PROPOSAL FOR THE FY2004 IFA RESEARCH COMPETITIVE GRANTS PROGRAM

Texas Imported Fire Ant Research and Management Project

Title of project:
Identification of Gene Targets and Delivery Systems for Fire Ant Control

Lead principal investigators, contact information and signatures:
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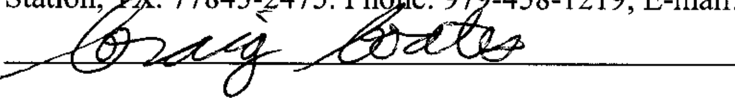

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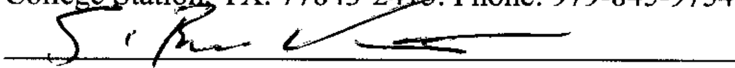


Abstract

There is a clear need to develop alternative control strategies to support existing measures for limiting the economic and social impacts of the imported fire ant. We have chosen to target the transition of fire ant queens from alates, prior to a mating flight, to dealates, following a mating flight. This transition is associated with numerous important physiological changes that are critical to fire ant reproduction and development. Interference with any of these processes would have a detrimental affect on fire ant populations. Our previous efforts have identified a number of candidate genes that are differentially expressed in these two developmental stages of fire ant queens. We propose to use gene-silencing techniques to further investigate the function of these target genes and to determine the effect on fire ant colonies if these genes are knocked out. One of our most interesting findings is the discovery of two novel vitellogenin genes that we believe are critically involved in the maturation of eggs in the dealate queen. We propose to investigate and confirm the function of these important gene products and determine if they will be suitable targets for control purposes. The other component of this research proposal is the development of transgenic bacteria, originally isolated from fire ant midguts, to ultimately deliver gene products into fire ant colonies. We propose to transform three different bacterial species with fluorescent marker genes.

Background Information

Imported Fire Ant (IFA) physiology, development and reproduction are poorly understood at a molecular and genetic level. The elucidation of the molecular mechanisms involved in these key processes will provide a number of novel targets that could be exploited for the purposes of fire ant control as part of an integrated program. We have made significant advances in this area through the construction of a suppression subtractive hybridization (SSH) library from alate and dealate IFA queens. Analysis of this library has revealed a number of candidate gene targets that are differentially expressed in these IFA queen developmental stages. The complete

characterization of this library will soon be finalized and we will have a number of suitable gene candidates available for further analysis. Of note was the discovery of two novel vitellogenin genes that had not previously been identified at a molecular level, although the presence of at least one potential additional gene had been suggested by experimental data obtained by Dr. L. Keeley's group. Our current experimental evidence suggests that the expression of these additional genes plays a key role in the final maturation and development of eggs within the dealate queens. We were also able to isolate a number of bacterial symbionts from IFA midguts, from both reproductive and worker larvae. Both gram positive and gram negative bacteria were obtained, which will allow the future development of delivery systems for a variety of potential gene targets. We propose the following specific objectives.

1. **Double Stranded RNA (dsRNA) Silencing of Fire Ant Genes.**
   Double stranded RNA (dsRNA) molecules will be produced for each of the differentially expressed gene fragments identified from the suppression subtractive hybridization (SSH) library. Introduction of the dsRNA molecules into the appropriate stage and caste of the fire ant will result in the homology-dependent silencing of the corresponding endogenous gene. The mutant phenotypes will be observed and used to assign a function to these gene fragments. This will enable the identification of genes that will be suitable targets for the design of novel control strategies.

2. **Characterization of the Vitellogenin Genes, Vg2 and Vg3.**
   As described in the preliminary data section of Objective 1, two additional vitellogenin (Vg) genes were discovered during the analysis of the suppression subtractive hybridization (SSH) library. Unlike the previously characterized gene (Vg1), Vg2 and Vg3 are only expressed in the fire ant queens. We propose to complete the characterization of Vg2 and Vg3 and determine their roles in fire ant reproduction. Experimental procedures to complete this objective will include double stranded RNA (dsRNA) silencing of the 3 genes and the production of specific antibodies to enable each protein to be detected independently of the others.

