A chromosome map of the Flavescence dorée phytoplasma

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The Flavescence dorée phytoplasma (FD-P), a non-cultivable, plant-pathogenic bacterium of the class *Mollicutes*, is the causal agent of a quarantine disease affecting vineyards of southern Europe, mainly in southern France and northern Italy. To investigate FD-P diversity and phytoplasma genetic determinants governing the FD-P life cycle, a genome project has been initiated. A physical map of the chromosome of FD-P strain FD92, purified from infected broad beans, was constructed by performing restriction digests of the chromosome and resolving the fragments by PFGE. Single and double digestions of the chromosome with the enzymes *Sa*II, *Bss*HI, *M*luI and *E*agI were performed and used to map 13 restriction sites on the FD-P chromosome. The size of the chromosome was calculated to be 671 kbp. Southern blot analyses using cloned phytoplasma probes were carried out to assist in the arrangement of contiguous restriction fragments and to map eight genetic loci, including the two rRNA operons, the *tuf*, *uvrB-degV* and *secY-map* (FD9) genes, the FD2 marker and two orphan sequences (FDDH29 and FDSH05) isolated through subtractive suppression hybridization.

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INTRODUCTION

Phytoplasmas are plant-pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less organisms phylogenetically related to low G+C content Gram-positive bacteria (Weisburg *et al.*, 1989). Phytoplasmas are associated with several hundreds of plant diseases that damage annual and perennial crops, including fruit trees and grapevines (Lee *et al.*, 2000; Seemüller *et al.*, 2002). These pathogens multiply within the phloem cells of the host plant and are transmitted from plant to plant by phloem-feeding insects (Christensen *et al.*, 2005; Weintraub & Beanland, 2006). The failure of numerous attempts to cultivate phytoplasmas in cell-free medium has hindered progress in their study. The development of molecular tools has enabled identification and preliminary

classification of phytoplasmas according to 16S rDNA gene phylogeny and RFLP profiling into 14 groups (Lee *et al.*, 2000). Several phytoplasmas have been assigned *Candidatus* status (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group, 2004; Lee *et al.*, 2000; Seemüller *et al.*, 2002). Whereas important progress has been made in phytoplasma classification and ecology, little is known about molecular determinants of phytopathogenicity and insect vector specificity. These areas should benefit from comparative analyses of phytoplasma genomes. The size of several phytoplasma chromosomes has been determined by PFGE, and ranges from 530 to 1350 kbp, revealing an important heterogeneity within and between phylogenetic groups that does not correlate with the phylogenetic classification (Marcone *et al.*, 1999; Neimark & Kirkpatrick, 1993). Physical and genetic maps of phytoplasma chromosomes have been determined for '*Candidatus* Phytoplasma pruni' (Firrao *et al.*, 1996), sweet potato little leaf phytoplasma (Padovan *et al.*, 2000), '*Candidatus* Phytoplasma mali' (Lauer & Seemüller, 2000) and '*Candidatus* Phytoplasma prunorum' (Marcone & Seemüller, 2001). Phytoplasma genomes have been partly

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Abbreviations: FD, Flavescence dorée; FD-P, Flavescence dorée phytoplasma; SSH, subtractive suppression hybridization.

sequenced for '*Candidatus Phytoplasma pruni*' (Liefting & Kirkpatrick, 2003) and '*Candidatus Phytoplasma solani*' (Cimerman *et al.*, 2006), and the complete genome sequences of two strains of '*Candidatus Phytoplasma asteris*' (860 and 706 kbp) have been described (Bai *et al.*, 2006; Oshima *et al.*, 2004).

Flavescence dorée (FD) is an epidemic, leafhopper-transmitted, quarantine disease of grapevines which has an important economic impact in Europe (Boudon-Padiou, 2002). This pathogen belongs to the 16SrV group, for which the genome of '*Candidatus P. asteris*' (16SrI group) can hardly serve as a reference due to the important phylogenetic distance between these two groups (Lee *et al.*, 2004a, b; Oshima *et al.*, 2004).

This paper describes the physical map of the FD phytoplasma (FD-P) chromosome as a preliminary step of a genome project. As phytoplasmas are not available in culture, the first step was to isolate phytoplasma DNA from plant extracts as described by Padovan *et al.* (2000). We constructed an FD92 strain chromosome map, employing restriction endonucleases with G+C-rich recognition sequences that produce a limited number of large restriction fragments from the AT-rich chromosomal DNA. Following the separation and the sizing of the restriction fragments under appropriate conditions, the fragments were separated by PFGE and organized along the circular chromosome by performing double digestion and Southern hybridization.

