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Host-symbiont specificity in insects: Underpinning mechanisms and evolution

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Abstract

How host organisms evolved and maintain specific mutualisms with microorganisms is a fundamental question that is subject to intensive research. In the large majority of insect mutualistic interactions, the host-microbe specificity is maintained by a “partner fidelity” mechanism, mainly through direct symbiont transmission from mother to offspring. Such a vertical manner of symbiont transmission is remarkably diverse in insects, including ovarian transmission, milk-gland transmission, coprophagy, egg-smearing, and capsule transmission. In contrast to the insect-microbe symbioses, many animals and plants do not vertically transmit their symbionts but acquire symbionts from ambient environments every generation. Sophisticated “partner choice” mechanisms are at play to maintain these mutualisms. This symbiont transmission mode, called horizontal transmission or environmental acquisition, is rarely found in insects, but recent studies have described this type of symbiosis in a few insect groups. The symbiosis between the bean bug *Riptortus pedestris* and its gut symbiont *Burkholderia insecticola* is one of the model systems that is intensively studied to understand how host-symbiont specificity and mutualistic interactions are maintained in insects with horizontal symbiont transmission. Phylogenetic analyses of symbionts in natural insect populations and bacterial inoculation tests in the laboratory revealed a high degree of specificity in this symbiosis while mutant screening of the symbiotic bacterium, genomics and transcriptomics, and histological observations have identified underpinning genetic and molecular bases. In this chapter, we focus on the symbiont

43 transmission modes and mechanisms observed in the amazing diversity of microbial

44 symbioses in insects and we highlight how they could have evolved.

45 (247 < 250 words)

46

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49 microbe-microbe competition, *Riptortus-Burkholderia* symbiosis

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Introduction

Many, if not most animals and plants are intimately associated with microorganisms. In these symbioses, the microbial partners contribute to the host fitness via various biological services such as the provision of essential nutrients, protection from antagonists, and degradation of toxins (Engel and Moran, 2013; Itoh et al., 2018b; Kikuchi, 2009). Since environments are filled with enormously diverse microorganisms including not only mutualists but also parasites and pathogens, hosts should winnow out these harmful microbes and selectively acquire the desired partners. To ensure the specific microbial partnership, host organisms have evolved sophisticated mechanisms for symbiont transmission and sorting. In the case of many insects that harbor specific gut or intracellular symbionts, the specific partnership is maintained by the “partner fidelity” mechanism that is based on the from-mother-to-offspring vertical symbiont transmission. The vertical transmission mechanisms in insects are remarkably diverse among taxonomic groups. In other cases, in a few insect groups, in marine invertebrates and in terrestrial plants, symbionts are not vertically transmitted but acquired from the ambient environment every generation (called horizontal transmission or environmental acquisition), wherein “partner choice” mechanisms facilitate the host-microbe specificity (Bright and Bulgheresi, 2010; Sachs et al. 2004). In this section, we will review the amazing diversity of symbiont transmission modes in insects and their underlying mechanisms, highlighting how the host-symbiont specificity is maintained and has evolved in this most diversified terrestrial animal group.

Vertical transmission

Insects that feed exclusively on a nutritionally imbalanced diet like plant saps or on indigestible materials like wood commonly harbor specific microbial symbionts that help the hosts to feed and grow on these specific nutritional sources. These symbionts reside in body cavities, gut crypts, or the cytoplasm of specialized cells called mycetocytes or bacteriocytes (Buchner, 1965; Kikuchi, 2009). Symbionts are mostly bacteria, while in some cases yeast-like symbionts, archaea and protist symbionts were also reported (Buchner, 1965; Brune and Dietrich, 2015; Engel and Moran, 2013; Kikuchi, 2009; Ohkuma and Brune, 2010; Sudakaran et al., 2017). The symbiotic microorganisms play a pivotal metabolic role in the insect hosts, such as the production of essential metabolites that are scarce in the diets and that the insect cannot synthesize or the degradation of plant polysaccharides that the insect cannot digest. In such nutritional and digestive symbioses, symbionts are commonly essential for survival, development, and reproduction of the hosts, and therefore, insects show high mortality when symbionts are removed by heat, ethanol and antibiotic treatment (Anbutsu et al., 2017; Brake, 1978; Douglas et al., 2001; Fukatsu and Hosokawa, 2002; Hosokawa et al., 2010; Itoh et al., 2016; Kikuchi et al., 2009; Salem et al., 2017; Sangare et al., 2016; Schlein, 1977; Srivastara and Auclair, 1976; Tada et al., 2011). To ensure the acquisition of the obligate partner by the next generation, most insects have evolved mechanisms for strict vertical transmission of the symbionts. It should be noted here

that the vertical manner of symbiont transmission guarantees not only absolute symbiont-acquisition by the offspring but also the host-symbiont specificity from generation to generation. A dramatic consequence of the vertical transmission is that the genomes of the microorganisms are usually strongly eroded and therefore, they are difficult or impossible to culture and manipulate genetically (Kikuchi, 2009; McCutcheon and Moran, 2012; Moran and Bennett, 2014; Moya et al., 2008). To date, various mechanisms for vertical symbiont transmission has been reported in insects. The transmission mechanisms are fundamentally different depending on the symbiont localization pattern, *i.e.* intracellular or extracellular.

Vertical transmission of intracellular symbionts

Intracellular symbionts are broadly known in diverse groups of insects, including the orders Blattaria, Hemiptera, Coleoptera, Hymenoptera and Diptera, in which symbiont harboring bacteriocytes or mycetocytes form clusters in the insect bodies, called bacteriome or mycetome. Intracellular symbionts are generally transmitted by ovarial transmission, while in some blood-sucking insects of the Diptera, milk-gland transmission has been reported.

Ovarial transmission

In the ovarial transmission mechanism, symbiotic bacteria directly infect the ovary and/or embryo from maternal bacteriocytes (Salem et al., 2015). The detailed infection process has been investigated in the pea aphid *Acyrtosiphon pisum* (Koga et al., 2012).

At the interface between a maternal bacteriocyte and embryo, the symbiont *Buchnera aphidicola* is exocytosed from the maternal bacteriocyte, momentarily released into the hemolymph, and then immediately endocytosed by the embryo. Although no detailed observation has been conducted in other insect groups so far, a similar exo- and endocytosis of symbiotic bacteria probably plays an important role in the ovarian transmission mechanisms.

Milk-gland transmission

In blood-sucking insects of the Diptera, such as tsetse flies and bat flies, symbiotic bacteria, *Wigglesworthia glossinidia* and *Aschnera chinzei*, respectively, are transmitted to offspring via the milk-gland, a specific organ that supplies “milk” to larvae (Hosokawa et al., 2012; Attardo et al., 2008). These blood-sucking insects are unique ovoviviparous species and females grow a single larva in a uterus-like organ with milk supplementation which contains the symbiotic bacteria, enabling to transmit the bacteria to the larva.

Vertical transmission of extracellular symbionts

Specific extracellular symbionts, most of which are gut symbionts, are reported in termites, stinkbugs, and beetles. The gut symbionts are vertically transmitted either by coprophagy, egg-smearing, or capsule transmission.

Coprophagy

Termites and wood-feeding cockroaches possess specific gut microbiota in the hindgut, consisting of not only bacteria but also specific protists (Brune and Dietrich, 2015; Ohkuma and Brune, 2010). In these social insects, aposymbiotic individuals such as newly-born hatchlings and newly molted nymphs acquire the specific microbiota by feeding feces excreted from parents or siblings. Such coprophagy is also reported in blood-sucking kissing bug *Rhodnius* spp., where the symbiont *Rhodococcus rhodonii* is transmitted through the feeding on feces (Beard et al., 2002). It was suggested that honey bees and ants transmit their specific gut microbiota through coprophagy and/or a specific food-exchange behavior called mouth-to-mouth trophallaxis (Koch et al., 2011; Onchuru et al., 2018; Powell et al., 2014).

Egg-smearing

Egg-smearing, or egg surface contamination by symbiotic bacteria, has been described in detail in stinkbugs (Bansal et al., 2014; Bistolas et al., 2014; Buchner, 1965; Dasch and Weiss, 1984; Hayashi et al., 2015; Hosokawa et al., 2013; Itoh et al., 2016; Karamipour et al., 2016; Kashkouli et al., 2019a, 2019b, 2019c; Kenyon et al., 2015; Kikuchi et al., 2012, 2009; Miyamoto, 1961; Prado et al., 2009, 2006; Tada et al., 2011; Taylor et al., 2014). Phytophagous species of stinkbugs, particularly members of the infraorder Pentatomomorpha, develop a symbiotic organ composed of rows of crypts, located in the posterior region of the midgut and housing specific extracellular bacterial symbionts (Buchner, 1965; Dasch and Weiss, 1984; Kikuchi et al., 2009; Miyamoto, 1961). Most species of the superfamily Pentatomoidea possess gammaproteobacterial

symbionts, which are smeared on the egg surface upon oviposition. In female insects, the last few crypts are bulbous compared with the other crypts and can discharge symbiotic bacteria from the crypt lumen. These crypts are thought to be specialized for housing symbionts for transmission (Hayashi et al., 2015). In the particular case of the stinkbug family Acanthosomatidae however, all crypt entrances are completely sealed in adult insects, making the direct smearing from crypts impossible (Kikuchi et al., 2009). Instead, female insects evolved a novel pair of organs, associated with the female ovipositor and called “lubricating organs”. From these sac-like organs, symbionts are smeared on the egg surface during oviposition (Kikuchi et al., 2009). It is still enigmatic when and how the symbiotic bacteria migrate from the gut to the lubricating organ and how the new organ specialized for symbiont transmission evolved in the stinkbug family. In the case of *Lagria* beetles, antibiotic-producing defensive *Burkholderia gladioli* symbionts are associated with female accessory glands. They are smeared on the egg surface, protecting host eggs from pathogenic fungi (Kaltenpoth and Florez, 2019).

