APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1990, p. 170–175
0099-2240/90/010170-06$02.00/0

Vol. 56, No. 1

Characterization of pXV10A, a Copper Resistance Plasmid in *Xanthomonas campestris* pv. *vesicatoria*†

CAROL L. BENDER,[1]* DEAN K. MALVICK,[1] KENNETH E. CONWAY,[1] STEVEN GEORGE,[2] AND PHILLIP PRATT[3]

Department of Plant Pathology, Oklahoma State University, Stillwater, Oklahoma 74078[1]; Research and Extension Center, Texas A&M University, Dallas, Texas 75252[2]; and 230 W. Okmulgee Street, Muskogee, Oklahoma 74401[3]

Received 24 July 1989/Accepted 19 October 1989

The efficacy of copper bactericides for control of *Xanthomonas campestris* pv. *vesicatoria* in eastern Oklahoma tomato fields was evaluated. Copper bactericides did not provide adequate control, and copper-resistant (Cu^r) strains of the pathogen were isolated. The Cu^r genes in these strains were located on a large indigenous plasmid designated pXV10A. The host range of pXV10A was investigated; this plasmid was efficiently transferred into 8 of 11 *X. campestris* pathovars. However, the transfer of pXV10A to other phytopathogenic genera was not detected. DNA hybridization experiments were performed to characterize the Cu^r genes on pXV10A. A probe containing subcloned Cu^r genes from *X. campestris* pv. *vesicatoria* E3C5 hybridized to pXV10A; however, a subclone containing Cu^r genes from *P. syringae* pv. *tomato* PT23 failed to hybridize to pXV10A. Further DNA hybridization experiments were performed to compare pXV10A with pXvCu plasmids, a heterogenous group of Cu^r plasmids present in strains of *X. campestris* pv. *vesicatoria* from Florida. These studies indicated that the Cu^r genes on pXV10A and pXvCu plasmids share nucleotide sequence homology and may have a common origin. Further experiments showed that these plasmids are distinctly different because pXV10A did not contain sequences homologous to IS*476*, an insertion sequence present on pXvCu plasmids.

The acquisition of resistance to copper bactericides by phytopathogenic bacteria has become an important problem in tomato and pepper production areas. Since copper sprays are the basis for bacterial disease control in many vegetable crops, the existence of copper-resistant pathogens may explain why disease incidence is high despite the application of these compounds. Resistance to copper has been identified in two pathogens of solanaceous crops: *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot on peppers and tomatoes, and *Pseudomonas syringae* pv. *tomato*, which causes bacterial speck on tomatoes (1, 2, 12). Moreover, the copper resistance (Cu^r) genes in some strains of *X. campestris* pv. *vesicatoria* (15) and *P. syringae* pv. *tomato* (2) have been localized on self-transmissible plasmids. Strains of *P. syringae* pv. *tomato* isolated from tomatoes grown in southern California contained Cu^r genes on a 35-kilobase (kb) plasmid (4). Although the 35-kb plasmid (pPT23D) was not shown to be self-transmissible, it was mobilized into other strains as a cointegrate with another plasmid, pPT23C (3). Stall et al. (15) found that strains of *X. campestris* pv. *vesicatoria* from Florida contained Cu^r genes on a conjugative plasmid designated pXvCu. Copper-resistant strains of the bacterial spot pathogen were also recovered from western and central Mexico (1), but the genetic basis of resistance was not studied. The association of Cu^r genes with self-transmissible plasmids may explain the increased recovery of Cu^r phytopathogenic bacteria in the field.

Although copper bactericides are heavily applied to tomato fields in eastern Oklahoma, adequate control of *X. campestris* pv. *vesicatoria* is not achieved. Therefore, a field trial was conducted to evaluate the efficacy of copper sprays for control of the bacterial spot pathogen in eastern Oklahoma. Strains of *X. campestris* pv. *vesicatoria* which were obtained from this study contained Cu^r genes on a conjugative plasmid which we have designated pXV10A. The host range of pXV10A was investigated by conducting mating experiments with various phytopathogenic bacteria. DNA hybridization experiments were performed to characterize the Cu^r genes on pXV10A and compare pXV10A with Cu^r plasmids present in strains of the pathogen from Florida.

(Preliminary reports of this work have appeared elsewhere [C. Bender, D. Malvick, S. George, K. Conway, and P. Pratt, Phytopathology **78**:625, 1988; D. Malvick and C. Bender, Phytopathology **78**:1587, 1988].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in the present study are listed in Table 1. Nutrient agar (NA [13]) was used for routinely subculturing *X. campestris* pathovars, *Erwinia herbicola*, and *Pseudomonas corrugata*. *P. syringae*, *Pseudomonas andropogonis*, and *Agrobacterium* spp. were maintained on mannitol-glutamate medium (9) supplemented with yeast extract at 0.25 g/liter (MGY). *Escherichia coli* cultures were grown in Luria-Bertani (11) broth at 37°C; all other bacteria were grown in MGY or nutrient broth at 25 to 30°C. Selective antibiotic concentrations were as follows: rifampin, 50 μg/ml; chloramphenicol, 50 μg/ml; nalidixic acid, 70 μg/ml; tetracycline, 12.5 μg/ml; ampicillin, 40 μg/ml; and streptomycin, 25 μg/ml.

