Sequence analysis of two plasmids from the phytoplasma beet leafhopper-transmitted virescence agent

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The complete nucleotide sequences of the two plasmids from the phytoplasma beet leafhopper-transmitted virescence agent (BLTVA) have been determined. The larger plasmid, pBLTVA-1, was 10 785 nt in length and contained 11 putative ORFs, almost all of which were duplicated or triplicated on the plasmid due to the presence of large repeated regions. The sequence contained a series of tandem repeats, the largest of which was 338 nt long. The sequences of ORFs 4 and 11 showed homology with the replication genes of plasmids from other phytoplasmas and from geminiviruses. ORF9, the only ORF present as a single copy, showed homology with DNA primase genes from bacterial chromosomes and contained the conserved zinc finger and topoisomerase/primase domains. None of the other eight ORFs showed homology with known sequences in the GenBank database. pBLTVA-2 was 2587 nt in length, and all of its sequence was nearly identical to sequences from pBLTVA-1, most of which spanned ORFs 10 and 11, including the 338 nt tandem repeat. Analysis of 30 strains of BLTVA showed that most of the 11 putative ORFs were present, but the size of the plasmids varied in these strains.

INTRODUCTION

Phytoplasmas belong to the class Mollicutes, a diverse group of wall-less prokaryotes with small, AT-rich genomes. Phytoplasmas are known to cause diseases in hundreds of plant species worldwide, including economically important food, fibre, forage, fruit and ornamental plants. Phytoplasmas multiply only within the phloem cells of their host plants and are transmitted by phloem-feeding insects, especially leafhoppers, in which they also multiply and may cause disease (Kirkpatrick, 1992). Phytoplasmas cannot be cultured in vitro and as a result they remain poorly characterized.

The beet leafhopper-transmitted virescence agent (BLTVA) phytoplasma causes economically important diseases of many vegetable crops. Specific diseases caused by BLTVA include the production of aerial tubers in potato (Smart et al., 1993), seed sterility in radish (Shaw et al., 1990) and big bud disease of tomato (Shaw et al., 1993). BLTVA has a wide host range, and its leafhopper vector, Circulifer tenellus, is prevalent in much of the western United States. Golino et al. (1989) determined that 43 plant species can be infected with BLTVA by C. tenellus. The predominant symptoms of BLTVA are virescence (greening of floral tissue), phyllody (leaf-like petals and sepals) and a premature induction of flowering in day-length-sensitive hosts which may involve alteration of normal gibberellic acid activity (Golino et al., 1988). The BLTVA type line FC-83-13, originally isolated from a single C. tenellus collected from Buena Vista, Kern County, California, in 1983 (Golino et al., 1987), has been well characterized. Its transmission biology was studied in detail (Golino et al., 1987) and two plasmids, pBLTVA-1 (11 kb) and pBLTVA-2 (2·7 kb), were identified (Shaw, 1991). Total DNA from FC-83-13-infected plants and C. tenellus leafhoppers was fractionated by centrifugation in caesium chloride-ethidium bromide (CsCl-EtBr) density gradients. The resulting supercoiled DNA fraction was subjected to agarose gel electrophoresis and only pBLTVA-1 and 2 were observed in EtBr-stained gels. Additional Southern blot analysis of total DNA from FC-83-13 hosts using cloned pBLTVA-1 as a probe showed only the presence of pBLTVA-1 and 2 (Shaw, 1991). These results suggested that pBLTVA-1 and 2 are the predominant plasmids present in the FC-83-13 type line.