Capsule transmission

Contrary to species of the other Pentatomoidea families, stinkbugs of the family Plataspidae deploy a unique mechanism for vertical transmission, called “capsule transmission” (Buchner, 1965; Fukatsu and Hosokawa, 2002). Together with the egg masses, mother insects oviposit brownish capsules containing the symbiotic bacterium *Ishikawaella capsulata* and hatchlings acquire the symbiont by sucking up the capsule

content. Females of the insect develop for capsule production a specific portion in the midgut, located just after the crypt-bearing symbiont-harboring region (Fukatsu and Hosokawa, 2002; Hosokawa et al., 2008; 2007; 2006; 2005). A similar mechanism, called “jelly transmission”, has been reported in another stinkbug family of the Pentatomoidea, the Urostylididae, in which mother insects oviposit eggs with a large amount of a symbiont containing jelly-like matrix (Kaiwa et al., 2014). Hatchlings acquire symbiotic bacteria by consuming the jelly.

In addition to the stinkbug symbioses, another unique type of capsule transmission was discovered in the tortoise leaf beetle *Cassida rubiginosa* (Salem et al., 2017). The leaf beetle harbors a pectin-degrading symbiont, *Candidatus Stammera capleta*, inside a pair of sac-like organs associated with the foregut. Female insects deposit a symbiont-containing capsule or “caplet” on the top of each egg and hatchlings acquire the symbiont by consuming the caplet compounds.

Partner choice in insect-microbe symbiosis with vertical transmission

Since insects develop highly sophisticated mechanisms for vertical symbiont transmission as shown above and symbionts generally show phylogenetic congruence with host phylogeny, the transmission mechanisms are commonly thought of as very rigorous without room for parasites and cheaters. However, several studies demonstrated that also partner choice plays a role to maintain the insect-microbe mutualism with vertical transmission. For example, the pea aphid is frequently infected with secondary intracellular symbionts, such as *Serratia symbiotica*, in addition to the

primary symbiont *B. aphidicola* (Moran et al., 2005; Oliver et al., 2010). Detailed microscopic observations revealed that *Buchnera* is selectively transmitted from maternal bacteriocytes to the embryo by the exo-/endocytosis process even when secondary symbionts co-colonize in the bacteriocytes (Koga et al., 2012).

Beewolf wasps, that harbor the defensive symbiont *Streptomyces philanthi* in antennal glands, transmit the symbiont by spreading out gland secretions in their nest, where larvae develop through the pupal stage until adult emergence (Goettler et al., 2007; Kaltenpoth et al., 2010, 2005). The bacteria in the nests provide protection against pathogenic fungi and bacteria by producing different antimicrobial compounds (Kroiss et al., 2010). Interestingly, experimental inoculation of a non-symbiotic actinomycetes bacterium to aposymbiotic adult females revealed that the related bacterium can stably colonize the antennal glands but cannot be secreted and transmitted from the antennae (Kaltenpoth et al., 2014), suggesting that a partner choice mechanism exists in the vertical transmission process. Both cases highlight that partner choice mechanisms reinforce partner fidelity to stabilize long-term, strictly specific insect-microbe symbioses.

Horizontal transmission of symbionts in animals and plants

Apart from the majority of insect-microbe mutualisms, most animals and also plants do not commonly transmit their symbionts vertically; instead, they acquire specific partners from the ambient environment every generation. Because of the enormous diversity of

microorganisms in environments, hosts should actively select their symbionts from the environmental microbiota in these symbioses with horizontal symbiont transmission. To efficiently and specifically choose a partner, host organisms develop sophisticated mechanisms for symbiont sorting. Such partner choice mechanisms have been well investigated and described in two model systems: the nitrogen-fixing *Rhizobium* symbiosis in leguminous plants and the bioluminescent *Vibrio* symbiosis in the Hawaiian bobtail squid (Nyholm and McFall, 2004; Wang et al., 2018). As mentioned above, the vertically transmitted insect symbionts have strongly reduced genomes, making them unculturable outside their host (Kikuchi, 2009; McCutcheon and Moran, 2012; Moran and Bennett, 2014; Moya et al., 2008). Because of their different lifestyles including the free-living state in the environment, the symbionts maintained by horizontal transmission are in contrast culturable and genetically manipulatable. These traits have been very useful to clarify the genetic and molecular bases of the symbiotic associations.

The legume-*Rhizobium* symbiosis

The legume-*Rhizobium* symbiosis is probably the best-characterized symbiotic system from different perspectives, including the understanding of the evolutionary ecology and the molecular mechanisms that govern the symbiosis. In response to nitrogen starvation and the presence of specific compatible rhizobium bacteria in the rhizosphere, legumes will form particular symbiotic organs on their roots, called nodules. The rhizobia are housed in large numbers inside these nodules as intracellular

symbionts and they convert atmospheric nitrogen gas into ammonia, which is used by the plant as a nitrogen source for its growth. The legume-*Rhizobium* interaction is usually extremely specific and a given *Rhizobium* strain will interact only with a narrow range of legume species and vice versa. The host-symbiont compatibility is monitored all along the symbiotic process, from the initiation of the interaction over the development and infection of the nodules till the nitrogen fixation in mature nodules. This is achieved by a continuous exchange of signals between the two partners, including plant-derived flavonoids and antimicrobial peptides, and bacterial molecules like lipochitooligosaccharides, extracellular polysaccharides, lipopolysaccharides, type III and type IV secretion effectors and even small RNAs (Poole et al., 2018; Mergaert, 2018). Most emblematic among these signals are the rhizobial Nod factors which are produced in the rhizosphere and upon recognition trigger the plant genetic nodulation program. Nod factors are lipochitooligosaccharides, which have a similar structure in all rhizobia but which still differ from each other by the presence of strain-specific chemical modifications (D'Haeze and Holsters, 2002). Nod factor signaling contributes to the specificity of the interaction by the presence of a matching Nod factor receptor complex in the plant. Similarly for the other signals, it is believed that a specific signal-receptor correspondence exists. Moreover, in a mature nodule, the host plants monitor the symbiont's nitrogen-fixation activity. If its performance is not optimal, the hosts control the oxygen supply to the nodule and limit the survival of the symbiont (Kiers et al., 2003). Such sanction mechanisms are thought to be important to prevent the evolution of cheaters and maintain the mutualistic association with horizontal symbiont

transmission (Archetti et al., 2011; Sachs et al., 2004). Thus, the legume-*Rhizobium* mutualism is stabilized by partner choice mechanisms based on signaling cues, avoiding legumes from initiating or completing interactions with incompatible rhizobia, as well as by sanctions applied in established interactions to reduce the costs of maintaining low-quality partners.

The squid-*Vibrio fischeri* symbiosis

In the case of the squid-*Vibrio* symbiosis, another type of host-symbiont chemical signaling has been reported (Nyholm and McFall-Ngai, 2004; Visick and Ruby, 2006). Depending on the presence of marine bacteria, squid hatchlings start to produce mucus at ciliated epithelia of the light organ, which traps *V. fischeri* from marine water. *V. fischeri* aggregates on the mucus and out-competes other bacteria, and then migrate into the light organ by passing through a narrow entrance using flagellar motility (Graf and Ruby, 1994; Millikan and Ruby, 2004, 2003; Nyholm et al., 2000). In the duct and crypt of the light organ, a certain concentration of nitric oxide and hypohalous acid is reached further winnowing out contaminating bacteria (Davidson et al., 2004; Small et al., 1999; Weis et al., 1996). Tracheal cytotoxin (TCT), a peptidoglycan fragment derived from *V. fischeri* and LPS are recognized by host receptors and stimulate a morphological alteration of the symbiotic organ (Koropatnick et al., 2004; Nyholm and Graf, 2012), leading to maturation of the light organ and establishment of the symbiosis. *V. fischeri*-derived outer membrane vesicles (OMVs) are also involved in the morphological alteration and apoptosis of the light organ (Aschtgen et al., 2016a, 2016b). From the

host side, peptidoglycan recognition proteins (PGRPs) and probably also LPS-binding proteins (LBPs) play important roles for recognition and interaction with the symbiont-derived signals (Chun et al., 2008; Krasity et al., 2011; Nyholm and Graf, 2012; Troll 2009; 2010).

Horizontal transmission of symbionts in insects

Recent studies have discovered a few exceptions to the general pattern of vertical symbiont transmission in the insects. These groups of insects acquire the symbionts from the environment (Bright and Bulgheresi, 2010; Salem et al., 2015), like in the legume-*Rhizobium* and squid-*Vibrio* symbioses. Among these insect-microbe symbioses with horizontal transmission, the symbiosis between the bean bug *Riptortus pedestris* and *Burkholderia insecticola* is a powerful model system (Kaltenpoth and Florez, 2019; Takeshita and Kikuchi, 2017) and it has been well investigated how host-microbe specificity has evolved and is maintained in this insect.

The bean bug-*Burkholderia* symbiosis

The bean bug *R. pedestris* (Heteroptera: Pentatomomopha: Coreoidea: Alydidae) (Fig. 1A) is a serious pest of leguminous crops in India, South-East Asia, China, Korea and Japan (Kikuhara, 2005; Schaefer and Panizzi, 2000). *R. pedestris* is a hemimetabolous insect and develops to adults via five instar stages in approximately 20 days (Kikuchi and Fukatsu, 2014). As is typical for stinkbugs, *R. pedestris* possesses numerous crypts at the posterior part of the midgut, in which *Burkholderia* symbionts are harbored (Fig.

1B-G) (Kikuchi et al., 2005). *Burkholderia insecticola* strain RPE64 is the type strain for the *R. pedestris* symbionts (Takeshita et al., 2018). The *Burkholderia* symbiont is beneficial for the host insect: symbiotic insects show a faster development, larger body size, and a higher number of eggs than aposymbiotic insects (Kikuchi et al., 2007; Kikuchi and Fukatsu, 2014). Transcriptomic analyses of the *Burkholderia* symbiont revealed that the symbiont recycles host's metabolic wastes and provides the host with essential amino acids and vitamins (Ohbayashi et al., 2019a). Among the five instar stages, *R. pedestris* acquires the *Burkholderia* symbiont from ambient soil mainly in the 2nd instar stage (Kikuchi et al., 2011b).