Efficacy of copper bactericides for control of bacterial spot. Tomato plants (cv. Jet Star) were planted at the Oklahoma State University Vegetable Research Station, Bixby, on 18 April 1987. Each plot contained six plants, and treatments were replicated five times. Plants received nine applications at weekly intervals (13 May to 9 July) of copper oleate (22.5 liters of active ingredient [a.i.] per ha); cupric hydroxide

* Corresponding author.

† Technical paper no. 5639 from the Oklahoma Agricultural Experiment Station.

TABLE 1. Bacterial strains used in the present study

Strain	Relevant properties and plasmids[a]	Source or reference[b]
E. coli		
HB101	pCOP2, recombinant plasmid containing Cu^r genes from *P. syringae* pv. *tomato*; Tc^r	3
JM101	pXvCu1-16, recombinant plasmid containing Cu^r genes from *X. campestris* pv. *vesicatoria* E3C5; Ap^r	B. Staskawicz
X. *campestris* pv. *vesicatoria*		
XV10	Cu^r; MIC, 2.4 mM; pXV10A	Copper oleate treatment
XV11	Cu^r; MIC, 2.4 mM	Kocide 101 treatment
XV12	Cu^r; MIC, 2.4 mM	Bravo C/M treatment
XV13	Cu^r; MIC, 2.4 mM	Dithane M-45–Kocide 101
XV14	Cu^r; MIC, 2.4 mM	Unsprayed control
E3C5	Cu^r; pXvCuE3C5	B. Staskawicz
81-23	Cu^r; pXvCu81-23	B. Staskawicz; 15
75-3	Cu^r; pXvCu75-3	B. Staskawicz; 15
68-1	Cu^r; pXvCu68-1	B. Staskawicz; 15
XV16	Rif^r; MIC, 0.6 mM; no plasmids	This study
XV16.1	Rif^r; MIC, 3.2 mM; pXV10A	XV10 × XV16
XV17	Nal^r Cm^r; MIC, 0.8 mM; no plasmids	This study
XV17.1	Nal^r Cm^r; MIC, 1.2 mM; pXvCu81-23	81-23 × XV17
XV17.2	Nal^r Cm^r; MIC, 1.6 mM; pXvCuE3C5	E3C5 × XV17
X. campestris pv. *vitians*		
QR33	MIC, 0.8 mM	W. Chun
XCV10	MIC, 0.8 mM	Rif^r QR33
XCV10.1	MIC, 2.4 mM; pXV10A	XV10 × XCV10
X. campestris pv. *malvacearum* 3PM1	Rif^r Sm^r; MIC, 0.6 mM	R. Gholson
X. campestris pv. *glycines* XG10	Rif^r Sm^r; MIC, 1.6 mM	This study
X. campestris pv. *translucens* B-430	Rif^r Sm^r; MIC, 0.8 mM	V. Mellano
A. radiobacter K-84	Sm^r Cm^r; MIC, 1.6 mM	A. Kerr
A. tumefaciens NT1	Rif^r Cm^r; MIC, 2.0 mM	D. A. Cooksey
Erwinia herbicola 13329	Rif^r Cm^r; MIC, 0.8 mM	American Type Culture Collection
P. syringae pv. *syringae* PS51	Rif^r Cm^r; MIC, 0.1 mM	2
P. corrugata 0682-12	Rif^r Cm^r; MIC, 2.0 mM	D. A. Cooksey
P. andropogonis A1044-1	Rif^r Cm^r; MIC, 2.0 mM	W. Chun
X. campestris pv. *campestris*		
0186-1	MIC, 0.8 mM; no plasmids	D. A. Cooksey
XC10	MIC, 0.8 mM; no plasmids	Sm^r 0186-1
XC10.1	MIC, 2.4 mM; pXV10A	XV10 × XC10
XC11	MIC, 0.8 mM; no plasmids	Nal^r Cm^r 0186-1
XC11.1	MIC, 2.4 mM; pXvCu68-1	68-1 × XC11
XC11.2	MIC, 1.6 mM; pXvCu75-3	75-3 × XC11
X. campestris pv. *dieffenbachiae*		
B-400	MIC, 0.8 mM	N. Schaad
XD10	MIC, 0.8 mM	Rif^r B-400
XD10.1	MIC, 2.0 mM; pXV10A	XV10 × XD10
X. campestris pv. *manihotis*		
QR32	MIC, 0.6 mM	W. Chun
XM10	MIC, 0.6 mM	Rif^r QR32
XM10.1	MIC, 2.4 mM; pXV10A	XV10 × XM10
X. campestris pv. *nigromaculans*		
0682-1	MIC, 0.8 mM	D. A. Cooksey
XN10	MIC, 0.8 mM	Rif^r 0682-1
XN10.1	MIC, 2.0 mM; pXV10A	XV10 × XN10
X. campestris pv. *pelargonii*		
0782-29	MIC, 1.2 mM	D. A. Cooksey
XP10	MIC, 1.2 mM	Rif^r 0782-29
XP10.1	MIC, 2.4 mM; pXV10A	XV10 × XP10
X. campestris pv. *phaseoli*		
QR60	MIC, 0.4 mM	W. Chun
XCP10	MIC, 0.4 mM	Rif^r QR60
XCP10.1	MIC, 1.6 mM; pXV10A	XV10 × XCP10