Extrachromosomal DNAs have been detected in a number of other phytoplasmas. However, only a few of them have been completely sequenced. These include the 2·6 kb plasmid of sugarcane white leaf phytoplasma (Nakashima & Hayashi, 1997), a 4·2 kb plasmid from peanut witches'-broom...
phytoplasma (unpublished GenBank entry, accession number
AY270152), a 4·3 kb plasmid from aster yellows phytoplasma
(unpublished GenBank entry, accession number
NC_003353) and seven plasmids, ranging in size from 3·1 to
7 kb, from the three strains of onion yellows phytoplasma
(Nishigawa et al., 2001, 2002a, b, 2003; Oshima et al., 2001).
To date, virtually nothing is known about the significance of
these plasmids to the phytoplasma, or whether they have a
potential role in pathogenesis. To better understand the
structure and function of the plasmids of BLTVA, we
completely sequenced the two plasmids, pBLTVA-1 and
pBLTVA-2, that occur in this phytoplasma. The 11 kb
pBLTVA-1 plasmid is the largest reported and sequenced for
a phytoplasma to date.

METHODS

Isolation and cloning of pBLTVA-1. The isolation and cloning
of pBLTVA-1 DNA was described by Shaw (1991). Briefly, supercoiled
plasmid DNA was separated from total plant and phytoplasma DNA
by CsCl-EtBr equilibrium density gradients. Restriction digests of
the resulting plasmid DNA identified unique restriction sites for
EcoRI and SalI. The linear and nicked phytoplasma plasmid DNA,
and phytoplasma and plant chromosomal DNA, from the CsCl-EtBr
gradient were subjected to CsCl-bisbenzimide density-gradient cen-
trifugation to separate the A+T-rich DNA from the denser plant
DNA. This DNA was digested with EcoRI to linearize pBLTVA-1 and
cloned into lambda EMBL3. pBLTVA-1 DNA was excised from the
EMBL3 vector by digestion with BamHI and SalI and the resulting
two fragments were subcloned into pUC18.

DNA extraction. Plasmid DNA was prepared from the pUC18
clones of pBLTVA-1 using the QIAprep Spin Miniprep Kit (Qiagen),
according to the manufacturer’s instructions. DNA from BLTVA
type line FC-83-13 for use in PCR and Southern blot hybridization
was extracted using the QIAprep Spin Miniprep Kit (Qiagen),
and cloned into lambda EMBL3. pBLTVA-1 DNA was excised from
the EMBL3 vector by digestion with BamHI and SalI and the resulting
DNA was used to create the plasmid diagrams. The alignment features
were created using BOXshade 3.21 (http://www.ch.embnet.org/
software/BOX_form.html).

PCR amplification. The ORFs of pBLTVA-1 were amplified using
the primer pairs listed in Table 1. The primer BLTVA2REV, 5’-GCTCTAGATATGTTATGC-3’, was designed from unique
sequence in pBLTVA-2. The sequence of primer 7291FOR (5’-
GTTCCATGGACATTTATCC-3’) started at nucleotide position
7291 of pBLTVA-1. Inverse PCR was performed using the primer
pair ORF11NVF (5’-CTATAGGTATCTGTAATCCAG-3’) and
ORF11NVR (Table 1). PCR was performed in 30 µl reactions
containing 10–50 ng template DNA, 150 µM dNTPs, 0·5 µM each
primer and 1 U Taq DNA polymerase with the recommended PCR
buffer containing MgCl2 (Qiagen). Amplification involved an initial
denaturation cycle at 95°C for 5 min, then 35 cycles of 95°C for
1 min, 50°C or 56°C for 1 min and 72°C for 2 min, followed by a
final 10 min extension at 72°C. Long-range PCR was performed
using the Expand Long Template PCR System (Roche Molecular
Biocchemicals) according to the manufacturer’s instructions. Long-
range PCR cycling conditions involved an initial denaturation at
94°C for 2 min, then 15 cycles of 94°C for 10 s, 56°C for 30 s and
68°C for 8 min, followed by 15 cycles with extension times
increased by 20 s for each cycle and a final extension time of 7 min.

