Linkage of Copper Resistance and Avirulence Loci on a Self-Transmissible Plasmid in *Xanthomonas campestris* pv. *vesicatoria*

R. E. Stall, D. C. Loschke, and J. B. Jones

Professor and assistant professor, respectively, Department of Plant Pathology, University of Florida, Gainesville 32611; and assistant professor, Gulf Coast Research and Education Center, University of Florida, Bradenton 34203.

Florida Agricultural Experiment Station Journal Series Paper 6365.
The authors wish to thank R. W. Rice for technical assistance.
Helpful council was provided by B. J. Staskawicz and D. Dahlbeck, University of California, Berkeley 94720.
Accepted for publication 16 September 1985 (submitted for electronic processing).

ABSTRACT

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.

Resistance to copper in strains of *Xanthomonas campestris* pv. *vesicatoria* was transferred by conjugation to strains sensitive to copper. A self-transmissible plasmid was associated with the transfer of copper resistance. Frequency of conjugation varied with donor and recipient and ranged from 0 to 1.6×10^{-2} transconjugants per donor cell. Strains that were resistant to copper and avirulent on breeding line 10R pepper plants were mated with strains that were sensitive to copper and virulent. Transconjugants, selected for copper resistance only, from these matings were also avirulent. Thus, loci for copper resistance and avirulence to 10R pepper plants are linked and both loci were transferred with the large self-transmissible plasmid. The size of the plasmid with copper resistance (pXvCu) varied, but with several strains it migrated in agarose-gel electrophoresis at nearly the same rate as the wild-type Ti plasmid in *Agrobacterium tumefaciens* strain 4013 which is approximately 193 kilobases in size.

Additional key words: bacterial spot of pepper, *Capsicum annuum*, microbial genetics.

Sprays of fixed copper have been recommended for control of bacterial spot of pepper since the disease was first described in 1922 (8). Resistance to copper was common among strains of the causal organism, *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye isolated from 1960 to 1980 from pepper (*Capsicum annuum* L.) plants in Florida. The level of resistance was sufficient to reduce disease control on pepper plants in field plots sprayed with copper (11).

An association between copper resistance and race 2 of the pepper strain of *X. c.* pv. *vesicatoria* was noted previously (11). Eleven of thirteen cultures were either copper-resistant and race 2 or copper-sensitive and race 1. The two remaining cultures were copper-resistant and race 1. When 25 additional strains were scored for copper resistance and race reaction, all strains of race 2 were resistant to copper and all race 1 strains were sensitive to copper (G. M. Marco and R. E. Stall, *unpublished*). Thus, only two of 38 cultures obtained from pepper plants from Florida were not consistent with an association of copper resistance and race 2 reaction.

Resistance to copper was reported in a culture of *Escherichia coli* obtained from pigs that were fed a copper-supplemented diet (15). Furthermore, the copper resistance was controlled by a self-transmissible plasmid. Resistance to zinc, but not to cadmium, cobalt, lead, mercury, or silver was conferred by plasmid pRJ1004 in *E. coli*.

The copper-resistant strains of *X. c.* pv. *vesicatoria* from Florida have a spectrum of heavy metal resistance that is similar to that reported for the strains of *E. coli* that have plasmid pRJ1004 (R. E. Stall, *unpublished*). Because of that similarity, it was decided to test the hypothesis that copper resistance in *X. c.* pv. *vesicatoria* is also controlled by a self-transmissible plasmid.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

MATERIALS AND METHODS

Bacterial strains. Eight strains of *X. c.* pv. *vesicatoria* were used in conjugation experiments. Four strains, XV E-3, XV 68-1, XV 81-23, and XV 83-3 were resistant to copper (Cu^R); three of these were race 2 of the pepper strain and strain XV 68-1 was race 1. Four other strains, XV 65-2, XV 69-1, XV 71-21, and XV 82-8 were sensitive to copper (Cu^S); three of these were race 1 and XV 65-2 was race 2. Nalidixic acid-resistant colonies (nal^R) of the Cu^S strains were selected and they maintained their original race designation.

