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Stable Transformation of the *Xylella fastidiosa* Citrus Variegated Chlorosis Strain with *oriC* Plasmids

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***Xylella fastidiosa* is a gram-negative, xylem-limited bacterium affecting economically important crops (e.g., grapevine, citrus, and coffee). The citrus variegated chlorosis (CVC) strain of *X. fastidiosa* is the causal agent of this severe disease of citrus in Brazil and represents the first plant-pathogenic bacterium for which the genome sequence was determined. Plasmids for the CVC strain of *X. fastidiosa* were constructed by combining the chromosomal replication origin (*oriC*) of *X. fastidiosa* with a gene which confers resistance to kanamycin (Kan^r). In plasmid p16KdAori, the *oriC* fragment comprised the *dnaA* gene as well as the two flanking intergenic regions, whereas in plasmid p16Kori the *oriC* fragment was restricted to the *dnaA-dnaN* intergenic region, which contains *dnaA*-box like sequences and AT-rich clusters. In plasmid p16K, no *oriC* sequence was present. In the three constructs, the promoter region of one of the two *X. fastidiosa* rRNA operons was used to drive the transcription of the Kan^r gene to optimize the expression of kanamycin resistance in *X. fastidiosa*. Five CVC *X. fastidiosa* strains, including strain 9a5c, the genome sequence of which was determined, and two strains isolated from coffee, were electroporated with plasmid p16KdAori or p16Kori. Two CVC isolates, strains J1a12 and B111, yielded kanamycin-resistant transformants when electroporated with plasmid p16KdAori or p16Kori but not when electroporated with p16K. Southern blot analyses of total DNA extracted from the transformants revealed that, in all clones tested, the plasmid had integrated into the host chromosome at the promoter region of the rRNA operon by homologous recombination. To our knowledge, this is the first report of stable transformation in *X. fastidiosa*. Integration of *oriC* plasmids into the *X. fastidiosa* chromosome by homologous recombination holds considerable promise for functional genomics by specific gene inactivation.**

Xylella fastidiosa is a fastidious gram-negative, xylem-limited bacterium (26) that causes a range of economically important plant diseases, including citrus variegated chlorosis (CVC) (3, 23); Pierce's disease (PD) of grapevine; alfalfa dwarf; leaf scorch of almond, coffee, elm, sycamore, oak, plum, mulberry, maple, and oleander; and periwinkle wilt (for reviews, see references 19 and 20).

CVC is a major problem in Brazil, where over 70 million sweet orange trees (34%) are affected. The disease also occurs in Argentina, under the name "pecosita" (7, 9). CVC affects all commercial sweet orange varieties. Affected fruits are small and hardened and thus of no commercial value. Rapid dissemination of CVC comes from the use of infected nursery trees and transmission of *X. fastidiosa* by several xylem-feeding sharpshooter insect vectors.

The genome sequence of the CVC strain of *X. fastidiosa*, clone 9a5c, was recently determined, and the nature of genes that were identified by annotation suggests a number of potential pathogenicity mechanisms, such as cell-wall hydrolysis, adhesion, intervessel migration, and toxicity (26). However, gene function must be determined experimentally. One way is through the study of relevant mutants. Production and analysis of such mutants require bacterial transformation with appropriate plasmids.

Broad-host-range and/or suicide plasmids have been used extensively in genetic studies of various gram-negative, phytopathogenic bacteria, including *Xanthomonas* spp., which are known to be phylogenetically related to *X. fastidiosa* (27). However, attempts to transform *X. fastidiosa* 9a5c by conjugal transfer from an *Escherichia coli* donor strain or by electrotransformation with plasmids pUFR047 (6), pLAFR6 (2), pVSP61 (1), pDSK602 (17), pSUP2021 (25), and pUIRM504 (14) were unsuccessful (P. B. Monteiro and J. Renaudin, unpublished data). Therefore, we have constructed plasmids containing the chromosomal replication origin (*oriC*) of *X. fastidiosa*, a strategy which we developed for transformation of *Spiroplasma citri*, a plant-pathogenic mollicute related to low-guanosine-plus-cytosine gram-positive bacteria (22, 28). Here we report: (i) the construction of plasmids containing all or part of the *oriC* region of *X. fastidiosa,* as well as a kanamycin resistance gene driven by an *X. fastidiosa* rRNA promoter; (ii) the transformation of two *X. fastidiosa* citrus isolates with these plasmids; and (iii) integration of the plasmids into the bacterial chromosome at the rRNA promoter by homologous recombination. The results suggest that plasmids based on the *X. fastidiosa oriC* are promising tools for specific gene targeting through homologous recombination in *X. fastidiosa*.

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TABLE 1. Origins of *X. fastidiosa* strains

Name	Origin	Host source
9a5c	Macaubal, São Paulo, Brazil	*Citrus sinensis* (L.) Osbeck, Natal sweet orange
PR1111	Londrina, Parana, Brazil	*Citrus sinensis* (L.) Osbeck, Valência sweet orange
AR1111	Bella Vista, Corientes, Argentina	*Citrus sinensis* (L.) Osbeck, Valência sweet orange
J1a12	Jales, São Paulo, Brazil	*Citrus sinensis* (L.) Osbeck, Pera sweet orange
B111	Bebedouro, São Paulo, Brazil	*Citrus sinensis* (L.) Osbeck, Pera sweet orange
CA1111	Matão, São Paulo, Brazil	*Coffea arabica*, Catuaí
CA3124	Matão, São Paulo, Brazil	*Coffea arabica*, Mundo Novo

MATERIALS AND METHODS

Bacterial strains. *E. coli* XL-1 Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *Z*ΔM15 Tn*10* (Tet^r)]} was used as the host strain for subcloning experiments and for propagation of plasmids. Strains of *X. fastidiosa* were isolated and subcultured from symptomatic twigs of sweet orange or coffee as previously described (3, 13). Their geographical origin and host species are indicated in Table 1. *X. fastidiosa* cells were grown in PW medium (5) at 29°C in the dark with low-speed, rotatory agitation (100 rpm).