METHODS

Phytoplasma source and plant material. Phytoplasma strains FD70 and FD92 were transmitted to broad bean (*Vicia faba* var. Aquadulce) (Caudwell *et al.*, 1970) using *Scaphoideus titanus* leafhoppers collected in FD-affected vineyards in the south-west of France in 1970 and 1992, respectively. FD92 has been maintained since this time by uninterrupted serial transmission from broad bean to broad bean using *Euscelidius variegatus* as an alternative leafhopper vector (Caudwell *et al.*, 1972). FD70 and FD92 were also transmitted by infected *E. variegatus* to periwinkle (*Catharanthus roseus*) and maintained by grafting (Caudwell *et al.*, 1973). FD70 and FD92 belong to types FD1 and FD2 of FD-P, respectively, as defined by Arnaud *et al.* (2007).

Nucleic acids extraction and preparation of phytoplasma chromosomes. Total nucleic acids were extracted from 1 g leaf midribs from broad bean or periwinkle plants by the CTAB method, according to Maixner *et al.* (1995). The resulting nucleic acid pellets were resuspended in 60 µl 1×TE (10 mM Tris/HCl, 1 mM EDTA, pH 8).

Phytoplasma chromosomes were prepared according to the method of Padovan *et al.* (2000). Two grams of fresh midribs, petioles and stems from the top 15 cm of FD92-infected broad beans were ground at 4 °C in polyethylene bags with a ball-crushing apparatus (Bioreba) in 12 ml ice-cold extraction buffer [0.1 M Na₂HPO₄, 0.03 M NaH₂PO₄, 10% (w/v) sucrose, 2% (w/v) polyvinylpyrrolidone (MW 40000), 10 mM EDTA, pH 7.6, with 0.15% (w/v) BSA and 1 mM isoascorbic acid] and then subjected to different centrifugation and filtration steps, as described by Padovan *et al.* (2000), to obtain 50 µl of a final phytoplasma suspension in TSE buffer [20 mM Tris/

HCl, 10% (w/v) sucrose, 0.05 M EDTA, pH 8]. The suspension was mixed at 55 °C with an equal volume of molten 2% (w/v) LMP agarose (Bio-Rad) dissolved in 2×TES buffer (0.2 M Tris/HCl, 0.2 M NaCl, 20 mM EDTA, pH 8) and was poured into 100 µl rectangular plastic moulds. The resulting agarose blocks were treated for 60 h at 50 °C in lysis buffer with four buffer changes [1% (w/v) N-lauroyl sarcosine, 500 mM EDTA, 1 mg proteinase K ml⁻¹]. Blocks were washed for 12 h in lysis buffer without proteinase K and stored at 4 °C in 0.5 M EDTA, pH 8. The same procedure was executed with healthy broad beans as a control.

Restriction endonuclease digestion and PFGE. Endonuclease digestion was carried out in agarose blocks. After two rinses with 10 vols 1×TE, the blocks were incubated overnight at 4 °C in 10 vols 1×TE plus 1 mM PMSF to inactivate the proteinase K, then rinsed four times for 2 h at 4 °C with 10 vols 1×TE. Blocks were first incubated for 1 h at 4 °C in 5 vols digestion buffer [4 mM DTT, 0.1 mg BSA ml⁻¹, 4 mM spermidine, 1×restriction enzyme buffer supplied by the manufacturer (Euromedex)], then transferred to 2 vols digestion buffer plus 30 units restriction enzyme (Euromedex) and left overnight at the recommended temperature. For double digestions, the first enzyme was inactivated by incubating the blocks for 2 h at 4 °C in 10 vols 0.5 M EDTA, pH 8 followed by three rinses, and the digestion procedure was repeated with the second enzyme. Rare-cutting restriction endonucleases *Apal*, *Bss*HII, *EagI*, *I-CeuI*, *KpnI*, *MluI*, *PvuII*, *Sall*, *SmaI* and *XhoI* 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. All single and double restriction digestions were performed separately and to completion.

For the identification of DNA fragments smaller than 10 kbp, 5 µg DNA extracted from FD92-infected broad beans by the CTAB method were also digested overnight at the recommended temperature with 20 units restriction enzyme in a final volume of 40 µl.

PFGE was performed by the clamped homogeneous electrical field (CHEF) technique using the CHEF-DR III system (Bio-Rad). Blocks were installed in a 0.8% (w/v) pulsed field certified agarose (Bio-Rad) gel. Electrophoresis was performed in 0.5×TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.3) at 6 V cm⁻¹ and 14 °C at an angle of 120° for 18 h, and with various ramped pulse times: 0.7–13 s for low-size-range resolution, 2–40 s and 2–60 s for medium-size-range resolution, and 5–90 s for high-size-range resolution.

Gels were stained with ethidium bromide (2 µg ml⁻¹) and destained by washing for 1 h in distilled water before visualization under UV light. The molecular mass of fragments was estimated by comparison to yeast chromosome PFG (*Saccharomyces cerevisiae* YPH80) and low-range PFG (mixture of λ DNA *Hind*III fragments and concatemers) markers from New England Biolabs using Quantity One 4-2-3 software (Bio-Rad). The mean size of each fragment was calculated from at least three different gels.

