Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA

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A method has been developed to amplify the 16S rRNA gene of plant-pathogenic mycoplasma-like organisms (MLOs) from infected plant material using the polymerase chain reaction (PCR). The procedure is dependent on the presence of a *BclI* restriction site in the 16S rDNA of chloroplasts but not in that of the MLOs. This difference permits the specific amplification of the 16S rDNA of the MLOs from *BclI*-digested total DNA from infected plants using primers from conserved regions of this gene. In this study 16S rDNA was obtained from 52 MLO isolates from herbaceous dicots and monocots as well as woody plants. Digestion of the 16S rRNA genes using *AluI* endonuclease revealed seven restriction patterns, which were used to group the isolates examined. Group I, which is also characterized by the presence of two *KpnI* sites, consisted of 31 isolates, most of which are from herbaceous dicots. Isolates assigned to groups II to VI were mostly from woody plants, while the isolates of group VII were from monocots or obtained from a leafhopper. The restriction patterns varied little within groups; however, four group I isolates and one group IV isolate differed slightly from the typical patterns of these groups as a result of a deletion or a slight shift of one restriction site. The groupings uncovered by *AluI* restriction were also obtained by digesting the 16S rDNA with *RsaI* endonuclease. However, some atypical patterns were observed within group V isolates. The groups described on the basis of restriction digest data were supported by sequence analysis. With one exception, the 16S rDNA of isolates within the same group exhibited 97.8 to 99.5% homology while those of different groups showed 89.6 to 92.0% homology.

Introduction

Mycoplasma-like organisms (MLOs) are nonculturable, parasitic prokaryotes of the class *Mollicutes* associated with diseases of several hundred plant species (McCoy *et al.*, 1989). Until recently, differentiation and characterization was mainly based on host range and the symptoms induced in natural hosts and in the experimental host *Catharanthus roseus* (L.) G. Don (periwinkle) (Marwitz, 1990). However, with the introduction of serological and nucleic acid hybridization methods into plant mycoplasmaology, more reliable and specific means are available to characterize MLOs. The development of techniques to obtain MLO DNA from infected plants and insect vectors and the cloning of MLO DNA have greatly enhanced this work. A number

of recent papers on dot and Southern hybridization has contributed to our better understanding of the relatedness of the MLOs (Bertaccini *et al.*, 1990; Bonnet *et al.*, 1990; Lee & Davis, 1988; Lee *et al.*, 1990; Kuske *et al.*, 1991). Based on Southern hybridization with a DNA fragment of an MLO associated with aster yellows, a differentiation between organisms inducing decline symptoms and those causing floral virescence has been proposed (Kuske *et al.*, 1991). Although several organisms have been differentiated using these methods they are limited by the fact that undefined DNA fragments have been used as probes.

In contrast to undefined genomic DNA fragments, the 16S rRNA gene is a universal character which provides valuable molecular information on MLOs. This gene shows regions which are highly conserved among the prokaryotes while other regions show considerable variation, thus permitting phylogenetic and taxonomic studies (Stackebrandt, 1991). Recently, 16S rRNA sequences have been used for the phylogenetic analysis and classification of culturable mollicutes (Weisburg *et al.*, 1989) and to elucidate the phylogeny of two MLOs (Lim

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Abbreviation: MLO, mycoplasma-like organism.

Table 1. Origin and source of the MLO isolates and the symptoms they induce in periwinkle