Primers and PCR amplification. The sequences of all primers used in this study are described in Table 2. Primers RP1 and RP2 were used to amplify the promoter region of rRNA operon 1 of *X. fastidiosa* 9a5c (fragment *rop* in Fig. 1A). Primers OR1 and OR2 were used to produce a 1,893-bp DNA fragment (fragment *oriC*) comprising the *dnaA* gene and the 5′ and 3′ flanking intergenic regions. Amplification with primers OR3 and OR2 yielded a 366-bp product corresponding to the region between the genes *dnaA* and *dnaN* (fragment *ori* in Fig. 1A). Primers KAX1 and KA2 were used to amplify the coding sequence of the Tn*903* kanamycin resistance (Kan^r) gene with plasmid pUC4K (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) as the template. For simplicity we called this fragment "kan^r " in Fig. 1A. Primer KAX1 contains the 10 nucleotides (underlined in Table 2), including the ribosome binding site (boldface characters in Table 2), immediately upstream of the coding sequence of the *X. fastidiosa dnaN* gene. PCRs were performed in a thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.) using *Pfu* turbo DNA polymerase as described by the supplier (Stratagene, La Jolla, Calif.). Amplification was achieved over 40 cycles of 30 s at 92°C, 1 min at 60°C, and 4 min at 72°C, with an additional step of 10 min at 72°C. For the amplification of fragments *rop*, *oriC,* and *ori*, we used purified genomic DNA of *X. fastidiosa* as the template. Cloned PCR products were sequenced with a T7 sequencing kit (Amersham Pharmacia Biotech, Inc.). Amplification reactions with primer pairs RP1-KA2 and M13 universal primer (M13U)-RP3 were carried out in a 20-μl reaction mixture containing 1 μl of *X. fastidiosa* culture or 50 ng of target DNA, 50 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 200 μg of bovine serum albumin per ml, 0.05% W1 detergent, 0.2 mM deoxynucleoside triphosphates, each primer at 1 μM, and 2.5 U of *Taq* DNA polymerase (GIBCO/BRL Life Technologies, Inc., Gaithersburg, Md.). After a denaturation step of 4 min at 94°C, amplification was achieved over 45 cycles of 1 min at 92°C, 50 s at 60°C, and 3 min at 72°C, with a final step of 10 min at 72°C. The PCR product obtained with primer pair M13U-RP3 was purified from agarose gel and directly sequenced. Primers OR1, OR2, OR3, RP1, RP2, and RP3 (Table 2) were designed from the *X. fastidiosa* genome sequencing data accessible online at http://www.lbi.dcc.unicamp.br/xylella/.

TABLE 2. Primers designed from the *X. fastidiosa* genome sequencing data

Primer name	Primer sequence
cKA2	5′-CGGGGTACCAGTGAATTCCCCGGATC-3′
KAX1	5′-CCGGAATTCA**AGGA**ACAAAATGAGCCATATTCAACGGGAA-3′
OR1	5′-CGCGGATCCGATAAGTACGCTTGGTGGCC-3′
OR2	5′-CGCGGATCCGCGCTGTAATCTGAAACGC-3′
OR3	5′-CGCGGATCCAGACTGGGATAAACTAATGCGA-3′
RP1	5′-GGGTACCGGATCCGTCGACGCCAGATATCGTATTCCATTG-3′
RP2	5′-CCGGAATTCTCAATGCATACGCCAAAA-3′
RP3	5′-AGATAAGGTAGATTCCGATGTAT-3′

Construction of plasmids p16K, p16KdAori, and p16Kori. The promoter region of the *X. fastidiosa* rRNA operon 1 was obtained as an 831-bp DNA fragment by PCR amplification of genomic DNA with primer pair RP1-RP2. The coding sequence of the kanamycin resistance gene was amplified with the primer pair KAX1-KA2 with pUC4K as the template. Both fragments were digested with *Eco*RI. These fragments were then ligated with T4 DNA ligase, and the resulting ligation products were restricted with *Kpn*I. The *Kpn*I-digested DNA was ligated to the dephosphorylated, *Kpn*I-linearized pBS+ vector (Stratagene, La Jolla, Calif.), and the final ligation mixture was used to electrotransform *E. coli* XL-1 Blue competent cells. Primer pair RP1-KA2 was used in a PCR to screen the transformed, kanamycin-resistant clones. The relative orientations of the promoter and Kan^r gene fragments were determined by double digestion of the recombinant plasmids with enzymes *Eco*RI and *Sma*I. The recombinant plasmid carrying the Kan^r gene downstream of the promoter and in the appropriate orientation for transcription was named p16K (Fig. 1A). To construct plasmid p16KdAori, a 1,893-bp *oriC* fragment containing the *dnaA* gene and its flanking regions was obtained by amplification of genomic DNA from *X. fastidiosa* 9a5c with primer pair OR1-OR2 (Fig. 1B). The PCR product was digested with *Bam*HI and inserted at the *Bam*HI site of the pBS+ vector. The cloned fragment was rescued from the recombinant plasmid as a *Sma*I-*Hin*cII fragment of 1,903 bp and inserted into the *Xba*I-linearized, Klenow-filled-in, plasmid p16K to produce the final plasmid construct, p16KdAori (Fig. 1A). The relative orientations of the *oriC* and the Kan^r fragments were checked by PCR with the primer pair KAX1-OR2. In plasmid p16KdAori, the *dnaA* and Kan^r genes are in the same orientation. To construct the p16Kori plasmid, a 366-bp fragment encompassing the *dnaA-dnaN* intergenic region was amplified with primer pair OR3-OR2 (Fig. 1B). The PCR product was digested with *Bam*HI, ligated to dephosphorylated, *Bam*HI-linearized pBS+ vector, and cloned into *E. coli*. The cloned fragment was rescued from the recombinant plasmid as a *Sma*I-*Hin*cII fragment of 376 bp and transferred to *Xba*I-linearized, Klenow-filled-in plasmid p16K to yield the recombinant plasmid p16Kori (Fig. 1A). Using the primer pair RP1-OR2, it was determined by PCR that the *dnaA* box region was cloned in an orientation reverse that of the Kan^r gene.

