

Sequence Heterogeneity in the Two 16S rRNA Genes of *Phormium* Yellow Leaf Phytoplasma

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***Phormium* yellow leaf (PYL) phytoplasma causes a lethal disease of the monocotyledon, New Zealand flax (*Phormium tenax*). The 16S rRNA genes of PYL phytoplasma were amplified from infected flax by PCR and cloned, and the nucleotide sequences were determined. DNA sequencing and Southern hybridization analysis of genomic DNA indicated the presence of two copies of the 16S rRNA gene. The two 16S rRNA genes exhibited sequence heterogeneity in 4 nucleotide positions and could be distinguished by the restriction enzymes *Bpm*I and *Bsr*I. This is the first record in which sequence heterogeneity in the 16S rRNA genes of a phytoplasma has been determined by sequence analysis. A phylogenetic tree based on 16S rRNA gene sequences showed that PYL phytoplasma is most closely related to the stolbur and German grapevine yellows phytoplasmas, which form the stolbur subgroup of the aster yellows group. This phylogenetic position of PYL phytoplasma was supported by 16S/23S spacer region sequence data.**

Phytoplasmas (formerly called mycoplasma-like organisms) are wall-less prokaryotes of the class *Mollicutes* that cause diseases in several hundred plant species (24). They occur in phloem cells of the host plant and are transmitted by phloem-feeding insects, especially leafhoppers. They replicate in both their insect and plant hosts. Characterization of these pathogens has been difficult because they cannot be cultured in vitro. Traditionally, phytoplasmas were differentiated on the basis of biological properties such as disease symptoms on plants, plant host range, and insect vector specificity. Such procedures are time-consuming and often subjective and unreliable. Substantial progress has been made in recent years towards improved differentiation and classification of phytoplasmas by the application of serological tests (5, 19) and molecular methods including nucleic acid hybridization and restriction fragment length polymorphism (RFLP) analysis of chromosomal DNA (16–18, 32).

While antibodies and DNA probes are suitable for differentiating phytoplasmas which belong to the same group, they are not able to determine the phylogenetic or taxonomic positions of all phytoplasmas in relation to each other and to other microorganisms. In contrast, the 16S rRNA gene is widely used in phylogenetic and taxonomic studies of prokaryotes because it has both highly conserved and sufficiently variable regions (37). Various classification schemes for phytoplasmas based on restriction site and sequence analysis of the 16S rRNA gene have been proposed. Several major groups and subgroups (RFLP groups) of related phytoplasmas have been identified by restriction analysis of the 16S rRNA gene (20, 29). Sequence analysis of the 16S rRNA gene divided the phytoplasmas into five (33) and then six (30) major phylogenetic groups, which were divided further into 11 subclades by Gundersen et al. (12). There are close parallels between the classification schemes based on RFLP and sequence analysis of the 16S rRNA gene.

The 16S/23S rRNA intergenic region has also been used as a phylogenetic marker for phytoplasmas (15). This region contains a highly conserved tRNA^{Leu} flanked by variable sequences. Kirkpatrick et al. (15) defined 12 phytoplasma groups based on 16S/23S spacer region sequences which agree closely with the 16S rRNA groups.

Phormium yellow leaf (PYL) phytoplasma causes a lethal disease of the large tufted monocotyledon New Zealand flax (*Phormium tenax*) and was a contributor to the eventual demise of a locally important natural fiber industry based on this species. PYL was first recognized over 80 years ago, but it was not until 1969, when Ushiyama et al. (35) observed phytoplasmas in the phloem of diseased plants and in the leafhopper vector (*Oliarus atkinsoni*) by electron microscopy, that the etiology of this disease was determined. PYL phytoplasma causes abnormal yellowing of leaves, stunted growth, and premature flowering in *P. tenax* (4). Other symptoms include degeneration of vascular bundles in the rhizome, root death, and rhizome rotting (4).

We have determined the phylogenetic position of PYL phytoplasma by sequencing the 16S rRNA genes and the 16S/23S spacer regions. Both DNA sequence analysis and Southern hybridization of the rRNA genes of PYL phytoplasma revealed the presence of two operons which exhibited sequence heterogeneity.

MATERIALS AND METHODS

Plant material. Healthy and PYL-diseased *P. tenax* plants were collected from three geographically distinct locations in New Zealand (Auckland, Turangi, and Wellington). The apical rhizome tissue was cut into small pieces and stored at –80°C.

Maryland aster yellows (AY1), New York elm yellows (EY1), beet leafhopper-transmitted virescence (VR), Canada peach X (CX), and tomato big bud (BB) phytoplasmas kindly provided by R. E. Davis, Beltsville, Md., were maintained in a greenhouse by periodic grafting onto a white-flowered clone of periwinkle (*Catharanthus roseus*).

