Bacterial Reservoir of an Agent Infectious to Fungi

G. D. Lindberg

Professor of plant pathology, Department of Plant Pathology and Crop Physiology, Louisiana Agricultural Experiment Station, Louisiana State University, Baton Rouge 70803.

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ABSTRACT

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An irreversible disease condition was produced in *Helminthosporium turcicum* grown on potato-dextrose agar (PDA) or nutrient-dextrose agar (NDA) with established colonies of a pseudomonad bacterium; subcultures of the affected *H. turcicum* also were diseased. The disease developed without physical contact between the mycelium of *H. turcicum* and the pseudomonad colonies, which suggested an extracellular causal agent. *Helminthosporium maydis* race T and several other species of Helminthosporium grown with the pseudomonad on PDA or NDA changed markedly, but the change was not permanent; subcultures of the affected *H. maydis* race T were always normal. Mycelium of *H. maydis* grown with 1-wk-old colonies of the pseudomonad on peptone-dextrose-yeast extract agar (PDYA) with 1.2% peptone was normal and symptomless after 1 wk, then abruptly began to collapse and lyse; most subcultures of the treated *H. maydis* made 1 wk after commencement of lysis failed to grow, but surviving mycelium was normal. Symptom development was delayed ~1 wk when single conidia of *H. maydis* were placed on the PDYA with either 4-, 7-, or 10-day-old colonies of the pseudomonad. The causal agent was transmitted from diseased to healthy *H. turcicum* only after hyphal contact.

Among colonies of *Helminthosporium turcicum* Pass. isolated from diseased corn, we observed some that were abnormal in culture plates that also contained colonies of a yellow bacterium (a *Pseudomonas* sp. hereafter referred to as "the pseudomonad"). Healthy *H. turcicum* mixed with the bacterium grew and emerged, bacteria free, onto the agar surface, but the mycelium that developed on the agar was not typical of *H. turcicum*. Subcultures of the altered *H. turcicum* were abnormal, indicating that the change was permanent; the abnormal colonies were so severely stunted that maintenance of an abnormal culture by serial transfer was difficult.

No plaques developed in active colonies of the pseudomonad but the absence of plaques did not eliminate the possibility of bacteriocin production. Bacteriocin particles are of two basic types, one a small bactericidal protein not sedimented by centrifugation while the other resembles bacteriophage particles. In 1965, Reeves (3) stated that bacteriocins appeared to be a natural class of antibiotics, but distinctive in that they were proteinaceous and, in contrast to all other antibiotics, act only on strains of the same or closely related species. Bacteriocins were thought to kill sensitive bacteria only after binding to receptors of the outer membrane, a point that has since been established with colicins, which are bacteriocins of *Escherichia coli* (1).

On the basis of these preliminary observations, a thorough investigation of production of this change in *H. turcicum* was undertaken. We proceeded to determine whether the causal agent of the change was transmissible from bacteria-free, diseased *H. turcicum* to healthy *H. turcicum* and to determine the reaction of other fungi to treatment with the pseudomonad.

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MATERIALS AND METHODS

Growth media. Potato-dextrose agar (PDA, 300 g of peeled diced potatoes, 25 g dextrose, and 15 g Difco agar in 2 L of distilled water) was the medium used to demonstrate the activity produced by the pseudomonad as well as for the routine cultivation of the fungi and the bacterium. Peptone-dextrose-yeast extract agar medium (PDYA, 5–12 g peptone, 4 g yeast extract, 12 g dextrose, and 8 g agar in 1 L of distilled water) and nutrient-dextrose agar (NDA, 23 g Bacto nutrient agar and 12 g dextrose in 1 L of distilled water) were used to demonstrate variations in activity of the agent produced by the pseudomonad.

Single conidia were isolated with finely drawn glass needles under a dissecting microscope at ×75.

RESULTS

Production of disease in *H. turcicum*. *H. turcicum* from culture plates mixed with mature colonies of the pseudomonad on PDA grew amidst the bacteria and emerged, bacteria-free, onto the agar surface. A permanent change or disease developed in the *H. turcicum* when small bits of fungus mycelium were mixed with the bacterium but symptoms often failed to develop in the *H. turcicum* when larger fungal inocula were used. The studies seemed to indicate that *H. turcicum* had a certain capacity to resist the change initiated by the pseudomonad.

Conidia of *H. turcicum* sporulating on diseased corn leaves were

harvested on the tip of a moistened transfer needle and streaked on water agar. They were transferred individually with glass needles and placed on PDA with 2- to 3-wk-old colonies of the pseudomonad. There were two bacterial colonies and five single conidia per plate, with four conidia placed adjacent to, and one conidium between, the two bacterial colonies.

