Mutations for Change of Race in Cultures of *Xanthomonas vesicatoria*

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ABSTRACT

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Suspensions of *Xanthomonas vesicatoria* at approximately 10^4 cells per milliliter were infiltrated into the mesophyll of pepper (*Capsicum annuum*) leaves that reacted hypersensitively to the bacterium. Mutants in the inoculum produced lesions after 3–4 wk of incubation and bacteria from those lesions produced a susceptible reaction in the leaves. With fluctuation analysis experiments it was determined that these mutations occurred randomly in nutrient broth cultures at an apparent rate of 4×10^{-4} mutants per cell per division. Within the populations of *X. vesicatoria* mutations occurred that converted the tomato race 1 to pepper race 2 and pepper race 2 to pepper race 1. No mutational conversion was observed from tomato race 1 to pepper race 1. It was postulated that the latter only would occur after a double mutation. The high rate of mutation for change of race did not occur with change in resistance to streptomycin. The latter occurred at an apparent rate of 1.9×10^{-9} mutants per cell per division.

Additional key words: bacterial genetics, *Capsicum annuum*, hypersensitivity, *Xanthomonas vesicatoria*.

Three races of *Xanthomonas vesicatoria* (Doidge) Dows have been distinguished by hypersensitive or susceptible symptoms in leaves of tomato (*Lycopersicon esculentum*) plants and of differential cultivars of pepper (*Capsicum annuum*) (3). All tomato cultivars were susceptible to isolates representing all three pathogenic groups of *X. vesicatoria*. All pepper cultivars were hypersensitive to isolates of group 1. The isolates of group 2 induced a hypersensitive reaction in pepper lines derived from PI 163192 (2) and a susceptible reaction in other pepper cultivars. All pepper cultivars were susceptible to isolates included in group 3. Isolates in group 1 were designated race 1 of the tomato strain, isolates in group 2 were designated as race 2 of the tomato strain, and the group 3 isolates were designated as race 1 of the pepper strain.

Only race 1 of the tomato strain and race 2 of the pepper strain have been isolated from commercial plantings of tomato and pepper in Florida. However, when pepper lines with the single-gene resistance to *X. vesicatoria* from PI 163192 were planted in commercial plantings of pepper, lesions with pepper race 1 types of bacteria developed on the plants within one season. Thus, race 1 of the pepper strain developed very rapidly under natural conditions.

Schnathorst (8) reported that cells of race 2 of *X. malvacearum* were isolated from cotton (*Gossypium hirsutum*) plants carrying a single major gene for resistance to race 1 and inoculated with the latter race. Transformation of bacteria in the host was suggested by Schnathorst as a possible explanation for the changes involved because uptake and incorporation of certain host antigens by cells of *X. malvacearum* might have occurred. *X. malvacearum* and *G. hirsutum* share some antigenic components (9).

The suggestion by Schnathorst and the apparent rapid development of race 1 of *X. vesicatoria* in the field revived our interest in the effect of the host on development of new races of bacterial pathogens. The purpose of this research was to measure the rate of change of race within populations of *X. vesicatoria* and to assess the role of the host plant in the change.

MATERIALS AND METHODS

Test plants. Plants used in these experiments were of pepper (*C. annuum* L.) cultivars Yolo Wonder 43 (YW) and Early Cal Wonder (ECW) (susceptible to races 1 and 2 of the pepper strain and hypersensitive to race 1 of the tomato strain) and the experimental lines 23-1 and 10R (hypersensitive to race 1 of the tomato strain and race 2 of the pepper strain, but susceptible to race 1 of the pepper strain). Lines 23-1 and 10R contain the single gene for hypersensitivity from PI 163192 and the latter cultivar is nearly isogenic to ECW. Plants were grown in steamed soil in a greenhouse bench.

Inoculum. The bacteria were cultured in nutrient broth, centrifuged from the medium at 3,000 *g* for 10 min, and resuspended in double-distilled water. The suspensions of bacteria were adjusted to OD 600 nm = 0.3 (approximately 10^8 cells per milliliter) in a Spectronic 20 spectrophotometer. Other concentrations of bacteria were prepared by dilution and those below 10^7 cells per milliliter were suspended in 0.85% buffered saline.

Pure culture technique. To avoid the use of mixed cultures in the experiments, a single colony from each isolate was selected after four successive subculturings. At each subculturing, a colony was selected from a plate with 10–100 colonies, transferred to nutrient broth, and dilutions were plated.

