JOURNAL OF BACTERIOLOGY, Apr. 1991, p. 2225–2230
0021-9193/91/072225-06$02.00/0

Vol. 173, No. 7

First Step toward a Virus-Derived Vector for Gene Cloning and Expression in Spiroplasmas, Organisms Which Read UGA as a Tryptophan Codon: Synthesis of Chloramphenicol Acetyltransferase in *Spiroplasma citri*

C. STAMBURSKI, J. RENAUDIN, AND J. M. BOVE*

Laboratoire de Biologie Cellulaire et Moléculaire, Institut National de la Recherche Agronomique et Université de Bordeaux II, B.P. 81, 33883 Villenave d'Ornon Cedex, France

Received 3 October 1990/Accepted 16 January 1991

Spiroplasmas are wall-less procaryotes in which the UGA codon serves not as a stop signal but as a code for the amino acid tryptophan. Spiroplasma genes that contain UGA codons thus cannot be studied in the usual *Escherichia coli* cloning and expression systems. Although this problem can be circumvented by using UGA-suppressor strains of *E. coli*, spiroplasmas themselves would provide a more efficient cloning and expression host. We have now successfully employed the replicative form (RF) of a filamentous spiroplasma virus (SpV1) to clone and express the *E. coli*-derived chloramphenicol acetyltransferase (CAT) gene in *Spiroplasma citri*. The CAT gene was inserted in one of the four intergenic regions of the SpV1 RF and introduced into cells by electroporation. Both the RF and the virion DNA produced by the transfected cells contained the CAT gene sequences. Northern blot analysis, primer extension, and S1 mapping showed that transcription of the CAT gene started from a promoter located on the SpV1 RF and was terminated downstream of the CAT gene, still within the viral RF. Expression of the CAT gene was demonstrated by acetylation of chloramphenicol by cell-free extracts from the transfected spiroplasmas.

Mollicutes (mycoplasmas) are wall-less procaryotes that have arisen by degenerative evolution from ancestors of gram-positive eubacteria with low guanine-plus-cytosine (G+C) content in the DNA, such as *Clostridium innocuum* and *Clostridium ramosum* (24, 28). With genome sizes of 600 to 1,700 kbp and G+C contents of 25 to 33%, they represent the smallest self-replicating cells. Spiroplasmas, with a 1,700-kbp genome, are unique among the mollicutes in possessing helical morphology and motility. They were first discovered as pathogens in plants; *Spiroplasma citri* is the causal agent for citrus stubborn disease (12, 20), and *Spiroplasma kunkelii* is the causal agent for corn stunt disease (26, 27). Since 1970, when *S. citri* was cultured for the first time, more than 50 different spiroplasmas have been isolated, primarily from insects and ticks. The frequent isolation of new *Spiroplasma* spp. from insects could make this genus the largest among the procaryotes (11). The cellular and molecular biology of the spiroplasmas has recently been reviewed (2).

Spiroplasmas are infected by four morphologically distinct viruses, designated SpV1, SpV2, SpV3, and SpV4 (2). Two of these viruses, SpV1 and SpV4, have single-stranded circular DNA genomes. SpV1 is a rod-shaped, nonlytic virus found in many different *Spiroplasma* species (2). SpV4, an isometric virus found only in the B63 strain of *Spiroplasma melliferum*, is lytic to *S. melliferum* but not to any other known spiroplasma (16). Bacterial equivalents of SpV1 and SpV4 are found in coliphages M13 and φX174, respectively. The entire nucleotide sequences of SpV1 and SpV4 DNAs have been determined (14, 18). The capsid protein gene of SpV4 was found to contain nine TGA triplets. In spiroplasmas (17) as well as in mycoplasmas (29), the UGA codon does not function as a stop codon but codes for the amino acid tryptophan. Spiralin, the major protein of the cell membrane of *S. citri*, could be cloned and expressed in *Escherichia coli* because it contains no tryptophan (3). Spiralin mRNA contains no UGA codons and is thus fully translated by the *E. coli* ribosomes. In contrast, the SpV4 capsid protein gene was not expressed in *E. coli* because bacterial ribosomes stop at the first UGA codon encountered on the SpV4 capsid protein mRNA. Since most *Mycoplasma* and *Spiroplasma* genes contain TGA triplets, these genes cannot be fully expressed in *E. coli* unless UGA-suppressor strains are used (19). However, suppressor strains may not be fully efficient. In this paper, we describe the first experiment toward the development of a system for the cloning and expression of foreign genes directly in *S. citri*.

