ARBITRARILY PRIMED PCR OF cDNA FOR INVESTIGATION OF DIFFERENTIAL PRESENCE AND EXPRESSION OF GENES IN THREE LINES OF *SPIROPLASMA CITRI* AND SEQUENCE ANALYSIS OF *SERRATIA MARCESCENS* STRAINS ASSOCIATED WITH CUCURBIT YELLOW VINE DISEASE

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AND

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MARCESCENS STRAINS

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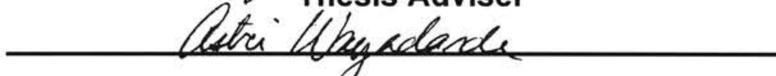

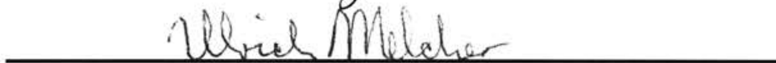

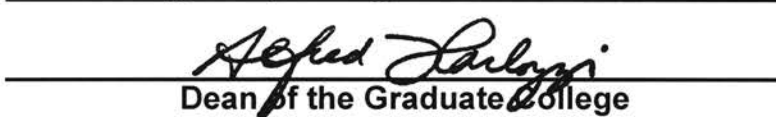

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CHAPTER I

INTRODUCTION

1. Arbitrarily Primed PCR of cDNA for Investigation of Differential Presence and Expression of Genes in Three Lines of *Spiroplasma citri*

Spiroplasma is one of several genera of the Class Mollicutes, a large taxonomic group of prokaryotic organisms notable for their lack of cell walls and the low guanidine-thymidine (G+C) content (~25%) of their genome. Spiroplasmas are unique among the mollicutes with respect to their helical physiology and a spiralling motility that is independent of flagella. Their genome sizes range from about 990-2200 kb, depending on species (Carle et al., 1995), and, like many other mollicutes, they utilize the triplet sequence UGA to encode tryptophan rather than as a stop codon as do other organisms.

Phytopathogenic spiroplasmas are transmitted in natural environments by phloem-feeding insects, predominantly leafhoppers in the order *Hemiptera*, and are found in the phloem of host plants. Although spiroplasmas were initially described by Davis and Worley (1973) in association with corn stunt disease, the agent of citrus stubborn disease, *Spiroplasma citri*, was the first spiroplasma to be cultured in artificial media (Saglio et al., 1973). This pathogen causes a lethal stunting disease in a number of host plants, including citrus and several brassicaceous crops and weed species (Kaloostian et al., 1976; Gumpf, 1988).

Three lines of *Spiroplasma citri* BR3, derived following different maintenance conditions over several years, differ in their ability to be transmitted by the beet leafhopper, *Circulifer tenellus* (Wayadande and Fletcher, 1995; Fletcher et al., 1998). The triply-cloned progenitor, BR3-3X, was originally isolated from horseradish plants with brittle root disease (Fletcher et al., 1981). The derivative line BR3-T (insect-transmissible) was maintained in turnip plants via leafhopper transmission, while BR3-G (graft-transmissible) was maintained exclusively in plants by graft transmission, and is now nontransmissible by insects.

Extensive genomic variations were found among these closely related lines (Ye et al., 1996), yet it was not known whether genes present in all three lines might be differentially expressed in different lines. Both of these circumstances should be reflected in differential mRNA presence. To investigate these possibilities, total RNA from BR3-3X, BR3-T, and BR3-G was isolated by cesium chloride ultracentrifugation and used to produce both single- and double-stranded cDNA. A variety of randomly selected (arbitrary) primers were used singly to prime the synthesis of polymerase chain reaction (PCR) products from each cDNA line using low-stringency PCR (arbitrarily primed, or AP-PCR). Certain primers were shown to prime synthesis of differential products, resulting in different "fingerprints" for each line. Some of these variant fragments were then cloned and sequenced, and resulting sequences compared to existing nucleotide databases using BLAST-X and BLAST-N. Selected differential

fragments were also used as probes against total DNA and RNA from the three spiroplasma lines.

2. 16S and *groE* Sequence Analysis of *Serratia marcescens* Strains Associated With Cucurbit Yellow Vine Disease

Yellow vine disease (YV) of cucurbits in Oklahoma was first described as a severe wilting pathology of pumpkins and squash in 1988 (Bruton et al., 1995). Exhibiting characteristic symptoms of yellowing foliage, wilting, and phloem discoloration, this disease has since caused severe losses in cantaloupe and watermelon crops as well. Disease incidence has varied annually from small, isolated outbreaks to complete crop loss, especially in early-planted fields (Avila et al. 1998). Bruton et al. (1998) reported consistent association of disease symptoms with the presence of rod-shaped bacteria in the phloem sieve elements of YV-symptomatic plants, detected using transmission electron microscopy. Avila et al. (1998) designed primers for detection of the YV organism by PCR and found that the deduced nucleotide sequence for 16S ribosomal DNA placed this organism within the gamma-3 proteobacteria. The nearest apparent relative to the YV bacterium was *Serratia marcescens*, a species not usually associated with plant diseases.

To substantiate the placement of the YV bacterium within the genus *Serratia* and to ascertain its phylogenetic position, we determined the nucleotide sequences of two highly conserved genomic regions for two yellow vine isolates,

as well as for eight bacterial strains tentatively identified as *S. marcescens* isolated from soil, plants, and clinical human infections.

The first conserved sequence chosen for study was the 16S rDNA, a region of approximately 1500 base pairs. Analysis of 16S rDNA sequences is one of the most useful and widely accepted means for assessment of the taxonomic relationships of microorganisms (Kolbert and Persing 1999; Woese 1987). The second region was the *groE* operon, which is highly conserved in the bacterial world and encodes stress proteins functioning as chaperonins, products essential for cell viability (Harada and Ishikawa, 1997). The *groE* operon has been effective in the classification of closely related bacterial species possessing 16S rDNA sequences too highly conserved to be useful for distinction (Harada et al., 1997).

Relevant 16S and *groE* sequences were obtained by PCR of genomic DNA from these ten isolates. These data were used to establish phylogenetic trees describing the relationships among these isolates as well as to other members of the Enterobacteria.

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CHAPTER II

LITERATURE REVIEW

I. MOLLICUTES AND SPIROPLASMAS

Spiroplasma is one of several genera of the Class *Mollicutes*, a large taxonomic group of prokaryotic organisms notable for their lack of cell walls and the low guanidine-thymidine (G+C) content (~25%) of their genetic makeup. The genome size of the genus *Mycoplasma*, as little as 600 kb for certain species, was believed by some (Fraser et al., 1995) to represent the absolute lowest limit of genetic material required for a self-replicating cellular organism, although the actual lowest limit now appears to be somewhat lower (Mushegian and Koonin, 1996). Certain acholeplasmas and spiroplasmas have genomes approximately three times that size (Carle et al., 1995), yet are still only one-third the size of genome of the prototypical prokaryote *Escherichia coli*. This minimal genetic quotient reflects the absence of several key pathways in mollicutes, including cell wall synthesis, purine biosynthesis, functional tricarboxylic acid cycle, and cytochrome-based electron transport functions (Dybvig and Voelker, 1996). Mollicutes lack many other genes normally present in other bacteria, such as genes functioning in DNA repair, as well as those necessary for the synthesis of certain amino acids, lipids, and fatty acids (Sears et al., 1989). The absence of these biochemical pathways in mollicutes underlies the complete dependence of these prokaryotes on higher organisms, and as a result of evolution they have developed a close co-existence with the cells of eukaryotes (Maniloff, 1983).

Mollicutes have among the lowest %G+C in their DNA of any bacteria (Woese, 1987). As noted by Ishikawa (1987), parasitism and endosymbiosis by microbes tend to result in the accumulation of adenosine plus thymidine (A+T) pairs, lowering the G+C content; that is, nonfunctional genetic elements tend to accumulate A+T. This seems somewhat paradoxical for mollicutes, which already have the smallest genomes known, but introduces the possibilities that, despite their small genomes, many mollicutes still carry unused genetic material, and that their genomes may continue to shrink. This should hold true particularly for those mollicutes with somewhat larger genomes and a lower % G+C, such as spiroplasmas. Nonfunctional genetic elements in mollicutes probably arose as a consequence of their close relationship to hosts, on which they rely to provide most of their basic anabolic needs.

Initial justification for the designation of *Mollicutes* as a new class was based on a number of factors that appeared to separate known mollicutes from other prokaryotes. In addition to the absence of a cell wall or its chemical precursors and low %G+C in their DNA, these features include pleiomorphic cellular morphology, filterability through membranes of 450 nanometer pore diameter or greater, small genomes ranging from approximately 500 to 1000 megadaltons (Mda) , and lack of reversion to walled forms (Freundt 1975). Mollicutes are believe to have evolved by genome reduction from the genus *Clostridium* or related walled antecedents (Woese, 1987).

Taxonomic classification of mollicutes has been greatly refined in recent years through advances in the genetics and molecular biology of these organisms, especially by exploitation of highly conserved entities such as ribosomal nucleic acid and DNA/DNA homology as evolutionary markers. The original classification of species within this class was based primarily upon phenotypic characteristics, such as morphology and biochemical and serologic properties, and is largely reinforced by these newer techniques, which rely primarily upon genetic relatedness (Dybvig and Voelker, 1996).

A proposed classification of the higher taxa of bacteria by Murray (1984, Bergey's Manual) suggested four divisions among the kingdom *Prokaryotae*: Division I, *Gracilicutes*, the gram-negative bacteria; Division II, *Firmicutes*, the gram-positive bacteria; Division III, the wall-less mollicutes; and Division IV, the archaebacteria. More widely accepted was a classification scheme proposed by Tully et al. (1993), which placed the class *Mollicutes* into four orders:

Order I, *Mycoplasmatales*, sterol-requiring mollicutes primarily associated with vertebrates; containing one family (*Mycoplasmataceae*) comprised of two genera, *Mycoplasma* and *Ureaplasma.*

Order II, *Entomoplasmatales*, mollicutes primarily associated with insects; containing Family I, the nonhelical *Entomoplasmataceae*, comprised of two genera (sterol-requiring *Entomoplasma* and non-sterol-requiring *Mesoplasma*), and Family II, the helical, sterol-requiring *Spiroplasmataceae*, comprised of a single genus, *Spiroplasma*.

Order III, *Acholeplasmatales*, non-sterol-requiring mollicutes having a wide range of animal and plant hosts; containing a single family (Acholeplasmataceae) comprised of a single genus (*Acholeplasma*).

Order IV, *Anaeroplasmatales*, obligately anaerobic mollicutes; containing a single family (*Anaeroplasmataceae*), comprised of two genera, *Anaeroplasma* and *Asteroleplasma*.

Although spiroplasmas were initially described by Davis in association with corn stunt disease, the first spiroplasma to be cultured in artificial media was the agent of citrus stubborn disease. This pathogen also was the first spiroplasma to receive a Latin binomial, *Spiroplasma citri* (Saglio et al., 1973). Currently, 34 different spiroplasma groups have been identified from various environments, including plants, mammals, insects, and abiotic substrates (McCammon et al.,1988; Williamson et al.,1998). A wide variety of agents previously thought to be spirochetes, viruses, or pleiomorphic mollicutes were subsequently found to be spiroplasmas (Tully et al.,1976,1977; Williamson and Whitcomb,1974). Spiroplasmas share many properties of mycoplasmas, including the formation of "fried egg" colonies on certain types of solid media, but produce motile, helical cells in liquid culture. Spiroplasma cells vary in shape depending on their living environment. The cells can be spherical or ovoid to helical, or branched, non-helical filaments, and reproduce asexually by binary fission (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes,* 1995).

It has been postulated (Hackett and Clark, 1989) that spiroplasmas, with their motile, chemotactic, and helical properties, evolved from Gram-

positive insect-gut inhabiting bacteria and are especially suited for survival in the insect gut. Motility gives them the means to maintain their position against nutrient flow, as well as penetrate the thin (typically ~200 nm) gut membrane, from where they may invade midgut cells, the hemocoel, and salivary glands, leading to their subsequent deposition and spread to host plants. In natural environments, this sort of transmission activity apparently requires a specific relationship between the spiroplasma and its insect vector, as evidenced by the fact that only leafhoppers of relatively few species are natural vectors for *S. citri*. However, the leafhopper species *Dalbulus maidis*, which is not known to be a vector for *S. citri* in nature, actually serves as a more efficient agent of transmission following hemolymph injection than the natural insect host *C. tenellus* (Wayadande, et al., personal communication). Although a particular insect species may transmit only certain species of spiroplasma, other, nontransmissible spiroplasma species may be detected within the insect by PCR and ELISA. This is not surprising, as nonphytopathogenic spiroplasmas appear to be widely distributed among arthopods, including flies, beetles, wasps, true bugs, ticks, and butterflies (Hackett and Clark, 1989), most apparently coexisting in a benign relationship with the insect (Whitcomb and Williamson, 1979). Notable cases of spiroplasmas causing disease in their insect hosts include the “sex ratio spiroplasma” which kills the male progeny of Drosophila species (Williamson et al., 1989); *S. melliferum* as a honeybee pathogen (Whitcomb and Williamson, 1979); and a number of spiroplasmas causing disease in mosquitoes (Humphrey-Smith et al., 1991). *S. citri* may cause decreased fecundity and longevity in its leafhopper vector, *C. tenellus*

(De Almeida, et al., 1997). Observations that the effects of mollicutes on host plants were more detrimental than those on their insect vectors led Nault and DeLong (1980) to suggest that the mollicute-insect relationship, having arguably arisen prior to mollicute-plant interactions, has become less deleterious to the insect over time.

Spiroplasmas are unique among the mollicutes with respect to their helical physiology and a motile nature that is independent of flagella. Their genomes range from about 990-2200 kb in size (Carle et al., 1995) depending on species, indicating a coding capacity for approximately 1000 proteins (Cordwell et al., 1997). Like many other mollicutes, they utilize the triplet sequence UGA to encode tryptophan rather than as a stop codon. The large variations in spiroplasma genome sizes suggest that genome size is not indicative of phylogenetic position (Ye et al., 1995). At approximately three times the size of the genome of *Mycoplasma genitalium*, which has the smallest known genome of all cellular organisms, some of this larger size is due to retention of genes in spiroplasmas that have been lost in *M. genitalium* (Razin et al., 1998). Some of the extra genetic material may represent IS elements, repeated genes, integrated copies of extrachromosomal elements, or genes specific for the interaction of the spiroplasma with its plant and insect hosts. Variation in size is not localized to any one chromosomal region since numerous large restriction
fragments vary among species and strains (Ye et al., 1995). The presence and location of this extra genetic material is thought to account for a

significant source of variation among *S. citri* strains (Melcher and Fletcher, 1999).

A large amount of the *S. citri* genome consists of integrated viral sequences. At least 17 copies of SpV1-R8A2 and 2 copies of SpV1-S102 DNA were present in the *S. citri* strain R8A2, comprising up to 150,000 base pairs, or one twelfth of the genome (Ye et al., 1992). In some instances, the incorporation of these extrachromosomal elements has phenotypic consequences; a comparatively higher growth rate was attributed to the presence of larger numbers of SV3-virus-derived sequences in some spiroplasma strains (Razin et al., 1987); integration of SVTS2 DNA confers immunity to SVTS2 infection (Sha et al., 1995); and erythromycin resistance has been reported to result from integration of plasmid DNA (Salvado et al., 1989). Additionally, variation in methylation of spiroplasma strains may be due to integration of viral sequences into a DNA methylase gene (Melcher et al., 1999).

Spiroplasmas are arguably among the most abundant microbes on earth (Hackett and Clark, 1989), and are primarily found in association with arthropods as pathogens, commensals, or endosymbionts. Whereas *Mycoplasma* species are ubiquitous parasites and pathogens of animal and human cells, members of the genera *Spiroplasma* and *candidatus Phytoplasma* are prevalent primarily among plants and insects. In plant diseases, both of these mollicutes are confined to and multiply in the phloem, with the former causing physiological symptoms ranging from stunting to phloem necrosis, and the latter inducing hormone imbalances resulting in

symptoms such as phyllody, virescence, and floral asymmetry. Phytopathogenic spiroplasmas are transmitted in natural environments by phloem-feeding insects in the class Homoptera, predominantly leafhoppers, and are found in the phloem of host plants. *S. citri*, *S. kunkelii*, and *S. phoenicium* are the only spiroplasma species known to be plant pathogens. Specifically, *S. citri* is responsible for the diseases horseradish brittle root (Fletcher et al., 1981) and citrus stubborn (Markham et al., 1974). Leafhopper transmission of *S. citri* in the United States occurs by members of the species *C. tenellus*, *Scaphytopius acutus*, and *S. nitridus* (Kaloostian et al., 1976; Oldfield et al., 1977a and 1977b). *S. citri* is able to infect plants of many families when introduced into functional sieve tubes by insect vectors. At least 35 species of plants in 12 families have been reported as natural hosts of *S. citri* (Calavan and Bove, 1989). Only two monocotyledons, leek and onion, are known to be host species (Oldfield et al., 1979).

