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Presence of Two Sets of Ribosomal Genes in Phytopathogenic Mollicutes

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DNA from 28 strains of phytopathogenic mycoplasmalike organisms that represented five primary taxonomic clusters was digested with restriction endonucleases and hybridized with several ribosomal probes. The results indicate the presence of two sets of ribosomal genes in all strains examined. Restriction maps of the two ribosomal operons for a group of 12 aster yellows mycoplasmalike organisms were constructed.

Nonhelical phytopathogenic mollicutes, usually known as mycoplasmalike organisms (MLOs), are a group of widely distributed, wall-less prokaryotes that are associated with diseases of several hundred plant species (10, 11). Only recently, through the introduction of molecular methods into plant mycoplasmology, has it become possible to differentiate and partially characterize the MLOs on a reliable basis. In particular, studies on the 16S rRNA gene have contributed to our knowledge about the phylogenetic and taxonomic relationship of the MLOs with one another and with other prokaryotes, especially other mollicutes (7–9, 14, 18). Both sequence and restriction site analyses of this gene were used to establish a classification system for the MLOs (8, 14, 18). In the work by Schneider et al. (14) and Seemüller et al. (18), the following five primary clusters were distinguished: (i) the aster yellows (AY) strain cluster (divided into AY and stolbur subgroups); (ii) the apple proliferation strain cluster; (iii) the western X-disease strain cluster (divided into western X-disease and sunhemp witches'-broom subgroups); (iv) the sugarcane white leaf strain cluster; and (v) the elm yellows strain cluster (divided into elm yellows and ash yellows subgroups).

Mollicutes have a small genome of about 600 to 1,800 kb in size that contains only one or two sets of ribosomal genes (13). The occurrence of either one or two rRNA operons has been observed in the genera *Mycoplasma* and *Spiroplasma*, while the acholeplasmas, to which the MLOs are more closely related than to any other mollicutes (9, 18), have two operons (2, 3). Hybridization studies on the *Oenothera* (OAY) MLO and severe AY (SAY) MLO, for which genome sizes of 870 and 1,185 kb, respectively, have been determined (12), indicated that these two organisms of the AY strain cluster have two sets of ribosomal genes (7, 9). In contrast, the western X-disease MLO, which has a chromosome of 640 kb (12), is reported to have only one rRNA operon (4). However, conclusive evidence on operon numbers has not been published for the MLOs. Moreover, no data on MLOs from taxonomic groups other than the AY and western X-disease strain clusters are available. Thus, the objective of this study was to examine representative strains of all primary clusters that were established by Schneider et al. (14) and Seemüller et al. (18) for their numbers of rRNA operons. From one group of the AY strain cluster, restriction maps of the two operons are provided.

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The MLO strains examined as well as their origins and taxonomic assignments are listed in Table 1. Further information on most of these strains is given elsewhere (14). All strains with the exception of strain EAY, which was maintained in *Coleus blumei*, and strains Md and P. com, which were collected from a naturally infected apple tree and pear tree, respectively, were maintained in periwinkle (*Catharanthus roseus*) by periodic grafting. DNA was isolated from MLO-infected plants by the cetyltrimethylammonium bromide method (5) or by an MLO enrichment procedure (1). For Southern blot hybridization, DNA was digested with *Eco*RI, *Hin*dIII, *Bgl*II (BRL-Life Technologies or Amersham), or a combination of two of these enzymes. The digested DNA was separated overnight in horizontal 1% agarose gels (containing ethidium bromide) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) at 2 V/cm. The DNA was visualized by irradiation with UV light. Following Southern transfer to a nylon membrane (Hybond N; Amersham), the DNA was fixed for 2 h at 80°C.

