Characterization of Two Plasmids from Spiroplasma Citri Strains MH and M4: Their Distribution Among Other Spiroplasma Strains or Species

C. Mouches, G. Barroso, A. Gadeau, and J. M. Bove

ABSTRACT. As a preliminary to the determination of the functions of *S. citri* plasmids, two such plasmids, pMH1 (7 Kbp) and pM41 (8 Kbp), have been purified from two *S. citri* strains, MH and M4 respectively. The plasmids were mapped by restriction endonucleases.

Using *in vitro* recombinant DNA technology, the linearized *S. citri* plasmid pMH1 could be introduced into the Hind III site of *E. coli* plasmid pBR328. The recombinant molecule was able to transform *E. coli* strain HB101 and replicate in the bacterial host. Cloning of a spiroplasma plasmid in a bacterial cell has thus been achieved.

Using a ^{32}P-labeled probe 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. Similarly, a radioactive probe specific for plasmid pMH1 allowed us to detect related DNA sequences among various other spiroplasmas.

Spiroplasmas are motile Mollicutes with helical morphology (3). *Spiroplasma citri* was the first such organism to have been cultured and characterized (11) ; it is the causal agent of citrus stubborn disease (6).

Many spiroplasmas, including several *S. citri* strains, contain plasmids of size between 2 and 50 kilobase pairs (kbp) (3, 9, 10). Plasmids play a significant role in the biology of microorganisms both by the functions for which they code (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, as a preliminary step to study the functions of spiroplasma plasmids and their role in genetic transfer among organisms, we have characterized two plasmids from *S. citri* by restriction mapping and, upon *in vitro* DNA recombination with plasmid pBR328 as a vector, we have been able to clone one of them in *E. coli*. In addition, radioactive probes specific for each plasmids were used to further investigate their distribution among various spiroplasmas.

MATERIALS AND METHODS

Spiroplasma Isolates

The designation and origin of the *S. citri* strains used in this work were described previously (2, 3, 7) except the spiroplasma isolates Arizona strain 608, provided by Dr. R. Allen, and ASP1 made available to us by P. Townsend.

The origin of the other spiroplasma strains used in this work is listed in table 1 according to the classification proposed by Junca *et al.* (5), and further extended by Whitcomb et al. (15). All spiroplasmas were triply cloned by conventional procedures.

Procedure for Plasmid Osolation

Growth of spiroplasmas. Spiroplasmas were grown in BSR

TABLE 1
ORIGIN AND CLASSIFICATION OF THE SPIROPLASMA STRAINS USED

Group	Name	Strains	Host of origin
I-1	*S. citri*	R8A2, M4, MH,.	Citrus or Periwinkles
I-2	—	BC3	Honey Bee
		B88, B63	Honey Bee
I-3	—	E275	Corn with Stunt Disease
I-4	—	277F	Rabbit Tick
I-5	—	LB12	Green Leaf Bug
I-6	—	M55	Surface of Flowers
I-7	—	N525	*Cocos Nucifera*
III	*S. floricola*	OBMG, BNR1	Surface of Flowers
IV	*S. apis*	B31	May Disease Affected Honey Bees
	—	F1	Surface of Flowers
VII	—	MQ1	Monobia Wasp
VIII	—	EA1	Syrphid Fly

medium at 32°C (2). Cells from a 250 ml culture were harvested at the end of the exponential phase of growth by centrifugation in a 250 ml plastic tube at 10,000xg for 20 minutes at 4 C. The pellet was frozen at –20 C for 2 hours before DNA extraction.

Preparation of DNA extracts by alkaline lysis and phenol extraction. The whole procedure was carried out at room temperature. Immediately before extraction, 50 ml of "lysing buffer" [0.05 M Tris-HCl, pH 7.4, 0.02 M ethylenediamine tetraacetic acid, disodium salt (EDTA), 1% (w/v) sodium dodecyl sulfate (SDS)], were adjusted with 4 M NaOH to pH 12.4 using a numerical pH-meter previously standardized at pH 10 with a Beckman Standard pH 10 buffer.

Ten ml of "lysing buffer" at pH 12.4 were added to the spiroplasma pellet. After partial resuspension using a 10 ml glass pipet, the suspension was transferred to a 125 ml glass-stoppered measuring cylinder. The volume of the lysate was adjusted to 50 ml using pH 12.4 "lysing buffer." The organisms were allowed to lyse for 20 minutes, with occasional homogenization of the suspension by passage through a 10 ml glass pipet.

