Physical Map of the Western X-Disease Phytoplasma Chromosome

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A physical map of the chromosome of the western X-disease phytoplasma was constructed and represents the first physical map of a phytoplasma chromosome. The western X-disease phytoplasma is a nonculturable, plant-pathogenic member of the class *Mollicutes* and is the causal agent of a severe disease of fruit trees in North America. The map was generated by performing restriction digests of the chromosome and resolving the restriction fragments by pulsed-field gel electrophoresis. Southern blot analysis using cloned phytoplasma probes confirmed the arrangement of contiguous restriction fragments. The locations of 20 restriction sites for the enzymes *Sall*, *XhoI*, *BssHII*, *RsrII*, *SmaI*, and *NotI* were mapped on the chromosome, which is circular and comprises approximately 670 kb. The locations of the two rRNA operons and of four previously cloned fragments of chromosomal DNA were also placed on the map.

Phytoplasmas (7) are a large and diverse group of the class *Mollicutes* and are associated with plant and insect diseases (10). Phytoplasmas infect a wide variety of agricultural and ornamental crops and in some cases cause severe damage (20). Although the phylogenetic relationship of phytoplasmas within the class *Mollicutes* has recently been established (6, 14, 28), very little is known about their physiological and genetic properties because of the inability to culture these organisms in vitro. The western X-disease (WX) phytoplasma causes a severe disease of many *Prunus* fruit and nut trees in North America. Several diagnostic tools for the detection of the WX phytoplasma exist, including monoclonal antibodies (5, 9) and cloned chromosomal DNA probes (15). More recently, regions of the WX rRNA operon, including the complete 16S rDNA, the 16S/23S rDNA spacer region, a portion of the 23S rDNA, and a promoter region upstream of the 16S rDNA, have been sequenced (11, 14, 25). These sequences have been used to develop PCR-based detection assays, which are specific for the WX group of phytoplasmas (13). In addition, a number of other phytoplasmas have been shown to be serologically, genetically, and phylogenetically similar to the WX phytoplasma (11, 12, 19). In order to expand our understanding of the phytoplasma genome and to provide a framework for characterizing those genes important in pathogenicity, we generated a physical map of the WX phytoplasma chromosome.

The G+C content and genome size of a number of phytoplasmas have been determined and were found to range from 23 to 29.5 mol% (16, 27) and from 650 to 1,200 kb (22), respectively. The low G+C content of phytoplasma chromosomal DNA suggested that digestion with restriction endonucleases with GC-rich recognition sequences would produce a limited number of large restriction fragments, which would differ substantially in size from fragments produced by digesting higher-G+C-content DNA of the host plant. These large phytoplasma restriction fragments could then be separated by

an appropriate gel electrophoresis method, and their arrangement could be determined by comparing the results of multiple and partial digests (2). We have successfully utilized this approach to generate a contiguous physical map of the WX phytoplasma chromosome.

The WX phytoplasma strain used for the construction of the map was isolated by Jensen (8) from a diseased peach tree in northern California and was maintained in celery by serial transmission from diseased to healthy plants by using *Collado-nus montanus* leafhopper vectors (8). Celery infected with this phytoplasma strain, as well as the healthy celery plants used as controls, was kindly provided by A. H. Purcell (University of California, Berkeley).

