MOLECULAR TYPING OF SPIROPLASMA SPECIES AND LINES USING REP-POLYMERASE CHAIN REACTION AND TRANSPOSOME MUTAGENESIS AND SELECTION OF NATURAL NON-ADHERENT MUTANTS OF *SPIROPLASMA CITRI*

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CHAPTER I

INTRODUCTION

Spiroplasmas constitute a group of wall-less prokaryotes with helical morphology. The genus *Spiroplasma*, in the class Mollicutes, is distinguished from other genera by morphology, motility, and absence of urease activity. Spiroplasmas have very small genomes ranging from about 780 to 2,200 Kb and a low guanine-cytosine (G+C) content of about 24 to 31% of their genome (Subcommitee on the Taxonomy of Mollicutes, 1995).

Spiroplasmas are harbored in various habitats, and are particularly important as pathogens of plants, as parasites of insects and ticks, and as saprophytes associated with the nectaries and surfaces of flowers (Markham & Townsend, 1981; Williamson et al., 1989). Most spiroplasmas show association with arthropods (Whitcomb, 1981), with which they often share a symbiotic or at least mutually non-detrimental co-existence reflecting a long period of evolution in association with each other (Fletcher, 1999). Because of these special relationships with so many insects, spiroplasmas were thought to be one of the most abundant groups of microbes on the earth (Hackett & Clark, 1989).

Phytopathogenic spiroplasmas, including *Spiroplasma citri* and *Spiroplasma kunkleii*, are associated with several important plant diseases. The bacteria reside in the phloem sieve tubes of plants and are naturally transmitted

by phloem-feeding insects, mainly leafhoppers in the order Homoptera (Kaloostian et al., 1978).

Spiroplasmas differ in biological properties, such as host range, transmissibility and vector specificity (Tully, 1995). Such differences may occur even within the same species. For example, several lines derived from *S. citri* BR3-3X, an insect transmissible isolate from horseradish, have altered biological and molecular characteristics after undergoing different maintenance regimes. These alternations may be due to chromosomal rearrangements (Ye et al., 1996). One of the derived lines (BR3-G), which became non-transmissible after long-term maintenance by graft transmission, also showed different molecular features from the parental strain. Extensive chromosomal rearrangement, including chromosomal inversion and deletion, occurred during the derivation of the non-transmissible line from the transmissible parent (Fletcher et al., 1998).

Rep-PCR-Based Characterization of Bacteria

Repetitive extra palindromic-polymerase chain reaction (rep-PCR) analysis, based on the occurrence and distribution of repetitive elements within the genome, has been widely used to assess genetic diversity in bacteria (Louws et al., 1999). Rep-PCR employs oligonucleotide primers designed to be complementary to interspersed repetitive elements within bacterial genomes. DNA regions intervening between two adjacent repetitive elements are amplified to provide multiple, different sized DNA sequences (Versalovic et al., 1991; Versalovic et al., 1998). If spiroplasmas have repetitive elements, their

presence or profiles might differ among species/lines or might correlate with their biological properties. However, because mollicutes have undergone significant genome size reductions, they were expected to lack non-essential sequences such as repetitive elements. Therefore, we did not know whether such repetitive elements occur in spiroplasmas, or whether the technique would be reliable for their molecular characterization.

In Search of Non-Adherent Spiroplasma

Various factors may contribute to the transmissibility of spiroplasmas by insect vectors, and different spiroplasma lines are transmitted with different efficiency. Specific cellular and molecular interactions, such as binding events and the nature and prevalence of adhesion proteins, may be involved during spiroplasma colonization of, movement within, the vector (Fletcher et al., 1998). The exact nature of these mechanisms, and the specific genes involved, are still unknown. One approach to characterize those factors involved in transmission is to investigate spiroplasmas that have lost the ability to bind to insect cells. Adherence-deficient spiroplasmas may be obtained by creating spiroplasmas whose genes encoding adhesion-related elements are knocked out, or by selection of natural non-binding mutants.

Interactions between a phytopathogenic spiroplasma and its insect vector that contribute to transmission putatively involve a specific adhesion protein (Yu et al., 2000). DNA transposable elements, present in eukaryotic and prokaryotic organisms, can move from one location to another in the genome. One type of

transposable element is transposon, which contains genes for its insertion into the genome and its mobilization to other locations in the genome. The transposon is characterized by the presence of two inverted repeats flanking a DNA sequence encoding an enzyme generically called transposase, which in most instances is the only requirement for transposition. The transposase catalyzes the transposition by mediating the processing of DNA at the donor and target sites (Labrador & Corces, 1997). Insertion of the transposon into a genome results in a mutant with changed or abolished gene function.

Although the utilization of transposons gives many advantages in the genetic analysis of an organism, its application for genetic study of mollicutes is challenging due to atypical properties of this taxon. Two properties characteristics of mollicutes are a relatively small genome size and an unusual base composition. Mollicutes possess a circular double-stranded DNA chromosome of about 600 to1800 kbp (Dybvig & Voelker, 1996). In addition, spiroplasmas and mycoplasmas have unusual nucleic acid codon usage. Whereas the triplet UGA is a universal stop signal in most organisms, mollicutes utilize the codon for encoding tryptophan (Dybvig, 1990). Such a difference in the genetic code has phylogenetic and practical implications. The fact that this outstanding property is shared by mollicutes and mitochondria is of great phylogenetic interest. From an experimental point of view, this property imposes a serious restriction on the expression of cloned mollicute genes in *Escherichia coli.* Since *E. coli* regards UGA as stop codon, translation of a mollicute message in *E. coli* may stop prematurely, resulting in a truncated mollicute

protein. Accordingly, to study protein function, cloned mollicute genes must often be expressed in UGA suppressor strains (Kannan & Baseman, 2000; Razin, 1992).

Transposon Mutagenesis of Spiroplasma

Transposon-mediated mutagenesis has been used as a valuable tool for genetic study since it provides random insertion and the generation of a number of mutants, allowing the construction of a mutant library and the screening for gene function (Goryshin et al., 2000). However, mollicutes, including spiroplasmas, remain recalcitrant to genetic manipulation. Systems for transposon mutagenesis that are suitable for genetic analysis of mollicutes are rare. Although the introduction of exogenous DNA into mollicute cells is not hampered by a cell wall, DNA transfer efficiency is usually rather low. The efficiency of mollicute transformation can be increased either in the presence of polyethylene glycol (PEG) or by using electroporation (Razin et al., 1998). The paucity of suitable selectable markers has also hampered genetic studies with mollicutes. Two selectable markers used most often in studies on gene transfer in mollicutes are *tet*M, whose tetracycline resistance determinant is found on Tn*916*, and the gentamicin-resistance determinant of Tn*4001*. Because mollicutes in general are sensitive to tetracycline, while not all mollicutes are sensitive to gentamicin, the *tet*M gene is most commonly used (Dybvig & Voelker, 1996).

DNA transposition studies usually involve strictly *in vivo* approaches, in which the transposon of choice and the transposase encoding gene are introduced into the cell to be studied. However, there are technical limitations in all *in vivo* systems. For instance, the transposase must be expressed in the target host, the transposon must be introduced into the host on a suicide vector, and the transposase usually is expressed in subsequent generations, resulting in potential genetic instability. To overcome the limitations of *in vivo* systems, Goryshin et al. (2000) developed a technique for transposition that involves the formation *in vitro* of released Tn*5* transposition complexes (Transposome^{TM}) followed by introduction of the complexes into the target cell by electroporation. This technique has been used successfully to generate high efficiency transposition in some bacterial species including *E. coli, Salmonella typhumurium, Proteus vulgaris*, and a phytopathogenic bacterium, *Xylella fastidiosa* (Guilhabert et al., 2001).

In this research, the transposome was used to generate random mutations in the insect transmissible *S. citri* BR-3-3X. The random mutagenesis may result in transposition anywhere on the spiroplasma genome, providing a large variety of mutants. The affected genes may be identified by the presence of the inserted transposon. The goal of this experiment was to generate mutants in which a transposon interrupted a gene encoding an adhesion protein or a gene involved in the spiroplasma-insect interaction. Those mutants were characterized with respect to their ability to bind to insect cells and to be transmitted.

Spiroplasma Adherence to Insect Vector Cells

The first physical interaction of *S. citri* with cells of the leafhopper vector is likely to be adhesion. It is hypothesized that a cell surface adhesin protein of the spiroplasma interacts specifically with vector cell membrane receptors. A cell line (CT-1), developed from the embryonic cells of the *S. citri* insect vector, *Circulifer tenellus,* is a reliable tool for assay of such spiroplasma-vector interactions (Yu et al., 2000). Using a cell line is more convenient than using living vectors, although it limits experimental conclusions due to the dissimilarity between cultured and *in vivo* insect cells (Wayadande & Fletcher, 1998). *S. citri* binds to CT-1 cells.

Research Objectives

Biological variation within a bacterial species is common. A population of wild type spiroplasmas in culture may contain not only individual cells that have ability to adhere but also natural non-adhering ones. We sought to select natural non-adherent *S. citri* mutants from a wild type population, by serial passages on CT-1 cell monolayers. Adherent spiroplasmas should bind to the CT-1 cells, while non-adherent ones would remain suspended in the aqueous medium. The unbound spiroplasmas could then be isolated simply by collecting the aqueous medium or supernatant after low speed centrifugation. The unbound spiroplasmas, putative natural variants lacking adherence to the insect vector cells, would be useful for understanding factors important factors in vector transmission.

The objectives of this study were: (1) to utilize the rep-PCR technique for molecular typing among spiroplasma species/lines (2) to evaluate electroporation-mediated random transposon mutagenesis of *Spiroplasma citri* BR3-3X using transposome system, and (3) to select and characterize natural spiroplasma variants, lacking the ability to adhere to tissue cultured *Circulifer tenellus* (CT-1) cells.

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CHAPTER II

LITERATURE REVIEW

Spiroplasma

Spiroplasma, a genus belonging to the family *Spiroroplasmataceae*, consists of mollicute species isolated from arthropods, animals, plant surfaces, or plant phloem, which have the following characteristics: helical cell morphology; not obligately anaerobic; require sterol (Subcommitee on the Taxonomy of Mollicutes, 1995); and have a small genome size of about 940 to 2220 kbp (Carle et al., 1995). Due to their biological uniqueness and ecological importance, spiroplasmas have challenged the interest of scientists to investigate many of their features, from the ecological to molecular levels.

Spiroplasmas were initially confused with both spirochetes and viruses since they share a number of basic similarities, such as helical form, filterability, and lack of a cell wall, with these other microbes (Tully & Whitcomb, 1992). Spiroplasmas were recognized as a unique group and placed in a separate genus, *Spiroplasma*, about three decades ago. Their discovery was preceded by a breakthrough by Japanese scientists, who demonstrated the presence of small, pleomorphic, wall-less microorganisms in the phloem cells of mulberry plants afflicted with a dwarfing disease (Markham & Townsend, 1981). Several other plant diseases previously considered to be caused by viruses were found associated with small, wall-less bacteria called mycoplasma-like organisms

(MLO, now known as phytoplasmas). Disease remission was correlated with tetracycline treatment of infected plants (Doi et al., 1967, Ishiie et al., 1967, Nasu et al., 1967).

The first spiroplasma observed microscopically was the helical sex-ratio organism (SRO), which eliminates male progeny from certain species of *Drosophila* (Poulson & Sakaguchi, 1961). That organism was suggested to be a spirochete until Williamson & Whitcomb (1974) showed ultrastructural features that eliminated that possibility by revealing the absence of periplasmic fibrils.

Observation using phase-contrast microscopy of sap from corn plants affected by the lethal corn stunting disease showed numerous tiny helical, motile cells. These mycoplasma-like structures did not appear to be pleomorphic but were consistently helical in form, so that the microrganism acquired the trivial name “spiroplasma” (Davis et al., 1972). After characterization of the cultivated mollicute causing corn stunt, that agent was designated *Spiroplasma kunkelii* (Whitcomb et al., 1986). The microbe *Spiroplasma citri,* discovered by two independent groups in France (Saglio et al., 1971) and the US (Fudl-Allah et al., 1971), was a subsequent addition to the diversity of prokaryotic organisms. Both research groups observed identical microorganisms from orange trees with citrus stubborn disease. These microorganisms showed many of the properties of mycoplasmas, including the formation of typical “fried egg” colonies on solid media, but they produced motile helical cells in liquid culture. This microorganism was later designated *S. citri* (Saglio et al., 1973). In addition, an organism isolated by Pickens et al. (1968) from rabbit ticks was initially thought to

be a spirochete, but eventually was identified by Brinton & Burgdorfer (1976) as a spiroplasma based on its ultrastructural features. Another organism isolated from rabbit ticks, called the suckling mouse cataract agent (SMCA), was previously considered to be a virus (Clark, 1964). Later, the organism was cultured on artificial medium, identified as spiroplasma (Tully et al., 1976), and designated *S. mirum* (Tully et al., 1982). Other spiroplasmas were identified over the next two decades, many from various taxa of arthropods, resulting in over 32 species known today (Williamson et al., 1998).

Taxonomy, Cultivation and Morphology of Spiroplasmas

The cell wall is considered a fundamental property of bacteria. The absence of a cell wall or of chemical precursors of cell walls was the initial justification for placing known mollicutes into a taxonomic group, the class *Mollicutes,* separated from other prokaryotes. Other critical features include the presence of a plasma membrane, pleomorphic cellular morphology, filterability through membrane filters of 450 nm pore diameter, lack of reversion to walled prokaryotes, and a small genome (500 to 1000 MDa). Certain unusual nutritional needs (for example, cholesterol and urea) and growth characteristics distinguished some mollicute families and genera (Dybvig, 1990; Dybvig & Voelker, 1996; Woese et al., 1985).

The class Mollicutes is grouped into four orders and consists of five families. Until recently there were seven recognized genera in the class Mollicutes, i.e. *Mycoplasma* and *Ureaplasma* (order Mycoplasmatales: family

Mycoplasmataceae); *Entomoplasma* and *Mesoplasma* (Entomoplasmatales: Entomoplasmataceae); *Spiroplasma* (Entomoplasmatales: Spiroplasmataceae); *Acholeplasma* (Acholeplasmatales:Acholeplasmataceae); and *Anaeroplasma* and *Asteroplasma* (Anaeroplasmatales:Anaeroplasmataceae). In addition, phytoplasmas (formerly known as mycoplasma-like organisms or MLOs), whose taxonomic status is undefined, also are included in the class Mollicutes Criteria used for the classification include serological relatedness, polyacrylamide gel electrophoretic (PAGE) pattern of proteins, guanine + cytosine (G+C) DNA base content, and DNA-DNA homology (Subcommitee on the Taxonomy of Mollicutes, 1995).

Designation of groups within the genus *Spiroplasma* has been largely based on serological evidence and the groups have been termed “serogroups” (Gasparich et al., 1993). A recent tally included 34 serogroups; for example *S. citri*, *S. melliferum, S. kunkelii, S. insolitum* and *S. phoeniceum* are members of serogroup I, whereas *S. floricola*, *S. apis* and *S. mirum* are members of serogroup III, IV and V, respectively (Williamson et al., 1998).

Unlike the related phytoplasmas, spiroplasmas have been successfully cultivated in artificial media. Spiroplasmas require complex nutrition for growth, including an absolute presence of a non-toxic sterol, which is normally supplied in axenic culture as horse serum or fetal bovine serum. The sterol is required for membrane synthesis and is thought to play an essential role in maintaining cell membrane permeability (Markham & Townsend, 1981). Various media have been formulated for isolating and maintaining spiroplasmas, based on either

media formulations for non-helical mollicutes or modified, specific and complex media (Tully & Whitcomb, 1992). Cultivation of spiroplasmas can also be accomplished using insect cell culture (Hackett & Lynn, 1995).

Spiroplasmas generate energy from fermentation of carbohydrates, which are also important for regulation of osmolarity. Amino acids are added to the medium to serve as protein building blocks or as nutrients (Whitcomb, 1981). In some circumstances, spiroplasmas degrade arginine to obtain ATP by the arginine dihydrolase pathway. Particular supplements may also be needed. For instance, corn stunt spiroplasma requires an exogenous supply of α-ketoglutaric acid (Markham & Townsend, 1981).

Some physical conditions are critical for optimum cultivation of spiroplasmas, such as high osmotic pressure, pH of the growth medium equal to that of the sieve tube sap, and a specific optimum temperature (Saglio et. al., 1973). For example, *S. citri*, which is osmotically fragile, requires an osmotic pressure of approximately 15 atm and its temperature optimum is usually 32 °C, conditions in which it achieves a titer of about 10^8 to 10^{10} cells/ml (Markham & Townsend, 1981).

The mollicute cell is surrounded by a cytoplasmic membrane, which in most species contains cholesterol as an indispensable component. Morphologically, mollicutes have two distinct cell types, i.e. non-helical and helical. The cell shapes vary from spherical or pear shaped structures (0.3 to 0.8 μm in diameter) to branched or helical filaments (as small as 0.2 μm in diameter). Some mollicute species are pleomorphic. The cell shape in many instances

appears to depend on both nutritional qualities and the osmotic pressure of the medium, as well as the age of the culture. Spiroplasmas are shaped as thin helical filaments under most circumstances in liquid medium, exhibiting rotatory motility with flexional and twitching movements (Razin, 1992).

Since a typical bacterial cell wall, characterized by peptidoglycan content, is absent in mollicutes, these microbes are Gram negative. However, from extensive molecular genetic data that reveal phylogenetic relationships, the mollicutes are considered to have evolved from Gram-positive bacteria, e.g. members of the Clostridia (Fox et al., 1980, Woese et al., 1980). The total lack of a cell wall explains many of the unique properties of the mollicutes, such as sensitivity to osmotic pressure and detergents, resistance to penicillin and other antibiotics affecting cell wall formation, and the production of peculiar fried-egg shaped colonies (Nienhaus & Sikora, 1979; Razin, 1992). Thin sections of mollicutes reveal that the cells are built essentially of three organelles: the cell membrane, ribosomes, and the characteristic prokaryotic genome. In this respect, the mollicutes are the microbes closest to the concept of “minimal self-replicating cells” proposed by Morowitz (Razin, 1992).

In some solid media, spiroplasmas form fried egg-like colonies similar to those of mycoplasmas. Due to their motility and plasticity, spiroplasmas are able to migrate and penetrate into the agar matrix, so that the colony appearance is a dense central core surrounded by a diffuse peripheral zone consisting of satellite colonies. The motility, colony shape and size are determined by factors such as temperature, nutrient status, pH and particularly by agar density. The ability of

an individual spiroplasma to form a colony is used as a basis for their enumeration, expressed in colony forming units (CFU)/ml (Markham & Townsend, 1981).

