Identification and Characterization of Phytoplasmal Genes, Employing a Novel Method of Isolating Phytoplasmal Genomic DNA

Sharon Melamed,¹ Edna Tanne,² Raz Ben-Haim,¹ Orit Edelbaum,¹ David Yogev,³ and Ilan Sela^{1*}

Institute for Plant Sciences, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100,¹ The Volcani Center, Agricultural Research Organization, Bet Dagan 50250,² and Department of Membrane Research, The Institute of Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem 91120,³ Israel

Received 14 July 2003/Accepted 18 August 2003

Phytoplasmas are unculturable, insect-transmissible plant pathogens belonging to the class *Mollicutes*. To be transmitted, the phytoplasmas replicate in the insect body and are delivered to the insect's salivary glands, from where they are injected into the recipient plant. Because phytoplasmas cannot be cultured, any attempt to recover phytoplasmal DNA from infected plants or insects has resulted in preparations with a large background of host DNA. Thus, studies of the phytoplasmal genome have been greatly hampered, and aside from the rRNA genes, only a few genes have hitherto been isolated and characterized. We developed a unique method to obtain host-free phytoplasmal genomic DNA from the insect vector's saliva, and we demonstrated the feasibility of this method by isolating and characterizing 78 new putative phytoplasmal open reading frames and their deduced proteins. Based on the newly accumulated information on phytoplasmal genes, preliminary characteristics of the phytoplasmal genome are discussed.

Phytoplasmas are a group of plant-pathogenic, phloem-restricted bacteria belonging to the class *Mollicutes* which represents the smallest self-replicating life forms on earth and are characterized by a lack of firm cell walls and remarkably small (580- to 1,350-kb), AT-rich genomes (40). Phytoplasmas are the causal agents of diseases in hundreds of plants (1, 22, 31) and are transmitted from plant to plant by grafting and other vegetative propagation techniques and by specific phloem-feeding insects, especially leafhoppers, planthoppers, and psyllids (31). Many diseases caused by phytoplasmas are of great economic importance, in particular those of trees, perennials, and high-value vegetable crops (1).

Since the phytoplasma is restricted to the phloem tissue, its concentration in infected plants is very low. Furthermore, in contrast to mycoplasmas, phytoplasmas cannot be cultured in vitro. Therefore, phytoplasmal DNA is obtained with a high host (plant or insect) background, hampering attempts to study structural features and genome organization. In the last 15 years, some progress has been made towards enabling the enrichment of phytoplasmal fractions from extracts of infected plants and insects (4, 20, 21, 42, 47, 48). In some cases, it became possible to produce phytoplasma-specific polyclonal and monoclonal antibodies, greatly facilitating diagnosis (6, 9). However, as detailed below, only a few phytoplasmal genes have been characterized. Currently, phytoplasma detection and characterization are based predominantly on PCR amplification of rRNA genes (rDNAs), especially the 16S rDNA (1, 11, 15, 19, 25, 48, 51).

The preferred methods for phytoplasma classification have become restriction fragment length polymorphism (RFLP) analysis and sequence analysis of rDNA. Sequence analysis of

16S rDNA revealed that phytoplasmas form a monophyletic clade within the mycoplasma phylogenetic tree. This method also allowed, for the first time, a comprehensive phytoplasma classification as the basis for an emerging taxonomy. Under the provisional status *Candidatus*, six species have been defined to date (16, 19, 26, 28, 49, 50, 55).

Extraction and purification of phytoplasmal DNA are critical and essential steps towards understanding the molecular biology of these unique microorganisms. Since the source of phytoplasmal genomic DNA is an infected plant or insect (and not cultured bacterial cells), resulting in a high background of host DNA, a preparation containing sufficient amount of phytoplasma DNA representing the entire phytoplasma genome was critically sought. At least two groups have made considerable, yet unpublished, progress in isolating and characterizing the phytoplasmal genome of the Western X phytoplasma (L. Leifiting and B. Kirkpatrick, Abstr. 14th Int. Congr. Int. Org. Mycoplasmol., abstr. 29, p. 27, 2002) and the apple proliferation phytoplasma (E. Seemuller, personal communication). However, only a few phytoplasmal non-tRNA or non-rRNA genes have been hitherto described in published papers (3–5, 10, 16, 18–20, 27, 28, 30, 33, 34, 37, 46, 54, 57); additional, as-yet-unpublished genes can be found in the GenBank database.

Recently, for the purposes of demonstrating the vector's ability to transmit phytoplasma, we devised a method of collecting insect saliva and showed, by PCR analysis of rRNA genes, that phytoplasmal DNA can be easily detected in that saliva (53). Based on those findings, we describe a novel approach to isolating phytoplasmas and their genomic DNAs and validate it by isolating and characterizing 78 new putative phytoplasmal open reading frames (ORFs).

MATERIALS AND METHODS

Insects as a source of phytoplasmas. Leafhoppers (*Orosius albicinctus*) were collected from and around phytoplasma-infested vineyards and were character-

* Corresponding author. Mailing address: The Hebrew University, Faculty of Agricultural, Food and Environmental Quality Sciences, Virus Laboratory, Rehovot 76100, Israel. Phone: 972-8-9489377. Fax: 972-8-9473402. E-mail: sela@agri.huji.ac.il.

TABLE 1. Primers used for PCR in this study.

Clone	Primer sequence (5' to 3')	Position	Annealing temp (°C)	Product length (bp)
A380	TCAACCCAATGTCGTATAATCG	373–394	62	500
	AACGGAGTTGAACCGCTTAAGG	873–852	62	
A182	GGGGAATACTTTAATTTCTGCA	169–190	60	
	TCCAACCGCTTCTAAAACTG	775–756	60	416
A182, nested	GGAACCTCTTAGGGAAGATAA	269–290	58	
	GTGCTCCACAATATTTGGCT	695–676	58	
A10	TACAGCTGGACCTTCTTCT TA	360–381	56	727
	TGCACAAGGTGTAGCATATTC	1245–1225	56	
A10, nested	CAGCACGTTGAGGAAATCTAA	461–481	58	
	GCCTATAACTACTCCCAAAACAA	1188–1167	58	1,034
A244	AGAAGATGCTGGACGTACAGAA	136–157	54	
	CATTACCATAGCGACCTGAC	1330–1311	54	
A244, nested	ATAAAAGCAGGGGCAACAGTAA	191–212	56	
	GTAATTCCAAGTTCAGGACCAA	1224–1203	56	

ized as carrying the aster yellows (AY)-type phytoplasma by PCR analysis of 16S rDNA sequences (25, 51). Samples of phytoplasma-carrying and phytoplasma-free leafhoppers were kept frozen for future validation assays.