[a] All MICs refer to copper sulfate.
[b] In all mating experiments, the order is donor-recipient; for example, XV10 (Cu^r donor) was mated with XV16 (Cu^s recipient).

(Kocide 101; 2.1 kg a.i. per ha); a combination of chlorothalonil, copper oxychloride, and maneb (Bravo C/M; 3.5 kg a.i. per ha); or a mancozeb-cupric hydroxide tank mix (Dithane M-45 [1.4 kg a.i. per ha], Kocide 101 [1.4 kg a.i. per ha], and Triton CS-7 [0.3 ml/liter], which was added as a sticker). One group of plants served as an unsprayed control. Tomatoes were harvested five times (18 and 25 June and 2, 9, and 16 July), and yield was recorded from the two center plants in each treatment replicate. On 16 July, all green fruits were harvested from the treatment groups and visually inspected for symptoms of bacterial spot. Putative strains of *X. campestris* pv. *vesicatoria* were isolated from lesions from all treatment groups, tested for Gram and oxidase reactions, and inoculated to tomato cv. Marglobe. The inoculation technique consisted of swabbing bacterial suspensions (5×10^8 CFU/ml) onto young leaves of 3- to 5-week-old tomato plants.

Copper tolerance evaluation. All *X. campestris* pv. *vesicatoria* strains and recipient strains used in mating experiments were tested for sensitivity or resistance to copper sulfate. Cultures to be screened were grown for 36 to 48 h on NA or MGY agar plates at 28°C. Strains were then streaked to MGY agar or NA plates containing copper sulfate at concentrations ranging from 0 to 4.0 mM. The MIC of copper sulfate was designated as the concentration of $CuSO_4$ which inhibited confluent growth of the culture after a 72-h incubation at 28°C.

Conjugation experiments. Matings between Cu^r *X. campestris* pv. *vesicatoria* strains and various recipients were done as described by Stall et al. (15) with slight modifications. Donor and recipient strains were prepared for mating by being cultured on NA or MGY agar for 1 to 3 days. Single colonies of donor and recipient cells were then transferred to separate 4-ml aliquots of nutrient broth and incubated on a rotary shaker at 26°C for 15 to 18 h (late log phase). The donor and recipient cells (500 μl of each) were then mixed and collected on 25-mm-diameter membrane filters with a 0.45-μm pore size. The filters were then placed onto mating medium (NA with a 1% water agar overlay) and incubated for 15 to 18 h at 26°C. Bacteria were then removed by vortexing the filters in 3 to 5 ml of nutrient broth, 10-fold dilutions were made, and 0.1-ml volumes of selected dilutions were plated onto various selective media to enumerate recipients and putative transconjugants. Transfer of Cu^r plasmids into copper-sensitive (Cu^s) recipients was selected at a level approximately 0.4 to 0.6 mM above the MIC for the particular recipient. Unused portions of the mating mixture were stored in 15% glycerol at −20°C and used in colony blotting experiments. These experiments were conducted to determine if conjugative transfer of pXV10A occurred but was not detected in the recipients after mating because Cu^r genes were not expressed. The stored mating mixtures from selected filter matings were plated to media containing antibiotics to select for the recipient and counterselect against the donor (XV10). Approximately 1,000 to 1,500 recipient colonies were blotted from each mating and probed with ^{32}P-labeled pXV10A.

