Microbiology (1997), 143, 3381–3389 Printed in Great Britain

Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas

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Primers designed from sequences of the gene encoding the elongation factor Tu (*tuf* gene) of several culturable mollicutes amplified most of the *tuf* gene from phytoplasmas of the aster yellows, stolbur and X-disease groups. About 85% of the *tuf* gene from two aster yellows strains and a tomato stolbur phytoplasma was sequenced. The nucleotide sequence similarity between these related phytoplasmas was between 87·8 and 97·0%, whereas the homology with other mollicutes was 66·3–72·7%. The similarity of the deduced amino acid sequence was significantly higher, ranging from 96·0 to 99·4% among the phytoplasmas and 78·5% to 83·3% between phytoplasmas and the culturable mollicutes examined. From the nucleotide sequences of the phytoplasma strains, two pairs of primers were designed; one amplified the phytoplasmas of most phylogenetic groups that were established, the other was specific for the aster yellows and stolbur groups. The phytoplasmas of the various groups that were amplified could be distinguished by RFLP analysis using *Sau*3AI, *Alu*I and *Hpa*II. The aster yellows group could be divided into five *Sau*3AI RFLP groups. These results showed that the *tuf* gene has the potential to be used to differentiate and classify phytoplasmas. Southern blot analysis revealed that the *tuf* gene is present as a single copy.

Keywords: phytoplasmas, differentiation, classification, elongation factor, PCR

INTRODUCTION

Phytoplasmas, formerly called mycoplasma-like organisms (MLOs), are plant-pathogenic prokaryotes of the class *Mollicutes* that cannot be cultured under axenic conditions. The inability to culture phytoplasmas has made it difficult to characterize these pathogens. Only recently, by the introduction of molecular methods into plant mycoplasmology, has it become possible to determine the phylogenetic and taxonomic relationships of the phytoplasmas to each other and to other prokaryotes. Currently, classification is based on sequence analysis of the 16S rRNA gene (Lim & Sears, 1989; Kuske & Kirkpatrick, 1992; Namba *et al.*, 1993; Gundersen *et al.*, 1994; Seemüller *et al.*, 1994; Schneider *et al.*, 1995b). This gene is present in all prokaryotes and the conserved and variable regions make it suitable for phylogenetic classifications. Other phytoplasma genes or DNA regions that have been used for classification are the ribosomal protein genes *rpl22* and *rps3* (Lim & Sears, 1992; Gundersen *et al.*, 1994; Toth *et al.*, 1994) and the 16S/23S rRNA spacer region (Kirkpatrick *et al.*, 1994; Schneider *et al.*, 1995b). The latter are considerably more variable than the 16S rRNA gene but phylogenetic analysis of all three sequence categories has resulted in a similar classification of the phytoplasmas in relation to each other and to other mollicutes (Lim & Sears, 1989, 1992; Kuske & Kirkpatrick, 1992; Namba *et al.*, 1993; Gundersen *et al.*, 1994; Seemüller *et al.*, 1994; Toth *et al.*, 1994).

Although the relationship between the phylogenetic classification of the phytoplasmas and pathological and other biological traits has not as yet been clearly elucidated, it appears that the most widely used sequence in phytoplasma differentiation and classification, the 16S rRNA gene, is insufficiently variable to allow distinction of phytoplasmas that differ in host or vector specificity. On the other hand, the more variable 16S/23S rDNA spacer region is too short a fragment to be suitable for RFLP analysis, the preferred method for routine differentiation and characterization of phyto-

The GenBank accession numbers for the sequences reported in this paper are L46368 (AAY), L46369 (KV) and L46370 (STOLF).

plasmas (Lee *et al.*, 1993b; Schneider *et al.*, 1993). To bring the classification into line with phenotypic diversity, a higher resolution of groups and subgroups is needed. Nucleic acid sequences of conserved protein-encoding genes are potential candidates due to the degeneracy of the genetic code (Gundersen *et al.*, 1994; Toth *et al.*, 1994). Parts of the ribosomal protein operon have already been used to differentiate closely related strains of the aster yellows and X-disease group which were indistinguishable in their 16S rDNA profiles (Gundersen *et al.*, 1996). The *tuf* gene was examined as a possible candidate for the classification of phytoplasmas. This conserved gene encodes the elongation factor Tu (EF-Tu) and has a central function in the translation process (Filer & Furano, 1980; Ludwig *et al.*, 1990). Analyses of EF-Tu sequences have proven to reflect phylogeny and gave essentially the same results when compared to 16S rDNA sequences (Ludwig *et al.*, 1993). The information on *tuf* genes within the mollicutes is very limited. At present, five genes from culturable mollicutes have been cloned and sequenced (Inamine *et al.*, 1989; Loechel *et al.*, 1989; Ladefoged & Christiansen, 1991; Lüneberg *et al.*, 1991). In Southern hybridization studies, this gene was useful to show genomic variation and to differentiate species and distinct strains within the mollicutes (Yogev *et al.*, 1988; Ladefoged & Christiansen, 1991). In this study, PCR-amplified *tuf* genes of phytoplasmas from various phylogenetic groups were examined by nucleotide sequence, RFLP analysis and Southern blot hybridization for their suitability to differentiate and classify phytoplasmas. The strains used consisted of the aster yellows (AY) group, the stolbur group (previously described as a subgroup of the AY group), the apple proliferation group, the faba bean phyllody group, the X-disease group, the sugarcane white leaf group, the elm yellows group and the ash yellows group (previously described as a subgroup of the elm yellows group). These groups were previously established by RFLP and/or sequence analysis of 16S rDNA (Schneider *et al.*, 1993, 1995a; Seemüller *et al.*, 1994).

