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Sequence Analysis of the Small Cryptic *Xanthomonas campestris* pv. *vesicatoria* Plasmid pXV64 Encoding a Rep Protein Similar to Gene II Protein of Phage I2-2

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The complete nucleotide sequence (1,851 bp) was determined for the *Xanthomonas campestris* pv. *vesicatoria* plasmid pXV64. Sequence analysis revealed an intergenic region (IG) of 355 bp and two oppositely running open reading frames, ORF1 and ORF2, encoding polypeptides of 39 and 16 kDa, respectively. While the function of ORF2 is not known, ORF1 is suggested to be the gene encoding Rep protein based on (i) similarity in amino acid sequence to that of the gene II protein (gIIP) of filamentous phage I2-2, (ii) presence of a sequence in the *ori*-containing region which is similar to the sequence around the Rep nicking site in some rolling circle-replicating replicons, and (iii) ability to support replication *in trans* of the region containing pXV64 *ori* (392 bp) which is located within the region including IG and a short stretch in the N-terminus of ORF2.

Xanthomonas campestris is an important species of gram-negative plant pathogenic bacteria consisting of more than 123 pathovars (1). Several of the pathovars have been shown to possess single or multiple plasmids (2-10). However, detailed studies on these plasmids are limited and none of their complete nucleotide sequences has been determined.

pXV64 (10) is a very small cryptic plasmid isolated from *X. campestris pv. vesicatoria*, the pathogen for foliage and fruit spot disease in peppers and tomatoes (11). This plasmid was shown to be compatible with the broad-host range IncP vector pLAFR1 (12), and stably maintained at high copy numbers in several strains representing different pathovars of *X. campestris* (10).

Since nucleotide sequence analysis is the first step towards understanding of the structure, function, organization and evolution of a gene/genome, sequence of pXV64 was determined in this study. Sequence analysis revealed an IG and two open reading frames. ORF1 was shown to possess Rep protein function, which was able to support replication of the pXV64 *ori*-containing sequence *in trans.*

MATERIALS AND METHODS

Strains and culture conditions. *X. campestris* pv. *vesicatoria* 64 is the strain harboring pXV64 (10). *X. campestris.* pv. *campestris* 17 (also called Xc17) (13) was used as the host for maintaining pXV64 derivatives. *E. coli* DH5α (14) was the host for DNA cloning. Cultures of *E. coli* and *X. campestris* were grown at 37 °C and 28 °C, respectively, in Luria broth (LB) or on L agar plates and were maintained and stored according to standard protocols (15). LB and L agar were supplemented with the following antibiotics as required: ampicillin (50 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml).

Plasmids. Plasmid p64PK was a derivative of pXV64 carrying a kanamycin resistance cartridge ligated into the unique *Pst*I site (10). p64PK-1 was derived from p64PK by deleting a 319-bp fragment (bp 1,254 to1,572). To express the proteins encoded by ORF1 and ORF2, pXV64 was cloned into pGEX-3X (16) to form pGEX-F. The construction was of two stages. Firstly, pXV64 was linearized with *Sal*I and cloned into the *Sal*I site of pUK21 (17), generating pUK21-I. The *Sal*I site of pUK21 is located in the middle of the multiple cloning sites (MCS); therefore, the *Sal*I-linearized pXV64 has the sites *Xba*I-*Eco*RV-*Bam*HI-*Sal*I running continuously at the upstream and *Sal*I-*Pst*I-*Eco*RI-*Sma*I-*Stu*I at the downstream, relative to the direction of ORF1. Then, the 1.85-kb *Bam*HI-*Eco*RI fragment from pUK21-I was cloned into compatible sites of pGEX-3X (16), resulting in plasmid pGEX-F which carried ORF1 in the same direction as *tac* promoter. pCPP33 (obtained from H.-C. Huang) is a broad-host-range, IncQ plasmid (7.5 kb, Tc^r) with MCS, *Eco*RI-*Sac*I-*Xba*I-*Bam*HI-*Pst*I-*Eco*RI-*Hind*III-*Xho*I-*Kpn*I-*Sph*I-*Hind*III, being placed between *lac* promoter and *lacZ′* allowing blue//white screening. For the complementation test, a 1.3-kb *Xba*I-*Pst*I fragment containing ORF1 from pUK21-I was ligated into the MCS of pCPP33 to form pCPP-1. The insert of pCPP-1 thus carried at its upstream a short *Xba*I-*Sal*I fragment from the pUK21 MCS, followed by the 1.26 kb *Sal*I-*Pst*I fragment from pXV64 (Fig. 1). In addition, the orientation of ORF1 was the same as that of the *lac* promoter in the vector. pOK (1.56 kb) was the pXV64 *ori*-containing plasmid constructed by two steps. Firstly, the large *Stu*I-*Pst*I fragment from p64PK-1 was cloned into

