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TERMITE GUT MICROBES AS TOOLS AND TARGETS FOR TERMITE CONTROL

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in
The Department of Entomology

By
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Abstract

The Formosan subterranean termite (FST), *Coptotermes formosanus*, is an invasive urban pest in the United States. Colonies of the FST are dependent on the symbiotic gut protozoa for cellulose digestion in the workers' guts, and the gut bacterial community is known to provide essential nutrients to the termite. The objectives of this PhD research were to develop and evaluate paratransgenesis and phage therapy for termite control.

During this study, a termite gut bacterium: *Trabulsiella odontotermitis* was genetically engineered and was evaluated as a 'Trojan horse' for paratransgenesis. We proved that *T. odontotermitis* can tolerate 50 times more concentration of ligand-Hecate than the concentration required to kill the gut protozoa. We also engineered *T. odontotermitis* to express Green Fluorescent Protein (GFP) and visualized the expression of GFP in the termite gut. We created a strain of *T. odontotermitis* expressing kanamycin-resistant gene using tn7 transposon. We used this strain to prove that once ingested, *T. odontotermitis* can stay in the termite gut for at least three weeks and it is horizontally transferred amongst nest mates. We also engineered *T. odontotermitis* to express functional ligand-Hecate-GFP fusion protein.

Removal of the bacterial community from the gut also has a negative impact on the survival of the termites. The presence of a diverse and rich bacterial community makes the termite gut a perfect niche for bacteriophages; viruses that infect bacteria. So far, there has been no research to study the presence and role of bacteriophages in the gut of the termite. Bacteriophages have the potential to be used in 'Phage therapy' targeting the essential termite gut bacteria.

During this study three novel bacteriophages were isolated and sequenced from the termite gut. A meta-virome sequencing of the termite gut was also done, which revealed the presence of

previously unknown bacteriophages and other viruses associated with the termites. This is the first study elucidating the presence of a diverse and largely unexplored bacteriophage community in the termite gut. The study suggests that termites can serve as a model system to study the effect of bacteriophages on bacteria and ultimately on the host harboring the microbial community.

Chapter 1: Introduction

Cellulose is considered the most abundant organic polymer on earth (Klemm et al., 2005). In the natural terrestrial environment, termites play an important role in recycling cellulose (Jouquet et al., 2011). There are approximately 3000 known species of termites in the world. In the natural ecosystems, termites are considered as the main macroinvertebrate decomposers and soil engineers (Bignell, 2006, Ulyshen, 2016). Termites build colonies, mounds, and galleries which also impact the local ecosystem. They have direct and indirect effects on microbes in the soil, plants, and animals [reviewed in (Jouquet et al., 2011)]. Termites are considered as the first animals to evolve eusociality (Korb, 2007, Wang et al., 2015). All the termite species universally display co-operative brood care, overlapping generations, and division of labor (Krishna and Weesner, 1969). All the termites harbor various microbial symbionts in their gut. Termites are broadly classified as higher and lower termites. Higher termites (family Termitidae) contain bacterial symbionts in their guts while the lower termites harbor protozoa in addition to the bacteria (families Mastotermitidae, Serritermitidae Kalotermitidae, Termopsidae, Rhinotermitidae, Hodotermitidae). Many termite species live in huge colonies and some subterranean termites can harbor more than a million individual termites at a time (Rust and Su, 2012). Out of all the termite species, only 6 % of the species have been reported as pests (Edwards and Mill, 1986). In the urban environments, some termite species can cause severe structural damage, making them an important urban pest.

Globally, termites are estimated to cause an economic damage of $40 billion annually (Rust and Su, 2012). The Formosan subterranean termite (FST), *Coptotermes formosanus* is the most destructive invasive urban pest in the United States. This species was first described from the island of Formosa (currently Taiwan, east China). (Shiraki, 1909). It is believed that FSTs were

first introduced to Hawaii from China and entered US mainland during the Second World War (Husseneder et al., 2012, Yates III and Tamashiro, 1999). The FST was first identified on the US mainland in 1957 in Charleston, South Carolina (Chambers et al., 1988). As of 2015, FSTs are found in ten states in the US (Su and Scheffrahn, 2016). Formosan subterranean termites are estimated to cause an annual economic damage of around $1 billion in the US (Pimentel et al., 2005) and recent data indicate that the number might be higher.

In Louisiana, FSTs are estimated to cause an economic loss of $500 million annually (Aluko and Husseneder, 2007). Along with the damage caused to the structures made up of dead wood, FSTs also infest live trees (Messenger and Su, 2005). In a recent study it was predicted that FSTs may further increase their range by 15-20% in the coming years (Buczkowski and Bertelsmeier, 2017). This new predicted range expansion is likely to cause major economic and ecological impacts which makes their control more important than ever. The termite control strategies can be broadly classified into chemical control and biological control.

1.1 Chemical methods used for termite control

Liquid termiticides are most widely used for the control of FSTs. It was estimated that 80% of the chemicals used for termite control are liquid-based termiticides (Rust and Su, 2012). These termiticides are applied to the soil around the structures. The main objective behind the application is to create a barrier between the structure and the termites. They act either by killing the termite on contact or by repelling the termites away from the structure (Su and Scheffrahn, 1990, Forschler, 2009). Permethrin, Cypermethrin, and Bifenthrin are pyrethroids and are widely used as repellent termiticides. Pyrethroids act by preventing the closure of the voltage gated sodium channels in the axons. Other widely used liquid termiticides such as neonicotinoids act on nicotinic acetylcholine receptors while fipronil acts by targeting the GABA-gated chloride

channels and glutamate-gated chloride (GluCl) channels. Liquid termiticides are used with the main aim of protecting the structures and may not achieve colony level elimination. All the subterranean termites form underground colonies and only a small number of foraging termites are affected by these liquid termiticides. Even though there has been evidence of horizontal transfer with liquid termiticides, due to interconnected nests and supplementary reproductives these termiticides may not reach far enough in the termite colony. Thus, when area-wide management is desired (not just individual structure protection) an alternative approach is desired.

To overcome this limitation, slow acting non-repellent metabolic or chitin synthesis inhibitors in the bait form are used (Su et al., 1995, Su, 2003). These insecticides are picked up by the foraging worker termites and are then horizontally transferred to other colony members. Metabolic inhibitors, such as hydramethylnon and sulfluramid which act by targeting the mitochondria, have been unsuccessful in achieving colony level elimination most likely due to their quick killing action (Su and Scheffrahn, 1998). Insect growth regulators like chitin synthesis inhibitors are considered to be more successful than metabolic inhibitors (Evans and Iqbal, 2015). Since chitin is not produced by plants, prokaryotes, and vertebrates, chitin synthesis inhibitors are considered comparatively safer in terms of non-target side effects (Merzendorfer, 2013).

Even though chemical termiticides in the liquid and bait forms have shown a mixed degree of success in achieving colony level elimination, they are known to have additional non-target side effects. Neonicotinoids (e.g. imidacloprid Premise ® and fipronil e.g., Termidor®) which are widely used for termite control in the liquid form have been shown to accumulate in soil and have a high potential to contaminate surface and ground waterbodies (Bonmatin et al., 2015, Pisa

et al., 2015). Both neonicotinoids and fipronil have also been shown to affect many non-target invertebrate species including bees (Whitehorn et al., 2012). Pyrethroids have been shown to be toxic to multiple non-target organisms (Thatheyus and Selvam, 2013). Even insect growth regulators like teflubenzuron and hexaflumuron have been shown to affect non-target insects in the environment (Campiche et al., 2006). Due to these unwanted side effects, it was believed that biological control can serve as a safer alternative for termite control.

1.2 Biological methods used for termite control

Biological control methods involve targeting the termites using pathogens. The delivery of pathogens in the termite colony is done either by using baits (Wang and Powell, 2004) or by trapping and treating termites with pathogens and releasing them back to their colony (Rath, 2000). Because termites live closely in large colonies, the biological control relies on the replication and horizontal transfer of the pathogen causing a colony level elimination [reviewed in (Chouvenc et al., 2011)] .

Fungal pathogens are the most studied biological control agents for termites. *Metarhizium anisopliae* and *Beauveria bassiana* have been successfully used in the lab to kill FSTs (Hänel and Watson, 1983, Wang and Powell, 2004, Jones et al., 1996). Bacteria like *Serratia marcescens* and *Bacillus* sp. have also been used successfully in the lab as biological control agents (Khan et al., 1977, Smythe and Coppel, 1965). It has been suggested that viruses can serve as ideal biological control agents for termites (Chouvenc et al., 2011, Chouvenc and Su, 2010), but very few reports on viruses infecting termites exist (Al Fazairy and Hassan, 1988). While biological control has a high likelihood to be successful in the lab, their commercial application for termite control remains unsuccessful so far (Chouvenc et al., 2011).

Termites have developed many behavioral traits to overcome the pathogens. Termites display grooming (Yanagawa and Shimizu, 2007), removal of infected nest mates, secretion of antifungal compounds (Bulmer et al., 2009), pathogen alarm behavior (Rosengaus et al., 1999a), and closing infected areas of colony (Rosengaus et al., 2011a). They have individual humoral and cellular immune systems which involves production of antimicrobial peptides and encapsulation (Chouvenc et al., 2009, Da Silva et al., 2003, Rosengaus et al., 2007, Rosengaus et al., 1999b). Along with the social behavior and immune responses, termites also harbor symbiotic bacteria in their nest wall, which protects them against fungal pathogens (Chouvenc et al., 2013). Overall, due the presence of this multilayered defense system, conventional biological control remains unsuccessful for termites (Chouvenc et al., 2011).

The unwanted side effects of chemical control and the failure of biological control create the need for a novel environment-friendly termite control strategy. Termites are highly dependent on their gut microbes for survival and thus the gut microbes can be used as tools and targets to develop novel termite control strategies.

1.3 Termite gut protozoa as potential targets for termite control

Termites are broadly classified as higher or lower termites based on their gut microbiology. Higher termites harbor bacteria in their guts while the lower termites harbor obligatory symbiotic protozoa along with the bacteria. Workers of the FST harbor three species of obligatory symbiotic protozoa, namely *Pseudotrichonympha grassi*, *Holomastigotoides hartmanni*, and *Spirotrichonympha leidyi* (Koidzumi, 1921). These protozoa help the termite by digesting dietary cellulose, and removal of gut protozoa results in the death of the termite due to starvation (Eutick et al., 1978). The obligatory symbiotic protozoa which are essential for the survival of

the termite are not found anywhere else in nature and thus can be used as targets to develop a highly specific termite control strategy.

Paratransgenesis is a strategy which uses genetically engineered gut bacteria to deliver and express foreign genes in a host organism (Coutinho-Abreu et al., 2010). Paratransgenesis has been developed for many medically important insects to eliminate the disease-causing protozoal parasites such as *Trypanosoma cruzi*, *Plasmodium* sp., and *Trypanosoma brucei* (Durvasula et al., 1997, Wang et al., 2012, Aksoy et al., 2008). On similar grounds, a strategy using engineered gut bacteria to kill the gut protozoa, ultimately killing the termite, can be developed for termite control (Husseneder et al., 2009).

Antimicrobial peptides can kill the protozoa by destroying their cell membranes (Hancock, 2001). Hecate is one of the broad-range antimicrobial peptides which has been synthesized in the lab to mimic insect antimicrobial peptides (Henk et al., 1995). Hecate can kill both bacteria and protozoa, but attachment of a small (7 amino acids) ligand makes it more specific for the protozoa and less toxic to the bacteria (Husseneder et al., 2010b). In a previous study it was shown that ligand-Hecate successfully killed the gut protozoa of the FST at a concentration of 1 µM. In the absence of gut protozoa, all the termites died in two weeks due to starvation (Husseneder et al., 2010b). For the proof of concept that paratransgenesis can be developed for termite control, a commercially available yeast *Kluyveromyces lactis* was engineered to express ligand-Hecate. When fed to the termites in the lab, engineered *K. lactis* killed the termites by eliminating the gut protozoa (Sethi et al., 2014). In a separate study, engineered *K. lactis* expressing melittin, a lytic peptide from bee venom, was successful in killing the termite gut protozoa (Husseneder et al., 2016). Even though lab experiments were successful, there are limitations to the use of *K. lactis* in the field; 1) *K. lactis* is often present in dairy products and

associated with fruits (Trindade et al., 2002, Delavenne et al., 2011, Arroyo-López et al., 2008) in nature and thus has the potential to spread to unwanted niches in the environment; 2) Also, *K. lactis* is not a part of the termites' natural gut flora and does not have suitable adaptations for the gut environment. A termite gut bacterium can be maintained in the gut for a longer time and being from the termite gut it is less likely to survive in the environment, thus providing an environmentally-friendly alternative.

Formosan subterranean termites have a complex and diverse bacterial community in their guts in addition to the protozoa. Previous studies have shown that at least 213 different species of bacteria are present in the gut of the FST worker (Husseneder et al., 2010a). The bacterial community carries out acetogenesis and provides the termites with essential nitrogenous compounds and vitamins (Husseneder, 2010, Brune, 2014). Bacteria from the FST gut have also been implicated in uric acid recycling (Thong-On et al., 2012). A termite gut symbiotic bacterium is well adapted to the termite gut environment and thus is less likely to cause environmental contamination. Also, a symbiotic bacterium from the termite gut will not be recognized as a pathogen by the termite and thus can pass the multilayered defense system of the termite colony like a 'Trojan Horse'. An ideal bacterial 'Trojan Horse' should have the following attributes.

1. It should be a termite gut symbiont, preferably specific to the termite gut and not known from the environment.
2. It should be tolerant to the toxic effects of ligand-Hecate
3. It should be able to express foreign proteins in the termite gut
4. It should be maintained in the termite gut when fed externally
5. It should be horizontally transferred to other nest mates in the colony

6. It should be able to express functional ligand-Hecate

1.4 First research goal

The first goal of this research is to genetically engineer a termite gut bacterium as a 'Trojan Horse' to express functional ligand-Hecate. Specific objectives for this research goal are described below.

Objective 1: To isolate bacteria from the termite gut

To complete this objective, termites were collected from three different colonies in New Orleans, LA. Their guts were dissected and bacteria were isolated using conventional isolation techniques. Isolated bacteria were identified by sequencing their 16s rRNA genes. All the details are described in Chapter 2 (Tikhe et al., 2016b).

Objective 2: To study the tolerance of isolated termite gut bacteria to ligand-Hecate

To complete this objective, five bacteria isolated from the termite gut were selected. Minimum inhibitory concentration of Hecate and ligand-Hecate were determined for five termite gut bacteria. Detailed procedure and results are described in Chapter 2 (Tikhe et al., 2016b).

Objective 3: To genetically engineer a termite gut bacterium *Trabulsiella odontotermitis* to express foreign proteins in the termite gut

For the proof of concept that a termite gut bacterium can be engineered and can express foreign proteins in the termite gut, *T. odondotermitis* was engineered to express green fluorescent protein (GFP). The engineered bacteria were fed to the termites and GFP expression in the termite gut was observed. Details of these experiments are described in Chapter 3 (Tikhe et al., 2016a).

Objective 4: To study the longevity of engineered *T. odontotermitis* in the termite gut

To complete this objective, *T. odondotermitis* was engineered at chromosomal level to express a kanamycin resistance gene using tn7 transposon based engineering. The engineered strain was fed to the termites from three different colonies and its longevity in the gut was monitored. The details of these experiments are described in Chapter 3 (Tikhe et al., 2016a).

Objective 5: To study the horizontal transfer of *T. odontotermitis* between nest mates

To complete this objective, donor termites (termites fed a diet containing genetically engineered *T. odontotermitis*) were mixed with recipients (termites that were never fed engineered *T. odontotermitis*). Horizontal transfer of engineered *T. odontotermitis* to the recipient termites was monitored throughout this experiment. The detailed procedure and results of this experiment are described in Chapter 3 (Tikhe et al., 2016a).

Objective 6: To engineer *T. odontotermitis* to express functional ligand-Hecate

To complete this objective *T. odontotermitis* was engineered with five plasmids containing ligand-Hecate attached to various signal peptides and one plasmid expressing ligand-Hecate-GFP fusion protein. Production and functionality of ligand-Hecate from all the engineered *T. odontotermitis* strains were checked via Western blot and anti-protozoal bioassays against *Tetrahymena* sp. All the details of these experiments are described in Chapter 4 (Tikhe et al., 2016a).

1.5 Termite gut bacteria as potential targets for termite control

As described previously, FSTs harbor a complex and diverse community of bacteria in their guts. Beacuase the gut bacterial community provides the termites with essential nutrients, it can be exploited as a potential target for termite control.

Previous studies have shown that removal of bacterial community from the termite gut affects the metabolism and reproduction of termites (Rosengaus et al., 2011b, Peterson et al., 2015). All of the lab studies carried out to manipulate the gut bacterial community used antibiotics which are not suiTable for field applications. Hence an alternative approach to target the termite gut bacterial community is needed.

Phage therapy, which involves the use of bacteriophages to target a desired bacterial strain, can be used instead of antibiotics. Phage therapy has been suggested as an alternative solution to treat a wide variety of bacterial infections (Miedzybrodzki et al., 2016, Oechslin et al., 2016). Due to increased antibiotic resistance, phage therapy has received a renewed attention in the recent years (Roach and Debarbieux, 2017).

The bacterial community in the in the termite gut makes it a perfect niche for the presence of a diverse bacteriophage population. Bacteriophages have been known to play an important role in the ecosystem by carrying out nutrient recycling via bacterial cell lysis (Wilhelm and Suttle, 1999). They also play a crucial role bacterial genome evolution via horizontal gene transfer (Ochman et al., 2000). Despite the many studies to decipher the taxonomic and functional diversity of bacterial community in the termite gut, the bacteriophages in the termite gut remain unstudied. It has been shown that bacteriophages from the same location as their host bacteria are more successful in infecting the host as compared to other bacteriophages (Vos et al., 2009). This suggests that termite gut is the best place to look for bacteriophages infecting the termite gut bacteria. Study of bacteriophages from the termite gut will improve understanding of their role in the termite gut and might also provide tools to target the gut bacterial community.

1.6 Second research goal

The second goal of this research is to characterize the bacteriophages from the termite gut using conventional isolation techniques and meta-virome sequencing to set the stage for development of phage therapy for termite control.

Objective 1: Isolation and characterization of bacteriophages from the termite gut

To complete this objective, bacteriophages were isolated from the termite gut infecting termite gut bacteria. The isolated bacteriophages were characterized and their genomes were sequenced. A total of three novel bacteriophages were isolated and sequenced from the termite gut. The details of each bacteriophage are described in three separate chapters (5-7).

Objective 2: Metavirome sequencing of the termite gut

Because most of the bacteria from the termite gut are difficult to cultivate in the lab using conventional techniques, their bacteriophages also remain unstudied. To overcome this limitation, viral DNA from the termite gut was directly sequenced circumventing the isolation requirement. All the experimental details and results are described in Chapter 8.

If successful, paratransgenesis and phage therapy can provide an alternative environment friendly approach for termite control. Depending upon its success, these novel methods can be used as standalone tools or in combination with chemical or biological control techniques. The study will also act as a model for developing novel microbe based insect control strategies.

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Chapter 2: Isolation and assessment of gut bacteria from the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), for paratransgenesis research and application [*]

2.1 Introduction

The Formosan subterranean termite (FST) *Coptotermes formosanus* Shiraki is an invasive urban pest in the United States. The FST is found at least in 11 states in the US and is responsible for an annual economic loss of $1 billion (Pimentel et al., 2005) but this number might be higher (Nagro, 2015). Chemical insecticides are widely used to control FST (Rust and Su, 2012). Recent studies have shown that chemical termiticides can have negative effects on the environment. Many non-target invertebrate species have been shown to be affected by fipronil and neonicotinoids, such as imidacloprid (Pisa et al., 2015). Chemical insecticides used for termite control have also been shown to be toxic to various bees and have been implicated in colony collapse disorder (Whitehorn et al., 2012).

Biological control methods involving entomopathogens have been evaluated as a non-chemical alternative for FST control (Chouvenc et al., 2011). Even though conventional biological control is considered to be environment friendly, it remains largely unsuccessful for termite control (Chouvenc et al., 2011) due to the termites' immune defenses and hygienic behavior (Rosengaus et al., 2000, Rosengaus et al., 2004, Hamilton et al., 2011). Apart from this, mutualistic association with actinobacteria has been shown to play a role in the termites' defense against pathogens (Chouvenc et al., 2013). To break through the termites' strong defense mechanisms against pathogen invasion, biological control agents need to be improved to avoid detection by the termites' defenses and facilitate efficient spread of potent control agents throughout a colony.

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It has been suggested that a strategy based on paratransgenesis can be developed to achieve these goals (Chouvenc et al., 2011).

Paratransgenesis uses genetically engineered microbial symbionts as “Trojan Horses” to deliver and express foreign genes in a host organism (Coutinho-Abreu et al., 2010). The concept of paratransgenesis was first developed to control Chagas disease by targeting *Trypanosoma cruzi* in triatomine vectors (Durvasula et al., 1997). Since then, the possible application of paratransgenesis has been suggested for many vectors like mosquitoes, sand flies, and tsetse flies (Aksoy et al., 2008, Hurwitz et al., 2011, Medlock et al., 2013, Wang et al., 2012). The main goal in these classical paratransgenesis approaches is not to kill the insect, but to disrupt disease transmission by killing the pathogen.

The first paratransgenesis system aiming at actually killing an insect pest is being developed for the control of the FST (Sethi et al., 2014, Husseneder et al., 2010b). Workers of the FST have a complex symbiotic microbial community in their guts which is comprised of protozoa, bacteria, and archaea (Noda et al., 2005, Inoue et al., 2008). The three species of gut protozoa, namely *Pseudotrichonympha grassi*, *Holomastigotoides hartmanni*, and *Spirotrichonympha leidyi* aid in cellulose digestion (Koidzumi, 1921). A termite worker’s ability to digest cellulose is hampered by the loss of gut protozoa, which ultimately results in the death of termites (Eutick et al., 1978).

Hecate is a synthetic antimicrobial peptide capable of killing both bacteria and protozoa (Henk et al., 1995). Attachment of a protozoa-specific hepta-peptide ligand increased its specificity towards protozoa minimizing non-target effects (Husseneder et al., 2010b). In a previous study, commercially available yeast (*Kluyveromyces lactis*) genetically engineered to express a targeted antiprotozoal fusion peptide (ligand-Hecate) has been shown to be successful in killing termites by eliminating their gut protozoa (Sethi et al., 2014). Even though the *K. lactis* based ‘Trojan

Horse' system was successful, a termite-specific bacterium would be a more environmentally safe alternative. A termite-specific bacterium's adaptations to life in the gut make it less likely to survive in the environment than, for example, a ubiquitous yeast.

The FST gut is an ideal source to acquire a termite specific bacterial 'Trojan Horse' because of the high diversity and density of bacteria residing there (Husseneder et al., 2010a, Shinzato et al., 2005). The bacteria in the termite gut have been shown to play a key role in metabolic processes such as: uric acid recycling, acetogenesis, and nitrogen fixation. Therefore, some of these bacteria are likely obligate to termite survival (Doolittle et al., 2008, Thong-On et al., 2012, Breznak et al., 1973, Schink et al., 1997). Many bacteria identified in the termite gut are not known to exist in the environment or in other insects and are likely specific to termites.

An ideal bacterial 'Trojan Horse' must satisfy the following criteria: The bacteria should be (1) termite-specific, (2) able to tolerate higher concentrations of ligand-Hecate than required to kill the gut protozoa, (3) genetically modifiable, and (4) readily ingested by the termite and able to survive in the termite gut. In this study, we assessed termite gut bacteria for their potential to be the "Trojan Horses" for paratransgenesis.

2.2 Materials and methods

2.2.1 Isolation of bacteria from the FST gut

One hundred worker termites were collected from three different FST colonies in New Orleans, Louisiana, USA in fall 2009 using untreated in-ground bait stations. Termite colonies were designated as colony 1, colony 2, and colony 3. Termites were brought back to the laboratory in plastic containers containing moist filter paper and were processed immediately. Fifty workers from each colony were surface-sterilized by dipping them in 70% ethanol twice and then in

sterile water. Termites were dried on clean KimWipes®. Termite guts were carefully extirpated using sterile forceps as described previously (Sethi et al., 2011) and were homogenized in a 1.5 ml microcentrifuge tube containing 500 µl sterile Brain Heart Infusion (BHI) broth. The gut homogenate was intermittently vortexed to separate the bacteria from the gut wall. Ten-fold serial dilutions of homogenized gut contents were prepared and plated in triplicate on two selective media, McConkey agar, (M7408 Sigma Aldrich, selective for gram negative), and MRS agar (69964 Fluka, selective for lactic acid producing bacteria), and plates were incubated at 30° C for 48 h. Bacterial isolates in each media were categorized into different morphological types (morphotypes) based on the size, shape, and color of the bacterial colonies. Morphologically distinct bacterial colonies were selected and further purified. Individual bacterial isolates were grown overnight in BHI broth at 30°C and were stored as glycerol (20%v/v) stocks at -80°C until further analysis.

2.2.2 Sequencing of the 16S rRNA gene

A total of 135 isolates were grown overnight in 500 µl of sterile BHI. 250 µl of culture was used to extract DNA using the DNeasy® 96 Blood & Tissue Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The 16S rRNA gene was amplified with universal bacterial primers 27F and 1492R (Lane 1991) using LongAmp™ Taq 2X Master Mix (New England BioLabs, Ipswich, MA). The annealing temperature was calculated using the NEB Tm calculator (http://tmcalculator.neb.com/#!/). PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and were run on 1% agarose gel to assess the quality and size. DNA concentration of the purified PCR products was measured using NanoDrop® ND1000. PCR products were bi-directionally sequenced at Beckman Coulter Genomics facility, MA using Sanger dideoxy DNA Sequencing technique. Nearly full length sequences of ~1500 bp were

obtained. Forward and reverse sequences were assembled into contigs and were manually checked for errors using ChromasPro (v. 1.5). Individual sequences were also checked for the presence of chimeras using Mallard 1.02. Good quality sequences were compared with those present in the GenBank/NCBI, rdp, and EzTaxon (Cultured) database. The 16S rRNA gene sequences of all the bacteria identified in this study were deposited in NCBI Genbank database. Accession numbers are presented in Table 2.1.

2.2.3 Minimum inhibitory concentration of Hecate and Ligand-Hecate

Both Hecate (FALALKALKKALKKLKKALKKAL) and ligand-Hecate (ALNLTLHFALALKALKKALKKLKKALKKAL) were synthesized using solid state peptide synthesis at the LSU AgCenter Biotechnology Laboratory's Protein Facility. The minimum inhibitory concentration (MIC) of Hecate and ligand-Hecate was determined for the following termite gut bacteria isolated in this study, *Enterobacter cloacae* CMC61A1, *Trabulsiella odontotermitis* AS-7737, *Citrobacter* sp. E710D3, *Lactococcus lactis* MC45F4 and *Pilibacter termitis* PE49A2, as previously described (Hancock, 1999). *Enterobacter cloacae*, *Citrobacter* sp. and *L. lactis* have been previously reported in various termite species (Harazono et al., 2003, Adams and Boopathy, 2005, Bauer et al.,2000, Schultz and Breznak, 1978), but are not unique to termites. *P. termitis* and *T. odontotermitis* were reported exclusively from the termite gut (Chou et al., 2007, Higashiguchi et al., 2006). Serial dilutions of Hecate and ligand-Hecate were prepared in 0.01% acetic acid, 0.2% BSA in polystyrene tubes. Bacteria were grown overnight in Müller-Hinton broth (MHB) and were approximately diluted to 10^6 Colony Forming Units (CFU)/ml. The diluted cultures were grown overnight in MHB at 30°C with serially diluted Hecate and Ligand-Hecate (final volume: 5 ml). The final concentration of the peptides ranged from 100 µM to 0.19 µM. For *P. termitis* PE49A2, MIC was carried out in an anaerobic gas jar

using AnaeroGen (Oxoid, Hampshire, UK). After 24 hrs of incubation, the lowest concentration at which no visible growth was observed was considered as the MIC. All the MIC experiments were carried out in triplicates.

2.2.4 Transformation of *Trabulsiella odontotermitis*

Trabulsiella odontotermitis AS-7737 were grown to 0.6 O.D and 1 ml of the culture was centrifuged at 10,000 g at 4°C. The cell pellet was washed two times with 1 ml ice cold sterile distilled water followed by two washes with 1 ml ice cold 10% glycerol solution. The cells were suspended in 50 µl of 10% glycerol and were immediately used for electroporation. For electroporation, 50 ng of plasmid PTrcHis 2-ELGFP6.1–TOPO containing the GFP gene and an Ampicillin resistance marker for selective growth (Kato et al., 2002) was mixed with the cells and cells were transformed via electroporation in a 2 mm gap electroporation cuvette (Eppendorf electroporator 2510 at 2.5 kV). After electroporation, cells were grown in 1 ml SOC medium for 1h at 37°C and were spread on LB agar with 100 µg/ml Ampicillin and IPTG plates in different dilutions. Plates were incubated at 37°C for 24 h and Ampicillin resistant colonies were selected for further analysis. Plates with colonies of transformed *T. odontotermitis* were observed under a UV light trans-illuminator to check for the presence of fluorescent colonies. Cells from individual bacterial colonies were observed under a fluorescent microscope (Leica DM RXA2 fluorescent microscope, 100x oil, N.A= 1.3).

2.2.5 Termite feeding bioassay

Transformed *T. odontotermitis* expressing GFP (*T. odontotermitis* –GFP) cells were grown overnight in LB-Ampicillin broth and 1 ml of cells were centrifuged and washed 3 times with 5 ml of sterile distilled water. The cells were suspended in 500 µl of sterile water and were added

to cellulose discs prepared as previously described (Sethi et al., 2014). 50 workers and 10 soldiers collected from the three different termite colonies were allowed to feed on cellulose discs containing *T. odontotermitis* –GFP at 25±2°C and 85 % R.H. For each termite colony, five replicates were used. For control, termites were fed on cellulose discs containing no bacteria and cellulose discs containing non engineered *T. odontotermitis*. After 48 hours of feeding on the diet containing *T. odontotermitis* –GFP, termites were moved to a new petri dish containing a sterile cellulose disc moistened with sterile tap water. After 48 hours, guts of ten randomly selected termite workers from each petri dish were extirpated and homogenized in 500 µl sterile LB broth. A part of the homogenate was observed under a fluorescent microscope (Leica DM RXA2 fluorescent microscope) to test whether the bacteria express GFP in the gut. Serial dilutions of the remaining gut homogenate were spread on LB agar containing 100 µg/ml Ampicillin and IPTG. After 24 hours, plates were observed under an UV trans-illuminator (UVP, Upland, CA) and fluorescent colonies were counted. The number of bacteria from three different colonies was analyzed using analysis of variance using SAS 9.3 (SAS Institute, Cary, NC). *T. odontotermitis* –GFP isolated from the termite gut were grown overnight in LB broth and were stored as glycerol stocks at -80°C.

