Dynamics and molecular mechanisms aiding symbiont establishment in *Lagria villosa* beetles

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Ramya Ganesan
Born on 02.11.1994 in Coimbatore, India.

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Dean:
Prof. Dr. Eckhard Thines

Reviewers:
1. Prof. Dr. Martin Kaltenpoth
2. Prof. Dr. Ralf Heermann

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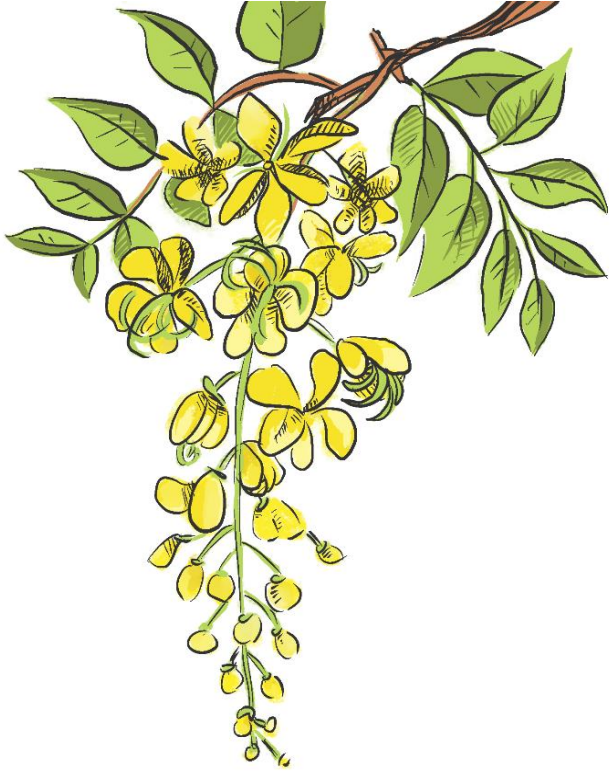


…It's a magical world, Hobbes, ol' buddy…Let's go exploring!

– Calvin (Bill Watterson)

SUMMARY

Many animals live in a symbiotic relationship with microbial partners. Among them, beneficial host-associated microorganisms may provide nutritional benefits, protect the host from antagonists, or help in detoxifying harmful compounds. To maintain a stable association, animal hosts vertically transmit symbionts to the offspring, acquire them horizontally from an external source, or carry out both transmission modes. In horizontal and mixed transmission modes, host molecular factors are necessary to screen for the right microbial partners from the environment, and microbial machineries help the symbionts gain entry and establish in the host. However, we know little about molecular factors that mediate symbiont establishment in invertebrates harbouring ectosymbionts. To expand our knowledge, I studied the defensive symbiotic association between *Lagria villosa* beetles and *Burkholderia* bacteria. Here, the female *Lagria* beetles smear symbionts on the egg surface during oviposition. The symbionts colonize three specialized cuticular invaginations on the dorsal surface of the larvae. On eggs and in the larvae, the symbionts produce antifungal compounds and protect the beetle from antagonists. Furthermore, the larvae can occasionally acquire symbionts from the external environment; therefore, they maintain a mixed transmission mode. Among the multiple strains of *Burkholderia* usually found in an individual, *Burkholderia* Lv-StB is the most dominant strain, but is uncultivable in the lab. However, *Burkholderia gladioli* Lv-StA that is occasionally found in the beetles, is cultivable. The ability to rear the beetles in the lab and cultivate one of the symbionts *in vitro* gives us the opportunity to study host and symbiont molecular factors involved in symbiont establishment. In this dissertation, first, I provide details on the dynamics and mechanisms of symbiont colonization. Symbionts colonize the dorsal structures of the larvae during hatching and a colonization time-window may restrict microbial entry into the host. Further, a transposon-insertion directed sequencing (Tn-seq) method was established in the lab using which 271 potential colonization factors in Lv-StA were identified, including motility, biofilm formation, cell wall structures, lipopeptides, oxidative stress response factors and iron scavenging molecules. Additionally, targeted mutagenesis and colonization assays with Lv-StA reveal that non-motile strains can still colonize the host, and this likely explains the loss of flagellar motility genes in Lv-StB, which has a more reduced genome than Lv-StA. Finally, by performing gene expression analysis across multiple developmental time points in symbiotic and aposymbiotic beetles, the host molecular responses in the presence of Lv-StB was investigated. It reveals that the host may not recognise symbiont presence, likely due to compartmentalization, or may have a constitutive response to the symbionts. Thus, combining our understanding of both host and symbiont molecular factors involved in establishment, interesting questions regarding how partner specificity is achieved in ectosymbioses, are revealed. This study gives us the opportunity to understand the mechanistic details of host – symbiont interactions and further to compare emerging patterns across other model and non-model systems.

ZUSAMMENFASSUNG

Viele Tiere leben in einer symbiotischen Beziehung mit mikrobiellen Partnern. Wirts-assoziierte Mikroorganismen können nützliche Funktionen haben und z.B. Nährstoffe liefern, den Wirt vor Antagonisten schützen oder bei der Entgiftung schädlicher Verbindungen helfen. Um eine stabile Verbindung aufrechtzuerhalten, übertragen tierische Wirte Symbionten vertikal an ihre Nachkommen, erwerben sie horizontal von einer externen Quelle oder führen beide Übertragungsarten durch. Bei der horizontalen und der gemischten Übertragung sind molekulare Faktoren des Wirts notwendig, um die richtigen mikrobiellen Partner aus der Umwelt herauszufiltern, und mikrobielle Mechanismen helfen den Symbionten, in den Wirt einzudringen und sich dort zu etablieren. Wir wissen jedoch nur wenig über die molekularen Faktoren, die die Ansiedlung von Symbionten in wirbellosen Tieren mit Ektosymbionten fördern. Um unser Wissen zu erweitern, habe ich die defensive symbiotische Verbindung zwischen *Lagria villosa* Käfern und *Burkholderia* Bakterien untersucht. Dabei schmieren die weiblichen *Lagria*-Käfer während der Eiablage Symbionten auf die Oberfläche der Eier. Die Symbionten besiedeln drei spezialisierte kutikuläre Einstülpungen auf der dorsalen Oberfläche der Larven. Auf den Eiern und in den Larven produzieren die Symbionten antifungale Verbindungen und schützen den Käfer vor Antagonisten. Darüber hinaus können die Larven gelegentlich Symbionten aus der äußeren Umgebung aufnehmen, so dass sie einen gemischten Übertragungsmodus beibehalten. Unter den verschiedenen Stämmen von *Burkholderia*, die normalerweise in einem Individuum vorkommen, ist *Burkholderia* Lv-StB der dominanteste Stamm, allerdings ist dieser im Labor nicht kultivierbar. *Burkholderia gladioli* Lv-StA, der gelegentlich in den Käfern vorkommt, ist jedoch kultivierbar. Die Möglichkeit, die Käfer im Labor aufzuziehen und einen der Symbionten *in vitro* zu kultivieren, erlaubt es uns molekulare Faktoren zu untersuchen, die bei der Etablierung der Symbionten eine Rolle spielen. In dieser Dissertation gehe ich zunächst auf die Dynamik und die Mechanismen der Symbiontenbesiedlung ein. Die Symbionten kolonisieren die dorsalen Strukturen der Larven während des Schlüpfens und ein Zeitfenster für die Kolonisierung kann den mikrobiellen Eintritt in den Wirt beschränken. Darüber hinaus wurde im Labor eine Transposon-Insertions-gesteuerte Sequenzierungsmethode (Tn-seq) etabliert, mit der 271 potenzielle Kolonisierungsfaktoren in Lv-StA identifiziert wurden, darunter Motilität, Biofilmbildung, Zellwandstrukturen, Lipopeptide, oxidative Stressreaktionsfaktoren und eisenbereitstellende Moleküle. Gezielte Mutagenese und Kolonisierungsversuche mit Lv-StA zeigen, dass auch nicht-motile Stämme den Wirt kolonisieren können. Dies erklärt wahrscheinlich den Verlust der Gene für die Flagellenmotilität in Lv-StB, dessen Genom stärker reduziert ist als das von Lv-StA. Schließlich wurde die molekulare Reaktion des Wirts in Gegenwart von Lv-StB untersucht, indem eine Genexpressionsanalyse über mehrere Entwicklungszeitpunkte bei symbiotischen und aposymbiotischen Käfern durchgeführt wurde. Dabei zeigte sich, dass der Wirt die Anwesenheit des Symbionten möglicherweise nicht erkennt, was wahrscheinlich auf die Kompartimentierung zurückzuführen ist, oder dass er konstitutiv auf den Symbionten reagiert. Durch die Kombination unseres Verständnisses der molekularen Faktoren von Wirt und Symbiont, die an der Etablierung beteiligt sind, ergeben sich interessante Fragen dazu, wie die Partnerspezifität in Ektosymbiosen erreicht wird. Diese Studie gibt uns die Möglichkeit, die mechanistischen Details der Wirts-Symbionten-Interaktionen zu verstehen und darüber hinaus aufkommende Muster in anderen Modell- und Nicht-Modellsystemen zu vergleichen.

TABLE OF CONTENTS

LIST OF PUBLICATIONS

CHAPTER – 2

Published in Microbiology and Molecular Biology Reviews

How It All Begins: Bacterial Factors Mediating the Colonization of Invertebrate Hosts by Beneficial Symbionts

R. Ganesan, J.C. Wierz, M. Kaltenpoth, L.V. Flórez

CHAPTER – 3

Published in Biology Letters

Colonization dynamics of a defensive insect ectosymbiont

R. Ganesan, R.S. Janke, M. Kaltenpoth, L.V. Flórez

CHAPTER – 4

Published in Journal of Visualized Experiments

Transposon-insertion Sequencing as a Tool to Elucidate Bacterial Colonization Factors in a Burkholderia gladioli Symbiont of Lagria villosa Beetles

R. Ganesan, M. Kaltenpoth, L.V. Flórez

CHAPTER – 5

In preparation

Mechanisms of colonization in a defensive ectosymbiont of Lagria villosa beetles

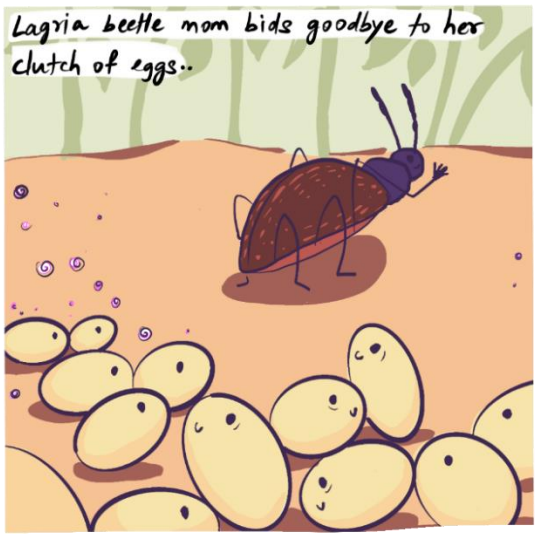
R. Ganesan, J. Krabbe, C. Hertweck, M. Kaltenpoth, L.V. Flórez

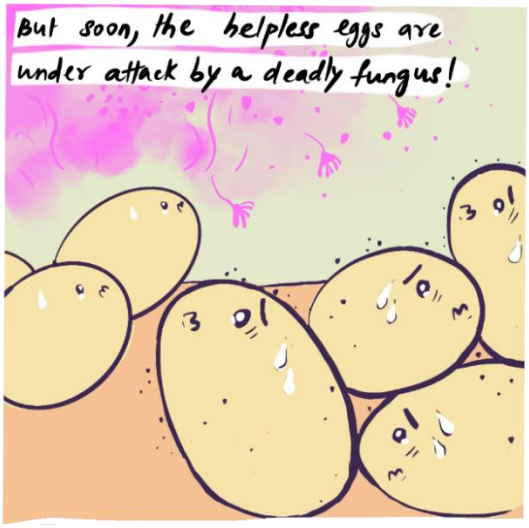
CHAPTER – 6

In preparation

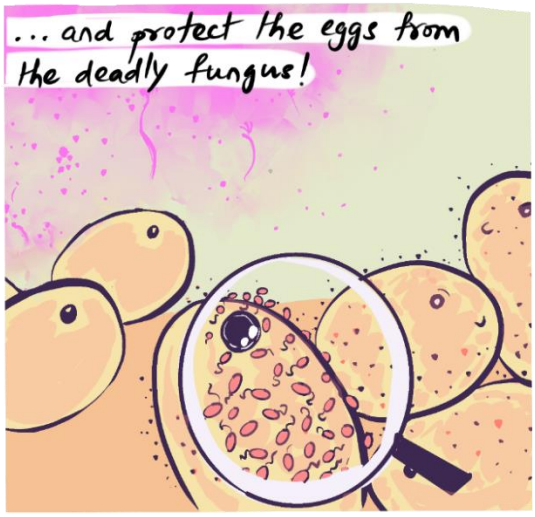
An insect host exhibits diminished systemic molecular response to the establishment of ectosymbionts

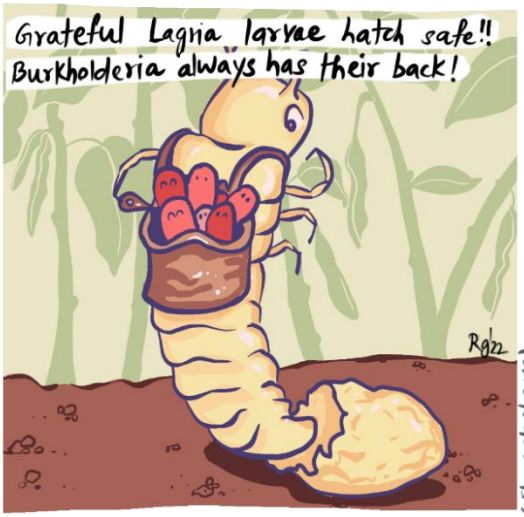
R. Ganesan, R. S. Janke, J. Keller, L.V. Flórez, M. Kaltenpoth





* Gram negative bacterium.





(Florez et al. 2018)

CHAPTER – 1

GENERAL INTRODUCTION

Over the centuries since Anton Von Leeuwenhoek first described "little animalcules" in rain water, the impact of microorganisms on ecosystems, human health and their economic significance have been well investigated. Microorganisms are drivers of carbon and nitrogen cycling in the soil and ocean (1,2). They degrade organic matter in the soil, improve nutrient availability and engineer ecosystems (3,4). Considering their evolutionary age predating the origin of eukaryotes, their ubiquity and functional capabilities, it is not surprising that microbial interactions with other organisms are commonplace in nature.

Originating as free-living entities, microbes found a new resource for nutrients, and a relatively safe haven within eukaryotic hosts. Anton de Bary coined the term "symbiosis" to describe such "living together of two unlike organisms" (5,6). In fact, the symbiotic integration of an alphaproteobacterium with an ancestral eukaryote-like cell or an archaeon gave rise to complex eukaryotic life-forms (7–9) and eons past this evolutionary landmark, most animals and plants have continued to interact with microorganisms in various contexts. Symbiosis has definitely shaped the evolution of life on earth as we know it today. These inescapable associations may have a positive, negative, or neutral fitness consequence on the host (10). But historically, microbiological research has centered around the pathogenic potential of microorganisms given that the consequences on health and economy are monumental. Only in the recent years has the beneficial impact of certain microbes on human health gained attention. Understanding symbiont function in a host, the factors that induce microbial transitions along the parasitism – mutualism continuum, and expanding on molecular tools and sequencing technologies, can provide us with the potential to harness host-associated microbes for further application in medicine and agriculture (11).

Along those lines, establishing a simplified model system where both the host and symbiont can be manipulated is essential to discern the molecular dialogue between the host and its microbial partners. The relatively minimalistic nature of invertebrate – microbial symbioses make them ideal models to study the details of microbe – host interactions. Microbial communities in invertebrates are often comparatively simple (12), and the host is equipped with an innate immune regulatory system to interact with its microbial partners. In addition, the diversity of invertebrate model systems available provides the opportunity to explore the ecology and evolution of host-microbe symbioses across several taxa. Having recognized this potential in the recent years, we have developed a new understanding of the functional importance and eco-evolutionary trajectories of beneficial animal-microbe interactions (13–15).

The following paragraphs in the general introduction provide a brief overview on functional benefits of harboring symbionts, symbiont localization, transmission, establishment, and summarizes information from previous work on the study system. In **chapter 2**, a detailed review on the molecular dialogue underlying symbiosis initiation is provided.

1. FUNCTIONAL BENEFITS OF MICROBIAL SYMBIONTS

Among all the long term mutualistic partnerships we observe in nature, symbiont capabilities have proved important for animals to occupy diverse ecological niches by expanding their nutritional range and overcoming antagonists (15). Symbionts supplement essential nutrients and enable host survival on nutritionally unbalanced diets. Some marine organisms, like the tubeworm *Riftia pachyptila* (16,17), and bivalve molluscs like *Myrtea spinifera* (18), thrive in the harsh environment of deep-sea hydrothermal vents by the aid of chemoautotrophic sulphur-oxidizing symbionts. Among insects, many hemipterans feed on nutrient poor plant sap diet and the intracellular symbionts provide amino acids or vitamins to the host (19–24). Similarly, symbionts help in breaking down complex substrates and improving host digestion, e.g. wood-feeding termites carry cellulose-digesting symbionts (25,26) and leaf-eating beetles are associated with pectin-degrading microbes (27,28). Furthermore, some termites and cockroaches are associated with nitrogen recycling gut microorganisms (24,29,30). These and many other nutritional symbioses are highly specialized, such that one partner cannot live without the other.

Apart from supplementing nutrients for hosts living on nutrient poor diets, symbionts can also help in detoxification (31). For example, extracellular symbionts in the bean-bug gut provide resistance to man-made pesticides (32). Harmful toxins may also occur in the host-plants that the insects feed on and these plant defenses are overcome by associating with symbionts that degrade phytotoxins (31,33). For example, the coffee-berry-borer beetle *Hypothenemus hampei,* harbours caffeine detoxifying gut bacteria, *Pseudomonas fulva,* that enables consumption of coffee beans that are exceptionally toxic to most insects (34).

Furthermore, by producing antibiotics, toxins or through bioluminescence, microbes also protect their animal hosts against predators (35–37), parasitoids (38) or microbial pathogens (39–43). For example, the Hawaiian bobtail squid harbours a bacterial symbiont *Vibrio fischeri* in light organs where symbiont bioluminescence acts as counter illumination against the moonlight at night and helps the squid camouflage from predators (35). In insects, a facultative bacterial symbiont *Hamiltonella defensa,* and its associated bacteriophage, APSE (*Acyrthosiphon pisum* secondary endosymbiont) can deter the development of parasitoids in aphids (38,44). Apart from defense against parasitoids and predators, microbial symbionts also deter pathogen infection in hosts. In the European beewolf, symbiotic *Streptomyces* is integrated on the cocoon wall spun by the larva and they protect the developing larva and pupa from fungal antagonists by production of bioactive agents (40,45). In the darkling beetle *Lagria villosa* and *Lagria hirta, Burkholderia* symbionts produce bioactive compounds and protect the developing embryo and larvae against entomopathogenic fungi (41–43).

Alternatively, the defensive symbionts can also deter colonization of other microbes by priority effects, direct microbe-microbe interactions, or by mediating colonization resistance (46). For example, in the honeybee gut, colonization by *Snodgrassella alvi* affects host immune gene expression and protects the host from the opportunistic pathogen, *Serratia marcescens* (47). In the carrion beetles, *Nicrophorus vespilloides,* offspring colonized first by the endogenous microbiome including *Providencia rettgeri* and *Morganella morganii* resist further colonization by *Serratia marcescens* and *Escherichia coli* (48). Therefore, the mechanistically diverse nutritional or defensive benefits provided by microbial symbionts improve host fitness.

2. SYMBIONT LOCALIZATION

Various morphological adaptations in host internal organs and external structures accommodate the microbial symbionts. Particularly, many sap-feeding and blood-feeding insects enclose symbionts in intracellular organs (19,49,50). These organs are called bacteriocytes, and multiple bacteriocytes clustered together collectively form the bacteriome **(Fig.1)**. The intracellular symbionts are usually specialized in provisioning nutrients like essential amino acids and vitamins, to the host (51). One of the advantages of hosting symbionts in a bacteriome is that the organ separates the beneficial symbionts from active immune effectors circulating in the haemolymph. For example, in the *Sitophilus* weevil, symbionts within the bacteriome are unaffected by upregulation of immune responses in the host **(Fig.1)** (52). Moreover, host molecular effectors can regulate symbiont titers in the bacteriome, *i.e.,* increase symbiont titers when dietary nutrients are low and vice versa (53). Most intracellular bacteriome-localized symbionts are in a highly integrated association with the host such that symbiont transmission is regulated by host molecular mechanisms (54–56). Such bacteriome – localized symbioses are termed closed systems since bacteria are protected from colonization by external microbes and cells are vertically transmitted to the offspring (49). Therefore, a clonal population of symbiont cells is maintained over time.

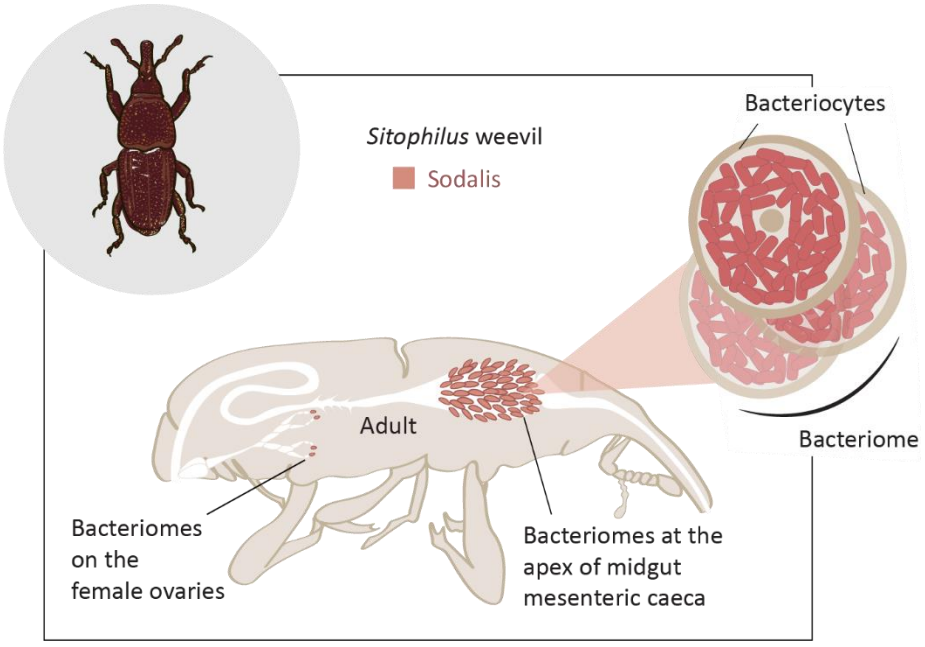


Fig. 1. *Sodalis* endosymbionts in bacteriomes of a *Sitophilus* weevil: Red highlights indicate the localization of *Sodalis* endosymbionts in bacteriomes at the apex of the midgut mesenteric caeca and on the female ovaries (Modified from Ganesan *et al.* 2022). Inset shows bacteria within individual bacteriocytes that collectively make up the bacteriome (Adapted from (Perraeu and Moran, 2022)).

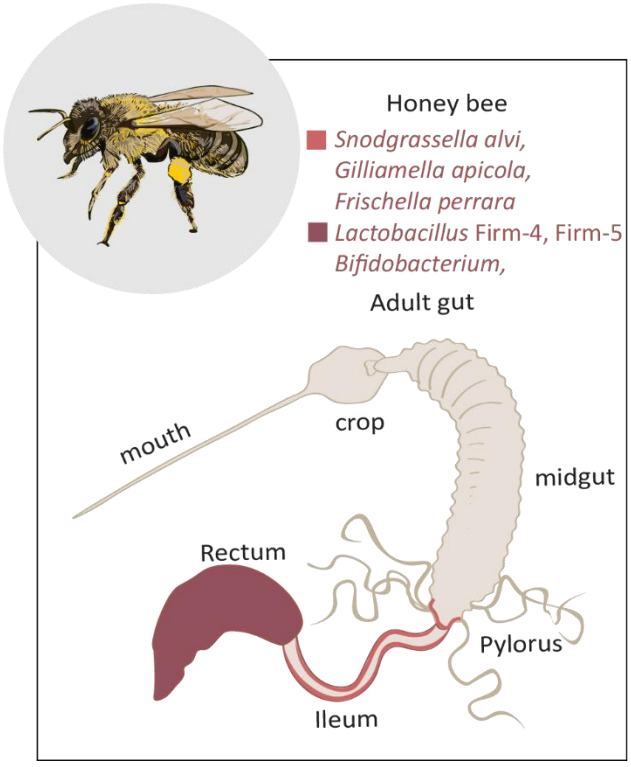


Fig.2. Gut-microbiota in an *Apis mellifera* honeybee: Red highlights indicate the localization of the bee gut microbes in the pylorus, ileum and rectum of the hind-gut (Modified from **chapter 2** (Ganesan *et al.* 2022)).

However, in many insects, the gut and the external cuticle are the most exposed structures that are frequently in contact with microbes (49). The gut environment is suitable for hosting beneficial microbes since it has a constant flow of nutrients and could provide bacteria protection from desiccation and other external stressors (49). In the honeybees a consortium of gut bacterial symbionts are localized in the hind-gut region; *(i.e.) Snodgrassella alvi* and *Gilliamella apicola* are found in the ileum, *Frischella perrara* in the pylorus, and *Lactobacillus* Firm-4, *Lactobacillus* Firm-5 and *Bifidobacterium* colonize the rectum(57) **(Fig.2)**. These gut microbes help in degrading complex food substrates, detoxification of harmful toxins, or even provide colonization resistance against pathogens **(Fig.2)**. A relatively less complex symbiotic association is found in the *Riptortus* bean-bugs (58). The bean-bugs are associated specifically with *Caballeronia insecticola,* which are acquired orally from the environment each generation and colonize the M4 mid-gut crypts of the host. *Caballeronia* is known to provide insecticide resistance and may be a source of nutrients for the host (32,58).

On the other hand, symbionts that provide protection against pathogens by secreting antimicrobial peptides are usually localized on external surface structures of the host. A well-known example of an ectosymbiotic association is that between the beewolf-digger wasp, *Philanthus* sp., and the antibiotic-producing *Streptomyces* symbionts (39,40,45). Here, the symbionts establish in antennal-reservoirs of the female wasps (59). Symbionts are smeared on the wall of the brood-cell where the female lays an egg and after hatching, the developing larva incorporates the symbionts into its cocoon where the symbionts produce antibiotics to protect the host from entomopathogens (39). **I**n another example, *Lagria villosa* beetles carry protective symbionts that are smeared on the egg surface during oviposition. The symbionts provide protection against fungal pathogens (41,42) and these protective benefits extend through the larval stages where the symbionts are housed in three dorsal surface pouches that are lined by the cuticle (41,43). The external localization of the defensive antibiotic- producing symbionts on the beewolf cocoon and the *Lagria* eggs and larvae, enables them to act directly at the first line of defense (60). Similarly, external localization of symbionts on the cuticle or associated structures can be found in other insects. For example, in some bark beetles, leaf-rolling weevils **(Fig.3)**, leaf-cutter ants **(Fig.3.)** and adult female beewolf wasps, symbionts are housed in mycangia (61), or on the cuticle (62) or within specialized antennal reservoirs of the host (59). These external symbiont – bearing structures help in

transmission and symbiont dispersal **(Fig.3)**. However, in animals that harbor microbial partners externally, like in the gut, cuticle or other symbiotic organs, the symbionts are exposed to the extracellular environment, and there is a threat of invasion by other microbes. Such associations can be referred to as "open" systems (49).

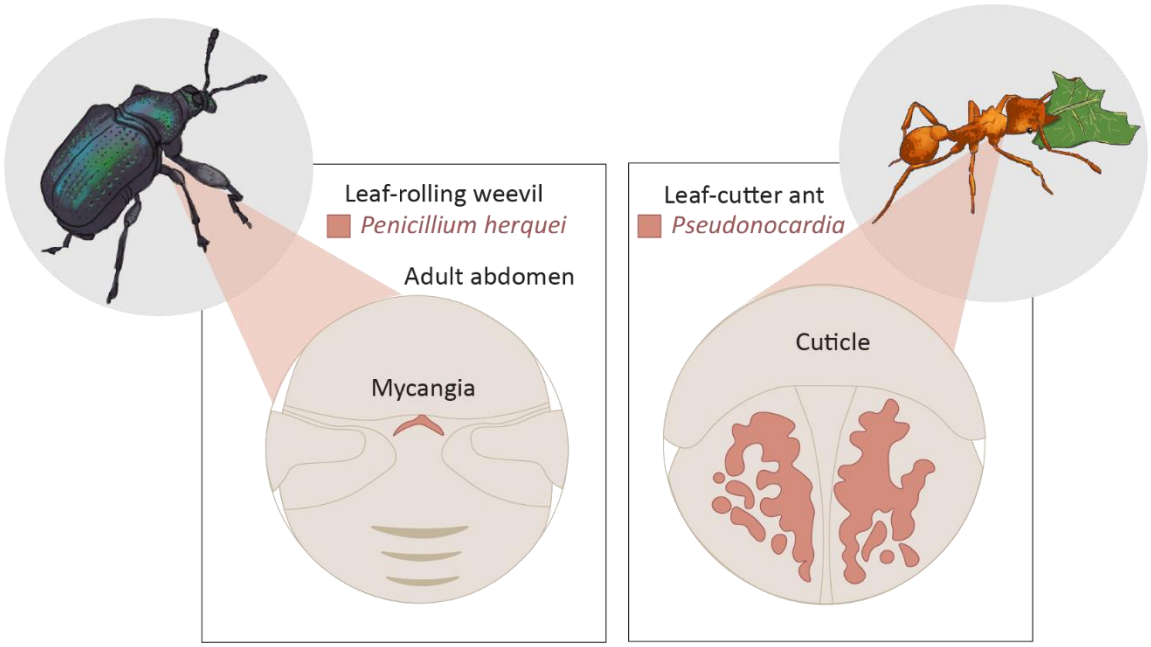


Fig.3. Insects with ectosymbionts: An adult leaf-rolling weevil, *Euops chinesis* **(left)** and leaf-cutter Attine ant **(right)**. Red highlights indicate the localization of the symbionts in the mycangia of the leaf-rolling weevil and the cuticle of the leaf-cutter ant. (Illustrations adapted from (Li *et al.* 2012, Goldstein *et al.* 2020))

In a few exceptional cases, symbionts that produce secondary metabolites are found within the body of the host, like in the *Paederus* rove beetles (63–65) and the Asian citrus psyllid, *Diaphorina citri* (66). The mechanism by which these symbiont metabolites are delivered externally to deter antagonists is unknown. Moreover, symbionts that provide protection against predators may be found either extracellularly in specialized organs, like *Vibrio fischeri* in the light-organs of the Hawaiian- bobtail squid (35), or intracellularly in bacteriomes like *Hamiltonella defensa* in the pea-aphids (38,67). Nevertheless, symbiont localization in specialized organs, the gut or the external cuticle gives the host direct or immediate access to the functional benefits provided by the microbes and further facilitates transmission.

3. INITIATION OF SYMBIOSES

3.1. Transmission

For animals to sustain a beneficial association with their mutualistic partner over time, the symbiont must be stably transmitted or acquired by the offspring. Transmission of symbionts could occur vertically, from mother to the offspring, or acquired from the external environment at every generation by the juvenile or naïve hosts. Simultaneously, it is in the interest of the microbial symbiont to propagate or transmit to novel hosts and find replenished resources (68).

During vertical transmission, symbiont colonization and establishment is tightly regulated by the host. A controlled vertical transmission route from parent to offspring reduces the risk of symbiont loss for the host, and ensures that partner specificity is maintained (69). Here, host adaptations ensure that the symbionts are provisioned and transmitted to the offspring. For example, in hemipteran bugs, symbionts may be deposited on the eggs, enclosed in a capsule or jelly, and the new hatchlings consume the symbiont-containing maternal provisions immediately after hatching (70–73). In whiteflies, symbionts are transferred to the developing embryo within the body of the host (55,74). Such intracellular symbionts face a population bottleneck during transmission, accumulate deleterious mutations and undergo genome erosion. Several microbial genes essential for a free-living lifestyle, like motility, chemotaxis, are redundant within host and are often lost from the genomes (75).Therefore most intracellular symbionts only retain genes that are necessary to maintain a symbiotic association with the host (76). However, in a few exceptional cases, intracellular symbionts retain transmission machineries. For example, in tsetse flies, intracellular *Wigglesworthia* symbionts activate flagellar motility during transovarial transmission (77) and during metamorphosis, *Sodalis pierantonus* colonize the cereal weevil adult bacteriomes using the type III secretion system (T3SS) (78).

In contrast, some hosts acquire symbionts horizontally from the environment or conspecifics each generation. The symbionts maintain a part of their lifecycle outside the host and may transition between different hosts. In this scenario, the host faces the risk of obtaining non-beneficial partners from the environment but also gains an opportunity to acquire novel symbionts or assemble a multispecies community with diverse beneficial traits. *Vibrio fischeri* and *Cabelleronia* are bacterial symbionts associated with the Hawaaiian bobtail squid and *Riptortus* bean bugs, respectively, and are acquired from the environment each generation. The symbionts retain autonomy and the microbial machineries for both, survival in the host, and in the free-living environment. For reliable colonization of the host, horizontally acquired symbionts usually retain molecular machineries like motility and chemotaxis, adhesion, biofilm formation, secretion systems and transporters (75,79,80). Remarkably, the squid and bean-bugs are consistently associated with the same partner taxa despite the horizontal route of transmission.

Finally, combining both scenarios, some symbionts are transmitted through mixed modes; i.e., maternally and can occasionally be acquired from the external environment. Here symbionts are immediately available for the offspring via maternal provisions and the host is also flexible to acquiring novel symbionts (75,80). In horizontal and mixed transmission modes, it is a challenge to ensure that the right partner is selected and maintained. Many symbiont-associated animals have resolved the matter by engaging in a highly specific molecular dialogue with the symbiont (81), imposing a developmental time-window during which symbionts are more welcome to colonize (82,83), or by screening for microbial symbionts from the environment (84). Likewise, symbiont encoded molecular factors and their interactions with co-colonizing microbes are also crucial factors that help colonize the host and for maintaining partner specificity in symbioses (80).

3.2. Molecular underpinnings of symbiosis establishment

Mutualistic symbionts resemble their pathogenic counterparts in some strategies and molecular tools that are utilized for colonization and proliferation within the host (85). For example, some pathogens and mutualists wield similar type III, type IV secretions systems, and modifications in the O-antigen structure that promote virulence or colonization in the host (85–87). Within the host, the context of symbiont's association shapes the host's response. Therefore, starting as a neutral symbiotic association and progressing into a mutualistic one requires coordinated adaptations of both the symbiont and host (80).

Firstly, initial contact and entry into symbiotic organs may rely on a combination of symbiont motility and host behaviour (79,80,88) (89,90). Further, within the symbiotic organs, microbial adhesion and the ability to form a biofilm layer, helps microbial cells attach to the tissue surface of the host, protects cells from host immune factors, and facilitates inter-microbial cooperation (80). In addition, cell-surface structures like lipopolysaccharide, peptidoglycan, tracheal cytotoxins, which are peptidoglycan fragements actively released by bacteria, and secretion systems engage with the host or help in evading host responses during colonization. Throughout this process, symbionts migrate through fluctuating conditions like changing temperature, oxygen concentration, availability of nutrients, and face oxidative stress. Therefore, symbiont molecular mechanisms help in reducing molecular stress, repairing DNA damage, or stabilizing protein - folding within the host. Adding another level of complexity, in a multispecies microbial consortium, inter-microbial interactions may also influence community assembly and composition. Contact-dependent inter-microbial competition (91) or the order of arrival in the symbiotic organ (priority effects) (92,93), could determine community composition during the initiation and establishment of a symbiosis. Therefore, microbial machineries for microbe-microbe and microbe-host interactions play a combined role in the assembly of multi-species/ strain symbiont communities. These symbiont colonization factors in invertebrate symbioses are described in more detail in **chapter 2**.

So far, a comprehensive view of the molecular underpinnings of symbiosis establishment has been obtained from a few well-studied model systems (80). Especially, studies on the association between the Hawaiian-bobtail squid and *Vibrio fischeri* symbionts have given us detailed insights into the process. Here, bacterial peptidoglycans released in the seawater induce the juvenile squid host to secrete a mucus that attracts the microbes towards the opening of the squid light organs (94). During this process, motility and chemotaxis help *Vibrio fischeri* reach the opening and enter the light organs (89,95). Further, after colonization, bacterial tracheal cytotoxins induce morphological changes at the opening of the light organ (96,97). Additionally, the host regulates *Vibrio* colonization by releasing nitric oxide (NO) within the symbiotic organs and symbiont factors combat this NO-mediated stress to establish in the host (98,99). Moreover, different strains of the *Vibrio fischeri* symbiont engage in a contact-dependent competition using the type VI secretion system (T6SS) (91). Multiple *Vibrio fischeri* strains co-exist in the same individual, however a dominant D-strain outcompetes the S-strains to spatially segregate and establish in separate crypts of the light organs (100). Therefore, an intricate system of host and symbiont molecular interactions mediate colonization and establishment in this symbiosis. Studies on other systems, including the association between *Riptortus* bean-bugs and *Cabelleronia* symbionts, the honeybee and *Snodgrassella alvi,* tsetse flies and *Sodalis glossinidius,* and *Heterorhabditis* nematodes and *Photorhabdus luminescens* bacteria have also contributed to our understanding of molecular factors

involved in establishment (87,90,101–107). Nevertheless, further research with diverse symbiotic systems, could help us gather information on emerging patterns across model and non-model systems (80).

3.3. Colonization window

Congruently, another strategy to control symbiont entry in open symbioses is for the host to maintain a specific time window for acquisition of symbionts. In the Hawaiian bobtail squid, presence of *Vibrio fischeri* within the crypts of the light organ leads to constriction of the bottleneck region preceding the crypt space and restricts further colonization (108). Similarly, in the *Riptortus* bean-bugs, *Cabelleronia* symbionts are more successful in colonizing second-instar nymphs than the third-, fourth- or fifth-instar nymphs (82). Here the symbionts pass through a constriction region to colonize symbiont-bearing crypts in the mid-gut. The symbionts induce closure of this constriction region within 12 – 18 hours post-colonization and prevent further entry of microbes (109). Similarly, in leaf cutter ants, defensive bacterial symbionts, *Psuedonocardia*, are acquired by trophyllaxis and contact with nest-mates only within the few hours post-eclosion (83). However, here, and in other ectosymbiotic associations, we are unsure of the mechanisms that induce restricted colonization beyond the time-window. Such a strategic colonization window and/or an accompanied molecular dialogue between symbiont and host could ensure that partner specificity is achieved during colonization even in horizontal or mixed modes of transmission.

4. SYMBIONT REGULATION BY THE HOST

Once inside the host, animals usually recognize microorganisms as foreign agents and inadvertently mount immune defenses against them. Invertebrates have an innate immune system that is equipped with pattern recognition molecules or receptors (PRRs) and respond to the presence of microbe associated molecular patterns (MAMPs) such as the lipopolysaccharide (LPS) and peptidoglycan (PGN) released from the outer surface of a bacterial cell (96,110). While this is an effective strategy to combat pathogenic infections, the host faces the threat of evicting beneficial partners if all microbes are treated as antagonists. Therefore, it is remarkable that the same innate immune system fighting off pathogens, can also redirect or modify immune responses to maintain the beneficial microbes (111). Multiple host strategies help in partner selection and regulating beneficial symbionts within the body. The host could differentiate between pathogens and beneficial symbionts through i) molecular signaling using PRRs and MAMPs (96), ii) compartmentalization in specialized organs (112) and/ or iii) preventing microbial escape into other host tissue (113). Alternatively, in some systems, the beneficial symbionts have the capability to camouflage themselves from an impartial immune response of the host (114).

The evolution of host immunity strongly depends on the mode of symbiont acquisition and transmission, and the complexity of the symbiotic community (115). First, in highly specialized and obligate symbioses with a primary endosymbiont that is vertically transmitted, symbiont titer and localization are tightly controlled by the host. For example, *Sodalis pierantonius* bacteria engage in a relatively recent endosymbiosis with *Sitophilus* weevils. In a clear indication of its pathogenic ancestry, *Sodalis* still retains its T3SS machinery and cell wall components (116) and symbionts control virulence in the host through quorum sensing (117). The weevil host segregates the beneficial symbiont in bacteriomes and thus protects them from the action of immune effectors, even when a systemic response is activated by

pathogen infection (52). Also, the host controls bacterial proliferation in the bacteriome by expression of an antimicrobial peptide (AMP), coleoptericin A, and a class of peptidoglycan recognition proteins, PGRP-LB, to cleave tracheal cytotoxins (TCTs), which are fragments of the bacterial peptidoglycan, and suppress immune stimulation (113). A combination of compartmentalization and bacteriome localized immune response helps in maintaining this recently established obligate intracellular symbiosis in the *Sitophilus* weevils (52).

Furthermore, among animals with extracellular symbionts, host physiological conditions could play a role in spatially segregating symbionts. Specifically, the gut is an open-ended system without strict compartments, however, differences in pH, food resources, and oxygen and hydrogen levels create a gradually changing environment (112). For example, in the honey bee gut, the crop and the mid-gut contain very few bacteria, while in the ileum of the hind-gut, *Snodgrassella alvi* is found on the gut wall, and *Giliamella apicola, Lactobacillus* Firm-4 and *Lactobacillus* Firm-5 are found in the lumen. Here, spatial segregation of the bee gut-microbes is likely because the microbes have their own metabolic niche and may be indicative of distinct selective conditions across the gut (57,112). In addition to the physiological conditions within the gut, immune effectors play a role in controlling gut microbial colonization and establishment. In fact, presence of the beneficial microbes in the honey bee gut triggers an immune response in the host. Immune stimulation prevents further infection by pathogens however, the beneficial symbionts are resistant to a few AMPs (47,118). In the African cotton-stainer bug, gut -associated symbionts seemingly evade the host's immune response (114). In contrast, other gut-symbionts seem to be more susceptible to the host's regulatory molecules. In the *Riptortus* bean bugs, *Cabelleronia* symbionts are housed in crypts of the mid-gut region. The symbionts develop an altered cell-envelop within the host which makes them susceptible to some host AMPs (87,119). Therefore, to prevent symbiont clearance, AMPs in the symbiotic M4 region of the midgut are possibly suppressed by the host.Thus, a combination of host physiological conditions and immune regulatory factors are usually essential for symbiont maintenance.

5. HOST – ASSOCIATED *BURKHOLDERIA*

Burkholderia s.l. (sensu lato) comprises a versatile group of pathogens (BCC) (*Burkholderia* s.str., i.e. sensu stricto), plant beneficial or environmental (PBE) (*Paraburkholderia)*, and stink bug beneficial or environmental (SBE) bacteria (*Caballeronia)* (120,121). The vast number of bioactive compounds produced by *Burkholderia* s.l., makes them effective beneficial symbionts and pathogens. For example, *Mycetohabitans rhizoxinica* (previously *Burkholderia rhizoxinica*) is associated with a fungal pathogen of plants, *Rhizopus microspores,* and the symbiont produces a bioactive compound, rhizoxin, that causes rice seedling blight (122). In *Ardisia crenata,* Candidatus *Burkholderia crenata* is a leaf-nodule symbiont and produces an insecticidal compound to possibly provide chemical defense to the plant (123). Similarly, in *Lagria* beetles, *Burkholderia* sp. are defensive microbial symbionts that produce several antifungal compounds to protect the beetle host during vulnerable stages of development (41–43). Moreover *Burkholderia glumae* produce a secondary metabolite, toxoflavin, that leads to panicle blight in rice and bacterial wilt in several plant crops (124,125). The soil-isolate, *Burkholderia rinogensis,* has insecticidal abilities (126). The *Burkholderia cepacia* complex comprise of bacterial species that have a wide host

range from the rhizosphere of plants all the way to human opportunistic pathogens isolated from cystic fibrosis patients (127).

The ecological diversity of *Burkholderia* sp. across these clades shows their ability to play the role of pathogens or mutualists in diverse host taxa (120). We should attribute their dynamic life-style to the breadth of molecular tools that lay tucked in their genomes. *Burkholderia* sp. genomes span from 0.8 Mbp to 11 Mbp in size and carry genes that promote virulence, like quorum sensing, biofilm formation, motility, and the ability to produce bioactive compounds like toxoflavin (120,124,128). These mechanisms may promote bacterial virulence across different hosts. For example, both in an alfalfa plant model and in the murine infection model, bacterial quorum sensing controls biofilm formation, swarming motility, production of extracellular proteases and siderophores in *Burkholderia cenocepacia* (127). Arguably, these molecular characteristics create the ideal circumstances for *Burkholderia* sp. to live in association with animal, plant, or fungal hosts (11).

The molecular factors involved during host-infection in pathogenic *Burkholderia* s. str. are well characterized, however, there is dearth of information about microbial mechanisms promoting colonization in their mutualistic counterparts. Understanding factors influencing their shifts along the parasitism – mutualism continuum, in the same or between different hosts, and the mechanisms that are brought into play during these transitions, would provide a comprehensive insight into the versatile life-style of *Burkholderia*. With this aim, we summarize past and recent findings about the mutualistic association of *Burkholderia* with *Lagria* beetles and the thesis further expands upon our recent findings about the molecular basis of this symbiotic association.

6. *LAGRIA* BEETLES AND THEIR MUTUALISTIC *BURKHOLDERIA* SYMBIONTS

The pioneering work of Hans Jürgen Stammer in 1929 gave us the first insight into the symbiotic interaction of *Lagria* beetles with certain rod-shaped bacterial symbionts (129). One of the species in this group, *Lagria hirta,* is widely distributed across Europe. Found usually on forest shrubs and bushes as a polyphagous herbivore, the *Lagria* larvae and pupae spend most of their lifecycle in the leaf litter and consume decaying plant material which often harbours bacteria and fungi. Their defensive microbial symbionts likely protect them from these frequent antagonistic encounters with pathogenic fungi in the damp, humid leaf litter. Stammer observed this symbiotic association in *Lagria hirta*, and described similar symbiotic partnerships across 83 different species in the group, showing the widespread distribution of the symbiosis in the tribe Lagriini (129).

Stammer's findings (129) and more recent studies (130), gave us insights into the ecology of *Lagria hirta* beetles. *Lagria hirta* lay approximately 50 eggs in a tightly packed clutch and the bacterial symbionts are smeared on the egg surface by the female adult beetle during oviposition. After 5- 10 days of development, first-instar larvae hatch, and the larvae have three specialized cuticular invaginations in the intersegmental regions between the two last thoracic and the first abdominal segments. According to Stammer, the symbionts likely migrate into these dorsal structures of the larvae during the last day of development through the micropyle, an opening on the egg surface that usually allows the entry of sperm. The bacterial symbionts are housed extracellularly in these dorsal surface structures that are

cuticle-lined (19,129). Male beetles lose the symbionts while female adults have a pair of globular structures next to the ovipositor and additional accessory glands where the symbionts are housed (131).

Fig. 4. Life-stages of *Lagria villosa* beetle: a-b) Eggs and first-instar larvae (Photograph credits: Devasena Thiagarajan), **c)** older larva, **d)** pupa and **e)** adult on a soybean leaf.

Another species, *Lagria villosa,* is native to Africa and was introduced to Brazil (132). It occurs similarly in the leaf litter and is a pest of agriculturally important crops like soybean and potato (133–135). In the laboratory, *Lagria villosa* lays clutches of 200 -300 eggs that develop over 5 days **(Fig. 4)**. Similar to *L. hirta,* symbionts are smeared on the eggs during oviposition and are found in the dorsal surface structures of the first-instar larvae (41,42) **(Fig. 4,5)**. Symbionts on the egg surface protect the developing embryo from fungal pathogens. Recently, Janke *et al.*, 2022 found that this protective benefit also extends to the larval stages (131). In pupae, the dorsal cuticular structures are more pronounced in females than in the males. Males in both *L. hirta* and *L. villosa* lose the symbionts probably because they are not beneficial in adults and are retained by females only for transmission (136). In the female *L. villosa* adults, a pair of four tubular extensions that are accessory glands on either side of the ovipositor house symbiotic bacteria **(Fig. 6)** (41,131).

The symbionts associated with the beetles are predominantly *Burkholderia* sp. Multiple strains of *Burkholderia* can be found associated with a single *L. hirta* or *L. villosa* host (43,136). Comparing the strain composition in *L. villosa* and *L. hirta* shows that both host species carry at least one *Burkholderia* strain, Lv-StB or Lv-StH respectively, that is yet unculturable. In *L. hirta,* the two *Burkholderia* strains, Lv-StG and Lv-StH frequently co-occur in the same individual and their relative abundance changes across the beetle life-cycle (137). By contrast, in *L. villosa, Burkholderia* Lv-StB is most dominant yet a facultative symbiont strain, and has a reduced genome size of ~2.3 Mbp, compared to the less abundant, yet frequently occurring *Burkholderia gladioli* Lv-StA which has a genome of 8.5 Mbp (138). The functional capabilities of the two strains are also quite different. Unlike Lv-StA, Lv-StB lacks genes for flagellar motility. However, Lv-StB is capable of producing a potent antifungal agent, lagriamide (42), while Lv-StA produces a large number of antifungal and potentially antibacterial compounds like icosalide, toxoflavin, caryoynencin, lagriene, burriogladin, haereogladin, sinapigladioside (41), gladiofungin (139) and gladiobactin (140). The beetles are capable of losing these *Burkholderia* strains in the lab, which shows that this symbiotic association is facultative, and occasional acquisition from the environment might be key for symbiont maintenance (136). Indeed, studies by Flórez *et al.* (41) and Wierz *et al.* (141) show that *Burkholderia* strains transition between the plant and insect hosts.

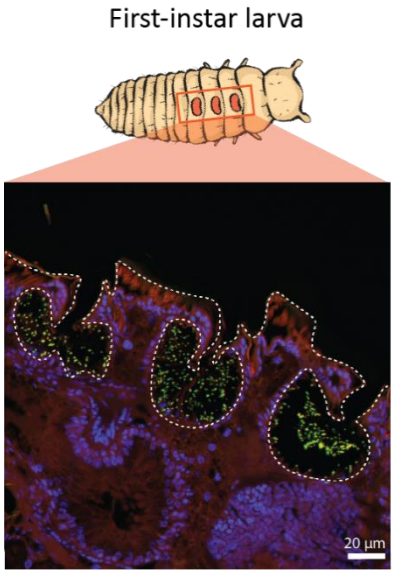


Fig. 5. Symbiotic dorsal structures of *Lagria villosa* larva: Fluorescent *in situ* hybridization on a sagittal section of a first-instar larva shows Lv-StA cells in the dorsal structures (white dotted-lines). *Burkholderia* are showed in green, eubacteria in red and host cell nuclei in blue. Adapted from **chapter 3** (Ganesan *et al.* 2023).

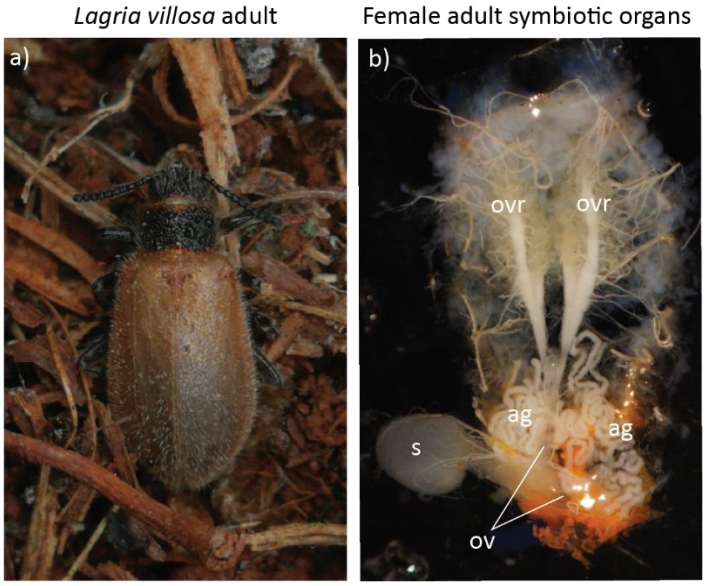


Fig. 6. A *Lagria villosa* adult and the female symbiont – bearing glands: a) An adult *Lagria villosa* beetle in the field (sex unknown), **b)** The symbiotic structures of the female *Lagria villosa* adult. (Ovr = ovaries, ag = symbiont containing accessory glands, s = spermatheca, ov = oviduct and ovipositor) (Credit: Rebekka Janke)

The external localization of defensive bacterial symbionts and specialized cuticular adaptations of the host are similar to symbiont – containing antennal reservoirs in beewolf wasps (59), mycangia of leaf-rolling weevils (142), and bark beetles (61). However, we know little about symbiont and host molecular factors that determine partner specificity and lead to establishment of such ectosymbiotic associations. The facultative nature of the association between *Lagria* beetles and *Burkholderia* bacteria allows us to separate the partners and generate aposymbiotic insects for experimental manipulation. While the dominant strain, Lv-StB, has not yet been culturable in the lab, the ability to cultivate Lv-StA (41) gives us the opportunity to do genetic manipulation, *in vitro* bioassays, and also study symbiont colonization dynamics by reestablishing the symbiosis in the lab.

7. THESIS OUTLINE

Over the past decades, the field of symbiosis has unravelled details about symbiont localization, transmission, and the functional benefits of symbionts in animals. However, the molecular mechanisms involved in symbiosis establishment remain known only for a small number of systems (90,101–103,106,143,144) in relation to the vast number of symbioses that have been so far functionally characterized, especially involving insect – microbe interactions (19,39,50,74,145–148). Difficulties in rearing field-collected insects under laboratory settings, in cultivating their symbionts, and performing genetic manipulation are hurdles for performing detailed molecular studies. The facultative nature of the symbiotic association between *Lagria* beetles and *Burkholderia* bacteria provides the ability to separate the ectosymbiotic bacteria from the host and generate aposymbiotic insects that can be reared in the laboratory. And while the predominant strain, Lv-StB, has not yet been cultivable in the lab, the ability to grow the closely related strain Lv-StA *in vitro* (41) gives us the opportunity to perform genetic manipulation, infect the host with modified symbiont strains, and study symbiont colonization dynamics. Therefore, using the *Lagria* – *Burkholderia* defensive symbiosis as a model system, my objective is to provide mechanistic insights into the establishment of an ectosymbiont in a beetle host.

Chapter 2 provides the background on known microbial molecular mechanisms important for symbiont colonization in invertebrate systems. We outline the process of initiation of a symbiosis and the evolutionary processes that decide the fate of the microbial symbiont. Further, in open symbiotic associations, we discuss the microbial molecular factors that aid colonization and try to derive general patterns that emerge across systems. We highlight that apart from molecular factors, understanding the ecological parameters and dynamics of symbiont colonization is essential to gain a complete understanding about how microbial symbionts colonize and establish themselves in invertebrate hosts.