Isolation of phytoplasma fractions from insect saliva. We adapted a method of phytoplasma isolation from artificial feeding medium (J. Zhang, S. Miller, C. Hoy, X. Zhou, and L. Nault, *Phytopathology* 88, abstr. S84, 1998), described by Tanne et al. (53), whereby phytoplasmas are isolated from the insect vector's saliva and their genomic DNA is extracted. White microcentrifuge tubes (1.5 ml) were used as insect chambers. The white tube caps were removed and replaced with insect-attracting yellow ones. The cylindrical cups in the yellow caps were filled with 200 µl of 5% sucrose in TE (10 mM Tris [pH 8.0], 1 mM EDTA) and sealed with Parafilm. The microcentrifuge tubes were then capped with the yellow caps carrying the sucrose-TE feeding medium. The bottom ends of the microcentrifuge tubes were cut, an individual insect was placed in each, and the cut end was sealed with cotton wool. Each tube, containing an individual leafhopper, was kept at 23 to 25°C for 48 to 72 h in a horizontal position with the cap facing a light source to attract the insects to the feeding medium.

Isolation of phytoplasma genomic DNA. Phytoplasma particles were pelleted out of the feeding solution by centrifugation at 12,000 × *g* for 15 min. Genomic DNA was extracted by adding 10 µl of 0.5 M NaOH, followed by the addition of 20 µl of 1 M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate and 20 mM EDTA. The mixture was incubated at 65°C for 15 min, precipitated with 2 volumes of ethanol, redissolved in 30 µl of TE, and kept at –80°C.

Molecular typing of phytoplasma DNA. A sample of the isolated phytoplasma DNA was subjected to PCR analysis with various type-specific 16S rDNA primers. The DNA reacted positively only with the AY-specific primers (data not shown). Phytoplasma typing results were corroborated by RFLP of the PCR amplicon with four distinct restriction enzymes (26, 36) (data not shown).

Construction of a phytoplasma genomic library. DNAs from 100 individual feeding tubes were pooled for the construction of a genomic library. The DNA was cleaved with the restriction enzyme *EcoRI* and ligated into the phagemid Lambda-Zap Express (Stratagene). Ligation and packaging were performed according to the manufacturer's protocol. About 5,000 plaques were obtained in this process, and blue-white selection indicated about 70% insert-carrying recombinant clones. Plasmids were excised from the phagemids by the addition of a helper phage as directed by the manufacturer. The recombinant plasmids were purified, and the nucleotide sequences of their inserts were determined by using the dideoxy chain termination method (44) with the automatic dye-terminator cycle sequencing model ABI PRISMA 377 sequencer (Perkin-Elmer, Foster City, Calif.). Sequence data were analyzed by using the BlastN, BlastX, and ORF finder programs from the National Center for Biotechnology Information and the FASTA program from the Genetics Computer Group.

Southern and Northern blot analyses. Total genomic DNA was extracted from plants as described by Maixner et al. (29). Leaf tissue was ground in extraction buffer (100 mM Tris-HCl [pH 8.0], 3% cetyl trimethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 0.2% 2-mercaptoethanol) at a tissue/buffer ratio of 1:5 (wt/vol). The slurry was incubated for 20 min at 65°C and then centrifuged for 10 min at 3,000 × *g*. The supernatant fluid was collected and extracted with an equal volume of chloroform-isoamylalcohol (24:1, vol/vol), followed by centrifugation, collection of the aqueous phase, and precipitation with 1 volume of isopropanol. The DNA pellet was washed with 70% ethanol, resuspended in 50 µl of 10 mM

Tris-HCl (pH 8.0)–1 mM EDTA. Total cell RNA was extracted from plant leaves with Tri reagent (Sigma) according to the manufacturer's protocol.

DNA was cleaved with the restriction enzyme *HindIII*, electrophoresed, and blotted onto nitrocellulose membranes. RNA was electrophoresed in formamide-formaldehyde gels and blotted onto membranes. Southern and Northern blot procedures were carried out by standard protocols (43).

Probes for the Southern and Northern blot hybridizations were prepared from selected clones with the Prime a Gene labeling kit (Promega). Due to the high A-T content of the phytoplasma DNA, hybridization was carried out at 56°C, and the membranes were washed at medium stringency, i.e., at 56°C in 0.5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2]).

PCR. Primers were designed from the nucleotide sequences of clones A10, A182, A244, and A380. The nucleotide sequences of the primers are shown in Table 1. Plant or leafhopper DNA (100 ng) served as a template. The reaction mixture was incubated at 95°C for 10 min and then subjected to 40 PCR cycles (denaturation at 92°C for 30 s, annealing for 30 s, and elongation and 72°C for 30 s), followed by 7 min of incubation at 72°C. The available amount of leafhopper DNA extracted from a single insect was scarce. To increase sensitivity and specificity, a nested PCR step was added to the analysis of DNA from several insects. For nested PCR, a sample from the primary PCR was diluted 1:100 and 2 µl of it served as a template for the nested PCR stage. PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

RESULTS

Characterization of phytoplasma clones. A collection of 32 recombinant phytoplasma clones, carrying inserts of about 300 to 4,000 kb in size, were subjected to sequence analysis. The information obtained is summarized in Table 2. These clones carry 78 putative ORFs. All but one (the gene for the elongation factor Tu) of the presently reported ORFs are new and have not hitherto been reported for phytoplasmas. The full nucleotide sequences of the various clones have been submitted to the GenBank database, and their accession numbers are given in Table 2.

