Identification of Bacterial Species in the Hemolymph of Queen *Solenopsis invicta* (Hymenoptera: Formicidae)

DANIELLE M. TUFTS[1] AND BLAKE BEXTINE

Department of Biology, University of Texas at Tyler, 3900 University Blvd., Tyler, TX 75799

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ABSTRACT Evidence that symbiotic microorganisms can impact the development and fitness of insects has been shown in many species. Hemolymph-associated symbiotic bacteria have been identified in larvae of *Solenopsis invicta* Buren, the red imported fire ant; however, their association with adult red imported fire ants and the mode by which these organisms are transmitted from queens to offspring are not well known. In this study, *Bacillus* spp. bacteria were routinely recovered in the hemolymph of queen *S. invicta*. Genetic analysis of the 16S gene confirmed the most common bacteria isolated were *Bacillus* spp.; several *Staphylococcus* species were also collected. Ovaries from reproductive and nonreproductive queens, freshly laid eggs, first-instar larvae, and hemolymph were collected from queens and analyzed for the presence of specific *Bacillus* spp. bacteria. It was indicated that these bacteria may be transmitted vertically from queen to progeny.

KEY WORDS insect symbiont, biological control, bacterial interaction

Solenopsis invicta Buren (Hymenoptera: Formicidae), the red imported fire ant, has successfully spread to much of North America's southeastern states as well as areas of southern California (Krieger 2004, Chen et al. 2006). Natural enemies of the red imported fire ant such as the microsporidian protozoan *Thelohania solenopsae* and *Vairimorpha invictae*, the fungus *Beauveria bassiana*, a parasitic ant *Solenopsis daguerrei*, and parasitoid phorid flies *Pseudacteon tricuspis* and *P. curvatus* are known and some have been manipulated in various areas in attempts to control red imported fire ant populations (Knell et al. 1977, Jouvenaz and Ellis 1986). Nonpathogenic relationships have been observed between red imported fire ants and various types of bacteria.

In general, a symbiotic relationship is the living together of unlike organisms (Wilkinson 2001). *Lactococcus garviae*, *Staphylococcus saprophyticus*, and *Enterococcus avium* are all symbiotic gram-positive bacteria that can be found in the midgut of fourth-instar larvae (Peloquin and Greenberg 2003). Additionally, several *Bacillus* species have been recovered from the hemolymph of red imported fire ant fourth-instar larvae (Gunawan et al. 2008).

Bacillus spp. have been associated with the digestive tract of many arthropods such as the sow bug (*Porcellio scaber*), mosquito larvae (Family: Culicidae), adult whiteflies (*Bemisia argentifolii*), and red imported fire ant larvae (Davidson et al. 2000, Luxananil et al. 2001, Swiecicka and Mahillon 2006, Gunawan et al. 2008). An assortment of bacterial biological control agents have been implemented to manage a variety of arthropod species. *B. thuringiensis* is an important insect pathogen and has been used to control certain pest orders such as Lepidoptera, Diptera, and Coleoptera. Various techniques (e.g., paratransgenesis or symbiotic therapy) may be used to alter the phenotype of a bacterium's host or to force the host cells to express an undesirable gene product that ultimately lead to host death (Beard et al. 2001). In this study, hemolymph-borne bacterial symbionts were recovered from red imported fire ant queens and identified through DNA sequence analysis. We also studied the mode of transmission by which these bacteria are transferred from queen to progeny.

Materials and Methods

Colony Collection and Soil Test. Red imported fire ant colonies were collected throughout the months of March and April in 2007 from Smith and Harrison, Texas counties. All colonies were maintained in a controlled environment in the laboratory (≈22.5°C, 12-h light:dark cycle). Colonies were offered half a Vienna sausage (Libby's, Chicago, IL) once a week and given an unlimited supply of water.

The soil collected with a colony was tested immediately for the presence of *Bacillus* by isolating it with mannitol salt agar (MSA). A tablespoon of soil was combined with 15 ml of tap water in a test tube and shaken vigorously for 15 s. The soil/water mixture was added to 15 ml of liquid MSA and incubated overnight at room temperature allowing the agar to cool and harden. After incubation, samples were gram stained

[1] Corresponding author, e-mail: dmtufts11@yahoo.com.

