Copper- and Streptomycin-Resistant Strains and Host Differentiated Races of *Xanthomonas campestris* pv. *vesicatoria* in North Carolina

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ABSTRACT

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Copper- and streptomycin-resistant strains of *Xanthomonas campestris* pv. *vesicatoria* were detected in diseased pepper and tomato plants. In surveys of 32 noncontiguous fields during four growing seasons, 63% of 70 strains were copper resistant and 30% were resistant to at least 100 μg/ml of streptomycin. All streptomycin-resistant strains were copper resistant. Strains of pepper races 1, 2, and 3, as well as strains from the tomato group, were detected. All tomato strains, all pepper race 2 strains, and 50% of pepper race 1 strains were copper resistant. Streptomycin resistance was detected only in the tomato strains and pepper race 2 strains. All strains of race 3 from the field were copper and streptomycin sensitive. Of 65 strains pathogenic to pepper (cv. Early Calwonder), 55, 31, and 12% were races 1, 2, and 3, respectively. A strain was isolated that was pathogenic on Early Calwonder (Xcv 19) but elicited a hypersensitive response in the three near-isogenic lines of Early Calwonder. It is proposed that this strain be designated race 0.

Xanthomonas campestris pv. *vesicatoria* (Doidge) Dye causes the economically important disease bacterial spot of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) (4,10). Copper is commonly used to control this disease, however, strains of this bacterium resistant to copper-containing compounds have been reported in Florida (13) and several other locations (1,3,8). Also, copper resistance occurs in several pathovars of *Pseudomonas syringae* van Hall (2,7,19) and some nonphytopathogenic bacteria (8,23). Streptomycin resistance in *X. c. vesicatoria* was observed in the late 1950s (21,22).

Three groups and several races of this pathogen can be differentiated using tomato, pepper cv. Early Calwonder (ECW), and three near-isogenic lines derived from ECW that contain the resistance genes *Bs1* (ECW-10R), *Bs2* (ECW-20R), and *Bs3* (ECW-30R) (15). Strains of the tomato group (XcvT) are pathogenic only on tomato, those in the pepper group (XcvP) only on pepper, and members of the pepper-tomato group (XcvPT) on both pepper and tomato (15). Strains in the XcvP and XcvPT groups can be further divided into races based on reaction on the near-isogenic lines of ECW (15).

Copper and streptomycin resistance and avirulence can be plasmid-encoded in strains of *X. c. vesicatoria* (3,8,14, 15,18,20). High frequencies of spontaneous changes of these phenotypes, especially of copper resistance and avirulence, have been reported in this pathogen and are attributed to the fact that the genes are plasmid-encoded (9,18,20).

The purpose of the research reported here was to determine whether copper- and streptomycin-resistant strains of *X. c. vesicatoria* were present in North Carolina, as well as what races of the pathogen were present, and to describe some of the characteristics of these strains.

MATERIALS AND METHODS

Bacterial strains. In 1986, 1987, 1989, and 1990, *X. c. vesicatoria* was isolated from infected pepper and tomato plants received in the Plant Disease and Insect Clinic at North Carolina State University in Raleigh or collected from diseased plants during field visitations. Survey emphasis was on pepper, and isolations from tomato were only incidental. Several lesions from each plant were macerated in 1 ml of sterile distilled water, and several loopfuls of the extraction mixture were streaked on YDC medium (10 g of yeast extract, 10 g of dextrose, 5 g of calcium carbonate, and 15 g of agar in 1 L of distilled water) and incubated at 28 C. After 36–72 hr, colonies of *X. c. vesicatoria* were selected based on morphology, color, and relative frequency of the colony type (16). Three to five colonies were pooled and restreaked on YDC. One or more single colonies from each plant sample were selected and tested for pathogenicity as described below. Cultures were stored in sterile distilled water at 4 C and in 20% glycerol at −70 C until used for experiments.

