# MOLECULAR CHARACTERIZATION OF SPIROPLASMA VIRUSES AND THE MECHANISM OF RESISTANCE OF *SPIROPLASMA CITRI* LINES TO INFECTION BY THE VIRUS SVTS2

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MOLECULAR CHARACTERIZATION OF
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OF *SPIROPLASMA CITRI*
LINES TO INFECTION
BY THE VIRUS SVTS2

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# CHAPTER I

# INTRODUCTION

Plant pathogenic spiroplasmas cause crop diseases including corn stunt and citrus stubborn disease. The spiroplasmas specifically inhabit phloem tissue in diseased plants and are transmitted in nature by phloem-feeding leafhoppers. Plant pathogenic spiroplasmas include _Spiroplasma_ _citri_, _S_. _kunkelii_ and _S_. _phoenicium_.

_S_. _citri_ causes citrus stubborn disease and horeseradish brittle root disease (Markham et al. 1974, Fletcher et al. 1981). Horseradish brittle root was first reported in Illinois in 1936, and caused severe crop losses in that year and also in 1953-1954, 1975, and 1979 (Fletcher et al. 1981). This disease also was epidemic in Maryland (Davis and Fletcher 1983). Stubborn disease affects numerous species of plants in at least 15 families (Saglio et al. 1973), and has been serious in California and Arizona. Three species of leafhoppers (_Circulifer_ _tenellus_, _Scaphytopius_ _nitridus_, and _S_. _acutus_) are known to transmit _S_. _citri_ (Kaloostian et al. 1975, 1979; Oldfield et al. 1976)

Spiroplasmas are helical, motile procaryotes which lack a cell well. The membrane is directly exposed to the environment and may have important physiological functions

in the interactions among spiroplasma, plant and leafhopper. Twenty-four distinct membrane proteins were distinguished by SDS-PAGE and 40 different membrane proteins were separated by 2-D IEF/SDS-PAGE (Wroblewski 1981, Simoneau et al. 1988). At least 12 membrane proteins have surface components (Fletcher et al. 1989), and may be involved in interactions with the plant host and insect vector.

Viruses often occur in spiroplasmas (Cole et al. 1979, Dickinson et al. 1984). These viruses have been investigated as a tool for studying the molecular biology of Mollicutes and have been applied as cloning vectors for expression of genes in Mollicutes (Bove et al. 1989; Stamburski et al. 1991; Marais et al. 1992). Replication of spiroplasma viruses is associated directly with a rapid decline of spiroplasma, giving rise to a hope of using viruses to control spiroplasma disease (Alivizatos 1982).

In this work, the mechanism of resistance of virus-resistant lines of S. citri was investigated. The possibility that viral DNA was methylated by the host spiroplasma and that a fragment of virus DNA acts as an insertion sequence was explored. The phenomenon of spiroplasma gene regulation by virus DNA integration was investigated. Finally, a new spiroplasma virus from S. citri strain BR3 was isolated and characterized.

# CHAPTER II

# LITERATURE REVIEW

## I. THE PLANT PATHOGENIC SPIROPLASMAS AND THEIR MOLECULAR BIOLOGY

### 1-1. *Spiroplasma citri* and the disease it causes.

Spiroplasmas are usually helical, motile procaryotes which lack a cell well and are closely related to mycoplasmas in the Class Mollicutes. These wall-less eubacteria are characterized by the small size of their genome and a low G + C base content. There are six genera of Mollicutes: *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Anaeroplasma*, *Acholeplasma* and *Asteroleplasma*. The helical Mollicutes, later designated spiroplasmas, were first described by Davis et al. (1972), and shown to cause corn stunt disease. Saglio et al. (1971) and Fudl Allah et al. (1972) were the first to culture the plant pathogenic mollicute, *Spiroplasma citri*. Three years later, the spiroplasma causing corn stunt, *S. kunkelii*, was cultivated (Williamson et al. 1975; Chen et al. 1975). More than 30 different spiroplasmas from more than 10 different environments, including plants, insects and abiotic substrates are known today (McCammon et al. 1988; Williamson et al. 1989). Plant pathogenic spiroplasmas cause several commercially important diseases in citrus,

cherry, maize, lettuce, turnip, broccoli, horseradish and others. They cause symptoms of stunting, yellowing, little leaf, abnormal flowers and fruits, wilting and necrosis. Spiroplasma infections may cause severe reductions in production efficiency of citrus, maize and horseradish (McCammon et al. 1988). Twenty-three spiroplasma groups and eight subgroups in Group I have been proposed based on cultural, serological, and biochemical comparisons, and on relatedness of genomic DNA (Williamson et al. 1989). The causal agent of citrus stubborn and horseradish brittle root diseases, S. citri, the agent of corn stunt disease, S. kunkelii, and the agent of yellowing disease of periwinkle, S. phoeniceum, are all in Group I (Jordan et al. 1989).

Stubborn disease of citrus is a serious problem in California and Arizona and is also widely distributed in many Mediterranean and Mideast countries including Algeria, Cyprus, Egypt, France, Iran, Iraq, Italy, Jordan, Lebanon, Morocco, Syria and Tunisia (Gumpf 1988). This pathogen is spread by leafhoppers. In the western United States the primary vectors are Circulifer tenellus (Baker), Scaphytopius nitridus (Delong) and S. acutus (Delongi Young) (Kaloostian et al. 1975, 1979; Oldfield et al. 1976). Symptoms include small, abnormal upright leaves, bunchy or rosetted foliage and numerous short leaf branches, twig dieback, unseasonal flowering, excessive fruit drop, small fruits with bitter flavor and low yield.

The genome of S. citri, about 1780 kbp in size, is the largest Mollicute genome tested so far (Ye et al. 1992). A physical and genetic map of S. citri DNA has been developed. The G + C values of this spiroplasma range from 24-31 mol %. (Bove et al. 1989). Comparisons of 16S and 5S ribosomal RNA sequences from different Mollicutes indicated that S. citri is phylogenetically related to Mycoplasma mycoides and its relatives (Weisburg et al. 1989).

**1-2. Spiroplasma membrane proteins.**

Because it lacks a cell wall, S. citri is extremely sensitive to osmotic shock. Its membrane, which is approximately 12-16 nm thick, contains lipid, cholesterol, phosphorus, RNA, DNA, carbohydrate, and hexosamine (Razin et al. 1973). Some of the mannose, glucose and galactose residues are exposed on the membrane surface and most of the carbohydrate residues are attached to proteins in the membrane, as glycoproteins (Kahane 1977).

Surface proteins of Mollicutes have been implicated in a variety of activities related to interactions with their host cells. For example, they may function as binding sites or as enzymes in the infection process. Surface proteins may prove to be critical components in the pathogenicity or transmissibility of plant Mollicutes (Fletcher et al. 1989). Wroblewski (1981) reported 24 bands in SDS-PAGE profiles of S. citri membrane proteins. He recognized seven major components: D5 (spiralin, 26 Kd), D7

(39 kd), D9 (51 kd), D13 (69 kd), D14b (76 kd), D16 (89 kd), and D17 (95 kd). Simoneau et al. (1989) detected a plasma membrane protein (84 kd), which binds concanavalin A and may be a glycoprotein. Studies by Townsend et al. (1980) revealed that a 39 kd membrane protein present in all helical strains was missing in the nonhelical S. citri strain, ASP-1. He suggested that this protein may be part of the structure connecting fibril elements to the membrane. Lee and Davis (1987) found that the poorly helical or "nonhelical" strain F32 of S. kunkelii lacked at least one protein (MW 38 Kd) present in helical strains. This missing protein was correlated with the lack of helicity. However, whether this membrane protein alone is responsible for the stability of cell helicity or whether other membrane components are involved is unknown. Simoneau et al. (1988) analyzed the plasma membrane proteins of S. citri by two-dimensional SDS-PAGE and found 40 different proteins in the membrane preparation. Recently, Fletcher et al. (1989) showed that twelve membrane proteins which have surface components are on the membrane of S. citri. These were designated SP1-SP12, and are 87, 85, 81, 77, 65, 62, 57, 55, 48, 40, 29 or 26 Kd.

Spiralin is the most abundant protein in the membrane of S. citri, contributing 22% of the total protein mass (Wroblewski 1977). It is the principal antigen of S. citri and was once considered to have a role in determination of

the helical shape of S. citri. It may be a fibrous protein. The amino acid composition of this protein is quite different from that of the whole membrane, as it lacks methionine and tryptophan (Wroblewski et al. 1984). It spans the membrane and has exposed sites on the surface of S. citri cells (Archer 1981). Its structure and/or functional integrity is necessary for maintaining the helical cell shape (Blanchard et al. 1985). Spiralin also has been detected in the cytoplasmic fraction, where it may perform a biological function or may be a precursor to that found in the membrane (Wagih and Fletcher 1988). Spiralin is not an extremely hydrophobic protein. It may be bound to the membrane by a sequential or a conformational hydrophobic anchor buried in the apolar region of the phospholipid bilayer (Wroblewski 1977). Disulfide bonds do not play a major role in the antigenicity of spiralin or the other integral membrane proteins of S. citri (Quilien 1986). Spiralin was cloned and expressed from its own promoter in Escherichia coli (Mouches et al. 1985). The cloned S. citri spiralin gene has been used to detect S. citri in diseased plants and insects (McCammon and Davis 1988). The gene has been sequenced, showing 241 amino acids and a molecular mass of 25,282 daltons (Chevalier et al. 1990). A weak serological relationship exists between the spiralin of S. citri and a spiralin-like protein of S. melliferum. Acylation of the S. melliferum spiralin-like protein was

demonstrated (Wroblewski et al. 1989). Chevalier et al. (1990) showed the presence of a gene in S. melliferum whose sequence was 88.6% identical to that of the S. citri spiralin gene. The deduced amino acid sequence was 75% identical to the spiralin sequence. The S. melliferum spiralin contains 242 amino acids and is 25,430 Da, slightly different from the S. citri spiralin. For both of these proteins, four amino acid resides, Ala, Val, Lys, and Thr, represent as much as 50% of all residues. Neither the S. citri spiralin nor the S. melliferum spiralin-like protein contains tryptophan (Chevalier et al. 1990). A change in one to twenty-two amino acids of the total 241 amino acids of spiralin causes a variation in electrophoretic mobility in several S. citri strains (Foissac et al. 1992). The physiological function of spiralin in spiroplasma cells is not yet known.

**1-3. Methylation in spiroplasma.**

Razin et al. (1980) were the first to detect methylated bases in mycoplasmal DNA. The DNA of several spiroplasma species and strains contains methylated cytosine, varying in its nearest neighbor relationship to either all of the four bases, to two of the four bases, or only to guanine in different species of spiroplasmas (Nur et al. 1985). Nur et al. (1985) and Bove et al. (1989) found that S. citri, S. melliferum, S. floricola, S. apis and a tick spiroplasma all appeared to contain $^{m5}$Cyt and $^{m6}$Ade in low amounts. The

extent of methylation was influenced by the age of the culture (Nur et al. 1985). The DNA methylases partially purified from S. apis strain B31 and Spiroplasma sp. strain MQ-1 can methylate the RF DNA of phage M13 in vitro. A CpG DNA methylase from Spiroplasma sp strain MQ1 (M.Sssl, Group VII) has been cloned and sequenced. The deduced amino acid sequence revealed that M.Sssl has all of the common domains characteristic of bacterial cytosine DNA methylases (Renbaum et al. 1990). Hershkovitz et al. (1990) showed that the methylase M.Sssl from spiroplasma can methylate all CpG sites in the pUC8CaMVCAT plasmid in vitro and completely inhibit CAT gene activity. Juettermann et al (1991) studied methylation effects on the RNA polymerase III- transcribed VAI gene of adenovirus type 2 DNA, and found that a CpG-specific DNA methyltransferase from Spiroplasma sp. methylated all 20 5'-CG-3' sequences in the VAI region and interfered with VAI transcription. Thus, methylation of CAT or viral genes by spiroplasma methylase can inactivate the expression of these genes.

## II. SPIROPLASMA VIRUSES

### 2-1. Spiroplasma virus characterization and classification.

Virus-like particles were first discovered in S. citri in 1973 (Cole et al.). A wide variety of viruses has since been associated with spiroplasmas. Of 48 spiroplasma strains examined, 92% carry one or more types of virus-like

particles. Most strains of the plant pathogen S. citri are persistently infected by a rod-shaped virus (Dickinson et al. 1984). Four types of spiroplasma viruses have been identified in different species and strains of spiroplasmas (Cole et al. 1974; Ricard et al. 1982; Townsend 1983; Dickinson et al. 1984; Renaudin 1988). Bove et al. (1989) divided spiroplasma viruses into groups designated SpV1, SpV2, SpV3 and SpV4 (Table 1). SpV1-type viruses of S. citri are rod-shaped particles containing a circular, single-stranded DNA (Dickinson et al. 1984). SpV2 is a polyhedron with a long tail and probably double-stranded DNA (Cole et al. 1973). SpV3 is a polyhedral short-tailed virus with double-stranded DNA (Cole et al. 1977). A small naked isometric virus with single-stranded circular DNA, SpV4, was discovered in 1982 (Ricard et al. 1982).

SpV1-type spiroplasma virus SVTS2, isolated from S. melliferum, is rod-shaped and contains circular, single-stranded DNA of about 6.5 kb. Virions or viral DNA can form plaques on lawns of the indicator host, S. citri strain M200H, which is a high passage subculture of the type strain of S. citri, R8A2 (McCammon and Davis 1987; Jordan et al. 1989).

SpV1-R8A2B was isolated from S. citri strain R8A2. The DNA of this virus contains 8,273 nucleotides and has a G + C content of 23 mol%. It has 12 ORF (open reading frames), of which ORF4 shows limited similarity to the integrase of

phage P22 (Renaudin et al 1990). Its ORF3 may be part of an insertion sequence (Renaudin et al. 1992; Dong et al. 1992).

SpV2-type spiroplasma virus SVC2 is a long-tailed polyhedron and has been isolated only from S. citri (Cole et al. 1973). This lytic virus has not been propagated in the laboratory, and therefore most of its properties are unknown (Cole et al. 1974).

SpV3-type spiroplasma virus ai was isolated from plants infected with S. citri strain Sp-V3 and propagated on S. citri strain Sp-A. Its host range is similar to that of SpV1- type spiroplasma virus aa. However, its isometric head and short tail are morphologically similar to SVC3 (SpV3), and it has a ds DNA genome. The virus contains seven structural proteins (MW 86, 65, 63, 55, 47, 45, 26 Kd), The 65-kd and 45-kd proteins are the major virion proteins. The SpV3-ai progeny virus is released by budding from the S. citri cell membrane. A restriction endonuclease cleavage map has been constructed (Dickinson et al. 1984).

Spiroplasma virus SpV4 is an isometric particle isolated from S. melliferum (Ricard et al. 1982). Its host range is limited to subgroup I-2 honeybee spiroplasmas. The isometric capsid is composed of one major protein of 60 kd and contains a circular, single-stranded DNA molecule (Renaudin et al. 1984). The nucleotide sequence of the SPV4 genome contains 4,421 nucleotides with a G + C content of 32 mol%, slightly higher than that of the S. melliferum host DNA

[26%]. Nine ORFs have been identified on its genome. ORF1 is the gene for the capsid protein, and ORF5 may encode a lysis protein (Renaudin et al. 1987).

Alivizatos (1982) first reported that viruses can be produced by spiroplasma within infected host plants, and that virus replication was associated with a rapid decline in spiroplasma viability. Spiroplasmas which supported a productive virus infection had substantially reduced pathogenic capabilities and were acquired and transmitted by leafhoppers with much lower frequency than those which were uninfected. Thus, it may be possible to use the viruses of plant-infecting spiroplasmas in biocontrol efforts.

**2-2. Mechanisms of virus-resistance in spiroplasmas.**

Dickinson et al. (1985) reported that SpV3-ai was able to lysogenise _S_. _citri_, and presented evidence of a correlation between lysogenisation and the attenuation of spiroplasma pathogenicity. They suggested that the lysogenisation of _S_. _citri_ SpA-MD by virus ai appears to result in a conversion of the cell surface, which is manifested by a loss of receptors for virus ai. They proposed that conversion of the cell surface is a general feature of lysogenisation of the spiroplasmas (Dickinson et al. 1985).

_S_. _melliferum_ strains BC-3, B63, G1R2 and G1R4 were found to be resistant to infection by SpV4 virions, but could be transfected with SpV4 DNA. This indicated that resistance to infection by whole virus is at the level of adsorption or

penetration of the virions (Bove et al. 1989).

Sequences of the SpV1-type and SpV3-type spiroplasma viruses occur in the chromosomal DNA of S. citri, S. kunkelii and S. phoeniceum (Bove et al. 1989). SVTS2 has been shown to hybridize to fragments of the genomes of both S. melliferum and S. citri (McCammon and Davis 1988). Probably the entire SpV1 virus genome inserted in the chromosome of the host spiroplasma (Renaudin et al. 1988, 1990). At least 17 copies of SpV1- R8A2B and 2 copies of SpV1-S102, which represents up to one 12th of the whole spiroplasma genome, have been found in the genome of S. citri strain R8A2HP. These viral-like sequences may be considered as repeated elements and may play a role in genomic rearrangements (Ye et al. 1992). The ORF3 of SpV1-R8A2 was considered to be an insertion sequence (IS) because it had up to 50% amino acid sequence identity with IS30 (Renaudin 1992) and 52% and 53% amino acid sequence identity with IS4351 and IS1086, respectively (Dong et al. 1992).

**2-3. Spiroplasma virus RF used as a molecular cloning vector.**

The complete RF DNAs of SVTS2, SpV1-R8A2 and SpV4 have been cloned in E. coli. When cloned RF DNA of SVTS2 or SpV4 was excised from the vector and recircularized, it was capable of transfecting susceptible spiroplasma hosts. However, the SVTS2::pUC19 cointegrate was not infectious

(Pascarel- Devilder et al. 1986; McCammon and Davis 1987; Renaudin et al. 1990). To address the problem of gene transfer, McCammon and Davis (1987) used 50% polyethylene glycol to enhance the introduction of viral DNA into spiroplasma, and obtained transfection efficiencies of 1 X $10^5$ transfectants/ug SVTS2 DNA. Transfection of S. citri R8A2 HP cells with SpV1-R8A2B RF DNA by electroporation resulted in 6 X $10^5$ transfectants /ug DNA (Stamburski et al. 1991). Recently, a promoter and a transcription terminator of SpV4 were characterized (Stamburiski et al. 1990). The first successful use of SpV1- R8A2B RF as a cloning vector for gene expression in S. citri has been reported (Stamburski et al. 1991). They showed that the CAT gene inserted into one of the four intergenic regions of the S. citri virus SpV1-R8A2 replication form and was introduced into S. citri cells by electroporation. The transfected spiroplasma cells transiently expressed CAT activity, but this activity was lost due to instability of the recombinant virus.

Stamburski et al. (1992) showed that mutagenesis of a tryptophan codon from TGG to TGA in the CAT gene does not prevent its expression in S. citri by the SpV1-RF vector. The G fragment of the cytadhesin P1 gene from Mycoplasma pneumoniae also was expressed in S. citri with the SpV1-RF vector system despite the 7 UGA codons present in the reading frame of the G fragment (Marais et al. 1992). These

results indicated that SpV1-RF can be used as a gene vector to transform S. citri cells, and that S. citri cells are able to express a foreign gene in which TGA codes for tryptophan. The SpV1-RF vector system can be applied in genetic research of Mollicutes.

**2-4. Mycoplasmavirus and bacteriophages.**

**a-1. Binding sites and receptors.**

Maniloff et al. (1979) found that the mycoplasma virus MVL 51 first attached or adsorbed to the cell surface and then the viral DNA penetrated into the cytoplasm. Putzrath (1977) showed that only about 1 in 10 of the potential virus-cell collisions leads to adsorption, suggesting that not all virus particles attach to the host cells. The mycoplasma cell may have a limited number of receptor sites, and only virus binding to a receptor site can adsorb. The involvement of protein in the cell receptor for mycoplasma virus L51 has been shown by Al-Shammari et al. (1982). Haberer et al. (1982) demonstrated binding of mycoplasmavirus L3 to the cell membrane of Acholeplasma laidlawii, and found that membrane proteins serve as the virus L3 receptors. Although mycoplasma membrane lipids do not form part of the receptor structure for L2 virus adsorption (Al-Shammari et al. 1982), the fatty acid composition of virus and cell membrane affect adsorption (Steinick et al. 1986), perhaps by modulating the functions of these membranes (Maniloff 1988). The oligosaccharide components of mycoplasma lipoglycan may form

part of the receptor for virus L2 adsorption (Al-Shammari et al. 1982)

Some bacteriophage receptors are components of the cell wall or lipopolysaccharide of host. A receptor for bacteriophages of Lactococcus lactis subsp. cremoris KH was located on the cell wall and not the cell membrane. Galactose was essential for binding of all phages to this bacterium (Valyasevi et al. 1990).

The lipopolysaccharides of the Pseudomonas phaseolicola cell membrane were shown to be responsible for binding to the bacteriophage (Samoilenko et al. 1985). The cell surface receptor for bacteriophage FC3-2 from Klebsiella pneumoniae was identified as a lipopolysaccharide, specifically an O-antigen. Mutants resistant to this phage were devoid of lipopolysaccharide O-antigen (Tomas et al. 1987), indicating that the absence of a virus receptor is the reason for virus resistance in this organism.

Adsorption of a virus to a host cell also may require the presence of a specific receptor protein on the virus. Studies showed that protein 38, located at the free ends of the long tail fibers of phage T2, K3 and Ox2, serves as an adhesin in the binding of virus to E. coli (Riede et al. 1987a). The sequences of residues 116-226 of the T2 protein and 116-223 of the K3 protein are similar to sequences present in phage receptors of E. coli outer membrane proteins (Riede et al. 1987b). Bamford (1987) showed that

bacteriophage 06, which lacked protein P3 or both proteins P3 and P6, did not adsorb to its host, Pseudomonas syringae. This indicated that a protein in the coat of the phage particles is required for binding to the host.

**a-2. Mechanisms of bacteriophage resistance in bacteria**

**(1). Adsorption**

Blokhina et al. (1985) found that in most cases the resistance of natural Pseudomonas aeruginosa strains to type phi K phages is due to disturbances in their adsorption. However, Budde-Niekiel et al. (1987) found that all phages of lactic acid Streptococci adsorbed to a variety of phage resistant strains, indicating that this phage resistance does not involve adsorption to the host. Dybvig et al. (1988) reported that a major surface antigen complex of Mycoplasma pulmonis, V-1, was missing in strains susceptible to virus P1. All of the resistant strains did have the V-1 antigen. The perfect inverse correlation between the presence of V-1 and adsorption of virus P1 to the host cell suggests that the V-1 antigen prevents virus adsorption.

**(2). Plasmid-mediated virus resistance**

Plasmid pTR 2030 was conjugated into Streptococcus cremoris M12R and its presence was correlated with resistance to lytic phage (Steenson et al. 1985). Jarvis and Klaenhammer (1987) also showed that a bacteriophage resistance plasmid, pTR2030, inhibited lytic infection by r

sub(1)t temperate bacteriophage but did not inhibit induction of prophage in Streptococcus cremoris R1. In Pseudomonas aeruginosa and P. putida, the infection ability of bacteriophages was limited primarily by plasmids belonging to the P-2 incompatibility group (Kulakov et al 1984). De Los Reyes-Gavilan et al. (1990) showed the presence of a restriction-modification (R/M) system against two bacteriophages, 320B1 and hv, in three Lactobacillus helveticus strains. The genes responsible for this R/M system were located in a 34 kb plasmid, indicating that this plasmid was involved in the bacteriophage resistance.

**(3). Mechanism of lysogeny**

Lysogeny implies viral DNA integration into the host cell chromosome. Dybvig and Maniloff (1983) showed that Acholeplasma laidlawii cells lysogenized by mycoplasma virus L2 have a single L2 DNA sequence integrated in the cell chromosome. A single integration site was found in both viral and cellular DNA. Viral DNA integration may also lead to rearrangement of host cell DNA. The chromosome of Mycoplasma pulmonis has a high frequency of rearrangements, some of which were correlated with changes in the susceptibility of the cells to mycoplasma virus P1 (Bhugra et al. 1992).

Bacteriophages integrated not only into different chromosomal sites of Pseudomonas aeruginosa, but also into its plasmid (Plotnikova et al. 1983). Van der Avoort et al.

(1984) showed that the intracellular presence of a recombinant plasmid containing the intercistronic region between genes H and A of bacteriophage Phi X174 strongly inhibits the conversion of infecting single-stranded Phi X DNA to parental replication-form DNA. This indicated that a phage reduction sequence functioned as a viral incompatibility element.

Proteins encoded by phage may also be involved with lysogeny. The CIII protein of lambdoid bacteriophages promotes lysogeny by stablizing the phage-encoded CII protein, a transcriptional activator of the repressor and integrase genes (Kornitzer et al. 1991)

In addition, lysogeny also modified host cell surface structure, resulting in the failure of virus to adsorb to the host. Lysogenization of Salmonella typhimurium with bacteriophages A3 and A4 results in O-acetylation of the L-rhamnose residues of the O-polysaccharide chain of the lipopolysaccharide of the bacterial cell envelope. This lysogenic conversion prevents the adsorption of the A3 and A4 phages (Wollin et al. 1987).

In summary, the absence or modification of a virus receptor, existence of a plasmid containing resistance genes, the integration of viral DNA and rearrangement of host DNA, or the role of a phage reduction sequence as a viral incompatibility element may all lead to virus resistance in the microbial host.

In this thesis, the mechanism of resistance of *Spiroplasma citri* lines to infection by the virus SVTS2 was studied to try to understand the interactions between virus and spiroplasma host.

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Table 1. Spiroplasma Virus Characterization and Classification.

| Group-Virus | Morphology | Genome | Host | Reference |
|---|---|---|---|---|
| SpV1-R8A2B | Rod-shaped | ss DNA 8.2 kb | S. citri R8A2B | Renaudin et al 1984 |
| SpV1-aa | Rod-shaped | ss DNA 8.5 kb | S. citri Sp-V3 | Dickinson et al 1984 |
| SpV1-SVTS2 | Rod-shaped | ss DNA 6.5 kb | S. melliferum TS2 | McCammon et al 1987 |
| SpV1-78 | Rod-shaped | ss DNA 8.4 kb | S. citri 78 | Renaudin et al 1988 |
| SpV1-SVBR3 | Rod-shaped | ss DNA 8.6 kb | S. citri BR3 | Sha et al 1992 |
| SpV2-C2 | Isometric head long tail | ds DNA | S. citri R8A2 | Cole et al 1974 |
| SpV3-ai | Isometric head short tail | ds DNA 16 kb | S. citri Sp-V3 | Dickinson et al 1984 |
| SpV3-C3 | Isometric head short tail | ds DNA 21 kb | S. citri | Cole et al 1974 |
| SpV3-SRO | Isometric head short tail | ds DNA 17 kb | Drosophila SRO | Cohen et al 1984 |
| SpV4 | Isometric no tail | ss DNA 4.4 kb | S. melliferum B63 | Ricard et al 1982 |

## CHAPTER III

Comparison of Total Protein Profiles and Identification of Membrane Proteins of S citri M200H and Two Virus SVTS2 Resistant Lines by One- and Two-Dimensional Gel Electrophoresis.

### ABSTRACT

Spiroplasma citri M200H is a triply cloned isolate derived from S. citri Maroc R8A2. Plaques are formed on agar lawns of M200H infected by spiroplasma virus SVTS2, which was isolated from S. melliferum TS2. Two virus- resistant S. citri lines, MR2 and MR3, were derived from colonies growing within cleared plaques on lawns of M200H inoculated at a high MOI with SVTS2. The resistant lines did not produce plaques when inoculated with SVTS2 virions. Protein profiles of the resistant lines were compared with each other and with those of the parent strain using 1- and 2-D polyacrylamide gel electrophoresis. There were no significant differences among the 1-D SDS-PAGE protein profiles. In 2-D gels, three proteins, P1, P2 and P3, seen in the parent strain M200H, were missing or significantly reduced in both resistant lines. P1 and P2 were membrane-associated proteins. By using antiserum against a membrane protein preparation from S. citri, 7 additional

membrane-associated proteins were identified in 2-D gels of proteins from M200H, MR2 and MR3. Genomic DNA fingerprints of M200H, MR2 and MR3 were similar. Electron microscopy showed SVTS2 particles bound to M200H cell surfaces. Pre-incubation of S. citri M200H with antiserum against total membrane protein of S. citri significantly reduced the number of plaques produced on subsequent inoculation with SVTS2, but pre-incubation with antisera specific for individual membrane proteins of 55 kd, 77 kd and 89 kd did not. Thus, these membrane proteins probably are not involved in virus attachment or invasion.