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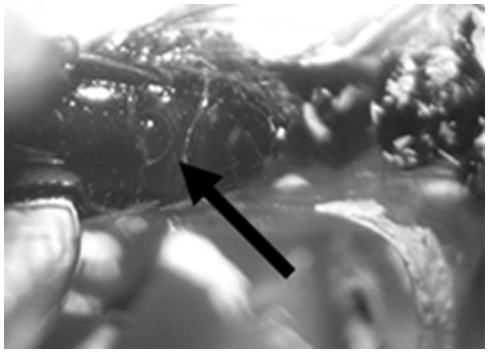

Fig. 1. View of queen hemolymph using a dissecting microscope. The clear hemolymph droplet can be seen emerging from the posterior alitrunk region of the queen (black arrow).

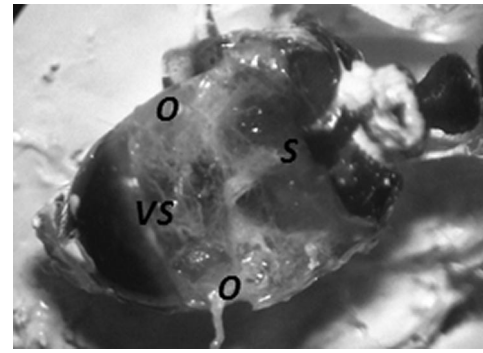

Fig. 2. View of the interior of a queen *S. invicta* using a dissecting microscope. The exoskeleton of the gaster has been removed to show the stomach (S), ovaries (O), and venom sac (VS).

and viewed under a light microscope for the presence of rod-shaped bacteria with an oval endospore at one end. Samples of soil from 10 different colonies were collected using a tablespoon and tested for the presence of *Bacillus* bacteria.

Hemolymph Retrieval From Queens and Bacterial Growth. Individual queens were removed from their colonies, washed for at least 10 s in 95% ethanol, and dried with a Kimwipe. Queens were adhered to a glass slide using double sided tape and a quick drying (≈15 s) adhesive (Liquid Paper, Oak Brook, IL). With a number 12 sterile surgical blade (HMD Healthcare, Cary, NC), a small transverse incision was made on the dorsal side of the posterior section of the alitrunk. Using forceps, slight pressure was applied to the alitrunk region to squeeze small droplets of clear hemolymph to emerge from the opening (Fig. 1). Only one or two droplets of clear hemolymph were extracted from each queen. Additional squeezing caused a milky white substance to emerging. The digestive tract runs directly ventral to the circulatory system in ants; therefore, this milky white substance was from the gut and not collected. Hemolymph was collected with a sterile cotton swab and inserted into a vial containing tryptic soy broth (TSB) and incubated for 24 h at 38°C. If bacterial growth was observed, a sample was inoculated onto tryptic soy agar (TSA) plates and again incubated for 24 h at 38°C. After the second incubation period, single bacterial colonies were collected and inoculated onto fresh TSA plates to produce pure cultures that were incubated for an additional 24 h at 38°C and used for DNA extractions. Samples were not incubated >72 h at any step during the bacterial growth phase.

To ensure that bacteria were not being collected from the exterior of the insect, samples were collected during different stages of the hemolymph collection process and tested. A sterile cotton swab was used to wipe the exterior of three different queens before being surface sterilized with 95% ethanol and placed in separate TSB tubes and incubated for 24 h at 38°C. In a second test, a sterile cotton swab was used to wipe the exterior of three different queens after being surface sterilized with 95% ethanol and placed in separate TSB tubes and incubated for 24 h at 38°C. A third test was conducted by collecting samples the same way as the first two; however, the samples were placed on separate TSA plates and incubated for 24 h at 38°C. This was also completed in triplicate for each process.

