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1 **Host-symbiont specificity in insects: Underpinning mechanisms and evolution**

2

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20

21

22 **Abstract**

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

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

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

45 **(247 < 250 words)**

46

47 **Keywords:**

48 vertical transmission, environmental acquisition, partner fidelity, partner choice,

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

50

51

52

## 53 **Introduction**

54

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

75

## 76 **Vertical transmission**

77

78 Insects that feed exclusively on a nutritionally imbalanced diet like plant saps or on  
79 indigestible materials like wood commonly harbor specific microbial symbionts that  
80 help the hosts to feed and grow on these specific nutritional sources. These symbionts  
81 reside in body cavities, gut crypts, or the cytoplasm of specialized cells called  
82 mycetocytes or bacteriocytes (Buchner, 1965; Kikuchi, 2009). Symbionts are mostly  
83 bacteria, while in some cases yeast-like symbionts, archaea and protist symbionts were  
84 also reported (Buchner, 1965; Brune and Dietrich, 2015; Engel and Moran, 2013;  
85 Kikuchi, 2009; Ohkuma and Brune, 2010; Sudakaran et al., 2017). The symbiotic  
86 microorganisms play a pivotal metabolic role in the insect hosts, such as the production  
87 of essential metabolites that are scarce in the diets and that the insect cannot synthesize  
88 or the degradation of plant polysaccharides that the insect cannot digest. In such  
89 nutritional and digestive symbioses, symbionts are commonly essential for survival,  
90 development, and reproduction of the hosts, and therefore, insects show high mortality  
91 when symbionts are removed by heat, ethanol and antibiotic treatment (Anbutsu et al.,  
92 2017; Brake, 1978; Douglas et al., 2001; Fukatsu and Hosokawa, 2002; Hosokawa et  
93 al., 2010; Itoh et al., 2016; Kikuchi et al., 2009; Salem et al., 2017; Sangare et al., 2016;  
94 Schlein, 1977; Srivastara and Auclair, 1976; Tada et al., 2011). To ensure the  
95 acquisition of the obligate partner by the next generation, most insects have evolved  
96 mechanisms for strict vertical transmission of the symbionts. It should be noted here

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

106

### 107 **Vertical transmission of intracellular symbionts**

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

114

#### 115 *Ovarial transmission*

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

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

125

#### 126 *Milk-gland transmission*

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

134

#### 135 **Vertical transmission of extracellular symbionts**

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

139

#### 140 *Coprophagy*



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

151

#### 152 *Egg-smearing*

153 Egg-smearing, or egg surface contamination by symbiotic bacteria, has been described  
154 in detail in stinkbugs (Bansal et al., 2014; Bistolas et al., 2014; Buchner, 1965; Dasch  
155 and Weiss, 1984; Hayashi et al., 2015; Hosokawa et al., 2013; Itoh et al., 2016;  
156 Karamipour et al., 2016; Kashkouli et al., 2019a, 2019b, 2019c; Kenyon et al., 2015;  
157 Kikuchi et al., 2012, 2009; Miyamoto, 1961; Prado et al., 2009, 2006; Tada et al., 2011;  
158 Taylor et al., 2014). Phytophagous species of stinkbugs, particularly members of the  
159 infraorder Pentatomomorpha, develop a symbiotic organ composed of rows of crypts,  
160 located in the posterior region of the midgut and housing specific extracellular bacterial  
161 symbionts (Buchner, 1965; Dasch and Weiss, 1984; Kikuchi et al., 2009; Miyamoto,  
162 1961). Most species of the superfamily Pentatomoidea possess gammaproteobacterial

163 symbionts, which are smeared on the egg surface upon oviposition. In female insects,  
164 the last few crypts are bulbous compared with the other crypts and can discharge  
165 symbiotic bacteria from the crypt lumen. These crypts are thought to be specialized for  
166 housing symbionts for transmission (Hayashi et al., 2015). In the particular case of the  
167 stinkbug family Acanthosomatidae however, all crypt entrances are completely sealed  
168 in adult insects, making the direct smearing from crypts impossible (Kikuchi et al.,  
169 2009). Instead, female insects evolved a novel pair of organs, associated with the female  
170 ovipositor and called “lubricating organs”. From these sac-like organs, symbionts are  
171 smeared on the egg surface during oviposition (Kikuchi et al., 2009). It is still enigmatic  
172 when and how the symbiotic bacteria migrate from the gut to the lubricating organ and  
173 how the new organ specialized for symbiont transmission evolved in the stinkbug  
174 family. In the case of *Lagria* beetles, antibiotic-producing defensive *Burkholderia*  
175 *gladioli* symbionts are associated with female accessory glands. They are smeared on  
176 the egg surface, protecting host eggs from pathogenic fungi (Kaltenpoth and Florez,  
177 2019).

