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Doctoral Thesis

Ecological and evolutionary traits of pathogenic bacteria hijacking the Stinkbug-*Caballeronia* symbiotic system

（カメムシ-*Caballeronia* 共生系を乗っ取る
病原性細菌の進化生態学的特性に関する研究）

March 2023

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Chapter 1
General Introduction

Symbiotic relationships are omnipresent on the earth

Symbiotic microorganisms widely distribute throughout the earth. Many plants and animals have evolved to coexist with microorganisms in diverse relationship types, including parasitism, commensalism, and mutualism. In particular, the interaction between host organisms and/or microbial symbionts has been recognized in recent years as one of the most important relationships in life on earth (1). In the interaction, both host and symbiont provide each other with numerous essential and beneficial effects. colonized in root nodules fix atmospheric nitrogen to ammonium and then the hosts assimilate ammonium into nucleotides, amino acids, vitamins, and hormones resulting in the promotion of growth and development (2, 3). In animals, the squid-*Vibrio* symbiosis is one of the most well-studied model systems. The Hawaiian bobtail squid *Euprymna scolopes* harbors the luminous symbiotic bacterium *Vibrio fischeri* in its symbiotic light organ (4). The squid camouflages itself in moonlit waters by using symbiont-derived bioluminescence to avoid predation from predators (5). In addition, many insects harbor specific microbial symbionts in specialized organs such as gut crypts or within specific cells called bacteriocytes (6, 7). Insects bacterial symbionts provide many benefits to their hosts, such as biogenesis and supplementation of essential nutrients that are lacking in the host's diet (8, 9). Overall, the organisms that we see do not exist on their own, but hidden symbionts play an important role. In order to truly understand the organism, we must investigate its relationship with the symbionts in order to get closer to the real character of the organism. Therefore, the study of symbioses is essential to understanding the organisms on the earth.

Transmission Mode of Symbionts

Symbiosis with microbial organisms could be mainly classified into two main types according to the mode of transmission of symbionts to the next generation: vertical

transmission, in which symbionts are transmitted from mother to offspring; and horizontal transmission, wherein the host acquires symbionts from the environment every generation.

ⅰ) Vertical Transmission

Symbioses with vertical transmission are found in a wide range of organisms including sponges, worms, bivalves, and insects. In most cases, symbionts are essential for host survival by providing essential nutrients and are directly or indirectly transmitted during embryogenesis or oviposition in many vertical transmissions, ensuring that the next generation acquire parental symbiotic bacteria (7, 9, 10) The interaction between the host's lineage and the symbiont's lineage is continuous in symbiosis with vertical transmission. Therefore, phylogenetic congruence was observed between host and symbionts in symbiosis with vertical transmission usually. As a result of this long-term coevolution, the symbionts lose genes that are needed to survive outside of the host, such as genes related to motility and environmental stress response, resulting in the symbionts cannot survive outside of the host body (11, 12). Moreover, the adaptation process to the host's environment may lead to an extreme reduction of genome size in symbiotic bacteria. The reduction of genome size ultimately results in too low a performance of the symbionts to maintain the symbiosis (11), which could be a risk of symbiotic systems maintained by vertical symbiont transmission.

Horizontal Transmission

In the case of horizontal transmission, the host acquires the from the environment every generation. A newborn offspring don't possess symbionts but obtains the free-living microbes as the symbionts from the environment, such as ambient soil or seawater (10). As can be imagined from the free-living phase of symbiotic bacteria, symbionts commonly possess large genomes and genes that are necessary for living outside hosts, and therefore, the symbiotic systems maintained by horizontal symbiont transmission could avoid symbionts' genetic deterioration which is frequently found in symbiotic systems with vertical symbiont

transmission. In addition, various species of the symbiont could be acquired from the environment and each symbionts confer different and sometimes novel benefits to the hosts. For example, previous studies demonstrated that some insects develop insecticide resistance by obtaining pesticide-degrading symbiotic bacteria. However, simultaneously, there are numerous parasitic and pathogenic microbes in the environment and the host organisms are exposed to infection by harmful microbes when they acquire the symbionts. Therefore, the host must select an appropriate symbiotic partner from diverse environmental microbes and indeed many host organisms have evolved to develop mechanisms for symbiont sorting with a complex interkingdom molecular crosstalk (4, 10). For example, legumes develop nodules each generation by acquiring specific rhizobia from the environmental soil (13, 14). Free-living rhizobia are attracted towards the plant's root by root exudates including nutrients and flavonoids, which are the host-specific signal molecules (10, 15), and the rhizobia produce the nodulation (Nod) factors, lipo-chitooligosaccharide molecules, in response to flavonoids (15, 16). The Nod factors are morphogens that initiate the nodulation of the host plant, allowing rhizobial symbionts to invade (10, 15, 16). The microstructure of Nod-factors differs among rhizobia species and host plants respond to their symbiont-specific Nod-factors, determining host-symbiont specificity (15–18). In addition, the host plants produce various nodule-specific cysteine-rich peptides (NCRs) that show antimicrobial activity to non-symbiotic bacteria (19, 20). In *Medicago truncatul-Shinorhizobium* symbiosis, a symbiotic peptide NCR247 shows high antibacterial activity against non-symbiotic bacteria, *Salmonella enterica* and *Listeria monocytogenes* (21), but is simultaneously important for bacteroid differentiation of native symbiont *Shinorhizobium* in host cells (22). In the case of squid-*Vibrio* symbiosis, the host squid acquires *Vibrio* symbionts from seawater and then the symbionts colonize the symbiotic light organ (4, 23). When the *Vibrio* cells start to colonize the light organ, they release a bacterial cell wall peptidoglycan fragment, tracheal cytotoxin

(TCT), and the swift morphogenesis of host's light organ occurs in response to it (24–26). In addition, the host squid secretes galaxin, an antimicrobial peptide (AMP), into the light organ by infection of *Vibrio*, modulating the symbiont growth followed by symbiotic maintenance (27).

Insect-Microbe Symbiosis

Insects are the most diverse group of organisms on the terrestrial ecosystem, consisting of more than half of the approximately 1.5 million species of organisms, and most of them possess symbiotic microorganisms within their bodies (1, 28). Insect symbionts provide many physiological benefits to the host insects, including supply of essential nutrients, promoting host immunity and fecundity, aiding to digest indigestible food, and detoxifying insecticides (29–32). For example, most insects that feed on nutritionally unbalanced foods, such as plant's phloem sap, have symbionts in their bodies that synthesize and provide essential nutrients to the host (33). One of the well-known examples of nutritional symbiosis is aphids-*Buchnera* symbiosis (31, 34). The *Buchnera* endosymbiotic bacteria are colonized in the host's specialized cells called bacteriocytes wherein the symbiont biosynthesize and provide essential amino acids to the host. The removal of the obligate endosymbiont by treatment of antibiotics or heat shock causes high mortality and small body sizes (31). Since the *Buchnera* has coevolved with the host aphid for a prolonged period via vertical transmission, it is highly adapted to the host's internal environment, lost most functional genes essential for free-living, and indeed can't survive outside of the host body (31, 35–37). In other words, the aphid and *Buchnera* establish an obligate mutualistic relationship. In addition, nutrient supply by the symbiotic bacteria contributes to the development of the host skin. For example, weevils have a γ-proteobacterial endosymbiont, *Nardonella*, and the symbiont's genome size is extremely small due to a long-term adaptation inside the host's body (38, 39). However, *Nardonella*

retains a full gene set for the synthesis of tyrosine, the primary precursor of the cuticle, which is a major component of insect skin (38, 40). The removal of the symbiotic bacteria by antibiotic treatment resulted in the host's cuticle becoming reddish, lower in tyrosine content, and softer, suggesting that the symbiotic bacteria is important for the hardness of the cuticle (38). In addition to nutritional symbiosis, insect symbionts have numerous other functions, such as aiding digestion and host protection. Wood-feeding termites and cockroaches have symbiotic bacteria and protists in their guts, and the symbiotic microbial communities help the host survival and development by degrading indigestible wood materials such as lignocellulose (41–44). In addition, *Serratia symbiotica*, one of the pea aphid's symbionts, is involved in a defensive symbiosis that mainly protects the host from high temperatures or parasitic wasps (45–47). Besides, *Rickettsiella* symbiont of the aphid regulates the host's body color from red to green resulting in modified predation pressure (48).

Stinkbug-Microbe Symbiosis

The infraorder Pentatomomorpha consists of 5 superfamilies (Aradoidea, Coreoidea, Lygaeoidea, Pentatomoidea, Pyrrhocoroidea) and 40 families, comprising over 14,000 species (49, 50). The Pentatomomorphan feeding nutritionally deficient or unbalanced diets, such as phytophagous bugs, have symbiotic organs in the digestive tract that is specialized to keep a large number of symbiotic bacteria.

Stinkbugs belonging to the superfamily Pentatomoidea possess gut symbionts of the Gammaproteobacteria that are essential for the host development and survival (51–59). The gut symbiotic bacteria are transmitted vertically from mother to offspring in various ways (60–62). Stinkbugs of the families Acanthosomatidae, Pentatomidae, Cydnidae, and Scutelleridae transmit their symbiotic bacteria by contaminating the egg surface with symbionts during oviposition, and hatchlings immediately acquire the maternal symbionts by feeding egg shells

(60). Elimination of the symbionts from the egg surface by sterilization results in high mortality and abnormal growth (52, 58, 60). Stinkbugs of the family Plataspidae harbor symbiotic bacteria, *Candidatus* Ishikawaella capsulata, in the crypt lumen of the posterior midgut (63). The adult female of the plataspid stinkbugs produces and lays symbiont-containing capsules underside of the egg masses (60, 64). Hatchlings then immediately probe the capsule and acquire the symbiotic bacteria by sucking up the capsule contents. When symbiotic capsules are artificially removed, the nymphs suffer from high mortality and abnormal growth (63, 64). The stinkbugs belonging to the family Urostylidae transmit the symbiotic bacteria with symbiont-supplemented jelly (61). The stinkbugs possess Gammaproteobacterial symbiont, *Candidatus* Tachikawaea gelatinosa, in the posterior midgut crypts. During the oviposition, the female insects cover the laid eggs with symbiont-containing jellies and hatchlings immediately consume the jellies resulting in the infection by the parental symbiont. Removal of the jelly cause high mortality and growth retardation in the newborn nymphs (61). These reports demonstrated that the vertically transmitting gammaproteobacterial symbionts broadly establish the obligate symbiotic associations with the Pentatomoidea stinkbugs.

On the other hand, numerous stinkbugs belonging to the superfamilies Coreoidea, Lygaeoidea and a few Phyrrhocoroidea (Largidae) harbor the betaproteobacterial symbionts of *Burkholderia* sensu lato (s.l.) group in their posterior midgut (65–73). The *Burkholderia* s.l. has recently been reclassified into seven genera; *Burkholderia*, *Paraburkholderia*, *Caballeronia*, *Robbsia*, *Mycetohabitans*, *Pararobbsia*, and *Trinickia* (74–79). Among them, *Burkholderia* sensu stricto (s.s) consists of diverse plant, animal, and human pathogens (80). The genus *Paraburkholderia* includes plant growth promoting rhizobia and some stinkbugs' symbionts (68, 75, 81). The Largidae stinkbugs establish the symbiotic relationship with *Paraburkholderia* bacteria (68). The genus *Caballeronia* is known as the "stinkbug-associated beneficial and environmental bacteria (SBE)", and most stinkbugs in the

superfamily Coreoidea (e.g. the families Coreidae and Alydidae) harbor *Caballeronia* symbionts in their midgut crypts (65–67, 82). In the Coreoidea stinkbugs, the *Caballeronia* symbionts are horizontally acquired from the environmental soil (66, 83). The *Caballeronia* symbionts provide numerous physiological benefits to the host stinkbugs, such as promoting host development, higher survival rate, larger body size, and enhancing fecundity (29, 67, 71, 82, 83). In addition, the *Riptortus pedestris* and *Cletus punctiger* (Coreoidea) show insecticide resistance, if they established the symbiotic relationships with insecticide-degrading *Caballeronia* strains (65, 84, 85). As in these examples, the *Caballeronia* symbionts give various benefits to Coreoidea stinkbugs. In contrast, the Lygaeoidea stinkbugs have been reported with histological studies about symbiotic organs and *Caballeronia* symbionts, but the details of their symbiosis have been rarely investigated.

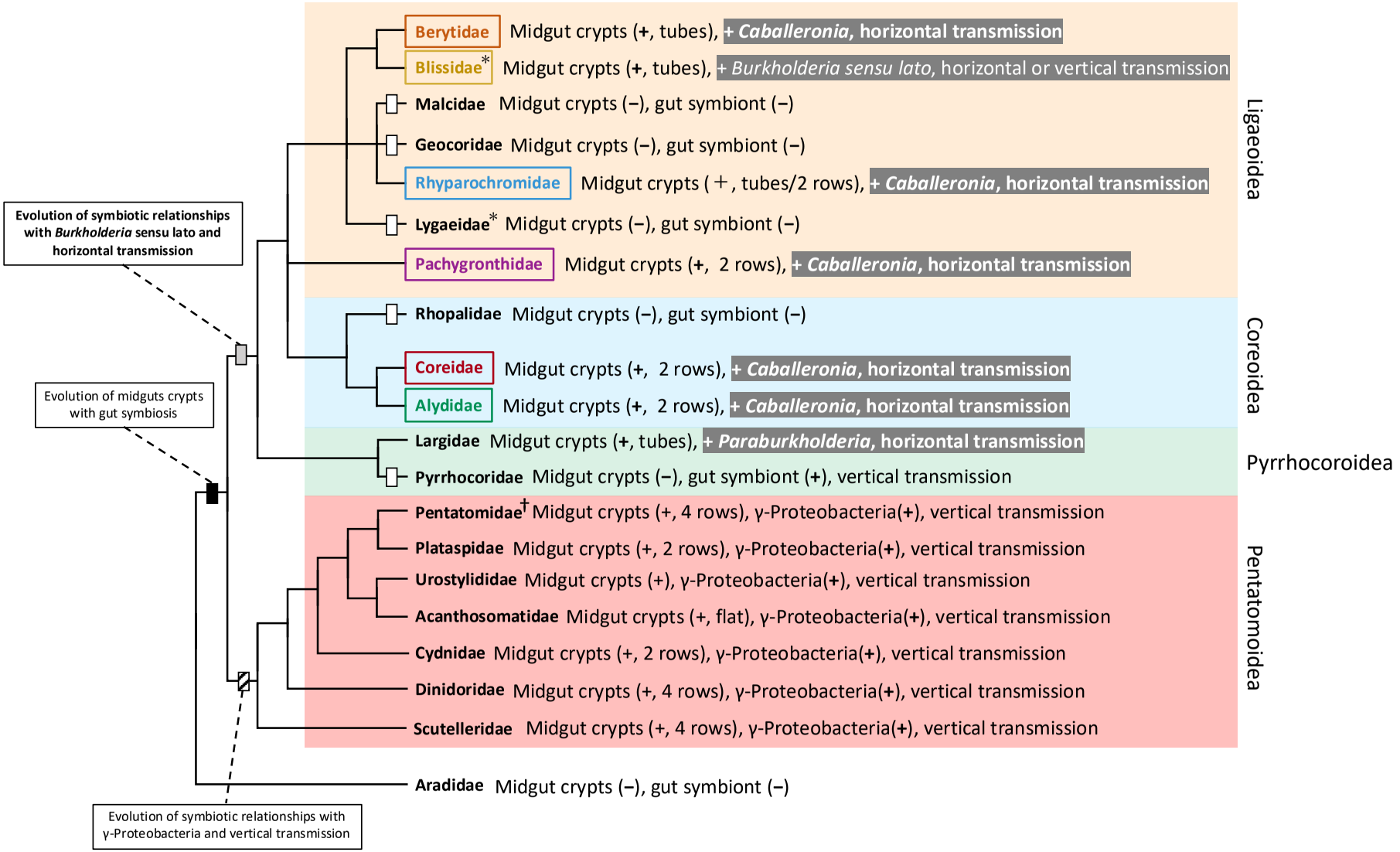
Evolution of symbiotic relationships with *Burkholderia* sensu lato and horizontal transmission
Evolution of midguts crypts with gut symbiosis
Evolution of symbiotic relationships with γ-Proteobacteria and vertical transmission
Berytidae Midgut crypts (+, tubes), + *Caballeronia*, horizontal transmission
Blissidae* Midgut crypts (+, tubes), + *Burkholderia sensu lato*, horizontal or vertical transmission
Malcidae Midgut crypts (−), gut symbiont (−)
Geocoridae Midgut crypts (−), gut symbiont (−)
Rhyparochromidae Midgut crypts (+, tubes/2 rows), + *Caballeronia*, horizontal transmission
Lygaeidae* Midgut crypts (−), gut symbiont (−)
Pachygronthidae Midgut crypts (+, 2 rows), + *Caballeronia*, horizontal transmission
Rhopalidae Midgut crypts (−), gut symbiont (−)
Coreidae Midgut crypts (+, 2 rows), + *Caballeronia*, horizontal transmission
Alydidae Midgut crypts (+, 2 rows), + *Caballeronia*, horizontal transmission
Largidae Midgut crypts (+, tubes), + *Paraburkholderia*, horizontal transmission
Pyrrhocoridae Midgut crypts (−), gut symbiont (+), vertical transmission
Pentatomidae† Midgut crypts (+, 4 rows), γ-Proteobacteria(+), vertical transmission
Plataspidae Midgut crypts (+, 2 rows), γ-Proteobacteria(+), vertical transmission
Urostylididae Midgut crypts (+), γ-Proteobacteria(+), vertical transmission
Acanthosomatidae Midgut crypts (+, flat), γ-Proteobacteria(+), vertical transmission
Cydnidae Midgut crypts (+, 2 rows), γ-Proteobacteria(+), vertical transmission
Dinidoridae Midgut crypts (+, 4 rows), γ-Proteobacteria(+), vertical transmission
Scutelleridae Midgut crypts (+, 4 rows), γ-Proteobacteria(+), vertical transmission
Aradidae Midgut crypts (−), gut symbiont (−)
Ligaeoidea
Coreoidea
Pyrrhocoroidea
Pentatomoidea

Figure 1-1. **Evolution of Burkholderia symbiosis in the Pentatomomorpha.**
Phylogeny of higher taxa in the Pentatomomorpha, inferred from phylogenetic studies based on molecular or morphological data (Henry 1997; Xie et al. 2005; Hua et al. 2008). Symbiotic systems of the pentatomomorphan families are based on published studies (Glasgow 1914; Miyamoto 1961; Buchner 1965; Abe et al. 1995; Fukatsu and Hosokawa 2002; Kikuchi et al. 2005; Hosokawa et al. 2006; Prado et al. 2006; Kikuchi et al. 2007; Kaltenpoth et al. 2009; Kikuchi et al. 2009; Prado and Almeida 2009; Kaiwa et al. 2010; Kikuchi et al. 2011a; Olivier-Espejel et al. 2011; Tada et al. 2011; Boucias et al. 2012; Kikuchi, Hayatsu et al. 2012; Kikuchi, Hosokawa, et al. 2012; Kuechler et al. 2012; Matsuura, Kikuchi, Hosokawa, et al. 2012; Sudakaran et al. 2012; Salem et al. 2013; Garcia et al. 2014; Itoh et al. 2014; Kaiwa et al. 2014; Matsuura et al. 2014; Salem et al. 2014; Hosokawa et al. 2015; Sudakaran et al. 2015; Hosokawa et al. 2019; Hosokawa and Fukatsu 2020.). Presence/absence of symbiotic bacteria, symbiont taxonomy, transmission mechanism, and crypt morphology are indicated. White rectangles on the phylogeny indicate the loss of the *Burkholderia* sensu lato symbionts and midgut crypts. Asterisks (*) indicate the existence of exceptional taxa that have lost the *Burkholderia* sensu lato symbionts and midgut crypts and evolved secondarily bacteriocytes carrying endocellular symbionts (Kuechler et al. 2012; Matsuura, Kikuchi, Hosokawa, et al. 2012). The dagger (†) indicates the existence of several groups that have lost midgut crypts and gut symbionts (Kikuchi et al. 2011a). Figure was adapted from Takeshita et al. 2015.

