



HOKKAIDO UNIVERSITY

Title	Elucidation of molecular bases underpinning insect-bacteria gut symbiosis
Author(s)	ジャン, ソンハン
Degree Grantor	北海道大学
Degree Name	博士(農学)
Dissertation Number	甲第14382号
Issue Date	2021-03-25
DOI	https://doi.org/10.14943/doctoral.k14382
Doc URL	https://hdl.handle.net/2115/81098
Type	doctoral thesis
File Information	Jang_Seonghan.pdf



Doctoral Thesis

Elucidation of molecular bases underpinning insect-bacteria gut symbiosis

(昆虫と細菌の腸内共生を支える分子基盤の解明)

March 2021

Division of Applied Bioscience
Graduate School of Agriculture
Hokkaido University

Seonghan JANG

TABLE OF CONTENTS

Table of contents	1
Chapter I. General introduction	2
Chapter II. Host–symbiont specificity determined by microbe–microbe competition in an insect gut.....	17
Abstract.....	18
Introduction	19
Materials and methods	31
Results and Discussion	37
Chapter III. Dual oxidase enables insect gut symbiosis by mediating respiratory network formation	68
Abstract.....	69
Introduction	70
Materials and methods	77
Results and Discussion	86
Chapter IV. General discussion.....	114
References	119
Acknowledgments	150

Chapter I

General introduction

Omnipresent microbial endosymbiosis

Many animals as well as plants have evolved to live together with microorganisms for a long time in diverse relationships including parasitic, commensal, and mutualistic associations (1). Especially, the mutualistic symbiosis between host organisms and microbial symbionts recently has gained recognition as one of the most important relationships in living organisms on the earth. In mutualism, both host and symbiont provide numerous essential or beneficial effects to each other. In plants, the major role of the microbial symbiont is a fixation of atmospheric nitrogen that converts stable nitrogen gas into biologically usable molecules such as ammonium and ammonia, which are an essential nutrient source to plants (2). Representatively, leguminous plants are forming a symbiotic association with symbiotic bacteria, *Rhizobium* species, in the nodules on the roots via a complex mechanism (3–5). The nodule-colonizing *Rhizobium* symbionts fix nitrogen gas, converting it into ammonia, which is then assimilated into nucleotides, amino acids, vitamins, hormones, and others, results in helping host growth and development (6, 7). In the case of animals, squid-*Vibrio* symbiosis is one of the most studied model systems. The Hawaiian bobtail squid, *Euprymna scolopes*, possesses glowing symbiotic bacteria *Vibrio fischeri* in the specialized location called light organ (8, 9). The *V. fischeri* allows the squid to produce light and, consequently, the nocturnal host can escape from the enemies by camouflage as the starlight or the moon (10). As well, insects harbor specific microbial symbionts in their compartment of the gut or specialized organ such as bacteriocyte (11, 12). The symbionts of insects provide tremendous benefits to the host, mainly supplying essential nutrients, which are scarce in the food source, by biosynthesis of them (13, 14). Moreover, it turns out that human gut

microbiota is also important to the host physiology such as absorption and decomposition of nutrients, thus directly linked to gut diseases (15–17). Recent studies have revealed that human gut microbiota even could affect hormone secretion and the nervous system (18–20). Taken together, microbial symbiosis is omnipresent from plant to human, thus research about the symbiotic association is important to understand the biological phenomenon of organisms on the earth.

Insect-microbe symbiosis

Insects are the most diverse group of organisms on the earth—over half of the estimated 1.5 million organism species described are classified as insects, and many of them have known to have symbiotic microbes in their body (1, 21). The role of insect symbionts has well-studied than any other model system due to its simplicity: lower diversity of symbiont than that of mammals or humans, and insect symbionts are living only in a specific host organ. Insect's symbiont confers plenty of physiological advantages to the host insects including the provision of the essential nutrients, enhancement of host immunity and fecundity, aiding digestion of the indigestible food ingredients, detoxifying insecticides and phytotoxins, and many others (22–26). In general, symbionts in the insect are vertically transmitted from mother to offspring via various routes, but some insects acquire symbionts horizontally from the environment (27–31).

Most endosymbiosis of insects has based on nutritional symbiosis that symbiont provides nutrient sources to the host (32). Pea aphid is one of the most famous and well-studied

model insects in nutritional symbiosis (24, 33). Pea aphid is a notorious pest to leguminous crops and harbors specific obligate intracellular symbiotic bacteria, *Buchnera*, in the specially evolved cells called “bacteriocyte” (12, 34, 35). *Buchnera* symbionts are vertically transmitted from mother insect to offspring directly during embryogenesis via transovarial infection (29). *Buchnera* mainly provides to host aphid the essential amino acids, which are scarce in plant’s phloem sap, thus symbiont is essential to host growth, fecundity, and even survival (24, 34, 36, 37). Indeed, symbiont-free aphids by artificial removal of *Buchnera* poorly grow and produce few or no offspring (24). On the contrary, aphid provides shelter to *Buchnera* symbiont. Since *Buchnera* has lived with the host aphid for a long time, they highly adapted in the environment of the host body, consequently lost a considerable number of genes that are unnecessary anymore, result in an extremely reduced genome, but retains genes for biosynthesis of amino acids (34, 38). *Buchnera* symbiont indeed has a small genome, 0.64 Mb in size, which lacks many important genes for free-living, and therefore unculturable *in vitro* (34). Besides, there are other many cases of nutritional symbiotic association in insects. Wood-feeding termite or cockroach harbors endosymbionts in the hindgut, and the diverse community of symbionts aid to digest food materials, which are difficult to degrade by the host itself such as lignocellulose, helping host survival and development (25, 39–42). The symbiotic bacteria of blood-feeders, including tsetse fly and lice, synthesize and provide B vitamins, which are scarce in the blood of vertebrates, to the host insects (43–45). All these nutritional symbioses are thus in principle vital to the host life.

Symbiotic bacteria have numerous different functions in addition to nutrient provision.

Besides primary symbiont *Buchnera*, several secondary symbionts (facultative symbionts) are occasionally infected in the bacteriocytes of pea aphid and confer several advantages to the host (46, 47). One of them, *Serratia symbiotica*, is the most frequent secondary symbiont and is mainly involved in a defensive symbiosis that protects aphids from adverse conditions such as high temperature and parasitoid wasps, but also known to provide nutrients (48–50). Another facultative symbiont of the aphid, genus *Rickettsiella*, changes the host's body color from red to green that influences prey-predator interaction (51). Besides, there are other secondary symbionts in aphids that play diverse roles. Weevils possess in the bacteriome γ -proteobacterial endosymbiont lineage *Nardonella*, which has an extremely reduced genome owing to long time adaptation in the host body (52, 53). Despite its small genome, *Nardonella* retains genes for the synthesis of tyrosine, which is the principal precursor of the hard cuticle of weevil (52, 54, 55). The elimination of endosymbiont by antibiotics treatment indeed caused reddish and soft cuticle of the host insect with low contents of tyrosine, demonstrating the importance of symbiotic bacteria in cuticle hardness (52). Symbiotic bacteria of beewolves and leafcutter ants produce an antibiotics cocktail and kill the parasitic or pathogenic microbes (56–59). In some insects, such as rove beetle and antlion, symbiotic bacteria produce toxins, which are utilized by host insects to capture prey (60, 61). Besides these functions, symbionts of insects possess even more diverse roles and are expected to be countless.

Endosymbiosis in stinkbugs

Stinkbugs, which are insects belonging to Heteroptera, consist of more than 38,000

species that are the largest groups of insects in the hemimetabolous insect (62). Their ecological traits including feeding types are enormously diverse. Among them, the infraorder Pentatomomorphan stinkbug families feeding plant sap or seed have revealed to possess symbiotic bacteria that mainly compensate for imbalanced nutritional sources of the foods to the host insects by biosynthesizing nutrients, but also symbionts provide numerous other benefits to host stinkbugs (11). Most phytophagous stinkbugs possess sac- or tube-like posterior midgut, wherein specific symbiotic bacteria are colonized. The families Pentatomidae, Plataspidae, Acanthosomatidae, Scutelleridae, and Parastrachiidae stinkbugs harbor the γ -proteobacteria as the gut symbiont. These endosymbionts are typically transmitted vertically from mother to offspring in a diverse way. On the other hand, the superfamilies Lygaeoidea and Coreoidea possess *Burkholderia* symbionts in the crypt-bearing posterior midgut region. In the molecular phylogeny of the Pentatomomorphan families, the Lygaeoidea/Coreoidea associated with β -proteobacterial *Burkholderia* symbionts formed a distinct major group from the Pentatomoidea associated with γ -proteobacterial symbionts (Fig 1). These *Burkholderia* symbionts are not detected on the egg surface, thus it could be acquired by the host insects from the environment every generation as it turns out in some stinkbugs.

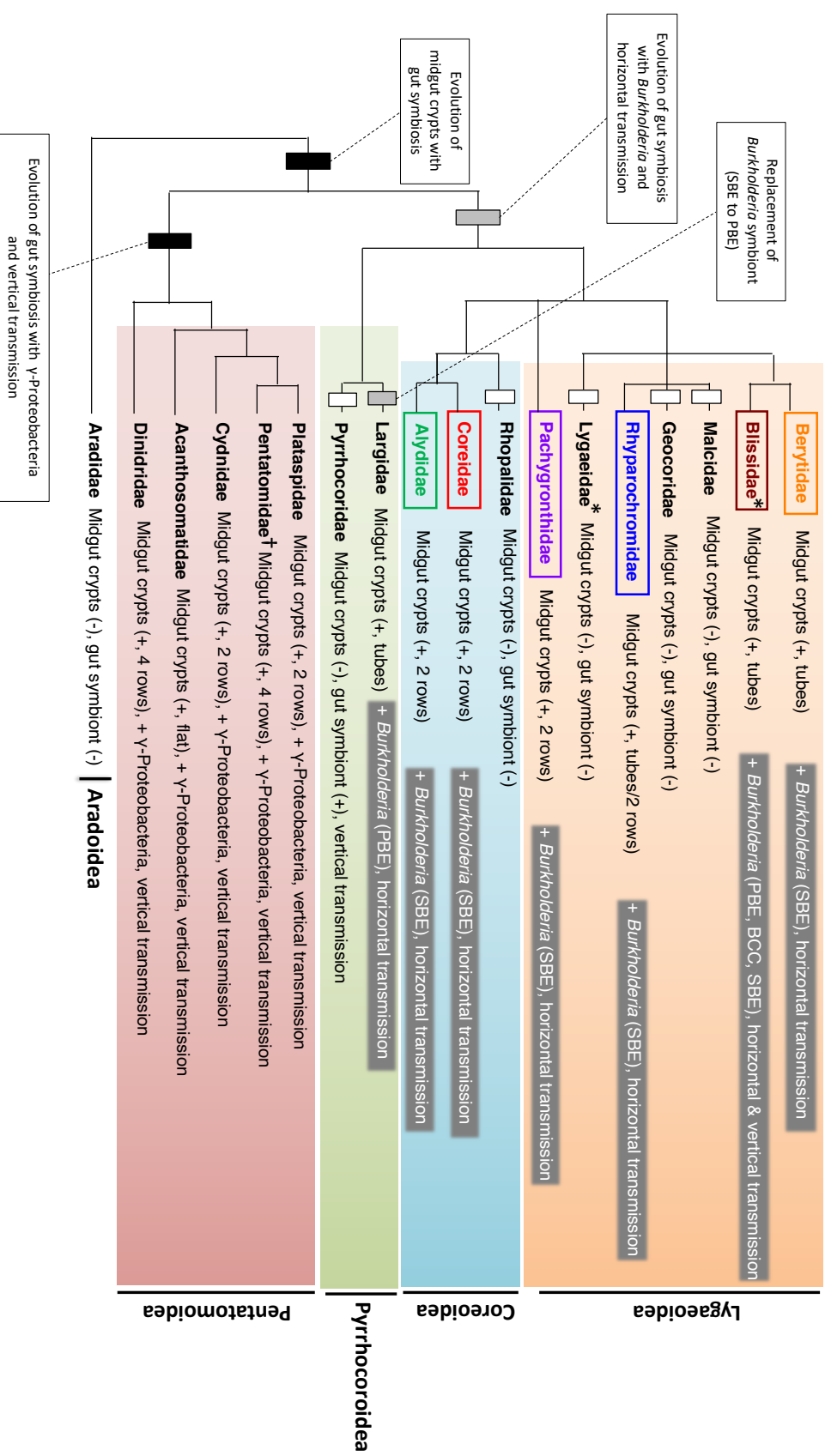


Fig. 1. Evolution of *Burkholderia* symbiosis in the Pentatomomorpha.

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). Presence/absence of symbiotic bacteria, symbiont taxonomy, transmission mechanism, and crypt morphology are indicated. Common insect names and their feeding habits are also shown. White rectangles on the phylogeny indicate the loss of the *Burkholderia* symbiosis and midgut crypts. Asterisks (*) indicate the existence of exceptional taxa that have lost the *Burkholderia* symbiosis 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).

Vertical transmission of symbiont in stinkbugs*Egg-smearing*

Many of the families Pentatomidae and Scutelleridae transfer symbiotic bacteria by laying symbiont-smearred eggs during oviposition of females, thus newborn nymphs directly acquire symbiont from (11, 27, 28, 63). Pentatomid stinkbugs, such as *Plautia stali*, *Plautia splendens*, and *Carbula crassiventris*, are the best-studied insects in egg-smearing transmission. Pentatomidae stinkbugs harbor specific symbiont of γ -proteobacteria in the extracellular cavity of crypts on the posterior midgut region which

consists of several rows (64–66). Sterilization of the egg surface of Pentatomidae stinkbugs causes high mortality and abnormal growth of these stinkbugs, demonstrating that the gut symbionts transmitted via eggshell are obligate symbiont (63, 65, 67, 68). Recent studies found that female-specific morphological trait of the midgut is related to lay symbiont-contaminated eggs (65). In contrast with male insects, several crypts at the end region of the posterior midgut are conspicuously enlarged and gut symbionts are often released to the main tract of the gut near widen crypts. This unique gut morphology of female, though speculative, could play an important role in vertical transmission and evolved in Pentatomidae (65, 69).

Capsule-transmission

The family Plataspidae vertically transfers endosymbionts by laying symbiont-containing capsules with eggs (70–72). This mode of transmission in plataspid stinkbug is first observed in *Coptosoma scutellatum*, which inhabits central and southern Europe (73). Upon hatching, the newly born nymphs immediately probe the capsule by using proboscis and ingest symbionts. The acquired endosymbionts are pass through the midgut and colonize the posterior midgut region, which is the symbiotic organ consists of numerous crypts. However, detailed research about symbiosis in *C. scutellatum* such as the investigation of microbiota in the capsule was scarce. More specific research has been executed in the Japanese common plataspid stinkbug, *Megacopta punctatissima*. *M. punctatissima* also lays egg masses with symbiont-containing capsules and newly hatched nymphs directly approached the capsule and probe it with their proboscis for a long time

to acquire symbiont (27, 70, 71). Based on sequencing and phylogenetic analysis, the endosymbiont in the capsule of *M. punctatissima* is revealed as γ -proteobacteria named “*Candidatus* Ishikawaella capsulate” (74). Deprivation of symbiont capsule caused high mortality, retarded growth, and sterility of insects, thus symbiotic bacteria in plataspid stinkbug is an obligate symbiont (71). In addition to these obligatory roles, the symbiont also alters the behavior of nymphal *M. punctatissima* that symbiont-free or symbiont-depleted stinkbug shows wandering behavior, but the symbiont-infected insect has rested in aggregation nearby eggs (75, 76). This behavioral alteration by symbiont is expected to minimize the energy and time spent on the activity, result in leading host benefits such as longevity. Lastly, capsule transmission of symbiont has observed in other plataspid stinkbug species, demonstrating that symbiont capsule has evolved in the lineage of the family Plataspididae (74).

Jelly-transmission

Stinkbugs of the family Urostylididae in Japan, *Urostylis westwoodii* and *Urostylis annulicornis*, possess γ -proteobacterial symbiont “*Candidatus* Tachikawaea gelatinosa” in the posterior midgut crypts and vertically transmit them via jelly-like substance (77). Female Urostylidid stinkbugs lay eggs on the bark of the host tree trunk and with jelly, which contains a large number of midgut symbionts. After hatching, nymphs immediately acquire midgut symbiont from jelly and endure until the third instar with consuming the only jelly (77). The reproductively mature female Urostylidid stinkbugs showing swollen abdomen is filled with an extremely developed ovary and oocytes are covered with

voluminous translucent liquid jelly. Although symbionts are transmitted by excreted jelly covering egg masses, no midgut symbionts are detected in the jelly embedding oocytes in the body (77). Female stinkbugs have a female-specific pair of organs, which is oval in shape and yellow in color, so-called “yellow organ” near the genital chamber, and a large number of symbionts are existed in this organ, suggesting that supplementation of the symbiont is occurred upon or after oviposition (77). According to results that midgut symbiont of Urostylidid stinkbug has an extremely reduced genome and removal of symbiont-containing jelly from newborn nymphs caused high mortality and retardation of growth, midgut symbionts are obligate in these stinkbugs (77).

***Riptortus pedestris*-*Burkholderia* gut symbiosis: an ideal model system**

The bean bug, *R. pedestris* (superfamily Coreoidea: family Alydidae), is a notorious pest of legumes. The bean bug harbors numbers of gut symbiotic bacteria of the genus *Burkholderia* in the posterior midgut region M4 that consists of hundreds of sac-like tissues called crypts (78). In contrast with most insects, bean bug acquires *Burkholderia* symbiont horizontally from the environmental soil via oral ingestion at the stage of second instar nymph every generation (31, 79). Gut-colonizing *Burkholderia* symbiont provides numerous physiological benefits to the host bean bug, such as promoting host growth, development, and fecundity, and enhancing immunity (22, 23, 30). Furthermore, recent studies have revealed that *Burkholderia* symbiont confers host resistance against insecticide by degrading it using microbial enzymes, identifying the importance of symbiont-mediated detoxifying in the bean bug (80, 81). However, bean bug also acquires

innumerable soil microbes thus is exposed to contamination of the symbiotic organ by non-symbiotic bacteria including pathogens. The midgut of the bean bug is organized into four distinct sections: M1, M2, M3, and M4 (82). In addition, there is a swollen region connected to the called M4 bulb (M4B) (83). To prevent infection of the symbiotic organ by non-symbiotic bacteria, bean bug has evolved a symbiont sorting organ in between M3 and M4B, called “constricted region (CR)”, which is extremely narrow and filled with a mucus-like matrix. The CR winnows out non-symbiotic bacteria (e.g. *Escherichia coli*), as well as other contaminants (e.g, food fluid), result in the selection of specific *Burkholderia* symbiont as a microbial partner(84). To pass the mucus-filled narrow passage, *Burkholderia* symbiont has a unique “screw-like” swimming motility. Indeed, the non-motile *Burkholderia* mutant did not pass the CR and failed to colonize the M4 (85). The infection process takes place quickly within 12-18 hours and the CR is physically closed after symbiont colonization, preventing any additional infection (86). Simultaneously, gut symbiont *Burkholderia* rapidly proliferates in the M4, occupying the lumen of the entire midgut crypts (86, 87). This tightly sealed CR is re-opened at the late developmental stage with aging, but it doesn’t affect gut symbiosis (88).

However, although the symbiont sorting organ winnows out diverse non-symbiotic bacteria, a recent study reported that some bacteria, which are closely related to natural *Burkholderia* symbiont, are able to colonize the M4 after passing through the CR (89). The bacterial genus *Burkholderia* is an ecologically diverse bacterial group that consists of over 100 species and is at least grouped as 3 distinct clades (90, 91): “*B. cepacia* complex and *B. pseudomallei*” (BCC&P) clade, which includes animal- and plant-

pathogens (92), “plant-associated beneficial and environmental” (PBE) clade, which contains plant growth-promoting *Burkholderia* (93, 94), “stinkbug-associated beneficial and environmental” (SBE) clade, which mainly consists of gut symbionts of stinkbugs (30, 95), and other *Burkholderiaceae*. Not only native SBE *Burkholderia* symbiont but also some non-symbiont PBE *Burkholderia* and even *Pandoraea* that is outgroup of *Burkholderia* colonizes the midgut well, providing beneficial effects to the host bean bug (89). However, the co-infection test of both native *Burkholderia* symbiont and non-symbiont revealed that *Burkholderia* in the SBE group always outcompetes other non-native bacteria in the midgut, thus SBE *Burkholderia* becomes predominance in natural bean bug populations (89). Taken together, microbe-microbe competition in the gut, as well as host-side morphological trait, contribute to build and develop *R. pedestris-Burkholderia* gut symbiosis.

Since most of the insect symbiont has a tremendously reduced genome (96)(38), thus they are uncultivable *in vitro*, *R. pedestris-Burkholderia* symbiosis model is more ideal to study molecular bases insect-microbe symbiosis than other insects. Whereas typically the genome size of an insect’s obligate symbionts is approximately 0.1 Mb to 0.8 Mb, the symbiont of the bean bug, *Burkholderia insecticola*, has a larger genome, 6.96 Mb in size, which is similar to environmental bacteria (97). Therefore, *Burkholderia* symbiont indeed easily culturable, and genetic manipulation is available, thus the investigation of critical symbiotic genes that are important to colonize the midgut of bean bug is possible by using diverse techniques (30). Indeed, through screening of *Burkholderia* mutant strains, several symbiosis factors have been identified. *Burkholderia* symbiont is gram-negative

bacteria, so its cell wall surface consists of lipopolysaccharide (LPS), which is the primary lipid layer embedded in the outer membrane and comprised of three distinct compartments: lipid A, core oligosaccharide, and O-antigen. O-antigen is the outermost component of LPS and plays a role as a primary protective barrier against the environment (98). LPS O-antigen-deficient *Burkholderia* mutant showed decreased infection rate in the bean bug probably due to increased susceptibility against host antimicrobial molecules (99–101). Similarly, *Burkholderia* symbiont, which has defects in the cell wall synthesis gene, became sensitive to diverse stressful environments and cannot properly colonize the M4 (102). When *Burkholderia* colonizes the midgut, it accumulates polyester, polyhydroxyalkanoate (PHA) granule, in the bacterial cell (103). Since the PHA granule is generally biosynthesized under nutrient stress (104), the environment of the host midgut is considered stressful to *Burkholderia* symbiont, thus PHA granule is important to colonization in the midgut. *Burkholderia* mutant, which cannot normally synthesize PHA granule, indeed did not colonize well the midgut crypts (103, 105). Besides, other symbiosis factors of *B. insecticola* symbiont, such as genes related to purine biosynthesis and peptidoglycan biosynthesis, have been identified (106–108). In addition, a recent study analyzed comparative transcriptomics of *in vitro* cultivated *Burkholderia* and gut-colonizing *Burkholderia* cells (109). This research suggests candidates of unrevealed important functional genes of *Burkholderia* symbiont based on the profile of highly up- and down-regulated genes. Moreover, *R. pedestris*-*Burkholderia* model system also has a host-side advantage that RNA-interference is perfectly working (110). Since transcriptomic data of *R. pedestris* is also available, the important genes of the bean bug for keep symbiosis with numbers of *Burkholderia* symbiont in the midgut could be found

by gene silencing method.

Chapter II

**Host–symbiont specificity determined by
microbe–microbe competition in an insect gut**

Abstract

Despite the omnipresence of specific host-symbiont associations with acquisition of the microbial symbiont from the environment, little is known about how the specificity of the interaction evolved and is maintained. The bean bug *Riptortus pedestris* acquires a specific bacterial symbiont of the genus *Burkholderia* from environmental soil and harbors it in midgut crypts. The genus *Burkholderia* consists of over 100 species showing ecologically diverse lifestyles and including serious human pathogens, plant pathogens, and nodule-forming plant mutualists, as well as insect mutualists. Through infection tests of 34 *Burkholderia* species and 18 taxonomically diverse bacterial species, we demonstrate here that non-symbiotic *Burkholderia* and even its outgroup *Pandoraea* could stably colonize the gut symbiotic organ and provide beneficial effects to the bean bug when inoculated on aposymbiotic hosts. However, co-inoculation revealed that the native symbiont always outcompeted the non-native bacteria inside the gut symbiotic organ, explaining the predominance of the native *Burkholderia* symbiont in natural bean bug populations. Hence, the abilities for colonization and cooperation, usually thought of as specific traits of mutualists, are not unique to the native *Burkholderia* symbiont but competitiveness inside the gut is on the contrary a derived trait of the native symbiont lineage only and was thus critical in the evolution of the insect gut symbiont.

Introduction

A number of animals and plants are associated with beneficial microorganisms, which provide diverse services to the host species, such as enhanced nutrition and protection from antagonists (111, 112). While some symbionts are vertically transmitted from mother to offspring, many animals and plants acquire in every generation the symbionts from the environment (113). Since microorganisms are abundant and diverse in the environment, hosts have evolved elaborate mechanisms for “partner choice” to ensure the specificity of the associations (114, 115). Well-studied model systems, the legume-*Rhizobium* and squid-*Vibrio* symbioses, have revealed that the partner specificity is achieved by various host mechanisms, such as signal recognition during initiation and secretion of antimicrobial agents during colonization (8, 116). In addition to such host control mechanisms, theoretical modelling studies have suggested the importance of microbe-microbe competition to ensure the specificity, wherein the host provides a demanding environment to let a favorable microorganism grow dominantly (117, 118). The “competition-based selection” is thought to be more powerful to eliminate contaminants or pathogens and maintain symbiont quality particularly in open systems such as the gut, the fungus-farming garden of insects and the plant rhizosphere, where microbial contamination can regularly occur and potentially disrupt the symbioses. Despite these theoretical studies, empirical support for the impact of microbe-microbe competition on the evolution and stabilization of host-symbiont specificity is very scarce.

The bean bug *Riptortus pedestris* (Heteroptera: Alydidae) is associated with a *Burkholderia* symbiont that is confined in symbiosis-specific crypts located in the posterior midgut region M4 (30). The *Burkholderia* symbiont is not essential but

beneficial for growth, reproduction, immunity homeostasis, and pesticide resistance of the insect host (31, 80, 119). While most insects vertically transmit their symbionts, the bean bug acquires it from the ambient soil in every generation (30, 31). To sort the symbiont from the enormously diverse soil microbiota, the stinkbug possesses a specific organ called the “constricted region”, a narrow passage filled with a mucus-like matrix, located in the midgut immediately upstream of the crypt-bearing region. The constricted region winnows out contaminating microorganisms and the *Burkholderia* symbiont specifically penetrates into the symbiotic region (120). In addition to this specific apparatus for bacterial sorting, the insect expresses diverse antimicrobial peptides in the symbiotic organ (82, 119), which may also play a role in the partner choice.

To be a bean bug symbiont, therefore, the *Burkholderia* species should be capable of penetrating the constricted region [initiation], stably colonizing the symbiotic organ [accommodation], and of course, behaving cooperatively with the host [cooperation]. Recent studies have revealed part of the symbiont’s mechanisms involved in these processes. The *Burkholderia* symbiont employs a unique corkscrew flagellar motility to pass through the constricted region (85); it undergoes cytological and metabolic alterations, such as flagella loss and polyhydroxyalkanoate accumulation, to cope with the crypt environment (103, 105, 109); and it rapidly proliferates to occupy entirely the available space in the lumen of the symbiotic M4 midgut region, notably by recycling host metabolic wastes into essential amino acids and B vitamins in the M4 crypts which are thought to be part of the symbiont services provided to the host (109).

The bacterial genus *Burkholderia* (Betaproteobacteria: Burkholderiaceae), comprises over 100 species with taxonomically validated names and is an ecologically

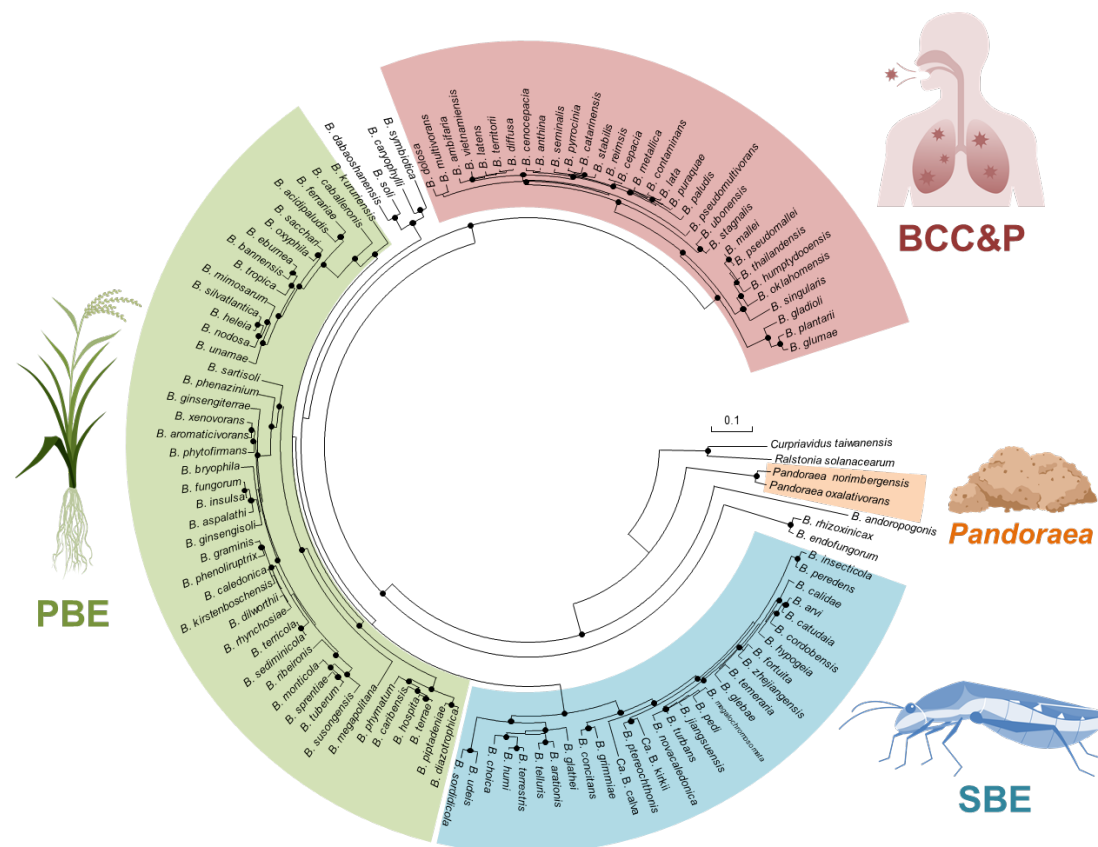


Fig. 2-1. Phylogenetic relationship of 113 species of the genus *Burkholderia*. A multi-locus tree based on 6,399 unambiguously aligned amino acid sites of 21 genes is shown. The 21 marker genes were commonly present in the 113 *Burkholderia* species and four beta-proteobacteria with well conserved alignment blocks (>60% of the alignment). Stinkbug-associated SBE, plant-associated PBE, and pathogenic BCC&P groups are highlighted with blue, green, and red, respectively. Recently proposed new genus names of SBE and PBE are also depicted under the group names. Closed circles indicate that the nodes are supported with > 70% bootstrap values. Illustrations by Matsuura Chiaki&Yu.

very diverse bacterial group (90). Based on genomic phylogeny, the genus *Burkholderia* is grouped into at least three distinct clades (91) (Fig. 2-1). The first clade consists of many human-, animal-, and plant-pathogens, including *B. cepacia*, *B. pseudomallei* and *B. mallei*, designated as the “*B. cepacia* complex and *B. pseudomallei*” (BCC&P) clade

(92). Some BCC&P species are also reported as fungal symbionts (121) and beetle symbionts (122). The second one includes a number of plant growth-promoting rhizobacteria and nodule symbionts of leguminous plants, and is designated as the “plant-associated beneficial and environmental” (PBE) clade (93). This clade, which also includes farming symbionts of the slime mold *Dictyostelium discoideum* (123), was recently even nominated as a new genus, *Paraburkholderia* (94). The third clade mainly consists of environmental species, leaf-nodule symbionts of Rubiaceae plants and gut symbionts of stinkbugs, and is called the “*Burkholderia glathei* clade” or the “stinkbug-associated beneficial and environmental” (SBE) clade (30, 95). Also for this clade, a new genus name, *Caballeronia*, was proposed (124). The outgroup of *Burkholderia* is the genus *Pandoraea* (Fig. 2-1), which are common soil bacteria, although some of them are opportunistic pathogens of humans (125). Almost all of the *Burkholderia* and *Pandoraea* species, except the leaf-nodule symbionts in SBE, are easy to culture and genetically manipulable, providing us an opportunity to clarify how symbiotic traits have evolved in a phylogenetically-well-defined bacterial lineage.

Genetically diverse *Burkholderia* species or strains are associated with natural *R. pedestris* populations but nearly all of them belong to the SBE clade (31, 78, 126) (Fig. 2-2) and only one study reported the infection with BCC&P species in overwintering *R. pedestris* (127). In addition, in insects reared on soil in the laboratory, we occasionally identified infections with PBE *Burkholderia* as well as *Pandoraea*, suggesting that infection to the symbiotic midgut organ is not strictly limited to SBE species (81). Although we revealed that the bacterial sorting organ in the bean bug gut winnows out symbionts from non-symbionts, only a limited number of bacterial species (i.e.

Escherichia coli, *Pseudomonas putida*, and *Bacillus subtilis*) have been tested as non-symbionts (120). Here we show by experimental inoculations of diverse bacterial species into *R. pedestris* that non-SBE *Burkholderia* and even *Pandoraea* are capable of passing through the constricted region, stably colonizing the gut symbiotic organ, and behaving cooperatively. We further demonstrate that, through competitive infection assays in conjunction with histological inspections, SBE always outcompetes these non-native symbionts inside the gut symbiotic organ, highlighting a pivotal role of symbiont competitiveness for stabilizing the insect-microbe gut symbiosis.

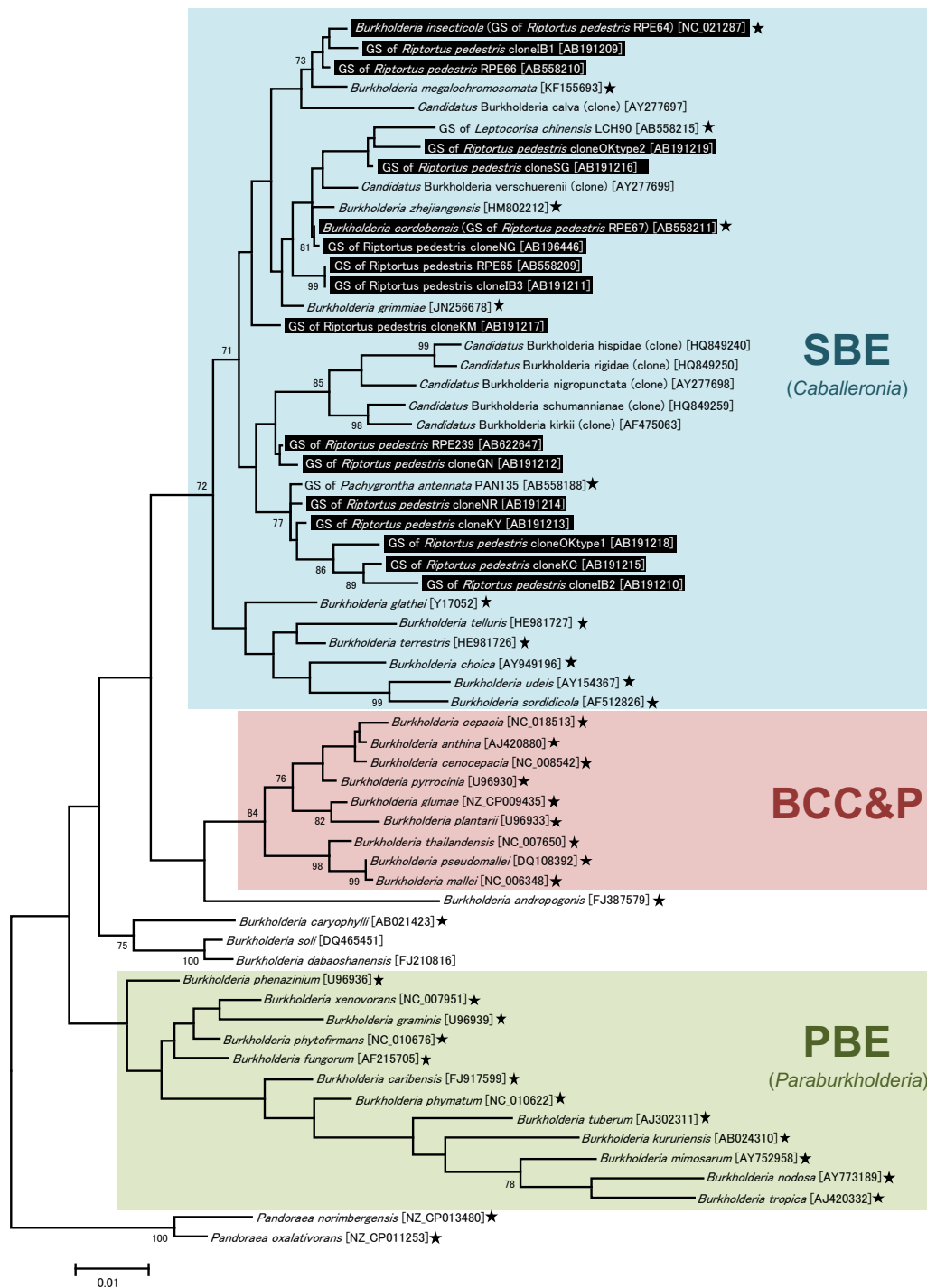


Fig. 2-2. Phylogenetic placement of symbiotic *Burkholderia* of the bean bug *R. pedestris*. A phylogenetic tree inferred from 1303 bp aligned sequences of the 16S rRNA gene is shown with higher than 70% bootstrap values at the nodes. OTUs shown on a black background are derived from cultured strains or sequence clones from the bean bug *R. pedestris* (Kikuchi et al., 2005, 2011, 2012). Stars indicate strains used for the inoculation test in this study

Table 2-1. Culture condition of bacterial strains used for the infection test

Strains	Culture medium [†]	Culture temperature (°C)
Burkholderia		
<i>Burkholderia insecticola</i> RPE64	YG	30
<i>Burkholderia fungorum</i> JCM 21562	YG	30
<i>Burkholderia plantarii</i> JCM 5492	YG	30
Pandoraea		
<i>Pandoraea norimbergensis</i> JCM10565	YG	30
Other Burkholderiaceae		
<i>Cupriavidus taiwanensis</i> DSM17343	YG	30
<i>Ralstonia solanacearum</i> AHU1949	YG	30
<i>Chitinimonas koreensis</i> DSM17726	R2A	30
Gammaproteobacteria		
<i>Pseudomonas putida</i> AHU1890	Nut	27
<i>Escherichia coli</i> DH5a	Nut	37
<i>Enterobacter cloacea</i> AHU1343	Nut	30
<i>Xanthomonas oryzae</i> AHU1700	Nut	27
Alphaproteobacteria		
<i>Agrobacterium tumefaciens</i> AHU1701	Nut	27
<i>Bradyrhizobium japonicum</i> JCM20679	MYE	30
<i>Acetobacter cerevisiae</i> AHU1581	PYG	30
Firmicutes		
<i>Bacillus subtilis</i> IAM12118	LB	30
<i>Bacillus thuringiensis</i> AHU1032	Nut	37
<i>Lactobacillus casei</i> AHU1110	MRS	37
<i>Enterococcus hirae</i> AHU1085	MRS	30
Actinobacteria		
<i>Arthrobacter globiformis</i> AHU1702	Nut	27
<i>Rhodococcus erythropolis</i> JCM20419	LB	30

[†]A Composition of each medium is as followings;

YG: 0.5% yeast extract, 0.4% glucose, 0.1% NaCl

Nut: 0.5% peptone, 0.3% yeast extract, 0.5% NaCl

R2A: R2A broth (Nippon Pharmaceuticals, Tokyo, Japan)

MYE: 0.5% mannitol, 0.05% yeast extract, 0.05% K₂HPO₄, 0.02% MgSO₄·7H₂O, 0.01% NaCl

PYG: 2% glucose, 0.5% peptone, 0.5% yeast extract, 0.27% Na₂HPO₄, 0.15% citric acid

LB: 1% trypton, 0.5% yeast extract, 0.5% NaCl

MRS: Lactobacilli MRS Broth (Difco Laboratories, Michigan, USA)

Table 2-2. Summary of the infection tests of *Burkholderia* species

Strains ^a	Infection to <i>R. pedestris</i> ^b	Area of M4B (mm ²)	Length of gut crypts (μm)	Index of M4B color (hue angle)
SBE clade				
<i>B. insecticola</i> RPE64	100% (10/10)	0.189	118.171	43.3
<i>B. cordobensis</i> RPE67	100% (10/10)	0.189	124.752	43.1
<i>B. zhejiangensis</i> CCTCC AB 2010354	90% (9/10)	0.277	122.549	41.5
<i>B. megalochromosomata</i> DSM 100850	100% (10/10)	0.204	125.647	43.5
<i>B. grimmiae</i> DSM 25160	90% (9/10)	0.169	117.827	30.5
<i>B. glathei</i> JCM 10563	100% (10/10)	0.127	113.986	37.6
<i>B. telluris</i> LMG 22936	100% (10/10)	0.207	124.254	44.6
<i>B. terrestris</i> LMG 22937	100% (10/10)	0.319	108.288	42.0
<i>B. choica</i> LMG 22940	100% (10/10)	0.316	129.508	40.9
<i>B. udeis</i> LMG 27134	100% (10/10)	0.278	114.437	41.3
<i>B. sordidicola</i> DSM 17212	80% (8/10)	0.149	117.819	43.0
<i>Burkholderia</i> sp. LCH90 ^c	100% (10/10)	0.180	116.93	41.9
<i>Burkholderia</i> sp. PAN135 ^d	100% (10/10)	0.188	118.451	34.7
PBE clade				
<i>B. fungorum</i> JCM 21562	100% (10/10)	0.446	93.742	40.5
<i>B. caribensis</i> DSM 13236	70% (7/10)	0.518	105.11	36.0
<i>B. phymatum</i> DSM 17167	100% (10/10)	0.465	93.868	33.8
<i>B. tuberum</i> DSM 18489	0% (0/10)	0.147	118.002	32.3
<i>B. graminis</i> DSM 17151	100% (10/10)	0.172	83.576	38.2
<i>B. phytofirmans</i> DSM 17436	50% (5/10)	0.207	101.351	32.7
<i>B. xenovorans</i> DSM 17367	40% (4/10)	0.442	108.556	41.5
<i>B. phenazinium</i> JCM 10564	60% (6/10)	0.435	111.657	26.7
<i>B. nodosa</i> DSM 21604	10% (1/10)	0.201	86.444	25.0
<i>B. mimosarum</i> DSM 21841	0% (0/10)	0.115	71.825	36.0
<i>B. tropica</i> DSM 15359	100% (10/10)	0.108	79.086	30.0
<i>B. kururiensis</i> JCM 10599	90% (9/10)	0.209	69.906	30.0
BCC&P clade				
<i>B. anthina</i> DSM 16086	10% (1/10)	0.114	61.555	24.0
<i>B. plantarii</i> JCM 5492	0% (0/10)	0.127	69.034	20.0
<i>B. glumae</i> DSM 9512	40% (4/10)	0.179	67.315	25.0
<i>B. pyrrocinia</i> DSM 10685	30% (3/10)	0.149	70.312	27.0
<i>B. thailandensis</i> DSM 13276	— ^e	— ^g	— ^g	— ^g
<i>B. pseudomallei</i> ATCC 23343	0% (0/10)	— ^g	— ^g	— ^g
<i>B. mallei</i> ATCC 23344	0% (0/10)	— ^g	— ^g	— ^g
Other <i>Burkholderia</i>				
<i>B. andropogonis</i> JCM 10487	0% (0/10)	0.094	67.115	24.0
<i>B. caryophylli</i> NBRC 13591	0% (0/10)	0.080	56.445	21.4
Pandoraea				
<i>Pandoraea norimbergensis</i> JCM10565	100% (10/10)	0.639	113.482	32
<i>Pandoraea oxalativorans</i> DSM23570	100% (10/10)	0.518	100.212	31.3
Other <i>Burkholderiaceae</i>				
<i>Cupriavidus taiwanensis</i> DSM17343	0% (0/10)	0.091	76.087	24
<i>Ralstonia solanacearum</i> AHU1949	0% (0/10)	0.124	82.621	26.7
<i>Chitinimonas koreensis</i> DSM17726	0% (0/10)	0.147	68.745	20

^a Strains except *Chitinimonas koreensis* DSM17726 were cultured in YG medium at 30°C.

