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DNA Probes for Detection of Copper Resistance Genes in *Xanthomonas campestris* pv. Vesicatoria†

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The copper resistance (Cu^r) genes encoded on pXV10A, a 190-kb plasmid in *Xanthomonas campestris* pv. vesicatoria XV10, were isolated on a 44-kb cosmid clone designated pCuR1. Tn*5* mutagenesis of pCuR1 indicated that a 4.0-kb region was required for copper resistance. Three restriction fragments located within the 4.0-kb region demonstrated high specificity for the Cu^r genes present in *X. campestris* pv. vesicatoria and will be useful in monitoring the presence of these genes in the environment.

Copper-resistant (Cu^r) strains of *Xanthomonas campestris* pv. vesicatoria, the causal agent of bacterial spot on tomato and pepper, have been isolated in Florida (16, 21), Mexico (1), Oklahoma (5), and California (8). However, the relatedness of Cu^r genes which occur in some of these strains is largely unknown. In combination with the selection pressure caused by the heavy use of copper sprays, the occurrence of Cu^r genes on conjugative plasmids has undoubtedly caused an increase in the number of copper-resistant bacteria in the environment. For example, Cu^r strains of *X. campestris* pv. vesicatoria isolated in Florida contained a heterogeneous group of conjugative Cu^r plasmids designated pXvCu (21). In Oklahoma, Cu^r genes in many strains of *X. campestris* pv. vesicatoria reside on pXV10A, a 190-kb transmissible plasmid which shares some nucleotide sequence homology with the Florida pXvCu plasmids (5). A host-range study indicated that pXV10A can be transferred into several other *X. campestris* pathovars, but transfer into other phytopathogenic genera was not detected (5).

In the present study, our objectives were twofold: (i) to characterize and localize the Cu^r genes on pXV10A by DNA cloning and transposon mutagenesis, and (ii) to develop nucleic acid probes specific for the Cu^r genes present on pXV10A and pXvCu plasmids. In future studies, nucleic acid probes will be used to monitor the presence of Cu^r genes in the environment and to determine the effects of copper bactericides on the incidence and evolution of Cu^r genes.

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in the present study are shown in Tables 1 and 2, respectively. *X. campestris* pv. vesicatoria was cultivated on either nutrient agar (NA) (19) or King's medium B (KMB) (14) at 28°C. *Escherichia coli* cultures were grown in Luria-Bertani (LB) (15) medium at 37°C. All other bacterial strains were maintained on KMB agar at 28°C. Minimal A medium (19) containing 1.0 mM $CuSO_4$ was used to screen for the expression of copper resistance in *E. coli*. Antibiotics were added as necessary to maintain selection for resistance markers. Selective antibiotic concentrations were as follows: tetracycline, 12.5 μg/ml; kanamycin, 25 μg/ml; ampicillin, 40 μg/ml. Lambda 467, the source of lambda::Tn*5*, was supplied by Nancy Kleckner, Harvard University.

All media used in MIC determinations were treated with Chelex 100 to remove heavy metals by using the batch method described by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Bacteria were grown on metal-free NA for 24 to 48 h prior to MIC testing. Cells were suspended in sterile water to an optical density of 0.1 at A_{600} and spotted in duplicate (5 μl per spot) onto NA containing different concentrations of copper sulfate (0 to 2.0 mM). The MIC of copper sulfate was expressed as the concentration that inhibited confluent growth of the culture after 72 h of incubation at 28°C.

Characterization of *X. campestris* pv. vesicatoria strains. In Oklahoma, tomato and bell pepper production areas occur primarily in eastern Oklahoma, whereas paprika and spice peppers are produced in western Oklahoma (Caddo County). Copper bactericides are heavily applied in eastern production areas but are often unnecessary in the western region. In August 1989, severe bacterial spot symptoms were observed on tomato and bell pepper cultivars grown at the Oklahoma State University Vegetable Research Station in Bixby (eastern region) and on paprika peppers cultivated in Caddo County (western region). Putative strains of *X. campestris* pv. vesicatoria were isolated from foliar lesions on tomato (seven cultivars), bell pepper (two cultivars), and paprika pepper (three unnamed breeding lines). These strains were tested for Gram stain, oxidase reaction, protease activity, and pathogenicity on tomato cv. Marglobe (5, 20).