Samples were prepared for pulsed-field gel electrophoresis (PFGE) as described by Neimark and Kirkpatrick (22), with minor modifications. Two samples of 2.5 g of fresh, symptomatic leaf tissue were ground separately in buffer as described previously (22). After an initial low-speed centrifugation (1,500 × g), the supernatants were filtered through cheesecloth and were centrifuged for 25 min at 18,000 × g (11,000 rpm in a Sorvall SS34 rotor). The pellets were suspended in 7 ml of Tris-sucrose (TS) buffer (22), and the suspensions were pooled and centrifuged at low speed repeatedly until no visible pellet was obtained. The supernatant was centrifuged again at high speed (18,000 × g); the final pellet was resuspended in 100 µl of TS, and the suspension was mixed at 40°C with an equal volume of 1% (wt/vol) InCert Agarose (FMC, Rockland, Maine) and pipetted into an ice-cold plastic mold. The resulting PFGE blocks were approximately 3.5 by 1.4 by 4 mm and contained 25 µl of the phytoplasma-agarose suspension. These blocks were then incubated in 0.5 M EDTA–10% (wt/vol) sodium dodecyl sulfate (SDS) buffer with proteinase K (0.5 mg/ml) for 3 days at 52°C as described previously (22). A volume of 500 µl of EDTA-SDS-proteinase K solution per block was used. Blocks were stored at 4°C for a maximum of 3 months in Sarkosyl-EDTA buffer as described previously (22).

Before digestion with restriction enzymes, the blocks were washed three times for at least 30 min per wash with TE (10 mM Tris-HCl–1 mM EDTA, pH 8) and once with 300 µl of the appropriate restriction buffer containing 100 µg of bovine se-

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FIG. 1. PFGE of DNA prepared from WX phytoplasma-infected celery digested with various restriction endonucleases. (A) Ethidium bromide-stained PFGE gel. Lanes: 1, lambda-*Hind*III; 2, mixture of linearized lambda and lambda-*Kpn*I; 3 and 12, lambda concatemers; 4, *Xho*I; 5, *Xho*I-*Sal*I; 6, *Sal*I; 7, *Bss*HII-*Sal*I; 8, *Bss*HII; 9, *Bss*HII-*Xho*I; 10, *Xho*I-*Sal*I-*Bss*HII; 11, *Rsr*II. (B) Southern blot hybridization of WX phytoplasma-infected celery DNA. Digestions were performed with *Xho*I (lane 1), *Sal*I (lane 2), and *Bss*HII (lane 3), separated by PFGE, and probed with a mixture of *Xho*I, *Sal*I and *Bss*HII fragments, which represent the whole WX phytoplasma genome.

rum albumin (Sigma, St. Louis, Mo.) per ml. The DNA in the blocks was then digested for 16 h in 100 μ l of restriction buffer containing 10 to 80 U of one or more of restriction enzymes *Bam*HI, *Bss*HII, *Eag*I, *Not*I, *Pvu*II, *Sac*II, *Sal*I, *Sma*I, *Rsr*II, and *Xho*I as directed by the manufacturer (New England Biolabs, Beverly, Mass.). After digestion, the blocks were washed once in TE and embedded in a 1.2% (wt/vol) GTG Gold agarose gel (FMC). *Saccharomyces cerevisiae* chromosomes and lambda concatemers (FMC) were used as DNA molecular size markers. The gel was electrophoresed in 0.25 $\times$ Tris-borate-EDTA (TBE) buffer (1) for 18 to 30 h at 160 V in a CHEF-DRII pulsed-field system (Bio-Rad, Hercules, Calif.), with switching times varying from 10 to 60 s depending on the size of the fragments to be resolved. Gels were stained with ethidium bromide, destained by washing for 3 to 12 h in several changes of distilled water, and photographed under UV light. A long destaining period was found to be critical for optimal visualization of digested fragments. When required, WX phytoplasma DNA bands were cut from the gel, washed first with TE and then with the appropriate restriction buffer, and then digested with a second restriction endonuclease.

In preparation for Southern transfer, restriction fragments were depurinated by washing the gel twice in 0.25 N HCl for 10 min and then denatured, neutralized, and transferred to a Hybond N nylon membrane (Amersham, Arlington Heights, Ill.) by standard techniques (1). Fragments used as probes were (i) four previously cloned WX phytoplasma chromosomal DNA fragments (15), (ii) a PCR-amplified DNA fragment containing the entire 16S rRNA gene and the 16S/23S rRNA spacer region (14), and (iii) DNA extracted from a phytoplasma-enriched fraction (15) of WX-infected celery. Probes were labeled with digoxigenin, and hybridizations were carried out by following the manufacturer's guidelines (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). In addition, WX DNA restriction fragments separated on low-melting-point agarose PFGE gels were purified from the gels with Gelase (Epicentre Technology, Madison, Wis.), labeled with digoxigenin, and used as probes.

