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Live imaging of symbiosis: spatiotemporal infection dynamics of a GFP-labelled *Burkholderia* symbiont in the bean bug *Riptortus pedestris*

YOSHITOMO KIKUCHI*† and TAKEMA FUKATSU‡

*Hokkaido Center, Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo 062-8517, Japan, †Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan, ‡Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

Abstract

Many insects possess endosymbiotic bacteria inside their body, wherein intimate interactions occur between the partners. While recent technological advancements have deepened our understanding of metabolic and evolutionary features of the symbiont genomes, molecular mechanisms underpinning the intimate interactions remain difficult to approach because the insect symbionts are generally uncultivable. The bean bug *Riptortus pedestris* is associated with the betaproteobacterial *Burkholderia* symbiont in a posterior region of the midgut, which develops numerous crypts harbouring the symbiont extracellularly. Distinct from other insect symbiotic systems, *R. pedestris* acquires the *Burkholderia* symbiont not by vertical transmission but from the environment every generation. By making use of the cultivability and the genetic tractability of the symbiont, we constructed a transgenic *Burkholderia* strain labelled with green fluorescent protein (GFP), which enabled detailed observation of spatiotemporal dynamics and the colonization process of the symbiont in freshly prepared specimens. The symbiont live imaging revealed that, at the second instar, colonization of the symbiotic midgut M4 region started around 6 h after inoculation (hai). By 24 hai, the symbiont cells appeared in the main tract and also in several crypts of the M4. By 48 hai, most of the crypts were colonized by the symbiont cells. By 72 hai, all the crypts were filled up with the symbiont cells and the symbiont localization pattern continued during the subsequent nymphal development. Quantitative PCR of the symbiont confirmed the infection dynamics quantitatively. These results highlight the stinkbug-*Burkholderia* gut symbiosis as an unprecedented model for comprehensive understanding of molecular mechanisms underpinning insect symbiosis.

Keywords: green fluorescence protein, gut symbiotic bacteria, insect symbiosis, midgut crypt, population dynamics, stinkbug, symbiont colonization

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Introduction

Symbiotic associations with microorganisms are omnipresent in nature and have substantially affected organismal evolution (Margulis & Fester 1991; Ruby *et al.* 2004; Moran 2007). Some microbes are harmful or even lethal to their hosts, being referred to as parasites or pathogens, while others may play pivotal biological roles for their hosts, being regarded as mutualists. Among the amazingly diverse symbiotic associations, the most cohesive forms are found in 'endosymbiosis', where the microbes inhabit the host body and the spatial intimacy facilitates intricate interactions between the symbiotic partners. Recent technological advancements in culture-independent molecular methods, including quantitative polymerase chain reaction (PCR), DNA sequencing, molecular phylogenetic analysis, *in situ* hybridization

Correspondence: Yoshitomo Kikuchi, Fax: +81 11 857 8980; E-mail: y-kikuchi@aist.go.jp

and secondary ion mass spectrometry (SIMS), as well as genomic and large-scale-omics analyses, have enabled deeper understanding of such endosymbiotic associations (Hentschel *et al.* 2012; McFall-Ngai *et al.* 2013). However, cultivation of symbiotic microbes is still crucial for investigating molecular mechanisms underlying host–symbiont interactions (Ruby 2008).

The arthropod class Insecta, consisting of over 1 000 000 described species, is the most diverse animal group. Insects that feed exclusively on nutritionally restricted diets such as plant sap, vertebrate blood and woody material usually possess obligate mutualistic symbionts inside their bodies. These symbiotic bacteria are often essential for hosts' survival and reproduction, and also affect hosts' biology and phenotypes in a variety of ways via, for example, providing essential nutrients (Douglas 1998), digesting food materials (Inoue *et al.* 2000), influencing host plant usage (Tsuchida *et al.* 2004), conferring resistance against parasites or pathogens (Oliver *et al.* 2003; Scarborough *et al.* 2005), changing host body colour (Tsuchida *et al.* 2010) and others. For ensuring such host–symbiont associations, many insects have evolved sophisticated mechanisms for vertical symbiont transmission, which may be ovarial transmission, egg surface contamination, coprophagy or other means (Kikuchi 2009; Bright & Bulgheresi 2010). Obligate insect–microbe associations, such as aphid–*Buchnera* and tsetse–*Wigglesworthia* symbioses, tend to exhibit host–symbiont phylogenetic congruence, reflecting strict vertical transmission and stable host–symbiont association over evolutionary time (Moran *et al.* 1993; Clark *et al.* 2000). Genomes of these symbionts tend to be drastically reduced, which severely hamper their capability of surviving outside their hosts (Shigenobu *et al.* 2000; Akman *et al.* 2002; Wernegreen 2002; Baumann 2005; Moran *et al.* 2008). While genomics of *Buchnera*, *Wigglesworthia* and other obligate symbionts has provided invaluable clues to the understanding of their biological roles and evolutionary features (Wernegreen 2002; Baumann 2005; Moran *et al.* 2008), their fastidious nature has hindered molecular studies on the mechanisms underpinning these symbiotic associations.

On the other hand, elaborate host–symbiont associations without vertical transmission are commonly found in nature, especially among plants and marine invertebrates (Ruby 2008; Bright & Bulgheresi 2010). For example, leguminous plants acquire *Rhizobium* or allied bacteria from the soil and form root nodules, where the symbiotic bacteria fix atmospheric dinitrogen and provide their hosts with nitrogenous nutrients (Brewin 1991; Schultze & Kondorosi 1998). Bobtail squids harbour a luminous bacterium, *Vibrio fischeri*, within their symbiotic light organs, which is selectively acquired from the ambient seawater by newborn squids for light production (Nyholm & McFall-Ngai 2004). Reflecting their capability of free life, these symbiotic bacteria are cultivable outside their hosts on standard microbiological media and, therefore, amenable to genetic manipulation (Ruby 2008). Hence, the legume and squid model systems have significantly contributed to our understanding of molecular mechanisms underlying symbiosis.