^c *C. koreensis* DSM17726 was cultured in R2A medium at 30°C.

^b Infection percentage (number of infected insects/total number of investigated insects).

Infection was confirmed by diagnostic PCR (See Materials and Methods in main text).

^c Isolated from the stinkbug, *Leptocoris chinensis* (Coreoidea: Alydidae) (Kikuchi et al., 2011).

^d Isolated from the stinkbug, *Pachygrontha antennata* (Lygaeoidea: Pachygronthidae)

(Kikuchi et al., 2011).

^e All insects died within 2-3 days after inoculation of this strain.

^g Numeric values were not measured from insect infected with biosafety level 3 (BSL3) pathogens

Table 2-3. Colonized area of SBE and competitors in symbiotic organ

Fig 16			
<i>B. insecticola</i> vs <i>Pandoraea</i>	Colonized area of SBE (% , red color)	Colonized area of competitor (% , green color)	Color ratio
#1	99.79050784	0.209492162	
#2	83.77002828	16.22997172	
#3	95.81708902	4.182910981	
#4	72.00127267	27.99872733	
#5	88.40950394	11.59049606	
<i>B. insecticola</i> vs <i>B. fungorum</i> (PBE)			
#1	100	0	
#2	99.81417096	0.185829037	
#3	99.3218536	0.6781464	
#4	100	0	
#5	100	0	
<i>B. insecticola</i> vs <i>B. phytofirmans</i> (PBE)			
#1	100	0	
#2	100	0	
#3	100	0	
#4	100	0	
#5	100	0	
<i>B. insecticola</i> vs <i>B. graminis</i> (PBE)			
#1	100	0	
#2	98.68421053	1.315789474	
#3	100	0	
#4	100	0	
#5	100	0	
<i>B. insecticola</i> vs <i>B. kururiensis</i> (PBE)			
#1	100	0	
#2	100	0	
#3	100	0	
#4	100	0	
#5	100	0	
Fig 17			
<i>B. cordobensis</i> vs <i>Pandoraea</i>			
#1	100	0	
#2	99.45855615	0.54144385	
#3	98.79119481	1.208805192	
#4	75.36146703	24.63853297	
#5	81.31033914	18.68966086	
<i>B. cordobensis</i> vs <i>B. fungorum</i> (PBE)			
#1	100	0	
#2	92.52868451	7.471315485	
#3	100	0	
#4	100	0	
#5	99.80928769	0.19071231	
<i>B. cordobensis</i> vs <i>B. phytofirmans</i> (PBE)			
#1	100	0	
#2	75.42650233	24.57349767	
#3	100	0	
#4	98.79371877	1.206281228	
#5	100	0	
<i>B. cordobensis</i> vs <i>B. graminis</i> (PBE)			
#1	80.37658397	19.62341603	
#2	98.95111111	1.048888889	
#3	78.50700441	21.49299559	
#4	100	0	
#5	98.25755232	1.742447675	
<i>B. cordobensis</i> vs <i>B. kururiensis</i> (PBE)			
#1	100	0	
#2	100	0	
#3	100	0	
#4	100	0	
#5	100	0	
Fig 18			
<i>B. insecticola</i> Δ vgrG vs <i>Pandoraea</i>			
#1	100	0	
#2	99.43109564	0.568904357	
#3	96.41025641	3.58974359	
#4	100	0	
#5	100	0	
<i>B. insecticola</i> Δ vgrG vs <i>B. fungorum</i> (PBE)			
#1	99.82156877	0.178431233	
#2	100	0	
#3	100	0	
#4	100	0	
#5	100	0	
<i>B. insecticola</i> Δ clpV vs <i>Pandoraea</i>			
#1	86.5265401	13.4734599	
#2	88.75510204	11.24489796	
#3	85.55858311	14.44141689	
#4	79.21000946	20.78999054	
#5	97.68655831	2.313441689	
<i>B. insecticola</i> Δ clpV vs <i>B. fungorum</i> (PBE)			
#1	100	0	
#2	100	0	
#3	98.35963455	1.640365449	
#4	100	0	
#5	100	0	

The colonized area of SBE and competitor was measured by ImageJ program and converted to percentage by [Colonized area of SBE or competitor] / [Total area of M4].

Table 2-4. Bacterial strains used for the phylogenetic tree shown in Fig. 2-1.

Name	Recently-proposed genus	Strain name	Isolation origin	Accession No.
SSE clade				
<i>Burkholderia insecticola</i>	<i>Caballeronia</i>	RPE64	Insect (stinkbug, <i>Riptortus pedestris</i>)	GCA_000402035.1
<i>Burkholderia cordobensis</i>	<i>Caballeronia</i>	RPE67	Insect (stinkbug, <i>Riptortus pedestris</i>)	GCA_000828875.1
<i>Burkholderia atlatlensis</i>	<i>Caballeronia</i>	LMG 29314	Soil	GCA_001544975.2
<i>Burkholderia anvi</i>	<i>Caballeronia</i>	LMG 29317	Soil	GCA_001544895.2
<i>Burkholderia calidiae</i>	<i>Caballeronia</i>	LMG 29321	Water (pond)	GCA_900044055.2
<i>Burkholderia caludata</i>	<i>Caballeronia</i>	LMG 29318	Soil (rhizosphere of <i>Fadogia homblei</i>)	GCA_001544755.2
<i>Burkholderia chloica</i>	<i>Caballeronia</i>	LMG 22940	Soil (rhizosphere of <i>Lotium perene</i>)	GCA_001544535.2
<i>Burkholderia concilians</i>	<i>Caballeronia</i>	LMG 29315	Human (lung tissue)	GCA_001544615.2
<i>Burkholderia fortuita</i>	<i>Caballeronia</i>	LMG 29320	Soil (rhizosphere of <i>Fadogia homblei</i>)	GCA_001544835.2
<i>Burkholderia galei</i>	<i>Caballeronia</i>	DSM 50014	Laticif soil	GCA_000698595.1
<i>Burkholderia glebae</i>	<i>Caballeronia</i>	LMG 29325	Soil	GCA_001545035.2
<i>Burkholderia grimmiae</i>	<i>Caballeronia</i>	R27	Plant (moss, <i>Grimmia montana</i>)	GCA_000698555.1
<i>Burkholderia humi</i>	<i>Caballeronia</i>	LMG 22934	Soil (rhizosphere of <i>Lotium perene</i>)	GCA_001544475.2
<i>Burkholderia hypogaea</i>	<i>Caballeronia</i>	LMG 29322	Soil	GCA_001544875.2
<i>Burkholderia jiangsuensis</i>	<i>Caballeronia</i>	MP-1	Methyl parathion contaminated soil	GCA_000648925.1
<i>Burkholderia megachromosomata</i>	<i>Caballeronia</i>	JC2949	Soil	GCA_001580565.1
<i>Burkholderia pedi</i>	<i>Caballeronia</i>	LMG 29323	Soil	GCA_001544915.2
<i>Burkholderia peregrina</i>	<i>Caballeronia</i>	LMG 29314	Soil	GCA_001544595.2
<i>Burkholderia pterochthonis</i>	<i>Caballeronia</i>	LMG 29326	Soil	GCA_001545075.2
<i>Burkholderia sordidicola</i>	<i>Caballeronia</i>	LMG 22029	Fungus (<i>Phanerochaete sordida</i>)	GCA_001544455.2
<i>Burkholderia telluris</i>	<i>Caballeronia</i>	LMG 22936	Soil	GCA_001544955.2
<i>Burkholderia teneraria</i>	<i>Caballeronia</i>	LMG 29319	Soil (rhizosphere of <i>Fadogia homblei</i>)	GCA_001544795.2
<i>Burkholderia terrestris</i>	<i>Caballeronia</i>	LMG 22937	Soil	GCA_001544715.2
<i>Burkholderia turbanis</i>	<i>Caballeronia</i>	LMG 29316	Human (pleural fluid)	GCA_001544655.2
<i>Burkholderia udeis</i>	<i>Caballeronia</i>	LMG 27134	Naphthalene contaminated soil	GCA_001544555.2
<i>Burkholderia zhejiangensis</i>	<i>Caballeronia</i>	OP-1	Sludge from a wastewater treatment system	GCA_000698575.1
<i>Candidatus Burkholderia calva</i>	-	UZhbo6	Plant (leaf nodules of <i>Psychotria calva</i>)	GCA_001184395.1
<i>Candidatus Burkholderia kirkii</i>	-	UZhbo1	Plant (leaf nodules of <i>Psychotria kirkii</i>)	GCA_000234195.1
PBE clade				
<i>Burkholderia acidipaludis</i>	<i>Paraburkholderia</i>	NBRC 101816	Plant (<i>Eleocharis dulcis</i>)	GCA_000684975.1
<i>Burkholderia aromaticivorans</i>	<i>Paraburkholderia</i>	BNS	Gasoline contaminated soil	GCA_002278075.1
<i>Burkholderia aspalathi</i>	<i>Paraburkholderia</i>	LMG 27731	Plant (root nodules of <i>Aspalathus abietina</i> Thuurb)	GCA_900116445.1
<i>Burkholderia bannensis</i>	<i>Paraburkholderia</i>	NBRC 103871	Plant (a root of <i>Panicum repens</i>)	GCA_000685015.1
<i>Burkholderia dryophila</i>	<i>Paraburkholderia</i>	LMG 23644	Plant (moss, <i>Sphagnum rubellum</i>)	GCA_003269035.1
<i>Burkholderia caballeronis</i>	<i>Paraburkholderia</i>	LMG 26416	Soil (rhizosphere of tomato plants)	GCA_900109675.1
<i>Burkholderia caledonica</i>	<i>Paraburkholderia</i>	NBRC 102488	Soil (rhizosphere)	GCA_000685095.1
<i>Burkholderia caribensis</i>	<i>Paraburkholderia</i>	MWAP64	Soil (vertisol)	GCA_001449005.1
<i>Burkholderia diazotrophica</i>	<i>Paraburkholderia</i>	LMG 26031	Plant (root nodules of <i>Mimosa candollei</i>)	GCA_900108945.1
<i>Burkholderia diwortii</i>	<i>Paraburkholderia</i>	WSM3556	Plant (root nodules of <i>Lebeckia ambigua</i>)	GCA_000472525.1
<i>Burkholderia eburnea</i>	<i>Paraburkholderia</i>	JCM 18070	Peat soil	GCA_002917095.1
<i>Burkholderia terrariae</i>	<i>Paraburkholderia</i>	NBRC 106233	Ore material	GCA_000685035.1
<i>Burkholderia fungorum</i>	<i>Paraburkholderia</i>	ATCC BAA-463	Fungus (<i>Phanerochaete chrysosporium</i>)	GCA_000961515.1
<i>Burkholderia ginsengisoli</i>	<i>Paraburkholderia</i>	NBRC 100965	Soil	GCA_000739735.1
<i>Burkholderia kisteboschensis</i>	<i>Paraburkholderia</i>	DCY85	Soil (rhizosphere)	GCA_001645125.1
<i>Burkholderia ginsengiterrae</i>	<i>Paraburkholderia</i>	CAD1M	Plant (senescent maize roots)	GCA_000172415.1
<i>Burkholderia gamsis</i>	<i>Paraburkholderia</i>	NBRC 101817	Plant (<i>Eleocharis dulcis</i>)	GCA_000739775.1
<i>Burkholderia hialea</i>	<i>Paraburkholderia</i>	DSM 17164	Soil	GCA_002902965.1
<i>Burkholderia hospita</i>	<i>Paraburkholderia</i>	LMG 28183	Upper sediments of an arsenic-rich shallow-sea hydrothermal area	GCA_003002115.1
<i>Burkholderia insulsa</i>	<i>Paraburkholderia</i>	KB15	Plant (root nodules of <i>Virgilia oroboides</i>)	GCA_001636975.1
<i>Burkholderia kisteboschensis</i>	<i>Paraburkholderia</i>	NBRC 107107	Soil (rhizosphere of <i>Virgilia oroboides</i>)	GCA_000739795.1
<i>Burkholderia kururensis</i>	<i>Paraburkholderia</i>	LMG 23650	Plant (rhizosphere of tobacco plant)	GCA_900113825.1
<i>Burkholderia megapollana</i>	<i>Paraburkholderia</i>	LMG 23256	Plant (moss, <i>Aulacomnium palustre</i>)	GCA_000472825.1
<i>Burkholderia mimosarum</i>	<i>Paraburkholderia</i>	JC2948	Plant (nodules of <i>Mimosa pigra</i>)	GCA_001580545.1
<i>Burkholderia monticola</i>	<i>Paraburkholderia</i>	DSM 21604	Soil	GCA_000519185.1
<i>Burkholderia nodosa</i>	<i>Paraburkholderia</i>	NBRC 105797	Plant (root nodules of <i>Mimosa scabrella</i>)	GCA_000685075.1
<i>Burkholderia oxyphila</i>	<i>Paraburkholderia</i>	LMG 2247	Acidic forest soil	GCA_900100735.1
<i>Burkholderia phenazinium</i>	<i>Paraburkholderia</i>	LMG 2247	Soil	GCA_900100735.1
<i>Burkholderia phenoliruptrix</i>	<i>Paraburkholderia</i>	AC1100	a mixed culture from a chemostat grown with 2,4,5-trichlorophenoxyacetic acid	GCA_000416445.1

(Continue on next page)

<i>Burkholderia phytatum</i>	<i>Paraburkholderia</i>	STM815	Plant (root nodules of <i>Medicago lunatum</i>)	GCA_000020045.1
<i>Burkholderia phytofirmans</i>	<i>Paraburkholderia</i>	PS-JIN	Plant (onion roots)	GCA_000020125.1
<i>Burkholderia pipedrianae</i>	<i>Paraburkholderia</i>	STM 7183	Plant (a nodule of <i>Pisipadonia gonocantha</i>)	GCA_900007165.2
<i>Burkholderia rhyzocissae</i>	<i>Paraburkholderia</i>	WSM_3937	Plant (root nodules of <i>Rhynchosia tenuifolia</i>)	GCA_002879865.1
<i>Burkholderia rhyzocissae</i>	<i>Paraburkholderia</i>	STM7296	Plant (a nodule of <i>Pisipadonia gonocantha</i>)	GCA_900019265.2
<i>Burkholderia sacchari</i>	<i>Paraburkholderia</i>	LMG_194.50	Soil (a sugarcane (<i>Saccharum officinarum</i>) plantation)	GCA_000785435.1
<i>Burkholderia sacchari</i>	<i>Paraburkholderia</i>	LMG_24000	Soil	GCA_900107895.1
<i>Burkholderia sediminicola</i>	<i>Paraburkholderia</i>	LMG_24238	Freshwater sediment	GCA_900104005.1
<i>Burkholderia silvaticola</i>	<i>Paraburkholderia</i>	PS-CR-88	— ^a	GCA_003096715.1
<i>Burkholderia spretniae</i>	<i>Paraburkholderia</i>	WSM5005	Plant (root nodules of <i>Lebeckia ambigua</i>)	GCA_00186575.1
<i>Burkholderia susongensis</i>	<i>Paraburkholderia</i>	LMG_295.40	weathered surfaces of rock	GCA_900177725.1
<i>Burkholderia terreae</i>	<i>Paraburkholderia</i>	DSM_1780.4	Soil	GCA_002902925.1
<i>Burkholderia terebra</i>	<i>Paraburkholderia</i>	LMG_20594	Soil	GCA_900142195.1
<i>Burkholderia tropica</i>	<i>Paraburkholderia</i>	LMG_222.74	Plant (stem of sugarcane)	GCA_900109265.1
<i>Burkholderia tuberum</i>	<i>Paraburkholderia</i>	DUS833	— ^a	GCA_900101795.1
<i>Burkholderia unamae</i>	<i>Paraburkholderia</i>	SC2a_39	Plant (stem of sugarcane)	GCA_003096875.1
<i>Burkholderia xenovans</i>	<i>Paraburkholderia</i>	LB400	PCB-contaminated soil	GCA_000013845.1
BC&P clade				
<i>Burkholderia ambifaria</i>	<i>Burkholderia</i>	AMMD	Soil (rhizosphere of pea plants)	GCA_000203915.1
<i>Burkholderia anthina</i>	<i>Burkholderia</i>	AZ-4-2-30-S1-D7	Soil	GCA_001523905.1
<i>Burkholderia californiensis</i>	<i>Burkholderia</i>	89	Soil	GCA_001883705.1
<i>Burkholderia cepacia</i>	<i>Burkholderia</i>	UCB 717	Plant (onion)	GCA_001471495.1
<i>Burkholderia cenocepacia</i>	<i>Burkholderia</i>	NCTC13227	Human (septum of a cystic fibrosis patient)	GCA_900446215.1
<i>Burkholderia contaminans</i>	<i>Burkholderia</i>	LMG_23361	Milk of a sheep	GCA_000987075.1
<i>Burkholderia diffusa</i>	<i>Burkholderia</i>	RF2-non-BP9	Soil	GCA_001718315.1
<i>Burkholderia dolosa</i>	<i>Burkholderia</i>	AU0158	Human (a cystic fibrosis patient, pediatric)	GCA_000959505.1
<i>Burkholderia gladioli</i>	<i>Burkholderia</i>	ATCC 10248	Plant (<i>Gladiolus</i> sp.)	GCA_000959725.1
<i>Burkholderia glumae</i>	<i>Burkholderia</i>	ATCC 33617	Plant (grain of <i>Oryza sativa</i>)	GCA_000960995.1
<i>Burkholderia humphreensis</i>	<i>Burkholderia</i>	MSMB122	Water (an automated water well)	GCA_001462435.1
<i>Burkholderia lata</i>	<i>Burkholderia</i>	383	Soil	GCA_000012945.1
<i>Burkholderia latens</i>	<i>Burkholderia</i>	AU17928	Human (maxillary sinus of a cystic fibrosis patient)	GCA_001718795.1
<i>Burkholderia mallei</i>	<i>Burkholderia</i>	ATCC 23344	Human (a glanders patient)	GCA_000011705.1
<i>Burkholderia mallei</i>	<i>Burkholderia</i>	FL-6-5-30-S1-D7	Soil	GCA_00171855.1
<i>Burkholderia multivorans</i>	<i>Burkholderia</i>	ATCC BAA-247	Human (a cystic fibrosis patient)	GCA_000959525.1
<i>Burkholderia novacaledoniae</i>	<i>Burkholderia</i>	LMG_286.15	Plant (root of <i>Costularia arundinacea</i>)	GCA_900258035.1
<i>Burkholderia oklahomaensis</i>	<i>Burkholderia</i>	CG786	Human (pelvic wound)	GCA_000959365.1
<i>Burkholderia paludis</i>	<i>Burkholderia</i>	MSH1	Soil	GCA_000732815.1
<i>Burkholderia plantarii</i>	<i>Burkholderia</i>	ATCC 43733	Plant (rice seedlings)	GCA_001411805.1
<i>Burkholderia pseudomallei</i>	<i>Burkholderia</i>	ATCC 23343	Human	GCA_001182285.1
<i>Burkholderia pseudomultivorans</i>	<i>Burkholderia</i>	SUB-INT23-BP2	Soil	GCA_001718415.1
<i>Burkholderia puraquae</i>	<i>Burkholderia</i>	CAMPA 1040	Water (a hemodialysis water reservoir)	GCA_002099195.1
<i>Burkholderia pyrocinia</i>	<i>Burkholderia</i>	DSM_10685	Soil	GCA_001028665.1
<i>Burkholderia reimsis</i>	<i>Burkholderia</i>	BE51	Soil (rhizosphere of maize)	GCA_003294055.1
<i>Burkholderia seminis</i>	<i>Burkholderia</i>	FL-5-4-10-S1-D7	Soil	GCA_001718535.1
<i>Burkholderia sigulensis</i>	<i>Burkholderia</i>	LMG_28154	Human (respiratory samples of a cystic fibrosis patient)	GCA_900176645.1
<i>Burkholderia stabilis</i>	<i>Burkholderia</i>	ATCC BAA-67	Human (septum of a cystic fibrosis patient)	GCA_001742165.1
<i>Burkholderia stagnalis</i>	<i>Burkholderia</i>	MSMB135WGS	Soil	GCA_001718985.1
<i>Burkholderia teitoni</i>	<i>Burkholderia</i>	RF8-non-BP5	Soil	GCA_001718335.1
<i>Burkholderia thailandensis</i>	<i>Burkholderia</i>	E264	Soil	GCA_000012365.1
<i>Burkholderia ubonensis</i>	<i>Burkholderia</i>	MSMB22	Soil	GCA_000959245.1
<i>Burkholderia vietnamiensis</i>	<i>Burkholderia</i>	LMG_10929	Soil (rhizosphere of <i>Oryza sativa</i>)	GCA_000959445.1
Other Burkholderia				
<i>Burkholderia andropogonis</i>	<i>Robbia</i>	ICMP2807	Plant (stripe disease in sorghum)	GCA_000970345.1
<i>Burkholderia rhizoxinica</i>	<i>Mycetohabians</i>	HK1_454	Fungus (<i>Rhizopus microsporus</i>)	GCA_000198775.1
<i>Burkholderia endolungorum</i>	<i>Mycetohabians</i>	HK1_456	Fungus (<i>Rhizopus microsporus</i>)	GCA_002927045.1
<i>Burkholderia symbiolica</i>	<i>Timicka</i>	JPY-345	Plant (root nodules of <i>Mimosa conduplicata</i>)	GCA_002934455.1
<i>Burkholderia canophylli</i>	<i>Timicka</i>	Ballard 720	Plant (carnation, <i>Dianthus caryophyllus</i>)	GCA_002879875.1
<i>Burkholderia soil</i>	<i>Timicka</i>	GP25-8	Soil	GCA_002879855.1
<i>Burkholderia debaoshanensis</i>	<i>Timicka</i>	GMN1_004	Mine soil	GCA_002879885.1
Pandoraea				
<i>Pandoraea norbergensis</i>	-	DSM_11628	Water column overlaying the sediment	GCA_001465545.3
<i>Pandoraea oxalicovorans</i>	-	DSM_23570	Soil	GCA_000972785.3
Other Burkholderiaceae				
<i>Cupriavidus taiwanensis</i>	-	LMG_194.24	Plant (a root nodule of <i>Mimosa pudica</i>)	GCA_000069785.1
<i>Ralstonia solanacearum</i>	-	GM11000	Plant (tomato)	GCA_000009125.1

^a No information is provided.

Table 2-5. Marker genes used for phylogenetic analysis of *Burkholderia* genomes in this study (Fig. 2-1)

COG ID	Description	Alignment length (aa)
COG0012	Predicted GTPase, probable translation factor	357
COG0016	Phenylalanyl-tRNA synthetase alpha subunit	332
COG0049	Ribosomal protein S7	145
COG0052	Ribosomal protein S2	228
COG0081	Ribosomal protein L1	230
COG0093	Ribosomal protein L14	122
COG0094	Ribosomal protein L5	179
COG0096	Ribosomal protein S8	118
COG0098	Ribosomal protein S5	171
COG0100	Ribosomal protein S11	102
COG0124	Histidyl-tRNA synthetase	298
COG0172	Seryl-tRNA synthetase	422
COG0200	Ribosomal protein L15	143
COG0201	Preprotein translocase subunit SecY	406
COG0202	DNA-directed RNA polymerase, alpha subunit/40 kD subunit	301
COG0256	Ribosomal protein L18	118
COG0495	Leucyl-tRNA synthetase	839
COG0522	Ribosomal protein S4 and related proteins	207
COG0525	Valyl-tRNA synthetase	913
COG0533	Metal-dependent proteases with possible chaperone activity	332
COG0541	Signal recognition particle GTPase (Ffh)	436
total		6399

Materials and methods

Insects and bacterial strains

The *R. pedestris* strain used in this study was originally collected from a soybean field in Tsukuba, Ibaraki, Japan, and maintained in the laboratory for over ten years. The insects were reared in Petri dishes (90 mm in diameter, 20 mm high) at 25°C under a long-day regimen (16 h light, 8 h dark) and fed with soybean seeds and distilled water containing 0.05% ascorbic acid. Bacterial species/strains used in this study and their culture conditions are listed in Table 2-1 and 2-2.

Oral administration of cultured bacteria

(i) Taxonomically diverse bacteria

Bacterial species were grown in specific optimal growth condition (Table 2-1) to an early log phase on a gyratory shaker at 150 rpm. Exponentially growing cells (approx. OD₆₀₀=0.5) were harvested by centrifugation and suspended in distilled water so that the final concentration was 10⁴ cells/μl. Newly-molted second instar nymphs were deprived of water overnight to make the insects thirsty and willing to ingest the bacteria-containing suspensions. The nymphs were fed with symbiont-containing water for 48 h, after which it was replaced by sterile water. Two days after the third instar molt (approximately five days after inoculation), the symbiotic organs were dissected under a binocular microscope (S8APO, Leica), rinsed twice in sterilized water, and homogenized in sterilized water. Dilution series of the solution were then plated on agar plates to examine the infection

status of the insects. If any colonies were detected, the number of CFUs was calculated.

(ii) Burkholderia and other Burkholderiaceae species

Burkholderia and other *Burkholderiaceae* species were grown at 30°C to an early log phase in yeast-glucose (YG) medium (0.5% yeast extract, 0.4% glucose, 0.1% NaCl) for *Burkholderia*, *Pandoraea*, *Cupriavidus*, and *Ralstonia*, or R2A broth (Nippon Pharmaceuticals) for *Chitinimonas* on a gyratory shaker at 150 rpm. Inoculations of the bacterial species were performed as described above. Two days after the third instar molt, the symbiotic organs were dissected and examined for infection with the *Burkholderia* or other *Burkholderiaceae* species by diagnostic PCR with specific primer sets for *Burkholderia* 16S rRNA gene (BF and BR) (128) or universal ones for the bacterial 16S rRNA gene (515F and 806R) (129) respectively, as previously described (126).

(iii) B. mallei and B. pseudomallei

Inoculation tests of two BSL3 pathogens, *B. mallei* and *B. pseudomallei*, were performed at the BSL3 facility of the Hokkaido University Research Center for Zoonosis Control. The two species were also grown at 30°C to an early log phase in YG medium on a gyratory shaker at 150 rpm as well as other *Burkholderia* species as described above. Oral administrations of the two species were performed as described above. Two days after the third instar molt, insects were preserved in acetone during one week so that eventual bacterial cells were completely killed. Total DNA was extracted from the abdominal part (without dissection) and subjected to diagnostic PCR to determine if the BCC&P species had colonized the gut symbiotic organ of *R. pedestris*.

GFP- or RFP-labeling of bacterial species

PBE species (*B. fungorum*, *B. phytofirmans*, *B. graminis*, *B. kururiensis*), BCC&P species (*B. plantarii*, *B. glumae*, *B. pyrrocinia*), *P. norimbergensis*, *Escherichia coli* and *Cupriavidus taiwanensis* were labeled with green fluorescent protein (GFP) by the Tn7 mini-transposon system, as previously described (120). For labeling SBE *Burkholderia* (*B. insecticola* and *B. cordobensis*) with red fluorescent protein (RFP), pMini-Tn7-kan-rfp (130) was used under the same experimental conditions.

Histological observations

Insects were inoculated with *Burkholderia* or *Pandoraea* species (listed in Table 2-2) at the 2nd instar, and dissected two days after the 3rd instar molt (approximately five days after inoculation). The whole midgut was dissected from each insect in PBS, and photographed by a digital camera (EC3, Leica) connected to a dissection microscope (S8APO, Leica). To investigate colonization properties in more detail, GFP-labeled *B. insecticola* (SBE), *B. fungorum* (PBE) and *P. norimbergensis* (*Pandoraea*) were inoculated, and infected crypts were observed under a confocal laser scanning microscope (TCS SP8, Leica). The dissected tissues were fixed with 4% paraformaldehyde for 10 min at room temperature, washed in PBS twice, incubated in PBS containing 0.1% Triton X-100 for 5 min, stained with 0.5 μ M of SYTOX Green and 5 units/ml of Alexa Fluor555 phalloidin (ThermoFisher) in PBS for 20 min, washed in PBS twice, and mounted on silane-coated glass slides for observations.

Competitive infection assays

(i) *In vitro* competition assay

For competition assays, a spontaneous rifampicin-mutant of *B. insecticola* (SBE) and spontaneous chloramphenicol-mutants of *B. fungorum* (PBE) and *P. norimbergensis* (*Pandoraea*) were used. Log-phase cultured cells of the SBE and a competitor (PBE or *Pandoraea*) were suspended in YG medium, adjusted to the same concentration at 5,000 CFU and mixed. The adjusted cells were diluted to 1 ml with YG medium in a 1.5 ml microtube, and incubated at 25°C without shaking. Two days after the inoculation, the medium was serially diluted and plated on YG agar plates containing either rifampicin or chloramphenicol. After three days incubation at 25°C, colonies of the SBE and competitor were counted on the plates. The competitive index (CI) was calculated by (output SBE CFU/ input SBE CFU)/(output competitor CFU/ input competitor CFU).

(ii) *In vivo* competition assay

Log-phase cultured cells of the SBE and a competitor (*B. fungorum* or *P. norimbergensis*) were suspended in distilled water, mixed and adjusted to the same concentration at either 250 CFU/μl or 5,000 CFU/μl infection dose. In each assay, newly molted second instar nymphs of *R. pedestris* were individually fed with 1 μl of the bacteria-containing suspensions, as previously described (80). Insects were reared at 25°C under a long-day regimen and fed with soybean seeds and distilled water. Two days after the inoculation, the symbiotic organ was dissected from each of the insects and homogenized with sterile distilled water. The output homogenate was serially diluted and plated on YG agar plates containing antibiotics. After three days incubation at 25°C, colonies of the SBE and

competitor were counted on the plates and CI values were calculated.

To visualize the *in vivo* competition, an RFP-labeled SBE *Burkholderia* (*B. insecticola* or *B. cordobensis*) and a GFP-labeled competitor (*B. fungorum*, *B. phytofirmans*, *B. graminis*, *B. kururiensis* or *P. norimbergensis*) were co-inoculated, and the colonization process was investigated by fluorescence microscopy (DMI4000B, Leica). Based on the microscope images, red and green fluorescent areas were calculated by ImageJ (131) and used to evaluate the competitiveness of the SBE *Burkholderia* species.

To clarify whether T6SS is involved in the competition ability, two T6SS genes, *vgrG* and *clpV* (Fig. 2-18A), were deleted in *B. insecticola* (SBE) by the homologous recombination technique, as previously described (103). *vgrG*, a tip protein playing a role as spike as well as an effector, is an essential gene for functional T6SS (132). *clpV*, encoding an AAA⁺ ATPase, is also one of the pivotal genes for the T6SS (132). The $\Delta vgrG$ and $\Delta clpV$ mutants were labelled with RFP and subjected to the *in vivo* competition assay, as described above.

Morphometrical measurements of the gut symbiotic organ

The whole midgut of 3rd instar was dissected in PBS and photographed by a digital camera (EC3, Leica) connected to a dissection microscope (S8APO, Leica). The length and area of M4B and M4 crypts were measured by ImageJ (131), as described in Fig. S2A. To evaluate M4B color, hue angles of M4B images were analyzed by using Adobe Photoshop CC 2018. 3 Ten pixels were randomly chosen from the M4B of each insect,

and the a^* and b^* values of each pixel's color were measured. By use of the values, hue angles were calculated by $[\text{Arc tan}(b^*/a^*)]$ (133), the average of which was used as the index of M4B color. To calculate the fraction of infected crypts, the numbers of infected- and uninfected-crypts were counted by the use of images of the confocal laser scanning microscope.

Statistical analysis

The Fisher's exact probability test and Mann-Whitney U-test were applied to the survival rate and the other fitness-parameters, respectively. Multiple comparisons were corrected by the Bonferroni method. CI values were statistically evaluated by the one sample t test. All statistical analyses were performed using the program R ver. 2.12.1 (134).

Results and Discussion

SBE, PBE, and *Pandoraea* species can pass through the constricted region and stably colonize the symbiotic organ

The midgut of *R. pedestris* consists of four distinct sections called M1, M2, M3 and M4. The SBE *Burkholderia* specifically colonizes the crypt-bearing M4 section. After symbiont infection, the M4 crypts swell and become whitish in color because of the symbiont colonization and proliferation in their lumen (30). In front of M4, a bulbous area called M4 bulb (M4B) develops, wherein symbiont cells, flowed backwards from M4, are entirely digested and the derived nutrients absorbed by the host (82, 102, 109). Probably because of the digested bacteria, the M4B becomes swollen and yellowish (30). The constricted region connects the M3 with the M4B (120). In order to analyze further the infection specificity of the stinkbug-*Burkholderia* gut symbiosis, we first experimentally inoculated 20 taxonomically diverse bacterial species of three major phyla into the bean bug and determined their colonization ability by counting colony forming units (CFU). The tested bacteria (Table 2-1) included species of the *Burkholderia* (SBE [*B. insecticola*], PBE [*B. fungorum*] and BCC&P [*B. plantarii*]), related species of the family *Burkholderiaceae* (*Ralstonia*, *Chitinimonas*, *Cupriavidus*, and *Pandoraea* species) as well as more distantly related species of the *Alpha*- and *Gamma*-*proteobacteria*. Inoculation was performed in early 2nd instar when the insects are competent for symbiont acquisition (135), and bacterial colonization was investigated at day 2 of the 3rd instar (*i.e.* 5 days post inoculation), which is two to three days after completion of the colonization process with *B. insecticola* (87). Whereas most of the tested species showed little or no infection in the symbiotic organ, the PBE and *Pandoraea*, as well as the SBE but not the

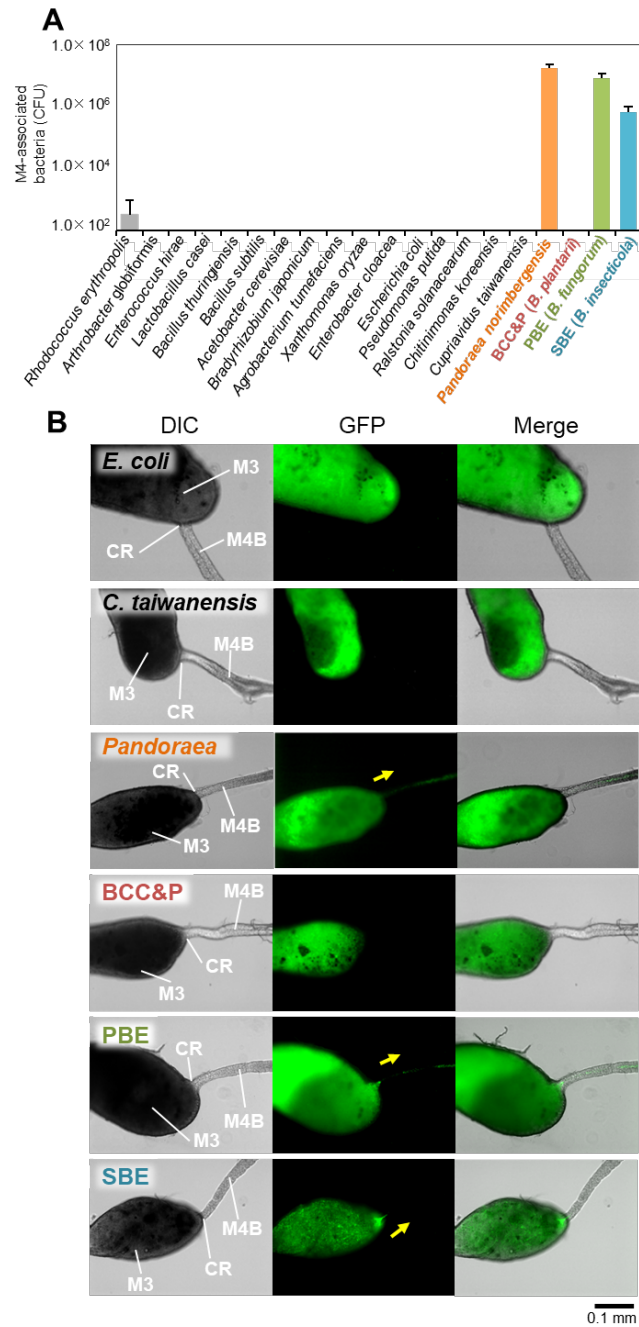


Fig. 2-3. Infection specificity of bacterial species in the gut symbiotic organ. (A) Infection ability of diverse bacterial species to the gut symbiotic organ. Five days after oral administration, the colonization level in M4 was evaluated by CFU counting. **(B)** Infection initiation process visualized by confocal microscopy. The infecting bacteria were labeled with GFP. Note that only *Pandoraea* (*P. norimbergensis*), PBE (*B. fungorum*), and SBE (*B. insecticola*) pass through the sorting organ, the constricted region. Arrows indicate an infection thread invading from M3 to M4B through the constricted region. Abbreviations: CR, constricted region; M3, midgut third section; M4B, M4 bulb

BCC&P, colonized the symbiotic organ to high density, around 10^7 CFU/organ (Fig. 2-3A). Microscopic observations with GFP-labeled strains of *E. coli*, *Burkholderia* and allied *Burkholderiaceae* species confirmed that while *E. coli*, *Cupriavidus* and BCC&P reached the M3 but did not pass through the constricted region, the SBE, PBE, and *Pandoraea* species could pass through the constricted region and penetrate into the symbiotic M4 region (Fig. 2-3B).