2.3 Results and discussion

2.3.1 Bacteria identified from the termite gut

Termites harbor a diverse bacterial population in their gut. Studies using culture independent techniques have shown the presence of at least 213 different bacterial species in the gut of FST (Husseneder et al., 2010a). For the paratransgenesis-based termite control method, a bacteria specific to the termite gut would be an ideal choice to be engineered as a ‘Trojan Horse’ to deliver detrimental gene products into a termite colony. Bacteria that are only known to occur in

the termite gut (and are thus not likely to survive in the environment) have special roles among the gut flora, which facilitates their retainment in the termite gut while reducing the risk of environmental contamination.

However, culturing bacteria from specialized environments, such as the termite gut, presents a unique challenge in the search of a 'Trojan Horse'. For paratransgenesis, a pure culture of bacteria is desired for further genetic manipulations. Most of the bacteria in the termite gut are uncultivable by conventional isolation techniques (Hongoh, 2010, Hongoh, 2011). Nevertheless, some termite-specific bacteria have been isolated from various species of termites (Chou et al., 2007, Higashiguchi et al., 2006, Pramono et al., 2015). Previous studies have shown that gram negative enteric bacteria and gram positive lactic acid bacteria are dominant among the cultivable bacteria from the termite gut (Adams and Boopathy, 2005, Bauer et al., 2000). In this study, we used MacConkey agar and MRS agar to isolate gram negative enteric bacteria and lactic acid bacteria from the termite gut, respectively.

After 48 hours of incubation, all McConkey agar and MRS agar plates showed the presence of various morphologically distinct bacterial colonies. From termite colony 1, colony 2, and colony 3, a total of 42, 55, and 38 morphologically distinct isolates were respectively processed for identification. 16S rRNA gene sequencing resulted in identification of nine different species from colony 1, twelve species from colony 2 and eight species from colony 3. Bacteria from the genera *Enterobacter*, *Klebsiella*, and *Pilibacter* were present in all three termite colonies. *Trabulsiella odontotermitis*, a termite-specific bacterium, was found in two of the three termite colonies. The detailed list of the bacteria identified from each termite colony along with their accession numbers in NCBI's GenBank is given in Table 2.1.

Table 2.1: List of bacteria isolated and identified from three different Formosan subterranean termite colonies along with the NCBI GenBank accession numbers of their 16S rRNA gene sequences.

Isolate	Closest match in NCBI database	Percent similarity	NCBI accession number of the match	NCBI accession number of the isolate	Termite colony
AMC81C9	*Klebsiella* sp. LB-2	99	DQ831003.1	KM878731	colony 1
AS-7737	*Trabulsiella odontotermitis* Eant 3-9	99	DQ453130.1	KJ563812.1	colony 1
CM42H5	Uncultured Firmicutes bacterium clone Cf4-97	99	GQ502570.1	KM878734	colony 1
CMC61A1	*Enterobacter cloacae* subsp. dissolvens strain 189 (P21Ms)	99	KF254602.1	KM878728	colony 1
CP41F12	*Enterobacter* sp. M.D.NA5-6	99	JF690888.1	KM878730	colony 1
CP51G3	*Serratia* sp. DCM0915	99	KC007128.1	KM878733	colony 1
EMC41C12	*Enterobacter hormaechei* strain IARI-NIAW2-34	99	KF054945.1	KM878729	colony 1
FP41H10	*Pilibacter termitis*	98	NR_042949	KM878732	colony 1
FP31H1	*Enterobacter aerogenes* EA1509E	99	FO203355.1	KM878727	colony 1
E710CC8	*Enterobacter* sp. A2	99	JX021670.1	KM878718	colony 2
E710D3	*Citrobacter koseri* ATCC BAA-895	97	CP000822.1	KM878715	colony 2
F510A12	*Trabulsiella odontotermitis* Eant 3-9	99	DQ453130.1	KM878725	colony 2
MC45F4	*Lactococcus lactis* strain KLDS4.0309	99	GU208281.1	KM878722	colony 2
MCC64A2	*Enterobacter* sp. WS05	98	JN210900.1	KM878719	colony 2
MCE64A9	*Enterobacter cloacae* subsp. cloacae ATCC 13047	99	JF775626.1	KM878717	colony 2
MCE84A10	*Enterobacter aerogenes* EA1509E	99	FO203355.1	KM878716	colony 2
MCF84A8	*Klebsiella oxytoca* strain ATCC 43863	99	KC155255.1	KM878721	colony 2
PA34H6	Uncultured Firmicutes bacterium clone Cf4-97	97	GQ502570.1	KM878726	colony 2
PC54C8	*Enterococcus faecalis* strain S4-15	99	KC478511.1	KM878720	colony 2
PF34D11	*Pilibacter termitis*	98	NR_042949	KM878724	colony 2

(Table 2.1 continued)

Isolate	Closest match in NCBI database	Percent similarity	NCBI accession number of the match	NCBI accession number of the isolate	Termite colony
PF44E9	*Lactococcus* sp. NBRC 106034	99	AB682336.1	KM878723	colony 2
C58B10	*Lactococcus garvieae* ATCC 49156	99	NR_102968.1	KM886376	colony 3
E47H2	*Enterobacter cloacae* strain T137	99	KC764978.1	KM886372	colony 3
E4S8H11	*Enterobacter asburiae* isolate PSB6	99	HQ242719.1	KM886371	colony 3
E67G11	*Enterobacter hormaechei* strain ASU-001	99	KC342256.1	KM886373	colony 3
MCC77A12	Enterobacteriaceae bacterium strain FGI 57	98	CP003938.1	KM886374	colony 3
McF67C3	*Enterobacter aerogenes* EA1509E	99	FO203355.1	KM886370	colony 3
McF77D1	*Klebsiella* sp. SR-143	98	KC455430.1	KM886375	colony 3
PE49A2	*Pilibacter termitis*	98	NR_042949.1	KM886377	colony 3

All the cultured bacteria identified during this study were closely related to the bacteria previously identified in the comprehensive culture independent 16S rRNA gene sequencing studies (Shinzato et al., 2005, Husseneder et al., 2010a). The identification of bacteria from three termite colonies revealed inter-colonial differences. This result is consistent with more in-depth studies showing differences in the composition of gut microbiota among termite colonies (Minkley et al., 2006, Husseneder et al., 2010a, Hongoh et al., 2005). Bacteria from the genera *Enterobacter*, *Klebsiella* from gram negative enteric bacteria and *Pilibacter* from the lactic acid bacteria were present in all three termite colonies and thus may be part of the core genera.

2.3.2 Susceptibility to Hecate and ligand-Hecate

Out of all the bacteria isolated, we selected *E. cloacae* CMC61A1, *T. odontotermitis* AS-7737, *Citrobacter* sp. E710D3, *L. lactis* MC45F4, and *P. termitis* PE49A2 to be tested for their tolerance against ligand–Hecate. The five bacteria strains were selected because they were previously described to play a role in the termite gut.

The ultimate goal in the future is to use an obligate and termite-specific bacterium as a 'Trojan Horse' to kill the cellulose-digesting protozoa in the FST gut via expressing antimicrobial peptides, such as Hecate, targeted to those protozoa. Previous studies have shown that 1 µM of ligand-Hecate, when injected into the gut of FST workers, was sufficient to kill the three species of gut protozoa, which ultimately resulted in the death of the termites (Husseneder et al., 2010b). Since the bacterial Trojan Horse's survival is critical for expressing sufficient antiprotozoal peptides within the FST gut and delivery of the peptides throughout the termite colony, it should be able to tolerate more than 1 µM of ligand-Hecate. The MIC of ligand-Hecate was higher than 1 µM in all the bacteria tested. The ligand was designed to bind to protozoa but does not bind to bacteria, which explains the decreased toxicity of ligand-Hecate toward bacteria compared to protozoa found in this and in previous studies (Husseneder et al., 2010b).

The minimum inhibitory concentration (MIC) for Hecate and ligand-Hecate was determined for five bacteria isolated from the termite gut: *Enterobacter cloacae* CMC61A1, *Trabulsiella odontotermitis* AS-7737, *Citrobacter* sp E710D3, *Lactococcus lactis* MC45F4, and *Pilibacter termitis* PE49A2. Out of the five bacteria tested *Citrobacter* sp E710D3 had the lowest MIC for both Hecate and ligand-Hecate, thus *Citrobacter* sp. E710D3 was the most susceptible bacteria strain. The largest difference between the MIC's of Hecate and ligand-Hecate was observed in *Lactococcus lactis* MC45F4. *Lactococcus lactis* MC45F4 was able to tolerate even 100 µM of

ligand-Hecate. For all the bacteria tested, the MIC for ligand-Hecate was always at least two times higher than that of Hecate. There was no difference in the MIC's among the replicates. The comparative MIC results of Hecate and ligand-Hecate for all the bacteria are shown in Figure 2.1.

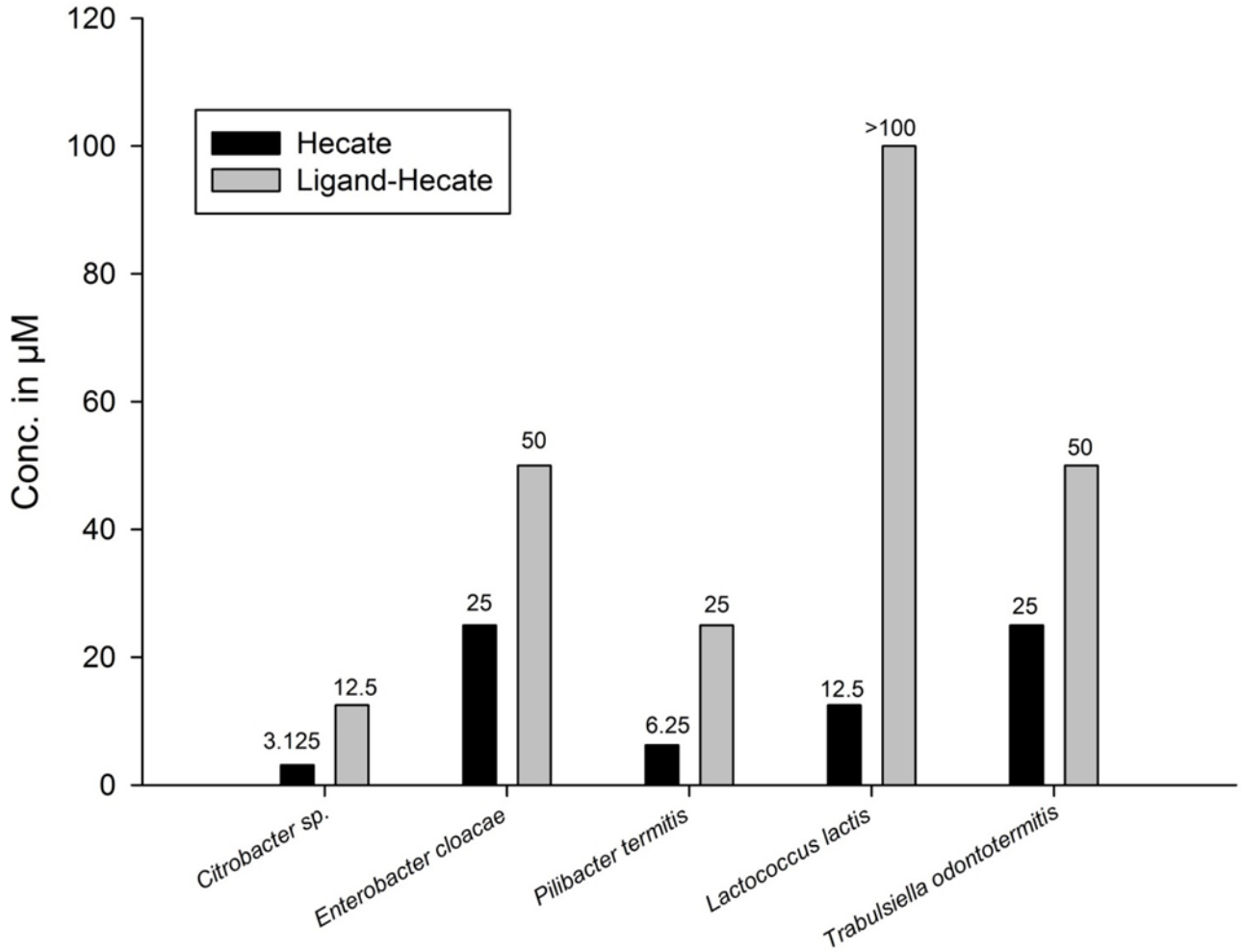

Figure 2.1: Minimum inhibitory concentration (µM) of Hecate and ligand-Hecate against the termite gut bacteria. There was no difference in the MIC's among the replicates.

Because these MIC results suggested that all the bacteria tested are fairly tolerant to our prospective antiprotozoal peptide, the advantages and disadvantages of each bacteria species concerning its usefulness as Trojan Horses are further weighed in the paragraphs below.

Although *E. cloacae* has been previously used as a 'Trojan Horse' for the proof of concept of paratransgenesis-based termite control (Husseneder and Grace, 2005) and bacteria of the genus *Enterobacter* have been found in many termite species (Adams and Boopathy, 2005, Husseneder et al., 2009), we did not consider *E. cloacae* as an ideal Trojan Horse because of its ubiquitous presence and potential to cause infections in humans and animals (Pages and Davin, 2015).

Citrobacter sp. has been shown to have important functions that probably make its presence beneficial to the termite gut and, thus, increase the likelihood of survival as potential 'Trojan Horse'. For example, *Citrobacter* sp. carries out nitrogen fixation in *Coptotermes lacteus*, *Mastotermes darwiniensis*, and *Nasutitermes exitiosus* (French et al., 1976). Aromatics-degrading *Citrobacter* sp. has been isolated from the gut of *Coptotermes formosanus*. Nevertheless, it was not considered for further use because this species showed the highest susceptibility to ligand-Hecate of all the bacteria tested.

Lactic acid bacteria represent the most abundant group of bacteria isolated from the termite gut and *L. lacti*s is found in the gut of many termite species (Tholen et al., 1997, Bauer et al., 2000). *Lactococcus lactis* was the most resistant bacteria to ligand-Hecate. However, *L. lactis* is not specific to termites and thus, its use might risk non-target effects (Yun et al., 2014).

The genus *Pilibacter* is represented by the sole member *Pilibacter termitis*. The type strain is fully described (Higashiguchi et al., 2006). *Pilibacter* is termite-specific and has been found frequently in the termite gut across different colonies and geographic regions (Shinzato et al., 2005, Husseneder et al., 2010a). Frequent and ubiquitous presence of these bacteria in the FST

gut suggests a close association with this termite, which would facilitate its application as 'Trojan Horse' across the distribution range of FST. Even though *P. termitis* has the advantage of being termite-specific, it grows very slowly on artificial media, forming a colony of approximately 1 mm diameter in about 72 hours and requiring anaerobic conditions, which makes genetic manipulation difficult.

We ultimately chose *T. odontotermitis* as a candidate to be developed as a 'Trojan Horse'. *Trabulsiella odontotermitis* is termite-specific and was originally isolated and described from a subterranean termite from the family Termitidae (*Odontotermes formosanus*) (Chou et al., 2007). It was also among one of the uricolytic bacteria isolated from the termite gut (Thong-On et al., 2012). The whole genome of *T. odontotermitis* has recently been sequenced, which has pointed out many adaptations specific for termite gut environment (Personal communication, Dr. Panagiotis Sapountzis). In this study, we found *T. odontotermitis* in two of the three termite colonies; its perceived absence in the third colony is most likely caused by the low number of bacteria sequenced. The MIC for ligand-Hecate against *T. odontotermitis* was 50 times higher than the concentration required to kill the gut protozoa. The bacterium belongs to the Enterobacteriaceae family, which makes the use of standard genetic transformation protocols feasible, as evidenced by our successful transformation with a Green Fluorescent Protein (GFP) expressing plasmid.

2.3.3 Termite feeding bioassay

After two days of feeding on cellulose diet containing *T. odontotermitis* –GFP cells, Ampicillin resistant fluorescent bacterial colonies were isolated from the gut homogenate of workers from all colonies. The average number of CFU/gut varied in the range of 3.912 to 4.327 x 10^3. Termites fed on cellulose diet without addition of any bacteria and non -engineered *T.*

odontotermitis did not show any presence of Ampicillin-resistant fluorescent colonies. There was no significant difference in the number of transformed *T. odontotermitis* isolated from the termites of the three different colonies [F (2, 12) =1.38, P=0.30]. Although culture proved the presence of *T. odontotermitis* –GFP in the gut, we were not able to observe single fluorescent bacterial cells in the homogenate under the fluorescent microscope. Detailed results of termite bioassay are shown in Figure 2.2.

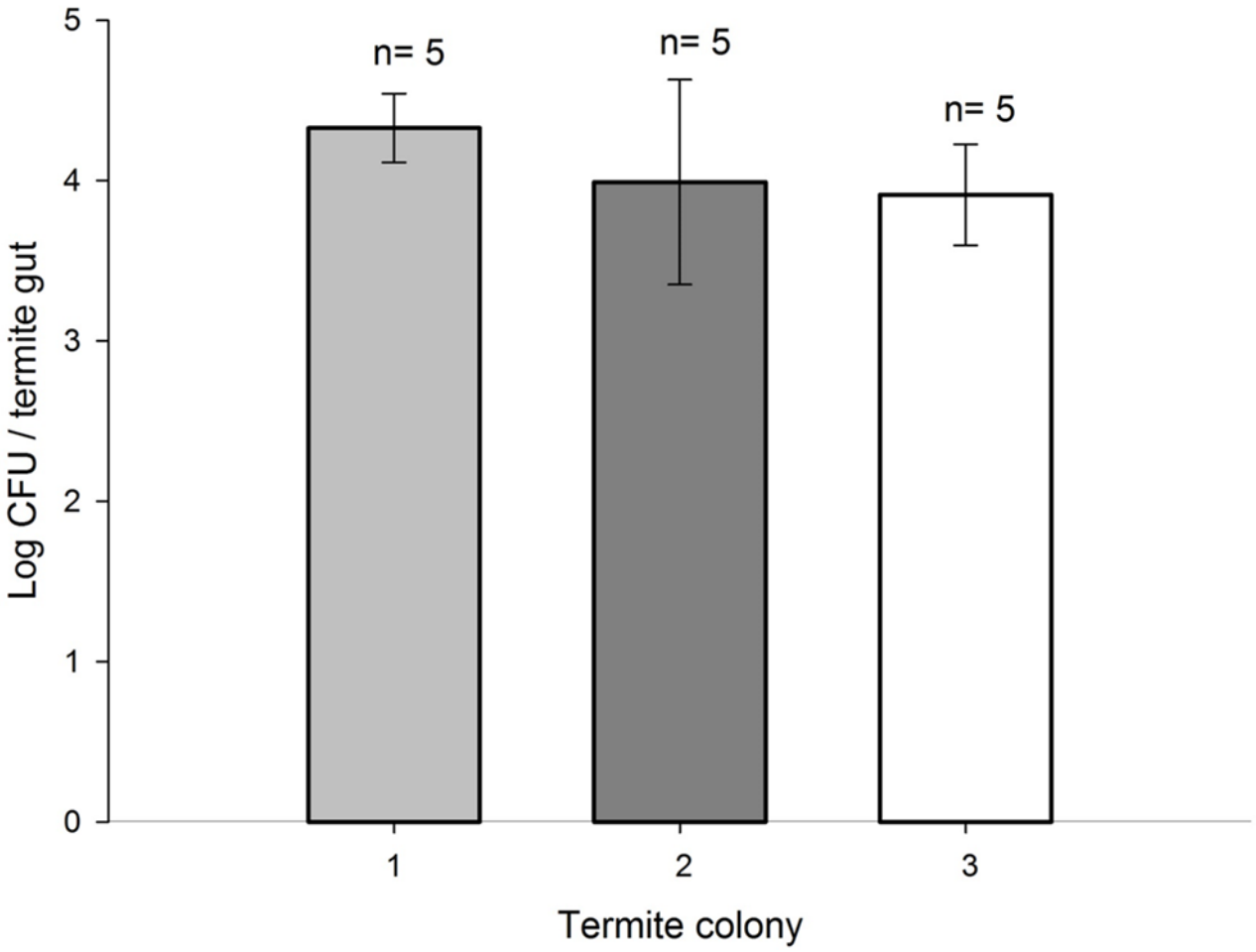

Figure 2.2**:** Number of transformed *Trabulsiella odontotermitis* colony forming units (CFU) recovered from the termite gut after 48 hrs of feeding on *T. odontotermitis* –GFP from three different termite colonies. No significant differences in the number of CFUs were found among the three termite colonies.

Due to the dense and diverse bacterial community in the termite gut, an externally ingested 'Trojan Horse' will have to make its own niche in the termite gut. Results suggest that there is no difference in the numbers of ingested *T. odontotermitis* among colonies, at least in the initial days post-feeding. Being a natural symbiont should facilitate survival in the termite gut (Husseneder and Grace, 2005). *Trabulsiella odontotermitis* –GFP was readily ingested and survived in the gut for at least 48 hrs at population sizes in between 3.912×10^3 and 4.327×10^3. Although this experiment did not track survival beyond the initial days, a prior study (Husseneder and Grace 2005) showed long-term survival (> 6 wks) of a genetically engineered bacterial symbiont of the FST gut (*E. cloacae)* sufficient to spread foreign genes throughout lab colonies.

To study the long term survival of *T. odontotermitis* in the termite gut, further experiments are needed. We were not able to observe single cells of *T. odontotermitis*-GFP when the gut homogenate was observed under a fluorescent microscope. In this study, GFP was expressed from a plasmid under the control of an inducible (trc) promoter, so the bacteria may not have expressed GFP in the gut and could only be detected *in vitro* when being cultured on agar containing the inducer. For future studies, we intend to express GFP using a constitutive promoter. Also, there is no selective antibiotic pressure in the termite gut, so it is likely that the bacteria will lose the plasmid. Thus, in the future, *T. odontotermitis* will be engineered at the chromosomal level for the study of long-term survival and transfer of the 'Trojan Horse' among colony mates and, ultimately, for the expression of protozoacidal ligand-Hecate for termite control.

Overall in this study we have shown that *T. odontotermitis* can tolerate a higher concentration of ligand-Hecate than is required to kill the gut protozoa and it can be transformed to express foreign genes. Both attributes are necessary requirements for *T. odontotermitis* to be used as expression system for ligand-Hecate for future termite control. Also, engineered *T. odontotermitis* is readily ingested by the termite when added to the diet and can thus be incorporated into a delivery system via bait. In summary, *T. odontotermitis* satisfies all the criteria of an ideal bacterial 'Trojan Horse' and thus can serve as a 'Trojan Horse' for a paratransgenesis-based termite control method.

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Chapter 3: Assessment of genetically engineered *Trabulsiella odontotermitis* as a 'Trojan Horse' for paratransgenesis in termites [*]

3.1 Introduction

Termites are eusocial insects displaying division of labor, overlapping generations, and cooperative brood care (Stuart, 1969). Termites depend on cellulose as their food source and play an important role in the natural ecosystem by carbon recycling (Bignell and Eggleton, 2000, Traniello and Leuthold, 2000). However, in the urban environment certain termite species are considered serious pests (Rust and Su, 2012). The Formosan subterranean termite (FST), *Coptotermes formosanus,* is an invasive urban pest from China and is estimated to cause an economic loss of $1 billion annually in the US (Lax and Osbrink, 2003). This termite species forms large underground colonies with tunnels and galleries; and, in a mature colony, the number of individual termites can exceed a million (King and Spink, 1969, Su and Scheffrahn, 1998). Chemical insecticides are widely used for termite control but are known to affect other non-target organisms (Pisa et al., 2015). Conventional biological control remains unsuccessful for termite control due the termites' hygienic behavior, such as grooming, removal of diseased individuals, and incorporation of antimicrobial substances into nest material, in addition to immune responses (Chouvenc et al., 2011). Paratransgenesis, a technique involving genetically engineered symbionts as 'Trojan Horses', can bypass a termite's various defense systems and is suggested as an alternative, chemical-free method for termite control (Chouvenc et al., 2011). In termite colonies, workers forage, digest the food, and feed the rest of the colony via stomodeal and proctodeal food exchange known as trophallaxis (Stuart, 1969). This social behavior aids the

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spread of the ‘Trojan Horse’ in the colony and makes termites a good model for paratransgenesis.

Workers of the FST harbor a complex and diversified microbial community of bacteria, protozoa, and archaea in their guts (Noda et al., 2005, Husseneder et al., 2010a). FSTs have an obligate symbiotic relationship with three species of gut protozoa, namely *Pseudotrichonympha grassi*, *Holomastigotoides hartmanni*, and *Spirotrichonympha leidyi* (Koidzumi, 1921). These gut protozoa assist the termite workers with the digestion of cellulose and are essential for the survival of the termite colony (Eutick et al., 1978). A targeted anti-protozoal peptide consisting of a ligand with affinity to protozoa fused to the lytic peptide Hecate has been shown to kill the gut protozoa (Husseneder et al., 2010b). In a previous study, genetically engineered yeast (*Kluyveromyces lactis)* expressing this ligand-Hecate fusion peptide was successfully used to kill termites by eliminating their gut protozoa (Sethi et al., 2014). Although the yeast, which is not a natural gut symbiont, provided proof for the ‘Trojan Horse’ concept, a termite-specific bacterium would be uniquely adapted to the gut environment and thus be more likely to survive for prolonged periods in the gut and less likely to cause environmental contamination. A carefully designed paratransgenesis approach utilizing genetically engineered termite-specific bacteria expressing an effector molecule that impacts the vitality of a termite colony directly (by killing termites) or indirectly (by killing obligate symbionts) could be developed as an alternative to conventional termite control or as a synergistic method in integrated pest management.

In a previous study, genetically engineered *Enterobacter cloacae* expressing an insecticidal toxin from *Photorhabdus luminescens* was shown to kill termites in lab experiments (Zhao et al., 2008). *Enterobacter cloacae* is frequently found in the termite gut and genetically engineered strains have been shown to be introduced effectively into termite colonies and survive long

enough to express foreign gene product and be transferred among nest mates (Husseneder and Grace, 2005). However, *Enterobacter cloacae* is not termite-specific and can be pathogenic in nature (Davin-Regli and Pages, 2015).

Trabulsiella odontotermitis is a termite-specific bacterium which was first isolated and described from the gut of the fungus-growing termite *Odontotermes formosanus* from southern Taiwan (Chou et al., 2007). A recent study showed that *T. odontotermitis* is frequently present in various species of fungus growing termites (Sapountzis et al., 2015). Genome sequencing of *T. odontotermitis* has shown many adaptations, such as the ability to switch between aerobic and anaerobic metabolism, increased capacity for bacterial competition, and possible aflatoxin degradation ability, suggesting that it is an important facultative symbiont of termites (Sapountzis et al., 2015). In a comparative study between bacterial flora of introduced and native FST populations using 16S rRNA gene sequencing, strains related to *T. odontotermitis* were found in FSTs from China (Husseneder et al., 2010a). In addition, *T. odontotermitis* was isolated from the gut of the FST from Japan as one of the uricolytic bacteria (Thong-On et al., 2012). In our previous study, we isolated *T. odontotermitis* from the gut of the FST from Louisiana, USA, and found that *T. odontotermitis* is 50 times more tolerant to ligand-Hecate than the concentration required to kill the gut protozoa (Tikhe et al., 2016). With the ultimate goal in mind to engineer *T. odontotermitis* in the future to express ligand-Hecate for termite control, we tested to determine whether genetically engineered *T. odontotermitis* was able to survive and express foreign proteins in the termite gut and be transferred among nest mates via trophallaxis (transfer of digestive fluids).

3.2 Materials and methods

3.2.1 Plasmid construction

DNA encoding ELGFP6.1, a variant of GFP (Kato et al., 2002), was amplified from plasmid pTrcHis2-ELGFP6.1 –TOPO using primers GFP6.1_KpnI_Fw 5'TTATGGTACCGATCATGAGTAAAGGAGAACTTTTC3' containing a *Kpn*I restriction site and a start codon and GFP6.1_XhoI_Rv 5'TTGACTCGAGATCATTTGTATAGTTCATCC3' with *Xho*I restriction site and a stop codon (restriction sites underlined). The product was digested with *Kpn*I and *Xho*I restriction enzymes and was ligated in frame with the Shine-Dalgarno sequence into plasmid pSF RecA Delta LexA constitutive (Product name- pSF-OXB20, Product Code: OG50, Oxford Genetics, UK) also digested with *Kpn*I and *Xho*I. The new plasmid was designated as pCT-ELGFP 6.1. Correct orientation of the insert was confirmed by PCR and sequencing using primers OGP-F2 5'TGTCGATCCTACCATCCA 3'and OGP-R2 5'AGTCAGTCAGTGCAGGAG 3'. Plasmid pCT-ELGFP 6.1 was maintained in *E.coli* DH5 alpha cells.

3.2.2 Confirmation of the *attTn7*site in the *Trabulsiella odontotermitis* chromosome

Trabulsiella odontotermitis AS-7737 was isolated from the FST gut in a previous study (Tikhe et al., 2016). To confirm presence of the *attTn7* site in the *T. odontotermitis* chromosome, *glmS* and *pstS* genes of *E. coli* MG1655, *Citrobacter koseri* ATCC BAA-895, *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2, *Klebsiella pneumoniae* subsp. *pneumoniae* HS1128, and *Enterobacter cloacae* EcWSU1 were aligned using ClustalX2 (Larkin et al., 2007). Two degenerate primers GLMS_CT_Fw and PSTS_CT_Rv were designed from the conserved regions of *glmS* and *pstS* genes, respectively (Figure 3.1). The primers were presumed to amplify

the C-terminus coding region of *glmS* gene, the inter-genic region between *glmS* and *pstS*, and the N-terminus coding region of the *pstS* gene. Genomic DNA of *T. odontotermitis* was extracted using the DNeasy Blood & Tissue Kit (Qiagen 69504) and was subjected to PCR using primers GLMS_CT_Fw and PSTS_CT_Rv.

