

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

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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 *Bss*HII, *Sma*I, *Eag*I and *I-Ceu*I. 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 & Seemüller, 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 (Jarusch *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; Marccone *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

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Abbreviations: 2-D, two-dimensional; PFG, pulsed-field gels; SPLL, sweet potato little leaf; TBB, tomato big bud; WX, Western X.

The EMBL accession number for the sequence determined in this work is AJ245515.

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 (*Lycopersicon esculentum*) with big bud disease was obtained from Adelaide (SA, Australia). The phytoplasmas in these field-collected plants were transmitted to *Catharanthus roseus* (periwinkle) plants using dodder (*Cuscuta australis*). Symptomatic periwinkles were maintained in a shadehouse by cleft-grafting onto healthy periwinkle rootstock, either obtained as seedlings or by cuttings. Periwinkles were harvested for chromosome isolation between 4 weeks and 3 months after grafting.

Preparation of phytoplasma chromosomes. Phytoplasma chromosomes were prepared as described by Neimark & Kirkpatrick (1993) with some modifications. All steps were performed at 4 °C. Approximately 2 g plant midribs and petioles from the top 10 cm of periwinkle plants was ground in 12 ml ice-cold isolation buffer [0.1 M Na₂HPO₄, 0.03 M NaH₂PO₄, 10% (w/v) sucrose, 2% (w/v) polyvinylpyrrolidone (molecular mass 40000), 10 mM EDTA, pH 7.6, with 0.15% BSA and 1 mM isoascorbic 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 micropestle 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 2 × 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 (Whatling & Thomas, 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*, *BssHII*, *BglI*, *BamHI*, *SmaI*, *SalI*, *KpnI*, *EagI*, *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 rDNA gene plus 16S/23S spacer region (Kirkpatrick *et al.*, 1994) and the *fus/tuf* gene (B. Schneider, unpublished); and mycoplasma gene probes for tRNA^{Arg} and tRNA^{Gly} (Samuelsson *et al.*, 1985), the rRNA operon (Amikan *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 tRNA^{Ile} (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

fragments was purified using a kit (Qiagen). Approximately 20 ng purified plasmid DNA, PCR product or gel-excised DNA was labelled with [α - 32 P]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).

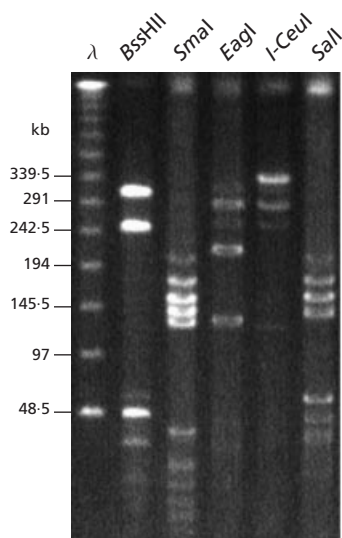


Fig. 1. Digestion of the SPLL-V4 chromosome with *BssHII*, *SmaI*, *EagI*, *I-CeuI* and *Sall*. λ , lambda ladder used as molecular mass marker. Pulse times were ramped from 2 to 40 s for 20 h.

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

Sizes of fragments were generally determined from three separate PFGs. Values are in kbp.

Enzyme/ fragment	1	2	3	4	5	6	7	Mean
I-CeuI								
I-CeA	331	334	334					333
I-CeB	291	281	282					285
Total	622	615	617					618
EagI								
EaA	286	291	281	291				287
EaB	210	210	209	215				211
EaC	128	127	127	128				128
Total	625	628	618	635				627
BssHII								
BsA	299	304			300	308		303
BsB	242	246			239	245		243
BsC	47	52	39	37	51	46		45
BsD			26	24	33			27
Total					624			620
SmaI								
SmA	166	175	163	171	170	174	169	170
SmB	139	152	141	140	148	150	141	145
SmC	126	135	125	117	136	135	127	129
SmD	114	127	114	97	126	124	114	116
SmE	28		29	27	38		29	30
SmF	20		19	18	24		20	20
SmG	18		15	14	18		18	16
Total	612		609	587	662		619	629
Sall								
SaA	172	187	188	172	177			179
SaB	152	163	160	152	155			157
SaC	135	143	131	136	135			136
SaD	59			59	57			58
SaE		51		45				48
SaF		36	38					37
Total								617
Total (\pm SD)								622 \pm 16