Codon usage table. Phytoplasmas were designated mycoplasma-like organisms due to their comparatively small size, lack of a cell wall, and being intracellular parasites. On the other hand, it was suggested that phytoplasmas are closer to acholeplasmas than to mycoplasmas. This designation was made on the basis of analysis of only a single phytoplasma operon (28) and two ribosomal protein genes (33). Acholeplasmas use the general codon usage table, while mycoplasmas use the mitochondrial codon usage table, in which the stop codon UGA is read as tryptophan (41). We have translated the obtained phytoplasma sequences according to both codon usage

TABLE 2. Summary of the characterized phytoplasmal (AY) clones^a

Clone information					Blast results			
Clone	GenBank accession no.	% GC	ORF no.	Base position and orientation ^b	Homologous protein	Organism	% Identity	% Similarity
A1	AY191277	54	1 2 3	89–988 + 153–1067 + 982–350 –	Hypothetical protein ^c Hypothetical protein Hypothetical protein			
A2	AY191278	36	1 2 3 4 5 6 7 8 9 10 11 12	605–315 – 648–818 + 1309–1127 – 1592–1443 – 1640–1822 + 2248–1901 – 2428–2261 – 2460–2636 + 2850–3200 + 3172–2996 – 3107–3370 + 3497–3823 +	Conserved hypothetical protein Hypothetical protein Hypothetical protein gtf2ird2 Hypothetical protein IpaD Hypothetical protein Similar to hornerin Putative dipeptidase protein Hypothetical protein Hypothetical protein agCP3554	<i>Helicobacter pylori</i> 26695 <i>Mus musculus</i> <i>Plasmodium falciparum</i> 3D7 <i>Shigella sonnei</i> <i>Mus musculus</i> <i>Sinorhizobium meliloti</i> <i>Anopheles gambiae</i>	26 40 46 31 40 50 51	47 59 64 58 52 56 69
A10	AY191279	24	1 2	2–238 + 1502–240 –	rRNA methylase Threonine dehydratase	<i>Thermoanaerobacter tengcongensis</i> <i>Bacillus halodurans</i>	40 56	60 75
A93	AY191280	25	1	214–1 –	NADH dehydrogenase 1	Mitochondrion of <i>Flexarida chaotica</i>	79	91
A115	AY191281	23	1 2 3 4 5	268–1 – 69–350 + 326–628 + 908–661 – 665–909 +	Hypothetical protein EF-G rpS10 Hypothetical protein rpL3	<i>Porphyromonas gingivalis</i> <i>Leptospira interrogans</i> <i>Thermoanaerobacter tengcongensis</i>	58 59 59	83 79 78
A167	AY191282	38	1 2	725–1 – 774–451 –	DNA polymerase Putative DNA polymerase	Mitochondrion of <i>Neurospora intermedia</i> Mitochondrion of <i>Porphyra</i> sp.	32 30	48 55
A182	AY191283	26	1 2 3	56–400 + 413–934 + 939–1105 +	rpL18 rpS5 rpL15	<i>Bacillus stremophilus</i> <i>Shewanella oneidensis</i> MR-1 <i>Neisseria meningitidis</i> MC58	45 46 76	57 64 85
A226	AY191284	29	1	230–1 –	Cytochrome <i>c</i> oxidase polypeptide III	Mitochondrion of <i>Spodoptera frugiperda</i>	67	77
A239	AY191285	24	1 2 3	8–361 + 349–609 + 600–1634 +	Acetohydroxy acid synthase II Hypothetical protein Ketol-acid reductoisomerase, mitochondrial precursor	<i>Escherichia coli</i> O157:H7 EDL933 <i>Neurospora crassa</i>	35 49	48 66
A244	AY191286	27	1 2	2–871 + 872–1885 +	2-Isopropylmalate synthase 3-Isopropylmalate dehydratase large subunit	<i>Aquifex aeolicus</i> <i>Oceanobacillus iheyensis</i>	53 49	71 68
A276	AY191287	34	1	78–164 +	Surface protein, putative	<i>Shewanella oneidensis</i> MR-1	39	58
A290	AY191288	18	1 2 3	95–493 + 450–647 + 651–963 +	Signal peptidase 1 rpS21 Shikimate 5-dehydrogenase	<i>Chlorobium tepidum</i> TLS <i>Myxococcus xanthus</i> <i>Clostridium perfringens</i>	38 44 36	57 68 63
A293	AY217338	33	1 2 3	617–378 – 629–883 + 1214–702 –	Hypothetical protein Hypothetical protein RNA polymerase I	<i>Plasmodium falciparum</i> 3D7	44	67
A294	AY191289	30	1	133–2 –	Putative transport protein	<i>Escherichia coli</i>	39	51
A295	AY1912290	34	1 2	188–1 – 13–210 +	Hypothetical protein Putative <i>N</i> -acetyltransferase Camello 4	<i>Leptospira interrogans</i> serovar Lai strain 56601 <i>Mus musculus</i>	29 31	52 54
A314	AY191291	25	1	5–981 +	NADH dehydrogenase subunit 5	Mitochondrion of <i>Pyrocoelia rufa</i>	51	66
A315	AY191292	48	1	128–2 –	Integrase protein	<i>Salmonella enterica</i> serovar Typhimurium	54	62
A328	AY191293	30	1	106–1197 +	Hypothetical protein			
A352	AY217339	33	1 2 3 4 5 3	1314–1084 – 1231–1551 + 1343–1510 + 1886–2071 + 2664–2825 + 508–359 –	Caspase-9 long chain Outer capsid protein Hypothetical protein Hypothetical protein Hypothetical protein Hypothetical protein	Mouse Porcine group C rotavirus	50 40	62 65

Continued on following page

TABLE 2—Continued

Clone information					Blast results			
Clone	GenBank accession no.	% GC	ORF no.	Base position and orientation ^b	Homologous protein	Organism	% Identity	% Similarity
A370	AY217340	40	1	534–1 –	Reverse transcriptase	<i>Picea glauca</i>	49	69
			2	1250–375 –	Putative Gag-Pol polyprotein	<i>Oryza sativa</i>	37	56
			3	1639–1328 –	Putative copia-type polyprotein	<i>Oryza sativa</i>	51	64
A380	AY191294	23	1	2–172 +	Cytochrome oxidase subunit III	Mitochondrion of <i>Manis tetradactyla</i>	45	60
			2	225–599 (+)	NADH dehydrogenase subunit 3	<i>Anopheles gambiae</i>	64	85
			3	1302–709(–)	Cyclophilin D	<i>Dictyostelium discoideum</i>	40	56
A424	AY191295	29	1	309–476 +	Hypothetical protein			
			2	322–471 +	Hypothetical protein			
A427	AY191296	22	1	2–160 +	Threonyl-tRNA synthetase	<i>Buchnera aphidicola</i> strain sg	92	94
A440	AY191297	29	1	4–429 +	Elongation factor Tu	<i>Cellulophaga lytica</i>	76	88
			2	652–891 +	rpL11	<i>Chlamydia muridarum</i>	63	86
A448	AY191298	22	1	194–1 –	rpS8	<i>Listeria innocuus</i>	62	81
			2	200–723 +	rpL6	<i>Oceanobacillus iheyensis</i>	43	60
A449	AY191299	31	1	743–1012 +	Hypothetical protein			
A450	AY191300	39	1	175–2(–)	Conserved hypothetical protein	<i>Agrobacterium tumefaciens</i>	33	53
			2	215–18 –	Nef attachable protein	<i>Homo sapiens</i>	38	58
A458	AY191301	45	1	222–1 –	Yjc1 protein	<i>Bacillus halodurans</i>	33	52
A463	AY191302	32	1	357–37 –	DNA-adenine methyltransferase	<i>Actinobacillus actinomycetemcomitans</i>	28	46
			2	269–436 +	Hypothetical protein			
			3	542–282 –	Hypothetical protein			
			4	530–781 +	Retroelement	<i>Tricholoma matsutake</i>	27	44
A526	AY217341	29	1	129–28 –	Fusolin	Nucleopolyhedrovirus of <i>Autographia californica</i>	48	68
A533	AY217342	34	1	655–825 +	Hypothetical protein			
			2	855–1061 –	ATPase subunit 6	Kinetoplast of <i>Trypanosoma brucei</i>	31	45
A540	AY217343	41	1	1–256 +	agCP1945	<i>Anopheles gambiae</i>	34	49

