DISTRIBUTION OF P58-LIKE GENES

AMONG MOLLICUTES

By

ANANDA SIVA CHANDRASEKARAN

Bachelor of Science

Bharathiar University

Coimbatore, India

1992

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July 2000

DISTRIBUTION OF P58-LIKE GENES

AMONG MOLLICUTES

Thesis Approved:

Ulrich Melcher

Thesis Advisor

Jacqueline Fletcher

Richard C Essenberg

Robert F Mott

Alfred Larkin

Dean of the Graduate College

ACKNOWLEGMENTS

I wish to thank my advisors, Dr. Ulrich Melcher and Dr. Jacqueline Fletcher for encouraging, supporting and guiding me throughout this study. I also appreciate my advisors patience for carefully correcting my thesis and providing useful suggestions to improve the quality of my thesis. I sincerely appreciate my other committee members, Dr. Richard Essenberg and Dr. Robert Matts for their encouragement and guidance. I would like to thank Dr. John Cushman for providing me numerous suggestions.

I wish to express my sincere gratitude to Dr. Astri Wayadande and Dr. Michael Berg for their assistance, encouragement and friendship. I would also like to give my appreciation to Ann Williams for her assistance.

I would like to thank fellow lab members for their suggestions and friendship. Thanks also go to my parents and my family for their support and encouragement. Finally, I would like to thank the Division of Agricultural Sciences and Natural Resources and the Department of Biochemistry and Molecular Biology for financial support during these two years of study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

A+T	adenine plus thymine
AY	aster yellows
BLTVA	beet leafhopper transmitted virescence agent
bp	base pair
cDNA	complementary DNA
cfu	colony forming unit
dNTP	deoxy nucleoside triphosphate
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
EF-Tu	elongation factor Tu
G+C	guanine plus cytosine
HAP	hydroxyapatite
hsp70	heat shock protein 70
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase pair
kDa	kiloDalton
Mb	Megabase pair
mRNA	messenger RNA
µM	micro molar

mm	millimeter
NADH	nicotinamide adenine dinucleotide, reduced form
ng	nanogram
nm	nanometer
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
PHYLIP	phylogeny inference package
rDNA	ribosomal DNA
SDS	sodium dodecyl sulphate
SRO	sex ratio organisms
ss	single stranded
TE	Tris-EDTA
u	unit
UV	ultra violet
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Chapter I

Introduction and Literature Review

The Class Mollicutes is comprised of prokaryotes lacking cell wall components, and includes mycoplasmas, spiroplasmas, phytoplasmas, ureaplasmas, acholeplasmas, anaeroplasmas and asteropleplasmas. The absence of a cell wall is responsible for various unique properties of mollicutes that distinguish them from other bacteria and contribute to their placement in a separate class (1). Spiroplasmas share the properties, including reduced genome size, low G+C (guanine plus cytosine) %, resistance to the antibiotic penicillin, 'fried egg' shaped colonies on solid medium, ability to pass through 220 nm filters and requirement of sterols (in most members of mollicutes, not all), with other members of mollicutes. However, spiroplasmas differ from other members in having a helical shape and exhibiting different types of motility in liquid media (2,3).

Spiroplasmas (and mollicutes in general) have evolved from low (G+C) gram-positive bacteria by genome reduction (4, 5). The genome sizes of spiroplasmas vary between about 800 and 2200 kilobase pairs (kb), while the closely related mycoplasmas have the smallest genomes (about 500 kb) of all living organisms. In spiroplasmas the (G+C) content is about 24 to 26 mol %. Probably due to high (A+T) content, spiroplasmas are more sensitive than other prokaryotes to UV irradiation, because of the

possibilities of thymidine dimerization (4, 25). Another interesting genetic feature of spiroplasmas is that the TGA codon is read as tryptophan instead of as a stop codon (6). This makes it difficult to use spiroplasma genes in *E. coli* cloning and expression systems. However, this problem can be overcome by using spiroplasmas as host cells since they can provide an efficient cloning and expression host (7).

'Mycoplasmalike' helical organisms initially cultured from stubborn-diseased citrus plants and from corn stunt-diseased plants were later called spiroplasmas (11, 12). Spiroplasmas inhabit plants, bees, ticks, flies, wasps, beetles and other arthropods; some spiroplasmas are pathogenic to plants and arthropods. Experimentally, tick and honeybee associated spiroplasmas are pathogenic to vertebrates (8, 9, 10). Both pathogenic and non-pathogenic spiroplasmas are found to be associated with arthropods in some part of their life cycle (3). After their discovery in the early 1970's a plethora of spiroplasmas were isolated from insects and plants; yet, to date, only three *Spiroplasma* species are identified as phytopathogens. *Spiroplasma citri* causes citrus stubborn disease and horseradish brittle root disease (13), *Spiroplasma kunkelii* is associated with corn stunt disease (14) and *Spiroplasma phoeniceum* infects periwinkle plants (in which the symptoms of the disease are similar to those of *S. citri* infection)(15). Among the non-phytopathogenic spiroplasmas, *S. melliferum* and *S. apis* inhabit honeybees and cause the disease spiroplasmosis or May disease (16). *S. mirum,* transmitted by ticks, experimentally causes cataracts and death in suckling rats (9). Whether it is naturally pathogenic to mammals is an intriguing question. Spiroplasmas associated with *Drosophila* kill the male progeny and hence are called sex ratio organisms (SRO) (17). Spiroplasmas are also known to be associated with mosquitoes (18). Other spiroplasmas

(*S. floricola*) are known to be present on the surface of certain flowers, probably as epiphytes (19).

Spiroplasma citri

S. citri is widely used as a model organism to study and understand more about mollicutes because it is cultivable (while some other mollicutes like phytoplasmas cannot be cultivated in media) and it grows faster than the other phytopathogenic spiroplasmas (*S. kunkelii* and *S. phoeniceum*). *S. citri* is a fastidious organism that needs complex ingredients in culture media for its growth; several factors like media composition, pH, temperature and osmolarity affect its growth (20). Like other mollicutes *S. citri* is resistant to penicillin, but it is very sensitive to tetracycline (28). Though most *S. citri* strains isolated are helical in shape, there are reports of the isolation and characterization of non-helical *S. citri* strains (21). The strains/species of spiroplasma are usually classified based on (G+C) %, serological and biochemical properties and also by DNA-DNA homology. The species *S. citri* and *S. kunkelii* are serologically closely related to each other, but based on DNA-DNA homology they have also been classified as different *Spiroplasma* species (22, 23). The DNA-DNA homology should be less than 70% to classify two prokaryotes as separate species; *S. citri* and *S. kunkelii* showed less than 70% homology in all the cases tested (14, 24).

S. citri contains many extrachromosomal elements, either as replicative forms of viruses or as plasmids. The extrachromosomal pattern varies with different strains of *S. citri*. Four different viruses, SpV1, SpV2, SpV3 and SpV4, are known to infect *S. citri*.

The replicative forms are present as extrachromosomal elements in the infected *S. citri* (39).

S. citri propagates in sieve cells of plant phloem, within which it is carried to different plant parts. In plants *S. citri* causes several disease symptoms, such as chlorosis, leaf mottling, tissue necrosis, yellowing, stunting and reduced size of leaves, flowers and fruits (26). Phytopathogenic spiroplasmas are transmitted from plant to plant by phloem-feeding leafhoppers. The transmission of spiroplasmas depends upon host-pathogen specificity. Particular leafhoppers may carry one or more different spiroplasmas and a particular *Spiroplasma* species is transmitted by one or more different leafhoppers (29). The natural vector for *S. citri* within the United States is the beet leafhopper, *Circulifer tenellus*. To be transmitted to new plant host after being ingested by the leafhopper, *S. citri* crosses the gut wall, multiplies in the hemocoel and passes into the salivary glands. The spiroplasmas multiply further in the salivary gland cells before being introduced into new plant phloem (27). The hemolymph occupying the coelomic cavities in arthropods and the phloem fluid in plants serve different transport functions pertaining to cellular metabolism. When the two fluids were compared, they were somewhat comparable with respect to ionic, carbohydrate, organic acid, lipid and protein compositions (28). Hence the hemolymph may serve as an ideal environment for *S. citri* multiplication inside the leafhopper.

Electron microscopic studies revealed that when natural vectors of *S. citri* and *S. kunkelii* were fed with their respective spiroplasmas, the phytopathogenic spiroplasmas could penetrate the gut and enter the hemocoel. When the non-phytopathogenic spiroplasmas like *S. floricola* were tested, however, they failed to cross the gut epithelium

of the vector (30). Whenever *S. citri* reaches its highest titer in the leafhopper *C. tenellus,* there is a maximum rate of *S. citri* transmission to host plants. During its multiplication inside the leafhopper *S. citri* loses its helical morphology and becomes spherical. In rare cases, phytopathogenic spiroplasmas may be pathogenic to their vector leafhoppers (31). Ultrastructural studies of *C. tenellus* salivary glands revealed that spiroplasmas have to cross three barriers (basal lamina, basal plasmalemma and apical plasmalemma) before being ejected in the saliva. The basal lamina of leafhoppers has no apparent pores or channels, and it is not known how spiroplasmas pass through this barrier (32).

It is hypothesized that leafhopper transmission of spiroplasmas is mediated by recognition of specific spiroplasma membrane proteins, a scenario that is consistent with electron microscopic observations following the incubation of *S. citri* with cultured leafhopper cells. Micrographs suggested that receptor-mediated endocytosis is involved in the invasion of *S. citri* into its vector (33). The mediation of mollicute surface proteins in the adherence to the host cells is a widely accepted concept. In *Mycoplasma pneumoniae* the P1 adhesin is present in a cluster at the tip of a stalk that attaches the microbe to the human cells, whereas in avirulent strains P1 is distributed all over the surface (27). In addition to P1 several other surface proteins of *M. pneumoniae* take part in the adherence process; P200 of *M. pneumoniae*, characterized as an adherence protein, shares some features with *M. pneumoniae* accessory proteins like HMW 1 and HMW 3 (35, 36). In *Mycoplasma gallisepticum*, the presence of multiple copies of a gene (multigene family) encoding the major surface protein pMGA has been reported (37). Many mycoplasma adhesins are members of multi-gene families, and the various

versions of these genes contribute to the mollicute's ability to avoid the host's immune recognition.

Identification of surface proteins in *S. citri* also has been reported (34), however, the involvement of these proteins in the interaction with leafhopper gut receptors is not fully understood. A microtiter plate adherence assay developed to study the binding of a *S. citri* transmissible line to cultured cells of *Circulifer tenellus* showed that a 89 kDa *S. citri* surface protein may be involved in the adherence. The adherence was tested by disrupting surface-exposed proteins with proteases and pre-incubating them with antibodies against individual *S. citri* surface proteins (38).

S. citri showed genetic variations after long-term maintenance under different conditions. The parent line, BR3-3X, when maintained by plant-to-plant grafting without involvement of its natural vector, lost its transmissibility, giving rise to a new line designated BR3-G. When BR3-3X was maintained by plant-to-plant transmission with the help of its natural vector, the transmissibility persisted, and the resulting line was designated BR3-T. The physical maps of BR3-T and BR3-G are different from that of the parent line BR3-3X, but the genetic orientation of the parent line is conserved in the transmissible line BR3-T and not in BR3-G. A large chromosomal inversion and deletions of about 10 kb at both the ends of inversion were observed in the non-transmissible line BR3-G (40). An open reading frame (ORF), P58, within the 10 kb deletion region had amino acid sequence similarity with at least two mycoplasma adhesins (41). The gene encoding P58 was found to be present in multiple copies (27). The presence of multigene families in the microbes (spiroplasmas) with reduced genomes is intriguing. It is possible to speculate, based on P58's sequence similarities with

mycoplasma adhesins and the presence of a potential transmembrane segment, that P58 is an adhesin of *S. citri*. The characterization of all the genes of the P58 multigene family might provide some clues to the *S. citri*-vector interactions.

The primary objective of this study was to analyze the presence and diversity of P58-like genes among mollicutes. Chapter I further discusses P58 genes and their presence in different strains of *S. citri* and in different species of spiroplasmas, mycoplasmas and phytoplasmas.

Circulifer tenellus Phylogeny

The advent of DNA sequencing methods in the early 1970s was one of the important milestones that formed the basis for the development of phylogenetic analyses using molecular characters (42). Mitochondrial DNA, which is widely used as a phylogenetic marker, has the same fundamental role in all the eukaryotes, i.e., it encodes a limited number of proteins and RNAs for the functional mitochondria. The mitochondrial DNA sequences of NADH dehydrogenase and cytochrome c oxidase, and mitochondrial DNA-specified components like large and small subunits of ribosomal RNA, are highly conserved among living organisms and proved to be effective subjects for phylogenetic analysis. In addition, the nuclear gene elongation factor EF-Tu gene, *tuf*, and the heat shock protein gene, *hsp70*, are also used as phylogenetic markers (43, 44).

Phylogenetically useful genes should meet certain criteria. The gene must be universally distributed, the gene product must have the same function in every organism tested, the gene must be readily isolated and sequenced and the gene base sequence must show clocklike behavior, accumulating small random base changes at a consistent rate that allows changes over long genealogical times to be preserved (42). In the study reported here, 16S rDNA was used as a phylogenetic marker to analyze the phylogeny of the leafhopper, *Circulifer tenellus*.

C. tenellus, commonly known as the beet leafhopper, is the primary natural vector of *S. citri* in the United States. The beet leafhopper is also a vector of beet curly top virus; it was the first reported leafhopper vector of a plant virus in North America. *C. tenellus* is a sun-loving insect that breeds in arid and semi-arid regions. Since 1896, the genus or species name of the beet leafhopper had at least 10 revisions until it was designated as *C. tenellus* in 1957 (45).

