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Immune responses in the haemolymph and antimicrobial peptide expression in the abdomen of *Apis mellifera* challenged with *Spiroplasma melliferum* CH-1

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ABSTRACT

Spiroplasma melliferum generally parasitizes honeybees and is one of main pathogens causing 'bee creeping disease' in China. *Spiroplasma melliferum* can be spread through honeybee pollination, which causes severe economic losses to apiculture. The design of this study was based on previous studies that utilized an *in vitro* bioassay to investigate the effects of *S. melliferum* CH-1 infection. We identified invasive *S. melliferum* CH-1 within *Apis mellifera* using transmission electron microscopy and investigated the immune response of honeybees infected with *S. melliferum* CH-1 by assaying the cellular immune response of the haemocytes, the plasma level of phenoloxidase activity and the transcript levels of 5 antimicrobial peptides, including the *Abaecin*, *Apidaecin*, *Defensin 1*, *Defensin 2*, and *Hymenoptaecin* gene products. The percentage of granulocytes in the haemolymph of infected honeybees was significantly higher than those of the controls during the early phase of infection, but the percentage of plasmatocytes was significantly higher than those of the controls at the fifth day post-infection. The phenoloxidase activity of the infected honeybees reached a maximum at the second day, and then decreased continuously. Moreover, the transcript levels of the 5 evaluated antimicrobial peptide genes were significantly increased during the early phase of infection and all 5 antimicrobial peptides were significantly decreased during the middle phase of infection. During the late phase of infection, only *Defensin 2* and *Hymenoptaecin* showed significantly increased transcription. These results suggest that the honeybee immune responses could be activated by *S. melliferum* CH-1 during the early phase of infection and that *S. melliferum* CH-1 is also capable of circumventing the host defensive mechanisms to complete its life cycle within the honeybee during the middle phase of infection.

1. Introduction

Spiroplasmas are helical, motile prokaryotes of the class *Mollicutes* [13], a group of microorganisms that lack a cell wall, although they are phylogenetically related to Gram-positive bacteria [60,62]. Spiroplasmas are mostly found in insects and crustaceans. Some of these organisms are symbionts of certain species of *Drosophila* and aphids and some are commensals that live in the gut of leafhoppers and beetles [1,33,41,44], whereas others are pathogenic for honeybees, *Drosophila*, mosquitoes, moths, crabs and shrimp [2,12,28,30,45,47,49,55,57,61]. *Spiroplasma melliferum* was first discovered in honeybees when they were examined for the presence of pathogenic microorganisms [12]. In China, *Spiroplasma melliferum* was first isolated from *Apis mellifera* individuals that were creeping and dying on the ground near their hives in 1984 [10].

Honeybees (*Apis mellifera*) are responsible for the production of approximately one third of the humans food supply [9]. The commercial-scale production of some fruits (apples, apricots, peaches, and cherries) and vegetables (cucumbers and melons) requires pollination by honeybees. In recent decades, the number of honeybee colonies has declined in most agricultural areas worldwide [15,50,54]. Since the 1980s, 'bee creeping disease' has spread widely in China, and this disease often co-occurs with sporidiosis and palsy disease, among other disorders. 'Bee creeping disease' has caused severe economic losses to the beekeeping industry and threatens agricultural production [3]. The innate defence strategies of insects have some similarities with those of mammals, having immune systems that can be broadly separated into cellular and humoral systems [56]. Compared with other insects, such as *Drosophila*, honeybees have only approximately one third the number of genes devoted to immunity [23]. However, all pathways are fully

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functional and a traditional defense of pathogens occurs in honeybees. Haemocytes are a central component of the insect cellular defence system, and these cells have been found to employ the mechanisms of phagocytosis, nodulation, encapsulation, and melanization for host defence [37,42]. Antimicrobial peptides (AMPs) are a set of diverse peptides that are important for insect host defence. AMPs are not only deployed in large quantities following immune activation, typically from the fat body located in the abdominal cavity of insects, but are also produced locally in tissues by gut epithelial cells and haemocytes [8]. Five well-characterized AMPs are encoded by the honeybee genome [23], which are important components of general barrier immunity in the honeybee abdomen [31], including the *Abaecin*, *Apidaecin*, *Defensin 1*, *Defensin 2*, and *Hymenoptaecin* gene products. In general, AMPs directly lyse microbial cells (e.g., defensin) but they also have cytostatic properties, as shown by their inhibition of the enzymatic activities necessary for parasite replication (e.g., apidaecin) [7,38,48]. Variations in the intensity of the AMP response have been linked to different honeybee genetic backgrounds and honeybee colonies, which reduce the levels of diseases caused by particular pathogens at a cost to colony productivity when the bees maintain a high AMP level [25].

We designed the experiments performed in this investigation to study the immune response in the haemolymph during the infection process and the interaction between the host and the pathogen. In this study, *S. melliferum* CH-1 was shown to colonize the midgut epithelial cells and pectoral muscle cells of *A. mellifera*. Both cellular and humoral responses were activated or inhibited by *S. melliferum* CH-1 at the different phases of infection. This study provides useful information about *Spiroplasma melliferum* CH-1 infections of *A. mellifera*, which might ultimately lead to the development of strategies for preventing this honeybee disease.