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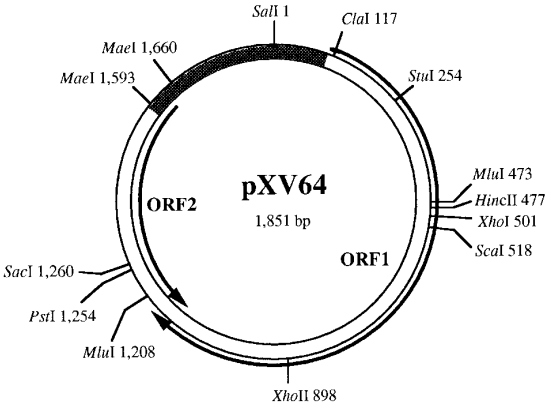

FIG. 1. The map of plasmid pXV64. Number beside a restriction site represents the nucleotide position counting from the unique *Sal*I site. Positions of ORF1 and ORF2 are indicated by arrows. Shaded portion indicates the suggested 392-bp region containing *ori* for pXV64 replication.

the multiple cloning sites of pUK21 forming pUK-OK. Secondly, the insert was recovered by cutting pUK-OK with *Stu*I plus *Eco*RV, followed by self-ligation. The resulting plasmid, pOK, thus carried an insert derived from pXV64 sequence corresponding to bp 1,573 to 256 and the Km^r cartridge.

Cloning procedures and standard techniques. Standard protocols (15) were followed for preparation of plasmid, restriction enzyme digestion, ligation, transformation of *E. coli,* and agarose gel (0.7%) electrophoresis. Competent cells of *X. campestris* were prepared and electroporated as described (18). The rapid screening method described by Weng et al. (10) was used to detect the presence of pXV64 derivatives.

DNA sequencing. The complete nucleotide sequence of pXV64 was determined on both strands by the chain termination method using Sequenase 2.0 according to the manufacturer's instructions (United States Biochemicals).

Expression of the protein encoded by ORF1 in E. coli maxicell. pGEX-F was used for maxicell expression in strain LCD44 (19). Preparation of maxicell, induction by IPTG (5 mM), and labeling the protein with [^{35}S]-methionine were carried out as described by Sancar et al. (20). The protein products were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography.

RESULTS AND DISCUSSION

Nucleotide Sequence Analysis of pXV64

The restriction map of pXV64 has been constructed previously (10; Fig. 1). In this study, different restriction fragments from pXV64 were cloned into M13mp18 and mp19, and the DNA sequences of both strands were determined. Totally 1,851 bp were obtained (Fig. 2; GenBank accession No. U78513). Computer search using PC/GENE program revealed two possible open reading frames, ORF1 (bp 114 to 1,172) and ORF2 (bp 1,609 to 1,136), running oppositely with an overlapping of 37 nucleotides at their C-termini. Lying between these two ORFs is an intergenic region (IG) of 355 nucleotides (bp 1,610 to 113) that contains no predictable open reading frame.

ORF1 encodes a putative 39,211-Da protein consisting of 352 amino acids. There is a consensus S/D sequence (21), 5′-GAGGAAG-3′, present at 8 nt upstream of the initiation codon ATG (Fig. 2). No sequence consensus to an *E. coli* type promoter was found in the region further upstream. Comparison of the deduced amino acid sequence of ORF1 product revealed 34.6% identity to that of the gene II product of phage I2-2 (22), a filamentous phage of *E. coli* (Fig. 3). In addition, the sizes of both proteins are similar. The gene II proteins of filamentous phages possess a topoisomerase activity that initiates the phage DNA replication, via rolling circle mechanism, by nicking the *ori* site in the viral strand of the double-stranded replicative form (23, 24). These similarities suggest that ORF1 product may be the replication initiation protein, Rep, of pXV64.