METHODS

Sources of phytoplasmas. The origin, source and group assignation of the phytoplasma strains examined are shown in Table 1. All strains were maintained in periwinkle [*Catharanthus roseus* (L.) G. Don], except strains AGY and PYL which were from their natural hosts and obtained from A. Padovan, Darwin, Australia and L. Liefting, Auckland, New Zealand, respectively.

DNA isolation. DNA for PCR analysis was isolated according to a phytoplasma enrichment procedure described by Ahrens & Seemüller (1992). DNA extraction for Southern blot analysis was according to a method described by Doyle & Doyle (1990). DNA from *Acholeplasma laidlawii*, *Spiroplasma citri* and *Mycoplasma mycoides* subsp. *mycoides* was provided by A. Padovan.

Primer selection. Two primers for the initial amplification of the phytoplasmal *tuf* gene were selected after multiple alignment of the *tuf* genes from *Mycoplasma pneumoniae*, *M. genitalium*, *M. gallisepticum*, *M. hominis* and *Ureaplasma urealyticum*. The forward primer fTuf1 (5′ CACATTGACCACGGTAAAAC 3′) and the reverse primer rTuf1 (5′ CCACCTTCACGAATAGAGAAC 3′) are located at positions 57–77 and 1073–1094, respectively, relative to the transcription start codon of *M. hominis*. The following internal primers were designed based on phytoplasma sequences and multiple alignments: fTufi1 (5′ CAAGTTGGTGTTCCAAA 3′), rTufi1 (5′ GTTGTCACCTGCTTGAGC 3′), fTufi (5′ AAACCTTTCTTAATGCC 3′), rTufi (5′ GGCATTAAGAAAGGTTT 3′), fTufAy (5′ GCTAAAAGTAGAGCTTATGA 3′), rTufAy (5′ CGTTGTCACCTGGCATTACC 3′), fTufu (5′ CCTGAAGAAAGAGAACGTGG 3′) and rTufu (5′ CGGAAATAGAATTGAGGACG 3′).

PCR amplification. The DNA was amplified in a 50 µl reaction volume containing 20–50 ng DNA, 0·25 µM each primer, 0·1 mM each nucleotide and 0·2 U Goldstar polymerase (Eurogentec). The reaction mixture was subjected to 35 cycles with the following incubations: 30 s denaturation at 95 °C, 30 s annealing at 45 °C and 60 s extension at 72 °C, for the primers fTuf1/rTuf1. All parameters were identical for the primers fTufAy/rTufAy and fTufu/rTufu, except for the annealing temperature which was 55 or 45 °C, and 55 °C, respectively.

Restriction enzyme digestion and Southern blot hybridization. About 10 µg DNA was digested with 20 U *Eco*RI, *Hin*dIII and *Bgl*II. A double digestion with *Eco*RI and *Hin*dIII was performed using 10 U of each enzyme according to the manufacturer's protocol. PCR products were digested with 0·1 U *Sau*3AI, *Alu*I and *Hpa*II and separated on 5–10% (w/v) polyacrylamide gels in TBE buffer (89 mM Tris/borate, 89 mM boric acid, 2 mM EDTA, pH 8·0). The DNA fragments for Southern blots were separated on a 1·0 or 1·5% (w/v) agarose gel containing 0·3 µg ethidium bromide ml^{-1} in TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8·0). Sizes of restriction fragments were determined from the gel by comparison with a 1 kb ladder (BRL Life Technologies). The DNA was blotted onto a nylon membrane and fixed by baking the membrane for 2 h at 80 °C (Sambrook *et al.*, 1989). The membrane was prehybridized for 2 h in a solution containing 6× SSPE (1× SSPE is 150 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7·6), 5× Denhardt's solution (1× Denhardt's solution is 0·02% Ficoll, 0·02% PVP, 0·02% BSA), 0·5% SDS and 100 µg denatured herring sperm DNA ml^{-1}. The membranes were hybridized overnight in the same solution containing the radiolabelled probe. The probe was labelled with [α-^{32}P]dATP using the megaprime labelling kit (Amersham). After hybridization the membranes were washed twice for 10 min at room temperature in a solution containing 2× SSC (1× SSC is 150 mM NaCl, 1·5 mM sodium citrate), 0·1% SDS and twice for 30 min in a solution containing 0·2× SSC, 0·1% SDS at 55 °C. The membranes were exposed to X-ray film at −80 °C using intensifying screens.

DNA sequencing. The PCR products were directly sequenced by cycle sequencing using the fmol DNA sequencing system and [γ-^{32}P]dATP end-labelled primers (Promega).

