Revised Subgroup Classification of Group 16SrV Phytoplasmas and Placement of *Flavescence Dorée*-Associated Phytoplasmas in Two Distinct Subgroups

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ABSTRACT

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The subgroup classification of phytoplasmas in 16S rRNA group 16SrV (elm yellows phytoplasma group) was revised and extended on the basis of enzymatic restriction fragment length polymorphism (RFLP) analysis of ribosomal (r) DNA and analysis of putative restriction sites in nucleotide sequences. A 1.85 kbp fragment of the rRNA operon from *flavescence dorée* (FD) phytoplasma strain FD70 from France was amplified and cloned, and its nucleotide sequence determined (GenBank acc. no. AF176319). Placement of FD70 in subgroup V-C was verified by analysis of amplified DNA and of the cloned sequence. Hemp dogbane phytoplasma HD1 (AF122912), a member of subgroup V-C, was distinguished from other subgroup V-C phytoplasmas by putative restriction site differences in the 16S-23S rRNA spacer region. A previously published FD phytoplasma sequence (GenBank accession no. X76560) differed from FD70 sequence AF176319 by at least eight nucleotide substitutions and differences in putative restriction sites. The X76560 FD phytoplasma was classified in a new subgroup (V-D). Based on analyses of 16S rDNA GenBank sequence Y16395, *Rubus* stunt phytoplasma was classified in new subgroup V-E. The revised classification was supported by sequence similarities, group 16SrV-characteristic sequences, and a phylogenetic tree constructed on the basis of 16S rDNA sequences.

Additional keywords: grapevine, mycoplasma

Flavescence dorée (FD) was the first grapevine yellows disease to be described (4). FD was distinguished from grapevine bois noir disease principally by the transmission of the FD pathogen by the leafhopper *Scaphoideus titanus* and the lack of transmission of bois noir pathogen by this insect species (5,7). Subsequently, FD disease has been defined as the grapevine yellows disease for which the causal agent is transmitted in nature by *S. titanus* (2,3,5-7,24). This biological definition of FD disease has seen broad application, and the term *flavescence dorée* has sometimes been generally applied to grapevine yellows in regions of Europe where *S. titanus* is known to occur.

In recent years, molecular methods have been used to differentiate and identify diverse phytoplasmas that are involved in

grapevine yellows diseases. Several phylogenetic groups representing different putative taxa are now known to be associated with grapevine yellows. Some appear to be limited in geographic distribution. On the basis of restriction fragment length polymorphism (RFLP) analysis of rDNA, FD phytoplasma has been classified in group 16SrV (elm yellows phytoplasma group), subgroup C; bois noir (*Vergilbungskrankheit*) phytoplasma has been classified in group 16SrXII (stolbur phytoplasma group) subgroup A; Australian grapevine yellows phytoplasma ("*Candidatus* Phytoplasma australiense") has been classified in group 16SrXII subgroup B; and phytoplasmas associated with grapevine yellows in Virginia, USA, have been classified in group 16SrI subgroup A and in group 16SrIII subgroup I (10,11,16). The ability to distinguish the varied grapevine yellows phytoplasma taxa through the use of molecular criteria supports the suggestion that the biological definition of FD proposed previously (5,6) should be supplanted by a definition based on molecular properties of the pathogen.

In the present study, we investigated a portion of the rRNA operon of FD phytoplasma strain FD70 that included most of the 16S rRNA gene, the 16S-23S rRNA spacer region, and the 5'-end of the 23S rRNA gene. This study was initiated following preliminary experiments in which we observed that the RFLP patterns of 16S

rDNA from FD phytoplasma strain FD70 were similar to, but not in complete agreement with, the putative restriction site maps that we constructed on the basis of a nucleotide sequence (GenBank accession no. X76560) of 16S rDNA from an FD phytoplasma. In this communication, we report results from RFLP analysis and nucleotide sequence analysis of a 1.85 kbp portion of the ribosomal (r) RNA operon from the phytoplasma strain FD70, and we compare these results with the nucleotide sequence X76560, as well as with rDNA sequences from other members of group 16SrV. Our findings resulted in a revised classification of subgroups in group 16SrV, and the placement of FD-associated phytoplasmas in two distinct subgroups.

MATERIALS AND METHODS

Polymerase chain reaction (PCR).