Southern blot hybridization. DNA in electrophoresed agarose
gels was transferred onto nylon membranes (Hybond-N+, Amersham
Biosciences) according to standard procedures (Sambrook et al.,
1989). Membranes were prehybridized in 0·5 M sodium phosphate,
ph 7·2, 7 % SDS and 1 mM EDTA (pH 8·0) at 65°C for 2–3 h. The
PCR product from ORF9 (daGA) was purified through a QIAquick
PCR purification column (Qiagen) and labelled using the random
hexamer priming method (Feinberg & Vogelstein, 1983) incorporating
[α-32P]dATP (NEN). The labelled probe was added to the prehy-
bridization solution and incubated at 65°C for at least 16 h.
Posthybridization washes (30 min each) consisted of two washes in
2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate)/
0·1% SDS at room temperature followed by two washes in 0·1×
SSC/0·1% SDS at 68°C. The membrane was exposed to X-ray film
at −80°C using intensifying screens.

RESULTS AND DISCUSSION

Sequence analysis of pBLTVA-1

The two pUC18 clones, representing two separate BamHI/
SalI fragments of pBLTVA-1, were completely sequenced.
PCR was performed on DNA template extracted from
BLTVA-infected leafhoppers using primers that face out-
wards from the ends of the two pUC18 clones to verify that
the clones had been assembled in the correct orientation.
The resulting PCR products were sequenced to check the
continuity of the sequence at the junctions of the two
BLTVA plasmid fragments that were cloned in pUC18.
The first base of the unique SalI site in pBLTVA-1 was designated
as the first nucleotide position in the sequence. Long-range
inverse PCR with the primer pair ORF6REV/ORF7FOR
(Table 1) amplified a 10·6 kb fragment, thereby confirming
that the plasmid is circular.

The complete nucleotide sequence of pBLTVA-1 was determined to be 10785 bp with a G+C content of
24·8 mol%. Analysis of the plasmid sequence predicted 11
ORFs encoding products larger than 50 amino acids, all of
which were orientated in the same direction (Fig. 1a, Table 2). Three sets of tandem repeats were identified. Two of these repeats were 10 bp repeated three times: one between ORF3 and ORF4 (AGGGGTGTGT), and the other between ORFs 9 and 10 (ATGGTGTCC). A 338 bp perfect direct repeat was present at position 9081–9762 (Fig. 1a). This large repeat started in the ORF9/ORF10 intergenic region and continued through ORF10 and into the first

<table>
<thead>
<tr>
<th>ORF</th>
<th>Primer pair</th>
<th>Sequence (5′–3′)</th>
<th>Product size (bp)</th>
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<td>ORF1FOR</td>
<td>TAATAAAGGTAATGCAGAACC</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>ORF1REV</td>
<td>ATTAGGAGCTTATCTGAGATAT</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ORF2FOR</td>
<td>AGGTGTTATTTGTATGGGATG</td>
<td>625</td>
</tr>
<tr>
<td></td>
<td>ORF2REV</td>
<td>TAGCCATATATAACCGCTTAC</td>
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</tr>
<tr>
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<td>ORF3FOR</td>
<td>TTAGGAGGTGTTATCTGAGATAT</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>ORF3REV</td>
<td>TGCTTATATATGTGCTGCTTAC</td>
<td></td>
</tr>
<tr>
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<tr>
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<tr>
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<tr>
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**Table 1.** Sequences of the oligonucleotide primers used for PCR amplification of the ORFs in pBLTVA-1 and the resulting product sizes

**Fig. 1.** Maps of the plasmids (a) pBLTVA-1 and (b) pBLTVA-2 of BLTVA. The first nucleotide in the unique SalI restriction site was designated position 1. The EcoRI site where pBLTVA-1 was linearized and cloned into the bacteriophage vector is indicated. The arrows indicate the putative ORFs and their direction of transcription. Genes with a putative function based on homology with genes in the database are named (see Table 2). DNA regions of pBLTVA-1 and pBLTVA-2 with identical sequence are shown in solid black tracks on the circular map of pBLTVA-1. The large perfect direct repeat (DR) and the three large imperfect repeats (R1, R2, R3) are marked around the outside of the map.
Table 2. Predicted ORFs in pBLTVA-1