Isolation and characterization of FD-P DNA fragments used as genetic markers. DNA fragments used for the realization of the genetic map are described in Table 1. FD2 and FD9 (*secY* gene) fragments were obtained from infected broad beans by cloning FD-P-enriched DNA fractions isolated from bisbenzimidazole-CsCl density gradients as described previously (Daire *et al.*, 1992, 1997). Their sequence was extended by genome walking using the Genomewalker kit (Clontech), as described by Arnaud *et al.* (2007). In this way, the *map* gene was characterized downstream of the *secY* gene (Arnaud *et al.*, 2007). The FDSH05 and FDDH29 fragments were obtained by subtractive suppression hybridization (SSH) and double-SSH, respectively, between *Hinc*II-digested DNA from infected and healthy periwinkle as described by Cimerman *et al.* (2006). *uvrB-degV* was obtained by SSH and genome walking as described by Arnaud *et al.*

Table 1. List of probes used for the establishment of the FD-P genetic map

Probe name	Sequence origin	Product	Accession no.	Reference
<i>map</i>	FD70-infected <i>C. roseus</i>	Methionine aminopeptidase	AM238512	Arnaud <i>et al.</i> (2007)
<i>uvrB-degV</i>	FD70-infected <i>C. roseus</i>	Subunit B of excinuclease ABC-DegV family protein	AM238511	Arnaud <i>et al.</i> (2007)
FD9 (<i>secY</i>)	FD92-infected <i>V. faba</i>	Preprotein translocase <i>SecY</i>	AF458384	Daire <i>et al.</i> (1992); Angelini <i>et al.</i> (2003)
	FD70-infected <i>C. roseus</i>		AM238512	Arnaud <i>et al.</i> (2007)
FD2	FD70-infected <i>C. roseus</i>	ABC-transporter ATP-binding protein	Not in databases	Daire <i>et al.</i> (1992)
FDDH29	FD70-infected <i>C. roseus</i>	Similarity with hypothetical protein from aster yellows witches' broom phytoplasma YP456430	AM889025	Arnaud (2006)
FDSH05	FD70-infected <i>C. roseus</i>	No homology in databases	AM889026	Arnaud (2006)
<i>tuf</i>	FD92-infected <i>V. faba</i>	Elongation factor EF-Tu	AM939565	Unpublished genome survey
16S rDNA	FD70-infected <i>C. roseus</i>	16S RNA	X76560	Seemüller <i>et al.</i> (1994)

(2007). The *tuf* gene was obtained from an unpublished genome survey of strain FD92 propagated in broad bean.

Southern hybridization. After depurination, denaturation and neutralization, DNA fragments from electrophoresis gels were submitted to capillary transfer onto a nylon membrane (Nytran Super Charge; Schleicher and Schuell) under neutral conditions, according to standard procedures (Sambrook *et al.*, 1989). Labelled DNA fragments used for Southern hybridization are described in Table 1 and PCR primers and DNA sources used for their synthesis are described in Table 2. Each probe except *secY* was labelled with digoxigenin by nested-PCR amplification. The first amplification was performed on 1 µl of either diluted DNA from phytoplasma-infected plant or purified recombinant pGEM-T Easy plasmid (Promega) containing the FD-P DNA insert in a volume of 25 µl with 1 µM each PCR primer, 2 mM MgCl₂, 200 µM dNTPs and 0.04 units *Taq* polymerase µl⁻¹ (Promega). Nested PCR was performed on 1 µl of a 1/100 dilution of the first PCR product, in a volume of 50 µl with 20 µM digoxigenin-dUTP (DIG-11-dUTP alkali-labile; Roche). PCR conditions are detailed in Table 2. The FD9 (*secY*) fragment was labelled with [α -³²P]dATP using the DNA Labelling System or Nick Translation System (Invitrogen), according to the manufacturer's instructions. Probes were purified using ProbeQuant G-50 micro columns (Amersham Biosciences), according to the manufacturer's instructions. The blotted membranes were pre-hybridized for 1.5 h and then overnight at 37 °C in hybridization solution [50% (v/v) formamide, 5 × SSC, 2% (w/v) blocking reagent (Roche), 0.1% (w/v) *N*-lauroyl sarcosine, 0.02% (w/v) SDS and 200 µg heat-denatured salmon sperm DNA ml⁻¹ (Stratagene)] and hybridized with 20 ng purified labelled probes ml⁻¹. Membranes were then washed four times for 15 min each in 2 × SSC/0.5% (w/v) SDS at ambient temperature and twice for 30 min each in 0.1 × SSC/0.1% (w/v) SDS at 50 °C. Probe hybridization was revealed using anti-digoxigenin FAb fragment and CDP-Star as substrate, according to the Dig DNA Labelling and Detection kit (Roche).

RESULTS

Separation of FD-P chromosome and restriction fragments resulting from single and double digestions

As shown in Fig. 1, PFGE performed with undigested DNA prepared from FD92-infected broad beans revealed a fragment around 680 kbp (lanes 1 and 2). No fragment

was detected with DNA prepared from control healthy broad beans (lane 3).

