Chromosome mapping of the sweet potato little leaf phytoplasma reveals genome heterogeneity within the phytoplasmas

Anna C. Padovan, Giuseppe Firrao†, Bernd Schneider‡ and Karen S. Gibb

To further understand the genomic diversity and genetic architecture of phytoplasmas, a physical and genetic map of the sweet potato little leaf (SPLL) strain V4 phytoplasma chromosome was determined. PFGE was used to determine the size of the SPLL-V4 genome, which was estimated to be 622 kb. A physical map was prepared by two-dimensional reciprocal digestions using the restriction endonucleases BssHII, Smal, Eagl and I-Ceu. Sixteen cleavage sites were located on the map. Southern hybridizations of digested SPLL-V4 chromosomal DNA were done using random clones and PCR-amplified genes as probes. This confirmed fragment positions and located the two rRNA operons and the linked fus/tuf genes encoding elongation factors G and Tu, respectively, on the physical map. An inversion of one of the rRNA operons was observed from hybridization data. Sequence analysis of one of the random clones identified a gid gene encoding a glucose-inhibited division protein. Digestions of the tomato big bud (TBB) phytoplasma chromosome with the same four enzymes revealed genome heterogeneity when compared to the closely related SPLL-V4, and a preliminary chromosome size for the TBB phytoplasma of 662 kb was estimated. This mapping information has revealed that significant genome diversity exists within the phytoplasmas.

Keywords: phytoplasma, genome mapping, ribosomal genes, pulsed-field gel electrophoresis, gid gene

INTRODUCTION

There is little information available on the genetic architecture of the non-helical, phytopathogenic mollicutes, the phytoplasmas. The G+C content of several phytoplasmas is at the lower end of the spectrum for mollicutes (Kollar & Seemueller, 1989; Sears et al., 1989). Phytoplasma genomes have been shown to contain two 16S rRNA genes (Schneider & Seemüller, 1994), ribosomal protein genes (Lim & Sears, 1992), a tuf gene encoding the elongation factor EF-Tu (Schneider et al., 1997), and genes encoding a major membrane protein gene (Barbara et al., 1998), an antigenic protein (Yu et al., 1998) and a nitroreductase (Jarausch et al., 1994). Extrachromosomal DNA has been reported for some phytoplasmas (Davis et al., 1988; Sears et al., 1989; Davis et al., 1990; Kuske & Kirkpatrick, 1990; Kuboyama et al., 1998), although its role remains speculative. The genome sizes of several phytoplasmas have been determined by PFGE and range from 530 to 1185 kb (Neimark & Kirkpatrick, 1993; Zreik et al., 1995; Firrao et al., 1996a, b; Marcone et al., 1999). Little else is known about the genetic structure of phytoplasmas, and genes involved with pathogenicity and other general housekeeping genes have not been identified.

With the determination of genome sizes for many mollicutes by PFGE, a great heterogeneity has been observed at the genus (Pyle et al., 1988; Neimark & Lange, 1990; Whitley et al., 1990; Carle et al., 1995), species and strain level (Pyle et al., 1990; Ladefoged & Christiansen, 1992; Ye et al., 1995). This heterogeneity has meant that chromosome size alone cannot be used
for the taxonomy of mollicutes (Neimark & Lange, 1990). Physical and genetic mapping of mollicute genomes has enabled inter- and intra-specific comparisons to be made. When both physical and genetic maps are known, genomes can be analysed for rearrangements, deletions, insertions or mutations. The first physical map of a phytoplasma chromosome was determined for the Western X (WX) disease phytoplasma showing the location of the two 16S rRNA genes (Firrao et al., 1996b).

This paper describes a method to obtain high-quality DNA from infected periwinkles for PFGE analysis. This method was used to characterize the phytoplasma associated with sweet potato little leaf (SPLL) disease strain V4 (SPLL-V4) by chromosome size estimation, physical mapping and the localization of characterized genes. Restricted chromosomal DNA of the closely related tomato big bud (TBB) phytoplasma chromosome was compared to that of SPLL-V4 to identify polymorphisms and thereby determine whether endonuclease recognition sequences were conserved between these two strains.

