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Zeeyong Kwong April 10th, 2023

Potential Mechanisms of Direct Competition Among Horizontally Transmitted Insect Symbionts

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Abstract

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Effective transmission is essential to the persistence of microbial symbioses that confer critical fitness advantages to their hosts. Contrasting to the direct methods of passage used by some partnerships, many symbioses depend on environmental transmission which hypothetically increases the potential for disruptions. Although the host organism has been demonstrated to impose mechanisms that filter out nonsymbiotic, environmental microbes, this is not fully sufficient to ensure the high-fidelity transmission dynamics that are observed. We hypothesize that horizontal symbiont transmission further depends on competitive dynamics between symbiont candidates. This was examined using the horizontally transmitted symbiosis between the squash bug *Anasa tristis* and its primary symbiont from the genus *Caballeronia*. The external culturability of *Caballeronia* enabled an extensive *in vitro* investigation into competitive dynamics between *Caballeronia* isolates. We first provide strong evidence of contact-dependent inhibition between a cohort of phylogenetically representative *Caballeronia* strains. This inhibition is strongly environmentally dependent. However, the *Caballeronia* strains do not demonstrate *in vitro* contact-dependent inhibition, which may be attributed to the highly regulated expression of the associated mechanisms that may require host conditions . We attempt to address this point by starting to create an *in vivo* model for competition by creating a *Caballeronia* mutant that is unable to compete through a known contact-dependent mechanism. Overall, this work provides a foundation for understanding how microbial symbionts ensure their passage and the evolutionary development of horizontal transmission.

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Furthermore, I would like to thank my parents, who have always been my biggest supporters, both in my personal and academic life. Your unwavering encouragement have been a constant source of inspiration, and I am grateful for everything you have done for me. Finally, I would like to express my appreciation to my girlfriend, Carissa Tan, who has supported me throughout this research journey. Your encouragement and unwavering support have meant the world to me.

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Potential Mechanisms of Direct Competition Among Horizontally Transmitted Insect Symbionts

Background

The near ubiquity of microbial mutualisms across all domains of life can be attributed to the unique advantages provided by microbes to their hosts. These advantages range from pathogen protection to the conversion of indigestible macromolecules into nutrient sources. (Clay, 2014; Pais et al., 2018). The pervasiveness and diversity of conferred benefits lends itself to a broad spectrum of transmission mechanisms that are bounded by vertical passage – directly from parent to offspring – on one end and horizontal passage – indirectly through the environment – on the other (Bright & Bulgheresi, 2010). The specific transfer methodology has critical evolutionary implications, such as the degree of interdependence and the capacity for microbial symbiont adaptation (Fisher et al., 2017; Shapira, 2016).

Horizontal mechanisms are of particular interest as their indirect nature appears to endanger transmission fidelity due to additional selective pressures from the environment and microbial cheaters (Sachs & Simms, 2006). Despite these disadvantages, compared to vertical transmission, horizontal transmission remains prevalent among symbioses, including the well characterized symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and its bioluminescent symbiont *Vibrio fischeri* that it requires from the ocean water every generation (Nyholm & McFall-Ngai, 2004). Deciphering the processes that underlie the horizontal transmission of these symbioses could provide further insight into the evolutionary development of these mutualisms (Mendiola et al., 2020).

Horizontal transmitted mutualisms were studied through the lens of the squash bug *Anasa tristis* and its microbial symbionts from the genus *Caballeronia* (syn. *Burkholderia*) (Figure 1A). *A. tristis* is an agricultural pest found throughout North America that primarily feeds on and inflicts damage to cucurbit crops such as squashes and cucumbers (Bonjour & Fargo, 1989). It can also transmit *Serratia marcescens*, the causative agent of cucurbit yellow vine disease (Bextine et al., 2001). Within specialized symbiont crypts in the gastrointestinal tract, *A. tristis* houses *Caballeronia*, a Gram negative aerobic betaproteobacteria that is acquired environmentally during the second nymphal stage of *A. tristis* maturation (Figure 1B). *Caballeronia* is considered a primary symbiont, as the nutritional and developmental benefits it provides are required for *A. tristis* to achieve adulthood (Acevedo et al., 2021). Based on unpublished work from the Gerardo Lab, *A. tristis* symbiont crypts exhibit low within-host diversity, often colonized with a single operational taxonomic unit (OTU) of *Caballeronia* (Figure 1C).

Distinct from some other members of its order (Hemiptera), *Caballeronia* is not transmitted vertically through a symbiont-containing capsule that is deposited on top of the eggs. (Taylor et al., 2014). Rather, recent experimentation from the Gerardo Lab has provided robust evidence that *A. tristis* uniquely transmits *Caballeronia* through a sophisticated excretion consumption behavior that ensures high fidelity transmission (Villa & Chen et al. under review). This robust behaviorally mediated horizontal transmission, combined with the external culturability of *Caballeronia*, makes this symbiosis an ideal model to explore factors that underlie horizontal transmission.

Horizontal transmission mechanisms can be broadly categorized into two factors: host selection and symbiont interactions. Although there has not been significant work in

understanding these factors in *A. tristis*, we can carefully draw parallels with an analogous symbiosis between the bean bug *Riptortus pedestris* and the symbiont *Caballeronia*. The host, *R. pedestris*, has been demonstrated to employ barriers such as a constricted region that precedes their symbiont crypts and adverse crypt conditions that filter out environmental microbes and advantage potentially symbiotic ones (Kikuchi et al., 2007, 2011, 2020). These structures have also been identified in *A. tristis* but have yet to be studied to the same extent. However, this work has also demonstrated that these host factors solely impose a coarse selection, potentially allowing for the introduction of potential cheaters that could disrupt the propagation of the symbiosis (Itoh et al., 2019). Consequently, microbe-microbe competition would be theoretically required to narrow down the crypt composition to a singular OTU and ensure colonization (Scheuring & Yu, 2012).

Although there is a theoretical framework for the role of microbial competition in symbiosis transmission, there has been a paucity of experimental work. An initial exploration in another agricultural pest, Western Flower Thrips (*Frankliniella occidentalis)*, has demonstrated that its symbiotic bacteria have a geographic-dependent competitive advantage over symbionts isolated from other *F. occidentalis* populations (Andongma et al., 2022). Within the order Hemiptera, competitive dynamics have been discovered in the *R. pedestris*-*Caballeronia* symbiosis. Symbiotic *Caballeronia* strains exhibited strong competitive inhibition against representative environmental microbes in both *in vitro* and *in vivo* contexts (Itoh et al., 2019). However, Itoh et al (2019) did not explored competitive interactions between symbiotic strains, which has greater translational applicability due to the inability of host factors to distinguish between them, or the mechanisms that underlie these allelopathic interactions were not explored.

My research aims to explore the avenues in which symbiotic *Caballeronia* compete to colonize the midgut crypts of *A. tristis*. We first demonstrate that two strains that were directly isolated from wild *A. tristis* exhibit contact-independent competitive dynamics. Identifying this individual relationship provided the foundation to examine permutations of a geographically diverse collection of *Caballeronia* isolates to highlight a more expansive pattern of interactions. We were not able to provide evidence that the two original *Caballeronia* isolates compete contact dependently *in vitro*. However, due to the energy cost of using contact-dependent allelopathic mechanisms and the specific nutritional and chemical conditions of the host crypts, we have begun to examine contact-dependent competition *in vivo*. Through this suite of experiments, we are able to demonstrate that symbionts use robust competition mechanisms to colonize *A. tristis*, providing insights into the underlying mechanics of horizontally transmitted symbiosis development.

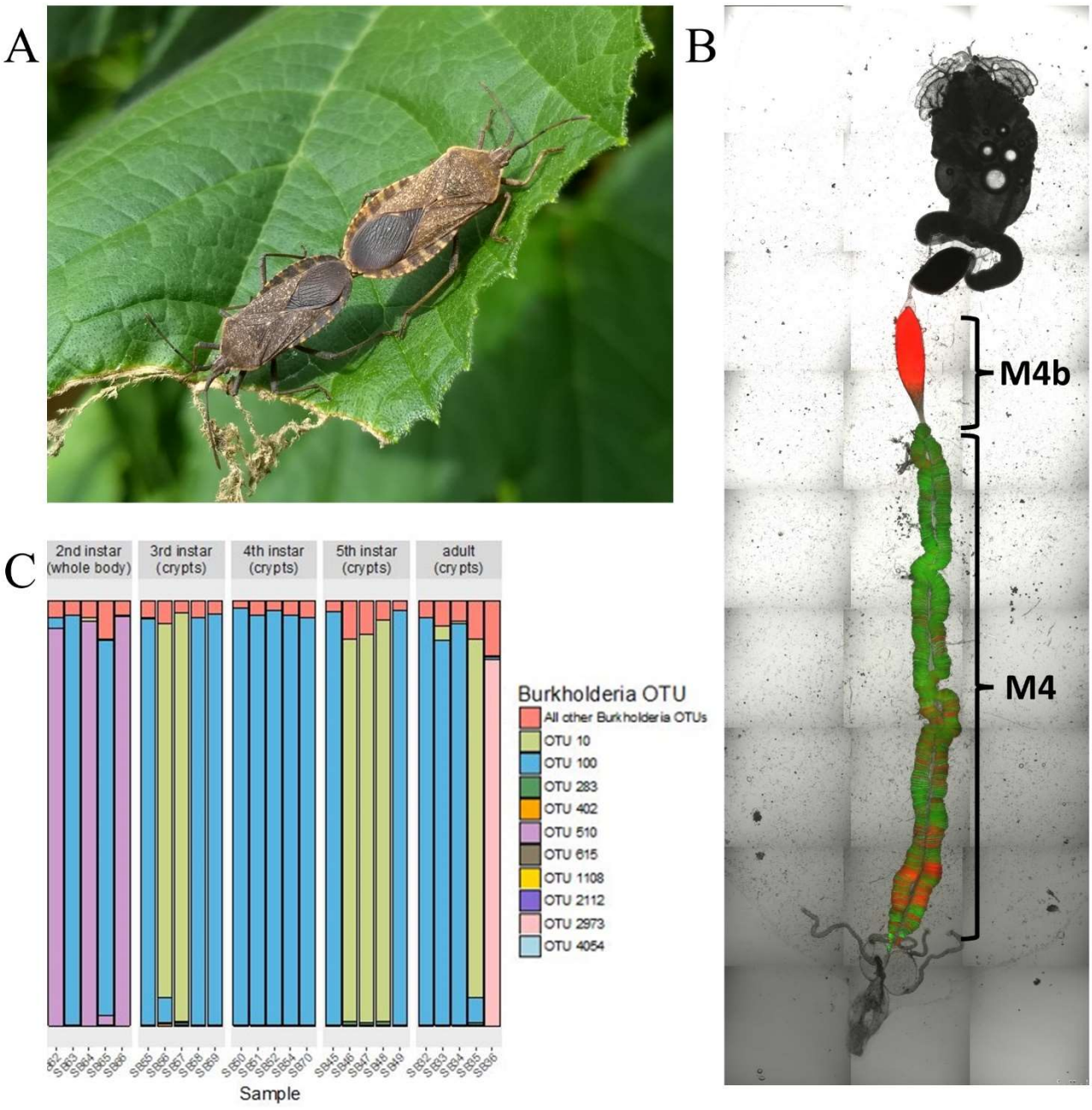


Figure 1. *Caballeronia* colonizes specialized symbiont crypts within *A. tristis*. A) Photograph of the host organism, *Anasa tristis* by Jason Chen B) Organization of *A. tristis* gastrointestinal tract with colonization in the M4 and M4b crypt by fluorescently tagged *Caballeronia*. Microscopy was done by Jason Chen C) Low diversity of colonized *A. tristis* midgut crypts in various developmental stages. Crypts were sequenced with 16S rRNA V4 (Garcia et al. unpublished work)

Chapter 1: Contact-Independent *Caballeronia* Strain Inhibition

Introduction

The variety of mechanisms that bacteria leverage to inhibit competitors can be broadly classified into contact-independent and contact-dependent processes. Due to the numerous potential environments that symbiotic *Caballeronia* travel through during transmission – the soil, squash plant, the oral and digestive regions of *A. tristis* and ultimately, the symbiotic crypts – it can be hypothesized that *Caballeronia* uses multiple forms of competition to ensure its colonization (Acevedo et al., 2021). This first chapter will primarily examine contact-independent dynamics.

Contact-independent competition is further divided into exploitative and interference competition. Exploitative competition is an indirect process where bacteria compete through their utilization of a shared limited resource. Superior utilization can be achieved by increased efficacy in resource uptake by modifying metabolic pathways or through the secretion of scavenger molecules, such as siderophores that chelate environmental iron (Ghoul & Mitri, 2016; Stubbendieck & Straight, 2016). Conversely, interference competition is the direct excretion of molecules that inhibit the growth of other microbial competitors. In *Escherichia coli*, the production of extracellular colicins, a form of bacteriocins, induces pore formation and cell death in closely related strains that occupy similar niches (Bayramoglu et al., 2017). Interference competition also involves the attenuation of another bacteria's competitive capacity by interfering with processes like quorum sensing (Christiaen et al., 2011).

Although identifying the specific mechanism of inhibition is the ultimate goal, the following set of experiments involving co-culture and conditioned media assays primarily assess for the presence of competition between symbiotic *Caballeronia* strains. We demonstrated that

Caballeronia from across a representative phylogeny of *Caballeronia* strains exhibit contact-independent inhibition.