3. **Genetic Transformation of Midgut-associated Bacteria.**
   We have isolated 3 different bacterial species from the midguts of fire ant larvae. The purpose of this objective is to develop a genetic transformation system for each of these bacterial species. This will enable the expression of exogenous proteins in these bacteria, such as fluorescent marker genes.

OBJECTIVE 1. Double Stranded RNA (dsRNA) Silencing of Fire Ant Genes.

Background and Preliminary Results

There is very limited information available for the imported fire ant at a molecular level. Only a small number of genetic database entries exist and few gene families have been investigated. While access to the complete genome sequences of the fruit fly and malaria mosquito does allow for some comparative analysis of identified gene fragments from the fire ant, this is not sufficient to assign a function or phenotype to these genes. Without this

information and experimental confirmation, it is not possible to contemplate the use of these genes as the basis for novel control strategies. The ability to produce dsRNA corresponding to target genes and introduce this dsRNA back into the species of interest, resulting in the silencing or knock-out of that gene, allows for the determination of the gene's function based on the mutant phenotype created by silencing the gene.

We successfully produced a suppression subtractive hybridization (SSH) library of gene fragments that are differentially expressed between alate and dealate fire ant queens. A variety of differential screening techniques were utilized to remove false positives and identify candidate gene targets (Figure 1). DNA sequence analysis of several clones has identified some potential target genes that could potentially be exploited for fire ant control purposes (Table 1). RT-PCR and Northern blot analysis procedures were used to confirm differential gene expression and to produce a developmental profile of each candidate gene (Figures 2 and 3).

Figure 1. Differential screening of the SSH library. 983 SSH clones were screened by dot blot hybridizations, 768 plasmid clones (left) and 215 PCR clones (right). Dealate and alate indicates that the probes were labeled respectively from dealate and alate queen mRNA.

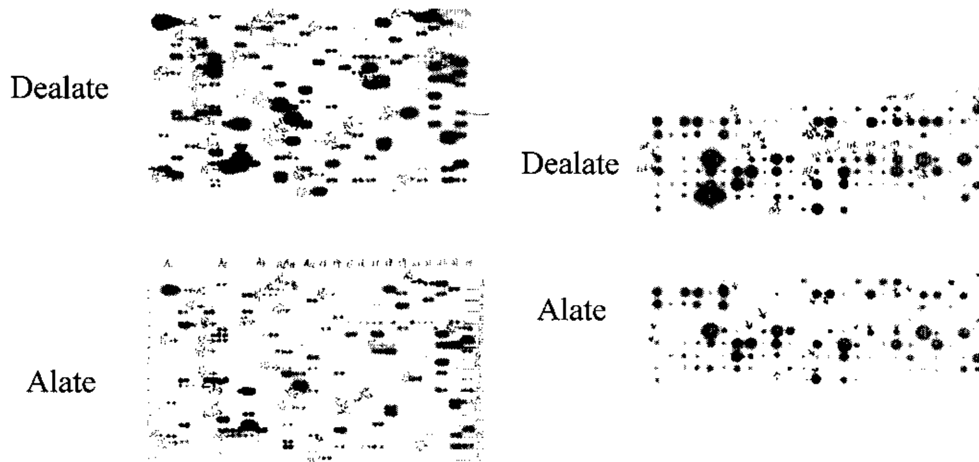
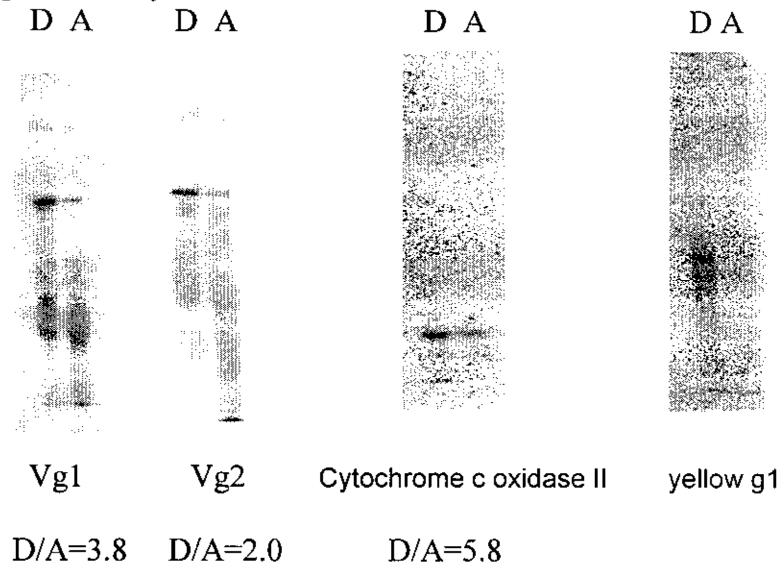