C. tenellus is classified in the family Cicadellidae and subfamily Deltocephalinae. According to the latest classification (46) *C. tenellus* is placed in the tribe Opsiini, within the subfamily Deltocephalinae. As said above, the second objective of this study was to determine the phylogenetic relationships among selected genera of leafhoppers in the subfamily Deltocephalinae. Chapter III deals more with *C. tenellus* phylogeny.

Chapter II

Distribution of P58-like Genes Among Mollicutes

Introduction

Spiroplasmas, wall-less prokaryotes, probably evolved by genome reduction from low (G+C) Gram-positive bacteria. Spiroplasmas belong to the Class Mollicutes and differ from the other members of the Class by their characteristic helical shape and motility in liquid media (3). The genome sizes of spiroplasmas are small and vary between 800 and 2200 kb. In spiroplasmas the nucleotide triplet TGA is not a stop codon, coding instead for tryptophan. Spiroplasma genes containing TGA codons are difficult to express or only partially expressed in *E. coli* strains (6).

Spiroplasma citri is the causative agent of stubborn disease in citrus and brittle root disease in horseradish plants. In the United States it is transmitted from plant to plant by its natural vector the beet leafhopper, *Circulifer tenellus*. When acquired by its natural vector, *S. citri* can penetrate the gut and enter the insects' hemocoel (30). The insect hemolymph, or circulating fluid, is an ideal environment for *S. citri* multiplication. From the hemocoel *S. citri* enters the insect salivary glands before being ejected into a new plant host during phloem feeding (31). Leafhopper transmission of *S. citri* is probably mediated by recognition of specific *S. citri* membrane proteins; a receptor-

mediated endocytosis is hypothesized for the invasion of *S. citri* (33). In a microtiter plate assay used to study the binding of *S. citri* to cultured leafhopper cells a protein of about 89 kDa was implicated in the adherence. The spiroplasmas' binding to tissue cultivated leafhopper cells was impaired after surface-exposed proteins were treated with proteases, and concomitantly, an 89 kDa protein band in the microbe's protein profile was eliminated or greatly reduced in intensity. When *S. citri* having disrupted surface proteins was grown in liquid medium it regenerated the disrupted P89 and the adherence to leafhopper cells was restored. So P89 may be one of the proteins responsible for adherence within the leafhoppers (47).

Transmission ability of *S. citri* changes when strains are maintained long-term under different conditions. When the parent *S. citri* line, BR3-3X, was maintained by plant-to-plant grafting the result was the eventual loss of its transmissibility (giving rise to the derivative non-transmissible line, BR3-G). The *S. citri* derivative line BR3-T was obtained by passing the parent line, BR3-3X, repeatedly from plant-to-plant via its natural vector, *C. tenellus*. The molecular analysis of non-transmissible BR3-G revealed many interesting facts. The genome size of BR3-G, 1.87 Mb, was bigger than that of the parent line, BR3-3X (1.60 Mb). In the BR3-G genome a large segment of chromosome had been inverted and at each end of the inverted region a fragment of about 10 kb had been deleted. One of the 10 kb fragments missing in BR3-G had four complete open reading frames encoding 58, 12, 54 and 123 kDa polypeptides. Though extensive rearrangement was also seen in the transmissible line BR3-T, the genetic organization of BR3-3X was maintained in BR3-T (40, 27).

A protein of about 58 kDa (P58), encoded by the gene located in the 10 kb deleted region, was shown to be a surface-exposed protein having limited amino acid sequence similarity with the adhesins of *Mycoplasma hominis* and *M. genitalium* (41). Southern blots of BR3 lines probed with P58 showed three copies of P58 genes in BR3-3X and BR3-T and two copies in BR3-G. The loss of one copy of the P58 gene in BR3-G may not be associated with loss of insect transmissibility, because the undeleted copies in BR3-G produce proteins recognizable by α-P58 antibodies, as shown by the western blots. The microtiter plate adherence assay showed that both BR3-G and BR3-T lines adhered to cultured *C. tenellus* cells to the same level (27, 41). As P58 is a multi-gene family member, it is possible that P58 functions as a spiroplasma adhesin. Many mycoplasma adhesins are multi-gene family members, and all are involved in both the mycoplasmas' evasion of their host's immune system and their anchoring to the host cells. Whether the P58 is a *S. citri* adhesin is still unclear. The characterization of all the copies of P58 genes may provide more clues.

Determination of the presence and number of copies of P58-like genes in different strains of *S. citri*, and in different species of spiroplasmas and other mollicutes, should reveal the diversity of this gene. All the spiroplasmas, whether phytopathogens or non-phytopathogens, are associated with arthropods in some part of their life cycle, and their association may be mediated by surface-exposed proteins. Identification and characterization of P58-like genes in different spiroplasmas will broaden the knowledge about P58 genes. In this study, in addition to different strains of *S. citri* and different spiroplasma species, phytoplasmas (closely related uncultivable phytopathogens) and mycoplasmas were also included. Different spiroplasmas were chosen to represent

diverse spiroplasma habitats; for example, *S. floricola* is an epiphyte on flower surfaces, *S. apis* and *S. melliferum* inhabit honeybees, *S. syrphidicola* is found in syrphid flies, and *S. kunkelii*, a phytopathogen, causes corn stunt disease.

Materials and Methods

Spiroplasma and Mycoplasma Cultivation

Spiroplasma citri strains (BR3-3X, R8A2, MDHR3, M200H, GO4, B105, Beni Mellal, Aceratagallia) and certain *Spiroplasma* species (*S. floricola* and *S. melliferum*) (Table 1) were cultured in a rich liquid medium, LD8 (48). *Spiroplasma kunkelii* was cultured in C3-G liquid medium (1.5% PPLO broth (w/v), 12% sucrose (w/v), 0.06 M HEPES (N'-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer at pH 7.4 and 20% horse serum (v/v) (49). Initial 5 ml cultures were maintained at 32°C and grown to log phase, approximately 10^8 cells/ml, as determined by direct counts using dark field microscopy. After checking for contamination under dark-field microscopy the entire volume was inoculated into 100 ml liquid medium and maintained as described for the 5 ml cultures. *S. apis, S. syrphidicola* and *S. corruscae* bacterial pellets were kindly provided by Dr. Gail Gasparich (Towson University, Baltimore, MD).

Mycoplasma species (*M. canis*, *M. felis*, *M. hyorhinis* and *M. bovis*) were obtained from Dr. Ronald Welsh at Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK and cultured in Friis liquid medium (50) by maintaining the cultures at 37°C under reduced oxygen or oxygen-free environments. The cultures were grown until the pH indicator in the liquid medium turned pink to pale orange. The cells were harvested before the medium turned yellow to avoid killing the cells.

The cultures were harvested by centrifugation at 12,000xg for 30 minutes. The pellets were the source of genomic DNA.

Species	Geographical area	Source	Isolated by/ obtained from
Spiroplasma citri strains			
BR3-3X	Illinois	Horseradish	J. Fletcher
R8A2	Morocco	Citrus	P. Saglio/R. E. Davis
MDHR3	Maryland	Horseradish	R. E. Davis
M200H	Experimental mutant of R8A2	Citrus	R. E. Davis
GO4	California	Citrus	G. Oldfield
B105	California	Citrus	R. Whitcomb
Beni Mellal	Israel	Citrus	J. M. Bove/ P. Markham
Aceratagallia	California	Leafhopper	R. Whitcomb
S. kunkelii	Mexico	Corn	K. Maramorosch/ R. E. Davis
S. melliferum	Maryland	Honeybee	T. B. Clark/ R. E. Davis
S. floricola	Maryland	Tulip poplar	R. E. Davis

Table 1. Selected *Spiroplasma* species and strains used in this study

Genomic DNA Isolation

Spiroplasma and mycoplasma bacterial pellets were suspended in 5 ml of Doyle buffer (1.4 M NaCl, 2.5% CTAB (hexadecyltrimethylammonium bromide)(w/v), 100 mM Tris-Cl pH 8.0 and 20 mM EDTA). The suspended cells were incubated at 55°C for 15 minutes to dissolve them completely in the buffer.

Genomic DNA was isolated by phenol: chloroform: isoamyl alcohol (25: 24: 1) extraction (once) followed by chloroform: isoamyl alcohol extraction (at least twice or until the interface between the chloroform and the aqueous phase disappeared) (51). The extracted DNA was precipitated by adding an equal volume of isopropyl alcohol and maintaining the mixture at –20°C overnight. The DNA was pelleted by centrifugation at 10,000xg for 25 minutes at 4°C. The pellet was vacuum dried and dissolved in 1 ml of Tris-EDTA (10 mM Tris-Cl and 1mM EDTA, pH 8.0) buffer with RNase, and the DNA was aliquoted and stored at –20°C.

Phytoplasma (aster yellows strains AY1 and AY2) and the beet leafhopper transmitted virescence agent (BLTVA, strains UCD and GOR)) DNAs were kindly provided by Dr. Mary Shaw (New Mexico Highlands University, Las Vegas, NM).

Restriction Digestion and Gel Electrophoresis

About 3 µg of spiroplasma, mycoplasma or phytoplasma DNA were digested with *Eco*RI (20 units) at 37°C in *Eco*RI buffer (REACT 3 buffer, GIBCO BRL) for 4 hours. Gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol (w/v)) was added in the ratio 1:5 to the digested mixture and loaded into an agarose gel (0.75% gel in 1x TAE (0.04 M Tris-acetate and 0.001 M EDTA)) to separate DNA

fragments. Electrophoresis, at 100 volts of constant current, was terminated when the gel-loading buffer crossed just over two thirds of the total gel distance (Hoefer submersible agarose gel electrophoresis unit, model HE33, Hoefer Scientific Instruments). After the gel was stained in ethidium bromide (1μg/ml) for about 45 minutes the DNA was blotted onto membranes as described below. *S. citri* strain BR3-3X DNA was used as the control throughout this study.

Southern Blotting

Ethidium bromide stained gels were rinsed with nanopure water and incubated in about 10 gel volumes of 0.25 M HCl for 20 minutes to partially depurinate the DNA fragments. The gels were rinsed again with nanopure water and incubated in about 10 gel volumes of denaturation solution (1.5 M NaCl and 0.5 M NaOH) for 20 minutes to convert the DNA to single strands suitable for hybridization. The pH of the denatured gels was adjusted to below 9.0 by incubating twice in about 10 gel volumes of neutralization solution (1.5 M NaCl and 0.5 M Tris-HCl pH 7.0).

The neutralized gels were rinsed with nanopure water and placed (gel bottom facing up) on 3 MM filter paper, supported on a glass plate over a reservoir containing transfer buffer (20x SSC (3 M NaCl and 0.3 M sodium citrate pH 7.0)). The ends of the filter paper were immersed into the reservoir. The area of the 3 mm filter paper outside the gel was covered with parafilm to prevent short-circuiting of transfer buffer. Nylon membrane (Hybond-N, Amersham) of gel size was submerged in nanopure water for 5 minutes and placed over the gel. The surface of the membrane was smoothed with a glass rod to remove any air bubbles in between the gel and the membrane. The surface of

the membrane was flooded with 20x SSC, and five sheets of 3 MM filter paper cut to the same size were placed over the membrane. Paper towels cut to the size of the membrane were stacked over the 3 MM filter papers to a height of about 4 cm. A glass plate and a weight of about 0.5 pound were placed over the paper towels. The setup was left at room temperature overnight (about 12 hours), undisturbed.

After transfer the positions of the wells were marked with a ballpoint pen by piercing through the wells to ensure the orientation of the membrane. The membranes separated from the gels were rinsed with 2x SSC and placed on a filter paper. The membrane was exposed to UV light (Stratalinker, Stratagene) to cross-link the DNA to the membrane, which was stored at room temperature until used (51, 52).

Hybridization Probe

The probe used in the hybridization was a 570 bp DNA fragment located at the 3' end of the already-characterized P58 gene (41), chosen because its encoded amino acid sequence was similar to adhesin sequences. The primers used for PCR amplification of the probe were 5'-GCTGAATATACACAC-3' and 5'-GTCCACAAACAGAC-3'. The PCR reaction mix contained 200 µM of deoxynucleoside triphosphates (Promega), 1 µM of each of the primers, 10mM Tris-HCl pH 8.8, 3.5 mM of $MgCl_2$, 25 mM KCl, 10 units of *Taq* DNA polymerase (Promega) and 2 µl of template P58 DNA.

The PCR amplified probe was purified on a preparative agarose gel (1%). A band of 550 bp was cut out of the gel and DNA was extracted from the gel using silica matrix.

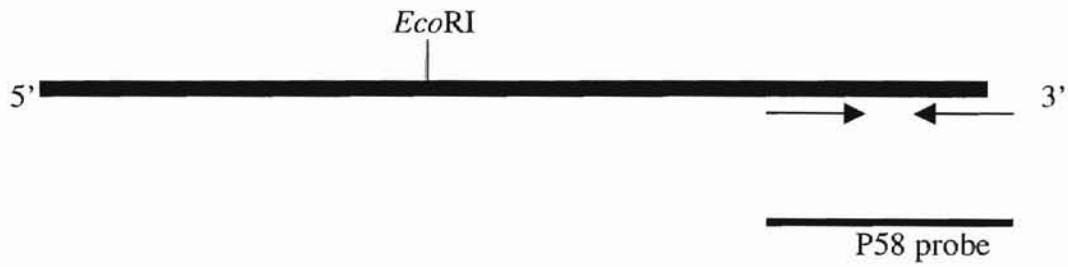

Fig. 1 P58 gene with *Eco*RI site.
P58 probe represents the 570 bp at the 3' end of the P58 gene.