MATERIALS AND METHODS

Bacteria, spiroplasmas, and viruses. *E. coli* HB101 was used for amplification of the plasmid vector pKK232-8 and its derivatives. *S. citri* R8A2 B is a low-passage-number (<10) subclone of strain R8A2 (ATCC 27556). Strain R8A2 B, like all other strains of *S. citri*, is naturally infected with an SpV1-type virus. The virus produced is called SpV1-R8A2 B. *S. citri* R8A2 HP is a high-passage-number (>500) subclone of strain R8A2 B and has permanently lost the ability to produce SpV1 virions spontaneously. However, it can be experimentally infected by SpV1-R8A2 B, after which it produces SpV1-R8A2 B virions. *S. citri* cells were grown at 32°C in SP4 medium (25).

Enzymes and chemical reagents. Restriction endonucleases and M-MLV reverse transcriptase were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). T4 DNA ligase and alkaline phosphatase were from Boehringer GmbH (Mannheim, Federal Republic of Germany). The random primed labeling kit and the labeled compounds [α-^{32}P]dCTP (3,000 Ci/mmol) and [^{14}C]chloramphenicol (60

* Corresponding author.

mCi/mmol) were from Amersham Corp. (Arlington Heights, Ill.). The chloramphenicol acetyltransferase (CAT) Genblock was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, N.J.).

Isolation of SpV1 RF DNA. *S. citri* R8A2 HP cells were grown to mid-logarithmic phase, infected with SpV1 virions at a multiplicity of infection of between 0.1 and 0.5, and harvested by centrifugation 16 h after infection. SpV1 replicative form (RF) DNA was prepared by the sodium dodecyl sulfate-NaCl lysis method (9) and was further purified by equilibrium centrifugation in a cesium chloride-ethidium bromide gradient.

In some experiments, RF DNA was prepared by the alkaline lysis method (1).

Transfection of *S. citri* cells. For electroporation, 10^9 *S. citri* cells were collected by centrifugation, suspended in 0.4 ml of 8 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4) containing 280 mM sucrose, and mixed with given amounts of DNA in 10 μl of H_2O. The mixture of cells and DNA was transferred to a cold 0.4-cm electroporation cuvette and kept on ice for 10 min. The pulse generator (gene pulser; Bio-Rad Laboratories, Richmond, Calif.) was set at 3 μF, 2.5 kV, and 1,000 Ω to generate 1-ms pulses. Two pulses were applied. After 10 min at room temperature, the cells were plated on a lawn of *S. citri* R8A2 HP grown on SP4 medium. Plaques were recorded within 30 h after plating.

Southern hybridization. Restricted or unrestricted RF DNA samples (about 100 ng) were analyzed by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with the CAT gene probe under stringent conditions (hybridization was at 42°C in 6× SSC buffer [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] containing 50% formamide, and washing was at 68°C in 1× SSC buffer). The probe was prepared by the random priming procedure (7, 8) with [α-^{32}P]dCTP as the labeled nucleotide.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated from SpV1-infected *S. citri* cells in culture by using the guanidium thiocyanate-cesium chloride method (4, 23). RNAs were separated by electrophoresis through denaturing formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with the CAT gene probe as described previously (22).

Primer extension analysis. The oligonucleotide primer (50 pmol) was hybridized to total RNA (60 μg) from SpV1-CAT-infected or CAT-free SpV1-infected *S. citri* cells and extended with reverse transcriptase as described previously (22). The reaction products were analyzed by electrophoresis through 6% denaturing polyacrylamide gels with sequencing reactions used as ladders.

S1 nuclease mapping. S1 nuclease mapping was used to determine the 3′ OH end of the CAT mRNA. The 3.1-kbp *Nco*I-*Eco*RI fragment of the SpV1-CAT RF, overlapping the putative termination site (Fig. 3B), was purified by agarose gel electrophoresis. The *Nco*I 3′ OH end was labeled by a fill-in reaction with [α-^{32}P]dCTP as the labeled nucleotide. The 3′ probe was hybridized to total RNA from SpV1-CAT-infected cells and treated with S1 nuclease as described by Stamburski et al. (22). The S1-protected fragments were analyzed by denaturing polyacrylamide gel electrophoresis.