Plasmid isolation procedures. Plasmid DNA was isolated from *E. coli* by standard procedures (11). When small amounts of plasmid DNA were to be isolated from *X. campestris*, the method of Crosa and Falkow (6) was used with slight modifications (2). In the present study, a preparative method for extracting plasmid DNA from *X. campestris* was developed from the Crosa and Falkow protocol. Overnight cultures of *X. campestris* (250 ml) were centrifuged, and the pellets were washed once in 40 ml of TE buffer (0.05 M Tris hydrochloride, 0.02 M EDTA [pH 8.0]). Washed cells were resuspended in 1.6 ml of TE buffer, and 17 ml of lysis buffer (4% sodium dodecyl sulfate in TE; pH 12.4) was added. After incubation for 30 min at 37°C, the mixture was neutralized with 1.2 ml of 2 M Tris hydrochloride (pH 7.0), and 9.2 ml of 5 M NaCl was added. After incubation on ice for 1 to 6 h, chromosomal DNA was pelleted by centrifugation at 17,000 × *g* for 15 min. The supernatant was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1; water saturated), and 22 ml of isopropanol was added to the aqueous layer. Plasmid DNA was precipitated at −20°C for at least 30 min and then centrifuged at 17,000 × *g* for 15 min. Plasmid DNA pellets were resuspended in 1.2 ml of TE, and the success of the procedure was checked on a 0.7% agarose gel.

Molecular genetic techniques. Agarose gel electrophoresis, DNA restriction digests, and Southern transfers were done by standard procedures (11). Prehybridizations (4 h at 68°C) and hybridizations (12 to 16 h at 68°C) were in aqueous solutions as described by Maniatis et al. (11). After hybridization, filters were washed twice (15 min per wash) at 25°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate and twice at 68°C with 0.1× SSC–0.5% sodium dodecyl sulfate (first wash, 1 h; second wash, 30 min). Southern transfers and colony blots were performed using nylon membranes purchased from Amersham Corp., Arlington Heights, Ill. Probe DNA was removed from nylon membranes as described by the manufacturer. When specific restriction fragments of cloned DNA were to be labeled with ^{32}P, they were separated from vector fragments on agarose gels and excised. Residual agarose was removed either as described previously (2) or with the Geneclean Kit manufactured by BIO101, La Jolla, Calif. Probe DNA was labeled with ^{32}P by using a nick translation kit purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. DNA fragments used as probes in the present study included the following: (i) the 4.4-kb *Pst*I insert in pCOP2 which contains Cu^r genes from *P. syringae* pv. *tomato* (3); (ii) the 4.8-kb *Bgl*II-*Hin*dIII fragment in pXvCu1-16 containing Cu^r genes from *X. campestris* pv. *vesicatoria* E3C5 (B. J. Staskawicz, unpublished data); and (iii) a 350-base-pair *Sal*I-*Sma*I fragment which is an internal portion of IS*476*, an insertion sequence present in *X. campestris* pv. *vesicatoria* strains isolated from diseased peppers and tomatoes in Florida (10).

Hybridization of Cu^r plasmids with selected DNA probes. A series of experiments was conducted to determine the relatedness of pXV10A (the Cu^r plasmid identified in the present study) and pXvCu, a heterogenous group of Cu^r plasmids present in strains of *X. campestris* pv. *vesicatoria* (15) from Florida. *X. campestris* pv. *vesicatoria* 68-1, 81-23, E3C5, and 75-3 are four Cu^r strains of the pathogen which originated in Florida and contain pXvCu (15). The Cu^r plasmid present in each of these four strains was mobilized into a plasmidless strain of *X. campestris* pv. *vesicatoria* (XV17) or *X. campestris* pv. *campestris* (XC11); this made it possible to isolate each pXvCu plasmid independent of smaller plasmids which resided in these strains. The Cu^r plasmids present in the transconjugants were designated pXvCuE3C5, pXvCu75-3, pXvCu81-23, and pXvCu68-1. Plasmid DNA was then isolated from the four transconjugants containing pXvCu (XV17.1, XV17.2, XC11.1, and XC11.2) and from XV16.1 (XV16 containing pXV10A) and digested with *Bgl*II, *Bam*HI, *Bgl*II-*Hin*dIII, and *Eco*RV-*Hin*dIII. These fragments were separated in 0.4, 0.7, and 1.0% agarose gels to resolve various fragment sizes. Selected gels were blotted