<table>
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<tr>
<th>ORF</th>
<th>Position</th>
<th>Size (aa)</th>
<th>Homologous protein (gene designation); E-value; organism showing best match (accession no.)</th>
</tr>
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<tbody>
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<td>187</td>
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</tr>
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<tr>
<td>3</td>
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<td>196</td>
<td>No homologues</td>
</tr>
<tr>
<td>4</td>
<td>2860–4101</td>
<td>413</td>
<td>Replication-associated protein (rep); 8e−49; peanut witches’-broom phytoplasma (AAP44293)</td>
</tr>
<tr>
<td>5</td>
<td>4468–5031</td>
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<td>5028–5807</td>
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<td>5861–6610</td>
<td>249</td>
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<td>8</td>
<td>6627–7166</td>
<td>179</td>
<td>No homologues</td>
</tr>
<tr>
<td>9</td>
<td>7725–8873</td>
<td>382</td>
<td>DNA primase (dnaG); 2e−76; peanut witches’-broom phytoplasma (AAP44294)</td>
</tr>
<tr>
<td>10</td>
<td>9185–9457</td>
<td>90</td>
<td>No homologues</td>
</tr>
<tr>
<td>11</td>
<td>9523–10773</td>
<td>416</td>
<td>Replication-associated protein (Rep); 6e−20; peanut witches’-broom phytoplasma (AAP44293)</td>
</tr>
</tbody>
</table>

232 nt of ORF11, resulting in the identity of this region of ORF11 with ORF10. Three large imperfect repeats were also present on pBLTVA-1, resulting in several of the ORFs sharing high homology at both the nucleotide and amino acid level (Fig. 1a). Apart from three amino acids, ORF1 and ORF5 were identical. One of the repeats, designated R2 (Fig. 1a), was repeated at a third location on the plasmid, resulting in ORFs 2, 6 and 7 sharing significant amino acid identity. ORF3 and ORF8 were almost identical apart from an insertion of 17 amino acids in ORF3. The online version of this paper (at http://mic.sgmjournals.org) contains a supplementary figure showing the sequence alignments of ORFs 1 and 5, ORFs 2, 6 and 7, and ORFs 3 and 8. All these ORFs, including ORF10, lacked significant homology to any protein sequence in the GenBank database. The occurrence of virtually identical ORFs on the same plasmid has also been described for the pCI65st plasmid from *Streptococcus thermophilus* (O’Sullivan et al., 1999).

The sequences of ORF4 and ORF11 were identical for 37% of their amino acids and both these ORFs shared homology with the replication (Rep) protein of the plasmids from peanut witches’-broom, onion yellows and *Vaccinium* witches’ broom phytoplasmas, and of geminiviruses (Table 2). The online version of this paper (at http://mic.sgmjournals.org) contains a supplementary figure showing the sequence alignment of ORF4 and ORF11 with homologous *rep* genes. The Rep protein from *Vaccinium* witches’ broom (Rekab et al., 1999) was not included in the amino acid alignment as it was a partial sequence consisting of 118 amino acids. The sequence similarity of Rep proteins from the plasmids of phytoplasmas to that of geminiviruses has been previously reported for sugarcane white leaf (Nakashima & Hayashi, 1997), *Vaccinium* witches’ broom (Rekab et al., 1999) and onion yellows (Nishigawa et al., 2001) phytoplasmas. Rep proteins from rolling circle replication plasmids, phages and geminiviruses fall into at least five different families, and almost all of them have three sequence motifs in common: motif 1 (UxkUTG; U), motif 2, termed the HUH motif, and motif 3, which contains one or two active-site tyrosine residues (Ilyina & Koonin, 1992). These conserved regions, including the conserved tyrosine residue, were not observed in the two putative replication proteins of pBLTVA-1. However, the putative proteins did contain the nucleotide-binding domain called the P-loop (GXXXXGKT/S) (Saraste et al., 1990). The P-loop of onion yellows phytoplasma did not align with the other amino acid sequences but was located at amino acid positions 269–276. As far as we are aware, this is the first example of two Rep proteins occurring on the same plasmid that use the rolling circle method of replication.