ORF2 is able to encode a polypeptide of 16,182 Da consisting of 157 amino acids. There is no sequence consensus to a promoter or S//D sequence in the upstream. Sequence homology was not found between ORF2 and other sequences in the database.

The region between bp 1,176 and 1,626 has potential to form 7 hairpin structures (designated A to G), among which the first 6 are within the ORF2 coding region (Fig. 2). In addition, hairpins C to G are similar to that present in the IG of M13 and the related filamentous phages containing the origins for replication of the viral strand and the complementary strand and the morphogenesis signal (25).

Identification of Regions Required for Replication

To identify the region required for autonomous replication, a series of p64PK derivatives were constructed by *Bal*31 deletion starting from the unique *Sac*I site (bp 1,260). p64PK-1 was one of the deletion clones which could maintain in *X. campestris* cells. Sequencing data showed that p64PK-1 had bp 1,254 to 1,572 (319 bp) being deleted from p64PK. This deletion caused a removal of hairpins C, D and E which are overlapping with ORF2, suggesting that these hairpin structures are not essential and ORF2 may not take part in pXV64 replication. These results also suggest that the *ori* for pXV64 replication maybe located in the region between bp 1,573 and ORF1.

To test whether the predicted *ori* region could replicate, plasmid pOK, carrying bp 1573 to 256 of pXV64, was electroporated into Xc17, Xc17(pCPP33), and Xc17(pCPP-1) for the maintenance test. Transformants resistant to tetracycline and kanamycin were obtained only in experiments using Xc17(pCPP-1) as the host but not in the other hosts. These results indicate that the region between bp1,573 to the *Stu*I site contains the *ori* for pXV64 replication that can only replicate in the presence of the cloned ORF1. Since the DNA fragment between bp 114 to 256 is overlapped with the

