Indigenous Miniplasmids in Strains of *Xanthomonas campestris* pv. *vesicatoria*

V. Dittapongpitch and D. F. Ritchie

Department of Plant Pathology, Box 7616, North Carolina State University, Raleigh 27695. Current address of the first author: Department of Plant Pathology, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand.

We thank R. G. Upchurch for helpful discussion and suggestions, especially in the early phases of this research. We also thank R. E. Stall (University of Florida, Gainesville) for graciously providing pepper seed of Early Calwonder and the three near-isogenic lines 10R, 20R, and 30R, and for *Xanthomonas campestris* pv. *vesicatoria* strains Xcv 2 (82-8, race 1) and Xcv 3 (E3, race 2) and clones with inserts for *avrBs1, avrBs3,* and the copper resistance gene. D. W. Gabriel (University of Florida, Gainesville) graciously provided vector pUFR047. We also acknowledge M. J. Dykstra, North Carolina State University College of Veterinary Medicine, for assistance with the electron microscopy.

This research was funded in part by the North Carolina Agricultural Research Service. The first author was a Freedom from Hunger Scholar supported in part by The Rotary Foundation of Rotary International.

Accepted for publication 19 May 1993.

ABSTRACT

Dittapongpitch, V., and Ritchie, D. F. 1993. Indigenous miniplasmids in strains of *Xanthomonas campestris* pv. *vesicatoria.* Phytopathology 83:959-964.

Four sizes of miniplasmids, estimated on the basis of migration distances in agarose gels, were observed in strains of *Xanthomonas campestris* pv. *vesicatoria.* Estimated sizes of these miniplasmids were 2.5, 2.28, 1.8, and 1.7 kilobase pairs (kbp). Only one size of miniplasmid was observed within a bacterial strain. There was DNA homology between the two larger miniplasmids, 2.5 and 2.28 kbp, and between the two smaller miniplasmids, 1.8 and 1.7 kbp, but not between the two size groups. The two larger miniplasmids were linearized by *Bam*HI; the two smaller miniplasmids were linearized by *Xho*I. Although all bacterial strains with miniplasmids also contained at least one larger plasmid, no homology of miniplasmid DNA to any larger plasmids or chromosomal DNA was detected. In bacterial strains isolated from diseased tissue, miniplasmids were detected only in race 2 strains and strains of the tomato group. Miniplasmids were observed in streptomycin-resistant and/or copper-resistant strains. However, not all strains resistant to copper contained a miniplasmid (e.g., race 1 strains) nor were all strains that contained a miniplasmid resistant to streptomycin. In the strains resistant to streptomycin, the size of the miniplasmid was inversely associated with the concentration of streptomycin tolerated by the strain. The miniplasmids did not hybridize with a copper-resistance determinant, *avrBs1* (specifies pathogen race 2), or *avrBs3* (specifies pathogen race 1). Miniplasmids that mobilized into copper- and streptomycin-sensitive strains of *X. c. vesicatoria* did not convert such strains to copper or streptomycin resistance.

Additional keywords: host-differentiated pathogen races, pepper.

Many phytopathogenic bacteria contain extrachromosomal DNA in the form of plasmids (8). Virulence and avirulence, resistance to antimicrobial chemicals, and production of antimicrobial products such as bacteriocins are examples of plasmid-encoded functions (4,6–8). In strains of the bacterial spot pathogen of pepper and tomato, *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye, several plasmid-encoded genes are present that enable this pathogen to adapt to different ecological niches. Genes that encode avirulence and subsequent host specificity are located on the self-transmissible pXvCu-type plasmid pXvCu1 (*avrBs1,* pathogen race 2) (27,28) and on plasmid pXV11 (45 kilobase pairs [kbp]) (*avrBs3,* pathogen race 1) (2). Plasmid pXvCu1 also contains three copies of an active transposable element, IS476, that can inactivate *avrBs1*, thus enabling the previously avirulent bacterial strain to evade the host defense response of pepper plants that contain the *Bs1* gene (15). On the basis of one or more avirulence genes in the pathogen and one of three resistance genes in near-isogenic lines of pepper (21), four host-differentiated races, designated 0, 1, 2, and 3, were detected (23).

Copper resistance in *X. c. vesicatoria* is associated with a large (approximately 200 kbp) self-transmissible plasmid, pXvCu (27). Other large plasmids in *X. c. vesicatoria,* including pXV10A, share homology with the pXvCu-type plasmids, but pXV10A does not show homology to IS476, which is present on the pXvCu-type plasmids (1). Resistance to streptomycin also is encoded on two nonconjugative plasmids in *X. c. vesicatoria,* a 68-kbp plasmid and a 15.9-kbp plasmid in strains BV5-4a and 85-10, respectively (20).

We have isolated strains of *X. c. vesicatoria* from diseased pepper and tomato plants in North Carolina and determined their host-differentiated races and sensitivities to copper and streptomycin (23). While analyzing the plasmid content of these strains in agarose gels, we observed that, in addition to large plasmids, some strains contained small plasmids that were estimated to be less than 3.0 kbp. We were unaware of any previous reports of such small plasmids in *X. c. vesicatoria* or in other phytopathogenic bacteria. However, small plasmids, termed miniplasmids, have been detected in several non–plant-pathogenic bacteria. Miniplasmids were reported in Gram-negative bacteria such as *Escherichia coli* strain 15, 2.3 kbp in size (5,9), and *Alcaligenes eutrophus,* 2.6 kbp (24), and in Gram-positive bacteria such as *Staphylococcus aureus,* 3.2 kbp (26), *S. epidermidis* (17), and *Streptococcus pneumoniae,* 2.6–3.1 kbp (25). A miniplasmid (1.8 kbp in size) also was detected in the archaebacterium *Halobacterium halobium* (12). These miniplasmids generally are multicopy plasmids, and with the exception of the 3.2-kbp miniplasmid in *S. aureus,* which is involved in resistance to cadmium (26), and the 2.4-kbp plasmid in *S. epidermidis,* which codes for resistance to macrolide-lincosamide-streptogramin B antibiotics (17), functions have not been attributed to them. Additionally, many methicillin-resistant strains of *S. aureus* often carry a small plasmid of approximately 1.7 kbp that appears to code solely for components involved with replication of the miniplasmid (30).

