Horizontal transmission of the obligate symbiont *Burkholderia* in the leaf-footed bug, *Leptoglossus zonatus* [Hemiptera: Coreidae]

[redacted]

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Brief Overview

In this study, the relationship between L. *zonatus,* better known as the Leaf-footed bug, and strains of environmentally transmitted bacteria *Burkholderia* will be explored. This research aims to determine if *Burkholderia* is horizontally transmitted from parent to offspring at the beginning of the insect's development. Vertical transmission is from mother to offspring. Direct vertical transmission is the administration of bacteria transmitted straight to offspring through physical interactions. Indirect vertical transmission of parental bacteria by picking it up from their environment. This can be transmitted through modes such as air dispersal, animate intermediaries, or picked up off surfaces. This will aid in understanding the complexities of environmentally acquired symbionts. This study will also provide a baseline for future research into this symbiotic relationship.

Previous Research

Symbiosis is an interaction seen in nature where two disparate organisms live in close contact with a typical positive outcome for both. Insect-bacteria symbiotic associations are widespread and long-standing. Symbiotic bacteria provide insects with multiple benefits, such as supplementing nutrition, protection from pathogens and parasitoids (Engel et al., 2013). Symbionts are acquired from the environment or are transmitted vertically from previous generations (Ohbayashi et al., 2015). With all interactions of symbioses, there are requirements to initiate the relationship. For instance, the initiation of symbiont and host association would start through physical contact with one another (Bright et al., 2010). Another aspect of symbioses is the maintenance of mutualistic associations, whether or not the host controls the relationship or if the symbiotic partner will control the impact. The transmission of a free-living symbiont like *Burkholderia* can protect an insect from environmental stresses (Kitkuchi et al., 2011). The outcome of the symbioses in any symbiotic relationship is to be surviving beneficially for both.

The abilities of an insect to utilize the microbial community also come into play for their development. Evolutionary specialization of both a host and symbiont can occur as they secure relations and biological changes to benefit their interactions (Engel et al., 2013). Bacterial symbionts may be obligate for the growth of the host and obligate symbionts are commonly vertically transmitted from parent to offspring. *Burkholderia* is essential for *L. zonatus* survival. It does not protect against predation or pathogens but allows for successful developmental and reproductive health. Without *Burkholderia*, L. *zonatus* have increased mortality, slower development, and an inability to reproduce.

Burkholderia is a diverse group of soil-dwelling bacteria that infects a wide range of living organisms. *Burkholderia* has three main clades, human-infecting *Burkholderia* cepacia complex and pseudomallei (BCC&P), plant-afflicting PBE (plant-associated beneficial and environmental), and stink bug-infecting SBE (stinkbug-associated beneficial and environmental) (Itoh et al., 2019). *L. zonatus* has specialized organs and gut regions that have evolved to allow *Burkholderia* to colonize the midgut. A constricted region preceding the M4 section restricts entry for everything other than the SBE *Burkholderia*. A 2019 study conducted on microbe competition within insect guts showed that "...*Burkholderia* symbiont is confined in symbiosis-specific crypts in the posterior midgut region M4"(Itoh et al., 2019, p. 1). The processes of this midgut interaction are a balance of biological development and gut structure.

Insects have different diets and gut structures than most other organisms, where each gut section develops specific traits that allow them to function and develop correctly for the insect. As previously mentioned, *Burkholderia* colonizes the mid-gut crypts inside the fourth midgut region of multiple stink bug species (Kikuchi et al., 2011). In this case, the microbial symbiont's interaction with external factors determines how the bacteria make it into the host.

While in the second instar, they will accumulate Burkholderia, *Leptoglossus* needs to pick up its microbial symbionts. The posterior regions M4B and M4 within the midgut of an insect's digestive tract serve as the breeding ground for the dominant bacterial species *Burkholderia* (Ohbayashi et al., 2015). As the symbiont survives within the insect the gut will determine how the bacteria manifests. It is important to understand how the gut functions to allow *Burkholderia* to become a dominant bacterial species within its host. Stink bugs contain a system of complex intestinal organs, one of which blocks food and non-symbiotic bacteria to allow the beneficial bacteria to flow through (Ohbayashi et al., 2015). Not to mention, the competition of bacteria seen within stink bug midgut is likely a result of SBE *Burholderia* evolving through gut specification. Multiple species of *Burkholderia* can live within the host at a time. SBE *Burkholderia* is the largest group of bacteria within populations, typically seen in M4 crypts in the stink bugs (Itoh et al., 2019).

