Chromosomal Heterogeneity among Various Strains of *Spiroplasma citri*

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The genomes of various *Spiroplasma citri* strains were digested with several restriction enzymes, and the fragments were analyzed by pulsed-field gel electrophoresis. Polymorphism of the restriction patterns was observed. The genome sizes of the strains obtained when we added the restriction fragment sizes ranged from 1,650 to 1,910 kbp. Physical and genetic maps of 12 strains were constructed by using several DNA probes as genetic markers. The relative positions of mapped loci were conserved in most of the strains; the main differences were differences in the order and number of restriction sites and differences in the sizes of certain fragments. The distribution of viral sequences, which occur at multiple sites in the *S. citri* genome and are homologous to the sequences of *S. citri* virus SpV1 strains R8A2B and S102, was also studied.

Since the first culture of *Spiroplasma citri* (38), the helical mollicute that causes stubborn disease of citrus, several hundred isolates of this agent have been obtained. These organisms have been isolated from infected citrus trees and herbaceous hosts and from insect vectors of the spiroplasmas, such as the leafhopper *Circulifer haematocaps* (15). The major characteristics of *S. citri* include helical morphology, motility, small genome size, and low genome G+C content (26 mol%).

Phylogenetic studies have shown that spiroplasmas, like all other organisms in the class *Mollicutes*, arose from the phylum of gram-positive bacteria by successive elimination of genomic DNA (46, 53, 54).

Physical and genomic maps of several mollicutes, including *S. citri* R8A2^T (T = type strain), have been published previously (2, 12, 21, 22, 29, 31, 43, 47–50, 55, 56). Polymorphism has been observed among strains of the same species with several organisms, including *Mycoplasma mycoides*, *Mycoplasma hominis*, *Mycoplasma hyopneumoniae*, and *Ureaplasma urealyticum* (16, 22, 31, 36), suggesting that these organisms belong to a group of prokaryotes that exhibits a high evolution rate, as suggested previously by 16S rRNA sequence data (54).

Several previous studies revealed that polymorphism could be found in several *S. citri* strains isolated from various hosts in different geographical areas; these studies were studies in which the researchers used serology, protein electrophoretic profiles, restriction fragment length polymorphism, and spiral gene nucleotide sequences (4, 5, 25). A striking feature of the *S. citri* genome is that it contains multiple copies of sequences homologous to the spiroplasma virus SpV1 sequence (34, 35, 56) and that the number and distribution of these sequences are variable from one strain to another (34).

In order to investigate further the chromosomal heterogeneity of various *S. citri* strains, we analyzed the genomic DNAs of these organisms by performing restriction enzyme digestion followed by pulsed-field gel electrophoresis (PFGE). Physical

and genomic maps of 12 strains were constructed, and the relative positions of restriction sites and genetic markers, including virus-like sequences, were compared.

MATERIALS AND METHODS

***S. citri* strains.** The 12 strains of *S. citri* which we studied are listed in Table 1. These strains were grown at 32°C in BSR medium (6). BSR medium (B medium supplemented with sorbitol and reduced) was derived from B medium of Ernø and Stipkovits (14) by adding 70g of sorbitol per liter, decreasing the concentration of horse serum from 20 to 10%, and omitting fresh yeast extract and DNA. The strains were identified as *S. citri* strains by an enzyme-linked immunosorbent assay in which a polyclonal antibody directed against *S. citri* R8A2^T was used (40).

Preparation and restriction of *S. citri* genomic DNA. *S. citri* genomic DNAs were prepared, included in low-melting-point agarose plugs, and digested with restriction endonucleases *Apa*I, *Bss*HI, and *Sal*I (Gibco/BRL, Gaithersburg, Md; Biolabs, Beverly, Mass.) as described previously (30, 56). The *S. citri* DNA was separated by PFGE by using a transverse alternating field electrophoresis system (Geneline II; Beckman, Palo Alto, Calif.) under the following conditions. For restricted genomic DNA, the pulse times were as follows: 45 s, 6 h (370 mA); 1 min, 6 h (390 mA); and 45 s, 6 h (390 mA). And for undigested genomic DNA the pulse times were as follows: 1 min, 12 h (350 mA); 2 min, 12 h (370 mA); and 3 min, 12 h (390 mA). The working buffer used was 0.25× TBE (1× TBE is 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA), and the migration temperature was maintained at 8°C.

