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A Plasmid from *S. citri* Strain M14 Hybridizes with Extrachromosomal DNAs from Other Spiroplasmas, Including Corn Stunt Spiroplasma E275, Tick Spiroplama 277F, and Coco Spiroplasma N525

C. MOUCHES, Ph.D., AND J.M. BOVÉ, Ph.D.

Laboratoire de Biologie Cellulaire et Moléculaire, INRA et Université de Bordeaux II, Domaine de la Grande Ferrade, Pont de la Maye, France

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A plasmid, pM41, has been isolated from the *Spiroplasma citri* strain M4 (group I-1) and characterized by restriction mapping. Using a ^{32}P-labeled probe specific of the plasmid, we have shown by DNA-DNA hybridization that a plasmid identical to pM41 or a closely related plasmid, is present in several, but not all, *S. citri* strains. DNA sequences that hybridize to pM41 were also identified in three other spiroplasmas not belonging to the *S. citri* species. Protein patterns of several *S. citri* strains have been compared in order to investigate the effect of pM41 on the spiroplasma protein profiles or maps. In fact, the presence of pM41 does not appear to modify the protein pattern.

INTRODUCTION

Many spiroplasmas contain extrachromosomal DNA molecules, the number and size of which may vary according to the spiroplasma strain considered [1,2,3,4]. Some of these DNA molecules have been shown to be covalently closed circular elements (ccc) and, in the absence of any detectable viruses in the cells harboring them, it was assumed they were plasmids [2,3,4].

Plasmids play a significant role in the biology of microorganisms both by the functions for which they code (e.g., pathogenicity, antibiotic resistance) and by their role in the dissemination of genetic information. However, so far, all spiroplasma plasmids are cryptic, i.e., none has yet been associated with a given phenotypic character. Also, the experimental transfer of a plasmid from one spiroplasma to another has not yet been achieved and, more generally, transfer of genetic material between mollicutes has not yet been described. In this paper, we describe the characterization of the pM41 plasmid from the *S. citri* strain M4 (group I-1, according to Junca et al. [5]) by means of restriction enzymes. Using a ^{32}P-labeled probe of this plasmid, we have identified by DNA hybridization not only several other spiroplasma strains containing DNA sequences that hybridize to pM41 but also spiroplasma strains lacking pM41-related sequences.

In addition, one- and two-dimensional protein patterns of various *S. citri* strains have been compared to investigate if the presence of the pM41 plasmid (or closely related plasmids) can affect the spiroplasma protein profiles and maps.

Address reprint requests to: Claude Mouches, Ph.D., Laboratoire de Biologie Cellulaire et Moléculaire, INRA et Université de Bordeaux II, Domaine de la Grande Ferrade, 33140 Pont de la Maye, France

RESULTS

Isolation and Endonuclease Restriction Map of Plasmid pM41

pM41 was isolated from the *S. citri* strain M4 cultured from a naturally infected periwinkle (*Vinca rosea*) in Morocco [6]. The plasmid was purified using the alkaline method of Casse et al. [7], cesium chloride centrifugation, and 0.5 percent agarose preparative gel electrophoresis. The purified plasmid pM41 has approximately 8.5 kilobase (Kb) pairs and a base composition of 22 mol % guanine plus cytosine. The pM41 restriction endonuclease map is shown in Fig. 1A. The plasmid was cut at a single site by restriction endonuclease Hind III and at three sites by MboI. The native plasmid (covalently closed circular supercoiled molecules: ccc), but not the open circular (oc) or linear forms (l), were cleaved at a unique site by the S1 endonuclease known to be specific for single-stranded regions. The plasmid was not cut by the restriction enzyme Sal I, Eco RI, Eco RV, Bam HI, or Pst I.

Occurrence of DNA Sequences That Hybridize to pM41 Among Various Spiroplasmas

A radioactive ^{32}P-pM41 probe was prepared by nick translation of the purified plasmid with *E. coli* DNA polymerase I using dCTP-^{32}Pα as radioactive substrate. The probe was used to detect the presence of pM41 hybridizable sequences in DNA from various spiroplasmas in the following way. Total DNA extracted from a given spiroplasma strain was submitted to electrophoresis on a 0.5 percent agarose gel. The separated DNA molecules were transferred from the agarose gel to a nitrocellulose sheet by "Southern blotting" [8]. The nitrocellulose sheet was then incubated for 15 hours at 65°C in the presence of the heat-denatured radioactive probe [9]. After washing off the excess probe, DNA hybrids on the sheet were detected by autoradiography.