When WX DNA was digested with either *Sma*I or *Not*I and

electrophoresed on a PFGE gel, a single DNA band of about 650 kb was detected (data not shown). The intensity of the ethidium bromide fluorescence of this band was stronger than that obtained from undigested blocks and was similar to that obtained from phytoplasma chromosomes linearized by gamma irradiation (22). Southern blot analysis of these single digests using DNA extracted from the phytoplasma-enriched fraction of WX-infected celery as a probe resulted in a single discrete band of about 650 kb (data not shown), indicating a single unique site for *Not*I and *Sma*I in the WX phytoplasma chromosome.

PFGE blocks digested with the restriction endonucleases *Xho*I, *Sal*I or *Bss*HII produced four to five discrete restriction fragments from each digest (Fig. 1A, lanes 4, 6, and 8). Digests with multiple enzymes were also performed, and the sizes of the resulting bands were used in constructing the map (Fig. 1A, lanes 5, 7, 9, and 10). Southern hybridization was used to identify DNA bands of low molecular weight which could not be detected in the ethidium bromide-stained gel and to distinguish them from bands arising from plant DNA contamination. The major WX DNA fragments of the *Xho*I, *Sal*I, and *Bss*HII digestions were purified from a low-melting-point agarose PFGE gel; the fragments from all three digests were pooled, labeled with digoxigenin, and used to probe a Southern blot of PFGE-separated *Xho*I, *Sal*I, or *Bss*HII restriction fragments. The hybridization pattern revealed four bands for *Bss*HII and five bands each for *Sal*I and *Xho*I (Fig. 1B), as was seen in ethidium bromide-stained gels (Fig. 1A). To demonstrate that the discrete banding pattern was derived from the phytoplasma DNA, *Not*I-digested blocks were run in PFGE gels and the phytoplasma DNA band was excised from the gel and digested with *Xho*I, *Sal*I, or *Bss*HII. The resulting DNA fragments were resolved in a new PFGE gel, transferred to a nylon membrane, and hybridized with labeled DNA extracted from WX phytoplasma-infected celery. Analysis of the sizes of hybridizing bands confirmed the patterns of bands of the single digests and indicated the position of the unique *Not*I site relative to the *Bss*HII, *Xho*I, and *Sal*I sites.

The four sites for *Bss*HII were the first to be placed on the map, because their positions could be quickly determined from the digestion of the *Bss*HII fragments with *Xho*I and from the digestion of the *Xho*I fragments with *Bss*HII (Table 1). Double digestions with *Xho*I-*Not*I, *Sal*I-*Not*I, *Bss*HII-*Not*I, *Xho*I-*Sma*I, *Sal*I-*Sma*I, and *Bss*HII-*Sma*I were used to locate the sites for the single-site enzymes *Not*I and *Sma*I relative to the *Bss*HII sites on the map. These double digests were also used to determine the positions of the *Sal*I and *Xho*I fragments containing the *Not*I and *Sma*I sites. The digestion of *Sal*I fragments with *Xho*I and *Xho*I fragments with *Sal*I provided the information necessary to complete the map for all five restriction endonucleases. Additionally, the digestions of *Bss*HII fragments with *Sal*I and of *Sal*I fragments with *Bss*HII were performed to confirm the locations of these sites on the map (Fig. 2).