MLO code	Origin	Country/state	Source*	Symptom group †
AAY	American aster yellows	Florida	1	A
ACLR	Apricot chlorotic leaf roll	Spain	2	A
AKV	Virescence of <i>Aquilegia alpina</i> L.	Germany	3	A
AP	Apple proliferation	Italy	4a	D
ASHY	Ash yellows	New York	5	D
AT	Apple proliferation	Germany	3a	D
AV2192	Aster yellows	Germany	3	B
AV2226	Aster yellows	Germany	3	B
AYW	Eastern American aster yellows	New Jersey	6	B
BGWL	Bermudagrass white leaf	Thailand	7	
BVK	Leafhopper-borne (<i>Psammotettix cephalotes</i>)	Germany	8	D
COL	Latent in <i>Cuscuta odorata</i> Ruiz et Pav.	?	8a	D
CVA	Leafhopper-borne (species not determined)	Germany	8	A
CVB	Leafhopper-borne (species not determined)	Germany	8	A
CVL	<i>Catharanthus</i> virescence	Peru	9	A
CVT	<i>Catharanthus</i> virescence	Thailand	7	A
DEV	Virescence of a <i>Delphinium</i> hybrid	Germany	3b	C
DIV	Virescence of <i>Diplotaxis erucoides</i> (L.) DC	Spain	10	B
EAY	Aster yellows	Germany	3	B
EY	Elm yellows	New York	5a	D
FDI	Flavescence dorée	Italy	4	A
GVX	Green Valley strain of X-disease	California	11	D
HYDP	Hydrangea phyllody	Belgium	12	A
KV	Clover phyllody	Germany	3	A
KVE	Clover phyllody	England	11	A
MOL	Molière's disease of cherry	France	13	A
OAY	Virescence of <i>Oenothera hookeri</i>	USA	14	
PARM	Apricot decline	Germany	7	
PER	Peach decline	Italy	15	A
PLN-V6	Plum leptonecrosis	Italy	4	A
PPER	Peach decline	Germany	7	
PRIVA	Virescence of primrose (<i>Primula</i> sp.)	Germany	3c	C
PRIVB	Virescence of primrose (<i>Primula</i> sp.)	Germany	3c	D
PRIVC	Virescence of primrose (<i>Primula</i> sp.)	Germany	3c	A
PSER	Decline of <i>Prunus serrulata</i> Lindl.	Germany	7	
PVM	Virescence of <i>Plantago coronopus</i> L.	Germany	3	B
PVW	Virescence of <i>Plantago major</i> L.	Germany	8	C
PYLR	Peach yellow leaf roll	California	11	D
RCAE	Rubus stunt of <i>R. caesius</i> L.	Germany	7	
RV	Rape virescence	France	16	B
SAFP	Safflower phyllody	Israel	17	A
SAS	Sandal spike	India	18	A
SAY	American western aster yellows	California	19	A
SBB	Big bud of <i>Solanum marginatum</i> L.	Ecuador	3d	A
SCWL	Sugarcane white leaf	Thailand	7	
STOL	Stolbur of <i>Capsicum annuum</i> L.	Croatia	20	A
STOLF	Stolbur of <i>Lycopersicon esculentum</i> Mill.	France	21	A
SUNHP	Sunhemp phyllody	Thailand	7	A
TBB	Tomato big bud	Australia	11	A
ULW	Witches' broom of <i>Ulmus carpinifolia</i> Gled.	France	22	D
VAC	Witches' broom of <i>Vaccinium myrtillus</i> L.	Germany	3e	D
WX	Western X-disease	California	23	

* Collected and/or transmitted to periwinkle or *Coleus blumei*, or provided by: 1, R. E. McCoy, University of Florida, Fort Lauderdale, USA (via 3); 2, G. Llacer, IVIA, Moncada-Valencia, Spain (via 13); 3, R. Marwitz, Biologische Bundesanstalt, Berlin, Germany (3a Marwitz *et al.*, 1974, 3b Marwitz & Petzold, 1976, 3c Marwitz & Petzold, 1983, 3d Marwitz *et al.*, 1979, 3e Marwitz *et al.*, 1987); 4, L. Carraro, Università degli Studi, Udine, Italy (4a Carraro *et al.*, 1988); 5, W. A. Sinclair, Cornell University, Ithaca NY, USA (5a Sinclair *et al.*, 1976); 6, R. F. Whitcomb, USDA-ARS, Beltsville MD, USA (via 11); 7, collected by the authors; 8, W. Heintz, Biologische Bundesanstalt, Dossenheim, Germany (8a Heintz, 1989); 9, C. E. Fribourg, International Potato Center, Lima, Peru (via 3); 10, P. Moreno, IVIA, Moncada-Valencia, Spain (Moreno *et al.*, 1985) (via 3); 11, M. F. Clark, Horticulture Research International, East Malling, UK; 12, W. Welvaert, Rijksuniversiteit, Gent, Belgium (Welvaert *et al.*, 1975) (via 3); 13, F. Dosba, INRA, Bordeaux, France; 14, B. B. Sears, MSU, East Lansing, USA (Sears & Klomparsens, 1989); 15, A. Ragozzino, Università di Napoli-Portici, Naples, Italy (via 13);