The pH of the lysate was lowered to 8.5-8.9 by adding 4 ml of 1 M Tris buffer pH 8.0 and stirring the mixture for 2 minutes. Six ml of 5 M NaCl were added and the mixture was slowly stirred with a magnetic stirrer for additional 20 minutes during which a white precipitate appeared.

Sixty ml of phenol saturated with 0.5 M NaCl were added and the resulting emulsion was stirred for 2.5 minutes, then centrifuged at 5,000xg for 10 minutes. The aqueous upper phase was transferred into a 125 ml glass stoppered measuring cylinder, and one volume of a chloroform-isoamyl alcohol (24:1) mixture was added. After stirring for 2.5 minutes, the emulsion was broken by centrifugation at 5,000xg for 5 minutes. The clear aqueous upper phase was transferred to glass centrifuge tubes and 2 volumes of cold (–20 C) 95% ethanol were added to precipitate the DNA from the aqueous phase. The tubes were kept at –20 C overnight.

The precipitated DNA was recovered by centrifugation at 5,000 xg at 0 C for 20 minutes. The ethanolic solution was removed from the tubes by decantation and the DNA pellets were dissolved in 3 ml of sterile water. Just before electrophoresis, 0.2 ml of SB buffer [5% (w/v) SDS, 20% (w/v) Ficoll, 0.2% (w/v) bromophenol

blue], were added to 0.8 ml of the DNA solution.

Preparative and analytical electrophoresis of DNA. Preparative or analytical electrophoresis on 0.7% agarose gel (25 cm long, 20 cm large, 0.5 cm thick) was carried out with the system for submerged electrophoresis from BioRob Labs (model H4). The gel was prepared from 1.4 g of agarose (BRL, gel electrophoresis grade) in 200 ml of Tris.acetate electrophoresis buffer [40 mM Tris base, 2 mM EDTA, 12 mM sodium acetate, pH 8.1]. Electrophoresis was carried out for 16 hours at a constant 40 volts. At the end of the electrophoretic run, the gel was stained for 30 minutes in an ethidium bromide solution (2 μg/ml) and the DNA bands were visualized by U.V. illumination at 302 nm. The bands of interest visualized under U.V. light were sliced out and the plasmid DNA was recovered from the agarose gel by a freeze-squeeze technique described elsewhere (8).

Mapping of Plasmids with Restriction Endonucleases

Restriction endonucleases were obtained from New England Biolabs. Reaction conditions were those specified by the supplier. When samples were digested with two or more enzymes, the digestion requiring the lowest salt concentration was done first. Electrophoresis on 1.2% agarose gel was in Tris-acetate buffer pH 8.1.

RESULTS

Isolation of S. Citri Plasmids pMH1 and pM41 and Mapping by Restriction Endonucleases. Two plasmids, pMH1 with 7 kbp and pM41 with 8 kbp were purified from the triply cloned *S. citri* strains MH and M4 respectively. The MH strain was cultured from a stubborn affected sweet orange tree growing in Morocco (3); strain M4 was isolated from a naturally infected periwinkle from Morocco also (2). Plasmids were characterized by agarose gel electrophoresis upon cleavage with restriction enzymes. As shown in figure 1, pMH1 contained a unique site for the restriction endonucleases Hind III, MboI (and its isoschizomer Sau III A I), and several sites for Hinc II; pM41 contained a unique Hind III restriction site and three MboI sites. The two plasmids were not cut by Bam HI, EcoRI, Pst I, Sal I or Sma I. From