The bean bug *Riptortus pedestris* (Hemiptera: Heteroptera: Alydidae; Fig. 1a) develops a number of sac-like tissues, called crypts, in a posterior region of the midgut, whose cavity is densely colonized by a betaproteobacterial symbiont of the genus *Burkholderia* (Kikuchi *et al.* 2005). The midgut of the bean bug consists of morphologically distinct regions designated as M1, M2, M3 and M4 (Fig. 1b). In addition, there is a slightly bulbous region between the M3 and M4 regions, called M4B region (Fig. 1b). The M4 region bearing numerous crypts is the principal symbiotic organ harbouring as many as 10^8 *Burkholderia* symbiont cells, and the M4B region bearing no crypts also contains some *Burkholderia* symbiont cells (Goodchild 1963; Futahashi *et al.* 2013; Kikuchi & Yumoto 2013). Infection with the *Burkholderia* symbiont improves growth rate and body size of the *Riptortus* host (Kikuchi *et al.* 2007), and some *Burkholderia* strains confer insecticide resistance to the host insect (Kikuchi *et al.* 2012). Notably, the bean bug does not transmit the *Burkholderia* symbiont vertically, but acquires the symbiont from the surrounding environment at an early nymphal stage every generation, and the symbiont is easily

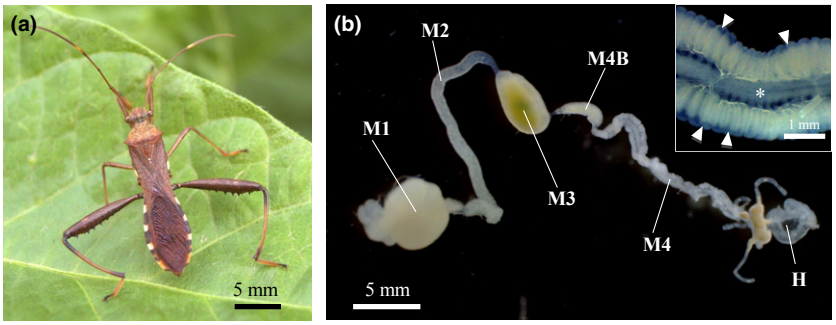

Fig. 1 Gut symbiosis in the bean bug *R. pedestris*. (a) Adult male. (b) Dissected midgut. Inset is an enlarged image of the symbiotic organ. Asterisk and arrowheads indicate midgut main tract and crypts, respectively. Abbreviations: M1, midgut first region; M2, midgut second region; M3, midgut third region; M4B, bulbous midgut region prior to M4 region; M4, midgut fourth region with crypts (symbiotic organ); H, hindgut.

cultivable on standard microbiological media (Kikuchi *et al.* 2007, 2011b). Under laboratory conditions, the bean bugs reared in clean containers with soybean seeds and sterilized water cannot acquire the *Burkholderia* symbiont and become aposymbiotic, whereas the insects reared in the same manner, but with water containing cultured *Burkholderia* cells, become symbiotic (Kikuchi *et al.* 2011b; Kikuchi & Yumoto 2013). Hence, the *Riptortus–Burkholderia* symbiosis represents a novel insect model system wherein the host–symbiont association is tractable experimentally.

Here, we report that, in the *Riptortus–Burkholderia* symbiosis, the symbiont is also tractable genetically. We established a genetically engineered strain of the *Burkholderia* symbiont that expresses a jellyfish-derived green fluorescence protein (GFP; Chalfie *et al.* 1994; Shimomura 2005), which enabled live imaging of the symbiont localization in the host symbiotic organ. By making use of symbiont visualization by GFP labelling in combination with symbiont quantification by quantitative PCR, we investigated detailed processes of the infection dynamics of the *Burkholderia* symbiont *in vivo* during the developmental course of the *Riptortus* host.

Materials and methods

Insects, bacterial strains and plasmids

The strain of *R. pedestris* used in this study was originally collected from a soybean (*Glycine max*) field in Tsukuba, Ibaraki, Japan, and maintained in the laboratory. The insects were reared in petri dishes (90 mm in diameter and 20 mm high) at 25 °C under a long-day regimen (16 h light, 8 h dark) and fed with soybean seeds and distilled water containing 0.05% ascorbic acid (DWA). *Burkholderia* symbionts used in this study are a rifampicin-resistant (Rf^r) spontaneous mutant strain RPE75 and a GFP-expressing mutant strain RPE225 (Table 1). These symbiont strains were preserved as frozen stocks at −80 °C and cultured at 25 °C with YG medium (0.5% yeast extract, 0.4% glucose, 0.1% NaCl) as described (Kikuchi *et al.* 2011b). *Escherichia coli* strains and plasmids used in this study are listed in Table 1.

Administration of Burkholderia *symbiont*

The symbiont strains were grown to an early log phase in YG medium (containing an adequate antibiotic) on a gyratory shaker (150 rpm) at 30 °C. Colony-forming unit (CFU) values were estimated by plating the culture media on YG agar plates containing adequate antibiotics. Symbiont cells were harvested by centrifuging the culture media, suspended in DWA and adjusted to 10^4 CFU/μL in DWA. Immediately after first instar nymphs moulted to the second instar, DWA was removed from the rearing containers so that the nymphs were kept without drinking water overnight. Then, DWA containing 10^4 CFU/μL symbiont cells was supplied to the rearing containers for 24 h, which the second instar nymphs immediately exploited, leading to the acquisition of *Burkholderia* symbionts. Then, the symbiont-containing DWA was replaced by symbiont-free DWA, and the nymphs were reared to adulthood. To clarify dose dependency of the colonization, symbiont cells were adjusted to 37, 370 and 3,700 CFU/μL in DWA, and inoculated as described above.