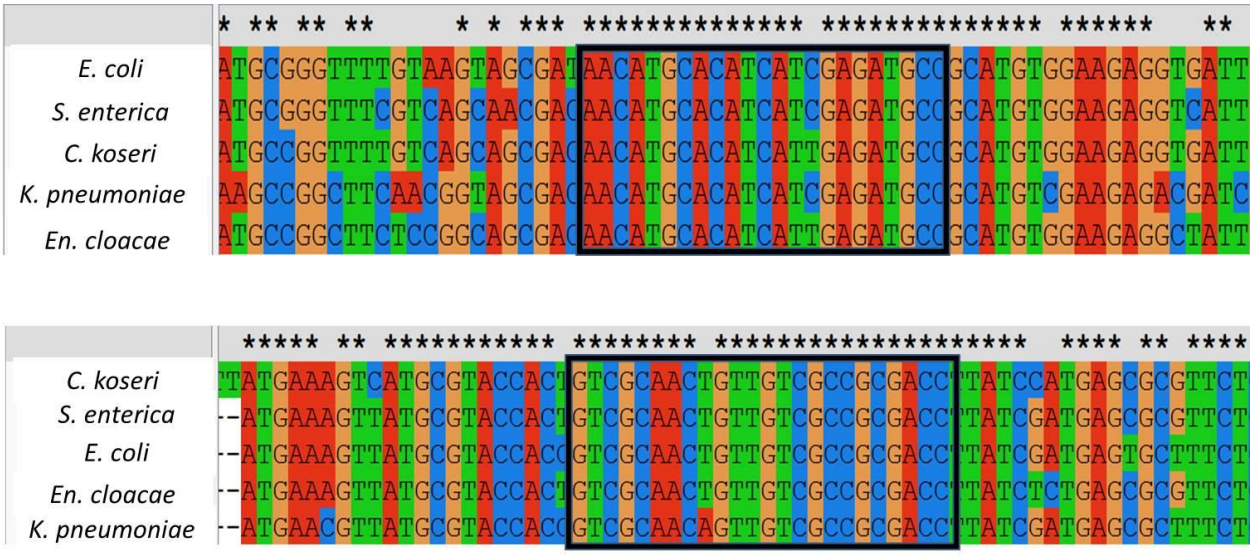

Figure 3.1: Multiple alignment of glmS (top) and pstS (bottom) genes of *E. coli* MG1655, *Citrobacter koseri* ATCC BAA-895, *Salmonella enterica* subsp. enterica serovar Typhimurium LT2, *Klebsiella pneumoniae* subsp. pneumoniae HS1128 and *Enterobacter cloacae* EcWSU1. Frames show the region used for designing primers GLMS_CT_Fw and PSTS_CT_Rv respectively.

The amplified product was cloned in pCR®2.1-TOPO® (Invitrogen K4660-01) according to manufacturer's instructions and was subsequently sequenced at Macrogen, MD, USA. The sequence obtained was used to confirm the presence of *attTn7* site by comparing it with the consensus *attTn7* site as described previously (Mitra et al., 2010). At the time of the experiment the whole genome sequence of *T. odontotermitis* was not yet published. However, we were able to confirm the sequence obtained from this experiment by comparing it to *T. odontotermitis glmS*

and *pstsS* genes obtained from the *T. odontotermitis* whole genome project made available to us by James Estevez, University of Puget Sound (Personal communication).

3.2.3 Preparation of electrocompetent cells and transformation of *Trabulsiella odontotermitis*

Trabulsiella odontotermitis culture was grown to O.D of 0.6 and 1 ml of the culture was centrifuged at 10,000 g at 4°C. The cell pellet was washed two times with 1 ml ice cold sterile distilled water followed by two washes with 1 ml ice cold 10% glycerol solution. The cells were then suspended in 50 µl of 10% glycerol and mixed with 50 ng of pCT-ELGFP 6.1 for electroporation in a 2 mm gap electroporation cuvette (Eppendorf electroporator 2510 at 2.5 kV). For transposition, cells were co-transformed with 100 ng each of PUC18R6KT-mini-Tn7T-Km and pTNS-3 (provided by Dr. Herbert Schweizer, Colorado State University) using the same electroporation conditions. PUC18R6KT-mini-Tn7T-Km is a plasmid with a Tn7 transposon containing a *KanR* cassette flanked by a FRT site within Tn7L and Tn7R sequences (Choi et al., 2005). pTNS-3 is a helper plasmid expressing *tnsABCD* (Choi et al., 2008). After electroporation, cells were grown in 1 ml SOC medium for 1-2 h and were spread on LB+ Kanamycin 50 µg/ml (LB+ Kan 50) plates in different 10 fold dilutions. Plates were incubated at 37°C for 24 h and Kanamycin resistant colonies were selected for further analysis. Plates were observed under a UV trans-illuminator (UVP) and *T. odontotermitis* transformed with pCT-ELGFP 6.1 was detected by the presence of fluorescent colonies. Cells from individual colonies were also observed under a fluorescent microscope (Leica DM RXA2 fluorescent microscope, 100x oil, N.A= 1.3, excitation 480 nm and emission 508 nm). For cells transformed with pUC18R6KT-mini-Tn7T-Km and pTNS-3, 100 Kanamycin-resistant colonies were re-streaked on LB+ Kan 50 plates and were subsequently stored as glycerol stocks at -80°C until further analysis. To check the utility of pCT-ELGFP 6.1 to express GFP in other wild type bacteria,

Klebsiella sp. AMC81C9, *Enterobacter cloacae* CMC61A1, *Enterobacter aerogenes* MCE84A10 and *Citrobacter koseri* E710D3 (all isolated previously from the termite gut) were also transformed (Tikhe et al., 2016).

3.2.4 Confirmation of insertion of *KanR* cassette at *attTn7*site

Genomic DNA was isolated from five Kanamycin-resistant isolates transformed with pUC18R6KT-mini-Tn7T-Km and pTNS-3 from the previous step using DNeasy Blood & Tissue Kit (Qiagen 69504). The DNA from these isolates and the DNA from wild type *T. odontotermitis* were used for PCR using GLMS_CT_Fw and PSTS_CT_Rv. PCR products were run on 1% agarose gel. Approximately 700 bp of the PCR product were sequenced from each end using GLMS_CT_Fw and PSTS_CT_Rv primers at Macrogen, MD, USA.

3.2.5 Termite collection

Workers and soldiers of the Formosan subterranean termite (FST) *Coptotermes formosanus* were collected from three different colonies in New Orleans, Louisiana using untreated inground bait stations. Colonies were designated as Colony A (collected from Canal Street, on 10/29/2013), Colony B (collected from Joe Brown Park 10/28/2013) and Colony C (collected from Little Woods, on 10/28/2013). Termites were brought back to the lab in plastic containers containing moist filter paper.

3.2.6 Feeding experiment

Feeding experiments were carried out with two different strains of *T. odontotermitis, T. odontotermitis*-pGFP and *T. odontotermitis*-Km^r:: *Tn7*. Strains *T. odontotermitis*-pGFP and *T. odontotermitis*-Km^r:: *Tn7* were grown to OD 0.6 in LB+ Kan 50 broth. Cells in 1 ml volume were pelleted down and washed three times with equal volume of sterile water. The cells were

suspended in 500 µl of sterile water and were added to cellulose discs prepared as previously described (Sethi et al., 2014).

For the feeding experiment with *T. odontotermitis*-pGFP, groups of 200 worker termites and 20 soldier termites were collected from each of three colonies (A, B, and C) and were fed on cellulose discs containing *T. odontotermitis*-pGFP for two days in a petri dish. All the experiments including the controls consisted of three replicates from each colony. After two days, guts of five randomly collected workers were dissected, pooled, and homogenized in sterile saline solution (0.85 % W/V NaCl). The homogenate was serially diluted and was spread on LB+ Kan 50 plates. The plates were incubated at 37°C for 24 h and fluorescent colonies were observed and counted under UV light (FirstLight® UV Illuminator, UVP). The numbers of bacteria per termite gut were estimated by dividing the bacterial colony count by five. After confirmation of bacterial intake in all the replicates, on day 3, termites were moved to a new petri dish containing a sterile cellulose disc moistened with sterile tap water. Every other day, 5 worker termites from each plate were dissected for bacterial isolation as described above. The experiment was carried out until no more fluorescent colonies were observed on LB+ Kan 50 plates (after 18 days). For the first four days after the termites were moved to a new petri dish, three worker guts from each plate were dissected and observed under the fluorescent microscope (Leica DM RXA2 fluorescent microscope).

For the feeding experiment with *T. odontotermitis*-Km^r :: *Tn7*, 400 termite workers and 40 termite soldiers from each colony were fed on cellulose discs containing *T. odontotermitis*-Km^r :: *Tn7*. After two days, five worker termites were randomly selected and were used for isolation of Kanamycin-resistant bacteria as described above. On the third day, 200 termite workers and 20 termite soldiers were moved to a new petri dish containing a sterile cellulose disc as soon as

presence of Kanamycin-resistant bacteria was confirmed in all the samples. Every two or three days, 5 worker termites from each petri dish were used to isolate and enumerate Kanamycin-resistant bacteria.

3.2.7 Bacterial horizontal transfer

For the bacterial transfer experiment, 200 termite workers and 20 termite soldiers from each colony were fed for two days on a cellulose disc containing 1% Sudan red G (91282 Fluka), which stains the fat body of the termites red (Lai et al., 1983). These termites were designated as recipient termites (no prior exposure to *T. odontotermitis*-Km^r :: *Tn7*). Termites fed on *T. odontotermitis*-Km^r :: *Tn7* were designated as donor termites. On the third day post-feeding, the uptake of *T. odontotermitis*-Km^r :: *Tn7* was confirmed in donors and they were mixed with the recipient termites in the ratio of 1:1 and 1:25 (Figure 3.2).

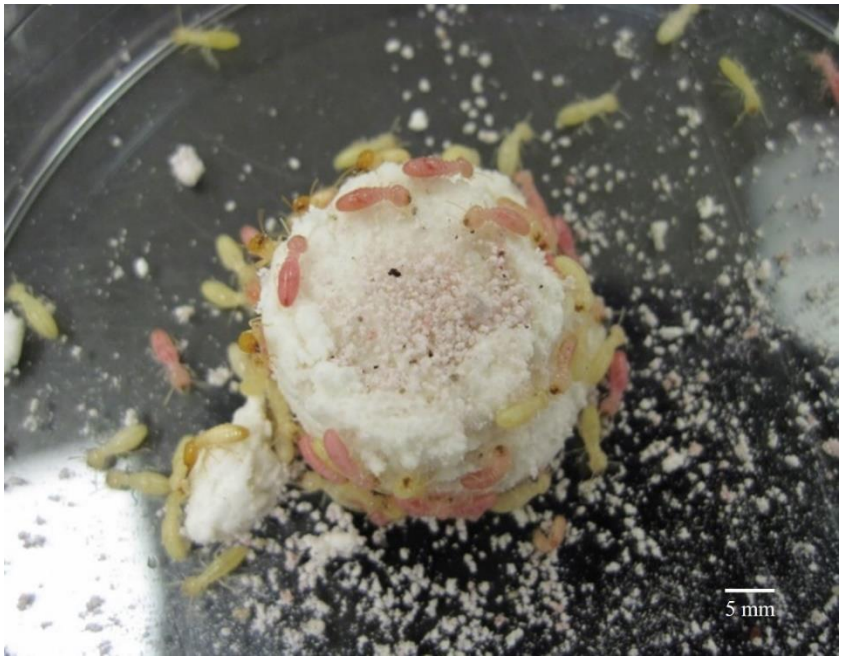

Figure 3.2: Termites feeding on a cellulose disc in bacterial transfer experiment; the white termites are the donor termites previously fed on cellulose diet with *T. odontotermitis*-Kmr :: Tn7, the pink termites are the recipient termites fed on cellulose diet with Sudan red, Donor: Recipient 1:1.

After every two days, five recipient worker termites were randomly selected and were dissected for isolation of Kanamycin-resistant bacteria as described above. The experiment was carried out for two weeks until recipient termites were indistinguishable from the donors due to the fading of the fat body stain. Two types of negative controls were used in the experiment; the first control contained 200 termite workers and 20 termite soldiers that were fed on cellulose containing non-engineered wild type *T. odontotermitis,* and the second control consisted of 200 worker termites and 20 soldier termites that were fed on moistened sterile cellulose discs. The controls were treated in the same way as described for the experiments involving *T. odontotermitis*-Km^r :: *Tn7* and *T. odontotermitis*-pGFP.

A total of 96 randomly selected isolates from the feeding and transfer experiments were subjected to PCR and 700 bp of the PCR product were sequenced from each end with primers GLMS_CT_Fw and GLMS_CT_Fw to confirm the isolates were in fact *T. odontotermitis*-Km^r :: *Tn7*. No Kanamycin-resistant bacteria could be isolated from any of the controls during the course of the experiment. PCR and sequencing of all the 96 isolates collected during the experiment confirmed that all tested the isolates were *T. odontotermitis*-Km^r :: *Tn7* .

3.2.8 Consumption and mortality analysis

All of the cellulose discs were weighed before the start of the feeding experiment for each of the four treatments (control, with no added bacteria, *T. odontotermitis* wild type, *T. odontotermitis*-pGFP, *T. odontotermitis*-Km^r :: *Tn7*, or with Sudan red). At the end of the feeding experiment, cellulose discs were dried and weighed again to measure the consumption. Termite mortality in each replicate was calculated by counting the live termite workers at the end of the experiment.

3.2.9 Statistical analysis

All statistical analysis was done using SAS 9.3 (SAS Institute, Cary, NC). PROC UNIVARIATE was used to check the data for normality. PROC MIXED with SLICE function was used to analyze the data from the feeding experiment from all days and all the replicates. PROC MIXED was used to analyze the data for consumption. PROC LOGISTIC adjusted with Tukey's test was used to calculate probabilities of termite mortality for various treatments.

3.4 Results and discussion

3.4.1 Transformation with a constitutively expressed plasmid leads to strong but transient GFP expression in the termite gut

In a previous study we transformed *Trabulsiella odontotermitis* with a lactose/ IPTG inducible GFP plasmid (Tikhe et al., 2016). We were able to retrieve engineered *T. odontotermitis* via culture from the termite gut, thereby confirming that the strain was ingested by the termites. However, we were not able to visually detect GFP expression in the termite gut (Tikhe et al., 2016). Failure to induce the promoter due to insufficient lactose concentration was the most likely cause for the lack of expression. Our previous experiments also showed that with a low copy number plasmid, it is difficult to observe GFP expression against the termite gut's auto-fluorescence (unpublished data). To overcome these issues, we constructed a new high copy number plasmid (pCT-ELGFP 6.1) in this study, which has a variant of GFP under the control of a strong constitutively expressed promoter RecA ΔLexA and *KanR* gene.

Transformation of *T. odontotermitis* with pCT-ELGFP6.1 conferred Kanamycin resistance. Transformed colonies showed fluorescent phenotype when observed under UV light. Even single cells from transformed colonies showed bright fluorescence when observed under a fluorescent

microscope (Figure 3.3), confirming the strong constitutive expression of GFP provided by this multicopy plasmid.

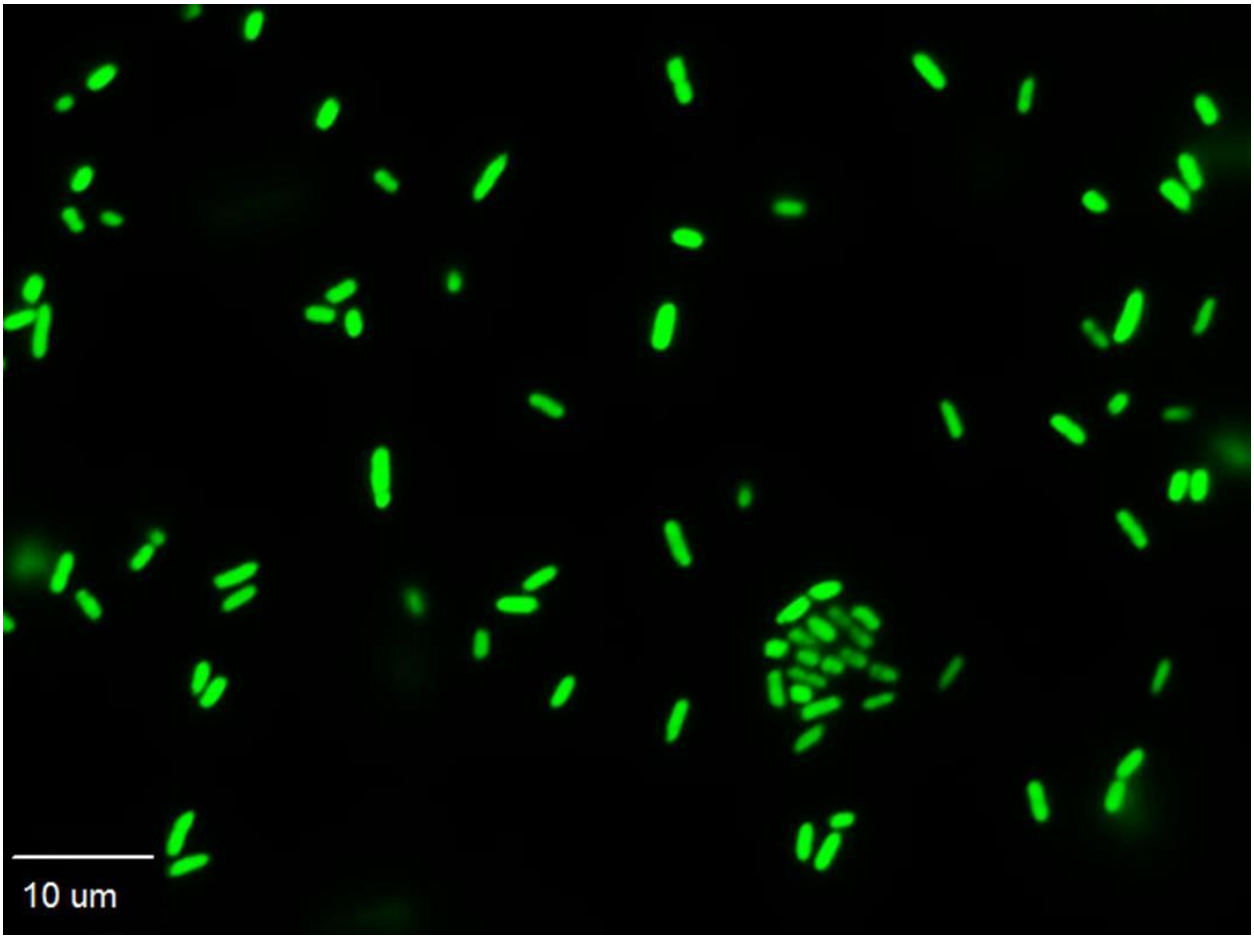

Figure 3.3: *T. odontotermit*s transformed with pCT-ELGFP 6.1, observed under Leica DM RXA2 fluorescent microscope, 100x oil, N.A = 1.3, excitation 480 nm and emission 508 nm

Trabulsiella odontotermitis harboring pCT-ELGFP 6.1 was designated as *T. odontotermitis*-pGFP. Three other bacteria species isolated from the termite gut (*Klebsiella* sp. AMC81C9, *Enterobacter cloacae* CMC61A1, *Enterobacter aerogenes* MCE84A10) also showed strong constitutive expression of GFP after being transformed with pCT-ELGFP6.1, which suggests that the plasmid can be used to tag a variety of wild-type bacteria. The results suggest that a

construct with RecA ΔLexA promoter can be utilized in our future goal of engineering *T. odontotermitis* to express ligand-Hecate.

After the termites were fed for two days on *T. odontotermitis*-pGFP, fluorescent Kanamycin-resistant colonies were isolated successfully from the gut homogenate of workers from all three termite colonies. The rapid uptake of *T. odontotermitis*-pGFP is consistent with the previous studies showing immediate presence of engineered bacteria and yeast in the termite gut, sometimes within hours after being added to the termite diet (Husseneder et al., 2005, Husseneder and Grace, 2005, Sethi et al., 2014).

Expression of GFP by *T. odontotermitis*-pGFP in the gut was observed directly via fluorescent microscopy. The *T. odontotermitis*-pGFP was concentrated in the hindgut region. In most instances, *T. odontotermitis*-pGFP appeared to have formed a biofilm around the hindgut paunch region, which contains the gut protozoa (Figure 3.4 A- Figure 3.4 F). Colonization of *T. odontotermitis* of the largely anaerobic hindgut region of termite workers suggests a preference for a niche with low oxygen levels in the gut (Brune et al., 1995). Similar results were observed in case of fungus-growing termites, where *T. odontotermitis* was predominately found in the hindgut paunch region (Sapountzis et al., 2015). A recent genome sequencing and gene expression study has shown that *T. odontotermitis* can switch between aerobic and anaerobic lifestyle (Sapountzis et al., 2015). The ability of *T. odontotermitis* to colonize the vicinity of the protozoa in the termite gut is an important attribute for a successful paratransgenesis system to achieve termite control via killing the cellulose-digesting protozoa (Husseneder et al., 2010b, Sethi et al., 2014). Colonization in the hindgut region would aid in the direct delivery of the protozoacidal peptide (ligand-Hecate) to the gut protozoa and would prevent the digestion of expressed ligand-Hecate by protease enzymes found in the termite midgut (Sethi et al., 2011).

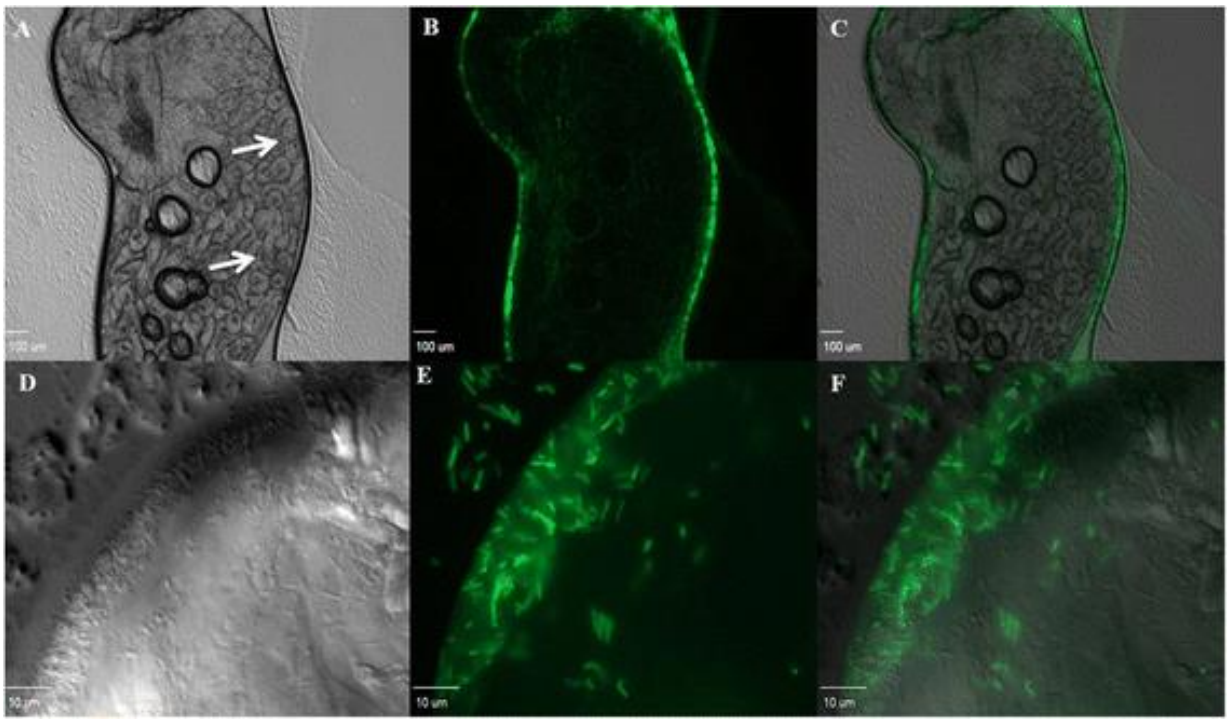

Figure 3.4: Termite hindgut observed under a Leica DM RXA2 fluorescent microscope after feeding on diet containing *T. odontotermitis*-pGFP. A) 5X Differential interference contrast (DIC), white arrows pointing at termite gut protozoa. B) 5X fluorescent, *T. odontotermitis*-pGFP seen concentrated at the hindgut wall. C) Overlay of A and B, *T. odontotermitis*-pGFP seen in the close vicinity of gut protozoa. D) 100X DIC, magnified image of the termite hindgut wall. E) 100X fluorescent, magnified image of the termite hindgut wall showing *T. odontotermitis*-pGFP cells expressing GFP. F) Overlay of D and E.

During the first two days of feeding on cellulose discs containing *T. odontotermitis*-pGFP, the number of *T. odontotermitis*-pGFP cells that could be isolated on Kanamycin media ranged from 3.96×10^4 to 6.49×10^4 per termite gut (Figure 3.5) and no significant differences were found in the bacterial counts from the three colonies ($P = 0.7696$, PROC MIXED with SLICE). After two days, termites were switched to a diet of sterile cellulose discs and the number of *T. odontotermitis*-pGFP cells isolated from the termite gut decreased rapidly. By day 7, no more Kanamycin-resistant bacteria could be isolated from the termites of colony C, and by Day 12 the number of *T. odontotermitis*-pGFP cells in guts of termites from colonies A and B also dropped

below a detectable threshold (Figure 3.5). Throughout the experiment, no Kanamycin-resistant bacteria could be isolated from the guts of the control termites.

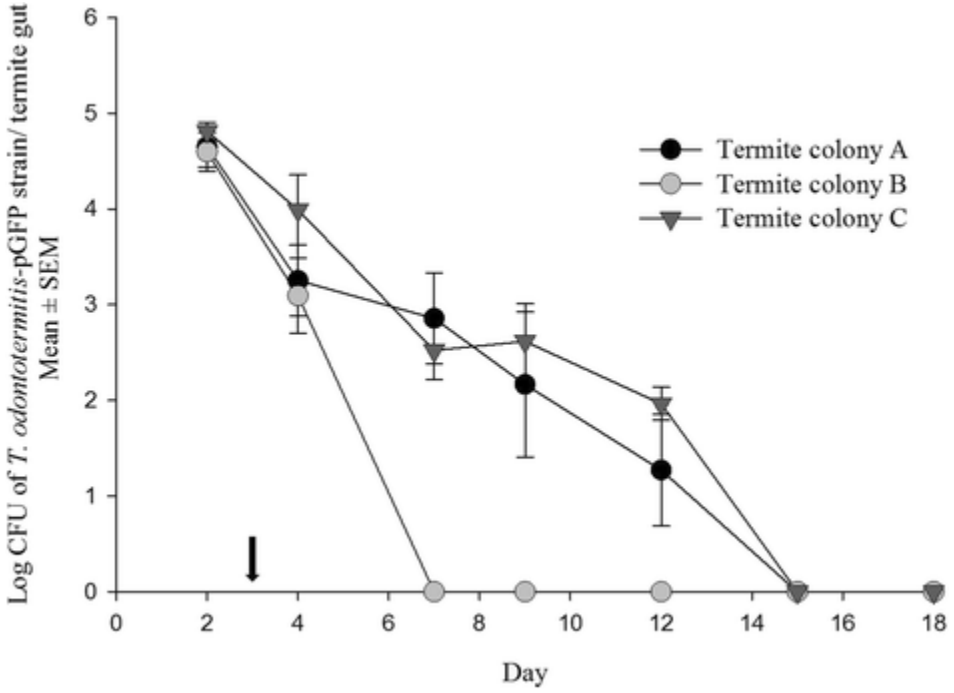

Figure 3.5: Number of *T. odontotermitis*-pGFP cells recovered from the gut of the termites of three different colonies after feeding for two days on cellulose discs containing *T. odontotermitis*-pGFP. The arrow indicates the day when the termites were moved to a sterile diet. The experiment had three replicates for each colony and 200 worker and 20 soldier termites were used for each replicate. Error bars indicate Standard Error of Mean (SEM).

Even though the use of pCT-ELGFP6.1 to transform *T. odontotermitis* improved expression in the termite gut compared to a previously used plasmid with a lactose/ IPTG inducible promoter (Tikhe et al., 2016), it is not suitable to study long-term survival of engineered bacteria in the termite gut and transfer among nest mates because GFP expression was lost too quickly. Loss of expression was most likely due to the loss of the plasmid by the bacteria in the absence of selective antibiotic pressure (Smith and Bidochka, 1998). Since the experiment was carried out in the laboratory, it is currently not known how fast and by what mechanisms plasmids might be lost in field colonies. However, the loss of the marker in the lab experiments prompted us to

construct *T. odontotermitis*-Km^r :: *Tn7*, a strain engineered to express *KanR* from the chromosome, to hopefully provide more stable expression.

3.4.2 *Trabulsiella odontotermitis* engineered at chromosomal level at the *attTn7* site

When engineering any wild bacterial strain with the goal of preserving its functionality, care needs to be taken not to disrupt any of its vital genes. The use of Tn5 and Mu transposons involves random transposition events (Lewenza et al., 2005, Pajunen et al., 2005) that can disrupt important genes required for efficient performance in the natural environment. A site-specific Tn7 transposon, however, inserts in the bacterial chromosome without disrupting any of the host genes (Peters and Craig, 2001). In most bacteria, the Tn7 transposon recognizes the *attTn7* site present within the C terminus region of a highly conserved glucosamine synthetase (*glmS*) gene (Mitra et al., 2010). Tn7 insertions take place 25 bp after the coding region without gene disruption (Mitra et al., 2010, Peters and Craig, 2001). These features make Tn7 transposon an ideal tool for tagging wild-type bacteria without any prior knowledge about the genome.

To utilize a Tn7 transposon system successfully, presence of *attTn7* in the chromosome at a neutral location is desired. A primer set GLMS_CT_Fw and PSTS_CT_Rv was designed with the goal to amplify a putative *attTn7* site present at the C-terminus coding region of the *glmS* gene. A PCR product with approximately 500 bp was obtained using primers GLMS_CT_Fw and PSTS_CT_Rv. Comparison of the sequenced PCR product to the sequences present in the NCBI GenBank database confirmed that this product contained the C-terminus coding region of the *glmS* gene, the inter-genic region between *glmS* and *pstS* and the N-terminus region of the *pstS* gene. Comparison of the sequence to a consensus *attTn7* sequence also revealed the presence of an *attTn7* site at the C-terminus region of *glmS* gene (Mitra et al., 2010). No known

gene or *Tn7* transposon was detected in the inter-genic region between the *glmS* and *pstS* genes. The sequence was further confirmed by comparing it with the whole genome sequence of *T. odontotermitis* (Sapountzis et al., 2015).

PCR amplification of the DNA of three isolates co-transformed with pUC18R6KT-mini-Tn7T-Km and pTNS-3, using primers GLMS_CT_Fw and PSTS_CT_Rv to confirm the insertion of *KanR* cassette in the *T. odontotermitis* chromosome, resulted in a PCR product of ~3000 bp. Amplification using control wild type *T. odontotermitis* resulted in a PCR product of ~500 bp (Figure 3.6 A, Figure 3.6 B).