(A)

```
GTCGACGCACTCAAGGTCATGGGCGATGTTCTTCGTCAGACAGCTATTGCAGCTAATCAGCTTGAGCTGGCTATCAACACTAAACGACGC    90
GCTGCCATGAGGAAGCGCAACAGATGATCGATTGGCTTTCGATGATTGTTCCTTGCTCTCATCCAACGCCAATTACTGGTGGACATGTTC  180
          S/D    (ORF1) M  I  D  W  L  S  M  I  V  P  C  S  H  P  T  P  I  T  G  G  H  V  L
TTTCGCTCACGCCTGATGGTGAGGTTTCTTGGCGTTCGTCTAAGCGTTCTACTGTTGAAGGATCGTTCGGCACAGGCCTTCAAATTCGAA  270
  S  L  T  P  D  G  E  V  S  W  R  S  S  K  R  S  T  V  E  G  S  F  G  T  G  L  Q  I  R  T
CATGCACACATACCGCTGACCCTTGCACGCATCTGGAAATAAGCGGAAATCCTGTCAAATTCTTTCAGGGTCACAACCTATGGGGCACTG  360
  C  T  H  T  A  D  P  C  T  H  L  E  I  S  G  N  P  V  K  F  F  Q  G  H  N  L  W  G  T  D
ATGATTTGCCCTCTCTTGCCATCGCAACGTTTAATCATCTGATCGGTGTTTTATCGTTGAATCCGACTGACACGGACCGTGCCTTGGTGC  450
  D  L  P  S  L  A  I  A  T  F  N  H  L  I  G  V  L  S  L  N  P  T  D  T  D  R  A  L  V  R
GGGCCGGAATGATCCAACTCACACGCGTTGACGTTACGGAATCTTTCCATCTCGAGAGCCGCGCGCAAGTACTCGCATGGCTTCGTGCTG  540
  A  G  M  I  Q  L  T  R  V  D  V  T  E  S  F  H  L  E  S  R  A  Q  V  L  A  W  L  R  A  A
CTGAGCAGACGGCACATCTTTCTCATCGTGGTCGAGGTCAGTTGGTTAAAGGCACAACGCTTTATTTCGGCAAGAACAGTCGCCGATGGG  630
  E  Q  T  A  H  L  S  H  R  G  R  G  Q  L  V  K  G  T  T  L  Y  F  G  K  N  S  R  R  W  G
GTCTTAAGCTGTATAGCAAAGGTCAGGAAATTCACGCCAAGGGCCATGGTCAAGATGCCATCTTGTCCCTGCCTCATGCTGTTGCCTGGG  720
  L  K  L  Y  S  K  G  Q  E  I  H  A  K  G  H  G  Q  D  A  I  L  S  L  P  H  A  V  A  W  A
CTGATAGAACCCTTCGAGCTGAGCTGACAATTCGAAGCATGGAGCTTAAGCGCATGGGACGCTCCATGGTTTCAGATTGGTTCTCTGACG  810
  D  R  T  L  R  A  E  L  T  I  R  S  M  E  L  K  R  M  G  R  S  M  V  S  D  W  F  S  D  D
ATGGGCTACCATGTGACGTAACTCGGGAGTTACTCCGCGAGCGTTTGCAGGGGATGACCATGACTACTACCGCACACTTGTCTGCTGAGA  900
  G  L  P  C  D  V  T  R  E  L  L  R  E  R  L  Q  G  M  T  M  T  T  T  A  H  L  S  A  E  I
TCCTCTCCACTTTTCGTCCGTCTTTGCGTCTTGCTTATCAGTCCTGGGAAGCCGGCACAGACCTTCGTTCTATCCTCCCTCACCGCACCT  990
  L  S  T  F  R  P  S  L  R  L  A  Y  Q  S  W  E  A  G  T  D  L  R  S  I  L  P  H  R  T  F
TTTACAAGTACCGTTCTGAGCTTCTGTCCCACGGCGTTGATATTGCAACGTTGGTTCCTCGCGAAGTCTCCAACGTAGTACCTCTCTTTA 1080
  Y  K  Y  R  S  E  L  L  S  H  G  V  D  I  A  T  L  V  P  R  E  V  S  N  V  V  P  L  F  R
GAACTCTTGAAGCTGTTCCCGCGCCTGTTCCTGATTGGGCTGTCGGGACACTGCTTTACTTCGAGCCTCCATCTGTCCGACTTCTCGCTT 1170
  T  L  E  A  V  P  A  P  V  P  D  W  A  V  G  T  L  L  Y  F  E  P  P  S  V  R  L  L  A  .
                                                                .  K  S  G  G  D  T  R  S  R  A
GACCCAACCCCGCTCCGGCGGGGGTTTTCTATTTCATGACGCGTCACGAAATGGGGTGCGGGGCGTGCCCCGCGGCTACCGCTCCTGCAGG 1260
          A                                                                   B
Q  G  L  G  A  G  A  P  N  E  I  E  H  R  T  V  F  H  P  A  P  R  A  G  R  S  G  S  R  C
AGCTCATCCCAACCCATGGATGCCTGGCGCGTGCCAGGTCGTTTCCCGGCCTACCCCCACCCCGCGCGCCTGCAAGCCGCTTGTCAGGGC 1350
S  S  M  G  V  W  P  H  R  A  R  A  L  D  N  G  P  R  G  G  G  R  A  G  A  L  R  K  D  P
TTCCAGGCGCCGCAGAGCTTGCAGCGCCAGCGGTGAGCGCCTGCGGCGCCTGGAAGCCCGTGCGTACCGCCGCAGTACCGTGGGAAGGGG 1440
                C
S  G  P  A  A  S  S  A  A  G  A  T  L  A  Q  P  A  Q  F  G  T  R  V  A  A  T  G  H  S  P
AGGGCAGGATACCGTCCTGTGTAGCCTCTGATGCCTCCCAGGAGCCGGTCAGCCCCCGCTTTGCTTGATACATGTGCCTAAGTAGCGGGC 1530
          D                                                  E
S  P  L  I  G  D  Q  T  A  E  S  A  E  W  S  G  T  L  G  R  K  A  Q  Y  M  H  R  L  L  P
AAACCCTCCTTCCATCCCTGAAACCCTTGCCTGGCTTTGCTCGGCCAACTCGATGTGCGGGGCTAGTGATACCGCACATTTGTGCACGCG 1620
                                                                F                 G
C  V  R  R  G  D  R  F  G  K  G  P  K  A  R  G  V  R  H  A  P  S  T  I  G  C  M  (ORF2)
GTGCACTGCCAATCCCCTTGCAGGCACTCCGCGTTACTCCTAGACTTGATCCATGCCATCTGACCAGACCCGCGTCAATCTCACCCTTCC 1710
CGCTGATGTAGTCCAGGTTTTGGACCGCCTCGGCAAGGTCACAGCGCCGGCCGCGCGACCATCATTCGCGAGTGGTTGATCGAGGGTCGC 1800
CCCATGTTTGCCGAGATGGCCACCGCCGCTGAAATGGCTCATGAGCGCCAG  1851
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(B)

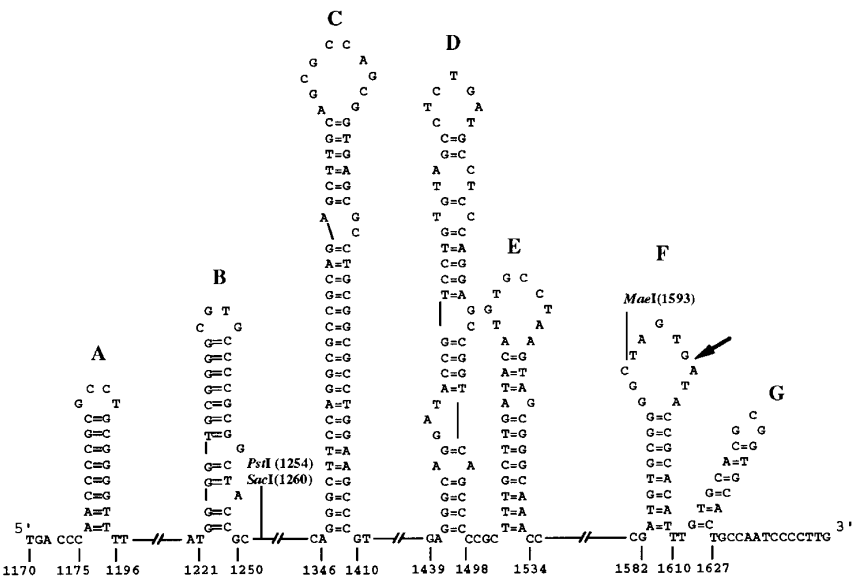

FIG. 2. (A) The complete nucleotide sequence of pXV64 and the deduced amino acid sequences for ORF1 and ORF2. The seven potential hairpin structures between bp 1,176 and 1,626, designated alphabetically from A to G, are indicated by arrows. (B) Schematic representation of the hairpin structures. Sites for restriction enzymes *Pst*I, *Sac*I, and *Mae*I are indicated. The predicted Rep nicking site is pointed out by an arrow.

