Group VIII *Spiroplasma* of Costa Rica

Kimberly M. Stewart
GROUP VIII SPIROPLASMA OF COSTA RICA

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A Thesis
Presented to
the College of Graduate Studies of
Georgia Southern University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
In the Department of Biology

by
Kimberly M. Stewart
July 2001
June 28, 2001

To the Graduate School:

This thesis entitled, “Group VIII Spiroplasma of Costa Rica,” and written by Kimberly M. Stewart is presented to the College of Graduate Studies of Georgia Southern University. I recommend that it be accepted in partial fulfillment of the requirements of the Master of Science Degree in Biology.

[Signature]
Frank E. French, Supervising Committee Chair

We have reviewed this thesis and recommend its acceptance:

[Signature]
Daniel V. Hagan, Committee Member

[Signature]
William S. Irby, Committee Member

[Signature]
Laura B. Regassa, Committee Member

Accepted for the College of Graduate Studies

[Signature]
G. Lane Van Tassell
Dean, College of Graduate Studies
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Curriculum Vitae
Kimberly Michelle Stewart

Personal:
Business Address:
Department of Biology, Georgia Southern University,
Statesboro, Georgia 30460-8042
Business Phone:
(912) 681-5901
E-mail:
kimberly_m_stewart@gasou.edu

Education:
1994-1998:
B.S. in Biology, Georgia Southern University, Statesboro, Georgia
Major: Biology
1999-2001:
M.S. in Biology, Georgia Southern University, Statesboro, Georgia
Major: Biology

Employment History:
Summer 2000:
Cotton Scout, High Cotton Consulting, Statesboro, Georgia
Spring 2000:
Teaching Assistant, Department of Biology, Georgia Southern University,
Statesboro, Georgia
1999-2000:
Graduate Student Assistant, Department of Biology, Georgia Southern University,
Statesboro, Georgia
1999-2000:
House Director, Kappa Delta Sorority, Statesboro, Georgia
1998-1999:
Veterinary Assistant, Metter Veterinary Clinic, Metter, Georgia
1997-1998:
Student Assistant, Payroll Department, Georgia Southern University,
Statesboro, Georgia
Memberships:
2000-present:
  Graduate Student Organization
1999-present:
  Parasitology Journal Club
1999-present:
  Georgia Entomological Society
1999-present:
  Entomological Society of America
1998-present:
  Kappa Delta Alumnae Organization, Delta Lambda Chapter
1999-2001:
  Chapter Advisory Board, Kappa Delta Alumnae Organization,
  Delta Lambda Chapter
1981-present:
  Gail Hursey School of Dance
1996-1998:
  Kappa Delta Sorority, Delta Lambda Chapter

Leadership Positions:
2001:
  Associate Host, Biting Fly Workshop, Alpine, Texas
2001-present:
  Biology Representative, Research/Travel Grant Review Committee
2000-present:
  Senator, Graduate Student Organization
2000-2001:
  Membership Advisor, Chapter Advisory Board, Kappa Delta Sorority,
  Delta Lambda Chapter
1999-2000:
  House Director, Kappa Delta Sorority, Delta Lambda Chapter
1997-1998:
  Coach Chairman, Kappa Delta Sorority, Delta Lambda Chapter

Honors:
1997-1998:
  Beta Beta Beta Biological Honor Society
Spring 1996:
  Dean’s List
1994-1998:
  Gamma Beta Phi Honor Society
1994-1998:
  HOPE Scholarship
Grants, Awards:
2001:
   Biology Department Travel Funds, Department of Biology, Georgia Southern University; $250
2000:
   Competitive Research Grant, Department of Graduate Studies, Graduate Student Professional Development Fund, Georgia Southern University; $450
2001:
   Winner of Spring 2001 Sigma Xi Research Competition. Georgia Southern University Chapter of Sigma Xi, Statesboro, Georgia. 24 April 2001; $50.
2001:
   Competitive Travel Grant, Department of Graduate Studies, Graduate Student Professional Development Fund, Georgia Southern University; $500
2001:
   1st Runner-up in Poster Competition at 5th Joint Meeting of the Georgia Entomological Society (65th Annual Meeting) and South Carolina Entomological Society (47th Annual Meeting). 5 April 2001. Gainesville, Georgia; $70
2000:
   Academic Excellence Award, College of Science and Technology, Georgia Southern University; $250

Field, Research Experience:
2000-2001:
   St. Catherine’s Island, Georgia
   − Relocated and Monitored sea turtle nests
1999-present:
   Department of Biology, Georgia Southern University, Statesboro, Georgia
   − Serological and Molecular Evaluation of Spiroplasma Bacteria Isolated from Costa Rican Tabanid Flies
   − Assistant with undergraduate research projects
1999:
   Highlands, North Carolina
   − Assistant in Field Entomology Class
   − Collected and processed tabanids
   − Prepared field cultures of tabanid fly spiroplasma
1999:
   Costa Rica
   − Collected and processed tabanids
   − Prepared field cultures of tabanid fly spiroplasma
1996:
   Department of Biology, Georgia Southern University, Statesboro, Georgia
   − Seed size variation in Ipomoea coccinea and I. quamoclit
Diving Certifications:
1997-present:
  Nitrox
1997-present:
  Open Water Diver

Publications:

Presentations:


“Serological Profiling and Molecular Characterization of Spiroplasma Bacteria Isolated From Costa Rican Tabanids.” 24 April 2001. Sigma Xi Research Competition, Georgia Southern University Chapter of Sigma Xi, Statesboro, Georgia.

“Serological and Molecular Evaluation of Costa Rican Tabanid Spiroplasma Bacteria.” 5 April 2001. 5th Joint Meeting of the Georgia Entomological Society (65th Annual Meeting) and South Carolina Entomological Society (47th Annual Meeting), Gainesville, GA.


“Seed Size Variation in Ipomoea coccinea and I. Quamoclit.” Fall 1996. Undergraduate Research Symposium, Department of Biology, Georgia Southern University.

