STUDY OF MIDGUT BACTERIA IN THE RED IMPORTED FIRE ANT,

***Solenopsis invicta* Büren (HYMENOPTERA: FORMICIDAE)**

A Dissertation

by

FREDER MEDINA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Entomology

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ABSTRACT

Study of Midgut Bacteria in the Red Imported Fire Ant, *Solenopsis invicta* Büren

(Hymenoptera: Formicidae). (May 2010)

Freder Medina, B. S., Universidad Central "Marta Abreu" de Las Villas

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Ants are capable of building close associations with plants, insects, fungi and bacteria. Symbionts can provide essential nutrients to their insect host, however, the development of new molecular tools has allowed the discovery of new microorganisms that manipulate insect reproduction, development and even provide defense against parasitoids and pathogens. In this study we investigated the presence of bacteria inside the Red Imported Fire Ant midgut using molecular tools and transmission electron microscopy. The midgut bacteria were also characterized by their morphology, biochemical activity, and antibiotic resistance profile.

After isolation, culture, and characterization of these bacteria, the molecular analysis revealed ten unique profiles which were identified to at least the genus level, *Enterococcus* sp.*/durans, Klebsiella ornithinolytica, Kluyvera cryocrescens, Lactococcus garvieae, Pseudomonas aeruginosa, Achromobacter xylosoxidans, Bacillus pumilus, Listeria innucua, Serratia marcescens,* and an uncultured bacterium from the Enterobacteriaceae. New SEM and TEM techniques revealed a possible functional association of endosymbiotic bacteria with the insect host, and it also showed the absence of

bacteriocytes in the epithelial cells of the midgut. The PCR results, from the bacteria abundance and distribution studies, showed that *Enterococcus* sp., *Kluyvera cryocrescens* and *Lactococcus garvieae* are the most abundant species, but they are not consistently found in all sites throughout the southeastern United States.

Kluyvera cryocrescens, *Serratia marcescens*, and an uncultured bacterium (isolate #38: Enterobacteriaceae) were genetically modified with the plasmid vector pZeoDsRed and successfully reintroduced into fire ant colonies. Strong fluorescence of DsRed was detected up to seven days after introduction. The transformed bacteria can still be rescued after pupal emergence; however most were passed out in the meconium. We further demonstrated that nurses contributed to the spread of the transformed bacteria within the colony by feeding the meconium to naive larvae.

Although the role of midgut bacteria in the fire ant is still unknown, we have no indication that they cause any pathology. Studies emphasizing the role of these bacteria in fire ant physiology are still ongoing. These results are the foundation for a fire ant biological control program using endosymbiotic bacteria as vectors to introduce foreign genes that express proteins with insecticidal properties.

DEDICATION

To my beloved grandparents, Maria, Angela, and Rolando.

To the United States of America,

I express my most profound gratitude for opening its doors and making my dreams come

true, and for defending democracy, freedom, and prosperity.

To motherland Cuba,

Let us sing our freedom to enjoy your beauty in the mountains and the sea.

"It is a sin not to do what one is capable of doing"

"Ser culto para ser libre"

José Martí

ACKNOWLEDGEMENTS

SPECIAL ACKNOWLEDGEMENT

I would like to express my profound gratitude to Haiwen Li for her help and support during the years we worked together. Our team work allowed us to learn from each other and, as a result, to succeed and publish our results in renowned scientific journals. She provided me not only with the knowledge needed to continue my research, but also with the gift to understand other cultures and to value the importance of collaboration. Our work has been presented at national and international meetings, obtaining several awards. Most importantly, I always made sure that Haiwen was properly given credit for all her contributions during these meetings.

Haiwen and I worked together from the beginning on the isolation, culture, and morphological and biochemical characterization of the midgut bacteria. Most importantly, she served as my coach and directly participated in the implementation of the biochemical and molecular biology tools that led to the identification of the bacteria species. She originally designed bacterial species-specific primers that were later redesigned, checked for specificity, and then were utilized to screen for these bacteria throughout the southeastern United States. Haiwen's molecular biology knowledge led to our successful completion of the genetic transformation of selected midgut bacteria.

Our greatest ideas came from our differences and debates, where our backgrounds and different points of view met in the middle to yield a complete piece of work. I wish Haiwen the best in her future scientific endeavors and that she might continue her successful scientific career.

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CHAPTER I

INTRODUCTION: SYMBIOSIS AND THE RED IMPORTED FIRE ANT

Introduction

An introduction to symbiosis and insects. In nature, organisms constantly interact with each other forming interspecific or intraspecific relationships. Intraspecific relations are defined by those involving organisms of the same species. Interspecific associations, also defined as symbiosis by Heinrich Anton de Bary in 1879, refers to as the living together of different organisms (Ahmadjian and Paracer 1986). Although a few years earlier in 1876, Pierre Joseph van Beneden on his book "Animal parasites and messmates" had already used the terms commensalism, mutualism, and parasitism, it was not until the printed speech titled "Die Erscheinung der Symbiose" by de Bary that those categories were incorporated into the term symbiosis (Ahmadjian and Paracer 1986). Commensalism is described as the type of association where one organism is neither harmed nor benefited. Mutualism is defined as when both symbionts are benefited, and parasitism when one is benefited at the expense of the other. To further complicate the matter, these relationships are not static and can change from one category to the other, being influenced by environmental and developmental factors within each organism (Ahmadjian and Paracer 1986, Bourtzis and Miller 2003).

These complex relationships make it harder to classified symbiosis, after years of conflict there was a need to standardize these terms. Today, we still used the same terms

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This dissertation follows the style and format of the Journal of Economic Entomology.

developed in the 1800's, but with the increasing knowledge, Symbionts are also classified as endosymbionts (living inside the host) or ectosymbionts (living outside the host). Or they can be referred as obligate or facultative depending on their ability to live with or without their host (Ahmadjian and Paracer 1986, Bourtzis and Miller 2003). After many years of investigation, symbiosis is becoming recognized as increasingly important, and more research is focused on the understanding of "Who is getting what from whom" (Hoffmeister and Martin 2003).

Insect species dominate the earth's landscapes, at least in part, due to their ability to digest a wide variety of food. A significant component of their success is the result of endosymbiotic associations with many microorganisms that live in internal organs, in many cases directly in the digestive system, thus playing an important role in host nutrition (Ahmadjian and Paracer 1986, Bourtzis and Miller 2003). Microorganisms can provide sources of critical nutrients such as essential amino acids, vitamins and lipids (Douglas 1998). With the development of new molecular tools, more endosymbionts have been identified and linked not only to digestive processes but also to developmental processes. Furthermore, some have been found to manipulate insect reproduction and even provide defense against parasitoids and pathogens (Werren 1997, Hurst et al. 1999, Stouthamer et al. 1999, Bourtzis and Miller 2003, Zientz et al. 2005, Brownlie and Johnson 2009, Haeder et al. 2009, Koukou et al. 2009, Vorburger et al. 2010).

As mentioned before, symbiotic associations range from pathogenic to mutualistic and from facultative to obligate (Lau et al. 2002) and symbionts are ubiquitously located in animal guts including many insects groups. For some insects, this relationship

had been studied to some extent, but for the majority, these associations are still unknown. Among those well studied, we find aphids with its primary and secondary endosymbionts, also cockroaches, termites, ants, flies, glassy-winged sharpshooters, triatomid bugs, weevils, among others (Buchner 1965, Goldberg and Pierre 1969, Ahmadjian and Paracer 1986, Aksoy et al. 1995, Baumann et al. 1995, Blattner et al. 1997, Beard et al. 1998, Douglas 1998, Boursaux-Eude and Gross 2000, Sauer et al. 2000, Bourtzis and Miller 2003, Moran et al. 2003, Moran et al. 2005). According to these authors, symbionts are commonly found in those insects feeding on poor and unbalanced diets, such as many blood and plant sap feeders.

In the classified genus proteobacteterium *Buchnera* sp., a symbiont of the pea aphid, the host typically consumes a single food source of sugar-rich phloem sap of higher plants which is generally poor in amino acids. The symbionts are thought to enable their hosts to survive on these restrictive diets by providing nutritional supplements such as amino acids and vitamins (Buchner 1965, Baumann et al. 1995, Blattner et al. 1997, Douglas 1998). Among insects, several systematic groups are frequently involved in symbiotic interactions with bacterial species, including: the genus *Wigglesworthia*, the well-characterized symbionts in testse flies (Aksoy et al. 1995, Chen et al. 1999), *Candidatus baumannia, Cicadellinicola* in *Homalodisca coagulate* (sharpshooters) (Moran et al. 2003) and *Blattobacterium* in cockroaches (Goldberg and Pierre 1969). These symbionts share a common ancestor and are systematically placed adjacent to the family Enterobacteriaceae (Autuori 1941, Kermarrec et al. 1986, Adams

et al. 2000). The potential use of these organisms for the biological control of insect pests has driven much of the current scientific research.

Social insects, such as ants, develop numerous interactions with different species of microorganisms at individual and population levels. These interspecies interactions often involve bacteria and fungi (Boursaux-Eude and Gross 2000). Certain protobacteria and other bacteria not yet identified by molecular methods had been found in some species of ants (Douglas 1989, Douglas 1998). These groups of bacteria are known to form bacteriocytes (bacteria specific mycetocyte) in two different tribes, the *Camponoti* and *Formicini* both members of the subfamily *Formicinae* (Bolton 1994). The genus *Camponotus* is classified in the subfamily *Formicinae*, and is a textbook model for symbiosis. In all *Camponotus* species investigated so far, intracellular bacteria are present within the midgut, even in the ovaries, and these gram-negative rods are classified within a single genus named *Candidatus blochmannia* that has been intensively studied (Sauer et al. 2000, Sauer et al. 2002).

Another well studied bacterium, *Wolbachia* sp., has been found in close association with 17% to 76% of all insects (Werren 1997, Jeyaprakash and Hoy 2000, Werren et al. 2008). In invertebrates, this bacterium can cause cytoplasmic incompatibility, parthenogenesis, and feminization of genetic males; they are also horizontally transmitted from mother to offspring (Werren 1997, Stouthamer et al. 1999). Particularly fire ants, in their natural South America habitat and in the United States, are also known to harbor this bacterium (Shoemaker et al. 2000, Dedeine et al. 2005, Bouwma et al. 2006).

Compared to fairly abundant studies on the relationship of other insects with microorganisms, very little is known about the relationship between fire ants and their symbionts. Recently, Peloquin and Greenberg (2003) found that *Solenopsis invicta*, a member of the subfamily Myrmicinae, carry endosymbiotic bacteria in their midguts. This relatively small ant has been considered one of the major pests in the United States for the last 75 years. Today, they have adapted to highly disturb environments in the country side and in urban areas, and they have no intention of leaving the comfort of our parks, roads and backyards. Therefore, this research project aims at the use of newly developed molecular tools in the study of endosymbiotic bacteria from the fire ant midgut, with the purpose of finding a more effective and environmentally safe alternative in a biological control program against the red imported fire ant in the United States.

History of *Solenopsis invicta.* It was 1929 in Alabama, when a florist and amateur entomologist from Mobile decided to go collecting insects in one of his "beetle trips" near the docks in the state port area. As he was searching for insects, he probably stumbled upon a different ant colony, and today, 70 years later, entomologists can still remember that day. His name, H. P. Löding was the first to discover the black imported fire ant, *Solenopsis richteri* Forel, near the docks in the port area of Mobile (Wilson 1959, Callcott and Collins 1996). This darker form was thought to match a racial variant from Argentina and Uruguay (Wilson 1959) and soon after, a lighter reddish-brown variant had also appeared (red imported fire ant- RIFA), which resembled a fire ant population from northern Argentina and southern Bolivia. It is not until 1972, when

William F. Büren (Buren 1972) described the lighter form as a different fire ant species, *Solenopsis invicta* (Hymenoptera: Formicidae). A year later, in 1973, a master student named F. E. Lennartz from the University of Florida at Gainesville determined that the lighter reddish-brown fire ant came in a second introduction between 1933 and 1945 (Wilson 1959, Lennartz 1973, Callcott and Collins 1996, Vinson 1997).

Spread of *S. invicta* in the United States. In the late 1950's, the red imported fire ant was already well adapted to their new environment on southeastern USA according to Wilson (Wilson 1959). Despite all the confusion on the early years of fire ant history, their time of arrival, origin, and taxonomy, one thing is for sure. "If left to spread unhampered, it will probably come to occupy most of the southeastern United States" (Wilson 1959). Edward O. Wilson was far from wrong in 1959. By 1975, *S. invicta* had already reached Texas, and they could be found from Texas to Florida up to North Carolina (Vinson and Sorensen 1986). Seventy years after their first incursion, there are five times more ants per acre in the United States than in the native habitats of South America (Calcaterra et al. 1999). The expansion of the RIFA in the USA is limited by humidity and temperature (Vinson and Greenberg 1986, Vinson 1997), but the real range can be much larger due to irrigation and current global warming. Today, over 56 million acres of land in Texas alone are infested with these ants (Lard 2002), but their range has extended to North Carolina, South Carolina, Florida, Georgia, Alabama, Tennessee, Mississippi, Louisiana, Arkansas, Oklahoma, New Mexico, Arizona, and California, adding up to over 128 million hectares (Drees and Gold 2003). As they keep

spreading, predictions suggest they will occupy Nevada, Oregon, and Washington in the west, and Virginia, Maryland, up to Delaware in the east (Korzukhin et al. 2001).

Many factors contributed to the rapid spread of these ants. Transporting nursery plants and grass from infested areas was probably the main spreading mechanism (Vinson and Greenberg 1986). Another means come from the fact that mature colonies can contain a large number of mature female and male alates (winged). These colony members are always ready waiting for a humid and warm day during the spring, summer, or fall, and just when winds are calm, they leave the colony and take off on their mating flights. Thousands of females and males rise up into the sky and disperse long distances for many miles (Banks et al. 1973, Wojcik 1983, Vinson and Greenberg 1986, Vinson 1997). Immediately after landing, the newly mated females will search for a perfect location to start a new colony. As if this was not enough, during flooding after heavy rains, the colonies can form rafts that float down the rivers until they can grab onto a branch, grass, or debris on the river banks and move to infest new areas (Hays 1959, Morrill 1974, Vinson and Greenberg 1986). And lastly, they are also moved by humans and human activities, especially after a mating flight, when thousands of RIFA queens are attracted to shiny surfaces landing on top of cars, trucks and trains (Vinson and Greenberg 1986).

Economic impact of *S. invicta*. RIFA can form large colonies that contain thousands of adult workers, which are very aggressive and armed with a powerful sting. Although their venom has the potential to kill small animals, in humans they cause the formation of small pustules that in some instances can cause acute infections

occasionally becoming life threatening (Vinson 1997). The problems associated with the red imported fire ant are not only limited to humans, they can be classified as, medical, nuisance, agricultural, domestic animals, wildlife, environmental, industrial, economic, legal, and political (Vinson 1997). These facts determined that in five major cities in Texas, the cost of control and management of fire ants can exceed $580 million per year, with just $526 million corresponding to the household sector in the urban areas (Lard 2002). In addition, in the Texan agriculture the costs add up to more than $90 million annually (Lard 2002).

Thus far, *S. invicta* has proven to be a successful competitor with the ability to change entire ecosystems by displacing the native fauna (Porter and Savignano 1990, Tschinkel 1993, Vinson 1994, Simberloff 1997, Vinson 1997). They can out compete, displace, and/or kill a variety of pests and beneficial arthropods (Porter and Savignano 1990, Stoker et al. 1995, Hu and Frank 1996, Forys et al. 2001, Cook 2003), including many natural enemies of economically important pests (Fleetwood et al. 1984, Lemke and Kissam 1988, Vinson 1997, Kaplan and Eubanks 2002). Vertebrates are mainly affected by these ants' sting and venom effects. The venom toxins with its necrotizing properties can produce alteration of behavior, injury, severe allergic reactions that could lead to death in birds, cattle, fish, sea turtles, and even humans (Caro et al. 1957, Lockey 1974, Rhoades 1977, Apperson and Adams 1983, Allen et al. 1994, Drees 1994, Drees et al. 1995, Stafford 1996, Contreras and Labay 1999, Allen et al. 2001, Parris et al. 2002). In addition, RIFA can have a devastating effect in important crops such as, corn,

sorghum, eggplants, peanuts, and soybeans (Adams 1983, Apperson and Powell 1983, Drees et al. 1991, Shatters and Vander Meer 2000, Vogt et al. 2001).

Following E. O Wilson's predictions in 1959, if a new environmentally safe and more effective control alternative is not developed soon, as fire ants keep spreading north and west, their damage will definitely lead to expenditure increases.

Earlier control methods of *S. invicta*. Since its introduction in the 1930's, the red imported fire ant (RIFA) has been a serious pest in the southeastern Unites States (Vinson and Sorensen 1986). In the early years of RIFA invasion calcium cyanide was a popular choice of control, and it was replaced in the 1950's by heptachlor and dieldrin (Eden and Arant 1949, Sauer et al. 1982). But it is not until the 1960's and 1970's that new alternative methods were introduced, including baits containing mirex (Lofgren et al. 1975, Wojcik et al. 1975). Today these baits are still very popular, and only the active ingredients have been changed to chemicals like hydramethylnon (AMDRO) and fenoxycarb (LOGIC) (Phillips Jr and Thorvilson 1989, Collins et al. 1992, Van der Meer et al. 2002).

According to Vinson and Greenberg (1986), there is a lot of controversy on how to control the RIFA. Even when some control programs may reduce the ant population, the long-term effects of insecticide use are uncertain (Vinson and Greenberg 1986). Early efforts for countrywide fire ant control began as far back as 1960 and were dependent on insecticides. Wetlands and nature reserves are environmental sensitive areas and among the prime fire ant habitats, however due to environmental risks they cannot be treated and therefore they serve as sources for re-infestation (Drees et al.

1996), which leads to failure of chemical control for fire ants. Today fire ant control is still heavily dependent on insecticides and the only way to maintain control is to apply insecticides one to two times a year (Drees 2002), at a cost of 6-12 billion dollars a year to treat all infested land. Furthermore, chemical insecticides are not biodegradable and residues pollute the environment.

Alternative control methods of *S. invicta.* As mentioned, expense, risks of insecticide treatments, and the inability to treat environmentally sensitive land, all make biological control a valuable research option. Therefore, it is also very important to have knowledge of all the natural enemies of fire ants in the United States and in their native habitat. Previous studies have identified a large number of arthropod species from fire ant nests; however, the vast majority has transient and non-specific relationships with the ants (Collins 1992). Insect predators, parasitoids and parasites such as viruses, rickettsia, nematodes, fungi, microspodiria, and bacteria have also been identified and used for biological control in association with fire ants (Whitcomb et al. 1973, Bedding and Akhurst 1975, Bulla 1975, Nichols and Sites 1991, Aronson and Shai 2001). Among parasitoids, phorid flies (*Pseudacteon* spp.), wasps (*Orasema* spp.), and a parasitic ant (*Solenopsis daguerri*) are well known examples. Although microorganisms are less studied than parasitoids, a microsporidian (*Thelohania solenopsae*), and a fungus (*Beauveria bassiana*) are also well know examples (Morrison et al. 1997, Knutson and Drees 1998, Williams et al. 1999) used against RIFA. However, none of the above has proven successful so far, and they were viewed as part of a combined alternative for a successful RIFA control program (Drees et al. 1996).

Of all these natural enemies, microorganisms may offer the greatest hope for biocontrol (Taber 2000). This challenging task has not been extensively investigated due to its complexity. For example, Jouvenaz (1986) affirmed that microbiological control of fire ants must overcome: 1) the care given to the queens by the workers, 2) the low susceptibility of the queen, whose gut is almost free of microbes, 3) the filter protecting the ant's digestive track and 4) the fire ants own control by fumigating their nests with venom (Jouvenaz 1986, 1990b). Another example of its complexity relates to the problem associated with the use of fungi, *Beauveria bassiana* to control fire ants and how the ant behavior affects its use was presented by Oi and Pereira (1993). The authors were able to determine that grooming removed the inocula from the ant body. The venom had antibiotic effects against the fungi and also necrophoresis and dispersal of infected ants prevented the transmission among nestmates. Bacteria play a key role in the biological control of insect pests and their possible advantages over the use of fungi is obvious, as bacteria live inside their host and propagate through direct contact among nest mates. The major species of bacteria involved in insect control are spore forming bacilli. One of the well-known examples is *Bacillus thuringiensis*, which has been used primarily for the control of both Diptera and Lepidoptera (Burges 1982).