Initially, miniplasmids were detected only in copper-resistant strains of *X. c. vesicatoria* (11). Thus, we were interested in determining whether these small plasmids may have a role in resistance to copper. We report here the occurrence of miniplasmids (less than 3.0 kbp in size) in strains of *X. c. vesicatoria* and describe some of their physical characteristics and the phenotypes of the bacterial strains that harbor them.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Approximately 70 strains of *X. c. vesicatoria* were isolated from diseased pepper and tomato plants obtained from production areas in North Carolina in 1986, 1987, 1989, and 1990 (23). In 1991, an additional 21 strains were isolated and included in this present study. Bacteria were isolated and grown on YDC medium (10 g of yeast extract, 10 g of dextrose, 5 g of calcium carbonate, and 15 g of agar in 1.0 L of distilled water) at 28 C, as described previously (23). SPA medium (20 g of sucrose, 10 g of peptone, 0.5 g of dibasic potassium phosphate, 0.25 g of magnesium sulfate, and 15 g of agar in 1.0 L distilled water) was used to perform the copper and streptomycin assays. Liquid culture was done in nutrient broth (NB) (8.0 g/L of distilled water, Difco Laboratories, Detroit, MI) at 28 C in a shaking water bath (80–100 oscillations per minute). The NB medium provided adequate bacterial growth without the excessive production of extracellular polysaccharides that is typically associated with xanthomonads. For long-term storage, cultures were placed in sterile distilled water at 4 C and in 20% glycerol at −80 C.

Copper and streptomycin assays. Assays were done on SPA amended with the appropriate chemicals, as described previously (23). Briefly, fresh stock solutions of copper (cupric sulfate, Sigma Chemical Co., St. Louis, MO) and streptomycin (streptomycin sulfate, Sigma) were prepared in sterile distilled water and filter sterilized. After SPA was autoclaved, appropriate concentrations of stock solutions were added to this medium (pH 7.0) and poured into petri plates. The bacteria were grown on YDC or SPA, suspended in sterile distilled water, and spotted on SPA amended with 1.25 mM (200 μg/ml) cupric sulfate or 20–500 μg/ml of streptomycin sulfate. The plates were incubated at 28 C, and the results were recorded after 36–72 h. Bacterial strains that grew on media amended with 200 μg/ml of cupric sulfate were considered resistant to copper (28), and strains that failed to grow on 20 μg/ml of streptomycin sulfate were considered sensitive to streptomycin.

Plant inoculation and pathogen race determination. Pathogenicity was verified on pepper (cv. Early Calwonder or Keystone Resistant Giant) or tomato (cv. Rutgers) as described (23). The pathogen race was determined by the infiltration of bacterial suspensions (10^8–10^9 cfu/ml) into pepper leaves of Early Calwonder (ECW) and into the near-isogenic lines ECW10R, ECW20R, and ECW30R (21).

Plasmid isolation and visualization. Plasmid minipreparations followed the alkaline-lysis procedure developed by Takahashi and Nagano (29) with several modifications. Cells of *X. c. vesicatoria* were collected by centrifugation from 1.5–3.0 ml of mid- to late-logarithmic growth phase (18–20 h at 28 C) cultures grown in 20 ml of NB in 125-ml flasks. The NaOH concentration in the lysing solution was 0.3 N. The supernatant containing the plasmid DNA was treated with two volumes of phenol:chloroform (1:1, v/v). The emulsion was broken by centrifugation for 10 min at 13,700 *g*. The supernatant was pipetted to a clean tube, treated with an equal volume of chloroform, then centrifuged 10 min at 13,700 *g*. This supernatant was pipetted to a clean tube, and the plasmid DNA ethanol was precipitated and dissolved in 20–30 μl of TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0). The sample was treated with 0.1 volume of 10 mg/ml RNase A (Sigma), as described by Maniatis (19), and the plasmid DNA sample was stored at 4 C.

For large scale plasmid DNA preparation, cells cultured in 300 ml of NB for 18–24 h were harvested by centrifugation. The cleared lysate procedure was used to remove the majority of chromosomal DNA before the use of two-step cesium chloride-ethidium bromide (CsCl-EtBr) density gradient centrifugation (13,22). After 24–30 h of centrifugation at 185,500 *g* in a fixed-angle rotor, DNA bands were visualized with long-wavelength UV, and the fractions containing the bands were removed from the centrifuge tube with an 18-gauge needle attached to a syringe. EtBr and CsCl were removed with isopropanol and dialysis against TE buffer, respectively (19,22).

Plasmids and restriction fragments were analyzed with horizontal agarose slab gels. The gel concentrations ranged from 0.5–1.0% agarose, depending on the application. Approximately 10 μl of each DNA suspension (1 μg/ml) was mixed with 0.5 volume of loading buffer (10% Ficoll 400, 0.1 M NaEDTA, and 10% sodium dodecyl sulfate, pH 8.0) and 0.1 volume of 0.1% bromophenol blue dissolved in 50% glycerol. Electrophoresis was done in TE buffer at 3–5 volts per centimeter for 3–5 h. The gels were stained with 0.5 μg/ml of EtBr and destained in deionized water for at least 2 h. Visualization and photographs were done with a transilluminator at a wave length of 366 nm.

The DNA of pXcv22-2, purified by CsCl-EtBr density gradient centrifugation, was prepared for electron microscopy as outlined by Burkardt and Lurz (3). Essentially, this consisted of mixing the DNA with 0.25 M ammonium acetate, heating (37 C) it for 5 min before the addition of cytochrome c (5–10 μg/ml), placing the mixture on carbon-coated Formvar grids, and shadow casting it with platinum and palladium. The length of the DNA was estimated from the relationship of contour length and distance between base pairs in double-stranded DNA (3).

Recovery of plasmid DNA from agarose. DNA bands were cut from agarose gels and soaked in electroelution buffer (20 mM Tris-HCl, 0.2 mM EDTA, 5.0 mM NaCl, pH 8.0) for 2 min. The DNA was electroeluted from the gels into high-salt buffer (10 M ammonium acetate containing 0.1% bromophenol blue) with an IBI Unidirectional Electroeluter Analytical Electroelution System, model UEA (International Biotechnologies, Inc., New Haven, CT). Current was applied at 130–150 volts for 3–5 min. DNA in the high-salt buffer was collected for at least 1 h by precipitation with two volumes of cold 95% ethanol, then centrifuged. A second procedure was performed with the Geneclean Kit (Bio 101, La Jolla, CA) according to the manufacturer's instructions.