## INTRODUCTION

Spiroplasmas are helical, wall-less procaryotes placed with mycoplasmas in the Class Mollicutes. Surface proteins of animal- and insect- infecting Mollicutes have been implicated in a variety of activities related to interaction with their host cells or with viruses that infect them. Surface proteins may prove to be critical components in similar activities of plant Mollicutes (Fletcher et al. 1989). Wroblewski et al. (1981) identified 24 membrane proteins in S. citri by 1-D SDS-PAGE. However, 40 different membrane proteins were distinguished in S. citri by 2-D IEF/SDS-PAGE (Simoneau et al. 1988). Fletcher et al. (1989) reported that at least twelve membrane proteins of S. citri have surface components. Spiralin (25-29 kd) is the most

abundant protein in the membrane of S. citri (Wroblewski et al. 1977). The spiralin gene has been cloned, expressed in E. coli, and sequenced (Mouches et al. 1985, Chevalier et al. 1990). This spiralin gene in S. citri shared 88.6% identity to the gene for a spiralin-like protein in S. melliferum (Chevalier et al. 1990). Spiralins of different S. citri strains may have different electrophoretic mobilities, due to changes in 1 to 20 amino acids (Foissac et al. 1992).

Four types of spiroplasma viruses are known: SpV1, SpV2, SpV3 and SpV4 (Bove et al. 1989). SpV1-R8A2 and SpV4 were completely sequenced (Renaudin et al. 1987, 1990). The SpV1-type spiroplasma virus SVTS2, isolated from S. melliferum strain TS2, is rod-shaped, contains circular, single-stranded DNA of 6.5 Kb and forms plaques on lawns of S. citri strain M200H (McCammon et al. 1987). To our knowledge, SVTS2 is the only spiroplasmavirus known to infect more than one spiroplasma species (Sha et al. 1993).

Dickinson et al. (1985) suggested that loss of a receptor for virus adhesion is a general feature of lysogenisation. Bove et al (1989) also hypothesized that for two spontaneous mutants of S. melliferum strain G1, G1R2 and G1R4, resistance to infection by the virus SpV4 is due to failure of the virion to adsorb to or to penetrate the spiroplasma.

In this work, two lines of S. citri which are resistant to SVTS2 infection were characterized. To determine whether

that the mechanism of resistance to the virus involved a failure of the virus particles to bind to the spiroplasma surface, the possibility was explored that a virus receptor protein was missing in the resistant spiroplasma lines by comparing protein patterns of susceptible and resistant lines.

## MATERIALS AND METHODS

**Isolation of virus-resistant lines and assay for virus resistance.** A lawn of S. citri strain M200H, which was derived from S. citri Maroc (R8A2) by subculture, was prepared by spreading 100 ul of LD8 broth (Davis 1979) culture (approximately $10^8$ cells/ml) onto a 6-cm LD8 agar plate. After overnight incubation at 31 $^oC$, 50 ul SVTS2 preparation ($10^{12}$ PFU/ml) was dropped into the center of the lawn and the plate incubated at 31 $^oC$ for 1-2 days for development of plaques. The presence of colonies within plaque boundaries suggested either spontaneous mutation or selection for virus-resistant spiroplasmas. Two resistant colonies, designated MR2 and MR3, were transferred to LD8 broth. This isolation of resistant S. citri lines was done by R. E. Davis and coworkers, U.S.D.A./ARS, Beltsville, MD.

For plaque assay, S. citri M200H was grown in LD8 broth to a titer of approximately 2 X $10^8$ cells/ml. One hundred ul of culture was spread onto 6 cm LD8 plates and allowed to dry,

covered, at room temperature for 1-2 hr. Fifty ul virus suspension at 4.2 X $10^4$ or 4.2 X $10^6$ PFU/ml was dropped onto the center of the lawn. Plates were inverted and incubated at 31 $^{O}$C until plaques were visible (2-4 days).

**Virus isolation and purification.** S. melliferum strain TS2 was grown to a titer of 5 X $10^8$ -1 X $10^9$ cells/ml in one L LD8 broth. Cells were pelleted at 10,000 x g, 4 $^{o}$C for 30 min. The supernatant was brought to 0.2% Triton X-100 and incubated overnight at 4 $^{o}$C. The suspension was centrifuged at 95,000 x g, 5 $^{o}$C for 2.75-3 hr, and the pellet was resuspended in 10 mM Tris-HCl (pH 7.9) overnight. For each ml of virus preparation, 0.387 g CsCl was added, and the mixture was centrifuged at 105,000 x g, 20 $^{o}$C for 24-48 hrs. The virus band was removed from the tube and dialyzed in 10 mM Tris-HCl (pH 7.9) at 4 $^{o}$C for 48-73 hrs with three changes of buffer. Virus titer was determined by plaque assay.

**Extraction of spiroplasma cell total proteins.** LD8 broth was inoculated with log phase cultures of strains M200H, MR2, and MR3 and incubated at 31 $^{o}$C until the titer reached approximately $10^{10}$ cells/ml. Cells were harvested by centrifugation at 15,000 x g, 20 min. Each pellet was resuspended in 25-30 ml HEPES-S buffer (0.07 M HEPES, 10% sucrose, pH 7.5) and centrifuged again. The washing was repeated twice. The final pellet was resuspended in 300 ul HEPES-S and sonicated for a few seconds until the cells were

lysed and the preparation was homogeneous. The protein concentration of the sample was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, California) and the sample was frozen at -20 °C.

**Extraction of spiroplasma membrane proteins.** The TX-114 phase separation method of Bordier (1981) was used. An S. citri protein preparation was thawed and the protein concentration determined by the Bio-Rad protein assay. To one ml of a 1 mg/ml protein solution, pre-condensed TX-114 was added to a concentration of 1% and mixed thoroughly. The solution was incubated at 4 °C, 5 min and then centrifuged at 13,000 x g to remove insoluble components. TX-114 solubilized material was transferred to a new microfuge tube and incubated 5 min, 37 °C, to condense the TX-114. After centrifuging 5 min, 13,000 x g, at room temperature, the upper (aqueous) phase was transferred to a second tube, which was brought to 1% TX-114 again. The lower (detergent) phase was brought to the original volume with phosphate buffered saline. Both tubes were incubated at 0-4 °C, 15 min. These steps were repeated twice.

**One-dimensional SDS-PAGE.** For 10% SDS-PAGE, cells were pelleted, solubilized and electrophoresed as previously described (Fletcher et al. 1981). Samples containing 10 ug protein/well (Bio-Rad Protein Assay) were electrophoresed 15 hr at 7-8 mA constant current. Bands were visualized with a silver stain (Mirrisley 1981), or proteins were transferred

to nitrocellulose for Western blotting.

**Two-dimensional IEF/SDS-PAGE.** For 2-D gels, proteins were subjected to isoelectric focusing (IEF) followed by SDS-PAGE (O'Farrell 1975). The spiroplasma total cell protein, dissolved in HEPES-S, was centrifuged at 15,000 x g and pellets were resupended in IEF buffer (9 M urea, 4% [w/v] chaps, 0.5% [w/v] dithiothreitol, 5% [v/v] Biolyte 3/10, 2% [v/v] Biolyte 5/7 [Bio-Rad Laboratories, Richmond, CA 94804]). Samples (25 ug protein/tube, Bio-Rad Protein Assay) were loaded onto IEF tube gels (0.25 x 8.5 cm) and electrofocused at 400 V for 12-18 hr, then at 800 V for 2 hr. Gels were extruded from the tubes, frozen on dry ice, and stored at -20 $^{o}$C. For the second dimension, an IEF gel was thawed and equilibrated in SDS reducing buffer (0.5 M Tris- HCl, pH 6.8, 10% glycerol, 2% SDS, 0.75% dithiothreitol and 0.005% bromophenol blue) and overlaid onto a 10% SDS-PAGE slab gel. The 2nd-dimension gel was electrophoresed 15 hr at 7-8 mA constant current in 16 cm long x 14 cm wide x 1.5 mm thick gel and proteins were visualized with silver stain (Mirrisley 1981).

**Western blotting.** After SDS-PAGE, the separated proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad Co., Richmond, California) using a transphor apparatus 9.5 cm between electrodes (Hoefer Scientific Instruments, San Francisco 94107) for 1.25 hr at 100 volts in transfer buffer (0.025 M Tris-HCl, 0.192 M

glycine, pH 8.3, and 20% [v/v] methanol). Filters were blocked overnight at 4 °C in 5% [w/v] Carnation powdered skim milk (5% [w/v] milk powder, 20 mM Tris-HCl , 500 mM NaCl, pH 7.5). After three washes in TBS (20 mM Tris-HCl, 500 mM NaCl pH 7.5), they were incubated for 3 hr at room temperature in antiserum diluted 1:300 in antibody buffer (TBS with 0.05% [v/v] Tween 20 and 1% [w/v] BSA). The antiserum against _S. citri_ BR3 total membrane protein preparation or antiserum against membrane protein p29 (spiralin) were prepared previously (Fletcher et al. 1989). After three additional washes, the nitrocellulose membranes were incubated for 1 hr in protein A-horseradish peroxidase (2 ug/ml in TBS). A final wash was followed by incubation in substrate (5 volumes TBS, 1 volume 4-chloro-1-naphthol at 3 mg/ml in methanol, and 0.018 volume 3% $H_2O_2$).

**Genomic DNA isolation and purification.** A modification of the method of Williamson et al. (1991) was used to isolate genomic DNA. A volume of 200 ml of log phase spiroplasma culture was centrifuged at 20,000 x g for 30 min. The cells were resuspended in 2 ml TGE buffer (25 mM Tris- HCl, pH 8.0, 50 mM glucose, 10 mM EDTA). Fifty ul of 20% SDS was added and cells were lysed at 60 $^{o}$C for 10 min. The solution was brought to 7.5 ml with TE buffer (100mM Tris- HCl, pH 7.4, 10 mM EDTA) and subjected to CsCl-EtBr gradient centrifugation (final density 1.55g/$cm^3$). After centrifugation at 239,120 x g (VTi 65 rotor, 50 k rpm) for

15 hr, the DNA band was removed and extracted with phenol. Two times the DNA volume of deionized water was added to extracted DNA. Two times the total DNA solution volume of 95% ethanol was added, and the mixture incubated at -70 °C for 1 hr. The DNA precipitate was collected at 14,000 x g for 10 min at 4 °C. The pellet was resuspended in 250 ul TEN buffer (10 mM Tris-HCl, pH 7.5, 10mM NaCl, 1 mM EDTA) and mixed 10-15 min. Six ul 5 M NaCl and 300 ul phenol (equilibrated with 0.1 M Tris buffer, pH >7.8.) were added and the mixture was microfuged 5 min. The aqueous phase was extracted with ether. Seven hundred fifty ul 95% ethanol was added and the suspension incubated at -20 °C overnight. After microfuging 15 min at 16,000 x g, the pellet was washed with 500 ul 70% ethanol, and the sample was microfuged for 5 min. The pellet was dried in a vacuum desiccator and dissolved in 100 ul TE buffer.

**Genomic DNA fingerprinting analysis.** The method of Herring et al. (1982) was used. Genomic DNA was digested with *Eco*RI 1 hr at 37 °C, and electrophoresed in 7% SDS-PAGE, for 28-29 hr at 22-25 mA constant current using electrode buffer (0.036 M Tris, 0.03 M sodium dihydrogen phosphate, 0.001 M EDTA, pH 7.8). For staining, the gels were washed with 10% [v/v] ethanol-0.5% [v/v] acetic acid for 30 min, soaked in 0.011 M silver nitrate for 2 hr, and rinsed briefly in distilled water. Reduction was performed with developing solution (0.75 M sodium hydroxide, 0.1 M formaldehyde, and

0.0023 M sodium borohydride).

**Electron microscopy.** Ten ml early log phase culture (1 x $10^9$ cells/ml) of M200H was centrifuged at 15,000 x g, 4 $^oC$ for 15 min. The pellet was resuspended in 1/4 volume TB buffer (15 mM Tris- HCl pH 7.5, 7% sorbitol, 0.1% fructose), and mixed with 50 ul virus SVTS2 (6.6 x $10^6$ PFU/ml). After incubating the mixture at 31 $^oC$ for 0.5-1 hr to allow for virus binding, the cells were fixed with 2.5 ml glutaraldehyde mixture (12.5% glutaraldehyde [v/v], 0.2 M cacodylate, 7% sorbitol [w/v], pH 7.0) and incubated on ice for 1 hr. The cells were collected at 15,000 x g for 20 min, washed with 2 ml Tris buffer (15 mM Tris-HCl, pH 7.5), and centrifuged again. The pellet was resuspended with 1/10 original volume of TB to give a cell titer of 1 x $10^{10}$ cells/ml. The sample was negatively stained with 3% [w/v] ammonium molybdate and examined using an JEOL 100-CX STEM electron microscope (Barile 1983).

**Blocking virus binding to the spiroplasma surface with antiserum against surface membrane protein.** A 5 ml culture of S. citri M200H (1.2 -1.8 X $10^9$ cell/ml) was centrifuged at 14,000 x g for 20 min. The cells were resuspended in 500 ul LD8 broth and incubated for 2 hr at room temperature with diluted preimmune rabbit serum (1:10, 1:50, 1:100, 1:200, and 1:300), with diluted antiserum against a S. citri total membrane protein preparation, or with antisera against a single membrane protein [P29, P55, P77, or P89] (Fletcher et

al. 1989). The cells were pelleted at 8,000 x g for 15 min, resupended in 500 ul LD8 broth, spread onto LD8 agar (100 ul/6 cm plate) and allowed to dry overnight at room temperature. Virus SVTS2 (6.6 X $10^6$ PFU/ml) was overlain at 50 ul/plate, and incubated overnight at room temperature, followed by 3-5 days at 31 $^{o}$C. The number of plaques was counted and statistical analysis was by the Student T-Test.

**Surface immunoprecipitation.** This technique was a modification of the one described previously (Fletcher et al. 1989). Thirty ml of a log phase spiroplasma culture was centrifuged at 10,000 x g for 15 min, and pellets were resupended in 3 ml of HEPES-S. A volume of 300 ul resuspended sample was inoculated with virus SVTS2 at MOI=1 for 20 min and centrifuged at 10,000 x g for 15 min. The pellet was resuspended in 300 ul HEPES-S. Samples were incubated for 2 h with 10 ul of undiluted anti-SVTS2 serum to allow binding of the antibody to the virus, then microcentrifuged for 2 min and washed twice with HEPES-S to remove unbound antibody. Pellets were resuspended in 500 ul of a hypotonic solution (10 mM Tris-HCl, pH 7.6, 10 mM NaCl, and 2 mM $MgCl_2$) and incubated at room temperature for 5-20 min. The membranes were solubilized by the addition of 100 ul of 5x lysis buffer (5% Triton X-100 [v/v], 5% Na deoxycholate [w/v], 0.5% SDS [w/v], 0.75 M NaCl, and 125 mM Tris-HCl, pH 7.5); surface membrane protein-SVTS2-antiserum complexes were then collected by the addition of 100 ul of

Staph A protein for 1 h at room temperature. After microcentrifugation, the Staph A pellets were resuspended in 25 ul HEPES-S buffer. Samples were electrophoresed in 10% SDS-PAGE, transferred to nitrocellulose, and probed with antiserum against S. citri membrane proteins as described above.

## RESULTS

**Resistance of S. citri lines MR2 and MR3 to SVTS2 infection.** After inoculation with SVTS2, cleared plaques formed in the lawns of S. citri strain M200H (Figure 1), but not in those of MR2 or MR3, even at high virus concentration [4.2 x $10^{6}$ PFU/ml] (Table 1). This experiment was replicated five times.

**Identification of protein differences among S. citri M200H, MR2 and MR3.** In one-dimensional SDS-PAGE, 46 bands were distinguished in lanes loaded with total proteins of S. citri M200H. No significant differences were seen between this profile and those of MR2 or MR3 (Figure 2A).

Two-dimensional IEF/SDS-PAGE patterns of S. citri M200H had 155 distinguishable protein spots, while MR2 had 153 and MR3 had 154 (Figures 2B, C, and D). Three protein spots (designated P1, P2, and P3), present in M200H, were missing or significantly reduced in MR2 and MR3. Spot P1 was significantly reduced in MR3 and missing in MR2, spot P2 was

missing in both MR2 and MR3, and spot P3 was significantly reduced in both MR2 and MR3. The test was repeated more than 20 times.

**Identification of membrane proteins of _S_. _citri_ in the 2-D gel profile.** Western blots of SDS-PAGE developed with antiserum against _S_. _citri_ BR3-3X membrane proteins showed no differences among M200H, MR2 (Figure 3A) and MR3 (data not shown). A 89 kd membrane protein was present in _S_. _citri_ BR3-3X, but missing in M200H, MR2 and MR3. Blots of two-dimensional gels of _S_. _citri_ M200H probed with antiserum against _S_. _citri_ strain BR3-3x membrane proteins, contained spots designated P4, P5, P6, P7, P8, P9 and P10 (Figure 3B). Spots P4, P5, P6, and P7 were more prominent than spots P8, P9 and P10. Blots probed with antiserum against _S_. _citri_ BR3 spiralin reacted with spot P7 (Figure 4). There were no significant differences in these probable membrane-associated proteins among M200H, MR2 and MR3 with four repeats. Spots P1 and P2 were not detected using this antiserum. Western blots were replicated 3 times.

2-D gels of purified membrane proteins of _S_. _citri_ separated into aqueous and detegent phases by TX-114 extraction, showed that P4 through P10 were detergent-soluble (data not shown).

**Genomic DNA fingerprinting analysis of M200H, MR2 and MR3.** Genomic DNA of M200H, MR2 and MR3, digested with _Eco_RI or _Hin_dIII and separated in 7% SDS-PAGE, showed no differences

in DNA restriction fingerprint patterns (Figure 5).

**Electron microscopy of virus-inoculated spiroplasmas.** Electron microscopy showed spiroplasma virus SVTS2 adsorbed to cells of S. citri M200H by one end of the rod- shaped virion (Figure 6). Many viruses bound to a single spiroplasma cell. Loss of the helical cell morphology was observed 15-24 hr after inoculation with virus. SVTS2 particles were not seen bound to cells of MR2 or MR3, but this experiment was not replicated.

**Blocking of virus binding on the spiroplasma surface with antiserum against spiroplasma surface proteins.** Lawns of M200H cells pretreated with antiserum against total S. citri BR3 membrane proteins had significantly fewer plaques (3.6 plaques/plate) than cells that were pretreated with preimmune serum or had no serum treatment (63 plaques/plate) [$t = 12-26 > t_{0.05} = 2.26$] (Table 2). No significant difference in plaque numbers occurred when M200H cells were pretreated with preimmune serum or with antiserum specific for P55, or P77, and then inoculated with SVTS2. However, significantly fewer plaques occurred in the lawns of cells pretreated with the antiserum against the 29 kd protein (spiralin) than in those pretreated with preimmune serum [$t=3-6 > t_{0.05}=2.26$] (Table 3). A negative significant difference occurred between 1;100 and 1:200 dilution, but not 1:50 or 1:300 treatment, of anti-P89 and preimmune serum treatment.

**Surface immunoprecipitation with anti-SVTS2 serum.** A S. citri M200H culture inoculated with SVTS2 was incubated with anti-SVTS2 serum. After cell lysis, the membrane protein-SVTS2-antiserum complex was collected with Staph A protein, electrophoresed in SDS-PAGE, and Western blotted using antiserum against total membrane protein of S. citri BR3 (Figure 7). The treated sample had a 29 kd protein band. However, this protein (29 kd) was absent in the controls: M200H cells + staph A (lane C), and M200H cells + SVTS2 (lane D). Positive control showed all membrane protein bands (lane E).

## DISCUSSION

Maniloff et al. (1979) found that the mycoplasma virus MVL 51 adsorbed to the mycoplasma cell surface prior to viral DNA penetration into the cytoplasm. Electron microscopy of SVTS2-inoculated S. citri cells suggest that virus binding to the host cell is a necessary step for virus infection. The fact that many viruses bound to a single spiroplasma cell indicated that many virus receptors occurred on the spiroplasma surface. Spiralin (29 kd), the most prevalent membrane protein, may be a candidate for this binding function.

The data presented a possiblity that the membrane protein spiralin (29 kd) is a virus receptor. However, anti-P29

serum deforms the spiroplasma and inhibits its growth (Whitcomb et al. 1983). Thus, the plaque reduction may be due to either cell deformation or to a block of virus adherence, or both. Use of the Fab fragment of anti- P29 serum may be useful in addressing this question (Fletcher 1992).

Both host protein receptors and virus coat proteins may be involved in adsorption of viruses to bacteria (Bamford 1987; Al-Shammari et al. 1982). That host membrane proteins serve as mycoplasmavirus L3 receptors has been demonstrated (Haberer et al. 1982). S. citri cells pretreated with anti-membrane protein serum or anti-spiralin serum showed decreased plaque numbers after inoculation with virus, suggesting that spiroplasma membrane proteins may be involved in virus binding. Antisera against membrane proteins of 55 kd, and 77 kd did not affect the plaque number, indicating that these three membrane proteins are not involved in virus binding. Anti-P89 serum treatment increased plaque number; perhaps membrane protein P89 has a nagative effect on virus binding.

Forty spots were identified in the plasma membrane preparation of S. citri strain R8A2 by 2-D gel electrophoresis (Simoneau et al. 1988). The two spots of plasma membrane proteins located at the intersection of pH 6.5 and MW 30-40 kd on their 2-D gel may correspond to spots P1 and P2 in our test, since their position relative to

other proteins is similar. If this is the case, then spots P1 and P2 in our 2-D gel of M200H, which are missing in MR2 and MR3, are probably membrane proteins.

P1 and P2 did not react with antiserum prepared against S. citri membrane proteins. S. citri M200H membrane protein preparations fractionating into the detergent phase by TX-114 extraction showed only spots P4-P10 in the 2-D gels. Therefore, our antiserum against S. citri BR3 membrane proteins, which was prepared using a TX-114 detergent fraction, may not contain anti-P1 and -P2 antibodies. Although most membrane proteins fractionate into the detergent phase, some do not (Razin 1983).

Oishi et al. (1984) reported that the presence of virus affects the surface properties of the Drosophila sex-ratio spiroplasma. Dickinson et al. (1985) suggested that conversion of the cell surface, which is manifested by the loss of a receptor for SpV3-type virus ai, is a general feature of lysogenisation of S. citri. Bove et al. (1989) hypothesized that for two spontaneous mutants, G1R2 and G1R4, of S. melliferum strain G1, resistance to infection by the virus SpV4 is due to failure of the virion to adsorb to or to penetrate the spiroplasma. Thus, the resistance of spiroplasmas to virus infection may involve surface interaction. In our work, it is possible that three proteins (P1, P2 and P3) of M200H, which are lacking or significantly reduced in virus-resistant lines MR2 and MR3, may be

involved in virus resistance. The membrane-associated proteins P1 and P2 may serve as receptors of virus or may be involved as co-factors for virus binding. However, other factors may also contribute to virus resistance. Liss et al. (1985) showed that Acholeplasma laidlawii strains JSY and JGCt, resistant to virus MVL51, still bound the virus. In this case, virus resistance may not involve surface protein receptors of A. laidlawii. An alternative resistance mechanism might involve the insertion of viral DNA into the chromosome of the resistant lines, or the presence of viral DNA in an extrachromosomal form within the resistant lines. In this scenario, the lack or reduction of three proteins in lines MR2 and MR3 may be due to disruption of the genes encoding them by integration of viral DNA.

In Western blots of 2-D gels, we identified 7 spots which reacted with an antiserum against S. citri BR3 membrane proteins. Spots P4, P5, P6, and P7 were very prominent. Spot P7 reacts with antiserum against spiralin (29 kd) of S. citri BR3. Comparing these spots with ones reported by Simoneau et al. (1988), spot P4 corresponds to D55, while spots P5 and/or P6 correspond to D50. Spots D55 and D50 were present in both membrane and cytoplasmic fractions of S. citri R8A2. All 7 membrane-associated proteins were present in all three lines, M200H, MR2 and MR3.

The similarity of the genomic DNA fingerprints among M200H, MR2 and MR3 indicated that in the derivation of lines

MR2 and MR3 no significant changes occurred in the genomes of these lines. However, a limitation of this fingerprinting method is that high molecular weight DNA fragments cannot be well separated in SDS-PAGE. Thus, differences in these DNA fragments may not be identified.

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Table 1. Plaque Assay for Infectivity of SVTS2 on *S. citri* Strains M200H, MR2 and MR3

| Lawn | Overlay | | | |
|---|---|---|---|---|
| | SVTS2 | | Tris buffer | None[b] |
| | $4.2X10^{4}$ PFU/ml | $4.2X10^{6}$ PFU/ml | | |
| M200H | +[a] | + | - | - |
| MR2 | - | - | - | - |
| MR3 | - | - | - | - |

[a] These data were compiled from three different experiments. Each combination was tested with a total of 26 plates.

[b] No virus or buffer was added.

Table 2. Plaque Assay after Inoculation of SVTS2 into *S. citri* M200H Pretreated with Antiserum Against Spiroplasma Surface Membrane Proteins.

| | Number of plaques/plate | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Test # | Antiserum against *S. citri* BR3 membrane proteins | | | | | Preimmune serum[a] | | | | |
| | 1:10 | 1:50 | 1:100 | 1:200 | 1:300 | 1:10 | 1:50 | 1:100 | 1:200 | 1:300 |
| | 0 | 0 | 5 | 7 | 9 | 56 | 57 | ND[c] | 52 | 65 |
| | 0 | 6 | 0 | 0 | 8 | 61 | 66 | ND | 68 | 71 |
| I[b] | 0 | 0 | 2 | 7 | 5 | 75 | 77 | ND | 75 | 93 |
| | 0 | 0 | 0 | 3 | 1 | 70 | 42 | ND | 73 | 78 |
| | 0 | 0 | 0 | 0 | 0 | 60 | 45 | ND | 58 | 75 |
| | 2 | 2 | 1 | 9 | 11 | 78 | 41 | 48 | 42 | 59 |
| | 2 | 4 | 4 | 11 | 18 | 76 | 65 | 52 | 59 | 66 |
| II | 4 | 3 | 4 | 7 | 15 | 80 | 18 | 65 | 46 | 65 |
| | 0 | 2 | 4 | 11 | 14 | 81 | 62 | 51 | 59 | 78 |
| | 2 | 4 | 4 | 0 | 0 | 75 | 78 | 60 | 59 | 42 |
| x[d] | 1[e] | 2[e] | 2[e] | 5[e] | 8[e] | 79[f] | 55[f] | 55[f] | 59[f] | 69[f] |
| t | 26 | 9 | 18 | 13 | 12 | | | | | |

[a] Preimmune serum was collected from the rabbit prior to injection with membrane protein of *S. citri* BR3 as antigen.

[b] Test I plaque assay was evaluated 3 days after inoculation. Test II plaque assay was evaluated 5 days after inoculation.

[c] Plates contaminated with bacteria.

[d] Average of number of plaques.

[e&f] Statistical analysis: $t_{0.05}$=2.26, $t_{0.01}$=3.25. Each antiserum dilution was compared only with the corresponding preimmune serum dilution. Different dilution of the same antiserum or of the preimmune serum were not compared with each other. Values within a row followed by the same letter do not differ (P=0.05) by Student's t test.