The complexity of the microbial communities in insects provides numerous opportunities for intervention for biological control purposes. Endosymbiont populations are normally in balance and controlled by their insect host, but this could be modified or provided with an advantage such that they become pathogenic to the host organism. Understanding these complex microbial communities has been greatly enhanced by the

development of molecular identification techniques based on the 16s rRNA subunit gene (Pace 1997). The 16s rRNA gene is a well-conserved, universal bacterial gene with constant and highly constrained functions that were established in the early stages of evolution and is relatively unaffected by environmental pressures (Woese 2000). This gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny) and more recently, it has also become an important means to identify unknown bacteria to genus or species level (Sacchi et al. 2002). Analysis of the 16s rRNA gene can potentially be applied to identify all bacteria including those not able to grow *in vitro*. In contrast to traditional microbiological methods, it provides at least two primary advantages: a rapid turn-around time, and improved accuracy in identification (Springer et al. 1996). Alternatively, the genetic modification of a bacterial species could be used to alter the normal behavior and/or physiology of the host and thus can be exploited for insect control purposes.

Following these intensive studies of symbiotic bacteria in insect species and with the recent developments in molecular biology and genetics, new approaches are being developed for biological control (Peloquin et al. 2000, Peloquin et al. 2002). Genetic engineering of well adapted symbiotic microorganisms, such as bacteria, can produce potential vectors for the introduction of specific genes into the fire ant genome. The possibility of biological control presented by this situation demands investigation, and their utilization for control of the RIFA populations depends on our ability to decipher complex biological, pathological, and epizootiological relationships between the microorganisms and their host (Jouvenaz 1986).

Alternative methods of fire ant control are desperately needed, particularly approaches that do not rely on the application of broad-based insecticides. As described before, the use of genetically modified bacteria that are associated with insect species is an emerging field of research that offers great promise.

Control methods using genetically transformed bacteria. Although the use of symbiotic bacteria in a biological control approach have been investigated, the information is still limited. One example is the vector-symbiont intervention project in Chagas disease, where a bacterial endosymbiont called *Rhodococcus rhodnii* have been transformed to express an anti-tripanosomal agent in the midgut of *Rhodnius prolixus* (Beard et al. 1992, Beard et al. 1998). Another example involves the introduction of a DsRed fluorescent protein into a bacterium (*Alcaligenes xylosoxidans denitrificans*) found inside the glassy-winged sharpshooter (*Homalodisca coagulate* Say) and the reintroduction of the transformed bacterium into the host foregut (Bextine et al. 2004). *A. xylosoxidans denitrificans* is an endophytic symbiont of grapes that competes with *Xylella fastidiosa* (Grape Pierce's disease pathogen) and colonizes the same area in the foregut of the glassy-winged sharpshooter. Therefore this bacterium is a good candidate for the delivery of an anti-*Xylella* agent (Bextine et al. 2004)

Biology and behavior of *Solenopsis invicta*. In order to develop more effective alternatives for the control of *S.invicta,* we must understand their biology and behavior. In addition, particular attention must be paid to those elements of their biology and behavior with direct effects on the ant-bacteria interactions.

As in all eusocial insects, the RIFA queen is at the center of the colony, and by the release of a pheromone complex she communicates with the rest of the colony thus maintaining its perfect balance. Fire ant colonies are classified as monogyne if there is only one queen and polygyne if more than one is present (Vinson 1997). Every day, each queen is able to lay hundreds of eggs with the ability to decide which eggs are fertilized becoming diploid workers and reproductive female, or not fertilized becoming haploid males. The tiny white eggs hatch within 7-10 days (Vinson 1997), and the larva goes through four different stages or instars before becoming pupa as described in detail by (Petralia and Vinson 1979). Depending on the temperature and caste, these four different instars last between 12-15 days (Vinson 1997). Worker pupae development can last between 9-16 days. The newly emerged lighter adults are referred to as callows and will darken within a couple of days (Vinson 1997). Adult ants vary in size; during the summer months, minor workers can live from 60 to 90 days, while major workers are able to live from 90 to 150 days. Overwintering ants can live longer, more than eight months, but queens can live up to seven years (Vinson 1997).

One distinguishing characteristic of social insects is the division of labor in the colony, as it is in the case of fire ants. This division of tasks depends on the age and size of the worker, as well as colony needs. For instance, younger workers or nurses are in charge of tending the queens and brood; as they age, they become the reserve of the colony and their task is more of maintenance and defense. The older ants are the foragers, but this is not always a rule, usually the reserve of the colony can take over the other tasks in case it is needed (Vinson 1997).

Feeding behavior is an important element in direct relation with the midgut bacteria. Fire ants are omnivorous and effective scavengers (Vinson 1997); therefore we can assume that their diet is not as poor as in other insects carrying symbionts. Adult workers can only consume liquids, and these are passed to other workers through trophallaxis (Vinson 1997). Although workers collect solid and liquid food, only the liquids are ingested after passing through the filtering system in their pharynx. Any particles bigger than 0.88 µm are screened out, collected and compacted in the infrabuccal pocket forming the so called buccal pellet (Glancey et al. 1981, Vinson 1997).

The first, second, and third larval instars are only fed liquid food by the nurses. The third instar larvae are a lot smaller in comparison to the fourth instar, this is due to the fact that fourth instar larvae are not only fed with liquids, but also with solid food, increasing their size very rapidly. These solid food that had been passed to the nurses inside the colony is placed as a buccal pellet in the antero-ventral pouch or praesaepium (Petralia and Vinson 1978). This structure is within the reach of the larval mandibles, and it is where an enzymatic digestion of proteins occurs (Petralia and Vinson 1978, 1980b, Sorensen et al. 1983). From this information, we can say that the fourth instar larvae play a central role as the food processor in the fire ant colony, including the digestion of proteins. According to Vinson (1997) the digestion product from the fourth instar larvae, now in a more liquid stage, is highly nutritious and is collected by the nurses to feed the younger instars as well as the queen. After gathering the previous information, it is valid to make an important assumption; this might well explain why

previous control methods had failed, the fire ant behavior is so well adapted to protect their most precious member, that all the royal food must be filtered several times and well digested before reaching the queen.

Anatomy of *S. invicta*. Electron microscopy had been used in the past to describe the ant's anatomy, and in fewer cases, to investigate ant-microorganism association. In 2001, Arab and Caetano (Arab and Caetano 2001) studied the midgut ultrastructure in *Solenopsis saevissima* Forel. And just recently, the first report of an endosymbiont in the digestive track of ponerine ants was published by Caetano et al. (2009).

Petralia and Vinson (Petralia and Vinson 1978, 1979, Petralia et al. 1980, Petralia and Vinson 1980a, b, Petralia et al. 1982) were among the first to use electron microscopy techniques to describe the ant external and internal anatomy. Based on their results, multiple factors suggested that only the fourth instar larvae can feed on solid food. According to Petralia and Vinson (1978), the head of the larvae of the first to third instars is positioned in a way that makes it impossible to reach the antero-ventral region, also their mandibles are not well defined and are not sclerotized. They also found that the fourth instar larvae have a special adaptation in the antero-ventral region that they called the food basket and its function is to hold the buccal pellet carried by the worker ants (Petralia and Vinson 1980b, Medina et al. 2007). In addition, the position of the head at this stage allows them to reach and consume the food deposited in the food basket; they also have well developed and sclerotized mandibles (Petralia and Vinson

1978). These adaptations are believed to play a role in the extra-oral digestion of proteins in the ventral pouch (Petralia and Vinson 1978).

Internally, the larvae does not have the filtering system found in the adults (Petralia and Vinson 1980b), meaning that they will be able to ingest larger food particles (e.i. vegetable fibers, fungal spores, bacteria, etc) deposited in the food basket by the adult worker ants. Although Petralia and Vinson (Petralia and Vinson 1980a) gave abundant detailed of the entire digestive system, our focus is only on the midgut. The midgut is lined with columnar epithelial cells that secrete thick peritrophic membranes. The midgut of fire ants, as in many higher Hymenopterans, is closed, meaning that all of its content is released in the meconium, right before entering the pupal stage (Petralia and Vinson 1980a, Petralia et al. 1982).

Regardless of all the information available today, there are no ultra-structural studies on the relationship between midgut bacteria and *S. invicta*, therefore our current project is the first attempt in the search for symbiotic bacteria inside the red imported fire ant midgut using SEM and TEM (Medina et al. 2007). This will provide knowledge about the relationship of bacteria with the ant host and possibly determine if any intracellular symbiont is present.

Bacterial microbiology of *S. invicta*. Donald P. Jouvenaz was probably one of the first entomologists to explore the red imported fire ant-microorganisms interaction with means of biological control (Jouvenaz et al. 1977, Jouvenaz 1983, 1986, 1990c, Jouvenaz et al. 1996). Beckham et al. (Beckham et al. 1982) emphasized the need for bacteria surveys in ants, but it is not until 2003 that the first report of bacteria living

inside the fire ant midgut was published (Peloquin and Greenberg 2003). The authors confirmed the presence of bacteria in the midgut of the fourth instar reproductive larvae. Their results inspired our project, which became the second effort to study these bacteria in the midgut (Li et al. 2005), with the isolation, culture, molecular identification, morphological and biochemical characterization of ten different species of bacteria from the fourth instar larvae. Later, Lee et al. (2008) published another study using culture independent methods, therefore listing more bacteria species in colonies collected from three different geographical locations in Louisiana (Baton Rouge, Rosepine, and Bogalusa). In the same year, another group published their work on symbiotic bacteria isolated from the hemolymph of *S. invicta* (Gunawan et al. 2008). And just recently, Tufts and Bextine (2009) isolated and identified bacteria from the hemolymph of the red imported fire ant queens and determined a possible vertical transmission of *Bacillus cereus* and *Bacillus thuringienses* in red imported fire ants.

Dissertation objectives. Although there are many more uncultured bacteria species in the red imported fire ants, only those growing *in vitro* have the potential for genetic transformation. Consequently we sustained the main objective for our project, to study only those ten midgut bacteria described in my previous publication (Li et al. 2005) with the purpose of selecting the best bacterial candidates for genetic transformation. This will potentially become part of a more effective and environmentally safe approach in a biological control program against the red imported fire ant in the United States by future introduction of foreign genes that express proteins with specific insecticidal properties.

The following research project must be structured and organized in such way that facilitates the comprehension of the complex RIFA-bacteria interactions. All information is presented as follow. *Chapter II*: isolation, identification, and biochemical and morphological characterization of the bacteria from the red imported fire ant midgut. *Chapter III*: investigate the internal anatomy of the midgut and its relation to the bacteria, their abundance, and the presence of any specialized structures, such as mycetocytes, meaning the host dependency to an obligate symbiont. *Chapter IV*: determine the abundance and distribution of symbiotic bacteria in colonies collected in southeastern United States, with means to select the best bacteria candidates for genetic transformation. *Chapter V*: the transformation of selected bacteria by introducing a DsRed fluorescent gene, thus allowing tracking of the bacteria throughout the ant developmental stages and studying their possible function as well as their relationship to the ant host. This last objective will provide the means for the introduction of foreign genes with insecticidal properties into the fire ant. *Chapter VI*: discussion and conclusions, with emphasis on future work.

CHAPTER II

ISOLATION, CHARACTERIZATION AND MOLECULAR IDENTIFICATION

OF BACTERIA FROM THE RED IMPORTED FIRE ANT

(*Solenopsis invicta* Büren) MIDGUT[*]

Introduction

The red imported fire ant, *Solenopsis invicta*, Büren (Hymenoptera: Formicidae) is native to the lowland areas of South America. After accidentally being introduced through the port of Mobile, Alabama in the 1930s (Vinson 1997), it has become one of the major pests in the United States. The red imported fire ant has spread throughout the Southeastern region of the USA. This dramatic spread has occurred through mating flights, colony movement, by rafting to new sites during periodic floods and by human activities (Vinson 1997). Seventy years after its first intrusion, there are five times more ants per acre in the United States than in the native habitats of South America (Calcaterra et al. 1999). The red imported fire ant results in serious health, economic, and environmental impacts to the communities it invades and causes billions of dollars of losses each year in urban and agricultural areas. In Texas alone, the damage and control costs exceed $90 million annually (Lard et al. 1999). Fire ant control is heavily dependent on insecticides and the only way to maintain control is to apply insecticides 1–2 times each year, at an estimated cost of 6–12 billion dollars per annum to treat all

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[*] Reprinted with permission from Li, H., F. Medina, S. B. Vinson, and C. J. Coates. 2005. Isolation, characterization, and molecular identification of bacteria from the red imported fire ant (*Solenopsis invicta*) midgut. J. Invertebr. Pathol. 89: 203-9. Copyright 2005 by Elsevier.

infested land (Drees 2002). Furthermore, the chemical insecticides used are not specific to fire ants and residues in the environment can impact non-target species. The expense of insecticides and environmental concerns leaves most infested land untreated and these areas boost the spread of the fire ant. Given the expense and hazard of insecticide treatments, as well as the presence of untreatable areas, the potential use of biological control for the fire ant is an important research avenue to pursue.

The major species of bacteria involved in insect control are spore-forming *Bacilli* with the best-known example being *Bacillus thuringiensis*, which has been used primarily for the control of Diptera and Lepidoptera (Burges 1982). Studies of symbiotic bacteria in insect species are allowing the development of new approaches for biological control. Symbiotic bacteria are ubiquitously located in animal guts with these symbioses ranging from pathogenic to mutualistic and from facultative to obligate (Lau et al. 2002). For *Buchnera* spp., the symbionts of the pea aphid, the host typically consumes a single food source of sugar-rich phloem sap of higher plants, which is generally poor in amino acids. The symbionts are thought to enable their hosts to survive on these restrictive diets by providing nutritional supplements such as amino acids and vitamins (Buchner 1965, Baumann et al. 1995, Blattner et al. 1997). Among insects, several systematic groups are frequently involved in symbiotic interactions with bacterial species, including: the genus *Wigglesworthia*, the well-characterized symbionts in testse flies (Aksoy et al. 1995, Chen et al. 1999), *Cicadellinicola* in *Homalodisca coagulate* (sharpshooters) (Moran et al. 2003) and *Blattobacterium* in cockroaches (Goldberg and Pierre 1969). These symbionts share a common ancestor and are

systematically placed adjacent to the family Enterobacteriaceae (Autuori 1941, Kermarrec et al. 1986, Adams et al. 2000). The potential use of these organisms for the biological control of insect pests has driven much of the current scientific research.

The vector-symbiont intervention (VSI) project was initiated as a novel means of control for Chagas disease, an insect vector-borne disease that affects 16–18 million people in regions of South and Central America (Beard et al. 1992, Beard et al. 1998). The Chagas disease vector, *Rhodnius prolixus*, harbors the symbiotic bacteria, *Rhodococcus rhodnii*. (Beard et al. 1992, Beard et al. 1998) found that the symbiotic bacteria could be genetically transformed to express an anti-trypanosomal agent in the gut. This discovery provides proof of principle for the use of symbionts as biological control agents.

Social insects develop numerous interactions with different species of microorganisms at individual and population levels. These interspecies interactions often involve bacteria and fungi (Boursaux-Eude and Gross 2000). The genus *Camponotus* is classified in the same subfamily as the fire ant, *Formicinae*, and is a textbook model for symbiosis. In all *Camponotus* species investigated so far, intracellular bacteria are present within the midgut, with these gram-negative rods being classified within a single genus, *Candidatus* Blochmannia gen. nov. (Sauer et al. 2000, Sauer et al. 2002). Previous studies have identified a large number of arthropod species from fire ant nests, however, the vast majority have transient and non-specific relationship with ants (Collins 1992). Insect predators, parasitoids, and parasites such as viruses, rickettsia, nematodes, fungi, and bacteria have also been identified and used for biological control

in association with fire ants (Bedding and Akhurst 1975, Bulla 1975, Aronson and Shai 2001). The red imported fire ant has now been a major pest in the United States for more than four decades. The effective and efficient control of fire ants remains a continuous challenge. Symbiotic bacteria found in other insect species may shed new light on the potential for biological control of the fire ant. The midguts of fourth instar reproductive fire ant larvae have been found to harbor bacteria (Peloquin and Greenberg 2003), providing an opportunity to identify host-specific bacteria for biological control of this pest species. Further studies of fire ant associated bacteria, particularly if obligate symbionts are found, may provide a long-term sustainable solution for fire ant biological control.

An understanding of complex microbial communities has been greatly enhanced by the development of molecular identification techniques based on the 16s rRNA subunit gene (Pace 1997). The 16s rRNA gene is a well-conserved, universal bacterial gene with constant and highly constrained functions that were established in the early stages of evolution and is relatively unaffected by environmental pressures (Woese 2000). This gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny) and more recently it has also become an important means to identify unknown bacteria to genus or species level (Sacchi et al. 2002). Analysis of the 16s rRNA gene can potentially be applied to identify all bacteria. In contrast to traditional microbiological methods, it provides at least two primary advantages: a rapid turn-around time and improved accuracy in identification (Springer et al. 1996).

The long-term goal of our research is to use genetically modified bacteria as an

alternative strategy for fire ant control. In this study, we describe the isolation and characterization of bacteria from the midguts of fourth instar fire ant worker larvae. Using PCR-RFLP (PCR followed by restriction fragment length polymorphism) and sequence analysis of the bacterial 16s rRNA gene, we identified ten bacterial isolates to at least the genus level. Antibiotic resistance profiles and biochemical activities were also determined for these species. This work provides the basis for a wider characterization of bacterial distributions in fire ant colonies and provides strains suitable for genetic manipulation to develop novel methods of fire ant control.

Materials and Methods

Red imported fire ant colonies. Red imported fire ant colonies used in this study were originally collected from different locations around Brazos County, Texas. These colonies were dug directly from the field along with soil and placed in 5 gallons plastic buckets, and then transported to the Entomology Research Laboratory (ERL) at Texas A&M University. After a day or two, the ants settled in and built new tunnels in the dirt inside the buckets; at this time, they were separated from the dirt using the drip-flotation method (Jouvenaz et al. 1977) and then moved into plastic sweater boxes with the inner wall coated with Fluon® to prevent any escapes. Colonies were maintained in the rearing room at the ERL and fed ad libitum with honey water, meal worms, and frozen crickets. There was no obvious pathogenesis associated with any of the samples.

Bacterial isolation and culture conditions. Several fourth instar worker larvae were randomly pulled from each colony, surface sterilized in 70% Et-OH, and then soaked in a 15% bleach solution (5.25% Sodium Hypochloride stock solution) for 1

minute, followed by 5 washes in sterilized ddH₂O and one wash in sterilized Phosphate

Buffered Saline (PBS). Each midgut sac was dissected out with sterilized probes,

individually homogenized, diluted 1,000-10,000 fold, and then spread on Blood agar

plates (containing 5% defibrinated sheep blood), Brain Heart Infusion (BHI) agar plates,

and nutrient agar plates (Becton Dickinson, Sparks, MD). The PBS used for the last

wash was also plated on the appropriate media as a negative control. After a 24 hour

incubation period at 37°C, or 3 days at 29°C, representative colonies, according to their

morphologies on the different culture media, were selected for further characterization.

Colonies were re-streaked on BHI agar plates until a pure culture was obtained.

Genomic DNA extraction. Well-isolated single colonies were selected and

inoculated in liquid BHI media at 37°C for 16 hours with shaking at 300 rpm. Bacterial

genomic DNA was isolated from the pure culture. Briefly, the cell pellet from a 1.5ml

culture was re-suspended in 464μl TE buffer (100mM Tris-HCl and 10mM EDTA, pH

8.0), then 30μl 10% SDS and 6μl proteinase K (10mg/ml) were added, mixed and

incubated at 37°C for 2 hours with occasional mixing. The mixture was extracted once

with 0.8 ml phenol-chloroform-isoamyl alcohol (25:24:1), and once with 0.8ml

chloroform-isoamyl alcohol (24:1), and precipitated with 2.5 volumes of 100% Et-OH.

After washing the DNA pellet with 70% Et-OH, the dried DNA pellet was re-suspended

in 50μl of ddH₂O.

Bacterial 16s rRNA gene amplification and RFLP analysis. To amplify the

bacterial 16s rRNA gene, the universal primers fD2 and rp2 described by Weisburg et al.