^a Sequences of over 50 amino acids lacking an initial methionine or a stop codon (usually ORFs at the ends of the clones) are also included.

^b The orientation from the T7 promoter end of the clone is arbitrarily designated +.

^c A sequence of at least 50 amino acids with no match by BlastX is termed a hypothetical protein.

tables. Applying the criteria for ORF definition as described below, we concluded that phytoplasmas read UGA as tryptophan. This was based on the following observations. (i) The general codon usage table generated 62 ORFs, compared to 78 ORFs generated by the mycoplasma codon usage table. For example, ORF 9 of clone A2, a homologue of a bacterial gene, would have disappeared if translated according to the general codon usage table, due to insertion of four stop codons. (ii) In several cases, alignments made with the homologous genes resulted in continuous stretches of aligned sequences when translated with the mycoplasma codon usage table, while the translation according to the general codon usage table resulted in only fragmented alignments (Fig. 1, top panel). (iii) Similarly, ORFs obtained by using the general codon usage table were shorter than those obtained by using the mycoplasma codon usage table. The latter continued to align with the same homologous gene, indicating that the reading of UGA as a stop codon interrupted the reading of the gene within its coding region (Fig. 1, bottom panel). Phytoplasmal ORFs were thus defined throughout this work according to the codon usage table of mycoplasmas-mitochondria. It should be noted that all of the phytoplasmal ribosomal protein genes reported above did not carry in-frame UGA codons, and therefore translation

FIG. 1. Schematic illustration of the translation of three phytoplasmal clones according to the mycoplasma-mitochondrion codon usage table and to the general codon usage table.

FIG. 2. Southern blot analysis of periwinkle DNA. Chromosomal periwinkle DNA (12 μ g) was digested with *Hind*III restriction enzyme, subjected to Southern blot hybridization, and probed with 32 P-labeled clone A1 (A) or with a 670-bp PCR product representing a conserved region of the tubulin gene (B). The size of the hybridization band is indicated on the left. The exposure time was 3 weeks (A) or 3 days (B). DNA was from healthy plants (lanes 1 and 2), from a Stolbur-infected plant (lanes 3), or from an AY-infected plant (lanes 4).

by either codon usage table resulted in the same deduced protein sequences.

Homology search. Initially, a BlastX homology search was performed for each cloned insert to find similarity to proteins in the GenBank database. At a later stage, ORFs were defined by ORF Finder, and each individual ORF was searched for homologies by using Blast and BlastX. Homology of the phytoplasma ORFs to known proteins is indicated in Table 2, provided that the level of identity is over 25%. ORFs with no homology to any known protein, or with indicated similarity below 25%, are included in Table 2 only if they represent a string of at least 50 continuous amino acids. These ORFs were designated as coding for hypothetical proteins. If the highest BlastX score was for a putative or uncharacterized gene while similarity to a defined gene was noted at a somewhat lesser score, the ORF was designated as being similar to that of the defined gene.

Analysis of the phytoplasma ORFs revealed that 12 ORFs code for ribosomal proteins, enzymes, and factors involved in protein synthesis, rRNA processing, and tRNA acylation. Eight ORFs putatively code for enzymes involved in energy metabolism (hydrogen transfer and oxidative phosphorylation). Five ORFs code for components of RNA and DNA polymerases and for enzymes involved in DNA modification and integration. Three ORFs code for retroelements (either retrotransposons or retroviruses) and for a protein reacting with a product of the *nef* retrogene. The other reported putative phytoplasma ORFs code for metabolic enzymes, surface and signal peptides, and various other proteins; some of these are discussed below. Notably, 25 of the 78 ORFs have no match in the GenBank database and are designated hypothetical proteins (Table 2). Four other ORFs putatively code for proteins similar to those found in other organisms but with no as-yet-defined function.

Although it is premature to draw conclusions about the phytoplasma genome organization, it should be noted that, based on the presently reported sequences, the gene density is very high: 27,407 bases, out of a total of 32,753 bases sequenced (83.7%), are ORFs. Several clones carry partially

overlapping ORFs at different frames, some of which are positioned on opposite strands.

Specificity of the phytoplasma clones. The following tests were carried out in order to ascertain that the above-described clones were of phytoplasma origin and did not represent host sequences of plants, insects, or other microorganisms. Genomic DNAs or RNAs of noninfected, Stolbur-infected, and AY-phytoplasma-infected plants [periwinkle; *Catharantus roseus* (L.) G. Don] were subjected to Southern or Northern blot analysis with several inserts of selected clones as probes. An example of a Southern blot analysis is given in Fig. 2, and an example of a Northern analysis is given in Fig. 3. In both analyses, the probes reacted with DNA (Fig. 2A) or with total RNA (Fig. 3A) from AY-infected plants and not with that from noninfected plants. In the Southern analysis, the A1 clone also reacted faintly with DNA from plants infected with Stolbur (a phytoplasma related to AY). When the insert of clone A2 served as the probe in the Northern blot analysis, a complex hybridization pattern was observed, which could be attributed to the fact that 12 putative ORFs (Table 2) were identified on that clone. In order to verify equal loading of the various lanes, hybridization with the housekeeping β -tubulin gene is shown for the Southern analysis, and an ethidium bromide-stained total RNA gel (prior to hybridization), showing the rRNA bands, is shown for the Northern analysis.

To further assess the specificity of the phytoplasma DNA inserts, primers were generated from clone A380 (Table 1) and used for PCR analysis of genomic DNA obtained from healthy or infected plants (Fig. 4). A PCR product was obtained only with DNA extracted from phytoplasma-infected plants (Fig. 4, lanes 2 and 3). The specific PCR product was obtained with DNA from AY- and Stolbur-infected plants. Healthy plants did not produce any band upon PCR (Fig. 4, lane 1).