Fitness measurement

To reveal fitness effects of the *Burkholderia* symbiont, 93 and 60 first instar nymphs were prepared for symbiont-infected and uninfected groups, respectively: the former group was inoculated with cultured cells of the symbiont at the second instar stage as described above, while the latter group was left untreated. Nymphs were reared in clean plastic containers (8 cm in diameter,

Table 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description	Reference or resource
Bacterial strain		
Burkholderia symbiont		
RPE75	Wild-type, spontaneous Rf^r mutant of *Burkholderia* symbiont RPE64	Kikuchi *et al.* 2011a,b;
RPE225	GFP mutant of *Burkholderia* symbiont RPE75	This study
Escherichia coli		
WM3064	*thrB1004 pro thi rpsL hsdS lacZΔM15 RP4–1360 Δ(araBAD) 567 ΔdapA 1341*::[*erm pir(wt)*]	DK. Newman, California Institute of Technology
Plasmid		
pURR25	Mini Tn7KsGFP, GFP driven by P_{lac} ($P_{A1/04/03}$) promoter, mobilizable *ori*T_{IncP}α, suicide *ori*$R_{R6K\gamma}$; Ap^r (*bla*)	Teal *et al.* 2006;
pUX-BF13	Tn7 transposase genes *tnsABCDE*, mobilizable *ori*T_{IncP}α, suicide *ori*$R_{R6K\gamma}$; Ap^r (*bla*)	Bao *et al.* 1991

5.5 cm in depth) to adulthood and subjected to measurements of survival rate (adult emergence rate), nymphal period, body length and thorax width. In each container, 10–11 nymphs were reared and fed with 10 soybean seeds and DWA. For recording time to reproduction and number of eggs, each newly emerged female was mated with two males that emerged from the same experimental group on the same day, and number of eggs the female laid was recorded for 2 weeks from its first oviposition. Each mating pair was reared in a clean plastic container and fed with three soybean seeds and DWA. The fitness parameters were statistically analysed using the software R version 2.12.1 (R development core team 2005). Survival rate was analysed by Fisher's exact test, and the others were analysed by a Mann–Whitney *U*-test. Colonization of the *Burkholderia* symbiont was confirmed by diagnostic PCR with specific primer sets (see Table S1, Supporting information), as previously described (Kikuchi *et al.* 2007).

Construction of GFP-labelled Burkholderia *symbiont*

The GFP-labelled *Burkholderia* symbiont strain RPE225, originating from the *Burkholderia* symbiont strain RPE75, was generated by inserting a mini-Tn7 transposon containing P_{lac}::GFP into the symbiont chromosome using triparental mating as described (Teal *et al.* 2006). The donor strain *E. coli* WM3064 (a diaminopimelic acid [DAP] auxotroph) containing either pURR25 (a plasmid containing the miniTn7KSGFP; Teal *et al.* 2006) or pUX-BF13 (encoding Tn7 transposase; Bao *et al.* 1991) was grown and mated with RPE75 cells by triparental mating on YG agar containing 300 μg/mL DAP. GFP-labelled recipient cells were selected for resistance to kanamycin (WM3064 requires DAP to grow) and screened for GFP fluorescence using an epifluorescence microscope (Axiophot, Carl Zeiss).

Observation of dissected symbiotic organ

The insects administered with the GFP-labelled symbiont strain RPE225 were dissected in phosphate-buffered saline (PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 [pH7.4]) using a fine forceps under a dissection microscope. The dissected midguts were observed under the epifluorescence microscope freshly, or after a brief fixation with 4% paraformaldehyde for 10 min and a PBS washing.

Insect rearing on soybean pots inoculated with the GFP-labelled Burkholderia *symbiont*

Each plant pot (diameter 90 mm, height 120 mm) was filled with sterilized vermiculite and planted with a soybean seed. After the seed budded, approximately 10^8 cells of the GFP-labelled symbiont strain RPE225 suspended in 10 ml of distilled water were evenly spread across the pot. Newly moulted second instar nymphs were transferred from a rearing cage to the soybean pot. In addition to the soybean plant, ten dry soybean seeds in a small petri dish were supplied to the soybean pot. The infection processes of the GFP-labelled symbiont were observed using the epifluorescence microscope as described above.

Quantitative PCR

Real-time quantitative PCR was performed using SYBR green and MX3000P QPCR system (Stratagene, La Jolla, CA) with primers BSdnaA-F and BSdnaA-R (Table S1, Supporting information) targeting a 0.15 kb region of the *dnaA* gene of the *Burkholderia* symbiont as described (Kikuchi *et al.* 2011b). Total DNA was extracted from M4 and M4B parts by using NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany), and the extracted DNA was eluted in 200 μL of TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). Each of the PCR mixtures (20 μL in total) consisted of 2 μL of 10× *Taq*Gold buffer (Applied Biosystems, Foster City, CA), 1.2 μL of 25 mM $MgCl_2$, 2 μL of dNTPs (2 mM each of dATP, dTTP, dGTP and dCTP), 1 μL of dimethyl sulphoxide, 0.2 μL of SYBR green I (1/1000 diluted solution; Molecular Probes, Eugene, OR), 0.6 μL of primer mixture (5 μM each of forward and reverse primers), 0.1 μL of AmpliTaq Gold DNA polymerase (Applied Biosystems), 8.9 μL of distilled water and 4 μL of DNA sample. The PCR temperature profile was 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 15 s. A standard curve for the *dnaA* gene was generated with standard samples that contained 10, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 copies per reaction of the target PCR fragment amplified with the primers BSdnaA-F and BSdnaA-R.