Infection states of *Burkholderia* and *Pandoraea* species in the bean bug

To further detail the specificity of the interaction and to clarify the infection states of *Burkholderia* and *Pandoraea* species in *R. pedestris*, infection rate (% of infected insects) and morphology of infected midguts were surveyed for 13 SBE species, 12 PBE species, 7 BCC&P species, two species of other *Burkholderia* clades and two *Pandoraea* species. Results of the infection assays, microscopic observations, and morphometric measurements are summarized in Fig. 2-4, Table 2-2, and Fig. 2-5, respectively. Details of the infection tests are described in the following sections and shown in Fig. 2-6 to 2-13.

SBE *Burkholderia*

All of the 13 tested SBE *Burkholderia* species or strains consistently showed high, 80%-100%, infection rates (Table 2-2). In aposymbiotic insects, M4 crypts and M4B were not well developed (Fig. 2-4A), the luminal space of each crypt was small and

its epithelium cells were thick (Fig. 2-4A). In contrast, the symbiotic organ infected with SBE (*B. insecticola*) was well developed (Fig. 2-4B and 2-5): M4 crypts were bulging by the bacterial proliferation and colonization (Fig. 2-5B and C); the M4B became bulbous in shape (Fig. 2-5D) and yellowish in color (Fig. 1-5E). Laser scanning microscopy (LSM) and transmission electron microscopy (TEM) observations confirmed that M4 crypts were entirely filled with SBE cells (Fig. 2-4B and 2-6), while no or partial colonization occurred in the M4B (Fig. 2-7). These morphological features of M4 and M4B were

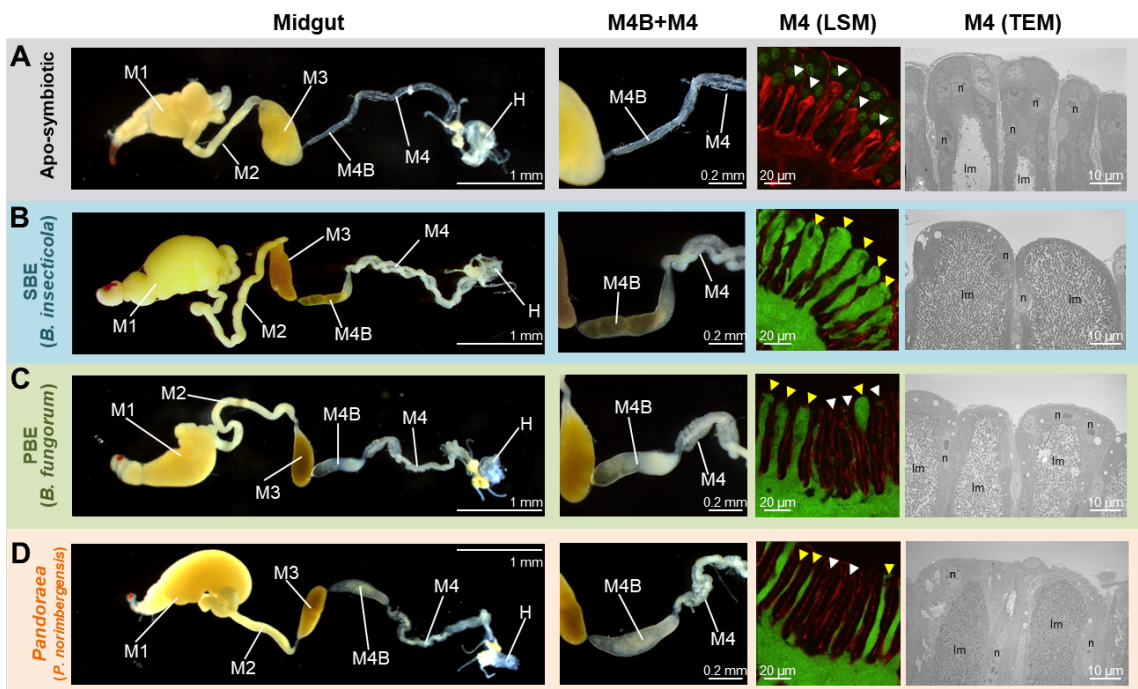


Fig. 2-4. Morphological alteration of the gut symbiotic organ after colonization of symbiotic and nonsymbiotic bacteria. Images of whole midgut, M4B and M4 region, LSM of M4, and TEM of M4 in an aposymbiotic insect (A), and insects inoculated with SBE (B), PBE (C), and Pandoraea (D). All images are of thirdinstar nymphs. Abbreviations: H, hindgut; M1, midgut first section; M2, midgut second section; M3, midgut third section; M4B, M4 bulb; M4, crypt-bearing midgut fourth section. In LSM images: green, SYTOX GREEN staining of colonizing bacteria and host nuclei; red, phalloidin staining of cytoskeleton; yellow arrowheads, infected M4 crypts; white arrowheads, uninfected M4 crypts. In TEM images: lm, luminal region of crypt; n, host nucleus.

consistently observed in insects infected with the other 12 species of SBE *Burkholderia* (Fig. 2-8, 2-9 and Table 2-2).

PBE *Burkholderia*

Among the 12 tested species of PBE *Burkholderia*, the infection rate was variable (Table 2-2). Five species showed a very high (90%-100%) infection rate; while four species showed moderate (40%-70%) and three species showed low (0%-10%) infection rates. The morphology of the symbiotic organ infected with the highly infective representative, *B. fungorum*, was observed in detail. This revealed that the symbiotic organ colonized by PBE *Burkholderia* (Fig. 2-4C) was markedly different from the organ colonized by SBE *Burkholderia*: the M4 crypts were infected but not well bulged (Fig. 2-5B and C); the M4B was significantly larger (Fig. 2-5D) but remained whitish and did not adopt the yellowish color characteristic for SBE infection (Fig. 2-5E). LSM and TEM observations revealed that this non-native symbiont occupied strongly the central tract of the M4 but had only partially colonized the crypt lumen (Fig. 2-4C and 2-5F). When colonized, the luminal space of each crypt was narrower and its epithelial cells were thicker than those of the crypts colonized by SBE (Fig. 2-4B&C). Colonizing PBE cells had a typical rod shape and were not notably different from colonizing SBE cells (Fig. 2-6). Although no or partial colonization of SBE *Burkholderia* occurred in the M4B, TEM observations showed that a high number of bacterial cells of *B. fungorum* colonized this region (Fig. 2-7), suggesting that the PBE is digestion tolerant or that the digesting enzymes are not properly produced and that the colonizing cells cause the swelling and

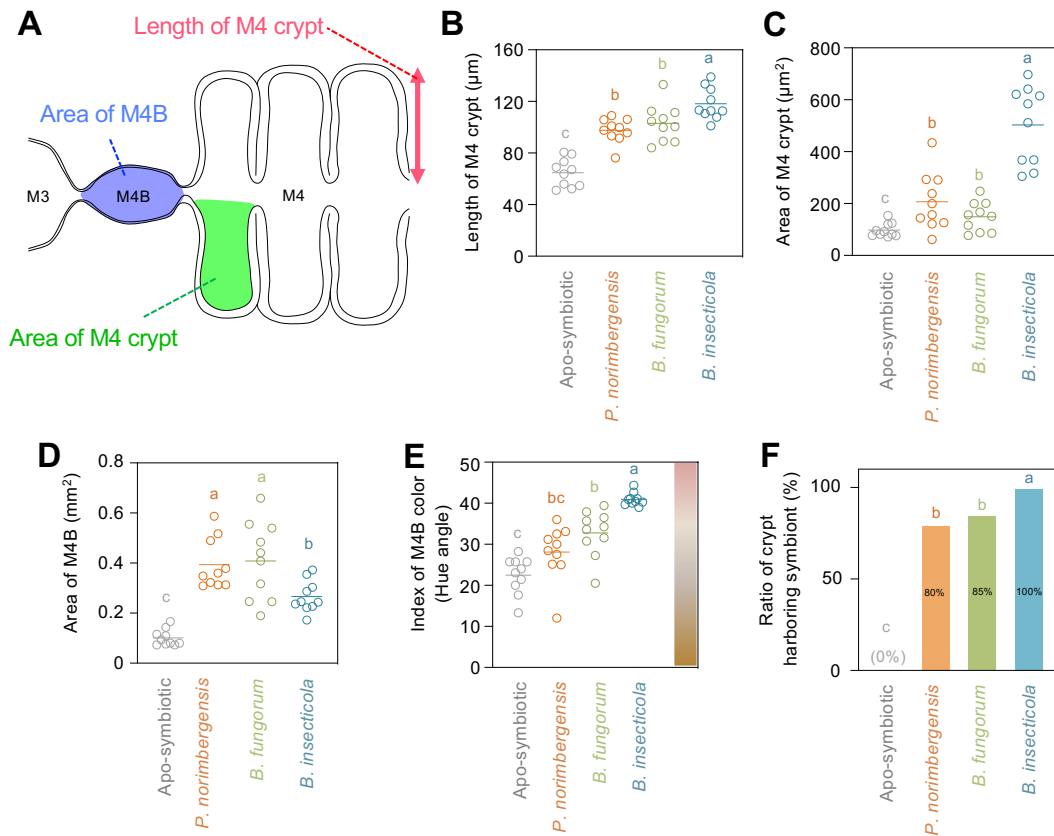


Fig. 2-5. Morphometric comparisons of M4B and M4 between insects infected with SBE, PBE, or *Pandoraea* species. Insects infected with either SBE (*B. insecticola*), PBE (*B. fungorum*), or *Pandoraea* (Pan: *P. norimbergensis*), as well as aposymbiotic insects (Apo), were compared. **(A)** A schematic image of morphometric measurements of the symbiotic organ. Length of M4 crypt (red arrow), area of M4 crypt (green part), and area of M4B (blue part) were measured by ImageJ. **(B)** Length of M4 crypts. Each dot indicates the average of ten randomly-selected crypts of an insect. **(C)** Area of M4 crypts. Each dot indicates the average of ten randomly-selected crypts of an insect. **(D)** Area of M4B. **(E)** Color index of M4B. Each dot indicates the average of hue angles of ten randomly-selected pixels of an M4B. The corresponding M4B color is shown on the right side. **(F)** Ratio of infected crypts. In LSM images of insects infected with a GFP-labelled strain, forty crypts were randomly selected and the infection ratio was calculated as (number of infected crypts/total number of crypts). Different letters indicate statistically significant differences ($P < 0.05$), which were calculated by Brunner-Munzel test **(B)**, Mann-Whitney U-test **(C-E)**, chi-square test **(F)** after the Bonferroni correction.



Fig. 2-6. TEM images of bacterial cells colonizing the M4 crypts. The crypt luminal region of 3rd instar nymphs (5 days post inoculation) infected with SBE (*B. insecticola*), PBE (*B. fungorum*), or *Pandora* (*P. norimbergensis*) is shown.

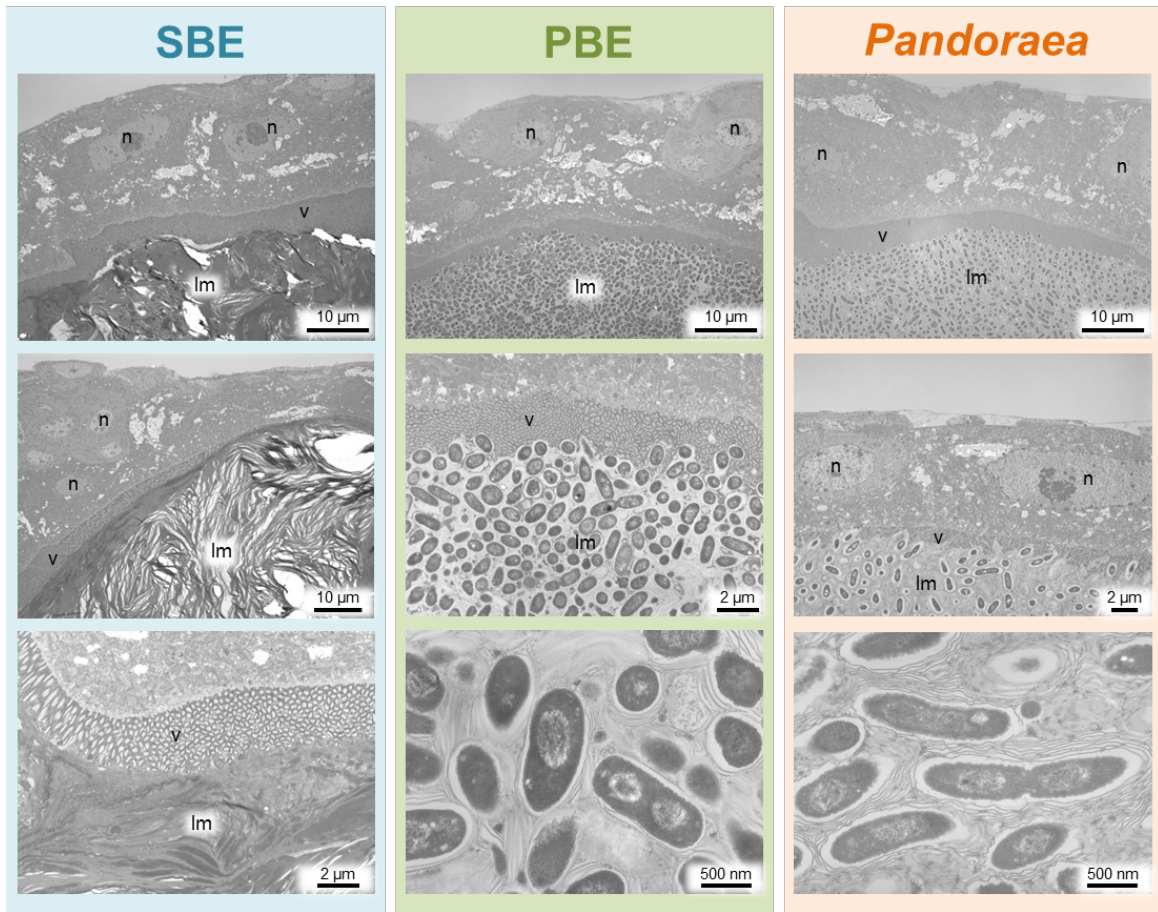


Fig. 2-7. TEM images of M4B infected with SBE, PBE, or *Pandoraea* species. M4B of 3rd instar nymphs (5 days post inoculation) infected with SBE (*B. insecticola*), PBE (*B. fungorum*), or *Pandoraea* (*P. norimbergensis*). Abbreviations: lm, luminal region; n, host nucleus; v, villus. In SBE-infected insects, no bacterial cells but mucus-like matrix is observed. Many PBE and *Pandoraea* cells are detected in M4B.

whitish color of M4B. These morphological features, *i.e.* smaller M4 crypts and swollen and whitish M4B, were consistently observed in insects infected with other PBE species (Fig. 2-8, 2-10 and Table 2-2). PBE species with low infection rates did not alter morphogenesis of M4B and M4, which resembled the aposymbiotic state. Thus, the infection rate and morphological alterations in M4 and M4B were highly correlated (Fig. 2-8D and E), suggesting that bacterial infection and proliferation in the gut symbiotic organ stimulate the morphological change.

BCC&P *Burkholderia* and other *Burkholderia* species

The seven tested BCC&P species, including two species of biosafety level 3 (BSL3) pathogens, *B. mallei* and *B. pseudomallei*, consistently showed a low, 0%-40%, infection rate (Table 2-2). In these BCC&P group, even when infection was detected by diagnostic PCR, the M4 and M4B were small and not well-developed as is the case in aposymbiotic insects (Fig. 2-8 and 2-11). GFP-labelled *B. glumae* and *B. pyrrocinia* confirmed that the BCC&P species can penetrate the constricted region and reach the M4 region (Fig. 2-13), although they did not stably accommodate inside the crypts. Other *Burkholderia* species of independent clades, *B. andropogonis* and *B. caryophylli*, showed no infection (Fig. 2-8, 2-11 and Table 2-2).

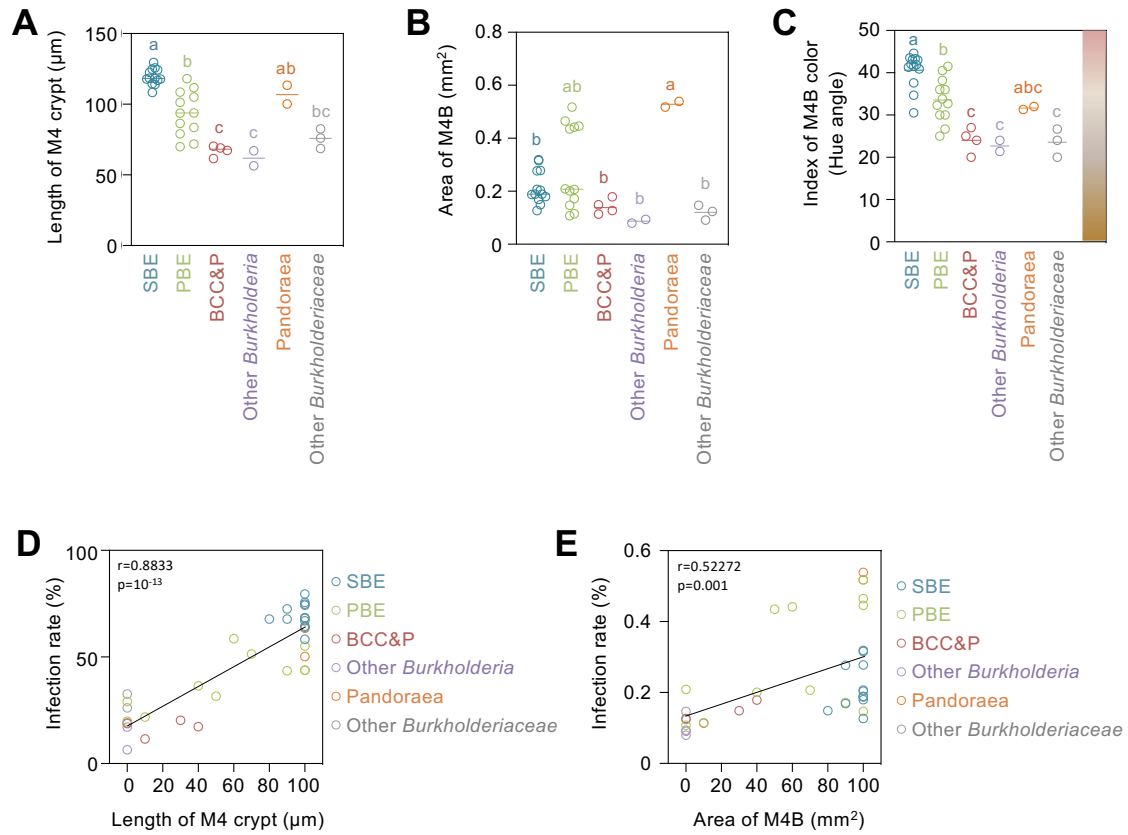


Fig. 2-8. Morphometric comparisons of M4B and M4 between insects infected with diverse *Burkholderiaceae* species. Insects infected with *Burkholderiaceae* species listed in Table 2-2 were subjected to morphometric measurements by Image J, as described in Fig 2-15. Each dot indicates a parameter measured from an insect infected with one of the *Burkholderiaceae* species. Results are summarized in Table 2-2. **(A)** Length of M4 crypt. Each dot indicates the average of ten randomly-selected crypts of an insect. **(B)** Area of M4B. **(C)** Color index of M4B. Each dot indicates the average of hue angles of ten randomly-selected pixels of an M4B. The corresponding M4B color is shown on the right side. Different letters indicate statistically significant differences ($P < 0.05$), which were calculated by Mann-Whitney U-test (**A** and **B**) and Brunner-Munzel test (**C**) after the Bonferroni correction. **(D)** Correlation between the infection rate and length of M4 crypts. **(E)** Correlation between the infection rate and area of M4B. R square (r) indicates Spearman's correlation coefficient.

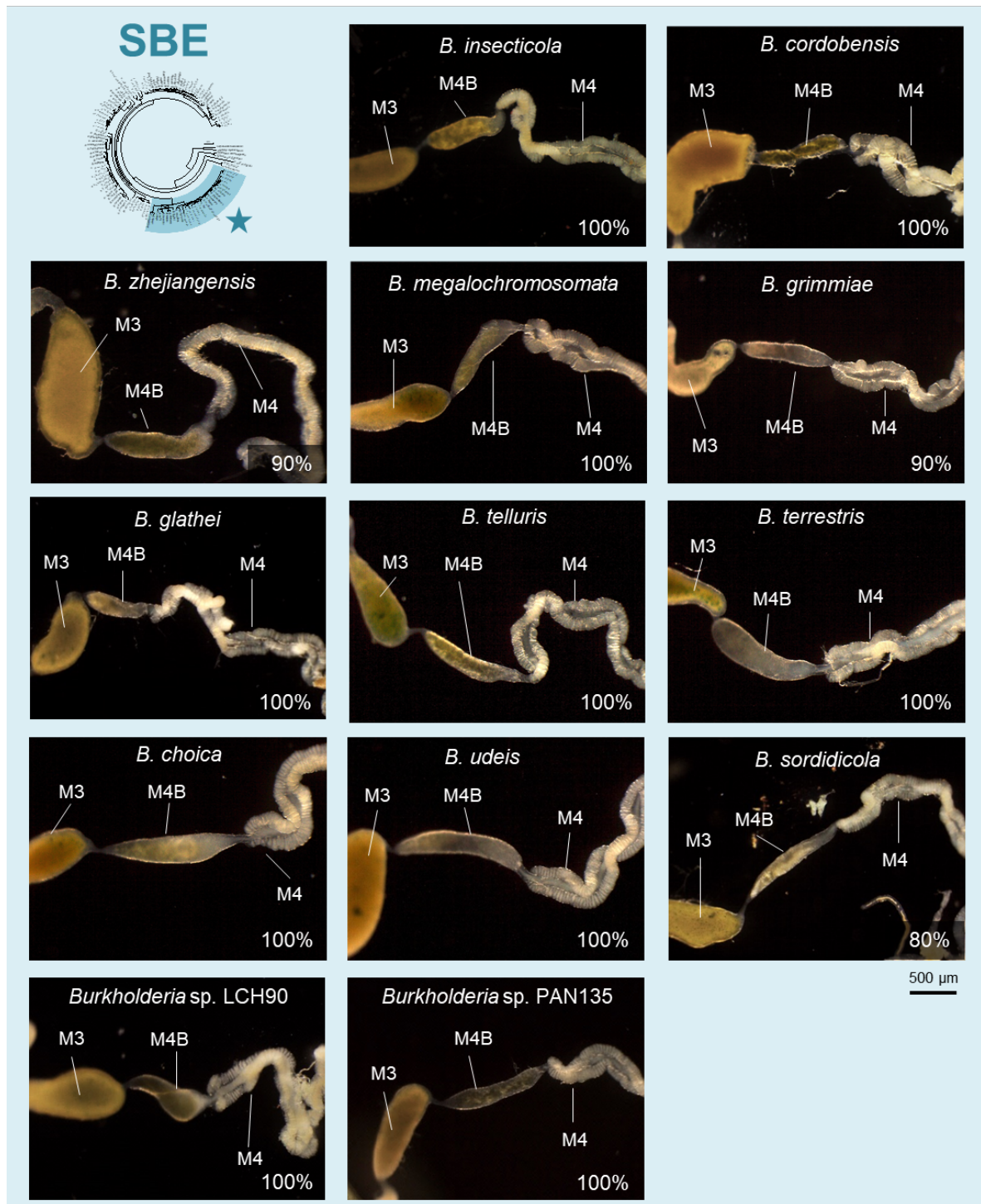


Fig. 2-9. Infection states of SBE species. The symbiotic region (M4B and M4) at five days post inoculation (5 dpi) of insects infected with 13 SBE species. Overall, the infection rate is remarkably high, M4B becomes bulbous and yellowish and M4 crypts are well-developed. The infection rate (%), which is depicted at the bottom right of each image, was determined by diagnostic PCR of 10 tested individuals.

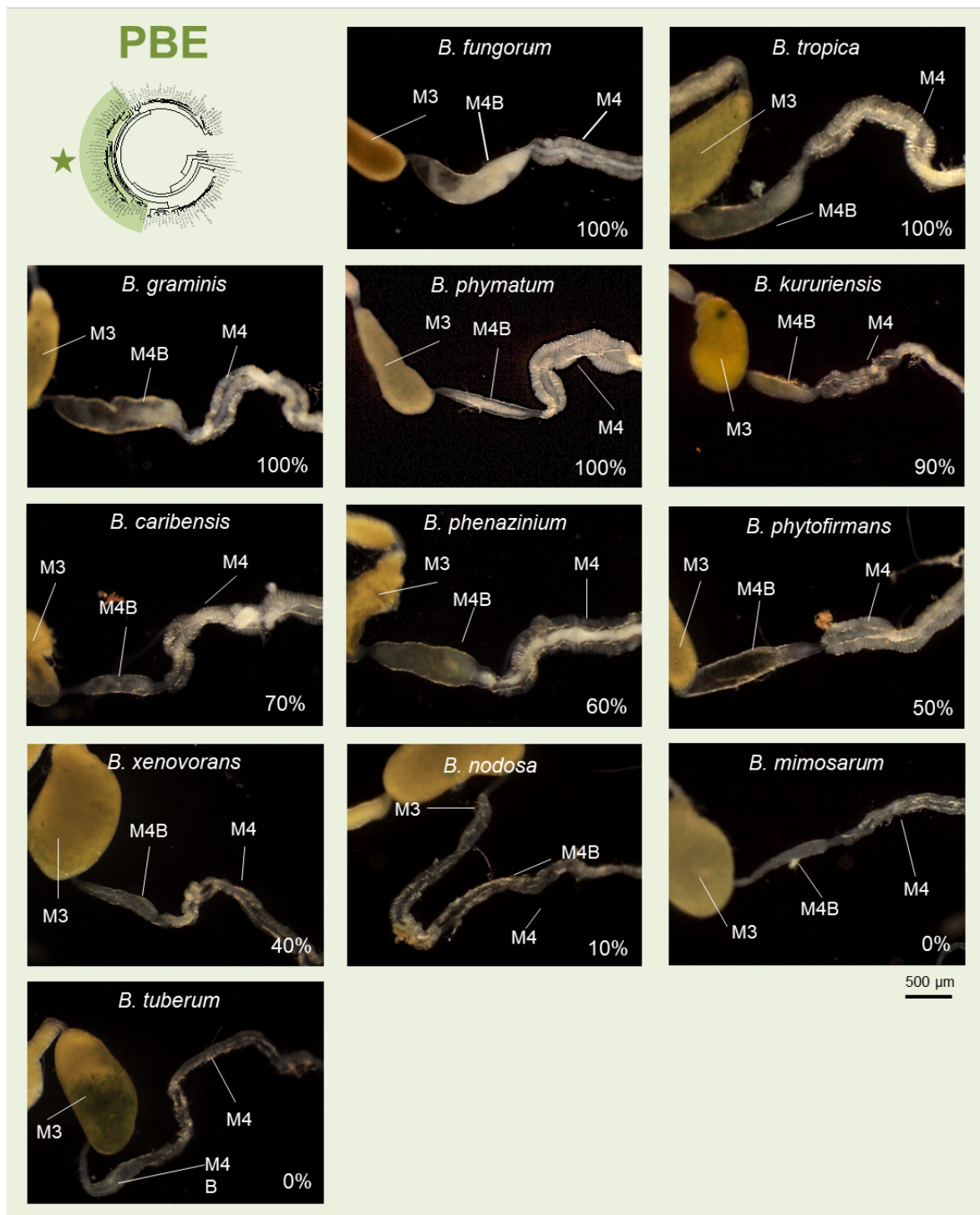


Fig. 2-10. Infection states of PBE species. The symbiotic region (M4B and M4) at five days post inoculation (5 dpi) of insects infected with 12 PBE species. The infection rate is variable among these species; if infection-positive, M4B tends to become bulbous and whitish, M4 crypts are infected but less-developed. The infection rate (%), which is depicted at the bottom right of each image, was determined by diagnostic PCR of 10 tested individuals.

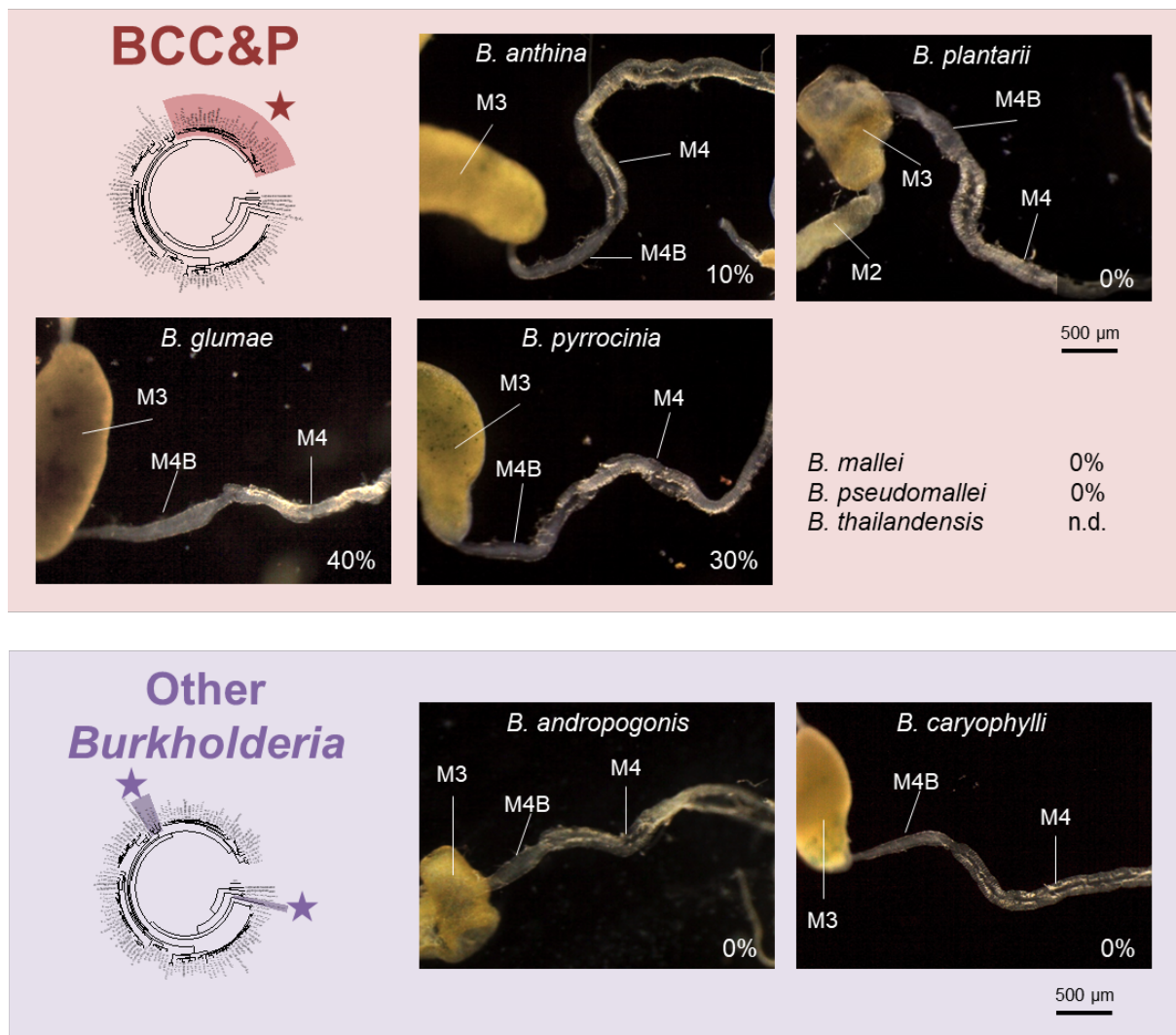


Fig. 2-11. Infection states of BCC&P and other *Burkholderia* species. The symbiotic region (M4B and M4) at five days post inoculation (5 dpi) of insects infected with 5 BCC&P species and 2 species of the other 2 clades of *Burkholderia*. The infection rate is very low and the organs are not developed as is the case with aposymbiotic ones. The infection rate (%), which is depicted at the bottom right of each image, was determined by diagnostic PCR of 10 tested individuals. No pictures are available for the BSL3 pathogens *B. mallei* and *B. pseudomallei*, and for the insecticidal species *B. thailandensis*.

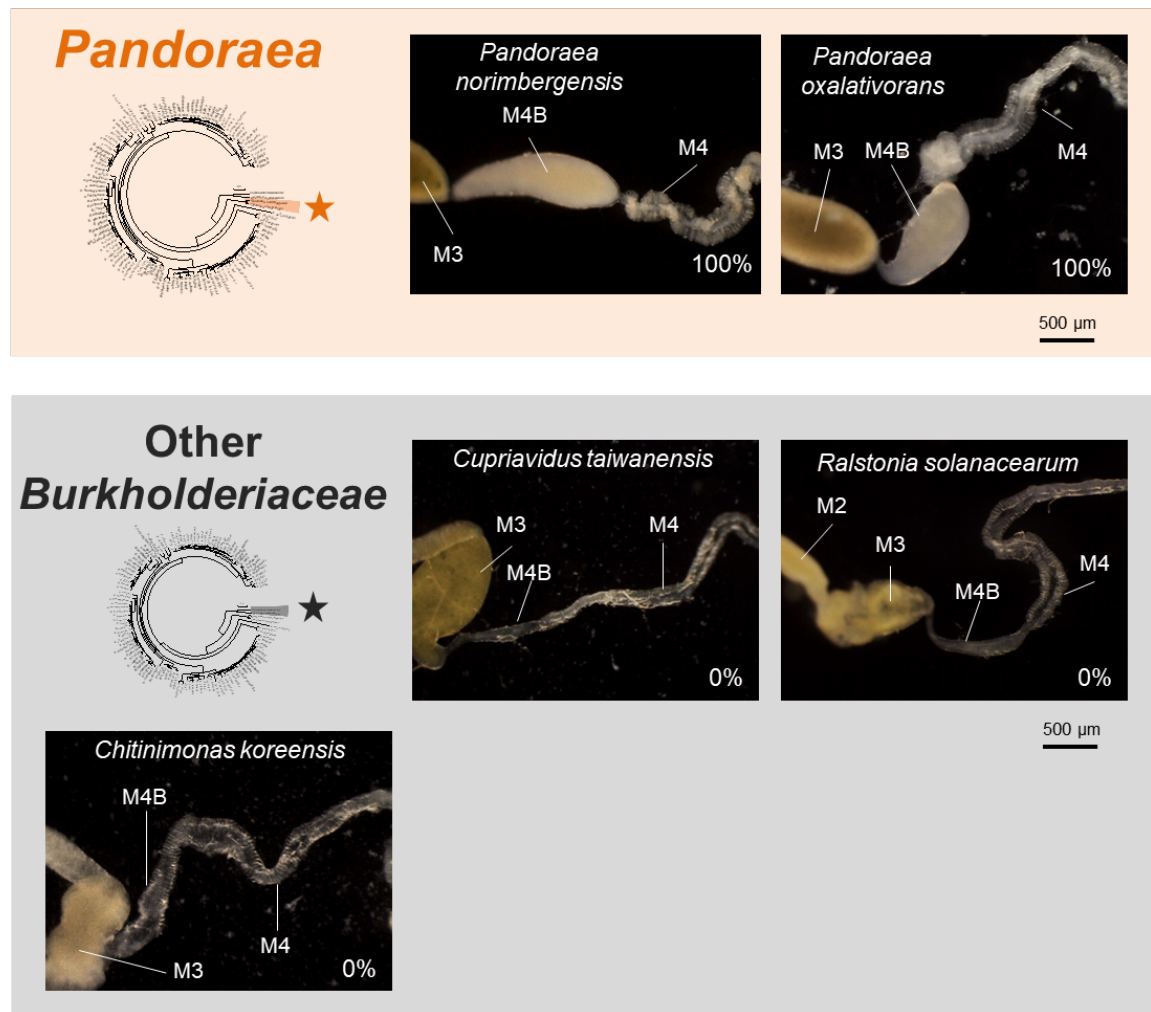


Fig. 2-12. Infection states of *Pandoraaea* and other *Burkholderiaceae* species. The symbiotic region (M4B and M4) at five days post inoculation (5 dpi) of insects infected with 2 *Pandoraaea* species and 3 species of other *Burkholderiaceae*. *Pandoraaea* species show 100% infection, the M4B is bulbous and whitish, and M4 is less-developed. Other *Burkholderiaceae* species show no infection. The infection rate (%), which is depicted at the bottom right of each image, was determined by diagnostic PCR of 10 tested individuals.

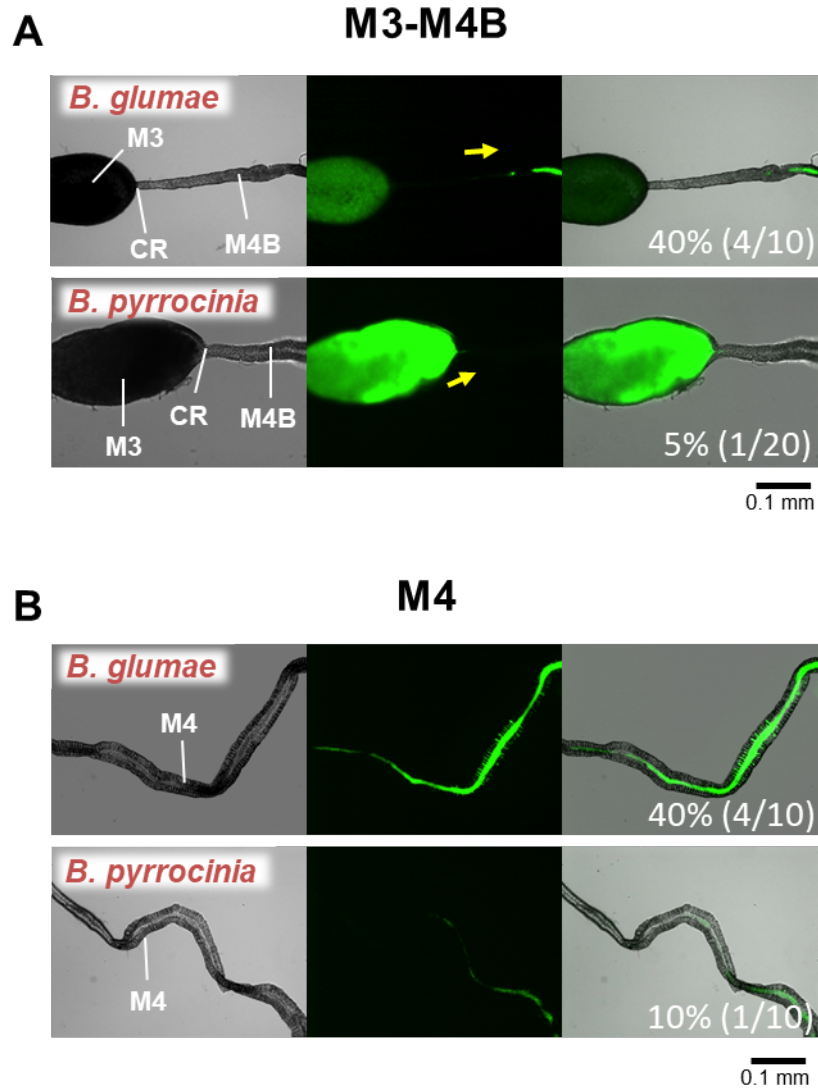


Fig. 2-13. Infection initiation process of *B. glumae* and *B. pyrrocinia*, visualized by confocal microscopy. (A) M3 and M4B 12 h after the oral administration of each strain. The infecting bacteria were labeled with GFP. *B. glumae* and *B. pyrrocinia* passed through the constricted region, although infrequently. Arrows indicate an infection thread invading from M3 to M4B through the constricted region. (B) M4 48 h after the oral administration of each strain. Left, middle, and right panels show the bright field, GFP, and merged images, respectively. The infection rate (%), which is depicted at the bottom right of right panels, was determined by GFP signal observation of 10 tested individuals. Abbreviations: M3, midgut 3rd section; M4B, M4 bulb; CR, constricted region.