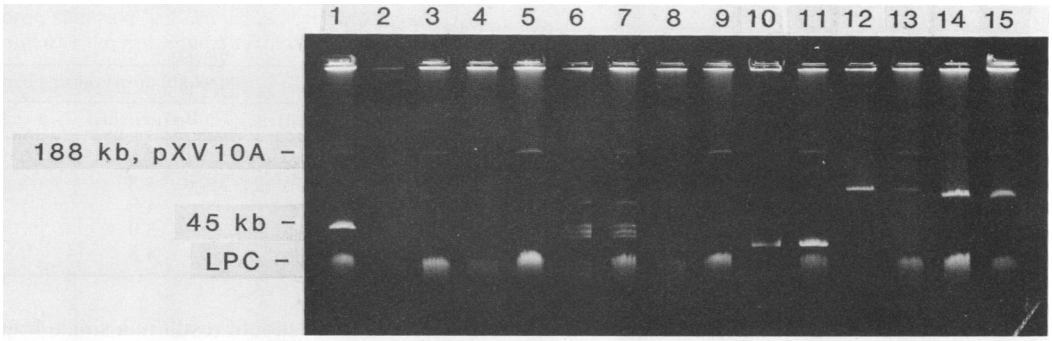

FIG. 1. Transfer of Cu^r plasmid, pXV10A, from XV10 to Cu^s *X. campestris* recipients. Plasmid DNA was isolated from each strain and subjected to agarose gel electrophoresis at 60 V for 2.5 h. LPC, Linearized plasmid and chromosome. Lanes: 1, pv. *vesicatoria* XV10; 2, Cu^s pv. *campestris* XC10; 3, Cu^r pv. *campestris* XC10.1; 4, Cu^s pv. *dieffenbachiae* XD10; 5, Cu^r pv. *dieffenbachiae* XD10.1; 6, Cu^s pv. *manihotis* XM10; 7, Cu^r pv. *manihotis* XM10.1; 8, Cu^s pv. *nigromaculans* XN10; 9, Cu^r pv. *nigromaculans* XN10.1; 10, Cu^s pv. *pelargonii* XP10; 11, Cu^r pv. *pelargonii* XP10.1; 12, Cu^s pv. *phaseoli* XCP10; 13, Cu^r pv. *phaseoli* XCP10.1; 14, Cu^s pv. *vitians* XCV10; 15, Cu^r pv. *vitians* XCV10.1.

and hybridized with the radiolabeled 4.8-kb fragment from pXvCu1-16 or the 350-base-pair *Sal*I-*Sma*I fragment internal to IS*476*.

RESULTS

Efficacy of copper bactericides. The percentage of green fruit showing visible symptoms of bacterial spot ranged from 52 to 67% with all treatments, suggesting that copper-tolerant strains of *X. campestris* pv. *vesicatoria* were present. Although there was no significant difference between treatments for either yield of healthy fruit or incidence of disease, visual inspection of the fruit suggested that the most effective control was achieved with a tank mix of Dithane M-45, Kocide 101, and the spreader-binder Triton CS-7.

Isolates of *X. campestris* pv. *vesicatoria* were recovered from lesions on tomato fruit in each treatment group and in the unsprayed control group. These yellow, mucoid isolates were gram negative and oxidase negative and reproduced symptoms on tomato cv. Marglobe which were typical of the bacterial spot pathogen. One strain of *X. campestris* pv. *vesicatoria* from each treatment group and the unsprayed control group (XV10 to XV14; Table 1) was assayed for tolerance to copper sulfate. Regardless of the treatment regimen, the strains behaved uniformly in their responses to copper sulfate and exhibited a MIC of 2.4 mM.

Plasmid involvement in copper resistance. Stall and coworkers previously demonstrated that Cu^r strains of *X. campestris* pv. *vesicatoria* isolated from diseased pepper plants in Florida contained Cu^r genes on a conjugative plasmid designated pXvCu (15). Therefore, an experiment was conducted to determine whether the strains of *X. campestris* pv. *vesicatoria* isolated in the present study contained Cu^r genes on a self-transmissible plasmid. The plasmid profiles of XV10, XV11, XV12, XV13, and XV14 were identical; each strain contained a large plasmid which comigrated with the 188-kb plasmid present in *Agrobacterium radiobacter* K84 and a smaller plasmid which was approximately 45 kb (see Fig. 1, lane 1). XV10 was arbitrarily chosen as a putative donor of Cu^r genes and mated with *X. campestris* pv. *vesicatoria* XV16, for which the MIC was 0.6 mM $CuSO_4$ (Table 1). Resistance to 1.2 mM $CuSO_4$ was transferred to XV16 at a frequency of 8.0×10^{-2}; this was approximately 10^6-fold higher than the frequency of spontaneous mutation to copper resistance in XV16. Agarose gel electrophoresis of Cu^r XV16 colonies indicated that the larger XV10 plasmid, designated pXV10A, had been transferred to the Cu^r transconjugants (data not shown).

Host range studies with pXV10A. Laboratory experiments were conducted to evaluate the transmissibility of pXV10A into various gram-negative recipients. After organisms were mated with XV10, the frequency of copper resistance in *X. campestris* pv. *campestris*, *dieffenbachiae*, *manihotis*, *nigromaculans*, *pelargonii*, *phaseoli*, and *vitians* was 10^3- to 10^9-fold higher than the frequency of spontaneous mutation to copper resistance. To determine whether XV10 was transferred to these pathovars, plasmid DNA was isolated from the various Cu^r recipients (10 colonies of each). Agarose gel electrophoresis of the isolated plasmids showed that each Cu^r transconjugant contained a single plasmid with a mobility identical to that of pXV10A (Fig. 1, lanes 3, 5, 7, 9, 11, 13, and 15). The MICs of $CuSO_4$ for Cu^s recipients and Cu^r transconjugants containing pXV10A are indicated in Table 1.