We assumed that hyphal growth from the single conidium would have to touch the bacterial colony before the process of the abnormal change in *H. turcicum* would start. Consequently, the single conidia were placed about 3 mm from the bacterial colonies. Hyphal growth of the conidia showed severe symptoms of the abnormal change, but it extended as vigorously in the direction of the bacterial colonies as in other directions. Subcultures of the fungus made from these plates after 1 wk were all diseased, even though it appeared that some of the diseased fungal colonies had not contacted the bacterial colonies. In all subsequent studies, the conidia were placed on the agar medium 10–12 mm from the bacterial colonies.

Single conidia of *H. turcicum* were placed on PDA plates that contained 1-, 2-, 3-, 4-, and 5-wk-old colonies of the pseudomonad (Fig. 1). Colonies of *H. turcicum* that developed from single conidia were largest in plates with 1-wk-old colonies of the bacterium followed in size by those in plates with 2-wk-old colonies of bacteria and smallest in plates with 3-wk-old colonies of bacteria. All of these fungus colonies showed severe symptoms of disease. Colonies of *H. turcicum* in plates with 4-wk-old colonies of the bacterium either grew fairly abundantly and without symptoms or grew very sparsely, producing only a few hyphal strands. Colonies of *H. turcicum* in plates with 5-wk-old colonies of the bacterium were invariably small with a few hyphal strands.

H. turcicum colonies in plates with 1-, 2-, or 3-wk-old colonies of the pseudomonad were subcultured 1 wk after placement of the conidia and all were diseased. A total of 150 colonies of *H. turcicum* were subcultured for each of the three treatments in three experiments. None of the colonies of *H. turcicum* from plates with 3-wk-old colonies of the bacterium and few of the colonies of *H. turcicum* in plates with 2-wk-old colonies of the bacterium had made contact with the bacteria when the transfers were made. Consequently, this disease in *H. turcicum* was probably produced without cell-to-cell contact.

One hundred thirty-five colonies of *H. turcicum* in plates with 4-wk-old colonies of the bacterium were subcultured 1 wk after placement of the conidia, but none of the subcultures was diseased (Table 1). One hundred ten colonies of *H. turcicum* in plates with 5-wk-old colonies of the bacterium were subcultured 1 wk after placement of the conidia, but none of the subcultures was diseased. One hundred twenty-four colonies of *H. turcicum* in plates with 4-wk-old colonies of the bacterium were subcultured 2 wk after placement of the conidia and 63 were diseased. Eighty-one colonies of *H. turcicum* in plates with 5-wk-old colonies of the bacterium were subcultured 2 wk after placement of the conidia and 24 were diseased. A remarkable difference in ability to produce disease in *H. turcicum* was seen between 3- and 4-wk-old pseudomonad colonies. These colonies were yellow, and for most of the first 3 wk of growth on PDA formed a colony (with a network of crinkles on its surface) that could be lifted intact from the agar surface with a transfer needle. After 3 wk of growth, the bacterial colonies became more fluid and the crinkled texture was lost.

Bacteria-free diseased *H. turcicum*. Subcultures of treated colonies of *H. turcicum* showed no growth of the pseudomonad and agar transfers made frequently from between the *H. turcicum* and bacterial colonies failed to show bacterial growth. Transfers from diseased colonies of *H. turcicum* seeded to 25 flasks with 25 ml each of peptone yeast extract broth (PDYB) in each of four separate trials, as well as many transfers placed in potato-dextrose broth (PDB), showed no bacterial growth. Two-hundred conidia taken from diseased colonies of *H. turcicum* were streaked on water agar, isolated individually, and placed on PDA. About 65% of the conidia germinated; 55% produced small, diseased colonies; and 10% produced apparently healthy colonies, which were all free of any sign of bacterial growth. The remaining 35% of the conidia failed to germinate and no bacterial growth developed in the agar where the conidia were placed. Conidia of normal, healthy *H. turcicum* always approached 100% germination.