& Sears, 1989; Kuske & Kirkpatrick, 1992). Both sequence and restriction enzyme analysis of 16S rDNA have been used in taxonomic studies on mollicutes (Göbel *et al.*, 1987; Laigret *et al.*, 1990; Taschke *et al.*, 1990) and other prokaryotes (Grimont & Grimont, 1986; Bouvet *et al.*, 1991; Gurtler *et al.*, 1991). The study of the 16S rRNA gene is greatly facilitated by the application of polymerase chain reaction (PCR) technology using primers that allow amplification of prokaryote 16S rDNA. Here we report a method which amplifies the 16S rRNA gene from MLOs and can be used to group them on the basis of restriction enzyme analysis of 16S rDNA.

Methods

Sources of MLOs and MLO 16S rDNA sequences. Sources of the MLOs examined and the codes used to describe them are listed in Table 1. Isolate EAY was maintained in the greenhouse in *Coleus blumei* Benth. by cuttings. PARM, PPER, PSER and RCAE were obtained from diseased apricot, peach, flowering cherry (*Prunus serrulata* Lindl.) and *Rubus caesius* L., respectively, grown in the experimental field of the Dossenheim institute. BGWL and SCWL were obtained from diseased bermudagrass and sugarcane, respectively, collected near Bangkok, Thailand. All other isolates were maintained in an insect-proof greenhouse in periwinkle by graft transmission. With the exception of the naturally infected isolates, CVL and CVT, the periwinkle-maintained MLOs were originally transmitted to this host with *Cuscuta* spp. bridges or by leafhoppers. The symptoms induced in periwinkle are indicated in Table 1. 16S rDNA sequences of the Oenothera (OAY-) (Lim & Sears, 1989) and western aster yellows (SAY-) MLOs (Kuske & Kirkpatrick, 1992) were included in the study for comparison.

Other prokaryotes. Isolates of *Agrobacterium tumefaciens* (strain At 1), *Clavibacter michiganensis* (strain C 2140), *Erwinia amylovora* (strain Ea. 6/6), and *Xanthomonas campestris* (strain Xc 314) (all obtained from W. Zeller, Biologische Bundesanstalt, Dossenheim) were grown on nutrient glycerol agar slants. *Escherichia coli* (strain XL1 blue, Stratagene) and *Spiroplasma citri* (strain R8A2, obtained from C. Saillard, INRA Bordeaux, France) were cultivated in LB medium (Maniatis *et al.*, 1982) and BSR medium (Bové & Saillard, 1979), respectively. Overnight growth from the cultures on solid media suspended in water and similar-aged liquid cultures of *E. coli* and *S. citri* were used without further treatment for *in vitro* amplification of 16S rDNA as described below.

PCR amplification. Five different primers from conserved regions of the 16S rRNA gene were used. The pair fD1 and rP1 (Weisburg *et al.*,

1991) primed proximal to the 5' and 3' termini, allowing the amplification of nearly the entire 16S rRNA gene. The three internal primers consisted of the forward primer fA extending from position 759 to 778 of the OAY-MLO (Lim & Sears, 1989), the reverse primer rA extending from position 1316 to 1297 of the same organism (Ahrens & Seemüller, 1992), and the reverse primer rC which is complementary to primer fA.

DNA from healthy and diseased plants was obtained by using an MLO enrichment procedure as described previously (Ahrens & Seemüller, 1992). Five microlitres of such DNA preparations were digested with 5 U of *Bcl*I restriction endonuclease (Amersham) in a total volume of 20 µl. Five microlitres of the digest or of bacterial suspension were used to amplify DNA in a 50 µl reaction containing 125 µM of the four dNTPs, 0.5 µM of each of the primers, and 1 U of *Taq* polymerase (Boehringer-Mannheim). PCR conditions consisted of 30 cycles of 30 s at 95 °C, 30 s at 50 °C, and 60 s at 72 °C, plus one additional cycle with a 4 min chain elongation. After amplification, 5 µl of the product was digested with *Bcl*I as described above and was then separated by electrophoresis in a horizontal 1% (w/v) agarose gel in TAE buffer (40 mM-Tris/acetate, 1 mM-EDTA, pH 8.0). From the band containing the desired 16S rDNA some material was removed from the gel with a hypodermic syringe and was, without further purification, amplified again as described above. The purity of the final product was examined by another *Bcl*I digestion followed by agarose gel electrophoresis. 16S rDNA of healthy plants was amplified without *Bcl*I digestion. All amplifications were performed using a Thermocycler 60 (bio-med).