Endonuclease digestions. Enzymes were purchased from BRL Life Technologies, Inc. (Gaithersburg, MD). Assay buffers, reaction temperatures, and procedures were used according to manufacturer's instructions.

Bacterial matings. Donor and recipient strains were grown in 0.8% NB until mid-log phase. One milliliter of each culture was centrifuged, and the bacterial cells were resuspended in 0.5 ml of fresh NB. Equal volumes (approximately 10^8 cfu/ml) of donor (Cu^r, Rif^s) and recipient (Cu^s, Rif^r) strains were mixed, and 10–25 μl was spotted on SPA and allowed to incubate at 28 C for 18 h. Bacteria were resuspended in sterile distilled water, diluted, and 100 μl was plated on selective media. Putative copper-resistant transconjugants were selected on SPA amended with 200 μg/ml of cupric sulfate plus 50 μg/ml of rifampicin, pH 6.8. Selection for streptomycin-resistant transconjugants (donor Cu^r, Sm^r, Rif^s, recipient Cu^s, Sm^s, Rif^r) was on SPA amended with 100 μg/ml of streptomycin plus 50 μg/ml of rifampicin. Transfer frequencies were expressed as the ratio of cfu of transconjugants detected at sampling time (after 18 h on SPA) to the number of cfu of the donor at the time of mixing, as described by Lai et al (16).

Hybridization and Southern blot analyses. Plasmid DNA was transferred from agarose gels to membrane filters by the method of Southern as described by Maniatis (19). The transfers were made either to Nitroplus 2000 hybridization filters (pore size 0.45 μm) or to Magnagraph nylon filters (pore size 0.45 μm, Micron Separations, Inc. Westboro, MA) by blotting for 24–30 h. Dot blots were done by spotting DNA samples (1 μl) on the nylon filters. Concentrations of DNA were determined with DNA Dipsticks (Invitrogen Corp., San Diego, CA). Purified DNA of the four miniplasmids was obtained by agarose gel electrophoresis and the Geneclean kit. The DNA was linearized with either *Bam*HI or *Xho*I and labeled with nonradioactive digoxigenin-deoxyuridine triphosphate, according to the Genius kit protocol (Boehringer Mannheim Biochemicals, Indianapolis, IN). Prehybridization and hybridization procedures were performed in hybridization solution containing 5× SSC, 0.5% (w/v) blocking reagent (Genius kit), 0.1% (w/v) *N*-lauroylsarcosine (Na-salt), and 0.02% SDS. The prehybridization procedure was performed for 3–12 h in the hybridization solution at 68 C, and hybridization

was performed at 68 C for 16–24 h. After hybridization, the filters were washed twice for 5 min at room temperature in 2× SSC and 0.1% (w/v) SDS and then washed twice for 15 min at 68 C with 0.1× SSC and 0.1% (w/v) SDS. Hybridized DNA was detected by the enzyme-linked immunoassay (Boehringer Mannheim Biochemicals), as directed by the manufacturer.

The *avrBs1* probe consisted of a 5.3-kbp *Bgl*II-*Pst*I insert of pXv2007 (28), *avrBs3* was a 3.2-kbp *Bam*HI insert from pEC83 (21), and the copper-resistance probe was a 4.8-kbp *Bgl*II-*Hin*dIII insert from pXvCu1-13 (1). Insert DNA used for labeling was obtained from the clone by enzyme digestion and extraction from agarose gels with the Geneclean kit. Nonradioactive labeling and detection was done according to the protocols of the Genius kit. Hybridizations were done as described above.

Cloning of miniplasmids pXcv22 and pXcv30. Miniplasmids pXcv22 and pXcv30, which can be linearized with *Bam*HI, were ligated into the 8.6-kbp shuttle vector pUFR047 (obtained from D.W. Gabriel), which has a *Bam*HI site in the *lac*Z region. Miniplasmid DNA was isolated from agarose gels, cleaned with the Geneclean kit, ligated into the vector, and transformed into DH5α cells prepared by calcium chloride treatment according to Maniatis et al (19). Transformed cells were selected on Luria-Bertani medium (LB) containing 50 μg/ml of ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plus isopropyl-thio-β-galactoside after incubation at 37 C for 24 h. Agarose gel plasmid profiles and hybridization with pXcv22 were used to verify the presence of recombinant DNA in bacteria taken from white colonies on selective LB medium. The recombinant DNA was then transferred to *X. c. vesicatoria* strain Xcv12 Rif^r by triparental conjugation with helper plasmid pRK2013, as described by DeFeyter et al (10). Transconjugants were selected on NA containing 50 μg/ml of rifampicin and 3 μg/ml of gentamicin. Incubation was at 30–34 C for 20–24 h. Yellow pigmented colonies that grew were selected and analyzed for plasmids and recombinant DNA with digoxigenin-labeled pXcv22 and pXcv30.

TABLE 1. Pathogen races, copper and streptomycin sensitivity, and the presence of miniplasmids in strains of *Xanthomonas campestris* pv. *vesicatoria*

Pathogen race[a]	Copper resistance[b]	Streptomycin resistance[c]	Size of miniplasmid (kbp)[d]	Strains examined (no.)
0	+	−	ND	1
1	−	−	ND	21
1	+	−	ND	25
2	+	−	2.5	6
2	+	+	2.28	7
2	+	++	1.8	14
2	+	+++	1.7	1
3	−	−	ND	9
3	+	−	ND	2
3	−	++	1.8	1[e]
T	+	++	1.8	6

[a] Races 0, 1, 2, and 3 are strains of the pepper or pepper/tomato groups. T indicates strains of the tomato group.

[b] Copper resistance was determined by the ability of strains to grow on SPA medium (20 g of sucrose, 10 g of peptone, 0.5 g of dibasic potassium phosphate, 0.25 g of magnesium sulfate, and 15 g of agar in 1.0 L of distilled water) amended with 200 μg/ml of cupric sulfate. + Indicates growth; − indicates no growth.

[c] − Indicates no growth on SPA medium ammended with at least 20 μg/ml of streptomycin sulfate; + indicates growth on medium amended with 100 μg/ml but not with 200 μg/ml; ++ indicates growth on medium amended with 200 μg/ml but not with 500 μg/ml; and +++ indicates growth on medium amended with 500 μg/ml.

