A chromosome map of the European stone fruit yellows phytoplasma

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A physical map of the European stone fruit yellows phytoplasma strain GSFY1 chromosome was constructed using PFGE-purified genomic DNA from diseased tobacco and tomato plants. The map was generated with single and double digestions of the chromosome with *Sma*I, *Bss*HI, *Apa*I, *Bam*HI and *Xho*I restriction endonucleases and the fragments were resolved by PFGE. Reciprocal double digestions were used to locate 26 restriction sites on the chromosome. Southern blot analysis was also used to assist in the arrangement of the contiguous restriction fragments obtained. From the restriction fragments generated by double digestion, the circular chromosome was calculated to be approximately 635 kb. Loci of two rRNA operons, the operon containing the *tuf* gene, genes encoding an immunodominant membrane protein and a putative nitroreductase, and randomly cloned DNA fragments IH184 and AT67 were placed on the map. Digestion of chromosomal DNA of strain GSFY1 with *Mlu*I gave a complex restriction pattern, suggesting that this isolate consists of a population with heterogeneity with respect to *Mlu*I restriction sites. The GSFY1 physical map was different from that of the closely related apple proliferation phytoplasma but the genetic arrangement was similar.

Keywords: prokaryotes, genome size, yellows diseases, pulsed-field gel electrophoresis, ribosomal genes

INTRODUCTION

The European stone fruit yellows (ESFY) phytoplasma is an important prokaryotic pathogen that infects most or all kinds of stone fruits in Europe and is known to cause apricot chlorotic leaf roll, leptonecrosis and decline of Japanese plum (*Prunus salicina*), as well as yellows and decline diseases of peach, almond, European plum (*Prunus domestica*) and flowering cherry (*Prunus serrulata*) (Lorenz *et al.*, 1994; Marcone *et al.*, 1996). This organism is closely related to other important fruit tree pathogens, the apple proliferation (AP), pear decline and peach yellows leaf roll agents, and forms with them a distinct phylogenetic cluster, the AP group (Seemüller *et al.*, 1998). In contrast to the progress made in differentiation, classification and detection of phytoplasmas, very little is known about how these pathogens induce disease. Understanding pathogenicity on the

molecular level requires construction of a physical map followed by information on the genomic organization of the pathogen. With culturable organisms, a physical map can easily be generated using PFGE of large fragments which can be assembled using hybridization and fingerprinting methods (Pyle & Finch, 1988; Colman *et al.*, 1990; Ladefoged & Christiansen, 1992). Unculturable phytoplasmas can be studied using these methods only when the colonization density of the pathogens in the host is high. Thus, only three phytoplasmas, the western X-disease (WX), AP and sweet potato little leaf (SPLL) agents, have been mapped so far, using PFGE resolution of large fragments (Firrao *et al.*, 1996; Lauer & Seemüller, 2000; Padovan *et al.*, 2000). As a first step towards a detailed genetic characterization of the ESFY phytoplasma, we constructed a physical map of its chromosome, employing an approach similar to that used by Firrao *et al.* (1996), who generated the first physical map of a phytoplasma chromosome, that of the WX agent.

METHODS

Phytoplasma sources and transmission experiments. Strain GSFY1 of the ESFY phytoplasma, which was previously

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Abbreviations: AP, apple proliferation; ESFY, European stone fruit yellows; SPLL, sweet potato little leaf; WX, western X-disease.

Table 1. Number and mean size (kb) of fragments obtained by single and double digestions of the chromosome of strain GSFY1 of the ESFY phytoplasma

The mean of the sums of the restriction fragments generated from double digestions was 635 kb.