Conferences, Workshops Attended:
2001:  
Joint Meeting of the North American Dipterists' Society and Biting Fly Workshop, Alpine, Texas

2001:  
Clarke Mosquito Control Workshop, Statesboro, Georgia

2001:  
5th Joint Meeting of the Georgia Entomological Society (65th Annual Meeting) and South Carolina Entomological Society (47th Annual Meeting), Gainesville, Georgia

2001:  
Georgia Academy of Science Annual Meeting, Brunswick, Georgia

2000:  
Southeastern and South Carolina Branches of the American Society for Microbiology, Statesboro, Georgia

1999:  
Entomological Society of America, Annual National Meeting, Atlanta, Georgia

1998:  
Biting Fly Workshop, Black Water Falls State Park, West Virginia
ABSTRACT

GROUP VIII SPIROPLASMA OF COSTA RICA

July 2001

Kimberly M. Stewart

BS IN BIOLOGY GEORGIA SOUTHERN UNIVERSITY
MS IN BIOLOGY GEORGIA SOUTHERN UNIVERSITY

Directed by: Professor Frank E. French

_Spiroplasma_ bacteria isolates are classified or identified through a series of serological tests that normally consists of screening, one-way deformation tests, cloning, antisera production, and reciprocal deformation tests. Serological tests on the spiroplasmas are followed by molecular analysis. The standard molecular analysis used for spiroplasmas has been 16S rRNA sequencing. The primary goal of my research was to evaluate serologically Group VIII spiroplasmas isolated from tropical Costa Rican tabanids (Diptera: Tabanidae) and to compare them to the temperate North American Group VIII spiroplasmas. A secondary goal was to evaluate both the temperate and tropical Group VIII strains by sequencing the 16S-23S rRNA intergenic spacer region.

_Spiroplasma_ cultures were obtained from Costa Rican tabanids and serologically screened. This screening procedure placed ten of the 72 cultures (GSU 5367, 5401, 5404, 5408, 5429, 5431, 5436, 5437, BARC 4898, and BARC 4899) in Group VIII. Further
serological procedures including one-way deformation tests, serocloning, dilution cloning, antisera production, and reciprocal deformation tests indicated that the isolates are serologically related to five serovars previously reported for temperate North American Spiroplasma. Five of the strains (GSU 5401, 5404, 5408, 5436, and BARC 4899) are serologically related to TAAS-1 and GSU 5367 is closely related to BARC 2649 of the southern United States. Four of the strains, GSU 5429, 5431, 5437, and BARC 4898, form a cluster of intermediate strains, linking TAAS-1 and BARC 1357. The frequency of the serovar TAAS-1 (5/10) in the Costa Rican sample was notably higher than the frequency of 7.7% in Tabanus lineola of temperate North America.

Since 16S rRNA analyses were found to be too conservative in Group VIII spiroplasmas, attempts were made to sequence the 16S-23S rRNA intergenic spacer region in an effort to separate this group. DNA was extracted from six isolates, two from the Costa Rican sample and four temperate North American Group VIII strains. Primers were developed for the 16S-23S rRNA spacer region and used in PCR amplification. Amplification was not achieved with the first set of primers. Consequently, a second set of primers has been designed for PCR amplification and will be used for future research.
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Chapter I

Introduction

Spiroplasma bacteria are members of Division Tenericutes, Class Mollicutes, Order Entomoplasmatales, Family Spiroplasmataceae, and Genus *Spiroplasma* (Tully et al. 1993). They are prokaryotic organisms, lacking a cell wall, and thought to be descendants of a branch of gram-positive bacteria (Woese 1987). At some point in their life cycle, generally during the exponential growth phase, all spiroplasma exhibit a helical morphology (Williamson et al. 1989, Vazeille-Falcoz 1997). Spiroplasma exhibit both rotatory and flexional movement despite their lack of flagella, periplasmic fibrils, and axial filaments (Razin 1978, Bové 1997). These organisms can be found as natural pathogens or commensals in association with a wide variety of insects, plants, and ticks, and as experimental pathogens in association with vertebrates (Clark 1982, Hackett and Clark 1989, Bové 1997).

Spiroplasma were discovered through studies on two plant diseases, citrus stubborn and corn stunt disease. The agent of citrus stubborn disease, *Spiroplasma citri*, was the first mollicute to be cultured (Saglio et al. 1973) and the first spiroplasma to be characterized and named. Two other plant-pathogenic spiroplasmas have been identified and classified since that time, *Spiroplasma kunkelli*, the agent of corn stunt disease (Whitcomb et al. 1986a), and *Spiroplasma phoeniciem*, the agent of periwinkle disease (Saillard et al. 1987). The three plant-pathogenic spiroplasmas are restricted to the sieve tube elements of the plant and are vectored by leafhoppers. Leafhoppers acquire
spiroplasmas by feeding on an infected plant. The spiroplasma multiply in the hemolymph, gut, and other organs of the insect. During subsequent feeding, infected saliva is transferred from the salivary glands of the leafhopper to the plant (Bové 1997).

Insects are bountiful sources of spiroplasma. Spiroplasmas have been isolated from representatives of nine insect orders: Coleoptera; Diptera; Hemiptera; Homoptera; Hymenoptera; Lepidoptera; Mecoptera; Odonata; and Trichoptera (Clark 1982, Hackett et al. 1990, Bové 1997). Spiroplasma are found in the gut, hemolymph, and organs such as salivary glands of most of these insects, however, isolations from Odonata came only from the gut (Hackett et al. 1990). Tabanids (Diptera: Tabanidae) are particularly abundant sources of spiroplasma (Clark et al. 1984, French et al. 1992). Multiple species of spiroplasma have been isolated from a single tabanid fly (Whitcomb and Hackett 1996). Adult tabanids are easy to maintain under laboratory conditions and can be artificially infected with spiroplasma rather easily, which makes them exceptional candidates for study (Wedincamp et al. 1997). The majority of the characterized and described spiroplasma have been isolated from tabanids; 11 of the 34 recognized spiroplasma groups were isolated from tabanids (Williamson et al. 1998, Whitcomb et al. 1999).

The life cycle of spiroplasma bacteria is not fully understood and there are varying opinions on modes of infection in insects. Hackett et al. (1990), after an analysis of spiroplasma isolations from representative Odonata that came only from the gut, concluded that the infections were contaminants acquired through predation rather than true infections. Wedincamp et al. (1997) tested the suggestion that spiroplasmas could be acquired through predation by infecting mealworm beetle pupae with spiroplasma and
then offering them to predaceous firefly larvae. The firefly larvae failed to become infected after feeding on the infected pupae. In further studies involving tabanids and fireflies, Wedincamp et al. (1996, 1997) demonstrated that spiroplasmas could be exchanged between the two species at common carbohydrate feeding sites. The fact that *Spiroplasma apis* could be cultured from plant surfaces up to 200 m away from infected beehives suggests that the spiroplasmas found on plant surfaces were most likely deposited there by other insects (Bové 1997), thus supporting the suggestion by Wedincamp et al. (1996) of oral transmission during carbohydrate feeding by tabanids.