Phytoplasma-universal primer pairs P1/P7 (12,25) and R16F2n/R16R2 (F2n/R2) (14) were used to prime amplification of DNA sequences from the rRNA operon in PCR assays. For nested PCR, products of PCR primed by P1/P7 were diluted 1:50 with HPLC grade water (Sigma Chemical Co., St. Louis, MO) and used as template in PCR primed by R16F2n/R2. Template DNA was extracted as previously described (23) from a lyophilized sample of *Vicia faba* L. containing phytoplasma strain FD70 (8) and from frozen (-80°C) samples of plant tissues infected by reference phytoplasmas ash yellows (AshY1), clover yellow edge (CYE = CYE-C), elm yellows (EY1), loofah witches'-broom (LfWB), pigeon pea witches'-broom (PPWB), apple proliferation (AP), stolbur (STOL), Mexican periwinkle virescence (MPV), and Bermuda grass white leaf (BGWL) (16). Each reaction was performed in 0.5 ml PCR tubes, in a final volume of 25 µl of reaction mixture as previously described (14). PCRs were carried out for 35 cycles using the following conditions: 1 min (3 min for first cycle) denaturation at 94°C, annealing for 2 min at 55°C, and primer extension for 3 min (10 min in final cycle) at 72°C. Negative controls consisted of reaction mixtures devoid of templates. PCR products were analyzed by agarose gel electrophoresis, staining with ethidium bromide, and visualization of DNA bands using a UV transilluminator. DNA fragment size standard was 1 kb ladder (Life Technologies, Gaithersburg, MD).

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GenBank accession numbers of DNA sequences: AF175304, AF176319, AF189214, AF189215, AF248956, AF248957, AF248958, AF248959, AF248960, AF248961.

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RFLP analyses of amplified DNA.

DNA amplicons from PCR primed by P1/P7 and from nested PCR primed by F2n/R2 were analyzed by single restriction endonuclease digestion with *AluI* (Life Technologies, Gaithersburg, MD), *BfaI*, *HpaII*, *MseI*, *Sau3AI*, *ThaI*, *TaqI*, and *Tsp509I* (New England Biolabs, Beverly, MA), following instructions provided by the manufacturers. The products of digestion were analyzed by electrophoresis through a 5% polyacrylamide gel followed by staining with ethidium bromide and visualization of DNA bands with UV transilluminator. DNA fragment size standard used was PhiX174 RF *HaeIII* digest (Life Technologies). Group and subgroup affiliations are designated according to the 16S rDNA-based classification system of Lee et al. (16).

Cloning of PCR products and sequence analysis of FD phytoplasma rDNA. A 1.85 kbp FD70 phytoplasma DNA sequence that had been amplified in PCR primed by P1/P7 was cloned in *Escherichia coli* using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. A cloned DNA fragment (termed FD70#1) was selected for nucleotide sequencing by use of automated DNA sequencing of both strands. The complete 1.85 kb sequence was assembled after 2X to 5X sequencing coverage for each base position. The nucleotide sequence determined in this study was deposited in the GenBank data library under accession number AF176319. For comparisons, a previously reported nucleotide sequence of DNA from the rRNA operon of a FD phytoplasma was obtained from GenBank (accession number X76560). Sequence alignments were generated and sequence similarities evaluated by using the DNASTAR program Align option (DNASTAR Inc., Madison, WI). Putative restriction site maps were generated by using the DNASTAR program MapDraw option.

Cloned rDNA sequences used as standards. To provide standards useful for comparisons of enzymatic RFLP patterns of rDNAs, we prepared DNAs from known control strains of phytoplasmas. DNA sequences were amplified from rRNA operons of AshY1, CYE = CYE-C, EY1, LfWB, PPWB, APU, STOL, MPV, and BGWL phytoplasmas by the use of PCRs primed by primer pair P1/P7. The amplified 1.8-kbp phytoplasma DNA sequences were then cloned, sequenced, and analyzed for putative restriction sites as described above. Putative restriction sites were examined for agreement with sites determined by enzymatic digestion of uncloned rDNA and of the cloned rDNA from each control phytoplasma. The nucleotide sequences of the cloned DNA standards were deposited in the GenBank data library; their GenBank accession numbers are given in Table 1

along with GenBank numbers of other phytoplasma, *Acholeplasma laidlawii*, and *A. modicum* 16S rDNA sequences used in this study.