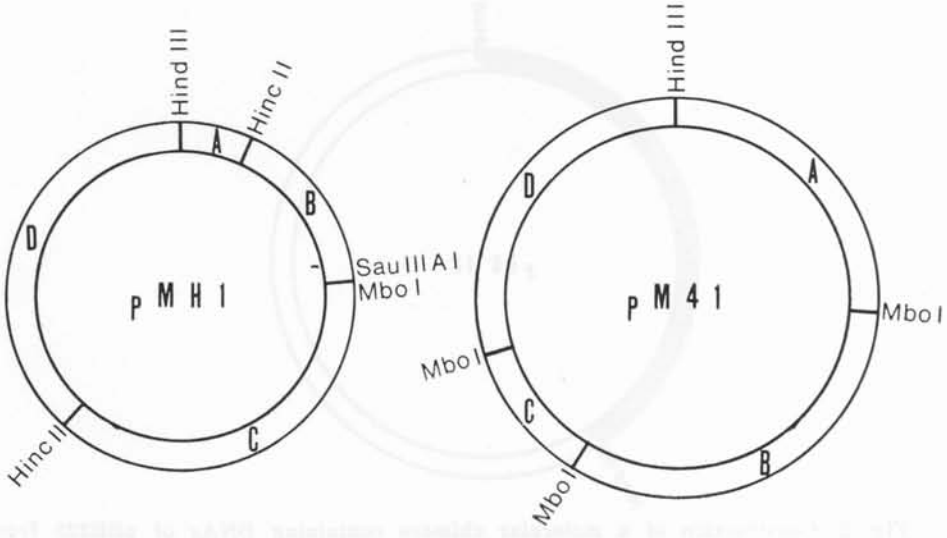

Fig. 1. Restriction cleavage maps of pMH1 and pM41.

these restriction maps, the two plasmids are clearly different.

Cloning of a Plasmid from S. Citri **in** E. Coli **Using** In Vitro **Recombinant DNA Technology.** Using *in vitro* recombinant DNA technology, we have constructed molecular chimeras containing plasmid DNA from *S. citri* and *E. coli* (figure 2). The *E. coli* plasmid used was pB328, a plasmid which confers resistance to ampi-

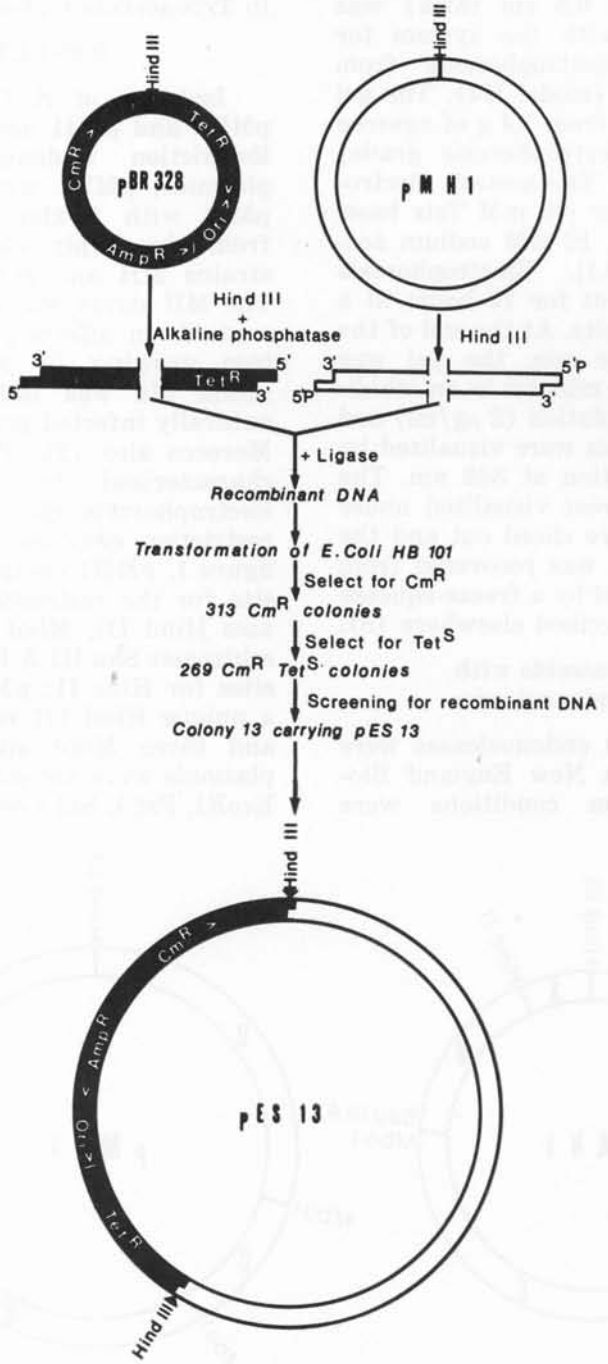

Fig. 2. Construction of a molecular chimera containing DNAs of pBR328 from E. coli and pMH1 from S. citri.

