Developmental and Comparative Immunology 69 (2017) 12–22

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Gut symbiotic bacteria stimulate insect growth and egg production by modulating *hexamerin* and *vitellogenin* gene expression

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ARTICLE INFO

Article history:
Received 5 October 2016
Received in revised form
19 November 2016
Accepted 19 November 2016
Available online 6 December 2016

Keywords:
Symbiosis
Riptortus pedestris
Burkholderia
Hexamerin
Vitellogenin
Symbiosis-affected host molecule

ABSTRACT

Recent studies have suggested that gut symbionts modulate insect development and reproduction. However, the mechanisms by which gut symbionts modulate host physiologies and the molecules involved in these changes are unclear. To address these questions, we prepared three different groups of the insect *Riptortus pedestris*: *Burkholderia* gut symbiont-colonized (Sym) insects, *Burkholderia*-non-colonized (Apo) insects, and *Burkholderia*-depleted (Sym^{Burk-}) insects, which were fed tetracycline. When the hemolymph proteins of three insects were analyzed by SDS-PAGE, the hexamerin-α, hexamerin-β and vitellogenin-1 proteins of Sym-adults were highly expressed compared to those of Apo- and Sym^{Burk-}-insects. To investigate the expression patterns of these three genes during insect development, we measured the transcriptional levels of these genes. The *hexamerin-β* gene was specifically expressed at all nymphal stages, and its expression was detected 4–5 days earlier in Sym-insect nymphs than that in Apo- and Sym^{Burk-}-insects. However, the *hexamerin-α* and *vitellogenin-1* genes were only expressed in adult females, and they were also detected 6–7 days earlier and were 2-fold higher in Sym-adult females than those in the other insects. Depletion of *hexamerin-β* by RNA interference in 2nd instar Sym-nymphs delayed adult emergence, whereas *hexamerin-α* and *vitellogenin-1* RNA interference in 5th instar nymphs caused loss of color of the eggs of Sym-insects. These results demonstrate that the *Burkholderia* gut symbiont modulates host development and egg production by regulating production of these three hemolymph storage proteins.

1. Introduction

The *Riptortus-Burkholderia* system is a powerful model system for analyzing molecular cross-talk between insects and gut symbionts at the molecular and biochemical levels and has several experimental advantages. For example, this system enabled mass production of *Burkholderia* gut symbiont-colonized insects (referred to as Sym-insects) and gut symbiont-non-colonized insects (referred to as Apo-insects) in the laboratory (Kim et al., 2013c, 2015). The second instar nymphs of *Riptortus* acquire the *Burkholderia* symbiont from the environment every generation (Kikuchi et al., 2011). Orally fed *Burkholderia* cells colonize the symbiotic organ, the midgut fourth region (M4) (Ohbayashi et al., 2015). Additionally, the *Burkholderia* symbiont is easily culturable on standard bacterial media and is genetically manipulable; thus, the biological functions of specific bacterial genes can be assessed *in vivo* using *Burkholderia* mutant strains (Kim et al., 2014, 2013b, 2013c; Lee et al., 2015; Ohbayashi et al., 2015). Moreover, because a large quantity of homogeneous naïve *Burkholderia* symbionts can be prepared from the host midgut, it is possible to study biochemical differences between *in vitro* cultured *Burkholderia* cells and *in vivo* colonized symbiotic cells (Byeon et al., 2015; Kim et al., 2013a, 2015). Therefore, using this model system, we aimed to

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http://dx.doi.org/10.1016/j.dci.2016.11.019
0145-305X/

extend our understanding of host-gut symbiont interactions at the molecular level.

In the association between diverse and evolutionarily successful insects and their resident symbionts within the gut, tissues and/or cells, the symbionts may benefit host fitness, growth and fecundity. For example, the depletion of symbiont by antibiotic treatment severely affected aphid development, growth and fecundity (Douglas, 1996), and the elimination of symbiont from *Megacopta punctatissima* caused retarded growth, mortality, and sterility of the insects (Fukatsu and Hosokawa, 2002; Hosokawa et al., 2006). In the bean bug *Riptortus pedestris*, experimental comparisons between symbiotic and aposymbiotic insects have revealed that symbiont colonization significantly improves host fitness (Kikuchi and Fukatsu, 2014; Kikuchi et al., 2007; Kim et al., 2013c). Despite these important beneficial effects of the symbiont on their hosts, it is still not clear how the symbionts modulates the fitness, development and egg production of host insects and which host molecules are affected by the presence of symbiont.

Most insects possess the biliverdin-associated hemolymph proteins hexamerin and vitellogenin. Insect hexamerins are the most abundant protein synthesized by the fat body and are exported to the hemolymph (insect blood) (Burmester, 1999). This protein is utilized by nymphs and adults during development and reproduction (Telfer and Kunkel, 1991). It is primarily considered a storage protein that provides amino acids and energy for non-feeding periods, such as molting and mating (Munn et al., 1967). However, recent studies have reported that this protein is also involved in other biological functions, such as hormone transportation, immune response, cuticle formation, and stem cell proliferation (Blackburn et al., 2004; Braun and Wyatt, 1996; Eliautout et al., 2016; Peter and Scheller, 1991; Wang et al., 2010). Vitellogenin is a precursor form of the major egg yolk protein, vitellin. This protein is synthesized in the fat body and circulates in the hemolymph before uptake through receptor-mediated endocytosis into the oocytes (Tufail et al., 2005). Vitellogenins are usually found only in reproductive females, and their mRNA expression is regulated by sex, developmental stage, and diapause conditions (Shinoda, 1993). Additionally, once vitellogenins accumulate as egg yolk proteins, they is stored as vitellin, which is the major nutritional reservoir for the developing embryo (Hirai et al., 1998). During these processes, vitellogenin and vitellin undergo limited proteolytic cleavage by proteases. Although the biological roles of hexamerin and vitellogenin in insect physiology have been elucidated, the molecular relationship between the symbiont, these two proteins, and host fitness, development and egg production is still unclear.