Transformation of *X. fastidiosa* and selection of transformants. A single colony from a freshly streaked PW agar plate was dispersed in 2 ml of liquid PW medium (minus $MgSO_4$, plus L-histidine [0.1%, wt/vol]) by vortexing, and the cell suspension was incubated at 29°C for 4 days. A sample (0.3 ml) of this culture was used to inoculate 30 ml of PW. After a 4-day incubation under the same conditions, the culture was transferred to a chilled polypropylene tube and the cells were collected by centrifugation (2,600 × *g* for 15 min) at 4°C. The pelleted cells were washed twice in 30 ml of chilled ultrapure (milli-Q; Millipore) water and once in cold 10% glycerol. The final cell pellet was resuspended in 0.3 ml of 10% glycerol and kept on ice. For electroporation, an 80-μl aliquot of cell suspension was mixed with 5 to 10 μg of DNA in 5 μl of TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA). The mixture of cells and DNA was transferred to a cold 0.2-cm electroporation cuvette and kept on ice for 1 min. The cells were electroporated at 2.5 kV, 200 Ω, and 25 μF to generate a pulse of approximately 6 ms. After electroporation, the cells were resuspended in 1 ml of PW plus L-histidine (0.1%, wt/vol) recovery medium and incubated at 29°C for 6 h without agitation. The transformants were selected by plating (250 μl of cell suspension per plate) on PW agar medium supplemented with kanamycin (5 μg per ml). The plates were wrapped with Parafilm to prevent desiccation and incubated for 20 days at 29°C. The kanamycin-resistant colonies were picked individually and grown in broth medium containing 5 μg of kanamycin per ml. During propagation, the antibiotic concentration was progressively increased to 20 μg/ml.

DNA isolation and Southern blot hybridization. Large-scale and small-scale preparations of plasmid DNA amplified in *E. coli* were carried out according to standard procedures (24). *X. fastidiosa* DNA was isolated as described by Chen and coworkers (4), with the following modification. After the cells were lysed