178

#### 179 *Capsule transmission*

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

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

192 In addition to the stinkbug symbioses, another unique type of capsule  
193 transmission was discovered in the tortoise leaf beetle *Cassida rubiginosa* (Salem et al.,  
194 2017). The leaf beetle harbors a pectin-degrading symbiont, *Candidatus Stammera*  
195 *capleta*, inside a pair of sac-like organs associated with the foregut. Female insects  
196 deposit a symbiont-containing capsule or “caplet” on the top of each egg and hatchlings  
197 acquire the symbiont by consuming the caplet compounds.

198

### 199 **Partner choice in insect-microbe symbiosis with vertical transmission**

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

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

211 Beewolf wasps, that harbor the defensive symbiont *Streptomyces philanthi* in  
212 antennal glands, transmit the symbiont by spreading out gland secretions in their nest,  
213 where larvae develop through the pupal stage until adult emergence (Goettler et al.,  
214 2007; Kaltenpoth et al., 2010, 2005). The bacteria in the nests provide protection against  
215 pathogenic fungi and bacteria by producing different antimicrobial compounds (Kroiss  
216 et al., 2010). Interestingly, experimental inoculation of a non-symbiotic actinomycetes  
217 bacterium to aposymbiotic adult females revealed that the related bacterium can stably  
218 colonize the antennal glands but cannot be secreted and transmitted from the antennae  
219 (Kaltenpoth et al., 2014), suggesting that a partner choice mechanism exists in the  
220 vertical transmission process. Both cases highlight that partner choice mechanisms  
221 reinforce partner fidelity to stabilize long-term, strictly specific insect-microbe  
222 symbioses.

223

## 224 **Horizontal transmission of symbionts in animals and plants**

225

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

229 microorganisms in environments, hosts should actively select their symbionts from the  
230 environmental microbiota in these symbioses with horizontal symbiont transmission. To  
231 efficiently and specifically choose a partner, host organisms develop sophisticated  
232 mechanisms for symbiont sorting. Such partner choice mechanisms have been well  
233 investigated and described in two model systems: the nitrogen-fixing *Rhizobium*  
234 symbiosis in leguminous plants and the bioluminescent *Vibrio* symbiosis in the  
235 Hawaiian bobtail squid (Nyholm and McFall, 2004; Wang et al., 2018). As mentioned  
236 above, the vertically transmitted insect symbionts have strongly reduced genomes,  
237 making them unculturable outside their host (Kikuchi, 2009; McCutcheon and Moran,  
238 2012; Moran and Bennett, 2014; Moya et al., 2008). Because of their different lifestyles  
239 including the free-living state in the environment, the symbionts maintained by  
240 horizontal transmission are in contrast culturable and genetically manipulatable. These  
241 traits have been very useful to clarify the genetic and molecular bases of the symbiotic  
242 associations.