Pandoraea and other *Burkholderiaceae*

Both tested *Pandoraea* species, *P. norimbergensis* and *P. oxalativorans*, showed a 100% infection rate (Table 2-2). Similar to PBE *Burkholderia*, M4 crypts were infected but not properly developed and the M4B was strongly swollen in shape but whitish in color (Fig. 2-4D, 2-5, and 2-12). LSM and TEM observations revealed that *Pandoraea* were abundant in the central duct of M4 but only partially colonized the crypt lumen (Fig. 2-4D and 2-5F). TEM observations revealed that M4 colonized cells were typical rods (Fig. 2-6) and *Pandoraea* cells densely colonized M4B (Fig. 2-7), similar to PBE *Burkholderia*, suggesting that the *Pandoraea* is also digestion tolerant and the colonizing cells cause the swelling and whitish color of M4B. Other *Burkholderiaceae* species, *Cupriavidus taiwanensis*, *Ralstonia solanacearum* and *Chitinomonas koreensis*, did not infect the symbiotic organ (Fig. 2-8, 2-12, and Table 2-2).

PBE *Burkholderia* and *Pandoraea* are beneficial for the bean bug

The inoculation tests revealed that many PBE *Burkholderia* species and even the outgroup, *Pandoraea*, passed through the constricted region and stably accommodated in the M4 region, although they did not fully colonize the crypt lumen. To determine if the PBE and *Pandoraea* species are beneficial for the bean bug, fitness parameters of the insect host (survival rate, growth period, body size and weight) were measured after infection. *B. insecticola* (SBE), *B. fungorum* (PBE) and *P. norimbergensis* (*Pandoraea*), all of which show a 100% infection rate, were tested. Compared with aposymbiotic insects, insects infected with either of these strains showed significantly higher survival rates, shorter

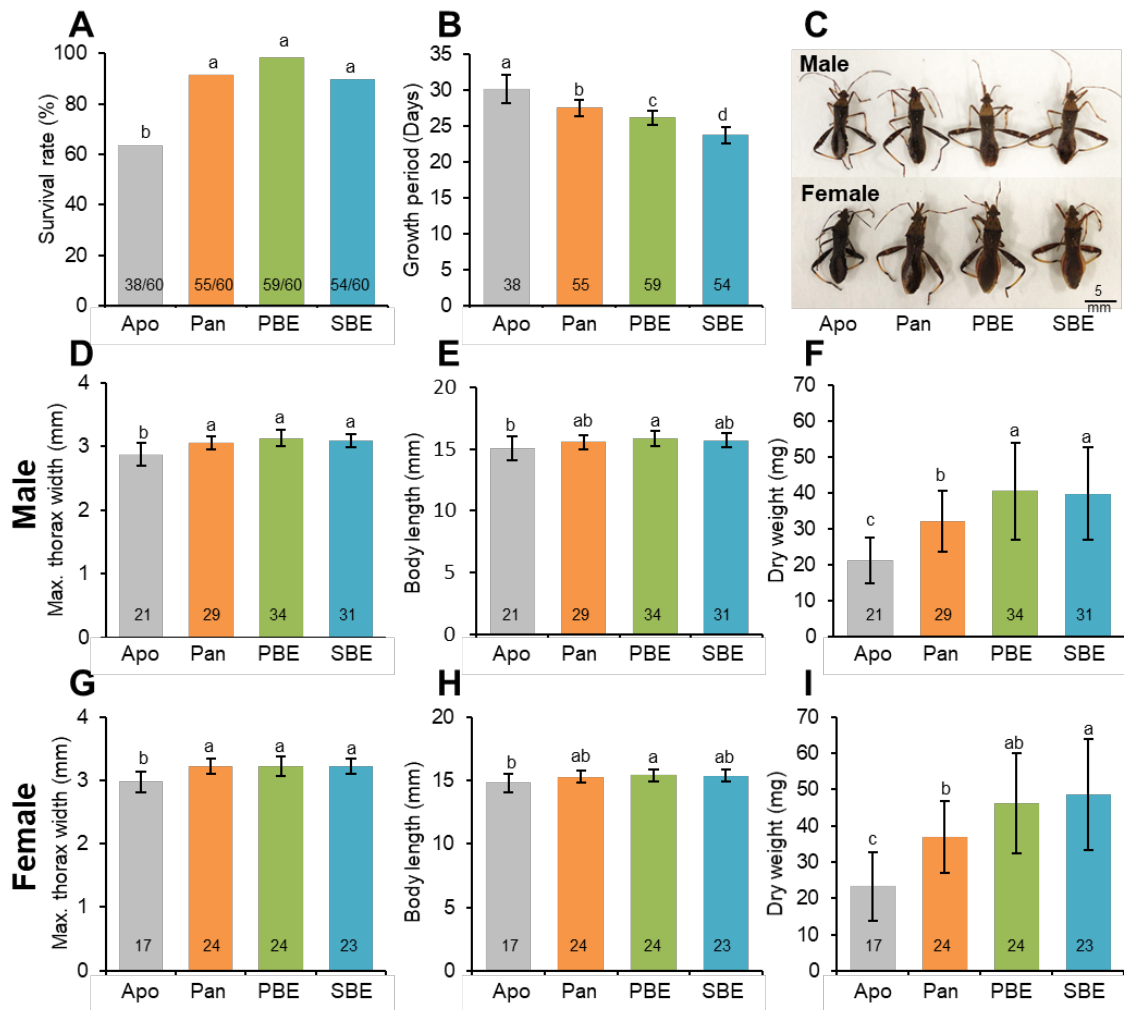


Fig. 2-14. Fitness effect of symbiotic and non-symbiotic bacteria in the bean bug. Comparison of fitness parameters between aposymbiotic insects (Apo) and insects infected with *Pandoraea* (Pan: *P. norimbergensis*), PBE (*B. fungorum*), and SBE (*B. insecticola*). (A) Survival rate (=adult emergence rate), (B) time to adulthood, (C) photographs of male and female adults. (D-F) Male insects: (D) maximum thorax width, (E) body length, (F) dry weight. (G-I) Female insects: (G) maximum thorax width, (H) body length, and (I) dry weight. Different letters indicate statistically significant differences ($P < 0.05$). Error bars indicate standard deviations. Number of replicates are depicted on the bars.

developmental time before adulthood, larger body size, and heavier body weight (Fig. 2-14). However, insects infected with the *Pandoraea* showed nevertheless a slower developmental time and smaller body weight than those infected with the SBE *Burkholderia* (Fig. 2-14) while insects infected with the PBE species showed an almost identical body size and weight to those with SBE *Burkholderia*, although the growth period was significantly delayed (Fig. 2-14). Together, the rearing experiments clearly demonstrated that the non-native bacteria *B. fungorum* and *P. norimbergensis* are not parasitic but enhance survivability, growth, and development of the host insect although to a slightly lesser extent than the native symbiont *B. insecticola*.

SBE *Burkholderia* always outcompete PBE and *Pandoraea* in the gut symbiotic organ in co-infection assays

PBE *Burkholderia* and *Pandoraea* species are common bacterial groups in soil environments, and detected frequently with SBE *Burkholderia* (81, 136, 137). Given the high infectivity and benefits of PBE and *Pandoraea*, why do only SBE *Burkholderia* prevail in field populations of the bean bug? To clarify this point, colonization competitiveness of the PBE and *Pandoraea* against the SBE was investigated. For this analysis, *B. insecticola*, *B. fungorum* and *P. norimbergensis* were used as representatives of SBE, PBE and *Pandoraea*, respectively. To investigate *in vitro* competitiveness, log-phase cultured cells of the SBE [rifampicin resistance] and a competitor (PBE or *Pandoraea* [chloramphenicol resistance]) were suspended in YG medium, and incubated at 25°C without shaking for two days. The incubated medium was plated on YG agar

plates containing either rifampicin or chloramphenicol, and the competitive index (CI) was calculated as (output SBE CFU/ input SBE CFU)/ (output competitor CFU/ input competitor CFU) (80). To determine the *in vivo* competitiveness, newly molted second instar nymphs of *R. pedestris* were individually fed with 1 µl of distilled water containing the same CFUs of the SBE and a competitor (*B. fungorum* or *P. norimbergensis*). Two days after rearing, the symbiotic organs were dissected, their homogenates were plated on YG agar plates containing antibiotics, and CI values were calculated based on the CFU counts.

While the SBE *Burkholderia* did not dominate the two other species *in vitro* (Fig. 2-15A), the *in vivo* competitive assay revealed that the SBE *Burkholderia* significantly outcompeted the PBE species in the gut symbiotic organ, even in a high inoculation dose condition (Fig. 2-15B). Although the *Pandoraea* was competitive against the SBE under a high, 5,000 cells, infection dose, the SBE became significantly dominant under a lower, 250 cells, dose condition (Fig. 2-15B). Microscopic visualization of the competition process by use of an RFP-labeled *B. insecticola* (SBE) and GFP-labeled *B. fungorum* (PBE) or *P. norimbergensis* (*Pandoraea*) revealed that firstly the SBE and the non-native bacteria co-colonized the M4 region in a mixed manner, that the SBE then gradually gained the upper hand and that it eventually solely occupied the symbiotic organ in 5 days after co-inoculation (Fig. 2-15C and D). Similar results were consistently observed when the SBE species was co-inoculated with other PBE *Burkholderia* species (Fig. 2-16 and Table 2-3) and when another SBE species, *B. cordobensis*, was tested against the PBE and *Pandoraea* species (Fig. 2-17 and Table 2-3). These observations clearly demonstrate that the exclusive prevalence of SBE

Burkholderia in natural bean bug populations is supported by the higher competitiveness of SBE species inside the gut symbiotic organ.

Competitiveness in the crypt lumen is critical for the *Riptortus-Burkholderia* specificity

Our previous studies have revealed that the bean bug *Riptortus pedestris* develops a specific sorting organ called the “constricted region” in the midgut and employs in the M4 unique antimicrobial peptides to select bacteria (82, 120). This study confirmed that diverse bacterial species are winnowed out by the constricted region. However, the selective apparatus is not perfect, and bacteria that are closely-related to the natural symbiont, including some PBE *Burkholderia* and *Pandoraea* species, can penetrate the constricted region and reach the gut symbiotic organ (Fig. 2-3 and Table 2-2). Although the PBE and *Pandoraea* did not fully colonize the gut crypts (Fig. 2-4 and 2-5), these non-native bacteria stably accommodated in the M4 region and, surprisingly, were not harmful, but on the contrary, beneficial for the host survival, growth rate, and body size (Fig. 2-14). In contrast, SBE *Burkholderia* are highly adapted to the internal environment of the gut symbiotic organ and always outcompete PBE and *Pandoraea* species (Fig. 2-15 to 2-17). These results clearly demonstrated that the exclusive prevalence of SBE *Burkholderia* in natural bean bug populations is underpinned not only by host-mediated partner choice but also by the higher competitiveness of SBE species inside the gut symbiotic organ.

In symbiotic associations with environmental transmission, host organisms have evolved elaborate mechanisms for partner choice and sanctions of cheaters (114).

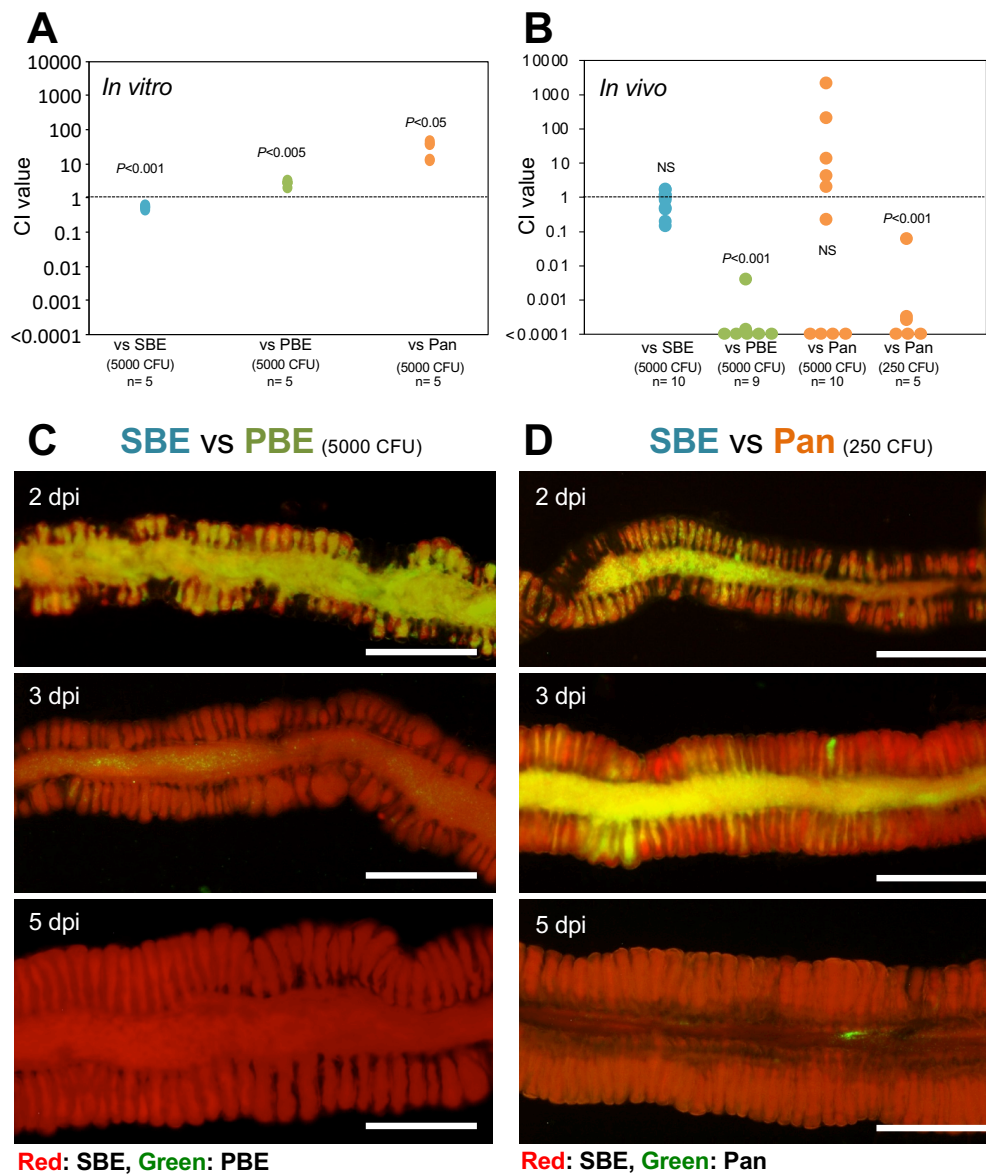


Fig. 2-15. Infection competitiveness of symbiotic bacteria against non-native bacteria. Competitiveness of SBE (*B. insecticola*) against PBE (*B. fungorum*) and *Pandora* (Pan: *P. norimbergensis*) in *in vitro* growth (A) and *in vivo* (B). Inoculum doses are indicated in CFU. Each dot represents a competitive index (CI) value from a microtube or an insect. The CI values are obtained by [output SBE colony count/input SBE colony count]/[output competitor colony count/input competitor colony count] and statistically evaluated by the one sample t test. NS, not significant. (C-D) Visualization of the *in vivo* competition dynamics. An RFP-labelled SBE strain was co-inoculated with a GFP-labelled strain of PBE (C) or *Pandora* (D). dpi: days post inoculation. Bars: 0.2 mm.

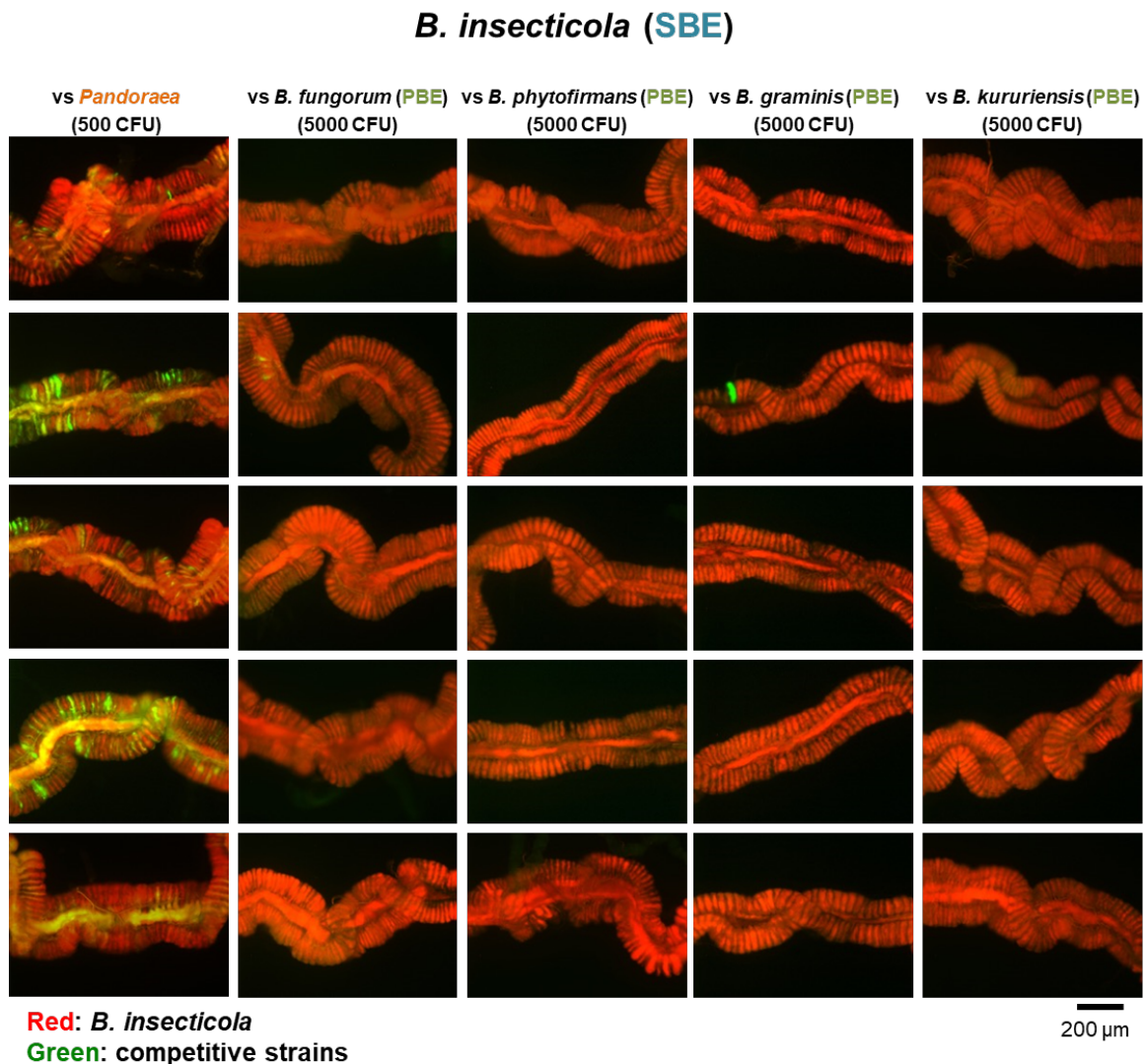


Fig. 2-16. Infection competitiveness of *B. insecticola* (SBE) against *Pandora* and PBE species. An RFP-labelled SBE species, *B. insecticola*, was co-inoculated with GFP-labelled *Pandora* (*P. norimbergensis*) or a PBE species. M4 was dissected at five days post inoculation (5 dpi; infected at 2nd instar and dissected at 3rd instar) and observed under a fluorescence microscope. Five individuals per competition assay were observed.

B. cordobensis (SBE)

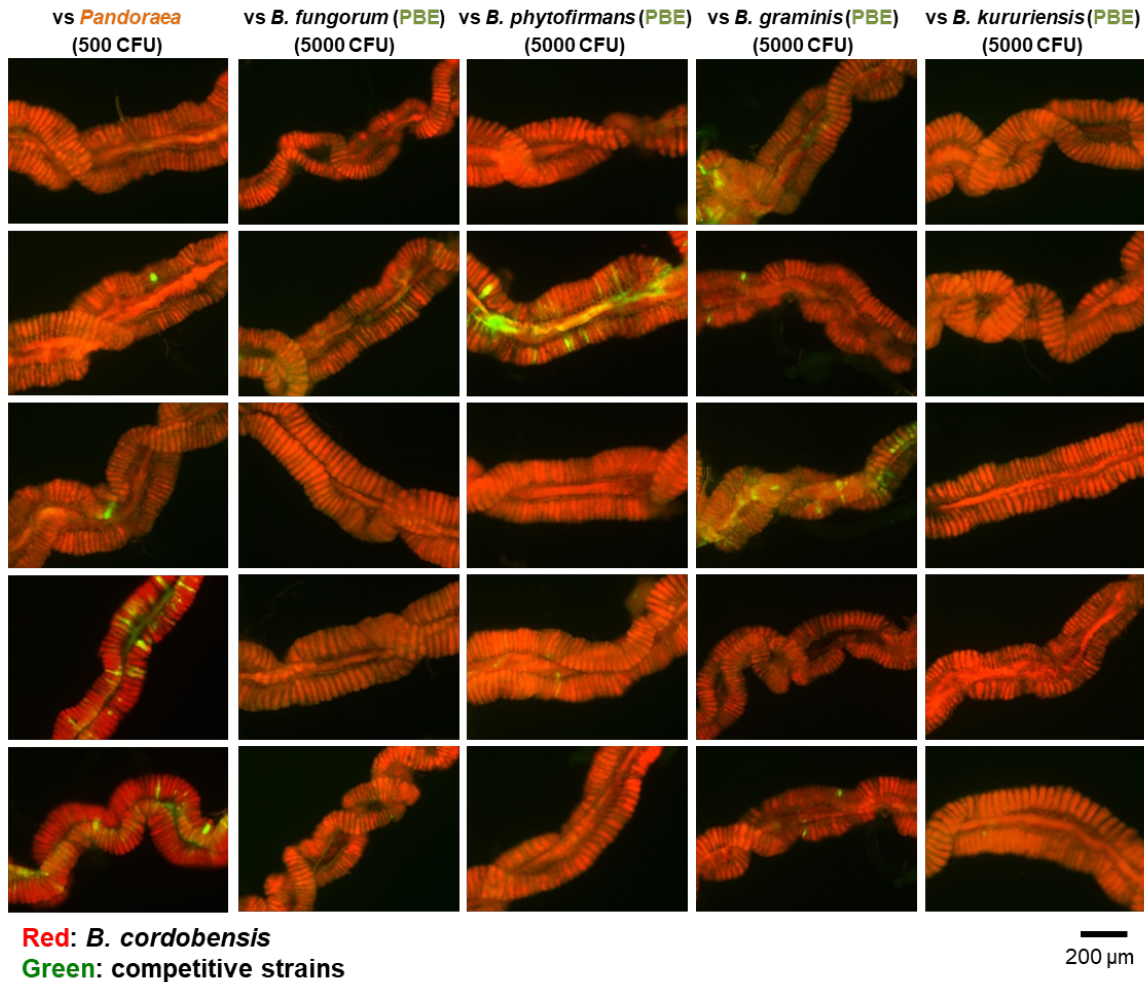


Fig. 2-17. Infection competitiveness of *B. cordobensis* (SBE) against *Pandoraea* and PBE species. The RFP-labelled SBE species *B. cordobensis* was co-inoculated with GFP-labelled *Pandoraea* (*P. norimbergensis*) or a PBE species. The M4 was dissected at five days post inoculation (5 dpi; infected at 2nd instar and dissected at 3rd instar) and observed under a fluorescence microscope. Five individuals per competition assay were observed.

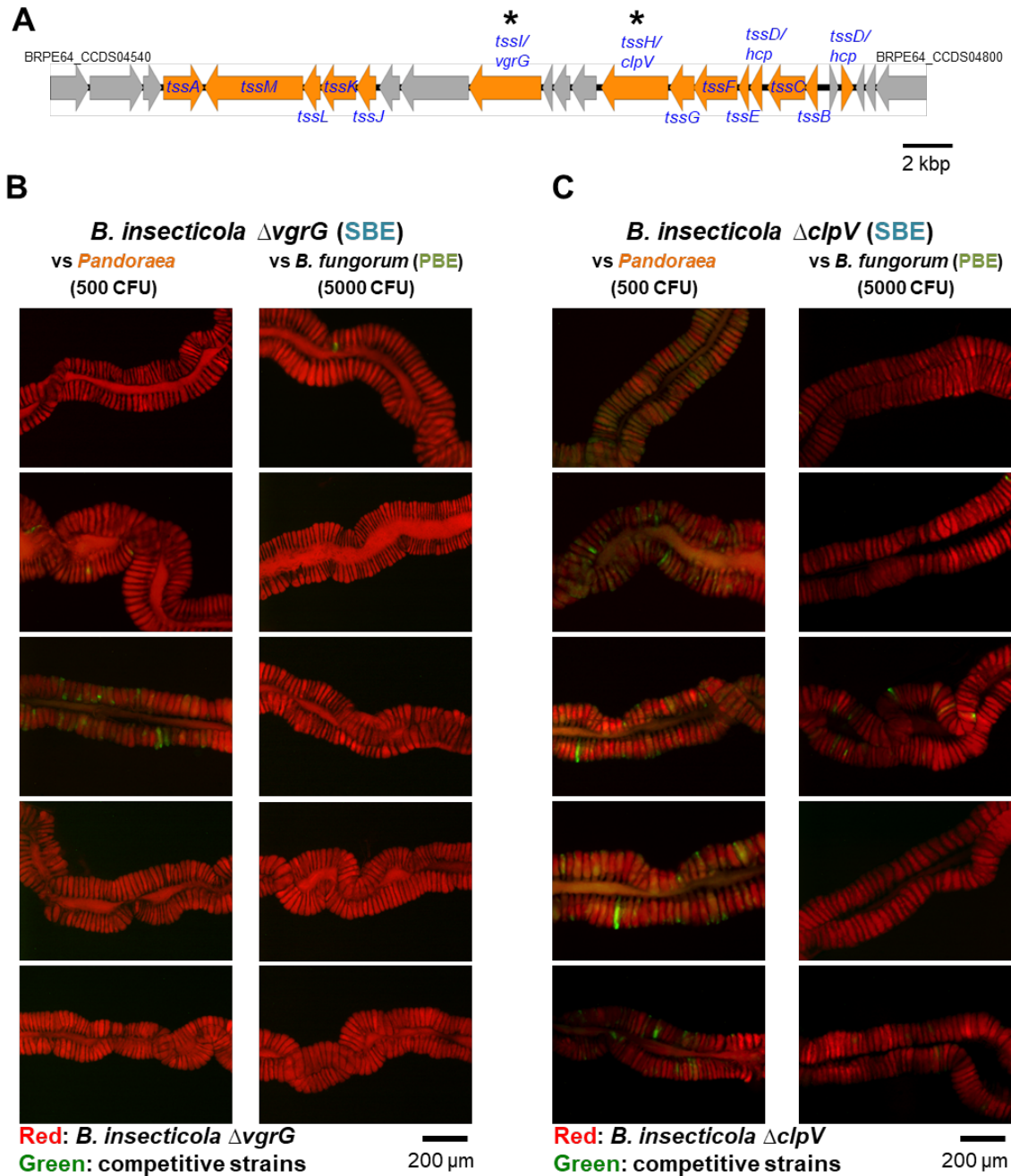


Fig. 2-18. T6SS mutants of *B. insecticola* (SBE) outcompete *Pandora* and PBE species.

(A) T6SS gene cluster of *B. insecticola* RPE64 (Localization: Chr. 3 (NC_021288): 455343..4898). RPE64 has only a single intact gene cluster for T6SS. In this study, two genes, *vgrG* and *clpV* (asterisks), were completely deleted. $\Delta vgrG$ mutant **(B)** and $\Delta clpV$ mutant **(C)** were RFP-labelled and then co-inoculated with GFP-labelled *Pandora* (*P. norimbergensis*) or PBE (*B. fungorum*). The M4 was dissected at five days post inoculation (5 dpi; infected at 2nd instar and dissected at 3rd instar) and observed under a fluorescence microscope. Five individuals per competition assay were observed.

Thus to establish a specific association with their hosts, symbiotic microorganisms have to be capable of passing a sorting mechanism [initiation], colonizing the symbiotic organ [accommodation], and behaving cooperatively in the hosts [cooperation]. Mutualists are generally characterized by these features. Strikingly, the inoculation of a broad range of *Burkholderia* and allied species revealed that these fundamental features of symbiotic microorganisms are not restricted to SBE but shared by PBE and even the outgroup *Pandoraea*, indicating that these symbiotic traits are ancestral (Fig. 2-19). By contrast, the advanced adaptation to and strong competitiveness in the gut symbiotic organ evolved exclusively in the SBE lineage (Fig. 2-19). These features enable the SBE *Burkholderia* to dominantly and stably occupy the symbiotic gut portion of the bean bug and underpin the host-symbiont specificity. Previous studies on the SBE species *B. insecticola* have revealed some of the genetic bases for the initiation, accommodation, and cooperation, all of which are conserved in PBE and *Pandoraea* species: flagellar motility for initiation (120); polyhydroxyalkanoate/purine/cell-wall/LPS biosynthesis for accommodation (100, 102, 103, 106); nitrogen and sulfur recycling for cooperation (109). In how far differential activity of these functions in competitive and non-competitive strains contributes to the colonization competitiveness will deserve attention in future studies.

At this stage, it remains also unclear where and how the competitive traits have evolved in the SBE lineage. It is plausible that the competitive traits have been selected in the insect gut. This scenario requires that the SBE *Burkholderia* gain fitness through the symbiotic association, for example by an efficient re-colonization of the soil environment from the insect midgut crypts. Although this aspect of the symbiosis is

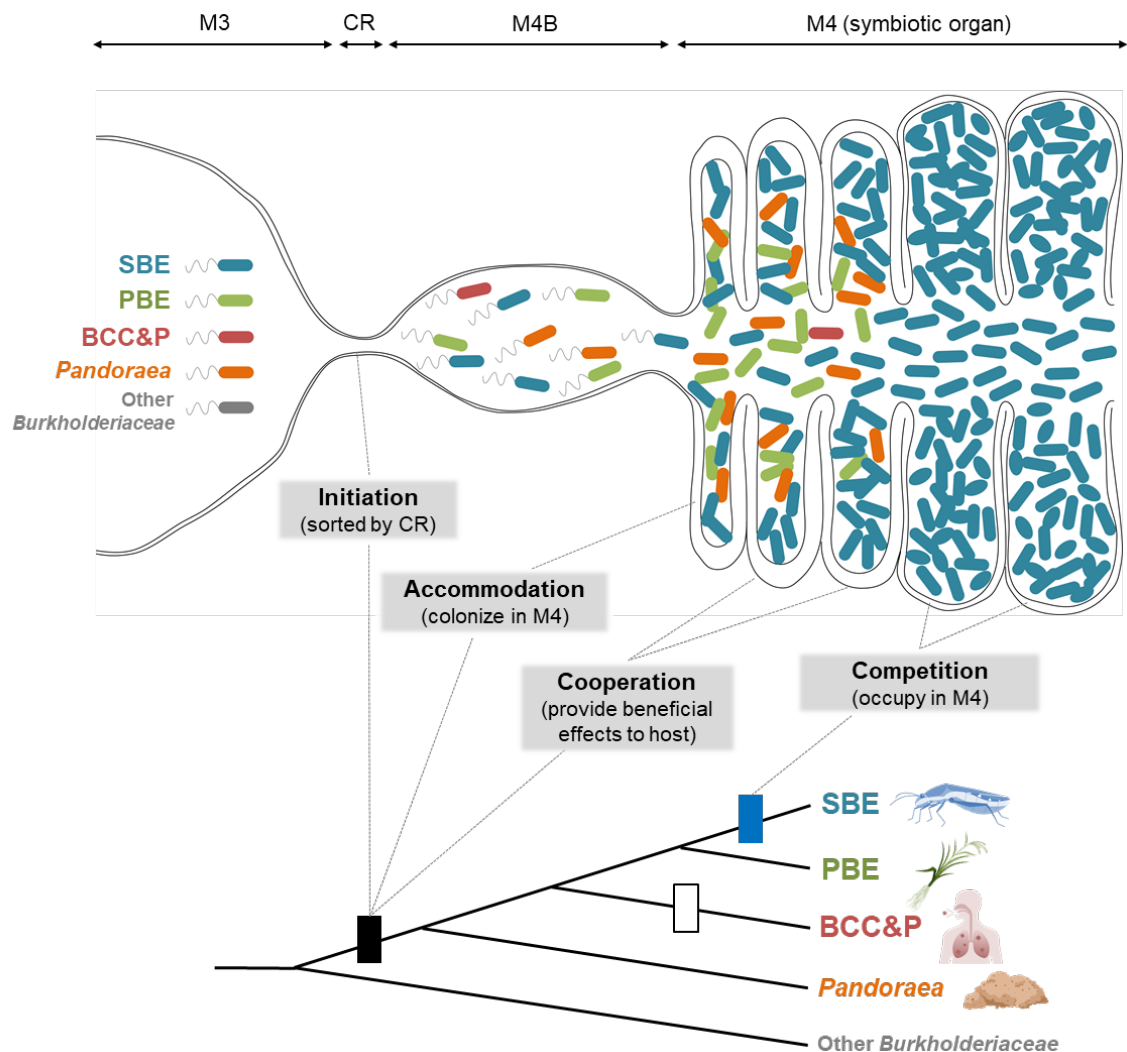


Fig. 2-19. Evolutionary hypothesis of the symbiotic traits in the *Burkholderiaceae*. Abilities for initiation, accommodation, and cooperation in the bean bug gut have evolved in the common ancestor of the genera *Pandoraea* and *Burkholderia* (black box in the dendrogram), and only the competitiveness in the gut symbiotic organ has evolved in the SBE lineage (blue box in the dendrogram). These traits, except the initiation ability, are lost in BCC&P (white box in the dendrogram).

currently poorly investigated (30), recolonization of the soil does not seem impossible because in all stages of the insect life cycle, a high number of viable bacteria are present in the posterior midgut. Alternatively, the competitive traits may have evolved due to selection on SBE *Burkholderia* lifestyles in a different environment, such as soil and rhizosphere, and the symbiont could outcompete other bacteria in the M4 incidentally. To clarify this point, the symbiont's benefit gained from the association should be investigated in forthcoming work.

Possible mechanisms underpinning the competitiveness

The molecular mechanisms supporting the competitiveness of SBE in the gut symbiotic organ are at present unclear. Possible mechanisms for the competitiveness could be categorized into two groups: direct and indirect mechanisms. In the former mechanism, symbionts directly interfere with competitors, while in the latter mechanism, symbionts are selected by host-served specific conditions, such as nutrients and antimicrobial agents. As for the former mechanism, the T6SS is known as a competition factor in the squid-*Vibrio* system and honey bee gut microbiota (138, 139). However, the T6SS of SBE *Burkholderia* is strongly repressed *in vivo* (109) and T6SS mutants outcompeted PBE and *Pandoraea* species similarly as the wild type (Fig. 2-18), strongly suggesting that the T6SS is not involved in the *in vivo* competitiveness of SBE *Burkholderia*. In addition, SBE species lack the contact-dependent growth inhibition (CDI) system, which is also known as a microbe-microbe toxin delivery system in some *Burkholderia* (140).

Taking into account the histological observations, the latter, indirect

competition mechanism, seems more plausible to the bean bug system. The microscopic observations revealed that PBE and *Pandoraea* colonize and proliferate well in M4B and the central tract of M4 but show a colonization defect inside crypts (Fig. 2-4), suggesting that the environment of the crypt lumen is different from the central tract of the M4 and is highly specialized for housing SBE *Burkholderia*. This could contribute to the *in vivo* competitiveness of the symbiont. Although speculative, SBE *Burkholderia* may efficiently utilize nutrients served by the host in the crypt lumen, and also adapt to specific chemical conditions (e.g. pH, osmolarity and/or oxygen concentration) of the luminal environment. Since diverse crypt-specific antimicrobial peptides are highly expressed (82), it is also plausible that SBE *Burkholderia* are more tolerant to the host gut immunity than the less-competitive species. Furthermore, theoretical studies have pointed out that the adhesion ability is an additional pivotal character to determine the competitiveness and stability of a bacterium in complex microbiota developing on a surface substrate, such as biofilms and intestinal flora (141). As an alternative mechanism, therefore, SBE *Burkholderia* may have an adhesin-mediated higher affinity to the crypt epithelia. Future genome comparisons in conjunction with gene-deletion or –transfer experiments could lead to the identification of key genes responsible for the SBE-specific colonization and competitiveness in the midgut crypts.

Diversity of the symbiont specificity in stinkbugs

In addition to the bean bug, other heteropteran species of the superfamilies Coreoidea and Lygaeoidea and the family Largidae are commonly associated with *Burkholderia* in the

midgut crypts. Although the bean bug and most members of the Coreoidea and Lygaeoidea are exclusively associated with SBE *Burkholderia* (126), some species are associated mainly with SBE but to a lesser extent also with PBE and even with BCC&P (142–146). In addition, members of the Largidae harbor a specific group of PBE *Burkholderia* (147, 148). These previous surveys support the hypothesis that the symbiotic traits were ancestral in the genus *Burkholderia*, and also suggest that the competition-based selection favors different *Burkholderia* clades in different stinkbug taxa.

Competition-based selection of an optimal symbiont

Theoretical studies predicted the importance of host control (i.e. partner choice and partner fidelity) for the evolution and maintenance of mutualistic associations (149). More recent modeling studies proposed, furthermore, competition-based mechanisms as an alternative to assure symbiont quality (117, 118, 141, 150), where the host serves as a selective environment to let a favorable microorganism grow preferentially. We here point out experimentally the importance of this mechanism in the *Riptortus-Burkholderia* symbiotic system. Recent transcriptomic analyses revealed that SBE *Burkholderia* recycle the host's metabolic wastes into utilizable nutrients such as essential amino acids and B vitamins (109). Combined with histological data, the gene expression patterns in the symbiotic organ further suggest that the *Burkholderia* symbiont proliferates in the M4 crypts and that excess symbiont cells move into the M4B region, where they are entirely digested and all the derived nutrients absorbed by the host (82, 109). In other words, the

bean bug cultivates the symbiont in the M4 and digests them in the M4B. In such a “cultivation symbiosis”, growth rate and regeneration efficiency of host metabolites in the symbiotic organ seem to be directly linked to the host fitness, whereby the host could gain a significant benefit from the better grower. If so, we could speculate that the host insect facilitates microbial competition inside the symbiotic organ not only for winnowing out potential parasites but also for selecting a better grower. In this context, it is notable that SBE showed greater benefits to the host than PBE and *Pandora* species in terms of growth rate and body weight (Fig. 2-14). Moreover, the apparent absence of bacterial degradation in the M4B in PBE or *Pandora* infected insects (Fig. 2-6) could be a further reason for the reduced benefits provided by these bacteria.

A competition-based symbiont selection plays also a pivotal role in the fungus-cultivating ambrosia beetles (151), suggesting that in “cultivation symbiosis”, where hosts harvest growing symbionts, the competition-based mechanism is commonly important not only for keeping symbiont quality but also maybe for selecting the better-growing symbionts. More generally, broader surveys could clarify the general impact of microbe-microbe competition in the evolution and stabilization of symbiotic associations maintained via environmental symbiont transmission as suggested by recent examples (138, 139).