DNA extraction. DNA for use in PCR was extracted by an intact phytoplasma enrichment procedure (11), which was modified slightly for PYL phytoplasma in that the flax rhizome tissue was ground in liquid nitrogen prior to addition of the isolation medium. The final pellet from 5 g of tissue was resuspended in 200 μ l of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA [pH 8.0]).

DNA for Southern blot hybridizations was prepared by a modification of the cetyltrimethylammonium bromide (CTAB) method (1). Flax rhizome tissue (10

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TABLE 1. Position and sequences of primers used for sequencing the 16S rRNA gene of *PYL* phytoplasma

Primer	Strand ^a	Sequence (5'-3')	Position ^b
m1	F	GAAGGCATCTTTTATC	181-197
m2	F	GGGAAGAAAAGATGG	450-464
ng	F	AGGCGGCTTGCTGGTCTT	725-743
r4	F	GGGTTAAGTCCCGCAACGAG	1073-1092
r5	R	CTGCTGCCTCCCGTAGGAGT	358-339
gr	R	AAGACCCAGCAAGCCGCTT	743-725
r8	R	CTCGTTGCGGGACTTAACCC	1092-1073
nr	R	AGCCATTGTATCACGTT	1231-1215

^a F, forward; R, reverse.

^b All positions correspond to positions in the sequence of OAY phytoplasma (21).

g) was ground in liquid nitrogen with a mortar and pestle. The powder was transferred to a 30-ml tube and mixed with 25 ml of isolation medium (100 mM Na₂HPO₄, 10% sucrose, and 2% polyvinylpyrrolidone, pH 7.6, to which 0.15% bovine serum albumin and 1 mM ascorbic acid were added just before use). The homogenate was centrifuged at 4°C for 5 min at 1,500 × g. The supernatant was decanted and recentrifuged at 4°C for 25 min at 18,000 × g. The pellet was resuspended in 15 ml of hot (60°C) CTAB buffer (5% CTAB, 100 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 1.4 M NaCl, 1% polyvinylpyrrolidone) and was incubated at 60°C for 30 min. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol). After centrifugation, the aqueous layer was precipitated with 2/3 volume of ice-cold isopropanol, incubated at -20°C for 1 h, and then centrifuged at 10,000 × g for 15 min. The pellet was washed with ice-cold 70% ethanol, dried under a vacuum, and resuspended in 200 μl of TE buffer.

PCR. Primer pairs P1/P6 and P1/NP6A were used to amplify the 16S rRNA gene of *PYL* phytoplasma. Primers P1 and P6, designed by Deng and Hiruki (6), bind to positions 6 to 30 and 1484 to 1509 of the 16S rRNA gene, respectively. Primer NP6A (5'-TTA CGA CTT AAC CCC AA-3'), designed in our laboratory, binds to positions 1477 to 1493 near the 3' end of the 16S rRNA gene.

Primers NP6AF and P7 were used to amplify the 16S/23S spacer region. The forward primer NP6AF (5'-TTG GGG TTA AGT CGT AA-3') is complementary to primer NP6A. Primer P7 (5'-CGT CCT TCA TCG GCT CTT-3'), kindly provided by C. Smart (University of California, Davis), binds to the 5' end of the 23S rRNA gene.

PCR amplification was performed with a 20-μl reaction mixture containing 4 μl (10 to 50 ng) of template DNA, 20 μM each primer, 200 μM deoxynucleoside triphosphates, 1.25 mM MgCl₂, and 1 U of *Taq* DNA polymerase with recommended PCR buffer (Amplitaq, Perkin-Elmer). Amplification involved an initial denaturation at 94°C for 2 min and then 25 cycles of 94°C for 30 s, 55°C for 15 s, and 72°C for 1 min in a Corbett FTS-1S Capillary Thermal Sequencer. PCR products were analyzed by electrophoresis through 1% agarose gels for 16S rRNA products or 2% agarose gels for 16S/23S spacer region products in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) buffer.

Restriction enzyme analysis of PCR products. PCR products (5 to 15 μl) of the 16S rRNA gene generated by the primers P1/NP6A or P1/P7 were digested with *Bpm*I or *Bsr*I according to the manufacturer's instructions (New England Biolabs) and were analyzed by electrophoresis through 1.5% agarose gels or 4% NuSieve GTG agarose gels.