(1991) were used. Polymerase chain reaction (PCR) amplification was performed in a

final volume of 100µl with the following reaction components: 50ng template DNA, 2mM dNTPs, 1µM each primer, 3 units PCL DNA polymerase (Continental Lab Products, San Diego, CA), and 1x reaction buffer (2.5mM MgCl2). PCR amplification was performed in a MJ Research PTC- 200 Thermal Cycler (GMI, Inc., Ramsey, MN). The reaction conditions included an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C) and extension (2 min at 72°C), with a final extension at 72°C for 5 min. The amplification products were separated by electrophoresis on a 1% agarose gel and purified using the QIAQuick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified PCR products were digested with *Hae*III and separated on a 2% agarose gel. By comparing the patterns revealed from the PCR-based RFLP, colonies showing the same restriction profile were grouped together. The bacterial isolates with similar *Hae*III restriction patterns were analyzed using a second restriction enzyme *Rsa*I. Based on these results, ten isolates were selected for further characterization by biochemical and DNA sequencing analysis.

Biochemical characterization of bacterial isolates. Following the PCR-RFLP analysis, the ten selected isolates were identified according to Gram reaction (Gram stain set, Fisher Scientific Co., Swedesboro, NJ) and oxidase activity (Oxidase test sticks, Hardy diagnostics, Santa Maria, CA). MacConkey Agar Plates (Becton Dickinson, Sparks, MD) were used to investigate lactose formation. Brain Heart Infusion agar plates supplemented with 5% de-fibrinated sheep blood were used to test the hemolytic activity of the bacteria. Motility was observed on API-M medium (bioMerieux, Hazelwood,

MO). Differentiated oxidative or fermentative metabolism of glucose was tested on API-OF medium. The following biochemical properties of the bacteria were determined using the commercial test system, Analytical Profile Indexes - API-20E (bioMerieux, Hazelwood, MO); arginine dihydrolase, citrate utilization, H_2S production, urease hydrolysis, indole production, acetoin production, gelatinase hydrolysis, fermentation/ oxidation of glucose, mannitol, sorbitol and arabinose. The bacterial isolate no. 38 was also analyzed by gas chromatography (GC) fatty acid profiling, using the MIDI identification system in the Plant Disease Diagnostic Lab, at Texas A&M University, College Station, Texas.

Antimicrobial susceptibility test. The antimicrobial susceptibilities of the 10 selected isolates were determined using the disk diffusion test procedure (Kirby-Bauer) based on the consensus standard of the National Committee for Clinical Laboratory Standards (NCCLS) (Bauer et al. 1966); NCCL Standard, 1997. Mueller Hinton Agar (Meat infusion 2.0g/L, casein hydrolysate 17.5g/L, starch 1.5g/L, agar 13g/L) was used as the growth medium. Antibiotic impregnated disks were applied according to the recommendations of the manufacturer (Hardy Diagnostic Lab, Santa Maria, CA, USA). Briefly, Mueller Hinton Agar plates were swabbed with properly adjusted inoculum, and then the antimicrobial sensitivity test disks were applied. Plates were incubated at 35°C and the inhibition zones were measured after 16-18h. Isolates were considered as resistant (R), intermediate (I) and susceptible (S) following the disk diffusion zone diameter chart provided by the Hardy Diagnostic Lab. The following antimicrobial agents were used: Ampicillin (10μg), Chloramphenicol (30μg), Doxycycline (30μg),

Erthromycin (30μg), Gentamycin (10μg), Kanamycin (30μg), Nalidixic acid (30μg), Spectinomycin (100μg), Streptomycin (10μg) and Tetracycline (10μg). Antibiotic resistance to Zeocin™ was also tested by selecting a single colony of the isolated bacteria and streaking on an LB medium plate containing 25mg/L Zeocin™. Growth of the colonies was scored as resistant, non-growth of the bacteria was considered as susceptible, due to the lack of reference for Zeocin™ on the Hardy Disk™ chart.

Isolate identification by 16s rRNA gene sequencing. Gel purified PCR products of the 16s rRNA gene fragments from the ten isolates were cloned directly into the TA cloning vector, Topo2.1 (Invitrogen, San Diego, CA) following the manufacturer's instructions. The nucleotide sequences of the recombinant plasmids were determined using the ABI 3100 sequencer at the Crop Biotechnology Center, Texas A&M University. Near-full length gene sequence of the 16s rRNA gene was determined using the M13 forward and reverse primers and a custom designed internal primer. All sequences were assembled using the vector NTI software (Inc., InforMax, Bethesda, MD) and the 16s rRNA gene sequence of each isolate was compared with known 16s rRNA gene sequences in the GenBank database using the BLAST search algorithm (Altschul 1990).

PCR screening to determine the distribution of the midgut bacteria. Near-full length 16s rRNA gene sequences from ten isolates were aligned with vector NTI software for multiple sequence alignment. Primers were designed to species-specific regions for each bacterial isolate (Table 1). PCR amplification was performed on all ten isolates for each specific PCR primer pair to confirm specificity. Two of the fire ant

colonies were maintained in our laboratory and used as laboratory conditioned colony PCR-based screening. These were colony #3789 collected from Brazos County on August 29, 2003 and colony #3839 collected from Burleson County on October 16, 2002. Another colony, from Burleson County, was collected two days before DNA extraction on April 16, 2004 and served as the field colony. Genomic DNA was extracted from 30-40 midguts of fourth instar larvae from the mentioned colonies. The fourth instar fire ant worker larvae were surface sterilized and the midgut sacs dissected as described previously. The extracted genomic DNA was purified using a phenol-chloroform protocol (modified from (Hoelzel and Green 1992). Approximately 100ng genomic DNA was used as the PCR template, and partial sequences of the 16s rRNA gene were amplified with ten pairs of bacteria-specific PCR primers.

<div align="center">**Results**</div>

Phenotypic characterization of isolates. Bacteria were cultured from the midgut of previously surface sterilized fire ant worker larvae. All isolates grew equally well on Brain Heart Infusion agar plates at 29°C and 37°C from the dissected gut homogenate. Most colonies grew up to 4 mm in diameter after 24 hours incubation at 37°C, however, cultures also typically contained some colonies that were only 1/3 the diameter of the majority of colonies. β and γ -hemolysis was observed when grown differentially on Blood Agar plates. No colonies grew on the negative control plates, ruling out contamination during manipulation. According to growth characteristics and colony morphology, 36 well-isolated colonies were selected for further characterization and identification.

Table 1. Midgut bacteria specific PCR primers from the fourth instar worker larvae of red imported fire ants.

Primer Name	Size of Amplification (base pairs)	Primer Sequence
Bac4-5'		5'-GGC TCC AAA AGG TTA CCT CA-3'
Bac4-3'	400	5'-ACT CTA GAG ATA GAG CTT CCC-3'
Bac22-5'B		5'-AGG CCT TCG GGT TGT AAA GT-3'
Bac22-3'C	850	5'-GTC GCT TCT CTT TGT ATG CG-3'
Bac27-5'B		5'-CTG GGA ACG TAT TCA CCG TA- 3'
Bac27-3'C	900	5'-AAA GTA CTT TCA GCG AGG AGG A-3'
Bac36-5'B		5'-CAT GAT TCT TAT TTG AAA GAA GCA A -3'
Bac36-3'B	900	5'-GTT TAT CAC CGG CAG TCT CAC -3'
Bac38-5'		5'-TAC GAC TTC ACC CCA GTC ATG-3'
Bac38-3'	500	5'-TCC ACA GAA GTT TCA GAG ATG A-3'
Bac42-5'C		5'-CAC GCT ATC AGA TG AGC CTA A -3'
Bac42-3'C	900	5'- TTG CA ACC CTT TGT ACC GA-3'
Bac48-5'		5'-TAC GAC TTC ACC CCA GTC ATG-3'
Bac48-3'	450	5'-ATG CCG AAG AGA TTG CA GTG-3'
Bac101-5'		5'-GGA GCTTGCTCC CGG ATG TT-3'
Bac101-3'	400	5'-TGC GAG CAG TTA CTC TCG CA-3'
Bac102-5'		5'-AGC TTG CTT CTC TGT CGG TG-3'
Bac102-3'B	500	5'-GAA GCT CTG TCT CCA GAG TG-3'
Bac104-5'		5'-GCACAA GAG CTT GCT CTC-3'
Bac104-3'	500 and 900	5'-TTG ATG AAC GTA TTA AGT TC-3'

PCR amplification of the 16s rRNA gene and RFLP analysis. Bacterial genomic DNA was extracted from each of the 36 selected isolates and the bacterial 16s rRNA genes were amplified using universal primers. An approximately 1.5 kb fragment was produced in all cases, indicating that the amplification product was essentially the full length 16s rRNA gene. All amplified 16s rRNA products were digested with *Hae*III

and separated on a 2% agarose gel. RFLP analysis resulted in 10 different restriction patterns for the amplified 16s rRNA gene products. Colonies showing the same RFLP pattern were grouped together and a representative of each group is shown (Fig. 1A). In the situation where the differences in restriction patterns between two groups were not clear, *Rsa*I digestion was used to determine whether they belong to the same group or different groups (Fig. 1B).
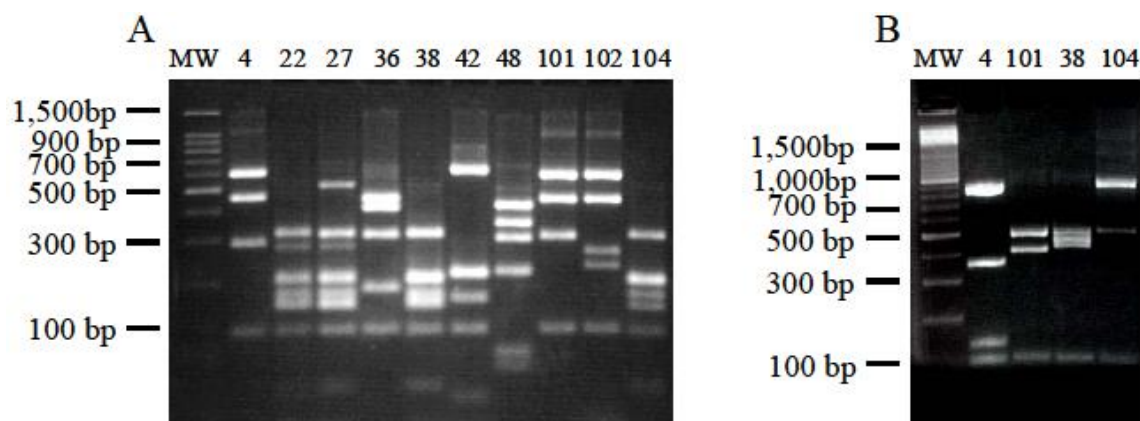
Fig. 1. PCR-RFLP profile of 16s rRNA genes from the midgut bacteria of the red imported fire ant obtained by *Hae*III and *Rsa*I digestion. (A) *Hae*III restriction digestion of amplicons from the 16s rRNA genes of ten selected isolates (as numbered). MW= 100bp molecular weight ladder. (B) *Rsa*I restriction digestion of 16s rRNA amplicons from isolates 4, 101, 38, and 104. MW= 100bp molecular weight ladder.

16s rRNA gene sequence analysis. An appropriately 1.5kb fragment of the 16S rRNA gene was obtained from all isolates using the universal bacteria primer set and the DNA sequence determined. The molecular identification using BLAST queries against the NCBI database (Table 2) indicated that the isolated bacteria from the fire ant midgut

belonged to 2 major groups. All the gram-positive bacteria, isolates 4, 36, 101, and 102, formed the first group- Bacilli. Based on these results, isolates 4 and 102 were identified to the genus level as likely *Enterococcus* and *Listeria*. Isolate 36 and 101 were identified to be most likely *Lactococcus garvieae* and *Bacillus pumilus*, respectively. The predominant bacterial group from the gram-negative bacteria was identified as protobacteria. Among all the gram-negative isolates, four belonged to the γ-subdivision. Isolates 27, 42, and 104 were most likely *Kluyvera cryocrescen, Pseudomonas aeruginosa* and *Serratia marcescens*, respectively, the *S. marcescens* we isolated here is a non-pigmentation strain (Data not shown).

Table 2. NCBI BLAST results for the 16s rRNA gene sequence from the ten fire ant midgut bacteria isolates.

BAC	Bacteria Identification	GenBank Accession No.	BLAST Match Accession No.	Identity (%)
4	*Enterococcus* sp.	AY946282	AY321376	99
22	*Enterobacter* sp.	AY946283	Z96078	99
27	*Kluyvera cryocrescens*	AY946284	AF310218	99
36	*Lactococcus garvieae*	AY946285	AY699289	99
38	Uncultured bacterium	AY946286	AJ487029	100
42	*Pseudomonas aeruginosa*	AY946287	AB126582	99
48	*Achromobacter xylosoxidans*	AY946288	AF411021	99
101	*Bacillus pumilus*	AY946289	AB020208	99
102	*Listeria* sp.	AY946290	AL596173	95
104	*Serratia marcescens*	AY946291	AB061685	99

Isolate 22 was identified to the genus level as *Enterobacter*. Isolate 48 belonged to the β-subdivision, which was identified as *Achromobacter xylosoxidans*. The E-value

of all BLAST results was 0. The identities between the bacterial isolates and the GenBank entries from the NCBI database (Table 2) were at least 98% in all cases. The BLAST result for isolate 38 indicates an uncultured bacterium with respect to the NCBI database. Identification from gas chromatography (GC) fatty acid profiling and the API-20E commercial kit resulted in an unreliable identification, indicating that isolate 38 has poor matches within the known bacterial databases, and may therefore be an unreported species.

Biochemical characterization and antimicrobial susceptibility of bacterial isolates. As shown in Table 3, isolate 22, 27, 38, 42, 48, and 104 grow well on MacConkey agar after 24 hours incubation at 37°C, while others (isolates 4, 36, 101, and 102) grew poorly, indicating that four of the ten isolates (4, 36, 101, and 102) are gram positive. These results were further confirmed by Gram-stain experiments. Out of six gram negative isolates, two (27 and 104) showed lactose fermentation on MacConkey agar plate, while the others (22, 38, 42, and 48) were colorless. This result revealed that isolates 22, 38, 42 and 48 are lactose non-fermenting strains.

The hemolytic status of all isolates was also determined. Isolates 42 and 101 showed a completely lysed clear zone around the colony on BHI Blood Agar plates, indicating they are β- hemolytic; isolates 4, 22, 27, 36, 38, 48, 102 and 104 showed non-hemolysis (γ -hemolytic) on the blood plates. No α- hemolysis was observed among these isolates.

In addition, all isolates were Oxidase negative except for isolates 42 and 48. Motility was observed for isolates 22, 27, 36, 38, 42, 48 and 104 on the API-M medium,

Table 3. Reactions and enzymatic activity results. Part of the biochemical characterization of ten bacteria isolates from the midgut of 4th instar larvae in red imported fire ant workers. (+) positive reaction, (-) negative reaction.

Reactions/Enzymatic Activity	Bacteria Isolates									
	4	22	27	36	38	42	48	101	102	104
Motility	+	-	-	-	-	-	-	+	+	-
MacConkey	-	+	+	-	+	+	+	-	-	+
Cytochrome-Oxidase	-	-	-	-	-	+	+	-	-	-
Arginine dihydrolase	+	+	-	+	-	+	-	-	-	-
Citrate utilization	-	+	-	-	-	+	+	-	-	+
H2S production	-	-	-	-	-	-	-	-	-	-
Urease hydrolysis	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	+	-	-	-	-	-	-	-
Acetoin production	-	+	-	+	-	-	-	-	-	-
Gelatinase hydrolysis	-	-	-	-	-	+	-	+	-	+
Glucose fermentation/oxidation	+	+	+	-	+	+	-	-	+	+
Mannitol fermentation/oxidation	-	+	+	-	-	-	-	-	-	+
Sorbtiol fermentation/oxidation	-	-	-	-	-	-	-	-	-	+
Arabinose fermentation/oxidation	-	+	+	-	-	-	-	-	-	-

while isolates 4, 101 and 102 were non-motile bacteria. Isolates 4, 22, 27, 36, 38, 102 and 104 displayed a fermentative metabolism of glucose, while isolate 42 used oxidative metabolism of glucose, and isolate 48 and 101 were inert for metabolism of glucose. The biochemical properties of the isolates were also determined using the API- 20E kit, with the results of the biochemical tests summarized in Table 3. All isolates produced H₂S and were urease hydrolysis negative. Except for isolate 27, the Indole reaction was negative for all isolates. Isolates 4, 27, 38, 42, 48, 101, 102, 104 were acetoin production negative. All isolates exhibit sorbitoil oxidation except isolate 104. Isolates 22 and 27 use Arabinose fermentation while the others use arabinose oxidation. Results from the

arginine dihydrolase, citrate utilization, gelatinase hydrolysis, metabolism manner of glucose and mannitol tests demonstrated that the ten isolated bacteria are biochemically distinct.

To determine the antibiotic susceptibility of the isolates, 11 antibiotic reagents were used (Table 4). Multiple-resistance phenotypes were observed among the ten isolates. Isolate 48 is resistant to all the antibiotics tested here except for intermediate resistance to Erythromycin.

Table 4. Antibiotic resistance test results for each bacterium isolate. (R) resistant, (I) intermediate, and (S) Susceptible.

Antibiotics\Bacteria Isolates	4	22	27	36	38	42	48	101	102	104
Ampicillin	S	R	I	S	S	R	R	S	S	R
Chloramphenicol	S	S	S	S	S	I	R	S	S	S
Doxycycline	S	S	R	R	S	R	R	S	R	R
Erythromycin	R	R	I	S	I	R	I	I	S	R
Gentamicin	S	S	S	S	S	S	R	S	S	S
Kanamycin	R	S	S	S	S	R	R	S	S	S
Nalidixic Acid	R	S	S	R	S	R	R	I	R	S
Neomycin	S	S	S	S	S	I	R	S	S	S
Spectinomycin	S	S	S	S	R	S	R	S	I	S
Streptomycin	S	S	S	S	S	I	R	S	S	S
Tetracycline	S	S	R	R	S	R	R	S	R	R
Zeocin	R	S	S	R	S	R	R	S	R	R

The grade of resistance to Gentamicin, Kanamycin, Nalidixic Acid and Streptomycin was high, with no zones of inhibition around the disks. In contrast, isolate 101 was susceptible on all the antibiotics except for intermediate resistance on Erythromycin and Nalidixic Acid. A similar result was observed for isolates 22 and 38,

which were susceptible on nine antibiotics and showed resistance or intermediate resistance on the other two (Ampicillin, Erythromycin resistance for isolate 22 and Erythromycin, Spectinomycin intermediate resistance for isolate 38).

Distribution of the bacteria in the midguts among fire ant colonies. Based on the sequence alignment of the 16s rRNA genes from these 10 worker midgut bacteria, species-specific primers were designed and tested on all isolates for their specificity. The results revealed that the predicted band was only amplified from the specific isolates and was absent in others. We then examined the distribution of these ten isolates on two laboratory maintained colonies and one colony directly collected from the field by using PCR amplification with these ten pairs of specific primers. As shown in Fig. 2, an expected 0.9 kb amplification fragment from isolate 27, *Kluyvera cryocrescens*, was presented in all 3 colonies, suggesting that this bacterium is common throughout all the colonies. The same result was also observed with the primer set specific for isolate 42, *Pseudomonas aeruginosa*. Another bacterial isolate, 36, *Lactococcus garvieae*, was present in only one lab maintained colony and the colony from the field. For bacteria isolate 48, *Achromobacter xylosoxidans*, the expected 0.5 kb fragment of the partial 16s rRNA gene is amplified in the field colony, however it was absent from the selected lab maintained colonies. The expected size of amplification products were not generated from DNA extracts of fire ant midguts with the primer sets for isolates 4, 22, 38, 101, 102, and 104, indicating that these bacteria may not be common in fire ant colonies.