Transmission is the first aspect of starting a symbiotic relationship. *Burkholderia* is known to be environmentally transmitted. Stink bugs are exposed to multiple *Burkholderi* clades in the soil of their given environment. Among the Burkholderia variants that are possible, the bug variants are most prominent. The question comes down to what kind of interaction *L. zonatus* initiates. A nonsocial interaction would likely imply that acquisition of Burkholderia is through fecal contamination or ingestion, whereas social insects could be picking up bacteria from contact with one another (Engel et al., 2013). Environmental infection of *Burkholderia* infection confined to be acquired around the second and third instar for *L. zonatus* (Ohbayashi et al., 2015), indicating that there may be developmental restrictions such as mobility and behavior required for symbiont acquisition in the Coreidae family of insects. Bacterial routes used for transmission during nymph development are unclear. The *Burkholderia* has a possibility to be excreted into the soil, or plant surfaces through waste products, there is also the possibility of transmission to the surface or deposited on the internal tissues of a plant as the adults feed, which occurs for other insect symbionts (Gonella et al., 2015; Xu et al., 2016). These various modes could be through close contact with other insects or the environment itself (soil). Symbiosis is also possible through fecal contamination on eggs or proximity to contaminated individuals of the same species (Kikuchi et al., 2011). This study will explore the possibility that the young leaf bugs are exposed to indirect horizontal transmission, resulting in infection of *Burkholderia* bacteria.

Hypothesis and Predictions

This study will provide new insight into the symbiotic relationship between *Burkholderia* and *Leptoglossus zonatus.* Developing nymphs of *L. zonatus* acquire *Burkholderia* from the soil, Whether the insects must obtain a new strain each time or if they pick up the parental strain (indirect, vertical transmission) is not well understood. Studying a possible mode of horizontal transmission will allow a clearer understanding of where the bacteria is coming from. Concentration on horizontal transmission will help provide new insight as it concerns interactions not previously targeted. The study will examine if there is evidence of horizontal transmission of the environmentally acquired symbiont, *Burkholderia* in the leaf-footed bug, *Leptoglossus zonatus* [Hemiptera: Coreidae]. If *Leptoglossus zonatus* is in proximity to natural parents, they will accumulate Burkholderia through horizontal transmission. The study connects to previous research on insect gut anatomy and the bacterial strains picked up from the soil to the role of Burkholderia in the developmental growth of leaf-footed bugs. . Based on the earlier research, the following predictions can be made for this study. Most of the *L. zonatus* offspring will accumulate the tagged parental bacteria from the horizontal transmission. The observable difference between environmental and horizontal transmission will be reflected in developmental strength and presence of GFP (green fluorescent protein) tagged *Burkholderia.*

Methods and Safety

Leptoglossus zonatus culture

Leptoglossus zonatus adults were collected in a pomegranate orchard near Mettler, Kern County, California, the USA in October 2017 and established in the laboratory at the University of Arizona (Tucson, AZ, USA) in large screened plexiglass cages (30 X 30 X 30 cm) in a walk-in incubator set at 27 C, 16L:8D. The cages contained whole cowpea plants *(Vigna unguiculata*) potted in Promix LP 15 potting mix in 15 cm pots, and raw Spanish peanuts as a food source glued in arrays to index cards.

Rearing *Leptoglossus zonatus* with GFP *Burkholderia*

Ten females and ten males from the main culture will be split equally between two starting jar cages (plastic gallon jars with mesh sides, 15cm pots with *V, unguiculata* plants, and raw peanuts). The jars will be checked daily for eggs and the egg clutches will be transferred to individual petri dishes with water vials until the insects molt to the 2nd instar. A total of 50 2nd

instar nymphs will be moved to 5 plexiglass boxes (11.33 cm x 11.33 cm x 4 cm, 10 nymphs per box) with mesh lids and peanuts, but deprived of water for 24hrs. After 24hrs, the nymphs will be fed with an aqueous suspension of GFP *Burkholderia* cells once a day for 2 days. After the second day, a single *V. unguiculate* seedling and water vial will be placed in each box and will be replaced as needed until the bugs reach adulthood.

Horizontal transmission experiment

Twelve adults (6 male and 6 female) inoculated with GFP *Burkholderia* will serve as the parental generation. The adults will be paired up and transferred to 6 individual small jar cages (quart-size plastic jar with mesh lid, *V, unguiculata* plant in 8cm pot, and peanuts) and an extra 3 jar cages will serve as the control. Each jar will serve as one of three treatments “A”, “B”, and “C” with three replicates each. Jar “A” will house or contain eggs, their parents, and soil with non-tagged Burkholderia bacteria to determine if nymphs pick up the GFP *Burkholderia* from their parents or a random strain from the soil. The eggs in jar “B” will be collected and replaced with eggs collected from jar “A” to see if the offspring also pick up the GFP *Burkholderia* from adults that are not their parents. And jar “C” will not have any adults, but eggs will be moved to the jars and serve as a control to let us know that the bugs can pick up *Burkholderia* from the soil.

***Burkholderia* GFP screening**

Progeny from the three jar cages will be collected once they reach adulthood, labeled, and stored in a -80 C° freezer. Their mid-gut M4 will be dissected and the presence of GFP Burkholderia will be confirmed with fluorescence microscopy. The presence of non-GFP Burkholderia will be confirmed with polymerase chain reaction (PCR). Collected data will be analyzed using mixed model ANOVA in R to determine if the offspring are preferentially picking up the parental strain of *Burkholderia*, which will support the indirect vertical transmission hypothesis.

Safety and risk factors

Risk factors for this experiment are minimal. None of the provided methods pose dangerous risk factors. GFP's or green fluorescent proteins are non-hazardous substances the human body can process and break down. PCR is also a safe and non-hazardous process. Not to mention the insects cannot harm, pass any bacteria or viruses to humans.

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