Several spiroplasmas are entomopathogens. *Spiroplasma apis* and *melliferum* both infect honeybees. *Spiroplasma melliferum* was first isolated from the hemolymph of "dead and moribund bees" (Clark 1977, Clark et al. 1985). *Spiroplasma apis* was cultured in 1981 from honeybees carrying "May disease" as well as from plants growing around beehives with infected bees (Bové 1997). *Spiroplasma poulsonii* is found in four related species of Neotropical fruit flies, vertically transmitted, and lethal to male progeny (Williamson et al. 1999).

*Spiroplasma mirum* is an experimental pathogen of chicken embryos and suckling rats and mice. When injected with *S. mirum*, suckling rats and mice experienced neurological and ocular disease. Embryonated chicken eggs injected with *S. mirum* developed lethal infections (Tully et al. 1982).

Currently spiroplasmas are classified according to a group system. To merit group status in the Genus *Spiroplasma*, the following requirements must be met (Anonymous 1995, Whitcomb et al. 1999): (1) triple cloning of the organism, (2) demonstration that the organism belongs to the Class Mollicutes, (3) confirmation that the organism belongs
to the genus *Spiroplasma*, Family Spiroplasmataceae, demonstration that the organism is serologically distinct from other spiroplasma groups and species by performing reciprocal DF tests and at least one other serological test, determination of guanine-plus-cytosine content is optional for group status but should be determined if there are plans to designate the organism as a new species, deposition of the candidate in a nationally recognized culture collection so that it is available to other workers, and determination of species according to the requirements established by the “minimal standards for description of new mollicute species” as previously established by the Subcommittee on the Taxonomy of *Mollicutes* (Anonymous 1995). *Spiroplasma*, which exhibit moderate amounts of DNA-DNA homology and serological interactions, can be designated as subgroups. Subgroups exhibit less than 70% homology with the related subgroups and therefore, like groups are eligible for species designation (Whitcomb et al. 1999).

In addition to the requirements for group designation, requirements for species designation as outlined by the International Subcommittee on Systematic Bacteriology are: (1) G+C base composition less than 40%, determination of genome size, and sequence analysis (16S ribosomal); (2) order and family determination which includes determination of sterol requirements, cellular and colony morphology, determination of the usage of aerobiosis or anaerobiosis, and the organisms usage of the UGA codon; (3) genus determination which for Spiroplasmataceae would include organisms cultured from arthropods, plant surfaces, or phloem that are helical, do not require anaerobic conditions, may or may not require sterols, and have a genome size ranging from 940 to 2,200 kbp; (4) determination of species which includes a species description and DNA-
DNA homology of less than 70% (Anonymous 1995). Genetically, the most defining characteristic for a species is less than 70% shared DNA-DNA homology (Gasparich et al. 1998).

Williamson et al. (1998) and Whitcomb et al. (1999) list 34 groups, 14 subgroups, and 34 published species descriptions. Group VIII contains three subgroups with two designated species, along with three serovars (strains), which are awaiting species designation. Members of this group have less than six turns per helix, reside in dipterans, require temperatures ranging from 30-37°C for growth, have the G+C content ranging from 29 to 30 +/- 1 mol%, and specific antigenic requirements (Gasparich et al. 1993). When the 16S rRNA gene was sequenced for Group VIII spiroplasmas, it failed to separate this tightly knit clade (Dodge et al. 1998).

In order to make evaluations of the phylogeny, biogeography, ecology, host distribution, and pathogenicity of spiroplasmas, they have to be identified and classified. They are identified through a series of serological tests that normally consists of screening, one-way deformation tests, cloning, antisera production, and reciprocal deformation tests (Whitcomb et al. 1999). Serological tests on the spiroplasmas are followed by molecular analysis.

Screening is a serological technique in which a spiroplasma is tested against antisera directed against known spiroplasmas. The screening process places the isolate into one of twelve recognized antigenic groups (Williamson et al. 1998). After screening, the deformation (DF) test (Williamson et al. 1978) is the most widely used method for further serological evaluation of spiroplasmas. Organisms to be tested are mixed with antisera at a wide range of dilutions. The mixtures are allowed to react and then
examined by dark field microscopy for clumping or deforming of the cells. The point at which half of the spiroplasmas are reacting with the antisera and half are not is called the endpoint titer of the reaction and should indicate the identity of the organism (Williamson et al. 1978, Whitcomb et al. 1999).

Dilution cloning is done when the identity of a spiroplasma isolate has not been clearly resolved through the screening and one-way DF tests. The purpose of dilution cloning a spiroplasma is to obtain a culture that originates from a single cell so that it reacts homogeneously with the antisera. Cloning is essential in many cases because the results of serological tests performed on mixed cultures (cultures containing more than one type of spiroplasma) are worthless. The dilution cloning procedure is normally repeated three times (Whitcomb et al. 1986b) followed by one-way DF tests to determine if the identity has been resolved. If an isolate has been triply-cloned and the identity cannot be determined with one-way DF tests, then it is assumed to represent a novel antigenic class and antisera is produced from the clone. A triply-cloned culture is the antigen used for antisera production. The new antisera is used to evaluate serologically the unidentified culture through homologous and reciprocal DF tests run against previously typed spiroplasma cultures (Williamson et al. 1978).

Molecular analysis is used to support identifications made through serological tests and to estimate phylogeny (Dodge et al. 1998). Spiroplasma subgroups with less than 70% shared DNA-DNA reassociation values with putative sister taxa are eligible for species designation. Previous experiments have shown that some Group VIII subgroups indeed share less than 70% genomic similarities among the matrix of species therefore making them candidates for molecular analysis based on 16S rRNA gene sequence
analysis (Whitcomb et al. 1999). However, when the 16S rRNA analyses were done, they demonstrated more than 99% sequence similarity, thus showing that the 16S rRNA is too conservative in the case of Group VIII spiroplasmas and therefore not effective for distinguishing between pairs of species that are closely related (Dodge et al. 1998).

Similar problems with the inability of 16S rRNA analysis to successfully resolve phylogenetic relationships were indicated by Schulenberg et al. (2000) through sequence analysis of three male-killing spiroplasmas. *Spiroplasma poulsonii*, from the *Drosophila willistoni* species group of fruit flies, and two other unnamed spiroplasmas found in the beetles, *Adalia bipunctata* and *Harmonia axyridis*, are lethal to male embryos. Analysis of 16S rRNA sequences from these spiroplasmas demonstrated that the male-killer spiroplasmas found in the beetles formed a monophyletic clade with *Spiroplasma ixodetis*, the representative strain for Group VI, distinct from *S. poulsonii* of Group II. The two male-killing spiroplasmas from the beetles and *S. poulsonii* fell into related clades. Although the 16S rRNA analysis indicated that these spiroplasmas did fall into related clades, it failed to provide enough detail to resolve exact relationships. The 16S-23S intergenic spacer region was then sequenced in an attempt to make evaluations of the specific relationships among the clade. The results suggest the sequence variation and length of the spacer region can be used in identification of spiroplasma infections as well as species-specific markers (Schulenburg et al. 2000). The 16S-23S intergenic spacer region has been demonstrated to be species-specific in studies conducted on ureaplasmas and mycoplasmas as well (Harawasa 1996, 1999, Harawasa et al. 1999).