Plants infected with spiroplasmas exhibit a variety of symptoms that vary according to host plant, environmental conditions, and spiroplasma strain. Characteristic symptoms include stunting, shortened internodes, abnormally small leaves, fruits and flowers, leaf mottling, proliferation of growing points, poor root growth, and/or death of roots followed by lethal wilting (Kaloostian et al., 1979). Hormonal imbalance, disruption of normal membrane permeabilities, occlusion of phloem, and other mechanisms may be involved in disease processes initiated by spiroplasmas (Lee and Davis, 1980). *S. citri* is often found as a component of multiple infections, especially in association with phytoplasmas and viruses. The beet leafhopper-

transmitted virescence agent phytoplasma tends to give some degree of protection against the pathogenic effects of *S. citri* in citrus, presumably due to competition (Oldfield et al., 1983). Protective effects are also evident in plants coinfected with *S. citri* and the aster yellows phytoplasma (Kloepper and Garrott, 1983). In coinfections with mosaic viruses of horseradish (Fletcher et al., 1984), however, neither invasion nor disease development by *S. citri* was limited.

S. citri can be transmitted from plant to plant neither mechanically nor through seed, but readily multiplies in suitable vector insects in a manner that is both propagative and persistent; that is, it multiplies within the insect, which then remains inoculative for life (Liu et al., 1983). To be transmitted to a potential host plant, spiroplasmas ingested from plant phloem adhere to the gut epithelium and move across this barrier into the hemocoel, where they multiply (Hackett and Clark, 1989; Kwon et al., 1999). The pathogen must traverse the membrane barriers in the leafhopper midgut to reach the hemolymph before crossing another membrane barrier to enter the salivary glands, where it again multiplies (Liu et al., 1983). It then exits the insect via saliva that is introduced into the plant phloem, from which it is translocated throughout the plant. As certain spiroplasma lines have lost the ability to be transmitted via leafhoppers, passage through membrane barriers in the leafhopper may potentially be an event requiring the appropriate ligand for specific recognition by membrane receptors mediating endocytosis.

Our laboratory is particularly interested in this last facet of the spiroplasma/ insect relationship. An *S. citri* line designated BR3 was originally

isolated from horseradish plants with brittle root disease (Fletcher et al., 1981). The triply cloned isolate, BR3-3X, was maintained in several ways over a period of approximately 15 years. *S. citri* line BR3-G was obtained through extended plant-to-plant transmission of the original isolate by grafting in Madagascar periwinkle, the pathogen at some point undergoing a phenotypic switch and losing insect transmissibility. The barriers to transmission of strain BR3-G lay at both the gut and salivary gland membranes, as this strain was able to multiply to high titers in the insect hemocoel upon injection, yet failed to cross the gut membrane into the hemocoel following insect feeding (Wayadande and Fletcher, 1995). Line BR3-T, on the other hand, was derived following repeated transmission from turnip to turnip via its natural insect vector, *C. tenellus.* This line, like the progenitor BR3-3X, remains insect transmissible (Fletcher et al., 1996). Characterization of surface proteins in these lines revealed at least 12 different surface-exposed proteins (Fletcher et al., 1989), two of which (approximately 92 and 144 kDa) were absent in the transmission-deficient BR3-G. Additionally, a continuous cell line originating from embryonic *C. tenellus* cells has been developed for use as an in vitro model to examine the mode of entry of *S. citri* into insect host cells (Wayadande and Fletcher, 1998). Adherence of protease-treated *S. citri* cells to this cell line was significantly reduced relative to untreated *S. citri* cells, suggesting a possible surface protein role in adhesion (Yu et al., 1997).

Investigations of *S. citri* BR3 strains at the genetic level in our laboratory have revealed a surprising degree of heterogeneity among these

closely related lines. Physical genome mapping by pulsed field gel electrophoresis (PFGE) of BR3-3X and derivatives BR3-T and BR3-G revealed that genetic organization was relatively conserved between BR3-3X and BR3-T, although the latter was now approximately 150 kb larger than the former (1750 kb versus 1600 kb). The nontransmissible BR3-G, however, had undergone a large (approximately half of the chromosome) chromosomal inversion with deletions of approximately 5-10 and 10-20 kb at each inversion border (Ye et al., 1996). Still, at 1870 kb, the genome of BR3-G had increased 270 kb relative to its BR3-3X progenitor. The identity and source of this additional genetic material remains unknown. These results would seem to be at odds with the general concept of mollicute evolution through genome reduction. Hybridization of spiroplasma virus SpV1 DNA to restricted genomic DNA from the three lines resulted in multiple bands and very different patterns for different lines, suggesting the direct or indirect involvement of SpV1-like sequences in the extensive chromosomal aberrations seen in these lines (Ye et al., 1996). Evidence for this hypothesis emerged upon further characterization of the inversion borders. A negative screening strategy enabled the identification of a 9.5 kb segment of BR3-3X DNA spanning the endpoints of the deletion (Ye et al., 1997). The inferred translation product of the far left truncated region resembled the transposase encoded by the spiroplasma virus SpV1 R8A2B (Renaudin et al., 1990), while the right truncated region showed evidence of homologous recombination between two C4-similar sequences from SpV1 on opposite sides of the chromosome (Melcher et al., 1997; Ye et al., 1996).

While interesting, these differences between transmissible and nontransmissible lines do not yet explain the molecular basis for the loss of insect transmissibility. Three primary objectives must be attained for successful insect transmission; a) recognition of and attachment to host cell barrier(s), as might be accomplished with an adhesin-like element, much like the P1 adhesin of *Mycoplasma pneumoniae* (Kahane et al., 1985); b) traversal of host cell barriers, possibly through receptor-mediated endocytosis; c) survival within the new environment encountered subsequent to crossing cell barriers, including survival of the host immunity response and ability to survive in environments peculiar to host (non-immune factors). With respect to these requirements for successful transmission, possible genetic mechanisms to account for loss of transmission may include a) truncation and subsequent inactivation (during the aforementioned chromosomal inversion), or deletion, of a gene coding for an adhesin-like product; b) a mutation resulting in the production of a product functioning as a repressor of production of such a product; c) the gene encoding the necessary product is commonly present but differentially expressed due to loss or alteration of a gene encoding, for example, an environmental recognition factor which in turn regulates production of an adhesin-like molecule; or d) loss or inactivation of a gene responsible for a product relating to the ability to survive and/ or multiply within the leafhopper.

We have hypothesized that spiroplasma strain BR3-G fails to produce a gene product relating to transmissibility, such as a putative adhesin, due to differential gene expression. In this study, we sought to identify potential

transmissibility-related genes induced only in the transmissible lines, using arbitrarily-primed PCR (AP-PCR) with mRNA (in the form of cDNA) from the three spiroplasma lines as templates. Gene products not made due to missing DNA sequences may be distinguishable using AP-PCR.

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II. ARBITRARILY PRIMED PCR

Polymerase chain reaction (PCR), originally described by Saiki et al. (1985), is most commonly employed as a means to amplify a specific, known sequence from a complex mixture of nucleic acids. The primers used, therefore, must be absolutely specific for the sequence that is to be amplified, as the highly stringent annealing temperatures used will allow binding of homologous sequences only. In contrast to this "conventional PCR", arbitrarily primed PCR (AP-PCR, sometimes referred to as "random PCR") (von Eggeling et al., 1995), utilizes primers with sequences that have no known homology to sequences present in the template nucleic acid, together with low-stringency annealing temperatures that allow primer binding to, and subsequent amplification of, regions having only partial similarity to the template sequence. Although there are usually several mismatches between the annealing sites and the primer, the best annealing sites are specific for each primer and nucleic acid mixture. When used to compare genomic DNAs from different individuals, varying results obtained using AP-PCR can represent point mutations as well as insertions and deletions of various sizes. Additionally, differences in AP-PCR products obtained from various subsets of RNA (or its equivalent cDNA) from an isogenic source can represent differentially expressed genes.

The earliest examples of AP-PCR in the literature are two papers published simultaneously in 1990 by Welsh and McClelland (1990) and by Williams et al. (1990) in *Nucleic Acids Research*. These two seminal papers introduced a quick and surprisingly simple means of establishing a set of

genomic fingerprints, represented as specific DNA polymorphisms, for different yet closely related organisms. A particularly attractive feature of both processes was that no prior sequence information was required for the individual organisms to be tested.

Welsh and McClelland investigated the use of single, arbitrarily chosen primers (20 and 34 base pairs) to differentiate DNA isolated from eleven strains of *Staphylococcus pyrogenes*; from twenty-four strains of *Staphylococcus* from five species; and from three subspecies of *Oryza sativa* (rice). Their PCR protocol included two cycles of low-stringency annealing at 40°C for five minutes, followed by ten cycles of high-stringency annealing at 60°C for one minute. At this point, additional Taq polymerase was added, along with additional dNTPs, one of which was radiolabeled, and the high stringency protocol was continued for an additional 20 to 30 cycles.

The authors' rationale for the principle of AP-PCR was that, at a sufficiently low annealing temperature, primers could be expected to bind at a number of sequences with a variety of mismatches. Some of these annealings would be within a few hundred base pairs of each other and on opposite strands, and thus amplifiable. The extent to which amplification occurred would depend upon the efficiency of priming at each annealing point, and on the efficiency of extension.

In this study, the authors identified five parameters affecting the reproducibility, number, and intensity of bands in these AP-PCR fingerprints: salt (magnesium chloride) concentration, primer length, primer sequence, annealing

temperature, and template concentration. Exploring the latter two variables in more detail, they found that consistent AP-PCR could be achieved over a fairly broad annealing temperature range of about 36°C to 48°C, with a template concentration between 30 picograms and 7.5 nanograms. Earlier experiments showed the lower level of reproducibility to be about 10 picograms, representing about 3000 molecules of *S. aureus* genome ($3x10^6$ base pairs). The authors assumed that even the best priming events are quite inefficient given the low template concentration, and that the probability of initiating each AP-PCR event during each of the two low temperature cycles was about one in 55 (the square root of 3000).

The protocols followed by the Williams group (Williams et al., 1990) were similar to those employed by Welsh and McClelland, but differed in critical ways. They chose to investigate the effects of using a set of shorter (9-10 base pairs) primers, synthesized specifically for these experiments, in PCR reactions using a lower (36°C) annealing temperature for 45 cycles, with no higher-stringency steps. Additionally, a much higher DNA template concentration (25 nanograms per 25 microliter reaction) was used in every case, and the effects of variant template concentrations were not explored. Much more effort, however, was devoted to determining the effects of primer composition than was the case for the Welsh group. Williams et al. suggested the acronym RAPD, standing for Random Amplified Polymorphic DNA, for this process.

In the first procedure of Williams et al. (1990), two different isolates or species each of human, soybean, corn, and fungal (*Neurospora crassa)* genomic

DNA were subjected to low-stringency PCR using a single, different primer in each case, with primer constraints being 9-10 base pairs in length; no palindromic sequences present; and G+C composition of 50-80%. Additionally, negative control samples were included in which genomic DNA was omitted from the reactions, to confirm that the observed bands were amplified genomic sequences and not primer artefacts. Several DNA products were apparent in each non-control reaction, and at least one polymorphic sequence was apparent between individuals in each subset.

To determine the contribution of each nucleotide to the specificity of the amplification reaction, a set of eleven related oligonucleotide 10-mers was synthesized for use with DNA from two different soybean species, *Glycine max* and *G. soja*. Each primer differed from the prototype 10-mer by substitution of a single nucleotide at a successive position in the sequence, with the G+C content of all primers maintained at 50%. Most nucleotide substitutions caused a complete change in the pattern of amplified DNA as compared to the original primer, and in many cases revealed new polymorphisms. In further amplification experiments using *G. max* and *G. soja*, and primers between six and ten base pairs in length, and of 10-mers with G+C% ranging from zero to one hundred, a nine-base pair oligonucleotide with a minimum of 40% G+C was required to yield detectable levels of amplification products.

"DNA fingerprinting" of the sort originally described in these two papers introduced a means by which closely related organisms could be differentiated, as well as a method which could potentially be used to pinpoint a particular gene

expressed differentially in closely related individuals. This type of technique was described first by Martin et al. (1991), when the RAPD protocol was used to identify a region closely linked to the *Pto* gene conferring resistance to *Pseudomonas syringae* pv. tomato in two near-isogenic lines of tomato. One hundred forty-four random primers were screened on these lines, and seven amplified products were identified that were present in one line but not the other. Of four products that were further investigated, three were confirmed by segregation analysis to be tightly linked to the *Pto* gene.

A further extension of the use of random PCR (Welsh et al., 1992) used arbitrary primers to fingerprint RNA populations from different tissues of the same mouse, and from the same tissues from mice of different strains. Two different 20-mers were used singly to prime the synthesis of cDNA from six concentrations of RNA (1.6 nanograms to 2.5 micrograms), using reverse transcriptase. The cDNA was then made double-stranded in a PCR reaction initiated by the same primer used for cDNA synthesis. The resulting products, having the same primer sequence at both ends, were subjected to high-stringency PCR with simultaneous radiolabelling, separated by acrylamide gel electrophoresis, and visualized by autoradiography. Five products appearing differentially in kidney and heart tissue were purified from the gel, reamplified, and used to probe Northern blots of heart and kidney. Four of these products hybridized primarily or exclusively to the expected tissue, while the fifth hybridized to RNAs in both tissues.

In a study that greatly extended the application of AP-PCR for the identification of differentially expressed genes, Liang and Pardee (1992) introduced the procedure of differential display. Here, a partially degenerate primer included a longer, anchored oligo-dT segment, along with a shorter arbitrary sequence. Subpopulations of mRNA defined by these primer pairs were amplified by PCR following reverse transcription, and multiple primer sets yielded reproducible patterns of amplified complementary DNA fragments that showed strong dependence on sequence specificity of either primer. Individual mRNAs exhibiting differential expression between different cells or tissues, or under altered conditions, could be isolated and cloned.

The primary objective of this project was to determine, if possible, the mechanism underlying the loss of insect transmissibility in the *Spiroplasma citri* line BR3-G. Three lines of *S. citri* BR3, derived following different maintenance conditions over several years, differ in their ability to be transmitted by the beet leafhopper, *Circulifer tenellus*. The triply-cloned progenitor, BR3-3X, was originally isolated from horseradish plants with brittle root disease (Fletcher et al., 1981). The derivative line BR3-T (transmissible) was maintained in turnip plants via leafhopper transmission, while BR3-G was maintained exclusively in plants by graft transmission, and is no longer insect- transmissible. Previous research in our lab had shown (Ye et al., 1996) that BR3-G had experienced extensive chromosomal rearrangements, including a large chromosomal inversion with deletions of about 10 kb at each inversion border. This suggested the possible loss of an expressed gene (or genes) now absent in the nontransmissible BR3-G

line. In addition, it is possible that genes present in all three lines may be differentially expressed in different lines.

AP-PCR of cDNA was selected to determine whether differential gene expression was a factor in the loss of insect transmissibility in *S. citri* line BR3-G. Its application allowed the potential identification of either a gene commonly present but differentially expressed between transmissible and nontransmissible lines, or, alternatively, an expressed gene that was now absent in the nontransmissible BR3-G line.

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CHAPTER II

LITERATURE REVIEW

III. *SERRATIA MARCESCENS* AND THE CUCURBIT YELLOW VINE DISEASE ORGANISM

III-1: Disease Pathology of the Yellow Vine Organism

Cucurbits are important horticultural crops in Oklahoma and Texas, yielding an annual value greater than $100 million (USD) and occupying approximately 40,000 hectares (Bruton et al. 1995b). In the past 20 years, vine declines of cucurbits have increased in number and severity to the extent that they have become a limiting factor in yields in many production areas worldwide. In 1988, a disease called yellow vine (YV) was first observed in pumpkin (*Cucurbita pepo* var *L.*) and squash (*C. pepo* var *melopepo*) in central Texas and Oklahoma (Bruton et al. 1995a). This disease has since caused severe losses in these two crops as well as in cantaloupe (*Cucumis melo* var. *canteloupensis*) and watermelon (*Citrullus lanatas*), yet has never been observed in cucumber. Early-planted crops are more severely affected by this disease, while plantings after mid-June usually have lower disease incidence.

Disease symptoms are similar in watermelon, squash, pumpkin, and cantaloupe, and generally appear 10-15 days prior to fruit maturity. The diseased plants gradually decline and manifest a blighted appearance within 7-10 days. Leaves undergo a color change from green to lime-yellow to bright yellow. Severely affected immature plants sometimes wilt and collapse in a

single day without undergoing a change in color. Flowers and fruit of diseased plants are not disfigured, but watermelon fruit may lose their chlorophyll very quickly (Bruton et al, 1995b). The symptom most clearly associated with YV is a honey-brown discoloration of the phloem tissue, which normally exhibits a clear to translucent appearance (Avila et al, 1998; Bruton et al., 1998).