The *Riptortus-Burkholderia* symbiosis is an ideal model to elucidate the molecular bases of host-microbe symbiosis for several reasons. The *Burkholderia* symbiont is easy to culture in standard bacterial media and to genetically manipulate by standard techniques and tools (Kikuchi and Fukatsu, 2014; Kim et al., 2013; Ohbayashi et al., 2015). The whole genome sequence of the *Burkholderia* symbiont is available (Shibata et al., 2013; Takeshita et al., 2014). In addition, the bean bug host is easily reared and produces large numbers of offspring in small containers, requiring only dried soybean seeds and distilled water containing ascorbic acid (Kikuchi et al., 2007). The symbiont inoculation method is well established and is based on adding the desired bacteria to the drinking water of the insects (Kikuchi and Fukatsu, 2014). This laboratory inoculation method closely mimics the natural infection process in which the insects acquire the symbionts through drinking or feeding. Although the bean bug genome is not completely sequenced, several sets of transcriptome data are available,

notably for symbiotic conditions and immune responses (Futahashi et al., 2013; Ohbayashi et al., unpublished data). RNA interference (RNAi) works very well in *R. pedestris* (Futahashi et al., 2011). Contrary to most insect-microbe symbioses where symbionts are essential and aposymbiotic insects have thus a high mortality, aposymbiotic insects of the bean bug show a growth delay but have high survivability (Kikuchi et al., 2007), enabling the genetic and physiological comparisons between aposymbiotic and symbiotic insects. This attractive model system has been used to investigate host-symbiont specificity and how it evolved and is maintained in insect-microbe symbiotic associations with horizontal transmission.

The bean bug-*Burkholderia* symbiosis is highly specific

The genus *Burkholderia* consists of over 100 species and is an ecologically diverse group (Eberl and Vandamme, 2016). Based on genomic phylogeny, the genus is grouped into at least three distinct clades. The first one includes human-, animal-, and plant-pathogens, named the “*B. cepacia* complex and *B. pseudomallei*” (BCC&P) clade (Mahenthiralingam et al., 2005). The second clade consists of many plant growth-promoting rhizobacteria and nodule symbionts of leguminous plants, designated as the “plant-associated beneficial and environmental” (PBE) clade (Suárez-Moreno et al., 2012). The third clade mainly consists of environmental species, leaf-nodule symbionts of Rubiaceae plants, and gut symbionts of stinkbugs, and is called the “stinkbug-associated beneficial and environmental” (SBE) clade (Takeshita and Kikuchi, 2017; Peeters et al., 2016). The BCC&P, PBE, and SBE clades are recently designated as

different genera called *Burkholderia* sensu stricto, *Paraburkholderia*, and *Caballeronia*, respectively (Beukes et al. 2017). The outgroup of the *Burkholderia* is the genus *Pandoraea*, mostly consisting of common soil bacteria.

Analyses of the M4 midgut crypt symbionts in natural populations of *R. pedestris* systematically demonstrated the colonization by *Burkholderia* species (Jung and Lee, 2019; Kikuchi et al., 2005; 2011a). These *Burkholderia* are genetically diverse but nearly all of them belong to the SBE clade, although infection with species of the more distant BCC&P clade was also reported in a particular study of overwintering *R. pedestris* specimens (Jung and Lee, 2019).

As mentioned above, the presence of bacterial symbiont-carrying M4 midgut crypts is very common in the heteropteran infraorder Pentatomomorpha (Kikuchi et al., 2011a). In many species of the superfamilies Coreoidea and Lygaeoidea of the Pentatomomorpha, these crypts are also colonized with SBE *Burkholderia* (Boucias et al., 2012; Itoh et al., 2014; Garcia et al., 2014; Kikuchi et al., 2011a, 2005; Kuechler et al., 2016; Ohbayashi et al., 2019b; Oliver-Espejel et al., 2011). Nevertheless, other *Burkholderia*, belonging to the BCC&P and PBE clades, and even *Cupriavidus* and *Pseudomonas* species were occasionally identified in some insect species (Boucias et al., 2012; Garcia et al., 2014; Itoh et al., 2014). However, it should be noted that, except for several SBE *Burkholderia* isolates from *R. pedestris* (Kikuchi et al., 2007), *Coreus marginatus* (Ohbayashi et al., 2019b) and *Blissus insularis* (Xu et al., 2016), for none of the other identified strains (*Burkholderia* or other species) the Koch's postulates were verified. These bacteria were not cultured and tested for their capacity to infect and

colonize the crypts of aposymbiotic hosts. Finally, also the M4 midgut crypts in species of the family Largidae of the superfamily Pyrrhocoroidea carry *Burkholderia* but they belong strictly to the PBE clade (Gordon et al., 2016; Sudakaran et al., 2015; Takeshita et al., 2015).

Together, the inspections of these natural stinkbug samples suggest a very strong or nearly exclusive colonization of the midgut crypts with SBE *Burkholderia* in the Coreoidea and Lygaeoidea superfamilies and with PBE *Burkholderia* in the family Largidae of the Pyrrhocoroidea. Similarly as what is firmly established for the *R. pedestris* symbionts (Kikuchi et al., 2007), environmental acquisition of the *Burkholderia* was postulated or demonstrated in all the examined insect species, even if occasional vertical transmission was suggested in some of them (Itoh et al., 2014; Xu et al., 2016). Thus, the infections of the M4 midgut crypts in *R. pedestris* and its allied stinkbug species should be controlled by efficient partner choice mechanisms that are specific at the broad taxonomic scale of *Burkholderia* groups. Nevertheless, because of the observed genetic diversity of the symbionts in each insect species, these mechanisms may have a more relaxed specificity at a finer scale of the species and strain level.

Two types of laboratory experiments in *R. pedestris* supported these conclusions. In laboratory insects reared on soil, infections with PBE *Burkholderia* as well as *Pandoraea* were occasionally identified in the crypts but the overwhelming majority of crypt bacteria belonged to the SBE clade (Itoh et al., 2018a). In a second type of experiments, infection tests of aposymbiotic *R. pedestris* were performed with a

broad range of bacteria in pure culture, including 34 *Burkholderia* species (13 species of SBE, 12 species of PBE, and 7 species of BCC&P) and an additional 18 taxonomically diverse non-*Burkholderia* bacterial species (Itoh et al., 2019). All tested SBE species were very efficiently colonizing the midgut crypts but also most tested PBE species and the tested *Pandoraea* species, although PBE and *Pandoraea* species did not fully colonize the M4 crypts. Moreover, the PBE and *Pandoraea* species enhanced the survival and development of the bean bug similar to SBE species, indicating that these bacteria are not harmful but beneficial to the insect host. On the contrary, the tested BCC&P *Burkholderia* species and all other bacterial species, which included other Burkholderiaceae (*Ralstonia*, *Chitinimonas*, *Cupriavidus*) and non-Burkholderiaceae, were unable to establish in the crypts. Thus overall, these laboratory infection experiments using soil or cultured bacteria recapitulated very well the observed natural specificity of *R. pedestris* for SBE *Burkholderia*, although the specificity seems to be broader in the laboratory than in nature. This then raises the question what the underlying partner choice mechanisms are that determine the symbiont selection in natural and laboratory conditions. This seems to be a particularly fundamental issue in light of the enormous diversity of bacteria in soils (Bahram et al., 2018; Delgado-Baquerizo et al., 2018), suggesting that these mechanisms must be particularly performant.

Partner choice mechanisms in *Riptortus pedestris*

The gut constricted region as a partner-choice apparatus

Histological observations and inoculation experiments have revealed that *R. pedestris* develops a marked symbiont sorting organ in the midgut (Ohbayashi et al., 2015). The midgut is separated in five morphologically different sections (Fig. 1C): a swollen first section (M1), a tubular second section (M2), an ovoid-shaped third section (M3), the crypt-bearing fourth section (M4) and in addition, a bulbous part in front of the M4 region, called the M4 bulb (M4B). The *Burkholderia* symbiont partially colonizes the M4B in addition to the M4 crypts. The M1 to M3 sections constitute the digestive regions of the intestine, while the M4B and M4 are symbiotic regions.

The junction between M3 and M4B is a remarkably narrow channel which has a diameter close to the dimensions of bacterial cells (Fig. 1C; Fig. 2C). This so-called “constricted region” is filled with a mucus-like matrix and strictly prevents food flow from M3 to the symbiotic gut sections (Fig. 2A) but this channel also constitutes the gate through which ingested bacteria pass to enter the symbiotic M4 region of the intestine (Fig. 2B and C). However, the constricted region is not a simple open gate allowing any bacteria to pass but it filters them and only bacteria with the right (still unknown) key can pass through. Indeed, co-inoculation tests of a green fluorescence protein (GFP)-labelled *B. insecticola* and a red fluorescent protein (RFP)-labelled *E. coli* revealed that while *E. coli* is sorted out by the constricted region, only the *Burkholderia* symbiont passes and reaches the symbiotic region (Fig. 2D). In addition to *E. coli*, typical soil bacteria like *Pseudomonas putida*, *Bacillus subtilis*, *Bradyrhizobium japonicum* as well as many other species, also cannot pass through the constricted region and are sorted out (Itoh et al., 2019; Ohbayashi et al., 2015).

In contrast to the crypt-bearing species of the Lygaeoidea and Coreoidea superfamilies and the Largidae family, which are commonly associated with *Burkholderia*, members of the Pentatomoidea, another superfamily of the Pentatomomorpha, possess gammaproteobacterial symbionts in midgut crypts which are maintained by strict vertical transmission (Bansal et al., 2014; Bistolas et al., 2014; Hayashi et al., 2015; Hosokawa et al., 2016, 2013; Itoh et al., 2016; Karamipour et al., 2016; Kashkouli et al., 2019a, 2019b, 2019c; Kenyon et al., 2015; Kikuchi et al., 2012; Prado et al., 2009, 2006; Tada et al., 2011; Taylor et al., 2014) using the earlier mentioned mechanisms of egg-smearing, capsule transmission or jelly transmission. Interestingly, the constricted region is broadly conserved, not only in the *Burkholderia*-associated lygaeoid, coreoid and largid species, but also in the Pentatomoidea (Gordon et al., 2016; Ohbayashi et al., 2015). This strongly suggests that the constricted region evolved in the common ancestor of the stinkbug superfamilies. Thus, even if in the Pentatomoidea, the symbiotic association is mainly maintained by partner fidelity mechanisms, *i.e.* vertical transmission, partner choice achieved by the constricted region may play a pivotal role in these species too.