Sequence alignment and phylogenetic analysis. 16S rRNA gene sequences from FD phytoplasma (strain FD70) and 25 other phytoplasma strains representing fifteen 16S rRNA phytoplasma groups (16), *Acholeplasma laidlawii*, and *A. modicum* were aligned using DNASTAR to identify nucleotide sequences characteristic of group 16SrV phytoplasmas. A second alignment of 16S rRNA gene sequences (1.2 kb in size, representing the sequence between annealing sites of primer pair F2n/R2 [F2n/R2 segment]) from FD phytoplasma (strain FD70) and 20 other phytoplasma strains representing twelve 16S rRNA phytoplasma groups (*sensu* ref. 16), and *Acholeplasma laidlawii* was produced using computer program ClustalX version 1.63b (28). Gaps and incomplete sequences were removed. A phylogenetic tree was constructed by the Neighbor-Joining method option in ClustalX, and the tree was viewed by using TreeViewPPC (22). *A. laidlawii* was selected as the outgroup to root the tree. Bootstrapping was performed 1000 times for estimation of stability and support for the clades.

RESULTS

Cloned rDNA standards. The cloned 1.8 kbp rDNAs from AshY1, CYE = CYE-C, EY1, LfWB, PPWB, APU, STOL, MPV, and BGWL phytoplasmas were sequenced and the sequences deposited in the GenBank database (Table 1). For each phytoplasma sequence, the DNA fragment sizes expected, on the basis of analysis of putative restriction sites in the F2n-R2 segment, were in excellent agreement with the fragment sizes that were obtained by enzymatic RFLP analysis of cloned and uncloned rDNAs (data not shown).

RFLP analysis of rDNA amplified from phytoplasma strain FD70. PCR primed by F2n/R2 was used to amplify a fragment of 16S rDNA from grapevine FD phytoplasma strain FD70. The resulting 1.2 kbp product was subjected to RFLP analysis (data not shown). Comparisons of the RFLP patterns with published RFLP patterns of other phytoplasma 16S rDNAs indicated that strain FD70 is a member of group 16SrV (elm yellows phytoplasma group, 16), subgroup C (FD phytoplasma subgroup), in agreement with previous classification of an FD-associated phytoplasma (16) (Table 2).

DNA was then amplified from strain FD70 in PCR primed by primer pair P1/P7,

Table 1. GenBank numbers of 16S rRNA gene sequences used in this study

Phytoplasma (strain)	16S rRNA group affiliation ^a	GenBank accession no.	Reference
Aster yellows (MIAY)	16SrI	M30970	18
Peanut witches' broom (PnWB)	16SrII	L33765	15
Witches' broom disease of lime (WBDL) ("Candidatus Phytoplasma aurantifolia")	16SrII	U15442	32
Western X-disease (WX)	16SrIII	L04682	26
Virginia grapevine yellows (VGYIII)	16SrIII	AF060875	11
Clover yellow edge (CYE, CYE-C)	16SrIII	AF175304	This paper
Coconut lethal yellowing (LY)	16SrIV	U18747	29
Elm yellows (EY1)	16SrV	AF189214	This paper
Elm yellows (WVEY)	16SrV	AF122911	13
Elm yellows (ULW)	16SrV	X68376	27
<i>Flavescence dorée</i>	16SrV	X76560	26
<i>Flavescence dorée</i> (FD70)	16SrV	AF176319	This paper
Alder yellows (AldY = ALY)	16SrV	Y16387	26
Hemp dogbane phytoplasma (HD1)	16SrV	AF122912	13
<i>Rubus</i> stunt (RS)	16SrV	Y16395	26
Clover proliferation (CP)	16SrVI	L33761 ^b	15
Ash Yellows (AshY) ("Candidatus Phytoplasma fraxini")	16SrVII	AF189215	This paper
Loofah witches' broom (LFWB)	16SrVIII	AF248956	This paper
Pigeon pea witches' broom (PPWB)	16SrIX	AF248957	This paper
Apple proliferation (APU)	16SrX	AF248958	This paper
Rice yellow dwarf (RYD)	16SrXI	D12581	21
Stolbur (STOL)	16SrXII	AF248959	This paper
Australian grapevine yellows (AUSGY) ("Candidatus Phytoplasma australiense")	16SrXII	L76865	10
Mexican periwinkle virescence (MPV)	16SrXIII	AF248960	This paper
Bermudagrass white leaf (BGWL)	16SrXIV	AF248961	This paper
Hibiscus witches' broom (HibWB) ("Candidatus Phytoplasma brasiliense")	16SrXV	AF147708	See note added in proof
<i>Acholeplasma laidlawii</i>	N. A. ^c	M23932	30
<i>A. modicum</i>	N.A.	M23933	30

^a Group assignments are based on RFLP analysis of DNA sequences corresponding to fragments amplified in PCRs primed by oligonucleotide pair R16F2n/R16R2.