In **chapter 3**, we investigated the dynamics of symbiont colonization in *Lagria villosa* beetles. We determined that symbiont colonization occurs during or immediately after hatching and that colonization efficiency declines after the beetles' first-instar larval stage. We hypothesize that host movements during hatching and microbial molecular factors aid colonization of the symbiotic structures in the larvae.

In **chapter 4**, we describe a protocol for transposon-insertion directed sequencing and provide details on how the method could be adapted to study colonization factors in an ectosymbiont of an insect host. Using the protocol, a library of *B. gladioli* Lv-StA mutants was generated. The mutants were infected *in*

vivo and simultaneously grown *in vitro.* Further, adapting an insertion-directed sequencing method, potential symbiont genes involved in colonization of the beetle host were identified. The results of the experiment are presented in **chapter-5** and provide insights into potential factors important for this ectosymbiont to colonize *Lagria* beetles. Based on genome comparisons of Lv-StA and Lv-StB, we speculate on potential similarities and differences in the colonization strategies of different symbiont strains. Additionally, using targeted mutagenesis and colonization experiments, we investigate the relevance of flagellar motility during colonization.

Having explored the microbial factors promoting establishment of the symbiosis, we turn our attention towards the host in **chapter-6.** A comparative RNA-seq experiment of symbiotic and aposymbiotic beetles across life-stages, provides insights into how the host responds to the symbionts during establishment. The experiment shows that in the early life stages, very few host genes are differentially expressed in the presence of symbionts compared to aposymbiotic individuals. Therefore it is likely that the cuticle-lined symbiotic structures of the *Lagria* larvae compartmentalize the symbionts and prevent systemic immune activation.

Finally, in **chapter-7,** results of the previous chapters are combined to provide a summary and discussion about potential mechanisms mediating partner selection in the *Lagria – Burkholderia* association. Using this knowledge, future directions for research are proposed.

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CHAPTER – 2

How it all begins: bacterial factors mediating the colonization of invertebrate hosts by beneficial symbionts

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Ramya Ganesan [1,2], Jürgen C. Wierz [1,2], Martin Kaltenpoth [1,2], Laura V. Flórez [1,3]

[1] Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg University, Mainz, Germany

[2] Department of Insect Symbiosis, Max Planck Institute for Chemical Ecology, Jena, Germany

[3] Department of Plant and Environmental Sciences, Section for Organismal Biology, University of Copenhagen, Copenhagen, Denmark.

CONTRIBUTIONS

R.G.: conceptualization, visualization, writing—original draft, review and editing. J.C.W.: conceptualization, writing—original draft, review and editing. M.K.: conceptualization, funding acquisition, resources, supervision, writing—review and editing. L.V.F.: conceptualization, funding acquisition, resources, supervision, writing—original draft, review and editing.

All authors commented on the final draft and gave final approval for publication.

1. ABSTRACT

Beneficial associations with bacteria are widespread across animals, spanning a range of symbiont localizations, transmission routes and functions. While some of these associations have evolved into obligate relationships with permanent symbiont localization within the host, the majority requires colonization of every host generation from the environment or via maternal provisions. Across the broad diversity of host species and tissue types that beneficial bacteria can colonize, there are some highly specialized strategies for establishment, yet also some common patterns in the molecular basis of colonization. This review focuses on the mechanisms underlying the early stage of bacteria-animal beneficial associations, from initial contact to the establishment of the symbionts in a specific location of the host's body. We first reflect on general selective pressures that can drive the transition from a free-living to a host-associated lifestyle in bacteria. Then, we cover bacterial molecular factors for colonization in symbioses from both model and non-model invertebrate systems where these have been studied, including terrestrial and aquatic host taxa. Finally, we discuss how interactions between multiple colonizing bacteria and priority effects can influence colonization. Taking the bacterial perspective, we emphasize the importance of developing new experimentally tractable systems to derive general insights into the ecological factors and molecular adaptations underlying the origin and establishment of beneficial symbioses in animals.

2. INTRODUCTION

Symbiosis is at least as old as eukaryotic cells (1) and has played a major role in the evolution of micro- and macro-organisms. In animals, there is compelling evidence that beneficial microorganisms can fuel major ecological innovations by conferring a diverse spectrum of functional benefits to the host (2–7). A range of bacteria are prone to interact with eukaryotes, as host associations have evolved independently in multiple different bacterial phyla (8, 9). Among these, beneficial bacteria have evolved either from environmental (8) or parasitic ancestors (10, 11). Both ecological and genomic preadaptations have likely been important for the evolution of tight interactions with eukaryotes (12).

Notably, the benefits to engage in symbiosis are often discussed from the perspective of the host, while the microbe's ecological drivers of mutualism with an animal are poorly understood (13–15). From the symbiont's perspective, a series of challenges must be overcome to successfully engage in a persistent association with a host (15–17). First, bacteria need to reach and establish contact. Second, the microbes must make their way to the housing tissues, persist in these, successfully compete with other microorganisms, and protect themselves against host immune reactions **(Box 1)**. And third, they need to disperse or relocate to new host individuals.

Box 1: The animal immune system as a key challenge for bacterial colonizers

Along with diverse microbial factors influencing the establishment of beneficial interactions, host immunity plays a crucial role in determining which microbes can colonize and persist. Importantly, shifts between a free-living and a host-associated lifestyle, as well as transitions across the parasite-mutualism continuum, usually require that bacteria evolve adaptations to cope with the host immune system.

The innate immune system of invertebrates comprises two different components for controlling antagonists (18,19). Through cellular immunity, haemocytes that are constantly circulating the host body become activated, resulting in encapsulation or phagocytosis of microorganisms (20,21). By contrast, humoral immunity describes the ability of pattern recognition receptors (PRRs) to recognize microorganism-associated molecular patterns (MAMPs) and subsequently trigger the expression of antimicrobial peptides (AMPs) and other effectors through complex signaling cascades (20). Notably, some invertebrates have evolved mechanisms to recognize previously encountered microorganisms (18,22). However, the memory component is more pronounced and fine-tuned in the vertebrate adaptive immune system, which relies on specialized lymphocyte populations forming a living record of pathogen encounters that can be rapidly re-activated upon recurrent infections.

Similar to pathogens, endosymbionts have evolved multiple strategies to cope with their hosts' immune defenses. Some can manipulate the host's immune system, for example by downregulating genes of defense pathways (23). Alternatively, symbionts can become more resistant to the host's antimicrobial activity, as is the case in the secondary symbiont of tsetse flies, *Sodalis glossinidius*, which is more resistant to an antimicrobial compound produced by the host compared to closely related non-symbiotic bacteria (24). Another widespread mechanism for immune evasion is to hinder recognition by the host. For example, bacterial peptidoglycan is generally recognized by PRRs, but bacteria can elude detection by modifying their MAMPs or by losing the peptidoglycan machinery altogether (25).

In addition to immune evasion by the symbionts, the host can evolve mechanisms to confine the symbionts, e.g. by compartmentalization in specific tissues, like **bacteriomes** or specialized gut regions, and downregulation of immune effectors in these compartments (26 – 29). Alternatively, some hosts develop defenses that spare beneficial symbionts, e.g., by reducing specific immune factors that target a desired symbiont while keeping other components unchanged (30). Furthermore, some eusocial insects have less immune-related genes, resulting in a reduced repertoire of antimicrobial peptides, and instead rely on social immunity (31,32).

The interaction between beneficial symbionts and the host's immune system has been covered in great depth elsewhere, and interested readers are referred to previous reviews on this topic (for examples, see references 18 and 33 to 34).

Both symbiont localization and transmission strongly affect the bacterial traits required for colonization of a host. Since symbionts can reside intra- or extra-cellularly in a variety of host tissues (8), gaining access to the final destination requires adaptations to achieve translocation and specific molecular interactions with host cells, as well as the ability to cope with stressful conditions in the host environment. While the original establishment of symbiotic interactions necessarily relies on acquisition from the environment (8), transfer over generations —or vertical transmission— has evolved in numerous symbioses (16, 35). For highly integrated partners involving consistent intracellular localization and a strict vertical transmission route, the microbial symbiont is likely to have a passive role in colonization, especially after the massive loss of functions due to genome erosion commonly associated with an ancient host-associated lifestyle (36). We will henceforth refer to these as **closed symbioses**, and to those relying on recurrent acquisition from the environment or unrelated hosts as **open symbioses**, based on categories of symbiosis proposed elsewhere (37). A combination of both vertical and horizontal transmission, called **mixed-mode transmission**, is likely a common phenomenon balancing some of the benefits and challenges of each mode (17,38) **(Box 2)**. Accordingly, we will here refer to such associations as **mixed symbioses** (37). A plethora of mechanisms has evolved across different bacteria to navigate these transmission routes, enabling colonization in taxonomically diverse hosts with varying morphologies and lifestyles (12, 39 – 41).

The increasing availability of molecular data has now opened new avenues to investigate factors facilitating symbiosis and evaluate common patterns across model and non-model systems. Host adaptations to accommodate symbionts

Box 2: Implications of symbiont transmission mode

The mode of symbiont transmission is usually related to the degree of dependence in the association, with vertical transmission being more likely in symbioses with increased dependence (42). Still, there are also systems in which obligate symbionts are acquired horizontally (43) and each transmission mode —vertical, horizontal or mixed-mode— entails a different set of trade-offs for both host and symbiont **(Table-1)**. Vertical transmission can result in an obligate association involving partner co-evolution and co-diversification, here referred to as a closed symbiosis. The symbiont can benefit from this through an ensured passage from one host generation to the next, a sheltered and nutritious environment, reduced competition and protection against antagonists. Faithful transmission is a mechanism to maintain specificity through partner fidelity (44). The aligned interests of host and symbiont in a system with vertical transmission also promote a shift from pathogenic to beneficial interaction. However, it is not clear whether the reduction in pathogenicity favors the evolution of vertical transmission or vice versa. Symbionts with a strict vertical transmission can suffer from genome erosion due to relaxed selection on genes unnecessary in the host environment in conjunction with repeated population bottlenecks (45 – 47).

By contrast, in systems relying on horizontal transmission, or open symbioses, the symbiont needs to be adapted to the host, while also maintaining the ability to survive in and transition to/from free-living conditions outside of the host or other unrelated hosts throughout the symbiont's lifecycle. The evolutionary trajectory of a symbiont in such a scenario depends on many factors, including the adaptations necessary for colonization

of the host and the non-host-environment(s), the frequency these environments are encountered, trade-offs between environments and the probability of transitioning between environments (15, 48). Also, horizontally-transmitted symbionts must deal with partner choice mechanisms imposed by the host to promote specificity and ensure cooperation (44). Advantages for bacteria with horizontal transmission include the potential to spread to other host individuals, populations or even species (49). Furthermore, secondary hosts might serve as a refuge if the primary host is not available (50). As a result of the capabilities needed to persist in varying environments, horizontally transmitted symbionts generally show less dramatic genome erosion (42), and contacts with other microorganisms can enable the acquisition of novel functions through horizontal gene transfer (51).

Symbionts experiencing mixed-mode transmission, *i.e.,* in a mixed symbiosis, potentially reap the benefits of both transmission modes. Ebert (17) speculates that this may be the most common mode of transmission and can sometimes occur as an intermediate stage in the transition towards strict vertical transmission. Strikingly, even occasional interactions with other bacteria can facilitate horizontal gene transfer and thus mitigate genome erosion caused by strict vertical transmission (52, 53).

and in particular host immunity have been actively investigated **(Box 1)** and are expanding subfields within symbiosis research, as highlighted in a number of studies and reviews (22, 33, 34, 54 – 57). In the present review, bacterial factors are in the spotlight. We aim to synthesize the current knowledge on molecular mechanisms enabling beneficial bacterial symbionts to successfully colonize invertebrate animal hosts. We chose to focus on symbionts that must colonize the host tissues each generation, either after maternal provisioning or environmental acquisition, i.e., open and mixed symbioses.

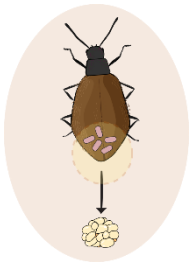
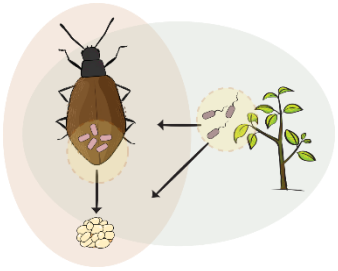
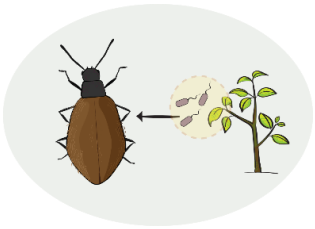
The review is structured in three sections that integrate ecological, molecular and evolutionary perspectives on the establishment of beneficial bacterial symbionts. First, we reflect on the selective pressures that could generally drive the transition from a free-living lifestyle to a consistent association with a host. Then, we review the prevalent mechanisms that facilitate symbiont entry and the underlying genetic factors orchestrating these early phases: initial contact with the host and establishment in the housing tissues **(Fig. 1)**. In a third and final section, we mention relevant ecological components of symbiont establishment, discussing multipartite interactions between bacteria and their prominent role in host colonization. The review does not aim to be exhaustive in covering the numerous studies on systems with a complex microbiota. We deliberately focus on a number of invertebrate animals from terrestrial, freshwater and marine environments in which bacterial molecular factors for establishment in the host have been described **(Table-2, Fig. 1),** and refer to a few examples in vertebrates and plants to complement key points.

3. SWITCHING BETWEEN FREE-LIVING AND HOST-ASSOCIATED LIFESTYLES

Symbionts taking part in open or mixed systems, which includes the majority of described symbioses (58), often experience short or extended periods outside of the host. The environmental conditions during this phase can be starkly different, and relevant challenges must be overcome for successful transition in either direction —from host to the environment and vice versa, as well as for persistence in

the environment (15) **(Box 2)**. What scenarios can be envisioned for initial transition from a free-living to a host-associated lifestyle? How can re-colonization in open and mixed symbioses persist at evolutionary timescales? From the symbiont's perspective, the following scenarios are possible: (i) a selection-driven transition, where the host as a habitat or a means of dispersal provides an overall advantage in comparison to a free-living lifestyle, (ii) capture, where living in the host has a neutral or negative impact on the fitness of the symbiont but an immediate benefit for the host, and (iii) a neutral start, where none of the partners experience benefits in early stages of symbiosis.

Table-1. Overview of potential benefits (+) and downsides (-) for hosts and symbionts depending on symbiont transmission mode.

Symbiotic partner		 Vertical	 Mixed	 Horizontal
Host	Benefits	Symbionts guaranteed in offspring Maximum control Selects for beneficial symbionts	Symbionts guaranteed in offspring Flexible acquisition of novel symbionts when environment changes	Flexible acquisition of novel symbionts when environment changes
	Downsides	Potentially costly adaptations for transmission Inflexible to changes Accumulation of mildly deleterious mutations can reduce symbiont benefits (Muller's ratchet)	Symbionts can become harmful Filtering and control mechanisms necessary Competition between symbionts may reduce benefit to the host	Symbionts may not always be available (resulting in uninfected offspring) Symbionts can become harmful Filtering and control mechanisms necessary Competition between symbionts may reduce benefit to the host
Symbiont	Benefits	No competition Reliable nutrients from the host Safe niche	Priority for vertically transmitted symbiont Horizontal Gene transfer possible Biphasic lifestyle can expand ecological potential Colonization of unrelated host individuals or new host species possible Reliable nutrients	Horizontal Gene Transfer possible Biphasic lifestyle can expand ecological potential Colonization of unrelated host individuals or new host species possible
	Downsides	Genome erosion and Muller's ratchet	High competition	High competition

	Increased dependence on host Limited or no possibility for spreading to unrelated hosts	In case of a biphasic lifestyle: costly adaptations to host and environment (trade-offs) Transition between environments potentially challenging	Hosts may not always be available In case of a biphasic lifestyle: costly adaptations to host and environment (trade-offs) Transition between environments potentially challenging

3.1. Selection-driven transition

In the first case, where the microorganism benefits from being housed or vectored, symbiont adaptations favoring effective host colonization are under positive selection. These will however vary in strength according to the availability of host individuals and the presence of microbial competitors, since these will challenge successful colonization of the host as a valuable niche. At the same time, traits for survival in a temporary host or persistence in the transitional free-living stage, like nutritional independence, might also be favored. An open or mixed symbiosis will also give room for genetic replenishment and the maintenance of variation (for example, see reference 53). From the host's perspective, environmental symbiont uptake entails the risk of failing to acquire beneficial symbionts or experiencing costly infections by symbionts shifting towards the detrimental end of the parasite-mutualism continuum (17, 35). However, an open symbiosis can also allow for rapid adaptation to changing conditions if the source of potential symbionts is reliable. Thus, while recurrent switching between host and environment can be a stage in the evolutionary transition leading to a closed system, it can also establish as a long-term viable strategy for symbiosis. This is possibly the case in the symbionts of squids, entomopathogenic nematodes, stinkbugs **(Table-2)**, and potentially many other open and mixed symbioses. Notably, while adaptations on the host side to maintain environmental acquisition over generations can occur, these are not always necessary (15). For instance, plant pathogenic microorganisms like phytoplasmas benefit from being vectored by various sap-sucking insects (59). While the insect does not necessarily benefit from hosting the microbe and acquires it passively, the latter hitchhikes on the insect's feeding habits and is likely to drive this insect-microbe association. In other words, the persistence of an open or mixed symbiosis can be driven solely by microbial traits. As recently discussed by Obeng and coauthors (15), the prolonged recurrence of host-environment switching enables selection to act on the different stages of the microbe's biphasic life cycle. In this framework, evolutionary processes will promote a microbial lifestyle that is optimized for entry and persistence in the host, but also for dispersal and survival under free-living conditions.

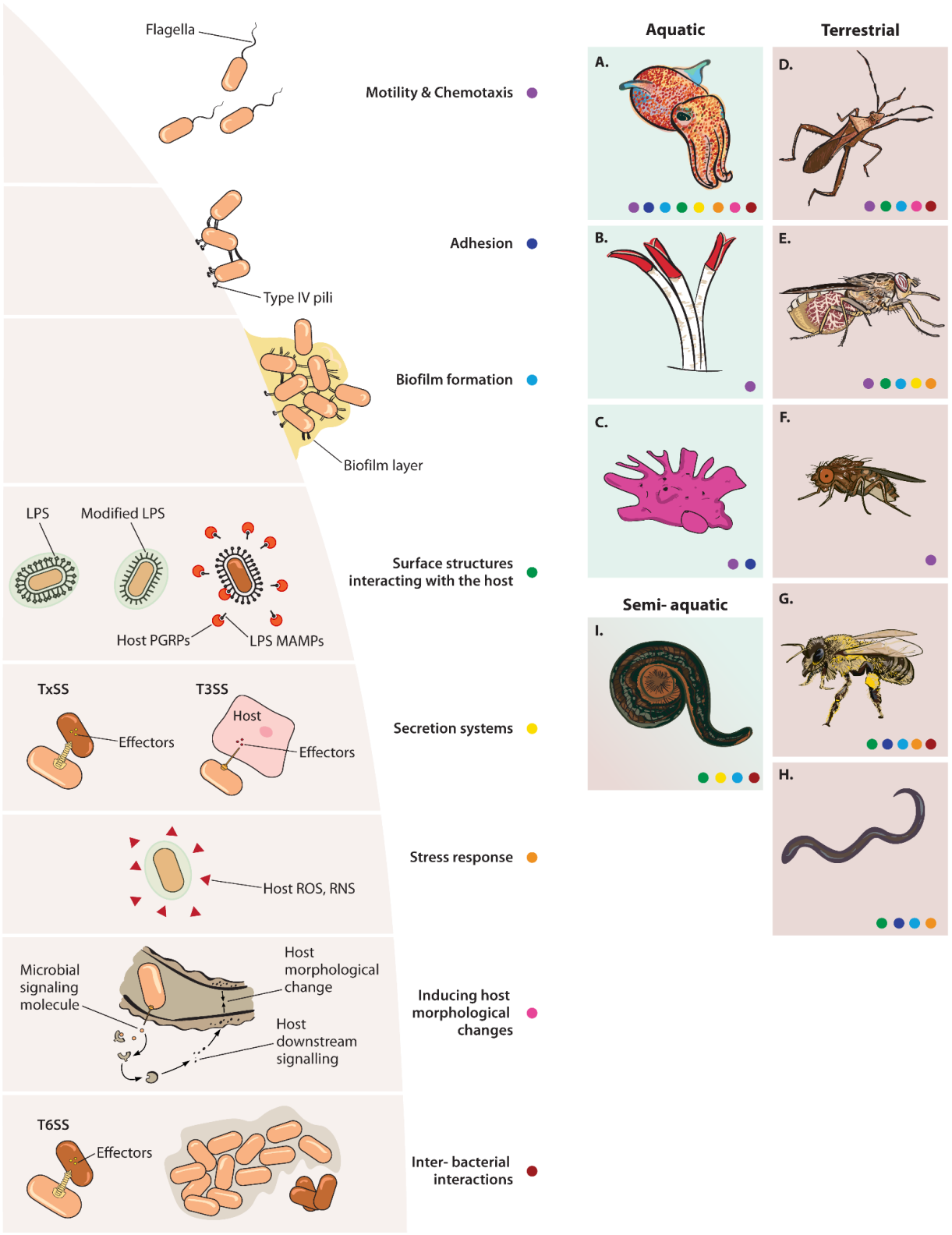

Flagella
Motility & Chemotaxis
Adhesion
Type IV pili
Biofilm formation
Biofilm layer
LPS
Modified LPS
Host PGRPs
LPS MAMPs
Surface structures interacting with the host
TxSS
T3SS
Effectors
Host
Effectors
Secretion systems
Host ROS, RNS
Stress response
Microbial signaling molecule
Host morphological change
Host downstream signalling
Inducing host morphological changes
T6SS
Effectors
Inter- bacterial interactions
Aquatic
Terrestrial
A.
D.
B.
E.
C.
F.
Semi- aquatic
I.
G.
H.

Fig. 1. Overview of known bacterial molecular mechanisms used for colonization of animal hosts in symbioses that require re-entry of the symbionts into the host tissues every generation from the parents or the environment. The mechanisms addressed in the review are depicted on the left and invertebrate-bacteria symbioses described in the review are depicted on the right. The coloured dots below the figures of the hosts match the list of mechanisms that their respective symbionts are known to use during colonization. The symbiotic systems include hosts living in marine, terrestrial and freshwater habitats: (A) bobtail squids, (B) giant tubeworms, (C) sponges, (D) broad-headed bean bugs, (E) tsetse flies, (F) fruit flies, (G) honey bees, (H) entomopathogenic nematodes and (I) leeches.

3.2. Capture

In a second scenario in which a host-associated lifestyle does not bring a net advantage to the microbial partner, symbiosis establishment is most likely asymmetrically driven by selection on the host level, favoring the capture of symbionts. Each transmission event between host individuals is thus an opportunity for the symbiont to escape and proliferate in the environment or other hosts. Therefore, traits that facilitate release into the environment are likely under selection in such a scenario. In this case, microbial adaptations enhancing establishment are unlikely, unless host-driven evolution of strict vertical transmission occurs, which would tie the symbionts' fitness to their host's. At this point, synergistic adaptations of host and symbiont could lead to a highly integrated association as observed in many **obligate symbioses** (45). For instance, in cases where bacteria are incorporated into specialized tissues or cells, the symbionts might be alleviated from the host immune system (for examples, see references 60 and 61) **(Box 1)**. Under these conditions the host can also regulate bacterial metabolism by controlling access to substrates or coupling of metabolic pathways (25, 45, 62, 63). However, the bacteria are most often no longer capable of a free-living lifestyle, as in symbioses that are currently highly integrated. For example, *Sitophilus* cereal weevils exert a strong control over their intracellular symbiont *Sodalis pierantonius*. In this symbiosis, knocking down host genes associated with the production of antimicrobial peptides in and around the symbiotic organ results in symbionts escaping the **bacteriocytes**, i.e. the cells usually harboring the symbionts (60, 64). Thus, the symbionts break free in the absence of host control. This suggests that, in this case, the insect host and not the symbiont, warrants maintenance in the symbiotic organ and might have driven symbiont confinement in the first place. More generally, it has been argued that intracellular symbioses are most often associations with unilateral benefits that are in many cases controlled by the host (14).

Table-2. Animal-bacteria symbioses in which symbionts must re-colonize host tissues each generation, and for which bacterial molecular factors involved in colonization have been identified. Included are taxonomic affiliation of each partner, localization, short title referring to the section occurrence and associated references.

Figure panel	Host	Symbiont Group Phylum / Family	Symbiont Species	Localization	Section	References
A	*Euprymna scolopes* (squid)	Proteobacteria Vibrionaceae	*Vibrio fischeri*	Light organ	"Motility & chemotaxis", "Surface structures interacting with the host", "Adhesion", "Biofilm formation", "Secretion systems", " Induction of host morphological responses", "Stress response", "Microbial competition or facilitation", "Priority effects"	85, 100, 104, 107, 119, 131, 133, 151, 165, 184, 187, 209, 213, 250, 251
B	*Riftia pachyptila* (giant tubeworm)	Proteobacteria sulfur-oxidizing symbionts	*Candidatus* Endoriftia persephone	Trophosome	"Motility & chemotaxis"	16, 109
C	*Petrosia ficiformis* (sponge)	Cyanobacteria Synechococcaceae	*Candidatus* Synechococcus feldmannii	Bacteriocytes,	"Motility & chemotaxis", "Adhesion"	110, 155
		Cyanobacteria Synechococcaceae	*Candidatus* Synechococcus spongiarum	Extracellularly	"Motility & chemotaxis", "Adhesion"	110
D	*Riptortus pedestris* (broad-headed bugs)	Proteobacteria Burkholderiaceae	*Caballeronia insecticola*	Midgut crypts	"Motility & chemotaxis", "Surface structures interacting with the host", "Biofilm formation", "Induction of host morphological responses", "Stress response", "Microbial competition or facilitation", "Priority effects"	44, 114, 115, 119, 136, 167, 193, 194, 226
E	*Glossina* spp. (tsetse flies)	Proteobacteria Erwiniaceae	*Wigglesworthia glossinidia*	Milk glands, Intrauterine larva, Bacteriome	"Motility & chemotaxis"	122,123

		Proteobacteria Pectobacteriaceae	*Sodalis glossinidius*	Body lumen, Milk glands, Intrauterine larva, Gut	"Surface structures interacting with the host", "Secretion systems","Stress response"	21, 177–179, 199, 208
F	*Drosophila melanogaster* (fruit fly)	Proteobacteria Acetobacteraceae	*Acetobacter thailandicus*	Gut	"Motility & chemotaxis"	128
G	*Apis mellifera* (honey bee)	Proteobacteria Neisseriaceae	*Snodgrassella alvi*	Gut	"Surface structures interacting with the host", "Adhesion", "Biofilm formation", "Stress response", "Microbial competition and facilitation", "Priority effects"	84, 145, 230
H	*Heterorhabditis bacteriophora* (nematodes)	Proteobacteria Morganellaceae	*Photorhabdus*	Gut, rectal gland cells	"Surface structures interacting with the host", "Adhesion", "Biofilm formation"	138, 148, 152–154, 166
I	*Hirudo verbana* (Leech)	Bacteroidetes Rikenellaceae	*Rikenella*-like	Gut	"Microbial competition and facilitation"	169
		Proteobacteria Aeromonadaceae	*Aeromonas veronii*	Gut	"Surface structures interacting with the host", "Biofilm formation", "Secretion systems", "Microbial competition and facilitation"	139, 169, 174

3.3. Neutral start

A third case has been recently discussed, in which host-microbe symbiosis can initially evolve without specific benefits for any of the partners (65). A modelling-based analysis focusing on *Drosophila melanogaster* and its microbiota highlighted the relevance of dispersal via the host and the impact of the microbes on host habitat, which in turn influences host development. While these aspects can in fact be considered indirect benefits, this approach is nonetheless a useful standpoint to experimentally assess whether a mutualistic association including bacterial adaptations enhancing host colonization can evolve under initially neutral conditions.

A spectrum spanning the three scenarios — selection-driven transition, capture or neutral start — likely exists based on the observations on dependence and genomic architecture across symbiotic systems. However, we know little about the general benefits of colonization traits for beneficial bacteria, and even less about their costs. Our understanding of molecular mechanisms for bacterial colonization in hosts has been heavily influenced by pathogen research and has only begun to actively expand to beneficial

Box 3: Genetic manipulation to unravel symbiont colonization factors

Mutations inducing loss or gain of functions are key to identify specific genes involved in a biological process, like host colonization. Targeted (66) and random mutagenesis techniques (67) have revealed a plethora of molecular mechanisms involved in bacterial infection and pathogenesis (68 – 70). For a number of beneficial bacterial symbionts, however, the inability to cultivate these in vitro hinders genetic manipulation (71). Cultivation has been nonetheless successful for several invertebrate symbionts like *Vibrio fischeri* (72), *Aeromonas veronii* (73), *Photorhabdus luminescens* (74), *Cabelleronia insecticola* (75), *Snodgrassella alvi* (76), and *Sodalis glossinidius* (77). In these systems, a combination of targeted and/or random mutagenesis techniques are now available. For example, a versatile toolkit for genetic manipulation using broad-host-range plasmids focusing on the honey bee and bumblebee gut microbiome was recently developed (78). This includes a set of tailored constructs allowing for disruption of specific genes using CRISPR-Cas9, as well as heterologous expression of fluorescent reporters, antibiotic resistance markers, or other genes of interest in various proteobacteria. The toolkit has also helped in engineering *S. alvi* symbionts to activate the host's internal RNAi machinery and modulate host gene expression (79).

Another method widely used in studying symbiont molecular mechanisms is the generation of random mutant libraries using transposon insertions. Transposons allow for individually disrupting genes across the genome through a "cut" and "paste" mechanism, and many mutants can be generated in a single transformation reaction. Once a diverse library of mutants is available, the fitness of all mutants can be assessed simultaneously under the desired experimental conditions. Based on this approach, the composition of mutants in a library infected into a host organism will change in diversity and frequency in comparison to that grown in vitro. Additionally, screening or negative selection can be used to separate individual mutants. For example, by plating the library on low percentage agar and screening for mutants that are less motile or hypermotile, novel genes involved in bacterial motility can be discovered (80, 81). Similar approaches have been used in a

number of invertebrate symbionts to identify other phenotypes, including a study in *S. glossinidius* in tse-tse flies (82) and *Caballeronia* in *R. pedestris* bean bugs (83). The advent of next generation sequencing technologies has furthered the development of efficient approaches to track relevant genes in these random mutant libraries. In particular, the use of Transposon insertion sequencing (Tn-Seq), is valuable in assessing the changes in individual mutant frequencies by insertion-directed sequencing in a high-throughput manner. Tn-seq experiments to identify symbiont genes essential for host colonization have been performed in various beneficial bacteria, including *S. alvi* symbionts of honey bees (84) and *V. fischeri* symbionts of squids (85), as well as in associates of vertebrates (86) and plants (87 – 89). Recent advances combining droplet-based microfluidics and Tn-seq have proven useful in studies where population effects and cell-cell competition may interfere with identifying specific single-cell phenotypes under certain conditions (90). Another advancement is the use of CRISPR/Cas9 genome editing for targeting genes in non-model microbes. For example, this approach was effective in knocking out the ompA gene and also in integrating fluorescence and gentamycin resistance markers in Cedecea neteri gut symbionts of Aedes mosquitos (91). CRISPRi is an emerging method yet to be applied in beneficial symbiosis. It enables the transcription repression of target genes without modifying the target site (92, 93). In CRISPR, a guide RNA complementary to a DNA sequence points to the target where the Cas9 nuclease protein must cleave. By contrast, in CRISPRi, the Cas9 protein is catalytically deactivated (dCas9) to downregulate the expression of the target gene. Since CRISPRi is inducible, growth essential genes (94, 95) and temporally essential genes (95, 96) may be studied, which is not possible with targeted or transposon mutagenesis techniques (93, 97, 98).

associations, or bacteria shifting across the parasite-mutualist continuum (57). While the phenotypic effects of successful colonization have been intensively studied for both host and bacteria, the ecological consequences and evolutionary drivers of initiating a host-associated lifestyle in beneficial bacteria remain poorly understood. However, there is considerable work on the molecular underpinnings of symbiosis establishment in a few symbiotic model systems and growing capacities to explore similar questions in non-model organisms **(Box 3)**. As outlined in the section "Molecular mechanisms for symbiont colonization", this work is paramount to better understand the drivers and constraints of transitioning to a host-associated lifestyle.

4. MOLECULAR MECHANISMS FOR SYMBIONT COLONIZATION

4.1. Reaching the host: motility and chemotaxis

In most open symbioses, initial contact and entry into the host can depend on the ability of the microbe to sense and move towards a chemical cue (99). In fact, from land to the deep-sea waters, most described bacterial symbionts having a free-living stage retain the genes essential for motility at least until they migrate into the symbiotic structures of the host. The role of these machineries has been experimentally addressed in *Vibrio fischeri* bacteria associated with *Euprymna scolopes,* the Hawaiian bobtail squid, a key model system for the molecular crosstalk in establishment of environmentally acquired symbionts. Bioluminescent *V. fischeri* is considered a beneficial symbiont as it serves for counter-illumination — a

form of camouflage – for the host to evade predators under the moonlight (100). During the first steps of the colonization process in the squids, *V. fischeri* from the seawater aggregate in mucus secreted by the squid host. Once *V. fischeri* cells have aggregated onto the epithelial surface of the squid, they navigate along a chitobiose gradient, which is a chemoattractant established by the host, and move towards the crypts of the light organ (101 – 103) where the bioluminescent cells are finally housed **(Fig. 2A)** (104 – 106). Concordantly, mutations in the cheY and cheR genes impaired colonization of the light organs, supporting the hypothesis that chemotaxis is required for *Vibrio* to navigate to its final destination in the squid (107). Motility is also key in this stage, as *V. fischeri* mutants that are immotile or contain a disrupted putative homolog of the *V. cholerae* **flagellar** response regulator, *flrC*, cannot colonize the host (107). In other systems, mucins and glycoproteins that are constituents of host mucus, can act as chemoattractants enabling pathogens to colonize the mammalian mucus-lined intestine (108). Also similar to the *V. fischeri* symbionts, related pathogens like *Vibrio cholerae, Helicobacter pylori* and *Campylobacter jejuni* rely on motility to penetrate the mucus layer of the intestinal epithelium in mammalian hosts (108).

In symbionts of other marine invertebrates, functional genomics and comparative studies suggest that motility and chemotaxis are also important for colonization initiation. A metagenome analysis of "*Candidatus* Endoriftia persephone" endosymbionts that colonize bacteriocytes in the giant tubeworm *Riftia pachyptila* **(Fig. 2B)** revealed that the symbionts encode a wide array of chemoreception and motility genes (16, 109). In *Petrosia ficiformis* sponges, the **facultative** intracellular symbiont "*Candidatus* Synechococcus feldmannii" **(Fig. 2C)** carries a motility related pilus retraction ATPase, *pilT* gene (110). Strikingly, this gene is absent in the obligate extracellular symbiont, "*Candidatus* Synechococcus spongiarum" **(Fig. 2C)**, pointing to a different way of colonization (110). However, direct evidence for the role of motility is lacking and challenging to obtain in this and other systems that are not amenable to manipulation.

Terrestrial environments present considerable limitations to bacterial movement. Hence, on land, the first encounter of free-living symbiotic bacteria with a host might be more spatially restricted. To initiate contact in these conditions, bacteria can benefit from alternative mechanisms, such as their host's behavior and biology. This is particularly observed in microbe-insect interactions, where the symbionts can take advantage of trophallaxis, coprophagy, and egg surface smearing (35, 111). Presumably, symbionts hitchhike on these behaviors that serve nutrient exchange although the evolutionary drivers could be both nutrition and bacterial transmission. In fact, the presence of bacteria might promote these behaviors in some cases, as observed in cockroaches. Feces from artificially created axenic individuals are less attractive to conspecifics due to differences in the volatile blend usually containing aggregation pheromones. Thus, the bacteria promote this gregarious behavior and thereby increase their chances for transmission among cockroach hosts (112).

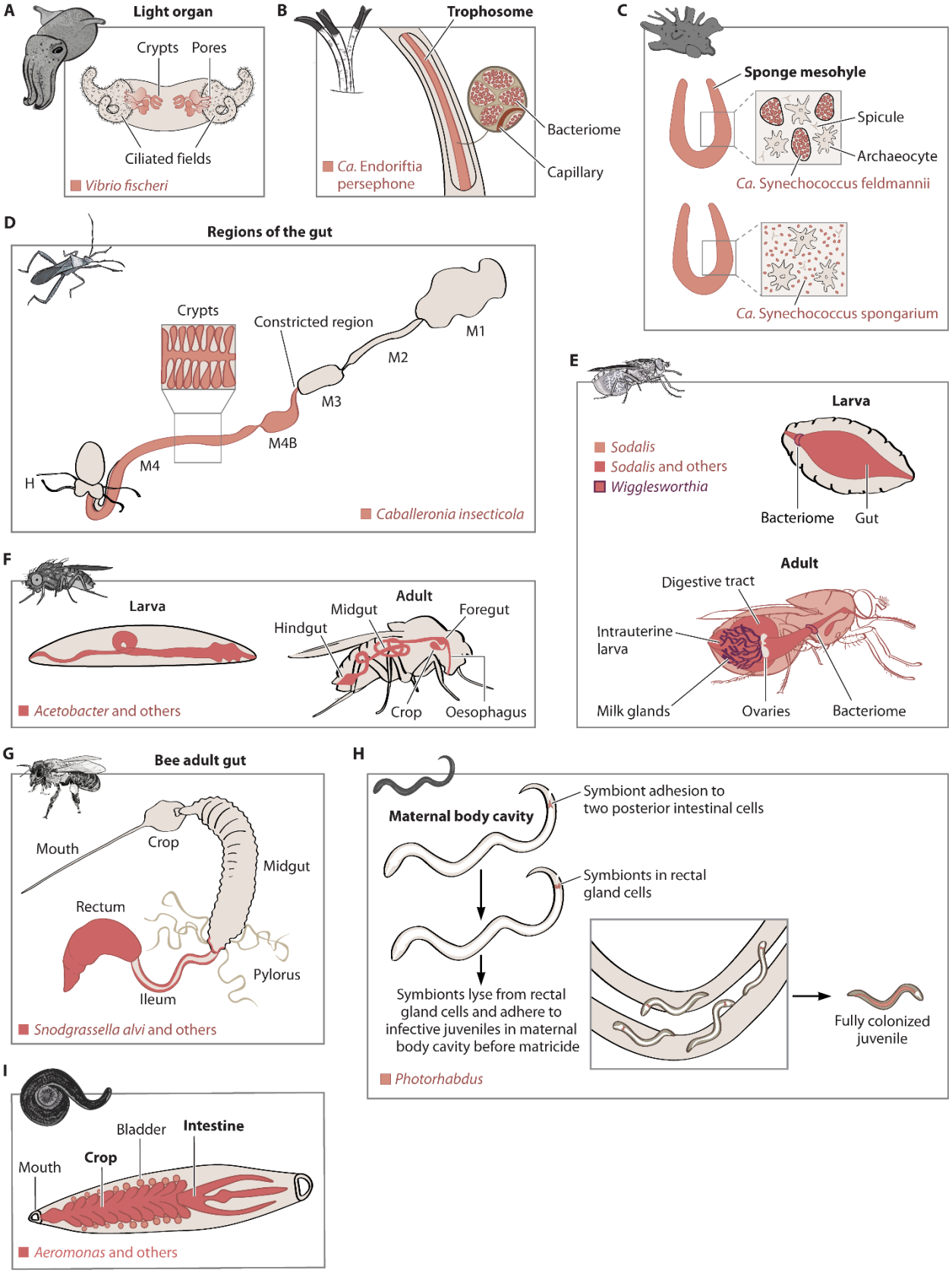

A
Light organ
Crypts
Pores
Ciliated fields
Vibrio fischeri
B
Trophosome
Bacteriome
Capillary
Ca. Endoriftia persephone
C
Sponge mesohyle
Spicule
Archaeocyte
Ca. Synechococcus feldmannii
Ca. Synechococcus spongarium
D
Regions of the gut
Crypts
Constricted region
M1
M2
M3
M4B
M4
H
Caballeronia insecticola
E
Larva
Sodalis
Sodalis and others
Wigglesworthia
Bacteriome
Gut
Adult
Digestive tract
Intrauterine larva
Milk glands
Ovaries
Bacteriome
F
Larva
Acetobacter and others
Adult
Midgut
Foregut
Hindgut
Crop
Oesophagus
G
Bee adult gut
Mouth
Crop
Midgut
Rectum
Pylorus
Ileum
Snodgrassella alvi and others
H
Maternal body cavity
Symbiont adhesion to two posterior intestinal cells
Symbionts in rectal gland cells
Symbionts lyse from rectal gland cells and adhere to infective juveniles in maternal body cavity before matricide
Fully colonized juvenile
Photorhabdus
I
Bladder
Intestine
Mouth
Crop
Aeromonas and others

Fig. 2. Localization of beneficial symbionts in the invertebrate systems discussed in the review: A) bobtail squids (based on reference 105), B) giant tubeworms (based on reference 264), C) sponges (based on references 265 and 266), D) broad-headed bean bugs (based on reference 267), E) tsetse flies (based on reference 268 and 269), F) fruit flies (based on reference 270), G) honey bees (based on reference 271), H) entomopathogenic nematodes (based on reference 152), I) leeches (based on reference 272). Red highlights indicate the specific location of a symbiont in an organ/ body of the host. Multiple symbiotic strains or species may sometimes co-localize together which is indicated in darker red.

After gaining entry, bacteria must still migrate to and/or enter the specific symbiotic organs or target niches to establish an association in terrestrial hosts. In *Riptortus pedestris* bean bugs, *Caballeronia* (previously *Burkholderia*) symbionts are orally acquired by nymphs (113) and they require functional flagellar motility to enter the M4 midgut crypts where they are housed (114, 115) **(Fig. 2D)**. Related opportunistic pathogens, like *Burkholderia cepacia and Burkholderia glumae,* also utilize flagellar motility to invade the host (65, 66), which is common across many pathogenic bacteria (118). Interestingly, ***in vitro*** assays demonstrate that the bug's *Caballeronia* symbionts have developed a specialized corkscrew-like motion (119), in which the flagellum wrapped around the cell-body thrusts the cell forward. The same study identifies a similar mechanism in a *Vibrio fischeri strain*, implying that this type of flagellar motility might also be relevant for other bacteria (119). The wrapped flagellum is not essential for penetrating the constriction region, but it is speculated that it improves the efficiency of movement through the mucus in this region (119).

While being motile can be —unsurprisingly— important for beneficial bacteria that recurrently colonize the host, it is widely observed that vertically transmitted endosymbionts lose motility genes or, like in *Buchnera aphidicola,* flagellar genes may be involved in protein transport (120, 121). There are exceptions, however, like the *Wigglesworthia* symbionts of tsetse flies. These bacteria reside intracellularly in bacteriocytes near the anterior midgut and are also found extracellularly in the milk gland lumen from where they are vertically transmitted to the intrauterine progeny via a milk gland (122) **(Fig. 2E)**. Here, expression of motility and flagellar genes is upregulated during maternal transmission and larval intrauterine development, suggesting motility as an important factor for colonization of the offspring (122, 123).

In the case of obligate, vertically transmitted endosymbionts, symbiont translocation is often achieved through transport mechanisms of the host (124, 125). Therefore, the endosymbionts might lose genes essential for motility and chemotaxis as a more consistent association with the host evolves (98). For example, free-living *Serratia symbiotica* that are transitioning from pathogenic to beneficial life-styles in aphids have lost swimming motility and chemotaxis, while retaining some genes important for interaction with the host (126). Similarly, a defensive symbiont of *Lagria villosa* beetles facing ongoing genome reduction lacks genes required for chemotaxis and flagellar motility (127). In a relatively open symbiotic association, a comparative analysis shows that flagellar motility is lost in *Acetobacter* bacteria that reside in the gut of laboratory reared *Drosophila* fruit flies **(Fig. 2F)**, while it is retained in those strains isolated from wild caught flies. This suggests that motility is not advantageous in the laboratory where strains remain in close contact with the host through several generations of lab rearing, but it may facilitate colonization and establishment in a more heterogenous natural environment (128). Loss of flagellar motility or the structural components may also be an evolutionary adaptation to avoid activation

of host innate and adaptive immune defenses since flagellins are recognized as immune activators in plants and mammalian cells (129, 130).

In summary, motility appears to be a valuable tool for beneficial bacterial colonizers both outside and inside an animal, yet can quickly become dispensable for symbionts evolving towards tighter associations with the host.

4.2. Engaging with the host

4.2.1. Surface structures interacting with the host

Bacterial surface structures are key during colonization as they mediate the cell's contact with the external environment. These structures are involved in partner recognition and signaling, symbiont immune evasion and mediating adhesion to host tissues (also see **Box 1**). Surface components like the **lipopolysaccharide (LPS)**, **tracheal cytotoxin (TCT)** from the **peptidoglycan** layer and exopolysaccharides are termed microbe-associated molecular patterns (MAMPs), as they can interact with MAMP-recognition proteins of the host (131, 132).

In the squid symbiont, *V. fischeri,* MAMPs are important for signaling bacterial presence to the squid host. Although unspecifically, bacterial surface peptidoglycans in the seawater can already trigger mucus secretion by the host before colonization (104). Also, despite being part of the cell wall peptidoglycan, TCT can be released and act at a distance. TCT triggers haemocyte infiltration into the ciliated epithelial fields and induces apoptosis of the epithelial cells that make up the fields of the squid light organ (133), leading to the restructuring of host ciliated appendages (131, 133) as also mentioned in "Making space: induction of host morphological traits relevant to colonization".

In other symbionts, surface structures and in particular LPS, are crucial for colonizers to avoid detection by the host immune components during entry. Anchored to the outer membrane of the cell, the LPS consists of three regions — lipid-A, core-oligosaccharide and O-antigen —, and acts as a protective barrier in pathogens and mutualists against a harsh external environment (134). The host immune system recognizes LPS when it is released from the outer membrane due to cell-death or removed from the outer membrane by the host Lipid-A binding protein (LBP). To avoid recognition by the host immune system, some bacteria exhibit changes in the chemical structure of the LPS, such as alterations in acylation patterns and phosphorylation of the lipid-A structure, or variations in the O-antigen polysaccharide structure (135). Phosphorylation changes the net charge exposed on the cell surface and thus affects the interaction of bacterial cells with the environment, AMPs and antibiotics (134). In the *Caballeronia* symbionts of the *Riptortus* bean bug, an intact O-antigen helps protect *Caballeronia* symbionts from cationic antimicrobial peptides of the host until the symbionts reach the symbiotic midgut region (136). It is speculated that, after colonization, the host induces loss of the O-antigen to maintain control over symbiont titers (137) implying that modification to the surface structure may be essential for symbiosis establishment. The LPS core, O-antigens, and genes involved in their assembly are also speculated to help in immune evasion or aid in colonization in other beneficial symbionts including *Snodgrasella alvi* in bees (84), *Photorhabdus* in entomopathogenic nematodes (138), *Sodalis glossinidius* in tsetse flies (20)

and *Aeromonas* in leeches (139).

4.2.2. Adhesion

Surface structures that help in attachment to the host are widely important for horizontally transmitted symbionts. This is especially true in the marine environment, where free-swimming cells face the threat of being washed away by currents, but also in the gut, where the same occurs with the passage of food by peristaltic movement (140). Therefore, adhesion mediated by **pili** or **fimbriae**, curli proteins, trimeric autotransporter adhesin (TAA), or through **biofilm** formation (see "Coping with a new environment: stress response") are important for mutualists and pathogens alike (141).

Pili or **fimbriae**, are hair-like appendages that are found on the bacterial cell surface. While these terms are often used interchangeably, there are some general distinctions: fimbriae are shorter than pili and may not be involved in transfer of DNA (142) (see the GLOSSARY). Among these structures, **Type IV pili** are especially relevant for adhesion to host cells, biofilm formation, twitching motility and protein transport. These structures have been widely studied in pathogens like *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Vibrio cholerae* and *Clostridium* sp. (143, 144). In a similar fashion, Type IV pili may aid host colonization in a number of host-beneficial bacteria of invertebrates. They are predicted to be essential for colonization by *S. alvi* symbionts in the gut of honey bee *Apis mellifera* **(Fig. 2G)** (84, 145), akin to the related pathogen *Neisseria gonorrhoeae* that also employs the Type IV pilus to adhere to the host epithelium (146). Among beneficial symbionts, the role of pili in adhesion to host tissues extends to those associated with earthworms (147), entomopathogenic nematodes (148), humans (149, 150), and sponges (110). Interestingly, in "*Ca.* Synechococcus" symbionts of sponges, the relevance of pili likely relates to the transmission route of the bacteria. Although intracellular, the facultative and presumably horizontally acquired "*Ca.* Synechococcus feldmannii" **(Fig. 2C)** retains Type IV pili genes (110). By contrast, these are absent in the congeneric "*Ca.* Synechococcus spongiarum", which is an obligate and vertically transmitted symbiont despite its extracellular location (110). It is likely that for the latter, the stable transfer over generations obviates the need for attachment mechanisms. A putative pili-encoding gene in *V. fischeri* offers a competitive advantage during cocolonization with other strains in the squid light organ, while the absence of the gene does not affect individual mutants during colonization (151). This diversity of systems underlines the relevance of pili-mediated adhesion for beneficial bacteria that must repeatedly colonize the host and thus retain this feature from free-living or pathogenic lifestyles.

In addition to Type IV pili, the role of fimbriae, chaperone-usher pili, curli fibers, and TAA for adhesion in host associated bacteria is well characterized (141). A relevant case study occurs in *Heterorhabditis bacteriophora* entomopathogenic nematodes, where the symbiont *Photorhabdus luminescens* is maternally transmitted to the developing juveniles in the mother's body cavity **(Fig. 2H)**. The symbionts use the *mad* ("maternal adhesion defective") fimbrial locus for binding to the intestine of the maternal nematode (152). Cells that can adhere and invade the adult rectal gland cells are preferentially transmitted to the offspring developing inside the body cavity (153). Notably, an invertible promoter controls an ON and OFF switch that regulates this fimbrial locus. This switch mediates the bacterial transition between a mutualistic form in the host nematode, and an insect-pathogenic form in prey insects, which is key for the entomopathogenic phase of the nematode's life cycle (154).

Other relevant adhesion factors in the context of mutualistic bacteria include Type Vc **secretion system** adhesins and eukaryote-like proteins such as fibronectin type III (FN_3), cadherin, and leucine-rich-repeat domain-containing proteins, which have been reported in bacteria from the sponge microbiome, although evidence for their role in symbiont colonization is so far indirect (110, 155). For example, FN_3 domains are enriched in sponge-associated cyanobacteria in comparison to free-living strains of the same group (110). FN_3-containing proteins are possibly involved in binding to glycoproteins and structural proteins on host cells (156). They are also known to be essential for attaching to the host epithelial cells in pathogenic bacteria like *C. jejuni* (157), as well as in probiotic *Lactobacillus* attaching to the mammalian gut (158).

Non-fimbrial or non-pili adhesins like TAA are secreted through the outer membrane in Gram-negative bacteria, specifically by the Type Vc secretion system (159). TAA are a common family of adhesion factors often associated with virulence in bacteria (160). In one of the gut symbionts of honey bees, *S. alvi*, TAA possibly aids in binding to the bee gut epithelium as predicted by a genome-wide screening analysis using a mutant library (84). TAA could be more commonly implicated in symbiont establishment, as homologous proteins have also been found in the genome of *Burkholderia* Lv-StB symbionts of *Lagria villosa* beetles (127), although the importance of TAA as colonization factors in these systems remains unexplored.

4.2.3. Biofilm formation

Adhesion often precedes the formation of a **biofilm**, an important strategy for microbial colonization on surfaces including those within a host (161). Subsistence in a biofilm can entail important benefits such as increased protection from antimicrobial substances including host immune factors **(Box 1)**, occupation of nutrient-rich areas, or facilitated cooperation (162, 163).

Both pathogenic and beneficial bacteria in a broad range of animal hosts —within and beyond invertebrates— rely on the formation of biofilms upon attachment to settle in the host tissues. In *Euprymna* squids, for instance, formation of a biofilm is crucial for the *V. fischeri* symbionts to pass through the squid ciliated epithelial area before entering the light organ pore **(Fig. 2A)**. The production of the extracellular "symbiosis polysaccharide" (Syp) by the bacteria (164), as well as several regulator molecules (85, 165) enables biofilm formation. In entomopathogenic nematodes, the formation of a biofilm allows *Photorhabdus* bacteria to establish in the posterior end of the gut before invading cells in the rectal gland epithelium (166) **(Fig. 2H)**. Similarly, there are indications that *S. alvi* gut symbionts of honey bees also form biofilms that facilitate host colonization and rely on adhesion factors for their formation (84). *Caballeronia* symbionts of the bean bug *R. pedestris* also rely on biofilm formation for proper establishment in the insect gut (167), similar to related pathogens of the genus *Burkholderia* that produce a biofilm for successful infection (168).

Polymicrobial settlement in biofilms can be another key phenomenon in the gut, or on other host surfaces. Several studies indicate that the presence of a polysaccharide matrix formed by already existing bacteria can facilitate the recruitment of new associates, even when these are themselves impaired in biofilm formation. The possibility to attach to other bacteria instead of directly to the host can thus spare the need of host-directed adhesins (161). As observed in leeches, and further discussed in "Dealing with

third parties: competition and facilitation among symbionts", the bacterial polymeric matrix might also promote important crosstalk between co-existing symbionts (169).

4.2.4. Secretion systems

Secretion systems have been widely studied in beneficial and pathogenic bacteria, and are known to enhance communication with the host or mediate inter-bacterial warfare.

The Type II secretion system has several evolutionary similarities to the Type IV pilus, flagella in archaea and competence pili in Gram-positive bacteria (170). Unlike the T3SS and T6SS, the T2SS secretes exoproteins into the extracellular environment for adhesion and biofilm formation, lysis of host tissue, or to remodel the environmental niche (171). An example for the use of T2SS by mutualistic bacteria includes *Aeromonas veronii* symbionts found in the leech crop **(Fig. 2I)**. *A. veronii* secretes haemolysin via a T2SS to lyse a part of the ingested erythrocytes in the blood meal of the leech and utilize it as a heme source. Absence of haemolysis activity in *A. veronii* renders them incapable of colonizing the leech crop. Similarly, T2SS in a related pathogen, *Aeromonas hydrophila*, is important for pathogenesis by secretion of putative virulence proteins, including hemolysin (172).