Assay of CAT activity in cell-free extracts of *S. citri* cells. Spiroplasma cells (10^9) were collected by centrifugation 16 h after infection, washed in 1.5 ml of 8 mM HEPES buffer (pH 7.4) containing 280 mM sucrose, and resuspended in 100 μl of 250 mM Tris hydrochloride buffer (pH 7.5). Cell-free extracts were prepared by four cycles of freezing in liquid nitrogen and thawing at 37°C. Cell debris was removed by centrifugation. The supernatant was heated at 65°C for 10 min to inactivate deacetylases, clarified by centrifugation, and kept at −20°C until use. Samples (30 μl) of the extracts were incubated at 37°C for 2 h with 170 μl of the following reaction mixture: 100 μl of 250 mM Tris hydrochloride (pH 7.5), 50 μl (1 μCi) of [^{14}C]chloramphenicol (specific activity, 60 mCi/mmol), and 20 μl of acetyl coenzyme A (3.5 mg/ml) (according to Gorman et al. [10]). The acetylated forms of [^{14}C]chloramphenicol were extracted by ethyl acetate. The organic layer was dried and taken up in 20 μl of ethyl acetate. Each sample (5 μl) was spotted on silica gel thin-layer plates and submitted to ascending chromatography with chloroform-methanol (95:5) as the solvent for 90 min. The plates were then dried and autoradiographed.

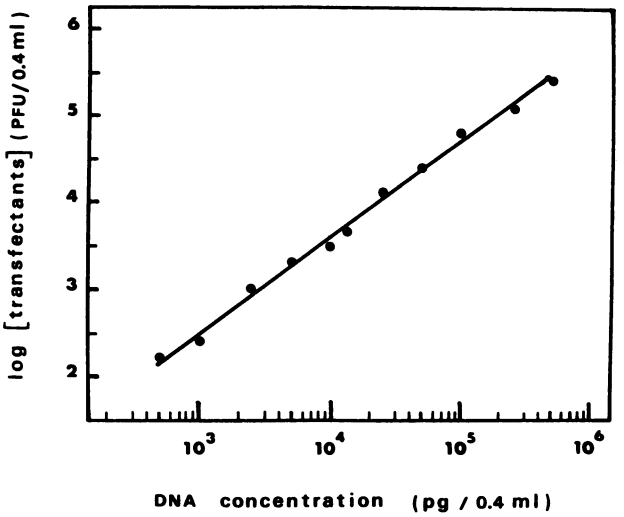

FIG. 1. Transfection of *S. citri* R8A2 HP with RF DNA of SpV1-R8A2 B. Transfectants were recorded as PFU.

RESULTS

Transfection of *S. citri* by electroporation. Transformation of spiroplasmas with viral DNA (transfection) was achieved previously with SpV4 DNA (13, 15), at which time polyethylene glycol-treated cells were used as first described by Sladek and Maniloff (21) for *Acholeplasma laidlawii* virus MVL2. However, with the polyethylene glycol technique, transfection of *S. citri* with SpV1 single-stranded virion DNA or double-stranded RF DNA was poorly reproducible and inefficient; fewer than 10^2 transfectants per μg of DNA were obtained in a variety of experimental conditions. When electroporation was used to promote penetration of DNA into the spiroplasma cells, a much higher efficiency of transfection was observed. As shown in Fig. 1, the number of transfectants is directly related to the amount of DNA used for electroporation and reaches 6×10^5 transfectants of *S. citri* R8A2 HP per μg of SpV1-R8A2 B RF DNA. Thus, electroporation is a reliable and reproducible technique for introducing SpV1 RF DNA into *S. citri* cells. Electroporation, but not the polyethylene glycol method described for *A. laidlawii* (6), also permitted successful transformation of *S. citri* R8A2 HP cells by plasmid pAM120, which carries the Tet M determinant in Tn*916*. Tetracycline-resistant *S. citri* transformants were obtained (results not shown).