Fragment	<i>Sma</i> I	<i>Bss</i> III	<i>Apa</i> I	<i>Bam</i> HI	<i>Xho</i> I	<i>Sma</i> I/ <i>Bss</i> III	<i>Sma</i> I/ <i>Apa</i> I	<i>Sma</i> I/ <i>Bam</i> HI	<i>Sma</i> I/ <i>Xho</i> I	<i>Bss</i> III/ <i>Apa</i> I	<i>Bss</i> III/ <i>Bam</i> HI	<i>Bss</i> III/ <i>Xho</i> I	<i>Apa</i> I/ <i>Bam</i> HI	<i>Apa</i> I/ <i>Xho</i> I	<i>Bam</i> HI/ <i>Xho</i> I
A	350	450	552	237	232	235	345	170	232	438	237	125	237	232	108
B	284	65	65	121	153	220	205	121	153	65	113	104	121	95	100
C		50	15	113	80	65	65	113	80	50	65	80	113	80	80
D		50		75	80	50	15	75	80	50	64	80	65	80	65
E		15		64	25	50	2*	64	25	15	50	65	64	40	64
F				23	25	15		64	25	15	50	35	23	25	56
G					12	1*		23	12	3*	23	35	10	25	52
H					12			2*	12	2*	15	25	5*	15	25
I					9				9		10	25	4*	12	22
J					9				9		4*	15		12	15
K									1*		1*	12		9	12
L									1*			12		9	12
M												9		1*	9
N												9			9
O												2*			4*
P															3*
Total size	634	630	632	633	637	636	632	632	639	638	632	633	642	635	636

* Fragments predicted by restriction and alignment of larger fragments but not identified in PFGE gels.

transmitted from a diseased apricot tree to *Catharanthus roseus* (periwinkle) via dodder bridges (*Cuscuta reflexa*) (Marccone *et al.*, 1999a), was used for mapping. In the course of this work, this strain was transmitted via dodder bridges to *Nicotiana tabacum* (tobacco) and from there by grafting to several *Nicotiana* species and other solanaceous plants, including tomato. The periwinkle-maintained strains ESFY1 (Loi *et al.*, 1995) and GSFY2 (Marccone *et al.*, 1999a) of the ESFY phytoplasma were also included in this study.

Chromosome isolation and restriction endonuclease digestion. Phytoplasma chromosomes were extracted from leaf midribs, cortical stem tissue or stem phloem preparations. Phloem was prepared as reported by Lauer & Seemüller (2000), while sample preparation for PFGE and PFGE analysis was carried out as described by Neimark & Kirkpatrick (1993), except that the lysis solution was changed several times, SeaPlaque agarose (FMC BioProducts) was used, and the blocks were not gamma-irradiated. PFGE was carried out in a CHEF DRIII apparatus (Bio-Rad Laboratories). Entire linearized ESFY phytoplasma chromosomes were isolated by PFGE, excised from the gel, washed twice for at least 30 min per wash with TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) on ice and once with the appropriate restriction buffer and then digested with rare cutting enzymes as reported by Lauer & Seemüller (2000). Double digestions were carried out sequentially in the same preparation. After the first digestion, each preparation was first washed twice with TE and then with the appropriate restriction buffer, and then digested with a second restriction endonuclease. In addition, most major restriction fragments were excised from the gel and digested with the second enzyme individually. A concatameric lambda DNA ladder, *Saccharomyces cerevisiae* chromosomes (Bio-Rad Laboratories) and a Low Range PFG Marker (New England BioLabs) were used as size markers.

Southern blot hybridization. Southern transfers were carried out as described by Neimark & Kirkpatrick (1993). The following probes were used for hybridization: (a) linearized chromosomal DNA of strain GSFY1 recovered from the pulsed-field gel; (b) a PCR-amplified 1800 bp rDNA fragment of the ESFY phytoplasma comprising the entire 16S rRNA gene and 16S/23S rDNA spacer region (Schneider *et al.*, 1995); (c) a cloned fragment containing a gene encoding an immunodominant membrane protein (Berg *et al.*, 1999); (d) a cloned fragment containing a gene encoding the elongation factor Tu (*tuf*) (Berg & Seemüller, 1999); (e) randomly cloned fragment IH184 (Bonnet *et al.*, 1990); (f) randomly cloned fragment AT67 (Schneider & Seemüller, 1994); and (g) cloned fragment IH196 containing a putative nitroreductase gene (Jarausch *et al.*, 1994). For probe preparation from entire GSFY1 chromosomes, DNA was recovered from the pulsed-field gel by agarase (Roche Diagnostics) digestion. PCR products as well as cloned fragments were eluted from conventional agarose gels using the QIAquick gel extraction kit (Qiagen). Probes were labelled with [α -³²P]dATP using the Prime-It II kit (Stratagene). Hybridization was carried out according to the protocol of Hoheisel *et al.* (1991).