Table 3. Plaque Assay after Inoculation of SVTS2 into Cultures of S. citri M200H Pretreated with Antiserum Specific for Membrane Protein Serum.

| Test # | Number of plaques/plate | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 29 kd AS[a] | | | | 55 kd AS | | | | 77 kd AS | | | | 89 kd AS | | | | Preimmune AS | | | |
| | A | B | C | D[b] | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D |
| | 14 | 9 | 20 | 18 | ND | 22 | 18 | 25 | 13 | 28 | 23 | 48 | 47 | 47 | 38 | 25 | 21 | 36 | 27 | 32 |
| | 8 | 2 | 23 | 0 | ND | 21 | 43 | 28 | 9 | 28 | 31 | 58 | 50 | 33 | 45 | 48 | 23 | 9 | 22 | 33 |
| I[c] | 0 | 12 | 19 | 23 | ND | 18 | 24 | 28 | 8 | 45 | 27 | 58 | 54 | 53 | 63 | 39 | 21 | 34 | 25 | 45 |
| | 6 | 15 | 8 | 20 | ND | 26 | 25 | 33 | 21 | 25 | 47 | 30 | 42 | 38 | 47 | 37 | ND | 31 | 22 | 20 |
| | ND | 7 | 30 | 30 | ND | 0 | 41 | 19 | 0 | 5 | 40 | 51 | 24 | 52 | 29 | 29 | ND | 30 | 22 | 30 |
| | 0 | 1 | 1 | 2 | 18 | 13 | 7 | 10 | 0 | 6 | 8 | 9 | 12 | 13 | 6 | 11 | 6 | 14 | 11 | 19 |
| | 0 | 0 | 2 | 3 | 9 | 6 | 10 | 5 | 0 | 5 | 5 | 7 | 3 | 17 | 16 | 10 | 9 | 11 | 5 | 10 |
| II | 0 | 0 | 2 | 1 | 7 | 10 | 9 | 4 | 0 | 2 | 5 | 6 | 7 | 18 | 7 | 8 | 9 | 19 | 4 | 9 |
| | 1 | 2 | 1 | 0 | 9 | 20 | 9 | 12 | 0 | 0 | 4 | 6 | 9 | 6 | 25 | 17 | 5 | 14 | 21 | 12 |
| | 0 | 0 | 2 | 2 | 7 | 5 | 6 | 5 | 2 | 0 | 4 | 1 | 4 | 6 | 18 | 11 | 2 | 8 | 12 | 8 |
| x[d] | 3[f] | 5[f] | 11[f] | 10[f] | 10[g] | 14[g] | 19[g] | 17[g] | 5[g] | 14[g] | 19[g] | 27[g] | 25[g] | 28[h] | 29[h] | 24[g] | 12[g] | 21[g] | 17[g] | 22[g] |
| t[e] | 5 | 6 | 3 | 3.7 | 1 | 1.7 | 0.6 | 1.9 | 1.3 | 1.5 | 0.5 | 1.3 | 2 | 2.3 | 2.9 | 0.5 | | | | |

[a] Specific anti-membrane protein serum.

[b] Dilution of the antiserum. A, 1:50; B, 1:100; C, 1:200; D, 1:300.

[c] Tests I and II were evaluated 3 days after virus inoculation.

[d] Average of number of plaques

[e] Statistical analysis: $t_{0.05}=2.26$, $t_{0.01}=3.25$. Each antiserum dilution was compared only with the corresponding preimmune serum dilution. Different dilutions of the same serum were not compared with each other.

[f,g&h] Values within a row followed by the same letter do not differ (P=0.05) by Student's t test.

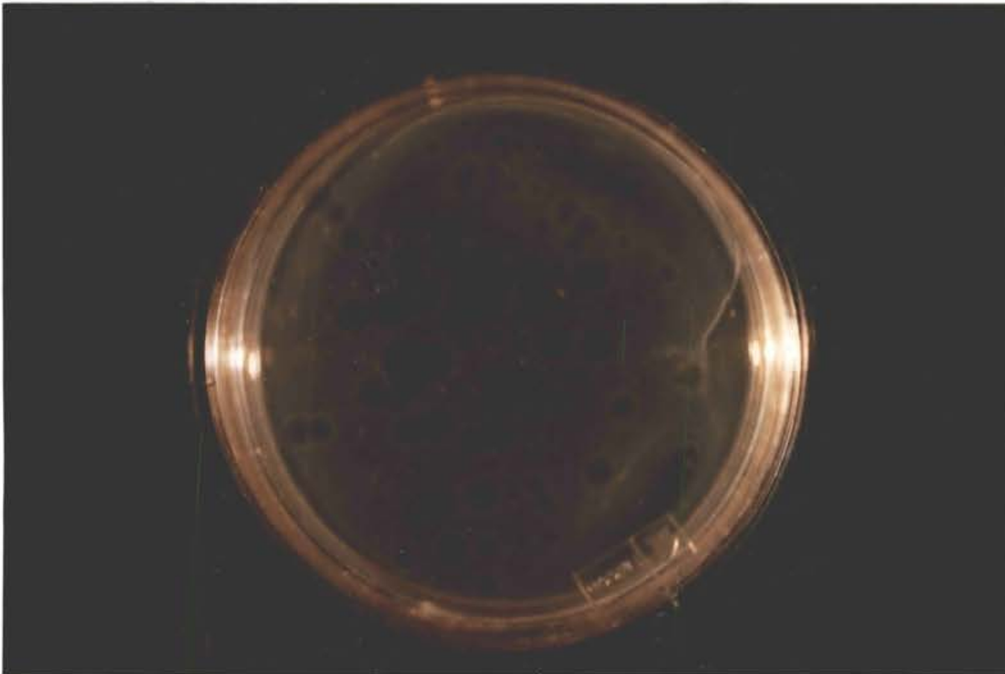


Figure 1. Plaques produced by spiroplasmavirus SVTS2 on a lawn of Spiroplasma citri strain M200H.

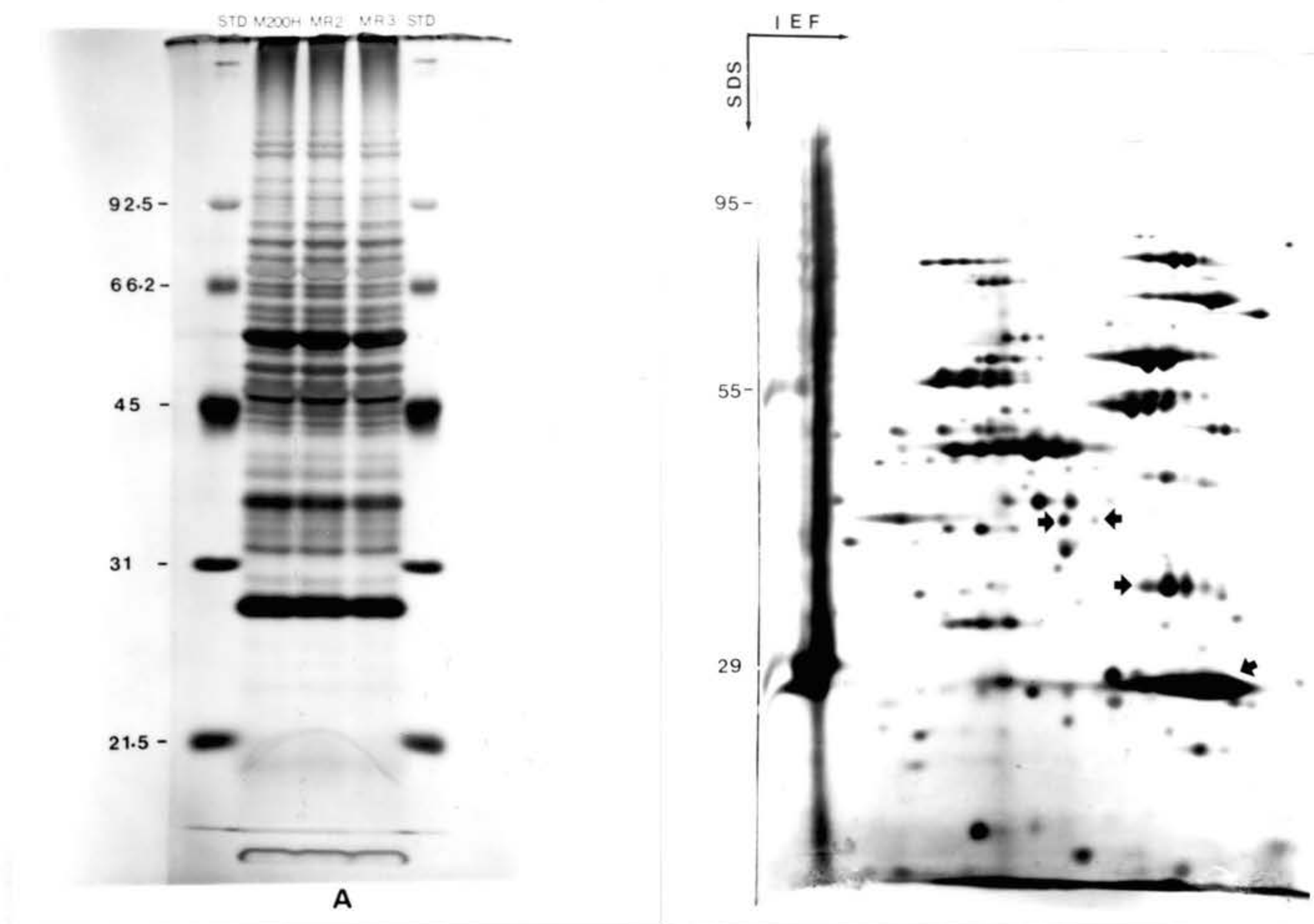
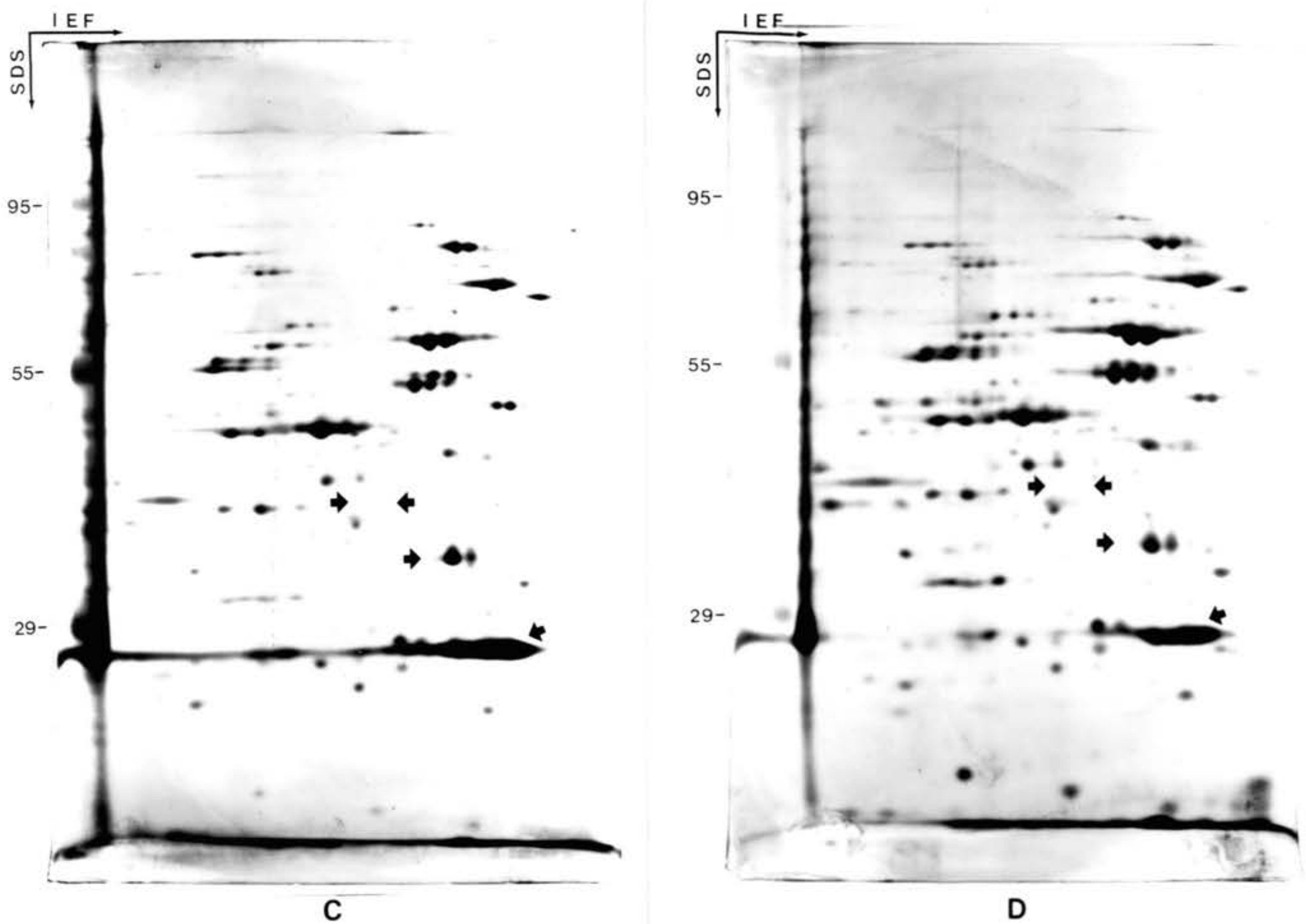



Figure 2. Electrophoresis of total proteins of S. citri M200H and two virus resistant lines, MR2 and MR3. Panel A, one-dimensional SDS-PAGE. STD, standard molecular marker. Panels B, C, and D, two-dimensional IEF/SDS-PAGE. B, M200H; C, MR2 and D, MR3. Arrows point to spots (a=P1, b=P2, and c=P3) not equally represented in all three lines.




Continuation of Figure 2. Electrophoresis of total proteins of S. citri strain M200H and two virus resistant lines, MR2 and MR3. Panels B, C, and D, two-dimensional IEF/SDS-PAGE. C, MR2. D, MR3. Arrows point to spots (a=P1, b=P2, and c=P3 ) not equally represented in all three lines.

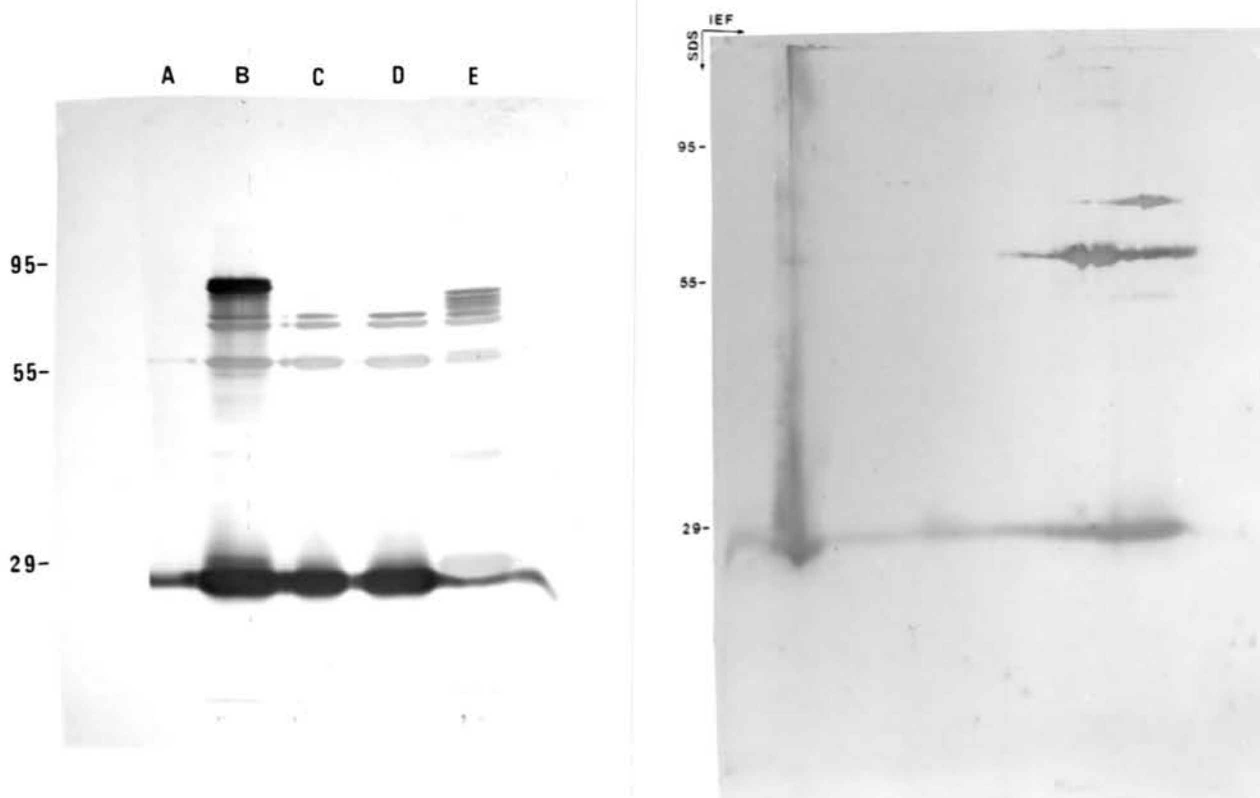



Figure 3. Western blot of 1-D SDS-PAGE and 2-D IEF/SDS-PAGE of S. citri lines M200H, MR2 and MR3 probed with antiserum specific for S. citri strain BR3-3X membrane proteins. 3A, 1- D SDS-PAGE: A, standard; B, S. citri BR3; C, S. citri M200H; D, S. citri MR2; E, S. melliferum MR5; 3B, 2-D SDS-PAGE of M200H.

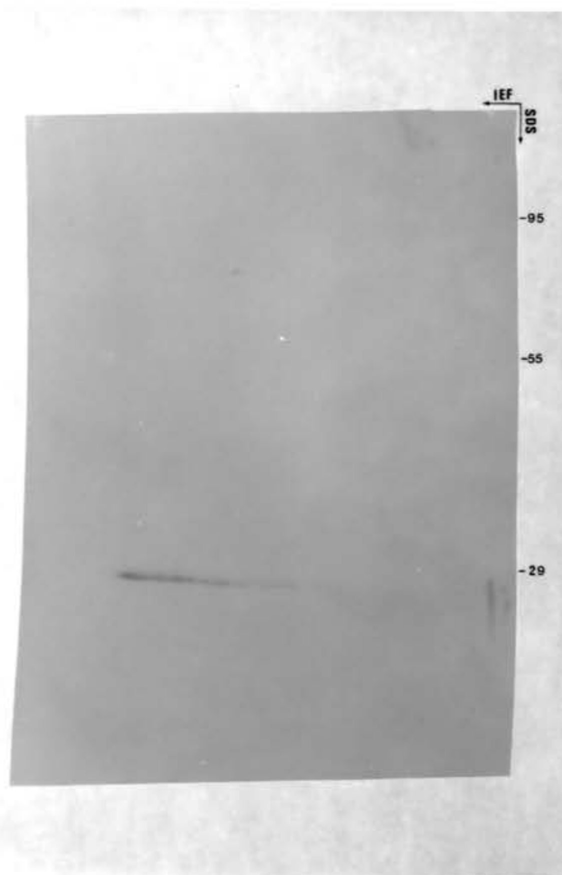



Figure 4. Western blot of a 2-D gel of _S_. _citri_ line MR2 proteins probed with antiserum specific for spiralin (25-29 kd) of _S_. _citri_ strain BR3-3X.

Figure 5. Genomic DNA fingerprinting of S. citri lines M200H, MR2 and MR3. A, MR3; B, MR2; C, M200H; D, R8A2. E, standard molecular marker.

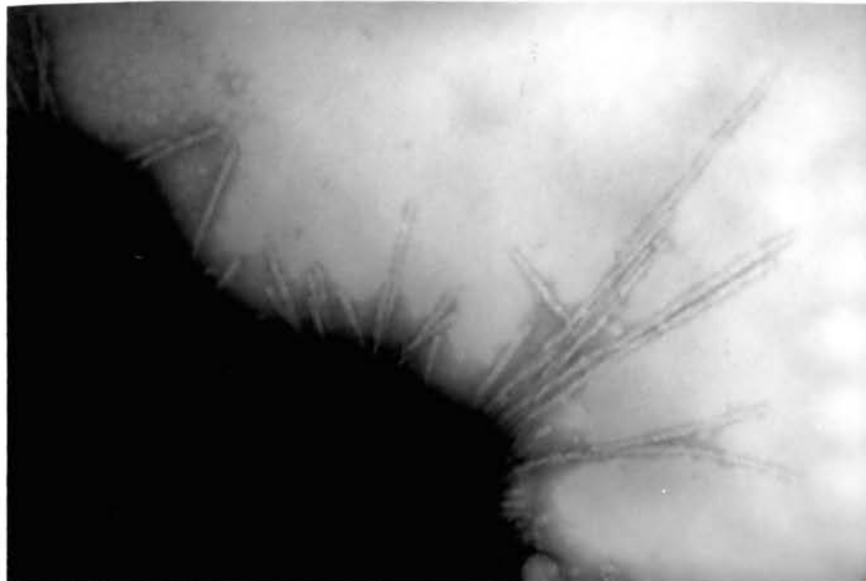


Figure 6. Electron microscopy of spiroplasma virus SVTS2 bound to S. citri strain M200H cells (x 58,000).

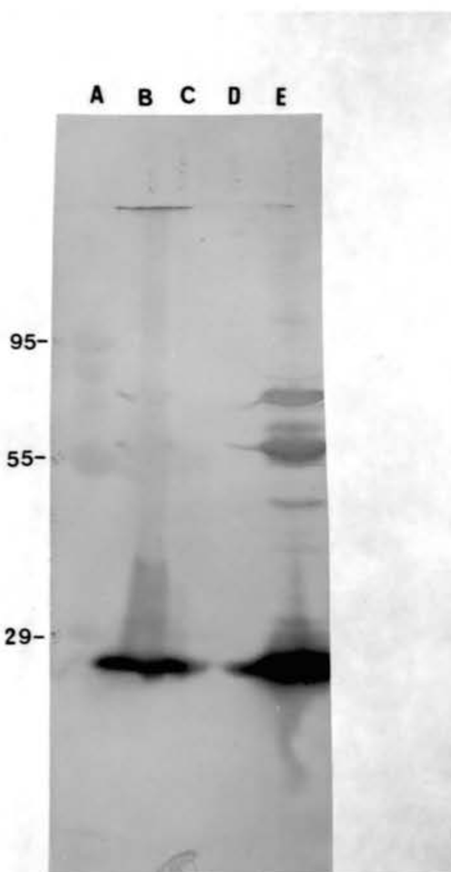



Figure 7. Western blot of surface immunoprecipitation of virus binding-associated protein probed with antiserum specific for S. citri strain BR3-3X total proteins. A, standard molecular marker. B, M200H membrane protein-SVTS2-anti-SVTS2 serum-staph A complex treatment. C, M200H cells and staph A treatment. D, M200H cells and SVTS2 treatment. E, M200H cells only.

## CHAPTER IV

## Studies on the Mechanism of Resistance of *Spiroplasma citri* Lines to Infection by the Virus SVTS2.

## ABSTRACT

Spiroplasmavirus SVTS2, isolated from *Spiroplasma melliferum* TS2, produces plaques when inoculated onto lawns of *S. citri* M200H. *S. citri* lines MR2 and MR3, originally selected as colonies growing within plaque boundaries on a lawn of M200H inoculated with SVTS2, were resistant to SVTS2. Electroporation of SVTS2 DNA into M200H produced 1.5 X $10^5$ transfectants/ug SVTS2 DNA. No transfection occurred in the M200H-derived lines MR2 or MR3. Native viruses isolated from M200H, MR2 and MR3 were designated SVM200H, SVMR2 and SVMR3. Restriction enzyme digestion patterns of the RF DNA of SVM200H, SVMR2, and SVMR3 were similar. RF DNAs of all the native viruses shared some homology with the SVTS2 DNA probe. Three bands (1.3 kb, 2.1 kb and 5.1 kb) not present in SVM200H hybridized strongly in SVMR2, and two of these bands (1.3 kb and 2.1 kb) also hybridized in SVMR3, indicated that a fragment of SVTS2 DNA was present in native virus RF DNA (extrachromosomal ds DNA) in MR2 and MR3, but not in M200H. Four weak hybridization bands (4.3, 6.5, 8.4

and 10.8 kb) were detected in the genomes of all three lines when probed with the SVTS2 DNA probe. There was also strong hybridization to 13.6 kb and 19.2 kb EcoRI fragments of the chromosomes of MR2 and MR3, but not to the chromosome of M200H. This indicated that SVTS2 DNA integrated into the genome of MR2 and MR3, but not M200H. When native virus SVMR3 RF, which contained a 2.1 kb SVTS2 DNA fragment, was transfected into M200H, the transformed spiroplasma was resistant to SVTS2. These results are consistant with the interpretation that SVTS2 fragments, inserted into the spiroplasma chromosome or extrachromosomal DNA, may function as a viral incompatibility element and provide immunity to superinfection by SVTS2.

## INTRODUCTION

Spiroplasma citri is a wall-less, prokaryotic phytopathogen of citrus and horseradish (Markham et al. 1974; Fletcher et al. 1981). Four groups of spiroplasma viruses (SpV1, SpV2, SpV3 and SpV4) have been described (Bove et al. 1989) since the first report of a virus of spiroplasma by Cole (1974). Recently the complete nucleotide sequences of SpV1-R8A2B from S. citri R8A2 and SpV4 from S. melliferum have been determined (Renaudin et al. 1987, 1990). SpV1-type viruses are rod-shaped particles containing circular, single-stranded DNA. The replicative form (RF) of

SpV1-R8A2B has been used as a cloning vector for gene expression in spiroplasma (Stamburski et al. 1991; Marais et al. 1992).

Insertion of spiroplasma virus sequences into the spiroplasma genome also has been reported (Renaudin et al. 1986, 1990; Bove et al. 1989; Ye et al. 1992). These viral-like sequences may be repeated elements and may play a role in genomic rearrangements (Ye et al. 1992). ORF3 of SpV1-R8A2B showed 49.1%, 52.2% and 53.4% identity with IS30, IS4351 and IS1086 putative transposase (Dong et al. 1992). ORF4 of SpV1-R8A2B had slight identity with Salmonella phage P22 integrase (Renaudin et al. 1990). Dong et al. (1992) suggested that the protein encoded by ORF3, or ORF4, of SpV1-R8A2B could act on the adjacent inverted repeat, or integrase, to catalyze integration of the virus genome into the host chromosome.

A SpV1-type spiroplasma virus, SVTS2, was isolated from S. melliferum strain TS2 (McCammon and Davis 1985). SVTS2, a rod shaped particle which contains 6.5 kb circular, single-stranded DNA, causes plaque formation when inoculated onto lawns of S. citri M200H (McCammon et al. 1990). Two lines of S. citri, MR2 and MR3, apparently resistant to SVTS2 infection, were isolated from plaques of lawns of strain M200H infected with SVTS2. Protein patterns of MR2 and MR3 were different from those of M200H (Chapter III; Sha et al. 1991). In the experiments reported here, we further

investigated the resistance mechanism of lines MR2 and MR3 to SVTS2 infection.

## MATERIALS AND METHODS

**Source of spiroplasma and viruses.** S. citri Maroc R8A2 was originally isolated from citrus in Morocco; M200H was derived from strain R8A2 by 200 subculture passages; MR2 and MR3 are virus-resistant lines derived from M200H. S. citri BR3-3X, BR5-3X, and BR6-3X were isolated from horseradish with brittle root symptoms in Illinois. S. melliferum TS2 and AS576 were isolated from honeybees. S. floricola 23-6 was isolated from a flower surface. All strains were cultured in LD8 broth (Davis 1979) and stored in broth at -70 $^{o}$C. Virus SVTS2 was isolated from S. melliferum TS2 and increased in S. citri M200H. Virus SVBR3 was isolated from S. citri BR3-3X.

**Spiroplasma virus multiplication and isolation.** S. citri strain M200H was grown to a titer of 5 x $10^{8}$ to 1 x $10^{9}$ cells/ml in 1 L LD8 broth. The cells were mixed with SVTS2 at MOI 100 and incubated at 31 $^{o}$C for 2-3 days. The cells were pelleted at 10,000 x g, 4 $^{o}$C for 30 min. Triton X-100 was added to the supernatant to a final concentration of 0.2% (v/v). After overnight incubation at 4 $^{o}$C, the liquid was centrifuged at 95,000 x g in a Beckman type 30 rotor at 4 $^{o}$C for 2.75 hr. The final pellet was resuspended in Tris

buffer (10 mM Tris-HCl, pH 7.9) and 0.387 g CsCl/ml virus preparation was added. The CsCl suspension was centrifuged at 105,000 x g, 20 $^{o}$C, for 24-48 hrs. The virus band was removed and dialysed for 48 hr in 4 L Tris buffer.