Ovary Collection. To determine whether bacteria were present and potentially transferred transovarially, ovaries were extracted from red imported fire ant queens. Reproductive and nonreproductive queens were washed with 95% ethanol and rinsed with deionized water. Queens were affixed to a clear glass microscope slide using double-sided tape and a quick drying adhesive (Liquid Paper, Oak Brook, IL). The top layer of the gaster was removed to expose the digestive tract, ovaries, and venom sac (Fig. 2). Ovaries were located and removed; washed in 95% ethanol two times; and rinsed in nanopure water. Ovaries from 40 individual queens were removed. Because ovaries provide little starting material, ovaries from five individual queens were combined and used for each sample. Therefore, a total of eight samples were used for DNA extractions and detection of bacteria by polymerase chain reaction (PCR) and sequencing.

DNA Extraction and Sequencing. After the incubation period, bacterial samples were placed in 1.5-ml microcentrifuge tubes. DNA extractions were performed for all samples using the DNeasy Tissue Kit according to the manufacturer's directions (Qiagen, Valencia, CA). An initial PCR was performed using primers of the 16S *r*RNA target gene, FD2 (DEG 16S), and RP1 (DEG 16S) (Table 1). PCR was executed under the following conditions: an initial denature step 95°C for 3 min; denatured at 95°C for 1 min, annealed at 55°C for 1 min, and elongated at 72°C for 1.3 s repeated 35 times; samples were held at 4°C until they were removed from the Bio-Rad machine (iCycler; Bio-Rad, Hercules, CA). Samples were tested in duplicate to confirm the validity of positive results.

DNA was extracted from queen ovaries, fresh eggs, and first-instar larvae using the DNeasy Tissue Kit according to the manufacturer's directions (Qiagen). Specific primers were designed to amplify a 150-bp

Table 1. Oligonucleotides used to detect the presence of bacteria in *S. invicta* queen hemolymph, queen ovaries, freshly laid eggs, and early-instar larvae

Oligonucleotide designation	Oligonucleotides (5′→3′)	Primers specific for
FD2 (DEG 16S)	AGAGTTTGATCATGGCTCAG	Proteobacteria
RP1 (DEG 16S)	ATGGTTACCTTGTTACGACTT	Proteobacteria
B. cereus F1 (16S)	ACTGGGATAACTCCGGGAAA	*B. cereus*
B. cereus F2 (16S)	AACATTTTGAACCGCATGGT	*B. cereus*
B. cereus R1 (16S)	AATGGACGAAAGTCTGACGG	*B. cereus*
B. cereus F3 (16S)	GGAGGCAGCAGTAGGGAATC	*B. cereus*
B. cereus R3 (16S)	TAATTCCGGATAACGCTTGC	*B. cereus*
B. cereus R4 (16S)	TACGCATTTCACCGCTACAC	*B. cereus*
GG + F1 (16S)	GCCACACTGGAACTGAGACA	General gram-postive bacteria
GG + R1 (16S)	GCAAGCGTTATCCGGAATTA	General gram-postive bacteria
GG + F2 (16S)	ACACGGTCCAGACTCCTACG	General gram-postive bacteria
GG + R2 (16S)	TTTTCCAGTTTCCCATGACC	General gram-postive bacteria
GG + F3 (16S)	GAAAGCCACGGCTAACTACG	General gram-postive bacteria
GG + R3 (16S)	CATTTCACCGCTACACATGG	General gram-postive bacteria
GyrB F1	CATCGTGTAGCTGCGAAAAA	*B. cereus*
GyrB R1	AAGTCGTGCCTTTTCCACAT	*B. cereus*
GyrB F2	AAAAGGTACGATGGCTGCAC	*B. cereus*
GyrB R2	GCGGTAAAATTGCTTGGAAA	*B. cereus*
GyrB F3	GGTGGATCTGCAAAACAAGG	*B. cereus*
GyrB R3	CACCATCATACATCGGCATC	*B. cereus*

segment of the Gyrase B gene, the *B. cereus* genome, and general gram-positive genome (GG+) (Table 1). Samples collected from queen ovaries (eight samples), fresh eggs (two samples), first-instar larvae (four samples), and hemolymph (eight samples) were subjected to the same 16S *r*RNA PCR conditions, as mentioned above, using the newly designed primers (Fig. 3). All samples that produced a band at ≈200 bp were sequenced using the CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA); sequence homology was determined using the National Center for Biotechnology Information (NCBI) and Basic Local Alignment Search Tool of nucleotides (BLAST*n*) programs (Table 2).