The colony appearance is strongly dependent on the composition of the medium. Although most mollicutes can grow beneath the surface of solid medium, this either does not occur or is difficult to demonstrate on some media with certain strains. In particular, the motility of spiroplasmas may prevent typical colony formation on conventional “soft” agar (containing 0.8 to 1% purified agar). Spiroplasmas usually form diffuse colonies, with a number of satellite colonies nearby, under such circumstance. However, the use of so-called “hard agar” (containing 2.0 to 2.5% purified agar) may permit demonstration of fried-egg spiroplasma colonies, particularly if the medium is suboptimal (Subcommitee on the Taxonomy of Mollicutes, 1995).

In sap of infected plants and during some phases of growth in culture, spiroplasmas produce typical spiral forms (helices), which distinguish them from all other mollicutes. Helical forms usually arise in liquid media but not in semi-solid agar culture (Maramorosch, 1981). Spiroplasma cells may lose helicity and motility in liquid media when they are adjusted to high osmolarity and viscosity, but these properties rapidly reappear when the cells are reintroduced into suitable medium, such as C-3G (Whitcomb, 1981). However, *S. citri* strain ASP-1 cells, isolated from diseased sweet orange trees, do not form helices, even in liquid media. This non-helical spiroplasma does not exhibit any of the distinguishable types of motility observed with normal spiroplasma strains.

Surprisingly, the strain could be transmitted to beans by leafhoppers injected with cultured organisms, and induce similar symptoms to those produced with the wild type (Townsend et al., 1977). This non helical strain was demonstrated, by polyacrylamide gel electrophoretic (PAGE) analysis, to lack a single protein of 39 kDa in its membrane. It was hypothesized that the missing protein corresponds to part of the mechanism responsible for connecting fibrils to the cell membrane required to form helicity (Markham & Townsend, 1981). In some cases, spiroplasma cells may lose their helical morphology (Hackett & Clark, 1989), becoming pleomorphic, after they invade insect tissues (Kwon et al., 1999).

In early log phase culture, dimensions of spiroplasma cells range from 0.15 to 0.50 μm in diameter by 2 to 15 μm in length, with a uniform wave and amplitude to the helical form (Davis & Worley, 1973). However, a series of morphological changes occurs in liquid culture. In late log phase, longer helical filaments to about 15 μm in length become prevalent and round bodies are often seen. In late and stationary phases, cells tend to elongate, lose helicity and form blebs and rounded or irregular bodies (Cole et al., 1973). Some spiroplasmas form aggregates ranging from “micelle”-like clusters to “medusa-like masses of intertwined helices” (Markham & Townsend, 1981). Spiroplasmas have no cell wall, but are bounded by a tri-laminar plasma membrane that separates the cytoplasm from its surroundings (Markham & Townsend, 1981). Recent study using negative staining transmission electron microscopy (TEM) shows that spiroplasma cells possess pilus-like structures on the outer surface of cell membrane (Ammar et al., 2004). It is not clear whether those structures

observed are bacterial pili or part of filamentous viruses infecting spiroplasmas. Ozbek et al. (2003) also reported the presence on *S. kunkelii* of those peritrichous structures of ~8 nm diameter, interpreted to be fimbriae protruding from the cell surface, and pilus-like appendages at one or both cells poles.

Spiroplasmas are motile bacteria despite lacking flagella, structures that commonly facilitate motility of prokaryotes. Spiroplasmas have a membrane-bound internal cytoskeleton, composed primarily of a unique 59 kDa protein, which affects the cell shape and is thought to act as a linear motor, in contrast to the rotary motor of flagella (Trachtenberg, 1998). The cytoskeleton exists as a seven fibril ribbon that extends the length of the cell and is attached to the cytoplasmic membrane. The cytoskeleton is involved in motility due to its linear contraction and its close interaction with the cytoplasmic membrane (Trachtenberg & Gilad, 2001).

Host Plant-Spiroplasma-Vector Relationships

Spiroplasmas are harbored in various habitats, including many terrestrial arthropods (Williamson et al., 1989), in which they are postulated to have evolved as a lineage adapted to exploit various habitats in the gut, hemocoel, and cells. Based on the natural history of spiroplasmas and the definition of habitat as the place where an organism normally lives and grows, the habitats of phytopathogenic spiroplasmas are mainly: (1) the phloem sieve tubes of plants, (2) the hemolymph and certain tissues and organs (ovaries, fat bodies, and salivary glands) of insects, and (3) the gut lumen of insects. In addition, non-

phytopathogenic spiroplasmas have been isolated from other natural sites, such as plant (flower and leaf) surfaces and soil surrounding ground-inhabiting immature insects (Hackett & Clark, 1989).

Phytopathogenic mollicutes, spiroplasmas and phytoplasmas, cause severe diseases of various important crops resulting in significant economic losses (Fletcher, 1999). Spiroplasmas affect host plants by interfering with the hormonal balances that control plant development and growth. The disease symptoms include shortening of internodes, stunting, breaking dormancy, and production of axillary shoots and adventitious roots (Maramorosch, 1981). *S. citri* also produces toxins that may be responsible for producing symptoms in infected plants. A toxin extracted from spiroplasma cultures, which broke down plant cells and protoplasts and inhibited seed germination, was a low molecular weight, polar, uncharged substance, possibly a polyhydroxy compound (Daniels et al., 1978).

Spiroplasmas are insect-transmitted pathogens associated with several important plant diseases. Three phytopathogenic spiroplasmas known today are *S. citri*, *S. kunkelii* and *S. phoeniceum*. *S. citri* is a causal agent of stubborn disease of citrus, and can be naturally transmitted by the leafhoppers *Circulifer tenellus* Baker*, Scaphytopius nitrides* Delong*, S. acutus delongi* (Kaloostian et al., 1978), and *Neoaliturus haematoceps* Mulsant & Rey (Fos et al., 1986) (now known as *Circulifer haematoceps* Mulsant & Rey), whereas *Macrosteles fascifrons* Stal can serve as an experimental vector (O'Hayer et al., 1984). The symptoms in citrus, which are usually expressed at high temperatures, are

chlorosis, stunting, bushy growth due to shortened internodes and axillary proliferation, and fruit malformation (Gumpf & Calavan, 1981). The same pathogen also causes a disease known as brittle root in horseradish, and at least six other brassicaceous species are susceptible (Fletcher et al., 1981, Raju et al., 1981). Other hosts include *Catharanthus roseus* (L.) G. Don*, Callistephus chinensis* (L.) Nees*, Phlox drummondi* Hook, *Vicia faba* L.*, Pisum sativum* L.*,* and *Trifolium repens* L. (Davis, 1978; Davis, 1979). *S. kunkelii,* the pathogen of corn stunt disease, is transmitted by the leafhoppers *Dalbulus maidis* Delong & Wolcott*, D. elimatus* Ball and *Graminella nigrifons* Forbes(Nault & Bradfute, 1979). *S. kunkelii* also infects teosinte (*Zea mays mexicana*) (Davis, 1979), but compared to *S. citri* its host range is much more focused. *S. phoeniceum*, which was discovered in Syria, is the causative agent of periwinkle yellows (Garnier et al., 2001).

The spread of phytopathogenic mollicutes from plant to plant in the field is facilitated by insect vectors. Distribution and spread of the diseases are correlated with the feeding habits and biology of the insect vectors as well as with the presence of alternative plant reservoirs (Garnier et al., 1999). To date, effective methods to control plant pathogenic mollicutes, whether directed at the control of the pathogen or its insect vector, or by providing resistant cultivars, are very limited (Fletcher, 1999).

Insect transmission of phytopathogenic spiroplasmas, as well as phytoplasmas, is circulative-propagative; that is, mollicutes ingested by the insect from phloem move from the insect gut lumen and traverse the gut wall,

propagate in the hemolymph, and move into the salivary glands from which they are discharged into a new plant during insect feeding activity (Fletcher et al. 1998; Melcher & Fletcher, 1999).

Spiroplasmal Adherence and Transmission

One of the earliest events in many bacterial infections is the molecular interaction that occurs between the pathogen and host cells. These interactions are required for extracellular colonization or internalization to occur. Colonization of host tissues is usually mediated by adhesins on the surface of the bacteria, which are responsible for recognizing and binding to specific receptor moieties of host cells (Soto & Hultgren, 1999). The definition of adhesion or adherence is the measurable union between a bacterium and a substratum, in which energy is required to separate the two. Two main mediators of the process of adhesion are adhesins and receptors. An adhesin is a bacterial surface molecule capable of binding to a receptor on a substratum, whereas the receptor is a substratum molecule that is complementary to an adhesin (Ofek & Doyle, 1994).

In some conditions, adhesion may not be beneficial for bacteria. For example, attachment of bacteria to phagocytes may lead to bacterial death due to the lack of nutrients at the site of adhesion and to phagocytosis. Subsequent detachment would permit migration toward a more favorable environment. Evolutionary pressures may have selected for populations of organisms containing both adherent and non-adherent phenotypes whose emergence may be due to complex genetic regulation of the expression of the adhesions or to

naturally occurring inhibitors or promoters of adhesion. Under natural conditions, the ability of the bacteria to adhere or not to adhere to a substratum is regulated by the environment and/or the bacterial genome (Ofek & Doyle, 1994).

Adherence of mollicutes to host cells is well studied in human and animal pathogenic mycoplasmas. Adherence is one of the main virulence factors in mycoplasmas and is essential in the initial stages of infection (Rosengarten et al., 2000) when the mollicutes fuse with epithelial cells (Razin and Jacobs, 1992; Roedinger & Macfarlane, 2002).

As self-replicating organisms, mollicutes carry a minimal set of genes involved in energy metabolism and biosynthesis. The microbes still have the essential genes for DNA replication, transcription, and translation, and the minimal number of rRNA and tRNA genes. The reduction in mycoplasmal genomes explains their need for host nutritional molecules. A significant number of mycoplasmal genes appear to be devoted to cell adhesion and attachment organelles as well as variable membrane surface antigens to maintain parasitism and evade host immune and non-immune surveillance systems (Razin et al., 1998).

Some pathogenic mycoplasmas use a specialized polar structure formed as a membrane-bound extension of the mycoplasma cell, called an attachment organelle or tip, for adherence to the host epithelium (Razin and Jacobs, 1992). This terminal structure is required for the polar localization of adhesins. The cytoskeleton is involved in the organization of the adhesions and the terminal tip structure of the other flask-shaped pathogenic species. The tip structure is

stabilized by internal proteins forming a filamentous cytoskeleton-like structure (triton shell) (Kahane & Jacobs, 1995; Layh-Schmitt & Harkenthal, 1999). Helical cell of spiroplasma showed one tapered end (tip structure) and one blunt or round end. The tip structure exhibit an electron-dense conical or rod shaped core and thought to be involved in orientation and attachment of the helices to their host cells (Ammar et al., 2004).

Transmission of a spiroplasma by a leafhopper insect vector from one host plant to another involves a process in which the pathogen is translocated through the insect body interior. After acquisition by the insect during feeding on a host plant, spiroplasma cells have to pass through several barriers. From the gut lumen, spiroplasmas traverse the insect gut epithelium to enter the hemocoel, where the pathogen multiplies in the hemolymph. The surviving spiroplasmas cross the cells of the salivary gland, from which the pathogens are introduced into next host plant phloem along with the saliva during leafhopper feeding (Fletcher et al., 1998).

Adherence preceding the invasion of spiroplasmas into the insect host cells is likely involved in transmission of the pathogen by its leafhopper vector, *C. tenellus*. A protein putatively responsible for binding, *S. citri* adhesion-related protein P89 (SARP1) was identified and characterized (Berg et al. 2001).

Genomic Features of Spiroplasmas

Mollicute genomes are not only very small, but their base composition is characterized by a low guanine plus cytosine (G + C) content (Razin, 1992).

Information on the G + C content of mollicute genomes is available for most species of mollicutes, and its determination is an obligatory test for the definition of new species (Subcommitee on the Taxonomy of Mollicutes, 1995) because it is an effective taxonomic measure. A difference in the G + C content greater than 1.5 to 2.0 mol% between the DNAs of two bacteria is considered sufficient to rule out their inclusion in the same species. The G + C data are very useful in spiroplasma classification (Bové et al., 1989), and the fact that the ratio in mollicutes is low supports the hypothesis that mollicutes evolved from low G + C Gram-positive bacteria (Woese et al., 1980).

A G+C value of 24 mol%, common to many mollicutes, corresponds to the minimum theoretical value enabling coding for proteins with a normal amino acid composition (Razin, 1992). In contrast, the adenine + thymine (A + T) content of mollicute genomes is high and the intergenic spacer regions can reach values as high as 80 mol% A + T. As a result, the mollicutes preferentially use codons rich in A and U in the third position (Razin et al*.,* 1998).

The high A+ T content of the genome of mollicutes could also be expected to increase their sensitivity to ultraviolet (UV) radiation, because of the greater likelihood of thymine dimerization. In fact, mollicutes are more sensitive to UV irradiation than other prokaryotes, when inactivation values are normalized per unit of DNA (Labarere & Barroso, 1984). The parasitic mode of life of mollicutes would be expected to offer some protection against radiation and in this way counterbalance the higher sensitivity of these organisms to UV irradiation (Razin, 1992).

The molecular and genome characteristics of mollicutes differ from those of other prokaryotes. Mollicute ribosomal RNAs have some unique features. The 5S rRNA component appears to be shorter than that of other prokaryotes, while the 16s RNA component contains distinct oligonucleotide sequences (Woese et al., 1980; Woese et al., 1985). Prokaryotic organisms may have several ribosomal RNA (rRNA) operons, for instance *E. coli* and *Bacillus subtilis* contain seven and ten sets of rRNA operons, respectively. In contrast, most mollicutes have only one or two operons, for instance, *Spiroplasma citri* has a single set (Amikam et al., 1984; Bové, 1989). The rRNA genes are considered to be the best-characterized mollicute genes. Sequences of these highly conserved genes, especially those of 16S rRNA, serve as the most important analytical tool for molecular diagnosis, taxonomy and phylogeny (Razin et al., 1998).

The mollicute chromosome is a genetically dynamic structure that undergoes frequent rearrangements, insertions, deletions, and inversions of genes or entire genomic segments (Melcher & Fletcher, 1999). The mollicute genome may carry repetitive elements of various types, such as insertion sequence, (IS)-like sequences, integrated viral or plasmid genomic segments, or repetitive elements of endogenous origin, consisting of segments of mycoplasmal genes. These are the elements which facilitate, through homologous recombination, chromosome rearrangements, as well as loss of genomic material by deletion of intervening sequences during recombination, a process presumably taking place during the reductive evolution of mollicutes (Melcher et al., 1999; Razin, 1998). SpV1-like viral DNA sequences may play a major role in

the genome variation of *S. citri*. The DNA of the SpV1 viruses SpV1-R8A2 B and SpV1-C74 are insertion elements of the IS3 and IS30 families, respectively. Both viral DNAs have genes putatively encoding transposases (Melcher et al., 1999).

Genomic Fingerprinting by PCR of Repetitive Elements

Prokaryotic and eukaryotic genomes may contain dispersed repetitive sequences separating longer, single copy DNA sequences. The interspersed repetitive DNA sequences are characterized as non coding, intercistronic, and relatively short (usually <500 bp) (Lupski & Weinstock, 1992). Prokaryotic moieties represent repetitive DNA that is not organized in tandem repeats but is dispersed evenly throughout the entire genome of microorganisms (Van Belkum et al., 1998). Short, interspersed, repetitive DNA sequences have also been isolated from *S. citri* (Nur et al., 1987).

Repetitive elements have a high degree of evolutionary conservation. It is thought that natural selection may constrain variation in these sequences because they represent sites of essential protein:DNA interaction. DNA replication proteins, *E. coli* DNA gyrase and polymerase I, specifically bind to repetitive sequences. These sequences may also propagate themselves as selfish DNA by gene conversion. Retroposons are thought to represent a significant source of genetic diversity in evolution by their multiplication and creation of retropseudogenes. Repetitive elements may be transcribed into RNA and dispersed through RNA intermediates. According to reverse transcriptase activity, which has been found in eubacteria, gene conversion through RNA

intermediates remains a possible mechanism accounting for the widespread, presence and conservation of these prokaryotic repetitive elements. The dispersion of repetitive elements in the genome is thought to be gene conversion, rather than random transposititon (Versalovic et al., 1991).

A family of repetitive elements identified in *E. coli* and *Salmonella typhimurium*, enterobacterial repetitive intergenic consensus (ERIC) sequences, also known as intergenic repeat units (IRUs), was defined using genomic sequence information. These ERIC elements, as large as 126 bp, contain a highly conserved central inverted repeat and are located in extragenic regions. ERIC-like sequences are found primarily in Gram-negative enteric bacteria and their close relatives in the same phylum (Versalovic et al., 1991; Versalovic et al., 1998). Another dispersed-repeat motif was identified in *Streptococcus pneumoniae*. This so-called BOX repeat is unrelated to the repeat types of ERIC. The BOX repeat forms stable secondary structures and is transcribed in some instances. Most of the BOX sequences were encountered in close proximity to genes, suggesting their potential role as a regulatory element controlling coordinate virulence- or competence-related gene expression (Van Belkum et al., 1998).

Because the repetitive DNA sequences are dispersed in bacterial genomes in different orientations and are separated by various distances, the sequences can be used as primer binding sites in the polymerase chain reaction (PCR). The rep-PCR would yield distinct patterns of varying sized DNA fragments among different bacterial species and strains (Van Belkum et al.,

1998; Versalovic et al., 1991). Direct rep-PCR amplification and agarose gel electrophoresis of PCR products provide genomic fingerprints of sufficient complexity to distinguish species and strains. Combined with rapid cell lysis methods rep-PCR techniques enable one to characterize different bacterial strains in several hours (Versalovic et al., 1991).

Most Gram-positive bacterial species, for example *Bacillus subtilis*, showed minimal ERIC-PCR amplification, which is consistent with previous computer search of ERIC in the DNA sequence databases and known phylogenetic distances between Gram-positive bacteria and Gram-negative enteric bacteria (Versalovic et al., 1991; Woese et al., 1980).

Transposon Mutagenesis of Spiroplasma

Transposable elements found in prokaryotes and eukaryotes are sequences of DNA having the ability to move from one site in a genome to another (Madigan et al., 1996). Transposable elements, including insertion sequences (IS), transposons and some special viruses, contribute significantly to genetic variation since their capacity to move can modify gene expression and promote genome rearrangements (Chandler, 1998). An insertional sequence is the simplest element, carrying only one type of genetic information other than that required for its own translocation to the new site, whereas a transposon is larger and more complex, and contains other genes (Madigan et al., 1996), such as those for antibiotic resistance (Chandler, 1998).

Transposon mutagenesis is generated by insertion of transposons into genes, causing their inactivation. The genes carrying the inserted transposons may be identified and mapped. The next step is to prove the predicted function of the inactivated gene by complementation with the same gene from the wild type (Razin et al., 1998).