Table 1. Description of differentially expressed fire ant clones

Clone	GenBank accession no.	expression dealate/alate	mRNA size, kb#	Sequence homologies *	life stage expression by RT-PCR
1A21	CB252015	5.8	0.9	cytochrome oxidase subunit II, E=e-52	all stages abundantly except little in reproductive larvae and pupae
2F18	CB252020	-	-	ATP synthase subunit 6 E=e-7 (BLASTn)	same as above
2J12	CB252016	-	-	cytochrome oxidase subunit I, E=e-44	all stages abundantly
A181	CB252013	3.0	1.3	STARS, E=e-24, e-8	all stages, adult more
A117	CB252008	3.8	6.0	Vitellogenin (Vg1), E=e-24	all stages abundantly
2N7	CB252009	2.0	6.0	Vitellogenin (Vg2), E=e-10	dealate and alate abundantly
A130	-	-	-	Vitellogenin (Vg3), E=e-24	dealate and alate abundantly
A48	CB252011	specific	1.6	yellow g (Yg1), E=e-29	pupae and dealate
A14	CB252010	specific	1.0	yellow g (Yg2), E=e-38	pupae and dealate more express in alate than Yg1
A101	CB252014	specific	0.8	abaecin precursor, E=e-6	all stages, eggs, worker larvae, worker and dealate express more
A45	CB252012	no signal	no	hymenoptaecin precursor, E=e-4	all stages, little in alate and reproductive larvae

Deduced on the mobility of mRNA and RNA ladder in Northern blots.
* Based on BLASTx homology comparisons to the nonredundant protein database; E, the best "Expect" value. Clone 2F18 matches ATP synthase subunit 6, E=0.003
-, No data.

Figure 2. Northern blot analysis for 7 different clones, D and A indicate dealate and alate queen total RNA (2.5μg/lane). Among these, 4 genes are expressed highly in dealate queens, while another 3 are expressed only in dealates.