The frequency of copper resistance after organisms were mated with XV10 was not significantly different from the spontaneous-mutation frequency in the following bacteria: *X. campestris* pv. *malvacearum*, *glycines*, and *translucens*; *A. radiobacter* and *Agrobacterium tumefaciens*; *P. andropogonis*, *P. corrugata*, and *P. syringae* pv. *syringae*; and *Erwinia herbicola*. The stored mating mixtures from these filter matings were plated to media containing antibiotics to select for each recipient and counterselect against XV10. ^{32}P-labeled pXV10A did not hybridize to colony blots from any of these recipients; therefore, transfer of pXV10A could not be detected.

Characterization of Cu^r genes on pXV10A. With respect to molecular weight, pXV10A resembled pXvCu, the Cu^r plasmid in strains of *X. campestris* pv. *vesicatoria* from Florida (15). The Cu^r genes present on pXvCu in *X. campestris* pv. *vesicatoria* E3C5 have been subcloned as a 4.8-kb *Bgl*II-*Hin*dIII fragment in pUC18. The clone containing these genes, pXvCu1-16, was supplied to us by Brian Staskawicz (University of California, Berkeley). This 4.8-kb fragment hybridized strongly to plasmid pXV10A in XV10 but did not hybridize to the smaller 45-kb plasmid present in this strain (data not shown). This result indicated that E3C5 and XV10 share related Cu^r genes.

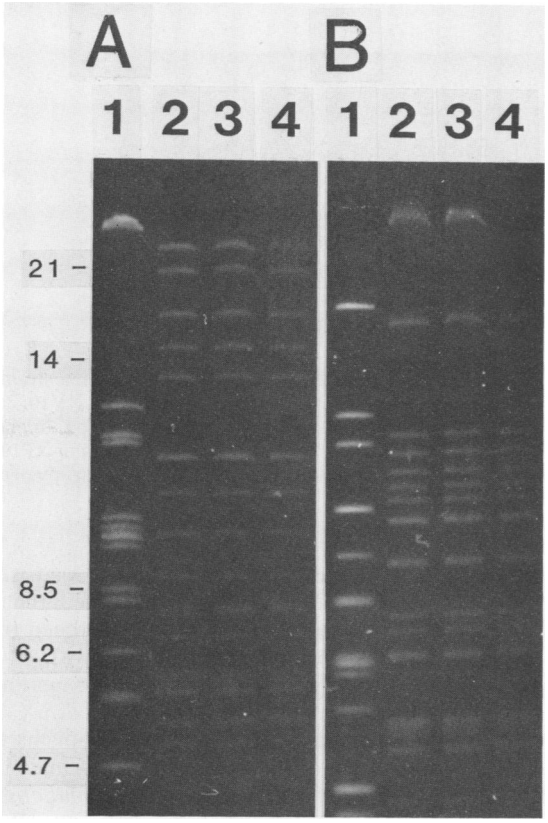

FIG. 2. *Bgl*II (A) and *Bam*HI (B) digests of Cu^r plasmids in *X. campestris* pv. *vesicatoria*. Lanes: 1, pXV10A; 2, pXvCuE3C5; 3, pXvCu75-3; 4, pXvCu81-23. Electrophoresis was for 12 h at 60 V.

The Cu^r genes from *P. syringae* pv. *tomato* PT23, another foliar pathogen of tomato which causes bacterial speck disease, have been subcloned from plasmid pPT23D as a 4.4-kb *Pst*I fragment (3). This fragment did not hybridize to pXV10A at the stringencies used in the present study.

Hybridization of Cu^r plasmids with selected DNA probes. As was found with pXV10A, plasmids pXvCuE3C5, pXvCu81-23, and pXvCu68-1 also comigrated with the 188-kb plasmid present in *A. radiobacter*; pXvCu75-3 was slightly larger. The five plasmids were isolated from transconjugants XV16.1, XV17.1, XV17.2, XC11.1, and XC11.2 and cut with various restriction enzymes. Digests of pXvCuE3C5, pXvCu75-3, and pXvCu81-23 were remarkably similar; however, digests of pXV10A were quite different from those of the other three plasmids. For example, Fig. 2 shows the *Bgl*II and *Bam*HI digests of pXV10A (lane 1), pXvCuE3C5 (lane 2), pXvCu75-3 (lane 3), and pXvCu81-23 (lane 4). The pattern of restriction fragments generated from digested pXvCu68-1 was different from the patterns with the other four plasmids in all digests (data not shown).