Restriction digest and gel electrophoresis. The final amplification products were digested with *Alu*I, *Rsa*I, *Eco*RI or *Kpn*I, following the manufacturer's instructions (Amersham). Five microlitres of *Alu*I and *Rsa*I digests were resolved on vertical 5 or 8% (w/v) polyacrylamide gels in TBE buffer (89 mM-Tris/borate, 89 mM-boric acid, 2 mM-EDTA, pH 8.0). The *Eco*RI and *Kpn*I digests were separated by electrophoresis in 1% (w/v) agarose as previously described. The DNA was visualized under UV light after staining with ethidium bromide.

DNA sequencing. PCR-amplified 16S rDNAs from the AAY-, ACLR-, ASHY-, AT-, EY-, PPER- and ULW-MLOs were cloned in Bluescript M13+ (Stratagene) using standard procedures; ligation was performed according to Marchuk *et al.* (1991). One strand of the cloned 16S rDNA was sequenced with the Sequenase kit (US Biochemical) following the manufacturer's instructions. Two universal primers priming near the multiple cloning site (T3 and SK, Stratagene), one internal primer designed by Lane *et al.* (1985) extending from position 532 to 515 of the 16S rRNA gene of *E. coli*, another reverse primer designed by F. Laigret (personal communication, position 1068 to 1049 of *E. coli*), and the two internal reverse primers rA and rC described previously were used.

Analysis of data. Sequences of the 16S rRNA genes of the following organisms were compared by multiple alignment using the Clustal program (Higgins & Sharp, 1988): OAY- (EMBL/GenBank accession

15, G. Marchoux, INRA, Avignon-Montfavet, France (Marchoux & Giannotti, 1971) (via 3); 17, M. Klein, Volcani Center, Bed Dagan, Israel (Klein, 1970) (via 3); 18, J. Dijkstra, Agricultural University, Wageningen, Netherlands (Dijkstra & Lee, 1972) (via 3); 19, B. C. Kirkpatrick, University of California, Davis, USA (Kuske & Kirkpatrick, 1992); 20, D. Sutic, University of Zagreb, Croatia (via 3); 21, M.-T. Cousin, INRA, Versailles, France; 22, G. Morvan, INRA, Avignon-Montfavet, France (via 13); 23, D. D. Jensen, University of California, Berkeley (Jensen, 1986).

† According to the predominant symptoms in periwinkle: A, virescence, phyllody (typical for clover phyllody and stolbur); B, virescence, phyllody, elongated and etiolated internodes (typical aster yellows symptoms); C, small and faintly coloured flowers, elongated and etiolated internodes (atypical aster yellows symptoms); D, reduced flower size, leaf and flower malformations, no virescence, phyllody or elongated and etiolated internodes ('decline MLOs').

no. M30970), WX- (L04682), SAY- (M86340), AAY- (X68373), ACLR- (X68338), ASHY- (X68339), AT- (X68375), EY- (X68377), PPER- (X68374) and ULW- (X68376) MLOs, and *S. citri* (M23942). Analysis for the presence of *Bcl*I, *Alu*I and *Rsa*I restriction sites was performed with 16S rDNA of *Spiroplasma apis* (M23937), *Mycoplasma hominis* (M2447), *M. hyopneumoniae* (Y00149), *M. capricolum* (X00921), *Acholeplasma laidlawii* (M23932), *Anaeroplasmata abactoclasticum* (M25050), *E. coli* (V00348), and *Clostridium innocuum* (M23732). The 16S rDNA of the chloroplasts of *Glycine max* (X06428), *Nicotiana tabacum* (V00165), *Pisum sativum* (M30826), *Spinacea oleracea* (I01440) and *Sinapis alba* (X04182) were also included in restriction site analysis. All sequences of the culturable prokaryotes and the chloroplasts, as well as those of the OAY- and SAY-MLOs, were available in the EMBL Data Library, Heidelberg, Germany.

