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Mini review

Genetic variation in *Spiroplasma citri*

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Abstract

Spiroplasmas are members of the Class Mollicutes, wall-less prokaryotes having a high adenosine–thymidine content in their small genomes. *Spiroplasma citri* is a plant pathogen that inhabits phloem. Like other phytopathogenic spiroplasmas and the related phytoplasmas, it is transmitted from plant to plant by phloem-feeding leafhoppers that serve as alternate hosts for the spiroplasma as well as vectors. Genetic information in spiroplasmas is carried on a circular chromosome, on plasmids and/or in virus genomes. A picture emerging from recent research on the *S. citri* genome is one of frequent and often extensive variation, resulting from a number of different mechanisms. Expansion and contraction events must continually be occurring in about equal proportions so that the net genome size varies within defined boundaries. Particularly impressive are large changes in genome size that can occur in only a few generations. As with most organisms, genetic variation in *S. citri* results from variation in extrachromosomal DNA content, changes due to DNA replication and repair processes and changes due to recombination. The implied flux of genetic information into and out of the *S. citri* genome should be beneficial to the bacterium, allowing it, with its small genome size, to adapt to new environments.

Introduction

The Class Mollicutes is comprised of several genera of prokaryotes that lack cell walls and have a high (up to 75%) adenosine–thymidine content in their small genomes. These microbes probably evolved by genome reduction from Gram-positive walled precursors (Woese et al., 1980). Mollicute sizes are correspondingly small and the genus *Mycoplasma*, which contains the smallest known cellular organisms, is a model for the definition of the molecular machinery of a minimal self-replicating cell. Complete genome sequences have been determined for two mycoplasmas, *M. pneumoniae* (Himmelreich et al., 1996) and *M. genitalium* (Fraser et al., 1995) and for a related mollicute, *Ureaplasma ureolyticum* (Glass et al., 1998). Key bacterial enzyme pathways missing in mollicutes include those for cell wall production, purine biosynthesis, a functional tricarboxylic acid cycle, and cytochrome-based electron transport (Pollack et al., 1997; Dybvig and Voelker, 1996). The resulting inefficiency of their metabolic activity at least partially explains the fastidious nutritional nature of the mollicutes. Although some mollicutes continue to resist cultivation in artificial media, many spiroplasmas can be grown *in vitro*, and the first isolation of *Spiroplasma citri* was reported in the early 1970s (Saglio et al., 1971; Fudl-Allah et al., 1972). Members of the genus *Spiroplasma* are unique among the mollicutes in their helical morphology. Spiroplasmas of different species have genomes of 990–2200 kb (Carle et al., 1985). Like most other mollicutes, spiroplasmas utilize UGA to encode tryptophan rather than as a ‘stop’ codon. Many mollicutes (including spiroplasmas) contain viruses and/or plasmid DNA.

Life/disease cycle

Most mollicutes are parasites and/or pathogens of eucaryotic organisms. *Mycoplasma* spp. cause numerous diseases of the respiratory and urogenital tracts of humans and animals, while phytoplasmas and spiroplasmas primarily infect insects and plants. Spiroplasmas inhabit plant phloem, where they absorb the nutrient-rich sap and multiply to high titers, possibly inhibiting movement of nutrients and often leading to phloem necrosis and collapse. Plant hosts, including citrus, brassicaceous plants such as horseradish, turnip and numerous weeds, and maize and its relatives, become chlorotic and stunted. Flowers and leaves may be smaller than usual, but otherwise appear normal. Phytopathogenic mollicutes may cause reductions in crop yield or even result in death of the host plant.

Phytopathogenic spiroplasmas and phytoplasmas are transmitted in nature by phloem-feeding Homopteran insects, predominantly leafhoppers (Purcell, 1982). The transmission is propagative; that is, mollicutes ingested from phloem traverse the insect gut wall, multiply in the hemolymph, and move into the salivary glands from which they are discharged into a new plant. The relationship between the insect and the mollicute is very specific. An insect species transmits only certain spiroplasmas or phytoplasmas, although non-transmissible types may be detected within the insect. Conversely, a particular mollicute is transmitted only by certain insect species. The insect often is affected adversely by the infection, as evidenced by reduced longevity and fecundity.

Insect-spiroplasma interactions are clearly crucial to the biology and epidemiology of the pathogen, yet little is known about the specific events at the microbe-vector interface that result in spiroplasma movement through physical barriers in the insect and contribute to transmission specificity (Fletcher et al., 1998). The genome of an insect nontransmissible line of *Spiroplasma citri*, which arose during generations of plant-to-plant grafting (Wayadande et al., 1995), differed from the transmissible parent line in a major chromosomal inversion and two deletions (Ye et al., 1996). An open reading frame in one of the deleted regions had sequence similarity to the adhesin protein of two zoopathogenic mycoplasmas and may encode a protein involved in the adherence of the spiroplasma to gut epithelial or salivary gland cells of the insect vector. Such adherence is hypothesized to precede spiroplasma invasion of the insect cells and traversal of these cellular barriers within the body of the vector.

Foissac et al. (1996, 1997), using the transposon Tn4001, developed two mutants of *S. citri* strain GII3 that failed to cause symptoms in plants caged with microinjected leafhoppers. In one mutant, which retained insect transmissibility but had a reduced multiplication rate in plants, the transposon had inserted into a gene within the fructose operon, coding for a protein that showed similarity with the repressor of the *E. coli* deoxyribonucleotide operon. No spiroplasmas could be cultured from plants exposed to insects carrying the second mutant line, nor could this spiroplasma be detected in such plants, indicating that transposon insertion had affected the spiroplasma's ability either to be transmitted or to multiply in the plant. This mutant failed to multiply in the leafhopper and displayed a lower growth rate and lower final titer in growth medium. Whether spiroplasmas were actually introduced into the test plants could not be determined. In this case, the transposon had inserted into a gene encoding a putative product with similarity to a calcium-transporting ATPase.

