Phytoplasmas Associated with Elm Yellows: Molecular Variability and Differentiation from Related Organisms

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ABSTRACT

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Restriction fragment length polymorphism (RFLP) analyses were performed on polymerase chain reaction (PCR) amplimers of phytoplasmal DNA from eight samples obtained from *Ulmus* spp. (elms) affected by elm yellows (EY) in Italy and the United States, from *Catharanthus roseus* infected with strain EY1, and from five other plant species infected with phytoplasmas of the EY group sensu lato (group 16SrV). RFLP profiles obtained with restriction enzyme *TaqI* from ribosomal DNA amplified with primer pair P1/P7 differentiated elm-associated phytoplasmas from strains originally detected in *Apocynum cannabinum*, *Prunus* spp., *Rubus fruticosus*, *Vitis vinifera*, and *Ziziphus jujuba*. RFLP profiles obtained similarly with *BfaI* differentiated strains from *A. cannabinum* and *V. vinifera* from other phytoplasmas of group 16SrV. Elm-associated strains from within the United States had two RFLP patterns in ribosomal DNA based on presence or absence of an *RsaI* site in the 16S-23S spacer. Elm-associated phytoplasma strains from Italy were distinguished from those of American origin by RFLPs obtained with *MseI* in the same fragment of non-ribosomal DNA. Strain HD1, which was discovered in *A. cannabinum* associated with EY-diseased elms in New York State, was unique among the strains studied.

The phytoplasmal disease elm yellows (EY) occurs in North America and Europe (8,15-17,23). EY causes decline of Eurasian elm species and hybrids in some European localities, notably in Italy (8,9,15). Numerous phytoplasma strains in elms in Europe have been identified, and the predominant strain in each case has been a member of the EY group sensu lato (= group 16SrV; 8-11,16). Strain ULW from a witches'-broom on *Ulmus minor* (European field elm) in France was the first to be identified (18,22).

Significant EY outbreaks occur sporadically in indigenous elms (*U. americana* L., *U. rubra* Mühl., *U. alata* Michx., *U. serotina* Sarg., and *U. crassifolia* Nutt.) in the United States and are lethal in these species (23). However, elms of Eurasian origin growing in North America have

rarely been damaged by EY. The reason for this escape is unknown. Understanding more about the relatedness of elm-inhabiting EY strains is important for determining host and epidemiological differences in EY between Europe and North America.

Phytoplasmas of the EY group sensu lato occur in diverse plant genera including, among others, *Apocynum*, *Prunus*, *Rubus*, *Vitis*, and *Ziziphus* in addition to *Ulmus* (1,6,7,10,12,14,16,22). In New York State, *A. cannabinum* (hemp dogbane) infected with a phytoplasma of group 16SrV was found growing on the site of an EY outbreak. This isolate was transferred to periwinkle and designated HD1 (6).

The aim of this study was to address the following two hypotheses: first, that phytoplasma strains of group 16SrV inhabiting *Ulmus* spp. in North America and Europe comprise a genetically coherent subgroup and, second, that HD1 differs from elm-inhabiting phytoplasmas. Abstracts describing part of this work have appeared (6,7).

MATERIALS AND METHODS

Phytoplasma collection and propagation. Nine samples of phytoplasma-infected *Ulmus* spp. representing *U. americana*, *U. rubra*, *U. minor* (*U. carpinifolia*),

U. chenmouii, and the hybrid clone 'Lobel' plus a sample from *A. cannabinum* were obtained from naturally infected plants in Italy and the United States (Table 1). Phytoplasmal infection was initially indicated by symptoms and, in *A. cannabinum*, by means of the DAPI (4', 6-diamidino-2-phenylindole·2HCl) fluorescence test (21,24). Five DNA samples previously collected from plants of other species infected with phytoplasmas of group 16SrV were used as standards (Table 1). Eight samples of diseased elm were utilized directly for DNA extraction. Phytoplasmas from the other two source plants (EY1 from elm and HD1 from hemp dogbane) were transmitted by *Cuscuta subinclusa* (dodder) to *Catharanthus roseus* (periwinkle) grown from seed and then were maintained in this species by serial grafting. Plants were grown in a screened greenhouse with periodic insecticide treatments to prevent unwanted introduction or transmission of phytoplasmas.