**Purification of spiroplasma virus DNA.** A modification of the method of McCammon and Davis (1987) was used. One L culture of S. citri M200H in early log phase was inoculated with SVTS2 at an MOI of about 2.3 x $10^{-6}$ and incubated at 31 $^{o}$C for 2-3 days. The spiroplasma cells were pelleted and the supernatant was passed through a 0.2 um filter. The filtrate was precipitated overnight at 4 $^{o}$C with 10% polyethylene glycol and 0.5 M NaCl. A fine precipitate was collected by centrifugation at 16,000 X g for 30 min, and the pellet was resuspended in 15 ml TES buffer (0.01 M Tris-HCl, 0.001 M EDTA, and 0.1 M NaCl, pH 8.0). This suspension was extracted two times with phenol and precipitated with ethanol. The final pellet was resuspended in 4.0 ml TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Four g CsCl and 0.4 ml ethidium bromide (10 mg/ml) were added. After the final density of the solution was adjusted to 1.55 g/ml, it was centrifuged at 239,000 x g in a Beckman VTi type-65 rotor for 15 hr. The DNA was recovered by ethanol precipitation, dried, resuspended in 50 ul TE buffer, and stored at -20 $^{o}$C. The ratio of absorbance at 260/280 nm of the SVTS2 DNA was 1.88-2.0.

**Purification of spiroplasma virus replication form (RF).**

To maximize virus yield, a 5 ml culture of M200H grown 24 hr was incubated with SVTS2 (1x $10^{14}$ PFU/ml) at MOI 100 for 1-2 days, and then transferred to 100 ml of cultured M200H. After similar transfers to 500 and 1000 ml cultures, the spiroplasma cells were pelleted at 13,000 X g for 35 min at 4 $^{o}$C. The pellets were alkaline-extracted by the method of Sambrook et al. (1989). The DNA pellet was dissolved in 4 ml TE buffer and subjected to a CsCl gradient as described above. The lower of two bands was collected from the tube. Two volumes of deionized water were added, followed by two volumes of 95% ethanol, and the mixture was incubated at -70 $^{o}$C, 1 hr. After centrifugation in a Beckman J17 rotor at 14,000 x g, 10-15 min at 4 $^{o}$C, the pellet was dissolved in 250 ul TEN buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl and 1 mM EDTA), phenol extracted and ethanol precipitated. The dried viral RF DNA was resuspended in 50 ul TE buffer and stored at -20 $^{o}$C. The ratio of absorbancy (260/280 nm) of SVTS2 RF was 2.0-2.1. Native virus RF was purified by the same protocol.

**Transfection of S. citri with viral DNA.**

**PEG treatment.** The method of McCammon and Davis (1987) was used. A volume of 5 ml of S. citri M200H cells, 1 X $10^{9}$ cells/ml, were collected by centrifugation at 14,000 x g for 30 min at 4 $^{o}$C and resuspended in 250 ul fresh LD8 medium lacking serum. Viral DNA was added and the suspension mixed with 2 ml polyethylene glycol 6000 (50% PEG, 0.5 M sucrose,

0.01 M Tris-HCl, pH 8.0) for 2 min. Ten ml 0.01 M Tris-HCl, pH 8.0 was added, and the cells were pelleted and resuspended in 1 ml basal LD8 medium (without serum), added to 1 ml of complete LD8 medium (with serum) containing 1% agar at 48 $^{o}$C. After mixing, 0.5 ml was spread onto agar-solidified LD8. The plate was incubated at 31 $^{o}$C for 2-3 days.

**Electroporation.** We used the procedure of Stamburski et al. (1991). S. citri cells (10 ml volume at 1 X $10^{9}$ cells/ml) were collected by centrifugation and resuspended in 0.8 ml of 8 mM HEPES buffer (N-2-hydroxyethylpiperazine-N -2-ethanesulfonic acid, pH 7.4) containing 280 mM sucrose. The suspension was mixed with $10^{4}$-$10^{6}$ pg SVTS2 DNA, placed into a chilled 0.4 cm cuvette and incubated on ice for 10 min. The Gene Pulser (Bio-Rad Lab, Richmond, CA.) conditions were: 3 uF capacitance, 2.5 KV set volts, 1000 ohms and 1 ms pulses. Two pulses were applied. After incubation for 10 min at room temperature, the cells (100 ul/plate) were dropped onto a previously prepared lawn of an S. citri test strain. Plates were checked for plaques 1-2 days after plating.

**Restriction enzyme digestion.** Restriction enzymes Sau3AI, HinfI, TaqI, AluI and DdeI (GIBCO BRL) were used. The reaction solution included 2 ul 10X enzyme buffer, 14 ul sterile $H_2O$, 3 ul viral DNA (2.13 ug) and 1 ul enzyme. The mixture was incubated at 37 $^{o}$C, 1 hr except those with TaqI, which were incubated at 65 $^{o}$C. The digested DNA was

electrophoresed in a 0.7% agarose gel with TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). The gel was stained with 5 ug/ml EtBr for 10 min and photographed using a UV light box.

**Molecular cloning of SVTS2 RF DNA.** After digestion of four ug Bluescript KS+ cloning vector DNA with BamHI for 1 hr at 37 $^{o}$C, 1/50 volume 5 M NaCl and 1 volume buffered phenol was added. The top phase was extracted with 3 volumes of ether, and three volumes of 95% ethanol were added to the aqueous phase, which was incubated at -70 $^{o}$C for 1 hr. The vector DNA was treated with calf intestine alkaline phosphatase (GIBCO BRL, Life Technologies, Inc. MD 20877) and phenol extracted again. SVTS2 RF DNA was digested with Sau3AI for 1 hr at 37 $^{o}$C and then phenol extracted. The 0.04 ug vector and 0.27 ug SVTS2 RF were ligated with 1 ul T4 DNA ligase (400 U/ul) at 12 $^{o}$C overnight. The recombinant plasmid was transformed into E. coli (Library Efficiency DH5a $^{TM}$ competent cells, Life Technologies Inc.). Twenty ul of competent bacteria were mixed with 1 ul of the diluted DNA (1 ul ligation mixture to 4 ul TE buffer) and incubated on ice for 30 min, at 42 $^{o}$C for 40 seconds, and on ice again for 2 min. Eighty ul LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl) was added and the mixture incubated at 37 $^{o}$C for 1 hr. Transformed bacteria were spread on TAXI plates (1% Bactotryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM $MgSO_4$, 1.5% agar, 5 ug/ml thiamine, 20 ug/ml ampicillin, 33

ug/ml X-gal and 8.1 ug/ml iPTG) and incubated at 37 °C for 24 hr. A white colony was purified by streaking on ampicillin plates (2% LB agar, 20 ug/ml ampicillin) and the insertion confirmed by 0.7% agarose gel electrophoresis in TAE buffer.

**$^{32}$P labeling of SVTS2 DNA probe and Southern blotting.** Recombinant plasmids were isolated by alkaline extraction and purified by phenol extraction, EtBr-CsCl gradient centrifugation and ethanol precipitation. Purified DNA was labeled with $^{32}$P by nick translation (Sambrook et al. 1989). For Southern blotting, the RFs of SVM200H, SVMR2, and SVMR3 were digested with _TaqI_ at 65 °C for 1 hr, while the chromosomal DNA of _S. citri_ was digested with _EcoRI_ at 37 °C for 1 hr. Both were electrophoresed on 0.7% agarose gels with TAE buffer. The DNA was transferred to nylon membrane sheets wet with 20 ml 4x SSC solution (20x SSC: 3 M NaCl, 0.3 M $Na_3$ citrate.2 $H_2O$, pH 7.0). Prehybridization was in a solution (10 ml/blot) of 5X SSC, 5x Denhardt's solution (50x: 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin in 1 L $H_2O$), 200 mg/L sonicated calf thymus DNA, and 50 mM sodium phosphate, incubated at 65 °C for 2 hr. Hybridization was in a solution (5 ml/blot) of 1X SSC, 1x Denhardt's solution, 100 mg/L sonicated calf thymus DNA, 20 mM sodium phosphate, 0.1% SDS, and 10-15 ul labeled SVTS2 DNA [0.1-0.5 uCi]. After overnight (>16 hr) incubation at 65 °C, the nylon was washed twice at room temperature for

30 min with 2x SSC and 0.1% SDS, then twice at 65 °C for 15 min with 0.1X SSC and 0.1% SDS. The blots were autoradiographed.

**Spiroplasma chromosomal DNA purification.** A modification (Chapter III, this thesis) of the method of Williamson et al. (1991) was used for purification of spiroplasma chromosomal DNA.

## RESULTS

**Transfection with SVTS2 viral DNA.** Using 50% PEG, 194 plaques/4 plates were observed in lawns of M200H transfected with total 1.4 ug SVTS2 DNA. The calculated transfection frequency of SVTS2 viral DNA into S. citri strain M200H was $1.3 \times 10^{2}$ transfectants/ug DNA. No transfection was obtained under comparable conditions in virus-resistant lines MR2 or MR3.

Using electroporation, 1621 plaques/10 plates, and 15,960 plaques/10 plates were produced in lawns of M200H transfected with total 0.011 ug and 0.55 ug SVTS2 DNA. This represents a transfection frequency of $1.5 \times 10^{5}$ transfectants/ug DNA, a much higher transfection frequency than with 50% PEG treatment. The number of plaques obtained increased linearly with amounts of SVTS2 DNA (Figure 1). No plaques were obtained with MR2 and MR3, although up to 1101 ng SVTS2 DNA was added; its transfection frequency was <1

transfectant/ug DNA. This experiment was repeated six times.

**Isolation of native virus from M200H, MR2 and MR3.** Filtrates of cell cultures of MR2, MR3 and M200H all produced plaques on lawns of the indicator strain S. citri M200H (Table 1), indicating that MR2, MR3 and M200H each contain a virus or viruses. We designated these native virus populations SVMR2, SVMR3 and SVM200H.

**Restriction digestion pattern of native virus RF.** Restriction digestion patterns of extrachromosomal DNA (including the RF DNAs of SVTS2, SVM200H, SVMR2 and SVMR3) incubated with Sau3AI, HinfI, AluI, TaqI or DdeI are shown in Figure 2. Following Sau3AI digestion, a 6.5 kb band occurred in SVM200H, SVMR2 and SVMR3. Two bands (8.6 and 15.3 kb) were present in SVMR2 (lane D) and SVMR3 (lane H), but not in SVM200H (lane F). A 9.4 kb band was common to SVM200H and SVMR3 but was not present in SVMR2. Following HinfI digestion, SVMR2 (lane E) and SVMR3 (lane I) each had four bands (4.9 kb, 4.0 kb, 2.1 kb and 1.9 kb) in common with SVM200H (lane G). However, SVMR2 had two additional bands (2.4 kb and 3.0 kb) absent in SVM200H and SVMR3, and SVMR3 had one additional band (1.5 kb) absent in SVM200H and SVMR2. A 2.4 kb band occurred uniquely in SVTS2. SVM200H RF DNA had one band (0.9 kb) not present in SVMR2 or SVMR3.

After AluI digestion, the 6.8 kb band was common to SVM200H and SVMR3, but other bands were different.

SVMR2 (lane P) and SVMR3 (lane Q) had two TaqI bands (2.1

kb and 1.3 Kb) not seen in SVM200H, although the three strains had five bands in common. Bands of 2.1 kb and 1.3 kb also were present in SVTS2, but SVM200H had no bands in common with SVTS2.

In DdeI digestion, three bands (1.8, 2.3 and 2.7 kb) were common for SVM200H, SVMR2 and SVMR3. Bands of 1.3 kb and 2.2 kb were present in SVM200H (lane S) and SVMR3 (lane U)but not SVMR2 (lane T). SVMR3 had one additional band (1.2 kb) which was not present in SVM200H and SVMR2. SVMR2 had one additional band (5.5 kb) not present in SVM200H and SVMR3.

Restriction digestion experiments were replicated three times.

**Molecular cloning of SVTS2 RF and construction of a DNA probe.** After ligation of the Sau3AI fragments of SVTS2 RF with BamHI digested vector Bluescript KS+ DNA and transformation of E. coli, two types of clones were recovered. The plasmid of one clone had an insert of 6.5 kb (designated clone pESV1-1) and the plasmid of the other clone had an insert of 3.2 kb (designated pESV1-43) (Figure 3). The 6.5 kb DNA fragment was labeled with $^{32}P$ for use as a DNA probe. The 6.5 kb DNA probe hybridized to all AluI- and DdeI-digested fragments of SVTS2 (Figure 4), indicating that pESV1-1 represents a clone of the complete SVTS2 genome.

**SVTS2 DNA in the extrachromosomal double stranded DNA of MR2 and MR3.** S. citri extrachromosomal DNA (including native

virus RF) was digested with restriction enzymes and a Southern blot was probed with SVTS2 DNA (Figure 5). The experiment was replicated three times. All the ds DNA preparations shared some identity with SVTS2, and for the purposes of this discussion, will be referred to as SVM200H, SVMR2 and SVMR3. Two bands (1.3 kb and 2.1 kb) not present in SVM200H hybridized strongly in SVMR2 and SVMR3, although these same two bands were faint in the corresponding EtBr-stained agarose gel. In addition, one probe-reactive band (7.0 kb) present in SVM200H was not present in SVMR2 and SVMR3. Instead an additional, more intensely reacting band, was present. The SVMR3 band (7.6 kb) was larger and the SVMR2 band (5.2 kb) was smaller than the SVM200H band (7.0 kb). The SVMR2 band (5.2 kb) very strongly hybridized to the SVTS2 DNA probe even though it was faint in the corresponding agarose gel. This suggests that this band has a high percentage of homology to SVTS2 or may be a concatameric form of the viral DNA. The SVMR3 band (7.6 kb) may be a native virus-SVTS2 junction fragment. Three bands (1.3 kb, 2.1 kb and 5.2 kb) not present in SVM200H corresponded to those obtained from digestion of SVTS2 RF DNA. This indicated that the fragments of SVTS2 DNA were present in an extrachromosomal double-stranded DNA molecule in MR2 and MR3, but not in the parent line, M200H.

**SVTS2 DNA fragment insertion into the chromosomal DNA of *S. citri*.** Chromosomal DNA was isolated from M200H, MR2 and

MR3 and digested with EcoRI. A Southern blot was probed with SVTS2 DNA (Figure 6). Two bands (13.6 and 19.2 kb) hybridized very strongly to the probe in MR2 and MR3 but not in M200H, suggesting that SVTS2 DNA inserted into the chromosome of MR2 and MR3, but not M200H. These inserted viral DNA fragments may be present in more than one copy in the chromosomal DNA; in EtBr-stained agarose gels, the inserted DNA fragments appear much denser than the genome of S. citri, possibly due to repetitive sequences. Four weak hybridization bands (4.3, 6.5, 8.4 and 10.8 kb) in M200H, MR2, MR3 and R8A2 probably reflected insertion of DNA of their native viruses, since M200H and R8A2 were not previously exposed to SVTS2.

**Susceptibility of MR2 and MR3 to the heterologous virus, SVBR3.** Spiroplasma virus SVBR3, isolated from S. citri strain BR3 (Chapter VI), formed clear plaques about 0.5-3.0 mm in diameter on lawns of M200H, MR2 and MR3 (Table 2). The morphology of the plaques was identical in these three lines. Southern blot hybridization with a cloned SVBR3 DNA probe (8.6 kb, the entire virus genome) showed no hybridization to the chromosomal DNA of M200H, MR2 or MR3 (Figure 7), indicating that the genomes of these lines had no SVBR3-related sequences.

**Transformation of M200H with native virus SVMR3 RF DNA by electroporation.** A log phase culture of M200H was transformed with SVMR3 RF DNA, which contained a 2.1 kb

SVTS2 fragment, by electroporation. Isolated colonies growing within plaque boundaries in M200H lawns were cultured in LD8 broth. One of these, designated M200H-SV1, was resistant to SVTS2 infection at 5.6 x $10^8$ PFU/ml, while control lawns of M200H became infected by SVTS2 at that concentration (Table 3). Using a higher concentration of SVTS2 (1 x $10^{14}$ PFU/ml), a lawn of M200H-SV1 had only a few plaques, while the lawn of M200H was almost cleared by confluent plaques.

**Correlation of viral DNA presence in the spiroplasma genome and susceptibility to virus infection.** DNA of SVTS2, which was originally isolated from S. melliferum TS2, strongly hybridized with the chromosomal DNA of S. melliferum strains TS2 and AS576, hybridized less strongly with the chromosomal DNA of S. citri, and did not hybridize with the chromosomal DNA of S. floricola strain 23-6 (Table 4). Host spiroplasma lawns developed plaques with S. citri strains R8A2, M200H, BR3-3X, BR5-3X, and BR6-3X. However, lawns of S. citri MR2 and MR3 and S. melliferum TS2 and AS576 did not develop plaques.

DNA of SVBR3, originally isolated from S. citri BR3-3X, hybridized very strongly with the chromosomal DNA of S. citri strain BR3-3X; less strongly with the chromosomal DNA of S. citri BR5-3X and BR6-3X; weakly with the chromosomal DNA of S. melliferum TS2 and AS576; and not at all with the chromosomal DNA of S. citri lines R8A2, M200H, MR2, MR3, or

that of S. floricola 23-6. SVBR3 infection resulted in plaque formation in R8A2, M200H, MR2 and MR3. No infection occurred in BR5-3X and BR6-3X.

The results of these experiments indicated that virus infection produced plaques on lawns of spiroplasmas that did not contain integrated viral DNA, but did not produce plaques when the host carried an integrated viral DNA fragment. There is a negative correlation between viral DNA hybridization to the spiroplasma chromosomal DNA and plaque production by that virus or a related virus.

## DISCUSSION

Dickinson et al. (1985) suggested that the lysogenisation of S. citri SpA-MD with SpV3-type virus ai appears to result in a conversion of the cell surface which is manifested by a loss of receptors for virus ai. Bove et al. (1989) reported that S. melliferum strains BC-3, B63, G1R2 and G1R4 were resistant to infection by SpV4 virions, but could be transfected by SpV4 DNA. They suggested that resistance to infection by whole virus is at the level of adsorption or penetration of the virions. The protein profiles of M200H, MR2, and MR3 showed a few differences in membrane-associated proteins (Chapter III; Sha et al. 1991). However, although SVTS2 DNA can transfect S. citri M200H at a high frequency ($1.5 \times 10^5$ transfectants/ug SVTS2 DNA) after

electroporation, it does not transfect lines MR2 and MR3. This suggests that SVTS2 DNA is capable of entry into the MR2 and MR3 cells, and that absence of plaques reflects some phenomenon other than the loss of virus-binding spiroplasma surface proteins, perhaps that viral DNA replication is blocked.

Sequences of the SpV1 type and SpV3 type spiroplasma viruses occur in the chromosomal DNA not only of S. citri, but also of S. kunkelii and S. phoeniceum (Bove et al. 1989). Probably the entire SpV1 virus genome is present in the chromosomal DNA of the spiroplasma host (Renaudin et al. 1986, 1990). Dickinson et al. (1985) found that spiroplasma SpA-MD (ai) contained a full complement of ai viral DNA integrated into its genomic DNA and suggested that insertion of a full complement of ai viral DNA may be the cause for lysogeny of S. citri SpV-MD (ai). Acholeplasma laidlawii cells lysogenized by mycoplasma virus L2 were found to have a single L2 genome integrated in the cell chromosome (Dybvig et al. 1983). These data indicated that in these cases lysogeny is correlated with viral integration into the host genome. The SVTS2-resistant S. citri lines MR2 and MR3 were derived from S. citri M200H inoculated with virus SVTS2 (Sha et al 1991). The opportunity existed for SVTS2 to enter some M200H cells and to leave a fragment of viral DNA or the whole viral genome behind. Our results are consistent with this explanation. The two EcoRI fragments (13.6 and 19.2

kb), present in the genomes of MR2 and MR3, but not M200H, hybridized strongly to the viral DNA probe, indicating that genome or fragments of SVTS2 integrated into the chromosomal DNA of MR2 and MR3, but not M200H.

Lambdoid phage that encode a repressor (cI gene) which determines the immunity to lambdoid or related phages, in addition to its function in maintaining lysogeny (Lewin 1990). Any phage that possesses this immunity region confers on its host immunity to any other phage of the same type. In our experiments, perhaps these integrated viral DNA fragments carried a gene for repression of viral DNA replication. When expressed in spiroplasma, the repressor gene would inhibit replication of the same or a closely related virus (Figure 8).

A plasmid extraction method was used to purify native virus RF DNA. It is possible that extrachromosomal DNA other than virus RF DNA is present in this preparation. However, Southern blotting with the SVTS2 DNA probe showed that most DNA fragments hybridized to the probe, indicating most of them contained virus RF DNA.

In this work, SVTS2 viral DNA fragments were found to exist as extrachromosomal double-stranded DNA molecules in MR2 and MR3, but not in M200H. van der Avoort et al. (1984) showed that phage Phi X reduction sequence functioned as a viral incompatibility element to inhibit viral DNA replication. It is possible that extrachromosomal SVTS2 DNA

fragments function as viral incompatibility elements to inhibit replication of the challange virus. Our transformation of M200H with viral DNA by electroporation rendered it resistant to virus infection, supporting the hypothesis that the presence of SVTS2 DNA fragments in the host may result in resistance to subsequent SVTS2 infection.

MR2 and MR3 were susceptible to a virus, SVBR3, which is serologically related to SVTS2, but does not hybridize with it (Chapter VI, Sha et al. 1992). This example is similar to the immunity of SpA-MD (ai) to SpV3-ai but not to the related viruses SpV3-ag and SpV3-AVa/3 (Dickinson et al. 1985). We found no hybridization to SVBR3 DNA in the genomes of MR2, MR3 or M200H. The SVTS2 DNA fragments present in the genomes of MR2 and MR3 apparently do not interfere with SVBR3 infection. Since there is no DNA sequence identity beween these two viruses at our level of detection, this further supports the hypothesis that viral DNA integration in the genome of S. citri is correlated to resistance to that virus but not to unrelated viruses. This observation also disproves the possibility that MR2 and MR3 are simply incompetent to replicate viral DNA.

Most temperate phages integrate into the genomes of their hosts, but some exist as extrachromosomal replication units or plasmids (Ikeda et al. 1968). The virus SVTS2 occurs in resistant spiroplasma lines as two forms, an extrachromosomal replication unit and an insertion in the

host chromosome. To our knowledge, this is the first demonstration that SpV1-type spiroplasma virus can exist simultaneously both as integrated DNA and as an extrachromosomal replication unit.

DNA hybridization showed that there is a correlation of viral DNA integration in the spiroplasma genome and susceptibility to virus infection. The absence of virus DNA fragments, or the presence of DNA fragments of a less-related virus, does not interfere with the challenge virus infection, while the presence of homologous viral DNA or that of a closely related virus, does interfere with the challenge virus infection. Thus, viral incompatibility elements are a major cause of virus resistance.

Nur et al. (1987) reported that a small fragment of DNA from so-called plasmid pRA1 (virus) is present as multiple copies in short, dispersed and repetitive sequences in the chromosome of the spiroplasma. In our experiments, the integrated fragments of SVTS2 had high copy numbers in the chromosome of MR2 and MR3, probably as repetetive sequences. Apparently SVTS2 integrates into the chromosomal DNA of the host at more than one site. The repetitive sequences may move from chromosomal DNA to extrachromosomal DNA, but their function is still unknown.

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Table 1. Plaque assay of native viruses isolated from S. citri lines M200H, MR2 and MR3.

| Filtrate[a] | lawn | | |
|---|---|---|---|
| | M200H | MR2 | MR3 |
| M200H | +[b] | - | + |
| MR2 | + | + | + |
| MR3 | + | ND[c] | + |
| Tris buffer | - | - | - |

[a] Filtrate: virus purified as described but without the CsCl gradient.

[b] Positive indicates plaques produced; negative indicates no plaques produced.

[c] Not done.

Table 2. Relationship of virus susceptibility and presence of viral DNA sequences in the host spiroplasma chromosome

| S. citri line | SVTS2 | | SVBR3 | |
|---|---|---|---|---|
| | Plaque formation | Viral DNA in chromosome[a] | Plaque formation | Viral DNA in chromosome[b] |
| M200H | + | - | + | - |
| MR2 | - | + | + | - |
| MR3 | - | + | + | - |

[a] Southern blot of spiroplasma chromosomal DNA probed with SVTS2 DNA. Strong hybridization was interpreted as integration of tested viral DNA, weak hybridization as integration of a related virus, and no hybridization as no viral integration.

[b] Southern blot of spiroplasma chromosomal DNA probed with SVBR3 DNA.

Table 3. Susceptibility of S. citri M200H-SV1 to SVTS2

| S. citri | SVTS2 | | Tris buffer |
|---|---|---|---|
| | $5.6 \times 10^{8}$ PFU/ml[a] | $1 \times 10^{14}$ PFU/m | |
| M200H-SV1 | -[b] | + | - |
| M200H | ++++ | ++++ | - |

[a] Virus concentration

[b] Plaque assay. "-" indicates no plaques produced. "+" indicates only a few plaques produced; "++++"indicates many plaques produced.

Table 4. Analysis of natural virus infection, transfection with viral DNA and viral DNA hybridization

| Species and Strains | SVTS2 | | | SVBR3 | | |
|---|---|---|---|---|---|---|
| | Natural infection | Trans-fection | DNA hybri-dization[a] | Natural infection | Trans-fection | DNA hybri-dization[b] |
| S. citri | | | | | | |
| R8A2 | +[c] | +[d] | + | | | - |
| M200H | + | + | + | + | + | - |
| MR2 | - | - | +++ | + | + | - |
| MR3 | - | - | +++ | + | + | - |
| BR3-3X | + | | + | | | +++ |
| BR5-3x | + | | + | - | | ++ |
| BR6-3X | + | | + | - | | ++ |
| S. melliferum | | | | | | |
| TS2 | - | | +++ | | | ± |
| AS576 | - | | +++ | - | - | ± |
| S. floricola | | | | | | |
| 23-6 | + | | - | - | | - |

[a] Using SVTS DNA probe.

[b] Using SVBR3 DNA probe.

[c] "+" indicates production of plaques; "-" indicates no plaques.

[d] "+++" indicates very strong hybridization; "+" indicates less strong hybridization; "±" indicates weak hybridization; "-" indicates no hybridization.

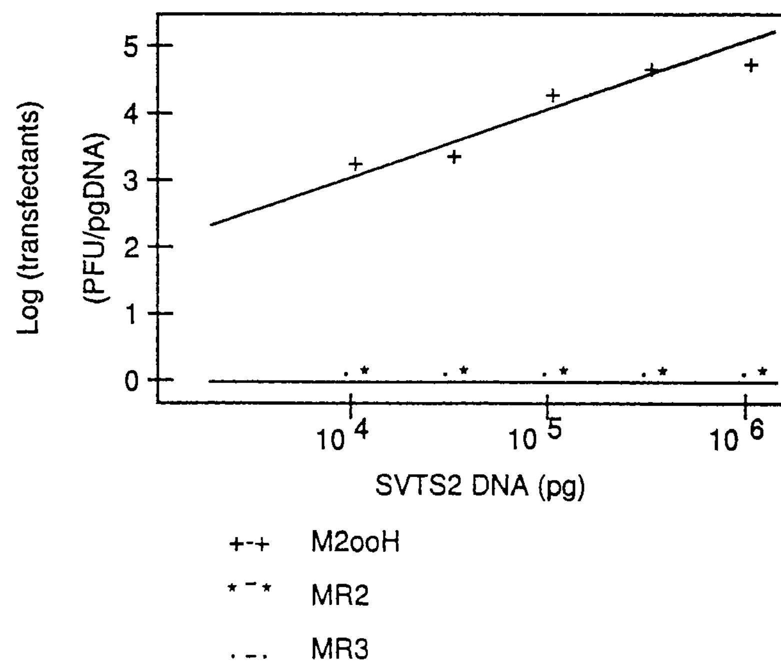



Figure 1. Transfection of S. citri M200H, MR2 and MR3 with SVTS2 DNA.