The only other ORF from pBLTVA-1 that had homology to sequences in the database was ORF9. The deduced amino acid sequence of ORF9 showed the greatest homology to a putative DNA primase (*dnaG*) from the plasmid of peanut witches’-broom phytoplasma (Table 2). ORF9 was also homologous to the primase genes from a large number of bacteria, including *Staphylococcus* sp., *Helicobacter* sp., *Enterococcus* sp. and *Buchnera* sp. The online version of this paper (at http://mic.sgmjournals.org) contains a supplementary figure showing the sequence alignment of the *dnaG* genes from two of these bacteria with the *dnaG* genes from the plasmids of BLTVA and peanut witches’-broom phytoplasmas. Apart from the plasmid-borne primase from peanut witches’-broom phytoplasma, no other bacterial primase genes from the BLAST results were plasmid-encoded. Primase is an essential enzyme in DNA replication. Its role is to catalyse the synthesis of RNA primers needed for DNA polymerase to initiate Okazaki fragment synthesis. Although a variety of conjugative plasmids of several different incompatibility groups encode DNA primases, there is no obvious sequence relationship between these primases and the host DnaG protein (Strack et al., 1992). ORF9 contains two conserved domains characteristic
of bacterial chromosomal primases. The zinc-binding domain at the N-terminal region contains two pairs of Cys(His) residues allowing formation of a zinc finger (Ilyina et al., 1992; Versalovic & Lupski, 1993). The topoisomerase/primase domain is at the C-terminal region (Pansegrau & Lanka, 1992; Aravind et al., 1998). This domain consists of two conserved motifs: one which centers at a conserved glutamate and the other one at two conserved aspartates (DxD) (Aravind et al., 1998). Housekeeping genes tend not to be located on plasmids (Koch, 1981); additional proteins required by the plasmid for rolling circle replication are recruited from the host (del Solar et al., 1998). Therefore, the significance of the putative primase gene on pBLTVA-1 is unclear, but its presence suggests that either the chromosomal DNA primase is not present or is nonfunctional, or a novel process is involved in pBLTVA-1 replication. Shaw (1991) determined that pBLTVA-1 plasmid sequences were not present in the phytoplasma chromosome by Southern blot analysis using the entire pBLTVA-1 plasmid as the hybridization probe. We confirmed this finding using only ORF9 (dnaG) as the hybridization probe (Fig. 2). However, this does not preclude the possibility that a dnaG gene is also present on the BLTVA chromosome composed of a sequence not detected under the stringent hybridization conditions used in our study. A primase gene was sequenced from both the plasmid and chromosome of peanut witches’-broom phytoplasma and they shared 45% amino acid identity but were not significantly homologous at the nucleotide level (pairwise BLAST analysis of unpublished GenBank entries with accession numbers AY271052 and AY270153). If this is also the case with BLTVA, then the dnaG gene from the chromosome would not be detectable by Southern blot hybridization or PCR. The complete genome of BLTVA is currently being sequenced and from this it will be possible to determine if a primase gene is also present on the chromosome.

**Sequence analysis of pBLTVA-2**

Shaw (1991) previously reported that there was a region of homology between pBLTVA-1 and pBLTVA-2 identified by Southern blot analysis. We hypothesized that the gene common to both plasmids was most likely to be that of the Rep protein (ORF4 or ORF11) because all phytoplasma plasmids that have been sequenced to date contain this gene. A Southern blot of BLTVA-infected leafhopper DNA determined that both the 10-8 kb band of pBLTVA-1 and the 2-6 kb band of pBLTVA-2 hybridized to ORF11 (data not shown). Almost the entire pBLTVA-2 plasmid was successfully amplified by inverse PCR using primers (ORF11INVF/ORF11INVR) designed from ORF11 of pBLTVA-1. The resulting PCR product of approximately 2.5 kb was directly sequenced on both strands by primer walking. In order to sequence the region between the inverse PCR primers, inverse PCR was performed again using the unique pBLTVA-2 primer, BLTVA2REV, in combination with primer 7291 For that occurred in the intergenic region between ORF8 and ORF9 of pBLTVA-1 (Fig. 1a). The size of the resulting PCR product was approximately 21 kb, indicating that it originated from pBLTVA-2 because the region between these two primers on pBLTVA-1 is approximately 6 kb. The region of this PCR product between the ORF11INVF and ORF11INVR primers was then sequenced.