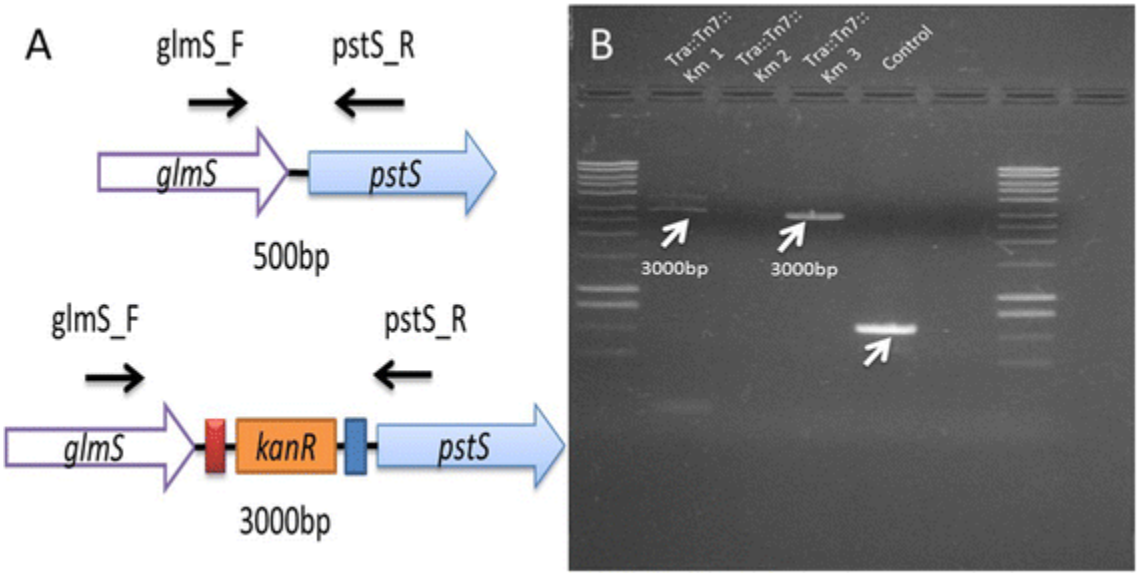

Figure: 3.6 A) Integration of *kanR* gene in the chromosome of *T. odontotermitis* using a *Tn7* transposon integration; glmS_F and pstS_R show the position and direction of primers used to confirm the integration B) PCR-based confirmation of integration of *kanR* gene in the *T. odontotermitis* chromosome using glmS_F and pstS_R primers. Tra:: Tn7:: km 1,2,3 are the three different isolates after a Tn7 transposition, control is the wild-type *T. odontotermitis*.

Partial sequencing of 3000 bp PCR product confirmed the correct orientation of the inserted *KanR* cassette. *Trabulsiella odontotermitis* containing a *KanR* cassette in the chromosome was

designated as *T. odontotermitis*-Km^r :: *Tn7*. The successful insertion of *KanR* cassette in the intergenic region between *glmS* and *pstS* proved its usefulness in a non-disruptive chromosomal tagging. This approach will be utilized in the future to insert a ligand-Hecate gene in the *T. odontotermitis* chromosome without disrupting any of its native genes. This is the first report of genetic manipulation in the genus *Trabulsiella* at the chromosome level.

3.4.3 Chromosomally engineered *T. odontotermitis* is maintained in the termite gut for three weeks after ingestion

Similar to the results showing a rapid intake of *T. odontotermitis*-pGFP strain by termites, *T. odontotermitis*-Km^r :: *Tn7* was also isolated from the gut of workers from all three colonies within two days of feeding. It is likely that bacteria were ingested within hours as shown previously (Husseneder et al., 2005, Husseneder and Grace, 2005). Only at the beginning of the experiment (at Day 2 of feeding), there was significant difference in the bacterial count among colonies ($P = 0.0349$, PROC MIXED with SLICE), with termites from Colony B having less Kanamycin-resistant bacteria compared to Colony A and C (Figure 3.7). However, once the termites were moved to sterile cellulose discs, no significant differences were found in the bacterial counts from all three colonies until Day 22 (PROC MIXED with SLICE). The bacterial count decreased sharply in all three colonies until Day 6 (Three days after the diet was switched to a sterile cellulose disc). From Day 6 to Day 22, the bacteria count oscillated between 10^3-10^4 bacteria/ termite gut in all three colonies. After Day 22, the bacterial counts from Colony C decreased steadily. However, in the other two colonies (A, B) the engineered bacteria strain persisted and even at the end of the experiment (Day 36) an average of 4.9×10^3 bacteria cells per termite gut were still detected in both colonies.

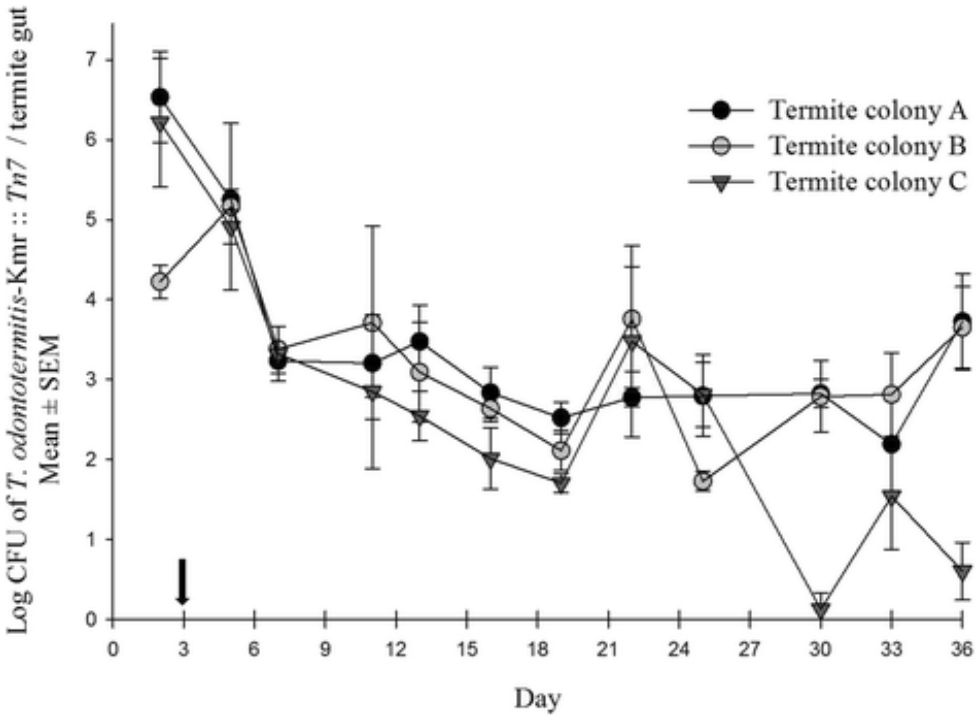

Figure 3.7: Number of Kanamycin-resistant *T. odontotermitis*-Kmr :: Tn7 recovered from the gut of the termites of three different colonies. The arrow on the X-axis indicates the day when termites were moved to a sterile diet. The experiment had three replicates for each colony and 200 worker and 20 soldier termites were used for each replicate. Error bars indicate Standard Error of Mean (SEM).

Results show that *T. odontotermitis* is maintained in the gut for at least three weeks irrespective of the colony. This should be more than enough time for a future *T. odontotermitis* 'Trojan Horse' that will be engineered to express lytic peptides to spread throughout a termite colony and kill the vital gut protozoa in workers. Previous studies have shown that 1 µM of ligand-Hecate can kill all the gut protozoa within 10 minutes *in vitro* (Husseneder et al., 2010b). Injection of 0.3 µL of 500 µM ligand-Hecate solution killed all three species of gut protozoa within 24 hours. Ingested genetically engineered *K. lactis* expressing ligand-Hecate also killed all the gut protozoa within three weeks. After the loss of gut protozoa, termites die within two weeks (Husseneder et al., 2010b, Sethi et al., 2014).

In this study the load of genetically engineered *T. odontotermitis* per termite gut was counted. This procedure differs from the previous studies in which the focus was on the number of termites ingesting the bacteria (Husseneder et al., 2005, Husseneder and Grace, 2005, Zhao et al., 2008). In case of genetically engineered *K. lactis*, the number of yeast cells isolated from the termite gut after three weeks was approximately ten times higher than that of *T. odontotermitis* (Sethi et al., 2014). However, in that study termites were continuously feeding on a diet containing the engineered yeast (Sethi et al., 2014). During this study, termites were allowed to feed on the cellulose diet containing *T. odontotermitis*-Km^r :: *Tn7* for only two days. The results show that even without continuous replenishing of engineered bacteria through feeding, the bacteria are maintained in the gut at moderate levels (10^3-10^4 bacteria/termite gut). This is a useful attribute for the future application of paratransgenesis where we intend to use a bait system to feed engineered bacteria to termites. Only a fraction of the termite workers forages at any point in time and foraging sites may change. Thus, continuous feeding on the bait cannot be assured and the success of paratransgenesis depends on fast and efficient uptake, and survival of engineered bacteria in the termite gut, in addition to efficient spread to colony members that did not directly feed on the bait.

3.4.4 Chromosomally engineered *T. odontotermitis* is horizontally transferred among nest mates

Horizontal transfer of termiticides is required to achieve a colony-level elimination and has been established with many termiticides (Ibrahim et al., 2003, Gautam et al., 2012). Previous studies have shown that termites can transfer bacteria and yeast horizontally via trophallaxis (Husseneder and Grace, 2005, Husseneder et al., 2005, Sethi et al., 2014). Horizontal spread of engineered *T. odontotermitis* throughout the colony was modelled experimentally by combining

donors (termites that ingested *T. odontotermitis*-Km^r :: *Tn7*) and recipients in two ratios 1:1 and 1:25.

In the transfer experiment with the donor: recipient ratio 1:25, Kanamycin-resistant bacteria could be isolated from the gut of the recipient termites from two of the three colonies after two days of mixing donor and recipient termites. There was no significant difference between the bacterial counts of the three termite colonies on Days 4, 10 and 13 (PROC MIXED with SLICE). However, on Days 2 and 7 the bacterial counts of the three termite colonies were significantly different from each other (PROC MIXED with SLICE). On Day 16, no bacteria were recovered from any of the replicates from Colony C and overall there was no significant difference between the bacterial counts from all the three termite colonies (P = 0.3991, PROC MIXED with SLICE). There were large differences in the bacterial counts among individuals even within the same colony (Figure 3.8).

In the transfer experiment with the donor: recipient ratio 1:1, Kanamycin-resistant bacteria were isolated from the gut of the recipient termites from all three colonies just two days after combining donor and recipient termites. Except for Day 7 (P = 0.04, PROC MIXED with SLICE), there was no significant difference in the bacterial counts in the guts of recipients from the three termite colonies for 16 days (PROC MIXED with SLICE). After Day 16 the experiment was discontinued because there was no clear distinction between the donor and recipient termites due to the loss of coloration in the fat body of the recipients. At Day 16, the average number of bacteria per recipient termite gut was around $7.5x10^3$ (Figure 3.9).

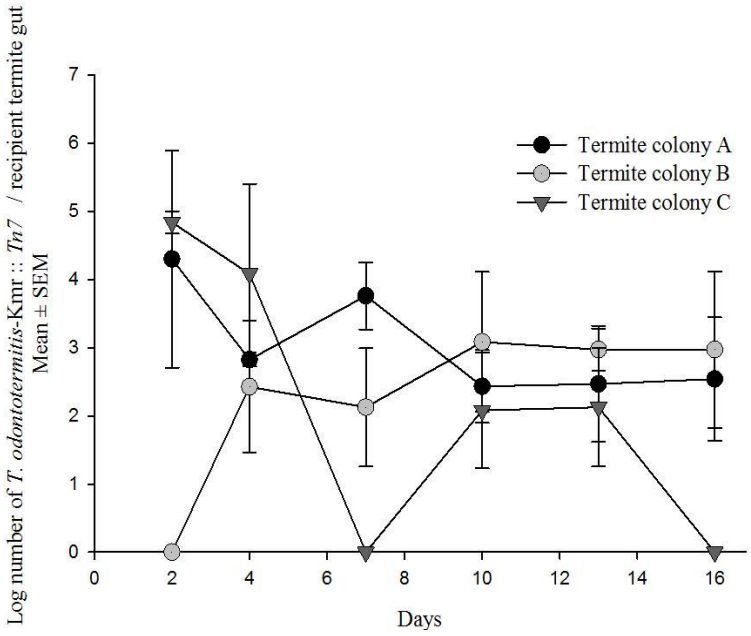

Figure 3.8: *T. odontotermitis*-Km^r :: *Tn7* recovered from the gut of the recipient termites (donor: recipient ratio 1:25) of three different colonies. The experiment had three replicates for each colony. Error bars indicate Standard Error of Mean (SEM)

In order to be successful in a bait system, *T. odontotermitis* must be transferable horizontally to the nest mates. These results suggest that *T. odontotermitis* has a better transfer efficiency among the nest mates than *K. lactis*. After two weeks the number of *T. odontotermitis* recovered from the termite gut was approximately five times higher in comparison to *K. lactis* (Sethi et al., 2014). A higher transfer efficiency of *T. odontotermitis* will aid in its spread throughout the termite colony which again is an important asset for the success of paratransgenesis.

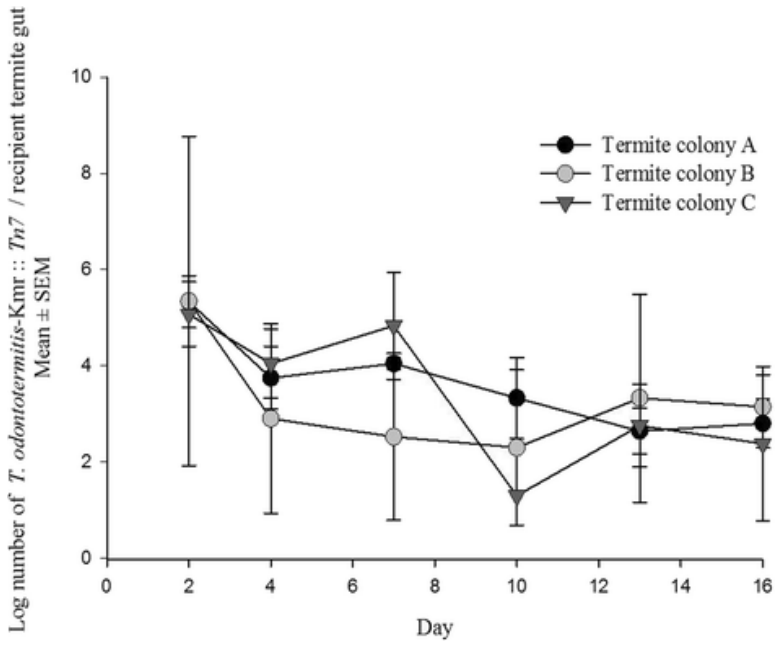

Figure 3.9: *T. odontotermitis*-Kmr :: Tn7 recovered from the gut of the recipient termites (donor: recipient ratio 1:1) of three different colonies. The experiment had three replicates for each colony. Error bars indicate Standard Error of Mean (SEM)

3.4.5 Consumption and Mortality

For future application of *T. odontotermitis* as a 'Trojan Horse' for termite control in a bait system, non-repellency is an important aspect to ensure ingestion of a lethal dose (Hu et al., 2005). Analysis of cellulose consumption suggests that addition of *T. odontotermitis* did not deter termites from feeding. The type of treatment, i.e. whether termites were fed with either strain of the genetically engineered bacteria (*T. odontotermitis*-pGFP or *T. odontotermitis*-Km^r :: *Tn7*), did not have any effect on the consumption of cellulose (P = 0.38,PROC MIXED). However, each colony reacted differently to different substrates and colony membership had a significant effect on the consumption (P = 0.004, PROC MIXED). In this study, there was no correlation between the probability of mortality and the type of treatment, suggesting that

addition of *T. odontotermitis* to the diet does not result in increased mortality of termites (PROC LOGISTIC) (Figure 3.10).

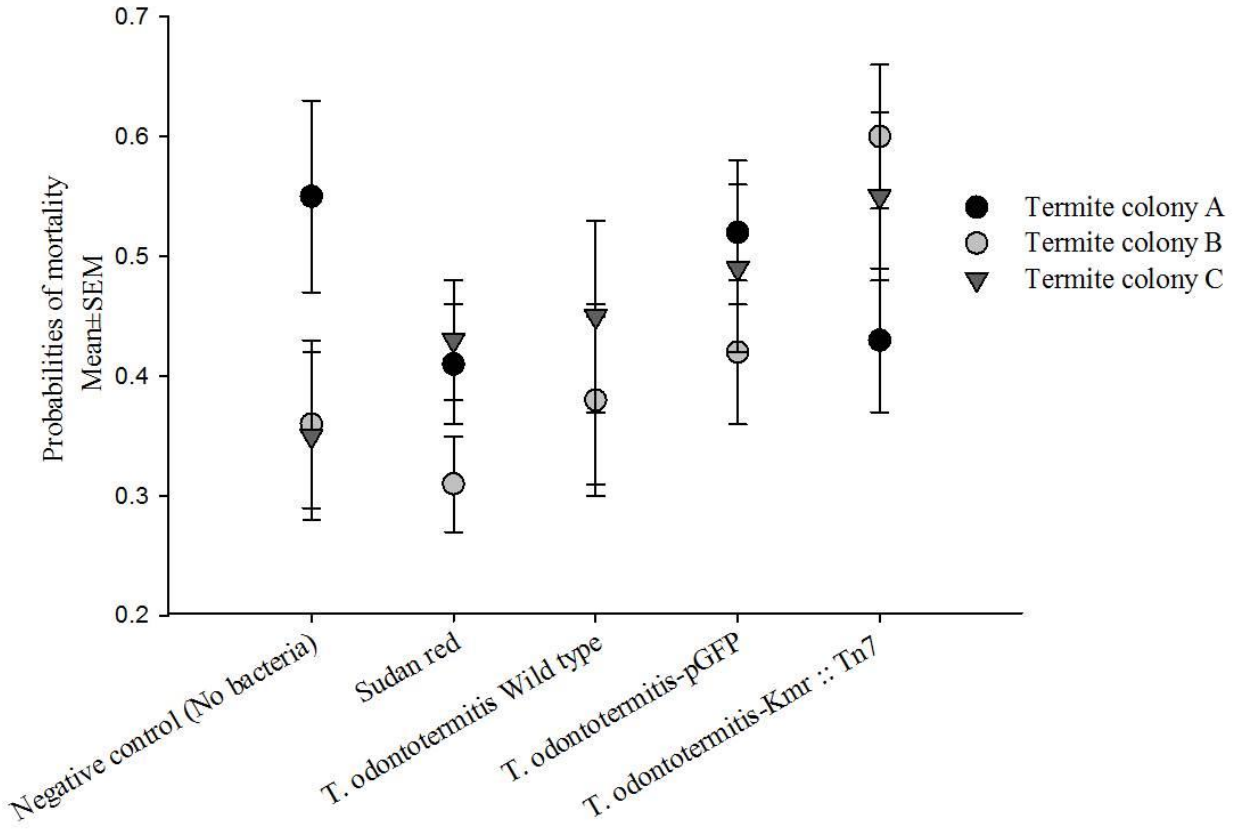

Figure 3.10: Probabilities of mortality of three termite colonies fed on cellulose diet with the addition of *T. odontotermitis*-Kmr :: Tn7, T. odontotermitis wild-type, *T. odontotermitis*-pGFP, and Sudan red. The negative control consisted of cellulose only. There was no significant difference among the probabilities of mortality based on the type of diet consumed (P = 0.21, PROC LOGISTIC).

The study proved: 1. A termite-specific bacterium, *T. odontotermitis,* can be engineered with a plasmid and at chromosome level using a non-disruptive *Tn7* transposon-based method to express foreign proteins in the termite gut; 2. Engineered *T. odontotermitis* was ingested by the termite and survived in the gut for at least 21 days; 3. Engineered *T. odontotermitis* is transferred

horizontally among nest mates via social interactions; 4. *T. odontotermitis* does not have an effect on termite mortality and diet consumption. This study provided proof of concept for the future development of genetically engineered termite gut bacteria for paratransgenesis-based termite control. In the future we intend to genetically engineer *T. odontotermitis* to express ligand-Hecate using a constitutive promoter and a Tn7 transposition. Ultimately, engineered *T. odontotermitis* expressing ligand-Hecate will be used in bait and will be assessed as a termite control agent.

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Chapter 4: A pilot study to genetically engineering of *Trabulsiella odontotermitis* to express functional ligand-Hecate

4.1 Introduction

The Formosan subterranean termite (FST) *Coptotermes formosanus* is the most destructive structural pest in the US. These termites cause billions of dollars of economic loss in damage control and repairs (Pimentel et al., 2005). Termite control relies heavily on chemical insecticides which are known to have unwanted side effects on the environment (Rust and Su, 2012, Pisa et al., 2015). Biological control efforts remain unsuccessful for most termites due to their behavioral and immune responses to the pathogens (Chouvenc et al., 2011). To overcome these limitations, paratransgenesis targeting the essential gut protozoa of the termites is being developed (Chouvenc et al., 2011).

Formosan subterranean termites rely on their symbiotic protozoa for cellulose digestion, making them an attractive target for termite control (Eutick et al., 1978, Husseneder and Collier, 2009). In previous studies we have shown that ligand-Hecate, a synthetic targeted anti-protozoal peptide, can kill termite workers efficiently by eliminating the gut protozoa (Husseneder et al., 2010). Engineered *Kluveromyces lactis* yeast capable of secreting ligand-Hecate, eliminated the gut protozoa, which resulted in the death of termite lab colonies (Sethi et al., 2014). Even though engineered *K. lactis* successfully killed termites, it is not adapted for the termite gut and may cause environmental contamination. In the search for a better alternative for *K. lactis*, we assessed a bacterium isolated from the termite gut, *Trabulsiella odontotermitis,* for its suitability as a 'Trojan Horse' (Tikhe et al., 2016b, Tikhe et al., 2016a). *Trabulsiella odontotermitis* is a termite-specific bacterium found in the guts of various termite species (Chou et al., 2007, Tikhe et al., 2016b). Whole genome sequencing of *T. odontotermitis* has revealed many adaptations for

the termite gut (Sapountzis et al., 2015). We have shown that *T. odontotermitis* can tolerate 50 times more concentration of ligand-Hecate than the concentration required to kill the gut protozoa (Tikhe et al., 2016b). In a previous study, genetically engineered *T. odontotermitis* was ingested by the termites and successfully expressed green fluorescent protein (GFP) in the gut. Once ingested, engineered *T. odontotermitis* was maintained in the termite gut for at least 21 days. We also showed that *T. odontotermitis* is transferred horizontally among other nest mates (Tikhe et al., 2016a). Overall, *T. odontotermitis* satisfied all the criteria of an ideal 'Trojan Horse' described previously (Tikhe et al., 2016b). The next step in the development of a paratransgenic 'Trojan Horse' is to engineer *T. odontotermitis* to express and secrete functional ligand-Hecate. In this pilot study we engineered *T. odontotermitis* with a plasmid and tested for expression and secretion of functional ligand-Hecate in bioassays.

4.2 Materials and methods

4.2.1 Designing of codon optimized genes for *T. odontotermitis*

The gene sequence of ligand-Hecate was obtained from previous studies (Sethi et al., 2014, Husseneder et al., 2010). Based on an extensive literature review, five signal secretion peptides were selected to be fused to the ligand-Hecate gene (Table 1). For detection purposes a Histidine tag (6X HIS) was chosen to be attached to each gene. Nucleotide sequences of all the protein coding open reading frames were obtained from the whole genome of *T. odontotermitis* (Sapountzis et al., 2015). A codon usage Table for *T. odontotermitis* was created using CUSP software available on EMBOSS server (http://www.hpa-bioinfotools.org.uk/pise/cusp.html). The final gene construct included a *T. odontotermitis* codon optimized ligand-Hecate gene followed by a six histidine tag (6xHis-tag) and a signal peptide either to the 5' or 3' of the gene (Table 1).

Table 4.1 A list of all the signal peptides and fusion proteins used in this study

Signal peptide	Origin of the signal peptide	Bacterial secretion system	Construct
PelB	Pectate lyase *Pectobacterium carotovorum*	Sec pathway	PelB-ligand-Hecate-6X-HIS
OmpA	Outer membrane protein A *E. coli* W3110	Sec pathway	OmpA-ligand-Hecate-6X-HIS
MalE	Maltose binding protein E *E. coli* W3110	Sec pathway	MalE-ligand-Hecate-6X-HIS
HlyA	Hemolysin A *E. coli* CFT073	Type I secretion system	ligand-Hecate-6X-HIS-HlyA
IgL	Immunoglobulin like protein *Trabulsiella odontotermitis*	Predicted Type I secretion	ligand-Hecate-6X-HIS-IgL
No		Intracellular expression	ligand-Hecate-6X-HIS
No		Intracellular expression	ligand-Hecate-GFP

Two gene constructs contained ligand-Hecate gene, one with a HIS- tag and the other fused to green fluorescent protein (LiHe-GFP) without any signal peptide. All the *T. odontotermitis* codon optimized genes were synthesized and cloned in a constitutively expressed plasmid pSF-OXB20 (OG50) at Oxford Genetics (Oxford, UK). Insertion and orientation of the synthesized gene in the plasmid was confirmed by PCR and sequencing according to manufacturer's instructions.

4.2.2 Transformation of *T. odontotermitis*

Once the gene insertion was confirmed in the correct location, all seven plasmids were used to transform electrocompetent *T. odontotermitis* cells as described previously (Tikhe et al., 2016a). Transformants were selected on LB + Kanamycin (50 µg/ ml) plates. From each transformation, ten isolates were selected and maintained as glycerol stocks. Plasmids were extracted from all the isolates using QIAprep Spin Miniprep Kit (QIAGEN 27106) to confirm successful transformation.

4.2.3 Protein expression

Trabulsiella odontotermitis cells transformed with the different plasmids (Table 1) were grown for 16 hours in LB broth containing Kanamycin (50 µg/ml). For all the experiments, cell lysis and centrifugation was carried out at 4°C. For intracellular protein expression, 1 ml of cells were centrifuged and the cell pellet was washed with sterile ice cold phosphate buffered saline (PBS) twice. The cell pellet was then suspended in 50 µl of PBS and was mixed with 50 µl of Tricine sample buffer (Bio-Rad 1610739). The mixture was vortexed for 2 mins and was boiled at 95°C for 5 minutes. The mixture was used to carry out SDS-PAGE using 20% Tris-Tricine precast gels (Bio-Rad 4563115). For analyzing extracellular secretion of ligand-Hecate, supernatant of transformed *T. odontotermitis* cells that were grown for 16 hours in LB broth with Kanamycin was used. The 15 ml supernatant was centrifuged in a Vivaspin® 15R Centrifugal Concentrator at 2500 X g for 30 minutes. Concentrated proteins from the supernatant were separated using SDS-PAGE as described above. SDS-PAGE gels were used for Western blots. Penta-His (Qiagen) (1:5,000) and goat-anti-mouse conjugated to horseradish peroxidase (HRP) (Pierce) (1:10,000) were used as primary and secondary antibodies respectively. ImmunStar HRP kit (Bio-Rad) was used for detection. For *T. odontotermitis* transformed LiHe-GFP plasmid, cells were observed

under a fluorescence microscope to confirm GFP expression (Leica DM RXA2, 100x oil, N.A = 1.3).

4.2.4 *Tetrahymena* sp. bioassays

This type of assay is widely used to test environmental pollutants and toxic chemicals. The protocol used below has been previously tested in our laboratory against variety of protozoa species (Husseneder et al., 2010).

Transformed *T. odontotermitis* cells were grown for 16 hours in LB broth containing Kanamycin (50 µg/ml). Cells were centrifuged and the pellet was washed with ice cold sterile PBS twice and was suspended in 5 ml of ice cold sterile PBS. Suspended cells were lysed with a sonicator (Q55 Sonicator, QSonica, CT, USA) with 10 bursts of 15 sec followed by intervals of 30 sec of cooling. The cell lysate was centrifuged at 10,000 X g for 15 mins at 4°C to obtain intracellular expressed but not secreted peptides. The supernatant was mixed with *Tetrahymena* sp. (purchased from Carolina Biological Supply Company, NC, USA) culture containing 10^4 cells /ml in the ratio 1:1. *Tetrahymena* sp. (suspended in Tetrahymena media) were observed under the microscope after 5, 10, 15 and, 60 mins for cell lysis. To study the extracellular antiprotozoal activity of secreted peptides, the supernatant of transformed *T. odontotermitis* cells grown for 16 hours in LB broth containing Kanamycin (50 µg/ ml) was used. The supernatant (15 ml) was concentrated using Vivaspin® 15R Centrifugal Concentrator at 2500 X g for 30 minutes. The concentrated supernatant was then added to *Tetrahymena* sp. culture containing 10^4 cells/ml in the ratio 1:1 (suspended in Tetrahymena media) and cell lysis was observed under the microscope after 5, 10, 15, and 60 mins. All the experiments were carried out in triplicate.

4.2.5 Improvised antiprotozoal BACTOX assay

To check the toxicity of transformed cells, an alternative method known as the BACTOX assay was carried out on *Tetrahymena* sp. (Schlimme et al., 1999). The BACTOX method uses complete bacterial cells instead of cell lysates or cell supernatants and the Tetrahymena media is replaced by sterile water.

Briefly *Tetrahymena* sp. cells were grown to a density of 10^4 cells/ml in Tetrahymena media. 1 ml of cells were briefly centrifuged at 200 X g and were re-suspended in 1 ml of sterile autoclaved tap water. Cells were allowed to acclimatize to the tap water osmolarity for 30 minutes. *T. odontotermitis* cells expressing ligand-Hecate-GFP were grown on LB+ Kanamycin (50 µg/ml) plates for two days. One single isolated colony was picked and was re-suspended in 1 ml of sterile tap-water. The bacterial suspension (1 ml) was added to 500 µl of *Tetrahymena* sp. previously suspended in sterile tap water (total volume 1.5 ml). *Tetrahymena* sp. cells were observed under the microscope and were scored as described previously (Schlimme et al., 1999). The scoring system grades the antiprotozoal activity of the bacteria in study between 1 and 5. Score of one is considered as no effect on the protozoa while five is considered as the most lethal. *Trabulsiella odontotermitis* expressing GFP was used as a control for these experiments.

4.3 Results and discussion

To be a successful 'Trojan Horse', *T. odontotermitis* must express functional ligand-Hecate to kill the termite workers' gut protozoa and, ultimately, the termite colony.