Electron microscopy of phloem tissue of YV-infected plants has revealed rod-shaped forms 0.25-0.5 μm in width and 1.0-3.0 μm in length, surrounded by a triple-layered cell envelope (Bruton et al., 1995, 1998). These structures, confined primarily to sieve tubes, are similar to those of the bacterium-like organisms (BLOs) associated with clover club leaf (Windsor and Black, 1973), citrus greening (Garnier et al., 1984), papaya bunchy top (Davis et al., 1996), and several other diseases. Comparatively little is known about BLOs, although a number of plant diseases have been attributed to them (Davis et al., 1983; Garnier et al., 1984; Hopkins 1977; Klein et al., 1979; Nourrisseau et al., 1993; Purcell and Hopkins 1996; Rumbos et al., 1977; Sha et al., 1986).

III-2: Etiology of the Yellow Vine Organism

Since YV was first observed in central Texas and Oklahoma in 1988, extensive efforts have been made to determine and isolate its causal agent and to identify the means by which it is acquired by host plants. Various fungal and bacterial pathogens are responsible for many important wilt and decline syndromes of cucurbits. Predominant among the causal agents are the fungi

Macrophomina phaseolina, *Fusarium oxysporum*, and *F. solani*, and certain species of the bacterial genus *Erwinia* (Bruton et al., 1988).

An exhaustive study by Bruton et al. (1998) included attempts to isolate fungal and prokaryotic organisms from surface-sterilized sections of infected cantaloupe and watermelon, and to specifically detect various candidate pathogens using serological and molecular methods. A large number of fungal (mostly of the genus *Fusarium*) and bacterial (22 species, largely *Enterobacter* and *Erwinia*, according to fatty acid profiles) isolates were recovered. Yet there was no consistent correlation between the prescence of any cultivable bacterium or fungus and the occurrence of symptoms in plants. Test inoculations of squash and watermelon with representative bacterial and fungal isolates failed to reproduce phloem discoloration or other characteristic yellow vine symptoms. However, successful inoculations of this type are often difficult, especially with phloem-limited pathogens.

Certain characteristics of the YV disease, such as the agent's colonization of host plant phloem tissues, circumstantial evidence of insect transmission, and inability to isolate an organism that was consistently associated with YV symptomology, suggested mollicutes as a potential causal agent. Yet Dienes' staining of cross sections of infected plant tissue (Deely et al., 1979), which might have implicated a spiroplasma or an uncultivable phytoplasma, were inconclusive (Bruton et al., 1998). More Dienes'-positive samples were found among symptomatic plants than healthy controls, but some plants showing typical

symptoms of yellow vine failed to stain and some asymptomatic plants stained positively.

Previous efforts (Bruton et al., 1995) to assess the role of insects as possible YV vectors showed that squash fields treated with the pesticides cypermethrin or esfenvalerate had a significantly lower YV incidence than untreated fields, yet fumigation with methyl bromide had little effect, essentially ruling out a soil-borne pathogen as the responsible agent. The use of aluminum and black plastic mulches significantly increased YV incidence, yet this effect was largely overcome in the presence of pesticide applications (Brurberg et al., 1995). Preliminary observations, which had suggested the involvement of the squash bug (*Anasa tristis*) or melon aphid (*Aphis gossypii*), were not definitively substantiated. Relatively high populations of squash bugs were observed in areas of high YV incidence. These insects are not known to transmit any disease-causing organism, yet can cause sudden plant wilting known as Anasa wilt through feeding (Robinson and Richards, 1931). However, evidence collected in insecticide-treated versus non-treated plots suggested that squash bug population and disease incidence were independent of each other.

Futher substantiation of an insect role in YV transmission was shown in an exclusion experiment (B. Bextine, personal communication), in which the incidence of YV was compared between uncovered squash plants and squash plants covered with fine mesh that excluded insects. Seven percent of uncovered plants and 0 % of covered plants tested positive using Dienes' stain, a relatively insensitive detection tool. In highly sensitive PCR assays using YV-

specific primers, 40% of uncovered plants and none of the covered plants were positive for the presence of YV. These differences were statistically significant ($p<0.001$), supporting the hypothesis that insects are involved in the transmission of the bacterium.

PCR primers designed to be YV-specific were originally developed by Avila et al. (1998) using primers known to amplify 1.5 kb 16S rDNA from both prokaryotes and chloroplasts. Amplified fragments resistant to restriction digestion with *Bcl* I, characteristic of prokaryotic but not chloroplast amplification products, were cloned and partially sequenced, and the resultant sequences were used to develop primers apparently specific for YV 16S sequences. A 1.5 kb fragment amplified from *Bcl* I-digested template from symptomatic plants was cloned and fully sequenced (GenBank accession number U82807). In a search of databanks for sequences closely related to the cloned sequence the highest-scoring matches were those of *Serratia marcescens*. Additionally, the predicted secondary structure of nucleotides 119 to 260 contained signature sequences characteristic of gamma-3 proteobacteria (Weisburg et al., 1991), confirming the placement of the YV bacterium in this group (Avila et al., 1998).

III-3: Bacterial Classification and Phylogeny

Completion of Koch's postulates, and confirmation of the role of a particular microorganism in the etiology of a plant disease, require characterization of the organism of interest. It may be necessary in some cases to include several closely related organisms in the analysis to assess their inter-

relationships. To properly characterize a microorganism, it is generally necessary to isolate and maintain pure cultures of the organism; otherwise one might examine the combined features of several interactive organisms or an anomaly based on environmental variations.

For classification purposes, phenotypic and genotypic criteria each have their own particular merits and drawbacks. In typing a bacterial isolate of medical interest, for example, it may be of much greater benefit to determine, preliminary to a finer degree of categorization, whether resistance to one or more antibiotics is present than to delimit the isolate by genus and species as defined by 16S or other sequence data. Conversely, it may be of greater advantage to phylogenetically characterize bacterial isolates observed repeatedly in a particular environment or relationship and/ or causing a similar symptomology, for instance, in cases of microbes having a commensal relationships with plants or animals, or causing disease in them. The seemingly intractable problem of precisely grouping bacteria on either phenotypic or phylogenetic bases stems from extensive lateral gene transfer between prokaryotes, whether by conjugation, bacteriophage lysogeny, or other means. The concept of phylogenetic analysis of the major prokaryotic groups is seen by some (Teichmann and Mitchison, 1999; Doolittle, 1999) as largely compromised by such gene transfer. This effect is compounded by the short generation time, high mutation rate, and high multiplication rate characteristic of most bacteria. One possible short-term effect of such lateral gene transfer might be two bacterial isolates having diverged from a common ancestor a short time ago (e.g.,

approximately 50 years), with each isolate having identical sequence information contained in molecules used by phylogenists as molecular "clocks" (e.g., rDNA), yet containing different assortments of metabolic, catabolic, and other genes through processes of lateral gene transfer and mutation. Such isolates might be expected to have strikingly different morphological and physiological characteristics, even though they would be considered "identical" if characterized solely by sequence data.

Presumably, classification systems based on genetic analyses reflect a natural evolutionary hierarchy more accurately than those based on phenotypic characteristics. However, for practical reasons, both phenotypic and genetic characteristics are still employed in most microbial identification and classification endeavors.

A: Nongenetically-Based Characteristics

i. Cultural

The most general and perhaps easiest method to begin bacterial classification involves the direct observation of bacterial isolates in pure culture, with emphasis on the characteristic color and morphology of bacterial colonies. Bacterial colonies are typically evaluated on the basis of form (circular, rhizoid, filamentous, spindle, punctiform, or irregular); elevation (raised, flat, convex, pulvinate, or umbonate); and margin (undulate, entire, lobate, erose, curled, or filamentous (Atlas, 1984).

ii. Morphological

Morphological characterization entails the direct microscopic observation of individual bacterial cells, noting cell size and morphology, motility, presence or absence of flagella (including number, type, and arrangement, if present), and positive or negative reaction with Gram and acid fast stains. Additional morphological variables include the presence or absence of capsules, which serve to protect bacterial cells from phagocytosis and desiccation, and endospores, which provide resistance to heat, drying, radiation, and other environmental insults (Towner and Cockayne, 1993).

iii. Physiological

Bacterial species and strains can sometimes be characterized on the basis of the optimum and extreme temperature, pH, and salinity ranges under which they can grow. Hemagglutination assays test the ability of bacterial isolates to agglutinate and/or lyse red blood cells. (Towner and Cockayne, 1993). Additional physiological criteria include oxygen relationships, osmotic tolerances, and sensitivity to various antibiotics (Atlas, 1984).

iv. Nutritional

Carbon source utilization and the ability to oxidize various carbohydrates are particularly well-characterized methods for bacterial identification. Isolates may be plated on differential media in which their ability to utilize the carbon

source present results in a color reaction. Alternatively, automated single substrate multi-test systems such as API (bioMerieux SA, La Balme-les-Grottes, France), Biolog (Biolog, Inc., Hayward, CA), and Vitek (bioMerieux Vitek, Inc., Hazelwood, MI, USA) allow the simultaneous screening of bacterial isolates for the ability to catabolize 20 or more different carbon sources. These companies also provide publicly accessible databases, allowing the comparison of the strains of interest with other bacterial species.

v. Serological

Most relevant for bacteria of clinical importance, serological methods include agglutination tests that differentiate bacterial isolates by their ability to recognize and bind to specific epitopes, or receptors, on cell surfaces (Stansfield, 1981). The common basis of most serological methods is the ability of bacterial proteins and lipopolysaccharides (LPS) to serve as antigens, invoking an immune response resulting in the production of active sera upon immunization of laboratory animals. Antibodies produced in the immune response can be used to reproducibly detect antigenic differences between different bacterial isolates. Immunodiffusion assays, for example, determine the *in vitro* ability of specific antibodies to bind to a particular antigen, or epitope, on the bacterial surface. Enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) rely on antibodies anchored to an inert surface, which have an affinity for specific antigens or surface epitopes present on bacterial cell surfaces.

vi. Chemotaxonomic

Chemotaxonomic properties reflect quantitative and/ or qualitative differences in the composition of various bacterial cellular components. For example, psychrophilic bacteria have an optimal growth temperature of 15°C or lower, a characteristic reflected in a high content of unsaturated fatty acids in the cytoplasmic membrane, allowing the retention of a semifluid state at low temperatures. Physical features of bacteria may thus serve as a means of identification and characterization, and may be measured and quantified by specialized methods. Bacterial chemotaxonomic characters may include fatty acids, polar lipids, LPS composition, proteins and lipopolysaccharides, amino acid and whole cell sugar composition of cell wall components, cellular pigments, quinone system features, and polyamine content, among others (Towner and Cockayne, 1993; Brock et al., 1994).

B: Genetically-based characteristics

Unlike the aforementioned non-genetic methods of bacterial characterization and classification, analysis of the genetic material of a microorganism presents a major advantage in that it is not subject to phenotypic variation, since it does not rely upon the expression of particular properties. Genetic methods of bacterial classification have therefore risen to the forefront in recent years, particularly as techniques such as polymerase chain reaction

(PCR) and automated sequencing have become increasiningly available and cost-effective.

I. Genotypic

a). Plasmid Analysis

Bacterial plasmids are autonomous genetic elements that replicate independently of the main chromosome. Plasmids have been found in the vast majority of bacterial genera in which they have been sought (Stanisich, 1988). Characterization of bacteria according to the size and structure of their plasmid DNA content is seldom useful for taxonomic identification of a particular bacterium, although four different phytoplasmas were reported to contain unique plasmid banding patterns and hybridization characteristics sufficient for identification (Denes and Sinha, 1991). Plasmid analysis currently has greater utility in the epidemiology and classification of bacterial strains and isolates than in species identification (Agata et al,1990; Gasparich et al, 1993).

b). DNA Base Ratio (%G+C)

Determination of the guanidine plus cytosine (G+C) base composition of an organism's DNA is required in order to name it as a new species. G+C values in prokaryotes can range from about 20% to 78% (Woese, 1987). Organisms with similar phenotypes often, but not always, have similar DNA base ratios. When two organisms thought to be closely related by phenotypic criteria are shown to have widely differing base ratios, further examination generally indicates that they are less closely related than was supposed. Also, two

organisms can have exactly the same base ratios yet be completely unrelated, as an astronomical number of possible sequences are possible with DNA of a given base composition. Therefore, GC ratios are considered an exclusionary determinant, helpful for determining unrelatedness, but not useful to support the claim that two organisms are related.

c). Randomly Amplified Polymorphic DNA (RAPD)

RAPD is a PCR procedure in which random primers (i.e., primers having no known homology to the organismal DNA of interest) are used to generate strain-specific or species-specific fingerprints. This method is based on the observation of Welsh and McClelland (1990) that a single arbitrarily chosen primer, when used in a low-stringency (low annealing temperature) PCR reaction, can generate a reproducible and discrete set of amplification products characteristic of particular genomes. At a sufficiently low annealing temperature, a primer can be expected to anneal to many sequences with a fairly large degree of mismatching. Some of these sequences will be sufficiently near each other and on opposite strands, so that the intervening sequence will be amplified. Arbitrary primers as short as 5 base pairs have been used to generate complex genomic fingerprints following PCR amplification (Caetano-Annolles et al., 1991).

d). RFLP (Restriction Fragment Length Polymorphism)

Like RAPD, RFLP analysis establishes a set of fingerprints that can be used to determine whether particular bacterial isolates are related. Using

carefully selected restriction endonucleases, the bacterial genome is cut into a series of appropriately-sized fragments amenable to gel electrophoresis. Differences in distance between restriction sites yield fragments of different sizes. Occasionally, the large number of fragments generated can limit reproducibility and make accurate interpretation of the results difficult. However, if the DNA of organisms having complex patterns of restriction fragments is denatured and tranferred by blotting techniques from the gel to a nylon or nitrocellulose membrane and then hybridized with a suitable labelled probe, a much simpler pattern results, consisting only of fragments capable of binding the probe. This increases the ability of RFLP analysis to be used for comparing bacterial isolates.

e). PFGE (Pulsed-Field Gel Electrophoresis)

A solution to genome characterization problems caused by complex chromosomal fingerprint patterns is to use 'rare-cutting' restriction enzymes to generate a limited number of DNA fragments. Conventional gel electrophoresis is incapable of separating fragments much larger than 50 kb (kilobases), but PFGE uses successively alternating electrical fields that force the migrating DNA fragments to repeatedly change their direction of migration. Separation thus is most likely based on the fact that larger DNAs change direction more slowly than smaller ones, resulting in separation due to size-based retardation. This technique is in contrast to the sieving-type separation employed by more conventional gel electrophoresis (Slater and Noolandi, 1989; Stellwagen and

Stellwagen, 1989). As with RFLP, the resulting fragments may be blotted and hybridized with selected probes. Pulsed-field gels have the additional advantage of permitting an estimation of genome size for the organism in question.

f). Specific-Primer PCR

Following the introduction of PCR, it was quickly realized that this method offered an extremely sensitive approach for the detection and identification of specific microorganisms in a variety of sample types. A characteristic DNA sequence from a single cell can potentially be amplified to detectable levels within a very short period of time.

An increasingly common method for quick bacterial identification, specific primer PCR utilizes primers targeted to a specific bacterial sequence, usually one that is putatively unique to a species or even strain. Most often this is a region within the highly conserved 16S ribosomal DNA, present in 2 to 11 copies per bacterial cell (Srivastava and Schlesinger, 1990), in a process sometimes called PCR ribotyping. This procedure is discussed in greater detail below.

II. Phylogenetic

Modern bacterial taxonomy is best served by a polyphasic approach, with 16S rDNA sequence-based phylogeny serving as a backbone (Hauben et al., 1998). Although comparisons based on multiple parameters may yield conflicting results, there may be greater degree of accuracy in characterizations which utilize more than a single approach.

a). DNA:DNA Hybridization

The percentage reassociation of denatured DNAs from remains an important parameter of microorganism species definition. In this procedure, whole-gene DNAs from similar bacteria are denatured separately, then combined and allowed to reassociate with each other. As recently as 1987, the definition of a bacterial species was a group of strains sharing 70% reassociation values and a high degree of phenotypic similarity (Wayne et al., 1987). However, it is increasingly evident that any particular cut-off value is arbitrary and not guaranteed to define bacterial groups that correspond to real ecological units (Vandamme et al., 1996). Additionally, it is not clear what determines the fraction of genomic segments that anneal in DNA:DNA hybridizations: the sequence similarity at shared gene loci, or the fraction of shared genes.

b). Sequence Analysis of 5S rDNA

The 5S ribosomal RNA molecule was once a popular choice as a molecular chronometer (Hori et al., 1979; Kuntzel, 1982). However, this molecule tends to be too small and constrained to be the ideal choice for phylogenetic characterization, having only 4-5 separate helical elements. Additionally, it has apparently undergone certain nonrandom sequence changes leading to a size increase in some *Bacillus* species (and likely other bacteria as well), which distorts attempts at phylogenetic determination (Hori et al., 1979).

c). Sequence Analysis of 23S rDNA and 16S-23S Spacer Region

Theoretically, the large subunit bacterial rRNA (23S) is the most informative of phylogenetic markers. Under optimal circumstances it provides a resolution of one evolutionary event per 0.2-0.3 million years (Ludwig and Schleifer, 1999). The greatest drawback for use of this marker is the relatively small number of bacterial isolates for which 23S data is available, although this number is increasing.