Discussion and Conclusions

In this study, we isolated bacteria from 4th instar larval midguts of red imported
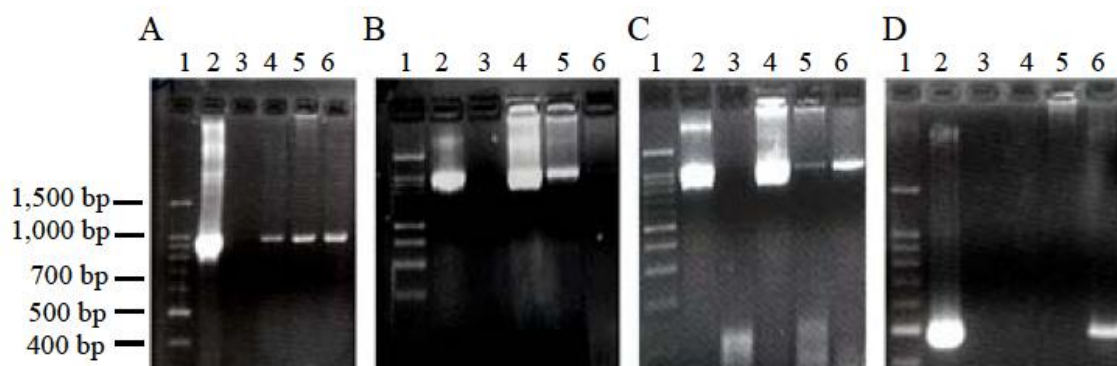
Fig. 2. Bacterial species-specific PCR screening of the midgut bacteria in red imported fire ant workers. From lab-maintained colonies and a field colony. (A), (B), (C), (D) Bacterial species-specific PCR screening for isolates 27, 36, 42 and 48, respectively. Lane 1, 100bp molecular size marker; Lane 2, Positive control for the specific bacteria; Lane 3, Negative control with ddH2O used as the template; Lane 4, Fire ant colony Burleson County #2; Lane 5, Fire ant colony #3789; Lane 6, Fire ant colony # 3839.

fire ant workers. By using PCR-RFLP analysis of the 16s ribosomal RNA gene and DNA sequencing of this gene, we were able to group and identify several isolated bacteria to at least the genus level. We also characterized the morphologies, biochemical activities, and antibiotic resistance profiles of these bacteria. We were able to efficiently select candidate bacteria from thousands of colonies recovered from the midgut homogenate. The general approach is based on PCR amplification and RFLP finger printing of the 16s rRNA gene. A significant advantage of this protocol is that candidate bacterial isolates can be identified within 2–3 days, without prior characterization and conventional selection using routine biochemical tests, which generally take several weeks. A number of reports have demonstrated that 16s rRNA gene sequence analysis improves the identification of bacteria compared to conventional phenotypic methods and that the 16s rRNA gene system was superior to conventional phenotypic

identification (Tang et al. 2000, Bosshard et al. 2003). A growing number of studies have reported the use of 16s rRNA sequencing for the identification of bacteria and their phylogenetic relationships in insects, (Ohkuma et al. 1999, Moran et al. 2003, Peloquin and Greenberg 2003). In this study, the near full length 16s rRNA gene was used to identify the isolated bacteria. In applying this method, we isolated and grouped ten bacterial species from the fire ant midgut.

Peloquin and Greenberg (Peloquin and Greenberg 2003) identified several gram-positive bacteria from the midgut of fourth instar reproductive larvae. By using partial 16s rRNA sequences, they identified two strains as *Lactococcus garviae* and *Staphylococcus saprophyticus*. In addition, they also grouped isolates to the genus, *Enterococcus*. In agreement with these findings, we also identified a strain as *L. garviae* and a strain to *Enterococcus*, indicating that they might be common to both the California and Texas fire ant samples. However, in contrast to (Peloquin and Greenberg 2003), where only gram-positive bacteria were isolated, we also recovered several gram-negative bacteria from the midgut of fourth instar worker larvae. Our study indicates that the isolated bacteria are closely related. Of the isolates, at least 40% belong to the γ-subdivision of Proteobacteria, with one bacteria belonging to the β-subdivision, which is in agreement with the results from previous insect studies (Aksoy et al. 1995, Chen et al. 1999). Recent molecular phylogenetic analysis of 16s rRNA genes also demonstrated that most insect symbionts belong to the Proteobacteria, primarily within the γ-subdivision (Moran and Telang 1998). The closest relatives amongst symbionts are the *Wigglesworthia* spp., the endosymbiotic bacteria of the tsetse fly and *Buchnera*

aphidicola, the symbionts of aphids, which together form a large cluster of symbiotic organisms and have a common ancestor with the Enterobacteriaceae (Aksoy et al. 1995, Chen et al. 1999).

Serratia marcescens is a bacterial species that commonly occurs in soil and water as well as animal intestines. Jouvenaz et al. (1996) fed fire ants food contaminated with this bacterial species to determine whether these bacteria were ingested. The pigmentation produced by the *S. marcescens* strain and lack of pathogenicity to the fire ants makes this strain an ideal candidate for feeding tests. In their experiments, they found that no bacteria were recovered from any of the queens and workers, indicating that all bacteria were effectively excluded from the gut. However, they found the bacteria in all midguts after feeding the fourth instar larvae with bacteria contaminated food. This result reinforced the finding that the pharyngeal filters of fire ant workers prevent particles greater than $0.88 \pm 0.02 \, \mu m$ in diameter from passing, however the larvae do not filter these small particles (Glancey et al. 1981). In the present study, we successfully isolated a strain identified as a *S. marcescens* species. The fourth instar is the only stage that is fed solid food and the *S. marcescens* strain we isolated here is a non-pigmented strain. Whether the *S. marcescens* we isolated is from the soil or a food source is unknown.

Kluyvera cryocrescens is a soil bacterium that is also found in the rumens and intestines of animals and humans (Farmer et al. 1981). Intestinal bacteria are important to the health of human beings; with similar functions being reported in insects (Tokuda et al. 2000), such as producing short-chain fatty acids from carbohydrates or

synthesizing amino acids. Based on these functions, it is expected that these bacteria may play a role in the nutritional physiology of their hosts. The midgut bacteria could be parasites living in the gut, relying on nutrients that host enzymes have digested (Vossen et al. 2004). While the role of these bacteria in the fire ant is unknown, we have no indication that they cause any pathology. Studies emphasizing the role of these bacteria in fire ant physiology will be addressed further.

Just recently, after publishing the results from this project (Li et al. 2005), culture independent methods were also used to explore the midgut bacteria diversity in RIFA colonies from three different geographical locations near Baton Rouge, LA (Lee et al. 2008). The authors identified a total of 68 different bacterial species from the RIFA midgut, with 36 species (52.9%) classified as uncultured bacteria. They also concluded that midgut bacteria were ubiquitous present in the three studied sites. *Klebsiella* sp., *Enterobacter* sp., a *Proteobacterium*, and an uncultured bacterium were the only species present in at least two of the sites (Lee et al. 2008). The results presented here (*Chapter II*), demonstrated that only ten of these 68 bacteria can be cultured *in vitro*, thus the only possible candidates for genetic transformation.

In summary, we have described the isolation of bacteria from the fire ant midgut. Bacterial isolates were grouped by 16s rRNA gene analysis using PCR-RFLP and gene sequencing. The characterization and identification of bacteria from the fire ant are important steps towards investigating the roles they play in fire ant physiology and the possibility for them to be used in a biological control strategy for the fire ant. Furthermore, a subsection of these bacteria are easy to cultivate, genetically modify, and

reintroduce back into the fire ant host. Studies aimed at the development of a robust method to genetically engineer these gut bacteria and re-introduce them into the fire ant colony are under way. The bacteria studied here will be investigated for their use as shuttle vehicles for the introduction of foreign genes and the expression of foreign proteins in the fire ant, allowing the bacteria to be used as an alternative tool for the biological control of fire ants.

CHAPTER III

APPLICATION OF SEM AND TEM IN MICROBIOLOGICAL STUDIES OF

THE RED IMPORTED FIRE ANT (*Solenopsis invicta* Büren) MIDGUT[*]

Introduction

Throughout evolution insects have evolved alongside microorganisms; and in many cases, insects have developed internal and/or external specialized structures to keep and protect endosymbionts (Bourtzis and Miller 2003). External structures containing bacteria have been recently described in European beewolves (Goettler et al. 2007). Internally, specialized cells called mycetocytes are known to harbor intracellular microorganisms regarded as obligate endosymbionts. These large cells are usually found lining the gut epithelium of the digestive track, in the Malpighian tubules, free in the haemocoel, or in the fat body, depending on the insect group (Douglas 1989). According to Douglas (1989), mycetocytes in the midgut are irregularly distributed among other bacteria free epithelial cells. But, in the specific case of bostrychid beetles, the mycetocytes are free in the midgut lumen while anchored to the wall through a filament of epithelial cells (Douglas 1989).

The association between microorganisms and insects has been reported in multiple occasions (Bourtzis and Miller 2003), and mostly referred as of the pathogenic type in the early studies. A review titled "Endosymbionts of Insects" by Dasch et al.

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[*] Reprinted with permission from Medina, F., E. A. Ellis, M. W. Pendleton, A. Holzenburg, S. B. Vinson, and C. J. Coates. 2007. Application of SEM and TEM in microbiological studies of the red imported fire ant (*Solenopsis invicta* Büren) midgut. Microsc. Microanal. 13: 234-235. Copyright 2007 by Cambridge University Press.

(1984) clearly demonstrates the interest of entomologists to investigate the relationship of endosymbionts with their insect host. In 1984, these authors recognized that our knowledge of symbionts has been limited by our inability to culture them. Therefore, all the efforts to investigate their distribution and abundance in different insect tissues had been based on staining, and on light and electron microscopy techniques (Dasch et al. 1984). Insects that are dependent on their symbiont usually feed on restricted diets (Dasch et al. 1984, Douglas 1989), which can be divided into several groups, blood sucking, plant sap sucking, cellulose and store grain feeders, and complex diet feeders (Dasch et al. 1984). As members of the later group, ants of the species *Camponotus ligniperda* and *Formica fusca* were the first insects reported carrying an endosymbiotic bacterium in their ovarian tissues (Blochmann 1882).

Microsporidia, yeasts, fungi, viruses, and bacteria, have all been identified from fire ants in the United States (Avery et al. 1977, Jouvenaz et al. 1977, Beckham et al. 1982, Jouvenaz 1984, Jouvenaz et al. 1984, Jouvenaz 1986, 1990d, a, Jouvenaz and Kimbrough 1991, Shoemaker et al. 2000, Peloquin and Greenberg 2003, Li et al. 2005, Bouwma et al. 2006, Baird et al. 2007, Gunawan et al. 2008, Lee et al. 2008). Since the first report on bacteria living inside fire ants, no studies have attempted to elucidate the fire ant-endosymbiont relationship (Jouvenaz 1990c, Peloquin and Greenberg 2003). Therefore our main objective was to investigate the abundance, distribution, ecology, and, most importantly, the ant-bacteria interactions and their potential use for genetic transformation as means for biological control. To help understand the ant-bacteria interactions and ecology, we must determine whether these bacteria can be found as free

living organisms in the fire ant, or if they are contained or associated with any specialized cells or structures. This last idea will provide proof that a true endosymbiont is present. For this purpose, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) will provide the power and resolution that allows a visual search of the internal anatomy of fire ants as well as any bacteria cells.

In the past, traditional methods failed to detect most microbes, especially bacteria. However, today not only the newly developed molecular tools have had an upper hand on the detection of microorganisms, but also advances in electron microscopy have played an important role on microbiological studies. This is due to the fact that most of endosymbiotic bacteria cannot be cultured *in vitro* and are only detectable by molecular methods (Douglas 1989, Corby-Harris et al. 2007) and by electron microscopy (Caetano et al. 2009).

Electron microscopy has been used to describe the ant internal anatomy, and in fewer cases, to investigate ant-microorganism association. However, it has never been used to study the relationship between midgut bacteria and *Solenopsis invicta*. Petralia and Vinson (Petralia and Vinson 1978, 1979, Petralia et al. 1980, Petralia and Vinson 1980a, b, Petralia et al. 1982) were among the first to use electron microscopy techniques to describe the ant external and internal anatomy. However, Jouvenaz and Ellis (Jouvenaz et al. 1984, Jouvenaz and Ellis 1986), investigated two microsporidia in fire ants using light microscopy and TEM. Also, Arab and Caetano (2001) studied the midgut ultrastructure in *Solenopsis saevissima* Forel. Finally, the first report of an endosymbiont in the digestive track of ponerine ants was recently published by Caetano

et al. (2009). Our current project is the first attempt to search for symbiotic bacteria inside the red imported fire ant midgut using SEM and TEM, thus investigating their relationship with the insect host.

Materials and Methods

Fire ant samples. Adult workers and fourth instar larvae were randomly selected from our laboratory reared colonies. These colonies were maintained in the Entomology Research Laboratory (ERL) as previously described in other chapters. Only healthier fire ant colonies, with plenty of brood in their nests, were selected for this investigation.

Sample preparation. Samples consisting of adult worker ants and fourth instar larvae were exposed to osmium vapors before being sputter coated with palladium-gold (50:50) and then observed in a JEOL JSM-6400 SEM at an accelerating voltage of 15 kV. Another group of samples was prefixed with osmium vapors followed by fixation in 2.5% glutaraldehyde-2.5% acrolein in 0.1M sodium cacodylate buffer, pH 7.4. Specimens were then post-fixed in 1% osmium tetroxide, dehydrated in methanol to propylene oxide and then infiltrated and embedded in epoxy resin. Hundreds of micro-sections were first examined under a light microscope, selecting the ones of most interest based on their location in the larval body (Fig. 3). The selected micro-sections were post stained with uranyl acetate and lead citrate and then examined in a JEOL 1200EX TEM at an accelerating voltage of 100 kV.

Results

In all the samples observed under SEM and TEM, only free living bacteria was
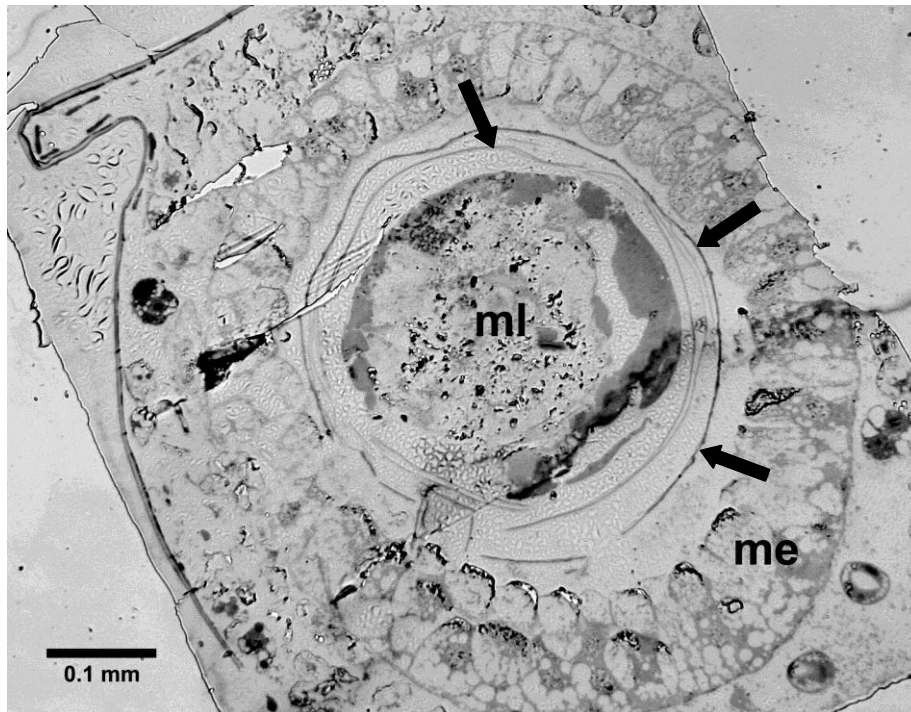
Fig. 3. Light microscopy image of midgut cross section of a fourth instar larvae. ml, midgut lumen; me, midgut epithelium; arrows, showing the peritrophic envelopes (Reichert-BioStar Inverted microscope 100X).

found within the midgut of the red imported fire ant. No specialized structures were identified at least in the midgut tissue or around the epithelial cells lining the gut.

Some bacteria attach themselves to the peritrophic membranes in the midgut lumen. SEM images confirmed several structural adaptations of fire ant to a social lifestyle as shown in previous work (Petralia and Vinson 1978, 1979, Petralia et al. 1980, Petralia and Vinson 1980a, Petralia et al. 1982). Nevertheless, it also provided us with new ideas about possible sites where bacterial activity might play an important role for the ants. These new sites include the ventral pouch used for the extra-oral digestion of proteins by the fourth instar larvae, the salivary glands, salivary reservoir, and foregut

and hindgut of the digestive system, which are all known to harbor microorganisms in other insects.

Images also provided us with a general structure of the midgut environment, the possible function of the peritrophic membrane in fire ants and their association with bacteria and other microorganisms. Fungal spores were also common in the midgut of ants, although we did not investigate further into this topic, it could definitely provide important information.

Discussion and Conclusions

Fire ants are social insects living in large colonies with a distinctive division of labor. Mature colonies are formed by a single (monogyne) or multiple (polygyne) queens, adult workers, adult reproductive females and males, eggs, first, second, third, and fourth instar larvae, pre-pupae, and pupae (Petralia and Vinson 1978, 1979, Vinson and Greenberg 1986, MacKay et al. 1990, Vinson 1997). Foragers, represented by the older workers, collect food and bring them to the nest where the younger adult workers (Fig. 4-1) are responsible of caring for and feeding the immature stages. Fire ant larvae go through four well defined stages of development as mentioned above (Petralia and Vinson 1979). A fully developed fourth instar larva (Fig. 4-2) is fed liquids and solid food by young worker ants. The food is brought as a buccal pellet (Fig. 4-3) and deposited in a ventral pouch (Fig. 4-4), where secreted enzymes and possibly bacteria help with the digestion of proteins (still under investigation).

Extra-orally digested food is both consumed by the larvae and picked up by other
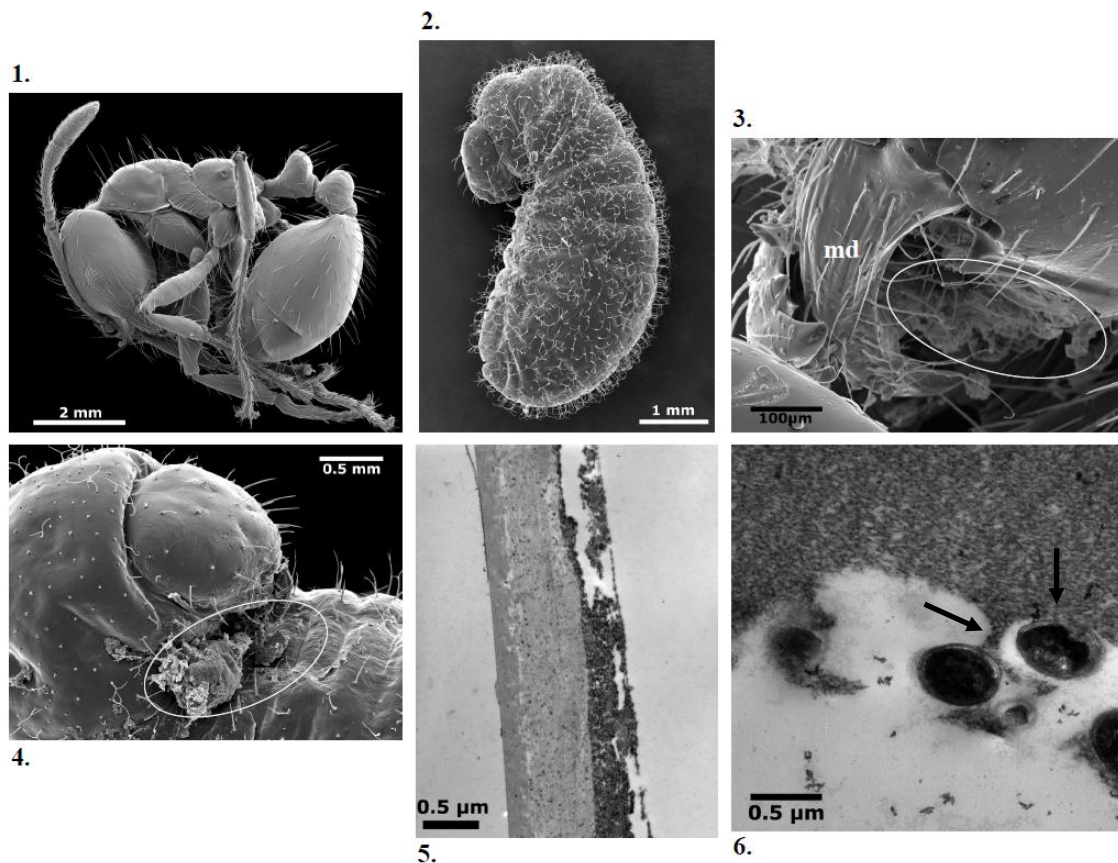
Fig. 4. Scanning and transmission electron microscopy images of RIFA. **1.** Adult fire ant worker (JSM-6400 15kV X60). **2.** Fourth instar larva (JSM-6400 15kV X85). **3.** Mouth parts of an adult worker showing the buccal pellet (circled) held by the mandibles (md) (JSM-6400 15kV x370). **4.** Antero-ventral view of the fourth instar larva (JSM-6400 15kV x160). The solid food (circled) deposited on the ventral depression is extraorally digested by a mixture of saliva and enzymes. **5.** Peritrophic envelope; these separate all the midgut content, including bacteria and other microorganisms, from the midgut epithelium (JEOL 1200EX TEM 100kV X25000). **6.** Free living bacteria found inside the midgut sac of the larva. Some bacteria were directly attached to the peritrophic envelope; the function of which is still unknown (JEOL 1200EX TEM 100kV X10000).

workers to feed the queens, therefore the great importance of fourth instar larvae as food processors in the colony. It is important to note, that at the larval stage and with each molt, the internal cavity or lumen of the midgut is lined with peritrophic envelopes or membranes forming multiple layers, probably each layer corresponding to each molt

(further investigation needed). These layers protect the epithelial cells not only from mechanical and chemical damage, but also from pathogens acquired with the food. Thin sections for TEM exposed part of the digestive system internal structure (Fig. 4-5), providing evidence of bacteria inside the midgut lumen (Fig. 4-6), and revealed a possible functional association of the bacteria with their insect host. It also showed that no specialized structures and only free living bacteria were found in the midgut. In adult ants, once again the pictures demonstrated that bacteria smaller than 0.8 µm (0.5 µm in our case) are able to reach the midgut. This is due to the presence of a filtering system in the adult workers (Glancey et al. 1981).

