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Distribution of the obligate endosymbiont *Blochmannia floridanus* and expression analysis of putative immune genes in ovaries of the carpenter ant *Camponotus floridanus*

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ABSTRACT

The bacterial endosymbiont *Blochmannia floridanus* of the carpenter ant *Camponotus floridanus* contributes to its hosts' ontogeny via nutritional upgrading during metamorphosis. This primary endosymbiosis is essential for both partners and vertical transmission of the endosymbionts is guaranteed by bacterial infestation of oocytes. Here we present a detailed analysis of the presence and localisation of *B. floridanus* in the ants' ovaries obtained by FISH and TEM analyses. The most apical part of the germarium harbouring germ-line stem cells (GSCs) is not infected by the bacteria. The bacteria are detectable for the first time in lower parts of the germarium when cystocytes undergo the 4th and 5th division and *B. floridanus* infects somatic cells lying under the basal lamina surrounding the ovarioles. With the beginning of cystocyte differentiation, the endosymbionts are exclusively transported from follicle cells into the growing oocytes. This infestation of the oocytes by bacteria very likely involves exocytosis–endocytosis processes between follicle cells and the oocytes. Nurse cells were never found to harbour the endosymbionts. Furthermore we present first gene expression data in *C. floridanus* ovaries. These data indicate a modulation of immune gene expression which may facilitate tolerance towards the endosymbionts and thus may contribute to their transovarial transmission.

1. Introduction

Insects are by far the most diverse and a highly successful animal group on earth, which is reflected not only in species richness and abundance but also in the variety of ecosystems they inhabit. Diversification and evolutionary success of insects are at least partially facilitated by symbiotic associations with microorganisms (Buchner, 1965). Bacterial endosymbionts may contribute to their hosts' biology in various ways (Feldhaar, 2011), e.g. via nutritional upgrading of unbalanced diets (Nogge, 1981; Zientz et al., 2004), enhanced resistance to pathogens or parasitoids and thermal tolerance (Dunbar et al., 2007; Kaltenpoth, 2009; Oliver et al., 2010). These host associated bacteria comprise so-called primary and secondary endosymbionts. The latter are facultative symbionts from the host's perspective and are found intracellularly and/or extracellularly in various host tissues (Dale and Moran, 2006). Secondary endosymbionts can be transmitted vertically or horizontally (Oliver et al., 2010; Russell et al., 2003).

In contrast, primary endosymbionts have obligate associations with their insect hosts that developed over long evolutionary timescales. Generally these bacteria are endowed with small genomes (<1 Mb) (Klasson and Andersson, 2010; McCutcheon and Moran, 2012). Accordingly, primary endosymbionts have lost the ability to multiply independently from their hosts (Baumann, 2005; Feldhaar and Gross, 2009). The localisation of primary endosymbionts within their insect hosts is most frequently restricted to specialised cells, the so-called bacteriocytes, which often form multicellular symbiotic organs called bacteriomes (Buchner, 1965). Due to the obligate nature of the association, primary endosymbionts are transmitted strictly vertically.

Though the transmission of primary endosymbionts occurs strictly vertically, transmission routes of different endosymbionts may be highly diverse. Host biology (ovipary vs. vivipary) and localisation of endosymbionts in their host have selected different

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transmission routes of endosymbiotic bacteria to the host's offspring. The viviparous tsetse fly harbours one primary and several secondary endosymbionts that are also transmitted vertically. The primary endosymbiont *Wigglesworthia glossinida* as well as the secondary endosymbiont *Sodalis glossinidius* are transmitted to the developing embryo via milk gland secretions. Remarkably, in the milk gland secretions *W. glossinidia* has an extracellular localisation. The secondary endosymbiont *Wolbachia* already infects trophocytes and developing oocytes (Balmand et al., 2013). The transmission of the primary endosymbiont of aphids, *Buchnera*, proceeds also including an extracellular stage. Within the parthenogenetic and viviparous aphids *Buchnera* is transmitted from maternal bacteriocytes into blastulae (early embryos) through a series of exo- and endocytotic processes (Koga et al., 2012).

Whilst the transmission of endosymbionts is most often established late during development of eggs and embryos in viviparous insects, the infection of oocytes during transovarial transmission in oviparous insects occurs either early or late during oogenesis. For example, the endosymbionts *Schneideria nysicola* of seed bugs of the genus *Nysius* (Hemiptera) are generally localised intracellularly in a pair of large bacteriomes which are in close association with the gonads. In adult female bugs *S. nysicola* was also found in the "infection zone" in the middle of each germarium, where the first egg-chamber forms within the telotrophic-meroistic ovarioles of the seed bugs. Within the "infection zone" the *Schneideria* endosymbionts preferentially localised in the ovarial bacteriocytes. Additionally, *S. nysicola* was observed in the "symbiont balls" at the anterior poles of each oocyte, suggesting that the endosymbionts enter the developing oocytes ensuring vertical transmission of the bacteria (Matsuura et al., 2012a, 2012b). In the scale insect *Marchalina hellenica*, an early infection of germ cells, the so-called cystocytes, was suggested. As a consequence both cell types deriving from the cystocytes, the trophocytes and oocytes, are infected with bacterial endosymbionts. During oogenesis, the endosymbionts are progressively transported from trophocytes into the developing oocyte (Szklarzewicz et al., 2013). Apart from such cases of very early infection of oocytes with bacterial endosymbionts there are several cases showing infection of oocytes through nurse cells in later stages of oogenesis or even early embryos. For example, it was shown that *Wolbachia* in chalcid wasps of the genus *Aphytis* are transmitted from the nurse cells into developing oocytes through cytoplasmic bridges between the two cell types (Zchori-Fein et al., 1998). The bacterial endosymbiont *Westeberhardia cardiocondylae* of the invasive ant *Cardiocondyla obscurior* is located in nurse cells and transmitted into late-stage oocytes during nurse cell depletion (Klein et al., 2016). The bacterial endosymbionts of the leafhopper *Macrosteles laevis*, which were located in a structure called "symbiotic ball" in the perivitelline space between the oolemma and the follicular epithelium, invade the oocyte at the posterior pole only after the end of oocyte growth (Kobialka et al., 2015).