Results

The *Bacillus* collected from queen hemolymph was not from contamination of the exterior of the insect. Individual queens were tested four different ways for contamination, and each test yielded negative results. All six vials containing TSB with swipes from queens before (three vials) and after (three vials) being surface sterilized with 95% ethanol had no bacterial growth after incubation. All six TSA plates of queens swiped before (three plates) and after (three plates) being surface sterilized with 95% ethanol had no bacterial growth after the incubation period. An experiment using mannitol salt agar tested positive for the presence of *Bacillus* in the soil medium. After a 24-h incubation period each vial (10 vials) was observed having large, white, and yellow bacterial growth. In addition, rod-shaped bacteria containing oval shaped endospores were observed under a light microscope, confirming the existence of *Bacillus* bacteria in the soil medium.

Two types of bacterial growth were observed during this experiment designated type A (*Staphylococcus*) and type B (*Bacillus* species), depending on the type of growth seen. After DNA extraction for both types of bacterial growth, PCR and gel electrophoresis showed single bands of ≈1,650 bp using the DEG 16S primer set (Table 1). Samples that produced bands of this size were sequenced and matched to homologous

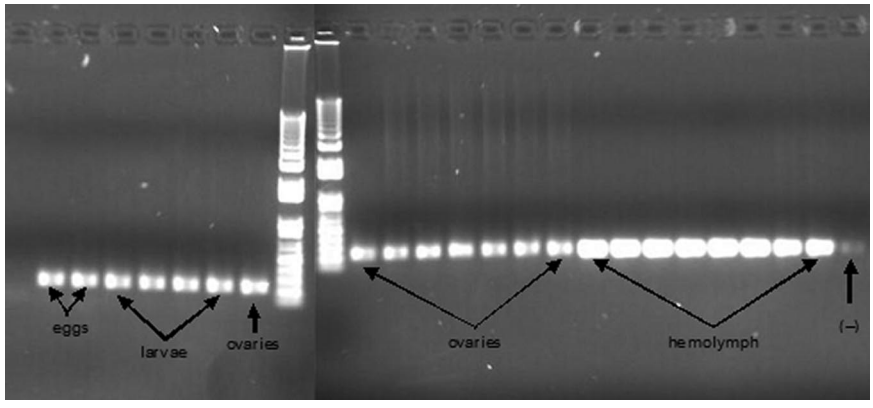

Fig. 3. Multiple gels showing positive results for samples using GG + F3 and R3 primer set. Bands observed at ≈200 bp.

Table 2. Molecular identification of individual samples of hemolymph collected from red imported fire ant queens, using the 16S rRNA gene

GenBank accession no.	Species	Closest match on GenBank	Percent match
FJ030623	*B. cereus*	DQ339665.1	98%
FJ030624	*Bacillus* sp.	EU779995.1	99%
FJ030625	*Staphylococcus hominis*	EU730935.1	97%
FJ030626	*B. pumilus*	EU912555.1	99%
FJ030627	*B. pumilus*	EU407552.1	97%
FJ030628	*Bacillus* sp.	AB126763.1	96%
FJ030629	*Bacillus* sp.	DQ993313.1	96%
FJ030630	*B. cereus*	EU816955.1	98%
FJ030631	*Bacillus* sp.	DQ993313.1	98%
FJ030632	*B. thuringiensis*	EF113616.1	98%
FJ030633	*Staphylococcus epidermidis*	EU834240.1	100%
FJ030634	*Bacillus* sp.	DQ104988.1	97%
FJ030635	*B. cereus*	DQ518612.1	98%
FJ030636	*Bacillus* sp.	EU010244.1	94%
FJ030637	*B. subtilis*	EU624321.1	96%
FJ030638	*Paenibacillus* sp.	AY556414.1	97%
FJ030639	*Staphylococcus epidermidis*	EU021221.2	97%
FJ030640	*Staphylococcus epidermidis*	EU834244.1	96%
FJ030641	*B. subtilis*	EU882849.1	98%
FJ030642	*Bacillus* sp.	DQ904622.1	95%