The complete nucleotide sequence of plasmid pBLTVA-2 was determined to be 2587 bp with a G+C content of 25.6 mol%. The sequence of pBLTVA-2 was composed of three separate fragments of pBLTVA-1 (Fig. 1). The sequences of the largest fragment shared between pBLTVA-1 and pBLTVA-2 were identical and there was a single-base substitution in each of the two smaller regions. Apart from the three large duplicated regions between the two plasmids, there were two very small regions that are not shown on Fig. 1(a). One was a 5 bp region at the stop codon of ORF1. The other was located in the intergenic region of ORF3 and ORF4 consisting of 35 bp of pBLTVA-2 dispersed over a 73 bp region of pBLTVA-1. This latter region was where the unique pBLTVA-2 primer, BLTVA2REV, was designed. The sequence of pBLTVA-2 contains the unique SalI site, one of the 10 bp tandem repeats and the large 338 bp direct repeat from pBLTVA-1 (Fig. 1b). ORFs 10 and 11 in pBLTVA-1 were exactly the same in pBLTVA-2 with no additional ORFs detected in the smaller plasmid.

Shaw (1991) determined that only pBLTVA-1 was detected in DNA extracted from recently infected periwinkle shoots and the concentration of pBLTVA-2 increases significantly late in the infection process. Also, pBLTVA-1 was in much higher concentration than pBLTVA-2 in all tissues except the roots, where the concentrations of the two plasmids were approximately equal. Therefore, both sequence analysis and biological studies strongly suggest that the smaller plasmid is derived from the larger one. Recombination between plasmids is a relatively common phenomenon and has also been reported to occur between the plasmids of the onion yellows phytoplasma (Nishigawa et al., 2002b). The
Presence of ORFs in other BLTVA strains

Primer pairs were designed to amplify each of the ORFs in BLTVA-1, BLTVA-2, and BLTVA-3 and BLTVA-4. The primers were used in PCR reactions to determine if their target was present in the DNA template. The PCR products were then analyzed by gel electrophoresis to determine if the ORF was amplified. The results were recorded as positive or negative for each ORF.

The ORFs were amplified from the DNA template using the primer pairs shown in Table 1. The PCR products were analyzed by gel electrophoresis to determine if the ORF was present in the DNA template. The results were recorded as positive or negative for each ORF.

There was no significant difference in the size of the PCR products between different strains. However, there was a difference in the intensity of the PCR product for some ORFs. This may be due to the differences in the template DNA used for the PCR reactions. The intensity of the PCR product was determined by visual inspection of the gel.

The results are shown in Table 1. All 30 strains were positive for ORFs 1, 2, 7, 9, and 11 in PCR with an annealing temperature of 56°C. A PCR analysis was also performed with an annealing temperature of 50°C. The results are shown in Table 2. All 30 strains were positive for ORFs 1, 2, 7, 9, and 11 in PCR with an annealing temperature of 50°C.