The primary goal of my thesis was to serologically evaluate Group VIII spiroplasmas isolated from Costa Rican tabanids and to compare them to temperate North
American Group VIII spiroplasma. A secondary goal was to evaluate both the temperate and tropical Group VIII strains through sequencing of the 16S-23S rRNA spacer region.
Chapter II

Materials and Methods

The hosts for the Group VIII spiroplasmas of this project were females, live trapped in a Gressitt Malaise trap in Costa Rica by Frank E. French in 1995 or 1998. From the 1995 sample of 13 flies, 12 isolations were obtained and from the 1998 sample of 99 flies, 66 isolations were transported safely to Georgia Southern University. Spiroplasma isolations were made as previously described by Markham et al. (1983) and Wedincamp et al. (1996). At Site A (Figure 1), in the central highlands of Santo Domingo, Heredia Province (N09°58.5’, W84°05.5’, ele. 1,100m) BARC 4898 and BARC 4899 were isolated from two Poecilodespus quadripunctatus (Fabricius) on August 12, 1995. From Site B, on August 6, 1998, on the east coast at Puenta Vargas National Park, Limón Province (N09°42.9’, W82°49.3’, ele. 2m), GSU 5367 was isolated from a Diachlorus curvipes (Fabricius). At Site C on the upper eastern coastal plain at the Hiltoc Cereré Biological Station, Limón Province (N09°4.4’, W83°01.4’, ele. 100m) isolations made on August 7, 1998 were GSU 5401 from Tabanus praeteritus Fairchild, GSU 5404 from Tabanus occidentalis Linnaeus, and GSU 5408 from Leucotabanus flavinotum (Kröber). At Site D four isolations were made on August 14, 1998 on the western coast near Curu, Puntarenas Province, (N9°48.17’, W84°55.52’, ele. 35m); they were GSU 5429 from Tabanus colombensis Macquart, GSU 5431 from Tabanus occidentalis Linnaeus, and GSU 5436 and 5437 from Tabanus pungens Wiedemann.
Figure 1. Host and Isolation Sites for Spiroplasma in Costa Rica
A- Central highlands of Santo Domingo, Heredia Province
B- East coast at Puenta Vargas National Park, Limón Province
C- Upper eastern coastal plain at Hiltoy Cereré Biological Station, Limón Province
D- Western coast near Curu, Puntarenas Province
Subcultures of all primary isolations of spiroplasma growth were screened using 1:10 diluted antisera to 12 known spiroplasma bacteria groups associated with tabanids. Antisera was placed in microtiter wells, an equal volume of culture was added to each well, allowed to react for 20 minutes, and then examined by dark field microscopy for deformation of the cells (Appendix A). On the basis of the screening reactions, ten isolates were chosen for further serological tests (Table 1). Each of the ten cultures reacted positively in the screening process with Group VIII antigens, with two isolates appearing to be mixed cultures based upon reactivity to other antisera groups.

BARC 4898 was isolated from a mix by serocloning the mixed culture against Screening Cocktail 12 (Appendix B). GSU 5404 was isolated by serocloning the mixed culture against TN-1. Following serocloning, the group VIII components of BARC 4898 and GSU 5404 were confirmed by DF tests.

The eight cultures, which reacted positively with Group VIII in the screening procedure, listed above, underwent DF tests. Each spiroplasma culture was tested against individual antisera to the five known Group VIII spiroplasma serovars from the United States, EA-1, DF-1, TAAS-1, BARC 1357, and BARC 2649. Equal amounts of culture were mixed with each of the five antisera at a wide range of dilutions, from 1:10 to at least 1:1280, allowed to react for 30 minutes, and then examined by dark field microscopy for clumping or deforming of the cells. The point at which half of the spiroplasma reacted with the antisera was recorded as the endpoint titer of the reaction and was used to indicate the identity of the isolate. Additionally, each of the isolates was subcultured in several types of media throughout the serological evaluation process to determine which growth medium facilitated the best morphology. The media utilized
Table 1. Host and Host Location of Group VIII Isolates Chosen for Cloning

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>BARC 4898</td>
<td><em>Poeciloderas quadripunctatus</em> (Fabricius)</td>
<td>Santo Domingo, Heredia Province</td>
</tr>
<tr>
<td>BARC 4899</td>
<td><em>Poeciloderas quadripunctatus</em> (Fabricius)</td>
<td>Santo Domingo, Heredia Province</td>
</tr>
<tr>
<td>GSU 5367</td>
<td><em>Diachlorus curvipes</em> (Fabricius)</td>
<td>Puentas Vargas National Park, Limón Province</td>
</tr>
<tr>
<td>GSU 5401</td>
<td><em>Tabanus praeteritus</em> Fairchild</td>
<td>Hiltoy Cereré Biological Station, Limón Province</td>
</tr>
<tr>
<td>GSU 5404</td>
<td><em>Tabanus occidentalis</em> Linnaeus</td>
<td>Hiltoy Cereré Biological Station, Limón Province</td>
</tr>
<tr>
<td>GSU 5408</td>
<td><em>Leucotabanus flavinotum</em> (Kröber)</td>
<td>Hiltoy Cereré Biological Station, Limón Province</td>
</tr>
<tr>
<td>GSU 5429</td>
<td><em>Tabanus colombensis</em> Macquart</td>
<td>Curu, Puntarenas Province</td>
</tr>
<tr>
<td>GSU 5431</td>
<td><em>Tabanus occidentalis</em> Linnaeus</td>
<td>Curu, Puntarenas Province</td>
</tr>
<tr>
<td>GSU 5436</td>
<td><em>Tabanus pungens</em> Wiedemann</td>
<td>Curu, Puntarenas Province</td>
</tr>
<tr>
<td>GSU 5437</td>
<td><em>Tabanus pungens</em> Wiedemann</td>
<td>Curu, Puntarenas Province</td>
</tr>
</tbody>
</table>
included M1D (Whitcomb 1983), and R2 (Liao and Chen 1977), and derivatives of these two including \( \frac{1}{2} R2 \), \( \frac{1}{2} M1D \), and M1D Horse Serum (M1D HS). Both \( \frac{1}{2} R2 \) and \( \frac{1}{2} M1D \) contain one half the amount of horse serum or fetal bovine serum, respectively, used to make R2 and M1D. In the M1D HS, horse serum was substituted for fetal bovine serum.