Cloning of PCR products. PCR products were gel purified with the BresaClean Kit (Bresatec) and ligated into the pGEM-T Vector (Promega) according to the manufacturers' instructions. The ligation mixture was used to transform competent cells of *Escherichia coli* MC1022 or DH5α and plated on Luria-Bertani medium containing ampicillin, X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), and IPTG (isopropyl-β-D-thiogalactopyranoside) (28). White colonies were screened by PCR as follows: colonies were resuspended in 50 μl of sterile water, boiled for 5 min, and spun in a microcentrifuge for 2 min, and 4 μl of the supernatant was used in PCR with the same primers used to generate the insert. Amplification was performed in a Corbett FTS-1S Capillary Thermal Sequencer by using the same parameters as described above.

DNA sequencing. Plasmid DNA was prepared for sequencing by the alkaline lysis method and purified by CsCl-ethidium bromide density-gradient centrifugation as described by Sambrook et al. (28) or by using the Wizard Minipreps DNA purification system (Promega) according to the manufacturer's instructions. Both strands of each DNA fragment were sequenced with an Applied Biosystems model 373A sequencer using the *Taq* dideoxy terminator cycle-sequencing method. 16S rRNA genes were sequenced by using universal forward and reverse primers and the internal primers shown in Table 1. The primers m1, m2, ng, gr (complement of ng), and nr are PCR primers designed by us. The primers r4, r5, and r8 are 16S rRNA sequencing primers designed by D. Saul (Centre for Gene Technology, University of Auckland, Auckland, New Zealand). The 16S/23S spacer region was sequenced by using the universal forward

and reverse primers only. All nucleotide positions given in this paper correspond to the numbering of *Oenothera* aster yellows (OAY) phytoplasma (21).

Data analysis. Computer analyses of sequences were performed with the GCG 8.1 (Genetics Computer Group, Madison, Wis.) software package of Devereux et al. (7). Sequences were aligned by the algorithm PILEUP (10), and the 16S rRNA sequence of *Clostridium innocuum* was included as an outgroup sequence so that the phylogenetic tree could be rooted. The choice of alignment was clear-cut, and no corrections for secondary structure were required. Phylogenetic trees were constructed from the aligned sequences with the computer program PAUP 3.1.1 (34) by a heuristic search via simple stepwise addition with the tree bisection and reconnection branch-swapping algorithm to find the optimal phylogenetic trees. A total of 100 bootstrapped trees were sampled to determine a measure of the support for each clade on the tree.

Preparation of 16S probes. The full-length 16S rRNA probe was constructed by PCR with primers P1 and NP6A. PCR products were electrophoresed in a 1% agarose gel in 1× TBE buffer and were eluted from the gel with the BresaClean kit (Bresatec). 5' and 3' probes were prepared by digesting the full-length 16S probe with *Hind*III, which cleaves the 16S rRNA gene at position 1,255. The resulting fragments of 1,255 bp (5' probe) and 245 bp (3' probe) were separated through a 1.5% gel and were eluted separately with the BresaClean kit. Probes were labeled with [α-³²P]dCTP with the Rediprime labeling kit (Amersham) according to the manufacturer's instructions.

Southern blot hybridization. Total DNA extracted from healthy and *PYL*-diseased flax plants was digested with *Hind*III (BRL-Life Technologies), *Ase*I (New England Biolabs), or a combination of the two enzymes, at 37°C overnight. Digested DNA was electrophoresed in 1% agarose gels in 1× TBE buffer at 2 V/cm, depurinated with 0.25 M HCl for 15 min, denatured with 1.5 M NaCl-0.25 M NaOH for 30 min, and finally neutralized with 0.5 M Tris-1.5 M NaCl for 30 min. DNA was transferred onto nylon membranes (Hybond N⁺, Amersham) as described by Sambrook et al. (28). The membranes were baked at 80°C for 2 h and then prehybridized at 65°C for 3 h in 0.5 M Na₂HPO₄ (pH 7.2) with H₃PO₄, 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA. Labeled probes were added to the prehybridization solution and incubated at 65°C for at least 16 h. Posthybridization washes (30 min each) were carried out under highly stringent conditions consisting of two washes in 2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate)-0.1% SDS at room temperature; this was followed by two washes in 0.1× SSC-0.1% SDS at 68°C. The membranes were exposed to X-ray film (Agfa RP1) at -80°C by using intensifier screens.

Nucleotide sequence accession numbers. The nucleotide sequences of *PYL* phytoplasma have been deposited in the GenBank database under the following accession numbers: U43569, 16S *ma*A; U43570, 16S *mb*B; and U43571, 16S/23S spacer region. The GenBank accession number of the 16S rRNA sequence of *C. innocuum* is M23732. The GenBank accession numbers of the 16S rRNA sequences of phytoplasmas used to construct phylogenetic trees are shown in Table 2.

RESULTS

Amplification of 16S rRNA gene and 16S/23S spacer region.