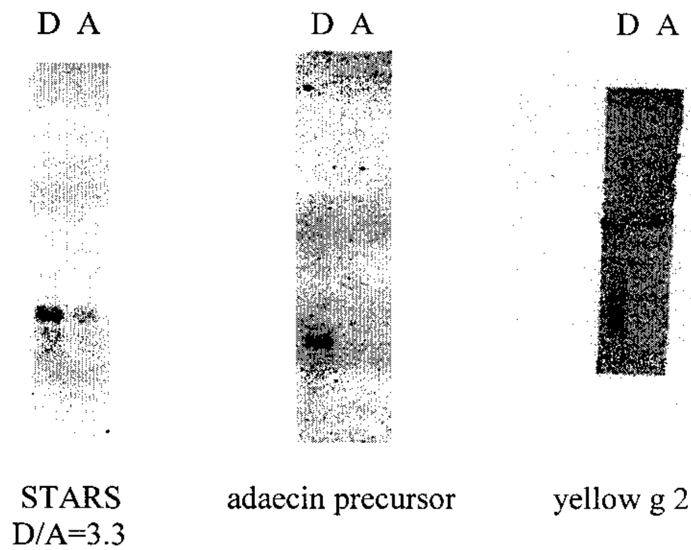
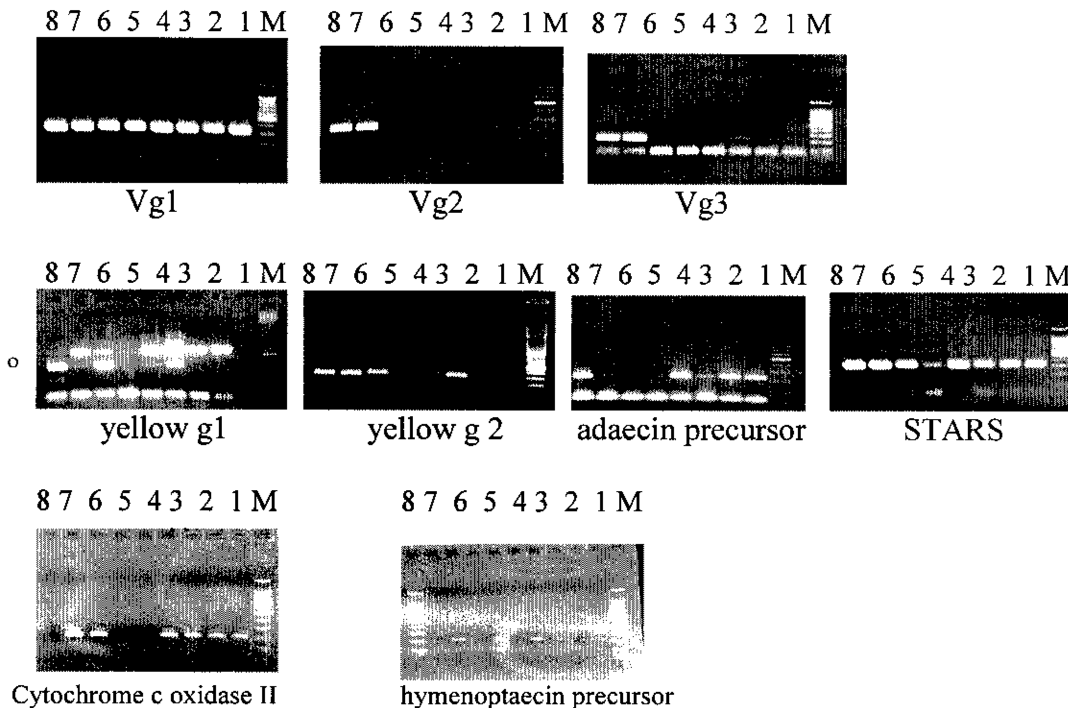


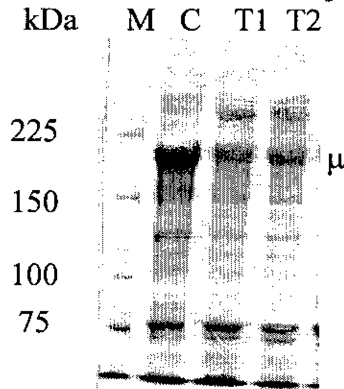
Figure 3. RT-PCR analysis for different clones.
1=egg; 2= worker larvae; 3=worker pupae; 4=worker;
5=reproductive larvae; 6=reproductive pupae; 7=alate; 8=dealate; M=100bp DNA ladder



Confirmation of putative gene function is required before these and other potential gene targets can be exploited. We propose to use double stranded RNA (dsRNA) interference (RNAi) or gene silencing experiments to investigate and confirm gene function and assess their potential use as target genes for population control or reduction. This requires the identification of suitable gene fragments, the design of specific oligonucleotide primers and subcloning of the target region into a double-T7 promoter vector. This vector is designed to allow the synthesis of double stranded RNA (dsRNA) molecules that correspond to target genes. The introduction of the dsRNA molecules into the appropriate life stage and caste will result in the induction of a gene silencing mechanism that will specifically degrade mRNA transcripts containing that gene sequence and thus inactivate the gene. The mutant phenotype of the treated individuals will be observed to determine the normal gene function and thus its potential for use as a target for fire ant control.