S. citri genomic DNAs from various strains were also extracted from cultured cells as described by Marmur (24), and about 2 µg of the DNA from each strain was digested overnight with *Eco*RI and *Hind*III by using the procedure recommended by the manufacturer (Gibco/BRL).

Mapping of the *S. citri* genomes. Restricted *S. citri* DNAs were blotted onto nylon membranes (Hybond N⁺; Amersham, Buckinghamshire, United Kingdom) with 0.4 N NaOH by using the standard alkaline Southern blotting procedure (32, 42), except that the PFGE gels were previously treated twice with 0.25 N HCl for 10 min. The orders of the restriction fragments of various *S. citri* genomes were determined by hybridization with the probes listed in Table 2. Some randomly cloned DNA fragments (fragments S2, S4, and p6) from *S. citri* R8A2^T were also used as probes to connect the restriction fragments. The DNA probes were labelled with [α -³²P]dATP by random priming with a Random Primers DNA Labelling System (Gibco/BRL).

RESULTS

Polymorphism at the *rrn* locus. *S. citri* has only one rRNA operon (*rrn*) (19). In order to study the level of heterogeneity at the single *rrn* locus in various *S. citri* strains, we hybridized the *Eco*RI- and *Hind*III-restricted *S. citri* genomic DNAs with the following two plasmid probes: pR136 containing the 16S ribosomal DNA (rDNA) of *S. citri* (23) and pMC5 containing

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TABLE 1. *S. citri* strains studied

Strain	Geographic origin	Animal or plant origin	Source(s) ^a	Reference
R8A2 ^T (= ATCC 27556 ^T)	Tadla, Morocco	Washington navel orange leaves	ATCC, LBCM	39
R8A2-B	Tadla, Morocco	Washington navel orange leaves	LMM	27
C-189 (= ATCC 27665)	California	Madame Vinous orange leaves	ATCC, UC	17
Hinckley	California	Hinckley orange leaves	LBCM	3
MH	Gharb, Morocco	Washington navel orange leaves	LBCM	37
M4	Rabat, Morocco	Periwinkle leaves	LBCM	6
Corse	Corsica, France	Leafhopper	LBCM	15
Israel (= NCPPB 2565)	Israel	Valencia late orange seeds	NCPBB, JII	13
78	Misis, Turkey	Washington navel orange leaves	LBCM	45
Alc-254	Alcanar, Spain	Washington navel orange fruits	LBCM	3
Aspl (= NCPPB 3095)	Israel	Washington navel orange leaves	NCPBB, JII	44
Palmyre	Palmyre, Syria	Leafhopper	LBCM	15

^a ATCC, American Type Culture Collection, Rockville, Md.; LBCM, Laboratoire de Biologie Cellulaire et Moléculaire, Institut National de la Recherche Agronomique, Villenave d'Ornon, France; LMM, Laboratory of Molecular Microbiology, Frederick, Md.; UC, University of California, Riverside; NCPBB, National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Food and Fisheries, Harpenden, United Kingdom; JII, John Innes Institute, Norwich, United Kingdom.

the 3' end of the 16S rRNA gene and the entire 23S and 5S rRNA genes of *Mycoplasma capricolum* (1). The first probe was chosen because it includes the highly conserved *Eco*RI site in the middle of the 16S rDNA, and the second probe was chosen because it includes two relatively conserved *Hind*III sites in the 23S rDNA (19).

As shown in Fig. 1, hybridization between the *Eco*RI- and *Hind*III-digested DNAs and the rDNA probes revealed strain polymorphism. Strains R8A2^T, R8A2-B, and C-189 (Fig. 1, lanes 1, 2, and 6, respectively) produced the same patterns with both enzymes. The other nine strains differed from these three strains in both their *Eco*RI fragment patterns and their *Hind*III fragment patterns; they produced identical *Eco*RI profiles but could be distinguished by their *Hind*III patterns. Strains Corse and Hinckley (lanes 4 and 10) produced the same *Hind*III pattern, which was distinct from the *Hind*III patterns of strains MH, M4, Israel, Aspl, 78, and Alc-254 (lanes 5, 12, 7, 3, 8, and 11 respectively). In addition, the rDNA gene hybridization pattern of *Hind*III-restricted strain Palmyre DNA (lane 9) was quite different from the patterns produced by all other strains.