DNA extraction and amplification.

The nucleic acid extraction procedure of Lee et al. (9) was used for elm samples, and the method of Dellaporta et al. (3) was used for periwinkle samples, beginning with the grinding of 0.75 g of fresh leaf midribs in liquid nitrogen. The final pellets were treated with RNase, and DNA concentrations were estimated spectrophotometrically using standard techniques (19).

Phytoplasmal DNA was amplified in polymerase chain reactions (PCRs) in which two primer pairs, P1/P7 and FD9f/r, were used routinely. P1/P7 (4,20) amplifies nearly the entire 16S rRNA gene, the 16S-23S spacer, and a small portion of the 23S rRNA gene (1.8-kilobase [kb] product). Primer pair FD9f/r (2) amplifies a 1.3-kb fragment of non-ribosomal DNA. A third primer pair, P4/P7 (523-base pair [bp] product; 20) was used with elm samples from Bismarck and Fargo, North Dakota, because DNA amplified from these samples with primer set P1/P7 was insufficient for restriction fragment length polymorphism (RFLP) analyses.

Amplifications with ribosomal and non-ribosomal primers were performed in 50- μ l mixtures containing 100 ng of nucleic acid

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and 1.25 U of *Taq* DNA polymerase (GIBCO BRL, Gaithersburg, MD) as described by Lee et al. (11) and Daire et al. (2). Parameters used for 35-cycle PCRs with primer pair P1/P7 were denaturation at 94°C for 30 s (90 s for the first cycle), annealing for 55 s at 55°C, and primer extension for 80 s (9 min for last cycle) at 72°C. Parameters used with primer pair P4/P7 for 35-cycle PCR were as previously described (20). Parameters used with primer pair FD9f/r for 40-cycle PCRs were denaturation at 92°C for 30 s (90 s for the first cycle), annealing for 30 s at 54°C, and primer extension for 80 s at 72°C. PCR products in 5-μl samples of reaction mixtures were detected and their sizes estimated by electrophoresis with size standards in 0.7% agarose gel in Tris-borate EDTA (TBE) buffer, followed by staining with ethidium bromide and visualizing the DNA bands with a UV transilluminator.

RFLP analyses. Aliquots of PCR products obtained with primer pair P1/P7 were digested overnight at 37°C with either *AluI*, *BamHI*, *HaeIII*, *HhaI*, *HpaII*, *MseI*, *RsaI*, *ThaI* (GIBCO BRL), or *BfaI* (New England Biolabs, Beverly, MA) or at 65°C overnight with *TaqI* (GIBCO BRL). PCR products obtained with primer pair P4/P7

were digested overnight at 37°C with *RsaI* or at 65°C overnight with *TaqI*. Each digest used 10 U of enzyme in the presence of spermidine (40 mM) in a 10-μl reaction, except that 5 U of enzyme was used with *MseI*, *ThaI*, and *BfaI*. Restriction digest products were separated by electrophoresis in 5, 6.5, or 9% acrylamide gels (concentration depending upon molecular weight of the expected bands) prepared in TBE (19) and were stained with ethidium bromide. DNA bands were visualized as described above. FD9f/r PCR products were digested overnight at 37°C with 5 U of *MseI* in a 10-μl reaction. The entire restriction digest was separated by electrophoresis in a 10% acrylamide gel prepared in TBE (19) and stained and visualized as described above.

Nucleotide sequencing and sequence alignment. The P1/P7 amplimers from strains EY1, EY-WV, and HD1 were sequenced, because these strains represented the three RFLP profiles encountered in rDNA of group 16SrV phytoplasmas from North America. The PCR products were purified using 0.7% low-melting-point agarose (Ultrapure LMP, GIBCO BRL) prepared in Tris-acetate EDTA (TAE) and extracted with the QiAquick gel extraction kit (Qiagen, Chatsworth, CA). The final

elution from the column was performed with sterile distilled water. DNA concentrations were estimated spectrophotometrically and adjusted for sequencing using sterile distilled water. Sequencing was performed by automated equipment in Cornell University's Biotechnology Center. Each strand was sequenced in both directions with at least five primers located along the template to allow overlapping of sequences and thus eliminate ambiguous regions. Sequence data were collated, aligned with the aster yellows sequence of Lim and Sears (13), and mapped using LASERGENE software (5). Locations of restriction enzyme recognition sites reported here are based on the aster yellows sequence.