cillin (AmpR), chloramphicol (CmR) and tetracycline (TetR) (12). This plasmid was converted to an efficient cloning vector (4.9 kbp) by linearization by the restriction enzyme Hind III and treatment with alkaline phosphatase (4). As pMH1 contained a unique site for Hind III, the chimeras were constructed by ligation of 3 μg of Hind III-cleaved pMH1 DNA with 5 μg of the pBR328-derived cloning vector. Twenty ng of the ligase reaction products were used to transform 10^9 *E. coli* HB101 cells (14). *E. coli* transformants that expressed chloramphenicol resistance (CmR) were selected on solid medium containing 25 μg of antibiotic per ml. They were further screened for their tetracycline sensitivity (TetS), since introduction of *S. citri* plasmid DNA into the unique Hind III site of pBR328 will inactivate the tetracycline resistance determinant coded by the *E. coli* plasmid.

In this way, 313 CmR colonies were obtained. Among them, 269 transformants had a CmR TetS phenotype. Nineteen colonies were selected and their plasmid DNA was extracted using a miniscreening method (4). They were further characterized by 0.7% agarose gel electrophoresis, before and after digestion by Hind III or EcoRI. Fourteen of the DNA extracts each contained a plasmid yielding, upon EcoRI linearization, a unique DNA fragment of about 12 kbp; upon treatment by Hind III, two fragments, one of which corresponded in size to the Hind III cleaved-pMH1 DNA of 7.0 kbp and the second to the 4.9 kbp pBR328-derived DNA vector. One such plasmid, plasmid pES13 from colony # 13, was further purified upon amplification by spectinomycin added at 300 μg/ml to the growth medium. The DNA fragments generated respectively from pES13 and pMH1 by the restriction nucleases BamH1, EcoRJ, Rinc II, Hind III, PstI, SaI1 and Sau IIIAI used alone or in combination were compared by electrophoresis on 1.2% agarose gels. From the patterns obtained, it was clear that indeed pE13 contains one pMH1 sequence as an insert (figure 2).

Occurrence of pM41 or pMH1 Hybridizable DNA Sequences in Other Spiroplasmas. A radioactive ^{32}P-pM41 probe was prepared by nick translation of the purified plasmid with *E. coli* DNA polymerase I. The probe was used to detect the presence of pM41 hybridizable sequences in DNA from various spiroplasmas. Total DNA extracted from a given spiroplasma strain was submitted to electrophoresis on a 0.5% agarose gel. The separated DNA molecules were transferred from the agarose gel to a nitrocellulose sheet by "Southern blot" (13).

The nitrocellulose sheet was then incubated for 15 hours at 65 C in the presence of the heat denatured probe (1). After washing off the excess probe, DNA hybrids on the sheet were detected by autoradiography.

As shown in figure 3, the pM41 probe hybridized strongly with two DNA bands from *S. citri* strain M4: one band is the covalently closed circular (ccc) form of pM41; the second band contains both the open circular (oc) and the linear (1) forms derived from the ccc pM41 plasmid. No detectable hybridization was observed with the chromosomal DNA (c). In addition, the pH41 probe hybridized strongly with some, but not all, extrachromosomal DNA bands of two other *S. citri* strains from 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). More recently, we found that the