Phytoplasma DNA was collected from insect saliva. Therefore, the possibility that the described clones were derived

FIG. 3. Northern blot analysis of periwinkle RNA. Total cellular RNAs were extracted, electrophoresed (15 μ g per lane), subjected to Northern blot hybridization, and probed with 32 P-labeled clone A2 (A) or stained with ethidium bromide (B). RNA was from healthy plants (lanes 1) or from an AY-infected plant (lanes 2). The base lengths of RNA are indicated on the left.

FIG. 4. PCR analysis of periwinkle chromosomal DNA (100 ng per reaction). PCR primers were derived from clone A380 (Tables 1 and 2), amplifying a 500-bp region. Template DNA was from a healthy plant (lane 1), from an AY-infected plant (lane 2), from a Stolbur-infected plant (lane 3), or from clone A380 as a positive control (lane 4). Lane M, molecular size markers.

from insect cells which were deposited in the saliva or from other insect microorganisms (viruses or bacteria) needed to be ruled out. To address this issue, we selected phytoplasma-carrying and phytoplasma-free insects and carried out four PCR analyses on both types of insects. The PCRs were based on four distinct cloned phytoplasma-like sequences obtained in our study. (Fig. 5). In order to increase sensitivity and specificity, nested PCR was performed. Primers for these PCR assays were designed from clones A10, A182, A244, and A380 (Table 1). In all cases, PCR products were obtained from phytoplasma-infested insects (Fig. 5, lanes 2, 5, 8, and 11) and not from phytoplasma-free insects (Fig. 5, lanes 3, 6, 9, and 12). The lack of the tested sequences in the saliva of phytoplasma-free leafhoppers minimizes the possibility of contamination by microorganisms that were injected into the sucrose upon feeding. Collectively, the data suggest that the clone inserts represent authentic phytoplasma genomic fragments.

Homology of phytoplasma clones to eukaryotic genes. Most of the listed ORFs bore homology to prokaryotic and mitochondrial genes, which are considered to be of prokaryotic origin. Ten ORFs, however, were found to be homologous, at a reasonable level, to nonmitochondrial eukaryotic genes. Eight of these eukaryotic genes were found to be located on the same physical unit with prokaryotic genes (clones A2, A239, A295, and A450). Interestingly, three ORFs were homologous to retrovirus-like sequences (clone A370; clone A463, ORF 4; and clone A450, ORF 2 [which codes for a protein that is attachable to the retroviral *nef* gene]), indicating the possible occurrence of retrotransposons in the phytoplasma genome. Notably, however, all of the eukaryote-homologous clones (clone A2, ORFs 4, 5, 8, and 12; clone A239, ORF 3; clone A295, ORF 2; clone A380, ORFs 2 and 3; clone A450, ORF 2; and clone A540) exhibited a low GC content (23 to 39%), and most of them were located on the same genomic fragment adjacent to prokaryotic genes. The implications of such homologies are discussed below.

DISCUSSION

Until now, it had not been possible to obtain phytoplasma DNA that was sufficiently free of its respective host DNA. Therefore, the study of the phytoplasma genome was ham-

pered, and detection and characterization of phytoplasma genes were performed randomly. Phytoplasmas were diagnosed almost solely on the basis of 16S rDNA sequences. Aside from the presently reported sequences, only 33 phytoplasma genes, or parts thereof, are currently listed in the GenBank database. Extrachromosomal (plasmid) phytoplasma sequences have also been reported (35, 41). The presently reported method for isolating background-free phytoplasma DNA enabled the construction of a phytoplasma genomic library and demonstrates the ability to systematically analyze phytoplasma genes. The usefulness of the method is demonstrated by its almost tripling the amount of phytoplasma gene information in a short time.

Sequence analysis of the phytoplasma clones indicated some unique mycoplasma-like features: they have a low GC content (less than 35% in most cases), and the best ORF designation was obtained through the mycoplasma-mitochondrion codon usage table. These features, in addition to the lack of a cell wall, small genome size, and intracellular location in the host tissues, indicate that phytoplasmas might be evolutionarily related to mycoplasmas. The entire genomes of five mycoplasma species are known (8, 12, 13, 17, 45). Surprisingly, however, only one of the presently reported phytoplasma ORFs was found to be homologous, at a reasonable score, to a mycoplasma gene (clone A448, ORF 1, is homologous to the mycoplasma ribosomal protein S8 [rpS8] gene, albeit at a lesser score than to the same gene from *Listeria innocuus*). Furthermore, none of the phytoplasma ORFs reported by others is homologous to mycoplasma genes. It was also reported that phytoplasmas are phylogenetically closer to achleplasmas than to mycoplasmas (33). The organization of the highly conserved ribosomal protein genes in these species also adds to the confusion. The gene order in clone A182 (rpL18, rpS5, rpL15) and in A448 (rpS8, rpL6) is similar to the gene order in *Mycoplasma genitalium*. On the other hand, the gene order in clone A115 (EF-G, rpS10, rpL3) resembles that of *Bacillus* (EF-G, EF-Tu, rpS10, rpL3) rather than those of mycoplasmas (*M. genitalium* and *Mycoplasma pneumoniae*, EF-G, rpS6, ssb, rpS18; *Mycoplasma pulmonis*, EF-G, EF-Tu, hypothetical protein, hypothetical protein). Analysis of the complete S10-spc operon of the onion yellows phytoplasma (33)

FIG. 5. PCR analysis of leafhopper DNA (100 ng per reaction). PCR primers derived from clones A10, A182, A244, and A380 (A380 is the same primer as was used for the plant PCR assay for Fig. 4) were used to amplify a segment of DNA extracted from leafhoppers. All reactions except that with A380 primers were nested PCR. Lanes 2, 5, 8, and 11, PCRs with template DNAs from phytoplasma-carrying leafhoppers. Lanes 3, 6, 9, and 12, PCRs with template DNAs from phytoplasma-free leafhoppers. Lanes 1, 4, 7, and 10, positive controls (with the cloned DNAs serving as templates) for PCRs with A182, A244, A380, and A10, respectively.

also led to the conclusion that phytoplasmal genes resemble those of achleoplasmas and *Bacillus* more than those of other *Mollicutes*. These data indicate that the phytoplasma phylogenetic association is still an unresolved matter, and the question of the relatedness of phytoplasmas to mycoplasmas remains open.