Two other strains of *X. c.* pv. *vesicatoria*, XV 75-3 and XV 81-18, were Cu^R and were used in tests to compare plasmid mobility in agarose gels during electrophoresis. A Cu^S strain was selected from XV 81-18 and its plasmids were compared with those of the parent. The strain 4013 of *Agrobacterium tumefaciens* (supplied by B. J. Staskawicz of the Plant Pathology Department, University of California, Berkeley) was a source of the wild type of Ti plasmid, and was used as a standard in electrophoresis.

Media. All bacteria were cultured routinely on nutrient agar (NA) or in nutrient broth (NB). Copper resistance was determined by growth on NA amended with 200 μg of $CuSO_4 \cdot 5H_2O$ per milliliter. The sodium salt of nalidixic acid (Sigma Chemical Co., St. Louis, MO) at 75 μg/ml in NA was used for selection of nal^R colonies. Sometimes NA contained both copper and nalidixic acid (CuNal) at the concentrations that each were used alone. Streptomycin sulfate (Sigma Chemical Co.) in NA at 200 μg/ml was used to select for colonies resistant to streptomycin (str^R). All antibacterial agents were added to cooled media (55–65 C). Isolation of the Cu^S strain from the Cu^R strain was facilitated by culturing the resistant bacterium in nutrient-yeast extract-dextrose broth (NYDB) amended with mitomycin (Sigma Chemical Co.) at 0.187 μg/ml. When NYDB plus 1.5% agar (NYDA) was used to determine Cu^R, $CuSO_4 \cdot 5H_2O$ was added at 200 μg/ml.

Race determinations. Race 2 of *X. c.* pv. *vesicatoria* is distinguished from race 1 by the initiation of a hypersensitive reaction (HR) in leaves of pepper plants that contain the Bs_1 gene for resistance (2). To determine races, a single colony was transferred to 2 ml of NB, allowed to grow for 24–36 hr, centrifuged at 1,500 *g* for 5 min, and the pellet was resuspended in 2 ml of sterile

tap water. Each suspension was injected into about 2 cm^2 of a leaf of a plant of breeding line 10R with a hypodermic syringe and needle. The plants were then kept at 30 C and development of necrosis within 12 hr after inoculation was indicative of race 2. Necrosis after 48 hr was indicative of race 1 (13).

Mating procedures. A modification of the procedure described by Curtis (3) was used for conjugation experiments. Appropriate strains were cultured in 2 ml of NB until the cells were in the late log-phase of growth. Cultures of two strains were mixed and bacteria in 1 ml were collected on a 13-mm-diameter membrane filter with 0.8 μm pore size. The loaded filters were then transferred to plates of NA with an overlay of 1% water agar. After overnight matings (16 hr) the bacteria on the filters were immersed in 1 ml of NB and suspended by agitation with a Vortex mixer. The concentrations of parent strains and possible transconjugants were determined by making 10-fold dilutions and transferring 0.05 ml of each dilution onto appropriate media.

Selection for copper sensitivity. A suspension containing about 10^6 cells of the Cu^R strain XV 81-18 per milliliter was prepared in NYDB and treated with mitomycin. After culture overnight in complete darkness at room temperature (23 C), the bacteria were plated on NYDA to obtain discrete colonies. Colonies were then transferred to NYDA and NYDA with copper. Colonies that grew on the former medium and not the latter were isolated.

Agarose gel electrophoresis. The alkaline lysis procedure of Kado and Liu (10) was used to examine plasmid DNA. The cells in 1 ml of a cell suspension were lysed with 0.8 ml of the lysing buffer. The lysed cells were kept at 55 C for 15 min prior to chloroform-phenol extraction. Fifty microliters of the aqueous phase, separated from emulsion by centrifugation, was mixed with 5 μl of tracking dye. Twenty microliters of the DNA-dye mix was placed in a 1 × 6-mm well in 0.7% agarose for horizontal electrophoresis in a mini-cell system (Bio-Rad Laboratories, Richmond, CA). The agarose bed was 4 mm thick. Electrophoresis was accomplished at 100V in a 0.04 M tris-acetate buffer, pH 7.8. The gels were stained with ethidium bromide at 0.5 μg/ml, viewed, and photographed with short-wave ultraviolet illumination.

