Characterization of the Bacterium Inciting Chocolate Spot of Corn

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ABSTRACT

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The bacterium that incites chocolate spot, a leaf blight disease of corn, is a fluorescent pseudomonad in group Ia of Lelliott et al. On the basis of its bacteriological properties and toxin production, this bacterium was shown to differ substantially from strains of *Pseudomonas syringae* that cause holcus spot of corn, and to conform with *P. coronafaciens* and with the causal organism of halo blight of timothy. Halo blight symptoms were induced on oats and timothy by inoculation with the corn chocolate spot strains. These strains synthesize three cholorosis-inducing toxins in culture, two of which are structurally identical with the tabtoxins reported for *P. coronafaciens* and the timothy halo blight bacterium. It is proposed that the casual organism of corn chocolate spot in Wisconsin be designated as *P. coronafaciens* pathovar *zeae* to distinguish it from other strains of this nomenspecies which are unable to infect corn and timothy.

Additional key words: oat halo blight, toxin, timothy.

A leaf blight disease of corn (*Zea mays* L.) called chocolate spot first appeared in 1971 at several locations in Wisconsin. Since then, the disease has occurred sporadically and only in fields with potassium-deficient soils (1, 12). The disease has not been reported from other states. The symptoms (dark brown, elongated spots up to 3 cm long) are restricted to the leaves (Fig. 1). Wounding of leaves by wind whipping appears to promote infection. The most characteristic symptom is a pronounced, broad yellow halo that surrounds each lesion, indicative of the possible effect of a toxin. The lesions are more numerous along the leaf edges and toward the tips, areas where potassium deficiency symptoms also first appear. Later, they may coalesce causing death of large areas.

The causal organism of corn chocolate spot provisionally has been designated as a strain of *Pseudomonas syringae* (1). Previously *P. syringae* [Syn. *P. holci* (Kendr.) Bergey et al.] was identified as the incitant of holcus spot of corn (13). However, the symptoms of holcus spot differ markedly from those of chocolate spot; the lesions of the former disease are characteristically smaller than those of chocolate spot, and are formed by tan to whitish papery spots with reddish margins. The narrow band of chlorotic tissue that sometimes occurs around older lesions is not considered important for diagnostic purposes. In 1976, Sellam and Wilcoxson (24) reported a leaf blight of wheat in Minnesota, and the causal agent was identified as a strain of *P. syringae;* it also was pathogenic on corn artifically inoculated. However, no description of the symptoms produced by this strain on corn was given.

The purpose of this paper is to establish the relationship of the Wisconsin chocolate spot organism to other closely related bacteria in terms of their physiological characteristics, including toxin production, and pathogenicity.

MATERIALS AND METHODS

The following organisms were used: five strains (ChS 1 to 5) from typical chocolate spot lesions on corn. These strains came from diseased specimens collected in 1975 and 1976 from Columbia and Walworth Counties, Wisconsin; *Pseudomonas coronafaciens,* PC-27 from M. P. Starr (Bacteriology Department, Univ. Calif., Davis); *P. tabaci,* six strains including ATCC#11528, ATCC#17914, and four tobacco strains from R. W. Fulton (Department of Plant Pathology, Univ. Wisconsin, Madison); *P. syringae,* three strains (BS 1 to 3) from brown spot of bean in Wisconsin, and two strains (B-397 and B-359) that incite holcus spot on corn from J. E. DeVay (Department of Plant Pathology, Univ. Calif., Davis); and two strains (Ti 1 and 2) from timothy (*Phleum pratense* L.) halo blight obtained in 1975 and 1976 from Columbia County, Wisconsin.