Chromosomal DNA of strain FD92 was digested individually with 10 different restriction enzymes possessing G + C-rich recognition sequences. Enzymes *I-CeuI* and *KpnI* did not digest the phytoplasma chromosome, and *XhoI*, *PvuII*, *Apal* and *SmaI* were not selected to construct the physical map as they produced only small or too many fragments, or gave smeary banding patterns (not shown). However, *BssHII*, *EagI*, *MluI* and *Sall* all generated a clear banding pattern with two or three fragments between 50 and 550 kbp and were consequently selected for mapping. No fragments with sizes larger than 50 kbp were observed with DNA prepared from healthy broad beans (Fig. 2a, lanes 12 and 13). Single restriction profiles are shown in Fig. 2(a, b), lanes 1–4. Mean sizes of fragments were determined from at least three independent PFGE experiments with running conditions optimized to resolution of low-, medium- or high-size-range, depending on the expected size of the fragments. These profiles are presented in Table 3. PFGE analysis revealed two fragments of 415.1 ± 8.2 and 193 ± 6.4 kbp for *Sall* digestions (lanes 1), two fragments of 552.1 ± 8.4 and 110.2 ± 2.7 kbp for *BssHII* digestions (lanes 2), three fragments of 404.9 ± 5.1 , 146.6 ± 6.5 and 109 ± 3.1 kbp for *EagI* digestions (lanes 3) and three fragments of 515.6 ± 6.4 , 80.1 ± 6.6 and 73.2 ± 6.2 kbp for *MluI* digestions (lanes 4). As shown in Fig. 2(a, b), *BssHII*, *EagI*, *MluI* and *Sall* digestions of strain FD92 DNA also produced fragments smaller than 50 kbp. Their identification and size determination were not easy to perform because of a background signal also present in healthy broad bean controls which might correspond to broad bean chloroplast or mitochondrial DNA contaminating the phytoplasma chromosome preparations. Therefore, fragments smaller than 50 kbp were identified by hybridization with FD-specific probes.

BssHII/Sall, *EagI/Sall*, *MluI/Sall*, *EagI/BssHII*, *MluI/BssHII* and *MluI/EagI* double digestions generated three, four, three, three, four and five fragments longer than 50 kbp, respectively (Fig. 2a and b, lanes 5–10). Their mean sizes

Table 2. List of primers and PCR conditions employed for the synthesis of probes used for mapping

Probe name	Size (bp)	DNA source	PCR and nested-PCR primers pairs	Primer reference or sequence (5'–3')	PCR conditions
<i>map</i>	803	FD70-infected <i>C. roseus</i>	FD9f5 MAPr1 FD9f6 MAPr2	Arnaud <i>et al.</i> (2007)	92 °C, 60 s; (92 °C, 30 s; 52 °C, 30 s; 66 °C, 90 s) × 35; 66 °C, 5 min
<i>uvrB-degV</i>	1126	FD70-infected <i>C. roseus</i>	UVRBf1 DEGVR4 UVRBf3 DEGVR3	Arnaud <i>et al.</i> (2007)	
FD9 (<i>secY</i>)	1174	FD92-infected <i>V. faba</i>	FD9f FD9r FD9f3b FD9r2	Daire <i>et al.</i> (1997) Clair <i>et al.</i> (2003); Angelini <i>et al.</i> (2001)	92 °C, 90 s; (94 °C, 40 s; 55 °C, 40 s; 72 °C, 70 s) × 35
FD2	506	Recombinant pGEM-T Easy plasmid	SP6 T7 Ff2 Fr8	ATTTAGGTGACACTATAGAA TAATACGACTCACTATAGGG GCTGTTGTTGGTTCTACCGG AATTGTGGATAAACGATGTGCG	94 °C, 3 min; 94 °C, 30 s; 55 °C, 30 s; 66 °C, 45 s) × 35; 66 °C, 5 min
FDDH29	351	Recombinant pGEM-T Easy plasmid	SP6/T7 SSH29f SSH29r	GCAAAAAAGTTGAATTTAAGGG GTATTAATATGTGATGACCTTC	
FDSH05	354	Recombinant pGEM-T Easy plasmid	SP6/T7 FDSH05f FDSH05r	TGGAAACTGTTAGTAAAGC TGGTTCTGGATCTTTTCTG	
<i>tuf</i>	1164	FD92-infected <i>C. roseus</i>	FDTUF-F1 FDTUF-R1 FDTUF-F2 FDTUF-R2	ATTGGTCATGTAGACCATGG GTTCTTCCGCCCTTACAGTAC TTGTCAGAACAAAGGATTAGC CTTGTTCTTCTTCGATCGC	92 °C, 60 s; (92 °C, 30 s; 56 °C, 30 s; 72 °C, 60 s) × 35; 72 °C, 5 min
16S rDNA	863	FD70-infected <i>C. roseus</i>	fP1/rP7 fU5/rU3	Deng & Hiruki (1991); Smart <i>et al.</i> (1996) Lorenz <i>et al.</i> (1995)	94 °C, 3 min; (94 °C, 30 s; 55 °C, 30 s; 66 °C, 45 s) × 35; 66 °C, 5 min

were determined from at least three independent PFGE experiments and are presented in Table 3. As for the single digestions, fragments smaller than 50 kbp were confirmed by hybridization.