243

#### 244 **The legume-*Rhizobium* symbiosis**

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

251 symbionts and they convert atmospheric nitrogen gas into ammonia, which is used by  
252 the plant as a nitrogen source for its growth. The legume-*Rhizobium* interaction is  
253 usually extremely specific and a given *Rhizobium* strain will interact only with a narrow  
254 range of legume species and vice versa. The host-symbiont compatibility is monitored  
255 all along the symbiotic process, from the initiation of the interaction over the  
256 development and infection of the nodules till the nitrogen fixation in mature nodules.  
257 This is achieved by a continuous exchange of signals between the two partners,  
258 including plant-derived flavonoids and antimicrobial peptides, and bacterial molecules  
259 like lipochitooligosaccharides, extracellular polysaccharides, lipopolysaccharides, type  
260 III and type IV secretion effectors and even small RNAs (Poole et al., 2018; Mergaert,  
261 2018). Most emblematic among these signals are the rhizobial Nod factors which are  
262 produced in the rhizosphere and upon recognition trigger the plant genetic nodulation  
263 program. Nod factors are lipochitooligosaccharides, which have a similar structure in all  
264 rhizobia but which still differ from each other by the presence of strain-specific  
265 chemical modifications (D’Haeze and Holsters, 2002). Nod factor signaling contributes  
266 to the specificity of the interaction by the presence of a matching Nod factor receptor  
267 complex in the plant. Similarly for the other signals, it is believed that a specific signal-  
268 receptor correspondence exists. Moreover, in a mature nodule, the host plants monitor  
269 the symbiont’s nitrogen-fixation activity. If its performance is not optimal, the hosts  
270 control the oxygen supply to the nodule and limit the survival of the symbiont (Kiers et  
271 al., 2003). Such sanction mechanisms are thought to be important to prevent the  
272 evolution of cheaters and maintain the mutualistic association with horizontal symbiont

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

278

### 279 **The squid-*Vibrio fischeri* symbiosis**

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

295 host side, peptidoglycan recognition proteins (PGRPs) and probably also LPS-binding  
296 proteins (LBPs) play important roles for recognition and interaction with the symbiont-  
297 derived signals (Chun et al., 2008; Krasity et al., 2011; Nyholm and Graf, 2012; Troll  
298 2009; 2010).

299

### 300 **Horizontal transmission of symbionts in insects**

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

309

### 310 **The bean bug-*Burkholderia* symbiosis**

311 The bean bug *R. pedestris* (Heteroptera: Pentatomomopha: Coreoidea: Alydidae) (Fig.  
312 1A) is a serious pest of leguminous crops in India, South-East Asia, China, Korea and  
313 Japan (Kikuhara, 2005; Schaefer and Panizzi, 2000). *R. pedestris* is a hemimetabolous  
314 insect and develops to adults via five instar stages in approximately 20 days (Kikuchi  
315 and Fukatsu, 2014). As is typical for stinkbugs, *R. pedestris* possesses numerous crypts  
316 at the posterior part of the midgut, in which *Burkholderia* symbionts are harbored (Fig.



317 1B-G) (Kikuchi et al., 2005). *Burkholderia insecticola* strain RPE64 is the type strain  
318 for the *R. pedestris* symbionts (Takeshita et al., 2018). The *Burkholderia* symbiont is  
319 beneficial for the host insect: symbiotic insects show a faster development, larger body  
320 size, and a higher number of eggs than aposymbiotic insects (Kikuchi et al., 2007;  
321 Kikuchi and Fukatsu, 2014). Transcriptomic analyses of the *Burkholderia* symbiont  
322 revealed that the symbiont recycles host's metabolic wastes and provides the host with  
323 essential amino acids and vitamins (Ohbayashi et al., 2019a). Among the five instar  
324 stages, *R. pedestris* acquires the *Burkholderia* symbiont from ambient soil mainly in the  
325 2<sup>nd</sup> instar stage (Kikuchi et al., 2011b).

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

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

348

#### 349 **The bean bug-*Burkholderia* symbiosis is highly specific**

350 The genus *Burkholderia* consists of over 100 species and is an ecologically diverse  
351 group (Eberl and Vandamme, 2016). Based on genomic phylogeny, the genus is  
352 grouped into at least three distinct clades. The first one includes human-, animal-, and  
353 plant-pathogens, named the “*B. cepacia* complex and *B. pseudomallei*” (BCC&P) clade  
354 (Mahenthiralingam et al., 2005). The second clade consists of many plant growth-  
355 promoting rhizobacteria and nodule symbionts of leguminous plants, designated as the  
356 “plant-associated beneficial and environmental” (PBE) clade (Suárez-Moreno et al.,  
357 2012). The third clade mainly consists of environmental species, leaf-nodule symbionts  
358 of Rubiaceae plants, and gut symbionts of stinkbugs, and is called the “stinkbug-  
359 associated beneficial and environmental” (SBE) clade (Takeshita and Kikuchi, 2017;  
360 Peeters et al., 2016). The BCC&P, PBE, and SBE clades are recently designated as