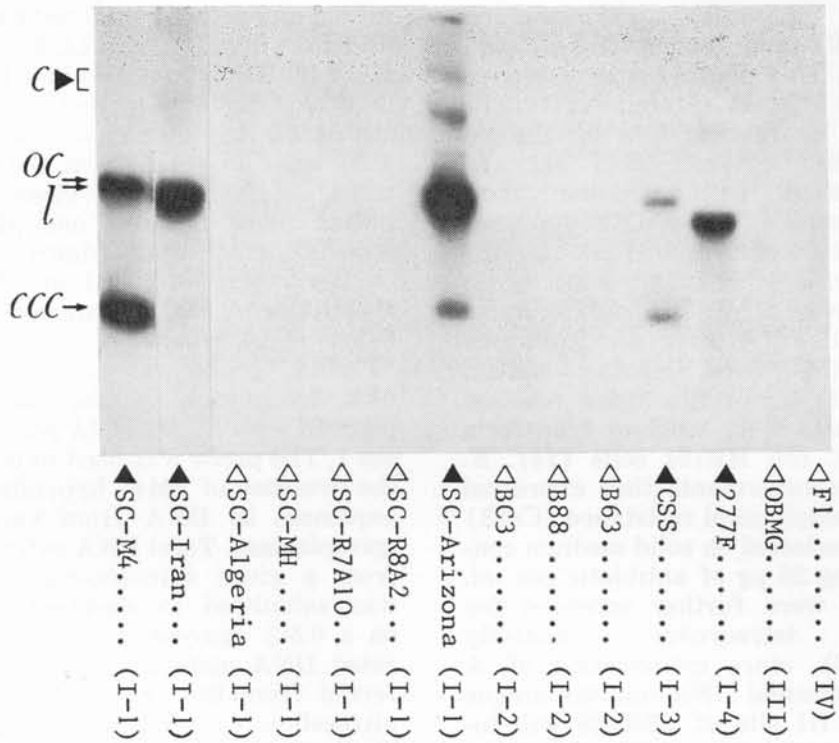

Fig. 3. Hybridization of the pM41 probe with plasmid DNA of various spiroplasma strains.

pM41 probe hybridized strongly also with extrachromosomal DNA bands from a third *S. citri* strain, ASP1, and with strain N525 from *Cocos nucifera* (group I-7) (Table II). No hybridization was observed with DNAs from several other *S. citri* strains, including the MH strain, or from various other spiroplasmas of different groups or subgroups (figure 3 and table 2).

In addition we have shown elsewhere (9) that the homologies that are detected in these hybridization experiments were not due only to short common sequences between various plasmids but that the complete plasmid pM41 of the *S. citri*

TABLE 2
HYBRIDIZATION OF CLONED pM41 PROBE FROM *SPIROPLASMA CITRI* WITH PLASMID DNA OF DIFFERENT SPIROPLASMA STRAINS

Group	Hybridization	No Hybridization
I-1	M4, Iran, Arizona, ASP1	R8A2, Algeria, MH, R7A10, Israel, CES 3033
I-2	—	BC3, B88, B63
I-3	E275	—
I-4	277F	—
I-5	—	LB12
I-6	—	M55
I-7	N525	—
III	—	OBMG, BNR1
IV	—	F1, B31
VII	—	MQ1
VIII	—	EA1

TABLE 3
HYBRIDIZATION OF pES13 PROBE WITH DNA FROM VARIOUS SPIROPLASMA STRAINS OR SPECIES

Group	Hybridization	No Hybridization
I-1	MH, M4, R7A10, Algeria	R8A2, Iran, ASP1
I-2	B88	—
I-3	E275	—
I-4	277F	—
I-5	LB12	—
I-6	—	M55
III	—	BNR1
IV	—	B31
VII	—	MQ1
VIII	—	EA1

strain M4, or at least a very closely related plasmid, was also present in other *S. citri* strains (but not all) and in spiroplasmas other than *S. citri*. Hence, the same plasmid can be shared by different spiroplasmas.

In recent experiments, we have used a ^{32}P-pES13 probe to detect the presence of pMH1 hybridizable sequences in various spiroplasmal DNA extracts. As shown in table 3, DNA sequences hybridizable with pMH1 from *S. citri* strain MH were indeed detected in a number of spiroplasmal DNA extracts. Hence, again, identical DNA sequences can be shared by different spiroplasmal plasmids DNAs.

CONCLUSION

On the basis of all these results we conclude that *S. citri* plasmid pMH1 was introduced upon linearization into the Hind III site of *E. coli* plasmid pBR 328; that the recombinant molecule was able to transform *E. coli* strain HB 101, and that it replicated in the bacterial host. Cloning of a spiroplasma plasmid in a bacterial cell has thus been achieved.

In parallel experiments, Hind III cleaved-pM41 was ligated to the Hind III-cleaved pBR 328 vector. The efficiency of ligation was checked by agarose gel electrophoresis of the ligated DNAs and the ligation products were used to transform *E. coli* HB 101. However, we failed to detect any recombinants carrying DNA from pM41 in pBR 328. This result is in contrast to that obtained with pM41 above.