RESULTS

Conjugation experiments. Evidence was accumulated that copper resistance in strains of *X. c.* pv. *vesicatoria* was transferred to Cu^S strains by conjugation. After the four Cu^R nal^S strains were mated with the four Cu^S nal^R strains in all possible combinations, colonies developed on the CuNal medium with 10 of 16 dual combinations. The number of colonies with some combinations was too high to be accounted for by mutation to copper or nalidixic-acid resistance by one or the other bacterium. Similar treatment of the eight parents yielded no colonies on the CuNal medium.

The number of cells growing on the CuNal medium increased more rapidly during incubation of Cu^R nal^S with Cu^S nal^R bacteria than the total number of cells. The number of colonies that developed on the CuNal medium from the mating of XV 81-23 (Cu^R nal^S) with XV 82-8 (Cu^S nal^R) for 0, 6, 12, or 24 hr was 0, 1.8×10^4, 6.2×10^4, and 1.8×10^6, respectively. The number of colonies that grew on NA medium was 4.6×10^8, 7.8×10^8, 5.8×10^8, and 1.6×10^9, respectively. This meant that resistance to either copper or nalidixic acid increased more rapidly in culture than the growth of the bacteria.

Strain XV 81-23 also has natural resistance to streptomycin which facilitated determination of the donor and recipient in matings. Thirty-six colonies from a mating of XV 81-23 (Cu^R nal^S str^R) with XV 82-8 (Cu^S nal^R str^S) were transferred from the CuNal medium to the str medium. Colonies of each parent were also transferred to the str medium. Only the parent, XV 81-23, grew. Thus, the streptomycin-sensitive parent, XV 82-8, was the recipient of the Cu^R determinant.

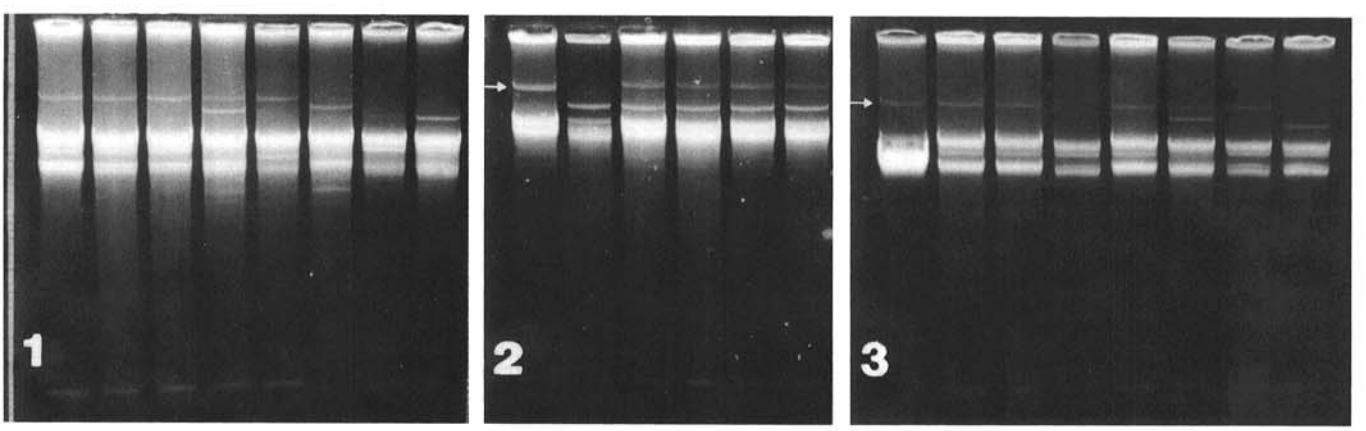
No colonies developed on the CuNal medium after cells of XV 69-1 nal^R, XV 71-2 nal^R, or XV 82-8 nal^R were incubated overnight in filter-sterilized supernatant of a culture of XV 81-23. Bacteria grew on the NA medium with all treatments. Incubating Cu^S strains with the filtrate of a Cu^R strain failed to transfer copper resistance.