The larval digestive system is closed (Petralia and Vinson 1980a), meaning that all ingested food remains in the midgut and it is only excreted in the meconium right before entering the pupal stage (Figs. 5 and 6). This also includes the bacteria within the midgut lumen (Medina et al. 2009)
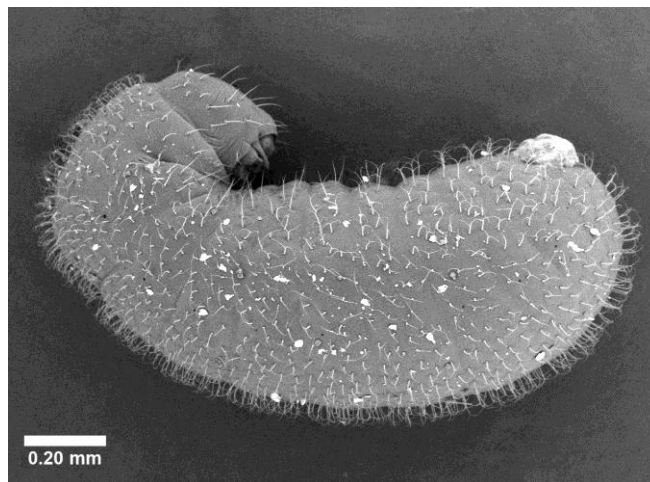
Fig. 5. Fourth instar larvae excreting the meconium right before entering the pre-pupal phase. This picture was acquired with a Hitachi Tabletop Microscope TM-1000.
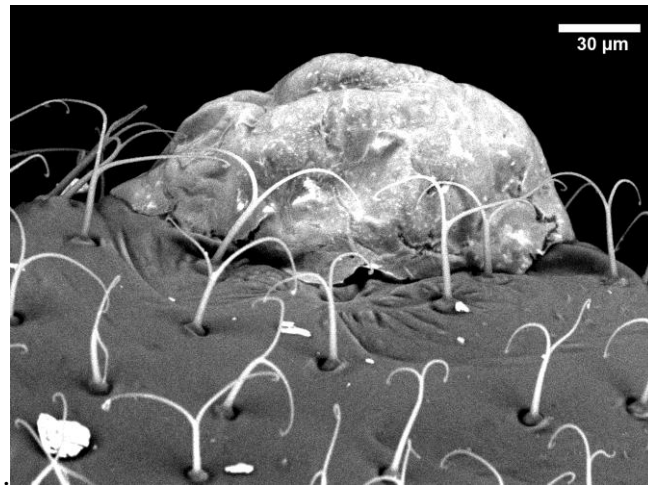
Fig. 6. Close up image of the meconium. Image acquired with the Hitachi Tabletop Microscope TM-1000.

The importance of bacteria in the spread of RIFA in the southeastern United States supports the potential uses of these bacteria as biological control agents. Results also confirm morphological adaptations of these ants to their social lifestyle, feeding behavior and potential association with symbiotic microorganisms.

CHAPTER IV

GEOGRAPHICAL DISTRIBUTION OF THE RED IMPORTED FIRE ANT

(*Solenopsis invicta* Büren) MIDGUT BACTERIA, SELECTED FOR GENETIC

TRANSFORMATION, IN SOUTHEASTERN UNITED STATES

Introduction

The potential use of bacteria in a biological control approach has been investigated to some extent in other insects, as shown in following examples. But, in the red imported fire ant, the information is still very limited. Further investigation is still needed before we can benefit from the full potential of endosymbiotic bacteria as a biological control agent. Fortunately, new developments in biotechnology may soon enable us to create new strains of microbial pathogens that are more virulent, easier to mass produce, and less sensitive to diverse climate conditions.

Investigations on a widely spread bacterium, *Bacillus sphaericus*, revealed they can produce protein toxins which are lethal to mosquito larvae (Thanabalu and Porter 1996). Chan et al. (1996) also demonstrated that the different toxins produced by this bacterium were particularly effective against certain mosquito species, proving its host specificity. The gram negative bacterium, *Asticcacaulis excentricus*, has been investigated as another potential candidate in a biological control program against mosquitoes due to their persistence, UV resistance, lack of toxin degrading proteases and low production costs (Liu et al. 1996). Another example comes from McKillip et al. (1997), after a successful culture and transformation of a midgut bacteria from the

leafroller, *Pandemis pyrusana*. Additionally, the bacterium, *Enterobacter cloacae*, had been transformed to express an ice nucleation gene (inaA), becoming another good candidate for a biological control program (Watanabe et al. 2000). There are two more examples worth mentioning. One is the vector-symbiont intervention project in Chagas disease, where *Rhodococcus rhodnii* have been transformed to express an anti-tripanosomal agent in the midgut of *Rhodnius prolixus* (Beard et al. 1992, Beard et al. 1998). And second, the introduction of a DsRed fluorescent protein into a bacterium found inside the glassy-winged sharpshooter and the reintroduction of the transformed bacterium into the host foregut (Bextine et al. 2004).

Just recently, molecular and electron microscopy tools allowed us to explore the real potential of bacterial candidates for a biological control program against the red imported fire ant, *Solenopsis invicta* Büren (Peloquin and Greenberg 2003, Li et al. 2005, Medina et al. 2007, Gunawan et al. 2008, Lee et al. 2008, Medina et al. 2009, Tufts and Bextine 2009). Microbes are the most abundant life form on earth, but not all are best suited for genetic transformation and/or biological control of insect pests (El Husseini 2006).

As our main purpose and by means of genetic transformation, the selected midgut bacteria will be use as a delivery agent to express a toxic gene product inside the red imported fire ant. But first, we must take into consideration certain characteristics that will make the midgut bacterium the best candidate (El Husseini 2006). Foremost, they must have a narrow host range specific to the red imported fire ant, not posing an environmental threat to humans and other animals, including the native ant populations.

Consequently, best bacteria candidates must be closely associated with their ant host; under this condition, a possible symbiotic association will be highly desirable. Candidates must adapt well to the climate conditions and ideally have a high reproductive potential with multiple generations per host. In addition, they must have a high infestation rate and ability to naturally infect new colonies.

From the midgut of the fourth instar larvae of the red imported fire ants, we identified ten bacteria species. Including, *Enterococcus* sp*./durans, Klebsiella ornithinolytica, Kluyvera cryocrescens, Lactococcus garvieae, Pseudomonas aeruginosa, Achromobacter xylosoxidans, Bacillus pumilus, Listeria innucua, Serratia marcescens,* and an unidentified bacterium from the family Enterobacteriaceae (Li et al. 2005). They were all well adapted to the environmental conditions inside the fire ant colonies, but they also readily grew in artificial media. This permitted their biochemically and morphologically characterization, and also testing for resistance against several antibiotics (Li et al. 2005).

In our samples we found the presence of several gram-positive and gram-negative bacteria. Among the later, some were closely related to each other, belonging to the γ-subdivision of Proteobacteria, and one bacterium in the β-subdivision. This seems exciting, due to the fact that most insect symbionts are members of the phylum Proteobacteria, primarily within the class γ-Proteobacteria (reviewed in Moran and Telang 1998). Supporting this idea, some well known symbiont members of this group, included: *Arsenophonus* spp*.,* an endosymbiont of insects (Gherna et al. 1991, Hypsa and Dale 1997, Trowbridge et al. 2006), *Buchnera* spp*.* a symbiont of aphids (Munson et

al. 1991, Clark et al. 1992), and the endosymbiotic bacteria of the tsetse fly*, Wigglesworthia* spp. (Aksoy et al. 1995); *Escherichia* spp. is also a member of this group.

Specifically from the midgut bacteria, we were able to find members of the Proteobacteria: Gammaproteobacteria: Enterobacteriales: Enterobacteriaceae, which included the following species: *Klebsiella ornithinolytica* (Sakazaki et al. 1989, Brenner et al. 2005)*, Kluyvera cryocrescens* (Farmer et al. 1981, Brenner et al. 2005)*, Serratia marcescens* (Grimont and Grimont 1978, Brenner et al. 2005), and an unidentified bacterium (isolate#38). Therefore close attention will be given to the distribution of these species in southeastern United States.

Our hypothesis states that, if any of these midgut bacteria are consistently present in all red imported fire ant colonies, regardless of geographical location, it would be an excellent indicator of a symbiotic association. Therefore, those bacteria can become potential vectors of foreign genes in a control program against the red imported fire ant. For that purpose, several fire ant colonies from Texas, Louisiana, Mississippi, and Alabama (including the Mobile State Port) were collected and screened for the presence of the ten midgut bacteria. The abundance of each bacteria species will be determined, in addition to their exact geographical distribution from global positioning systems (GPS) data.

These results will constitute the basis for a successful genetic transformation of only the best bacteria candidates. Future experiments will include the introduction of a gene to express a red fluorescent protein, and finally, the introduction of exogenous

genes with specific insecticidal properties (still under investigation). Results will potentially become part of a more effective and environmentally safe approach in a biological control program against the red imported fire ant in the United States.

Materials and Methods

Collecting sites. On August 2004, 17 different sites were sampled along the route from College Station, TX to Mobil, AL. These sites included the nearby areas to the docks at the Alabama State Port in Mobile, which is the point of entry for RIFA into the USA (Creighton 1930, Wilson 1959, Lennartz 1973). 12 more locations were sampled on a second trip in the summer of 2005 from College Station, TX to Brownsville, in south Texas. During the summer and fall of 2008, all the eight counties surrounding Brazos Valley, near Texas A&M University campus, were studied. Due to its proximity, the Brazos Valley area was more intensively sampled and its 14 collecting sites were grouped into eight counties. At least two to three fire ant colonies were collected per site on the other trips, and coordinates from a Global Positioning System (GPS) device were recorded for all the sites to map and track the bacteria populations. Maps were created with ArcView, Geographic Information Systems software developed by the Environmental Systems Research Institute (ESRI) in Redlands, California.

Fire ant colonies. More than 80 fire ant colonies were collected from different sites in Texas, Lousiana, Mississippi, and Alabama including the port area in Mobile, Al. These colonies were dug directly from the field along with soil and placed in plastic buckets, then transported to the Entomology Research Laboratory (ERL) at Texas A&M University. Once the colonies settled and built new tunnels inside the buckets, they were

separated from the dirt using the drip-flotation method (Jouvenaz et al. 1977) and then placed inside plastic sweater boxes with the inner walls previously coated with Fluon®. Inside each tray, the nests or queen chambers were built of a Petri dish (different sizes) filled half way with dental stone (Castone®, Dentsply International Inc, York, PA), and with two holes drilled on the top plate for easy access. Colonies were ad libitum fed crickets, mealworm larvae and/or pupae, and 10% honey water solution every other day. Water was also readily available in the colony and supplied in a test tube with a cotton stopper.

Sample processing. Immediately after separating the colonies from the dirt, several fourth instar larvae and adult workers were taken from each colony, placed in a 2mL centrifuge vial, and properly labeled to identify their specific collecting site. These were surface sterilized following the methodology described by Li et al. (2005) and placed into new sterile vials, then dipped into liquid nitrogen for a few seconds and stored at -80°C.

Midgut extraction. Following the procedures described by Li et al. (2005), midguts were extracted under sterile conditions inside a biological safety cabinet. With the aid of a dissecting microscope, the cuticle of the larva was carefully removed with forceps in a sterilized PBS solution to expose the midgut sac. To acquire enough DNA, a total of 20 larvae per sample were dissected. The 20 resulting midguts were then placed into a new vial; this procedure was repeated for all of the studied sites.

DNA Isolation. Samples were thawed and quickly placed into a VWR® Screw-Cap Microcentrifuge Tube 2.0mL with 20 to 30 glass beads (1.0mm- BioSpec Products

Inc.). Each tube is then placed in the MINI-BeadBeater™ BioSpec Products Inc. for 70 seconds at 5000 rpm; when completely homogenized, it was transferred to an ice bin for DNA extraction. The Qiagen DNeasy Blood & Tissue Kit protocol for DNA isolation from gram-positive bacteria was slightly modified. The protocol included an incubation step at 56°C for 30 min after adding the proteinase K and Buffer AL, this timing was increased from 30 min to 1h. Such modification helped with the digestion of protein from the fire ant midgut tissue. The animal tissue (spin-column) protocol completed the bacterial DNA isolation, with another small change on step 7, where only 60μL of Buffer AE was used to increase the final DNA concentration.

Primer design. Ten pairs of species-specific bacteria primers from RIFA midguts were designed by selecting distinct/unique regions of rDNA sequence after amplification with universal 16S rDNA primers and multiple sequence alignment software Vector NTI Suite 7 from Invitrogen (Li et al. 2005). However, two of the ten primer pairs (for Bac22 and Bac27) that were not at first species-specific were redesigned based on a unique base insertion or deletion on the 3'end of the primer and checked for specificity against all of the other midgut bacteria cultured in this study (Table 5). The primers were then tested and confirmed for specificity against all ten midgut bacteria alongside negative and positive PCR controls (larval PBS and genomic DNA from midgut bacteria previously cultured from each of the ten species, respectively). After the specificity of the primers was confirmed, the primers were then used to test for the presence/absence of these bacteria in midguts of numerous fourth instar larvae collected from RIFA colonies throughout the southeastern United States.

Table 5. New set of species-specific bacteria primers from RIFA midguts. These were designed by selecting distinct/unique regions of rDNA sequence after amplification with universal 16S rDNA and tested for specificity.

Primer Name	Size of Amplification (base pairs)	Primer Sequence	Bacteria Identification
BAC4-5'	453	5'- TGG CTC CAA AAG GTT ACC TCA- 3'	*Enterococcus* sp./*durans*
BAC4-3'		5'- ACT CTA GAG ATA GAG CTT CCC- 3'	
BAC22-5'	793	5'- CTT GTC GAT TGA CGT TAC CCC- 3'	*Klebsiella ornithinolytica*
BAC22-3'		5'- GTC GCT TCT CTT TGT ATG CG- 3'	
BAC27-5'	957	5'- CTG GGA ACG TAT TCA CCG TA- 3'	*Kluyvera cryocrescens*
BAC27-3'		5'- AAA GTA CTT TCA GCG AGG AGG A- 3'	
BAC36-5'	984	5'- CAT GAT TCT TAT TTG AAA GAA GCA A- 3'	*Lactococcus garvieae*
BAC36-3'		5'- GTT TAT CAC CGG CAG TCT CAC- 3'	
BAC38-5'	500	5'- TAC GAC TTC ACC CCA GTC ATG- 3'	Enterobacteriaceae
BAC38-3'		5'- TCC ACA GAA GTT TCA GAG ATG A- 3'	
BAC42-5'	1046	5'- CAC GCT ATC AGA TGA GCC TAA- 3'	*Pseudomonas aeruginosa*
BAC42-3'		5'- TTG GCA ACC CTT TGT ACC GA- 3'	
BAC48-5'	450	5'- TAC GAC TTC ACC CCA GTC ATG- 3'	*Achromobacter xylosoxidans*
BAC48-3'		5'- ATG CCG AAG AGA TTG GCA GTG- 3'	
BAC101-5'	406	5'- GGA GCT TGC TCC CGG ATG TT- 3'	*Bacillus pumilus*
BAC101-3'		5'- TGC GAG CAG TTA CTC TCG CA- 3'	
BAC102-5'	958	5'- AGC TTG CTT CTC TGT CGG TG- 3'	*Listeria* sp./*innocua*
BAC102-3'		5'- GAA GCT CTG TCT CCA GAG TG- 3'	
BAC104-5'	407	5'- GCA CAA GAG AGC TTG CTC TC- 3'	*Serratia marcescens*
BAC104-3'		5'- TTG ATG AAC GTA TTA AGT TC- 3'	
27f	1432	5'- AGA GTT TGA TCM TGG CTC- 3'	Universal Primers 16s rDNA
1492r		5'- GGT TAC CTT GTT ACG ACT T- 3'	

PCR reactions and gel electrophoresis. Each reaction (total volume: 25 µL) included a GoTaq® Green Master Mix 2X (12.5 µL) from Promega (Madison, WI) with a forward (2.5 µL) and a reverse primer (2.5 µL), PCR ready MilliQ water (Nuclease free), and 0.5 µL of the DNA template (concentration: 20ng/µL). Reactions were performed at the same time for each of the collecting sites to avoid any variations in the reaction preparation. Amplifications were performed in a Bio-Rad MyCycler, Personal Thermal Cycler programmed as follow: 2 min at 95 °C for one cycle, 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C for 30 cycles and a final 5 min at 72°C cycle. Gel electrophoresis was conducted in a 2% agarose gel, and stained with ethidium bromide. Bands in the gels were visualized with UV illumination and the digitally captured images were labeled and stored electronically. A 100bp DNA ladder marker from Promega (Madison, WI) was used for size comparisons.

Results

In this study, ten bacteria species previously studied by Li et al. (2005) were screened for their presence or absence in red imported fire ant colonies throughout the southeastern United States. Results from our first trip, from College Station, Texas to Mobile in Alabama, are summarized on Table 6. Notice that a sample collected on site 1 (Vidor, TX) did not provide enough DNA (from midgut of 4th instar larvae); nevertheless it was still included on the screening process. None of the ten species were detected on sites 5, 7, and 11. Gel electrophoresis results obtained for *Kluyvera cryocrescens* on that trip are shown on Fig. 7. The ethidium bromide stained bands are revealed after UV light exposure of the gels, each lane on the gel represents the 17 different collecting sites, plus

a positive and a negative control (Fig. 7). Tables 7 and 8 summarize, in the same manner, the results from the gel images corresponding to the collecting trips to south Texas and Brazos Valley respectively. Notice again that on the south Texas trip (Table 7), sites 3 and 11 did not provide enough bacterial DNA due to the small number of 4th instar larvae present in those colonies. The bacterium *Lactococcus garvieae* was abundant in the south Texas samples, gel electrophoresis results are shown on Fig. 8. An uncultured bacterium belonging to the Enterobacteriaceae family was also abundant in the Brazos Valley samples when compared to the other species at the same sites, gel electrophoresis results are shown on Fig. 9.

Maps of the collecting sites and the bacterial populations were created with ArcView (GIS software, ESRI, Redlands, CA). Each map represents the exact geographical location for each collecting site in southeastern United States (Fig. 10) and Brazos Valley (Fig. 11). Some multiple sites may appear as a single red dot on the maps due to the fact that some sites were relatively near to each other and due to the large scale used on the maps construction.