Methods

Caballeronia Strains

All *Caballeronia* strains used in the assays were initially isolated from wild squash bugs and have been demonstrated to provide a primary benefit to their hosts by enabling them to reach maturity. The phylogeny of the *Caballeronia* strains can found in Figure S1. The co-culture assay employed two well characterized strains: GA-OX1 (*Caballeronia zhejiangensis*) and SQ4a (*Caballeronia concitans*). In terms of growth, *C. zhejiangensis* tends to have a shorter lag phase and a higher stationary phase than *C. concitans* in Lysogeny Broth (LB) and M9 minimal medium supplemented with glucose media (Figure S2). Previous work from Jason Chen, a graduate student in the Gerardo and Vega Labs, genomically integrated fluorescent cassettes bearing super folding green folding protein (sfGFP) and dTomato into *Caballeronia* strains through triparental conjugations using a miniTn7 system (Wiles et al., 2018).

Medium Preparation

Three widely used media – Lysogeny Broth (LB) medium, Nutrient medium and M9 minimal medium – were used to test the impact of nutritional environment on competitive dynamics. Lennox's version of Lysogeny Broth (LLB) medium, a low salt formulation, was prepared from powdered stock produced by Sigma-Aldrich. A high salt formulation of LB (LB+S) that had 10% NaCl concentration was also produced. Nutrient medium was made using

3% yeast extract and 5% peptone. M9 minimal medium was made according to the protocol from Cold Spring Harbor ("M9 Minimal Medium (Standard)," 2010).

Squash Juice Medium

Although the competition assays were all conducted *in vitro*, *in vivo* (though not directly in host) conditions were imitated by conducting the assays in squash juice, the primary food source of *A. tristis*. Organic squash was purchased from the grocery store and juiced using a commercial juicing machine. Since sterilization with the autoclave would denature most chemical compounds, sterilization was done by filtration. To remove larger particles, the squash juice was aliquoted into VWR International 250 mL polycarbonate centrifuge bottles and placed into a Sorvall RC-5B Refrigerated Superspeed centrifuge for 4 hours at 12,500 rpm and 4 ˚C. The supernatant was filtered through a Corning 0.22-micron bottle top vacuum filter and was stored at -20˚C.

In vitro Co-Culture Assay

sfGFP and dTomato strains of GA-OX1 and SQ4a were initially streaked from frozen 20% glycerol stocks onto nutrient agar plates and incubated at 30˚C for 48 hours. Individual colonies from each plate were inoculated into 3 mL of LB+S medium and incubated in a New Brunswick Scientific Excella E25 at 30˚C for 12 hours with shaking at 225 rpm. All cultures were equalized to an optical density (OD) of 1 by adding 100 uL of each culture to a 96-well plate and taking readings with the Synergy HTX multimode plate reader. The equalized cultures were spun down with the Eppendorf centrifuge 5424 R and washed with 1 mL of PBS three times. Monocultures of GA-OX1 and SQ4a were subsequently combined to form self vs. self and

self vs. competitor co-cultures for a total of four combinations. Co-cultures that contained the same *Caballeronia* strain, but with contrasting fluorescence, acted as a control to ensure that there was not a difference in the relative fitness cost of expressing a fluorophore for the *Caballeronia* strains. The experimental treatment, a co-culture of fluorescent GA-OX1 and SQ4a was also counterbalanced to further ensure a lack of confounding effects. All co-cultures were subsequently incubated for 24 hours at 30˚C with shaking at 225 rpm. At 24 hours, colony forming units (CFUs) of each microbe in the co-culture were counted through dilution plating and fluorescence microscopy to distinguish colony type based on fluorophore differences. A visual representation of this protocol can be seen in Figure 2A.

In vitro Conditioned Media Assay

Due to high throughput nature of conditioned media assays, seven *Caballeronia* strains were used. All seven strains were streaked from frozen 20% glycerol stocks onto nutrient agar plate and incubated at 30˚C for 48 hours. Individual colonies from each plate were inoculated into two vials containing either LB+S medium, M9 medium supplemented with glucose or squash juice medium and incubated at 30˚C for 12 hours with shaking at 225 rpm. To make the conditioned media, one of the cultures was run through a VMR international syringe filter with a 0.2-micron cellulose acetate membrane. A ratio was created by dividing a strain's OD in conditioned medium by the strain's OD in unconditioned mediaum (Equation 1). Thus, a ratio greater than one would indicate that the conditioned media facilitates the strain's growth while a ratio less than one implies that the conditioned media inhibits the strain's growth.

$$\text{OD600 Ratio} = \frac{\text{OD600 in Conditioned Medium}}{\text{OD600 in Unconditioned Medium}} \qquad \text{(Equation 1)}$$

Results

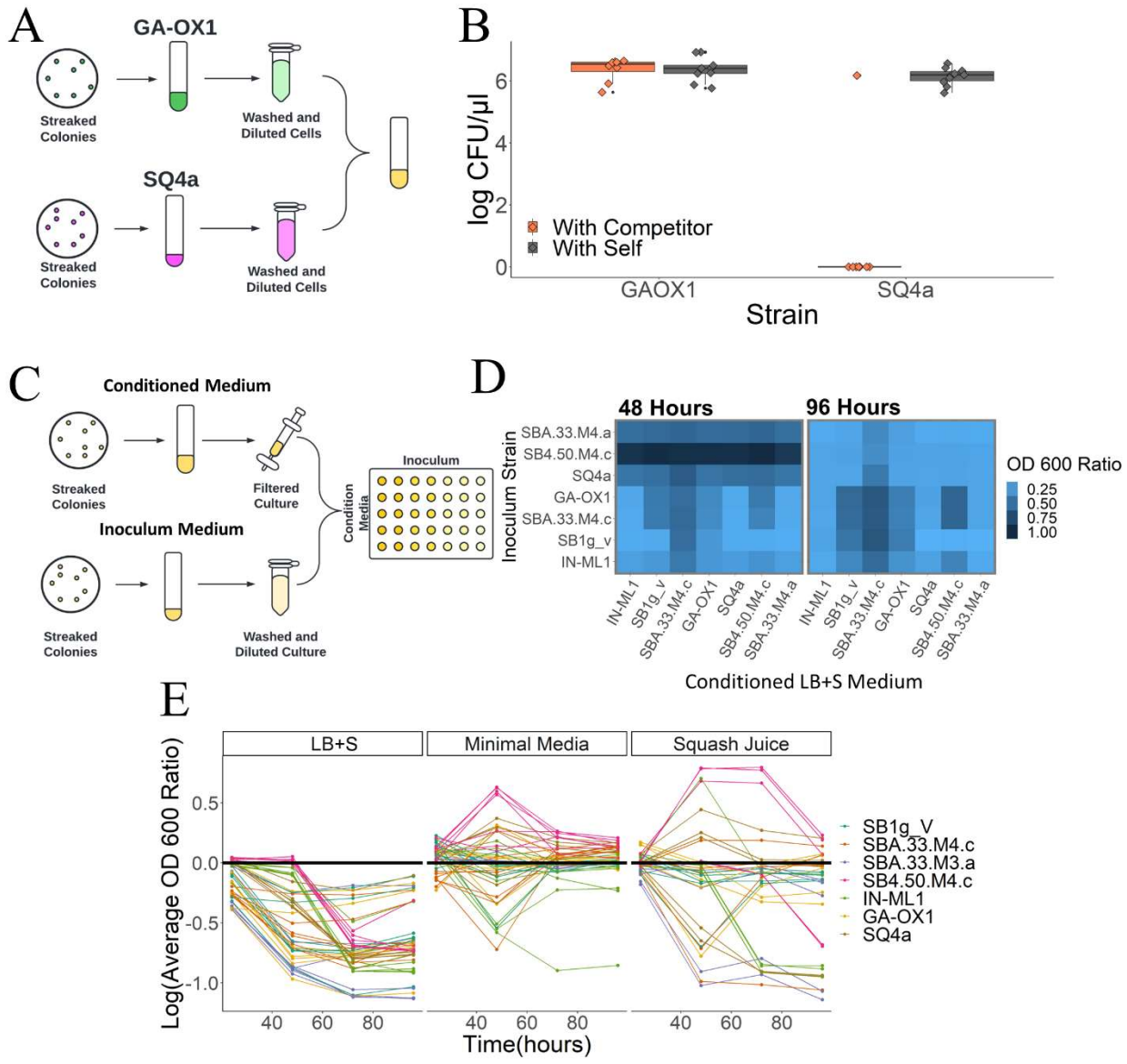


Figure 2. Contact independent inhibition between *Caballeronia* strains. A) Schematic of the co-culture assay used to assess competition between GA-OX1 (*C. zhejiangensis*) and SQ4a (*C. concitans*). B) CFU/μL at 24 hours for each strain in the co-culture with a competitor or self (n = 8 per bacterial pairing). C) Schematic of the high throughput conditioned media assay used to assess competition between seven *Caballeronia* strains. D) Matrix of average *Caballeronia* growth in various conditioned LB+S medium at 48 and 96 hours as a proportion of the positive control growth (n=14 per treatment). E) The conditioned media assay with permutations of the seven *Caballeronia* strains was repeated with minimal medium supplemented with glucose (n=2) and squash juice medium (n=1) over 96 hours with timepoints every 24 hours. Each line represents the OD600 ratio for each strain-conditioned medium combination. Lines are colored by the inoculating strain.

GA-OX1 and SQ4a exhibit contact independent inhibition in co-culture. As an exploratory experiment to determine if *Caballeronia* strains demonstrate competitive capacities, two strains, GA-OX1 and SQ4a, were placed into an *in vitro* co-culture with another (Figure 2A). After 24 hours, *Caballeronia* in both control treatments – competition with self – grew to a stationary phase of approximately 10^9 CFU/mL with minimal variation between trials. Similarly, GA-OX1 in co-culture with SQ4a demonstrated similar stationary phase carrying capacity to one another. Contrastingly, SQ4a in co-culture competition exhibited near zero growth with the exception of a single trial where SQ4a grew to a CFU/mL that was comparable to the control (Figure 2B). Examining the initial data, it does not appear that this growth was due to experimental error as the CFU/mL of GA-OX1 was comparable to the other trials. Thus, while the control conditions demonstrated congruent outcomes, the experimental competition conditions lead to disparate differences in growth between GA-OX1 and SQ4a.

***Caballeronia* strains demonstrate broad patterns of contact independent inhibition in conditioned LB media.** A high throughput conditioned media assay allowed for the expansion of the co-culture results to test for interactions between other *Caballeronia* strains. Rather than putting strains into direct competition with one another, strains were placed into

medium conditioned with chemical excretions from another strain. The nature of a contact-independent interaction was measured as a proportional inhibition of growth in conditioned medium (Figure 2C). The absolute optical density curves of each inoculum in their respective conditioned medium can be found in Figure S1. At 48 hours, inoculum strains display a diversity of responses to conditioned media. ANOVAs of all medium-inoculum combinations at 48 hours and at 96 hours demonstrated significant variation, ($F(71, 908) = 33.99$, $p < 2*10^{-16}$). Regardless of which conditioned medium it was inoculated in, SB4.50.M4.c grew to the same optical density as the positive control, while SB1g_V growth was inhibited in nearly all conditioned medias except for SBA.33.M4.c. GA-OX1 demonstrated a strong sensitivity to the conditioned medium strain, as its growth in SBA.33.M4.c and SM4.50.M4.c conditioned media was comparable to the positive control, while it was suppressed in SB1g_V and IN-ML1 medium. For the conditioned media, SBA.33.M4.c and SB4.50.M4.c media were the most permissive to growth, regardless of the inoculating strain. At 96 hours, there appeared to be variation in the patterns identified at 48 hours. Although SBA.33.M4.c conditioned media continued to remain permissive to inoculum growth, SB4.50.M4.c conditioned media demonstrated novel inhibition towards SBA.33.M4.c, SB4.50.M4.c and SQ4a inocula. Further, SBA.33.M4.c and SB4.50.M4.c inocula, which demonstrated unaffected growth at 48 hours, were considerably inhibited at 96 hours compared to their respective positive control. However, some patterns, such as the inhibition of SB1g_V growth in most conditioned media was constant at both 48 and 96 hours (Figure 2D).

Medium composition shapes inoculum-condition medium interactions. The previously described conditioned media experiments were conducted in rich, complex LB+S medium. To explore the importance of nutrient conditions, the experiment was repeated in M9

minimal medium supplemented with glucose – a simple, defined medium – and squash juice medium – a environmental medium (Figure 2E). The differential influences of the medium were apparent by 48 hours. For the LB medium, there was evidence of overarching inhibition in all inoculum-condition medium permutations. This suppression of growth reached a maximum at 72 hours and remained constant to the last 96-hour time point. Contrarily, both the minimal and squash juice mediums exhibit both inhibition and facilitation inoculum-conditioned medium relationships at 48 hours. The squash juice medium demonstrates greater variation in the spectrum of interactions compared to the minimal medium ranging from a one log decrease in OD to a half of a log increase in OD. This wider variation remained constant for the remainder of the experiment, particularly at 96 hours as the various combinations in M9 minimal medium tightly clustered around an OD ratio of one, while they demonstrated higher dispersal in the squash juice medium.

Discussion

In these experiments, we provide evidence of contact-independent inhibition across a phylogenetically diverse group of *Caballeronia* isolates. The first experiment which pitted two *Caballeronia* isolates – GA-OX1 and SQ4a – against one another in an LB liquid co-culture revealed nearly complete competitive inhibition of SQ4a by GA-OX1 (Figure 2B). Since co-culture incubation is done with constant shaking, the bacterial cells are in constant movement and do not have the capacity to come in direct contact, likely making the inhibition contact independent. The observed inhibition is not likely due to differences in growth, as their growth curves at 24 hours are only marginally different from each other (Figure S2). Rather it is more

likely that GA-OX1 imposes some form of interference competition to suppress the growth of SQ4a.