Data analysis and nucleotide accession numbers. The multiple sequence alignment was performed with the program CLUSTAL (Higgins & Sharp, 1988). The overall nucleic acid and amino acid similarities were calculated with the program GAP (Needlman & Wunsch, 1970) as part of the GCG 8.1.0 (Genetics Computer Group, Madison, WN, USA) software package provided by the Australian National Genomic Information Service, Sydney. The following *tuf* gene sequences were used in multiple alignments or similarity analysis: *M. hominis* (M57136), *M. genitalium* (X16463), *M. gallisepticum* (X16462), *M. pneumoniae* (X55768), *U. urealyticum* (Z34275)

Table 1. Phytoplasma code, origin, source and group assignation of the isolates used in this study

Phytoplasma code	Origin	Country/state	Group*	Reference†
AAY	American aster yellows	Florida, USA	AY	1, 2, 3
ACLR	Apricot chlorotic leaf roll	Spain	AY	1, 2, 3
AGY	Australian grapevine yellows	Australia	STOL	5, 6
AKV	Virescence of *Aquilegia alpina* L.	Germany	AY	1
AT	Apple proliferation	Germany	AP	1, 2, 3
ASHY	Ash yellows	New York, USA	ASHY	1, 2, 3
AV2192	Aster yellows	Germany	AY	1
AV2226	Aster yellows	Germany	AY	1
BLL	Brinjal (eggplant) little leaf	India	ASHY	3
BVK	Blütenverkleinerung (small flowers)	Germany	SCWL	1, 2
CHRYM	Yellows of *Chrysanthemum frutescens* L.	Germany	AY	1
COL	Latent in *Cuscuta odorata* Ruiz et Pav.	Germany	AY	1
CVA	Leafhopper-borne (species not determined)	Germany	AY	1
CVB	Leafhopper-borne (species not determined)	Germany	AY	1
CVT	Catharanthus virescence	Thailand	AY	1, 3
EY	Elm yellows	New York, USA	EY	1, 2
FBP	Faba bean phyllody	Sudan	FBP	1, 2
GVX	Green Valley strain of X-disease	California, USA	X-disease	1, 2
HYDP	Hydrangea phyllody	Belgium	AY	1
KV	Clover phyllody	Germany	AY	1
KVE	Clover phyllody	UK	AY	1
KVF	Clover phyllody	France	AY	–
KVM	Clover phyllody	France	AY	–
MOL	Molière disease of cherry	France	STOL	1, 2
PYL	Phormium yellow leaf	New Zealand	STOL	7
PYLR	Peach isolate	California, USA	X-disease	1, 4
SAS	Sandal spike	India	AY	1
SUNHP	Sunn-hemp (*Crotalaria juncea* L.) phyllody	Thailand	FBP	1, 2, 3
STOL	Stolbur of *Capsicum anuum* L.	Serbia	STOL	1, 2
STOLF	Stolbur of *Lycopersicon esculentum* Mill.	France	STOL	1
TBB	Tomato big bud	Australia	FBP	3
ULW	Witches' broom of *Ulmus carpinifolia* Gled.	France	EY	1
VAC	Witches' broom of *Vaccinium myrtillus* L.	Germany	X-disease	1, 2

* AY, aster yellows group; AP, apple proliferation group; ASHY, ash yellows group; EY, elm yellows group; FBP, faba bean phyllody group; SCWL, sugarcane white leaf group; STOL, stolbur group; X-disease, X-disease group.

† 1, Schneider *et al.* (1993); 2, Seemüller *et al.* (1994); 3, Schneider *et al.* (1995a); 4, Schneider *et al.* (1995b); 5, Padovan *et al.* (1996); 6, Davis *et al.* (1997); 7, Liefting *et al.* (1996).

and *Escherichia coli* (X57091). In addition, the 16S rRNA sequences of the following organisms were used for similarity analysis: phytoplasma strains AAY (X68373), KV (X83870) and STOL (X76427), and *M. hominis* (M24473), *M. genitalium* (MGU39693), *M. gallisepticum* (M22441), *M. pneumoniae* (M29061), *U. urealyticum* (M23935) and *E. coli* (V00348).

RESULTS

Amplification of phytoplasma *tuf* genes

Phytoplasma strains AAY, AT, BVK, GVX, STOL, ASHY and EY were selected for initial amplification of the *tuf* gene with primer pair fTuf1/rTuf1. The primers amplified a fragment of about 1·1 kb from the strains AAY, STOL and GVX. From the other strains and healthy periwinkle, three to four faint bands were amplified, ranging in size from 500 bp to 1·5 kb. These non-specific PCR products were not visible in the amplification products obtained from strains AAY, STOL and GVX. In subsequent experiments with fTuf1/rTuf1, in which all remaining strains were included (see Table 1), the target DNA from all AY (including strain ACLR) and stolbur group strains was amplified. From the X-disease group, strain PYLR was amplified but not strain VAC. No specific amplification product was obtained from strains of the other groups (data not shown).