Transposition has become a powerful tool in genetic analysis, with application in creating insertional knockout mutations, generating gene-operon fusions to reporter functions, providing physical or genetic landmarks for the cloning of adjacent DNAs, and locating primer binding sites for DNA sequence analysis (Goryshin et al., 2000).

Little is known about the presence of naturally occurring transposons in mollicutes. The microbes may obtain transposons from other bacterial sources (Mahairas & Minion, 1989). A streptococcal transposable element Tn*916,* containing a tetracycline resistance determinant and carried by the plasmid pAM120, was successfully inserted into the *Mycoplasma pulmonis* and *M. hyorhinis* chromosomes at numerous sites by polyethylene glycol (PEG)-mediated transformation (Dybvig & Alderette, 1988). Tn*4001,* a small (4.7 kb) transposable element of the composite class I, originally isolated from *Staphylococcus aureus*, encodes a bifunctional peptide specifying both kanamycin and gentamicin resistance in separate domains flanked by a 1.3 kb insertion element, IS*256* (Lyon et al., 1984; Lyon & Skurray, 1987). The transposon was capable of being randomly inserted into *M. pulmonis*

chromosome using an *E. coli*-derived vector and PEG-mediated transformation (Mahairas & Minion, 1989).

Transformation of *S. citri* GII3, a European strain capable of being transmitted by the Old World leafhopper *C. haematoceps* to periwinkle, with the Tn*4001* transposon yielded a number of mutants. One mutant had lost the ability to multiply, or multiplied poorly, in the leafhopper and became non-transmissible by the insect vector. Another mutant was still transmissible to the plant by the vector and reached a titer in the plant as high as the wild type strain, but took twice as much time to do so. This mutant also caused delayed symptoms in the host plant; symptoms began to develop only as the mutant lost the transposon (Foissac et al., 1997).

An approach using so-called transposomes to analyze gene mutations randomly with *in vivo* insertion of transposable elements into bacterial chromosome was recently developed. A transposome is a complex composed of hyperactive Tn*5* –based transposase and transposon DNA, with 19 bp inverted repeat mosaic ends. The bond-breaking process of transposon excision from the donor DNA and its insertion into the target DNA requires magnesium. In the absence of this element, the stable complexes (transposomes) are formed between the hyperactive transposase and the excised transposon DNA fragments. Transposition of the transposome will occur when it is introduced by electroporation into the cell environment and into the genome in the presence of magnesium and the target DNA. The use of the transposome also facilitated direct DNA sequencing of the insertion region in the bacterial genome, without

endonuclease restriction and cloning steps (Goryshin et al. 2000, Hoffman et al. 2000).

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CHAPTER III

MOLECULAR TYPING OF SPIROPLASMA SPECIES AND LINES USING REPETITIVE EXTRAGENIC PALINDROMIC-POLYMERASE CHAIN REACTION (REP-PCR)

Abstract

Repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) methods using BOXA1R and ERIC1R/ERIC2 primers were performed to analyze the presence of repetitive elements in spiroplasma and to differentiate among spiroplasma species and lines. Both rep-PCRs were able to generate DNA sequences from *Spiroplasma citri* lines/strains (BR3-3X, BR3-T, BR3-G, R8A2, and ASP-1), *S. melliferum* TS-2, *S. floricola* 23-6, *S. kunkelii* CR2-3X and *S. phoeniceum.* The DNA patterns were simple, with one to several bands, and distinguished spiroplasma species but did not discriminate at the line/strain level. However, because of the limited number of DNA bands and poor reproducibility among experiment replications, the rep-PCR was not sufficient for specific identification and relatedness determination among *Spiroplasma* strains/lines.

Introduction

The genus *Spiroplasma*, of the class Mollicutes, comprises wall-less prokaryote species that are typically distinguished from other mollicutes by having a helical cell morphology and motility (Tully & Whitcomb, 1992).

Spiroplasmas have a small genome size of about 940 to 2220 kbp and a low guanine-cytosine (G+C) content ranging from 24 to 31 mol%. These prokaryotes are genetically and phenotypically diverse, as manifested by 34 group species designations within this taxonomic group (Carle et al., 1995; Williamson et al., 1998). Spiroplasmas play important roles as pathogens or microflora in association with various hosts and habitats, including arthropods and plants (Tully & Whitcomb, 1992).

The mollicute chromosome is a genetically dynamic structure that undergoes frequent rearrangements, insertions, deletions, and inversions of genes or entire genomic segments, processes that have taken place during reductive evolution of mollicutes. The genome may carry repetitive elements of various types, such as insertion sequences, (IS)-like sequences, integrated viral or plasmid genomic segments, or repetitive elements of endogenous origin, consisting of segments of mycoplasmal genes (Melcher & Fletcher, 1999; Razin et al., 1998).

Several lines of *S. citri* derived through different maintenance regimes from an insect transmissible strain, BR3-3X, have altered biological characteristics, i.e. insect transmissibility. The derived line BR3-G, which became non-transmissible after maintenance by continuous graft transmission, also showed molecular features different from those of the parental strain. Extensive chromosomal rearrangement occurred between the transmissible and non transmissible lines, including chromosomal inversion and deletion (Fletcher et al., 1998; Ye et al., 1996).

For assessing the diversity of bacteria, methods that rely on phenotypic properties of the microbes are useful but often lack the necessary resolution potential for strain discrimination, such as minor changes in the bacterial genome that do not influence particular features (Beyer et al., 1998). Repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) analysis, based on the occurrence and distribution of repetitive elements within the genome, has been widely used to assess genetic diversity in bacteria (Louws et al., 1999). Rep -PCR employs oligonucleotide primers designed to be complementary to interspersed repetitive elements within bacterial genomes. DNA regions intervening between two adjacent repetitive elements are amplified to provide multiple, different sized DNA sequences (Versalovic et al., 1991; Versalovic et al., 1998). Rep-PCR fingerprinting is relatively simple, rapid and sensitive for discriminating between closely related strains of many bacteria (Van Belkum et al., 1998; Versalovic et al., 1991).

In this study, we used a rep-PCR approach that may be useful to analyze the existence of repetitive element in spiroplasmas and to assess genetic variation in different species, strains and lines.

Materials and Methods

Spiroplasmas

Spiroplasma species and lines used in this experiment are listed in Table 1. All spiroplasmas were cultured in LD8 broth medium (Lee & Davis, 1984) at

31 °C, except *S. kunkelii,* which was cultured in LD8A3 broth medium (Lee & Davis, 1984) at 33 °C.

Genomic DNA isolation

Template DNA was isolated using a method adapted from Doyle & Doyle (1990). Fifteen ml of log phase culture (approximately 10^9 cells/ml) was centrifuged at 27,000 g for 20 min at 4 °C. The pellet was resuspended in 5 ml preheated (60 °C) 2.5% CTAB buffer (2.5 % cetyltrimethyl-ammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, pH 8) and incubated 30 minutes at 60 °C. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and the mixture centrifuged at 5,000 g for 10 at minutes, 20 °C. The upper phase was removed, mixed with an equal volume of chloroform:isoamyl alcohol as above and centrifuged at 9,000 g for 5 minutes at 20 °C. The upper phase was removed and DNA was precipitated with cold isopropanol (-20 °C). DNA pelleted at 30,000 g for 20 minutes was washed twice with prechilled 70% ethanol, air dried and resuspended with 200 μl distilled H_2O.

Rep-Polymerase Chain Reaction

The oligonucleotide primers for rep-PCR were synthesized by the Oklahoma State University Recombinant DNA/Protein Resource Facility. The primers and their nucleotide sequences were:

(1) BOXA1R: 5'- CTACGGCAAGGCGACGCTGACG-3' (Versalovic et al., 1998)

(2) ERIC1R: 5'-ATGTAAGCTCCTGGGGATTCAC-3' (Louws & Cuppels, 2001)

(3) ERIC2: 5'- AAGTAAGTGACTGGGGTGAGCG-3' (Louws & Cuppels, 2001).

Rep-PCRs employing BOXA1R and ERIC1R/ERIC2 oligonucleotides were performed as described previously (Louws & Cuppels, 2001) with a minor modification of using $MgCl_2$ at a concentration 3.35 mM instead of 6.7 mM in 5X Gitschier buffer (Kogan et al., 1987). PCRs were carried out with 2-4 μl template DNA and 2 units of *Taq* polymerase (Life Technologies, Grand Island, New York) in 25 μl Gitschier buffer, 160 μg/ml BSA, 10% DMSO, 1% Tween-20, 1.25 mM each dNTP, and 0.3 μg primer. Reactions were incubated for 2 min at 95 ºC followed by 30 cycles of 94 ºC for 30 seconds, 92 ºC for 30 seconds, 50 ºC for 1 minute, 65 ºC for 8 minutes with a final extension time of 65 ºC for 8 minutes. Rep PCR products were electrophoresed at 80 Volt DC in 1.5% agarose in 0.5X TAE buffer with a 1 kbp DNA ladder as a size standard.

Results and Discussion

Rep PCR performed using either the ERIC/ERIC2 or BOXA1R oligonucleotides amplified DNA fragments from the *Spiroplasma* genomic DNA of all the lines tested. In repeated experiments, the PCR products generated with the ERIC1R/ERIC2 and BOXA1R primers yielded several bands ranging mainly from 0.4 to 4 kbp and 0.35 to 1 kbp, respectively. Examples of patterns generated by rep-PCR are shown in Figure 1, panels A and B. Rep-PCR with BOXA1R (Figure 1, panel A) produced a band of about 1.0 kbp that was shared by all spiroplasmas tested. *S. citri* BR3-T, BR3-G, BR3-3X, and R82A and *S. melliferum* TS-2 showed three common prominent bands of 1.0, 0.5 and 0.35

kbp, a pattern distinguishable from that of *S. kunkelii* CR2-3X, which had prominent bands of 1.0, 0.85 and 0.3 kbp and from that of *S. floricola* 23-6 and *S. phoeniceum*, both of which had only a single band of 1.0 kbp. *S. citri* R8A2 had also an additional prominent band of 1.8 kbp, which was not present in any other spiroplasmas.

DNA patterns from rep-PCR with ERIC1A/ERIC2 DNA patterns (Figure 1, panel B) showed complexity similar to that with BOXA1R. All spiroplasmas except strain 23-6 shared a common band of about 1.6 kbp. *S. citri* strains BR3-T, BR3-G, BR3-3X, and R82A and *S. melliferum* TS-2 showed two common bands (1.6 and 0.5 kbp) and a rather faint band of 1.5 kbp. These patterns differentiate the latter spiroplasmas from *S. floricola* strain 23-6, *S. kunkelii* strain CR3-3X and *S. phoeniceum*. The latter spiroplasmas also gave distinctive patterns.

However, the DNA patterns produced from experiment to experiment were generally inconsistent, either in size, number or intensity of individual bands in each pattern. Only one band of 1 kbp, generated with the BOXA1R primer, was generally consistent among all experiments.

Pattern variation within species was relatively limited; however, the *S. melliferum* TS-2 pattern was very similar to those of *S. citri* strains, whereas the *S. floricola* 23-6, *S. kunkelii* CR2-3X and *S. phoeniceum* patterns were slightly different from those of *S. citri*. Modification of the $MgCl_2$ and DMSO concentrations used for the rep PCR optimized the intensity of DNA fragments (Figure 2) but did not enhance reproducibility of the DNA patterns.

The rep-PCR results revealed the presence of repetitive elements in several spiroplasma genomes. The band patterns among the *Spiroplasma* species suggested that the elements varied in number and distribution in different locations in the genomes. The DNA patterns generated with rep-PCR/BOXAIR were quite simple and had less power to differentiate *S. citri* strains/lines. A distinction among strains of *S. citri* resulting from the PCR was that strain R8A2 differed from the rest lines/strains. *S. citri* R8A2 also differs from *S. citri* BR3-3X and its derived lines also in terms of host plant, insect vector and continent origin of isolation. *S. citri* R8A2 was isolated from citrus with stubborn disease in Morocco, whereas *S. citri* BR3-3X was isolated from horseradish with brittle-root in the USA. It is likely that these strains arose from different evolutionary paths which led to different genetic properties.

The PCR was capable of showing distinction among *Spiroplasma* species; those tested in this study had different rep-PCR DNA patterns except that *S. melliferum* TS-2 was indistinguishable from *S. citri*. Both *S. floricola* 23-6 and *S. phoeniceum* had a single identical band.

Although rep-PCR with both primers produced DNA patterns of low complexity, use of the ERIC primers allowed slightly greater distinction among spiroplasma species. The different species of spiroplasmas tested in this study showed unique DNA patterns. According to the revised group classification of the genus *Spiroplasma* (Williamson et al., 1998), *S. citri*, *S. melliferum*, *S. kunkelii* and *S. phoeniceum* were placed in different sub-groups within Group I, while *S. floricola* is in Group III. The rep-PCR from this study showed

differentiation of *Spiroplasma* species that support the above classification, in that the different species had distinguishable DNA patterns.

Because of the poor reproducibility and limited number of DNA fragments amplified, rep-PCR was not sufficient for specific identification or determination of relatedness among *Spiroplasma* lines/strains. Both BOX and ERIC primers were designed to amplify repetitive sequences identified originally in Gram-negative bacteria. The rep-PCR has been successfully used for typing and differentiating among many Gram-negative bacteria and some Gram-positive bacteria (Versalovic et al., 1991; Versalovic et al., 1993), but it has not yet been previously reported to be used for spiroplasmas or other mollicutes, which are believed to have evolved from Gram-positive bacteria. Repetitive elements in spiroplasmas may be present in lower numbers than in other bacteria. It is also speculated that the rep-PCR primers might act as primers in randomly amplified polymorphic DNA (RAPD)-PCR or as arbitrary primers for AP-PCR. Gillings & Holley (1997) reported that the reproducibility and complexity of sequence patterns generated by rep-PCR was affected by stringency, higher stringency resulting in fewer amplified bands.

Conclusions

Repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) using BOXA1R and ERIC1R/ERIC2 primers have shown the presence of repetitive elements in spiroplasmas. Both rep-PCRs were able to generate DNA sequences from *Spiroplasma citri* lines/strains (BR3-3X, BR3-T, BR3-G, R8A2,

and ASP-1), *S. melliferum* TS-2, *S. floricola* 23-6, *S. kunkelii* CR2-3X and *S. phoeniceum*, which were simple, with one to several bands. The discrimination power of rep-PCRs for spiroplasma was at species level but not at the line/strain. Due to the limited number of DNA bands and poor reproducibility among experiment replications, the rep-PCRs were not sufficient for specific identification and relatedness determination among *Spiroplasma* strains/lines.

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Table 1. List of spiroplasma species and lines characterized with rep-PCR

Species	Group designation (Williamson et al., 1998)	Strain or line	Original source/properties	Reference
S. citri	I-1	BR3-3X	Isolated from horseradish with britleroot disease and triply cloned, transmissible line	Fletcher et al., 1981
		BR3-T1	Derived from BR3X strain, maintained in turnip by *C. tenellus* transmission, transmissible line	Wayadande et al., 1993
		BR3-G1	Derived from BR3-3X strain, maintained in plants by periodic graft transmission, have became non-transmissible	Wayadande et al., 1993
		Maroc R8A2	Isolated from citrus with stubborn disease, ATCC 27556^T	Saglio et al., 1973
		ASP-1	Non helical strain, isolated from orange	Townsend et al., 1977
S. melliferum	I-2	TS-2	Isolated from honeybees	Clark et al., 1985
S. kunkelii	I-3	CR2-3X	Isolated from corn with stunt disease	Castro et al., 1992
S. phoeniceum	I-8	-	Isolated from periwinkle with yellows disease	Garnier et al., 2001
S. floricola	III	23-6	Isolated from surface of flowers, ATCC 29989^T	Davis, 1978

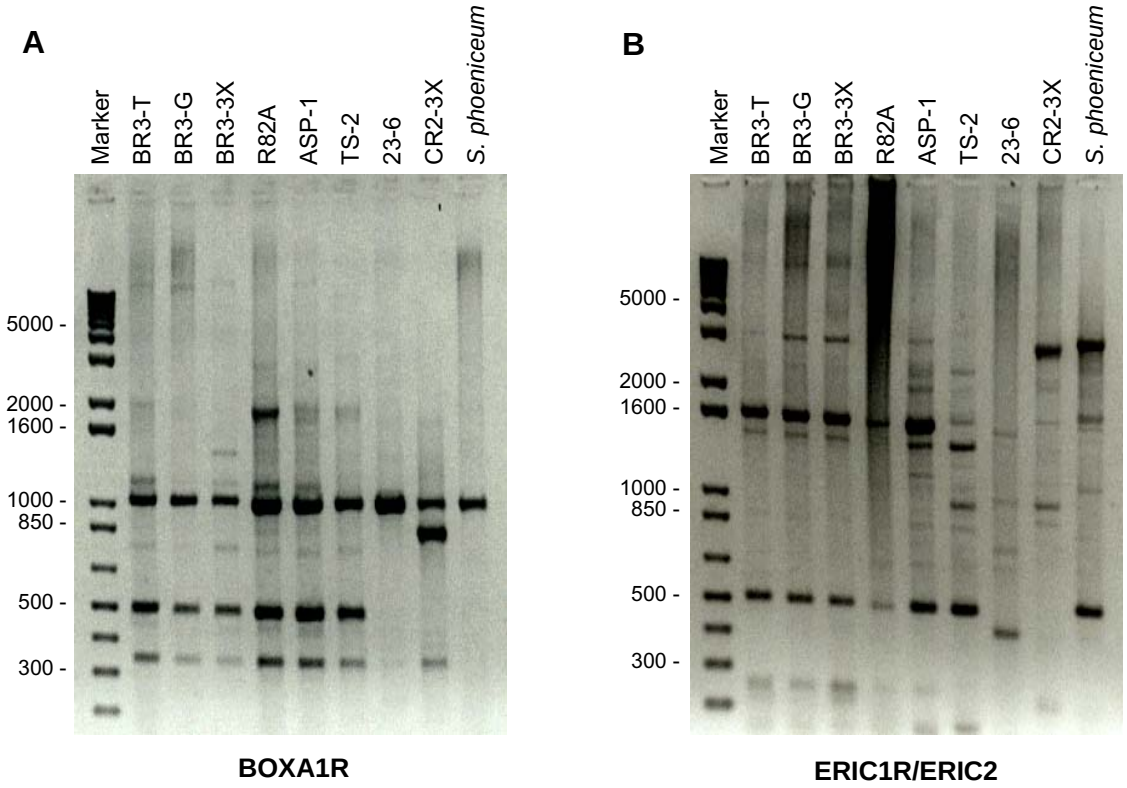

Figure 1. Rep-PCR DNA patterns of spiroplasmas generated with BOX1R **(A)** and ERIC1R/ERIC2 **(B)** primers