The spacer region between the 16S and 23S rRNA genes contains either one or two tRNA genes. This region is extremely variable in size and sequence even within closely related taxonomic groups (Gurtler and Stanisch, 1996). Phylogenetic analysis based on spacer region data is, like that for 23S rDNA, limited somewhat by the relative paucity of gene sequences available. However, Gurtler and Stanisch (1996) note three factors which may encourage the use of this molecule, which would in turn generate more available sequences for comparison. First, at least half of the bacterial species studied (both Gram-negative and Gram-positive) carry multiple copies of the rRNA operon, with some (e.g., *Haemophilus* and *Bacillus*) possessing as many as ten. Second, the length of the 16S-23S spacer region varies both between species and between alleles of a given species. Third, variations in the length of this spacer region are related, in part, to the number and type of tRNA genes that it contains. These factors generate a degree of heterogeneity that make the 16S-23S spacer region a suitable target for typing.

d). Sequence Analysis of 16S rDNA

The bacterial 16S rDNA molecule posesses a number of attributes that make it almost ideal for bacterial characterization and classification (Fox et al., 1980). Ribosomal RNAs exhibit a high degree of functional constancy, ensuring a good representation of the overall rate of evolutionary change in a line of descent (Woese, 1985). They are present in all bacteria, and different positions in their sequences tend to mutate at very different rates, allowing a measurement of virtually all phylogenetic relationships, both near and distant (Woese, 1987). It was 16S RNA sequencing, for example, that led to the current division of living organisms into the *Bacteria*, the *Eucarya*, and the *Archaea* (Woese et al., 1990). Their large size (about 50 helical domains) implies that they are less constrained thoughout their sequence than a smaller sequence would be, so that changes introduced into the sequence over time affects a smaller portion of the molecule, thus introducing less uncertainty into phylogenetic measurement.

Ribosomal analysis, as presently used for bacterial characterization, was made possible with the advent of PCR and automated sequencing techniques (Kharsany et al., 1999). The utility of comparing 16S rRNA gene sequences for determining phylogenetic relationships between bacteria was recognized as early as 1978 with the completion of the complete 16S ribosomal RNA gene sequence of *Escherichia coli* (Brosius et al, 1978). Popularization of this technique was greatly enhanced by the incorporation of PCR as a means by which to directly sequence 16S rDNA fragments, thus obviating the need

for cloning the gene sequence (Bottger, 1989), and by the availability of universal primers that amplify 16S rDNA sequences from a wide variety of bacteria (Weisburg et al., 1991). Prior to these advances, the relevant sequence had to be cloned, or the RNA was directly sequenced using reverse transcriptase. The former method entailed the creation of large cDNA libraries, while the latter had the drawbacks of resulting in frequent sequence abnormalities, requiring large numbers of bacteria, and necessitating large amounts of intact bacterial RNA (Olsen et al., 1986). In contrast, DNA is much more stable and easily obtained.

e). *groE* Sequence Analysis

Functional homologs of the proteins GroES and GroEL (15 and 65 kilodaltons, respectively), first identified as products of the *gro*E operon in *Escherichia coli*, occur widely in nature and are the most intensively studied proteins having a molecular chaperone function (Lund, 1995; Vezina et al., 1997). Also known as Hsp60 (heat shock) or Cpn60 proteins, they are essential at all temperatures and prevent protein aggregation *in vitro* by binding unfolded or partially-folded proteins (Circuela et al., 1997). Production of GroE proteins is also vital for cell wall synthesis; cells deprived of GroE lyse due to a lack of diaminopimelic acid, synthesis of which is GroE-dependent (McLennan and Masters, 1998). GroES and GroEL proteins are highly conserved in bacteria and almost all bacterial species have a single copy of the *gro*E operon in their genomes (Truscott and Scopes, 1998). Therefore, *groE* gene sequences are increasingly used in the assessment of bacterial phylogenetic relationships, often

in combination with 16S rDNA analysis (Ericsson et al., 1997; Dale et al., 1998). For example, DNA sequence comparison of the *gro*EL and 16S rRNA genes in an isolate of the Human Granulocytic Ehrlichiosis (HGE) agent with the homologous genes in *Ehrlichia chaffeensis* indicated that the *gro*EL gene was more variable than the 16S rDNA, yet it contained enough conservation to allow the creation of a new strain designation for this pathogen (Dumler et al., 1995).

g). Sequence Analysis of Other Protein-Coding Genes

DNA sequences of certain "housekeeping" protein-coding genes (as is *gro*E) have been more effective than DNA-DNA hybridizations for classifying the ecological diversity of bacteria (Palys et al., 1997). Apparently exclusive to *S. marcescens*, the major metalloprotease gene has been found in all all strains thus far examined and has been characterized by several researchers (Matsumoto et al., 1984; Nakahama et al., 1986; Braunagel and Benedik, 1990). The discriminatory capacity of metalloprotease genotyping has been shown equal or superior to that of ribotyping for identification of epidemiological S. marcescens isolates (Jayaratne, 1996).

III-4: Sequence Alignment Analysis

Accumulated raw sequence data is only the starting point for phylogenetic characterization. The sequence of interest is usually sequenced twice (sometimes more) in both directions to insure accuracy. The resultant data is then processed by any of a number of available alignment utilities, such as

CLUSTAL W (Thompson et al., 1994; Aiyar, 2000), or PileUp (Feng and Doolittle, 1997), which assemble the pieces into a contig, or contiguous unit. Most of these utilities are now freely available on the internet. Assembled contigs of the bacterial isolates of interest may then be analyzed using software which analyzes and assembles the data to interpret an evolutionary, or cladistic, relationship.

A: Distance Matrix

In distance matrix analyses an evolutionary distance between two organisms is calculated using the number of positions in the sequence in which the two differ (Fitch and Margoliash, 1967; Felsenstein, 1982). The possibility that multiple changes might have occurred, which would result in the same sequence in both, is accounted for using a statistical correction factor. A matrix of evolutionary distances is then analyzed using a computer algorhythm designed to produce phylogenetic trees from these measurements, resulting in an evolutionary distance. The end results are (1) an examination of all possible branching arrangements for the set of distances compared, (2) arrangement of branch lengths for each branching arrangement best fitting the data, and (3) indentification of the best resulting arrangement (as defined by the algorithm used) as the "correct" phylogenetic tree. The evolutionary distance separating any two organisms is thus directly proportional to the total length of the branches separating them.

B: (Maximum) Parsimony

The parsimony method of phylogenetic analysis generates evolutionary trees based on the assumption that only the minimal amount of evolutionary change needed to cause the divergence of two lineages from a common ancestor occurred during their evolution. Unlike distance matrix methods, parsimony does not reduce differences among sequences to a single number or distance; each position is treated individually (Felsenstein, 1982). It makes the assumption that the correct phylogenetic tree is the one for which the least number of mutations must be postulated to have occurred. A computer algorithm is used to decide upon the most parsimonious tree from among all possible resulting branching arrangements.

C: Cluster Analysis

Cluster analysis, the third major method used for sequence data analysis, groups sequences based on similarity to one another or to other groups of sequences (Sneath, 1962). It is the least computationally intensive of the three methods, but also the least accurate (Woese, 1987).

The primary obstacle in all analyses of sequence data relates to the fact that different lineages and different sequence positions can evolve at substantially different rates. Distance corrections made on the assumption that all positions in a sequence change at the same rate underestimates the degree of correction required (Golding 1983; Jukes and Cantor, 1969). Rapidly evolving lineages are often not positioned correctly by parsimony analysis, nor are rapidly

changing positions adequately evaluated; these problems are slightly less severe with distance treeing (Olsen et al., 1986). Cluster analysis is even more sensitive to these problems. Improvements in analysis will likely derive from the establishment of a database large enough to evaluate the pattern of changes at given positions in RNA molecules, so that computational analyses focused more specifically on these 'hot spot' positions may be developed (Woese, 1987).

III-5: ***Serratia marcescens***

Serratia marcescens is a Gram negative, coliform bacterial species of the family *Enterobacteriaceae*. Formerly known by a variety of names including *Chromobacterium prodigiosum* (Sleigh, 1983), the assigned type strain of *S. marcescens* is that originally described by Bizio in 1823 (Gaughran, 1968). *S. marcescens* is a non-spore-forming facultative anaerobe with peritrichous flagella. Host cell adhesion is facilitated by two types of pili, which may differ in width, sensitivity to mannose, and ability to agglutinate erythrocytes. Pili have also been associated with virulence in these bacteria (Guentzel, 1999).

S. marcescens is distinguished from other enterobacteria by the production of a number of interesting compounds that have been the focus of intensive research. These include the surfactant sarawettin, various marcescins, extracellular proteases and chitinases, the antibiotic carbapenem, and red pyrrole-containing pigments known as prodigiosins. Prodigiosin biosynthesis in *S. marcescens* decreases respiration rates (Kobayashi and Ichikawa, 1985) and is dependent on growth conditions, increasing in the presence of light, NaCl,

(Rjazantseva et al., 1994) and other factors. Pigment formation is also inversely related to flagella formation (Kobayashi and Ichikawa, 1990). Other novel functions of this compound include the uncoupling of mitochondrial and bacterial F-ATPases (Konno et al., 1998) and T-cell specific immunosuppression (Han et al., 1998). Interestingly, pigmented and non-pigmented *S.marcescens* strains have sharply different fatty acid profiles, with pigmented types showing a reduction of total lipids and a high percentage of unsaturated fatty acids (Pizzimenti et al., 1999).

Many compounds produced by *S. marcescens* are of interest for their potential as biocontrol agent, industrial catalysts, and clinical products. An epiphytic *S. marcescens* strain (MSU-97), found growing on aquatic plants in Venezuela, produces a novel macrocyclic lactone, oocydin A, which may have potential in biocontrol. This compound has demonstrated a high degree of efficacy in the control of such phytopathogenic oomycetes as *Pythium ultimum*, *Phytopthora parasitica*, *P. cinnamomi*, and *P. citrophora* (Strobel et al., 1999). Certain strains have demonstrated biocontrol potential through their production of extracellular chitinases, which which can degrade chitinous structures in fungi and insects.

For many years, *S. marcescens* was considered to be an innocuous, saprophytic microorganism of soil and water habitats. However, it was recognized by some as early as 1896 that this bacterium was responsible for more deaths than were many pathogenic bacteria (Hejazi and Falkiner, 1997). Sites of human infection include the bloodstream (bacteremia), lungs

(pneumonia), urinary tract, surgical wounds (sepsis), cornea of the eye (keratitis), and others (Guentzel et al, 1999). Infections caused by *S. marcescens* can be difficult to treat due to increasing multi-drug resistance. Many clinical isolates of *S. marcescens* are extended-spectrum β-lactamase-producing organisms and thus are able to inactivate most β-lactam antibiotics (Naas et al., 1994). As many as 90% of *S. marcescens* clinical isolates are resistant to aminoglycoside antibiotics such as gentamycin as well (Garcia et al., 1995), either through alterations of the bacterial cell envelope or through chemical modification of the drug (Sleigh, 1983). At least four plasmid-encoded β-lactamases, or carbapenemases, have been characterized in *S. marcescens* (Yano et al, 1999).

In association with plants, *S. marcescens* is chiefly noted for its positive effects. The oocydin A-producing Venezuelan strain noted above affords plant protection against *Pythium*. In addition, researchers at the International Rice Research Institute (IRRI) have demonstrated a rice endophyte, identified as *S. marcescens* by 16S sequence homology, which fixes nitrogen (Gyaneshwar, et al., 1999). In the plant rhizosphere, soil-colonizing strains of *S. marcescens* perform a role as plant growth-promoting rhizobacteria (PGPR), facilitating plant nutrition and affording protection against certain plant pathogens (Ordentlich et al., 1987). Production of extracellular chitinases is one aspect of particular interest in PGPR activity; these chitinases may degrade chitinous structural elements of fungi (Kalbe et al., 1996), insects (Lysenko, 1976), and nematodes. The synthesis of plant compounds operative in induced systemic resistance (ISR), such as peroxidases, glucanases, phytoalexins, chitinases, and

pathogenesis-related (PR) proteins, can be triggered or enhanced by certain *S. marcescens* strains as well (Lawton and Lamb, 1987; Kloepper et al., 1992).

Only two cases have been reported in which *S. marcescens* has been described as a plant pathogen; crown rots of the grain legume sainfoin (Sears et al., 1975) and alfalfa (Lukezik et al., 1982; Turner and Van Alfen, 1983). In both cases, *S. marcescens* was found as part of a pathogen complex in which the involvement of extracellular endotoxins or enzymes was suggested to the authors by the dark, dry necrotic effects caused by this disease. In this paper, we propose that cucurbit yellow vine disease is also caused by a strain of *S. marcescens*.

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CHAPTER III

Arbitrarily Primed PCR of cDNA for Investigation of Differential Presence and Expression of Genes in Three Lines of *Spiroplasma citri*

INTRODUCTION

Spiroplasma is one of several genera of the Class *Mollicutes*, a large taxonomic group of prokaryotic organisms notable for their lack of cell walls and the low guanidine-thymidine (G+C) content (~25%) of their genomes (Woese, 1987). Although spiroplasmas were initially described by Davis in association with corn stunt disease, the first spiroplasma to be cultured in artificial media was the agent of citrus stubborn disease. This pathogen was also the first spiroplasma to receive a Latin binomial, *Spiroplasma citri.* Currently, 34 different spiroplasma groups have been identified from various environments, including plants, mammals, insects, and abiotic substrates (McCammon et al.,1988; Williamson et al.,1998).

For successful transmission to occur, spiroplasmas ingested by phloem-feeding leafhoppers move into the insect gut, cross the gut membrane into the hemocoel where they multiply, then traverse a second membrane barrier into the salivary glands, from which they may be ejected upon subsequent insect feedings. An *S. citri* line designated BR3 was originally isolated from horseradish plants with brittle root disease (Fletcher et al., 1981). The triply cloned isolate, BR3-3X, was maintained in several ways over a period of approximately 15

years. *S. citri* line BR3-G was obtained through extended plant-to-plant transmission of the original isolate by grafting in periwinkle, at some point undergoing a phenotypic switch and losing insect transmissibility. The barriers to transmission of strain BR3-G lay at both the gut and salivary gland membranes, as this strain was able to multiply to high titers in the insect hemocoel upon injection, yet failed to cross the gut membrane into the hemocoel following insect feeding (Wayadande and Fletcher, 1995). Line BR3-T, on the other hand, was derived following repeated transmission from turnip to turnip via its *C. tenellus* insect vector. This line, like the progenitor BR3-3X, remains insect transmissible.

Investigations of *S. citri* BR3 strains in our laboratory at the genetic level have revealed a surprising degree of heterogeneity among these closely related lines. Physical genome mapping by pulsed field gel electrophoresis (PFGE) of BR3-3X and derivatives BR3-T and BR3-G revealed that genetic organization was relatively conserved between BR3-3X and BR3-T, although the genome of the latter was approximately 150 kb larger than that of BR3-3X (1750 kb versus 1600 kb). The nontransmissible BR3-G, however, had undergone a large (approximately half of the chromosome) chromosomal inversion with deletions of approximately 5-10 and 10-20 kb at each inversion border (Ye et al., 1996). At 1870 kb, the genome of BR3-G is 270 kb larger than its BR3-3X progenitor.

While interesting, these differences between transmissible and nontransmissible lines did not explain the molecular basis for the loss of insect transmissibility. To identify potential transmissibility-related genes induced only in the transmissible lines, a strategy using arbitrarily-primed PCR (AP-PCR) with

mRNA (in the form of cDNA) from the three spiroplasma lines as template was chosen as a method that could identify transmissibility-related products formed only in transmissible lines. Alternatively, this method might discern a negatively-acting regulatory product made only in the nontransmissible line. Finally, gene products not made due to missing DNA sequences would also be identified using AP-PCR.

MATERIALS AND METHODS

Bacterial Isolates and Cultivation

Spiroplasma citri lines BR3-3X, BR3-T, and BR3-G were grown in LD8 broth (Davis, 1979) at 30°C. Each line was grown to late log phase, as determined microscopically by enumeration.