Sequence analysis

The nucleotide sequence of both strands from strains AAY, KV and STOLF was determined from the fTuf1/

Table 2. Nucleic acid (na) and amino acid (aa) sequence similarities of the elongation factor Tu of phytoplasmas, culturable mollicutes and *E. coli*

Genotype*	Sequence similarity (%) to strains:		
	AAY [na/aa (16S rDNA)]	KV [na/aa (16S rDNA)]	STOLF [na/aa (16S rDNA)]
KV	97·0/99·4 (98·2)	–	–
STOL	88·3/96·3 (95·1)	87·8/96·0 (96·4)	–
M. hom	70·0/82·4 (76·5)	69·7/82·7 (76·2)	69·1/82·5 (75·8)
M. gen	68·4/81·0 (76·6)	68·4/80·9 (76·2)	68·6/81·0 (77·2)
M. pne	66·5/78·5 (77·1)	66·3/78·5 (76·9)	66·8/78·5 (76·4)
M. gal	69·9/82·0 (76·4)	69·1/82·0 (77·4)	69·3/81·8 (77·8)
U. ure	70·5/81·7 (76·6)	70·4/82·0 (76·5)	72·7/83·3 (76·5)
E. col	65·1/80·2 (72·0)	64·2/79·5 (70·8)	65·0/78·5 (72·0)

*M. hom, *Mycoplasma hominis*; M. gen, *M. genitalium*; M. pne, *M. pneumoniae*; M. gal, *M. gallisepticum*; U. ure, *Ureaplasma urealyticum*; E. col, *Escherichia coli*.

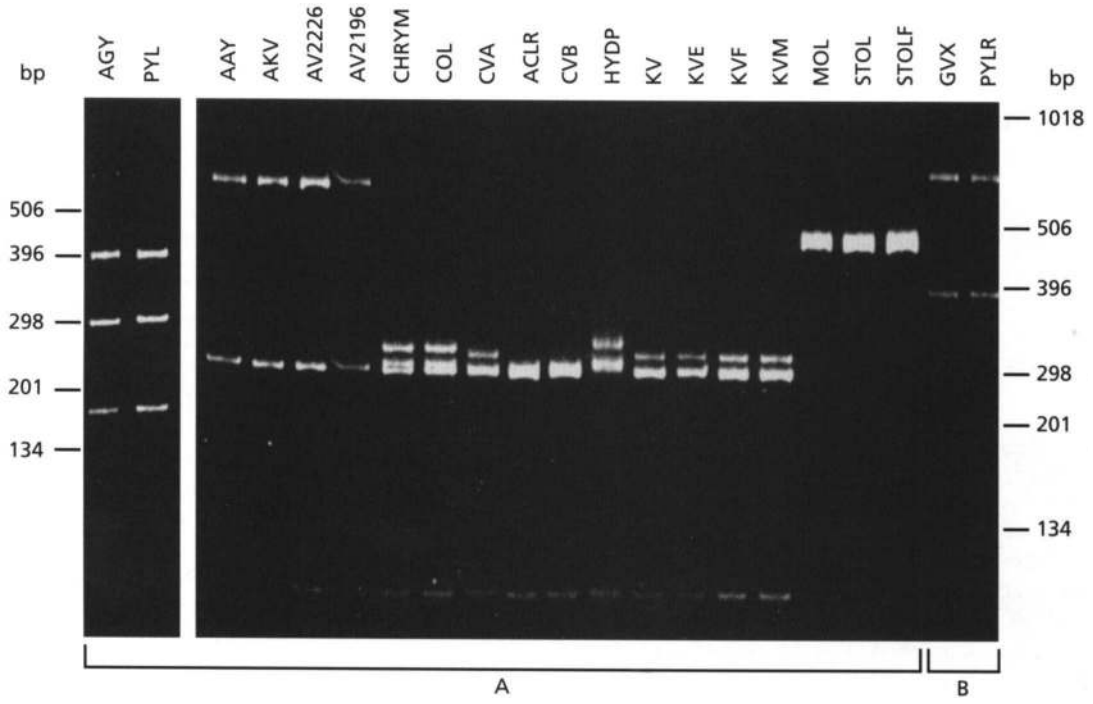

Fig. 1. *Sau*3AI RFLP pattern of AY, STOL and X-disease group phytoplasmas. The fragment was amplified with the primer pair fTufAy/rTufAy (A) or fTuf1/rTuf1 (B). The AY group phytoplasmas show five different RFLP patterns. The pattern of the stolbur type phytoplasmas (MOL, STOL, STOLF, AGY and PYL) is distinct from the AY group members. A 1 kb ladder was used as a size marker. See Table 1 for phytoplasma codes.

rTuf1 PCR product. These primers were also used to sequence the distal ends of the amplimers. The internal primers fTufi1, rTufi1, fTufi and rTufi were selected after progressive sequencing of the 5′ and 3′ ends. The internal primers were highly homologous to the corresponding regions of *M. hominis*, except rTufi1 which had four mismatches. Between 977 and 1007 bases from strains AAY, KV and STOLF were determined, comprising 82·6–85 % of the *tuf* gene. About 200 bp from the 5′ and 3′ end of strain GVX were determined. The G + C content was 37·4 mol % for AAY, 36·4 mol % for KV and 37·2 mol % for STOLF. The nucleic acid similarity was 97 % between strains AAY and KV and both strains were about 88 % similar to strain STOLF. The similarity to other mollicutes and *E. coli* ranged between 72·7 and 64·2 % (Table 2).