Expression of recombinant proteins in non-model organisms can be challenging due to the lack of knowledge about their biology. *Trabulsiella odontotermitis* is a termite gut bacterium isolated from the guts of various termite species. In 2015, the whole genome of *T. odontotermitis* was

sequenced and it was shown that it has many adaptations for the termite gut (Sapountzis et al., 2015). It was also shown that the genome of *T. odontotermitis* is very different from a closely related species *Trabulsiella guamensis* (Sapountzis et al., 2015). *Trabulsiella guamensis* is not found in the termite gut and does not appear to have specialized adaptations required for the environment in the termite gut. Even though genome sequencing revealed some insights into the genetic makeup, the biology of *T. odontotermitis* remains unstudied so far. Hence, engineering *T. odontotermitis* was based on our previous knowledge and a detailed literature review. Previously, we successfully engineered *T. odontotermitis* to express foreign marker proteins (Tikhe et al., 2016a). When introduced in the termite gut, *T. odontotermitis* was able to express foreign proteins without the need of any inducers (Tikhe et al., 2016b). In our previous study, we constructed a novel constitutively expressed GFP plasmid. The plasmid pCT-ELGFP contains a constitutive promoter and a Kanamycin resistant gene (Tikhe et al., 2016a). Due to successful expression with pCT-ELGFP, we decided to use the same backbone for this study (pSF-OXB20, OG50).

In a previous study we showed that *T. odontotermitis* expressing GFP colonizes the hindgut wall of termite workers (Tikhe et al., 2016a). The gut protozoa are located exclusively in the hindgut and colonization of the hindgut brings *T. odontotermitis* in a close proximity to the gut protozoa.

In order to target the gut protozoa effectively, *T. odontotermitis* should be able to express ligand-Hecate in the termite gut continuously. To test whether this can be achieved, we transformed *T. odontotermitis* in this pilot study with a plasmid capable of constitutively expressing ligand-Hecate (LiHe-HIS). Successful transformation of *T. odontotermitis* cells was confirmed by extracting the plasmid from multiple transformants. However, we were unable to detect ligand-Hecate-6XHIS expression in the cell lysate using Western blots. The cell lysate also did not have

any effect (cell lysis, change in cell morphology) on *Tetrahymena* sp. Due to these results we hypothesized that ligand-Hecate may be getting degraded in the intracellular environment.

Ligand-Hecate is a short peptide of 31 amino acids and most likely remains in an unfolded state in the cell. Many studies have shown that recombinant proteins expressed intracellularly are prone to bacterial proteases (Choi and Lee, 2004, Jin et al., 2006). The susceptibility is even higher for small unfolded peptides (Skosyrev et al., 2003a, Piers et al., 1993). Also, intracellular recombinant proteins may be toxic to the bacterial cells (Li, 2011). To overcome these above mentioned limitations, extracellular secretion of recombinant proteins is used as a tool (Choi and Lee, 2004). It should be noted that the secretion of recombinant ligand-Hecate is not a mandatory requirement for an ideal 'Trojan Horse', but it has many benefits such as protection from bacterial proteases and direct delivery of ligand-Hecate to the gut protozoa without the need of cell lysis over intracellular expression.

4.3.1 Sec-dependent secretion signals

Secretion signals from proteins that are dependent on the Sec-pathway guide the protein to the periplasmic space (Tsirigotaki et al., 2016). Attachment of these signal peptides to recombinant proteins has been shown to process and export the proteins to the periplasmic space (Mergulhao et al., 2005, Choi and Lee, 2004). Recombinant proteins are protected from intracellular proteases in the periplasmic space. Overexpression of recombinant proteins in the periplasmic space leaks the proteins in the media (Mergulhao et al., 2005). Multiple recombinant proteins have been expressed successfully and secreted using Sec-dependent signal sequences (Chen et al., 2012).

The signal sequences PelB from *Erwinia carotovora* pectate lyase B gene, OmpA, and MalE from *E. coli* outer membrane protein A and Maltose binding protein E, respectively, have been widely used with various recombinant proteins (Ni and Chen, 2009, Choi and Lee, 2004, Mergulhao et al., 2005). Thus, we decided to test these three signal peptides for their usefulness to express and secrete ligand-Hecate in *T. odontotermitis*.

Plasmid extraction from transformed *T. odontotermitis* cells confirmed successful transformation with PelB and OmpA plasmids. However, transformation of *T. odontotermitis* using MalE plasmid was not successful despite multiple attempts evidenced by the lack of growth on selective Kanamycin media with MalE plasmid. *Escherichia coli* DH5α cells transformed with MalE plasmid produced very small pinpoint colonies on selective media. Reduced colony size of recombinant bacteria is considered as an indication of toxicity (Rosano and Ceccarelli, 2014). This indicates that overexpression of MalE-ligand-Hecate fusion protein is toxic to *T. odontotermitis* cells. Thus, we did not use MalE plasmid for further experiments.

Although transformed successfully, we were not able to detect PelB-ligand-Hecate and OmpA-ligand-Hecate in the Western blots. The cellular lysate and the concentrated supernatant from the cultures did not have any effect on *Tetrahymena* sp. There were no changes in cell morphology, cell motility or the number of live *Tetrahymena* cells as compared to the control. We hypothesized that attachment of signal peptides targeting the Sec-pathway did not protect ligand-Hecate from intracellular proteases by transporting it to the periplasmic space. Therefore, we tested whether a different secretion system, i.e., signal peptides from Type I secretion system, could be used to express ligand-Hecate.

4.3.2 Type I secretion system

In a previous paratransgenesis study targeting *Plasmodium* sp., antiprotozoal peptides were expressed and secreted successfully in *Pantoea agglomerans* using Type I secretion system (Wang et al., 2012). The *E. coli* hemolysin secretion system is the most studied Type I secretion system (Thomas et al., 2014). It requires two accessory proteins HlyB and HlyD to form a channel, which can secrete HlyA protein directly into the extracellular environment (Costa et al., 2015). TolC forms an outer membrane channel which is linked to HlyB via HlyD and is also required for successful secretion using Type I system. Genome analysis of *T. odontotermitis* confirmed the presence of *TolC, HlyB* and *HlyD* genes. Instead of *HlyA*, the *T. odontotermitis* genome contains a gene that expresses a big immunoglobulin-like protein (Sapountzis et al., 2015). A similar protein observed in *Salmonella* sp. is shown to be an adhesin protein, which is exported using Type I system (Gerlach et al., 2007). We attached the predicted signal peptides of *E. coli* HlyA and *T. odontotermitis* IgL like protein to the C-terminus end of ligand-Hecate to test if this fusion protein will lead to the extracellular secretion of ligand-Hecate. However, similar to our previous results, we were not able to detect the presence of ligand-Hecate with Western blot or *Tetrahymena* sp. bioassays.

It was surprising that we were not able to detect ligand-Hecate expressed by any of the constructs. Smaller peptides are much harder to detect using Western blots (Tomisawa et al., 2013). Failure to detect ligand-Hecate in the Western blot may be due to some of the physical attributes of the peptide, e.g. high positive charge (Henk et al., 1995). Further, standardization of the procedure may help to overcome this issue.

Even though Western blots were not successful with engineered *T. odontotermitis*, there was the possibility that expression of functional ligand-Hecate can be detected by the antiprotozoal

activity of the peptide. In a previous study in which *K. lactis* was engineered to express ligand-Hecate, it was not detected in Western blots either, although the strain successfully killed the termite gut protozoa and the termites (Sethi et al., 2014). However, our bioassays with *Tetrahymena* sp. to check the antiprotozoal activity did not show any effect on the protozoa. This pilot study indicates that *T. odontotermitis* was not able to express functional ligand-Hecate, most likely due to the small size of the peptide.

4.3.3 Ligand-Hecate-GFP fusion protein

To overcome the issue of the small peptide size, we decided to construct a fusion peptide. Our previous studies have shown that *T. odontotermitis* can express functional GFP (Tikhe et al., 2016a). Moreover, GFP has been used previously in many other studies as a fusion partner to express recombinant peptides (Skosyrev et al., 2003b). GFP has also been shown to protect peptides from bacterial proteases (Soundrarajan et al., 2016). Therefore, we decided to construct a fusion protein ligand-Hecate-GFP. Our results showed that *T. odontotermitis* transformed with LiHe-GFP plasmid produced fluorescent colonies. Individual *T. odontotermitis* cells also showed GFP expression under a fluorescent microscope (Figure 4.1). As compared to GFP (Tikhe et al., 2016a), LiHe-GFP showed less fluorescence. To determine whether LiHe-GFP fusion protein had antiprotozoal activity, we carried out BACTOX antiprotozoal assay (Schlimme et al., 1999).

BACTOX assays showed a clear difference between the control (*T. odontotermitis* expressing GFP) and *T. odontotermitis* expressing LiHe-GFP. *Tetrahymena* sp. incubated with *T. odontotermitis* expressing LiHe-GFP showed decreased motility as compared to the control after five minutes (watch the video at: https://www.youtube.com/watch?v=0KHogrhKSwo). After 15 minutes of incubation, approximately 80 % of the *Tetrahymena* cells appeared to be dead as

compared to the control in which no mortality was observed. These qualitative observations indicate that *T. odontotermitis* expressing LiHe-GFP has antiprotozoal activity.

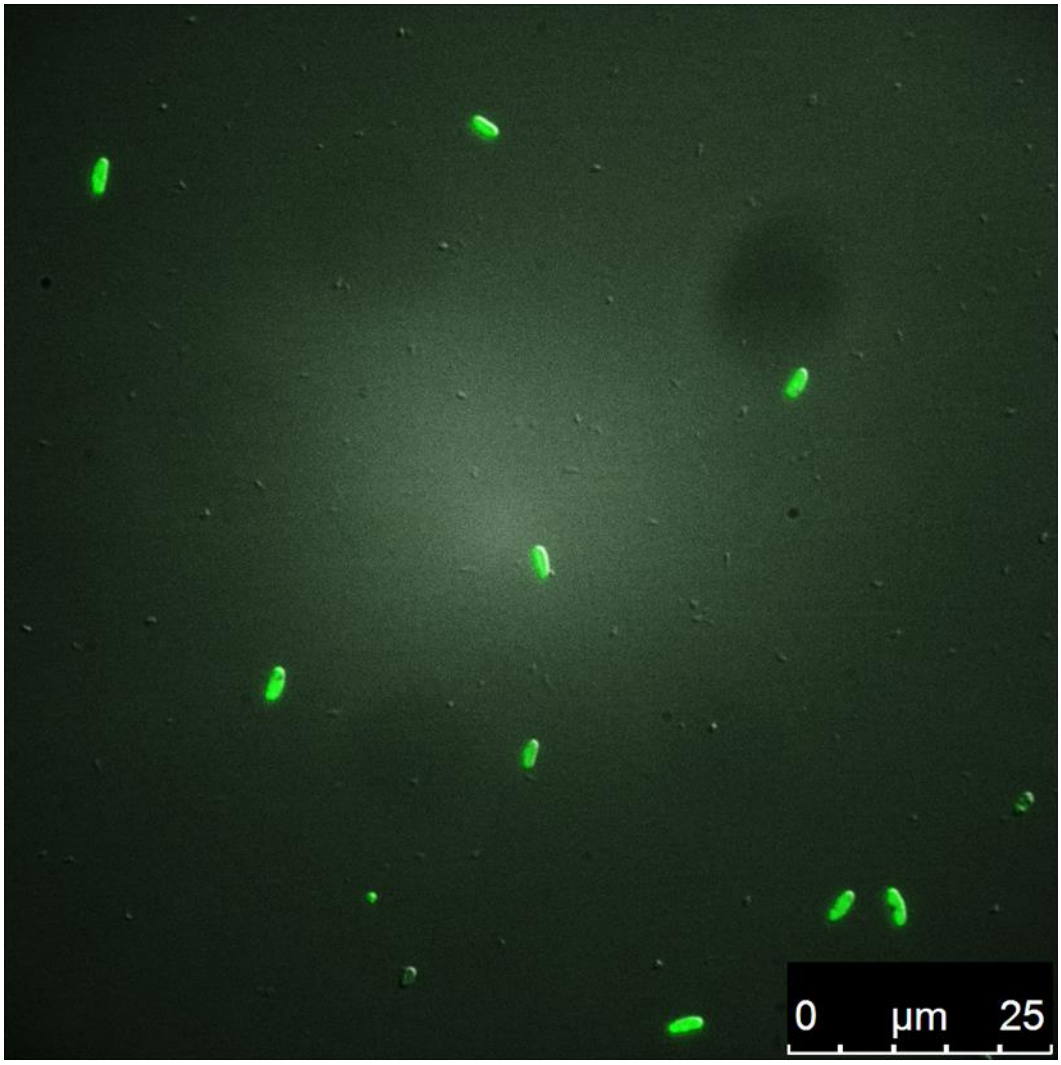

Figure 4.1: *Trabulsiella odontotermitis* expressing ligand-Hecate-GFP (Image taken on Leica DM RXA2, 100x oil, N.A= 1.3)

In conclusion, *T. odontotermitis* was able to express functional ligand-Hecate when attached to GFP. We hypothesize that attachment of GFP protects ligand-Hecate from intracellular protease.

Previously, the TAT pathway has been used successfully to target GFP to the periplasmic space and leak it into the extracellular medium (Thomas et al., 2001, Albiniak et al., 2013). In the future, LiHe-GFP fusion protein can be directed to the periplasmic space using signal peptides like GIII and TorA which use the TAT pathway (Barrett et al., 2003, Wendel et al., 2016).

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Chapter 5: Complete genome sequence of *Citrobacter* phage CVT22 isolated from the gut of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki *

5.1 Introduction

The Formosan subterranean termite is an invasive pest in the United States. Diverse bacteria and protozoa in the gut of its workers provide essential nutrients to the termite colony. The multifarious bacterial community makes the termite gut a niche for bacteriophages. However, the bacteriophages of the termite gut remain unexplored to date. Here we report the first genome sequence of a termite gut bacteriophage (CVT22). CVT22 infects *Citrobacter* sp. TM1552 (GenBank accession number KP765691), also isolated from the termite gut.

5.2 Materials and methods

Termite gut homogenate was filtered through a 0.22 µm syringe filter to isolate bacteriophage CVT22, which infected *Citrobacter* sp. TM1552 with clear plaque morphology. DNA was purified from high titer lysates of CVT22 using phenol-chloroform-isoamyl alcohol extraction and sequenced using the Illumina MiSeq platform (2x300 bp) at MR DNA (Molecular Research LP Shallowater, TX). Sequencing resulted in 2,012,032 reads, with an average read length of 300 bp with approximately 12,000X genome coverage. Illumina reads were assembled using DNAstar SeqMan NGen DNA assembler. The assembled contig contained terminally redundant repeats and the genome was confirmed to be circularly permuted by restriction enzyme analysis. Gene predictions were generated using Glimmer (Delcher et al., 1999, Salzberg et al., 1998) and GeneMark (Besemer and Borodovsky, 1999) and manually annotated with DNA Master (http://cobamide2.bio.pitt.edu/). Phage morphology was determined using electron microscopy

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(EM) at the Socolofsky Microscopy Center at LSU. Phage family search was carried out using VIRFAM (Lopes et al., 2014).

5.3 Results and discussion

The genome of CVT22 is 47,636 bp with a G+C content of 41.6% and is circularly permuted. We organized the CVT22 genome into two convergent transcriptional units. Whole genome nucleotide blast using High Similarity criteria against the GenBank nucleotide nr database did not result in any matches. Less stringent discontiguous megablast showed a match to *Pseudomonas* phage PA11 (Query coverage 11%, Identity 69%) and *Salinivibrio* phage CW02 (Query coverage 11%, Identity 67%). The genome contains 82 predicted protein coding genes with 37 (45.12%) exhibiting similarity to phage genes in the GenBank nr protein database. Of those, 14 were similar (identity between 33-68%) to *Pseudomonas* phage PA11 (Kwan et al., 2006), while 11 showed similarity (25-65%) to *Salinivibrio* phage CW02 (Shen et al., 2012). Twenty-five genes (30.48%) could be assigned a putative function based on homology. In addition to structural genes, we could identify a terminase gene and a lysis cassette consisting of endolysin, holin, and o- and i-spanin genes. Other putative proteins encoded in the genome include DNA polymerase, primase/helicase, ATP grasp protein, sigma transcription factor, amidoligase, S-adenosylmethionine-dependent methyltransferase superfamily protein, and aspartate aminotransferase superfamily protein. Two copies each of exonuclease, endonuclease, and amidotransferase encoding genes were also identified. BLAST analysis did not identify synteny to prophage genomes and we could not identify integrase genes or other genes encoding proteins involved in lysogeny. This, along with the clear plaque morphology, suggests that CVT22 may have a lytic life cycle. VIRFAM predicted CVT22 to be a member of the Podoviridae type3 group and clustered with *Pseudomonas* phage PA11 [3, 5]. The overall size

and genome organization of CVT22 is similar to PA11 and EM analysis supports the assignment of CVT22 to the Podoviridae family (Figure 5.1).

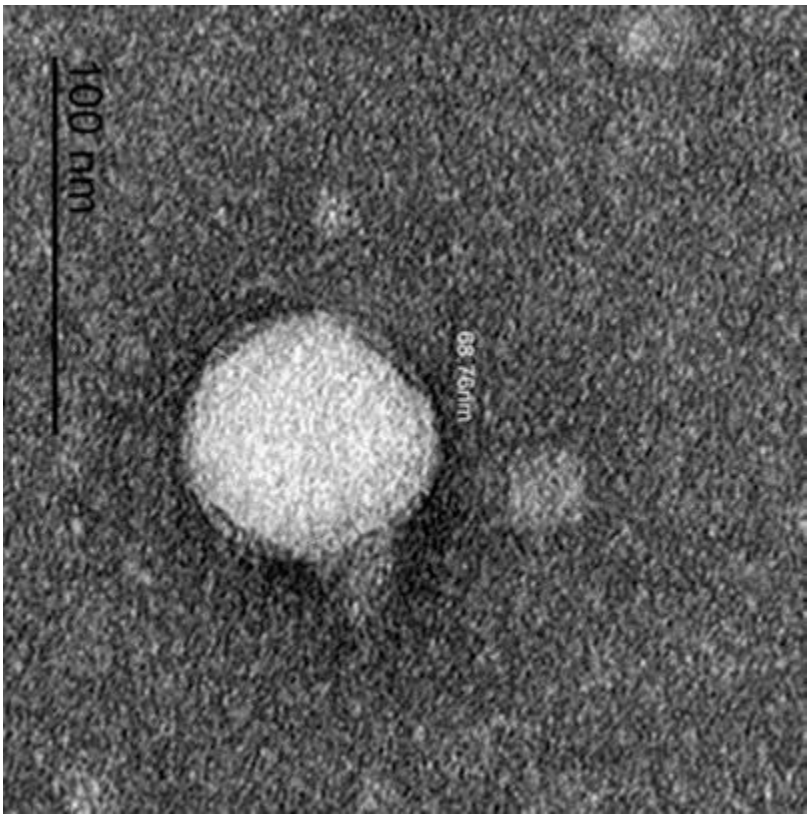

Figure 5.1: Electron micrograph of *Citrobacter* phage CVT22 (Image taken on JEOL JEM- 2011 Transmission Electron Microscope)

We have described here for the first time a genome of a bacteriophage isolated from the termite gut. Further studies of CVT22 will reveal its role in the termite gut microbial ecosystem.

The complete annotated sequence of the *Citrobacter* phage CVT22 genome can be accessed under the GenBank accession number KP774835.

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Chapter 6: Whole genome sequencing of a novel temperate *Enterobacter* bacteriophage Tyrion isolated from the termite gut

6.1 Introduction

Even though the gut bacterial community of various termite species has been well studied, their bacteriophages remain understudied. We previously isolated and sequenced a novel bacteriophage from the termite gut predicted to represent a novel cluster of lytic bacteriophages (Tikhe et al., 2015, Casjens and Grose, 2016). Here, we report the genome sequence of a novel circularly permuted bacteriophage Tyrion infecting *Enterobacter* sp. CT7 (KT204538.1), both isolated from the gut of the Formosan subterranean termite *Coptotermes formosanus*.

6.2 Materials and methods

Bacteriophage isolation and DNA extraction was carried out as described previously (Tikhe et al., 2015). Purified phage DNA was sequenced at Molecular Research LP (Shallowater, TX) using the Illumina Mi-seq (2X300 bp) platform. The raw reads were checked for quality and adapter contamination using Trim Galore (Krueger) and then assembled into contigs using SPAdes genome assembler (Bankevich et al., 2012). The end of the DNA were conformed via PCR. Genes were predicted using GeneMark (Lukashin and Borodovsky, 1998) and were manually annotated using NCBI protein nr database. The Family of phage Tyrion was predicted using VIRFAM analysis (Lopes et al., 2014). Electron microscopy was carried out at Socolofsky Microscopy Center at Louisiana State University.

6.3 Results and discussion

Bacteriophage Tyrion produced small turbid plaques on *Enterobacter* sp. CT7, indicating its temperate nature. Genome sequencing produced a contig with terminal repeats and PCR analysis

confirmed the DNA ends to be circularly permuted. The genome of phage Tyrion is 41,760 basepairs with a G+C content of 51%. At nucleotide level, segments of phage Tyrion genome showed similarity to multiple *Escherichia coli*, *Salmonella,* and *Klebsiella* genomes (query coverage 64-28%, identity 87-76%), and multiple *Escherichia coli* and *Salmonella* bacteriophages (query coverage 63-24%, identity 86-78%). We were not able to detect any tRNA genes in the genome. The genome of phage Tyrion contained 52 predicted protein coding genes, of which 51 matched to proteins from prophage-like regions in multiple *Citrobacter, Escherichia, Salmonella,* and *Enterobacter* strains. Of 52 genes, 27 genes encoded for hypothetical proteins with unknown functions. The bacteriophage genome architecture was similar to that of *Salmonella* phage SPN1S (Shin et al., 2012) and *Escherichia* phage phiV10 (Perry et al., 2009).

The genome of Tyrion contained a DNA packaging module comprised of a small and a large terminase subunit. The structural module contained a single copy each of the head-tail connecting protein, major capsid protein, head closure protein, and an adaptor protein. The lysis cassette was composed of endolysin, holin, and spanin. The genome also harbored an integrase gene and a gene encoding a recombineering protein confirming its temperate nature. The replication module was comprised of a primosomal protein and a replication associated protein. In addition, the genome had a gene encoding an acyltransferase. This acyltransferase has been shown to alter host surface antigens and provide superinfection immunity in *Escherichia* phage phiV10 (Perry et al., 2009). Electron microscopy and VIRFAM analysis confirmed phage Tyrion to be member of the *Podoviridae* family (Figure 6.1).

The wide distribution of bacteriophage Tyrion-like genes in multiple pro-phages in the *Enterobacteriaceae* family and its potential ability to provide superinfection immunity makes it a good candidate to study bacteriophage-host interactions (Boyd and Brüssow, 2002).

The complete annotated genome sequence of the *Enterobacter* phage Tyrion can be accessed under the GenBank accession no. KX231829.1.

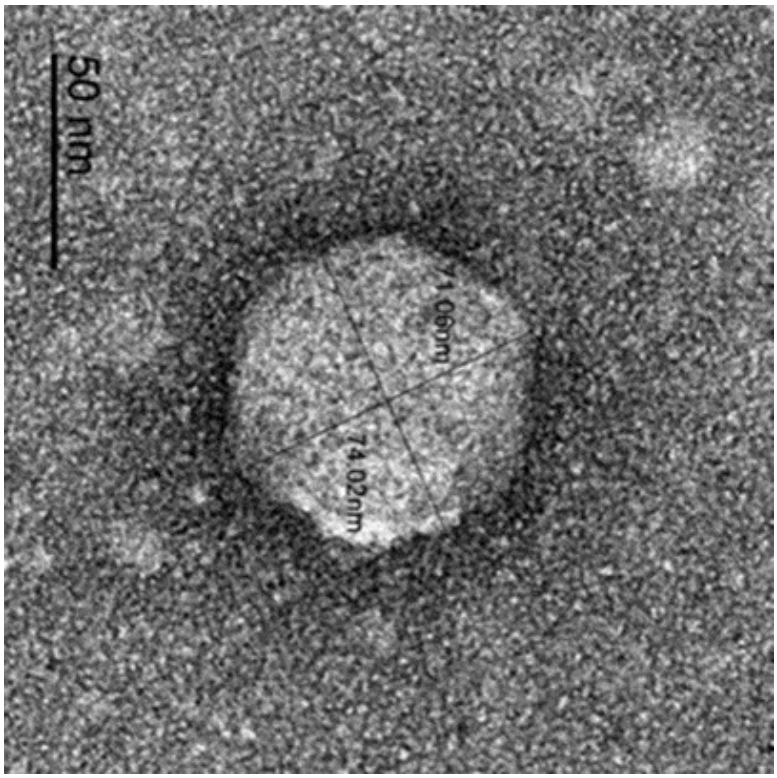

Figure 6.1: Electron micrograph of *Enterobacter* phage Tyrion (Image taken on JEOL JEM-2011 Transmission Electron Microscope)

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Chapter 7: Whole genome sequencing of a novel *Enterobacter* bacteriophage Arya with a truncated integrase, isolated from the termite gut

7.1 Introduction

In our previous studies we isolated two novel bacteriophages from the termite gut indicating a population of unexplored novel bacteriophages (Tikhe et al., 2015). In this study we isolated a novel bacteriophage Arya infecting *Enterobacter* sp. CT7 (KT204538.1), making it the third bacteriophage isolated from the termite gut and the second to infect the termite gut bacterium *Enterobacter* sp. CT7.

7.2 Materials and methods

Bacteriophage Arya was isolated from the gut of the Formosan subterranean termite *Coptotermes formosanus*. Phage isolation, electron microscopy, DNA extraction, sequencing, and annotation were carried out as described previously (Tikhe et al., 2015).

7.3 Results and discussion

Bacteriophage Arya produced small but clear plaques on *Enterobacter* sp. CT7. Sequenced DNA produced a linear contig with terminal repeats. PCR and restriction digestion confirmed the DNA to be circularly permuted. Arya has a genome length of 41,918 bp with a G+C content of 54%. The genome has a total of 64 predicted protein coding sequences and an Arginine tRNA gene. Of the 64 genes, 55 produced a match in the NCBI GenBank protein nr database. Based on the sequence similarity to the database, 22 proteins could be assigned a function. The overall genome architecture is conserved with a packaging module, a structural module, a DNA replication-metabolism module, and a lysis cassette. The genome also has a putative predicted integrase gene. The genome of phage Arya showed high level of synteny to *Escherichia* phage

vB_EcoM_ECO1230-10 (Santos and Bicalho, 2011), *Escherichia* phage vB_EcoM-ep3 (Lv et al., 2015) and *Pseudomonas* phage PPpW-3 (Park et al., 2000). Both the *Escherichia* bacteriophages vB_EcoM_ECO1230-10 and vB_EcoM-ep3 are lytic and no integrase or other lysogenic genes are present in their genomes (Lv et al., 2015, Santos and Bicalho, 2011). Despite of being lytic, phage PPpW-3 harbors a predicted integrase gene and an arginine tRNA next to the integrase gene like phage Arya. Similar to phage PPpW-3, Arya did not show the presence of other essential lysogenic genes (repressor, anti-repressor genes). The presence of integrase gene in phage PPpW-3 is hypothesized to be a result of a horizontal gene transfer event (Santos and Bicalho, 2011). The predicted integrase from phage Arya encodes for a 43 amino acid product which is very small compared to other functional integrase genes and thus is most likely non-functional (Groth and Calos, 2004). Presence of a similar arrangement of integrase gene and an arginine tRNA in phage Arya and PPpW-3 suggests an evolutionary relation between the two. Electron microscopy and VIRFAM (Lopes et al., 2014) analysis confirmed phage Arya to be a member of the *Myoviridae* family (Figure 7.1).

In a previous study we have isolated bacteriophage Tyrion, which also infects *Enterobacter* sp. CT7. Phage Tyrion is lysogenic and is predicted to alter the host cell receptors to provide superinfection immunity against the host. Isolation and sequencing of bacteriophage Arya provides us with a model system to study superinfection immunity and the dynamics between a bacterial host, a lytic phage, and a lysogenic phage.

The complete annotated sequence of the *Enterobacter* phage Arya genome can be accessed under the GenBank accession no. KX231828.1.

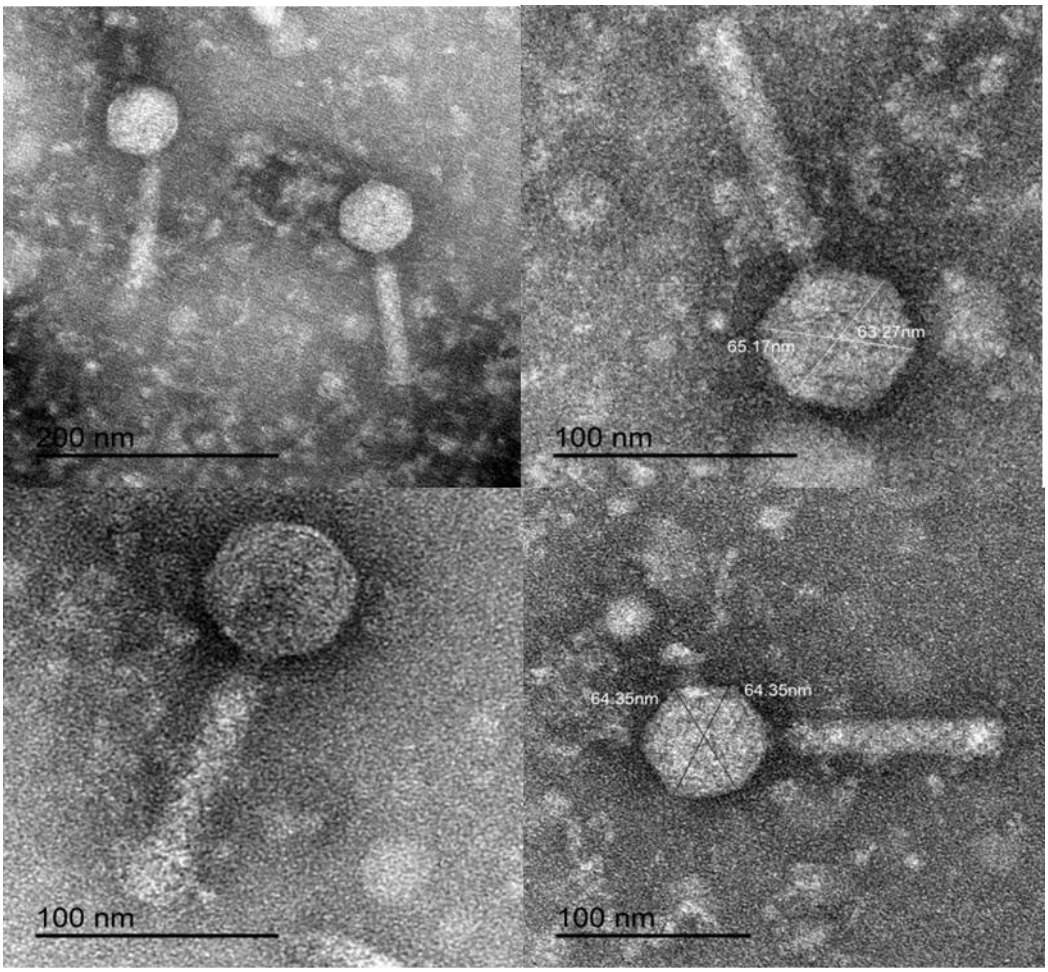

Figure 7.1: Electron micrographs of *Enterobacter* phage Arya (Images taken on JEOL JEM-2011 Transmission Electron Microscope)

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Chapter 8: Meta-virome sequencing of the termite gut gives insight into a quadripartite relationship between the termite-protozoa-bacteria and bacteriophages

8.1 Introduction

Bacteriophages are considered as the most abundant biological entities on earth with their total number estimated to be 10^{31} (Wommack and Colwell, 2000, Suttle, 2005). Bacteriophages play an important role in the ecosystem by carrying out nutrient recycling via bacterial cell lysis (Wilhelm and Suttle, 1999). Bacteriophages also carry out horizontal gene transfer and are considered as a driving force behind bacterial genome evolution (Ochman et al., 2000, de la Cruz and Davies, 2000). Along with horizontal gene transfer, bacterial-phage antagonistic co-evolution is hypothesized to have a major impact on bacterial and bacteriophage diversification (Buckling and Rainey, 2002, Paterson et al., 2010). Previous studies have shown that the majority of the differences observed in the genomes of different bacterial strains of the same species in the human gut were related to restriction-modification systems, and glycosyltransferases, which play a key role in phage defense (Zhu et al., 2015). Bacteriophage-resistant strains have been shown to possess modifications in their surface receptors like o-antigens and outer membrane proteins (Labrie et al., 2010, Bassford et al., 1977). These surface receptors also play an important role in cell surface adhesion (Shin et al., 2005) and thus may alter the interactions of bacterial strains with their host (Lerouge and Vanderleyden, 2002). Co-evolution of bacteriophages and bacteria has been shown to alter the metabolic capacities of bacteriophage-resistant strains including the ability to utilize certain carbon sources (Middelboe et al., 2009). All these observations show that phage-bacterial interactions can have a profound effect on the ecology of the microbial community and can in turn affect the host harboring the community.