^b For analyses in the present work, sequence L33761 was modified by insertion of corrected bases in place of n's in the original GenBank sequence.

^c N. A. = not applicable.

and the 1.85 kbp product was cloned in *E. coli*. Further analyses were carried out with cloned insert FD70#1. For analysis, this cloned insert was amplified from the recombinant plasmid in PCR primed by P1/P7, and the 1.85 kbp product was subjected to enzymatic RFLP analysis. The RFLP patterns of the cloned rDNA were indistin-

guishable from those of (uncloned) PCR products (rDNA) generated from template DNA extracted from infected plant tissue (Fig. 1). These results verified the identity of cloned FD70#1 rDNA as originating from FD phytoplasma strain FD70.

Nucleotide sequence analysis of cloned FD70 rDNA. The nucleotide se-

quence of clone FD70#1 rDNA was determined and deposited in the GenBank data library under accession number AF176319. A map of putative restriction sites in sequence AF176319 (Fig. 2; some data not shown) was in excellent agreement with the RFLP patterns obtained from enzymatic RFLP analysis of rDNA PCR products and of cloned rDNA fragment FD70#1. These data, and the agreement in multiple coverage of positions in the nucleotide sequencing of both strands of the DNA, indicated that the nucleotide sequence AF176319 accurately reflects the sequence of the FD70 phytoplasma rDNA. Alignment of FD70 sequence AF176319 with FD sequence X76560 revealed that the two sequences differed at 11 positions, including eight base substitutions.

Putative restriction site analysis and revised subgroup classification. Sequence AF176319 (from FD phytoplasma FD70) and the FD phytoplasma sequence X76560 were subjected to putative restriction site analysis (Fig. 2). Comparison of putative restriction sites revealed several differences between the two sequences. For example, within the sequence corresponding to that amplified in PCR primed by primer pair F2n/R2 (segment F2n-R2), the putative restriction site map for sequence X76560 contained a site for *HpaII* that was absent in sequence AF176319. Sequence AF176319 (and enzymatically digested rDNA of strain FD70, as noted above) contained a *ThaI* site and a *Tsp509I* site that were absent in sequence X76560. Since these sites are within the F2n-R2 segment of the 16S rRNA gene (the sequence on which group and subgroup classifications are assigned in the system of Lee et al. [16]), the data are consistent with the concept that strain FD70 and the X76560 phytoplasma belong to two different subgroups in group 16SrV. Since FD phytoplasma FD70 is assigned to subgroup V-C (16; this paper), we assign the phytoplasma that was the source of sequence X76560 to a new subgroup, subgroup V-D (Table 2). The difference between the two sequences also included the presence, outside of the F2n-R2 segment, of a *MseI* site in the 16S rRNA gene sequence of X76560 that was not present in sequence AF176319 (Fig. 2).

The restriction site maps for sequences AF176319 and X76560 were compared with those of 16S rDNA sequences obtained from GenBank for EY1, Rubus stunt, alder yellows, and hemp dogbane yellows (HD1) phytoplasmas (Fig. 2). On the basis of putative restriction sites in the sequences corresponding to that amplified in PCR primed by F2n/R2 (F2n-R2 segment), alder yellows and HD1 phytoplasmas are classified in subgroup V-C, in agreement with previous work (13,16) (Table 2). The sequence from Rubus stunt phytoplasma was distinguished from the other sequences analyzed by the presence

Table 2. Subgroup classification of several phytoplasmas belonging to group 16SrV (elm yellows phytoplasma group)^a

Phytoplasma strain, geographic origin	Classification based on	
	RFLP analysis of F2n-R2 rDNA ^a	Putative restriction site analysis ^b
Elm yellows (EY1), New York State	A (16)	A (this paper)
<i>Flavescence dorée</i> (FD70), France	C (16)	C (this paper)
Alder yellows (AldY = ALY), Germany	Not reported	C (16)
Hemp dogbane phytoplasma (HD1), New York State	C (13)	C (this paper)
<i>Flavescence dorée</i> (FD X76560), France	Not reported	D (this paper)
Rubus stunt (RS), Italy	C (16)	E (this paper)

^a Subgroup classifications are based on RFLP analysis by enzymatic digestion of 1.2 kbp rDNA sequences corresponding to the DNA fragment (F2n-R2 segment) amplified in PCR primed by primer pair R16F2n/R16R2, according to the classification system of Lee et al. (16). Reference for the subgroup classification is given in parentheses.