Following the serocloning and initial DF tests, the identity of two of the isolates was resolved. The eight unresolved cultures were dilution-cloned in broth medium utilizing serial dilutions of \( 10^6 \) through \( 10^{11} \) in microtiter plates (Appendix C) (Whitcomb et al. 1986b). The isolates were dilution-cloned at least three times and deformation tests were made after each cloning. Following the dilution cloning, the identities of only three triply cloned isolates, GSU 5367, GSU 5408, and GSU 5431, were not satisfactorily resolved and antisera was produced for these spiroplasma clones as previously described (Williamson et al. 1978). Spiroplasma cells for antigen were grown in at least 500 ml broth medium from a triply cloned culture, that was centrifuged at 21,000 x g, combined with RIBI® adjuvant (Sigma Chemical Co., St. Louis, MO), and injected into a rabbit. GSU 5367 antigen was grown in R2 and GSU 5431 antigen was grown in \( \frac{1}{2} R2 \). GSU 5408 antigen was grown in two different batches of M1D and one batch of \( \frac{1}{2} HS R2 \). Each of the 500 ml samples for GSU 5408 was centrifuged, and the pellets were combined for the antigen. The animals were cared for in accordance with approved guidelines set forth in the Guide for the Care and Use of Laboratory Animals and their use was reviewed and approved by the Institutional Animal Care and Use Committee of Georgia Southern University. The antisera was recovered when homologous titers of 1:320 or higher were observed and used in reciprocal DF tests
against the five Group VIII type cultures and against all of the Group VIII isolates from Costa Rica.

Following the serological evaluation process, six strains were chosen for molecular analysis, GSU 5367 and GSU 5431 from Costa Rica, and four United States Group VIII strains, DF-1, TAAS-1, BARC 2649, and BARC 1357. DNA was isolated and extracted from the six isolates as previously described (Appendix D) (Duret et al. 1999). The candidate cultures were passed into two 10 ml aliquots of M1D medium and incubated at 30°C until growth was observed. The cultures were then examined by dark field microscopy to determine morphology and concentration. When prolific cells with good morphology were observed, the cultures were transferred to 50 ml centrifuge tubes and harvested by centrifugation in a Fisher Scientific Marathon 21K/BR centrifuge at 12,000 x g. The pellets obtained were resuspended in STE buffer and the cells were lysed with sodium dodecyl sulfate. The lysate was heated to 65°C, treated with RNase, and then subjected to phenol-chloroform-isooamyl extraction, and ethanol precipitation. The purified products were run on an agarose gel, using λ Hind III as a standard, to demonstrate DNA had been extracted (Figure 2).

Primers were developed for the 16S-23S intergenic spacer region. For primer development, the 16S, 23S, and 16S-23S spacer region sequences from Genbank for the isolates from Drosophila willistoni, Entomoplasma freundtii, Harmonia axyridus, and Ixodes pacificus and sequences from the spiroplasma S. citri, S. diabroticae, S. gladiatoris, S. ixodetis, S. mirum, S. monobiae, S. poulsonii, and S. taiwanense were aligned using the Genetic Data Environment Program (Smith et al. 1994). The aligned sequences were then compared and primers were developed.
Figure 2. Representative agarose gel of DNA isolation.  
Lane 1- λ Hind III,  
Lane 2- GSU 5431.
manually from the most conserved areas of the 16S and 23S rRNA genes. The forward primer, 5'GCACTTATGCAAGAGC, was chosen from the most conserved region near the end of the 16S rRNA. The reverse primer, 5'CAAGGCATTCCACCATAAC, was chosen from the most conservative region of the 23S rRNA gene (Figures 3-4). The primers were used in PCR amplification under the conditions outlined by Duret et al. 1999. Specifically, amplification was carried out in 50 µl reaction mixtures containing 1 µl (9-20 ng) of target DNA, 1X Cloned *Pfu* Buffer (200 mM Tris-HCL, pH 8.8, 20 mM MgSO₄, 100 mM KCl, 100 mM (NH₄)₂SO₄, 1% Triton X-100, 1 mg/ml nuclease-free BSA) (Stratagene Cloning Systems, La Jolla, CA), 0.2 mM deoxynucleoside triphosphates (Promega Corp., Madison, WI.), 1 µM of each primer, and 2.5 U of Cloned *Pfu* DNA polymerase (Stratagene Cloning Systems, La Jolla, CA). PCR reactions were performed in a PE 2400 Thermal Cycler (Perkin-Elmer, Foster City, CA). The parameters for the PCR were an initial denaturation at 92°C for 2 minutes, followed by 35 cycles of 92°C for 45 seconds, 48°C for 45 seconds, and 72°C for 1 minute with a final extension at 72°C for 10 minutes. When these conditions did not amplify the targeted product, the conditions were adjusted which included altering annealing temperatures, DNA concentrations, and MgCl₂ concentrations. Concentrations of MgCl₂ were varied from 2.0 mM to 4.0 mM and 8.0 mM using the Cloned *Pfu* DNA Polymerase and 10X Cloned *Pfu* Buffer. Additionally, *Taq* DNA Polymerase (Promega Corp., Madison, WI.) was ordered along with a 10X buffer (100 mM Tris-HCL, pH 9.0 at 25°C, 500 mM KCL, 1% Triton® X-100) (Promega Corp., Madison, WI.), which did not contain MgCl₂, and MgCl₂ titrations ranged from 1.0 mM to 3.0 mM. Additional annealing temperatures
Figure 3. Gene map depicting approximate values for 16S, 16S-23S ITS, and 23S genes for *Spiroplasma* sp. Arrows indicate areas where primers were chosen. ITS is an abbreviation used for the 16S-23S intergenic spacer region. F indicates the location from which the forward primer was chosen and R represents the location from which reverse primer was chosen. The dashed line following the first 200 base pairs of the 23S rRNA gene indicates that complete sequences were not available for this region but it should extend 2,300 base pairs.
Figure 4. Sequence alignments used for primer development. (A) Aligned sequences showing the conservative region at the end of the 16S rRNA gene from which the forward primer was chosen. The specific bases that were selected are in bold. (B) Aligned sequences showing the conservative region at the end of the 23S rRNA gene from which the reverse primer was chosen. The specific bases that were selected are in bold. The Genbank accession numbers represent the following species and strains: AJ130955-Spiroplasma sp. from Drosophila willistoni, AF036954- Entomoplasma freundii, AJ130953- Spiroplasma sp. from Harmonia axyridus, AJ130954- Spiroplasma sp. from Drosophila willistoni, AF005327- Spiroplasma citri spacer region, M24476- Spiroplasma taiwanense, M24662- Spiroplasma mirum, M24481- Spiroplasma monobiae, M24483- Spiroplasma poulsonii, M24475- Spiroplasma gladiatoris, AJ245996- Male-killing Spiroplasma sp., M24482- Spiroplasma diabroticae, and M24476- Spiroplasma ixodetis.