Termites rely on their symbiotic gut microbial community for cellulose digestion and acquisition of essential nutrients [reviewed in (Brune, 2014)]. The microbial community of the higher termites (family: Termitidae) is comprised of bacterial symbionts while the lower termites (Rhinotermitidae, Mastotermitidae, Hodotermitidae, Kalotermitidae, Termopsidae, Serritermitidae) harbor flagellated protozoa in addition to the bacterial symbionts. Workers of the Formosan subterranean termite (FST), *Coptotermes formosanus* (Family: Rhinotermitidae), harbor three species of protozoa in the hindgut paunch, which are essential for the survival of the termite colony (Eutik et al., 1978). The worker caste specializes in providing nutrition for the entire termite colony by digesting lignocellulose and the protozoa enhance the endogenous cellulolytic capability of the worker gut. The protozoa also harbor endo and ecto-symbiotic bacteria which carry out nitrogen fixation and amino acid production (Hongoh et al., 2008, Desai and Brune, 2012). The termite gut microbial community is responsible for many complex biochemical processes, providing the termite host with essential nutrients. In a previous study based on cloning of 16S rRNA gene amplicons, at least 213 different bacterial ribotypes were reported from the gut of the FST (Husseneder et al., 2010). A closely related species, *Coptotermes gestroi* was estimated to harbor a bacterial community of 1460 different species using next-generation sequencing (Do et al., 2014). The termite gut presents a unique scenario in which the host is heavily dependent on the microbial community and any potential changes can have detrimental effects on the host (Rosengaus et al., 2011). The presence of a multifaceted complex bacterial community makes the termite gut a perfect ecological niche for the presence of a diverse bacteriophage population which remains unstudied so far.

We previously isolated and sequenced the first bacteriophage of a termite, i.e., CVT22, which infects *Citrobacter* sp. from the gut of the FST (Tikhe et al., 2015). Two additional novel

bacteriophages, Tyrion and Arya (NC_031077.1 and NC_031048.1), infecting *Enterobacter* sp. were also isolated and sequenced from the termite gut (unpublished data). In a recent study, a bacteriophage infecting "Candidatus *Azobacteroides pseudotrichonymphae*" was discovered making it the first bacteriophage to be associated with an obligate intracellular mutualistic endosymbiont (Pramono et al., 2017). These reports of novel bacteriophages in the termite gut indicate the presence of an unexplored bacteriophage population. The main obstacle for studying the bacteriophages from the termite gut using conventional isolation technique is the unculturable bacterial community (Hongoh, 2010). Metavirome sequencing circumvents the requirement of isolation and therefore, we chose this approach to study the bacteriophage population in the FST gut in its entirety.

In this study we report the virome sequencing of FST gut with the intention of exploring the unstudied bacteriophage diversity. This study is the first effort focused entirely on uncovering the bacteriophages and any other possible viruses associated with a termite species. Studying the virome of the termite gut will help us to understand the complex quadripartite relationship between the termite host, protozoa, and bacteria symbionts as well as associated bacteriophages.

8.2 Materials and methods

8.2.1 Termite collection

Workers of the (FST) were collected from three different colonies in New Orleans, Louisiana using untreated in-ground bait stations. Termite colonies were designated as Colony 1 (collected from City Park, on 06/21/2013), Colony 2 (collected from Hayne Blvd., lakefront on 06/18/2013) and Colony 3 (collected from Cypress St. on 06/21/2013). All the termites were

brought back to the lab in a plastic container containing moist filter paper. Termites were processed immediately for viral community DNA extraction.

8.2.2 Extraction of viral community DNA

A total of 500 worker termites from each colony were dissected and their guts were suspended in 3 ml sterile phosphate buffered saline (pH 7.5) kept on ice. The guts were homogenized vigorously using a sterile pestle until a uniform solution was formed. The homogenate was centrifuged at 10,000 g and the supernatant was filtered twice through a 0.22 µm syringe filter. The filtered homogenate was then treated with 2.5 units per µl of RNase A and DNase I at 37°C for 6 h. The filtrate was then mixed with with 200 µl of 0.5M EDTA, and DNA was isolated using phenol-chloroform-isoamyl alcohol extraction. Concentration and quality of the extracted DNA was checked with NanoDrop® ND1000. Bacterial DNA contamination was checked via a PCR using 27f and 1492r universal 16S rRNA gene primers. The initial concentrations of the extracted DNA from Colony 1, Colony 2 and Colony3 were 4 ng/ µl, 1.8 ng/ µl, and 2.5 ng/ µl, respectively. The extracted DNA was amplified using illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life sciences, Pittsburgh, USA). The amplified DNA was then ethanol precipitated and was dissolved in sterile distilled water.

8.2.3 Next generation sequencing and bioinformatics analysis

From each of the three colonies, 50 ng of DNA was used to prepare the libraries using Nextera DNA Sample Preparation Kit (Illumina). The insert size was determined by Experion Automated Electrophoresis Station (Bio-Rad). The insert size of the libraries ranged from 300 to 850 bp (average 500 bp). Individual libraries were sequenced at Molecular Research LP, Shallowater, Texas, on the Illumina MiSeq platform (2 × 250 bp) for Colony 1, while for Colony 2 and

Colony 3 MiSeq platform (2 × 300 bp) was used. Quality of the DNA reads was checked using FASTQC (Andrews, 2010). DNA reads were checked for Illumina adaptor contamination and reads below the Phred score of 20 were removed using Trim Galore (Krueger, 2015). DNA reads were assembled into contigs using SPAdes Genome Assembler (Version 3.0) available on Illumina BaseSpace platform with the default parameters using error correction and assembly mode (Bankevich et al., 2012). The contigs obtained were uploaded on the Metavir server for taxonomic assignments of the predicted ORFs using RefSeq complete viral genome protein sequence database from NCBI (released on 01/11/2017) (Roux et al., 2014). All the predicted protein coding genes were also blasted against the protein sequences from bacteriophage ProJPt-1Bp1 (Pramono et al., 2017). Contigs available on Metavir were screened for the presence of *VP1*, *TerL*, and *Rep* genes. For the construction of phylogenetic trees, we used full length amino acid sequences of terminase large subunit TerL (terminase_1, terminase_6, terminase_3, terminase_GPA, and terminase_1), Microviridae VP1, and Circoviridae Rep proteins. The sequences were aligned using MUSCLE (Edgar, 2004). Maximum likelihood trees were constructed using PhyML algorithm with a WAG substitution model (Guindon et al., 2010). For *Microviridae* subfamily assignment, full-length amino acid sequences of VP1 protein from (Quaiser et al., 2015) were used to construct a phylogenetic tree. Contigs containing *VP1, TerL, Rep* genes were analyzed manually for the presence of other putative viral genes. Contigs were classified as of a viral origin using the parameters described previously with the POG13 database used instead of POG10 (Bellas et al., 2015) . Putative partial or full phage genomes were annotated manually and comparative genomic diagrams were generated using Easyfig (Sullivan et al., 2011). Putative viral genomes were visualized using CGview (Grant and Stothard, 2008) and SnapGene® (from GSL Biotech; available at snapgene.com). PHACTS analysis was carried

out to determine the lifestyle and host of the putative phages (McNair et al., 2012). Family assignment of putative phage genomes was performed with VIRFAM using the ACLAME database (Lopes et al., 2014). For contig LSPY100002, RNA polymerase beta and beta' subunit sequences from phiKZ like bacteriophages were used to construct phylogenetic trees (Lavysh et al., 2016). Functional annotation was carried out using the MG-RAST automated pipeline with an integrated M5NR database (Keegan et al., 2016). Orthologous genes from the three colonies were compared against each other using Orthovenn (Wang et al., 2015). All the assembled contigs have been submitted to NCBI GenBank under the accession numbers LSPY0000, LSQA0000, and LSPZ0000. Fully annotated contigs from this study are available publicly on Metavir server under the study named "termite gut metavirome". (http://metavir-meb.univ-bpclermont.fr).

8.3 Results and discussion

8.3.1 Next generation sequencing and bioinformatics analysis

No bacterial contamination was detected when the products from PCR with 27f and 1492r universal 16S rRNA primers were run on a 1% agarose gel.

The sequencing data, predicted genes by Metavir and MG-RAST, and the number of circular contigs predicted by Metavir are summarized in Table 1.

Table 8.1: Sequencing data, gene prediction, and number of circular contigs from viral DNA isolated from the guts of the FST workers from three different colonies**.**

	Colony 1	Colony 2	Colony 3
# raw reads	2,693,057 [a]	1,670,422 [b]	1,293,080 [b]
# contigs (pre NCBI/ MG-RAST QC)	4,413	10,539	9,440
N50/N75	5,157/1,216	4330/1000	3202/949
# contigs (post NCBI/MG-RAST QC)	4347	10022	9190
Largest contig	251606	299025	246064
GC (%)	41±9	45±10	40±9
# genes predicted (Metavir)	9497	22389	21850
# predicted proteins (MG-RAST)	6523	14282	14723
# of circular contigs	79	104	132

[a]Illumina MiSeq platform (2 × 250 bp), [b] Illumina MiSeq platform (2 × 300 bp)

8.3.2 Taxonomic assignment

Metavir assigned 27.13% of the genes from Colony 1, 27.85 % from Colony 2, and 28.9 % from Colony 3 as virus affiliated genes with an e-value of 10^{-5} or less. Compared to Metavir, MG-RAST classified fewer genes as viral. Percentage of genes classified as viral for Colony 1 was 9.57%, for Colony 2 was 3.8%, and for Colony 3 was 3.53 %. Taxonomically MG-RAST classified between 93-79 % of the genes as bacterial. The difference between the taxonomic assignments by MG-RAST and Metavir has been previously observed in the metavirome sequencing of Antarctic soils (Zablocki et al., 2014). The taxonomic assignment of phage DNA as bacterial is likely due to the fact that reference databases classify pro-phages as bacterial when

they are integrated into a bacterial chromosome at the time of genome sequencing. The number of sequenced bacterial genomes is reaching the 100,000 mark while the bacteriophage genomes still remain poorly represented in the NCBI database (around 2000 Caudovirales genomes). Sequencing more phage genomes is paramount to improve recognition of pro-phage sequences in bacterial genomes and will improve taxonomic assignments in all virome studies.

Previous studies showed that Bacteroidetes form around 70% of the bacterial flora of the *Coptotermes formosanus* gut (Noda et al., 2005, Shinzato et al., 2005). For this reason we expected the Bacteroidetes phages to dominate the virome composition. The virome composition, however, was different from what we expected, with Proteobacteria and Firmicutes comprising at least 40% of the identified genes. This difference could be explained by the small number of sequenced Bacteroidetes bacteriophages. Since we sequenced filtrate from the termite gut it was not surprising that viral sequences were at least 42 times enriched in our data as compared to the unfiltered metagenome of a higher termite, *Nasutitermes* species (Warnecke et al., 2007). We also observed that functionally, phage-related sequences were at least 10 times enriched in our data. The overall enrichment in phage-related genes indicates a successful separation of bacterial contaminants during viral DNA purification.

Taxonomic assignment using Metavir showed that dsDNA virus related genes were dominant among all the viral genes (Figure 8.1 A). The dsDNA viral genes predominantly belonged to the tailed bacteriophages from the order Caudovirales (Figure 8.1 B). Genes related to all three families of the order Caudovirales i.e., Myoviridae, Siphoviridae, and Podoviridae were present in all three termite colonies (Figure 8.1 D). Apart from the genes related to Caudovirales, genes related to large eukaryotic dsDNA viruses and other unclassified viruses were also present in all the three termite colonies. The single stranded DNA viruses (ssDNA) contributed between 1-

10% of the total virus related genes. The ssDNA viruses were dominated by Microviridae phages, contributing between 62-65% (Figure 8.1 C). Percentage of each virus group, present in the three different termite colonies is described in Figure 8.1.

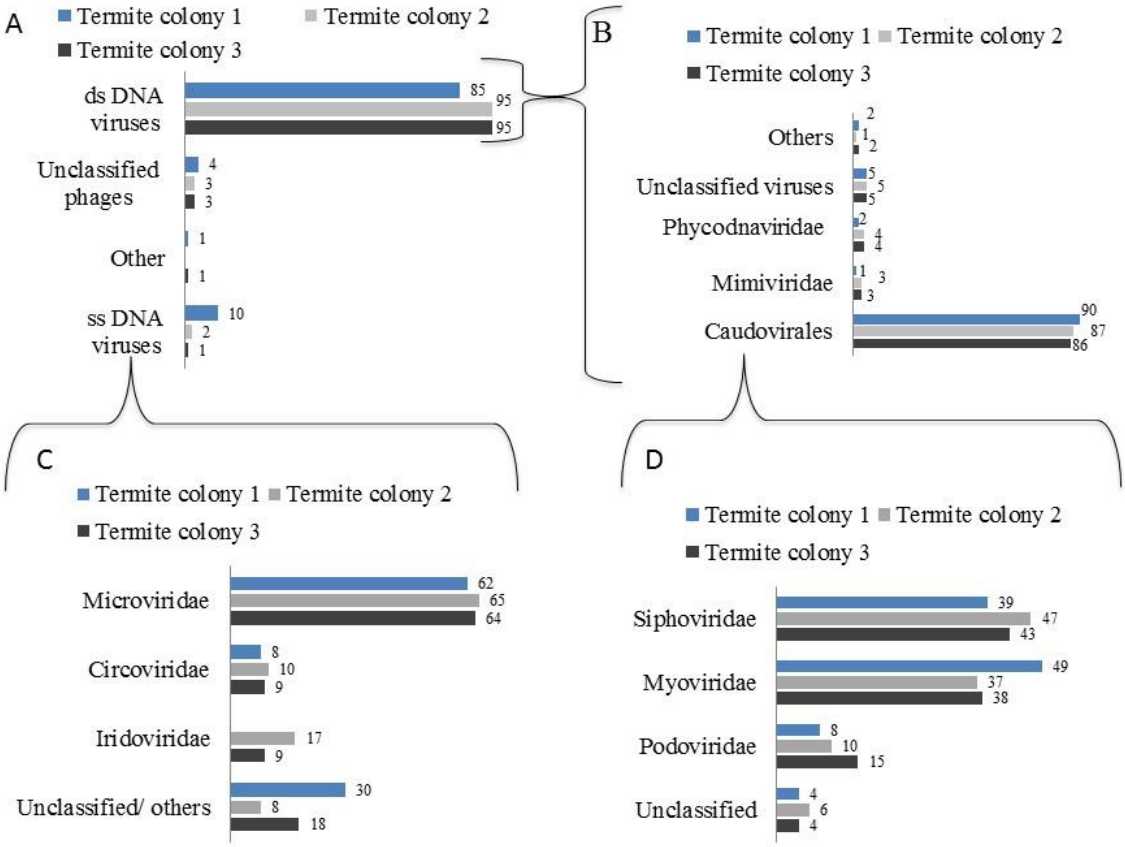

Figure 8.1: Classification and abundance of various virus types observed in the guts of the termites from three different colonies. The data was generated using Metavir-2 server

8.3.3 Diversity of tailed bacteriophages

Among all three termite colonies, Colony 3 was the most diverse in terms of tailed bacteriophages, with genes related to 712 different bacteriophages followed by Colony 2 (598)

and colony 1 (389). Genes related to a total of 960 different tailed bacteriophages were observed across all three termite colonies.

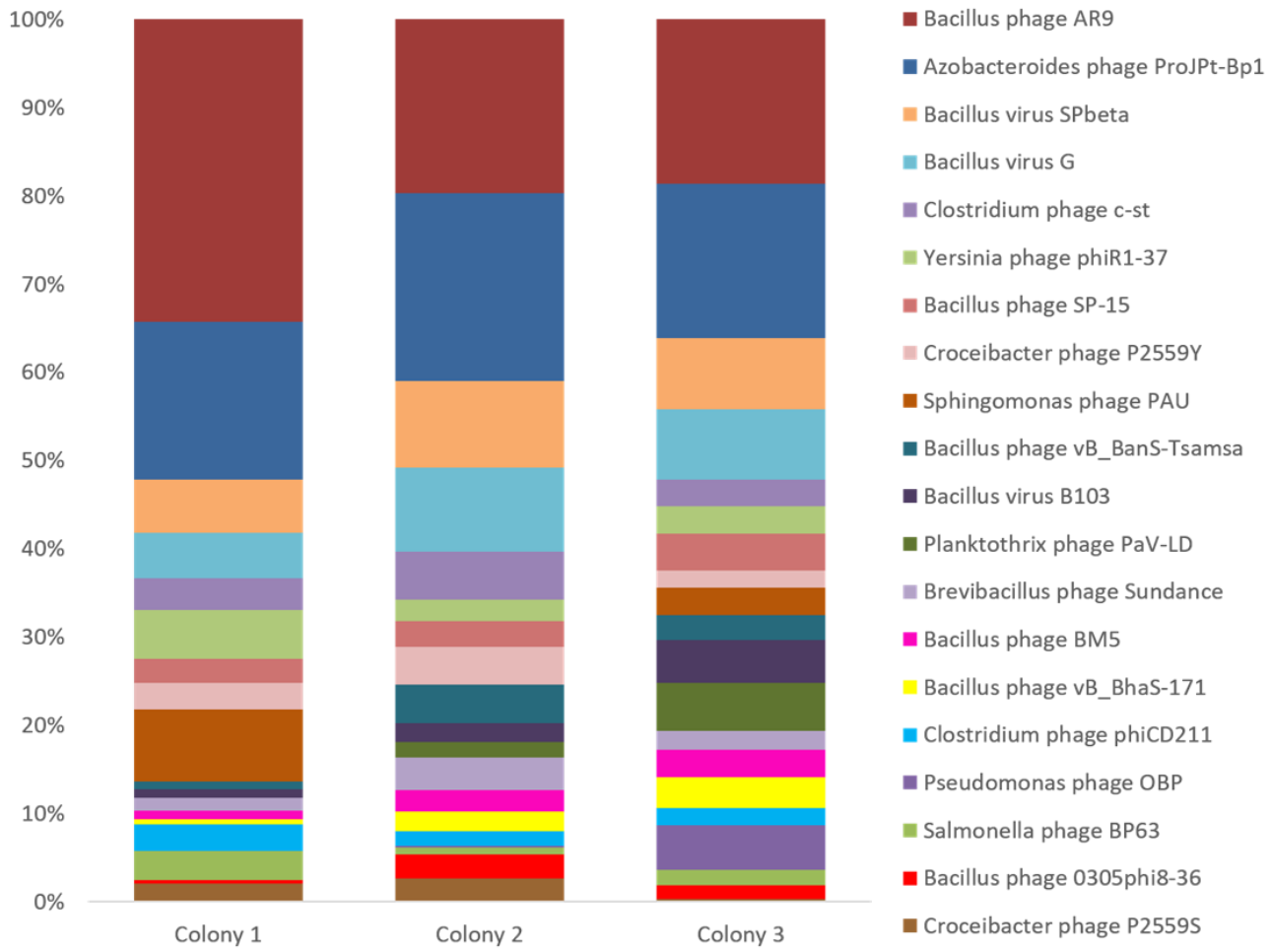

Figure 8.2: Percent distribution of the predominant dsDNA bacteriophage species from the guts of the termites from three different colonies. The data was generated using Metavir-2 server by comparing the predicted proteins to the NCBI virus protein database. Only top BLAST hits with an e value of 10^{-5} or less were used

This number accounts for 48.65 % of all tailed bacteriophages whose complete genomes are available in the NCBI Genbank database (04/12/2017). Of these 960 bacteriophages,

Siphoviriadae-related bacteriophages were the most diverse representing 483 different bacteriophage species, followed by Myoviridae (335) and Podoviridae (142). A considerable number of bacteriophages (218) were shared by all three termite colonies, with *Bacillus* phage AR9 (Lavysh et al., 2016) and *Azobacteroides* phage ProJPt-1Bp1 (Pramono et al., 2017) related genes being present in the highest proportions (Figure 8.2). In Colony 1, 10% of all the classified sequences belonged to *Bacillus* phage AR9 followed by Colony 2 (6%) and Colony 3(4%). *Azobacteroides* phage ProJPt-1Bp1 related genes constituted 6.45% of all the classified genes in Colony 1, 5.61 % in Colony 2, and 3.92% in Colony 3. Out of the top 20 most dominant tailed bacteriophage related genes from all the three termite colonies, 12 bacteriophages had Firmicutes as their host out of which 9 bacteriophages were infecting *Bacillus* spp.

The analysis of phage diversity of the three termite colonies suggests the presence of a core virome of tailed bacteriophages in the termite gut. Although there is some degree of inter-colonial variation, nearly a quarter of all tailed bacteriophages (23%) were present in all three colonies. This hypothesis, however, needs to be confirmed by a larger study including more colonies from the introduced and native distribution areas of the FST.

8.3.4 Phylogenetic analysis of terminase genes

From all three termite colonies together, 51 unique full length terminase large subunit amino acid sequences were obtained. Out of the 51 sequences, 25 contained terminase_6 (pfam03237) domains, 14 contained terminase_3 (pfam04466.8) domains, 9 had terminase_gpa (pfam05876) domains, 2 had terminase_1 (pfam03354) domains, and one sequence could not be classified (and was, therefore, not used in phylogenetic analysis). Our data of terminase diversity in the termite gut is comparable to the results from virome sequencing of the deep sea, where 52 unique terminase sequences were identified (Mizuno et al., 2016). Most of the terminase sequences from

the termite gut matched closely to the terminase genes from the many prophage regions in the bacterial genomes (Figure 8.3).

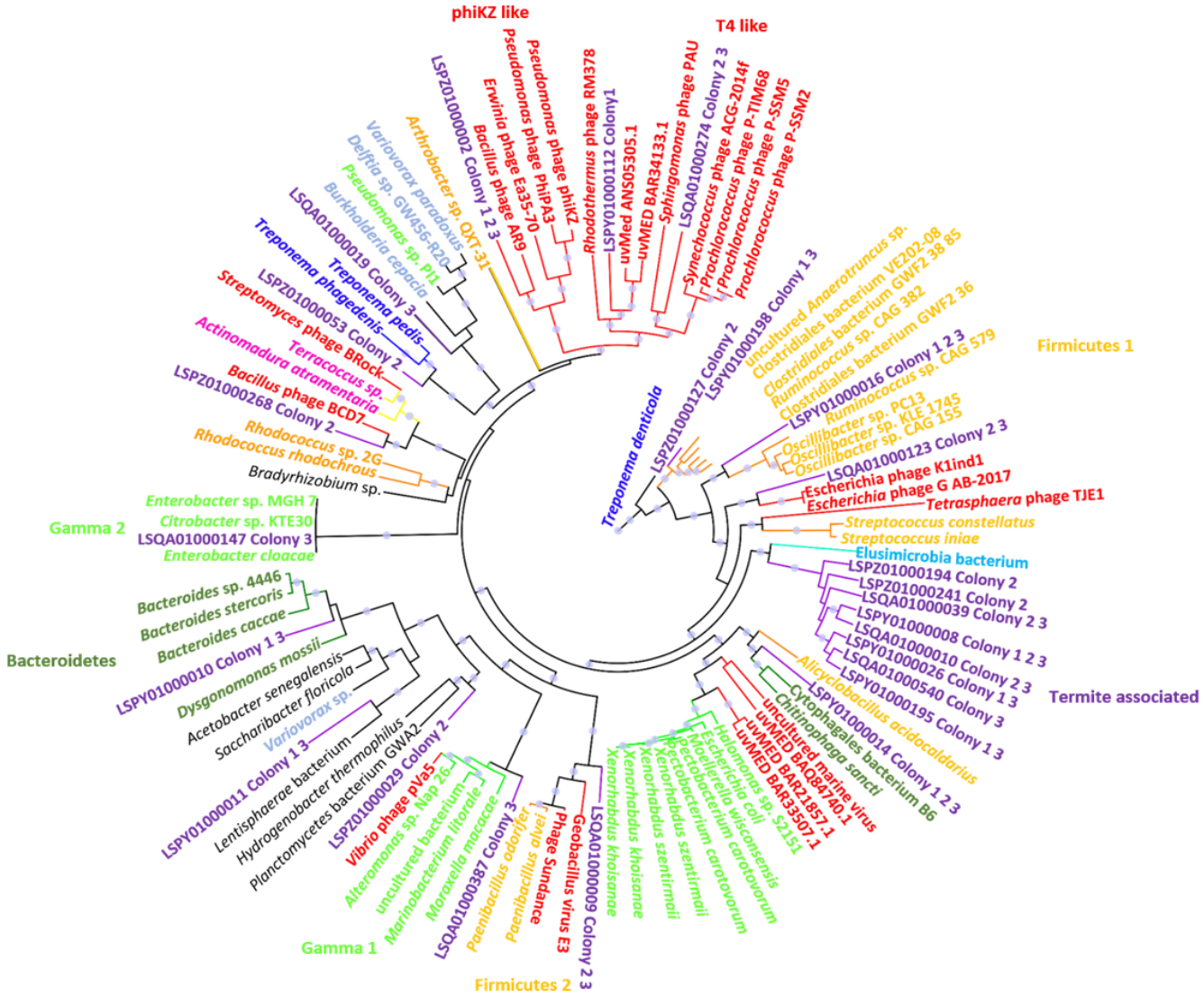

Figure 8.3: A maximum likelihood phylogenetic tree of large terminase subunit of type terminase_6. The nodes with a bootstrap value of 70% or more are indicated by a circular symbol. Sequences from the termite gut are colored purple. Bacteriophages: red. Firmicutes: orange. Spirochetes: dark blue. Gammaproteobacteria: bright green. Bacteroidetes: dark green. Actinomycetes: pink. Alphaproteobacteria: light blue. Termite Group I bacterium: sky blue. Others: black.

Phylogenetic analysis of terminase_6 showed a vast diversity with matches to prophages associated with a wide range of bacterial taxa. These results were also similar to the deep sea sequencing data, where terminase_6 domain was the most abundant type (Mizuno et al., 2016). Eight terminases from the virome formed a separate clade comprising a terminase gene from a Termite Group 1 bacterium. Two terminase genes were clustered with two separate Firmicute clades, another two were clustered with two separate Gammaproteobacteria clades, and one was clustered with a Bacteroidetes clade. Another clade was comprised entirely of bacteriophages from the Myoiridae family and three genes from the termite gut. The clade was further subdivided in phiKZ like bacteriophages and T4 like bacteriophages (Figure 8.3). Remaining genes were present in multiple clades comprised of terminases from taxonomically diverse bacterial phyla.

Phylogenetically, terminase 3 genes were divided in four clearly separated clades. The Alphaproteobacteria clade and the Bacteroidetes clade each contained three terminases associated with the termite gut virome, while both the Enterobacteriaceae and the Firmicute clade contained one. Two other terminases found in the termite gut formed separate branches while one was grouped with *Clostridium* sp. CAG 306 (Figure 8.4). A number of subunits of Terminase GPA from the termite gut virome were assigned to two distinct clades of Spirochetes and Alphaproteobacteria. The Spirochetes cluster contained five terminase GPA subunits from the termite gut while another subunit formed a sister clade with Alphaproteobacteria. (Figure 8.5).

The phylogenetic analysis of the terminase gene indicated that most of the genes matched to prophage genomes rather than sequenced bacteriophage genomes. The results suggest that most of the termite gut bacteriophages might be temperate in nature.