**METHODS**

**Plant material.** Sweet potato (Ipomoea batatas) with little leaf disease was collected at Middle Point (Darwin, NT, Australia) and tomato (Lycopersicum esculentum) with big bud disease was obtained from Adelaide (SA, Australia). The phytoplasmas in these field-collected plants were transmitted to Catharanthus roseus in a shadehouse by cleft-grafting onto healthy periwinkle plants was ground in 2 M NaCl, 20 mM EDTA, pH 8.0) maintained at 4°C 12 ml ice-cold isolation buffer [0.1 M NaHPO₄, 0.03 M NaCl, 10% (w/v) sucrose, 2% (w/v) polyvinylpyrrolidone (molecular mass 40000), 10 mM EDTA, pH 7.6, with 0.15% BSA and 1 mM isosaccharic acid added fresh] using a pre-chilled mortar and pestle. The extract was centrifuged at 1500 g for 5 min and the supernatant filtered through a double layer of cheesecloth to remove any dislodged pellet pieces before recentrifugation at 18000 g for 25 min. The resulting green pellet was gently resuspended in 10 ml TSE (20 mM Tris/Cl, 10% sucrose, 0.05 M EDTA, pH 8.0) using a glass rod and homogenized by trituration using a 1 ml plastic transfer pipette. The low- and high-speed centrifugation steps were repeated, then the centrifuge tubes were inverted and left to drain for 1 min. The final pellet was resuspended in 50 µl TSE and placed in a 2 ml microfuge tube. If necessary, a micropette was used to homogenize any remaining clumps. The phytoplasma suspension was warmed to 37°C for approximately 3–5 min and quickly mixed with an equal volume of molten 2% (w/v) InCert agarose (FMC) dissolved in 2x TES (0.2 M Tris/Cl, 0.2 M NaCl, 20 mM EDTA, pH 8.0) maintained at 40°C. The phytoplasma agarose suspension was mixed by gentle trituration using a truncated micropipette tip, quickly pipetted into rectangular, 100 µl plastic moulds taped at the base and allowed to set on ice for 5–15 min. Once set, the agarose blocks were expelled into lysis buffer (0.5 M EDTA, 1% N-lauroylsarcosine, pH 8.0) in a 25–50 ml plastic tube or glass jar using a plastic spatula. Approximately 1 ml lysis buffer was used per 100 µl block. Proteinase K (Amresco) was added to the blocks in lysis buffer from a freshly prepared 20 mg ml⁻¹ stock dissolved in sterile distilled water, to give a final concentration of 1 mg ml⁻¹. The blocks were left on ice for 0.5 h before incubating at 50°C for 2 d, with three changes of lysis buffer and proteinase K per day, the last one just before leaving overnight. If the blocks were still green at the end of the second day, they were left overnight in fresh lysis buffer with proteinase K. At the end of the digestion period, blocks were clear and sometimes had a lime/yellow tinge. Unless used immediately, the blocks were stored at 4°C in a minimal volume of lysis buffer without proteinase K.

**Restriction endonuclease digestion of DNA in agarose blocks and PFGE.** Low-molecular-mass DNA was removed from agarose blocks by electrophoresis prior to restriction enzyme digestion (Whaling & Swayne, 1993). Agarose blocks were digested in 100 µl 1× restriction enzyme buffer supplied with the enzyme, DTT (40 µM final concentration), BSA (60 µg ml⁻¹ final concentration), and 20–40 U enzyme at the recommended temperature. Rare-cutting restriction endonucleases were selected on the basis of the length and G+C content of their recognition sequence to generate a small number of fragments suitable for restriction mapping by PFGE. Genomic grade enzymes used for the digestions were NotI, SfiI, XhoI, ApaI, BssHI, BglII, BamHI, SmaI, SacI, XbaI and I-CeuI. PFGE was performed by the contour-clamped homogeneous-electric field (CHEF) technique using the CHEF-DR III system (Bio-Rad) with 1% Seakem agarose (FMC). When digested fragments were to be excised from the gel and used either as probes or for a second digestion, Seaplaque agarose (FMC) was used. Electrophoresis was performed at 6 V cm⁻¹ for 18–22 h at an included angle of 120° in 0.5× TBE buffer (0.045 M Tris/borate, 1 mM EDTA) maintained at 14°C with varying ramped pulse times. Molecular masses were estimated by comparisons to yeast chromosome (YC) standards (Saccharomyces cerevisiae YNN295; Bio-Rad), lambda (λ) DNA concatemers (lambda ladder; Bio-Rad) or lambda DNA digested with HindIII (λ/HindIII; Promega). Two-dimensional (2-D) digestions of the genome with BssHII, SmaI, EagI and I-CeuI were performed as described by Bautsch (1988).