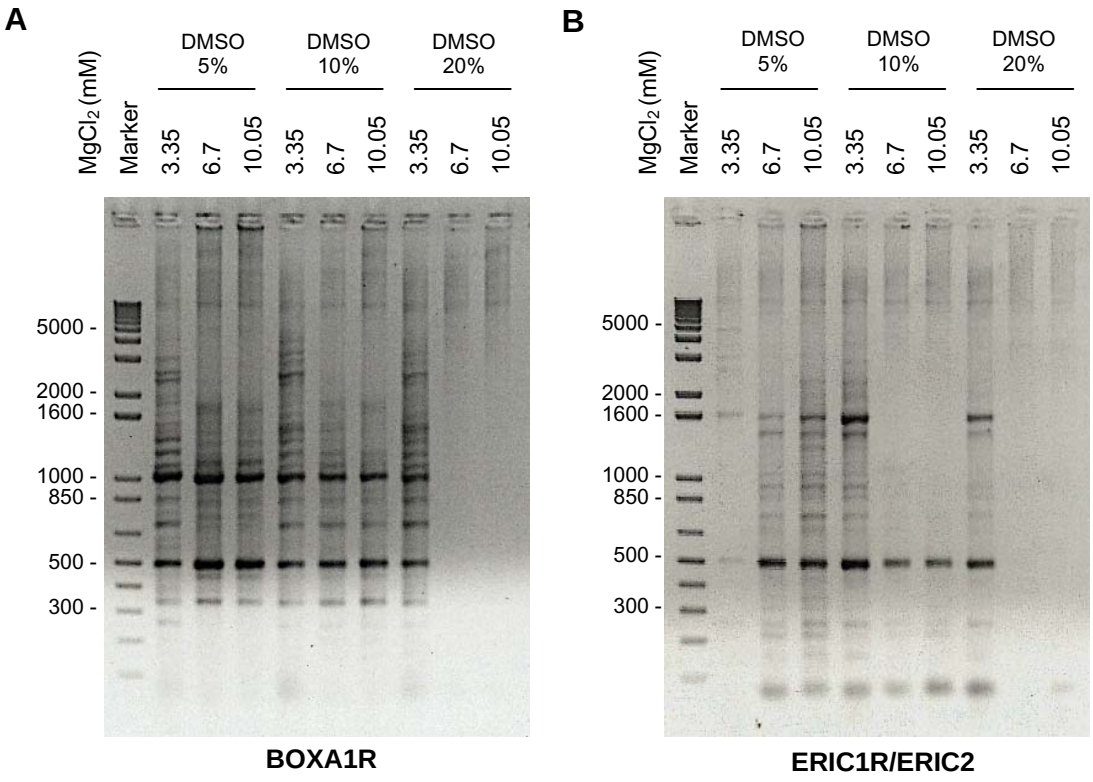

Figure 2. Rep-PCR optimization employing BOX1R (**A**) and ERIC1R/ERIC2 (**B**) primers by adjusting concentration of $MgCl_2$ and DMSO with *S. citri* BR3-G DNA as a template.

CHAPTER IV

RANDOM TRANPOSON MUTAGENESIS OF *SPIROPLASMA CITRI* USING TRANSPOSOME SYSTEM

Abstract

The application of many powerful tools of molecular genetics to mollicutes, such as *Spiroplasma citri,* is challenging. In this study a recently developed, transposome system, which has been used successfully to mutagenize bacteria, was evaluated for its effectiveness in the transformation of *S. citri* BR3-3X. Using the EZ::TN^{TM} <DHFR-1>Tnp Transposome^{TM} (Epicentre®) system, mediated by electroporation, the efficiency of transformation averaged 28.8 CFUs/ng transposome when 15 ng transposome and 10^9 spiroplasma cells were used. Many transformants appearing on the initial selection plates were growth impaired when transferred to broth medium. Altering the broth composition, adding common cell culture enhancement factors, and conditioning the medium with cultured cells of the *S. citri* vector, *Circulifer tenellus* Baker*,* did not improve their growth. However, placing colonies into a reduced broth volume (50 μl) resulted in robust growth and successful subsequent passages. PCR using primers for the dihydrofolate reductase (DHFR) gene confirmed the transposon's presence in the genome of selected transformants, and Southern-blot hybridization showed its insertion to be random, mostly single and chromosome-located. The insertions were stable as indicated by the consistent presence of

the gene and sustainability of transformants sub-cultured to high passages. All of the transformants retained the ability to adhere to *Circulifer tenellus* (CT-1) cell line, but showed slower growth compared to their parental wild type *S. citri*.

Introduction

Spiroplasmas of the class Mollicutes are helical, wall-less, cultivable phytopathogenic prokaryotes (Tully & Whitcomb, 1992) that have small genomes and utilize the UGA codon for encoding tryphtophan, instead of as a start codon (Bové, 1993; Dybvig & Voelker, 1996). *Spiroplasma citri* BR3-3X, a phytopathogenic mollicute isolated from horseradish with brittle root disease, is transmitted by a phloem-feeding leafhopper, *Circulifer tenellus* Baker.

Transposon-mediated mutagenesis has been used as a valuable tool for genetic study since it provides random insertion and the generation of a number of mutants, allowing the construction of a mutant library and the screening for gene function (Koide et al., 2004). However, genetic manipulation of mollicutes remains challenging. Systems for transposon mutagenesis that are suitable for genetic analysis of mollicutes are quite rare. To date, there are only few reports describing successful genetic manipulation of mollicutes, for example the use of TN*916* for mycoplasmas (Dybvig & Alderette, 1988) and TN*4001* for *S. citri* (Foissac et al., 1997).

A transposon mutagenesis system employing a novel construct designated a transposome, was recently developed and used to mutagenize a number of prokaryotes. The transposome is a stable complex of the EZ::TN

transposase and the EZ::TN <DHFR> transposon. The transposon contains the dihydrofolate reductase (DFHR) gene conferring trimethoprim resistance, which is flanked by hyperactive 19 bp mosaic end (ME) EZ::TN transposase recognition sequences (Goryshin et al., 2000).

The objective of this work was to evaluate the use of the transposome system mediated by electroporation to transform *S. citri* BR3-3X, to obtain mutants useful for study of its biology and host interactions.

Materials and Methods

Spiroplasma

Spiroplasma citri BR3-3X was isolated originally from horseradish with brittle-root symptoms (Fletcher et al., 1981). The culture was stored in LD8 liquid at -80 °C. The *S. citri* culture was grown in fresh broth medium to log phase (10^8 to 10^9 cells/ml) at 31 °C for two days before use.

Sensitivity of *S. citri* to Trimethoprim

Trimethoprim is an antimicrobial compound that binds to dihydrofolate reductase (DHFR) and inhibits formation of tetrahydrofolic acid (Madigan et al., 1996). The antibiotic has broad range activity against Gram-negative and Gram-positive bacteria. The objectives of this experiment were to measure the optimum concentration and inhibitory effect of trimethoprim on *S. citri* BR3-3X. A concentration of trimethoprim that completely inhibits spiroplasma growth was

used for selection of spiroplasma transformants resulting from transposon mutagenesis with a transposome containing a trimethoprim resistance gene.

S. citri BR3-3X was grown in LD8 broth to log phase (2 days old, approximately 10^8 cells/ml). The cells were pelleted by centrifugation at 8000 g for 5 minutes at 4 °C. The pellet was resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 10% sucrose. The titer of the cell suspension was determined by direct counting using a dark-field microscope (Olympus, BH2, Olympus Optical Co., Tokyo, Japan). PBS was added to give a titer of approximately 10^4 cells/ml. A volume of 50 μl cell suspension was plated onto agar solidified LD8 medium in 3 cm plastic Petri dishes amended with trimethoprim at a concentration of 0, 0.1, 5.0, 10 or 20 μg/ml medium. The plates were incubated at 31 °C for 7 days. The number of colony forming units (CFUs) on plates was counted seven days after plating using a stereo microscope (Olympus CK2, Olympus Optical Co., Tokyo, Japan). The inhibitory effect of trimethoprim on spiroplasma was calculated as:

$$\text{Percent inhibition (\%)} = \left(1 - \frac{CFU_{tn}}{CFU_{t0}}\right) \times 100\%$$

Where CFU_{tn} = CFU from the concentration of trimethoprim treatment, CFU_{t0} = CFU from the control (medium without trimethoprim). The lowest concentration of trimethoprim causing 100% inhibition was used for selection of transformants.

Persistence of trimethoprim in LD8 plates at different concentrations and of 31 °C-incubation periods of time preceding cell plating were evaluated**.** The trimethoprim concentrations tested were 0 (control), 20, 50 and 100 μg/ml, and

the pre-incubation periods were from 0 to 24 days with 2 day-interval. Fifty μl suspension of approximately 10^5 *S. citri* BR3-3X cells in HEPES buffer (0.008 M HEPES, pH 7.4 in 0.28 M sucrose) was plated onto the pre-incubated LD8 agar containing trimethoprim at concentration indicated and incubated at 31 °C. CFUs of *S. citri* BR3-3X on the LD8 plates were counted at 10 days after plating.

Electroporation and Selection of Transformants

The method used for electroporation-mediated transformation of spiroplasmas was based on those of Dybvig et al. (1995) and Sha et al. (1995). The transposome, supplied in a commercial kit (EZ::TN™ <DHFR-1> Tnp Transposome™; Epicentre®, Madison, Wisconsin), is a stable complex formed between the EZ::TN transposase enzyme and the EZ::TN <DHFR> transposon. This transposon's dihydrofolate reductase (DFHR) gene, which confers trimethoprim resistance, is flanked by hyperactive 19 bp mosaic end (ME) EZ::TN transposase recognition sequences (Figure 1). The DHFR gene is 474 bp with an ATG start codon and a TAA terminal codon at each end, located at bases 208 and 679, respectively. Only 18 restriction endonucleases were predicted to cut the gene at a restriction site (Figure 1), although 94 endonucleases can restrict the transposon DNA, each at 1 to 4 restriction sites. A pair of reverse and forward primers derived from positions 69 to 90 and 805 to 624, respectively, is available for sequencing (Epicentre®). A second pair of PCR primers, derived from the same position, prime in the opposite direction.

S. citri BR3-3X was grown in LD8 liquid broth to a titer of approximately 10^9 cells/ml and harvested by centrifugation at 10,000 g for 30 minutes at 4 °C. The pellet was washed by resuspension in electroporation buffer (0.008 M HEPES, pH 7.4 in 0.28 M sucrose) and centrifugation. The electroporation buffer was added to a titer of 10^9 cells/ml and the suspension was incubated on ice for 10 to 30 min. A volume of 400 μl of cell suspension was added to pre-chilled 0.2 cm gap electroporation cuvettes (Dybvig et al., 1995; Sha et al., 1995). One μl of the EZ::TN <DHFR> Tnp Transposome™ (15 ng/μl) was added and electroporation was performed using a Gene Pulser (Bio-Rad, Hercules, California). Optimization was carried out by testing voltages at 8.0, 10.0 and 12.5 kV, and resistance at 100, 800 and 1000 ohm. The capacitance was 25 μFD and two pulses were applied.

Immediately after electroporation, 600 μl pre-warmed LD8 broth was added and suspension was incubated at 31 °C for 3 hours. Each 50 μl of suspension was plated on semisolid selection medium (LD8 + 1% Noble agar containing 100 μg/ml trimethoprim in a 3 cm diameter Petri dish). Transformant colonies were recovered and grown in 1 ml LD8 broth to log phase, aliquoted into small vials and stored at -80 °C until used.

Optimization of Transformant Growth in Broth Medium

Although ample numbers of transformant colonies appeared on selection agar, colonies that were subsequently transferred to broth grew sluggishly. An experiment was designed to enhance the growth of these transformants by

plating after (1) varying the composition of fetal bovine serum in the LD8 broth (5, 10 [standard], 15, or 20%); (2) adding a growth enhancement factor, i.e. hybridoma cloning factor (Origen®, Igen International, Inc., Gaithersburg, MD), a growth factor used in insect cell culture, to a concentration of 0.5, 1, 2 or 5%; (3) conditioning the LD8 broth by adding Noble agar at low concentration (0.01, 0.05, 0.1, and 0.2%); (4) conditioning the LD8 broth by adding filtrates of cultured *S. citri* BR3-3X cells to 0.5, 1, 10, or 25%; (5) amending the LD8 broth by adding *C. tenellus* (CT-1) cell line suspension (5 X 10^5 cells/ml) to 0.5, 1, 5, or 10% v/v; (6) replacing the LD8 broth with LD8A3 or M1D broth; and (7) variation of the volume of LD8 broth used for initial cultivation of transformant colonies by gradual addition of the medium and different container used, and the final addition of 5 ml in a 12 ml glass bottle.

Isolation of Total Genomic, Chromosomal and Plasmid DNAs

Total genomic DNA was isolated using a method adapted from Doyle & Doyle (1990). Fifteen ml of log phase spiroplasma culture (10^9 cells/ml) was centrifuged at 27,000 g for 20 minutes at 4 °C. The pellet was resuspended in 5 ml preheated (60 °C) buffer (2.5% cetyltrimethyl-ammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, pH 8) and incubated 30 minutes at 60 °C. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and the mixture centrifuged at 5,000 g for 10 minutes at 20 °C. The aqueous phase was removed, mixed with an equal volume of chloroform:isoamyl alcohol as above and centrifuged at 9,000 g for 5 minutes at 20 °C. The upper phase was

removed and DNA was precipitated with cold isopropanol (-20 °C). DNA was pelleted at 30,000 g for 20 minutes and washed twice with pre-chilled 70% ethanol, air-dried and resuspended with 200 μl distilled H_2O.

For extraction of chromosomal DNA, 25 ml bacterial culture was centrifuged at 27,000 g for 20 minutes. The supernatant was discarded, and 4 ml preheated (60 °C) CTAB buffer (2.5% cetyltrimethyl-ammonium bromide (CTAB), 1.4 M NaCl, 100 mM, 20 mM Tris-HCl, EDTA, pH 8) was added. The suspension was incubated at 60 °C for 10 minutes, and 6 ml chloroform:isoamyl alcohol (24:1 v/v) was added and gently mixed. After centrifugation at 5,000 g for 10 minutes at 20 °C, the aqueous phase was removed to new tube, and 4 ml chloroform:isoamylalcohol was added. After centrifuging as before, the final aqueous phase was removed to a new tube and an equal volume of pre-chilled isopropanol was added. The suspension was shaken gently, incubated overnight at -20 °C, and centrifuged at 27,500 g for 15 minutes at 4 °C. The supernatant was discarded and the pellet was washed twice with pre-chilled 70% ethanol to remove CTAB and salts. The suspension was centrifuged at 27,500 g for 10 minutes at 4 °C, and pellet was air dried and dissolved in TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8).

S. citri plasmid DNA was isolated using an alkaline lysis method adapted from Horowicz & Burke (1981). One ml *S. citri* culture was centrifuged at 27,000 g for 1 minute. The pelleted cells were resuspended in 100 μl solution I (50 mM glucose, 25 mM Tris HCl, 10 mM EDTA, pH 8.0) by vortexing, incubated at room temperature for 10 minutes, then placed on ice. Two hundred μl solution II (2%

of 10 N NaOH, 1% sodium dodecyl sulphate (SDS), water) was added and mixed by inverting the tubes gently. The suspension was incubated on ice for 5 minutes and 150 μl solution III (3 M potassium acetate, 12.6% glacial acetic acid, water, pH 5.4) was added and mixed by gentle inversion. The suspension was incubated on ice for 5 minutes and centrifuged at 27,000 g for 2 minutes. Four hundred μl upper phase liquid was removed to a new tube containing 250 μl isopropanol, mixed by gentle inversion, and incubated at room temperature for ≥ 10 minutes. After centrifugation at 27,000 g for 5 minutes, the supernatant was discarded and the pellet resuspended in 400 μl of 70% ethanol and centrifuged at 27,000 g for 5 minutes. The tube was drained and the dried pellet dissolved in 30 μl TEN buffer (10 mM Tris HCl pH 7.5, 10 mM NaCl, 1 mM EDTA).

Detection of the DHFR Gene in Transformant Using PCR

PCR primers were designed complementary to sequences that flank a region of approximately 750 bp within the transposon. The nucleotide sequences of the PCR primer pair were:

(1) Forward primer: 5'-CGATTTGTAATAACAGAGTGTC-3'

(2) Reverse primer: 5'-CCGCCTTTGTAACCTACGCC-3'

A standard PCR assay was performed to detect the presence of the DHFR gene inserted in the transformant genome. Total genomic DNA was isolated as previously described and used as template.

PCR was carried out in a 25 μl reaction mixture containing 1 to 5 μl template DNA and 1 unit of *Taq* polymerase (Life Technologies, Grand Island,

New York) 2.5 μl of 10X PCR buffer containing 15 mM $MgCl_2$, 0.1 mM each dNTP, 0.4 μg of each primer and sterile H_2O up to 25 μl. Reactions were incubated for 1 minute at 92 °C followed by 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1.5 minutes with a final extension time of 72 °C for 10 minutes. Alternatively, PCR was conducted using whole cells, instead of isolated DNA, as a template. The 25 μl reaction mixture for this alternative PCR contained 1-5 μl bacterial culture (template), 1 unit of *Taq* polymerase, 4 μl of 5X Gitschier buffer, 1.2 μl of 25 mM $MgCl_2$, 0.1 mM each dNTP, 0.4 μg of each primer, 3.2 μg bovine serum albumin (BSA) and sterile H_2O up to 25 μl. Reactions were incubated for 5 minutes at 95 °C followed by 35 cycles of 95 °C for 1 minute, 54 °C for 1 minute, and 72 °C for 1.5 minutes with a final extension time of 72 °C for 10 minutes. PCR products were electrophoresed at 80 V DC in 1.0% agarose in 0.5X TAE buffer with a 1 kbp DNA ladder as a size standard.

Southern Hybridization Analysis

Genomic, chromosomal and plasmid DNAs were extracted from transformants using methods previously described. From each transformant 2 to 3 μg DNA was separately digested with *Hind*III and *Rsa*I (20 μl reaction mixture) and electrophoresed in a 1.5% agarose gel. The gel was gently rocked in 0.25 N HCl at room temperature for 10 minutes, then in denaturation solution (1.5 M NaCl and 0.5 M NaOH) at room temperature for 45 minutes. The gel was neutralized in 1 M Tris-HCl, pH 8.0 containing 1.5 M NaCl for 45 minutes and blotted onto nylon membranes (Amersham Biosciences, Piscataway, NJ)

following the manufacturer's instructions, for 36 hours at room temperature. The membrane was exposed on a UV transilluminator (Fischer Biotech FBTIV-614, Fischer Scientific, Pittsburgh, PA) at 320 nm for 5 minutes to immobilize the DNA.