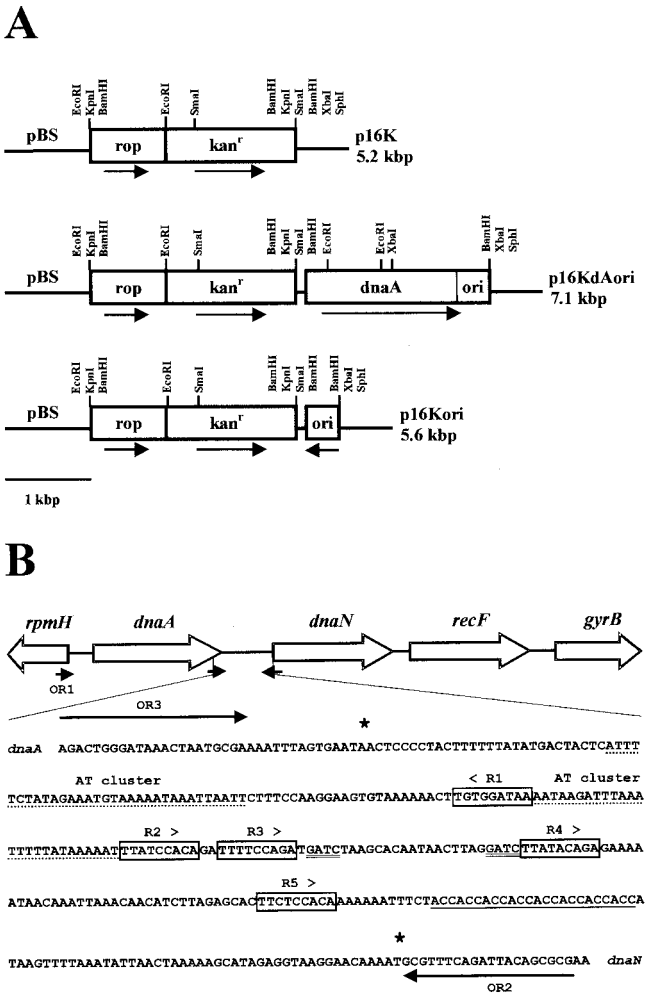

FIG. 1. (A) Partial restriction maps of plasmids p16K, p16KdAori, and p16Kori. pBS, pBluescript; rop, promoter region of *X. fastidiosa* ribosomal operon 1; kan^r, kanamycin resistance gene from Tn*903*; ori, *X. fastidiosa dnaA-dnaN* intergenic region. The arrows indicate the direction of transcription. (B) Gene organization of the *X. fastidiosa oriC* region and nucleotide sequence of the *dnaA-dnaN* intergenic region. Genes *rpmH*, *dnaA*, *dnaN*, *recF*, and *gyrB* are indicated by open arrows. Positions of primers OR1, OR2, and OR3 are indicated by black arrows. Boxed sequences R1 to R5 are putative *dnaA* boxes. The two GATC sites are double underlined; the stretch of ACC triplets is single underlined. The AT clusters are underlined with a dotted line. The upper and lower asterisks indicate the translational stop codon of *dnaA* and the initiation codon of *dnaN*, respectively.

with lysozyme, the preparation was heated at 60°C for 1 h in the presence of 0.5% sodium dodecyl sulfate and 0.1 mg of proteinase K/ml of solution. *X. fastidiosa* extrachromosomal DNA was prepared by the alkaline lysis method (24). For Southern blot hybridizations, restricted DNA was denatured in 0.4 N NaOH and blotted to positively charged nylon membranes by the capillary transfer procedure with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridizations with biotinylated probes were carried out according to the procedures outlined in the labeling kit, BioPrime, and the detection kit, Blue Gene (GIBCO/BRL Life Technologies, Inc.). The biotinylated probes used in this study were obtained by PCR amplification with the following primer pairs: KA1-KA2 for the kanamycin resistance gene (probe *kan*), OR2-OR3 for the replication origin (probe *ori*), and RP1-RP2 for the promoter region of rRNA operon 1 (probe *rop*).

RESULTS

***oriC* region of *X. fastidiosa*.** The *oriC* region used in this work was selected from the *X. fastidiosa* genome sequence (26). This region comprises the *dnaA* gene and the two flanking intergenic regions, one between genes *rpmH* and *dnaA* and the other between *dnaA* and *dnaN* (Fig. 1B). Plasmid p16KdAori contains the entire *oriC* region, while in plasmid p16Kori, only the 366-bp intergenic region downstream of *dnaA* is present (Fig. 1A). This fragment shares features with the *oriC* region of other gram-negative bacteria. In particular, it contains five *dnaA* boxes (consensus sequence TTATCCACA), two GATC Dam methylation sites in between *dnaA* boxes R3 and R4, and two AT-rich sequences of approximately 35 nucleotides each (Fig. 1B). In addition, the region downstream of the *dnaA* boxes contains a distinctive stretch of 24 nucleotides consisting of 8 repeats of the ACC triplet that could represent the counterpart of the 13-mer repeats in the *E. coli oriC* region (18).

Transformation of *X. fastidiosa* isolates with plasmids p16K and p16KdAori. Five citrus strains and two coffee strains of *X. fastidiosa* (see Table 1 for origins of the strains) were electrotransformed with plasmids p16K and p16KdAori. Transformation assays with all seven strains, using 5 or 10 μg of plasmid p16K, yielded no kanamycin-resistant transformants, indicating that p16K did not replicate or integrate into the host chromosome and that spontaneous resistance was not detected for the level of kanamycin used in the experiments. In contrast, kanamycin-resistant transformants were obtained for the citrus strains J1a12 and B111 electroporated with plasmid p16KdAori but not for the other five strains of *X. fastidiosa* tested. With both strains J1a12 and B111, the transformation efficiency was found to be very low, approximately 10 transformants per μg of plasmid DNA. Ten kanamycin-resistant colonies of each strain were grown separately in liquid PW medium containing 5 μg of kanamycin per ml. The presence of p16KdAori sequences in these transformants was subsequently demonstrated by PCR amplification with primer pair RP1-KA2 and by hybridization of total DNA with the *kan*, *ori*, and *rop* probes (data not shown).

Maintenance of plasmid p16KdAori in *X. fastidiosa* transformants. To determine whether the plasmid was maintained as free extrachromosomal DNA or had integrated into the *X. fastidiosa* chromosome, the transformants carrying p16KdAori were subcultured twice and grown in liquid medium for a period corresponding to approximately 40 generations. The maintenance of the plasmid was monitored by Southern blot hybridization of total and extrachromosomal DNA with various probes. In the experiment represented in Fig. 2, total and extrachromosomal DNAs of two independent *X. fastidiosa* J1a12 transformants were digested with *Sph*I or *Bam*HI and hybridized with the *kan* probe. The bands of 7.1 kbp and 2 kbp correspond, respectively, to the *Sph*I and *Bam*HI fragments of the purified plasmid (Fig. 2A, lanes 1 and 7) and were not detected in the transformed cells, either in extrachromosomal DNA (Fig. 2A, lanes 2 and 6) or in the total DNA (Fig. 2A, lanes 3 and 5). Instead, an 11.1-kbp *Sph*I fragment and an 8.4-kbp *Bam*HI fragment were detected in the transformants (Fig. 2A, lanes 3 and 5) but not in untransformed cells (Fig. 2A, lanes 4). The absence of p16KdAori as a free extrachromosomal element was confirmed by the inability to transform