The symbioses between bacteria of the genus *Blochmannia* and carpenter ants of the genus *Camponotus* was the first endosymbiosis between bacteria and animals ever described (Blochmann, 1882; Buchner, 1965). *Blochmannia floridanus* resides free within the cytoplasm of bacteriocytes in the midgut tissue as well as in matured oocytes of *Camponotus floridanus* (Blochmann, 1882; Sauer et al., 2002; Schröder et al., 1996). The bacteria upgrade the diet of their insect hosts (Feldhaar et al., 2007; Gil et al., 2003). During metamorphosis of the ants the numbers of bacteria and of bacteriocytes in the midgut increase dramatically and peak in the late pupal stage, transforming the entire midgut tissue into a huge symbiotic organ. Subsequently, in adult animals the number of bacteriocytes and, concomitantly, of *Blochmannia* continuously decrease, since the symbiosis is apparently of less relevance for older animals (Stoll et al., 2010). Interestingly, during the period of bacterial proliferation in the pupal stages two negative immune-regulators, the peptidoglycan recognition proteins PGRP-LB and PGRP-SC2 are highly expressed only in the midgut suggesting a down-modulation of the immune response possibly contributing to symbiont tolerance within the midgut tissue (Ratzka et al., 2013).

Blochmann (1882), Buchner (1918) as well as Hecht (1923) focussed on the localisation of the bacteria (previously also called "Blochmann bodies", (Koch, 1960)) mainly during embryogenesis of *Camponotus ligniperdus*. The bacteria were found in the developing bacteriocytes within the determined midgut tissue within embryos, but also in cells which come into close contact with the predestined ovarian tissue during embryogenesis (Hecht, 1923). However, very young oocytes were not found to be infected with the endosymbionts, while developing oocytes in fertile animals were described to become infected via follicle cells (Blochmann, 1882; Buchner, 1918). Recent analyses showed that oocytes in posterior parts of the ovarioles of *C. floridanus* are the only cells infected by the endosymbionts (Sauer et al., 2002; Schröder et al., 1996).

To date, the understanding of the mechanism of transovarial transmission of *Blochmannia* remained limited. Thus, in the present manuscript we investigated the localisation of *B. floridanus* in ovaries of queenless *C. floridanus* workers during oogenesis based on imaging techniques using fluorescent *in situ* hybridisation (FISH) and transmission electron microscopy (TEM). In addition, we present first expression data of immune-related genes in the ovarian tissue possibly contributing to endosymbiont control or tolerance.

2. Material and methods

2.1. Ants

C. floridanus colonies C90 (derived from a mated queen from Florida, Orchid Island, 2001) and C152 (derived from a mated queen from Florida, Orchid Island, 2002) were maintained in a climatic chamber at 25 °C, 70% humidity and a 12 h light–dark cycle. Animals were fed twice to three times a week with cockroaches and honey water (50% w/w). Workers normally have small and underdeveloped ovaries. However, when workers are isolated from their colonies, in the absence of a fertile queen and queen-laid eggs they activate oocyte development and begin to lay haploid eggs after several weeks (Endler et al., 2004). Therefore, orphaned (queenless) workers could be used for our investigations after two to four months. Respective sub-colonies consisted of 60–80 major workers and at least 200 minor workers. To maintain nursing behaviour of workers, larvae (at least stage L2) and pupae (stages P1-P3) with stages defined according to Stoll et al., 2010 were provided.

2.2. Fluorescent in situ hybridisation (FISH)

For analysing the distribution of *B. floridanus* in worker ovaries FISH was performed as previously described (Feldhaar et al., 2007). Endosymbionts were visualised with a probe Bfl172 targeting a specific region of the *B. floridanus* 16S rRNA (5′-CCTATCTGGGTTCATCCAATGGCATAAGGC-3′). Oligonucleotides were labelled with the fluorophore Alexa488 by the manufacturer (Sigma–Aldrich Chemie GmbH, Munich, Germany). The respective sense probe Bfl172sense (5′-GCCTTATGCCATTGGATGAACCCAGATAGG-3′) was used as a negative control for specificity.

Ovaries of workers were dissected in ice-cold PBS (pH7.4). Ovaries with at least one matured egg at the posterior end were transferred in small glass vials for fixation in 4% (w/v) paraformaldehyde in PBS. After 2 h of fixation at room temperature (RT) the fixative was exchanged by PBS by three 5 min washes. Then samples were

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