CAT gene cloning in *S. citri*. The gene arrangement of

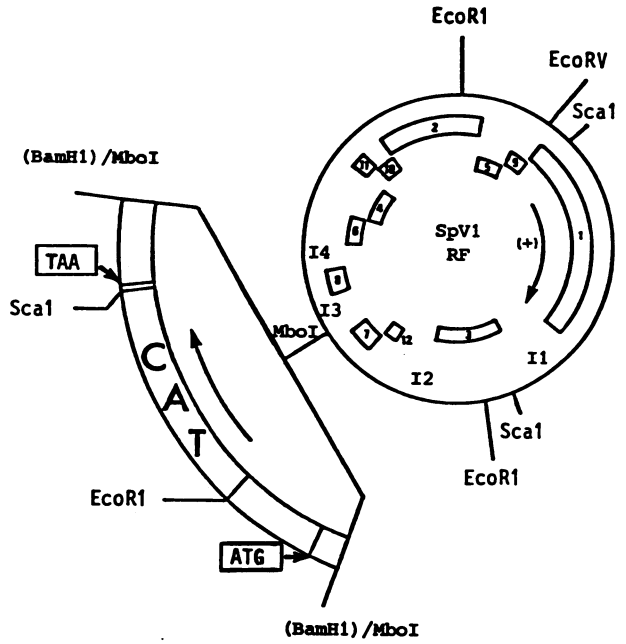

FIG. 2. Putative gene organization of the SpV1-R8A2 B RF and strategy for insertion of the CAT gene into the viral RF. ORFs 1 to 12 (boxed numerals) are distributed in all three reading frames. The four intergenic regions (I1 to I4) are indicated. Relevant restriction sites are also given. The CAT Genblock contains the complete polypeptide-coding sequence and two ribosome-binding sites but lacks the transcription promoter and terminator sequences. The coding sequence starts with ATG and ends with TAA. Arrows indicate the direction of transcription.

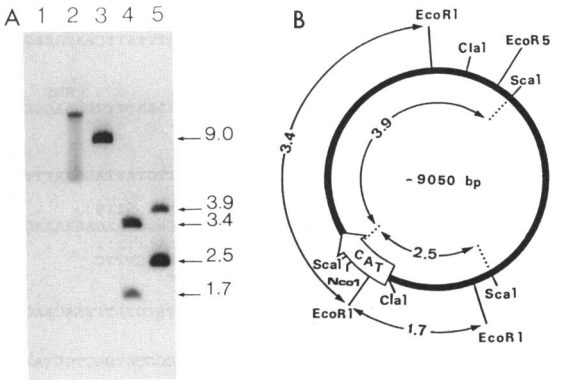

FIG. 3. Southern blot hybridization (A) and restriction map (B) of the SpV1-CAT RF. (A) Lane 1, Unrestricted RF DNA from cells infected with CAT-free SpV1 virions; lanes 2 through 5, RF DNA from cells infected with SpV1-CAT virions (lane 2, unrestricted DNA; lanes 3, 4, and 5, DNAs restricted with *Eco*RV, *Eco*RI, and *Sca*I, respectively). (B) Restriction map of the SpV1-CAT RF. The theoretical size of the RF is 9,065 bp (8,273 bp [SpV1-R8A2 B RF] plus 792 bp [CAT Genblock]). The sizes of restriction fragments are indicated.

SpV1-R8A2 B (Fig. 2) has been deduced from the nucleotide sequence (14). Twelve open reading frames (ORFs 1 to 12) and four intergenic regions (I1 to I4) have been identified. There is a unique *Mbo*I restriction site in the intergenic region I3. This *Mbo*I site was selected for the insertion of foreign DNA into the RF of SpV1-R8A2 B. The CAT gene was chosen as the foreign DNA (Fig. 2). The *Mbo*I-linearized RF (about 50 ng) and the *Bam*HI-limited CAT Genblock (about 100 ng) were ligated, and the ligation mixture was used to transfect *S. citri* R8A2 HP cells by electroporation. Virus production by the transfected cells was determined by plating the transfection mixture onto a lawn of *S. citri* cells and recording plaques. In this experiment, 71 plaques were obtained. When the CAT gene was omitted from the ligation mixture, only 17 plaques were produced. Sixteen of the 71 plaques were examined for the presence of CAT sequences in the viral DNA. For that purpose, virions from individual plaques were propagated in *S. citri* R8A2 HP cells grown in broth medium. The infected spiroplasma cells were sedimented by centrifugation, and the SpV1 virions were collected from the supernatant by high-speed centrifugation. The DNAs extracted from the infected cells and from the free virions were examined for the presence of CAT sequences by dot blot hybridization with the CAT Genblock as the probe. Fifteen of the 16 SpV1 clones gave positive hybridizations with DNA from the infected cells as well as with virion DNA, indicating that CAT sequences were present (virions carrying CAT sequences in their DNA will be called SpV1-CAT). The hybridization observed with DNA from the infected cells must be due to the presence of the CAT sequences in the viral DNA and, in particular, in the RF DNA. Therefore, the RFs of 6 of the 15 SpV1-CAT clones were purified from cells infected with these viral clones and analyzed by Southern blot hybridization with the CAT Genblock as the probe. The results were the same for all six clones, one of which is illustrated in Fig. 3A. They show that the CAT gene is inserted at the *Mbo*I site with the orientation given in Fig. 3B. Indeed, digestion of the recombinant RF with *Eco*RI or *Sca*I yields two hybridizing DNA fragments of 1.7 and 3.4 kbp for *Eco*RI (Fig. 3A, lane 4) and 2.5 and 3.9 kbp for *Sca*I (Fig. 3A, lane 5). No hybridization was obtained with the RF corresponding to the CAT-free transfection mixture (Fig. 3A, lane 1). In all six recombinant RFs examined, the CAT gene had the orientation shown in Fig. 3B. We do not yet know if the opposite orientation also occurs. These experiments show that the CAT gene has been inserted into the RF of SpV1 and cloned in *S. citri* R8A2 HP cells.