Although determining the specific mechanism of inhibition was beyond the scope of these experiments, we can use work done in related *Burkholderia* species to hypothesize about potential mechanisms. *Burkholderia pseudomallei*, the causative agent of melioidosis, has been demonstrated to excrete a flagella-degrading protein that excludes the growth of *Burkholderia thailandensis* which occupies a similar ecological niche (Ngamdee et al., 2015). Additionally, *B. pseudomallei* further produces secondary metabolites called 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs) that inhibit environmental Gram positive bacteria (Mou et al., 2021). *Caballeronia* may also be capable of other well characterized interference competition mechanisms, such as the excretion of bacteriocins, antibiotics or siderophore production (Eickhoff & Bassler, 2020; Stubbendieck & Straight, 2016). To determine whether this interaction between GA-OX1 and SQ4a is indicative of a broader, conserved pattern, we subsequently examined a matrix of interactions between a collection of *Caballeronia* strains.

The conditioned medium assay, which assessed combinations of seven environmentally acquired *Caballeronia* strains, indicated that *Caballeronia* strains demonstrate temporally dependent and isolate-specific patterns of inhibition (Figure 2D). Conditioned media were used instead of co-cultures as they bypass the need for a way to differentiate or selectively isolate one competitor from the other and limit the effects of exploitative competition by replenishing the conditioned medium with fresh medium, while still preserving the secondary metabolites and other excreted products that potentially underlie *Caballeronia* conflict. This increased efficacy enabled a comprehensive exploration of every combination of interaction between seven *Caballeronia* isolates. The resulting matrix illustrated a prevailing theme of inhibition as the

best-performing inocula only grew to an OD equal to the positive control. There was clear strain specific variation in inhibition with some condition media such as SBA.33.M4.c being more permissive to growth while SB1g_v conditioned medium was inhibitive to the majority of strains.

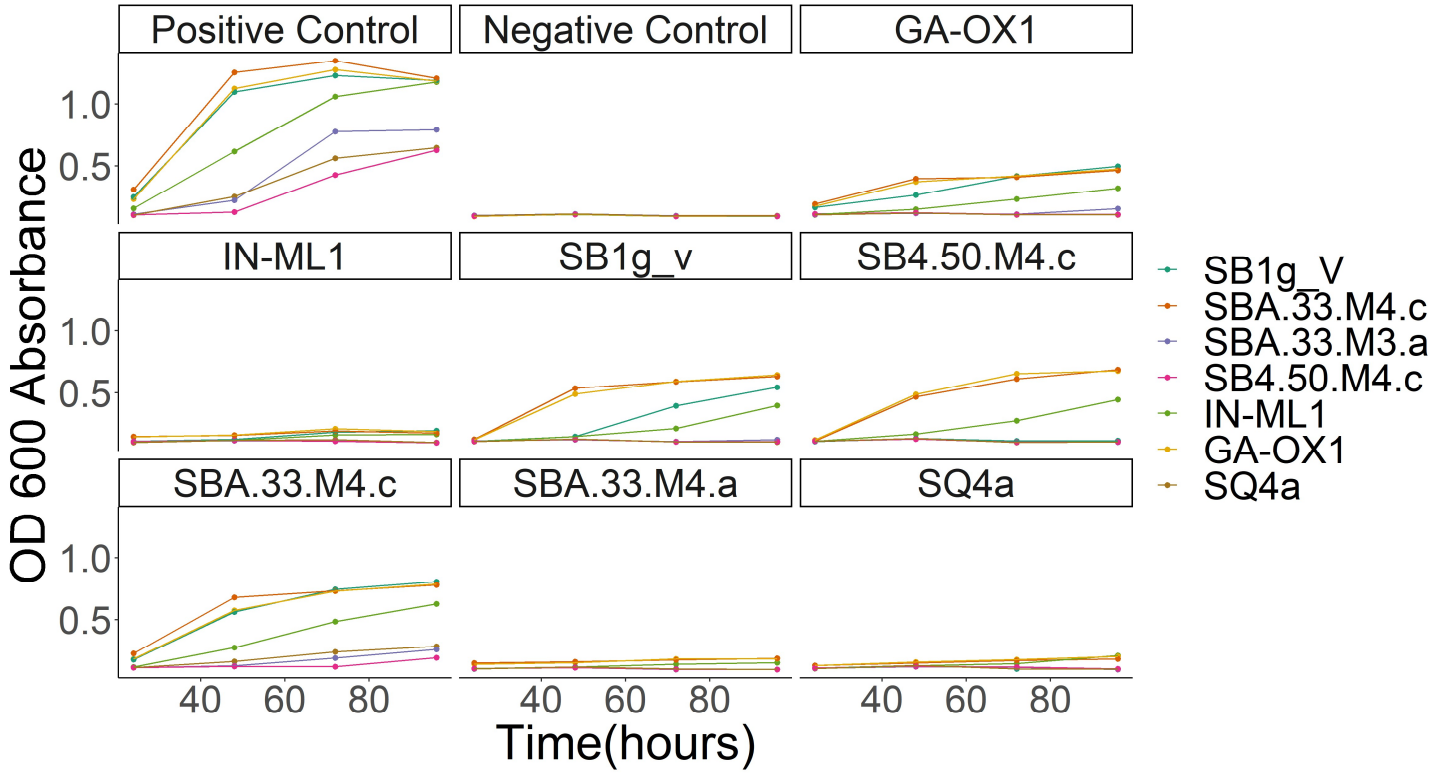
A natural deduction would be that this competitive variation would be correlated with the degree of benefit a strain provides to the host. In the horizontally transmitted *E. scolopes – V. fischeri* symbiosis, a strain of *V. fischeri* with heightened bioluminescence, which would likely provide a greater benefit to the host, demonstrates a competitive advantage over another strain of *V. fischeri in vivo* (Sun et al., 2016). However, we currently do not have any evidence to support the notion that the *Caballeronia* isolates differ in the benefit that they provide to *A. tristis*. Based on the phylogenetic relationships between the seven isolates (Figure S1), there does not appear to be a simple genetic explanation for the differences in competition. Factors such as horizontal gene transfer could complicate the inheritance of a competitive advantage. Consequently, more genomic, and ecological work is required to fully explain the patterns seen in the conditioned medium assay.

The previously discussed conditioned medium assay was done in the rich complex medium of LB+S, which includes many compounds that are not found in *Caballeronia*'s natural environment. Further, work in other members of Hemiptera have established that it is likely that the intestinal crypts of *A. tristis* are nutritionally deficient, a possible selection mechanism employed by the host (Kim et al., 2014). Additionally, before colonization of the crypt, *Caballeronia* is likely suspended in squash juice, the primary nutrient source of *A. tristis*, which is a highly complicated medium with multiple complex sugars and fibers. To mimic these environments, the conditioned medium assays were repeated in two additional media, minimal

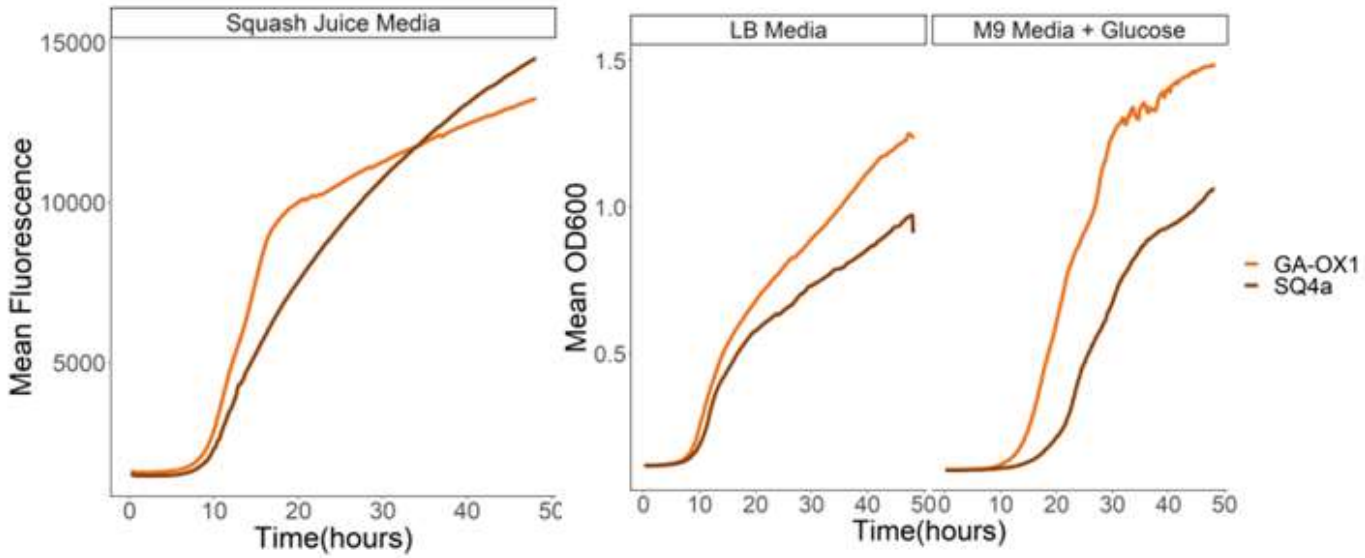
media supplemented with glucose and squash juice media. Compared to their dynamics in LB medium, inoculum-conditioned medium relationships exhibited a broad spectrum of interaction behavior, including facilitative ones. This trend agrees with ecological theory which posits that rich environments facilitate competition, while depleted ones favor cooperation (Hoek et al., 2016; Hu et al., 2022). This may imply a potential role for symbiont cooperation during colonization, as a symbiont reproducing to a higher titer because it cooperatively interacts with another *Caballeronia* strain may allow it to outcompete other microbes in the intestinal tract. However, a better understanding of host crypt conditions is required to fully characterize the interactions between *Caballeronia* strains due to the evident nutrient-dependent nature of their dynamics (Ohbayashi et al., 2019)

This series of experiments has revealed that *Caballeronia* species demonstrated robust inhibitive dynamics in a contact-independent setting. Although we were not able to identify an underlying basis of this competition, the specific mechanisms are likely dependent on the specific nutrient and chemical environment in which the *Caballeronia* is located. In reference to the environmental backdrop of these interactions, it is also critical to consider the spatial constriction within the crypts, which increases the probability of bacteria coming in direct contact with one another (Ohbayashi et al., 2015). Consequently, contact-dependent inhibition must also be characterized to develop a thorough understanding of the role of competition in shaping *Caballeronia* colonization of *A. tristis*.

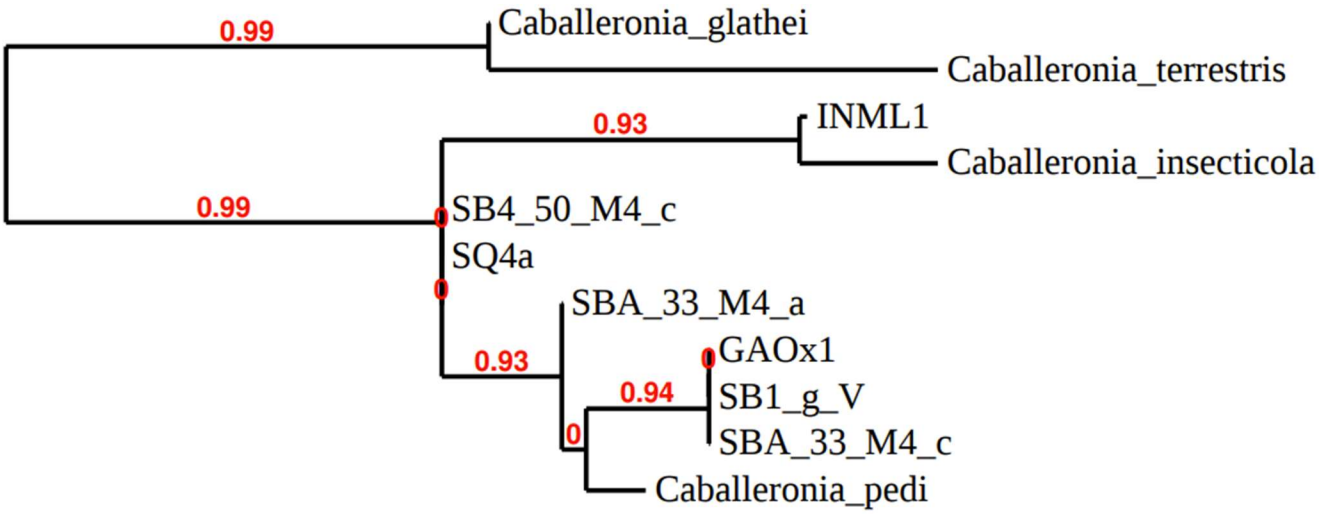
Supplemental Figures



Supplemental Figure 1. Absolute OD600 Absorbance of each conditioned media combination at 4 time points (0-96 hours). Each facet represents a different conditioned media, and each line a different bacterial strain.



Supplemental Figure 2. Growth curves of GA-OX1 and SQ4a in LB, M9 supplemented with glucose and squash juice medium. Growth was measured with OD600 for LB and M9 media and fluorescence for squash juice media due to the turbidity of the media.



Supplemental Figure 3. Phylogeny of *Caballeronia* strains used in conditioned media assays based on 16S V4 rRNA sequencing and made with the ggtree package in RStudio

Chapter 2: Contact-Dependent *Caballeronia* Strain Inhibition

Introduction

With contact-independent inhibition identified, the use of contact-dependent mechanisms was subsequently investigated. Access to the symbiotic crypts in *A. tristis* is restricted by a constricted passage with a single cell width. Consequently, the preceding region, the M4b crypt, exhibits a high concentration of environmental and symbiotic bacteria attempting to enter the constricted region (Figure 1B). This accumulation of bacteria in the crypts likely brings cells into direct contact with one another, rationalizing an investigation into *Caballeronia*'s use of contact-dependent inhibition.

A likely mechanism is the Type VI Secretion System (T6SS). T6SSs can be broadly found across Gram negative bacteria and excrete toxins through a structure analogous to the bacteriophage tail (Cianfanelli et al., 2016). Although T6SSs have been shown to have various functions from the collection of metals to biofilm formation, our focus is on their role in direct microbial interactions (Chen et al., 2019). In *Pseudomonas aeruginosa*, activation of toxic effector release from a T6SS is activated by membrane disturbance caused by the injection of a T6SS from an opposing bacterium (Basler et al., 2013).