Isolates of *X. campestris* pv. vesicatoria were recovered from foliar lesions on all three hosts. These yellow, mucoid isolates were gram negative and oxidase negative, produced protease, and reproduced symptoms on tomato cv. Marglobe which were typical of the bacterial spot pathogen. Strains XV18, XV19, XV20, XV21, XV22, XV23, and XV24 originated from tomato cultivars Gibraltor, President, Carnival, Summer Flavor 5000, Bingo, Mountain Pride, and Jet Star, respectively. These strains were identical in their response to copper sulfate, which had a MIC of 1.4 mM. *X. campestris* pv. vesicatoria strains which were isolated from bell pepper cultivars Big Bertha Bell (XV29) and Early Calwonder (XV30) and from three unnamed paprika breeding lines (XV31, XV32, and XV33) were sensitive to copper sulfate, which had MICs ranging from 0.3 to 0.4 mM. These results indicated that the *X. campestris* pv. vesicatoria population at the Oklahoma State University Vegetable Research Station (eastern region) was heterogeneous, because Cu^r and Cu^s strains were isolated from tomato and pepper cultivars, respectively. Only Cu^s strains were recov-

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TABLE 1. Bacterial strains used in the present study

Bacterium (no. of strains examined)	Strain designation	Source or reference
Agrobacterium radiobacter (1)	K-84	A. Kerr
A. tumefaciens (2)	C58, NT1	D. A. Cooksey
Clavibacter michiganense subsp. *michiganense* (3)	982-1, 683-15, 683-16	D. A. Cooksey
C. michiganense subsp. *sepedonicum* (1)	9850	ATCC[a]
Erwinia amylovora (1)	682-3	D. A. Cooksey
E. carotovora (1)	76-11	L. W. Moore
E. herbicola (3)	785-2, 785-3	D. A. Cooksey
	13329	ATCC
E. quercina (1)	389	J. Sims
E. rhapontici (1)	1184-8	D. A. Cooksey
Escherichia coli (3)	K-12	2
	HB101	15
	CSH25a	19
Pseudomonas aeruginosa (3)	B4, B5	M. Delgado
	PA02700	NCPPB[b]
P. andropogonis (1)	A1044-1	W. Chun
P. cepacia (2)	945	NCPPB
	389-4	K. Conway
P. cichorii (2)	284-10, 1082-2	D. A. Cooksey
P. corrugata (1)	682-12	D. A. Cooksey
P. fluorescens (1)	785-17	D. A. Cooksey
P. putida (1)	882-9	D. A. Cooksey
P. syringae pv.:		
atropurpurea (1)	1304	M. Sato
glycinea (1)	4180	ICMP[c]
maculicola (1)	438	D. Cuppels
mori (1)	1413	NCPPB
morsprunorum (2)	3714, 567	ICMP
phaseolicola (1)	285-1	D. A. Cooksey
pisi (1)	1086-2	C. Bender
savastanoi (1)	13526	ATCC
syringae (1)	B310	D. Gross
tabaci (1)	1086-1	C. Bender
tomato (3)	PT23.2, OK-1	C. Bender
	4325	ICMP
P. viridiflava (6)	5782-2, 5787-3, PV-4	R. Stall
	PV-1, PV-10	J. Hunter
	SF312	C. Liao
Xanthomonas campestris pv.:		
campestris (1)	186-1	D. A. Cooksey
dieffenbachiae (1)	B400	N. Schaad
glycines (1)	1086-4	C. Bender
malvacearum (1)	3PM1	R. Gholson
manihotis (1)	QR32	W. Chun
nigromaculans (1)	682-1	D. A. Cooksey
pelargonii (1)	782-29	D. A. Cooksey
phaseoli (1)	QR60	W. Chun
translucens (1)	B-430	V. Mellano
pruni (4)	588-9, 688-18, 688-19, 688-3	C. Bender
vesicatoria Cu^s strains (8)	1083-12	D. A. Cooksey
	B202	N. Schaad
	XV16	5
	XV29, XV30, XV31, XV32, XV33	This study
vesicatoria Cu^r strains (15)	81-23, 75-3, 68-1, E-3	B. Staskawicz; 21
	81-18, H7998-11	J. Jones; 21
	XV10	5
	XV18, XV19, XV20, XV21, XV22, XV23, XV24	This study
	XV16.1	5