The type III secretion system (T3SS) is known in symbiosis as an important colonization determinant. Several T3SS components are similar to that of the flagellar proteins, and it spans the bacterial cell membrane. When in contact with the host cell membrane, the T3SS injects effector molecules into the eukaryotic cell (173). It helps bacteria gain entry into the host cells, and modulate signaling processes in the host, or avoid host innate immune factors. An example of a microbial symbiont that uses T3SS for immune evasion is *A. veronii.* There, the T3SS helps avoid phagocytosis by leech hemocytes circulating in the intraluminal fluid of the crop, and is additionally involved in pathogenesis in mammalian hosts, showing that T3SS has a dual role as a colonization factor and a virulence factor in different hosts (174). Among the *Aeromonadaceae*, apart from the beneficial symbiont of leeches, pathogens such as *Aeromonas salmonicida* and *A. hydrophila* carry a T3SS (175). The use of T3SS for modulation of the host immune response is also observed in systems involving taxonomically distant hosts, like leguminous plants. There, nitrogen fixing rhizobia use the T3SS to inject Nops (Nodulation outer protein) effectors to suppress host immune responses, and establish in the root nodules of host plants. However, in incompatible rhizobia – legume combinations, Nops effectors can also affect nodulation negatively and prevent infection, showing their role in specific colonization and their similarity to plant pathogenic effectors (176). Other bacteria are capable of inducing changes to the host-cytoskeleton and interrupting host signaling processes for immune suppression using the T3SS, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Yersinia pestis* (173, 177).

S. glossinidius symbionts of tsete flies use the T3SS machinery to gain entry into host cells. There, the T3SS regulated by the PhoP-PhoQ two component system affects the bacteria's ability to colonize the host fly (178, 179). Pathogens like *Salmonella, Shigella,* and *Chlamydia* (173) employ a similar factor as the *S. glossinidius* symbiont of tsetse flies in establishing an intracellular lifestyle using the T3SS. Interestingly, *S. glossinidius* has only recently transitioned to an endosymbiotic lifestyle in the tsetse flies, and it retains a T3SS machinery that shares a common ancestry with *Salmonella enterica* and *Shigella flexneri* invasins. This machinery now facilitates the symbiont's vertical transmission from the

hemolymph to the intrauterine progeny (82). The T3SS also seems to be a conserved feature in a close relative of *S. glossinidius, S. pierantonius*. This intracellular symbiont of cereal weevils uses the T3SS for translocation during host metamorphosis. The endosymbiont undergoes a transient extracellular phase as a few cells migrate from larval bacteriocytes to stem cells that are precursors of adult bacteriocytes (61).

Other than the T3SS, in the squid symbiont, *V. fischeri* the two-component secretion system, TamAB, is likely to be involved in host colonization (85). Furthermore, as addressed in detail in "Dealing with third parties: competition and facilitation among symbionts", the T6SS is also now recognized as an important machinery for animal-associated microbes. It mediates interactions with both the host and cocolonizers in mutualistic and pathogenic bacteria (180).

4.3 Making space: induction of host morphological traits relevant to colonization

In many symbioses, spatial confinement or compartmentalization of the microbial partners in host tissues or organs is important to isolate and regulate the symbionts, maintain partner fidelity, and/or avoid direct conflict between different symbionts in a single host (28, 181). In some hosts, the formation of symbiont-housing organs is hardwired, potentially due to coadaptation during the evolution of symbiosis, and does not require cross-talk with the symbionts to trigger its development (for examples, see references 182 and 183). However, in other cases such morphological alterations are induced in the presence of a specific symbiont enabling successful establishment. Over two decades ago, it was shown that *V. fischeri* symbionts trigger the postembryonic development of the light organ, where the bacteria will later reside (184) **(Fig. 2C)**. The colonization of the crypts induces major changes in the light organ tissues, including swelling of the epithelial cells, increased microvillar density and later apoptosis and regression of the ciliated field where symbionts are recruited. This process begins within hours after first contact between host and bacteria, and is orchestrated through an impressive molecular dialogue between symbiont and host, which has been characterized in detail (105, 185 – 187). During this process, actin rearrangements trigger the light organ formation in squids by *V. fischeri* (185, 187). Similarly, some pathogens of mammals (188, 189) or plants (190, 191) also target actin in the host cell cytoskeleton by secreting proteases and other effectors that manipulate the cellular framework, promoting invasion and dissemination (192). While defined and in some cases highly specialized symbiont-housing organs are present in many other animal and plant hosts (181), the role of symbiont molecular factors that might induce their formation is known for only a few model systems.

Symbiont-induced morphogenetic changes can also enhance partner specificity during colonization. In the bean bug *R. pedestris*, the passage of *Caballeronia* symbionts or related strains through a constricted region leading to the crypt-bearing section of the midgut provokes its closure **(Fig. 2D)**. This blocks the subsequent entry of nonspecific bacteria and thus reinforces partner choice during colonization (193, 194). As noted above, a similar finding was reported in *Euprymna* squids, where *V. fischeri* triggers the constriction of a passage through which symbionts must navigate to reach the crypts. As in the bugs, this bacteria-induced mechanism is relevant for temporal and spatial regulation of colonization, in addition to increased specificity and compartmentalization of the symbionts (185).

4.4. Coping with a new environment: stress response

Even though the host might offer a stable environment for some symbionts, colonizing bacterial partners can experience extreme and/or fluctuating conditions in terms of temperature, pH, oxygen concentration, nutrient supply, and oxidative stress. Bacteria can mitigate stress with the help of chaperones (195, 196). In intracellular symbionts, genes encoding chaperones are commonly the highest expressed genes (197), which indicates that symbionts experience a stressful environment. However, extracellular symbionts that experience some degree of genome erosion and related suboptimal codon usage seem to require increased assistance of protein refolding via chaperones (198). For example, the tsetse fly symbiont *S. glossinidius* must react to rapid fluctuations in temperature due the intake of warm blood by the fly, which causes thermal stress. As a response to elevated temperatures the symbiont upregulates the expression of the chaperones *dnaK*, *dnaJ*, and *grpE* (199), which are known to aid bacterial cells to cope with raised temperatures (200) by stabilization and refolding of denatured proteins (201).

A common threat to bacteria residing within host tissues are reactive oxygen species (ROS) that can originate from the environment, the host, or the bacteria themselves (202). Highly reactive molecules such as hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet 2}$), and hydroxyl radicals ($OH^{\bullet}$) have a dramatic effect on the structure and activity of proteins, DNA, and membrane lipids (203). Consequently, most organisms utilize enzymes to transform ROS into a nonharmful state and repair cellular damage (204–206). For the tsetse fly symbiont, the high temperatures related to blood intake could additionally result in such oxidative stress (207), to which *S. glossinidius* reacts with the upregulation of genes involved in the breakdown of ROS, repair of oxidative damage, transport of iron and manganese, and protein refolding (208), under the control of the regulatory N-(3-oxohexanoyl) homoserine lactone (OHHL).

In a second example, the Hawaiian bobtail squid *E. scolopes* regulates bacterial colonization by releasing nitric oxide synthase (NOS) and its product, nitric oxide (NO), at the epithelia of the superficial ciliated fields, ducts, and crypt antechambers (209). Furthermore, it provides aggregation mucus with vesicles containing NO and NOS to limit the number of bacteria (209). *V. fischeri* uses the flavohemoglobin Hmp for protection against the inhibition of aerobic respiration caused by NO (210). In the absence of NO, Hmp is expressed at a low level but is upregulated up to 120-fold in its presence (211). Flavorubredoxin produced by *V. fischeri* can also combat NO, albeit to a lesser extent and only under anaerobic conditions (210). Additionally, *V. fischeri* detoxifies ROS-damaged peroxidized membrane lipids by upregulating multiple genes during colonization stages inside the host (212). Once inside the light organ crypts, high expression of multidrug efflux pump genes suggests that the symbiont might actively expel antimicrobial compounds present in the crypts, a process that is probably coordinated by quorum sensing (212, 213). The aforementioned NO also plays a role during colonization in other symbiotic systems, e.g., in the legume-rhizobium symbiosis (214) and possibly in the beewolf-*Streptomyces* symbiosis (215). This suggests that withstanding oxidative stress is likely important for establishment in several host-associated microbes.

S. alvi bacteria in the honey bee gut must also cope with various environmental challenges. Powell et al. (84) deduced that during the first 5 days of colonization, multiple stress response mechanisms are likely important and speculate on candidate genes that might enable these mechanisms based on infections

with a random mutant library. This includes genes involved in modifying rRNA and tRNA, which could improve translation efficiency and fidelity and thereby mitigate nutritional and temperature stress (84). Furthermore, the expression of different enzymes responsible for amino acid synthesis and amino acid transporters can potentially be adjusted to help safeguard cell survival during nutrient limitation, a strategy previously shown in *E. coli* (216, 217). Additionally, factors involved in protein recycling and stabilization might be essential to ensure protein quality. *S. alvi* might also counteract ROS-inflicted DNA damage and repair DNA breaks by expressing a pathway that includes genes for the SOS response, recombinational repair, D-loop extension, resolution of Holliday junctions, and postrepair chromosomal separation. Furthermore, the antioxidant glutathione might play an important role in gut colonization, as suggested by the expression of genes for its synthesis and activity (84, 218).

Cell wall integrity can also play an important role in stress tolerance during establishment in the host. In *S. alvi*, outer membrane stress appears to be mitigated by the regulation of LPS synthesis and export (137). In the case of *Caballeronia* symbionts of *Riptortus* bean bugs, the deletion of a crucial gene for cell wall biosynthesis (*uppP*) results in an altered peptidoglycan structure, a higher sensitivity to lysozyme activity, and environmental stressors *in vitro* and failed initiation of symbiosis *in vivo* (219). This implies that the absence of the gene may expose the cells to bactericidal agents in the gut, although direct evidence is lacking (219). However, other examples of cell wall integrity changes related to stress in beneficial bacteria while colonizing extracellularly are to our knowledge lacking. In highly integrated intracellular symbionts of some insects, the host has horizontally acquired bacterial genes for cell wall metabolism and thereby gains increased control over the host-microbe interaction (for examples, see references 220 and 221).

5. MULTIPARTITE MICROBIAL INTERACTIONS AND THEIR ROLE IN COLONIZATION

5.1. Dealing with third parties: competition and facilitation among symbionts

Besides the interaction with the host, colonization success and its specificity also depend on the interplay between bacterial competitors. Inter- or intraspecific interactions between bacterial colonizers can be facilitating, when one individual positively influences another directly, for example, by supplying nutrients or collective protection in a shared biofilm (222) or indirectly by modifying hosts resources, behavior, or immune responses (223). Alternatively, antagonistic interactions can involve exploitative competition through higher growth rate, enhanced motility or resource utilization, or interference competition in the form of antibiotic production, signaling disruption, or predation (224, 225). Importantly, as with host-microbe interactions, the outcome of an interaction between bacterial players can be context dependent and vary along a continuum from mutualism to antagonism (10).

Work in the *Riptortus-Caballeronia* symbiosis directly demonstrates how competition can play an important role in preventing other bacteria from colonizing and increasing specificity in the association (44, 226). While some closely related *Pandoraea* bacteria are able to bypass the selective machinery imposed by the host, these bacteria are quickly outcompeted by the *Caballeronia* symbiont in the M4 gut upon coinfection (226). While the specific mechanisms are unclear, Itoh et al. (226) speculate that the

host environment is tailored toward housing its beneficial *Caballeronia* symbiont, providing it with a competitive advantage over other microbes.

In other cases, molecular mechanisms mediating interference competition by co-colonizing bacteria have been identified. For example, effector proteins associated with the T6SS are known to facilitate antagonistic interactions between bacterial strains in some symbiotic systems (227). The T6SS is a contractile apparatus built by a sheath tube of protein subunits (TssB and TssC), within which are stacks of hemolysin coregulated protein (Hcp) that help to transport effector molecules. At the tip, the Valine-glycine repeat protein G (VgrG) syringe serves to puncture bacterial competitors and deliver effectors (227). Direct competition between coinfecting bacteria influenced by the T6SS has been described at the molecular level in the squid-*Vibrio* symbiosis. Here, multiple strains compete over establishing a symbiosis within the crypts of the nascent light organ. At least three strains utilize a T6SS to eliminate competitors (228). This is promoted by upregulating the expression of Hcp and controlled by the alternative sigma factor *s* 54 and the bacterial enhancer binding protein VasH (229). In bees, *S. alvi* and coinfecting *Gilliamella apicola* symbionts conceivably engage in inter-bacterial competition during colonization, as suggested by upregulation of genes coding for a T6SS and various recombination hot spot (Rhs) toxins with antimicrobial activity (84, 230). However, other *S. alvi* and *G. apicola* strains lack the T6SS or Rhs genes, indicating that interbacterial interactions of core symbionts in the bee gut may also involve alternate mechanisms. The T6SS machinery also interacts with the host and cocolonizing bacteria in pathogenic bacteria. Pathogenic relatives of beneficial symbionts, such as *Burkholderia thailandensis*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*, target host macrophages during infection and/or mediate bacterial warfare using different classes of T6SS (180).

Bacterial competition also operates as a screening process during symbiont colonization. Such a scenario has been modeled (231) and experimentally supported (232) for the defensive symbionts of attine ants. In this system, antibiotic-producing actinomycete bacteria are hosted on the insect cuticle and protect the ants' fungus garden from parasites (233).

While the beneficial actinobacterial symbionts can be vertically transmitted, this mixed symbiosis allows for the entry of multiple other bacterial strains. Nonetheless, rich host-provided resources may fuel interference competition, and the priority effects of vertical transmission also play an important role, as discussed in the following section: "Impact of priority effects on symbiont colonization". The antibiotic producers thereby likely prevent nonproducers from successful colonization (232). However, other antibiotic producers can be picked up horizontally and subsequently benefit the host by adding their metabolites to the antibiotic cocktail. This shows that at least for antibiotic-based defensive symbioses, competition not only drives community assembly but also may screen for beneficial symbionts.

In contrast to the antagonistic scenarios described above, facilitation aids cocolonization of *A. veronii* and a *Rikenella*-like bacterium in the digestive tract of the medical leech *Hirudo verbana* (169) **(Fig. 2I)**. Mixed microcolonies of the two bacteria are covered by a polysaccharide matrix that provides an oxygen-depleted environment for the obligate anaerobe *Rikenella*-like bacterium. In addition, the matrix promotes nutrient transfer between the two cocolonizing bacteria (234, 235). Thus, both facilitative and

competitive interactions can shape the assembly, composition, and specificity of host-associated microbial communities.

5.2. The impact of priority effects on symbiont colonization

While competition can heavily influence colonization success, other factors like the order and timing of strain arrival, or priority effects, are also relevant for open and mixed symbioses. The recurring entry of microbes might make hosts more susceptible to the entry of various nonsymbiotic or harmful microorganisms, demanding regulation mechanisms that favor a stable symbiosis. In this context, the temporal aspect of symbiont colonization can be decisive for the structure and function of the microbiota, as is true for biological communities in general (236), and can have important ecological and evolutionary consequences (231).

Microbes colonizing specialized glands or crypts are likely to experience such impacts intensively, since priority effects are usually stronger in environments that promote rapid changes in the size or structure of a local population, i.e., fast local population dynamics (236). How does that pertain to symbiotic organs? High growth rates in relation to immigration rate are usually tied to fast local population dynamics, as likely occurs in partially confined symbiotic organs. Several factors relevant in comparable scenarios might affect dynamics in symbiotic organs. First, stable conditions can promote fast growth (237). Second, the carrying capacity is reached more quickly because of the small habitat size in comparison to alternative environments (238, 239). Third, in systems in which symbionts are acquired during a specific host life stage or time point, immigration is usually bounded. Also, priority effects might be especially influential during symbiont colonization due to the capability for fast adaptation to local conditions after arrival (240). Experiments testing sequential infection of *Hydra vulgaris* by two types of the same bacterial symbiont, distinguished by fluorescent labeling, reveal a clear impact of priority effects (241). This study demonstrates that provided a sufficient time lag in colonization, a secondary colonizer will be at a disadvantage, given that the primary colonizer is already closer to reaching the total carrying capacity. Other studies have put forward specific mechanisms underlying such effects in the case of unrelated colonizers. In the human gut, for example, priority effects during community assembly in early life are thought to have long-lasting consequences (242). An early entry of *E. coli* can deplete oxygen levels, hindering colonization by facultative aerobes and facilitating colonization of obligate anaerobes such as *Bacteroides* spp. (243). In newborns, *Bifidobacterium* spp. deplete breast milk oligosaccharides and thereby limit the colonization of other species requiring these carbon sources (244). Similar effects might hamper the establishment of pathogenic bacteria, as suggested by manipulative experiments in model animals. Work by Litvak et al. (245) in mice and chicks suggests that the combined effects of carbohydrate breakdown by *Clostridia*—which stimulates oxygen consumption in the gut epithelium—and oxygen consumption by gut-associated *Enterobacteriaceae* result in colonization resistance against the pathogen *Salmonella enteritidis*. Also in mice, gut-associated *Clostridia* and *Erysipelotrichia* deplete sugar alcohols, which are required for the colonization of some opportunistic enteric pathogens (246).

Priority effects can impact symbiotic communities at the species or strain level and should be especially common among symbiont strains with a high phenotypic overlap. This is particularly relevant if recurrent exposure to a free-living phase prevents sympatric evolution and thereby niche differentiation (247). Notably, priority effects at the strain level might be often overlooked. In honey bees, assessment of the gut bacterial community composition using the customary 97% similarity threshold in the 16S rRNA gene shows an apparently high consistency across bees. However, a more rigorous assessment of the strain-level composition revealed that each bee carries a single strain of the core bacterial taxa but that there is a high variation in strain identity across individuals. Strikingly, most of the strains were found to be both dominant and rare in the guts of different bees from the same apiary (248). This suggests that priority effects play an important role in this system, although the mechanisms underlying the dominance of single strains in the honey bee microbiota are not fully understood. Along similar lines, controlled experiments in mice show that higher taxonomic levels remained unchanged independent of arrival order, but specific bacterial types were overor underrepresented depending on the timing of host exposure (249). In *Euprymna* squids, the earlier arrival of so-called dominant beneficial strains can inhibit the entry of other strains. However, secondary colonization by a dominant strain occasionally persists (250). Therefore, other factors in addition to priority effects are likely to play a role in determining strain dominance in this system. These might be genetically encoded in *V. fischeri* strains, yet none of the candidate genes have been conclusively linked to colonization dominance so far (186, 251). In summary, priority effects may have a strong impact on establishment success and specificity in open and mixed symbioses and should be considered when investigating the dynamics and molecular mechanisms underlying host colonization by multipartite microbial communities.

6. CONCLUDING REMARKS AND OUTLOOK

There is now a wealth of knowledge on the identity of beneficial symbiotic bacteria and their fitness impact on the host. However, the field has yet to catch up on understanding key factors that drive the establishment of these associations, especially from the perspective of the microbe. By spanning a number of symbionts from invertebrate hosts, we aimed to provide an overview of the known strategies used by beneficial bacteria to colonize invertebrates **(Fig. 1)**. While doing so, parallels between the bacterial factors regulating the establishment of beneficial and pathogenic infections in animal hosts become readily apparent. The examples of related bacteria that engage in these two different lifestyles using similar strategies for colonization are useful for comparative studies investigating shifts across the mutualism-parasitism continuum and their mechanistic basis.

There are yet a few challenges and outstanding questions in the field that merit attention. First, the difficulty in culturing many host-associated bacteria acts as a barrier in advancing molecular symbiosis studies. In some symbioses, multiple symbiotic species or strains coexist in the host and probably mediate interbacterial cross talk, and studies with singular symbiont taxa and host may not represent the true nature of these associations or the factors involved in their establishment (248, 252). Second, being able to separate and genetically modify both the symbiont(s) and the host to experimentally determine the mediators of colonization and establishment has so far been possible in only a handful of systems (57, 71). Symbioses that enable us to cross this barrier are those in which host-symbiont integration is

mild or the microbial partner retains its free-living abilities. Due to the limited systems that can be studied, there is a potential bias. Already known molecular factors like flagellar genes, modifications to the LPS, type IV pilus adhesion factors, and genes related to biofilm formation arise in many host-associated bacteria as important colonization features. Therefore, we risk overlooking other relevant factors in beneficial bacteria that may sometimes be particular to a group of microbes or hosts. The *syp* locus in *V. fischeri* and the *mad* fimbrial locus in *Photorhabdus* are examples of such factors unique to a system, and there is likely more to be explored in other beneficial symbionts. Another promising aspect for future research is the role of phages aiding in colonization through genetic innovation and dynamic transfer between strains, as well as their impact on the establishment of microbial communities by providing competitive advantages to some bacteria while hindering others (for examples, see reference 253). So far, a few recent reports discuss the effects of phages in animal microbiomes, including those of bees (254), aphids (255), corals (256), sponges (257, 258), and avian and mammalian pathogens (253). While prophages are increasingly recognized as relevant players in host-associated microbiomes, they remain an underexplored aspect of microbial evolution in the context of animal colonization.

The expanding availability of sequencing and imaging tools to study complex microbial communities, including nonculturable symbionts, will certainly be fundamental to the further exploration of the diversity of microbial molecular factors involved in animal colonization. For most microbes that still experience both free-living and host-associated conditions, a direct comparison of the implications of each lifestyle and the knowledge of the drivers to transition between these are lacking. Experimental evolution and modeling are valuable tools to address this knowledge gap (15, 259) and have been used to evaluate associated questions, such as host fitness consequences of horizontal versus vertical transmission (260 – 262). Monitoring systems that allow us to track symbionts in their free-living state and quantify fitness have also been proposed as a promising approach (263). Importantly, understudied symbioses need to be transformed into experimentally tractable systems, with targeted or genome-wide genetic manipulation representing particularly promising approaches **(Box 3)**. Thereby, we are likely to derive detailed and generalizable insights into the microbial adaptations for establishing as beneficial or pathogenic symbionts in animal hosts.

7. GLOSSARY

Bacteriocyte/ bacteriome: Bacteriocytes are animal cells that are specialized to house endosymbiotic microbes. Bacteriomes are organs formed by bacteriocytes.

Biofilm: Assemblage of microbial cells attached to a surface and embedded in a self-produced polymer matrix predominantly composed of extracellular polysaccharides.

Closed symbioses: those involving strict vertical transmission of microbial symbionts, often via a transovarial route.

Facultative symbiosis: One or both of the partners involved in symbiosis can live independently without a drastic effect to fitness, or only a context-dependent impact on survival.

Fimbriae: The term is often interchangeable with pili for historical reasons (94). They are hair-like structures mediating adhesion and biofilm formation, and are usually distinguished from pili for being shorter and not directly involved in DNA transfer.

Flagella: Filamentous structures found in some microorganisms that help in locomotion.

In vivo: an experiment (or process) carried out in a living organism, in this case in an animal host.

In vitro: an experiment (or process) performed in laboratory culture conditions, not in the animal host.

Lipopolysaccharide (LPS): Outer membrane components of gram-negative bacteria that induce an immune response in eukaryotic hosts. They are composed of 1. O-antigen, 2. Core Oligosaccharide and 3. Lipid A components.

Local population: a set of individuals within a delimited area smaller than the geographic range of the species, which is often within a population or is a disconnected population.

Mixed symbioses: those involving both vertical and horizontal symbiont transmission.

Obligate symbiosis: One or both the organisms involved in symbiosis cannot survive without the other.

Open symbioses: those in which microbial symbionts are acquired horizontally, i.e. from the environment or unrelated hosts.

Peptidoglycan: Polymer forming a layer in the outer surface of bacteria, which supports cellular integrity. Gram-positive bacteria have a thicker peptidoglycan layer compared to Gram-negatives.

Pili: Tubular appendages found on the surface of many Gram-negative and Gram-positive bacteria that are important for adhesion, twitching motility, transfer of DNA and protein secretion. They are formed by pilin protein subunits.

Population dynamics: how and why populations change in size and structure over time. Key factors affecting these changes are reproduction, death and migration rates.

Secretion system: Complex protein structure found embedded in the external membrane of Gram-negative and Gram-positive bacteria. They help in the transport of substances from the cytosol to the extracellular environment or the delivery of effector molecules to other organisms.

Sympatry: the condition in which species or populations share the same habitat or geographical range. In the context of this review, it refers to microbial strains or species persistently sharing the same host tissue or symbiotic organ.

Tracheal Cytotoxin (TCT): Soluble peptidoglycan components in the cell wall of gram negative bacteria comprised of a disaccharide and a peptide chain. TCTs released by some pathogens cause damage to ciliated epithelial tissue and hinder the removal of foreign microbes and mucus from the tissue surface.

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CHAPTER – 3

Colonization dynamics of a defensive insect ectosymbiont

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Ramya Ganesan [1, 2], Rebekka S. Janke [1, 3], Martin Kaltenpoth [1, 2], Laura V. Flórez [1, 4]

[1] Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg University, 55128 Mainz, Germany

[2] Department of Insect Symbiosis, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany

[3] Department of Symbiosis, Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany

[4] Department for Plant and Environmental Sciences, Section for Organismal Biology, University of Copenhagen, 1871 Frederiksberg C, Denmark

CONTRIBUTIONS

R.G.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualization, writing—original draft, writing—review and editing. R.S.J.: conceptualization, investigation, methodology, visualization, writing—review and editing. M.K.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—review and editing. L.V.F.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

1. ABSTRACT

Beneficial symbionts are horizontally or vertically transmitted to offspring, relying on host- or microbe-mediated mechanisms for colonisation. While multiple studies on symbionts transmitted internally or by feeding highlight host adaptations and dynamics of symbiont colonisation, less is known for beneficial microbes colonising host external surfaces, such as the insect cuticle. Here, we investigate the colonisation dynamics of a bacterial symbiont that protects eggs and larvae of Lagria villosa beetles against pathogens. After maternal application to the egg surface, symbionts colonise specialized cuticular invaginations on the dorsal surface of larvae. We assessed the colonisation time point and investigated the involvement of the host during this process. Symbionts remain on the egg surface before hatching, providing protection. Immediately after hatching, cells from the egg surface colonise the larvae and horizontal acquisition can occur, yet efficiency decreases with increasing larval age. Additionally, passive or host-aided translocation likely supports colonisation of the larval symbiotic organs. This may be especially important for the dominant non-motile symbiont strain, while motility of additional strains in the symbiont community might also play a role. Our findings provide insights into colonisation dynamics of cuticle-associated defensive symbionts and suggest alternate or complementary strategies used by different strains for colonisation.

2. BACKGROUND

Transmission of microbial symbionts ensures that symbiont-derived benefits such as provision of nutrients, protection against pathogens or breakdown of harmful chemicals are sustained through generations (1,2). Transmission may be horizontal from the environment or unrelated hosts, vertical from parent to offspring, or a mixture of both (1). In insects, some symbionts are localized intracellularly and are vertically transmitted through the germline (3–5). By contrast, most extracellular symbionts associated with insects experience a phase of environmental exposure during transmission and are acquired after egg hatching, e.g. by probing the symbiont-contaminated egg surface or specialized symbiont-containing secretions (2). In some cases, the host provides the extracellular symbionts with protection from the environment during transmission (2,6–8). Other extracellular symbionts are horizontally acquired from the environment each generation (9), and many of these symbionts retain machineries like motility and chemotaxis to enter the host and reach its symbiotic organs (10,11). Therefore, host and symbiont adaptations can both contribute to successful colonisation and establishment (12).

During transmission, the timing of symbiont entry has important implications for the establishment and the evolutionary trajectory of the symbiosis. The timing of colonisation can enhance specificity (13) or synchronize host development with the initiation of symbiosis (14). Ectosymbionts of beewolves (15) and fungus-growing ants (16) are transmitted via maternal provisions or nestmates, respectively, and a colonisation window restricts transmission of *Pseudonocardia* symbionts in the fungus-growing ants (16). However, how precisely the bacteria invade the cuticular structures of adult beewolves and fungus-growing ants has not been described. Similarly, *Lagria villosa* larvae (Coleoptera, Tenebrionidae) carry several strains of *Burkholderia* bacteria in three specialized cuticular invaginations on the dorsal surface

of the body (17–20). However, the process by which symbionts colonise the cuticular structures of the beetles from the egg surface has not been investigated.

As adults, female *L. villosa* beetles host defensive bacterial symbionts in accessory glands associated to the reproductive system (17). About two million *Burkholderia* cells are smeared onto the egg surface during oviposition, where they produce bioactive secondary metabolites that protect the developing embryo and the larvae from fungal infection (17–19). Among the different *Burkholderia* strains found in these beetles (17,21), *Burkholderia* Lv-StB, henceforth "Lv-StB", is the most abundant and prevalent strain across individuals which can produce the antifungal polyketide lagriamide (18). Yet, Lv-StB remains uncultivated *in vitro* and has a reduced genome in comparison to its close relatives (22). It is presumably restricted in motility, as functional genes for flagellar biosynthesis are absent from the genome (22). The closely related strain, *Burkholderia gladioli* Lv-StA, henceforth called "Lv-StA", was isolated from *L. villosa* and is capable of producing a range of bioactive compounds that protect the insect host and is motile. However, Lv-StA is only sporadically present in the beetles (17–19). In the congeneric species *Lagria hirta*, it was proposed that the symbionts enter the egg shortly before hatching (23) and colonise the dorsal structures in the embryo as part of the vertical transmission route. However, direct evidence for this route is lacking. Notably, the dorsal structures in *L. villosa* larvae and pupae remain open to the outside (19,20) and the larvae are also capable of acquiring Lv-StA from the environment and successfully transfer them to the adult female glands during metamorphosis (24).

Here, we carried out manipulative assays using the culturable strain, Lv-StA (17), to determine the timing of symbiont entry into the dorsal structures and investigate the efficiency of symbiont acquisition during different time points in early *L. villosa* larvae. To better understand the colonisation mechanism of the cuticle-associated symbionts, we additionally tested host involvement in the process by mimicking acquisition with fluorescent beads.

3. METHODS

3.1. Insect collecting and rearing

L. villosa beetles were collected in soybean plantations within the state of São Paulo, Brazil (2019, 2020). Adults were fed soybean, lettuce, and kidney bean leaves and kept under a 16/8 h day/night regime at 26-28°C and 55-60% humidity. Water was provided in centrifuge tubes with a cotton plug.

3.2. Preparing B. gladioli Lv-StA culture for infection

Lv-StA was grown in King's B (KB) media (soybean peptone 20g/L, K_2HPO_4 1.5 g/L, $MgSo_4.7H_2O$ 1.5 g/L; agar 15 g/L for solid media) and incubated at 30°C, 300 rpm for liquid cultures. Bacterial cells were centrifuged at 10,000 rpm for 6 min, and the pellet was washed twice with 1 ml 1x PBS each. The final pellet was resuspended in 500-1000 µl of 1x PBS. The cell concentration was determined using a Neubauer cell counting chamber and adjusted to 2×10^6 cells/µl in 1x PBS before infecting 2.5 µl of the suspension per egg or larva.

3.3. Estimating the bacterial colonization time point

Lv-StA was inoculated on half of a surface-sterilized, freshly laid egg clutch containing approximately 200-300 eggs (day 0). Egg sterilization was performed as previously described (17). The other half of the eggs was not infected and used as aposymbiotic control. Six individual mid-time eggs (day 4 after oviposition), late eggs (day 5), first-instar larvae (day 6) and second-instar larvae (day 7) were collected from three replicate clutches. To quantify the cells that entered the eggs or the larval cuticular structures, individuals were surface washed with 100 µl 1% SDS (thrice) and 1x PBS (twice) to ensure that cells that had not colonised were washed off. Individuals were crushed in 100 µl 1x PBS and diluted (1:10 and 1:100) before plating on KB agar plates. Lv-StA Colony Forming Units (CFUs) per individual were counted after 24-hours.

3.4. Comparing colonisation efficiency across time points

Freshly laid eggs from five replicate clutches were surface sterilized and split into four groups. Lv-StA cells were infected on eggs on day 4 (group 1), first-instar larvae on day 5 (group 2), or second-instar larvae on days 6 (group 3) or 7 (group 4). Larvae were collected from the infected groups 24-hours post-infection, embedded in 1% agar and stored in 4% formaldehyde for 3-4 months at 4°C. After histological sectioning as previously described (25) we performed fluorescence *in situ* hybridization (FISH).

3.5. Fluorescence in situ hybridization (FISH)

Using FISH (25), we assessed the presence of Lv-StA in the dorsal structures of first and second-instar larvae. Semi-thin sections (8 µm) of larvae were hybridized with fluorescently labelled probes **(Supp. Table-2)** and imaged using an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany) or a Leica DMi8 imager (Leica Microsystems, Wetzlar, Germany). We did a single-blinded assessment of microscopy images to compare Lv-StA colonisation efficiency before and after hatching. At least three clearly identifiable cells in the dorsal structures were counted as symbiont presence.

3.6. Simulated symbiont transmission from eggs to larvae using fluorescent beads

Individual *L. villosa* eggs were exposed to 2.5µl (10^6 beads/µL) of fluorescent microparticles (Sigma Aldrich, latex beads, amine-modified polystyrene, fluorescent red, 1.0 µm mean particle size). After hatching, six first-instar larvae were either imaged alive as whole mount (four individuals), after freezing at –20 °C, or embedded in 1 % agar (two individuals) and fixed in 4% formaldehyde for histological sectioning as described previously (25). Localization of the beads on eggs and larvae was assessed using epifluorescence microscopy.

3.7. Data availability

The data are provided in the supplementary section.

4. RESULTS AND DISCUSSION

4.1. When do symbionts enter the larval dorsal structures?

Female *L. villosa* adults vertically transmit on average 2×10^6 symbiont cells onto the egg surface (17), where they remain over a period of four days until hatching. In larvae, the symbionts are housed in three invaginations of the dorsal cuticle (17–20). After transmission onto the egg surface, it is possible that a) symbionts enter the late egg to colonise the dorsal structures of the embryo as described for the related species *L. hirta* (23), or b) larvae acquire the symbionts from the egg surface during or after hatching. To

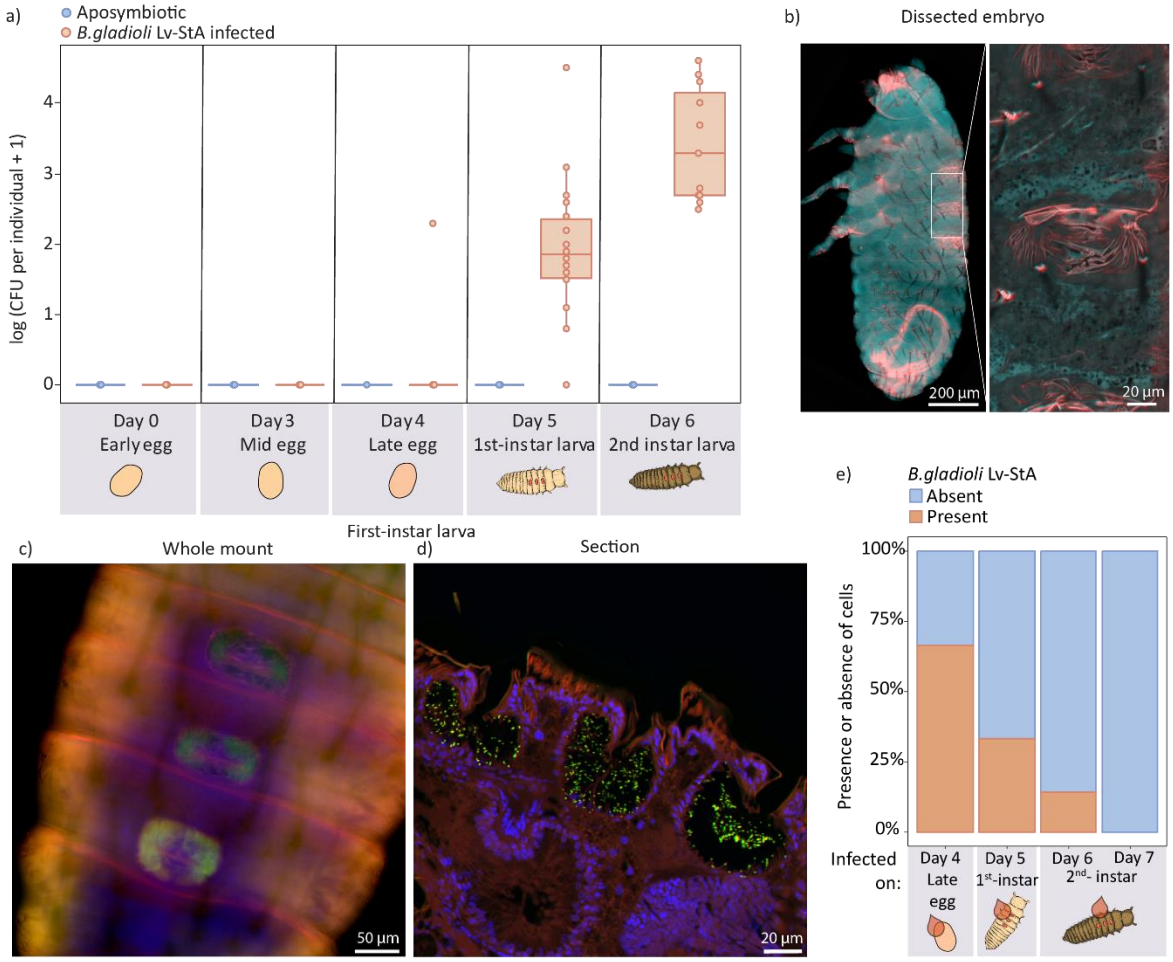

Fig.1. Lv-StA symbionts colonize *Lagria* larvae during or after hatching from the eggs: a) Estimation of internal Lv-StA cells per individual in eggs (day-0, 3, 4), first and second-instar larvae. The lack of cells in eggs shows that the beetles are colonized only in the larval stages during or after hatching ($\chi^2 = 70.822$, df = 4, $p < 0.001$). First-instars had significantly less cells than second-instar larvae (likelihood ratio = 19.393, $p < 0.001$). **b)** FISH of a dissected embryo close before hatching from a symbiont-infected egg (autofluorescence of the eGFP channel in cyan). The inset shows the larval organs not yet occupied by symbionts. **c)** Whole mount FISH image of a field-collected first-instar larva showing Lv-StB within the organs. **d)** Lv-StA cells in the three dorsal organs of an infected first-instar larva. **c,d)** *Burkholderia* specific staining is shown in green, eubacteria in red, host cell nuclei in blue. **e)** Percentage of larvae showing presence (orange) or absence (blue) of Lv-StA in the dorsal structures based on FISH images ($\chi^2 = 7.5208$, df = 3, p-value = 0.057). Larvae were collected for FISH 24-hours after Lv-StA infection on late eggs (n=6), first-instar (n=6) or second-instar larvae (day 6 (n=7) and day 7 (n=6)).

evaluate which route Lv-StA follows in *L. villosa*, we infected freshly laid eggs with 2×10^6 cells and assessed the presence of cells internalized in eggs or larvae by CFU counting after removal of residual symbionts on the surface. We did not detect any colonies on plate when we sampled eggs, with a single exception that may have resulted from incomplete surface sterilization. However, significantly higher CFU counts were obtained from first-instar larvae (n=18) after hatching ($\chi^2 = 70.822$, df = 4, p-val < 0.001), and, on average 2044 cells (min= 0, max= 33600) **(Fig. 1a, Supp. methods)** were estimated to colonise the dorsal structures. In second-instar larvae (n=11), CFU counts amounted to 9431 on average (min= 290, max=40300), which is significantly higher than in first-instar larvae (likelihood ratio = 19.393, p-val < 0.001) **(Fig. 1a, Supp. methods)**. This indicates that symbionts colonise during or after hatching, colonising cell number is highly variable across different host individuals and symbiont titer increases over time. A complementary FISH experiment on a dissected embryo and early first-instar larvae supports the finding that bacteria are absent in the embryo and colonize the structures after hatching **(Fig. 1b-d, Supp. Fig. 1a-b)**. In conclusion, colonisation occurs post-hatching for Lv-StA in *L. villosa* and differs from Stammer's observation of symbionts in *L. hirta* (23), however, we cannot exclude the possibility that other strains associated with *L. villosa* colonise at an earlier time point.

In the lab-reared second-instar larvae, symbiont cell numbers may be overestimated since 8 out of 10 sampled-larvae consumed infected exuvia after moulting and FISH images show cells adhering to the exuvia within the gut **(Supp. fig. 1c,d)**. Therefore, CFU counts in infected second-instars possibly represent the sum of cells that colonised the dorsal structures and those present in the gut. However, these cells are probably transient as they are not consistently present at later time points and do not seem to adhere to the gut lining.

4.2. Symbiont acquisition efficiency declines over time

Aposymbiotic larvae are capable of acquiring Lv-StA symbionts when repeatedly exposed to infected leaf-litter (24). To understand the efficiency of symbiont acquisition in younger larvae in a defined time frame after hatching, we performed inoculations on late eggs (day 4), first-instar (day 5) and second-instar larvae (day 6 and 7) and assessed the presence or absence of Lv-StA 24-hours post-exposure. A comparatively low number of samples could be assessed through the laborious microscopy technique, which limits the statistical power of this dataset. However, there is a clear and marginally significant trend for lower efficiency of symbiont acquisition with increasing age of the larvae ($\chi^2 = 7.5208$, df = 3, p-val = 0.057) **(Fig. 1e, Supp. methods)**. This suggests that symbiont acquisition from the egg surface is more likely to succeed compared to acquisition by larvae one or more days after hatching. While this early time window might impose constraints, previous studies suggest that horizontal acquisition is also possible later in larval development (24). An environment with high Lv-StA abundance, and increased frequency of exposure may encourage horizontal acquisition in larvae (24). However, in natural populations, priority effects and inter-microbial interactions may also hinder entry by a successive colonizer (26–29).

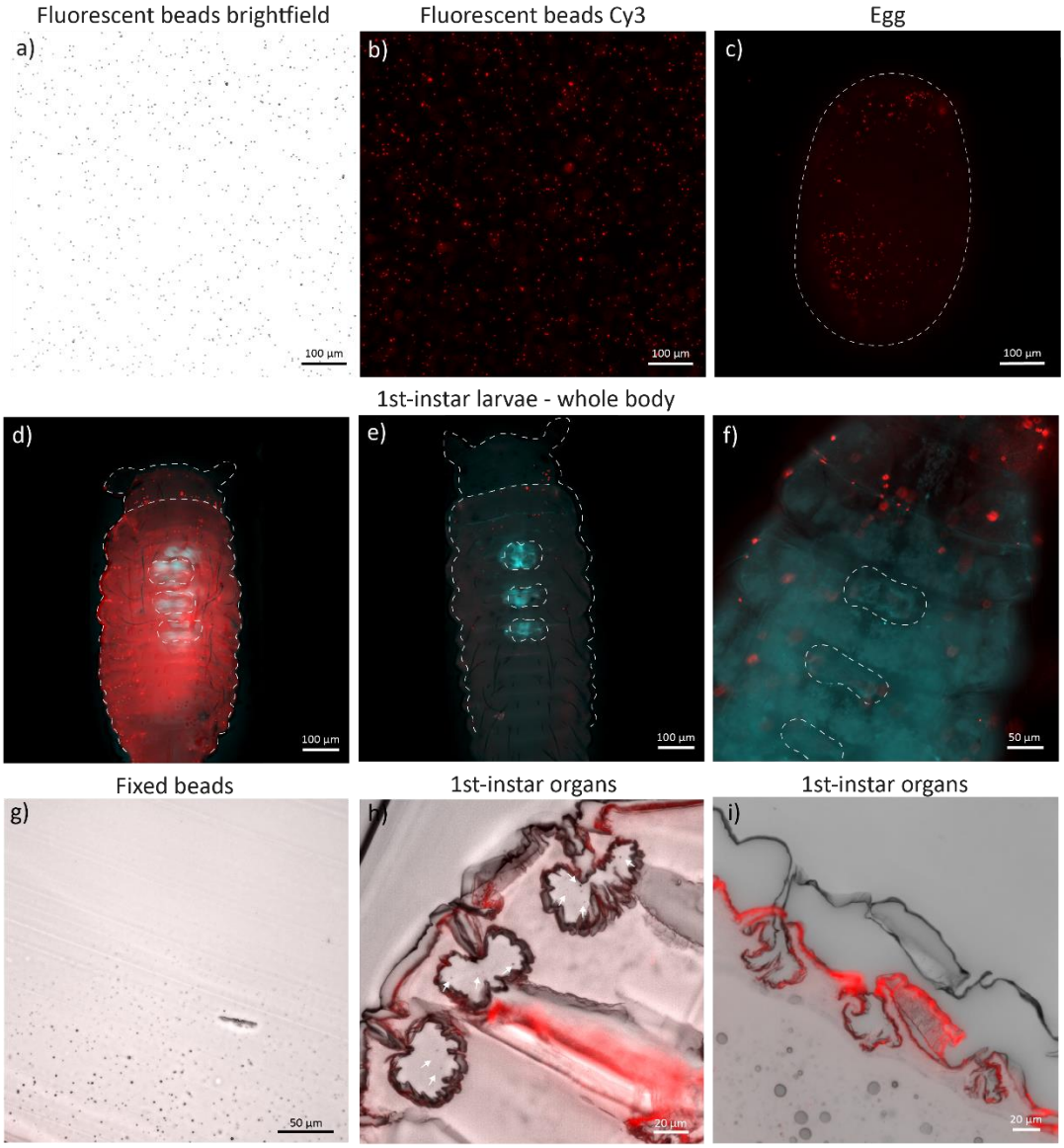

Fig. 2. Simulating transfer of symbionts from eggs to the larval surface using fluorescent beads: a-b) Fluorescent beads in a PBS suspension imaged with bright field **(a)** and in the Cy3-channel **(b)**. **c)** A beetle egg after inoculation with fluorescent beads. **d-f)** Whole first-instar larvae hatched from infected eggs carrying beads on the outer surface. **c-f)** Dotted lines indicate specimen profiles and the dorsal structures based on the DAPI signal (not shown) or EGFP channel (cyan) autofluorescence of the larval tissues. **g)** A section of a 1% agar block with fixed beads. The beads lost fluorescence when fixed in 4% formaldehyde and dehydrated but are visible in bright field. **h-i)** Sagittal section of a hatched first-instar larvae after bead infection showing a few (arrow heads in **(h)**) or no beads **(i)** within the dorsal structures. Overlay of bright field (black and white) and Cy3-channel (red). Red signal shows the autofluorescence of the cuticle.

4.3. Are host movements enough for symbiont acquisition?

The dominant symbiotic strain, Lv-StB is likely incapable of swimming motility, as it lacks genes for flagellar synthesis and chemotaxis (22). It is puzzling how a strain lacking a flagellum can successfully

colonise and dominate the host-associated community, given that entry into the dorsal structures of the host is necessary (19,20). By simulating symbiont transmission using fluorescent beads **(Fig. 2a-b)**, we tested if the larva's movements while hatching are sufficient to direct particles, and thus potentially cells of comparable size, into the dorsal structures. Beads infected on *L. villosa* eggs, with a concentration similar to natural symbiont infections (17), adhered successfully to the egg surface **(Fig. 2c)**. After hatching, the surface of first-instar larvae was covered with the beads, including regions close to the symbiotic organs **(Fig. 2d-f)**. While we could not detect a large number of beads within the dorsal structures in whole larvae **(Fig. 2d-f)**, very few beads were occasionally seen in histological sections **(Fig. 2g-i)**. This suggests that immotile beads can successfully translocate from eggs onto the larval outer surface, and some passively reach symbiotic structures.

In a complimentary experiment to visualize the colonization process, eggs were infected with GFP-tagged motile Lv-StA **(Supp. methods, Supp. Table-1)**, and hatching larvae were imaged under a light-sheet microscope **(Supp. methods)**. Although we could not yet observe cells entering from the egg surface into the dorsal structures, we visualized motile Lv-StA in the dorsal structures of the first-instar larva shortly after hatching **(Supp. vid.1, Supp. methods)**.

Since only a few immotile beads seem to enter the organs, and the light sheet imaging (**Supp. vid.1, Supp. methods)** shows actively moving Lv-StA cells in the larval organs, it is probable that some symbiont-mediated cellular mechanisms, likely including motility, are important factors enhancing colonisation efficiency. It is puzzling how an immotile Lv-StB colonises and dominates the symbiotic organs after the larvae hatch. We suspect that Lv-StB might make use of an alternative approach, like inter-bacterial hitchhiking (30) or a host-controlled mechanism to navigate into the organs. Alternatively, a few immotile cells of Lv-StB entering the organs by chance due to larval movements may have a competitive advantage over Lv-StA in utilizing host-provided resources, leading to strain dominance.

5. CONCLUSION

Symbiont colonisation of a host usually involves considerable changes in effective population size, translocation to a new habitat and re-establishment of the host-microbe interaction. As such, it is a key stage determining symbiosis stability and evolutionary fate. We show that *L. villosa* larvae can acquire cells from the surface of the egg, most likely when the larva brushes against the egg surface as it hatches. Infection experiments at different time points indicate that direct transfer of symbionts from the egg surface is a more effective colonisation strategy than horizontal acquisition during later larval instars. However, further experiments investigating the mechanics of colonisation with the most dominant strain, Lv-StB, and interactions between multiple symbiont strains during colonisation are necessary to understand the natural strain dynamics. Our results also reveal that host movements are sufficient for symbionts to spread over the larval external surface, but navigation into the specialized dorsal structures is probably aided by symbiont molecular factors that are yet to be investigated.

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9. SUPPLEMENTARY METHODS

9.1. Construction of GFP-labelled symbiont strain Lv-StA-GFP

The GFP-labelled strain *B. gladioli* Lv-StA-GFP originated from the symbiont strain previously isolated from *L. villosa, B. gladioli* Lv-StA (1). It was generated via a mini-TN7 transposon insertion containing P_{lac}:GFP into the symbiont chromosome by triparental mating, as previously described (2,3) using the strains listed in **Supp. table S1.** Diaminopimelic acid (DAP) auxotroph *E. coli* donor strains WM3064 containing either the plasmid for the MiniTn7KsGFP transposon (pURR25,(2)) or the plasmid for the Tn7 transposase genes tnsABCDE (pUXBF13) (4) were individually grown on Luria-Bertani (LB) medium (Tryptone 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L) containing, 300 µg/mL DAP and ampicillin (Amp, 100 µg/mL) at 30 °C. The recipient symbiont strain Lv-StA was grown in LB at 30 °C. Donor and recipient strains were conjugated by triparental mating on LB-agar containing 300 µg/mL DAP for 24 h at 30 °C. Afterwards, GFP-labelled recipient cells were selected on LB-agar containing kanamycin (Km, 50 µg/mL) and screened for GFP-fluorescence using an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany). *B. gladioli* Lv-StA-GFP cells were preserved as frozen glycerol stocks at -80 °C and cultured either in LB or KB medium.

9.2. Light sheet microscopy

Egg sterilization was performed as previously described in (1). Aposymbiotic eggs were infected with a culture of Lv-StA-GFP (2×10^6 cells/µL in PBS) and enclosed in a 2% low melting agarose column. We imaged the symbiotic structures of a first-instar larva using a ZEISS Lightsheet.Z1 microscope taking a single-plane time series of 300 frames over 30 seconds with an exposure time of 90 ms and a light sheet thickness of 5.6 µm.

9.3. Statistical analysis

A test of equality of proportions was used to investigate the probability of colonization in different time points and for comparing acquisition efficiency across time points. To assess which life stages differed from each other in their colonization status, pairwise tests for equality of proportions were applied, and obtained p-values were corrected for the false discovery rate (FDR).

To test for differences in CFUs between life stages where bacteria did colonize, a linear mixed effects model was used (lme of the nlme library (5,6)), with life stage as fixed effect and clutch as random intercept. Only life stages with at least three infected samples were used for the analysis. To achieve homogeneity and normality of residuals the data were log-transformed. The influence of the fixed effect was obtained by removing the fixed effect and comparing the simpler with the more complex model with a likelihood ratio test (7). All analyses were done in R 4.2.0 (8).

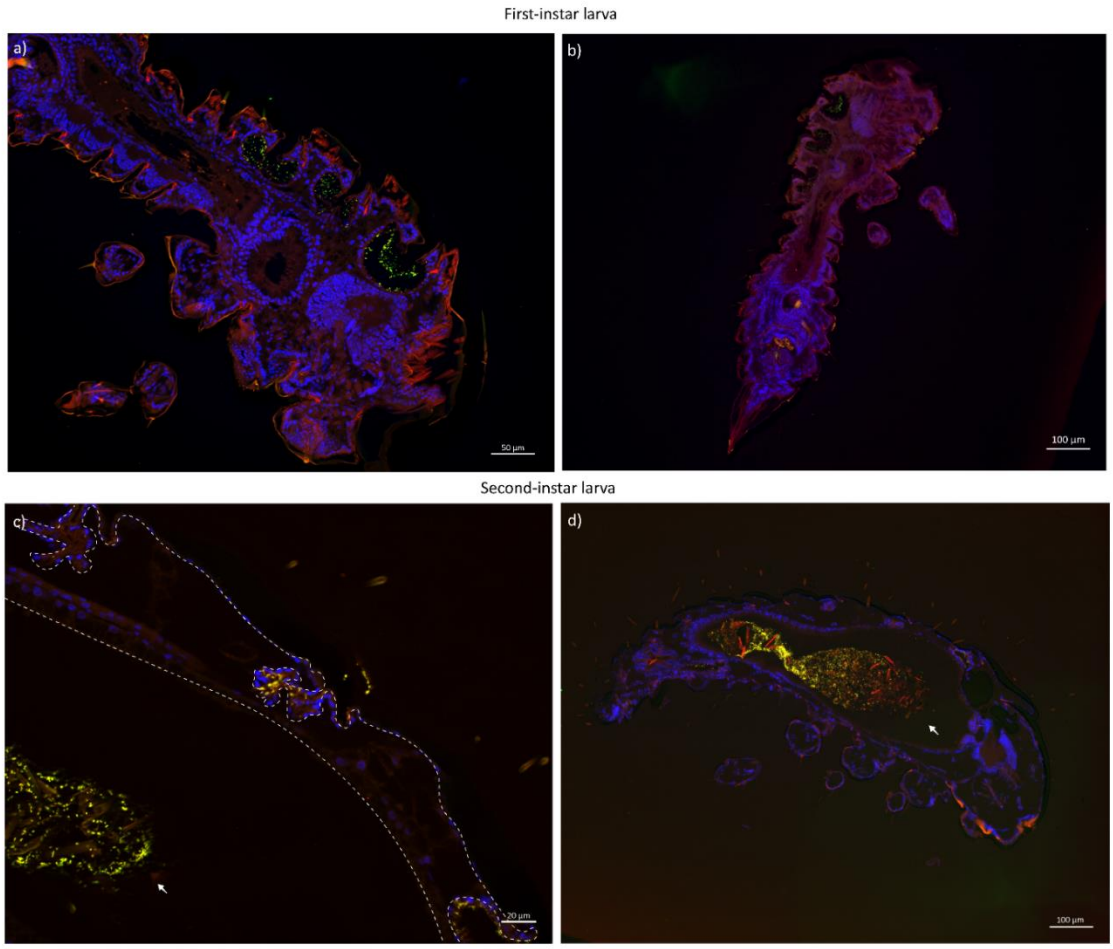
10. SUPPLEMENTARY TABLES AND FIGURES

Table-S1: Bacterial strains and plasmids used in this study

Bacterial strain	Plasmid	Function	Description	Reference
E. coli WM3064	pURR25	Donor containing transposon	Mini Tn*7*KsGFP, GFP driven by P_{lac} ($P_{A1/04/03}$) promoter, mobilizable $oriT_{IncP}\alpha$, suicide $oriR_{R6K\gamma}$; Ap^r (bla)	(2)
E. coli WM3064	pUX-BF13	Helper encoding transposases	Tn*7* transposase genes tnsABCDE, mobilizable $oriT_{IncP}\alpha$, suicide $oriR_{R6K\gamma}$; Ap^r (bla)	(4)
B. gladioli Lv-StA	-	Wild type	Isolate from *L. villosa*	(1)
B. gladioli Lv-StA-GFP	-		GFP-mutant of *B. gladioli* Lv-StA	This study

Table-S2: Probes and stain for Fluorescence *in situ* hybridization

Probe	Sequence	Specificity	References
Eub338_cy3	5'-cy3-GCTGCCTCCCGTAGGAGT-3'	Eubacteria	(9)
Burk_16s_cy5	5'-cy5- TGCGGTTAGACTAGCCACT -3'	*Burkholderia gladioli*	(1)
4',6-diamidino-2-phenylindole (DAPI)		Host cell nuclei	



Supp. figure S1. Lv-StA may be transiently present in the second-instar larval gut aside from the dorsal structures. Lv-StA cells stained in green, Eubacteria (red), host nuclei (DAPI, blue) in the three dorsal organs of a **(a-b)** first and **(c-d)** second-instar larva (surface washed with 1% SDS). **c-d)** Bacterial cells in the second-instar larva gut are indicated by white arrows and the dotted line marks the dorsal organs and gut lining. Cells in the gut are likely transient and acquired from the exuvia that the larva can consume after moulting.