Total RNA Extraction

RNA was isolated from the three *S. citri* lines using the guanidinium/cesium chloride method described by Sambrook et al. (1989) (Figure 1). Briefly, cells grown to late log phase in 150 ml LD8 broth were centrifuged at 20,000 x g for 30 min at 4° C in a Beckman JA-14 rotor, and resuspended in 10 ml HEPES-sucrose buffer (0.1 M HEPES, 0.3 M sucrose, pH 7.5). Following a second centrifugation for 15 min, the cells were resuspended in 2 ml GIT buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate [pH 7.0], 0.1 M β-mercaptoethanol, 0.5% Sarkosyl), and thoroughly lysed by repeated

passages through a 27-gauge needle. Cesium chloride (0.8 g) was added to the lysate, which was then layered onto a 1.2 ml cushion of 5.7 M cesium chloride in 0.1 M EDTA (pH 7.0), and centrifuged 15 hr, 4°C in a Beckman VTi 65 rotor at 117,000 x g. The supernatant, containing cellular debris and DNA, was decanted and the pelleted total RNA was dissolved in 2 ml TES buffer (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1% SDS) and extracted with a 4:1 mixture of chloroform and 1-butanol. The organic phase was reextracted with an equal volume of TES buffer, and the two aqueous phases were combined and precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.2 volumes ethanol at -20°C for 2 hrs. The RNA was recovered by 10 minutes centrifugation at 12,400 x g and stored at -80°C in 95% ethanol. Denaturing agarose electrophoresis showed the RNA to be of satisfactory quality, as determined by the presence of intact, undegraded 23S and 16S ribosomal RNA bands.

cDNA Synthesis

cDNA was prepared using a RiboClone® cDNA Synthesis Kit (Promega, Madison, WI) according to manufacturer's instructions. Briefly, 5 μg aliquots of heat-denatured total RNA were converted to single-stranded DNA using random hexamers to prime DNA synthesis by AMV (avian myoblastosis virus) reverse transcriptase. For second-strand synthesis, a portion of the first-strand reaction was combined with second-strand buffer, DNA Pol I, RNAse H, and water. The mixture was incubated for 20 min at 14°C, followed by 10 min at 70°C, centrifuged briefly and placed on ice. T4 DNA polymerase was added and the

mixture was incubated 10 min at 37°C. The reaction was stopped by addition of 200 mM EDTA. cDNA was then extracted by addition of an equal volume of TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1 by volume) (TE: 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and brief vortexing followed by centrifugation for 2 min (12,400 x g, 4°C). The aqueous layer was transferred to a new tube and 0.1 volume 2.5 M sodium acetate (pH 5.2) and 2.5 volumes ice-cold 95% ethanol were added and briefly vortexed. cDNA was precipitated 30 min at -20°C, centrifuged (12,400 x g, 4°C) for 5 min, washed with 70% ethanol, dried briefly, and resuspended in 100 μl nuclease-free H_2O. The cDNA was then quantified by spectrophotometer and frozen in 10 μl aliquots of 50 ng/μl.

Arbitrarily-Primed PCR

Each reaction was performed using single or paired arbitrarily-chosen primers having no known homology to spiroplasma nucleic acid. Twenty-seven different primers (Table 1) were tested for their efficiency at generating large numbers (15-20 or more) of DNA fragments.

Several low-stringency PCR protocols (Rawadi et al., 1995; Coelho et al., 1995; Fan et al., 1995) were compared for their effectiveness at generating large numbers of arbitrarily-primed products; one adapted from Solokov et al. (1994) was chosen as the most effective for these purposes, as it was found to produce greater numbers of DNA fragments for most template/primer combinations than were produced by the other low-stringency methods tested. Approximately 50 ng double-stranded (DS) or single-stranded (SS) cDNA was subjected to PCR in a

total volume of 50 μl containing 5 μl 10X buffer, 5 μl 25 mM magnesium chloride, 0.5 μl of 2.5 mM each dNTP, 50 pM primer, and 1.5 units Taq polymerase (Promega, Madison, WI). Amplification was performed with 45 cycles (one minute each of 95°C, 34°C, and 72°C), followed by 10 min at 72°C for extension.

For analysis of the PCR products, samples were electrophoresed in 1.5% MetaPhor® agarose (FMC BioProducts, Rockville, MD) containing 5 μg ethidium bromide per 100 ml in the presence of 1X TAE electrophoresis buffer (0.04 M Tris-acetate, 0.001 M EDTA), and band sizes were estimated by comparison with 100 bp DNA molecular weight markers. DNA fragments unique to particular spiroplasma lines were isolated by stabbing into the band of interest with a disposable pipette tip from which the terminal 3 mm was trimmed, and performing high-stringency PCR (5 min at 95°C; 25 cycles of 94° C, one min, 55°C, one min, 72°C, 2 min; followed by extension for 10 min at 72°) on the 2-3 μl of cored material in the presence of the primer originally used in the low-stringency protocol. The products of these reactions were analyzed by electrophoresis for size identification.

Cloning of PCR Products

DNA from the high-stringency PCR reactions described above was then cloned directly from each band using an Original TA Cloning Kit (Invitrogen Corporation, San Diego, CA), according to manufacturer's instructions. Briefly, approximately 10 ng of DNA in a volume of 1 μl was added to a reaction containing 5 μl sterile water, 1 μl ligation buffer, 2 μl (10 ng) pCR 2.1® vector, and

1 μl T4 ligase, and incubated overnight at 14°C. The ligation reaction mixture was centrifuged briefly at 12,400 x g, 4°C, then placed on ice until stored at -4°C or used for transformation. One μl of reaction mixture was added to 50 μl One Shot™ competent INVαF' *E. coli* cells and 2 μl β-mercaptoethanol. The mixture was incubated on ice 30 min, heat shocked for 30 sec at 42°C, then placed on ice for 2 min. SOC medium (250 μl) was added, and the cells were shaken horizontally for 1 hr, 225 rpm, and placed on ice for 5 min. Transformed cells (50 μl) were spread on LB agar plates (10 g each of Bacto-tryptone and NaCl, 5 g Bacto-yeast extract, and 10 g agar noble dissolved in 1 liter H_2O, adjusted to pH 7.5 with NaOH) containing 50 μg/ml ampicillin and 40 μl/ml of 40 mg/ml X-Gal for selection, and incubated at 37°C for 18 hr. Plates were then incubated at 4°C for 3 hr to allow color development, and 5-10 white transformants were selected and grown separately in 2 ml LB medium containing 5 μg/ml ampicillin for 8 hrs at 37°C with shaking.

Plasmid Isolation and Analysis

Plasmid isolation was performed according to the alkaline lysis method described by Sambrook et al. (1989). Transformed *E. coli* cells from 2 ml cultures were pelleted in a microcentrifuge tube and resuspended in 200 μl TGE (25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA, pH 8.0) by pipetting. A volume of 300 μl freshly prepared 0.2 M NaOH/1% SDS was added, and the samples mixed by inversion and placed on ice for 10 min. A volume of 300 μl sodium acetate (3 M, pH 4.8) was added, mixed, and the tubes incubated for 5 min on

ice. Supernatant was collected after a 10 min centrifugation at 12,400 x g, and samples incubated with RNAse A (20 μg/ml final concentration) for 20 min at 37°C. Preparations were extracted twice with an equal volume of phenol/chloroform, precipitated with an equal volume of isopropanol and centrifuged immediately (12,400 x g, 4°C) for 10 min, then washed with 70% ethanol. DNA was dissolved in 32 μl H_2O, followed by addition of 8 μl of 4 M NaCl and 40 μl PEG 8000 [polyethylethylene glycol, avg. MW 8000, 13% (w/v)], mixing, and 20 min incubation on ice. Plasmid DNA was collected by 15 min centrifugation (12,400 x g, 4°C), washed in 70% ethanol, and resuspended in 100 μl TE buffer. Following quantification by spectrophotometry, 5 μg of each sample was digested with *Eco*RI (Gibco-BRL, Gaithersburg, MD) and subjected to gel electrophoresis to verify the sizes of the cloned sequences.

Sequencing and Sequence Analysis

Plasmid inserts were sequenced at the OSU Nucleic Acids and Protein Core Facility using an Applied Biosystems/Perkin-Elmer Model 373 automated sequencer (PerkinElmer Inc., Wellesley, MA). The sequences were compared to existing databases using Blast-N and Blast-X (Altschul et al., 1990).

RESULTS AND DISCUSSION

RNAs isolated by cesium chloride centrifugation from the three *S. citri* lines, BR3-3X, BR3-T, and BR3-G, were revealed by denaturing agarose gel electrophoresis to be of satisfactory quality, as determined by the presence of intact, undegraded 23S and 16S ribosomal RNA bands (Figure 1). The use of more than one primer yielded fewer DNA fragments than the use of a single one (Figure 2). For example, a fairly large number of fragments are produced using the primer CON singly (Figure 2, lanes 4-6); a lesser number are produced using primer OV singly (lanes 1-3). However, using the two in combination (lanes 7-9) dramatically reduced the total number of fragments amplified. Henceforth primers were used singly. Preliminary experiments showed that double- and single-stranded cDNAs served equally well as templates for producing equally large numbers of PCR products for each "fingerprint" (data not shown). Therefore, only single-stranded forms were used subsequently.

The three low-stringency PCR protocols tested for their effectiveness at producing large numbers of arbitrarily-primed products included a ramped method (Coelho et al., 1995), a dual phase protocol of five low-stringency cycles followed by 30 high-stringency cycles (Rawadi et al., 1995), and a protocol adapted from Solokov et al. (1994), that used 35 cycles, all of low stringency. The latter strategy yielded the greatest number of bands for any given

template/primer combination, and therefore was chosen for all subsequent reactions. Several frequently-formed fragments, as well as some more infrequently-occurring products, were selected for further study via cloning and sequencing. Differences in PCR products formed between different lines were evident (Figures 2 and 3), and many of these products were cloned and sequenced.

Among the 27 different arbitrarily-chosen primers used to prime the production of PCR products under low-stringency conditions, twelve consistently yielded large numbers (15-20 or greater) of DNA fragments as revealed by agarose gel electrophoresis. Primers were initially selected based on a low G+C content, corresponding to that of *S. citri*; however, no differences in effectiveness in producing large numbers of fragments were observed based on this selection. For example, primer CON, with low % A+T (17.8%), generated a greater number of fragments than did either primer OV (44.5% A+T) or γ (70% A+T) (Figure 2B). Banding patterns produced for any given set of AP-PCR reactions varied, sometimes substantially, according to input quantity and quality of cDNA template, different batches of cDNA synthesized from like mRNAs, and unknown parameters. Certain primers (i.e., primer PE6) were found to prime synthesis of the same differential products on a fairly regular basis, while others (i.e., primer ZR), gave inconsistent results from one experiment to another. Differences among the lines in PCR products formed were often evident, with unique fragments associated with a single or often two lines.

Because the cloning method used (Invitrogen TA Cloning Kit, Invitrogen Corporation, Carlsbad CA, USA) recommended the use of PCR products that had not been purified from gels, it was deemed necessary to obtain each DNA band of interest as the single product of a PCR reaction. Achieving this objective entailed "stabbing" a portion of the band of interest from the gel with a micropipet tip from which the terminal 3 mm had been trimmed (Figure 4). This product was used as template in a high-stringency PCR reaction in the presence of the primer that initially gave rise to it; often this procedure was repeated several times before a single product of the correct size was produced (Figure 5).

Selected single-product PCR fragments were cloned and amplified in *E. coli* and subjected to restriction digestion with *Eco*RI as described (Figure 6), and twelve cloned fragments were sequenced using a PerkinElmer automated sequencer (PerkinElmer Inc., Wellesley MA, USA).

Database searches using Blast-X and Blast-N revealed six clones with some degree of homology to previously identified genes (Table 2); these are of interest due to the possibility that the identified function might suggest a means by which insect transmissibility (or its absence) might be explained. For example, a gene product potentially relating to insect transmissibility or its loss might be a transmembrane protein functioning as an adhesin, such as the P1 adhesin described in *Mycoplasma pneumoniae* (Kahane et al., 1985). Adhesins are likely required for recognition of and adherence to cognate receptors at membrane barriers within the insect prior to traversal of the membrane. No adhesin-like proteins were identified as differentially produced among these

spiroplasma lines, however. One high-scoring clone, T-2.4-500, resembled the *E. coli cut*C gene, which encodes a product involved in copper homeostasis. Absence or insufficient production of such a gene product functioning to keep intercellular copper levels in balance might result in the death or debilitation of spiroplasma cells, particularly in environments of high copper concentration. If high copper levels are present within the leafhopper vector in zones within which spiroplasma survival and multiplication must occur for successful transmission to result, lack of a gene product regulating copper homeostasis could subsequently hinder or prevent insect transmission. Cloned sequence PVR2-G-600 had the highest degree of homology to a known gene, resembling a *Mycoplasma gallisepticum* 5S ribosomal RNA sequence (Scamrov and Beabealashvilli, 1991). Clone PE6-X-800 had sequence homology to the tRNA hydroxylase-encoding *miaE* gene of *Salmonella typhimurium* (Persson and Bjork, 1993). Other homologous sequences found included *S. citri* elongation factor *Ts* (Chevalier et al., 1990), a *Mycoplasma pneumoniae* hypothetical protein, and a pre-protein translocase from *Mycoplasma capricolum* (Miyata et al., 1993).

Differences revealed by AP-PCR might result from differences in *S. citri* mRNA expression or from differences in gene content. Northern hybridization could reveal whether the reflected transcript was actually expressed and simply failed to be revealed using this technique. Alternatively, Southern hybridization might reveal whether the failure of a particular mRNA to be made in a line or lines reflects the absence of the gene sequence responsible for its production in the other line(s).

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Figure 1. Schematic representation of *Spiroplasma citri* RNA isolation and agarose gel electrophoresis. Isolated total RNA is shown in lower righthand corner.

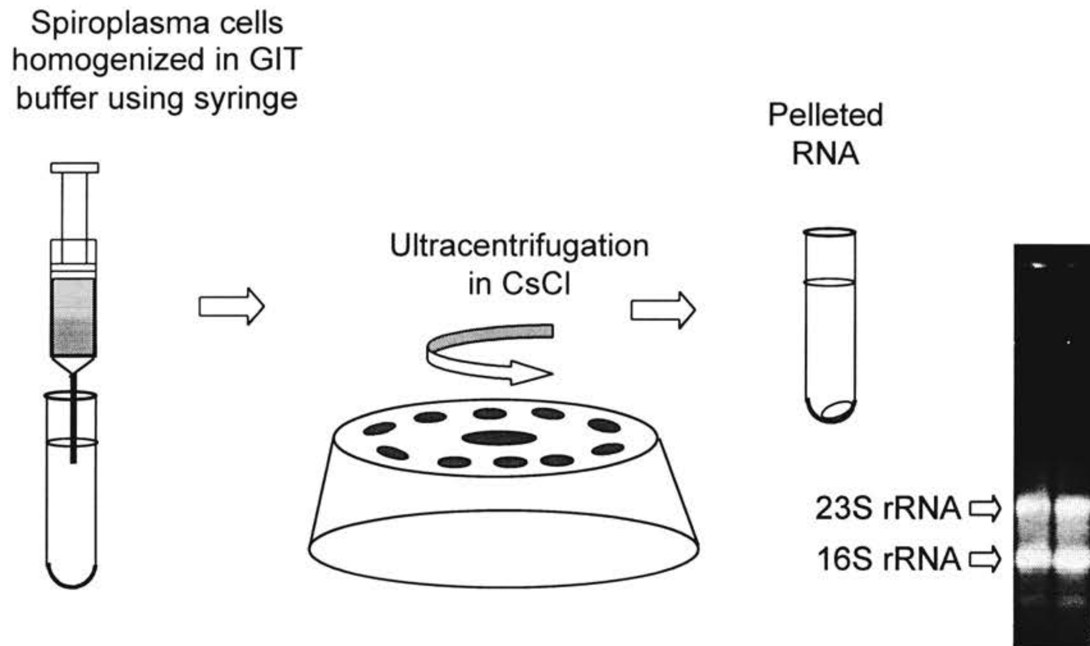


Figure 2. AP-PCR showing greater numbers of fragments generated using single primers than with paired primers. T, BR3-T. G, BR3-G. X, BR3-3X. MW, molecular weight marker.