Within the context of symbiosis maintenance, T6SS expression in *V. fischeri* is upregulated when entering the light organ of *E. scolopes*, which results in the dominant colonization of the symbiotic strain (Speare et al., 2021). With T6SSs structures identified in the *Caballeronia* strains that participate in the *R. pedestris* symbiosis, it can be hypothesized that T6SS has an indispensable function in the elimination of competing microbes during *Caballeronia* colonization in *A. tristis* (Ohbayashi et al., 2019).

In the following experiment, a coarse examination of contact-dependent inhibition in *Caballeronia* was conducted, similar to the contact-independent experiments. However, we were unable to provide conclusive evidence of contact-independent inhibition with *in vitro* co-culture assays on solid agar.

Methods

Escherichia coli Strain

For the contact-dependent inhibition experiments, a lab strain of a K12 derived *E. coli*, MC4100, was used. M4100 has no pathogenicity islands, does not secrete colistin, and is an overall poor competitor (Peters et al., 2003). For the experiments, MC4100 was streaked onto a nutrient agar plate from glycerol stock, incubated at 30 ˚C for 24 hours, and then inoculated into 3 mL of LLB medium for 8 hours at 30˚C and shaking of 225 rpm. MC4100 does have stably integrated gentamycin resistance, which allows for its selection during the dilution plating and counting process.

Vibrio cholerae Strains

Two strains of *V. cholerae* (C6706) from the Goldberg Lab were used in the contact-dependent inhibition experiments. The first strain was a T6SS knockout (T6SS-), while the second strain had a constitutively active T6SS (T6SS+). Based on the growth curves of the strains (Figure S4), *V. cholerae* T6SS- demonstrates a higher optical density at stationary phase than *V. cholerae* T6SS+. For the experiments, *V. cholerae* variants were streaked onto a nutrient agar plate from glycerol stock, incubated at 30 ˚C for 24 hours, and then inoculated into 3 mL of LB+S medium for 8 hours at 30˚C and shaking of 225 rpm.

In vitro Monolayer Killing Assay

In vitro T6SS killing was assessed through a monolayer killing experiment. MC4100, *V. cholerae* T6SS+, *V. cholerae* T6SS-, GA-OX1 and SQ4a were prepared as described for the co-culture assay in Chapter 1. All were streaked from their frozen glycerol stock and incubated at 30˚C on a nutrient agar plate. A colony from each plate was inoculated into nutrient medium and incubated at 30˚C with shaking at 225 rpm. *Caballeronia* strains were incubated for 12 hours, while MC4100 and the *V. cholerae* strains were incubated for 8 hours due to their faster doubling time (Figure S4). All cultures were washed with PBS before being equalized to an optical density of 1.

Based on the combinations listed in Table 1, the "predator" and "prey" strain were combined in a 10:1 ratio and a co-culture aliquot of 20 uL was made on the corresponding agar plate. The predator strain had an approximate inoculum of 10^8 CFU/mL, and the prey strain had an inoculum of 10^7 CFU/mL (Figure S6 & Figure S7). The aliquot was incubated at 30 ˚C after fully drying and removed four hours later for harvesting. The competition spots were subsequently cut from the agar and resuspended in PBS using a Fisher Brand vortexer. CFU/mL of the prey strains were found through dilution plating on gentamycin nutrient medium plates, as all prey strains stably expressed fluorescence and gentamycin resistance. This value was divided by CFU/uL of the prey strain grown in a monolayer alone to calculate the inhibition index (Equation 2). For this measure of competition, an index value of 1 indicates no inhibition.

$$\text{Inhibition Index} = \frac{\text{Prey } \frac{\text{CFU}}{\mu\text{L}} \text{ with Predator}}{\text{Prey } \frac{\text{CFU}}{\mu\text{L}} \text{ Grown Alone}} \qquad \text{(Equation 2)}$$

Table 1. Predator and prey strain combinations and media types used in the *in vitro* killing assay

Purpose	Predator Strain	Prey Strain	Different Media Tested
Upper Killing Bound	*V. cholerae* T6SS+	MC4100	LB Media, Nutrient Media, Squash Juice Media
Lower Killing Bound	*V. cholerae* T6SS-	MC4100	LB Media, Nutrient Media, Squash Juice Media
Control	MC4100	MC4100	LB Media, Nutrient Media, Squash Juice Media
Control	GA-OX1	GA-OX1	Nutrient Media, Squash Juice Media
Experimental	GA-OX1	MC4100	LB Media, Nutrient Media, Squash Juice Media
Control	SQ4a	SQ4a	Nutrient Media, Squash Juice Media
Experimental	SQ4a	MC4100	LB Media, Nutrient Media, Squash Juice Media
Experimental	GA-OX1	SQ4a	Nutrient Media, Squash Juice Media
Experimental	SQ4a	GA-OX1	Nutrient Media, Squash Juice Media

Results

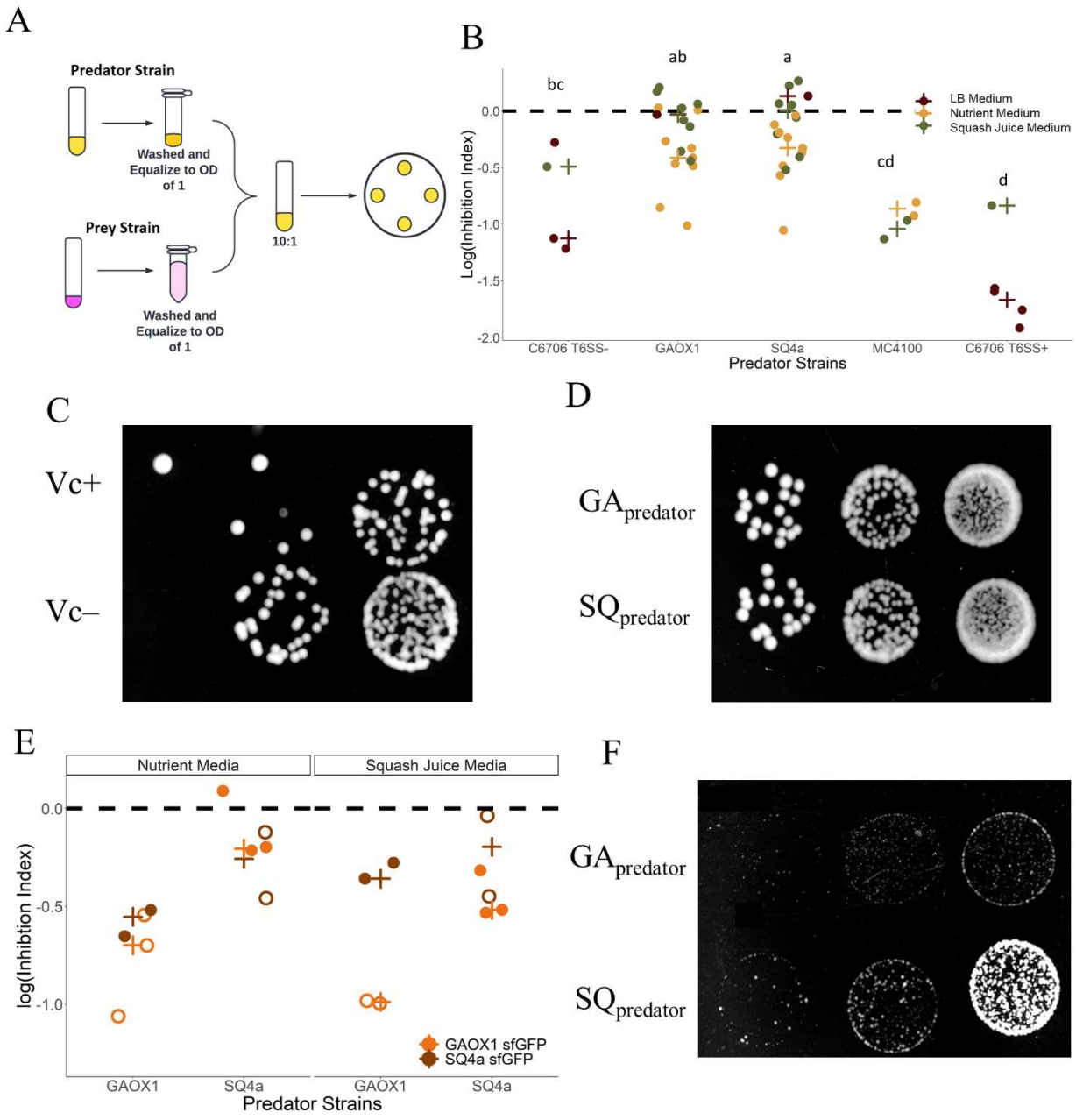

A
Predator Strain
Washed and Equalize to OD of 1
Prey Strain
Washed and Equalize to OD of 1
10:1
B
bc
ab
a
cd
d
LB Medium
Nutrient Medium
Squash Juice Medium
Log(Inhibition Index)
0.0
-0.5
-1.0
-1.5
-2.0
C6706 T6SS-
GAOX1
SQ4a
MC4100
C6706 T6SS+
Predator Strains
C
Vc+
Vc–
D
GApredator
SQpredator
E
Nutrient Media
Squash Juice Media
log(Inhibtion Index)
0.0
-0.5
-1.0
GAOX1 sfGFP
SQ4a sfGFP
GAOX1
SQ4a
GAOX1
SQ4a
Predator Strains
F
GApredator
SQpredator

Figure 3. No *in vitro* evidence of contact dependent inhibition between *Caballeronia* strains. A) Graphical schematic of monolayer killing assay protocol. Monolayers were made by combining predator and prey strains in a 10:1 ratio. Prey strains were selected for with gentamycin after 4 hours. B) Degree of MC4100 inhibition by various predator strains of *Caballeronia* and *V. cholerae* in three media. Unless specified otherwise, each point represented one trial, and each cross represents the median CFU/mL for the respective media type. Letters represent the results of a one-way ANOVA of the log transformed inhibition index ($F(4, 44) = 19.52$, $p < 2.68*10^{-9}$) and a post-hoc Tukey Test. C) Brightfield microscopy of MC4100 prey strains dilutions after monolayer incubation with *V. cholerae* T6SS+ (Vc+) and *V. cholerae* T6SS- (Vc-). All microscopy was done with an Olympus SZX16 microscope. D) Brightfield microscopy of MC4100 prey strains dilutions after monolayer incubation with GA-OX1 (GA) and SQ4a (SQ). E) Degree of prey *Caballeronia* strain inhibition by predator *Caballeronia* strain on two media (nutrient and squash juice media). Open circles represent monolayer competition against self and closed circle represents monolayer competition against competing strain. F) Brightfield microscopy of SQ4a and GA-OX1 dilutions after monolayer incubation with GA-OX1 (GA) and SQ4a (SQ), respectively.

***Caballeronia* strains do not exhibit contact-dependent inhibition against attenuated *E. coli*.**

To determine whether *Caballeronia* strains exhibit contact-dependent inhibition, MC4100 – a lab strain of *E. coli* – was used as the prey strain in a modified version of the co-culture assay from the contact-independent experiments (Figure 3A). High density co-cultures of strains were dried on agar plates to create spatial structure and facilitate contact-dependent interactions (Figure 3A). In addition, two strains of *V. cholerae* with contrasting T6SS functionality were used as positive and negative controls. Due to *V. cholerae's* requirement for high salt content, LB media instead of nutrient medium was used. (Figure 3B).

When grown in a monolayer by itself, the predator strains achieved comparable CFU/ml to one another after the four-hour incubation period with approximately 10^8 CFU/mL. There did not appear to be a difference in growth based on whether nutrient or squash juice medium was used. (Figure S5)

In LB medium, *V. cholerae* T6SS+ demonstrated the strongest killing of MC4100 with an average inhibition index of 0.02. Conversely, *V. cholerae* T6SS- exhibited a decreased killing

capacity with an average killing index of 0.1. This is visually apparent as there were fewer colonies of MC4100 in each of the *V. cholerae* T6SS+ dilutions compared to *V. cholerae* T6SS- (Figure 3C). However, in the one trial that was done in squash juice medium, there was both a reduction in both strains' killing index and in the difference between the two *V. cholerae* variants (Figure 3B).

Caballeronia inhibition against MC4100 was primarily done in nutrient or squash juice agar. In nutrient agar, the average killing indices for GA-OX1 and SQ4a were similar to one another at 0.31 with individual trials ranging from a killing index of 0.1 to 1. Inhibition was further decreased when the *Caballeronia* predations of MC4100 were done on squash juice agar with an average inhibition index of 1, which indicates that there was no difference in growth between the control growth and predation growth (Figure 3B). These observations were further reinforced visually where the dilution growth of MC4100 was comparable for GA-OX1 and SQ4a. Further, there were qualitatively more CFUs of MC4100 in the *Caballeronia* predation trials compared to the *V. cholerae* trials. All of *Caballeronia* inhibition indices were higher than the inhibition index of *V. cholerae* T6SS- (Figure 3D).

Lack of clear *in vitro* contact-dependent inhibition between *Caballeronia* strains. When grown in a monolayer by itself, the predator *Caballeronia* strains achieved a comparable CFU/mL to each other after the four-hour incubation period with approximately 10^8 CFU/μL. There did not appear to be a difference in growth based on whether nutrient or squash juice was use (Figure S5).