TABLE 1. Frequency of conjugation of some strains of *Xanthomonas campestris* pv. *vesicatoria*

Donor[a]	Recipient[a]	Conjugation frequency[b]
E-3 (Cu^RHR^+)	82-8 (Cu^SHR^-)	0
68-1 (Cu^RHR^-)	82-8 (Cu^SHR^-)	1.6×10^{-2}
81-23 (Cu^RHR^+)	82-8 (Cu^SHR^-)	5.0×10^{-4}
E-3 (Cu^RHR^+)	71-21 (Cu^SHR^-)	1.0×10^{-8}
68-1 (Cu^RHR^-)	71-21 (Cu^SHR^-)	3.0×10^{-4}
81-23 (Cu^RHR^+)	71-21 (Cu^SHR^-)	1.3×10^{-5}
E-3 (Cu^RHR^+)	65-2 (Cu^SHR^+)	0
68-1 (Cu^RHR^-)	65-2 (Cu^SHR^+)	0
81-23 (Cu^RHR^+)	65-2 (Cu^SHR^+)	0

[a] All donors were sensitive to nalidixic acid (nal^S) and all recipients were resistant to nalidixic acid (nal^R). Symbols in parentheses refer to strain reaction to copper (Cu) and hypersensitivity (HR) in leaves of pepper having the Bs_1 gene for resistance.

[b] Transconjugants were selected on a medium amended with copper and nalidixic acid. Frequency is expressed as transconjugants per donor cell.



Figs. 1–3. 1, DNA in lysates of four copper-resistant strains (four lanes to the left) and four copper-sensitive strains (four lanes to the right) of *Xanthomonas campestris* pv. *vesicatoria*. From left to right, lanes contained lysates of strains XV E-3, XV 68-1, XV 81-23, XV 83-3, XV 65-2, XV 69- 1, XV 71-21, and XV 82-8. **2,** DNA in lysates of a copper-resistant, copper-sensitive, and transconjugant strains of *X. c.* pv. *vesicatoria*. From left to right, lanes contained lysates of strains XV 81-23 (Cu^R nal^S), XV 82-8 (Cu^S nal^R), and four randomly selected transconjugants. The largest plasmid (arrow) moved from the copper-resistant parent to the transconjugants. **3,** DNA in lysates of *Agrobacterium tumefaciens* strain 4013 and seven strains of *X. c.* pv. *vesicatoria*. From left to right, lanes contained lysates of strains AT 4013, XV E-3, XV 81-23, XV 75-3, XV 68-1, XV 83-3, XV 81-18, and XV 81-18 (Cu^S). The latter strain was selected from mitomycin-treated cells of XV 81-18 and was sensitive to copper. The size of the Ti plasmid (arrow) is approximately 193 kilobases.

This result apparently ruled against transformation or transduction as mechanisms for transfer of copper resistance. Conjugation seemed to be the mechanism for transfer of copper resistance, because physical contact of strains seemed to be essential.

The frequency of conjugation was determined with the same donors and recipients as in earlier experiments, excluding XV 83-3 as a donor and XV 69-1 nal^R as a recipient (Table 1). Cells of strains XV 81-23 and XV 68-1 were more frequent donors than were cells of strain XV E-3. Cells of strain XV 82-8 nal^R were the best recipients. Strain XV 65-2 nal^R did not function as a recipient in these conjugation experiments. This strain was unique among the copper-sensitive strains in that it reacted as race 2 rather than race 1.