Growth media. Three media were prepared to check for mutagenicity of the media on isolate E-3. These media were nutrient broth (Difco), mineral broth (0.1% $NH_4H_2PO_4$, 0.02% KC1, 0.02% $MgSO_4 \cdot 7H_2O$, 1.0% glucose, and 0.1% yeast extract), and lima bean broth (100 g of ground frozen lima beans in 300 ml of H_2O were steamed for 15 min, filtered, from the broth with cheese cloth, and the broth was brought to a volume of 1 L).

Inoculation. The technique described by Klement (5) was used to infiltrate the intercellular spaces of leaves with inoculum. In some cases, whole leaves were inoculated and in others only half-leaves were used. In the latter, different bacterial suspensions were infiltrated into the two halves of a leaf. Care was taken to avoid infiltration past the midvein.

Lesion counts. Lesions in leaves were counted 3–4 wk after inoculation. In addition, the infiltrated areas of the leaves were determined by the dot-counting method (7). From these data, the number of lesions per 10 cm^2 of leaf area (MLSA) was calculated.

Isolation of pathogen from lesions. A section of leaf tissue containing a lesion about 1 mm in diameter was placed in 0.5 ml of buffered saline and ground with a sterile glass rod. The resulting suspension was streaked onto nutrient agar plates. Isolations were checked for resistance to streptomycin by placing onto the streaked plates a disk (6 mm in diameter) which was previously soaked in 100 μg/ml of streptomycin sulfate (Merck and Co., Rahway, NJ 07065).

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Dilution plating. Tenfold dilutions were made by transferring 0.05 ml of a bacterial suspension with a diluter (Cooke Engineering Co., Alexandria, VA 22313) to 0.45 ml of 0.85% buffered saline. After serial transfers, appropriate dilutions were poured onto plates of nutrient agar and spread over the surface with a glass rod.

Antibiotic resistance. Sensitivity to 27 antibiotics was tested on lawns of isolates on nutrient agar. The test antibiotics were distributed in Multidisks® (Consolidated Laboratories, Inc., Glenwood, IL 60425).

RESULTS

Cultures from a single colony of a race 2 isolate, E-3, were placed in water suspension and adjusted to $\sim 10^4$ cells per milliliter and the suspensions were infiltrated into leaves of 10R plants. Although the 10R leaves reacted hypersensitively to E-3, that inoculum concentration produced no confluent necrosis and the leaves remained visibly healthy. Four weeks after the inoculation date, a few lesions were noticed in the infiltrated leaves and bacteria from those lesions were found to be of the race 1 type. Because a relatively low number of bacteria were infiltrated into the leaves, it was postulated that a very high spontaneous rate of change of race had occurred.

Fluctuation analysis. A fluctuation analysis experiment patterned after that of Luria and Delbruck (6) was undertaken to determine whether changes of race occurred in the culture medium or in the leaf. Isolate E-3 was used again because it contained a streptomycin-resistance marker that enabled differentiation from extraneous latent bacteria in the leaves.

Seventy cultures were started with the addition of an average of 20 cells per milliliter per tube. After incubation, a suspension of each culture was adjusted to about 0.8×10^4 cells per milliliter. Each suspension was infiltrated into half of one leaf. Half of the suspensions were infiltrated into leaves of 10R and the other half were infiltrated into leaves of 23-1. The other half of each leaf was infiltrated with a standard suspension containing a mixture of the remainder of each of the 70 suspensions. The standard suspension also was infiltrated into three whole leaves of YW, which is susceptible to the isolate.

Four weeks after inoculation, lesion counts on the leaves of YW averaged 730 lesions per MLSA. The assumption was made that 730 bacterial cells had been infiltrated into MLSA because the number of lesions should be directly proportional to the number of cells that entered the leaf. A pepper leaf averages 0.25 mm thick and 33% of leaf volume is intercellular space. (The latter value was obtained by comparisons of weights of equal areas of water-infiltrated and noninfiltrated leaves.) On that basis, an inoculum containing 0.8×10^4 cells per milliliter would provide 800 cells per MLSA.

Lesions developed in leaves of both 10R and 23-1 and the mean number per MLSA with the test cultures and the standard culture were similar for both cultivars. However, the fluctuation of lesion numbers among the half leaves was much larger for the test cultures than for the standard culture (Table 1). Thus, the calculated variances were greater for the test cultures than for the standard culture. These data supported the conclusion that the race changes occurred in the cultures prior to inoculation into the leaves.