[d] Size estimate of a miniplasmid is based on the distance of migration in agarose gels (Fig. 1). kdp = Kilobase pairs. ND = No miniplasmid detected.

[e] This strain was selected as a spontaneous mutant of strain Xcv43 (race 2, Cu^r, Sm^r) in the laboratory. All other strains were isolated from diseased plant tissue from field samples.

RESULTS

Occurrence of miniplasmids in bacterial strains. Native-form miniplasmids were visualized in all strains of race 2 and in all tomato strains but not in pepper race 1 strains (Table 1). All race 2 and all tomato strains examined also were resistant to copper. All streptomycin-resistant strains contained native-form miniplasmids, but not all strains of *X. c. vesicatoria* that contained miniplasmids were resistant to streptomycin (Table 1). No bacterial strain contained more than one miniplasmid.

Observations based on migration distances in agarose gels revealed four different miniplasmids (Fig. 1). Their sizes were estimated to be 2.5, 2.28, 1.8, and 1.7 kbp by comparison of their migration distances to the linear size-standards of the 1-kbp DNA ladder (BRL Life Technologies). The sizes of the miniplasmids were inversely associated with the concentrations of streptomycin sulfate tolerated by the strains that harbored them (Table 1).

Physical characteristics of miniplasmids. Two to three UV-fluorescent bands were observed after density gradient centrifugation of plasmid DNA in discontinuous two-step CsCl-EtBr gradients. Supercoiled miniplasmid DNA was concentrated in the fastest migrating band (bottom band) when the CsCl-EtBr-gradient fractions where analyzed by agarose gel electrophoresis. The two slower moving bands (top two bands) contained only a trace of miniplasmid DNA.

Miniplasmid pXcv22 (2.5 kbp in size) also was examined using electron microscopy and found to be a typical small circular plasmid (data not shown). Direct measurements from electron micrographs also indicated the size of pXcv22 to be approximately 2.5 kbp.

Analysis of miniplasmids with restriction endonucleases. Four restriction endonucleases (*Eco*RI, *Bam*HI, *Hin*dIII, and *Xho*I) that recognize six base-pair cutting sites and three endonucleases (*Sau*3AI, *Taq*I, and *Alu*I) that recognize four base-pair cutting sites were used. None of the four miniplasmids had restriction sites for *Eco*RI or *Hin*dIII. The 2.5- and 2.28-kbp miniplasmids were linearized by *Bam*HI but not by *Xho*I (Fig. 1, lanes 6–11).

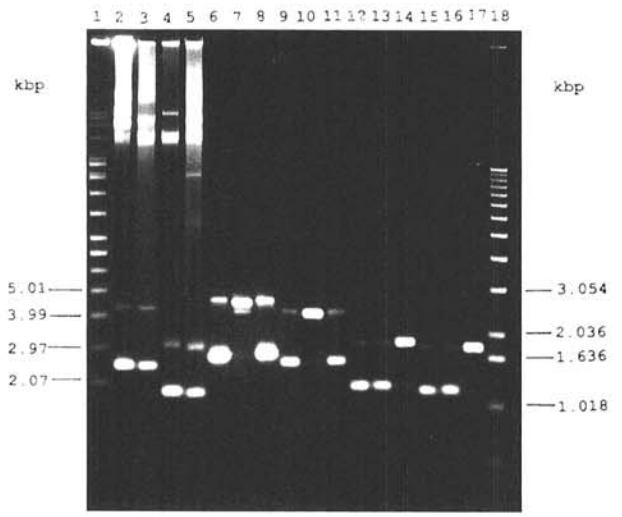

Fig. 1. Plasmid profiles of four strains of *Xanthomonas campestris* pv. *vesicatoria* in 0.7% agarose gel representing the four sizes of miniplasmids. Lane 1, supercoiled DNA-ladder standard; lanes 2–5, nondigested plasmid profiles of strains Xcv22-2 (Cu^r, Sm^s), Xcv30 (Cu^r, Sm^r, 100 μg/ml), Xcv8 (Cu^r, Sm^r, 200 μg/ml), and Xcv26 (Cu^r, Sm^r, 500 μg/ml), respectively. Lanes 6–8, DNA of miniplasmid pXcv22, *Bam*HI-digested pXcv22, and DNA of miniplasmid pXcv22 incubated with *Xho*I, respectively. Lanes 9–11, DNA of miniplasmid pXcv30, *Bam*HI-digested pXcv30, and DNA of miniplasmid pXcv30 incubated with *Xho*I, respectively. Lanes 12–14, DNA of miniplasmid pXcv8, DNA of miniplasmid pXcv8 incubated with *Bam*HI, and *Xho*I-digested pXcv8, respectively. Lanes 15–17, DNA of miniplasmid pXcv26, DNA of miniplasmid pXcv26 incubated with *Bam*HI, and *Xho*I-digested pXcv26, respectively. Lane 18, 1-kbp linear DNA-ladder standard.

In contrast, the 1.8- and 1.7-kbp miniplasmids were linearized by *Xho*I but not by *Bam*HI (Fig. 1, lanes 12–17). Incubation of the miniplasmids with *Alu*I, *Sau*3AI, or *Taq*I produced several small fragments (data not shown).

Southern hybridization and dot blots. There was homology between the two larger miniplasmids (2.5 and 2.28 kbp) and between the two smaller miniplasmids (1.8 and 1.7 kbp) but not between the two size groups (Fig. 2). Furthermore, no significant homology was detected with any of the larger plasmids in *X. c. vesicatoria* strains and the 2.5-kbp miniplasmid (Fig. 3E, Fig. 4).