Linkage of avirulence with copper resistance. Race 2 is determined by an avirulence gene in the pathogen. The linkage of avirulence with copper resistance was determined in matings of the donor, XV 81-23, which is of race 2 with XV 82-8 nal^R which is of race 1. Nine transconjugants, selected for copper resistance only, were tested along with the parents for avirulence in leaves of 10R pepper plants. All nine transconjugants and XV 81-23 were avirulent (race 2), In another test, all of 80 transconjugants and XV 81-23 were avirulent, even though the recipient, XV 82-8, was virulent. The avirulence was linked with copper resistance and transferred to the transconjugants.

In some tests, however, not all transconjugants were avirulent. The proportion of avirulent to virulent transconjugants was not constant between tests. The linkage apparently was broken during some conjugations.

Electrophoresis. The eight strains of *X. c.* pv. *vesicatoria* used in matings were examined for plasmids by agarose gel electrophoresis. Each of the four Cu^R strains had a large plasmid that migrated similarly in the gel (Fig. 1). Copper-sensitive strains contained smaller plasmids except for XV 65-2 nal^R, which also had the large plasmid. Strain XV 65-2 nal^R may indeed have the self-transmissible plasmid found in other race 2 strains, but with the copper-resistance gene absent or modified. The fact that XV 65-2 nal^R did not function as a recipient with copper-resistant strains supports that view.

The plasmid profiles of four transconjugants from the mating of XV 81-23 and XV 82-8 nal^R were compared with the plasmid profiles of the parents (Fig. 2). Each parent had one large plasmid, but their sizes differed. The transconjugants had two large plasmids. The largest plasmid in the transconjugants comigrated with the donor plasmid, XV 81-23. Thus, the largest plasmid in Cu^R strains appears to be the self-transmissible plasmid.

The size of the large self-transmissible plasmid (designated pXvCu) varied in some strains of *X. c. pv. vesicatoria* (Fig. 3). The plasmid in strains XV E-3, XV 81-23, XV 68-1, XV 83-3, and XV 81-18 was nearly the same size as the Ti plasmid in *A. tumefaciens* strain 4013, which is about 193 kilobases. The pXvCu plasmid in XV 75-3 is larger than the Ti plasmid, however.

One Cu^S strain, XV 81-18 Cu^S, was screened from 756 colonies of XV 81-18 after exposure to mitomycin. The Cu^S strain also lost the avirulence locus even though there was no selection for virulence. The comparison of the plasmids in the Cu^S strain and the parent revealed a smaller plasmid in the Cu^S mutant (Fig. 3). Possibly, both copper resistance and avirulence were lost simultaneously by DNA deletion.

DISCUSSION

The major contribution of this paper is the discovery of a self-transmissible plasmid, pXvCu, in some strains of *X. c.* pv. *vesicatoria*. The plasmid contains loci for copper resistance and avirulence to pepper plants that have the Bs_1 gene for resistance to bacterial spot. The discovery of the plasmid was dependent upon the identification of the copper-resistance marker on the plasmid which allowed selection in culture. Loci that confer resistance to copper have been reported for other plasmids (9,15), but plasmid-determined resistance to copper is not common in bacteria (7). Although an avirulence gene was recently cloned into a cosmid (14), to the authors knowledge, this is the first report of an avirulence gene naturally located on a plasmid in a plant pathogen. By contrast, in *A. tumefaciens*, virulence genes rather than avirulence genes are located on the Ti plasmid (16). The presence of the avirulence gene on the same plasmid as the copper resistance was not surprising, because race 2 and copper resistance were closely associated in wild-type strains.

Change of race 2 to race 1 occurs in culture at a very high frequency of 4×10^{-4} per cell per division (5). The change of race involves loss of avirulence which is dominant to virulence (12). A preliminary screening of strains of the two races for plasmids failed to associate loss of avirulence with loss of a plasmid (4). It was then assumed that loss of avirulence was due to point mutation (5). Evidence exists in this report, however, that avirulence may be lost by deletion rather than by point mutation. The size of the self-transmissible plasmid, pXvCu, is not consistent, which may mean that deletions do occur. Also, the loss of copper resistance and avirulence in a strain selected after mitomycin treatment was associated with the presence of a much smaller plasmid. Whether deletions, point mutations, or other genomic rearrangements account for the high frequency of change of avirulence can be determined by molecular analysis.

