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Spiroplasma citri Virus SpV1-Derived Cloning Vector: Deletion Formation by Illegitimate and Homologous Recombination in a Spiroplasmal Host Strain which Probably Lacks a Functional *recA* Gene

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We have previously described the use of the replicative form (RF) of *Spiroplasma citri* virus SpV1 as a vector for expressing an epitope of the P1 adhesin protein from *Mycoplasma pneumoniae* in *S. citri* (A. Marais, J. M. Bové, S. F. Dallo, J. B. Baseman, and J. Renaudin, J. Bacteriol. 175:2783–2787, 1993). We have now studied the structural instability of the recombinant RF leading to loss of the DNA insert. Analyses of viral clones with deletions have shown that both illegitimate and homologous recombination were involved in deletion formation. For one such clone, deletion has occurred via a double crossing-over exchange between the circular free viral RF and SpV1 viral sequences present in the *S. citri* host chromosome. The homologous recombination process usually requires the RecA protein. However, characterization of the *recA* gene of the *S. citri* R8A2 host strain revealed that over two-thirds of the open reading frame of the *recA* gene was deleted from the C-terminal part, indicating that this particular strain is probably RecA deficient.

Spiroplasmas are wall-free prokaryotes with helical morphology and motility. They belong to the class Mollicutes, a group of organisms which have derived by regressive evolution from ancestors of gram-positive bacteria with low-level guanine-plus-cytosine DNA (66, 70). The high-level adenine-thymine content has resulted in a peculiar codon usage. For instance, in mycoplasmas, ureaplasmas, mesoplasmas, entomoplasmas, and spiroplasmas, UGA is read as tryptophan and not as a termination signal. As a result, expression of mollicute genes in *Escherichia coli* has been limited to genes without the UGA tryptophan codon (8, 26, 42) or has led to truncated translation products (43). Cloning in a mycoplasma or a spiroplasma host would overcome this difficulty and, in addition, would open the way to genetic analysis of these organisms. Gene function can be identified only by analysis of their mutations, and complementation of gene mutations requires that genes be transferred between mutants. For members of the class Mollicutes, genetic studies have been hampered by the lack of metabolic markers and the lack of suitable vectors for transferring genes between isolates. Several approaches have been examined for the development of gene transfer systems (5, 17). Recently, potential cloning vectors based on the pKMK1 plasmid of *Mycoplasma mycoides* have been constructed (31).

Plasmids have also been isolated from spiroplasma cells (see reference 6 for a review). However, a combination of the *Spiroplasma citri* pMH1 plasmid and the chloramphenicol acetyl transferase (*cat*) gene of pBR328 was found to be highly unstable, as the recombinant plasmid could not be recovered from the spiroplasmal transformants (56). A more successful approach was the use of the replicative form (RF) of *S. citri* virus SpV1, a rod-shaped virus with a circular, single-stranded DNA genome. A peculiar feature of SpV1 is the presence of many viral sequences integrated into the *S. citri* host chromosome. The viral sequences consist of both full-length and deletion forms of the viral genome (49).

In previous experiments, we have used the RF of SpV1 as a vector for the cloning and expression of the *cat* gene in spiroplasma cells (59, 60). The *S. citri*-SpV1 cloning system was also used to express an epitope carried on the G fragment of the cytadhesin P1 gene from *Mycoplasma pneumoniae* (37). However, we now report the structural instability of the recombinant viral DNA carrying the G fragment. The finding that, in addition to illegitimate recombination, homologous recombination is very probably involved in deletion formation led us to characterize the *recA* gene of the *S. citri* R8A2 host strain.

MATERIALS AND METHODS

Bacterial strains, viruses, plasmids, and media. *E. coli* TG1 [*hsd*Δ*5* F′ (*traD36 proAB*$^+$ *lacZ*αM15) *supE thi* Δ(*lac-proAB*)] was used as the host for amplification of plasmid vector pBS$^+$ (Stratagene Cloning Systems, La Jolla, Calif.) and its derivatives and for propagation of the M13 recombinant viruses. *E. coli* cells were grown in Luria-Bertani medium (52). *S. citri* R8A2 (ATCC 27556) was used for propagation of *S. citri* virus SpV1-R8A2 B (49). *Spiroplasma melliferum* BC3 (ATCC 33219) and *S. citri* cells were grown at 32°C in SP4 medium (67). Experimental infection of *S. citri* R8A2 by SpV1 viruses has been previously described (59). Construction and restriction maps of the SpV1-O-P1G RF are described in Marais et al. (37).

Transformation of *E. coli* and *S. citri*. Transformation of *E. coli* cells was performed by the method of Hanahan (29). *S. citri* cells were transformed by electroporation as previously described (59).

Propagation of the SpV1 recombinant clones. Virions from a single plaque were used to infect 1 ml of an early-log-phase culture of *S. citri* R8A2 and incubated at 32°C for 24 h. For successive propagations of the virus, the infected culture was filtered through a 0.22-μm-pore-size filter to remove spiroplasma cells, and then the filtrate was used as the viral inoculum to infect a fresh mid-log-phase culture of *S. citri*. Such successive propagations (named passage numbers 1, 2, etc.) were used to test the stability of the recombinant SpV1 RF DNA.

DNA isolation. Large-scale and small-scale preparations of plasmids and M13 RFs amplified in *E. coli* were done according to standard procedures (52).

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Spiroplasmal genomic DNA isolation (68) as well as purification of the SpV1 RF (59) has been described elsewhere.

Southern blot hybridization. DNA was blotted to positively charged nylon membranes by the alkali transfer procedure (52). Hybridization and washing conditions were those described by Stamburski et al. (59). When heterologous probes were used, the stringency level was reduced by decreasing the hybridization temperature from 42 to 37°C. Washings were performed in 2× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C. Hybridization with oligonucleotide probes was performed as described by Zeff and Geliebter (71). DNA probes were prepared by the random priming procedure (21, 22) with [α-^{32}P]dATP (110 TBq/mmol) as the labelled nucleotide. Oligonucleotide probe CH1 (nucleotide sequence: 5′-CACTATATACTGCCTACAAGTTT-3′) was 5′ end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Maxam and Gilbert (39).

In situ hybridization of SpV1 plaques was performed according to the standard protocol described for coliphage M13 plaques (52), except that because of the small size of SpV1 plaques, an amplification step, the transfer of initial plaques to a new indicator lawn, was added.