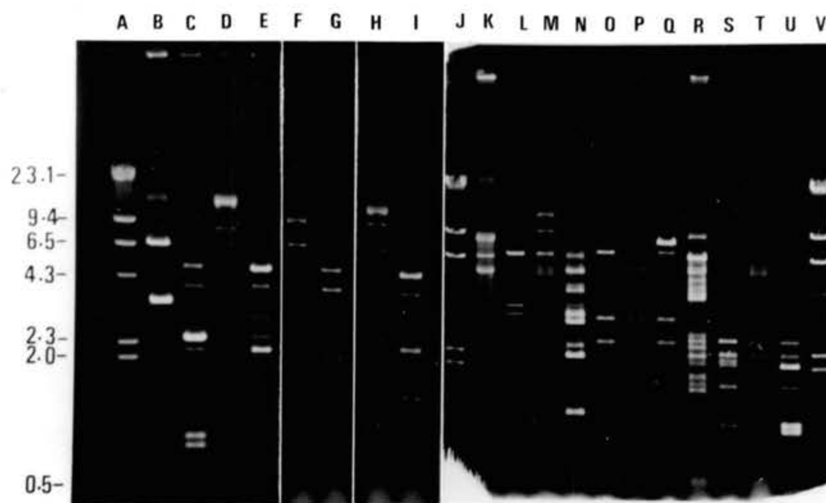
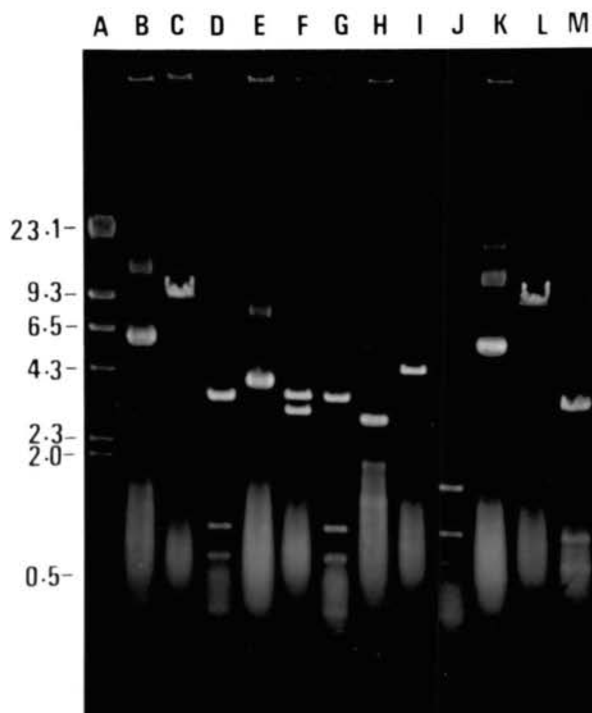



Figure 2. Restriction enzyme digestion patterns of native virus RF DNA. A,J and V, standard molecular marker (lambdoid DNA/HindIII fragments); B, SVTS2 RF cut with Sau3AI; C, SVTS2 RF cut with HinfI; D, SVMR2 cut with Sau3AI; E, SVMR2 RF cut with HinfI; F, SVM200H RF cut with Sau3AI; G, SVM200H RF cut with HinfI; H, SVMR3 RF cut with Sau3AI; I, SVMR3 cut with HinfI; K, SVTS2 cut with AluI; L, SVM200H cut with AluI; M, SVMR3 cut with AluI; N, SVTS2 cut with TaqI; O, SVM200H cut with TaqI; P, SVMR2 cut with TaqI; Q, SVMR3 cut with TaqI; R, SVTS2 cut with DdeI; S, SVM200H cut with DdeI; T, SVMR2 cut with DdeI; U, SVMR3 cut with DdeI.



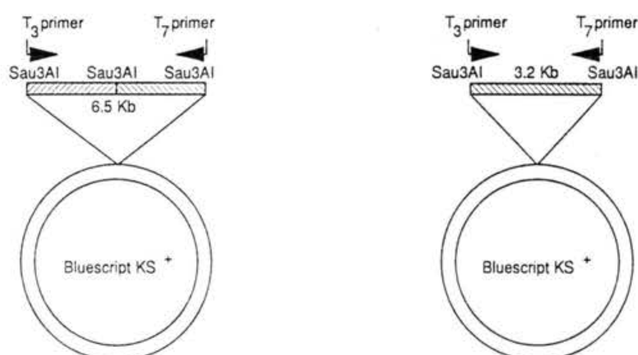




Figure 3. Molecular cloning of SVTS2 RF DNA fragment and restriction digestion. A, standard molecular marker; B, uncut 6.5 kb insertion (pESV1-1) recombinant plasmid; C and D, 6.5 kb insertion plasmid digested with BamHI and Sau3AI, respectively; E, uncut 3.2 kb insertion (pESV1-43) recombinant plasmid; F and G, 3.2 kb insertion plasmid digested with BamHI and Sau3AI, respectively; H, I, J, uncut pESV1-41, digested with BamHI and Sau3AI, respectively; K, L, and M, uncut pESV1-31, digested with BamHI and Sau3AI, repspectively.

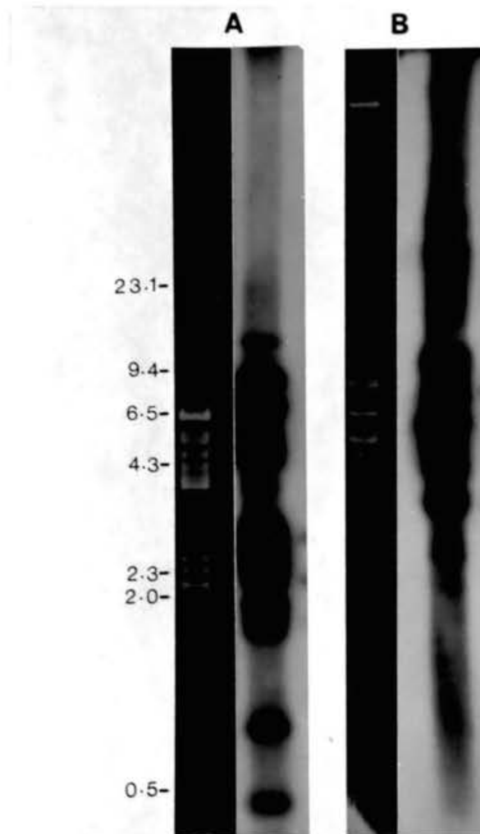



Figure 4. Southern blot of SVTS2 RF digested with AluI or DdeI. Left panel was stained with EtBr. Right panel was probed with SVTS2 6.5 kb (pESV1-1) recombinant plasmid DNA probe. A, SVTS2 RF DNA digested with DdeI; B, SVTS2 RF DNA digested with AluI.

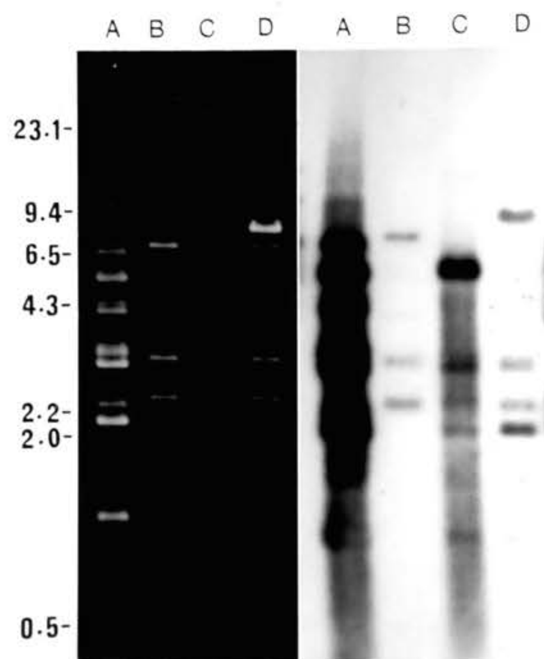



Figure 5. Agarose gel (left panel) and Southern blot (right panel) of native viral RF DNA digested with TaqI and hybridized with SVTS2 DNA probe. A, SVTS2 RF DNA; B, SVM200H RF DNA; C, SVMR2 RF DNA; D, SVMR3 RF DNA.

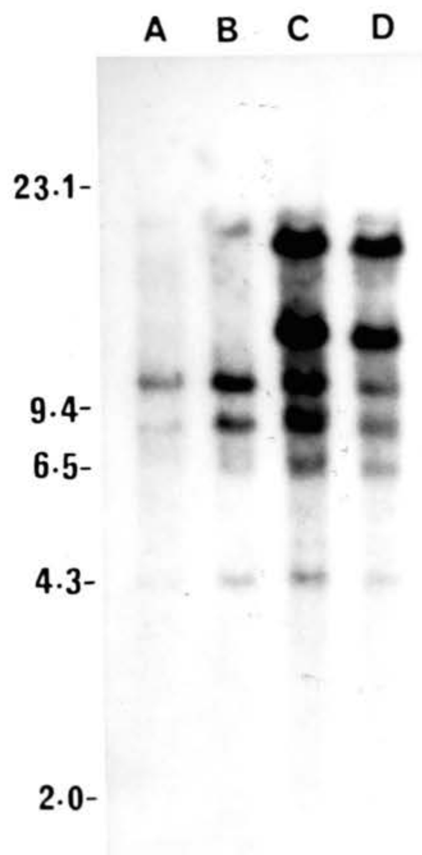



Figure 6. Southern blot of S. citri chromosomal DNA digested with EcoRI and probed with SVTS2 DNA. A, R8A2; B, M200H; C, MR2, D, MR3.

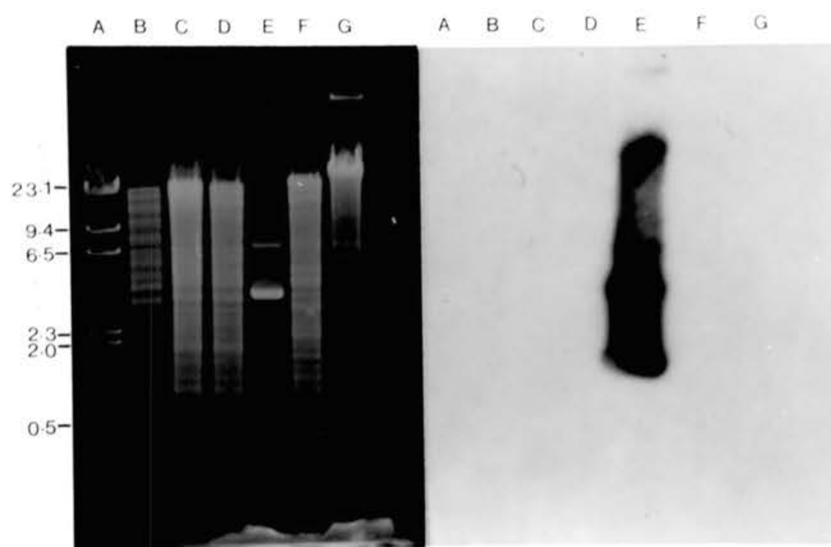



Figure 7. Agarose gel (left panel) and Southern blot (right panel) of S. citri chromosomal DNA digested and probed with SVBR3 RF DNA. A, Standard molecular marker; B-D, digested with EcoRI; B, M200H; C, MR2; D, MR3; E, SVBR3 clone A17. F and G, MR3 chromosomal DNA digested with HindIII and BamHI, respectively.

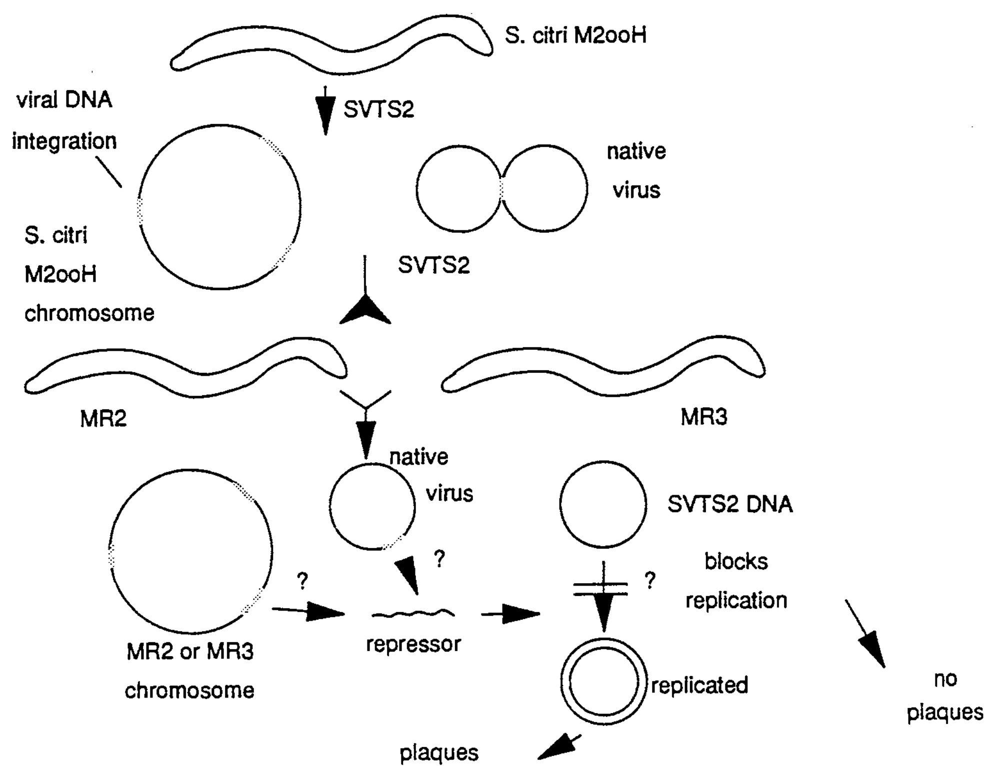



Figure 8. A model of the mechanism of resistance of S. citri lines to infection by the virus SVTS2.

## CHAPTER V

## Molecular Characterization and DNA Sequence Analysis of Spiroplasma Virus SVTS2

## ABSTRACT

Virus SVTS2, isolated from the honeybee spiroplasma, Spiroplasma melliferum TS2, can infect the phytopathogen S. citri. SVTS2 was found to infect S. floricola and all except two S. citri strains tested. SVTS2 was determined by Western blotting to have five proteins (60, 107, 140, 153 and 181 kd). A restriction map of SVTS2 RF indicated one EcoRI, one HpaII, two BstyI/ Sau3AI, four HinfI, and four TaqI sites. This map differs from those of viruses SpV1-78 and SpV1-aa, both isolated from S. citri. Clones of the complete SVTS2 RF DNA (6.5 kb) and a fragment (3.2 kb) were obtained. Partial DNA sequencing showed that the 3.2 kb fragment was identical to that of a segment of the 6.5 kb DNA. Methylation of one of the Sau3AI sites of SVTS2 RF DNA in S. citri M200H was observed. DNA hybridization showed that five EcoRI fragments of the genome of S. melliferum TS2 hybridized to the SVTS2 DNA probe, indicating that SVTS2 DNA was integrated into the genome of S. melliferum TS2 in at least three sites. DNA sequence analysis showed that SVTS2 had 56% identity to SpV1

R8A2 and 47.9% identity to SpV4 in a 1.2 kb segment of SVTS2 DNA. Thus, SVTS2 is different from, but closely related to, SpV1-R8A2. The possibility that a TaqI fragment of SVTS2 is an insertion sequence was investigated.

**INTRODUCTION**

Since the first spiroplasma virus was described (Cole et al. 1973), four different types of spiroplasmaviruses, designated SpV1, SpV2, SpV3 and SpV4, have been reported (Bove et al. 1989). SpV1 is a rod-shaped, nonlytic virus found in many different Spiroplasma species. SpV4 is an isometric virus found only in the B63 strain of S. melliferum. Recently, the complete nucleotide sequences of SpV1-R8A2B and SpV4 have been determined (Renaudin et al. 1987, 1990). The use of SpV1-R8A2B as a cloning vector for gene expression in spiroplasma has been reported (Stamburski et al. 1991). DNA sequences from two regions of the S. citri "plasmid" pRA1 (now considered as a SpV1-type virus by Bove et al. 1989) are present as short, dispersed, repetitive sequences in the chromosomal DNA of S. citri Maroc and S. kunkelii E275 (Nur et al. 1987). The ORF3 of SpV1-R8A2B was thought to be an insertion element (Renaudin 1992; Dong et al. 1992). ORF4 of SpV1-R8A2B displayed limited homology with the integrase of Salmonella phage P22 (Renaudin et al. 1990). Ye et al. (1992) found that SpV1-R8A2 integrated into the genome of S. citri strain R8A2HP and suggested that this

viral-like sequence may be present as a repeated element and may play a role in genomic rearrangements.

The SpV1-type virus SVTS2 was first isolated from the honeybee spiroplasma, S. melliferum TS2, by McCammon and Davis (1985). This virus was rod-shaped, 150-180 X 7-12 nm, containing single-stranded circular DNA of 6.5 Kb. The RF of SVTS2 has been cloned (McCammon et al. 1987). Cloned SVTS2 RF DNA was recircularized with T4 DNA ligase and was found to yield 9 x $10^{-9}$ transfectants/DNA molecule in S. citri M200H after PEG treatment, and 3 x $10^{-6}$ transfectants/DNA molecule after electroporation (McCammon et al. 1990).

In the present work, the spiroplasma virus SVTS2 was further characterized, the interaction of virus and host examined, and the possibility that a TaqI fragment of SVTS2 acts as an insertion element was explored.

## MATERIALS AND METHODS

**Spiroplasma sources and maintenance.** S. citri Maroc R8A2 was originally isolated from citrus in Morocco. S. citri M200H was passage 200 of strain R8A2. S. citri MR2 and MR3 are SVTS2-resistant lines derived from M200H. S. citri strains BR3-3X, BR5-3X, BR6-3X, BR12-3X and BR18-3X were isolated from horseradish in Illinois and triply cloned. ASP-1 is a non-helical strain of S. citri. S. citri Sp-V3 was isolated in Israel. S. melliferum strains AS576 and TS2 were isolated from honeybees. S. floricola strain 23-6 was

isolated from a flower surface. All spiroplasma strains were cultured in LD8 broth and stored at -70 °C. Escherichia coli DH5a™ competent cells (Life Technologies Inc.) were stored at -70 °C.

**Purification of SVTS2 DNA and its replication form (RF).** A modified method of McCammon et al. (1987) was used. One liter of S. citri strain M200H, about 1 x $10^{8}$ cells/ml in LD8 broth (Davis 1979), was inoculated with SVTS2 at an M.O.I. of about 2.3 X $10^{-6}$ and allowed to incubate at 31 °C for 2-3 days. The spiroplasma cells were pelleted and the supernatant was passed through a 0.2 um filter. The filtrate was treated with 10% polyethylene glycol and extracted with phenol. The DNA was precipitated with ethanol and the final pellet was resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). This was subjected to EtBr-CsCl gradient centrifugation, and the DNA was recovered by ethanol precipitation.

The viral RF DNA was purified by alkaline lysis and CsCl-EtBr gradient centrifugation, phenol extraction, and ethanol precipitation, as described (Chapter IV).

**Restriction enzyme double digestion.** TaqI, HincIII, HpaII, HinfI, BstyI, Sau3AI, and EcoRI were used to digest the SVTS2 DNA. The reaction mixture (2 ul 10x enzyme buffer, 14 ul sterile water, 3 ul viral DNA and 1 ul enzyme) was incubated at 37 °C, 1 hr except those with BstyI and TaqI, which were incubated at 60 °C or 65 °C. The reaction solution

was mixed with 180 ul sterile water, 4 ul 5 M NaCl and 200 ul phenol (buffered), and centrifuged at 16,000 x g for 5 min. The upper aqueous layer was transferred to a new tube, and three volumes of ether were added. This mixture was centrifuged at 16,000 x g for 3 sec and the bottom layer was saved. Three volumes of 95% ethanol were added and incubated at -70 $^{o}$C for at least 1 hr. The sample was centrifuged at 16,000 x g for 15 min. The pellet was washed with 500 ul 70% ethanol and centrifuged at 16,000 x g for 5 min, then dried in a desiccator. Fourteen ul water was added, and the sample was digested with the second enzyme under the same conditions. Digested viral DNA was electrophoresed in 0.7% agarose gels with TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA).

**SDS-PAGE and Western blotting.** Standard methods were used (Chapter III). Western blots were developed with anti-SVTS2 antiserum which was a gift from R. E. Davis (USDA/ARS, Beltsville, MD).

**Molecular cloning and sequencing of SVTS2 DNA.** The vector Bluescript KS+ was digested with BamHI. SVTS2 RF DNA was digested with Sau3AI and ligated to the vector with T4 DNA ligase. The recombinant plasmids were transformed into E. coli DH5a$^{TM}$ competent cells by heat shock (Sambrook et al. 1989), and transformants were selected and screened on TAXI plates (LB agar containing thymine, ampicillin, X-gal, and IPTG ) (Chapter IV).

The Sanger method of dideoxy-mediated chain termination was used for sequencing SVTS2 DNA (Sambrook et al. 1989; Chen and Seeburg 1985). The Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemical, Cleveland, Ohio) was used as recommended by the manufacturer. The recombinant plasmid DNA was extracted by alkaline lysis (Sambrook et al. 1989). Three to six ug purified plasmid DNA were mixed with 50 ul alkaline reagent (0.2 N NaOH, 0.2 mM EDTA) for 5 min at room temperature. Five ul neutralizing solution (2 M ammonium ion, 5 M acetate ion: 15.42 g $NH_4OAC$, 12.0 ml acetic acid, volume brought to 100 ml with water) and 125 ul 95% ethanol were added, and the mixture was incubated overnight at -20 $^{o}C$. DNA was pelleted by centrifugation for 15 min at 16,000 x g (4 $^{o}C$). The pellet was rinsed with 500 ul cold 70% ethanol, centrifuged at 16,000 x g for 1 min, dried in a vacuum desiccator, and dissolved in 7 ul $H_2O$. In the annealing reaction, this DNA sample was added to 2 ul 5X sequencing buffer and 1 ul primer, incubated 2 min at 65 $^{o}C$, slowly cooled to below 30 $^{o}C$, and then placed on ice. In the labeling reaction, the sample was mixed with 1 ul 0.1 M DTT, 2 ul 1x labeling mix, 0.5 ul $^{35}S$ dATP, and 2 ul diluted Sequenase Version 2.0 (1 part enzyme mix: 7 part enzyme dilution buffer), and incubated at room temperature 2-5 min. In the termination reaction, 3.5 ul of the labeling reaction mixture was transferred to each termination tube (one each containing ddGTP, ddATP, ddTTP and ddCTP), mixed and

incubated for 5 min at 37 °C. The reaction was stopped by adding 4 ul stop solution. The sample was electrophoresed in an 8% acrylamide sequencing gel at 55 watts for 3-6 hr. The sequencing gel was fixed (25% ethanol, 10% acetic acid) for 20-30 min, dried for 45-60 min at 80 °C and autoradiographed. Derived sequences were compared to known sequences in GenBank by computer.

**$^{32}$P labeling of SVTS2 DNA probe and Southern blot.** After SVTS2 RF DNA was cloned in E. coli, the recombinant plasmid was isolated and labeled with $^{32}$P by nick translation (Sambrook et al. 1989). Standard methods were used for preparation and hybridization of Southern blots (Chapter IV).

## RESULTS

**SVTS2 host range.** SVTS2 produced plaques in all but two tested strains of S. citri, and in S. melliferum AS576 and S. floricola (Table 1). Lines MR2 and MR3 of S. citri and S. melliferum TS2 and MR5 were not infected.

**SVTS2 polypeptide profile.** A Western blot probed with anti-SVTS2 serum is shown in Figure 1. SVTS2 had five protein bands (60 kd, 107 kd, 140 kd, 153 kd and 181 kd; lane B). A 153 kd protein band was present in native viruses SVMR2 and SVMR3, which were isolated from S. citri lines MR2 and MR3 (lanes C and D). The 60 kd, 107 kd and 181 kd bands

had greater signal than those at 140 kd and 153 kd. These experiments were replicated 3 times.

**Restriction enzyme digestion and viral restriction map.** Restriction enzymes AluI, BstyI, DdeI, DraI, EcoRI, HinfI, HincII, MspI, and Sau3AI digested SVTS2 RF DNA. Others (BamHI, BglII, BstxI, ClaI, HindIII, HhaI, PvuII, SacI, and SmaI) did not digest SVTS2 RF DNA.

A restriction map of SVTS2 was constructed by double digestion with restriction enzymes (Figure 2). SVTS2 RF DNA had one EcoRI site, one HincIII site, one HpaII site, four HinfI sites, two Sau3AI/BstyI sites, and four TaqI sites. This map was different from those of SpV1-78 and SpV1- aa (Figure 3). SpV1-aa has two EcoRI sites but no Sau3AI site (Dickinson et al. 1984). SpV1-78 has two EcoRI sites and one Sau3AI site (Renaudin et al. 1986). SVBR3 has one Sau3AI site but no EcoRI site (Sha et al. 1992). SVTS2 has only one EcoRI site and two Sau3AI sites. SVTS2 has four HinfI sites, while SpV1-R8A2 and SpV1-aa have only two HinfI sites. SVTS2 has two BstyI sites, but SpV1-R8A2 has none.

**Molecular cloning and partial sequencing of SVTS2, and comparison to SpV1-R8A2 DNA sequence.** After cloning SVTS2 DNA into *E. coli* DH5a, we obtained clones with 6.5 kb inserts (designated pESV1-1) and clones with 3.2 kb inserts (designated pESV1-43). Restriction enzyme digestion showed that the 6.5 kb inserts (pESV1-1) had two Sau3AI fragments (3.2 and 3.3 kb) and the 3.2 kb inserts (pESV1-43) had one

Sau3AI fragment (3.2 kb) (Chapter IV, Figure 3).

The recombinant plasmid containing the 6.5 kb insert was partially sequenced (Figure 8). Sequencing data showed that the SVTS2 DNA (1.2 kb) has a low G + C content (28%). The positions of the Sau3AI/BstyI and EcoRI, and one of four TaqI sites, obtained from sequence analysis, agreed with the restriction map of SVTS2. Computer analysis of DNA sequence data showed that SVTS2 DNA had 56% residue identity to SpV1-R8A2B and 47.9% identity to SpV4 based on 1.2 kb of SVTS2 DNA.

**Methylation of SVTS2 RF DNA in *S. citri* M200H.** SVTS2 RF DNA purified from *S. citri* M200H was digested with EcoRI and/or Sau3AI. The EcoRI digest showed one 6.5 kb band, while the Sau3AI digest showed three bands, 6.5 kb, 3.2 kb, and 3.3 kb (Figure 4). After cloning in *E. coli*, clone pESV1-1 (containing the 6.5 kb insertion) was digested with Sau3AI, producing one 3.2 kb and one 3.3 kb band (Chapter IV, lane D). A Southern blot of viral RF DNA showed that all viral Taq I fragments hybridized to the SVTS2 6.5 kb probe but only two fragments hybridized to the SVTS2 3.2 kb probe (Figure 5). Partial sequencing showed that clone pESV1-43 (containing a 3.2 kb insertion) had more than 98% DNA residue identity with clone pESV1-1 (containing a 6.5 kb insertion). This showed that the 3.2 kb fragment is a portion of the 6.5 kb viral genome of SVTS2. To investigate the observation that some of the viral DNA had one Sau3AI

site while others had two Sau3AI sites, SVTS2 RF was digested with MboI, which can digest the sequence GAT$^{m5}$C. The 6.5 kb band disappeared, and only 3.2 kb and 3.3 kb band were present. SVTS2 RF digested with Sau3AI, which can digest GATC or G$^{m6}$ATC but not GAT$^{m5}$C, had all 3 bands (6.5 kb, 3.2 kb, and 3.3 kb; 4.9 kb band is SVTS2 ssDNA) (Figure 6). This indicated that a cytidine of one Sau3AI site in SVTS2 DNA was partially methylated by a host methylation system.

**SVTS2 DNA integration into the genome of its host, S. melliferum TS2.** Five EcoRI fragments of the genome of S. melliferum TS2 (2.7 kb, 6.5 kb, 8.9 kb, 14.5 kb and 21 kb) and three EcoRI fragments of the genome of S. melliferum AS576 (2.7 kb, 6.5 kb and 14.5 kb) strongly hybridized with the SVTS2 DNA probe (Figure 7). The viral integration patterns in TS2 and AS576 were similar except for the 8.9 kb and 21 kb bands.