The bacteria isolated from lesions in leaves of 10R and 23-1 caused a susceptible reaction in leaves of 10R, as is characteristic of race 1. The race of the isolated bacteria did not revert through five successive subcultures. The isolates were resistant to streptomycin, the colony type of the bacteria was indistinguishable from that of the parent colony type, and susceptibility or resistance to 27 test antibiotics of the isolated race 1 type and parent race 2 type cultures was unchanged.

Effect of medium. Isolate E-3 was cultured in three media to test for possible mutagenic effects of nutrient broth. Fifteen cultures in each medium (nutrient broth [NB], mineral broth [MB], and lima bean broth [LBB]), were seeded with an average of 19 cells per milliliter per culture. After incubation and growth, suspensions with an average of 1.8×10^4 cells per milliliter from each culture were prepared and each was infiltrated into a single half-leaf of a 10R plant. A standard suspension, obtained by combining the remainder of the suspensions from cultures in nutrient broth, was infiltrated into the other half of each half-leaf and in three whole leaves of ECW plants.

The lesion counts from ECW leaves averaged 1,280 per MLSA. The mean number of lesions per MLSA that developed in 10R leaves for test cultures in NB, MB, and LBB were 5.6, 2.0, and 2.4, respectively. The mean number of lesions per MLSA with the standard inoculum on leaves that had received the three types of test cultures were 4.2, 4.1, and 5.1, respectively. Twelve isolates from the 10R leaves were resistant to streptomycin and were of race 1 type. Thus, change of race occurred in all three media.

Effect of isolate. Fifteen cultures of each of three isolates (E-3, 69-20, and 76-4), which represented race 2 of the pepper strain, were started in nutrient broth by transferring an average of 20 cells per milliliter to each tube. A suspension from each culture was adjusted to 10^4 cells per milliliter and each was infiltrated into a whole leaf of a 10R plant. Lesions containing bacteria of race 1 type developed in 10R leaves inoculated with each isolate. The mean number of lesions per MLSA was 2.4, 9.2, and 1.8 for isolate E-3, 69-20, and 76-4, respectively. Thus, the high rate of change of race occurred in isolates other than E-3.

Change of tomato race 1 to pepper race 2 and pepper race 1. Fifteen cultures of isolate 75-1 (tomato race 1) were started in nutrient broth. After suspensions of each culture were adjusted to 10^4 cells per milliliter, a whole leaf of 10R and YW plants and a leaflet of a Bonny Best tomato plant were infiltrated with each suspension. Confluent necrosis developed in the inoculated tomato leaflets and lesions could not be counted. A mean of 1.8 lesions per MLSA developed in leaves of YW. No lesions were observed in leaves of 10R plants. Isolates from lesions of YW were characterized as pepper race 2.

This experiment was repeated with isolate 75-1 which caused a mean of 16.3 lesions per MLSA in leaves of YW and no lesions in leaves of 10R plants. With isolate 76-3 (another isolate of the tomato race 1) a mean of 11.5 lesions per MLSA was obtained in leaves of YW but none in leaves of 10R plants. All isolates from lesions in these latter tests were characterized as pepper race 2.

Changes of tomato race 1 to pepper race 2 occurred at about the same frequency as those of pepper race 2 to pepper race 1. However, changes of tomato race 1 to pepper race 1 were never detected.

Estimation of rate of change of race. The frequency of change per cell division from race 2 to race 1 of the pepper strain for isolate E-3 was estimated from data of the fluctuation analysis experiment by the procedure of Luria and Delbruck (6). The change of cells of the isolate E-3 from race 2 to race 1 was estimated at 4×10^{-4} changes per cell per division.

Estimation of mutation rate for streptomycin resistance. The mutation rate for streptomycin resistance was determined for an isolate of *X. vesicatoria* from a fluctuation analysis experiment. One hundred and ten NB cultures were started by adding 10^3 cells per milliliter to each tube. When the cultures reached 7.4×10^8 cells per milliliter, 1 ml from each culture was transferred to plates of nutrient agar containing 500 ug/ml of streptomycin sulfate. Colonies of bacteria that were resistant to streptomycin developed on 14 plates. The apparent mutation rate was 1.9×10^{-9} per cell per division.