Previous papers (see, for instance, reference 28) have indicated that phytoplasmas employ the general codon usage table. These findings were based on analysis of only a few ribosomal protein genes and the *tuf* gene. We analyzed 78 ORFs, and in about half of them (including all ribosomal protein genes) it did not matter which codon usage table was employed for translation. This may explain the previous reports of the use of the general codon table by phytoplasmas. However, in 16 out of the 32 reported clones (30 out of the reported 78 ORFs) a better translation was obtained with the mitochondrion-mycoplasma codon usage, while in the rest of the clones it did not matter which codon usage table was employed. This indicated that phytoplasmas use the mitochondrion-mycoplasma codon usage table, where UGA is read as tryptophan (three such cases are shown in Fig. 1). This is a large enough sample to allow a preliminary conclusion that at least the AY phytoplasma reads UGA as tryptophan. However, it uses this triplet infrequently, so that in many cases it does not matter which codon usage table is in effect.

Ten of the aforementioned ORFs are homologous to non-mitochondrial eukaryotic genes, and two ORFs (clones A352 and A526) are homologous to viral genes. It is worth noting that in addition to the eight eukaryotic genes mentioned above, four of the eukaryote-homologous ORFs are retroelements, implying the presence of transposons, or retrovirus infection, in phytoplasmas.

The eukaryote-homologous ORFs are not contaminants brought into the system from insect cells, as they are of low GC content and some are positioned on the same clone with prokaryote-homologous ORFs (for example, clones A2, A239, and A450). The close intracellular relationship between phytoplasmas and their host cells might have resulted from exchanges of genetic material (perhaps due to recombination) between the host and the pathogen. The host-homologous genes were adapted by the pathogen and underwent modifications (for instance, although they remain homologous to eukaryotic genes, they exhibit a low GC content in the phytoplasma). In this regard, it is worth mentioning that several ORFs are homologous to insect genes (mitochondrial or unassigned genes), and none (except for clone A340, which carries transposable elements) is of plant origin. The baculovirus-homologous ORF (clone A526) is also of special interest. This ORF is homologous to fusolin (also known as spindle-body protein or GP37 [38]). Fusolin has been found in baculoviruses and in insect-infecting poxviruses and has been shown to enhance infection by a factor of 10^4 . Fusolins are missing from poxviruses that do not infect insects (32, 52). It is of special interest that some fusolins bear distant homology to chitin- and cellulose-binding proteins (38). The insect's peritrophic membrane is composed of protein and chitin, and a major component of plant cell walls is cellulose. Phytoplasmas are pathogens of both insects and plants, and it is possible that a fusolin-like protein is required for establishing phytoplasmal infection. It is also worth noting that a gene for a caspase (caspases are cysteine

proteases involved in apoptosis following mitochondrial signaling [7, 24]) was found in phytoplasma (clone A352). In eukaryotic cells caspases are recruited as antibacterial agents (14); however, their origin is believed to be prokaryotic (2, 23).

Currently, phylogenetic analyses of phytoplasmas and their assignment to taxons and clades are based on the similarity or diversity found in their rDNA sequences, the gene for elongation factor Tu (*tuf* gene), and some ribosomal protein genes (16, 26, 28, 30, 49). For better reliability, taxonomy should rely on similarities and diversities in more than a single operon. In this respect, it is noteworthy that none of the sequences found for the isolated genes of the presently reported AY phytoplasma are homologous at their highest score to other phytoplasmal genes. Similar observations, which might challenge the present rDNA-based classification, were also made with other systems. The immunodominant membrane proteins from two phytoplasmas belonging to the same clade (as determined by 16S rDNA analysis) were found to be highly divergent in their hydrophilic region (3), and phytoplasmas diagnosed by 16S rDNA analysis as various types of apple proliferation were also found to be quite variable when PCR-RFLP analysis was performed on a nonribosomal fragment (18).

To validate the results, a determination of gene order for some other clones is also important. For example, all three ORFs in clone A380 are of mitochondrial origin (cyclophilin-D is a mitochondrial protein [56]).

Phytoplasma diagnoses are currently based on rDNA analyses. In this report, we provide PCR analyses, specific to AY, that are based on non-rDNA sequences. The information gathered here, and more sequences to come, will allow a better classification of phytoplasmas.

Many phytoplasmal clones carry ORFs in different frames and even in opposite orientations. This is to be expected when a relatively large amount of information is condensed into a small genome. This is consistent with the situation in *M. genitalium*. Almost every clone (accession numbers U39679 to U39729) carries overlapping ORFs in different frames, and short overlaps in opposite orientations are quite common. In some cases, longer opposite overlaps are also found; for example, the gene for O-sialoglycoprotein endopeptidase and that for signal recognition particle protein (accession no. U39684) overlap for 230 bp in opposite orientations. When two ORFs are partially overlapping and are located in opposite orientations, a sense-antisense silencing of expression, due to the formation of double-stranded RNA (39), probably takes place, unless the expression of each ORF is regulated at a different time.

The expected size of the phytoplasma genome is only 500 to 1,200 kb (50). Therefore, the above-described genomic library (about 5,000 clones with an average insert size of approximately 2,000 bp) seems to be representative of the entire phytoplasmal genome, allowing the launch of a project to decipher the entire phytoplasmal genome.

REFERENCES

1. Ahrens, U., and E. Seemüller. 1992. Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology* **82**:828–832.
2. Aravind, L., and E. V. Koonin. 2002. Classification of the caspase-hemoglobin fold: detection of new families and implications for the origin of the eukaryotic separins. *Protein Struct. Funct. Genet.* **46**:355–367.
3. Barbara, D. J., A. Morton, M. F. Clarck, and D. L. Davies. 2002. Immuno-