^b Putative restriction site analysis of nucleotide sequences of the F2n-R2 segment of 16S rDNA. New subgroup classifications are in **bold** type. Reference for the subgroup classification is given in parentheses.

Fig. 1. Restriction fragment length polymorphism (RFLP) analysis of rDNA amplified in polymerase chain reaction (PCR) primed by oligonucleotide pair P1/P7 from *flavescence dorée* (FD) phytoplasma strain FD70. DNA products from PCRs were digested with restriction endonucleases *AluI*, *BfaI*, *TaqI*, *Tsp509I*, *HpaII*, *MseI*, and *ThaI*. D, DNA amplified from *V. faba* infected by FD phytoplasma strain FD70. C, DNA amplified from cloned rDNA of FD phytoplasma strain FD70. S, PhiX174 *HaeIII* digest size standard, fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

of a site for *Tsp509I* in the F2n-R2 segment of the rDNA (Fig. 2), indicating that this phytoplasma, although previously classified in subgroup V-C, represents a separate subgroup, *sensu* Lee et al. (16). Therefore, on this basis we tentatively reclassify the *Rubus* stunt phytoplasma in a new subgroup, designated subgroup E (V-E) in group 16SrV, until the presence of the

differentiating *Tsp509I* site can be verified (Table 2). The absence of one site for *BfaI* in the F2n/R2 segment from EY1 distinguished subgroup V-A phytoplasmas from other subgroups in group 16SrV, as noted previously (16; this paper).

Comparison of putative restriction sites outside of the F2n-R2 segment also differentiated FD70 from hemp dogbane (HD1)

phytoplasma (absence of one site each for *AluI* and *Tsp509I* and presence of one additional site each for *Sau3AI* [data not shown] and *Tsp509I* in HD1), from alder yellows (AldY = ALY) (absence of one site for *Tsp509I* in AldY), and from elm yellows EY1 (presence of an additional site for *Tsp509I*, corresponding to the additional site in HD1 noted above, and a miss-

Fig. 2. Analysis of putative restriction sites in rRNA operon sequences from phytoplasmas belonging to group 16SrV (elm yellows phytoplasma group). The sequences correspond to fragments amplified in polymerase chain reactions (PCRs) primed by primer pair P1/P7. Maps were generated using the MapDraw option of the DNASTAR program (DNASTAR) and manually aligned for comparison of recognition sites for restriction nucleases. GenBank accession numbers of the sequences are in parentheses. Solid bar represents the fragment of DNA amplified in PCR primed by F2n/R2. Arrows indicate putative restriction sites that differentiate sequence AF176319 from the other sequences.

ing *TaqI* site in EY1 that is present in all other group 16SrV sequences analyzed) (Fig. 2). Analysis of cloned rDNA from EY1 showed the presence of a *Sau3A1* (data not shown) that was not observed in the enzymatic RFLP analysis, possibly due to a polymerase error or due to the cloning of a sequence variant in the EY1 population.

Characteristic sequences in the rRNA operon of group 16SrV phytoplasmas. Nucleotide sequences characteristic of phytoplasmas classified in group 16SrV were determined by alignment of the nucleotide sequences from *A. laidlawii*, *A. modicum*, and phytoplasmas classified in 15 groups listed in Table 1. All group 16SrV characteristic sequences were present in phytoplasmas classified in subgroups V-C, V-D, and V-E (Table 3). For example, the characteristic sequence 5'-gtaac-3', which was present in positions 828-832 of FD70 sequence AF176319, was also found in alder yellows (Y16387), hemp dogbane (HD1), FD (X76560), and Rubus stunt (Y16395) phytoplasma 16S