PCR amplification. The *recA*$_i$ fragment was obtained by PCR amplification of *S. melliferum* genomic DNA with primers SM1 and SM2. Nucleotide sequences of these primers were 5′-GCTGAACACGCTTTAGACCC-3′ and 5′-TCCCGC TGTTGTTTCAGG-3′, respectively. For PCR amplification, approximately 0.2 to 1 ng of genomic DNA of *S. citri* or *S. melliferum* was added to a 50-μl mixture containing 70 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 17 mM ammonium sulfate, 10 mM β-mercaptoethanol, 200 μg of bovine serum albumin per ml, 0.05% W1 detergent, 0.2 mM deoxynucleoside triphosphates, 0.2 to 1 μM each primer, and 2.5 U of *Taq* DNA polymerase (GIBCO/BRL Life Technologies, Inc., Gaithersburg, Md.). Amplification was performed in a thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.) in over 40 cycles, each of 1 min at 92°C, 1 min at 57°C, and 2 min at 72°C, with a final step of 10 min at 72°C for chain termination. PCR amplification with primers SC11 (5′-GGAAGATAATAAACCAGTTCA CCCT-3′) and SC16 (5′-TCATTGTCTATCTTGGGG-3′) was achieved in over 35 cycles, each of 1 min at 92°C, 1 min at 50°C, and 2 min at 72°C, with an additional step of 10 min at 72°C.

DNA sequencing and nucleotide sequence analyses. Double-stranded or single-stranded DNA was sequenced by the dideoxy chain termination technique (53), by following standard procedures (52). Nucleotide and amino acid sequences used for comparison were imported from GenBank (Los Alamos, N. Mex.) with the retrieve electronic mail server of the National Center for Biotechnology Information at the National Library of Medicine, National Institutes of Health, Bethesda, Md. Sequence analyses were performed with the Seqaid II codon bias program (58), BLAST program (2), and the Genetic Computer Group software package (13).

Nucleotide sequence accession numbers. The sequence data reported here have been assigned GenBank accession numbers U31909 for the *recA* region of *S. citri* chromosomal DNA and U31910 for the 1.6-kbp *Hin*dIII chromosomal DNA fragment containing SpV1-related sequences.

RESULTS

Instability of the SpV1-O-P1G RF. The SpV1-O-P1G recombinant RF carries the G fragment of the cytadhesin P1 gene inserted at the *Mbo*I site in intergenic region I_3 of the SpV1 RF (37). To assess the stability of the recombinant RF DNA, we checked for the presence of the G fragment in the RF DNA during successive propagations of the SpV1-O-P1G recombinant virus. For each passage, the virions present in the culture filtrate were diluted and various dilutions were spotted onto a lawn of spiroplasma cells to produce plaques. Viral DNA from individual plaques was screened for the presence of G sequences by in situ hybridization with a G probe. In Fig. 1A, passage number 0 corresponds to the triply cloned recombinant virus SpV1-O-P1G. As expected, 100% of the viral plaques hybridized with the G probe, indicating the homogeneity of the initial viral inoculum. However, the figure shows that the percentages of plaques hybridizing with the probe decreased with increasing passage numbers. Starting from 100%, this percentage decreased to 80% for the third passage and 30% for the fifth passage and was down to 2% for the seventh passage. After 10 successive propagations, no hybridization could be detected, indicating that most, if not all, of the virions had lost the G fragment at this stage. This result was further documented by the results of the experiment shown in Fig. 1B in which the viral RF was extracted from infected cells after various passages and analyzed by agarose gel electrophoresis. Digestion of the native SpV1 RF by *Eco*RI yielded two fragments of 3.9 and 4.4 kbp (Fig. 1B, lane 1). The SpV1-O-P1G recombinant RF (Fig. 1B, lane 2) also yielded two fragments, one of 3.9 kbp identical to a fragment of the native RF and one of 5 kbp containing the G fragment. The viral RF isolated at passage numbers 1 (Fig. 1B, lane 3) and 3 (lane 4) gave the same *Eco*RI profile as that of the original recombinant RF (lane 2). In contrast, at passages 7, 9, and 10 (Fig. 1B, lanes 6 to 8, respectively), the RF DNA preparation yielded two *Eco*RI fragments of 3.9 and 4.4 kbp, i.e., the *Eco*RI profile of the native SpV1 RF (Fig. 1B, lane 1). It should be noticed that the RF DNA from passage number 5 (Fig. 1B, lane 5) yielded three fragments of 5, 4.4, and 3.9 kbp. This suggests the presence, at this stage, of a mixture of two viral RFs, the SpV1-O-P1G recombinant RF and a deletion form similar to the native SpV1 RF. As expected, and in agreement with the results shown in Fig. 1A, Southern blot hybridizations revealed that only the 5-kbp fragment hybridized with the G probe (data not shown). These results indicate that the SpV1-O-P1G recombinant RF is unstable and that the G fragment DNA insert was lost within the first 10 passages of the recombinant virus. These data also suggest that the recombinant virus carrying the G fragment replicates less efficiently than the native SpV1 virus or the virus that lost the G fragment DNA insert. Indeed, a comparison of growth curves of the native SpV1, the recombinant SpV1-O-P1G, and a deletion virus clone showed that the growth rate of the recombinant virus carrying the G fragment was reduced compared with the growth rate of the native SpV1 RF or the deletion virus (data not shown).

Thirteen SpV1 clones that lost the G fragment DNA insert were selected, and the corresponding RFs were characterized

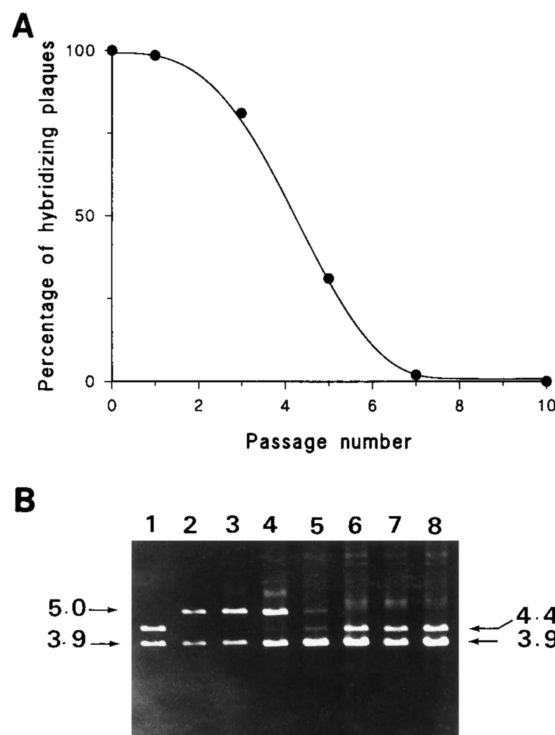


FIG. 1. (A) Percentages of viral plaques hybridizing with the G probe as a function of viral passage numbers. (B) Agarose gel electrophoresis of the SpV1 and SpV1-O-P1G RFs restricted with *Eco*RI. Lane 1, SpV1 RF; lanes 2 to 8, SpV1-O-P1G RF from the original recombinant virus (lane 2) and from passage numbers 1, 3, 5, 7, 9, and 10 (lanes 3 to 8, respectively). The sizes of restriction fragments are indicated in kilobase pairs.