**Southern hybridizations.** DNA fragments in pulsed-field gels (PFGs) were either UV-irradiated (UV Stratalinker; Stratagene) or soaked in 0.1 M HCl, and then denatured, neutralized and blotted according to standard procedures (Sambrook et al., 1989). Probes used for Southern hybridizations included random probes from SPLL-V4 (Davis et al., 1997), apple proliferation (provided by B. Mogen) and WX (Firrao et al., 1996b) phytoplasmas; gel-purified PFGE fragments; PCR-amplified products containing the SPLL-V4 16S rRNA gene plus 165/235 spacer region (Kirkpatrick et al., 1994) and the ftsuf/tuf gene (B. Schneider, unpublished); and mycoplasma gene probes for tRNA¹⁰⁸ and tRNA¹⁰⁹ (Samuelsson et al., 1985), the rRNA operon (Amkan et al., 1982), serine hydroxymethyltransferase and RNA polymerase B subunit (Rasmussen & Christiansen 1990), ATPase (Rasmussen & Christiansen 1987), elongation factor EF-Tu (Inamine et al., 1989) and tRNA¹⁰⁸ (Guindy et al., 1989). Inserts less than 2 kb were amplified from a 1:1000 dilution of spin-purified plasmid DNA (Qiagen) using the universal primers M13f/M13r. Otherwise, entire plasmids were used. DNA from excised gel
Phytoplasma chromosome analysis fragents was purified using a kit (Qiagen). Approximately 20 ng purified plasmid DNA, PCR product or gel-excised DNA was labelled with [α-32P]dATP using a random priming kit (Promega). Hybridizations and washes were performed at 55 °C for homologous probes and 40 °C for heterologous probes as described by Sambrook et al. (1989). Filters were exposed to Hyperfilm MP film (Amersham) either overnight or for several days depending on signal intensity and developed according to standard procedures (Sambrook et al., 1989).

**Sequencing.** The random clone pH4 was purified using a plasmid mini kit (Qiagen) and the sequencing reactions were performed using the Big Dye Terminator kit as described by the manufacturer (Perkin-Elmer). Forward and reverse M13 primers were used. A BLAST (Basic Local Alignment Search Tool) search (Altschul et al., 1990) was performed to identify alignments with protein sequences available in the database using software provided by the Australian National Genomic Information Service, Sydney.

**RESULTS**

**Symptoms and relatedness of strains SPLL-V4 and TBB**

Symptoms of SPLL in periwinkle were reduced flower size and virescence, whereas symptoms of TBB in periwinkle were extreme phyllody and lack of normal flower production. As the plants became older, periwinkles with SPLL became more chlorotic, whereas periwinkles with TBB tended to show proliferation. rDNA from SPLL in sweet potato and SPLL transmitted from sweet potato to periwinkle was polymorphic, with one slight band shift in AluI- and RsaI-restricted 16S rDNA (Davis et al., 1997), and the pattern attributed to the phytoplasma in periwinkle was called SPLL-V4 to distinguish it from that in the field-collected sweet potato. The SPLL-V4 pattern has been found in a variety of other naturally infected plants (Schneider et al., 1998).