Arthropods that do not transmit plant pathogens also may harbor wall-less prokaryotes. Hackett and Clark (1989) noted that nonphytopathogenic spiroplasmas are widely distributed in arthropods, and speculated that spiroplasmas may be among the most abundant microorganisms on earth. Some cause disease in their insect hosts (Humphery-Smith et al., 1991; Whitcomb and Williamson, 1979; Williamson et al., 1989), although most apparently coexist in a benign relationship with the insect (Whitcomb and Williamson, 1979). The spiroplasmas may be confined to the intestine or may colonize the hemolymph as well (Hackett et al., 1990). The physical and physiological factors that determine whether phytopathogenic mollicutes are transmissible by a certain insect may also determine whether an insect can harbor a particular mollicute and whether that microorganism is able to penetrate the intestine wall and colonize the hemolymph.

Genetic variation

Protein profiles

The genome size of spiroplasmas indicates coding capacity for approximately 1000 proteins (Cordwell et al., 1997). Although electrophoretic protein patterns differ between spiroplasma species (Whitcomb et al., 1986), overall protein profiles of strains within

a species are relatively similar (Fletcher et al., 1981). Yet, divergence has occurred. Spiralin, the major *S. citri* membrane protein, usually migrates to 26 kDa, but Mouches et al. (1979) and Foissac et al. (1996) noted different spiralin mobilities among strains of *S. citri*. Foissac's group, investigating protein sequences deduced from corresponding DNA, noted that although all but one of the spiralins had 241 amino acids, amino acid substitutions had occurred in one to 22 positions. These changes could not account for the differences in mobility, however, and differences in acylation were also ruled out as responsible (Foissac et al., 1996). Fletcher et al. (1996) reported an anomaly in spiralin mobility among lines of *S. citri* derived from a common parent (BR3) after long-term propagation under different maintenance regimes. These derived lines differed in their ability to be transmitted by the leafhopper, *Circulifer tenellus*. In most of the lines, spiralin had the expected 26 kDa mobility, but one line had proteins of both 23.5 and 26 kDa mobilities, and subclones of this line expressed only one or the other of the two bands. Probable spiralin homologues have been detected in other spiroplasma species (Zaaria et al., 1990; Wroblewski et al., 1987; Archer and Townsend, 1981; Bove et al., 1993; Whitcomb et al., 1983), indicating that a spiralin-like protein is probably ubiquitous in spiroplasmas (Bove et al., 1993).

Other protein differences also were noted in BR3 derived lines. Two insect-transmissible derivatives each contained two proteins missing in the non- or rarely-transmitted lines (Fletcher et al., 1996). In addition, one protein was unique to each of two still-transmissible lines. Mowry (1986) reported the loss of a different protein in a nontransmissible BR3 line. Whether any of these protein differences contributed to the changes in insect transmissibility is not known. It is clear that changes in genome sequence or gene expression, or both, occur relatively frequently in spiroplasmas. Variation in spiroplasma surface proteins also is implied by the resistance of some *S. melliferum* strains to SpV4 virions, but not to DNA of these viruses (Renaudin and Bove, 1994).

The variation in electrophoretic mobility noted for spiralin in BR3-G is reminiscent of surface protein variation in several human and animal pathogenic mycoplasmas. Phenotypic switching, including differential presence or absence of a protein and protein size variation, occurs. Affected proteins have been termed variable lipoproteins (VLPs), variable surface proteins (VSPs), or variable adherence-associated antigens (VAAs). Phenotypic switching may be triggered by different environmental conditions or exposure to different host cells or tissues, and may result in the adaptation of the mycoplasma to diverse host environments and to evasion of the host immune response. The phenomenon results, in different mycoplasmas, from different molecular events. These include the gain or loss of intragenic repetetive sequences (Zhang and Wise, 1996; Citti and Wise, 1995; Lysnyansky et al., 1996), deletion/insertion mutations (Citti and Wise, 1995; Theiss and Wise, 1997), DNA inversion (Simmons et al., 1996; Lysnyansky et al., 1996), differential presentation of expressed surface proteins (Theiss et al., 1996), and presence of multigene families or multiple copies of partial genes in the mycoplasma chromosome (Behrens et al., 1994). Such variation in surface protein expression has not been reported for spiroplasmas.

Conserved genes

Contrasting with the strain variability among spiralin genes is the conservation of other genes. An *Apa*I site in the p6 sequence is conserved in all *S. citri* strains (Ye et al., 1995). *Eco*RI sites in the *fib* gene are conserved between *S. citri* and *S. melliferum* (Williamson et al., 1991). The *dnaA–dnaN–gyrB* region of strains R8A2 and ASP1 are 100% identical (Renaudin et al., 1995), as are the remnants of a *recA* gene in these two strains (Marais et al., 1996a). Analysis by PCR of the *recA* genes of six strains revealed no differences. The remnant can encode the N-terminal one-third of the recA protein (Marais et al., 1996b). The sequence truncating the *recA* gene is a partial degenerated copy of a gene, characterized in strain BR3-3X, for a 123 kDa protein (Melcher et al., 1999). At the species level, the truncation represents a variation since the truncated *recA* gene was not found in *S. melliferum*, *S. phoeniceum* or *S. apis* (Marais et al., 1996a). *S. melliferum* has a complete gene, though it is interrupted by a termination codon that also appears in the RNA transcript. Occasional read through of this termination codon is implied by the observation of both full-length and truncated recA protein products in the proteome of *S. melliferum* (Cordwell et al., 1997). The absence of strain-dependent sequence variation in an apparently non-functional *S. citri* gene suggests that such variation in the *S. citri* spiralin gene may be of biological significance. This view is supported by the presence in that gene of almost twice as many non-synonymous as synonymous substitutions (Foissac et al., 1996).