Selected bacteriological tests performed were: Levan formation and ability to induce potato rot (19); activity of

oxidase (16), arginine dihydrolase (28), β-glucosidase (10), and tyrosinase (7); ability to induce pitting on polypectate gel (2), necrotic lesions on cowpea (17), and a hypersensitive reaction on tobacco leaves (15); and utilization of organic acids as sole carbon sources (20). The production of syringomycin was determined with the bioassay technique of Gross and DeVay (8); *Geotrichum candidum* Link ex Pers. was used as the test organism. An arthrospore suspension of the fungus was sprayed on petri dishes containing PDA plus 0.4% casamino acids that had been spot-inoculated previously (6 days) with the bacterial isolates. The occurrence of inhibition zones around the bacterial colonies was considered indicative of syringomycin synthesis. The in vitro formation of chlorosis-inducing toxins was tested with filtrates from cultures grown in Woolley's medium (26). After incubation for 5 days on a rotary shaker at 24 C, cultures were centrifuged and the supernatant fraction was filter-sterilized (Millipore, 0.22 μm pore size) and then bioassayed. In some cases, this fraction was further concentrated and purified by passage through a column containing Amberlite CG-120 (H+ form). After washing the column with distilled water, the toxins were eluted with 5% NH_4OH. To minimize breakdown, the eluent was evaporated quickly in vacuo at 35 C and the residue resuspended in ethanol. The resulting precipitate was removed by centrifugation and the toxins in the supernatant fluid then were separated by ion-exchange chromatography as previously described (26). Chlorosis-inducing activity of crude filtrates and partially purified preparations was tested by prick inoculations of primary or first true leaves of bean seedlings, cultivar Bush Blue Lake-274, through 10-μliter droplets. The plants were then placed in a controlled environment chamber (12-hr day at 26 C and 10,000 lux; night temperature of 21 C); the results were recorded 48 hr later.

Pathogenicity tests were performed in the greenhouse (20-30 C) on plants that were grown in a mixture of compost soil, peat moss, and sand (3:1:1, v/v). The species used were: corn hybrids A619×W64A and W64A×A554; cowpea cultivar California Blackeye 3; oat cultivars Portage and Dal; timothy cultivar Verdant; and tobacco cultivars Xanthi and Havana 38. Inocula consisted of suspensions containing about 10^6 viable cells per ml prepared from 1-day-old cultures on glycerol nutrient agar slants. Bacterial cells were washed by centrifuging and resuspending twice in sterile, distilled water. Triton X-100 (0.1% final conc.) was added to the suspensions used in the spray inoculations. Incoculations were performed by pricking moistened leaves with a sterilized needle through droplets of inoculum, or by spraying the inoculum onto plants by means of a Ceccato 2701-2711 (1.5 nozzle) paint gun, without causing apparent water soaking. Inoculated plants were kept in a mist chamber for 24 hr and then transferred to the greenhouse.

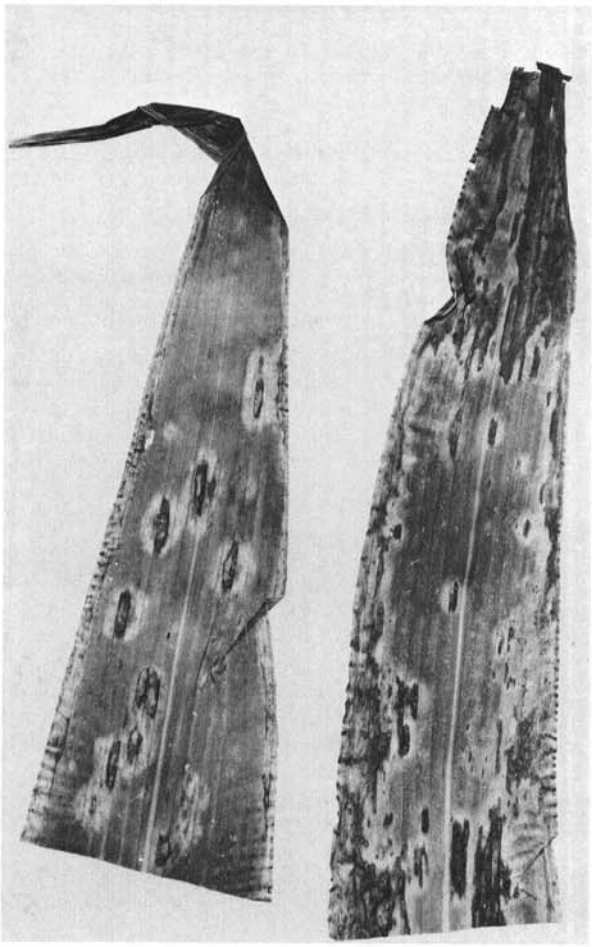

Fig. 1. Symptoms of chocolate spot on naturally infected corn leaves. Note the pronounced halos surrounding the necrotic lesions and their coalescence mainly along leaf edges and tips.