On nutrient medium agar, GA-OX1 and SQ4a suppressed their respective prey to different degrees. GA-OX1 with SQ4a as the prey had an average inhibition index of

approximately 0.25 with one trial that exhibited a stronger inhibition index of approximately 0.1. Contrastingly, SQ4a as the predator strain demonstrated less inhibition of GA-OX1 with an average inhibition index of approximately 0.56 with one trial where GA-OX1 had a higher CFU/μL compared to the positive control. Similarly, the controls of GA-OX1 and SQ4a competing against themselves demonstrated similar inhibition patterns, where GA-OX1 inhibited itself to a greater degree than SQ4a inhibited itself (Figure 3E). A visual representation of the GA-OX1 and SQ4a with their respective prey reaffirmed these observations. At the lowest dilution shown, GA-OX1 as the prey visually demonstrated a higher CFU/μL compared to prey SQ4a (Figure 3F).

For squash juice medium, SQ4a continued this pattern with minimal difference in the inhibition index between self and GA-OX1. However, for GA-OX1, it demonstrated reduced inhibition of prey SQ4a with an average inhibition index that was approximately equal to SQ4a's predation of GA-OX1. Further, GA-OX1 exhibited relatively strong inhibition against itself with an average index of 0.1. Thus, GA-OX1 as a predator in squash juice medium exhibited increased self-inhibition compared to inhibition of the prey SQ4a strain (Figure 3F).

Discussion

As *Caballeronia* symbionts move from the environment into the rostrum and intestinal tract of *A. tristis*, they enter a spatially limited environment that is further exacerbated by the single-cell wide constricted region (Ohbayashi et al., 2015). Consequently, it is hypothesized that *Caballeronia* will employ contact-dependent mechanisms of inhibition, to increase the likelihood of colonizing the crypts. This series of experiments aimed to provide initial evidence that *Caballeronia* participate in constant-dependent inhibition utilizing a monolayer inhibition assay.

However, we were ultimately unable to provide significant evidence of these processes being used by *Caballeronia* in an *in vitro* context.

To assess contact-dependent inhibition, a monolayer assay that facilitates microbial contact through the drying of the co-culture on an agar plate was used (Crisan et al., 2021). Since the co-cultures were thoroughly mixed before plating, there was likely a homogenous mixture of the predator and prey strains, increasing the probability that the predator and prey strains come into direct contact with one another rather than interacting with themselves in aggregates.

To maximize potential inhibition, a lab strain of *E. coli*, MC4100, that expresses limited pathogenicity was selected as an ideally defenseless prey. Additionally, the two variants of *V. cholerae* would theoretically set the boundaries of inhibition, as one variant has a constitutively expressed T6SS and the other is a T6SS knockout (KO). The results mirror this assumption, setting an inhibition index boundary ranging from 0.1 to -0.02, or approximately one log difference when done in LB media. The difference in inhibition index disappears for squash juice media as the lower salt content likely affects the growth and function of *V. cholerae* (Faruque et al., 1998). This constructed boundary was further confirmed by the condition where MC4100 was put into co-culture with itself as it had an average inhibition index similar to *V. cholerae* T6SS-. Theoretically, both MC4100 and *V. cholerae* T6SS- are not able to participate in contact-dependent inhibition and any inhibition that is seen is due to exploitative competition. With an established boundary to distinguish T6SS killing from other forms of competition such as exploitative competition, the inhibition indices of *Caballeronia* with MC4100 as the prey can be interpreted (Figure 3B).

GA-OX1 and SQ4a had inhibition distributions that were statistically insignificant from one another. Both strains were found to have inhibition indices that were outside of the lower

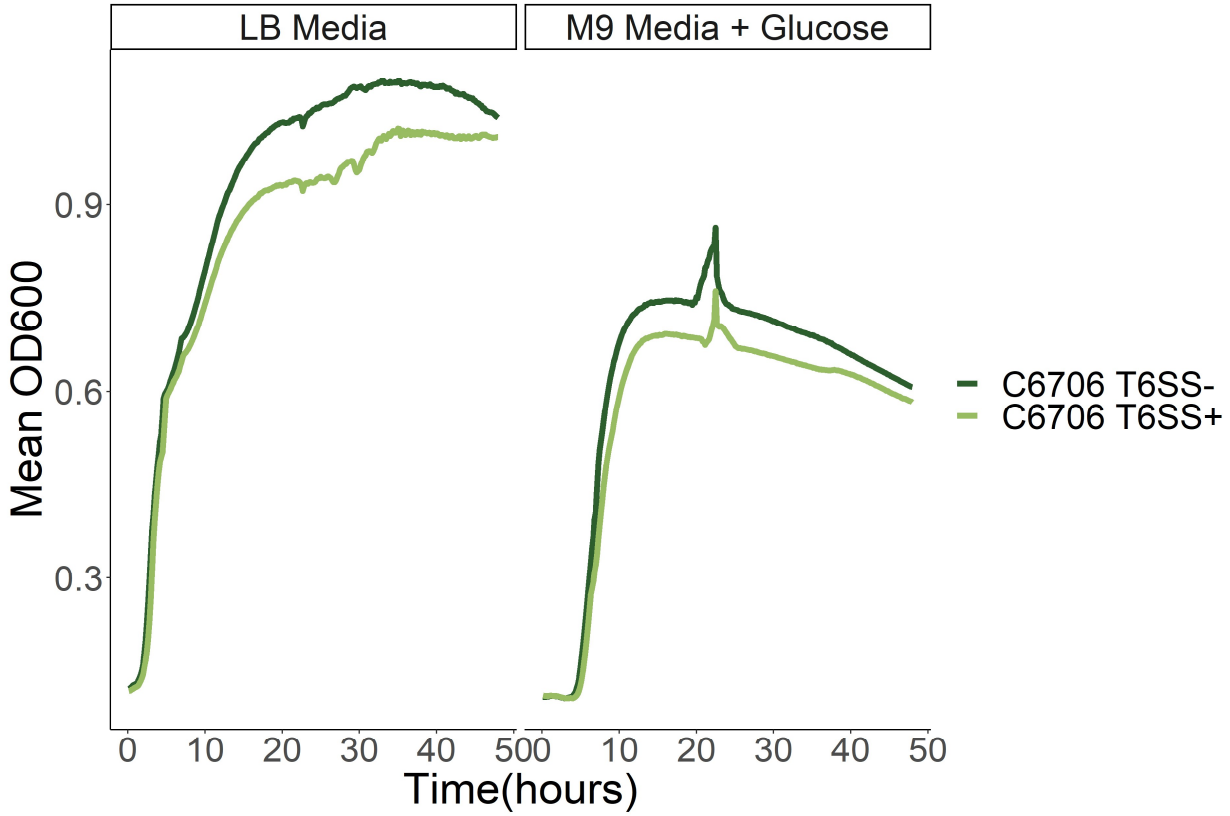
boundary, indicating that they were not likely expressing T6SS killing or other forms of contact-dependent inhibition *in vitro* against MC4100. This is further demonstrated by the statistical insignificance between GA-OX1's and the KO variant of *V. cholerae's* inhibition of MC4100. There appeared to be a slight influence of media type for both *Caballeronia* strains, as trials done in squash juice media demonstrate decreased inhibition compared to trials done in nutrient medium (Figure 3B). These observations do not fully reject the hypothesis that *Caballeronia* uses contact-dependent mechanisms in the colonization process. T6SS expression is a highly regulated process due to the energetic expenditure of sheath assembly and effector protein expression, which potentially has a detrimental effect on microbial fitness (Basler et al., 2013; Gupta et al., 2021; Lazzaro et al., 2017). This phenomenon is also visible within the two *V. cholerae* variants, as the T6SS+ mutant demonstrated decreased carrying capacity and doubling rate compared to the T6SS knockout (Figure S4). Consequently, *Caballeronia* T6SS expression may be dependent on specific environmental signals. One potential type of signal is interactions with other competitive microbes. For instance, *Serratia marcescens* upregulates its expression of T6SS in the presence of effector molecules excreted by a competitor such as *Acinetobacter* (Lazzaro et al., 2017). In *P. aeruginosa*, T6SS expression is a form of "counterattack" to T6SS activity from competing strains such as *V. cholerae* (Basler et al., 2013). These examples of armed competitor induced T6SS expression raise the possibility that the lack of *Caballeronia* T6SS expression was due to the defenseless nature of MC4100. By not having any form of allelopathy, such as antimicrobial peptides or T6SS, MC4100 may have been unable to induce the expression of *Caballeronia* T6SS. To test this hypothesis, contact-independent inhibition between GA-OX1 and SQ4a was considered, as they both contain competition machinery.

In the subsequent experiments, GA-OX1 and SQ4a contact-dependent inhibition capacity was also assessed through monolayer co-cultures. Although GA-OX1 significantly inhibit the growth of SQ4a compared to SQ4a's inhibition of GA-OX1 in nutrient medium, GA-OX1 inhibited itself to greatest degree. Thus, there did not appear to be a strain-specific difference in inhibition for either GA-OX1 or SQ4a. Further, their average inhibition indices lay outside of the *V. cholerae* T6SS- lower boundary, indicating that there was likely no T6SS expression. In squash juice medium, this pattern continued for SQ4a. However, for GA-OX1, the reverse was observed, where there was greater inhibition of itself compared to the opposing strain (Figure 3E). The exact reason for the reversal is unknown. However, a potential explanation may be due to the complex carbon sources present in the squash juice medium, as they may lead to strong niche partitioning, which would prevent a prey GA-OX1 from being able to establish in an environment dominated by a predator GA-OX1 compared to SQ4a, which may be able to occupy a different metabolic niche. Nevertheless, it is evident that direct competition between T6SS possessing strains does not activate T6SS expression in *Caballeronia* in an *in vitro* context.

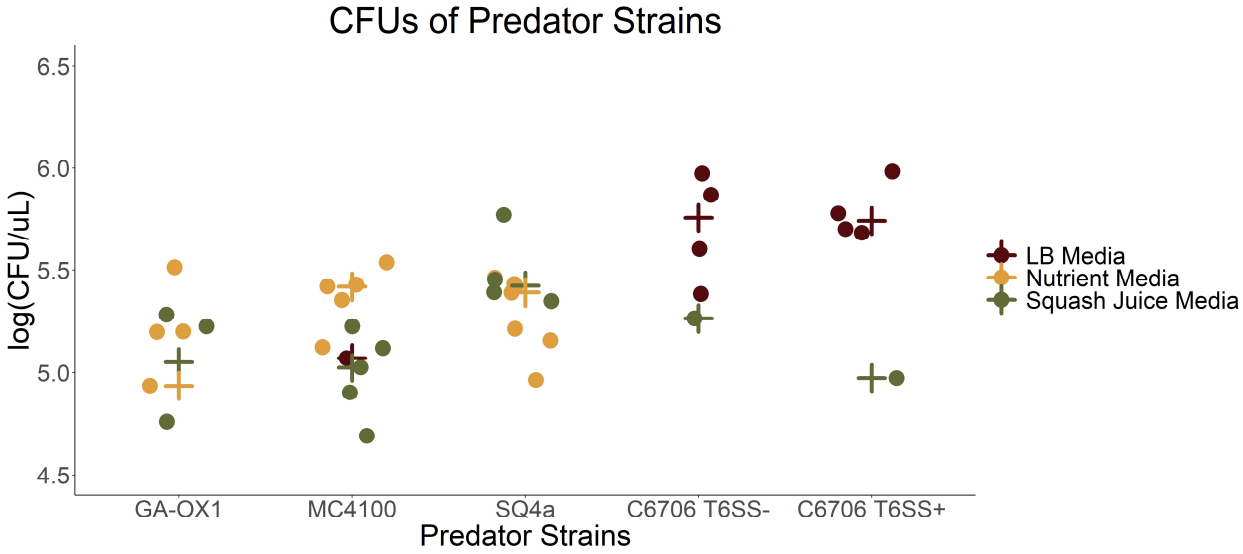
The other form of signals that could activate T6SS expression are specific metabolic or chemical cues. In the *E. scolopes – V. fischeri* symbiosis, *V. fischeri* expression of T6SS is dependent on the presence of host-specific conditions such as viscosity and pH, as they will not express the machinery in a planktonic state (Speare et al., 2021). Further a pathogen of fish, *Francisella noatunensis*, demonstrates upregulated expression of T6SS genes at lower temperatures and under oxidative stress (Lewis & Soto, 2019). To investigate whether symbiotic *Caballeronia* T6SS expression is dependent on similar ecological cues, *in vivo* assays will be the primary form of experimentation as the specific conditions in the crypts of *A. tristis* have yet to be fully elucidated. Although we have created squash juice medium to mimic the environment

inside of the host, it is likely that the host excretes specific compounds that could be critical to the expression of T6SS. Thus, the next stage of experiments will focus on examining *Caballeronia* T6SS expression within a host by creating KOs to use for *in vivo* competition assays.