Nucleotide sequence accession numbers. Sequences of the P1/P7 amplimers of phytoplasma strains EY1, EY-WV, and HD1 were deposited in GenBank with accession numbers AF 122910, AF 122911, and AF 122912, respectively.

RESULTS

P1/P7 amplimers and RFLPs. Profiles from strains EY1, EY-NYII, and ITEY were identical with 10 enzymes (Table 2). Restriction sites in the P1/P7 amplimer of strain EY-WV, identified from sequence

Table 1. Phytoplasma strain, geographic origin, host origin, and source

Strain name (16S rRNA subgroup) ^x	Geographic origin	Host origin	Strain source
EY1 (A) ^y	Ithaca, New York	<i>Ulmus americana</i>	Sinclair
ITEY (A) ^{y,z}	Northern Italy	<i>U. minor</i>	Lee
EY-Pisa ^z	Pisa, Tuscany, Italy	<i>Ulmus</i> 'Lobel'	Bertaccini
EY-Presiola ^z	Presiola, Emilia-Romagna, Italy	<i>Ulmus chenmoui</i>	Bertaccini
EY-NYI	Ithaca, New York	<i>U. americana</i>	Griffiths & Sinclair
EY-NYII	Ithaca, New York	<i>U. americana</i>	Griffiths & Sinclair
EY-WV	Martinsburg, West Virginia	<i>U. rubra</i>	Sinclair
EY-Bismarck	Bismarck, North Dakota	<i>U. americana</i>	Walla
EY-Fargo	Fargo, North Dakota	<i>U. americana</i>	Walla
FD70 (C) ^{y,z}	France	<i>Vitis vinifera</i>	Boudon-Padieu
RS (C) ^{y,z}	Northern Italy	<i>Rubus fruticosus</i>	Lee
HD1	Trumansburg, New York	<i>Apocynum cannabinum</i>	Griffiths & Sinclair
JWB (B) ^{y,z}	Northern China	<i>Ziziphus jujuba</i>	Lee
CLY (B) ^{y,z}	China	<i>Prunus</i> spp.	Lee

^x Subgroups delineated by Lee et al. (10) for characterized strains.

^y Samples previously identified, used as standards.

^z Nucleic acid sample provided by collector.

Table 2. Arbitrary restriction fragment length polymorphism group designations given to P1/P7 amplimers obtained from 11 strains of phytoplasma group 16SrV after digestion with 10 restriction enzymes

Strain	Restriction enzyme									
	<i>AluI</i>	<i>BamHI</i>	<i>BfaI</i>	<i>HaeIII</i>	<i>HhaI</i>	<i>HpaII</i>	<i>MseI</i>	<i>RsaI</i>	<i>TaqI</i>	<i>ThaI</i>
EY1	I	I	I	I	I	I	I	I	I	I
EY-NYII	I	I	I	I	I	I	I	I	I	I
ITEY	I	I	I	I	I	I	I	I	I	I
EY-Pisa	...	...	I	...	...	I	...	I	I	...
EY-Presiola	...	...	I	...	...	I	...	I	I	...
EY-WV	I	I ^z	I ^z	I ^z	I ^z	I ^z	I ^z	II	I	I ^z
FD70	I	I	II	I	I	I	I	I	II	I
HD1	I	I	II	I	I	I	I	I	II	I
RS	I	I	II	I	I	I	I	I	II	I
CLY	I	I	I	I	I	II	I	III	III	I
JWB	I	I	I	I	I	II	I	III	III	I

^y ... indicates no data.