Supp. video. S1) Real-time video showing an *L. villosa* egg clutch in which larvae are hatching (top left corner) or already hatched (middle) but remain on the eggs and are in contact with the egg chorions through repeated body movements. Link to video: https://doi.org/10.6084/m9.figshare.22698318.v1

Supp. video. S2) Time-lapse showing GFP-tagged *B.gladioli* Lv-StA in the symbiotic structures and dorsal cuticle of a 1st-instar larva acquired using a ZEISS lightsheet.Z1 Microscope. Link to video: https://doi.org/10.6084/m9.figshare.22698273.v1

11. SUPPLEMENTARY REFERENCES

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CHAPTER – 4

Transposon-insertion Sequencing as a Tool to Elucidate Bacterial Colonization Factors in a *Burkholderia gladioli* Symbiont of *Lagria villosa* Beetles

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Ramya Ganesan [1], Martin Kaltenpoth [1, 2], Laura V. Flórez [1, 3]

[1] Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg University, Mainz, Germany

[2] Department of Insect Symbiosis, Max Planck Institute for Chemical Ecology, Jena, Germany

[3] Department of Plant and Environmental Sciences, Section for Organismal Biology, University of Copenhagen, Denmark

CONTRIBUTIONS

R.G.: conceptualization, experimental work—generating mutants, PCRs, *in vivo* and *in vitro* experiments, DNA extraction, sequencing library preparation, analysis, visualization, writing—original draft, review and editing. M.K.: conceptualization, funding acquisition, resources, supervision, writing—review and editing. L.V.F.: conceptualization, funding acquisition, resources, supervision, writing—review and editing.

All authors commented on the final draft and gave final approval for publication

1. ABSTRACT

Inferring the biological function of genes by manipulating their activity is an essential tool for understanding the genetic underpinnings of most biological processes. Advances in molecular microbiology have seen the emergence of diverse mutagenesis techniques for the manipulation of genes. Among them, transposon-insertion sequencing (Tn-seq) is a valuable tool to simultaneously assess the functionality of many candidate genes in an untargeted way. The technique has been key to identifying molecular mechanisms for the colonization of eukaryotic hosts in several pathogenic microbes and a few beneficial symbionts.

Here, Tn-seq is established as a method to identify colonization factors in a mutualistic *Burkholderia gladioli* symbiont of the beetle *Lagria villosa*. By conjugation, Tn5 transposon-mediated insertion of an antibiotic-resistance cassette was carried out at random genomic locations in *B. gladioli*. To identify the effect of gene disruptions on the ability of the bacteria to colonize the beetle host, the generated *B. gladioli* transposon-mutant library is inoculated on the beetle eggs, while a control is grown *in vitro* in a liquid culture medium. After allowing sufficient time for colonization, DNA is extracted from the *in vivo* and *in vitro* grown libraries. Following a DNA library preparation protocol, the DNA samples are prepared for transposon-insertion sequencing. DNA fragments that contain the transposon-insert edge and flanking bacterial DNA are selected, and the mutation sites are determined by sequencing away from the transposon-insert edge. Finally, by analyzing and comparing the frequencies of each mutant between the *in vivo* and *in vitro* libraries, the importance of specific symbiont genes during beetle colonization can be predicted.

2. INTRODUCTION

Burkholderia gladioli can engage in a symbiotic association with *Lagria villosa* beetles, playing an important role in defense against microbial antagonists of the insect host (4,5,6). Female beetles house several strains of *B. gladioli* in specialized glands accessory to the reproductive system. Upon egg-laying, females smear *B. gladioli* cells on the egg surface where antimicrobial compounds produced by *B. gladioli* inhibit infections by entomopathogenic fungi (4,6). During late embryonic development or early after the larvae hatch, the bacteria colonize cuticular invaginations on the dorsal surface of the larvae. Despite this specialized localization and vertical transmission route of the symbionts, *L. villosa* can presumably also acquire *B. gladioli* horizontally from the environment (4). Furthermore, at least three strains of *B. gladioli* have been found in association with *L. villosa* (4,6). Among these, *B. gladioli* Lv-StA is the only one that is amenable to cultivation *in vitro*.

B. gladioli Lv-StA has a genome size of 8.56 Mb[6] and contains 7,468 genes. Which of these genes are important for *B. gladioli* bacteria to colonize the beetle host? To answer this question, we used transposon-insertion sequencing (Tn-seq), an explorative method to identify conditionally essential microbial genes (1,2,3). A mutant library of *B. gladioli* Lv-StA was created using a Tn5 transposon. Through conjugation from *Escherichia coli* donor cells to *B. gladioli* Lv-StA, a pRL27 plasmid carrying the Tn5 transposon and an antibiotic resistance cassette flanked by inverted repeats was transferred **(Fig.1)**. Thereby, a set of mutants that individually carry disruptions of 3,736 symbiont genes was generated **(Fig.**

2).

The mutant pool was infected onto beetle eggs to identify the colonization factors and—as a control—was also grown *in vitro* in King's B (KB) medium. After allowing sufficient time for colonization, hatched larvae were collected and pooled for DNA extraction. Fragments of DNA containing the transposon insert and the flanking genomic region of *B. gladioli* Lv-StA were selected using a modified DNA library preparation protocol for sequencing. Read quality processing followed by analysis with DESeq2 was carried out to identify specific genes crucial for *B. gladioli* Lv-StA to colonize *L. villosa* larvae when transmitted via the egg surface.

3. PROTOCOL

3.1. Media and buffer preparation

3.1.1. Prepare KB and LB media and agar plates as given in **Table-1**, and autoclave at 121 °C, 15 psi, 20 min.

3.1.1.1. Add 50 µg/mL filter-sterilized kanamycin and 300 µM filter-sterilized 2,6-diaminopimelic acid (DAP) to the autoclaved LB medium before culturing *E.coli* WM3064 + pRL27.

3.1.1.2. Add 50 µg/mL filter-sterilized kanamycin to the autoclaved KB agar to pour plates needed for selecting successful *B. gladioli* Lv-StA transconjugants.

3.1.2. Prepare 1x phosphate-buffered saline (PBS) by mixing the following components: NaCl 8 g/L, KCl 0.201 g/L, Na_2HPO_4 1.42 g/L, and KH_2PO_4 0.272 g/L. Dissolve the salts in distilled water and autoclave the mixture at 121 °C, 15 psi, 20 min before use. Store at room temperature.

3.1.3. Prepare a 2x Bind-and-wash buffer by dissolving the following components: 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediamine tetraacetic acid (EDTA), and 2 M NaCl in distilled water. Filter-sterilize the mixture before use. Store at room temperature.

3.1.4. Prepare 1x Low-TE by dissolving 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA in double-distilled water. Sterilize by autoclaving at 121 °C, 15 psi, 20 min. Store at room temperature.

3.2. Conjugation to generate the transposon mutant library

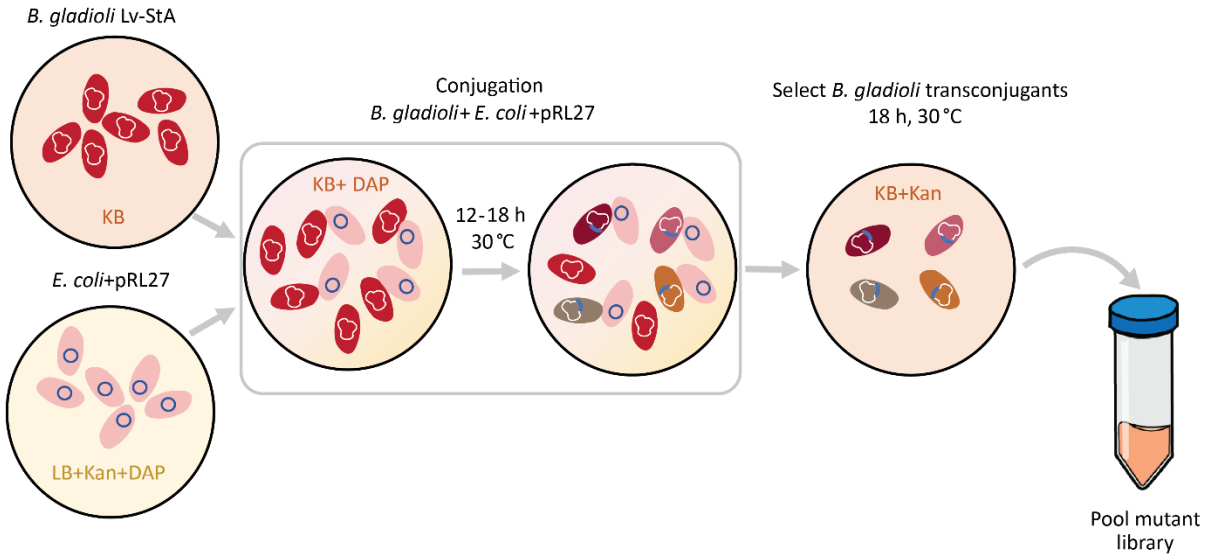


Fig. 1. Conjugation protocol steps: The conjugation recipient Burkholderia gladioli Lv-StA (red) and donor Escherichia coli containing the pRL27 plasmid (pink) are grown in KB agar and LB, respectively, supplemented with kanamycin and DAP. After conjugative transfer of the plasmid for 12–18 h at 30 °C, the transconjugant B. gladioli cells are selected on KB containing kanamycin and pooled together. Abbreviations: DAP = 2,6-diaminopimelic acid; Kan = kanamycin.

3.2.1. Under a sterile hood, inoculate a fresh donor culture of *Escherichia coli* WM3064 + pRL27 in 10 mL of LB medium supplemented with kanamycin and DAP. Inoculate *Burkholderia gladioli* Lv-StA recipient cells in 5 mL of KB medium. Incubate the cultures at 30 °C overnight on a shaker at 250 rpm.

3.2.2. After overnight growth, centrifuge 4 mL of each of the cultures at 9,600 × *g* for 6 min to pellet the cells. Discard the supernatant.

3.2.3. Under a sterile hood, wash the pelleted cell cultures in KB medium containing DAP and finally resuspend the cultures separately in 4 mL of KB + DAP medium.

3.2.4. In a fresh 15 mL tube, mix 250 µL of the washed *E. coli* donor cells with 1 mL of the washed *B. gladioli* Lv-StA recipient cells.

3.2.5. Spot 10 µL of this conjugation cell mixture on KB agar plates containing DAP. Allow the plate to rest undisturbed in the sterile hood at room temperature for 1 h. Then, incubate the plates with the conjugation spots at 30 °C for 12–18 h.

NOTE: The conjugation period can be adjusted according to the target species. However, a long conjugation period increases the risk for double insertions or plasmid integration into the genome. For slow-growing bacteria, allow for longer conjugation periods.

3.2.6. After incubation, add 2–4 mL of 1x PBS into the plates under a sterile hood and use a cell scraper to release the grown bacterial conjugation spots from the agar. Pipette the conjugated-cell–mix into 2

mL microfuge tubes.

3.2.7. Pellet the cells by centrifuging at 9,600 × *g* for 2 min. Discard the supernatant and wash the pellet twice in 1 mL of 1x PBS by pipetting up and down. Resuspend the final pellet in 1200 µL of 1x PBS. Make dilutions before plating if the number of cells in the mixture is above 1×10^4.

3.2.8. Mix well and spread 200 µL of the cell mixture on large KB agar plates (6 or more, if required) supplemented with kanamycin and incubate at 30 °C overnight.

NOTE: Target mutant colonies appear within 30 h on the selective agar plates. Due to the antibiotic resistance marker, only mutated colonies appear on the selective agar plate. Therefore, all colonies are expected to be successful transconjugants.

3.2.9. Count the total number of transconjugant colonies on three plates and extrapolate to calculate the approximate number of mutants obtained in all the plates. To increase the chances of obtaining a representative library, ensure that the total number of colonies is several fold higher than the total number of genes in the genome. To confirm the success of conjugation, perform a PCR targeting the insertion cassette using 10–20 sample colonies, as described in section 3.

NOTE: The aim is to ensure that the number of colonies is at least 10-fold the number of genes in the whole genome, in this case, >75,000 mutants. However, it is generally challenging to accurately estimate the number of colonies that would correspond to a fully representative library. Given that disruptions in essential genes are not captured, there are often multiple different mutation sites for the same gene, and mutations generated with Tn5 transposons are not entirely random.

3.2.10. Under a sterile hood, scrape colonies from the plates by adding 1–2 mL of 1x PBS on the agar. Pool the cell mixture scraped off from the plates into 50 mL tubes. Vortex the library to mix thoroughly and then split 1 mL of the pooled mutant library into several cryotubes. Add 1 mL of 70% glycerol to the tubes and store at -80 °C.

3.3. PCR and gel electrophoresis to confirm successful insertions in B. gladioli Lv-StA

3.3.1. To confirm the presence of the insertion, pick individual mutant colonies from the selection plates in step 2.9 and perform a PCR targeting the insertion cassette using the primers listed in **Table-2**. Prepare the PCR master mix according to **Table-3** and set conditions in the thermal cycler as described in **Table-4**.

3.3.2. Run the PCR products on a 1.6% agarose gel by electrophoresis (250 V, 40 min) to check if the amplified DNA fragments are of the expected length of 1580 bp.

3.4. Mutant pool infection on beetle eggs

3.4.1. Library washing steps

3.4.1.1. Thaw an aliquot of the prepared mutant library on ice. Centrifuge at 2,683 × *g* for 10 min

and remove the supernatant. Under a sterile hood, wash the cells with 4 mL of 1x PBS to remove any remaining medium from the cells. Resuspend the cells in 4 mL of 1x PBS.

3.4.1.2. Count the number of cells in an aliquot of the library using a cell counting chamber. Dilute a part of the library to 2×10^6 cells/µL in 1x PBS.

3.4.1.3. Vortex the library aliquot thoroughly to mix the whole library homogenously before taking the required volume.

3.4.2. Egg clutch sterilization and in vivo infection

3.4.2.1. Select an *L. villosa* egg clutch. Count the number of eggs and continue if the clutch contains more than 100 eggs.

3.4.2.2. Sterilize the entire egg clutch.

3.4.2.2.1. Add 200 µL of 70% ethanol and gently wash the eggs for 5 min. Remove the ethanol and wash the eggs twice with autoclaved water.

3.4.2.2.2. Add 200 µL of 12% bleach (NaOCl) and gently wash the eggs for 30 s. Remove the bleach immediately and wash the eggs again three times with 200 µL of autoclaved water.

3.4.2.3. Infect 2×10^6 cells/µL of the washed mutant library on the sterilized egg clutch (2.5 µL per egg).

3.4.2.4. Two days after the infected beetle larvae hatch, collect 100 2nd instar larvae per 1.5 mL microfuge tube and store at -80 °C.

3.4.3. In vitro mutant library control

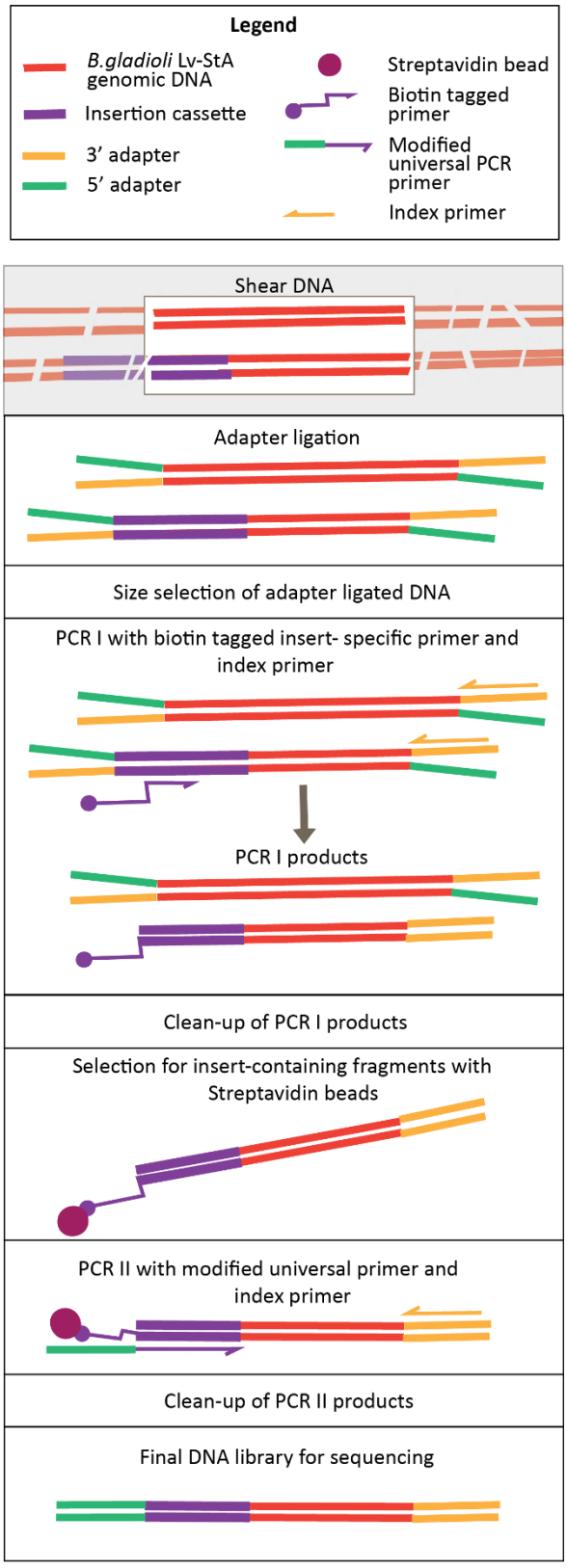
3.4.3.1. Under a sterile hood, inoculate 250 µL of 2×10^6 cells/µL in 10 mL of KB medium containing kanamycin.

3.4.3.2. Incubate the *in vitro* mutant culture at 30 °C for 20 h.
NOTE: Calculate the duration of incubation to match the approximate number of generations of WT *B. gladioli* Lv-StA *in vivo* during colonization.

3.4.3.3. After the 20 h incubation, add an equal volume of 70% glycerol to the *in vitro* mutant culture and store it at -80 °C.

3.5. Infected beetles and in vitro mutant library DNA extraction

NOTE: DNA extractions were performed using a DNA and RNA purification kit according to the manufacturer's protocol briefly outlined below.



3.5.1. Homogenize pooled larvae (maximum of 4 mg per microfuge tube) by adding 1–2 mL of liquid nitrogen.

3.5.2. Thaw the *in vitro* grown mutant cultures from glycerol stocks on ice. Pellet the cells by centrifuging at 9,600 × *g* for 10 min before cell lysis.

3.5.3. Add 300 µL of Tissue and Cell lysis solution to the *in vitro* and *in vivo* samples. Add 5 µL of 10 mg/mL Proteinase K, incubate the mix at 60 °C for 15 min, and then place on ice for 3–5 min.

3.5.4. Add 150 µL of protein precipitation reagent to the lysates and vortex thoroughly. Pellet the protein debris by centrifuging at 9,600 × *g* for 10 min.

3.5.5. Transfer the supernatant to a 1.5 mL microfuge tube. Add 500 µL of isopropanol to the supernatant and gently invert the tubes at least 40 times before incubating at -20 °C for 1 h or overnight.

3.5.6. Pellet the precipitated DNA by centrifuging at 9,600 × *g* for 10 min. Discard the supernatant and add ice-cold 70% ethanol to the DNA pellet.

3.5.7. Centrifuge at ≥10,000 × *g* for 5 min.

Fig. 2. Schematic of the DNA library preparation steps: After shearing and adapter ligation, the modified protocol includes a streptavidin bead-selection step to enrich DNA fragments containing the insertion cassette.

Discard the supernatant and leave the samples to air-dry for at least 1 h.

3.5.8. Resuspend the DNA from the *in vitro* and *in vivo* samples in 100 µL of Low-TE buffer.

3.5.9. Store the samples at -20 °C.

3.6. Sequencing library preparation

NOTE: The protocol and reagents for DNA library preparation are adapted and modified from the instructions provided by the manufacturer of the DNA library preparation kit.

3.6.1. Dilute the samples to 20 ng/µL concentration and volume of 100 µL and keep them on ice.

3.6.2. Shear *in vivo* and *in vitro* sample DNA using an ultrasonicator. Set the ultrasonicator at 70% power. Vortex the samples briefly and shear for 1 min 30 s.

NOTE: The settings for the ultrasonicator will differ among instruments. In this case, the fragment size was 200–400 bp, which is appropriate for this sequencing approach of 150 bp, paired-end (see step. 9.1). The shearing parameters can be adjusted according to the experimenter's requirements.

3.6.3. Check if the DNA was sheared to the desired size range (in this case, 200–400 bp). Load 5 µL of the unsheared and sheared DNA after mixing with gel loading dye in a 1:1 ratio on a 1.6% agarose gel run at 250 V for 40 min (**Fig. 3 A, B**).

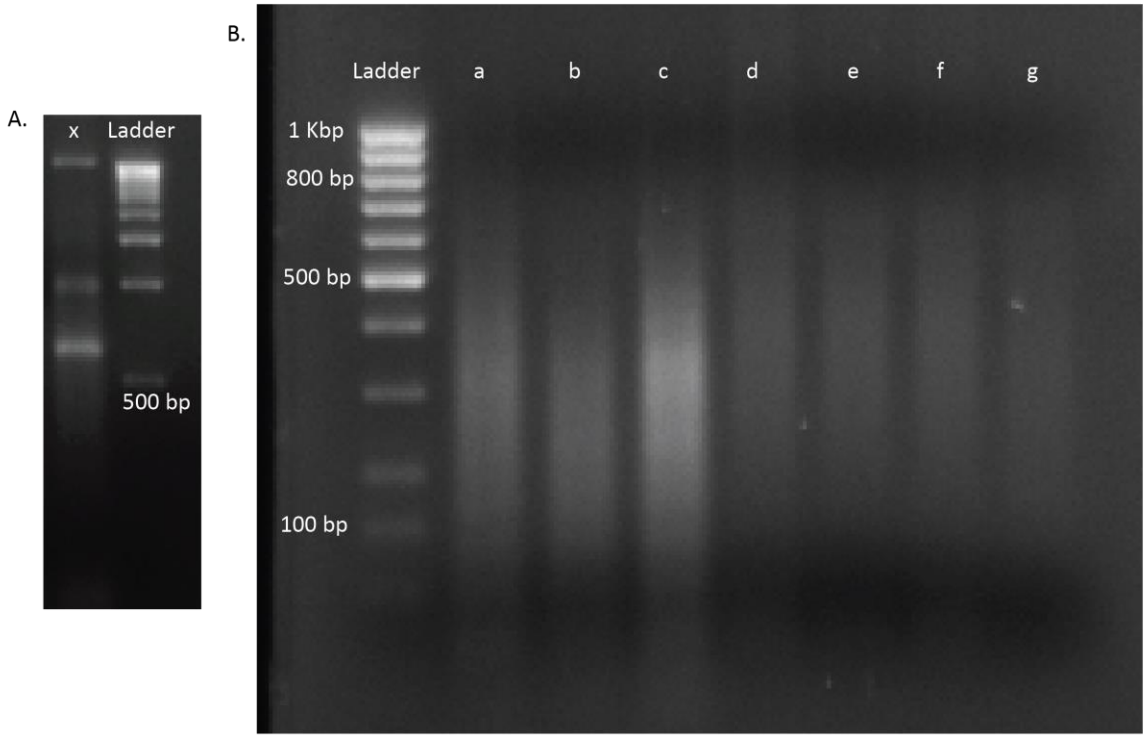


Fig. 3. Agarose gels of a mutant and DNA libraries: (A) Agarose gel with unsheared DNA of a mutant in lane x and a 1 kbp ladder for scale. (B) Gel with sheared DNA library. The band sizes of the ladder in the first lane are indicated on the left side. The first three lanes a, b, and c contain sheared DNA fragments of the in vivo libraries. Lanes d, e, f, and g contain sheared DNA fragments of the in vitro libraries.

3.6.4. Preparation of fragment ends required for adapter ligation

3.6.4.1. To 50 µL of the sheared DNA, add the end preparation reagents given in the library preparation kit: 3 µL of the enzyme mix and 7 µL of reaction buffer and mix well by pipetting. Set a thermal cycler with a heated lid at ≥ 75 °C and incubate the samples for 30 min at 20 °C and 30 min at 65 °C. Hold at 4 °C.

3.6.5. Adapter ligation

3.6.5.1. For adapter ligation, add the following reagents to the products of the end preparation step: 30 µL Ligation Master Mix, 1 µL Ligation Enhancer, and 2.5 µL diluted Adapter. Mix thoroughly by pipetting and incubate the sample for 15 min at 20 °C in the thermal cycler with the heated lid off.

3.6.5.2. After 15 min, add 3 µL of the enzyme (uracil DNA glycosylase + DNA glycosylase-lyase Endonuclease VIII) (see the **Table of Materials**). Mix well by pipetting and incubate the sample for 15 min at 37 °C in a thermal cycler with the lid heated at ≥47 °C.

NOTE: The protocol can be paused at this step, and the samples can be stored at -20 °C.

3.6.6. Size selection of adapter-ligated DNA targeting fragments of 250 bp

3.6.6.1. Vortex the magnetic bead solution (see the **Table of Materials**) and place it at room temperature for 30 min before use.

3.6.6.2. Add 0.3x of beads to 96.5 µL of the ligated DNA mixture and mix by pipetting thoroughly. Incubate the bead mixture for 5 min.

NOTE: The presence of salts and polyethylene glycol in the bead mixture facilitates the precipitation of DNA fragments on the beads. A low ratio of beads to DNA molecules leads to the binding of only larger DNA fragments to the beads. In this case, DNA fragments above 250 bp in length are bound to the beads.

3.6.6.3. Place the tubes on a magnetic stand to pull down the beads and remove DNA fragments of unwanted size. Let the beads settle for 5 min and then transfer the clear supernatant to a new microfuge tube (keep the supernatant).

3.6.6.4. Add 0.15x of fresh beads to the supernatant and mix by pipetting well. Incubate the bead mixture for 5 min and then place the tubes on a magnetic stand to pull down the beads bound to

the target DNA. Wait for 5 min and then discard the supernatant (keep the beads).

NOTE: This ratio of beads to DNA leads to the binding of fragments of the desired 250 bp size.

3.6.6.5. With the beads on the magnetic stand, add 200 µL of 80% ethanol (freshly prepared) and wait for 30 s. Pipette out and discard the ethanol wash carefully without disturbing the beads on the magnetic stand. Repeat this step.

3.6.6.6. After the last wash, remove traces of ethanol from the beads and then air-dry the beads for 2 min until they appear glossy but not completely dried out. Do not over-dry the beads.

3.6.6.7. Remove the tubes from the magnetic stand and add 17 µL of 10 mM Tris-HCl or 0.1x TE (Low-TE). Mix by pipetting ~10 times and incubate the mixture at room temperature for 2 min.

3.6.6.8. Place the tubes back on the magnetic stand and wait for 5 min. Once the beads have settled down, transfer the DNA supernatant to a new tube.

3.6.7. PCR I to add biotin tag to DNA fragments containing the insertion cassette

3.6.7.1. Add a biotinylated primer tag to the DNA fragments containing the Tn5-insertion cassette by using the transposon-specific biotinylated primer (**Table-5**) and an index primer. Prepare the PCR master mix according to **Table-6** and follow the PCR conditions for the thermal cycler listed in **Table-7**.

3.6.8. Clean-up of PCR I without size selection

3.6.8.1. Vortex 0.9x beads and place them at room temperature for at least 30 min before clean-up.

3.6.8.2. Add 0.9x beads to the PCR products and mix thoroughly.

3.6.8.3. Place the beads on a magnetic stand to pull down the beads.

3.6.8.4. Remove the clear supernatant and wash the bead-bound-DNA with 200 µL of freshly prepared 80% ethanol twice.

3.6.8.5. Remove the ethanol after the wash steps and air dry the beads until they look glossy but not too dry.

3.6.8.6. Add 32 µL of 10 mM Tris-HCl or 0.1X TE (Low-TE) and incubate the beads for 5 min. Place the mixture back on the magnetic stand and transfer the supernatant to a fresh microfuge tube.

3.6.9. Binding biotinylated DNA fragments to streptavidin beads

3.6.9.1. Resuspend 32 µL of streptavidin beads in 1x Bind-and-wash buffer. Wash the beads with

the buffer three times while placed on a magnetic stand.

3.6.9.2. Add 32 µL of 2x Bind-and-wash buffer and resuspend the beads. To this, add 32 µL of the cleaned-up PCR 1 products. Mix thoroughly and incubate at room temperature for 30 min.

3.6.9.3. Place the bead-DNA mixture on a magnetic stand for 2 min. Pipette out the supernatant as biotin-tagged DNA containing the insertion edge binds to streptavidin on the beads.

3.6.9.4. Wash the beads with 500 µL of 1x Bind-and-wash buffer and then wash the beads with 200 µL of Low-TE. Resuspend the DNA-bound beads in 17 µL of Low-TE.

3.6.10. PCR II to add adapters to the fragments containing the insertion cassette edge

3.6.10.1. Prepare a master mix, as shown in **Table-8,** using the index primers and modified universal PCR primers listed in **Table-5**. Add 15 µL of the DNA-bound streptavidin beads from the previous step to the PCR mix. See **Table-7** for the thermal cycler conditions.

3.6.11. Clean up the PCR products without size selection as given in step 3.6.8 of this protocol. Elute the final DNA products in 30 µL of molecular-grade water.

3.6.12. Store the samples at -20 °C and use them for sequencing.

3.7. Sequencing and analysis

3.7.1. Sequence the library using high-throughput sequencing technology. Adjust the sequencing depth depending on the transposon library size, as noted below. Assess the read quality with FastQC (7). Select reads containing the Tn5-insertion edge on the 5' end of the read and remove the insertion edge sequence using Cutadapt(8) and/or Trimmomatic(9).

NOTE: Here, a paired-end sequencing approach was used to target 150 bp per read and a total of 8 Mio reads. To obtain a representative dataset, ensure that the total number of sequenced reads exceeds the maximum possible number of mutants in the library, i.e., the total estimated number of colonies from step 3.2.9. As a reference, this protocol aimed for 40-fold of the maximum possible library size. Other successful studies using Tn-seq for a similar purpose sequenced a total number of reads close to 25-fold of the actual number of unique insertions in the corresponding mutant library (22,23).

3.7.2. Considering that mutations at the ends of genes are not functionally disruptive, trim 5% off both ends of gene annotations of the reference genome GFF file. Map the trimmed reads to the reference genome using Bowtie2 (10).

3.7.3. Calculate the number of insertions from the number of unique 5' positions in the alignment BAM file.

3.7.4. Using FeatureCounts (11), obtain the number of hit genes for each replicate sample.

3.7.5. Using the DESeq2 (12) package in RStudio, calculate the difference in mutant abundances between different conditions.

4. REPRESENTATIVE RESULTS

Host-associated bacteria can employ several factors to establish an association, including those mediating adhesion, motility, chemotaxis, stress responses, or specific transporters. While factors important for pathogen–host interactions have been reported for several bacteria (13–18), including members of the genus *Burkholderia* (19,20), fewer studies have explored the molecular mechanisms used by beneficial symbionts for colonization(21–23). Using transposon insertion sequencing, the aim was to identify molecular factors that enable *B. gladioli* to colonize *L. villosa* beetles.

Transposon-mediated mutagenesis was performed using the pRL27 plasmid, which carries a Tn5 transposon and a kanamycin resistance cassette flanked by invert repeat sites. The plasmid was introduced into the target *B. gladioli* Lv-StA cells by conjugation with the plasmid donor *E. coli* WM3064 strain (as shown in **Fig. 1**). After conjugation, the conjugation mix containing *B. gladioli* recipient and *E. coli* donor cells were plated on selective agar plates containing kanamycin. The absence of DAP on the plates eliminated the donor *E. coli* cells, and the presence of kanamycin selected for successful *B. gladioli* Lv-StA transconjugants. The pooled *B. gladioli* Lv-StA mutant library obtained from harvesting the 100,000 transconjugant colonies on the plate was prepared for sequencing using a modified DNA library preparation kit and custom primers. **Fig. 2** highlights the DNA library preparation steps. Sequencing yielded 4 Mio paired reads; 3,736 genes out of 7,468 genes in *B. gladioli* Lv-StA were disrupted.

To identify mutants that were colonization-defective in the host, the *B. gladioli* Lv-StA mutant library was infected on the beetle eggs and grown *in vitro* in KB medium as a control. The *in vivo* colonization bottleneck size was calculated before the experiment. A known number of *B. gladioli* Lv-StA cells was infected on beetle eggs, and the number of colonizing cells in freshly hatched first instar larvae was obtained by plating a suspension from each larva and counting colony-forming units per individual. These calculations were done to ensure that the number of colonizing cells is enough to assess all or a high percentage of the mutants in the library for their ability to colonize the host. Additionally, the growth time between *in vitro* and *in vivo* conditions was normalized based on the number of bacterial generations to make these samples comparable.

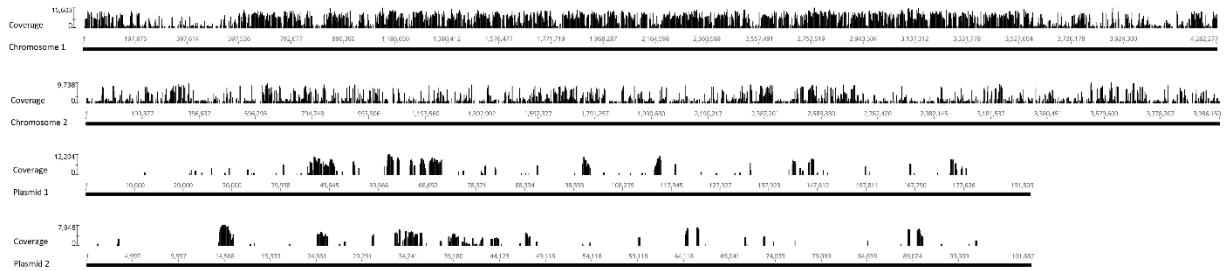


Fig. 4: Location of unique insertion sites in the original library across the four replicons in the Burkholderia gladioli Lv-StA genome. Each bar along the x-axis is located at a site of insertion. The height of a bar along the y-axis corresponds to the number of reads associated with that site. Note that the two chromosomes and two plasmids are shown in full length and thus have different scales on the x-axis.

After the eggs hatched, 1,296 larvae were collected in 13 pools. The corresponding *in vitro* mutant cultures were grown and stored as glycerol stocks. DNA of the *in vivo* and *in vitro* grown mutant libraries was extracted and fragmented in an ultrasonicator. **Fig. 3** shows the size distribution of the sheared DNA, where the majority of the fragments span between 100 and 400 bp, as expected. This step was followed by the modified DNA library preparation protocol for sequencing. At each step of the protocol, the concentration of remaining DNA was checked to ensure that the steps were performed correctly and to track losses of DNA. A quality check (see the **Table of Materials**) before sequencing revealed that the DNA libraries contained unexpectedly large (>800 bp) DNA fragments, and this was more pronounced in the *in vivo* libraries. Given the difficulty in optimizing the clustering of fragments in the sequencing lanes, it was necessary to increase the sequencing depth to 10 Mio paired reads in the *in vivo* libraries to attain the desired number of reads. The analysis of the sequencing results revealed that an average of 4 Mio reads in the *in vivo* libraries and 3.1 Mio reads in the *in vitro* libraries contained the Transposon edge in the 5' end of Read-1 (**Table-9**), which was satisfactory for this experiment. The distribution of the 24,224 unique insertions across the *B. gladioli* genome in the original library is shown in **Fig. 4**. An analysis carried out using DESeq2 revealed that the abundances of 271 mutants were significantly different between the *in vivo* and *in vitro* conditions.

5. DISCUSSION

A *B. gladioli* transposon mutant library was generated to identify important host colonization factors in the symbiotic interaction between *L. villosa* beetles and *B. gladioli* bacteria. The major steps in the protocol were conjugation, host-infection, DNA library preparation, and sequencing.

As many strains of *Burkholderia* are amenable to genetic modification by conjugation(24,25), the plasmid carrying the transposon and antibiotic insertion cassette was conjugated successfully into the target *B. gladioli* Lv-StA strain from *E. coli*. Previous attempts of transformation by electroporation yielded very low to almost no *B. gladioli* transformants. It is advisable to optimize the transformation technique for the target organism to efficiently yield a large number of transformants.

One round of conjugation and 40 conjugation spots disrupted 3,736 genes in *B. gladioli* Lv-StA. In hindsight, multiple rounds of conjugation would be necessary to disrupt most of the 7,468 genes and

obtain a saturated library. Notably, the incubation time during conjugation was not allowed to exceed 12–18 h, which is the end of the exponential growth phase of *B. gladioli*. Allowing conjugation beyond the exponential growth phase of bacterial cells reduces the chances of success of obtaining transconjugants(26). Therefore, the conjugation period should be adjusted according to the growth of the target bacterial species.

To successfully carry out an experiment involving the infection of mutant libraries in a host, it is important to assess the bacterial population bottleneck size during colonization and the diversity of mutants in the library before infection(4,5,27). In preparation for the experiment, we estimated the minimum number of beetles that must be infected to have a high chance that each mutant in the library is sampled and allowed to colonize. The approximate *in vivo* bacterial generation time and the number of generations for the duration of the experiment were also calculated. The *in vitro* culture was then grown up to a comparable number of generations by adjusting the incubation time. For a similar infection experiment in other non-model hosts, the ability to maintain a laboratory culture and a constant source of the host organisms is desirable.

Following the growth of the mutant library *in vivo* and *in vitro* and sample collection, a modified DNA library preparation protocol for transposon insertion sequencing was carried out. The modification in the protocol involved designing custom PCR primers and adding PCR steps to select for DNA fragments containing the insertion cassette. Because the protocol was customized, additional PCR cycles in the protocol increased the risk of overamplification and obtaining hybridized adapter-adapter fragments in the end libraries. Hence, a final cleanup step (without size selection) after the two PCRs is recommended, as it helps in removing these fragments. The size distribution of the DNA libraries was still broader than expected. However, increasing the sequencing depth provided sufficient data that were filtered during bioinformatics analysis, obtaining satisfactory results.

As transposon-mediated mutagenesis generates thousands of random insertions in a single experiment, it is possible to generate a saturated library of mutants that contains all except those mutants where genes essential for bacterial growth have been disrupted. We most likely did not work with a saturated mutant library, given the estimations of essential genes in other studies on *Burkholderia sp.*(28,29). A non-saturated library nevertheless helps in exploring various candidate genes for further studies using targeted mutagenesis. Before the experiments, it is also important to remember that some transposons have specific insertion target sites that increase the abundance of mutants at certain loci in the genome(30). Mariner transposons are known to target AT sites for insertion(31), and Tn5 transposons have a GC bias(32,33). Including steps during bioinformatics analysis to recognize hotspots for transposon insertions will help in assessing any distribution bias.

Although prone to setbacks, a well-designed transposon insertion sequencing experiment can be a powerful tool to identify many conditionally important genes in bacteria within a single experiment. For example, a dozen genes in *Burkholderia seminalis* important for the suppression of orchid leaf necrosis were identified by combining transposon mutagenesis and genomics(34). Beyond *Burkholderia,* several adhesion and motility genes and transporters have been identified as important colonization factors in

Snodgrassella alvi symbionts of *Apis mellifera* (Honeybee)(22), and in the *Vibrio fischerii* symbionts of *Euprymna scolopes* (Hawaiian bobtail squid)(23) using the Transposon-insertion mutagenesis approach. As an alternative approach, transposon mutagenesis may be followed by screening for individual mutants using selective media instead of sequencing. Phenotypic screening or bioassays to identify deficiencies, such as motility, production of bioactive secondary metabolites, or specific auxotrophies, are feasible.

For example, screening of a *Burkholderia insecticola* (reassigned to genus *Caballeronia*(35)) transposon mutant library has been key in identifying that the symbionts employ motility genes for colonizing *Riptortus pedestris,* their insect host(36). Furthermore, using transposon mutagenesis and phenotypic screening, the biosynthetic gene cluster for the bioactive secondary metabolite caryoynencin was identified in *Burkholderia caryophylli*(37). An auxotrophic mutant of *Burkholderia pseudomallei* was identified following transposon mutagenesis and screening and is a possible attenuated vaccine candidate against melioidosis, a dangerous disease in humans and animals(38). Thus, transposon mutagenesis and sequencing is a valuable approach in studying the molecular traits of bacteria that are important for the interactions with their respective hosts in pathogenic or mutualistic associations.

6. ACKNOWLEDGMENTS

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7. DISCLOSURES

The authors declare that they have no conflict of interest pertaining to the study.

8. TABLES

Table-1. Media components

King's B medium/ agar	
Peptone (soybean)	20 g/L
K_2HPO_4	1.5 g/L
$MgSO_4.7H_2O$	1.5 g/L
Agar	15 g/L
Dissolved in distilled water	

LB medium / agar	
Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

Table-2. Primers to confirm the success of conjugation

No.	Primers	Sequence	PCR annealing temp. (°C)
1	tpnRL17–1RC	5′-CGTTACATCCCTGGCTTGTT-3′	58.2
2	tpnRL13–2RC	5′-TCGTGAAGAAGGTGTTGCTG-3′	

Table-3. PCR master mix to confirm the success of conjugation.

PCR mix	(µl)
HPLC purified water	4.92
Buffer S (high specificity)	1
$MgCl_2$ - 0.2µL	0.2
dNTPs	1.2
Primer 1	0.8
Primer 2	0.8
Taq	0.08
Mastermix total	9
Template	1

Abbreviations: HPLC = high-performance liquid chromatography; dNTPs = deoxynucleoside triphosphate.

Table-4. PCR conditions to confirm the success of conjugation.

Steps	Temperature °C	Time	Cycles
Initial Denaturation	95	3 min	1
Denaturation	95	40 s	
Annealing	Tm	40 s	30 to 35
Extension	72	1-2 min	
Final Extension	72	4 min	1
Hold	4	∞	

Table-5. Primers and adapter for PCR I and II during DNA library preparation.

Primers	Sequence	Tm °C	Use	source
Transposon specific biotinylated primer	5′-Biotin-ACAGGAACACTTAACGGCTGACATG-3′	63.5	6.7.1. PCR I	Custom
Modified Universal PCR primer	5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAATTCATCGATGATGGTTGAGATGTGT – 3′	62	6.10.1. PCR II	Custom
Index primer	Refer to the manufacturer's manual		6.7.1. PCR I & 6.10.2. PCR II	NEBNext Multiplex Oligos for Illumina (Index primers set 1)
Adapter	Refer to the manufacturer's manual		6.5. Adapter ligation	NEBNext Ultra II DNA library prep kit for Illumina

Table-6. DNA library preparation—PCR I master mix.

PCR mix	(µl)
Adaptor ligated DNA fragments	15
NEBNext Ultra II Q5 master mix	25
Index primer	5
Transposon specific biotinylated primer	5
Total volume	50

Table-7. DNA library preparation—PCR I and II conditions.

Steps	Temp	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	6 to 12
Annealing	65°C	30 s	
Extension	72°C	30 s	
Final Extension	72°C	2 min	1
Hold	16°C	∞	

Table-8. DNA library preparation—PCR II master mix.

PCR mix	(µl)
Bead-selected DNA	15
NEBNext Ultra II Q5 master mix	25
Index primer	5

Modified universal PCR primer	5
Total volume	50

Table 9. Summary of sequencing output and transposon insertion frequency per library.

Libraries	Invivo-1	Invivo-2	Invivo-3	Invitro-1	Invitro-2	Invitro-3	Original library
No. of reads (PE)	56,57,710	39,19,051	30,65,849	35,73,494	28,83,440	36,61,956	46,09,410
No. of reads containing Tn – edge on 5' end of Read-1	54,15,880	37,31,169	29,36,247	33,00,499	27,35,705	33,50,402	41,53,270
Bowtie2 overall alignment rate (%) (Read-1 only)	95.53%	83.71%	89.87%	80.79%	78.00%	73.06%	74.92%
Number of unique insertions	8,539	4,134	7,183	18,930	18,421	20,438	24,224
Number of genes hit	1575	993	1450	2793	2597	3037	3736

Abbreviation: PE = paired-end.

9. TABLE OF MATERIALS

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2,6- Diaminopimelic Acid	Alfa Aesar	B22391	For *E.coli* WM3064+ pRL27
Agar - Agar	Roth	5210	
Agarose	Biozym	840004	
AMPure beads XP (magentic beads + polyethylene glycol + salts)	Beckman Coulter	A63880	Size selection in step 6.6
Bleach (NaOCl) 12%	Roth	9062	
Buffer-S	Peqlab	PEQL01-1020	For PCRs
Cell scraper	Sarstedt	83.1830	
DNA ladder 100 bp	Roth	T834.1	
dNTPs	Life Technology	R0182	PCR for confirming success of conjugation
EDTA, Di-Sodium salt	Roth	8043	
Epicentre MasterPure Complete DNA and RNA Purification Kit	Lucigen	MC85200	
Ethidium bromide	Roth	2218.1	
Glycerol	Roth	7530	
K_2HPO_4	Roth	P749	
Kanamycin sulfate	Serva	26899	
KCl	Merck	4936	
KH_2PO_4	Roth	3904	
$MgSO_4.7H_2O$	Roth	PO27	
Na_2HPO_4	Roth	P030	

NaCl	Merck	6404	
NEBNext Multiplex Oligos for Illumina (Index primers set 1)	New England Biolabs	E7335S	
NEBNext Ultra II DNA library prep kit for Illumina	New England Biolabs	E7645S	
Peptone (soybean)	Roth	2365	For *Burkholderia gladioli* Lv-StA KB-medium
peqGOLD 'Hot' Taq- DNA Polymerase	VWR	PEQL01-1020	PCR for confirming success of conjugation
Petri plates - 145 x 20 mm	Roth	XH90.1	For selecting transconjugants
Petri plates - 90 x 16 mm	Roth	N221.2	
Streptavidin beads	Roth	HP57.1	
Taq DNA polymerase	VWR	01-1020	
Tris -HCl	Roth	9090.1	
Tryptone	Roth	2366	For *Escherichia coli* WM3064+pRL27 LB medium
Ultrasonicator	Bandelin	GM 70 HD	For shearing
Yeast extract	Roth	2363	
USER enzyme (uracil DNA glycosylase + DNA glycosylase-lyase Endonuclease VIII)	New England Biolabs	E7645S	Ligation step 6.5.2
FastQC v.0.11.8			Bioinformatic tool for assessing the quality of sequencing data. Reference 7 in main manuscript.
Cutadapt v.2.10			Bioinformatic tool for removing specific adapter sequences from the reads. Reference 8 in main manuscript.
Trimmomatic v.0.36			Bioinformatic tool for trimming low quality reads and also adapter sequences. Reference 9 in main manuscript.
Bowtie2 v.2.4.2			Bioinfromatic tool for read mapping. Reference 10 in main manuscript.
FeatureCounts v.2.0.1			Bioinformatic tool to obtain read counts per genomic feature. Reference 11 in main manuscript.
DESeq2			RStudio package for assessing differential mutant abundance. Usually used for RNAseq analysis. Reference 12 in main manuscript.
Qiaxcel (StarSEQ GmbH, Germany)			Quality check after DNA library preparation

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CHAPTER – 5

Mechanisms of colonization in a defensive ectosymbiont of *Lagria villosa* beetles

Manuscript in preparation

Ramya Ganesan [1, 2], Jana Krabbe [3], Christian Hertweck [3], Martin Kaltenpoth [1, 2], Laura. V. Flórez [1, 4]

[1] Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg University, Mainz, Germany

[2] Department of Insect Symbiosis, Max Planck Institute for Chemical Ecology, Jena, Germany

[3] Hans Knöll institute, Jena, Germany

[4] Department of Plant and Environmental Sciences, Section for Organismal Biology, University of Copenhagen, Denmark

CONTRIBUTIONS

R.G.: conceptualization, experimental work—generating transposon-insertion directed mutants and targeted mutants, PCRs and sequencing library preparation, Tn-seq experiments and analysis, targeted mutant colonization assays, analysis, FISH and microscopy, visualization, writing—original draft, review and editing. J.K.: experimental work—generating targeted mutants. C.H.: resources, supervision. M.K.: conceptualization, funding acquisition, resources, supervision, writing—review and editing. L.V.F.: conceptualization, funding acquisition, resources, supervision, writing—review and editing.

1. ABSTRACT

Microbes that colonize the host from an external environment, or that spend part of their life cycle outside the host, encode molecular machineries that help them during colonization and establishment, like motility, chemotaxis, biofilm formation, stress response factors, secretion of effectors and transporters for metabolite exchange. However, most of our knowledge about microbial molecular factors is gained from studies on pathogens, while host-beneficial microbes have received much less attention. In order to expand our understanding about colonization factors of beneficial microbes, we performed genetic manipulation and colonization assays with a cultivable defensive *Burkholderia gladioli* Lv-StA symbiont of *Lagria villosa* beetles and compared our findings to the eroded genome of the co-occurring, but yet uncultivated *Burkholderia gladioli* Lv-StB. Transposon-insertion sequencing revealed genes involved in motility or biofilm formation, formation of the cell wall and related structures, membrane transporters, lipopeptides, oxidative stress response factors, and iron metabolism as candidate colonization factors in Lv-StA. However, targeted mutagenesis and individual colonization assays show that non-motile mutants can still colonize the host as efficiently as the wild type. This likely explains the loss of flagellar motility genes in the more dominant and consistently associated Lv-StB. Genome comparison of the two symbiont strains shows similarities and a few differences in the genetic capabilities of Lv-StA and Lv-StB. Both strains likely have similar LPS structures and uptake of iron-bound siderophores, however, Lv-StB lacks the genes for siderophore biosynthesis and flagellar motility. We speculate that Lv-StB may have streamlined genetic capabilities, retain the bare necessities, utilize public goods, and thus, dominate the symbiotic structures of the beetle. Thus, the *Lagria-Burkholderia* symbiosis not only presents a genetically tractable system to study the molecular basis of symbiosis establishment, but also provides a rare opportunity to compare colonization strategies among closely related bacteria and assess their interactions in a multipartite symbiosis.

2. INTRODUCTION

In many insects, the presence of microbial symbionts affects host fitness, and successful colonization by the microbial partners is key for host survival or reproduction. Symbionts provide various benefits such as nutrition (1,2), defence (3,4) or detoxification (5,6), which enables the host to exploit novel dietary resources, enhances protection against a broad range of pathogens, predators and parasitoids, or confers the ability to cope with abiotic stressors. In order to sustain the microbial partnership through generations, insect-associated symbionts are vertically transmitted to the offspring, horizontally taken up from the environment or exchanged among unrelated individuals, or undergo a mixed mode of transmission (7). During transmission and symbiont acquisition, an interplay of host and symbiont factors mediates establishment and specificity in insects (8–14) as in other animals (15–19).

The molecular factors aiding colonisation have been investigated only for a small number of experimentally and genetically tractable symbioses (20), like the association between the Hawaiian bobtail squid, *Euprymna scolopes,* and *Vibrio fischeri* (19,21,22), *Riptortus* bean bugs and *Caballeronia* (8,23–25), and honey bees and *Snodgrassella alvi* (12). Some of the key molecular factors essential for symbiont colonization include motility to reach the symbiotic organs of the host (11,26) and biofilm formation to adhere to the host epithelial surface (27,28). However, the molecular factors underlying the establishment of cuticular ectosymbionts remain unknown, despite their wide distribution across insect

taxa. For example, *Actinobacteria* in Attine ants, are harboured in cuticular crypts on the thorax (29). The symbionts produce antibiotics that protect the fungal cultivars of the ants against pathogenic fungi of the genus *Escovopsis* (30). In another example, female beewolf digger wasps carry antibiotic-producing *Streptomyces* in antennal glands (31). In the subterranean brood-cell, the symbionts are transferred to the cocoon and protect the offspring from pathogenic fungi (31–33). In both leaf-cutter ants and beewolf wasps, we are aware of the host behavioural mechanisms for symbiont uptake (31,34), but the molecular factors involved in symbiosis establishment remain unknown.

In *Lagria villosa* beetles, defensive bacterial symbionts, predominantly *Burkholderia gladioli*, are found in specialized accessory glands of the female reproductive system (35,36). The female beetles deposit symbionts on the egg surface during oviposition (35,36), where the bacteria produce a cocktail of bioactive compounds that protect the developing embryo from entomopathogenic fungi. Once the larvae hatch, the symbionts colonize three specialized cuticle-lined structures on the dorsal surface of the larvae (35–37), and symbiont mediated defence extends to the vulnerable phases after larval molting events (38).

Multiple strains of *Burkholderia* can be found associated with a single beetle host (35,38). Among the strains, Lv-StB is the most consistent and abundant in the community. Lv-StB has a reduced genome (39) and produces an antifungal compound, Lagriamide that is detrimental to fungal growth on eggs (36). A second strain, Lv-StA, is less prevalent in natural populations, but has been successfully cultivated *in vitro* (35). This strain exhibits a larger genome compared to Lv-StB and can synthesize several antifungal and antibacterial agents (35,40–42). The bacterial strains are housed in the dorsal structures of the larvae, which are open to the outside environment, and can be exchanged between the host and the environment by horizontal transmission (35,43). Despite this relatively open and facultative symbiotic association (44), we find that *Burkholderia* are the most abundant and consistent bacteria among the microbial community of the *Lagria* beetles (38). Thus, it is likely that microbial and/or host molecular factors play a crucial role during the initial establishment of the symbiosis and help maintain a consistent community of *Burkholderia* strains in the dorsal structures of the larvae and the accessory structures to the female reproductive system.