```
XV64   MIDWLSMIVPCSHPTPITGGHVLSLTPDGEVSWRSSKRSTVEGSFGTGLQIRT-CTHTADP  60
       MIDW:: ::PC H .P:.:G:VLS::PDG.V.W S K S V.GSF ::: :R:  ::..:
I2-2   MIDWVTAVLPCLH-VPVDAGRVLSVAPDGSVEWESVKFSRVTGSFQSSISVRSQGSDGNGK  60

XV64   CTHLEISGNPVKFFQGHNLWGTDDLPSLAIATFNHLIGVLSLNPTDTDRALVRAGMIQLTR  121
        THL :.GNP K.:QGHN: G:DDL :L IA :.:::::L:: .  ..   V :G  :L.R
I2-2   ATHLYVDGNPSKWLQGHNIVGSDDLNGLMIAFYARMLSLLNIPHHLESYRQVLSGQYELKR  121

XV64   VDVTESFHLESRAQVLAWLRAAEQTAHLSHRGRGQLVKGTTLYFGKNSRRWGLKLYSKGQE  182
       VD:.  F:L :  :V :WL:AAE .A. :::GR . .KGT LYFGKNSRRW::K YSK :E
I2-2   VDINYMFELPTLIDVRSWLHAAEFKAK-TRHGRPATAKGT-LYFGKNSRRWSIKAYSKYDE  180