Results

Amplification of 16S rDNA

Sequence comparisons with the 16S rRNA genes of the OAY-, WX-, AT- and AAY-MLOs and the chloroplasts examined revealed the presence of a *Bcl*I restriction site in the chloroplasts but not in the gene of the MLOs. To obtain a specific PCR amplification of the 16S rDNA of the MLOs, the DNA from infected plants was digested with *Bcl*I before amplification. As the digest was usually incomplete, the amplification products were also *Bcl*I-restricted. Gel electrophoresis resolved one fragment approximately 1500 bp in size representing the 16S rRNA gene of the MLOs. The cleaved 16S rDNA of the chloroplasts appeared as two fragments approximately 800 and 700 bp in length.

Restriction and sequence analysis

The 52 isolates examined were grouped according to the presence of *Alu*I and *Rsa*I restriction sites. All sites of groups I to VI of which complete (AAY, ACLR, AT, OAY, PPER, SAY, WX) or partial (ASHY, EY, ULW) sequences of representative isolates were available could be determined. With the exception of the *Rsa*I sites yielding very small fragments, all sites of group VII were also determined using internal primers. The sizes of the amplified rDNA sequences and of the restriction fragments differed slightly due to small deletions or insertions. However, the position of the restriction sites could be determined by aligning the sequences with the analogous sequence of the OAY-MLO to which all MLO positions given in this paper correspond.

Restriction digestion of the amplified 16S rDNA with *Alu*I revealed seven different profiles among the MLO isolates (Figs 1 and 2), which were used to divide the isolates into seven major groups (Table 2). Group I is the largest and includes 31 isolates. With the exception of the periwinkle-maintained isolates HYDP, MOL, SAS and PER from woody hosts, they all originate from her-

baceous dicots or were obtained from leafhoppers (Table 1). All isolates of group I showed five restriction sites at positions a, d, e, f and g (Fig. 1). Group I contains isolate MOL and the stolbur-type isolates STOL, STOLF and TBB, which differ from the other group members by a

Fig. 1. *Alu*I restriction map of 16S rDNA depicting the seven (I-VII) different restriction profiles that occur among the MLOs examined. Representative isolates of the seven groups are given in parentheses (see Table 1 for MLO code). The figures given in group I correspond to the sequence positions of the OAY-MLO.

Fig. 2. *Alu*I restriction profiles of 16S rDNA from MLOs representing six of the seven groups established. AT to PARM, group I; RCAE to EY, group IV; ACLR and PLN-V6, group II; ASHY, group III; AAY, group I; VAC to SUNHP, group VI; *C. ros.*, healthy periwinkle; *S. citri*, *Spiroplasma citri*. See Table 1 for MLO codes.

Fig. 3. *AluI* restriction profiles of 16S rDNA of MLO isolates of group I showing that one fragment (arrow) of the isolates TBB to STOLF is slightly smaller than those of the typical isolates PRIVA to SBB. See Table 1 for MLO codes.

Table 2. Grouping of the MLO isolates examined based on restriction analysis of *AluI*- and *RsaI*-digested 16S rDNA

Group	Typical isolates*	Group members showing atypical results	
		<i>AluI</i> digest	<i>RsaI</i> digest
I	AAV, AKV, AV2192, AV2226, AYW, COL, CVA, CVB, CVL, CVT, DEV, DIV, EAY, HYDP, KV, KVE, OAY, PER, PRIVA, PRIVB, PRIVC, PVM, PVW, RV, SAFF, SAS, SAY, SBB	MOL, STOL, STOLF, TBB	MOL, STOL, STOLF, TBB
II	ACLR, PLN-V6		
III	ASHY		
IV	EY, RCAE, ULW		
V	AP, AT		PARM, PPER, PSER†, SUNHP
VI	FDI, GVX, PYLR, VAC, WX	SUNHP	
VII	BGWL, BVK, SCWL		

* See Table 1 for MLO code.

† *AluI* restriction profiles of these isolates are identical to that of the AP- and AT-MLO.

slightly shorter 3' fragment (Fig. 3). Restriction analysis of a fragment amplified with the internal primers fA and rA revealed that these isolates have a deletion of estimated size 10 bp near restriction site g.