The probes used for hybridization included a cloned *Hin*dIII fragment (MR16) of OAY MLO, approximately 5 kb in length, that comprised a 2.8-kb fragment upstream of the 16S rRNA gene, the entire 16S rRNA gene of about 1.5 kb in length, the spacer region between the 16S and 23S rRNA genes of about 0.3 kb in length, and the 5′ region of the 23S rRNA gene of about 0.4 kb in length (9). By digesting this fragment with *Eco*RI-*Hin*dIII and *Nru*I-*Hin*dIII (there is an *Nru*I site after position 1340 of the 16S rRNA gene of OAY MLO [18]), a probe (upstream probe) comprising the region upstream of the 16S ribosomal DNA (rDNA) and a probe (downstream probe) consisting of the portion downstream of the *Nru*I site were obtained. Probes from the 16S rRNA gene of the OAY MLO were constructed by amplifying the entire gene with a PCR procedure using primers fD1 and rP1 as described previously (14) and by digesting the amplification product with *Eco*RI. The portion upstream of the conserved *Eco*RI site after position 669 of the 16S rRNA gene (all positions given below correspond to positions of the sequence of the OAY MLO [9]) was designated the 5′ probe, and the downstream portion was designated the 3′ probe. The probes were radiolabeled with [^{32}P]dATP by random priming. Southern blot hybridization was performed under moderately (0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate [SDS], 55°C) or highly (0.1× SSC–0.1% SDS, 68°C) stringent washing conditions as described previously (15).

Probe MR16 and the MR16-derived 5′, 3′, and downstream probes hybridized to rDNA of all 28 MLO strains that were

TABLE 1. Restriction fragments obtained by Southern blot hybridization of *Eco*RI-, *Hin*dIII-, and *Bgl*II-digested DNA from various MLOs with probes from the 5′ and 3′ portions of the 16S rRNA gene of the OAY MLO

Strain	Disease or origin	Group[a]	Source[b]	Fragment size(s) (kb)			
				*Eco*RI digestion		*Hin*dIII digestion, 5′ probe	*Bgl*II digestion, 5′ probe
				5′ probe	3′ probe		
AAY	American aster yellows	AY	1	2.5, 1.0	12, 8.0	5.0, 2.4	ND[c]
AKV	Virescence of *Aquilegia alpina*	AY	2	2.5, 1.0	12, 8.0	5.0, 2.4	ND
AV2192	German aster yellows	AY	2	2.5, 1.0	12, 8.0	5.0, 2.4	ND
CVL	Virescence of *Catharanthus roseus*	AY	3	2.5, 1.0	12, 8.0	5.0, 2.4	ND
DEV	Virescence of *Delphinium* hybrid	AY	2	2.5, 1.0	12, 8.0	5.0, 2.4	ND
DIV	Virescence of *Diplotaxis erucoides*	AY	2	2.5, 1.0	12, 8.0	5.0, 2.4	ND
EAY	German aster yellows	AY	2	2.5, 1.0	12, 8.0	5.0, 2.4	ND
PRIVA	Virescence of *Primula* sp.	AY	2	2.5, 1.0	12, 8.0	5.0, 2.4	ND
PRIVC	Virescence of *Primula* sp.	AY	2	2.5, 1.0	12, 8.0	5.0, 2.4	ND
RV	Rape virescence	AY	4	2.5, 1.0	12, 8.0	5.0, 2.4	ND
SAFP	Safflower phyllody	AY	5	2.5, 1.0	12, 8.0	5.0, 2.4	ND
SAS	Sandal spike	AY	6	2.5, 1.0	12, 8.0	5.0, 2.4	12, 10
ACLR	From diseased apricot	AY	7	2.5, 6.0	6.0	4.0, 2.4	ND
PRIVB	Virescence of *Primula* sp.	AY	2	2.5, 6.0	6.0	4.0, 2.4	ND
KV	Clover phyllody	AY	2	2.5, 8.0	8.0	5.0, 4.0	ND
CVA	Leafhopper borne (unknown species)	AY	8	2.5, 8.0	8.0	5.0, 4.0	ND
CVB	Leafhopper borne (unknown species)	AY	8	2.5, 8.0	8.0	5.0, 4.0	ND
HYDP	Hydrangea phyllody	AY	9	2.5, 9.0	10, 12	8.0, 2.4	ND
STOL	Stolbur of pepper	STO	10	6.0, 4.5	12, 8.0	3.5, 2.4	12
AP	Apple proliferation	AP	11	ND	12, 7.0	6.0, 5.5	ND
AT	Apple proliferation	AP	2	12, 10	12	7.0, 6.5	ND
Md	Apple proliferation	AP	12	ND	ND	6.0, 5.5	ND
P. com	Pear decline	AP	12	ND	ND	6.0, 5.5	ND
PYLR	Western X-disease	WX	13	9.0, 2.0	9.0, 2.0	12, 9.0	8.2, 7.3
VAC	Vaccinium witches'-broom	WX	2	11, 2.0	12, 9.0	12, 8.0	7.0
BVK	*Psammotettix cephalotes* (leafhopper)	SCWL	8	9.0, 6.5	12, 7.0	9.0, 5.0	11, 8.0
EY	Elm yellows	EY	14	8.2, 7.0	4.5	7.0, 5.0	12, 10
ASHY1	Ash yellows	ASHY	14	9.0, 1.5	10, 6.0	9.0, 5.3	8.0, 7.5