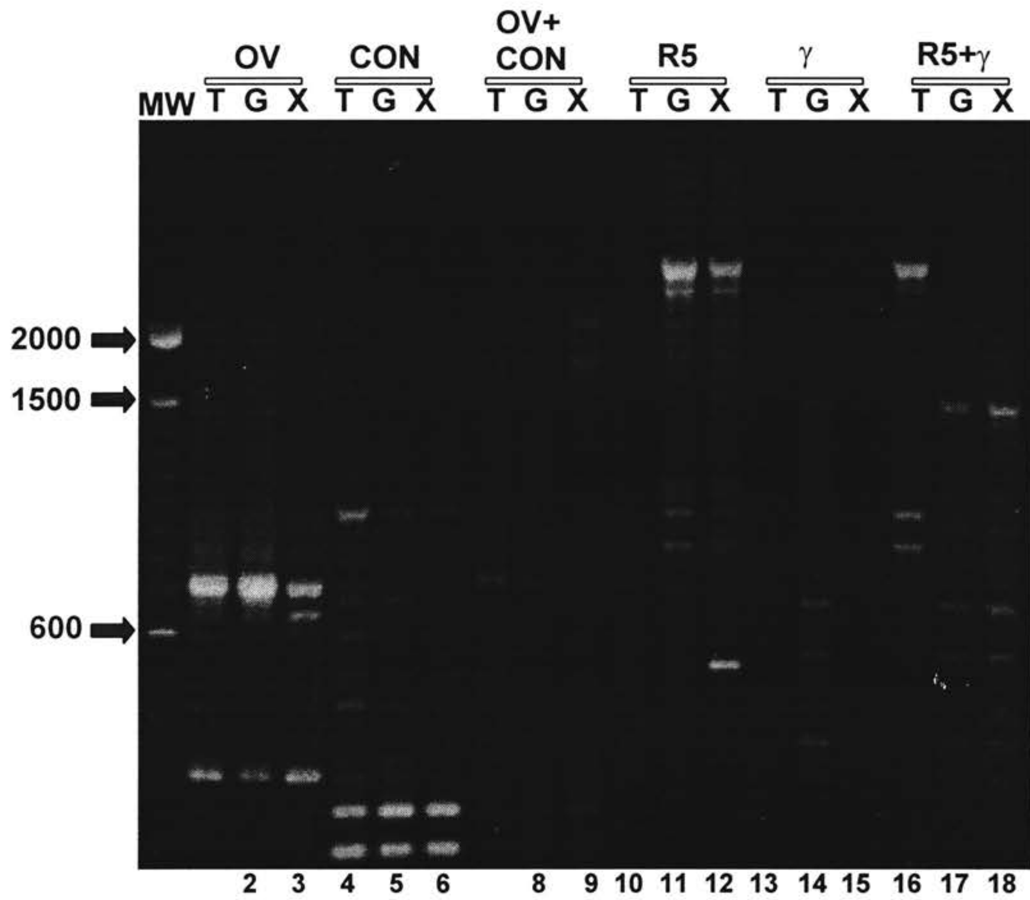


Figure 3. Examples of AP-PCR of cDNA from *Spiroplasma citri* BR3 lines using various arbitrary primers.

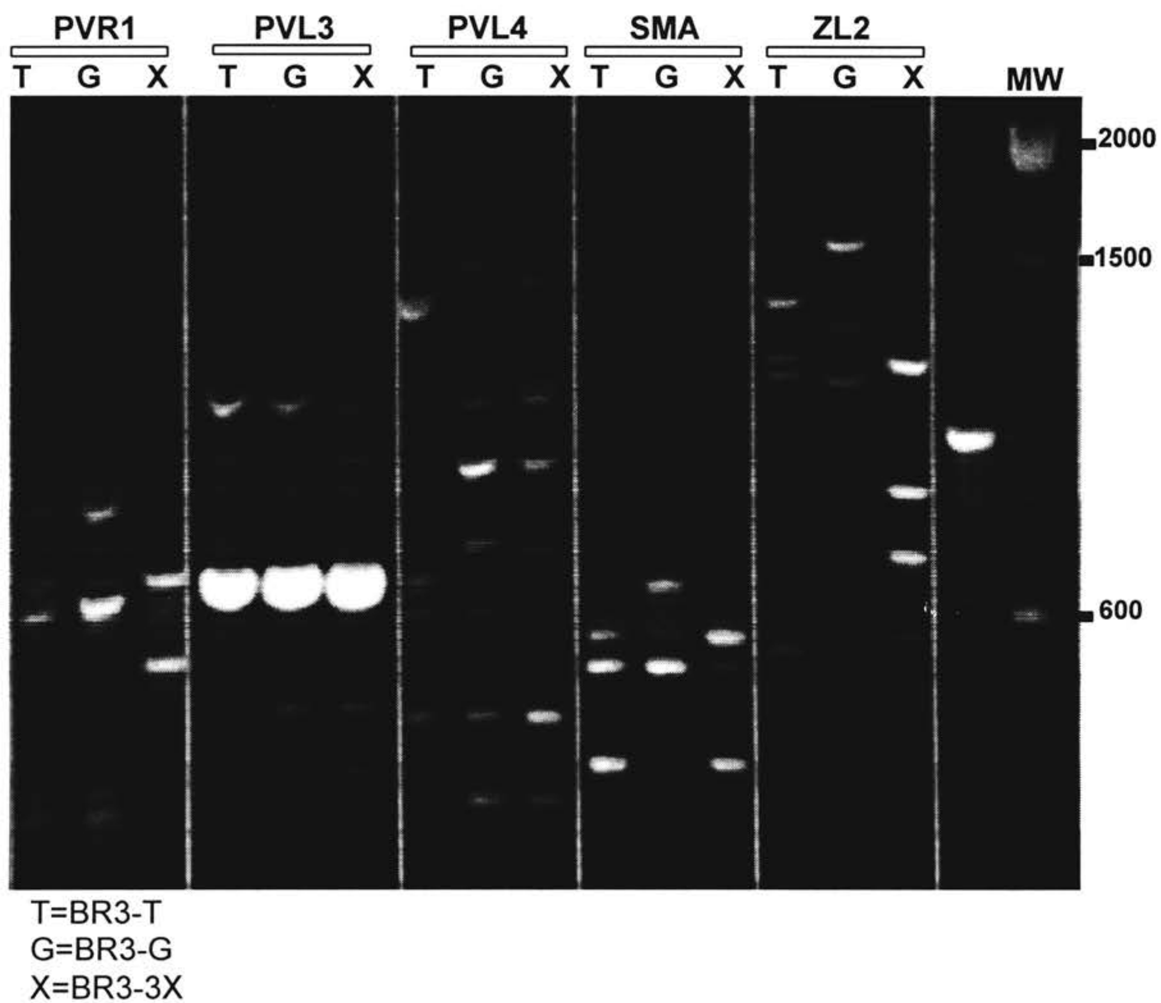
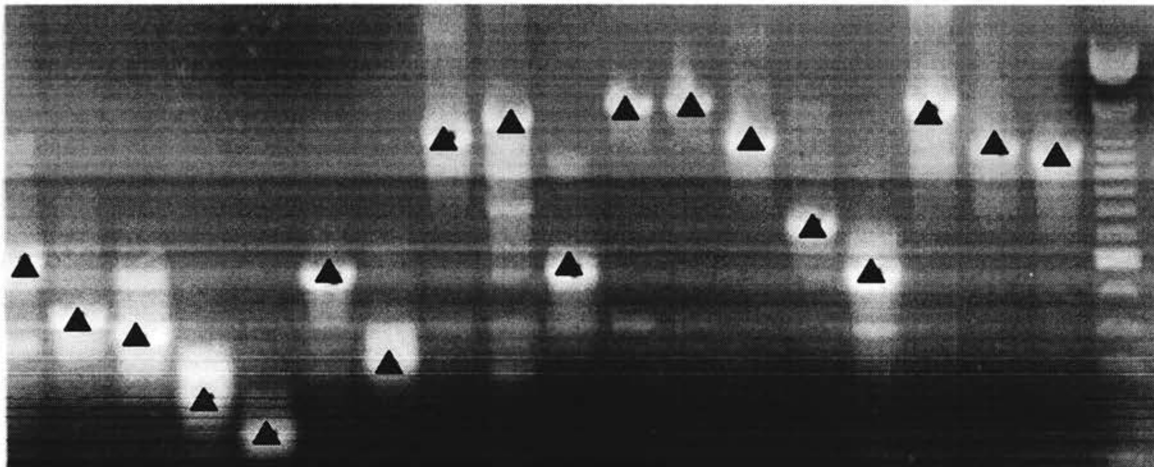
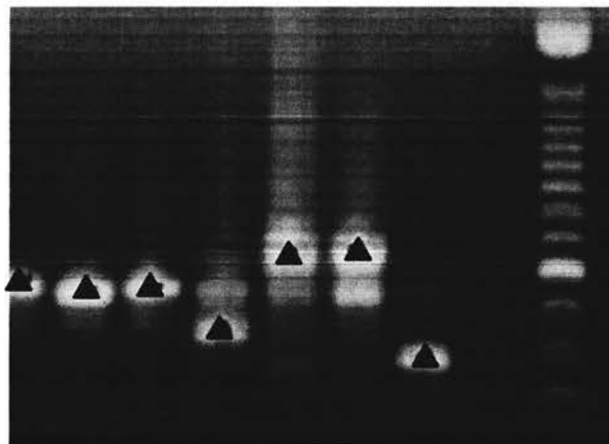


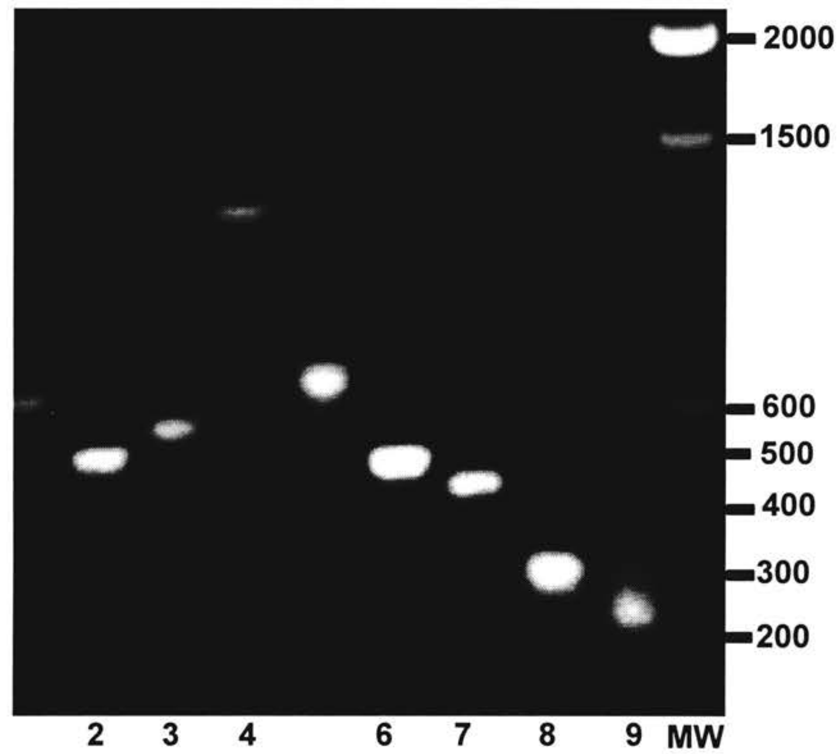
Figure 4. "Stabbing" of electrophoretically separated products regenerated by PCR to yield isolated fragment.





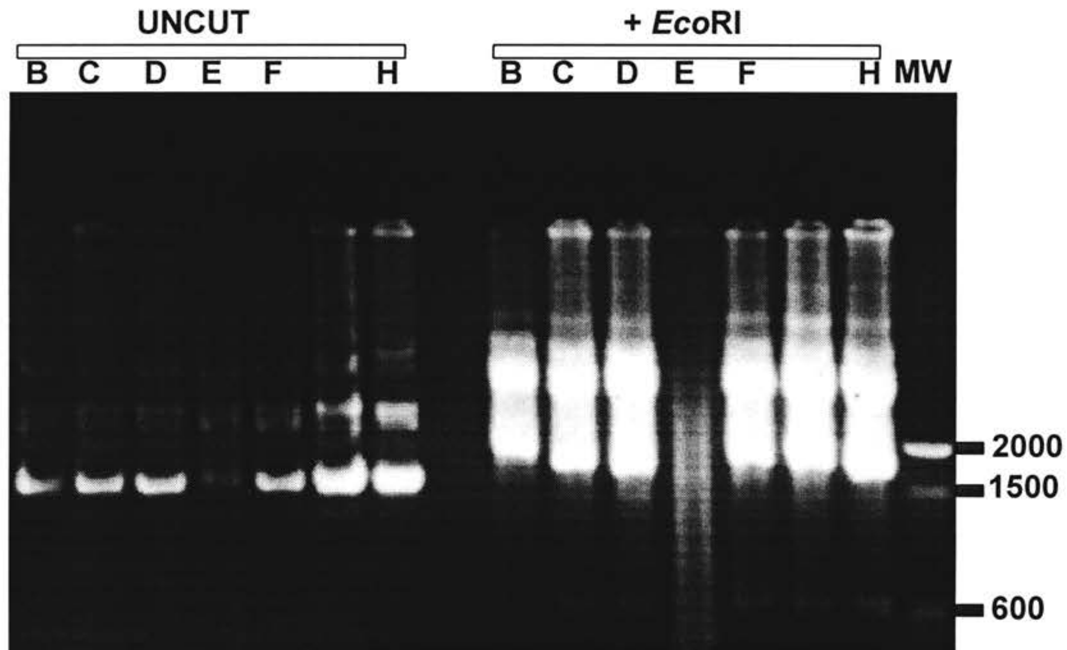
▲Denotes stabbed band

Figure 5. Single-sized products generated from re-PCR of excised AP-PCR fragments.



Lane 1: PE6-X-800
Lane 2: ZL2-X-550
Lane 3: PVR2-G-600
Lane 4: PVL4-T-1400
Lane 5: CON-X-800
Lane 6: CON-X-550
Lane 7: CON-T-500
Lane 8: CON-G-300
Lane 9: CON-T-250

Figure 6. AP-PCR clone PVR2-G-650 before and after restriction digest. Digestion with *Eco*RI generates the appropriate size 650 bp product.



CHAPTER IV

16S and *groE* sequence analysis of *Serratia marcescens* strains associated with cucurbit yellow vine disease

Introduction

Yellow vine disease (YV) of cucurbits was first observed in Oklahoma in 1988 in pumpkins and squash (Bruton 1995). Exhibiting characteristic symptoms of yellowing foliage, wilting, and phloem discoloration, this disease has since caused severe losses in canteloupe and watermelon crops as well. Disease incidence has varied annually from small, isolated outbreaks to complete crop loss, especially in early-planted fields (Avila et al. 1998). Confined largely to central Oklahoma and Texas, YV has since been confirmed in Tennessee as well (Bost et al., 1999). Bruton et al. (1998) reported consistent association of disease symptoms with the presence of rod-shaped bacteria in phloem sieve elements, detected using transmission electron microscopy.

Avila et al. (1998) found that the YV organism was detectable by polymerase chain reaction (PCR) and that the deduced nucleotide sequence for 16S ribosomal DNA placed this organism within the gamma-3 proteobacteria, with the nearest apparent relative being *Serratia marcescens*. Capable of thriving in diverse habitats, *S. marcescens* has been isolated from soil, water, plants, food products, and human infections (Hejazi and Falkiner, 1997; Hauben et al., 1998; Sproer et al., 1999). The various roles filled by this species range from helpful rhizobacteria to innocuous colonizer to disease-causing pathogen.

Although more generally benign than otherwise, the potential for members of this species to cause disease in humans, animals and plants may present concerns regarding human health and food safety.

The initial failure of yellow vine bacteria cultivation attempts and the apparent limitation of the bacterium to phloem tissue in infected plants led to its preliminary classification as an uncultivable BLO. However, recent efforts at cultivation have been successful (F. Mitchell, personal communication), and the ability to grow the bacterium in pure culture has greatly facilitated the characterization of this organism.

Two highly conserved genomic regions are commonly used in taxonomic investigations. Sequence analysis of 16S rDNA, a region of approximately 1500 base pairs, is one of the most useful and widely accepted means for assessment of the natural and taxonomic relationships of microorganisms (Kolbert and Persing 1999; Woese 1987). For example, determination of the 16S rDNA sequence of the bacterium-like organism associated with marginal chlorosis in strawberry, which is also a phloem-limited bacterium, allowed the taxonomic placement of this organism as a new genus in group 3 of the gamma subclass of proteobacteria (Zreik et al. 1998).

The second conserved region is the *groE* operon, which encodes stress proteins functioning as chaperonins, and is known to be highly conserved in the bacterial world, as well as essential for cell viability (Harada and Ishikawa, 1997; McLennan and Masters 1998). The *groE* operon has been effective in the classification of closely related bacterial species, especially those in the

Enterobacteriaceae, possessing 16S rDNA sequences too highly conserved to be useful for distinction (Harada and Ishikawa, 1997).

To more precisely place the YV bacterium phylogenetically, we determined the nucleotide sequences of these two genomic regions from two recently isolated yellow vine strains as well as from eight isolates from different habitats preliminarily characterized as *S. marcescens*.

MATERIALS AND METHODS

Bacterial Isolates. Two YV-derived strains, and eight bacterial isolates obtained from other investigators and preliminarily characterized as *S. marcescens*, were used in this study. Their sources and characteristics are summarized in Table 1.