The genus *Burkholderia* (*Burkholderia*/*Paraburkholderia*/*Caballeronia*) includes a variety of symbiotic lifestyles, such as the nodule symbionts in leguminous plants and farming symbionts in the social amoeba as well as the gut symbionts in stinkbugs, in which the molecular mechanisms underpinning the specificity (and probably importance of symbiont’s competitiveness) seem different between each system (152,

153). The amazing diversity of symbiotic lifestyle in this monophyletic group would provide a great opportunity to find out the different paths a microorganism can take to become a symbiont in the competitive microbial world.

Chapter III

**Dual oxidase enables insect gut symbiosis by
mediating respiratory network formation**

Abstract

Most animals harbor a gut microbiota that consists of potentially pathogenic, commensal and mutualistic microorganisms. Dual oxidase (Duox) is a well-described enzyme, involved in gut mucosal immunity by the production of reactive oxygen species that antagonizes pathogenic bacteria and maintains gut homeostasis in insects. However, despite its non-specific harmful activity on microorganisms, little is known about the role of Duox in the maintenance of mutualistic gut symbionts. Here we show that in the bean bug *Riptortus pedestris*, Duox-dependent ROS did not directly contribute to epithelial immunity in the midgut in response to its mutualistic symbiont, *Burkholderia insecticola*. Instead, we found that the expression of Duox is tracheae-specific and its downregulation by RNAi results in the loss of dityrosine crosslinks in the tracheal protein matrix, a collapse of the respiratory system and a disruption of the gut symbiosis. Downregulation of the hypoxia-responsive transcription factor Sima or the regulators of tracheae formation Trachealess and Branchless produces similar phenotypes. Thus, in addition to known roles in immunity and in the formation of diverse extracellular matrices, Duox is also a crucial enzyme for tracheae stability by establishing a dityrosine network of luminal matrix proteins, which maintains mutualistic symbionts and gut homeostasis. We expect that this is a conserved function in insects.

Introduction

Many animals possess a gut microbiota that is composed of pathogenic, commensal and mutualistic microorganisms (154, 155). In insects, gut symbiotic microorganisms play a broad range of physiological roles in host metabolism and immunity through digestion of food, supplementation of essential nutrients that are scarce in food, degradation of phytotoxins and pesticides, and prevention of pathogen invasion by stimulation of the host immune system, production of antibiotics or competition for limited nutrients and colonization sites (26, 154, 156–158). Keeping symbiotic populations in the gut poses specific challenges: the hosts should specifically sustain the beneficial microbes, while at the same time and same place, they need to winnow out pathogens and parasites acquired through feeding. To maintain gut homeostasis, some insects have evolved a symbiont sorting organ or filter in the gut (84, 159), or develop a specific gut compartment called crop or crypt to harbor beneficial microbes (1).

Studies in *Drosophila* have revealed that gut mucosal immunity plays an important role to eliminate unfavorable bacteria and to keep gut homeostasis (160, 161). In addition, other physiological conditions in the gut lumen, such as pH, osmotic pressure, and oxygen concentration probably contribute to create the favorable conditions for gut symbiosis. Except for gut symbionts in wood-feeding insects and honeybees, most insects' gut symbionts are aerobic bacteria belonging to the Proteobacteria (42, 162), suggesting that oxygen supplementation is critical to maintain quality and quantity of the gut symbiont population. However, it is poorly understood how the oxygen supply to the gut is

established and how it affects the gut symbiosis.

Ingested bacteria, especially pathogens that come in contact with the gut epithelia, activate immune responses such as the production of AMPs and reactive oxygen species (ROS) (163, 164). The activated gut immunity maintains the gut homeostasis by eliminating the invading environmental microbes, which may be harmful to the host. Especially, bactericidal ROS, produced by the membrane-bound enzyme dual oxidase (Duox) is thought to antagonize bacterial growth and to play a major role in gut mucosal immunity (160, 161, 165–169). Duox generates ROS via its C-Terminal NADPH oxidase domain that produces H_2O_2 in the gut lumen and its N-terminal peroxidase-homology domain that converts the H_2O_2 into highly bactericidal hypochlorous acid. In *Drosophila* fed with a gut pathogen, silencing of *Duox* led to an increased pathogen load in the gut and the death of the insect (160). It was proposed that the Duox and ROS-dependent immunity in the gut is dominant over AMP production that serves as a fail-safe system in case of infection with ROS-resistant microbes (170).

In addition to the direct immune function, Duox-mediated ROS also contributes to gut homeostasis in insects by stabilizing the peritrophic membrane of the midgut through the formation of covalent dityrosine bonds between matrix proteins (171, 172). The peritrophic matrix of insects forms a layer composed of chitin and glycoproteins that lines the midgut lumen. This layer protects the midgut epithelium from abrasive food particles and microbes. In the mosquito *Anopheles gambiae*, *Duox*-RNAi leads to the disruption

of a dityrosine network (DTN), which cross-links proteins in the peritrophic membrane, and a loss of the matrix's barrier function (172). Consequently, gut microbes gain direct contact with gut epithelial cells and activate chronic AMP expression, which provokes on its turn a dramatic reduction of the bacterial midgut population. A similar function of Duox was reported in the tick *Ixodes scapularis* (173). Thus, Duox can interact in the insect gut with ingested microbes in two ways, by producing bactericidal ROS or by generating a physical barrier. In contrast to these well-described functions in mucosal immunity, little is known about the impact of the Duox enzyme on mutualistic bacteria.

The bean bug *Riptortus pedestris* (Fig. 3-1A), a notorious pest of legume crops, has a particular symbiotic organ located in the posterior midgut (30, 174, 175) (Fig. 3-1B). This region, called M4, is composed of several hundred crypts, organized in two parallel rows. The lumen of these crypts is entirely filled with a specific gut symbiont of the genus *Burkholderia*. This is a nutritional symbiosis based on recycling by the gut symbiont of insect metabolic wastes into high-value metabolites used by the host (109). This interaction is essential for optimal development and reproduction of the bean bug host (30). Every new generation of *R. pedestris* acquires the symbiont from the environmental soil by oral intake during feeding and drinking. A narrow channel located in the midgut, at the entrance of the M4, as well as microbe-microbe competition in the gut crypts sorts out the specific partner from the ingested soil microbiota, resulting in the specific colonization of the symbiotic organ (84, 89). The few hundreds of bacterial cells that are taken up by the symbiotic organ during infection multiply in a few days to a population of several ten millions that completely occupy the luminal space of all the crypts in the

M4 (86). The crypts control this very large symbiont population using immune-related AMPs as well as M4-specific symbiotic peptides (82, 109, 119, 176).

Given the cardinal role of ROS produced by Duox in insect gut immunity and the fact that the *Burkholderia* symbionts stay in direct contact with the crypt epithelial cells (78), we postulated in this study that besides the AMPs, Duox and ROS are also involved in the management of the symbiosis in the midgut crypts of *R. pedestris*. However, our findings did not support an antimicrobial immune function of Duox, but revealed an unanticipated pivotal role for the enzyme in the formation of a tracheal network enveloping the symbiotic organ, which is on its turn essential for supplying sufficient oxygen for respiration to the symbiotic organ and the aerobic gut symbiont.

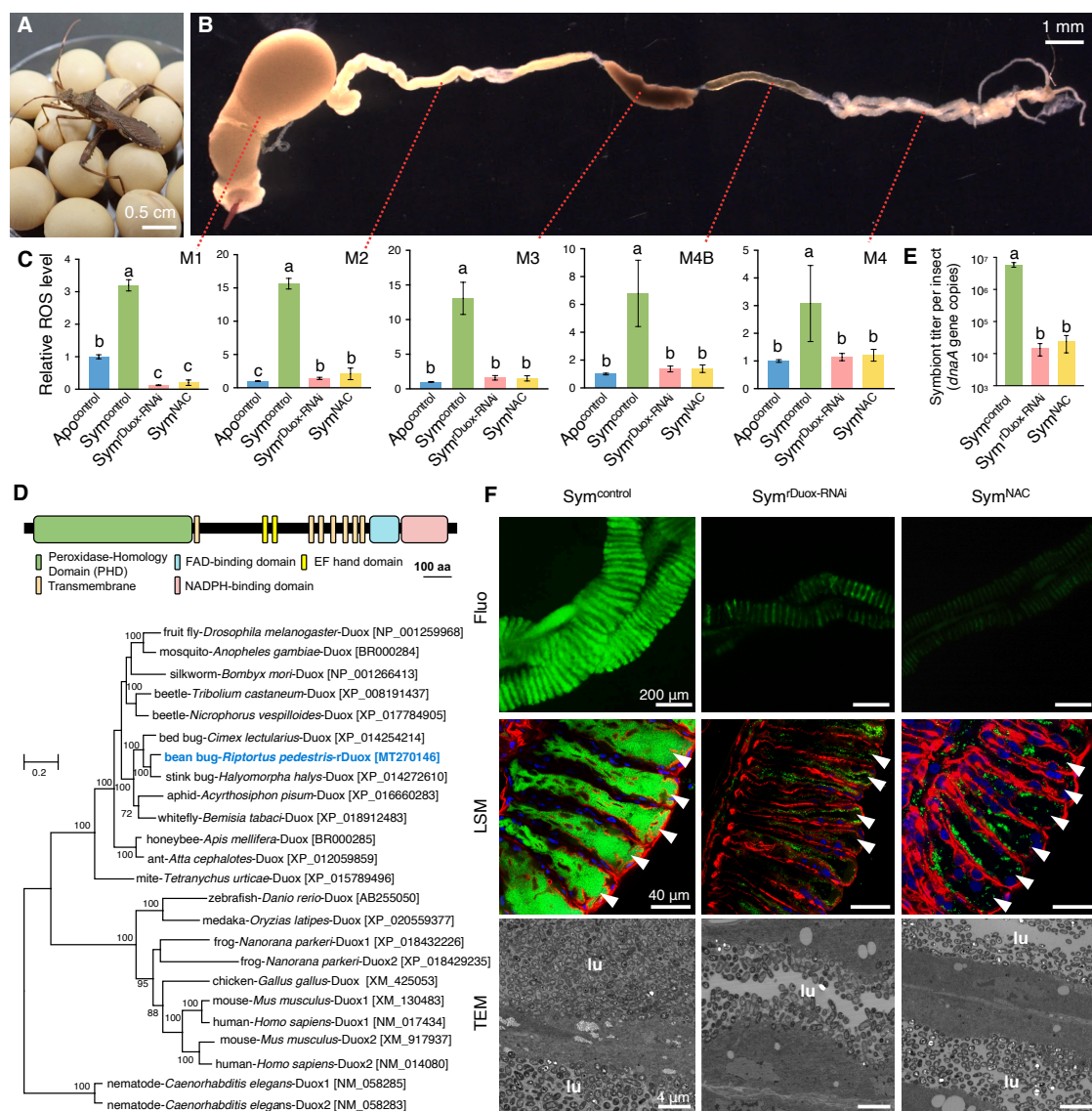


Fig. 3-1. rDuox-mediated ROS is important for the gut symbiosis. (A) An adult male *R. pedestris* feeding on soybeans. (B) The dissected midgut of *R. pedestris*. (C) Relative ROS level in the different midgut regions of Apo (Apo^{control}), Sym (Sym^{control}), *rDuox*-RNAi (Sym^{rDuox-RNAi}), and NAC-fed (Sym^{NAC}) insects. All values were normalized by the ROS level of Apo insects. (D) The predicted domains of the rDuox protein (top). Maximum likelihood phylogeny based on amino acid sequences (bottom). Numbers in parentheses indicate accession numbers. Bootstrap values (>60%) are shown near each node. (E) The number of symbionts colonizing the M4. Data shown in (C and E) are mean $\pm$ s.d. $n = 9$ and 6 insects, respectively. Error bars indicate statistically significant differences ($P < 0.05$). The statistical significance was analyzed by the Mann-Whitney U test with Bonferroni correction. (F) Microscopy images of the M4. Epifluorescence microscopy (first row). Laser scanning confocal microscopy (second row). Green, symbionts; blue, host nuclei; red, host cytoskeleton; white arrowheads, midgut crypts. Transmission electron microscopy (third row). lu; luminal region of the midgut crypt.

Table 3-1. Bacteria strains

Bacteria	Description	Fluorescent	Reference ^c
<i>Burkholderia insecticola</i> symbiont			
RPE75	Spontaneous Rf ⁺ mutant of wild-type <i>Burkholderia insecticola</i> symbiont RPE64	-	(1)
RPE225	GFP-labeled mutant of <i>Burkholderia</i> symbiont RPE75	GFP ^a	(2)
<i>resB::pVO-GFP</i>	Cytochrome c biogenesis mutant	GFP ^b	This study
<i>ΔuppP</i>	Cell wall synthesis mutant	GFP ^a	(3)
<i>purl::Tn5</i>	Purine biosynthesis mutant	GFP ^a	(4)
<i>fljC::Tn5</i>	Flagella mutant	GFP ^a	(5)
<i>Escherichia coli</i>			
DH5α	F- ϕ 80d <i>lacZ</i> ΔM15 Δ(<i>lacZ</i> Y _A -argF)U169 <i>deoR recA1 endA1 hsdR17</i> (K ⁻ , m ⁺) <i>phoA supE44 λ-thi-1 gyrA96 relA1</i>		Toyobo
<i>Serratia marcescens</i>			
Db11	Spontaneous Rf ⁺ mutant of <i>S. marcescens</i> Db10		(6)

^aGFP was labelled by labelled by the Tn7 mintransposon system

^bGFP was labelled by insertion of GFP-expressing plasmid pVO-GFP into *resB* gene

^cReferences: **(1)** Kikuchi Y, Meng XY, Fukatsu T. Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocoris chinensis* (Heteroptera: Alydidae). *Appl Environ Microbiol* 2005; 71: 4035-4043; **(2)** Kikuchi Y, Fukatsu T. Live imaging of symbiosis: spatiotemporal infection dynamics of a GFP-labelled *Burkholderia* symbiont in the bean bug *Riptortus pedestris*. *Mol Ecol* 2014; 23: 1445-1456; **(3)** Kim JK, Lee HJ, Kikuchi Y, Kitagawa W, Nikoh N, Fukatsu T et al. Bacterial cell wall synthesis gene *uppP* is required for *Burkholderia* colonization of the stinkbug gut. *Appl Environ Microbiol* 2013; 79(16): 4879-4886; **(4)** Kim JK, Jang HA, Won YJ, Kikuchi Y, Han SH, Kim CH et al. Purine biosynthesis-deficient *Burkholderia* mutants are incapable of symbiotic accommodation in the stinkbug. *J SIME J* 2014; 8: 552-63; **(5)** Ohbayashi T, Takeshita K, Kitagawa W, Nikoh N, Koga R, Meng XY et al. Insect's intestinal organ for symbiont sorting. *Proc Natl Acad Sci USA* 2015; 112: E5179-E5188; **(6)** Kurz CL, Chauvet S, Andr s E, Aurouze M, Vallet I, Michel GP et al. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. *EMBO J*. 2003 1;22(7):1451-60.

Table 3-2. Primers

Primers	Sequence (5'-3')	Tm (°C)	Description	Product size (bp)
bsDnaA_F	AGCGCGAGATCAGACGGTGTGGAT	70.7	Measurement of symbiont <i>dnaA</i> gene copies by qPCR	150
bsDnaA_R	TCCGGCAAGTGTGGCAGCGCA	66.6		
T7_pTbR20	TAATACGACTCACTATAGGGGATCTACTAGTCATATGGAT	74	Used for dsRNA synthesis	Depend on the insert size
T7_pTbU19_2	TAATACGACTCACTATAGGGGACGGCCAGTGAAT	74.1		
Duox_dsF	TGCCCTGCTGACATTCCTCA	58.4	Used for dsRNA synthesis targeting <i>rDuox</i>	451
Duox_dsR	AGTTGATCCTCCCGCTCTG	60.5		
Duox_qF	CATTGGCGGGATGTTAGAGT	51.8	Used for qPCR targeting <i>rDuox</i>	139
Duox_qR	TTCTTCGGGTTGAAATATCC	58.4		
Bnl_dsF	TCCCTCAGCTAGCTCACAAT	60.5	Used for dsRNA synthesis targeting <i>rBnl</i>	490
Bnl_dsR	TGGGTCTAGGTGAGCTGAG	62.5		
Bnl_qF	AACAGAAAGATCAGGCACCG	60.5	Used for qPCR targeting <i>rBnl</i>	117
Bnl_qR	TGtGTGGTATGGTCTCTCT	60.5		
Tth_dsF	CCATCACCTOCCAAACTGGAC	62.5	Used for dsRNA synthesis targeting <i>rTth</i>	581
Tth_dsR	GAAGGTTGACTTCATCCGCA	60.5		
Tth_qF	GACGCACACAGACTGAGGAA	60.5	Used for qPCR targeting <i>rTth</i>	151
Tth_qR	GCTCGTCTGCGTTTTTCGAA	58.4		
Sima_dsF	TGTCACCTGAGAGCAGTCAAG	60.5	Used for dsRNA synthesis targeting <i>rSima</i>	516
Sima_dsR	GGTTTCTCCCTTTGCTGGAT	58.4		
Sima_qF	AGGATCATTTCCGGTTTGtG	56.4	Used for qPCR targeting <i>rSima</i>	134
Sima_qR	CATCCTTTCATCCGCATAGG	58.4		
Eti_a_F	CCTGCATCCGTTGCTTTTGT	58.4	Used for qPCR targeting <i>rEti a</i>	150
Eti_a_R	GGCATCGAGGGCTTTCATTA	58.4		
Relish_dsF	GCTGGCACGAAAACACTACTAA	59.5	Used for dsRNA synthesis targeting <i>rRelish</i>	525
Relish_dsR	TGTACATTCTGAGGGCCAAAG	59.5		
Relish_qF	GCCAGATGAATATTGTGCAATG	58.4	Used for qPCR targeting <i>rRelish</i>	107
Relish_qR	TAACAGCCGACTGCCAGAG	59.5		
RipIocin_R	TCCGAAGCTGAGGGTCTTCCCG	67.9	Used for qPCR targeting <i>rIpicin</i>	128
RipIocin_F	TCGGATCCAAAGTTGGCGTCC	65.3		
Defensin_F	TCGGTCGAGCTGAGACTGAA	60.5	Used for qPCR targeting <i>rDefensin</i>	108
Defensin_R	TTGCCGCTTTGTATCCCTT	58.4		
Thanatin_F	GTCTGCCTTGTGGAAGACG	60.5	Used for qPCR targeting <i>rThanatin</i>	108
Thanatin_R	ATTGCTTGCAAAACGCCG	56.3		
BmFp49_F	CAGGCATCAATCGGATCGCT	60.5	Used for qPCR targeting <i>BmFp49</i>	150
BmFp49_R	GGGAGCATATGACGGGTCTT	60.5		
BmDuox_F	GGTGGGATGCTGGAAGTCTAA	60.5	Used for qPCR targeting <i>BmDuox</i>	103
BmDuox_R	TTTGAACCAAAATCGATCA	52.3		
GbEti_a_F	ATTGATGCCCCGTGTCACAG	60.5	Used for qPCR targeting <i>GbEti a</i>	104
GbEti_a_R	TCAAATCAGCAGTACCCCG	60.5		
GbDuox_F	CCAAAGATTGCTCTGAAAG	58.4	Used for qPCR targeting <i>GbDuox</i>	149
GbDuox_R	TACCGATTGGTTGAGGTTCC	58.4		
TcRps18_F	CTGCTGAAGTGAAGTCTGGG	62.5	Used for qPCR targeting <i>TcRps18</i>	141
TcRps18_R	CGCAATTTGGAAGTCAAGCGT	58.4		
TcDuox_F	GTATTGCTACCCCGACAAT	58.4	Used for qPCR targeting <i>TcDuox</i>	147
TcDuox_R	GCCGATGAAAAAATAGCCAAA	54.3		

Materials and methods

Insect rearing and infection with symbiont

The *R. pedestris* strain used in this study was originally collected from a soybean field in Tsukuba, Ibaraki, Japan, and maintained in the laboratory for over ten years. The bean bugs were reared in Petri dishes (90 mm in diameter and 20 mm high) under a long-day regimen (16 h light and 8 h dark) at 25°C and fed with soybean seeds and distilled water containing 0.05% ascorbic acid (DWA). To infect insects with symbionts, *B. insecticola* (RPE75), GFP-expressing *B. insecticola* (RPE225), or mutant *Burkholderia* strains (Table 3-1) were orally administrated to second instar nymphs by supplying the bacteria at a density of 10^7 cells/ml to the DWA. To feed NAC, DWA with 10 mg/ml of NAC was supplied to the bean bugs. *T. castaneum* was a gift of Dr. Ryo Futahashi (AIST Tsukuba Center, Japan). *G. bimaculatus* and *B. mori* were purchased from a pet shop (Sapporo, Japan).

Detection of ROS levels

Fourth instar nymphs of bean bugs were dissected in phosphate-buffered saline (PBS), pH 7.4, using fine forceps under a dissection microscope (S8APO, Leica; MZ FZ III, Leica), and each part of their midguts was transferred to 1.5 ml microcentrifuge tubes and homogenized by a pestle. The homogenized midgut samples were centrifuged at 15,000 rpm for 1 min to remove tissue debris, and the supernatant was transferred to the 96-well black polystyrene microplate and incubated with 50 μ M 2',7'-dichlorofluorescein diacetate (DCF-DA) (Thermo Fisher Scientific) for 20 min in the dark. The ROS level in

each sample was measured by a microplate reader, Infinite F200 PRO (Tecan), using an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

Synthesis of dsRNA and RNA-interference

Gene sequences of *Duox*, *rRel*, *rTrh*, *rBnl* and *rSima* were identified by an RNA-sequencing analysis of symbiotic insects and BLAST identification using the *Drosophila* proteins as search term (Accession number MT270146-MT270150). Fragments of target genes for RNAi knock down were obtained from *R. pedestris* cDNA. The fifth instar nymphs of bean bugs were dissected in PBS and total RNA was extracted from the M4 using the RNAiso Plus (Takara Bio Inc) and the RNeasy Mini Kit (Qiagen Inc). The cDNA was synthesized from 1 µg of extracted total RNA by PrimeScript™ RT reagent Kit with gDNA Erase (Takara Bio Inc).

To synthesize dsRNA for RNAi, approximately 500 bp fragments of the target gene were amplified from cDNA with primers listed in Table 3-2. The PCR products were cloned in the pT7Blue T-vector and transformed into *Escherichia coli* DH5α cells. Clones were verified by sequencing. Next, the target gene was amplified by PCR from these plasmids using specific upstream and downstream primers, each extended with the T7 promotor sequences (Table 3-2), to create a template for RNA polymerization. The dsRNA was synthesized from these PCR products by the MEGAscript™ RNAi kit (Thermo Fisher Scientific).

To silence a target gene, 100 ng of the corresponding dsRNA was injected into the hemolymph, between the torso and hind leg of the symbiont-infected second instar nymph of bean bugs after 2 days of symbiont infection, using a glass capillary and a Femtojet microinjector (Eppendorf). For triggering a septic shock, 10^7 *E. coli* DH5a cells were similarly injected in the hemolymph. The phenotypic alteration in the insects was observed a week after RNAi. The detailed timeline of the experiment is described in (Fig. 3-12).

Estimation of infection levels of symbiotic organs by quantitative PCR

To measure the number of symbionts present in the M4, DNA was extracted from the dissected M4 by the QIAamp DNA Mini Kit (Qiagen Inc) and real-time quantitative PCR was performed using primers BSdnaA-F and BSdnaA-R, which target the *dnaA* gene of the *Burkholderia* symbiont, the KAPA SYBR FAST qPCR Master Mix Kit (KAPA Biosystems Inc) and the Roche LightCycler® 96 System (Roche) (Table 3-2). The number of gut symbionts was calculated based on a standard curve for the *dnaA* gene containing 10 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , and 10^7 copies per reaction of the target PCR product.

Gene expression determination by reverse transcriptase-quantitative PCR

To measure the mRNA expression level of the target genes, total RNA was extracted from

the M4 or the fat body of the bean bugs and reverse transcribed to cDNA as described above. The real-time quantitative PCR for target genes was performed as described above using primers listed in (Table 3-2). The efficiency of the RNAi process on target genes and change of gene expression patterns of immune genes was measured three days after RNAi (Fig. 3-12). The mRNA levels were normalized by the elongation factor 1- α (EF1 α) housekeeping gene.

Microscopy analyses

To analyze the infection level of control, RNAi or NAC treated insects, the bean bugs were infected with GFP-expressing *Burkholderia* symbionts and injected with dsRNA or fed with NAC (Fig. 3-12). After a week, the M4 organs of fourth instar bean bugs were dissected in PBS, transferred to a glass-bottom dish (Matsunami) and covered with a cover glass. The GFP-signal derived from the M4-colonizing gut symbionts was observed with an epifluorescence microscope (DMI4000B, Leica).

Alternatively, to observe the colonization of the M4 by the gut symbiont with confocal microscopy, the bean bugs harboring GFP-expressing symbionts were dissected in PBS and the midgut was transferred to 2 ml microcentrifuge tube. The gut samples were fixed with 4% paraformaldehyde (pH 7.4) for 10 min at 25°C. The fixed samples were washed three times by PBS and permeabilized by PBS with 0.1% Triton X-100 (PBST) for 10 min at 25°C. After permeabilization, the gut samples were washed again three times by PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) and Alexa Fluor™ 647

Phalloidin for 30 min at 25°C, to stain respectively the nucleus and cytoskeleton of host cells. Then, the samples were transferred to a glass-bottom dish, mounted with ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific), and observed under a laser scanning confocal microscope (TCS SP8, Leica).

Dityrosine networks were revealed by immunostaining. The whole gut of the fourth instar of control or RNAi bean bugs was dissected in PBS and fixed with 4% paraformaldehyde. The fixed midgut was permeabilized and incubated with blocking buffer (1% BSA in PBST) for 30 min at 25°C. Then, the gut was stained with 1:400 dilution of anti-dityrosine monoclonal antibody (MyBioScience) overnight at 4°C. Subsequently, the midgut was washed three times by PBS, each for 10 min, and then stained with 1:500 dilution of goat anti-mouse IgG H&L conjugated with Alexa Fluor® 555 (Abcam) for 1 hour at 25°C in the dark. The midgut was washed three times by PBS, each for 10 min, and then incubated with DAPI and phalloidin. The midgut was transferred to a glass-bottom dish, mounted, and observed with a laser scanning confocal microscope. The 3D image of the dityrosine network was reconstructed from z-stacked images using the Leica LAS X software.

To observe the tracheal network by chitin fluorescent labelling, dissected midguts were stained with the fluorescent brightener 28 (Sigma-Aldrich) at a final concentration of 0.1 mg/ml for 5 min in the dark. Then, the midgut was washed three times by PBS, transferred to a glass-bottom dish, and observed with a confocal or epifluorescence microscope.

To analyze the hypoxic condition of the midgut, dissected M4 were stained with 10 μ M Image-iT™ Green Hypoxia Reagent for 30 min at 25°C and observed with a confocal microscope. To label active mitochondria, dissected midguts were incubated with 100 nM MitoTracker™ Green FM for 30 min at 25°C and observed with a confocal microscope.

For transmission electron microscopy (TEM) observations, the bean bugs were dissected with fine forceps in the fixative solution (0.1 M sodium phosphate buffer containing 2.5% glutaraldehyde, pH 7.4). The isolated M4 was pre-fixed in the fixative solution at 4°C overnight and post-fixed in the 2% osmium tetroxide at 4°C for 1 hour. After a series of dehydration steps by ethanol, the gut was embedded in Epon812 resin (TAAB Ltd). Ultrathin sections were made by using an ultramicrotome (EM UC7, Leica), mounted on a copper mesh, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (H-7600, Hitachi).

Colony forming unit assay

Cultured *S. marcescens* (10^7 cells/ml) were orally administrated to the third instar of Sym and *rDuox*-RNAi bean bugs. The midgut M3 regions were dissected 3 days after infection and homogenized with a pestle in 100 μ l of PBS. After the serial dilution of these M3-lysates, 10 μ l of the dilutions were spotted on a LB agar plate with 30 μ g/ml of rifampicin and plates were incubated at 30°C for 1 day to obtain bacterial growth. To measure the symbiont titer in the M3 of Sym and *rDuox*-RNAi bean bugs, dsRNA of *rDuox* was

injected to second instar nymphs of Apo insects, and then newly molted third instar nymphs were infected with cultured *Burkholderia* symbionts (10^7 cells/ml) and the M3 was dissected 24 h after infection. The dissected M3 was homogenized and dilution series were spotted on a YG agar plate with 30 µg/ml of rifampicin, which was incubated for 2 days at 30°C.

Bacterial growth and insect rearing in hypoxia and hyperoxia conditions

To culture the *Burkholderia* symbiont in anaerobic or microaerophilic conditions, agar plates were placed in a 2.5 L W-zip standing pouch jar (Mitsubishi Gas Chemical), containing respectively an Anaero Pack-Anaero ($O_2 < 0.1\%$) or an Anaero Pack-MicroAero ($6\% < O_2 < 12\%$) (Mitsubishi Gas Chemical). Plates were incubated for 48 h at 30°C and subsequently for a further 48 h at 30°C in normoxia condition.

To rear insects in a hypoxia environment, Petri dishes containing second instar nymphs were placed in the 2.5 L rectangular jar containing an Anaero Pack-MicroAero. To construct the hyperoxia condition, Petri dishes containing the second instar nymphs were placed in a culture jar and oxygen was injected daily via a valve in order to maintain 39-42% oxygen as shown in (Fig. 3-7C). The oxygen content (%) in the jar was monitored by an oxygen indicator OXY-M (JICKO).

Measurement of the insect survival rate

To estimate the pathogenicity of entomopathogenic bacterium *S. marcescens*, third instar nymphs of Sym and *rDuox*-RNAi bean bugs were orally infected with 10^7 cells/ml of *S. marcescens* and the number of dead insects was counted every day. The number of carcasses of *rBnl*-, *rTrh*-, *rSima*-, and *rDuox*-RNAi insects was counted every day to measure the mortality of insects upon the silencing of these genes. For each experiment, 20 insects were used.

RNA-sequencing analysis

Total RNA was extracted from the dissected symbiotic organ of Sym and *rDuox*-RNAi insects using the RNAiso Plus and RNeasy Mini Kits. The cDNA libraries were prepared by TruSeq RNA Sample Preparation Kit v2 (Illumina) and sequenced by Illumina HiSeq-2000 (Illumina). The adapter sequences of raw reads (Accession number DRR215696-DRR215701) were trimmed by Trimmomatic (177) and the reference contigs were constructed by *de novo* assembly using Trinity (178). To identify the genes used in this study, assembled sequences were compared with the protein database of *Drosophila* by blastx. Transcripts of each sample were quantified by RSEM with Bowtie2 Aligner and differential expression of genes between Sym and *rDuox*-RNAi insects was determined with DESeq2 (179). To visualize relatively up- or down-regulated genes, heatmap presentations were produced with the gplots package in program R ver. 3.6.2.

Protein domain and phylogenetic analysis

The transmembrane and functional domains of rDuox were predicted by Phobius and InterPro. The amino acid sequences of Duox proteins of selected organisms were retrieved from DDBJ/ENA/GenBank database and aligned using the MAFFT version 7 (180). Phylogenies were inferred based on the maximum likelihood methods with the Jones-Taylor-Thornton model (181) and 1,000 bootstrap replications, using the MEGA version 7.0.26 (182).

Statistical analyses

All statistical analyses were performed using the program R ver. 3.6.2 and GraphPad Prism ver. 8.3.1.

Results and Discussion

Gut symbionts induce the generation of rDuox-dependent ROS in the *R. pedestris* midgut

To determine if the gut symbiont *Burkholderia insecticola* induces ROS in the symbiotic organ of *R. pedestris*, we measured the relative ROS level in the lysate of dissected gut compartments of apo-symbiotic (Apo) and symbiotic (Sym) insects using the fluorescent dye 2',7'-Dichlorofluorescein Diacetate. An approximately threefold higher level of ROS was detected in the symbiotic organ of Sym insects compared to that of Apo insects, confirming that the colonization of the crypts by the *B. insecticola* symbiont stimulates ROS production (Fig. 3-1C). Moreover, the ROS level in the digestive tract of the midgut, the M1, M2 and M3 anterior regions (Fig. 3-1B), equally increased after symbiont colonization, indicating that the colonization by *B. insecticola* induces systemic high ROS levels throughout the whole midgut regions even if the symbiont resides only in the posterior symbiotic midgut region M4 (Figs. 3-1B and C). To demonstrate whether Duox generates the symbiont-mediated ROS, the homologous gene of *R. pedestris* (*rDuox*) was down-regulated by RNAi (Fig. 3-2A). The *rDuox* transcript was identified based on homology with the *Drosophila melanogaster* protein in a *R. pedestris* transcriptome dataset generated from the infected symbiotic organ. We found one *rDuox* copy in *R. pedestris*, displaying 74.7% homology and the encoded protein had all the functional domains of typical Duox proteins from insects to humans (171) (Fig. 3-1D). *rDuox*-RNAi reduced the ROS level in the symbiotic organ, as well as in the other midgut regions of Sym insects, to a similar level of Apo insects (Fig. 3-1C), demonstrating that induction of ROS by symbiont colonization is due to Duox activity. Similarly, the feeding of Sym

insects with the antioxidant compound *N*-acetylcysteine (NAC) (183), added to the drinking water, also decreased the ROS levels of the midgut regions to similar concentrations as in Apo insects (Fig. 3-1C). Taken together, these results demonstrate that the infection of *B. insecticola* induces production of ROS in the entire midgut region via the rDuox enzyme.

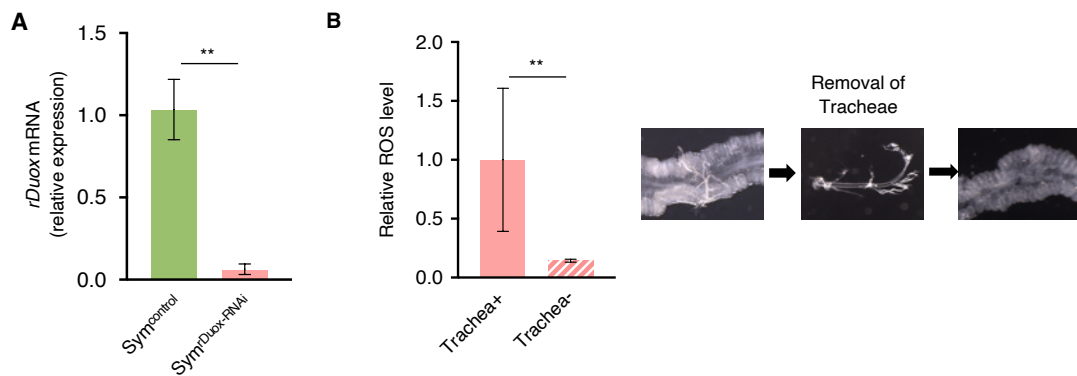


Fig. 3-2. Duox-mediated generation of ROS from the tracheal network in *R. pedestris*. (A) The efficiency of *rDuox*-RNAi. The expression level of *rDuox* was quantified by RT-qPCR after the injection of *rDuox* dsRNA. (B) Relative ROS levels in the M4 of Sym^{control} insects before and after removing tracheae (left); photographs showing the removal of the tracheae (right). Data shown in (A and B) are mean $\pm$ s.d. $n = 5$ insects. Asterisks indicate statistically significant differences (**, $P < 0.01$). The statistical significance among samples was analyzed by the Mann-Whitney *U* test.

rDuox-produced ROS is important for the gut symbiosis but not as a microbicidal molecule

The production of infection-associated ROS is compatible with a mucosal immune response directed against the gut symbiont in the symbiotic organ. To test this hypothesis, we estimated the population size of symbionts in the M4 of Sym, *rDuox*-RNAi (Sym^{*rDuox*-RNAi}), and NAC-fed (Sym^{NAC-fed}) insects by qPCR. In *Drosophila*, silencing of *Duox* led to a reduced microbicidal ROS production and a concomitant increased total number of gut microbes (160). However, contrary to *Drosophila*, the titer of the *Burkholderia* symbiont in the M4 of *R. pedestris* was decreased more than 100 times in *rDuox*-RNAi insects compared to Sym insects (Fig. 3-1E). Also feeding of the antioxidant NAC to Sym insects reduced the number of gut symbionts to similarly low levels (Fig. 3-1E). Microscopy analysis confirmed that *rDuox*-RNAi and NAC-feeding caused the collapse of the symbiosis (Fig. 3-1F). Laser scanning microscopy (LSM) and transmission electron microscopy (TEM) images showed that in the symbiotic organ of *rDuox*-RNAi and NAC-fed insects, gut symbionts were present close to the epithelial surface of the midgut crypts, but few symbionts were observed in the interior part of the crypt lumen (Fig. 3-1F). These results indicate that rDuox plays a pivotal role in controlling gut symbiosis but by a mechanism that is unrelated to a bactericidal action of ROS against the symbiont.

We next investigated the possible relation of rDuox activity and bacterial infection and multiplication in the anterior digestive regions of the midgut. Although the ROS-level was significantly decreased in M3 by *rDuox*-RNAi (Fig. 3-1C), we found that the survival

rate of *R. pedestris* after oral infection with the entomopathogen *Serratia marcescens* was not affected (Fig. 3-3A), suggesting that removing Duox-mediated ROS production did not render the insects more susceptible to the pathogen. Accordingly, the number of *S. marcescens* cells in the M3 region of the midgut was not significantly changed after *rDuox* RNAi (Fig. 3-3B). Moreover, the number of *B. insecticola* cells that were transiently present in the M3 region after symbiont ingestion was also not altered in *rDuox*-RNAi insects (Fig. 3-3C). Together, these results demonstrate that Duox-mediated ROS generation is not important to regulate the bacterial titer of pathogens or symbionts in the non-symbiotic midgut regions.