[a] AY, aster yellows subgroup of aster yellows strain cluster; STO, stolbur subgroup of aster yellows strain cluster; AP, apple proliferation strain cluster; WX, western X-disease subgroup of western X-disease strain cluster; SCWL, sugarcane white leaf strain cluster; EY, elm yellows subgroup of elm yellows strain cluster; ASHY, ash yellows subgroup of elm yellows strain cluster.

[b] 1, R. E. McCoy, University of Florida, Fort Lauderdale (via R. Marwitz); 2, R. Marwitz, Biologische Bundesanstalt, Berlin, Germany; 3, C. E. Fribourg, International Potato Center, Lima, Peru (via R. Marwitz); 4, G. Marchoux, Institut National de la Recherche Agronomique, Avignon-Montfavet, France (via R. Marwitz); 5, M. Klein, Volcani Center, Bed Dagan, Israel (via R. Marwitz); 6, J. Dijkstra, Agricultural University, Wageningen, The Netherlands (via R. Marwitz); 7, G. Llacer, Instituto Valenciano de Investigaciones Agrarias, Moncada-Valencia, Spain; 8, W. Heintz, Biologische Bundesanstalt, Dossenheim, Germany; 9, W. Welvaert, Rijksuniversiteit, Gent, Belgium (via R. Marwitz); 10, D. Sutic, University of Beograd, Serbia (via R. Marwitz); 11, L. Carraro, Universita delgi Studi, Udine, Italy; 12, collected by us; 13, M. F. Clark, Horticulture Research International, East Malling, United Kingdom; 14, W. A. Sinclair, Cornell University, Ithaca, N.Y.

[c] ND, not determined.

examined (Table 1 and data not shown). Hybridization also occurred with rDNA of plant chloroplasts, sometimes to fragments of the same length as the MLO rDNA fragments. However, most or all of the plant reactions were removed by high-stringency washes, while the hybridization signals with MLO rDNA remained. This stable hybridization with MLO DNA reflects the high sequence similarity of at least 89.5% among MLO 16S rRNA genes (18). Hybridization signals that were doubtful because of similar-size MLO and chloroplast DNA fragments were clarified by the use of additional restriction enzymes. Especially digestion with *Bgl*II facilitated interpretation of the hybridization results because the size of the major fragment of chloroplast DNA was different from that obtained by *Eco*RI and *Hin*dIII digestions. Furthermore, double digestion was used to clarify and support data obtained with one enzyme.

Table 1 summarizes the data obtained by hybridization of *Eco*RI-, *Hin*dIII-, and *Bgl*II-digested DNA with the 5′ and 3′ probes. Hybridization of *Eco*RI-digested DNA with the 5′ probe yielded fragments whose sizes were mostly different from those obtained with the 3′ probe. This result indicates the presence of the conserved *Eco*RI site in the 16S rDNA of most or all MLOs. In contrast, hybridization of *Hin*dIII- and *Bgl*II-digested DNA with the two probes (as well as the downstream probe) usually resulted in identical patterns. For that reason, the data obtained with the 3′ probe were omitted in Table 1. The similarity of the hybridization results with the two probes and the downstream probe shows that there are no *Hin*dIII and *Bgl*II sites between the 5′ terminus of the 16S rRNA gene and the *Hin*dIII site in the 5′ region of the 23S rRNA gene, which is conserved in many mollicutes (3) and corresponds to the cloning site of MR16. The only exceptions to this pattern were strain STOL of the stolbur subgroup, which has a *Hin*dIII site after position 1255 of the 16S rRNA gene, and strain PYLR (but not VAC) of the western X-disease subgroup, which has a *Bgl*II site after position 203 (Fig. 1 and 2). These restriction sites resulted in different profiles following hybridization of *Hin*dIII- and *Bgl*II-digested DNA with the 5′ and 3′ probes. All *Eco*RI, *Hin*dIII, and *Bgl*II fragments hybridizing with the 5′, 3′, and downstream probes as well as additional fragments from regions upstream of the 16S rRNA gene were recovered by hybridization with probe MR16 (Fig. 2 and data not shown).