We have made significant progress towards this goal using a vitellogenin gene (Vg1) fragment as a test case. A cDNA fragment specific to the Vg1 gene was PCR amplified and cloned into the double-T7 promoter vector. dsRNA molecules were produced and injected into alates. Subsequent protein analysis of the injected individuals confirmed that the levels of Vg1 protein had been reduced compared to individuals injected with control dsRNA (Figure 4). This supports the hypothesis that the function of candidate and novel genes can be investigated in the fire ant using dsRNA mediated gene silencing techniques.

Figure 4. RNA interference for the expression of the Vitellogenin 1 protein (Vg1)



The 5% acrylamide SDS-PAGE of hemolymph (20Гg in each lane) from alate queens injected dsRNA 9 days later
C: hemolymph from 4 alate queens injected dsRNA of EGFP
T1: hemolymph from 4 alate queens injected 286bp dsRNA of Vg1
T2: hemolymph from another 3 alate queens injected 286bp dsRNA of Vg1
M: Broad Range Protein Molecular Weight Markers (Promega)
Arrow indicates the band of Vg1

Methodology

As described in the preliminary results, candidate gene fragments will be cloned into the double-T7 promoter vector using standard recombinant DNA and PCR technologies. Double-stranded RNA (dsRNA) will be synthesized for each gene and introduced into fire ant eggs, larvae, pupae or adults as appropriate, using microinjection techniques. Control individuals are injected with dsRNA corresponding to the green fluorescent protein (GFP). Individuals will be marked and reintroduced into colonies and observed for phenotypic effects. Molecular analysis of these individuals will include Northern blot and RT-PCR analysis as well as protein analysis and Western blotting where appropriate.

Expected Outcome

The completion of this objective will increase our knowledge of the IFA at a molecular and genetic level and will provide novel targets for future control efforts. The preliminary stage of this objective will identify multiple gene targets for further investigation. The potential for these gene targets to form part of a fire ant control strategy will initially be evaluated through the completion of this objective, in which these genes will be silenced to determine their effect on fire ants at the individual and colony level. Genes that have a negative effect on colony reproduction and survival when silenced, represent potential targets for control.

The successful demonstration and use of the dsRNA technology in fire ants will also benefit other fire ant research projects, such as those involved with investigations of the vitellogenin receptor (Pietrantonio, Vinson) and venom gland protein products (Renthal). This technology can be applied to validate these and other genes as potential fire ant control targets.

In the future these gene products can be produced as purified proteins, or in cell culture, thus providing screening targets for suites or arrays of novel chemical compounds that may be useful control agents for fire ants. This research would be performed in conjunction with private companies that have access to large libraries of chemical compounds that could be tested for

their effects on the identified target genes products. The long term potential of this research would be the identification of novel chemical compounds that could be used to specifically interfere with fire ant reproduction and development.

Timeline

OBJECTIVE 2. Characterization of the Vitellogenin Genes, Vg2 and Vg3.

Background and Preliminary Results

During the preliminary analysis of the SSH library described above, we recovered several gene fragments that had similarity to a previously characterized vitellogenin gene from the imported fire ant. However, these gene sequences were sufficiently different as to suggest that they may represent two, as yet uncharacterized vitellogenin genes, which we represent as Vg2 and Vg3. RT-PCR and Northern blot analysis of these gene fragments confirmed that the imported fire ant does indeed contain three vitellogenin genes and that the developmental and caste-specific expression of these genes differs. The previously characterized vitellogenin gene, Vg1, is expressed at all life stages and in both reproductive and worker individuals. The newly obtained vitellogenin genes, Vg2 and Vg3, are only expressed in IFA queens. All Vg genes are more highly expressed in dealates compared to alates. These results suggest that Vg1 may play a more general role in IFA development and nutrition, whereas Vg2 and Vg3 may be solely involved in IFA reproduction.