Results

Experimental administration and fitness effects of the Burkholderia *symbiont*

Second instar *Riptortus* nymphs were orally administrated either with DWA containing cultured *Burkholderia* cells of the strain RPE75 or sterile DWA without bacteria. When some of the insects were subjected to dissection of the midgut and diagnostic PCR detection of the symbiont at the third instar stage, the former insects were all symbiont positive (30/30) while the latter insects were all symbiont negative (0/30). These insects were reared to adulthood, and their fitness parameters were inspected. While survival rates to adulthood were

not different between the symbiotic insects and the aposymbiotic insects (Fig. 2a), the symbiotic insects exhibited shorter time to adulthood, larger body length and greater thorax width than the aposymbiotic insects (Fig. 2b–d) as previously reported (Kikuchi *et al.* 2007). In addition, the symbiotic female insects started reproduction earlier and produced more eggs than the aposymbiotic female insects (Fig. 2e, f).

GFP labelling of the Burkholderia *symbiont*

We generated a GFP-labelled *Burkholderia* symbiont strain RPE225 by transforming the strain RPE75 with a Tn7 transposon mutagenesis system. The Tn7:GFP plasmid, pURR25 (Teal *et al.* 2006), encodes a Tn7 cassette that contains a *lac* promoter, a GFP gene and a kanamycin resistance (Km^r) gene. Tn7 specifically inserts into the bacterial chromosome at a Tn7 attachment (*att*Tn7) site located at a downstream region of the *glmS* gene that encodes glucosamine-6-phosphate synthetase (Choi *et al.* 2006). Because the *att*Tn7 site encodes no functional gene, the Tn7 insertion at the site is expected to be selectively neutral, retaining the intact gene set of the original strain. Reflecting the chromosomal Tn7GFP insertion, the recombinant symbiont strain RPE225 displayed bright and stable fluorescence both *in vitro* and *in vivo*,

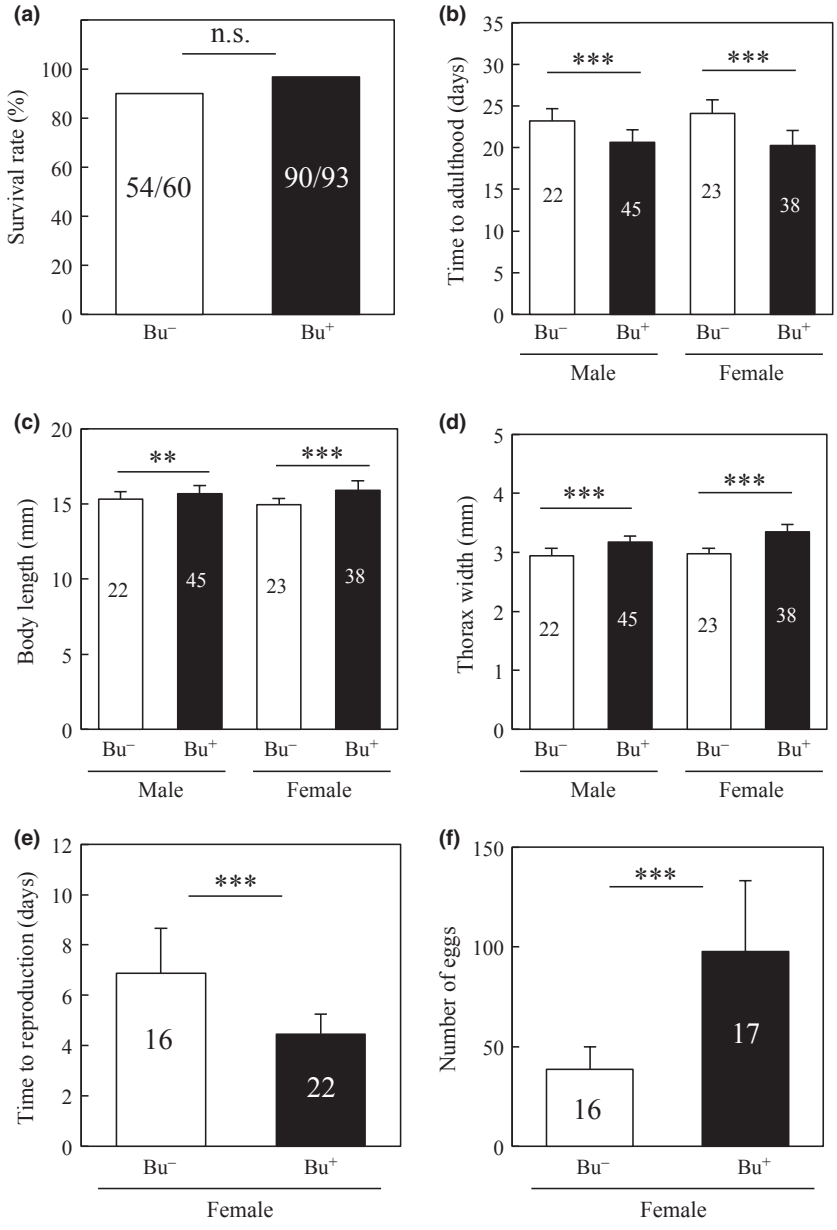

Fig. 2 Comparison of fitness parameters between the *Burkholderia*-infected (Bu^+) and uninfected (Bu^-) adult insects of *R. pedestris*. (a) Survival rate (=adult emergence rate) (b) time to adulthood, (c) body length, (d) maximum thorax width, (e) time to reproduction, (f) number of eggs. Numbers inside bars indicate sample sizes. Asterisks indicate statistically significant differences (**, $P < 0.001$; ***, $P < 0.0001$).

which required neither antibiotic selection nor induction by isopropyl β-D-1-thiogalactopyranoside (IPTG).

Symbiont colonization visualized by GFP labelling

When newly moulted second instar nymphs were administrated with the GFP-labelled *Burkholderia* symbiont cells, infection of the symbiotic organ was observed around 6 h after inoculation (Fig. 3a). At the beginning, the symbiont cells formed an aggregate at the entrance of the M4B region and then gradually migrated into the M4B region. By 24 h after inoculation, dense populations of the symbiont cells appeared in the main tract of the M4 region, and some symbiont cells were detected inside the crypts (Fig. 3b). The majority of the crypts were filled with the symbiont cells by 48 h after inoculation (Fig. 3c, d). By 72 h after inoculation, all the crypts were filled up with the symbiont cells (Fig. 3e), which remained during the subsequent nymphal development (Fig. 3f–h).