Multiple gene families

The smallest characterized mollicute genome, that of *M. genitalium* (Fraser et al., 1995), has a set of repeated sequences related to the gene for the major surface antigen, which serves as adhesin for the bacteria's attachment to host cells. The larger size of the *M. pneumoniae* genome is mostly due to families of lipoprotein and variable surface antigen genes (Himmelreich et al., 1997). Multiple gene families in mycoplasmas function in the bacteria's evasion of mammalian host defense responses. *S. citri* does not have a known mammalian host, but it still may contain multiple gene families. Two copies of the *fib* gene for fibrillin are found in strain Alc-254 but not in other strains (Ye et al., 1995). During investigation of possible insect transmissibility genes (Ye et al., 1996), a gene for a 58 kDa polypeptide with sequence resemblance to mycoplasma adhesins (Ye et al., 1997) was identified. Southern blotting and hybridization suggested the presence of two further copies of this gene in the BR3-3X strain from which it was isolated (Ye et al., 1996). A small survey of other *S. citri* strains revealed variation in copy number (1–3) of this gene (Ye, Melcher and Fletcher, unpublished). Hybridization probes generated by arbitrarily primed PCR from *S. citri* cDNA (Rascoe et al., 1996), when used to probe Southern blots, revealed multiple copies of some of the corresponding genes. It is not immediately obvious why *S. citri* multiple gene families should survive selection pressure for small genome size, if indeed they do.

Gene regulation and expression variations

The potential for differential regulation of gene expression is a possible reason for having multiple gene families. We have already noted that differential gene expression under different environmental conditions may lead to the phenomenon of phase switching of variable surface antigens in mycoplasmas. There is some evidence for differential regulation of expression among lines of a spiroplasma species. When arbitrarily primed PCR was used to amplify cDNA of several *S. citri* BR3-derived lines that differ in biological activity, line-dependent banding patterns were obtained with several primers (Rascoe et al., 1998). Northern and Southern blots suggested the possibility that some of the bands represented genes present in all the lines but expressed only in a subset of them (unpublished data), and such gene regulation may account for the observed differences in spiroplasma behavior.

Gene expression in mollicutes apparently is controlled in a manner similar to that of walled, Gram-positive bacteria (Bove, 1993; Dybvig and Voelker, 1996). At the transcription level, gene expression can be modulated by differential synthesis of RNA polymerase sigma factors, by the action of repressors and activators or by differential termination of transcription. The spiroplasma core RNA polymerase has the eubacterial subunit organization $\alpha_2\beta\beta'$ (Gadeau et al., 1986). To date, all characterized spiroplasma genes have promoters that conform to the consensus sequence TATAAT (-10) and TTGACA (-35) and only a single sigma factor gene has so far been characterized (Ye et al., 1992). Repressors were not known in mollicutes (Bove, 1993) until recently, when a *M. genitalium* MG205 transcription repressor (hrcA) was identified (Fraser, 1995). Differential regulation of transcription termination may operate in *S. citri* since the *rpsB* gene sequence is present in two transcripts, one monocistronic and the other polycistronic (Le Dantec et al., 1998a). The potential termination transcript for the monocistronic signal is a binding site for a protein that probably is ribosomal protein L29 (Le Dantec et al., 1998b), suggesting that L29 regulates termination. Previous suggestions for regulation in this region of the genome (Chevalier et al., 1990) have since been discounted (Le Dantec et al., 1998a). An additional potential regulatory mechanism is the methylation of the DNA template for transcription. In eukaryotes, methylation of bases at specific nucleotide sequences is a mechanism for regulation of gene expression (Nur et al., 1985). Although most prokaryotes methylate both adenine and cytosine, most spiroplasma species resemble eucaryotes in methylating only cytosine (Nur et al., 1985). Methylation is discussed further below.

At the translation level, Shine–Dalgarno sequences for ribosome binding to mRNA have been identified in mollicutes 5–10 nucleotides upstream of translation start sites. Variation in their sequence will lead to variation in the efficiency of translation initiation. Translation normally is terminated by an inverted repeat followed by a terminator codon (Bove, 1993); in most mollicutes, including spiroplasmas, only two termination codons (UAA and UAG) are possible since UGA encodes tryptophan. The possibility of suppression of termination codons was mentioned above in discussion of the *recA* gene.

rRNA operon number, rRNA polymorphisms, tRNA genes

The organization of mollicute rRNA operons is similar to that of bacteria, with the genes (5′) 16S, 23S, and 5S (3′) in that order. As in *E. coli* and *B. subtilis*, transcription of the *S. citri* rRNA operon is initiated by one of two adjacent promoters, P1 and P2, just upstream of the 16S rDNA gene. Most bacteria have several rRNA operons; *Escherichia coli* has seven and *Bacillus subtilis* ten (Bove, 1993). In contrast, most mycoplasmas and phytoplasmas, and a few spiroplasmas, have only two copies (Amikan et al., 1984; Schneider and Seemuller, 1994). Most spiroplasmas, including the single *S. citri* strain tested (Grau et al., 1990; Bove, 1993), have only one rRNA operon. More important in the consideration of genomic change is the fact that the rRNA gene complex of *S. citri* strains shows variation in the mobilities of probe-reactive *Eco*RI and *Hin*dIII fragments (Ye et al., 1995).

Although tRNA genes of most bacteria are located between the 16S and 23S genes and within the rRNA operon, the phytoplasma–acholeplasma branch of the mollicutes is the only one known to have this arrangement (Razin et al., 1998). A small number of mycoplasma species have tRNA genes located between the two promoters, but in most other mollicutes, including the spiroplasmas, they are located completely outside the rRNA operon. *M. capricolum* has 29 different tRNA species, similar in number to the 31 in *B. subtilis* (*E. coli* has 45). These 29 mycoplasma tRNAs, however, are encoded by only 30 different tRNA genes (only the lysine gene is duplicated), whereas gene duplication is far more prevalent in the two other bacteria.