Hybridization profiles of the genetic markers

Hybridization profiles of the different genetic markers are presented Fig. 2(c, d, e, f, g, h, i). The mean sizes of fragments are presented in Table 3. Hybridizations using probes FDSH05 (Fig. 2d, lanes 1–10), FDDH29 (Fig. 2e and h, lanes 1–10), *map* (Fig. 2f, lanes 1–10), *uvrB-degV* (Fig. 2g, lanes 1–10) and *tuf* (Fig. 2h, lanes 1–10) each revealed a single fragment. No fragments were revealed following hybridization to healthy control DNA (lanes 12 and 13). This indicated that these markers were present in a single copy on the strain FD92 chromosome. As expected, hybridization with the *secY* gene to *Bss*HII, *Mlu*I, *Sal*I *Bss*HII/*Sal*I, *Mlu*I/*Sal*I and *Mlu*I/*Bss*HII restriction fragments (data not shown) presented the same profile as hybridization with the *map* gene which is already known from sequence data to be located next to the *secY* gene

(Arnaud *et al.*, 2007; Arnaud, 2006). Hybridization to *Eag*I, *Eag*I/*Sal*I, *Eag*I/*Bss*HII and *Mlu*I/*Eag*I restriction fragments was not performed. Hybridization with the FD2 fragment (data not shown) also produced the same profile, showing that this marker is located on the same restriction fragment. The FDDH29 probe gave the same hybridization profile as the *tuf* gene, showing that both markers are located on the same restriction fragment. Hybridization performed with the 16S rDNA (Fig. 2c) probe revealed two fragments for each single and double digestion, except for *Eag*I/*Bss*HII. This suggests the presence of two *rrn* operons in the strain FD92 chromosome. The strong signal observed in lane 6 (Fig. 2c) for *Eag*I/*Sal*I digestion corresponds to a doublet of 14 and 15 kbp. The 16S rDNA probe also hybridized with control DNA from healthy plants (lanes 12 and 13), but the sizes of the revealed fragments were different from those of the infected plant DNA digested with the same enzymes. This might be due to non-specific hybridization of broad bean chloroplast rDNA. As no fragment was revealed after the hybridization for the *Eag*I/*Bss*HII double digestion (lane 8) and as small fragments could possibly have run out of the

Fig. 1. PFGE of DNA prepared from FD-P-infected broad beans (lanes 1 and 2) and healthy broad beans (lane 3). M, low-size-range PFGE marker (kbp).

gel, a Southern blotting experiment was carried out after conventional agarose gel electrophoresis. As expected, two small fragments of 3.5 and 3.8 kbp were revealed when DNA from FD92-infected broad bean was digested by *EagI*/*Bss*HII and hybridized with the 16S rDNA probe (Fig. 2i, lane 5).

Estimation of the size of the strain FD92 chromosome

The size of the chromosome of strain FD92 was estimated to be 671 ± 14 kbp. This represents the mean of the sums of the size of all fragments generated by single digestions with *Bss*HII, *EagI* and *MluI* enzymes and double digestion with *MluI*/*Bss*HII for which every fragment was directly visible on PFGE gels and hybridization patterns.

Mapping of the strain FD92 chromosome

Data from all independent single or double restrictions and from hybridization profiles were assembled to derive a restriction map for the strain FD92 chromosome, showing locations of the different genetic markers (Fig. 3). Thirteen restriction sites and nine genetic markers located on six restriction fragments were mapped on the chromosome. The FDDH29 marker and *tuf* gene were positioned on one *SalI* restriction fragment of 35.6 ± 2.7 kbp. The *map* gene and FD2 marker were located on one *EagI*/*SalI* restriction fragment of 211.1 ± 4.3 kbp. The *secY* gene, already known from sequence data to be located next to the *map* gene (Arnaud *et al.*, 2007; Arnaud, 2006), also mapped to this restriction fragment. The FDSH05 marker and *uvrB-degV*

genes were positioned on one *EagI* fragment of 146.6 ± 6.5 kbp and on one *SalI*/*MluI* fragment of 91.9 ± 5.7 kbp, respectively. Genome walking, performed using the 16S rDNA sequence present in databases (accession number X76560) (Seemüller *et al.*, 1994) confirmed the presence of two copies of the *rrn* operon on the FD-P chromosome (Arnaud, 2006). They were located on two *EagI*/*Bss*HII restriction fragments of 3.5 and 3.8 kbp. The presence of a *Bss*HII site at the beginning of both 16S rDNA sequences enabled the operons to be orientated. With the exception of the 16S rDNA gene, none of the known FD-P sequences contained a restriction site used to build up the map and no genetic marker could therefore be precisely positioned on the chromosome. For the *SalI* single digestion and *MluI*/*SalI*, *MluI*/*EagI*, *Bss*HII/*SalI*, *Bss*HII/*EagI* and *EagI*/*SalI* double digestions, some fragments smaller than 28 kbp, presented in *italics* in Table 3 (column 3), were not distinguished by either PFGE profiles or hybridization patterns, but instead were deduced from mapping. The size of the smaller restriction fragment was estimated to be 3.5 kbp and the larger, with no mapped internal sites, was estimated to be 211 kbp.