Table 3. RFLP groups of AY and STOL phytoplasmas based on *Sau*3AI digestion

Group	Strains
AY-I	AAY, AKV, AV2196, AV2226, CVT, SAS
AY-II	CHRYM, COL
AY-III	CVA, KV, KVE, KVF, KVM
AY-IV	ACLR, CVB
AY-V	HYDP
STOL-I	MOL, STOL, STOLF
STOL-II	AGY, PYL

The phytoplasma sequences contain one ORF encoding 334 (AAY), 335 (KV) and 325 (STOLF) aa. As a result of the high A + T content, the nucleotide in the second and third position of the codon is strongly biased towards A and T. Codon analysis revealed that the three strains have 64·4–64·8 % A or T at the second position and 79·9–81·8 % A or T at the third position. Glutamine, for example, is only represented by the codon GAA but never by GAG. From the possible 61 codons, only 45 are used. The GTP-binding elongation factor signature was found in all three sequences between amino acids 16 and 30 (50 and 64 in *M. hominis*) and was fully homologous to the consensus sequence. The GTP-binding site at position 19–26 of *M. hominis* was beyond the sequence determined. However, it is likely to be present because the primer fTuf1, which amplified the target from these strains, is derived from this region.

Amplification and RFLP analysis of the phytoplasmal *tuf* gene

Results from sequencing analysis led to the design of primer pair fTufAy/rTufAy for the specific amplification of phytoplasmas of the AY and stolbur group. Of these primers, oligonucleotide fTufAy is fully homologous to the target sequence of strains AAY, KV and STOLF, whereas oligonucleotide rTufAy shows one mismatch with the corresponding sequence of strain STOLF. In comparison to GVX (corresponding sequence reads 5′ GATAAACAAAGTTACGA 3′) and *M. hominis*, fTufAy has eight and seven mismatches, respectively. Primer rTufAy has five mismatches with GVX (corresponding sequence reads 5′ CTAAATCACCAGGCATTACC 3′) and *M. hominis*. The primer pair amplified a fragment of about 940 bp from all strains of the AY and stolbur groups. No amplification product was obtained from phytoplasmas of the other groups. Following RFLP analysis of the amplified fragment with *Sau*3AI, five restriction profiles were discernible in the AY group. The pattern of the stolbur strains was distinct from those of the AY strains. The pattern of the stolbur type phytoplasmas AGY and PYL was different from the stolbur strains MOL, STOL and STOLF from Europe (Fig. 1, Table 3). Fig. 1 shows also the *Sau*3AI pattern of the fTuf1/rTuf1 amplification products from strains GVX and PYLR which is different from the AY and

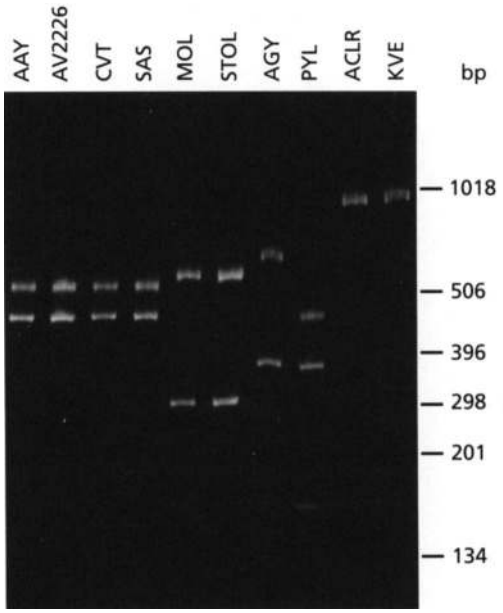

Fig. 2. *Hpa*II RFLP pattern of AY and STOL group phytoplasmas. The fragment was amplified with the primer pair fTufAy/rTufAy. The phytoplasmas ACLR and KVE lack a *Hpa*II restriction site. The RFLP patterns of the stolbur phytoplasmas AGY and PYL differ from each other and from MOL and STOL. A 1 kb ladder was used as a size marker. See Table 1 for phytoplasma codes.

stolbur patterns. The *Sau*3AI pattern of the fTuf1/rTuf1 product of the AY phytoplasmas is similar to the fTufAy/rTufAy pattern (data not shown). A further restriction site for this enzyme exists at the 5′ end of the fTuf1/rTuf1 amplimer but none at the 3′ end, creating an additional 40 bp band and shifting the largest band up by 60 bases. PCR products from ten AY and stolbur phytoplasmas with a different RFLP pattern were selected and digested with the restriction enzymes *Alu*I and *Hpa*II. As with *Sau*3AI, the pattern of strains AGY and PYL was different from the European stolbur strains and they were different from each other (Fig. 2, and data not shown). Following *Alu*I restriction all AY strains showed an identical pattern, whereas after *Hpa*II digestion ACLR and KVE differed from the otherwise homogeneous strains of the AY group (Fig. 2, and data not shown).

The primers fTufu and rTufu were designed for the universal amplification of the phytoplasmal *tuf* gene. The primer fTufu is fully homologous to the corresponding region of AAY, KV and STOLF, and has one mismatch with the sequence of GVX (corresponding sequence reads 5′ CCTGAAGAAAAAGAACGTGG 3′), two mismatches to *U. urealyticum* and three or more mismatches with the other culturable mollicutes. The primer rTufu is fully homologous to AAY, KV and *M. genitalium* and has one mismatch with STOLF, two mismatches with GVX (corresponding sequence reads 5′ CTTAAATAGAATTGAGGACG 3′) and two or more mismatches with the other culturable mollicutes. The