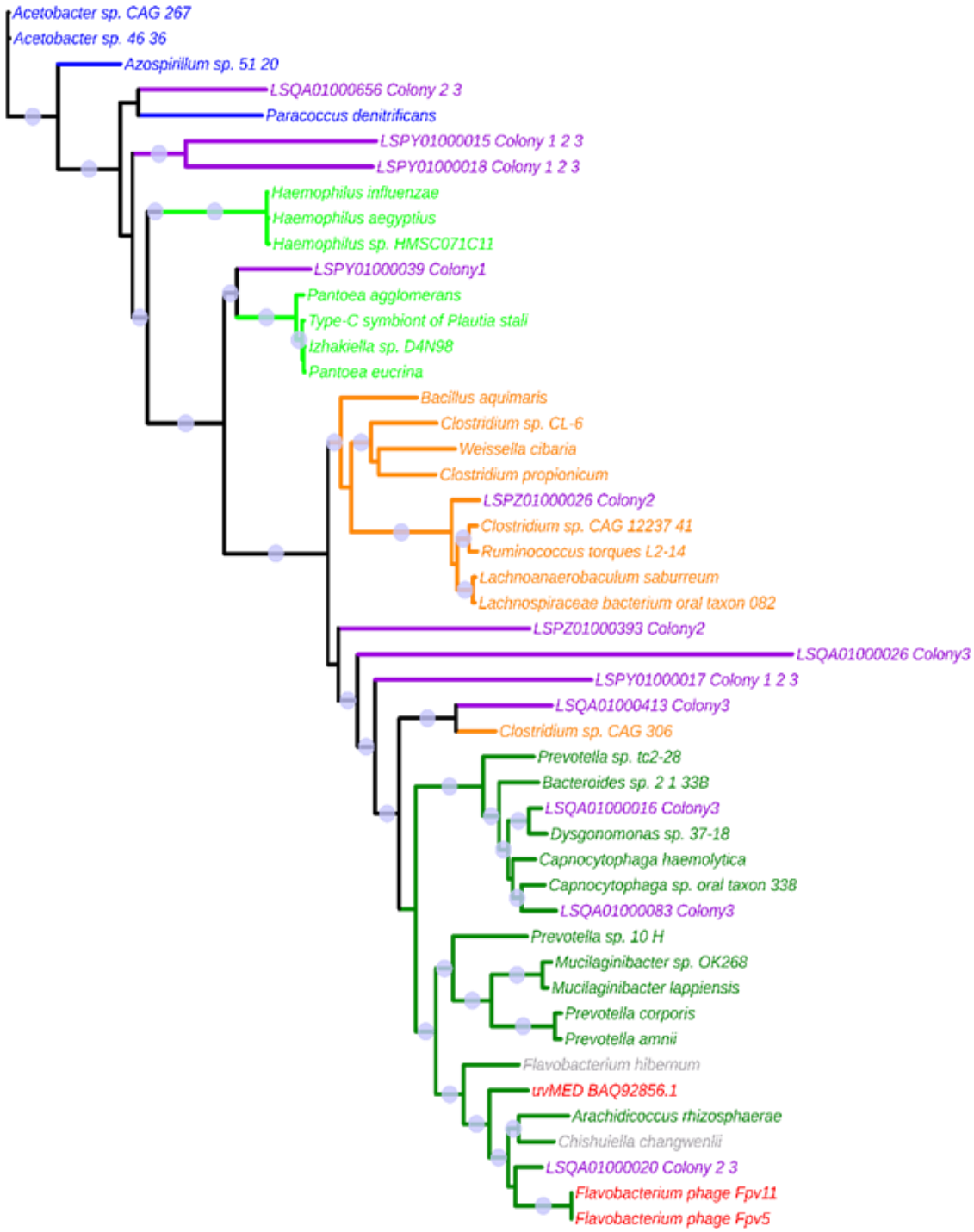

Figure 8.4: A maximum likelihood phylogenetic tree of large terminase subunit of type terminase_3. The nodes with a bootstrap value of 70% or more are indicated by a circular symbol. Sequences from the termite gut are colored purple, Bacteriophages: red. Firmicutes: orange. Spirochetes: dark blue. Gammaproteobacteria: bright green. Bacteroidetes: dark green.

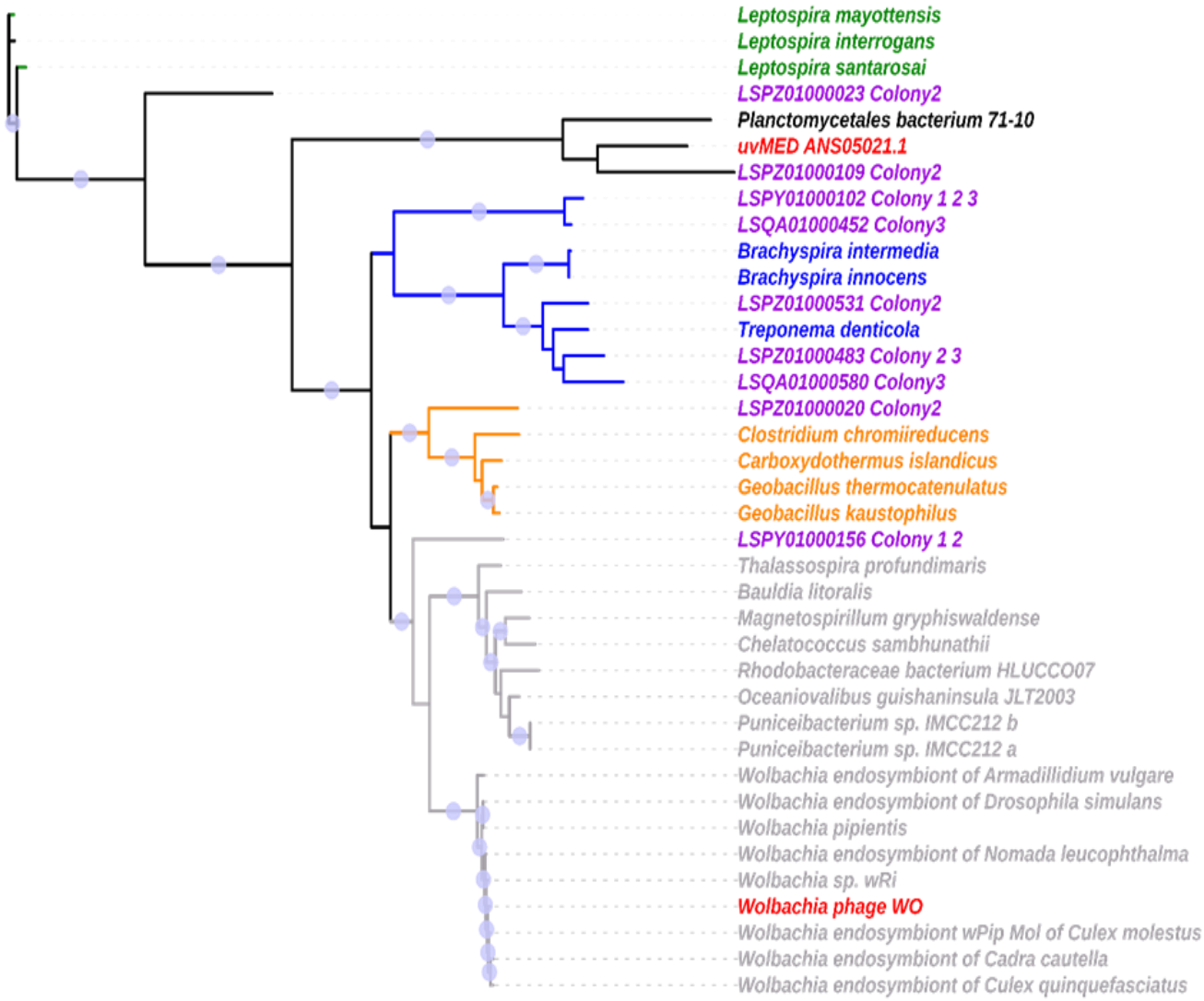

Figure 8.5: A maximum likelihood phylogenetic tree of large terminase subunit of type terminase_GPA. The nodes with a bootstrap value of 70% or more are indicated by a circular symbol. Sequences from the termite gut are colored purple. Firmicutes: orange. Bacteroidetes: dark green Spirochetes: dark blue. Bacteriophages: red. Alphaproteobacteria: grey. Others: black.

8.3.5 Phylogenetic analysis of the integrase genes

The integrase gene is used by the temperate bacteriophages to enter the lysogenic life cycle. It has been shown that prophage integrates in the host genome with a minimum impact on the

overall chromosomal architecture. The bacteriophage also undergoes numerous adaptations according to the host genome in order to successfully establish a lysogenic life cycle (Brüssow et al., 2004). It can be assumed that temperate bacteriophages are likely to infect closely-related bacteria or bacteria where the overall genome architecture is conserved. Thus, phylogenetic analysis of phage integrase is likely to yield more information about its host. A total of 31 unique phage integrase sequences were identified from three termite colonies. Phylogenetic analysis of phage integrase genes also showed that sequences from the termite gut are clustered with a wide range of bacterial taxa (Figure 8.6). Five termite gut integrases were clustered within a Spirochete clade, six were associated with Firmicutes, another six were distributed in two clades comprised of Spirochetes and Bacteroidetes, and four were associated with two clades comprised of Bacteroidetes and Firmicutes. The remaining genes were distributed in clades comprised of diverse bacterial phyla. The results suggest the presence of temperate bacteriophages capable of infecting all the major bacteria taxa in the termite gut. The same integrase genes were also found to be present in multiple termite colonies, which points toward a conserved temperate bacteriophage population.

8.3.6 Putative contigs of dsDNA bacteriophage origin

Based on the criteria described previously (Bellas et al., 2015), many contigs were considered to represent putative complete or partial bacteriophage genomes.

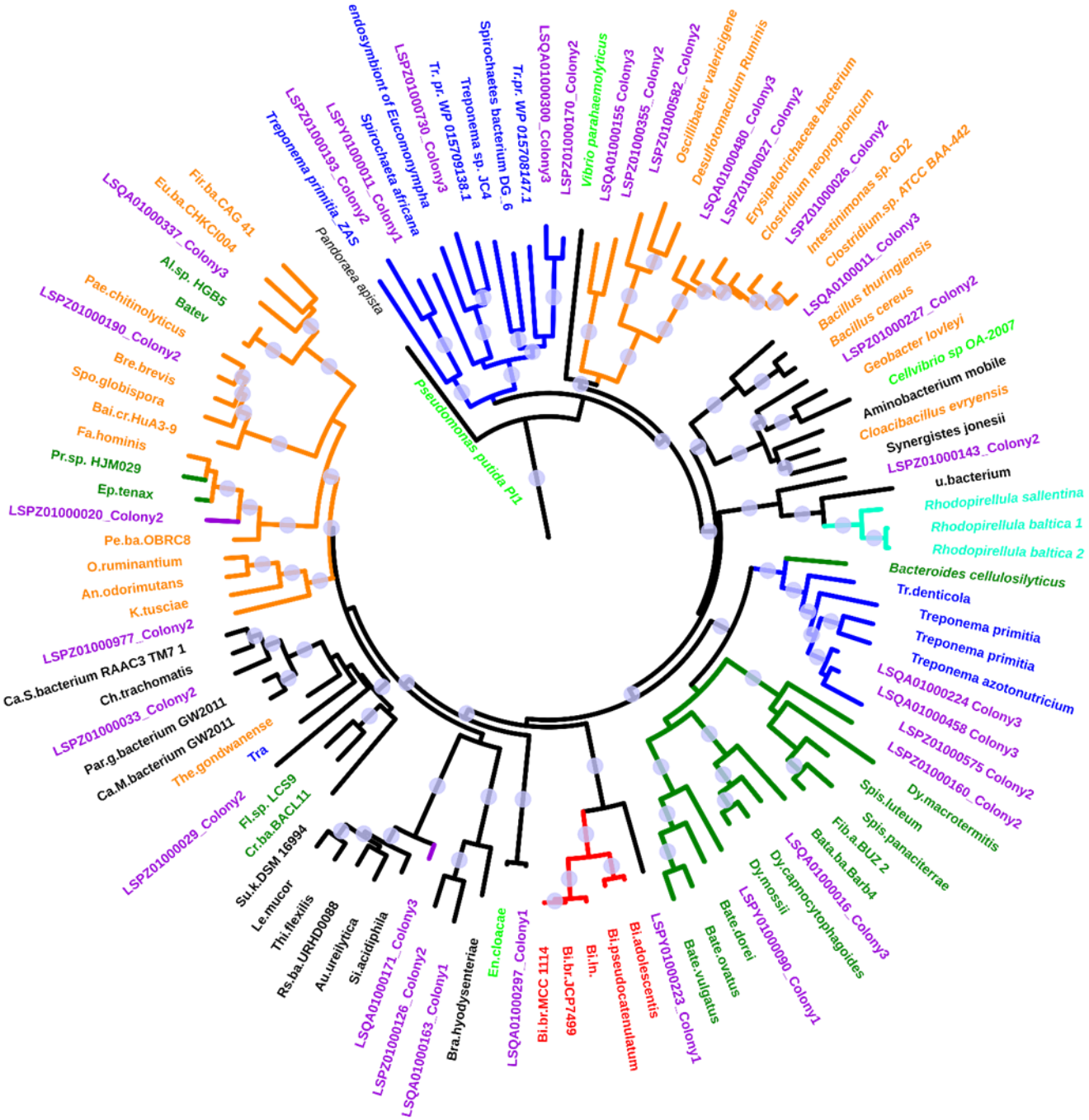

Figure 8.6: A maximum likelihood phylogenetic tree of phage integrase genes. The nodes with a bootstrap value of 70% or more are indicated by a circular symbol. Sequences from the termite gut are colored purple. Firmicutes: orange. Actinobacteria: red. Spirochetes: dark blue. Gammaproteobacteria: bright green. Bacteroidetes: dark green. Planctomycetes: sky blue. Others: black.

8.3.6.1 LSPY01000004 and LSPY01000006 represent genomes of bacteriophages infecting the symbiotic bacteria of the gut protozoa

Both LSPY01000004 and LSPY01000006 were predicted as circular contigs in termite Colony 1. Generally circular contigs are indicative of a complete genome. Contigs mapping onto LSPY01000004 and LSPY01000006 were present in all the three termite colonies, suggesting an inter-colonial conserved distribution. Out of the 68 predicted genes in LSPY01000004, 30 produced a match in NCBI nr protein database with an e-value of 10^{-5} or less. Of those 30, 21 genes matched only to *Azobacteroides* phage ProJPt-1Bp1, a bacteriophage infecting an obligatory intracellular bacterium Candidatus *Azobacteroides pseudotrichonymphae* of the termite gut protozoa (Pramono et al., 2017). Out of the remaining genes, five genes matched to two different plasmids from Ca. *A. pseudotrichonymphae* and one gene matched to the genome (Hongoh et al., 2008). A total of 65 genes were predicted in contig LSPY01000006, of which 36 produced a match in the NCBI database. Out of those 36, 22 matched phage ProJPt-1Bp1, 7 genes matched the plasmid of Ca. *A pseudotrichonymphae* and 1 gene matched the genome of Ca. *A pseudotrichonymphae*. There was very little similarity at nucleotide level in the genomes of LSPY01000004 and LSPY01000006 (73% match over 5 % of the genome); most of the similarity was observed in the region of conserved hypothetical proteins also found in the genome of ProJPt-1Bp1. Overall the genome arrangement of LSPY01000004, phage ProJPt-1Bp1, and LSPY01000006 was alike with areas of high similarity and synteny (Figure 8.7 A). LSPY01000004 and LSPY01000006 showed many differences in the hypothetical proteins. Notably LSPY01000006 harbored a gene similar to dihydrofolate reductase (DHFR) which was absent in LSPY01000004 and phage ProJPt-1Bp1. T4 bacteriophage DHFR has been predicted to play an important role in DNA metabolism and was also predicted to be a part of the virion particle (Mosher et al., 1977).

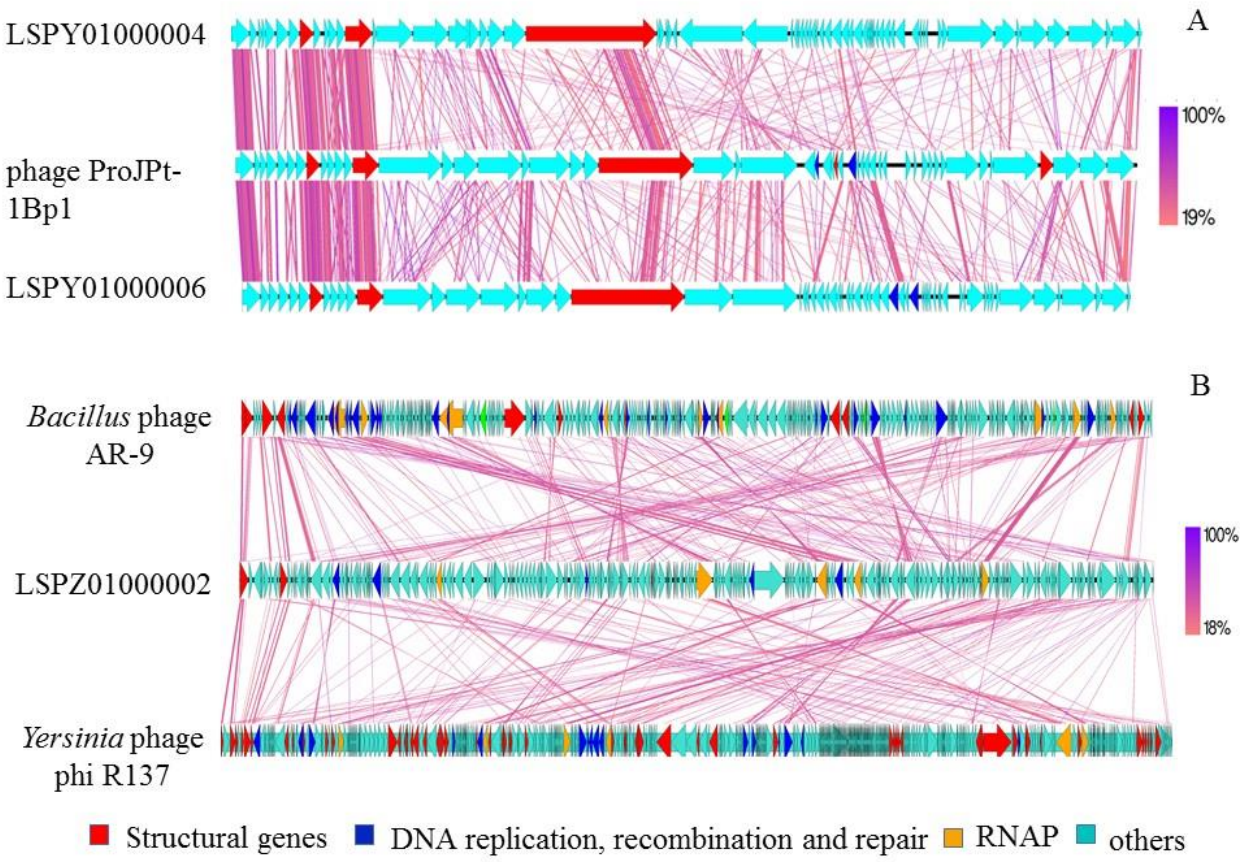

Figure 8.7: A) Comparative genomic analysis of LSPY01000004 and LSPY01000006 with *Azobacteroides* phage ProJPt-1Bp1. All three circular genomes have been rearranged so that the start codon of a conserved hypothetical protein is the first base in the sequence. B) Comparative genomic analysis of LSPZ01000002 with *Bacillus* phage AR9 and *Yersinia* phage phi R137. All three circular genomes have been rearranged so that the start codon of the large terminase subunit is the first base in the sequence. The Figures were generated using Easyfig software with tblastx. The structural genes are indicated in red, DNA metabolism related genes are indicated in blue and RNA polymerase genes are indicated in orange. All the other genes are indicated in sky blue.

However, at this moment the function of DHFR in bacteriophages remains to be studied.

VIRFAM analysis of ProJPt-1Bp1, LSPY01000004, and LSPY01000006 predicted them to be the members of Caudovirales; however, the contigs could not be assigned to any family. The genome of phage ProJPt-1Bp1 was sequenced while sequencing the host bacterial genome and a

t-RNA detected in the phage genome also matched to the host (Pramono et al., 2017). We were not able to identify any t-RNA in LSPY01000004 and LSPY01000006 or any other genes which could point toward the potential host of these phage-like genomes. However, based on the similarity of genes to phage ProJPt-1Bp1 and Ca. *A. pseudotrichonymphae*, LSPY01000004, and LSPY01000006 most likely infect Ca. *A. pseudotrichonymphae* or a closely related bacterial symbiont of the obligatory gut protozoa. Also, it should be noted that phage ProJPt-1Bp1 was isolated from Ca. *A. pseudotrichonymphae* from the gut of *Prorhinotermes japonicas* (Bellas et al., 2015). It has been demonstrated for Ca. *A. pseudotrichonymphae* that their protozoa hosts and the termites have co-diversified (Noda et al., 2007, Ikeda-Ohtsubo and Brune, 2009, Desai et al., 2010). This presents us with a possibility of co-diversification of bacteriophages along with their hosts. The observed differences and the conserved genome architecture between phage ProJPt-1Bp1, LSPY01000004 and LSPY01000006 can be explained by the bacteriophage co-diversification hypothesis. A study of more similar phage genomes from various termite species will shed more light on the association and co-diversification of bacteriophages, protozoa, their symbiotic bacteria, and the termite host. One interesting feature that was notable in LSPY01000004 and LSPY01000006 is the apparent absence of conserved phage genes like terminase, endolysins, and phage integrases. According to the best of our knowledge, no Caudovirales bacteriophage without the presence of a terminase gene has been found prior to our study. There are two explanations for the missing genes in LSPY01000004 and LSPY01000006. One is that these bacteriophages have lost their signature genes and are maintained in the bacteria as plasmids. Whether this relationship is symbiotic, as hypothesized in the case of phage ProJPt-1Bp1 (Pramono et al., 2017), remains to be explored. The other possibility is that these types of bacteriophages have a completely new method of carrying out essential functions such

as DNA packaging and host lysis. Gene expression analysis would answer the question of whether these bacteriophages are dormant or play any role in the bacterial metabolism, or enter an infective cycle. If ProJPt-1Bp1-like phages enter an infective cycle, studying their mode of infectivity would aid in finding the mechanism by which intracellular phages penetrate two different types of cells. It has been shown that *Wolbachia* phage WO can form virions and it has been suggested that phage WO can infect other *Wolbachia* cells from neighboring infected cells (Bordenstein et al., 2006) (Kent and Bordenstein, 2010). The mechanism by which phage WO penetrates both the insect and bacterial cells remains elusive.

8.3.6.2 LSPZ01000002 represents the genome of a PhikZ-like bacteriophage

The large contig LSPZ01000002 of 252,037 basepairs harboring a terminase gene was discovered in colony 2. Further analysis of all the three termite colonies showed multiple contigs mapping against LSPZ01000002. Phylogenetic analysis of the terminase gene showed that LSPZ01000002 clustered with phiKZ-like bacteriophages (Figure 8.3). Of the predicted 238 genes, 71 genes had a match in the NCBI database with an e-value of 10^{-5} or less. Of those 71 genes, 35 genes matched to the phiKZ-like bacteriophage *Bacillus* phage AR9 (Lavysh et al., 2016). In all three termite colonies, *Bacillus* phage AR9-like genes were present in the highest abundance, suggesting that the bacteriophage, possibly along with its host, has an important role in the termite gut. PhiKZ-like bacteriophages encode two multi-subunit RNA polymerases (RNAPs); one of them is packed in the virion while the other is expressed in infected cells (Krylov et al., 2007, Ceyssens et al., 2014). These RNAPs are considered as the signature genes of phiKZ-like bacteriophages. LSPZ01000002 had six predicted genes encoding RNAP; three were predicted to encode beta subunit, while three encoded beta' subunit. Phylogenetic analysis of predicted virion (Figure 8.8) and non-virion RNAPs (data not shown) along with RNAPs from

the other phiKZ-like phages produced a similar phylogenetic topology as described previously (Lavysh et al., 2016).

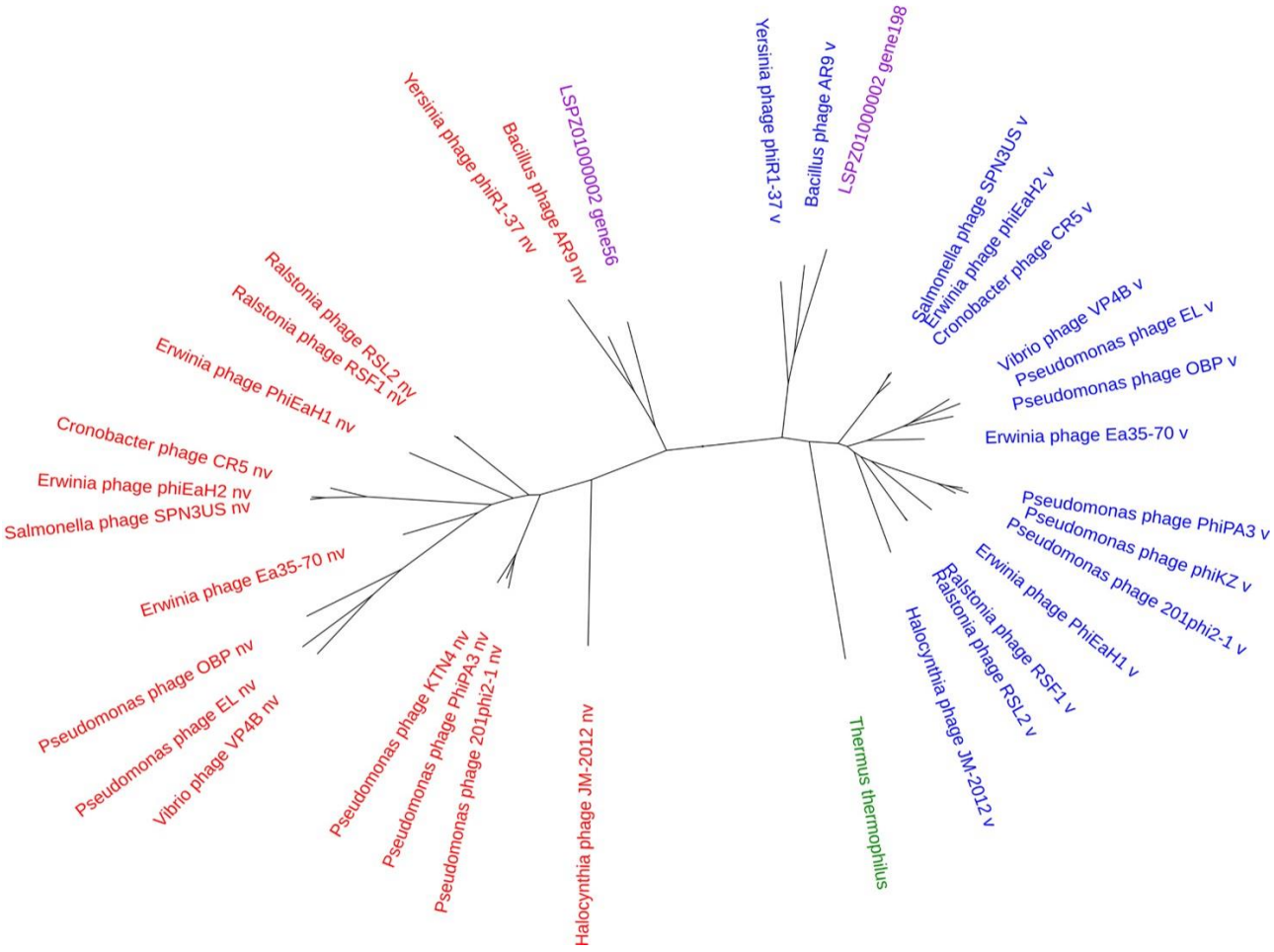

Figure 8.8: Unrooted maximum likelihood phylogenetic tree of RNAP beta subunit of virion and non-virion subunits from phiKZ like bacteriophages. RNAPs from LSPZ01000002 are shown in purple. RNAP from *Thermus thermophilus* is used as an outgroup.

LSPZ01000002 RNAPs were clustered with *Bacillus* phage AR9 and *Yersinia* phage phiR-137. Comparative genomic analysis of LSPZ01000002, *Bacillus* phage AR9, and *Yersinia* phage phiR137 showed considerable similarities but no synteny in the genomes (Figure 8.7 B). Lack of synteny in the genomes of closely-related phiKZ-like bacteriophages has been observed

previously (Cornelissen et al., 2012, Jang et al., 2013). Most of the PhiKZ-like bacteriophages are considered lytic in nature. However, some phages have been shown to be pseudolysogenic (Lavysh et al., 2016, Pletnev et al., 2010). Whether LSPZ01000002 is lytic or pseudolysogenic remains unknown. Unlike the genome of *Bacillus* phage AR9 which had multiple introns, RFAM BLAST did not predict any intron sequences in LSPZ01000002. One open reading frame (ORF) encoded a Group I intron-like endonuclease which showed similarities to many Group I introns from Firmicutes. One of the most important differences observed between Bacillus phage AR9 and LSPZ01000002 is the difference between ribonucleotide reductase (RNR) genes. Bacillus phage AR9 contains an operon of class I RNR genes (*nrdE and nrdF*). This class of RNR is dependent on oxygen and is found in organisms, which can grow aerobically (Dwivedi et al., 2013). LSPZ01000002, on the other hand, contains an operon of class III RNR genes. This class is sensitive to oxygen and bacteriophages infecting strict anaerobes like *Clostridium* sp. harbor only this class of RNR genes (Dwivedi et al., 2013). The RNR genes found in LSPZ01000002 showed a high degree of similarity to *Treponema primitia* RNR genes, which is a strict anaerobic spirochete isolated from the gut of a damp wood termite *Zootermopsis angusticollis* (Graber et al., 2004). Many spirochetes have been previously reported from the gut of various termite species and some have been known to be ectosymbionts of the gut protozoa (Noda et al., 2003, Hongoh et al., 2007). These data suggest that LSPZ01000002 most likely infects a strict anaerobe from the termite gut, possibly a spirochete.

8.3.6.3 LSPY01000009 and LSQA01000015 represent partial genomes of *Lactococcus lactis* phage 1706-like phages

The three contigs LSPY01000009, LSPZ01000022, and LSQA01000015 were identified in termite Colony 1, 2, and 3 respectively. LSPY01000009 and LSPZ01000022 showed 99%

similarity at genome level and hence were considered as genomes from the same phage species. A moderate nucleotide level similarity was observed between LSPY01000009 and LSQA01000015 mostly at the ends of the two contigs (46% query coverage, 67% identity). All the three contigs showed a high degree of similarity to proteins from *Rhodococcus* phage ReqiPepy6 (Summer et al., 2011) and *Arthrobacter* phage Mudcat. As observed in the *Rhodococcus* phage ReqiPepy6 and *Arthrobacter* phage Mudcat, LSQA01000015 lacked reverse transcriptase in the genome. However, LSPY01000009 and LSPZ01000022 harbored a reverse transcriptase enzyme belonging to Group II introns. Another important difference observed between the three termite gut contigs, *Rhodococcus* phage ReqiPepy6 and *Arthrobacter* phage Mudcat, is the presence of anaerobic ribonucleotide reductase gene (*nrdD*) which has been previously observed in the genomes of bacteriophages infecting anaerobes (Dwivedi et al., 2013). No other class of RNR gene was observed in any of the contigs. Interestingly RNR genes in LSPY01000009 and LSQA01000015 showed very little similarity to each other at amino acid level. Comparative genomic analysis showed segments of synteny in structural, DNA metabolism-related genes and segments of variable small hypothetical proteins (Figure 8.9 A). It has been shown that *Rhodococcus* phage ReqiPepy6, along with other closely-related phages from *Lactococcus lactis* phage 1706-like phages have segments of genome expansion (Summer et al., 2011). In this phenomenon, closely-related bacteriophages have segments of conserved genes but differ from each other in genome segments where multiple small hypothetical proteins are observed (Lavigne et al., 2009). Another characteristic of *Lactococcus lactis* phage 1706-like phages is the enrichment of membrane related proteins (14-23%) (Garneau et al., 2008, Summer et al., 2011).