[a] ATCC is the American Type Culture Collection, Rockville, Maryland.
[b] NCPPB is the National Collection of Plant Pathogenic Bacteria, Harpenden, England.
[c] ICMP is the International Collection of Micro-organisms from Plants, Auckland, New Zealand.

ered from paprika breeding lines cultivated in Caddo County (western region). *X. campestris* pv. vesicatoria 1083-12 and B202, which were obtained from other investigators (Table 1), were found to be Cu^s, and MICs of $CuSO_4$ for them were 0.4 mM.

Copper resistance in strains 81-23, 75-3, 68-1, E-3, and 81-18, which contain the Cu^r plasmid pXvCu, was previously demonstrated by Stall et al. (21); H7998-11 is a Cu^r strain obtained from J. Jones, Bradenton, Fla. In the present study, the MIC of $CuSO_4$ was 1.4 mM for XV10, a strain

TABLE 2. Plasmids

Plasmid	Relevant properties	Source or reference
pXV10A	Cu^r	5
pLAFR3	Tc^r; cosmid vector	22
pRK415	Tc^r; RK-2 derived cloning vector	13
pUC119	Ap^r	23
pCuR1	Tc^r; pLAFR3 Cu^r clone	This study
pCuR2	Tc^r; contains a 6.4-kb *Bgl*II fragment cloned into pRK415	This study
pCuR2.A	Ap^r; contains a 2.2-kb *Hin*dIII-*Bam*HI fragment cloned into pUC119	This study
pCuR2.B	Ap^r; contains a 0.8-kb *Bam*HI fragment cloned into pUC119	This study
pCuR2.C	Ap^r; contains a 1.0-kb *Bam*HI fragment cloned into pUC119	This study

which contains the Cu^r plasmid pXV10A and was previously isolated from tomatoes grown at the Oklahoma State University Vegetable Research Station (5). The MIC for XV16.1, a Cu^r transconjugant obtained by transferring pXV10A into the copper-sensitive recipient XV16, was also 1.4 mM $CuSO_4$; however, the parent strain XV16 was inhibited by 0.3 mM $CuSO_4$. The MICs obtained for XV10, XV16, and XV16.1 were slightly lower than those observed previously (5). This discrepancy can be explained by the different method used for MIC evaluation in the present study and by variations in the composition of NA.

Cloning copper resistance genes from pXV10A. The copper resistance plasmid pXV10A was isolated from XV16.1 by a preparative isolation procedure which has been described previously (5). Preparative methods for extracting plasmid DNA from *E. coli* were used as described by Maniatis et al. (15). All preparative plasmid DNA isolations were subjected to at least one purification on CsCl-ethidium bromide gradients. Rapid, small-scale plasmid DNA isolations from *E. coli* were performed by boiling method 2 as described by Crouse et al. (10). Small amounts of plasmid DNA were isolated from *X. campestris* pv. vesicatoria by the method of Crosa and Falkow (9), with slight modifications (3). Agarose gel electrophoresis, DNA restriction digests, Southern transfers, and colony lifts were done by standard procedures (15). All hybridizations were conducted under conditions of high stringency as described previously (5). Methods for labeling specific DNA fragments with ^{32}P (6, 12) and for constructing cosmid DNA libraries in pLAFR3 (4, 22) have also been described. pLAFR3 clones and pRK415 subclones were transferred into the Cu^s recipient, *X. campestris* pv. vesicatoria XV16, by electroporation. XV16 was prepared for electroporation by a procedure described by Cooksey (7), except that KMB broth and 0.5 M sucrose were utilized for growing and washing the cells, respectively. Electroporation was performed at 8 kV/cm with a single pulse delivered with a 25-μF capacitor at 200 Ω.