Transfer of the avirulence gene from bacterium to bacterium by conjugation is further evidence that resistance to bacterial spot in pepper is controlled by a gene-for-gene system (6). For resistance, a dominant gene, Bs_1 must be present in the host and can be transferred to progeny by classical breeding techniques (1). Also, for resistance a dominant gene for avirulence must be present in the pathogen (12), and in this case, can be transferred to a virulent bacterium by conjugation. The transconjugant is avirulent on the plant with the resistance gene.

The presence of the avirulence gene on a self-transmissible plasmid will add to the value of this system as a model for host-parasite studies. Host lines that are near-isogenic, but differ in resistance and susceptibility have been developed (5). Now, near-isogenic strains of the pathogen that are virulent and avirulent can be developed by transferring plasmid pXvCu. Thus, a host and parasite system that is relatively homogeneous, except for the genes for resistance and avirulence, can be developed .

LITERATURE CITED

1. Cook, A. A., and Stall, R. E. 1963. Inheritance of resistance in pepper to bacterial spot. Phytopathology 53:1060-1062.
2. Cook A. A., and Stall, R. E. 1969. Differentiation of pathotypes among isolates of *Xanthomonas vesicatoria*. Plant Dis. Rep. 53:617- 619.
3. Curtis, R. 1981. Gene transfer. Pages 243-265 in: Manual of Methods for General Bacteriology. E. W. Nester, ed. American Society of Microbiology, Washington, DC. 524 pp.
4. Dahlbeck, D., Pring, D. R., and Stall, R. E. 1977. Detection of covalently closed circular DNA's from *Xanthomonas vesicatoria*. (Abstr.) Proc. Am. Phytopathol. Soc. 4:176.
5. Dahlbeck, D., and Stall, R. E. 1979. Mutations for change of race in cultures of *Xanthomonas vesicatoria*. Phytopathology 69:634-636.
6. Flor, H. H. 1955. Host-parasite interactions in flax rust—Its genetics and other implications. Phytopathology 45:680-685.
7. Foster, T. J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. 47:361-409.
8. Higgins, B. B. 1922. The bacterial spot of pepper. Phytopathology 12:501-516.
9. Ishihara, M., Kamio, Y., and Terawaki, Y. 1978. Cupric ion resistance as a new genetic marker of a temperature-sensitive R plasmid, Rts 1, in *Escherichia coli*. Biochem. Biophys. Res. Commun. 82:74-80.
10. Kado, C. I., and Liu, S. T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.
11. Marco, G. M., and Stall, R. E. 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.
12. Stall, R. E., Bartz, J. A., and Cook, A. A. 1974. Decreased hypersensitivity to xanthomonads in pepper after inoculations with virulent cells of *Xanthomonas vesicatoria*. Phytopathology 64:731-735.
13. Stall, R. E., and Cook, A. A. 1966. Multiplication of *Xanthomonas vesicatoria* and lesion development in resistant and susceptible pepper. Phytopathology 56:1152-1154.

14. Staskawicz, B. J., Dahlbeck, D., and Keen, N. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.). Merr. Proc. Nat. Acad. Sci. USA 81:6024-6028.
15. Tetaz, T. J., and Luke, R. K. J. 1983. Plasmid-controlled resistance to copper in *Escherichia coli*. J. Bacteriol. 154:1263-1268.
16. Van Larebeke, N., Genetello, C., Schell, J., Schilperoot, R. A., Hermans, A. K., Hernalsteens, J. P., and van Montagu, M. 1975. Acquisition of tumor-inducing ability by non-oncogenic bacteria as a result of plasmid transfer. Nature (Lond.) 255:742-743.