Riptortus pedestris–Caballeronia symbiosis: an ideal symbiosis with horizontal transmission

The bean bug *R. pedestris*, a notorious pest of leguminous crops, is the most well-studied model system for the stinkbug-*Caballeronia* symbiosis. The midgut of *R. pedestris* is divided into four sections, M1, M2, M3, and M4 (86, 87) and the *Caballeronia* symbionts are specifically in a bulbous region called M4B in the anterior of the M4 region and M4 (88). The M4 is fully occupied by millions of monospecific gut symbionts of the genus *Caballeronia* (89)(Kikuchi and Yumoto). The *Caballeronia* symbiont provides many benefits to the host *R. pedestris*, such as promoting host growth, development, fecundity, and immunity, but does not affect host survival (29, 83–85). As in other symbioses with horizontal transmissions, *R. pedestris* has evolved to develop symbiont sorting mechanisms to establish the monospecific symbiotic relationship with *Caballeronia* symbiont. To prevent infection by non-symbiotic bacteria in the symbiotic organ, *R. pedestris* have a narrow organ filled with mucous-like matrix between M3

and M4B, called the constricted region (CR) (86). Non-symbionts, such as *E. coli*, are unable to pass through the CR (86). In addition, Screening of the *C. insecticola* mutants revealed that the motility of bacteria is essential to pass CR (86). Although various bacteria possess motility in soil, The *Caballeronia* symbiont has a unique swimming motility in order to pass through the mucous CR (90). *Caballeronia* symbionts wrap flagella around their own body in highly mucous environments (90). In addition to this specific sorting mechanism, symbiotic organs, M4B and M4, express diverse immune-related AMPs and specific symbiotic peptides (87, 91–93) such as the light organ of squid and the nodule of legume. Therefore, these immunity products also probably work to sort the specific symbionts. Moreover, competition between symbiotic bacteria after reaching the symbiotic organ M4 ultimately leads to the establishment of a relationship with a particular symbiont (94). When *R. pedestris* is fed non-symbiont *Paraburkholderia* and *Pandoraea* (outgroup of *Burkholderia* sensu lato) at 2nd instar, the bacteria can colonize the symbiotic organ well and give beneficial effects to the host without any harmful effects (94). However, the co-infection test of both a *Caballeronia* symbiont and the non-symbiont *Paraburkholderia*/*Pandoraea* revealed that *Caballeronia* symbionts always outcompete the other non-symbionts in the midgut crypts (94). These symbiont-sorting and competition-based mechanisms have plausibly evolved to prevent pathogenic bacteria from the host.

Theoretical studies have shown that symbionts that help their partner despite the cost lose out in competition to selfish symbionts (ex. cheater) that steal benefits from the host and maximize their own benefits (95–97). However, there are countless examples of mutualism in nature, and diverse approaches have been taken to overcome the contradictions with the previous theoretical studies (96–100). Partner choice is one way to prevent cheaters in mutualism. In this case, the host identifies cheaters and excludes them from the symbiosis and then establishes a symbiotic relationship only with beneficial symbionts (96, 97). In the

R. pedestris-*Caballeronia* symbiosis, symbionts are selected in diverse ways, as described above. Previous studies have not found cheaters that can pass through these sorting systems, and the *R. pedestris*-*Caballeronia* symbiosis is considered a robust relationship. However, there are often evolutionary arms races between cheaters/parasites and host (101–107). The host develops mechanisms to prevent the cheaters/parasites, but the cheaters/parasites evolve toward overcoming those mechanisms (101, 104, 106, 107). Usually, the symbiotic organs of the host are the ideal environment for symbiotic bacteria because the symbionts are supplied with nutrients and can dominate the niche. Indeed, *Caballeronia* grows explosively in the symbiotic organ of *R. pedestris*, from a few cells at the start of infection to as many as $10^{7\sim8}$ cells (89). In addition, once the symbiotic organ of *R. pedestris* is colonized by the symbiotic bacteria, the CR is completely closed, making it impossible for other bacteria to invade (108). Taken together, the symbiotic organ of *R. pedestris* is an ideal environment for cheaters as well, and thus an evolutionary arms race may exist between the symbiote selection mechanism of *R. pedestris* and cheaters. Therefore, there is a possibility that cheaters that can colonize M4, but the cheaters have not been reported in *R. pedestris*-*Caballeronia* symbiosis.

Objectives of the PhD work

Compared to the abundant knowledge of the Coreoidea-*Caballeronia* symbiosis, research on the Lygaeoidea-Caballeronia symbiosis has lagged behind. Therefore, in this doctoral thesis, first, I elucidated the symbiotic relationship between *Paradieuches dissimilis* (Lygaeoidea) and *Caballeronia*. 16S rRNA gene amplicon sequencing analysis was performed on the gut symbiotic organs of *P. dissimilis* collected from 10 locations in Hokkaido, Japan, to investigate gut symbiotic bacteria, and rearing experiments were conducted to investigate the mode of transmission of symbiotic bacteria and the effects of the symbiont on their hosts.

Second, in general, a "cheater" appears in a mutualistic symbiosis, but no cheater has been found in the *R. pedestris*-*Caballeronia* symbiosis. Here, I report the finding of the pathogenic *Burkholderia* species that can colonize the symbiotic organ of *R. pedestris* and has the ability to evade the host's partner choice mechanisms from the symbiotic organ of *P. dissimilis*.

This doctoral thesis consists of four chapters. This chapter 1 provides the general introduction of this thesis. In chapter 2, I investigated that Lygaeoidea stinkbugs, *P. dissimilis,* harbor *Caballeronia* symbionts in the posterior region of midgut, and the symbionts are essential for host survival and development. In chapter 3, I discovered the *Burkholderia* sp. SJ1 that isolated from the symbiotic organs of the *P. dissimilis* colonize the symbiotic organ of *R. pedestris* and kill the host. In addition, I experimentally demonstrated that the pathogenic bacteria have the ability to evade the host's symbiont sorting mechanisms (CR, immunity, competition). Finally, a general discussion of my work is provided in chapter 4.

Chapter 2

Obligate gut symbiotic association with *Caballeronia* in the mulberry seed bug *Paradieuches dissimilis* (Lygaeoidea: Rhyparochromidae)

Introduction

Many insects establish symbiotic associations with microorganisms and harbor symbionts in their body cavities, gut lumen, or within cells (1, 109). Such symbionts play pivotal metabolic roles in host insects by providing nutrients that are barely contained in the diet but are nevertheless important for growth and survival, aiding digestion of indigestible food materials, and recycling metabolic wastes (41, 43, 110, 111). In addition, symbionts confer additional functions on host insects, including detoxification of insecticides and phytotoxins (32, 65, 85), tolerance to natural pathogens and enemies (46), cuticle hardening (38), and modification of body color (48).

Most phytophagous species of the heteropteran infraorder Pentatomomorpha possess a large number of symbiotic bacteria in the posterior midgut region, which bears hundreds of sac-like tissues called "crypts" (1, 66, 112). Among the infraorder Pentatomomorpha, many stinkbugs of the superfamily Pentatomoidea harbor symbiotic gut bacteria of Gammaproteobacteria that are essential for host development and survival (51–59, 113–115). The gut symbiotic bacteria of these stinkbugs are transmitted vertically from the mother to offspring by either symbiont-containing capsules/jelly or egg-smearing with the symbionts (60–62). In contrast, the majority of the superfamilies Coreoidea and several clades of Lygaeoidea stinkbugs possess betaproteobacterial gut symbionts of *Burkholderia sensu lato* group in the posterior midgut crypts (65–68, 70–73, 116). *Burkholderia sensu lato* has recently been reclassified into six genera (*Paraburkholderia, Caballeronia, Robbsia, Mycetohabitans, Pararobbsia,* and *Trinickia*) (74–79). Among them, the genus *Caballeronia* was previously referred to as a "stinkbug-associated beneficial and environmental" (SBE) clade of *Burkholderia sensu lato*, which is subdivided into four groups: SBE-α, SBE-β, SBE-γ, and Coreoidea clade (82), and most stinkbugs belonging to the Coreoidea and Lygaeoidea harbor *Caballeronia* (*i.e.* SBE-*Burkholderia*) as gut symbionts (66, 74, 117). *Caballeronia* gut

symbionts are not vertically transmitted, but are horizontally acquired by nymphs from the environmental soil of every host generation (83, 118). In the superfamily Coreoidea, the beneficial effects of *Caballeronia* symbionts have been well investigated, wherein the symbiont enhances host development, survival rate, fecundity, and immunity (29, 30, 67, 71, 82, 83). However, little is known regarding the effects of *Caballeronia* gut symbionts in the superfamily Lygaeoidea, because there are few useful model species that can be maintained in the laboratory.

The mulberry seed bug *Paradieuches dissimilis* (Lygaeoidea: Rhyparochromidae) (Figure 2-1A) is widely distributed in East Asia (119, 120) and is known to mainly feed on mulberry plants (121), whereas little is known concerning its feeding ecology. In this study, I succeeded in rearing *P. dissimilis* by feeding seeds of sunflower, wheat, and buckwheat under laboratory conditions, even if they were not native food sources. In addition, I revealed the diversity of the *Caballeronia* gut symbiont and putative facultative symbionts, *Symbiopectobacterium*, *Wolbachia*, and *Rickettsiella*, associated with field populations of *P. dissimilis*, by amplicon sequencing of the bacterial 16S rRNA gene. In addition, I demonstrated that the *Caballeronia* gut symbiont is essential for host growth and survival by inoculating *in vitro* cultured symbiont cells into laboratory-maintained *P. dissimilis*.

Materials and Methods

Insect rearing and symbiont infection

Field populations of *P. dissimilis* were collected from mulberry trees (*Morus australis*) from 10 different locations in Hokkaido, Japan (Table 1-1). Although adult *P. dissimilis* feeds on the fruit part of the mulberry tree in nature, it is difficult to rear insects using fresh mulberry fruits because of their rapid decomposition. The collected insects were reared in a plastic Petri dish at 25°C by feeding on sunflower, wheat, and buckwheat (*Helianthus annuus*, *Triticum aestivum,*

and *Fagopyrum esculentum* seeds, respectively), and distilled water containing 0.05% ascorbic acid (DWA), which is an important vitamin for insect growth and development (122, 123), under a long-day regimen (16 h light and 8 h dark). The seeds and distilled water were changed every 7 days. Laid eggs from laboratory-maintained adults were collected daily and transferred to a new plastic Petri dish. To preserve the laboratory population of *P. dissimilis*, 10^7 cells/ml of cultured *Caballeronia* symbiont, which was isolated from the midgut of wild-captured *P. dissimilis* (see the following section "Isolation and identification of *Caballeronia* symbiont") or unsterilized (naturally symbiont-containing) environmental soil were supplied to newly hatched insects so that the hatchlings became symbiont-harboring insects.

Table 2-1. Details of *Paradieuches dissimilis* samples inspected in this study

Collection site	Sample identifier	Collector	Collection Date	Diagnostic PCR[a]
11-chome-2-5, Tsukisamuhigashi, Toyohiraku, Sapporo, Hokkiado, Japan	TS	K. Ishigami, K. Kawano, S. Jang	30th June, 2020	100% (10/10)
8-chome-1, Misono 11 Jo, Toyohiraku, Sapporo, Hokkiado, Japan	TK	K. Ishigami, S. Jang, H. Itoh	1st July, 2020	100% (4/4)
18-chome-1, Hiragishi 1 Jo, Toyohiraku, Sapporo, Hokkiado, Japan	TZ	K. Ishigami, S. Jang, H. Itoh	1st July, 2020	100% (6/6)
487 Nishioka, Toyohiraku, Sapporo, Hokkiado, Japan	NO	K. Ishigami, S. Jang, H. Itoh	1st July, 2020	100% (9/9)
781 Kurokawacho, Yoichi, Hokkaido, Japan	YI-1	K. Ishigami, S. Jang, Y. Kikuchi	28th June, 2021	100% (3/3)
12-chome-61 Higashimachi, Niki, Yoichi, Hokkaido, Japan	YI-2	K. Ishigami, S. Jang, Y. Kikuchi	28th June, 2021	100% (3/3)
380 Yamadacho, Yoichi, Hokkaido, Japan	YI-3	K. Ishigami, S. Jang, Y. Kikuchi	28th June, 2021	100% (10/10)
1-chome-1-1, Kawazoe 5 Jo, Minamiku, Sapporo, Hokkaido, Japan	MK	K. Ishigami, S. Jang, Y. Kikuchi	28th June, 2021	100% (6/6)
2-chome-4-21 Manachi, Chitose, Hokkaido, Japan	CT-1	K. Ishigami, S. Jang, Y. Kikuchi	28th June, 2021	100% (10/10)
2-chome-2-3 Yamato, Chitose, Hokkaido, Japan	CT-2	K. Ishigami, S. Jang, Y. Kikuchi	28th June, 2021	100% (8/8)
				Total: 100% (69/69)

[a] Infection rate of *Burkholderia* in the midgut M4. In parentheses, the number of infected insects per total number of insects investigated are shown.

Diagnostic PCR

To detect the presence of *Caballeronia* symbiont in the midgut of field-captured *P. dissimilis*, 69 adults of *P. dissimilis* were dissected in phosphate-buffered saline (PBS) with a pair of fine tweezers under a stereomicroscope (DMI4000B, Leica). The dissected symbiotic organ was transferred to a 1.5 ml microcentrifuge tube with 100 µl of PBS and homogenized using a mortar and pestle. One microliter of gut lysate was used as DNA template in diagnostic PCR. To investigate whether the *Caballeronia* symbiont is vertically transmitted in *P. dissimilis* insects, a total of 10 eggs laid by laboratory-maintained adult insects were separately homogenized in each tube with 100 µl of PBS, and 1 µl of homogenate was used in diagnostic PCR amplifications as a DNA template. A 0.46 kb region of the 16S rRNA gene was amplified

using the *Caballeronia*-specific primer set, Bf and Br (Table 2-2), as previously described (124). To rule out potential technical issues in diagnostic PCR, the cytochrome c oxidase subunit 1 mitochondrial gene (*COI*) of the host insect was amplified as a control (Table 2-2).

Table2-2. Primers used in this study

Target group	Target gene	primer/probe name	Nucleotide sequence (5'-3')	Approximate product size (kb)	Annealing temp (°C)	Reference
Primers						
Eubacteria	16s rRNA	515F	GTGCCAGCMGCCGCGGTAA	0.3	54	Caporaso et al. 2012
		806R	GGACTACHVGGGTWTCTAAT			
Burkholderia	16s rRNA	Bf	TAGCCCTGCGAAAGCCG	0.5	56	Tago et al. 2014
		Br	GCCAGTCACCAATGCAG			
Invertebrates	*COI*	LCO1490	GGTCAACAAATCATAAAGATATTGG	0.7	48	Folmer et al. 1994
		HCO2198	TAAACTTCAGGGTGACCAAAAAATCA			

Caporaso JG, Lauber CL, Walters WA, et al (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*. 6:1621–1624.

Tago K, Itoh H, Kikuchi Y, et al (2014) A fine-scale phylogenetic analysis of free-living *Burkholderia* species in sugarcane field soil. *Microbes Environ*. ME14122.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol*. 3:294–299.

Isolation and identification of *Caballeronia* symbiont

The gut symbiont of *P. dissimilis* used in this study, *Caballeronia* sp. PDI-1, was isolated from the symbiotic organ of an adult female *P. dissimilis* that was collected from the Tsukisappu (TS) region in 2020 (Table 2-1). The symbiotic organs were dissected in PBS and incubated in 100 µl of yeast extract-glucose (YG) medium (0.5% yeast extract, 0.4% glucose, and 0.1% NaCl) at 25°C for 1 d. The incubated symbiotic organs were homogenized and spread on YG agar. The plate was incubated for 2 days at 25°C, and colonies were isolated by streaking them on fresh YG agar and incubating for 2 more days. To identify the isolated bacteria, 1.5 kb of the bacterial 16S rRNA gene was amplified by PCR using the primer sets 16SA1 and 16SB1 (Table 2-2) (125) and AmpliTaq Gold™ 360 Master Mix (Applied Biosystems). The PCR product was cleaned with Exo-SAP-IT (GE Healthcare), reacted with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and then purified using the FastGene Dye Terminator Removal Kit (Nippon Genetics Co.). Prepared DNA was sequenced using an ABI 3130xl DNA sequencer (Applied Biosystems). The 16S rRNA sequence of the isolated *Caballeronia* symbiont was identified using a BLASTn search against the NCBI 16S rRNA database.

Construction of RFP-expressing *Caballeronia* symbiont

To construct a red fluorescent protein (RFP)-expressing *Caballeronia* symbiont, the plasmid pIN29 carrying the *dsRed* gene was transferred into *Caballeronia* symbiont cells by electroporation using the Ec2 mode of a MicroPulser Electroporator (Bio-Rad) (126). pIN29 has a chloramphenicol-resistance marker; thus, an RFP-expressing *Caballeronia* symbiont was selected by spreading electroporated cells on YG agar containing 15 μg/ml of chloramphenicol. Since the *dsRed* gene of pIN29 is under the control of the highly active *pTAC* promoter, *Caballeronia* cells with pIN29 intensely express dsRed, resulting in the bacterial cells themselves turning red. A red signal from successfully transformed *Caballeronia* colonies was confirmed using LED-EXTRA with a red filter (OptoCode). RFP-expressing *Caballeronia* symbionts (10^7 cells/ml) were inoculated into host insects as described above, and a fluorescent signal from the midgut crypts harboring the RFP-expressing symbionts was observed under an epifluorescence microscope (DMI4000B, Leica).

Measurement of host survival rate and fitness

Development and survival rates were measured among three different groups of *P. dissimilis*: aposymbiotic, soil-infected, and cultured symbiont-infected insects. Aposymbiotic insects (n = 59) were supplied with microbe-free DWA and sterilized seeds after hatching. To generate symbiont-harboring *P. dissimilis*, insects were supplied with environmental soil originally containing native *Caballeronia* symbionts (soil-infected insects) or 10^7 cells/ml of cultured *Caballeronia* sp. PDI-1 symbionts (cultured symbiont-infected insects) for one week (n = 82 and 25, respectively) after hatching. After seven days, the soil or bacterial solution was changed to microbe-free DWA. The survival rate of the host insects was measured by calculating the adult emergence rate. The body length and luminance of the pronotum of fifth-instar insects were measured using ImageJ software after acquiring images under a microscope (n = 4, 10,

and 5 for aposymbiotic, soil-infected, and cultured symbiont-infected insects, respectively). The relative luminance of the insect pronotum was measured by calculating the mean gray value using ImageJ. The mean gray value ranged from 0 to 255. A lower number indicates darker, and a higher number indicates lighter. The luminance of the pronotum of the aposymbiotic insects was set as a standard. The acquired data were statistically analyzed using GraphPad Prism 9.1.0 (GraphPad Software). The statistical significance of the host body length and color were calculated using the Mann-Whitney *U* test with Bonferroni correction, and the statistical significance of adult emergence rates was determined using Fisher's exact test followed by Bonferroni correction.