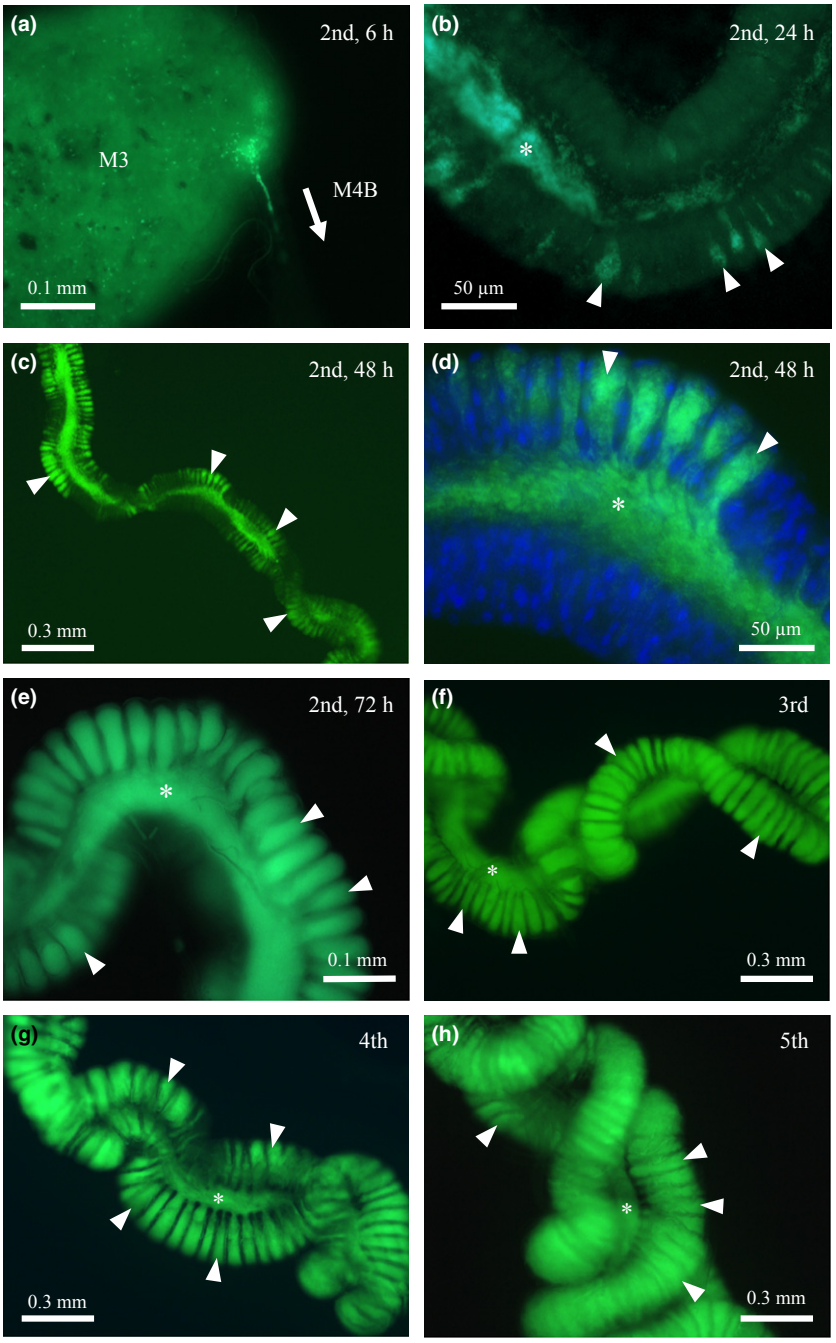

Fig. 3 Infection processes of the GFP-labelled *Burkholderia* symbiont visualized by epifluorescence microscopy. (a) The junction of the M3 and M4B regions of a second instar nymph, 6 h after inoculation. (b) The M4 region of a second instar nymph, 24 h after inoculation. (c, d) The M4 region of a second instar nymph, 48 h after inoculation. (e) The M4 region of a second instar nymph, 72 h after inoculation. (f) The M4 region of a third instar nymph. (g) The M4 region of a fourth instar nymph. (h) The M4 region of a fifth instar nymph. Asterisks and arrowheads indicate the main tract and crypts of the midgut, respectively. In (d), host nuclear DNA was counterstained by 4′,6-diamidino-2-phenylindole.

Population dynamics of the Burkholderia *symbiont during colonization*

Figure 4 shows the titres of the *Burkholderia* symbiont in the dissected symbiotic organ (M4 plus M4B) during colonization. Quantitative PCR targeting the symbiont *dnaA* gene revealed that the symbiont titres in the infected insects exponentially increased as the host development proceeded and reached a plateau around 10^7 per individual by the third instar stage.

Dose dependency of the symbiont colonization process

To clarify the dose dependency of the symbiont colonization process, newly moulted second instar nymphs were inoculated with different doses (37, 370 and 3700 CFU/μL) of the GFP-labelled *Burkholderia* symbiont. At the low inoculum dose (37 CFU/μL), the second instar nymphs exhibited delayed symbiont infection to the midgut crypts in comparison with the insects inoculated at higher doses (370 and 3,700 CFU/μL; Fig. 5). At the third instar, all the insects possessed symbiotic organs that were full of symbiont cells irrespective of the inoculum doses (data not shown).