RESULTS

The incitant of corn chocolate spot is a Gram-negative rod that produces fluorescent pigment on King's B Medium containing glycerol (14), is motile by one to five polar flagella, and has an oxidative mode of glucose metabolism. It forms levan, is oxidase- and arginine dihydrolase-negative, and induces a hypersensitive reaction when infiltrated into tobacco leaves. According to the LOPAT characteristics it is a fluorescent pseudomonad in group Ia of Lelliott et al. (19) which includes a number of nomenspecies, such as *P. coronafaciens, P. tabaci,* and *P. syringae.* On the basis of these and additional tests (Table 1), the bacterium could not be differentiated from *P. coronafaciens* (*sensu* reference 11) and the closely related pseudomonad that incites timothy halo blight (27). The chocolate spot organism differed from strains of *P. syringae* by its inability to utilize DL-lactate, inability to induce necrotic lesions on cowpea, and by its tyrosinase activity. The lack of utilization of L(+) tartrate as a sole source of carbon, in addition to its inability to cause pitting on polypectate gel, differentiated the chocolate spot bacterium from *P. tabaci.*

All *P. syringae* strains inhibited the growth of *G. candidum* (indicating the production of syringomycin), but the corn chocolate spot, timothy halo blight, *P. coronafaciens,* and *P. tabaci* strains did not. On the other hand, culture filtrates from strains of the chocolate spot

organism contained toxins that induced chlorotic lesions with necrotic centers, when injected into bean or tobacco leaves. This effect was similar to that obtained with filtrates from *P. coronafaciens, P. tabaci,* and the pathogen of timothy. The toxic material was heat-labile (100 C for 10 min) and the chlorotic effect was light-dependent, as is characteristic of the tabtoxins (4, 25). Ion-exchange chromatography of partially purified preparations from the chocolate spot bacterium revealed two peaks of chlorosis-inducing activity (Fig. 2). The first active peak (50-60 min retention time) upon acid hydrolysis (6 N HCl, 1 hr at 100 C) yielded only tabtoxinine, as determined by chromatographic and mass spectral procedures (26). Its complete structure is currently being determined. The second peak corresponded in elution time (100-120 min) to the two previously reported tabtoxins (26). Acid hydrolysis of this fraction yielded threonine, serine, and tabtoxinine as determined by the same procedures. In addition, this peak cochromatographed with authentic tabtoxins in ion-exchange and thin-layer chromatographic systems (26), and upon standing, its contents partially degraded into the biologically inactive isotabtoxins.

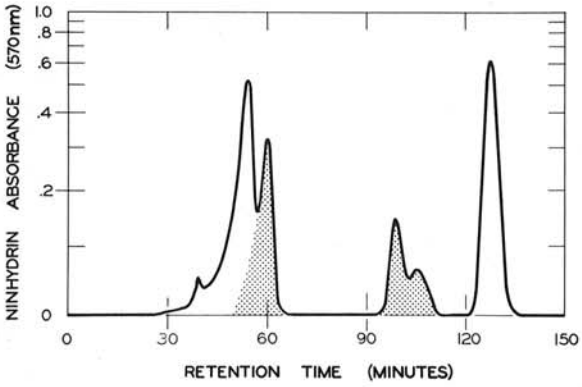

Fig. 2. Amino acid elution profile of a partially purified preparation from the corn chocolate spot pseudomonad. Hatched areas indicate zones with chlorosis-inducing activity in bean leaves.