- dominant membrane proteins from two phytoplasmas in the aster yellows clade (chlorante aster yellows and clover phyllody) are highly divergent in their major hydrophilic region. *Microbiology* **148**:157–167.
4. Berg, M., D. L. Davies, M. F. Clark, H. J. Vetter, G. Maier, C. Marcone, and E. Seemüller. 1999. Isolation of the gene encoding an immunodominant membrane protein of the apple proliferation phytoplasma, and expression and characterization of the gene product. *Microbiology* **145**:1937–1943.
 5. Blomquist, C. L., D. J. Barbara, D. L. Davies, M. F. Clark, and B. C. Kirkpatrick. 2001. An immunodominant membrane protein gene from western X-disease phytoplasma is distinct from those of other phytoplasmas. *Microbiology* **147**:571–580.
 6. Boudon-Padieu, E., J. Larrue, and A. Caudwell. 1989. ELISA and dot blot detection of Flavescence dorée-MLO in individual leafhopper vectors during latency and inoculative state. *Curr. Microbiol.* **19**:357–364.
 7. Chai, J., C. Du, J.-W. Wu, S. Kyin, X. Wang, and Y. Shi. 2000. Structural and biochemical basis of apoptosis activation by Smac/DIABLO. *Nature* **406**:855–862.
 8. Chambaud, I., R. Heilig, S. Ferris, V. Barbe, V. Samson, F. Galisson, I. Moszer, K. Dybvig, H. Wroblewski, A. Viari, E. P. C. Rocha, and A. Blanchard. 2001. The complete genome sequence of the murine respiratory pathogen *Mycoplasma pulmonis*. *Nucleic Acids Res.* **29**:2145–2153.
 9. Chen, K. H., J. R. Guo, X. Y. Wu, N. Loi, L. Carraro, Y. H. Guo, Y. D. Chen, R. Osler, R. Pearson, and T. A. Chen. 1993. Comparison of monoclonal antibodies, DNA probes and PCR for detection of the grapevine yellows disease agent. *Phytopathology* **83**:915–922.
 10. Chu, Y. R., and C. P. Lin. 1997. Cloning and characterization of RecA gene of phytoplasma associated with peanut witches' broom. *Zhiwu Binglixue Huikan* **6**:207–208.
 11. Davis, R. L., B. Schneider, and K. S. Gibb. 1997. Detection and differentiation of phytoplasmas in Australia. *Aust. J. Agric. Res.* **48**:535–544.
 12. Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, J. D. Fritchman, J. F. Weidman, K. V. Small, M. Sandusky, J. Fuhrmann, and D. Nguyen. 1995. The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**:397–403.
 13. Glass, J. I., E. J. Lefkowitz, J. S. Glass, C. R. Heiner, E. Y. Chen, and G. H. Cassell. 2000. The complete sequence of the mucosal pathogen *Ureaplasma urealyticum*. *Nature* **407**:757–762.
 14. Gramse, H., V. Jendrosseck, and E. Glubins. 2001. Molecular mechanisms of bacteria induced apoptosis. *Apoptosis* **6**:441–445.
 15. Griffiths, H. M., W. A. Sinclair, C. D. Smart, and R. E. Davis. 1999. The phytoplasma associated with ash yellows and lilac witches' broom: "*Candidatus* Phytoplasma fraxini." *Int. J. Syst. Bacteriol.* **49**:1605–1614.
 16. Gunderson, D. E., I. M. Lee, S. A. Rehner, R. F. Davis, and D. T. Kingsbury. 1994. Phylogeny of mycoplasma-like organisms (phytoplasma): a basis for their classification. *J. Bacteriol.* **176**:5244–5254.
 17. Himmelreich, R., H. Hilbert, H. Plagens, E. Pirkel, B. C. Li, and R. Herrmann. 1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Res.* **24**:4420–4449.
 18. Jarausch, W., C. Saillard, B. Heliott, M. Garnier, and F. Dosba. 2000. Genetic variability of apple proliferation phytoplasmas as determined by PCR-RFLP and sequencing of a non-ribosomal fragment. *Mol. Cell. Probes* **14**:17–24.
 19. Jomantiene, R., R. E. Davis, J. Maas, and E. L. Dally. 1998. Classification of new phytoplasma associated with diseases of strawberry in Florida, based on analysis of 16S rRNA and ribosomal protein gene operon sequences. *Int. J. Syst. Bacteriol.* **48**:269–277.
 20. Kakizawa, S., K. Oshima, T. Kuboyama, H. Nishigawa, H. Jung, T. Sawayanagi, T. Tsuchizaki, S. Miyata, M. Ugaki, and S. Namba. 2001. Cloning and expression analysis of phytoplasma protein translocation genes. *Mol. Plant-Microbe Interact.* **14**:1043–1050.
 21. Kirkpatrick, B. C., D. C. Stenger, T. J. Morris, and A. H. Purcell. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. *Science* **238**:197–200.
 22. Kirkpatrick, B. C. 1991. Mycoplasma-like organisms: plant and invertebrate pathogens, p. 4050–4067. In A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes*. Springer-Verlag, New York, N.Y.
 23. Koonin, E. V., and L. Aravind. 2002. Origin and evolution of eukaryotic apoptosis: the bacterial connection. *Cell Death Differ.* **9**:394–404.
 24. Kroemer, G. 1997. Mitochondrial implication in apoptosis. Towards an endosymbiont hypothesis of apoptosis evolution. *Cell Death Differ.* **4**:443–456.
 25. Lee, I.-M., R. W. Hammond, R. E. Davis, and D. E. Gunderson. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* **83**:834–842.
 26. Lee, I.-M., D. E. Gunderson-Rindal, R. E. Davis, and I. M. Bartoszyk. 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol.* **48**:1153–1169.
 27. Lim, P. O., and B. B. Sears. 1991. DNA sequence of the ribosomal protein genes rpl2 and rpl9, from a plant-pathogenic mycoplasma-like organism. *FEMS Microb. Lett.* **84**:71–74.
 28. Lim, P. O., and B. B. Sears. 1992. Evolutionary relationship of a plant pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *J. Bacteriol.* **174**:2606–2611.
 29. Maixner, M., U. Ahrens, and E. Seemüller. 1995. Detection of German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *Eur. J. Plant Pathol.* **101**:241–250.
 30. Marcone, C., I. M. Lee, R. E. Davis, A. Ragozzino, and E. Seemüller. 2000. Classification of aster yellows-group phytoplasmas based on combined analysis of rRNA and tuf gene sequences. *Int. J. Syst. Evol. Microbiol.* **50**:1703–1713.
 31. McCoy, R. E., A. Caudwell, C. J. Chang, T. A. Chen, L. N. Chikowski, M. T. Cousin, J. L. Dale, G. T. N. de Leeuw, D. A. Golino, K. J. Hackett, B. C. Kirkpatrick, R. Marvitz, H. Petzold, R. C. Sinha, M. Sugiura, R. F. Whitcomb, I. L. Yang, B. M. Zhu, and E. Seemüller. 1989. Plant diseases associated with mycoplasma-like organisms, p. 545–640. In R. F. Whitcomb and J. C. Tully (ed.), *The mycoplasmas*, vol. 5. Spiroplasmas, achleplasmas, and mycoplasmas of plants and arthropods. Academic Press Inc., San Diego, Calif.
 32. Mitsuhashi, W., Y. Furuta, and M. Sato. 1997. Complete nucleotide sequence of the fusulin gene of an entomopoxvirus in the cupreous chafer, *Anomala cuprea* Hope (Coleoptera, Scarabaeidae). *Insect Biochem. Mol. Biol.* **27**:869–876.
 33. Miyata, S. I., K. I. Furuki, K. Oshima, T. Sawayanagi, H. Nishigawa, S. Kakizawa, H. Y. Jung, M. Ugaki, and S. Namba. 2002. Complete nucleotide sequence of the S10-spc operon of phytoplasmas: gene organization and genetic code resemble those of *Bacillus subtilis*. *DNA Cell Biol.* **21**:527–534.
 34. Montano, H. G., R. E. Davis, E. L. Dally, S. Hogenhout, J. P. Pimentel, and P. S. T. Brioso. 2001. "*Candidatus* Phytoplasma brasiliense", a new phytoplasma taxon associated with hibiscus witches' broom disease. *Int. J. Syst. Evol. Microbiol.* **51**:1109–1118.
 35. Nishigawa, H., K. Oshima, S. Kakizawa, H. Y. Jung, T. Kuboyama, S. Miyata, M. Ugaki, and S. Namba. 2002. A plasmid from a non-insect transmissible line of phytoplasma lacks two open reading frames that exist in the plasmid from the wild-type line. *Gene* **298**:195–201.
 36. Orenstein, S., A. Franck, L. Kuznetsova, I. Sela, and E. Tanne. 1999. Association of phytoplasmas with yellows diseases of carrot in Israel. *J. Plant Pathol.* **81**:193–199.
 37. Padovan, A. C., G. Firrao, B. Schneider, and K. S. Gibb. 2000. Chromosome mapping of the sweet potato little leaf phytoplasma reveals genome heterology within the phytoplasmas. *Microbiology* **146**:893–902.
 38. Phanis, C. G., D. P. Miller, S. C. Cassar, M. Tristem, S. M. Thiem, and D. R. O'Reilly. 1999. Identification and expression of two baculovirus gp37 genes. *J. Gen. Virol.* **80**:1823–1831.
 39. Plasterk, H. A. 2002. RNA silencing: the genome's immune system. *Science* **296**:1263–1265.
 40. Razin, S., D. Yegorov, and Y. Naot. 1998. Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev.* **62**:1094–1156.
 41. Rekar, D., L. Carraro, B. Schneider, E. Seemüller, J. Chen, C. J. Chang, R. Loc, and G. Firrao. 1999. Gemini virus-related extrachromosomal DNAs of the X-clade phytoplasmas share high sequence similarity. *Microbiology* **145**:1453–1459.
 42. Saillard, C., and J. M. Bové. 1990. Isolation of the DNA of various plant pathogenic mycoplasma-like organisms from infected plants. *Phytopathology* **80**:233–237.
 43. Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 44. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 45. Sasaki, Y., J. Ishikawa, A. Yamashita, K. Oshima, T. Kenri, K. Furuya, C. Yoshino, A. Horino, T. Shiba, T. Sasaki, and M. Hattori. 2002. The complete genomic sequence of *Mycoplasma penetrans*, an intracellular bacterial pathogen in humans. *Nucleic Acids Res.* **30**:5293–5300.
 46. Sawayanagi, T., N. Horikoshi, T. Kanehira, M. Shinohara, A. Bertaccini, M. T. Cousin, C. Hiruki, and S. Namba. 1999. "*Candidatus* phytoplasma japonicum," a new phytoplasma taxon associated with Japanese hydrangea phyllody. *Int. J. Syst. Bacteriol.* **49**:1275–1285.
 47. Sears, B. B., P.-O. Lim, N. Holland, B. C. Kirkpatrick, and K. L. Klomparens. 1989. Isolation and characterization of DNA from a mycoplasma-like organism. *Mol. Plant-Microbe Interact.* **2**:175–180.
 48. Seed, E., E. Seemüller, B. J. Schneider, C. Saillard, B. Blanchard, Y. Bertheau, and M. T. Cousin. 1994. Molecular cloning, detection of chromosomal DNA of the MLO associated with Faba bean (*Vicia faba* L.) phyllody by Southern blot hybridization and the polymerase chain reaction (PCR). *J. Phytopathol.* **144**:97–106.
 49. Seemüller, E., C. Marcone, U. Lauer, A. Ragozzino, and M. Göschl. 1998. Current status of molecular classification of the phytoplasmas. *J. Plant Pathol.* **80**:3–26.
 50. Seemüller, E., M. Garnier, and B. Schneider. 2002. Mycoplasmas of plants and insects, p. 91–116. In S. Razin and R. Herrmann (ed.), *Molecular biology*