The results obtained at 56°C were used to determine the presence of each ORF in the DNA template. The results obtained at 50°C were used to determine the presence of each ORF in the DNA template. The results obtained at 56°C were used to determine the presence of each ORF in the DNA template. The results obtained at 50°C were used to determine the presence of each ORF in the DNA template.
 Samples were scored as: +, positive; ±, weak positive; or −, negative. Samples that produced a negative or weak positive result at an annealing temperature of 56 °C were retested at a PCR annealing temperature of 50 °C and results are shown in parentheses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Host</th>
<th>Location/field*</th>
<th>ORF1</th>
<th>ORF2</th>
<th>ORF3</th>
<th>ORF4</th>
<th>ORF5</th>
<th>ORF6</th>
<th>ORF7</th>
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*Location and field number, if more than one field was sampled, where plant sample was collected. CA, California; ID, Idaho; OR, Oregon; WA, Washington.
†Number of ORFs positive and weak positive by PCR for each strain at an annealing temperature of 56 °C or 50 °C.
‡Total number of strains positive and weak positive by PCR for each ORF at an annealing temperature of 56 °C or 50 °C.
The low incidence of pBLTVA-1 in field-collected isolates of BLTVA could be attributed to the observed tendency of larger plasmids to slow host reproduction more than smaller ones (Zünd & Lebak, 1980), thereby selecting against propagation of strains containing pBLTVA-1. Genes on plasmids tend to evolve more rapidly in response to environmental changes than those on chromosomes (Eberhard, 1990). A classic example of this occurring in phytoplasmas is the loss of vector transmissibility over a relatively short period of time. Extensive rearrangement of the clover phyllody phytoplasma plasmid DNA was observed in clover plants regenerated by tissue culture and the phytoplasma could no longer be transmitted by insects (Denes & Sinha, 1992). Similarly, Oshima et al. (2001) isolated a non-insect-transmissible line of onion yellows phytoplasma after 2 years of maintenance by plant grafting without insect vectors and Nishigawa et al. (2002a) determined that the plasmid from this line lacks a single-stranded DNA-binding protein and an uncharacterized putative membrane protein that exist in the wild-type line. Although these studies demonstrated a correlation between changes in phytoplasma plasmids and insect transmissibility, there was no detailed analysis of potential changes in phytoplasma chromosomal DNA whose gene products may mediate insect transmission. Indeed, the plasmid profiles of numerous BLTVA strains varied considerably, but all of these strains were transmissible by the beet leafhopper (Shaw, 1991). Changes that occurred in EcoRI restriction profiles of plasmid DNA in the BLTVA type strain (FC-83-13) over a 2.5 year period of insect and graft transmission (Shaw, 1991), together with our PCR analyses of plasmid-encoded ORFs in 30 field strains, provide strong evidence that plasmid size and genetic composition undergo numerous and frequent rearrangements in this phytoplasma.

Concluding remarks

Interesting features of the two plasmids from the type strain of BLTVA were revealed from the sequence analysis in this study. pBLTVA-1 contains an abundance of repeated sequences, which could mediate genetic recombination in this plasmid. This suggestion is consistent with the sequence of pBLTVA-2, which appears to have been derived directly from pBLTVA-1 through loss of 75% of its length. The dynamic nature of BLTVA plasmids was further illustrated by analysis of their sizes in 30 strains of BLTVA. In half of these strains the plasmid was only 60% as large as pBLTVA-1, and most of the remaining strains contained a plasmid only slightly larger or smaller than this. Only one other field strain of BLTVA contained a 10.8 kb plasmid that was the same size as pBLTVA-1. These findings suggest that the 10.8 kb form of pBLTVA-1 is unstable, perhaps because of the large regions of repeated sequences, and the majority of plasmids in the other BLTVA strains had lost these repeats.

The dynamic variability of the plasmids in BLTVA suggests that the genes it carries are not essential to phytoplasma viability and replication, but may encode virulence or other factors that are required at various levels or during different phases of its life cycle. This was seen with the two plasmids in the type strain of BLTVA, where their ratios varied between different plant host tissues, with the smaller plasmid rising late in infection. However, due to the inability to culture phytoplasmas in vitro and the lack of molecular genetic tools to mutate or complement phytoplasma genes of interest, the functional role of these genes cannot be determined at this time.

ACKNOWLEDGEMENTS

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REFERENCES


Sequence of BLTVA plasmids