The primer pair P1/P6 (6), designed to amplify virtually the entire 16S rRNA gene of phytoplasmas, produced amplification products from DNA samples of both diseased and healthy flax plants. We therefore used a modified primer, NP6A, which with P1 produced a 1.5-kb product only from diseased samples (Fig. 1A). Primer NP6A is very similar to the primer P6 (6), differing in that it is located 7 nucleotides 5' of P6. Primer NP6A binds to a region that is highly conserved among phytoplasmas, whereas primer P6 extends outside this conserved region into a slightly variable region.

The 16S/23S spacer region of *PYL* phytoplasma was amplified with primers NP6AF and P7, which yielded a product of approximately 325 bp (Fig. 1B). Using primers NP6AF and P7, we obtained products of approximately 350 bp for VR and BB, 360 bp for CX, 375 bp for AY1, and 385 bp for EY1 (Fig. 1B). No product was obtained from DNA samples of healthy plants or from minus-template controls.

Sequence analysis. Eight 16S rRNA clones of *PYL* phytoplasma representing two independent clones from each of four plants were sequenced. One plant was obtained from Auckland, one plant was Turangi, and two plants came from Wellington. Sequence heterogeneity was noted at four positions (Table 3). The eight sequences could be divided into two classes. In the first class (I), the variable nucleotides at positions 371, 543, 1159, and 1441 were (5' to 3') G, G, C, and A, respectively, whereas in the second class (II), the nucleotides

TABLE 2. Names and accession numbers of phytoplasma 16S rRNA sequences used to construct phylogenetic trees

Abbreviation	Full name and origin	Accession no.
ACLR	Apricot chlorotic leafroll, Spain	X68338
APS	Apple proliferation, Spain	X76426
ASHY	Ash yellows, USA	X68339
AY1	Maryland aster yellows, Maryland	L33767
BB	Tomato big bud, Arkansas	L33760
CP	Clover proliferation, Canada	L33761
CX	Canadian peach X-disease, Canada	L33733
EY	Elm yellows, New York	L33763
FBP	Faba bean phyllody, Sudan	X83432
FD	Flavescence dorée, France	X76560
LDT	Coconut lethal yellowing substrain Tanzanian lethal disease	X80117
LDY	Yucatan coconut lethal decline	U18753
LFWB	Loofah witches'-broom, Taiwan	L33764
LY	Coconut lethal yellowing	U18747
OAY	<i>Oenothera</i> aster yellows	M30790
OY	Onion yellows, Japan	D12569
PnWB	Peanut witches'-broom, Taiwan	L33765
PPER	European stone fruit yellows of peach, Germany	X68374
PPWB	Caribbean pigeon pea witches'-broom	U18763
RYD	Rice yellow dwarf, Japan	D12581
SCWL	Sugarcane white leaf, Thailand	X76432
STOL	Stolbur of pepper, Serbia	X76427
SUNHP	Sunn hemp or crotalaria witches'- broom, Thailand	X76433
TWB	Tsuwabuki witches'-broom, Japan	D12580
VAC	Vaccinium witches'-broom, Germany	X76430
VK	Grapevine yellows, Germany	X76428

were A, A, U, and G. Clones 1, 4, 5, and 8 were of class I, and clones 2, 3, 6, and 7 represented class II. Each of the four plants therefore contained a representative of each class of sequence. The variable positions were unlikely to be caused by PCR errors because of their nonrandom distribution. This 16S rRNA sequence heterogeneity suggests that infected *Phormium* plants contain either one strain with two distinct ribosomal operons or two strains of PYL phytoplasma.

The 16S rRNA sequence of PYL phytoplasma was manually aligned with the secondary structure of *Mycoplasma capricolum* (22). Variable position 371 occurred in the middle of a helical region (Fig. 2A). G (class I) was involved in correct base pairing whereas A (class II) disrupted base pairing and therefore weakened the stem. Position 543 occurred at the beginning of a stem and neither of the nucleotides (G or A) paired

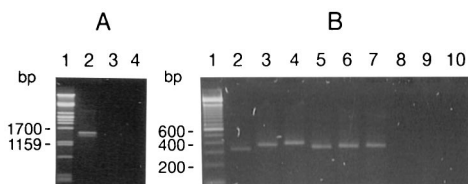


FIG. 1. (A) Agarose gel electrophoresis of 16S rRNA PCR products obtained with primers P1 and NP6A. Lane 1, *Pvu*I-digested λ DNA. Templates for PCR were as follows: lane 2, DNA from PYL-diseased flax; lane 3, DNA from healthy flax; lane 4, minus-template control. (B) Agarose gel electrophoresis of 16S/23S spacer region PCR products obtained with primers NP6AF and P7. Lane 1, 100-bp ladder (BRL-Life Technologies). Templates for PCR were as follows: lane 2, DNA from PYL-diseased flax; lanes 3 to 7, DNAs from AY1-, EY1-, VR-, CX-, and BB-infected periwinkle plants; lane 8, DNA from healthy flax; lane 9, DNA from healthy periwinkle; lane 10, minus-template control. Abbreviations for phytoplasmas are defined in Materials and Methods.