We used 5' and 3' rapid amplification of cDNA ends (RACE) techniques to obtain full-length cDNA sequences for these additional vitellogenin genes (see multiple sequence file in Appendix 1). Comparison of the gene sequences has allowed us to identify unique regions of the genes such that we can to design dsRNA molecules specific to each gene. This will enable gene silencing experiments to be performed that knock out the activity of either or both genes as described in objective 1 for the Vg1 gene. A final use of this sequence information will be to assist in the production of antigens that are unique to each protein such that specific antibodies can be produced to independently detect each gene product.

A protein expression vector for Vg1 has been constructed and purification of a specific Vg1 antigen has been completed. A similar approach will be followed for the Vg2 and Vg3 proteins, resulting in the production of 3 independent antigens that can be used to produce antibodies specific to each protein. The availability of these anti-sera's will enable a full determination of the respective roles that each protein plays in fire ant reproduction and development.

Methodology

Polymerase Chain Reaction (PCR) amplification will be used to generate Vg gene-specific cDNA fragments. These will be cloned into the protein expression vector and the completed

constructs will be used to produce purified antigens. The antigens will be introduced into rabbits to enable the production of anti-sera that is specific to each Vg gene.

Expected Outcome

These experiments will be coordinated with Dr. Pietrantonio and Dr. Vinson, and will complement their proposed fire ant research objectives. The outcome of the combination of these experiments will be a greatly enhanced understanding of the specific mechanisms involved in fire ant reproduction and development. These physiological processes are clearly suitable targets for the development of control strategies and novel insecticides, which would proceed much along the same pathway as described for Objective 1.

Timeline

OBJECTIVE 3. To genetically transform the midgut-associated bacteria

Background and Preliminary Results.

During the previous funding period we were able to identify three different bacterial species inhabiting the midgut of 4th instar fire ant larvae. One of these species was gram negative and the other two were gram positive bacteria. The two gram positive bacterial species exhibited different colony morphologies, of which one was capable of hemolysis. Further characterization of these bacterial species is continuing and includes lipid and biochemical profiling. Furthermore, we have successfully amplified a section of a rRNA gene (data not shown) and will clone and sequence the PCR amplification products and compare these with known sequences in the genetic databases. We believe that these bacterial species represent potential delivery systems for the expression of target genes directed at negatively impacting fire ant populations.

We have obtained genetic transformation plasmids that express fluorescent marker genes and antibiotic resistance genes such that we will be able to transform these species, select on antibiotic plates and then screen for the fluorescence marker genes.. This work was performed and experimental procedures derived from a collaborative effort with Dr. J. Peloquin at U.C. Riverside. An additional set of bacterial transformation vectors have been obtained from Dr. D. Lampe at Duquesne University. These vectors provide the ability to integrate transgenes into bacterial chromosomes, increasing the stability of the transformants and reducing any potential fitness costs.

Methodology

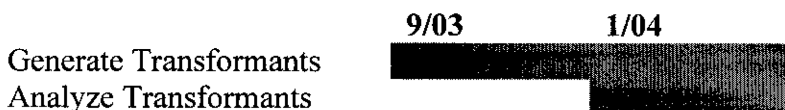
The lipid and biochemical profiling will follow standard procedures as provided by the manufacturers of the test kits. PCR amplification products will be cloned into TA-cloning vectors and subjected to DNA sequence analysis. Database querying and comparison will be

performed with standard software in the Vector NTI suite. Cultures of the bacterial strains will be prepared for use in electroporation protocols to enable the introduction of the genetic transformation plasmids. Genetic transformants will be selected on Zeocin antibiotic plates and frozen stocks prepared. Molecular analysis of the transformants will include Southern blot and Inverse-PCR (IPCR) analysis.