CAT gene transcription in *S. citri*. Transcription of the CAT gene was studied in *S. citri* cells infected with the SpV1-CAT virions by Northern blot hybridization. Figure 4

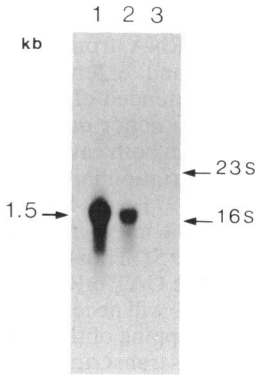

FIG. 4. Northern blot analysis of RNAs from *S. citri* R8A2 HP cells infected with SpV1-CAT or CAT-free SpV1 virions. Lanes 1 and 2, 7 and 3 μg, respectively, of RNA from SpV1-CAT-infected cells; lane 3, 5 μg of RNA from CAT-free SpV1-infected cells. Positions of 16S and 23S rRNAs are indicated.

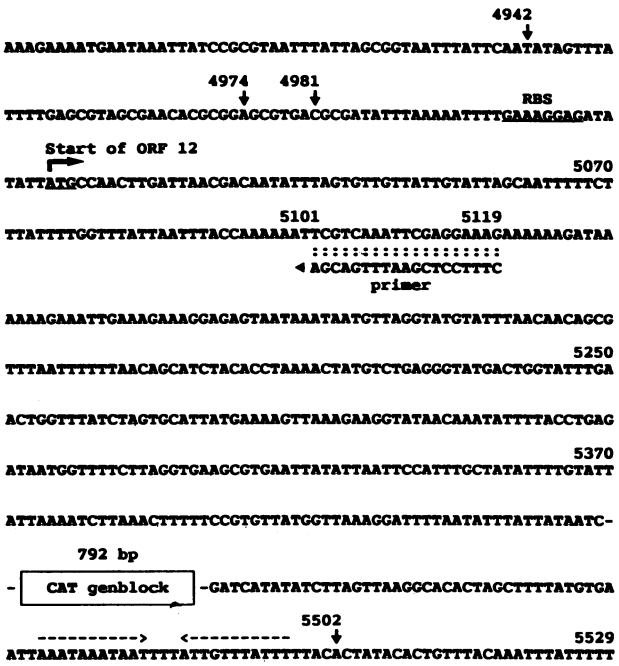

FIG. 5. Nucleotide sequence surrounding transcription initiation and termination sites of the SpV1 transcript carrying the CAT sequences. Transcription initiation and termination sites are indicated by vertical arrows. The location of the oligonucleotide used in primer extension, the start of ORF 12, the position of the CAT Genblock, and inverted repeat sequences of the transcription terminator (horizontal arrows) are also indicated. RBS, Ribosome binding site.

shows that SpV1-CAT-infected cells contain a 1.5-kb RNA that hybridizes with the CAT probe (Fig. 4, lanes 1 and 2). No such RNA is present in cells infected with the CAT-free SpV1 virions (Fig. 4, lane 3). These results indicate that the CAT gene is transcribed from the SpV1-CAT RF. Since the CAT Genblock inserted in the SpV1 RF lacks promoter and terminator sequences, transcription of the CAT gene must involve the regulatory signals present on the SpV1 RF.