Most isolates of groups II to VI were from woody hosts (Table 1). Group II differs from group I by the lack of *AluI* restriction site e. The ASHY-MLO, which

Table 3. *RsaI* restriction maps of PCR-amplified 16S rDNA of MLOs representing the seven groups evidenced by *AluI* digestion

Site and position*		Groups and representative isolates:						
		I AAV	II ACLR	III ASHY	IV EY	V AT	VI WX	VII SCWL
k	425	+	—	—	—	(—)	+	—
l	482	+	+	—	—	—	+	—
m	819	+	+	+	+	+	+	+
n	843	—	—	+	+	—	+	?
o	863	+	+	+	+	+	+	?
p	879	+	+	+	+	+	—	?
q	883	+	+	+	+	+	+	+
r	956	+	+	—	+	—	—	—
s	1381	+	+	+	+	+	+	+

* Corresponding to the OAY-MLO (Lim & Sears, 1989). +, —, restriction site present or missing, respectively; ?, site could not be determined because restriction fragments too small; (—) site present in isolates PARM, PPER, and PSER of Group V.

represents group III, lacks restriction sites d and f but shows restriction sites b and i. Groups IV to VII lack restriction site a. In addition, group IV lacks restriction sites d and f and is the only group with restriction sites h and j. Restriction site c occurs in group V only. Group VI is characterized by the presence of only three *AluI* restriction sites, at positions b, f and g (Figs 1 and 2). The SUNHP-MLO differs, probably due to a downstream shift of restriction site b, from the other isolates of group VI by a smaller fragment between sites b and f and a larger 5' fragment. Group VII (data not shown in Fig. 2) includes two MLOs from the monocot hosts sugarcane and bermudagrass as well as the leafhopper-borne isolate BVK. With the exception of the missing restriction site c, this group shows the same pattern as group V. The only *AluI* restriction site common to all the MLOs is g (Fig. 1).

RsaI restriction analysis recovered identical groupings to those revealed by *AluI* digestion (Tables 2 and 3, Fig. 4). With the former enzyme, a total of nine restriction sites (k to s) were found, of which the three at positions m, q and s were common to all MLOs examined. The isolates of group I have eight restriction sites and show the same profile. As with the patterns obtained after *AluI* digestion, isolate MOL and the stolbur-type isolates STOL, STOLF and TBB differ from the other MLOs of this group by a deletion near the 3' terminus, resulting in a shorter 425 bp-fragment between sites r and s. The isolates within the groups II and IV, respectively, were homogeneous. Isolate ASHY (group III) differs from group IV by the lack of restriction site r. In group V, the *Prunus* isolates PARM, PPER and PSER differ from the apple isolates AP and AT by an additional restriction site at position k. In group VI, all isolates show the same

Fig. 4. *RsaI* restriction profiles of 16S rDNA from MLOs representing the seven groups established. AAY and STOL, group I (AAY has two fragments 425 bp in length while for STOL one of them is slightly smaller); ACLR, group II; ASHY, group III; EY, group IV; AT and PPER, group V (PPER has an additional restriction site); VAC and SUNHP, group VI (SUNHP varies in the two uppermost fragments); SCWL, group VII; *C. ros.*, healthy periwinkle. See Table 1 for MLO codes.

Table 4. Sequence homology (%) of the 16S rDNA of various MLOs

See Table 1 for MLO code.

	OAY	SAY	ACLR	AT	PPER	WX
AAY	98.4	98.7	97.9	90.0	91.4	89.6
OAY		99.5	98.4	92.0	92.6	90.7
SAY			98.0	90.4	91.4	90.0
ACLR				90.1	90.9	90.0
AT					97.8	90.6
PPER						90.9

profile except SUNHP, which showed slight differences in the *AluI* digestion and has a 5' fragment that is approximately 15 bp longer than that of the other isolates while the fragment between position q and s is shorter to the same degree. SCWL shows the same major fragments as AT and AP. However, the position of *RsaI* sites at the *Rsa* site aggregation in the middle of the gene could not be determined with the electrophoresis methods used.

All isolates showed a unique *EcoRI* restriction site in the 16S rDNA at position 669 of the OAY-MLO. Also, a unique *NruI* site at position 1340 was detected in all organisms from which sequencing data were available. Isolates of group I showed two *KpnI* sites at positions 488 and 959 which were absent in the other groups. The only exception was CVB (group I), which has one *KpnI*

site at either position 488 or 959. In addition, the two organisms of group II have one *KpnI* site at position 959.