The distribution of bacteria was determined and results are shown for Mobile (Fig. 12), South Texas (Fig. 13), and Brazos Valley (Fig. 14). An overall value was also calculated for each bacteria species in southeastern United States (Fig. 15). To calculate the overall values, the total number of screened sites was adjusted to 34 because no genomic DNA from bacteria was detected in three of the 37 screened locations.

Discussion and Conclusions

In nature, the bacterial diversity is determined by numerous abiotic and biotic

Table 6. Bacterial distribution in RIFA colonies collected in 17 geographical locations from College Station, TX to Mobile, AL. Including collecting sites near the docks of the Alabama State Port, the RIFA point of entry to the USA. Samples collected on August 2008. BAC, bacterium isolate; +, Present; blank, absent.

BAC	Bacteria Identification	Collecting Sites (College Station, TX - Mobile, AL)																	Size (base pairs)
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
4	*Enterococcus* sp./*durans*		+	+	+		+		+	+	+			+	+	+	+	+	453
22	*Klebsiella ornithinolytica*		+				+		+								+		793
27	*Kluyvera cryocrescens*		+	+	+		+		+	+	+		+	+	+	+	+	+	957
36	*Lactococcus garvieae*		+	+	+		+		+	+	+			+	+	+		+	984
38	Enterobacteriaceae		+		+				+							+			500
42	*Pseudomonas aeruginosa*		+	+			+			+			+	+	+				1046
48	*Achromobacter xylosoxidans*						+						+	+					450
101	*Bacillus pumilus*																		406
102	*Listeria* sp./*innocua*												+						958
104	*Serratia marcescens*																		407
16S	Bacterial 16S Ribosomal DNA		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1432

Fig. 7. Gel electrophoresis results for *Kluyvera cryocrescens* (957 base pairs) from the trip to Mobile, AL. Lanes 1 through 17 represent each collecting site; +C, positive control from pure culture; –C, negative control.

Table 7. Bacterial distribution in RIFA colonies collected in 12 geographical locations from College Station, TX to Brownsville, TX. Samples collected on July 2005. BAC, bacterium isolate; +, Present; blank, absent.

BAC	Bacteria Identification	Collecting Sites (College Station, TX - Brownsville, TX)												Size
		1	2	3	4	5	6	7	8	9	10	11	12	(base pairs)
4	*Enterococcus* sp./*durans*	+	+			+	+		+	+			+	453
22	*Klebsiella ornithinolytica*												+	793
27	*Kluyvera cryocrescens*	+	+		+		+	+	+				+	957
36	*Lactococcus garvieae*	+	+			+	+	+	+	+	+		+	984
38	Enterobacteriaceae		+				+							500
42	*Pseudomonas aeruginosa*				+				+		+			1046
48	*Achromobacter xylosoxidans*					+					+		+	450
101	*Bacillus pumilus*													406
102	*Listeria* sp./*innocua*													958
104	*Serratia marcescens*													407
16S	Bacterial 16S Ribosomal DNA	+	+		+	+	+	+	+	+	+		+	1432

Table 8. Bacterial distribution in RIFA colonies collected from eight adjacent counties in the Brazos Valley, TX. Geographical locations represent Brazos, Burleson, Washington, Grimes, Leon, Madison, Robertson, and Milam counties respectively. Samples were collected throughout the year 2008. BAC, bacterium isolate; +, Present; blank, absent.

BAC	Bacteria Identification	Collecting Sites (Brazos Valley, TX)								Size (base pairs)
		1	2	3	4	5	6	7	8	
4	*Enterococcus* sp./*durans*	+	+	+	+	+	+	+	+	453
22	*Klebsiella ornithinolytica*									793
27	*Kluyvera cryocrescens*								+	957
36	*Lactococcus garvieae*	+	+	+	+	+	+	+	+	984
38	Enterobacteriaceae/unculture		+		+		+	+	+	500
42	*Pseudomonas aeruginosa*									1046
48	*Achromobacter xylosoxidans*									450
101	*Bacillus pumilus*									406
102	*Listeria* sp./*innocua*									958
104	*Serratia marcescens*									407
16S	Bacterial 16S Ribosomal DNA	+	+	+	+	+	+	+	+	1432

Fig. 8. Gel electrophoresis screening results for *Lactococcus garvieae* (984 base pairs) from the South Texas trip. +C, positive control from pure culture; -C, negative control.
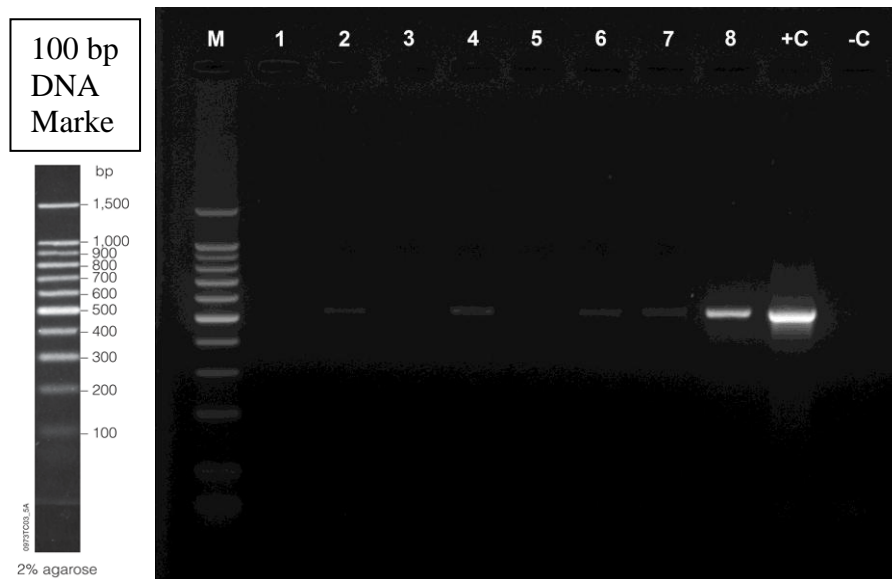
Fig. 9. Gel electrophoresis screening results for Enterobacteriaceae/uncultured (≈500 base pairs) from the Brazos Valley. Sites 2, 4, 6, 7, and 8 showed a weak band on the gel, but they still screened as positive for the presence of that bacterium (isolate 38-Enterobacteriaceae). +C, positive control from pure culture; -C, negative control.
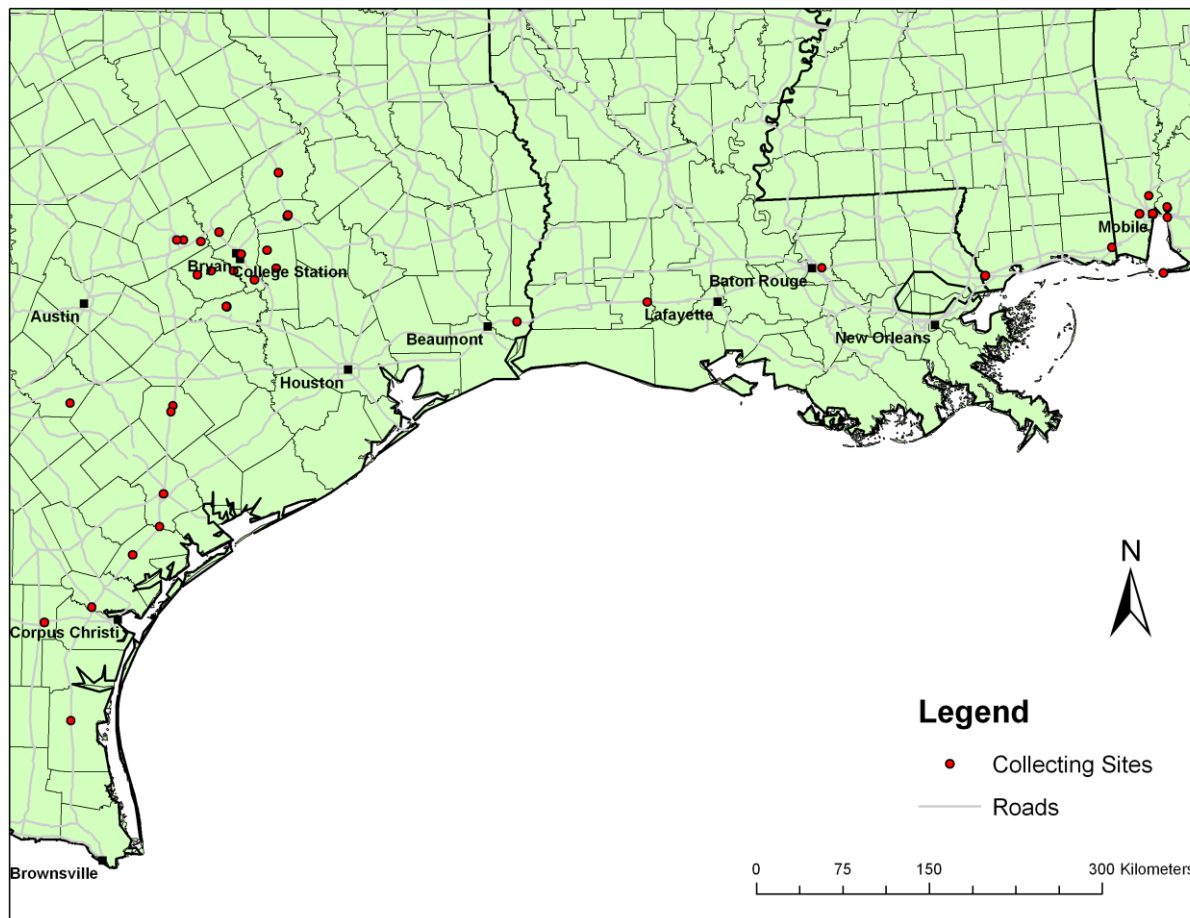
Fig. 10. Map of all collecting sites obtained with ArcView software (ESRI) from GPS data. A total of 43 sites covered an area from south Texas, near the border with Mexico, to the docks area in the State Port of Albama in Mobile.
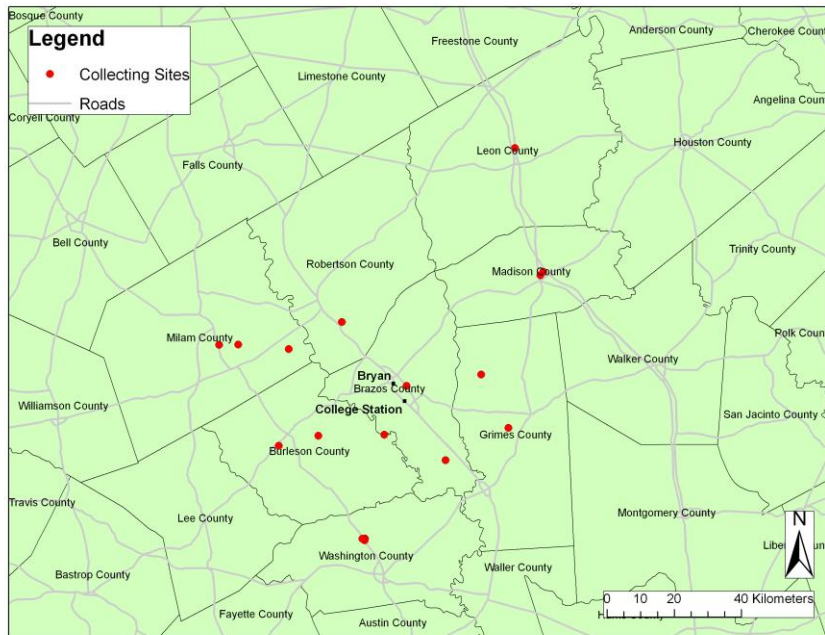
Fig. 11. Map represents the 14 collecting sites within eight counties in the Brazos Valley, TX (Brazos, Burleson, Washington, Grimes, Leon, Madison, Robertson, and Milam) from GPS data.

factors (Franklin and Mills 2003, Horner-Devine et al. 2004). The abiotic elements include, seasonal climate changes in temperature, relative humidity, and precipitations; also different types of soils with its physical properties, pH, chemistry, humidity, and organic matter content; and lastly, the pollutants from animal and human activities in particular. Among biotic factors, other bacteria are very important from the ecological standpoint, since they are able to keep certain bacteria in check or in perfect balance by restricting the growth of certain bacteria populations more than others. Antagonistic micro-organisms also present in the soils will inhibit or benefit certain species of bacteria. The plant composition of an ecosystem also carries its own array of specific microbes. Animals and humans, especially the later, also have an important impact on
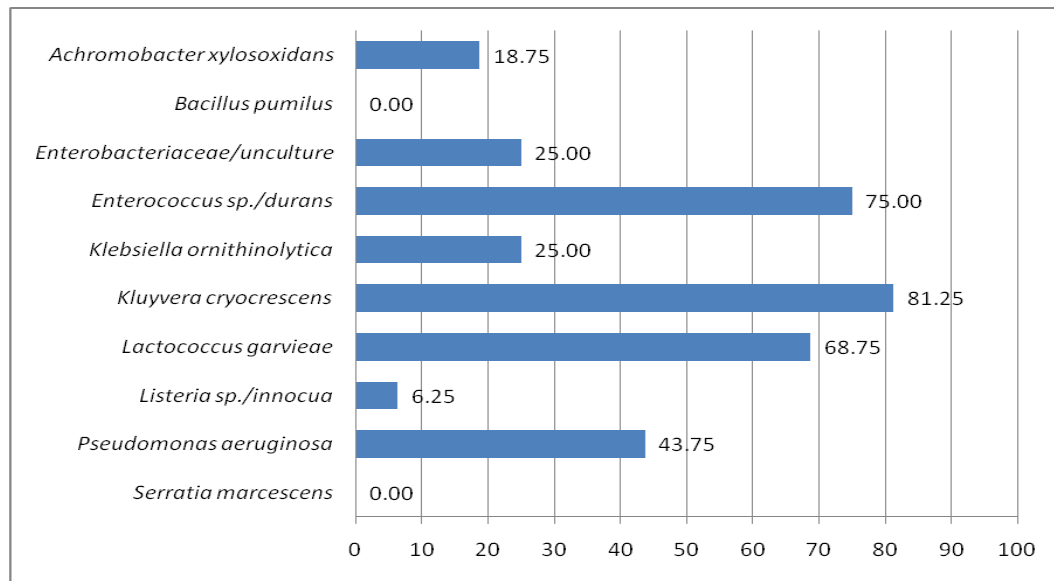
Fig. 12. Abundance of midgut bacteria in fire ant colonies collected from College Station, TX to Mobile, AL. All data are presented as a percentage of the total number of sites where that bacterium was present. For this trip, 17 sites equal 100%.
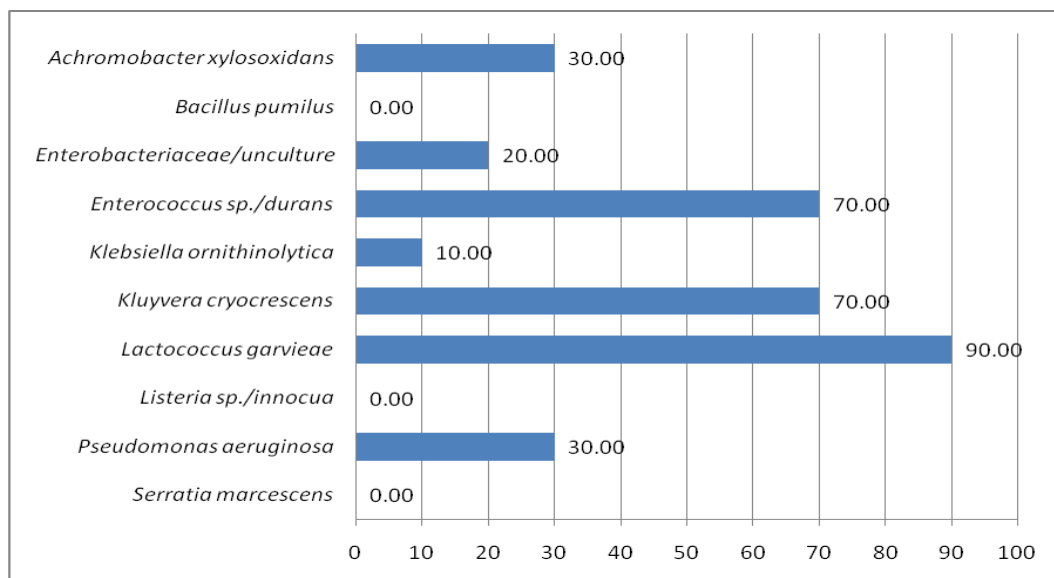
Fig. 13. Abundance of midgut bacteria in fire ant colonies collected from College Station, TX to Brownsville, TX. All data are presented as a percentage of the total number of sites where that bacterium was present. For this trip, 10 sites equal 100%.

Fig. 14. Abundance of midgut bacteria in fire ant colonies collected from eight counties in the Brazos Valley, TX. All data are presented as a percentage of the total number of sites where that bacterium was present. For this trip, 8 counties equal 100%.
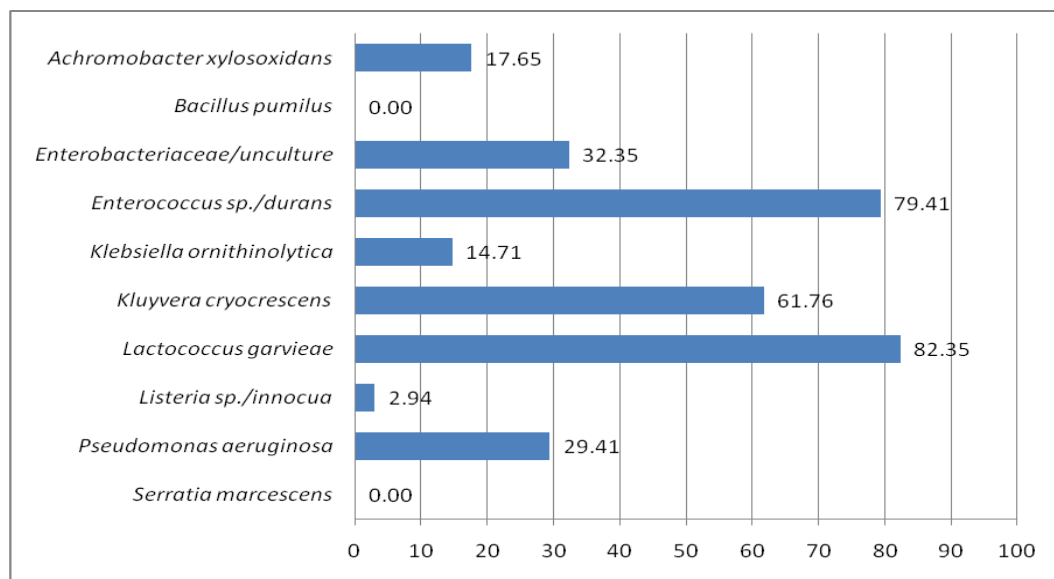
Fig. 15. Abundance of bacteria found in the midgut of fourth instar larvae in fire ant colonies collected from southeastern United States. All data are presented as a percentage of the total number of sites where that bacterium was present. For this trip, 34 sites equal 100%.
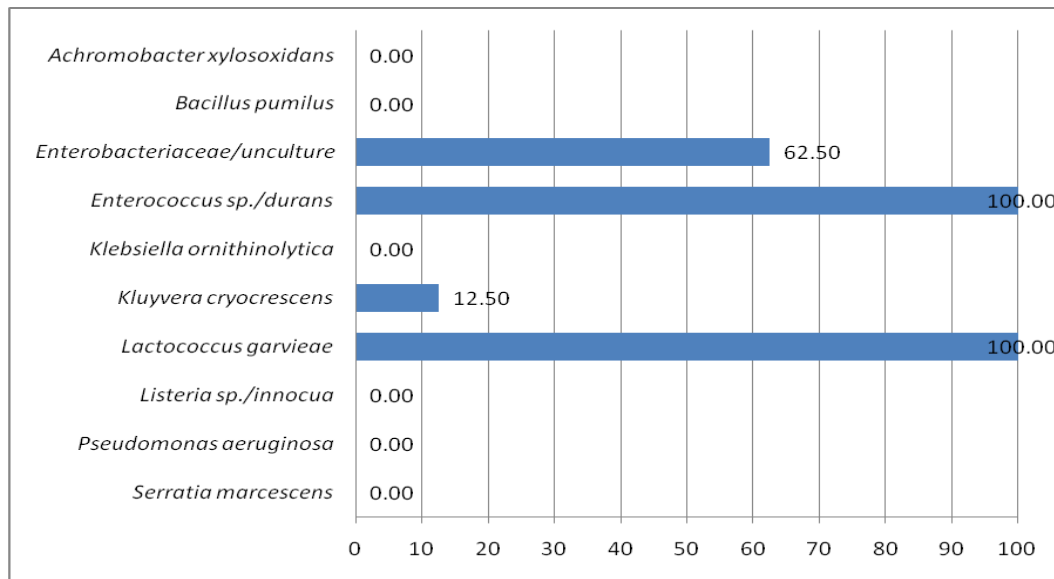
the bacteria composition through the environment (Horner-Devine et al. 2004). In the case of fire ants, an omnivorous insect, the type of food available is also very important, directly affecting the bacterial fauna in the midgut lumen.