As shown in Fig. 1B, the pM41 probe hybridized strongly with two DNA bands from *S. citri* strain M4: one band is the ccc form of pM41; the second band contains both the open circular (oc) and the linear (l) forms derived from the ccc pM41 plasmid. No detectable hybridization was observed with the chromosomal DNA (c). In addition, the pM41 probe hybridized strongly with some but not all extrachromosomal DNA bands of two other *S. citri* strains: Iran and Arizona. Furthermore, hybridization was also obtained with strain E275 of the corn stunt spiroplasma (CSS, group I-3) and strain 277F of the tick spiroplasma (group I-4). In another experiment, we found that the pM41 probe also hybridized strongly with extrachromosomal DNA bands from the *S. citri* strain ASP1 and with strain N525 from *Cocos nucifera* (group I-7). As shown in Fig. 1B and Table 1, no hybridization was observed with DNAs from several other *S. citri* strains: R8A2, Algeria, MH, R7A10, Israel, CES 3033, or from various other spiroplasmas of different groups or subgroups. Additional DNA bands showed some radioactivity only after much longer exposure times of the X-ray film on the nitrocellulose sheet. The significance of this label remains to be investigated. It may be due to some short common sequences between various plasmids, such as the origin of replication, for instance.

To determine the extent of DNA-DNA homology between the various extrachromosomal DNAs, we undertook the following experiment. An aliquot of each total DNA preparation was digested until completion with Hind III and MboI, then submitted to electrophoresis on 1.2 percent agarose gels. Under these conditions, the pM41 plasmid generated four fragments, A, B, C, D (Fig. 1A). The separated DNA restriction fragments were transferred on nitrocellulose by "Southern blotting," then

TABLE 1
pM41 Probe Hybridizes with Extrachromosomal DNA of Only Certain Spiroplasma Strains

Group	Name	Host of Origin	Hybridization	No Hybridization
I-1	*S. citri*	Citrus or periwinkles	M4, Iran, Arizona, ASP1	R8A2, Algeria, MH, R7A10, Israel, CES 3033
I-2		Honeybee		BC3, B88, B63
I-3		Corn with stunt disease	E275	
I-4		Rabbit tick	277F	
I-5		Green leaf bug		LB12
I-6		Surface of flowers		M55
I-7		*Cocos nucifera*	N525	
III	*S. floricola*	Surface of flowers		OBMG, BNR1
IV	*S. apis*	May disease-affected honeybees		B31
		Surface of flowers		F1
VII		Monobia wasp		MQ1
VIII		Syrphid fly		S1

hybridized with the ^{32}P-pM41 probe. With DNA of the homologous *S. citri* strain M4, the probe hybridized with each of the four pM41-derived restriction fragments (Fig. 1C, track 3). The same hybridization pattern was also obtained with restricted DNA of the *S. citri* strain ASP1 (result not shown). With restricted DNAs of the *S. citri* strains Iran and Arizona, the probe also hybridized strongly with four fragments, of which three had the same electrophoretic mobility as fragments A, D, and C from the pM41 digest (Fig. 1C, tracks 6 and 7); the fourth fragment B* had a molecular weight only slightly higher than the B fragment generated from pM41. In contrast, the DNA bands of spiroplasma 277F (Fig. 1C, track 5) or of the corn stunt spiroplasma (result not shown) that hybridized with pM41 were not split under the same conditions by Hind III or MboI, probably because of base replacement or methylation within the sequences recognized by the restriction enzymes.

From these results we conclude that plasmid pM41 of the *S. citri* strain M4, or a closely related plasmid, is also present in *S. citri* strains Iran, Arizona, and ASP1. The same plasmid is also very probably present in strain E275 of the corn stunt spiroplasma (I-3), in the 277F tick spiroplasma (I-4), and in strain N525 from *Cocos nucifera* (I-7). On the contrary, several *S. citri* strains and many other spiroplasmas do not contain DNA sequences identical or nearly identical to those of plasmid pM41 or, if they do, they do so only at a very low level number.

Influence of Plasmid pM41 on Spiroplasma Protein Pattern

Many spiroplasmas contain extrachromosomal DNA molecules and especially plasmid DNA [1,2,3,4]. The presence of such extrachromosomal DNA drastically affects the electrophoretic profile of the restriction endonuclease fragments of DNA from several *S. citri* strains [1]. Also, as discussed elsewhere [10], it may affect the results of DNA-DNA hybridization experiments. When spiroplasma proteins were analyzed on polyacrylamide gels, about 50 bands were seen on the protein profile upon one-dimensional SDS-PAGE and more than 300 spots on the map resulting from two-dimensional analyses [11].

The question arises as to whether some of these spots or bands might be due to the presence of extrachromosomal DNA.