Deep sequencing

Deep sequencing of the bacterial 16S rRNA gene was performed using symbiotic organs from 69 field-collected insects. The symbiotic organ was dissected in PBS and total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). The variable region (V4) of the bacterial 16S rRNA gene was amplified by diagnostic PCR using 515F and 806R universal primers for deep sequencing on an Illumina platform (Table 2-2) (127). The PCR mixture consisted of 0.4 μM primer 515F with Illumina P5 sequences, 0.4 μM primer 806R (Illumina) extended with a 6-base index and Illumina P7 sequences, Q5 High-Fidelity 2X Master Mix (New England BioLabs), and the extracted DNA as the template. The PCR conditions were as follows: initial denaturation at 98°C for 90 s, followed by 35 cycles at 98°C for 10 s, 54°C for 30 s, and 72°C for 30 s. The PCR products were purified using AMPure XP beads (Beckman Coulter). Target PCR products were confirmed by gel electrophoresis and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). DNA libraries containing all tagged amplicons and internal controls (phiX) were prepared for paired-end sequencing using iSeq 100 i1 Reagent v2 (Illumina) and sequenced on an Illumina iSeq 100 sequencer according to the manufacturer's

instructions.

Data analyses

The paired-end (2 × 151 bp) reads obtained were further analyzed using USEARCH (128). Consensus forward and reverse reads were merged and sequences with low quality (quality score <39) were filtered out by the '-fastq_mergepairs' and '-fastq_filter' commands, respectively. Then, unique sequences were identified by '-fastx_uniques' command and clustered into Operational Taxonomic Units (OTUs) using the -cluster_otus' option. Then, incorrectly joined chimeras were removed and biologically meaningful zero-radius OTUs (zOTUs) were identified using the UNOISE3 algorithm (128). Bacterial taxonomy was assigned by investigating the similarity of the V4 region of 16S rRNA sequences with the EzBioCloud database (https://www.ezbiocloud.net/resources/16s_download) using the amplicon sequence as a query.

Phylogenetic analyses

A 254 bp corresponding to the V4 regions of the zOTUs and other publicly available bacterial 16S rRNA sequences were aligned using the MAFFT program (55), and maximum likelihood phylogenies were inferred using MEGA X software (130). Bacterial *16S* rRNA sequences (1,288 bp) of the isolated gut symbiont *Caballeronia* sp. PDI-1 and other *Caballeronia* bacteria were aligned, and their phylogenies were analyzed as described above. Bootstrap tests were performed with 1,000 replications. The final phylogenetic trees were visualized using iTOL (131).

Data availability

All sequence data generated by the deep sequencing were have been deposited in the National

Center for Biotechnology Information under the BioProject accession number PRJNA810207 (SRX14284164 - 14284232). The 16S rRNA sequence of isolated gut symbiont, *Caballeronia* sp. PDI-1, has been deposited under the accession number LC726282.

Results

Morphological characterization of symbiotic organ

The dissected midgut of *P. dissimilis* was divided into five sections from M1 to M4 (Figure 2-1B), as previously described for other stinkbug species (15). The junction between M3 and M4B was remarkably constricted, as observed in other stinkbug species (86). Among the midgut regions, many rod-shaped bacteria were observed in M4 (Figure 2-1B), suggesting that this posterior midgut section may represent a symbiotic organ similar to other Rhyparochromidae family stinkbugs (66). Morphologically, the midgut M4 consists of several ramified long tubular crypts (Figure 2-1B).

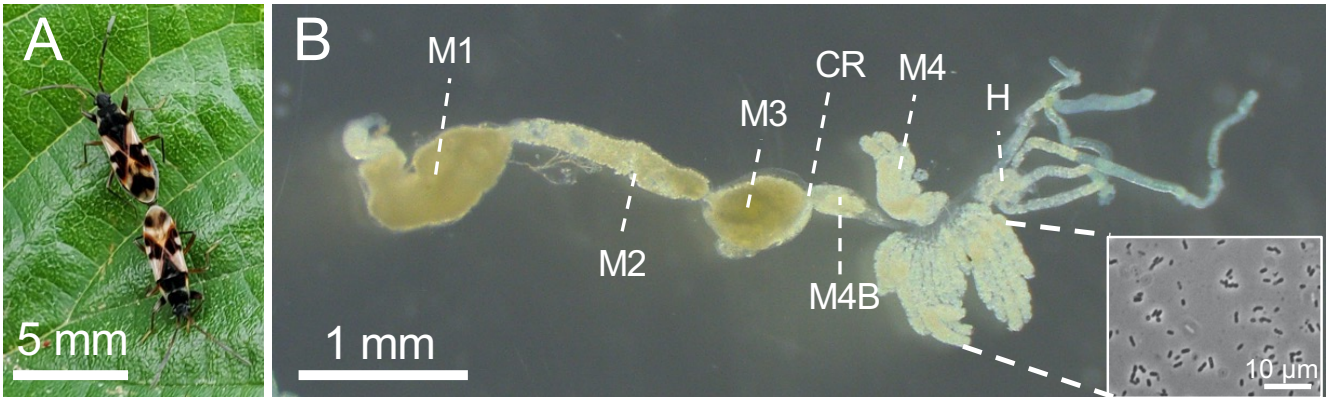

Figure 2-1. Mulberry seed bug *P. dissimilis* and its midgut structure

(A) A pair of mating *P. dissimilis* on a mulberry tree leaf. (B) Dissected alimentary tract of an adult *P. dissimilis* female. M1, midgut first section; M2, midgut second section; M3, midgut third section; CR, constricted region; M4B, bulbous region prior to M4; M4, midgut fourth section bearing crypts; H, hindgut. The box at bottom left shows gut-colonizing bacteria in M4.

Diversity of *Caballeronia* symbionts in natural populations of *P. dissimilis*

To clarify the prevalence and diversity of *Caballeronia* gut symbionts, as well as other bacteria among natural *P. dissimilis* populations, bacterial communities of the symbiotic organs of 69 *P. dissimilis* individuals collected from 10 different sites in Hokkaido prefecture in Japan were analyzed by Illumina deep sequencing. A total of 13,664 to 100,000 valid reads were generated depending on the sample (Table 2-3), and 139 biologically meaningful bacterial zOTUs were obtained using the USEARCH-UNOISE3 algorithm. Bacterial diversity varied slightly depending on the capture location, but was not significantly different, except for samples from Tsukisamukoen (TK) and Chitose (CT) (Figure 2-2). Overall, the genus *Caballeronia* was predominant across all investigated insect midguts, comprising > 80% of the entire gut bacterial community in 66 individuals (Figure 2-3). In total, eleven zOTUs belonging to the genus *Caballeronia* were detected in the midgut of *P. dissimilis*, among which zOTU1 was dominant in almost all individuals (61.8 ± 23.2%), followed by zOTU3 (18.6 ± 7.0%) (Figure 2-4). The two dominant *Caballeronia* species (zOTU1 and zOTU3) were placed in the SBE-β subclade in the phylogenetic tree (Figure 2-5). Interestingly, in the host midgut, where zOTU1 and zOTU3 were less colonized, other subgroups of *Caballeronia* were predominant (Figure 2-5). A small number of *P. dissimilis* insects possessed zOTU2 and zOTU4, which belong to the SBE-γ subgroup, as the main gut symbionts (Figures 2-4 and 2-5). Some insects harbored zOTU5 and zOTU6 (Coreoidea clade), and one individual had zOTU7 and zOTU16 (SBE-α) as the dominant symbionts (Figures 2-4 and 2-5). These results imply that a single subclade of *Caballeronia* generally monopolizes the host midgut, although some individuals retain two co-dominant subgroups.

Table 2-3. Information of data obtained from 16S rRNA amplicon sequencing

Collected site	ID	Total reads	Valid reads	Min reads	Max reads	Avg reads	Min Val Read	Max Val Reads	Avg Val Reads
TS	q001	17,112	15,760	13,665	100,000	27,286	11,989	93,750	24,174
	q002	19,761	17,953						
	q003	34,607	32,695						
	q004	15,416	14,398						
	q005	22,201	18,681						
	q006	17,169	14,733						
	q007	17,791	15,416						
	q008	20,658	18,271						
	q009	22,286	19,700						
	q010	19,906	17,373						
TK	q011	19,192	16,054						
	q012	21,049	19,119						
	q013	14,955	11,989						
	q014	18,949	17,198						
TZ	q015	22,385	19,471						
	q016	19,086	16,390						
	q017	16,597	14,503						
	q018	22,872	20,829						
	q019	20,089	17,181						
	q020	18,246	16,618						
NO	q021	19,765	17,849						
	q022	20,517	17,786						
	q023	25,039	23,392						
	q024	14,199	13,091						
	q025	26,783	23,917						
	q026	24,446	21,090						
	q027	25,258	22,462						
	q028	34,785	30,165						
	q029	27,812	25,480						
YI	q030	21,940	19,290						
	q031	55,419	44,611						
	q032	20,086	18,892						
	q033	27,056	24,630						
	q034	67,690	61,211						
	q035	16,002	13,576						
	q036	23,462	21,516						
	q037	27,253	25,062						
	q038	29,203	25,335						
	q039	28,611	26,256						
	q040	24,299	22,068						
	q041	26,338	21,992						
	q042	29,112	25,340						
	q043	23,064	20,053						
	q044	22,429	19,412						
	q045	20,958	17,599						
MK	q046	24,613	20,823						
	q047	28,469	25,853						
	q048	31,041	26,922						
	q049	26,736	23,924						
	q050	13,665	12,190						
	q051	32,523	29,029						
CT	q052	26,343	23,944						
	q053	31,752	27,921						
	q054	32,843	28,824						
	q055	100,000	93,750						
	q056	61,850	53,186						
	q057	26,619	24,553						
	q058	25,818	22,450						
	q059	25,912	22,547						
	q060	41,942	37,421						
	q061	35,237	29,221						
	q062	61,028	53,069						
	q063	32,805	29,178						
	q065	27,136	24,465						
	q066	27,059	24,410						
	q068	16,677	14,789						
	q069	37,767	34,521						
	q070	15,005	13,179						
	q071	18,061	15,438						

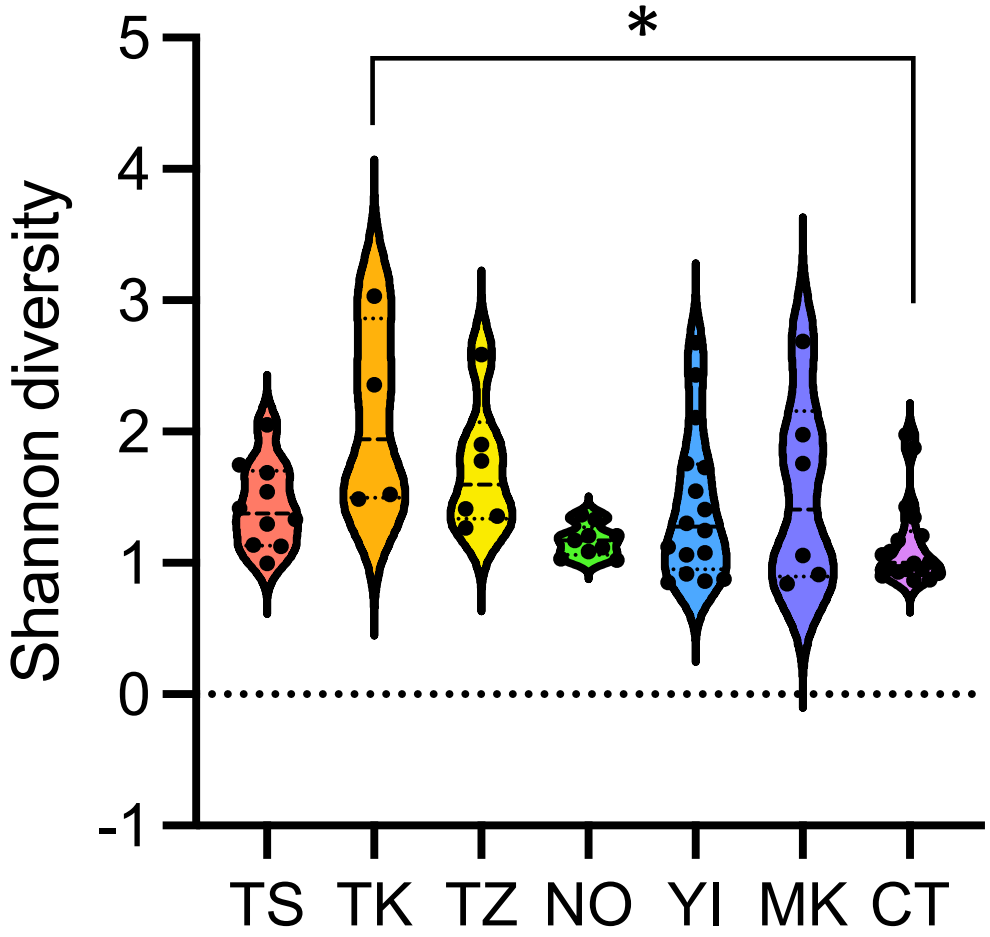

Figure 2-2. Shannon diversity of bacterial community in *P. dissimilis* midgut

Bacterial alpha diversity was measured using the Shannon index. Statistical significance was calculated using the Kruskal-Wallis test with Dunn's correction ($P < 0.05$). Statistical significance was detected only between TK and CT, indicating that the diversity of gut microbial communities is similar among host populations. TS, Tsukisappu; TK, Tsukisamukoen; TZ, Tenzinzan; NO, Nishioka; YI, Yoichi; MK, Makomanai; CT, Chitose.

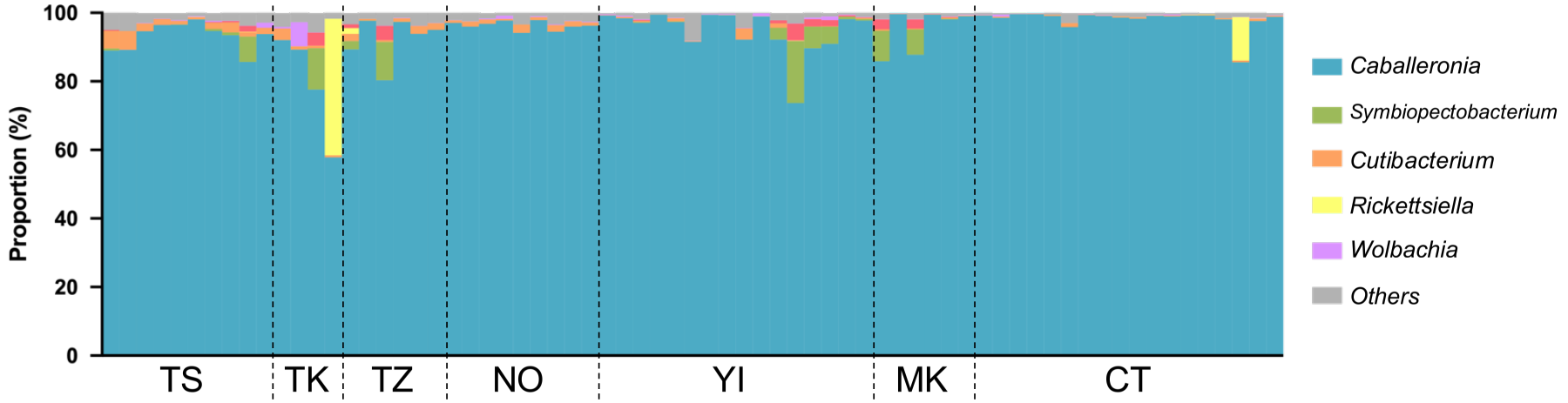

Figure 2-3. Bacterial community composition of the symbiotic organ at the genus level.

The bacterial community composition of M4 was analyzed by bacterial 16S rRNA gene amplicon sequencing. A total of 69 individual insects were captured at 10 different sites in Hokkaido, Japan. The taxa of gut-colonizing bacteria were classified based on the EzBioCloud 16S database version PKSSU4.0 (CJ Bioscience, Korea). TS, Tsukisappu; TK, Tsukisamukoen; TZ, Tenzinzan; NO, Nishioka; YI, Yoichi; MK, Makomanai; CT, Chitose.

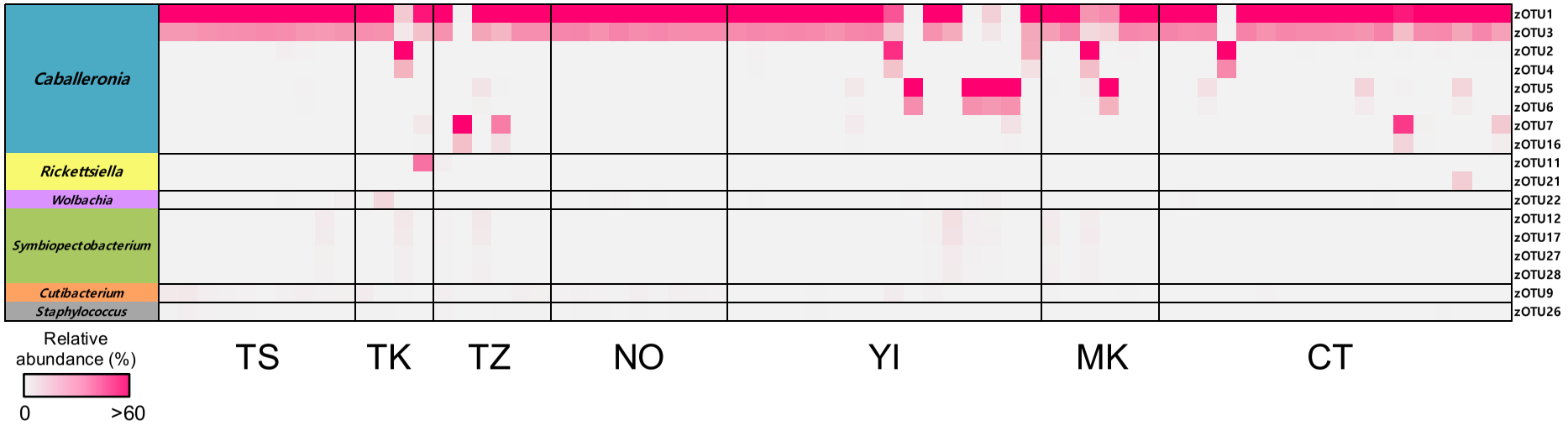

Figure 2-4. Abundance and prevalence of major zOTUs.

Zero-radius OTUs (zOTUs) with > 0.1% relative abundance in the gut bacterial community are shown in the heatmap. A total of 17 zOTUs belonging to six bacterial genera were selected: *Caballeronia* (n = 8), *Rickettsiella* (n = 2), *Wolbachia* (n = 1), *Symbiopectobacterium* (n = 4), *Cutibacterium* (n = 1), and *Staphylococcus* (n = 1). Each column represents a single insect species. TS, Tsukisappu; TK, Tsukisamukoen; TZ, Tenzinzan; NO, Nishioka; YI, Yoichi; MK, Makomanai; CT, Chitose.