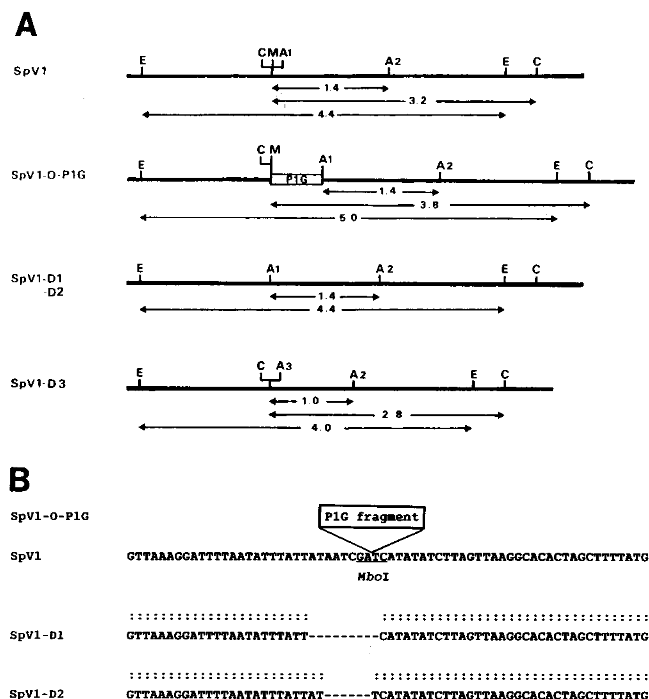


FIG. 2. (A) Comparison of the partial restriction maps of the SpV1, SpV1-O-P1G, SpV1-D1, SpV1-D2, and SpV1-D3 RFs. The sizes of fragments are indicated in kilobase pairs. Restriction sites: E, *Eco*RI; C, *Cla*I; M, *Mbo*I; A A1 to A3, *Alu*I. (B) Partial nucleotide sequences of the RFs of SpV1-O-P1G, SpV1, SpV1-D1, and SpV1-D2 around the *Mbo*I cloning site. Colons indicate nucleotides that are identical to those of the SpV1 RF. Dashes indicate nucleotides that are deleted in SpV1-D1 or in SpV1-D2.

by restriction mapping and partial sequencing. The results showed that these deletion RFs were of three types, named SpV1-D1, SpV1-D2, and SpV1-D3 (Fig. 2). The RFs of types 1 (seven clones) and 2 (one clone) had the same restriction map as the native SpV1 RF except that the *Cla*I site and the *Mbo*I site, where the G fragment had been inserted, were absent (Fig. 2A). Partial sequencing of the region around the *Mbo*I cloning site revealed a 9-nucleotide deletion upstream of the C of the GATC *Mbo*I site for SpV1-D1 and a 6-nucleotide deletion upstream of the T of the GATC *Mbo*I site for SpV1-D2 (Fig. 2B). In the case of SpV1-D3, restriction mapping showed that the SpV1-D3 RF was approximately 400 bp shorter than the native SpV1 RF. In particular, the RF DNA restricted by *Cla*I and *Eco*RI yielded fragments of 2.8 and 4.0 kbp, respectively, compared with the 3.2- and 4.4-kbp fragments of the native SpV1 RF (Fig. 2A).

To further characterize the SpV1-D3 RF, the 4-kbp *Eco*RI fragment was subcloned and partially sequenced. The comparison of this sequence with that of the native SpV1 RF showed that the nucleotide sequence of SpV1-D3 did not fully match that of the native SpV1 (Fig. 3). The 654-nucleotide region in SpV1, starting immediately downstream of the GATC *Mbo*I site (nucleotide 127 in Fig. 3) and ending at nucleotide 781, was partly absent in SpV1-D3. In addition, the SpV1-D3 sequence present in this region (254 nucleotides) showed only partial homology with that of SpV1 (Fig. 3). The 254-nucleotide region of SpV1-D3 is clearly different from the 654-nucleotide region of SpV1. In addition, it is well known that many SpV1-related sequences are present in the chromosomal DNA of *S. citri* (49). These observations led us to hypothesize that the 254-bp fragment of SpV1-D3 may originate from the chromosomal DNA of *S. citri*. To test this hypothesis, an oligonucleotide specific to the 254-bp region of SpV1-D3 (named CH1; Fig. 3) was designed and used to probe the chromosomal DNA of *S. citri* by Southern blot hybridization under stringent conditions (Fig. 4). As expected, in control experiments the probe hybridized with a 1-kbp *Alu*I fragment of the SpV1-D3 RF (Fig. 4, lane 1) from which the oligonucleotide was designed, but no hybridization was detected with the native SpV1 RF (lane 3) nor with the SpV1-O-P1G RF (data not shown). Interestingly, the probe did hybridize with the *S. citri* chromosomal DNA and more precisely with a 1.6-kbp *Hin*dIII fragment (Fig. 4, lane 2). These results show that oligonucleotide CH1 present in SpV1-D3 is also present in the chromosomal DNA of *S. citri* and strongly suggest that the 254-bp region of SpV1-D3 is of chromosomal origin. If so, the 1.6-kbp chromosomal DNA fragment should contain the 254-bp region of SpV1-D3. To test this conclusion, the 1.6-kbp *Hin*dIII fragment was subcloned in *E. coli* and sequenced. The nucleotide sequence was analyzed for the presence of open reading frames (ORFs) and was compared with the nucleotide sequences of SpV1-O-P1G and SpV1-D3 (Fig. 5). The 1.6-kbp *Hin*dIII chromosomal DNA fragment was shown to contain 1,584 nucleotides, 1,184 of which consisted of SpV1-related sequences. In particular, it carries the SpV1 ORFs 7, 8, and 12. Even though these ORFs are highly homologous to those of SpV1, they differ by several single base mutations. In addition, ORF 8 was found to be 60 nucleotides shorter than that of SpV1. Comparison of the gene organizations in SpV1-O-P1G, in SpV1-D3, and in the chromosomal DNA fragment revealed that ORFs 7 and 12 of SpV1-D3 had sequences identical to those of SpV1-O-P1G but not to those of the chromosomal DNA. In addition, SpV1-D3 does not carry ORF 8. From these results, it can be deduced that the sequences of chromosomal origin, present in SpV1-D3, are those located between ORF 7 and ORF 8 in the 1.6-kbp DNA fragment. They also suggest that the loss of the insert of SpV1-O-P1G has resulted from a double crossing-over exchange between the SpV1-O-P1G recombinant RF and the chromosomal DNA to yield the SpV1-D3 deletion RF. As indicated in Fig. 5, the crossing-over breakpoints would be located upstream of the G fragment and downstream of ORF 8 in SpV1-O-P1G and in between ORF 7 and ORF 8 in the chromosomal DNA (Fig. 5). In an attempt to precisely locate the sites of the crossing-over events, the nucleotide sequences of SpV1-O-P1G, SpV1-D3, and the 1.6-kbp chromosomal DNA fragment were aligned (Fig. 3). In SpV1, two regions were found to be identical to regions of SpV1-D3 (100% matches). One region was at the 5′ end, from nucleotides 1 to 126, and the other was at the 3′ end, from nucleotides 782 to 989. Interestingly, the 254-bp region of SpV1-D3, which was found to be different from the 654-bp region of SpV1 (see above), was totally present in the 1.6-kbp chromosomal DNA fragment. The two sequences were identical except for 4 mismatches corresponding to positions 142, 161, 460, and 461 of SpV1 (Fig. 3). In addition, the nucleotide sequences of SpV1, SpV1-D3, and the chromosomal DNA fragment were all found to be identical from nucleotides 79 to 126 and from nucleotides 782 to 791. These data indicate that the two crossing-over events must have occurred in the region between nucleotides 79 and 126 at the 5′ end and in the region between nucleotides 782 and 791 at the 3′ end (Fig. 3). The locations of the crossing-over breakpoints cannot be defined more precisely because of the identity of the three sequences in these two regions. According to these data, the model presented in Fig. 5 shows that deletion of the G fragment of SpV1-O-P1G probably occurred by homologous recombination between the free SpV1-O-P1G RF and SpV1-related se-