In many organisms, the terminal nucleotide base in a triplet codon may vary without changing the target amino acid ('wobble'). *M. capricolum* lacks tRNA having the anticodon CCG, which is required for translation of CGG in eubacteria, suggesting that CGG may be an unassigned codon in this mycoplasma (Andachi et al., 1989). In contrast, *S. citri* had three copies of the codon CGG within a region of 1776 codons (Bove, 1993). These two mollicutes are in the same phylogenetic group, and the CGG codon difference is among the evidence pointing to a divergence of the mycoplasmas from a spiroplasmal precursor. In addition, the fact that UGA is used for tryptophan in mycoplasmas and spiroplasmas, but not in acholeplasmas, is consistent with the conclusion that acholeplasmas were early derivatives of walled bacteria, with spiroplasmas evolving from that group later on (Bove, 1993).

Genome size

The size of the *S. citri* chromosome varies among strains from 1.6 to 1.9 Mbp (Ye et al., 1995), a range somewhat narrower than that for the species (Carle et al., 1995). These large variations suggest that genome size is not an indicator of phylogenetic position (Ye et al., 1995). The genome is about three times as large as that of *M. genitalium*. Some of this larger size is due to the retention in *S. citri* of genes that have been lost in *M. genitalium* (Razin et al., 1998). In addition, some extra material in *S. citri* chromosomes could represent repeated genes, IS elements, integrated versions of extrachromosomal elements or genes specific to *S. citri* interaction with its two disparate hosts, the plant and the leafhopper. The size variation is not localized to one region of the chromosome since numerous large restriction fragments vary, but one area is more variable than others (Ye et al., 1995). Only some of the variation in restriction fragment lengths is due to variation in the number of restriction sites. Considerable variation in the sizes of restriction fragments generated by frequently cutting enzymes was noted in a comparison of lines derived from strain BR3-3X (Fletcher et al., 1996).

Size variation of *S. citri* chromosomes is a continuing process with major changes occurring during laboratory propagation (Ye et al., 1996). During derivation of BR3-T from BR3-3X by repeated leafhopper transmission, most *Sal*I fragments increased in length and no decreases were apparent. Thus, the *S. citri* chromosome readily undergoes expansion. The use of triply cloned individuals for the propagation reduced competition between variants in the population. It is possible that, in the long range and in nature, expansion is balanced by selection for contraction, resulting in a constant mean size for the chromosome.

Gene order

Except for size variation, the restriction maps available for *S. citri* strains (Ye et al., 1995) are, for the most part, consistent with one another. Their construction included the probing by hybridization with selected cloned genes or chromosomal fragments (Ye et al., 1992), suggesting that genetic maps, had they been

constructed, would also show little variation. Thus the *S. citri* chromosome has not undergone frequent major rearrangements. However, one major inversion event has been described (Ye et al., 1996). It occurred during the derivation of BR3-G by repeated graft transmission from BR3-3X. Approximately half the chromosome was inverted relative to the other half. The inversion was accompanied by deletions of several kbp of sequence at each end, evidence that genome contraction can occur. DNA rearrangements may have biological consequences. In *M. agalactiae* they are associated with variable expression of surface antigens (Glew et al., 1998). In *S. citri*, loss of leafhopper transmissibility may be associated with the BR3-G inversion (Wayadande and Fletcher, 1995). If there is a causal relationship, it may be that inversions occur frequently in nature but are seldom detected because of negative selection. Major rearrangements of genes are seen in interspecies comparisons. *M. genitalium* and *M. pneumoniae* consist of six genome segments arranged in two different ways (Himmelreich et al., 1997). *S. citri* and *S. melliferum* differ by an inversion (Ye et al., 1994a) equivalent in size to that that occurred in the derivation of the BR3-G line. In an intergeneric comparison, no conservation of gene order was found between the *Mycoplasma* species and *Ureaplasma urealyticum* (Glass et al., 1998).

Modification methylation

A puzzling variation in the *S. citri* genome is the degree of methylation modification. One restriction enzyme and one modification enzyme have been isolated and characterized from spiroplasmas. The restriction enzyme is *Sci*NI from *S. citri* strain ASP2, a type II enzyme recognizing GCGC (Stephens, 1982). Presence of a cognate modification methylase activity is made doubtful by the finding that in the one *S. citri* strain tested mAde, but no mCyt, could be found (Nur et al., 1987). This contrasts with other *Spiroplasma* species, which methylate only cytosine (Nur et al., 1987). The modification enzyme, isolated from *S. monobiae*, methylates the CpG dinucleotide (Renbaum et al., 1990). Because of the importance of CpG methylation of mammalian genomes, it has become an important tool (Renbaum and Razin, 1992). Whether there is a corresponding restriction enzyme is unclear. Sequence analyses have led to identification of a *S. citri* gene, *hsdS*, for the specficity component of a type I restriction-modification system (Laigret et al., 1996) and an inactive copy of a DNA adenine modification methylase (Melcher et al., 1999). That spiroplasma virus RF DNA prepared from an *E. coli* strain lacking the *Eco*K restriction-modification system was infectious to spiroplasma, while DNA from strains with the system was not infectious (Pascarel-Devilder et al., 1986; McCammon et al., 1990; Renaudin and Bove, 1994), suggests that modification of adenine may tag molecules for restriction.

DNA modification is a source of genetic variation in *S. citri* in two ways. First, some individuals in a population may have a site methylated while others do not. Variation in modification affects extrachromosomal DNA. Variability in restriction site methylation in the RF DNA of SVTS2 virus facilitated cloning of the full length DNA (Sha et al., 1995). Since variable protection against restriction has not yet been documented for chromosomal DNA, some of the observations of multiple bands, detected by Southern blotting (Rascoe et al., 1996), may result from incomplete restriction. The degree of methylation is also variable with target site, suggesting that methylation specificity may be degenerate and that restriction specificity is higher. Though *Sal*I cleaves *S. citri* DNA, the number of cleavage sites is far lower than expected (Ye et al., 1992, 1994b). One site, in the gene for 16S rRNA, is completely blocked, as is a site for *Eco*RI in the rDNA (Ye et al., 1995). Second, the ability to methylate specific sequences varies among strains. Of the DNAs of 12 strains examined, three were completely resistant to *Sal*I digestion and one was resistant to *Bss*HII (Ye et al., 1995). That *S. citri* strains vary greatly in their transformability with genetically engineered DNA extracted from *E. coli* has been attributed to the strain variable activity of uncharacterized type I restriction enzymes (Renaudin et al., 1995). Padovan and Gibb (1998) speculated that differences in expected and observed restriction patterns in phytoplasmas also may be due to base modifications such as methylation.