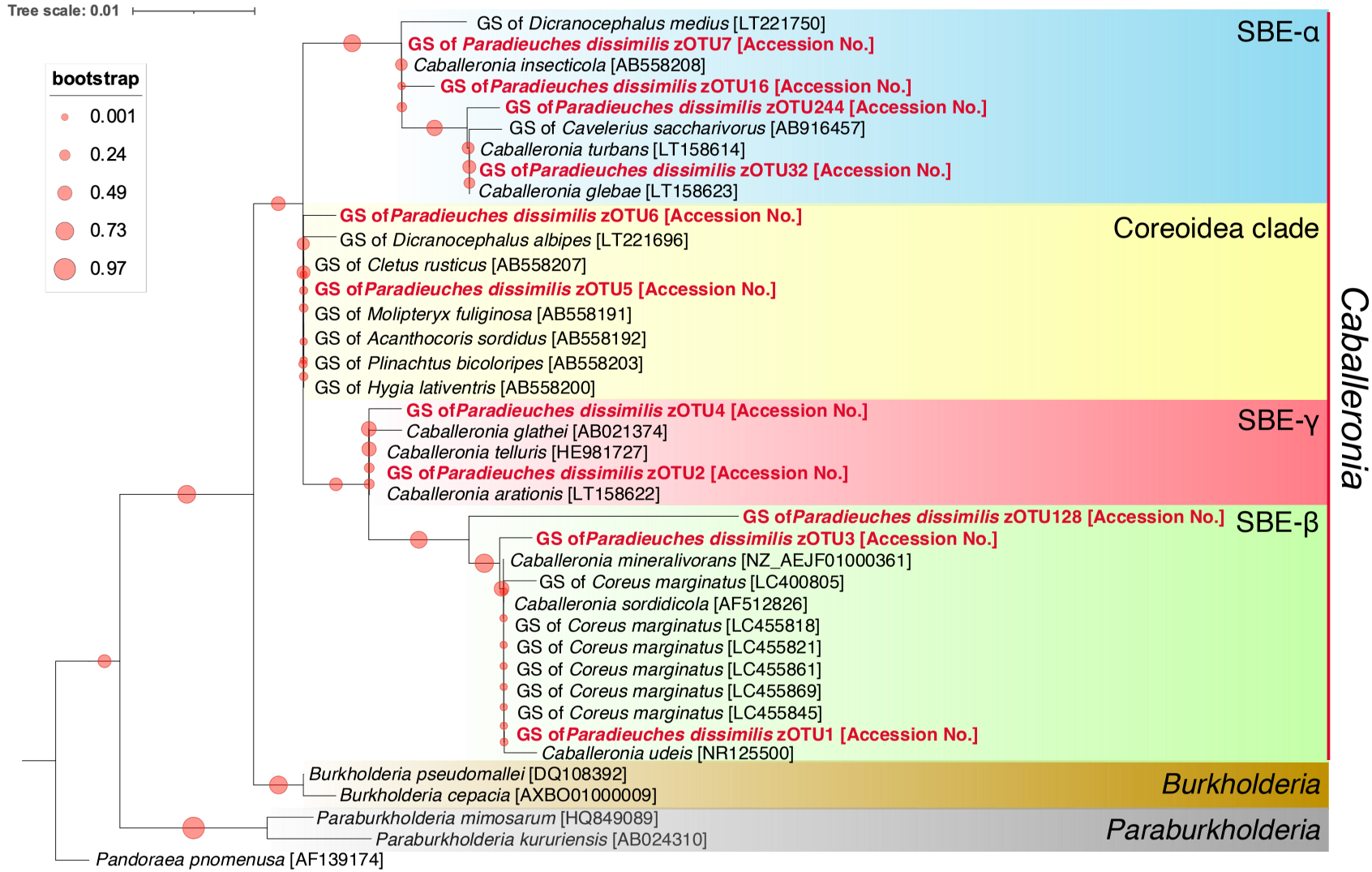

Figure 2-5. Phylogenetic tree of zOTUs belonging to the genus *Caballeronia*.

A maximum likelihood tree of zero-radius OTUs (zOTUs) belonging to the genus *Caballeronia* was constructed based on partial 16S rRNA gene sequences (254 bp). Bootstrap values (1,000 repetitions) are shown at the nodes. zOTUs obtained in this study are shown in bold red letters. GS, gut symbiont.

Because there were many zOTUs other than *Caballeronia* in the midgut of *P. dissimilis*, I investigated the top 20 zOTUs with high relative abundance. Four zOTUs belonging to the genus *Symbiopectobacterium*, which is known as an intracellular symbiont of nematodes (132) and insects (133–135), were detected in a small proportion of individuals (Figures 2-2 and 2-3). All *Symbiopectobacterium* colonizing *P. dissimilis* formed a monophyletic group with an endosymbiont clade, rather than a free-living *Pectobacterium* (Figure 2-6A). In addition, two zOTUs of *Rickettsiella*, known as secondary endosymbionts of insects, including the pea aphid (48, 136–138), were present in high abundance in two individuals of *P. dissimilis* (26.5% and 8.9%, respectively) (Figures 2-2 and 2-4) and formed a monophyletic group with endosymbionts of other insects (Figure 2-6B). In the midgut of one *P. dissimilis* individual, zOTU belonging to *Wolbachia* was detected (7.2%), and it was phylogenetically close to the endosymbiont of mosquitos and seed bugs (Figures 2-2, 2-4, and 2-6C). Taken together, these results suggest that *P. dissimilis* harbors *Caballeronia* as a primary symbiont, but is occasionally associated with putative facultative symbionts.

Isolation and identification of *Caballeronia* gut symbiont

To determine the physiological roles of the *Caballeronia* gut symbiont in *P. dissimilis*, I isolated one of the gut-colonizing bacterial strains from *P. dissimilis* by spreading homogenized gut lysates on YG agar. Bacterial taxonomy was identified by 16S rRNA sequence of the isolated colony after BLASTn searches, and the gut symbiont of *P. dissimilis* belonged to the genus *Caballeronia*, thus the gut-colonizing symbiont is named "*Caballeronia* sp. PDI-1". Phylogenetic analysis revealed that *Caballeronia* sp. PDI-1 symbiont was located in the SBE-γ subgroup of *Caballeronia* (Figure 2-7).

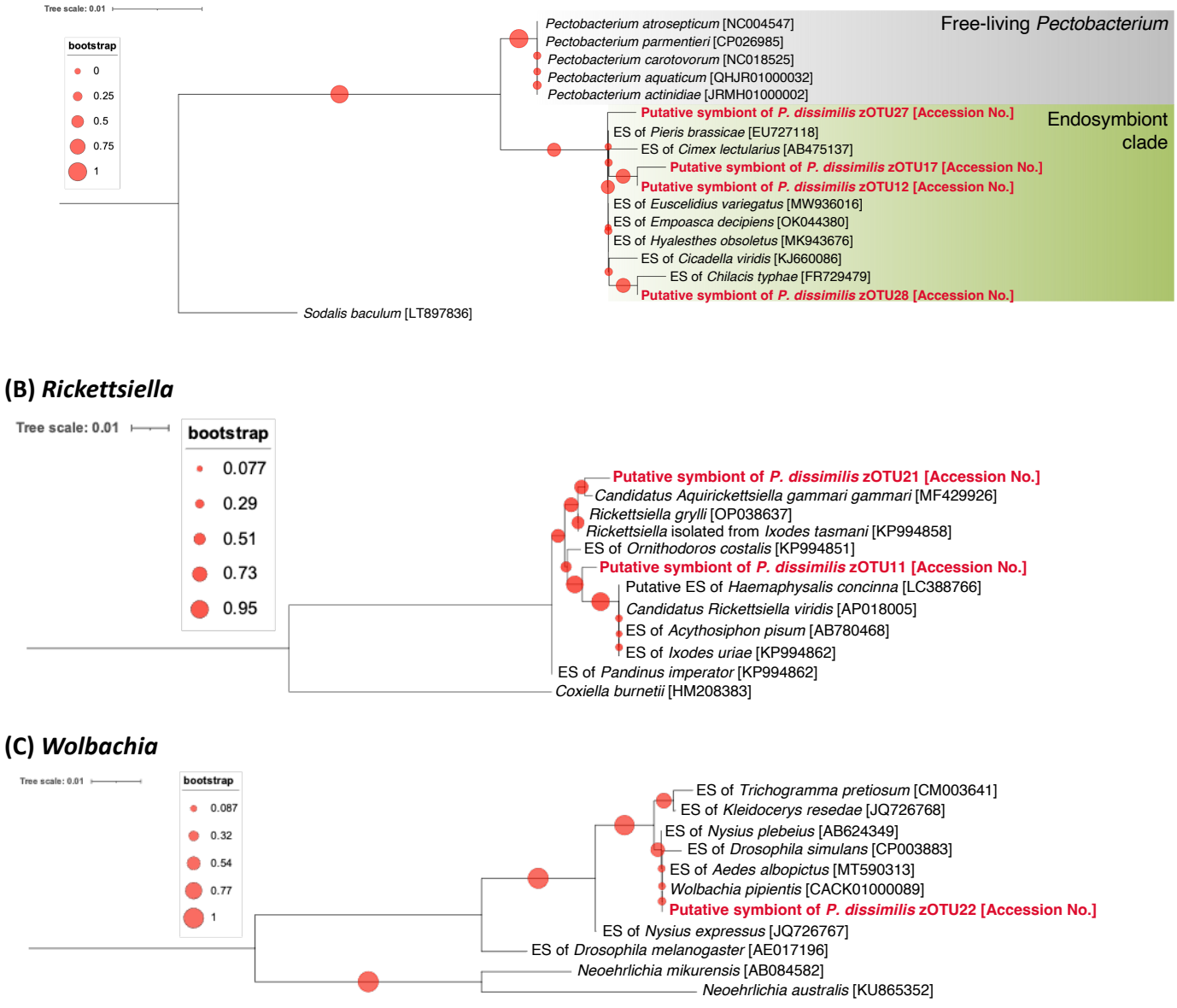

Figure 2-6 Phylogenetic tree of putative facultative symbionts.

Phylogenetic trees of zOTUs belonging to (A) *Symbiopectobacterium*, (B) *Rickettsiella,* and (C) *Wolbachia* were constructed based on partial sequences of the 16s rRNA gene (254 bp) using a bootstrapped maximum-likelihood tree (1,000 repetitions). ES, endosymbiont. Bootstrap values are shown at the nodes.

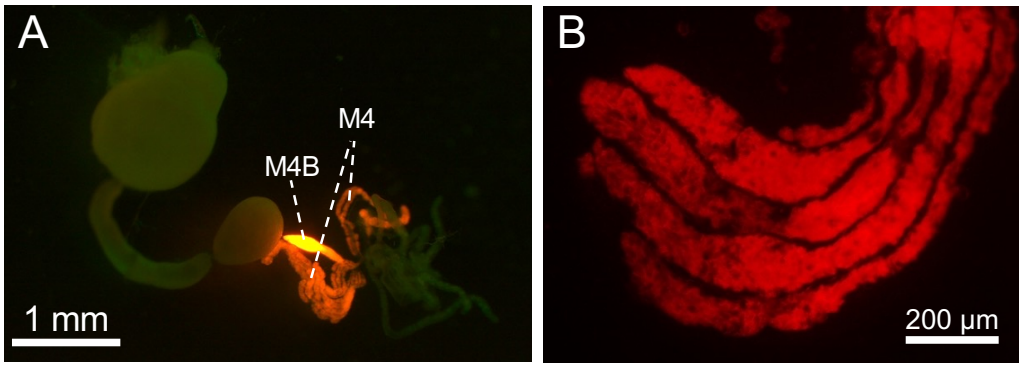

Figure 2-7. Colonization of RFP-expressing *Caballeronia* symbiont in the symbiotic organ. (A) Dissected midgut of a fifth instar nymph infected with red fluorescence-labelled *Caballeronia* sp. PDI-1 symbiont. (B) Enlarged image of symbiont-harboring M4 crypts. Red fluorescence signal indicates RFP-expressing *Caballeronia* sp. PDI-1 cells.

In general, *Caballeronia* gut symbionts in many stinkbugs (e.g., *C. insecticola* symbiont of the bean bug *Riptortus pedestris*) are acquired by host insects from environmental soil in each generation (118). To verify whether gut-colonizing *Caballeronia* sp. PDI-1 in *P. dissimilis* is indeed horizontally obtained from the soil, the 16S rRNA gene of *Caballeronia* from the following three samples was investigated by diagnostic PCR: (1) eggs laid by laboratory-maintained symbiont-harboring insects, (2) M4 crypts of insects reared with microbe-free DWA, and (3) M4 crypts of insects reared with symbiont-containing unsterilized soil. Although no *Caballeronia* was detected in the eggs (detected/investigated = 0/10) and insects reared with DWA (0/10), the midgut of insects reared with soil was 100% infected with *Caballeronia* symbiont (10/10), demonstrating that the *Caballeronia* symbiont was horizontally acquired from the soil in *P. dissimilis* as well. I then constructed RFP-expressing *Caballeronia* sp. PDI-1 cells which were used to investigate symbiont localization. RFP-labelled symbionts were orally administered to newly hatched nymphs, and the ingested fluorescence-expressing symbiont cells colonized the midgut crypts (Figures 2-8A and B). These results demonstrated that *P. dissimilis* is associated with environmentally acquired

symbionts in tubular-shaped M4 crypts.

Fitness effects of *Caballeronia* symbiont on *P. dissimilis*

Using the laboratory-reared line of *P. dissimilis*, the fitness effects of the *Caballeronia* symbiont on the host were investigated. Most of the symbiont-free aposymbiotic insects died during the second-to fourth-instar nymph stages, and only a few individuals reached fifth-instar nymphs. Aposymbiotic insects that successfully reached the fifth instar stage exhibited reduced body size and brighter body color (Figures 2-8A and B). Although the overall body color was remarkably different between the aposymbiotic and symbiotic insects, I measured and compared the color of the pronotum as an index. The color of the pronotum was much darker in normal symbiotic insects than in aposymbiotic insects (Figures 2-8A, B, C). In addition, the total body length of aposymbiotic insects was significantly shorter than that of symbiotic insects (Figure 2-8D). Most importantly, although some aposymbiotic *P. dissimilis* molted to fifth-instar nymphs, all failed to emerge as adult insects (0/59) (Figure 2-8E). However, the survival rates of *Caballeronia*-harboring *P. dissimilis* following infection with symbiont-containing soil or cultured *Caballeronia* sp. PDI-1 cells were 56% (46/82) and 48% (12/25), respectively (Figure 2-8E). *Caballeronia*-dependent host survival is generally observed in other Lygaeoidea and Coreoidea stinkbugs as well (67, 139). For example, the southern chinch bug *Blissus insularis* (Blissidae) suffers from low growth and smaller body size due to antibiotic treatment (140, 141). The omnivorous bug genus *Jalysus* (Berytidae) also possesses *Caballeronia* symbionts, wherein aposymbiotic insects exhibit delayed development and lower fecundity (142). These results demonstrated that *Caballeronia* is an obligate gut symbiont in *P. dissimilis*.

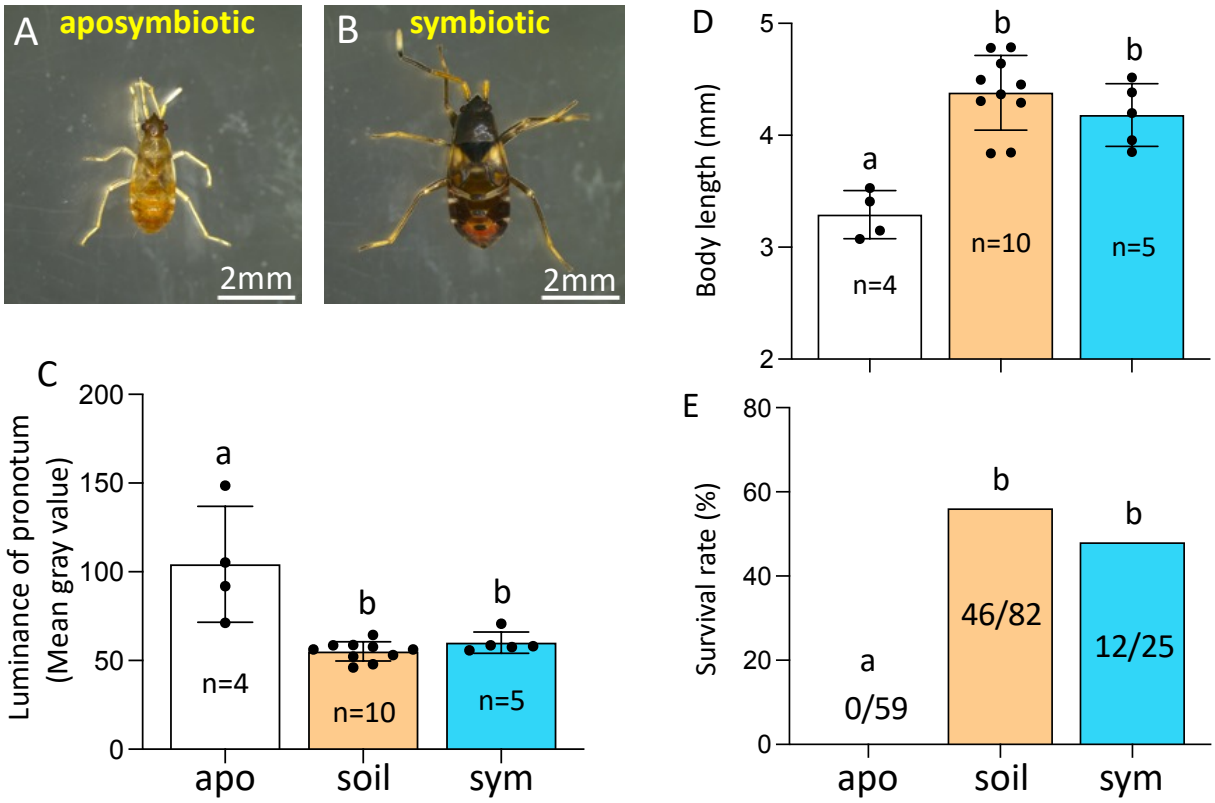

Figure 2-8. Comparison of fitness parameters between aposymbiotic and symbiotic *P. dissimilis*.

(A and B) Photographs of fifth-instar of symbiont-free aposymbiotic and *Caballeronia*-harboring symbiotic insects. (C) Relative luminance of the pronotum of fifth-instar nymphs measured by mean gray value. Luminance values ranged from 0 (black) to 255 (white); thus, lower values indicate darker color. Luminance of the pronotum of aposymbiotic insects was set as a standard. (D) Body length of fifth-instar nymphs and (E) survival rate (adult emergence rate). Error bars indicate standard deviations. The statistical significances of luminance of the pronotum and body length were analyzed by the Mann-Whitney *U* test with Bonferroni correction ($P < 0.05$). The statistical significance of survival rate was analyzed using Fisher's exact test with Bonferroni correction ($P < 0.05$). Apo, aposymbiotic insect; Soil, *Caballeronia*-harboring soil-infected insect; Sym, insect infected with cultured *Caballeronia* sp. PDI-1 cells.

Discussion

By conducting a field survey in conjunction with laboratory rearing experiments, I demonstrated that (1) *P. dissimilis* possesses the *Caballeronia* symbiont in the crypt-bearing posterior midgut region (Fig. 1 and 5); (2) the *Caballeronia* symbiont is horizontally acquired from the soil of each host generation; and (3) the gut-colonizing *Caballeronia* is an obligate symbiont that is essential for host survival and development (Figure 2-9). In addition to several species of the Coreoidea (66, 83, 143), our findings of *P. dissimilis*-*Caballeronia* gut symbiosis demonstrate that stinkbugs belonging to the Lygaeoidea, which have a posterior midgut consisting of crypts, also establish an obligate symbiotic relationship with members of the genus *Caballeronia*, strongly suggesting the omnipresence of horizontally transmitted obligate symbiosis in this insect group.