* indicates nucleotide difference
used included 37°C, 39°C, 41°C, 43°C, 45°C, 47°C, and 54°C. DNA concentrations of 50 ng and 100 ng were also used.
Chapter III

Results

Ten strains of Group VIII were examined serologically in this study (BARC 4898, BARC 4899, GSU 5367, GSU 5401, GSU 5404, GSU 5408, GSU 5429, GSU 5431, GSU 5436, and GSU 5437) and compared serologically with five recognized strains of Group VIII spiroplasmas from the United States. Cultures examined by dark field microscopy revealed short helices with six or less turns and were often in excess of $10^{10}$ per ml broth. All strains grew well in MID broth medium. Additionally, each strain grew in R2 broth medium; however, the DF test in R2 was more difficult to read perhaps due to a combination of a different refractive index and morphology. Some strains also grew in $\frac{1}{2}$ HS MID and $\frac{1}{2}$ R2. Antisera pellets for GSU 5367, GSU 5408, and GSU 5431 were grown in R2, R2 $\frac{1}{2}$ HS MID and M1D, and $\frac{1}{2}$ R2 respectively.

The ten strains examined are serologically related to five serovars previously reported for temperate North American Spiroplasma. The homologous DF test titer for GSU 5367 was 1:2560 (Table 2) and the reciprocal tests with BARC 2649 were both strong with titers of 1:640. GSU 5367 cells reacted at very low levels (1:20 to none) with antisera of GSU 5431 and the five strains from the United States.

GSU 5431 had a homologous DF titer of 1:1280. The GSU 5431 antisera DF titers were strong against cells from GSU 5429 and GSU 5437 (1:1280 and 1:640, respectively), which also were from the same collection at Curu. GSU 5431 cells had strong one–way DF titers against the antisera of TAAS-1 (1:640) and BARC 1357.
Table 2. Results of One-way and Reciprocal Deformation Tests for Costa Rican and Temperate North American Group VIII Spiroplasmas.

<table>
<thead>
<tr>
<th>Antigens</th>
<th>GSU 5367</th>
<th>BARC 2649</th>
<th>BARC 1357</th>
<th>GSU 5431</th>
<th>VIII-3</th>
<th>GSU 5408</th>
<th>VIII-2</th>
<th>VIII-1</th>
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<tbody>
<tr>
<td>GSU 5367</td>
<td>2560</td>
<td>640</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>nd</td>
<td>N</td>
<td>20</td>
</tr>
<tr>
<td>BARC 2649</td>
<td>640</td>
<td>320</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>BARC 1357</td>
<td>20</td>
<td>N</td>
<td>1280</td>
<td>20</td>
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<td>640</td>
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<td>80</td>
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<td>20</td>
<td>320</td>
<td>640</td>
<td>160</td>
<td>nd</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
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<td>N</td>
<td>N</td>
<td>80</td>
<td>1280</td>
<td>320</td>
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<td>5120</td>
<td>nd</td>
<td>N</td>
<td>N</td>
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<td>GSU 5436</td>
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<td>N</td>
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<td>160</td>
<td>2560</td>
<td>nd</td>
<td>80</td>
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<td>N</td>
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<td>40</td>
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<td>N</td>
</tr>
<tr>
<td>BARC 4899</td>
<td>N</td>
<td>N</td>
<td>20</td>
<td>20</td>
<td>2560</td>
<td>nd</td>
<td>40</td>
<td>N</td>
</tr>
<tr>
<td>DF-1</td>
<td>N</td>
<td>N</td>
<td>160</td>
<td>20</td>
<td>40</td>
<td>nd</td>
<td>2560</td>
<td>N</td>
</tr>
<tr>
<td>EA-1</td>
<td>20</td>
<td>N</td>
<td>20</td>
<td>80</td>
<td>160</td>
<td>N</td>
<td>80</td>
<td>1280</td>
</tr>
</tbody>
</table>

N = No Reaction
nd = not done
TAAS = TAAS-1

Homologous Reactions

Intermediate Strains
(1:320). However, the reciprocal DF titers were only 1:160 for TAAS-1 cells, and 1:20 for BARC 1357. For cells of other strains from Costa Rica or the United States, only moderate (1:160) to no DF titers were obtained with the GSU 5431 antisera. Cells of GSU 5431 had DF titers of 1:20 or none to the other three antisera (Table 2).

In addition to strong one-way reactions with antisera GSU 5431, GSU 5437 cells had DF titers of 1:320 (BARC 1357) and 1:160 (TAAS-1), and GSU 5429 cells had a titer of only 1:80 for BARC 1357 but a 1:320 titer with antisera of TAAS-1.

TAAS-1 antisera reacted very strongly with cells of BARC 4898, BARC 4899, GSU 5401, GSU 5404, GSU 5408, and GSU 5436, with titers of 1:1280-1:5120. Reciprocal DF titers of GSU 5408 and TAAS-1 were 1:1280 and 1:2560, respectively.

DNA was extracted and purified from GSU 5367, GSU 5431, BARC 1357, BARC 2649, DF-1, and TAAS-1. The primers chosen were used for PCR amplification and the PCR conditions were altered numerous times; however, only primer dimers were obtained.
Chapter IV

Discussion

Serology, in particular the DF test, has been the main method by which new spiroplasma isolates have been characterized (Williamson et al. 1998). The five Group VIII strains from the United States have been repeatedly differentiated by reciprocal DF tests in this research project and others (Whitcomb et al. 1997, 1999, Williamson et al. 1998). When antiserum is not available for an isolate, only one-way DF tests are possible. While one-way DF tests are not always indicative of close relationships, titers of 1:1280 or greater have not been associated with reciprocal titers of less than 1:320.

Reciprocal DF tests among the five Group VIII strains (EA-1, DF-1, TAAS-1, BARC 1357, and BARC 2649) and 16 other strains associated with tabanids from the United States produced no reactions above 1:160 except for homologous reactions (Whitcomb et al. 1997). The one–way DF reactions of 1:1280 to 1:5120 for cells of BARC 4899, GSU 5401, GSU 5404, and GSU 5436 with TAAS-1 antisera are indicative of a close relationship with TAAS-1.