Genome sizes and restriction patterns. Nonrestricted genomic DNAs of the 12 *S. citri* strains were analyzed by PFGE and were compared with the yeast chromosome, which was used as a size marker. We found that the genome sizes of the various strains ranged from 1,600 to 1,900 kbp (data not

shown). In order to determine the genome sizes more accurately, the genomic DNAs were cleaved with restriction enzymes *Apa*I, *Bss*HII, and *Sal*I, and the resulting fragments were separated by PFGE. The restriction patterns are shown in Fig. 2. The sizes of the genomes were calculated by adding the sizes of the restriction fragments; the resulting values, as well as the numbers of restriction sites, are shown in Table 3. The genome of strain R8A2^T was almost 1,820 kbp long, 50 kbp longer than the genome of R8A2-B or C-189. The two Morocco strains, strains MH and M4, not only had identical genome sizes (1,820 kbp) but also produced the same restriction patterns. The same was true for strains Israel and Aspl, which had the smallest genomes (1,650 kbp). The genome of strain Alc-254 was the largest genome (1,910 kbp).

On the basis of the restriction profiles, it appeared that differences occurred preferentially in certain restriction fragments. For example, the smaller the genome, the smaller fragment ApA was. Strains R8A2^T, R8A2-B, and C-189 produced similar patterns except that fragment ApA or BsC of R8A2^T was 50 kbp longer. With *Apa*I or *Bss*HII, the patterns obtained for strains MH, M4, Israel, Aspl, 78, and Alc-254 were very similar (Fig. 2, lanes 5, 6, 8, 11, 9, and 10, respectively). With *Sal*I, a single fragment was obtained for strains MH, M4, and Alc-254; double digestion with *Apa*I and *Sal*I or *Bss*HII and *Sal*I revealed that the genomes of these three strains were not

TABLE 2. Probes used for *S. citri* genome mapping

Probe no.	Gene(s)	Product(s)	Source	Reference
1	<i>rpsB</i> - <i>tsf</i> - <i>x-spi</i> - <i>pfkA</i> - <i>pyk</i>	Ribosomal protein S2, EF-Ts, protein X, spiralin, 6-phosphofructokinase, pyruvate kinase	<i>S. citri</i>	9
2	<i>fib</i>	Fibrillar protein	<i>S. citri</i>	52
3	<i>rm</i>	16S rRNA	<i>S. citri</i>	23
4	<i>rm</i>	16S, 23S, and 5S rRNAs	<i>M. capricolum</i>	1
5	<i>pyrG</i> - <i>purA</i> - <i>purB</i>	CTP synthetase, adenylosuccinate synthetase and lyase	<i>S. citri</i>	10
6	<i>rpoD</i>	RNA polymerase σ subunit	<i>S. citri</i>	56
7	<i>dnaA</i> and <i>oriC</i>	DNA replication protein A, chromosomal replication origin	<i>S. citri</i>	57
8	<i>trpT</i> - <i>trpU</i> - <i>serT</i>	tRNA ^{Trp} (UCA), tRNA ^{Trp} (CCA), tRNA ^{Ser} (CGA)	<i>S. citri</i>	11
9	<i>trnB</i>	tRNA ^{Val} (UAC), tRNA ^{Thr} (UGU), tRNA ^{Tyr} (GUA), tRNA ^{Gln} (UUG), tRNA ^{Lys} (UUU), tRNA ^{Leu} (UAA)	<i>S. citri</i>	10
10	SpV1-S102	Spiroplasma virus 1	<i>S. citri</i>	35
11	SpV1-R8A2B	Spiroplasma virus 1	<i>S. citri</i>	33
12	p6	Unknown	<i>S. citri</i>	This study
13	S2	Unknown	<i>S. citri</i>	This study
14	S4	Unknown	<i>S. citri</i>	This study