In addition to *Caballeronia*, the genera *Paraburkholderia* and *Burkholderia* can colonize the midgut crypts of certain Lygaeoidea stinkbug species (73, 116). For example, the oriental chinch bug *Cavelerius saccharivorus* (Lygaeoidea: Blissidae) occasionally harbors *Paraburkholderia* bacteria in the midgut as a sole symbiont (116). In addition, some *C. saccharivorus* individuals possess double or triple symbionts (e.g., *Caballeronia* + *Paraburkholderia*) in the symbiotic organ (33). However, no such cases were observed in this study; bacteria other than *Caballeronia* monopolized the midgut crypts of *P. dissimilis* (Figures 2-2 and 2-4). Nevertheless, other bacteria, such as *Symbiopectobacterium, Rickettsiella*, *Wolbachia*, and *Cutibacterium*, coexisted with the *Caballeronia* symbiont in many individuals of *P. dissimilis* (Figures 2-2 and 2-4). A few individuals harbored a high percentage of *Symbiopectobacterium*, *Rickettsiella*, or *Wolbachia*, which are symbiotic bacteria of diverse insects (132, 136, 144–147). The detected zOTUs belonging to these bacteria in the midgut of *P. dissimilis* formed monophyletic groups with the insect endosymbionts (Figure 2-6). *Symbiopectobacterium* is an obligate endosymbiont of sap- and seed-feeding hemipteran

insects (135, 148, 149) and a facultative symbiont of bed bugs (133), kissing bugs (150), and leafhoppers (134). *Symbiopectobacterium* is a putative nutritional symbiont among these insects. *Rickettsiella* endosymbionts have been reported to protect aphids from pathogenic fungi (144). *Wolbachia*, a sex ratio distorter (151), sporadically infects stinkbug species (115, 152). In addition, the *Wolbachia* symbiont of the bedbug *Cimex lectularius* is known to supply B vitamins to their hosts (146). Thus, these co-infected bacteria, besides the *Caballeronia* gut symbiont, could act as facultative symbionts in *P. dissmillis*, although this requires further investigation.

In the well-studied *Caballeronia*-associated model stinkbug *R. pedestris*, host-symbiont specificity is determined by bacterial selection at the constricted region of the midgut (86) and by microbe-microbe competition inside the crypt-bearing M4 region (94). *P. dissimilis* also developed a constricted region between M3 and M4B (Figure 2-1B), which is plausibly involved in symbiont sorting. The bean bug *R. pedestris* possesses *C. insecticola* in the midgut crypts as a native gut symbiont, but non-native symbionts, such as *Paraburkholderia* or even its outgroup *Pandoraea*, can pass through the constricted region and stably colonize the symbiotic organ (94). However, *C. insecticola* symbionts outcompete non-native symbionts in the symbiotic organ, although the molecular basis of this competitive mechanism remains unclear. Nevertheless, a small amount of non-symbiotic bacteria has been detected in the midgut of soil-infected adults or wild populations of *R. pedestris* by deep sequencing or diagnostic PCR (153, 154). This could be due to the re-opening of the symbiont sorting organ at the adult stage, giving an opportunity for non-symbiotic bacteria to colonize the symbiotic organ (155). However, it should be noted that the predominant gut-colonizing bacteria are *Caballeronia* symbionts. Similar to *R. pedestris*, most *P. dissimilis* had overwhelmingly dominant *Caballeronia* in the midgut crypts (Figures 2-2 and 2-4), although a relatively high proportion of putative facultative symbionts was detected in a few individuals. A recent study

demonstrated that microbe-microbe interactions in the midgut are also important in competition among subgroups of *Caballeronia* (SBE clade). In the conifer bug *Leptoglossus occidentalis*, each subgroup of *Caballeronia* colonized the midgut in mono-infection tests, but one subclade symbiont outperformed the other in competition assays (82). *P. dissimilis* also possessed multiple zOTUs belonging to *Caballeronia*; however, a single subclade, especially SBE-β, was predominant in the midgut (Figures 2-4 and 2-5). These results suggest that in addition to bacterial filtering by the constricted region, microbe-microbe competition is also critical in *P. dissimilis*-*Caballeronia* gut symbiosis to choose a symbiotic partner.

In the bean bug *R. pedestris*, the *Caballeronia* symbiont is beneficial for growth and fecundity but is not essential for host survival (83). In contrast, the *Caballeronia* symbiont presented an obligate nature in *P. dissimilis*, wherein no aposymbiotic insects could reach adulthood (Figure 9E). A similar obligate nature of the *Caballeronia* symbiont has also been reported in Coreoidea stinkbugs such as *C. marginatus* and *Anasa tristis* (67, 139). Although the biological function(s) of the *Caballeronia* symbiont in stinkbug species are not fully understood, host dependency seems likely to be related to the diet of the host insects. For example, *R. pedestris* feeds on the seeds of leguminous plants that are rich in amino acids (156). Meanwhile, *C. marginatus, A. tristis*, and *P. dissimilis* feed on *Rumex* plants, squash, and mulberry, respectively (67, 121, 139). Indeed, the nutrient composition of mulberries is much lower than that of soybean seeds (91, 157). In addition to effects on survival, the body color of the aposymbiotic insects was much brighter than that of the symbiotic insects (Figures 2-9A, B, C), which strongly suggests that the *Caballeronia* symbiont is involved in melanization and hardness of the host exoskeleton, as shown in diverse beetles (158). Thus, even though cuticle hardness was not measured in the present study, gut symbiotic *Caballeronia* may be able to biosynthesize and supply *P. dissimilis* with essential nutrients that are important for cuticle hardening as well as for host survival. Indeed, the symbiotic

bacteria of beetles are involved in host cuticle hardening by providing tyrosine (38, 158). Tyrosine and its derivative, L-DOPA, are important principal substrates for initiating chemical reactions related to cuticle tanning, polymerization, and melanization (159). Symbiont elimination by antibiotic treatment caused a low tyrosine titer, resulting in a pale, soft cuticle. To determine the specific biological functions of the *Caballeronia* symbiont in *P. dissimilis*, whole-genome sequencing of the isolated *Caballeronia* symbiont and comparative transcriptome analysis between *in vivo* gut-colonizing and *in vitro* cultured *Caballeronia* should be performed in future studies.

Chapter 3

A lethal pathogen hijack insects-microbe symbiosis

Introduction

Many animals and plants have symbiotic microorganisms within their body and they interact intimately with each other (1). Symbiosis is a continuum of relationships from parasitism to mutualism. Many organisms stably establish mutualistic interactions with microbes in nature (1). However, in mutualism, cheaters, which derive benefits from the host and simultaneously cause harm, threat the evolutionary stability of mutualism (96, 98, 160–165). Therefore, countermeasures against cheaters have been theoretically and experimentally investigated (96, 98, 164, 166, 167). Theoretical studies of countermeasures against cheaters have presented two major options: one is partner fidelity feedback in which the host gives more benefits to the partner who provides greater benefits (96, 97, 167), and the other is a mechanism called partner choice wherein the host interacts only with beneficial symbionts and applies punishment or sanction to cheaters (96, 97). These countermeasures have been theoretically found to contribute to the evolutionary stability of mutualism.

Experimental studies of cheaters have been well performed in the symbiosis with horizontal transmission, in which the host acquires symbionts from the environment (160, 161, 163, 166, 167). In the horizontal transmission, the host selects symbiotic partners every generation from various environmental microbes from harmful to beneficial, and thus, the host is always threatened by the infection of cheaters. Indeed, legumes are occasionally infected by cheaters lacking nitrogen-fixing ability but the host sanctions such parasitic cheaters by halting the supply of nutrients (165, 167–169).

The bean bug *R. pedestris* (Heteroptera: Alydidae) harbors *Caballeronia* symbionts in the posterior midgut. The midgut of *R. pedestris* consists of four distinct sections called the M1, M2, M3, and the symbiotic organ M4, in which millions of *Caballeronia* symbionts are housed in the midgut crypts (71). The *R. pedestris*-*Caballeronia* symbiosis is not an obligate relationship but the *Caballeronia* symbiont is beneficial for growth, fecundity, immunity, and

detoxification in the host insects (29, 30, 71, 83–85). The *R. pedestris* newly acquires the *Caballeronia* symbionts from the ambient soil every generation after hatching (83). To acquire the specific symbiont from complex soil microbiota, the *R. pedestris* have evolved to develop specialized symbiont selection mechanisms. In the anterior part of the symbiotic organ, there is the symbiont sorting organ called the "constricted region (CR)", which is a narrow organ filled with a mucous-like matrix (86). Most non-symbionts such as *E. coli* and *Salmonella* cannot pass the CR (86), whereas *Caballeronia* symbiont rapidly passes the narrow sorting organ using a unique gliding motility, in which the symbiont wraps flagella around its cell body and rotates showing a screw-like motion (90). This unforeseen motility is considered one of the important factors to pass the CR. However, a few non-symbionts, such as *Paraburkholderia fungorum* and *Pandoraea norimbergensis*, can pass the CR and colonize the symbiotic organ, but the native *Caballeronia* symbiont always outcompetes the non-symbionts in microbe-microbe competition, resulting in that the *R. pedestris*-*Caballeronia* monospecific symbiosis is maintained (94). Besides the microbial competition, the genes encoding antimicrobial substances and digestive enzymes are highly expressed in the midgut and these molecules are also considered to contribute to the selection of the *Caballeronia* symbiont by killing non-symbionts (87, 92, 170). Indeed, midgut-derived antibacterial substances effectively killed the non-symbionts but not the *Caballeronia* symbionts (92, 170–172). These symbiont selection mechanisms are fundamental to the *R. pedestris*-*Caballeronia* specificity. However, cheaters, which evade the symbiont sorting organ, successfully colonize the symbiotic organ and harm the host insect, have not been found yet in *R. pedestris*.

Theoretical studies of " cheaters" include symbionts that actively harm the host, but in mutualisms between host-microorganisms, no symbiont has been found that actively kills the host while colonizing the symbiotic organ (163). This is thought to be because, theoretically, if a symbiont kills a host that provides benefits to the symbiont, the symbiont abandons any

future benefits from the host. In addition, the host has mechanisms to eliminate cheaters, preventing cheater s from establishing themselves in the symbiotic organ (96). However, in the *R. pedestris*-*Caballeronia* symbiosis, I discovered a lethal pathogen that kills the host even though the symbiont is able to colonize and proliferate in the symbiotic organ same as the native symbiont, *Caballeronia*. In this study, I investigated the mechanism of how the pathogen kills the host in the symbiotic organ and evade the host's symbiont sorting mechanisms.

Materials and Methods

Insects rearing and symbiont inoculation

The bean bug *R. pedestris* used in this study were collected from soybean fields in Tsukuba, Ibaraki, Japan, and maintained in the laboratory for more than 10 years. Insects were reared in Petri dishes (90 mm in diameter and 20 mm high) at 25°C under long-day conditions (16 hours light, 8 hours dark) and fed with soybean seeds and distilled water containing 0.05% ascorbic acid (DWA). The gut symbiont *Caballeronia insecticola* was cultured in yeast-glucose (YG) medium (0.5% yeast extract, 0.4% glucose, 0.1% NaCl) with antibiotics (100 μg/ml of rifampicin and 30 μg/ml of chloramphenicol) at 30°C with vigorous shaking (150 rpm) until the mid-log phase. Bacterial cells were then harvested by centrifugation at 4,000 rpm for 10 min and diluted in DWA to a final concentration of 10^7 cells/ml. To inoculate symbiont, cotton pads were soaked with bacterial suspension and provided to newly molted second instar nymphs.

Isolation and identification of entomopathogen *Burkholderia* sp. SJ1

A bean bug-killing pathogen *Burkholderia* sp. SJ1 was isolated from the midgut of the mulberry seed bug *Paradieuches dissimilis* (Lygaeoidea: Rhyparochromidae) collected from Sapporo,

Japan (173). The midgut of *P. dissimilis* was isolated, homogenized in a phosphate-buffered saline (PBS), and spread on YG agar for 2 days at 30°C. A 1.5 kb of the 16S rRNA gene of a pure isolate was amplified by PCR using AmpliTaq Gold™ 360 Master Mix (Applied Biosystems, USA) and primers 16SA1 and 16SB1 (Table 3-1) (125). The amplified PCR product was cleaned with Exo-SAP-IT (GE Healthcare, USA), reacted with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), and purified by FastGene Dye Terminator Removal Kit (Nippon Genetics Co., Japan). The purified DNA was then sequenced by ABI 3130 XL Genetic Analyzer (Applied Biosystems, USA). The isolated bacterium was identified as *Burkholderia* species by BLASTn search against the NCBI 16S rRNA database.

Table3-1. Primers used in this study

Target group	Target gene	primer/probe name	Nucleotide sequence (5'-3')	Approximate product size (kb)	Annealing temp (°C)	purpose	Reference
Primers							
Burkholderia	16s rRNA	BCC370F	TTTTGGACAATGGGCGAAAG	0.8	55	Diagnostic PCR	Itoh et al. 2014
		Burk16SR	GCTCTTGCGTAGCAACTAAG				
	16s rRNA	Bf	TAGCCCTGCGAAAGCCG	0.5	56	Sequencing	Tago et al. 2014
		Br	GCCAGTCACCAATGCAG				
	16s rRNA	BSdnaA-F	AGCGCGAGATCAGACGGTCGTCGAT	0.15	60	Quantitative PCR	Kikuchi et al. 2011
		BSdnaA-R	TCCGGCAAGTCGCGCACGCA				

Itoh H, Aita M, Nagayama A, et al. (2014) Evidence of environmental and vertical transmission of Burkholderia symbionts in the oriental chinch bug, *Cavelerius saccharivorus* (Heteroptera: Blissidae), *AEM,* 80:5974-5983

Tago K, Itoh H, Kikuchi Y, et al (2014) A fine-scale phylogenetic analysis of free-living *Burkholderia* species in sugarcane field soil. *Microbes Environ*. ME14122.

Kikuchi Y, Hosokawa T, Fukatsu T (2011) Specific Developmental Window for Establishment of an Insect-Microbe Gut Symbiosis. *AEM*, 77:4075-4081

Phylogenetic analysis

The full-length of 16S rRNA genes of *Burkholderia* sp. SJ1 and its related bacterial genera (*Burkholderia*, *Caballeronia,* and *Paraburkholderia*) were aligned using the MAFFT multiple sequence alignment software (129). A maximum likelihood phylogeny of bacteria was inferred using MEGA X software with 1,000 replications of bootstrap tests (130).

Colonizing ability of *Burkholderia* sp. SJ1 in the symbiotic organ

To measure the colonizing ability of *Burkholderia* sp. SJ1 in the symbiotic organ of *R. pedestris*, one μl (10^4 cells/μl) of the cultured green fluorescent protein (GFP)-labeled *Burkholderia* sp.

SJ1 cells was supplied to the second instar nymphs. Two hours after feeding bacteria, the *Burkholderia* sp. SJ1 cells passing the constricted region, which is located in front of the symbiotic organ, were observed under an epifluorescence microscope (DMI4000B, Leica, Germany). To examine the presence of midgut-colonizing *Burkholderia* sp. SJ1, DNA was extracted from the symbiotic organs 3 days after bacteria infection using QIAamp DNA Mini Kit (QIAGEN, Germany), and diagnostic PCR was performed using *Burkholderia*-specific primers (Table 3-1) (n = 10) (116). Simultaneously, the number of midgut-colonizing *Burkholderia* sp. SJ1 was measured every day after oral infection (n =4 insects per day) by real-time quantitative PCR of *dnaA* gene using primers BSdnaA-F (5′-AGC GCG AGA TCA GAC GGT CGT CGA T-3′) and BsdnaA-R (5′-TCC GGC AAG TCG CGC ACG CA-3′) (Table 3-1) (88), KAPA SYBR FAST qPCR Master Mix Kit (Kapa Biosystems Inc., USA), and LightCycler 96 System (Roche Diagnostics, Switzerland). As the control group, the number of *dnaA* gene copies of *C. insecticola* symbiont was measured from only symbiont-infected insects by qPCR.

Measurement of bacterial pathogenicity

The pathogenicity of *Burkholderia* sp. SJ1 to *R. pedestris* during oral and systemic infections was measured. In the case of oral infection, different concentrations of serially diluted bacterial solution (1 to 10^7 cells/ml) were fed to the second instar nymphs and the survival rate was measured by counting carcasses every day. To measure the LD50 (50% lethal dose), one μl of various concentrations of serially diluted bacterial solution (1 to 10^4 cells/μl) was fed to the second instar nymphs as previously described (89), and the host survival rate was measured every 12 hours. The LD50 was measured by using the equation probit as previously described (89). To measure the pathogenicity of *Burkholderia* sp. SJ1 in systemic infection, one μl of various concentrations of serially diluted bacterial solution (1 to 10^7 cells/μl) was directly

injected into the hemocoel using a glass capillary and a FemtoJet microinjector (Eppendorf, Germany), and the host survival rate was measured every 12 hours.

Histological observations

Insects were fed with 10^7 cells /ml of GFP-labelled gut symbiont *C. insecticola* or *Burkholderia* sp. SJ1 at the second instar nymphal stage and the whole midgut region was isolated 5 days after infection. The isolated whole midgut was observed under a stereomicroscope (S8APO, Leica, Germany) and photographed with a connected digital camera (EC3, Leica, Germany). The morphology of the M4B or symbiotic organ infected by *C. insecticola* gut symbiont or *Burkholderia* sp. SJ1 was observed under a confocal laser scanning microscope (TCS SP8, Leica, Germany). The isolated organs were fixed in 4% paraformaldehyde for 10 min at 25°C and washed twice with PBS. The fixed samples were permeabilized by PBS containing 0.1% Triton X-100 (PBST) for 10 min at 25°C. Then, the samples were stained with 4′ 6-diamidino-2-phenylindole (DAPI) and Alexa Fluor™ 647 phalloidin for 20 min at 25°C to stain host cells and F-actin of the cytoskeleton, respectively. After that, the samples were placed on a glass-bottom dish (Matsunami, Japan), mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, USA), and observed under a confocal laser scanning microscope.

Observation of midgut melanization

The 10^7 cells /ml of gut symbiont *C. insecticola* or *Burkholderia* sp. SJ1 was orally administrated to insects as described above. Three days after infection, the symbiotic organs were isolated and midgut morphology and melanization were observed under a stereomicroscope (S8APO, Leica, Germany) and a confocal laser scanning microscope (TCS SP8, Leica, Germany). The melanized parts of the midgut turned black color. The percentage of insect individuals with melanized midgut was calculated by the formula [the number of

melanized individuals/the number of observed individuals (n = 6)].

Colony-forming unit (CFU) assay

The number of bacterial cells in the M4B or hemolymph was counted by CFU assay. The second instar nymphs of the bean bugs were infected by the GFP-labelled *Burkholderia* sp. SJ1 or *C. insecticola* gut symbiont, and the M4B was isolated 5 days after oral administration. The isolated M4B was completely homogenized by a pestle in 100 μl of PBS, and the serially diluted lysate was spread on YG agar with antibiotics (*Burkholderia* sp. SJ1: 30 μg/ml of kanamycin, *C. insecticola*: 100 μg/ml of rifampicin). The hemolymph was collected by cutting off the legs of the bean bugs 7 days after infection of the GFP-labelled *Burkholderia* sp. SJ1 or *C. insecticola* symbiont and suspended in 100 μl of PBS. The hemolymph solution was spread on YG agar with antibiotics (*Burkholderia* sp. SJ1: 30 μg/ml of kanamycin, *C. insecticola*: 100 μg/ml of rifampicin). The plates were incubated at 30°C for 2 days and the number of grown colonies was counted.