GSU 5367 shares a close serological relationship to BARC 2649. BARC 2649 was isolated from Tabanus lineola Fabricius, Bulloch Co., Georgia and is considered rare with an 8.6% frequency of isolation from 104 of the type host and locality (F. E. French, unpublished). GSU 5408, although initially obscure, shows reciprocally, a close serological relationship with TAAS-1. Four other isolates, BARC 4899, GSU 5401, GSU 5404, and GSU 5436 had very high one–way DF titers of 1:1280 to 1:5120 to TAAS-1.
antisera. Among the Group VIII isolates from Costa Rica, five of ten reacted strongly with TAAS-1 antisera; thus, the strain TAAS-1 is perhaps the most prevalent Group VIII form in Costa Rica. These five cultures were obtained from five different tabanid species from three sites, Curú, Hiltoy Cereré, and Santo Domingo with a range in elevation from 35 to 1,100m. In Bulloch Co. Georgia, 7.7% of Group VIII spiroplasmas isolated from 104 *T. lineola*, reacted strongly with TAAS-1 (F. E. French, unpublished).

GSU 5431, with a homologous titer of 1:1280, reacted strongly with both TAAS-1 (1:640) and BARC 1357 (1:320) in one-way DF tests but in reciprocal DF tests it only reacted at 1:20 with BARC 1357 and moderately with TAAS-1 (1:160). GSU 5431 may represent an intermediate strain between BARC 1357 and TAAS-1, and perhaps is a more serologically basic strain. GSU 5431 and three other strains, BARC 4898, GSU 5429, and GSU 5437 appear to form a small cluster of bridge strains that tie BARC 1357, GSU 5431, and TAAS-1 to a common near ancestor (Table 2).

At the outset of this research project the prediction was made that there would be many new spiroplasma strains in the tropics. However, the evaluation of the samples in this study failed to reveal any new Group VIII strains. The ten isolates from Group VIII showed at least a strong one-way relationship with isolates from the United States, unlike the long form spiroplasmas from Costa Rica in which there are at least seven isolates that show moderate to no reciprocal reactions with any strain from the United States (R.F. Whitcomb and F.E. French, unpublished). Since the Group VIII spiroplasma isolations were obtained from seven different species of tabanids, captured in four different locations in Costa Rica in two separate years, it is likely that there is no widely distributed undiscovered Group VIII spiroplasma in Costa Rica.
DNA was isolated from GSU 5367, GSU 5431, BARC 1357, BARC 2649, DF-1, and TAAS-1. Amplification with the first set of primers was not achieved despite varying annealing temperatures, DNA concentrations, and MgCl₂ concentrations. Therefore, a second set of primers was designed. Amplification with a hot start was attempted concurrently for both sets of primers. A dominant product, approximately 900 base pairs in length, was obtained at 1.0, 1.5, and 2.5 mM MgCl₂ concentration with the first set of primers. The second set of primers produced a dominant product only with the 2.5 mM MgCl₂ concentration; the product was approximately 900 base pairs long. This has led to the assumption that perhaps the spacer region is longer than the approximately 300 base pairs previously estimated. The MgCl₂ concentrations will be altered once again and if the same size product is obtained, then the five remaining samples will be PCR amplified. Following amplification the products for each of the six samples will be purified on an agarose gel, excised, and then sent to the appropriate facilities for sequencing. After the sequences are obtained, they will be analyzed by alignment with the sequences used for primer development to determine if the 16S-23S spacer region has been amplified. If the spacer region has indeed been amplified, the sequences will then be compared to determine if the spacer region is species specific within the Group VIII spiroplasmas.
Literature Cited


Appendices
Appendix A

Screening Test for Spiroplasmas

I. Materials

1. Microtiter Plate
2. Culture (antigens)
3. 12 Screening Cocktails of 1:10 antiserum of each serovar

(1) B31, PPS1
(2) EA-1, DF-1 TAAS-1, BARC, 1357, BARC 2649
(3) EC-1
(4) Tab4C, HYOS-1
(5) TN-1
(6) TG-1
(7) TABS-2
(8) TAUS-1
(9) BARC 4906, BARC 4907, BARC 4908
(10) BARC 4886
(11) BARC 4900
(12) BARC 4903, TALS-2

II. Procedure

1. Check culture to be screened to be certain that the spiroplasma cells are free of deformation.
2. Label 12 wells along the x-axis with the numbers 1-12. Label the y-axis with the appropriate culture number.
3. Add 20 \( \mu \)l of the appropriate screening cocktail to the microtiter well whose number corresponds to the number on the cocktail.

4. Add 20 \( \mu \)l of the appropriate culture to each well.

5. Cover the plate and allow it to react for 30 minutes at room temperature before beginning the readings.

6. Make slides and observe cells for deformation at 1200 magnification with dark field illumination.
Appendix B

Serocloning

(Protocol based on R. F. Whitcomb personal communication)

50μl of culture

50μl of antisera 1:10 (single or combined screen)

1. Mix, after one hour add about one ml of medium.

2. Gently pass through 0.45μm filter without foaming and examine microscopically for estimate of concentration.

3. Serially dilute by 1/10, i.e. $10^{-1} = 1.8$ ml medium and 0.2ml of filtered culture (or 0.9 ml medium and 0.1 ml culture).

For very low concentrations, do $10^{-1}$ through $10^{-5}$ tubes.

For short spiroplasmas in heavy concentration, do $10^{-1}$ through $10^{-11}$ tubes.
Appendix C

Triple Cloning Spiroplasmas

(Protocol based on Whitcomb et al. 1986 and R. F. Whitcomb personal communication)

1. Select cultures to be cloned carefully.
2. Resuscitate chosen culture, and serially pass it until the spiroplasmas exhibit morphology free of deformation.
3. When the culture has stabilized and generates 200 helices per field or so, it is ready to be cloned.
4. If the culture has serological reactivity, it is a good idea to do a confirmatory deformation titer.

Prepare dilution series for cloning.

The first part of the dilution series will be in 3.0 ml snap-cap tubes, and the final tubes will be large sterile centrifuge tubes.

For dilutions of: $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$, add 2.7 ml of broth medium to 3.0 ml tubes.

For dilutions of: $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$, $10^{-10}$, and $10^{-11}$, add 18 ml of broth medium.

Filtration

Filter the culture through a 0.45μm filter.
Dilutions

Now prepare the dilution series. Make tenfold dilutions (0.3 filtrate to 2.7 ml of medium, and then serially dilute). Specifically, transfer 0.3 ml filtrate by a sterile 1 ml pipette, discard pipette, mix with vortex mixer for 30 seconds, and then transfer 0.3ml to next dilution. When you get to the $10^{-6}$ tube, add 2 ml of diluted culture (using a sterile 2ml pipette) from the $10^{-5}$ tube to the 18 ml of medium in the centrifuge tubes.