In a microfuge tube, two-gel volumes of 6 M guanidine-thiocyanate were added to the gel slice and melted at 55°C for 3 minutes. To the melted gel, 15 µl of silica suspension was added and incubated at 55°C for 10 minutes with shaking from time to time (silica suspension preparation - 10 g of silica was added to 100 ml of phosphate-buffered saline, mixed well and allowed to stand for 2 hour; the supernatant containing fine particulates was removed and the process was repeated; the silica pellet was resuspended in 6 M guanidine-thiocyanate at 100 mg/ml working concentration.). The DNA, now bound to the silica, was pelleted and washed twice with wash buffer (50 mM NaCl, 10 mM Tris-Cl pH 7.5, 2.5 mM EDTA and 50% v/v ethanol). The pellet was vacuum dried and suspended in 30 µl of nanopure water. The DNA was recovered from the silica by incubating at 55°C for 5 minutes and pelleted; the supernatant containing the DNA was recovered and precipitated using 2.5 recovered DNA volumes of 95% ethanol. The DNA precipitate was dissolved in 20 µl of TE buffer and stored at –20°C for later use in labelling.

Oligolabelling and Hybridization

The labelling reaction was carried out in a total volume of 50 µl. The probe was heated in boiling water for 3 minutes and immediately placed in ice to denature the probe; about 30 ng of denatured probe was used for radioactive ^{32}P labelling. To the denatured probe 10 µl of reaction mix (Oligolabelling Kit, Amersham Pharmacia Biotech), 50 µCi of α-^{32}P dCTP (Sp. Act. ~3000 Ci/mmol, ICN Biomedicals) and 1 µl of Klenow fragment of DNA polymerase I (Amersham Pharmacia Biotech) were added and the reaction was carried out at 37°C for 45 minutes.

The membrane was pre-hybridized in 15 ml Church buffer (0.5 M Na_2PO_4 pH 7.2, 7% sodium dodecyl sulphate (SDS) and 1 mM EDTA) containing denatured salmon sperm DNA (100 µg/ml) at 55°C in a revolving hybridization chamber for about 1 hour. The labeled reaction mix was added to the pre-hybridized membrane without changing the buffer. Hybridization was carried out in the same buffer overnight at 55°C.

Volumes of 30 ml of wash buffer (40 mM Na_2PO_4 pH 7.2 and 0.1% SDS) were used for washing the hybridized membrane four times, each 20 minutes, at 55°C. The membrane was wrapped in a plastic film and exposed to a X-ray film overnight or 24 hrs. The films were developed and the signals were analyzed by comparing with the agarose gel photograph.

Results

Restriction digestion and Southern blotting

The *Eco*RI restriction digestion gel images and Southern blots of the mollicutes selected for this study are shown in Figures 2-5. *S. citri* strain BR3-3X was used as the control (lane 2, Fig. 2-5) of this study. The blots showed that in BR3-3X there were two high intensity P58 probe-reactive bands (~9 kb and ~4 kb), and two faint bands (~6 kb and just over 9 kb). The intensity of the 4 kb probe-reactive was higher than that of the 9 kb probe-reactive band (lane 2, Fig. 2-5).

When the whole P58 gene (41) was used as the probe in the Southern hybridization, BR3-3X had four probe reactive bands (~2kb, ~4kb and two of undetermined size) (data not shown). The presence of an *Eco*RI site in the P58 gene may be the reason for the additional bands when the whole gene was used as the probe.

S. citri strains

There was no major variation in the *Eco*RI digestion patterns of *S. citri* strains (Figs. 2 & 4). In corresponding Southern blots strains R8A2, B105 and M200H had only one probe-reactive band, corresponding to the 9 kb band of the control BR3-3X (lanes 3, 4 & 5, Fig. 2). The strain MDHR3 had a single probe-reactive band (slightly larger than 9 kb), which did not correspond to any of the bands of the control (lane 6, Fig. 2). The strain GO4 showed a probe-reactive band that corresponded to the 4 kb band of the control (lane 7, Fig. 2). Since the intensity and thickness of the GO4 probe-reactive band

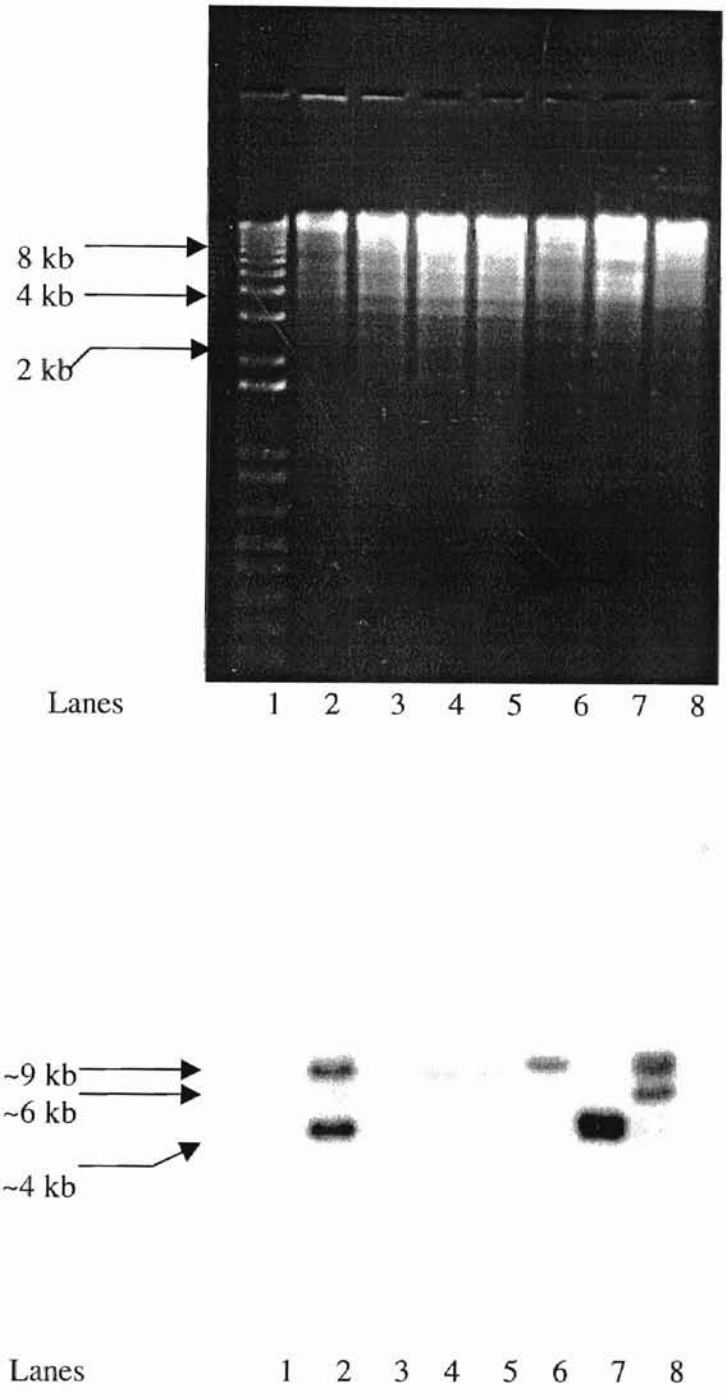

Fig. 2 Restriction digestion pattern and Southern blot of *S. citri* strains (high stringency - hybridization at 55°C).
Lane **1**. 1 kb+ DNA marker, **2**. Control BR3-3X, **3**. R8A2, **4**. B105, **5**. M200H, **6**. MDHR3, **7**. GO4 and **8**. Aceratagallia

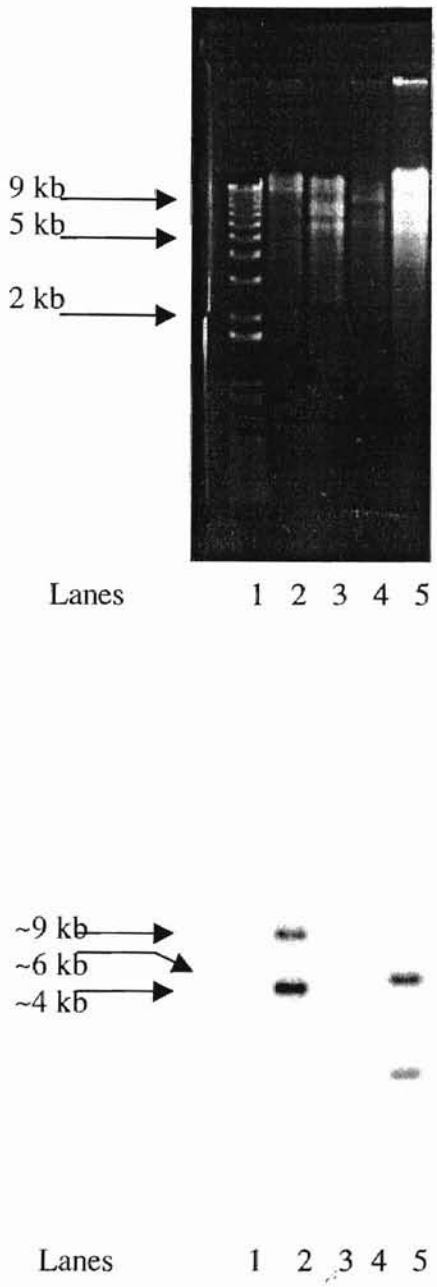

Fig. 3 Restriction digestion pattern and Southern blot of *S. kunkelii* and selected non-phytopathogenic spiroplasmas (high stringency - hybridization at 55°C).
Lane **1**. 1 kb+ DNA marker, **2**. control BR3-3X, **3**. *S. melliferum*, **4**. *S. apis* and **5**. *S. kunkelii*

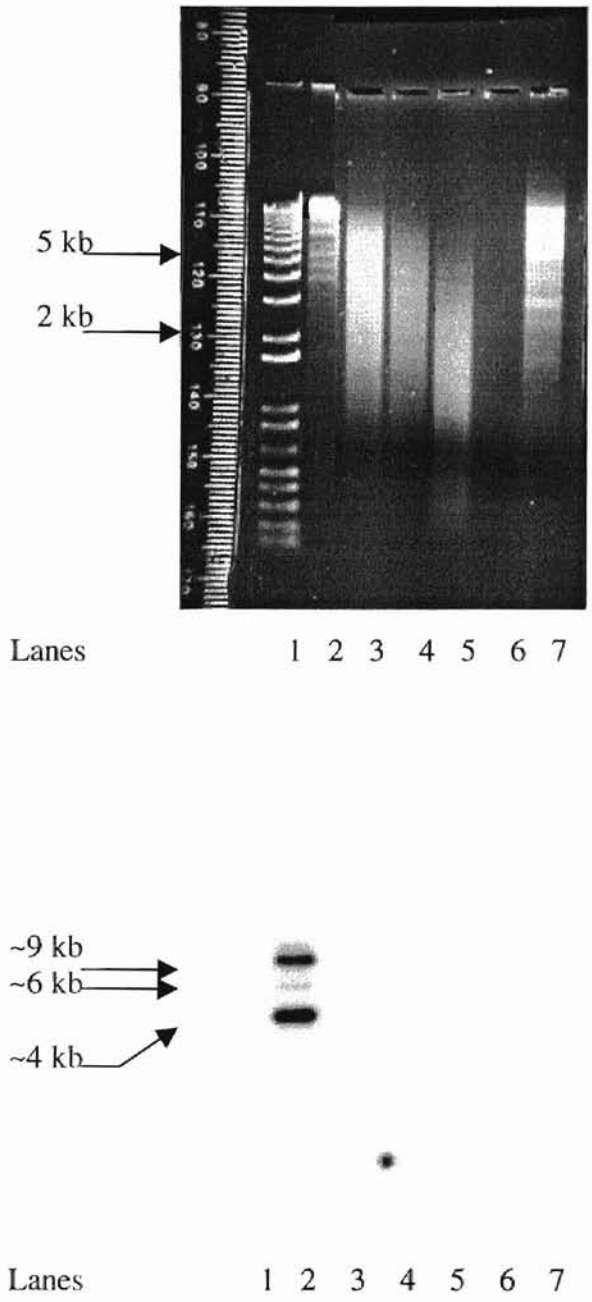

Fig. 4 Restriction digestion pattern and Southern blot of selected phytoplasmas and mycoplasmas (high stringency - hybridization at 55°C).
Lane **1.** 1 kb+ DNA marker, **2.** Control BR3-3X, **3.** Aster yellows (AY1), **4.** Aster yellows (AY2), **5.** *M. canis*, **6.** *M. bovis* and **7.** *S. citri* strain Beni Mellal.