TABLE 3. Variable 16S rRNA positions between PYL phytoplasma clones

Position ^a	Plant 1 clones		Plant 2 clones		Plant 3 clones		Plant 4 clones	
	1	2	3	4	5	6	7	8
371	G	A	A	G	G	A	A	G
543	G	A	A	G	G	A	A	G
1159	C	U	U	C	C	U	U	C
1441	A	G	G	A	A	G	G	A

^a Numbers correspond to positions in the sequence of OAY phytoplasma (21).

(Fig. 2B). At position 1159, C (class I) paired correctly while U (class II) was involved in GU pairing (Fig. 2C), which is commonly found in the secondary structure of 16S rRNA. The variable position 1441 is either an A or a G and occurred in an unpaired region, that is, in a loop (Fig. 2D). Overall, we conclude that the four single-base differences do not significantly affect the 16S rRNA secondary structure.

The 16S/23S spacer region of PYL phytoplasma was found to be 224 bp long and to contain the same highly conserved tRNA^{Leu} of 76 nucleotides which occurs in 49 other phytoplasmas (15). The lengths of the other phytoplasma 16S/23S spacer regions vary from 189 to 282 bp (sequences kindly provided by C. Smart, University of California, Davis), which accounts for the different sizes of the PCR products for the phytoplasmas shown in Fig. 1B. Three independent clones of the 16S/23S spacer region of PYL phytoplasma were sequenced and were all identical.

Operon analysis. Several phytoplasmas have been shown to contain two rRNA operons (31). The number of ribosomal operons was determined for PYL phytoplasma by Southern blot hybridization (Fig. 3). Sequence data indicated that the 16S rRNA gene of PYL phytoplasma has one *Hind*III site but no *Ase*I sites. Three products of 6.5, 4, and 0.75 kb were detected when DNA from PYL-diseased plants was digested with *Hind*III and probed with the full-length 16S rRNA gene (Fig. 3A, lane 1). Both the 5' probe (which was produced by using a PCR product in the region upstream of the 16S rRNA *Hind*III site) and the 3' probe (downstream of the 16S rRNA *Hind*III site) hybridized with a 6.5-kb product. This indicated that the 6.5-kb band observed in lane 1 of Fig. 3A (by using the full-length 16S rRNA sequence as a probe) was a doublet, representing two distinct products which hybridized with both the 5' and 3' probes. In addition to the 6.5-kb band, the 5' probe also hybridized with a 4-kb product (Fig. 3B, lane 1), and the 3' probe hybridized with a 0.75-kb product (Fig. 3C, lane 1). These two products corresponded in size to the bands which hybridized with the full-length probe (Fig. 3A, lane 1). Two products of 4.9 and 3.8 kb hybridized with all three probes following digestion with *Ase*I (Fig. 3, lane 2). Three products of 3.2, 1.3, and 0.75 kb hybridized to the full-length probe from DNA digested with both *Hind*III and *Ase*I (Fig. 3A, lane 3). Products of 3.2 and 1.3 kb hybridized with the 5' probe (Fig. 3B, lane 3), and products of 3.2 and 0.75 kb hybridized with the 3' probe (Fig. 3C, lane 3). These results indicated that the 3.2-kb fragment seen in lane 3 of Fig. 3A was also a doublet. Additional faint bands were presumed to be the products of incomplete digestion. The 0.75-kb product shown in Fig. 3A, although not readily visible following photographic reproduction, was clearly visible on the autoradiograms. DNA extracted from healthy plants did not hybridize with any of the probes under the stringency conditions used (data not shown). These results suggest that PYL phytoplasma contains two copies of