The differences between the groups and the homogeneity within individual groups was also shown by comparing the homology of the total sequences of the amplified rRNA genes (Table 4). Thus, isolates of the same group exhibit 98.4 to 99.5% homology while those of different groups usually differ by about 10%. However, isolate ACLR of group II is highly homologous to the group I MLOs. Isolate PPER, which produced an atypical *RsaI* restriction profile, showed a homology of 97.8% with AT-MLO, another group V isolate.

Sequence analysis showed that all the walled and wall-less prokaryotes examined differ in the *AluI* and *RsaI* restriction patterns of the 16S rDNA from those of the MLOs included in this study. Also, the 16S rRNA genes of the plant pathogenic bacteria *Xanthomonas campestris*, *Erwinia amylovora*, *Clavibacter michiganensis* and *Agrobacterium tumefaciens* showed different profiles than the MLOs. The *RsaI* patterns of *E. coli* and *S. citri* are shown in Fig. 4.

In both the *AluI* and *RsaI* restriction profiles of a number of MLOs, a DNA fragment approximately 100 bp in size became evident which is not part of the 16S rRNA gene (Figs 2 and 4). Its origin remains obscure.

Discussion

The 16S rRNA gene is the most widely used sequence in taxonomic studies on the prokaryotes. However, the study of 16S rRNA sequences in MLOs presents problems as they have not, as yet, been cultured *in vitro*. With the method described in this paper, 16S rDNA of the MLOs can readily be obtained by PCR amplification without recourse to *in vitro* cultivation. Examination of the amplified fragment by restriction and sequence analysis showed that it represents the authentic 16S rDNA of the MLOs and not the gene of other plant-associated prokaryotes or chloroplasts. Also, there is strong evidence that the amplified sequence was from one organism or at least from organisms of the same group because the restriction patterns of the isolates assigned to a group or a subgroup were uniform and there was no indication of mixed patterns. However, the presence of a second organism from a different group cannot be excluded, if it was present in low numbers relative to the organism that was amplified. The results obtained do not give information on the number of rRNA operons. The fact that 16S rDNA of any MLO would have been amplified with the primers used and that the restriction patterns were typical for one rRNA gene indicates that the MLOs examined have either only one rRNA operon or the copies of the 16S rRNA gene of

one organism yield similar restriction patterns. The culturable mollicutes have either one or two rRNA operons (Razin, 1989), and two occur in the OAY-MLO (Lim & Sears, 1989).

The MLOs examined originate from four continents and are associated with diseases of woody plants and of herbaceous monocots and dicots. In addition, the organisms induce very different symptoms in periwinkle. On the basis of the restriction patterns of the 16S rDNA, the organisms included in this study could be divided into seven groups. About 60 % of the isolates were assigned to group I, in which MLOs from all four symptom groups are represented (Table 1). Most of these MLOs were from herbaceous dicots and include the agents of aster yellows, clover phyllody, periwinkle virescence, and stolbur and/or big bud of solanaceous plants.

There are results from hybridization experiments which support the interrelatedness of the obviously diverse MLOs of group I. B. Schneider (unpublished results) hybridized Southern blots of DNA from most of the organisms included in this study with chromosomal DNA probes of the AAY-MLO. Under moderate stringency conditions these probes hybridized to most of the MLOs included in group I. Kuske *et al.* (1991) found homology between DNA probes from an AY-MLO and the stolbur isolate STOL as well as several isolates that induce AY symptoms in periwinkle. Also, probes from another AY-MLO cross-hybridized with DNA of the tomato big bud (BB)-MLO and of an MLO causing virescence in *C. roseus* (Lee & Davis, 1988). On the other hand, DNA fragments of the BB-MLO hybridized with DNA of the MLOs associated with clover phyllody and a virescence of *C. roseus* (Lee *et al.*, 1990).