Immune stimulation by *rDuox*-RNAi is not responsible for symbiosis collapse

In addition to its bactericidal activity, Duox-mediated ROS is also known to contribute to the construction of the midgut peritrophic membrane, as shown in *A. gambiae* (172). Knock-down of *duox* in *A. gambiae* leads to a decreased barrier function of the peritrophic matrix and enhanced immune stimulation and AMP expression by gut microbes coming in close contact with epithelial cells. However, in contrast with the majority of insects, most hemipteran insects, including *R. pedestris*, does not possess a peritrophic membrane in the midgut (184). Nevertheless, the gene expression profile comparison between the symbiotic organs of Sym and *rDuox*-RNAi insects showed that expression of immunity-related genes, especially genes encoding AMPs (99), was increased in the midgut of *rDuox*-RNAi insects (Figs. 3-3D and E), but not in the fat body (Fig. 3-3F). It should be noted however that this up-regulated AMP gene expression in the midgut by *rDuox*-RNAi

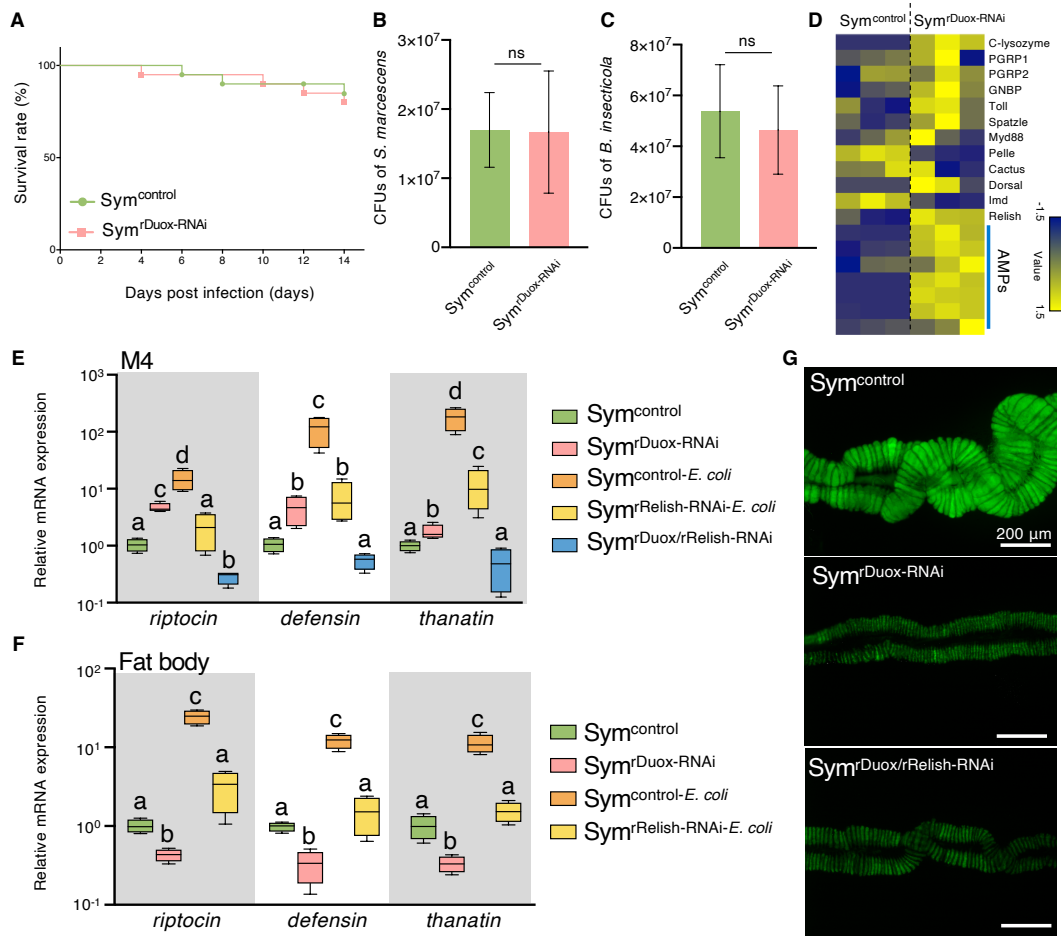


Fig. 3-3. rDuoX function is not related to gut mucosal immunity in the bean bug. (A) The Survival rate of Sym (Sym^{control}) and *rDuoX*-RNAi (Sym^{rDuoX-RNAi}) insects after oral infection with 10^7 cells/ml of *Serratia marcescens*. $n = 20$ insects. (B) Number of *S. marcescens* colonies from the M3 region of third instar nymph Sym^{control} and Sym^{rDuoX-RNAi} insects. (C) The number of *Burkholderia* symbionts in the M3 of the third instar nymph Sym^{control} and Sym^{rDuoX-RNAi} insects; CFU, colony-forming units. (D) Expression profile from RNA-seq data of immunity-related genes in the M4 of Sym^{control} and Sym^{rDuoX-RNAi} insects. Each column is a replicate experiment. (E) The expression level of AMPs in the M4 of Sym insects without immune stimulation (Sym^{control}), Sym^{control} with septic shock by *E. coli* injection in the hemolymph (Sym^{control-E. coli}), *rRelish*-RNAi with *E. coli* injection (Sym^{rRelish-RNAi-E. coli}), *rDuoX*-RNAi without bacterial injection (Sym^{rDuoX-RNAi}), and double *rDuoX/rRelish*-RNAi insects without bacterial injection (Sym^{rDuoX/rRelish-RNAi}). (F) The expression level of AMPs in the fat body. Data shown in (B and C) are $n = 5$ and (E and F) are $n = 4$ insects. Different letters indicate statistically significant differences ($P < 0.05$). The statistical significance was analyzed by the Mann-Whitney *U* test with Bonferroni correction. Error bars indicate SDs; ns, not statistically significant. (G) Effect of *rDuoX/rRelish*-double RNAi on symbiosis. Green color indicates the GFP signal from gut-colonizing *Burkholderia* symbionts.

was significantly lower than in immune-stimulated insects by septic shock (Figs. 3-3E and F). Because induced immunity in the symbiotic organ could antagonize the gut symbiont and thus in principle be at the origin of the symbiosis defect in *rDuox*-RNAi insects, we aimed at knocking down the expression of the AMP genes in the bean bug. In the brown-winged green bug *Plautia stali*, a stinkbug related to *R. pedestris* in the infraorder Pentatomomorpha but belonging to a different family, the NF- κ B transcription factor Relish of the Imd pathway is an essential regulator of AMP gene expression (185). *rRelish*-RNAi in *R. pedestris* reduced the expression of all three tested AMP genes in the symbiotic organ as well as the fat body, indicating that also in *R. pedestris* Relish is a critical regulator of the expression of the AMP immune effectors (Figs. 3-3E and F). After simultaneously silencing of both *rRelish* and *rDuox*, the AMP expression was not up-regulated anymore in the symbiotic organ (Fig. 3-3E), showing that the induced AMP expression in the M4 by knockdown of *rDuox* is dependent on the canonical Imd immune pathway. Importantly, the GFP signal of *Burkholderia* gut symbionts in double *rDuox/rRelish*-RNAi insects were not restored to control levels but remained similar to single *rDuox*-RNAi insects (Fig 3-3G). Therefore, we can conclude that the up-regulation of the immune response does not directly contribute to disrupting the symbiotic relationship in *rDuox*-RNAi insects.

Symbiont colonization triggers tracheal development and rDuox-mediated ROS stabilizes the respiratory network by forming a dityrosine network

Thus, contrary to our initial expectation, the role of rDuox and the ROS produced by it in

the *R. pedestris*–*Burkholderia* symbiosis is not related to mucosal gut immunity. To find a clue leading to the specific function of *rDuox* in the symbiosis, we measured its tissue-specific expression by RT-qPCR. Surprisingly, the expression of the *rDuox* gene was incomparably high in the tracheae relative to all other tested tissues, suggesting that the *rDuox*-mediated generation of ROS may have an important function in the respiratory system for maintaining gut symbiosis (Fig. 3-4A). The insect tracheal system consists of a tubular epithelial network containing a luminal chitin and protein matrix (172, 186–188). High *rDuox* expression levels in the tracheae could be in agreement with the previously documented function of Duox-mediated ROS in shaping extracellular matrices by forming dityrosine cross-links between proteins and a DTN (171). Immunostaining of a dissected midgut by a dityrosine-specific antibody revealed the presence of a DTN enveloping the symbiotic organ of *R. pedestris* that likely corresponds to a respiratory network composed of tracheae and tracheoles (Fig. 3-4B). In addition to protein, chitin is another major constituent of the tracheal matrix (188). Chitin staining with calcofluor-white Fluorescent Brightener 28 revealed a tracheal network around the symbiotic organ that colocalized with the network revealed with the dityrosine-specific antibody (Fig. 3-4C), confirming that the DTN highlights tracheae and that dityrosines are abundant in them. Interestingly, both chitin and dityrosine staining revealed that the tracheal network enveloping the symbiotic organ and the tracheoles connecting inside the midgut crypt tissue were much stronger developed in Sym insects compared to Apo insects (Fig. 3-4D). This morphological response suggests that colonization of the crypts by the large number of the *Burkholderia* gut symbiont is an oxygen-requiring process that induces the development of the respiratory network to supply enough oxygen to the symbiotic organ.

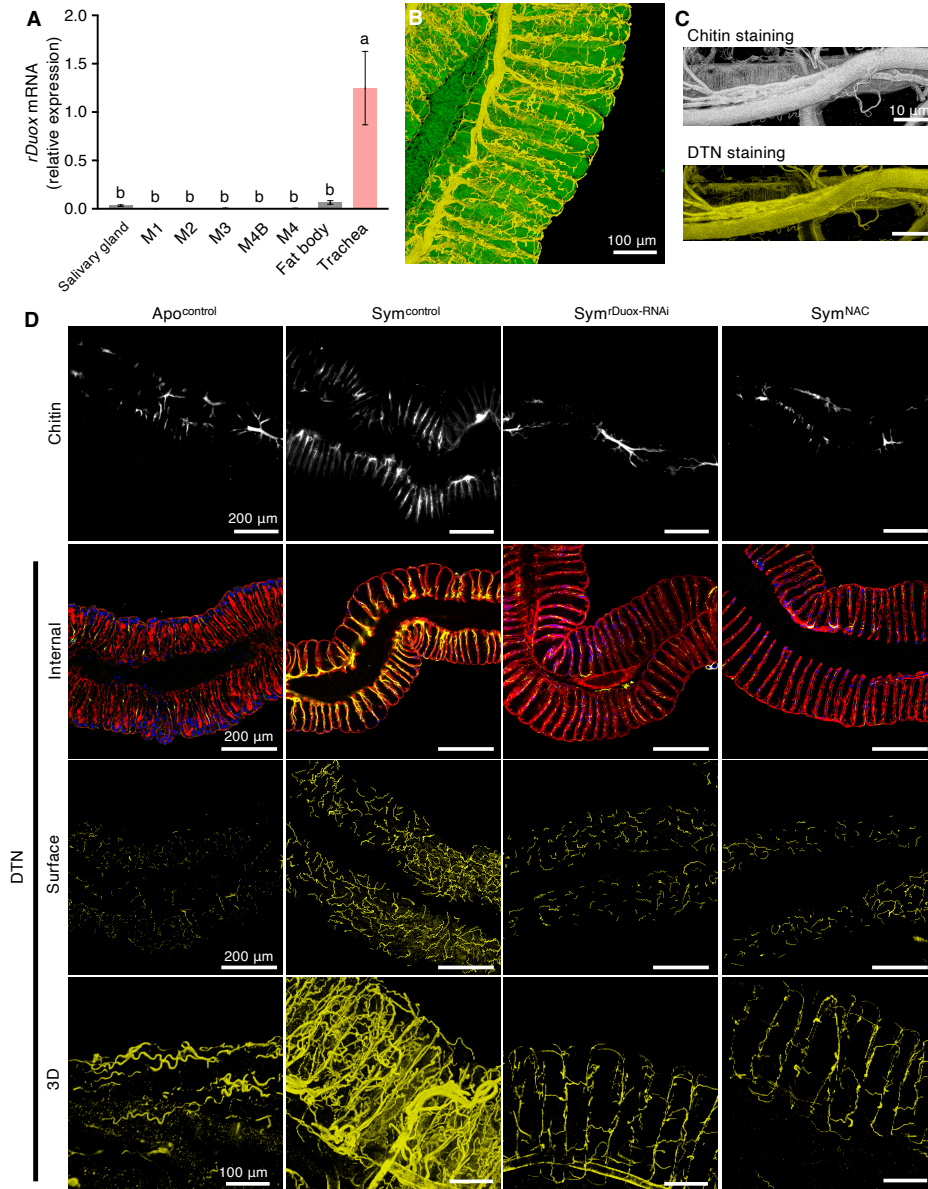


Fig. 3-4. Symbiont colonization triggers tracheal development and *rDuox*-mediated ROS stabilizes it by forming a dityrosine network. (A) The relative expression of *rDuox* in different tissues. Data shown are mean $\pm$ s.d. $n = 4$ insects. Different letters indicate statistically significant differences ($P < 0.05$). The statistical significance among the samples was analyzed by the Mann-Whitney U test with Bonferroni correction. **(B)** 3D image of the tracheal network in the M4 of a *Sym*^{control} insect. **(C)** Staining of the chitin and dityrosine network (DTN) of tracheae detached from the M4. **(D)** The tracheal network in the M4 of Apo (*Apo*^{control}), *Sym* (*Sym*^{control}), *rDuox*-RNAi (*Sym*^{*rDuox*-RNAi}), and NAC-fed (*Sym*^{NAC}) insects. The tracheal network, stained with fluorescent brightener 28 (calcofluor-white) to visualize chitin (first row); DTN in the internal section of the M4 (second row); DTN located on the surface of the M4 (third row); 3D reconstitution of DTN of the tracheal network (fourth row). The colors in the images of panels (B-D) are: white, chitin; yellow, the DTN; red, host cytoskeleton; blue, host nuclei; green, GFP signal derived from gut-colonizing symbionts.

Moreover, the tracheal network, especially the ramified tracheoles, did develop in response to the colonization of the gut symbiont *Burkholderia* not only around the crypt-bearing M4 but also in the other midgut regions (Fig. 3-5), indicating that the activity of the symbiotic organ provokes a systemic oxygen stress. This is furthermore in agreement with the ROS accumulation in all midgut regions (Fig. 3-1C). Accordingly, the higher ROS levels in the midgut of Sym insects compared to Apo insects, resulted from the strongly developed attached tracheal network since their careful removal from the symbiotic organ significantly reduced the detected ROS level (Fig. 3-2B). Strikingly, the DTN as well as the chitin mesh in the symbiotic organ of Sym insects was strongly reduced by *rDuox*-RNAi or feeding of the antioxidant NAC (Fig. 3-4D). Thus, rDuox is indispensable for the establishment of the symbiosis-induced tracheal network in *R. pedestris* and the increased ROS level in the midgut, produced by rDuox after symbiont infection, is due to the development of a tracheal network along the different midgut regions.

Destabilization of the respiratory network by *rDuox*-RNAi causes hypoxia in the midgut

Based on these findings, we made the hypothesis that the collapse of the respiratory system by *rDuox*-RNAi will cause a hypoxic environment in the symbiotic organ. We first measured in the crypt epithelial cells the activity of the mitochondria using the MitoTracker dye. In Sym insects, a high number of active mitochondria was detected in the epithelial cells of the crypts of the symbiotic organ, which was drastically decreased

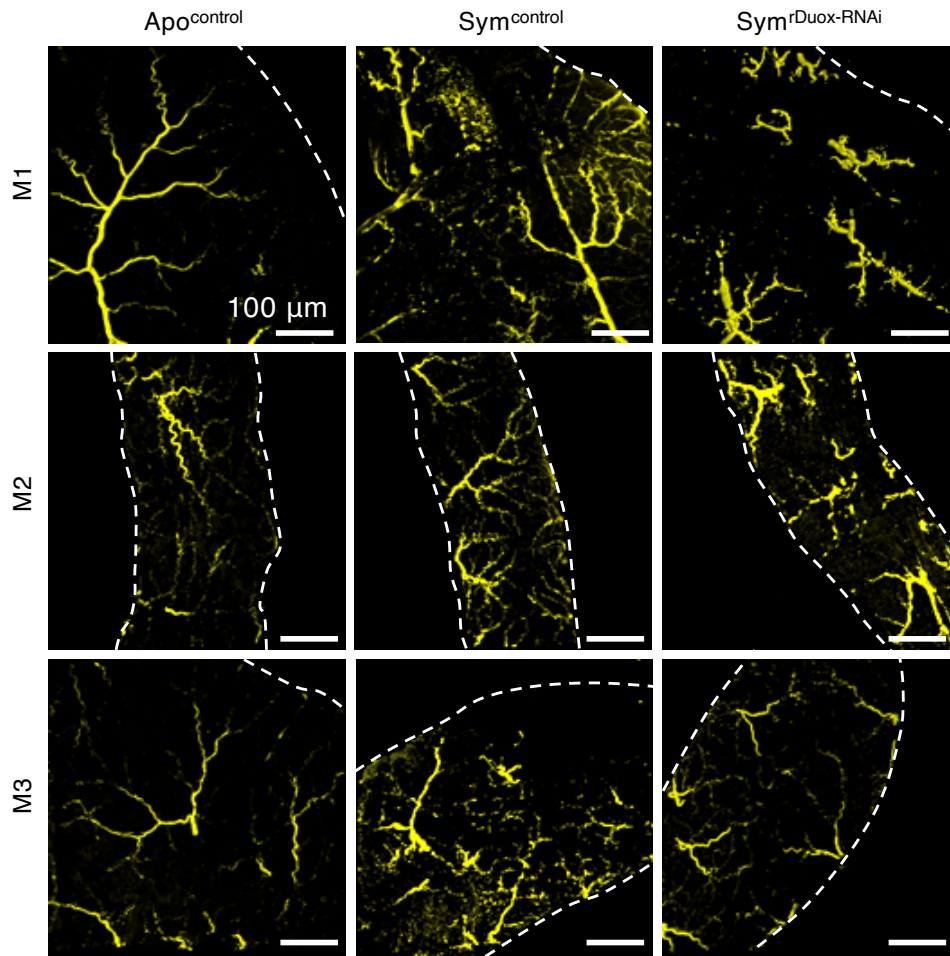


Fig. 3-5. Dityrosine network (DTN) in the tracheae of the M1, M2, and M3. Dissected M1, M2, and M3 of Apo (Apo^{control}), Sym (Sym^{control}) and *rDuox*-RNAi (Sym^{*rDuox*-RNAi}) insects were stained by anti-dityrosine antibody. The tracheoles were much more ramified in each midgut region of Sym insects, compared to Apo or *rDuox*-RNAi insects. Yellow color indicates the DTN of tracheae.

after *rDuoX*-RNAi (Fig. 3-6A). Correspondingly, the expression of genes encoding the mitochondrial respiratory chain was down-regulated in *rDuoX*-RNAi insects (Fig. 3-6B), confirming that the symbiotic organ entered into a hypoxic condition after *rDuoX*-RNAi. Moreover, staining the symbiotic organ with the Image-IT dye, which is a real-time hypoxia indicator, revealed a high fluorescence in the epithelia of the midgut crypts after *rDuoX*-RNAi compared with control Sym insects, further supporting that the disorganization of the respiratory network by *rDuoX*-silencing causes hypoxia of the symbiotic organ (Fig. 3-6A). Of note, our finding that AMP-encoding genes are up-regulated in the hypoxic M4 of *rDuoX*-RNAi insects in a Relish dependent manner (Figs. 3-3D and E), mirrors previous findings in *Drosophila*, demonstrating that a hypoxic environment activates the expression of AMPs via the Toll and IMD pathways (189, 190).

Hypoxia could reduce the metabolic activity (190) of the crypt epithelial cells and their capacity to feed the bacterial symbiont, thereby provoking the collapse of the symbiosis. Some insect genes related to glycolysis and most genes of the tricarboxylic acid (TCA) cycle were down-regulated in the M4 after *rDuoX*-RNAi, indicating that the metabolic activity of the crypt epithelial cells was indeed lower in *rDuoX*-RNAi insect than in Sym insects (Fig. 3-7A). Furthermore, hypoxia is expected to affect also directly the proliferation of the *Burkholderia* symbiont. Species of the genus *Burkholderia* are obligate aerobic bacteria, although they can tolerate microaerobic and anaerobic conditions (191–193). Accordingly, the *Burkholderia* symbiont grew up normally in normoxia (21% O₂) but did not grow well in hypoxia (6-12% O₂) and anoxia (<0.1% O₂) (Fig. 3-7B). However, the bacteria incubated first in hypoxia or anoxia grew up again

when transferred to normoxia and cultured for an additional 48 hours (Fig. 3-7B), suggesting that *B. insecticola* needs enough oxygen for proliferation *in vitro* as well as in the midgut crypts. Furthermore, a *B. insecticola* mutant in the *resB* gene, essential for cytochrome c maturation and respiration, cannot properly colonize the M4 crypts (Fig. 3-6C). A reduced flux of oxygen and nutrients into the lumen of the crypts of *rDuoX*-RNAi insects could create a rapid depletion of these molecules resulting in a gradient from the surface of the crypts towards their interior. Such a gradient could explain the presence of a relatively high number of symbionts close to the epithelial surface of the midgut crypts, and a lack of them in the interior part of the crypt lumen, as observed by TEM (Fig. 3-1F). Thus, the symbiosis defect in the *rDuoX*-RNAi insects is likely due to a lack of sufficient oxygen in the symbiotic organ that can sustain the respiratory activity of the symbiont.

Conversely, the high bacterial respiration in the M4 is the probable trigger of the extensive tracheal network in Sym insects. *B. insecticola* mutants in *fliC*, encoding the flagellin protein, and *uppP*, involved in cell wall synthesis, cannot colonize the M4 (84, 102), while a mutant in *purL*, involved in purine biosynthesis, can only weakly colonize the crypts (106) (Fig. 3-6C). The formation of tracheae was not or less stimulated in insects infected with these mutants (Fig. 3-6C), confirming that the mere presence of *Burkholderia* in the midgut is not sufficient and that bacterial proliferation in the M4 crypts is required to trigger tracheal development.

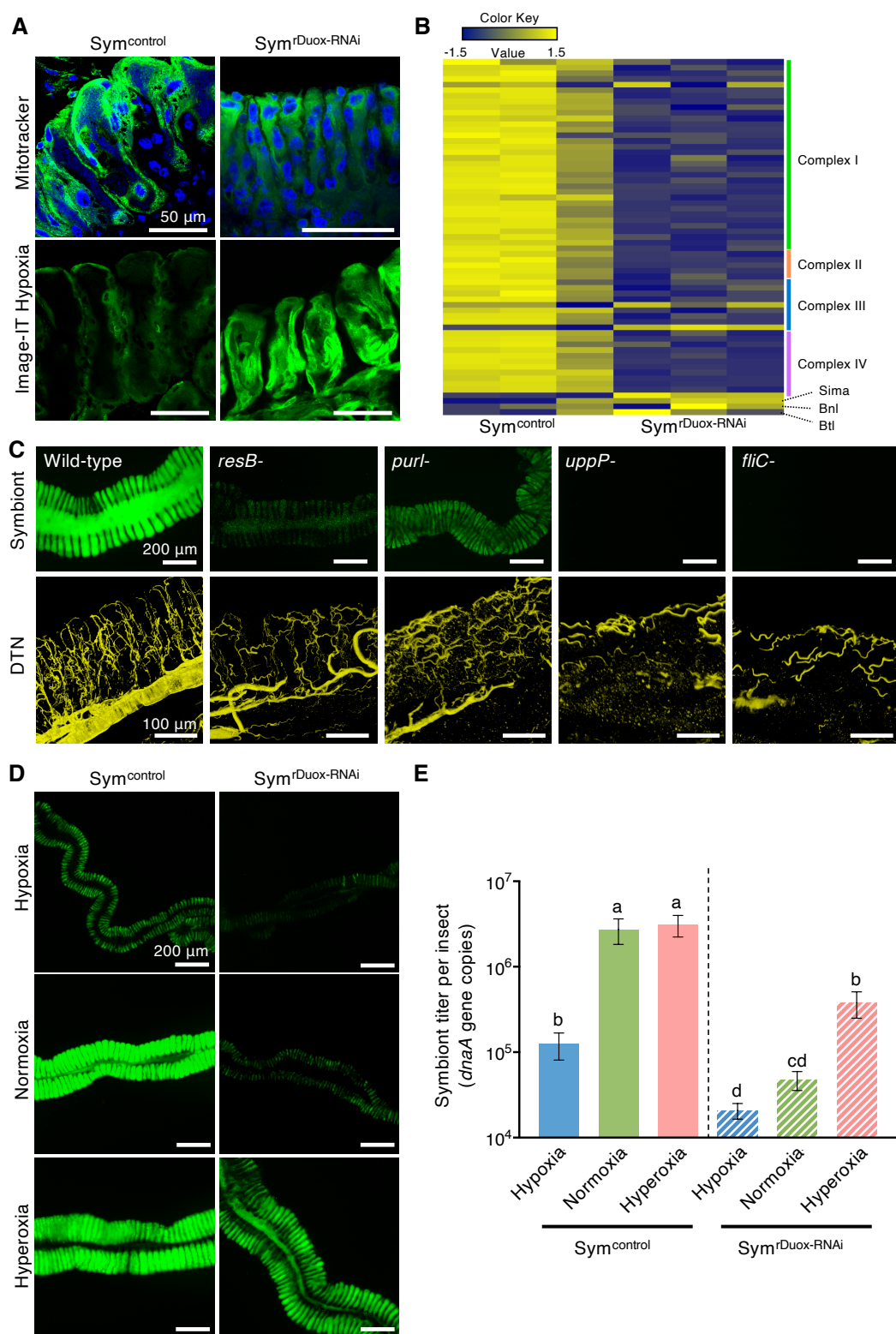


Fig. 3-6. Destabilization of the respiratory network by rDuox-RNAi causes hypoxia in the midgut. (A) Active mitochondria in Sym (Sym^{control}) and *rDuox*-RNAi (Sym^{rDuox-RNAi}) insects stained by Mitotracker, a membrane potential fluorescence dye, which stains active mitochondria in live cells. Green, Mitotracker; blue, host nuclei (top). Hypoxia in the M4 revealed by staining with the Image-IT hypoxia fluorescence dye, a real-time oxygen detector (green, bottom). (B) Heatmap of relative expression levels of genes related to the host respiratory chain, Sima, Branchless (bnl), and Breathless (btl) in Sym^{control} and Sym^{rDuox-RNAi} insects. Each column is a replicate experiment. (C) Colonization level of the wild type, *resB*-, *purL*-, *uppP*-, and *fliC*-deficient *B. insecticola* mutants in the symbiotic organ (top panel). The DTN of the tracheal network from the symbiotic organs infected with wild type and *Burkholderia* mutants (lower panel). Yellow color indicates the DTN signal of the tracheal network. (D) Observation by epifluorescence microscopy of the midgut of Sym^{control} and Sym^{rDuox-RNAi} insects reared in hypoxia (6-12% O₂), normoxia (21% O₂), and hyperoxia (39-42% O₂) environments. Green is the GFP signal derived from *Burkholderia* gut symbionts. (E) The number of gut symbionts in the M4 of Sym^{control} and Sym^{rDuox-RNAi} insects reared in hypoxia, normoxia, and hyperoxia condition. Data shown in (E) are mean $\pm$ s.d. $n = 10$ insects. Different letters indicate statistically significant differences ($P < 0.05$). The statistical significance of differences between samples was analyzed by the Mann-Whitney *U* test with the Bonferroni correction.

Next, we confirmed the crucial role of oxygen in gut symbiosis by rearing Sym and *rDuoX*-RNAi insects in hypoxia (6-12% O₂), normoxia (21% O₂) and hyperoxia (39-42% O₂) conditions (Fig. 3-7C). The GFP signal of gut symbionts was diminished in Sym insects reared in a low oxygen environment (Fig. 3-6D). In agreement with the microscopy observation, qPCR confirmed the decreased number of *Burkholderia* in the M4 crypts (Fig. 3-6E). In contrast, when Sym insects were reared in hyperoxia, the number of gut-colonizing symbionts was not significantly increased, suggesting that the oxygen supply to maintain symbiosis is sufficient in normoxia (Figs. 3-6D and E). As described above, *rDuoX*-RNAi insects harbor only few symbiotic bacteria under normoxia, and when they were reared in hypoxia, their symbiont titer was decreased even further (Figs. 3-6D and E). However, the M4 crypt population of gut symbionts in *rDuoX*-RNAi insects considerably recovered in a hyperoxia environment (Figs. 3-6D and E). These results demonstrate that the availability of sufficient oxygen in the symbiotic organ is critical for a successful symbiosis and that a deficient oxygen supply is the major factor underpinning the symbiotic defect of *rDuoX*-RNAi insects.

Silencing of regulators of tracheal development simulates the collapse of symbiosis

To confirm the importance of oxygen distribution by the respiratory network in gut symbiosis, we silenced *branchless* (*rBnl*) and *trachealess* (*rTrh*), two known genes important for the tracheal development in *Drosophila* (194–196), as well as *rSima*, encoding the Hypoxia-Inducible Factor (HIF)-1 α , which is a central regulator of the transcriptional response to hypoxia (197). The *rTrh*-RNAi insects showed a high

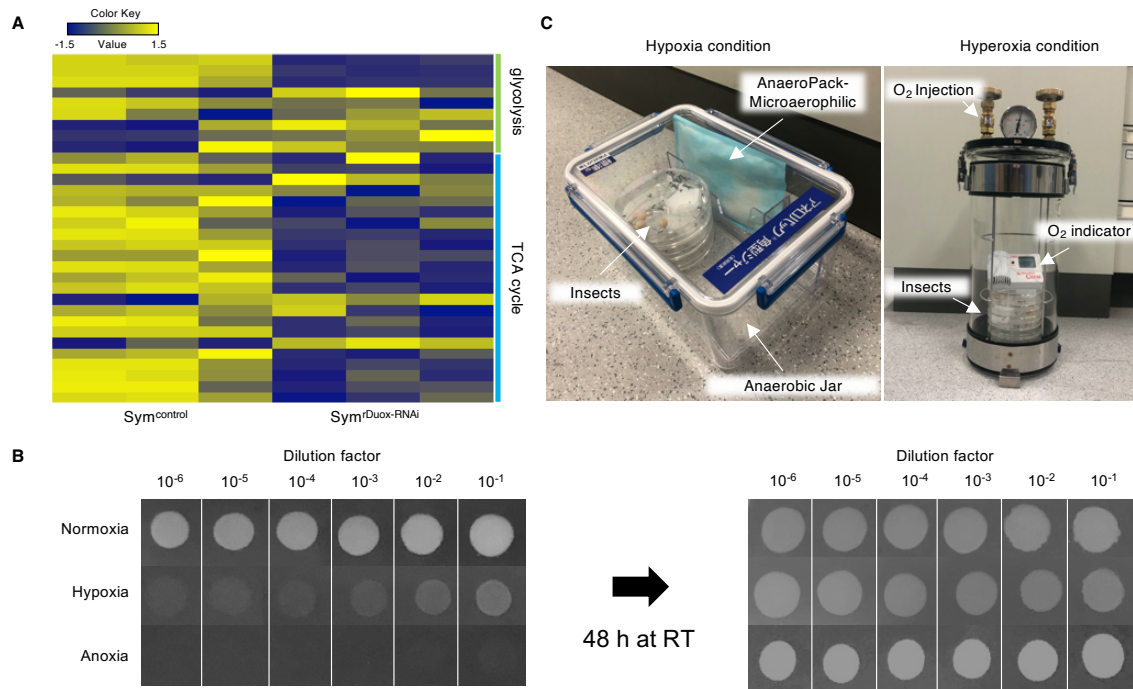


Fig. 3-7. Effect of oxygen level on host metabolism and *Burkholderia* symbiont. (A) The expression level of the genes related to glycolysis and TCA cycle in *Sym^{control}* and *Sym^{rDuox-RNAi}* insects. Each column is a replicate experiment. (B) Growth of the wild-type *Burkholderia* gut symbiont on agar plate in aerobic (21% O_2), microaerophilic (6-12% O_2), and anaerobic (<0.1% O_2) environment for 48 h (left panel) and growth after transfer of plates to normoxia and further incubation for 48 h (right panel). (C) Images of the hypoxia (6-12% O_2) and hyperoxia (39-42% O_2) set ups.

mortality (Fig. 3-9B), but the alive insects and those silenced for the other two genes had an underdeveloped respiratory network in the M4 (Fig. 3-8), in agreement with the expected role of *rBnl* and *rTrh* and further showing that oxygen-depletion signaling via Sima controls the tracheal development in the symbiotic organ. Crucially, the defect in the tracheal development in these RNAi insects is accompanied with a failure to establish the symbiosis with a full infection of the crypts as revealed by the strongly decreased GFP-signal derived from gut symbionts in the symbiotic organ (Fig. 3-8 and 3-9A). Thus, the downregulation of these three regulators of the respiratory system affected symbiosis in a very similar way as rDuox-RNAi.

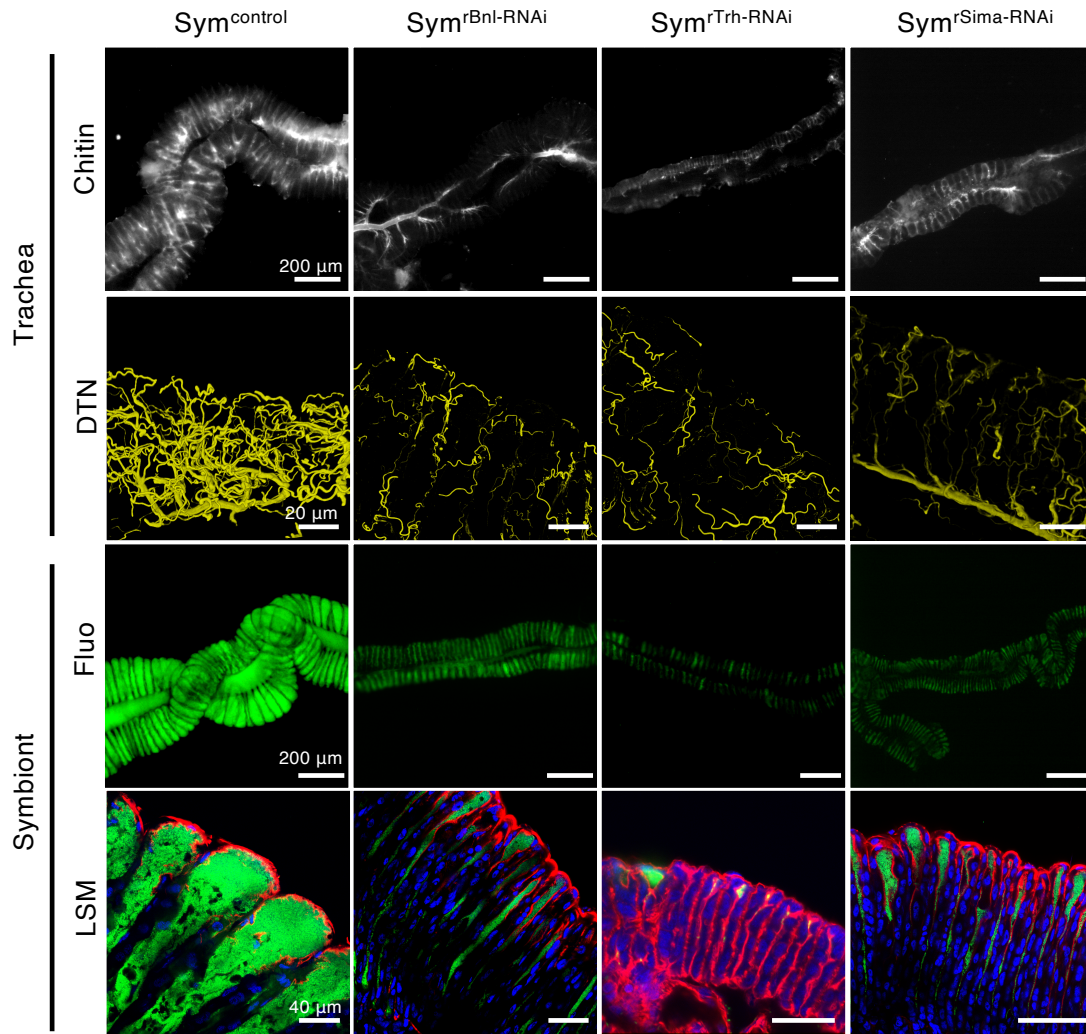


Fig. 3-8. Oxygen supply to the symbiotic organ via the tracheal network is important for gut symbiosis. Chitin staining (first row), 3D reconstruction of the dityrosine network (second row), colonization by *Burkholderia* (third row) and crypt morphology (fourth row) in the M4 of Sym (Sym^{control}), *rBnl*-RNAi (Sym^{rBnl-RNAi}), *rTrh*-RNAi (Sym^{rTrh-RNAi}), and *rSima*-RNAi (Sym^{rSima-RNAi}) insects. Images were obtained by epifluorescence microscopy in the first and third row and by laser scanning confocal microscopy in the second and fourth rows. White, chitin; yellow, DTN; green, gut symbiont; blue, host nuclei; red, host cytoskeleton.

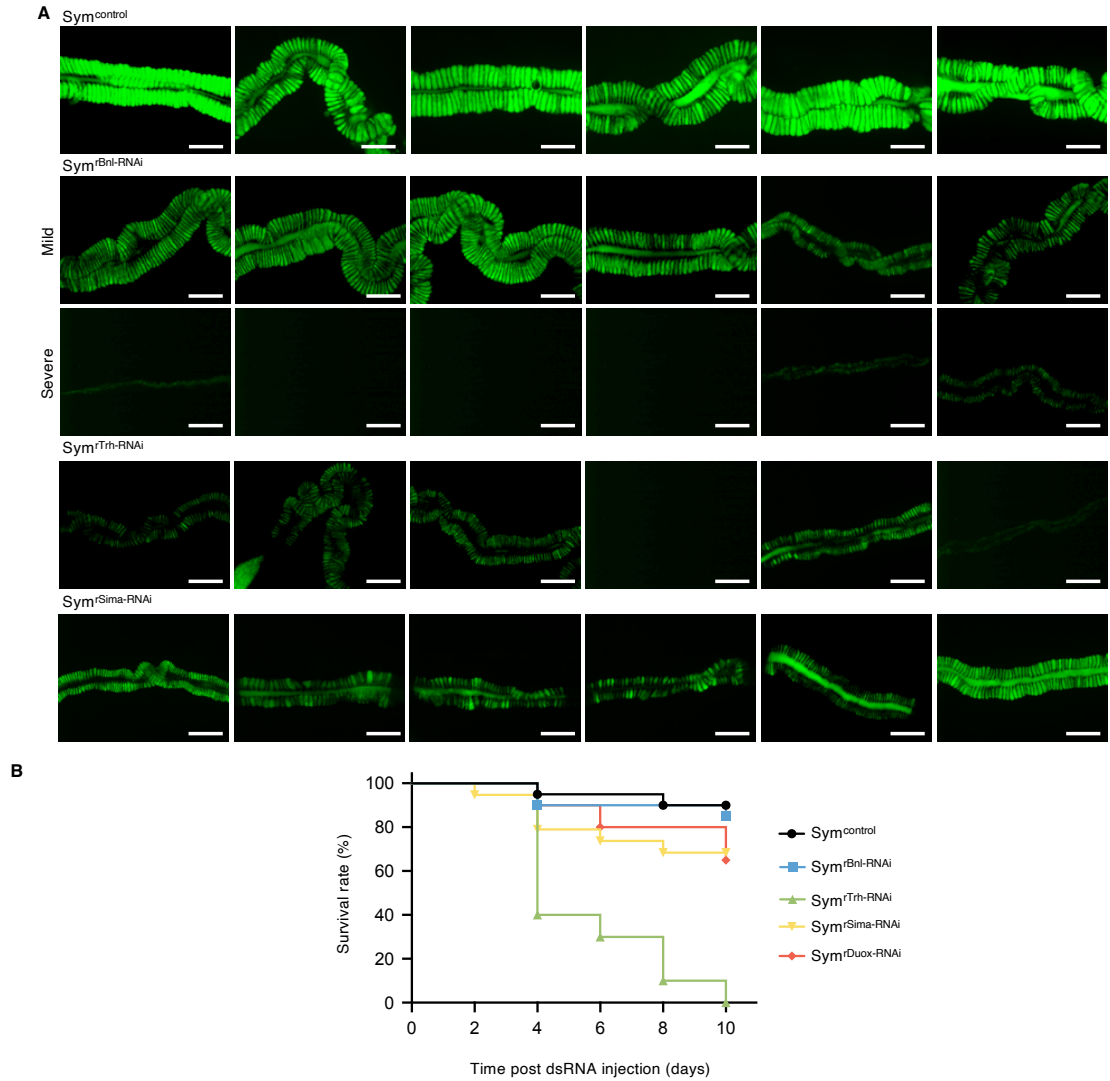


Fig. 3-9. Effect of *rBnl*-, *rTrh*-, and *rSima*-RNAi on symbiosis and survival of insects. (A) Fluorescence microscopy images of the symbiotic organ of Sym (Sym^{control}), *rBnl*-RNAi (Sym^{rBnl-RNAi}), *rTrh*-RNAi (Sym^{rTrh-RNAi}), and *rSima*-RNAi (Sym^{rSima-RNAi}) insects. In Sym^{rBnl-RNAi} insects, “Mild” are examples in which symbiosis is moderately affected by RNAi and “Severe” are examples in which symbiosis is seriously affected by RNAi. **(B)** The survival rate of Sym^{control}, Sym^{rBnl-RNAi}, Sym^{rTrh-RNAi}, and Sym^{rSima-RNAi} insects in days after the silencing of the target genes. $n = 20$ insects.