**Possibility that part of a TaqI fragment of SVTS2 acts as an insertion sequence (IS) in the S. citri chromosome.** Southern blotting showed the presence of SVTS2 fragments or of the whole viral genome in the spiroplasma chromosome and as extrachromosomal DNA in two SVTS2-resistant S. citri lines, MR2 and MR3 (Chapter IV, this thesis). Hybridization of EcoRI digests of the spiroplasma chromosome showed more than one copy of inserted viral DNA in the host chromosome. The 2.1 kb TaqI fragment of SVTS2 occurred in the

extrachromosomal DNA of MR2 and MR3. Perhaps the TaqI fragment of SVTS2 (Figure 2) contains an insertion sequence which can insert into the spiroplasma genome, or even move into the extrachromosomal DNA of its host. Our DNA sequencing data (Figure 8) indicated that a 579 bp SVTS2 DNA sequence shares 55% residue identity with the insertion element ISM1 of Methanobrevibacter smithii DNA (Hamilton et al. 1985), more specifically there was 58.9% identity to ORFIS of the insertion element ISM1, based on comparison to the 438 bp SVTS2 DNA sequence.

SVTS2 DNA shares 55% DNA residue identity to the S. aureus transposon 4001 aacA-aphD aminoglycoside resistance gene (located between bp 1725-3164, Rouch et al. 1987) which is near the transposase gene of this transposon (located between bp 102-1274).

SVTS2 DNA also has 49% DNA sequence identity to the ORF3 of Mycoplasma incognitus insertion sequence (Hu et al. 1990) based on comparison 292 bp of SVTS2 DNA.

These results suggested that TaqI fragment of SVTS2 may contain an insertion sequence, but more sequence data is needed to support this.

## DISCUSSION

The SpV1-type virus SVTS2 can produced plaques on strains of S. citri, S. floricola and S. melliferum. To our

knowledge, this is the only spiroplasmavirus reported to infect more than one spiroplasma species. The wide host range and small genome of this virus may be useful for its application as a cloning vector for Mollicutes.

The restriction map of SVTS2 differs from those of SpV1-R8A2, SpV1-78, SpV1-aa and SVBR3, although these viruses all belong to the SpV1 group.

Partial DNA sequence analysis showed that SVTS2 had 56% identity to SpV1-R8A2B. Ye et al. (1992) reported that 17 copies of SpV1-R8A2B integrated into the chromosome of its host, S. citri R8A2HP, comprising up to one 12th of the whole spiroplasma genome. Our hybridization showed that the genome of S. citri R8A2 hybridized only weakly to a SVTS2 DNA probe (Chapter IV, Figure 6). These findings further indicated that SVTS2 is different from SpV1-R8A2B, but that the two viruses are closely related.

Strong hybridization of the genome of S. melliferum TS2 to the SVTS2 DNA probe indicated that SVTS2 DNA integrated into the spiroplasma genome in three or more sites. SVTS2 also inserted into the genome of S. citri M200H, resulting in the selection of SVTS2-resistant lines MR2 and MR3 (Chapter IV, this thesis). This type of integration may involve a specific integration site. ORF3 of SpV1-R8A2 is an insertion element (IS) (Renaudin 1992; Dong et al. 1992). Dong et al. (1992) suggested that the protein encoded by ORF3 may act to catalyze integration of the virus genome. In our case, the

sequence of the 2.1 kb TaqI fragment showed 55% DNA identity with transposon 4001 of S. aureus and 55% DNA identity with ISM1 of M. smithii. Thus, the SVTS2 2.1 kb TaqI fragment may contain a gene (integrase) for integration, which could explain why the viral DNA insertion site was specific and stable.

The similar pattern of SVTS2 insertion in the genome of two different strains of S. melliferum could indicate that these two host strains have a close evolutionary relationship, or that there are very limited numbers of sites for insertion.

DNA probes of clone pESV1-1 (6.5 kb) and pESV1-43 (3.2 kb) all hybridized to SVTS2 DNA and share more than 98% DNA sequence identity, indicating that the 3.2 kb fragment was part of the 6.5 kb SVTS2 DNA. McCammon et al. (1990) reported that SVTS2 RF DNA, which was cloned into pUC19 and passed through E. coli strain JM109, was methylated in the adenine of the sequence GATC by E. coli JM109. In our studies, SVTS2 RF digested with Sau3AI produced 6.5 kb, 3.3, and 3.2 kb bands, but SVTS2 RF digested with MboI produced only 3.3 and 3.2 kb bands. This indicated that a cytidine of one Sau3AI site in SVTS2 genome DNA (6.5 kb) was methylated. SVTS2 RF DNA digested with BstyI separated into 3.3 kb and 3.2 kb fragments, further indicating that the Sau3AI site was methylated (BstyI and Sau3AI both recognize the sequence [GATC]).

CpG methylase was characterized in *Spiroplasma* sp. (Renbaum et al. 1990). Our results showed that SVTS2 RF DNA, purified from *S. citri* M200H, was methylated in one of two *Sau*3AI sites. The sequence data showed that clone pESV1-1 had the sequence TG **GATC** TC in a *Sau*3AI site at the 5′ end, while the 3′end of clone pESV1-43 had sequence 5′- **GATC** CT -3′. The 5′ end was identical to that of clone pESV1-1. These *Sau*3AI sites were not methylated by CpG methylase. It is possible that the 3′ end of clone pESV1-43 has the sequence 5′-CGATC CT-3′ which is easily methylated by CpG
3′-GCTAG GA-5′
methylase in spiroplasma. The SVTS2 RF DNA was methylated in one *Sau*3AI site of the mature virion ($GAT^{m5}C$). That some viral DNA was not methylated at this site may be because methylation requires extra time after new virus DNA replication. Or, the extent of methylation of spiroplasma is influenced by the age of the culture (Nur et al. 1985). *S. citri* M200H may have a CpG methylase modification system which protects it from foreign DNA invasion.

Juettermann et al. (1991) showed a CpG-specific DNA methyltransferase from *Spiroplasma* sp. methylated 5′-CG-3′ sequences in the RNA polymerase III- transcribed VAI gene of adenovirus type 2 DNA. McCammon et al. (1990) suggested that methylation of SVTS2 RF cloned in *E. coli* JM109 would inhibit the infectivity of the virus. This suggested that methylation of viral DNA or genes by spiroplasma methylase inactivates viral gene expression. Perhaps methylation of

SVTS2 in *S. citri* M200H is related to inhibition of viral infection.

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Table 1. Host range of spiroplasma virus SVTS2.

| Host | | Production of plaques after inoculation with SVTS2 virus particles |
|---|---|---|
| Species | Strain | |
| S. citri | M200H | + |
| | MR2 | - |
| | MR3 | - |
| | R8A2 | + |
| | BR5-3X | + |
| | BR6-3X | + |
| | BR12-3X | + |
| | BR18-3X | + |
| | ASP-1 | + |
| | SpV3 | + |
| | Cal-3X | + |
| S. melliferum | AS576 | ±[a] |
| | TS2 | - |
| | MR5 | - |
| S. floricola | 23-6 | + |
| | 01-3X | + |

[a] " ± " indicates variable results after 4 replications.

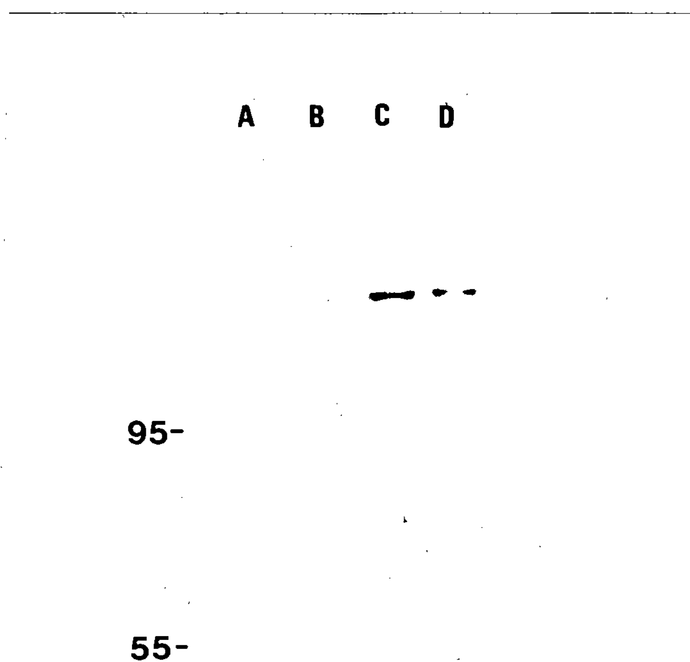


Figure 1. Western blot of spiroplasma virus SVTS2 polypeptides probed with SVTS2-specific antiserum. A, standard molecular size markers; B, SVTS2 polypeptides; C, SVMR2 polypetides; D, SVMR3 polypeptides.

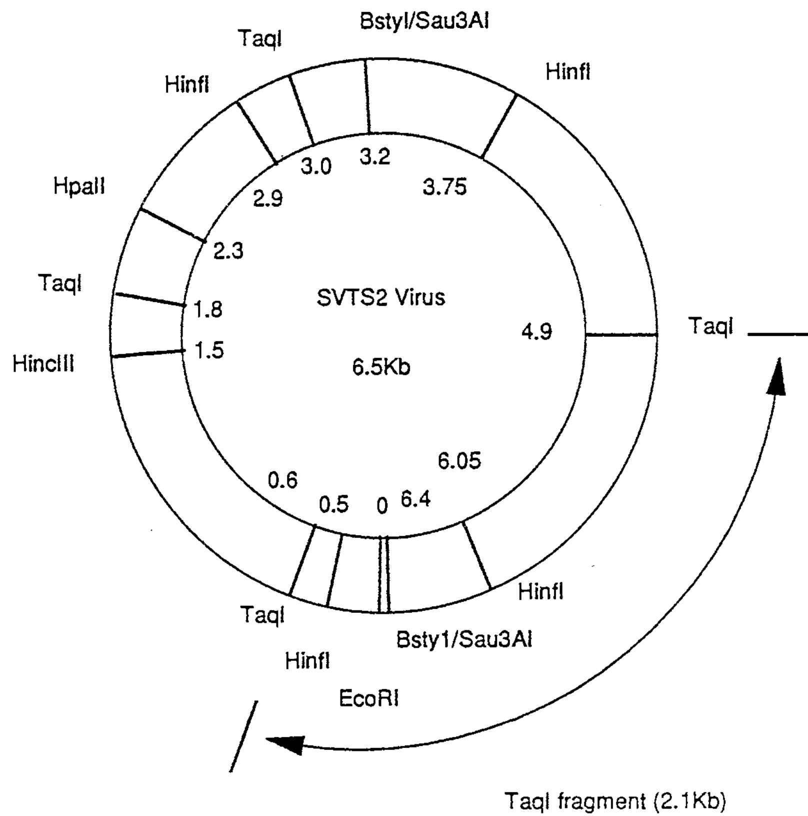


Figure 2. Restriction map of spiroplasma virus SVTS2.

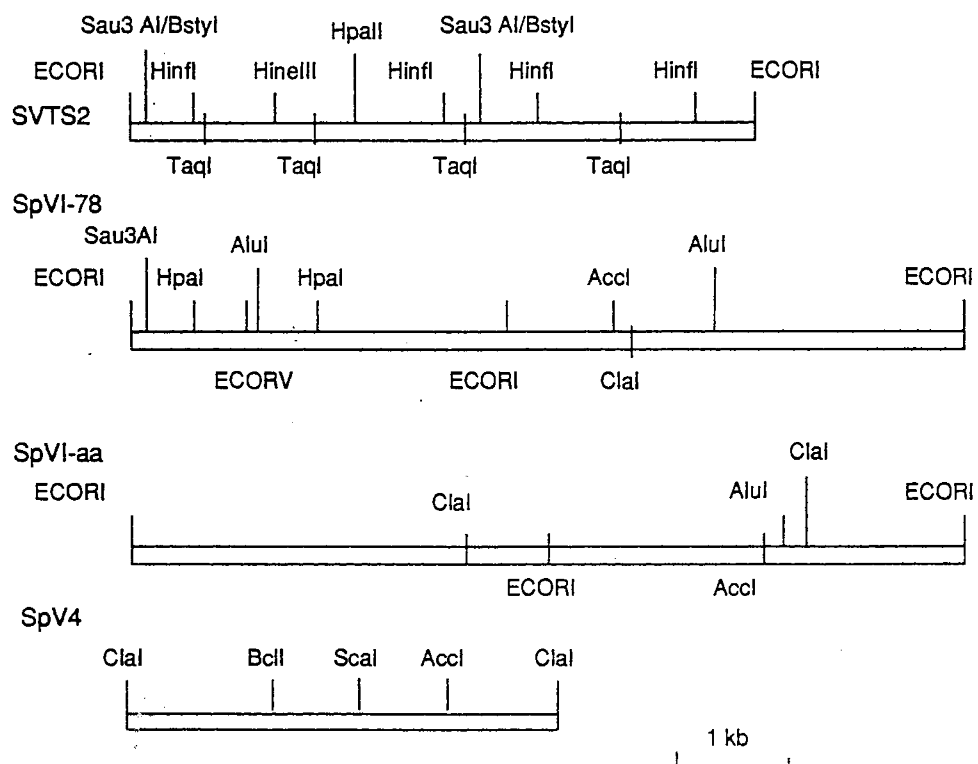


Figure 3. Comparison of restriction maps of SVTS2, SpV1-78, SpV1-aa and SpV4.

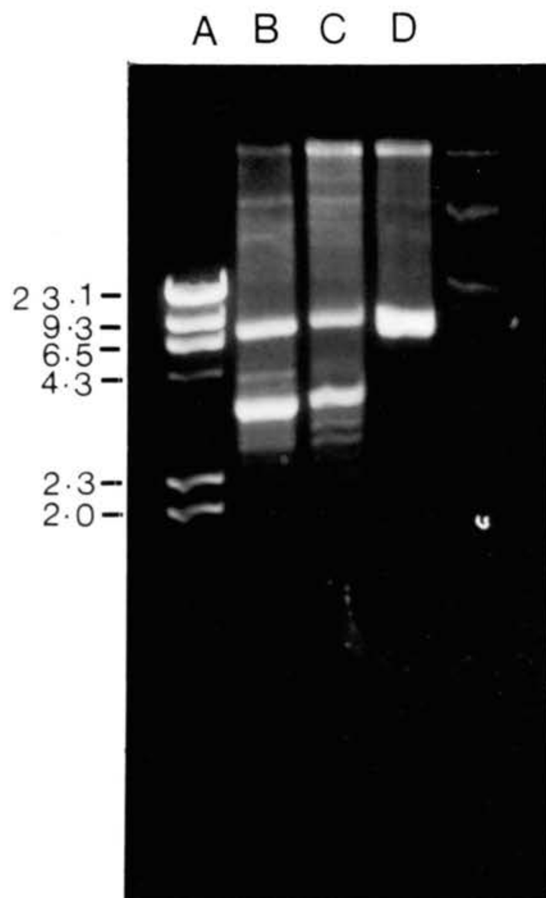


Figure 4. Restriction enzyme digestion of SVTS2 RF DNA electrophoresed in 1.5% agarose. A, standard molecular size markers; B, viral RF DNA digested with _Sau_3AI and _Eco_RI; C, viral RF DNA digested with _Sau_3AI; D, viral RF DNA digested with _Eco_RI.

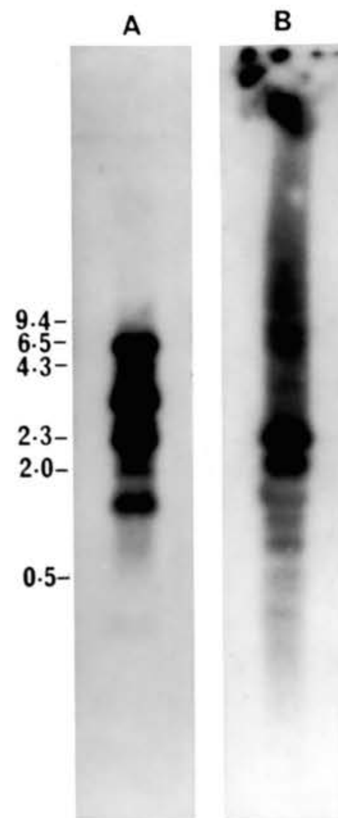


Figure 5. DNA hybridization of TaqI fragments of SVTS2 RF DNA with a SVTS2 DNA-specific probe. A, 6.5 kb SVTS2 DNA probe (pESV1-1); B, 3.2 kb SVTS2 DNA probe (pESV1-43).

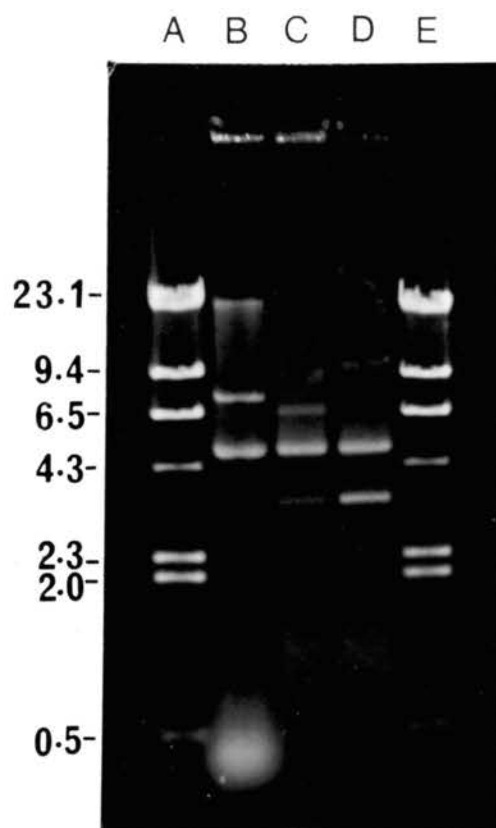


Figure 6. Restriction digestion of SVTS2 RF DNA. A and E, standard molecular size markers; B, uncut SVTS2 RF DNA; C, SVTS2 RF DNA digested with Sau3AI; D, SVTS2 RF DNA digested with MboI.

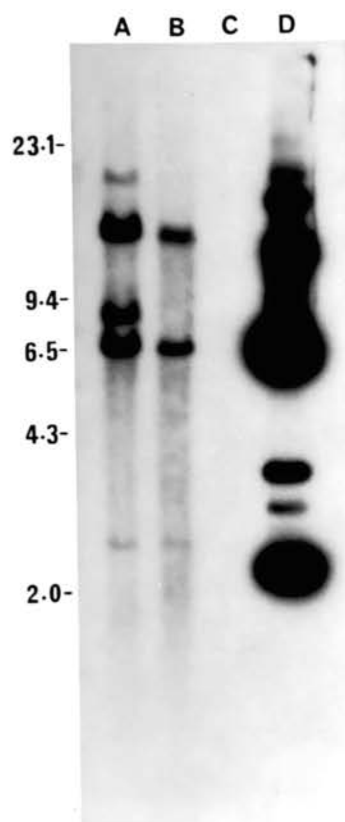


Figure 7. DNA hybridization of the genomes digested with <u>Eco</u>RI of spiroplasma strains with a probe specific for the virus SVTS2 DNA. A, <u>S</u>. <u>melliferum</u> TS2; B, <u>S</u>. <u>melliferum</u> AS576; C, <u>S</u>. <u>floricola</u> 23-6; D, SVTS2 DNA (clone pESV1-1).

```
LOCUS        #1T3+T7+06    961 BP DS-                     UPDATED   06/17/93
BASE COUNT       387 A     97 C    162 G     315 T       0 OTHER
ORIGIN       POSITION 1 OF #1T3+T7+063 (2)
        1 TATATTAAGT CACCTGATCT TTAAGAATCT ATATAGATTT TAAAAGAAAG AGAAATCTCT
       61 ATGAATGATT TCTCATGTGA TCACGTGTGT ATAGATATTG CACACGGGTA TAAATCGCAC
      121 GGTATTAAAG ATAGTACCAA GAGTGATATG TAGAAGTGAA ATGTAGAAGT ATGATGTATA
      181 TCACTTTATC ACCACGATGT ATTAAATATA TGAAGAAATG TGTGATCTCG TGTATAAGAG
      241 TGATCACAAA GAAGTATTGT TGCTACAAAT ACTACAAATG CGACTAGTCG TATAAATATA
      301 CATCTGATCG AGAAATACTG GACCTGATGT ATTTAGAGAT ATAAACCTCT ATATTATATA
      361 TGTATAGTGT ATATATTATA TTATAGTGTT CTGCGTCTAT AATAGTGGAT AGGTAAAAAT
      421 ATATACTATA TGTTGAACGC TAAATGATGA AAATTAATAT TGTTGAACAT ATACAGAAAT
      481 TATAACGAGC AAGAATTCAT TAACAATATT CTAATAATTC ATCAATAACA GAAGCAATAG
      541 TTATATGAAA CAACAATTAT GTATTGTTTG TATTAATGAT ACTGTTTGAG AAATGGATCT
      601 CGTTATTGTT GAAATTGTGT TAATGAAAAT GTTATTTTAA TTAACAATAT ATCTAGGTTA
      661 GAAAATGACT AAGAAAAAAA CAGATTGTTA AGGTTATAAT GGCAAAGAAT AAAGATAAAT
      721 TGATTATAAA TTAGAAAAAT TAATGTTAGG TCATATCAGT TTCAGCAGAT GAATACTAAT
      781 GAACATGAGC ATAGTAGAGA AAAACGAGAA TCCGAAAAAA GCAAACTAAG TTATATACAA
      841 CGATTAAATA TTGGTTAATG TTATCTCAAT GTTTACTACT AAATACATAT TTATGTAGAA
      901 GTATCAGGTA TTCTACATAG TATGATTGGT ATGAGACCAT AGTATAGTAG ACAGTAATAG
      961 T
```

Figure 8. Nucleotide sequence of the TaqI fragment of SVTS2.

## CHAPTER VI

Characterization of SVBR3, A Virus from
Spiroplasma citri Strain BR3-3X.

## ABSTRACT

A virus designated SVBR3, isolated from Spiroplasma citri BR3-3X (one strain of the spiroplasma which causes horseradish brittle root disease), is rod-shaped and 6-8 X 198-215 nm. Purified SVBR3 had a density of 1.26 g/cm$^3$ in CsCl. Virus DNA sensitivity to mung bean nuclease indicated a single-stranded form. These features are consistent with those of group I spiroplasma viruses (SpV1). The replicative form of SVBR3 is approximately 8.6 Kb. The virus infected most strains of S. citri tested, but not other spiroplasma species. Many restriction enzymes specific for G + C rich DNA did not digest SVBR3 RF DNA, suggesting a low G + C content. SVBR3 RF DNA restriction digestion patterns differed from those of another SpV1-type virus, SVTS2 (isolated from S. melliferum). Western blotting using anti-SVTS2 serum showed that SVBR3 had two polypeptides in common with SVTS2. Hybridization of Southern blots showed that SVBR3 had no detectable DNA sequence relatedness to SVTS2. The viral DNA was cloned in E. coli DH5a. Partial DNA

sequencing indicated that SVBR3 had 51% residue identity with DNA of SpV1-R8A2 and 37% identity to SpV4 (two previously characterized spiroplasmaviruses). DNA hybridization showed that viral DNA integrated into the genome of _S_. _citri_ BR3-3X at nine or more sites. Different viral DNA integration patterns in _S_. _citri_ lines of the BR group and those of the R8A2 group may be useful genetic markers for spiroplasma classification.

## INTRODUCTION

Since the first spiroplasma virus was described (Cole et al. 1974), four types of spiroplasmaviruses, designated SpV1, SpV2, SpV3 and SpV4, have been reported (Bove et al. 1989). SpV1-type viruses are rod-shaped particles containing circular, single-stranded DNA (Dickinson et al. 1984); the SpV2-type virus is a polyhedron with a long tail and probably double-stranded DNA (Cole et al 1974); SpV3-type viruses are polyhedral, short tailed viruses with double-stranded DNA (Cole et al. 1974); and SpV4 is a small, naked isometric virus with circular, single-stranded DNA (Ricard et al. 1982). Recently, the nucleotide sequences of SpV1-R8A2B and SpV4 have been completely determined (Renaudin et al. 1987, 1990). SpV1-R8A2B RF has been used as a cloning vector for Mollicute gene expression in spiroplasma (Stamburski et al. 1991, Marais et al. 1992).

SpV1 had a major capsid protein and several minor proteins; SpV3 had 5-7 proteins; and SpV4 had a major capsid protein of about 60 kd (Bove et al. 1989). Spiroplasma viruses have been found in *Spiroplasma citri*, *S. melliferum*, and the *Drosophila*-infecting sex-ratio spiroplasma (Cole et al. 1974; Dickinson et al. 1984; Ricard et al. 1984). Alivizatos (1982) first reported that viruses may occur in spiroplasmas within infected host plants, and that replication of cloned virus was associated with a rapid decline in spiroplasma viability. Spiroplasmas which supported a productive virus infection had substantially reduced pathogenic capabilities and were acquired and transmitted with much lower frequency than those which were uninfected. Dickinson et al. (1985) presented evidence of a correlation between lysogenisation of the SpV3-type virus ai and attenuation of pathogenicity of *S. citri* strain Sp-V3. Thus, it may be possible to use spiroplasmaviruses in biocontrol of spiroplasma-induced plant disease.

In the present work, a previously undescribed spiroplasma virus, designated SVBR3, was isolated from *S. citri* strain BR3-3X and characterized with respect to the viral polypeptides and viral genome. Viral DNA integration into the host genome also was investigated. This virus may have potential as a cloning vector or as a tool to investigate spiroplasma pathogenicity or transmissibility factors.

## MATERIALS AND METHODS

**Spiroplasma sources and maintenance.** *S. citri* strains BR3-3X, BR5-3X, BR6-3X, BR12-3X, BR18-3X were isolated from brittle root diseased horseradish in Illinois and triply cloned (Fletcher et al. 1981). *S. citri* strains Maroc R8A2 (originally isolated from stubborn diseased citrus in Morocco), M200H (subcultured 200 times from strain R8A2), MR2 and MR3 (virus-resistant lines derived from M200H), ASP-1 (a non-helical strain), Sp-V3 (originally isolated in Israel); *S. melliferum* strains AS576 and TS2 (isolated from honeybees); and *S. floricola* strain 23-6 (isolated from flower surfaces) were gifts of R. E. Davis, USDA, Beltsville, MD. All strains were cultured in LD8 (Davis 1979) broth at 31 $^{o}$C and stored at -70 $^{o}$C.

**Spiroplasma virus SVBR3 isolation and purification.** A triply-cloned line of *S. citri* strain BR3 (passage 30) was grown to 5 X $10^{8}$ -1 X $10^{9}$ cells/ml in 1 liter LD8 broth. Cells were pelleted at 10,000 x g, 4 $^{o}$C for 30 min. Triton X-100 was added to the supernatant to 0.2% (v/v) and the mixture incubated overnight at 4 $^{o}$C. The solution was centrifuged at 95,000 x g, 5 $^{o}$C, for 2.75-3 hr. The pellet was resuspended in 10 ml of 10 mM Tris buffer (pH 7.9) overnight. For each ml of virus preparation, 0.387 g CsCl was added. The solution was centrifuged at 105,000 x g, 20 $^{o}$C, for 24-48 hrs. The virus band was withdrawn and dialyzed

in 10 mM Tris buffer (pH 7.9) at 4 °C for 48-73 hrs with three changes of buffer. Virus titer was determined by plaque assay on S. citri M200H (Chapter III).

Virus SVTS2 was isolated from S. melliferum TS2 and multiplied in S. citri M200H. The virus purification method was same as above for SVBR3.

**Electron microscopy.** The negative staining method of Barile (1983) was used for EM observation. A small drop of virus suspension was applied to the grid for 1-2 min and then was drawn off with No.1 Whatman filter paper. A drop of 2% ammonium molybdate was immediately applied to the grid. After 30 to 60 seconds, the stain was withdrawn and the grid was allowed to dry for 1-2 min and examined under a JEOL 100-CX STEM electron microscope.

**Purification of SVBR3 and SVTS2 DNA and their replicative forms.** A modification of the method of McCammon and Davis (1987) was used. A log-phase culture of S. citri strain BR3 or S. melliferum strain TS2 was pelleted and the supernatant was precipitated with 10% polyethylene glycol (PEG) and extracted with phenol. The DNA was precipitated with ethanol and subjected to CsCl-EtBr gradient centrifugation, and the DNA was recovered by ethanol precipitation (Chapter IV).

For purification of viral RF DNA, 500 ml log phase culture of S. citri strain BR3 or S. melliferum TS2 (for SVTS2) in LD8 broth was pelleted at 13,000 x g for 30 min at 4 °C. All procedures were described earlier (Chapter IV).