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

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

370 As mentioned above, the presence of bacterial symbiont-carrying M4 midgut  
371 crypts is very common in the heteropteran infraorder Pentatomomorpha (Kikuchi et al.,  
372 2011a). In many species of the superfamilies Coreoidea and Lygaeoidea of the  
373 Pentatomomorpha, these crypts are also colonized with SBE *Burkholderia* (Boucias et  
374 al., 2012; Itoh et al., 2014; Garcia et al., 2014; Kikuchi et al., 2011a, 2005; Kuechler et  
375 al., 2016; Ohbayashi et al., 2019b; Oliver-Espejel et al., 2011). Nevertheless, other  
376 *Burkholderia*, belonging to the BCC&P and PBE clades, and even *Cupriavidus* and  
377 *Pseudomonas* species were occasionally identified in some insect species (Boucias et  
378 al., 2012; Garcia et al., 2014; Itoh et al., 2014). However, it should be noted that, except  
379 for several SBE *Burkholderia* isolates from *R. pedestris* (Kikuchi et al., 2007), *Coreus*  
380 *marginatus* (Ohbayashi et al., 2019b) and *Blissus insularis* (Xu et al., 2016), for none of  
381 the other identified strains (*Burkholderia* or other species) the Koch's postulates were  
382 verified. These bacteria were not cultured and tested for their capacity to infect and

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

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

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

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

424

425 **Partner choice mechanisms in *Riptortus pedestris***

426 *The gut constricted region as a partner-choice apparatus*

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

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

449           In contrast to the crypt-bearing species of the Lygaeoidea and Coreoidea  
450 superfamilies and the Largidae family, which are commonly associated with  
451 *Burkholderia*, members of the Pentatomoidea, another superfamily of the  
452 Pentatomomorpha, possess gammaproteobacterial symbionts in midgut crypts which are  
453 maintained by strict vertical transmission (Bansal et al., 2014; Bistolos et al., 2014;  
454 Hayashi et al., 2015; Hosokawa et al., 2016, 2013; Itoh et al., 2016; Karamipour et al.,  
455 2016; Kashkouli et al., 2019a, 2019b, 2019c; Kenyon et al., 2015; Kikuchi et al., 2012;  
456 Prado et al., 2009, 2006; Tada et al., 2011; Taylor et al., 2014) using the earlier  
457 mentioned mechanisms of egg-smearing, capsule transmission or jelly transmission.  
458 Interestingly, the constricted region is broadly conserved, not only in the *Burkholderia*-  
459 associated lygaeoid, coreoid and largid species, but also in the Pentatomoidea (Gordon  
460 et al., 2016; Ohbayashi et al., 2015). This strongly suggests that the constricted region  
461 evolved in the common ancestor of the stinkbug superfamilies. Thus, even if in the  
462 Pentatomoidea, the symbiotic association is mainly maintained by partner fidelity  
463 mechanisms, *i.e.* vertical transmission, partner choice achieved by the constricted region  
464 may play a pivotal role in these species too.

465

#### 466 *Corkscrew flagellar motility*

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

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

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



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

495

#### 496 *Midgut closure stimulated by symbiont colonization*

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

510

#### 511 *Competition-based selection in the gut*

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

515 *Burkholderia fungorum* are capable to colonize the midgut crypts of *R. pedestris* (Fig.  
516 3A-D), even though these common soil bacteria are usually not associated with the bean  
517 bug in natural conditions. An additional partner choice mechanism, called “competition-  
518 based selection”, was put forward to explain this observed symbiont specificity (Itoh et  
519 al., 2019). It was shown that the SBE *Burkholderia* always outcompete PBE  
520 *Burkholderia* or *Pandoraea* species in the M4 region when these species are co-  
521 infecting the symbiotic organ, achieving the predominance of SBE *Burkholderia* in the  
522 bean bug gut (Fig. 3E-G). These experimental data clearly demonstrate that the abilities  
523 for colonization and cooperation, usually thought of as specific traits of mutualists, are  
524 not unique to the *Burkholderia* symbiont (*i.e.* SBE *Burkholderia*). On the contrary,  
525 competitiveness inside the gut is a derived trait of the bean bug symbiont lineage only  
526 (Fig. 3H) and has thus played a critical role in the evolution of the insect gut symbiont.