Here, we explored the defensive symbiotic association between *Lagria villosa* beetles and *Burkholderia* bacteria to gain insights on microbial molecular factors important for establishing a cuticle associated symbiosis. We took advantage of the cultivability and genetic tractability of one of the symbiont strains, Lv-StA, to study colonization factors of an ectosymbiont. In order to explore many colonization factors simultaneously, we generated a transposon mutant library of this strain (45), and assessed the ability of the mutants to colonize the larval dorsal structures by high-throughput transposon-directed sequencing. This approach yielded 271 candidate colonization factors, including genes that are involved in motility & biofilm formation, maintain the cell-wall and membrane, aid in uptake or transport of nutrients and iron, respond to oxidative stress and some Lv-StA specific lipoptides. Screening for these putative colonization genes of Lv-StA in the eroded genome of Lv-StB indicates shared but also unique factors underlying symbiont colonization across the two strains. We followed up the Tn-seq experiment with targeted mutagenesis and colonization assays to determine whether flagellar motility and a lipopeptide, which is a swarming inhibitor, are important colonization factors. However, the results indicate that non-

motile mutants are able to successfully colonize the larval dorsal structures in isolation as well as in competition with the wild type Lv-StA. This likely explains the loss of flagellar motility genes in Lv-StB and indicates that host-movements may direct non-motile strains towards the dorsal organs of the larvae. Further comparative genome studies with Lv-StB could point us towards identifying processes that may be essential for establishing this ectosymbiotic association.

3. METHODS

3.1. Beetle rearing

Lagria villosa adults and larvae were collected from soybean fields near Rio Claro, Brazil in 2019 - 2023 and transported to Germany. Beetles were reared at 23-26°C, 55-60% humidity and 16 h day: 8 h night cycle. They were fed on a diet of lettuce and soybean leaves, and were provided autoclaved tap water in 50ml- tubes with a cotton lid. Adult females laid clutches of approximately 250- 300 eggs, which were collected for experiments.

3.2. Bacterial culture conditions

The *B. gladioli* Lv-StA bacterial symbiont was previously isolated from *Lagria villosa* beetles (35). This strain was inoculated from glycerol stock into King's B (KB) liquid or solid media (soybean peptone 20g/L, K_2HPO_4 1.5 g/L, $MgSO_4.7H_2O$ 1.5 g/L; agar 15 g/L for solid media) before each experimental assay. The medium or agar was supplemented with kanamycin (50 µg/ml) for culturing mutant strains. Media was usually sterilized by autoclaving at 121°C, 15 psi for 20 mins. Liquid cultures were incubated at 30°C, with shaking at 200-220 rpm, overnight.

For targeted mutagenesis, Lv-StA was cultured in MGY media (yeast extract 1.25 g/L , glycerol 10 g/L, M9 salts, part A: 70 g/L K_2HPO_4, 20 g/L KH_2PO_4; part B: 0.58 g/L tri-sodium citrate dihydrate, 1 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4$). Double selection agar for selecting mutants was prepared by first autoclaving a mixture of Glycerol 10 g, M9 salt A 20 ml, M9 salt B 20 ml, L-amino acid mix 2 g, D,L-4-chlorophenylalanine 1 g and agar 24 g. The following filter sterilized solutions were added to this autoclaved mixture: vitamin solution 2ml, trace element solution 1 ml, L-Leucine solution 100mM 16.8 ml, L-histidine solution 60 mM 5ml, L-lysine solution 100mM 10 ml, L-tryptophan solution 40mM 10 ml, L-methionine solution 40 mM 10 ml)(46) and supplemented with 300 µg/ml kanamycin. Glycerol stocks of all cultures were made by adding an equal volume of 70% glycerol to the freshly grown cultures and stored at -80°C.

3.3. Generating an Lv-StA transposon mutant library

To identify candidate colonization factors, we used a transposon insertion sequencing approach. Tn5 transposon mediated insertions were introduced into *B. gladioli* Lv-StA by conjugation with an *E. coli* WM3064 strain carrying the plasmid pRL27. The detailed protocol is provided in (Ganesan, Kaltenpoth and Flórez, 2021) (45).

3.4. PCR and sequencing

3.4.1. Isolating individual mutants from selective plates

DNA was extracted from the 20 individual mutant colonies that were isolated from the selective antibiotic plates after generating the transposon mutant library. DNA from each mutant was used for PCRs to check i) for the presence of the kanamycin resistance cassette, ii) if the isolated mutants were Lv-StA and not contaminants, and iii) to identify the mutated genes. PCRs were performed on a Biometra Tprofessional Thermocycler (Biometra, Germany).

3.4.2. PCR to test for successful trans-conjugants:

We tested whether the kanamycin cassette was successfully inserted into *B. gladioli* Lv-StA using a PCR with the following primer pairs tpnRL17_1RC (5′-CGTTACATCCCTGGCTTGTT-3′) and tpnRL13–2RC (5′-TCGTGAAGAAGGTGTTGCTG-3′) **(Table-1)**. A protocol of the PCR is provided in (45). The outcome of the PCR was evaluated by a standard agarose gel electrophoresis.

3.4.3. PCRs for the identification of Burkholderia sp.

To ensure that the mutants isolated from the selective plates after conjugation were indeed *Burkholderia* and not contaminants or the conjugation donor *E. coli* strain, we performed a PCR targeting the 16s rRNA gene of *B. gladioli* using the *Burkholderia*-specific primers Burk16s_1_F (5′- GTTGGCCGATGGCTGATT-3′) and Burk16s_1_R (5′- AAGTGCTTTACAACCCGAAGG-3′) **(Table-1)**. The 10µl PCR mix contained 4.92 µl HPLC purified water, 1 µl Peqlab Buffer S (10x high specificity), 0.2 µl MgCl2 (25 mM), 1.2 µl dNTPs (2mM), 0.8 µl of each primer (10 µM), 0.08 µl VWR Taq polymerase (5 U/µl) and 1 µl template DNA. PCR conditions are initial denaturation (95°C, 3 min, 1 cycle), followed by 30 – 35 cycles of denaturation (95°C,40 s), annealing (58.2°C, 40s) and extension (72°C, 4 min), and a final extension step (72°C, 4 min). The thermocycler was paused at 4°C after the end of the cycles. The result of the PCR reaction was evaluated by a standard agarose gel electrophoresis.

3.5. PCR to identify individual mutants and verify the insertions

We performed PCRs to identify if the insertion locations in the individual mutants were in random genomic locations. Since the location of the transposon-mediated insertions of the kanamycin cassette are unknown, we designed primers such that the last five base pairs (highlighted in **bold**) at the 3′-end of the random primers targets one of the many repeating Kmers that occur in the Lv-StA genome. These were paired with a primer that targets and amplifies outwards from the edge of the kanamycin cassette. The first step of the PCR was performed with the random primer, here, Burkh_arb5F (5′ **GCCACGCGTCGACTAGTAC**NNNNNNNNNN**CAGCT** – 3′) (28) and the Tn5- Kanamycin insert specific primer, tnp-K13 (5`- AGCTCTCATCAACCGTGGC -3′) **(Table-1)**. In a second PCR reaction, products of the first PCR were amplified using primers tnpRL13-2 (5′- CAGCAACACCTTCTTCACGA- 3′)(47) and Arbstep2 (5′- **GCCACGCGTCGACTAGTAC** - 3) (28) **(Table-1)** where Arbstep2 falls on the tail of the Burkh_arb5F (overlapping regions are highlighted in **blue**). Other random primers designed for this purpose are in **Table-1**. A 10 µl PCR mix was prepared and contained 4.92 µl water, 1 µl Peqlab Buffer Y (10x High Yield), 0.2 µl MgCl2 (25 mM), 1.2 µl dNTPs (2 mM), 0.8 µl primer 1 and primer 2 (10 µM) each, and 0.08 µl VWR

Taq polymerase (5 U/µl), and 1 µl template DNA. PCRs I and II were performed under the following conditions: lid temperature = 94°C, initial denaturation (94°C, 3 min), and 35 cycles of denaturation (94°C, 40s), annealing (PCR1 = 58°C, PCR II = 59°C, 30s) and extension (72°C, 40s), followed by final extension at 72°C for 4 min and the products were held at 4°C. Results of the PCR reaction was evaluated by a standard agarose gel electrophoresis.

3.6. Sanger sequencing to identify Tn-insertion site in selected mutants

Amplified DNA fragments from the individual mutants were purified using Analytik Jena Innuprep PCR pure kit (Analytik Jena AG, Jena, Germany) according to the manufacturer's protocol. The clean PCR products were then prepared for Sanger sequencing. The reaction mixture was prepared by mixing 1 µl of tnpRL13-2 or ArbStep2 (10µM) **(Table-1)**, 1µl of the purified PCR product, 5µl of ddH2O to a total volume of 7µl. Sanger sequencing was done at StarSEQ GmbH, Mainz, Germany.

3.7. Confirmation of neutral mutant insertion site:

A mutant identified from the previous PCR had the Tn5-mediated Kanamycin insertion in an intergenic site. To confirm the site of the insertion, we used primers targeting the Lv-StA genomic region, or the insertion cassette and the flanking genomic region.

Using the primers specific for Lv-StA wild type strain, Lv-StA_m10_fwd (5`-GCTCGCGGTCAGATGGATTA – 3`) paired with Lv-StA_m10_rev (5'- GATGAGACGTCCGTCCGTAC – 3') **(Table-1)** we expect a product of 310 bp. Using primers targeting the insertion in the mutant and the flanking genomic region, *i.e.*, Tn5_m10_fwd (5' - ACACCTTCTTCACGAGGCAG – 3') paired with Lv-StA_m10_rev **(Table-1)**, we expect a product of 282 bp size for the mutant.

The mutant was streaked from glycerol stock on a fresh KB plate containing kanamycin. Single colonies (×4) of the m10 mutant were picked using a sterile toothpick and dissolved in 4 µl water in the PCR tubes. PCR was performed using the Qiagen Taq PCR master mix kit (Qiagen, Netherlands). 5 µl of Taq PCR master mix (2×), and 0.5 µl each of the PCR primer pairs were added to the 4 µl colony mix to a final volume of 10 µl. The PCR was run with the given conditions: Initial denaturation (94°C, 3 min, 1 cycle), followed by 35 cycles of Denaturation (94°C, 40 sec), Annealing (55 – 62°C gradient, 30 sec), and Extension (72°C, 40 sec), and finally, one cycle of final extension at 72°C for 40sec, and then the reaction was paused at 4°C. Results of the PCR reaction were evaluated by standard agarose gel electrophoresis.

3.8. Egg surface sterilization

For infecting the Tn-mutants and the mutants generated by targeted mutagenesis, freshly laid *Lagria villosa* eggs were collected and surface sterilized to remove symbionts. Eggs were washed once with 70% ethanol for 5 min, 12% bleach for 30 s and water between each step. Sterilized eggs were counted and split into equal-sized groups based on the number of treatments for each experiment. They were placed in a petri dish lined with wet vermiculite and a filter paper. For the targeted mutant infections, each group of eggs within a petri dish was further split into clusters of 10 eggs to ensure an even symbiont infection on all the eggs.

3.9. Tn-mutant library infection and DNA extraction

The generated library contained 2×10^4 unique mutants (exactly 24,224) (45) and, on average, 2×10^3 Lv-StA cells colonize a single first-instar larva (37). *Lagria villosa* beetles lay egg clutches of 250- 300 eggs. To ensure that the complete mutant library was properly represented in the experiment, we calculated the number of eggs necessary so that each mutant was sampled approximately 200 times. We infected a homogenous aliquot of the Tn-mutant library on 13 clutches and collected 100 second-instar larvae per clutch. Before infection, the pooled mutant library was split into 13 aliquots in 15 ml-tubes, and replicate clutches were infected with one aliquot each. Infection on eggs and growth *in vitro* were performed as given in (45). DNA extractions were performed using the Epicentre MasterPure™ Complete DNA and RNA extraction kit according the manufacturer's instructions and as given in (45). After extraction, the samples were air-dried for 15-30 mins. The DNA was suspended in Low-TE buffer (1:10 dilution of TE) and stored at -20°C. (**Fig. 1)** gives a summary of the steps involved.

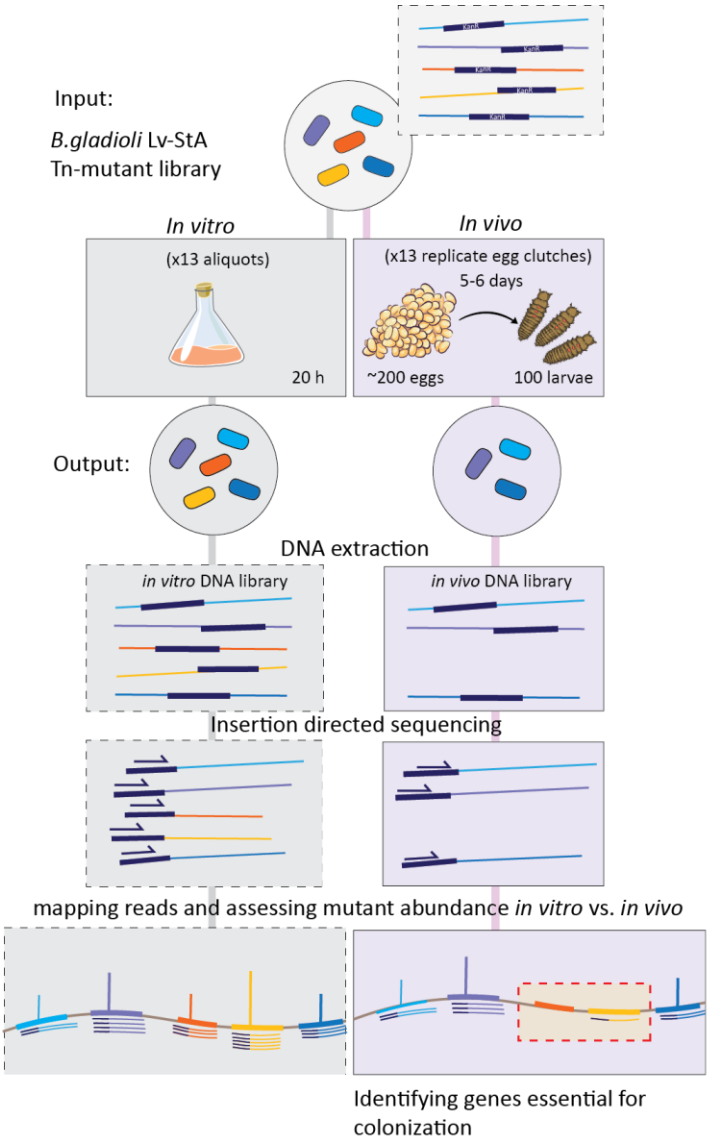


Fig.1. Schematic of the Tn-seq experiment. Tn-mutant library was infected on eggs and grown *in vitro* as a control. To assess which mutants were lost while colonizing the larvae, we extracted the DNA from *in vitro* and *in vivo* infected samples, sequenced outwards from the site of Tn-insertion, and mapped reads to the genome of Lv-StA. Genes possibly involved for colonization were identified by comparing the mutant abundance in the two conditions.

3.10. DNA library preparation and insertion-directed sequencing

DNA libraries were prepared for sequencing using the NEBNext® Ultra™ II DNA library prep kit for Illumina® (New England Biolabs) with some slight modifications as given in (45). Using an ultrasonicator (Bandelin electronic GmbH & Co, Berlin, Germany), with 70% power, the DNA samples were sheared on ice to fragment the DNA to 200 – 400 bp size. Using the NEBNext End prep protocol, Illumina sequencing adapters were added to the sheared DNA. Adapter-ligated DNA fragments were mixed with magnetic beads (AMPure

beads XP (magnetic + polyethylene gylcol + salts), Beckman Coulter, USA) to select for fragments around 250 bp in length.

We performed a PCR to add a biotin tag to select for fragments containing the Tn5-inserted kanamycin cassette. A 50 µl PCR mix was prepared with 15 µl of the Adapter-ligated DNA fragments, 25 µl of the NEBNext Ultra II Q5 master mix (New England Biolabs), 5 µl of each Primer – 1. Transposon-specific biotinylated primer, Biotin_pRL27_2255F (5′-Biotin- ACAGGAACACTTAACGGCTGACATG -3′) and 2 **(Table-1)**. Index primers from the NEBNext Multiplex Oligos for Illumina Index primers set 1 (New England Biolabs) **(Table-1)**. Conditions for PCR are given in (45).

The resulting fragments from PCR I were cleaned using magnetic beads without size selection and then streptavidin beads (Roth, Germany) were used to select the fragments containing the biotin tag. A second PCR was done on the DNA bound to the streptavidin beads to complete the NEBNext adapters attached to the selected fragments. The 50 µl PCR mix contained 15 µl of the bead –selected DNA, 25 µl of the NEBNext Ultra II Q5 master mix (New England Biolabs), 5 µl index primers (NEBNext Multiplex Oligoes for Illumina Index primers set 1) and 5 µl of the modified universal PCR primer, Univ_pRL27_2296F, (5′- AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC TTCCGATCTGAATTCATCGATGAT GGTTGAGATGTGT – 3′) **(Table-1)**. The conditions are the same as mentioned before. After DNA library preparation, samples were stored at -20°C.

3.11. Illumina sequencing and analysis

Before sequencing, we performed another clean-up with magnetic beads as recommended by the sequencing company (StarSEQ GmbH, Mainz, Germany) to remove adapter-adapter fragments that can occur due to overamplification during the PCR cycles. The resulting DNA library was sequenced by StartSEQ GmBH, with paired-end Illumina technology, targeting 150 bp reads, and to a depth of 20 Mio reads per replicate. Read quality was assessed with FastQC (48), and reads containing the transposon-insertion edge on the 5′end were selected. The insertion cassette was trimmed using Cutadapt (49) and Trimmomatic (50). We considered that the mutations at the edge of genes are not disruptive functionally and trimmed 5% off both ends in the GFF annotations of the Lv-StA reference genome. Trimmed reads were mapped using Bowtie2 (51). The number of unique insertions was calculated from the unique 5′positions of the alignments from the BAM file in Galaxy (52). Using featureCounts (53) we obtained the number of genes hit for each replicate sample and calculated the mutant abundances between different conditions using DESeq2 (54).

3.12. Kegg pathway reconstruction and functional comparison of Lv-StA and LvStB genomes.

Reference genomes of Lv-StA and Lv-StB were available from previous studies and manual annotations were used for the analysis (35,39). Using BlastKOALA (55), KO terms were assigned to two subsets of genes: 1. candidate colonization factors (Deseq2, padj < 0.05) and 2. genes that were non-essential for colonization (DEseq2 padj > 0.05 and had at least 1 read count in all treatments and replicate libraries). Using the Kegg reconstruct pathway tool, pathways and the potential colonization genes were visualised. Similarly, KO terms for all annotated genes in Lv-StA and Lv-StB genomes were obtained and compared using the Kegg pathway reconstruction tool.

3.13. Fluorescence in situ hybridization (FISH)

We collected second-instar larvae infected with the mutant library to check for the presence of mutants in the dorsal structures using a standard FISH protocol (56,57). The DNA probe Burk_16s-cy5 (5'- Cy5-TGCGGTTAGACTAGCCACT-3') was used to tag the 16s rRNA of Lv-StA, EUB338-Cy3 (5'- Cy3 – GCTGCCTCCCGTAGGAGT- 3') was used to target the 16s rRNA of general bacteria, and host cell nuclei were counter-stained using DAPI (4',6-diamidino-2-phenylindole) **(Table-1)**. Images were captured on AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany).

3.14. Targeted mutagenesis

3.14.1. Plasmid construction

The knockouts in Lv-StA were designed based on genes featured in the Tn-Seq results as candidate colonization factors or previously reported as colonization factors in other symbioses. We targeted genes for flagellin biosynthesis (*fliC*), flagellar hook-length control (*fliK*), polyhydroxybutyrate granule formation (*phbF*) and an FLP pilus assembly related secretin (*cpaC*). Primers used to amplify the flanking homologous regions of the target genes are given in **Table-2**. The homologous regions were amplified using KAPA2G Robust HotStart ReadyMix (2×, with dye, Sigma Aldrich). The 20 µl master mix contained 7.5 µl ddH2O, 10 µl KAPA2G Robust HotStart ReadyMix, 0.5 µl of gDNA and 1µl of each primer (10 µM). PCR was performed with initial denaturation (95°C, 90 s), 35 cycles of denaturation (95°C, 15 s), annealing (60 or 65°C, 10s) and extension (72°C, 15 s), followed by final extension (72°C, 5 min) and hold at 14°C. The kanamycin cassette was amplified from pJK347 using primers JK583 and JK1032 designed for a previous study (42).

To ensure double cross-over mutants were obtained, we used a plasmid containing the *epheS* gene in the backbone. The *epheS* gene encodes a phenylalanine t-RNA synthetase that charges t-RNAs erroneously with 4-chloro-phenylalanine instead of phenylalanine. Single cross-over mutants that retain the backbone of the plasmid, and therefore the *epheS* gene, can be removed by double selection on media containing 4-chloro-phenylalanine (58–60). The *epheS* (60) was previously introduced into the *Hind*III restriction site of pJK347. pJK347-epheS plasmid backbone was *Xho*I/*Xba*I-digested (5 µl NEB Cutsmart® buffer (New England Biolabs), 2 µl XbaI, 2 µl XhoI, 41 µl pJK347 plasmid incubated at 37°C for 1 hour) to generate sticky ends.

Using the NEBuilder Hifi DNA assembly kit (New England Biolabs), the plasmid was built in a 4- fragment assembly. The master mix included 2 µl NEBuilder master mix, 1 µl plasmid backbone, 0.5 µl KanR cassette, 0.25 µl front homologous region and 0.25 µl back homologous region and was incubated at 50°C for 60 min and stored at -20°c after the reaction. After assembly, 1-2 µl of the plasmid was transferred into *E.coli* TOP10 and transformants were selected on LB plates containing kanamycin (50µg/ml) and ampicillin (100 µg/ml) after 16 hours. Glycerol stocks of the *E.coli* TOP10 carrying the transformation plasmids were prepared using a 1:1 mixture of culture and 70% glycerol and stored at -80°C. Before transforming Lv-StA, the NEB Monarch® plasmid miniprep kit (New England Biolabs) was used to extract the plasmid from the stocks, following the protocol given by the manufacturer. The plasmid was verified by test-digest and Sanger sequencing of the homologous region.

3.14.2. Transformation into B.gladioli Lv-StA

Lv-StA cells were grown in 2ml MGY media to prepare a pre-culture and grown overnight at 30°C. After overnight growth, 2 ml of the pre-culture was inoculated in 20 ml pre-incubated sterile media in a conical flask. The freshly inoculated culture was incubated up to an OD of 0.6-0.8. The 20 ml culture was transferred to 50-ml tubes and pelleted by centrifugation at 7,500 rpm for 5 min at 21°C. The pellet was washed once with 35 ml autoclaved water followed by a wash with 20 ml water. The pellet was finally suspended in 150 µl of ddH2O. 5 µl of plasmid and 50 µl of washed cells were added to the 1 mm-gapped electroporation cuvette and electroporated at 2500 V (Eppendorf, Hamburg, Germany). 500 µl of MGY medium was added to recover the cells and incubated for 2-3 hours at 30°C with shaking. The cell mixture was then plated on NAG agar (Nutrient agar (Bacto,BD), 1 % glycerol) containing 300 µg/ml kanamycin and incubated at 30°C until colonies appeared on plate. Transformants were picked and re-streaked on double selection agar (46) supplemented with 300 µg/ml kanamycin.

We confirmed the presence of double-crossover insertions by doing colony PCRs using the primers mentioned in **Table-3** and Sanger sequencing. **Table-4** shows the expected product sizes of the colony PCR for successful knockouts, wild type strains, and single-cross overs. Mutant cultures were stored in glycerol stocks at -80°C.

3.15. Testing strain and mutant motility

Motility of Δ*fliC*, Δ*fliK* strains was assessed using a hanging drop motility test. The mutant strains and wild type Lv-StA were grown overnight in given culture conditions. On a glass slide, a ring of parafilm was constructed and placed in the middle to make a well-like structure. Using a sterile inoculating loop, a drop of the liquid culture was placed in the centre of a clean cover slip. The cover slip was slowly inverted over the glass slide to let the drop hang within the well. Using a Leica DMi1 inverted microscope we observed the motility of the mutant strains and wild type at 6 h and 24 h post inoculation.

3.16. In vivo mono-colonization assays

Targeted mutants Lv-StA:Δ*fliC*, Δ*fliK* and Δ*icoS* and the wild type Lv-StA (WT) were infected on eggs to test the success of colonization. Eggs from field-collected beetles (2022, 2023) were collected within 24 hours of egg-laying. A fresh culture of mutants and the WT strains were inoculated in 10 ml KB media and grown overnight. 4 ml of the cell cultures were aliquoted into 1.5 ml- tubes and centrifuged at 10,000 rpm for 5 min. Cell pellets from each aliquot were washed with 750 µl 1x PBS three times and pooled to make a final pellet. The final pellet was resuspended in 100 – 150 µl 1x PBS. The number of cells in the suspension was assessed using a Neubauer cell-counting chamber. Each mutant and WT strain was diluted to 2.5×10^7 cells/µl concentration in 100-200 µl 1x PBS. Mutant and WT cells were infected on the sterilized eggs from four clutches (0.5 µl/egg), and the eggs were incubated under normal rearing conditions, until they hatched. Individual first-instar larvae were collected, surface-washed with 1% SDS (3 times), 1x PBS (2 times) and crushed in 100 µl 1x PBS. The crushed suspension was diluted 1:100 and 1:1000 times before plating on KB and KB plates containing kanamycin (50 µg/ml). After 24-30 hrs, cells appearing on each plate were counted and CFUs/individual were calculated as follows:

$$CFUs\ per\ individual = \frac{CFU\ counts\ \times\ dilution\ factor}{\frac{Volume\ plated\ (80\ \mu l)}{Volume\ of\ suspension\ (100\ \mu l)}}$$

3.17. In vivo competition assays

Targeted mutants Lv-StA:Δ*fliC*, Δ*fliK* and the WT were cultured and cells were counted as mentioned above, and the cell count in 1x PBS was adjusted to 1-2 × 10^7 cells/µl. Serial dilution of the 10^7 cell mix was done to get concentrations of 10^5 and 10^4 cells/µl for each strain. Equal volumes of the mutant and WT strains were mixed to generate a 1:1 cell mixture (10^7 each) for the first competition experiment. In a second competition experiment, 1:1 cell mixtures of lower concentrations (Wild type: mutant 10^4:10^4 and 10^5:10^5) were generated. The respective competition cell mixtures were then inoculated on eggs from one replicate clutch as a pilot experiment (0.5 µl/egg). Similar to the mono-colonization assays, first-instar larvae from the infected eggs were collected, surface washed with 1% SDS and plated on KB plates with and without kanamycin (50 µg/ml). The WT and mutant colonies can grow on KB plates, however, the KB plates with kanamycin can only support the growth of the mutant colonies since the WT is not resistant to the antibiotic. Therefore, to obtain WT counts, total counts of colonies on KB plates containing kanamycin were subtracted from those on KB plates. Thus, we calculated the CFU per individual for both WT and the mutant from each infected larva.

3.18. Statistics and sequencing analysis

Statistical analysis was done in RStudio (61). For estimating the number of colonizing cells, the experimental and statistical analysis details are given in (Ganesan *et al.* 2023)(37). Tn-seq analysis was performed with DESeq2 as given in (Ganesan *et al.* 2021) (45).

A linear mixed effects model (LME) was used for statistical analysis of differences in CFU count data between mutant strains and the wild type in the mono-colonization and competitive colonization assays, using the nlme package (62). For the mono-colonization assay and the 1:1 competitive colonization assay with 10^7 cells/µl of wild type and mutant, the strain type was set as fixed effect and clutch-ID was included as the random effect. Normality of the residuals was tested with a Shapiro-Wilk test. The influence of the fixed effect was tested by comparing it to a null model using a likelihood ratio test. For the 1:1 competitive colonization assay with one clutch (MC13) with different inoculum sizes, 10^4, 10^5, 10^7 cells/µl of wild type and mutant each, strain type was set as fixed effect, larva-ID was set as random effect since wild type counts were paired to mutant counts. If the residuals were not normally distributed, data was transformed. A likelihood ratio test comparing the full-model with the null model was used to assess the influence of the fixed effect.

3.19. Data availability

Reference genome of Lv-StA was obtained from the NCBI database (Genbank assembly accession: GCA_026651625.1) and manual annotations were used for the Tn-seq analysis.

4. RESULTS

4.1. Identifying mutants with PCRs

We first performed PCRs on twenty randomly chosen Tn-mutants to identify the site of insertion. 8 out of 20 samples could be successfully amplified, and among the 8 mutants, sequencing results showed that one carried an insertion at an intergenic site roughly between 3,844,516 bp and 3,844,849 bp on chromosome-1. An insertion at an intergenic site is unlikely to have any fitness consequences for the cell. Furthermore, we performed colony PCR to confirm an insertion at this site. The results showed the presence of mutant and wild type genomic DNA in a single colony. This indicated that *in vivo* infection of the library included a mixture of mutants and wild type cells.

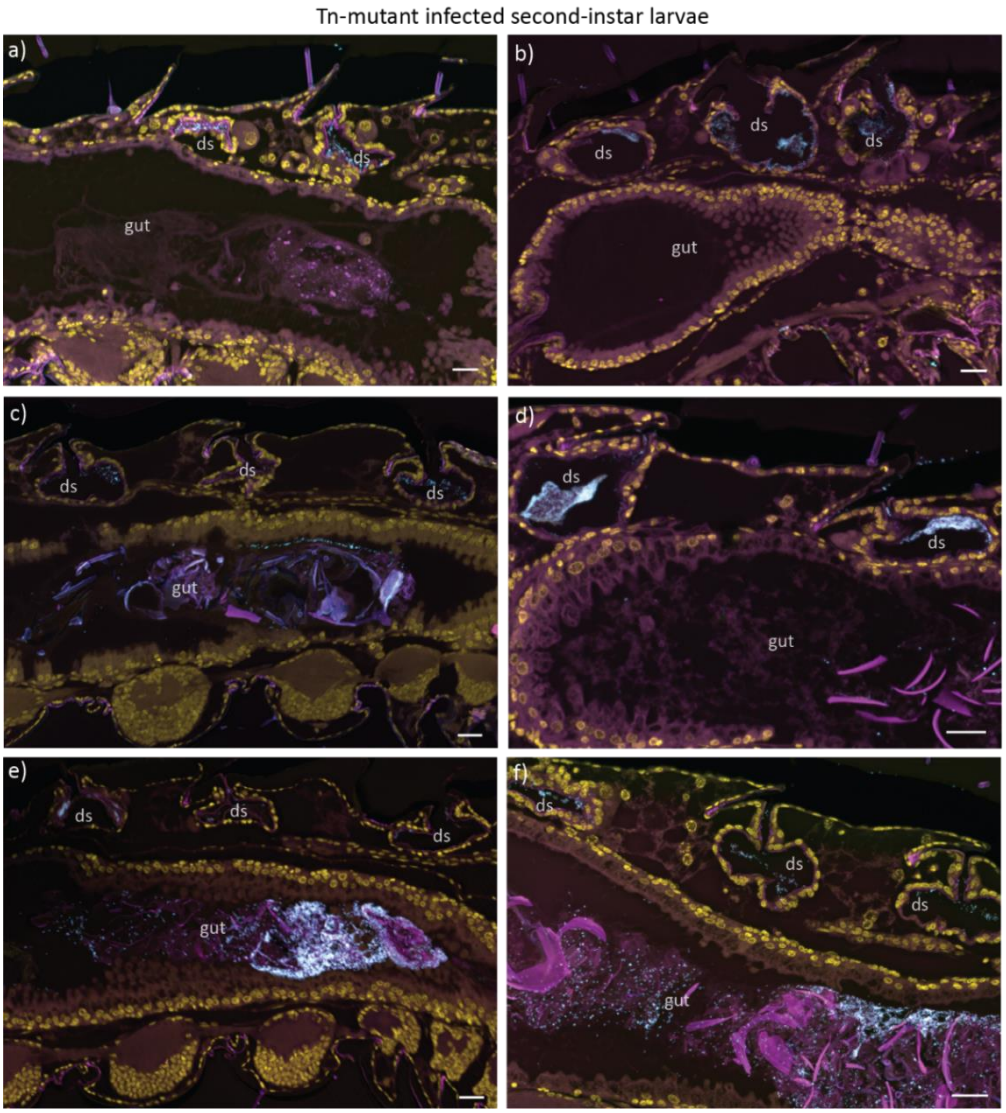


Fig.2. FISH images of Tn-mutant-infected second-instar larvae: Six second-instar larvae carrying varying numbers of Burkholderia sp. **(a-f)** in the dorsal structures (ds) **(c-f)** and the gut. In total, Tn5-mutants were present in the gut in 8 out of 10 larvae.

4.2. Fluorescent in situ hybridization to confirm mutant library infections

We imaged second-instar larvae to confirm that the bacteria colonize the dorsal structures and that any cells on the larval cuticular surface were shed along with the first-moult. We performed FISH using probes specific for *Burkholderia* sp. (Cy5 – shown as Turquoise), eubacteria (Cy3 – shown as Violet) and host cell nuclei are stained with DAPI (shown in yellow). *Burkholderia* cells colonized the dorsal structures **(Fig. 2)** and in 8 out of 10 cases, were also found in the gut. This indicates that in the laboratory setting, second-instar larvae likely consume the exuvia after molting. This implies that among ~1,300 larvae infected, close to 80% of the larvae may contain transient Tn-mutants in the gut. Presence of the cells in the gut may result in failure to recognize some genes involved in colonization, *i.e.*, we may have false negatives in our Tn-seq analysis.

4.3. Potential colonization factors in Lv-StA

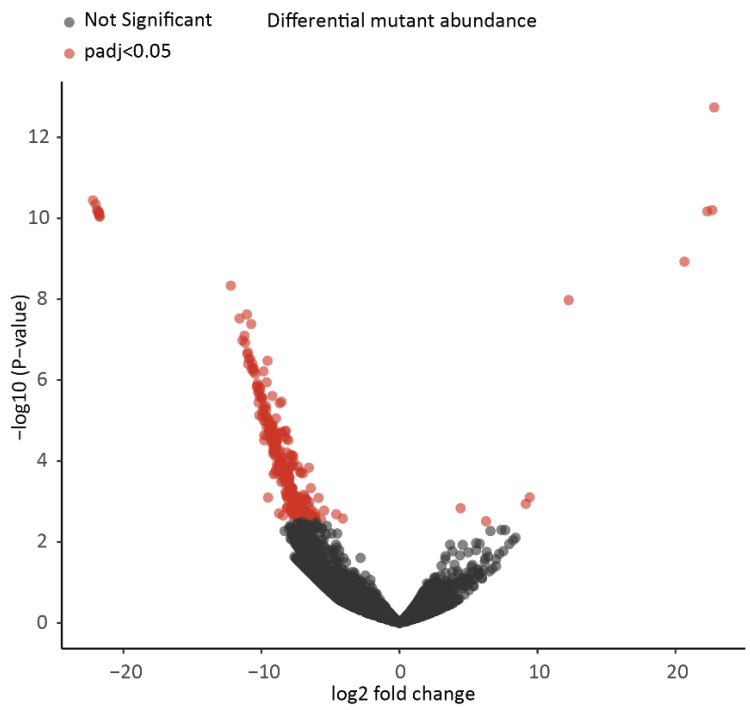


Fig.3. Volcano plot shows differential mutant abundance in vivo: The red dots depict mutants that were significantly lower or higher in the *in vivo* infected library compared to the *in vitro* grown library.

Results from the Tn-seq analysis show that a total of 24,224 transposon mediated insertions hit 3,736 genes from a total of 7,468 annotated genes in the Lv-StA genome, possibly rendering them non-functional (45) **(Fig. 3)**. Among the 3,736 genes hit in the original Tn-seq library, we estimated that 271 genes **(Table-5)** could have some functional importance *in vivo*, since disruption of the genes changed mutant abundance *in vivo* compared to the *in vitro* growth condition (45). Among the 271 mutants that were differentially abundant, 262 were depleted *in vivo* and 9 were relatively more abundant *in vivo* **(Table-5, Fig.3)**. Notably, the candidate colonization factors included traits previously implicated in host colonization across other symbioses, *i.e.*, motility, biofilm formation, iron metabolism and stress response and we highlight these genes in the following sections. Furthermore, to assess whether the two strains of *Burkholderia* associated with the beetles use similar strategies for colonization, we compared the KO-terms and used Megablast to find orthologs of Lv-StA-candidate colonization genes in Lv-StB. Interestingly, 91 of the 271 factors showed hits for orthologous genes within two of the three circular chromosomes of Lv-StB **(Table-5)**.

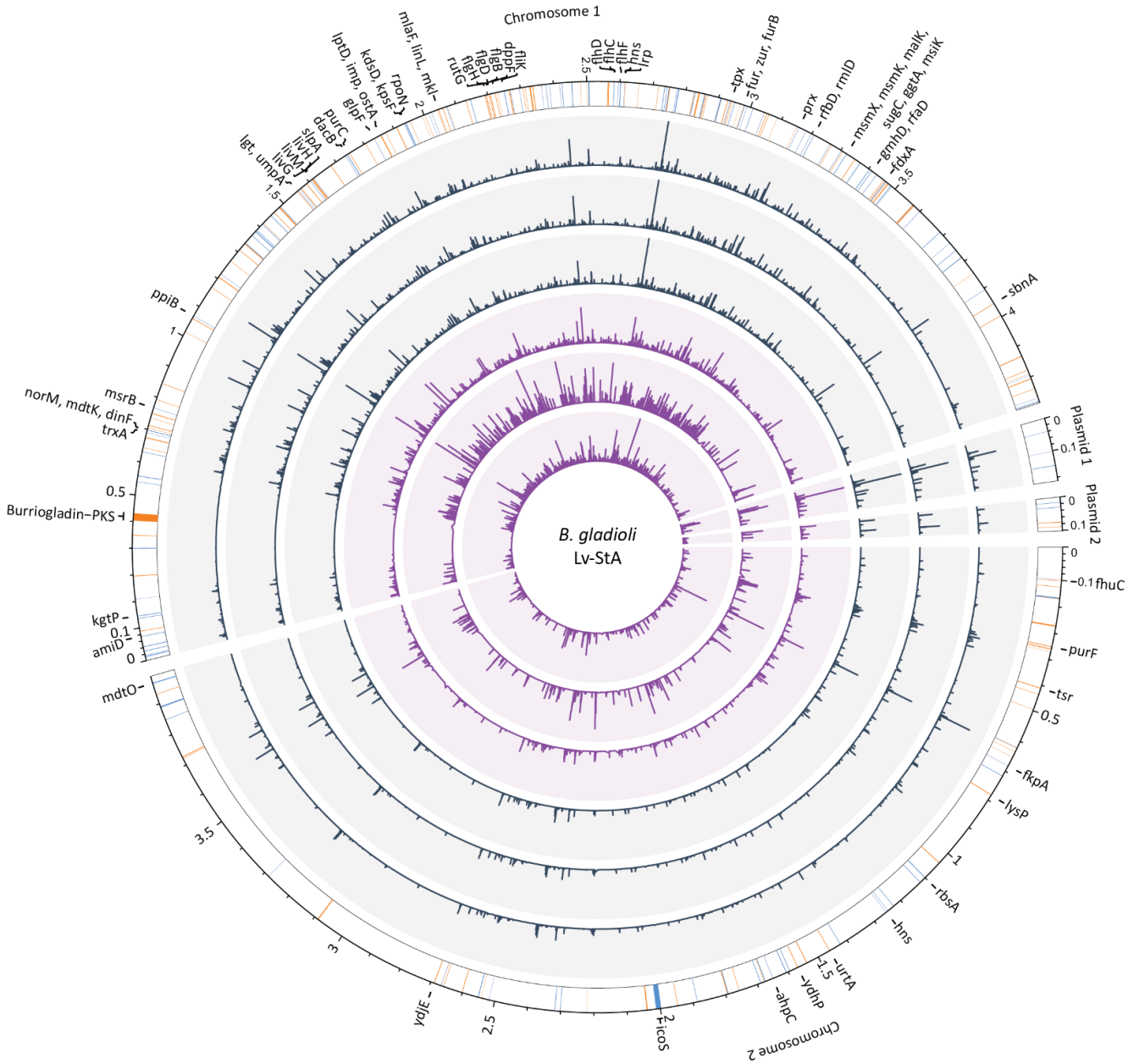


Fig.4. **Representation of the Lv-StA genome highlighting potential colonization factors:** Lv-StA genome includes two chromosomes and two plasmids. The 271 potential colonization essential genes are indicated in the outer track. Genes in the negative strand direction are indicated as orange bars and those in the positive strand direction are in blue. Only the genes that are mentioned in the following text are labelled. The following three tracks (light grey) show the coverage of Tn-insertions (normalized cpm) in the *in vitro* replicate libraries and the three inner tracks (purple) show the coverage of Tn-insertions (normalized cpm) in the *in vivo* replicate libraries.

4.3.1. Motility and biofilm formation

The female *Lagria villosa* beetle smears the symbionts onto the egg surface during oviposition and the bacteria colonize the dorsal structures of a first-instar larva during or after hatching (35,37). We suspected that a combination of host movements and bacterial motility could mediate symbiont entry into the dorsal structures. In the Tn-mutant library, several genes involved in flagellar biosynthesis were hit by insertions that possibly rendered the genes non-functional (**Fig. 5** all coloured genes). The candidate genes that are likely essential for Lv-StA during colonization are *fliK*, *flgD*, *flgH*, *flgB*, *flhC*, *flhD* and *rpoN* (**Fig. 5** genes in red) since Tn-mutants of these targets were depleted *in vivo* but survived *in vitro*. Other genes (*fliD*, *flgL*, *flgK*, *flgF*, *flgG*, *flhA*, *fliA*, *fliT*, *fliY*) showed no differential representation during *in vivo*

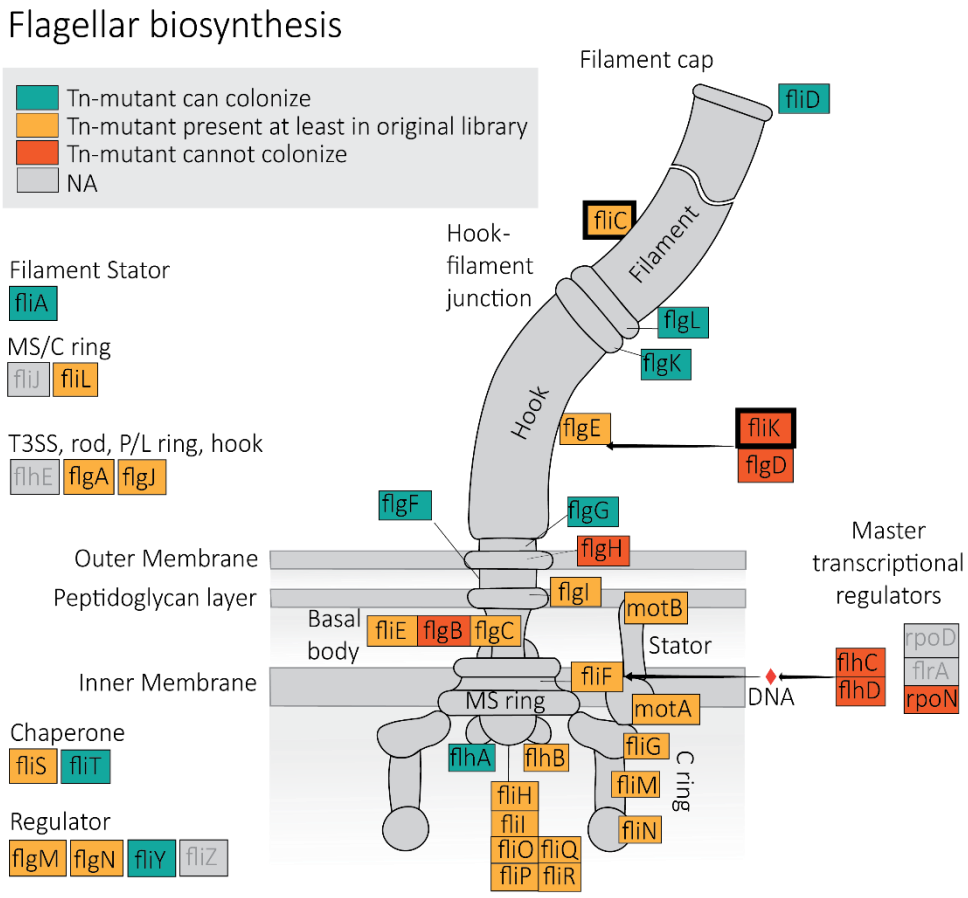


Fig.5. Schematic of genes involved in flagellar biosynthesis: Genes in red were present *in vitro* but not *in vivo*, and therefore, may be important for colonization. Genes in green were present *in vitro* and *in vivo* and therefore may not have differential impact in either conditions. Genes in yellow were hit in the original Tn5-mutant library but were not present in the *in vivo* or *in vitro* sequencing results. Their involvement in colonization is unknown.

colonization and *in vitro* growth (**Fig. 5** in green) and are therefore not important for symbiont establishment. Genes highlighted in yellow (**Fig.5)** were mutated in the original Tn-mutant library but the mutants were absent in both *in vitro* and *in vivo* sequencing outputs. Therefore, their involvement in colonization is unknown. The genes in grey (**Fig. 5)** were not mutated in the original library.

Among the genes potentially important for colonization (**Fig. 5** genes highlighted in red), are first the flagellar transcription regulators (*flhC*, *flhD*, *rpoN*). The flagellar transcriptional regulator, *flhCD*, is a class I gene or the flagellar master regulator that induces or inhibits flagellar gene expression, biofilm formation and production of secondary metabolites (63,64). *flhDC* initiates class II genes that are responsible for the synthesis of the flagellar hook-basal body and include the regulators *flgM* and *fliA*. Class III genes synthesize the complete flagellum and are involved in chemotaxis. Mutation in *flhCD* would possibly affect flagellar assembly and motility in Lv-StA and also downstream processes like biofilm formation, and production of secondary metabolites.

Apart from *flhCD*, we identified four more flagellar assembly genes that were important for Lv-StA during colonization - *flgD*, *flgB*, *flgH*, *fliK*. *flgD* forms the hook-cap and is transiently associated during basal rod and hook polymerization, *flgB* encodes the external proteins for the proximal rod, *flgH* is part of the flagellar hook basal body and *flik* controls the length of the hook by acting as a molecular ruler (63,65).

Mutations in the following flagellar biosynthesis genes - *fliD*, *flgL*, *flgK*, *flgF*, *flgG*, *flhA*, *fliA*, *fliT*, *fliY* did not affect colonization. *fliD* is the filament cap that inhibits *fliT* and also inhibits class II gene transcription while class III promotors are transcribed (63). *flgL* and *flgK* form the hook-filament junction, *flgF* is part of the proximal rod, *flgG* is part of the distal rod, and *flhA* encodes the integral membrane protein. Binding of *flgM* to *fliA* could inhibit flagellin gene transcription and *fliT* is another regulator that inhibits *flhDC* activation and prevents activation of class II promoters after the hook-basal body is assembled (63,65). Therefore, regulators at check-points of flagellar assembly in Lv-StA were mutated, but the mutations did not affect colonization.

Furthermore, several environmental factors affect flagellar biosynthesis and biofilm formation, like leucine-responsive regulator protein, Lrp (66), DNA-binding protein, Hns (67), and a methyl accepting chemotaxis protein, Tsr (68). In Lv-StA, these genes could possible regulate multiple microbial factors for host colonization. We also find that Tn5-mutants carrying insertions in the genes, *purF* and *purC*, are unable to colonize the *in vivo* environment, and speculate that this may be due to altered biofilm formation (10) in the dorsal structures.

4.3.2. Cell wall components

In gram negative bacteria, like Lv-StA, the outer membrane is riddled with membrane structures, mainly lipoproteins and lipopolysaccharides (LPS). The O-antigen part of the LPS consists of repeating oligosaccharide units linked together and may differ between strains and species (69). In several host-associated bacteria, these cell surface structures interact with host recognition factors (20).

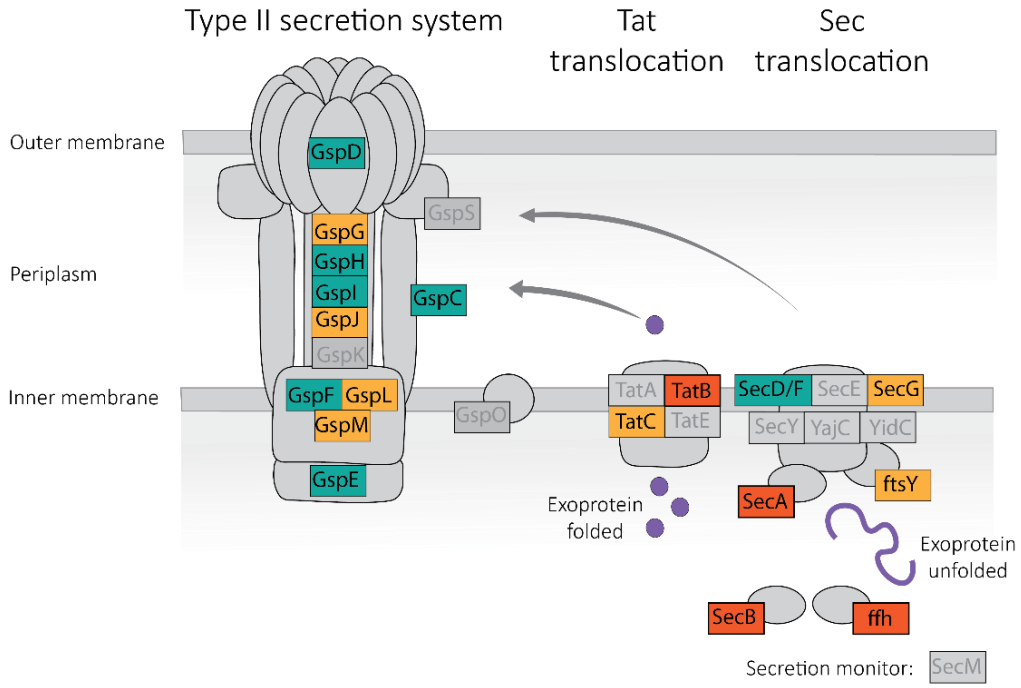
In Lv-StA, a dTDP-L-Rhamnose oligosaccharide is likely a part of the O-antigen structure. The enzymes RfbABCD catalyse the conversion of, D-Glucose 1-phosphate to dTDP-L-Rhamnose. In Lv-StA, a Tn5-mutant of the *rfbB* gene, had no impediment during colonization. However, a disruption of the gene, *rfbD*, in the last step, affects Lv-StA during colonization **(Table-5)**. The shared presence of genes underlying the biosynthesis of dTDP-L-Rhamnose and GDP-6-deoxy-D-talose glycans suggest that Lv-StA and Lv-StB may possibly have similar O-antigen structures.

Furthermore, disruption of *lptD*, an essential gene involved in flipping LPS molecules into the outer membrane, appeared to have negative consequences for colonization by Lv-StA. Another gene involved in the biogenesis of lipoproteins, *lgt*, may also be essential during colonization. More genes involved in LPS biosynthesis (*kdsD* and *gmhD)* and in peptidoglycan biosynthesis (*dacB* and *amiD*) were important *in vivo.* All of the above mentioned genes, except *amiD* have similar genes in Lv-StB, indicating that the genome-reduced strain possibly retains capabilities to build a complete cell wall similar to Lv-StA **(Table-5)**.

4.3.3. Transporters and secretion systems

Bacterial transporters are essential for importing and exporting substances like toxins, antibiotics, amino acids, etc. In Lv-StA, we found several were important for colonizing the *Lagria* larva **(Table-5)***;* a niacin transporter YdjE, a Na+ driven multidrug efflux pump, NorM, a putative transmembrane protein, MdtO, an alpha-ketoglutarate symporter, KgtP, lysine symporter, LysP, putative transporter, YdhP, a glycerol uptake facilitator, GlpF, and a pyrimidine symporter RutG.

Several ABC transporters that are involved in ATP-mediated translocation of proteins across the bacterial membrane, were also potentially relevant during colonization for Lv-StA, but absent from the genome of Lv-StB, like the urea ABC transporter, UrtA, a high-affinity branched chain amino acid transporter – LivG, accompanying permease, livM, and the ATP-binding cassette LivH. A sugar transport system, MsmX, and a ribose ABC transporter (ATP binding subunit), RbsA were other genes important in the *in vivo* environment. Other genes that were both important for colonization of Lv-StA and present in the genome of Lv-StB are the phospholipid/cholesterol ABC transporter (ATP binding subunit), MlaF, and the dipeptide ABC transporter (ATP binding subunit), DppF.



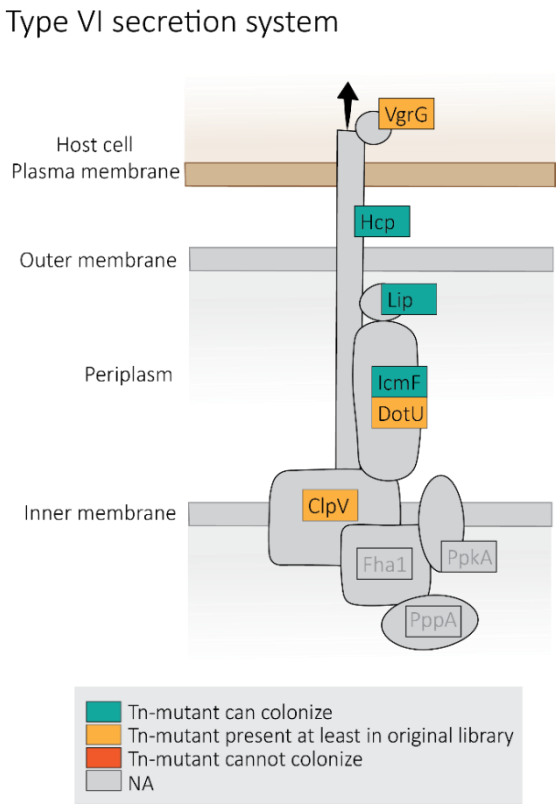


Fig.6. Schematic of the T2SS, Sec & Tat translocation system, and T6SS in Lv-StA: Genes in red were present *in vitro* but not *in vivo*, and therefore, may be important for colonization. Genes in green were present *in vitro* and *in vivo* and therefore may not have differential impact in either conditions. Genes in yellow were hit in the original Tn5-mutant library but were not present in the *in vivo* or *in vitro* sequencing results. Their involvement in colonization is unknown.