RESULTS

Effect of plant sources on chromosome isolation

Chromosomal DNA of strains ESFY1, GSFY1 and GSFY2 of the ESFY phytoplasma was initially extracted from midribs, cortical tissue and phloem preparations of periwinkle plants showing different stages of disease. However, only faint chromosomal DNA bands, insufficient for restriction analysis, were resolved by PFGE (data not shown). Higher concentrations of

Fig. 1. PFGE of chromosomal DNA of the ESFY phytoplasma. (a) Ethidium-bromide-stained gel of single and double digests with various rare-cutting enzymes. S1 and S2, *SmaI* restriction fragments which were previously separated under appropriate PFGE conditions ($1-21$ s, 6 V cm^{-1} , 19 h) and excised from the gel in order to be used either undigested or digested separately with the second enzyme. M, concatameric lambda DNA ladder. PFGE parameters were $0.7-12.5$ s, 6 V cm^{-1} , 18.8 h. (b) Southern blot hybridization of DNA fragments obtained by single and double digestion with cloned DNA fragment probe AT67.

chromosomal DNA of the closely related AP phytoplasma were obtained from phloem preparations of *N. tabacum* cv. Samsun (Lauer & Seemüller, 2000) so strain GSFY1 was transmitted to this plant via dodder bridges. However, this plant, too, was found to be an unsuitable host for this work, as were several other *Nicotiana* genotypes, including *N. tabacum* cv. White Burley, *Nicotiana occidentalis*, *Nicotiana rustica*, *Nicotiana edwardsonii*, *Nicotiana debneyi*, *Nicotiana glaucum*, *Nicotiana silvestris*, *Nicotiana benthamiana*, *Nicotiana quadrivalvis* and *Nicotiana rusticana*, as well as several tomato cultivars, including Rheinländer, Tigerella, Harzfeuer, Hellfrucht, Goldene Königin and St. Pierre. Red pepper and eggplant proved unsuitable. At certain stages of disease, moderate amounts of chromosomal

Fig. 2. Southern blot hybridization of DNA fragments in Fig. 1(a) probed with (a) randomly cloned DNA fragment IH184 and (b) a cloned DNA fragment containing a gene encoding the elongation factor Tu (*tuf*).

DNA were obtained from phloem preparations of *Nicotiana glutinosa* and the tomato cultivars Roma, Olivade, Hildares and Frühzauber. These genotypes were used for phytoplasma extraction.

Restriction endonuclease digestion

Strain GSFY1 chromosomal DNA was digested individually with 11 different restriction enzymes possessing GC-rich recognition sequences. Restriction enzymes *NotI*, *BglI* and *Cfr42I* failed to cleave the chromosomal DNA whereas digestions with *SalI* or *HpaII* yielded more than 10 fragments and were not used for mapping. Restriction enzymes *SmaI*, *ApaI*, *BssHII*, *BamHI* and *XhoI* produced two, three, five, six and ten fragments, respectively, and were chosen to construct the map (Table 1; see Figs 1, 3, 4, 6 and 7). PFGE-purified chromosomal DNA of strain GSFY1 was used to probe Southern blots of PFGE-separated *SmaI*, *BssHII*, *ApaI*, *BamHI* and *XhoI* restriction fragments. The banding pattern revealed two, five, three, six and ten bands for *SmaI*, *BssHII*, *ApaI*, *BamHI* and *XhoI*,

Fig. 3. PFGE of chromosomal DNA of the ESFY phytoplasma. (a) Ethidium-bromide-stained gel of single and double digests with various rare-cutting enzymes. PFGE parameters were 0.7–12.5 s, 6 V cm⁻¹, 18.8 h. (b) Southern blot hybridization of DNA fragments obtained by single and double digestion with cloned DNA fragment probe IH184.