Co-infection tests

Kanamycin-resistant *Burkholderia* sp. SJ1 and rifampicin-resistant *C. insecticola* were cultured until the mid-log phase in YG medium containing the appropriate antibiotics. The cultured cells were harvested by centrifugation at 4,000 rpm for 10 min, washed twice with sterilized PBS, and suspended in DWA. One μl of 5,000 cells/μl of each bacterial solution or mixture was fed to the freshly molted second instar nymphs. After single- or co-inoculation, the survival rate was daily measure by counting dead individuals. To identify an inhibitory priority effect, the 10^7 cells/ml of the gut symbiont *C. insecticola* was fed to the freshly molted second instar nymphs for 1 day, allowing the gut symbiont priorly colonizes the symbiotic organ, and then the same concentration of *Burkholderia* sp. SJ1 cells was fed to the symbiont-

harboring insects for additional 3 days. As a control, insects were infected by only *Burkholderia* sp. SJ1 or *C. insecticola* as well. The survival rate of insects was measured every day as above.

Competition assays

In vitro competition assay

The mid-log phase of kanamycin-resistant *Burkholderia* sp. SJ1 and rifampicin-resistant *C. insecticola* were harvested, washed twice with sterilized PBS, and lastly suspended in YG medium. Five thousand of each bacterial cell were mixed together and the total volume of the mixture was adjusted up to 1 mL with YG medium. The mixed bacterial solution was then incubated at 25°C without shaking. Three days after standing culture, the bacterial solution was serially diluted, plated on YG agar containing appropriate antibiotics (30 μg/ml of kanamycin for *Burkholderia* sp. SJ1 and 100 μg/ml of rifampicin for *C. insecticola*), and incubated for 2 days at 25°C. After 2 days of incubation, the number of each bacterial colony was counted and the competitive intelligence (CI) was calculated by the formula (output *Burkholderia* sp. SJ1 CFU/input *Burkholderia* sp. SJ1 CFU)/(output *C. insecticola* CFU/input *C. insecticola* CFU).

In vivo competition assay

Five thousand cells/μl of each kanamycin-resistant *Burkholderia* sp. SJ1 and rifampicin-resistant *C. insecticola* were mixed and one μl of the bacterial mixture was fed to the freshly molted second instar nymphs. Three days after infection, the symbiotic organs were isolated from alive insects, homogenized by a pestle, serially diluted, and plated on YG agar containing each kanamycin and rifampicin. Two days after incubation at 25°C, the number of colonies of *Burkholderia* sp. SJ1 and *C. insecticola* were counted and the CI values were calculated. Since *Burkholderia* sp. SJ1 shows high pathogenicity to the bean bug, some individuals are dead after oral infection. The bean bugs that died on the third day were dissected, and the symbiotic organ was isolated and the CI values of two bacterial species were calculated as above.

Antibacterial sensitivity tests

To measure the sensitivity of the *Caballeronia* symbiont and *Burkholderia* sp. SJ1 against antibacterial substances, the symbiotic organ colonized by the *Caballeronia* or *Burkholderia* sp. SJ1 were collected from the third instar nymphs 5 days after bacterial infection and completely homogenized by a pestle in PBS. To harvest *in vivo* gut-colonizing bacterial cells, the homogenized gut lysates were filtrated through a 0.45 μm filter and harvested by centrifugation. The bacterial cells were washed and diluted with PBS to adjust the number of cells to approximately 1,000 cells /50 μl. The prepared cells were treated with M4B gut lysate or commercial antimicrobial peptides, polymyxin B, or bleomycin. To prepare M4B lysate, the M4B was collected from 10 fifth instar nymphs of *R. pedestris* in 100 μl of PBS and homogenized by a pestle. Then the organ-derived debris are precipitated by centrifugation at 20,000 x g for 15 mins, the total protein concentration of the supernatant was measured by the NanoDrop and the final protein concentration of the M4B lysate was adjusted to 20 μg/ml. The concentrations of polymyxin B and bleomycin used for the experiment are 25 μg/ml and 10μg/ml, respectively. Fifty μl of the prepared bacterial solutions are incubated with 50 μl of each M4B lysate or antimicrobial peptides at 27°C for 30 mins, as a control PBS was used. After incubation, the bacterial cells were spread on YG agar with 30 μg/ml of kanamycin for *Burkholderia* sp. SJ1 or 100 μg/ml of rifampicin for *C. insecticola*, and cultured for 2 days at 27°C. The number of grown colonies on agar was counted as described above.

Observation of swimming motility

The screw-like motility of the *Burkholderia* sp. SJ1 was observed as described in the previous study (90). To label bacterial flagella, the *Burkholderia* sp SJ1 cells were harvested by centrifugation at 5,000×g for 4 min at 25°C, resuspended in 1 ml of 0.1M sodium phosphate

buffer (pH 7.8) containing Cy3-NHS-ester (GE Healthcare), and incubated for 10 min at room temperature. After labeling, the excess fluorescent dye was removed by washing twice with PBS, and fluorescence-labeled cells were resuspended in YG medium. Viscous agents including Ficoll PM 400 (F4375, Sigma Aldrich) and methylcellulose (M0512, Sigma Aldrich) were infused into the flow chamber containing the bacterial cells, and the motility of the *Burkholderia* sp. SJ1 was observed under a total reflection fluorescence microscope (IX71; Olympus).

statistical analysis

All statistical analyses were performed by GraphPad Prism 9.1.0 (GraphPad Software) and R ver 4.1.3 (174). The statistical significance of symbiont's titer within symbiotic organs, CFU of M4B, and each stress-sensitivity test were calculated using the Mann-Whitney *U* test with Bonferroni correction, and the statistical significance of melanization rates and proportion of individuals with *Burkholderia* sp. SJ1 in hemolymph was determined using Fisher's exact test. The statistical significance of the survival rate was analyzed using the Log-rank test with Bonferroni correction. To estimate the $LD_{50,}$ the results of oral administration were evaluated by probit analysis using the statistical program R. The CI values were statistically evaluated by the 1-sample t-test against CI = 1.0.

Result

Symbiotic organ-invading pathogen kills the bean bug

During infection tests of newly isolated bacteria belonging to *Burkholderia* s.l. group to the bean bug, I observed one species named *Burkholderia* sp. SJ1, which is isolated from the midgut of the mulberry seed bug *P. dissimilis*, fully colonizes the symbiotic organ of the bean bug. The infection rate of *Burkholderia* sp. SJ1 confirmed by a diagnostic PCR using

Burkholderia-specific primers was 100% (10/10). Phylogenetic analysis of the 16S rRNA sequences revealed that the *Burkholderia* sp. SJ1 is belonging to the *Burkholderia cepacia* complex (BCC) (Fig. 3-1).

Next, the efficiency of gut colonization was investigated by estimating the number of

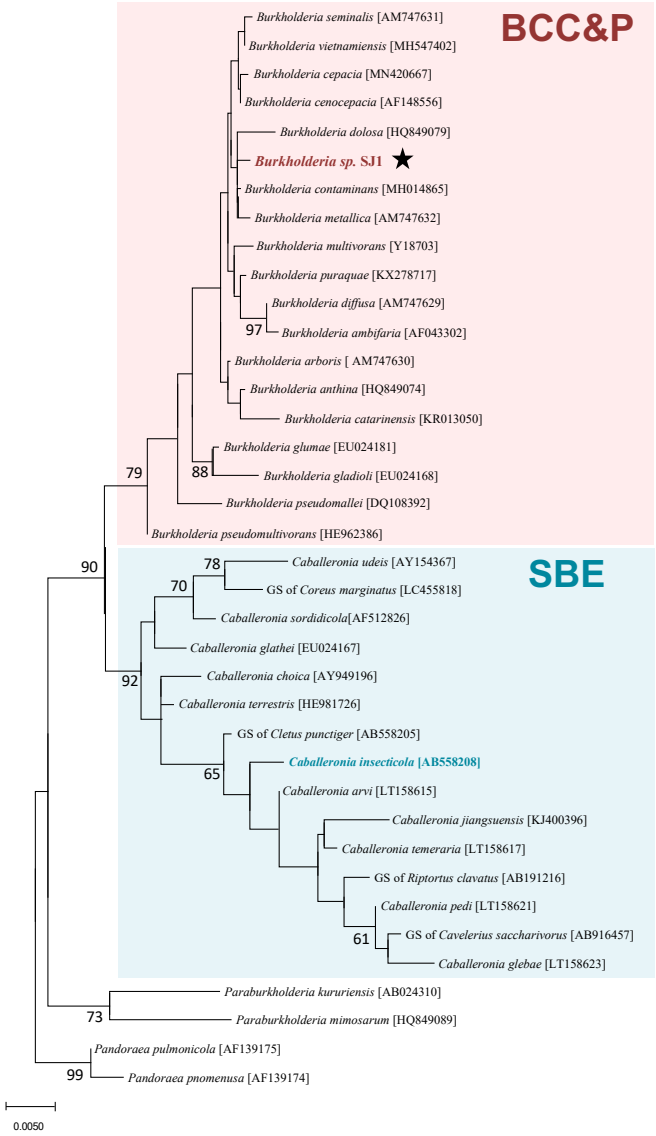

Figure 3-1. Phylogenetic placement of *Burkholderia* strains based on 16S rRNA gene sequences, shown by a maximum likelihood tree.

Stars indicate strains isolated from *P. dissimilis* and used for the infection experiment in this study. SBE, stinkbug-associated beneficial and environment group; BCC&P, *B. cepacia* complex and *B. pseudomallei* group; Bootstrap values higher than 60% are depicted at the nodes. *Pandoraea pulmonicola* AF 139175 and *P. pnomenusa* AF139174 were used as an outgroup.

M4-colonizing bacteria (Fig. 3-2a). The gut symbiont *C. insecticola* rapidly proliferated into around a million cells in the symbiotic organ within the third day after infection and the number of gut-colonizing cells was maintained until the seventh day (Fig. 3-2b). The number of *Burkholderia* sp. SJ1 also increased in the symbiotic organ after oral infection but was significantly lower than that of *C. insecticola* symbiont until the sixth day after inoculation (Fig. 3-2b). However, the *Burkholderia* sp. SJ1 cells reached a similar number of symbiont cells on the seventh day after infection (Fig. 3-2b). These results indicate that the non-native symbiont, *Burkholderia* sp. SJ1, colonizes the symbiotic organs of *R. pedestris* swiftly enough, but the proliferation rate or adaptation ability in the symbiotic organ is slightly lower than the native gut symbiont.

Pathogenicity of gut-invaded *Burkholderia* sp. SJ1

I observed a phenomenon during the infection test that the gut-colonizing *Burkholderia* sp. SJ1 kills the bean bugs. To identify the pathogenicity of *Burkholderia* sp. SJ1, various concentrations of cultured *Burkholderia* sp. SJ1 cells were orally fed to the bean bugs and the mortality of insects was measured. Infection of fewer than 10,000 cells didn't show any insecticidal activity against the bean bugs as equal to the DWA-fed insects (Fig. 3-2c). However, the bean bugs rapidly succumb to more than 10^5 cells of *Burkholderia* sp. SJ1, resulting in most insects dying within 10 days after oral infection (Fig. 3-2c). LD50 of *Burkholderia* sp. SJ1 to kill the bean bugs was $10^{3.5}$ (3,162) cells, underpinning the potent pathogenicity of gut-invaded *Burkholderia* sp. SJ1 (Fig. 3-2d).

In addition, observation of the symbiotic organs infected by *Burkholderia* sp. SJ1 by a stereomicroscope showed gut melanization, which generally occurred as an immune response of insects (Fig. 3-3a). Melanization of the symbiotic organ never happens in insects infected

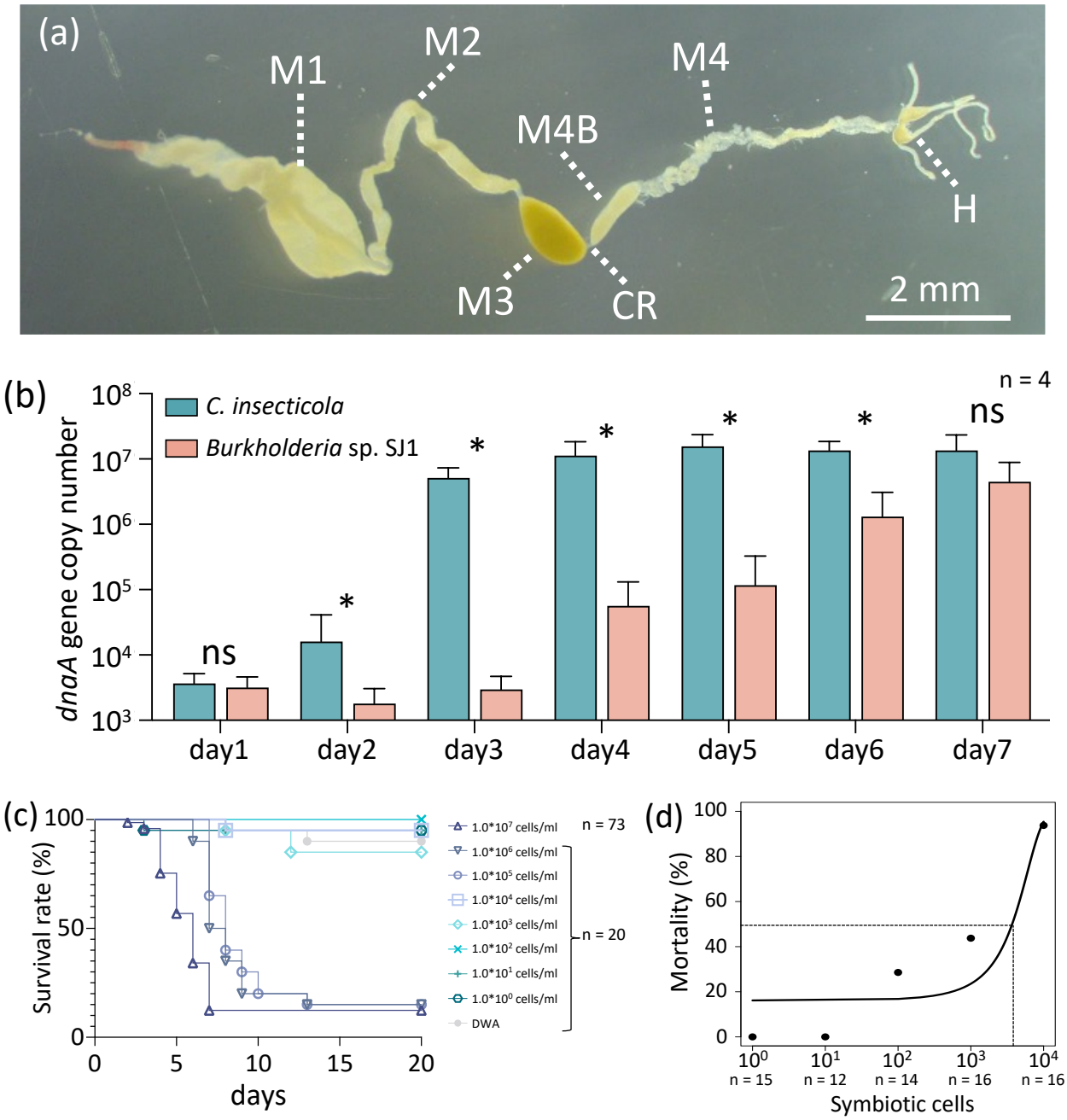

Figure 3-2. Fitness effect and colonization in the symbiotic organ of *Burkholderia* sp. SJ1 isolated from *P. dissimilis* in *R. pedestris.*

(a) Dissected gut of *R. pedestris* infected with *Burkholderia* sp. SJ1: M1, midgut first section; M2, midgut second section; M3, midgut third section; M4B, the bulbous region prior to M4; M4, midgut fourth section. (b) Titer of gut-colonizing *Burkholderia* sp. SJ1 measured by qPCR. The statistical significance was analyzed by the Mann-Whitney U test. ns, non-significant. n = 4 insects were used. (c) The survival rate of *R. pedestris* after fed with *Burkholderia* isolated from *P. dissimilis* several concentrations. (d) LD_{50} of *Burkholderia* sp. SJ1 to *R. pedestris.* In the oral administration experiments, second-instar nymphs were fed with different numbers of *Burkholderia* sp. SJ1 cells. The LD50 indicated by the dotted lines, was determined using the equation probit by R version 4.1.3.

by the gut symbiont *C. insecticola*, while it occurred in the symbiotic organ of 80% of individuals (16/20) infected with *Burkholderia* sp. SJ1 (Figs. 3-3a and b). However, gut melanization didn't occur in the non-symbiotic organs (M1, M2, and M3) of the bean bugs infected with *Burkholderia* sp. SJ1 cells (data not shown), indicating that the melanization is M4-specific phenomenon. The laser scanning confocal microscopic images of the symbiotic organ clearly confirmed the presence of gut melanization in the symbiotic organ and the melanized region of the midgut crypt lost its intact sac-like morphology, showing that the crypt cells are crumbling down into the luminal side (Fig. 3-3c). The *C. insecticola* is an ectosymbiont which colonizes the gut lumen and thus it can't enter the host's cells (Fig. 3-4d). In contrast, the *Burkholderia* sp. SJ1 cells were detected in the midgut epithelium and even inside of the host cells, suggesting the gut-invaded *Burkholderia* sp. SJ1 can penetrate the gut layer of the symbiotic organ after colonization and could migrate to the hemolymph of the bean bug despite the melanization, which occurred as an immune response (Fig. 3-3d). Indeed, the *Burkholderia* sp. SJ1 cells were detected in the hemolymph from 66.7% of alive bean bugs (4/6) after 7 days of infection, whereas no *C. insecticola* cells were observed in the hemolymph (Fig. 3-3e).

Since the gut-invaded *Burkholderia* sp. SJ1 efficiently kills the bean bug with migrating to the hemolymph, the pathogenicity of *Burkholderia* sp. SJ1 upon systemic infection was measured by injecting various concentrations of *Burkholderia* sp. SJ1 cells into the hemocoel. The *Burkholderia* sp. SJ1 cells showed intense virulence in the bean bugs that injection of only 10 cells killed 60% of the bean bugs within 6 days, and moreover, all insect individuals succumbed if a higher dose of *Burkholderia* sp. SJ1 cells were injected (Fig. 3-3f), underpinning that a few bacterial cells migrated from the midgut after oral infection are enough to kill the bean bugs.