The *Bgl*II, *Bgl*II-*Hin*dIII, and *Eco*RV-*Hin*dIII digests of the five plasmids were probed with the 4.8-kb *Bgl*II-*Hin*dIII fragment in pXvCu1-16 which contains the subcloned Cu^r genes from pXvCuE3C5. The results of this experiment indicated both similarities and differences among the five plasmids. The bands which hybridized to the probe in the *Bgl*II-*Hin*dIII digests varied only slightly in size (4.8 or 5.0 kb; see Table 2); this indicates conservation of this fragment in all five Cu^r plasmids. Since the 4.8-kb *Bgl*II-*Hin*dIII fragment does not contain an internal *Bgl*II site, hybridization to *Bgl*II digests should result in a single fragment which includes DNA flanking the target sequences. Again, little variation was observed among the five plasmids; the size of the hybridizing band was either 6.6 or 6.9 kb (Table 2). The subcloned fragment was also hybridized to *Hin*dIII-*Eco*RV digests to study the conservation of flanking sequences. In the four pXvCu plasmids, little variation was noted; the probe hybridized either to an 8.0-kb band (pXvCuE3C5, pXvCu75-3, and pXvCu81-23) or to an 8.5-kb band (pXvCu68-1). In the pXV10A digest, a much larger band (21 kb) hybridized to the probe. These results indicate that sequences flanking the Cu^r gene(s) are conserved in some, but not all, of the Cu^r plasmids. All digests and hybridizations were repeated with similar results.

TABLE 2. Size of hybridizing bands in selected digests of *X. campestris* pv. *vesicatoria* Cu^r plasmids probed with Cu^r genes cloned from *X. campestris* pv. *vesicatoria* E3C5

Plasmid	Size (kb) of hybridizing band of:		
	*Bgl*II	*Bgl*II-*Hin*dIII	*Eco*RV-*Hin*dIII
pXV10A	6.9	5.0	21.0
pXvCuE3C5	6.6	4.8	8.0
pXvCu75-3	6.6	4.8	8.0
pXvCu81-23	6.6	5.0	8.0
pXvCu68-1	6.6	4.8	8.5

IS*476* is a 1.2-kb insertion sequence present in many Cu^r strains of *X. campestris* pv. *vesicatoria*; it was originally isolated from pXvCu81-23 (10). It was used as a probe in the present study in an attempt to distinguish pXV10A from pXvCu plasmids. Since IS*476* does not contain a *Bam*HI site (B. Kearney, personal communication), the five Cu^r plasmids were digested with *Bam*HI and probed with the 350-base-pair *Sal*I-*Sma*I fragment internal to the element to detect the presence of the element and determine the copy number per plasmid. This fragment hybridized to three *Bam*HI fragments of approximately 38, 10.3, and 7.6 kb in pXvCuE3C5, pXvCu75-3, and pXvCu81-23. Detection of three copies of IS*476* in pXvCu81-23 agreed with previous data reported by Kearney et al. (10). The *Sal*I-*Sma*I fragment from IS*476* did not hybridize to *Bam*HI digests of pXV10A or pXvCu68-1 (data not shown).

DISCUSSION

Copper-resistant strains of *X. campestris* pv. *vesicatoria* were isolated from all treatment groups in the field study, including the unsprayed control. Although none of the treatments provided complete control, plants sprayed with the Dithane M-45–Kocide 101–Triton CS-7 tank mix had the least amount of bacterial spot. Several previous reports have noted the enhanced efficacy of similar tank mixes for control of bacterial spot (1, 5, 12).

While there are studies involving antibiotic resistance plasmids which are resident in clinical bacterial pathogens, the host ranges of plasmids indigenous to phytopathogenic bacteria have not been extensively investigated. To our knowledge, this is the first instance in which the host range of a plasmid indigenous to *X. campestris* has been investigated. Plasmids resident in phytopathogenic bacteria are likely to have a limited host range because of the long history of coadaptation among plasmid, bacterial chromosome, and host plant (19). pXV10A readily entered a majority of *X. campestris* pathovars in the present study, indicating that these recipients share common chromosome factors necessary for the maintenance and replication of this plasmid.

The possibility for plasmid transfer between *X. campestris* pathovars exists on both host and nonhost plants. Timmer et al. (18) reported that *X. campestris* pv. *alfalfae*, *campestris*, *translucens*, and *pruni* can multiply on tomato leaves under conditions of high relative humidity. Conversely, *X. campestris* pv. *vesicatoria* populations were capable of multiplying on the leaves of nonhost plants such as plum and peach. Providing the right conditions and bacteria are present, interpathovar transfer of pXV10A could occur in nature. This would be especially important in a nursery setting, where many different plants are grown, multiple pathovars of *X. campestris* may be present, and copper sprays are used heavily.

Our results indicate that the Cu^r genes present on pXV10A are closely related to those on the pXvCu plasmids. The 4.8-kb *Bgl*II-*Hin*dIII fragment containing the subcloned Cu^r genes from E3C5 hybridized to similar-sized fragments in the *Bgl*II-*Hin*dIII digests of the other four plasmids, indicating strong conservation of this fragment. Sequences flanking the subcloned Cu^r genes were conserved in some, but not all, of the Cu^r plasmids. The *P. syringae* pv. *tomato* Cu^r genes did not hybridize to pXV10A in the present study. However, all hybridizations and washes were conducted at very stringent levels. D. A. Cooksey (personal communication) has observed hybridization of the *P. syringae* pv. *tomato* Cu^r genes to a 100-kb plasmid in a Cu^r strain of *X. campestris* pv. *vesicatoria*. It is possible that hybridizations at reduced stringency levels would reveal relatedness between the Cu^r genes on pXV10A and those cloned from *P. syringae* pv. *tomato*.