respectively, as seen in ethidium-bromide-stained gels. Reciprocal double digestions resulted in restriction fragments with sizes between 9 and 438 kb (Table 1; see Figs 1a, 3a, 4a, 6a and 7a). Analysis of the restriction data indicated that for each double digestion there must be, in addition to the clearly identified fragments, one or two fragments between 1 and 5 kb that were too small to be detected due to insufficient amounts of phytoplasma DNA in the gel (see Table 1). Different pulse parameters and running times were used so that the size of each restriction fragment could be determined under optimal electrophoretic conditions. Pulse times were varied between 0.7 and 120 s, with different extents of ramping depending on the size range of molecules to be resolved. The mean size of restriction fragments, which was established from a minimum of three different gels, is shown in Table 1. From the sums of restriction fragments generated from all double digests, the size of the strain GSFY1 chromosome was calculated to be 635 kb (see Fig. 8). This value is slightly larger than the mean calculated from single digests (633 kb) as well as that estimated from the migration of entire chromosomes in the PFGE gels (630 kb) (Marccone *et al.*, 1999b).

Digestion of chromosomal DNA of strain GSFY1 with *MluI* generated fragments whose combined size was larger than the expected 630–635 kb (Fig. 1a). On the basis of the difference in fluorescence intensity shown by the restriction fragments, two different profiles could be constructed. One of the profiles, designated *MluI*-A, consisted of seven fragments 194, 145, 122, 58, 48, 32 and 32 kb in size. The second profile, designated *MluI*-B, comprised the 194, 48, 32 and 32 kb fragments of profile *MluI*-A and contained two other fragments of 170 and 160 kb. The phytoplasma nature of the latter two *MluI* fragments was confirmed by hybridization. In most preparations obtained from both tobacco and tomato, fragments of profile *MluI*-A showed a brighter fluorescence than the fragments of 170 and 160 kb of

profile *MluI*-B (Fig. 1a). In only a few preparations, fragments of profile *MluI*-B showed a more intensive fluorescence than those of profile A, or fragments of both profiles showed the same fluorescence intensity (data not shown). Due to difficulties encountered in aligning *MluI* fragments, the *MluI* sites were not placed on the map.

Map construction

Data obtained on fragment sizes from single and reciprocal double digestions as well as from hybridization analyses were used to locate the restriction sites on the map shown in Fig. 8. Construction of the map started with double digestion of the strain GSFY1 chromosome with *SmaI* and *BssHII*, which showed that one of the two *SmaI* sites was located in the large *BssHII* fragment (Fig. 1a) whereas the second was very close to one of the *BssHII* sites. This *BssHII* site was chosen as the starting point of the map. From summation of resulting restriction fragments as well as from staining intensity, there was evidence that digestion of the strain GSFY1 chromosome with *BssHII* resulted in two fragments of 50 kb which could not be resolved by PFGE. In order to arrange these two fragments on the map, the 350 and 284 kb *SmaI* fragments, designated S1 and S2, respectively, were separated under appropriate PFGE conditions, excised from the gel and digested separately with *BssHII*. From each of the *SmaI* fragments, one 50 kb *BssHII* fragment was obtained (Fig. 1a). Southern blot hybridization with probe IH184 revealed that the two 50 kb *BssHII* fragments were linked (Fig. 2a). The linking restriction site was chosen as the starting point of the map.

Double digestion of the chromosome with *SmaI*/*ApaI* and *BssHII*/*ApaI* and digestion of *SmaI* fragments with *ApaI*, *ApaI* fragments with *SmaI* and *BssHII* fragments with *ApaI* (Figs 1a, 3a and data not shown) allowed

Fig. 4. PFGE of chromosomal DNA of the ESFY phytoplasma. (a) Ethidium-bromide-stained gel of single and double digests with various rare-cutting enzymes. S1, S2 and M, see Fig. 1 for abbreviations; M1, low range PFGE marker. PFGE parameters were 0.7–12.5 s, 6 V cm⁻¹, 18.8 h. (b) Southern blot hybridization of DNA fragments obtained by single and double digestion with cloned DNA fragment probe IH184.

positioning of *ApaI* sites on the map. However, the *ApaI* site at position 632 proved to be very close to both *SmaI* and *BssHII* sites while the *ApaI* site at position 62 was close to one of the *BssHII* sites. Southern blot hybridization with probe IH184 revealed that the *ApaI* site at position 632 was located near the *BssHII* restriction site at the start point of the map (Figs 2a and 3b). Double digestions *SmaI/BamHI*, *BssHII/BamHI* and *ApaI/BamHI* were used to locate the sites for

Fig. 5. Corresponding Southern blot hybridization of DNA fragments in Fig. 4(a) probed with a PCR-amplified 1800 bp rDNA fragment of the ESFY phytoplasma containing the entire 16S rRNA gene and 16S/23S rDNA spacer region.