rRNA gene sequences. This sequence distinguished FD70 and phytoplasmas in subgroups V-C, V-D, and V-E from phytoplasmas classified in other subgroups in group 16SrV, as well as from phytoplasmas in other groups and from the two *Acholeplasma* species used in this study. Within group 16SrV, the characteristic sequence 5'-ctagttg-3' (positions 1100-1106) was present in 16S rRNA gene sequences in subgroup V-C (flavescence dorée FD70 [AF176319], alder yellows [Y16387], and hemp dogbane [AF122912] phytoplasmas), subgroup V-D (FD [X76560] phytoplasma), and subgroup V-E (Rubus stunt [Y16395] phytoplasma) but not in 16S rDNA from elm yellows phytoplasma (subgroup V-A). Interestingly, this sequence, which contains a site for *BfaI* that distinguishes subgroups V-C, V-D, and V-E from subgroup V-A, is found in other phytoplasmas, including Bermuda grass leaf phytoplasma (acc. no. AF248961) and several other phytoplasmas not classified in group 16SrV (data not shown), and in *A. modicum*. In addition, sequence AF176319

contains the sequence for an *AluI* site (5'-agct-3') that is present in all, and only in, members of group 16SrV among phytoplasmas but that is also present in equivalent position in *A. modicum* (see Table 3). All of the group 16SrV characteristic sequences found in sequence AF176319 from phytoplasma strain FD70 were also found in FD phytoplasma sequence X76560.

Sequence similarities. As noted above, the rDNA sequence AF176319 from phytoplasma FD70 (this paper) and the sequence X76560, also from an FD phytoplasma (27), differed from each other in putative restriction sites. Therefore, for further comparisons we aligned the regions of these sequences, and of 16S rDNA sequences from other group 16SrV phytoplasmas, that corresponded to fragments amplified in PCR primed by F2n/R2 (F2n-R2 segments). Sequence similarities among the group 16SrV phytoplasmas ranged from 98.3 to 100% (Table 4). Interestingly, HD1 and AldY share 99.0% 16S rDNA sequence similarity and are classified in the same subgroup, whereas, EY1 and FD70 share 99.8% sequence similarity but are classified in different subgroups. The 16S rDNA sequence similarity between phytoplasma FD70 and phytoplasma X76560 was 99.3%. This level of sequence similarity is consistent with the classification of both the phytoplasma (FD70) and the X76560 FD phytoplasma in the same 16S rRNA group. The sequence similarity between phytoplasma FD70 and alder yellows phytoplasma was 100%, indicating that the FD70 and alder yellows phytoplasmas are closely related to one another.

Phylogenetic analysis. Phylogenetic analysis of 21 phytoplasmas and *A. laidlawii* yielded the consensus tree illustrated in Figure 3. This tree is in good agreement with trees constructed recently. The bootstrapping values strongly supported the branches, indicating a robust tree whose branching order is in general agreement with previous findings (10,15,16,27,31). Strains from phytoplasmas belonging to 12 groups were included for comparison with phytoplasma FD70. In agreement with classification of phytoplasmas into groups and subgroups based on RFLP profiles of 16S rDNA (16), the phylogenetic analysis confirmed the close relatedness among the phytoplasma strains in group 16SrV and emphasized the concept that they form a coherent group. However, the subgroups in group 16SrV were not well resolved by this phylogenetic tree, which is based on analysis of 16S rRNA gene sequences.

DISCUSSION

This communication presents properties of rDNA that was amplified and cloned from phytoplasma strain FD70. This strain is one of two (FD70 and FD88) that were originally isolated from yellows-diseased cultivated grapevines in southwestern France and maintained in *V. faba* through

Table 3. Characteristic sequences in 16S rDNA of group 16SrV phytoplasmas^a

Sequence position ^b	Nucleotide sequence
828-832	5'-gtaac-3' ^c
1100-1106	5'-ctagttg-3' ^d
1271-1283	5'-ggtagtctcagta-3' ^e
253-274	5'-gaggtaaaggcttaccagatt-3' ^f
138-149	5'-atcggaacggt-3' ^g
1314-1317	5'-agct-3' ^g

^a Characteristic sequences in the 16S rRNA gene.

^b Position in sequence AF176319.

^c Sequence present in 16S rRNA genes of subgroup V-C [*Flavescence dorée* FD70 (AF176319), alder yellows (Y16387), and hemp dogbane (AF122912)], subgroup V-D [FD (X76560)], and subgroup V-E [Rubus stunt (Y16395)] phytoplasmas and absent from 16S rRNA genes of other phytoplasmas in group 16SrV, as well as from phytoplasmas in other groups and the two *Acholeplasma* species used in this study.

^d Sequence present in subgroups V-C, V-D, and V-E, distinguishing them from other subgroups in group 16SrV. This sequence is also found in Bermuda grass whiteleaf phytoplasma, in other non-group 16SrV phytoplasmas, and in *A. modicum*.