FIG. 1. Southern blot of genomic DNAs from 12 *S. citri* strains digested with *EcoRI* and hybridized with probe 3 (B) or digested with *HindIII* and hybridized with probe 4 (A). Lanes 1, R8A2^T; lanes 2, R8A2-B; lanes 3, Asp1; lanes 4, Corse; lanes 5, MH; lanes 6, C-189; lanes 7, Israel; lanes 8, 78; lanes 9, Palmyre; lanes 10, Hinckley; lanes 11, Alc-254; lanes 12, M4.

cleaved by *SalI* (data not shown). Similarly, the genomic DNA of strain Hinckley was not cut by *BssHII*. The restriction pattern of strain Palmyre was completely different from the restriction patterns of all other strains.

Construction and comparison of genomic maps. In order to compare the genomes of the 12 strains, we constructed physical and genetic maps for these strains. Restriction fragments of each strain generated by enzymes *ApaI* and *BssHII* were aligned and linked by hybridization with several probes. Examples of the hybridization experiment results are shown in Fig. 3. When *fib* was used as a probe, extensive polymorphism was observed. This polymorphism correlated with the variation in the size of fragment ApA. For strain Alc-254 (Fig. 3, lane 10), two hybridization signals were obtained after digestion with *ApaI* or *BssHII*. Since *fib* does not contain any restriction site for *ApaI* or *BssHII*, it is likely that this gene is duplicated in the chromosome of strain Alc-254. When p6 was used as a probe, the hybridization patterns obtained were more conserved among strains. The DNA fragment of p6 contains an *ApaI* restriction site, and this site was present in all of the strains tested since in all instances two *ApaI* restriction fragments were obtained when p6 was used as a probe. The sizes of the *BssHII* fragments that hybridized with p6 appeared to be almost identical for all of the strains tested except strain Hinckley (whose DNA is not cut by *BssHII*) and strain Palmyre.

Eleven genetic markers were located on the restriction maps (Fig. 4). We did not construct maps for *SalI* fragments because

TABLE 3. Genome sizes of 12 *S. citri* strains and numbers of *ApaI*, *BssHII*, and *SalI* restriction sites

Strain(s)	Genome size (kbp)	No. of restriction sites for:		
		<i>ApaI</i>	<i>BssHII</i>	<i>SalI</i>
R8A2 ^T	1,820	9	9	8
R8A2-B, C-189	1,770	9	9	8
Corse	1,870	9	7	10
Hinckley	1,840	8	0	10
Alc-254	1,910	10	7	0
78	1,760	9	6	9
MH, M4	1,820	9	7	0
Israel, Asp1	1,650	9	7	10
Palmyre	1,820	8	5	14

the genomes of three strains were not cut by this enzyme. The maps of strains R8A2^T, R8A2-B, and C-189 were almost identical except that fragment ApA of R8A2^T was 50 kbp larger than the corresponding ApA fragments of the two other strains. For the following strains, the genomes within a pair were similar, but differed from the genomes of the other organisms: MH and M4; Israel and Asp1; and 78 and Alc-254. Strain Hinckley had no *BssHII* site, but its genome reassembled the genome of strain Corse on the basis of the *ApaI* restriction map. In contrast, the map of strain Palmyre was different from the maps of all other strains.

Even though the different strains exhibited obvious chromosomal heterogeneity, it seemed that the gene order for most genes examined was conserved in all of the strains. Certain restriction sites were also conserved. For instance, the *ApaI* site connecting fragment ApC to fragment ApF or ApG (depending on the strain) and contained in probe p6 was present in the genomes of all of the strains (Fig. 4). Similarly, the two adjacent *BssHII* sites that accounted for approximately 150 kbp (fragment BsE or BsF) were conserved in most strains. The same was true for fragments ApB and BsA carrying the *dnaA* gene and the chromosomal replication origin (*oriC*).

Viral sequences in the genome. Most strains of *S. citri* are infected with one or several strains of SpV1, a rod-shaped virus

FIG. 2. PFGE of restricted genomic DNAs from various *S. citri* strains. Lanes 1, R8A2^T; lanes 2, R8A2-B; lanes 3, C-189; lanes 4, Hinckley; lanes 5, MH; lanes 6, M4; lanes 7, Corse; lanes 8, Israel; lanes 9, 78; lanes 10, Alc-254; lanes 11, Asp1; lanes 12, Palmyre; lanes A, size marker (lambda DNA ladder).