Bacterial DNA Isolation. Bacterial isolates were grown in 3 ml Luria broth (LB) (Sambrook et al., 1989) for 8-12 hrs with shaking at 28° C. DNA was extracted using a modified version of the hexadecyl trimethyl ammonium bromide (CTAB) method (Ausubel et al., 1987). Briefly, 3 ml of log-phase cells were centrifuged for 5 min at 10,000 x g, 4° C, supernatant was removed and cells resuspended by pipetting in 200 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The mixture was incubated 30 min at 60° C, then 100 μl 5 M NaCl and 80 μl CTAB/NaCl buffer (10% hexadecyl trimethyl ammonium bromide in 0.7 M NaCl) were added and mixed, and the suspension incubated at 60° C for 10 min. One

hundered μl phenol plus 100 μl chloroform/ isoamyl alcohol (24:1) were added, and the mixture was vortexed thoroughly and centrifuged at 12,000 x g, 4° C for 15 minutes. The supernatant was removed to a new tube and DNA precipitated with addition of an equal volume of isopropanol, 30 min incubation at -20° C, 15 min centrifugation at 4° C, and washing in 100 μl 70% ethanol.

PCR. Amplification and sequencing of the *gro*E region, approximately 1900 base pairs, was performed with three primer sets A1/ B1, A2/ B2, and A3/ B3 as described by Harada and Ishikawa (1997) (Figure 1). These primers annealed to the *gro*E region as depicted in Figure 1B. Each reaction was performed using 30 ng template DNA in a volume of 25 μl containing 2.5 μl 10X buffer, 3.5 μl 25 mM $MgCl_2$, 0.5 μM of 2.5 mM each dCTP, dATP, dTTP, and dGTP, 200 pM each primer (A1/B1, A2/B2, and A3/B3), and 1.5 Units Taq polymerase (Promega, Madison, WI). The DNA was amplified under the following conditions: 3 min at 95°C; 35 cycles of 30 s at 94°C, 60 s at 55°C, and 60 s at 70°C; and final elongation at 72°C for 10 min.

Amplification and sequencing of 16S rDNA was achieved using universal primers fD1, rP2 (Weisburg et al., 1991), as well as α, β, and γ (Figure 2) (C. Sproer, personal communication). Each reaction was performed using 30 ng DNA, isolated from organisms as previously described, in a total volume of 25 μl containing 2.5 μl 10X buffer, 3.5 μl 25 mM magnesium chloride, 0.5 μl each dCTP, dATP, dTTP, and dGTP, 200 pM each primer, and 1.5 units Taq

polymerase. Amplification was performed as follows: 95°C for 3 min; 35 cycles of 60 s at 92°C, 45 s at 47°C, and 90 s at 72°C, followed by 10 min at 72°C.

For analysis of the PCR products, samples were electrophoresed in 1.5% MetaPhor® agarose (FMC BioProducts, Rockville, MD) containing 5 μg ethidium bromide per 100 ml, and band sizes were estimated by comparison with 1 Kb Plus DNA Ladder molecular weight markers (Gibco-BRL, Rockville MD).

The resultant PCR products were excised from the gel and prepared for direct sequencing using a QIAquick gel extraction kit (Quiagen Inc., Valencia, CA). The gel-purified DNAs were quantified by electrophoresis and compared with herring sperm DNA of known quantity. Automated sequencing was performed by the OSU Nucleic Acids and Proteins Core Facility using an Applied Biosystems/PerkinElmer 373 sequencer (PerkinElmer Inc., Wellesley, MA).

Sequence Editing and Phylogenetic Tree Construction. Resulting sequences were aligned and edited using BioEdit software version 4.7.3 (Tom Hall, North Carolina State University, USA). Phylogenetic trees based on 16S rDNA (Figures 3 and 4) and *gro*E (Figures 5 and 6) sequences were compiled using the PHYLIP programs (Felsenstein, 1993). 16S rDNA and groE sequences of additional plant and/or insect-related members of the *Enterobacteriaceae,* selected for inclusion because either their habitat or taxonomic position inferred possible relevance to *S. marcescens*, were obtained from Genbank (Table 2A and 2B). Alignments were bootstrapped 1000 times using SEQBOOT. Distance matrices were calculated with DNADIST and used to

construct trees by neighbor joining as implemented in NEIGHBOR. A consensus tree of the data set was produced by the CONSENSE program. The phylogenetic trees were arranged and edited using TreeView (Roderic D.M. Page, University of Glasgow, UK.

RESULTS AND DISCUSSION

Phylogenetic trees were generated using genomic sequences of yellow vine bacterial isolates and eight other bacterial strains preliminarily characterized as *S. marcescens* (Figures 3 through 6). Trees generated in this study from 16S rDNA sequences were very similar, but not identical, to those generated from *groE* sequences. A number of methods for generating phylogenetic trees (Fitch, Kitsch, and DNA Parse), as implemented within Phylip, were tested. The Phylip programs DNADIST and NEIGHBOR, with the endosymbiont *Sitophilus oryzae* as an outgroup, yielded branching motifs and species groupings in which the relationships among the included members of the *Enterobacteriaceae* most resembled results similar to those achieved by others. Parsimony, as implemented in Dnapars, gave unsatisfactory results, most likely due to the fact that this program compares only at "informative" sites, or sites where more than one sequence has the same altered nucleotide. Few such cases of this appear in these sequences. Harada and Ishikawa (1997), who characterized a number of the *Enterobacteriaceae* on the basis of their *groE* sequences, omitted the spacer region between the *groES* and *groEL* genes to

obtain a reliable alignment for phylogenetic comparison. In our study, including or deleting this spacer region made no difference in the resulting phylogenetic trees, even though there were clear base differences in this segment (trees depicted in Figures 5 and 6 are compiled from sequence data with spacer regions omitted).

Figures 3 and 5 depict trees generated using a large number of sequences (16S rDNA and *groE*, respectively) from various members of the *Enterobacteriaceae*. Phylogenetic trees generated in Figures 4 (16S rDNA) and 6 (*groE*) are more concise, involving a lesser number of sequences from isolates outside this study. All four trees confirm that the causal bacterium of cucurbit yellow vine (Bruton et al., 1998) is indeed *S. marcescens*. Both sequences place YV isolates W01-A and Z01-A, which are almost identical to each other, within branches that clearly reflect their close relationship to the human clinical isolates, H01-A and H02-A, as well as to the type-strain, *S. marcescens* 13880. Also included in this grouping are soil isolates CP01(4)CU and 98A-742, as well as plant endophytes 90-166 (cotton) and R01-A (rice). Cotton root endophyte JM-983, identified at one time as *S. plymuthica* by fatty acid analysis (J. Kloepper, personal communication), appears to have some relationship with *S. ficaria*, particularly using *groE* sequence data. However, a Blast-N search of the *gro*E sequence from JM-983 yields *S. marcescens* JCM 1239 as the best fit, with 1239 of 1292 nucleotides identical. Blast-N analysis of the 16S rDNA sequence data shows *S. grimesii* DSM 30063 to have by far the greatest homology to this isolate, with 1406 of 1410 nucleotides identical. *S. grimesii* was not included in

our phylogenetic trees because no *groE* sequence data are available for this organism. Isolate JM-965, a cotton root endophyte, appears most distantly related to the other isolates characterized. Here again, *groE* and 16S sequence data yield somewhat different results; *Enterobacter asburiae* JCM 6051 is the species having greatest homology to the JM-965 *groE* sequence by Blast-N search (1233 of 1292 nucleotides), while *Enterobacter cloacae* (1405 of 1407 nucleotides) is the most similar 16S rDNA sequence available in the database.

The phylogenetic placement of the two YV strains W01-A and Z01-A by 16S rDNA and *groE* sequencing data is in surprising contrast with placement based on automated Biolog, Vitek, and API-20 methods, as well as fatty acid analysis (J. Fletcher, personal communication). These two isolates were unidentifiable by Vitek. W01-A was classified as *Alcaligenes xylosoxydans* by three separate fatty acid analyses (FAME, performed by E. Dickstein, University of Florida), and as *Aeromonas veronii* using Biolog (Biolog Inc., Hayward, CA, also performed by E. Dickstein). Isolate Z01-A was identified as *Pantoea agglomerans* using API-20, as *A. xylosoxydans*, *Edwardsiella ictaluri*, and *Proteus vulgaris* in three separate fatty acid analyses, and as *Vibrio cholerae* using Biolog. Our results indicate that the metabolic capabilities and fatty acid profiles of these cucurbit bacteria are much different from those of the type strain of *S. marcescens*, although they are clearly identified as this species by both *groE* and 16S rDNA sequence data. The majority of cases in which the YV isolates differed from *S. marcescens* in Biolog analysis were instances where W01-A and Z01-A were unable to metabolize substrates usually considered

diagnostic for *S. marcescens*. For example, neither YV isolate tested positive for the presence of DNAse and oxidase. YV isolates W01-A and Z01-A also differ somewhat from each other in their physiological characteristics as determined by Biolog and FAME analysis, as shown by their identification as different species by these techniques.

The substantial disparity between characterization of these YV isolates based on 16S rDNA and *groE* sequence data versus characterizations made using FAME and Biolog identification techniques may stem from a lack of the genes responsible for proteins and/or enzyme pathways normally found in *S. marcescens*. Alternatively, regulatory mechanisms present in the YV isolates may be responsible for the cessation of these gene products even though they posess the required functional genes. Determination of the genome size of the YV pathogen might be a first step toward explaining this discrepancy.

Serratia marcescens strains have been found to occupy a wide range of roles as epiphytes, endophytes, soil inhabitants, and insect pathogens (Someya and Kataoka, 2000; Strobel et al., 1999; Ahrenholtz et al, 1994; Schalk et al., 1987; Grimont and Grimont, 1982), and some researchers have investigated the potential of specific *S. marcescens* strains as biocontrol agents. Identification of the causal agent of cucurbit yellow vine disease as *S. marcescens* introduces a question of health and safety, since *S. marcescens* is a nosocomial human pathogen of increasing interest (Hejazi and Falkiner, 1997). Grimont and Grimont (1982) reported finding *S. marcescens* in nearly 30% of the fresh salads inspected in a hospital setting. *Burkholderia cepacia,* another bacterium

increasingly used as a biocontrol agent, can also function both as a human and plant pathogen. This microbe has recently been the focus of some discussion (Govan et al., 2000) centering on whether biocontrol strains may be capable of occupying a pathogenic role. Whether YV strains have the capacity to serve as human pathogens is unknown at this time.

The means by which the YV pathogen is transferred to healthy plants is as yet unknown, but circumstancial evidence suggests that an insect vector may be required. Previous efforts (Bruton et al., 1995) to assess the role of insects as possible YV vectors showed that squash fields treated with the pesticides cypermethrin or esfenvalerate had a significantly lower YV incidence than untreated fields. Futher substantiation of an insect role in YV transmission was shown in an exclusion experiment (B. Bextine et al., unpublished data), in which the incidence of YV was compared between uncovered squash plants and squash plants covered with fine mesh that excluded insects. In PCR assays using YV-specific primers, up to 40% of uncovered plants, but none of the covered plants, were positive for the YV bacterium. These differences were statistically significant ($p<0.001$), supporting the hypothesis that insects are involved in the transmission of the bacterium.

This study illustrates the difficulty inherent in assigning classifications to bacterial isolates solely on the basis of either sequence, physiological, or morphological similaries to other bacteria. Taking into account the large degree of horizontal gene transfer that is likely to occur in prokaryotes, whether by conjugation, mutation, viral lysogeny, or other mechanisms, most bacterial

isolates are best described as products of both their genes and their environment. Those individuals whose genetic complement is modified in such a manner as to give them a competitive advantage in a particular environment will thrive relative to those not benefitting from such modifications.

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Table 1. Bacterial isolates characterized by sequence analysis.

Strain designation	Original identification	Isolated from:
W01-A	YV bacterium	watermelon (Oklahoma)
Z01-A	YV bacterium	zucchini squash (Oklahoma)
90-166[1]	*S. marcescens*	cotton root (Alabama)
98A-742[1]	*S. marcescens*	soil treated with DiTera (Florida)
CP01(4)CU[1]	*S. marcescens*	golf course rhizosphere (Florida)
JM-983[1]	*S. plymuthica*	cotton roots (Florida)
JM-965[1]	*S. marcescens*	cotton stems (Florida)
H01-A[2]	*S. marcescens*	human (Oklahoma)
H02-A[2]	*S. marcescens*	human (Oklahoma)
R01-A[3]	*Serratia* ssp.	rice (Philippines)

(1) Provided by J. Kloepper, Univ. of Alabama
(2) Provided by D. Adamson, Medical Arts Laboratory, Oklahoma City, OK
(3) Provided by G. Prasad, International Rice Research Institute, Los Banos, The Philippines

Table 2. Genbank accession numbers of bacterial isolates used in treemaking.

A. *groE* genes

Bacterial species and strain (if available)	GenBank Accession Number
Serratia marcescens W01-A	(in submission process)
Serratia marcescens Z01-A	(in submission process)
Serratia marcescens R01-A	(in submission process)
Serratia marcescens H01-A	(in submission process)
Serratia marcescens H02-A	(in submission process)
Serratia marcescens 90-166	(in submission process)
Serratia marcescens 98A-742	(in submission process)
Serratia marcescens CP01(4)CU	(in submission process)
Serratia marcescens JM-983	(in submission process)
Enterobacter asburiae JM-965	(in submission process)
Eschericha coli	X07850
Enterobacter asburiae strain JCM6051	AB008137
Enterobacter intermedius strain JCM1238	AB008138
Enterobacter gergoviae strain JCM 1234	AB008139
Enterobacter amnigenus strain JCM 1237	AB008140
Enterobacter aerogenes strain JCM 1235	AB008141
Enterobacter agglomerans strain JCM 1236	AB008142
Serratia rubidaea strain JCM 1240	AB008143
Serratia ficaria JCM 1241	AB008144
Serratia marcescens JCM 1239	AB008145
Klebsiella pneumoniae JCM 1662	AB008146
Klebsiella planticola JCM 7251	AB008148
Klebsiella oxytoca JCM 1665	AB008147
Klebsiella ornithinolytica JCM 6096	AB008149
Erwinia herbicola JCM 7000	AB008150
Pantoea ananas JCM 6986	AB008151
Erwinia carotovora IAM 12633	AB008152
Erwinia aphidicola IAM14479	AB008153
Sitophilus oryzae principal endosymbiont	AF005236

B. 16S rDNA genes

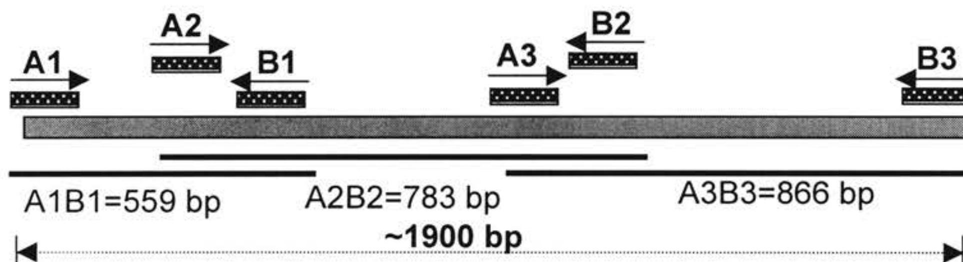
Bacterial species and strain (if available)	GenBank Accession Number
Serratia marcescens W01-A	(in submission process)
Serratia marcescens Z01-A	(in submission process)
Serratia marcescens R01-A	(in submission process)
Serratia marcescens H01-A	(in submission process)
Serratia marcescens H02-A	(in submission process)
Serratia marcescens 90-166	(in submission process)
Serratia marcescens 98A-742	(in submission process)
Serratia marcescens CP01(4)CU	(in submission process)
Serratia marcescens JM-983	(in submission process)
Enterobacter asburiae JM-965	(in submission process)
Eschericha coli	J01859
Enterobacter agglomerans strain A11	AF130890
Enterobacter intermedius JCM1238	AB004747
Enterobacter gergoviae strain JCM 1234	AB004748
Enterobacter amnigenus strain JCM 1237	AB004749
Enterobacter aerogenes strain JCM 1235	AB004750
Enterobacter agglomerans strain A11	AF130890
Serratia rubidaea strain DSM 4480	AJ233436
Serratia ficaria strain DSM 4569	AJ233428
Serratia marcescens	AF124035
Klebsiella pneumoniae strain Klebs919	Y17669
Klebsiella planticola strain DR3	X93216
Klebsiella oxytoca strain ATCC 13182T	AF129440
Klebsiella ornithinolytica strain JCM6096T	AJ25146
Erwinia herbicola strain JCM 7000	AB004757
Pantoea ananas strain JCM 6986	AB004758
Erwinia carotovora strain LMG 2386	Z96090
Sitophilus oryzae principal endosymbiont	AF005235

Figure 1. PCR strategy for amplification and direct sequencing of *groE* region.