The Sec and Tat machineries that transport folded or unfolded proteins across the inner membrane into the periplasmic space, are present in both Lv-StA and Lv-StB. The Sec and Tat transporters seem important for Lv-StA *in vivo* according to the results of the Tn-seq analysis **(Fig. 6)**. In connection to the Sec transport system, folding-proteins like *ppiB* (peptidyl-propyl cis- trans isomerase B), *slpA* and *fkpA* may be also play a role during colonization in Lv-StA. Although Lv-StA is equipped with a T2SS and a T6SS, the Tn-seq results suggest that it is not involved in colonization **(Fig. 6)**. Proteins translocated to the periplasmic space by Sec and Tat transporters are usually secreted through the outer membrane by the T2SS, T4SS or the T5SS.

4.3.4. Secondary metabolites - Lipopeptides

Secondary metabolites produced by *Burkholderia gladioli* strains may help in biofilm formation and swarming (40,70), show protective benefits to insects (35,38,39) and also mediate pathogenesis in humans (71). In our Tn-seq experiment, mutants that had an insertion in a gene involved in icosalide biosynthesis, *icoS*, and another one involved in Burriogladin biosynthesis (fig|6666666.542543.peg.4026) were depleted *in vivo* compared to the *in vitro* condition **(Table-5)**. Since icosalide is a swarming inhibitor (40), in its absence, the Tn5-mutant may swarm more and Burriogladin promotes biofilm formation (70), therefore, the Tn5-mutant may not be able to adhere to the host surfaces or gain the protection of a biofilm matrix on the egg surface or in the dorsal structures of the larvae. Mutants which had Tn-insertions in other polyketide synthase related genes were either too low in abundance in the original library (*pksE*, *pksI*, *pksE*, *pksN*), or showed no significant difference in the *in vivo* or *in vitro* growth conditions. Moreover, these lipopeptides are encoded only by Lv-StA and are absent in Lv-StB.

4.3.5. Iron metabolism

When grown under iron limiting conditions, bacteria produce siderophores to capture free, but insoluble ferric iron (Fe^{3+}), and import the iron-bound siderophores into the cell (72). Antismash (73) results show that in the *Lagria* system, Lv-StA encodes non-ribosomal peptide synthase (NRPS) modules that are involved in synthesis and uptake of megapolibactin-like and plantaribactin-like siderophores that may also have additional functions as nitric oxide (NO) donors (74). Among this cluster of genes involved in plantaribactin-like siderophore synthesis and transport, a protein associated with the ferric hydroxamate ABC transporter (FhuC), seems to be important during colonization in Lv-StA. Interestingly, the strain Lv-StB seems to lack the genes necessary for synthesis of megapolibactin or plantaribactin-like siderophores but only encodes Fe^{3+}-hydroxamate importers (FhuBCD). Apart from this, another protein important for colonization by Lv-StA is a 2,3 – diaminopropionate biosynthesis protein, SbnA, that encodes a precursor for secondary metabolites, including some siderophores.

Furthermore, an iron regulatory gene, *fur,* was also implicated as an essential regulatory component for Lv-StA *in vivo*. In Lv-StA, four distinct copies of the ferric uptake regulatory gene, fur, are distributed across the genome, and one of them has an ortholog that is 91.6% similar in Lv-StB.

4.3.6. Oxidative stress response factors

Moreover, in animal hosts, a strategy to control microbial infection is to produce oxidants including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH.)(75). Bacteria neutralize damages by oxidants using catalases, peroxiredoxins and superoxide dismutases. The amino acid cysteine, when damaged by oxidative radicals, can be repaired by thioredoxins (*trx*), and methionine residues are repaired by methionine sulfoxide reductases (*msr*)(75) both of which; *trxA* and *msrB*, were found important for Lv-StA in the *in vivo* condition. Others that may have similar roles to control oxidative stress in Lv-StA during colonization include *fdxA*, *tpx*, *prx*, and *ahpC*. Tn-mutants that had a disruption in *fdxA* were more abundant *in vivo* than in the *in vitro* condition. All except a *tpx,* and *msrB* have orthologous genes in Lv-StB, indicating that there is a need to counteract ROS/RNSs in the symbiotic structures of the *Lagria* host.

4.4. Targeted mutagenesis and colonization assays with mutants relevant for flagellar motility

As a complementary approach to corroborate our findings from the Tn-seq experiment and to specifically investigate the importance of flagellar motility and swarming during colonization, we performed targeted mutagenesis and colonization experiments with individual motility mutants. We generated mutants targeting genes involved in flagellar biosynthesis – Δ*fliC*, Δ*fliK*. First, to confirm if the mutants Δ*fliC*, Δ*fliK* were indeed non-motile, we performed a hanging drop motility assay. We found that Δ*fliC* was non-motile at 6 h and 24 h post-inoculation, while Δ*fliK* still retained the ability to swim without any obvious impairments. Further assessing the genome of Lv-StA, we found that Lv-StA carries two copies of *fliK*, which likely explains how the deletion mutant Δ*fliK* remains motile. Nevertheless, mono-colonization assays were performed with the mutants Δ*fliC* and Δ*fliK* and the wild type Lv-StA **(Fig. 5)**. The Δ*icos* mutant, which has enhanced swarming abilities, was generated in a previous study (40).

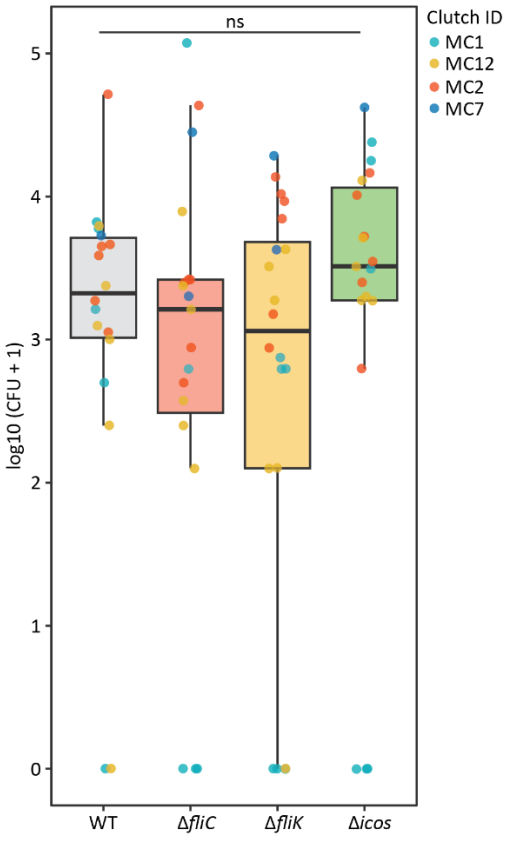


Fig.5. Mono-colonization experiment: The log_{10}(CFU+1) values of the WT (Wildtype) and mutants Δ*fliC*, Δ*fliK* and Δ*icoS* in four replicate clutches show that mutants colonize the dorsal structures of the *Lagria villosa* larvae to the same efficiency as the wild type Lv-StA strain (LME, likelihood ratio = 2.26, p-val = 0.52).

After infecting eggs, we assessed the individual colonization efficiencies of the wild type and mutants in the dorsal structures of first-instar larvae by CFU counting. The colonization efficiency of the mutants and wild type strains were not significantly different (LME, likelihood ratio = 2.26, p-val = 0.52) **(Fig. 5)**. Therefore, motility defects did not appear to influence individual colonization efficiency of Lv-StA.

To further investigate if the mutants Δ*fliC* and Δ*fliK* have a competitive disadvantage when co-colonizing with the wild type strain, we co-inoculated mutant and wild type in equal proportions (WT: ΔfliC = 10^7:10^7 cells/µl) and assessed the CFUs of each strain in the dorsal structures of first-instar larvae. Both *ΔfliC* and *ΔfliK* cells colonized the dorsal structures equally well as the wild type **(Fig. 6)**. (LME, WT : *ΔfliC*, likelihood ratio = 0.46, p-val = 0.49), (LME, WT : *ΔfliK*, likelihood ratio = 1.44, p-val= 0.23).

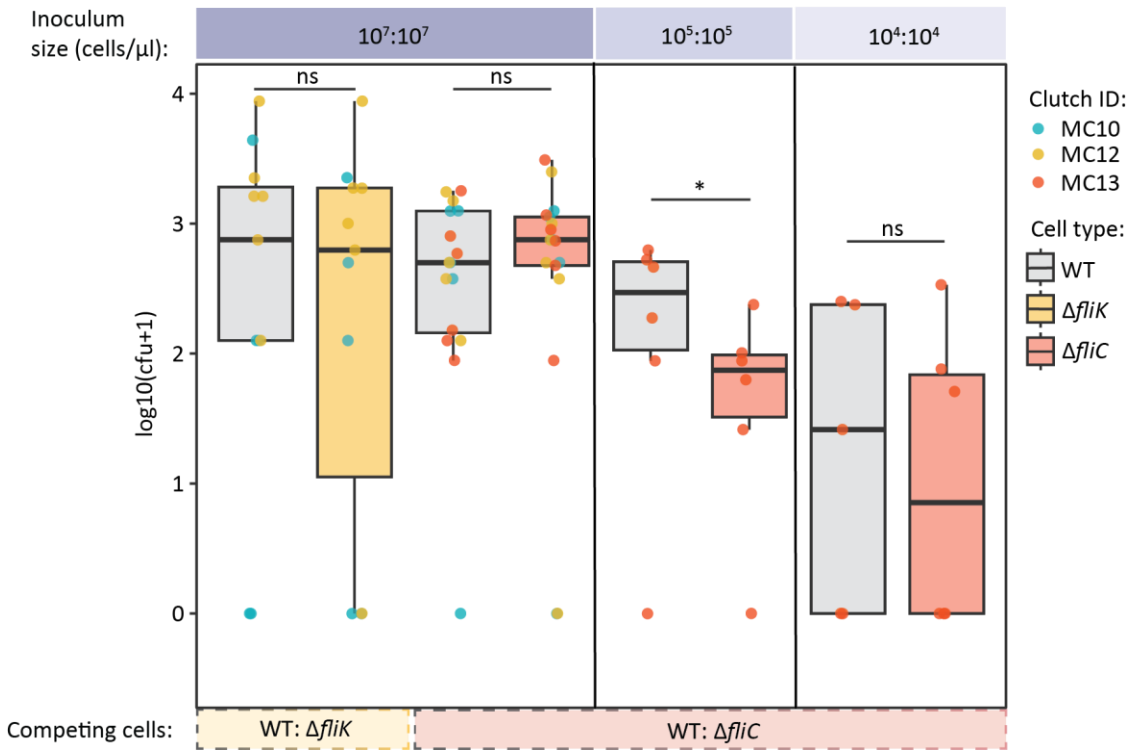


Fig.6. Competition experiment: WT and the mutants (Δ*fliC*, Δ*fliK*) were co-inoculated at different ratios (10^4:10^4, 10^5:10^5, 10^7:10^7 cells/µl) on egg clutches and first-instar larvae were collected. By plating crushed larvae on agar plates with and without kanamycin $\log_{10}$ (CFU+1) of each cell type within the dorsal structures of a larva was obtained. The mutants Δ*fliC* and Δ*fliK* colonize the dorsal structures of the larvae to similar proportions as Lv-StA (LME, WT : Δ*fliC*, 10^7:10^7 cells/µl, likelihood ratio = 0.46, p-val = 0.49), (LME, WT : Δ*fliK*, 10^7:10^7 cells/µl, likelihood ratio = 1.44, p-val= 0.23). Moreover, co-inoculation of WT and Δ*fliC* under different total inoculum sizes (10^4:10^4, 10^5:10^5 cells/µl) shows that at lower inoculum, WT and Δ*fliC* colonize equally well (LME, WT: Δ*fliC*, 10^4:10^4 cells/µl, likelihood ratio = 0.11, p-val = 0.73), but more WT cells colonize the larva at a medium inoculum ratio (10^5:10^5 cells/µl) (LME, WT: Δ*fliC*, 10^5:10^5 cells/µl, likelihood ratio = 5.64, p-val = 0.017).

We further performed a preliminary experiment with one clutch, where the wild type and the non-motile mutant Δ*fliC* were mixed in 1:1 proportions, testing different total cell counts (10^4: 10^4, 10^5: 10^5) **(Fig. 6)**. As expected, total CFU of wild type and Δ*fliC* in the 10^7:10^7 treatments were significantly higher than the CFU counts of each strain from the other treatments with the lower inoculum ratios (LME, likelihood ratio = 20.51, p-val < 0.001). This shows that higher number of strains on the egg surface allows more cells to colonize the dorsal structures of the first-instar larvae. However, we wanted to know if a low inoculum size makes it more favourable for motile cells to outcompete non-motile cells. Within each inoculum size, we tested if the final CFU counts of wild type or Δ*fliC* are equal, therefore, maintaining the initial 1:1 inoculum ratio. The average CFU of wild type is similar to that of Δ*fliC* in the 10^4: 10^4 inoculum (LME, likelihood ratio = 0.11, p-val = 0.73) **(Fig. 6)**. However, after inoculating 10^5: 10^5 ratio of wild type: Δ*fliC*, the average CFU of wild type was more than 200-fold higher than the non-motile mutant in the dorsal structures (LME, likelihood ratio = 5.64, p-val = 0.017) **(Fig. 6)**. More replicates need to be collected to confirm if lower inoculum ratios improve the colonization efficiency of motile cells.

5. DISCUSSION

We used a random mutant library coupled with transposon directed sequencing to identify potential colonization factors in a defensive bacterial ectosymbiont of a beetle. The library was constructed using Tn5 transposon-mediated mutagenesis from the so far only cultured symbiotic strain of *L. villosa* beetles, *Burkholderia* Lv-StA (35). We generated 24,224 insertions across the genome of Lv-StA that disrupted 3,736 genes (45). The 271 genes that we found important for colonization in Lv-StA included those involved in regulation of motility and biofilm formation, maintaining cell wall integrity and formation of cell-surface structures, transporters, lipopeptides, iron metabolism and oxidative stress responses.

In several animal-microbe symbioses, flagellar motility is essential for bacterial cells colonizing the host from the environment and/or for migration into the symbiotic organs of the host (15,76,77). Moreover, in a previous study, we speculated that Lv-StA probably relies on flagellar motility to colonize the dorsal structures of the larvae and have also observed Lv-StA cells swimming inside the dorsal structures (37). We were intrigued by this observation in Lv-StA, since one of the other host-associated strains, Lv-StB, that is more dominant and widespread across field-collected individuals, does not encode genes responsible for flagellar biosynthesis. However, motility may not be strong requirement as suggested by the Tn-seq experiment. The results from the colonization assays with targeted mutants instead indicated that motility mutants can still colonize alone or in competition with the wild type **(Fig.5,6)**. Furthermore, we would need more replicates at the lower inoculum ratios to conclusively state if inoculum size affects colonization efficiency of motile or non-motile strains **(Fig. 6)**. We speculate that the regulators of flagellar motility, *flhCD,* may perform the role of switching Lv-StA cells to form a biofilm during after immediate colonization, and we would need further experiments to collect direct evidence for this. Moreover, a previous microbial community profiling of the beetles across life-stages shows the presence of other symbiont species, Acinetobacter, Luteibacter and Variovorax in the host (38). Genomic data suggests that Luteibacter and Acinetobacter are capable of flagellar biosynthesis, however, Acinetobacter does not encode genes for flagellar motility **(Table-6)**. Therefore, the *Lagria villosa* symbionts may establish in the host aided by host-movements during hatching, or the symbionts may have mechanisms for motility that do not require formation of a flagellum (37).

In addition to symbiont motility, we hypothesized that the production of biosurfactants like burriogladin and icosalide could regulate swarming motility and biofilm formation on host surfaces. The lipoptide, icosalide, has an unusual role as a swarming inhibitor since most biosurfactants are known to enhance swarming (78). Therefore, in its absence, the mutant Δ*icoS* likely exhibits enhanced swarming capabilities (40). However, in our mono-colonization assay, the colonization efficiency of the mutant is similar to the wild type, so enhanced swarming does not seem to inhibit, or even promote colonization. However, the inability of Δ*icoS* to colonize the host in the Tn-seq experiment indicates that the mutant may have reduced competitive colonization efficiency. This may indicate that icosalide has alternate roles other than being a biosurfactant, or that the mutant is not able to competitively colonize the host possibly due to slower growth, and this needs further experimental validation. Furthermore, icosalide was proven to have antibacterial activity against two gram-positive bacteria, *Bacillus thuringiensis* and *Paenibacillus larvae* (40) therefore, it may be more relevant for microbe – microbe interactions during colonization. In addition, we need to test the colonization abilities of a burriogladin mutant which is another candidate

colonization factor from the Tn-seq experiment. Contrary to icosalide, the lipopeptide, burriogladin is essential for swarming *in vitro* and promotes biofilm formation (70). On the egg surface and inside the larval dorsal structures, symbiont strains likely form a biofilm layer to adhere to the cuticular lining which could prevent loss during moulting and also help in exchange of metabolites between strains and microbial species. Therefore, the role of the burriogladin and the factors involved in biofilm formation during establishment has to be investigated further.

Moreover, the symbiont cell wall is an interface that provides contact with the host. In *Riptortus* bean-bugs, gut-associated *Caballeronia* symbionts are acquired orally from the environment, and the LPS O-antigen possibly helps in protecting the symbiont cells from cationic anti-microbial peptides in the saliva of the host (23). Concordantly, the integrity of the peptidoglycan cell wall is essential to protect symbionts from lysozymes (8). Similarly, in a nematode, mutants of *Photorhabdus* symbionts that are defective in O-antigen assembly are susceptible to cationic antimicrobial peptides and unable to colonize the nematode host or mount a pathogenic infection in the nematode's prey (79). In the *Lagria* symbiont, Lv-StA, several cell wall and membrane structures were found to be important *in vivo* and they help maintain cell integrity and/or construct a complete LPS structure. It is likely that the external environment on the egg surface is exposed to abiotic stresses, or the larval dorsal structures may contain immune effectors secreted by the host. Therefore, an intact cell wall may be essential for symbionts to survive these conditions. Based on the genomic data, it seems likely that Lv-StA and Lv-StB have similar LPS structures, which may enable the host to recognize beneficial *Burkholderia* symbionts from other microorganisms.

Furthermore, several membrane transporters seem necessary for Lv-StA colonization of the beetle. In the cuticle-lined dorsal structure of the *Lagria* larvae it is conceivable that certain host secreted metabolites, or break-down products of the cuticle, may be a source of nutrients for symbiont growth, so symbiont-encoded membrane transporters may be essential for nutrient uptake. In *Acromyrmex* leaf-cutter ants, nutrients released by the host seem to be taken up by cuticle-associated *Pseudonocardia* symbionts (14). Further studies are necessary to understand how the insect hosts support symbiont growth on the cuticular surface.

For most host-associated bacteria, iron is one among the many limited resources (80) and its role during symbiont establishment remains unexplored in beneficial symbioses. The *Lagria* symbiont, Lv-StA, encodes non-ribosomal polyketide synthase clusters that are likely involved in the synthesis of siderophores for iron-scavenging. Interestingly, the genome of Lv-StB encodes only a siderophore uptake transporter (*fhuBCD*), while siderophore biosynthesis clusters are absent. Lv-StB may thus be using transporters to scavenge iron-bound siderophores from other strains or species in the natural community and dominate the community by reducing the cost of producing its own siderophores. The dynamics of strain interactions through iron scavenging is an interesting aspect that remains to be explored in beneficial symbioses.

Furthermore, oxidative and nitrosative stressors, e.g. NO, can act as effectors that regulate gene expression in bacterial symbionts and mediate interactions between an animal host and the symbiont (81,82). In the Hawaiian-bobtail squid, a proteomics analysis of the adult light organ environment shows antioxidants are abundant in the symbiont proteome, including MsrA that is also implicated as an Lv-StA

colonization factor in our experiment, indicating that the symbiont protects itself from host ROSs during establishment (83). In line with this, in the *Lagria* system, we find that several Tn5-mutants of genes related to oxidative stress response were not able to associate with the beetle host. Further investigation is necessary to understand if the *Lagria* beetle produces ROSs or RNSs in response to bacterial colonization or to regulate microbial colonization in the dorsal structures of the larvae.

We were not yet able to completely validate the results of the Tn-seq experiment with the individual colonization experiments. It is important to note that there are limitations to a Tn-seq experiment. Firstly, in the *Burkholderia* genome, some sites seem to be hot-spots for Tn-insertions and therefore lead to generation of multiple copies of the same mutant. Multiple mutants carrying an insertion within same gene could outnumber other low-abundant ones. Unequal abundances of mutants in the mutant library cannot be resolved by homogenized mixing before infection. To support this claim, we find that reads for mutants involved in biosynthesis of chitinase, biosynthesis of capsular polysaccharides, other NRPSs, were present in the original library at less than 10 read counts, but were absent in the *in vivo* and *in vitro* conditions. It is likely that these mutants were outnumbered by others during colonization. Secondly, mutants that cannot biosynthesize public goods like siderophores, quorum sensing molecules or extracellular molecules like the chitinases and NRPSs may be able to utilize those released by neighbouring cells (84) and compensate for the loss in function of the gene due to the Tn-insertion. Therefore, the importance of these mutants in colonization could not be tested. Targeted knock-outs and mono-colonization experiments would be helpful in identifying the role of the extracellular enzymes during host colonization. However, it is surprising that our Tn-seq experiment indicates extracellular lipoptides like icosalide and burriogladin are potentially important for colonization. It is probable that these lipopeptides may not diffuse far from the producing cells, or may be false-candidates that showed up from the Tn-seq experiment. And lastly, mutants that are auxotrophic for primary metabolites may be able to compensate for the loss by taking up nutrients from the environment. However, symbiont cells that usually rely on external metabolites, but lack functioning uptake transporters, could probably have a stronger depletion *in vivo.* We were probably able to identify several such nutrient uptake transporters in the Tn-seq experiment. To support our findings, targeted mutagenesis and colonization assays with biologically relevant gene candidates chosen from the Tn-seq results is necessary.

6. CONCLUSIONS

Lagria villosa beetles are associated with *Burkholderia* symbionts that provide defence against fungal pathogens during vulnerable stages of host development (35,36,38). Symbionts are smeared on the egg surface during oviposition (35), and colonize the cuticle-lined dorsal structures of the larvae during hatching (37). Multiple strains of *Burkholderia* (Lv-StA, Lv-StB, etc.) are associated with a single host and one of them, *Burkholderia* Lv-StA, has been cultured in the lab and is amenable to genetic manipulation. However, Lv-StB is the more prevalent and abundant strain in field collected beetles and has a highly reduced genome. Using transposon insertion directed sequencing, we knocked-out 3,736 genes in Lv-StA and tested their relevance for host colonization. We find genes involved in regulation of motility and biofilm formation, cell wall biosynthesis, transporters, lipoptides, and factors regulating oxidative stress may be relevant during colonization. Furthermore, we find that some of these genes are shared with the genome-eroded Lv-StB, while other are not, suggesting that the two strains may use different

mechanisms to colonize the host. However, contradicting the Tn-seq results, targeted mutagenesis and colonization experiments with Lv-StA show that non-motile mutants can colonize the dorsal structures of the larvae to the same extent as a motile wild type Lv-StA. It is likely that motility is an accessory factor for Lv-StA during colonization and loss of flagellar motility biosynthesis genes in Lv-StB helped streamline its genome and adapt better to a host-associated life-style. Thus, *Lagria- Burkholderia* symbiosis is a valuable model system where we can explore the molecular basis of symbiosis establishment using a cultivable and genetically tractable strain and also gain insights on genetic capabilities across different strains of symbionts associated with a single host.

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9. SUPPLEMENTS

Table-1. General primers and probes used in the study

Name	Sequence	Annealing temp (°C)	fwd/rev	Target	Reference	Usage
tpnRL17_1RC	5′-CGTTACATCCCTGGCTTGTT-3′	58.2	rev	Kanamycin insertion cassette from pRL27	(47)	PCR to test for successful transconjugants
tpnRL13–2RC	5′-TCGTGAAGAAGGTGTTGCTG-3′		fwd		(47)	
Burk16s_1_F	5′- GTTGGCCGATGGCTGATT-3′	59°C	fwd	*Burkholderia sp.*16s rRNA gene	(38)	PCR to identify *Burkholderia* sp.
Burk16s_1_R	5′- AAGTGCTTTACAACCCGAAGG-3′		rev		(38)	
Burk_Arb2F	5`-GCCACGCGTCGACTAGTACNNNNNNNNNNGATGC- 3′	58°C	fwd	PCR1- Lv-StA random genomic location.	(28)	PCR to identify individual mutants and verify insertions
Burk_Arb3F	5′-GCCACGCGTCGACTAGTACNNNNNNNNNNGATGG- 3′		fwd			
Burkh_arb5F	5′ GCCACGCGTCGACTAGTACNNNNNNNNNNCAGCT – 3′		fwd			
Burkh_arb7F	5′-GCCACGCGTCGACTAGTACNNNNNNNNNNACGCT- 3′		fwd			
Burkh_arb8F	5′-GCCACGCGTCGACTAGTACNNNNNNNNNNAATCG- 3′		fwd			
tnp-K13	5`- AGCTCTCATCAACCGTGGC -3′		rev	PCR1- transposon insertion cassette		
tnpRL13-2	5′- CAGCAACACCTTCTTCACGA- 3′	59°C	fwd	PCR2- transposon insertion cassette amplified from PCR 1	(47)	
Arbstep2	5′- GCCACGCGTCGACTAGTAC - 3		rev	PCR2- Lv-StA genomic location amplified from PCR 1	(28)	

Name	Sequence	Annealing temp (°C)	fwd/rev	Target	Reference	Usage
Lv-StA_m10_fwd	5`- GCTCGCGGTCAGATGGATTA – 3`	55 - 62°C gradient	fwd	310 bp intergenic genomic DNA in Lv-StA	Designed for this study	Confirmation of neutral mutant insertion site
Lv-StA_m10_rev	5′- GATGAGACGTCCGTCCGTAC – 3′		rev			
Tn5_m10_fwd	5′ - ACACCTTCTTCACGAGGCAG – 3′		fwd	282 bp Tn-insertion cassette and genomic DNA in Lv-StA		
Biotin_pRL27_2255F	5′-Biotin- ACAGGAACACTTAACGGCTGACATG -3′	65°C	fwd	Tn-insertion cassette	(45)	DNA library preparation and insertion-directed sequencing
NEBNext Multiplex Oligos for Illumina Index primers set 1 (E7335G)	5′-CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′		rev	Index region		
	5′-CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′		rev			
	5′-CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′		rev			
	5′-CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′		rev			
	5′-CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′		rev			
	5′-CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′		rev			
	5′-CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′		rev			
	5′-CAAGCAGAAGACGGCATACGAGATTCAAGTG		rev			

Name	Sequence	Annealing temp (°C)	fwd/rev	Target	Reference	Usage
	TGACTGGAGTTCAGACGTGTGCTCTTCCGATC -s-T-3′					
	5′- CAAGCAGAAGACGGCATACGAGATCTGATCG TGACTGGAGTTCAGACGTGTGCTCTTCCGATC -s-T-3′		rev			
	5′- CAAGCAGAAGACGGCATACGAGATAAGCTAG TGACTGGAGTTCAGACGTGTGCTCTTCCGATC -s-T-3′		rev			
	5′- CAAGCAGAAGACGGCATACGAGATGTAGCCG TGACTGGAGTTCAGACGTGTGCTCTTCCGATC -s-T-3′		rev			
	5′- CAAGCAGAAGACGGCATACGAGATTACAAGG TGACTGGAGTTCAGACGTGTGCTCTTCCGATC -s-T-3′		rev			
Univ_pRL27_2296F	5′- AATGATACGGCGACCACCGAGATCTACACTCT TTCCCTACACGACGCTC TTCCGATCTGAATTCATCGATGAT GGTTGAGATGTGT – 3′		fwd	Modified universal adapter for Illumina sequencing	(45)	
NEBNext Adaptor for Illumina	5′-/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT C dU A CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT C-s-T-3′	-	-	-	-	
Burk_16s-cy5	5′- Cy5-TGCGGTTAGACTAGCCACT-3′			Burkholderia 16s rRNA gene	(38)	Fluorescent *in situ* hybridization
EUB338-Cy3	5′- Cy3 – GCTGCCTCCCGTAGGAGT- 3′			Eubacteria 16s rRNA gene	(85)	

Table-2. Primers used for targeted mutagenesis on Lv-StA

Name	Sequence	Tm (°C)	fwd/rev	Target	Reference	Usage
fliC_back_P9_42bp	5′-CTTCTTGACGAGTTCTTCTGAGGCTGTCGCGGTACTGCTTTG-3′	68.5	fwd	fliC flanking homologous region	designed for this study	Amplify front and back homologous regions of the fliC gene in Lv-StA
fliC_back_P10_40bp	5′-ATTGTAGGAGATCTTCTAGACGAGACCGTGACCTGGTAGG-3′	65.4	rev			
fliC_front_P7_43bp	5′-GCCAGATCTTCCGGATGGCTCGAGAATCGTCGTCATGTCGTTC-3′	70.1	fwd	fliC flanking homologous region		
fliC_front_P8_40bp	5′-CTACGTGTTCAATCACTAGTGGAAATTCTCCTAAGTGAGC-3′	62.5	rev			
fliK_back_P3_38bp	5′-CTTCTTGACGAGTTCTTCTGACCCGTCGGCCCGCCGCG-3′	73	fwd	fliK flanking homologous region		Amplify front and back homologous regions of a fliK gene in Lv-StA
fliK_back_P4_40bp	5′-ATTGTAGGAGATCTTCTAGATCAGGCGGTCTTGTTGCCGC-3′	66.9	rev			
fliK_front_P1_44bp	5′-GCCAGATCTTCCGGATGGCTCGAGCTACCTGCTCGGCTTCGACG-3′	73.9	fwd	fliK flanking homologous region		
fliK_front_P2_41bp	5′-CTACGTGTTCAATCACTAGTGGCAAGGACTCCCCAGTAGAG-3′	66.3	rev			
phbF_back_P19_39bp	5′-CTTCTTGACGAGTTCTTCTGAGCGGCAGCGGGGCCATCG-3′	71.6	fwd	phbF flanking homologous region		Amplify front and back homologous regions of a phbF gene in Lv-StA
phbF_back_P20_40bp	5′-ATTGTAGGAGATCTTCTAGAGCGACATCGACACGCGCACG-3′	68.1	rev			
phbF_front_P17_42bp	5′-GCCAGATCTTCCGGATGGCTCGAGGCCGTGATCGACACGAAC-3′	72.6	fwd	phbF flanking homologous region		
phbF_front_P18_40bp	5′-CTACGTGTTCAATCACTAGTGGAACACCTGTGAGCGCCGG-3′	68.8	rev			
cpaC_back1_40bp	5′-TTCTTGACGAGTTCTTCTGAAGCGCCGGACCATGGGGTAG-3′		fwd	cpaC flanking homologous region		Amplify front and back homologous regions of a cpaC gene in Lv-StA
cpaC_back2_40bp	5′-ATTGTAGGAGATCTTCTAGAGAGCAGCGACAGCACCTTGC-3′	66.7	rev			

cpaC_front1_42bp	5′-CAGATCTTCCGGATGGCTCGAGCGTCGACGTGTTCGTCAACC-3′	71.6	fwd	cpaC flanking homologous region		
cpaC_front2_40bp	5′-CTACGTGTTCAATCACTAGTTGTGTCGGAGGTCCGCAGCC-3′		rev			
Kan_seq_fwd	5′-GGCTACCCGTGATATTGC-3′	56	fwd	pJK347 Kanamycin cassette	(42)	PCRs to confirm KanR insertion
Kan_seq_rev	5′-GCTTCCCAACCTTACCAGAG-3′	59.3	rev			
JK583	5′-ACTAGTGATTGAACACGTAG-3′	53.2	fwd	pJK347 Kanamycin cassette		To obtain KanR amplicon for plasmid construction
JK1032	5′-TCAGAAGAACTCGTCAAGAAG-3′	55.9	rev			
pJET1.2_FP	5'-CGACTCACTATAGGGAGAGCGGC-3'	66	fwd	pJET1.2 plasmid backbone		PCR to confirm double cross-overs
pJET1.2_RP	5'-AAGAACATCGATTTTCCATGGCAG-3'	59	rev	pJET1.2 plasmid backbone		PCR to confirm double cross-overs

Table-3. Primers used for confirming knockouts after targeted mutagenesis on Lv-StA

Name	Sequence	Tm (°C)	fwd/rev	Target	Reference	Usage
fliC_P16	5'-CTCTTGCTGCCGACCTGCAG-3'	61.4	rev	right flank to fliC homologous region	designed for this study	PCRs to confirm Knock-outs
fliC_P15	5'-ACTCGACCAACCTGTCGTCG-3'	61.4	fwd	fliC gene		
fliC_P14	5'-GTGATGGCTTGCGACAGAGC-3'	61.4	rev	fliC gene		
fliC_P13	5'-TCACGCAGCTCAGCGATCAG-3'	61.4	fwd	left flank to fliC homologous region		
fliK_P8	5'-CTCGTGAGGGCGGCCGGTTC-3'	67.6	rev	right flank to fliK homologous region		
fliK_P7	5'-GAAGAGTCAGGCCGCATCGG-3'	63.4	fwd	fliK gene		
fliK_P6	5'-GCGACAGGGTGAGCAACAGC-3'	63.4	rev	fliK gene		
fliK_P5	5'-CTACAAGGTGGCGGTGGTGC-3'	63.4	fwd	left flank to fliK homologous region		
phbF_P24	5'-GGCTGCATGGCGAATCGGTC-3'	63.4	rev	right flank to phbF homologous region		
phbF_P23	5'-CAGCTCGTTCCCGTTCAAGC-3'	61.4	fwd	phbF gene		
phbF_P22	5'-GTCGTACAGCCGGCGATTCG-3'	63.4	rev	phbF gene		
phbF_P21	5'-AGCTCGCCTTCGACAAGGTC-3'	61.4	fwd	left flank to phbF homologous region		
cpaC_P31	5'-CACCAAGCTGCCGAGCACG-3'	64.5	fwd	cpaC gene		

Name	Sequence	Tm (°C)	fwd/rev	Target	Reference	Usage
cpaC_P32	5'-CAGCGCGTGTTGAGGAACAGC-3'	64.9	rev	right flank to cpaC homologous region		
cpaC_P29_new	5'-GTGGTCGAGAGTTCGCTGTC-3'	60.7	fwd	left flank to cpaC homologous region		
cpaC_P30_new	5'-AGCGTCAAGGCACTCAATGC-3'	61.6	rev	cpaC gene		

Table-4. Colony PCR product sizes to confirm successful knock-outs in Lv-StA

Primer pairs	Knock-out	WT	SCO-front	SCO-back
Δ*fliC*				
fliC_P13 + Kan-seq_rv	914 bp	-	914 bp	(6255 bp)
fliC_P16 + Kan-seq_fw	847 bp	-	(6188 bp)	847 bp
fliC_P13 + fliC_P14	-	781 bp	-	781 bp
fliC_P15 + fliC_ P16	-	881 bp	881 bp	-
fliC_P13 + pJET1.2_RP	-	-	2481 bp	2527 bp
fliC_P16 + pJET1.2_FP	-	-	2529 bp	2483 bp
fliC_P13 + fliC_ P16	2524 bp	2570 bp	(7865 bp)	(7865 bp)
Δ*fliK*				
fliK_P5 + Kan-seq_rv	853 bp	-	853 bp	(bp)
fliK_P8 + Kan-seq_fw	797 bp	-	(bp)	797 bp
fliK_P5 + fliK_P6	-	674 bp	-	674 bp
fliK_P7 + fliK_P8	-	707 bp	707 bp	-
fliK_P5 + pJET1.2_RP	-	-	bp	bp
fliK_P8 + pJET1.2_FP	-	-	bp	bp
fliK_P5 + fliK_P8	2413 bp	1952 bp	(bp)	(bp)
Δ*phbF*				
phbF_P21 + Kan-seq_rv	889 bp	-	889 bp	(bp)
phbF_P24 + Kan-seq_fw	829 bp	-	(bp)	829 bp
phbF_P21 + phbF_P22	-	736 bp	-	736 bp
phbF_P23 + phbF_P24	-	757 bp	757 bp	-
phbF_P21 + pJET1.2_RP	-	-	bp	bp

Primer pairs	Knock-out	WT	SCO-front	SCO-back
phbF_P24 + pJET1.2_FP	-	-	bp	bp
phbF_P21 + phbF_P24	2481 bp	1945 bp	(bp)	(bp)
Δ*cpaC*				
cpaC_P29_new + Kan-seq_rv	876 bp	-	876 bp	(6360 bp)
cpaC_P32 + Kan-seq_fw	791 bp	-	(6275 bp)	791 bp
cpaC_P29_new + cpaC_P30_new	-	766 bp	-	766 bp
cpaC_P31 + cpaC_P32	-	780 bp	780 bp	-
cpaC_P29_new + pJET1.2_RP	-	-	2333 bp	2665 bp
cpaC_P32 + pJET1.2_FP	-	-	2726 bp	2394 bp
cpaC_P29_new + cpaC_P32	2430 bp	2762 bp	(7914 bp)	(7914 bp)

Table-5. Potential colonization factors in Lv-StA and orthologs in Lv-StB.

The first six columns show Lv-StA genes identified as potential colonization factors from the Tn-seq experiment. Genes in bold were more abundant *in vivo* compared to the *in vitro* grown library. Last three columns (red headers) show if orthologs of these Lv-StA colonization factors are also present in Lv-StB.

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.1045	K10441	rbsA	ABC transporter, ATP-binding protein (cluster 2, ribose/xylose/arabinose/galactose) / ABC transporter, ATP-binding protein (cluster 2, ribose/xylose/arabinose/galactose)	0.000805281	0.0179451			
fig|6666666.542543.peg.1066		NA	hypothetical protein	3.78E-05	0.0016409			
fig|6666666.542543.peg.1129		NA	Transcriptiol regulator, LysR family	6.17E-11	3.29E-08			
fig|6666666.542543.peg.115		NA	hypothetical protein	0.001508326	0.0285412			
fig|6666666.542543.peg.1154		NA	FIG00457171: hypothetical protein	0.000556722	0.0135341			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.1159	K03746	hns	DNA-binding protein H-NS	0.000297118	0.0080021			
fig|6666666.542543.peg.116	K07497	K07497	Transposase	0.000180871	0.0054583			
fig|6666666.542543.peg.1320	K21703	cbbR, cmpR, ndhR	Phosphote uptake and metabolism regulator, LysR-family	6.99E-11	3.29E-08			
fig|6666666.542543.peg.1350	K11959	urtA	hypothetical protein	8.98E-11	3.29E-08			
fig|6666666.542543.peg.1427	K07644	cusS, copS, silS	hypothetical protein	0.001289234	0.0258436			
fig|6666666.542543.peg.1449	K19577	ydhP	Uncharacterized MFS-type transporter	0.001336008	0.0264193			
fig|6666666.542543.peg.1475	K06987	K06987	Succinylglutamate desuccinylase/aspartoacylase	0.000589755	0.0141477			
fig|6666666.542543.peg.1492	**K00425**	**cydA**	**Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)**	**6.28E-11**	**3.29E-08**			
fig|6666666.542543.peg.1531	K24119	ahpC	Alkyl hydroperoxide reductase protein C (EC 1.11.1.15)	1.65E-05	0.0009761	84.70%	6.36E-161	99.65%
fig|6666666.542543.peg.1551		NA	hypothetical protein	0.002375466	0.0401088			
fig|6666666.542543.peg.1552		NA	Rhodanese-related sulfurtransferase	2.29E-05	0.0011869			
fig|6666666.542543.peg.1554		NA	Transcriptiol regulator, Xre family	3.02E-07	5.30E-05			
fig|6666666.542543.peg.1565		NA	Radical SAM protein required for addition of adenosine to hopane skeleton, HpnH	0.001644816	0.0303393	91.20%	0	100.00%
fig|6666666.542543.peg.1623	K03561	exbB	MotA/TolQ/ExbB proton channel family protein	0.002879875	0.0469987			
fig|6666666.542543.peg.1657	K03496	parA, soj	Chromosome (plasmid) partitioning protein ParA	0.000603367	0.0143955			
fig|6666666.542543.peg.1658	K03497	parB, spoOJ	Chromosome (plasmid) partitioning protein ParB	0.000152635	0.0049262			
fig|6666666.542543.peg.1659		NA	Protein involved in initiation of plasmid replication	7.97E-08	1.84E-05	89.80%	0	100.00%
fig|6666666.542543.peg.1744		NA	Transcriptiol regulator, LysR family	0.000168331	0.0052034			
fig|6666666.542543.peg.1819		NA	Transcriptiol regulator, LysR family	0.002961798	0.0481566			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.1870		NA	Polyketide synthase modules and related proteins	0.001590294	0.029708			
fig|6666666.542543.peg.1895		NA	hypothetical protein	4.75E-05	0.0020036			
fig|6666666.542543.peg.203	K18138	acrB, mexB, adeJ, smeE, mtrD, cmeB	RND efflux system, inner membrane transporter	0.001457418	0.0280037			
fig|6666666.542543.peg.2065		NA	hypothetical protein	0.000182772	0.0054583			
fig|6666666.542543.peg.2135		NA	hypothetical protein	5.50E-05	0.0022791			
fig|6666666.542543.peg.2150	K07793	tctA	hypothetical protein	6.16E-05	0.0024345			
fig|6666666.542543.peg.2328		NA	FIG00456462: hypothetical protein	3.61E-11	3.29E-08			
fig|6666666.542543.peg.2348		NA	Rubredoxin	0.000130044	0.0043598			
fig|6666666.542543.peg.2357	K00128	ALDH	Aldehyde dehydrogese B (EC 1.2.1.22)	0.002431676	0.0407445			
fig|6666666.542543.peg.2401		NA	L-threote/D-erythrote MFS-type transporter	1.55E-06	0.0001746			
fig|6666666.542543.peg.2456		NA	hypothetical protein	0.001638099	0.0303393			
fig|6666666.542543.peg.2464		**NA**	**hypothetical protein**	**6.77E-11**	**3.29E-08**			
fig|6666666.542543.peg.2465		NA	hypothetical protein	0.001908077	0.033776			
fig|6666666.542543.peg.2489	K08369	ydjE	Niacin transporter NiaP	4.59E-06	0.0003876			
fig|6666666.542543.peg.258	K00764	purF, PPAT	Amidophosphoribosyltransferase (EC 2.4.2.14)	1.91E-06	0.0002044			
fig|6666666.542543.peg.265	K01696	trpB	Tryptophan synthase beta chain (EC 4.2.1.20)	3.42E-06	0.000313	93.50%	0	98.74%
fig|6666666.542543.peg.266	K01817	trpF	Phosphoribosylanthranilate isomerase (EC 5.3.1.24)	2.38E-08	6.53E-06	90.00%	0	98.43%
fig|6666666.542543.peg.273		NA	Entericidin	0.000244377	0.0068332			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.2780	K03724	lhr	Uncharacterized ATP-dependent D helicase PA3272	0.001845031	0.0330098			
fig|6666666.542543.peg.2948		NA	hypothetical protein	0.000141656	0.0046408			
fig|6666666.542543.peg.3356	K06075	slyA	Transcriptiol regulator, MarR family	6.13E-07	8.16E-05			
fig|6666666.542543.peg.3361		NA	Maebl	5.60E-05	0.002297	86.90%	0	100.00%
fig|6666666.542543.peg.3470	K13766	liuC	Methylglutaconyl-CoA hydratase (EC 4.2.1.18)	0.001498543	0.0284788			
fig|6666666.542543.peg.3511		NA	CzcABC family efflux RND transporter, transmembrane protein	0.002425203	0.0407445			
fig|6666666.542543.peg.3549	K02483	K02483	Two-component transcriptiol response regulator, OmpR family	1.08E-05	0.0007314			
fig|6666666.542543.peg.3585	K15547	mdtO	Putative transmembrane protein	0.002226322	0.038178			
fig|6666666.542543.peg.3608	K02600	nusA	Transcription termition protein NusA	0.000916809	0.01935	91.90%	0	78.37%
fig|6666666.542543.peg.3610	K02834	rbfA	Ribosome-binding factor A	2.30E-05	0.0011869	91.60%	1.50E-145	100.00%
fig|6666666.542543.peg.3619	K00658	DLST, sucB	Dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogese complex (EC 2.3.1.61)	0.000251554	0.0069894	90.40%	0	100.00%
fig|6666666.542543.peg.3631	**K02280**	**cpaC, rcpA**	**Type II/IV secretion system secretin RcpA/CpaC, associated with Flp pilus assembly**	**0.003078747**	**0.0498734**			
fig|6666666.542543.peg.3659	K11066	E3.5.1.28D, amiD	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28) @ 1,6-anhydro-N-acetylmuramyl-L-alanine amidase	0.001971561	0.0344827			
fig|6666666.542543.peg.3679	K05539	dusA	tR-dihydrouridine(20/20a) synthase (EC 1.3.1.91)	4.44E-05	0.001891	89.20%	0	98.35%
fig|6666666.542543.peg.3724	K00852	rbsK, RBKS	Ribokinase (EC 2.7.1.15)	0.000949264	0.0198441			
fig|6666666.542543.peg.3728	K03761	kgtP	Alpha-ketoglutarate permease	0.000366044	0.0095651			
fig|6666666.542543.peg.3775		NA	hypothetical protein	0.002670957	0.0439529			
fig|6666666.542543.peg.3847	K18929	lldF	Predicted L-lactate dehydrogese, Iron-sulfur cluster-binding subunit YkgF	0.00046747	0.0117268			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.386	K05874	tsr	Methyl-accepting chemotaxis sensor/transducer protein	0.002103717	0.0363595			
fig|6666666.542543.peg.3933		NA	hypothetical protein	9.20E-06	0.0006516			
fig|6666666.542543.peg.3937		NA	hypothetical protein	3.02E-05	0.0014072			
fig|6666666.542543.peg.3982	K00925	ackA	Acetate kise (EC 2.7.2.1)	0.000559267	0.0135341			
fig|6666666.542543.peg.4026		NA	Polyketide synthase modules and related proteins	0.002673219	0.0439529			
fig|6666666.542543.peg.404		NA	Acetyltransferase, GT family	0.000153745	0.0049262			
fig|6666666.542543.peg.4055	K16137	nemR	Transcriptiol regulator, AcrR family	1.98E-05	0.0010626			
fig|6666666.542543.peg.4072		NA	Xanthine/uracil permeases	0.001097575	0.0226214			
fig|6666666.542543.peg.4136	K01079	serB, PSPH	Phosphoserine phosphatase (EC 3.1.3.3)	0.001438622	0.0278218	89.40%	0	100.00%
fig|6666666.542543.peg.4145		NA	PhbF	1.08E-05	0.0007314	88.80%	0	100.00%
fig|6666666.542543.peg.4170	K15539	rodZ	Cytoskeleton protein RodZ	4.32E-06	0.0003717	85.60%	0	100.00%
fig|6666666.542543.peg.4172	K00940	ndk, NME	Nucleoside diphosphate kise (EC 2.7.4.6)	0.000813871	0.0179542	92.00%	1.30E-171	99.77%
fig|6666666.542543.peg.4187	K03671	trxA	Thioredoxin	5.37E-07	7.60E-05	92.40%	2.89E-132	100.00%
fig|6666666.542543.peg.4194	K03327	TC.MATE, SLC47A, norM, mdtK, dinF	+-driven multidrug efflux pump	3.00E-05	0.0014072			
fig|6666666.542543.peg.4199	K13771	nsrR	Nitrite-sensitive transcriptiol repressor NsrR	0.001374003	0.026923			
fig|6666666.542543.peg.4207	K14260	alaA	Alanine transamise (EC 2.6.1.2)	0.001744667	0.0317604	92.10%	0	99.92%
fig|6666666.542543.peg.4231	K02963	RP-S18, MRPS18, rpsR	SSU ribosomal protein S18p @ SSU ribosomal protein S18p, zinc-independent	0.001475177	0.0281566	94.90%	2.45E-122	100.00%
fig|6666666.542543.peg.4246		NA	hypothetical protein	3.61E-06	0.0003231			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.4248	K03424	tatD	Uncharacterized metal-dependent hydrolase YcfH	7.56E-06	0.0005726	91.80%	0	100.00%
fig|6666666.542543.peg.4264	K07305	msrB	Peptide-methionine (R)-S-oxide reductase MsrB (EC 1.8.4.12)	0.000466458	0.0117268			
fig|6666666.542543.peg.4283	K01358	clpP, CLPP	ATP-dependent Clp protease proteolytic subunit ClpP (EC 3.4.21.92)	0.002122212	0.0365353	91.90%	0	100.00%
fig|6666666.542543.peg.4335	K01297	ldcA	Muramoyltetrapeptide carboxypeptidase (EC 3.4.17.13)	0.001964951	0.0344827			
fig|6666666.542543.peg.448	K07062	fitB	VapC toxin protein	0.00010712	0.0037924			
fig|6666666.542543.peg.4485		NA	hypothetical protein	0.000893586	0.0189509			
fig|6666666.542543.peg.4529	K00928	lysC	Aspartokise (EC 2.7.2.4)	6.99E-07	9.03E-05	91.80%	0	98.96%
fig|6666666.542543.peg.4536	K03768	PPIB, ppiB	Peptidyl-prolyl cis-trans isomerase PpiB (EC 5.2.1.8)	0.000499779	0.0124661	89.60%	6.31E-66	39.23%
fig|6666666.542543.peg.4638	K13643	iscR	Iron-sulfur cluster regulator IscR	2.99E-08	7.73E-06	90.70%	0	99.63%
fig|6666666.542543.peg.4671	K01915	glnA, GLUL	Glutamine synthetase type I (EC 6.3.1.2)	0.000146347	0.004759	92.30%	0	100.00%
fig|6666666.542543.peg.4672		NA	hypothetical protein	0.000827734	0.0181688			
fig|6666666.542543.peg.4680		NA	FIG001341: Probable Fe(2+)-trafficking protein YggX	2.98E-07	5.30E-05	93.00%	1.16E-110	97.83%
fig|6666666.542543.peg.4731	K11477	glcG	Protein GlcG	0.000088923 7	0.0189509			
fig|6666666.542543.peg.4772	K00337	nuoH	DH-ubiquinone oxidoreductase chain H (EC 1.6.5.3)	0.000178653	0.0054464	92.90%	0	100.00%
fig|6666666.542543.peg.4796	K01652	E2.2.1.6 L, ilvB, ilvG, ilvI	Acetolactate synthase large subunit (EC 2.2.1.6)	3.29E-05	0.0014725	93.20%	0	99.49%
fig|6666666.542543.peg.4798		NA	Putative transmembrane protein	4.62E-09	1.45E-06			
fig|6666666.542543.peg.4800		NA	Putative transmembrane protein	0.000561095	0.0135341			
fig|6666666.542543.peg.4827	K00228	CPOX, hemF	Coproporphyrinogen III oxidase, aerobic (EC 1.3.3.3)	8.13E-05	0.0030471	91.10%	0	98.49%
fig|6666666.542543.peg.4845		NA	hypothetical protein	0.000320241	0.0085723			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.4848		NA	Uncharacterized MFS-type transporter	0.000284949	0.0077697	90.60%	0	96.69%
fig|6666666.542543.peg.4853		NA	Glycosyltransferases involved in cell wall biogenesis	0.000409759	0.0105814	91.80%	0	100.00%
fig|6666666.542543.peg.4870	K03616	rnfB	Iron-sulfur cluster-binding protein	6.81E-11	3.29E-08	90.60%	0	55.99%
fig|6666666.542543.peg.4875		NA	Putative periplasmic cytochrome type-C oxidoreductase sigI peptide protein (EC 1.-.-.-)	1.23E-06	0.0001499			
fig|6666666.542543.peg.4903		NA	Integrase	0.000294768	0.0079878			
fig|6666666.542543.peg.4919	K07497	K07497	Transposase	0.000158044	0.0049915			
fig|6666666.542543.peg.4946		NA	hypothetical protein	1.46E-06	0.0001688			
fig|6666666.542543.peg.4948	K07497	K07497	Transposase InsD for insertion element IS2	0.000834 71	0.0182307			
fig|6666666.542543.peg.5011	K13292	lgt, umpA	Prolipoprotein diacylglyceryl transferase	8.19E-05	0.0030471	91.40%	0	94.95%
fig|6666666.542543.peg.5014	K08738	CYC	Cytochrome c551/c552	0.002352507	0.0398745			
fig|6666666.542543.peg.5020	K03795	cbiX	Sirohydrochlorin cobaltochelatase CbiX(small) (EC 4.99.1.3)	4.92E-07	7.45E-05	88.80%	1.56E-130	100.00%
fig|6666666.542543.peg.5024	K00390	cysH	Adenylyl-sulfate reductase [thioredoxin] (EC 1.8.4.10)	0.000336794	0.0088534			
fig|6666666.542543.peg.5025		NA	Oxidoreductase probably involved in sulfite reduction	1.91E-05	0.0010399			
fig|6666666.542543.peg.5041		NA	Integrase	0.001541997	0.0289289			
fig|6666666.542543.peg.5057	K06183	rsuA	SSU rR pseudouridine(516) synthase (EC 5.4.99.19)	0.000206523	0.0059257			
fig|6666666.542543.peg.5064	K01995	livG	Branched-chain amino acid ABC transporter, ATP-binding protein LivG (TC 3.A.1.4.1)	2.58E-06	0.0002558			
fig|6666666.542543.peg.5065	K01998	livM	Branched-chain amino acid ABC transporter, permease protein LivM (TC 3.A.1.4.1)	2.37E-05	0.00121			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.5066	K01997	livH	Branched-chain amino acid ABC transporter, permease protein LivH (TC 3.A.1.4.1)	0.001175333	0.0238876			
fig|6666666.542543.peg.5069	K03774	slpA	FKBP-type peptidyl-prolyl cis-trans isomerase SlpA (EC 5.2.1.8)	0.002335875	0.0397951	93.80%	1.73E-21	14.25%
fig|6666666.542543.peg.5070	K03630	radC	UPF0758 family protein	0.000125181	0.0042952			
fig|6666666.542543.peg.5133	K00075	murB	UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.3.1.98)	0.000185161	0.0054923			
fig|6666666.542543.peg.5183	K03577	acrR, smeT	Transcriptiol regulator, AcrR family	2.04E-07	4.07E-05			
fig|6666666.542543.peg.5190	K07259	dacB	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	0.000689737	0.0161922	89.30%	0	99.94%
fig|6666666.542543.peg.5198	K01923	purC	Phosphoribosylaminoimidazole-succinocarboxamide synthase (EC 6.3.2.6)	0.000125236	0.0042952			
fig|6666666.542543.peg.5255		**NA**	**hypothetical protein**	**0.000790372**	**0.0177935**			
fig|6666666.542543.peg.5279	K02440	GLPF	Glycerol uptake facilitator protein	0.000761838	0.0173289			
fig|6666666.542543.peg.5301	K04744	lptD, imp, ostA	LPS-assembly protein LptD Organic solvent tolerance protein precursor	0.000748773	0.01721	89.90%	0	100.00%
fig|6666666.542543.peg.5314	**K08310**	**nudB, ntpA**	**Dihydroneopterin triphosphate pyrophosphohydrolase type 2**	**1.05E-08**	**3.08E-06**	**88.30%**	**8.87E-164**	**100.00%**
fig|6666666.542543.peg.5346		**NA**	**Outer membrane factor (OMF) lipoprotein associated wth EmrAB-OMF efflux system**	**0.001460785**	**0.0280037**			
fig|6666666.542543.peg.5367	K06041	kdsD, kpsF	D-arabinose-5-phosphate isomerase (EC 5.3.1.13)	0.000137191	0.0045283	91.90%	0	100.00%
fig|6666666.542543.peg.5372	K03092	rpoN	R polymerase sigma-54 factor RpoN	0.000799407	0.0179051			
fig|6666666.542543.peg.5373	K05808	yhbH	Ribosome hibertion promoting factor Hpf	0.001681131	0.0308793	91.80%	2.48E-143	99.45%
fig|6666666.542543.peg.5388	K00954	E2.7.7.3 A, coaD, kdtB	Phosphopantetheine adenylyltransferase (EC 2.7.7.3)	0.000921658	0.0193592	91.20%	0	100.00%
fig|6666666.542543.peg.5426		NA	putative membrane protein	0.000326 86	0.008644 1			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.5435	K03071	secB	Protein-export protein SecB (maintains pre-export unfolded state)	1.39E-06	0.0001652	91.30%	0	100.00%
fig|6666666.542543.peg.5449	K09806	ubiK	FIG005902: hypothetical protein	4.83E-05	0.00202	90.90%	5.11E-94	97.67%
fig|6666666.542543.peg.5456		NA	hypothetical protein	0.000461688	0.0117268			
fig|6666666.542543.peg.5476		NA	Ferredoxin, 2Fe-2S	7.85E-05	0.0029691			
fig|6666666.542543.peg.5481	K02065	mlaF, linL, mkl	ABC transporter, ATP-binding protein (cluster 9, phospholipid)	0.000194953	0.0056678	91.60%	0	94.79%
fig|6666666.542543.peg.5493	K16171	faaH	Fumarylacetoacetate hydrolase family protein	4.50E-11	3.29E-08			
fig|6666666.542543.peg.5504		NA	Oxidoreductase, short-chain dehydrogese/reductase family	0.000123732	0.0042952			
fig|6666666.542543.peg.5508		NA	hypothetical protein	0.001949653	0.0343734			
fig|6666666.542543.peg.5563		NA	hypothetical protein	2.47E-05	0.0012479			
fig|6666666.542543.peg.5578	K01640	E4.1.3.4, HMGCL, hmgL	Pyruvate:Oxaloacetate transcarboxylase domain protein	0.001750891	0.0317604			
fig|6666666.542543.peg.5626		NA	Outer membrane porin protein 32 precursor	9.51E-05	0.0034796			
fig|6666666.542543.peg.5632	K09016	rutG	Pyrimidine permease	0.001863221	0.0331155			
fig|6666666.542543.peg.5638	K02393	flgH	Flagellar L-ring protein FlgH	2.02E-05	0.0010709			
fig|6666666.542543.peg.5642	K02389	flgD	Flagellar basal-body rod modification protein FlgD	5.35E-06	0.0004348			
fig|6666666.542543.peg.5644	K02387	flgB	Flagellar basal-body rod protein FlgB	1.68E-05	0.0009805			
fig|6666666.542543.peg.5660	K12372	dppF	Dipeptide ABC transporter, ATP-binding protein DppF (TC 3.A.1.5.2)	0.00040075	0.01041	91.20%	0	100.00%
fig|6666666.542543.peg.5666		NA	High potential iron-sulfur protein	6.75E-05	0.0026471			
fig|6666666.542543.peg.5687	K02414	fliK	Flagellar hook-length control protein FliK	6.96E-05	0.0027023			
fig|6666666.542543.peg.5701	K00789	metK	S-adenosylmethionine synthetase (EC 2.5.1.6)	1.70E-05	0.0009826	92.00%	0	97.64%
fig|6666666.542543.peg.5704	K07334	higB-1	Toxin HigB	0.002814576	0.0461044			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.5706	K09921	K09921	Protein of unknown function DUF484	6.06E-07	8.16E-05	87.40%	0	100.00%
fig|6666666.542543.peg.572		NA	hypothetical protein	0.00023567	0.006632	90.50%	3.22E-122	91.60%
fig|6666666.542543.peg.5734	K03571	mreD	Rod shape-determining protein MreD	1.05E-07	2.30E-05	92.20%	0	100.00%
fig|6666666.542543.peg.574	K09117	K09117	Transamidase GatB domain protein	0.00103113 8	0.0213523	89.80%	1.08E-157	96.20%
fig|6666666.542543.peg.5744		NA	Probable proline rich sigl peptide protein	0.000326 21	0.008644 1			
fig|6666666.542543.peg.5753	K01462	PDF, def	Peptide deformylase (EC 3.5.1.88)	4.89E-06	0.000404 9	88.80%	9.20E-179	100.00%
fig|6666666.542543.peg.5764	K01153	hsdR	Type I restriction-modification system, restriction subunit R (EC 3.1.21.3)	0.00137039 6	0.026923			
fig|6666666.542543.peg.5769	K01919	gshA	Glutamate--cysteine ligase (EC 6.3.2.2)	1.26E-05	0.000826 8	90.10%	0	98.39%
fig|6666666.542543.peg.5799		NA	Predicted transcriptiol regulators	1.91E-05	0.0010399			
fig|6666666.542543.peg.5810	K07497	K07497	Transposase InsD for insertion element IS2	0.00071383 8	0.016668 9			
fig|6666666.542543.peg.5818		NA	R polymerase sigma-54 factor RpoN	3.07E-05	0.0014072			
fig|6666666.542543.peg.5849		NA	Putative IpgF protein	3.99E-07	6.25E-05			
fig|6666666.542543.peg.585	K03602	xseB	Exodeoxyribonuclease VII small subunit (EC 3.1.11.6)	1.36E-05	0.0008687			
fig|6666666.542543.peg.5854	K06048	gshA, ybdK	Glutamate--cysteine ligase-like protein YbdK	8.03E-06	0.0005977	91.70%	0	99.82%
fig|6666666.542543.peg.5880	K03496	parA, soj	Chromosome (plasmid) partitioning protein ParA	5.98E-05	0.002408 6	87.50%	0	99.87%
fig|6666666.542543.peg.5881	K03497	parB, spo0J	Chromosome (plasmid) partitioning protein ParB	6.72E-06	0.000526 6	89.10%	0	99.55%
fig|6666666.542543.peg.5902		NA	Transcriptiol regulator, MerR family	0.00010330 8	0.0036872			
fig|6666666.542543.peg.5918	K02437	gcvH, GCSH	Glycine cleavage system H protein	4.11E-08	1.00E-05	89.60%	7.32E-139	100.00%
fig|6666666.542543.peg.5923	K03656	rep	ATP-dependent D helicase Rep	0.000162 279	0.005088 6			
fig|6666666.542543.peg.5980		NA	Phenylpropiote dioxygese and related ring-hydroxylating dioxygeses, large termil subunit	0.00176578 1	0.0317696			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.5982		NA	Dehydrogeses with different specificities (related to short-chain alcohol dehydrogeses)	0.00016801	0.0052034			
fig|6666666.542543.peg.5983	K00648	fabH	putative ketoacyl-ACP synthase	1.02E-05	0.00071			
fig|6666666.542543.peg.5984	K02078	acpP	hypothetical protein	0.000515457	0.0127127			
fig|6666666.542543.peg.6000	K02403	flhD	Flagellar transcriptiol activator FlhD	2.56E-05	0.0012781			
fig|6666666.542543.peg.6001	K02402	flhC	Flagellar transcriptiol activator FlhC	3.64E-05	0.0015974			
fig|6666666.542543.peg.601		NA	hypothetical protein	0.001026231	0.0213514			
fig|6666666.542543.peg.6013		NA	hypothetical protein	3.08E-05	0.0014072			
fig|6666666.542543.peg.6018	K02404	flhF	Flagellar biosynthesis protein FlhF	0.001309222	0.0261249			
fig|6666666.542543.peg.6030	K03746	hns	D-binding protein H-NS	0.002632841	0.0436157			
fig|6666666.542543.peg.6032	K03719	lrp	Transcriptiol regulator, AsnC family	0.000182036	0.0054583			
fig|6666666.542543.peg.6075	K02610	paaB	1,2-phenylacetyl-CoA epoxidase, subunit B (EC 1.14.13.149)	9.99E-05	0.0035952			
fig|6666666.542543.peg.6136	K03978	engB	GTP-binding protein EngB	1.49E-05	0.0009111	88.50%	0	100.00%
fig|6666666.542543.peg.6153	K01129	dgt	dNTP triphosphohydrolase, broad substrate specificity	7.78E-11	3.29E-08	89.90%	0	99.92%
fig|6666666.542543.peg.6160	K07491	K07491	Transposase and ictivated derivatives	0.000089055	0.0189509	90.30%	0	94.77%
fig|6666666.542543.peg.6162	K00266	gltD	Glutamate synthase [DPH] small chain (EC 1.4.1.13)	0.001619451	0.0301245	91.10%	0	100.00%
fig|6666666.542543.peg.6163	K02471	bacA	Efflux ABC transporter, permease/ATP-binding protein	5.78E-06	0.0004611	90.80%	0	99.20%
fig|6666666.542543.peg.6191	K03117	tatB	Twin-arginine translocation protein TatB	1.70E-06	0.0001862	93.50%	0	100.00%
fig|6666666.542543.peg.6196	K00412	CYTB, petB	Ubiquinol-cytochrome C reductase, cytochrome B subunit (EC 1.10.2.2)	8.71E-05	0.0032127			
fig|6666666.542543.peg.6197	K00413	CYC1, CYT1, petC	Ubiquinol-cytochrome C reductase, cytochrome C1 subunit	2.62E-06	0.0002558	90.30%	2.24E-32	13.52%