FIG. 3. Southern blots of gels after PFGE of *Apa*I- or *Bss*HII-restricted genomic DNAs from various *S. citri* strains with probe 2 (*fib*) and probe 12 (*p6*). Lanes 1, R8A2^T; lanes 2, R8A2-B; lanes 3, C-189; lanes 4, Hinckley; lanes 5, MH; lanes 6, M4; lanes 7, Corse; lanes 8, Israel; lanes 9, 78; lanes 10, Alc-254; lanes 11, Asp1; lanes 12, Palmyre.

FIG. 4. Physical and genetic maps of various *S. citri* strains obtained with restriction enzymes *Apa*I (Ap) and *Bss*HII (Bs). The positions of several gene markers are indicated at the top of each map. Abbreviations: *trp*, *trpT-trpU-serT*; *pyrG*, *pyrG-purA-purB*. The position of *p6* is strongly conserved. The asterisks indicate the positions of Spv1-S102-like sequences.

with single-stranded circular DNA (33–35). The genome of SpV1 strain R8A2B has been sequenced and contains 8,273 bases (33). A striking feature of *S. citri* is the presence in its genome of multiple sequences homologous to SpV1 sequences (34, 35, 56). SpV1 strains R8A2B and S102 are two distinct isolates. The genomes of these viruses are similar in size (about 8 kbp) but exhibit almost no cross hybridization (35). Sequences homologous to one of the two viral genomes were detected in the chromosomes of all of the *S. citri* strains tested (35).

The *S. citri* chromosome contains more sequences that are homologous to SpV1 strain R8A2B sequences than sequences that are homologous to SpV1 strain S102 sequences (35, 56). As shown in Fig. 5, most chromosomal restriction fragments reacted with the SpV1 strain R8A2B probe, suggesting that viral sequences may be scattered along the whole genome. In contrast, SpV1 strain S102 sequences were present in only two or three sites on the *S. citri* chromosome (data not shown). These sequences were located on the genomic maps, and one was located in the vicinity of the spiraline gene (*spi*) in all of the strains tested.

DISCUSSION

Polymorphism of *S. citri* rDNA genes was correlated with chromosomal heterogeneity; in other words, the strains that

FIG. 5. Hybridization of *ApaI*-restricted *S. citri* genomic DNA fragments with the SpV1-R8A2B probe. Lane 1, R8A2^T; lane 2, R8A2-B; lane 3, C-189; lane 4, Hinckley; lane 5, MH; lane 6, M4; lane 7, Corse; lane 8, Israel; lane 9, 78; lane 10, Alc-254; lane 11, Asp1; lane 12, Palmyre.

had similar genomic maps produced identical hybridization patterns with rDNA probes. This was the case for strains R8A2^T, R8A2-B, and C-189, which produced identical rDNA profiles and very similar PFGE patterns; the only difference was an *Apa* fragment that was 50 kbp shorter in R8A2-B and C-189 than in R8A2^T. In a previous study, *S. citri* strains were divided into groups on the basis of the mobility of spiralin (the major protein component of the membrane) during polyacrylamide gel electrophoresis (4). As a general rule, the strains that produced identical rDNA profiles and similar PFGE patterns contained spiralin that had the same apparent molecular weight. Genomic variations did not affect serologic reactivity against an antiserum to type strain R8A2. The only difference that has been observed is the lack of reactivity in Western blots of the spiralin of strain Palmyre to a monoclonal antibody against *S. citri* Israel (4). This lack of reactivity was related to the amino acid sequence of the spiralin itself (4).