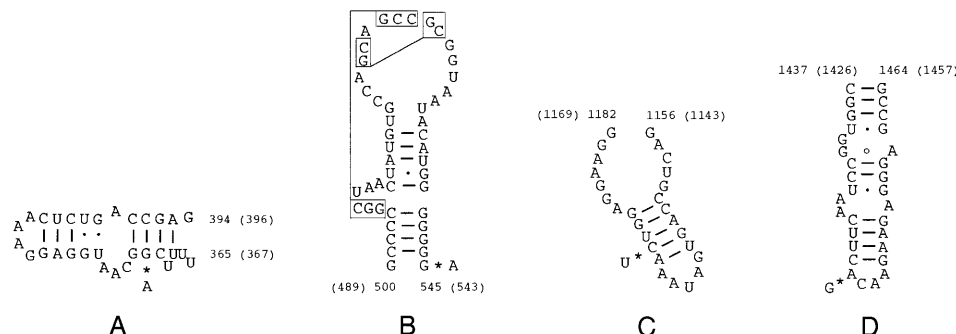


FIG. 2. Proposed secondary structure of the four variable regions (A to D) of PYL phytoplasma 16S rRNA adapted from that proposed for *M. capricolum* (23). Nucleotide differences between *rmA* and *rmB* (*), canonical (-), G · U (.), and A · G (o) base pairs are indicated. The nucleotide numbering corresponds to *E. coli* (13), with PYL phytoplasma numbering shown in parentheses.

the 16S rRNA gene. Therefore, it is assumed that the two classes of sequence correspond to the two operons of PYL phytoplasma. The two sequence classes I and II will be referred to as ribosomal operons *rmA* and *rmB*, respectively.

Computer analysis of PYL phytoplasma 16S rRNA sequences revealed that the restriction sites for *BpmI* and *BsrI* differed between the two operons. *BpmI* has a single recognition site at position 1179 in *rmA* but lacks a site in *rmB* (Fig. 4A, compare lanes 3 and 4). *BsrI* has recognition sites at positions 169, 839, and 1149 in both operons, with an extra recognition site at position 1163 in *rmA* (Fig. 4B, compare lanes 3 and 4). The extra fragment of 15 bp in *rmA* was too small to be visualized by gel electrophoresis, but the difference was observed by the size of the 3'-most fragment, which was reduced from 344 to 329 bp. Both 16S rRNA operons were amplified from genomic DNA, as shown by digestion of genomic DNA PCR products with *BpmI* and *BsrI*, which resulted in a combination of the bands produced by the *rmA* and *rmB* operons (lane 2 in Fig. 4A and B).

These restriction site differences were utilized to identify the 16S/23S spacer region of both operons prior to sequencing. The entire 16S rRNA gene, the spacer region, and part of the 23S rRNA gene of PYL phytoplasma were amplified by PCR with primers P1 and P7. Clones of the amplified fragment were digested with *BpmI* to distinguish the two operons. The 16S/23S spacer regions of three clones from each operon were sequenced with the primers NP6AF and universal reverse. This region did not show sequence heterogeneity.

Phylogenetic analysis of PYL phytoplasma. Phylogenetic analysis of 16S rRNA gene sequences of 27 phytoplasmas

identified 15 equally parsimonious trees. One of these trees, which corresponded to the strict consensus tree, is shown in Fig. 5. This tree corresponded to the trees constructed by Seemüller et al. (33) and Schneider et al. (30) and showed that PYL phytoplasma is most closely related to the stolbur and grapevine yellows phytoplasmas. These three phytoplasmas form a subgroup of the aster yellows group which was designated the stolbur subgroup by Seemüller et al. (33).

Another phylogenetic tree was constructed on the basis of 16S/23S spacer region sequences kindly provided by C. Smart. The phylogenetic position of PYL was the same as that shown by 16S rRNA sequence data (data not shown).

To determine the effect of 16S rRNA sequence variation on phylogeny, two trees were constructed by using both classes of PYL phytoplasma sequences. The 4 nucleotide differences had no effect on the phylogenetic position of PYL phytoplasma.

DISCUSSION

There has been little study of the phytoplasma associated with PYL since its first report in 1969 (35), in part because it has not been transmitted to a more amenable host such as *C. roseus*. Over the past few years many other phytoplasmas have been classified by using restriction site and sequence analysis of the 16S rRNA gene (12, 20, 29, 33). In this approach, the 16S rRNA genes are amplified by PCR, which has facilitated the

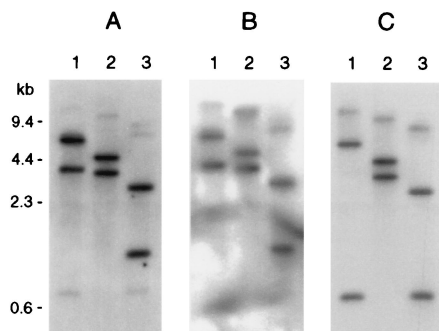


FIG. 3. Southern blot analysis of DNA extracted from a PYL-diseased plant digested with *HindIII* (lane 1), *AseI* (lane 2), and *HindIII-AseI* (lane 3). Blots were probed with the full-length 16S rRNA gene (A), the 5' region of the 16S rRNA gene (B), and the 3' region of the 16S rRNA gene (C).