Extrachromosomal elements

S. citri strains contain numerous extrachromosomal elements including the replicative forms (RF) of several viruses and poorly characterized circular DNAs, tentatively designated cryptic plasmids. The cryptic plasmids may be plasmids with unidentified functions or RFs of uncharacterized viruses. Plasmid gene functions have not been definitively identified. A plasmid of the erythromycin resistant M4+ strain, when transformed

into the erythromycin sensitive R8A2 strain, did confer erythromycin resistance on R8A2, but the free plasmid was not detected in the transformants. An integrated version was found, leaving open the possibility that erythromycin resistance was caused by the integration event rather than by a plasmid gene (Salvado et al., 1989). Almost every *S. citri* strain has a unique pattern of extrachromosomal DNA and some elements are common to several strains but not to all (Barber, 1983). The results of a survey of strains for the presence of plasmids pMH1 and pM41 are consistent with these conclusions (Mouches et al., 1984). These plasmids were also found in other spiroplasma species. The patterns of *Eco*RI fragments of extrachromosomal DNA of lines derived from BR3-3X varied, as did patterns from sister clones of the same line (Fletcher et al., 1996), indicating that variation in extrachromosomal DNA is a continuing process.

One plasmid whose strain distribution was characterized early, pRA1, was subsequently identified as the RF of a SpV3 virus, SV3 (Ranhand et al., 1987). Three types of viruses, SpV1, SpV2, and SpV3, are known to infect *S. citri* . The RF forms are present in infected *S. citri* strains as extrachromosomal elements. Nucleotide sequences of three SpV1 viruses have been determined (Renaudin and Bove, 1994; Renaudin et al., 1990; Sha et al., 1998) (Figure 1). Two of these are closely related, allowing detailed assessment of the variation between them. Since their replication occurs in *S. citri* cells, it is likely that insights gained from their analysis also apply to variation in the *S. citri* chromosome. The common regions of SpV1-R8A2 B and SpV1-C74 differ by scattered nucleotide substitutions. In addition, several short length differences have characteristics of strand slippage during replication (Melcher et al., 1999). Each of the two viruses also has some unique coding regions. Of particular importance are two different transposase genes and associated inverted repeat target sequences (Melcher et al., 1999). Curiously, the region corresponding to the transposase genes in the third virus, SVTS2, isolated from *S. melliferum* (Sha et al., 1995), has still a third sequence segment in it, but no similarity to transposases could be identified (Sha et al., 1998). Nevertheless, the structures of the SpV1 viruses suggests that they are packaged versions of IS element transposition intermediates and that virus-related sequences can be expected in the chromosome as well.

Integrated extrachromosomal elements

Because of the active transposase genes carried by the viruses, one expects chromosomes of *S. citri* strains to contain numerous integrated extrachromosomal

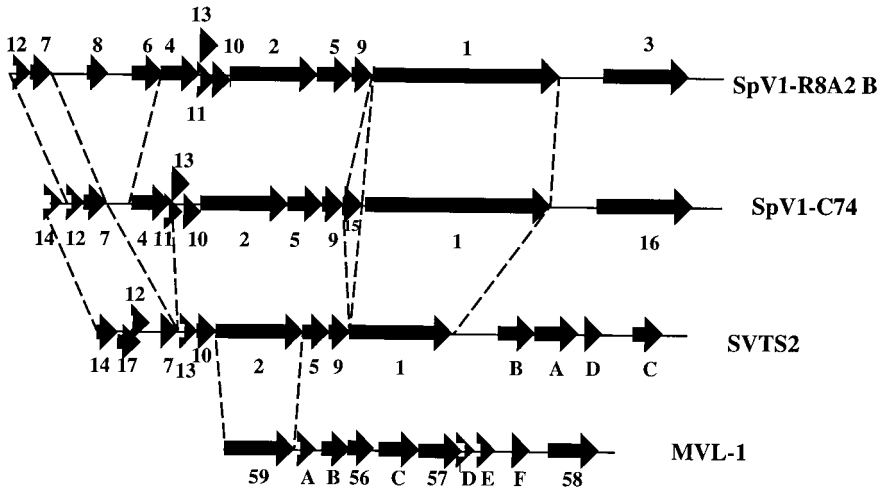

Figure 1. Comparison of the arrangement of open reading frames in the DNAs of four plectroviruses, SpV1-R8A2 B and SpV1-C74 from *S. citri*, SVTS2 from *S. melliferum* and MVL-1 from *Acholeplasma laidlawii*. The circular DNAs are displayed in linear fashion to facilitate comparison. Linearization for spiroplasma viruses was at the border between common sequences (left) and virus-specific sequences (right). ORFs 3 and 16 putatively encode transposases. ORFs of spiroplasma viruses with the same number are homologous as are MVL-1 ORF 59 and spiroplasma virus ORF 2, as indicated by areas between pairs of dashed lines. Numbered MVL-1 ORFs are those previously identified (X58839). Additional ORFs are designated with letters.

DNA-related sequences and that the presence or absence, position and number of such sequences is a significant source of variation among strains. Sequences related to one cryptic plasmid, pMH1, isolated from a citrus strain of *S. citri*, are present in the chromosomes of several strains (Mouches et al., 1984). In contrast, DNA from plasmid pM41 from a *Vinca* strain could not be found in chromosomal DNA of any strains examined (Mouches et al., 1984). In addition to SpV1 sequences, SpV3 sequences are expected in the chromosome. SpV3 viruses are temperate phages that integrate their DNA in the host chromosome (Dickinson and Townsend, 1984). Razin et al. (1987) found multiple inserts derived from SV3 in the *S. citri* chromosome, and estimated that as much as 10% of the chromosome could be SV3-related sequences. It is surprising that no sequences attributable to this viral DNA have been found in sequences of numerous segments of genome obtained to date.