Upon hybridization with the 5′ and 3′ probes, cleaved DNA from all strains examined showed in at least one blot two fragments whose sizes clearly showed that the organisms have two sets of ribosomal genes, regardless of the sizes of their

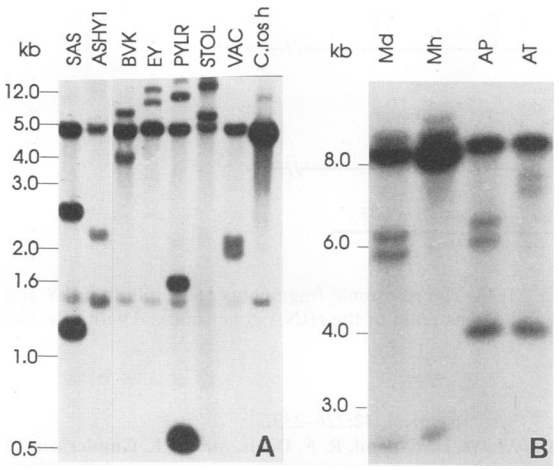

FIG. 1. Southern blots of DNA from representative MLO strains from several taxonomic groups hybridized under moderately stringent conditions of the posthybridization washes. All strains show two MLO-specific fragments. (A) *Eco*RI-*Bgl*II-digested DNA hybridized with a ribosomal probe from the 5′ portion of the 16S rRNA gene (5′ probe). SAS, sandal spike MLO; ASHY1, ash yellows MLO; BVK, leafhopper-borne MLO; EY, elm yellows MLO; PYLR, western X-disease MLO; STOL, stolbur MLO; VAC, vaccinium witches'-broom MLO; C.ros h, healthy *Catharanthus roseus*. The 0.5-kb fragment in the lane for strain PYLR indicates the presence of a *Bgl*II site in the 5′ region of the gene. (B) *Hin*dIII-digested DNA from strains of the apple proliferation MLO hybridized with the 3′ probe. Md, apple proliferation-diseased apple; Mh, healthy apple; AP and AT, periwinkle-maintained strains. The bands at 2.5, 4, and 8 kb are plant reactions.

chromosomes (Table 1 and data not shown). Two such fragments were also obtained in several hybridizations with DNA from the western X-disease strain PYLR, which was thought to have only one rRNA operon (4). Hybridization results obtained with representative strains of all MLO groups examined are shown in Fig. 1 and 2. In several hybridizations, some of the strains showed only one fragment. It is possible that in such cases the two fragments were similar in size. Also, the possibility cannot be excluded that in some strains the conserved *Eco*RI site in the 16S rDNA is present in only one of the two genes or is modified. An example of modification of the *Eco*RI site in both rRNA genes appears to be strain AT of the apple proliferation MLO. In this case, similar fragments were recovered when *Hin*dIII- and *Hin*dIII-*Eco*RI-digested DNA was hybridized with the 5′ probe (data not shown). However, when PCR-amplified DNA of this strain was examined, the *Eco*RI site was always present (16).

Analysis of the restriction data showed that strain AAY and 11 other strains of the AY subgroup yield similar restriction profiles when hybridized with probe MR16 and the 5′ and 3′ probes (Table 1 and data not shown). Restriction patterns of strains from this group are shown in Fig. 1 (SAS) and Fig. 2 (AAY). Comparison of these profiles with data from other workers revealed that the *rrnA* operon of the AAY-like MLOs corresponds to fragment MR16 from OAY MLO, which has been cloned and partially characterized by Lim and Sears (9) and Sears (17). On the other hand, operon *rrnB* is similar to the ribosomal sequence of the SAY MLO that has been described and partially sequenced by Kuske (6) and Kuske and Kirkpatrick (7). The restriction profiles show that the *Eco*RI and *Hin*dIII sites flanking the 16S rRNA genes of both operons are similar in all AAY-like strains (Fig. 3). However, hybridization of *Eco*RI-digested DNA of these strains with the upstream probe resulted in fragments of different sizes, thus indicating that the outermost *Eco*RI sites upstream of the 16S rRNA genes shown in Fig. 3 are not conserved. Operon *rrnB*, but not *rrnA*, is also present in strains ACLR, PRIVB, and HYDP of the AY subgroup. The other strains of the AY subgroup, which are clover phyllody-type organisms, showed a pattern that was different from those of the AY strain described above (Table 1). These results indicate that closely related organisms from