Corkscrew flagellar motility

Although the molecular mechanism underpinning the bacterial sorting in the constricted region remains unclear, a screening for colonization-defect mutants of *B. insecticola* revealed that flagellar motility is a key factor for the symbiont's ability to colonize the M4 crypts (Ohbayashi et al., 2015). Although non-motile mutants reach the M3, they

cannot enter the constricted region and never reach M4B and M4, clearly demonstrating the importance of flagellar motility for passing through the mucus-filled narrow passage. However, this finding raises the question why only the *Burkholderia* symbiont can pass through the constricted region, in spite of active motility also in the tested *E. coli*, *P. putida*, and *B. subtilis*.

To date, two types of flagellar motility have been described in bacteria (Jarrel and McBride, 2008): run-and-tumbling motility, described well in bacteria having peritrichous flagella, such as *E. coli* and *Pseudomonas* species, but also common in bacteria having polar flagella; and forward-and-reverse motility, found in bacteria having polar flagella, such as *Vibrio* species. In addition, some peritrichous bacteria show swarming motility on a surface of semi-solid agar (Jarrel and McBride, 2008). *B. insecticola*, that has one to three polar flagella (Fig. 1B), shows normal run-and-tumbling motility in a liquid environment. However, in a viscous condition, a unique, novel type of flagellar motility has been found in the *Burkholderia* symbiont, called “corkscrew flagellar motility”. In this particular swimming mode, the symbiont wraps the flagellar filaments around the cell body and moves like a drill in the viscous environment (Fig. 2F and G) (Kinoshita et al., 2018). Considering that this type of novel motility has been reported in a few polar-flagellated bacteria including *Vibrio fischeri* and *Shewanella putrefaciens* (Kinoshita et al., 2018; Kuhn et al., 2017), it is tempting to speculate that the corkscrew motility contributes to the specific penetration of the mucus-filled constricted region in the *Burkholderia* symbiont. To prove this hypothesis, it will thus be of interest to analyze the corkscrew flagellar motility in other

Burkholderia or *Pandorea* strains that can move into the M4 crypts or to identify the genetic determinants of corkscrew motility in *B. insecticola*.

Midgut closure stimulated by symbiont colonization

In addition to the partner choice achieved by the selective passage through the constricted region, a second layer mechanism for maintaining the *Riptortus-Burkholderia* specificity was recently discovered. This mechanism also involves the constricted region and is called the “midgut closure” (Kikuchi et al. submitted). In a few hours after the *Burkholderia* symbiont starts penetrating into the constricted region and colonizing the M4 crypts, the constricted region and the M4B, which are at first permeable for the bacteria, become closed, completely preventing the entrance of additional bacteria. Later on, after the full occupation of the M4 crypts with the symbionts, the M4B region is re-opened and surplus symbiont cells from the crypts flow back to the M4B, filling it with symbiont cells. Notably, even if the re-opening of M4B occurs, the constricted region is kept firmly closed. This midgut closure prevents additional colonization of the symbiotic region by symbiotic bacteria or any possible contaminants and it thus contributes to preserve the specific colonization of the crypts.

Competition-based selection in the gut

As mentioned above, the capacity to pass through the constricted region and the infection of the M4 crypts is not limited to the natural symbiont group of SBE *Burkholderia* (Itoh et al., 2019). For example, *Pandoraea norimbergensis* and

Burkholderia fungorum are capable to colonize the midgut crypts of *R. pedestris* (Fig. 3A-D), even though these common soil bacteria are usually not associated with the bean bug in natural conditions. An additional partner choice mechanism, called “competition-based selection”, was put forward to explain this observed symbiont specificity (Itoh et al., 2019). It was shown that the SBE *Burkholderia* always outcompete PBE *Burkholderia* or *Pandoraea* species in the M4 region when these species are co-infecting the symbiotic organ, achieving the predominance of SBE *Burkholderia* in the bean bug gut (Fig. 3E-G). These experimental data clearly demonstrate that the abilities for colonization and cooperation, usually thought of as specific traits of mutualists, are not unique to the *Burkholderia* symbiont (*i.e.* SBE *Burkholderia*). On the contrary, competitiveness inside the gut is a derived trait of the bean bug symbiont lineage only (Fig. 3H) and has thus played a critical role in the evolution of the insect gut symbiont.

Although at present the molecular bases of the *in vivo* competitiveness of the *Burkholderia* symbiont remains unclear, the following four types of mechanisms can be considered.

(i) *Nutrient-based mechanism.* Auxotrophic mutants of the *Burkholderia* symbiont, such as purine biosynthesis mutants, show a severe crypt colonization defect (Kim et al., 2014), suggesting that the M4 environment is nutritionally poor or there is a selective repertoire of available nutrients. In fact, transcriptomic analyses revealed that the *Burkholderia* symbiont actively proliferates by assimilating host’ metabolic wastes, such as sulfate and allantoin/urea, in M4 (Ohbayashi et al., 2019a). Adaptive abilities to

the M4 nutritional environment probably affect the competitiveness and the selective colonization of the *Burkholderia* symbiont.

(ii) *Antimicrobial agent-based mechanism*. Previous studies demonstrated that the AMP resistance of *B. insecticola* plays an important role in stable colonization of the symbiont in M4. For example, a deletion mutant of *uppP* (undecaprenyl pyrophosphate phosphatase; involved in cell wall biosynthesis) becomes susceptible to lysozymes *in vitro* and also shows a severe colonization defect in the M4 crypts (Kim et al., 2013). Similarly, lipopolysaccharide (LPS) biosynthesis mutants, such as $\Delta wabO$, $\Delta waaC$ and $\Delta waaF$, also show decreased colonization ability in M4 (Kim et al., 2016; Kim et al., 2017). It should be noted that transcriptomic analyses of M4 revealed that a novel type of antimicrobial peptides (AMPs), called crypt-specific cysteine-rich peptides (CCRs), are highly and specifically expressed in the midgut crypts (Futahashi et al., 2013). Although the principal role of these crypt-specific AMPs remains unclear, they may play a role in the symbiont's competitiveness and the *in vivo* selection process.

(iii) *Adhesion-based mechanism*. Theoretical studies have predicted that bacterial adhesion to gut epithelial cells has a selective advantage for competitive colonization in the gut (McLoughlin et al., 2016). Genomic data indicates that the *Burkholderia* symbiont possesses some Tad pilli and type I fimbriae, which may contribute to the efficient colonization and competitiveness of the *Burkholderia* symbiont.

(iv) *Direct inhibition-based mechanism*. Type VI secretion system (T6SS) is one of the well-known bacterial systems to directly inject anti-bacterial effectors and inhibit competitors (Russell et al., 2014). The importance of T6SS in internal bacterial competition has been reported in the squid-*Vibrio* symbiosis (Speare et al., 2018) and the honey bee gut symbiosis (Steele et al., 2017). Although SBE *Burkholderia* have a single type of T6SS, co-inoculation tests of PBE *Burkholderia* or *Pandoraea* and T6SS deletion mutants of *B. insecticola* demonstrated that T6SS is not involved in the M4 competitiveness of the symbiont (Itoh et al., 2019). Some BCC&P *Burkholderia* dispose of another type of microbe-microbe toxin-delivery system, called “contact-dependent growth inhibition (CDI)” (Garcia et al., 2016). However, the SBE *Burkholderia* do not have any CDI genes. Hence, at this stage these direct systems do not seem to be involved in the symbiont’s *in vivo* competitiveness.

Conclusion

Although theoretical studies have proposed mechanisms for host-microbe mutualisms, such as partner fidelity feedback, partner choice and sanction, competition-based selection, and public goods theory (Archetti et al., 2011), it is not entirely clear how mutualistic associations have evolved and are maintained. Furthermore, the genetic and molecular bases of interspecific mutualisms are almost totally unclear in most of the symbiotic systems, except a few model systems such as the legume-*Rhizobium* and squid-*Vibrio* symbioses. The recent works on the *Riportus-Burkholderia* symbiosis have greatly improved our knowledge concerning the molecular bases of insect-microbe

symbiosis with horizontal symbiont transmission. This useful model system contributes to clarify how host-symbiont specificity evolved and is maintained in insect gut symbiosis. In contrast, the genetic and molecular bases of the vertical symbiont transmission remain unknown, despite the omnipresence of this type transmission in insects. At this stage, only a few studies have tackled the question because reverse-genetic approaches like RNAi and genome-editing do not efficiently work in many insect models of endosymbiosis. Stinkbugs and beetles may be promising models because they possess well-developed symbiotic systems and RNAi experiments are possible in most cases. In fact, by use of the RNAi technique, recent studies have succeeded in identifying a transcription factor and immune-related genes that play pivotal roles in the maintenance of intracellular symbioses (Login et al., 2011; Maire et al., 2019; Matsuura et al., 2015).

In insects, as summarized in this section, there are diverse vertical transmission mechanisms involving highly sophisticated morphological features and behaviors, as well as environmental acquisition mechanisms with well-developed partner choice and competition-based mechanisms. It remains totally unknown how such complex adaptive traits could evolve. The amazing diversity of microbial symbioses in insects, in conjunction with the established molecular tools and the rapidly progressed omics techniques, provides us a great opportunity to tackle this fundamental but still enigmatic question.

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605

606 **Conflict of Interest**

607 The authors declare that they have no competing interests.

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References

- Anbutsu, H., Moriyama, M., Nikoh, N., Hosokawa, T., Futahashi, R., Tanahashi, M., Meng, X. Y., Kuriwada, T., Mori, N., Oshima, K., Hattori, M., Fujie, M., Satoh, N., Maeda, T., Shigenobu, S., Koga, R. and Fukatsu, T., 2017. Small genome symbiont underlies cuticle hardness in beetles. *Proc. Natl. Acad. Sci. U. S. A.* 114, E8382-E8391.
- Archetti, M., Scheuring, I., Hoffman, M., Frederickson, M. E., Pierce, N. E. and Yu, D. W., 2011. Economic game theory for mutualism and cooperation. *Ecol. Lett.* 14, 1300-1312.
- Aschtgen, M. S., Lynch, J. B., Koch, E., Schwartzman, J., McFall-Ngai, M. and Ruby, E., 2016a. Rotation of *Vibrio fischeri* flagella produces outer membrane vesicles that induce host development. *J. Bacteriol.* 198, 2156-2165.
- Aschtgen, M. S., Wetzel, K., Goldman, W., McFall-Ngai, M. and Ruby, E., 2016b. *Vibrio fischeri*-derived outer membrane vesicles trigger host development. *Cell. Microbiol.* 18, 488-499.
- Attardo, G. M., Lohs, C., Heddi, A., Alam, U. H., Yildirim, S. and Aksoy, S., 2008. Analysis of milk gland structure and function in *Glossina morsitans*: Milk protein production, symbiont populations and fecundity. *J. Insect. Physiol.* 54, 1236-1242.
- Bahram, M., Hildebrand, F., Forslund, S. K., Anderson, J. L., Soudzilovskaia, N. A., Bodegom, P. M., Bengtsson-Palme, J., Anslan, S., Coelho, L. P., Harend, H., Huerta-Cepas, J., Medema, M. H., Maltz, M. R., Mundra, S., Olsson, P. A., Pent,

632 M., Polme, S., Sunagawa, S., Ryberg, M., Tedersoo, L. and Bork, P., 2018.
 633 Structure and function of the global topsoil microbiome. *Nature* 560, 233-237.