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*, *ApaI*, *BamHI* and *XbaI*. The enzymes *BssHII*, *SmaI*, *EagI*, *I-CeuI* and *Sall* generated four, seven, three, two and six fragments, respectively (Fig. 1), which were

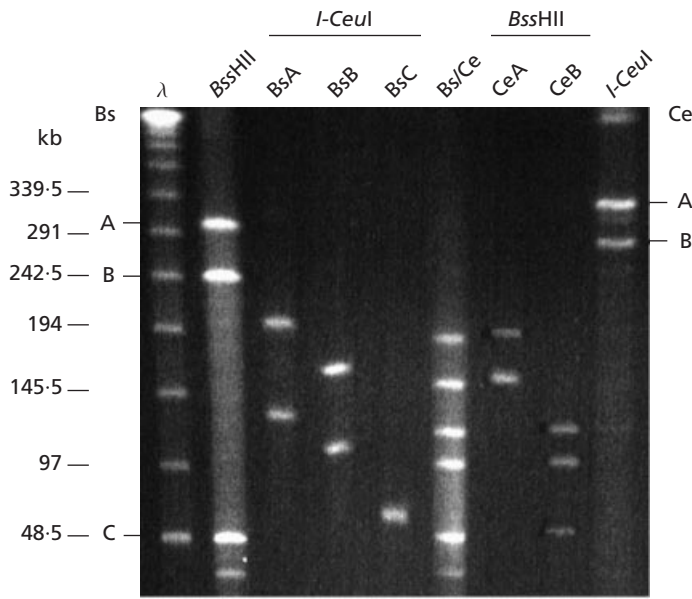


Fig. 2. 2-D reciprocal digestions of SPLL-V4 chromosomal DNA digested with *BssHIII* and *I-CeuI*. Double digestion products (*Bs/Ce*) resolved in one dimension are shown in the middle. Fragments resulting from single digestions are indicated by letters at the sides and above the lanes. The enzyme used in the second digestion is shown above the corresponding fragments. λ , lambda ladder used as molecular mass marker. Pulse times were ramped from 5 to 30 s for 14 h.

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.

Enzyme	Fragment	Size (kb)	Second enzyme:			
			<i>BssHIII</i>	<i>SmaI</i>	<i>EagI</i>	<i>I-CeuI</i>
<i>BssHIII</i>	BsA	303	–	140 + 13 + 26 [296]	122 + 115 + 65 [302]	180 + 122 [302]
	BsB	244	–	104 + 97 + 20 + 11 + 10 [242]	146 + 91 [237]	150 + 98 [248]
	BsC	46	–	37 = BsC [37]	35 = BsC [35]	48 = BsC [48]
	BsD	28	–	15 + 12 [27]	25 = BsD [25]	25 = BsD [25]
<i>SmaI</i>	SmA	170	104 + 35 + 15 + 12 [166]	–	150 + 35 [185]	160 + 30 [190]
	SmB	145	145 = SmB [145]	–	115 + 45 [160]	130 + 40 [170]
	SmC	129	130 = SmC [130]	–	80 + 50 [130]	140 = SmC [140]
	SmD	117	104 + 10 [114]	–	120 = SmD [120]	130 = SmD [130]
	SmE	31	35 = SmE [35]	–	40 = SmE [40]	25 = SmE [25]
	SmF	21	26 = SmF [26]	–	25 = SmF [25]	20 = SmF [20]
	SmG	17	20 = SmG [20]	–	20 = SmG [20]	15 = SmG [15]
<i>EagI</i>	EaA	288	115 + 91 + 35 + 20 [261]	150 + 115 + 25 [290]	–	288 = EaA = CeB [288]
	EaB	211	146 + 65 [211]	120 + 50 + 35 + 20 [225]	–	211 = EaB [211]
	EaC	127	122 = EaC [122]	80 + 40 [120]	–	127 = EaC [127]
<i>I-CeuI</i>	CeA	334	180 + 150 [330]	140 + 130 + 40 + 30 + 25 [365]	211 + 127 [338]	–
	CeB	285	122 + 98 + 48 + 25 [293]	160 + 125 [285]	288 = CeB [288]	–