Symbiont colonization in soybean plant pot

The colonization process of the *Burkholderia* symbiont to the *Riptortus* host was investigated in soybean plant

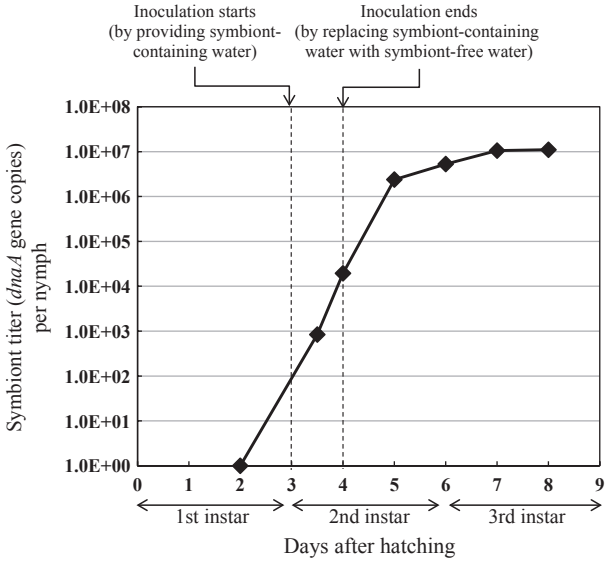

Fig. 4 Population dynamics of the *Burkholderia* symbiont during colonization to and proliferation in the midgut symbiotic organ. Dissected midguts were subjected to quantitative PCR of symbiont *dnaA* gene copies. Mean of 17 or 18 insects is indicated at each data point. The symbiont inoculation window is indicated by dotted lines.

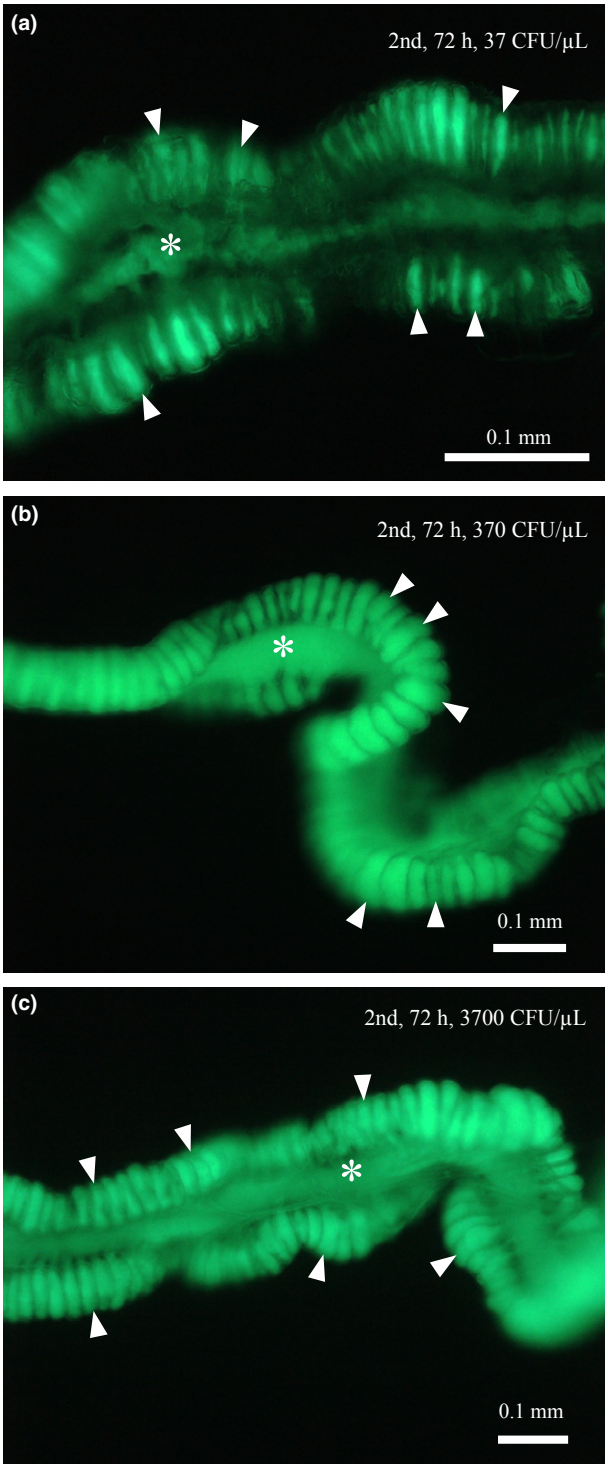

Fig. 5 Infection processes of the GFP-labellled *Burkholderia* symbiont in second instar nymphs of *R. pedestris* inoculated at different doses (37, 370 and 3700 CFU/μL). The insects were dissected 72 h after inoculation, and their midgut M4 regions were observed by epifluorescence microscopy. (a) 37 CFU/μL, (b) 370 CFU/μL and (c) 3700 CFU/μL. Asterisks and arrowheads indicate the main tract and crypts of the midgut M4 region, respectively.