The one- and two-dimensional protein patterns of the *S. citri* strains which contained pM41 sequences have been compared with those of the *S. citri* strains lacking

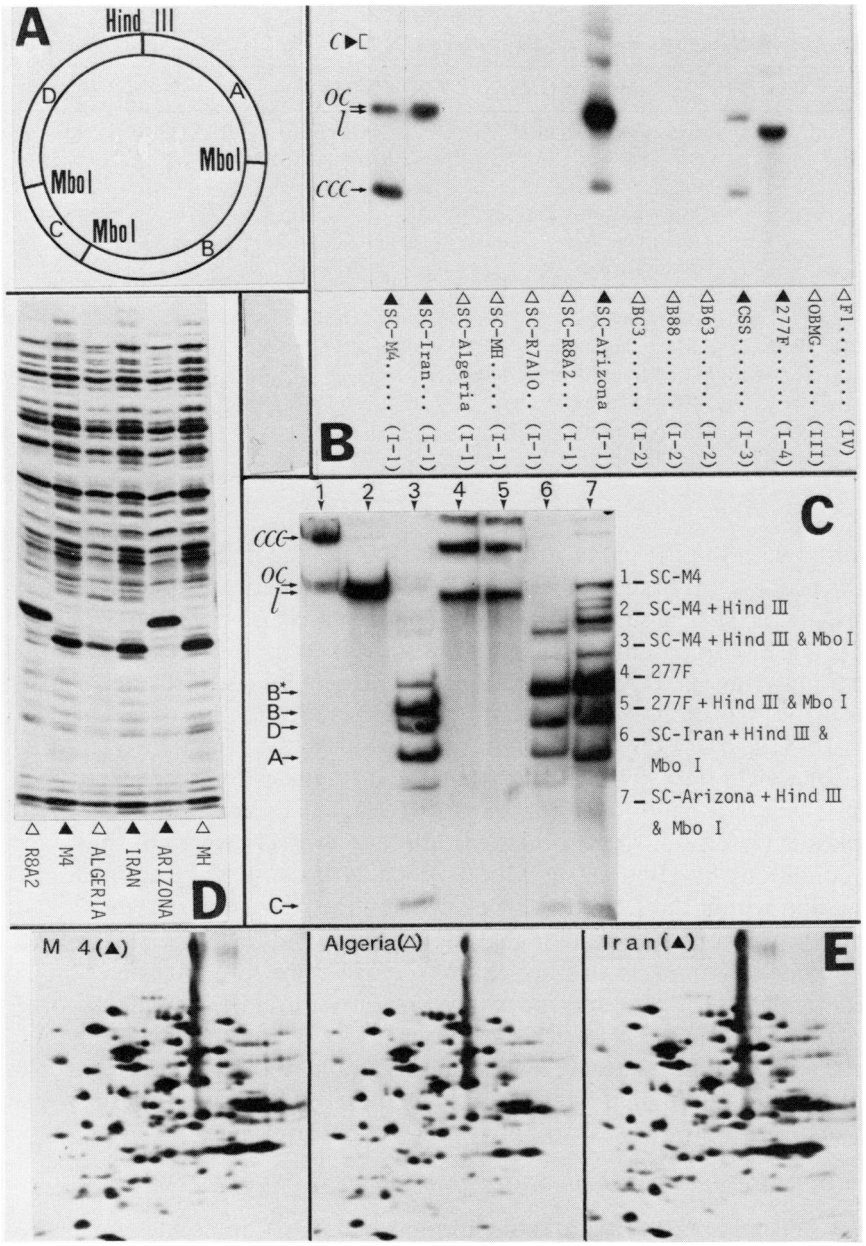

FIG. 1. **A** Endonuclease restriction map of plasmid pM41. **B** Radioautography showing the patterns of hybridization between the ^{32}P-pM41 probe and various DNAs separated on a 0.5 percent agarose gel and immobilized on a nitrocellulose sheet upon "Southern blotting." Two μg of each DNA preparation were submitted to electrophoresis. The specific radioactivity of the probe was 2×10^8 cpm/μg. Autoradiography was carried out at 20°C for two hours using Cronex type 4 X-ray film and Cronex intensifying screens. **C** Radioautography showing the patterns of hybridization between the ^{32}P-pM41 probe and various DNAs and DNAs restriction fragments separated on a 1.2 percent agarose gel and transferred to a nitrocellulose sheet. Twenty μg of DNA or endonuclease DNA digests were submitted to electrophoresis. The specific radioactivity of the probe was 2×10^7 cpm/μg. The autoradiography exposure time was 72 hours. **D** and **E** One- and two-dimensional analysis of proteins from *S. citri* strains containing plasmid pM41 or a closely related plasmid (▲) and from *S. citri* strains lacking pM41-related sequences (△).

such DNA sequences. As shown in Fig. 1D and E, no specific protein(s) could be associated with the presence of the plasmid pM41. Thus, the presence of plasmid pM41 does not appear to modify the protein pattern as seen on the gels.

CONCLUSION

Using a ^{32}P-labeled probe specific of pM41, we have shown by DNA-DNA hybridization that a plasmid identical to pM41 or a closely related plasmid, is present in several, but not all, *S. citri* strains and also in three other spiroplasmas not belonging to the *S. citri* species. Hence, the same plasmid can be shared by different spiroplasmas. So far, all spiroplasma plasmids are cryptic, i.e., none has yet been associated with a given phenotypic character. No specific protein(s) could be associated with the presence of plasmid pM41. However, the identification of certain spiroplasma strains harboring the same or nearly the same plasmid will be useful in finding a phenotypic marker specific of this plasmid.

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