Reciprocal inoculations with the strains from corn chocolate spot and timothy halo blight were positive (Table 2); the symptoms (necrotic spots with yellow halos) induced by all these strains appeared identical on the inoculated leaves of both hosts. In addition, they produced typical halo blight symptoms on oats. Strains of *P. coronafaciens* and *P. tabaci* were pathogenic on their natural hosts (oats and tobacco, respectively) but not on corn and timothy. Holcus spot symptoms were produced on corn by the *P. syringae* strains B-359 and B-397; neither strain produced symptoms on oats or timothy.

DISCUSSION

According to Doudoroff and Palleroni's revision of the genus *Pseudomonas*, as published in the eighth edition of the Bergey's Manual of Determinative Bacteriology (3),

TABLE 2. Comparison of pathogenicity to selected hosts of corn chocolate spot and timothy halo blight strains with three species of *Pseudomonas*

Strain or nomenspecies	Test Plant[a]			
	Corn	Timothy	Oat	Tobacco
ChS 1 to 5	+[b]	+	+	–
Ti 1 and 2	+	+	+	–
P. tabaci[c]	–	–	–	+
P. coronafaciens	–	–	+	–
P. syringae[d]	+	–	–	–

[a]Light spray inoculation with about 10^6 washed viable cells/ml.

[b]Symbols: + = virulent; – = avirulent.

[c]Strain ATCC #17914 was nonpathogenic. The remaining strains reacted identically.

[d]"Holcus spot" strains B-359 and B-397. Strains BS 1 to 3 were nonpathogenic on all four test plants.

TABLE 1. Comparison of bacteriological properties of corn chocolate spot (ChS) and timothy halo blight (Ti) strains with three species of *Pseudomonas*

Test	Strain or nomenspecies				
	ChS 1 to 5	Ti 1 and 2	*P. coronafaciens*	*P. syringae*[a]	*P. tabaci*[a]
Growth on DL-lactate	–[b]	–	–	+	–
Growth on L(+) tartrate	–	–	–	–	+
Pitting on polypectate gel	–	–	–	–	+
β-Glucosidase production	+	+	+	+	+
Necrotic lesions on cowpea	–	–	–	+	–
Tyrosinase activity	+	+	+	–	NT

[a]All strains reacted identically.

[b]Symbols: + = positive reaction; – = negative reaction; NT = not tested.

all fluorescent plant-pathogenic pseudomonads that are negative for the oxidase test should be considered within *P. syringae*. However, this proposal was considered provisional because of insufficient information at that time on the nomenspecies which comprise the group of plant pathogenic, oxidase-negative, fluorescent pseudomonads. It was even recognized that some of the nomenspecies included in *P. syringae* might deserve independent specific rank. In support of this, Pecknold and Grogan (21) suggested that genetic differences, as evidenced by DNA homology studies with a number of such nomenspecies, were great enough to justify their separation into several groups.

Hayward (9) stated that "any distinct pathological entity must receive a convenient label" for quarantine purposes; Schroth and Hildebrand (23) pointed out that the distinctive pathogenic capabilities of the organisms should be considered part of a determinative system. According to Dye et al. (5), organisms such as *P. tabaci* and others that induce "characteristic and reproducible host reactions" require names indicating their "different pathogenicities". If these alternative viewpoints are followed, the chocolate spot bacterium should not be designated *P. syringae*. This is based on the following points: (i) holcus spot of corn is a disease considerably different from chocolate spot; and (ii) the chocolate spot strains produce tabtoxins in culture, two of which are identical with those already reported. These toxins have not been associated with strains of *P. syringae*, but are produced by strains of *P. coronafaciens*, *P. tabaci*, and *P. garcae* Amaral et al. (26). Tabtoxins are capable of reproducing part of the symptoms (yellow halos) characteristic of the diseases incited by these nomenspecies and therefore can be considered of taxonomic relevance. Conversely, the corn chocolate spot pathogen produces no detectable syringomycin. This is especially significant because syringomycin synthesis, determined via the *G. candidum* bioassay, is a characteristic of *P. syringae* strains which can incite holcus spot and its in situ production has been considered an essential requirement for disease development on grass hosts (8).