TABLE 1. The fluctuation, mean, and variance of mutations for change of pepper race 1 to pepper race 2 of *Xanthomonas vesticatoria* detected with two pepper lines

Factor	Line 10R[b]		Line 23-1[c]	
	Test[d]	Standard[d]	Test	Standard
Fluctuation (lesions/MLSA[a])	0.0–30.5	0.2–3.5	0.0–56.1	0.0–2.1
Mean (lesions/MLSA)	1.7	0.9	2.6	1.6
Variance	31.7	0.3	87.8	0.7

[a]MLSA = 10 cm^2 of leaf surface area.
[b]Of lesions in 34 leaves.
[c]Of lesions in 31 leaves.
[d]Test = half-leaves inoculated with different cultures of isolate E-3 of *Xanthomonas vesticatoria* , and standard = the other half of the same leaves inoculated with a mixture of all cultures.

DISCUSSION

The suggestion of Schnathorst (8) that pathogenicity of bacterial cells might be transformed in leaves by uptake of host factors seemed a plausible explanation for the rapid nullification of resistance to *X. vesicatoria* in pepper. Although transformations of that kind cannot be discounted, results of the fluctuation analysis experiments in this research strongly support the hypothesis that race changes of *X. vesicatoria* were spontaneous and occurred in the cultures prior to inoculation rather than in the leaf. Therefore, the role of the host was to selectively reproduce the low numbers of cells that had changed race.

The estimated mutation frequency of 4×10^{-4} per cell per division was based on the number of lesions that developed in the leaf tissue. It is not known, however, if each mutant cell in the inoculum produced a lesion. Also, some multiplication of bacteria occurs in the leaf after inoculation of low numbers of bacteria before cessation of growth in the HR (10). The latter was not taken into account in calculations of the frequency of change of race.

The changes in race of cells in the cultures may be the result of phenomena other than classical mutation; however, the assumption was made that the changes were mutational events. An apparent mutation frequency of 4×10^{-4} per cell per division is very high. Rates of 1×10^{-4} per cell per division for loss of pigmentation of *Serratia marcescens* and 1×10^{-5} per cell per division for radiation resistance in *Escherichia coli* have been reported (1). Thus, even though the rate of change of race in *X. vesicatoria* is high, it is within the range for mutation in other bacteria. The apparent rate of 1.9×10^{-9} per cell per division for streptomycin resistance of an isolate of *X. vesicatoria* was low and nearer the expected rate (1). Therefore, the high mutation rate for change of race in *X. vesicatoria* does not apply to some of its other characteristics.

Change of race in *X. vesicatoria* was not associated with a change in colony type, which was noted by Schnathorst (8) in *X. malvacearum*. The lack of correlation of change of race with gain or loss of resistance to the antibiotics may mean that race change is not associated with loss of a plasmid. However, a plasmid association with change of race is under investigation (4).

The fact that pathogenic mutants in cultures of race 1 of the tomato strain of *X. vesicatoria* were not detected in leaves of 10R plants, but were detected in leaves of Yolo Wonder 43, may be related to the number of resistance genes in the plants. The frequency of 4×10^{-4} per cell per division for change of race was detected with plants that had a single-gene resistance factor. Nearly the same frequency of change of race was detected after cells of race 1 of the tomato strain were injected into Yolo Wonder 43 and Early Cal Wonder pepper leaves. This may mean that most pepper cultivars have a single gene for resistance to the tomato strain of the pathogen. If that assumption is made, then the cultivars 10R and 23-1 may have two genes for resistance to the tomato strain; ie, the common gene in pepper and the gene from PI 163192. Under those conditions, a double mutation would have to occur for pathogenic mutants to 10R to occur among cells of the tomato strain. The rate for a double mutant should be near 1.6×10^{-9} per cell per division if each mutation was independent. At that frequency, double mutants could not be detected by the techniques employed in our experiments.

The sequence of change from tomato race 1, to pepper race 2, to pepper race 1 also is consistent with the race pattern observed in the field in Florida. All of our field isolates were pathogenic to tomato regardless of their origin. Only a few isolates from tomato, however, were pathogenic to pepper. It is tempting to speculate that those isolates from tomato that were pathogenic to pepper were disseminated to tomato from diseased pepper plants. All isolates of pepper race 1 obtained from nature were pathogenic to tomato and all pepper cultivars. Thus genes for virulence in bacteria seem to be cumulative in nature.

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