^e Sequence present in subgroups V-A, V-C, V-D, and V-E in group 16SrV, and not found in other organisms in this study.

^f Sequence specific to, and found only in, group 16SrV phytoplasmas among organisms in this study.

^g *AluI* site unique to group 16SrV among phytoplasmas in this study, but also present in *Acholeplasma modicum*.

Table 4. Sequence similarities among 16S rDNAs from phytoplasmas classified in group 16SrV (elm yellows phytoplasma group)^a

Phytoplasma (strain) [subgroup]	Sequence similarity (%) with indicated phytoplasma DNA				
	X76560	AldY	HD1	RS	EY1 ^b
<i>Flavescence dorée</i> (FD70) [V-C]	99.3	100	99.0	99.4	99.8
<i>Flavescence dorée</i> (X76560) [V-D]		99.3	98.3	98.7	99.0
Alder yellows (AldY) [V-C]			99.0	99.4	99.8
Hemp dogbane (HD1) [V-C]				98.5	98.8
Rubus stunt (RS) [V-E]					99.2

^a Sequence similarities of 1.2-kbp sequences of 16S rDNA were determined using the Align option of DNASTAR program. The 1.2-kbp sequences extend from the annealing site of polymerase chain reaction (PCR) primer R16F2n through the annealing site of PCR primer R16R2.

^b EY1 is a member of group V, subgroup A (V-A).

transmission by the leafhopper *E. variegatus* (8). The two strains were previously differentiated from one another by Southern hybridization analysis and by RFLP analysis of a DNA sequence amplified in PCR primed by chromosomal DNA primer pair FD9f/r (9). To our knowledge, nucleotide sequences of the rRNA operons of strains FD70 and FD88 have not been compared. Nevertheless, previous work indicates that strain FD70 fits established biological definitions of FD phytoplasma (5,8). The present work is the first to present the nucleotide sequence of a segment of the rRNA operon from phytoplasma FD70. On the basis of RFLP patterns and putative restriction sites in rDNA sequences, phytoplasma FD70, as well as alder yellows and hemp dogbane yellows (HD1) phytoplasmas, belong to rRNA RFLP subgroup C in group 16SrV (subgroup V-C).

Sequence X76560, derived from an FD phytoplasma (27), contains the group 16SrV characteristic sequences that were found in sequences AF176319, AF122912,

and Y16387, which were derived from the subgroup V-C phytoplasmas (strain FD70, hemp dogbane, and alder yellows phytoplasmas, respectively) (Table 3). However, the putative restriction site analysis of the FD sequence X76560 indicated that the phytoplasma source of this DNA is not a member of subgroup V-C, the subgroup which contains strain FD70. Therefore, we classified the X76560 phytoplasma in a new 16S rDNA subgroup, subgroup V-D. Similarly, sequence Y163954 from *Rubus* stunt phytoplasma also contains the group 16SrV characteristic sequences found in sequence AF176319, but it is tentatively classified in a separate subgroup (V-E) on the basis of putative restriction site analysis of sequence Y16395 (this paper). Phytoplasmas belonging to subgroups V-C, V-D, and V-E could be distinguished from one another on the basis of putative restriction sites in the F2n-R2 segment, but they could not be distinguished on the basis of group 16SrV characteristic sequences that are present in 16S rDNA. Thus, sequences from other regions may be more useful in

distinguishing separate species that may comprise these subgroups. Notably, the 16S-23S spacer region in sequence AF122912 from hemp dogbane phytoplasma (HD1) distinguished this subgroup V-C phytoplasma from others that are classified in the 16S rRNA subgroup V-C, as well as in subgroup V-D, indicating that the HD1 phytoplasma may belong to a taxon different from that containing FD phytoplasma strains.

The phylogenetic tree underscored the close relationship among the group 16SrV phytoplasmas, a close relatedness which was indicated also by the high levels of 16S rDNA sequence similarities and by the characteristic sequences shared by the group 16SrV phytoplasmas. Although the tree did not well resolve subgroups classified in group 16SrV, it is possible that alder yellows and FD70 phytoplasmas represent a lineage that is distinct from those of *Rubus* stunt, elm yellows, hemp dogbane, and FD X76560 phytoplasmas, and that hemp dogbane phytoplasma is distinct from FD70 and other subgroup V-C phytoplas-

Fig. 3. Phylogenetic tree constructed by the Neighbor-Joining Method of 16S rRNA gene sequences from 21 phytoplasmas and *Achleplasma laidlawii*, employing *A. laidlawii* as the outgroup. The numbers on the branches are bootstrap (confidence) values. 16S rRNA RFLP group affiliations are given in Roman numerals. Subgroup affiliations for group V phytoplasmas are given in brackets.

mas, as well as from FD X76560 phytoplasma. Additional analyses incorporating other conserved genes, such as ribosomal protein genes, should help to clarify the phylogeny of group 16SrV phytoplasmas, and aid in the development of a molecular definition of FD phytoplasma and the eventual naming of taxa in group 16SrV.