In addition, the chocolate spot strains differ from *P. syringae* strains in certain biochemical tests considered useful for the separation of fluorescent pseudomonad nomenspecies. Thus, the chocolate spot strains show tyrosinase activity and are unable to utilize DL-lactate as a sole carbon source, whereas strains of *P. syringae*, including those that incite holcus spot on corn, give opposite reactions. The incitant of chocolate spot does not induce necrotic local lesions on cowpea primary leaves, whereas strains of *P. syringae*, regardless of the host of origin, induce a positive reaction. Furthermore, others have considered this test to be reliable for identifying strains of *P. syringae* (17).

The chocolate spot bacterium is probably identical with the organism that incites halo blight of timothy in Wisconsin (27). Their behavior in vitro, the types of toxins produced (tabtoxins), and the symptoms they induce on certain host plants are identical. They do, however, differ from strains of *P. coronafaciens* in host range. As first mentioned by Elliott (6), strains that cause oat halo blight do not infect corn. In addition, Taylor et al. (27) found *P. coronafaciens* to be nonpathogenic on timothy although their isolates from timothy induced halo blight symptoms on oats. Also, *P. coronafaciens* var. *atropurpurea* from various grasses is unable to attack corn or timothy (22, 29). On the basis of these considerations the organism which incites corn chocolate spot and timothy halo blight should be regarded as a distinct forma specialis of *P. coronafaciens*. In conformity with the recommendations of the International Code of Nomenclature of Bacteria (18), we propose that this bacterium be designated as *P. coronafaciens* pathovar *zeae*; because it differs from other strains of *P. coronafaciens* by its virulence on corn and timothy.

LITERATURE CITED

1. ARNY, D. C., S. SAAD, W. H. HUGHES, and G. L. WORF. 1972. Is sick corn related to nutrition? Better Crops Plant Food 72:20-21.
2. CUPPELS, D., and A. KELMAN. 1974. Evaluation of selective media for isolation on soft-rot bacteria from soil and plant tissue. Phytopathology 64:468-475.
3. DOUDOROFF, M., and N. J. PALLERONI. 1974. Genus I Pseudomonas Migula 1894. Pages 217-243 *in* R. E. Buchanan and N. E. Gibbons, eds. Bergey's manual of determinative bacteriology, 8th edition. Williams and Wilkins, Baltimore, Maryland. 1268 p.
4. DURBIN, R. D., and S. L. SINDEN. 1967. The effect of light on the symptomatology of oat halo blight. Phytopathology 57:1000-1001.
5. DYE, D. W., J. F. BRADBURY, R. S. DICKEY, M. GOTO, C. N. HALE, A. C. HAYWARD, A. KELMAN, R. A. LELLIOTT, P. N. PATEL, D. C. SANDS, M. N. SCHROTH, D. R. W. WATSON, and J. M. YOUNG 1975. Proposals for a reappraisal of the status of the names of plant-pathogenic Pseudomonas species. Int. J. Syst. Bacteriol. 25:252-257.
6. ELLIOTT, C. 1920. Halo-blight of oats. J. Agric. Res. 19:139-172.
7. GARRETT, C. M. E., C. G. PANAGOPOULOS, and J. E. CROSSE. 1966. Comparisons of plant pathogenic pseudomonads from fruit trees. J. Appl. Bacteriol. 29:342-356.
8. GROSS, D. C., and J. E. DEVAY. 1977. Population dynamics and pathogenesis of Pseudomonas syringae in maize and cowpea in relation to the in vitro production of syringomycin. Phytopathology 67:475-483.
9. HAYWARD, A. C. 1972. The impact of changes in nomenclature on plant quarantine measures. Pages 293-294 *in* H. P. Maas-Geesteranus, ed. Proc. Third Int. Conf. Plant Pathogenic Bacteria. Centre Agric. Publ. Doc. (PUDOC), Wageningen, The Netherlands. 365 p.
10. HILDEBRAND, D. C., and M. N. SCHROTH. 1964. Beta-glucosidase activity in phytopathogenic bacteria. Appl. Microbiol. 12:487-491.
11. HILDEBRAND, D. C., and M. N. SCHROTH. 1972. Identification of the fluorescent pseudomonads. Pages 281-287 *in* H. P. Maas-Geesteranus, ed. Proc. Third Int. Conf. Plant Pathogenic Bacteria, Centre Agric. Publ. Doc. (PUDOC), Wageningen, The Netherlands. 365 p.
12. KARLEN, D. L., D. C. ARNY, and L. M. WALSH. 1973. Incidence of chocolate spot (Pseudomonas syringae), northern corn leaf blight (Helminthosporium turcicum), and lodging of corn as influenced by soil fertility. Comm. Soil Sci. Plant Analysis 4:359-368.
13. KENDRICK, J. B. 1926. Holcus bacterial spot of Zea mays and Holcus species. Iowa Agric. Exp. Stn. Res. Bull. 100:303-334.
14. KING E. O., M. K. WARD, and D. E. RANEY. 1954. Two

simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.
15. KLEMENT, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature (Lond.) 199:299-300.
16. KOVACS, N. 1956. Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature (Lond.) 178:703.
17. LAI, M. and B. HAAS. 1973. Reaction of cowpea seedlings to phytopathogenic bacteria. Phytopathology 63:1099-1103.
18. LAPAGE, S. P., P. H. A. SNEATH, E. F. LESSEL, V. B. D. SKERMAN, H. P. R. SELIGER, and W. A. CLARK. 1975. International Code of Nomenclature of Bacteria. Bacteriological Code (1976 Revision). American Society for Microbiology. Washington D.C. 180 p.
19. LELLIOTT, R. A., E. BILLING, and A. C. HAYWARD. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.
20. MOUSTAFA, F. A., and R. WHITTENBURY. 1970. A comparison of some phytopathogenic and nonphytopathogenic pseudomonads. Phytopathol. Z. 67:63-72.
21. PECKNOLD, P. C., and R. G. GROGAN. 1973. Deoxyribonucleic acid homology groups among phytopathogenic Pseudomonas species. Int. J. Syst. Bacteriol. 23:111-121.
22. REDDY, C. S., and J. GODKIN. 1923. A bacterial disease of bromegrass. Phytopathology 13:75-86.
23. SCHROTH, M. N., and D. C. HILDEBRAND. 1972. Current taxonomic thinking on the genus Pseudomonas, with emphasis on the plant pathogens. Pages 261-267 *in* H. P. Maas-Geesteranus, ed. Proc. Third Int. Conf. Plant Pathogenic Bacteria, Centre Agric. Publ. Doc. (PUDOC), Wageningen, The Netherlands. 365 p.
24. SELLAM, M. A., and R. D. WILCOXSON. 1976. Bacterial leaf blight of wheat in Minnesota. Plant Dis. Rep. 60:242-245.
25. SINDEN, S. L., and R. D. DURBIN. 1969. Some comparisons of chlorosis-inducing pseudomonad toxins. Phytopathology 59:249-250.
26. TAYLOR, P. A., and R. D. DURBIN. 1973. The production and properties of chlorosis-inducing toxins from a pseudomonad attacking timothy. Physiol. Plant Pathol. 3:9-17.
27. TAYLOR, P. A., D. P. MAXWELL, and R. D. DURBIN. 1971. Occurrence in Wisconsin of halo blight of timothy incited by a Pseudomonas species. Plant Dis. Rep. 55:361-362.
28. THORNLEY, M. J. 1960. The differentiation of Pseudomonas from other gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. 23:37-52.
29. TOMINAGA, T. 1968. Halo blight of ryegrass, bromegrasses, and fescues. Ann. Phytopathol. Soc. Japn. 34:242-249.