According to Rosenzweig (1995), the habitat heterogeneity concept underlines the directly proportional relationship between number of species and sampling area. That is, the bigger the sampled area, the more species diversity would be found. Results definitely support this idea; 14 collecting sites in only eight adjacent counties in the Brazos Valley (Fig. 11), provided the smaller number of bacteria species for a given area with only 4 species represented (Fig. 14). However, the number of bacteria increased to 7 species with only 12 collecting sites from south Texas, in part because of the larger sampling area extending from College Station to Brownsville (Fig. 10). This increase was even more evident in the Mobile trip where 8 bacteria species were isolated from fire ant colonies (Fig. 12), corresponding with the largest sampled area in southeastern United States (Fig. 10).

The data from the Mobile and south Texas trips (Fig. 12 & 13), showed that the three most abundant species were *Enterococcus* sp.*/durans*, *Kluyvera cryocrescens*, and *Lactococcus garvieae,* but when looking at the Brazos Valley sites (Fig. 14), *Kluyvera cryocrescens* was only present in 12.5% of the sites, and it was replaced by the uncultured bacterium of the family *Enterobacteriacea*, found in 62.5% of the sites. Overall, *Lactococcus garvieae* was the most abundant of all, but it was only present in 82.35% of all sites (Fig. 15). The second two most abundant species, *Enterococcus* sp. (79.41%) and *Kluyvera cryocrescens* (61.76%) were also inconsistently found (Fig. 15).

Bacillus pumillus and *Serratia marcescens* were not found at any of the sites (Fig. 15); however, these two missing bacteria species were isolated and cultured from RIFA midguts collected in Brazos county in our first publication (Li et al. 2005). This can be explained from the fact that some bacteria species found at much lower population densities are harder to detect, and when growing the bacteria in artificial media, these low population densities can rapidly increase.

In summary, the midgut bacteria distribution in red imported fire ant colonies is determined by the ecosystem on which they develop, including its micro- and macro-geographical variations (Franklin and Mills 2003, Horner-Devine et al. 2004, Bouwma et al. 2006, Remenant et al. 2009). Therefore, in this study conducted in Texas, Louisiana, Mississippi, and Alabama, the population distribution of a selected group of bacteria is very diverse and did not show a repetitive pattern even when sampled at a much larger scale in southeastern United States. Similar results have been shown in other insect groups such as in the well studied dipteran, *Drosophila melanogaster* (Corby-Harris et al. 2007). Although we could not conclude that there are obligate symbionts in the midgut of fire ants, we can assert that the entire bacterial community plays an important role in fire ant reproduction and overall fitness. Our recent experiments demonstrated that, after feeding fire ant queens with antibiotics and sterile food, they stopped laying eggs; but were able to recover after feeding them with the same type of food containing bacteria (unpublished data).

This project was a continuation of our previous work (Li et al. 2005), and provided key information to reach our final goal of selecting the best bacteria candidates

for genetic transformation. The bacterial selection process was not only based on geographical distribution results, but also based in other factors including their biochemical and biological properties, as well as the bacteria background history.

For example, *Serratia entomophila*, had been developed as a biological control agent against the grass grub (*Costelystra zealandica*), a pest of pastures in New Zealand (Federici 2007). According to the author, this bacterium is easy to grow and mass produced. The bacterium, *Serratia marcescens*, producing the red pigment called prodigiosin, is also a known pathogen of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Connick 2001, Osbrink 2001), and other insects including hymenopterans (Grimont and Grimont 1978). In addition, *Serratia marcescens* have also been successfully used in previous feeding experiments with red imported fire ants (Jouvenaz et al. 1996).

From the same family Enterobacteriaceae, the bacterium *Enterobacter amnigenus*, have been used as a host to express the *cry4*B gene of *Bacillus thuringiensis,* and toxic genes from *B. sphaericus* (Khampang et al. 1999) for mosquito control. As stated in the introduction, *Enterobacter cloacae* transformed with an ice nucleation gene have been used in biological control strategies (Watanabe et al. 2000).

Although we chose only one of the most abundant species, *Kluyvera cryocrescens*, the other two selected species (Enterobacteriaceae/isolate #38 and *Serratia marcescens*) have both demonstrated to be good candidates for the introduction of foreign genes into the red imported fire ant colonies.

CHAPTER V

GENETIC TRANSFORMATION OF MIDGUT BACTERIA FROM THE RED IMPORTED FIRE ANT, *Solenopsis invicta* **Büren**[*]

Introduction

Today the red imported fire ant has become one of the most important agricultural and urban pests in the United States (Vinson and Sorensen 1986, Vinson 1997). In the five major Texan metroplexes alone, Lard (Lard 2002) estimated the annual cost for fire ant control to exceed 581 million dollars in 1998, while in the agricultural sector they exceeded 90 million dollars per year in 1999.

Early efforts for countrywide fire ant control began as far back as 1960 and were dependent on insecticides. Among the prime fire ant habitats, wetlands and nature reserves are environmental sensitive areas and they cannot be treated due to environmental risks, therefore they serve as sources for re-infestation (Drees et al. 1996), which leads to failure of chemical control for fire ants. The use of other management strategies such as biological control is currently being investigated. The introduction and establishment of pathogens can potentially result in the suppression of fire ant populations and research on several biological control agents has been reported (Thorvilson et al. 1987, Drees et al. 1992, Durvasula et al. 1997, Porter 1998, Porter and Alonso 1999, Williams et al. 1999, Peloquin et al. 2000, Sauer et al. 2000, Peloquin et

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[*] Reprinted with permission from Medina, F., H. Li, S. B. Vinson, and C. J. Coates. 2009. Genetic transformation of midgut bacteria from the red imported fire ant (*Solenopsis invicta*). Curr. Microbiol. 58: 478-82. Copyright 2009 by Springer.

al. 2001, Bextine and Thorvilson 2002, Peloquin et al. 2002, Dotson et al. 2003). Recently, the use of symbiotic bacteria as biological control agents has been proposed and is considered a long-term sustainable solution (Durvasula et al. 1997, Beard et al. 2002, Dotson et al. 2003, Bextine et al. 2004). One prominent example is the vector symbiont intervention (VSI) project that was initiated by Beard et al. (2002), see also (Durvasula et al. 1997, Dotson et al. 2003), to control Chagas disease. In studies aimed at evaluating the potential use of fire ant gut symbiotic bacteria for control applications, DsRed was used as a reporter gene for DNA introduction, a commonly used marker for transforming bacteria (Peloquin et al. 2000).

Symbiotic associations are widespread among invertebrates and it is estimated that at least 15-20% of all insects live in symbiotic relationships with microorganisms (Buchner 1965). According to our previous study of the red imported fire ant (Li et al. 2005), we were able to culture at least ten different bacteria species from the midgut of the fourth instar larvae. Other uncultured species have also been identified from their midgut (Lee et al. 2008). Although the function and relationship of the gut bacteria with the ant host are still under investigation, they are excellent candidates for genetic transformation therefore a good alternative towards biological control applications.

In this research project we demonstrated that the some bacterial strains found in the midgut of the red imported fire ant, can be genetically transformed with a shuttle vector encoding DsRed. The results imply the use of the genetically transformed bacteria to monitor their natural spread and transmission within the colony, and suggest further investigation to exploit these species as potential biological control agents.

Materials and Methods

Bacterial strains, recombinant plasmid and red imported fire ant colonies.
Previously isolated strains, *Kluyvera cryocrescens*, *Serratia marcescens,* and isolate #38
(Li et al. 2005), were re-streaked from glycerol stocks onto BHI media and incubated at
37°C overnight. pZeoDsRed has been used previously for transforming insect associated
bacteria (Peloquin et al. 2000, Peloquin et al. 2002). The red imported fire ant colonies
used in this study were maintained at the Entomology Research Laboratory (College
Station, TX).

Competent cell preparation and bacterial transformation. Overnight cultures
of *K. cryocrescens, S. marcescens* and isolate #38 strains were re-inoculated into fresh
low salt LB broth and incubated at 37°C with shaking until obtaining an OD590 of 0.56.
The cells were chilled on ice and subjected to repeated washes in decreasing volumes of
cold sterilized double distilled water and 10% glycerol solution until being finally re-
suspended in 1/500 of original volume of 10% glycerol. For transformation, 50µl
aliquots of competent bacteria were mixed with 30ng of pZeoDsRed and subjected to
electroporation (2.5 kV, 25 µF and 129Ω). After recovering at 37°C for 1hr with shaking
in 1ml SOC, cells were plated on LB supplemented with 100mg/L Ampicillin and
50mg/L Zeocin. Antibiotic-resistant colonies of the three transformed bacterial strains
were examined for DsRed expression by observation with an Axiovot 100 fluorescence
stereomicroscope equipped with a Rhodamine filter set (Carl Zeiss, Germany).

Plasmid detection of pZeoDsRed in transformed bacterial strains. Plasmid
DNA from the transformed bacteria was extracted using the Wizard® *Plus* SV Mini-

preps DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. Undigested DNA was separated by gel electrophoresis on a 0.8% agarose gel. Plasmid DNA preparations from untransformed bacterial strains were used as controls. For Southern blot hybridization, approximately 1µg of plasmid DNA from each recombinant bacterial strain was digested with *Eco*RI, separated on a 0.8% agarose gel and transferred to a positively charged nylon membrane. Linearized pZeoDsRed was labeled with $[P^{32}]$-α-dATP using the Prime-a-Gene®-labeling system (Promega, Madison, WI), according to the manufacturer's protocols. Hybridizations were performed in aqueous hybridization buffer containing 5xSSC, 5x Denhart's solution and 1% SDS at 65°C overnight and then washed twice in 0.2xSSC, 0.1% SDS and once in 0.1x SSC, 0.1% SDS at 65°C for 15min each. Autoradiography was performed by exposure to Kodak X-Omat film at -70°C.

Plasmid stability test. The plasmid stability of pZeoDsRed in the three transformed bacterial strains was tested as described by Peloquin (2000) at both room temperature and 37°C.

Re-introduction, detection and isolation of transformed bacteria in fire ant larvae. Six independent small fire ant colonies with fourth instar larvae and adult worker ants were prepared in artificial nests. Three colonies were selected to be fed with transformed bacteria, while the other three were fed with wild type untransformed bacteria. Bacterial cultures were grown at 37°C with shaking for 40hr (adding fresher medium after 16hr). Cells were collected and re-suspended in 10^{-3} volumes of fresh LB or 2xYT medium. Cell density was assayed by serial dilution of the bacteria and plating

on appropriate media. For each fourth instar larvae, a small droplet of bacterial solution was placed at the antero-ventral region, called "food basket" or praesaepium (Petralia and Vinson 1978), with the aid of a fine glass needle. Three days post feeding, 8-10 larvae from each fire ant colony were surface sterilized in a 1.5 ml centrifuge tube by washing in 70% EtOH for 30 seconds, followed by two rinses in sterilized ddH$_2$O and once in 300µl sterilized phosphate buffered saline (PBS). 150µl PBS from each tube was plated on LB+ Zeo^{50}+Amp^{100} as a negative control. Larva, in the remaining 150µl PBS, were ground and plated on the same media for bacterial isolation. Surface sterilized larvae were also placed on glass slides and viewed with a MBIOII fluorescence microscope with a Rhodamine filter (Carl Zeiss, Germany). Images were acquired with a Carl Zeiss color digital camera and AxioVision 2.05 software system (Carl Zeiss, Germany). Similarly, seven days post-bacterial feeding, 8-10 newly emerged pupae were collected from each fire ant colony for sterilization, bacterial isolation and DsRed visualization as described above.

Upon pupation the larval gut and its contents are purged and expelled as meconium. The bacteria-fed larvae, pre-pupae and meconium were selected for UV-microscopy observations by following the protocol described above. Meconia were also collected, grounded and plated on LB+ Zeo^{50}+Amp^{100} medium for bacterial isolation. Some of the meconia containing the transformed bacteria were placed inside colonies with only wild type bacteria, in which adult worker ants followed their natural behavior of feeding 4th instar larvae with meconia. 8-10 larvae were selected three days post-meconial treatment, surface sterilized and bacterial isolation was performed by following the

protocol described above.

Results

Introduction and expression of the DsRed gene in fire ant midgut bacteria.
The pZeoDsRed plasmid was introduced into *K. cryocrescens, S. marcescens* and isolate
#38 strains by electroporation and DsRed was expressed in all three transformed strains.
The transformation efficiency was 2.48×10^8 cfu/µg DNA for *K. cryocrescens* and
2.7×10^6 cfu/µg DNA for *S. marcescens,* significantly higher than isolate #38, which was
3.1×10^3 cfu/µg DNA. The plasmid DNA was isolated and analyzed by agarose gel
electrophoresis which revealed that the transformed bacterial strain contained an
additional DNA fragment that co-migrated with intact pZeoDsRed plasmid. Southern
blot DNA hybridization showed that only the transformed strains contained the
hybridizing fragments. The DsRed florescence intensity in transformed cells of isolate
#38 was higher than in *K. cryocrescens* and *S. marcescens* in either liquid culture or on
agar plates with Zeocin and Ampicillin selection.

Plasmid stability test. The stability of the pZeoDsRed plasmid in the fire ant
midgut bacteria was assayed at both 37°C and 22-24°C, without Zeocin selection
pressure. When transformed strains were maintained without antibiotic selection at 37°C
for 48hr, all colonies of *K. cryocrescens*/ pZeoDsRed and isolate #38/ pZeoDsRed were
fluorescence positive, indicating high stability (100%), while 98% of the *S. marcescens/*
pZeoDsRed colonies fluoresced following this incubation period. pZeoDsRed was stable
without Zeocin selective pressure after subculture and growth for nine days at room
temperature (22-24°C) in isolate #38, whereas the pZeoDsRed expression level in *K.*

cryocrescens was too low to observe in single colonies. However, the pZeoDsRed plasmid was still maintained in the transformed bacteria as confirmed by re-culturing the plate at 37°C, or re-streaking the colonies on LB+Zeo[50] media. Stability of pZeoDsRed in the *S. marcescens* strain was low at room temperature without selection, with most of the colonies being non-fluorescent after nine days re-streaking on LB plates.

Re-introduction of transformed midgut bacteria into fire ant colonies. Strains of *K. cryocrescens, S. marcescens* and isolate #38 carrying the pZeoDsRed plasmid were successfully re-introduced into the fire ant larvae by individually feeding a high cell density bacterial solution (10^{10-12} cfu/ml). The transformed bacteria survived in the fourth instar larvae with the DsRed being highly expressed in the midgut for 72 hrs post-feeding.

After feeding the larva with the transformed bacteria, *K. cryocrescens*/pZeoDs-Red, *S. marcescens*/pZeoDsRed, and isolate#38*/*pZeoDsRed, a strong DsRed fluorescence was observed at the larval stage and meconia for all the cases, compared to a low level of fluorescence in the pre-pupae (Fig. 16). Bacterial isolation from meconium obtained a large number of highly fluorescent bacterial colonies, confirming that most of the transformed bacteria came out with the meconium. Still, a small number of *K. cryocrescens*/ pZeoDsRed were successfully isolated from the pupae. DsRed-bacteria could not be isolated from the pupae in colonies fed with strain#38*/* pZeoDsRed and *S. marcescens*/ pZeoDsRed, however, approximately 500 DsRed fluorescent positive colonies were isolated from the pre-pupae in colonies fed with *S. marcescens*/ pZeoDs-Red. DsRed-bacteria were not isolated from the PBS solution (control), indicating a
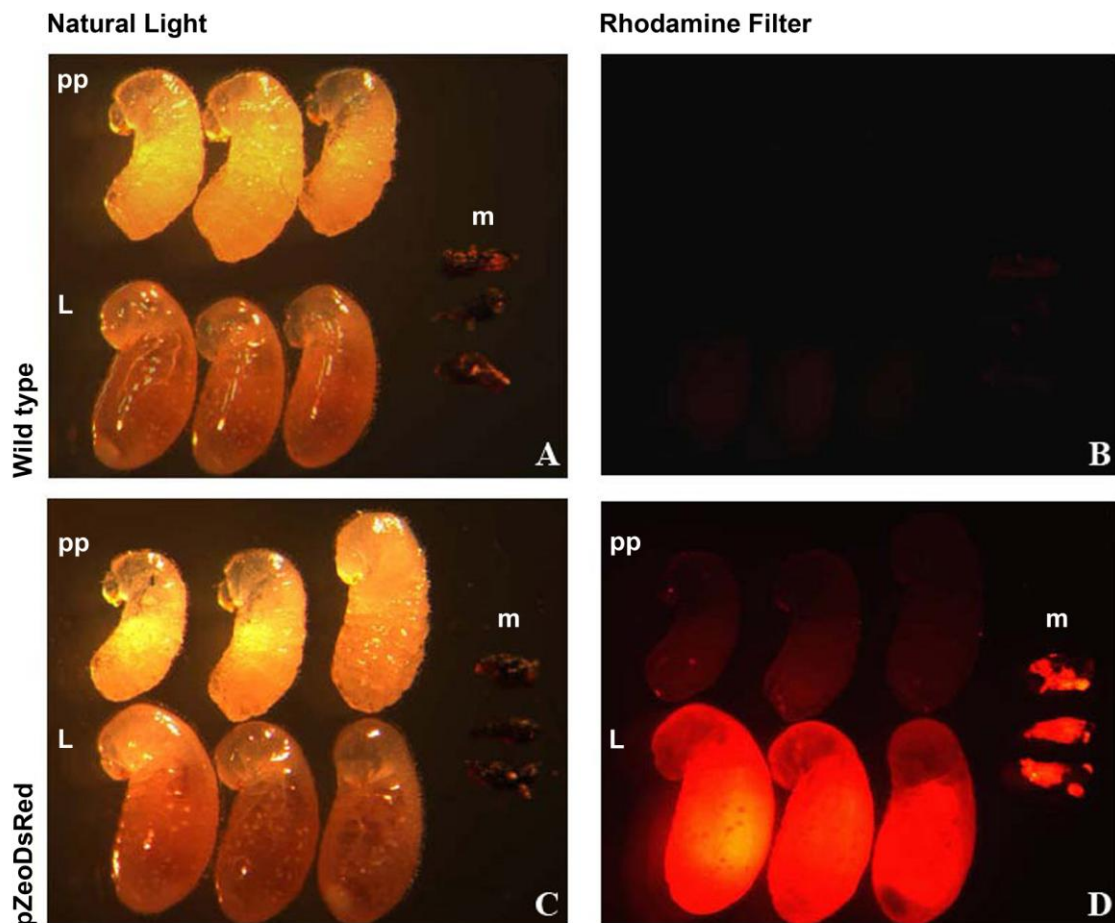
Fig. 16. Results from transformed bacteria feeding experiments in RIFA. Late fourth instar larvae (L) excretes the meconium (m), including most of the midgut bacteria, right before becoming prepupae (pp). Images A and B are the same photographs of individuals fed the wild type *K. cryocrescens*. Images C and D are the same photographs, this time of individuals fed the transformed *K. cryocrescens / pZeoDsRed*. No fluorescence at all can be seen in the wild type bacteria under the rhodamine filter (Image B), but it is highly expressed in the transformed bacteria (Image D). Notice the fluorescence levels in the meconium (m) after being excreted. Some transformed bacteria can still be isolated from the prepupae (pp) and pupae (not shown), see small fluorescent spots in the prepupae (pp) in image D.

successful surface sterilization protocol.

The meconia produced in the transformed bacteria-fed colonies was collected and placed inside naive colonies to investigate a potential infection rate. Seventy-two hours after feeding, 8-10 surface sterilized larvae were used for bacterial isolation. The isolation resulted in 500 to 1,000 bacteria colonies with strong DsRed fluorescence on each of the samples.