No restriction enzyme was found to cut the chromosome once to give a linear molecule; consequently, the *Bss*HII restriction site on the first copy of the 16S rDNA was arbitrarily chosen as the beginning of the map.

DISCUSSION

The recent sequencing of 12 kbp of the FD-P genetic *rplC-infA* locus, encompassing the *secY-map* locus, revealed that this fragment has a G+C content of 27 mol% (Arnaud, 2006). This locus is highly conserved in most microbial genomes and its G+C content is linearly correlated with the G+C content of the whole genome. This permitted us to estimate the G+C content of the FD-P genome as being close to 22–23 mol% (Arnaud, 2006). According to this result, one can estimate the theoretical frequency of the different restriction sites for the enzymes used in this study. *EagI* (CGGCCG) and *Bss*HII (GCGCGC) should cut the genome with a frequency of $(0.115)^6 = 1/430\,000$ bp, i.e. one to two times. The observed frequency was a little higher, with four sites for the enzyme *EagI* and two for *Bss*HII. This can be explained by the fact that these sites were mainly located in the vicinity of the *rrn* operons which are known to have a higher G+C content – 47 mol% in the case of the FD-P 16S rDNA, as is the case for other mollicute genomes (Barré *et al.*, 2004; Razin *et al.*, 1998; Sirand-Pugnet *et al.*, 2007). The concentration of G+C-rich restriction sites in the vicinity of the *rrn* operons has also been observed on physical maps of *Mycoplasma hominis* (Ladefoged & Christiansen, 1992) and *Spiroplasma citri* strains (Ye *et al.*, 1992).

The theoretical frequency of *MluI* (ACGCGT) and *SalI* (GTCGAC) restriction sites should be $(0.115)^4 \times$

Fig. 2. (a, b) PFGE of DNA prepared from FD-P-infected (lanes 1–11) and healthy (lanes 12–13) broad beans, single- or double-digested with various rare-cutting enzymes. Conditions of electrophoresis were adapted for resolution of low- (a) or medium- (b) size-range DNA. Lanes: 1, *SalI*; 2, *BssHII*; 3, *EagI*; 4, *MluI*; 5, *BssHII/SalI*; 6, *EagI/SalI*; 7, *MluI/SalI*; 8, *EagI/BssHII*; 9, *MluI/BssHII*; 10, *MluI/EagI*; 11, undigested; 12, *SalI*; 13, *BssHII*; M, low-size-range PFGE marker (kbp). (c–h) Southern blot hybridization profiles of low-size-range PFGE gels probed with 16S rDNA (c), FDSH05 (d), FDDH29 or *tuf* (e, h), *uvrB-degV* (g) and medium-size-range PFGE gels probed with *map* (f). (i) Southern blot hybridization of a conventional electrophoresis gel probed with 16S rDNA. Lanes 1, recombinant pGEM-T Easy with 16S rDNA insert; 2–5, DNA from FD-infected broad beans, undigested (2) or digested with *BssHII* (3), *EagI* (4), *BssHII* and *EagI* (5); 6–9, DNA from healthy broad beans, undigested (6) or digested with *BssHII* (7), *EagI* (8), *BssHII* and *EagI* (9).

$(0.39)^2 = 1/37\,600$ bp, i.e. 18 sites on the FD-P genome. However, *MluI* and *SalI* sites were found only three and four times, respectively. Such a discrepancy between the theoretical and observed frequency has also been found for *S. citri* where the *SalI* restriction sites were 10 times under-represented on its physical map (Ye *et al.*, 1992). In addition, the *Mycoplasma mycoides* subsp. *mycoides* Y and *M. hominis* physical maps showed frequencies of restriction sites containing one A and one T (*BamHI*, *KpnI*, *SalI* and *XhoI*) six to ten times lower than the theoretical ones (Ladefoged & Christiansen, 1992; Pyle & Finch, 1988). This phenomenon could be due to methylation of the majority of these restriction sites. However, our analyses performed *in silico* on the sequenced genome of different mollicutes,

using the MolliGen database (Barré *et al.*, 2004) and pDRAW32 software, showed that frequencies of *SalI* and *MluI* sites are also under-represented. For example, in the *Mycoplasma capricolum* genome (Wise *et al.*, 2006) (accession number NC_007633, 23.77 mol% G + C), only two *MluI* sites and no *SalI* sites were found, despite the 31 theoretically calculated. In addition, the *M. mycoides* genome (Westberg *et al.*, 2004) (NC_005364.2, 23.96 mol% G + C) presented only six *MluI* sites instead of 37. So, another explanation could be that the di- or trinucleotides that make up these sites could be under-represented in these genomes. This can be explained because small genomes of mollicutes have a high proportion of coding sequences, ranging from 73% for

