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THE RELATIONSHIP OF PATHOGENICITY IN PSEUDOMONAS SOLANACEARUM TO COLONY APPEARANCE ON A TETRAZOLIUM MEDIUM[1]

Arthur Kelman

SUMMARY

Mutants of *Pseudomonas solanacearum* differing in colony morphology from the normal or wild type were detected readily when bacterial suspensions from stock cultures were streaked on a medium containing tetrazolium chloride and examined with obliquely transmitted light. The most common mutant formed a round, butyrous, deep red colony with a narrow bluish border. In contrast, the normal or wild type formed an irregularly-round, fluidal, white colony with a pink center. Inoculation tests on tomato seedlings demonstrated that colony appearance on the tetrazolium medium could be related to pathogenicity. Cultures derived from butyrous red colonies were either weakly pathogenic or non-pathogenic, whereas cultures from fluidal white colonies with pink centers were highly pathogenic.

In connection with a study of colony morphology in isolates of *Pseudomonas solanacearum* E.F.S., causal agent of southern bacterial wilt, 3 main colony types were observed. Colonies of the normal or wild type, which characterize fresh isolates obtained from diseased plants, were irregularly-round, fluidal, and opaque. In contrast, the mutant type most frequently encountered in stock cultures formed uniformly round, butyrous, and translucent colonies. In both types the colony surface was smooth. A third type that formed butyrous translucent colonies with a rough surface was observed infrequently. These colony types were similar to certain of those described by Okabe (6, 7) in isolates of *P. solanacearum* obtained in Formosa and Japan.

Although the main colony type in a given isolate could be determined by examination of streaked plate cultures with a dissecting microscope, detection of small numbers of variants in the wild type culture was often difficult. In order to facilitate a study of variation in *P. solanacearum* it was apparent that a medium was needed that would aid in the differentiation of colony types. Tetrazolium chloride had been used in solid media for detection of certain biochemical mutants in *Escherichia coli* (4) and for detection of morphological variants in *Pasteurella pestis* (5) and certain other bacteria. The investigations reported in this paper were initiated to determine 1) whether differences among colony types would be accentuated on a medium containing tetrazolium and 2) whether pathogenicity to tomato of a given colony type could be correlated with its appearance on a tetrazolium medium. A preliminary report on certain phases of these studies has been presented (2).

EXPERIMENTAL METHODS.—Dilute bacterial suspensions were streaked on a medium of the following composition: 1.0 per cent peptone, 0.1 per cent casein hydrolysate (Difco), 0.5 per cent glucose, 1.7 per cent agar, and 0.005 per cent triphenyl tetrazolium chloride in 1 liter of distilled water. Proper aliquots of a sterile 1 per cent aqueous solution of tetrazolium were added

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aseptically to the melted medium to obtain the desired concentration prior to pouring the plates. The streaked plates were incubated at 32°C. for 36 hours and then examined under a dissecting microscope with obliquely transmitted light, following the procedure described by Henry (1).

Bacterial suspensions were prepared from stock cultures which had been stored under sterile mineral oil for periods ranging from 6½ years to 6 months. The method used in preparing these cultures for storage has been reported previously (3). The cultures used represented isolates from diseased potato, tobacco, tomato, peanut and pepper plants collected in different areas in North Carolina. Tomato plants of either the Bonny Best or the Rutgers variety were inoculated by placing a drop of bacterial suspension at the third node below the stem tip and forcing a needle into the stem through the drop. At the time of inoculation plants were usually 14-16 in. in height with 6-7 expanded leaves. Disease readings based on a numerical rating scale previously described (6) were taken at 7 day intervals.

Single cell isolations were made with the Chambers micromanipulator. Growth of single cells was obtained in a sterile medium of the following composition: 1.0 per cent tryptone, and 0.5 per cent glucose in 1 liter of distilled water.

EXPERIMENTAL RESULTS.—In all cultures, marked differences in the intensity of red coloration were evident between colonies of wild and variant types. Colonies of the wild type were either entirely white or white with a light red area in the center of the colony, whereas the colonies of mutant types were deep red with a very narrow light bluish border. These differences were most apparent when plates were inverted and the undersides of colonies examined. In cultures containing small mutant populations the colonies of mutants could be detected as tiny red circular spots, even when completely surrounded by the coalescing fluidal white growth of the wild type. Differences in color intensity between colonies decreased after 72 hours.

In order to confirm the assumption that the atypical colony types found in cultures were variants of *P. solanacearum*, single cells were isolated from a fresh culture of the wild type obtained from an inoculated tomato plant. When streaked on the tetrazolium medium, the cultures of single cell origin initially formed only the typical fluidal white colonies with pink centers. However, after 3-5 days of growth in 1 per cent tryptone broth, mutants could be detected that formed non-fluidal red colonies identical in appearance on the tetrazolium medium to those formed by the mutants observed in stock cultures.

Tests were then initiated to compare the virulence of cultures derived from mutant and wild type colonies. In repeated inoculations of tomato plants in greenhouse experiments, cultures derived from single mutant colonies that showed a dark red pigmentation on the tetrazolium medium were either weakly virulent or avirulent. In contrast, cultures from wild type colonies which remained relatively free of coloration during the initial 36 hours of growth were consistently highly pathogenic.