**Table 1. Size estimation of digested DNA from PFGs for the SPLL-V4 chromosome**

<table>
<thead>
<tr>
<th>Enzyme/fragment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>6</th>
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<td>334</td>
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<td></td>
<td></td>
<td>333</td>
</tr>
<tr>
<td>I-CeA</td>
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<td>281</td>
<td>282</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>285</td>
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<td>617</td>
<td></td>
<td></td>
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<td>618</td>
</tr>
<tr>
<td>EagI</td>
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<td>291</td>
<td>291</td>
<td>291</td>
<td></td>
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<td>287</td>
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<td>215</td>
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<td>EaC</td>
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<td>128</td>
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<td></td>
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<tr>
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<td>26</td>
<td>24</td>
<td>33</td>
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<td>Total</td>
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<td>628</td>
<td>617</td>
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<tr>
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<td>SmG</td>
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<td>14</td>
<td>18</td>
<td>18</td>
<td>16</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>612</td>
<td>609</td>
<td>587</td>
<td>662</td>
<td>619</td>
<td>629</td>
<td></td>
<td>629</td>
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<tr>
<td>Total (±SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>622±16</td>
</tr>
</tbody>
</table>

**Restriction endonuclease digestion and size estimation of the SPLL-V4 phytoplasma chromosome**

Failure to pre-electrophorese blocks resulted in smeared lanes and digested bands were difficult to distinguish (results not shown). Enzymes which did not cut the chromosome were NotI, SfiI and KpnI. Enzymes which cut the chromosome into more than eight fragments, generated fragments which contained very large and very small fragments, or which generated similar-sized fragments that were difficult to separate were XhoI, Apal, BamHI and XbaI. The enzymes BssHII, SmaI, EagI, I-CeuI and SalI generated four, seven, three, two and six fragments, respectively (Fig. 1), which were
never observed in preparations from healthy periwinkle. The sizes of fragments for any one restriction enzyme digestion were summed to give an estimated size of 622 kb for the SPLL-V4 chromosome (Table 1). Digestions with BssHII at 50 °C were always complete. Smal occasionally produced only partial digests, and double the amount of enzyme (40 U) was generally added to improve digestions. Digestions with Smal, EagI and I-CeuI also generated faint bands which did not add up to products of partial digestions.

**Physical mapping of the SPLL-V4 chromosome**

2-D reciprocal digestions were performed using BssHII, Smal, EagI and I-CeuI, to give six 2-D PFGs, an example of which is given in Fig. 2. Fragments produced by

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**Table 2. Summary of fragment overlap from 2-D reciprocal digestions**

Values are sizes in kb of digestion products of fragments restricted with a second enzyme. Double digestion products which were predicted to fit the map but not actually observed on PFGs are given in italics. The sum of the generated fragments is given in square brackets.

<table>
<thead>
<tr>
<th>Enzyme Fragment</th>
<th>Size (kb)</th>
<th>Second enzyme:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BssHII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EagI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I-CeuI</td>
</tr>
<tr>
<td>BssHII</td>
<td>BsA 303</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bb 244</td>
<td>104 + 97 + 20 + 11 + 10 [242]</td>
</tr>
<tr>
<td></td>
<td>BbC 46</td>
<td>37 = BbC [37]</td>
</tr>
<tr>
<td></td>
<td>BbD 28</td>
<td>15 + 12 [27]</td>
</tr>
<tr>
<td>Smal</td>
<td>SmA 170</td>
<td>104 + 35 + 15 + 12 [166]</td>
</tr>
<tr>
<td></td>
<td>SmB 145</td>
<td>145 = SmB [145]</td>
</tr>
<tr>
<td></td>
<td>SmC 129</td>
<td>130 = SmC [130]</td>
</tr>
<tr>
<td></td>
<td>SmD 117</td>
<td>104 + 10 [114]</td>
</tr>
<tr>
<td></td>
<td>SmE 31</td>
<td>35 = SmE [35]</td>
</tr>
<tr>
<td></td>
<td>SmF 21</td>
<td>26 = SmF [26]</td>
</tr>
<tr>
<td></td>
<td>SmG 17</td>
<td>20 = SmG [20]</td>
</tr>
<tr>
<td>EagI</td>
<td>EaA 288</td>
<td>115 + 91 + 35 + 20 [261]</td>
</tr>
<tr>
<td></td>
<td>EaB 211</td>
<td>146 + 65 [211]</td>
</tr>
<tr>
<td></td>
<td>EaC 127</td>
<td>122 = EaC [122]</td>
</tr>
<tr>
<td>I-CeuI</td>
<td>CeA 334</td>
<td>180 + 150 [330]</td>
</tr>
<tr>
<td></td>
<td>CeB 285</td>
<td>122 + 98 + 48 + 25 [293]</td>
</tr>
</tbody>
</table>
digestion of the chromosome with a single enzyme were designated Bs, Sm, Ea and Ce to identify the enzymes BssHIII, SmaI, EagI and I-CeuI, respectively. Products of double digestions were named in a similar fashion.