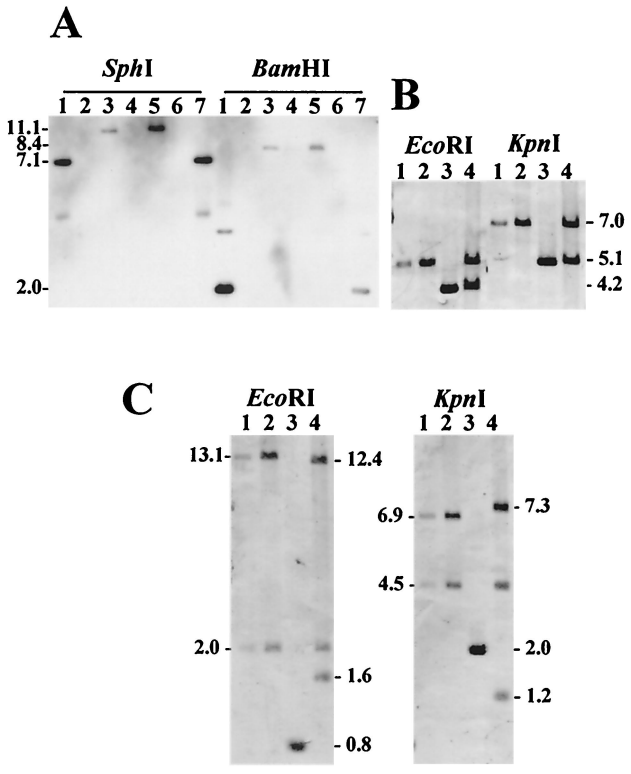

FIG. 2. (A) Southern blot hybridization between *Sph*I- or *Bam*HI-digested DNA extracted from *X. fastidiosa* transformants and the *kan* probe. Lanes 1 and 7, p16KdAori; lanes 2 and 6, extrachromosomal DNA extracted from transformant clones 1 (lanes 2) and 2 (lanes 6); lanes 3, 4, and 5, total DNA extracted from transformant clone 1 (lanes 3), from untransformed cells (lanes 4), and from transformant clone 2 (lanes 5). (B) Southern blot hybridization between *Eco*RI- or *Kpn*I-digested DNA extracted from *X. fastidiosa* transformants and the *ori* probe. Lanes 1 and 2, total DNA extracted from untransformed cells of strains 9a5c (lanes 1) and J1a12 (lanes 2); lanes 3, p16KdAori; lanes 4, total DNA extracted from *X. fastidiosa* transformant clone 1. (C) Southern blot hybridization of *Eco*RI- or *Kpn*I-digested DNA from untransformed and p16KdAori-transformed *X. fastidiosa* with the *rop* probe. Lanes 1, total DNA extracted from *X. fastidiosa* 9a5c; lanes 2, total DNA extracted from untransformed strain J1a12; lanes 3, p16KdAori; lanes 4, total DNA extracted from J1a12 transformant clone 1. Sizes are indicated in kilobase pairs.

E. coli with DNA extracted from the *X. fastidiosa* transformants. These results suggested that the plasmid or part of the plasmid had integrated into the bacterial chromosome.

Mapping the p16KdAori insertion site in *X. fastidiosa* transformants. To determine the position at which p16KdAori had integrated into the chromosome, genomic DNAs from three independent clones were digested with various enzymes and hybridized with the *ori* and the *rop* probes (Fig. 2B and C, respectively). Identical results were obtained with each of the three transformants. In Fig. 2B, the hybridization patterns of DNA extracted from the *X. fastidiosa* transformant are compared to those of the DNA extracted from untransformed cells from the same strain (J1a12). For comparison, DNA from strain 9a5c was also included. In the case of the transformant (Fig. 2B, lanes 4), the *ori* probe was found to hybridize with two *Eco*RI fragments, of 5.1 kbp and 4.2 kbp, or two *Kpn*I fragments, of 7 kbp and 5.1 kbp. One fragment from each pair (5.1-kbp *Eco*RI fragment or 7-kbp *Kpn*I fragment) comigrated with that detected in the untransformed cells (Fig. 2B, lanes 1 and 2), whereas the other comigrated with the fragment obtained from the purified p16KdAori. Having determined (from the experiment that produced Fig. 2A) that there was no free plasmid in the transformants, these results indicate that the plasmid was not integrated at the *oriC* region of the chromosome. To determine whether plasmid integration occurred by recombination at the rRNA promoter region, the restricted DNA was hybridized with the *rop* probe (Fig. 2C). As was expected from the occurrence of two rRNA operons in the *X. fastidiosa* genome (26), the probe hybridized with two DNA fragments in untransformed cells: two *Eco*RI fragments of 13.1 kbp and 2 kbp and two *Kpn*I fragments of 6.9 kbp and 4.5 kbp (Fig. 2C, lanes 1 and 2). However, with the DNA extracted from transformed cells, three fragments hybridized with the probe regardless of the enzyme (*Eco*RI or *Kpn*I) used to restrict the DNA (Fig. 2C, lanes 4). Interestingly, none of these three fragments is found in the purified plasmid (Fig. 2C, lanes 3). The 2-kbp *Eco*RI fragment or the 4.5-kbp *Kpn*I fragment corresponds to one of the two fragments detected in the untransformed cells, whereas the other two fragments, with sizes of 12.4 kbp and 1.6 kbp for *Eco*RI and 7.3 kbp and 1.2 kbp for *Kpn*I, differ in size from those in the untransformed cells. These data indicate that, in the transformants, the promoter region of one of the two rRNA operons is duplicated. This suggests that the p16KdAori plasmid has integrated into the chromosome by homologous recombination via a single crossover event, leading to the duplication of the insertion site sequences. Indeed, further Southern blot analyses showed that the restriction fragments hybridizing with the *rop* probe (Fig. 3A) had sizes that matched those predicted from the map in Fig. 3B, showing recombination between the rRNA promoter fragment carried by the plasmid and the promoter region of rRNA operon 1 of the chromosome. As shown in Fig. 3A, the 1.6-kbp *Eco*RI fragment, 1.2-kbp *Kpn*I fragment, 7.3-kbp *Sma*I fragment, 6.2-kbp *Hin*dIII fragment, and 3.9-kbp *Xba*I fragment (lanes 3, 12, 18, 21, and 24, respectively) were not detected in either the purified plasmid (lanes 1, 10, 16, 19, and 22, respectively) or the untransformed cells (lanes 2, 11, 17, 20, and 23, respectively). This indicates that plasmid integration did occur in the promoter region of the rRNA operon 1.