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 *BssHIII* at 50 °C were always complete. *SmaI* occasionally produced only partial digests, and double the amount of enzyme (40 U) was generally added to improve digestions. Digestions with *SmaI*, *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 *BssHIII*, *SmaI*, *EagI* and *I-CeuI*, to give six 2-D PFGs, an example of which is given in Fig. 2. Fragments produced by

digestion of the chromosome with a single enzyme were designated Bs, Sm, Ea and Ce to identify the enzymes *Bss*HIII, *Sma*I, *Eag*I and *I-Ceu*I, 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 *Sma*I 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. *Eag*I was found to cut the chromosome three times, with two of the restriction sites located within the *I-Ceu*I recognition sequence, as observed in double digestions (results not shown).

Five random probes generated from SPLI-V4 DNA (H4, H21, H30, H80 and E21) hybridized to single bands on membranes of enzyme-digested SPLI-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 *Bss*HIII-, *Sma*I-, *Eag*I- and *I-Ceu*I-digested chromosomal DNA. For each of the *Bss*HIII and *Sma*I digests, two fragments hybridized, indicating two copies of the 16S rRNA gene (Fig. 4b). However, digestions of the chromosome with *Eag*I and *I-Ceu*I 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 *Bss*HIII-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,

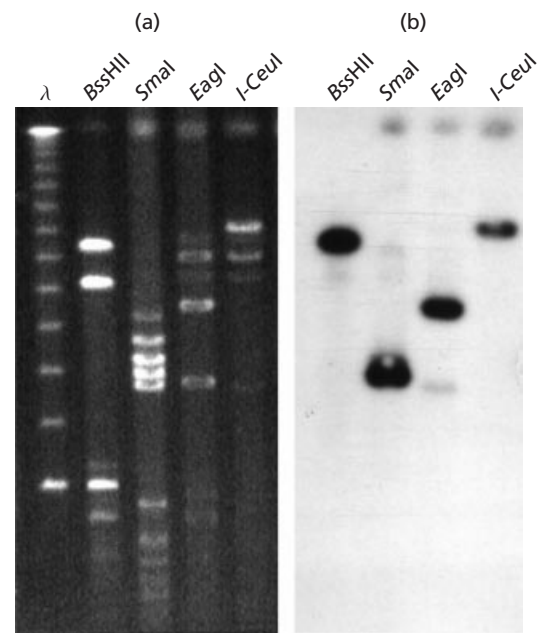


Fig. 3. Hybridization of digested SPLI-V4 chromosomal DNA with the random clone H30 (b). SPLI-V4 DNA used in these hybridizations was digested with *Bss*HIII, *Sma*I, *Eag*I and *I-Ceu*I as shown in (a). λ , lambda ladder used as molecular mass marker.

even at 40 °C. Chromosomal probes derived from the WX or apple proliferation phytoplasmas also did not hybridize to membranes containing SPLI-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 SPLI-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 *Bss*HIII 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*, *Borrellia burgdorferi* and *Mycoplasma genitalium*.

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 *SmaI*-, *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 *SmaI*, *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

genome polymorphisms compared to *S. citri* passaged by insect transmission (Ye *et al.*, 1996).