- and pathology of mycoplasmas. Kluwer Academic/Plenum Publishers, London, United Kingdom.
51. **Smart, C. D., B. Schneider, C. L. Blomquist, L. J. Guerra, N. A. Harrison, U. Ahrens, K.-H. Lorenz, E. Seemuller, and B. C. Kirkpatrick.** 1996. Phytoplasma-specific PCR primers based on sequences of the 16S–23S rRNA spacer region. *Appl. Environ. Microbiol.* **62**:2988–2993.
 52. **Sriskantha, A., R. J. Osborne, and D. J. Dall.** 1997. Mapping of the *Heliothis armigera* entomopoxvirus (HaEPV) genome, and analysis of genes encoding the HaEPV spheroidin and nucleoside triphosphate phosphohydrolase I proteins. *J. Gen. Virol.* **78**:3115–3123.
 53. **Tanne, E., E. Boudon-Padieu, D. Clair, M. Davidovich, S. Melamed, and M. Klein.** 2001. Detection of phytoplasma by polymerase chain reaction of insect feeding medium and its use in determining vectoring ability. *Phytopathology* **91**:741–746.
 54. **Wagner, M., C. Fingerhut, H. J. Ross, and A. Schon.** 2001. The first phytoplasma RNase P RNA provides new insight into the sequence requirement of this ribozyme. *Nucleic Acids Res.* **19**:2661–2665.
 55. **White, D. T., L. L. Blackall, P. T. Scott, and K. B. Walsh.** 1998. Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in “*Candidatus* Phytoplasma australiense” and a new taxon, “*Candidatus* Phytoplasma australasia.” *Int. J. Syst. Bacteriol.* **48**:941–951.
 56. **Woodfield, K. Y., N. T. Price, and A. P. Halestrap.** 1997. cDNA cloning of rat mitochondrial cyclophilin. *Biochem. Biophys. Acta* **1351**:27–30.
 57. **Yu, Y. I., K. W. Yeh, and C. P. Lin.** 1998. An antigenic protein gene of a phytoplasma associated with sweet potato witches’ broom. *Microbiology* **144**:1257–1262.