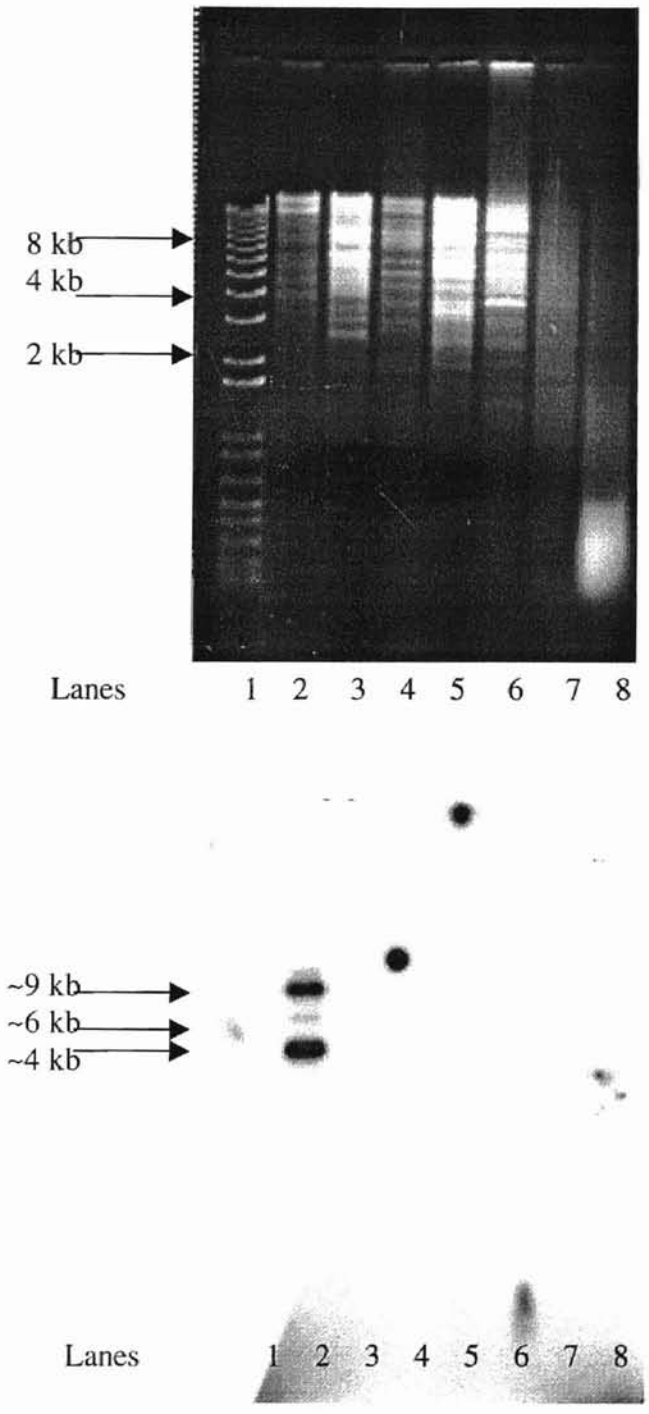

Fig. 5 Restriction digestion pattern and Southern blot of selected phytoplasmas and non-phytopathogenic spiroplasmas (low stringency - hybridization at 50°C).
Lane **1**. 1 kb+ marker, **2**. Control BR3-3X, **3**. *S. melliferum*, **4**. *S. syrphidicola*, **5**. *S. floricola*, **6**. *S. apis*, **7**. BLTVA strain GOR and **8**. BLTVA strain UCD.

were so high there may be two bands that lie close to each other. The Southern profile of the strain Aceratagallia showed two intense bands (one ~7kb but size cannot be determined for the other, broader band, which may have two bands lie close to each other) and a faint band that correspond to the 4 kb band of the control (lane 8, Fig. 2). Beni Mellal was the only *S. citri* strain that failed to show any probe-reactive bands (lane 7, Fig. 4).

S. kunkelii

Fig. 3 shows the restriction digestion profile of the phytopathogenic spiroplasma species *S. kunkelii* (lane 5) and the corresponding Southern blot. Two probe-reactive, high intensity bands (~1.3 kb and ~7 kb) and one faint band of undetermined size were present in *S. kunkelii* blot, none of which corresponded to any bands of the control. The intensity of the bands in the digestion pattern of *S. kunkelii* (lane 5, Fig. 3) was greater than those of the other mollicutes in Fig. 3 (though a uniform amount (3 µg) of DNA was digested and loaded into each well, the intensity varies). When genomic DNAs of the control and *S. kunkelii* were adjusted to give patterns of similar intensity, the blot still showed the presence of two intense probe-reactive bands in *S. kunkelii* (data not shown).

Phytoplasmas

Two different phytoplasmas were used in this study, aster yellows (strains AY1 and AY2)(lanes 3 & 4, Fig. 4) and BLTVA (Beet Leafhopper Transmitted Virescence Agent)(lanes 7 & 8, Fig. 5). Neither aster yellows nor BLTVA showed any P58-reactive bands in the blots. One of the BLTVA strains (UCD) showed an unusual restriction

digestion pattern (lane 8, Fig. 5); this streak of bands (size below 500 bp) may be RNA. BLTVA failed to show any probe-reactive bands (lanes 7 & 8, Fig. 5) even with reduced stringency of hybridization (hybridization temperature was lowered to 50°C; for high stringency the temperature was 55°C).

Non-phytopathogenic mollicutes

Amounts of genomic DNA isolated from the non-phytopathogenic species, *S. corruscae*, were insufficient for Southern analysis. None of the other non-phytopathogenic spiroplasmas, *S. melliferum* (lane 3, Fig. 3 and lane 3, Fig. 5 (at reduced stringency)), *S. apis* (lane 4, Fig. 3 and lane 6, Fig. 5 (at reduced stringency)) and *S. syrphidicola* (lane 4, Fig. 5 (at reduced stringency)) showed P58 probe-reactive bands in the blot. Similarly, the non-phytopathogenic species, *S. floricola* (lane 5, Fig. 5) showed no probe-reactive bands.

From the mycoplasma species *M. felis* and *M. hyorhinis*, the genomic DNA isolated was insufficient for Southern analysis. A sufficient amount of *M. bovis* DNA was obtained and digested, but no bands were visible in the gel (lane 6, Fig. 4). *M. canis* (lane 5, Fig. 4) produced a blurred digestion profile in the gel but did not show any probe-reactive bands in the Southern blot.

Restriction digestion and Southern hybridization for all the above species and strains were carried out at least twice and the results were reproducible. A summary of the results is presented in Table 2.

Species	Reaction with P58 probe	Number of probe-reactive bands	Probe-reactive band size
Spiroplasma citri strains			
BR3-3X	+	2	4 & 9 kb
BR3-T*	+	3**	1.8, 4 & 16 kb**
BR3-G*	+	2**	1.8 & 16 kb**
SPA*	+	1**	1.8 kb**
SPV3*	+	1**	1.8 kb**
ASP1*	+	1**	1.8 kb**
R8A2	+	1	9 kb
B105	+	1	9 kb
M200H	+	1	9 kb
MDHR3	+	1	>9 kb
GO4	+	1	4 kb
Aceratagallia	+	2	7 & >9kb
Beni Mellal	-	-	-
S. kunkelii	+	2	1.3 & 4kb
S. melliferum	-	-	-
S. apis	-	-	-
S. floricola	-	-	-
S. syrphidicola	-	-	-
Phytoplasmas			
Aster yellows			
strain (AY1)	-	-	-
strain (AY2)	-	-	-
BLTVA			
strain GOR	-	-	-
strain UCD	-	-	-
Mycoplasma canis	-	-	-
M. bovis	-	-	-

Table 2. Summary of P58-like genes in selected mollicutes

*S. *citri* strains tested in a previous P58 related study (63) using a different P58 probe. BR3-T - transmissible derivative of BR3-3X, BR3-G - non-transmissible derivative of BR3-3X, SPV3 - *S. citri* strain from citrus, SPA - non-transmissible derivative of SPV3 and ASP1 - non-helical derivative of strain R8A2.

**results from a probe derived from the 5' end of the P58 gene (63).

Discussion

The adherence of *S. citri* to the gut cells of *C. tenellus* is an important step for *S. citri's* entry into the leafhopper hemocoel (27). Little information is available about spiroplasma adhesins, while mycoplasma adhesins have been extensively studied and some of them are products of multi-gene families. The presence of multiple copies of the P58 gene (40, 27) in *S. citri* and its distant similarity to mycoplasma adhesins support the consideration of P58 as a putative adhesin. Knowledge of the distribution of P58-like genes in other mollicutes will provide additional understanding of P58. Restriction digestion of mollicute genomic DNA with *Eco*RI, and Southern blot analysis using a P58 probe (Fig. 1), is a logical way to screen for the presence of P58-like genes.

The characterized P58 gene is about 1.5 kb in size, with an *Eco*RI site at the 315th base pair position (40, 41). The probe (Fig. 1) used for this study was a 570 bp segment at the 3' end of the characterized P58 gene. Using this probe, the control *S. citri* strain, BR3-3X, showed two high intensity reactive bands, one of about 9 kb and the other of about 4 kb (lane 2, Fig. 2 to 5). In a previous study (63), when a 200 bp segment of the 5' end of the characterized P58 gene was used as probe, *S. citri* strain BR3-3X showed three probe-reactive bands, ~1.8 kb, ~4 kb and ~16 kb. All three reactive bands were also seen in the BR3-3X derivative transmissible line, BR3-T, but only two (1.8 kb and 16 kb) were seen in BR3-3X derivative non-transmissible line, BR3-G. The sequence of a 9.5 kb fragment of BR3-3X containing the P58 copy deleted in BR3-G was previously determined (63, 41). The sequence predicts that the 570 bp 3' end probe should react with a 4 kb *Eco*RI fragment. Comparing the P58-bands in BR3-3X of this study (lane 2, Fig. 2 to 5) with that of the previous study (63), the number of P58-bands varies in the

two studies (two reactive bands in this study and three in the previous). The intensity of the 4 kb P58-band in this study (lane 2, Fig. 2 to 4) was higher when compared to that of the 9 kb P58-band of this study. This trend was also seen in the low stringency blots (lane 2, Fig. 5), so it is possible that there may be two copies of P58 at the 4 kb position and the total, three P58 copies in BR3-3X, would then agree with data from the previous study.

In this study, all *S. citri* strains (lanes 3 to 8, Fig. 2) except the strain Beni Mellal (lane 7, Fig. 4) had at least one P58-reactive band. Interestingly, *S. citri* strain Aceratagallia (lane 8, Fig. 2) showed two probe-reactive bands, this is the only *S. citri* strain tested (besides the BR3 control) that showed multiple probe-reactive bands of different sizes. In the strain GO4, the probe-reactive band intensity (lane 7, Fig. 2) suggests that there were two bands migrating very close to each other, or maybe two or more reactive fragments of the same size. The reactive bands of strains R8A2, B105 and M200H (lanes 3, 4 & 5, Fig. 2) were of low intensity when compared to the control and other strains in Fig. 2. This may be due to lesser similarity of the P58 probe to DNA of these three strains. The variations in the number of P58 probe-reactive bands in different *S. citri* strains is difficult to explain, but in strains having more than one band, isolation of bands, sequencing and comparison of the sequences (with BR3-3X P58 gene sequences) would provide information on the degree of similarity between the two copies (bands) and with the previously-characterized P58 gene.

S. kunkelii had two intense probe-reactive bands (lane 5, Fig. 3), ~900 bp and ~5 kb, and a faint band of undetermined size. *S. kunkelii* is serologically closely related to *S. citri* (14), and DNA hybridization experiments between *S. citri* and *S. kunkelii* showed

60% DNA-DNA homology, confirming that they are closely related but low enough to consider these two as separate species (24). Other spiroplasmas were reported to have 30-40% homology with *S. citri*. The presence of P58-like genes in *S. kunkelii* thus may be attributed to its close relatedness to *S. citri*, but according to the 16S rDNA phylogenetic data (see below-phytoplasmas, non-phytopathogenic spiroplasmas, for details) (42), the close relatedness of mollicutes to *S. citri* may or may not be a factor related to the presence of P58-like genes in mollicutes.

Two phytoplasmas, aster yellows (lanes 3 & 4, Fig. 4) and BLTVA (lanes 7 & 8, Fig. 5), lacked P58-like bands. According to 16S rDNA data, phytoplasmas are distantly related to spiroplasmas (42), this fact may or may not contribute to the lack of P58-like bands in phytoplasmas. Yet another possible reason for lack of probe-reactive bands is that phytoplasmas are uncultivable in media and the phytoplasma DNAs were extracted directly from the infected plants. Extraction of pure or homogenous phytoplasma DNA without plant DNA contamination is a difficult task, and for this study no control DNA (BR3-3X) was extracted from infected plants similar to phytoplasma DNA extraction. Without such a control we cannot be sure that the extracted DNA was from the phytoplasma rather than from the plant DNA.

The non-phytopathogenic spiroplasmas *S. apis*, *S. melliferum*, and *S. syrphidicola*, and mycoplasma species *M. canis*, had no probe-reactive bands. Despite *S. apis* being closely related to *S. citri*, as determined by 16S rDNA phylogenetic studies (42), no P58-like genes were found in *S. apis*. The degree of relatedness of these other mollicutes to *S. citri*, whether based on DNA-DNA homology (*S. kunkelii*) or 16S rDNA phylogeny (*S. apis*), may or may not have any correlation with the presence of P58-like bands.

The restriction digestion profiles of *S. citri* strains (Fig. 2) and selected phytoplasmas and mycoplasmas (Fig. 4) showed *Eco*RI fragments of larger size in the former and smaller size in the latter. The variation may reflect a greater number of *Eco*RI sites in phytoplasmas and mycoplasmas than in *S. citri* strains. Alternatively, some of the *Eco*RI sites might have been modified by methylases in *S. citri* strains.

In some species P58-like genes may exist but have sequences diverged too far to be detected. The nucleotide sequences of the P58 probe, when translated, showed low similarity and identity with the P50 adhesin of *M. hominis*. When the nucleotide sequences of the P58 gene were compared to those of the already sequenced mycoplasma genomes, *M. pneumoniae* and *M. genitalium*, no alignments were achieved.

Another fully sequenced mollicute genome is that of *Ureaplasma urealyticum*. Amino acid sequence identity of about 25% was found between P58 and a *U. urealyticum* putative product. The gene encoding this product is in a cluster of 4 open reading frames very similar to that of P58 (40). This finding shows that there may be some common functions (probably involved in adherence process) between these two proteins.

In summary, recognizable P58-like genes were present in both of the tested phytopathogenic spiroplasmas, but not in non-phytopathogenic spiroplasmas that colonize insects, nor in phytopathogenic phytoplasmas, nor in animal mycoplasmas for which adhesins are well characterized. Clearly, a P58-like protein is not highly conserved among mollicutes of different habitats, nor is P58 likely to be a major housekeeping gene necessary for general mollicute function. The correlation between P58 presence and microbe identity as a plant pathogenic spiroplasma could reflect a role for P58 in some aspect of plant colonization and disease development, or of insect

transmission, that is either not required or is satisfied in some other way by phytopathogenic phytoplasmas. However, such a correlation is tenuous because only two phytopathogenic spiroplasmas were available for this study, and even the addition of the third (and only other) plant pathogenic spiroplasma, *S. phoeniceum*, would not constitute convincing evidence. In addition, as noted previously, some mollicutes may have P58-like genes but have diverged too far to be detected. And the P58 gene may or may not correlate with pathogenicity or transmissibility of mollicutes. Thus, the data is insufficient to conclude P58 as an adhesin.