Copper and streptomycin assays. Sensitivity was tested on SPA medium (20 g of sucrose, 5 g of peptone, 0.5 g of dibasic potassium phosphate, 0.25 g of magnesium sulfate, and 15 g of agar in 1 L of distilled water) amended with appropriate chemicals. Fresh stock solutions of copper (cupric sulfate, Sigma, St. Louis, MO) and streptomycin (streptomycin sulfate, Sigma) were prepared in sterile distilled water, filter-sterilized (0.22-μm pore size), and appropriate concentrations were added to SPA after autoclaving and cooling to 45 C before pouring into petri plates. In media containing copper, pH was adjusted with 2 N NaOH to 7 before autoclaving and again checked before sensitivity assays were done (11). Bacterial cultures were grown on YDC or SPA for 36–72 hr, suspended in sterile distilled water, and the concentration was adjusted to 10^8 colony-forming units (cfu) per milliliter. One to three microliters of the suspension was spotted on SPA and SPA amended with 200 μg/ml of cupric sulfate or different concentrations of streptomycin sulfate (20–500 μg/ml). Plates were incubated for 36–48 hr at 28 C, and the presence or absence of growth was recorded. Bacteria that grew on SPA amended with the cupric sulfate or 20 μg/ml or more of streptomycin sulfate were considered resistant to copper or streptomycin, respectively.

Determination of bacterial races. Bacteria were grown on YDC for 36–48 hr, suspended in sterile distilled water to 10^9 cfu/ml, and infiltrated into leaves of ECW, ECW10R, ECW20R, and ECW30R as previously described (15). Plants were incubated in the lab at 22–24 C under diurnal light. Under these conditions, tissue collapse within 12–36 hr indicated a hypersensitive reaction, whereas the development of water-soaked, chlorotic lesions after 3–5 days indicated a susceptible reaction. Strains that caused a hypersensitive reaction in ECW and the near-isogenic lines of ECW

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were tested for pathogenicity on tomato (cv. Rutgers) by inoculating leaves with cotton swabs saturated with bacterial suspensions (10^9 cfu/ml). Plants were incubated in clear plastic bags for 3 days and maintained on the greenhouse bench.

RESULTS

Strains and sensitivity to copper and streptomycin. Seventy strains of *X. c. vesicatoria* were obtained during the four survey years. Forty-four strains (63%) grew on SPA amended with 200 μg/ml of cupric sulfate (Table 1). Twenty-one strains (30%) were resistant to streptomycin sulfate (Table 1). Sensitivity to different concentrations of streptomycin was detected. Sensitive strains failed to grow on media amended with 20 μg/ml, 21 strains grew on 100 μg/ml, 15 grew on 200 μg/ml, and three grew on 300 μg/ml or more. All streptomycin-resistant strains were copper resistant but not all copper-resistant strains were streptomycin resistant (Table 1).

Races detected. Strains of the tomato group (XcvT) and pepper races 1, 2, and 3 were detected in North Carolina (Table 2). Thirty-six strains were pepper race 1, 20 were pepper race 2, eight were pepper race 3, and five were of the tomato group. One strain (Xcv 19) isolated in 1987, produced a hypersensitive response in the three near-isogenic lines of ECW and a susceptible reaction in ECW (Table 2). Only the XcvT group and pepper race 2 strains were detected in 1986. In 1987, pepper races 1 and 2 were detected. All three pepper races were detected in 1989 and 1990.

Races and sensitivity to copper and streptomycin. All 20 strains of pepper race 2, strain Xcv 19, and the five strains of the XcvT group grew on SPA amended with 200 μg/ml of cupric sulfate (Table 1). Copper resistance also was detected in 50% of the strains of pepper race 1 but not in strains of race 3. The five strains of XcvT grew on SPA amended with 200 μg/ml of streptomycin sulfate but not on SPA amended with 300 μg/ml. Of the pepper strains, streptomycin resistance was detected only in race 2. No streptomycin- or copper-resistant strains of race 3 were detected in the strains isolated from the field.

DISCUSSION

Copper resistance in *X. c. vesicatoria* has been reported in California (8), Florida (13), northwestern Mexico (1), and Oklahoma (3). In our study, copper resistance was detected in all strains of the XcvT group, all strains of pepper race 2, and 50% of the strains of pepper race 1 (Table 1). These results are similar to those reported by Marco and Stall in 1983 in which race 2 and some race 1 strains from Florida were copper resistant (13).