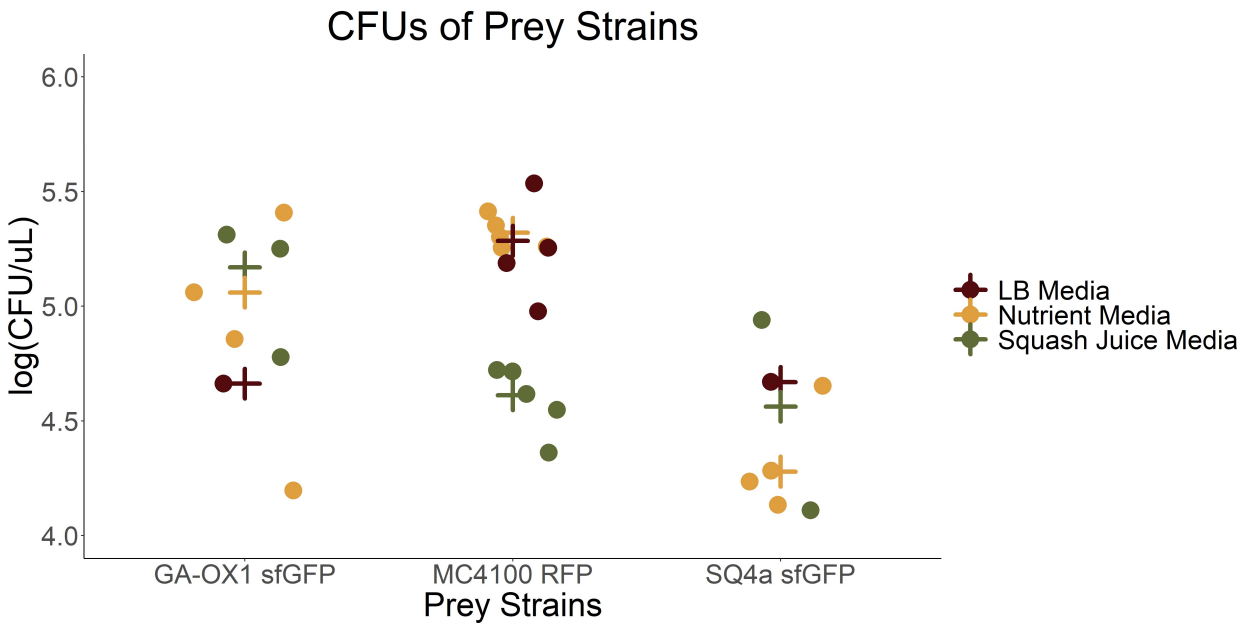
Supplemental Figures



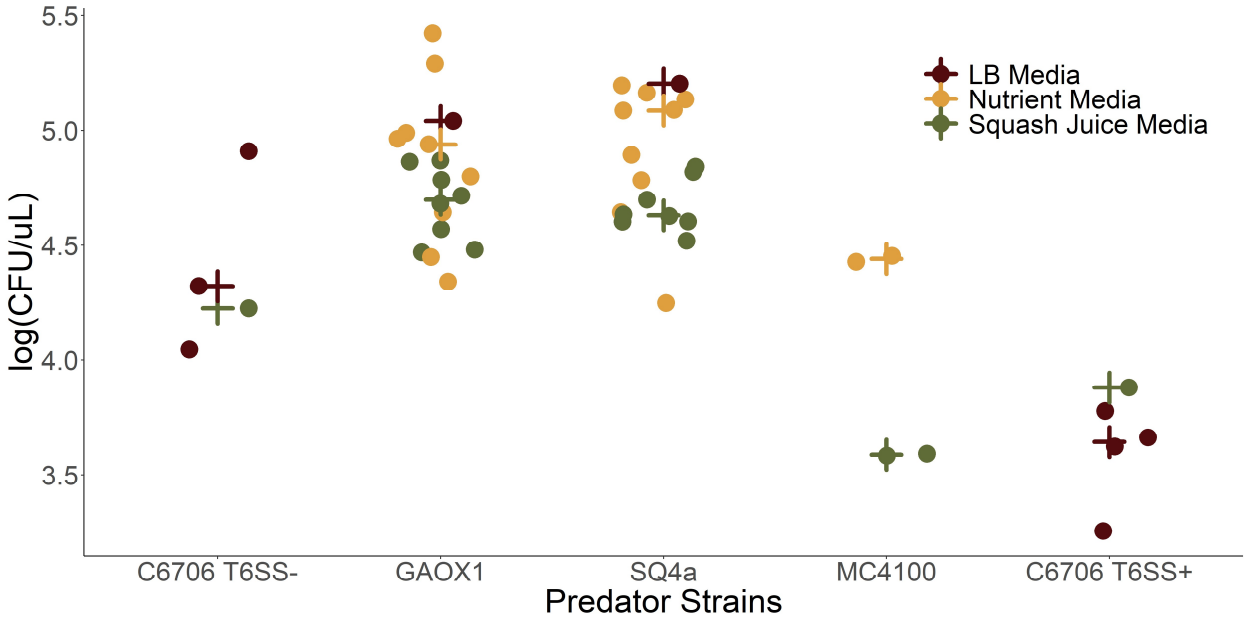
Supplemental Figure 4. Growth Curves of *V. cholerae* variants in LB and M9+glucose media. Growth is measured through OD600



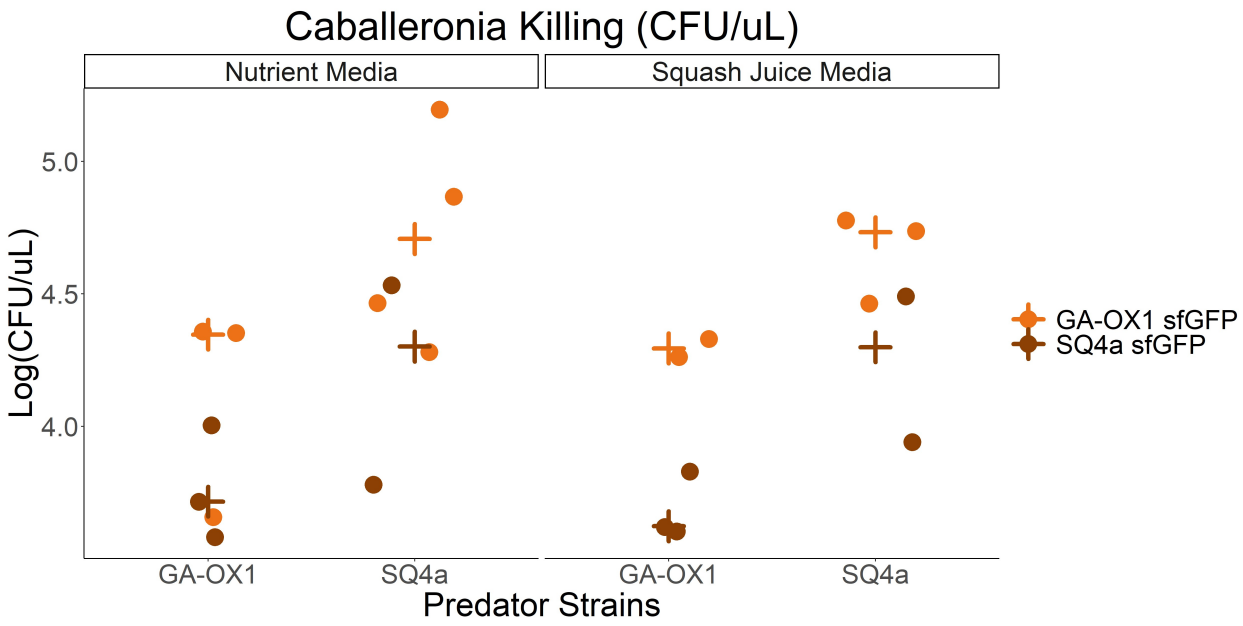
Supplemental Figure 5. The starting CFU/uL of predator strains in the three types of media used.



Supplemental Figure 6. The starting CFU/uL of the prey strains in the three type of media used



Supplemental Figure 7. Absolute CFU/uL values of MC4100 prey strain when placed in co-culture with various predator strains for 4 hours. This is the same data as Figure 3B



Supplemental Figure 8. Absolute CFU/uL values of *Caballeronia* prey strain when placed in co-culture with various predator strains for 4 hours. This is the same data as Figure 3E

Chapter 3: Constructing *Caballeronia* T6SS KOs for *in vivo* Competition Assays

Introduction

The lack of evidence of *Caballeronia* T6SS expression against an attenuated *E. coli* strain *in vitro* does not rule out any T6SS secretion; rather it raises questions about the particular context(s) that leads to its activation. The previous chapter demonstrated that the presence of competitors, both with and without functioning T6SS, do not alter expression in *Caballeronia*. The next goal would be to determine whether host conditions, such as nutritional environment, pH or viscosity, play key roles in the activation of *Caballeronia* T6SS. The most straightforward way to assess these factors is through *in vivo* competition assays inside of the *A. tristis* midgut. However, this assay requires the creation of a T6SS secretion system knockout (KO).

T6SS are encoded by thirteen core genes that form the transmembrane secretion apparatus (Cascales, 2008). The apparatus is composed of three distinct regions: the baseplate, the sheath complex, and the membrane complex. The baseplate, which is composed of four structural proteins acts as the origin for the synthesis of the sheath complex by building around VgrG, a protein that forms the tip of the needle (Cherrak et al., 2019). The sheath complex has two layers, an inner tube that is composed of Hcp protein polymers and topped with VgrG and a PAAR domain and an outer layer that is made of two structural proteins (Cascales & Cambillau, 2012). The membrane complex is of particular importance, as it anchors the secretion system in the membranes of the bacterial cell and creates a channel for the needle to pass through. The membrane complex is further subdivided into three proteins, TssJ, TssM and TssL. TssM works together in conjunction with TssL to connect the membrane complex to the baseplate (Cherrak et al., 2019). The critical role of TssM to proper functioning of T6SS makes it an ideal candidate for a KO.

Although there is evidence that *Caballeronia* that form insect symbioses encode T6SS motifs, it has not yet been confirmed in the strains within the Gerardo Lab collection (Itoh et al., 2019; Ohbayashi et al., 2019). Consequently, before creating KOs, T6SS genes must first be characterized within the *Caballeronia* strains of interest: GA-OX1, SBA.33.M4.c and SQ4a. Subsequently, the plasmid and strains can be assembled to create T6SS KOs through allelic exchange. Despite not being able to accomplish this second task, we have provided a strong foundation for further biotechnology work in *Caballeronia*, a non-model organism.

Methods

Identifying T6SS Motifs in *Caballeronia* Genomes

Genomes of GA-OX1, SQ4a and SBA.33.M4.c were previously sequenced by the Gerardo Lab. SQ4a and SBA.33.M4.c has fully assembled genomes while the GA-OX1 genome was sequenced, but the contigs have not yet been assembled. However, it is still possible to use the contigs to search for T6SS motifs.

T6SS motifs in *Caballeronia* were identified through a BLAST search of highly conserved structural proteins. Using the annotated genome of *Burkholderia* strain RPE67, an isolate from *R. pedestris*, DNA sequences of highly conserved T6SS proteins ClpV1 and Hcp were determined. These sequences were subsequently used in a BLAST search within each of the three genomes to identify regions of homology. Regions of significant homology, as calculated by BLAST, were confirmed with a tBLASTn search that uses the nucleotide sequence to search for a matching translated protein. Confirmed sites were then explored to map out the entire motif. The genomic region was first annotated with its open reading frames using ORFfinder program from NCBI. Based on the open reading frames, genes that neighbor the highly conserved regions could be identified with tBLASTn searches that identify >99%

homology. The orientation, identity and length of each gene were manually noted and mapped. The boundaries of the motif were determined when the identities of genes no longer had any previously identified association with the T6SS secretion system.

Allelic Exchange Overview

The *Caballeronia* KO will be constructed by knocking out the transmembrane protein TssM through allelic exchange (Figure 4C). Allelic exchange first requires a recombinant strain that contains the homologous regions bordering the target gene, but not the gene itself. The first step of the exchange process is the integration of the plasmid in the host genome, leading to a single recombinant (Lehman et al., 2016). With *Caballeronia*, this process will be done with a triparental mating step that parallels the procedure used to integrate fluorescence expression (Wiles et al., 2018). The single recombinants would subsequently be cultured to allow for the second recombination event that will excise the plasmid. Since the second recombination event can either lead to the restoration of the original genotype or create the KO, the KO will be selected for through a loss of antibiotic resistance (Lehman et al., 2016).

TssM Homologous Region Primers for Gibson Assembly

The *Caballeronia* KO will be constructed by knocking out the transmembrane protein TssM through allelic exchange. The first step is to create the homologous exchange plasmid that contains the homologous regions that border TssM, but does not contain TssM, through Gibson Assembly. The backbone of this plasmid is pAX2 which contains gentamycin resistance, ampicillin resistance and GFP fluorescence which will be critical in the selection process during the matings (Figure 4B). Using the SmaI cut site, two set of upstream and downstream

homologous regions of approximately 1 kb that surround TssM would be inserted through Gibson assembly.

To integrate these homologous regions into pAX2 plasmid, two primers sets that code for the homologous regions and meet the structural overlap that is critical for Gibson assembly must be created. This was primarily done with SnapGene which has a Gibson assembly primer functionality. Constructed primer sets were further analyzed with SECentral which identifies potential off target binding and primer dimers. This ensured that the primers would have the highest probability of replicating properly. For this set of experiments, two primer sets were created (Table 2). However, to ensure that these primers are indeed functional, they must be verified experimentally with PCR.

Table 2. Sequences and Tm of two sets of TssM homologous region primers.

Primer Title	Primer Sequence	Tm (˚C)
TssM 3' HR Forward 1	taggacaaatccgccgctaggagtcgcgagtttaaaccccCCCTCGCCGATGAGCC	78
TssM 3' HR Reverse 1	CGCGAAGCCCGGGTCCTGACCGGCGGCTGTCGGAC	79
TssM 5' HR Forward 1	AACGCGTCCGACAGCCGCCGGTCAGGACCCGGGCTT	81
TssM 5' HR Reverse 1	ggggattttccccgcgcatttctcgagtctggtcgaccccTGGCGATGTACATCGCTGGC	78
TssM 3' HR Forward 2	taggacaaatccgccgctaggagtcgcgagtttaaaccccCAGGATGATGTTGTCCGGCG	77
TssM 3' HR Reverse 2	ACTGGGAGCTGTCGCAGGAATCGGCGATTTCGCGGC	75
TssM 5' HR Forward 2	CCGCGCCGCGAAATCGCCGATTCCTGCGACAGCTCCC	78
TssM 5' HR Reverse 2	ggggattttccccgcgcatttctcgagtctggtcgaccccTGTTGCAGGTGCCGCTG	79

Although verifying primer replication is a fundamentally simple process, the lack of previous biotechnological work with *Caballeronia* combined with *Caballeronia's* relatively high GC content requires an initial exploration to define optimal parameters before being able to move forward with the development of the KO. The optimization process began with a base PCR formulation that includes a Thermofisher PCR Master Mix composed of 0.05U/uL Taq polymerase, 0.4 mM of each dNTPs, 4 mM of $MgCl_2$, and reaction buffer. To test various conditions, not only were the concentration of the materials in the Master Mix varied, but the

PCR cycle conditions, template DNA concentration, addition of Dimethyl sulfoxide (DMSO) was additionally varied. The total volume of the reaction along with the amount of master mix added remained constant between trials. Template DNA was acquired by subject *Caballeronia* cells to freeze-boil cycle using a thermocycler. Completed reaction mixtures were subsequently placed into a thermocycler and underwent a touchdown PCR, except for Reactions 17 and 18. For the touchdown PCR, the annealing temperature began at 65 ˚C and decreased by a degree every cycle until it reached a final temperature of 55 ˚C and completed a dozen more cycles (Green & Sambrook, 2018). An aliquot of the reaction was combined with New England BioLabs 6x gel loading dye in a 1:6 ratio and loaded into a 1% agrose gel stained with Biotium GelRed Nucleic Acid Stain along with a 1 kb ladder and run for approximately an hour at 150V.