634 Bansal, R., Michel, A. P. and Sabree, Z. L., 2014. The crypt-dwelling primary bacterial
 635 symbiont of the polyphagous pentatomid pest *Halyomorpha halys* (hemiptera:
 636 Pentatomidae). *Environ. Entomol.* 43, 617-625.

637 Beard, C. B., Cordon-Rosales C. and Durvasula, R. V., 2002. Bacterial symbionts of the
 638 Triatominae and their potential use in control of Chagas disease transmission.
 639 *Annu. Rev. Entomol.* 47, 123-141.

640 Beukes, C. W., Palmer, M., Manyaka, P., Chan, W. Y., Avontuur, J. R., van Zyl, E.,
 641 Huntemann, M., Clum, A., Pillay, M., Palaniappan, K., Varghese, N., Mikhailova,
 642 N., Stamatis, D., Reddy, T. B. K., Daum, C., Shapiro, N., Markowitz, V., Ivanova,
 643 N., Kyrpides, N., Woyke, T., Blom, J., Whitman, W. B., Venter, S.
 644 N. and Steenkamp, E. T., 2017. Genome data provides high support for generic
 645 boundaries in *Burkholderia* sensu lato. *Front. Microbiol.* 8, 1154.

646 Bistolas, K. S., Sakamoto, R. I., Fernandes, J. A. and Goffredi, S. K., 2014. Symbiont
 647 polyphyly, co-evolution, and necessity in pentatomid stinkbugs from costa rica.
 648 *Front. Microbiol.* 5, 349.

649 Boucias, D. G., Garcia-Maruniak, A., Cherry, R., Lu, H., Maruniak, J. E. and Lietze, V.
 650 U., 2012. Detection and characterization of bacterial symbionts in the
 651 heteropteran, *Blissus insularis*. *FEMS Microbiol. Ecol.* 82, 629-641.

652 Bracke, J. W., Cruden, D. L. and Markovetz, A. J., 1978. Effect of metronidazole on the
653 intestinal microflora of the american cockroach, *Periplaneta americana* L.
654 Antimicrob. Agents Chemother. 13, 115-120.

655 Bright, M. and Bulgheresi, S., 2010. A complex journey: Transmission of microbial
656 symbionts. Nat. Rev. Microbiol. 8, 218-230.

657 Brune, A. and Dietrich, C., 2015. The gut microbiota of termites: Digesting the
658 diversity in the light of ecology and evolution. Annu. Rev. Microbiol. 69, 145-
659 166.

660 Buchner, P., 1965. Endosymbiosis of animals with plant microorganisms. Interscience,
661 New York.

662 Chun, C. K., Troll, J. V., Koroleva, I., Brown, B., Manzella, L., Snir, E., Almabrazi, H.,
663 Scheetz, T. E., de Fatima Bonaldo, M., Casavant, T. L., Soares, M. B., Ruby, E.
664 G. and McFall-Ngai, M. J., 2008. Effects of colonization, luminescence, and
665 autoinducer on host transcription during development of the squid-vibrio
666 association. Proc. Natl. Acad. Sci. U. S. A. 105, 11323-11328.

667 D'Haeze, W. and Holsters, M., 2002. Nod factor structures, responses, and perception
668 during initiation of nodule development. Glycobiology 12, 79R-105R.

669 Dasch, G., E. and Weiss, C.K., 1984. Endosymbionts of insects. In Krieg, N. A. (ed.),
670 Bergey's Manual of Systematic Bacteriology, Vol. I, Williams and Wilkins,
671 Baltimore, pp. 881-883.

672 Davidson, S. K., Koropatnick, T. A., Kossmehl, R., Sycuro, L. and McFall-Ngai, M. J.,
 673 2004. No means 'yes' in the squid-vibrio symbiosis: Nitric oxide (NO) during
 674 the initial stages of a beneficial association. *Cell. Microbiol.* 6, 1139-1151.
 675 Delgado-Baquerizo, M., Oliverio, A. M., Brewer, T. E., Benavent-González, A.,
 676 Eldridge, D. J., Bardgett, R. D., Maestre, F. T., Singh, B. K. and Fierer, N., 2018.
 677 A global atlas of the dominant bacteria found in soil. *Science* 359, 320-325.
 678
 679 Douglas, A. E., Minto, L. B. and Wilkinson, T. L., 2001. Quantifying nutrient
 680 production by the microbial symbionts in an aphid. *J. Exp. Biol.* 204, 349-358.
 681 Eberl, L. and Vandamme, P., 2016. Members of the genus *Burkholderia*: Good and bad
 682 guys. *F1000Res.* 5, 1007.
 683 Engel, P. and Moran, N. A., 2013. The gut microbiota of insects-diversity in structure
 684 and function. *FEMS Microbiol. Rev.* 37, 699-735.
 685 Fukatsu, T. and Hosokawa, T., 2002. Capsule-transmitted gut symbiotic bacterium of
 686 the japanese common plataspid stinkbug, *Megacopta punctatissima*. *Appl.*
 687 *Environ. Microbiol.* 68, 389-396.
 688 Futahashi, R., Tanaka, K., Matsuura, Y., Tanahashi, M., Kikuchi, Y. and Fukatsu, T.,
 689 2011. Laccase2 is required for cuticular pigmentation in stinkbugs. *Insect*
 690 *Biochem. Mol. Biol.* 41, 191-196.

691 Futahashi, R., Tanaka, K., Tanahashi, M., Nikoh, N., Kikuchi, Y., Lee, B. L. and
692 Fukatsu, T., 2013. Gene expression in gut symbiotic organ of stinkbug affected by
693 extracellular bacterial symbiont. PLoS One 8, e64557.

694 Garcia, E. C., Perault, A. I., Marlatt, S. A. and Cotter, P. A., 2016. Interbacterial
695 signaling via *Burkholderia* contact-dependent growth inhibition system proteins.
696 Proc. Natl. Acad. Sci. U. S. A. 113, 8296-8301.

697 Garcia, J. R., Laughton, A. M., Malik, Z., Parker, B. J., Trincot, C., S, S. L. C., Chung,
698 E. and Gerardo, N. M., 2014. Partner associations across sympatric broad-headed
699 bug species and their environmentally acquired bacterial symbionts. Mol. Ecol.
700 23, 1333-1347.

701 Goettler, W., Kaltenpoth, M., Herzner, G. and Strohm, E., 2007. Morphology and
702 ultrastructure of a bacteria cultivation organ: The antennal glands of female
703 European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae).
704 Arthropod Struct. Dev. 36, 1-9.

705 Gordon, E. R., McFrederick, Q. and Weirauch, C., 2016. Phylogenetic evidence for
706 ancient and persistent environmental symbiont reacquisition in Largidae
707 (Hemiptera: Heteroptera). Appl. Environ. Microbiol. 82, 7123-7133.

708 Graf, J., Dunlap, P. V. and Ruby, E. G., 1994. Effect of transposon-induced motility
709 mutations on colonization of the host light organ by *Vibrio fischeri*. J. Bacteriol.
710 176, 6986-6991.

711 Hayashi, T., Hosokawa, T., Meng, X. Y., Koga, R. and Fukatsu, T., 2015. Female-
 712 specific specialization of a posterior end region of the midgut symbiotic organ in
 713 *Plautia splendens* and allied stinkbugs. Appl. Environ. Microbiol. 81, 2603-2611.
 714 Hosokawa, T., Hironaka, M., Inadomi, K., Mukai, H., Nikoh, N. and Fukatsu, T., 2013.
 715 Diverse strategies for vertical symbiont transmission among subsocial stinkbugs.
 716 PLoS One 8, e65081.
 717 Hosokawa, T., Kikuchi, Y. and Fukatsu, T., 2007. How many symbionts are provided
 718 by mothers, acquired by offspring, and needed for successful vertical transmission
 719 in an obligate insect-bacterium mutualism? Mol. Ecol. 16, 5316-5325.
 720 Hosokawa, T., Kikuchi, Y., Meng, X. Y. and Fukatsu, T., 2005. The making of
 721 symbiont capsule in the plataspid stinkbug *Megacopta punctatissima*. FEMS
 722 Microbial. Ecol. 54, 471-477.
 723 Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M. and Fukatsu, T., 2006. Strict host-
 724 symbiont cospeciation and reductive genome evolution in insect gut bacteria.
 725 PLoS Biol. 4, e337.
 726 Hosokawa, T., Kikuchi, Y., Shimada, M. and Fukatsu, T., 2008. Symbiont acquisition
 727 alters behaviour of stinkbug nymphs. Biol. Lett. 4, 45-48.
 728 Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X. Y. and Fukatsu, T., 2010. *Wolbachia* as
 729 a bacteriocyte-associated nutritional mutualist. Proc. Natl. Acad. Sci. U. S. A.
 730 107, 769-774.
 731 Hosokawa, T., Matsuura, Y., Kikuchi, Y. and Fukatsu, T., 2016. Recurrent evolution of
 732 gut symbiotic bacteria in pentatomid stinkbugs. Zoological Lett. 2, 24.

733 Hosokawa, T., Nikoh, N., Koga, R., Sato, M., Tanahashi, M., Meng, X. Y. and Fukatsu,
 734 T., 2012. Reductive genome evolution, host-symbiont co-speciation and uterine
 735 transmission of endosymbiotic bacteria in bat flies. *ISME J.* 6, 577-587.