Bacterial mating. Four copper-resistant or copper- and streptomycin-resistant strains were tested for the presence of self-mobilizable plasmids (Table 2). Copper resistance was detected in Xcv2 and Xcv12 after incubation with Xcv22-2 (Cu^r, Sm^s, 2.5-kbp miniplasmid), Xcv3 and Xcv30 (Cu^r, Sm^r [100 μg/ml], 2.28-kbp miniplasmid) at frequencies of 10^{-2} to 10^{-3} (Table 2). There was little difference in frequency of transfer when either of the two recipient strains, Xcv2 and Xcv12, were used (Table 2). Frequency of transfer of copper resistance to Xcv2 and Xcv12 was the same as the spontaneous mutation frequencies when Xcv26 (Cu^r, Sm^r [500 μg/ml], 1.7-kbp miniplasmid] was tested as a potential donor (Table 2). Miniplasmids characteristic of the donor strains were observed in the recipient strains of at least 50% of the transconjugants examined from each mating, except when strain Xcv26 was evaluated as a potential donor (Table 2). Of the four donor strains used, strain Xcv22-2 consistently yielded transconjugants containing a miniplasmid similar to that in the donor strain (Fig. 3E). Miniplasmid pXcv22 DNA hybridized with the miniplasmid found in the transconjugants (Fig. 3E) in which strain Xcv22-2 was used as the donor. When donor strains containing the 2.28-kbp miniplasmid were used, the miniplasmid was not observed in all the copper-resistant transcon-

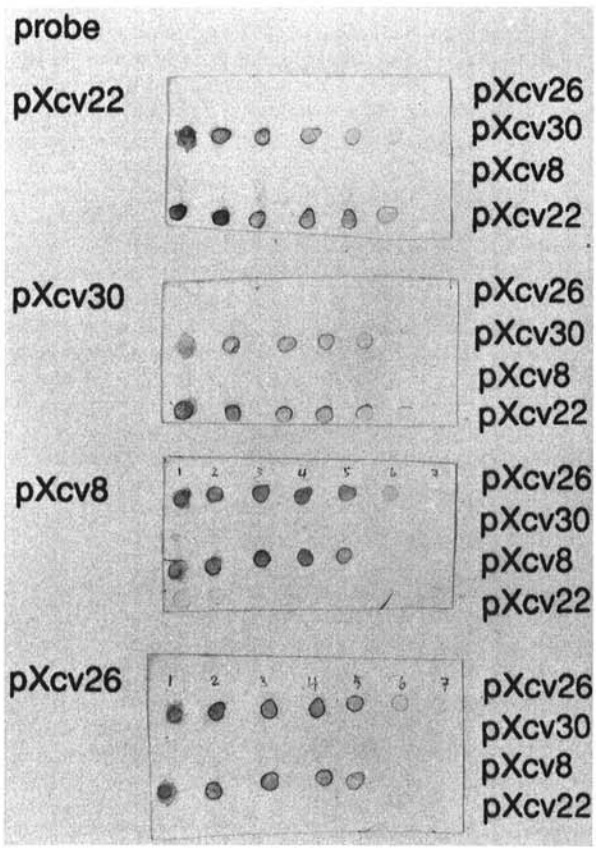

Fig. 2. Homology among the four sizes of miniplasmids. DNA from miniplasmids pXcv26 (1.7 kbp), pXcv30 (2.28 kbp), pXcv8 (1.8 kbp), and pXcv22 (2.5 kbp) is designated on the right. Digoxigenin-labeled miniplasmid DNA used as the probe is designated on the left. Miniplasmid DNA was recovered from agarose gels, and 1.0-μl samples of a dilution series were spotted on membranes. DNA concentrations were 250, 60, 20, 10, 5, 1, and 0.1 ng/μl (not detectable), from left to right, respectively.

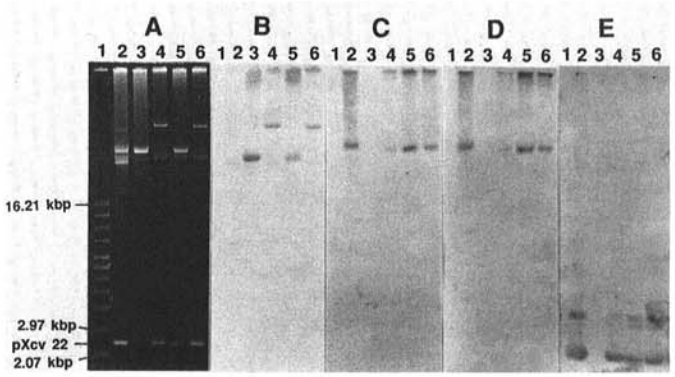

Fig. 3. Southern blot analysis of plasmid DNA of three transconjugant strains of *Xanthomonas campestris* pv. *vesicatoria,* lanes 4–6, (race 0, Cu^r, Rif^r) obtained from mating donor strain Xcv22-2 (race 2, Cu^r, 2.5-kbp miniplasmid), lane 2, with recipient strain Xcv33 Rif^r (race 1, Cu^s, lacks miniplasmid), lane 3. Lane 1 is the supercoiled DNA-ladder standard. **A,** Plasmid profiles in 0.7% agarose gel; **B,** hybridized with a 3.2-kbp *Bam*HI insert of pEC83 (*avrBs3* probe); **C,** hybridized with a 5.3-kbp *Bgl*II-*Pst*I insert of pXv2007 (*avrBs1* probe); **D,** hybridized with a 4.8-kbp *Hin*dIII-*Bgl*II insert of pXvCu1-13 (Cu^r resistance determinant); and **E,** probed with 2.5-kbp *Bam*HI linearized miniplasmid pXcv22.