In all the three contigs, between 20-23% of the predicted proteins were found to contain at least one transmembrane domain. Based on this analysis, LSPY01000009, LSPZ01000022 and LSQA01000015 represent partial genomes of *Lactococcus lactis* phage 1706-like phages, which most likely infects a Firmicutes bacterium.

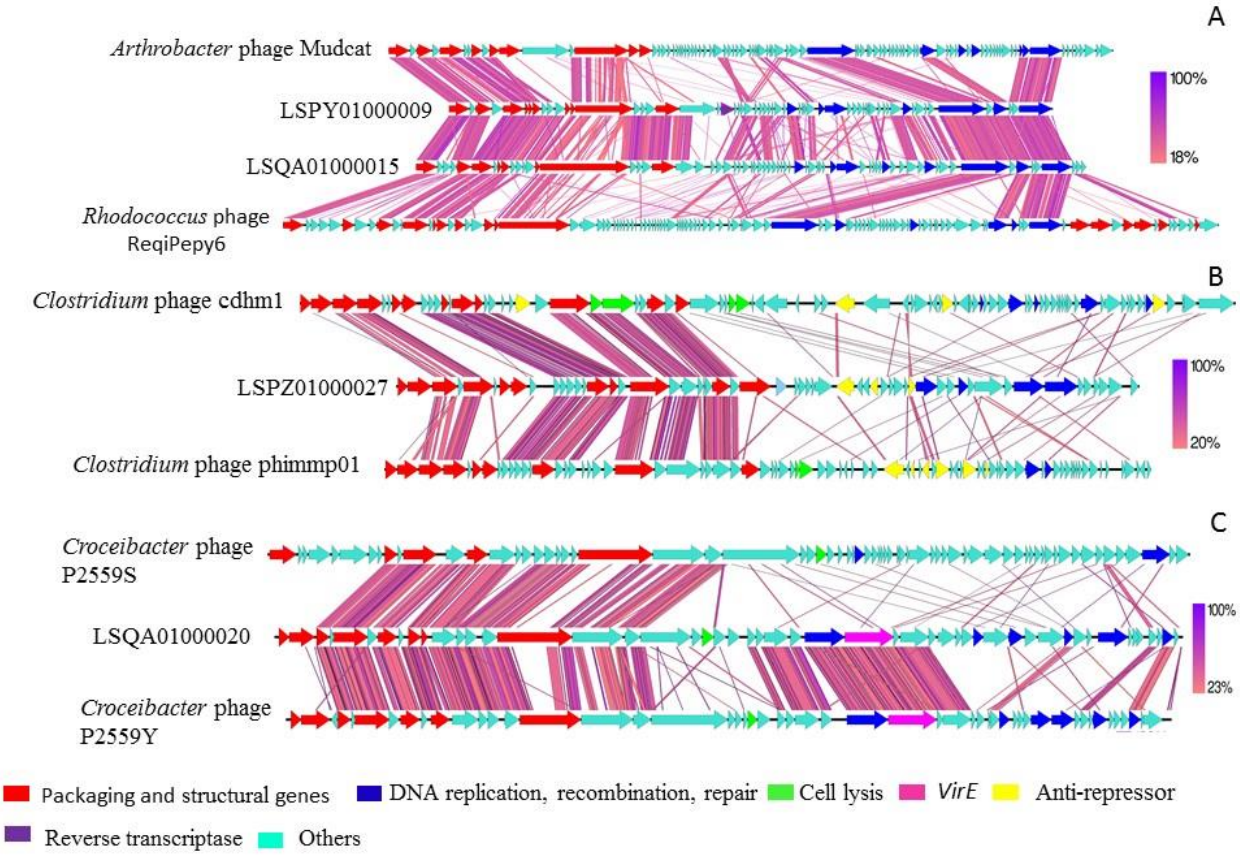

Figure 8.9: A) Comparative genomic analysis of LSPY01000009 and LSQA01000015 with *Arthrobacter* phage Mudcat and *Rhodococcus* phage ReqiPepy6. B) Comparative genomic analysis of LSPZ01000027 with *Clostridium* phage cdhm1 and *Clostridium* phage phimmp01. C) Comparative genomic analysis of LSQA01000020 with *Croceibacter* phage P2559S and *Croceibacter* phage P2559Y. All the genomes have been rearranged so that the start codon of the large terminase subunit is the first base in the sequence. The Figures were generated using Easyfig software with tblastx. The structural genes are indicated in red color, DNA metabolism related genes in blue, cell lysis green, virulence associated protein pink, anti-repressor yellow, reverse transcriptase purple. All the other genes are indicated in sky blue color.

8.3.6.4 LSPZ01000027 represents a full genome of a lysogenic phage

LSPZ01000027 was identified as a circular contig in termite Colony 2. Other multiple contigs could be mapped against LSPZ01000027 from all the three termite colonies. LSPZ01000027 genome showed high level of synteny to structural genes of *Clostridium* phage phiCDHM1 (Hargreaves et al., 2014) and *Clostridium* phage phiMMP01 (Boudry et al., 2015) (Figure 8.9 B). PhiCDHM1 genome was found to harbor a cassette of bacterial quorum sensing genes (Hargreaves et al., 2014), but no such cassette was identified in LSPZ01000027. PhiCDHM1 is considered a member of phiCD119-like bacteriophages with the presence of a signature DNA replication cassette. In LSPZ01000027 this signature DNA replication cassette was not observed. Also the G+C content of LSPZ01000027 was much higher (42%) than that of phiCDHM1 and phiMMP01the (G+C content 14-29%). Similar to the genome of phiCDHM1 and phiMMP01, LSPZ01000027 genome has many proteins annotated as putative anti-repressor proteins. The true identity of these anti-repressor proteins remains unknown at this moment. LSPZ01000027 also had a group II intron encoded reverse transcriptase. The presence of an integrase gene and multiple anti-repressor like proteins indicate that LSPZ01000027 is most likely a lysogenic bacteriophage.

8.3.6.5 LSQA01000020 represents the genome of a lytic Siphovirus

LSQA01000020 was identified as a linear contig in termite colony 3 and multiple contigs from colony 2 could be mapped against LSQA01000020. The first and the last gene of the linear contig encoded the same partial gene, indicating an almost complete circular bacteriophage genome. Phylogenetic analysis of the terminase gene from LSQA01000020 placed it in the

Bacteroidetes cluster in a sister clade with *Flavobacter* bacteriophages. Comparative genomic analysis showed that LSQA01000020 genome shows a high level of similarity to the genomes of two bacteriophages P2559S and P2559Y infecting a Bacteroidetes species *Croceibacter atlanticus* (Kang et al., 2012, Kang et al., 2016). Even though both P2559S and P2559Y are lytic Siphoviruses infecting the same species they show similarity only in the structural module of the genome (Kang et al., 2016). LSQA01000020 showed similarity in the structural module to both the phages and similarity to some extent in the replication module to P2559Y (Figure 8.9 C). LSQA01000020 contained thymidylate synthase and asparagine synthase genes, which were absent in P2559S and P2559Y. Based on the similarity to P2559S and P2559Y and phylogenetic placement of the terminase gene, LSQA01000020 most likely infects a Bacteroidetes species.

Contigs similar to the first bacteriophage (CVT22) isolated from the termite gut were also observed in Colony 3, suggesting some association with the termite gut (Tikhe et al., 2015). It has been suggested that CVT22 may represent a founding member of a new cluster of lytic bacteriophages (Casjens and Grose, 2016) and the termite gut might represent a niche of a diversity of CVT22-like bacteriophages.

There were many contigs observed in the termite with signature phage genes. However, due to the presence of a large proportion of previously unknown proteins, their origin remains unclear. As more phage genomes will be sequenced we believe that the virome uncovered from the termite gut will be better annotated.

8.3.7 Termite gut microviruses represent a putative new sub-family

As compared to double-stranded DNA viruses, single-stranded DNA (ssDNA) viruses were present in a low amount (1-10%). Our results did not indicate selective enrichment of ssDNA

bacteriophages reported in other studies (Kim and Bae, 2011). Colony 1 had the most diverse community of ssDNA viruses, with genes from 38 different types of ssDNA viruses, followed by Colony 2 (26) and Colony 3 (15). Most of the dominant ssDNA viruses were conserved in all the three colonies, along with some inter-colonial differences (Figure 8.10).

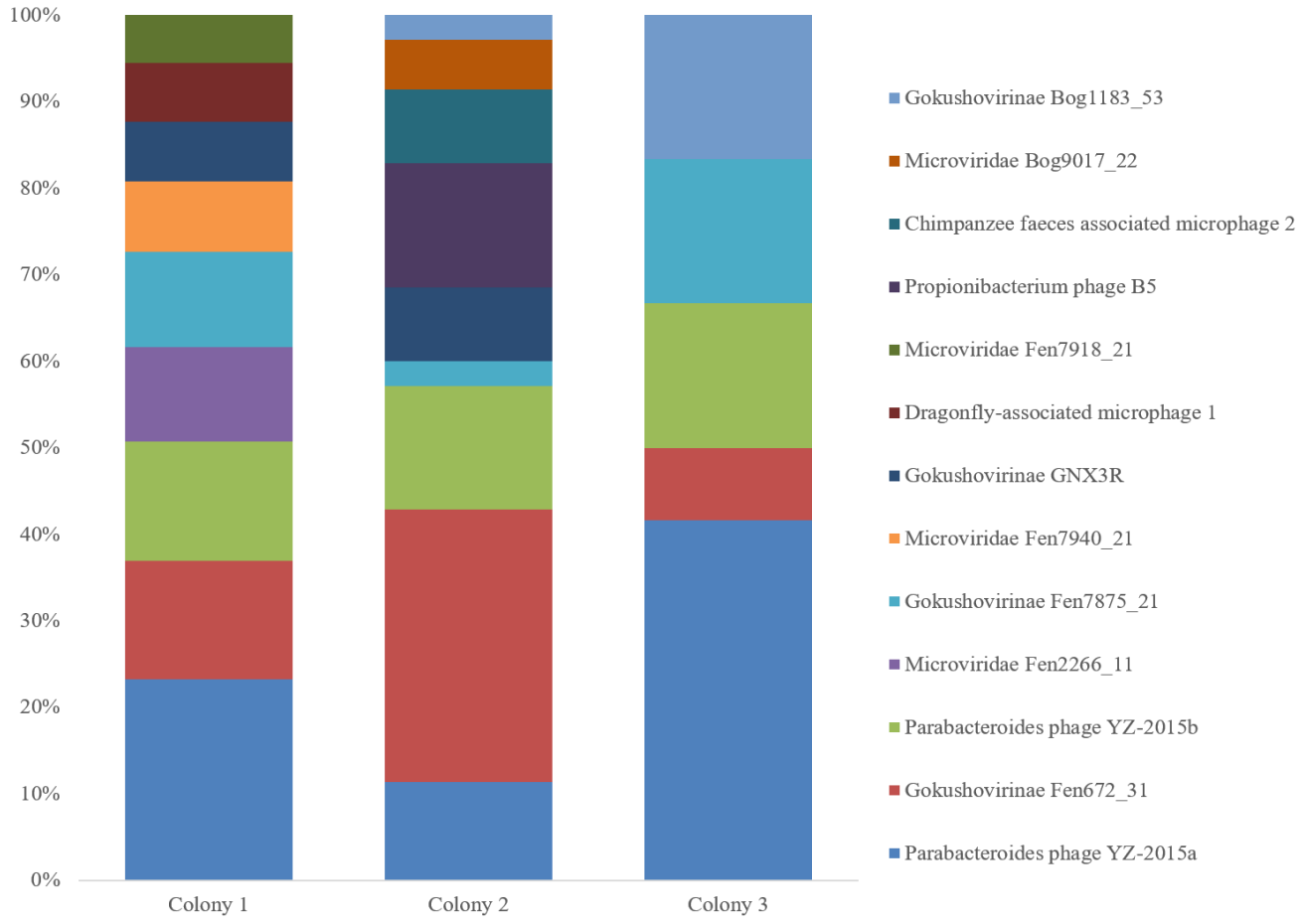

Figure 8.10: Percent distribution of the predominant ssDNA bacteriophage species from the guts of the termites from three different colonies. The data were generated using Metavir-2 server by comparing the predicted proteins to NCBI virus protein database. Top BLAST hits with an e-value of 10^{-5} or less were used.

Phylogenetic analysis of VP1 major capsid gene indicated a diverse population of Microviridae in the termite gut. We were able to construct 12 novel complete genomes of Microviridae from

all three termite colonies. Phylogenetically, VP1 from LSPY01000110 was placed in a cluster with *Dysgonomonas macrotermitis*, a bacterium of the phylum Bacteroidetes, which was previously isolated from the gut of a higher termite (*Macrotermes barneyi*) (Yang et al., 2014). Bacteria of this genus are also known to be a part of the gut community of the FST (Husseneder et al., 2010, Husseneder et al., 2009). LSPY01000110 showed synteny to a contig from the genome of a *D. macrotermitis* (Figure 8.11). It has been previously reported that microviridae bacteriophage can undergo a temperate life cycle in Bacteroidetes (Krupovic and Forterre, 2011). The *D. macrotermitis* pro-phage-like sequence and LSPY01000110 showed the same gene order (VP1-ORF2-VP2-VP4) followed by five ORFs encoding hypothetical proteins in *D. macrotermitis* and four ORFs in case of LSPY01000110. The hypothetical proteins showed no similarity to each other.

Phylogenetic analysis of the VP1 sequences of LSPZ01000262 and LSPY01000148 placed them in the Gokushovirinae subfamily (Figure 8.11). The genome arrangement of LSPZ01000262 and LSPY01000148 were different from previously described Gokushovirinae sequences (Quaiser et al., 2015). No clear distinguishable VP5 sequences were observed in LSPZ01000262 and LSPY01000148.

VP1 from nine contigs formed a completely separate cluster from all of the other Microviridae. The genome arrangement also showed a conserved order (VP1-ORF1-VP2-VP4-ORF2). In this cluster, the ORF present after theVP1 gene encoded for a hypothetical protein. This protein did not match any known protein in the NCBI database but showed a high similarity among the nine contigs. The protein encoded by the ORF after VP4 showed a loose similarity to Gokushovirinae VP5. Based on the genome arrangement and the VP1 phylogeny, we propose a new subfamily

Sukshmavirinae (Sukshma is the Sanskrit word for “small”) for the sequences observed in the termite gut virome.

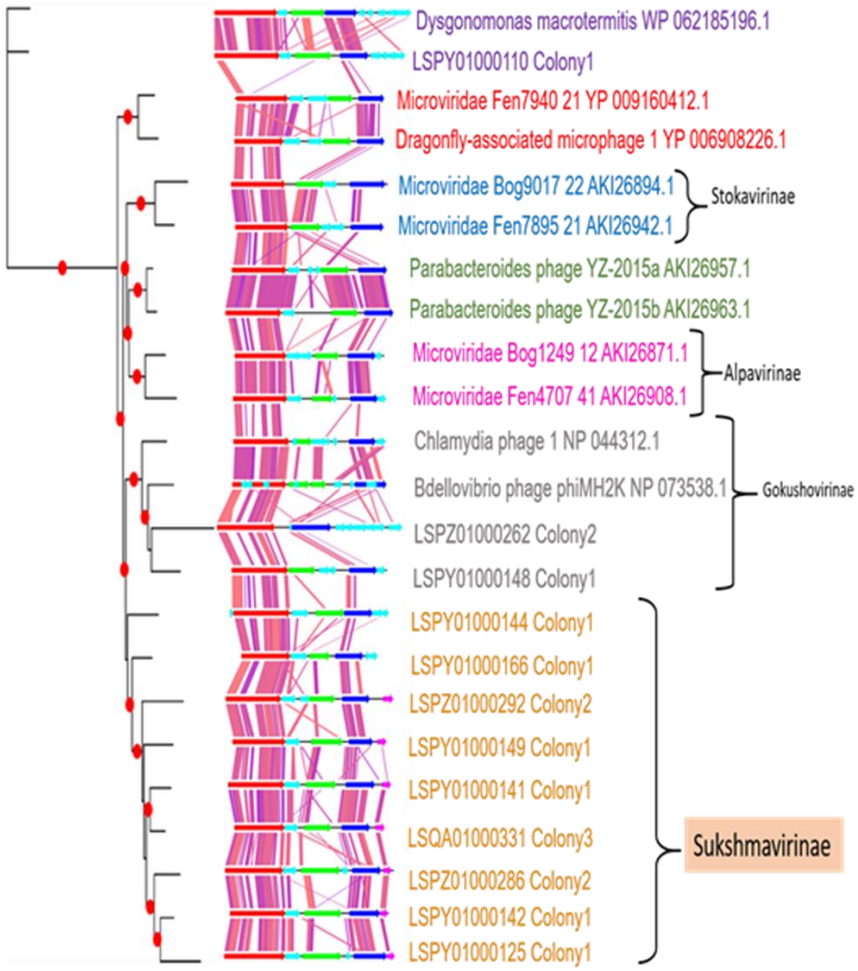

Figure 8.11: A maximum likelihood phylogenetic tree of Microviridae VP1 from the termite gut of the three colonies along with the comparative genomic analysis of closely related Microviruses. Nodes with a bootstrap score of more than 70% are indicated by red circles. For this Figure only VP1 genes from putative full Microviridae genomes were used. VP1 is colored red, VP2: green, VP4: blue. An extra ORF found after the VP4 gene of Sukshmavirinae is colored pink.

8.3.8 A diverse population of circoviruses is present in the termite gut

Circoviruses are small ssDNA viruses known to infect a number of higher eukaryotes (Todd et al., 2001). In recent years Circovirus-like genomes have been identified to be associated with a variety of animals including many insects (Rosario et al., 2011, Garigliany et al., 2015). The exact role of Circoviruses associated with various animals is currently not understood completely.

From all three termite colonies, 10 novel Circoviridae like genomes were assembled. The genome size ranged between 1,388 bp to 5,851 bp. All the genomes encoded the Circoviridae Rep protein which is considered as the signature gene of the family. Phylogenetic analysis of the Rep gene showed two distinct groups, one belonging to the Cycloviruses and the other to the Circoviruses (Figure 8.12). There was no correlation between the host of these viruses and the phylogenetic placement of the Rep proteins. Termite gut Rep proteins were distributed all over the phylogenetic tree with only one sequence clustered in the Cyclovirus group.

So far, there have been very limited data on viruses capable of infecting termites (Al Fazairy and Hassan, 1988). It has been suggested that a virus infecting termites would be an ideal candidate for biological control (Chouvenc et al., 2011). The origin of the Circoviruses found in the termite gut remains unclear. Whether these viruses are environmental and were picked up by the termites or they actually infect the termites is an area of future research.

8.3.9 Functional analysis shows a high degree of conservation in the gut virome

MG-RAST functional annotation indicated that genes belonging to phage, prophages, transposable elements, and plasmids were present in the highest abundance in all the three termite colonies (Figure 8.13).

Figure 8.12: Unrooted maximum likelihood phylogenetic tree of Circoviridae replication initiation protein. The hosts of the Circoviruses are displayed in a picture next to the sequence. Environmental Circoviruses are shown in green. Insect related Circoviruses are shown in red.

To study the conservation of functional genes in the gut virome, we studied the orthologous gene clusters present in the termite colonies. Orthologous genes may represent a difference in the sequence but the function is generally conserved. The orthovenn analysis of the gut virome from all the three termite colonies showed that all the 53,000 protein sequences formed a total of 9,625 clusters.

Figure 8.13: Percent distribution of the functional categories of predominant genes from the guts of the termites from three different colonies. The data were generated using MG-RAST server by comparing the predicted proteins to MD5nr database.

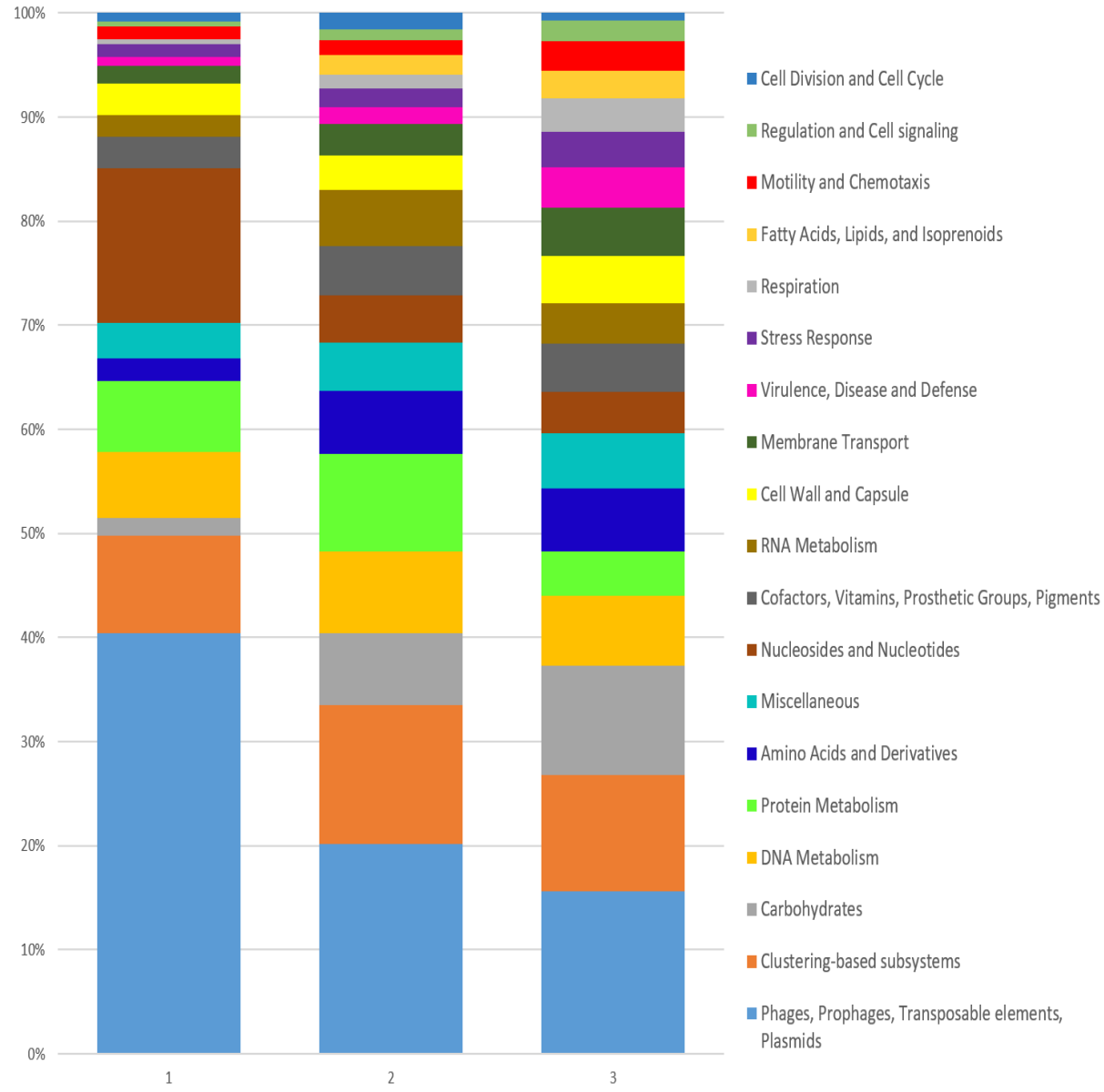

Figure 8.12: Unrooted maximum likelihood phylogenetic tree of Circoviridae replication initiation protein. The host of the Circoviruses are displayed in a picture next to the sequence. Environmental Circoviruses are shown in green color. Insect related Circoviruses are shown in red color.

Out of these clusters, 8,317 orthologous clusters contained genes from at least two of the three termite colonies. A total of 3,823 clusters comprised of 12,295 protein sequences were shared by all three colonies. The data suggest that the virome has a core set of functional genes that is conserved between all the three termite colonies. The presence of a conserved core is not surprising since most of the bacteriophages carry out the same core basic functions such as replication, lysis, packaging, and host integration during their life cycle.

8.3.10 Termites harbor a core virome and may follow piggyback-the-winner model

Based on the taxonomic and functional overlap among the three different termite colonies evidenced by shared phage species and gene functions, we hypothesize that termites harbor a highly conserved core virome. The obligatory symbionts of the gut protozoa form the core of the conserved bacterial community of the FST gut. The presence of bacteriophages infecting obligatory symbionts further corroborates our hypothesis of the conserved core virome. Phylogenetic analysis of the terminase and integrase genes further indicated that termite gut viruses show a high degree of similarity to pro-phage genes rather than lytic bacteriophages. Termites are highly dependent on their gut bacteria to complement their own metabolism, and changes in the bacterial population have been shown to negatively affect the termite host. The impact of bacteriophage pressure on the bacteria is known to alter their metabolic processes. It would be essential for the termite and the gut bacteria to maintain a functionally conserved set of biochemical pathways despite the presence of bacteriophage pressure. Hence, it would be advantageous to the termite host and the symbiotic gut community it relies upon, if the bacteriophage is temperate in nature rather than being lytic. We hypothesize that the termite gut virome follows the piggyback-the winner model, which predicts that bacteriophages become temperate in nature in higher host abundance and thus maximize their replication without

disrupting the balance (Knowles et al., 2016, Silveira and Rohwer, 2016). Manipulating the host abundance and studying the viruses in the termite gut would further help us understand the strategy followed by the termite bacteriophages. Termites are soil dwelling and the gut bacteria must be encountering a number of environmental bacteriophages. It would be interesting to study whether termite gut bacteriophages prevent the gut bacteria from environmental bacteriophages via superinfection immunity. Termites also present us with an ideal model system to study the effects of bacteriophages on the bacterial hosts and ultimately on the termites. In the future, we intend to develop termites as a model system to study the complicated quadripartite relationship between bacteria, bacteriophages, gut protozoa, and the termites themselves.

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Chapter 9: Conclusion and future directions

The aim of this research was explore and exploit the termite gut microbes for termite control. The first goal was to target the symbiotic gut protozoa of the termites using a genetically engineered termite gut bacterium as a 'Trojan Horse'. To choose an ideal 'Trojan Horse' we set six criteria. A bacterium fulfilling all the six criteria was to be used as the 'Trojan Horse'.

1. It should be a termite gut symbiont

   To satisfy the first criterion, we isolated *Trabulsiella odontotermitis*, which is a termite gut symbiont.

2. It should be tolerant to the toxic effects of ligand-Hecate

   We showed that *T. odontotermitis* is fifty times more tolerant to ligand-Hecate than the gut protozoa.

3. It should be able to express foreign proteins in the termite gut

   Engineered *T. odontotermitis* was able to express GFP in the termite gut, satisfying our third criterion.

4. It should be maintained in the termite gut when fed externally

   Chromosomally engineered *T. odontotermitis* strain was maintained in the termite gut for at least 21 days.

5. It should be horizontally transferred to other nest mates in the colony

   We showed that *T. odontotermitis* is transferred horizontally among nest mates

6. It should be able to express functional ligand-Hecate

   T. odontotermitis was able to express ligand-Hecate-GFP fusion protein which displayed antiprotozoal activity.

Overall, we showed that *T. odontotermitis* satisfies all the criteria of an ideal 'Trojan Horse' (Chapter 1, 2). As the results from a pilot study using *T. odontotermitis* engineered with a plasmid expressing LiHe-GFP fusion protein (Chapter 3) were promising, we are currently conducting the final experiments of measuring the antiprotozoal activity of the engineered *T. odontotermitis* strain quantitatively. Once protozoacidal activity of the gene construct is confirmed, *T. odontotermitis* will be engineered at the chromosomal level using a tn7 transposon to express and possibly secrete LiHe-GFP fusion protein (see Chapter 2). The engineered strain will be assessed for its ability to kill the termites by eliminating the gut protozoa. In the future, the engineered *T. odontotermitis* can be used in bait form as a standalone tool or in combination with the current termite control strategies.

Overall, this study established a platform for a novel termite control strategy. At each step in the study, strict criteria (choosing a target specific to the FSTs, choosing a lytic peptide causing minimal side effects to the non-target organisms, choosing a bacterium specific to the termite gut, reducing the risk of environmental contamination) were used, keeping environmental safety a top priority. In the future, this study can serve as a model for developing novel paratransgenesis-based insect control strategies. Even though the main focus of the study was to target termite protozoa for pest control, the techniques and findings established through this study have broader applications in a variety of fields, including targeting medically important protozoa.

The second goal was to study the bacteriophages from the termite gut to set the stage for developing phage therapy for termite control targeting the gut protozoa. To achieve this goal we isolated, identified, and sequenced three novel bacteriophages from the termite gut. CVT22, Tyrion, and Arya provide us with a potential tool to target the termite gut bacteria that these

phages infect. The extent to which the termite gut bacterial composition is altered after feeding these bacteriophages to the termites remains to be examined. As the survival of subterranean termite colony is dependent on many essential nutrients provided by the bacteria in their workers' guts, we hypothesize that changes in the bacterial flora might have detrimental effects on a colony.

Sequencing the metavirome of the termite gut indicated the presence of a diverse bacteriophage population. Many novel bacteriophage genomes were sequenced from the termite gut. This is the first study in termites focusing entirely on uncovering the bacteriophages and other associated viruses. The study also indicated a potential core virome present in the termite gut. We also predicted that the termite gut virome is dominated by lysogenic bacteriophages. These data indicate that the termite gut might follow 'piggy back the winner' model. This model suggests that the higher the abundance of host bacteria, the higher the number of temperate bacteriophages. This study establishes a first step toward developing the termite gut as a model to study the interactions between bacteriophages, bacteria, gut protozoa, and the termite. The study also showed the presence of novel circoviruses possibly infecting termites.

In summary, the study of bacteriophages provided an insight into the previously unknown aspect of the termite gut microbiology. The study opens up a new area of future research.

Appendices: Letters of permission

A Letter of permission for Chapter 2

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ASM Journals <Journals@asmusa.org> Tue, May 30, 2017 at 8:14 AM

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Vita

Chinmay Tikhe was born in Pune, India. Chinmay received a Bachelor's degree in Microbiology from University of Pune in 2008. After graduation Chinmay enrolled in a Master's program in Microbiology in University of Pune. For his Master's thesis Chinmay worked at National Center for Cell Science. His research was focused on studying the gut bacteria of various mosquito species from Dengue endemic and non-endemic areas of India.

In August 2011, Chinmay started his PhD program in the Department of Entomology at Louisiana State University Agricultural center. Currently, Chinmay is a PhD candidate in Dr. Claudia Husseneder's lab. His research is focused on developing new techniques for termite control using termite gut microbes. Chinmay intends to pursue a career in insect-microbe interactions.