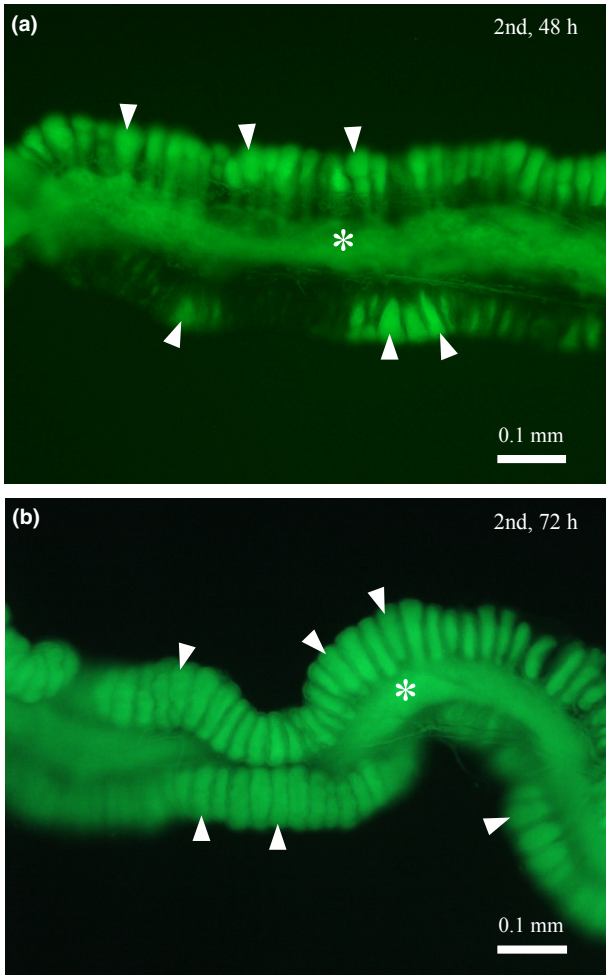

Fig. 6 Infection processes of the GFP-labelled *Burkholderia* symbiont in *R. pedestris* reared in soybean pots. Cultured symbiont cells were inoculated to the soybean pots, to which newly moulted second instar nymphs were introduced. The insects were dissected, and their midgut M4 regions were observed by epifluorescence microscopy. (a) 48 h after introduction and (b) 72 h after introduction. Asterisks and arrowheads indicate the main tract and crypts of the midgut M4 region, respectively.

pots inoculated with the GFP-labelled *Burkholderia* symbiont. Under the simulated seminatural condition, the symbiont colonization to the midgut crypts proceeded as in the petri dish experiments of the artificial symbiont administration (see Fig. 3). The majority of the crypts were filled with the symbiont cells by 48 h after the second instar moult (Fig. 6a), and all crypts were filled up with the symbiont cells by 72 h after the second instar moult (Fig. 6b).

Discussion

Like many insect symbionts of mutualistic nature thus far reported, the *Burkholderia* symbiont of *R. pedestris* exhibited beneficial effects on the host insect (Fig. 2) and specific localization to the symbiotic organ (Figs 1b and 3). Distinct from other symbionts, however, the *Burkholderia* symbiont is easily cultivable outside the host on standard microbiological media and therefore genetically manipulatable. In this study, we successfully established a GFP recombinant strain of the *Burkholderia* symbiont by making use of standard plasmid and conjugation systems commonly employed for genetic studies of *E. coli* and other proteobacteria. The GFP-labelled symbiont strain enabled live imaging of individual symbiont cells within dissected midgut symbiotic organs of the host insect, which offered an unprecedented opportunity to closely inspect the symbiont infection dynamics in the insect–microbe symbiosis.

Our observations of the infection processes using the GFP-labelled *Burkholderia* strain revealed that the symbiont colonization proceeds rapidly in second instar *Riptortus* nymphs, initiating within a few hours and completing by 72 h after inoculation. The colonization patterns under a laboratory rearing condition in petri dishes where the symbiont was administrated via drinking water (Fig. 3) were similar to the colonization patterns under a simulated seminatural condition in soybean plant pots where soil-mediated symbiont acquisition occurred (Fig. 6). Previous studies on the *Riptortus–Burkholderia* symbiosis have demonstrated that the host insect orally acquires the specific symbiont from the complex soil microbiota (Kikuchi *et al.* 2007), the symbiont acquisition preferentially occurs at the second instar stage (Kikuchi *et al.* 2011b) and even less than 100 symbiont cells are sufficient for establishing the symbiotic association (Kikuchi & Yumoto 2013). On the basis of these results, it seems likely that the symbiont infection processes are finely programmed and regulated in the host–symbiont interactions.

Our rearing experiments clearly showed beneficial effects of the *Burkholderia* symbiont on the *Riptortus* host (Fig. 2). However, it is currently unclear what the symbiont actually does for the host. A number of plant-sucking hemipteran insects possess beneficial symbionts inside their bodies, which are primarily located in the cytoplasm of specific cells called bacteriocytes (Buchner 1965; Kikuchi 2009). Recent genomic analyses of these symbiotic bacteria, in parallel with conventional nutritional and physiological studies, have shown that these symbionts provide their hosts with essential amino acids and some vitamins that are scarce in their plant sap diets (Douglas 1998; International aphid genomics consortium 2010; McCutcheon & Moran 2012; Husnik *et al.* 2013). While *R. pedestris* is a seed feeder rather than a sap feeder, it seems plausible, although speculative, that the *Burkholderia* symbiont is provisioning some nutritional factors that are deficient in food sources of

the *Riptortus* host. It is notable that the recently determined genome of the *Burkholderia* symbiont encodes complete metabolic pathways for essential amino acids and most vitamins (Shibata *et al.* 2013). Further nutritional and physiological studies are required to clarify biological roles of the *Burkholderia* symbiont for the *Riptortus* host.

The quantitative PCR analysis unveiled the initial and subsequent infection dynamics of the *Burkholderia* symbiont in the development of *Riptortus* nymphs: after inoculation, the symbiont titres sharply increased during the second instar period and reached a plateau at around 10^7 *dnaA* gene copies towards the third instar (Fig. 4). It should be noted that the symbiont titres in the symbiotic organ exhibited continuous increase even after the symbiont-containing drinking water was replaced with sterile drinking water (Fig. 4), indicating that the symbiont population growth within the second instar nymphs is mainly attributable to within-host proliferation of the symbiont cells. The symbiont population plateau towards the third instar is probably relevant to the filling up of the crypts in the host symbiotic organ (Fig. 3e, f).