To confirm the site of plasmid integration in the transformants, DNA was amplified with primers M13U, specific for pBS+, and RP3, specific for a site at the 5′ end of the 16S rRNA gene (Fig. 3B). As expected, a 1-kbp fragment was specifically amplified with the DNA from the transformants but not with DNA from untransformed cells. Sequence analyses showed that the amplification product did contain pBS+ sequences, the rRNA promoter region, and 200 bp of the 5′ end of the 16S rRNA gene. It should be noted that the nucleotide sequence of the rRNA promoter of *X. fastidiosa* strain J1a12 was found to be identical to that of strain 9a5c (26). Interestingly, analysis of 12 additional transformants of strain J1a12 revealed that plasmid integration occurred at the same location in the chromosome for all 12 transformants.

To assess the stability of plasmid integration, the p16KdAori transformants were propagated in liquid medium with or without kanamycin for more than 50 generations and then plated on 1% PW agar containing 20 μg of kanamycin per ml. No

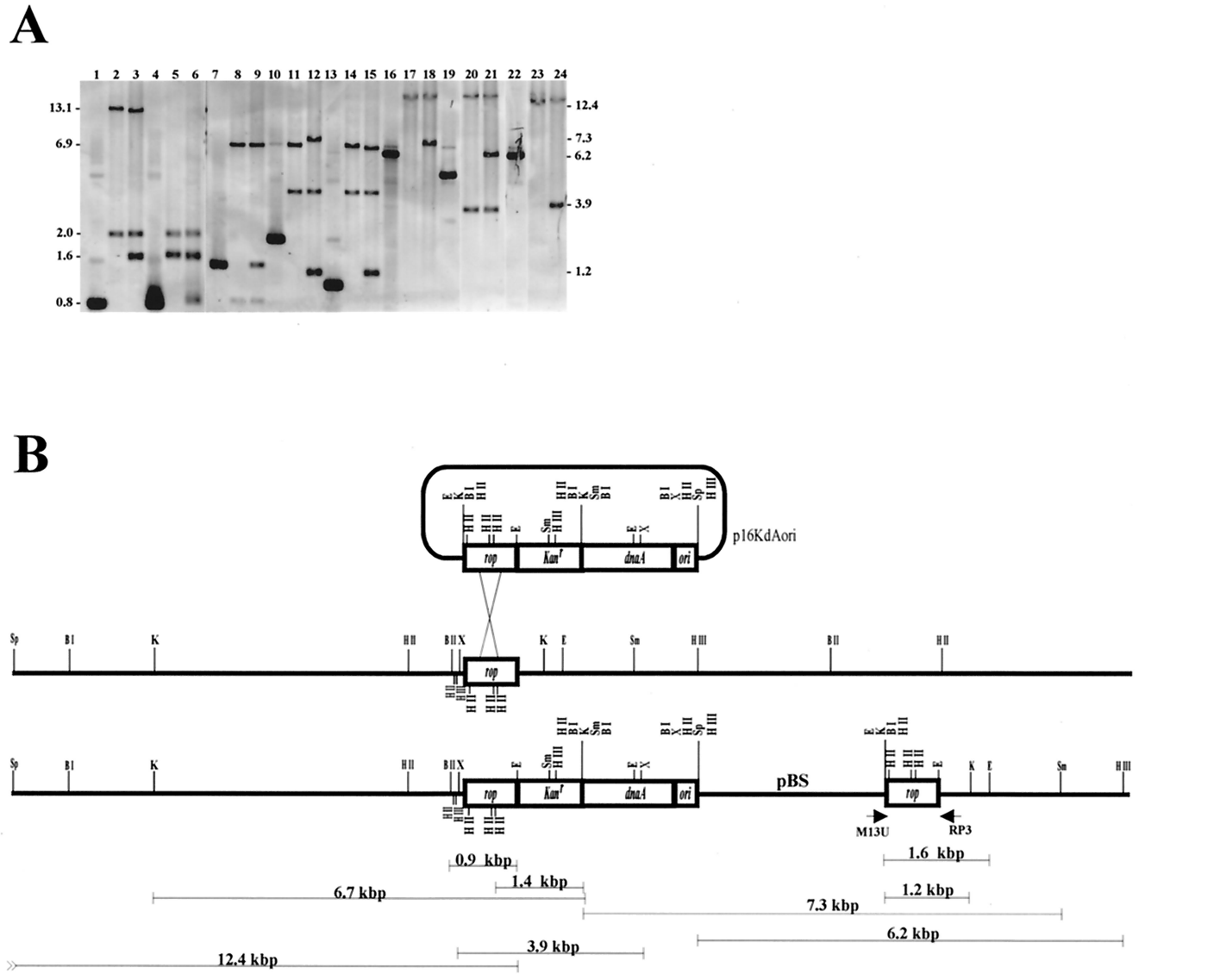

FIG. 3. (A) Southern blot hybridization of DNA from untransformed and p16KdAori-transformed *X. fastidiosa* J1a12 (clone 1) with the *rop* probe. Genomic DNA from untransformed cells (lanes 2, 5, 8, 11, 14, 17, 20, and 23) and from cells transformed by p16KdAori (lanes 3, 6, 9, 12, 15, 18, 21, and 24), as well as purified p16KdAori (lanes 1, 4, 7, 10, 13, 16, 19, and 22), were digested with *Eco*RI (lanes 1 to 3), *Eco*RI plus *Bgl*II (lanes 4 to 6), *Hin*cII (lanes 7 to 9), *Kpn*I (lanes 10 to 12), *Sma*I plus *Kpn*I (lanes 13 to 15), *Sma*I (lanes 16 to 18), *Hin*dIII (lanes 19 to 21), and *Xba*I (lanes 22 to 24), respectively. Sizes are indicated in kilobase pairs. (B) Schematic representation of p16KdAori integration by recombination at the promoter region of rRNA operon 1 of *X. fastidiosa*. The 1.6-kbp and 12.4-kbp *Eco*RI fragments, 0.9-kbp *Eco*RI-*Bgl*II fragment, 1.4-kbp *Hin*cII fragment, 6.2-kbp *Hin*dIII fragment, 1.2-kbp and 6.7-kbp *Kpn*I fragments, 7.3-kbp *Sma*I fragment, and 3.9-kbp *Xba*I fragment are indicated. The positions of primers M13U and RP3 are indicated by short thick arrows. Abbreviations: BI, *Bam*HI; BII, *Bgl*II; E, *Eco*RI; HII, *Hin*cII; HIII, *Hin*dIII; K, *Kpn*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I. For other abbreviations, see the legend to Fig. 1.