A summary of fragment overlaps deduced from the PFGs is provided in Table 2. For any one single-enzyme-generated fragment, the sum of the corresponding double digestion fragments from the 2-D gel was determined and compared to the size of the fragment calculated from single enzyme digests. Discrepancies where the sum of the double digestion products was smaller than that of the single fragment from which they were derived (Table 2) were probably due to small bands in the double digests which had either run off the PFG or which were too faint to be observed.

Based on the 2-D reciprocal digestion data, overlapping fragments were identified, and along with size determination of the single and double digests, relative cleavage positions could be located. The smaller SmaI fragments did not contain restriction sites for the other three enzymes, and thus the position of these fragments remains tentative. Overall, the size of the fragments determined in double digests was consistent with sizes determined from one-dimensional PFGs. EagI was found to cut the chromosome three times, with two of the restriction sites located within the I-CeuI recognition sequence, as observed in double digests (results not shown).

Five random probes generated from SPLL-V4 DNA (H4, H21, H30, H80 and E21) hybridized to single bands on membranes of enzyme-digested SPLL-V4 phytoplasma DNA. An example of a hybridization using probe H30 is shown in Fig. 3. These probes were located on the physical map and confirmed fragment arrangements based on reciprocal digestions. Southern hybridizations using some gel-purified fragments were also performed to check the arrangement of fragments on the map.

**Gene mapping**

Hybridizations using the 16S rRNA gene probe were performed on BssHIII-, SmaI-, EagI- and I-CeuI-digested chromosomal DNA. For each of the BssHIII and SmaI digests, two fragments hybridized, indicating two copies of the 16S rRNA gene (Fig. 4b). However, digestions of the chromosome with EagI and I-CeuI only resulted in one strong hybridization signal each. The fus/tuf gene probe hybridized to single bands of digested chromosomal phytoplasma DNA indicating one copy of these linked genes (Fig. 4c). The hybridization signal obtained for the BssHIII-digested DNA was unclear as the DNA was partially degraded. Nevertheless, the fus/tuf genes could be located on the map.

Apart from the mycoplasma ribosomal operon gene probe (pMC5) which gave faint hybridization signals, no other mycoplasma probes hybridized to membranes, even at 40 °C. Chromosomal probes derived from the WX or apple proliferation phytoplasmas also did not hybridize to membranes containing SPLL-V4 DNA, even at lower temperatures.

**Map construction**

The information derived from the reciprocal digestions and hybridizations was assembled to derive a restriction map for the SPLL-V4 phytoplasma, with locations of the random clones and the rrn and fus/tuf loci superimposed (Fig. 5). Because no restriction enzyme was found to cut the chromosome once to give a linear molecule, the BssHIII site between BsD and BsA was arbitrarily chosen as the beginning of the map. The probe sizes are not to scale and their locations lie somewhere within the smallest fragment bounded on each side by a cleavage site. The best resolution of the chromosome map was approximately 28 kb with the largest fragment of 108 kb (between positions 433 and 541) having no internal sites mapped.

**Sequence analysis**

Translation of the DNA sequence of clone pH4 (EMBL accession number AJ245515) identified one ORF and the BLAST search of the protein database revealed a high level of similarity to the gidA gene (glucose-inhibited division protein) from several bacteria including *Bacillus subtilis*, *Borrelia burgdorferi* and *Mycoplasma genitalium*. 