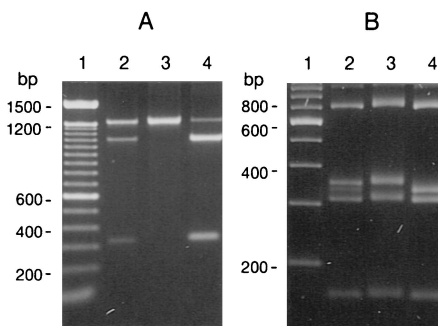


FIG. 4. Analysis of the ribosomal operons of PYL phytoplasma by restriction enzyme digestion. PCR products of the 16S rRNA gene generated by the primers P1/NP6A were digested with *BpmI* and separated through a 1.5% agarose gel (A) or digested with *BsrI* and separated through a 4% NuSieve GTG agarose gel (B). Lane 1, 100-bp ladder (BRL-Life Technologies). Templates for PCR were as follows lane 2, genomic DNA; lane 3, *rmB* operon plasmid DNA; and lane 4, *rmA* operon plasmid DNA. The uppermost band in lane 4 of panel A represents undigested DNA and the bands above the 800-bp marker in lanes 2 to 4 of panel B represent partially digested DNA.

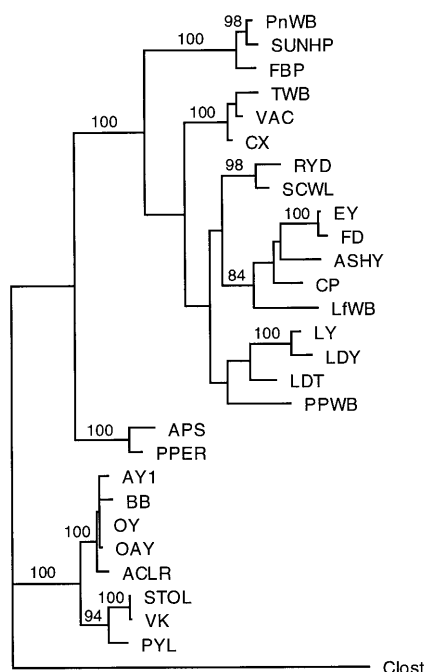


FIG. 5. Phylogenetic tree constructed by parsimony analysis comparing 16S rRNA gene sequences of PYL phytoplasma with other phytoplasmas obtained from GenBank, with *C. immitium* (Clost) as the outgroup. Branch lengths are proportional to number of inferred character state transformations. The numbers above the branches are bootstrap values obtained for 100 replicates (only values greater than 80% are shown). Abbreviations for phytoplasmas are defined in Table 2.

characterization of phytoplasmas which occur in low titers, as in the case of PYL phytoplasma.

In this paper, we have concluded that the two types of 16S rRNA sequence, which differ in 4 nucleotide positions, represent the two ribosomal operons of PYL phytoplasma. An alternative explanation, that there are two phytoplasma strains present in the same plant, seems unlikely. The isolation of both types of sequence from all four plants collected from three geographically separate locations suggests that the sequence variation represents the two operons rather than two strains, as not all plants would be expected to be infected by a mixed phytoplasma population. By coincidence, one clone from each plant was of class I (*rnmA*) and the other clone was of class II (*rnmB*). When sequence was obtained across the variable positions of a further six clones from three plants (two clones from each plant), five clones were of class I and one clone was of class II (data not shown).

Members of the class *Mollicutes* have been shown to have only one or two copies of rRNA genes, compared with 8 to 10 copies in most bacteria (27). The acholeplasmas and phytoplasmas have two rRNA operons, while the spiroplasmas and mycoplasmas have either one or two copies (2, 31). It has widely been assumed that the rRNA sequences are identical or nearly identical from operon to operon. However, in only a few examples has the nucleotide sequence of more than one rRNA gene from the genome of an organism been determined. The three rRNA sequences of the bacterium *Rhodobacter sphaeroides* were identical to one another (8), whereas the archaeobacterium *Haloarcula marismortui* had two nonadjacent rRNA operons which differed at 5% of the positions (25). In most instances, however, the sequences between rRNA operons differed at less than 0.5% of the nucleotide positions (9, 14, 26,

36). Interoperon sequence variation has also been shown to occur in members of the class *Mollicutes*. Partial sequence analysis of the two 16S rRNA genes of *Mycoplasma* sp. strain F38 revealed that 1.6% of the nucleotide positions differed (3). Therefore, the finding that the two rRNA genes of PYL phytoplasma differed in four (0.27%) of the nucleotide positions was not unexpected, although PYL is the first phytoplasma for which sequence heterogeneity in its 16S rRNA genes has been demonstrated by sequence analysis. Sequence heterogeneity in the 16S rRNA gene could also explain the results of RFLP analysis of the clover phyllody phytoplasma (20).