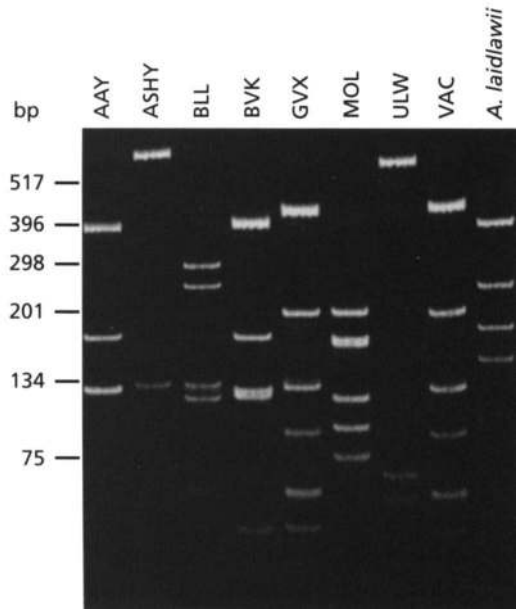

Fig. 3. *AluI* RFLP pattern of *tuf* gene from various phytoplasmas and *A. laidlawii*. The fragment was amplified with the primer pair fTufu/rTufu. A 1 kb ladder was used as a size marker. See Table 1 for phytoplasma codes.

primers amplified a fragment of about 850 bp from phytoplasma strains AAY, ASHY, BLL, BVK, GVX, MOL, ULW and VAC as well as from *A. laidlawii*, *S. citri* and *M. mycoides* subsp. *mycoides*. While from most strains the target DNA was amplified at an annealing temperature of 55 °C, a clear amplification product from strains ASHY, ULW, *A. laidlawii*, *S. citri* and *M. mycoides* subsp. *mycoides* was only obtained at 45 °C. At 50 °C the product obtained from these strains was low and at 55 °C missing. The *Alu*I digestion of the PCR fragments resulted in different patterns for the major phylogenetic clusters (Fig. 3, and data not shown). Identical patterns were obtained from strains GVX and VAC of the X-disease group. However, after calculating the sum of the fragment sizes for GVX and VAC and comparing the intensity of the fluorescence, it was apparent that the fragments of about 100 and 40 bp were not part of the gene. Electrophoresis of undigested PCR product on a high resolution gel showed an additional fragment of about 140 bp. An additional band of about 150 bp was also amplified for *A. laidlawii*. The primers failed to amplify the target DNA of the faba bean phyllody and apple proliferation group phytoplasmas even at an annealing temperature of 45 °C. Healthy periwinkle DNA was not amplified at 55 °C but a faint band was sometimes visible at an annealing temperature of 45 °C.

Southern blot hybridization

The fTuf1/rTuf1 PCR fragments from strains AAY and GVX were hybridized to DNA from strains of all major phylogenetic groups except the faba bean phyllody

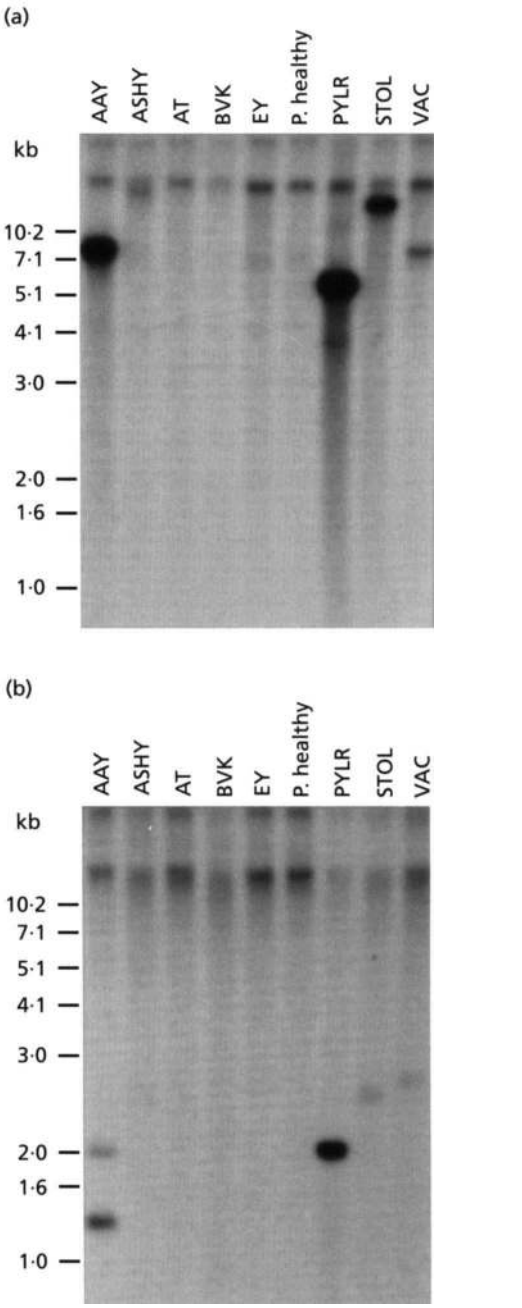

Fig. 4. Southern blot hybridization of (a) *Eco*RI- and (b) *Hin*dIII-digested DNA from infected periwinkle probed with the *tuf* gene of strains AAY and GVX. A 1 kb ladder was used as a size marker. See Table 1 for phytoplasma codes.