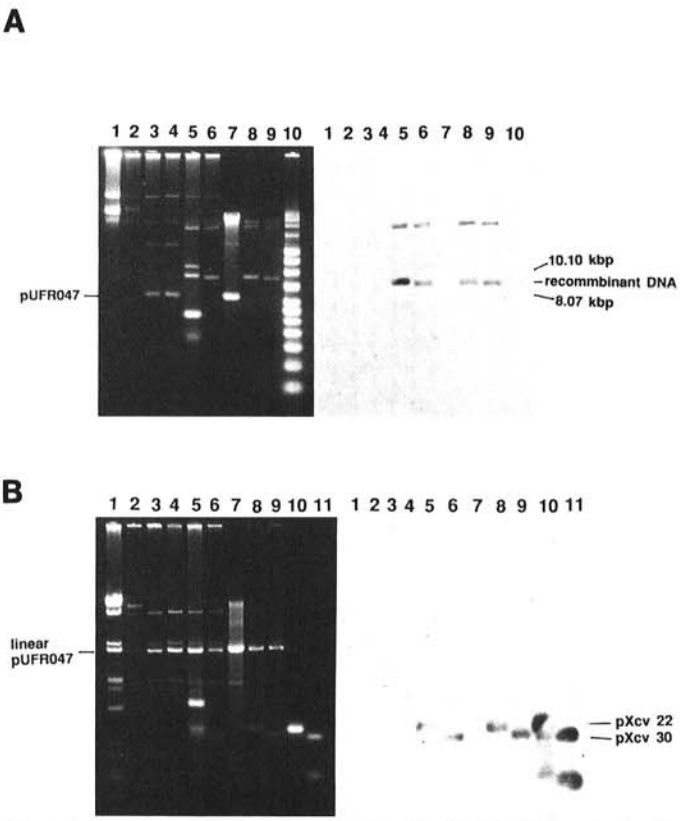

Fig. 4. A, Results of ligating miniplasmids pXcv22 (2.5 kbp) and pXcv30 (2.28 kbp) into the vector pUFR047, transforming this construct into *E. coli* DH5α, then mobilizing it by conjugation into *Xanthomonas campestris* pv. *vesicatoria* strain Xcv12 Rif^r and Southern blotting of plasmid DNA hybridized with 2.5-kbp linearized pXcv22 probe. Lane 10 contains a supercoiled DNA size standard. **B,** Miniplasmids excised from vector pUFR047 using *Bam*HI and Southern blotting of the *Bam*HI fragments hybridized with 2.28-kbp linearized pXcv30 probe. Lanes 1 and 2, Xcv12 and a Rif^r mutant of Xcv12 used as recipients, respectively; these strains are Cu^s and lack a miniplasmid. Lanes 3 and 4, two Xcv12 Rif^r transconjugants containing the pUFR047 vector. Lanes 5 and 6, two Xcv12 Rif^r transconjugants with miniplasmids pXcv22 and pXcv30 ligated into vector pUFR047, respectively. Lanes 7–9, *Escherichia coli* DH5α containing vector pUFR047, pUFR047 with insert pXcv22, and pUFR047 with insert pXcv30, respectively. Lanes 10 and 11 are linearized pXcv22 and pXcv30 DNA, respectively.

jugants (Table 2). No transfer of the streptomycin-resistance phenotype was detected.

Cloned miniplasmids pXcv22 and pXcv30. The two larger miniplasmids, pXcv22 and pXcv30, were linearized with *Bam*HI, ligated into vector pUFR047, transformed into *E. coli* DH5α, and then conjugated into the copper- and streptomycin-sensitive strain of *X. c. vesicatoria,* Xcv12 Rif^r (Fig. 4). These two miniplasmids also could be excised from pUFR047 in both DH5α and Xcv12 Rif^r with *Bam*HI (Fig. 4). Strains into which these two miniplasmids were mobilized exhibited no detectable change in sensitivity to copper or streptomycin.

Homology of miniplasmids with avirulence and copper-resistance genes. No homology was detected between the DNA of miniplasmid pXcv22 and *avrBs3, avrBs1,* or the Cu^r resistance determinant (Fig. 3B–D). When Xcv22-2 (Cu^r, *avrBs1,* race 2) was mated with Xcv33 (Cu^s, Rif^r, *avrBs3,* race 1), transconjugants of Xcv33 were obtained that contained the miniplasmid pXcv22, were Rif^r and Cu^r, and contained the *avrBs1* and *avrBs3* genes (Fig. 3A–E). These transconjugants also elicited a hypersensitive response in pepper lines ECW10R, ECW20R, and ECW30R, and a susceptible response in ECW.

DISCUSSION

Strains of *X. campestris* have been reported to contain plasmids ranging in size from 3.0 to at least 200 kbp (18). Most, if not all, strains of *X. c. vesicatoria* contain several indigenous plasmids, and the plasmids with which specific phenotypes have been associated range in size from 15.9 to approximately 200 kbp. These plasmids contain loci that encode products that determine host specificity and resistance to copper and streptomycin (1,2,6,7,15,20,21,27,28). However, this is the first report and description of indigenous, small plasmids in strains of *X. c. vesicatoria* or other strains of *Xanthomonas.* Because these plasmids are similar in size to small plasmids, termed miniplasmids, in several other bacteria such as *E. coli* strain 15 (5) and *A. eutrophus* (24), we have used similar terminology.

There are four sizes of miniplasmids determined on the basis of migration distances of both native-form and linearized miniplasmid DNA (Fig. 1). However, the two larger miniplasmids apparently are more similar to each other than to either of the two smaller miniplasmids, which have similarities to each other based on restriction-enzyme analysis and homology (Figs. 1 and 2).

The two larger miniplasmids, but not the two smaller miniplasmids, could be mobilized to copper-sensitive strains of *X. c. vesicatoria* by conjugation (Table 2). The use of strain Xcv22-2 as a donor, which contains the 2.5-kbp miniplasmid, resulted in high transfer frequencies and the detection of this miniplasmid in all transconjugants examined (Table 2, Fig. 3). The use of donor strains containing the 2.28-kbp miniplasmid also resulted in high transfer frequencies of the copper-resistance phenotype, but the miniplasmid was not detected in all transconjugants (Table 2). The use of strain Xcv26 as a potential donor, which contains the smallest miniplasmid (1.7 kbp), resulted in transfer frequencies that could not be separated from spontaneous mutation frequencies (Table 2). Furthermore, none of the suspected transconjugants examined contained a detectable miniplasmid. Strains that harbored a miniplasmid also contained one or more larger plasmids such as pXvCu. Thus, it is likely that the miniplasmids are cotransferred under the direction of one or more of the larger plasmids (e.g., pXvCu) or integrate into one of the larger plasmids and then are cotransferred. In *A. eutrophus,* the 2.6-kbp miniplasmid, pTOM3, was assumed to form part of a hybrid plasmid during the conjugation process and was not observed as a separate plasmid in the transconjugants (24). During conjugation, similar rearrangement in plasmids may occur in some strains of *X. c. vesicatoria.* However, if the *X. c. vesicatoria* miniplasmids are mobilized with a larger plasmid, they are capable of maintaining their entity, unlike the miniplasmid in the transconjugants of *A. eutrophus.* The unidentified bands in one of the Xcv12 Rif^r transconjugants (Fig. 4A, lane 5) may be the result of plasmid rearrangement. We have observed similar plasmid profiles in other transconjugants from matings of race 2 strains with race 1 strains; however, we have not detected miniplasmids integrated into any of the rearranged plasmids (Dittapongpitch and Ritchie, *unpublished*). Furthermore, we have observed that mobilization of vector pUFR047 into some race 1 strains of *X. c. vesicatoria* has been associated with the loss of a large indigenous plasmid, such as with strain Xcv12 Rif^r (Fig. 4A, lanes 3–6). Also, the *avrBs3* gene in the transconjugants apparently can occur on different plasmids, or plasmid rearrangement has occurred during conjugation (Fig. 3B). The occurrence of *avrBs3* on at least two plasmids was reported previously (2,4).