736 Itoh, H., Aita, M., Nagayama, A., Meng, X. Y., Kamagata, Y., Navarro, R., Hori, T.,
 737 Ohgiya, S. and Kikuchi, Y., 2014. Evidence of environmental and vertical
 738 transmission of *Burkholderia* symbionts in the oriental chinch bug, *Cavelerius*
 739 *saccharivorus* (Heteroptera: Blissidae). *Appl. Environ. Microbiol.* 80, 5974-5083.

740 Itoh, H., Hori, T., Sato, Y., Nagayama, A., Tago, K., Hayatsu, M. and Kikuchi, Y.,
 741 2018a. Infection dynamics of insecticide-degrading symbionts from soil to insects
 742 in response to insecticide spraying. *ISME J.* 12, 909-920.

743 Itoh, H., Jang, S., Takeshita, K., Ohbayashi, T., Ohnishi, N., Meng, X. Y., Mitani, Y.
 744 and Kikuchi, Y., 2019. Host-symbiont specificity determined by microbe–microbe
 745 competition in an insect gut. *Proc. Natl. Acad. Sci. U. S. A.* 116, 22673-22682.

746 Itoh, H., Matsuura, Y., Hosokawa, T., Fukatsu, T. and Kikuchi, Y., 2016. Obligate gut
 747 symbiotic association in the sloe bug *Dolycoris baccarum* (Hemiptera:
 748 Pentatomidae). *Appl. Entomol. Zool.* 52, 51-59.

749 Itoh, H., Tago, K., Hayatsu, M. and Kikuchi, Y., 2018b. Detoxifying symbiosis:
 750 Microbe-mediated detoxification of phytotoxins and pesticides in insects. *Nat.*
 751 *Prod. Rep.* 35, 434-454.

752 Jarrell, K. F. and McBride, M. J., 2008. The surprisingly diverse ways that prokaryotes
 753 move. *Nat. Rev. Microbiol.* 6, 466-476.

754 Jung, M. and Lee, D. H., 2019. Abundance and diversity of gut-symbiotic bacteria, the
 755 genus *Burkholderia* in overwintering *Riptortus pedestris* (Hemiptera: Alydidae)
 756 populations and soil in South Korea. PLoS One 14, e0218240.

757 Kaiwa, N., Hosokawa, T., Nikoh, N., Tanahashi, M., Moriyama, M., Meng, X. Y.,
 758 Maeda, T., Yamaguchi, K., Shigenobu, S., Ito, M. and Fukatsu, T., 2014.
 759 Symbiont-supplemented maternal investment underpinning host's ecological
 760 adaptation. Curr. Biol. 24, 2465-2470.

761 Kaltenpoth, M. and Flórez, L. V., 2019. Versatile and dynamic symbioses between
 762 insects and burkholderia bacteria. Annu. Rev. Entomol. doi: 10.1146/annurev-
 763 ento-011019-025025. [Epub ahead of print].

764 Kaltenpoth, M., Gottler, W., Herzner, G. and Strohm, E., 2005. Symbiotic bacteria
 765 protect wasp larvae from fungal infestation. Curr. Biol. 15, 475-479.

766 Kaltenpoth, M., Roeser-Mueller, K., Koehler, S., Peterson, A., Nechitaylo, T. Y.,
 767 Stubblefield, J. W., Herzner, G., Seger, J. and Strohm, E., 2014. Partner choice
 768 and fidelity stabilize coevolution in a cretaceous-age defensive symbiosis. Proc.
 769 Natl. Acad. Sci. U. S. A. 111, 6359-6364.

770 Kaltenpoth, M., Schmitt, T., Polidori, C., Koedam, D. and Strohm, E., 2010. Symbiotic
 771 streptomycetes in antennal glands of the south american digger wasp genus
 772 *Trachypus* (Hymenoptera, Crabronidae). Physiol. Entomol. 35, 196-200.

773 Karamipour, N., Fathipour, Y. and Mehrabadi, M., 2016. Gammaproteobacteria as
 774 essential primary symbionts in the striped shield bug, *Graphosoma lineatum*
 775 (Hemiptera: Pentatomidae). Sci. Rep. 6, 33168.

776 Kashkouli, M., Fathipour, Y. and Mehrabadi, M., 2019. Habitat visualization,
 777 acquisition features and necessity of the gammaproteobacterial symbiont of
 778 pistachio stink bug, *Acrosternum heegeri* (Hem.: Pentatomidae). Bull. Entomol.
 779 Res. 1-12.

780 Kashkouli, M., Fathipour, Y. and Mehrabadi, M., 2019. Heritable
 781 gammaproteobacterial symbiont improves the fitness of *Brachynema germari*
 782 Kolenati (Hemiptera: Pentatomidae). Environ. Entomol. 48, 1079-1087.

783 Kashkouli, M., Fathipour, Y. and Mehrabadi, M., 2019. Potential management tactics
 784 for pistachio stink bugs, *Brachynema germari*, *Acrosternum heegeri* and
 785 *Acrosternum arabicum* (hemiptera: Pentatomidae): High temperature and
 786 chemical surface sterilants leading to symbiont suppression. J. Econ. Entomol.
 787 112, 244-254.

788 Kenyon, L. J., Meulia, T. and Sabree, Z. L., 2015. Habitat visualization and genomic
 789 analysis of "*Candidatus Pantoea carbekii*," the primary symbiont of the brown
 790 marmorated stink bug. Genome Biol. Evol. 7, 620-635.

791 Kiers, E. T., Rousseau, R. A., West, S. A. and Denison, R. F., 2003. Host sanctions and
 792 the legume-rhizobium mutualism. Nature 425, 78.

793 Kikuchi, Y., 2009. Endosymbiotic bacteria in insects: Their diversity and culturability.
 794 Microbes and environments *Microbe. Environ.* 24, 195-204.

795 Kikuchi, Y. and Fukatsu, T., 2014. Live imaging of symbiosis: Spatiotemporal infection
 796 dynamics of a GFP-labelled *Burkholderia* symbiont in the bean bug *Riptortus*
 797 *pedestris*. Mol. Ecol. 23, 1445-1456.

798 Kikuchi, Y., Hosokawa, T. and Fukatsu, T., 2007. Insect-microbe mutualism without
799 vertical transmission: A stinkbug acquires a beneficial gut symbiont from the
800 environment every generation. *Appl. Environ. Microbiol.* 73, 4308-4316.

801 Kikuchi, Y., Hosokawa, T. and Fukatsu, T., 2011a. An ancient but promiscuous host-
802 symbiont association between *Burkholderia* gut symbionts and their heteropteran
803 hosts. *ISME J.* 5, 446-460.

804 Kikuchi, Y., Hosokawa, T. and Fukatsu, T., 2011b. Specific developmental window for
805 establishment of an insect-microbe gut symbiosis. *Appl. Environ. Microbiol.* 77,
806 4075-4081.

807 Kikuchi, Y., Hosokawa, T., Nikoh, N. and Fukatsu, T., 2012. Gut symbiotic bacteria in
808 the cabbage bugs *Eurydema rugosa* and *Eurydema dominulus* (Heteroptera:
809 Pentatomidae). *Appl. Entomol. Zool.* 47, 1-8.

810 Kikuchi, Y., Hosokawa, T., Nikoh, N., Meng, X. Y., Kamagata, Y. and Fukatsu, T.,
811 2009. Host-symbiont co-speciation and reductive genome evolution in gut
812 symbiotic bacteria of acanthosomatid stinkbugs. *BMC Biol.* 7, 2.

813 Kikuchi, Y., Meng, X. Y. and Fukatsu, T., 2005. Gut symbiotic bacteria of the genus
814 *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocoris*
815 *chinensis* (Heteroptera: Alydidae). *Appl. Environ. Microbiol.* 71, 4035-4043.

816 Kikuhara, Y., 2005. The Japanese species of the genus *Riptortus* (Heteroptera,
817 Alydidae) with description of a new species. *Jpn. J. Syst. Entomol.* 11, 299-311.

818 Kim, J. K., Jang, H. A., Kim, M. S., Cho, J. H., Lee, J. B., Lorenzo, F. D., Sturiale, L.,
819 Silipo, A., Molinaro, A. and Lee, B. L., 2017. The lipopolysaccharide core

820 oligosaccharide of *Burkholderia* plays a critical role in maintaining a proper gut
 821 symbiosis with the bean bug *Riptortus pedestris*. J. Biol. Chem. 292, 19226-
 822 19237.

823 Kim, J. K., Jang, H. A., Won, Y. J., Kikuchi, Y., Heum Han, S., Kim, C. H., Nikoh, N.,
 824 Fukatsu, T. and Lee, B. L., 2014. Purine biosynthesis-deficient *Burkholderia*
 825 mutants are incapable of symbiotic accommodation in the stinkbug. ISME J. 8,
 826 552-563.

827 Kim, J. K., Lee, H. J., Kikuchi, Y., Kitagawa, W., Nikoh, N., Fukatsu, T. and Lee, B.
 828 L., 2013. Bacterial cell wall synthesis gene *uppP* is required for *Burkholderia*
 829 colonization of the stinkbug gut. *Appl. Environ. Microbiol.* 79, 4879-4886.

830 Kim, J. K., Park, H. Y. and Lee, B. L., 2016. The symbiotic role of o-antigen of
 831 *Burkholderia* symbiont in association with host *Riptortus pedestris*. Dev. Comp.
 832 Immunol. 60, 202-208.

833 Kinoshita, Y., Kikuchi, Y., Mikami, N., Nakane, D. and Nishizaka, T., 2018. Unforeseen
 834 swimming and gliding mode of an insect gut symbiont, *Burkholderia* sp. Rpe64,
 835 with wrapping of the flagella around its cell body. ISME J. 12, 838-848.

836 Koch, H. and Schmid-Hempel, P., 2011. Socially transmitted gut microbiota protect
 837 bumble bees against an intestinal parasite. Proc. Natl. Acad. Sci. U. S. A. 108,
 838 19288-19292.