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**Fig. 3.** Hybridization of digested SPLL-V4 chromosomal DNA with the random clone H30 (b). SPLL-V4 DNA used in these hybridizations was digested with BssHIII, SmaI, EagI and I-CeuI as shown in (a). λ, lambda ladder used as molecular mass marker.
A. C. PADOVAN and OTHERS

Fig. 4. Hybridization of digested SPLL-V4 chromosomal DNA with the 16S gene probe (b) and with the fus/tuf probe (c). SPLL-V4 DNA used in these hybridizations was digested with BssHII, Smal, Eagl and I-Ceu as shown in (a). λ, lambda ladder used as molecular mass marker.

Fig. 5. Physical and genetic map of the chromosome of the SPLL-V4 phytoplasma. Restriction sites for BssHII, Smal, Eagl and I-Ceu are indicated. Fragment sizes are drawn to scale, giving a total genome size of 622 kb. Map units are in kb, with the 5' end of the BsaA fragment arbitrarily designated as the beginning of the map at position 0/622. The location of random probes and genes are indicated but are not to scale. The orientation of the 16S/tRNAIle/23S genes are indicated by arrows. The asterisks indicate that the order of the fus/tuf gene, the gidA gene and probe H80 have not been determined. Restriction enzyme digestion of the TBB chromosome

Restriction enzyme digestion of the TBB chromosome

Enzymatic digestion of the TBB chromosome using BssHII, Smal, Eagl and I-Ceu revealed a different pattern to that obtained with SPLL-V4 (Fig. 6). Digestion of TBB with BssHII generated six bands compared with four for SPLL-V4, whilst digestion with Smal and Eagl resulted in five bands each for TBB compared to seven and three bands, respectively, for SPLL-V4. Digestion with I-Ceu generated three bands. Based on BssHII digestion products, a chromosome size of 662 kb was estimated for the TBB phytoplasma.

Fig. 6. Comparison of TBB and SPLL-V4 chromosome digestion patterns using BssHII, Smal, Eagl and I-Ceu. λ, lambda ladder used as molecular mass marker. Pulse times were ramped from 2 to 40 s for 20 h.

DISCUSSION

At 622 kb, the SPLL-V4 chromosome is one of the smallest of the phytoplasmas with only two Bermuda grass white leaf (BGWL) isolates having a smaller
The chromosome size of 530 kb (Marcone et al., 1999). The size of the TBB chromosome was estimated to be 662 kb, which is smaller than the 690 kb estimated by Marcone et al. (1999) from full-length chromosomes. This discrepancy could be due to different electrophoresis conditions, anomalies in the separation of larger compared to smaller, digested fragments, or to different isolates being used. Within the faba bean phyllody (FBP) phytoplasma group to which strains SPLL-V4 and TBB belong, chromosome sizes fall within a narrow range and include 660 kb for the faba bean phyllody phytoplasma (Marcone et al., 1999), 720 kb for witches' broom disease of lime (Zreik et al., 1995) and 790 kb for the phytoplasma associated with cotton phyllody (Marcone et al., 1999). The size of the SPLL-V4 phytoplasma genome is the smallest within this group. Genomes of the aster yellows (AY) phytoplasmas were found to be much larger (Neimark & Kirkpatrick, 1993), but recent genome size estimations of 29 AY isolates have shown that chromosome sizes within this group are heterogeneous and range from 660 kb to 1130 kb (Marcone et al., 1999). Interestingly, phytoplasmas in the AY group are widespread and have been reported in several Continents, whereas phytoplasmas in the FBP group have only been identified in Australia and Asia (Seemüller et al., 1998). As more phytoplasma genomes are studied, a relationship between geographic distribution and chromosome size range may emerge to reflect changing genomes required for the adaptation of certain phytoplasma groups to varying conditions which include different plant hosts and insect vectors.