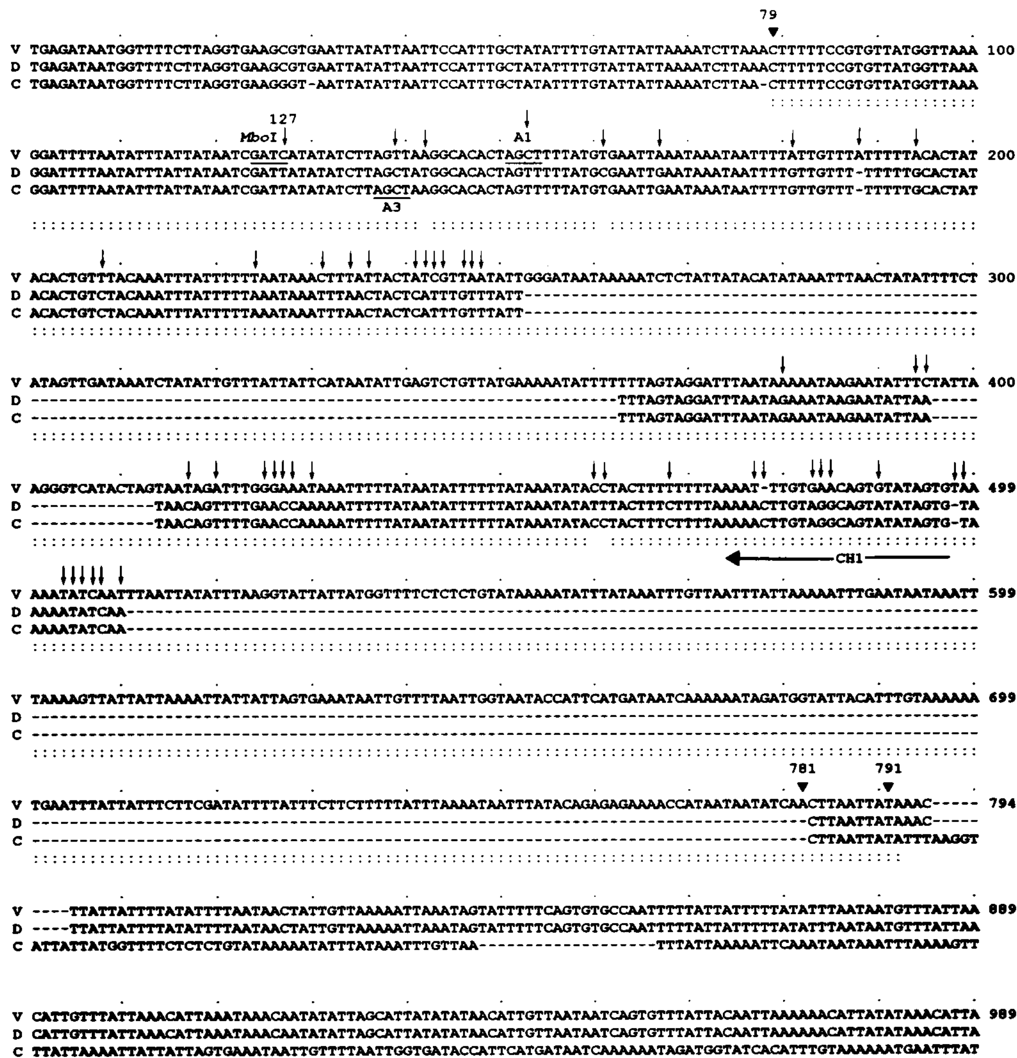


FIG. 3. Alignment of partial nucleotide sequences around the *Mbo*I site of the SpV1 (V) and the SpV1-D3 (D) RFs and the 1.6-kbp chromosomal DNA fragment (C). Numbers refer to the nucleotide sequence of the SpV1 RF. Dashes indicate the absence of sequence. Colons indicate nucleotide identity between the SpV1-D3 RF and the chromosomal DNA fragment. Vertical arrows indicate base changes between SpV1 and SpV1-D3. Arrowheads indicate nucleotides 79, 781, and 791. The location of oligonucleotide CH1 is indicated with a horizontal arrow. In comparison with Fig. 5, chromosomal ORF 7 ends at position 106 and chromosomal ORF 8 begins at the nucleotide corresponding to the position 801 in the SpV1 sequence. Restriction sites: M, *Mbo*I; A1 and A3, *Alu*I.

quences present in the host chromosomal DNA. As a result, a fragment of a size between 1,254 and 1,312 bp carrying the G fragment has been replaced by a chromosomal DNA fragment of a size between 254 and 312 bp, leading to the deletion clone SpV1-D3.