Although miniplasmids were observed in all streptomycin-resistant strains, transfer of the miniplasmid(s) by conjugation and attempts to select for the streptomycin-resistance phenotype were unsuccessful. Minsavage et al (20) also were unable to transmit, by conjugation, plasmids carrying the gene(s) for streptomycin resistance.

Transconjugants from the mating of strain Xcv22-2 (race 2, Cu^r, Rif^s, 2.5-kbp miniplasmid) with strain Xcv33 (race 1, Cu^s, Rif^r, no miniplasmid) were copper-resistant, contained a miniplasmid, and contained DNA that hybridized with *avrBs1* and *avrBs3* probes (Fig. 3). These transconjugants also elicited a hypersensitive reaction in ECW10R, ECW20R, and ECW30R and a susceptible reaction in ECW, which fits the characteristics of race 0 (23). Thus, the reaction of race 0 is the result of a composite of several avirulence genes. Hibberd et al (14) suggested the determinants for races 1 and 2 could occur within the same strain of *X. c. vesicatoria.* The results in Figure 3 support their

TABLE 2. Transmisson frequencies of the copper-resistance phenotype to copper-sensitive strains of *Xanthomonas campestris* pv. *vesicatoria* and detection of miniplasmids

Donor[a]	Miniplasmid size (kbp)[b]	Recipient[c]	Spontaneous mutation frequencies[d]		Transfer frequencies[e]	Transconjugants containing miniplasmids/ number tested
			Donor	Recipient		
Xcv22-2	2.5	Xcv2	7.9×10^{-9}	3.4×10^{-9}	4.1×10^{-3}	4/4
Xcv22-2	2.5	Xcv12	9.7×10^{-9}	3.3×10^{-9}	1.2×10^{-3}	4/4
Xcv3	2.28	Xcv2	3.2×10^{-9}	1.3×10^{-9}	1.9×10^{-2}	3/5
Xcv3	2.28	Xcv12	1.1×10^{-9}	5.1×10^{-9}	1.9×10^{-3}	Not tested
Xcv30	2.28	Xcv2	5.0×10^{-9}	5.8×10^{-9}	8.4×10^{-3}	2/4
Xcv30	2.28	Xcv12	5.0×10^{-9}	6.0×10^{-9}	1.3×10^{-3}	4/4
Xcv26	1.7	Xcv2	4.0×10^{-9}	2.0×10^{-9}	3.0×10^{-6}	0/4
Xcv26	1.7	Xcv12	1.2×10^{-10}	6.0×10^{-9}	3.0×10^{-9}	0/4

[a] Donor strains were Cu^r, Rif^s.
[b] Kilobase pairs.
[c] Recipient strains were Cu^s, Rif^r.
[d] Frequency of donor phenotype to rifampicin resistance and of recipient to copper resistance.
[e] Transfer frequencies are expressed as the ratio of the number of cfu of transconjugants detected at sampling time (after 18 h on YDC medium [10 g of yeast extract, 10 g of dextrose, 5 g of calcium carbonate, and 15 g of agar in 1.0 L of distilled water]) to the number of cfu of the donor at the time of mixing.

hypothesis. Given that a strain can change host range by losing avirulence activity (21), it is tempting to speculate that strains of race 0 could be a source of any of the three other races by deletion or inactivation of one or more avirulence genes.

The miniplasmids appear to be very stable in the *X. c. vesicatoria* strains in which they occur. Although most strains containing a miniplasmid were subcultured weekly, none was observed to lose its miniplasmid. Attempts to cure the 2.5-kbp miniplasmid from strain Xcv22-2 and transconjugants obtained with Xcv22-2 as a donor were unsuccessful when acridine orange, mitomycin C, and exposure to elevated growth temperature (37 C) were used. Attempts to cure the 2.6-kbp miniplasmid from strain 10A of *A. eutrophus* with mitomycin C and elevated growth temperature also were unsuccessful (24).

The miniplasmids appear to be different because DNA hybridizations indicated no significant homology with other plasmid DNA, even under conditions of low stringency (Fig. 3E and Fig. 4). Also, we failed to detect homology using genomic DNA in dot blots of strains of *X. c. vesicatoria* in which miniplasmids were not observed by gel electrophoresis. Furthermore, we have not visualized by gel electrophoresis or detected by dot blots the presence of miniplasmids in strains of other *X. campestris* pathovars such as *zinniae, pruni,* and *campestris.*

More than 90 strains of *X. c. vesicatoria* isolated during a 5-yr period were examined for miniplasmids. Miniplasmids were observed only in strains resistant to copper (both race 2 and tomato strains) but not in copper-resistant race 1 strains (Table 1). One exception to this was in a race 2 strain (Xcv43), which was isolated from a diseased pepper plant and was observed as being Cu^r, Sm^r. After being subcultured frequently in the laboratory, it spontaneously lost the copper-resistance phenotype, changed virulence from race 2 to race 3, but retained streptomycin resistance and the miniplasmid. Comparisons were made to the original strain that had been stored at −80 C. Also, mobilization of miniplasmids pXcv22 and pXcv30 into a copper-sensitive strain, Xcv12 Rif^r, did not result in increased resistance to copper. These results suggest that miniplasmids, even though commonly associated with copper-resistant race 2 and tomato strains but not with race 1 strains, are not essential for copper resistance. A high percentage of race 2 strains also are pathogenic in tomato plants, while a low percentage of race 1 strains are pathogenic in tomato plants (4). Thus, in the case of this characteristic, the tomato strains and pepper race 2 strains are more similar to each other than either is to pepper race 1. It also is of interest that tomato and pepper race 2 strains harbor miniplasmids, but no miniplasmids have been detected in race 1 strains. Thus, the miniplasmids described in *X. c. vesicatoria,* the first for plant-pathogenic bacteria, join a growing group of very small plasmids in certain strains of bacteria whose function(s) remains mostly unknown (5,9,24–26,30,31).