Streptomycin resistance in this pathogen was observed in the 1950s (21). Thayer and Stall also observed that strains of *X. c. vesicatoria* were sensitive to different concentrations of streptomycin (21,22). In our study, 30% of the strains were streptomycin resistant (Table 1) and exhibited sensitivity to several concentrations of streptomycin. All streptomycin-resistant strains were copper resistant, but no copper-sensitive strains were streptomycin resistant. This suggests that streptomycin resistance in these strains of *X. c. vesicatoria* may be linked to copper resistance. In *Staphylococcus aureus* Rosenbach, resistance to

Table 1. Number of strains of *Xanthomonas campestris* pv. *vesicatoria* isolated from pepper and tomato plants in North Carolina in 1986, 1987, 1989, and 1990 and sensitivity to copper and streptomycin sulfate

Year	Host	Number of fields	Number of strains	Race	Copper resistant	Copper sensitive	Streptomycin concentration (μg/ml)			Copper and streptomycin resistant
							100	200	>300	
1986	Tomato	1	5	XcvT[a]	5	0	5	5	0	5
	Pepper	1	2	2	2	0	2	2	0	2
	Pepper	1	5	2	5	0	5	0	0	5
Total			12		12	0	12	7	0	12
1987	Pepper	1	2	1	0	2	0	0	0	0
	Pepper	2	7	1	0	7	0	0	0	0
	Pepper	1	2	1	0	2	0	0	0	0
	Pepper	3	4	2	4	0	2	1	1	2
	Pepper	1	2	2	2	0	0	0	0	0
	Pepper	1	1	0[b]	1	0	0	0	0	0
Total			18		7	11	2	1	1	2
1989	Pepper	8	15	1	15	0	0	0	0	0
	Pepper	2	6	1	0	6	0	0	0	0
	Tomato	1	1	2	1	0	1	1	1	1
	Pepper	1	3	2	3	0	3	3	0	3
	Pepper	1	4	3	0	4	0	0	0	0
	Pepper	1	1	3	0	1	0	0	0	0
Total			30		19	11	4	4	1	4
1990	Pepper	1	2	1	2	0	0	0	0	0
	Pepper	1	1	1	1	0	0	0	0	0
	Pepper	1	1	1	0	1	0	0	0	0
	Pepper	1	2	2	2	0	2	2	0	2
	Tomato	1	1	2	1	0	1	1	1	1
	Pepper	1	3	3	0	3	0	0	0	0
Total			10		6	4	3	3	1	3
4-yr totals			70		44	26	21	15	3	21
Reference strains from R. E. Stall[c]										
	Pepper	Xcv 2 (82-8)		1	−	+	−	−	−	−
	Pepper	Xcv 3 (E3)		2	+	−	+	+	+	+
	Pepper	Xcv 4 (81-23)		2	+	−	−	−	−	−

[a] XcvT indicates *Xanthomonas campestris* pv. *vesicatoria* tomato group.
[b] This strain (Xcv 19) caused a hypersensitive reaction in ECW10R, ECW20R, and ECW30R and a susceptible reaction in ECW.
[c] + = Positive reaction for the characteristic evaluated, − = negative reaction for the evaluated characteristic.

Table 2. Source, number, and reaction of strains of *Xanthomonas campestris* pv. *vesicatoria* on Early Calwonder (ECW) and three near-isogenic lines of ECW pepper

Year isolated	Host	Number of fields	Number of strains	Reaction in pepper leaves[a]				Race
				ECW	ECW10R	ECW20R	ECW30R	
1986	Tomato	1	5	HR	HR	HR	HR	XcvT[b]
	Pepper	2	7	+	HR	HR	+	2
1987	Pepper	4	6	+	HR	HR	+	2
	Pepper	4	11	+	+	HR	HR	1
	Pepper	1	1[c]	+	HR	HR	HR	0
1989	Tomato	1	1	+	HR	HR	+	2
	Pepper	10	21	+	+	HR	HR	1
	Pepper	2	3	+	HR	HR	+	2
	Pepper	2	5	+	+	HR	+	3
1990	Tomato	1	1	+	HR	HR	+	2
	Pepper	1	2	+	HR	HR	+	2
	Pepper	1	3	+	+	HR	+	3
	Pepper	2	4	+	+	HR	HR	1
Reference strains from R. E. Stall								
	Pepper	Xcv 2 (82-8)		+	+	HR	HR	1
	Pepper	Xcv 3 (E3)		+	HR	HR	+	2
	Pepper	Xcv 4 (81-23)		+	HR	HR	+	2