The reliability of colony appearance on the tetrazolium medium as a criterion of virulence was further demonstrated in the following experiment. This test was initiated in order to determine whether virulent cultures could be obtained consistently by colony selection from isolates containing mixtures of virulent and weakly virulent or avirulent types. Dilute bacterial suspensions of the isolates listed in Table 1 were made from stock cultures preserved under mineral oil. When these suspensions were streaked on 1 day-old plates of the tetrazolium medium, it was apparent that colony types were present to a varying degree in all stock cultures. One well-isolated single colony of the wild type was selected from each of 17 different isolates; a single colony of the mutant type was selected from each of 5 isolates. These single colonies were suspended in sterile water blanks and streaked on the tetrazolium medium. The procedure of re-streaking a single colony from each of the 22 cultures was repeated 5 times at 72 hour intervals. Single colonies selected from the fifth series of streaked plates were re-streaked on a nutrient medium of the following composition: 1.0 per cent tryptone, 0.1 per cent yeast extract, 0.5 per cent glucose, and 1.7 per cent agar in 1 liter of distilled water. At the end of 72 hours, 1 loop of bacteria was removed from each of these cultures and suspended in 2 ml. of sterile tap water. Eight Rutgers tomato plants were inoculated for each isolate.

Results are summarized in Table 1. All cultures considered to be pathogenic on the basis of the characteristic appearance of colonies on the tetrazolium medium brought about very rapid wilting of inoculated plants. At the end of 14 days, all plants inoculated with these virulent cultures were either severely wilted or dead. In marked contrast, none of the mutant type cultures had produced pronounced wilting symptoms or plant death 21 days after inoculation. However, stunting, vascular discoloration, and adventitious root formation appeared in plants inoculated with the mutant type cultures of isolates 5 and 16.

The tetrazolium medium has also been evaluated as an aid in detecting the wild colony type in isolations from diseased tobacco, tomato, potato, and eggplant. In those instances in which bacterial suspensions were obtained from severely diseased plants and streaked on the tetrazolium medium, many different kinds of colonies often appeared, including those of various saprophytic bacteria. Because of the distinctive appearance of the wild type colonies of *P. solanacearum* on this medium, however, selection of these typical white colonies from mixed cultures was greatly facilitated. Single colonies selected in this manner were re-streaked until examination with the dissecting microscope indicated that the cultures were free of other bacteria. All isolates obtained by this procedure were highly pathogenic to tomato.

TABLE 1.—*Pathogenicity to tomato of cultures of Pseudomonas solanacearum classified on basis of colony appearance on the tetrazolium medium*

Cultures Tested			Disease Index[a]	
Number	Host from which isolated	Number of months stored under sterile mineral oil	Colony appearance Fluidal-white	Non-fluidal-red
3	Potato	78	85	
4	Potato	78	98	
5[b]	Potato	66	—	20
9	Potato	30	100	
51	Potato	6	90	0
16	Tobacco	66	95	20
17	Tobacco	66	100	
18	Tobacco	66	88	
40	Tobacco	42	100	
41	Tobacco	42	98	
45	Tobacco	42	100	
46	Tobacco	30	100	
47	Tobacco	30	100	
48[b]	Tobacco	6	—	0
25	Tomato	42	100	
60	Tomato	3	100	0
60-R[c]	Tomato	0	100	
31	Peanut	66	100	
80	Pepper	6	93	

[a] Disease index 14 days after inoculation.
[b] Wild type colony was not found in stock culture.
[c] 60-R was a fresh re-isolate of 60 following passage through a moderately resistant tobacco plant (Dixie Bright 101).

DISCUSSION.—Isolates of *P. solanacearum* often lose virulence rapidly when maintained in culture by routine methods. The procedure of storing cultures under sterile mineral oil has been found to aid in maintaining isolates in a pathogenic state for 4 years (3). In these studies it was found that storage under mineral oil did not prevent the development of mutants of reduced virulence; however, the virulent wild type had not been completely eliminated in most instances. Furthermore, pathogenic cultures could be recovered from isolates stored for as long as 6½ years under mineral oil.

Evidence was also obtained that loss in virulence of cultures could be attributed to mutation and selection that resulted in populations dominated by weakly virulent types, differing in colony morphology from the wild form. This conclusion confirms in part results obtained by Okabe (7) in his studies on the pathogenicity of colony types of *P. solanacearum* in Japan. In macroscopic observations of morphological characteristics of colonies, Okabe noted that weakly pathogenic or avirulent mutants differed in colony morphology from the fluidal wild type. However, he also reported in tests on tomato and tobacco that all fluidal colonies were not necessarily highly pathogenic. Moreover, virulence was variable among morphologically similar colonies derived from a single virulent fluidal colony. This latter finding differs from results obtained thus far in pathogenicity tests on tomato with isolates from North Carolina. All cultures have been highly pathogenic that were derived from wild type colonies selected on the basis of their characteristic appearance on the tetrazolium medium.

The application of these results has facilitated the initiation of a more detailed study of the relationship between colony morphology and virulence of *P. solanacearum*.

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