The level of sequence similarity between the two 16S rRNA genes (99.73%) in one strain is comparable to the level of homology between different phytoplasma strains. For example, the typical aster yellows strains, American aster yellows, Western aster yellows, and OAY, exhibit levels of homology of 99.46 to 99.74% (33). Because phytoplasmas as a group show such a high level of sequence homology, it is important to compare sequences corresponding to genes from the corresponding operon (3) or compare data obtained from direct sequencing of PCR products (9), as has been done for the majority of the phytoplasma 16S rRNA gene sequences.

Comprehensive phylogenetic trees based on phytoplasma 16S rRNA gene sequences have been constructed by Gundersen et al. (12) and Seemüller et al. (33). The latter tree has since been updated by Schneider et al. (30). These trees correspond well to each other except that different phytoplasmas have been represented, which resulted in slightly different phylogenetic groupings. Seemüller et al. (33) divided the phytoplasmas into the following five primary clusters: (i) AY; (ii) AP; (iii) WX; (iv) SCWL; and (v) EY. The FBP strain cluster was subsequently added as group vi (30). Five major phylogenetic groups which were divided into 11 distinct subclades were recognized by Gundersen et al. (12). The subclades were designated as follows: (i) AY1; (ii) AP; (iii) PnWB; (iv) CX; (v) RYD; (vi) PPWB; (vii) LY; (viii) ASHY; (ix) CP; (x) EY; and (xi) LfWB.

The phylogenetic tree presented in this paper can be divided into the same groupings proposed by Gundersen et al. (12) and Schneider et al. (30), but additional groups can be recognized due to the different phytoplasmas being represented. Following the groupings proposed by Schneider et al. (30), the phytoplasmas causing LY, LDY, LDT, and PPWB form a seventh new primary cluster (see Table 2 for definitions). Two new subclades were formed according to the classification scheme of Gundersen et al. (12). Continuing with the same numbering, these subclades and representative phytoplasmas are proposed as follows: (xii) LDT and (xiii) STOL, VK, and PYL. These groupings will continue to evolve as more phytoplasma 16S rRNA genes are sequenced.

PYL phytoplasma clusters with the stolbur and German grapevine yellows (Vergilbungskrankheit [VK]) phytoplasmas. However it is not as closely related to these two phytoplasmas as they are to each other. The stolbur and grapevine yellows phytoplasmas have been considered to be strains of the same organism by Maixner et al. (23) because of their occurrence in the same weed species, their transmission by the same leafhopper, and a 16S rRNA sequence homology of 99.8%. PYL phytoplasma was 98% (*rnmA*) (or 98.21%, *rnmB*) and 97.94% (or 98.15%) homologous to the stolbur and the VK phytoplasmas, respectively. Maixner et al. (23) designed PCR primers which specifically amplify the stolbur and VK phytoplasmas. These primers are unlikely to amplify PYL phytoplasma due to the fact that although the forward primer matches the PYL phytoplasma sequence exactly, the reverse primer has four mismatches at the 3' end. The branching pattern on the phyloge-

netic tree also indicated that PYL does not share the close relationship between STOL and VK in that it formed a separate branch from these other two phytoplasmas.

PYL phytoplasma adds to the small number of phytoplasmas which have been isolated and sequenced from monocotyledon hosts. Of the 40 or so phytoplasma 16S rRNA gene sequences which are now available, only 7 are from monocotyledon hosts. These are LDT, LDY, LY, OY, PYL, RYD, and SCWL (see Table 2 for definitions). Except for OY, these phytoplasmas have not been transmitted to an experimental host, indicating possible host and/or insect specificity. There is no correlation between the monocotyledonous nature of the host and its phylogenetic grouping.

The 16S rRNA gene is forming a basis for classifying phytoplasmas by restriction site or sequence analysis. Overall, sequence heterogeneity between the two ribosomal operons does not appear to affect this classification scheme. Many phytoplasmas have been characterized by RFLP analysis of the 16S rRNA gene by using a range of common restriction enzymes with both operons yielding the same restriction patterns, clover phyllody phytoplasma digested with *AluI* being the exception (20). The 0.27% interoperon sequence variation between the 16S rRNA genes of PYL phytoplasma did not affect its phylogenetic classification by sequence analysis probably because the closest relatives of PYL phytoplasma were sufficiently heterogeneous. However, operon heterogeneity may pose a problem for determining the relationships between phytoplasmas which belong to the same group, for example, the aster yellows group, where the members are more than 99% homologous. Therefore, comparison of 16S/23S spacer region sequence data, which has greater variability than the 16S rRNA gene between different phytoplasmas, is a useful genetic marker to support the phylogenetic groupings of phytoplasmas determined by 16S rRNA sequences.

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