The genome sizes of the *S. citri* strains examined ranged from 1,650 to 1,910 kbp. This genome size polymorphism may have resulted from genomic rearrangements (deletions or duplications). One example is the 50-kbp difference between the genome of high-passage strain R8A2^T and the genome of low-passage strain R8A2-B; these two strains originated from the same *S. citri* isolate, and the difference in genome sizes occurs between the *fib* and *spi* loci in fragment ApA, a region where most of the variations in size were observed. Other examples of rearrangements were found in the genomes of strains MH, M4, Israel, Asp1, 78, and Alc-254. The genome of strain Alc-254 was the largest genome examined (1,910 kbp), and the genomes of strains Asp1 and Israel were the smallest (1,650 kbp). Between these two extremes were the genomes of strains 78, MH, and M4 (1,760, 1,820, and 1,820 kbp, respectively). A comparison of the genomic maps revealed that approximately 100 kbp in fragment ApA of strain 78, 80 kbp in the same region of strains MH and M4, and 250 kbp in the genomes of strains Israel and Asp1 were missing. These deletions accounted for the differences in the genome sizes of these strains. In addition, it seemed that part of fragment ApA of strain Alc-254, which contained *fib*, was duplicated.

Chromosomal heterogeneity of *S. citri* strains was observed not only in genome size, but also in restriction patterns. Compared with other genomic regions, the region containing the chromosomal replication origin (*oriC*) seemed to be the most stable region. In contrast, the large region of fragment ApA,

corresponding to the putative replication terminus, was apparently the most variable region. This seems to be consistent with the results of studies on enterobacteria which suggested that genes far away from *oriC* have a higher mutation rate than genes near *oriC* (41).

We have reported previously that SpV1 strain R8A2B viral sequences are present in at least 17 locations on the *S. citri* R8A2^T genome (56). This seemed to be true for all of the strains tested in this study. Viral sequences are distributed all along the genome except for one region in the vicinity of the *rrn* locus (56). Although nothing is known about the origin and role of these sequences, we speculate that their occurrence in such large numbers has biological significance. In contrast, SpV1 strain S102 viral sequences were present in only two or three regions on the genome, and one was always located near the *spi* fragment.

Chromosomal rearrangements cannot account for all of the heterogeneity that was observed among the strains which we studied. Mutations may be responsible for a certain number of differences in restriction sites, especially when genome size and gene order are similar. DNA modifications, such as base methylation, may also be responsible for some of the differences observed. It has been observed previously that the number of *SalI* restriction sites is always much lower than the number predicted by statistics not only in *S. citri*, but also in *Spiroplasma melliferum* (55, 56), *M. mycoides*, and *M. capricolum* (48–50). The same thing is true for *M. hyopneumoniae* when restriction enzyme *XhoI* is used (16). It has been suggested that a *SalI* site (or a *XhoI* site) might be a methylation target in these organisms (16, 56). In this study we found that most *S. citri* strains which we examined have a low number of *SalI* sites and that three strains, MH, M4, and Alc-254, have no *SalI* sites. Because this is unlikely statistically, we propose that strains MH, M4, and Alc-254 have a more efficient methylation system than the other strains which we studied. *EcoRI* sites might also be targets of modifications. Indeed, when the *EcoRI*-restricted DNAs of strains R8A2^T, R8A2-B, and C-189 were hybridized with the rDNA probe, no 1.2-kb DNA fragment was observed (Fig. 1), although such a fragment was identified in all other strains. Evidence that the sites had been modified came from a sequence analysis of the rDNA region of strain R8A2^T; the single *EcoRI* and *SalI* sites were present in the sequence but were not revealed by a Southern blot analysis of the genomic DNA (18).

Our results support the contention that genome size is not an important marker for taxonomic classification of organisms in the class *Mollicutes*. Variations in genome size have been observed previously at the genus, species, and strain levels (8, 16, 20, 22, 26, 28, 31, 36, 48, 49, 55). In *S. citri* the sizes of the smallest and largest genomes may differ by up to 260 kbp. Polymorphism in the genome sizes of different strains has also been observed in *M. mycoides* (31), *U. urealyticum* (36), *M. hominis* (22), and *M. hyopneumoniae* (16), as well as their walled eubacterial relatives *Clostridium perfringens* (7) and *Clostridium acetobutylicum* (51). It has been suggested on the basis of the results of 16S rRNA sequence comparisons that mollicutes have high mutation rates (53, 54); in addition, these organisms seem to have significant rearrangements in large parts of their genomes. However, there is no biological trait which has been associated with variations in the genome size of *S. citri* strains.

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