Chapter III

Circulifer tenellus Phylogeny

Introduction

Circulifer tenellus, commonly known as the beet leafhopper, is approximately 20 – 30 mm long, wedge shaped, and pale green to gray or brown in color. It may have dark markings on the dorsal surface of the body. It is a phloem-feeding insect and the natural vector of *Spiroplasma citri*, a pathogen of citrus and horseradish plants (13), in the United States. It is also the vector of beet curly top virus (45). *C. tenellus* is a sun loving insect and breeds well in arid and semi-arid regions. Acquisition of spiroplasmas from the phloem of plants by leafhoppers involves specific feeding behavior in which the stylets probe through outer plant tissues and pierce the sieve tube elements. The minimum acquisition access period varies with spiroplasma-infected plant species, vectors, pathogens and conditions, but in general the efficiency of *S. citri* acquisition by *C. tenellus* is relatively low (1-5%). For comparison, the corn leafhopper, *Dalbulus maidis,* can acquire the phytopathogen *Spiroplasma kunkelii* (causal agent of corn stunt disease) with 100% efficiency (53).

The family Cicadellidae (leafhoppers) consists of more than 25, 000 species. *C. tenellus* is classified in the family Cicadellidae and subfamily Deltocephalinae, but its

tribe affiliation has been modified overtime. Previously, *C. tenellus* was placed in the tribe Euscelini (55), but according to the latest classification (46) it is in the tribe Opsiini. In the past most of the phylogenetic relationships among leafhoppers were characterized mainly on morphological features such as color, shape of male genitalia, length and placement of setal hairs, shape of frontal sutures and wing venation (57). These morphological differences are often subtle and fail to provide character-based differences between the species for phylogenetic analysis.

Molecular data provide some additional characters useful for testing the morphology-based phylogenetic analyses (54). Molecular markers like 16S rDNA is commonly used in the phylogenetic analyses. Only very few studies (54, 56) have been reported about leafhopper phylogeny based on ribosomal DNA sequences, and *C. tenellus* has yet to be studied using this type of genetic analysis. The purpose of this study was to use ribosomal DNA sequences to derive the phylogenetic relationship of *C. tenellus* with other selected genera of leafhoppers in the subfamily Deltocephalinae.

Materials and Methods

Specimens

Leafhoppers used in this study were in the subfamily Deltocephalinae. Insect species, their collection locality and conditions of storage are listed in Table 3.

DNA Extraction

DNA was extracted from hind legs of frozen individual leafhoppers. Insects were mounted ventral side up on a dish containing wax; with the aid of a dissecting microscope and using 0.5 mm pointed forceps the hind legs were carefully removed. The body was frozen for later species identification. The hind legs were crushed using a sterile toothpick in a 0.5 ml microfuge tube containing 30 µl of homogenization buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1% Nonidet P-40 and 100 µg/ml proteinase K). After crushing, 30 µl of sterile nanopure water was added and the contents were heated at 95°C for 3 minutes. The contents were briefly spun (14,000xg) to pellet the debris; the supernatant was the source of DNA. For dried specimens, the whole body was used for DNA isolation as described above, with an additional step of incubating the specimens at 37°C for 1 hr before isolation.

Primers for PCR Amplification

The primers used for the amplification of 16S ribosomal DNA were 5'-CGCCTGTTTAACAAAAA-3' (forward primer) and 5'-CCGGTCTGAACTCAGATCAAGT-3' (reverse primer). These were designed from

Species	Collection location	Collection date	Storage condition
Circulifer tenellus	Payne County, Oklahoma	April 2000	Frozen
Dalbulus elimatus	Payne County, Oklahoma	April 2000	Frozen
Dalbulus maidis	Payne County, Oklahoma	April 2000	Frozen
Endria inimica	Payne County, Oklahoma	May 2000	Frozen
Exitianus exitiosus	Payne County, Oklahoma	April 2000	Frozen
Flexamia reflexa	Northwestern Oklahoma	1959	Dried
Macrosteles quadrilineatus	Payne County, Oklahoma	April 2000	Frozen
Paraphlepsius irroratus	Payne County, Oklahoma	May 2000	Frozen
Scaphoideus sp.	Wyandotte, Oklahoma	1939	Dried
Empoasca sp. (out group)	Payne County, Oklahoma	May 2000	Frozen

Table 3. Leafhopper species used for 16S rDNA phylogeny study

conserved sequences of the 16S ribosomal DNA sequences of the fruit fly, *Drosophila yakuba,* as well as of *Locusta migratoria* and *Magicicada* (56). The predicted size of the amplified product was 550 bp.

PCR Amplification

The PCR amplification was carried out in a total volume of 50 µl of reaction buffer (50 mM KCl, 10 mM Tris-Cl, pH 9.0, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% TritonX-100, 200 µM dNTPs and 1 µM of each primer) in a microfuge tube with 25 µl of mineral oil (Perkin Elmer) layered on top of the reaction buffer. The tubes were exposed to an UV light source at a distance of about 5 cm for 10 minutes to destroy any contaminating DNA. A volume of 2 µl of template DNA (isolated from leafhopper hind legs) was added through the oil and heated to 80°C in a thermal cycler (Perkin Elmer) prior to the addition of 5 units of *Taq* DNA polymerase (Promega Biotech).

The program for PCR amplification (frozen specimens) consisted of 10 cycles of 1 min at 92°C, 1 min at 48°C and 1.5 min at 72°C, followed by 25 cycles of 1 min at 92°C, 35 seconds at 52°C and 1.5 min at 72°C. A final extension reaction at 72°C for 7 min was also carried out. After amplification the microfuge tubes were frozen at –20°C; the mineral oil layered over the frozen reaction buffer was carefully removed (56).

The PCR reaction conditions for dried specimens consisted of 10 cycles of 1 min at 92°C, 1 min at 46°C and 1.5 min at 72°C, followed by 30 cycles of 1 min at 92°C, 35 seconds at 52°C and 1.5 min at 72°C. The final extension and storage conditions were as with frozen specimens (56).

PCR Reamplification

The PCR-amplified products were electrophoresed and the target band was sliced out and crushed in TE buffer (pH 8.0) and centrifuged briefly. The supernatant was used as the source of DNA for PCR reamplification. The program for PCR reamplification consisted of 30 cycles of 1 min at 92°C, 35 seconds at 58°C and 1.5 min at 72°C (56).

PCR Product Purification

The PCR products were purified by adding the reaction mixture to purification columns (PCR Select-II, Eppendorf 5 prime) and centrifuging at 1500xg for 5 minutes using a swinging bucket centrifuge. The purified PCR products (column eluent) were ethanol precipitated by adding 2.5 volumes of ethanol and incubating overnight at –20°C. The precipitated DNA pellet was resuspended in 20 µl of TE (pH 8.0) buffer.

The purified PCR products were checked for the presence of the predicted 550 bp of 16S rDNA by agarose gel electrophoresis (1% gel in 1x TAE (0.04 M Tris-acetate and 0.001 M EDTA)). To 5 µl of the purified and precipitated DNA, 1 µl of gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol (w/v)) was added and the samples were electrophoresed along with 5 µl of 1 Kb+ DNA ladder (GIBCO BRL) and 4 µl of high mass ladder (GIBCO BRL). The gel was stained with ethidium bromide (1 µg/ml) for 45 minutes and viewed under an UV light source.

DNA Sequencing

Double stranded DNA (the PCR products) was sequenced using thermal cycle sequencing with a DNA Sequencing System (ABI Prism 3700 DNA Analyzer) at the

OSU Recombinant DNA and Protein Resources (Core) Facility. DNA sequenced was about 20 –25 ng/µl and sequenced in both directions (5' and 3') using the forward and reverse PCR primers (50 nM) as described above.

Sequence Alignment

The DNA sequences obtained using forward and reverse primers for each of the DNA samples were aligned pair-wise (AssemblyALIGN software, Oxford Molecular Biology group) and manually corrected for any misaligned nucleotides. The consensus sequences of the pair-wise aligned sequences from each of the samples were used for the multiple sequence alignment using Internet based software (Bionavigator) (65).

Phylogenetic Analysis

Since different tree-building algorithms make different evolutionary assumptions, two methods, maximum parsimony (MP) and neighbor joining (NJ), were used to estimate phylogenetic relationships. The assumptions of the MP method are "each site evolves independently, different lineages evolve independently, the probability of a base substitution at a given site is small over the lengths of time involved in a branch of the phylogeny, the expected amounts of change in different branches of the phylogeny do not vary by so much that two changes in a high-rate branch are more probable than one change in a low-rate branch, and the expected amounts of change do not vary enough among sites that two changes in one site are more probable than one change in another (64, 65)". NJ method is very fast and can handle larger data sets. "NJ is a distance matrix method producing an unrooted tree without the assumption of a clock. NJ

constructs a tree by successive clustering of lineages, setting branch lengths as the lineages join. The tree is not rearranged thereafter (64, 65)".

Internet based (Bionavigator) software was used to perform maximum parsimony (DNA Parsimony, Bionavigator) and neighbor joining (DNA Distance Matrices, Bionavigator) (65). The sequences were bootstrapped with 100 replications. The consensus bootstrapped data were used for tree construction using PHYLIP software program.

Results and Discussion

DNA Isolation and Amplification

When 16S ribosomal DNA isolation and amplification were carried out with thawed hind-leg samples of three leafhopper species (*Circulifer tenellus*, *Dalbulus elimatus* and *Exitianus exitiosus*), a band of the expected size (550 bp) occurred in samples from all three species (Fig. 6). The amplified DNA was sequenced (see below).

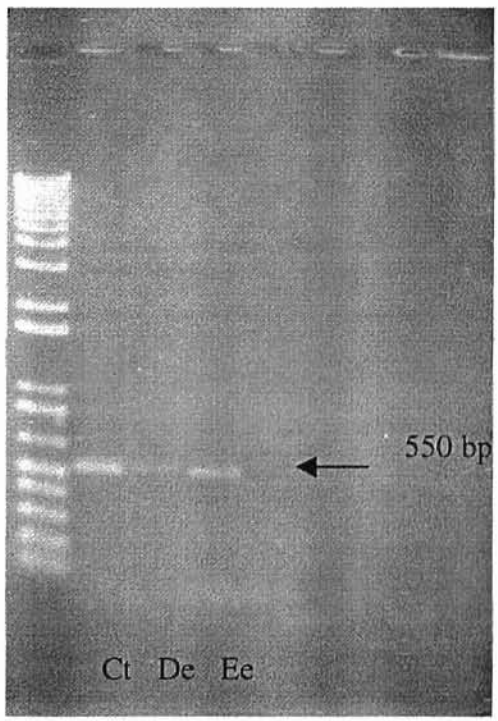

Fig. 6 Gel image of PCR-amplified 550 bp 16S rDNA
Ct-*Circulifer tenellus*, De-*Dalbulus elimatus*, Ee-*Exitianus exitiosus*

PCR amplification of dried specimens (*Flexamia reflexa* and *Scaphoideus* sp.) failed to yield any DNA, possibly because of nucleic acid degradation during storage. Isolation of DNA and PCR amplification from other frozen species (*Dalbulus maidis*,

Endria inimica, *Macrosteles quadrilineatus* and *Empoasca sp.*) yielded insufficient DNA for sequencing. DNA sequencing (ABI Prism 3700 DNA Analyzer) requires at least 150-200 ng of DNA at a concentration of 20 ng/μl, and the yields from these insects were marginally below that required. However, it should be possible to improve yields and add these species to the phylogeny development.

Fig. 7 shows the initial PCR-amplified bands from frozen specimens of six species. PCR-amplified DNAs from these six species were reamplified to gain more DNA for sequencing. The individual bands were sliced out from the gel, crushed

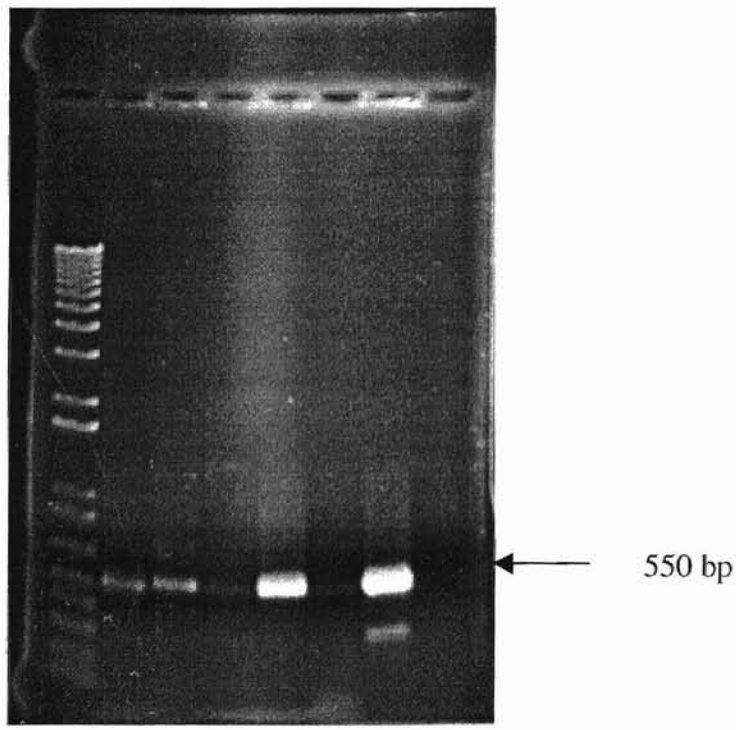

Fig. 7 16S rDNA PCR product from frozen specimens
Ct-*Circulifer tenellus*, De-*Dalbulus elimatus*, Dm-*Dalbulus maidis*, Ei-*Endria inimica*, Ee-*Exitianus exitiosus*, Mq-*Macrosteles quadrilineatus*

in TE buffer (pH 8.0), and briefly centrifuged. The supernatant was used as the DNA source for reamplification. An important difference in the PCR conditions between initial amplification and reamplification was the annealing temperature. The annealing temperature in the initial PCR amplification was 48°C, but during reamplification the annealing temperature was increased to 58°C, to increase the stringency.