Listed species were recently added to GenBank; accession numbers are provided.

sequences found at NCBI's BLAST*n* program. From the 79 individual *S. invicta* queens tested, nine types of bacteria were isolated from the hemolymph (data not shown). Eight species of *Bacillus* bacteria were identified: *B. anthracis*, *B. amyloliquefaciens*, *B. cereus*, *B. megaterium*, *B. pumilus*, *B. subtilis*, *B. thuringiensis*, and *Paenibacillus* species. The genus *Staphylococcus* was also identified in queen fire ant hemolymph. The focus of this experiment was the *Bacillus* genus; therefore, *Staphylococcus*-positive samples were excluded. Most individuals had several forms of bacteria present in their hemolymph; the most common forms of bacteria discovered were homologous to *B. cereus* (nine individuals), *B. thuringiensis* (nine individuals), and *Staphylococcus* (seven individuals).

Queen ovaries, freshly laid eggs, first-instar larvae, and queen hemolymph tested positive for the presence of bacteria. Single bands at ≈200 bp were observed in positive samples using the specific primer sets for the 16S gene and general gram-positive genome (Fig. 3). Sequence data were also obtained from samples that produced bands of ≈200 bp to produce additional confidence in results, using the specific primer sets. Homologous sequences resulting from positive samples retrieved from NCBI's BLAST*n* program were most similar to *B. cereus*, *B. thuringiensis*, and *Staphylococcus.*

Discussion

Numerous strains of bacteria were recovered from queen *S. invicta* hemolymph, as well as freshly laid eggs, first-instar larvae, and queen ovaries. However, *B. cereus* was found most frequently; therefore, we focused mostly on this type of bacteria. Queens were chosen for this experiment because they are physically the largest caste members in a colony and have the potential for transovarial transmission of hemolymph symbionts. Transovarial transmission is a form of vertical transmission in arthropods wherein a female passes a symbiotic organism through her ovaries to her offspring (Murray et al. 2005). Presence of *B. cereus* in the ovarioles indicated the potential for transovarial transmission of this bacterium. Individuals in the larval stage of development are in direct contact with their environment and have begun feeding; consequently, this can be considered an additional route of exposure to bacteria. Freshly laid eggs are in contact with the soil; however, they do not feed therefore transmission cannot be attributed to ingestion. The presence of the bacterium in eggs is an important factor to definitively determine transovarial transmission of bacteria to offspring. Similarly, queen ovaries are isolated from the outside environment; therefore, the discovery of *Bacillus* in the ovaries also strongly indicates that transovarial transmission of bacteria is occurring.

Queens used in this experiment were of the polygyne phenotype and most likely alates or nonreproductive alates. Colonies were collected in the spring months (March and April) before the mating flight, and ≈80 individuals were used in this experiment. Some individuals did not provide any useable sequencing data, whereas others did not show any bacterial growth after the first incubation period. *Staphylococcus* and *Bacillus* spp. are ubiquitous and widespread organisms; however, greater attention was given to the *Bacillus* genus as a possible biological control bacterium. Samples were not incubated >72 h ensuring only fast-growing bacteria such as *Bacillus* were being collected and analyzed. However, slower-growing bacteria may be of more interest in the future as a possible control agent for *S. invicta.*

A successful method for collection of hemolymph from queen *S. invicta* was developed. Before this experiment hemolymph had only been collected from fourth-instar larvae (Gunawan et al. 2008). In addition, a novel and successful method for retrieving ovaries was developed. *B. cereus* was the main type of bacteria found in *S. invicta* fourth-instar larvae (Gunawan et al. 2008) and was prominent in the hemolymph of queen red imported fire ants as well. *B. thuringiensis* was another bacterium that was quite prominent in the hemolymph of queen red imported fire ants. Cry toxins produced by *B. thuringiensis* have proven to be effective in mosquito control by binding to epithelial cell membranes and preventing insect resistance to insecticides (Bravo et al. 2006). The discovery of *B. thuringiensis* in queen hemolymph might have similar implications for *S. invicta.*

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