Table 3. Number, mean sizes and probe hybridization of fragments obtained by single and double digestions of the FD-P chromosome

Enzyme	Fragment name	Mean size $\pm$ SD* (kbp)	Hybridizing probes	Sum of mean sizes (kbp)†
<i>Bss</i> HII	A	552.1 $\pm$ 8.4	<i>map</i> , <i>secY</i> , <i>uvrB-degV</i> , FD2, FDSH05, 16S rDNA	662.3
	B	110.2 $\pm$ 2.7	FDDH29, <i>tuf</i>	
<i>Eag</i> I	A	404.9 $\pm$ 5.1	<i>map</i> , <i>uvrB-degV</i> , FD2‡	673.4
	B	146.6 $\pm$ 6.5	FDSH05	
	C	109 $\pm$ 3.1	16S rDNA, FDDH29, <i>tuf</i>	
	D	14	16S rDNA	
<i>Mlu</i> I	A	515.6 $\pm$ 6.4	<i>map</i> , <i>secY</i> , <i>uvrB-degV</i> , FD2, FDSH05, 16S rDNA	668.9
	B	80.1 $\pm$ 6.6	FDDH29, <i>tuf</i>	
	C	73.2 $\pm$ 6.2		
<i>Sal</i> I	A	415.1 $\pm$ 8.2	<i>map</i> , <i>secY</i> , FD2, FDSH05, 16S rDNA	671.2
	B	193 $\pm$ 6.4	<i>uvrB-degV</i> , 16S rDNA	
	C	35.6 $\pm$ 2.7	FDDH29, <i>tuf</i>	
	D§	27.5		
<i>Bss</i> HII/ <i>Sal</i> I	A	365.7 $\pm$ 5.4	<i>map</i> , <i>secY</i> , FD2, FDSH05	673.5
	B	181.2 $\pm$ 3.9	16S rDNA, <i>uvrB-degV</i>	
	C	53 $\pm$ 2.8	16S rDNA	
	D	38.2 $\pm$ 7.4	FDDH29, <i>tuf</i>	
	E§	27.5		
	F§	8		
<i>Eag</i> I/ <i>Sal</i> I	A	211.1 $\pm$ 4.3	<i>map</i> , FD2‡	683.6
	B	180.9 $\pm$ 6.4	<i>uvrB-degV</i>	
	C	146.2 $\pm$ 5.6	FDSH05	
	D	52 $\pm$ 2.6		
	E	38.9 $\pm$ 5.8	FDDH29, <i>tuf</i>	
	F§	27.5		
	G	14	16S rDNA	
	H	13	16S rDNA	
<i>Mlu</i> I/ <i>Sal</i> I	A	418.1 $\pm$ 5.2	<i>map</i> , <i>secY</i> , FD2, FDSH05, 16S rDNA	683.3
	B	91.9 $\pm$ 5.7	<i>uvrB-degV</i>	
	C	81.8 $\pm$ 6.4		
	D	38.6 $\pm$ 8.2	FDDH29, <i>tuf</i>	
	E	26	16S rDNA	
	F§	11		
	G§	16		
<i>Eag</i> I/ <i>Bss</i> HII	A	398 $\pm$ 10.2	<i>map</i> , <i>uvrB-degV</i> , FD2‡	671.6
	B	146.9 $\pm$ 6.2	FDSH05	
	C	110.5 $\pm$ 4.3	FDDH29, <i>tuf</i>	
	D§	9		
	E	3.8	16S rDNA	
	F	3.5	16S rDNA	
<i>Mlu</i> I/ <i>Bss</i> HII	A	465 $\pm$ 11.6	<i>map</i> , <i>secY</i> , <i>uvrB-degV</i> , FD2, FDSH05	675.5
	B	80.7 $\pm$ 7.6		
	C	59.1 $\pm$ 4	FDDH29, <i>tuf</i>	
	D	52.7 $\pm$ 3.4	16S rDNA	
	E	18	16S rDNA	
<i>Mlu</i> I/ <i>Eag</i> I	A	313.3 $\pm$ 7.9	<i>map</i> , <i>uvrB-degV</i> , FD2‡	686.2
	B	145.5 $\pm$ 4.6	FDSH05	
	C	82 $\pm$ 6		
	D	58.7 $\pm$ 3.6	16S rDNA, FDDH29, <i>tuf</i>	
	E	50.7 $\pm$ 2.5		
	F	21 $\pm$ 1	16S rDNA	
	G§	15		

*Means were calculated from at least three separate PFGE experiments. Italic numbers indicate fragments smaller than 28 kbp that were not distinguished by either PFGE profiles or hybridization patterns, but instead were deduced from mapping.