A cosmid library of pXV10A was constructed in pLAFR3, transferred to XV16 by electroporation, and screened for the expression of copper resistance on NA containing 0.8 mM $CuSO_4$. Out of 200 clones, one 44-kb cosmid, pCuR1, conferred copper resistance to XV16; when pCuR1 was transferred to XV16, the MIC of copper sulfate increased from 0.3 to 1.4 mM. To determine whether the pCuR1 Cu^r genes could be expressed in *E. coli*, the cosmid was transformed into competent cells of the prototrophic strain K-12. Tc^r transformants were unable to grow on minimal A medium containing 1.0 mM copper sulfate, indicating that the *Xanthomonas* Cu^r genes were not expressed in K-12 under these conditions.

A restriction map of the 22-kb insert in pCuR1 was generated by using *Hin*dIII, *Eco*RI, and *Bgl*II (Fig. 1A). To identify which regions on pCuR1 were involved in the copper resistance phenotype, this cosmid was mutagenized with Tn*5*. Bacteriophage stock of lambda::Tn*5* was prepared as described by De Bruijn and Lupski (11), and titers were determined on *E. coli* CSH25a as described by Miller (19). Approximately 500 μl of phage stock (2×10^{10} PFU/ml) was combined with 1 ml of HB101(pCuR1), and mutagenesis was performed as described previously (11). Plasmid DNA was isolated from Tc^r Km^r HB101(pCuR1) cells and purified on CsCl-ethidium bromide gradients. pCuR1 cosmids containing Tn*5* insertions were selected by transforming *E. coli* HB101 with this plasmid preparation and selecting Tc^r Km^r transformants. The location of Tn*5* insertions on pCuR1 was mapped by digesting mutated cosmids with *Eco*RI, *Hin*dIII, and *Bgl*II. Selected pCuR1 cosmids containing Tn*5* mutations were electroporated into XV16, and the MIC of $CuSO_4$ for the resulting transformants was assessed.

Eleven insertions in the 4.5-kb *Hin*dIII-*Bgl*II fragment (Fig. 1A, fragment 7) completely eliminated Cu^r and reduced

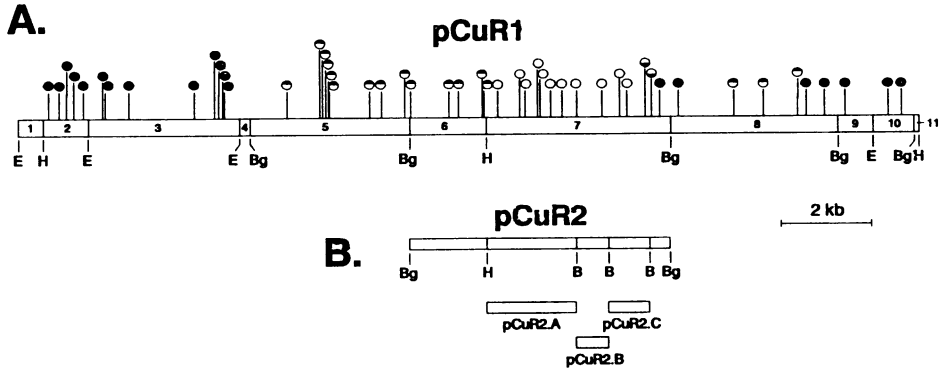

FIG. 1. (A) Restriction endonuclease map of pCuR1. The vertical lines represent the positions of Tn*5* insertions which affect the MIC of $CuSO_4$ as follows: ○, MIC = 0.3 to 0.4 mM; ◒, MIC = 0.6 mM; ◓, MIC = 1.0 to 1.2 mM; ●, MIC = 1.4 mM. (B) Restriction map of pCuR2, a 6.4-kb *Bgl*II fragment cloned in pRK415. pCuR2.A, pCuR2.B, and pCuR2.C were constructed by subcloning internal fragments of pCuR2 in pUC119 (see text). Restriction sites are shown for *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), and *Hin*dIII (H).