The SPL-4 map shows the locations of the two unlinked *rrn* operons containing the 16S rRNA, intergenic tRNA^{le} 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 SPL-4 phytoplasma *rrn* 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 *rrn* 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 SPL-4 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 SPL-4 genome, the clustering of two random probes, the *gidA* gene, the *fus/tuf* genes and one of the *rrn* 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 *rrn* 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 SPL-4 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 restriction endonuclease cleavage site conservation between WX and SPL-4 indicated that phytoplasmas are a diverse group of organisms. Even between the two phylogenetically closely related strains SPL-4 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 *Sma*I, *Eag*I and I-CeuI digests of TBB DNA, which were different to those generated with SPL-4 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 SPL-4 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 SPL-4 DNA with TBB- and SPL-4-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

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* 215, 403–410.
- Amikan, D., Razin, S. & Glaser, G. (1982). Ribosomal RNA genes in Mycoplasmas. *Nucleic Acids Res* 10, 4215–4222.
- Barbara, D. J., Davies, D. L. & Clark, M. F. (1998). Cloning and sequencing of a major membrane protein from chlorante (aster yellows) phytoplasma. *12th International Organisation of Myco-*

- plasmology Conference, 22–28 July 1998, Sydney, Australia (programme and abstracts), poster G.04.
- Bautsch, W. (1988).** Rapid physical mapping of the *Mycoplasma mobile* genome by two-dimensional field inversion gel electrophoresis techniques. *Nucleic Acids Res* **16**, 11461–11467.
- Berg, M. & Seemüller, E. (1999).** Chromosomal organization and nucleotide sequence of the genes coding for the elongation factors G and Tu of the apple proliferation phytoplasma. *Gene* **226**, 103–109.
- Bhugra, B., Voelker, L. L., Zou, N. X., Yu, H. L. & Dybvig, K. (1995).** Mechanism of antigenic variation in *Mycoplasma pulmonis* – interwoven, site-specific DNA inversions. *Mol Microbiol* **18**, 703–714.
- Carle, P., Laigret, F., Tully, J. G. & Bové, J. M. (1995).** Heterogeneity of genome sizes within the genus *Spiroplasma*. *Int J Syst Bacteriol* **45**, 178–181.
- Davis, M. J., Tsai, J. H., Cox, R. L., McDaniel, L. L. & Harrison, N. A. (1988).** Cloning of chromosomal and extrachromosomal DNA of the mycoplasma-like organism that causes maize bushy stunt disease. *Mol Plant–Microbe Interact* **1**, 295–302.
- Davis, R. E., Lee, I.-M., Douglas, S. M. & Dally, E. L. (1990).** Molecular cloning and detection of chromosomal and extra-chromosomal DNA of the mycoplasma-like organism associated with little leaf disease in periwinkle (*Catharanthus roseus*). *Phytopathology* **80**, 789–793.
- Davis, R. I., Schneider, B. & Gibb, K. S. (1997).** Detection and differentiation of phytoplasmas in Australia. *Aust J Agric Res* **48**, 535–544.
- Firrao, G., Scott, S., Smart, C., Carraro, L., Chang, C.-J., Seemüller, E. & Kirkpatrick, B. C. (1996a).** Serological and molecular genetic characterization of five members of the X-disease phytoplasma clade. *Inst Organ Mycoplasma Lett* **4**, 278–279.
- Firrao, G., Smart, C. D. & Kirkpatrick, B. C. (1996b).** Physical map of the western X-disease phytoplasma chromosome. *J Bacteriol* **178**, 3985–3988.
- Fraser, C. M., Gocayne, J. D., White, O. & 26 other authors (1995).** The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397–403.
- Guindy, Y. S., Samuelsson, T. & Johanssen, T.-I. (1989).** Unconventional codon reading by *Mycoplasma mycoides* tRNAs as revealed by partial sequence analysis. *Biochem J* **258**, 869–873.
- Inamine, J. M., Loechel, S. & Hu, P.-C. (1989).** Nucleotide sequence of the *tuf* gene from *Mycoplasma gallisepticum*. *Nucleic Acids Res* **17**, 10126.
- Jarusch, W., Saillard, C., Dosba, F. & Bové, J. M. (1994).** Differentiation of mycoplasma-like organisms (MLOs) in European fruit trees by PCR using specific primers derived from the sequence of a chromosomal fragment of the apple proliferation MLO. *Appl Environ Microbiol* **60**, 2916–2923.