A probe was made from the PCR product amplified from the DHFR gene and labeled with digoxygenin (DIG DNA labeling kit, Roche, Indianapolis, IN). Pre-hybridization in 100 ml pre-hybridization solution (1% blocking agent, 5X SSC, 1% SDS, 10% laurylsarcosine) was at 68 °C for 1 hour. Hybridization was done by incubating the membrane in hybridization solution (probe and pre-hybridization mix) at 68 °C overnight. The membrane was washed twice with solution I (2X SSC and 0.1% SDS) at room temperature and twice with solution II (0.1X SSC and 0.1% SDS) at 68 °C. The membrane was air-dried and the digoxygenin-labeled probe was detected with CSPD (DIG Luminescent Detection Kit, Roche, Indianapolis, IN) and exposed to Kodak X-ray films for 5 to 30 minutes.

Stability of Transformants through Passages

To assess the stability of the transformants, bacteria were cultured consecutively into trimethoprim-containing LD8 broth and incubated at 31 °C for 2 to 4 days to log phase. From each passage, titer of bacteria was checked by direct count and presence of DHFR gene was detected using PCR as mentioned above. Stable transformants should grow successively in LD8 broth and give PCR positive results.

Adherence Assay of Transformants Using CT-1 Monolayer

A microtiter plate adherence assay using leafhopper *C. tenellus* (CT-1) cell monolayers (Yu et al., 2000) was used to examine the adherence of the transformants to the insect cells.

Microtiter plates were coated with CT-1 cells, grown in NT medium, at room temperature for 48 hours or until a monolayer was formed. The NT medium was discarded and the monolayers were washed twice with PBS, fixed with glutaraldehyde (0.25%, w/v) in PBS for 10 minutes, washed three times with PBS and blocked with PBS containing 0.1 M glycine and 1% BSA (w/v) for 2 hours at room temperature. After two washes in PBS containing 0.05% Tween 20 (v/v), a suspension of spiroplasma transformants in PBS containing 10% sucrose (w/v), at a titer of 10^8 cells/ml, was added to each well (100 μl/well). The plate was incubated at 30 °C for 2 hours and washed three times with PBS-sucrose. The binding of transformant spiroplasmas to CT-1 cells was quantitated by an ELISA-based method (Clark & Adams, 1977). Alkaline-phosphatase-conjugated anti-*S. citri* whole cell IgG was added to the wells, and color development was measured as for ELISA (Ofek et al., 1986).

Growth Rate of Transformants in LD8 Broth Medium

The growth rates of *S. citri* BR3-3X and its transformants in LD8 broth were evaluated by cultivation to log phase in LD8 broth amended with trimethoprim (100 μg/ml). Ten milliliters of each culture (approximately 5 X 10^7 cells/ml) was added to 9 ml fresh LD8 broth in tubes. The cultures were

incubated at 31 °C for 48 hours. One hundred ml samples of culture were taken every 2 hours (from 0 to 48 hours) for direct count under a dark field microscope and plating onto LD8 agar containing trimethoprim at 100 μg/ml. These values represented the titer of bacteria as cells/ml and CFUs, respectively. The titer values were plotted to show the growth rate.

Results and Discussion

Sensitivity of *S. citri* to Trimethoprim

Since trimethoprim was the selectable marker in the transposome used for transformation of *S. citri*, an assay of trimethoprim sensitivity was carried out to ensure that wild-type spiroplasma was sensitive to the antibiotic. The sensitivity was expressed as percent reduction of CFUs, as shown in Table 1. The lowest trimethoprim concentration to almost totally inhibit spiroplasma growth was 5 μg/ml. Within the concentration range of 5 to 100 μg/ml, the percent reduction was higher than 99% and not significantly different among treatments, although higher concentrations did cause greater inhibition, approaching 100%. However, at each of these concentrations, a small number of colonies formed (< 0.1%), suggesting that the BR3-3X population used in this assay contained trimethoprim-resistant individuals resulting from spontaneous mutation. In subsequent experiments trimethoprim selection was achieved with an antibiotic concentration of 100 μg/ml, to allow for some loss of antibiotic potency during the incubation.

Electroporation Optimization and Transformation Efficiency

Assessment of the viability of *S. citri* after electroporation and results of the experiments to optimize transformation by testing electroporation parameters are shown in Table 2. All combinations of electroporation reduced viability (expressed as percentage of CFUs) of electroporated bacteria compared to that of untreated controls (Table 2 panel A). In almost every case, the higher the voltage applied, the lower the bacterial viability measured. The resistance level significantly affected spiroplasma viability. At a resistance of 800 ohm and different voltages viability ranged from 25.7% (8.0 kV) to 2.9% (12.5 kV), values lower than those measures at 100 ohm.

Different electroporation parameters resulted in different efficiencies of transformation (Table 2, panel B). The number of colony forming units and transformation efficiency could be enumerated following electroporation with a resistance of 100 ohm, but not of 800 ohm. Although colonies were actually present at 800 ohm, they were aggregated and could not be counted or isolated. At 100 ohm, transformation efficiency ranged from 8.16×10^{-7} to 1.48×10^{-6} depending on the voltage. Based on these data, parameters chosen for most subsequent electroporations were 8.0 kV, 100 ohm, 25 μF and two pulses (parameter set A).

Further comparisons of electroporation parameters are shown in Table 3. Among three treatments, the use of parameter set A resulted in the highest number of CFUs, as many as 580 CFUs or 38.7 CFUs/ng transposome. The average of transformation efficiency was 28.8 CFUs/ng transposome. This

efficiency was higher than that of *Xylella fastidiosa* (5.8 CFUs/ng transposome) (Guilhabert et al., 2001), but much lower than that of electrocompetent *E. coli* (>1000 CFUs/ng transposome), provided as a standard by the kit manufacturer. Parameter sets B and C, with voltages of 10.0 and 12.5 kV, respectively, yielded fewer CFUs than did parameter set A. This result was consistent among subsequent experiments (data not shown).

Growth Enhancement of Transformants in Broth Medium

Transformation of *S. citri* via electroporation yielded numerous colonies on trimethophrim-amended LD8 plates. A constraint during these experiments was that transformed bacteria grew poorly in LD8 broth medium after transfer from a selection plate colony. Attempts to enhance growth included testing alternative medium formulations (M1D and LDA3 instead of LD8), varying the concentration of fetal bovine serum, adding enhancement factors (i.e. cloning factor, commonly used for tissue culture), conditioning the broth by adding wild-type culture filtrate, and co-cultivating with CT-1 cells of *C. tenellus*. These changes did not significantly improve the bacterial growth rate (Table 4).

The maximum titer of transformed spiroplasmas in M1D and LD8 broth was indistinguishable. However, because M1D contains an acid-base indicator, phenol red, bacterial growth could be monitored by the change in broth color. This feature was useful when working with a large number of cultures, and in subsequent experiments phenol red was added to LD8 broth for culturing and

growth monitoring of spiroplasma. The chemical did not detectably affect spiroplasma growth.

A method (Table 4, parameter E) that significantly improved spiroplasma growth was the reduction of the volume of LD8 broth into which transformant colonies were transferred (Figure 2). Optimum growth occurred in a medium volume of 50 μl in an Eppendorf tube, with gradual additions of fresh medium after 48 hours during incubation (increasing volumes of 0.4, 1 and 5 ml [Table 4, Figure 2]). These conditions resulted in robust growth and were used in subsequent experiments. By this method, 35% of single colonies recovered from the initial transformant selection plates were grown in LD8 broth to a final volume of 5 ml. The percentages of colonies grown successively in LD8 broth with others parameters (A, B, C, and D) were generally less than 10%.

The age of the transformant colonies on agar plates was considered to be critical for transferring to broth medium. Persistence of trimethoprim in LD8 plates over the amount of time of incubation at 31 °C is shown in Table 5. The trimethoprim is spiroplasmastatic, not spiroplasmacidal, and it began to lose its activity, indicated by colonies starting to form, after incubation for 10, 14 and 18 days, at a concentration of 20, 50 and 100 μg/ml, respectively. On the other hand, colonies were visible only after 10 to 12 days after plating. In addition to true transformants, and possibly spontaneous mutants, colonies formed on plates after 14 days might have included untransformed bacteria that grew as the activity of trimethoprim declined. Such undesired spiroplasmas might have been

co-isolated in the selection, since isolation was usually done 10 to 14 days after plating.

DHFR Gene Detection in Transformants and Stability of Transformation

Detection by PCR of the DHFR gene in transformants, conferring bacterial resistance to trimethoprim, is shown in Table 6 and Figure 3. A DNA fragment of about 750 bp was amplified by PCR in positive samples. All positive controls (transformation mixture remaining in the cuvette after electroporation, resuspended in electroporation buffer) tested were positive by PCR, whereas the negative controls (H_2O) were consistently negative. The parameters used for PCR proved to be reliable for detection of the transposon within the transformants.

Among the transformants successfully grown in LD8 broth to the first or second passage, 19 were PCR positive, indicating that the transposon was present in these populations (Table 6). However, only 5 transformants remained viable to higher passages and retained the transposon as shown by PCR (Figure 3). The other transformants failed to grow further or grew very poorly, hindering continued sub-culture. The PCRs on these transformants were negative, indicating that resistance, and the presence of the transposon, were temporary.

The stability of the insert within the spiroplasma genome was evaluated by sequential subculture of five transformants in antibiotic-containing broth. To date, transformants XTA-10, XTB-1 and XTC-4 have sustained 20 passages,

whereas transformants XTA-96 and XTA-238, isolated more recently, have reached the 10^{th} passage.

Southern Hybridization Analysis of Transformants

The stable transformants were characterized further. Patterns resulting from restriction digestion of transformant genomic DNA with *Hind*III and *Rsa*I were almost not distinguishable from that of the parental strain BR3-3X (Figure 4, panel A). The BR3-3X showed a prominent band of 5 kb. However, this band was only present very faintly in the transformants. The PCR products, used as positive controls, were uncut by these enzymes. Southern blot analysis showed five transformants consistently containing target DNA. The facts that only single bands were amplified from most transformants, except from XTA-A96, and that each of these bands was in a different position, indicate that the insertions of the transposon into the spiroplasma genome were usually single and random (Figure 4, panel B). Interestingly, transformant XTA-96 shows two amplified bands, possibly reflecting insertion at two locations in the genome or co-isolation of two different transformants. It is unlikely that the two bands resulted from the enzyme cleavage at the transposon region, since the transposon sequence does not have a restriction site for either *Hind*III or *Rsa*I. From many studies of the use of transposomes with various kinds of bacteria, insertion was usually single and random in the bacterial genome (Guilhabert et.al., 2001; Hoffman et al., 2000; Koide et al., 2004; Rieß et al., 2003).

In general, *Hind*III-generated DNA fragments containing the transposon (as detected by Southern hybridization) were larger (about 1.0 kbp or larger) than those generated with *Rsa*I (about 1.0 kbp or smaller). This information would be useful for further characterization, i.e. sequencing of the region flanking transposon, and deciding which endonuclease to use in constructing DNA clones for sequencing.

The size and number of DNA bands detected in each transformant was consistent among culture passages and among four replications of the experiment (summarized in Table 6). These results indicate that the transposon remained stable in the genome at the same location once it was inserted.

The same Southern hybridization methods were used to determine whether the transposon insertion was chromosomal or plasmid-located. In all cases, the gene was detected in the chromosomal fragment (Figure 5), but not in plasmid preparations (Figure 6). For each transformant, the sizes and numbers of bands detected by Southern hybridization in both chromosomal and genomic DNAs were identical, whether digestion was by *Hind*III or *Rsa*I.

Growth Rate of Transformants

Growth rates of all transformants, compared to the wild-type parental *S. citri* BR3-3X, are shown in Tables 6, 7 and 8 and Figure 7. The growth rates over a period of 48 hours, at 2-hour observation intervals, are shown as bacterial titer (cells/ml determined by direct counts and CFUs/ml determined by dilution plating). Both methods showed similar growth rates among the transformants.

The curves generally reflected typical microbial growth phases, i.e. periods of lag phase from hour 0 to about hour 24, followed by a log phase of about 14 hours (hour 24 to hour 38), and stationary phase for about 4 hours. A death phase was detected at the end of the period of observation for *S. citri* BR3-3X, but was not clearly observed for the transformants, probably because the slower growth of transformants compared to BR3-3X resulted in delay of the death phase to beyond 48 hour. In comparison to the wild-type, transformant spiroplasmas showed significantly slower growth, with the exception of XTA-96, which showed little delay to reach log phase. Transformant XTA-238 showed the slowest growth. These results indicate that transposon insertion in the transformant genome might be located in a gene involved in metabolism, causing a phenotypic change expressed as slower growth. This phenomenon also may explain the difficulty we encountered in producing transformants able to grow in LD8 broth.

Adherence of Transformants to CT-1 Cells

The ability of all positive transformants to adhere to a CT-1 cell monolayer was examined by the microtiter plate adherence assay (MPA). The $O.D._{450}$ readings in wells to which transformants were added were indistinguishable from those in wells containing wild-type spiroplasmas, suggesting that none of the former had lost the ability to adhere to the insect cells (summarized in Table 6, Figure 8).

Conclusions

The transposome (EZ::TN™ <DHFR-1>Tnp Transposome™, Epicentre®) system was used successfully to transform *Spiroplasma citri* BR3-3X mediated by electroporation. The electroporation was optimized with the following parameters: the voltage 8.0 kV, the resistance 100 ohm, and the capacitance 25 μFD. The efficiency of transformation averaged 28.8 CFUs/ng transposome when 15 ng transposome and 10^9 spiroplasma cells were used. The insertion of the transposon in the bacterial genome was usually single and random, stable and choromosome-located. The transformants were shown to retain their ability to adhere to CT-1 cells, and exhibited a delay of their growth curve in LD8 broth. The transposome system provides a potential tool for study of the genetics of spiroplasma and other mollicutes.

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Table 1. *Spiroplasma citri* BR3-3X CFUs grown on LD8 agar medium containing trimethoprim at various concentrations

Replication	CFUs on medium amended with trimethoprim at concentrations indicated					
	0 μg/ml	0.1 μg/ml	5 μg/ml	10 μg/ml	20 μg/ml	100 μg/ml
1	97.2	188.2	1.0	0.6	0.0	0.2
2	221.8	56.6	1.2	1.2	0.4	0.0
3	132.4	183.2	0.6	0.0	0.2	0.2
4	51.0	52.8	0.2	0.8	0.2	0.2
5	87.0	138.8	1.6	1.0	0.0	0.0
6	125.6	173.2	0.4	0.2	0.0	0.0
7	66.0	118.6	0.4	0.0	0.0	0.0
8	67.2	65.6	0.6	0.6	0.2	0.0
9	71.8	36.8	1.6	0.0	0.0	0.0
10	175.6	66.8	0.4	0.4	0.0	0.0
Average	109.6	107.7	0.8	0.48	0.1	0.06
% inhibition	0.0	2.28	99.27	99.56	99.91	99.95

Table 2. Transformation of *Spiroplasma citri* BR3-3X with transposome using different electroporation parameters

A. Without transposome (post-electroporation viability assay)[a]

Parameter set	Voltage (kV)	Resistance (ohm)	Capacitance (μF)	Pulse duration (msec)	CFUs	
					Average CFUs/ plate[b]	Viability (%)[c]
A	8.0	100	25	2.3, 2.3	78	44.6
B	10.0	100	25	2.3, 2.3	105	60.0
C	12.5	100	25	2.3, 2.3	54	30.9
D	8.0	800	25	10.1, 10.0	45	25.7
E	10.0	800	25	8.4, 8.7	10	5.7
F	12.5	800	25	9.2, 8.8	5	2.9
Untreated control					175	

[a] Electroporated cells were plated onto LD8 agar
[b] Number is an average of 3 replications
[c] Percentage of number CFUs in treatment to CFUs in untreated control

B. With application 3.75 ng transposome per 100 μl *S. citri* of 10^9 cell/ml[a]

Parameter set	Voltage (kV)	Resistance (ohm)	Capacitance (μF)	Pulse duration (msec)	Total CFUs	Transformation frequency
A	8.0	100	25	2.4, 2.4	371	1.48×10^{-6}
B	10.0	100	25	2.4, 2.4	296	1.18×10^{-6}
C	12.5	100	25	2.4, 2.4	204	8.16×10^{-7}
D	8.0	800	25	14.4, 14.2	*	-
E	10.0	800	25	13.6, 13.1	*	-
F	12.5	800	25	12.0, 12.6	*	-

[a] Electroporated cells were plated onto LD8 agar containing trimethoprim
* CFUs were present in small numbers but were aggregated and mixed with debris
- Could not be determined

Table 3. Efficiency of electroporation-mediated transposome mutagenesis of *Spiroplasma citri* BR3-3X

Experiment	CFUs yielded in electroporation parameter sets[a] indicated					
	total			per ng transposome		
	A	B	C	A	B	C
1	741	522	343	49.4	34.8	22.9
2	592	371	289	39.5	24.7	19.3
3	408	392	227	27.2	26.1	15.1
Average	580	428	286	38.7	28.6	19.1

[a] Electroporation was performed at 100 Ω, 25 μFD, 2 pulses and different voltages, i.e. A (8 KV), B (10 KV) and C (12.5 KV) with application of 1μl (containing 15 ng) transposome to 400μl *S. citri* BR3-3X cell suspension (10^9 cells/ml). The electroporated cells were plated onto LD8 agar containing trimethoprim (50 μg/ml).

Table 4. Methods of culturing *Spiroplasma citri* transformants from a single colony on plate into LD8 broth

Method	Amount of LD8 broth (ml) added at time indicated and container used				Percentage of colonies grown successfully in the broth
	Day 0 (initial)	Day 2	Day 4	Day 6	
A	1 (12 ml bottle)	2 (12 ml bottle)	5 (12 ml bottle)	-	1%
B	0.2 (2.5 ml bottle)	1 (2.5 ml bottle)	5 (12 ml bottle)	-	2%
C	0.2 (Microtiter plate well)	0.2 (Microtiter plate well)	1 (2.5 ml bottle)	5 (12 ml bottle)	7%
D	0.1 (Microtiter plate well)	0.2 (Microtiter plate well)	0.5 (2.5 ml bottle)	5 (12 ml bottle)	9%
E	0.05 (1.5 ml Eppendorf)	0.4 (1.5 ml Eppendorf)	1 (1.5 ml Eppendorf)	5 (12 ml bottle)	35%

Table 5. Persistence of trimethoprim activity in LD8 agar over time[a]

Pre-incubation of LD8 plates at 31 °C (days)	CFUs/ml[b] on LD8 plate amended with trimethoprim at concentrations and pre-incubations preceding plating indicated			
	0 μg/ml	20 μg/ml	50 μg/ml	100 μg/ml
0	TNTC[c]	0	0	0
2	2.66×10^8	0	0	0
4	2.76×10^8	0	0	0
6	2.52×10^8	0	0	0
8	1.82×10^8	0	0	0
10	2.07×10^8	6.67×10^4	0	0
12	2.48×10^8	3.67×10^5	0	0
14	1.63×10^8	1.20×10^6	1.33×10^5	0
16	1.25×10^8	9.67×10^5	0	0
18	9.69×10^7	7.00×10^5	3.33×10^4	6.67×10^4
20	8.54×10^7	1.27×10^6	1.00×10^5	0
22	8.82×10^7	7.33×10^5	3.33×10^4	3.33×10^4
24	6.83×10^7	6.33×10^5	1.00×10^5	3.33×10^4

[a] *Spiroplasma citri* BR3-3X CFUs grown on LD8 plates to which trimethoprim had been added at various concentrations and varying times prior to plate use. Initial titer of spiroplasma was 5×10^8 cells/ml and 50 μl was applied per plate.