2. Materials and methods

2.1. Spiroplasma melliferum CH-1 cultures

Spiroplasma melliferum CH-1 was originally isolated from *Apis mellifera* honeybees with 'bee creeping disease' in China. The organism was cultured at 32 °C in R-2 liquid medium [46,57]. When incubated for 72 h, *S. melliferum* CH-1 reached the exponential growth phase (10^8 spiroplasma cells ml^{-1}), as well as its most potent level of pathogenicity and was used for the infection experiments [16,18].

2.2. Experimental animals

Apis mellifera honeybees were obtained from a commercial apiary in Nanjing, Jiangsu province of China. Honeybees were collected from the top edge of the honey combs, above and distant from the brood-rearing area. This location was chosen for consistency and to reduce the chance of collecting hatchlings and nurse bees. For each experiment, we consulted a reference of standard methods used in honeybee research for guidance on hive and bee sampling and for anaesthesising the bees by chilling them [29].

2.3. Spiroplasma melliferum CH-1 inoculation and sample collection

Apis mellifera individuals were removed from a single colony to control for their genetic background and were isolated in an incubator at 28 °C for < 24 h prior to selecting age-matched honeybees. These honeybees were fasted in clean bee cup cages for 4–5 h [24]. The *A. mellifera* immune challenge was performed by inoculating the bees with *S. melliferum* CH-1. Each honeybee was infected per os with a 5-μl cell suspension (10^3 cells/μl) by hand using a 10-μl pipette. As a control, honeybees were inoculated with 5 μl of R-2 medium. The expression of the spiralin gene was utilized to confirm whether the spiroplasma exist in honeybees from different groups at each time point [53]. We discarded the non-corresponding samples to guarantee the honeybees tested were free of spiroplasma before inoculation. The honeybees readily consumed the inoculum, after which they were isolated by treatment group in sterile cages (to prevent contact with the other treatment groups), provided 1:1 sterile sugar water and maintained in an incubator at 28 °C and 55% relative humidity. For haemocyte analysis, 5 honeybees were harvested from each treatment group at each time point, chilled and dissected, after which the haemocytes collected were used for microscopic analysis and determination of the level of phenoloxidase activity. For abdominal analysis, 5 honeybees were harvested from each treatment group at each time point and were immediately frozen at −80 °C, after which the abdomens were removed.

2.4. Electron microscopy of tissue samples

According to the incidence of 'Bee creeping disease' in pilot experiments, we took samples of honeybees every 24 h after inoculation. We measured *S. melliferum* in honeybees from different groups at each time point by qRT-PCR as previously described [53]. And then honeybees from the treatment groups and the control groups were dissected and were prepared for examination using a transmission electron microscope (TEM). Samples (1–2 mm^3) of the midgut and pectoral muscle were fixed in 4% glutaraldehyde for 2 h at 4 °C. The fixed samples were then washed 3 times for 15 min each time with phosphate buffer at 4 °C. The midgut and pectoral muscle samples were fixed again in 2% osmium tetroxide for 2 h at 4 °C and then were washed 3 times with phosphate buffer. Then, the tissues were dehydrated through a graded series of ethanol and were subsequently embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate and were observed using a Hitachi H-7650 TEM.

2.5. Haemocyte extraction and staining

To characterize the haemocytes, 5 honeybees were anaesthesised by chilling them at −20 °C. After carefully rupturing the ventral cuticle under a stereomicroscope, diluted haemolymph was mounted on slides and was directly observed by optical microscopy using differential interference contrast [43]. Approximately 5 μl of raw haemolymph was then spread on a small area of a coated slide and was air dried prior to Wright staining according to the directions of the manufacturer. The types of haemocytes present were determined based on the body size and cytoskeletal structure of the cells, as revealed by Wright staining. The number of plasmatocytes and granulocytes was determined by counting the total number of these cells in 100 microscopic fields per preparation. All of the microscopic examinations were performed using an OLYMPUS BX43 microscope, and images were collected using a Canon EOS600D camera. The digital images were processed and analysed using Adobe Photoshop CS6 software.

2.6. Phenoloxidase activity

The phenoloxidase (PO) activity in the plasma was measured as previously described [14]. Briefly, the haemolymph of 5 honeybees was collected as described above and was diluted 4 times using fresh sterile phosphate buffered solution (PBS). Then, the haemolymph samples were centrifuged at 2500 g for 5 min to separate the haemocytes from the plasma. A total of 20-μl of plasma was added to a microtube containing 140 μl of 12.5% PBS solution at pH 6.5 to measure PO activity. Then, 140 μl of a 2 mg/ml L-dopa solution (Sigma) was added to the microtube. The reaction was allowed to proceed at room temperature (20 °C-25 °C) for 30 min, and then the optical density (OD) at 490 nm was determined every minute using a UV–visible spectrophotometer. One unit of PO activity was defined as a change of 0.001 OD_{490nm} in 1 min. The specificity of the reaction was tested using phenylthiourea (PTU) at 2, 4 and 8 mg/ml to specifically inhibit PO activity.