group. The PCR fragments hybridized strongly with the homologous phytoplasmas (AAY) or the same pathogen (PYLR), and faintly with the strains STOL, VAC and ASHY. The *tuf* gene probes hybridized to only one fragment when the DNA was restricted with *Eco*RI or *Bgl*II (Fig. 4a, and data not shown), indicating the absence of an internal restriction site for these enzymes. Following digestion with *Hin*dIII, two bands were present in the lane of AAY and one band in the lane of

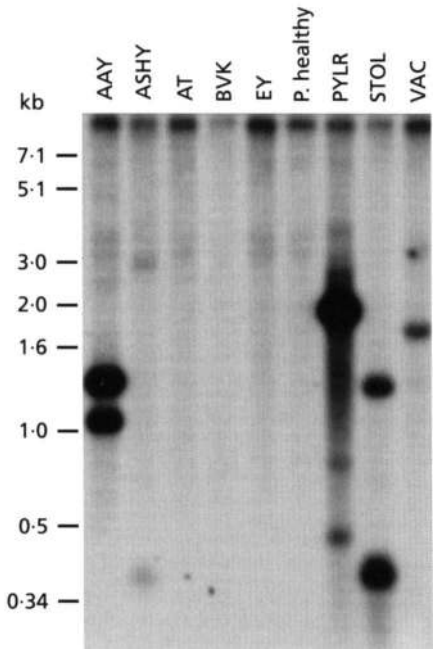

Fig. 5. Southern blot hybridization of *Eco*RI/*Hin*dIII double-digested DNA from infected periwinkle probed with the *tuf* gene of strains AAY and GVX. A 1 kb ladder was used as a size marker. See Table 1 for phytoplasma codes.

STOL, PYLR and VAC (Fig. 4b). Following double digestion with *Eco*RI and *Hin*dIII, strains AAY, STOL and ASHY showed two bands, and strain VAC one band. PYLR showed one strong hybridization and two faint bands (Fig. 5). In none of the blots did the other phytoplasmas show hybridization signals, even after long exposure times.

DISCUSSION

The analysis of conserved macromolecules is important in molecular taxonomy and phylogeny, especially of unculturable organisms such as the phytoplasmas. RFLP analysis of the conserved 16S rRNA gene was the basis for the first molecular classification system of phytoplasmas (Lee *et al.*, 1993b; Schneider *et al.*, 1993). Later sequence analysis of 16S rDNA was used to characterize phytoplasmas in more detail and to determine their phylogenetic relationships to each other and to other prokaryotes (Lim & Sears, 1989; Kuske & Kirkpatrick, 1992; Namba *et al.*, 1993; Gundersen *et al.*, 1994; Seemüller *et al.*, 1994). The availability of sequences from 16S rDNA and the adjacent 16S/23S rDNA spacer region has made rDNA a prime target for phytoplasma detection by PCR (Ahrens & Seemüller, 1992, 1994; Lee *et al.*, 1994; Gibb *et al.*, 1996; Smart *et al.*, 1996).

To broaden the basis for molecular characterization and classification of the phytoplasmas and to explore other targets for detection of these pathogens by PCR amplification, we examined the gene encoding the elongation factor Tu. This gene proved to be suitable for these purposes in work with culturable mollicutes and other bacteria (Yogev *et al.*, 1988; Ladefoged & Christiansen, 1991). With primers derived from conserved *tuf* gene regions of culturable mollicutes, only phytoplasmas from the AY group, the related stolbur group and the X-disease group were amplified. This result confirms the phylogenetic data obtained from 16S rDNA analyses that the members of the AY and stolbur groups are the closest phytoplasma relatives of the culturable mollicutes (Gundersen *et al.*, 1994; Seemüller *et al.*, 1994). Sequence analysis of the *tuf* gene from two strains of the AY group (AAY and KV) and from one strain of the stolbur group (STOLF) revealed a similarity of 66·3–72·7 % with the culturable mollicutes examined. These values are 4·5–11·7 % lower than the 16S rDNA similarity. Among the phytoplasma strains that we examined, the homology of the *tuf* gene sequences was also lower than that of the 16S rDNA. While, for instance, the *tuf* gene sequence of strain AAY differs from that of strain KV in 3 % of the positions and from that of strain STOLF in 11·7 % of the positions, the differences in the 16S rDNA sequences were only 1·8 and 4·9 %, respectively. This shows that the *tuf* gene is considerably more variable than the 16S rRNA gene. This higher variability proved to be useful in distinguishing and classifying phytoplasmas that are difficult to differentiate on the basis of 16S rDNA data. So, the comparison of strains AAY, KV and STOLF shows that, according to *tuf* gene sequences, the stolbur group is more distantly related to the AY group than indicated by 16S rDNA analysis. This greater phylogenetic distance justifies placement of the stolbur phytoplasmas into a separate group from the AY group and not as a subgroup as previously proposed (Seemüller *et al.*, 1994). Such a distinction on a higher taxonomic level is supported by data obtained through sequence analysis of the 16S/23S rDNA spacer region (Kirkpatrick *et al.*, 1994).