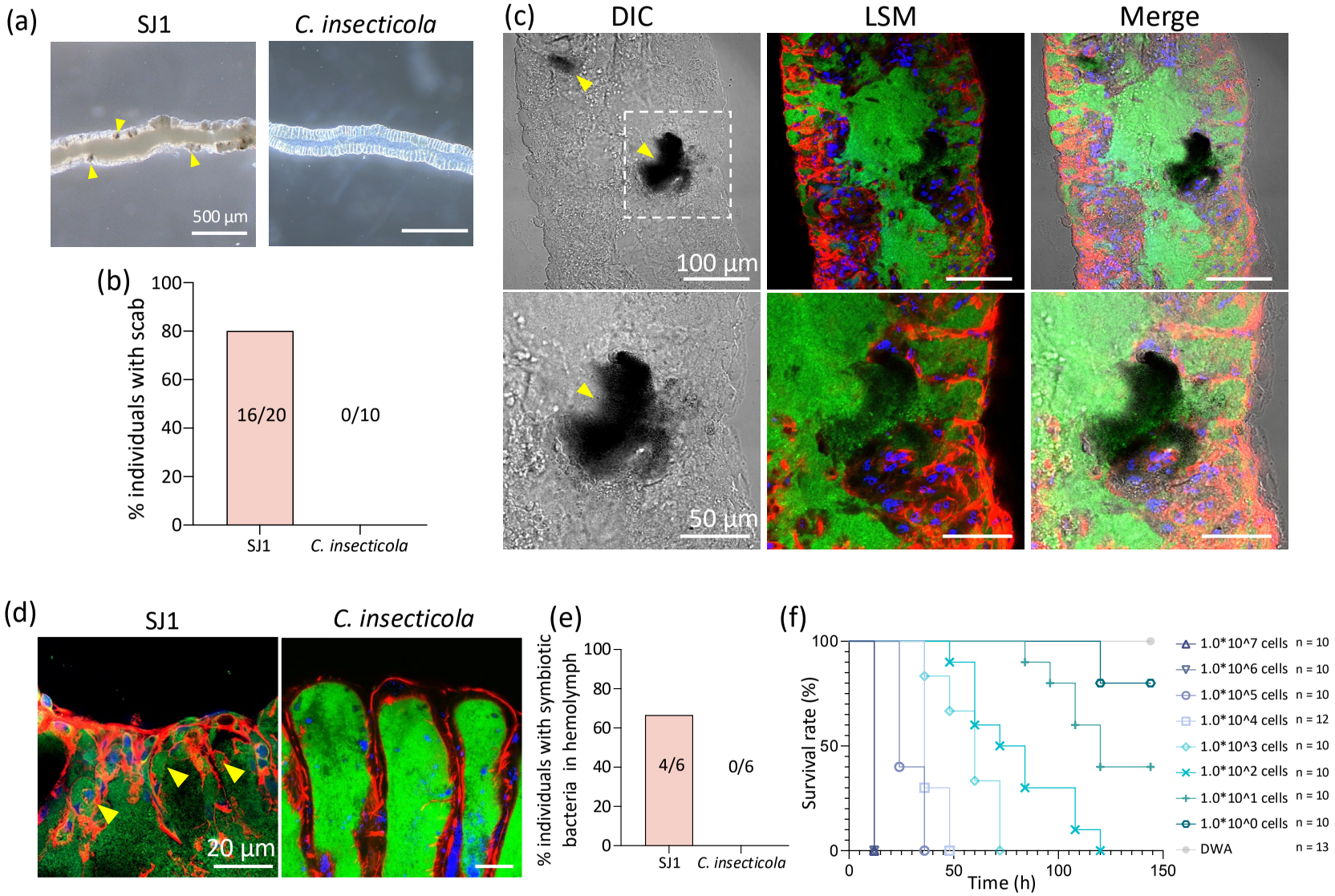

Figure 3-3. Symbiotic organ of *R. pedestris* infected with *Burkholderia* sp. SJ1.

(a) Dissected symbiotic organ of *R. pedestris* infected with SJ1 or *C. insecticola*. (b) Melanization rate of the symbiotic organ of *R. pedestris* (c) Confocal microscopy images of symbiotic organ infected with SJ1. Yellow arrows mean Melanization area. (d) Confocal image of a symbiotic organ infected by SJ1. Yellow arrows indicate host cells that SJ1 has entered. The white dotted line indicates the enlarged area. (e) Percentage of individuals with SJ1 detected in hemolymph. (f) The survival rate of *R. pedestris* injected *Burkholderia* sp. SJ1 at several concentrations. The statistical significance of Melanization rate and proportion of individuals with *Burkholderia* sp. SJ1 present in hemolymph were analyzed using Fisher's exact test

Symbiont-like motility of *Burkholderia* sp. SJ1

Burkholderia sp. SJ1 has to pass the CR to colonize the symbiotic organ followed by killing the insects. The GFP-labelled *Burkholderia* sp. SJ1 cells were orally administrated to the second instar nymphs of the bean bugs and the CR-M4B regions were observed under an epifluorescence microscope 2 hours after bacterial infection. A few *Burkholderia* sp. SJ1 cells were already passing the M4B part and migrating toward the symbiotic organ (Figs. 3-4a and b). Interestingly, *Burkholderia* sp. SJ1 has similar swimming motility with the native symbiont of the bean bug, *C. insecticola*, that wraps its flagella around the cell body and shows a screw-like motion (Fig. 3-4c). This symbiont-like motility could be a key factor for the *Burkholderia* sp. SJ1 to invade the CR.

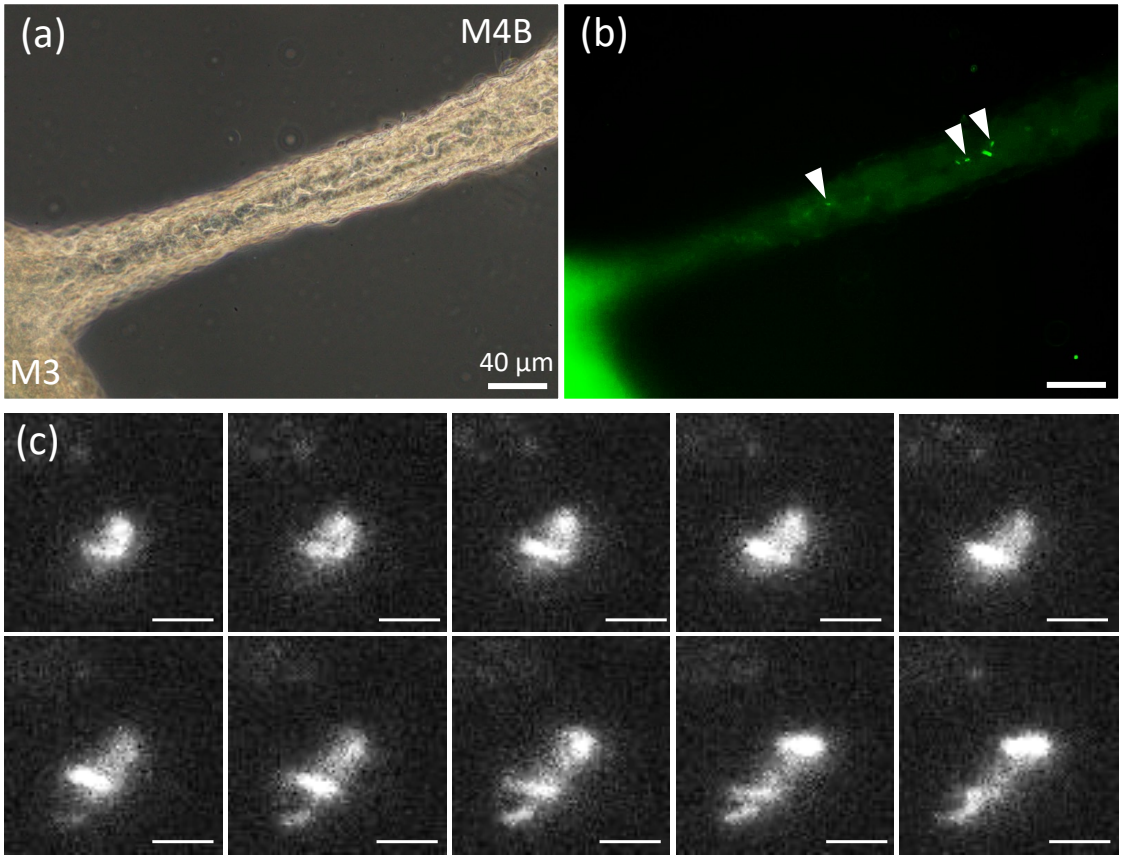

Figure 3-4. Observation of *Burkholderia sp.* SJ1's motility.

(a, b) *Burkholderia* sp. SJ1 passes through the CR section. White arrows indicate GFP-labeled *Burkholderia* sp. SJ1. (c) Wrapping motion of *Burkholderia* sp. SJ1. Sequential fluorescent images of a wrapping motion in a free-swimming state at 25 ms-intervals. The tip of the flagella (hook) was located at the top of the cell body. The flagellar wave propagated in a direction from the top end to bottom end of the cell body. Scale bar is 2 μm

Resistance of *Burkholderia* sp. SJ1 to host immune products

When M4B was observed under laser scanning confocal microscopy, *C. insecticola* was present within M4B at low densities (Figure 3-5a). However, *Burkholderia* sp. SJ1 was observed in M4B with a large number of cells maintaining their shape (Figure 3-5a). In addition, colonies of about $10^{5\sim6}$ CFU grew on the medium spread with M4B infected with *Burkholderia* sp. SJ1, while *C. insecticola* grew only a significantly smaller number of colonies, about 10^2 CFU (Figure 3-5b). These results indicate that *Burkholderia* sp. SJ1 is able to survive in the host's digestive organs to a higher degree compared to *C. insecticola*.

In addition, while *C. insecticola* was barely survivable by all antimicrobial substances (M4B lysate, Polymyxin B, Bleomycin), *Burkholderia* sp. SJ1 was highly resistant to all antimicrobial substances (Figure 3-5c). In the case of M4B lysate and Polymyxin B, there were no surviving *C. insecticola*, whereas *Burkholderia* sp. SJ1 was not negatively affected (Figure 3-5c).

Co-infection of *C. insecticola* and *Burkholderia* sp. SJ1

When *C. insecticola* and *Burkholderia* sp. SJ1 were infected together with *R. pedestris*, the survival rate of the stink bugs was significantly lower (survival rate: 36%, 27/76) (Figure 3-6a) than the survival rate when only *C. insecticola* was infected (100% 20/20). However, the survival rate was significantly higher than when *Burkholderia* sp. SJ1 was fed alone (12%, 8/69) (Figure 3-6a). This indicates that the pathogenicity of *Burkholderia* sp. SJ1 is suppressed when *Burkholderia* sp. SJ1 is infected with *C. insecticola* at the same time. In addition, When *C. insecticola* was fed first and then *Burkholderia* sp. SJ1, the survival rate of R. pedestris was significantly higher (77%, 23/30) than when DW was fed followed by *Burkholderia* sp. SJ1 (20%, 6/30) (Figure 3-6a).

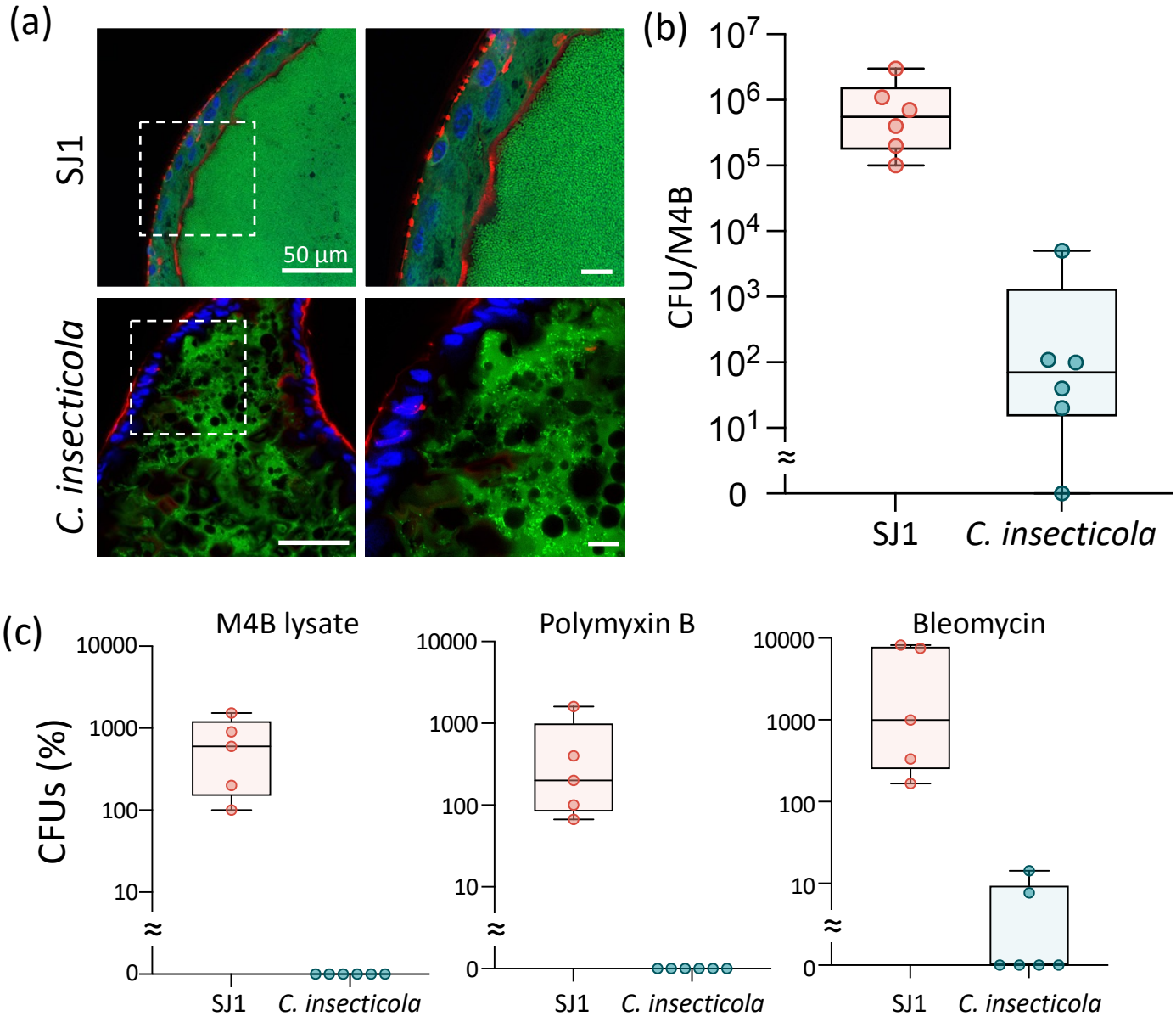

Figure 3-5. Resistance of *Burkholderia* sp. SJ1 and *C. insecticola* to immunity and digestive substances.

(a) Confocal microscopy images of M4B infected with *Burkholderia* sp. SJ1 and *C. insecticola*. Scale bar is 50 μm. (b) Comparison of the CFU of M4B between *Burkholderia* sp. SJ1 and *C. insecticola*. (c) The specific antimicrobial activity of M4B lysate(10 μg/ml), Polymyxin B(12.5 μg/ml) and Bleomycin(5μg/ml) against the symbiotic *Burkholderia* sp. SJ1 and *C. insecticola*. Cell suspensions of the *Burkholderia* sp. SJ1 and *C. insecticola* symbiont were subjected to CFU assays after incubation with M4B lysate samples.

The competitiveness between *C. insecticola* and *Burkholderia* sp. SJ1

In the case of the in vitro competition assay, there was no significant difference in competitive ability between *Burkholderia* sp. SJ1 and *C. insecticola* (Figure 3-6b). On the other hand, in vivo, *C. insecticola* was dominant in M4 of individuals that were alive after 3 days, and *Burkholderia* sp. SJ1 was present in only a very small percentage. However, in M4 of individuals that died on day 3, *Burkholderia* sp. SJ1 and *C. insecticola* were present in equal proportions (Figure 3-6b).

priority effect in symbiotic organ

In cases where *R. pedestris* was first infected with *C. insecticola* and then fed *Burkholderia* sp. SJ1, the survival rate of *R. pedestris* recovered significantly (about 76.7%, 23/30) compared to *Burkholderia* sp. SJ1 only (20%, 6/30) and was not significantly different than when only *C. insecticola* was fed (100%, 20/20) (Figure 3-6c). This indicates that *Burkholderia* sp. SJ1 cannot be pathogenic to the host if *C. insecticola* first colonizes the symbiotic organ.

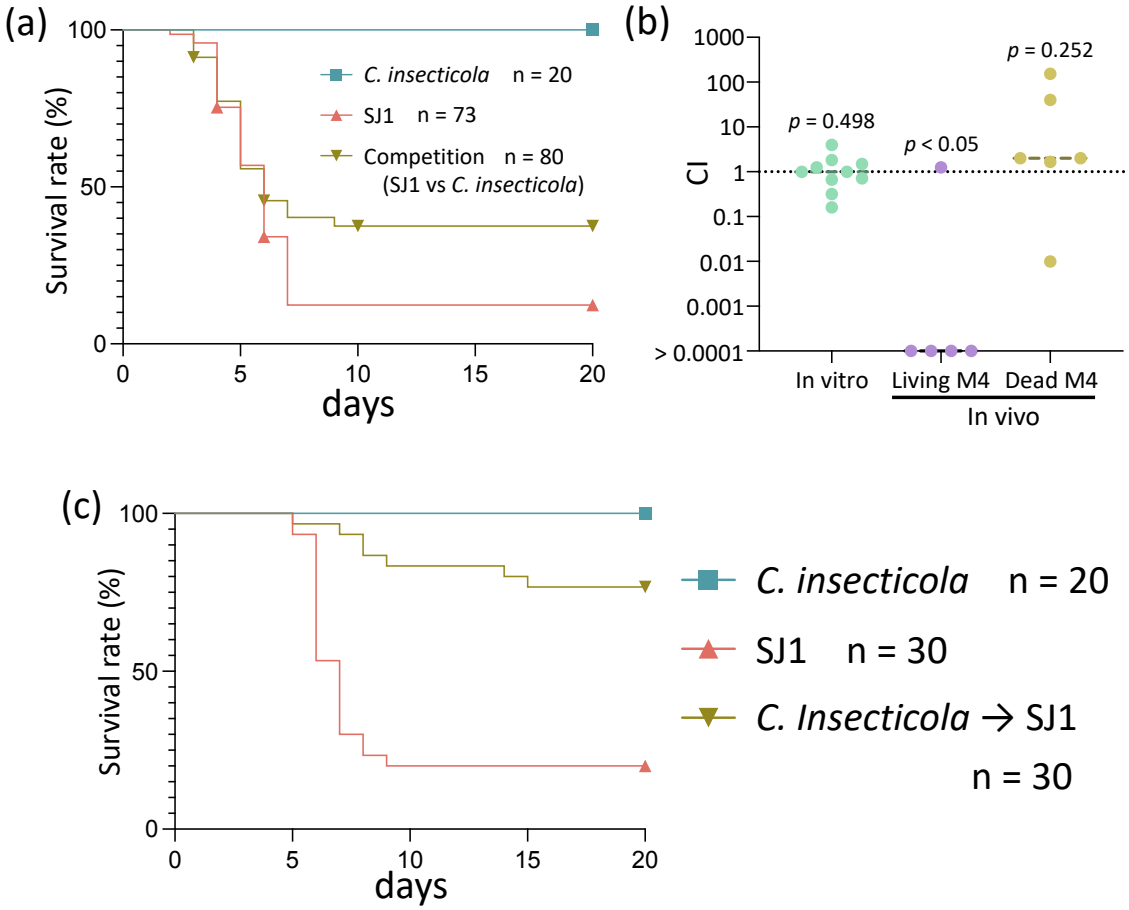

Figure 3-6. Competitive and priority effect between *Burkholderia* sp. SJ1 and natural symbiont, *C. insecticola*.

(a) Survival rate of *R. pedestris* fed with *C. insecticola, Burkholderia* sp. SJ1 or Co-infection. The survival rates were analyzed using the Log-rank test with Bonferroni correction. (b) Competitiveness *Burkholderia* sp. SJ1 against *C. insecticola* in vitro growth and in vivo. CI value from a microtube or an insect. The CI values were obtained by (output *Burkholderia* sp. SJ1 CFU/input *Burkholderia* sp. SJ1 CFU)/(output *C. insecticola* CFU/input *C. insecticola* CFU) and statistically evaluated by the 1-sample t test(against CI = 1.0). (c) Survival rate of *R. pedestris* fed with firstly *C. insecticola,* then *Burkholderia* sp. SJ1. The survival rates were analyzed using the Log-rank test with Bonferroni correction.