Reamplification was expected to produce a single band of 550 bp with high intensity, but many smaller bands in addition to the 550 bp band occurred, as shown in Fig. 8. The complexity of the banding pattern precluded DNA sequencing.

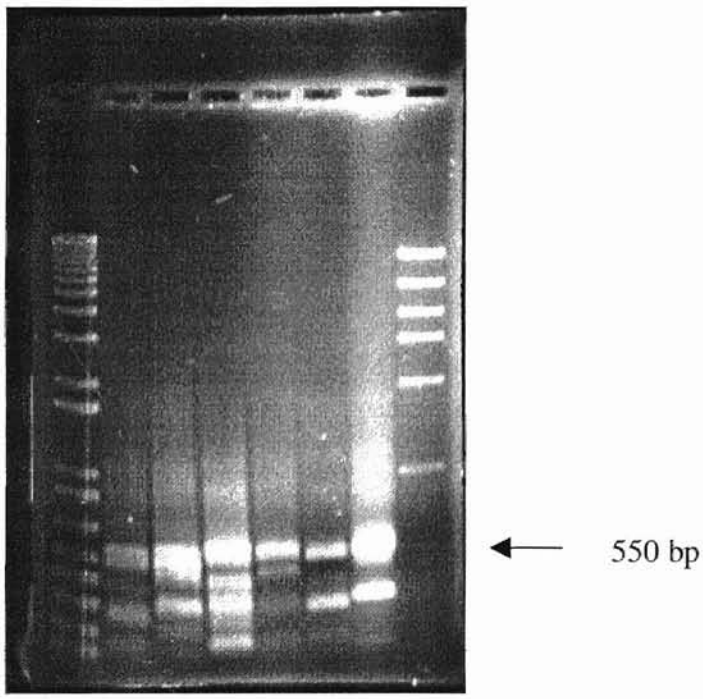

Fig. 8 Reamplified 16S rDNA
Ct-*Circulifer tenellus*, De-*Dalbulus elimatus*, Dm-*Dalbulus maidis*, Ei-*Endria inimica*, Ee-*Exitianus exitiosus*, Mq-*Macrosteles quadrilineatus*

One possible reason for the presence of unexpected bands could be mispriming at several positions in the template DNA. During the initial PCR amplification the reaction mix was maintained at 80°C before the addition of *Taq* polymerase, but during reamplification *Taq* was added along with other reagents without preheating. The low stringency conditions during the initial heating may have caused the anomalous bands. Another possible explanation for the unexpected bands is that the gel was exposed to UV light for a long time after initial amplification, while the bands were sliced from the gel. UV exposure may have caused nicks in the target DNA (in the gel slice). During reamplification the primer then might have bound to the nicked fragments, resulting in the amplification of fragments below the expected size of 550 bp. If these are the real causes for the extra bands seen after reamplification, then careful handling will overcome these problems.

Sequencing of 16S rDNA

Amplified 16S rDNA from the specimens of *C. tenellus*, *D. elimatus* and *E. exitiosus*, shown in Fig. 6, were sequenced using the same primers used for PCR amplification. The sequences were aligned using ClustalW (Bionavigator) (Fig. 9). The sequence alignment (Fig. 9) shows that *C. tenellus* is more closely related to *D. elimatus* than it is to *E. exitiosus*. According to the latest classification (46), *C. tenellus* is in Opsiini tribe, *D. elimatus* is in Macrostelini tribe and *E. exitiosus* is in Doraturini tribe (all tribes are in the subfamily Deltocephalinae). In the literature the relationship of tribal phylogeny of the subfamily Deltocephalinae has already reported

```
                1         11        21        31        41
consensus       CTATGGTT-T    AACTAAATGGCTGCAGTAT-----ATTGACGG GCA
C.tenellus      .........-----..........C...........T.......G...
D.elimatus      ........G.TATA.............................T.TA..
E.exitiosus     T..A.AA...CTGCTT..T...CTT.C.TTA..TCAAC..C..G.TC...

                51        61        71        81        91
consensus       AAGGTAGCATAGT ATTAGCTC TTAATTAGAA---GCTGG ATGAATGG
C.tenellus      ............A........T......G.G.........A........
D.elimatus      ...........A.C.....T.TC..................C........
E.exitiosus     ..AT..AT....AT..G.A...GCC...A.A.TTAC....TT..CCC.AA

                101       111       121       131       141
consensus       G TA-ATGAGAT TA ATTTTATTAAGATTAATATTAAAATTTA AATTT
C.tenellus      CA.GT.......T..A..............T.T...G......A.T...
D.elimatus      .T..........A..T........T.AT................G.....
E.exitiosus     .G....CT.AT.C.TG...CC.AA.G...CT.A.CA..C.AAA.T....-

                151       161       171       181       191
consensus       GAGTTAAAATACT AA T T  -TTTAGGGACGATAAGACCCTATAGAAT
C.tenellus      ..........G..C..A.GGCC..A.......................C
D.elimatus      A............T..T.A.TT.....................G......
E.exitiosus     ..TA.......A.A..G.T.AAA....TCC..C-----....C.GC....

                201       211       221       231       241
consensus       TTTATAAAATC TTTT-AA AGTATTTATGTTATATTA TTTA A AT--
C.tenellus      ....ATT....G....T..TTTA....T.T.A......G....C.-.C..
D.elimatus      ..........TA.......C..................T...TA.G....
E.exitiosus     CA.C.....A.CC.AG...G.....AA.A....A.A.CAAC..G.A..TA

                251       261       271       281       291
consensus       GAT-TATATTT G TGGGGTG TAG TAAATAA  T AACTTTAACTTTT
C.tenellus      .T.....T...A.T.......ACT.T.......--.C.............
D.elimatus      .....T.....T.C.......G...G......TAT.TG......T.....
E.exitiosus     ...G..A.G..CTA.A....CT..TCGTCCC..GAAA.C......GCA..

                301       311       321       331       341
consensus       TT TTCA TTATATATGTAA TTTTGATCTTTAAAAGGAAAAAAGATATA
C.tenellus      ..A....A......T.....-........C..........TG......A.
D.elimatus      ..T....T....T.......G...........TT............A...
E.exitiosus     ..----..C...-.A..T...A..CCA..A..A...TTA.T....T....T

                351       361       371       381       391
consensus       ----ATTACCTCAGGGATAACAGCGTAATTTTAATGGGGAGTTCATATCT
C.tenellus      ....G......T.....................................
D.elimatus      .....................................................
E.exitiosus     CCTC....AT...TTC...C....T.CTAA....--.AA.C.A-..TA..
                                                          contd.....
```

Fig. 9 Alignment of *C. tenellus, D. elimatus* and *E. exitiosus* sequences
Consensus of the three sequences is at the top, '.' represents similar bases, '-' represents no bases

```
                401         411         421         431         441
consensus       ATATTAA TTTTGCGACCTCGA-----TGTTGAATTA AGATAA  ATAA
C.tenellus      .......A.............................-......TGTC..
D.elimatus      .......T.............................C......AT....
E.exitiosus     ..GC..CC...-..ACAG..A.AATAC..CA.CCA.TT..TATCCC...G

                451         461         471
consensus       GACG AGCTTTCTT TT TTAAG
C.tenellus      ..G.T..AG.....GA.T.....
D.elimatus      ....C.........A..G.....
E.exitiosus     .G.AG.A...A.C.T..A.A..A
```

(55). The tribes are given in the sequence of most primitive (Scaphytopiini) to most advanced tribes (Deltocephalini). Tribes Macrostelini and Opsiini (previously, Euscelini) ranked together as moderately advanced tribes within selected tribes; the other selected tribes are Acinopterini, Deltocephalini and Scaphytopiini. Other than this, there is no other data is available in the literature to explain the relationship of *C. tenellus* and *D. elimatus* and nor about *E. exitiosus* with the other two species.

The sequences of just three species are insufficient to perform meaningful phylogenetic analyses. These will await the resolution of the reamplification problems and the successful completion of DNA sequencing from all the specimens.

Bibliography

1. Neimark, H. (1979) Phylogenetic relationships between mycoplasmas and other prokaryotes. Pages 43-61 in: The Mycoplasmas, vol. I, M. F. Barile and S. Razin, eds. Academic Press, New York
2. Bove, J. M. and Saillard, C. (1979) Cell biology of spiroplasmas. Pages 83-153 in: The Mycoplasmas, vol. III, R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York
3. Whitcomb, R. F. (1981) The biology of Spiroplasmas. Ann. Rev. Entomol. 26: 397-425
4. Razin, S. (1989) Molecular approaches to mycoplasma phylogeny. Pages 33-69 in: The Mycoplasmas vol. V, R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York
5. Woese, C. R., Maniloff, J. and Zablen, L. B. (1980) Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. USA 77: 494-498
6. Yamao, F., Muto, A., Kawauchi, Y., Iwami, M., Iwagami, S., Azumi, Y. and Osawa, S. (1985) UGA is read as tryptophan in *Mycoplasma capricolum*. Proc. Natl. Acad. Sci. USA82: 2306-2309
7. Stamburski, C., Renaudin, J. and Bove, J. M. (1991) First step toward a virus derived vector for gene cloning and expression in spiroplasmas, organisms which read

UGA as a tryptophan codon: Synthesis of chloramphenicol acetyltransferase in *Spiroplasma citri*. J. Bacteriol. 173: 2225-2230

8. Tully, J. G., Whitcomb, R. F., Clark, H. F. and Williamson, D. L. (1977) Pathogenic mycoplasmas: Cultivation and vertebrate pathogenicity of a new Spiroplasma. Science 195: 892-894

9. McGarrity, G. J., Megraud, F. and Gamon, L. (1984) Rabbit lens cell cultures in the characterization of *Spiroplasma mirum* pathogenicity. Ann. Microbiol. (Inst. Pasteur) 135A: 249-254

10. Chastel, C., Legoff, F. and Humphery-Smith, I. (1991) Multiplication and persistence of *Spiroplasma melliferum* strain A56 in experimentally infected suckling mice. Res. Microbiol. 142: 411-417

11. Davis, R. E. and Worley, J. F. (1973) *Spiroplasma*: Motile, helical microorganisms associated with corn stunt disease. Phytopathology 63: 403-408

12. Fudl-Allah, A. E-S. A. and Calavan, E. C. (1973) Effect of temperature and pH on growth in vitro of a mycoplasmalike organism associated with stubborn disease of citrus. 63: 256-259

13. Fletcher, J. (1983) Brittle root of horseradish in Illinois and the distribution of *Spiroplasma citri* in the United States. Phytopathology 73: 354-357

14. Whitcomb, R. F. (1989) *Spiroplasma kunkelii*. Pages 487-544 in: The Mycoplasmas, vol. V, R. F. Whitcomb and J. G. Tully eds. Academic press, New York

15. Saillard, C., Vignault, J. C., Bove, J. M., Raie, A., Tully, J. G., Williamson, D. L., Fos, A., Garnier, M., Gadeau, A., Carle, P. and Whitcomb, R. F. (1987) *Spiroplasma phoeniceum* sp. nov., a new plant-pathogenic species from Syria.

Int. J. Syst. Bacteriol. 37: 106-115

16. Yongxuan, C., Baodi, X. and Yonghong, G. (1988) Studies on the characteristics of the honeybee spiroplasma in China. Scientia Sinica (series B) 31: 1073-1079

17. Williamson, D. L. and Pulson, D. F. (1979) Sex ratio organisms (spiroplasmas) of *Drosophila*. Pages 176-208 in: The Mycoplasmas, vol. III, R. F. Whitcomb and J. G.Tully eds. Academic Press, New York

18. Shaikh, A. A., Johnson, JR., W. E., Stevens, C. and Tang, A. Y. (1987) The isolation of spiroplasmas from mosquitoes in Macon county, Alabama. J. Amer. Mosq. Con. Assoc. 3: 289-295

19. Tully, J. G. and Whitcomb, R. F. (1981) The Genus Spiroplasma. Pages 2271-2284 in: The Prokaryotes, M. P. Starr, H. Stolp, H. G. Truper, A. Balows and H. G. Schlegel eds. Springer -Verlag, Berlin

20. Chen, T. A. and Davis, R. E. (1979) Cultivation of spiroplasmas. Pages 65-82 in: The Mycoplasmas, Vol. III, R. F. Whitcomb and J. G. Tully eds. Academic Press, New York

21. Townsend, R., Markham, P. G., Plaskitt, K. A. and Daniels, M. J. (1977) Isolation and characterization of a non-helical strain of *Spiroplasma citri*. J. Gen. Microbiol. 100: 15-21

22. Williamson, D. L., Whitcomb, R. F., Tully, J. G., Gasparich, G. E., Rose, D. L., Carle, P., Bove, J. M., Hackett, K. J., Adams, J. R., Henegar, R. B., Konai, M., Chastel, C., and French, F. E. (1998) Revised group classification of the genus Spiroplasma. Int. J. Bacteriol. 48: 1-12

23. Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, A. D., Kandler, O.,

Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrant, E., Starr, M. P., and Truper, H. G. (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37: 463-464

24. Liao, C. H. and Chen, T. A. (1981) Deoxyribonucleic acid hybridization between *Spiroplasma citri* and corn stunt spiroplasma. Curr. Microbiol. 5: 83-86

25. Marais, A., Bove, J. M. and Renaudin, J. (1996) Characterization of the recA gene regions of *Spiroplasma citri* and *Spiroplasma melliferum*. J. Bacteriol. 178: 7003-7009