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

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

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

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

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

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

569

## 570 **Conclusion**

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

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

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

599

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605

606 **Conflict of Interest**

607 The authors declare that they have no competing interests.

608

609

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1024 **Figures legends**

1025 **Figure 1. Gut symbiosis between the bean bug *Riptortus pedestris* and its symbiont**  
1026 ***Burkholderia insecticola*.**

1027 (A) A female adult of the bean bug *R. pedestris*. (B) *B. insecticola*. (C) Whole midgut of  
1028 *R. pedestris*. (D-G) A GFP-labeled strain of *B. insecticola* colonizes the posterior region  
1029 of the midgut (D-E), in which GFP signals are specifically detected in the crypt-bearing  
1030 symbiotic region (E-G). Abbreviations: M1, midgut first section; M2, midgut second  
1031 section; M3, midgut third section; M4, midgut fourth section with crypts; M4B, M4  
1032 bulb; CR, constricted region; H, hindgut. Each crypt is indicated by arrowheads in F  
1033 and G. Panels B and C are from Ohbayashi et al. 2015.

1034

1035 **Figure 2. Partner-choice in the gut of *R. pedestris*.**

1036 (A) A midgut fed with water containing 0.05% food coloring (congo red). Note that the  
1037 coloring is blocked at the constricted region. (B) A midgut infected with GFP-labeled *B.*  
1038 *insecticola* showing that the bacteria can flow from M3 into M4B by passing through  
1039 the constricted region. (C) Electron microscopy image of the narrow channel in the  
1040 constricted region. The lumen of the channel is bordered by a layer of microvilli (ML)  
1041 and contains a few bacterial cells (S) that are passing through the channel. (D) The  
1042 constricted region in an insect co-inoculated with a GFP-labeled *B. insecticola* and an  
1043 RFP-labeled *E. coli*. At the constricted region, *E. coli* is sorted out and only the  
1044 *Burkholderia* symbiont passes through the channel. (E) Normal swimming mode of *B.*  
1045 *insecticola* in a liquid environment. (F) Corkscrew motility of *B. insecticola* in a

1046 viscous environment. Flagellar filaments are visualized by covalent coupling to the  
1047 fluorescent dye Cy3-NHS-ester. Panels **A**, **C** and **D** are from Ohbayashi et al. 2015.

1048

1049 **Figure 3. Microbe-microbe competition in the gut of *R. pedestris*.**

1050 Midgut M3-M4B-M4 regions of **(A)** an aposymbiotic insect; **(B)** an insect infected with  
1051 a SBE *Burkholderia* (*B. insecticola*); **(C)** an insect infected with a PBE *Burkholderia*  
1052 (*B. fungorum*); **(D)** an insect infected with *Pandoraea* (*P. norimbergensis*). **(E-G)** M4  
1053 region of an insect inoculated with an RFP-labeled SBE *Burkholderia* (*B. insecticola*)  
1054 and an RFP-labeled *Pandoraea* (*P. norimbergensis*) at 2, 3, and 5 days post inoculation  
1055 (dpi). **(H)** The schematic image of a symbiont specificity in the midgut of the bean bug  
1056 *R. pedestris*. In the early infection stage, SBE-*Burkholderia*, PBE *Burkholderia*, and  
1057 *Pandoraea* co-colonize the M4 (left). In the mature stage, SBE *Burkholderia*  
1058 outcompetes the other strains and becomes dominant in the M4 (right). Panels **A-G** are  
1059 from Itoh et al. 2019.