A. Representation of *E. coli* gro*E* operon.

SD= Shine-Dalgarno box
Phsp= Heat shock promoter
Pcon= Constitutive promoter
Term= Termination

B. Chromosomal location and arrangement of primers used for *groE* sequencing.



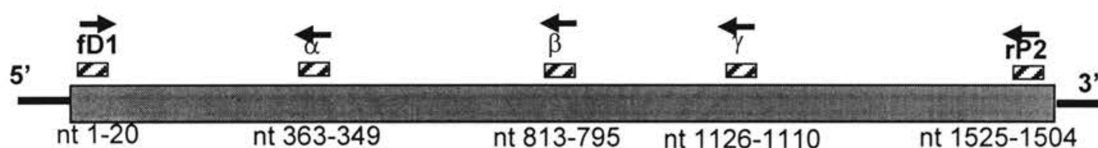
C. Sequences of *groE* primers.

Primers used for *groE* PCR and sequencing	Annealing position
A1 (5'-ATATTCGTCCATTGCATGATCG-3')	nt 5-26
B1 (5'-CGTTTGCTTTAGAGGCAACTTC-3')	nt 584-563
A2 (5'-CCATCACCAAAGATGGTGTTTCCGT-3')	nt 480-504
B2 (5'-GCTCCATACCGATCTCTTCAGAGA-3')	nt 1262-1239
A3 (5'-ACCCTGGTGGTTAACACCATGC3')	nt 1118-1140
B3 (5'-TACATCATGCCGCCCATGCCACCC-3')	nt 1983-1960

Figure 2. PCR strategy for amplification and direct sequencing of 16S rDNA region.

A. Arrangement of ribosomal RNA genes.

B. Arrangement of primers used for 16S rDNA amplification and sequencing.



C. Sequences of 16S rDNA primers.

Primers used for 16S rDNA PCR and sequencing	YV sequence annealing
fD1 (5'-AGAGTTTGATCCTGGCTCAG-3')	nt 1-20
rP2 (5'-ACGGCTACCTTGTTACGACTT-3')	nt 1525-1504
α (5'-CTGCTGCCTCCCGT-3')	nt 349-363
β (5'-CTACTCGGGTATCTAATC-3')	nt 795-813
γ (5'-AGGGTTGCGCTCGTTG-3')	nt 1110-1126

Figure 3. Extended phylogenetic distance tree compiled from 16S rDNA sequence data, using DNADist, Neighbor, and Consense, and the endosymbiont *Sitophilus oryzae* as outgroup. Branches with bootstrap values less than 500 (out of 1000) are collapsed.

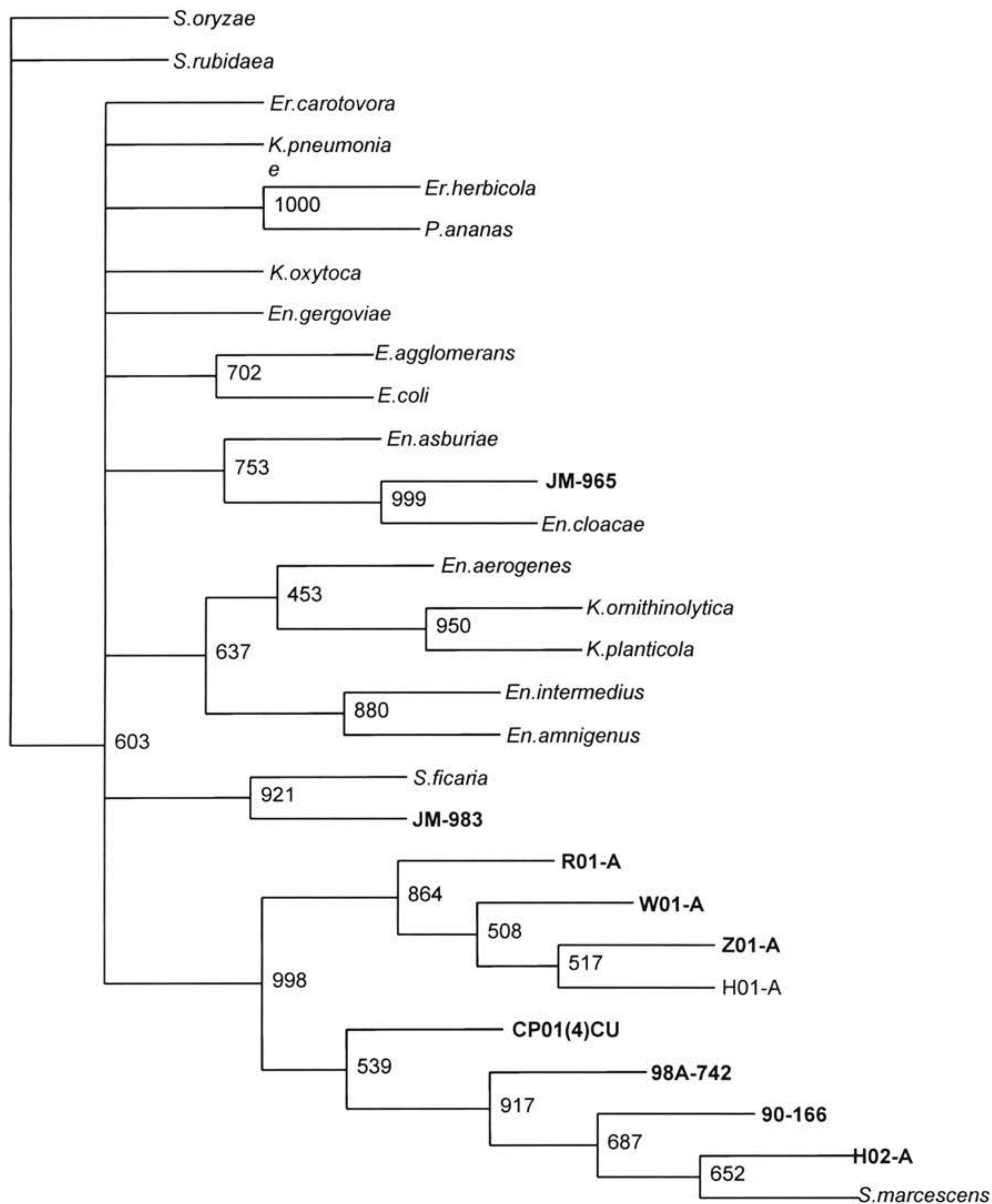


Figure 4. Concise distance tree compiled from 16S rDNA sequence data using DNAdist and Neighbor, *Sitophilus oryzae* endosymbiont as outgroup. Branches with bootstrap values less than 500 (out of 1000) are collapsed.

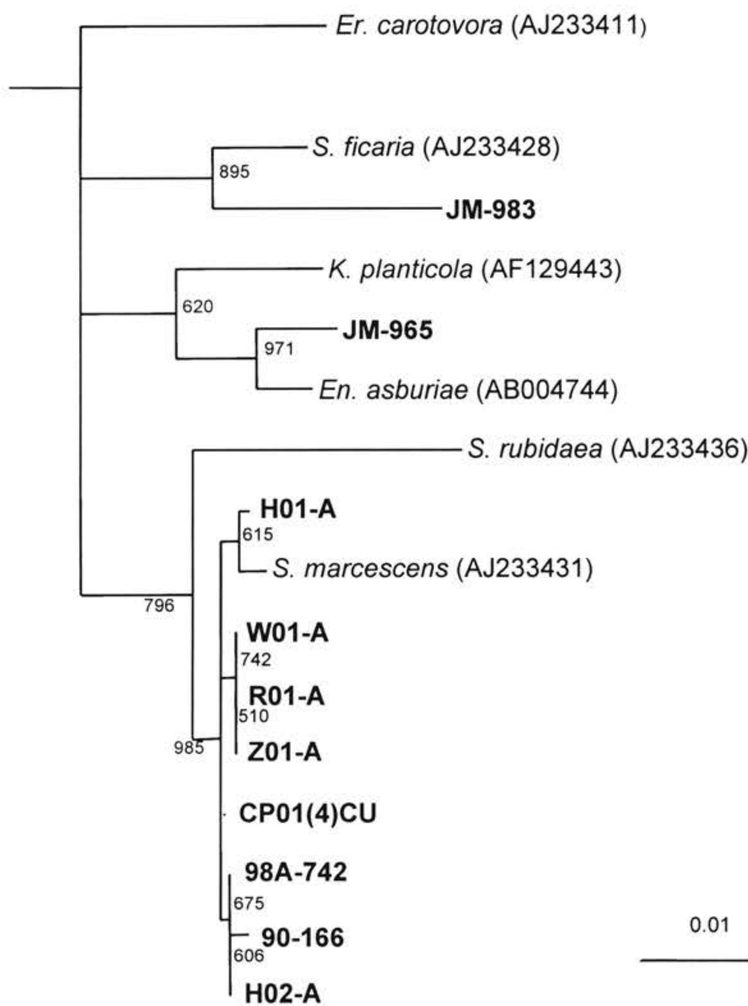


Figure 5. Extended phylogenetic distance tree compiled from groE sequence data, using DNADist, Neighbor, and Consense, and the endosymbiont *Sitophilus oryzae* as outgroup. 100 Bootstraps were performed, with branch values less than 50 left uncollapsed.

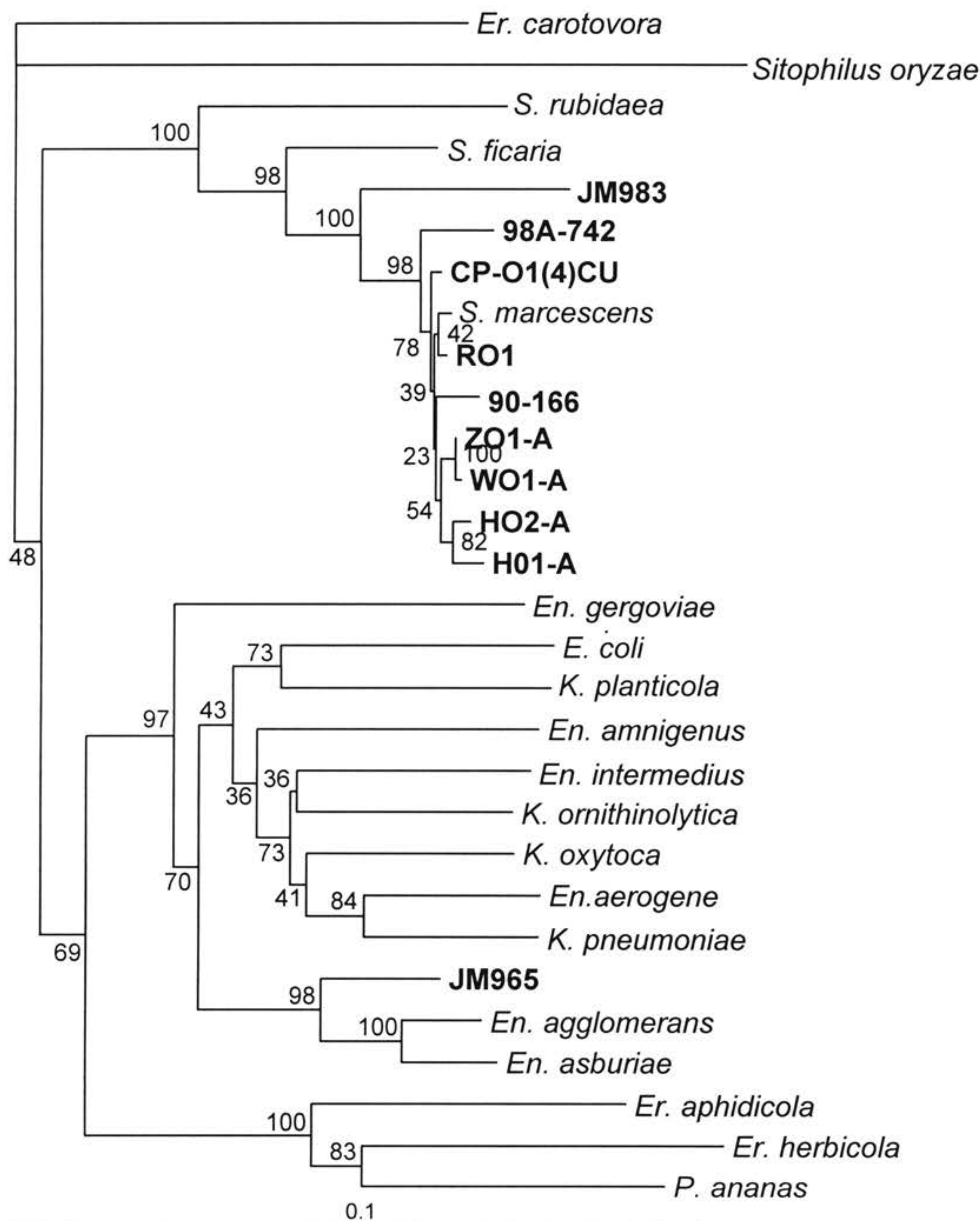
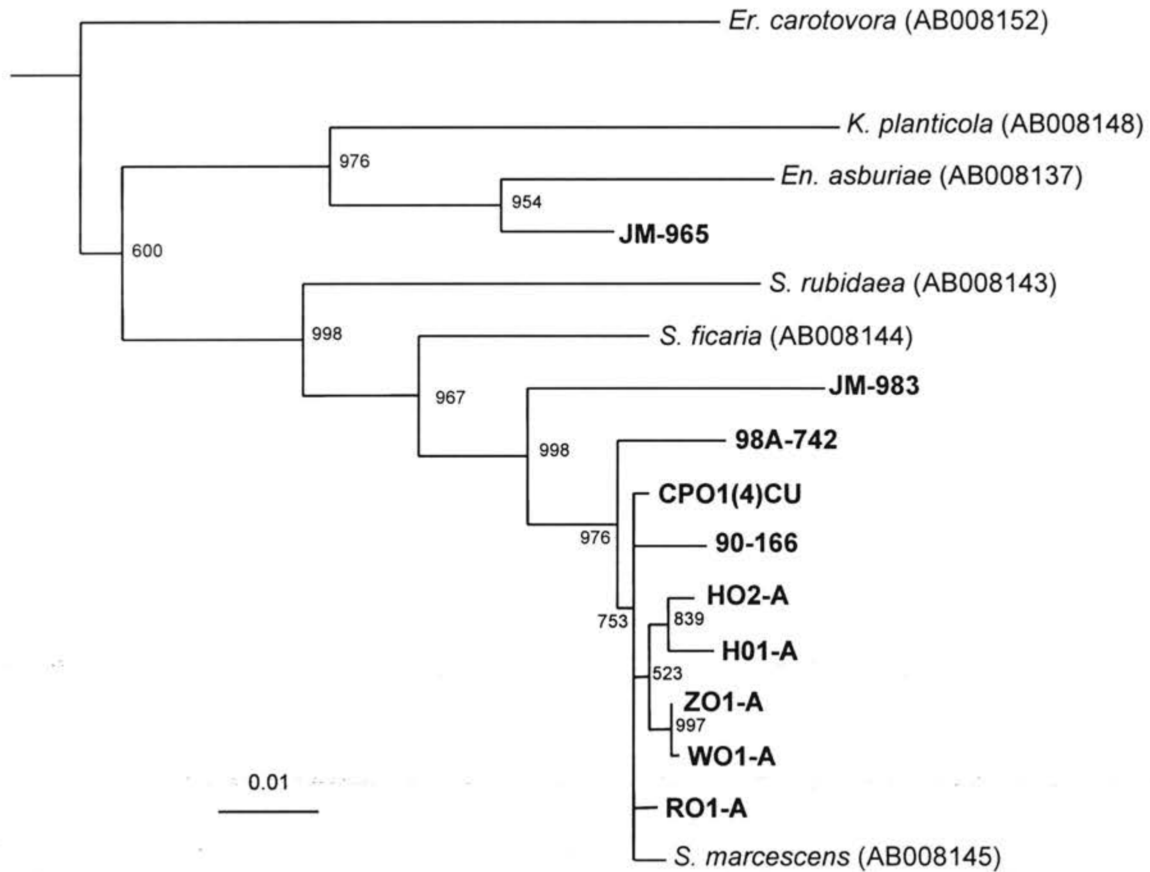


Figure 6. Concise phylogenetic distance tree compiled from *groE* sequence data, using DNADist and Neighbor, with the endosymbiont *Sitophilus oryzae* as outgroup. Branches with bootstrap values less than 500 (out of 1000) are collapsed.



2

VITA

John E. Rascoe

Doctor of Philosophy

Thesis: ARBITRARILY PRIMED PCR OF cDNA FOR INVESTIGATION OF DIFFERENTIAL PRESENCE AND EXPRESSION OF GENES IN THREE LINES OF *SPIROPLASMA CITRI*

and

SEQUENCE ANALYSIS OF *SERRATIA MARCESCENS* STRAINS ASSOCIATED WITH CUCURBIT YELLOW VINE DISEASE

Major Field: Plant Pathology

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