LITERATURE CITED

1. Bender, C. L., Malvick, D. K., Conway, K. E., George, S., and Pratt, P. 1990. Characterization of pXV10A, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria.* Appl. Environ. Microbiol. 56:170-175.
2. Bonas, U., Stall, R. E., and Staskawicz, B. 1989. Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria.* Mol. Gen. Genet. 218:127-136.
3. Burkardt, H., and Lurz, R. 1984. Electron microscopy. Pages 281-302 in: Advanced Molecular Genetics. A. Puhler and K. N. Timmis, eds. Springer-Verlag, New York.
4. Canteros, B., Minsavage, G., Bonas, U., Pring, D., and Stall, R. 1991. A gene from *Xanthomonas campestris* pv. *vesicatoria* that determines avirulence in tomato is related to *avrBs3.* Mol. Plant-Microbe Interact. 4:628-632.
5. Chang, A. C. Y., and Cohen, S. N. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141-1156.
6. Cooksey, D. A. 1990. Genetics of bactericide resistance in plant pathogenic bacteria. Annu. Rev. Phytopathol. 28:210-219.
7. Cooksey, D. A., Azad, H. R., Cha, J.-S., and Lim, C.-K. 1990. Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. Appl. Environ. Microbiol. 56:431-435.
8. Coplin, D. L. 1989. Plasmids and their role in the evolution of plant pathogenic bacteria. Annu. Rev. Phytopathol. 27:187-212.
9. Cozzarelli, N. R., Kelly, R. B., and Kornberg, A. 1968. A minute circular DNA from *Escherichia coli* 15. Proc. Natl. Acad. Sci. U.S.A. 60:992-999.
10. DeFeyter, R., Kado, C. I., and Gabriel, D. W. 1990. Small, stable shuttle vectors for use in *Xanthomonas.* Gene 88:65-72.
11. Dittapongpitch, V., Ritchie, D. F., and Upchurch, R. G. 1989. Small, conjugatable plasmid in copper-resistant strains of *Xanthomonas campestris* pv. *vesicatoria.* (Abstr.) Phytopathology 79:1192.
12. Ebert, K., Goebel, W., and Pfeifer, F. 1984. Homologies between heterogeneous extrachromosomal DNA populations of *Halobacterium halobium* and four new halobacterial isolates. Mol. Gen. Genet. 194:91-97.
13. Garger, S. J., Griffith, O. M., and Grill, L. K. 1983. Rapid purification of plasmid DNA by a single centrifugation in a two step cesium chloride ethidium bromide gradient. Biochem. Biophys. Res. Commun. 117:835-842.
14. Hibberd, A. M., Stall, R. E., and Bassett, M. J. 1987. Different phenotypes associated with incompatible races and resistance genes in bacterial spot disease of pepper. Plant Dis. 71:1075-1078.
15. Kearney, B., and Staskawicz, B. J. 1990. Characterization of IS476 and its role in bacterial spot disease of tomato and pepper. J. Bacteriol. 172:143-148.
16. Lai, M., Panopoulos, N. J., and Shaffer, S. 1977. Transmission of R plasmids among *Xanthomonas* spp. and other plant pathogenic bacteria. Phytopathology 67:1044-1050.
17. Lampson, B. C., and Parisi, J. T. 1986. Nucleotide sequence of the constitutive macrolide-lincosamide-streptogramin B resistance plasmid pNE131 from *Staphylococcus epidermidis* and homologies with *Staphylococcus aureus* plasmids pE194 and pSN2. J. Bacteriol. 167:888-892.
18. Lazo, G. R., and Gabriel, D. W. 1987. Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris.* Phytopathology 77:448-453.
19. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
20. Minsavage, G. V., Canteros, B. I., and Stall, R. E. 1990. Plasmid-mediated resistance to streptomycin in *Xanthomonas campestris* pv. *vesicatoria.* Phytopathology 80:719-723.
21. Minsavage, G. V., Dahlbeck, D., Whalen, M. C., Kearney, B., Bonas, U., Staskawicz, B. J., and Stall, R. E. 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria*-pepper interactions. Mol. Plant-Microbe Interact. 3:41-47.
22. Priefer, U. 1984. Isolation of plasmid DNA. Pages 14-25 in: Advanced Molecular Genetics. A. Puhler and K. N. Timmis, eds. Springer-Verlag, New York.
23. Ritchie, D. F., and Dittapongpitch, V. 1991. Copper- and streptomycin-resistant strains and host differentiated races of *Xanthomonas campestris* pv. *vesicatoria* in North Carolina. Plant Dis. 75:733-736.
24. Schmidt, T., and Schlegel, H. G. 1989. Nickel and cobalt resistance of various bacteria isolated from soil and highly polluted domestic and industrial wastes. FEMS Microbiol. Ecol. 62:315-328.
25. Sibold, C., Markiewicz, C., Latorre, C., and Hakenbeck, R. 1991. Novel plasmids in clinical strains of *Streptococcus pneumoniae.* FEMS Microbiol. Lett. 77:91-96.
26. Solh, N. E., and Ehrlich, S. D. 1982. A small cadmium resistance plasmid isolated from *Staphylococcus aureus.* Plasmid 7:77-84.
27. Stall, R. E., Loschke, D. C., and Jones, J. B. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria.* Phytopathology 76:240-243.
28. Swanson, J., Kearney, B., Dahlbeck, D., and Staskawicz, B. 1988. Cloned avirulence gene of *Xanthomonas campestris* pv. *vesicatoria* complements spontaneous race-change mutants. Mol. Plant-Microbe Interact. 1:5-9.
29. Takahashi, S., and Nagano, Y. 1984. Rapid procedure for isolation of plasmid DNA and application to epidemiological analysis. J. Clin. Microbiol. 20:608-613.
30. Walters, J. A., and Dyke, K. G. H. 1990. Characterization of a small cryptic plasmid isolated from a methicillin-resistant strain of *Staphylococcus aureus.* FEMS Microbiol. Lett. 71:55-64.
31. Yoon, K. H., and Pack, M. Y. 1987. Construction of a shuttle vector between *Escherichia coli* and *Zymomonas anaerobia.* Biotechnol. Lett. 9:163-168.