Hybridization studies also led to the prediction that up to one-twelfth of the *S. citri* chromosome is made of sequences related to SpV1-R8A2 B (Ye et al., 1992). Restriction mapping identified a minimum of 17 separate sites of insertion with only the rDNA not showing invasion of viral DNA (Ye et al., 1992, 1995). Not all these inserts are derived from SpV1-R8A2 B, since at least one, originally identified as from this source (Marais et al., 1996), is more closely related to SpV1-C74 (Melcher et al., 1999). Fewer large restriction fragments (two or three) bear sequences complementary to SpV1-S102 (Ye et al., 1992, 1995).

Spiroplasma strains vary in their content of integrated extrachromosomal DNA. A pair of probes derived from SV3 detected related sequences in one quarter of the *Spiroplasma* strains examined, including non-*citri* species (Nur et al., 1987). The amounts detected were strain dependent. Sequences related to SpV1-R8A2 B were found in all *S. citri* strains examined and in *S. melliferum* (Ye et al., 1995, 1994a). *S. citri* strains also harbor sequences derived from SVTS2 (Sha et al., 1995; Davis and Dally, 1998). But, sequences derived from extrachromosomal DNA of BR3-3X were not so evidently wide ranging, not being found in the non-BR strains investigated (Sha et al., 1995).

Variation in what parts of the extrachromosomal DNA are found in chromosomes occurs. Razin et al. (1987) concluded from Southern blotting and hybridization that SV3 sequences in the chromosomes are mostly small and derived from different parts of the circular DNA. On the other hand, Bebear et al. (1996) found that all *S. citri* strains examined had a major 3.9 kbp *Eco*RI probe-reactive fragment derived from complete SpV1 viral DNA, representing half the viral genome. All large SpV1-R8A2-hybridizing fragments reacted with probes from two separate regions of the viral DNA, leading to the conclusion that inserts were full-length (Ye et al., 1992). DNA sequence information revealed that at least one insert is indeed full length (Bebear et al., 1996) (though, even this is a non-functional element, substitutions having interrupted coding regions), but others are fragments (Laigret et al., 1996; Marais et al., 1996; Ye et al., 1996; Melcher et al., 1999). Partial sequences found in the chromosome may have arisen by deletion after integration or to incomplete integration. Putative examples of each exist. The variety of mechanisms involved in deletions and integrations were reviewed recently (Melcher et al., 1999). The pattern of SVTS2-derived fragments integrated in the virus-resistant lines MR2 and MR3 suggests that only a portion of that genome was transferred (Sha et al., 1995).

Thus there is considerable variation in the presence or absence of particular integrated extrachromosomal DNAs and in the part of the extrachromosomal DNA integrated. There may be little variation in the site of integration. Razin et al. (1987) observed that the pattern of SV3-reactive restriction fragments from different strains were highly similar, suggesting limited numbers of target sites. Though, except for fragments from the *rrn* region, all large fragments reacted with a SpV1-R8A2 B probe (Ye et al., 1992), the pattern of reactive fragments generated by more frequently cutting enzymes was strain dependent (Ye et al., 1995). Two of the DNA SpV1 S102-related sequences were found in different positions in *S. citri* and *S. melliferum* (Ye et al., 1994a). Variation in the number and position of S102-reactive fragments was also encountered among strains of *S. citri* (Ye et al., 1995).

Three phenotypic effects of integration of extrachromosomal DNA into the chromosome have been documented. A relatively higher growth rate has been attributed to the presence of larger numbers of SV3-derived sequences in strains (Razin et al., 1987). Immunity to SVTS2 infection is a consequence of integration of part of SVTS2 DNA into the chromosome (Sha et al., 1995). As discussed above, erythromycin resistance results from plasmid DNA integration (Salvado et al., 1989). A phenotypic effect of viral sequence integration can be inferred from

the observation that one of the characterized viral sequences is flanked by remnants of a DNA modification methylase gene (Melcher et al., 1999). That the gene was interrupted precisely by the viral DNA insertion, suggests that the original event caused inactivation of the methylase. Perhaps some of the strain variation in DNA methylation is due to events like this.

Sources of genetic variation

As with most organisms, genetic variation in *S. citri* results from variation in extrachromosomal DNA content, changes due to DNA replication and repair processes and changes due to recombination. Variation in extrachromosomal DNAs results from the selective acquisition of DNAs by some strains and not others and the loss of the extrachromosomal DNAs.

DNA acquisition and loss

As with other bacteria, DNA can enter a spiroplasma cell by several means. Mollicute transformation was first reported using Tn916 in *Acholeplasma laidlawii* and *M. pulmonis* (Dybvig and Cassell, 1987). Transformation of *S. citri* by naked DNA was first demonstrated in 1989 by transfer of the M4+ erythromycin resistance (Salvado et al., 1989). As in that case, transformation with pMH1 bearing a *cat* marker gene produced an unstable plasmid (Simoneau and Labarere, 1990). Similarly, attempts to use SpV1 viral DNA sequences as a vector resulted in unstable transformants (Marais et al., 1996). Stable transformation has since been achieved with engineered plasmids containing the origin of *S. citri* DNA replication (Ye et al., 1994b). Successful transformation is highly dependent on the strain, indicating that transformation may be a minor contributor to genetic variation in *S. citri*, in particular for the range of plasmid variation among strains of *S. citri* (Barber, 1983). Nevertheless, transformation has been a useful tool, allowing transposon mutagenesis and the analysis of transposon tagged genes (Foissac et al., 1997a,b; Jacob et al., 1997).

DNA carried by viruses also enters spiroplasma cells. The mechanisms of virion uncoating and entry are not well understood. SpV1, belonging to the *Inoviridae*, presumably attaches by one end of the rod-shaped particle to the cell and uncoats at the membrane as the nucleic acid enters the cell. Other spiroplasma viruses may use different attachment-injection mechanisms. Receptors for phages have not been clearly identified. Genetic transduction of marker genes from one strain to another via a virus has not been demonstrated. Each SpV1-R8A2 B, SpV1-C74 and SVTS2 have genes not present in the other viruses. Whether these genes are of viral or bacterial origin is not known.