^z Data obtained by sequence analysis of 16S rRNA gene.

data and confirmed for *RsaI* and *TaqI* by RFLP analyses, were identical to those of EY1 for all enzymes except *RsaI*, which had an additional recognition site in the spacer region (Table 2, Figs. 1A and 2). Restriction enzymes *BfaI*, *HpaII*, and *RsaI* were used in analysis of amplicons

Fig. 1. Restriction fragment length polymorphism profiles of 1.8-kilobase (kb) fragments of rDNA, amplified by polymerase chain reaction (PCR) with primer pair P1/P7, from sources of phytoplasmas in group 16SrV: *Catharanthus* infected with elm yellows strain EY1, *Ulmus* sp., Italy (ITEY), *U. rubra*, West Virginia (EY-WV), *U. americana*, New York (EY-NYII), *Apocynum cannabinum* (HD1), *Rubus fruticosus* (RS), *Vitis vinifera* (FD70), and *Ziziphus jujuba* (JWB). PCR products were digested with (A) *RsaI*, (B) *TaqI*, or (C) *BfaI*. Std: 1-kb DNA ladder (Gibco-BRL, Grand Island, NY).

from strains EY-Pisa and EY-Presiola, because these enzymes had restriction sites that varied among group 16SrV phytoplasmas (10). The profiles from these digests were identical to those observed with digests of amplicons from EY1 and ITEY (Table 2).

PCR products of the expected size but insufficient for RFLP analysis were obtained with primers P1/P7 and templates from samples EY-NYI, EY-Bismarck, and EY-Fargo. However, with template EY-NYI and primers P4/P7, sufficient product (523 bp) was obtained to establish that EY-NYI had an extra *RsaI* site at the same location within the spacer region as observed for strain EY-WV (*data not shown*). P4/P7 amplicons from strains EY-Bismarck and EY-Fargo, digested with *RsaI*, had the same profiles as EY1 (*data not shown*). *TaqI* profiles were the same for all seven strains obtained from elm trees and resembled that of EY1 (Fig. 1B and *data not shown*).

Digestion of the P1/P7 amplicon of strain HD1 with the 10 restriction enzymes gave profiles identical to those observed with strains FD70 and RS (Table 2). The *TaqI* and *BfaI* RFLPs differed from those observed with EY1 (Figs. 1B, C, and 2). Previous work (10) showed that strains FD70 and RS belong to the same subgroup, 16SrV-C. Analysis of sequence data from strains FD70 and RS (GenBank numbers X76560 and Y16395, respectively) and from HD1 obtained in this study (GenBank number AF 122912) confirmed the RFLP findings. FD70 and RS have

99% similarity, FD70 and HD1 98% similarity, and RS and HD1 98% similarity. Therefore, strain HD1 may also be assigned to subgroup 16SrV-C.

Nucleotide sequences. Alignment of a 1,710-bp sequence of the 16S rRNA gene and 16S-23S spacer of strains EY1, EY-WV, and HD1 revealed three genotypes corresponding to the RFLP profile groups detected with *BfaI*, *RsaI*, and *TaqI*. The sequences varied only at three positions, where single base substitutions defined or abolished recognition sites. These sites were a *BfaI* site (CTAG) at position 1112–1115 in strain HD1, an *RsaI* site (GTAC) at position 1550–1553 in strain EY-WV, and a *TaqI* site (TCGA) at position 1756–1759 in strain HD1 (Fig. 2). Positions 1550 and 1756 are within variable parts of the spacer; position 1112 is in a conserved region of the 16S rRNA gene (13).

FD9f/r amplicons and *MseI* profiles. FD9f/r (non-ribosomal) amplicons from strains EY1, EY-WV, and EY-NYII were identical when digested with *MseI* (Fig. 3). Insufficient amplicon was obtained from EY-NYI for analysis. FD9f/r amplicons from strain EY-Pisa, when digested with *MseI*, gave a profile different from those observed with the North American EY strains. The FD9f/r amplicon from strain HD1, when digested with *MseI*, gave a unique profile. *MseI* analysis of amplicons obtained with primer set FD9f/r also differentiated elm-inhabiting strains from others in group 16SrV (*data not shown*), as previously demonstrated by Daire et al. (2).