[b] An average counted from 3 replications

[c] TNTC = too numerous to count due to too low dilution plating

Table 6. Properties of *Spiroplasma citri* transformants

Transformants		Sub-culture in LD8 broth		MPA	Growth rate compared to *S. citri* BR3-3X	Southern hybridization analysis
		Highest passage attained	Highest passage that PCR positive			
1.	XTA-7	3, poor[a]	2	+[b]	nd[c]	nd
2.	XTA-10	20	20	+	slower	+[d], 1 band
3.	XTA-12	3, poor	2	+	nd	nd
4.	XTA-32	3, poor	2	+	nd	nd
5.	XTA-46	3, poor	2	nd	nd	nd
6.	XTA-91	2, poor	1	nd	nd	nd
7.	XTA-92	2, poor	1	nd	nd	nd
8.	XTA-93	2, poor	1	nd	nd	nd
9.	XTA-94	2, poor	1	nd	nd	nd
10.	XTA-95	2, poor	1	nd	nd	nd
11.	XTA-96	10	10	+	slower	+, 1 band
12.	XTA-97	2, poor	1	nd	nd	nd
13.	XTA-98	2, poor	1	nd	nd	nd
14.	XTA-99	2, poor	1	nd	nd	nd
15.	XTA-102	2, poor	1	nd	nd	nd
16.	XTA-103	2, poor	1	nd	nd	nd
17.	XTA-238	10	10	+	much slower	+, 2 bands
18.	XTB-1	20	20	+	slower	+, 1 band
19.	XTC-4	20	20	+	slower	+, 1 band

[a] poor = poor growth and failed to be grown in next passage

[b] (+) indicates positive microtiter plate adherence assay (MPA) result, the bacteria retain their adherence to insect cell line

[c] nd = not determined

[d] (+) indicates positive result, showing either single or double band

Table 7. Growth rate within 48 hours of *Spiroplasma citri* BR3-3X and its transformants in LD8 medium counted directly under dark-field microscope

Hour	Titer (cells/ml)					
	BR3-3X	XTA-10	XTB-1	XTC-4	XTA-96	XTA-238
0	5.8×10^6	5.4×10^6	6.6×10^6	6.6×10^6	5.0×10^6	5.4×10^6
2	5.0×10^6	7.3×10^6	5.8×10^6	8.9×10^6	5.8×10^6	3.9×10^6
4	7.0×10^6	9.7×10^6	7.0×10^6	8.5×10^6	1.9×10^7	3.9×10^6
6	1.5×10^7	1.2×10^7	1.0×10^7	1.3×10^7	2.2×10^7	7.0×10^6
8	1.6×10^7	1.4×10^7	1.2×10^7	2.4×10^7	4.0×10^7	8.1×10^6
10	2.6×10^7	1.4×10^7	1.6×10^7	2.2×10^7	5.3×10^7	2.1×10^7
12	8.7×10^7	3.6×10^7	3.1×10^7	3.9×10^7	5.4×10^7	4.3×10^7
14	8.7×10^7	4.3×10^7	2.8×10^7	5.3×10^7	7.7×10^7	4.6×10^7
16	6.6×10^7	4.0×10^7	3.9×10^7	7.1×10^7	9.5×10^7	4.6×10^7
18	1.3×10^8	4.1×10^7	7.0×10^7	1.1×10^8	9.7×10^7	6.6×10^7
20	1.3×10^8	9.1×10^7	5.3×10^7	1.4×10^8	1.5×10^8	1.0×10^8
22	1.8×10^8	9.4×10^7	7.0×10^7	1.4×10^8	1.3×10^8	1.4×10^8
24	2.1×10^8	1.6×10^8	8.1×10^7	1.5×10^8	1.7×10^8	1.3×10^8
26	1.8×10^8	2.0×10^8	1.0×10^8	1.7×10^8	2.1×10^8	7.3×10^7
28	5.4×10^8	3.6×10^8	1.7×10^8	2.6×10^8	2.9×10^8	1.5×10^8
30	7.3×10^8	3.9×10^8	2.2×10^8	3.2×10^8	4.8×10^8	2.6×10^8
34	7.7×10^8	5.2×10^8	3.6×10^8	4.5×10^8	7.4×10^8	2.6×10^8
36	6.3×10^8	5.3×10^8	4.2×10^8	5.1×10^8	5.6×10^8	3.5×10^8
38	6.7×10^8	5.3×10^8	3.9×10^8	5.2×10^8	7.3×10^8	4.1×10^8
40	5.4×10^8	5.1×10^8	4.1×10^8	5.1×10^8	9.1×10^8	6.5×10^8
42	5.9×10^8	5.8×10^8	3.2×10^8	5.6×10^8	8.0×10^8	5.0×10^8
44	8.0×10^8	7.5×10^8	3.1×10^8	6.0×10^8	8.5×10^8	4.4×10^8
46	6.8×10^8	5.7×10^8	4.6×10^8	5.8×10^8	6.9×10^8	4.6×10^8
48	7.3×10^8	6.8×10^8	3.0×10^8	6.3×10^8	6.3×10^8	4.7×10^8

Table 8. Growth rate within 48 hours of *Spiroplasma citri* BR3-3X and its transformants in LD8 medium counted as CFUs/ml from plates

Hour	Titer (CFUs/ml)					
	BR3-3X	XTA-10	XTB-1	XTC-4	XTA-96	XTA-238
0	6.4×10^6	4.5×10^6	6.4×10^6	4.0×10^6	4.4×10^6	4.6×10^6
2	8.1×10^6	4.8×10^6	5.9×10^6	4.2×10^6	4.3×10^6	4.1×10^6
4	7.7×10^6	4.6×10^6	6.7×10^6	3.9×10^6	4.7×10^6	4.9×10^6
6	9.2×10^6	5.0×10^6	7.6×10^6	4.4×10^6	5.2×10^6	4.5×10^6
8	9.4×10^6	8.2×10^6	9.1×10^6	5.6×10^6	5.4×10^6	5.8×10^6
10	1.2×10^7	6.1×10^6	1.2×10^7	2.4×10^6	8.3×10^6	7.2×10^6
12	3.1×10^7	6.8×10^6	2.3×10^7	5.5×10^6	2.1×10^7	1.1×10^7
14	4.2×10^7	8.0×10^6	2.2×10^7	4.5×10^6	1.5×10^7	1.7×10^7
16	6.2×10^7	1.2×10^7	3.4×10^7	4.8×10^6	1.7×10^7	1.6×10^7
18	5.8×10^7	1.3×10^7	5.8×10^7	7.3×10^6	2.4×10^7	2.2×10^7
20	7.9×10^7	1.6×10^7	5.5×10^7	1.1×10^7	2.5×10^7	5.3×10^7
22	1.6×10^8	3.9×10^7	6.3×10^7	8.3×10^7	7.3×10^7	5.0×10^7
24	2.5×10^8	4.6×10^7	7.3×10^7	1.0×10^8	8.3×10^7	8.5×10^7
26	3.3×10^8	5.5×10^7	7.6×10^7	1.1×10^8	1.7×10^8	6.8×10^7
28	4.8×10^8	5.6×10^7	1.5×10^8	1.7×10^8	1.8×10^8	1.2×10^8
30	4.9×10^8	7.7×10^7	1.6×10^8	2.1×10^8	3.6×10^8	1.7×10^8
34	7.4×10^8	1.1×10^8	2.6×10^8	2.9×10^8	4.3×10^8	2.8×10^8
36	7.7×10^8	1.2×10^8	4.6×10^8	3.3×10^8	5.3×10^8	3.1×10^8
38	6.6×10^8	2.2×10^8	5.2×10^8	5.4×10^8	6.4×10^8	3.5×10^8
40	6.7×10^8	2.2×10^8	5.5×10^8	5.5×10^8	7.7×10^8	5.7×10^8
42	5.7×10^8	3.3×10^8	5.4×10^8	5.7×10^8	6.9×10^8	5.9×10^8
44	6.6×10^8	3.7×10^8	6.3×10^8	6.9×10^8	6.9×10^8	5.6×10^8
46	5.6×10^8	3.2×10^8	5.5×10^8	6.4×10^8	7.4×10^8	4.2×10^8
48	6.1×10^8	3.2×10^8	5.4×10^8	6.9×10^8	7.0×10^8	5.2×10^8

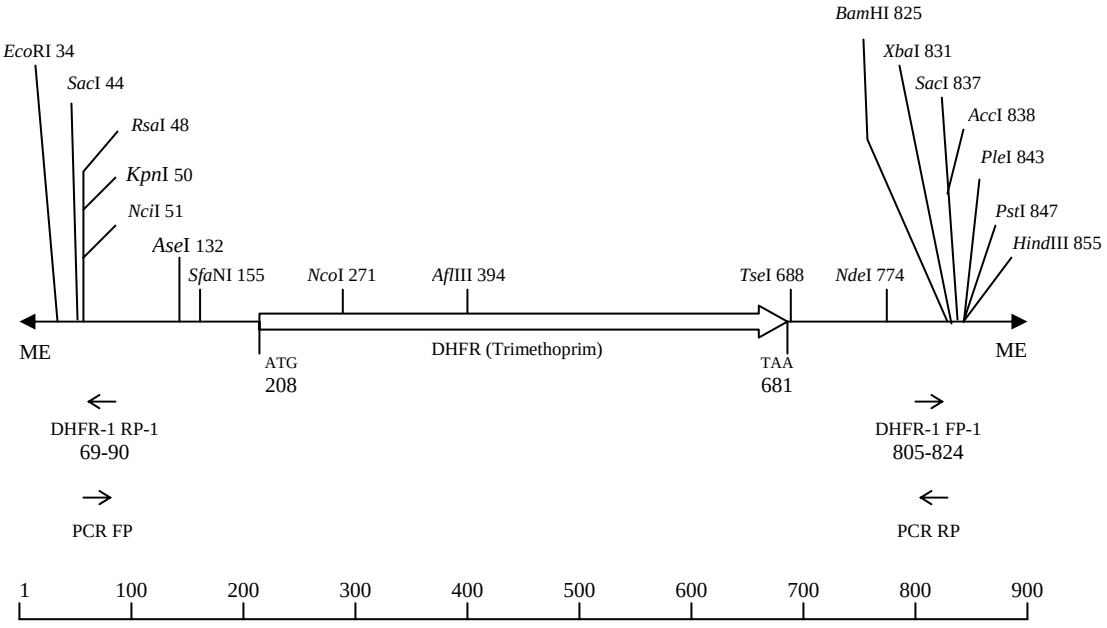

Figure 1. EZ::TN <DHFR-1> Transposon map
ME = Mosaic element, DHFR = Dihydrofolate reductase, FP = forward primer, RP = reverse primer, numbers indicate the position of sequences

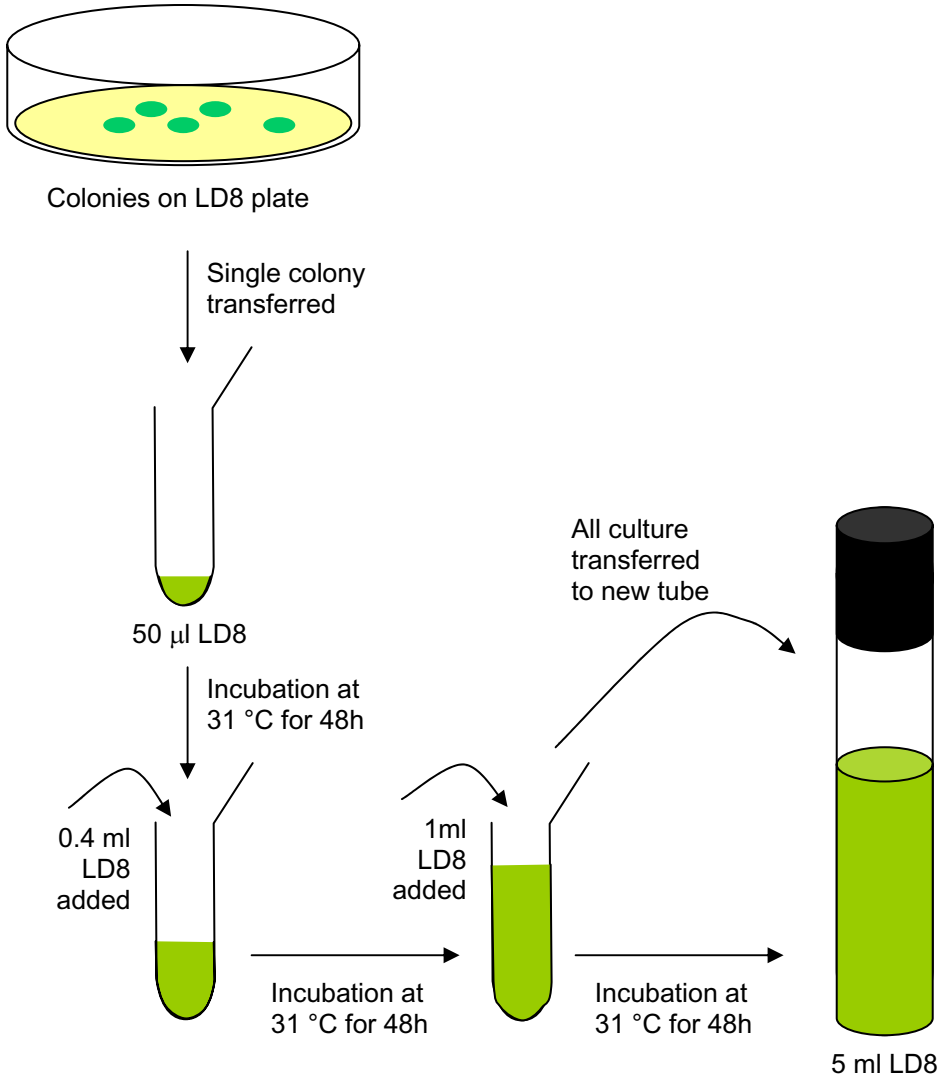

Figure 2. Initial sub-culturing of transformants into LD8 broth from single colony on plate

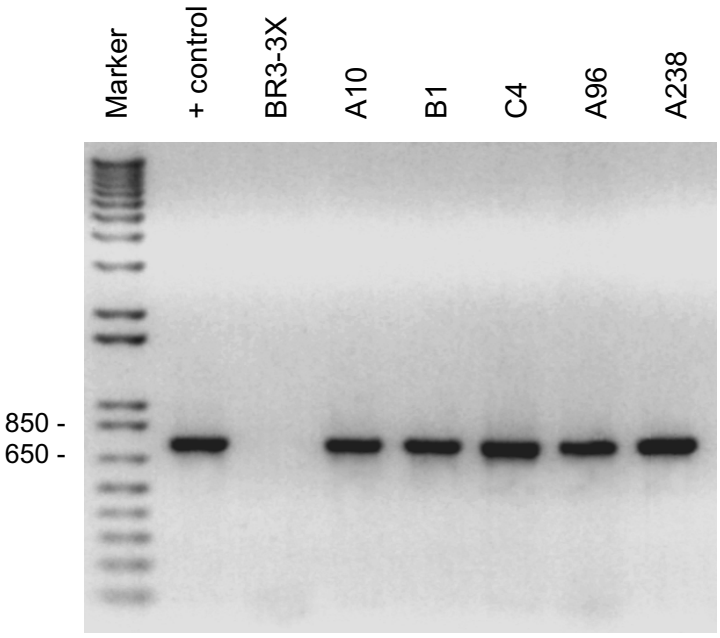

Figure 3. PCR amplification of DHFR gene of *S. citri* transformants
Marker = 1 Kb Ladder, (+) control = transposome suspension

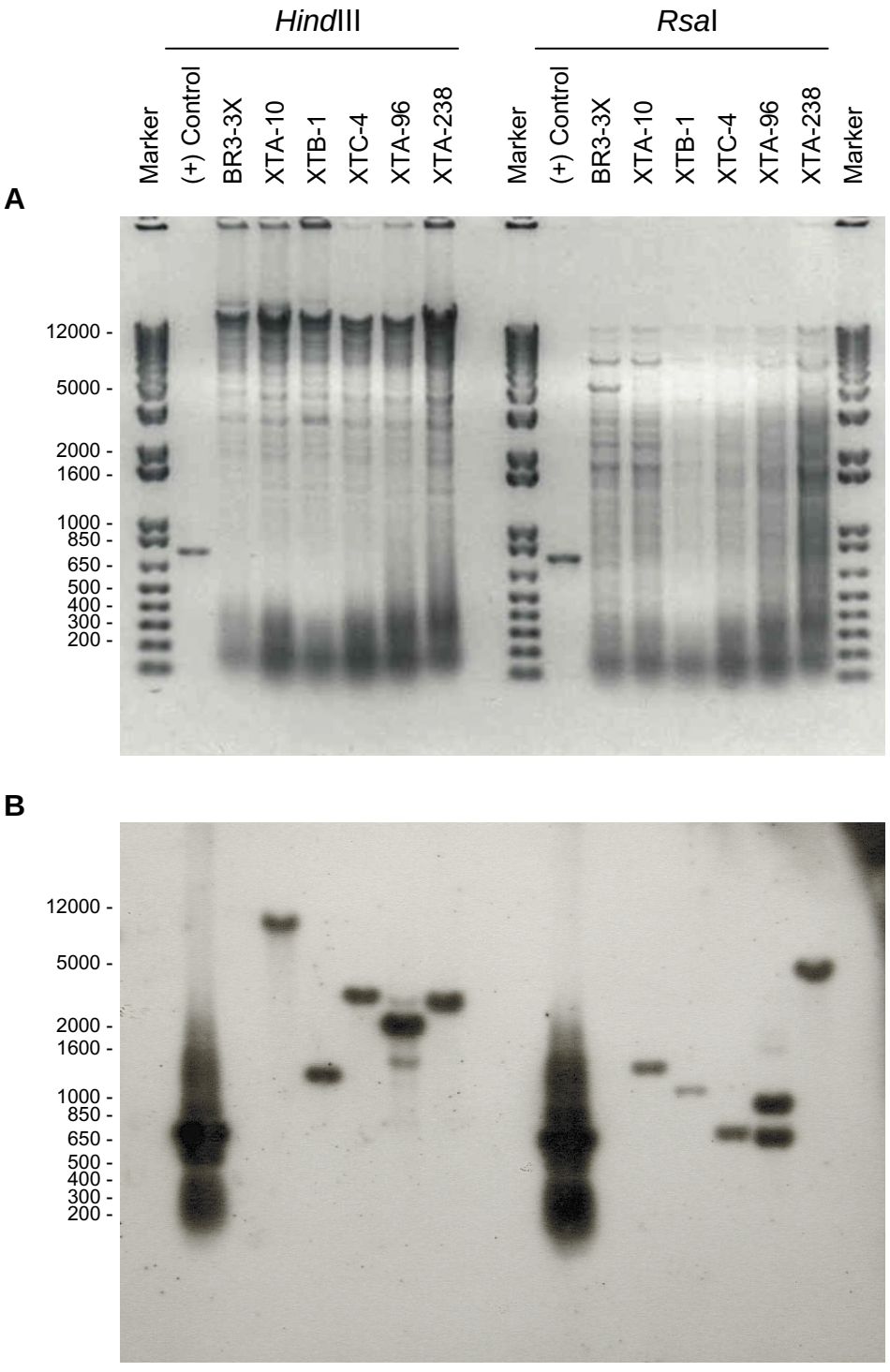

Figure 4. Restriction digestion of *S. citri* transformant genomic DNAs by *Hind*III and *Rsa*I (A), followed by Southern hybridization using the PCR product as a probe (B)