Conjugal transfer of DNA has been detected in *Mycoplasma* (Roberts and Kenny, 1987). Transfer of DNA from one strain of *S. citri* to another also has been reported (Barroso and Labarere, 1988). Doubly resistant clones were recovered from a mixed culture of strains marked for single resistance to vanadium oxide or to arsenic acid. Their appearance was not diminished by the presence of DNase in the medium, suggesting that the transfer was by conjugation. It is surprising that little use has been made of this observation.

Whatever the mechanism of DNA entry into the cell, the DNA must either have or acquire an origin of replication. The functioning of viral origins of replication is conditioned by the presence or absence of related viruses. Cells already infected with one virus are immune to superinfection by closely related viruses (Sha et al., 1995; Dickinson and Townsend, 1984). The molecular mechanisms of this immunity in *S. citri* are not yet understood. Though plasmid incompatibility has not been specifically demonstrated to function in *S. citri*, in other bacteria it is a uniform phenomenon, whereby cells can harbor additional plasmids only if the new and existing plasmids are of different incompatibility groups. Extrachromosomal DNAs are lost from cells if they fail to accumulate by replication to high enough levels to ensure segregation to both daughter cells and/or lack a specific segregation mechanism.

DNA replication and repair

Nucleotide substitutions result from failure correctly to repair changes to the DNA resulting either from environmental damage to the DNA or mistakes during DNA polymerization. Measurement of the sensitivity of *S. citri* to UV radiation (Labarere and Borroso, 1989) suggests two mechanisms of handling DNA damage, the less sensitive of which is active in only a minor percentage of any *S. citri* population. That low doses of UV, capable of inducing DNA damage, neither affect the viability of *S. citri* nor induce mutations suggests an effective DNA damage repair system in this bacterium. Since *uvrABC, mucB, ung* and *fpg* genes are

found in the smaller *M. genitalium* and *M. pneumoniae* genomes, these genes would also be expected in *S. citri*. The repair system is less effective in *S. citri* and *S. melliferum* than in *A. laidlawii* (Marais et al., 1996) as judged by a higher sensitivity to UV in the former two. At higher UV doses, the extent of decrease in viability correlates with the frequency of appearance of mutations, consistent with the notion that DNA damage, uncorrected, leads to mutation. Though the absence of apparently functional *recA* genes in the *Spiroplasma* species and the presence of one in *A. laidlawii* was proposed as a possible reason for the difference in UV sensitivity (Marais et al., 1996), this is unlikely since proteome studies identified a full length recA protein in *S. melliferum* (Cordwell et al., 1997). A more likely explanation is absence of *recF* genes in the *Spiroplasma* (Ye et al., 1994a). The *recF* gene product is known to participate in repair of UV damage.

Genes for some of the components needed for *S. citri* DNA replication (*dnaA, dnaN, gyrB* and *gyrA*) have been isolated and sequenced (Ye et al., 1994a). There is no reason to suspect that the DNA polymerase and associated enzymes have fundamentally different properties than the better studied enzymes, such as those of *E. coli*. The DNA, template for replication, however, is considerably different from that of *E. coli*. Its A + T richness has two consequences. First, *S. citri* DNA duplexes, under equivalent conditions, are less stable than *E. coli* duplexes. Second, the chances that two random sequences are identical is higher for genomes with strongly asymmetric base compositions. These facts conspire to increase the likelihood of slipped strand pairing during replication, leading to small insertions and deletions. Numerous examples of sequence variation among SpV1-derived sequences can be accounted for by invoking slipped strand pairing mechanisms during replication (Melcher et al., 1999; Marais et al., 1996). These events produce recombination limited to nearby short sequences.

Homologous recombination

In *S. citri*, recombination that does not require interaction of neighboring sequences is a major source of variation. The targets of recombination are the chromosome and extrachromosomal elements. Recombination in *S. citri* occurs by homologous recombination, transposition, and site-specific recombination.

Homologous recombination has occurred in *S. citri* between homologous positions on two DNAs or between similar sequences at non-homologous positions on the same DNA. The plasmid instability in transformants with the pBOT1 vector was due to recombination between homologous *dnaA* sequences in the plasmid and the chromosome (Renaudin et al., 1995). SpV1-D3 arose by a pair of recombination events between an engineered SpV1-R8A2 B viral DNA and an integrated chromosomal sequence (Marais et al., 1996) related to the highly similar SpV1-C74 (Melcher et al., 1999). Recombination at one end was at a homologous sequence (Marais et al., 1996). At the other end, recombination was between highly similar sequences but at non-homologous positions (Melcher et al., 1999). About 250 bp of significant but reduced similarity to SpV1-R8A2 B at one end of an SpV1-like element in the *hsdS-rpoB* region of the chromosome, suggested that this end was formed by recombination between an internal element sequence and a chromosomal sequence resembling it (Melcher et al., 1999). This type of recombination at non-homologous positions results in deletion of the intervening DNA. One of the two junctions resulting from the chromosome inversion-deletion found in the BR3-G chromosome most likely involved pairing of two SpV1-like sequences from the C4 region of the genome (Melcher et al., 1999; Ye et al., 1996). The presence of multiple copies of viral DNA sequences at dispersed positions in the *S. citri* chromosome should provide the opportunity for many rearrangement events in this genome.

Although homologous recombination likely has occurred frequently, evidence suggests that *S. citri* lacks a functional *rec*A gene (Marais et al., 1996). Such lack is not inconsistent with the observation of frequent recombination. It merely implies that a *rec*A independent recombination pathway is active in *S. citri*.