Table 3. TssM Homologous Regions Primers PCR Protocol Optimization Permutations

Reaction Number	Reaction Modification	Details
Reaction 1	Base PCR composition	25 uL of 2x Master Mix 5 uL of 5 uM TssM forward primer 5 uL of 5 uM TssM reverse primer 2.5 uL of Template DNA
Reaction 2	DMSO	5 uL DMSO
Reaction 3	Higher Primer Concentration	5 uL of 10 uM TssM forward primer 5 uL of 10 uM TssM reverse primer
Reaction 4	Additional dNTPs	5 uL of 40 uM dNTPs
Reaction 5	Template DNA concentration	2.5 uL of Template DNA (Double Concentration)
Reaction 6	DMSO + Higher Primer Conc.	5 uL of 10 uM TssM forward primer 5 uL of 10 uM TssM reverse primer
Reaction 7	DMSO + Additional dNTPs	5 uL DMSO 5 uL of 40 uM dNTPs
Reaction 8	DMSO + Higher Template DNA Conc	2.5 uL of Template DNA (Double Concentration) 5 uL DMSO
Reaction 9	Higher Primer Conc + Additional dNTPs	5 uL of 10 uM TssM forward primer 5 uL of 10 uM TssM reverse primer 5 uL of 40 uM dNTPs
Reaction 10	Higher Primer Conc + Higher Template DNA Conc	5 uL of 10 uM TssM forward primer 5 uL of 10 uM TssM reverse primer

		2.5 uL of Template DNA (Double Concentration)
Reaction 11	Additional dNTPs + Higher Template Conc	2.5 uL of Template DNA (Double Concentration) 5 uL of 40 uM dNTPs
Reaction 12	DMSO + Higher Primer Conc + Additional dNTPs	5 uL of 10 uM TssM forward primer 5 uL of 10 uM TssM reverse primer 5 uL DMSO 5 uL of 40 uM dNTPs
Reaction 13	DMSO + Higher Primer Conc + Higher Template Conc	5 uL of 10 uM TssM forward primer 5 uL of 10 uM TssM reverse primer 2.5 uL of Template DNA (Double Concentration) 5 uL DMSO
Reaction 14	DMSO + Higher Template Conc+ Additional dNTPs	2.5 uL of Template DNA (Double Concentration) 5 uL DMSO 5 uL of 40 uM dNTPs
Reaction 15	Higher Primer Conc + Higher Template Conc + Additional dNTPs	5 uL of 10 uM TssM forward primer 5 uL of 10 uM TssM reverse primer 2.5 uL of Template DNA (Double Concentration) 5 uL of 40 uM dNTPs
Reaction 16	DMSO + Higher Template Conc+ Additional dNTPs + Higher Primer Conc	5 uL of 10 uM TssM forward primer 5 uL of 10 uM TssM reverse primer 2.5 uL of Template DNA (Double Concentration) 5 uL DMSO 5 uL of 40 uM dNTPs
Reaction 17	Non-touchdown PCR Base PCR Composition	Annealing temperature of 55 ˚C
Reaction 18	Non-touchdown PCR Template DNA concentration	Annealing temperature of 55 ˚C 2.5 uL of Template DNA (Double Concentration)

Results

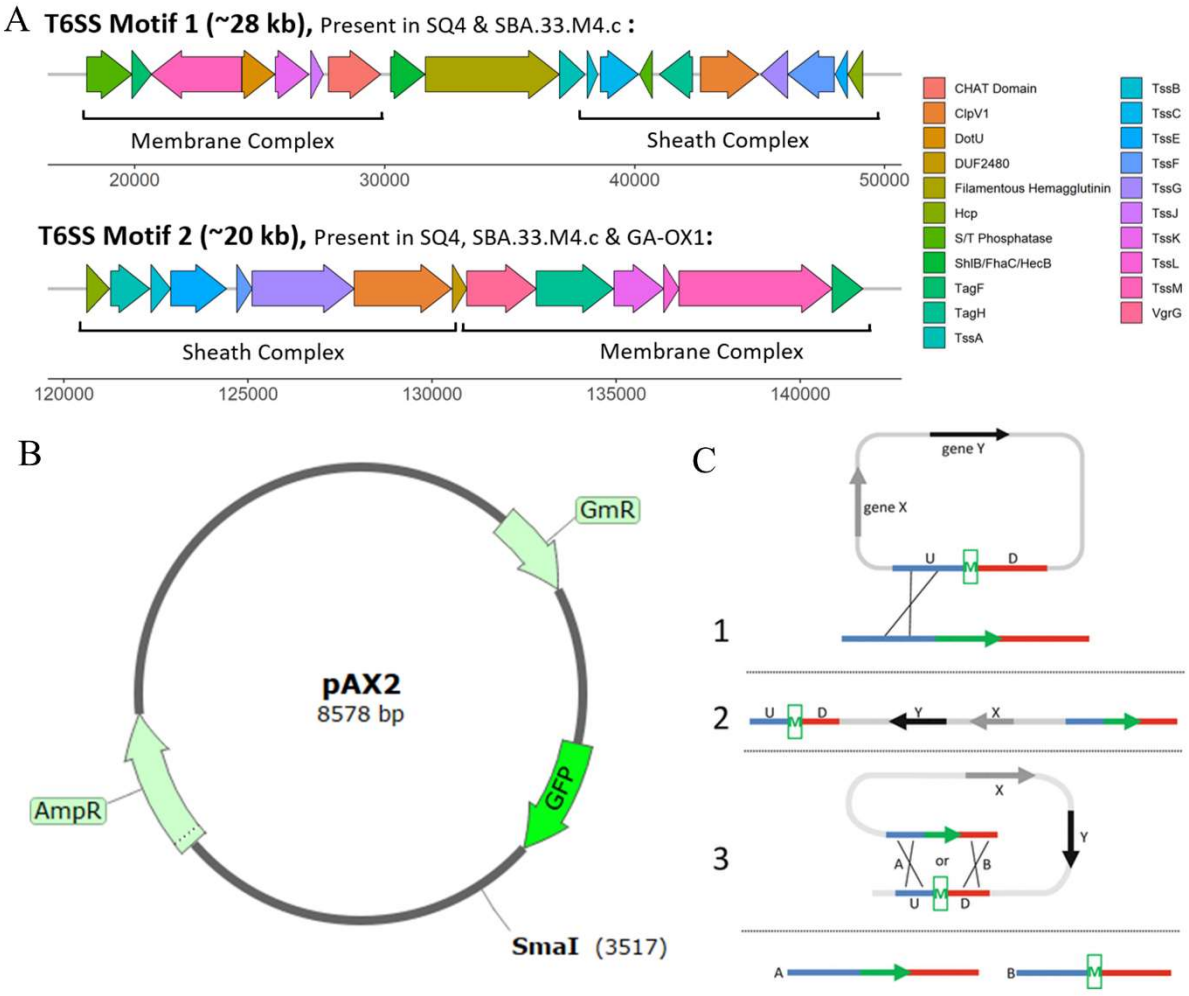


Figure 4. Preparatory steps for T6SS TssM KO. A) Visual representation of the two T6SS motifs found in the genomes of *Caballeronia* strains GA-OX1, SQ4a, and SBA.33.M4.c. B) The pAX2 plasmid use as the backbone for Gibson Assembly. The plasmid contained a GFP, Ampicillin and Gentamycin resistance. C) Visual representation of the allelic exchange process that will be used to create an inactivated T6SS in *Caballeronia* (Lehman et al. 2016)

Two distinct T6SS motifs found in *Caballeronia*. Using bioinformatics, primarily BLAST, two T6SS motif were identified between the three strains of *Caballeronia* analyzed. The first motif was found in both SQ4a and SBA.33.M4.c, while the second motif was found in all three strains.

GA-OX1 was the only strain to have one T6SS motif. Although both motifs contained the thirteen canonical structural genes necessary for T6SS expression, they exhibit multiple differences. Motif 1 was approximately 8 kb larger than motif 2 mostly due to the inclusion of a large filamentous hemagglutinin gene in the middle of the motif. Additionally, motif 2 contained a domain of unknown function (DUF) 2480 that was not found in the first motif. Further, while all of the genes in motif 2 had the same orientation, there was significant variation in the gene orientation in motif 1 with no clear patterns.

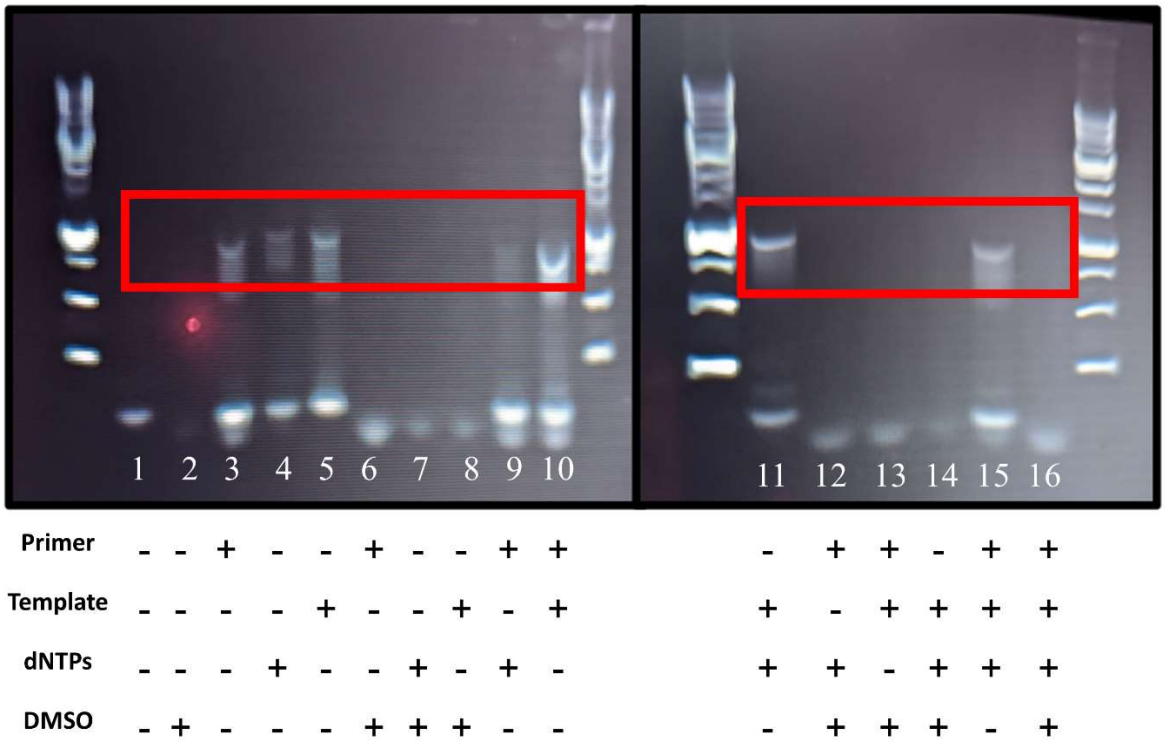


Figure 5. 3' homologous region primers amplify correctly under multiple conditions The numbers underneath each lane represent the specific reaction combination (Table 3). A 1 kb ladder was used for reference. The gel electrophoresis results of all 16 PCR combinations test

3' homologous region first primer set amplifies properly. For the first array of PCR reaction conditions in Figure 5A, nearly all of the trials contained banding at the end of the gel that extend past the ladder. However, reaction combinations 3, 4, 5, 9, 10, 11, 15 all demonstrated different degree of banding at the same approximate level of the 1 kb band in the ladder. Reaction combinations 10 and 11 had the strongest banding at the 1 kb level while reaction combination 4 had diffuse banding (Figure 5A). This result was repeated in Figure 5B, with the exception of the removal of reaction combinations that included DMSO. Interestingly, reaction combination 1 which did not produce banding in Figure 5A, produced the strongest bands in Figure 5B. All the reaction combinations produced banding that were at the same approximate level to the 1 kb ladder band with reaction combinations 5, 9 and 10 have the greatest degree of diffusion