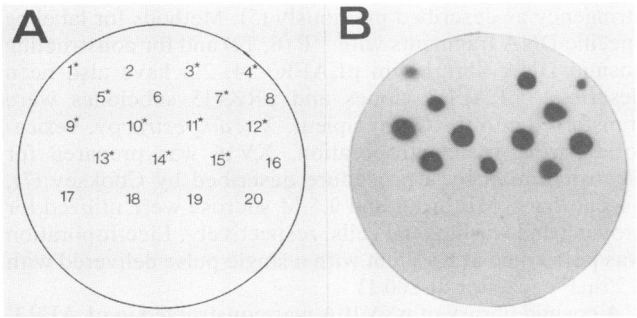

FIG. 2. Hybridization of pCuR2.C with Cu^s and Cu^r *X. campestris* pv. vesicatoria strains. (A) Key to location of colonies hybridized with pCuR2.C. Cu^r strains are indicated with asterisks. Strains are as follows: 1*, 81-23; 2, XV16; 3*, 75-3; 4*, 68-1; 5*, E3; 6, 1083-12; 7*, 81-18; 8, B202; 9*, XV10; 10*, XV18; 11*, XV19; 12*, XV20; 13*, XV21; 14*, XV22; 15*, H7998-11; 16, XV29; 17, XV30; 18, XV31; 19, XV32; 20, XV33. (B) Hybridization of the colony blot shown in panel A with the ^{32}P-labeled 1.0-kb *Bam*HI fragment cloned in pCuR2.C.

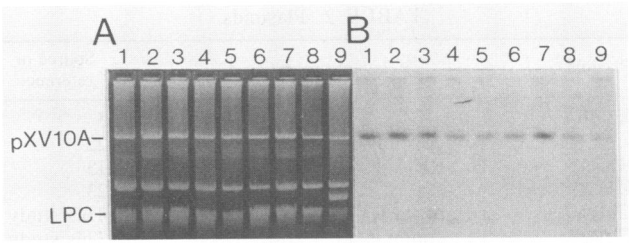

FIG. 3. Agarose gel electrophoresis and Southern blot analysis of plasmid DNA present in copper-resistant strains of *X. campestris* pv. vesicatoria. (A) Indigenous plasmids in copper-resistant strains. Electrophoresis of plasmid DNA proceeded for 2.5 h at 60 V in 0.7% agarose. Lanes: 1, XV10; 2, XV18; 3, XV19; 4, XV20; 5, XV21; 6, XV22; 7, XV23; 8, XV24; 9, H7998-11. The migration of pXV10A, the copper resistance plasmid in XV10, is indicated to the left of the gel. LPC, linearized plasmid and chromosomal DNA. (B) Hybridization of the ^{32}P-labeled 1.0-kb *Bam*HI fragment in pCuR2.C to a nylon membrane containing the plasmid DNA shown in panel A.

the MIC of copper sulfate from 1.4 mM to 0.3 or 0.4 mM. Two insertions in the right side of fragment 7 reduced the MIC to 0.6 mM. An insertion located adjacent to the *Bgl*II site in fragment 7 had no effect on resistance (MIC, 1.4 mM). Insertions in the 3.8-kb *Bgl*II, 1.9-kb *Bgl*II-*Hin*dIII, and 4.1-kb *Bgl*II fragments (Fig. 1A, fragments 5, 6, and 8, respectively) slightly reduced the MIC of $CuSO_4$. Insertions in fragments 2, 3, 9, and 10 did not affect the level of copper resistance conferred by pCuR1. These MIC evaluations for the various insertions were repeated three times with similar results.