Transposition

IS elements have been identified in several mollicutes. The structures of the RFs of SpV1-R8A2 B and SpV1-C74 DNAs suggest that they are circular extrachromosomal IS elements belonging, respectively, to the IS30 (Dong et al., 1992) and IS3 (Melcher et al., 1999) families of insertion elements. This assignment to IS family also implies that the elements transpose using mechanisms described for other members of the family. For IS3-family members, transposition may occur through a circular intermediate with abutted inverted repeat target sites, separated by a fragment of the size of the target site duplication (Polard et al., 1996). This

is precisely the structure of the viral RF and it may thus be regarded as an intermediate in the transposition process. For IS30-family members, a dimer is an important intermediate in transposition (Olasz et al., 1993, 1997). The transposase may have a preference for sites that resemble the inverted repeat sequences as targets of transposition (Olasz et al., 1997). That this may also be true of SpV1-C74 is indicated by the observation that the end of the chromosomal progenitor to the SpV1-D3 sequence ends at a viral repeat but is separated by a three bp spacer from another sequence, in inverted orientation, closely resembling the viral repeat sequence (Melcher et al., 1999). However, the expected duplication of target site sequences has not been identified (Renaudin and Bove, 1994). Though sequences related to these two viruses occur in the host chromosome and are probably the source of those sequences, no direct evidence for transposition has been presented. On the other hand, the SVTS2 virus (Sha et al., 1998), has been shown to transpose part of its sequence during the derivation of virus resistant lines MR2 and MR3 from MR200H (Sha et al., 1995). The SpV3 virus ai integrates its DNA into the chromosome probably using an Int-Flp type of recombinase (Dickinson and Townsend, 1984).

A restricted target site specificity for the integration of the SpV1 IS elements provides one possible explanation of the observation of common patterns of reactive fragments (Razin et al., 1987). On the other hand the observations could also be accounted for by the integration events having occurred long before the divergence of the analyzed strains from one another. Divergence could also account for the failure to identify duplicated target sequences.

RF DNA can transpose into the chromosome. It is not clear whether, once integrated, the DNAs retain the ability to transpose. A transposase ORF adjacent to the BR3-G inversion border may be functional since it lacks frameshifts and nonsense codons even though it has diverged considerably from the transposase of SpV1-R8A2 (Ye et al., 1996). On the other hand, nucleotide sequences of the L1 and L10 elements, though much less diverged from SpV1-R8A2, bear such interruptions suggesting that these elements are no longer autonomous (Bebear et al., 1996). There may be active transposases unrelated to those of the characterized SpV1 viruses. The *hsdS-rpoB* region of the chromosome contains an IS1202-related transposase gene inactivated by insertion of a small SpV1-like sequence (Laigret et al., 1996). If there are active transposase genes in the chromosome, their expression must be regulated to maintain a low or non-existent level of expression. This regulation has not been investigated.

An IS3-family member, *IS1138*, has been found in *M. pulmonis*, but not in other mycoplasmas (Bhugra and Dybvig, 1993). Instead, a different IS3-family member, *IS1296*, has been found exclusively in *M. mycoides* subsp. *mycoides* (Frey et al., 1995). *IS1138* was observed to move during laboratory culture (Bhugra and Dybvig, 1993). Horizontal transmission of such elements is suggested by the presence of similar elements in three swine infecting species of mycoplasma but not in otherwise more closely related mycoplasmas (Taylor et al., 1988).

The insertions of viral DNA by lysogenic or insertion element transposition mechanisms are site-specific recombination events. Additional site-specific recombination events not directly involving viral DNA encoded enzymes may also play a role in shaping *S. citri* chromosomes. The L1 element derived from SpV1-R8A2 B is missing a large chunk internal to the IS inverted repeat ends, relative to the L10 element (Bebear et al., 1996). The deletion junction is flanked by a series of short repeated sequences, suggestive of targets of site-specific recombination (Melcher et al., 1999). Similarly arranged (but different in sequence) inverted repeats in the BR3-3X sequence (Ye et al., 1996) are suggestive of recognition by a site-specific recombination enzyme (Melcher et al., 1999). It is puzzling that some recombination junctions have generated mirror repeats suggestive of triple helical pairing at some point in the mechanism of recombination (Melcher et al., 1999).

Site-specific recombination mechanisms may contribute to sequence variation of surface antigens of *M. hominis* (Boesen et al., 1998) and to inversions controlling phase variable expression of a restriction-modification system of *M. pulmonis* (Sitaraman and Dybvig, 1997; Dybvig and Yu, 1994). These inversions coincide with inversions affecting the expression of variable surface antigens (Bhugra et al., 1995). That restriction modification systems in *M. pulmonis* are phase variable may indicate that variation in restrictability within *S. citri* strains, noted above, is due to heterogeneity in the population resulting from phase switching (French et al., 1998). Such elaborate regulatory mechanisms have yet to be identified in spiroplasma. The electrophoretic variants of spiralin hint that at least a rudimentary variation system may exist. The existence of multiple gene families suggested by

Southern blotting and hybridization results provides further potential for regulated variation in expression of surface genes.

Implications

The picture that emerges from research on the *S. citri* genome and its variation is one of frequent and often extensive variation. That picture arises both from the inference of a number of mechanisms for genome variation and documentation of actual variation. Among the more impressive variations are the large changes in genome size that can occur within only a few generations. Expansion and contraction events must be occurring continually in about equal proportions so that the net genome size varies within defined boundaries.

The picture of rapid genome variation in *S. citri* contrasts with what is expected from its life cycle. Having two hosts in different kingdoms should impose constraints on the rapidity of evolution. Genes not necessary for successful replication in plant phloem, but needed for replication in the leafhopper, must be preserved in the absence of immediate selection pressure while the bacterium is replicating in the phloem. Conversely, genes not needed for successful replication in leafhoppers, but needed for replication in the plant, must be preserved while the bacterium is in the leafhopper, despite the absence of immediate selection pressure.

On the other hand, a large flux of genetic information into and out of the *S. citri* genome should be beneficial to the bacterium, allowing it, with its minimal genome size, to adapt to new environments. Bacteria with larger genomes contain a variety of genes whose expression is called upon only under certain circumstances. *S. citri* may be able to accomplish the same regulation by importing or rearranging DNA.

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