Primer extension has been used to confirm that transcription initiation occurs upstream of the insertion site of the CAT gene on the SpV1 RF whether the CAT gene is present or not. A 19-mer oligonucleotide primer complementary to nucleotides 5119 to 5101 of the SpV1 RF (Fig. 5) was chosen, hybridized to the total RNA from SpV1- or SpV1-CAT-infected *S. citri* cells, and extended with reverse transcriptase. Three major extended cDNAs (139, 146, and 178 nucleotides long) and two minor ones (135 and 136 nucleotides long) were detected in both cases (Fig. 6, lanes 1 and 2). The corresponding transcription initiation sites are at nucleotides 4985, 4984, 4981, 4974, and 4942, respectively, of the SpV1 RF, all five within the intergenic region I2 immediately upstream of ORF 12 (Fig. 5). Transcription of an mRNA of 1.5 kb (i.e., the size of the CAT mRNA [Fig. 4]) from either one of these initiation sites will necessarily involve the whole CAT gene. Indeed, S1 mapping of the 3′ OH end of the CAT mRNA has shown that transcription of the CAT gene terminates within the SpV1 RF, downstream of the CAT gene. Figure 7 shows that the major S1-protected fragment is 312 nucleotides long, corresponding to a transcription stop at nucleotide 5502 of the SpV1 RF (i.e., 73 nucleotides downstream of the CAT Genblock). Interestingly, this transcription termination site immediately follows an inverted repeat sequence (nucleotides 5473 to 5497) that could be involved as a transcription terminator.

Translation of the CAT gene in *S. citri*. To determine if the CAT gene is translated in the SpV1-CAT-infected spiroplasma cells, we looked for CAT activity in cell-free extracts prepared from these cells. Figure 8 shows that, indeed, acetylation of [^{14}C]chloramphenicol occurred in the SpV1-CAT-infected cells (Fig. 8, lane 3) but not in those infected with the CAT-free SpV1 (Fig. 8, lane 2). The level of CAT activity in spiroplasma cells (Fig. 8, lane 3) seems to be similar to that obtained in *E. coli* cells transformed by promoter selection plasmid pKK232-8 carrying the CAT gene under the control of *S. melliferum* SpV4 promoter P0 (22) (Fig. 8, lane 4).

DISCUSSION

Electroporation has permitted reproducible transfection of *S. citri* with SpV1 RF DNA. The relatively high efficiency of transfection (close to 10^6 transfectants per μg of DNA) allowed us to investigate the use of the SpV1 RF as a vehicle to clone and express foreign genes in spiroplasmas. A major advantage in using SpV1 RF DNA is that spiroplasmas transfected by the SpV1 RF yield virions, the presence of which is revealed by turbid plaque formation on a lawn of indicator cells. Plaque formation is not due to cell lysis but results from the slower growth of the infected cells than of the noninfected cells. Virion production by transfected cells is not prevented by a foreign DNA fragment inserted in the RF DNA, provided that insertion does not abolish an essential viral function. Moreover, the increase in size of the viral DNA due to the presence of an insert does not prevent its

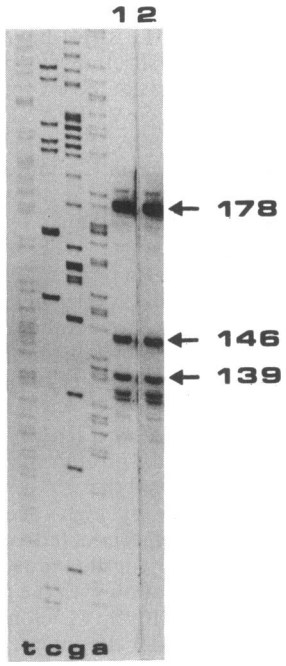

FIG. 6. Primer extension analysis of the 5′ terminus of the CAT mRNA transcript. Lanes 1 and 2, primer hybridized to total RNA extracted from *S. citri* cells infected with SpV1-CAT (lane 1) or CAT-free SpV1 (lane 2) virions and extended by reverse transcription. t, c, g, and a indicate sequencing reactions used as ladders.

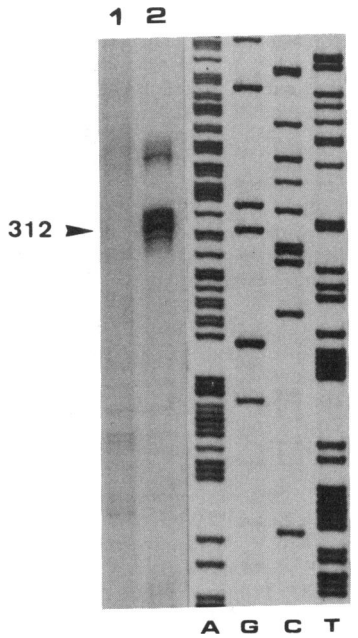

FIG. 7. S1 mapping of the 3′ terminus of the CAT mRNA transcript and analysis of S1-protected DNA fragments by denaturing polyacrylamide gel electrophoresis. Lanes 1 and 2, total RNA extracted from *S. citri* cells infected with SpV1 (lane 1) or SpV1-CAT (lane 2). A, G, C, and T indicate sequencing reactions used as ladders.

encapsidation into virions, since SpV1 is a rod-shaped virus with no strict constraint for viral DNA packaging.