In addition to *P. syringae* pv. *tomato* and *X. campestris* pv. *vesicatoria*, Cu^r plasmids have also been identified in *Mycobacterium scrofulaceum* (7) and *E. coli* (8, 14, 17). Very little work has been done to characterize the Cu^r plasmids which reside in different bacterial hosts. Cooksey (4) found that a 35-kb Cu^r plasmid was conserved among 12 different Cu^r strains of *P. syringae* pv. *tomato*. *Eco*RI and *Pst*I digests of this plasmid were identical, and a cloned Cu^r gene hybridized to the same location on the 35-kb plasmid of all 12 strains. However, we have demonstrated that *X. campestris* pv. *vesicatoria* Cu^r plasmids can differ in their restriction digest profiles. In the present study, these plasmids could also be distinguished by polymorphisms which resulted when digests were probed with the cloned Cu^r genes from E3C5 and by the presence or absence of IS*476*. In addition to these differences, some strains of *X. campestris* pv. *vesicatoria* contain the avirulence gene *avr*Bs_1 on pXvCu (10, 16). Kearney et al. have demonstrated that at least one copy of IS*476* is an active transposable element in strain 81-23 and can inactivate *avr*Bs_1, thus affecting host range (10). Insertion sequence-mediated rearrangements could explain some of the differences apparent among Cu^r plasmids in *X. campestris* pv. *vesicatoria*.

ACKNOWLEDGMENTS

We are especially grateful to Brian Staskawicz and Brian Kearney for providing bacterial strains, clones, sequence data, and helpful suggestions.

LITERATURE CITED

1. **Adaskaveg, J. E., and R. B. Hine.** 1985. Copper tolerance and zinc sensitivity of Mexican strains of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. Plant Dis. **69**:993–996.
2. **Bender, C. L., and D. A. Cooksey.** 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. J. Bacteriol. **165**:534–541.
3. **Bender, C. L., and D. A. Cooksey.** 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. **169**:470–474.
4. **Cooksey, D. A.** 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. *tomato*. Appl. Environ. Microbiol. **53**:454–456.
5. **Cox, R. S.** 1982. Control of bacterial spot of tomato in southern Florida. Plant Dis. **66**:870.
6. **Crosa, J. H., and S. Falkow.** 1981. Plasmids, p. 266–282. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
7. **Eradi, F. X., M. L. Failla, and J. O. Falkinham III.** 1987. Plasmid-encoded copper resistance and precipitation by *Mycobacterium scrofulaceum*. Appl. Environ. Microbiol. **53**:1951–1954.
8. **Ishihara, M., Y. Kamio, and Y. Terawaki.** 1978. Cupric ion resistance as a new genetic marker of a temperature sensitive R plasmid, Rts1 in *Escherichia coli*. Biochem. Biophys. Res. Commun. **82**:74–80.
9. **Keane, P. J., A. Kerr, and P. B. New.** 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. **23**:585–595.
10. **Kearney, B., P. C. Ronald, D. Dahlbeck, and B. J. Staskawicz.** 1988. Molecular basis for evasion of plant host defence in bacterial spot disease of pepper. Nature (London) **332**:541–543.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Marco, G. M., and R. E. Stall.** 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Dis. **67**: 779–781.
13. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Rouch, D., J. Camakaris, B. T. O. Lee, and R. K. J. Luke.** 1985. Inducible plasmid-mediated copper resistance in *Escherichia coli*. J. Gen. Microbiol. **131**:939–943.
15. **Stall, R. E., D. C. Loschke, and J. B. Jones.** 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **76**:240–243.
16. **Swanson, J., B. Kearney, D. Dahlbeck, and B. Staskawicz.** 1988. Cloned avirulence gene of *Xanthomonas campestris* pv. *vesicatoria* complements spontaneous race-change mutants. Mol. Plant Microbe Interact. **1**:5–9.
17. **Tetaz, T. J., and R. K. J. Luke.** 1983. Plasmid-controlled resistance to copper in *Escherichia coli*. J. Bacteriol. **154**: 1263–1268.
18. **Timmer, L. W., J. J. Marois, and D. Achor.** 1987. Growth and survival of xanthomonads under conditions nonconducive to disease development. Phytopathology **77**:1341–1345.
19. **Young, J. P. W., and M. Wexler.** 1988. Sym plasmid and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. J. Gen. Microbiol. **134**:2731–2739.