Expected Outcome

The completion of this objective will provide a potential biological delivery and control system for the fire ant. Further research in this area will need to be performed in conjunction with regulatory bodies to enable the completion of risk assessment studies for the potential release of transgenic bacteria into the environment. This research will complement that of other research groups that are investigating micro-organisms associated with the imported fire ant (Wilson, Vinson).

Timeline



Relevance to the Texas Imported Fire Ant Research and Management Plan

We propose to utilize molecular genetic technologies to provide additional knowledge and tools to be used as components of an integrated approach to control the imported fire ant. We will identify gene products that are involved in developmental processes critical to the success of fire ant colonies. Genetically modified symbionts will be produced and evaluated for their ability to deliver target gene candidates. These technologies and advances will assist in the development of novel control strategies to reduce the economic and social impacts of the imported fire ant.

Annual Budget

Principal Investigator	2004
C.J. Coates	$82,000
S.B. Vinson	$49,00
Total	**$131,000**

C.J. Coates	2004
Principle Investigator	$0
Postdoctoral Researcher[1]	$34,000
Research Associate[2]	$26,000
Materials and Supplies[3]	$20,000
Publication Costs[4]	$2,000
Total	**$82,000**

Budget Justification

1. Haisheng Tian (Postdoctoral Researcher) is a key component of this research project. He has expertise in the construction and screening of the SSH libraries and in the generation and testing of dsRNA constructs. He has been primarily responsible for all the preliminary data generated to date. He will continue to be a key investigator on this project.
2. Haiwen Li is a research associate that has been assisting with this project for the past 6 months. She has developed expertise in the microinjection of fire ants and has excellent molecular biology skills. She will be involved in the continued testing and validation of gene targets and the generation of the transgenic bacterial strains.
3. The requested funds for materials and supplies primarily cover the kits and reagents required for the molecular component of this research and include radioactive nucleotides, labeling kits, filters, film, dsRNA vectors and reagents, RNA supplies and reagents, bacterial media and supplements, standard molecular biology reagents and supplies. This is the minimal amount required for the work involved in a project of this magnitude.
4. Much of the preliminary research results that have been generated are close to reaching the publication and dissemination stage. Increasing page charges and publication costs are being used by many of the scientific journals and societies for publication. This cost will also cover the need for detailed colored images to be included in the publications.

S.B. Vinson	**2004**
Principle Investigator	$0
Graduate Student[1]	$17,400
Technician (50% time)[2]	$16,000
Ant Maintenance[3]	$8,000
Materials and Supplies[4]	$7,600
Total	**$49,000**

Budget Justification

1. Freder Medina is a Masters student who has been working on this project. He has been supplying fire ant stages of the correct age and caste for the preliminary research. He will be involved in the re-introduction of the transgenic bacteria into the fire ant colonies and the monitoring and analysis of the spread of these organisms.
2. A half-time technician is required for this project to assist in the collection, maintenance and provision of appropriate ant samples and colonies for these projects. This is a labor-intensive task that is essential to the project.
3. Ant Maintenance costs includes travel to and from field sites, equipment and supplies for the collection and maintenance of field and laboratory ant colonies.
4. Further materials and supplies are required for the downstream experiments involved with the tracking of transgenic bacteria within the experimental laboratory fire ant colonies.

Outside/Leveraged Related Funding

The technologies and expertise that were developed over the past funding period for the genetics fire ant project were transferred to other projects in the Coates and Vinson laboratories and have directly resulted in the submission of 2 grant proposals to the USDA-CREES-NRI program and 2 grant proposals to the NSF. The preliminary data generated for the submission of these proposals would not have been possible without the experimental techniques and results generated from the genetics fire ant project. We will continue to leverage fire ant project funding to obtain both state and federal funds.

Appendix 1. Multiple Sequence Alignment of 3 Fire Ant Vitellogenin Genes.