012; Duret et al., 2014; Killiny et al., 2005; Yu et al., 2000). Spiralin acts as a lectin, binding glycoproteins on insect cells (Duret et al., 2014; Killiny et al., 2005). The receptor of ScARPs has not been identified. Active multiplication of spiroplasmas in the leafhopper occurs with no apparent damage on fitness of insects (Almeida et al., 1997; Liu, 1983a), suggesting that *C. haematoceps* activates an immune response to control spiroplasma multiplication, and that spiroplasma circumvents these host defence mechanisms to complete its life cycle within the insect. To date, there have been no studies about the immune response of *C. haematoceps* to spiroplasma infection. However, it has been shown that the leafhopper *Macrosteles fascifrons* kills sex ratio organism (SRO) spiroplasmas, which are known to colonize *Drosophila* in only two days (Whitcomb and Williamson, 1975).

Insects have evolved several immune strategies to detect and control microbial infections. First are microbial recognition mechanisms through cellular and humoral receptors that include peptidoglycan recognition proteins (PGRPs), gram negative binding protein (GNBP) and lectins that bind microbial-associated molecular patterns (MAMPs), i.e., lipopolysaccharides (LPSs) and peptidoglycans (PGs) from bacteria or β-glucans from fungi (Aggrawal and Silverman, 2007; Lemaitre and Hoffmann, 2007). This recognition leads to the activation of humoral processes that include production of antimicrobial peptides by the fat body and melanisation. The insect immune response also involves cellular processes that include phagocytosis, nodulation and encapsulation of pathogens by haemocytes. The *S. citri* vector *C. haematoceps* is a leafhopper belonging to the family Cicadellidae of the order Hemiptera. There have been very few studies dealing with the immune response of *Cicadellidae*. Nevertheless, RNA-seq differential expression studies revealed ESTs having significant homology with insect immune databases in the leafhopper *Graminella nigrifrons*, including genes encoding receptors (gram-negative bacteria binding proteins, C-type lectin and scavenger receptor), proteins involved in the Toll and IMD pathways, and one antimicrobial peptide (defensin) (Chen et al., 2012). Some of these immune related genes were temporarily induced when the leafhoppers fed on virally infected maize (Cassone et al., 2014). However, *S. citri* lacks both LPS and PG, which are bacterial antigens highly recognized by the arthropod immune system. Instead, a set of adhesins, including the ScARPs and the lipoprotein spiralin, are known to be exposed at the spiroplasma cell surface (Béven et al., 2012; Townsend and Plaskitt, 1985). The finding that spiralin induced lymphocyte responses (IL-1, IL-6 and TNF-a secretion, splenocyte proliferation, IgG and IgM secretion), strongly suggested that it was recognized by the mammalian immune system (Brenner et al., 1997), supporting the hypothesis that the insect immune system would also recognize the spiroplasmas. However, other studies in *Drosophila* showed that the NSRO spiroplasma, from *Drosophila nebulosa*, was not recognized and did not induce the insect immune response (Herren and Lemaitre, 2011; Hurst et al., 2003; Hutchence et al., 2011). Similarly, *S. citri* did not induce the production of antimicrobial peptides when injected in *Drosophila,* and infection resulted in insect mortality, suggesting that the immune system of *Drosophila* is unable to control *S. citri* growth (Herren and Lemaitre, 2011). The question then becomes whether and how the *C. haematoceps* immune system recognizes *S. citri* and sets up a specific response directed against spiroplasma infection.

In this work, we showed that both cellular and humoral responses are activated following insect challenge with *S. citri*, the Gram-negative *Escherichia coli* and the Gram-positive *Micrococcus luteus.* Interestingly, this immune response exhibited different expression profiles according to the bacterial type. In *S. citri*-infected *C. haematoceps*, several genes, including hexamerin, are up regulated, suggesting their contribution in controlling infection. RNA interference assays showed that hexamerin is required for optimal PO activity and for *C. haematoceps* to survive *S. citri* infection.

2. Materials and methods

2.1. Bacterial challenge and leafhoppers

S. citri GII3 was originally isolated from its leafhopper vector *C. haematoceps* captured in Morocco (Vignault et al., 1980); it was cultivated at 32 °C in SP4 medium without fresh yeast extract (Whitcomb, 1983). *E. coli* (TOP10, Invitrogen) and *M. luteus* strains were cultivated in LB medium. *E. coli* DH5-alpha expressing the red fluorescent protein (DsRed) (Mo Bio Laboratories) was cultivated in LB medium supplemented with ampicillin 100 μg ml^{-1} (Sigma−Aldrich) and used for phagocytosis experiments.

C. haematoceps leafhoppers were reared on stock (*Matthiola incana*) plants in insect-proof cages at 28 °C. Leafhoppers were challenged by ventral microinjection of culture medium (medium injected) or living bacteria (infected) as previously described (Foissac et al., 1996) and kept in a moist atmosphere on stock plants at 28 °C. Unchallenged (healthy insects) insects were treated in parallel as a control.

2.2. Bacterial growth in insects

Approximately 10^5 living bacteria (CFU) were injected into *C. haematoceps*. For *E. coli* and *M. luteus*, a 0.5 OD_{550} culture (about 10^8 bacteria/ml) was centrifuged at 2500 g for 10 min and the pellet was suspended in the same volume of fresh LB medium before injection. Similarly, the *S. citri* culture (about 10^8 bacteria/ml) was centrifuged at 10,000 g for 20 min and the pelleted bacteria were dispersed in the same volume of fresh SP4 medium. To determine bacterial growth in the leafhoppers, pools of three insects were ground in culture medium; serial dilutions of homogenates were plated onto agar plates to count colony-forming units (CFU). In these experiments, three pools of three insects were analyzed for each time point. Spiroplasma growth in dsRNA injected insects was estimated from CFU counts as described above. Insects were first injected with dsRNA GFP or dsRNA Hex (see below), and then with 10^3 *S. citri* per insect six days later.

2.3. Total RNA extraction and cDNA synthesis

Total RNA from healthy, SP4 medium or *S. citri*-injected insects was extracted from a single insect with TRIzol® Reagent (Invitrogen, Cergy-pontoise, France), following the manufacturer's instructions. RNA extraction was performed at various times ranging from 30 min to 6 days after injection. RNA was treated with RNase-Free DNase (Promega) as described in the manufacturer's procedures. After protein removal by phenol-chloroform treatment, the RNA concentration of each sample was measured using an Epoch-Microplate Spectrophotometer (Biotech® Instrument, Inc, Winooski, Vermont USA) and RNA quality was checked by electrophoresis. Reverse transcription into first strand cDNA was carried out using the First-Strand Synthesis System for RT-PCR kit (Invitrogen) with oligo(dT).