Search for *recA*-like sequences in *S. citri*. Duwat and coworkers have described the use of degenerate oligonucleotide primers for PCR amplification of *recA* sequences from various gram-positive bacteria (16). They used these primers to amplify genomic DNA of *S. melliferum*. A 364-bp DNA fragment was obtained and cloned (15). The cloned PCR product was kindly made available to us for sequencing. A search for similarity to previously reported proteins showed the translational product of the sequenced fragment to be highly similar to RecA proteins. From the *recA* fragment of *S. melliferum* (Fig. 6A), we have designed two oligonucleotide primers (SM1 and SM2) to amplify the corresponding fragment in genomic DNA of *S. citri* R8A2. The results are shown in Fig. 6B. The expected 351-bp product was obtained with the DNA of *S. melliferum*

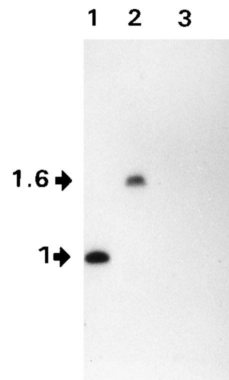


FIG. 4. Southern blot hybridization of the SpV1 RF, the SpV1-D3 RF, and genomic DNA of *S. citri* with the CH1 oligonucleotide probe. Lane 1, SpV1-D3 RF restricted with *Alu*I; lane 2, *S. citri* DNA restricted with *Hin*dIII; lane 3, SpV1 RF restricted with *Alu*I. Sizes of restriction fragments are indicated in kilobase pairs.

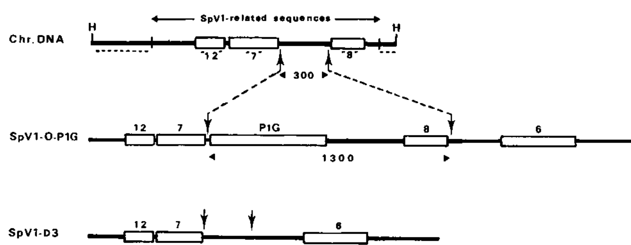


FIG. 5. Comparison of the ORFs (numbered boxes) present in the chromosomal DNA fragment (Chr. DNA), in the SpV1-O-P1G RF, and in the SpV1-D3 RF. Vertical arrows indicate the positions of the crossing-over events, and the lengths of the exchanged fragments are noted between arrowheads. Dashes indicate chromosomal DNA sequences which show no homology with SpV1 sequences. H, *Hin*dIII restriction site.

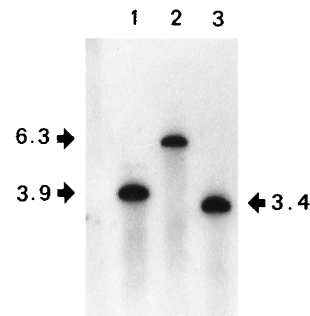
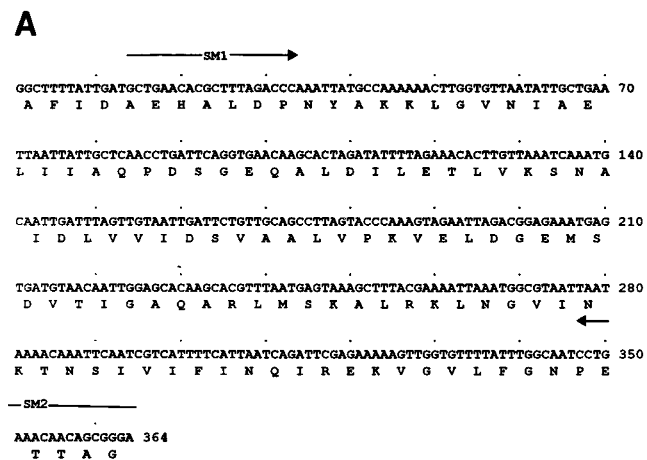


FIG. 7. Southern blot hybridization of restricted genomic DNA of *S. citri* R8A2 with the $recA_i$ probe. DNA was restricted with *Taq*I (lane 1), *Eco*RI (lane 2), and *Alu*I (lane 3). Sizes of restricted fragments are indicated in kilobase pairs.

used as a positive control (Fig. 6B, lanes 4 to 6); however, no amplification product could be detected when *S. citri* R8A2 DNA was used (lanes 1 to 3). To further investigate the *recA* situation with *S. citri*, the 351-bp *recA* fragment of *S. melliferum*, named $recA_i$, was used to probe Southern blots of restricted *S. citri* DNA under low-stringency hybridization conditions (Fig. 7). Restriction with *Taq*I (Fig. 7, lane 1), *Eco*RI (lane 2), and *Alu*I (lane 3) yielded in each case only one DNA fragment hybridizing with the $recA_i$ probe with sizes of 3.9, 6.3, and 3.4 kbp, respectively. These results suggested that *recA* sequences were present in the chromosome of *S. citri* and that only one copy of the $recA_i$ probe was present.



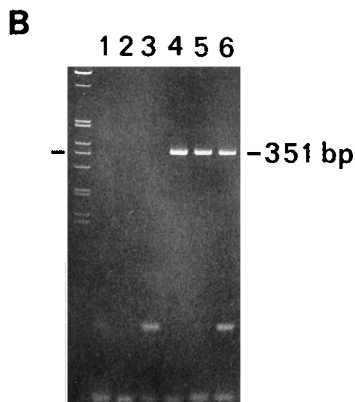


FIG. 6. (A) Nucleotide sequence and predicted amino acid sequence of the 364-bp *recA* fragment from *S. melliferum*. Positions of primers SM1 and SM2 are indicated with arrows. (B) Ethidium bromide-stained 2% agarose gel of DNA from *S. citri* (lanes 1 to 3) and *S. melliferum* (lanes 4 to 6) amplified with *S. melliferum* SM1 and SM2 primers. Lanes 1 and 4, 60 ng of DNA; lanes 2 and 4, 6 ng of DNA; lanes 3 and 6, 0.6 ng of DNA.