Colony hybridizations with selected DNA fragments. Our results indicated that a 4.0-kb region extending from the central *Hin*dIII site into fragment 7 was absolutely required for copper resistance. Consequently, restriction fragments from this region were examined for their usefulness as DNA probes for the detection of the pXV10A Cu^r genes. A 6.4-kb *Bgl*II fragment (Fig. 1A, fragments 6 and 7) containing the region essential for copper resistance was subcloned into the *Bam*HI site of pRK415; the clone which resulted was designated pCuR2 (Fig. 1B).

pCuR2 was further mapped with *Bam*HI to identify fragments within the 4.0-kb Cu^r region which could be used as Cu-specific hybridization probes. The restriction map which resulted (Fig. 1B) revealed three restriction fragments internal to the 4.0-kb region: a 2.2-kb *Hin*dIII-*Bam*HI fragment and 0.8- and 1.0-kb *Bam*HI fragments. These three fragments were subcloned in pUC119 and designated pCuR2.A (2.2-kb *Hin*dIII-*Bam*HI fragment), pCuR2.B (0.8-kb *Bam*HI fragment), and pCuR2.C (1.0-kb *Bam*HI fragment). The inserts in these three subclones were labeled with ^{32}P and used as probes in colony hybridizations with all of the bacteria listed in Table 1 except *E. coli*. All three probes hybridized strongly to Cu^r strains of *X. campestris* pv. vesicatoria from Florida and Oklahoma. Figure 2 shows the results obtained when the 1.0-kb *Bam*HI fragment in pCuR2.C was hybridized to Cu^r and Cu^s *X. campestris* pv. vesicatoria strains; identical results were obtained with the ^{32}P-labeled insert fragments cloned in pCuR2.A and pCuR2.B (data not shown). When plasmid DNA was isolated from these Cu^r strains and separated by agarose gel electrophoresis, the three probes hybridized to a large indigenous plasmid in each strain. Figure 3 shows the results obtained when the ^{32}P-labeled insert in pCuR2.C was hybridized to selected Cu^r strains; identical results were obtained with probes constructed from pCuR2.A and pCuR2.B (data not shown). No hybridization was observed between the three probes and genomic DNA of the following bacteria: (i) Cu^s strains of *X. campestris* pv. vesicatoria (Fig. 2), (ii) other *X. campestris* pathovars (data not shown), and (iii) bacteria belonging to the genera *Agrobacterium*, *Clavibacter*, *Erwinia*, and *Pseudomonas* (data not shown).

Origin of *X. campestris* pv. vesicatoria Cu^r genes. Resistance to copper has also been investigated in *Pseudomonas syringae* pv. tomato, which causes bacterial speck disease on tomato. Copper resistance genes from a California strain of *P. syringae* pv. tomato were subcloned from a 35-kb plasmid, pPT23D, as a 4.5-kb *Pst*I fragment (4). Sequence analysis indicated that the subcloned fragment contained four tandemly positioned open reading frames and was organized as an operon (17, 18). Homology to the *cop* operon has been found in chromosomal DNA of both Cu^r and Cu^s *Pseudomonas* spp., suggesting that the *cop* operon evolved from chromosomal genes (8). Consequently, the lack of homology between the three DNA probes constructed in the present study and genomic DNA isolated from Cu^s *X. campestris* pv. vesicatoria strains may indicate that the resistance genes encoded on pXvCu and pXV10A were introduced into this pathogen instead of evolving from endogenous genes. Although *P. syringae* pv. tomato and *X. campestris* pv. vesicatoria occur together on tomatoes grown in similar environments, there is no DNA homology between the *cop* operon and the Cu^r genes which occur on pXV10A and pXvCu (4, 5).

To our knowledge, no other DNA probes have been constructed for detecting specific Cu^r genes. Cooksey et al. (8) reported that the *cop* operon hybridized to Cu^s bacteria, thereby limiting its efficacy for the specific detection of Cu^r bacteria. The use of nucleic acid probes for the detection of specific Cu^r genes may be superior to detection by plating to selective media since different bacterial strains show a wide range of tolerance to copper.

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