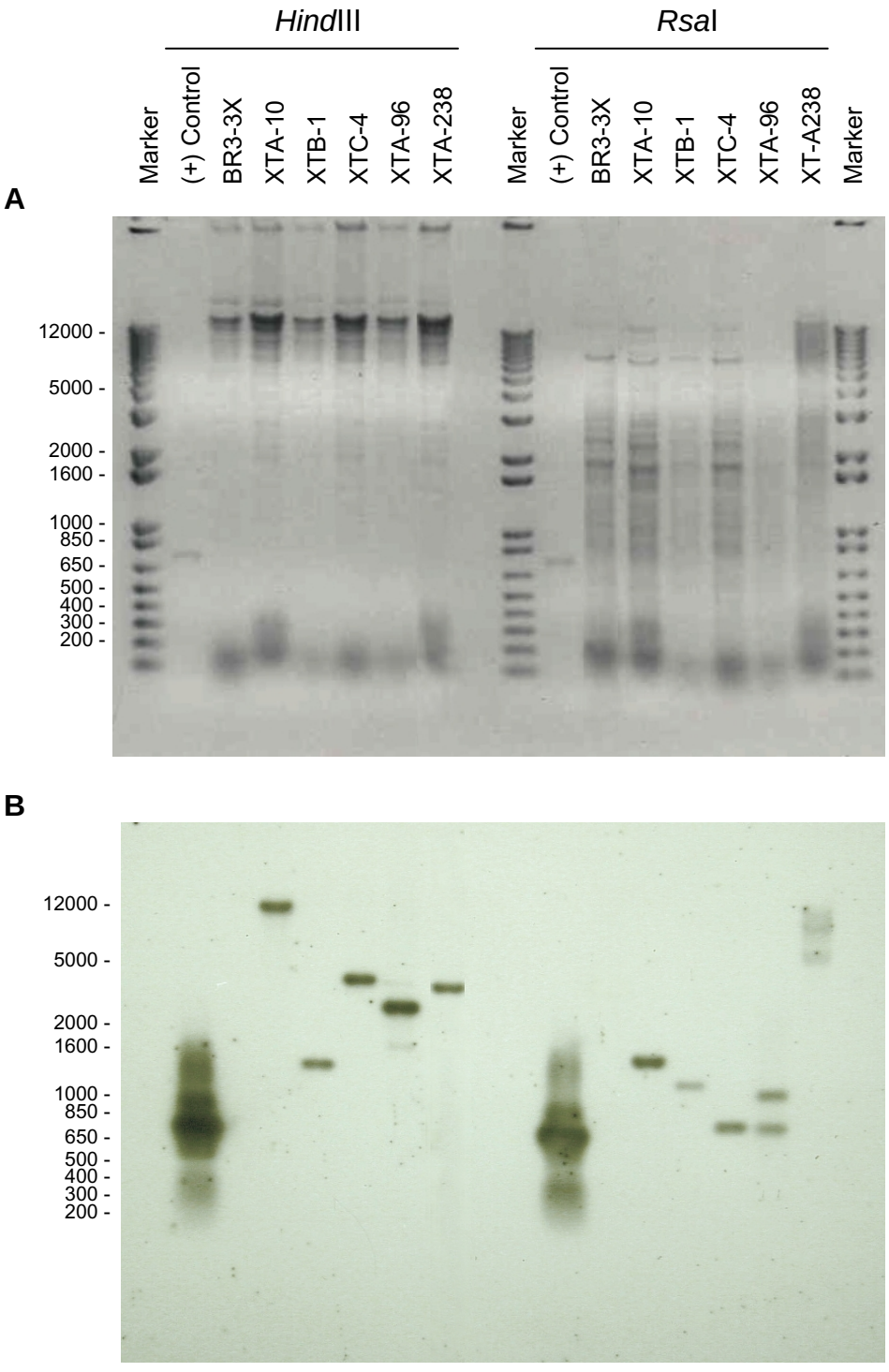

Figure 5. Restriction digestion of *S. citri* transformant chromosomal DNAs by *Hind*III and *Rsa*I (A), followed by Southern hybridization using the PCR product as a probe (B)

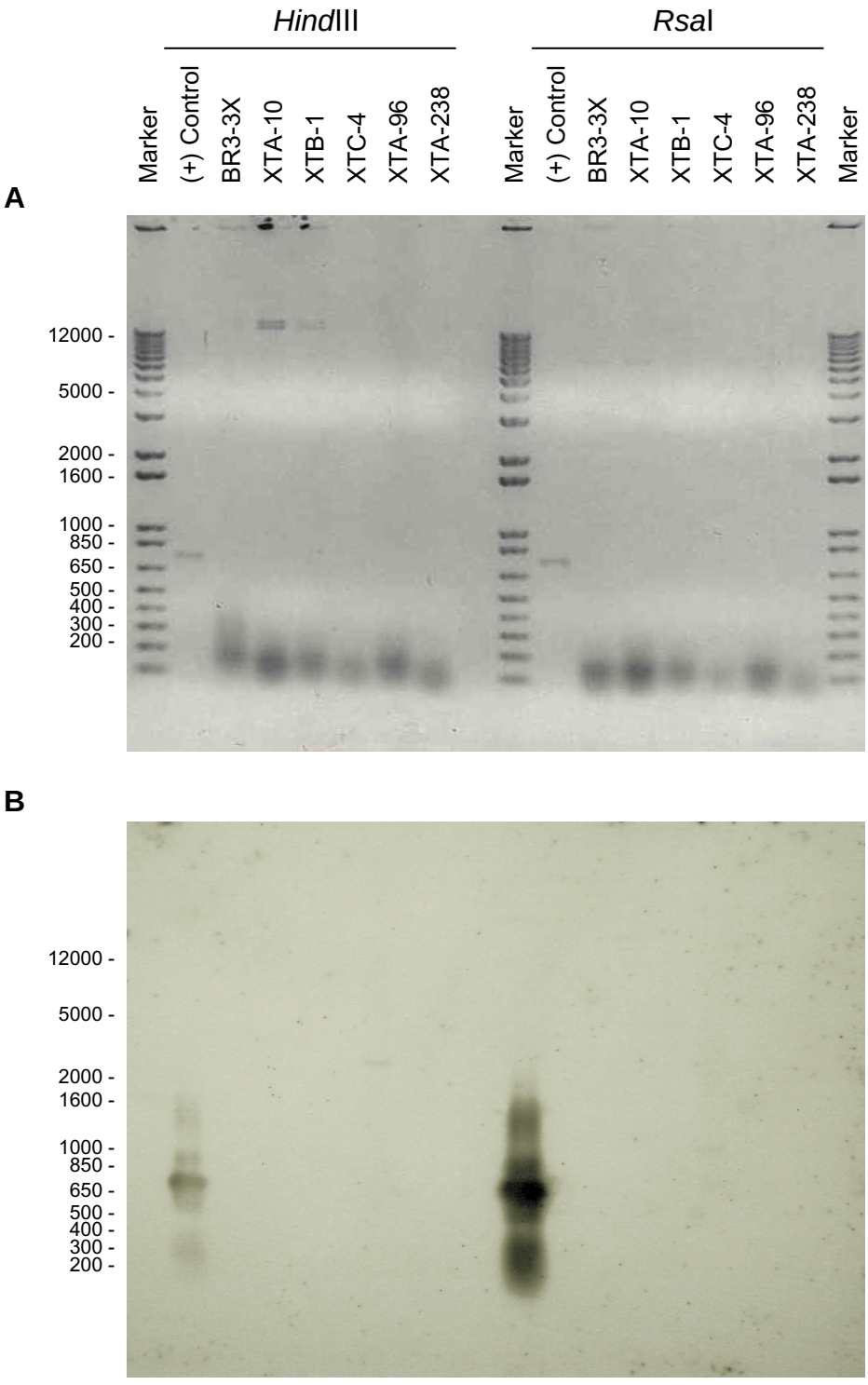

Figure 6. Restriction digestion of *S. citri* transformant plasmid DNAs by *Hind*III and *Rsa*I (A), followed by Southern hybridization using the PCR product as a probe (B)

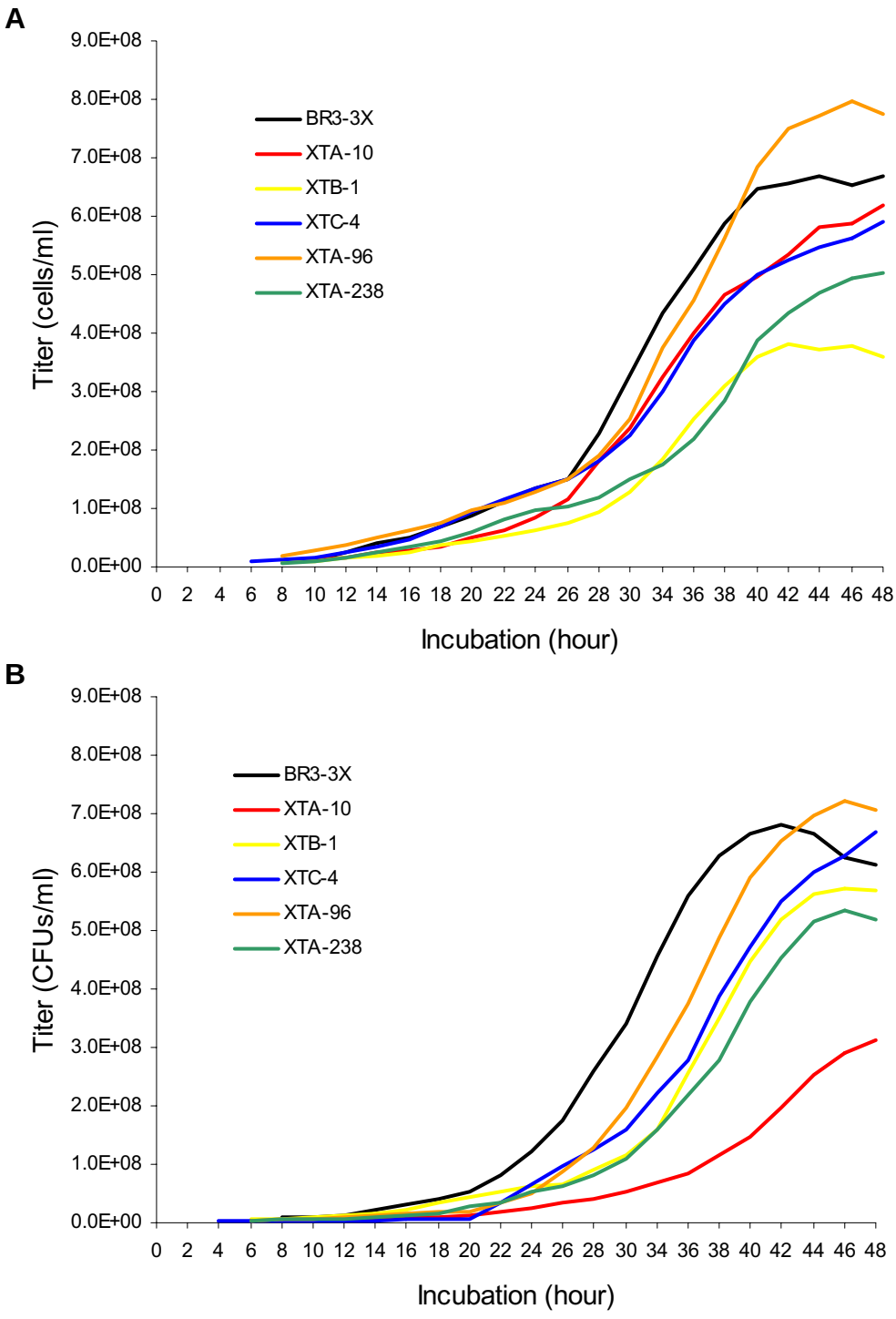

Figure 7. Growth rate curves of *S. citri* and its transformants within 48 hours based on direct count of number of cells per ml **(A)**, and colony forming units (CFUs) formed on LD8 plates **(B)**

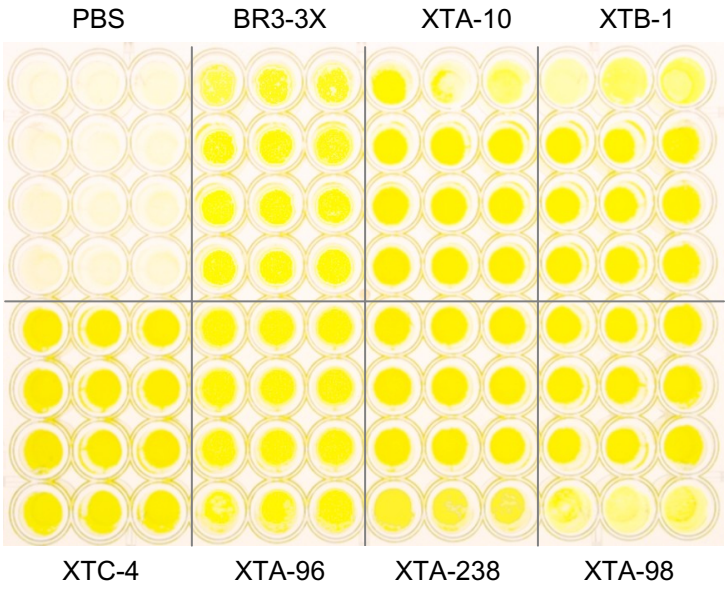

Figure 8. Microtiter plate assay to assess adherence of *S. citri* BR3-3X and its transformants to CT-1 cells

Yellow dot = positive reaction

CHAPTER V

ENRICHMENT FOR NATURAL BINDING-DEFICIENT *SPIROPLASMA CITRI* VARIANTS USING THE CT-1 CELL LINE OF *CIRCULIFER TENELLUS*

Abstract

Transmission of phytopathogenic spiroplasmas involves adherence to cells within their insect vectors. Identification of non-adhering lines would facilitate identification of genetic and phenotypic determinants of adhesion. We sought adherence-defective *Spiroplasma citri* via selection from a natural population. Enrichment for naturally occurring, binding deficient variants of *S. citri* BR3-3X was done by selection on tissue-cultured cells of the vector, *Circulifer tenellus* Baker, to which BR3-3X is known to adhere. The selection was carried out by two methods, i.e. by spiroplasma passages on immobilized CT-1 monolayers and by incubation of mixed suspensions of spiroplasmas and CT-1 cells. Regardless of the method used, the number of unbound spiroplasma cells declined with each passage until the 8^{th} to 10^{th} passage, after which it stabilized at less than 0.5% of the number of cells initially applied. The leveling of the population suggests that spiroplasmas remaining in the suspension by the 8^{th} to10^{th} passage were non-adherent.

Introduction

The mechanism of transmission of spiroplasmas by insects is thought to involve specific molecular interactions, especially during the event of spiroplasma traversal of the insect membrane barriers (Fletcher et al., 1998). During their movement within the insect body, spiroplasmas have to pass through the gut and salivary gland. Initially, they move into the midgut, adhere to receptors on the gut epithelial cells' apical plasmalemma and are taken into the cytoplasm by endocytosis. After spiroplasmas pass through and escape from the epithelial cells, they cross the basal lamina into the hemocoel. After multiplication in the hemolymph, spiroplasmas pass through the salivary gland basal lamina and adhere to receptors on the plasmalemma. They are taken up by endocytosis and escape into the salivary ducts (Fletcher et al., 1998; Kwon et. al., 1999).

Insect transmissibility of spiroplasmas can be lost due to selection pressure during continuous maintenance of spiroplasmas in the absence of the insect vector, either by subculture in artificial medium or by continuous grafting in host plants (Wayadande & Fletcher, 1995). It was suggested that the cell-free culture condition is unfavorable for spiroplasma to maintain its ability to adhere and penetrate insect cells or cuticle and to function metabolically in the insect host cell environment. However, the ability of spiroplasmas to survive and multiply is not affected by *in vitro* passages. The loss of transmissibility may result from the loss of elements necessary for interaction with insect receptors during translocation through the insect membrane (Mowry et al., 1986).

Phenotypic and genotypic variation in transmissible and non-transmissible spiroplasma lines has been revealed (Fletcher et al., 1996: Melcher & Fletcher, 1999; Ye et al., 1996). Also, the adherence of spiroplasmas to insect cells is suggested to play a role in transmission (Berg et al., 2001; Fletcher et al., 1998; Yu et al., 2000). For better understanding of the mechanism of the transmission, we sought to investigate a correlation between the ability of spiroplasmas to bind to insect cells and their insect transmissibility, an experimental approach that required non-binding spiroplasmas. A *C. tenellus* (CT-1) cell line has been established and utilized for the study of spiroplasma-insect interactions including spiroplasma binding ability (Wayadande & Fletcher, 1998; Yu et al., 2000). A population of spiroplasmas may contain natural variation. In this case, we sought to enrich for naturally occurring non-binding spiroplasmas by screening a natural *S. citri* BR3-3X population using the CT-1 cell line.

Materials and Methods

Spiroplasma

S. citri BR3-3X was grown in LD8 broth (Lee & Davis, 1984) to log phase (2 days old, approximately 10^9 cells/ml). The cells were pelleted by centrifugation at 8,000 g for 5 minutes at 4 °C, and resuspended in PBS-S (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4 containing 10% sucrose).