BamHI relative to *SmaI*, *BssHII* and *ApaI* sites (see Figs 6a and 7a).

As there was evidence that *XhoI* digestion resulted in four doublets 80, 25, 12 and 9 kb in size, their presence was verified by separate digestion of the two *SmaI* fragments with *XhoI* (Fig. 4a). Each of the resulting restriction patterns contained fragments of these four sizes. Southern blot hybridization with probe IH184 revealed that the 12 kb *XhoI* fragments were linked and were located near the *BssHII* site at position 0 (Figs 4b and 8). This region also contained *ApaI*, *SmaI* and *BamHI* restriction sites (Figs 2a, 3b, 4b, 6d and 7d). Southern blot hybridization with the other gene probes allowed positioning of the other *XhoI* sites within the S1 and S2 fragments and confirmed the position of other sites on the map. However, it was impossible to unambiguously allocate the restriction sites *XhoI*, *SmaI*, *BamHI* and *ApaI* clustering with the *BssHII* site at position 0 because these sites were too close together and the fragments too small to be observed in pulsed-field gels. A similar situation was observed for the *XhoI* sites adjacent to the *SmaI* site at position 283.

The alignment of the restriction fragments revealed that the chromosome of strain GSFY1 was circular, like that of all other mycoplasmas previously examined. Several genetic loci were mapped by Southern hybridization of restriction fragments with various probes (Figs 1, 2, 3, 4, 5, 6, 7 and data not shown). It could be shown that the two rRNA operons that seem to be present in all phytoplasmas were not linked (Fig. 5). The locus of the gene encoding the elongation factor Tu (*tuf*) was located on the same fragment as one of the rRNA operons while the randomly cloned fragment AT67 was located on the same fragment as the other rRNA operon (Figs 1b, 2b, 5,

Fig. 6. PFGE and Southern blot hybridization of chromosomal DNA of the ESFY phytoplasma. (a) Ethidium-bromide-stained gel of single and double digests with various rare-cutting enzymes. PFGE parameters were 1–21 s, 6 V cm⁻¹, 18.8 h. (b–d) Corresponding Southern blot hybridization of DNA fragments obtained by single and double digestion probed with (b) a cloned DNA fragment containing a gene encoding the elongation factor Tu (*tuf*), (c) a cloned DNA fragment containing a gene encoding an immunodominant membrane protein and (d) randomly cloned DNA fragment IH184.

6b, 7b, 8 and data not shown). However, the position of both *tuf* and fragment AT67 relative to the positions of rRNA operons was not determined. The locus of the gene encoding an immunodominant protein as well as that of a putative nitroreductase gene were identified on the 65 kb *Bss*HII and 56 kb *Bam*HI–*Xho*I fragments, respectively (Figs 6c, 7c and 8). The randomly cloned fragment IH184 was identified within the 12 kb *Xho*I fragment containing the *Bss*HII restriction site at position 0 (Figs 4b and 8).

DISCUSSION

Sufficient chromosomal DNA of strain GSFY1 of the ESFY phytoplasma was obtained from a broad range of host plants including periwinkle and several solanaceous plants. Phloem preparations of *N. glutinosa* and the tomato cultivars Roma, Olivade, Hildares and Frühzauber were useful for genetic analysis only at certain stages of disease. *N. glutinosa* plants proved better than tomato for phloem preparations. The importance of suitable host plants was also emphasized

by Firrao *et al.* (1996) and Lauer & Seemüller (2000), who succeeded in mapping the WX and the AT phytoplasma chromosomes, respectively, using material extracted from infected celery and tobacco but not infected periwinkle plants. In contrast, infected periwinkle plants were used to map the chromosome of the SPLI phytoplasma (Padovan *et al.*, 2000).