Tentative locations of the restriction sites for the endonuclease *Rsr*II are also reported in Fig. 2. Since *Rsr*II was inefficient in digesting DNA prepared in agarose blocks, most of the digestions performed with this enzyme were incomplete (Fig. 1A, lane 11). The map for *Rsr*II was obtained by analyzing the sizes of partial *Rsr*II and *Rsr*II-*Not*I digests and by digesting isolated *Rsr*II fragments with other restriction enzymes. Although the locations of these *Rsr*II sites are consistent with all of our results, the presence of additional undetected sites cannot be excluded.

The WX phytoplasma chromosomal DNA digested with *Bam*HI, *Eag*I, *Pvu*II, or *Sac*II individually produced eight or

TABLE 1. Results of restriction digests of the WX phytoplasma chromosome

Enzyme	Fragment name	Map size (kb) ^a	Observed size (kb) ^b	Second enzyme (product size[s]) ^c
<i>Bss</i> HII	B1 ^d	257	239	<i>Xho</i> I (139, 96, 14), <i>Sal</i> I (131, 110)
	B2 ^e	185	179	<i>Xho</i> I (142, 40), <i>Sal</i> I (110, 77)
	B3	149	142	<i>Xho</i> I (80, 65, 6), <i>Sal</i> I (86, 34, 26)
	B4	86	88	<i>Xho</i> I (88), <i>Sal</i> I (88)
<i>Xho</i> I	X1 ^e	294	275	<i>Sal</i> I (141, 77, 74), <i>Bss</i> HII (142, 138)
	X2	202	195	<i>Sal</i> I (155, 34, 18), <i>Bss</i> HII (88, 80, 40)
	X3	96	96	<i>Sal</i> I (96), <i>Bss</i> HII (96)
	X4	65	65	<i>Sal</i> I (65), <i>Bss</i> HII (65)
	X5	20	20	<i>Sal</i> I (20), <i>Bss</i> HII (14, 6)
<i>Sal</i> I	S1 ^e	228	219	<i>Xho</i> I (155, 74), <i>Bss</i> HII (110, 88, 26)
	S2 ^d	196	183	<i>Xho</i> I (96, 65, 20, 18), <i>Bss</i> HII (110, 86)
	S3	142	142	<i>Xho</i> I (141), <i>Bss</i> HII (131) ^f
	S4	77	77	<i>Xho</i> I (77), <i>Bss</i> HII (77)
	S5	34	34	<i>Xho</i> I (34), <i>Bss</i> HII (34)

^a Theoretical size of fragments calculated by placing the restriction sites on the 677 kb genome map.

^b Estimated mean size of restriction fragment based on relative migration in PFGE gels compared with a lambda concatemer standard.

^c Observed sizes (in kilobases) of the subfragments arising from the digestion of each fragment with the indicated second enzyme.

^d Contains the *Not*I site.

^e Contains the *Sma*I site.

^f Digestion of S3 with *Bss*HII resulted in a 131-kb fragment, yet no expected 10-kb or smaller fragments were observed in the gel.

more fragments. Because of the large number of these restriction sites, these enzymes were not used in the construction of the map.

Hybridization of restriction-digested WX phytoplasma chromosome with a PCR-amplified rDNA fragment gave a complex pattern, which is consistent with the presence of two target sites for the hybridization of the probes (Fig. 2). This result is in agreement with the observations of Schneider and Seemüller (26), who found two sets of rRNA genes in many phytopathogenic members of the class *Mollicutes*. Additionally, four previously cloned fragments of the WX phytoplasma chromosome (15) were mapped by Southern hybridization (Fig. 2).

The mean size of the WX phytoplasma chromosome was found to be 675 kb, which is in agreement with previous results (22). This size was calculated as a sum of the restriction fragments generated from double digests and was slightly larger than that calculated from the sum of the single digests. In general, the size of the larger fragments was underestimated compared with the sum of the subfragments arising from their digestion with a second enzyme. This may be due to anomalous migration of the large, compared with the small, genome fragments in PFGE or to differences in the G+C content of the DNA of the marker compared with that of the WX phytoplasma.