Discussion

The Stinkbugs-*Caballeronia* symbiosis has been studied for more than a decade, however, no symbiont has been identified that is harmful to the host. Most of the *R. pedestris* that were fed *Burkholderia* sp. SJ1 died within 10 days (Figure 3-2c). how is *Burkholderia* sp. SJ1 killing its host? *Burkholderia* sp. SJ1 eventually proliferated in the symbiotic organ to the same population size as the native symbiont *C. insecticola* (Figure 3-2b), but the mode of proliferation in the symbiotic organ was different from *C. insecticola*. The crypts infected by *Burkholderia* sp. SJ1 were very swollen, and the bacteria were observed to invade into the host cells (Figure 3-3). The presence of numerous scabs (melanization) in the symbiotic organ and the detection of *Burkholderia* sp. SJ1 in the hemolymph of the host suggest that *Burkholderia* sp. SJ1 grows violently in the symbiotic organ of the host, invades the hemolymph of the host, and causes septic shock in the host (Figure 3-3). In addition, because *Pandoraea*, an outgroup of *Burkholderia* and *Caballeronia*, can infect *R. pedestris* and also benefit the host (94), the *Burkholderia* sp. SJ1 is considered to have evolved a harmful trait from a beneficial strain.

Usually, mechanisms to prevent cheaters are important for the stable evolution of mutualism (96, 97, 161, 166, 175). Theoretically, there are two main mechanisms to prevent cheaters: partner choice and partner fidelity feedback (96, 97). In the *R. pedestris*-*Caballeronia* symbiosis, three mechanisms have been studied to prevent cheaters, including CR (86), which selects bacteria by their motility, AMPs highly expressed in the symbiotic organ (87, 88), and selection by competition among bacteria (94). Then, how does *Burkholderia* sp. SJ1 evade the mechanisms of the host? The first obstacle is a very narrow, viscous material-filled organ called the CR, located in the anterior part of the symbiotic organ (86). *Burkholderia* sp. SJ1 infects the symbiotic organ by passing through the CR, and also possesses the screw-like motion to move by wrapping its flagellum around its body, which is important for passing through the CR (90) (Figure 3-4). In addition, *Burkholderia* sp SJ1 was highly resistant to AMPs, and many

bacteria survived in M4B, where digestive enzymes and AMPs are highly expressed (87, 88) (Figure 3-5). Finally, after colonization of the symbiotic organ, *R. pedestris*-*Caballeronia* specificity is maintained by competition among other symbionts infected at the same time (94), but *Burkholderia* sp. SJ1 is not overcome by competition in the symbiotic organ with the native symbiont, *C. insecticola* (Figure 3-6). These results indicate that Burkholderia sp. SJ1 has the ability to evade the partner choice of the host. AMPs produced by the host are also used to manage symbiotic bacteria, legumes use NCRs to transform rhizobia into nitrogen-fixing specialized form, bacteroid (176, 177), and squid use galaxin to control *Vibrio* symbionts growth (27). *Burkholderia* sp. SJ1 grows aberrantly in symbiotic organs and is highly resistant to AMPs, suggesting that *Burkholderia* sp. SJ1 escapes the host's control.

In considering the evolution of mutualism, the occurrence of selfishly behaving symbionts called "cheaters" is very important (96, 97). However, in recent years, it has been pointed out that most of the reports of cheaters are only low-quality symbionts, and very few of them are net/serious cost to the host (163). Moreover, no harmful cheater, such as *Burkholderia* sp. SJ1 had been found that kills the host, even though the cheater invades and proliferates in the host's specific symbiotic organs (163). One reason why a cheater that actively kills the host has not been found is that killing the host would mean abandoning any future benefits from cooperators (96). Theoretical studies on parasite toxicity suggest that highly toxic parasites killing their hosts gradually decrease and parasites that do not kill their hosts increase (178–180). What is the reason for the emergence of a lethal pathogen such as *Burkholderia* sp. SJ1 in the *R. pedestris*-*Caballeronia* symbiosis? One possible reason for the occurrence of host-killing cheaters is the intensity of the host's exploitation against the symbionts. Theoretically, as exploitation becomes stronger, sanctions and punishments against the exploiter tend to become severe, and eventually, the selfish partner is rejected or eliminated (96, 97, 163, 181). Therefore, considering the *R. pedestris*-*Caballeronia* symbiosis from a

symbiotic bacteria, the *Caballeronia* symbiont grows in M4 and flows back into M4B, where the symbiont cells are digested and absorbed by the host (88, 91, 172). Furthermore, in *R. pedestris*-*Caballeronia* symbiosis, the symbiont is not expelled from the host during the lifetime of the host (83), thus, from the symbiont side, the host is a dead end, and the net benefit of the symbiont may be very low. Conversely, legumes do not digest rhizobia directly, but only exchange nutrients within the nodules (2, 13), and reproductive rhizobia are released from the nodules into the environment (10, 104). Furthermore, in squid-*Vibrio* symbiosis, the only cost paid by the vibrio is luminescence, which is not digested. In addition, the squid daily releases a part of the population of *Vibrio* symbiotes grown in the symbiotic organ into the seawater (4, 10, 23, 182, 183). Therefore, from the symbiont side, *R. pedestris* is likely the most exploitative host and does not seem to consistently provide benefits to the symbiont. Thus, speculatively, the very high virulence of *Burkholderia* sp. SJ1 against insect hosts may have evolved as an antagonistic response against a strongly exploitative host.

Chapter 4

General discussion

In this study, I elucidated that *P. dissimilis* possess symbiotic organs colonized by *Caballeronia*, and the *Caballeronia* symbiont is obligate and gives beneficial effects to *P. dissimilis*. I also revealed that *Burkholderia* sp. SJ1 isolated from *P. dissimilis* is a lethal pathogen and has the ability to evade the host's sorting mechanism and colonized the host symbiotic organ.

In Chapter 2, I showed that *P. dissimilis* harbors *Caballeronia* in the gut and the symbionts enhance the growth of the host and are essential for survival. Based on 16S rRNA gene amplicon sequencing of *P. dissimilis* symbiotic organs collected at 10 populations in Hokkaido, I found that the *Caballeronia* were dominated in the symbiotic organ. In addition, I demonstrated that the symbiotic bacteria are absent in the eggs and that newly hatched nymphs do not possess the symbiotic bacteria but acquire them from the soil. These findings indicate that this symbiotic system, like other stinkbugs-*Caballeronia* symbioses, is maintained by horizontal symbiont transmission. The results of infection and rearing experiments with symbiotic bacteria indicate that symbiotic bacteria are important for the normal development of host body coloration. This suggests that, like in the weevil-*Nardonera* symbiosis (38), the *Caballeronia* symbiont could supply tyrosine which is important for the development of cuticle in insects. Gut symbioses in the Lygaeoidea have been only investigated histologically and taxonomically due to the lack of a useful model system, compared with the Coreoidea wherein the bean bug *R. pedestris* has been well studied. While *R. pedestris* survives and shows normal body color without *Caballeronia*, aposymbiotic *P. dissimilis* suffers severe mortality and abnormal body color. Based on these findings, *P. dissimilis* would be a useful model system of the Lygaeoidea to clarify the functional differences of *Caballeronia* symbionts between Lygaeoidea and Coreoidea stinkbug hosts.

In Chapter 3, I discovered that *Burkholderia* sp. SJ1 colonizes the gut symbiotic organ and ultimately kills the bean bug host. This lethal pathogen colonized the symbiotic

organs by evading the partner choice mechanisms of *R. pedestris*, including the CR, immunity (ex. antimicrobial peptides), and *in vivo* environment enhancing microbe-microbe competition. Once it reached the gut symbiotic organ, *Burkholderia* sp. SJ1 explosively proliferated in the symbiotic organ, invaded into the hemolymph, and eventually killed the host by septic shock. Although various *Burkholderia* sensu lato bacteria have been examined for their ability to infect *R. pedestris* (94), no bacteria like *Burkholderia* sp. SJ1 have been found.

What are the abilities required to hijack the symbiosis and kill the host? The first key ability is motility through the very narrow and mucous-filled CR of *R. pedestris* (86). In order to pass through the CR and reach the symbiotic organ, the symbiotic bacteria need a screw-like motion that wraps the flagella around their body (90), and *Burkholderia* sp. SJ1 has the drill motility. Second, because the symbiotic organs of *R. pedestris* highly express AMPs and digestive enzymes (87, 92), resistance to these host immunities also is critical. *Burkholderia* sp. SJ1 was resistant to AMP and M4B lysate, which exhibits antimicrobial activity (88), suggesting that *Burkholderia* sp. SJ1 can resist host immunity. Finally, competitiveness in the symbiotic organs is also important. In the symbiotic organ of *R. pedestris*, competition occurs among the arriving symbiotic bacteria, and the non-native symbionts, *Paraburkholderia* and *Pandoraeai*, eventually are eliminated from the symbiotic organ by losing this competition with native symbionts, *Caballeronia* (94). *Burkholderia* sp. SJ1 is able to grow in the symbiotic organ as well as *Caballeronia* and is not outcompeted by competition with *Caballeronia*. Taken together, specialized motility to infect the symbiotic organ, resistance to the host's immunity, and competitiveness in the symbiotic organ are important for the ability to evade the host's symbiote selection mechanism and hijack the symbiosis.

Theoretically, the evolution of mutualism often involves the emergence of "cheaters", which avoid their costs but receive the benefits (96, 98, 160, 163–165, 184), and

in fact, such cheaters have been reported in diverse mutualistic systems including the well-developed legume-rhizobium symbiosis (165). Although there is a great number of studies on cheaters and the emergence of cheaters in mutualism is thought to be natural, it has recently been noted that cheaters with net/serious costs to their hosts are actually very rare (163, 181). Furthermore, there is no report of the existence of cheaters that kill their hosts while colonizing and proliferating in symbiotic organs (163, 181). One of the reasons that such cheaters have not been found is because killing hosts means abandoning the benefits from the cooperator in the future (96). In addition, the hosts can invoke countermeasures against cheaters, such as imposing sanction/punishment or changing the symbiotic partner (96, 97). Theoretical studies on the virulence of parasites indicate that parasites with high virulence killing the host gradually decrease and parasites not killing the host increase in host populations (178–180, 185). In summary, the existence of cheaters that cost the host is itself very rare, and cheaters that actively kill the host are very unlikely to emerge, theoretically and realistically.

Then, why does the lethal pathogen, *Burkholderia* sp. SJ1 emerges in the stinkbug gut symbiosis? One of the possible factors in the emergence of cheaters that kill the host is the intensity of exploitation of the host against symbionts. Theoretically, the stronger exploitation of the symbiotic partner, sanctions or punishments against the partner tend to be more severe, and finally, the victims reject or eliminate the selfish partner (96, 97, 178, 179). Although these points are generally discussed from the host side, here I would like to note that the same logic can also be applied in symbionts. *Caballeronia* symbionts proliferate in M4 and flow back into M4B, wherein symbiont cells are digested and their nutrients are absorbed by the host (88, 91, 172). Such a type of symbiosis is called “cultivation symbiosis” (91, 94). To manage symbiont population in the gut, host insects express AMPs and digestive enzymes specifically in symbiotic organs, M4 and M4B (87). Indeed, *in vivo* cells of *C.*

insecticola are swollen in shape compared to *in vitro* ones (91, 186), the cell membrane is altered (91), and the M4B lysate shows antimicrobial and digestive activities more to *in vivo* cells than *in vitro* cells of *C. insecticola* (88). Additionally, in *R. pedestris*-*Caballeronia* symbiosis, the symbionts are not expelled from the host during the lifetime of the host (83). Therefore, although there is a possibility that the symbiont can escape after the death of the host, from the symbiont side, the host would be a dead end and the net benefits of symbionts could be very low. On the other hand, legumes do not directly digest rhizobia, but only exchange the nutritional substances within the nodule (2, 13), and the rhizobia with the ability to reproduce are released from the nodules into the environment (10). Additionally, in the squid-*Vibrio* symbiosis, the cost paid by the *Vibrio* is only luminescence, not being digested. Moreover, the squids release part of the population of the *Vibrio* symbionts grown in the symbiotic organs into the seawater every day (4, 10, 23, 182, 187). Considering those symbioses from the view of the symbiont, *R. pedestris* is the most exploitative host and tames their symbionts. Therefore, although speculative, the very high virulence of *Burkholderia* sp. SJ1 against the insect host was probably evolved as an antagonistic response to the strongly exploitative host. Indeed, the high resistance of *Burkholderia* sp. SJ1 to M4B lysate and AMPs, and its survival in M4B, strongly suggests that *Burkholderia* sp. SJ1 resists the host's management.

Differences in the host's management intensity against symbionts may also affect the emergence of cheaters. The legume-rhizobium symbiosis is an intracellular symbiosis, in which rhizobia are transformed into a specialized form called bacteroid within the host cells (20, 104). In this process, the NCRs (nodule-specific cysteine-rich peptides) produced by the plant host promote the rhizobia to become the bacteroid, a cell-state specialized for dinitrogen fixation (20, 188, 189). In the *R. pedestris*-*Caballeronia* symbiosis, although the crypt-specific cysteine-rich peptides (CCR) are expressed (87), *Caballeronia* does not change like

rhizobia, but only alters its cell surface to make it easier to digest (91, 186). Therefore, compared to legume-rhizobia symbiosis, the *Caballeronia* symbionts may be less controlled by the *R. pedestris*, allowing the symbionts to "counter-punishment" more easily.

On the other hand, why are docile strains against exploitative hosts maintained in the *Riptortus-Caballeronia* system? A possible reason why is likely that the intensity of exploitation differs among host stinkbug species. In an environment where beneficial hosts are more common than exploitative hosts, symbionts likely gain more benefit by being docile rather than antagonistic to their hosts. In legumes, the degree of exploitation against rhizobia differs among host species (104, 167). Considering that a very wide variety of stinkbugs coexist with *Caballeronia*, the intensity of host exploitation of *Caballeronia* likely is diverse (66). Indeed, the chinch bug, *Cavelerius saccharivorus* (Lygaeoidea), harbors *Caballeronia* symbionts in the midgut and partially transmits the symbionts to the next generation via eggs (69). In the case of vertical transmission, with the increase in the host population, the symbiotic bacteria also increase and establish a symbiotic relationship with the same host strain, thus the symbiotic bacteria evolve cooperative traits (190). Theoretical studies also indicated that even an exploitative relationship can evolve into a mutualism if the host vertically transmits the symbiotic bacteria in part (100, 191). Therefore, the degree of exploitation of *C. saccharivorus* is considered to be lower than *R. pedestris* which does not transmit any symbiont to the next generation, and it is likely that the presence of such beneficial hosts allows docile lineages to be maintained.

In addition, theoretical studies have indicated that cheaters and symbionts can coexist if the host can provide an environment in which the symbiont has a competitive advantage over the cheater (192–194). In legume-rhizobium symbiosis which has been confirmed the presence of cheaters (165), the hosts sanction cheaters and provide a favorable environment for the beneficial symbionts to compete with cheaters (167, 169, 184). Co-

infection of *Burkholderia* sp. SJ1 and *C. insecticola* with *Metochus uniguttatus*, a member of Lygaeoidea same as *C. saccharivorus* and *P. dissimilis*, resulted in a recovery of host survival to the same level as when only *C. insecticola* was infected (unpublished data). Therefore, the mechanism is not firmly clear, but certainly a countermeasure against *Burkholderia* sp. SJ1 is evolving in some stinkbug species, and the hosts may provide an environment that allows the beneficial symbionts to dominate competitively.

The host also evolves to avoid incorporating cheaters such as *Burkholderia* sp. SJ1. In the *R. pedestris*-*Caballeronia* symbiosis, *Pandoraea*, which belongs to the outgroup of *Burkholderia* sensu lato, is able to infect symbiotic organs and is also beneficial to the host (94). This suggests that the *R. pedestris*-*Caballeronia* symbiosis originally started with a beneficial partner for the host and that *Burkholderia* sp. SJ1 evolved from a beneficial partner in a detrimental direction. The presence of cheaters promotes further evolution in the host's partner choice mechanisms (96, 98, 160, 164, 167, 184), and taking into consideration the inability of many *Burkholderia* to infect symbiotic organ of *R. pedestris*, evolutionary arms race exists between the sorting mechanisms of *R. pedestris* and the *Burkholderia.* In addition, the host of cultivation symbiosis has a self-contradiction: the host wants the symbiote (food) to proliferate and does not want the symbiote to proliferate too much, uncontrollable by the host. Basically, in the host of a cultivation symbiosis, the symbiotic organ should be maintained in an environment that allows the symbionts to increase since the growth of the symbiont is the same as an increase in food for the host. However, there is a risk that cheaters such as *Burkholderia* sp. SJ1 enter and colonize, and thus, the hosts need to sort out symbionts at the previous region of the symbiotic organ. Indeed, the selective exclusion of *Burkholderia*, which would be detrimental, occurs mainly in the CR prior to the symbiotic organ (94). Moreover, salivary gland secretions of *R. pedestris* contain AMPs (170),

suggesting that the secretions may assist in the preliminary sorting of symbionts before the CR.

When cheaters emerge, depending on the condition, the hosts may evolve to abandon the symbiotic relationship (160, 184). If the cheaters dominate the environment, it would be advantageous for the host to abandon the symbiotic relationship and be independent. Since *R. pedestris* can survive without the *Caballeronia*, the evolution toward waiving the symbiotic relationship is not impossible, and in fact, Rhopalidae stinkbugs that belong to superfamily Coreoidea same as *R. pedestris* lost the gut symbiotic organ and the symbiotic association with *Burkholderia* sensu lato (Fig. 1-1). Perhaps the presence of the cheaters such as *Burkholderia* sp. SJ1 was important in the decision to abandon this symbiotic relationship. Moreover, in the stinkbugs-*Burkholderia* sensu lato symbiosis, there are several strains that abandoned the symbiotic relationship, transferred the symbiotic partner, or shifted to vertical transmission from the horizontal transmission, all of which may have been due to the presence of cheaters such as *Burkholderia* sp SJ1.

Burkholderia sp. SJ1 has the potential to impact the agricultural field. *R. pedestris* is notorious as a pest of soybeans. Insecticides have been used to control the pest, but overuse of insecticides promotes the emergence of insecticide-resistant insects. *R. pedestris* with the insecticide degrading *Caballeronia* have insecticide resistant (32, 84, 85). In addition, the application of insecticides to soil causes a rapid increase in the percentage of insecticide-degrading *Caballeronia* (153). Because *Burkholderia* sp. SJ1 is highly pathogenic to R. pedestris even when infected simultaneously with *Caballeronia*, *Burkholderia* sp. SJ1 has the potential to be used as a biopesticide against insecticide-resistant *R. pedestris*.

Taken together, during my doctoral course, I have revealed the ecological characteristics of a lethal bacteria that hijacks the symbiotic system by evading the symbiont partner sorting mechanism of *R. pedestris*. Despite the recognition of microorganisms as an

important force in eukaryotic evolution (195), recognition of the impact of eukaryotes on microbial evolution has lagged behind (196). Interspecific interactions between microorganisms and eukaryotic hosts are continuous, ranging from parasitism to mutualism. To understand how these interactions evolve, it is necessary to consider perspectives on the symbiosis from both partners. Although it has long been recognized that symbionts must be more thoroughly investigated (197–199), the microbial perspective has been mostly ignored in symbiotic systems that are considered mutualism. The discovery of a lethal pathogen in mutualisms, *Burkholderia* sp. SJ1 brings new perspectives from microorganisms and contributes to the understanding of the evolution of symbiosis.

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