**Restriction enzyme digestion.** *DdeI*, *BclI*, *HinfI*, *Sau3AI*, *TaqI* and other enzymes were incubated with SVBR3 RF or SVTS2 RF at 37 °C or 65 °C (*TaqI* only) for 1 hr. Mung bean nuclease (25 units, 50 U/ul, BRL) was incubated with SVBR3 DNA (260 ng) or M13 mp18 (200 ng, USB) at 37 °C for 5 min. Digested samples were electrophoresed in 0.7% agarose with TAE buffer (Chapter IV).

**SDS-PAGE and Western blotting.** Standard methods (Fletcher et al 1988) were used. The gels were 10% acrylamide, and SVTS2-specific antiserum (from R. E. Davis) was used to develop Western blots (Chapter III).

**Southern blotting.** The SVBR3 or SVTS2 RF DNA was digested with *DdeI*, *HinfI* or *TaqI* and separated on 0.7% agarose gels. Southern blotting was done using standard methods (Chapter IV). SVBR3 and SVTS2 DNA, previously cloned in *E. coli* (Chapter IV), were labelled with $^{32}P$ by nick translation (Sambrook et al. 1989) for use as probes. In a 1.5 ml Eppendorf tube on ice, 0.1 ug viral DNA was mixed with 1 ul 10x H buffer (66 mM Tris-HCl, pH 7.4, 66 mM $MgCl_2$, 10 mM DTT, and 0.5 M KCl), 1 ul 0.5 mM dATP, 1 ul 0.5 mM dGTP, 1 ul 0.5 mM dTTP, 1 ul $^{32}P$ dCTP (1-10 uCi), and 1 ul DNAse (1 x $10^{-5}$ ug/ul) and incubated at room temperature for 1 min. A volume of 0.33 ul DNA polymerase holoenzyme (10000 U/ml, Promega) was added and the mixture incubated at 12 °C for 3 hr. The mixture was placed on ice and 20 ul calf thymus DNA (2 mg/ml), 20 ul 1 M Tris-HCl, pH 8.0, and 4 ul 5 M NaCl

were added. Water was added to bring to the volume to 200 ul. The sample was mixed with an equal volume of phenol and extracted as usual, followed by ethanol precipitation.

**Transfection of S. citri with viral DNA.** Transfection was performed using 50% PEG following the method of McCammon and Davis (1987) or electroporation as described by Stamburski et al. (1991) (Chapter IV).

**Molecular cloning and sequencing of SVBR3 RF DNA.** The vector Bluescript KS+ was digested with BamHI, SVBR3 RF DNA was digested with Sau3AI, and ligated to the vector with T4 DNA ligase. The recombinant plasmids were transformed into E. coli DH5a™ competent cells, and transformants were selected and screened on TAXI plates containing thiamine, ampicillin, X-gal, and IPTG (Chapter IV). The Sanger method of dideoxy-mediated chain termination (Sambrook et al. 1989) was used for sequencing SVBR3 DNA. The SVBR3 DNA was denatured and annealed to T7 or T3 primers. Labeling was with [$^{35}$S]dATP. After termination, the reaction mix was electrophoresed on 8% urea-polyacrylamide gels and autoradiographed (Chapter V).

**Spiroplasma chromosomal DNA purification.** A modification (Chapter III) of the method of Williamson et al. (1991) was used for purification of spiroplasma DNA.

## RESULTS

**Isolation, purification and electron microscopy of spiroplasmavirus SVBR3.** A spiroplasma virus, designated SVBR3, was isolated from _S. citri_ strain BR3. The purified virus had a density of 1.26 $g/cm^3$ in CsCl and yielded $10^{10}$ -$10^{11}$ PFU/ml on lawns of the indicator strain, _S. citri_ M200H. Plaques were clear and measured 0.1 - 2.0 mm in diameter on M200H. Electron microscopy of SVBR3 revealed rod-shaped particles, 6-8 X 198-215 nm. One end of the particles appeared rounded and the other end flat. Virus particles were often adsorbed to the spiroplasma membrane surface (Figure 1).

**Virus host range.** SVBR3 produced plaques on lawns of most strains of _S. citri_ tested, but did not produce plaques on _S. melliferum_ AS576 or _S. floricola_ 23-6 or 01-3X (Table 1). SVBR3 could infect the _S. citri_ M200H-derived lines MR2 and MR3, which are resistant to another SpV1-type virus, SVTS2 (Sha et al. 1992).

**Characterization of SVBR3 DNA and its RF DNA.** Digestion of SVBR3 DNA with mung bean nuclease, which is specific for digestion of single-stranded DNA, is shown in Figure 2. Both SVBR3 DNA and M13 DNA were digested by this nuclease, indicating that SVBR3 DNA is single-stranded.

The purified SVBR3 RF DNA had a density of 1.6 $g/cm^3$ in CsCl. Three DNA forms of the viral RF, supercoiled, open circular and linear, were seen on 0.7% agarose gels. The SVBR3 RF DNA was approximately 8.6 kb (Figure 3).

Restriction enzymes BclI, DdeI, HinfI, Sau3AI and TaqI digested the SVBR3 RF DNA. However, restriction enzymes specific for G + C rich DNA (KpnI, MluI, SmaI, PstI and HaeII) did not digest the SVBR3 RF DNA, suggesting a low G + C content in the viral DNA. Other enzymes (ApaI, EcoRI and HindIII) also did not digest SVBR3 RF DNA. Compared to RF DNAs of SVTS2, the digestion patterns of SVBR3 with HinfI, TaqI and DdeI were quite different (Figure 4). There were three restriction sites for HinfI and TaqI, but only two for DdeI.

**SVBR3 polypeptides and serological relation to other SpV1-type viruses.** Following 10% SDS-PAGE of the SVBR3 virus, a Western blot was probed with SVTS2-specific polyclonal antiserum. SVBR3 had two polypetides (153 kd and 60 kd) in common with SVTS2, but SVTS2 had additional protein bands not present in SVBR3 (Figure 5). The 153 kd protein may be a major protein of SVBR3.

**DNA hybridization of viruses SVBR3 and SVTS2.** SVBR3 RF DNA digested with HinfI, TaqI or DdeI was separated by electrophoresis and hybridized with a SVTS2 DNA probe under high stringency conditions (65 °C for > 16 hr, washed in 2x SSC at room temperature and 0.1x SSC at 65 °C). The Southern blot showed no hybridization of SVBR3 RF DNA with the SVTS2 DNA probe, while the control SVTS2 RF DNA strongly hybridized (Figure 4). Also SVTS2 DNA did not hybridize to a SVBR3 DNA probe (pESV1-A7) when SVTS2 DNA, digested with

Sau3AI (pESV1-1, Chapter IV), was probed. These data indicate that SVBR3 DNA has little or no DNA sequence relatedness to SVTS2 DNA under our hybridization conditions.

**Transfection of S. citri with viral DNA.** After 50% PEG treatment, S. citri MR2 and MR3 were transfected with SVBR3 viral RF DNA at frequencies of $2.1 \times 10^2$ transfectants/ug RF DNA and $2.3 \times 10^2$ transfectants/ug RF DNA, respectively. When electroporation was used, the transfection frequency in MR2 increased to $2.1 \times 10^3$ transfectants/ug DNA.

**Molecular cloning and sequencing of SVBR3 DNA.** Sau3AI digested SVBR3 RF DNA was cloned in a plasmid vector (Figure 6). Clones of viral DNA fragments of 8.6 kb (designated pESV1-A7, lane F), 6.9 kb (designated pESV1-A1, lane B), 5.1 kb (designated pESV1-A2, lane C), and 3.5 kb (designated pESV1-A3, lane D) were obtained. DNA of pESV1-A7 (8.6 kb) was labelled with $^{32}P$ and hybridized to the Sau3AI-digested DNA of pESV1-A1, pESV1-A2, and pESV1-A3. Hybridization occurred in all three, indicating that these three fragments were subsets of the whole virus pESV1-A7 (8.6 kb). Also, a pESV1-A7 DNA probe hybridized to all fragments of SVBR3 RF DNA digested with Sau3AI, indicating that pESV1-A7 carried the whole SVBR3 genome [8.6 kb] (data do not shown). These cloned fragments were partially sequenced (Table 2). These DNA sequences (about 2302 bp total) showed 51% residue identity to segments of the complete sequence of SpV1-R8A2 and 37% residue identity to SpV4. The G + C content is 32%.

**Viral DNA integration into the genome of the host and the use of its integration patterns in spiroplasma classification.** We used cloned SVBR3 RF DNA (clone pESV1-A7) as a DNA probe and hybridized it to the genomes of _S_. _citri_ lines BR3-3X and BR5-3X (Figure 7) and BR6-3X (data not shown). The viral DNA probe strongly hybridized to the genomes of the former two lines, indicating that these lines have viral integration into their genomes, in 7-9 integration sites. The hybridization banding patterns in these three lines are similar (Figure 3, Chapter VII). However, when the genomes of _S_. _citri_ lines in the BR group (BR3, BR5, and BR6) and the R8A2 group (R8A2, M200H, MR2 and MR3) were hybridized with the SVBR3 or SVTS2 DNA probes, the hybridization patterns showed significant differences. For BR group strains with the SVBR3 probe, hybridization was strong and the pattern was identical in BR3, BR5 and BR6. No hybridization to this probe occurred in _S_. _citri_ R8A2 group strains, _S_. _floricola_ 23-6 and only weak hybridization occurred in _S_. _melliferum_ strains TS2 and AS576. However, when the SVTS2 DNA probe was used, weak hybridization occurred in BR group strains, an intermediate level of hybridization occurred in R8A2 group strains (Chapter IV) and strong hybridization occurred in _S_. _melliferum_ (Chapter V). The hybridization patterns of these three groups were quite different.

**DISCUSSION**

SVBR3 is a rod-shaped virus with single-stranded DNA, features which are consistent with spiroplasma viruses of the SpV1 group. SVBR3 infects most tested S. citri strains, which come from different geographical areas, but not S. melliferum or S. floricola. This host range is different from that of SVTS2 (Sha et al. 1993).

Dickinson and Townsend (1984) reported that the RF of SpV1- type virus aa can be digested by EcoRI but not by Sau3AI. SpV1-78 had two EcoRI sites and one Sau3AI site (Renaudin et al. 1988). SVTS2 has one EcoRI site and two Sau3AI sites (Sha et al. 1993). In contrast, SVBR3 can be digested by Sau3AI but not by EcoRI. This indicates that the SpV1-type viruses have different DNA restriction digestion patterns.

Bove et al. (1989) described a major capsid protein and several minor proteins in the SpV1-type viruses. SVBR3 had a major 153 kd protein, a minor 60 kd protein, and possibly several other minor proteins.

In Western blots, the presumptive SVBR3 protein reacted with anti-SVTS2 antibody. However, the SVBR3 RF DNA did not hybridize with an SVTS2 DNA probe. This may be explained if the two virus DNA sequences had little or no homology, but they translate antigenically related proteins from different codes. A similar case was reported for turnip vein-clearing

virus (TVCV) and tobacco mosaic virus (TMV) vulgare. Lartey et al. (1993) reported that antiserum against TVCV has a 256 dilution end point with TMV vulgare. However, RNA of TMV 204 did not hybridize to the TVCV cDNA probe (Hartson, 1991). These phenomena would be consistent with a common evolutionary origin for the two viruses.

SVBR3 RF DNA can transfect spiroplasma cells, indicating that this extracted DNA was a virus DNA, and not a plasmid. The ability of the viral RF DNA to transfect spiroplasma cells shows the possibility of its use as a cloning vector.

SVBR3 DNA does not hybridize to an SVTS2 DNA probe, and SVTS2 DNA does not hybridize to an SVBR3 DNA probe. This indicated that SVBR3 and SVTS2 are different viruses although they both belong to the SpV1-type group.

Viral DNA sequencing further supports the premise that SVBR3 is an SpV1-type virus which differs from SpV1-R8A2, although these viruses share approximately 51% DNA residue identity. Viruses of the SpV1 type may be derived from a common ancestor.

The banding pattern on Southern blots of S. citri lines BR3-3X, BR5-3X, and BR6-3X are consistent with the conclusion that SVBR3 integrates into the genome of S. citri BR3 lines at 7-9 sites. Viral DNA insertion into the host genome may affect host DNA arrangement and result in disruption of host gene expression.

S. citri R8A2 was originally isolated from stubborn

diseased sweet orange in Morrocco (Saglio et al. 1971), while S. citri BR3 was isolated from brittle root diseased horseradish in Illinois (Fletcher et al. 1981). Previous results (Fletcher et al. 1989) and our data (Chapter III, Figure 3A) showed that a 89 kd membrane protein was present in S. citri BR3-3X, but missing in S. citri R8A2 and M200H. Genomic DNA fingerprints of S. citri BR3, BR5, and BR6 (BR group) also showed different restriction patterns than S. citri R8A2, M200H, MR2 and MR3 (R8A2 group). Viral DNA integration patterns were similar in members of the S. citri BR group, but were different from those of the S. citri R8A2 group and of S. melliferum. The differences in membrane proteins, genomic DNA fingerprints, viral DNA integration patterns and original host can be used to divide these spiroplasmas into two different subgroups: S. citri R8A2 and S. citri BR.

Riley et al. (1991) found that bacteriophage lysed all strains of the Clavibacter sp. associated with annual ryegrass toxicity, but not strains of the related bacteria, C. iranicum, C. tritici or C. rathayi. He suggested that this bacteriophage could serve as a tool for rapid identification of bacterial strains suspected of being this toxigenic Clavibacter sp. Although phage sensitivity tests can offer greater specificity in identifying bacteria than physiological or even serological test (Cuppels 1983), spiroplasma virus sensitivity may not be adequate to

identify spiroplasma species. For example, SVBR3 can produce plaques in most S. citri strains, but not in S. citri BR5 and BR6. SVTS2 can infect S. citri, S. melliferum and S. floricola (Chapter V). However, viral DNA integration patterns in the spiroplasma host genome are stable and may be useful as genetic markers for quick identification of a newly isolated spiroplasma.

Using the S. citri 8 kb "plasmid" pRA1 (later shown to be virus RF, Bove et al 1989) as a DNA probe (Nur et al. 1986), it detected less than 10 pg of S. citri DNA, which is equivalent to about $10^3$ organisms, in plants and insects. In our work, SVBR3 DNA strongly hybridized to the genome of all of S. citri strains from brittle root-diseased horseradish in Illinois, suggesting that this virus DNA probe will be useful for spiroplasma disease diagnosis and insect transmission study.

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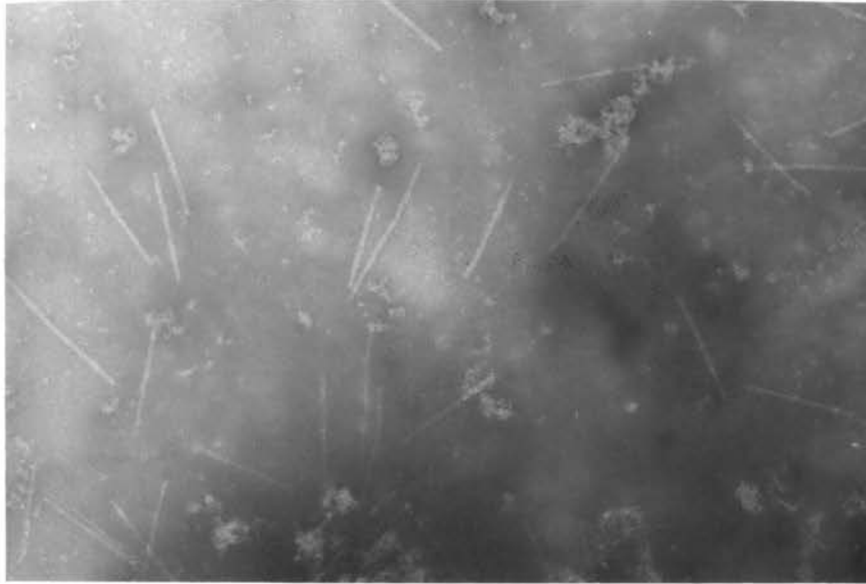
Table 1. Host Range of Spiroplasmavirus SVBR3.

| Host | | | SVBR3 |
|---|---|---|---|
| Species | Strain | Geographical area | |
| S . citri | R8A2 | Morocco | +[a] |
| | M200H | Morocco | + |
| | MR2 | | + |
| | MR3 | | + |
| | BR5-3X | Illinois | - |
| | BR6-3X | Illinois | - |
| | BR12-3X | Illinois | + |
| | BR18-3X | Illinois | + |
| | ASP-1 | Israel | + |
| | SpV3-3X | Israel | + |
| | Cal-3X | California | + |
| S. melliferum | AS576 | Maryland | - |
| | TS2 | | - |
| | MR5 | | - |
| S. floricola | 23-6 | | - |
| | 01-3X | | - |

[a] A positive indicates the formation of plaques on the tested strain. Negative indicates no plaques.

Table 2. Comparison of the sequences of SVBR3, SpV1-R8A2B and SpV4

| SVBR3 DNA fragment | Sequencing length (bp) | G + C | % Identity to SpV1-R8A2 | % Identity to SpV4 |
|---|---|---|---|---|
| pESV1-A1 (6.9 kb) | 447 | 28.4 | 54.1 | 45.1 |
| pESV1-A2 (5.1 kb) | 659 | 36.5 | 51.2 | 22.9 |
| pESV1-A3 (3.4 kb) | 570 | 32.8 | 50.9 | 49.4 |
| pESV1-A7 (8.6 kb) | 626 | 30.9 | 48.5 | 33.5 |
| Total | 2302 | | | |
| Average | | 32.2 | 51.2 | 37.7 |



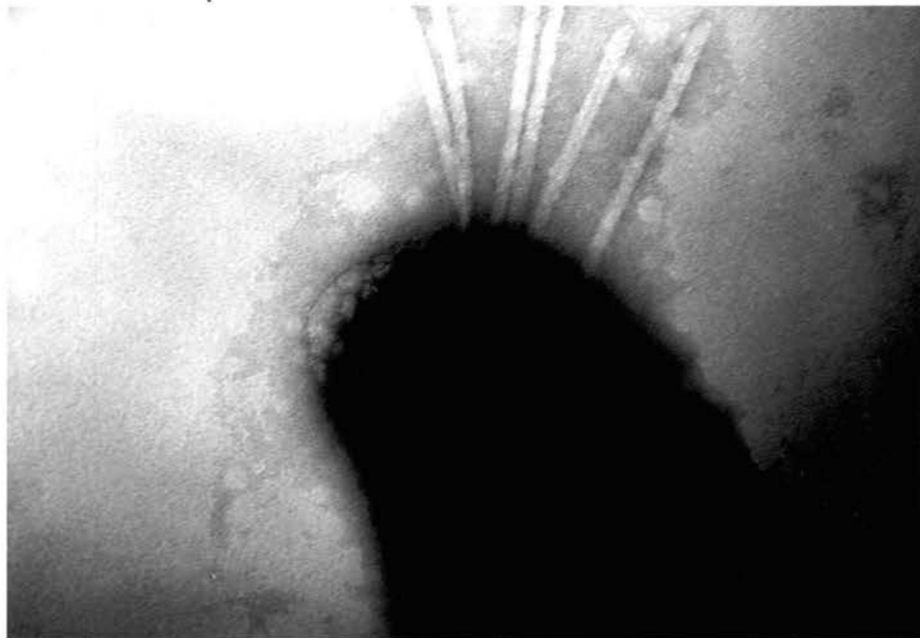


Figure 1. Electron microscopy of spiroplasma virus SVBR3. A, 58,000x; B, 190,000x.

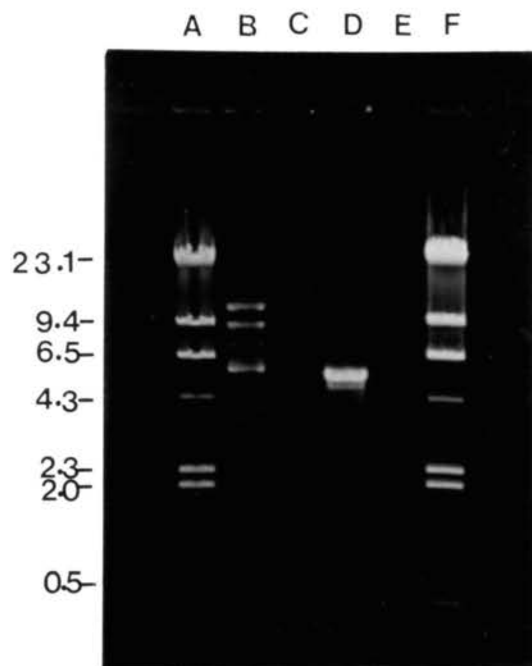



Figure 2. Agarose gel (0.7%) of spiroplasma virus SVBR3 DNA digested with mung bean nuclease. A, F, standard molecular size markers; B, undigested SVBR3 DNA; C, digested SVBR3 DNA; D, undigested M13 mp18 DNA; E, digested M13 DNA.

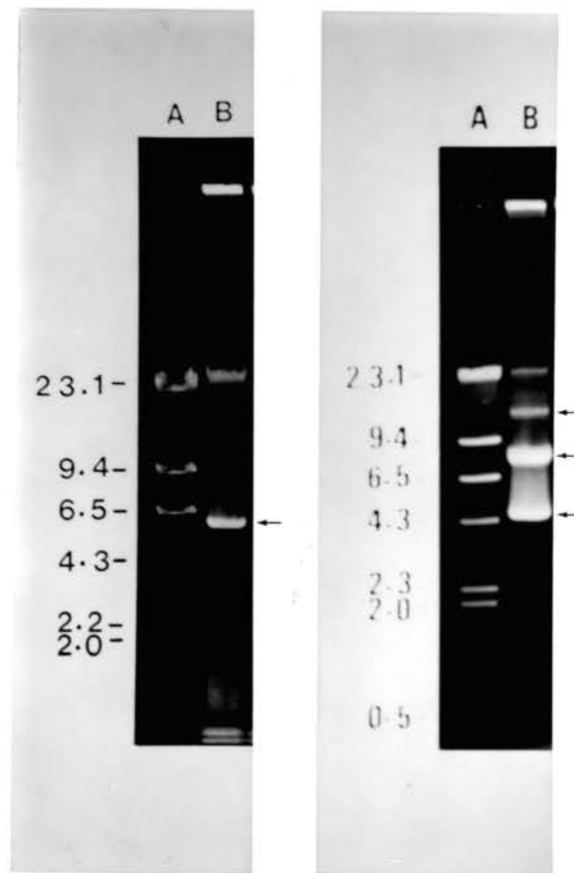



Figure 3. Agarose gel (0.7%) of spiroplasma virus SVBR3 DNA (left panel) and RF DNA (right panel). 3A, arrow indicates SVBR3 ssDNA; 3B, arrows indicate three forms of SVBR3 RF DNA.

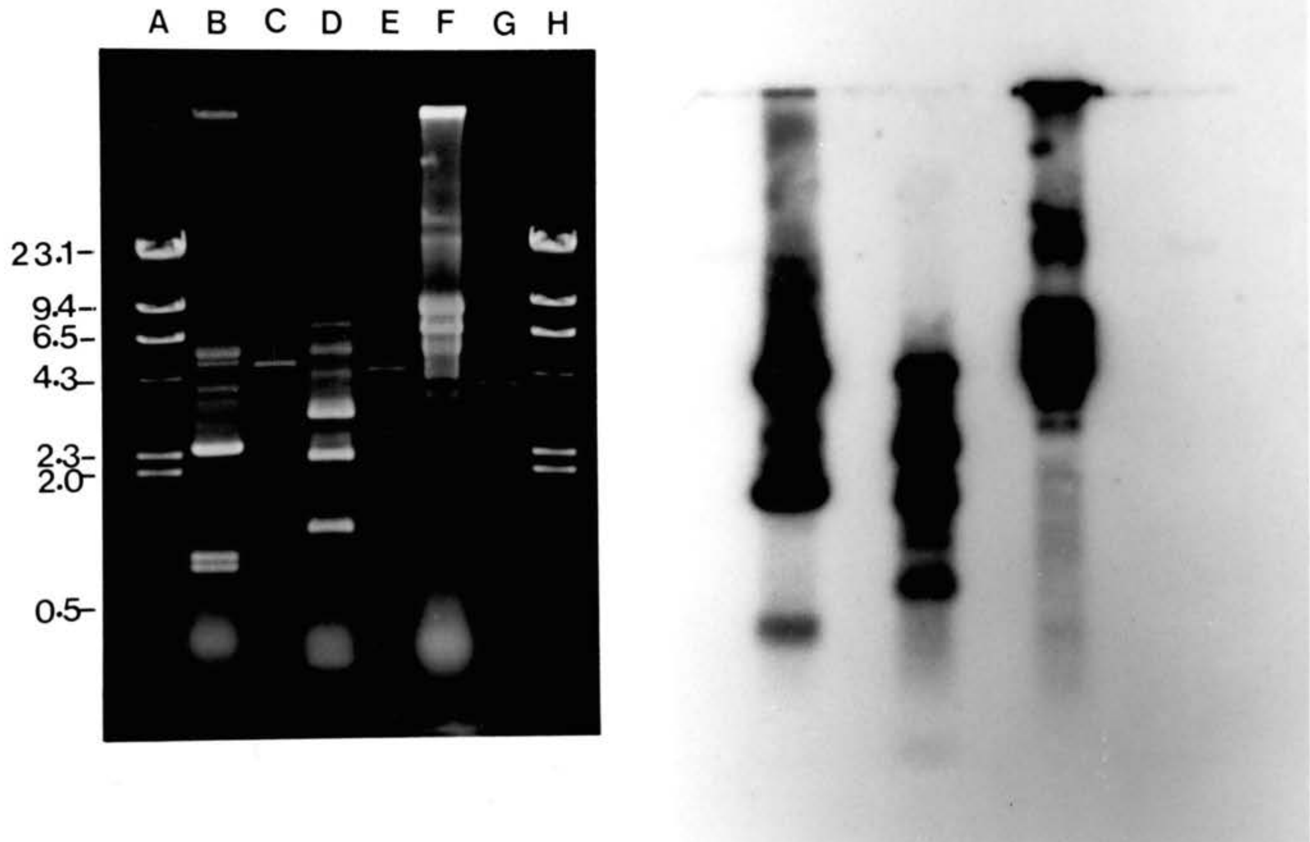



Figure 4. Restriction digestion patterns of SVBR3 and Southern blot developed with an SVTS2 DNA probe. 5A: A, H, standard molecular size markers; B, D, F, SVTS2 RF DNA digested with HinfI, TaqI and DdeI, respectively; C, E, G, SVBR3 RF DNA digested with HinfI, TaqI and DdeI, respectively. 5B, Southern blot of the same gel developed with SVTS2 DNA probe.

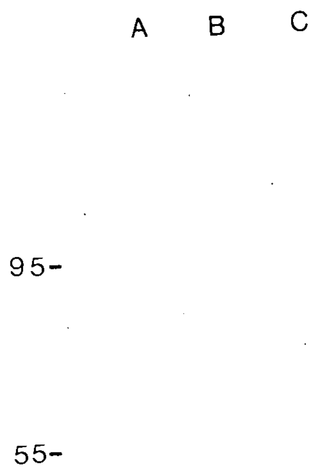



Figure 5. Western blot of spiroplasma virus SVBR2 polypeptides probed with SVTS2-specific antiserum. A, standard molecular size markers; B, SVTS2 polypeptides (30 ul/well); C, SVBR3 polypeptides (15 ul/well).

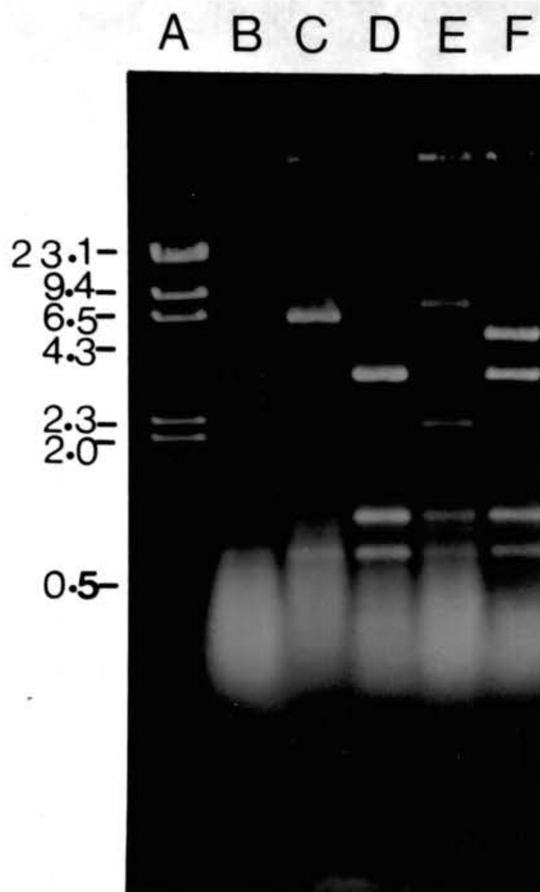



Figure 6. Molecular cloning and Sau3AI digestion of SVBR3 DNA. A, standard molecular size markers; B, clone pESV1-A1 (6.9 kb insertion); C, clone pESV1-A2 (5.1 kb insertion); D, clone pESV1-A3 (3.4 kb insertion); E, clone pESV1-A8 (12.6 kb insertion, maybe a spiroplasma plasmid); F, clone pESV1-A7 (8.6 kb insertion).