reversion to kanamycin sensitivity was noticed, regardless of the presence or absence of kanamycin as the selection pressure.

Transformation of *X. fastidiosa* with plasmid p16Kori. The *oriC* fragment of plasmid p16KdAori comprises the *dnaA* gene as well as the 366-bp *dnaA-dnaN* intergenic region. This region contains five *dnaA*-box-like sequences and two AT-rich clusters and is thought to represent the genuine replication origin of the *X. fastidiosa* chromosome (26). To determine whether this region alone is able to function as an autonomous replicating sequence, we constructed plasmid p16Kori by inserting this DNA fragment into plasmid p16K (see Materials and Methods). Transformation of *X. fastidiosa* strain J1a12 with p16Kori did yield kanamycin-resistant colonies, suggesting that the 366-bp *oriC* fragment was able to promote plasmid replication in *X. fastidiosa*. Three colonies were picked and grown in the presence of kanamycin. After two passages in PW medium containing 5 μg of kanamycin/ml, total DNA as well as extrachromosomal DNA was prepared and analyzed by Southern blot hybridization with the *ori* and *rop* probes (Fig. 4A and B). As shown in Fig. 4, the 5.1-kbp *Eco*RI fragment hybridizing with the *ori* probe in the untransformed cells (Fig. 4A, lane 1)

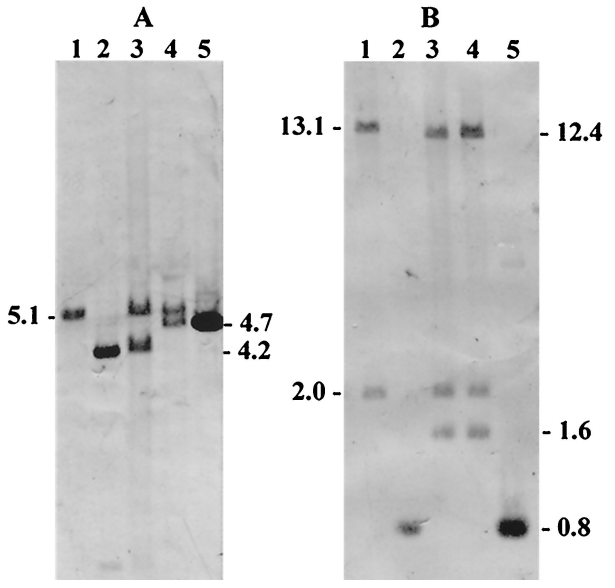

FIG. 4. Southern blot hybridization between *Eco*RI-restricted DNA extracted from *X. fastidiosa* J1a12 transformed with plasmids p16KdAori or p16Kori and the *ori* (A) or *rop* (B) probe. Lanes 1, total DNA from untransformed cells; lanes 2, plasmid p16KdAori; lanes 3 and 4, total DNAs from cells transformed with p16KdAori (lanes 3) or with p16Kori (lanes 4); lane 5, plasmid p16Kori. Sizes are indicated in kilobase pairs.

is still detected in the transformant (Fig. 4A, lane 4). In contrast, the 13.1-kbp *Eco*RI fragment hybridizing with the *rop* probe (Fig. 4B, lane 1) is not detected (Fig. 4B, lane 4). These results indicate that, as previously described for plasmid p16KdAori, p16Kori has also integrated in the rRNA promoter region. In agreement with this conclusion, the hybridization patterns of DNAs from p16Kori and p16KdAori transformants are identical (Fig. 4B, lanes 3 and 4). In addition, attempts to purify extrachromosomal DNA from the p16Kori transformant by the alkaline lysis procedure failed to reveal the presence of free plasmid (data not shown).