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.6244	K02492	hemA	Glutamyl-tR reductase (EC 1.2.1.70)	0.000190328	0.0056076	92.10%	0	100.00%
fig|6666666.542543.peg.6245		NA	FIG00452780: hypothetical protein	1.62E-05	0.0009721			
fig|6666666.542543.peg.6247	K01560	E3.8.1.2	Hydrolase	0.0008605	0.0186088			
fig|6666666.542543.peg.6257	K18307	mexI	RND efflux system, inner membrane transporter	0.00173679	0.0317604			
fig|6666666.542543.peg.6275		NA	Putative protease	0.000126629	0.0043093			
fig|6666666.542543.peg.6278	K18446	ygiF	Adenylate cyclase (EC 4.6.1.1)	0.001849749	0.0330098			
fig|6666666.542543.peg.6279	K01609	trpC	Indole-3-glycerol phosphate synthase (EC 4.1.1.48)	1.13E-06	0.000142	90.60%	0	100.00%
fig|6666666.542543.peg.6284	K01783	rpe, RPE	Ribulose-phosphate 3-epimerase (EC 5.1.3.1)	0.000130098	0.0043598	92.30%	0	100.00%
fig|6666666.542543.peg.630	K03772	fkpA	Peptidylprolyl isomerase, FKBP-type (EC 5.2.1.8)	1.92E-05	0.0010399			
fig|6666666.542543.peg.6316	K03070	secA	Protein translocase subunit SecA	0.000447714	0.0114939	91.40%	0	94.61%
fig|6666666.542543.peg.6317		NA	hypothetical protein	0.001257748	0.0253281			
fig|6666666.542543.peg.6339	K03106	SRP54, ffh	Sigl recognition particle protein Ffh	0.001334051	0.0264193	91.30%	0	100.00%
fig|6666666.542543.peg.6344		NA	hypothetical protein	0.002062188	0.0359246			
fig|6666666.542543.peg.6347		NA	hypothetical protein	0.000194495	0.0056678			
fig|6666666.542543.peg.6350	K11065	tpx	Thiol peroxidase, Tpx-type (EC 1.11.1.15)	7.41E-06	0.0005707			
fig|6666666.542543.peg.6354	K02160	accB, bccP	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	7.56E-11	3.29E-08	85.10%	1.98E-130	100.00%
fig|6666666.542543.peg.6361	K00014	aroE	Shikimate 5-dehydrogese I alpha (EC 1.1.1.25)	0.002501166	0.0417495	89.20%	0	98.63%
fig|6666666.542543.peg.6377		NA	hypothetical protein	2.01E-06	0.0002097			
fig|6666666.542543.peg.6390	K03711	fur, zur, furB	Ferric uptake regulation protein FUR	7.53E-05	0.0028762	91.60%	1.69E-170	100.00%
fig|6666666.542543.peg.6392	K00215	dapB	4-hydroxy-tetrahydrodipicolite reductase (EC 1.17.1.8)	0.002511898	0.0417698	90.00%	0	100.00%

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.6399	K08973	hemJ	Protoporphyrinogen IX oxidase, novel form, HemJ (EC 1.3.-.-)	3.54E-05	0.0015716	91.70%	7.77E-169	100.00%
fig|6666666.542543.peg.6418		NA	Putative transmembrane protein	0.002082206	0.03613			
fig|6666666.542543.peg.6454		NA	Cytochrome B561	0.000223538	0.0063312			
fig|6666666.542543.peg.6463	K07568	queA	S-adenosylmethionine:tR ribosyltransferase-isomerase (EC 2.4.99.17)	0.001379878	0.026923	90.50%	0	100.00%
fig|6666666.542543.peg.6470	K00286	proC	Pyrroline-5-carboxylate reductase (EC 1.5.1.2)	0.001517338	0.0285885	89.60%	0	99.26%
fig|6666666.542543.peg.6491		NA	hypothetical protein	0.000134083	0.0044593			
fig|6666666.542543.peg.6547		NA	FIG00453066: hypothetical protein	1.44E-05	0.0008953			
fig|6666666.542543.peg.6559	K24158	prx	Alkyl hydroperoxide reductase subunit C-like protein	1.45E-05	0.0008953	92.50%	0	100.00%
fig|6666666.542543.peg.658		NA	Uncharacterized UPF0118 membrane protein	0.000780672	0.0176657	92.30%	0	99.34%
fig|6666666.542543.peg.6582		NA	Predicted periplasmic or secreted lipoprotein	0.000523556	0.0128403			
fig|6666666.542543.peg.6598	K07735	algH	UPF0301 protein YqgE	3.06E-05	0.0014072			
fig|6666666.542543.peg.6608	K00067	rfbD, rmlD	dTDP-4-dehydrorhamnose reductase (EC 1.1.1.133)	0.001758037	0.0317604	87.60%	0	98.93%
fig|6666666.542543.peg.6653		NA	hypothetical protein	3.89E-05	0.0016733			
fig|6666666.542543.peg.6661	K03439	trmB, METTL1, TRM8	tR (guanine(46)-N(7))-methyltransferase (EC 2.1.1.33)	1.19E-07	2.49E-05	89.60%	0	98.84%
fig|6666666.542543.peg.6693	K18234	vat	Acetyltransferase (isoleucine patch superfamily)-like	0.000681923	0.0161819			
fig|6666666.542543.peg.6700	K00793	ribE, RIB5	Riboflavin synthase eubacterial/eukaryotic (EC 2.5.1.9)	8.61E-06	0.0006299	89.40%	0	93.40%
fig|6666666.542543.peg.6713	K10112	msmX, msmK, malK, sugC, ggtA, msiK	ABC transporter, ATP-binding protein (cluster 1, maltose/g3p/polyamine/iron); ABC transporter, ATP-binding protein (cluster 10, nitrate/sulfote/bicarbote)	0.000210892	0.0060118			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.6743		NA	Outer membrane porin	2.28E-07	4.35E-05			
fig|6666666.542543.peg.6764	K07010	K07010	Glutamine amidotransferase, class I	3.91E-07	6.25E-05	86.00%	0	98.09%
fig|6666666.542543.peg.6793	K00800	aroA	3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19)	3.72E-06	0.0003269	90.20%	0	100.00%
fig|6666666.542543.peg.6801	K03274	gmhD, rfaD	ADP-L-glycero-D-manno-heptose-6-epimerase (EC 5.1.3.20)	0.000759268	0.0173289	91.40%	0	99.70%
fig|6666666.542543.peg.6819	K02860	rimM	16S rR processing protein RimM	1.77E-05	0.0010063	87.50%	0	97.48%
fig|6666666.542543.peg.6849		NA	hypothetical protein	8.82E-06	0.0006346			
fig|6666666.542543.peg.6854	**K05524**	**fdxA**	**4Fe-4S dicluster fused with DUF3470**	**1.82E-13**	**7.98E-10**	**94.10%**	**1.31E-140**	**100.00%**
fig|6666666.542543.peg.6858		NA	putative anion permease	0.000260291	0.0071866			
fig|6666666.542543.peg.6861		NA	Acetyltransferase, GT family	0.000174518	0.0053576			
fig|6666666.542543.peg.6867	K08300	rne	Ribonuclease E (EC 3.1.26.12)	1.89E-05	0.0010399	90.00%	0	85.80%
fig|6666666.542543.peg.6879	K00645	fabD, MCAT, MCT1	Malonyl CoA-acyl carrier protein transacylase (EC 2.3.1.39)	6.14E-05	0.0024345			
fig|6666666.542543.peg.6883		NA	FIG00454574: hypothetical protein	0.000466207	0.0117268			
fig|6666666.542543.peg.69		NA	hypothetical protein	2.69E-06	0.0002564	91.10%	1.12E-42	100.00%
fig|6666666.542543.peg.6962	K07799	mdtA	Multidrug efflux system MdtABC-TolC, membrane fusion component MdtA	0.000685983	0.0161907			
fig|6666666.542543.peg.6964		NA	hypothetical protein	0.0012515	0.0253184			
fig|6666666.542543.peg.6965	K07093	K07093	Alkaline phosphatase (EC 3.1.3.1)	9.89E-05	0.0035876			
fig|6666666.542543.peg.6984		NA	hypothetical protein	7.28E-05	0.0028029			
fig|6666666.542543.peg.7061		NA	Transcriptiol regulator, LysR family	1.26E-05	0.0008268			
fig|6666666.542543.peg.7105		NA	hypothetical protein	2.67E-05	0.0013004			
fig|6666666.542543.peg.7139	K01069	gloB, gloC, HAGH	Hydroxyacylglutathione hydrolase (EC 3.1.2.6)	0.000154857	0.0049262	87.50%	1.14E-10	6.69%

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.7161	K01524	ppx-gppA	Exopolyphosphatase (EC 3.6.1.11)	2.86E-05	0.0013781	90.00%	0	100.00%
fig|6666666.542543.peg.720	K11733	lysP	Lysine-specific permease	0.000738215	0.0170566			
fig|6666666.542543.peg.7225		NA	hypothetical protein	3.33E-07	5.62E-05			
fig|6666666.542543.peg.7304		NA	Oxidoreductase probably involved in sulfite reduction	1.37E-05	0.0008687			
fig|6666666.542543.peg.7352	K21949	sbnA	2,3-diaminopropiote for siderophore biosynthesis protein Sb	3.27E-05	0.0014725			
fig|6666666.542543.peg.7385	K03436	fruR2, fruR	Transcriptiol regulator, DeoR family	0.000858832	0.0186088			
fig|6666666.542543.peg.74	K10829	fhuC	Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), ATP-binding protein FhuC	0.000730364	0.0169645	93.50%	0	100.00%
fig|6666666.542543.peg.7503	K01886	QARS, glnS	Glutaminyl-tR synthetase (EC 6.1.1.18)	0.000202622	0.005852	90.30%	0	100.00%
fig|6666666.542543.peg.7545	K00963	UGP2, galU, galF	UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	2.90E-06	0.0002705	92.60%	0	100.00%
fig|6666666.542543.peg.7553	K21695	aaeX	Tetrapartite efflux system component, FusD-like => FusD of FusABCDE system	0.002338753	0.0397951			
fig|6666666.542543.peg.7557		NA	hypothetical protein	2.47E-06	0.0002522			
fig|6666666.542543.peg.7567	K00311	ETFDH	Electron transfer flavoprotein-ubiquinone oxidoreductase (EC 1.5.5.1)	0.000880823	0.0189509	91.90%	0	100.00%
fig|6666666.542543.peg.7608		NA	Phage repressor protein cI	0.00081268	0.0179542			
fig|6666666.542543.peg.7647		NA	hypothetical protein	5.72E-05	0.0023255			
fig|6666666.542543.peg.7659	K02600	nusA	Transcription termition protein NusA	0.001165511	0.0237981	92.00%	0	100.00%
fig|6666666.542543.peg.7707		NA	hypothetical protein	5.22E-07	7.60E-05			
fig|6666666.542543.peg.7774		NA	hypothetical protein	0.00027428	0.0075256			
fig|6666666.542543.peg.78		**NA**	**FIG00456081: hypothetical protein**	**0.001141229**	**0.0234112**			
fig|6666666.542543.peg.7820		NA	hypothetical protein	0.000509132	0.0126276			

Lv-StA Gene ID	KO term	Gene name	Function in Lv-StA	DESeq2 P-value	DESeq2 P-adj	Lv-StB % Pairwise identity	Lv-StB E value	Lv-StB Query coverage
fig|6666666.542543.peg.7889	K12055	K12055, parA	Chromosome (plasmid) partitioning protein ParA	0.001401292	0.0272198			
fig|6666666.542543.peg.7904	K03497	parB, spoOJ	hypothetical protein	2.60E-05	0.0012812			
fig|6666666.542543.peg.7954		NA	hypothetical protein	8.98E-11	3.29E-08			
fig|6666666.542543.peg.7969		NA	hypothetical protein	0.0001226 34	0.0042952			
fig|6666666.542543.peg.985	**K00128**	**ALDH**	**Aldehyde dehydrogese B (EC 1.2.1.22)**	**1.18E-09**	**3.98E-07**			

Table-6. Genes involved in flagellar assembly and biosynthesis in *L. villosa* symbionts.

First column shows if the gene is essential during colonization (Green – not essential, red – essential) and whether they were absent in both conditions and/or from the original Tn-seq library (grey). Genes present in *Burkholderia* Lv-stA, Lv-StB, and in the other potential symbionts of *Lagria villosa* are highlighted in blue. (*Lutei* =*Luteibacter*, *Acin* = *Acinetobacter*, *Var* = *Variovorox*)

	Tn-seq	Genomes				
Basal body/hook	StA	StA	StB	*Lutei*	*Acin*	*Var*
MS/C ring, T3SS						
fliE						
fliF						
fliG						
fliH						
fliI						
fliJ						
fliK						
fliL						
fliM						
fliN						
fliO						
fliP						
fliQ						
fliR						
T3SS						
flhE						
flhA						
flhB						
Rod, P/L ring, hook						
flgA						
flgB						
flgC						
flgD						
flgE						
flgF						
flgG						
flgH						
flgI						
flgJ						
Hook-filament junction						
flgK						
flgL						

		Tn-seq	Genomes				
Basal body/hook		**StA**	**StA**	**StB**	***Lutei***	***Acin***	***Var***
H ring							
	flgO						
	flgP						
	flgQ						
	flgT						
T Ring							
	motX						
	motY						
Filament							
	fliC						
Filament cap, chaperone							
	fliD						
	fliS						
	fliT						
Stator							
	motA						
	motB						
	motC						
	motD						
Regulator							
	flgM						
	flgN						
	fliY						
	fliZ						
Global regulator							
	rpoD						
	flrA						
	flhD						
	flhC						
	rpoN						
Regulator							
	flrC						
	fliA						

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CHAPTER – 6

An insect host exhibits diminished systemic molecular response to the establishment of ectosymbionts

Manuscript in preparation

Ramya Ganesan [1,2], Rebekka S. Janke [1, 3], Jean Keller [2], Laura V. Flórez [1, 4], Martin Kaltenpoth [1, 2]

[1] Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg University, Mainz, Germany

[2] Department of Insect Symbiosis, Max Planck Institute for Chemical Ecology, Jena, Germany

[3] Department of Symbiosis, Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany

[4] Department for Plant and Environmental Sciences, Section for Organismal Biology, University of Copenhagen, 1871 Frederiksberg C, Denmark

CONTRIBUTIONS

R.G.: conceptualization, experimental work—PCRs, RNA extraction, qPCRs, RNA-sequencing analysis, visualization, writing—original draft, review and editing. R.S.J: conceptualization, experimental work—sample acquisition. J.K.: analysis, writing—review and editing. L.V.F.: conceptualization, funding acquisition, resources, supervision, writing—review and editing. M.K.: conceptualization, funding acquisition, resources, supervision, writing—review and editing.

1. ABSTRACT

Studies on insects harbouring intracellular symbionts and extracellular gut-associated symbionts, show that the innate immune system and physiological factors in the host can regulate symbiont colonization and establishment in a specific manner. Alternatively, some symbionts have evolved mechanisms to evade host immunity and establish in specific symbiotic organs of the host. However, we know little about how host regulatory factors determine partner specificity in ectosymbioses. To expand our knowledge, we studied the association between a darkling beetle and the extracellular defensive bacterial symbionts that are localized in specialized symbiotic structures on the dorsal surface of the larval cuticle. We performed an RNA-seq experiment across early beetle developmental stages; from eggs, first-instar, early to mid second-instar larvae, and compared gene expression patterns in individuals containing the natural community of symbionts *vs.* those that were made aposymbiotic. We find that in eggs, first-instar and early second-instar larvae, symbiotic individuals show almost no significant difference in gene expression compared to aposymbiotic beetles. At the mid second-instar stage, a small number of host genes (n=7) are differentially expressed. Therefore, it is likely that the host lacks partner recognition mechanisms during colonization, or that, host molecular factors are constitutively expressed in the symbiont-bearing structures of the beetle larvae. Further experiments will help confirm if a localized response can be observed in the dorsal structures of the larvae, or if presence of non-native or pathogenic microorganisms induce a more specific immune reaction in the beetles.

2. INTRODUCTION

A multi-layered landscape of innate immune mechanisms protect insects from pathogenic or opportunistic microorganisms (1,2). The cuticle and the epithelial layer lines the external surface, alimentary tract and tracheae, and act as the first line of defense. The cuticle provides protection against abiotic stress and antagonists like fungal pathogens or predators (3). Along the epithelial lining, antimicrobial peptides (AMP) prevent microbial entry into the body of the host. Beyond this barrier, haemocytes phagocytose and encapsulate microorganisms that infiltrate the haemolymph (1) and they form part of the cellular immunity of the host. Furthermore, microbe associated molecular patterns (MAMP), like peptidoglycan or lipopolysaccharide shed by the bacteria trigger humoral responses in the host. The MAMPs are recognised by the host's pattern recognition receptors (PRR) and lead to the activation of the Toll or IMD pathways and the production of antimicrobial peptides that disrupt the bacterial cell wall or interrupt cellular processes. Therefore, in most insects, the cuticle, epithelial layer, cellular and humoral components of immunity offer multiple layers of protection against invading pathogenic microbes.

Yet, how do host immune effectors recognize and accommodate beneficial symbionts that occur in many insects? In insect symbioses we find that i) beneficial symbionts evade or ii) resist immune activity in the host, iii) or the host may compartmentalize symbionts in organs to prevent contact with the immune effectors. For example, in tsetse flies, the presence of *Sodalis glossinidius* symbionts does not trigger an immune response in the host, whereas pathogenic *Escherichia coli* or a related free-living strain, *Sodalis preacaptivus*, induce an immune reaction (4). In addition, experiments with the African cotton-stainer, *Dysdercus fasciatus,* show that the gut symbionts are not drastically affected by alterations in host antimicrobial peptide gene expression (5). Here, *Sodalis preacaptivus* and the gut symbionts of African

cotton-stainer bug possibly evade or resist the immune activity of the host. Alternatively, compartmentalization of intracellular symbionts in designated symbiotic organs, like the bacteriome, prevent systemic immune activation in the host (6). Furthermore, in *Sitophilus* weevils, the antimicrobial peptide coleoptericin-A prevents the escape of the *Sitophilus* primary endosymbiont, *Sodalis pierantonius,* from the bacteriome (7). The host also cleaves Tracheal Cytotoxins (TCT) released from the symbiont cell wall and prevents a systemic immune activation (8). Therefore, compartmentalization and molecular policing of the symbionts in the organs help in maintaining a stable association. Apart from these scenarios, in an extreme case of host – symbiont integration, degeneration of the host immune system protects the primary symbiont in the pea-aphids (9) and costly immune functions are outsourced to facultative symbionts (10). Therefore, various host and symbiont strategies help in controlling or maintaining mutualistic symbioses. Apart from studies on symbionts housed in the insect gut, or bacteriomes, (4,5,7,8,11–13) it is not clear how symbionts localized on the external cuticle are assembled or maintained by the host.

To find if host molecular factors possibly recognize and regulate colonization of ectosymbionts, we studied the mutualistic interaction between *Lagria villosa* beetles and *Burkholderia* bacteria. *L. villosa* beetles are associated with symbionts that provide defensive benefits to the host (14–16). The beetles are invasive and potential crop pests (17,18) and at least part of their lifecycle is spent in the leaf-litter where they are exposed to fungal pathogens. During oviposition, female beetles smear the eggs with *Burkholderia* symbionts that produce antifungal compounds and prevent infection by entomopathogenic fungi (14,15). The most prevalent and abundant strain in the beetles is Lv-StB. It has a reduced genome, and is capable of producing the antifungal compound lagriamide (15,19). When the beetle larvae hatch, symbionts colonize specialized dorsal symbiotic structures that are invaginations of the cuticle (20), where they are housed until the pupal stage (21). Male adults progressively lose the symbionts during pupation and eclosion and the female beetles retain them in glandular structures accessory to the reproductive system (14,21,22).

We performed a transcriptomic study across the developmental stages of *Lagria villosa* beetles and analysed differences in gene expression between aposymbiotic and symbiotic beetles within each time point. The results show that very few genes are differentially expressed in the presence of symbionts in late eggs, first-instar larvae, second-instar larvae and mid second-instar larvae. The native symbionts of the *Lagria villosa* beetle likely do not induce an immune response in the host during colonization. When symbionts have established themselves in the dorsal structures in the mid second-instar larvae, a few immune related genes, including serine-protease inhibitors and serpins, are upregulated. We speculate that the initial assembly of the symbionts either occurs in an unspecific manner or is regulated by constitutively expressed host genes as well as the physiochemical environment of the dorsal cuticular organs.

3. METHODS

3.1. Beetle rearing

We collected *Lagria villosa* larvae and adults from three different locations near Rio Claro, Sao Paulo, Brazil in 2019. Beetles were transported live back to Germany and reared at 23-26°C, 55-60% 8-16 h day-

night cycle. Adult beetles were fed soybean leaves, lettuce and supplied with 50-ml tubes filled with water and closed with a cotton plug. Larvae were fed dried soy bean leaves and given water in 1.5 ml epis closed with a cotton plug. Female adults laid clutches of 200-300 eggs that were collected for further treatment and RNA-seq sampling. Since Lv-StB decreases in abundance in the lab (16) and is subsequently lost in the later generations, it was prudent to collect egg clutches laid by field-collected females in the lab and sample within the first-generation.

3.2. PCR of egg wash to check for symbiont presence

To obtain bacterial DNA from the egg wash for diagnostic PCRs, we collected four eggs from a freshly laid egg clutch and washed them with 100 µl sterile SDS (0.1%). The SDS was recovered and centrifuged at 10,000 x g for 5 min. The supernatant was removed and the (invisible) pellet was resuspended in 20 µl bacterial cell lysis solution (67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)2SO_4$, 5 mM beta-mercaptoethanol, 6.7 mM $MgCl_2$, 6.7 µM EDTA (pH 8) and 1.7 mM SDS) and incubated at 90°C for 5 min. 1 µl of this suspension was used for diagnostic PCR to check for the presence of *Burkholderia* sp. The 10 µl reaction mixture was prepared with 4.92 µl distilled water, 1 µl VWR Buffer S, 0.2 µl $MgCl_2$, 1.2 µl dNTPs, 0.8 µl Burk16s_1_F (5'- GTTGGCCGATGGCTGATT -3') primer and 0.8 µl Burk16s_1_R (5'- AAGTGCTTTACAACCCGAAGG -3') primer and 0.08 µl taq polymerase, as well as 1 µl of the egg wash suspension. A standard gel electrophoresis was used to assess the results. If the egg wash tested positive for *Burkholderia* presence, we proceeded to treating it for RNA-seq sampling.

3.3. Treatment and sampling for RNA-seq

A freshly laid egg clutch was collected, and after confirming the presence of *Burkholderia* sp., the clutch was split into different groups. One-third of the clutch was left untreated. The remaining eggs were washed with sterile 1x PBS (2.5 µl/egg) to collect the symbiont cells on the egg surface, and the eggs were subsequently surface-sterilized. Surface sterilization was performed by washing the eggs with 70% ethanol for 5 min, and with 12% sodium hypochlorite for 30 seconds. At the end of each step, eggs were washed with sterile water twice to remove the chemicals. The sterilized eggs were split into two groups for aposymbiotic and reinfection treatments.

To incubate the eggs, a thin-layer of vermiculite was added to a petridish (⌀ 90 cm) and sprayed with water to moisten it. The wet vermiculite was covered with a filter paper. Each group of eggs was added into separate plates. For the reinfection treatment, the PBS symbiont suspension washed off previously was applied onto the eggs (2.5 µl/egg). For the aposymbiotic treatment, we added 1x PBS to the eggs (2.5 µl/egg).

The eggs were incubated for 1 hour at 26°C, 60% humidity, and then 10 eggs were collected in 1.5 ml epis and frozen at -80°C for the first time point. We collected early eggs (t1), late eggs (t2), first-instar (t3), early second- instar (t4), mid second-instar (t5), late seventh-instar (t6), early pupa (t7), late pupa (t8) on day 0, day 5, ~day 6, ~day 7, ~day 10, day x-1, day x, day x+6 counted from the day of egg laying. For each egg and larval stage up to the seventh-instar, 10 individuals were collected and pooled together in a 1.5 ml tube before freezing at -80°C. In the seventh-instar and pupal stages, one individual was collected per 1.5 ml tube, flash frozen in liquid nitrogen, and stored in the -80°C.

3.4. RNA extraction workflow

RNA extraction was performed with the InnuPrep DNA/RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol for extracting RNA and DNA from tissue samples. Pooled egg and larval samples were homogenized in liquid nitrogen before extraction. Seventh instar larvae and pupae were split into three parts, processed separately to prevent clogging of the columns, and the nucleic acids from each part were combined in the end. All extractions were performed on ice. In the final step, RNA was eluted by adding 30 µl RNAse free water, and DNA was eluted in 30 µl of elution buffer. RNA samples were DNase treated according to the protocol in Quantabio PerfeCTa DNase I kit (Qiagen) to remove any contaminating DNA. According to the manufacturer's recommendations, we added 4 µl of reaction buffer and 4 µl DNase-I to 30 µl of the RNA aliquot. Samples were incubated at 37°C for 30 min. After incubation, 4 µl of the stop buffer was added and incubated at 65°C for 10 min. RNA and DNA concentrations were measured on a Qubit fluorometer (Thermo fisher) using the RNA High sensitivity assay kit (Thermo fisher). 5 µl of the extracted RNA was aliquoted for cDNA synthesis and qPCR. DNA and RNA samples were stored at -80°C.

3.5. cDNA synthesis and qPCR to confirm Lv-StB presence

To assess the presence or absence of Lv-StB before RNA-sequencing in all untreated, reinfected and aposymbiotic samples, we performed qPCRs. RNA aliquots were first converted to cDNA using FIREScript Reverse Transcription cDNA synthesis mix with random primers from Solis Biodyne. A 20 µl master mix was prepared with 2 µl 10x reaction premix with random primers, 1.5 µl FIREScript Enzyme Mix and 14.5 µl nuclease free water and 2 µl of the RNA template. The mixture was incubated at 25°C for 10 min for primer annealing, 50°C for 60 min for reverse transcription, and 85°C for 5 min for inactivating the enzyme. cDNA samples were stored at -20°C.

qPCR was performed using primers StB16S_1051_F (5'- CAAGCGGTGGATGATGTGGA - 3') and StB16S_1148_R (5' – TTCCGAGCACCCTCAGATTTT -3') that specifically target the Lv-StB 16s rRNA gene. The 10 µl reaction mix was prepared using 5x HOT FIREPol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia) by adding 6 µl water, 2 µl EvaGreen mix, 0.5 µl of each primer, and 1 µl template. The qPCR cycles were performed on a RotorGene-Q cycler (Qiagen) with the following conditions: initial denaturation at 95°C for 10 s, denaturation at 95°C for 20 s, annealing at 65 °C for 20 s, and extension at 72°C for 15 s for 75 cycles. We used the threshold cycle values to assess the presence or absence of Lv-StB. A fewer number of cycles in the qPCR reaction is enough to amplify bacteria which is more abundant in a sample. Therefore, samples in the early time points (t1 – t5) that amplified earlier than Ct = 30 were assumed to contain Lv-StB. To ensure that the difference in Lv-StB abundance between symbiotic and aposymbiotic samples was pronounced, those with a Ct above 35 were taken to be aposymbiotic. Lv-StB abundance also seems to reduce in the later time-points in the lab (t6- t8), and so, we adjusted the Ct thresholds for t6 – t8. Those that had a Ct of less than 40 were assumed symbiotic and above 60 were assumed aposymbiotic for Lv-StB.

3.6. Sequencing and analysis

To assemble a *Lagria villosa* reference genome, DNA was extracted from selected beetle specimens using the Nanobind Big DNA kit (Circulomics, Baltimore, MD, USA) followed by enrichment for high molecular weight DNA using the Short Read Eliminator kit XS (Circulomics), following the manufacturer's instructions. Sequencing was performed on a MinION platform (Oxford Nanopore Technologies). The resulting reads were de novo assembled using Flye v2.7.1 (43) followed by four rounds of polishing with Racon v1.3.3 (44). Reads were re-aligned to the resulting assembly with minimap2 v2.17 (45). A final round of polishing was performed using Medaka v1.2.0 (https://github.com/nanoporetech/medaka). After polishing, haplotype redundancies were merged using Purge haplotigs v1.0.4 (46) and collapsed duplicated haplotigs using Haplomerger2 v2.01 (47). Additionally Illumina short-read sequencing was performed at the Max Planck-Genome Centre (Cologne, Germany). Paired-end reads (2 x 150 bp) were generated by sequencing the library on an Illumina HiSeq2500 platform in Rapid Mode. Polishing of the final MinIon assembly with Illumina short reads was performed using ntHits ver. 0.1.1 (https://github.com/bcgsc/nthits) and ntEdit ver. 1.3.2 (48) using default settings. The relative contig coverage, GC content and contig taxonomic classification were scanned after genome assembly using Blobtools (https://github.com/DRL/blobtools) and TaxonAnalysis to enable the identification and removal of potential microbial symbionts or contaminants.

For the gene expression analysis from the aposymbiotic and untreated groups six replicate RNA samples were sequenced from time points t2 to t5 (late eggs to mid second-instar larvae), two replicates from t6 (late seventh-instar larvae), and three replicate pupae at t7 and t8 (early and late pupae). RNA-sequencing was performed at the Max Planck- Genome –Centre in Cologne. Samples were first subjected to dual rRNA depletion of insect and bacterial rRNAs. For insect rRNA depletion, a collection of oligos designed based on *Aedes albopictus* was used. The prepared libraries were sequenced paired-end, 150 bp read length, and at a depth of 25 Mio reads per sample on an Illumina HiSeq3000 platform. Raw reads were trimmed and low quality reads were removed using Trim Galore v0.6.10 (https://github.com/FelixKrueger/TrimGalore) with default parameters and quality threshold set to 30. Read quality was assessed with fastQC (23) from the Trim Galore package. The Nextflow (v22.10.7) RNASeq r3.12 (10.5281/zenodo.1400710) was used to estimate the differentially expressed genes. The pre-processing of reads (cleaning and removing adapters) was deactivated with the option --no-trimming. Ribosomal RNA were removed using SortMeRNA v4.3.4 (49). Read mapping and counting were performed using STAR v2.7.10a (50) and Salmon v1.10.1 (51). Raw counts were used to assess differential expression of genes on EdgeR v3.42.24 (24) and results of time points t2, t3, t4, t5 are shown. An exact test was applied to estimate differentially expressed genes with the aposymbiotic condition as reference.

4. RESULTS

To investigate how the host responds to the presence of the symbionts, we performed an RNA-seq experiment and analysed differences in gene expression between symbiotic and aposymbiotic beetles across different life stages. We initially sampled beetles that were i) untreated so that they retained their natural symbiotic community, ii) sterilized to make them aposymbiotic and iii) reinfected with the natural symbionts. However, qPCRs showed that reinfection with the natural symbiont community did not

successfully lead to reestablishment of the microorganisms and so we removed these samples from the RNA-sequencing procedure **(Fig. 1)**. Eggs that were collected immediately after they were laid on day 0 did not contain enough RNA, and so, were not sequenced. We sequenced aposymbiotic and untreated samples from late eggs (n=6), first-instar larvae (n=6), early second-instar larvae (n=6), mid second-instar larvae (n=6), late seventh-instar larvae (n=2), early pupae (n=3), and late pupae (n=3) **(Fig. 1)**. To identify differences in host gene expression during symbiont establishment, we compared symbiotic and aposymbiotic late eggs (t2), first-instar (t3), early (t4) and mid-second-instar larvae (t5). Read counts from 28,259 CDS encoded by *L. villosa* were filtered to retain 10204, 10164, 10304, 10477 CDS in t2, t3, t4, and t5, respectively. Among them, at time points, t2, t3, t4 and t5, only 4, 0, 5 and 7 genes respectively, showed differential regulation in the presence of the symbionts. The genes differentially expressed to the presence of symbionts within each of the time-points are shown in **Table-1.**

In the presence of symbionts on the eggs, a gene with DNA methyl transferase activity (Lagvil_g13340) and another gene with an unknown function (Lagvil_g4257) were upregulated. Likely, due to the presence of some bacterial contigs in the reference genome, we also find two bacterial genes upregulated in the symbiont containing samples, a LysR transcription regulator with a winged-helix DNA binding domain (Lagvil_g7179) and an iron-sulphur domain containing protein (Lagvil_g4672). Surprisingly, in first-instar larvae, we found no differentially expressed genes.

In early second-instar larvae, five genes were upregulated in response to symbionts - a gene encoding a ribosomal protein (Lagvil_g14828), a gene of unknown function (Lagvil_g8831), a translation elongation factor (Lagvil_g12535), a bacterial RNA polymerase (Lagvil_g7881), and a gene with potential oxidoreductase activity (Lagvil_g14008). Again, the possible presence of contaminating bacterial contigs in the reference genome might explain the presence of a bacterial RNA polymerase in the list of differentially expressed genes.

In mid second-instar larvae, the presence of symbionts induced the expression of some innate immunity related genes - a serine protease inhibitor (Lagvil_g20140), a gene encoding a serpin (Lagvil_g26415), and another serine protease inhibitor (Lagvil_g20139). We also found three more upregulated genes - two genes encoding proteins of unknown function (Lagvil_g8831, Lagvil_g25255), a type-B carboxylesterase (Lagvil_g17683) and a multicopper oxidase (Lagvil_g4130). Across different time points, we identified only one commonly occurring gene, Lagvil_g8831 (in bold in **Table-1)**, upregulated in the presence of symbionts in both t4 and t5 in second-instar larvae; however its function remains unknown.

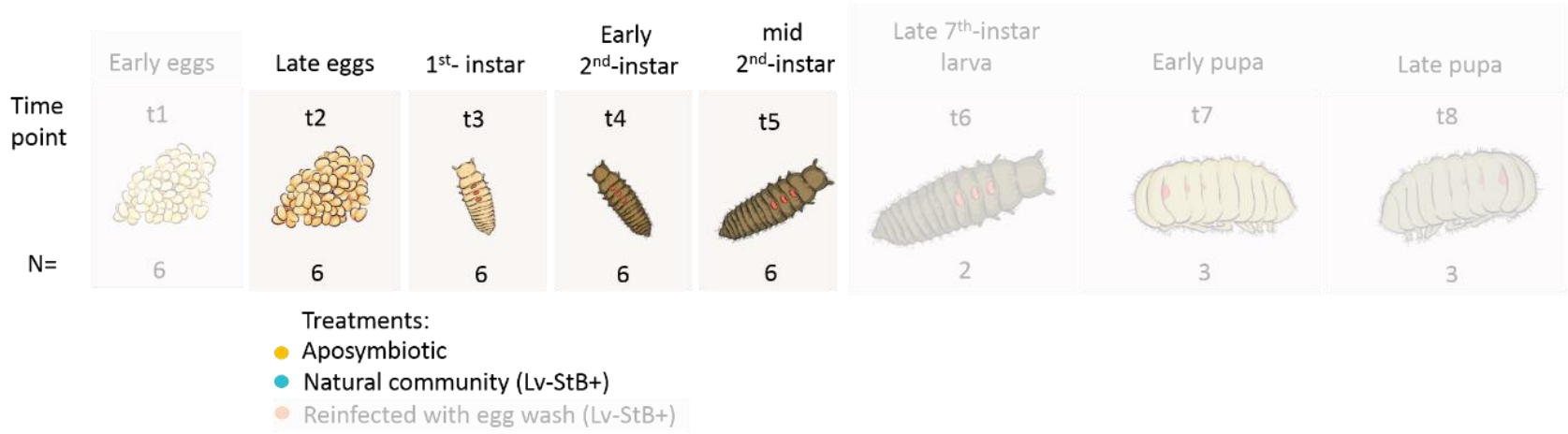

Fig.1. RNA sequencing experiment: Samples for RNA-sequencing were collected from early egg to late pupal stages. Three treatments were applied to the insects before sampling – eggs were surface sterilized to generate aposymbiotic beetles, or left untreated to maintain the natural symbiont community (that includes Lv-StB) or reinfected with the egg wash that contains the natural symbiont community (including Lv-StB). Due to loss of Lv-StB under laboratory conditions, or low RNA quantity in certain samples, we sequenced samples from t2 to t8 and included only the aposymbiotic and untreated treatments. Due to low number of replicates for t6, t7, t8, only results of RNA-seq analysis from t2-t5 (aposymbiotic vs. untreated) are presented in this chapter.

Table-1: *L.villosa* differentially expressed genes in response to the presence of a natural community of symbionts

Time point	Genes	Log Fold Change	Log CPM	P-value	FDR (False Discovery Rate)	Interpro code	Description	GO terms
Late eggs	Lagvil_g4257	9.40	5.38	5.96919E-36	6.09096E-32			
	Lagvil_g13340	10.33	4.45	5.21845E-30	2.66245E-26	IPR018117;IPR008593;IPR001525;IPR029063	S-adenosyl-L-methionine-dependent methyltransferase superfamily	GO:0003677|GO:0009007|GO:0032775;GO:0008168
	Lagvil_g7179	7.16	2.54	6.94662E-22	2.36278E-18	IPR005119;IPR000847;IPR036390;IPR036388	LysR transcription regulator; Winged helix-like DNA-binding domain superfamily	GO:0003700|GO:0006355

Time point	Genes	Log Fold Change	Log CPM	P-value	FDR (False Discovery Rate)	Interpro code	Description	GO terms
	Lagvil_g4672	7.37	3.29	2.46E-18	6.27951E-15	IPR015879;IPR017941;IPR001663;IPR036922	Aromatic-ring-hydroxylating dioxygenase ;Rieske [2Fe-2S] iron-sulphur domain superfamily	GO:0005506|GO:0044237|GO:0051537;GO:0051537;GO:0044237|GO:0051537
First-instar larvae								
Early second-instar larvae	Lagvil_g14828	7.35	3.27	2.77E-13	2.85E-09	IPR012340;IPR035104;IPR003029;IPR022967;IPR000110	Ribosomal protein S1	GO:0003676;GO:0003723|GO:0003735|GO:0005840|GO:0006412
	Lagvil_g8831	2.31	5.47	1.59E-11	8.19E-08			
	Lagvil_g12535	4.81	3.82	2.95E-11	1.01E-07	IPR004541;IPR009022;IPR027417;IPR004540;IPR005517;IPR035647;IPR004160;IPR041095;IPR009001;IPR005225;IPR000795;IPR004161;IPR000640;IPR009000;IPR020568;IPR031157;IPR033720;IPR041709;IPR035649;IPR014721	Translation elongation factor EFTu/EF1A, bacterial/organelle	GO:0003746|GO:0005525|GO:0006414;GO:0005525;GO:0003924|GO:0005525
	Lagvil_g7881	2.83	3.12	5.30E-06	0.013649612	IPR007121;IPR037034;IPR007642;IPR007644;IPR037033;IPR014724;IPR007645;IPR019462;IPR042107;IPR015712;IPR007641;IPR007120;IPR010243	RNA polymerase, beta subunit, conserved site, bacterial-type	GO:0003677|GO:0003899|GO:0006351;GO:0003899;GO:0003899|GO:0006351;GO:0003899|GO:0006351|GO:0032549

Time point	Genes	Log Fold Change	Log CPM	P-value	FDR (False Discovery Rate)	Interpro code	Description	GO terms
	Lagvil_g14008	2.41	2.91	1.49E-05	0.030711087	IPR017896;IPR037943;IPR044906;IPR006963;IPR009010;IPR036259;IPR027467;IPR006547;IPR029263;IPR006656;IPR006655;IPR028189;IPR006657;IPR006468;IPR038262	4Fe-4S ferredoxin-type; Molybdopterin oxidoreductase	GO:0016491;GO:0051539;GO:0008940|GO:0009325|GO:0042126;GO:0016491|GO:0043546
Mid second-instar larvae	Lagvil_g20140	3.96	2.82	3.39E-15	3.56E-11	IPR036084;IPR002919	Serine protease inhibitor-like superfamily	
	Lagvil_g8831	3.04	5.41	9.23E-13	4.83E-09			
	Lagvil_g26415	2.47	4.13	1.04E-11	3.63E-08	IPR023795;IPR042185;IPR000215;IPR023796;IPR036186;IPR042178	Serpin superfamily, domain 1	GO:0004867|GO:0005615
	Lagvil_g17683	1.96	3.36	1.77E-10	4.63E-07	IPR002018;IPR019826;IPR029058;IPR019819	Carboxylesterase type B	
	Lagvil_g25255	1.48	4.62	7.74E-09	1.62E-05			
	Lagvil_g20139	3.12	2.27	3.91E-06	0.006824139	IPR036084;IPR002919	Serine protease inhibitor-like superfamily	
	Lagvil_g4130	2.43	3.16	1.70E-05	0.025417273	IPR002355;IPR001117;IPR008972;IPR045087;IPR011706;IPR033138;IPR011707	Multicopper oxidase	GO:0005507;GO:0005507|GO:0016491

5. DISCUSSION

The results of the RNA-seq experiment show that only a few genes are differentially expressed in response to the presence of the natural community of symbionts across early developmental stages of the beetle. In insect eggs, the serosa is capable of mounting an innate immune response to phagocytose and encapsulate pathogens, or produce antimicrobial peptides and melanin to deter them (25–27). However, it seems like in symbiont-infected eggs of *Lagria* beetles, only a DNA methylase is upregulated in the presence of symbionts. DNA methylation can alter gene expression of the host and can possibly help in adaptation to environmental changes or stressors. For example, *Wolbachia,* a bacterial symbiont found in many insects, expresses miRNAs in *Aedes aegypti* during infection and suppresses a host cytosine methyltransferases, thereby ensuring the symbionts' maintenance in the mosquito host (28,29). However, we do not yet know the targets of the upregulated DNA methylase in the *Lagria* beetles. Apart from the DNA methylase, we do not find differential regulation of any other host genes in symbiotic *Lagria* eggs compared to the aposymbiotic eggs. Similarly, lack of host response to bacterial infection is also seen in eggs of the carrion beetle, *Nicrophorus vespilloides* (30).

Furthermore, after hatching, the first-instar larvae in *Lagria villosa* have no differential response to the presence of symbionts. One can speculate that this would allow bacterial colonization from the egg surface to the dorsal structures of the first-instar larva in a highly unspecific manner. Lack of melanization in first-instar larvae possibly promotes colonization by natural symbionts and other microorganisms. A similar pattern seems to exist immediately after first-instar larvae moult into the second-instar stage since the four upregulated genes identified from the RNA-seq analysis **(Table-1)** regulate essential cellular processes and lack relevance to microbial colonization or, originate from reads belonging to prokaryotes in the dataset.

However, ten days after egg laying, when the host is at the mid second-instar stage, the presence of symbionts upregulates host immune related genes like serpins and serine protease inhibitors. In arthropods, a serine protease cascade is triggered by the presence of pathogens (31) and it activates the prophenoloxidase pathway (proPO) leading to melanization, wound healing, and cuticle sclerotization. In *Drosophila*, peptidoglycans from bacterial cell wall activate a serine protease cascade and lead to the activation of Spätzle in the toll pathway (31). Regulation of the downstream pathways to prevent an overactive immune response is done by serpins, or serine protease inhibitors that have a protease inhibition activity (32). The serine proteases and the regulatory serpins seem to be expressed in some symbiont- carrying insects and in response to parasites. For example, in the honeybee gut, presence of the *Frischella perrara* upregulates host PRRs, serine proteases and regulatory serpins leading to a melanisation response which is seen as a scab phenotype at the pylorus region of the bee gut (33). Conversely, in the whitefly, *Bemisia tabaci,* parasitization by a wasp, *Eretmocerus mundus,* downregulates the regulatory serpins in the whitefly, and induces melanization and wound healing responses (34). In the *Lagria villosa* beetles, among the genes involved in the melanisation response, only the immune regulatory serpins and serine protease inhibitors are upregulated in mid second-instar larvae. This suggests that, in the presence of the symbionts, the host suppresses an overactive immune response and prevents symbiont removal.

Furthermore, a multi copper oxidase is upregulated in symbiotic mid second-instar larvae. Multi-copper oxidases are involved in a wide range of activities including pigmentation, sclerotization of the insect cuticle, lignin synthesis and degradation, iron homeostasis and morphogenesis (35,36). Apart from serpins and a multicopper oxidase, we find that a carboxylesterase is upregulated in the symbiotic host. Carboxylesterases are involved in detoxifying pesticides and xenobiotics (37). In *Bombyx mori* injection with lipopolysaccharide or infection with *E.coli* seem to induce the expression of homologs of carboxylesterase in the hemolymph and the authors speculate that they may help in detoxifying molecules released during pathogenesis (38). In *Galleria mellonella,* fungal infection induced the appearance of pigmentation spots on the cuticle of larvae and increased esterase activity in the hemolymph possibly due to the damage caused to the cuticle during infection (39). Apart from a few such examples, the mode of action and target of carboxylesterases during microbial infections in insects remains poorly understood. It is likely that a similar detoxification of microbial-breakdown products is essential in *Lagria* larvae as the symbionts establish themselves in the dorsal structures.