In contrast to group I, the MLOs within the other groups are more uniform with regard to symptom induction and host range. The two isolates of group II (ACLR and PLN-V6) are considered to be identical or closely related because they induce similar symptoms in periwinkle and showed close relationship in Southern hybridization experiments (Ahrens *et al.*, 1992). The ASHY- and the EY-MLOs induce similar symptoms in periwinkle but showed some differences in the restriction patterns of 16S rDNA and were, for that reason, assigned to different groups (III and IV). This distinction appears appropriate as Bertaccini *et al.* (1990) and Davis *et al.* (1992) found little cross-hybridization between these two isolates. Probes from the ASHY- and the EY-MLO did not cross-hybridize to DNA of the MLOs associated with AY, BB, and virescence of *C. roseus*. The ULW-MLO, the second elm isolate of group IV, causes similar symptoms in periwinkle and had Southern hybridization restriction patterns identical to the EY-MLO (Mäurer & Seemüller, 1992).

Group V comprises European fruit isolates. DNA probes from the apple proliferation isolate AT cross-hybridized with DNA of isolate AP and with those of the three stone fruit isolates PARM, PPER and PSER (Ahrens *et al.*, 1992). However, the hybridization profiles of the stone fruit isolates were different from that of the apple MLOs, as observed by *RsaI* restriction analysis in this study. Genomic probes from the AT-MLO did not hybridize with isolates of group I, II, VI and VII (Bonnet *et al.*, 1990; B. Schneider, unpublished results). The isolates of group VI are more heterogeneous than those of groups II to V, which were all obtained from woody plants and induce, depending on the group, either virescence or non-virescence symptoms in periwinkle. The MLOs assigned to group VI were from woody and herbaceous hosts and cause virescence or non-virescence diseases. The relationship of the group VI MLOs to the virescence MLOs was also shown by Southern blot hybridization experiments in which genomic probes from the VAC-MLO hybridized with DNA of the ACLR- and the PLN-V6-MLOs as well as with several isolates of group I which mostly induce virescence symptoms (Ahrens *et al.*, 1992; B. Schneider, unpublished results). Group VII, includes the only MLOs examined (SCWL and BGWL) that are known to originate from monocots.

Although the taxonomic rank of the seven groups established is not clear, their complexity differs considerably. The small groups II to V and VII include only one organism or a few closely related MLOs. These groupings are supported by hybridization results, host range, and the symptoms induced. The MLOs of group VI are more heterogeneous and remain to be further differentiated. This is especially true of isolate SUNHP, which may be sufficiently different from the other group VI MLOs to form a subgroup or a group of its own. The situation in group I is more complex because it includes organisms which can be distinguished by their association with diseases such as aster yellows, clover phyllody, sandal spike, or stolbur. Thus, several agents of group I were, in the phenotypic classification of Marwitz (1990), considered to be distinct. The slight variation in the restriction pattern of the MLOs clustering with the stolbur isolate STOL may indicate the presence of a subgroup.

The AAY-, OAY- and SAY-MLOs, the three organisms of group I for which 16S rDNA sequencing data are available, exhibit a sequence homology of at least 98.4 %. Therefore, they appear to be closely related although they belong to two different symptom groups. However, in their recent contribution on the significance of 16S rRNA data for differentiation of prokaryotes, Fox *et al.* (1992) reported on bacteria which are distinguished at the species level despite an even higher degree of sequence homology than found in these three MLOs. The results

of these authors indicate that a high 16S rDNA sequence similarity is not necessarily a sufficient criterion to guarantee species identity. To further differentiate the MLOs, especially those of the heterogeneous groups, additional tools such as other restriction endonucleases for 16S rDNA analysis, specific genomic probes used in Southern hybridization, or serological methods must be applied. Monoclonal antibodies and polyclonal antisera proved to be highly specific and allowed the differentiation of, for instance, the AY- and the clover phyllody MLOs or even strains of the AY-MLO (Lin & Chen, 1985; Clark *et al.*, 1988).

The taxonomic distance of MLOs from different groups was also examined by comparing the 16S rDNA sequencing data. Except for group II, which is closely related to group I, the homology is between 89.6 and 92.6%. This is considerably lower than between the sequenced isolates of groups I or V. Despite the low number of organisms on which these figures are based, they indicate that the differences in the nucleotide sequence are expressed in the restriction patterns. These values also show a relatively close relationship between the MLOs examined, which may have arisen from a common ancestor. In other mollicutes, such as those of the genus *Mycoplasma*, the differences are considerably greater. For instance, the sequence homology of the 16S rDNA of *M. capricolum* and *M. hyopneumoniae* is only 77.7% (Taschke *et al.*, 1987). These two organisms were assigned to different phylogenetic groups by Weisburg *et al.* (1989).

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