Discussion and Conclusions

Among the ten bacteria species cultured from the fire ant midgut (Li et al. 2005), only three species, *K. cryocrescens, S. marcescens* and isolate #38, were successfully transformed with a DsRed-encoding shuttle vector, pZeoDsRed. Although all three strains were successfully transformed with this vector, the transformation efficiencies and DsRed fluorescence levels were significantly different. For isolate #38, the transformation efficiency was significantly lower than that of the other two species, *K. cryocrescens* and *S. marcescens*. However, the expression of DsRed fluorescence was much higher in this species than in *K. cryocrescens* and *S. marcescens*. DsRed expression was higher at 37°C than at room temperature in the *K. cryocrescens* strain, however the pZeoDsRed plasmid was not lost during the lower temperature culture period and DsRed expression can be observed upon re-culturing at 37°C, or under antibiotic selection. pZeoDsRed was considerably stable in the absence of antibiotic selection at 37°C in all three isolated strains and was stable in *K. cryocrescens* and isolate #38 at room temperature. Possible explanations are that the growth and storage conditions evoke changes in the transformed population and that environmental

conditions could influence bacterial fitness. Expression differences could also be due to changes in protein secondary structure, post-translational modification of the hetero-logous protein and the relative promoter strength in each species (Gerdes et al. 1986).

The use of genetically modified bacteria to combat the red imported fire ant is a potentially powerful tool. To reach this goal, we provide here some initial studies towards a possible field application for control of the fire ant. Firstly, bacteria which are closely associated with the fire ant midgut can be readily isolated and cultured *in vitro*. Secondly, a robust method for genetic transformation of these bacteria exists, and genetically transformed bacteria can be maintained with minimal loss of the foreign gene both *in vitro* and *in vivo*. Also, genetically transformed bacteria can be successfully re-introduced and can survive in the fire ant for at least seven days. In addition, the normal function and maturation of the fire ant was apparently not affected by the genetic manipulation of the midgut bacteria. Finally, we demonstrated that the infected meconium produced upon pupation of bacteria-fed larvae can be fed to uninfected larvae by worker ants, thus the transformed bacteria can be spread throughout the colony; even potentially reaching the reproductive queen.

The successful introduction of the pZeoDsRed shuttle vector into fire ant midgut bacteria suggests that derivatives of this plasmid could serve as vectors for the expression of toxic proteins by these bacteria. This will allow the rapid screening of candidate effectors genes for use in fire ant control. We are now investigating candidate genes encoding toxic products for expression in *K. cryocrescens, S. marcescens* and isolate #38.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

Alternative methods for the control of the red imported fire ant are desperately needed, particularly approaches that do not rely on the application of broad-based insecticides. As described in the *Introduction*, the use of genetically modified bacteria associated with insect species is an emerging field of research offering great promises. Prior to my first publications (Li et al. 2005, Medina et al. 2007), little research had been conducted on the relationship of red imported fire ants with endosymbiotic bacteria (Jouvenaz et al. 1977, Jouvenaz 1990c, b, Peloquin and Greenberg 2003). Only recently, within the past four years, entomologists are paying more attention to this association (Gunawan et al. 2008, Lee et al. 2008, Tufts and Bextine 2009).

Until now, no research had attempted to genetically transform the Red Imported Fire Ant (RIFA) midgut bacteria for their reintroduction into RIFA colonies as a means of biological control. Therefore, this doctoral research project proposed a thorough investigation of the association between midgut bacteria and their RIFA host. Specifically, the objectives included: chapter II) isolation, culture, identification, and characterization of the midgut bacteria from the RIFA fourth instar larvae; chapter III) study the internal anatomy of the RIFA digestive system, and investigate bacterial abundance, and distribution, as well as the ant-bacteria interactions at the structural and ultra-microscopic level in the midgut; chapter IV) determine the abundance and distribution of RIFA bacteria in southeastern United States, and selection of best bacteria candidates for genetic transformation; and chapter V) genetically transform the selected

bacteria with a DsRed fluorescent protein, including their re-introduction into the RIFA colonies, and tracking and isolation of transformed bacteria from different RIFA developmental stages.

After successful isolation and culture of bacteria living in the midgut of RIFA fourth instar worker larvae, the DNA was extracted from the pure bacteria cultures and analyzed by PCR-RFLP. The near-full length 16s ribosomal RNA gene and DNA sequencing results revealed the presence of the following ten cultured species from the midgut: *Enterococcus* sp.*/durans, Klebsiella ornithinolytica, Kluyvera cryocrescens, Lactococcus garvieae, Pseudomonas aeruginosa, Achromobacter xylosoxidans, Bacillus pumilus, Listeria* sp.*/innocua, Serratia marcescens,* and an uncultured bacterium from the family Enterobacteriaceae (Li et al. 2005). These bacteria were also characterized by their morphology, biochemical activity, and antibiotic resistance, thus providing important information for the selection of the best candidates for genetic transformation. Also, species specific primers were designed by Li et al. (2005) to later screen for the presence of these bacteria in field collected samples.

The above results showed the presence of gram-negative and gram-positive bacteria in the midgut of worker larvae. Some of these bacteria were closely related to each other, and at least 40% belonged to the γ-subdivision of Proteobacteria with one bacterium in the β-subdivision. This increased my hope of finding a true symbiont, due to the fact that most insect symbionts are members of the phylum Proteobacteria, primarily within the class γ-proteobacteria (Moran and Telang 1998).

The following midgut bacteria species, *Klebsiella ornithinolytica* (Sakazaki et al. 1989, Brenner et al. 2005)*, Kluyvera cryocrescens* (Farmer et al. 1981, Brenner et al. 2005)*, Serratia marcescens* (Grimont and Grimont 1978, Brenner et al. 2005), and an uncultured bacterium are all members of the Proteobacteria (Gammaproteobacteria: Enterobacteriales: Enterobacteriaceae). These species can be found in the intestines of many organisms, including insects and humans, but also in soil, water, fruits, meats, eggs, vegetables, grains, flowering plants and trees. As noted, this group presents great heterogeneity in its ecology, host range, and pathogenic potential. Some members of this group are well known species, such as *Escherichia* spp., but most importantly, there are also well known insects symbionts. These include, *Arsenophonus* spp., an endosymbiont of insects (Gherna et al. 1991, Hypsa and Dale 1997, Trowbridge et al. 2006), *Buchnera* spp., a symbiont of aphids (Munson et al. 1991, Clark et al. 1992), and the endosymbiotic bacteria of the tsetse fly*, Wigglesworthia* spp. (Aksoy et al. 1995). These symbiotic bacteria form a large group with a common ancestor in the Enterobacteriaceae (Aksoy et al. 1995, Chen et al. 1999).

The genus *Listeria*, and specifically *L. innocua* (Firmicutes: Bacilli: Bacillales: Listeriaceae) is commonly found in soil, vegetation, wild and domesticated animals, humans, and food sources. Members of this genus also have the ability to survive extreme pH and temperature, as well as high salt concentrations. Some species are very common in fish, squids, crustaceans and other seafood. One species, *Listeria monocytogenes*, causes a severe food-borne disease called listeriosis (Seeliger and Schoofs 1977, Glaser et al. 2001).

From the genus *Bacillus, B. pumilus* (Firmicutes: Bacilli: Bacillales: Bacillaceae) was also isolated from the midguts. This genus is one of the most representative microorganisms in the soil (Parvathi et al. 2009). Due to their ability to form spores, they are highly resistant to extreme environmental conditions (Nicholson et al. 2000). For example, *B. pumilus* is one of the most abundant bacterial specie found in the interior and exterior surface of the International Space Station (La Duc et al. 2004). While some species have been isolated from the digestive track of insects, such as saw bugs and mosquito larvae, other *Bacillus* species have recently been found in the hemolymph of fire ants (Gunawan et al. 2008, Tufts and Bextine 2009).

Another bacterium living in the midgut, *Enterococcus* sp.*/durans* (Firmicutes: Bacilli: Lactobacillales: Enterococcaceae) had been isolated from milk and dairy products (Collins et al. 1984), and are tolerant to heat and desiccation. Although *E. durans* is found in the gastrointestinal track of humans and animals, it is not pathogenic (Sherman and Wing 1937, Devriese et al. 1987).

The ubiquitous gram positive bacterium, *Lactococcus garvieae* (Firmicutes: Bacilli: Lactobacillales: Streptococcaceae), originally described as *Streptococcus garvieae,* was first isolated from cases of bovine mastitis. It is also found in human infections, blood, skin, urine, and wounds (Elliott et al. 1991), and had been isolated from various species of fish where it is considered a major pathogenic agent (Collins et al. 1983, Eldar et al. 1996).

The common bacterium species, *Pseudomonas aeruginosa* (Proteobacteria: Gammaproteobacteria: Pseudomonadales: Pseudomonadaceae) is widely distributed in

nature. Some members of this genus are pathogenic to humans, animals, or plants. In humans, they can be commonly found in wounds, burns, and urinary tract infections (Garrity et al. 2005).

Lastly, another midgut bacterium isolated from the RIFA midgut, *Achromobacter xylosoxidans* (Proteobacteria: Betaproteobacteria: Burkholderiales: Alcaligenaceae), can be found in the water, soil, hospital environments, and in human clinical specimens as contaminants and/or pathological agents. Among human clinical specimens, they are found in blood, sputum, wounds, purulent ear discharge, spinal fluid, cerebral tissue, urine, feces, and, in a few cases, also from disinfectant solutions (Busse and Auling 2005). This species is also important in the biodegradation of aromatic and halogenated compounds in nature (Boivin-Jahns et al. 1995).

Although the role of these bacteria in the RIFA midgut is still under investigation, we have no indication that they cause any pathology. Studies emphasizing the role of these bacteria in fire ant physiology will be available in the future. Due to the history of insect symbiosis in the family Enterobacteriaceae, it offers great expectations for genetic transformation.

The presence of 10 bacterial species in the midgut of fire ants left me with the question of whether there is or there is not a true symbiont. Therefore, I recognized the importance of finding specialized structures in relation to the bacteria in the midgut. Equally important was the need to determine their presence within the RIFA midgut tissues. By taking advantage of newly developed techniques in electron microscopy I studied the internal and external anatomy of the fire ant. I investigated the presence of

any specialized structures, such as mycetocytes (bacteriocytes), as an indicator of an obligate symbiont. I also determined the bacterial abundance and distribution inside the midgut, becoming the first application of SEM and TEM to study the bacteria-ant relationship in *S. invicta*.

Thin sections for TEM exposed part of the digestive system's internal structure, provided evidence of free living bacteria inside the midgut lumen, and revealed a possible functional association of the bacteria with their insect host. They also showed that bacteria are not contained within specialized structures in the epithelial cells of the midgut, discarding the possibility of a bacteriocyte-forming symbiont in the midgut. Once again, the TEM images demonstrated that bacteria were able to reach the midgut in adult worker ants. Due to the presence of a filtering system in the adult workers (Glancey et al. 1981) any particles > 0.9 μm in diameter would be filtered out, but in this case the midgut bacteria found were only 0.5 μm in diameter. These data supported the findings of Jouvenaz et al. (1990a, 1996).

The importance of bacteria in the spread of RIFA in the United States and their potential use as biological control agents will require further investigation. Nevertheless, results confirmed the morphological adaptations of RIFA to its social lifestyle, feeding behavior and potential association with symbiotic microorganisms.

The results clearly support the idea that in nature, the bacterial diversity is determined by numerous abiotic and biotic factors (Franklin and Mills 2003, Horner-Devine et al. 2004) which also determine the habitat heterogeneity. The habitat heterogeneity concept (Rosenzweig 1995) states that the larger the screened

geographical area is, the more bacteria species would be found. For example, an analysis of the overall results revealed that the three most abundant species, *Enterococcus* sp*./durans*, *Kluyvera cryocrescens*, and *Lactococcus garvieae*, were found in > 60% of all sites, while only two species, *Bacillus pumillus* and *Serratia marcescens* were not found at any of the sites. On a smaller sampled area, corresponding to the "Texas trip", *Bacillus pumillus, Listeria* sp*./innocua,* and *Serratia marcescens* were not found. Whereas in the smallest sampled area of Brazos Valley, *Achromobacter xylosoxidans*, *Bacillus pumilus*, *Klebsiella ornithinolytica*, *Listeria innocua*, *Pseudomonas aeruginosa*, and *Serratia marcescens* were not found at any site. Therefore, in this study conducted in Texas, Louisiana, Mississippi, and Alabama, the population distribution of the ten bacteria from the RIFA midgut was very diverse and did not show a repetitive pattern even when sampled at a much larger scale in southeastern United States. Although I could not conclude that there are obligate symbionts in the midgut of fire ants, we can assert that the entire bacterial community plays an important role in RIFA reproduction and overall fitness. Recent experiments demonstrated that, after feeding fire ant queens with antibiotics and sterile food, they stopped laying eggs; but were able to recover after feeding them with the same type of food containing bacteria (unpublished data).

Results from the study of the abundance and distribution of RIFA midgut bacteria are important for selecting candidates for genetic transformation. Nevertheless, the bacterial species biology, and their biochemical and morphological characteristics are also equally important; all of them must be taken into consideration during the selection of the best candidates.

As noted previously, members of the family Enterobacteriaceae, might offer the best expectation as candidates. In addition, the history and biology of the bacteria is very important. For example, *Serratia entomophila*, has been studied as a biological control agent against *Costelystra zealandica*, a pest of pastures in New Zealand (Federici 2007). According to the author, this bacterium is easy to grow and mass produce. The bacterium, *Serratia marcescens*, producing the red pigment called prodigiosin, is a known pathogen of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Connick 2001, Osbrink 2001), and other insects including hymenopterans (Grimont and Grimont 1978). Also, *S. marcescens* has been successfully used in previous feeding experiments with red imported fire ants (Jouvenaz et al. 1996). From the same family, Enterobacteriaceae, the bacterium *Enterobacter amnigenus* has been used as a host to express the *Cry4*B gene of *Bacillus thuringiensis* and toxic genes from *B. sphaericus* (Khampang et al. 1999) for mosquito control. *Enterobacter cloacae* is another bacterium that has been transformed with an ice nucleation gene and used in biological control strategies (Watanabe et al. 2000).

Although I chose only one of the most abundant species, *Kluyvera cryocrescens*, the other two selected species (Enterobacteriaceae/isolate #38 and *Serratia marcescens*) have both demonstrated to be good candidates for the introduction of foreign genes into the red imported fire ant colonies. These three species, *K. cryocrescens, S. marcescens* and isolate #38 (Enterobacteriaceae), were successfully transformed with a DsRed-encoding shuttle vector, pZeo-DsRed. These genetically transformed bacteria were fed to naïve larvae and monitored over time. My results demonstrated that RIFA workers

collected the meconia, containing the transformed bacteria, and fed them to larvae. The natural behavior of feeding meconia to 4th instar larvae and to the queen/queens has proven to be very effective in the spread of transformed bacteria within the colony (Medina et al. 2009). Some of the transformed bacteria were recovered at the pupal stage, indicating that not all are excreted with the meconium.

Based on results, it is important to determine and quantify to what extent transformed bacteria can reach the queen or queens when fed to a RIFA colony. Also, I must investigate how long these transformed bacteria can survive after reaching the RIFA adult stage. Moreover, future investigation must address the environmental risks of using transformed bacteria, and most importantly its effects on native ant communities. My ongoing research focuses on the role of midgut bacteria in RIFA reproduction and its effects over colony fitness. In feeding experiments, antibiotic treatments had a negative effect on egg production. Fire ant queens completely stopped laying eggs after feeding them antibiotics under sterile conditions. After 38 days, two selected fire ant colonies were fed with the same food, but unsterilized and containing bacteria, which resulted in a complete recovery of the queen's egg laying capabilities until reaching the same level of brood production as in a control treatment. Further investigation of antibiotic effects will require multiple replications of each treatment and a thorough statistical data analyses. In addition, PCR screening of bacteria in the RIFA queens before and after antibiotic treatment will provide knowledge on how much antibiotics are affecting the bacterial populations, and thus queen reproduction.

The liquid part of the meconium has proven to increase egg production in the fire ant queen (Tschinkel 1995), therefore it is important to study the bacterial diversity in the meconia, and how this diversity is affected before and after antibiotic treatment. Based on the preliminary results from our antibiotic experiments, one could design an experiment to determine if any of the 10 midgut bacteria might actually be causing the effects on queen's reproduction. This can be achieved first, by creating ten aseptic fire ant colonies (bacteria free) and independently feeding them with a specific bacterium from pure culture.

During my research I made other observations which revealed that bacterial populations can change over time under laboratory conditions. This is determined by changes in RIFA's diet; from a diverse bacterial community in the field, to the lack of diversity under laboratory conditions. My preliminary investigation demonstrated that some bacteria species were acquired through the diet in the laboratory (e.g. *Listeria* sp./*innocua*), while other species tended to disappear. Further investigation of these observations might explain one of the factors associated with the deterioration of the fire ant colony fitness under laboratory conditions. Supporting this idea, I observed the following effects on RIFA colonies previously fed with antibiotics in my experiments. After completion of the antibiotic experiments, these colonies were reintroduced into a rearing room and fed only with the regular diet (containing bacteria). Over time the queens not only recovered their ability to lay eggs, but also the colony's fitness improved having a healthier appearance than other colonies of the same age.

To complement my research, the presence and role of bacteria in other tissues, using both molecular and electron microscopy tools must be investigated. It is especially important in those tissues because they are known to harbor symbionts in other insect groups. Particularly in RIFA, I suggest studying the hindgut tissue, the salivary glands and reservoir, the hemolymph, (Gunawan et al. 2008, Tufts and Bextine 2009), the ovaries, and fat bodies. The salivary glands and reservoir are directly involved in extra-oral digestion of proteins, thus colony food processing, while highlights the important role of proteases. In the midgut, the attachment of some bacteria to specific molecules in the surface of the peritrophic envelopes indicates an association and a possible function. In *Calliphora*, these molecules are lectin proteins that bind to mannose carbohydrates, and according to Chapman (1998) some bacteria are known for binding to these sites. The function of this association is still unknown to science according to sources, and it was shown for the first time in RIFA in my research.

To summarize, I have completed the identification and characterization of ten bacteria species which are closely associated with the fire ant midgut, these bacteria can be readily isolated and cultured *in vitro*. I have also investigated the internal structure of the fire ant midgut, and although there is no evidence of an obligate symbiont and/or specialized structures supporting endosymbiotic bacteria (bacteriocytes), they may still play an important role in fire ant digestion (under investigation). I have also, determined the distribution and abundance of the ten midgut bacteria species in fire ant colonies from southeastern United States. The results indicated a ubiquitous presence of the bacteria in the midgut of the red imported fire ant.

An effective method for genetic transformation of bacteria was confirmed with the transformation of three selected midgut bacteria species (Medina et al. 2009). These bacteria species can be genetically modified through the use of a transposable element vector that stably integrates transgenes into the bacterial chromosomes. The bacteria can be maintained with minimal loss of the foreign gene both *in vitro* and *in vivo*. My research demonstrated that transformed bacteria can be successfully re-introduced and survive in the RIFA for at least seven days. No apparent changes on the normal function and development of RIFA were observed after the introduction of transformed midgut bacteria. Finally, the meconia produced upon pupation from transformed bacteria-fed larvae is collected by nurses in the colony and naturally fed to uninfected larvae, thus the transformed bacteria can easily spread throughout the colony and potentially reach the queen or queens.

The use of genetically modified bacteria in a battle against the red imported fire ant is a potentially powerful tool. The current investigation provided preliminary results towards a biological control alternative in the red imported fire ant management program in the United Sates.

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Awards: Raleigh & Clara Miller Memorial Award, August 2007. 65th Annual Meeting of the Microscopy Society of America, 41st Annual Meeting of the Microbeam Analysis Society, and 40th Annual Meeting of the International Metallographic Society, Fort Lauderdale, Florida.

First Place Winner, 2007 Image Salon, Photomicroscopy, Department of Entomology, Texas A&M University.

First Place Winner, Student Poster Competition for the President's Prize Award. December, 2006 Entomological Society of America National Meeting, Indianapolis, Indiana.

Grants: Lead writer of the peer reviewed proposal "Development of an Environmentally Safe Gene Delivery System for the Control of the Red Imported Fire Ant", Submitted to the Texas Imported Fire Ant Initiative by Dr. Coates and Dr. Vinson. Grant received $105,000/year for 2 years.