[It is vital to change pipettes after each transfer. If pipettes are not changed after each transfer, "Pipettenfehlen" will occur in which organisms are retained on the pipette surface, and the endpoint goes out (irregularly) one, two, or maybe even three tenfold dilutions farther than it ought. You could miss the endpoint, and the data are no good for analysis.]

Preparing slides

The purpose of this step is to do an enumeration, so the number of organisms in the filtrate can be imperically determined. This step is usually done after loading the microtiter plates, if only one person is doing the cloning. Obviously it is best to do it as soon as possible. Prepare slides of the filtrate; examine microscopically to determine the number of organisms per field. Then prepare four slides of the filtrate, or of a dilution of the filtrate, so there are no more than 25 helices per field. Usually the filtrate itself, or the $10^{-1}$ dilution is used but if it is Group VIII, it might be necessary to use the $10^{-2}$ dilution. Carefully place, without bubbles, a 22mm square no.1 cover slide over a 5μl drop of culture. Record quantity of drop, size of cover glass, and magnification used for
counting. Be sure to record what dilution is counted. With a hand-counter, count 25 fields of each of the four slides for helix number and then determine an average number of helices per field for the prep.

**Loading the microtiter plates**

To load the microtiter plates, use 250µl pipette tips with aerosol filters and an 8-channel pipettor set to 200µl. Place the spiroplasma sample in a sterile trough and distribute the sample into a 96 well microtiter plate. Usually a few wells are empty (maybe 88 or 89 wells). The plates should be loaded in reverse sequence starting with the 10⁻¹¹ plate.

**Sealing the plates**

The plates have to be covered with a semitransparent plastic to keep them from drying out. There is an expensive lab product designed for this, but transparent 3-M Tape ® will also work.

**Storing the Plates**

The plates are stacked and incubated at 30°C.

**Examining the Plates**

The plates should be examined daily for color change in any of the microtiter wells. The medium used in the cloning procedure contains phenol red that will change from red to yellow when cell growth is present. It generally takes 4-5 days for the first wells to exhibit color changes. The 10⁻⁵ plate should exhibit color changes first; almost certainly
all of the wells will exhibit spiroplasma growth. The other plates will undergo color changes in turn, but as soon as below 100% of the wells on the plates exhibit growth, the wells exhibiting growth will more and more tend to be initiated by single helices; of course, these will all exhibit color changes at the same time, so there will be a final flood of growth, and then darkness.

Selection of Clones

This is an art. It is necessary to watch the plates, and pick at least one clone early on to be sure you don’t miss out altogether. Obviously, it is ideal to have one plate with only one clone. A well most remote from other wells exhibiting growth, on the plate that contains the fewest wells exhibiting spiroplasma growth should be selected. To pull the well contents, pierce the plastic with a sterile dental tool, withdraw well contents with a pipettor, and inoculate a tube of fresh medium.

Counting the Wells

It is good to keep a daily record of number of wells exhibiting a color change. Also keep track of the first tubes of the series; that will alert you when to expect the wells to change color. A point will quickly be reached where everything that will exhibit growth has done so. Then quit. Don’t wait for a final straggler. If the spiroplasma has struggled to grow, there is something abnormal about it. Maybe it is not even a spiroplasma, it could be a mutant that grows slowly. Neither of those things would be welcome.
*Triple-cloning*

Obviously, this procedure must be repeated twice, for a total of three clonings. Always save the clones, all of them, until the final clone is at hand. When the final clone is at hand, the early candidates can be discarded. Next, concentrate on growing up and lyophilizing the final. It is a good idea to DF or screen the final clone, to see if it reacts the way the primary culture did. Be sure to lyophilize a lot of vials of the final product.
Appendix D

DNA Extraction and Purification for 16S-23S rRNA Sequencing

(Procedure modified from Duret et al. 1999 and Molecular Biotechniques Notebook)

I. Materials

1. STE Buffer (100mM NaCl, 10mM Tris-HCL [pH 8.0], 1mM EDTA)
2. 10% Sodium Dodecyl Sulfate
3. Phenol
4. Chloroform-Isoamyl
5. 95% Ethanol
6. 70% Ethanol
7. Sodium Acetate
8. Microcentrifuge tubes
9. Medium

II. Methods

1. Grow 20 mls of appropriate culture.
2. Examine culture by dark field microscopy (5µl under 22mm cover glass). If spiroplasmas exhibit good morphology and are prolific, then proceed with extraction and purification immediately.
3. Place 1.5 mls in each of 6 microcentrifuge tubes.
4. Collect cells by centrifugation. Spin at 12,000-14,000 x g for 5 minutes. (If using a Fisher Scientific Marathon 21K/BR centrifuge, spin at 12,200 RPM and 4°C).

5. After centrifugation, pour off supernatant being careful not to disturb the pellet. Repeat process until all of culture has been harvested.

6. Resuspend each pellet in 600μl of STE buffer.

7. Lyse cells by adding 67μl of 10% SDS per tube.

8. Heat the lysate at 67°C for 15 minutes.

9. Treat lysate with 2.3μl (10mg/ml concentration) of RNase for 30 minutes at 37°C.

10. Add cold phenol in an amount equivalent to 1/2 of the total volume of the tube to each tube. Mix gently and pulse spin.

11. Add an equivalent amount of chloroform-isoamyl (24:1 concentration) to each tube and spin at 12,000-14,000 x g for 10 minutes.

12. Remove the top layer of each tube after centrifugation and place in new, sterilized microcentrifuge tubes. (If particles remain in the top layer of the solution then the chloroform-isoamyl precipitation can be repeated.)

13. Add 0.1 volumes of 3M Sodium Acetate to each tube and mix thoroughly by vortexing.

14. Add 2.0 volumes of ice-cold 95% ethanol to each tube and mix thoroughly. Allow to sit at room temperature or on ice for 10 minutes.
15. Spin at 12,000-14,000 x g for 10 minutes. Be sure that the hinge on the microcentrifuge tube is pointing out because you might not be able to see the pellet after the spin.

16. Carefully decant the supernatant and remove the last traces with a micropipettor. DO NOT DISTURB THE PELLET!

17. Add 0.5-1.0 ml of 70% ethanol to each tube and gently rock to rinse the pellet. Centrifuge at 12,000-14,000 RPM for 5 minutes.

18. Remove the supernatant as described in step 16. Set the tubes on benchtop for at least an hour to ensure that the pellets are completely dry.

19. Dissolve each pellet in 50µl of TE buffer and then combine the contents of the tubes into one tube.

20. Run the products out on an agarose gel at 100V for 45 minutes. Load 8µl of λ Hind III in well one as the standard. Load 10µl of sample plus 2µl of loading dye in well 2. The product in well 2 should be 23 KB.