Therefore, by comparing gene expression in aposymbiotic and symbiotic beetles across the early life-stages of the *Lagria villosa* beetles, we find that very few host genes are differentially regulated in the presence of the symbionts during and after establishment. It is likely that symbiont localization on the external surface of the eggs and in the cuticle-lined dorsal structures prevents contact with the host haemolymph. Therefore, the host effectively avoids immune activation by the symbionts by compartmentalizing them in external symbiotic structures (6). However, host molecular regulation of symbionts is usually necessary to ensure partner specificity. In the *Lagria villosa* beetles, in order to ensure partner specificity during establishment, it is possible that host molecular factors within the dorsal structures are expressed constitutively. In our experiment, by comparing whole individuals that were symbiotic and aposymbiotic, constitutively expressed tissue-specific genes might have gone undetected. To understand if the conditions within the symbiotic structures are selective for *Burkholderia* symbionts, we need to learn about the physiological and immunological conditions within the symbiotic structures. It is also likely that host control over symbiont establishment is minimal and, given a nutrient-rich environment within the symbiotic structures, microbe-microbe competition may drive colonization and prevent non-beneficial microbes from entering the structures. Such symbiont-directed competitive colonization is observed in the *Riptortus* bean-bugs and the *Acromyrmex* leaf-cutter ants (40–42). Moreover, further experiments would be necessary to understand if the beetle distinguishes the presence of the other *Burkholderia* strains, including Lv-StA. Along these lines, experiments with non-symbiotic bacteria, or pathogens might help us understand how the *Lagria* host responds to other bacteria or antagonists. Nevertheless, using the *Lagria villosa* – *Burkholderia* sp. defensive symbiosis we obtain insights into how partner specificity could be established in an ectosymbiotic association.

6. CONCLUSION

Lagria villosa beetles are associated with a defensive bacterial symbiont that resides on the external surface of the eggs and in specialized dorsal surface structures in the larval and pupal stages. We performed an RNA-sequencing experiment and differential gene expression analysis to understand if host molecular factors on the cuticular surface recognise the presence of the symbionts especially during symbiont colonization and establishment. Comparing beetles containing the natural symbiont

community with aposymbiotic individuals, we show that in late eggs, first-instar larvae and early second-instar larvae, the host shows no differential response to the presence of the symbionts, so the symbionts may go undetected during colonization. In mid second-instar larvae, we find upregulation of serpins, serine protease inhibitors, a carboxylesterase and a multicopper oxidase, that may regulate toll and melanization pathways of the host. It is surprising that such a small number of genes are differentially expressed in the presence of the symbionts. We speculate that the host may not regulate early colonization in the beetles, or that compartmentalization on the external symbiotic structures prevents immune activation in larvae, and a constitutive host response might be localized in the dorsal structures of the larvae, altering the symbiotic environment, and allowing only native symbionts to colonize and establish themselves in the host.

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CHAPTER – 7

GENERAL DISCUSSION

In several open systems, where the symbionts are acquired from the external environment or from conspecifics, a molecular cross-talk between the host and symbionts lead to establishment **(Chapter 2)**. However, in ectosymbiotic associations the involvement of host and symbiont molecular factors during establishment is yet to be determined. The aim of this thesis was to expand our knowledge using the *Lagria villosa – Burkholderia* symbiosis as a study system. First, we looked into the dynamics of symbiont colonization and identified that symbionts are acquired during or shortly after hatching by the beetle larvae **(Chapter 3)**. Next, by establishing a transposon-insertion directed sequencing (Tn-seq) method in the lab **(Chapter 4)**, putative microbial mechanisms directing symbiont colonization in the dorsal structures of the *Lagria villosa* larvae were identified **(Chapter 5)**. Similar to the more dominant strain in the natural system, Lv-StB, the cultivable strain, Lv-StA does not seem to rely on flagellar motility during colonization. And lastly, looking across the early developmental stages of the beetle, we find that the host's molecular response to symbiont presence is minimal. This indicates that microbial factors and microbe-microbe interactions may play a role in determining colonization success **(Chapter 6)**. This chapter provides a summary and discussion about how *Burkholderia* symbionts establish themselves in *Lagria* beetles and how partner specificity may be achieved in this ectosymbiosis. In addition, symbiont mechanisms that may be involved in transitions across the parasitism – mutualism continuum and between different hosts are discussed. Finally, the advantages and limitations of using Tn-seq to identify symbiont factors mediating colonization are summarized.

1. PARTNER SPECIFICITY IN OPEN SYMBIOSES

A symbiosis that maintains a mixed transmission modes enjoys the availability of a stable symbiotic partner and is flexible to welcoming novel partners (1) **(Chapter 2)**. When symbionts colonize from the environment and are maintained extracellularly, the host risks losing them and encountering antagonistic partners during establishment. Therefore, host molecular mechanisms help ensure that the right partner is selected during colonization. In open symbioses, which are systems where the symbionts are acquired from the external environment or from conspecifics (2,3), microbial molecular mechanisms help the symbionts migrate across the external and internal host environment (4,5) **(Chapter 2)**. Symbiont machinery including motility, chemotaxis, and cell-surface molecules, provide the ability to detect and respond to host and environmental conditions. The ability to deal with oxidative stress, withstand the host's immune reactions and interact with co-infecting microbes may decide the fate of the symbiont in the host.

In the *Lagria villosa* beetles partner specificity might be achieved in several ways. These may include mechanisms that act before or during acquisition, like i) host control over the timing of colonization, ii) behavioural mechanisms to acquire partner symbionts (6) **(Chapter 3)**, or iii) host physiological and immune **(Chapter 6)** adaptations may provide a selective environment for the symbionts. Correspondingly, symbiont molecular factors **(Chapter 4,5)** and competition-based selection (7–9) could play equally important roles in establishing partner specificity. **Chapter 7** expands upon each of these

aspects in the *Lagria – Burkholderia symbiosis* while drawing examples from other animal – microbe symbioses and from the field of microbial ecology.

2. SYMBIONT ACQUISITION - HOST BEHAVIOURAL MECHANISMS AND SYMBIONT MOTILITY

In the terrestrial ecosystem, host behavioural traits and microbial adaptations work simultaneously or successively to initiate contact and direct symbiont translocation into the symbiotic organs. Previous studies show that the female *Lagria* beetles smear microbial symbionts onto the egg surface during oviposition (10,11). Although Stammer observed pre-hatch colonization of the larval dorsal structures in *Lagria hirta* beetles (10), we found that, at least in *Lagria villosa* species, the larvae take up the symbionts only during or after hatching (6) **(Chapter 3) (Fig. 1)**. During hatching, larval movements likely transfer the symbionts from the eggs onto the cuticular surface **(Fig. 1)**. Correspondingly, host behavioural adaptations to acquire symbionts from maternally provisioned symbiont-bearing substances on the egg are observed in several non-social stink – bugs. Here, symbionts are smeared onto eggs or enclosed in capsules or a jelly deposited on the egg surface (12–14) and the newly emerged juvenile nymphs search for the symbiont-bearing capsules on the egg shells and ingest them. Sometimes, symbiont-released semiochemicals could also attract the host and promote transmission. In the German cockroach, *Blattella germanica,* gut symbionts mixed with the faeces produce volatile carboxylic acids that promote aggregation of nymphs and lead to transmission (15). Similarly, in *Anasa tristis,* newly emerged nymphs are attracted to faeces of conspecifics that contain *Caballeronia* symbionts, although the chemical nature of this attraction is yet unknown (16). In contrast to these examples, in *Lagria villosa,* symbionts smeared on the egg surface are not ingested and likely reach the external symbiotic dorsal cuticular structures of the larvae through an external transmission route (6) **(Chapter 3)**. When the larva hatches from the egg, head- first, bacterial cells are transferred from the egg shell onto the larval cuticular surface. Whether these behavioural traits are adaptive, and induced in response to any volatiles released by the symbionts is unknown. By observing and comparing the duration and the movements of the larva during hatching, in the presence and absence of symbionts, we could test if the host senses the presence of the symbionts on the egg surface and responds to it.

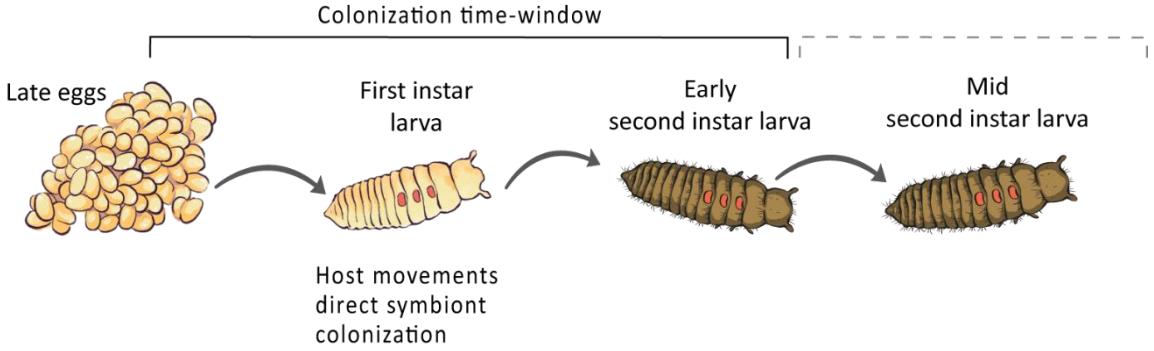

Fig. 1. Symbiont colonization in *Lagria villosa* beetles: Symbionts colonize larvae during hatching and colonization efficiency may decrease over developmental time. In older larvae, host molecular mechanisms may restrict symbiont colonization (dotted lines). Thus a colonization time-window may help establish partner specificity.

Furthermore, *Lagria villosa* beetles have a mixed mode of symbiont transmission (17). Vertical transmission via the egg surface reduces the risk of losing the symbiont and ensures that the faithful

partners are retained through the generations. However, *Lagria* larvae can occasionally acquire *Burkholderia* symbionts from the environment under frequent exposure to the symbionts (17). Environmental acquisition of symbionts may help stabilize the symbiotic association. However, it is likely that the rate of horizontal acquisition may be lesser in the field than in the lab conditions (17) and may differ across developmental stages in the larvae. In **chapter 3** (6), we find that the efficiency of symbiont acquisition may reduce by the seventh day after hatching **(Fig. 1)**. For the beetle host, confining symbiont acquisition within a time window may help avoid symbiont replacement or infection by antagonists beyond this period. Similarly, among other symbiont –associated invertebrates, leaf-cutter ants (18), Hawaiian bobtail squids (19), and the *Riptortus* bean-bugs (20) have established a specific time-window for acquiring symbionts from conspecifics or the external environment. In the Hawaiian bobtail squid and the *Riptortus* bean-bug, the entrance to the symbiotic organs constricts following symbiont colonization, thereby preventing further entry of microbes (19,21). In the *Lagria* beetles, the mechanism that controls the microbial colonization time-window and conditions that favour occasional horizontal acquisition from the environment are unknown **(Fig. 1)**. It may involve temporal changes in the expression of host molecules within the symbiotic structures **(Section – 3.2, Chapter 7)** and/or changes in morphology or closure of the entrance to the larval dorsal structures. Comparing gene expression within the organs and in the rest of the body, and imaging to measure the size of the organs between first-, second-instar, and older larvae might help us understand changes within the dorsal structures over time in response to symbiont presence.

Furthermore, during hatching, symbiont migration into the dorsal symbiotic organs may rely on bacterial molecular mechanisms like motility and chemotaxis (5,6) **(Chapter 2,3)**. Microbial motility plays an essential role in several open symbioses for initiating contact and establishing in the symbiotic organs host (22). For example, in the *Riptortus* bean-bug, *Caballeronia* symbionts acquired orally from the external environment, migrate through the constriction region between the M_3 to the M_4B in the mid-gut using a specialized cork-screw like motility and establish in the host (21,23). Similarly, given that Lv-StA can migrate between beetle – plant hosts (11), and is related to the above-mentioned *Caballeronia* sp., our initial hypothesis was that microbial flagellar motility guides Lv-StA into the dorsal structures. However, mono-colonization assays with non-motile Lv-StA shows that motility is not crucial for this process in **(Chapter 5)**.

However, under natural conditions, Lv-StB is the most dominant symbiont in the beetles and it lacks the genes essential for flagellar biosynthesis (11,24,25). In **(Chapter 5),** we tested if differences in motility between strains contribute to competitiveness during colonization. However, under head-to-head competition, *i.e.* after co-inoculation of motile and non-motile Lv-StA mutants, both strains colonize the larval structures equally well **(Chapter 5)**. A few symbiont cells gain may entry into the symbiotic structures by host behavioural mechanisms which makes flagellar motility less relevant for this ectosymbiosis. It is in line with the loss of flagellar motility in Lv-StB (24) and indicates that loss of flagellar motility does not reduce or improve Lv-StB's competitive abilities in the natural community. We could speculate that alternative forms of bacterial motility may help Lv-StB colonize the dorsal structures. On solid surfaces, twitching and gliding do not require a flagellum (22) and these mechanisms may guide Lv-StB into the dorsal structures of the larvae. Alternatively, host movements may be sufficient to direct Lv-StB cells into the dorsal structures.

Moreover, we find other microbial taxa, including *Luteibacter, Variovorox,* and *Acinetobacter,* in the microbial community within the beetles, albeit at a lower abundance compared to *Burkholderia* sp. (25). Among them, *Acinetobacter* does not seem to encode genes for flagellar motility **(Chapter 5)**. Therefore, it is likely that the other microbial strains or species enter into the symbiotic structures using alternative mechanisms of motility or are guided by host movements during hatching. *Burkholderia* sp. may dominate the community by outcompeting other microbes due to faster-growth dynamics., and/or antibiotic-mediated competition, or the type VI secretion system and toxins. A comparative genome analysis of the factors encoded in the different microbial species associated with the *Lagria* beetles might give us insights into the common colonization factors for establishment in the host.

Therefore, in ectosymbiotic associations, host behavioural traits may be sufficient to initiate the entry of a few symbiont cells into the symbiont- bearing structures. The few founder-cells that colonize the symbiotic structures may compete and proliferate to fill the space up to full capacity. Moreover, in ectosymbioses like that in the leaf-cutter ants and European beewolves, the symbionts belong to the genus Actinomycetes, which comprise gram-positive, non-motile bacteria that form hyphae and propagate by sporulation (26,27). In the leaf-cutter ants, *Pseudonocardia* is acquired by trophallaxis, and in the beewolves, the larva incorporates *Streptomyces* symbionts into the cocoon while spinning it (28). In many symbioses where symbionts are housed on the external surface, including bark beetles (29) and the leaf-rolling weevil (30), host behaviours may be essential for initial contact and colonization by symbionts.

3. CREATING A SELECTIVE ENVIRONMENT FOR THE BENEFICIAL SYMBIONTS

While symbiont transmission during oviposition gives the offspring immediate access to its protective benefits, the egg surface is an open environment that could be colonized by any other microbes (31,32). In the *Lagria* beetles, a multispecies/strain community of symbionts is found on the egg surface and colonize the larval symbiotic structures (11,25). Moreover, the larvae can acquire symbionts occasionally from the environment (17). Despite this, *Burkholderia* make up 65-86% of the bacterial taxa in an individual adult (11). Among the *Burkholderia* sp., multiple strains are found in the beetles; Lv-StA is occasionally prevalent in some individuals, and Lv-StB is the most frequent and dominant strain (25). It is possible that host physiological and immunological factors set an environment favourable for *Burkholderia* sp. to establish in the symbiotic organs of the host **(Fig. 2)**.

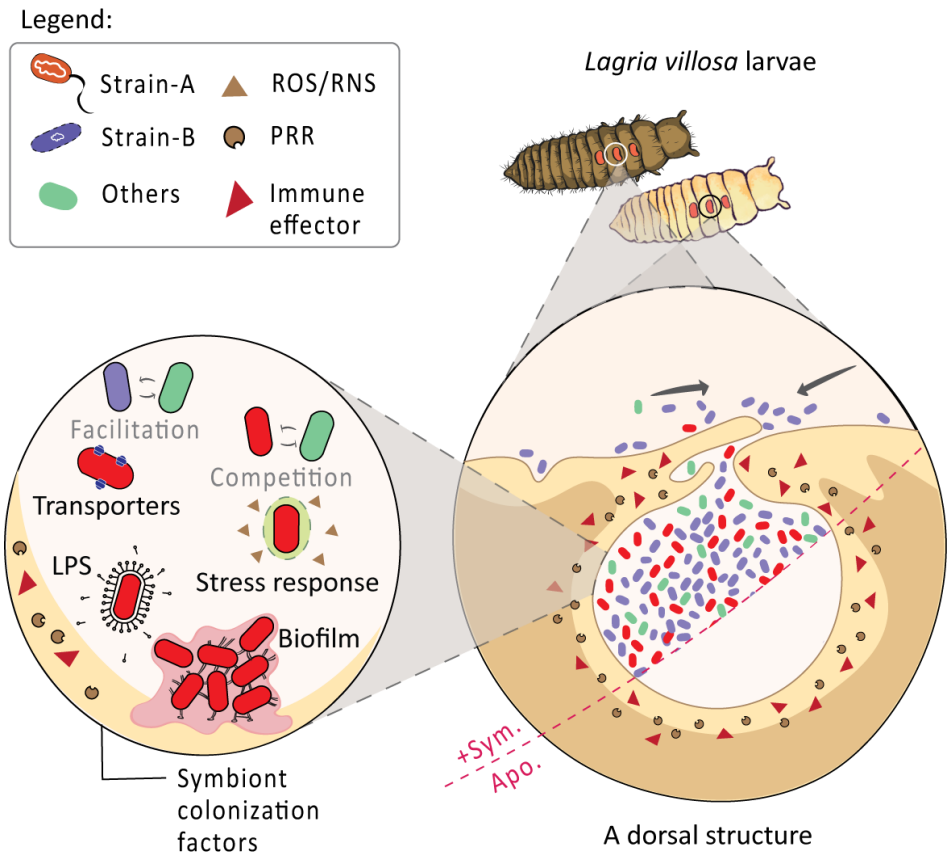

Fig. 2. Selective environment in the symbiotic structures and mirobial colonization factors. Larval dorsal structures are colonized by microbial symbionts. **Right circle:** A specific physiological condition and constitutive expression of immune factors in the symbiotic structures may be selective for *Burkholderia* sp.. Additionally, compartmentalization of the symbionts within the dorsal structures may prevent systemic immune activation. **Left circle:** Symbiont colonization factors may help them establish in the host. The conditions within the dorsal structures may promote cooperative or competitive interactions between the microbes. (Abbreviations: ROS/RNS = Reactive Oxygen or Nitrogen Species, PRR = Pattern Recognition Receptor, LPS = Lipopolysaccharide, +Sym. = Symbiotic, Apo. = Aposymbiotic)

3.1. Physiological conditions determining symbiont establishment

Female *Lagria villosa* beetles smear symbionts onto each egg during oviposition (11). As in other symbioses, the *Lagria* beetle symbionts may be exposed to environmental stressors, like changes in temperature and humidity, UV-radiation and the harsh conditions could be selective for microbial strains that can resist the stress and colonize the host (33). Therefore, apart from bacteria, the host secretions may contain other substances to protect the cells, act as an adhesive, and provide essential nutrients for the microbes to produce bioactive metabolites. In some hemipteran bugs (14,34) and tortoise leaf-beetles (35),symbiont-bearing capsules, caplets, and jelly-like substances likely protect the genome-reduced symbiont cells from external physiological stress. A recent study on a Plataspid stink bug shows that an abundant protein in the capsules deposited on the egg surface protect the enclosed *Ishikawaella* symbionts (34). Likewise, on the *Lagria* egg surface, host secretions smeared along with the symbionts

during oviposition may contain nutrients or a stress-resistant substance for the *Burkholderia* symbionts to persist and produce bioactive compounds.

The *Burkholderia* strains may be well adapted to withstand nutrient stress on the egg surface. In support of this idea, we find that in Lv-StA, Tn-mediated mutation of a gene, *phbF*, involved in polyhydroxybutyrate (PHB) granule biosynthesis (36) seems to affect establishment in the host **(Chapter 5).** PHB granules are bacterial endocellular storage polymers that provide resistance against nutrient depletion and environmental stress (37,38), and they may help Lv-StA persist on the egg surface before colonization. An analogous gene are also found in Lv-StB. A similar strategy to withstand nutrient stress is observed in *Caballeronia* symbionts associated with the *Riptortus* bean-bugs (38). In addition to the PHB granules, the Tn-seq experiment **(Chapter 5)** indicates that, *lrp*, a transcription regulator for amino acid and metabolism (39), may detect nutrient limitation and modulate cellular processes in Lv-StA. Similarly, *lrp* is indicated as an important colonization factor for *Snodgrasella alvi*, a symbiont in the honeybee gut (40). Therefore, on the egg surface, host secretions and bacterial factors may help the strains withstand nutrient and environmental stress before entry into the dorsal structures of the larvae. To identify factors that promote symbiont survival and growth when in association with the *Lagria* host, symbiont genes essential for synthesizing the PHB granules, transporters, and other amino acid biosynthesis genes could be knocked out by targeted mutagenesis, or auxotrophs could be screened using selective media from the Tn-mutant library **(Chapter – 4)**. Following this, *in vivo* mono- and co-colonization experiments might help understand their role when the symbionts colonize the symbiotic structures of the *Lagria* beetles.

Furthermore, in *Lagria villosa* larvae and female adults, we have observed the presence of glandular cells beside the symbiotic structures **(no data available)**, similar to those reported in *Lagria hirta* (10). These glands may be important for releasing nutrients, or immune-active effectors, to regulate the symbionts after colonization. However, in the RNA-seq experiment, sampling across several developmental stages **(Chapter 6)**, we do not find differential expression of host genes related to nutrient transport in aposymbiotic vs. symbiotic individuals **(Chapter 6)**. Nevertheless, there might be a constitutive expression of host genes in the symbiotic structures or eggs, which is not evident from our experimental design **(Fig. 2)**. Moreover, the symbiont community composition in the laboratory differs from that of the field-collected *Lagria hirta* and *Lagria villosa* beetles (41). It is possible that differences in the food diversity and quality between the natural and lab environments could affect host health, which in turn could alter the symbiont community composition (17). In other insects, like the pea-aphid, titers of the intracellular symbiont, *Buchnera,* correlate with the availability of nitrogen in the host (42). Host nutritional support for symbionts is evident in obligate symbioses since the genome- reduced symbionts tend to lose their ability to biosynthesize metabolites that are enriched within host tissues (43). In an ectosymbiotic association, like that seen in the *Acromyrmex* leaf-cutter ants, bacterial symbionts that colonize the ant cuticle are provisioned by nutrients from the host (9). However, other than the leaf-cutter ants, relatively less known about how the host provides for the symbionts that reside on the cuticular surface.

A possibility is that symbionts may establish on the insect cuticle by utilizing chitin and its derivatives as a source of nutrients. For example, *Xylella fastidiosa* is a bacterial plant-pathogen vectored in the cuticle-

lined foregut of plant sap-sucking insects, like sharpshooters (44). In the insect host, a chitinase produced by *X. fastidiosa* is important for degrading the cuticle to obtain nutrients and colonize the insect vector. Similarly, in the *Lagria* beetles, *Burkholderia* symbionts may express a chitinase and transporters to break-down chitin and take up nutrients within the dorsal structures of the larvae. However, from the Tn-seq experiment (**Chapter 5)**, we do not find evidence for the role of chitinases during establishment of Lv-StA. The absence of chitinases within the list of potential colonization factors is unsurprising, since chitinases are extracellular enzymes, and Tn-mutants that are unable to produce it, could still take advantage of neighbouring chitinase-producers to establish in the dorsal structures. Nevertheless, the experiment suggests that, MsiK, a transporter for oligosaccharides, including cellobiose and chitobiose, which are breakdown products of chitin, may have a role during establishment. Therefore, to identify if any chitinase is indeed released by the symbionts, targeted mutagenesis followed by *in vivo* colonization assays, and *in vitro* chitinase detection assays may be more insightful. Another possibility is that host-derived chitinases, like those expressed during molting (45), may help degrade chitin for the ectosymbionts.

3.2. Host immunity and compartmentalization of symbionts

Besides physiological parameters, host immune effectors usually play a crucial role in eliminating or maintaining symbionts (46–49). Surprisingly, in the *Lagria villosa* beetles, the presence of the natural community of symbionts on the egg surface and in the dorsal symbiotic structures of first-instar and early second-instar does not seem to elicit a strong systemic molecular response in the host **(Chapter 6)**. From our observations, a few scenarios could explain the role of immunity in establishing specific partners in an ectosymbiosis.

In insects, compartmentalization of intracellular endosymbionts in bacteriomes is an effective strategy for controlling symbionts and regulating proliferation (50). In the cereal weevil *Sitophilus oryzae,* immune effectors in the bacteriome of the host prevent symbiont escape and promote tolerance. Also, a systemic immune response against other microbes does not affect endosymbiont titers in the bacteriome (48). Therefore, bacteriome confinement may protect endosymbionts from host immune responses. In the *Lagria* beetles, at least during the early stages of development, extracellular compartmentalization within the larval structures lined by a cuticle may prevent immune activation in the host **(Fig. 2)**.

Furthermore, in leaf-cutter ants, *Lagria villosa* beetles and beewolf wasps, external cuticle-lined structures house the defensive symbionts that produce antibiotics. We are yet to determine if the antibiotics also have insecticidal effects and if the host is resistant to them. Nevertheless, compartmentalization could help protect the host from the presumed detrimental effects of the microbial bioactive compounds. In *Lagria villosa*, the external transmission route from eggs to larvae may help the host avoid toxic side effects and simultaneously spread the antibiotics at the first-line of defence against pathogens (51). To understand the effect of the compounds on the host, we would have to extract the antifungal compounds produced by the symbionts and test the survival of the host when they are exposed to the antifungals at different dosages and when it is applied internally *vs.* externally.

While compartmentalization reduces contact of beneficial symbionts with the immune factors in the haemolymph and may help the host avoid symbiont toxicity, the host must also have a strategy to create

a selective environment favourable to establishing a symbiotic association. The symbiotic compartments likely express a localized constitutive immune response that went undetected in our RNA-seq experiment. Constitutive localized immune factors in the dorsal structures may create an environment selective specifically for the defensive symbionts **(Fig. 2)**. The insect- associated *Burkholderia* sp. may resist the localized immunity in the symbiotic structures by secreting proteases, forming a biofilm **(Chapter 5)**, or using efflux pumps **(Fig. 2)**. Furthermore, the LPS exhibits variable immunogenic properties in several bacteria and helps in immune evasion (52). In the *Lagria* symbiosis, a comparison of the genes in Lv-StA and Lv-StB, indicates that the LPS structures of Lv-StA and Lv-StB may comprise similar glycan molecules **(Chapter 5) (Fig. 2)**. Whether this helps the strains evade host immune effectors is unknown and could be answered by knocking out the bacterial LPS components in Lv-StA and screening the mutant strains in selective plates containing antimicrobial peptides.

Furthermore, different species in the microbial community associated with the host may elicit varying responses from the host. For example, in the honey bee gut, mono-association with *Snodgrasella alvi* upregulated the expression of an antimicrobial peptide, apidaecin. However, infecting the bee gut with the natural symbiont community, upregulated both apidaecin and hymenoptaecin (53). Also in aphids, *Hamiltonella defensa* and *Regiella insecticola* facultative symbionts cause a decrease in host phenoloxidase activity however, a combination of other facultative symbionts *Serratia symbiotica* and *Rickettsiella viridis* does not show the same effect (54). Similarly, in *Lagria villosa* beetles, different symbiont species or strains may elicit distinct responses. Presence of the native *Burkholderia* symbionts may not be recognized on the eggs and larvae during the early developmental stages. Testing differences in host gene expression in beetles infected individually with *Burkholderia* sp., the non-dominant symbiont species, and other bacterial species *e.g. Escherichia coli, Bacillus thuringiensis*, might provide clues on differential specificity of host immune recognition and responses.

Alternatively, accompanied by spatial confinement, a temporal expression of immune genes may help restrict symbiont movement and entry beyond the early stages of the association. We find that Lv-StA colonize the dorsal structures of second-instar larvae at a lower efficiency than the first-instar larvae (6) **(Chapter 3) (Fig. 1)**. Some changes in physiological or immune conditions within the organs may restrict colonization in older larvae. Congruently, we observe that symbiont-induced immune response of the host is absent in egg – early first-instar stages **(Chapter 6)**, when symbionts colonize the dorsal structures (6)**(Chapter 3)**. Later, when the symbionts have established themselves in the mid second-instar larvae, serine protease inhibitors that regulate the activation of the toll pathway are upregulated. An attenuated immune response between eggs to the early second-instar stage may allow symbiont colonization within that developmental time-window, and in older larvae, restricted entry due to upregulation of molecular responses may help maintain partner specificity **(Section 2, Chapter 7) (Fig. 1)**. In the Hawaiian bobtail squids, host responses to the symbionts are coupled to development and induced by symbiont presence (5,55,56). Similarly, in the *Lagria* beetles localized and temporal variations in immune responses could help regulate symbiosis establishment.

4. SCREENING FOR BENEFICIAL SYMBIONTS

The stress-gradient hypothesis in microbial ecology predicts that a stressful environment creates positive interactions between microbial species and a nutrient-rich environment would promote competition

(57). Therefore, in animals harbouring ectosymbionts, the host could screen for effective antibiotic-producing symbionts by creating a nutrient-rich environment, or could allow cooperation and facilitated colonization of multi-species symbiont communities by creating a harsh *in vivo* environment **(Fig. 2)**. Under these scenarios, partner selection may be passive for the host (7,9,50,58). In the following sub-sections, the two scenarios are considered to discuss how microbial interactions may influence the success of symbiont establishment and determine community composition in the symbiotic structures of the *Lagria* beetle. First **(Section 4.1)** let's consider that, within the compartments of the dorsal structures, excess availability of nutrients fuels microbial competition and partner selection. Next, **(Section 4.2)** let's consider the alternative scenario where the dorsal structures may contain stressors that promote facilitation between the symbiotic strains when they establish in the host.

4.1. Symbiont competitive colonization and the molecular factors enabling it

The symbiotic structures in *Lagria villosa* larvae are open to the external environment, potentially allowing several environmental microbes for colonization (25). Consider a theoretical model for the assembly of antibiotic-producing defensive symbionts proposed by Scheuring and Yu (7); if the conditions within the symbiotic structures are nutrient-rich, the high resource availability would fuel production of antibiotics in beneficial symbionts. Antibiotic production in combination with priority effects, would promote competition and establishment of the beneficial symbiont in the host (7). Beneficial symbionts can gain priority for colonization due to vertical transmission and/or high immigration rate from the environment to the host. This theoretical model was substantiated empirically in Attine ants (9). Here, experiments show that the host provides nutrients to the symbionts on the cuticular surface. Vertically transmitted *Pseudonocardia,* and occasional horizontally acquired *Streptomyces,* were the predominant taxa on the cuticular surface. Moreover, the *Pseudonocardia* species produces antibiotics on the cuticular surface, and likely creates a stressful environment for other species.

Similarly, in the *Lagria* beetles, vertical transmission (11) likely provides priority to the antibiotic-producing symbionts during colonization. Despite their co-occurrence with other microbial species, *Burkholderia* Lv-StB is predominant in the community and Lv-StA is prevalent in a few individuals (25). It is unclear whether the secondary metabolite, Lagriamide, which has antifungal properties, can also act against other bacteria. Nevertheless, while the dorsal structures can hold other microbes, possibly priority effects, likely faster growth, and antibiotic-mediated warfare might lead to Lv-StB's dominance. However, Lv-StB is uncultivable *in vitro,* therefore it is challenging to probe Lv-StB's competitive colonization abilities. Therefore, given that Lv-StA is culturable in the lab and can produce a wide range of secondary metabolites (11), we could use this strain to perform further experiments to gain first insights into the competitive colonization abilities of the *Burkholderia* strains in cuticular structures of the beetle.

Among the secondary metabolites produced by Lv-StA, some lipopeptides and siderophores may mediate microbe - microbe competition and promote interactions with the host during establishment **(Chapter 5)**. Lv-StA produces lipopeptides like icosalide and Burriogladin that are known to suppress swarming and promote biofilm formation, respectively (59,60). Moreover, in the natural community, icosalide could inhibit the growth of gram-positive bacteria (59). In **Chapter 5**, the Tn-seq analysis shows

that icosalide and Burriogladin Tn-mutants were unable to outcompete the rest of the Tn-mutant library to colonize the dorsal structures of the *Lagria* larvae. However, in mono-colonization assays, the icosalide mutant, Δ*icoS*, was able to colonize the dorsal structures of the larvae to the same extent as the wild type Lv-StA. The mutant, Δ*icoS,* exhibits increased swarming abilities, therefore, it is possible that Δ*icoS* could colonize the dorsal structures individually by making use of its swarming capabilities. However, we need to test if the growth rate of the mutant in relation to the wild type. It is likely that the icosalide mutant may have a reduced growth rate which affects its competitive colonization ability when co-inoculated with other cells. Furthermore, the role of Burriogladin and other secondary metabolites of Lv-StA in aiding symbiont establishment in the beetles is yet to be tested with competitive colonization assays.

Furthermore, in the insect host, metals like iron are important resources for colonizing pathogens and beneficial bacteria (61). The host could also suppress pathogen infection by limiting iron availability or increasing iron-mediated oxidative stress on the invading microbial cells. Therefore, iron scavenging through siderophores may be essential for beneficial microbes *in vivo* and under iron-limited conditions, siderophores may mediate competition between microbial species in the community. In the *Lagria villosa* beetle, Lv-StA and the other symbiont species, *Luteibacter*, *Variovorax*, *Acinetobacter,* encode non-ribosomal polyketide synthase clusters for producing siderophores and transporting them (*fhuBCD*, *tonB*). Moreover, the Tn-seq experiment suggested that the transporter, *fhuC,* was also important for Lv-StA during colonization **(Chapter 5) (Fig. 2)**. On the other hand, Lv-StB encodes only the transporters to uptake iron-bound proteins **(Chapter 5)**. This suggests that Lv-StB may "cheat" to scavenge iron-bound siderophores from the co-occuring strains or species. Similarly, other metal ions like copper and zinc may also be involved in interactions between the host and beneficial microbes and could mediate competition between microbes (61). Other than the lipopeptides and siderophores produced by Lv-StA, effectors released by the type VI secretion system may mediate contact-dependent warfare **(Fig. 2)**.

Moreover, the *Burkholderia* strains associated with *Lagria villosa* are closely related to each other (24). Such closely related microbial strains usually compete for nutritional resources (62). In line with this, in the *Lagria villosa* beetles, Lv-StB and Lv-StA rarely co-exist within the same individual (25). On the contrary, in the *Lagria hirta* beetles, the two *Burkholderia* strains, Lh-StG and Lh-StH, frequently coexist within the same individual (63). Lh-StH has a reduced genome and Lh-StG has a larger genome size. It is surprising that although the symbionts seem to colonize similar cuticle-lined, dorsal surface symbiotic structures in the *Lagria* larvae, the strain pairs (Lv-StA *vs.* Lv-StB in *L. villosa* and Lh-StG *vs.* Lh-StH in *L. hirta*) may have different dynamics. These strain interaction dynamics may be influenced by differences in strategies for resource utilization within the symbiotic structures. For example, four closely related *Lactobacillus* species in the honey bee gut, utilize different substrates of the host's pollen diet and coexist in the same individual. However, the four *Lactobacillus* sp. shift to antagonism when only a single carbon source is available for all (64). Similarly, the functional capabilities and of each *Burkholderia* strain and differences in resource availability within the symbiotic structures of the two *Lagria* host species may determine whether the strain pairs coexist or compete during establishment.

4.2. Physiological stressors in Lagria villosa leading to symbiont facilitation

The bacterial species in the dorsal structures may facilitate each other's growth. Let us first assume that the nutritional conditions within the dorsal structure or female glands of the *Lagria villosa* beetle are limited or that there are immunological or oxidative stressors in the symbiotic organs. In such an environment, facilitation between microbial strains and species is expected (65,66). Microbial facilitation may occur in different forms, *i.e.,* in a multispecies microbial community, one species could degrade antibiotics, counteract oxidative stressors, or reduce toxic metal accumulation, and these traits confer benefits to the neighbouring cells and improve community fitness (67). For example, in toxic metal working fluid, microbial species coexist and facilitate each other's growth when at least one of them can detoxify the environment. (65). Also, in a defined medium and under the stress of oxidative compounds, the presence of species that are able to reduce the reactive oxygen species (ROS) seems to rescue the growth of others that are sensitive to ROS (66). When the oxidative stress is removed or reduced, microbial interactions switch from facilitation to competition. In an animal host, symbiont interactions may also be regulated by inducing such changes in physiological conditions.

Within the *Lagria* system, a laboratory infection with Tn-mutant strains of Lv-StA, thioredoxins and other oxidative stress response related genes seem essential for colonization **(Chapter 5) (Fig. 2)**. We are not aware if the host constitutively releases reactive oxygen/ nitrogen species in the dorsal structures. In the Hawaiian bobtail squid, the host releases nitric oxide synthase (NOS) and nitric oxide (NO) in the light organ duct and crypts where, the symbiont, *Vibrio fischeri,* utilizes hmp, a flavohaemoglobin for protection against the NO stress (68,69). Also, co-colonization in the *Lagria* symbiotic structures might be beneficial to reduce the RO/NSs effectively. Beyond this, we can speculate whether the host might modulate microbial interactions within the symbiotic structures by regulating the level of nutrient / oxidative stressors in the symbiotic structures.

Nevertheless, microbial interactions within the symbiotic structures might be more complex. A macrolide produced by Lv-StA, toxoflavin generates hydrogen peroxide in aerobic environments which is toxic to *E.coli* (70). Also, the megapolibactin-like and plantaribactin-like siderophores synthesized by Lv-StA **(Chapter 5)**, help in chelating iron and additionally seem to have NO-donating properties (71). Therefore, if Lv-StA colonizes the dorsal structures under the natural conditions, the strain could possibly alter the oxidative state of the symbiotic structures and promote competition between the symbionts. The expression of toxoflavin or the siderophores has not yet been detected within the *Lagria* host. If that is the case, we could still speculate that microbial competitors may have mechanisms to overcome Lv-StA in the dorsal structures. The relatively low prevalence of Lv-StA (3 out of 58 samples) (25) point towards such competitive dynamics during symbiosis establishment in the natural environment. Experiments testing the fitness of the natural *Lagria* symbiont isolates when they are co-inoculated in different *in vitro* media, and under varying conditions of oxidative and antibiotic stress, could provide initial clues to symbiont interaction dynamics in this system.

5. HOST AND LIFESTYLE TRANSITIONS – A STRANGE CASE OF DR. JEKYLL AND MR. HYDE?

Microbial interactions with animal hosts are context-dependent, and several factors like temperature, availability of resources, community composition, etc., may direct microbial transitions along the

parasitism – mutualism continuum (72). In the association between *Lagria villosa* beetles and *Burkholderia gladioli*, we have delved into understanding the dynamics of symbiont colonization **(Chapter 3)** and identified potential molecular mechanisms that help *Burkholderia* sp. establish in the beetles **(Chapter 4,5)**. However, besides being a defensive mutualist in the *Lagria* beetles, *Burkholderia gladioli* is also known as a plant pathogen or mutualist (73–76). In the *Lagria* system, previous work shows that *B. gladioli* beetle symbionts can infect plants and that the beetles can acquire them occasionally from the environment (11,17). The effects of the infection on plant-fitness are not strong, however, we could speculate that the *Lagria* beetle host could be a vector for *Burkholderia* and yet unknown ecological conditions may drive beetle – plant transmission. Moreover, the open loop that enables bacterial transition to and from plants may also help the beetles replenish their pool of beneficial *Burkholderia* when there is a dysbiosis and to sustain the symbiosis (17). The factors that help *Burkholderia gladioli* maintain alternate life styles in dual hosts are yet unknown. Using targeted mutagenesis **(Chapter 4)**, one could generate mutants for genes of interest, including those involved in the synthesis of toxoflavin, siderophores, or other secondary metabolites, and perform infection assays in plants and beetles. Additionally the molecular factors involved in host - transitions could be identified by testing mutant transmission efficiency across plant – beetle hosts.

It is also possible that the *Burkholderia* symbionts, especially Lv-StA, impose a cost on the host. Observations during experiments suggest that infecting Lv-StA at a high concentration (2×10^7-10^8 cells/µl) on the egg surface causes melanisation spots **(no data)**. Similar melanisation spots are observed in pupae infected with fungi (25). The beetle host may upregulate the phenoloxidase pathway and induce melanisation in response to fungal pathogens and when beneficial bacteria symbionts overpopulate the beetle surface. Therefore, symbiont factors may suppress virulence factors when the population density is low in the beetle host. The expression of *Burkholderia* virulence factors may be regulated by its quorum-sensing machinery. In other insects, like the *Sitophilus* weevils, bacterial quorum sensing attenuates virulence in *Sodalis* symbionts and promotes establishment in the host as a mutualist (77). Similarly, *Photorhabdus luminescens* are mutualistic symbionts in *Heterorhabditis bacteriophora* nematodes. The symbionts switch to a pathogenic state within insect prey that the nematodes penetrate (78,79). The pathogenic *Photorhabdus* kills the insect prey and provides the nematodes with nutrients for reproduction. The *Photorhabdus* symbionts are mutualistic when they express maternal adhesion (Mad) fimbriae and adhere to the intestine of the nematode host. This fimbrial apparatus is controlled by an invertible operon and mediates the switch between a mutualist to a pathogen in the nematode host vs. prey, correspondingly (79). Therefore, further experiments with *Burkholderia gladioli* symbionts of the beetles could help us understand the molecular factors that mediate these transitions within or across hosts along the mutualism – parasitism spectrum.

6. TRANSPOSON –INSERTION DIRECTED SEQUENCING TO STUDY HOST –MICROBE SYMBIOSES

Transposon-insertion directed sequencing (Tn-seq) is a high-throughput method that would help in identifying multiple conditionally essential genes in a single experiment (80–83) **(Chapter 4)**. Transposon- mediated mutagenesis allows disruptions in random locations in the genome and many such random mutants can be generated in experiment to generate a mutant library. Using the pooled

mutant library, mutant fitness under a certain growth condition can be tested simultaneously. Thus, genes essential for bacterial growth or survival under a test condition can be identified **(Chapter 5)**. However, the Tn-seq method to study symbiont establishment in an animal host has its own limitations, *i.e.,* i) natural isolates of bacteria may be difficult to manipulate, as is the case for any genetic manipulation technique, ii) transposon-insertion bias may generate multiple mutants at certain genomic hotspots (84–86), leaving other loci unhit, thereby generating an unsaturated Tn-seq library (83) **(Chapter 4)**, iii) establishing a modified sequencing protocol may pose technical challenges (83) **(Chapter 4)**, and iv) while infecting the host with the mutant library, an infection bottleneck limits the number of mutants that can be assayed at once (80–82). Nevertheless, once established, the Tn-seq method helps in identifying a broad number of microbial gene targets in a high-throughput manner. Combining Tn-seq and targeted mutagenesis techniques would help corroborate the findings **(Chapter 5)**. Alternatively, mutants from the Tn-seq library can be isolated, individually or in smaller pools, and screened under selective conditions. For example, the genes involved in motility can be identified from slower moving cells on an agar plate (82), or the genes important for establishment can be identified by isolating individual Tn-mutants and infecting the host (87).

The Tn-seq method has been applied in a few animal -microbe symbioses to identify microbial factors essential during the symbiont establishment. In the Hawaiian bobtail squid, the Tn-seq method helped identify 380 potential colonization factors in the *Vibrio fischeri* symbiont (88). Additionally, targeted mutagenesis and colonization experiments corroborated the results of the Tn-seq experiment. They showed that dnaJ/K genes, which encode chaperone proteins for protein folding and biofilm matrix formation, are important for initiating host colonization. Similarly, in the honey bee gut, Tn-seq was applied to one of the gut-associated bacteria, *Snodgrasella alvi* to identify 290 potential colonization factors (40). Factors including those important for adhesion, stress response and adaptation to nutrient-limiting conditions seem important for establishment of the symbiont in the honeybee gut. In this thesis, applying the Tn-seq method to the *Lagria* – *Burkholderia* symbiosis (83) **(Chapter 4)**, we identified 271 potential colonization factors for an ectosymbiont to establish in the dorsal structures of a larva **(Chapter 5)**. Therefore, by applying the technique to systems where the symbionts are amenable to genetic manipulation, we could identify the microbial factors that mediate establishment in different symbiotic structures of animal hosts, and characterize emerging patterns across systems.

7. CONCLUSIONS AND OUTLOOK

The aim of this thesis was to gain insights into the mechanistic basis of symbiont establishment in a beetle. The dynamics and timing of symbiont colonization were first investigated using a culturable symbiont strain, *Burkholderia gladioli* Lv-StA, and by establishing a transposon-insertion directed sequencing approach, we investigated the molecular factors that mediate colonization of the specialized dorsal cuticular structures of the larvae. Further, across early developmental stages of the host, we find that the host's molecular response to symbiont presence is not pronounced. We speculate that microbial adaptations to a specific environment within the symbiotic structures of the host and microbial interactions during colonization determine partner specificity in this ectosymbiotic association. Furthermore, this study raises interesting questions regarding the localized physiological and immune environment within the symbiotic structures of the host, what host factors support symbiont growth in

the host, and what factors mediate symbiont – symbiont interactions during establishment. These questions could be answered by taking advantage of the genetic tractability of the study system and by making use of the wide range of sequencing techniques, imaging tools, and experimental assays that are available. Furthermore, the role of the bacterial partners can be studied under different settings. For example, beyond their association with the beetles, *Burkholderia gladioli* sp. are able to defend germinating plants against fungal antagonists (76). They are also known pathogens that cause bacterial grain rot and leaf-sheath browning in rice (73,74,89). Therefore, broadening our studies to identify molecular and ecological factors necessary for establishment in plants and insects, for microbial transmission between hosts, and during transitions across the mutualism - parasitism continuum could help us develop avirulent strains for biocontrol (90) or harness antimicrobial factors to control plant pathogens (72,76). Thus, exploring the dynamics of microbial colonization in hosts and the factors that mediate host – microbe and microbe – microbe interactions might help us generate creative solutions for applications in agriculture.

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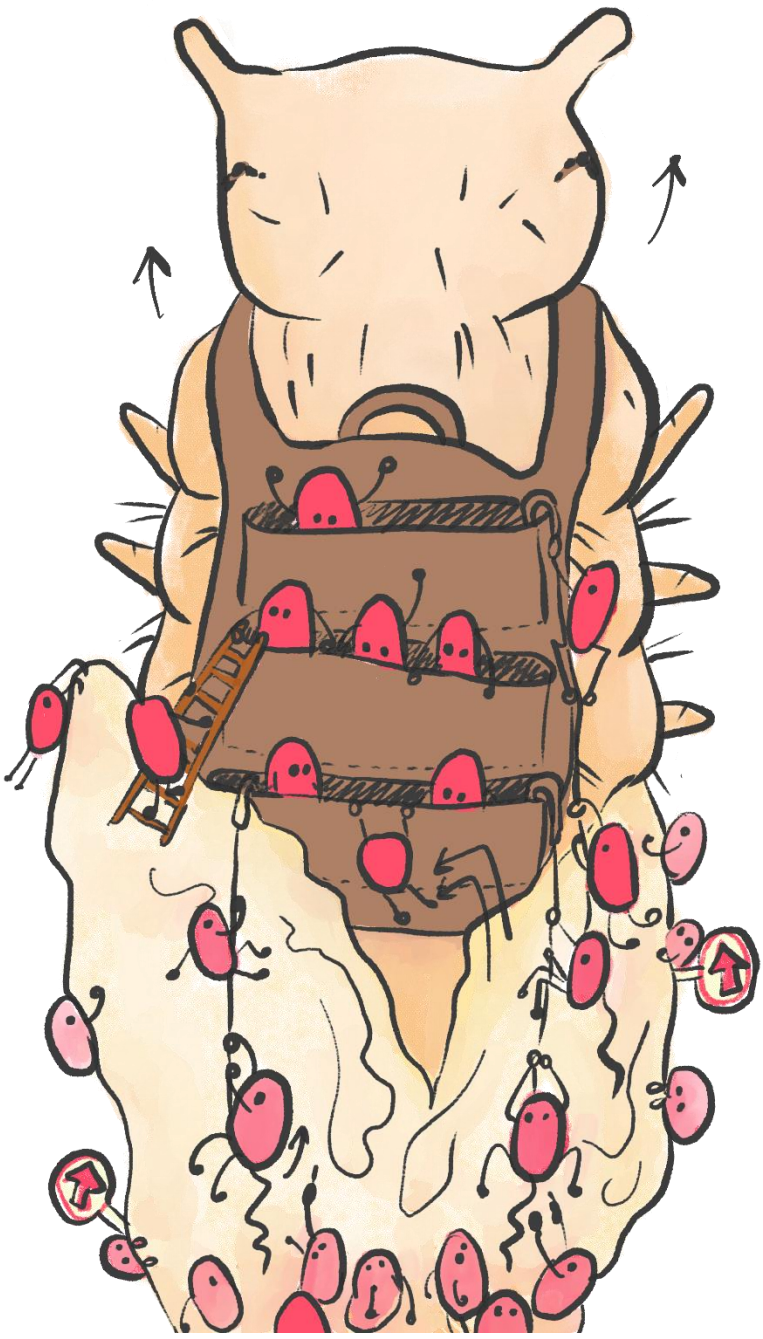

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CURRICULUM VITAE

Ramya Ganesan

EDUCATION

Nov 2017 – current	**Doctoral thesis** *Johannes Gutenberg university Mainz*
July 2012- May 2016	**B.Tech Biotechnology** *SASTRA University, Tanjore, India.*
June 2010- May 2012	**12th Higher Secondary School Certificate** *Bharathi Matriculation Higher Secondary school, Coimbatore, India*

RESEARCH EXPERIENCE

Nov 2017 - current	**Doctoral research** *JGU Mainz, MPI-CE Jena, Germany* *Supervisors:* Prof.Dr.Martin Kaltenpoth**,** Dr.Laura V. Flórez
January 2016- June 2017	**Bachelor's thesis & Research internship** *MPI-CE, Jena, Germany* *Supervisor:* Prof.Dr.Christian Kost
May 2015- July 2015	**Undergraduate research fellowship** *M S University of Baroda, Vadodara, India* *Supervisor:* Prof.Dr. Bharat Chattoo

PUBLICATIONS

2023	"Colonization dynamics of a defensive insect ectosymbiont" ***Ganesan R**, Janke RS, Kaltenpoth M, Flórez LV.***,** Biol. Lett.**,** 19**,** 20230100**,** (2023).
2022	"How it all begins: Bacterial factors mediating the colonization of invertebrate hosts by beneficial symbionts" ***Ganesan R**, Wierz JC, Kaltenpoth M, Flórez L V***,** *Microbiol. Mol. Biol. Rev.* 86 (4)**,** e00126-21**,** (2022).
2021	"Transposon-insertion sequencing as a tool to elucidate bacterial colonization factors in a *Burkholderia gladioli* symbiont of *Lagria villosa* beetles." ***Ganesan R**, Kaltenpoth M, Flórez LV.*, J.Vis. Exp. 174**,** e62843**,** (2021).

TEACHING EXPERIENCE

2020	Co-supervision of master's practical project: "In silico prediction of essential genes of *Burkholderia gladioli* Lv-StA and Lv-StB" *Krüsemer R*

	Co-supervision of master's thesis project: "Effects of motility on the establishment of a culturable bacterial symbiont in *Lagria villosa* beetles." *Mibes A*

ORAL PRESENTATIONS

January 2023	Dynamics and mechanisms of colonization in a beetle associated bacterial symbiont *Instituto Gulbenkian de Cienca – SymbNET seminar talk*
July 2022	Dynamics and molecular mechanisms of colonization in a defensive bacterial symbiont of Lagria villosa beetles *10th Congress of the International Society for Symbiosis (ISS – Holobiont)*

POSTER PRESENTATIONS

June 2023	Dynamics and mechanisms aiding colonization in a darkling beetle *Gordon conference – Animal microbe symbioses*
August 2022	Dynamics and molecular mechanisms of colonization in a defensive bacterial symbiont of Lagria villosa beetles *18th International Symposium on Microbial Ecology*
October 2021	Factors mediating colonization of Burkholderia defensive symbionts in Lagria villosa beetles *Gutenberg workshops - The rise and fall of mutualisms – Ecological and Evolutionary dynamics of host-microbe symbioses*
May 2021	Mechanisms of colonization in symbiotic *Burkholderia gladioli* associated with *Lagria villosa* beetles *World microbe forum (online)*
August 2020	Molecular factors important for symbiosis establishment in the defensive symbiosis between *Lagria* beetles and *Burkholderia* bacteria *Ecological Immunology Workshop: Resistance tolerance and symbionts (online)*
June 2019	Molecular mediators of symbiosis establishment in the association between Lagria beetles and Burkholderia bacteria *Gordon conference and seminar – Animal microbe symbioses*

WORKSHOPS

October 2021	Gutenberg workshops - The rise and fall of mutualisms – Ecological and Evolutionary dynamics of host-microbe symbioses
November 2020	Thinking your way into research articles – writing workshop by Ian Pattern.

January 2019	Workshop on genomics, Český Krumlov, Czech Republic

COMPLEMENTARY WORK

2022	"*Lagria- Burkholderia* defensive symbiosis" *Ganesan, R.* *ISME18 – Discover the microverse scientific image exhibition*
2019	"Can microbes read the clock" – article *Equal contributions by Ganesan R, Flórez L V, Köbler C, Scheurer N.* *VAAM – Microbiology information portal – short questions!*

ACHIEVEMENTS

2023	Best poster award at the GRC for Animal microbe symbiosis
2023	Travel grant awarded for best poster at the 22nd IMPRS symposium
2021	DAAD scholarship for socially active doctoral students
2020	Winner – Workshop on genomics - design contest
2016	SASTRA University semester abroad program scholarship
2015	Indian Academy of Sciences undergraduate summer research fellowship

ORIGINALITY STATEMENT

In accordance with § 11, paragraph 3d of the doctoral degree regulations

of the faculty of biology at the Johannes Gutenberg-Universität Mainz

I hereby certify that I have written this dissertation independently and have not used any references or sources other than those indicated. I have not submitted this work previously or concurrently to any other faculty or university for the award of any degree, or for any scientific examination. The names of all the collaborators who contributed to the work are mentioned in each chapter and appropriately acknowledged in the thesis.

ERKLÄRUNG

gemäß § 11, Abs. 3d der Promotionsordnung
des Fachbereiches Biologie der Johannes Gutenberg-Universität Mainz

Ich versichere hiermit, dass ich diese Dissertation selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Ich habe diese Arbeit weder vorher noch gleichzeitig an einer anderen Fakultät oder Universität zur Erlangung eines Abschlusses oder für eine wissenschaftliche Prüfung eingereicht. Die Namen aller Mitarbeiter, die an der Arbeit mitgewirkt haben, werden in jedem Kapitel genannt und in der Dissertation in angemessener Weise gewürdigt.