26. Calavan, E. C. and Oldfield, G. N. (1979) Symptomatology of spiroplamal plant diseases. Pages 37-64 in: The Mycoplasmas, Vol. III, R. F. Whitcomb and J. G. Tully eds. Academic Press, New York

27. Fletcher, J., Wayadande, Melcher, U. and Ye, F. (1998) The phytopathogenic mollicute-insect vector interface: a closer look. Phytopathology, 88: 1351-1358

28. Saglio, P. H. M. and Whitcomb, R. F. (1979) Diversity of wall-less prokaryotes. Pages 1-36 in: The Mycoplasmas, Vol. III, R. F. Whitcomb and J. G. Tully eds. Academic Press, New York

29. Clark, T. B. (1982) Spiroplasmas: diversity of arthropod reservoirs and host-parasite relationships. Science 217: 57-59

30. Markham, P. G. (1983) Spiroplasmas in leafhoppers: A review. Yale J. Biol. Med. 56: 745-751

31. Liu, H.-Y., Gumph, D. J., Oldfield, G. N. and Calavan, E. C. (1983) The relationship of *Spiroplasma citri* and *Circulifer tenellus*. Phytopathology, 73: 585-590

32. Wayadande, A. C., Baker, G. R. and Fletcher, J. (1997) Comparative ultrastructure of the salivary glands of two phytopathogen vectors, the beet leafhopper, *Circulifer tenellus* (Baker), and the corn leafhopper, *Dalbulus maidis* delong and wolcott (Homoptera : Cicadellidae). Int. J. Insect Morphol. & Embryol. 26: 113-120

33. Wayadande, A. C. and Fletcher, J. (1998) Development and use of an established cell line of the leafhopper *Circulifer tenellus* to characterize *Spiroplasma citri*-vector interactions. J. Invertebr. Pathol. 72: 126-131

34. Fletcher, J., Wills, J. W. and Denman, S. E. (1989) Identification of surface proteins of *Spiroplasma citri* with protease and antibody probes. Curr. Microbiol. 19: 383-391

35. Proft, T., Hilbert, H., Plaggens, H. and Herrmann, R. (1996) The P200 protein of *Mycoplasma pneumoniae* shows common features with the cytadherence – associated proteins HMW 1 and HMW 3. Gene 171: 79-82

36. Dirksen, L. B., Proft, T., Hilbert, H., Hermann, R. and Krause, D. C. (1996) Sequence analysis and characterization of the *hmw* gene cluster of *Mycoplasma pneumoniae*. Gene 171: 19-25

37. Markham, P. F., Glew, M. D., Sykes, J. E., Bowden, T. R., Pollocks, T. D., Browning, G. F., Whitaker, K. G. and Walker, I. D. (1994) The organization of multigene family which encodes the major cell surface protein, pMGA of *Mycoplasma gallisepticum*. FEBS Lett. 352: 347-352

38. Yu, J., Wayadande, A. C. and Fletcher, J. (1997) Surface proteins of *Spiroplasma citri* in its adherence to vector insect cells. Phytopathology 87: S107

39. Melcher, U. and Fletcher, J. (1999) Genetic variations in *Spiroplasma citri*. Eu. J.

Plant Pathol. 105: 519-533

40. Ye, F., Melcher, U., Rascoe, J. E. and Fletcher, J. (1996) Extensive chromosomal aberrations in *Spiroplasma citri* strain BR3. Biochem. Genet. 34: 269-285

41. Ye, F., Melcher, U. and Fletcher, J. (1997) Molecular characterization of a gene encoding a membrane protein of *Spiroplasma citri*. Gene 189: 95-100

42. Maniloff, J. (1992) Phylogeny of mycoplasmas. Pages 549-559 in: Mycoplasmas - Molecular biology and pathogenesis, J. Maniloff, R. N. McElhaney, L. R. Finch and J. B. Baseman eds. American Society of Microbiology, Washington D. C.

43. Gray, M. W., Burger, G. and Lang, B. F. (1999) Mitochondrial evolution. Science 283: 1476-1481

44. Razin, S., Yogev, D. and Naot, Y. (1998) Molecular biology and pathogenicity of mycoplasmas. Microbiol. Mol. Biol. Rev. 62: 1094-1156

45. Nielson, M. W. (1975) The Leafhopper vectors of phytopathogenic viruses (Homoptera, Cicadellidae) taxonomy, biology, and virus transmission. Technical Bulletin 1382: Pages 290-295. United States Dept. of Agriculture, Washington, D.C.

46. Bishop Museum, Hawaii State Museum of Natural and Cultural History, Entomology Department, http://www.bishop.hawaii.org/bishop/ento/cicadel.html

47. Yu, J., Wayadande, A. C. and Fletcher, J. (2000) Membrane protein P89 of *Spiroplasma citri* implicated in adhesion to cultured cells of the vector *Circulifer tenellus*. Phytopathology 90: (in press)

48. Davis, R.E. (1979) Spiroplasmas: Helical cell wall-free prokaryotes in diverse

habitats. In: Proc. ROC-US Coop. Sci. Seminar on Mycoplasma Diseases of Plants, Natl. Sci. Council, Republic of China, Taiwan, pp. 59-64

49. Liao, C. H. and Chen, T. A. (1977) Culture of corn stunt spiroplasma in a simple medium. Phytopathology, 67: 802-808

50. Friis, N. F. (1975) Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare* a survey. Nord. Vet. Med. 27: 337-339

51. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. E3-E4

52. Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517

53. Calavan, E. C. and Bove, J. M. (1989) Ecology of *Spiroplasma citri*. Pages 425-485 in: The Mycoplasmas, Vol. V, R. F. Whitcomb and J. G. Tully eds. Academic Press, New York

54. Dietrich, C. H., Whitcomb, R. F. and Black IV, W. C. (1997) Phylogeny of the grassland genus *Flexamia* (Homoptera: Cicadellidae) based on mitochondrial DNA sequences. Mol. Phyl. Evol. 8: 139-149

55. Nielson, M. W. (1979) Taxonomic relationships of leafhopper vectors of plant pathogens. Pages 3-28 in: Leafhopper Vectors and Plant Disease Agents, K. Maramorosch and K. F. Harris eds. Academic Press, New York

56. Fang, Q., Black IV, W. C., Blocker, H. D. and Whitcomb, R. F. (1993) A phylogeny of new world Deltocephalus-like leafhopper genera based on mitochondrial 16S ribosomal DNA sequences. Mol. Phyl. Evol. 2: 119-131

57. Triplehorn, B. W. and Nault, L. R. (1985) Phylogenetic classification of the genus *Dalbulus* (Homoptera: Cicadellidae), and notes on the phylogeny of the macrostelini. Ann. Entomol. Soc. Am. 78: 291-315

58. Raju, B. C., Nyland, G., Backus, E. A. and McLean, D. L. (1981) Association of spiroplasmas with brittle root of horseradish. Phytopathology 71: 1067-1072

59. Wayadande, A. C. and Fletcher, J. (1995) Transmission of *S. citri* and their ability to cross gut and salivary gland barriers within the leafhopper vector *C. tenellus*. Phytopathology 85: 1256-1259

60. Tanaka, T., Ogiwara, A., Uchiyama, I., Takagi, T., Yazaki, Y. and Nakamura, Y. (1996) Construction of a normalized directionally cloned cDNA from adult heart and analysis of 3040 clones by partial sequencing. Genomics 35, 231-235

61. Patanjali, S.R., Parimoo, S., Weissman, S.M. (1991) Construction of uniform-abundance (normalizes) cDNA library. Proc. Natl. Acad. Sci. 88: 1943-1947

62. Bonaldo, M.F., Lennon, G. and Soares, M.B. (1996) Normalization and subtraction: Two approaches to facilitate gene discovery. Genome Res. 6: 791-806

63. Ye, F., Melcher, U. and Fletcher, J. (Unpublished)

64. Felsenstein, J. (1989) PHYLIP - Phylogeny inference package (version 3.2). Cladistics 5: 164-166

65. BioNavigator by eBioinformatics Pty Ltd http://www.ebioinformatics.com

Appendix

Appendix I

Sequencing of a normalized cDNA library from *Circulifer tenellus*

Introduction

Circulifer tenellus, commonly known as the beet leafhopper, breeds well in arid and semi-arid regions like deserts and abandoned, uncultivated wastelands. *C. tenellus* is the principal vector of *Spiroplasma citri*, which is the causative agent of citrus stubborn disease and of horseradish brittle root disease (13, 58). The geographical range of *S. citri*-induced diseases is related to the seasonal migration of *C. tenellus* from arid regions to cultivated lands. *S. citri* adheres to and invades the gut of the leafhopper, crossing the gut cell membranes and entering the hemocoel (59). It is hypothesized that the attachment of *S. citri* to receptors of the host leafhopper is mediated by adhesins.

The genomic sequences from almost fifty thousand organisms including viruses, bacteria and many eukaryotes (including insects) have been stored in databases and serve many useful purposes. Among insects, the sequences of *C. tenellus* have yet to find a place in these databases.

About 50% of clones selected at random from a human heart cDNA library were derived from single gene (60). In any cell the expression level of a particular gene may

vary from 1 copy to 200, 000 copies (61) and many genes are present in high copy numbers; so the probability of randomly picking and sequencing the same gene is high. An ordinary cDNA library may contain a high frequency of undesirable 'junk' clones, such as clones with poly (A) tails of mRNA alone, clones with primer sequences that were used in the first strand synthesis of the cDNA library and clones with very small inserts (62). For efficient identification of novel genes expressed in low copy numbers it is desirable to normalize or equalize the cDNA library such that the library contains equal amounts of cDNA from each gene expressed in a given cell. Using a normalized cDNA library as the source of clones for sequencing may enhance the probability of identifying novel genes like those encoding receptors of *S. citri* adhesion proteins.

There are two general approaches to obtaining normalized cDNA libraries. The first approach depends on hybridization selection with genomic DNA such that the relative abundance of cDNAs would be proportional to the abundance of genes complementary to that cDNA in the genomic DNA. The other approach depends on reannealing or reassociation kinetics; reannealing follows second order kinetics such that rare species anneal less rapidly than common ones, so that the single-stranded fraction of cDNA becomes progressively more normalized (61). As many as four different types of normalization based on the kinetic approach were reported (62). PCR based normalization is one of the efficient methods to obtain a normalized library with moderate and large size inserts. This study deals with PCR based normalization (reassociation kinetics approach) using *C. tenellus* cDNA library prepared from crushing whole insect bodies.

Methods and Materials

cDNA Library

The cDNA library was made by Mr. Sravan Mallu (former Ph. D. student, Entomology and Plant Pathology, OSU) using a ZAP-cDNA synthesis kit (Stratagene). About 300 mg of *C. tenellus* was used to begin library construction. The vector used for the library construction was the Uni-ZAP XR vector (or λ ZAP vector (Fig. 10)). This Uni-ZAP vector combines the efficiency of library construction and the plasmid system with the blue-white color selection. The vector was double digested with *Eco*RI and *Xho*I to accommodate the inserts.

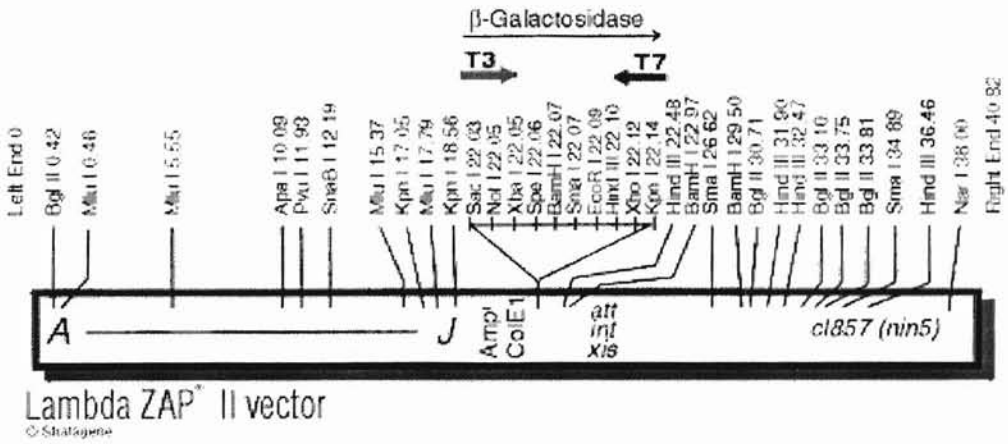

Fig. 10 Map of Uni-ZAP XR insertion vector
(from stratagene.com)

Mass Excision Protocol

The Uni-ZAP vector is designed to allow in vivo excision and recircularization of cloned insert within the ZAP vector to form a phagemid containing a cloned insert. The phagemid formed here is the pBluescript phagemid containing the insert. The polylinker of the pBluescript phagemid has unique cloning sites flanked by T3 and T7 promoters. The theory behind this excision strategy is that when we incubate the library with a strain of *E. coli* and a helper phage (VCS M13 or M13K07), the helper phage proteins recognize the initiator DNA within the ZAP vector and nick one of the two DNA strands (Fig. 11). At the nicking site DNA synthesis begins and duplicates whatever DNA exists in the λ ZAP vector downstream of the nicking site until it recognizes the termination signal on the ZAP vector. The gene II products of helper phage proteins circularize the new single stranded DNA; this DNA is called the phagemid DNA. The library (10^7 pfu) and *E. coli* (strain XI1 Blue MRF-10^8 pfu) are mixed in the ratio 1:10, and the helper phage (10^9 pfu) and *E. coli* cells are added in the ratio of 1:10. The mixture is incubated at 37°C for 15 minutes for absorption of cells and helper phage, and then 2 ml of LB broth is added and incubated at 37°C for 2.5 hrs. Then the mixture is heated to 65°C to kill the *E. coli* cells, and centrifuged at 1000g at 4°C to recover the supernatant containing the phagemid. For determination of phagemid titer, 100 µl of phagemid is mixed with 100 µl of SOLR strain *E. coli* cells (grown overnight, centrifuged, pellets dissolved in 10 mM $MgSO_4$, and diluted to OD_{600} value of 1.00). The mixture is incubated at 37°C for 15 minutes and diluted in the ratio 1:50 with LB broth; 135 µl of diluted mixture is plated on LB plates (with ampicillin 150 µg/ml, 100 mM IPTG 40 µg/ml and X-gal 40 µg/ml).