DISCUSSION

Originally, *oriC* plasmids were developed to study the initiation mechanism of chromosome replication by isolating chromosomal fragments with autonomous replication (*ars*) activity (12, 15). In our studies, transformation of CVC strains of *X. fastidiosa* to kanamycin resistance by electroporation was obtained with plasmids containing all (p16KdAori) or part (p16Kori) of the *X. fastidiosa oriC* region, but not with plasmid p16K, which lacks the *oriC* sequences. These results suggest that the two plasmids do possess *ars* activity even though, in the experiments reported here, we failed to detect the plasmids as free extrachromosomal DNA at the time when DNA was harvested from the transformed cells for analysis. To get enough material, the cells were harvested after two passages, which represented about 40 generations. We propose that during this period the *oriC* plasmids do replicate before integration. In agreement with this assumption, we have detected faint bands in some Southern blot experiments that could represent free plasmid DNA. Also, the *oriC* fragment of p16Kori, i.e., the 366-bp intergenic region downstream of the *dnaA* gene, has features in common with the *oriC* regions of other gram-negative bacteria, in particular five *dnaA* boxes and two AT-rich sequences. The requirement of these elements for *ars* activity has not been tested so far. However, our results represent the first experimental indication that sequences in the intergenic region downstream of the *dnaA* gene do possess *ars* activity.

In gram-negative bacteria, *oriC* plasmids are usually maintained extrachromosomally. In *E. coli*, incompatibility between *oriC* plasmids and the chromosome is seen only under very special conditions, such as reduced activity of DnaA protein (for a review, see reference 12). In contrast to *E. coli*, the gram-positive bacterium *Bacillus subtilis* strictly regulates the number of *oriC* copies within a cell (16). In this organism, *oriC* plasmids cannot be maintained and have a tendency to integrate into the chromosome. Surprisingly, in *X. fastidiosa* transformants, we found that plasmids p16Kori and p16KdAori were not maintained as free extrachromosomal elements but, instead, had integrated into the host chromosome. As indicated above, no free plasmid was detected in the transformed cells after two subcultures in liquid medium. These results suggest that, in *X. fastidiosa*, DnaA expression could be more tightly regulated than in *E. coli* and that therefore the limited level of DnaA expression does not allow extrachromosomal replication of *oriC* plasmids. Interestingly, in all 17 transformants tested, plasmid integration was found to occur by homologous recombination involving a single crossover event between the rRNA promoter region carried on the plasmid and the homologous sequences present in the chromosome. No recombination at *oriC* was observed, in contrast to the situation found to occur in the plant pathogen *S. citri* (22). In this case, the *oriC* plasmid pBOT1 integrates into the host chromosome by homologous recombination at the *oriC* region. The reason for which, in *X. fastidiosa*, plasmid integration preferentially occurred at the rRNA promoter region rather than at *oriC* is not known.

Five CVC strains and two coffee strains of *X. fastidiosa* were subjected to transformation assays, but only two CVC strains, J1a12 and B111, could be transformed. Thus, transformation of *X. fastidiosa* seems to be strain dependent for reasons that have to be investigated further. In the early stages of this work, attempts to transform *X. fastidiosa* 9a5c with several conjugative or suicide plasmids were unsuccessful. In these assays we used only CVC strain 9a5c. The experience gained with the *oriC* plasmids suggests that strains other than 9a5c could perhaps be transformed with some of the plasmids described above.

Transformation of CVC *X. fastidiosa* strain LAR20 has recently been reported (21). However, the shuttle plasmid pEcoR#10 used in these studies proved to be unstable. In contrast, in our case, we have determined that p16KdAori and p16Kori, once integrated into the chromosome, are stably maintained regardless of the presence or absence of kanamycin as the selection pressure.

X. fastidiosa is the first plant-pathogenic bacterium whose genome sequence has been determined (26). Although several putative virulence genes are present in *X. fastidiosa*, very little is known experimentally about the pathogenicity mechanisms compared with those of other gram-negative bacteria, such as *Ralstonia solanacearum* or *Xanthomonas campestris,* for which

the availability of gene transfer systems was a key factor in the development of genetic studies. In the plant-pathogenic mollicute *S. citri*, an organism related to low-guanosine-plus-cytosine gram-positive bacteria, *oriC* plasmids were used as vectors for both expression of cloned genes in spiroplasma cells (11, 22) and gene inactivation through homologous recombination (8, 10). The stable transformation of CVC strains of *X. fastidiosa* by *oriC* plasmids represents the first step toward functional genomic studies. It opens the way for the expression of cloned genes in *X. fastidiosa*. In addition, it can be expected that the integrative property of *X. fastidiosa oriC* plasmids will contribute to the construction of mutants by specific gene inactivation through homologous recombination. In this respect, the experimental infection of the herbaceous plant *Catharanthus roseus* by *X. fastidiosa* 9a5c that we have recently described (14a) should help us to evaluate the pathogenicity of the mutants.

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