The use of PFGE to separate very large DNA fragments by size has made possible the construction of physical maps for a number of bacterial chromosomes (reviewed in reference 18). Because of their comparatively small size, which allows the use of the mapping techniques proposed by Bautsch (2), chromo-

somes of members of the class *Mollicutes* have been studied extensively by PFGE. Chromosomal restriction maps of representatives of the major phylogenetic clades within the class *Mollicutes* have been determined, including *Acholeplasma oculi* (31), *Mycoplasma gallisepticum* (30), *Mycoplasma genitalium* (4, 29), *Mycoplasma mycoides* and relatives (23, 24), *Mycoplasma pneumoniae* (17), *Spiroplasma citri* (34), *Spiroplasma melliferum* (33), and *Ureaplasma urealyticum* (3). We report the construction of a physical map for the chromosome of a member of the phytoplasma group, a newly recognized and possibly autonomous clade within the class *Mollicutes*. The chromosomal map of the WX phytoplasma presents features common to other mycoplasmas, such as the presence of two unlinked rDNA operons and fewer *Sal*I sites than expected. The theoretically expected average size of fragments generated by the restriction endonuclease *Sal*I (four GC base pairs and two AT base pairs in the recognition sequence) in a DNA with a G+C content of 26 mol% is 25 kb, accounting for 20 to 80 sites in chromosomes ranging in size from 500 to 2,000 kb. However, a considerably lower number of *Sal*I-generated fragments has been reported for *M. mycoides*, *Mycoplasma capricolum* and relatives (21, 32), *S. citri* (34), and WX phytoplasma (this paper). Ye et al. (34) investigated this phenomenon further and suggested that the majority of *Sal*I sites could be part of a sequence that is methylated, a common feature of many members of the class *Mollicutes*.

A. oculi is phylogenetically the closest organism to the WX phytoplasma for which a physical map has been published (31). Interestingly, the mean sizes of the fragments generated by digestion with three of the four restriction endonucleases used to construct the WX phytoplasma map were significantly different between *A. oculi* and the WX phytoplasma. The mean sizes were, respectively, 400 and 50 kb for *Eag*I, 230 and 650 kb for *Sma*I, and 1,600 and 150 kb for *Bss*HII in *A. oculi* and WX phytoplasma (31). This difference in site frequencies may represent differences in codon bias.

In the physical map presented in this report, 20 sites were identified on the WX phytoplasma chromosome by using six different restriction endonucleases. It may be possible that

FIG. 2. Schematic representation of the physical map of the chromosome of the WX phytoplasma.

additional sites for some of these enzymes are present in the WX phytoplasma genome if the sites are close together. Closely linked sites would generate restriction fragments that would be too small to observe on PFGE gels. There are at least one and possibly more *Bss*HII sites in fragment S3 (Table 1), because digestion of S3 with *Bss*HII reduced the size of S3 from 141 to 131 kb and yet no 10-kb fragment was observed in the PFGE gel. We chose these six restriction enzymes because they would infrequently cut the AT-rich phytoplasma genome and at the same time they would generate numerous small fragments from any plant DNA, which is an unavoidable contaminant in the phytoplasma DNA preparations. The same map resolution could be theoretically achieved by using only one or two more frequently cutting enzymes such as *Eag*I or *Pvu*II, which were used to construct a genetic map of *A. oculi* (31); however, these enzymes might also produce large plant DNA fragments, which could complicate the interpretation of the PFGE patterns. The ability to readily identify PFGE restriction fragments in ethidium bromide-stained gels was due to the fact that the WX phytoplasma is present in high titers in celery plants (15). Similar preparations of other phytoplasma strains from other host plants, such as periwinkle, generally do not reach such high titers, and phytoplasma PFGE restriction fragments need to be identified by Southern blot hybridization (22). If reasonably enriched fractions of organisms can be produced, the techniques used in this study could be used to characterize the genomes of other nonculturable prokaryotes.

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