Fig. 2. Restriction maps of restriction fragment length polymorphism profile types obtained by digestion of rDNA of elm yellows (EY) or hemp dogbane (HD) phytoplasma strains with *BfaI*, *RsaI*, or *TaqI*.

DISCUSSION

RFLP analysis of 16S rDNA plus the 16S-23S spacer region of six strains of elm-inhabiting phytoplasmas from North America revealed variation only at one *RsaI* recognition site, located in the 16S-23S spacer region. Strains possessing this site were found in New York State and West Virginia. Presence or absence of this site may have no biological significance, because it is in a noncoding region. Moreover, strains EY-NYI and EY-NYII differed at this restriction enzyme site, even though these strains had been detected in adjacent EY-affected trees near Ithaca, New York, during 1997 and 1998, respectively.

Analysis of rDNA revealed no difference between type strain EY1 and Italian phytoplasma strains. However, using the non-ribosomal primer pair FD9f/r and restriction enzyme *MseI*, we found that the EY phytoplasmas inhabiting elm trees in North America are distinct from those inhabiting elm trees in Italy. The Italian EY strain RFLP profiles appeared identical to elm strain ULW from France, as characterized by Daire et al. (2). This suggests that the Italian strains from Pisa and Presiola may be more similar to the French strain ULW than to the North American elm strains.

Phytoplasma-infected *A. cannabinum* was found on the site of an EY outbreak; therefore, it was important to know if the phytoplasma (HD1) obtained from *A. cannabinum* was related to the EY agents found in elm trees. Strain HD1 is indeed a member of the EY group sensu lato. How-

ever, it seems more closely related to grapevine- and bramble-inhabiting strains (group 16SrV-C of Lee et al.; 10) than to typical elm-inhabiting strains (group 16SrV-A) and is therefore unlikely to be significant in an EY epidemic. On the basis of the 16S rRNA gene data alone, HD1 would be classified as a member of group V-C. However, use of the non-ribosomal primer pair FD9f/r and restriction enzyme *MseI* revealed that strain HD1 differs not only from the elm strains but also from strains FD70 and RS. Analysis of FD9f/r amplicons with restriction enzyme *MseI* had previously been valuable for differentiating among grapevine phytoplasmas belonging to the EY group and between two strains of EY (2).

Lee et al. (9) and Bertaccini et al. (1) reported heterogeneity of phytoplasmas of the EY group in elms, whereas Mäurer et al. (18) and Marcone et al. (15) reported that elm-inhabiting phytoplasmas of this group seemed to be homogeneous. Results of analyses of ribosomal and non-ribosomal DNA support the concept that elm-inhabiting phytoplasmas compose a taxonomically discrete but non-uniform subgroup (16SrV-A). Phenotypic variation in this subgroup should be assessed.

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Fig. 3. Restriction fragment length polymorphism profiles, obtained with *MseI*, of 1.3-kilobase (kb) fragments of non-ribosomal DNA amplified by polymerase chain reaction (PCR) with primer pair FD9f/r from sources of phytoplasmas in group 16SrV: *Ulmus americana*, New York (EY-NYII); *U. rubra*, West Virginia (EY-WV); *Ulmus* 'Lobel' Pisa, Italy (EY-Pisa); *Apocynum cannabinum* (HD1); and *Catharanthus* infected with elm yellows strain EY1. Std: 1-kb DNA ladder (Gibco-BRL, Grand Island, NY).

In the article "Phytoplasmas Associated with Elm Yellows: Molecular Variability and Differentiation from Related Organisms" by H. M. Griffiths, W. A. Sinclair, E. Boudon-Padiou, X. Daire, I.-M. Lee, A. Sfalanga, and A. Bertaccini, on pages 1101 to 1104, Figure 2 should appear as follows:

Fig. 2. Restriction maps of restriction fragment length polymorphism profile types obtained by digestion of rDNA of elm yellows (EY) or hemp dogbane (HD) phytoplasma strains with *BfaI*, *RsaI*, or *TaqI*.