839 Koga, R., Meng, X. Y., Tsuchida, T. and Fukatsu, T., 2012. Cellular mechanism for
 840 selective vertical transmission of an obligate insect symbiont at the bacteriocyte-
 841 embryo interface. Proc. Natl. Acad. Sci. U. S. A. 109, E1230-E1237.

842 Koropatnick, T. A., Engle, J. T., Apicella, M. A., Stabb, E. V., Goldman, W. E. and
 843 McFall-Ngai, M. J., 2004. Microbial factor-mediated development in a host-
 844 bacterial mutualism. *Science* 306, 1186-1188.

845 Krasity, Benjamin C., Troll, Joshua V., Weiss, Jerrold P. and McFall-Ngai, Margaret J.,
 846 2011. LBP/BPI proteins and their relatives: Conservation over evolution and roles
 847 in mutualism. *Biochem. Soc. Trans.* 39, 1039-1044.

848

849 Kroiss, J., Kaltenpoth, M., Schneider, B., Schwinger, M. G., Hertweck, C., Maddula, R.
 850 K., Strohm, E. and Svatos, A., 2010. Symbiotic streptomycetes provide antibiotic
 851 combination prophylaxis for wasp offspring. *Nat. Chem. Biol.* 6, 261-263.

852 Kuechler, S. M., Matsuura, Y., Dettner, K. and Kikuchi, Y., 2016. Phylogenetically
 853 diverse *Burkholderia* associated with midgut crypts of spurge bugs,
 854 *Dicranocephalus* spp. (Heteroptera: Stenocephalidae). *Microbes Environ.* 31,
 855 145-153.

856 Kuhn, M. J., Schmidt, F. K., Eckhardt, B. and Thormann, K. M., 2017. Bacteria exploit
 857 a polymorphic instability of the flagellar filament to escape from traps. *Proc. Natl.*
 858 *Acad. Sci. U. S. A.* 114, 6340-6345.

859 Login, F. H., Balmand, S., Vallier, A., Vincent-Monegat, C., Vigneron, A., Weiss-
 860 Gayet, M., Rochat, D. and Heddi, A., 2011. Antimicrobial peptides keep insect
 861 endosymbionts under control. *Science* 334, 362-365.

862 Mahenthiralingam, E., Urban, T. A. and Goldberg, J. B., 2005. The multifarious,
 863 multireplicon *Burkholderia cepacia* complex. *Nat. Rev Microbiol.* 3, 144-156.

864 Maire, J., Vincent-Monegat, C., Balmand, S., Vallier, A., Herve, M., Masson, F.,
 865 Parisot, N., Vigneron, A., Anselme, C., Perrin, J., Orlans, J., Rahioui, I., Da Silva,
 866 P., Fauvarque, M. O., Mengin-Lecreulx, D., Zaidman-Remy, A. and Heddi, A.,
 867 2019. Weevil *pgrp-lb* prevents endosymbiont TCT dissemination and chronic host
 868 systemic immune activation. Proc. Natl. Acad. Sci. U. S. A. 116, 5623-5632.
 869 Matsuura, Y., Kikuchi, Y., Miura, T. and Fukatsu, T., 2015. Ultrabithorax is essential
 870 for bacteriocyte development. Proc. Natl. Acad. Sci. U. S. A. 112, 9376-9381.
 871 McCutcheon, J. P. and Moran, N. A., 2012. Extreme genome reduction in symbiotic
 872 bacteria. Nat. Rev. Microbiol. 10, 13-26.
 873 McLoughlin, K., Schluter, J., Rakoff-Nahoum, S., Smith, A. L. and Foster, K. R., 2016.
 874 Host selection of microbiota via differential adhesion. Cell Host Microbe 19, 550-
 875 559.
 876 Mergaert, P., 2018. Role of antimicrobial peptides in controlling symbiotic bacterial
 877 populations. Nat. Prod. Rep. 35, 336-356.
 878 Millikan, D. S. and Ruby, E. G., 2003. FlrA, a 54-dependent transcriptional activator in
 879 *Vibrio fischeri*, is required for motility and symbiotic light-organ colonization. J.
 880 Bacteriol. 185, 3547-3557.
 881 Millikan, D. S. and Ruby, E. G., 2004. *Vibrio fischeri* flagellin a is essential for normal
 882 motility and for symbiotic competence during initial squid light organ
 883 colonization. J. Bacteriol. 186, 4315-4325.
 884 Miyamoto, S., 1961. Comparative morphology of alimentary organs of Heteroptera,
 885 with the phylogenetic consideration. Sieboldia 2, 197-259.

886 Moran, N. A. and Bennett, G. M., 2014. The tiniest tiny genomes. *Annu. Rev.*
 887 *Microbiol.* 68, 195-215.

888 Moran, N. A., Russell, J. A., Koga, R. and Fukatsu, T., 2005. Evolutionary relationships
 889 of three new species of Enterobacteriaceae living as symbionts of aphids and other
 890 insects. *Appl. Environ. Microbiol.* 71, 3302-3310.

891 Moya, A., Pereto, J., Gil, R. and Latorre, A., 2008. Learning how to live together:
 892 Genomic insights into prokaryote-animal symbioses. *Nat. Rev. Gen.* 9, 218-229.

893 Nyholm, S. V. and Graf, J., 2012. Knowing your friends: Invertebrate innate immunity
 894 fosters beneficial bacterial symbioses. *Nat. Rev. Microbiol.* 10, 815-827.

895 Nyholm, S. V. and McFall-Ngai, M., 2004. The winnowing: Establishing the squid-
 896 *Vibrio* symbiosis. *Nat. Rev. Microbiol.* 2, 632-642.

897 Nyholm, S. V., Stabb, E. V., Ruby, E. G. and McFall-Ngai, M. J., 2000. Establishment
 898 of an animal-bacterial association: Recruiting symbiotic vibrios from the
 899 environment. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10231-10235.

900 Ohbayashi, T., Futahashi, R., Terashima, M., Barriere, Q., Lamouche, F., Takeshita, K.,
 901 Meng, X. Y., Mitani, Y., Sone, T., Shigenobu, S., Fukatsu, T., Mergaert, P. and
 902 Kikuchi, Y., 2019a. Comparative cytology, physiology and transcriptomics of
 903 *Burkholderia insecticola* in symbiosis with the bean bug *Riptortus pedestris* and
 904 in culture. *ISME J.* 13, 1469–1483.

905 Ohbayashi, T., Itoh, H., Lachat, J., Kikuchi, Y. and Mergaert, P., 2019b. *Burkholderia*
 906 gut symbionts associated with European and Japanese populations of the dock bug
 907 *Coreus Marginatus* (Coreoidea: Coreidae). *Microbes Environ.* 34, 219-222.

908 Ohbayashi, T., Takeshita, K., Kitagawa, W., Nikoh, N., Koga, R., Meng, X. Y., Tago,
 909 K., Hori, T., Hayatsu, M., Asano, K., Kamagata, Y., Lee, B. L., Fukatsu, T. and
 910 Kikuchi, Y., 2015. Insect's intestinal organ for symbiont sorting. *Proc. Natl.*
 911 *Acad. Sci. U. S. A.* 112, E5179–E5188.

912 Ohkuma, M. and Brune, A., 2010. Diversity, structure, and evolution of the termite gut
 913 microbial community. Bignell DE, Roisin Y, & Lo N (Eds.), *Biology of Termites:*
 914 *A Modern Synthesis.* Springer Netherlands, Dordrecht, pp 413-438.

915 Oliver, K. M., Degnan, P. H., Burke, G. R. and Moran, N. A., 2010. Facultative
 916 symbionts in aphids and the horizontal transfer of ecologically important traits.
 917 *Annu. Rev. Entomol.* 55, 247-266.

918 Olivier-Espejel, S., Sabree, Z. L., Noge, K. and Becerra, J. X., 2011. Gut microbiota in
 919 nymph and adults of the giant mesquite bug (*Thasus neocalifornicus*)
 920 (Heteroptera: Coreidae) is dominated by *Burkholderia* acquired de novo every
 921 generation. *Environ. Entomol.* 40, 1102-1110.

922 Onchuru, T. O., Javier Martinez, A., Ingham, C. S. and Kaltenpoth, M., 2018.
 923 Transmission of mutualistic bacteria in social and gregarious insects. *Curr. Opin.*
 924 *Insect Sci.* 28, 50-58.

925 Peeters, C., Meier-Kolthoff, J. P., Verheyde, B., De Brandt, E., Cooper, V. S. and
 926 Vandamme, P., 2016. Phylogenomic study of *Burkholderia glathei*-like
 927 organisms, proposal of 13 novel *Burkholderia* species and emended descriptions
 928 of *Burkholderia sordidicola*, *Burkholderia zhejiangensis*, and *Burkholderia*
 929 *grimmiae*. *Front. Microbiol.* 7, 877.

930 Poole, P., Ramachandran, V. and Terpolilli, J., 2018. Rhizobia: From saprophytes to
 931 endosymbionts. *Nat. Rev. Microbiol.* 16, 291-303.

932 Powell, J. E., Martinson, V. G., Urban-Mead, K. and Moran, N. A., 2014. Routes of
 933 acquisition of the gut microbiota of the honey bee *Apis mellifera*. *Appl. Environ.*
 934 *Microbiol.* 80, 7378-7387.

935 Prado, S. S., Golden, M., Follett, P. A., Daugherty, M. P. and Almeida, R. P., 2009.
 936 Demography of gut symbiotic and aposymbiotic *Nezara viridula* L. (Hemiptera:
 937 Pentatomidae). *Environ. Entomol.* 38, 103-109.

938 Prado, S. S., Rubinoff, D. and Almeida, R. P. P., 2006. Vertical transmission of a
 939 pentatomid caeca-associated symbiont. *Annu. Entomol. Soc. Am.* 99, 577-585.

940 Russell, A. B., Peterson, S. B. and Mougous, J. D., 2014. Type VI secretion system
 941 effectors: Poisons with a purpose. *Nat. Rev. Microbiol.* 12, 137-148.

942 Sachs, J. L., Mueller, U. G., Wilcox, T. P. and Bull, J. J., 2004. The quarterly review of
 943 biology. *Q. Rev. Biol.* 79, 135-160.