†Sums of the mean sizes of the different restriction fragments generated.

‡Hybridization not demonstrated for *secY*.

§Fragment not observed on PFGE gels for which the theoretical size was deduced by placing the restriction sites on the 671 kbp genome map.

Fig. 3. Physical and genetic map of the chromosome of the FD-P. Restriction sites for *BssHII*, *EagI*, *MluI* and *SalI* are indicated on the circular scale. Restriction fragments hybridizing with 16S rDNA, *tuf*, *FDDH29*, *uvrB-degV*, *secY*, *map*, *FD2* and *FDSH05* probes are delimited by bars. The orientation of *rrn* genes is symbolized by arrows.

‘*Candidatus P. asteris*’ to 92% for *Mesoplasma florum*, according to MolliGen (Barré *et al.*, 2004), in which nucleotide distribution is not random, but is determined by codon usage bias. As an example, our analyses showed that the ACG trinucleotide of the *MluI* site, which should theoretically appear 5233 times in the *M. capricolum* genome, was only found 97 times. In addition, the CGT trinucleotide of *MluI* was found at only 908 instead of 5233 positions, whereas the GCT trinucleotide with the same theoretical frequency appeared 8141 times.

Only four of the 10 restriction enzymes assayed (*BssHII*, *EagI*, *MluI* and *SalI*) produced fragments that could be used for mapping the strain FD92 chromosome. They revealed 13 restriction sites of which 10 were concentrated in a 140 kbp region, suggesting a heterogeneous repetition of di- and trinucleotides that make up their cutting sites on the phytoplasma chromosome. Similar results have been observed on 120–200 kbp of the chromosome maps of *S. citri* (Ye *et al.*, 1992), ‘*Candidatus P. mali*’ (Lauer & Seemüller, 2000), ‘*Candidatus P. prunorum*’ (Marcone & Seemüller, 2001) and *Mycoplasma pneumoniae* (Krause & Mawn, 1990).

The two copies of the *rrn* operon were shown to have the same orientation and to be 100 kbp apart on the FD-P chromosome, with the *tuf* gene lying about 30 kbp upstream from *rrnB*. Our analyses, performed with the MolliGen database (Barré *et al.*, 2004), revealed a similar arrangement in the genomes of aster yellows witches’ broom phytoplasma (Bai *et al.*, 2006; Oshima *et al.*, 2004) and *Mycoplasma synoviae* (Vasconcelos *et al.*, 2005). The *rrnB* operon had an opposite orientation in the genomes of

sweet potato little leaf and onion yellows phytoplasmas (Oshima *et al.*, 2004; Padovan *et al.*, 2000). The *secY* and *map* genes were found to be adjacent on the FD-P chromosome. They make up part of the 12 kbp *rplC-infA* locus characterized by Arnaud (2006) which also contains genes encoding ribosomal proteins and translation initiation factor 1 (*infA*). This gene cluster is well conserved among mollicutes, except in *Mycoplasma gallisepticum* and *Mycoplasma synoviae* where it has been divided by chromosomal rearrangements (Sirand-Pugnet *et al.*, 2007). In the *rplC-infA* locus of all sequenced mollicutes, the *secY* and *map* genes are not adjacent, but are always separated by the *adk* gene which encodes an adenylate kinase.

The size of the strain FD92 chromosome was estimated to be 671 kbp. This is in accordance with the chromosome size of phytoplasmas from the 16SrV phylogenetic group determined by Marcone *et al.* (1999) which ranges from 680 kbp for ‘*Candidatus Phytoplasma ulmi*’ strain ULW to 820 kbp for *Rubus* stunt phytoplasma. Chromosome size is not related to phylogenetic relationships in this group since FD-P is phylogenetically closer to alder yellows phytoplasma (strain Aly), which has a chromosome size of 750 kbp, than to ULW, with a chromosome of 680 kbp (Angelini *et al.*, 2003; Arnaud *et al.*, 2007; Lee *et al.*, 2004b). The absence of a correlation between the genome size of mollicutes and their phylogenetic position has been described (Marcone *et al.*, 1999; Neimark & Kirkpatrick, 1993; Sirand-Pugnet *et al.*, 2007).

In conclusion, 13 restriction sites and nine genetic markers located on six restriction fragments were mapped on the 671 kbp chromosome of the FD92 epidemic strain of FD-

P. This physical and genetic map will be useful for the assembly steps of the genome sequencing project which is underway for this strain. Furthermore, it will be interesting to compare this map with those of other FD-P strains which present different biological properties to strain FD92 in terms of interaction with hosts, colonization and/or multiplication. In addition, this will add new data to the growing body of sequenced data for comparative genomic studies among phytoplasmas and mollicutes.

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