Bands were occasionally observed in PFGs prior to enzymic digestion, suggesting that mechanical processes during the extraction or digestion process linearized sufficient molecules for the chromosomes to enter the gel. Earlier preparations of digested phytoplasma DNA produced only faint or no bands and much effort was invested in increasing DNA yields to a level suitable for mapping work. Gamma irradiation did not increase the amount of linearized DNA (results not shown), suggesting that low phytoplasma titres rather than DNA immobilization was the problem, so some modifications were made to the original extraction procedure of Neimark & Kirkpatrick (1993). These changes included (i) the addition of EDTA to the extraction and resuspension medium to reduce possible DNase activity, which is known to be high in mycoplasmas (Razin et al., 1964); (ii) filtration of the supernatant from the first low-speed centrifugation through a double layer of cheesecloth to prevent any pieces of dislodged pellet being transferred to a fresh tube for recentrifugation; (iii) resuspension of the final phytoplasma solution in a minimal volume of TSE to increase the overall concentration of phytoplasmas in the agarose blocks; and (iv) increased proteinase K buffer changes (2–3 times a day), to give clear blocks within 2 d. It is most likely that a combination of these modifications contributed to an improvement in overall phytoplasma DNA yield, which suggests that the presence of plant endonucleases may have contributed to the low yields. Catharanthus roseus plants are the most commonly used host for maintaining phytoplasmas in laboratories worldwide because they are easy to grow, can be readily grafted and display a range of symptoms. However, extraction of high-molecular-mass phytoplasma DNA from infected Catharanthus roseus plants generally gives poor yields such that alternative plant hosts or insects have been used as a richer source of DNA for chromosome extraction (Neimark & Kirkpatrick, 1993; Firrao et al., 1996b; Lauer & Seemüller, 1998). It is possible that the method modifications described here will improve chromosome extractions from phytoplasmas maintained in Catharanthus roseus, which would allow many more phytoplasma genomes to be studied.

No restriction endonuclease was found to cut the SPLL-V4 chromosome only once to give a linear molecule. A total of 16 restriction sites were localized on the map, with two of the three EagI sites falling within the two I-CeuI sites. The presence of EagI sites within I-CeuI sites has also been reported for Borrelia species (Ojaimi et al., 1994) with the I-CeuI recognition sequence tolerating some degeneracy. There appeared to be no conservation of restriction sites between the SPLL-V4 and WX chromosomes, which have different digestion patterns for all of the four enzymes used. Of the 16 mapped cleavage sites for SPLL-V4, most seem to be fairly evenly spread throughout the genome and this is also the case for the WX map which contains 20 cleavage sites (Firrao et al., 1996b).

Faint bands were often observed in PFGs of Smal-, EagI- and I-CeuI-digested SPLL-V4 DNA. Based on the estimated sizes of these faint bands, they could not be designated as products from partial digestions. The faint bands were never observed in undigested or BssHII-digested SPLL-V4 DNA, or in preparations of healthy periwinkle. Southern hybridizations using gel-purified faint bands as probes showed hybridization to distinct SPLL-V4 bands (results not shown), suggesting that these faint bands are of phytoplasma origin, and similarly, SPLL-V4 random probes hybridized with these bands, supporting the conclusion that they represent phytoplasma DNA rather than a different pathogen present in the preparations. If two phytoplasma chromosomes (represented by the strong bands and the faint bands) were present, they would be polymorphic for Smal-, EagI- and I-CeuI but not for BssHII. It is unlikely that two separate chromosomes from two phytoplasma strains would have an identical BssHII pattern and different patterns for the other enzymes, so the results indicate the presence of two chromosome types in a phytoplasma population rather than two chromosomes from two different types of phytoplasma. This could occur if there was a chromosome rearrangement, which is a relatively common event in mollicutes (Bhugra et al., 1995; Lysnyansky et al., 1996), within one of the BssHII fragments. This would not alter the BssHII pattern, but would dramatically change the sizes of the fragments generated by the other enzymes. Chromosomal rearrangements have been reported for Spiroplasma citri where continued grafting of infected plants resulted in...
The SPLL-V4 map shows the locations of the two unlinked rrr operons containing the 16S rRNA, intergenic tRNA^{Ile} and 23S rRNA genes, in similar positions to the WX map (Firrao et al., 1996b). I-CeuI is a rare-cutting restriction enzyme which recognizes sequences in the 23S rRNA genes of many bacteria (Liu & Sanderson, 1995; Katayama et al., 1995; Toda & Itaya, 1995). The hybridization of only one band in I-CeuI-digested phytoplasma DNA to the 16S rDNA probe implies that both 16S rRNA genes are located within that fragment and therefore that one of the operons is inverted. The inversion of one of the SPLL-V4 phytoplasma rrr loci described in this study has not been reported before for phytoplasmas. The proposed orientation of the phytoplasma ribosomal genes would be consistent with a putative origin of replication (oriC) located between these two genes, somewhere between map positions 433 and 104 in a clockwise direction, with bidirectional replication and transcription. This is the case for Escherichia coli where most of the rrr loci are located in the half of the genetic map centred around oriC and are oriented such that transcription occurs in the same direction as replication (Lindahl & Zengel, 1986). Sequence analysis of the entire M. genitalium chromosome showed that genes upstream from the origin are transcribed in the antisense direction (Fraser et al., 1995). This is most likely the case for the phytoplasmas.