944 Salem, H., Bauer, E., Kirsch, R., Berasategui, A., Cripps, M., Weiss, B., Koga, R.,
 945 Fukumori, K., Vogel, H., Fukatsu, T. and Kaltenpoth, M., 2017. Drastic genome
 946 reduction in an herbivore's pectinolytic symbiont. *Cell* 171, 1520-1531 e1513.

947 Salem, H., Florez, L., Gerardo, N. and Kaltenpoth, M., 2015. An out-of-body
 948 experience: The extracellular dimension for the transmission of mutualistic
 949 bacteria in insects. *Proc. Biol. Sci.* 282, 20142957.

950 Sangare, A. K., Rolain, J. M., Gaudart, J., Weber, P. and Raoult, D., 2016. Synergistic
 951 activity of antibiotics combined with ivermectin to kill body lice. *Int. J.*
 952 *Antimicrob. Agents* 47, 217-223.

953 Schaefer, C. W. and Panizzi, A. R., 2000. Heteroptera of economic importance. CRC
 954 press, Boca Raton.

955 Schlein, Y., 1977. Lethal effect of tetracycline on tsetse flies following damage to
 956 bacterioid symbionts. *Experientia* 33, 450-451.

957 Shibata, T. F., Maeda, T., Nikoh, N., Yamaguchi, K., Oshima, K., Hattori, M.,
 958 Nishiyama, T., Hasebe, M., Fukatsu, T., Kikuchi, Y. and Shigenobu, S., 2013.
 959 Complete genome sequence of *Burkholderia* sp. strain RPE64, bacterial symbiont
 960 of the bean bug *Riptortus pedestris*. *Genome announc.* 1, e00441–13.

961 Small, A. L. and McFall-Ngai, M. J., 1999. Halide peroxidase in tissues that interact
 962 with bacteria in the host squid *Euprymna scolopes*. *J. Cell. Biochem.* 72, 445-457.

963 Speare, L., Cecere, A. G., Guckes, K. R., Smith, S., Wollenberg, M. S., Mandel, M. J.,
 964 Miyashiro, T. and Septer, A. N., 2018. Bacterial symbionts use a type VI secretion
 965 system to eliminate competitors in their natural host. *Proc. Natl. Acad. Sci. U. S.*
 966 *A.* 115, E8528-E8537.

967 Srivastava, P. and Auclair, J., 1976. Effects of antibiotics on feeding and development
 968 of the pea aphid, *Acyrtosiphon pisum* (Harris) (Homoptera: Aphididae). *Can. J.*
 969 *Zool.* 54, 1025-1029.

970 Steele, M. I., Kwong, W. K., Whiteley, M. and Moran, N. A., 2017. Diversification of
 971 type VI secretion system toxins reveals ancient antagonism among bee gut
 972 microbes. *mBio* 8, e01630-01617.

973 Suárez-Moreno, Z. R., Caballero-Mellado, J., Coutinho, B. G., Mendonca-Previato, L.,
 974 James, E. K. and Venturi, V., 2012. Common features of environmental and
 975 potentially beneficial plant-associated *Burkholderia*. *Microb. Ecol.* 63, 249-266.

976 Sudakaran, S., Kost, C. and Kaltenpoth, M., 2017. Symbiont acquisition and
 977 replacement as a source of ecological innovation. *Trends Microbiol.* 25, 375-390.

978 Sudakaran, S., Retz, F., Kikuchi, Y., Kost, C. and Kaltenpoth, M., 2015. Evolutionary
 979 transition in symbiotic syndromes enabled diversification of phytophagous insects
 980 on an imbalanced diet. *ISME J.* 9, 2587-2604.

981 Tada, A., Kikuchi, Y., Hosokawa, T., Musolin, D. L., Fujisaki, K. and Fukatsu, T.,
 982 2011. Obligate association with gut bacterial symbiont in Japanese populations of
 983 the southern green stinkbug *Nezara viridula* (Heteroptera: Pentatomidae). *Appl.*
 984 *Entomol. Zool.* 46, 483-488.

985 Takeshita, K. and Kikuchi, Y., 2017. *Riptortus pedestris* and *Burkholderia* symbiont:
 986 An ideal model system for insect-microbe symbiotic associations. *Res. Microbiol.*
 987 168, 175-187.

988 Takeshita, K., Matsuura, Y., Itoh, H., Navarro, R., Hori, T., Sone, T., Kamagata, Y.,
 989 Mergaert, P. and Kikuchi, Y., 2015. *Burkholderia* of plant-beneficial group are
 990 symbiotically associated with bordered plant bugs (Heteroptera: Pyrrhocoroidea:
 991 *Largidae*). *Microbes Environ.* 30, 321-329.

992 Takeshita, K., Shibata, T. F., Nikoh, N., Nishiyama, T., Hasebe, M., Fukatsu, T.,
 993 Shigenobu, S. and Kikuchi, Y., 2014. Whole-genome sequence of *Burkholderia*
 994 sp. strain RPE67, a bacterial gut symbiont of the bean bug *Riptortus pedestris*.
 995 Genome Announc. 2, e00556-14.

996 Takeshita, K., Tamaki, H., Ohbayashi, T., Meng, X.-Y., Sone, T., Mitani, Y., Peeters,
 997 C., Kikuchi, Y. and Vandamme, P., 2018. *Burkholderia insecticola* sp. nov., a gut
 998 symbiotic bacterium of the bean bug *Riptortus pedestris*. Int. J. Syst. Evol.
 999 Microbiol. 68, 2370-2374.

1000 Taylor, C. M., Coffey, P. L., DeLay, B. D. and Dively, G. P., 2014. The importance of
 1001 gut symbionts in the development of the brown marmorated stink bug,
 1002 *Halyomorpha halys* (Stal). PLoS One 9, e90312.

1003 Troll, J. V., Adin, D. M., Wier, A. M., Paquette, N., Silverman, N., Goldman, W. E.,
 1004 Stadermann, F. J., Stabb, E. V. and McFall-Ngai, M. J., 2009. Peptidoglycan
 1005 induces loss of a nuclear peptidoglycan recognition protein during host tissue
 1006 development in a beneficial animal-bacterial symbiosis. Cell. Microbiol. 11,
 1007 1114-1127.

1008 Troll, J. V., Bent, E. H., Pacquette, N., Wier, A. M., Goldman, W. E., Silverman, N. and
 1009 McFall-Ngai, M. J., 2010. Taming the symbiont for coexistence: A host PGRP
 1010 neutralizes a bacterial symbiont toxin. Environ. Microbiol. 12, 2190-2203.

1011 Visick, K. L. and Ruby, E. G., 2006. *Vibrio fischeri* and its host: It takes two to tango.
 1012 Curr. Opin. Microbiol. 9, 632-638.

1013 Wang, Q., Liu, J. and Zhu, H., 2018. Genetic and molecular mechanisms underlying
 1014 symbiotic specificity in legume-rhizobium interactions. *Front. Plant Sci.* 9, 313.
 1015 Weis, V. M., Small, A. L. and McFall-Ngai, M. J., 1996. A peroxidase related to the
 1016 mammalian antimicrobial protein myeloperoxidase in the *Euprymna*-
 1017 *Vibrio* mutualism. *Proc. Natl. Acad. Sci. U. S. A.* 93, 13683-13688.
 1018 Xu, Y., Buss, E. A. and Boucias, D. G., 2016. Environmental transmission of the gut
 1019 symbiont *Burkholderia* to phloem-feeding *Blissus insularis*. *PLoS One* 11,
 1020 e0161699.
 1021
 1022
 1023

Figures legends

Figure 1. Gut symbiosis between the bean bug *Riptortus pedestris* and its symbiont *Burkholderia insecticola*.

(A) A female adult of the bean bug *R. pedestris*. (B) *B. insecticola*. (C) Whole midgut of *R. pedestris*. (D-G) A GFP-labeled strain of *B. insecticola* colonizes the posterior region of the midgut (D-E), in which GFP signals are specifically detected in the crypt-bearing symbiotic region (E-G). Abbreviations: M1, midgut first section; M2, midgut second section; M3, midgut third section; M4, midgut fourth section with crypts; M4B, M4 bulb; CR, constricted region; H, hindgut. Each crypt is indicated by arrowheads in F and G. Panels B and C are from Ohbayashi et al. 2015.

Figure 2. Partner-choice in the gut of *R. pedestris*.

(A) A midgut fed with water containing 0.05% food coloring (congo red). Note that the coloring is blocked at the constricted region. (B) A midgut infected with GFP-labeled *B. insecticola* showing that the bacteria can flow from M3 into M4B by passing through the constricted region. (C) Electron microscopy image of the narrow channel in the constricted region. The lumen of the channel is bordered by a layer of microvilli (ML) and contains a few bacterial cells (S) that are passing through the channel. (D) The constricted region in an insect co-inoculated with a GFP-labeled *B. insecticola* and an RFP-labeled *E. coli*. At the constricted region, *E. coli* is sorted out and only the *Burkholderia* symbiont passes through the channel. (E) Normal swimming mode of *B. insecticola* in a liquid environment. (F) Corkscrew motility of *B. insecticola* in a

viscous environment. Flagellar filaments are visualized by covalent coupling to the fluorescent dye Cy3-NHS-ester. Panels **A**, **C** and **D** are from Ohbayashi et al. 2015.

Figure 3. Microbe-microbe competition in the gut of *R. pedestris*.

Midgut M3-M4B-M4 regions of (**A**) an aposymbiotic insect; (**B**) an insect infected with a SBE *Burkholderia* (*B. insecticola*); (**C**) an insect infected with a PBE *Burkholderia* (*B. fungorum*); (**D**) an insect infected with *Pandoraea* (*P. norimbergensis*). (**E-G**) M4 region of an insect inoculated with an RFP-labeled SBE *Burkholderia* (*B. insecticola*) and an RFP-labeled *Pandoraea* (*P. norimbergensis*) at 2, 3, and 5 days post inoculation (dpi). (**H**) The schematic image of a symbiont specificity in the midgut of the bean bug *R. pedestris*. In the early infection stage, SBE-*Burkholderia*, PBE *Burkholderia*, and *Pandoraea* co-colonize the M4 (left). In the mature stage, SBE *Burkholderia* outcompetes the other strains and becomes dominant in the M4 (right). Panels **A-G** are from Itoh et al. 2019.

Fig. 1

Fig. 2

Fig. 3

Red: SBE, Green: *Pandoraea*