Cloning and sequencing the *recA* gene of *S. citri*. Chromosomal DNA of *S. citri* R8A2 was restricted by *Alu*I, and restriction fragments were separated by agarose gel electrophoresis. Fragments with a size range of 3 to 4 kbp were ligated "in gel" with the *Hin*cII-linearized pBS^+ vector, and the ligation mixture was used to transform *E. coli*-competent cells. *E. coli* TG1 was used for these experiments because it carries a functional *recA* gene, which is supposed to increase the stability of plasmids containing a foreign *recA* gene (18). The transformants were screened by in situ colony hybridization with the *S. melliferum* $recA_i$ fragment as the probe. One positive clone was selected. Its plasmid contained a 3.4-kbp *Alu*I DNA insert which hybridized with the probe (data not shown). Sequencing this fragment revealed that the first 246 nucleotides at the 5′ end showed significant homology with the *recA* genes of *M. mycoides* subsp. *mycoides* (32), *Acholeplasma laidlawii* (18), *Mycoplasma pulmonis* (32), and an unidentified gram-positive bacterium (reference 4; accession number U20900). The percentages of identity were 73, 65, 62, and 71%, respectively.

In order to sequence the 5′ terminus of the *recA* gene upstream of the *Alu*I fragment, the 3.9-kbp *Taq*I fragment overlapping the 5′ end of the *Alu*I fragment was cloned and partially sequenced (Fig. 8).

Sequence analysis by Seqaid II codon bias (58) revealed the presence of seven potential coding sequences (ORFs); ORF 1 from nucleotides 3 to 335, ORF 2 from nucleotides 365 to 754, ORF 3 from nucleotides 741 to 1619, ORF 4 from nucleotides 1625 to 2041, ORF 5 from nucleotides 2045 to 2620, ORF 6 from nucleotides 2548 to 3018, and ORF 7 from nucleotides 3469 to 3933 (Fig. 8). A search in the data bank for similarities

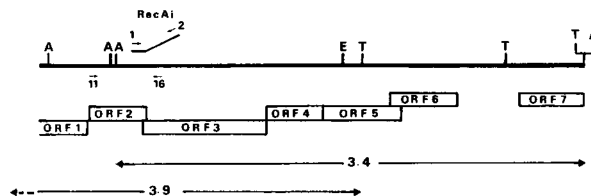


FIG. 8. Partial restriction map and positions of ORFs in the *recA* region of *S. citri*. The positions of primers SC11 (11) and SC16 (16) used for PCR amplification are indicated with arrows. The positions of the $recA_i$ probe defined by the SM1 (1) and SM2 (2) primers are also indicated. Restriction sites: A, *Alu*I; E, *Eco*RI; T, *Taq*I. Sizes of restricted fragments are indicated in kilobase pairs.

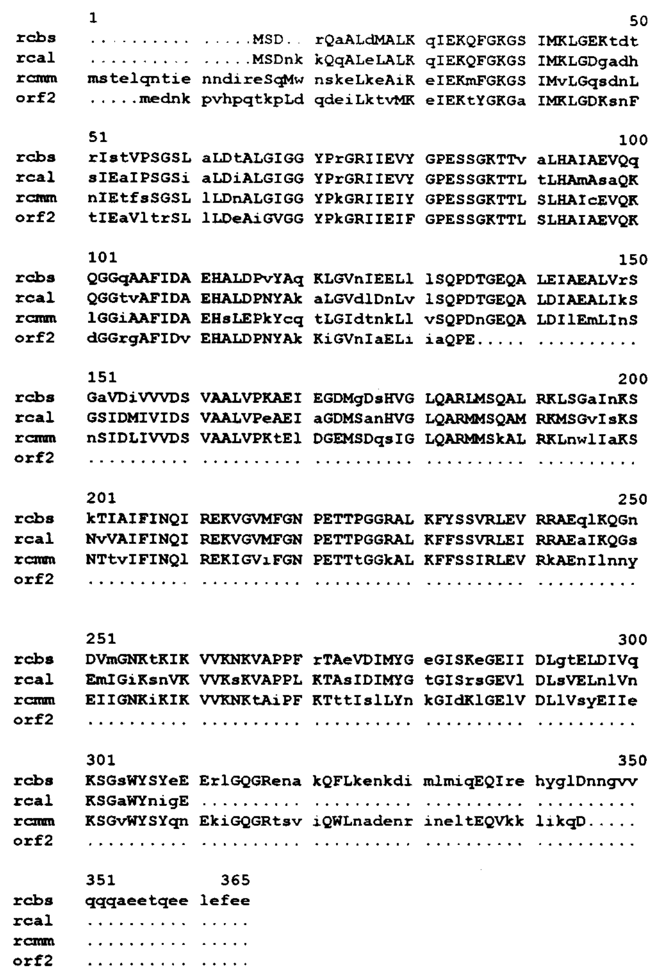


FIG. 9. Alignment of amino acid sequences of RecA proteins from *B. subtilis* (rcbs), *A. laidlawii* (rcal), *M. mycoides* (rcmm), and ORF 2 of *S. citri* R8A2. The alignments were performed with the PILEUP program available with version 7 of the Genetics Computer Group (University of Wisconsin) sequence analysis software package (13). Identical and conserved amino acids are indicated with uppercase letters. Dots indicate gaps introduced into the sequences to improve alignment. Amino acid numbers begin with the number 1, which was assigned to the first amino acid of the *M. mycoides* RecA protein.

between the putative translation products and known proteins showed that ORF 2 had striking similarity to RecA proteins. The alignment of the sequence of the ORF 2 translation product with those of the RecA proteins of *Bacillus subtilis* (62), *M. mycoides* (32), and *A. laidlawii* (18) is shown in Fig. 9. The percentages of identical amino acids were 62, 62, and 59%, respectively. However, with a coding capacity of 130 amino acids (Fig. 9), ORF 2 represents only one-third of the usual *recA* genes and, more precisely, the region corresponding to the N-terminal part of RecA proteins.

ORF 2 starts with an ATG initiation codon located 9 nucleotides downstream of a typical gram-positive ribosome binding site and ends with a TAA stop codon. No further nucleotide or amino acid homology was found downstream of the stop codon, indicating that the *recA* gene of *S. citri* strain R8A2 is truncated.

As indicated above (Fig. 6B), PCR amplification of *S. citri* DNA gave negative results when the *S. melliferum* primers SM1 and SM2 were used. This is due to the fact that the occurrence of a truncated *recA* gene in *S. citri* did not allow hybridization of primer SM2, even though primer SM1 did hybridize. As shown in Fig. 8, the overlapping region of the *S. citri recA* sequence and the $recA_i$ fragment of *S. melliferum* is only 80 bp long and does not contain the SM2 sequence.