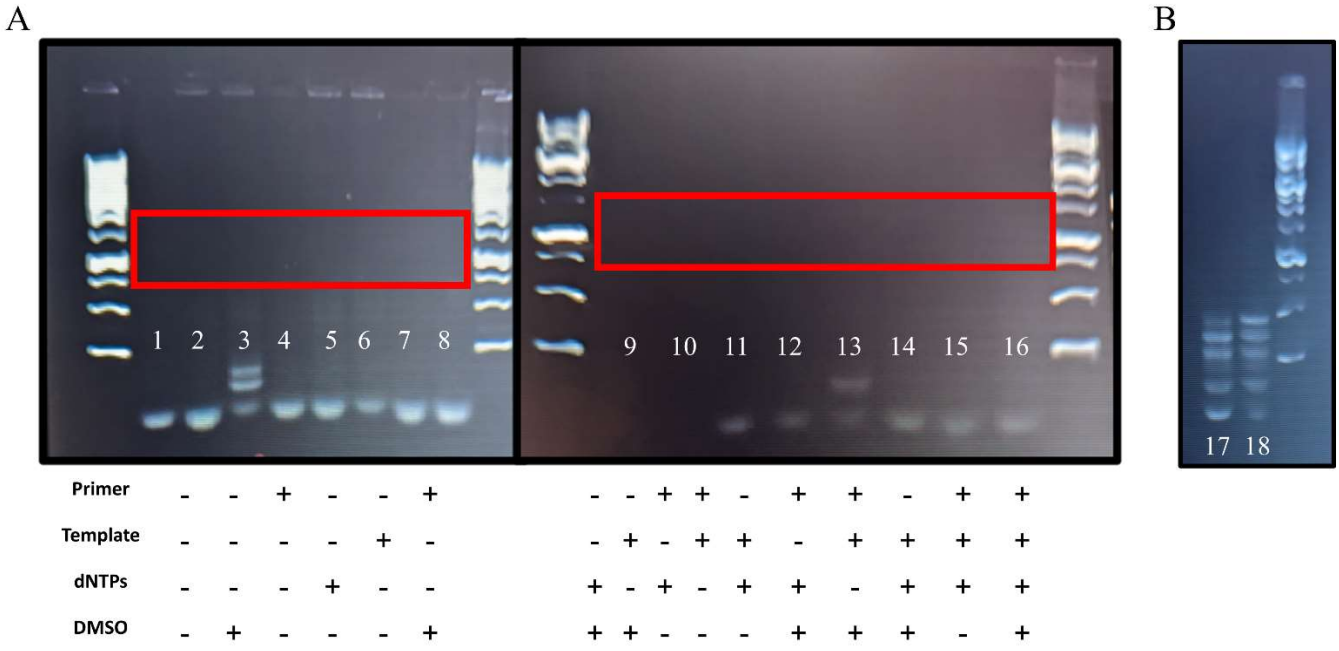


Figure 6. 5' homologous region primers do not amplify correctly under a variety of PCR conditions The numbers underneath each lane represent the specific reaction combination (Table 3). A 1 kb ladder was used for reference. A) The gel electrophoresis results of all 16 PCR combinations were tested B) The gel electrophoresis results of non-touchdown PCR thermocycler protocol.

5' homologous region primers do not demonstrate proper amplification. For the comprehensive 16 different PCR compositions, there was banding at the bottom of the gel in nearly all of the trials. Reaction 3 and 13 demonstrate stratified banding in the lower region rather than a single diffuse band that is present in the other lanes. However, none of the PCR conditions produced bands in the region that corresponds with the 1 kb band in the ladder (Figure 6A). Similar to the 3' homologous region primer, these results were reproduced in Figure 6B with the exception of trials including DMSO as they were excluded. The second attempt focused on the thermocycler conditions by changing from a touchdown to a non-touchdown protocol and beginning the annealing temperature at 65 ˚C. However, the pattern from previous PCR runs held constant as there was banding in lower region, but none in the 1 kb region of the gel. Interestingly, it appeared that there was a greater stratification of the banding with six distinct bands visible in the two trials (Figure 6B).

Figure 7. Second set of 3' and 5' homologous region primers reciprocate pattern of first primer set. The numbers underneath each lane represent the specific reaction combination (Table 3). A 1 kb ladder was used for reference. The first three lane tested the 3' primer set, and the second three lanes tested the 5' homologous regions.

Second set of 3' and 5' homologous region primers replicate pattern of first primer set. Due to lack of successful PCR replication with the 5' homologous region primers, the second set of homologous region primers were initially explored. Only three PCR conditions were tested for each of the primer pairs. There was significant banding at the bottom of the gel for each of the runs with the first run uniquely having two stratified bands. All of the 3' homologous region primer trials demonstrated banding in the 1 kb region. However, reaction 4 had the weakest banding while reactions 1 and 3 had equally strong banding. The 5' homologous region once again did not have any banding in the previously mentioned region.

Discussion

To examine whether contact-dependent mechanisms of inhibition, specifically T6SS, require specific host conditions, an *in vivo* competition assay must be used. However, a major preparatory step to executing this assay is the development of a T6SS knockout strain. The work in this chapter aimed to lay the groundwork for creating a *Caballeronia* T6SS knockout by identifying T6SS motifs and testing components used in the allelic exchange process. Due to *Caballeronia* being a non-model organism with little previous biotechnological work done on it, progress was hindered by the significant effort required to define optimal parameters.

An analysis of the genomes of three *Caballeronia* strains found two predominant T6SS motifs. These motifs both contained the canonical thirteen structural genes that encode the major complexes, indicating that they each likely encode a functional T6SS. A major difference between the two motifs is the relative size as the first motif which is approximately eight kilobases larger than the second motif. This is due to the inclusion of a large filamentous hemagglutinin encoding gene in the middle of the first motif. The function of filamentous hemagglutinin is best characterized in species of *Bordetella* where it acts as a virulence factor that mediates adherence to cells by binding to glycosphingolipids. Its importance in *Bordetella* infection has made it a target of multiple pertussis vaccines (Julio & Cotter, 2005; Weyrich et al., 2012). Although there has been no previous work in *Caballeronia*, filamentous hemagglutinin genes previously identified in *B. pseudomallei* isolates have been hypothesized to play a role in pathogenicity, as the protein demonstrated a positive correlation with positive blood cultures (Sarovich et al., 2014; Tuanyok et al., 2008). Since the protein's main function is to mediate cell adhesion, it possibly may help *Caballeronia* bind to other bacteria to employ contact-dependent mechanisms of inhibition. Contrastingly, multiple subtypes of T6SS have been demonstrated in

P. aeruginosa to be involved in eukaryotic virulence (Lesic et al., 2009). Thus, it may be possible that this T6SS motif serves this purpose and interacts with *A. tristis* intestinal and immunity cells during colonization.

The identification of the T6SS motifs enabled the development of the allelic exchange vector. This process was begun by identifying and inserting approximately one kilobase homologous regions up- and downstream of TssM, the gene of target. From these homologous regions, we developed two sets of Gibson assembly primers in anticipation of difficulty with primer expression in *Caballeronia*. Genomic work from the Gerardo Lab has shown that *Caballeronia* has genomes with a high GC content, which translates to primers with high Tm values, the temperature at which half of the DNA will dissociate to become single stranded. For both set of primers, the Tm had a high and narrow range between 75 to 81 ˚C, which increases the possibility of issues like secondary priming. To minimize mispriming and increase the yield of correct product, a touchdown PCR that sequentially decreases the annealing temperature was used (Green & Sambrook, 2018).

The issue of the Tm's was also addressed by varying the exact recipe of the PCR reactions. Four variables – 5% Dimethyl sulfoxide (DMSO), dNTP concentration, primer concentration, and template DNA concentration – were identified as important contributors to the success of high GC content PCRs and were tested in every permutation. DMSO acts as an enhancer of PCR specificity and yield by decreasing the formation of secondary structures and preventing annealing (Hung et al., 1990). dNTPs were also added in addition to the ones present in the Master Mix due to the possibility that the dNTPs in the mix degraded due to their relatively unstable nature. Increased concentration of primers and template DNA has been demonstrated to increase PCR amplification yield (Czerny, 1996).

For the 3’ homologous region of the first primer set, the 16 reaction combinations produced diffuse banding with peak intensity at the 1 kb region which matches with the hypothetical length of the homologous region (Figure 5A). The strongest banding was in reactions 10 (higher primer concentration + higher template concentration) and 11 (additional dNTPs + higher template concentration), which indicate that higher template concentration may work synergistically to increase the specificity and yield of the banding. There was an absence in banding in every reaction combination that included DMSO which may be attributed to too high of a DMSO concentration that potentially inhibited the function of the Taq polymerase (Hung et al., 1990). Thus, the reaction combinations were repeated with the exclusion of DMSO as the other reaction combinations were already relatively successful (Figure 5B). This once again produced clear and intense banding in the 1 kb region indicating proper amplification of the 3’ homologous region in this primer set without highly specific requirements for the composition of the PCR mix.

This was contrasted by the gel electrophoresis results of the 5’ homologous region. Out of the 16 reaction combinations, there was a complete lack of banding in every single combination. There were bands further down the gel, but those were likely the primers replicating rather than amplification of the homologous region (Figure 6A). These results were recapitulated in the next set of reactions that excluded DMSO combinations (Figure 6B). As a final attempt, the PCR cycle was changed from a touchdown to a constant annealing temperature of 65 ˚C. This once again did not produce bands in the region of interest (Figure 6C). This differing result from the 3’ homologous region led to analysis of a primer pair using SECentral’s primer annotation tool. The software found that the 5’ homologous region primers formed an

extensive primer dimer that may inhibit the formation of the desired product and only produced the side product that is visible at the bottom of the gel (Garafutdinov et al., 2020).

Consequently, our attention moved to the next primer set, which produced larger homologous regions of approximately 1.8 kb, but did not have any significant primer dimers that would likely inhibit proper amplification. A pilot experiment that tested three PCR combinations for both the 5' and 3' regions of the second primer set demonstrated banding in the 3' region that was consistent with the correct product size, while there continued to be no banding in the 5' region (Figure 7). Since there were no significant secondary structure issues with the 5' homologous region, the absence of banding could be due to a non-optimized PCR reaction mixture as only three conditions were tested, an incorrect annealing temperature or issues with the Taq polymerase. Since *Caballeronia* DNA inherently has a high GC content, a high fidelity Taq polymerase should ideally be used instead of the conventional Taq polymerase that was used in this experiment. However, due to time constraints, these experiments will be left as future directions.

These set of experiments set the foundation for creating a functioning strain of *Caballeronia* T6SS KO to be used in *in vivo* assays by characterizing important motifs and beginning to create the components that are necessary for the allelic exchange process. However, the novelty and minimal previous biotechnological work with *Caballeronia* proved to be a challenge as some components like the 5' homologous region were unable to properly amplify under a variety of conditions. The subsequent steps in the process could follow two paths. The first would be to continue testing different parameters for the replication of the 5' homologous region such as high fidelity Taq polymerases, increased extension time or other adjuvants that could increase priming specificity. Another path would be to move our focus from TssM and use

another highly conserved structural gene as the target of the KO as the continual inability to replicate the 5’ region may be indicative of a larger issue with that specific region of the genome. Either way, the end goal would be to integrate the chosen homologous regions into the pAX2 plasmid and develop KO strains that could be used to determine whether host conditions are important to T6SS activation.

Conclusions

The ubiquity of microbial symbioses across all scales of life and their importance to host fitness has made understanding their transmission between generations a critical area of research. This importance is further amplified by the evolutionary puzzle posed by the horizontal transmission of symbioses due to the magnified potential for a disruption in transmission and potential loss of the symbiont. These questions were addressed through the symbiosis between *A. tristis* and species from the genus *Caballeronia* due to its primary benefit and known horizontal transmission. Due to the wealth of previous work in related systems that examined how host factors filter out environmental microbes, the microbial symbiont and whether it is an active or passive participant in ensuring its passage between generations was the primary focus. Through a ranging examination of symbiont interactions, we have provided evidence that *Caballeronia* symbionts not only demonstrate inhibitive relationships with other strains *in vitro*, but that the inhibition is highly context dependent and may require specific host conditions for activation.

Competitive interactions were primarily determined in a contact-independent context through co-culture and conditioned media assays that produced inhibitive relationships between a variety of phylogenetically distinct *Caballeronia* strains. These relationships were strongly influenced by the nutritional environment, as a rich LB medium facilitated mainly inhibitive dynamics, while a minimal medium enabled greater variation that even encompassed cooperative interactions. This competitive ability may allow them to outcompete other microbes outside of the host as they wait to be picked up aposymbiotic *A. tristis* nymphs. Once inside, the symbionts face a spatially constricted gut region that is bottlenecked by a single cell wide passage. Thus, the next logical step was to analyze the contact-dependent competitive capacity of *Caballeronia*. An *in vitro* examination of contact-dependent inhibition between *Caballeronia* strains did not

produce evidence of competition. However, due to the energetic cost of contact-dependent inhibition machinery, it is likely that its expression is not constitutive and is instead regulated by particular conditions such as those within the host's gut. Without the ability to exactly replicate the host gut conditions, we instead aimed to create a contact-independent inhibition knockout that could be employed in *in vivo* assays. Due to the inherent difficulties of working with a non-model organism like *Caballeronia*, progress has been limited by the need to define parameters for conventional processes such as PCR. Despite this, we have been able to characterize secretion system motifs and develop primers that will be used to eventually create the knockouts.

It is clear that there is more work to be done to determine whether *Caballeronia* use contact-dependent inhibition during colonization. The next steps in creating a *Caballeronia* T6SS KO were outlined in the previous chapter. This KO strain could then be utilized in *in vivo* experiments to determine if host conditions are critical for T6SS expression. This work could be confirmed with other methods such as RNA-seq to quantitatively measure if T6SS expression is upregulated during colonization of *A. tristis* gut. For the contact-independent experiments, it would be intriguing to identify the chemical compounds that underlie the observed competitive outcomes using high pressure liquid chromatography or mass spectroscopy. Additionally, with the novel insights about *A. tristis's* excretion transmission mechanism, it would be interesting to explore how competition dynamics change in a semi-structured environment like a gel. This would potentially create spatial structure that could impact or alter the competitive dynamics that were previously observed.

However, the results presented thus far have provided strong evidence that *Caballeronia* strains are able to exhibit inhibition against one another in a variety of contexts. Furthermore, these results are indicative of *Caballeronia* taking on an active role in its horizontal transmission rather

than relying on the host mechanisms to weed out other microbes and enable sole colonization. Thus, these results may not only help to explain the transmission of the *A. tristis*-*Caballeronia* symbiosis, but also help to answer coevolutionary questions about the propagation of horizontally transmitted symbioses as a whole.

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