The only other phytoplasma genes positioned on the SPLL-V4 chromosome map were fus and tuf, encoding the elongation factors G and Tu, respectively. These two genes are linked, as shown by sequence analysis of the PCR product used to generate the probe (B. Schneider, unpublished) and as recently shown for other phytoplasmas (Berg & Seemüller, 1999). This is not the case for M. genitalium where the fus and tuf loci are approximately 217 kb apart (Fraser et al., 1995). In other bacteria, including E. coli and Bacillus subtilis, fus and tuf genes are adjacent (Post & Nomura, 1980; Yasumoto et al., 1996). The tuf locus has been mapped for other mollicutes and usually occurs in association with other important genes involved with DNA replication, repair and transcription (Ladefoged & Christiansen, 1992; Peterson et al., 1995). These genes are usually clustered around the origin of replication. For the SPLL-V4 genome, the clustering of two random probes, the gidA gene, the fus/tuf genes and one of the rrr loci suggests that the putative origin of replication for this phytoplasma, oriC, is upstream of the location of pH80. Once more restriction sites are mapped and more phytoplasma genes cloned and mapped, particularly those associated with DNA replication and repair, transcription and translation, then a better understanding of the location of the phytoplasma origin of replication will emerge.

The only mollicute gene probe which hybridized to phytoplasma DNA was pMC5, containing the highly conserved rrr locus, with no other gene probes having sufficient similarity to give a signal. This shows that the similarities at the gene sequence level between mycoplasmas and phytoplasmas are very limited. Even random chromosomal probes from other phytoplasmas did not share sufficient similarity with SPLL-V4 to give a hybridization signal. Further gene mapping of phytoplasmas should therefore focus on genes isolated from the phytoplasma being studied.

These results and the observed lack of restrictionendonuclease cleavage site conservation between WX and SPLL-V4 indicated that phytoplasmas are a diverse group of organisms. Even between the two phylogenetically closely related strains SPLL-V4 and TBB there appears to be no conservation of restriction sites, with no common fragments observed between them for any of the four restriction enzymes used. Faint bands were generated for Smal, EagI and I-CeuI digests of TBB DNA, which were different to those generated with SPLL-V4 DNA. The presence of three bands in I-CeuI digests of TBB DNA may suggest the presence of another chromosome or non-specific digestion, but this needs to be investigated further. The varied restriction patterns between SPLL-V4 and TBB were unexpected because of their close phylogenetic association based on 16S rDNA sequence comparisons, and also because dot-blot hybridizations of TBB and SPLL-V4 DNA with TBB- and SPLL-V4-derived clones indicated that they share a great deal of sequence homology (Schneider et al., 1998). Other comparisons of chromosome restriction patterns between closely related phytoplasmas in the WX phytoplasma group have also revealed polymorphisms (Firrao et al., 1996a).

Gene order in several mollicutes is conserved (Peterson et al., 1995; Ye et al., 1995) and this may be a better way to look at inter- and intra-species diversity. To determine whether the same also holds for phytoplasmas, more organisms, closely and distantly related, need to be studied, and more genes need to be mapped. Genetic rearrangements, such as deletions or insertions in particular areas of the phytoplasma genome, may be found to be correlated with biological differences, such as the range of symptoms expressed, host range and transmissibility. Areas of genomic rearrangements found in phytoplasmas could then be targeted for more detailed sequencing studies to identify key genes.

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REFERENCES