Our experiments show that the RF of *S. citri* SpV1 can be used as a vector for cloning and expressing the CAT gene in *S. citri*. In addition to the CAT gene, we have also used the SpV1 RF to clone in *S. citri* the kanamycin resistance gene

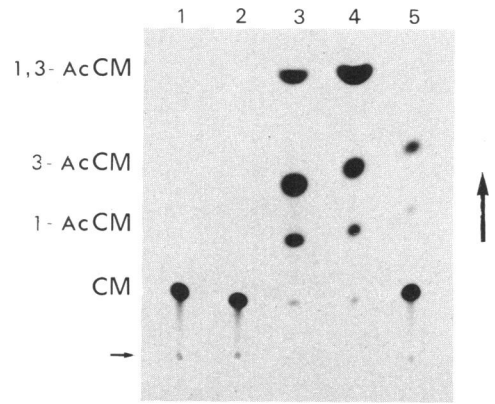

FIG. 8. CAT activity in cell-free extracts from *S. citri* R8A2 HP cells infected with SpV1-CAT or CAT-free SpV1 virions. Horizontal arrow indicates origin. Vertical arrow indicates direction of solvent migration. 1-AcCM, 3-AcCM, and 1,3-AcCM are acetylated forms of [^{14}C]chloramphenicol (CM). Lanes: 1, reaction mixture in which cell-free extract was replaced by SP4 medium; 2 and 3, reaction mixture with cell-free extracts from cells infected with CAT-free SpV1 (lane 2) or SpV1-CAT (lane 3) virions; 4 and 5, reaction mixtures with cell-free extracts from *E. coli* cells transformed with the plasmid vector pKK232-8 carrying the CAT gene free of promoter (lane 5) or under the control of promoter P0 of SpV4 (22) (lane 4).

and restriction fragments of the RF of coliphage M13 (results not shown). Thus, our results indicate that it is possible to develop the SpV1 RF into a general vector for cloning and expressing genes in *S. citri*, similar to the way the M13 RF vector is used in *E. coli*. Since *S. citri* uses UGA as a tryptophan codon, an essential advantage of our system is that any genes with such codons can be cloned and expressed with the SpV1 cloning system. *S. citri* high-passage subclone R8A2 HP was selected as the cloning host in this work for the following reasons. In contrast to low-passage subclones, it does not produce SpV1 virions spontaneously; however, it can be experimentally infected with SpV1-type viruses, upon which virion production occurs. In addition, subclone R8A2 HP has lost the ability to be transmitted to plants by its leafhopper vector (8a) and can therefore be used without danger for the environment.

Besides *S. citri*, most if not all other *Spiroplasma* spp. are naturally infected with an SpV1-type virus (2, 5), and it should be easy to transfer the experience gained with the SpV1 system of *S. citri* to other SpV1 systems of other spiroplasmas. In addition, the SpV1 system described here might be engineered to function not only in *S. citri* but in other *Spiroplasma* spp. Finally, the SpV1 RF seems to be, so far, the only multicopy vector with which foreign genes can be efficiently introduced into *S. citri* and expressed from extrachromosomal DNA. Similar systems have not yet been described for other mollicutes.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Conseil Regional d'Aquitaine.

We are grateful to D. L. Williamson and J. G. Tully for critical review of the paper.

REFERENCES

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Bové, J. M., P. Carle, M. Garnier, F. Laigret, J. Renaudin, and C. Saillard.** 1989. Molecular and cellular biology of spiroplasmas, p. 243–364. *In* R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. V. Academic Press, Inc., New York.
3. **Chevalier, C., C. Saillard, and J. M. Bové.** 1990. Organization and nucleotide sequences of the *Spiroplasma citri* genes for ribosomal protein S2, elongation factor Ts, spiralin, phosphofructokinase, pyruvate kinase, and an unidentified protein. J. Bacteriol. **172**:2693–2703.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
5. **Cole, R. M.** 1983. Isolation and characterization of spiroplasma viruses. Methods Mycoplasmol. **2**:425–431.
6. **Dybvig, K., and G. H. Cassell.** 1987. Transposition of Gram-positive transposon Tn916 in *Acholeplasma laidlawii* and *Mycoplasma pulmonis*. Science **235**:1392–1394.
7. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
8. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Addendum. Anal. Biochem. **137**:266–267.