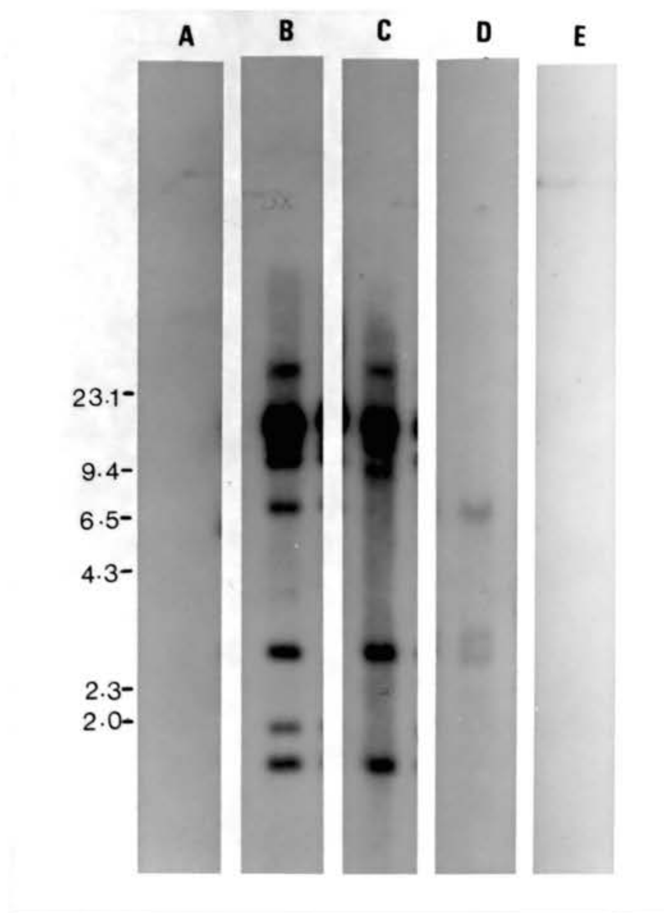



Figure 7. Southern blot of the genomic DNA of spiroplasmas digested with <u>Eco</u>RI, blot developed with SVBR3 DNA probe. A, <u>S</u>. <u>citri</u> R8A2; B, <u>S</u>. <u>citri</u> BR3; C, <u>S</u>. <u>citri</u> BR5; D, <u>S</u>. <u>melliferum</u> AS576; E, <u>S</u>. <u>floricola</u> 23-6.

## CHAPTER VII

DNA Fingerprinting Analysis and Viral DNA Integration into the Genome of Spiroplasma citri Lines Differing in Insect Transmissibility.

## ABSTRACT

Genomic DNA fingerprint analysis was used to compare three lines of Spiroplasma citri. BR3-T is insect transmissible and pathogenic; BR3-G is insect nontransmissible, but pathogenic; BR3-P is either insect nontransmissible or nonpathogenic. All three lines were derived from strain BR3-3X by different maintenance conditions during the past 10 years. Although the genomic DNA fingerprints of all three lines were similar, when compared to the transmissible line (BR3-T), BR3-G lacked three EcoRI fragments (0.4 kb, 0.5 kb, and 0.8 kb), while BR3-P lacked one EcoRI fragment (0.5 kb). BR3-P contains one EcoRI fragment (0.9 kb) not seen in either BR3-T or BR3-G. The genomes of these S. citri lines were digested with EcoRI and probed with a DNA probe of virus SVBR3, which naturally infects S. citri BR3. Viral DNA hybridized to the genome of all test lines indicating viral DNA integrated into the genome of all three lines at 6-9 sites. Viral DNA hybridization bands of 3.1 kb and 25.4 kb

occurred in BR3-T, but not in BR3-G and BR3-P. However, a band of 7.1 kb occurred in BR3-G and BR3-P, but not in BR3-T. These results indicated that the viral DNA integrates at different sites in insect transmissible and non-transmissible lines. In these limited tests, the presence of 3.1 kb and 25.4 kb fragments correlate with insect transmissibility and the presence of a 7.1 kb fragment correlates with non-transmissibility. Viral DNA integration in the spiroplasma genome may cause disruption or rearrangement of a gene or genes required for spiroplasma insect transmission.

## INTRODUCTION

Spiroplasmas cause corn stunt, citrus stubborn and horseradish brittle root disease (Davis et al. 1972; Markham et al. 1974; Fletcher et al. 1981). Three species of leafhoppers (*Circulifer tenellus*, *Scaphytopius nitridus*, and *S. acutus*) are known to transmit *Spiroplasma citri* (Kaloostian et al. 1975, 1979; Oldfield et al. 1976). *S. citri* strain BR3-3X, isolated from brittle root diseased horseradish plants in Illinos, has a SDS-PAGE protein pattern similar to that of *S. citri* strain R8A2 (Fletcher et al. 1981), but it has a surface protein of 89 kd which is missing in R8A2 (Fletcher et al. 1989). Wayadande et al. (1993) tested insect transmission of *S. citri* lines to

turnip plants using the beet leafhopper, C. tenellus. Three S. citri lines BR3-T, BR3-G, and BR3-P were derived from S. citri strain BR3-3X. Line BR3-T, which has been transferred from plant to plant for 10 years by leafhopper transmission, was transmitted with an overall efficiency of 22%, but BR3-G and BR3-P were not transmitted to test plants. For insect transmission to occur, Liu et al. (1983) suggested that spiroplasmas must cross from the lumen of the gut into the hemocoel and migrate to the salivary glands, moving across the membrane barrier and into salivary ducts after multiplication.

Spiroplasma viruses were first found in 1973 (Cole et al.). Viral DNA was shown to integrate into the genome of the host spiroplasma (Renaudin et al. 1986, 1990; Ye et al. 1992; Sha et al. 1992). The effect of the integration still is unknown, but it may cause rearrangement of spiroplasma DNA (Ye et al. 1992). In this work, genomic DNA fingerprints (7% SDS-PAGE, Herring et al. 1982) of S. citri lines BR3-T, -G, and -P were compared to each other and to BR3-3X and other horseradish isolates. Viral DNA integration patterns in the spiroplasma genomes, and their correlation with insect transmissibility, were investigated.

## MATERIALS AND METHODS

**Spiroplasma sources and maintenance.** S. citri strains BR3-

3X, BR5-3X and BR6-3X were isolated from horseradish plants with brittle root symptoms in Illinois and triply cloned (Fletcher et al. 1981). BR3-G, BR3-T, and BR3-P were derived from strain BR3-3X. BR3-3X was subcultured 30 times in LD8 broth prior to this study (Chapter VI); BR3-T is a line transmitted by the leafhopper C. tenellus for more than 10 years and is insect transmissible and pathogenic; BR3-G was continuously transmitted from plant to plant by grafting for more than 10 years, and has lost its insect transmissibility but is still pathogenic; BR3-P was subcultured more than 130 times in LD8 broth, and has lost either its insect transmissibility or its pathogenicity or both (Wayadande et al. 1993). All spiroplasma lines were cultured in LD8 broth and stored at -70 °C.

**Genomic DNA isolation and purification.** A modification of the method of Williamson et al. (1991) was used to isolate spiroplasma genomic DNA. A volume of two hundred ml of log phase spiroplasma culture was centrifuged at 20,000 x g for 30 min. The cells were resuspended in 2 ml TGE buffer (25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA). Fifty ul of 20% SDS was added and cells were lysed at 60 °C for 10 min. The solution was brought to 7.5 ml with TE buffer (100 mM Tris-HCl, pH 7.4, 10 mM EDTA) and subjected to CsCl-EtBr gradient centrifugation (final density 1.55 g/cm$^3$). After centrifugation at 239,000 x g for 15 hr, the DNA band was removed and extracted with phenol. Two times the DNA volume

of deionized water was added to the extracted DNA. Two times the total volume of 95% ethanol was added, and the mixture incubated at -70 °C for 1 hr. The precipitated DNA was collected by centrifugation at 14,000 x g for 10 min at 4 °C. The pellet was resuspended in 250 ul TEN buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA) and incubated 10-15 min at room temperature. Six ul 5 M NaCl and 300 ul phenol (buffered) were added and the mixture was centrifuged at 16,000 x g 5 min. The aqueous phase was extracted with ether. Seven hundred fifty ul 95% ethanol was added and the suspension incubated at -20 °C overnight. After centrifuging 15 min at 16,000 x g, the pellet was washed with 500 ul 70% ethanol, and the sample was centrifuged at 16,000 x g for 5 min. The pellet was dried in a vacuum desiccator and dissolved in 100 ul TE buffer.

**Genomic DNA fingerprinting analysis.** The method of Herring et al. (1982) was used. Spiroplasma genomic DNA was digested with EcoRI or HindIII 1 hr at 37 °C, and electrophoresed in 7% SDS-PAGE, for 28-29 hr at 22-25 mA using electrode buffer (0.036 M Tris, 0.03 M sodium dihydrogen phosphate, 0.001 M EDTA, pH 7.8). For staining, the gels were washed with 10% ethanol-0.5% acetic acid for 30 min, soaked in 0.011 M silver nitrate for 2 hr, and rinsed briefly in distilled water. Reduction was performed with developing solution (0.75 M sodium hydroxide, 0.1 M formaldehyde, and 0.0023 M sodium borohydride).

**$^{32}P$ labelling of spiroplasma virus SVBR3 DNA probe and Southern blotting.** After SVBR3 RF DNA was cloned in E. coli (Chapter VI), the recombinant plasmid (clone A7, 8.6 kb insertion, Sha et al. 1992) was isolated by alkaline extraction (Sambrook et al. 1989) and purified by phenol extraction, EtBr-CsCl gradient centrifugation and ethanol precipitation. The purified SVBR3 DNA was labeled with $^{32}P$ by nick translation (Sambrook et al. 1989). For Southern blotting, the genomic DNA of BR3-3X, BR3-T, BR3-G, BR3-P, BR5-3X, and BR6-3X were digested with EcoRI at 37 °C for 1 hr and were electrophoresed on 0.7% agarose gels with TAE buffer. The DNA was transferred to a nylon membrane, wet with 20 ml 4x SSC solution (20x SSC: 3 M NaCl, 0.3 M $Na_3$ citrate, pH 7.0). Prehybridization was in a solution (10 ml/blot) of 5x SSC, 5x Denhardt's solution [50x: 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin in 1 L $H_2O$], 200 mg/L sonicated calf thymus DNA, and 50 mM sodium phosphate, incubated at 65 °C for 2 hr. Hybridization was in a solution (5 ml/blot) of 1x SSC, 1x Denhardt's solution, 100 mg/L sonicated calf thymus DNA, 20 mM sodium phosphate, 0.1% SDS, and 10-15 ul labeled SVBR3 DNA [0.1-0.5 uCi]. After overnight (>16 hr) incubation at 65 °C, the nylon was washed twice at room temperature for 30 min with 2x SSC, then twice at 65 °C for 15 min with 0.1x SSC. The blots were autoradiographed.

## RESULTS

**Genomic DNA fingerprinting analysis of *S. citri* lines.** Purified spiroplasma genomic DNA was digested with *Eco*RI or *Hin*dIII and electrophoresed in 7% SDS-PAGE. *Eco*RI digested DNA from *S. citri* BR3-3X, BR3-T, BR5-3X, and BR6-3X had similar DNA fingerprints (Figure 1). However, *S. citri* lines BR3-G and BR3-P differed from the above lines. DNA bands of 0.4 kb, 0.5 kb, and 0.8 kb, present in BR3-T, were missing in BR3-G. Line BR3-P lacked the 0.5 kb band, but had an additional band of 0.9 kb not present in BR3-G or BR3-T. Using *Hin*dIII digestion (Figure 2), BR3-G had a band of lower molecular weight (0.38 kb) and one of higher molecular weight (0.52 kb), than those (0.39 kb and 0.51 kb) of BR3-T, BR3-P, BR5-3X and BR6-3X. BR3-G and BR3-P also lacked a 0.55 kb band which was present in BR3-T, BR5-3X and BR6-3X.

**Virus DNA integration pattern in *S. citri* lines.** Cloned SVBR3 RF DNA was used as DNA probe and hybridized to the genomic DNA of *S. citri* lines BR3-3X, BR3-G, BR3-T, BR3-P, BR5-3X and BR6-3X, *S. melliferum* strains AS576 and TS2 and *S. floricola* strain 23-6. Viral DNA hybridized to the genome of all lines of *S. citri* and *S. melliferum*, but not *S. floricola* (Figure 3), indicating that viral DNA integrated into the genomes of the former two species. There were at least 9 hybridization bands in BR3-3X, and fewer in the others. The integration patterns were somewhat different

among the three lines, although most of the hybridization bands were the same. BR3-3X has nine hybridization bands (1.5, 1.9, 3.1, 3.7, 7.1, 11.3, 15.5. 19.6, and 25.4 kb; lane A); of these, the hybridization bands of 3.1 kb, 7.1 kb and 25.4 kb are notable. BR3-T (lane C) and BR5-3X (lane E) had 3.1 kb and 25.4 kb bands, but not the 7.1 kb band; BR3-G (lane B) and BR3-P (lane D) had the 7.1 kb band, but lacked the 3.1 kb and 25.4 kb bands; BR3-3X and BR6-3X (lanes A and F) have all three bands (3.1, 7.1 and 25.4 kb). The 19.6 kb band hybridized very strongly and occurred in all test lines. Weak hybridization occurred in genomic DNA of S. melliferum (lanes G and H), and no hybridization occurred in genomic DNA of S. floricola (lane I).

## DISCUSSION

Denes and Sinha (1992) reported that the loss of vector transmissibility of the clover phyllody (CP) MLO was associated with either the loss or extensive rearrangement of a 4.8 kb extrachromosomal DNA band present in CP-MLO maintained in infected plants by vector transmission in the greenhouse. They suggested that this extrachromosomal DNA element affected the gene products necessary for the establishment of MLOs in insects. In our genomic fingerprint of spiroplasma lines, three genomic DNA EcoRI or HindIII fragments (0.4, 0.5 and 0.8 kb), present in BR3-T, were

missing in BR3-G and BR3-P. Whether these DNA fragments are involved in insect transmission is unknown, but it is possible that insect transmission-associated genes located in these DNA fragments were lost in BR3-G and BR3-P due to lack of selection pressure created by long-term maintenance in habitats other than the leafhopper or plant. Alternatively, mutation in an EcoRI or HindIII restriction site may yield fragments not cut by EcoRI or HindIII. Perhaps, if these mutations occur in insect transmission-associated genes, disruption of these genes may follow.

Virus SVBR3, isolated from S. citri BR3-3X, has a genome of about 8.6 kb (Sha et al. 1992). At least 17 copies of a related virus, SpV1-R8A2B, together constituting up to one 12th of the whole spiroplasma genome, have been found in the genome of S. citri strain R8A2HP, but the effects of these viral DNA insertions are unknown (Ye et al. 1992). Virus SVBR3 DNA integrates into its host genome at nine or more sites (Figure 3). A 19.6 kb band hybridized very strongly and occurred in all test lines. Because of its size and greater DNA sequence identity to the virus, this band may contain the whole virus genome inserted into the host genome.

BR3-T was maintained in a manner similar to that in the natural environment, having been transmitted plant to plant by leafhoppers for more than 10 years, and it retains transmissibility and pathogenicity (Wayadande et al. 1993).

BR5-3X was subcultured in LD8 broth only five times. We assume these two lines behave similarly to spiroplasmas in natural environments. Line BR3-G has lost its insect transmissibility but is still phytopathogenic after continuous graft transmission for 10 years. Line BR3-P has lost either its transmissibility or its pathogenicity after subculture in LD8 medium more than 130 times (Wayadande et al. 1993). The differences in genomic hybridization patterns of these three lines when probed with SVBR3 DNA could be explained by the excision of virus DNA (3.1 kb band) and its insertion into a new site (7.1 kb band) in lines BR3-G and BR3-P, a change which might have been retained due lack of selection pressure. The change in viral DNA integration sites in BR3-G and BR3-P may have disrupted a gene or genes necessary for insect transmission (Figure 4).

Considering the BR3-G, -P and -T lines, the presence of 3.1 kb and 25.4 kb bands correlates with transmissibility, these bands being absent in BR3-G and -P lines. Similarly, the presence of the 7.1 kb band correlates with non-transmission. That the parental BR3-3X containes all 3 bands (3.1, 7.1 and 25.4 kb) suggests that BR3-3X may be a mixture of transmissible and non-transmissible _S_. _citri_.

If selection for insect transmissibility can be shown to lead to selection for the 3.1 kb and 25.4 kb bands and against the 7.1 kb band, genes essential for transmission may be present in the 3.1 kb and 25.4 kb fragments. If the

non-transmissible lines are derived from the transmissible one, a possible mechanism could be that shown in Figure 5. In BR3 lines, viral integration may lead to host gene rearrangement. The genes for insect transmission may be located between EcoRI fragments c and i (3.1 and 25.4 kb) in BR3-3X or BR3-T. After viral DNA integrated into the c and the i bands of the host genome, identical integrated viral DNA fragments may recombine with each other, causing loss of fragments necessary for insect transmission. This could explain the lack of bands c and i in BR3-G and BR3-P.

Wayadande et al. (1993) suggested that *S. citri* lines BR3-G and BR3-P may be unable to traverse physical barriers in the leafhopper vector to reach the salivary ducts in *C. tenellus*. Perhaps this process requires a specific spiroplasma protein, which may be involved in recognition or invasion of the salivary gland cell membranes or basal lamina. Viral DNA integration in the spiroplasma genome may interfere with transmission protein gene expression and result in loss of insect transmissibility in BR3-G and BR3-P. Such viral DNA integration may be useful in isolating the insect transmission-associated gene.

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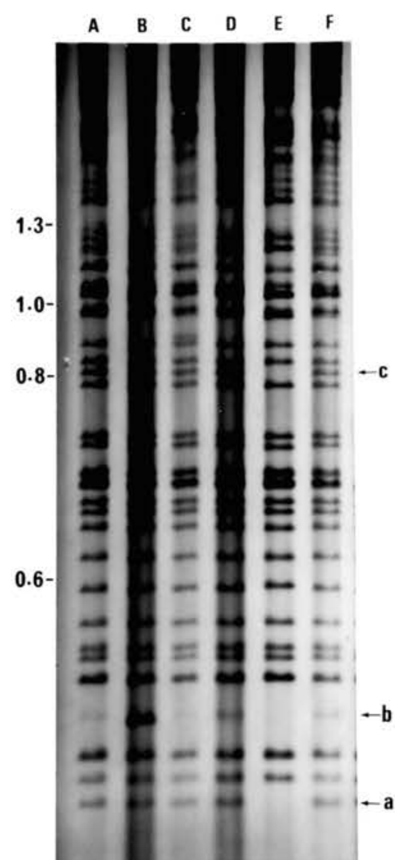


Figure 1. Genomic DNA fingerprint of S. citri. A, BR6-3X; B, BR5-3X; C, BR3-P; D, BR3-T; E, BR3-G; F, BR3-3X.

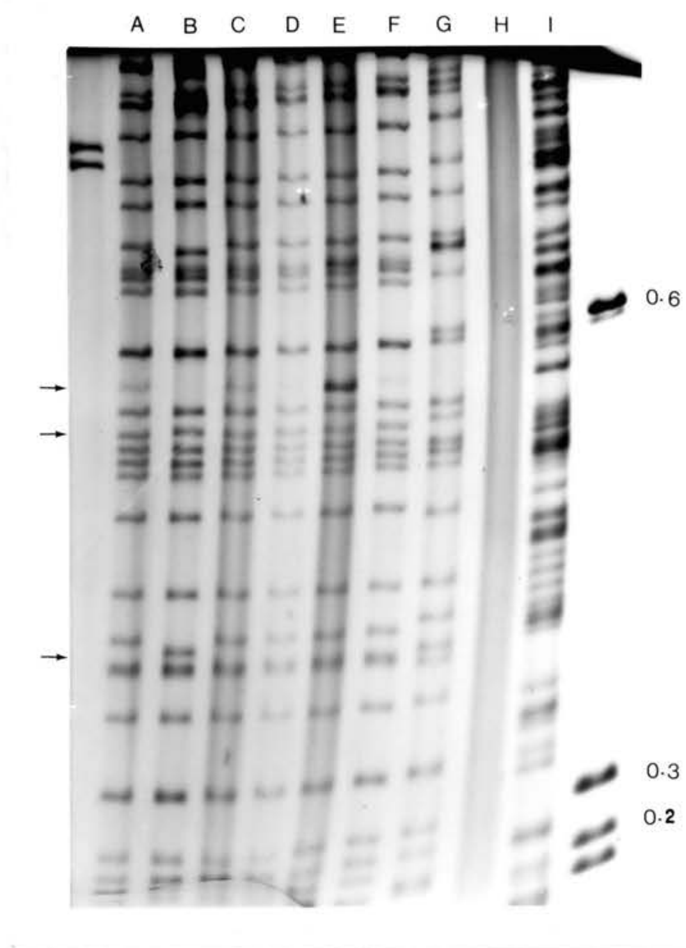


Figure 2. Genomic DNA fingerprint of S. citri, digested with HindIII. A, BR3-3X; B, BR3-G; C, BR3-T; D, BR3-P; E, BR5-3X; F, BR6-3X; G, R8A2; H, S. melliferum TS2; I, S. floricola 23-6.

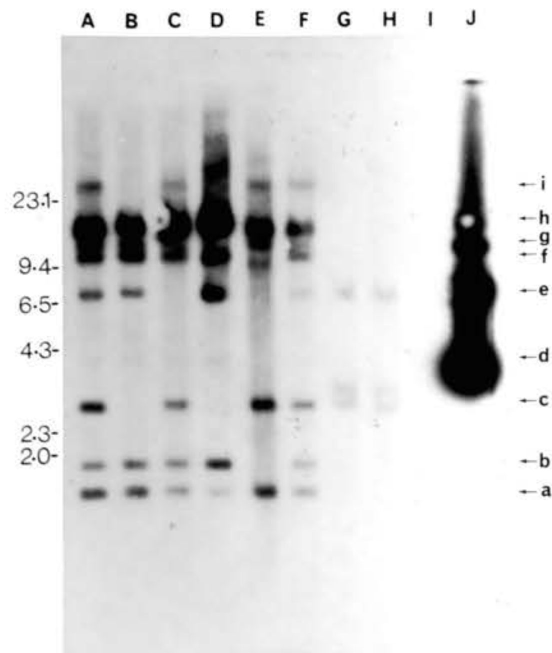


Figure 3. Southern blot of genomic DNA of spiroplasma strains probed with spiroplasma virus SVBR3 DNA. A-F, S. citri lines. A, BR3-3X; B, BR3-G; C, BR3-T; D, BR3-P; E, BR5-3X; F, BR6-3X; G-H, S. melliferum. G, AS576; H, TS2; I, S. floricola 23-6; J, SVBR3 DNA (clone A7).

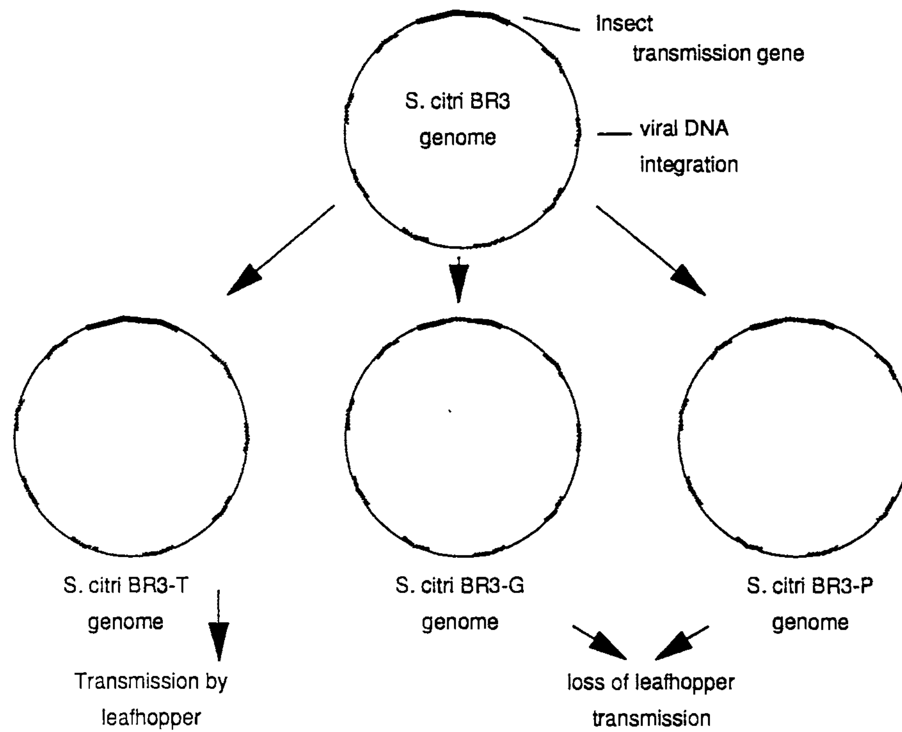


Figure 4. A model of disruption of genes involved with insect transmission by viral integration in the genome of spiroplasma.

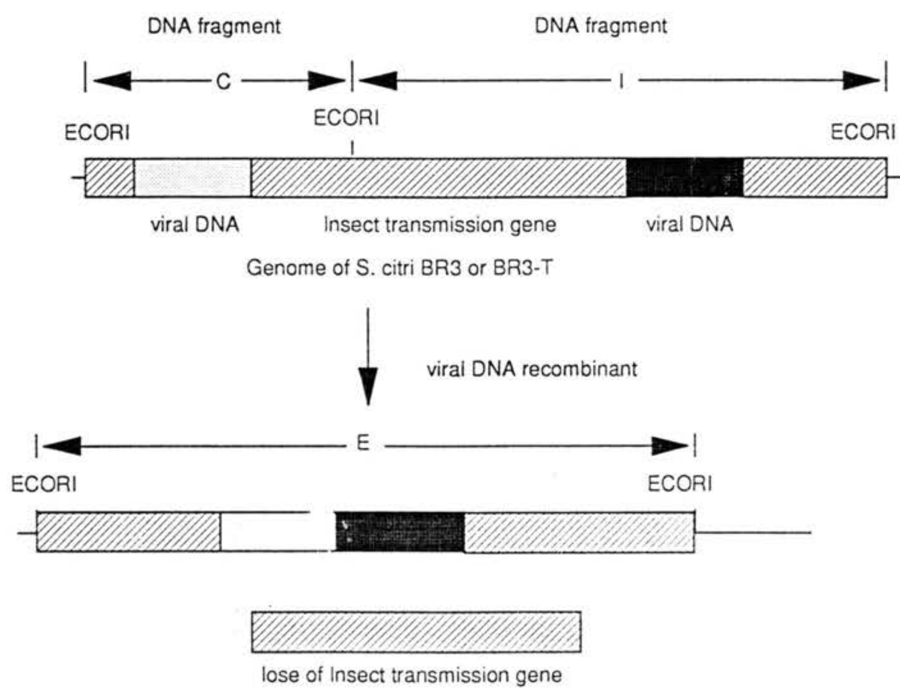


Figure 5. A model of spiroplasma DNA rearrangement by integrated viral DNA recombinant.

VITA

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Doctor of Philosophy

Thesis: MOLECULAR CHARACTERIZATION OF SPIROPLASMA VIRUSES AND THE MECHANISM OF RESISTANCE OF SPIROPLASMA CITRI LINES TO INFECTION BY THE VIRUS SVTS2

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