To ensure that the finding of a truncated *recA* gene was not a cloning artifact, the gene organization of this region was further characterized by PCR amplification of the *S. citri* chromosomal DNA with the following primers. Primer SC11 starts at the third nucleotide of ORF 2, and primer SC16 is 70 nucleotides downstream of ORF 2 (Fig. 8). We found the nucleotide sequence of the 457-bp PCR product to be identical to that of the cloned chromosomal DNA fragment containing ORF 2. This result indicates that no DNA rearrangement has occurred during cloning in *E. coli.*

Organization of the *S. citri recA* region. As shown in Fig. 8, seven ORFs could be deduced from the 3,938-nucleotide sequence of the *recA* region of *S. citri*. ORF 2 (130 amino acids) was shown to encode the N-terminal part of a RecA-like protein (see above). ORF 1 encodes the C-terminal part (111 amino acids) of a polypeptide having similarities with three previously reported proteins. One is a hypothetical 18.3-kDa protein (ORF 172) derived from the sequence in the 3′ region of the *marRAB* operon of *E. coli* (3), the second is an ORF from *Enterobacter agglomerans* (reference 48; accession number S313480), and the third is the carboxy terminus of Exp10 from *Streptococcus pneumoniae* (38, 45). An alignment of these four polypeptides is presented in Fig. 10. Percentages of similarity between *S. citri* ORF 1 and the other polypeptides were as follows: 61% with *E. coli* ORF 172, 59% with the *E. agglomerans* ORF, and 63% with the Exp10 protein of *S. pneumoniae*. Genetic studies have shown the *marRAB* operon to be involved in the adaptive response to oxidative stress and in multiple-antibiotic resistance (3, 9, 10). *S. pneumoniae* Exp10 and the *E. agglomerans* ORF are located immediately upstream of their respective *recA* genes. Interestingly, the same situation occurs with *S. citri* in the case of ORF 1. Pearce et al. (45) and Martin et al. (38) have shown that Exp10 was involved in the natural competence of *S. pneumoniae*. ORF 3 (293 amino acids), ORF 4 (139 amino acids), ORF 5 (192 amino acids), ORF 6 (157 amino acids), and ORF 7 (155 amino acids) showed no similarity with any of the protein sequences in the data banks.

DISCUSSION

The replicative form of the virus SpV1 has been used as a cloning vector to express foreign genes in *S. citri* (37, 59, 60), an organism which reads UGA as a tryptophan codon. The *E. coli*-derived *cat* gene was fully translated in *S. citri* to a functional polypeptide (59, 60). The *S. citri*-SpV1 cloning system was also used to express mycoplasmal genes containing UGA codons. An epitope carried on the G fragment of cytadhesin P1 from *M. pneumoniae* was produced in *S. citri* with the SpV1 RF as the cloning vector (37). In this study, we have examined the stability of the recombinant SpV1-O-P1G RF and we have found that the G fragment DNA insert was quickly lost during virus propagation. Such structural instability has been extensively described for the small plasmids of gram-positive bacteria which replicate by the rolling circle model (25, 63, 64). In this case, illegitimate recombination is the predominant process leading to deletion formation. A key event of the recombination process is the contact between direct repeats (1, 46, 54, 55, 57). Several properties of the *S. citri*-SpV1 cloning system could be involved in deletion of the DNA insert. (i) The SpV1-O-P1G RF carries many repeated sequences which are potential target sites for recombination. (ii) SpV1 replicates by

```
           1                                                  50
E.a.orf    .......... .......... .......... .......... ..........
Orf172     .......... .......... .......... .......... ..........
Exp10      mkaeiiavgt eiltgqivnt naqflsekla eigvdvyfqt avgdnevrll
Orf1       .......... .......... .......... .......... ..........

           51                                                100
E.a.orf    .......... .......... .......... .......... ..........
Orf172     .......... .......... .......... .......... ..........
Exp10      slleiasqrs slviltgglg ateddltkqt lakflgkalv fdpqaqekld
Orf1       .......... .......... .......... .......... ..........

           101                                               150
E.a.orf    .......... .......... .......... .......... ..........
Orf172     .......... .......... .......... .......... ..........
Exp10      iffalrpdya rtpnnerqaq ivegaiplpn etglavggkl evdgvtyvvl
Orf1       .......... .......... .......... .......... ..........

           151                                               200
E.a.orf    .......... .......... .......... .......... ..........
Orf172     .......... .......... .......... .......... ..........
Exp10      pgppselkpm vlnqllpklm tgsklysrvl rffgigesql vtiladlidn
Orf1       .......... .......... .......... .......... ..........

           201                                               250
E.a.orf    .......... .......... ....mninrd kivqlvDtdt iE........
Orf172     .......... .......... .......... .....Msekr Lh........
Exp10      qidptlapya ktgevtlrls tkassqeean qaldiLEnqi LDcqtfegis
Orf1       .......... .......... .......... .......... ..........

           251                                               300
E.a.orf    .......... ..nLtSalsq rLiAdqlRlT tAESCTGGkL AsAlcaaedt
Orf172     .......... ..eLsveIga aLKAkGwWVT cAESCTGGLi AkAItdiaGs
Exp10      lrdfcygyge etsLaSiVve eLKrqGktIa aAESlTaGLF qatVanfsGv
Orf1       .......... .......... .......... .......... ..........

           301                                               350
E.a.orf    pkFYgaGFVT FtdqAKmKiL sVSQqsLErY saVSEkvAaE MAtGAierAd
Orf172     SaWFDrGFVT YSnaAKhelL gVSeStLEqY GaVSEqvvhE MALGvlhaAg
Exp10      SsiFEgGFVT YSleeKsrmL dIpaknLEeh GvVSEftAqk MAeqArsktq
Orf1       .......... ......iKvg hVSQSiitkY GvVSketAea MAFacqkqfn

           351                                               400
E.a.orf    aDvsIaiTGy gGPEGgEdGt PaGTVWFaWh iK.GqnyTAV mhFaGDcEtV
Orf172     aDvAVSvsGI AGPDGgsaek PVGTVWFGFA gKdGrvlTAk qqFsGDrEaV
Exp10      sDfgISlTGV AGPDslE.Gh PVGTVFiGLA qdqGtevikV niggrsqaDV
Orf1       sDlAVSfTGn AGPgqlD.nl PIGTgFatil fqneattfAl tfnpnwsrEq

           401                   427
E.a.orf    laLAVrFALa qLLQlLL... .......
Orf172     RlqAaVFsLq taLreFiKn. .......
Exp10      RhiAVmhAFn lvrkaLLsD. .......
Orf1       ikFAtVttii kLqQiLeKEq ginskka
```

FIG. 10. Alignment of amino acid sequences of *S. citri* ORF 1 with the Exp10 protein from *S. pneumoniae*, Orf 172 from the *E. coli MarRAB* locus, and an Orf from *E. agglomerans* (E.a.orf). The alignments were performed with the PILEUP program (13). Amino acid numbers begin with the number 1, which was assigned to the first amino acid of the *S. pneumoniae* Exp10 protein. Dots indicate gaps in the sequences.

the rolling circle model, a replication process known to favor illegitimate recombination (40). (iii) In addition, homologous recombination could occur between the SpV1 RF and the SpV1 viral sequences present in the chromosomal DNA of the *S. citri* host (49).