8a. **Fos, A.** Personal communication.

9. **Godson, G. N., and D. Vapnek.** 1973. A simple method of preparing large amounts of ϕx174 RFI supercoiled DNA. Biochim. Biophys. Acta **299**:516–523.
10. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.

11. **Hackett, K. J., and T. B. Clark.** 1989. Ecology of spiroplasmas, p. 113–200. *In* R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. V. Academic Press, Inc., New York.
12. **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.
13. **Pascarel-Devilder, M. C., J. Renaudin, and J. M. Bové.** 1986. The spiroplasma virus 4 replicative form cloned in *Escherichia coli* transfects spiroplasmas. Virology **151**:390–393.
14. **Renaudin, J., P. Aullo, J. C. Vignault, and J. M. Bové.** 1990. Complete nucleotide sequence of the genome of *Spiroplasma citri* virus SpV1-R8A2 B. Nucleic Acids Res. **18**:1293.
15. **Renaudin, J., M. C. Pascarel, and J. M. Bové.** 1987. Cloning and sequencing of the genome of spiroplasma virus 4. Isr. J. Med. Sci. **23**:427–428.
16. **Renaudin, J., M. C. Pascarel, M. Garnier, P. Junca-Carle, and J. M. Bové.** 1984. SpV4, a new spiroplasma virus with circular, single stranded DNA. Ann. Virol. **135E**:343–361.
17. **Renaudin, J., M. C. Pascarel, C. Saillard, C. Chevalier, and J. M. Bové.** 1986. Chez les spiroplasmes le codon UGA n'est pas non-sens et semble coder pour le tryptophane. C. R. Acad. Sci. Ser. III Sci. Vie **303**:539–540.
18. **Renaudin, J., M. C. Pascarel-Devilder, and J. M. Bové.** 1987. Spiroplasma virus 4 (SpV4): nucleotide sequence of the viral DNA, regulatory signals, and proposed genome organization. J. Bacteriol. **169**:4950–4961.
19. **Renbaum, P., D. Abrahamove, A. Fainsod, G. G. Wilson, S. Rottem, and A. Razin.** 1990. Cloning, characterization and expression in *Escherichia coli* of the gene coding for the CpG DNA methylase from *Spiroplasma* sp. strain MQ1 (M.Sssl). Nucleic Acids Res. **18**:1145–1152.
20. **Saglio, P., M. Lhospital, D. Laflèche, G. Dupont, J. M. Bové, J. 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.
21. **Sladek, T. L., and J. Maniloff.** 1983. Polyethylene glycol-dependent transfection of *Acholeplasma laidlawii* with mycoplasma virus L2 DNA. J. Bacteriol. **155**:734–741.
22. **Stamburski, C., J. Renaudin, and J. M. Bové.** 1990. Characterization of a promoter and a transcription terminator of *Spiroplasma melliferum* virus SpV4. J. Bacteriol. **172**:5586–5592.
23. **Ullrich, A., J. Shine, J. Chirgwin, R. Pictet, E. Tischer, W. J. Rutter, and H. M. Goodman.** 1977. Rat insulin genes: construction of plasmids containing the coding sequences. Science **196**:1313–1319.
24. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. **171**:6455–6467.
25. **Whitcomb, R. F.** 1983. Culture media for spiroplasmas. Methods Mycoplasmol. **1**:147–158.
26. **Whitcomb, R. F.** 1989. *Spiroplasma kunkelii*: biology and ecology, p. 487–544. *In* R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. V. Academic Press, Inc., New York.
27. **Whitcomb, R. F., J. M. Bové, T. A. Chen, J. G. Tully, and D. L. Williamson.** 1987. Proposed criteria for an interim serogroup classification for members of the genus *Spiroplasma* (class *Mollicutes*). Int. J. Syst. Bacteriol. **37**:82–84.
28. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.
29. **Yamao, F., A. Muto, Y. Kawauchi, M. Iwami, S. Iwagami, Y. Azumi, and S. Osawa.** 1985. UGA is read as tryptophan in *Mycoplasma capricolum*. Proc. Natl. Acad. Sci. USA **82**:2306–2309.