[a] HR = Hypersensitive reaction within 12–36 hr, + = a susceptible reaction within 3–5 days after leaf infiltration.
[b] XcvT indicates *Xanthomonas campestris* pv. *vesicatoria* tomato group.
[c] Strain Xcv 19.

the metal cadmium is linked to penicillin and mercury resistance (17). However, if there is linkage in *X. c. vesicatoria*, it is not complete because a strain (Xcv 43) isolated as race 2 and found to be copper and streptomycin resistant, in the laboratory spontaneously changed to copper sensitive and race 3 while retaining streptomycin resistance (D. F. Ritchie, *unpublished*). The results of the spontaneous change in strain Xcv 43 further substantiates, at least in race 2, that copper resistance and the avirulence gene *avrBs1* are linked (18).

Pepper races 1, 2, and 3 and the tomato group of *X. c. vesicatoria* were detected in North Carolina. Although pepper race 1 was detected in more fields and represented 55% of the pepper strains isolated, race 2 was detected in all four survey years, whereas race 3 was detected only in 1989 and 1990. Our detection of all three pepper races in 1989 and 1990 may have resulted from more intensive surveys conducted in these 2 yr than in 1986 and 1987. Thus, in most years, all three races are likely to occur in North Carolina. No copper- or streptomycin-resistant strains of pepper race 3 were detected in the field (Table 1). However, strains of race 3 were also the least frequently detected, and this conclusion is based on only eight strains from 2 yr.

Strain Xcv 19, isolated from pepper in 1987, does not conform to profiles of current races using ECW and the three near-isogenic lines of ECW (Tables 1 and 2) (15). The fact that it causes a hypersensitive response in the three near-isogenic lines of ECW currently would not be a disadvantage for pathogenicity in North Carolina where almost all commercially grown pepper cultivars do not contain genes for resistance to *X. c. vesicatoria* (D. F. Ritchie, *unpublished*). Strain Xcv 19 appears to be similar to strains constructed by introducing the *avrBs3* gene into race 2 strain 81-23 (15). Also, we have been able to select a strain similar to Xcv 19 by culturing, on SPA, a copper-resistant strain of race 2 with a copper-sensitive strain of race 1 and selecting for copper-resistant race 1 strains (D. F. Ritchie, *unpublished*). This suggests the potential for a strain such as Xcv 19 to evolve if races 1 and 2 occurred in the same area. We propose that strain Xcv 19 and other strains causing similar reactions in ECW and the near-isogenic lines of ECW be designated race 0.

Recently, pepper races 1, 2, and 3 were reported in Taiwan as components of the natural bacterial population (12). In 1982, races 1 and 2 were the only two races reported in the United States with race 2 considered to be limited to Florida (6). Races and strains of *X. c. vesicatoria* may now be more widespread or can easily be distributed more widely than previously thought. Overwintering populations of *X. c. vesicatoria* in North Carolina fields are not considered a major source of primary inoculum. Primary inoculum is considered to enter North Carolina fields on contaminated seed and transplants. Thus, it is not unexpected that different strains were detected in our survey. Races and strains detected in a given area would likely be influenced by the races and strains present in the areas where seed and/or transplants are produced. Thus, there is the potential for all races and strains to occur wherever peppers and tomatoes are grown and seed and/or transplants are imported. Minsavage et al (15) suggested that peppers should have multiple genes for resistance to *X. c. vesicatoria* because each of the three currently known avirulence genes is subject to loss of activity. Additionally, as reported here, all races of this pathogen have the potential to occur in any pepper-growing area where the environment is favorable for bacterial spot. If any or all races contaminate pepper transplants or seed, multiple genes for resistance are essential if bacterial spot is to be reliably controlled by host resistance.

The relatively high frequency of detection of copper- and streptomycin-resistant strains and the occurrence of all three races on pepper has a negative impact on disease management strategies and indicates the importance of having pathogen-free seed and transplants. The apparent widespread occurrence of copper and streptomycin resistance is further compounded by the possible loss of the use of maneb and mancozeb, which enhance the effectiveness of copper for the management of both copper-sensitive and especially resistant strains of this pathogen (5,13).

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