Characterization of SpV1-D3, a deletion clone of SpV1-O-P1G having a genome size 400 bp shorter than that of SpV1, showed that, in this particular clone, deletion of the G fragment has occurred very probably by homologous recombination. The finding in SpV1-D3 of a 300-bp fragment of chromosomal origin indicates that a double crossing-over exchange has occurred between the SpV1-O-P1G RF and SpV1-related sequences present in the *S. citri* chromosomal DNA. In addition, the fact that ORF 8 is not present in SpV1-D3 agrees well with the location of the crossing-over breakpoints as determined by sequence comparison (Fig. 3 and 5). The 300-bp fragment of chromosomal origin present in SpV1-D3 and the corresponding fragment in the *S. citri* chromosome have the same sequence except for 4 nucleotides (Fig. 3). These base changes are not due to sequencing artifacts but probably result from point mutations that occurred during replication of the SpV1-D3 virus.

Several pathways of homologous recombination have been described for *E. coli*, and most of them require the *recA* gene product (34, 41, 44). In addition, it has been shown that the frequency of homologous recombination events was dramatically reduced in the *recA* mutant (41). Therefore, in order to increase the stability of our viral SpV1 cloning vector, we intended to develop an *S. citri* host strain deficient in homologous recombination. The rationale was to construct a *recA* mutant strain of *S. citri* by gene disruption, a strategy which has been successfully used with many bacteria (12, 47, 65), including the mollicute *A. laidlawii* (19). Surprisingly, we found that *S. citri* R8A2 used in this study, in which homologous recombination occurred, contained a truncated *recA* gene. The 390-nucleotide sequence related to *recA* genes encodes a polypeptide of 130 amino acids. This polypeptide corresponds to the N terminal part of the RecA protein. It contains the domain (amino acids 76 to 83) involved in nucleotide binding, in particular the A-site motif of the *E. coli* RecA protein 70-GPESS GKT-77, (61; for a review, see reference 34). However, the domains thought to be involved in DNA binding are lacking. These domains lie within two regions (loops 1 and 2) of the *E. coli* RecA protein (14, 20, 51). Searches for additional *recA* sequences elsewhere in the *S. citri* chromosome by Southern blot hybridization gave negative results. The *S. citri recA* probe used in these experiments represented the most conserved part of the *recA* gene; in particular it contains the highly conserved ATP binding site. For these reasons, it seems very unlikely that a second *recA* gene could not be detected in our hybridization stringency conditions. From these observations, it is improbable that *S. citri* R8A2 produces a functional RecA enzyme. Therefore, *S. citri* R8A2 must be RecA deficient, even though homologous recombination occurs in this strain, as shown by the data for SpV1-D3. Homologous recombination has also been reported for *S. citri* R8A2 and Asp1 for integration of the pBOT1 *oriC* plasmid into the spiroplasmal host chromosome (50). In this case, the plasmid integrates into the chromosome probably by a single crossing-over event within the *oriC* region.

With *E. coli*, Fishel et al. (23) and Luisa-DeLuca et al. (36) showed that *recE* pathway-mediated plasmid recombination could occur in the absence of a functional RecA protein. Moreover, recent data (27, 28, 33) showed that the *E. coli* RecT protein could promote homologous pairing and subsequent DNA strand-exchange reactions in the presence of exonuclease VIII, the *recE* gene product. Whether a *recE* pathway exists in spiroplasmas is unknown.

For members of the class Mollicutes, *recA* genes from *A. laidlawii* (18), *M. mycoides* (32), and *M. pulmonis* (32) have been characterized and only one natural *recA* mutant (strain 8195 of *A. laidlawii*) has been described (18). This strain harbors a higher level of UV sensitivity than the JA1 parent strain.

The RecA protein is known to play an important role in DNA repair (11, 41, 69). For *S. citri*, the finding of a nonfunctional *recA* gene in strain R8A2 agrees with the high level of sensitivity of this organism to UV irradiation compared with that of *A. laidlawi* B (35). The characterization of the *recA* gene as well as the UV sensitivities of various strains of *S. citri* is under investigation.

Besides homologous recombination, which accounts for the formation of SpV1-D3 deletion clones, illegitimate recombination probably accounts for the formation of SpV1 deletion clones SpV1-D1 and SpV1-D2. In SpV1-D1, the deleted sequence consists of the G insert together with 9 nucleotides upstream of the *Mbo*I cloning site. In SpV1-D2, it consists of the insert together with 6 nucleotides upstream of the *Mbo*I

site. No new, foreign sequences were found in the resulting RFs. In these cases, deletions might be due to intramolecular recombination between direct repeated sequences. Indeed, as a result of the cloning strategy used to construct the SpV1-O-P1G RF (37), the G fragment was flanked by short direct repeats. In particular, the *Mbo*I (GATC) and *Xba*I (TCTAGA) restriction sites are present at both ends of the G fragment DNA insert. During replication, these short repeats could also be targets for a copy choice process (1, 55), leading to the loss of the sequence in between the direct repeats. Such recombination events between short repeated sequences are characteristic of illegitimate recombination (7). Furthermore, the small sizes of the repeats distinguishes illegitimate recombination events from homologous recombination, which requires extensive regions of homology. The extent of homology necessary to promote homologous recombination has been estimated to be approximately 70 bp in *B. subtilis* (30) and 30 bp in *E. coli* (24).

In summary, the characterization of SpV1-O-P1G deletion clones, in which the G fragment DNA insert has been lost, shows that besides illegitimate recombination, homologous recombination is also responsible for deletion in the SpV1-O-P1G RF in *S. citri* R8A2.

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