1060

Fig. 1

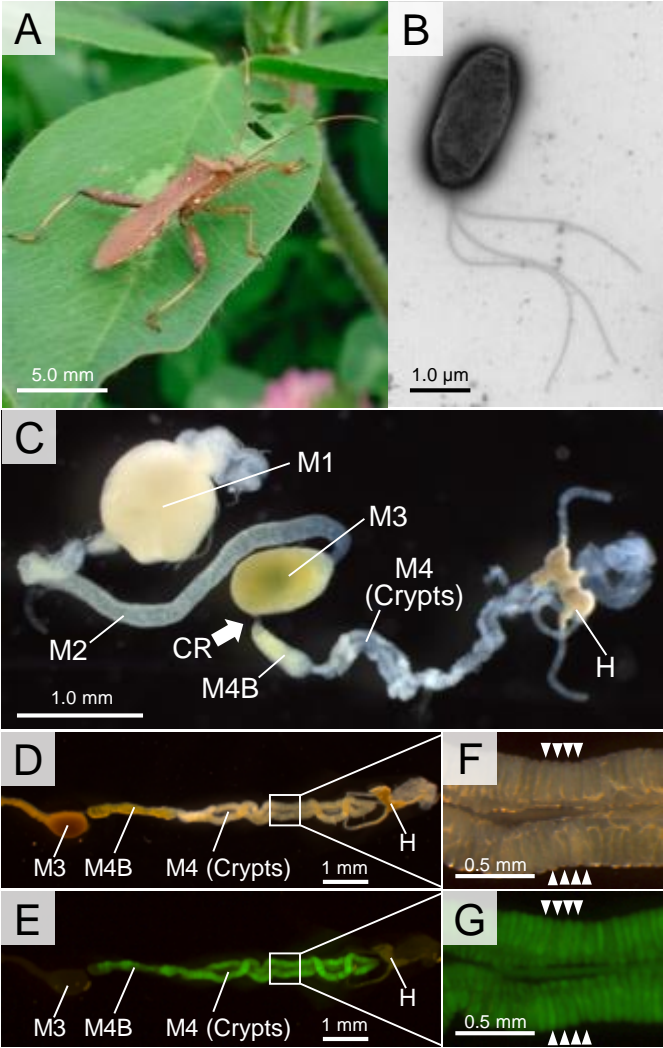


Fig. 2

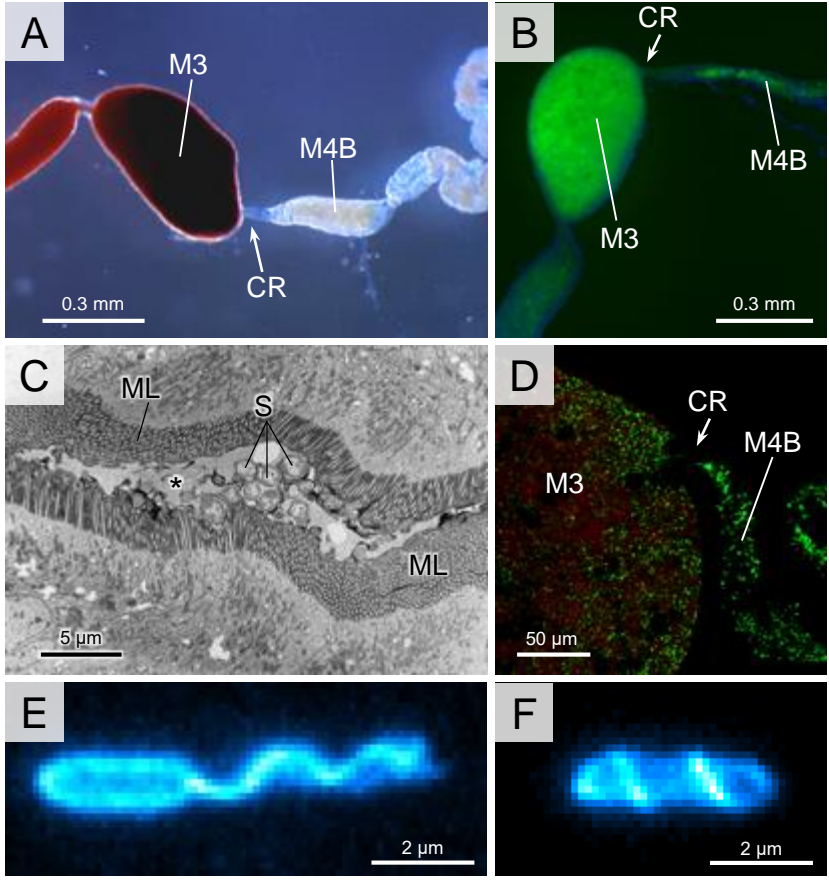


Fig. 3