Discussion

Our results demonstrate that the large population of the *Burkholderia* symbiont present in the body of the bean bug is an oxygen sink and stimulates the sprouting of tracheal branches towards the symbiont-infected M4 crypts (Fig. 3-10). We highlight a previously unknown function of the Duox enzyme in this process, catalyzing the formation of a newly discovered tracheal DTN, which is essential for the stabilization of the respiratory network. The tracheal system consists of a tubular epithelial network containing an extracellular luminal chitin and protein matrix (186, 187). Chitin is an essential component of this matrix, providing rigidity to the tracheal tubes, and mutants in chitin synthesis genes form misshapen tracheal tubes in *Drosophila* (188, 198). The matrix contains also proteins, including the zona pellucida domain proteins Piopio (Pio) and Dumpy (Dp), chitin-binding and remodeling proteins and other unidentified protein components. They are thought to provide, together with the chitin scaffold, a structural network in the luminal space, regulating tube length, width, branching and integrity (199–201). The requirement for Duox in the formation of new tracheae indicates that dityrosine-cross-linking these matrix proteins is crucial for their structural function. Similar as in *R. pedestris*, we found that the expression of *Duox* is much higher in the tracheae than in the midgut in the phylogenetically diverse insects *Gryllus bimaculatus* (Orthoptera), *Bombyx mori* (Lepidoptera), *Tribolium castaneum* (Coleoptera), and *D. melanogaster* (Diptera) (Fig. 3-11).

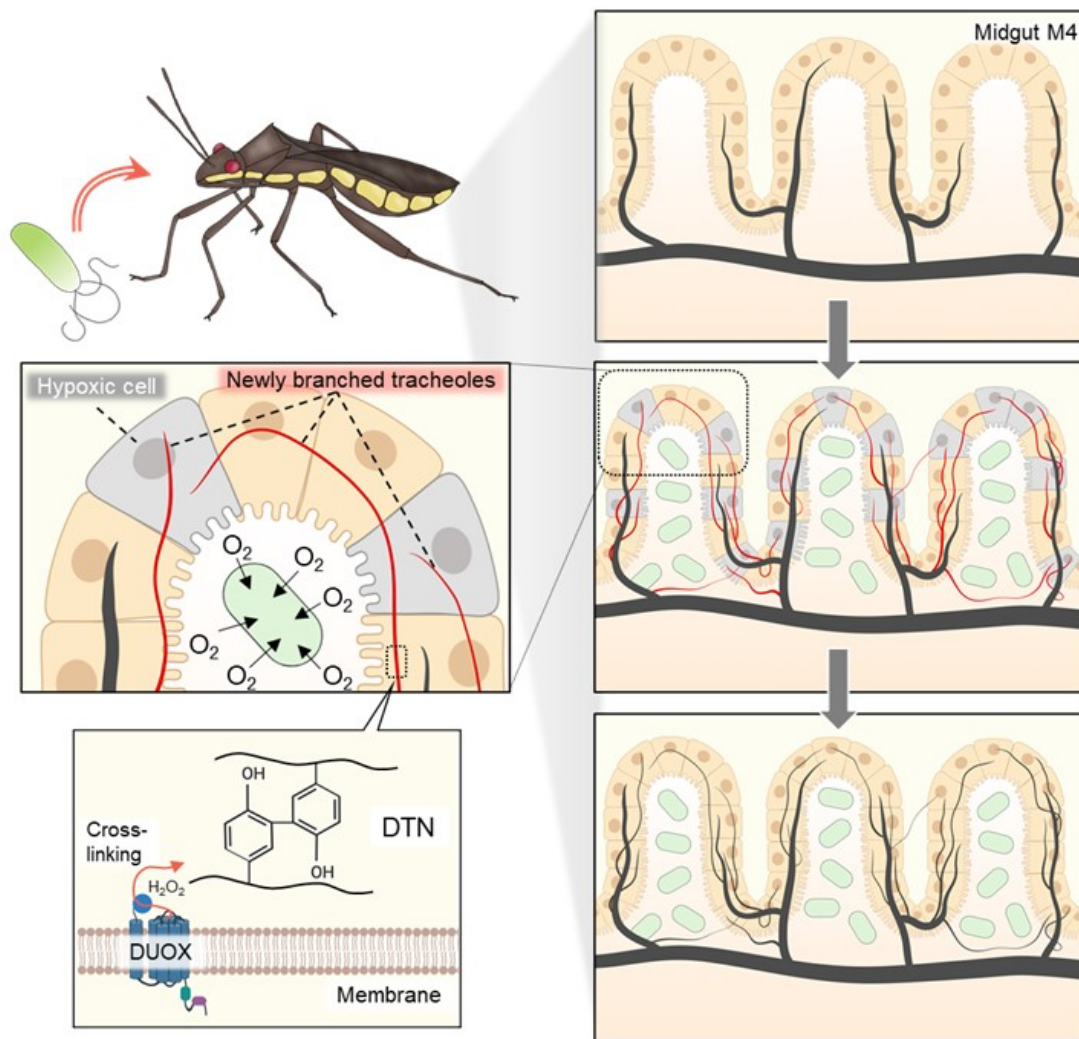


Fig. 3-10. *rDuox* mediates symbiosis and tracheal network stability. Tracheal network in the M4 before symbiont infection (top right). Development of the tracheal network is stimulated to supply oxygen to the M4 when colonized by the symbiont, which consumes high levels of oxygen (middle left and right); red, newly ramified tracheoles; green, gut symbiont; grey epithelial cells experiencing reduced oxygen levels and attracting growing tracheoles. The branched tracheoles are stabilized by *rDuox* (bottom right). *rDuox* stabilizes the DTN of the tracheal cuticle layer, which is critical for structurally maintaining trachea (bottom left). The developmental order is in the direction of the arrows.

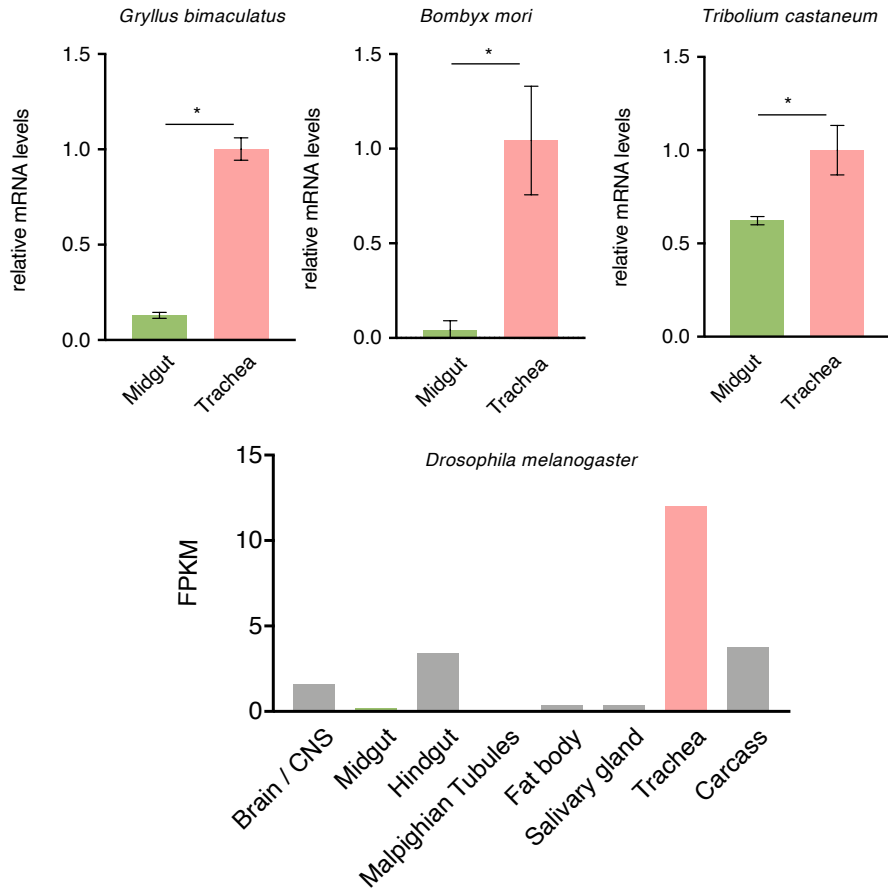


Fig. 3-11. The expression level of *Duox* in phylogenetically different orders of insects. The mRNA levels of *Duox* were measured from extracted midgut and tracheae of nymphal *G. bimaculatus* (Orthoptera), larval *B. mori* (Lepidoptera), and adult *T. castaneum* (Coleoptera), by RT-qPCR (top). Data shown in a,b are mean $\pm$ s.d. $n = 4$ insects. The statistical significance among samples was analyzed by the Mann-Whitney U test. Asterisks indicate statistically significant differences ($P < 0.05$). The FPKM (fragments per kilobase of exon model per million reads mapped) values of the *Duox* gene in larval *D. melanogaster* (Diptera) were extracted from the FlyAtlas 2 database (<http://flyatlas.gla.ac.uk/FlyAtlas2/index.html>) (bottom). CNS, central nerval system.

This conserved expression pattern suggests that the role of Duox in tracheae is common in the insects. Moreover, the here proposed pivotal role of Duox-mediated ROS in the formation of the tracheal cuticle adds up to a number of other structures composed of extracellular matrices requiring Duox for DTN formation. These structures include the earlier mentioned peritrophic matrix of the gut in the mosquito *A. gambiae* as well as in the tick *Ixodes scapularis* (172, 173), the eggshell of the kissing bug (202), the cuticle structure of wings in *Drosophila* (203, 204), the collagenous extracellular cuticle matrix that forms the outer covering of nematodes (205) and the hardened extracellular matrix of sea urchin eggs after fertilization (206). Therefore, the importance of Duox in forming DTNs as part of the biogenesis of extracellular cuticle structures is conserved and not limited to the insects. This function could be the ancestral and primary function of Duox enzymes.

The formation of dityrosine cross-links in cuticle requires a NADPH oxidase to generate H_2O_2 and a heme peroxidase to catalyze the formation of a covalent bond between two neighboring tyrosine residues via tyrosil radical formation and the reduction of the H_2O_2 . Potentially both these functions are provided by Duox, via its C-terminal NADPH oxidase domain and N-terminal peroxidase homology domain, respectively. In *Caenorhabditis elegans*, Duox is such a self-contained catalyst that mediates tyrosine cross-linking independently (207). However, in mosquito, fly and ticks, the peroxidase domain is only partially functional in DTN formation and an additional secreted heme peroxidase protein is required for the formation of the dityrosine cross-links (172, 173, 203). The weak peroxidase activity of Duox in these organisms is believed to result from a mutation in a

glutamic acid residue in the N-terminal peroxidase domain, involved in heme binding (173). In the case of the *R. pedestris* rDuox protein, the same point mutation is present, suggesting that the functionality of rDuox may be similar to the tick, fly and mosquito Duox enzymes. Therefore, we predict that the rDuox enzyme works in concert with an additional, unidentified heme peroxidase to form the dityrosine bridges in the matrix proteins.

Often, the production of bactericidal ROS is presented as the principal function of Duox in insect physiology (163, 164, 171). In *Drosophila*, knock-down of *Duox* or *Mesh*, a positive regulator of *Duox* expression, leads to an increase of the gut microbiome load or to the persistence in the gut after oral infection of the entomopathogenic bacterium *Erwinia carotovora* ssp. *carotovora* strain *Ecc15* (169, 208). A similar role in direct killing of gut microbes was proposed in *C. elegans* (168). In striking contrast, we did not observe after *Duox*-RNAi in the bean bug an increase of the pathogen *S. marcescens* in the M3 digestive region of the midgut or of the symbiont in M3 or the M4 crypts (Figs. 3-1E, 3-3A and 3-3C). On the contrary, we noticed a strongly reduced symbiotic population in M4 (Figs. 3-1E and F). In the mosquito *A. gambiae* and the tick *I. scapularis*, silencing *Duox* or the gene encoding the Duox partner-peroxidase, decreased the microbial load in the midgut rather than increasing it (172, 173). Together, these results indicate that the direct bactericidal activity of Duox as proposed originally in *Drosophila* is not universal in the insects and other arthropods. In light of this conclusion, we propose that the exclusive emphasis on the role of Duox in the production of antimicrobial ROS in the gut of *Drosophila* (163, 164, 171) should be revisited. It is likely that *Drosophila*

Duox is involved also in the synthesis of the peritrophic matrix in the midgut and contributes through this activity as well to gut immunity. In agreement with this hypothesis, a mutation of the *Drosophila dcy* gene, encoding the Drosocrystallin chitin-binding protein, results in a thinner but not completely abolished peritrophic matrix, which renders the flies more sensitive to gut infections with entomopathogenic bacteria (209, 210). This phenotype reminds the Duox RNAi flies although the latter were more severely affected in the immune response (160). Transglutaminase is another enzyme that affects protein-protein cross-linking and permeability in the peritrophic matrix of *Drosophila*, as well as the tolerance of the flies to the gut microbiota (211). Thus, Duox could have a dual contribution to the gut immune homeostasis in the case of *Drosophila*. On the other hand, the load of aerobic bacteria in the *Drosophila* midgut is much lower than in the *R. pedestris* symbiotic organ (212), even after sustained pathogen infection (160). Therefore, Duox-mediated development of tracheae enveloping the midgut could possibly have a lesser importance for sustaining the microbiota or permitting pathogen development in *Drosophila*.

The trigger for the formation of the respiratory network enveloping the crypts could be in principle a bacterial signal or a host signal generated indirectly by the colonization of the crypts. Alternatively, low oxygen levels created by the metabolic activity of the proliferating symbionts and the crypt epithelial cells that nurture the symbiont, could be the direct cause of tracheae genesis. In agreement with the latter possibility, we find that Sima, Trh and Bnl are required for tracheae development in a similar way as Duox. The *bnl* gene in *Drosophila* encodes a fibroblast growth factor homolog whose expression is

highly increased in oxygen-starved non-tracheal cells. Bnl exerts chemoattraction of tracheal cells via binding to the trachea-specific receptor Breathless (Btl) that controls the ramification of tracheae towards the Bnl-producing cells (194, 213). The transcription factor Trh is a central regulator of tracheal cell identity, controlling trachea-specific gene expression, including *btl* (195, 196). The Hypoxia-Inducible Factor (HIF)-1 α homolog Sima in *Drosophila* is a central regulator of the transcriptional response to hypoxia. The Sima transcription factor is actively degraded in normoxia, dependent on the oxygen sensor Fatiga, but is stable and active in hypoxia (197, 214). Active Sima in hypoxia up-regulates the expression of *btl* in tracheal cells and *bnl* in non-tracheal cells, thereby stimulating the development of the respiratory network (215). Thus in *R. pedestris*, oxygen depletion in the symbiotic organ triggered by symbiont colonization is potentially enough as a signal to stabilize Sima in both the crypt epithelia and the local tracheal cells. Sima subsequently activates the transcription of respectively *rBnl* and *rBtl*, the latter requiring also Trh. This signaling cascade controls the development of the tracheal network around the crypts.

In a remarkable analogous process to the tracheal development in the colonized midgut of *R. pedestris*, colonization of the mammalian small intestine by microbiota promotes angiogenesis and the development of the blood vessel network around the gut epithelia. This enhanced vascularization increases oxygenation of the villi, which have a modified morphology after colonization and an increased nutrient absorption activity (216–218). Thus from insects to mammals, colonization of the gut by symbionts can create a local oxygen sink, triggering an analogous postembryonic developmental response.

Besides the midgut crypts of *R. pedestris* and related stinkbugs, other types of symbiotic organs in other insects, such as bacteriomes (1, 12), are equally enveloped by a tracheal network, presumably to provide oxygen to the respiring symbiotic bacteria (219, 220). These symbioses might therefore depend on Duox activity as well. Feeding *Drosophila* and *C. elegans* with different types of antioxidant chemicals inhibits Duox-produced ROS (168, 208). Here, we show that feeding *R. pedestris* with the antioxidant NAC is sufficient to prevent the accumulation of ROS produced by Duox and the sprouting of the tracheae and this blocks the establishment of symbiosis. Since many insects obligatorily depend on their symbioses, triggering its collapse by the specific inhibition of the respiratory network with antioxidants could be a new route to fight insect pests.

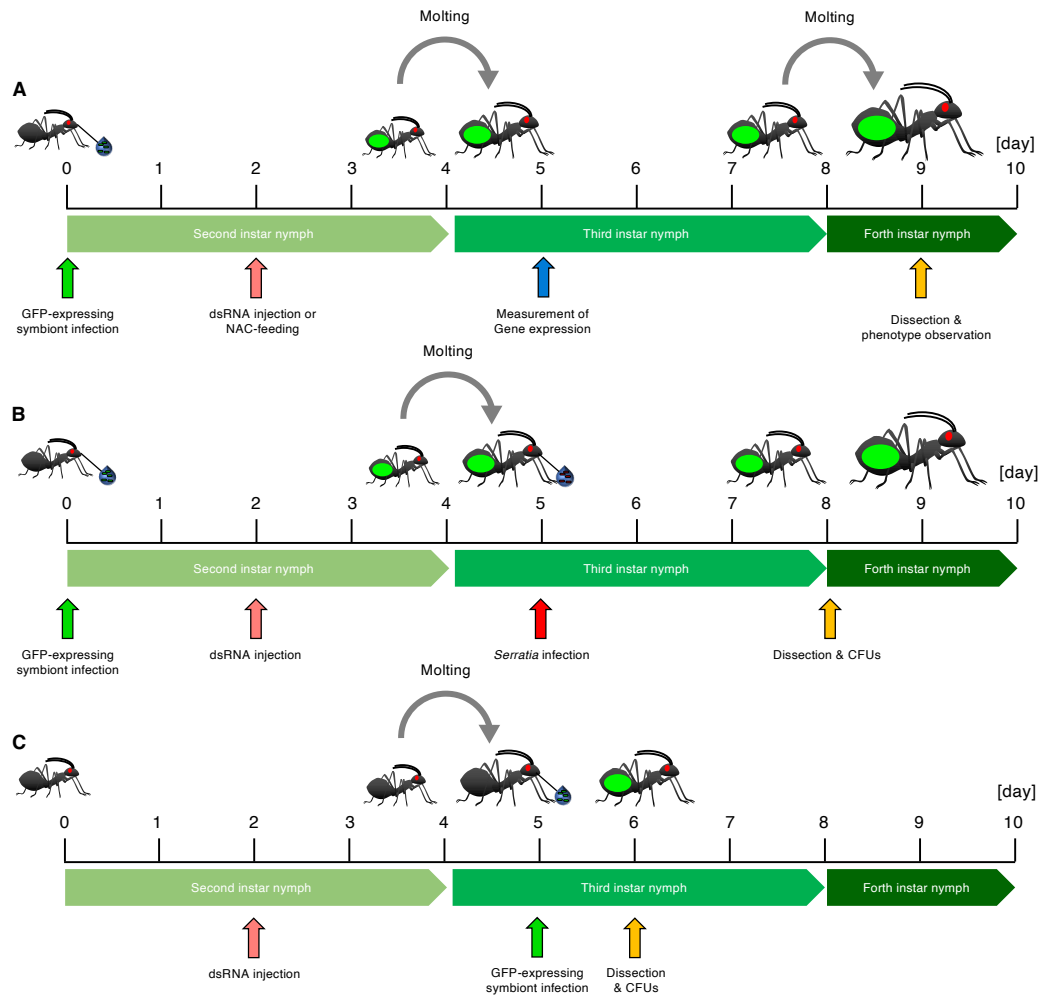


Fig. 3-12. The timeline of experiments used in this study. (A) The timeline of the experiments for measuring gene expression and phenotypic observation. **(B)** The timeline of the experiment for *Serratia* CFU. **(C)** The timeline of the experiment for *Burkholderia* CFU; CFU, colony-forming units.

Chapter IV

General discussion

This study has focused on the elucidation of host-symbiont specificity, especially the mechanism of how symbiont colonizes and adapts to the host midgut crypts and how host *R. pedestris* keeps a large number of symbiont in the midgut. Since *R. pedestris* acquires gut symbiont *Burkholderia* from the environmental soil every generation at the early nymphal stage, there always is a danger of contamination of symbiotic midgut organ by non-symbiotic soil microbes including pathogens. *R. pedestris* have evolutionarily developed a special symbiont sorting organ called “CR”, which is a very narrow and sticky region, in front of the symbiotic organ (120). The previous study has revealed that the CR physically winnows out non-symbiotic bacteria, but let only *Burkholderia* symbiont pass resulting in colonization to the midgut crypts. However, the previous study tested only three bacterial species (*E. coli*, *Pseudomonas putida*, and *Bacillus subtilis*), thus doubt about microbial contamination in the symbiotic organ has not been completely solved.

In this study (Chapter 2), I inoculated 34 *Burkholderia* species 18 taxonomically diverse bacterial species to *R. pedestris* and revealed that non-native symbiont *Burkholderia* of PBE group and even its outgroup *Pandoraea* colonize the midgut crypts, providing beneficial effects to the host along with native symbiont *B. insecticola*. This result implies that the CR is not perfect as filtering organ. However, the field-captured *R. pedestris* overwhelming has been infected by native *Burkholderia* of SBE clade even though some non-symbionts can successfully colonize the midgut crypts. Through co-infection tests of native *Burkholderia* symbiont and non-symbiotic bacteria, I demonstrated that microbial competition is critical to occupy the symbiotic organ. In other words, native symbiont SBE *Burkholderia* outcompetes non-native symbionts inside the

gut and then monopolizes the midgut crypts. This study is the first report on insects that the host's symbiotic partner is selected by microbial competition in the symbiotic organ besides the host-side factors.

Furthermore, I constructed T6SS genes-deleted mutant strains of *B. insecticola* to understand the mechanism of microbe-microbe competition in the gut. The T6SS is a commonly known factor that is important in the competition of bacteria by killing each other via the secretion of toxins (221). Recent reports show that T6SS is indeed a critical element of symbiotic microbes in squid and honey bee to occupy the niches (138, 139). *Burkholderia insecticola* possesses only one intact T6SS gene cluster, but $\Delta vgrG$ and $\Delta clpV$ *Burkholderia* mutants, which are the strains lacking spike protein or ATPase respectively, did not show any differences in competitive abilities compared to wild-type in the gut, demonstrating T6SS of *B. insecticola* is not involved in the microbial competition. Thus, more mutant strains should be tested to disclose the molecular bases mechanism of competition of gut symbiont versus non-symbiotic bacteria in the future study. A recent study compared the gene expression levels between *in vitro* cultured *B. insecticola* and *in vivo* gut-colonizing *Burkholderia* (109). Candidate genes, which are up-regulated *in vivo* compared to *in vitro*, could be deleted and those mutants can be used for co-inoculation with non-symbionts. However, if candidate genes are only highly expressed when *B. insecticola* co-exists with non-symbiont, further transcriptomic analyses are needed, such as the comparison of RNAseq between solely infected gut-colonizing *B. insecticola* and *B. insecticola* co-infected with non-symbiotic bacteria.

In Chapter 3, I determined that infection in the midgut by aerobic symbiont *B. insecticola* is a high oxygen-consuming process and thus extremely provokes the

development of the trachea, enveloping whole midgut crypts wherein a large number of gut symbionts are colonized. The cuticle layer of the trachea consists of a dityrosine network and it is stabilized by ROS produced by the Duox enzyme. Destabilization of the respiratory network by Duox-RNAi indeed caused a collapse of the tracheal development results in gut dysbiosis due to deficiency of oxygen in the midgut. Although Duox-mediated ROS production is well-studied in the *Drosophila* gut mucosal immunity, it should be reconsidered that the stabilization of the tracheal network by Duox is also important to keep beneficial microbiota of *Drosophila* through the delivery of enough oxygen to the gut. The higher expression level of Duox in the trachea than the midgut in diverse orders of insects underpins that oxygen transfer by trachea, which is stabilized by Duox-mediated ROS via forming DTN, could be highly conserved in insects and indispensable to maintain gut homeostasis. Besides the midgut crypts of *R. pedestris*, the symbiont-possessing compartments of other insects are also surrounded by the dense trachea and it should supply oxygen to the aerobic symbiont. This underpins that oxygen delivery to the symbiotic organ through the stabilized tracheal network by Duox is important to keep symbionts in the insect.

I also showed in this study (chapter 3) that the antioxidant NAC also disrupts a DTN of the tracheal network. It could be worth to use antioxidants in the agricultural fields to control pest insects that possess obligate symbiotic bacteria. Insecticides have been used to eradicate pests for a long time, but insecticide-resistant insects have appeared due to the overuse of pesticides. The biopesticides then have been developed to control resistant pests as an alternative product that are made by microorganisms themselves or microbe-derived toxins. My discovery in this study strongly suggests that the possibility

of an antioxidant NAC as a novel insecticide by demolishing essential symbiotic association between pests and symbionts. Numerous stinkbug species harbors obligate gut symbiont that is essential for host growth and survival, in other words, these bugs can't live without symbiotic bacteria. As a further study, I tested the insecticidal activity of NAC by feeding it to diverse stinkbugs possessing obligate symbiont. All tested nymphal insects were dead within 2 weeks after oral administration of NAC, indicating that an antioxidant could be used on a broad range of insects (unpublished data). However, the insecticidal ability of NAC should be tested on adult insects as well in the further study because of their fecundity. Besides the insecticidal activity, the effect of NAC on host plants of pest insects also has to be tested in further research although there are some reports that NAC gives beneficial effects to plants including promoting growth and enhancing resistance against environmental stresses.

Taken together, I revealed during the doctoral course “how host *R. pedestris* and symbiont *Burkholderia* maintains gut symbiosis” at the molecular level. Since *R. pedestris-Burkholderia* model system has been successfully studied over a decade, numerous fruitful information has been accumulated such as omics data. Many key symbiosis factors of *Burkholderia* symbiont indeed have been identified based on genomics and transcriptomics data and I determined the host-side symbiosis factor firstly in this study. Additional elements for gut symbiosis will be disclosed in further studies and those will contribute to understanding insect-bacteria or even eukaryote-prokaryote symbiosis.

References

1. P. Buchner, "Endosymbiosis of animals with plant microorganisms." (1965).
2. P. Mylona, K. Pawlowski, T. Bisseling, Symbiotic nitrogen fixation. *Plant Cell* (1995) <https://doi.org/10.1105/tpc.7.7.869>.
3. W. M. Chen, *et al.*, Legume Symbiotic Nitrogen Fixation by β -Proteobacteria Is Widespread in Nature. *J. Bacteriol.* (2003) <https://doi.org/10.1128/JB.185.24.7266-7272.2003>.
4. N. Garg, Geetanjali, Symbiotic nitrogen fixation in legume nodules: Process and signaling. A review. *Agron. Sustain. Dev.* (2007) <https://doi.org/10.1051/agro:2006030>.
5. K. M. Jones, H. Kobayashi, B. W. Davies, M. E. Taga, G. C. Walker, How rhizobial symbionts invade plants: The Sinorhizobium - Medicago model. *Nat. Rev. Microbiol.* (2007) <https://doi.org/10.1038/nrmicro1705>.
6. J. K. Vessey, Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* (2003) <https://doi.org/10.1023/A:1026037216893>.
7. B. Lugtenberg, F. Kamilova, Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* (2009) <https://doi.org/10.1146/annurev.micro.62.081307.162918>.
8. S. V. Nyholm, M. J. McFall-Ngai, The winnowing: Establishing the squid - Vibrios symbiosis. *Nat. Rev. Microbiol.* (2004) <https://doi.org/10.1038/nrmicro957>.
9. M. J. McFall-Ngai, The importance of microbes in animal development: Lessons

- from the squid-vibrio symbiosis. *Annu. Rev. Microbiol.* (2014)
<https://doi.org/10.1146/annurev-micro-091313-103654>.
10. B. W. Jones, M. K. Nishiguchi, Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar. Biol.* (2004)
<https://doi.org/10.1007/s00227-003-1285-3>.
 11. Y. Kikuchi, T. Hosokawa, T. Fukatsu, “Diversity of bacterial symbiosis in stinkbugs” in *Microbial Ecology Research Trends*, (2008).
 12. P. Baumann, Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* **59**, 155–189 (2005).
 13. A. E. Douglas, The molecular basis of bacterial-insect symbiosis. *J. Mol. Biol.* (2014) <https://doi.org/10.1016/j.jmb.2014.04.005>.
 14. A. E. Douglas, The microbial dimension in insect nutritional ecology. *Funct. Ecol.* (2009) <https://doi.org/10.1111/j.1365-2435.2008.01442.x>.
 15. H. J. Flint, K. P. Scott, P. Louis, S. H. Duncan, The role of the gut microbiota in nutrition and health. *Nat. Rev. Gastroenterol. Hepatol.* (2012)
<https://doi.org/10.1038/nrgastro.2012.156>.
 16. R. Krajmalnik-Brown, Z. E. Ilhan, D. W. Kang, J. K. DiBaise, Effects of gut microbes on nutrient absorption and energy regulation. *Nutr. Clin. Pract.* (2012)
<https://doi.org/10.1177/0884533611436116>.
 17. F. Karlsson, V. Tremaroli, J. Nielsen, F. Bäckhed, Assessing the human gut microbiota in metabolic diseases. *Diabetes* (2013) <https://doi.org/10.2337/db13->

0844.

18. A. C. Gomes, C. Hoffmann, J. F. Mota, The human gut microbiota: Metabolism and perspective in obesity. *Gut Microbes* (2018)
<https://doi.org/10.1080/19490976.2018.1465157>.
19. Q. Ma, *et al.*, Impact of microbiota on central nervous system and neurological diseases: The gut-brain axis. *J. Neuroinflammation* (2019)
<https://doi.org/10.1186/s12974-019-1434-3>.
20. S. S. Yarandi, D. A. Peterson, G. J. Treisman, T. H. Moran, P. J. Pasricha, Modulatory effects of gut microbiota on the central nervous system: How gut could play a role in neuropsychiatric health and diseases. *J. Neurogastroenterol. Motil.* (2016) <https://doi.org/10.5056/jnm15146>.
21. N. E. STORK, Insect diversity: facts, fiction and speculation. *Biol. J. Linn. Soc.* (1988) <https://doi.org/10.1111/j.1095-8312.1988.tb00474.x>.
22. J. B. Lee, *et al.*, Gut symbiotic bacteria stimulate insect growth and egg production by modulating hexamerin and vitellogenin gene expression. *Dev. Comp. Immunol.* **69**, 12–22 (2017).
23. J. K. Kim, *et al.*, Burkholderia gut symbionts enhance the innate immunity of host Riptortus pedestris. *Dev. Comp. Immunol.* **53**, 265–269 (2015).
24. A. E. Douglas, Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annu. Rev. Entomol.* **43**, 17–37 (1998).
25. A. Brune, Symbiotic digestion of lignocellulose in termite guts. *Nat. Rev.*

- Microbiol.* **12**, 168–180 (2014).
26. H. Itoh, K. Tago, M. Hayatsu, Y. Kikuchi, Detoxifying symbiosis: microbe-mediated detoxification of phytotoxins and pesticides in insects. *Nat. Prod. Rep.* **35**, 434–454 (2018).
 27. T. Hosokawa, Y. Kikuchi, T. Fukatsu, How many symbionts are provided by mothers, acquired by offspring, and needed for successful vertical transmission in an obligate insect-bacterium mutualism? *Mol. Ecol.* (2007)
<https://doi.org/10.1111/j.1365-294X.2007.03592.x>.
 28. S. S. Prado, D. Rubinoff, R. P. P. Almeida, Vertical transmission of a Pentatomid caeca-associated symbiont. *Ann. Entomol. Soc. Am.* (2006)
[https://doi.org/10.1603/0013-8746\(2006\)99\[577:VTOAPC\]2.0.CO;2](https://doi.org/10.1603/0013-8746(2006)99[577:VTOAPC]2.0.CO;2).
 29. R. Koga, X. Y. Meng, T. Tsuchida, T. Fukatsu, Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. *Proc. Natl. Acad. Sci. U. S. A.* (2012)
<https://doi.org/10.1073/pnas.1119212109>.
 30. K. Takeshita, Y. Kikuchi, *Riptortus pedestris* and *Burkholderia* symbiont: an ideal model system for insect-microbe symbiotic associations. *Res. Microbiol.* **168**, 175–187 (2017).
 31. Y. Kikuchi, T. Hosokawa, T. Fukatsu, Insect-microbe mutualism without vertical transmission: A stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl. Environ. Microbiol.* (2007)

- <https://doi.org/10.1128/AEM.00067-07>.
32. N. Y. D. Ankrah, A. E. Douglas, Nutrient factories: metabolic function of beneficial microorganisms associated with insects. *Environ. Microbiol.* (2018) <https://doi.org/10.1111/1462-2920.14097>.
 33. S. Richards, *et al.*, Genome sequence of the pea aphid *Acyrtosiphon pisum*. *PLoS Biol.* (2010) <https://doi.org/10.1371/journal.pbio.1000313>.
 34. S. Shigenobu, H. Watanabe, M. Hattori, Y. Sakaki, H. Ishikawa, Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* (2000) <https://doi.org/10.1038/35024074>.
 35. N. A. Moran, A. Telang, Bacteriocyte-Associated Symbionts of Insects. *Bioscience* (1998) <https://doi.org/10.2307/1313356>.
 36. S. Shigenobu, D. L. Stern, Aphids evolved novel secreted proteins for symbiosis with bacterial endosymbiont. *Proc. R. Soc. B Biol. Sci.* (2013) <https://doi.org/10.1098/rspb.2012.1952>.
 37. N. A. Moran, P. H. Degnan, Functional genomics of *Buchnera* and the ecology of aphid hosts. *Mol. Ecol.* (2006) <https://doi.org/10.1111/j.1365-294X.2005.02744.x>.
 38. R. Gil, B. Sabater-Muñoz, A. Latorre, F. J. Silva, A. Moya, Extreme genome reduction in *Buchnera* spp.: Toward the minimal genome needed for symbiotic life. *Proc. Natl. Acad. Sci. U. S. A.* (2002) <https://doi.org/10.1073/pnas.062067299>.
 39. M. Slaytor, Cellulose digestion in termites and cockroaches: What role do

- symbionts play? *Comp. Biochem. Physiol. -- Part B Biochem.* (1992)
[https://doi.org/10.1016/0305-0491\(92\)90194-V](https://doi.org/10.1016/0305-0491(92)90194-V).
40. A. Brune, M. Ohkuma, “Role of the termite gut microbiota in symbiotic digestion” in *Biology of Termites: A Modern Synthesis*, (2011)
https://doi.org/10.1007/978-90-481-3977-4_16.
 41. Z. L. Sabree, S. Kambhampati, N. A. Moran, Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. *Proc. Natl. Acad. Sci. U. S. A.* (2009) <https://doi.org/10.1073/pnas.0907504106>.
 42. S. Jang, Y. Kikuchi, Impact of the insect gut microbiota on ecology, evolution, and industry. *Curr. Opin. Insect Sci.* (2020)
<https://doi.org/10.1016/j.cois.2020.06.004>.
 43. A. K. Snyder, J. W. Deberry, L. Runyen-Janecky, R. V. M. Rio, Nutrient provisioning facilitates homeostasis between tsetse fly (Diptera: Glossinidae) symbionts. *Proc. R. Soc. B Biol. Sci.* (2010)
<https://doi.org/10.1098/rspb.2010.0364>.
 44. L. Akman, *et al.*, Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nat. Genet.* (2002)
<https://doi.org/10.1038/ng986>.
 45. B. M. Boyd, *et al.*, Primates, Lice and Bacteria: Speciation and Genome Evolution in the Symbionts of Hominid Lice. *Mol. Biol. Evol.* (2017)
<https://doi.org/10.1093/molbev/msx117>.

46. J. Guo, *et al.*, Nine facultative endosymbionts in aphids. A review. *J. Asia. Pac. Entomol.* (2017) <https://doi.org/10.1016/j.aspen.2017.03.025>.
47. K. M. Oliver, P. H. Degnan, G. R. Burke, N. A. Moran, Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu. Rev. Entomol.* (2010) <https://doi.org/10.1146/annurev-ento-112408-085305>.
48. J. A. Russell, N. A. Moran, Costs and benefits of symbiont infection in aphids: Variation among symbionts and across temperatures. *Proc. R. Soc. B Biol. Sci.* (2006) <https://doi.org/10.1098/rspb.2005.3348>.
49. K. M. Oliver, J. A. Russell, N. A. Morant, M. S. Hunter, Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl. Acad. Sci. U. S. A.* (2003) <https://doi.org/10.1073/pnas.0335320100>.
50. T. L. Wilkinson, R. Koga, T. Fukatsu, Role of host nutrition in symbiont regulation: Impact of dietary nitrogen on proliferation of obligate and facultative bacterial endosymbionts of the pea aphid *Acyrtosiphon pisum*. *Appl. Environ. Microbiol.* (2007) <https://doi.org/10.1128/AEM.01211-06>.
51. T. Tsuchida, *et al.*, Symbiotic bacterium modifies aphid body color. *Science* **330**, 1102–1104 (2010).
52. H. Anbutsu, *et al.*, Small genome symbiont underlies cuticle hardness in beetles. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E8382–E8391 (2017).
53. T. Kuriwada, *et al.*, Biological role of *Nardonella* endosymbiont in its weevil host. *PLoS One* (2010) <https://doi.org/10.1371/journal.pone.0013101>.

54. C. Dale, Evolution: Weevils Get Tough on Symbiotic Tyrosine. *Curr. Biol.* (2017) <https://doi.org/10.1016/j.cub.2017.10.031>.
55. H. Anbutsu, T. Fukatsu, “Symbiosis for insect cuticle formation” in *Cellular Dialogues in the Holobiont*, (2020) <https://doi.org/10.1201/9780429277375-12>.
56. C. R. Currie, A community of ants, fungi, and bacteria: A multilateral approach to studying symbiosis. *Annu. Rev. Microbiol.* (2001) <https://doi.org/10.1146/annurev.micro.55.1.357>.
57. C. R. Currie, J. A. Scottt, R. C. Summerbell, D. Malloch, Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* (1999) <https://doi.org/10.1038/19519>.
58. J. Kroiss, *et al.*, Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nat. Chem. Biol.* (2010) <https://doi.org/10.1038/nchembio.331>.
59. M. Kaltenpoth, W. Göttler, G. Herzner, E. Strohm, Symbiotic bacteria protect wasp larvae from fungal infestation. *Curr. Biol.* (2005) <https://doi.org/10.1016/j.cub.2004.12.084>.
60. N. Yoshida, *et al.*, Chaperonin turned insect toxin. *Nature* (2001) <https://doi.org/10.1038/35075148>.
61. J. Piel, I. Höfer, D. Hui, Evidence for A Symbiosis Island Involved in Horizontal Acquisition of Pederin Biosynthetic Capabilities by the Bacterial Symbiont of *Paederus fuscipes* Beetles. *J. Bacteriol.* (2004)

<https://doi.org/10.1128/JB.186.5.1280-1286.2004>.