In our previous studies, we observed that Sym-insects had higher expression of *hexamerin* and *vitellogenin* than that of Apo-insects. This difference disappeared following depletion of the *Burkholderia* gut symbiont by administration of an antibiotic. Based on these observations, we examined how *hexamerin* and *vitellogenin* gene expression was controlled by the *Burkholderia* gut symbiont in the host midgut. Here, we found that hexamerin-α and -β and vitellogenin-1 proteins in the hemolymph of *Riptortus* insects are involved in host fitness, development and fecundity, suggesting that the production is modulated by the gut symbiotic bacteria.

2. Materials and methods

2.1. Insect rearing

R. pedestris was reared as described previously (Kim et al., 2013c, 2014). Briefly, *R. pedestris* was maintained at 27 °C under a photoperiod of 16 h light and 8 h dark on dry soybean seeds and was supplemented with distilled water containing 0.05% ascorbic acid (DWA). The containers were cleaned every day, and the soybean seeds and DWA were replaced every two days. Upon reaching adulthood, the insects were transferred to larger containers (35 cm long by 35 cm wide and 40 cm high) in which soybean plant pots were provided for feeding and cotton pads were attached to the walls for egg laying. Eggs were collected daily and transferred to new cages for hatching.

2.2. Inoculation of Burkholderia symbiont

Burkholderia symbiont strain RPE75, which is a spontaneous rifampin-resistant mutant derived from strain RPE64 (Kikuchi et al., 2011) or its GFP-labeled mutant RPE225 (Kikuchi and Fukatsu, 2014), was cultured until mid-log phase at 30 °C in YG-RIF medium (0.5% yeast extract, 0.4% glucose, and 0.1% NaCl, containing 30 μg/ml rifampicin) (Kim et al., 2015). A cotton dish soaked with 10^7 cell/ml of cultured *Burkholderia* in DWA was provided to second instar nymphs 12 h after molting to generate *Burkholderia*-harboring Sym-insects. After the insects were fed inoculum solution for two days, fresh DWA was provided to the insects instead of the inoculum solution. In other experiments, Sym^{Burk-}-insects were generated after administration of *Burkholderia* inoculum solution for 48 h, followed by tetracycline-containing DWA (64 μg/ml) for three days. For confirmation of the depletion of symbiotic *Burkholderia* in the M4 crypt after tetracycline treatment, diagnostic PCR was performed to estimate colony forming units (CFUs), and fluorescence microscopy was used for the detection of orally fed *Burkholderia* cells.

2.3. Analyses of hemolymph proteins in Apo-, Sym- and Sym^{Burk-}-insects

One hundred microliters of hemolymph samples from Apo-, Sym- and Sym^{Burk-}-insects 3 days after adult emergence was collected in 1.5 ml microtubes containing 400 μl of decoagulation buffer (15 mM NaCl, 30 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, containing 5% glycerol, pH 5.0). The concentration of total proteins was measured by the Bradford method, and a protein sample was lysed in 2 × Laemmli sample buffer (120 mM Tris-HCl, 10% SDS, 20% glycerol, and 0.05% bromophenol blue, pH 6.8) or 3 × native sample buffer (200 mM Tris-HCl, 30% glycerol, and 0.05% bromophenol blue, pH 6.8). Thirty micrograms of hemolymph proteins was analyzed using 10% SDS-PAGE and a 4–15% native gradient gel (Bio-Rad, USA). The protein bands on the gels were stained with Coomassie Brilliant Blue R250. The 68 kDa and 50 kDa hemolymph proteins of Apo-, Sym- and Sym^{Burk-}-insects were transferred to a polyvinylidene difluoride membrane, the bands were cut from the membrane, and their N-terminal amino acid sequences were determined by an automatic gas-phase amino acid sequencer (Applied Biosystems, USA).

2.4. Purification of Hex-α and -β proteins from the egg or hemolymph of Riptortus

For purification of Hex-α protein from eggs, five hundred eggs from *Riptortus* female adults were collected in 10 ml of decoagulation buffer containing a protease inhibitor cocktail (Sigma Aldrich, USA). After homogenization with a plastic bar, the supernatant was collected after centrifugation at 3000 × *g* for 15 min at 4 °C. Subsequently, the supernatant was replaced with buffer A [10 mM phosphate buffer (PB) containing 100 mM NaCl, pH 7.0] using Amicon 30 K centrifugal filters (Millipore, USA). The obtained solution (32.78 mg protein) was loaded onto a Sephacryl S-200 column (GE Healthcare, UK) equilibrated with buffer A, and the fractions were eluted (10 ml) at a flow rate of 0.5 ml/min at 4 °C.