The variability of the *tuf* gene also permitted further differentiation of the strains of the AY group, the largest subclade of the phytoplasmas (Gundersen *et al.*, 1996). All AY strains included in our work showed the same restriction profiles when PCR-amplified 16S rDNA was digested with the frequently cutting and commonly employed endonucleases *Alu*I or *Rsa*I (Schneider *et al.*, 1993, and unpublished results). Five different restriction profiles were obtained when the *tuf* gene of the AY strains was digested with *Sau*3AI. In comparison with the rDNA, this reflects the higher mutation rate in a translated gene because modifications may not be apparent in the protein. Diversity within the AY group has previously been observed when several enzymes were employed in RFLP analysis of PCR-amplified 16S rDNA (Lee *et al.*, 1993b; Vibio *et al.*, 1996). The AY group could be further divided into subgroups by RFLP analysis of a fragment amplified from the ribosomal protein operon (Gundersen *et al.*, 1996). Strains that belong to the same 16S rDNA subgroup could be distinguished and more accurately reflected the genetic variation elsewhere in the genome. Based on the comparison of the restriction map derived from the 16S rDNA sequence of the phytoplasmas AAY, KV, ACLR

and STOL with the RFLP patterns of the AY subgroups established by Lee *et al.* (1993b) and Vibio *et al.* (1996), four AY subgroups were included in this work. Strain AAY represents the 16S rRNA subgroup I-B, strain KV belongs to subgroup I-C, and ACLR and STOL represent subgroups I-F and I-G, respectively. The subgrouping based on the *Sau*3AI pattern of the *tuf* gene from AAY (AY-I) and KV (AY-III) is consistent with the subgrouping based on the ribosomal protein gene fragment (Gundersen *et al.*, 1996). The other subgroups (AY-II, IV, V and STOL-I and II) are difficult to compare to the classifications of Gundersen *et al.* (1996) because different strains were used. However, a further subgrouping is likely when strains from both sources are examined with the same method. Differences within the AY group were also observed in serological studies when polyclonal and monoclonal antibodies raised to 'typical' AY strains reacted with some but not all phytoplasmas of the AY group. Among the phytoplasmas not recognized by AY antibodies were clover phyllody strains (Clark *et al.*, 1989; Lee *et al.*, 1993a). Antibodies to the clover phyllody phytoplasma in turn did not react with antigens of typical AY strains (Clark *et al.*, 1989). Such differences were also observed in this study in which the clover phyllody strains showed *Sau*3AI profiles that were distinct from that of the 'AY strains *sensu stricto*' (e.g. AAY and AV2226). However, the pathological significance of differences in restriction sites and serological reaction has yet to be elucidated, although it is likely that there are different pathogens within the AY group, as it is defined at present.

The primer pair fTufu/rTufu was designed to amplify the target DNA from all phytoplasma groups established by Seemüller *et al.* (1994) and Schneider *et al.* (1995a). However, they failed to amplify DNA from phytoplasmas of the faba bean phyllody and apple proliferation groups but amplified DNA from *A. laidlawii*, *S. citri* and *M. mycoides* subsp. *mycoides*, although the annealing temperature had to be reduced to obtain a PCR product from the culturable mollicutes. The amplification range of the primers fTufu/rTufu most certainly limits their use as diagnostic primers, although the cross-reaction with healthy plant DNA was low. The reason for the failure to amplify the *tuf* gene of the apple proliferation and faba bean phyllody groups is not clear because all phylogenetic investigations to date have shown that the phytoplasmas represent a monophyletic group and, according to these data, they are more closely related to each other than to their nearest relatives, *A. laidlawii* and other acholeplasmas of the anaeroplasma clade (Gundersen *et al.*, 1994; Seemüller *et al.*, 1994). A recent analysis of the EF-Tu and 16S rDNA sequence of five mollicute species resulted in a different phylogenetic relationship between the organisms compared (Kalma *et al.*, 1996). According to these results the phylogeny based on EF-Tu reflects the phenotypic characteristics better than that based on 16S rDNA. Thus, it would be interesting to analyse the sequences of the *tuf* gene from phytoplasmas of the faba bean phyllody and apple proliferation groups to see whether the phylogeny derived from *tuf* gene sequences differs from that based on ribosomal data.

Southern hybridization studies indicate that the phytoplasmas have only one copy of the *tuf* gene, like other mollicutes and Gram-positive bacteria (Loechel *et al.*, 1989; Sela *et al.*, 1989). The AAY and GVX PCR fragment hybridized only with closely related strains under the conditions applied, a fact that could be used to differentiate related phytoplasmas such as group members or members of related groups. The hybridization of the probe with two additional bands in the lane of PYLR (Fig. 5) is most likely caused by DNA fragments with a partial homology to the probe. The size of the main fragment (about 2·0 kb) and the results from the *Eco*RI and *Hin*dIII digests indicate that it is unlikely that these fragments represent the 5′ or 3′ part of the gene. For strain AAY the number of bands visible was in agreement with the number of restriction sites found after sequence analysis. The *tuf* gene of STOL, however, has two internal *Hin*dIII sites and three hybridization signals should be expected, but only one (Fig. 4) or two (Fig. 5) were visible. Probably because the internal *Hin*dIII fragment is only 400 bp it ran off the gel in Fig. 4. In the case of the double digest (Fig. 5) a small fragment (>200 bp) could have been produced or the lowermost hybridization signal represents two fragments.

ACKNOWLEDGEMENTS

The work was supported in part by funding from the European Commission, project AIR1 CT92-0659 and the Australian Centre for International Agricultural Research.

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Received 24 January 1997; revised 9 June 1997; accepted 2 July 1997.