62. C. Weirauch, R. T. Schuh, Systematics and evolution of heteroptera: 25 years of progress. *Annu. Rev. Entomol.* (2011) <https://doi.org/10.1146/annurev-ento-120709-144833>.
63. T. Hosokawa, *et al.*, Diverse Strategies for Vertical Symbiont Transmission among Subsocial Stinkbugs. *PLoS One* (2013) <https://doi.org/10.1371/journal.pone.0065081>.
64. T. Hosokawa, *et al.*, Obligate bacterial mutualists evolving from environmental bacteria in natural insect populations. *Nat. Microbiol.* **1**, 15011 (2016).
65. T. Hayashi, T. Hosokawa, X. Y. Meng, R. Koga, T. Fukatsu, Female-specific specialization of a posterior end region of the midgut symbiotic organ in *Plautia splendens* and allied stinkbugs. *Appl. Environ. Microbiol.* (2015) <https://doi.org/10.1128/AEM.04057-14>.
66. Y. Abe, K. Mishiro, M. Takanashi, Symbiont of Brown-Winged Green Bug, *Plautia stali* Scott. *Japanese J. Appl. Entomol. Zool.* (1995) <https://doi.org/10.1303/jjaez.39.109>.
67. S. S. Prado, R. P. P. Almeida, Role of symbiotic gut bacteria in the development of *Acrosternum hilare* and *Murgantia histrionica*. *Entomol. Exp. Appl.* (2009) <https://doi.org/10.1111/j.1570-7458.2009.00863.x>.
68. A. Tada, *et al.*, Obligate association with gut bacterial symbiont in Japanese populations of the southern green stinkbug *Nezara viridula* (Heteroptera:

- Pentatomidae). *Appl. Entomol. Zool.* (2011) <https://doi.org/10.1007/s13355-011-0066-6>.
69. S. Oishi, M. Moriyama, R. Koga, T. Fukatsu, Morphogenesis and development of midgut symbiotic organ of the stinkbug *Plautia stali* (Hemiptera: Pentatomidae). *Zool. Lett.* **5**, 16 (2019).
 70. T. Hosokawa, Y. Kikuchi, Y. M. Xien, T. Fukatsu, The making of symbiont capsule in the plataspid stinkbug *Megacopta punctatissima*. *FEMS Microbiol. Ecol.* (2005) <https://doi.org/10.1016/j.femsec.2005.06.002>.
 71. T. Fukatsu, T. Hosokawa, Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. *Appl. Environ. Microbiol.* **68**, 389–396 (2002).
 72. T. Fukatsu, T. Hosokawa, “Capsule-transmitted obligate gut bacterium of plataspid stinkbugs: A novel model system for insect symbiosis studies” in *Insect Symbiosis*, (2008) <https://doi.org/10.1201/9781420064117>.
 73. H. J. Müller, Experimentelle studien an der symbiose von *Coptosoma scutellatum* Geoffr. (Hem. heteropt.). *Zeitschrift für Morphol. und Ökologie der Tiere* (1956) <https://doi.org/10.1007/BF00407170>.
 74. T. Hosokawa, Y. Kikuchi, N. Nikoh, M. Shimada, T. Fukatsu, Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. *PLoS Biol.* **4**, e337 (2006).
 75. T. Hosokawa, Y. Kikuchi, M. Shimada, T. Fukatsu, Symbiont acquisition alters

- behaviour of stinkbug nymphs. *Biol. Lett.* (2008)
<https://doi.org/10.1098/rsbl.2007.0510>.
76. T. Hosokawa, T. Fukatsu, Relevance of microbial symbiosis to insect behavior. *Curr. Opin. Insect Sci.* (2020) <https://doi.org/10.1016/j.cois.2020.03.004>.
 77. N. Kaiwa, *et al.*, Symbiont-supplemented maternal investment underpinning host's ecological adaptation. *Curr. Biol.* **24**, 2465–2470 (2014).
 78. Y. Kikuchi, X.-Y. Meng, T. Fukatsu, Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocoris chinensis* (Heteroptera: Alydidae). *Appl. Environ. Microbiol.* **71**, 4035–4043 (2005).
 79. Y. Kikuchi, I. Yumoto, Efficient colonization of the bean bug *Riptortus pedestris* by an environmentally transmitted *Burkholderia* symbiont. *Appl. Environ. Microbiol.* **79**, 2088–2091 (2013).
 80. Y. Kikuchi, *et al.*, Symbiont-mediated insecticide resistance. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 8618–8622 (2012).
 81. H. Itoh, *et al.*, Infection dynamics of insecticide-degrading symbionts from soil to insects in response to insecticide spraying. *ISME J.* (2018)
<https://doi.org/10.1038/s41396-017-0021-9>.
 82. R. Futahashi, *et al.*, Gene expression in gut symbiotic organ of stinkbug affected by extracellular bacterial symbiont. *PLoS One* **8**, e64557 (2013).
 83. J. K. Kim, *et al.*, Specific midgut region controlling the symbiont population in

- an insect-microbe gut symbiotic association. *Appl. Environ. Microbiol.* **79**, 7229–7233 (2013).
84. T. Ohbayashi, *et al.*, Insect's intestinal organ for symbiont sorting. *Proc. Natl. Acad. Sci. U. S. A.* (2015) <https://doi.org/10.1073/pnas.1511454112>.
 85. Y. Kinoshita, Y. Kikuchi, N. Mikami, D. Nakane, T. Nishizaka, Unforeseen swimming and gliding mode of an insect gut symbiont, *Burkholderia* sp. RPE64, with wrapping of the flagella around its cell body. *ISME J.* (2018) <https://doi.org/10.1038/s41396-017-0010-z>.
 86. Y. Kikuchi, T. Ohbayashi, S. Jang, P. Mergaert, *Burkholderia insecticola* triggers midgut closure in the bean bug *Riptortus pedestris* to prevent secondary bacterial infections of midgut crypts. *ISME J.* (2020) <https://doi.org/10.1038/s41396-020-0633-3>.
 87. Y. Kikuchi, T. Fukatsu, Live imaging of symbiosis: spatiotemporal infection dynamics of a GFP-labelled *Burkholderia* symbiont in the bean bug *Riptortus pedestris*. *Mol. Ecol.* **23**, 1445–1456 (2014).
 88. S. Jang, Y. Kikuchi, Re-opening of the symbiont sorting organ with aging in *Riptortus pedestris*. *J. Asia. Pac. Entomol.* **23** (2020).
 89. H. Itoh, *et al.*, Host-symbiont specificity determined by microbe-microbe competition in an insect gut. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 22673–22682 (2019).
 90. L. Eberl, P. Vandamme, Members of the genus *Burkholderia*: good and bad guys.

- F1000Research* (2016) <https://doi.org/10.12688/f1000research.8221.1>.
91. C. W. Beukes, *et al.*, Genome data provides high support for generic boundaries in *Burkholderia* sensu lato. *Front. Microbiol.* (2017) <https://doi.org/10.3389/fmicb.2017.01154>.
 92. E. Mahenthiralingam, T. A. Urban, J. B. Goldberg, The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat. Rev. Microbiol.* (2005) <https://doi.org/10.1038/nrmicro1085>.
 93. Z. R. Suárez-Moreno, *et al.*, Common Features of Environmental and Potentially Beneficial Plant-Associated *Burkholderia*. *Microb. Ecol.* (2012) <https://doi.org/10.1007/s00248-011-9929-1>.
 94. A. Sawana, M. Adeolu, R. S. Gupta, Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: Proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. *Front. Genet.* (2014) <https://doi.org/10.3389/fgene.2014.00429>.
 95. C. Peeters, *et al.*, Phylogenomic study of *Burkholderia glathei*-like organisms, proposal of 13 novel *Burkholderia* species and emended descriptions of *Burkholderia sordidicola*, *Burkholderia zhejiangensis*, and *Burkholderia grimmiae*. *Front. Microbiol.* (2016) <https://doi.org/10.3389/fmicb.2016.00877>.
 96. A. Nakabachi, *et al.*, Defensive bacteriome symbiont with a drastically reduced genome. *Curr. Biol.* (2013) <https://doi.org/10.1016/j.cub.2013.06.027>.

97. K. Takeshita, *et al.*, *Burkholderia insecticola* sp. Nov., a gut symbiotic bacterium of the bean bug *Riptortus pedestris*. *Int. J. Syst. Evol. Microbiol.* (2018) <https://doi.org/10.1099/ijsem.0.002848>.
98. L. Leive, THE BARRIER FUNCTION OF THE GRAM-NEGATIVE ENVELOPE. *Ann. N. Y. Acad. Sci.* (1974) <https://doi.org/10.1111/j.1749-6632.1974.tb43261.x>.
99. J. K. Kim, *et al.*, Insect gut symbiont susceptibility to host antimicrobial peptides caused by alteration of the bacterial cell envelope. *J. Biol. Chem.* (2015) <https://doi.org/10.1074/jbc.M115.651158>.
100. J. K. Kim, *et al.*, The lipopolysaccharide core oligosaccharide of *Burkholderia* plays a critical role in maintaining a proper gut symbiosis with the bean bug *Riptortus pedestris*. *J. Biol. Chem.* (2017) <https://doi.org/10.1074/jbc.M117.813832>.
101. J. K. Kim, H. Y. Park, B. L. Lee, The symbiotic role of O-antigen of *Burkholderia* symbiont in association with host *Riptortus pedestris*. *Dev. Comp. Immunol.* (2016) <https://doi.org/10.1016/j.dci.2016.02.009>.
102. J. K. Kim, *et al.*, Bacterial cell wall synthesis gene *uppP* is required for *Burkholderia* colonization of the stinkbug gut. *Appl. Environ. Microbiol.* (2013) <https://doi.org/10.1128/AEM.01269-13>.
103. J. K. Kim, *et al.*, Polyester synthesis genes associated with stress resistance are involved in an insect-bacterium symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* (2013)

<https://doi.org/10.1073/pnas.1303228110>.

104. L. K. Goh, R. K. Purama, K. Sudesh, Enhancement of stress tolerance in the polyhydroxyalkanoate producers without mobilization of the accumulated granules. *Appl. Biochem. Biotechnol.* (2014) <https://doi.org/10.1007/s12010-013-0634-z>.
105. S. H. Jang, *et al.*, PhaR, a negative regulator of PhaP, modulates the colonization of a Burkholderia gut symbiont in the midgut of the host insect, Riptortus pedestris. *Appl. Environ. Microbiol.* **83** (2017).
106. J. K. Kim, *et al.*, Purine biosynthesis-deficient Burkholderia mutants are incapable of symbiotic accommodation in the stinkbug. *ISME J.* (2014) <https://doi.org/10.1038/ismej.2013.168>.
107. J. K. Kim, *et al.*, Purine biosynthesis, biofilm formation, and persistence of an insect-microbe gut symbiosis. *Appl. Environ. Microbiol.* (2014) <https://doi.org/10.1128/AEM.00739-14>.
108. J. B. Lee, *et al.*, Bacterial cell motility of Burkholderia gut symbiont is required to colonize the insect gut. *FEBS Lett.* (2015) <https://doi.org/10.1016/j.febslet.2015.08.022>.
109. T. Ohbayashi, *et al.*, Comparative cytology, physiology and transcriptomics of Burkholderia insecticola in symbiosis with the bean bug Riptortus pedestris and in culture. *ISME J.* **13**, 1469–1483 (2019).
110. R. Futahashi, *et al.*, Laccase2 is required for cuticular pigmentation in stinkbugs.

Insect Biochem. Mol. Biol. **41**, 191–196 (2011).

111. T. Cavalier-Smith, Symbiosis as a source of evolutionary innovation: Speciation and morphogenesis. *Trends Ecol. Evol.* (1992) [https://doi.org/10.1016/0169-5347\(92\)90028-a](https://doi.org/10.1016/0169-5347(92)90028-a).
112. E. Ruby, B. Henderson, M. McFall-Ngai, We Get by with a Little Help from Our (Little) Friends. *Science* (80-.). (2004) <https://doi.org/10.1126/science.1094662>.
113. M. Bright, S. Bulgheresi, A complex journey: Transmission of microbial symbionts. *Nat. Rev. Microbiol.* (2010) <https://doi.org/10.1038/nrmicro2262>.
114. J. L. Sachs, U. G. Mueller, T. P. Wilcox, J. J. Bull, The evolution of cooperation. *Q. Rev. Biol.* (2004) <https://doi.org/10.1086/383541>.
115. E. L. Simms, *et al.*, An empirical test of partner choice mechanisms in a wild legume-rhizobium interaction. *Proc. R. Soc. B Biol. Sci.* (2006) <https://doi.org/10.1098/rspb.2005.3292>.
116. E. Kondorosi, P. Mergaert, A. Kereszt, A Paradigm for endosymbiotic life: Cell differentiation of rhizobium bacteria provoked by host plant factors. *Annu. Rev. Microbiol.* (2013) <https://doi.org/10.1146/annurev-micro-092412-155630>.
117. M. Archetti, *et al.*, Economic game theory for mutualism and cooperation. *Ecol. Lett.* (2011) <https://doi.org/10.1111/j.1461-0248.2011.01697.x>.
118. I. Scheuring, D. W. Yu, How to assemble a beneficial microbiome in three easy steps. *Ecol. Lett.* (2012) <https://doi.org/10.1111/j.1461-0248.2012.01853.x>.
119. K. E. Park, *et al.*, The roles of antimicrobial peptide, rip-thanatins, in the midgut

- of *Riptortus pedestris*. *Dev. Comp. Immunol.* (2018)
<https://doi.org/10.1016/j.dci.2017.09.009>.
120. T. Ohbayashi, *et al.*, Insect's intestinal organ for symbiont sorting. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E5179-88 (2015).
 121. A. Levy, *et al.*, Invasion of Spores of the Arbuscular Mycorrhizal Fungus *Gigaspora decipiens* by *Burkholderia* spp. *Appl. Environ. Microbiol.* (2003)
<https://doi.org/10.1128/AEM.69.10.6250-6256.2003>.
 122. L. V. Flórez, *et al.*, Antibiotic-producing symbionts dynamically transition between plant pathogenicity and insect-defensive mutualism. *Nat. Commun.* (2017) <https://doi.org/10.1038/ncomms15172>.
 123. S. Disalvo, *et al.*, *Burkholderia* bacteria infectiously induce the protofarming symbiosis of *Dictyostelium amoebae* and food bacteria. *Proc. Natl. Acad. Sci. U. S. A.* (2015) <https://doi.org/10.1073/pnas.1511878112>.
 124. A. P. Dobritsa, M. Samadpour, Transfer of eleven species of the genus *Burkholderia* to the genus *Paraburkholderia* and proposal of *Caballeronia* gen. nov. to accommodate twelve species of the genera *Burkholderia* and *Paraburkholderia*. *Int. J. Syst. Evol. Microbiol.* (2016)
<https://doi.org/10.1099/ijsem.0.001065>.
 125. S. E. Jeong, H. J. Lee, B. Jia, C. O. Jeon, *Pandoraea terrae* sp. nov., isolated from forest soil, and emended description of the genus *Pandoraea* Coenye et al. 2000. *Int. J. Syst. Evol. Microbiol.* (2016) <https://doi.org/10.1099/ijsem.0.001229>.

126. Y. Kikuchi, T. Hosokawa, T. Fukatsu, An ancient but promiscuous host-symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts. *ISME J.* **5**, 446–460 (2011).
127. M. Jung, D. H. Lee, Abundance and diversity of gut-symbiotic bacteria, the genus *Burkholderia* in overwintering *Riptortus pedestris* (Hemiptera: Alydidae) populations and soil in South Korea. *PLoS One* (2019) <https://doi.org/10.1371/journal.pone.0218240>.
128. K. Tago, *et al.*, A fine-scale phylogenetic analysis of free-living *burkholderia* species in sugarcane field soil. *Microbes Environ.* (2014) <https://doi.org/10.1264/jsme2.ME14122>.
129. J. G. Caporaso, *et al.*, Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* (2012) <https://doi.org/10.1038/ismej.2012.8>.
130. M. H. Norris, Y. Kang, B. Wilcox, T. T. Hoang, Stable, site-specific fluorescent tagging constructs optimized for *burkholderia* species. *Appl. Environ. Microbiol.* (2010) <https://doi.org/10.1128/AEM.01188-10>.
131. M. D. Abràmoff, P. J. Magalhães, S. J. Ram, Image processing with imageJ. *Biophotonics Int.* (2004) <https://doi.org/10.1201/9781420005615.ax4>.
132. F. R. Cianfanelli, L. Monlezun, S. J. Coulthurst, Aim, Load, Fire: The Type VI Secretion System, a Bacterial Nanoweapon. *Trends Microbiol.* (2016) <https://doi.org/10.1016/j.tim.2015.10.005>.

133. M. R. MCLELLAN, L. R. LIND, R. W. KIME, HUE ANGLE DETERMINATIONS AND STATISTICAL ANALYSIS FOR MULTIQUADRANT HUNTER L_a,b DATA. *J. Food Qual.* (1995) <https://doi.org/10.1111/j.1745-4557.1995.tb00377.x>.
134. R. R Development Core Team, *R: A Language and Environment for Statistical Computing* (2011) <https://doi.org/10.1007/978-3-540-74686-7>.
135. Y. Kikuchi, T. Hosokawa, T. Fukatsu, Specific developmental window for establishment of an insect-microbe gut symbiosis. *Appl. Environ. Microbiol.* (2011) <https://doi.org/10.1128/AEM.00358-11>.
136. N. Stopnisek, *et al.*, Genus-wide acid tolerance accounts for the biogeographical distribution of soil Burkholderia populations. *Environ. Microbiol.* (2014) <https://doi.org/10.1111/1462-2920.12211>.
137. H. Itoh, *et al.*, Bacterial population succession and adaptation affected by insecticide application and soil spraying history. *Front. Microbiol.* (2014) <https://doi.org/10.3389/fmicb.2014.00457>.
138. L. Speare, *et al.*, Bacterial symbionts use a type VI secretion system to eliminate competitors in their natural host. *Proc. Natl. Acad. Sci. U. S. A.* (2018) <https://doi.org/10.1073/pnas.1808302115>.
139. M. I. Steele, W. K. Kwong, M. Whiteley, N. A. Moranb, Diversification of type VI secretion system toxins reveals ancient antagonism among bee gut microbes. *MBio* (2017) <https://doi.org/10.1128/mBio.01630-17>.

140. E. C. Garcia, A. I. Perault, S. A. Marlatt, P. A. Cotter, Interbacterial signaling via Burkholderia contact dependent growth inhibition system proteins. *Proc. Natl. Acad. Sci. U. S. A.* (2016) <https://doi.org/10.1073/pnas.1606323113>.
141. K. McLoughlin, J. Schluter, S. Rakoff-Nahoum, A. L. Smith, K. R. Foster, Host Selection of Microbiota via Differential Adhesion. *Cell Host Microbe* (2016) <https://doi.org/10.1016/j.chom.2016.02.021>.
142. , Evidence of environmental and vertical transmission of Burkholderia symbionts in the oriental chinch bug, Cavalerius saccharivorus (Heteroptera: Blissidae). *Appl. Environ. Microbiol.* (2014) <https://doi.org/10.1128/AEM.01087-14>.
143. D. G. Boucias, *et al.*, Detection and characterization of bacterial symbionts in the Heteropteran, Blissus insularis. *FEMS Microbiol. Ecol.* (2012) <https://doi.org/10.1111/j.1574-6941.2012.01433.x>.
144. J. R. Garcia, *et al.*, Partner associations across sympatric broad-headed bug species and their environmentally acquired bacterial symbionts. *Mol. Ecol.* (2014) <https://doi.org/10.1111/mec.12655>.
145. S. M. Kuechler, Y. Matsyuura, K. Dettner, Y. Kikuchi, Phylogenetically diverse Burkholderia associated with midgut crypts of spurge bugs, Dicranocephalus spp. (Heteroptera: Stenocephalidae). *Microbes Environ.* (2016) <https://doi.org/10.1264/jsme2.ME16042>.
146. T. Ohbayashi, H. Itoh, J. Lachat, Y. Kikuchi, P. Mergaert, Burkholderia gut symbionts associated with European and Japanese populations of the dock bug

- Coreus marginatus (Coreoidea: Coreidae). *Microbes Environ.* (2019)
<https://doi.org/10.1264/jsme2.ME19011>.
147. K. Takeshita, *et al.*, Burkholderia of plant-beneficial group are symbiotically associated with bordered plant bugs (Heteroptera: Pyrrhocoroidea: Largidae). *Microbes Environ.* (2015) <https://doi.org/10.1264/jsme2.ME15153>.
148. E. R. L. Gordon, Q. McFrederick, C. Weirauch, Phylogenetic evidence for ancient and persistent environmental symbiont reacquisition in Largidae (Hemiptera: Heteroptera). *Appl. Environ. Microbiol.* (2016)
<https://doi.org/10.1128/AEM.02114-16>.
149. J. J. Bull, W. R. Rice, Distinguishing mechanisms for the evolution of co-operation. *J. Theor. Biol.* (1991) [https://doi.org/10.1016/S0022-5193\(05\)80072-4](https://doi.org/10.1016/S0022-5193(05)80072-4).
150. J. Schluter, K. R. Foster, The Evolution of Mutualism in Gut Microbiota Via Host Epithelial Selection. *PLoS Biol.* (2012)
<https://doi.org/10.1371/journal.pbio.1001424>.
151. C. M. Ranger, *et al.*, Symbiont selection via alcohol benefits fungus farming by ambrosia beetles. *Proc. Natl. Acad. Sci. U. S. A.* (2018)
<https://doi.org/10.1073/pnas.1716852115>.
152. M. Lardi, S. B. de Campos, G. Purtschert, L. Eberl, G. Pessi, Competition experiments for legume infection identify Burkholderia phymatum as a highly competitive β -rhizobium. *Front. Microbiol.* (2017)
<https://doi.org/10.3389/fmicb.2017.01527>.

153. T. S. Haselkorn, *et al.*, The specificity of Burkholderia symbionts in the social amoeba farming symbiosis: Prevalence, species, genetic and phenotypic diversity. *Mol. Ecol.* (2019) <https://doi.org/10.1111/mec.14982>.
154. N. A. Moran, H. Ochman, T. J. Hammer, Evolutionary and Ecological Consequences of Gut Microbial Communities. *Annu. Rev. Ecol. Evol. Syst.* (2019) <https://doi.org/10.1146/annurev-ecolsys-110617-062453>.
155. R. E. Ley, *et al.*, Evolution of mammals and their gut microbes. *Science* (80-.). (2008) <https://doi.org/10.1126/science.1155725>.
156. A. E. Douglas, Multiorganismal insects: Diversity and function of resident microorganisms. *Annu. Rev. Entomol.* (2015) <https://doi.org/10.1146/annurev-ento-010814-020822>.
157. H. Vogel, *et al.*, The digestive and defensive basis of carcass utilization by the burying beetle and its microbiota. *Nat. Commun.* (2017) <https://doi.org/10.1038/ncomms15186>.
158. R. J. Dillon, V. M. Dillon, The Gut Bacteria of Insects: Nonpathogenic Interactions. *Annu. Rev. Entomol.* (2004) <https://doi.org/10.1146/annurev.ento.49.061802.123416>.
159. M. C. Lanan, P. A. P. Rodrigues, A. Agellon, P. Jansma, D. E. Wheeler, A bacterial filter protects and structures the gut microbiome of an insect. *ISME J.* (2016) <https://doi.org/10.1038/ismej.2015.264>.
160. E. M. Ha, C. T. Oh, Y. S. Bae, W. J. Lee, A direct role for dual oxidase in

- Drosophila gut immunity. *Science* (2005)
<https://doi.org/10.1126/science.1117311>.
161. K. A. Lee, *et al.*, Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in drosophila. *Cell* (2013)
<https://doi.org/10.1016/j.cell.2013.04.009>.
 162. J. H. Yun, *et al.*, Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Appl. Environ. Microbiol.* (2014) <https://doi.org/10.1128/AEM.01226-14>.
 163. N. Buchon, N. Silverman, S. Cherry, Immunity in *Drosophila melanogaster*-from microbial recognition to whole-organism physiology. *Nat. Rev. Immunol.* (2014)
<https://doi.org/10.1038/nri3763>.
 164. T. Kuraishi, A. Hori, S. Kurata, Host-microbe interactions in the gut of *Drosophila melanogaster*. *Front. Physiol.* (2013)
<https://doi.org/10.3389/fphys.2013.00375>.
 165. E. M. Ha, *et al.*, Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in drosophila gut. *Nat. Immunol.* (2009) <https://doi.org/10.1038/ni.1765>.
 166. E. M. Ha, *et al.*, Regulation of DUOX by the Gαq-Phospholipase Cβ-Ca²⁺ Pathway in *Drosophila* Gut Immunity. *Dev. Cell* (2009)
<https://doi.org/10.1016/j.devcel.2008.12.015>.
 167. Y. S. Bae, M. K. Choi, W. J. Lee, Dual oxidase in mucosal immunity and host-

- microbe homeostasis. *Trends Immunol.* (2010)
<https://doi.org/10.1016/j.it.2010.05.003>.
168. V. Chávez, A. Mohri-Shiomi, D. A. Garsin, Ce-Duox1/BLI-3 generates reactive oxygen species as a protective innate immune mechanism in *Caenorhabditis elegans*. *Infect. Immun.* (2009) <https://doi.org/10.1128/IAI.00627-09>.
 169. X. Xiao, *et al.*, A Mesh-Duox pathway regulates homeostasis in the insect gut. *Nat. Microbiol.* (2017) <https://doi.org/10.1038/nmicrobiol.2017.20>.
 170. J. H. Ryu, *et al.*, An essential complementary role of NF- κ B pathway to microbicidal oxidants in *Drosophila* gut immunity. *EMBO J.* (2006)
<https://doi.org/10.1038/sj.emboj.7601233>.
 171. S. H. Kim, W. J. Lee, Role of DUOX in gut inflammation: Lessons from *Drosophila* model of gut-microbiota interactions. *Front. Cell. Infect. Microbiol.* (2014) <https://doi.org/10.3389/fcimb.2013.00116>.
 172. S. Kumar, A. Molina-Cruz, L. Gupta, J. Rodrigues, C. Barillas-Mury, A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. *Science* (2010) <https://doi.org/10.1126/science.1184008>.
 173. X. Yang, A. A. Smith, M. S. Williams, U. Pal, A dityrosine network mediated by dual oxidase and peroxidase influences the persistence of lyme disease pathogens within the vector. *J. Biol. Chem.* (2014) <https://doi.org/10.1074/jbc.M113.538272>.
 174. T. Ohbayashi, P. Mergaert, Y. Kikuchi, “Host-symbiont specificity in insects: Underpinning mechanisms and evolution” in *Advances in Insect Physiology*,

- (2020) <https://doi.org/10.1016/bs.aiip.2020.03.002>.
175. M. Kaltenpoth, L. V. Flórez, Versatile and Dynamic Symbioses Between Insects and Burkholderia Bacteria . *Annu. Rev. Entomol.* (2020) <https://doi.org/10.1146/annurev-ento-011019-025025>.
 176. P. Mergaert, Y. Kikuchi, S. Shigenobu, E. C. M. Nowack, Metabolic Integration of Bacterial Endosymbionts through Antimicrobial Peptides. *Trends Microbiol.* (2017) <https://doi.org/10.1016/j.tim.2017.04.007>.
 177. A. M. Bolger, M. Lohse, B. Usadel, Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* (2014) <https://doi.org/10.1093/bioinformatics/btu170>.
 178. B. J. Haas, *et al.*, De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* (2013) <https://doi.org/10.1038/nprot.2013.084>.
 179. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* (2014) <https://doi.org/10.1186/s13059-014-0550-8>.
 180. K. Katoh, D. M. Standley, MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* (2013) <https://doi.org/10.1093/molbev/mst010>.
 181. D. T. Jones, W. R. Taylor, J. M. Thornton, The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* (1992)

<https://doi.org/10.1093/bioinformatics/8.3.275>.

182. S. Kumar, G. Stecher, K. Tamura, MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* (2016)
<https://doi.org/10.1093/molbev/msw054>.
183. O. I. Aruoma, B. Halliwell, B. M. Hoey, J. Butler, The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Biol. Med.* (1989)
[https://doi.org/10.1016/0891-5849\(89\)90066-X](https://doi.org/10.1016/0891-5849(89)90066-X).
184. A. J. P. GOODCHILD, STUDIES ON THE FUNCTIONAL ANATOMY OF THE INTESTINES OF HETEROPTERA. *Proc. Zool. Soc. London* (1963)
<https://doi.org/10.1111/j.1469-7998.1963.tb01631.x>.
185. Y. Nishide, *et al.*, Functional crosstalk across IMD and Toll pathways: Insight into the evolution of incomplete immune cascades. *Proc. R. Soc. B Biol. Sci.* (2019) <https://doi.org/10.1098/rspb.2018.2207>.
186. S. Hayashi, T. Kondo, Development and function of the drosophila tracheal system. *Genetics* (2018) <https://doi.org/10.1534/genetics.117.300167>.
187. J. Schottenfeld, Y. Song, A. S. Ghabrial, Tube continued: Morphogenesis of the Drosophila tracheal system. *Curr. Opin. Cell Biol.* (2010)
<https://doi.org/10.1016/j.ceb.2010.07.016>.
188. W. P. Devine, *et al.*, Requirement for chitin biosynthesis in epithelial tube morphogenesis. *Proc. Natl. Acad. Sci. U. S. A.* (2005)

<https://doi.org/10.1073/pnas.0506676102>.

189. D. Bandarra, J. Biddlestone, S. Mudie, H. A. Muller, S. Rocha, Hypoxia activates IKK-NF- κ B and the immune response in *Drosophila melanogaster*. *Biosci. Rep.* (2014) <https://doi.org/10.1042/BSR20140095>.
190. D. Zhou, *et al.*, Mechanisms underlying hypoxia tolerance in *Drosophila melanogaster*: Hairys a metabolic switch. *PLoS Genet.* (2008) <https://doi.org/10.1371/journal.pgen.1000221>.
191. M. A. Hamad, *et al.*, Adaptation and antibiotic tolerance of anaerobic *Burkholderia pseudomallei*. *Antimicrob. Agents Chemother.* (2011) <https://doi.org/10.1128/AAC.00953-10>.
192. A. M. Sass, *et al.*, The unexpected discovery of a novel low-oxygen-activated locus for the anoxic persistence of *Burkholderia cenocepacia*. *ISME J.* (2013) <https://doi.org/10.1038/ismej.2013.36>.
193. G. Pessi, *et al.*, Response of *Burkholderia cenocepacia* H111 to micro-oxia. *PLoS One* (2013) <https://doi.org/10.1371/journal.pone.0072939>.
194. D. Sutherland, C. Samakovlis, M. A. Krasnow, branchless encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* (1996) [https://doi.org/10.1016/S0092-8674\(00\)81803-6](https://doi.org/10.1016/S0092-8674(00)81803-6).
195. S. Y. Chung, C. Chavez, D. J. Andrew, Trachealess (Trh) regulates all tracheal genes during *Drosophila* embryogenesis. *Dev. Biol.* (2011) <https://doi.org/10.1016/j.ydbio.2011.09.014>.

196. R. Wilk, I. Weizman, B. Z. Shilo, trachealess encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.* (1996) <https://doi.org/10.1101/gad.10.1.93>.
197. S. Lavista-Llanos, *et al.*, Control of the Hypoxic Response in *Drosophila melanogaster* by the Basic Helix-Loop-Helix PAS Protein Similar. *Mol. Cell. Biol.* (2002) <https://doi.org/10.1128/mcb.22.19.6842-6853.2002>.
198. A. Tønning, *et al.*, A transient luminal chitinous matrix is required to model epithelial tube diameter in the *Drosophila* trachea. *Dev. Cell* (2005) <https://doi.org/10.1016/j.devcel.2005.07.012>.
199. S. Luschnig, T. Bätz, K. Armbruster, M. A. Krasnow, serpentine and vermiform encode matrix proteins with chitin binding and deacetylation domains that limit tracheal tube length in *Drosophila*. *Curr. Biol.* (2006) <https://doi.org/10.1016/j.cub.2005.11.072>.
200. S. Wang, *et al.*, Septate-junction-dependent luminal deposition of chitin deacetylases restricts tube elongation in the *Drosophila* trachea. *Curr. Biol.* (2006) <https://doi.org/10.1016/j.cub.2005.11.074>.
201. A. Jaźwińska, C. Ribeiro, M. Affolter, Epithelial tube morphogenesis during *Drosophila* tracheal development requires Piopio, a luminal ZP protein. *Nat. Cell Biol.* (2003) <https://doi.org/10.1038/ncb1049>.
202. F. A. Dias, *et al.*, Ovarian Dual Oxidase (Duox) activity is essential for insect eggshell hardening and waterproofing. *J. Biol. Chem.* (2013)

<https://doi.org/10.1074/jbc.M113.522201>.

203. T. R. Hurd, F. X. Liang, R. Lehmann, Curly Encodes Dual Oxidase, Which Acts with Heme Peroxidase Curly Su to Shape the Adult *Drosophila* Wing. *PLoS Genet.* (2015) <https://doi.org/10.1371/journal.pgen.1005625>.
204. N. T. T. Anh, M. Nishitani, S. Harada, M. Yamaguchi, K. Kamei, Essential role of duox in stabilization of *Drosophila* wing. *J. Biol. Chem.* (2011) <https://doi.org/10.1074/jbc.M111.263178>.
205. W. A. Edens, *et al.*, Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox. *J. Cell Biol.* (2001) <https://doi.org/10.1083/jcb.200103132>.
206. J. L. Wong, G. M. Wessel, Extracellular matrix modifications at fertilization: Regulation of dityrosine crosslinking by transamidation. *Development* (2009) <https://doi.org/10.1242/dev.030775>.
207. J. L. Meitzler, R. Brandman, P. R. Ortiz De Montellano, Perturbed heme binding is responsible for the blistering phenotype associated with mutations in the *Caenorhabditis elegans* dual oxidase 1 (DUOX1) peroxidase domain. *J. Biol. Chem.* (2010) <https://doi.org/10.1074/jbc.M110.170902>.
208. E. M. Ha, *et al.*, An antioxidant system required for host protection against gut infection in *Drosophila*. *Dev. Cell* (2005) <https://doi.org/10.1016/j.devcel.2004.11.007>.

209. T. Kuraishi, O. Binggeli, O. Opota, N. Buchon, B. Lemaitre, Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* (2011) <https://doi.org/10.1073/pnas.1105994108>.
210. A. Hori, S. Kurata, T. Kuraishi, Unexpected role of the IMD pathway in *Drosophila* gut defense against *Staphylococcus aureus*. *Biochem. Biophys. Res. Commun.* (2018) <https://doi.org/10.1016/j.bbrc.2017.11.004>.
211. T. Shibata, *et al.*, Transglutaminase-catalyzed protein-protein cross-linking suppresses the activity of the NF- κ B-like transcription factor relish. *Sci. Signal.* (2013) <https://doi.org/10.1126/scisignal.2003970>.
212. C. Ren, P. Webster, S. E. Finkel, J. Tower, Increased Internal and External Bacterial Load during *Drosophila* Aging without Life-Span Trade-Off. *Cell Metab.* (2007) <https://doi.org/10.1016/j.cmet.2007.06.006>.
213. J. Jarecki, E. Johnson, M. A. Krasnow, Oxygen regulation of airway branching in *Drosophila* is mediated by Branchless FGF. *Cell* (1999) [https://doi.org/10.1016/S0092-8674\(00\)81652-9](https://doi.org/10.1016/S0092-8674(00)81652-9).
214. R. K. Bruick, S. L. McKnight, A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* (2001) <https://doi.org/10.1126/science.1066373>.
215. L. Centanin, *et al.*, Cell Autonomy of HIF Effects in *Drosophila*: Tracheal Cells Sense Hypoxia and Induce Terminal Branch Sprouting. *Dev. Cell* (2008) <https://doi.org/10.1016/j.devcel.2008.01.020>.

216. F. Sommer, F. Bäckhed, The gut microbiota-masters of host development and physiology. *Nat. Rev. Microbiol.* (2013) <https://doi.org/10.1038/nrmicro2974>.
217. C. Reinhardt, *et al.*, Tissue factor and PAR1 promote microbiota-induced intestinal vascular remodelling. *Nature* (2012) <https://doi.org/10.1038/nature10893>.
218. T. S. Stappenbeck, L. V. Hooper, J. I. Gordon, Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc. Natl. Acad. Sci. U. S. A.* (2002) <https://doi.org/10.1073/pnas.202604299>.
219. P. Łukasik, *et al.*, Multiple origins of interdependent endosymbiotic complexes in a genus of cicadas. *Proc. Natl. Acad. Sci. U. S. A.* (2017) <https://doi.org/10.1073/pnas.1712321115>.
220. B. Weiss, M. Kaltenpoth, Bacteriome-localized intracellular symbionts in pollen-feeding beetles of the genus *Dasytes* (Coleoptera, Dasytidae). *Front. Microbiol.* (2016) <https://doi.org/10.3389/fmicb.2016.01486>.
221. B. T. Ho, T. G. Dong, J. J. Mekalanos, A view to a kill: The bacterial type VI secretion system. *Cell Host Microbe* (2014) <https://doi.org/10.1016/j.chom.2013.11.008>.
222. X. Hu, *et al.*, Molecular Cloning and Functional Characterization of the Dual Oxidase (BmDuox) Gene from the Silkworm *Bombyx mori*. *PLoS One* (2013) <https://doi.org/10.1371/journal.pone.0070118>.

Acknowledgments

First, I appreciate my supervisor, Prof. Dr. Yoshitomo Kikuchi. I cannot forget memories with Dr. Kikuchi from my first arrival to Japan until now. Because of his great assistance, I can graduate doctoral course without any problem, and even further I could grow up as one of a biologist and a scientist. Scientific discussion with him was always enjoyable and constructive. Based on the frequent discussion, I can develop my research and successfully published my studies in high impact journals. Besides, he introduced many nice Japanese as well as international scientists to me, and it was very helpful to widen my perspective. Besides science, he helped me with lots of personal things. Especially, it strongly remains in my memory that he always invited me to his home on special holidays such as the New Year. Thank you very much!

I also appreciate members of Kikuchi team. I learned a passion for research from Dr. Hideomi Itoh. He is always doing numerous things including his research and caring for students. He has many great ideas about research, thus discussion with him is very helpful to improve my research theme. As well, when I have some trouble in the laboratory, he kindly helped me to solve the problem. I want to follow his diligence and kindness in my future career. Keisuke Kawano has helped me with lots of things from trivial things to big help, such as support for moving home. I could not adjust to Japan without him. He got the JSPS DC1 fellowship and will keep research as a doctoral course student. I believe that he will do a good job. Kota Ishigami, who entered our laboratory in March 2020, is my first real doctoral junior student but also a good friend. He is a passionate and very kind person. During 1 year, he also has helped me in many ways. Especially, he rarely refuses someone's request (but I think he should refuse sometimes).

He is doing great work in our lab and I don't have any doubt that he will publish his research in one of the great journals during his doctoral course. Also, many thanks to Ms. Haruka Ooi, Aika Sawaguchi, and Yuumi Sugisawa for insect caring and helping experiments.

As well, I deeply appreciate Dr. Peter Mergaert (CNRS, France) who is the collaborator of Kikuchi team. Peter has helped me with tons of things including co-advising my research via weekly Skype meetings and constructing papers although the meeting time is very early morning in France. Because of his huge assistance, I can publish my paper in a high-impact journal. Dr. Tsubasa Ohbayashi (NARO), who was the postdoctoral researcher in Peter's lab, also has helped lots of things such as giving scientific and daily advice via Skype meeting. I also would appreciate Prof. Yu Matsuura (University of the Ryukyus) and Prof. Kazutaka Takeshita (Akita Prefectural University). They gave many fruitful advice and comments on my study for 3 years via weekly web-seminar.

I also would like to appreciate Dr. Wataru Kitagawa and Dr. Souichiro Kato (AIST Hokkaido center), and Prof. Teruo Sone (Hokkaido University). They positively evaluated my entrance interview of Hokkaido University, thus I can start the doctoral course in Japan. I also have received numerous advice and comments from them at the university/institute seminar. I also feel many thanks to researchers of AIST; Dr. Yasuo Mitani, Dr. Takashi Narihiro, Dr. Kensuke Igarashi, Dr. Kyosuke Yamamoto, Dr. Ryosuke Nakai, Dr. Shusei Kanie, and Dr. Maiko Furubayashi. Thanks to students and alumni of AIST as well; Motoyuki Watanabe, Tu Zhihao, Samia Quaiyum, Helena de Fátima Silva Lopes, Yan Huang, Xie Ruoyun, Tomoya Numadate, Shota Fukukawa, Putcha Jyothi,

Hiyu Kanbe, Yuki Sano, and Kokoro Nishioka. I would like to express thanks to all members of the laboratory of Applied Microbiology of Hokkaido University.

I also would appreciate other collaborators or people of close connection with me during the doctoral course. Dr. Takema Fukatsu, Dr. Ryuichi Koga, Dr. Ryo Futahashi, Dr. Yuya Sato, Dr. Xian-Ying Meng, Mr. Genta Okude, Mr. Bin Hirota (AIST Tsukuba center); Dr. Shuji Shigenobu (NIBB); Dr. Andreas Brune (Max Planck, Germany); Prof. Doohyung Lee, Mr. Minhyung Jung, Mr. Jooyoung Kim (Gachoen University, Korea); Dr. Atsushi Nagayama (Okinawa Prefectural Agricultural Research Center); Mr. Antoine-Olivier Lirette (McGill University, Canada); Dr. Lachat Joy, Mr. Jouan Romain, and Ms. Lextrait Gaëlle (CNRS, France).

I would like to appreciate my friends, Changguen Lim (banner maker), Minhuck Park (GPS repairman), and Jaemin Kim (Receipt collector). I also many thank my girlfriend, Sunhwa Kim, to support me for 3 years. Lastly, my deepest appreciation goes to my family (father, mother, sister, and puppy Ruri). I was able to finish 3 years safely because of them.

This study was supported by JSPS Research Fellowships for Young Scientists to Seonghan Jang (201911493); JSPS Grants-in-Aid for Scientific Research to Hideomi Itoh (19K15724); by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) KAKENHI to Yoshitomo Kikuchi (18KK0211 and 20H03303); and by a JSPS-CNRS Bilateral Open Partnership Joint Research Project and the CNRS IRP project “Ménage à Trois” to Yoshitomo Kikuchi and Peter Mergaert.