LITERATURE CITED

1. BOTCHAM, M., W. TOPP, and S. SAMBROOK
   1976. The arrangement of Simian virus 40 sequences in the DNA of transformed cells. Cell 3: 276-287.
2. BOVE, J. M., J. C. VIGNAULT, M. GARNIER, C. SAILLARD, O. GARCIA-JURADO, C. BOVE, and A. NHAMI
   1978. Mise en évidence de *Spiroplasma citri*, l'agent causal de la maladie du "stubborn" des agrumes dans les pervenches (*Vinca rosea L.*) ornementales de la ville de Rabat, Maroc. C. R. Acad. Sci., Sér. D, 286: 57-60.
3. BOVE, J. M. and C. SAILLARD
   1979. Cell biology of spiroplasmas. P. 83-153, *In* R. F. Whitcomb and J. G. Tully (eds.), "The Mycoplasmas," Vol. III. Plant and insect mycoplasmas. Academic Press, Inc. New York.
4. GOODMAN, H. M. and R. J. MAC DONALD
   1979. Cloning of hormone genes from a mixture of cDNA molecules. Methods in Enzymology 68: 75-90.
5. JUNCA, P., C. SAILLARD, J. G. TULLY, O. GARCIA-JURADO, J. R. DEGORCE-

DUMAS, C. MOUCHES, J. C. VIGNAULT, R. VOGEL, R. E. McCOY, R. F. WHITCOMB, D. L. WILLIAMSON, J. LATRILLE, and J. M. BOVE
1980. Caractérisation de spiroplasmes isolés d'insectes et de fleurs de France continentale, de Corse et du Maroc. Proposition pour une classification des spiroplasmes. C. R. Acad. Sci., Sér. D 290: 1209-1212.

6. MARKHAM, P. G., R. TOWNSEND, M. BAR-JOSEPH, M. J. DANIELS, A. PLASKITT, and B. M. MEDDINS
1974. Spiroplasmas are the causal agents of citrus little-leaf disease. Ann. Appl. Biol. 78: 49-57.

7. MOUCHES, C., J. C. VIGNAULT, J. G. TULLY, R. F. WHITCOMB, and J. M. BOVE
1979. Characterization of spiroplasmas by one- and two-dimensional protein analysis on polyacrylamide slab gels. Current Microbiol. 2: 69-74.

8. MOUCHES, C.
1983. Occurrence and identification of plasmids in spiroplasmas. Proc. 2nd Mycoplasma Technique Course, Bordeaux.

9. MOUCHES, C. and J. M. BOVE
1983. A plasmid from *S. citri* strain M4 hybridizes with extrachromosomal DNAs from other spiroplasmas, including corn stunt spiroplasma E275 and tick spiroplasma 277F. J. Biol. Med. (in press).

10. RANHAND, J. M., W. O. MITCHELL, T. J. POPKIN, and R. M. COLE
1980. Covalently closed circular deoxyribonucleic acids in spiroplasmas. J. Bacteriol. 143: 1194-1199.

11. SAGLIO, P., M. L'HOSPITAL, D. LAFLECHE, G. DUPONT, J. M. BOVE, T. G. TULLY, and E. A. FREUNDT
1973. *Spiroplasma citri* gen. and sp. n.: a mycoplasma-like organism associated with "stubborn" disease of citrus. Int. J. Syst. Bacteriol. 23: 191-204.

12. SOBERON X., L. COVARRUBIUS, and F. BOLIVAR
1980. Construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. Gene 9: 287-305.

13. SOUTHERN, E.
1979. Gel electrophoresis of restriction fragments. Methods in Enzymology 68: 152-176.

14. VILLA-KOMAROFF, L., A. EFSTRATIADIS, S. BROOME, P. LOMEDICO, R. TIZARD, S. P. NABER, W. L. CHICK, and W. GILBERT
1978. A bacterial clone synthesizing proinsulin. Proc. Nat. Acad. Sci. 75: 3727-3731.

15. WHITCOMB, R. F., J. G. TULLY, T. B. CLARK, D. L. WILLIAMSON, and J. M. BOVE
1982. Revised serological classification of spiroplasmas, new provisional groups, and recommendations for serotyping of isolates. Current Microbiol. 7: 291-296.