A total of 26 sites of five different restriction endonucleases were placed on the map of the GSFY1 chromosome of the ESFY phytoplasma. The mapped restriction sites were not uniformly distributed on the genome but clustered in certain areas. In particular, half of the restriction sites are located in the 100 kb region between positions 615 and 80, flanking the start point. A similar pronounced clustering of restriction sites is known for the chromosome of the phylogenetically closely related AP phytoplasma (Lauer & Seemüller, 2000) and for several other mollicute genomes (Pyle & Finch, 1988; Cocks *et al.*, 1989; Colman *et al.*, 1990; Ye *et al.*, 1992). In contrast, the restriction sites mapped on the WX and SPLI phytoplasma chromosomes are evenly distributed (Firrao *et al.*, 1996; Padovan *et al.*, 2000).

Fig. 7. PFGE and Southern blot hybridization of chromosomal DNA of the ESFY phytoplasma. (a) Ethidium-bromide-stained gel of single and double digests with various rare-cutting enzymes. PFGE parameters were 0.7–12.5 s, 6 V cm⁻¹, 18.8 h. (b–d) Corresponding Southern blot hybridization of DNA fragments obtained by single and double digestion probed with (b) randomly cloned DNA fragment AT67, (c) cloned DNA fragment IH196 containing a putative nitroreductase gene and (d) randomly cloned DNA fragment IH184.

Clustering in the mapped restriction sites may indicate differences in the overall base composition of the regions involved. It is possible that the 100 kb region flanking the arbitrary starting point is richer in G + C than other parts of the genome.

Most of the restriction enzymes we used were the same as those employed in the AP phytoplasma chromosome mapping. Comparisons of the maps showed that numbers and relative positions of restriction sites are conserved in only some cases. The three *Apa*I sites and the two *Sma*I sites are in the same or similar positions as those reported for the AP phytoplasma chromosome. In contrast, strain GSFY1 has more *Bss*HII sites than strain AT (five versus two) of which only one is at the same position (Lauer & Seemüller, 2000). Also, there are only six *Bam*HI sites in the GSFY1 chromosome while there are about 12 in the AT phytoplasma chromosome (E. Seemüller, unpublished). Different restriction patterns of closely related organisms are known for the SPL and tomato big bud phytoplasmas (Padovan *et al.*, 2000).

The same applies for five *Mycoplasma hominis* strains (Ladefoged & Christiansen, 1992). Although only some of the restriction sites are conserved in strains GSFY1 and AT, the order of identified genes in the GSFY1 chromosome is similar to that of the AT phytoplasma chromosome. In both cases, the two rRNA operons are unlinked. Such a chromosomal arrangement is also known from the WX and SPL phytoplasmas and some culturable mollicutes (Pyle & Finch, 1988; Cocks *et al.*, 1989; Pyle *et al.*, 1990; Firrao *et al.*, 1996; Padovan *et al.*, 2000). As there is an indication that the gene order is conserved in several mollicutes (Pyle *et al.*, 1990; Ladefoged & Christiansen, 1992; Peterson *et al.*, 1995; Himmelreich *et al.*, 1997), this marker could be more appropriate than restriction sites in studying genomic diversity of mollicutes.

The presence of different *Mlu*I restriction patterns, the fragments of which proved to be of phytoplasma nature, could be explained by the fact that strain GSFY1 consists of a phytoplasma population showing a sequence

Fig. 8. Physical map of the chromosome of the ESFY phytoplasma. Restriction sites for *SmaI*, *BssHII*, *ApaI*, *BamHI* and *XhoI* are indicated. Loci of the two rRNA operons, the operon containing the *tuf* gene, an immunodominant membrane protein gene, a putative nitroreductase (IH196) gene and randomly cloned DNA fragments IH184 and AT67 were also mapped.

heterogeneity with respect to *MluI* restriction sites. Similar observations have recently been reported for the SPL and tomato big bud phytoplasma chromosomes (Padovan *et al.*, 2000).

The physical map of the ESFY phytoplasma chromosome will further enhance genomic studies on this phytopathologically important phytoplasma, and will aid genome comparisons within the '*Candidatus Phytoplasma*' genus.

ACKNOWLEDGEMENTS

We thank Luigi Carraro for providing strain ESFY1 of the ESFY phytoplasma and Felix Hergenhausen for performing the transmission work. The work was supported by a grant from the Commission of the European Communities to the first author (Research Training Grant FAIR-BM-974428).

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Received 29 September 2000; revised 2 February 2001; accepted
12 February 2001.