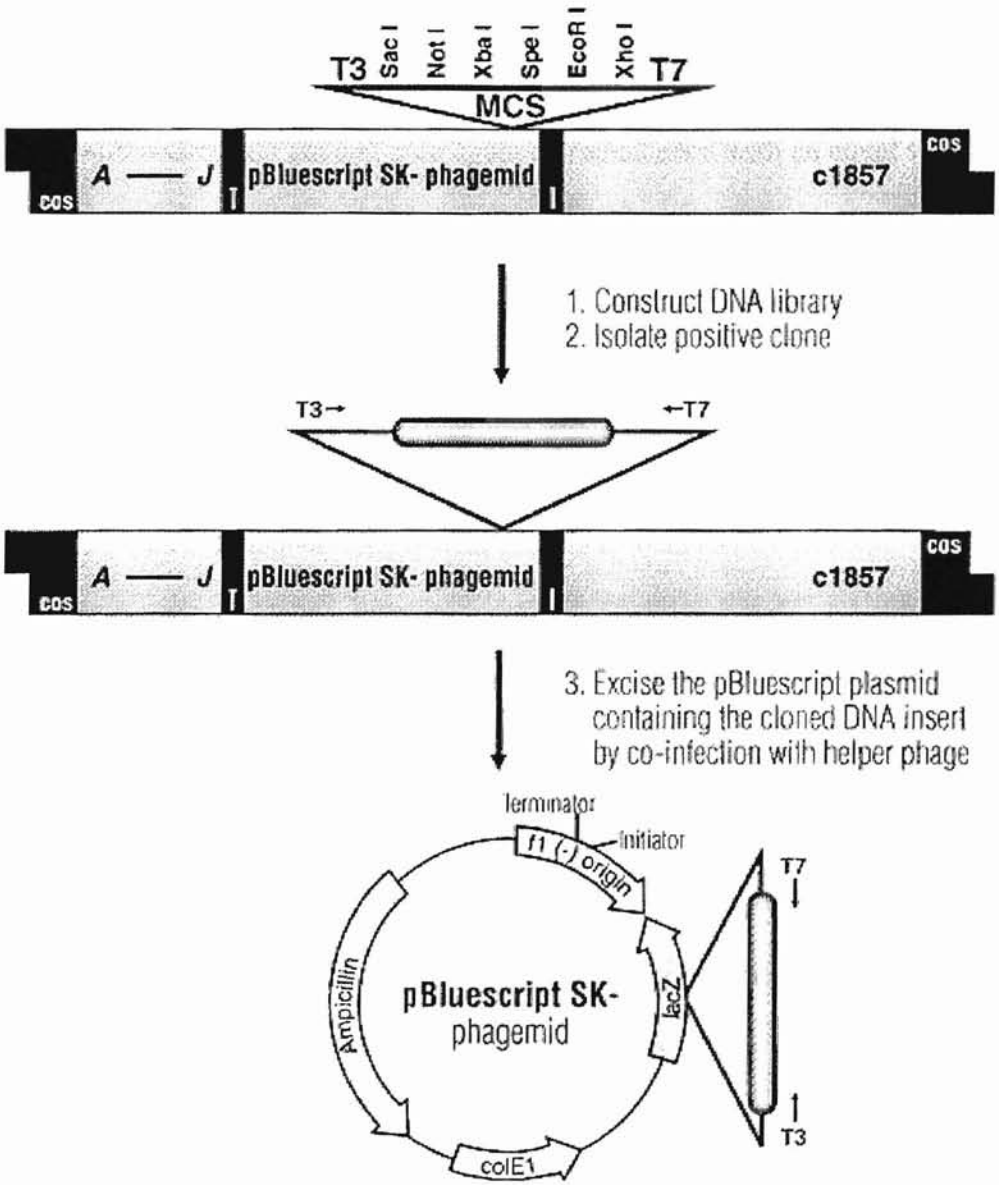

Fig. 11 Mass Excision Protocol
(from stratagene.com)

Extraction of Single Stranded DNA

The phagemid suspension is mixed with 40% PEG/2.5 M NaCl solution in the volume ratio of 4:1, kept on ice for 1hr and centrifuged to harvest the pellet containing the DNA. The pellet is dissolved in 100 ml TE and 0.02% of SDS and extracted once using an equal volume of buffer-saturated phenol, then again extracted once with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). To the extracted solution 3 volumes of ethyl alcohol and 10 µl of 3 M sodium acetate are added and the mixture is stored overnight at –20°C to precipitate the DNA. Then the mixture is centrifuged at 14,000g at 4°C to obtain a DNA precipitate, which is dissolved in 100 ml TE.

PCR

The PCR reaction mixture was composed of *Taq* polymerase (3 u), single stranded DNA (5-10 ng), $MgCl_2$ (3.5 mM), dNTPs (0.25 mM) and the primers (20 mM each) T7 (5'-AATACGACTCACTATAG-3') and SK (5'-CGCTCTAGAACTAGTGGATC-3') with 10X PCR buffer and made up to 50µl using sterile water. The PCR reaction consisted of 3 min ramping time from room temperature to 94°C and 20 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C. The PCR products were purified using commercially available PCR product purification columns (Eppendorf 5prime).

Reassociation

The purified PCR products (5 ng) were mixed with single stranded DNA (extracted from the phagemid (20 ng)) and blocking oligonucleotides T7 (5'-

AATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAG-3') and SK (5'-CTCGTGCCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGA-3') and incubated at 80°C. The blocking nucleotides were synthesized to be homologous to the pBluescript vector containing the inserts. The mixture was then hybridized using hybridization buffer (0.1 M Tris pH 8.0, 1 M NaCl and EDTA 0.5 M) at 30°C for 24 hrs. The DNA concentration (5 ng) and the time (24 hrs) for incubation were selected to get a Cot value (Co-conc. of DNA, t-time) of about 5.0 moles nucleotides x sec/L.

HAP Chromatography

The hydroxyapatite (HAP) column elution parameters were optimized by adding calf thymus DNA (a mixture of denatured ss DNA and ds DNA) and eluting the DNA using different concentrations of phosphate buffer. The hybridized sample mixture was then allowed to flow through the column to purify the remaining unhybridized ss DNA from the hybridized ds DNA. The ds and ss DNA can be eluted separately using different molarities of phosphate buffer (0.12 M – 0.16 M for ss DNA and 0.32 M – 0.40 M for ds DNA). The eluted ss DNA was extracted twice with 2-butanol and precipitated with ethanol.

Conversion of ss DNA to ds DNA

The ss DNA (from the HAP column) was later converted into ds DNA using a thermal cycler and a program consisting of no denaturation step, annealing at 55°C for 5 min and extension at 72°C for 30 min. The primer used was the T7 primer; except for the

primer and the template, other reaction mixture components were the same as in the PCR reaction described previously.

Plasmid Purification and Sequencing

The ds DNA was transformed into the *E. coli* strain XL1 Blue MRF' and selected colonies were grown overnight in 2x Yeast Tryptone broth. The cells were pelleted and lysed to obtain plasmid DNA using an alkaline lysis mini-prep DNA purification kit and following the protocol supplied by the manufacturer (Qiagen). The purified ds DNA was sequenced by thermal cycle sequencing reactions using BIG dye labeled dideoxy nucleotide terminators (Applied Biosystems). Standard procedure for sequencing was followed as recommended by the manufacturer (Perkin Elmer).

Results and Discussion

Initially, eleven clones from an unnormalized *C. tenellus* cDNA library were selected and sequenced to estimate the overlap of identical, high copy number genes within the selection group. Eleven clones from the *C. tenellus* cDNA library were randomly picked and sequenced; the results are summarized in Table 4. About 40% of the clones sequenced were identical, 80% were known sequences while the remaining 20% were unknown sequences. Since selection of random clones of the *C. tenellus* cDNA library resulted in 40% identical sequences, there was justification for normalization of the library.

Sequences	# of clones	Size in bp	GenBank accession #
NADH dehydrogenase*	1	497	-
ATP synthase	2	561	AF176696
16S rRNA*	4	391	-
Cytochrome c oxidase	1	652	AF176697
NADH-Ubiquitin oxidoreductase	1	429	AF177277
Unknown #1**	1	627	AF177777
Unknown #2**	1	184	AF177778

Table 4. Sequences of cloned DNA, selected randomly from unnormalized *C. tenellus* cDNA library
*Not submitted to GenBank
**No match was found in the database

A normalization of the cDNA library created from the leafhopper, *Circulifer tenellus*, was carried out to ensure that the library contained equal amounts of cDNA from each gene expressed. A normalized library is very useful in exploring novel genes when the clones are randomly picked and sequenced, because the normalization process results in all the genes being present in approximately equal numbers such that the probability of randomly choosing and sequencing different genes is very high.

The cDNA library from the leafhopper was constructed using the Uni-ZAP insertion vector; the titer of the library was $1x10^7$ pfu/ml. The phagemid containing the cloned insert from the Uni-ZAP vector was excised using a mass excision protocol mass excision protocol, and the titer of excised phagemid was $3x10^8$ cfu/ml.

Two different helper phages (VCS M13 and M13 KO7) were tried in the mass excision protocol to extract maximum amount of ss DNA from the phagemid. The concentration of ss DNA extracted from the phagemid varied from 20 – 80 µg/ml (regardless of which helper phage was used). However, the yield and the pattern of the ss DNA profile after PCR amplification was different when phage VCS M13 was used, compared to when M13 KO7 was used. VCS M13 yielded more amplified ss DNA than M13 KO7, and the ss DNA (VCS M13) profile showed that different sizes of ss DNA were amplified (Fig. 12, lanes 2-5). No visible pattern of M13 KO7 ss DNA was produced (Fig. 12, lanes 6-8). In VCS M13-produced ss DNA the presence of a ~1.2 kb band of greater intensity than the rest of the bands is intriguing. Because the profile should be like a streak of bands throughout the lane such that different sizes of DNA amplified are in equal amount to proceed with normalization.

When the ss DNA (from the HAP column – normalized library) was converted to ds DNA and transformed, it yielded no transformants. Changing the method of transformation from chemical to electroporation also failed to produce any colonies.

It is possible that the phosphate buffer used for eluting ss DNA from the HAP column might have an inhibitory effect on transformation.

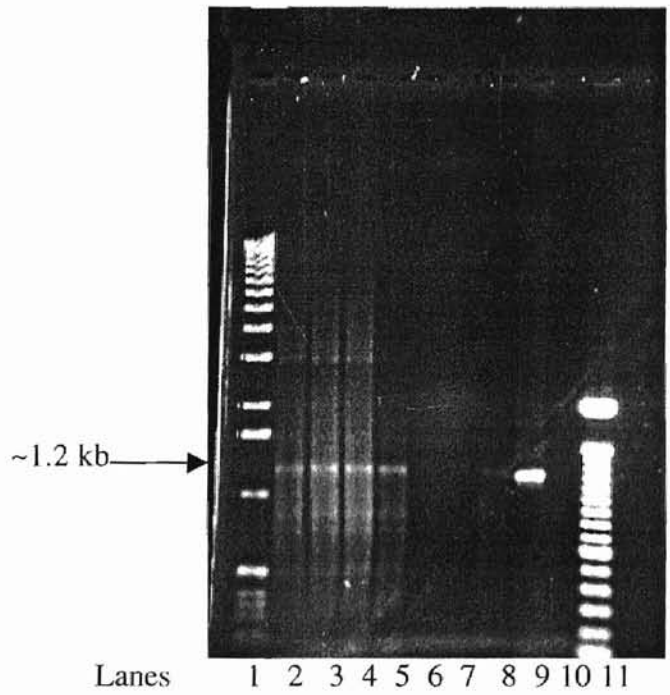

Fig. 12 PCR amplified ss DNA from phagemid
Lane 1. 1 kb DNA marker, 2-5 ss DNA obtained using VCS M13 helper phage, 6-8 ss DNA obtained using M13 KO7 helper phage, 9. positive control, 10. negative control and 11. 100 bp DNA ladder

To test the quality of the ss DNA that was initially extracted from the phagemid (and subsequently used for normalization), it was converted to ds DNA and transformed. Only few transformants were seen on the LB agar plate. It is necessary to investigate whether all the DNAs extracted from the phagemids are ss DNA, or just the helper phages. One useful approach may be to convert the ss DNA extracted from phagemid to ds DNA, then make the ds DNAs single stranded, for use in subsequent normalization steps. In this way we can be sure that only ss DNAs are used for normalization.

VITA 2

ANANDA S CHANDRASEKARAN

Candidate for the Degree of

Master of Science

Thesis: DISTRIBUTION OF P58-LIKE GENES AMONG MOLLICUTES

Major Field: Biochemistry and Molecular Biology

Biographical:

Education: Graduated from Bharathi Vidya Bhavan Matriculation Higher Secondary School, Erode, India in June 1988; received Bachelor of Science degree in Zoology from Bharathiar University, Coimbatore, India in December 1992; received Master of Science degree from Bharathidasan University, Tiruchirappalli, India in October 1995. Completed the requirements for the Master of Science degree with a major in Biochemistry and Molecular Biology at Oklahoma State University in July 2000.

Professional Experience: Senior Research Fellow, Center for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, India May 1996 to December 1997; Research Assistant, Department of Biochemistry and Molecular Biology, Oklahoma State University, January 1998 to present.