- Katayama, S., Dupuy, B., Garnier, T. & Cole, S. T. (1995).** Rapid expansion of the physical and genetic map of the chromosome of *Clostridium perfringens* cpn50. *J Bacteriol* **177**, 5680–5685.
- Kirkpatrick, B. C., Smart, C. D., Gardner, S. L. & 9 other authors (1994).** Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA spacer sequences. *Inst Organ Mycoplasma Lett* **3**, 228–229.
- Kollar, A. & Seemüller, E. (1989).** Base composition of the DNA of the mycoplasma-like organisms associated with various plant diseases. *J Phytopathol* **127**, 177–186.
- Kuboyama, T., Huang, C. C., Lu, X., Sawayanagi, T., Kanazawa, T., Kagami, T., Matsuda, I., Tsuchizaki, T. & Namba, S. (1998).** A plasmid isolated from phytopathogenic onion yellows phytoplasma and its heterogeneity in the pathogenic phytoplasma mutant. *Mol Plant–Microbe Interact* **11**, 1031–1037.
- Kuske, C. R. & Kirkpatrick, B. C. (1990).** Identification and characterization of plasmids from the western aster yellows mycoplasma-like organism. *J Bacteriol* **172**, 1628–1633.
- Ladefoged, S. A. & Christiansen, G. (1992).** Physical and genetic mapping of the genomes of five *Mycoplasma hominis* strains by pulsed-field gel electrophoresis. *J Bacteriol* **174**, 2199–2207.
- Lauer, U. & Seemüller, E. (1998).** Physical map of the apple proliferation phytoplasma genome. *12th International Organisation of Mycoplasma Conference, 22–28 July 1998, Sydney, Australia* (programme and abstracts), poster D.59.
- Lim, P.-O. & Sears, B. B. (1992).** Evolutionary relationships of a plant-pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *J Bacteriol* **174**, 2606–2611.
- Lindahl, L. & Zengel, J. M. (1986).** Ribosomal genes in *Escherichia coli*. *Annu Rev Genet* **20**, 297–326.
- Liu, S.-L. & Sanderson, K. E. (1995).** I-CeuI reveals conservation of the genome of independent strains of *Salmonella typhimurium*. *J Bacteriol* **177**, 3355–3357.
- Lysnyansky, I., Rosengarten, R. & Yogev, D. (1996).** Phenotypic switching of variable surface lipoproteins in *Mycoplasma bovis* involves high-frequency chromosomal rearrangements. *J Bacteriol* **178**, 5395–5401.
- Marcone, C., Neimark, H., Ragozzino, A., Lauer, U. & Seemüller, E. (1999).** Chromosome sizes of phytoplasmas composing major phylogenetic groups and subgroups. *Phytopathology* **89**, 805–810.
- Neimark, H. & Kirkpatrick, B. C. (1993).** Isolation and characterisation of full-length chromosomes from non-culturable plant-pathogenic mycoplasma-like organisms. *Mol Microbiol* **7**, 21–28.
- Neimark, H. C. & Lange, C. S. (1990).** Pulse-field electrophoresis indicates full-length mycoplasma chromosomes range widely in size. *Nucleic Acids Res* **18**, 5443–5448.
- Ojaimi, C., Davidson, B. E., Saint Girons, I. & Old, I. G. (1994).** Conservation of gene arrangement and an unusual organization of rRNA genes in the linear chromosomes of the Lyme disease spirochaetes *Borrelia burgdorferi*, *B. garinii* and *B. afzelii*. *Microbiology* **140**, 2931–2940.
- Peterson, S. N., Lucier, T., Heitzman, K., Smith, E. A., Bott, K. F., Hu, P.-C. & Hutchison, C. A., III (1995).** Genetic map of the *Mycoplasma genitalium* chromosome. *J Bacteriol* **177**, 3199–3204.
- Post, L. E. & Nomura, M. (1980).** DNA sequences from the *str* operon of *Escherichia coli*. *J Biol Chem* **255**, 4660–4666.
- Pyle, L. E., Corcoran, L. N., Cocks, B. G., Bergemann, A. D., Whitley, J. C. & Finch, L. R. (1988).** Pulse-field electrophoresis indicates larger-than-expected sizes for mycoplasma genomes. *Nucleic Acids Res* **16**, 6015–6025.
- Pyle, L. E., Taylor, T. & Finch, L. R. (1990).** Genomic maps of some strains within the *Mycoplasma mycoides* cluster. *J Bacteriol* **172**, 7265–7268.
- Rasmussen, O. F. & Christiansen, C. (1987).** Identification of the proton ATPase operon in *Mycoplasma* strain PG50 by heterologous hybridization. *Isr J Med Sci* **23**, 393–397.
- Rasmussen, O. F. & Christiansen, C. (1990).** A 23 kb region of the *Mycoplasma* strain PG50 genome with three identified genetic structures. *Zentbl Bakteriol Suppl* **20**, 315–323.
- Razin, S., Knyszynski, A. & Lifshitz, Y. (1964).** Nucleases of mycoplasma. *J Gen Microbiol* **36**, 323–331.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular*

Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Samuelsson, T., Elias, P., Lustig, F. & Guindy, Y. S. (1985). Cloning and nucleotide sequence analysis of transfer RNA genes from *Mycoplasma mycoides*. *Biochem J* **232**, 223–338.

Schneider, B. & Seemüller, E. (1994). Presence of two sets of ribosomal genes in phytopathogenic mollicutes. *Appl Environ Microbiol* **60**, 3409–3412.

Schneider, B., Gibb, K. S. & Seemüller, E. (1997). Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. *Microbiology* **143**, 3381–3389.

Schneider, B., Gibb, K. S., Padovan, A., Davis, R. I. & De La Rue, S. (1998). Comparison and characterization of tomato big bud- and sweet potato little leaf-group phytoplasmas. *J Phytopathol* **147**, 1–64.

Sears, B. B., Lim, P.-O., Holland, N., Kirkpatrick, B. C. & Klomparens, K. L. (1989). Isolation and characterisation of DNA from a mycoplasma-like organism. *Mol Plant–Microbe Interact* **2**, 175–180.

Seemüller, E., Marccone, C., Lauer, U., Ragozzino, A. & Göschl, M. (1998). Current status of molecular classification of the phytoplasmas. *J Plant Pathol* **80**, 3–26.

Toda, T. & Itaya, M. (1995). I-CeuI recognition sites in the *rrn* operons of the *Bacillus subtilis* 168 chromosome: inherent landmarks for genome analysis. *Microbiology* **141**, 1937–1945.

Whatling, C. A. & Thomas, C. M. (1993). Preelectrophoresis of

agarose plugs containing bacterial chromosomal DNA prepared for analysis by pulsed field gel electrophoresis can improve the clarity of restriction patterns. *Anal Biochem* **210**, 98–101.

Whitley, J. C., Muto, A. & Finch, L. R. (1990). A physical map for *Mycoplasma capricolum* cal. Kid with loci for all known tRNA species. *Nucleic Acids Res* **19**, 399–400.

Yasumoto, K., Liu, H. T., Jeong, S. M., Ohashi, Y., Kakinuma, S., Tanaka, K., Kawamura, F., Yoshikawa, H. & Takahashi, H. (1996). Sequence analysis of a 50 kb region between *spoOH* and *rrnH* on the *Bacillus subtilis* chromosome. *Microbiology* **142**, 3039–3046.

Ye, F., Laigret, F., Carle, P. & Bové, J. M. (1995). Chromosomal heterogeneity among various strains of *Spiroplasma citri*. *Int J Syst Bacteriol* **45**, 729–734.

Ye, F., Melcher, U., Rascoe, J. E. & Fletcher, J. (1996). Extensive chromosome aberrations in *Spiroplasma citri* strain BR3. *Biochem Genet* **34**, 269–286.

Yu, Y.-L., Yeh, K.-W. & Lin, C.-P. (1998). An antigenic protein gene of a phytoplasma associated with sweet potato witches' broom. *Microbiology* **144**, 1257–1262.

Zreik, L., Carle, P., Bové, J. M. & Garnier, M. (1995). Characterization of the mycoplasma-like organism associated with witches' broom disease of lime and proposition of a *Candidatus* taxon for the organism, "*Candidatus* Phytoplasma aurantifolia". *Int J Syst Bacteriol* **45**, 449–453.

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