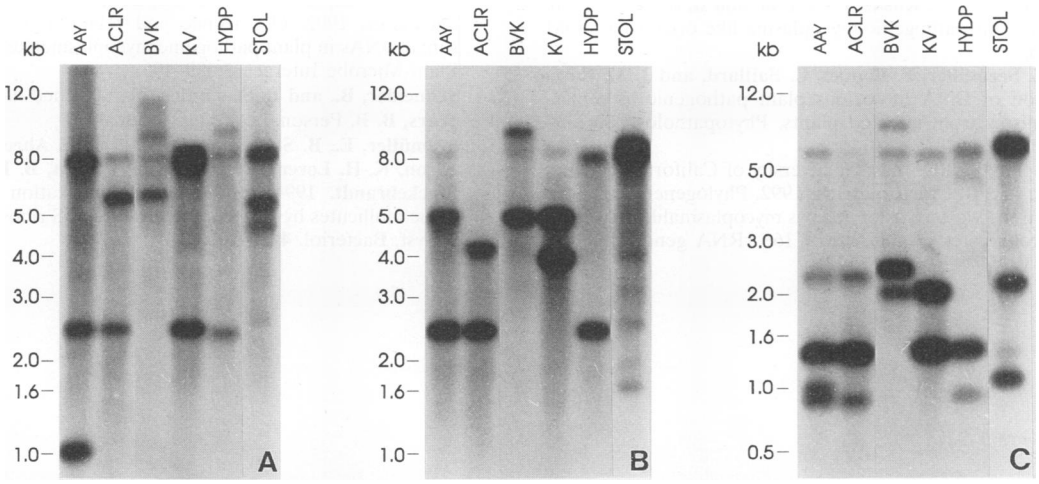

FIG. 2. Southern blot hybridization of *Eco*RI (A)-, *Hin*dIII (B)-, and *Hin*dIII-*Eco*RI (C)-digested DNA from MLO-infected plants with ribosomal probe MR16. The blots were washed at high stringency. See Table 1 and Fig. 1 for definitions of the MLO strains. Weak bands at approximately 8.0 kb in blots of *Eco*RI- and *Hin*dIII-digested DNA (e.g., strain BVK) and at approximately 6 kb in the blot of *Hin*dIII-*Eco*RI-digested DNA represent plant reactions, while strong bands at these positions (e.g., strain STOL) represent ribosomal MLO fragments. The four fragments in the lane for *Hin*dIII-digested DNA of strain STOL indicate the presence of a *Hin*dIII site in the 5′ portion of both 16S rRNA genes. There are two fragments of about 0.9 and 1.0 kb in size in *Hin*dIII-*Eco*RI-digested DNA of strain AAY.

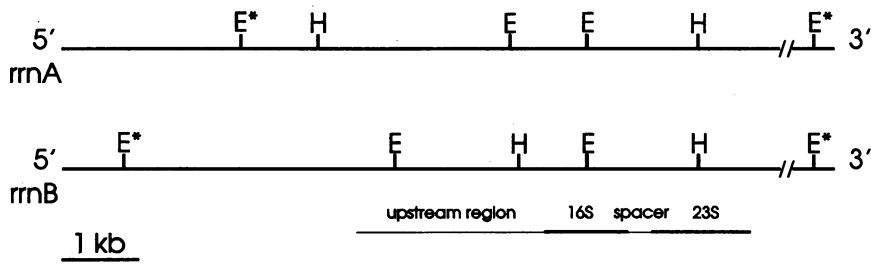

FIG. 3. Restriction maps of the two ribosomal operons of strain AAY of the AY MLO. The ribosomal fragments of several other AY strains show similar arrangements but may differ in the positions of *Eco*RI sites upstream and downstream of the rRNA genes marked with asterisks. E and H, *Eco*RI and *Hin*dIII restriction sites, respectively.

the same taxonomic cluster can further be differentiated by Southern blot hybridization with ribosomal probes.

There is evidence that the 16S rRNA genes of the two MLO rRNA operons are similar: the 16S rDNAs of operons *rrnA* of the OAY MLO and *rrnB* of the SAY MLO show a sequence similarity of 99.6% (7). Also, repeated digestions of PCR-amplified 16S rDNA of numerous MLOs with the frequently cutting enzymes *Alu*I and *Rsa*I always yielded similar restriction profiles (16, 18).

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