Source and Maintenance of CT-1 Cell Line

The *Circulifer tenellus* (CT-1) cell line used in this assay was established by Wayadande & Fletcher (1998) and stored in liquid nitrogen. A frozen CT-1 cell suspension was thawed quickly in a water bath at 37 °C and removed to a 50 ml Corning tube. NT medium (40% Schneider's Drosophila medium, 4% medium 199 + Earle's salts (10X), 2% medium CMRL 1066, 12% fetal bovine serum, 40% of 0.057M histidine buffer, pH 6.2) was added dropwise, swirling repeatedly, to a total volume of 2 ml. Then, consecutive two-drop volumes of NT medium were added and swirled, to a total volume of 5 ml. Finally, five drops of NT medium were added and swirled to obtain a 10 ml suspension. The addition of NT medium was done gradually to facilitate gentle separation of the aggregated CT-1 cells, in order to provide a homogenous cell suspension. The cells were pelleted at 900 rpm for 5 minutes at 4 °C, resuspended with 5 ml fresh NT medium, transferred to a 25 cm^2 flat-bottomed tissue-culture flask and incubated at 28 °C for 2 to 3 weeks to form a cell monolayer. Every 2 weeks, the NT medium was replaced with fresh medium and the CT-1 cells were split into two new flasks as follows. Old medium was gently aspirated off, the cells were rinsed twice with 3 ml PBS, and 250 μl trypsin-EDTA was added. After 5 minutes incubation at room temperature, the flask was tapped several times to detach the cells. Trypsinization was stopped by adding 3 ml NT medium. The cells were dislodged from the flask surface by gentle aspiration, and pelleted at 900 rpm for 10 minutes. After discarding the supernatant, the pellet was resuspended in 10 ml NT medium and transferred to two new flasks.

Selection of Naturally Occurring Non-Binding Spiroplasma

Selection of natural non-adherent *S. citri* variants using the CT-1 cell line was done by three techniques: (1) consecutive passages of spiroplasma cells on immobilized, confluent CT-1 monolayers, (2) consecutive rocked incubations of mixed spiroplasma and CT-1 cell suspension (3) consecutive un-rocked incubations of mixed spiroplasma and CT-1 cell suspension.

For technique (1), 5 ml spiroplasma cell suspension (5 X 10^8 cells/ml) was incubated successively with 10 CT-1 monolayers in 25 cm^2 flasks. Just before being used, the CT-1 monolayers were washed twice with 3 ml PBS-S. After PBS-S removal the remaining spiroplasma suspension was transferred into the flask and rocked for 2 hours. A volume of 50 μl spiroplasma cell suspension was removed for direct cell counts using dark-field microscopy and CFU enumeration by plating onto agar-solidified LD8. The remaining suspension was immediately transferred onto a new pre-washed CT-1 monolayer for the next passage. This process was repeated for a total of ten consecutive passages. The final suspension, which should be enriched for natural non-binding spiroplasma mutants, was plated onto agar-solidified LD8 and incubated at 31 °C for 7 days. Each single colony was recovered and grown in LD8 broth to log phase. The culture was aliquoted into small vials (1 ml) and stored at -80 °C until used.

In technique (2), a CT-1 cell suspension was prepared as follows. NT medium was removed from a CT-1 cell monolayer in a 25 mm^2 flask. The monolayer was washed twice with 2.5 ml PBS-S, and trypsinized with 250 μl

trypsin-EDTA for 5 minutes. The CT-1 cell suspension in trypsin was diluted with 2.5 ml PBS-S and centrifuged at 1000 rpm for 10 minutes at 4 °C. The pellet was resuspended in 5 ml spiroplasma cell suspension (2.5 X 10^8 cells/ml), briefly vortexed, and incubated for 1 hour at room temperature on a rocking platform (Red Rocker model PR-50, Hoefer Scientific Instrument, San Francisco) at speed level 5. After centrifugation (as above) a sample (200 μl) of the supernatant was removed for counting unbound spiroplasma cells. The remaining supernatant was placed into a new CT-1 cell pellet. These steps were repeated for a total of ten times. Technique (3) was similar to technique (2), except that the mixture was not rocked during incubation. Determination of the cell titer for approaches (2) and (3) was done as in approach (1).

Results and Discussion

In the selection of putative natural non-adherent variants, the results were similar regardless of the methods of selection (Table 1). The number of spiroplasma cells not bound to CT-1 cells decreased from the 1st to the 7th passage. The number of unbound spiroplasma cells in the 8th to the 10th passages appeared to be constant, which suggested that the remaining cells were incapable of adhering to CT-1 cells (Figure 1).

The titers of spiroplasma cells and CFU after each passage (incubation) on CT-1 monolayers are shown in Table 1 and in Figures 1 and 2. The highest percentage of spiroplasmas bound to insect cells occurred in the 1st and 2nd passages. It is likely that during these two incubations, most of the adherence-

capable spiroplasma cells attached to the insect cells. In the following passages, spiroplasma titers remained relatively constant. However, the titer curve showed a slight increase over time, which may be the result of propagation of spiroplasma cells, including the small portion of non-binding cells, during the incubation periods, which lasted about 20 hours. Consistent with this explanation was that the final titer of non-binding spiroplasma cells after 10 consecutive incubations was 1.09×10^8 (82.1%). This titer was surprisingly high, similar to that measured in the first incubation.

In these experiments, one disadvantage of direct spiroplasma cell counts was that broken insect cell appendages (fibrils), which resemble spiroplasma cells in shape and size, were present in the spiroplasma cell suspension, resulting in confusion in enumeration of spiroplasma cells. An alternative and possibly more reliable method to determine the proportion of the non-binding cells, is CFU enumeration by plating of spiroplasma. Using this method, the number of spiroplasma CFUs in suspension proportionally decreased from the 1st to 7th passage indicating that the number of cells binding to insect cells is constant. We speculated that the number of binding sites on the insect monolayer used in each passage/incubation was about one seventh of the initial number of spiroplasma cells incubated. The CT-1 cell binding sites appeared to be saturated by attachment of binding spiroplasma cells during 1st to 7th passages. In other words, approximately one seventh of the adherent spiroplasma cells was bound to CT-1 binding sites available at each passage within seven passages. The remaining spiroplasmas that did not bind to insect

cells may be considered as natural non-binding spiroplasmas (from 8th to 10th passages).

The unbound spiroplasmas provided by the selection methods were plated onto LD8 agar. However, they grew poorly when grown in LD8 broth. Poor growth of the selected spiroplasmas might be because their cell viability decreased during incubations/passages in the PBS-S environment, which took about 20 hours. The PBS-S is not a suitable medium for culturing either CT-1 cells or spiroplasmas, but was used during the selection to maintain viability without multiplication. However, maintenance in PBS-S might not sustain the spiroplasma viability over a long time. To minimize the loss of the spiroplasma viability during selection, we might be able to improve the methods of selection by reducing the number of passages by lowering the ratio of the number of CT-1 binding sites to spiroplasmas (in selection method 1) or by low centrifugation of the CT-1-spiroplasma mixture without incubation (method 2).

Once the non-binding *S. citri* variants can be selected and grown successfully, the following step will be to test them using the microtiter plate adherence assay to confirm whether their adherence is affected. The non-adherent variants would be useful for study of biology and genetics of insect-vector relationships.

Conclusions

The method of selection of naturally occurring non-binding *S. citri* variants using CT-1 cells is an entirely new strategy. It apparently worked, as indicated

by leveling of the titers at late passages (eighth to tenth). The method of selection needs to be improved, possibly by shortening the period of incubation of the cells in PBS-S. The microtiter plate adherence assay (MPA) can then be performed for those natural variants successfully selected to see whether the binding ability is affected.

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Table 1. Titer and number of unbound *Spiroplasma citri* after serial 2-hour incubations with the *Circulifer tenellus* (CT-1) cell line

Passage	Cell titer			Percentage of *S. citri* cells remained unbound (%)		
	Incubation on monolayer	Low speed centrifugation		Incubation on monolayers	Low speed centrifugation	
		Rocked[a]	Unrocked[b]		Rocked	Unrocked
A. Direct count (cells/ml)						
0	1.34×10^8	7.52×10^7	7.52×10^7	-	-	-
1	1.10×10^8	4.52×10^7	4.84×10^7	82.09	60.11	64.36
2	8.57×10^7	3.82×10^7	2.36×10^7	63.95	50.80	31.38
3	8.41×10^7	1.28×10^7	2.27×10^7	62.72	17.02	30.19
4	9.63×10^7	1.34×10^7	1.85×10^7	71.87	17.82	24.60
5	8.49×10^7	1.13×10^7	6.79×10^6	63.34	15.03	9.03
6	9.51×10^7	5.63×10^6	5.09×10^6	70.91	7.49	6.77
7	1.05×10^8	4.68×10^6	5.50×10^6	78.63	6.22	7.31
8	8.96×10^7	4.20×10^6	3.30×10^6	66.87	5.59	4.39
9	1.07×10^8	4.40×10^6	2.39×10^6	79.86	5.85	3.18
10	1.09×10^8	4.04×10^6	2.56×10^6	81.17	5.37	3.40
B. Plating on LD8 (CFUs/ml)						
0	3.58×10^8	2.73×10^8	2.41×10^8	-	-	-
1	2.73×10^8	1.27×10^8	1.35×10^8	76.28	46.57	56.03
2	2.41×10^8	9.78×10^7	5.53×10^7	67.23	35.83	22.92
3	1.56×10^8	2.65×10^7	3.29×10^7	43.71	9.71	13.64
4	1.19×10^8	2.00×10^7	2.88×10^7	33.37	7.34	11.95
5	4.98×10^7	6.49×10^6	8.25×10^6	13.91	2.38	3.42
6	1.43×10^7	3.33×10^6	2.87×10^6	3.99	1.22	1.19
7	2.60×10^6	2.24×10^6	1.25×10^6	0.73	0.82	0.52
8	1.67×10^5	7.64×10^5	2.65×10^5	0.05	0.28	0.11
9	1.84×10^5	3.55×10^5	1.45×10^5	0.05	0.13	0.06
10	5.00×10^4	3.00×10^5	1.93×10^5	0.01	0.11	0.08

[a] low centrifugation following rocked-incubation

[b] low centrifugation following unrocked-incubation

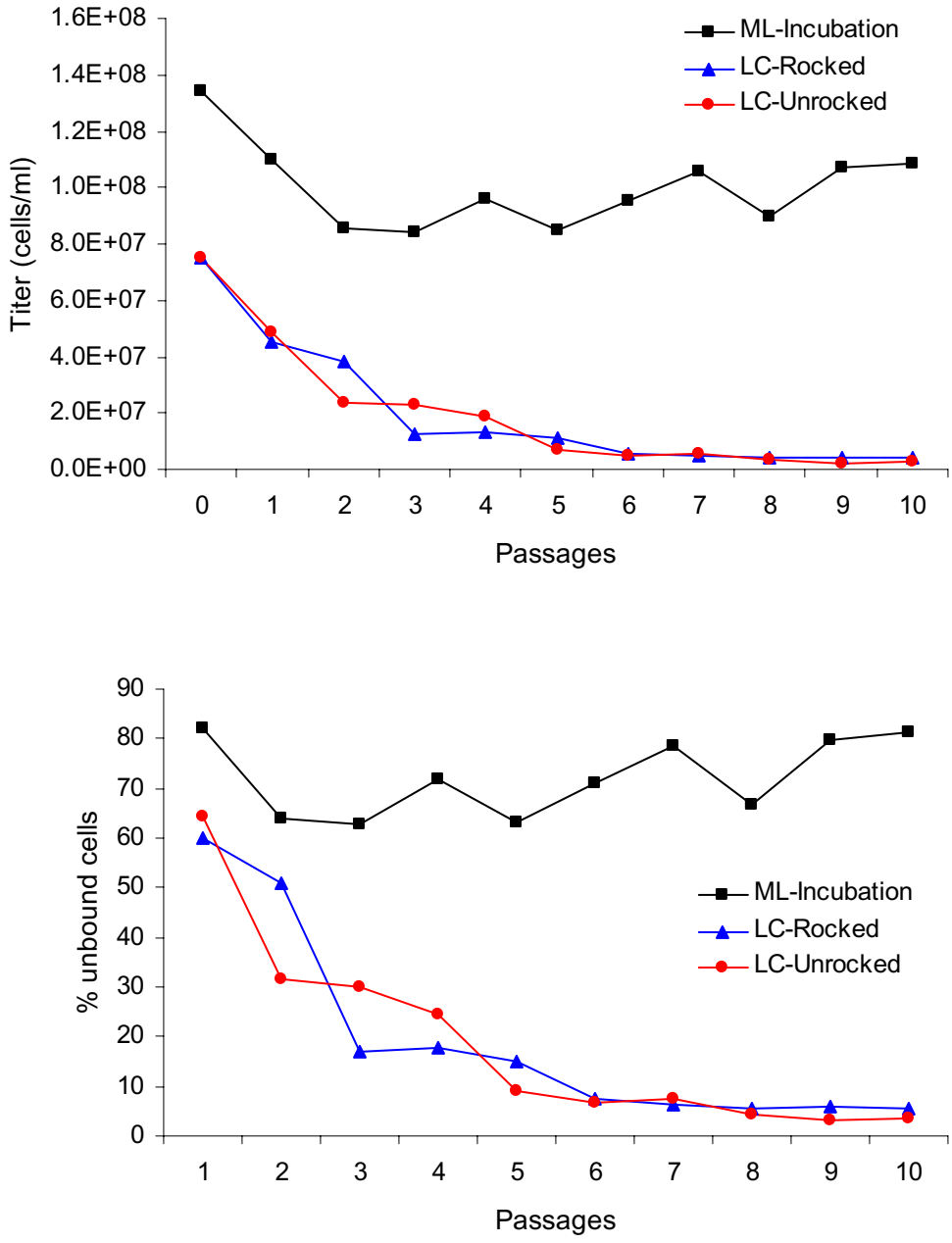

Figure 1. Number of unbound *Spiroplasma citri* cells selected using monolayers of *Circulifer tenellus* (CT-1) cell line during serial 2-hour-incubations (ML-Incubation), low centrifugation following rocked-incubation (LC-Rocked) and low centrifugation following unrocked-incubation (LC-Unrocked), represented as number of cells observed by direct count **(A)** and as the percentage of unbound cells **(B)**

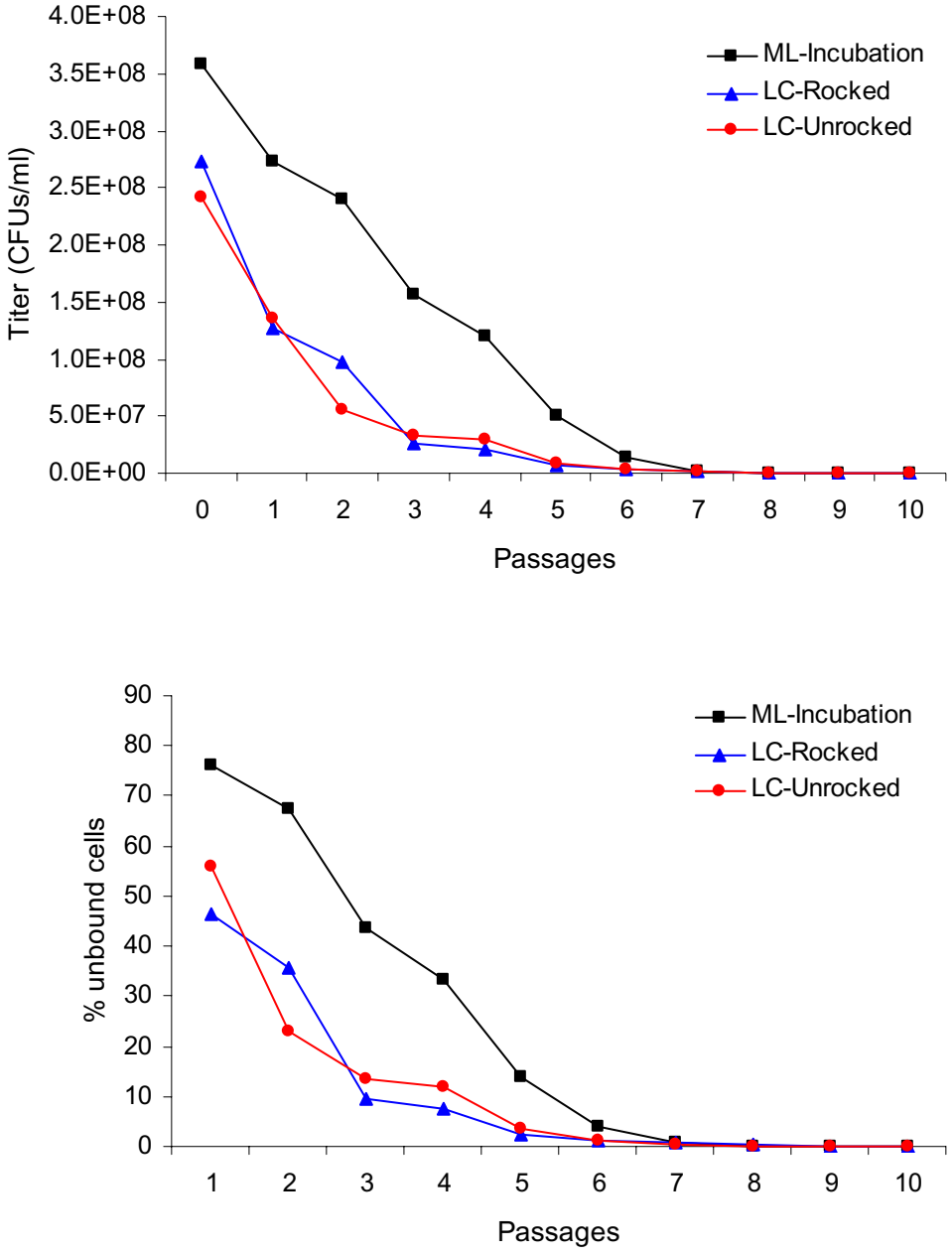

Figure 2. Number of unbound *Spiroplasma citri* cells selected using monolayers of *Circulifer tenellus* (CT-1) cell line during serial 2-hour-incubations (ML-Incubation), low centrifugation following rocked-incubation (LC-Rocked) and low centrifugation following unrocked-incubation (LC-Unrocked), represented as CFUs observed on plates **(A)** and as the percentage of unbound cells **(B)**

VITA

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Doctor of Philosophy

Thesis: MOLECULAR TYPING OF SPIROPLASMA SPECIES AND LINES USING REP-POLYMERASE CHAIN REACTION AND TRANSPOSOME MUTAGENESIS AND SELECTION OF NATURAL NON-ADHERENT MUTANTS OF *SPIROPLASMA CITRI*

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