Vertical symbiont transmission from mother to offspring is among the most pivotal processes for maintaining intimate host–symbiont associations (Bright & Bulgheresi 2010). Hence, researchers have paid considerable attention to the infection processes of symbiotic bacteria within their host insects (Buchner 1965). One of the best-described systems is the aphid–*Buchnera* symbiosis, wherein the symbiont cells are transmitted from maternal bacteriocytes to early embryos by specific exo- and endocytotic mechanisms (Buchner 1965; Miura *et al.* 2003; Koga *et al.* 2012). Such transovarial mechanisms for vertical symbiont transmission have also been described from many other systems including mealybug–*Tremblaya*, bedbug–*Wolbachia*, louse–*Riesia* and other endosymbiotic associations (Buchner 1965; Fukatsu & Nikoh 2000; Sasaki-Fukatsu *et al.* 2006; Hosokawa *et al.* 2010). In the tsetse–*Wigglesworthia* symbiosis, the symbiont cells are not transovarially transmitted but passed to the fly larva via milk gland secretion *in utero* (Attardo *et al.* 2008). In these systems, the symbiont transmission processes occur within the insect bodies and tissues, and the symbionts are genetically intractable due to their obligatory dependence on the intrahost environment. Hence, visualization of these symbiont transmission processes has been conducted on fixed specimens using such histological techniques as light microscopy, *in situ* hybridization, immunohistochemistry, and scanning and transmission electron microscopy.

On the other hand, some insects like stinkbugs have evolved posthatch symbiont transmission mechanisms outside their bodies. The stinkbugs or true bugs, belonging to the insect suborder Heteroptera with over 40 000 described species (Schuh & Slater 1995; Weirauch & Schuh 2011), are often in intimate association with specific symbiotic bacteria in the lumen of the midgut (Buchner 1965; Kikuchi 2009). These symbiotic associations are generally mutualistic, and symbiont-deprived insects often suffer growth defects, higher mortality, reduced fecundity and/or complete sterility (Buchner 1965; Abe *et al.* 1995; Fukatsu & Hosokawa 2002; Hosokawa *et al.* 2006, 2012; Kikuchi *et al.* 2007, 2009; Prado *et al.* 2009; Tada *et al.* 2011; Boucias *et al.* 2012; Salem *et al.* 2013). In the families Pentatomidae, Acanthosomatidae, Scutelleridae and Pyrrhocoridae, vertical symbiont transmission occurs upon oviposition via egg surface contamination with symbiont-containing excretions (Buchner 1965; Abe *et al.* 1995; Fukatsu & Hosokawa 2002; Kikuchi *et al.* 2007, 2009; Kaltenpoth *et al.* 2009; Prado *et al.* 2009; Kaiwa *et al.* 2010; Boucias *et al.* 2012). In the family Plataspidae, vertical symbiont transmission also occurs upon oviposition, but uniquely, the symbiont is provided within mother-made symbiont-containing capsules (Schneider 1940; Fukatsu & Hosokawa 2002; Hosokawa *et al.* 2006). In some subsocial species of the families Cydnidae and Parastrachiidae, mother insects stay with and take care of their offspring and provide the newborn nymphs with symbiont-containing fluid from the anus (Schorr 1957; Hosokawa *et al.* 2012). In these systems, the symbiont transmission processes occur outside the insect body, and, therefore, can be observed directly, often as specialized behaviour, ecology and structure of the host stinkbugs (Hosokawa *et al.* 2008, 2012). In the stinkbug superfamilies Lygaeoidea and Coreoidea (including the family Alydidae to which *R. pedestris* belongs), the uniqueness of the symbiosis is highlighted by the exceptional features that the symbiont is not vertically transmitted but acquired from the environment, and is easily cultivable, experimentally tractable and genetically manipulatable (Kikuchi *et al.* 2007, 2011a,b; Kikuchi & Yumoto 2013).

Genetically modified symbiotic bacteria labelled with GFP or other fluorescent proteins have provided a powerful tool for understanding spatiotemporal infection dynamics of the symbiont as well as intrahost ecological interactions of the symbiotic microbiota in legume–*Rhizobium*, squid–*Vibrio*, nematode–*Photorhabdus*/*Xenorhabdus*, zebrafish–*Pseudomonas* and other model symbiotic systems (Gage 2002; Dunn *et al.* 2006; Rawls *et al.* 2007; Ciche *et al.* 2008). The introduction of the GFP-labelled symbiont into the *Riptortus–Burkholderia* system facilitates the utility of the emerging model for insect symbiosis studies. The *Riptortus–Burkholderia* system has been regarded as promising in that (i) the symbiont is easily cultivable on standard microbiological media, which is

exceptional among insect symbiotic bacteria of a beneficial nature; (ii) the symbiont is orally acquired by young nymphs from the environment every generation, which allows easy preparation of symbiotic and aposymbiotic insects under controlled genetic backgrounds; (iii) both symbiotic and aposymbiotic insects are able to reach sexual maturity and reproduce in the laboratory, which are suitable for comparative physiological, transcriptomic, proteomic and metabolomic analyses on the effects of symbiosis; (iv) RNA interference of the host gene expression works beautifully, which enables straightforward functional analyses of host insect genes involved in symbiosis (Kikuchi *et al.* 2007, 2011b; Futahashi *et al.* 2011; Kikuchi & Yumoto 2013). Recently, in addition, both the symbiont genome sequence (Shibata *et al.* 2013) and the host transcriptome of the midgut symbiotic organ (Futahashi *et al.* 2013) have been determined, and a homologous recombination procedure to disrupt the symbiont genes has been established (Kim *et al.* 2013a,b). The accumulated knowledge and tools will enable approaches to investigate molecular mechanisms and associated genetic bases of the insect–microbe symbiosis from both the host side and the symbiont side. A number of important questions still remain unanswered. Why does the *Burkholderia* symbiont specifically infect and stably colonize the gut symbiotic organ of the *Riptortus* host? What factors affect the population dynamics of the symbiont in the intrahost ecosystem? How does the symbiont interact (and probably cope) with host immunity? We expect that molecular, genetic, cytological, physiological and ecological work with the *Riptortus–Burkholderia* model system will lead to deeper and wider understanding of the mechanisms underlying the insect–microbe symbiosis in the near future.

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Y.K. designed the study and performed experiments and analyses. Y.K. and T.F. wrote the manuscript.

Data accessibility

Data of the fitness experiments and quantitative PCR: DRYAD doi:10.5061/dryad.q1r19.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primer sets used for diagnostic and quantitative PCR.