The differences between strain FD70 and the X76560 FD phytoplasma in 16S rDNA are important, since both have been reported as strains of FD phytoplasma (8,27). The differences in restriction sites and the level of nucleotide sequence similarity are consistent with classification of the FD70 and X76560 phytoplasmas as members of two distinct subgroups in group 16SrV, as designated above. The data thus indicate that two different group 16SrV phytoplasmas are associated with symptoms characteristic of FD disease of grapevines.

It is apparent from other work also that at least two different group 16SrV phytoplasmas are associated with symptoms of FD in grapevines in Europe (1,8,9,20). Bianco et al. (1) found that two different phytoplasmas belonging to group 16SrV were present in diseased grapevines in Italy, and that these phytoplasmas could be distinguished by *Bfa*I RFLP analysis of 16S rDNA amplified in the PCR. According to criteria previously established by Lee et al. (16,17), the differing *Bfa*I RFLP patterns indicated that the phytoplasmas reported by Bianco et al. (1) belonged to two separate, undesigned subgroups in group 16SrV. Daire et al. (9) were able to distinguish strains FD70 and FD88 of FD phytoplasma by RFLP analysis of a non-ribosomal DNA fragment amplified from the phytoplasmas in PCRs, but they did not classify these strains according to 16S rRNA group/subgroup affiliations.

Other studies have distinguished among group 16SrV phytoplasmas on the basis of analysis of rDNA. In one study, Marcone et al. (19), using seven restriction enzymes, resolved three groups among phytoplasmas related to elm yellows phytoplasma, although they were not placed in the context of the classification system of Lee et al. (16). In another study, Martini et al. (20) determined that diseased grapevines exhibiting symptoms of FD disease in Italy were infected by distinct group 16SrV phytoplasmas which they classified in two different subgroups. Their subgroup classification is different from the classification system of Lee et al. (16) used in our work, since it was based on a restriction site polymorphism outside of the F2n-R2 segment of 16S rDNA. Thus, the subgroups described by Martini et al. (20) are not equivalent to the subgroups described in the present communication. However, we interpret the published *Bfa*I RFLP patterns to indicate a possibility that all of the group 16SrV strains examined by Martini et al. (20) could be classified in subgroup

V-C (16). None of the group 16SrV phytoplasma 16S rDNA sequences used in our study contained the *Taq*I site used by Martini et al. (20) to differentiate two FD phytoplasmas in Italy. Interestingly, only one of the group 16SrV phytoplasmas reported by Martini et al. (20) was found in individuals of the known FD insect vector, *S. titanus*, emphasizing that rDNA polymorphisms outside, as well as inside, the F2n-R2 segment may signal biologically significant differences among closely related phytoplasmas.

In order to understand and manage the grapevine yellows problem, it is important to clearly establish the identities of phytoplasmas involved in outbreaks of this complex disease. The recent findings related to FD (9,20; this paper) underscore this need. For example, the epidemiology of grapevine yellows associated with phytoplasmas belonging to subgroup V-D (this paper) may differ from that associated with subgroup V-C strains. Indeed, different insect vectors may be important in the spread of group V phytoplasma strains infecting grapevine. The data presented here and elsewhere (1,9,20) emphasize the importance of reexamining the role of *S. titanus* in the spread of FD-associated phytoplasmas, and of learning whether other insect species are involved in the spread of group V phytoplasmas in *V. vinifera*. The use of appropriate molecular criteria and accurate data to identify the pathogens should facilitate this task.

NOTE ADDED IN PROOF

Since acceptance of this report, the following paper has been published (see Table 1): Montano, H. G., Davis, R. E., Dally, E. L., Hogenhout, S., Pimentel, J. P., Brisso, P. S. T. 2001. "*Candidatus* Phytoplasma brasiliense," a new phytoplasma taxon associated with hibiscus witches' broom disease. *Int. J. Syst. Evol. Microbiol.* 51:1109-1118.

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