XV64   IH--AKGHGQDAILSLPHAVAWADRTLRAELTIRSMELKRMGRSMVSDWFSDDGLPCDVTR  241
       ::   K:HG  . :. .  V.W:...LR ELT:R:::L. :. :...:W ::.:
I2-2   VNCGKKAHGVPEEIKKTGLVEWSKNKLRLELTLRALQLTDINLNFAKNWSTETAY------  235

XV64   ELLRERLQGMTMTTTAHLSAEILSTFRPSLRLAYQSWEAGTDLRSILPHRTFYKYRSELLS  302
       .:::E :  :.M:..: L:.. : .:.::LR::Y .W..G. : .:::: T:::.R. L .
I2-2   TVFKEYMGRIEMSGNTLLTDTQVINLPSALRMTYVCWKQGICVTDMVSRATYFRHRKVLKE  296

XV64   HGVDIATLVPR-EVSNVVPLFRTLEAVPAPVPDWAVGTLLYFEPPSVRLLA  352
        G:DIA. V R : SNVVPL:R.LEA PA::P. . :.L:: . .  .:
I2-2   FGIDIAVTVDRVDNSNVVPLIRVLEAKPASIPS-QYDNLIFSSNRVSSF  344
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FIG. 3. Comparison of the deduced amino acid sequence of pXV64 ORF 1 (upper lines) with that of the filamentous phage I2−2 gene II protein (lower lines). Dots indicate a conservative substitution. Dashes are introduced for better alignments.

N-terminus of ORF1, this region may not be required for plasmid replication. If this prediction is correct, the *ori* can be narrowed down to be within the 392-bp region between bp 1,573 and 113, including hairpins F and G. In addition, ORF1 was thus demonstrated to possess Rep protein functions by these *in trans* tests.

A conserved sequence, TGATT(A)A, is present around the nicking site within the *ori* regions of the plus-strand replication of some phages and plasmids replicating by rolling circle mechanism (RCM); cleavage at 5′-G ↓ AT in this sequence by Rep proteins initiates replication (26). A similar sequence, 5′-TGATAC-3′, was found in hairpin F at bp 1,597 of pXV64 (Fig. 2). Viewing together the homology in amino acid sequence of the predicted ORF1 product to that of the I2-2 gIIP, and the similarity in DNA sequence in the putative nicking site to those occurring in the rolling circle replicating replicons, it is predicted that pXV64 may replicate by RCM. Should this prediction be true, pXV64 deserves further study in depth, because only a few plasmids of gram-negative bacteria have been known thus far to use RCM for replication (27-29).

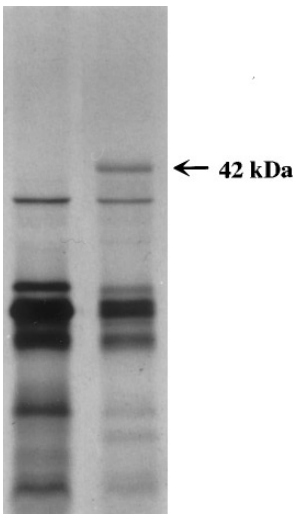

FIG. 4. Expression of a 42-kDa product encoded by ORF1 in *E. coli* maxicell. The cells of LCD44(pGEX-3X) (left) and LCD44(pGEX-F) (right) were induced with 5 mM IPTG.

Maxicell Expression of ORF1

To express the product encoded by ORF1, plasmid pGEX-F which carried the ORF1 under control of the *tac* promoter was used for maxicell expression in *E. coli* LCD44. A unique protein band with MW of 42 kDa was visualized in the autoradiogram after SDS-polyacrylamide gel electrophoresis (Fig. 4). The molecular size of this protein is similar to the value predicted for ORF1.

Some Implications in Evolution of pXV64

The G+C contents of ORF1 and ORF2 are 51.8 and 65%, respectively. It is noteworthy that one of the values is similar to that of the *X. c.* pv. *vesicatoria* chromosome (63.5%; 30), whereas the other is not. Deviation in G+C content implies that the region encompassing ORF1 may have originated from a source outside *X. campestris*. Thus, pXV64 appears to have evolved by fusion of a fragment of indigenous DNA containing the *ori* with an acquired sequence encoding the Rep protein.

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