In addition to demonstrating that the change or disease persisted in *H. turcicum* in the absence of the pseudomonad, it was shown that the pseudomonad alone could not produce the permanent change in *H. turcicum*. When cubes of agar containing mycelium of *H. turcicum* were touched to the surface of a pseudomonad colony and plated on PDA, both organisms grew. The bacterial growth dominated for 48–72 hr before the *H. turcicum* mycelium grew beyond the edge of the bacterial colonies. After about 1 wk, the mycelium of *H. turcicum* covered the agar surface and showed no disease symptoms. Transfers of the treated colonies of *H. turcicum* were taken two or more centimeters from the initial inocula and plated on PDA. Five subcultures were made from each of 10 replicate plates of the treated *H. turcicum* in three separate experiments and all 150 subcultures grew normally. Thus, there was an abundance of bacteria, but no production of disease in *H. turcicum*. An interval between initial growth of the pseudomonad and production of an agent responsible for the permanent change in *H. turcicum* appeared to account for absence of disease in the

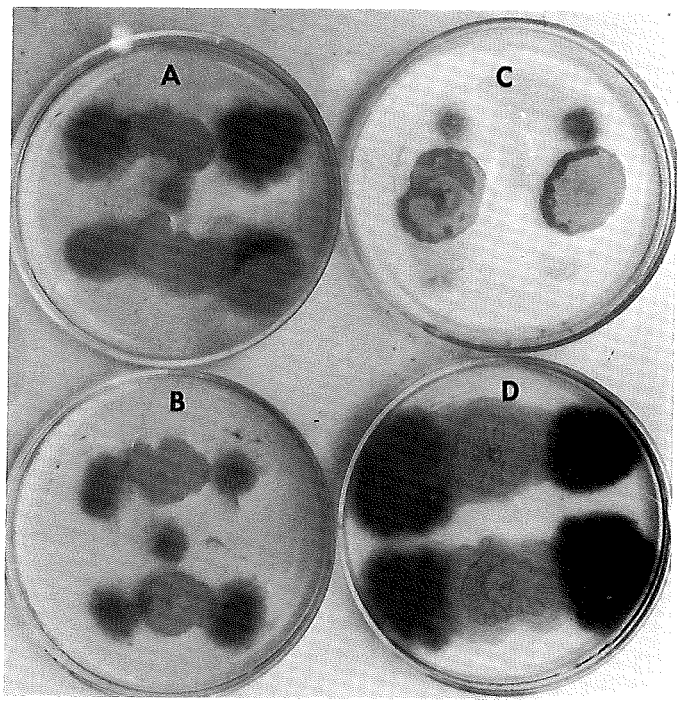

Fig. 1. Colonies from single conidia of *Helminthosporium turcicum* on PDA after 7 days with: **A,** 1-; **B,** 2-; **C,** 3-; and **D,** 4-wk-old colonies of the pseudomonad. Subcultures of treated *H. turcicum* made after 1 wk of fungus growth with 1-, 2-, and 3-wk-old colonies of the pseudomonad were always diseased, whereas those of *H. turcicum* with 4-wk-old colonies of the pseudomonads were healthy. Subcultures of the latter were about 50% diseased after 2 wk of growth.

TABLE 1. Production of change or disease in colonies of *Helminthosporium turcicum* started from single conidia placed in plates of PDA with 1-, 2-, 3-, 4-, and 5-wk-old colonies of a pseudomonad

Age of pseudomonad colonies (wk)	Subcultures of treated *H. turcicum* (no. diseased/no. subcultured)	
	Treated 1 wk	Treated 2 wk
1	150/150	···
2	150/150	···
3	150/150	···
4	0/135	63/124
5	0/110	24/81

fungus when the pseudomonad and *H. turcicum* were placed simultaneously on PDA.

Transmission from diseased to healthy *H. turcicum*. Transmission of the disease of *H. turcicum* was attempted according to the hyphal contact inoculation method of Lindberg (2). Small bits of mycelium and conidia of healthy *H. turcicum* were placed near fresh subcultures of the diseased fungus on PDA plates. Symptoms of disease developed first in the mycelium nearest the area of contact with the diseased fungus then spread through the younger cells around the entire edge of the colony. However, disease development was observed in only a small percentage of the inoculated colonies; usually the healthy mycelium grew over the diseased inoculum, showed no symptoms, and continued to grow and cover the agar surface. To increase the percent transmission from diseased to healthy *H. turcicum*, the healthy mycelium and conidia were placed adjacent to 4-day-old colonies of diseased *H. turcicum*. Disease developed in all 100 inoculated colonies of *H. turcicum* in five experiments. All 100 control transfers of *H. turcicum* grew normally. Even though genetically marked strains were not used, we feel confident that disease transfer did occur between diseased and healthy strains.

The disease failed to develop in *H. turcicum* when single conidia were placed on PDA that supported growth of diseased *H. turcicum*, but at distances that prevented hyphal contact. The diseased colonies of *H. turcicum* varied in age from 1 to 4 wk. The colonies of *H. turcicum* treated in this way grew normally and all 100 subcultures from 100 treated colonies were normal (Fig. 2).

Other media. All of the above studies on the reaction of *H. turcicum* to the activity produced by the pseudomonad were made on PDA. We wondered whether reaction of the fungus was the same on other media. The pseudomonad produced yellow, smooth

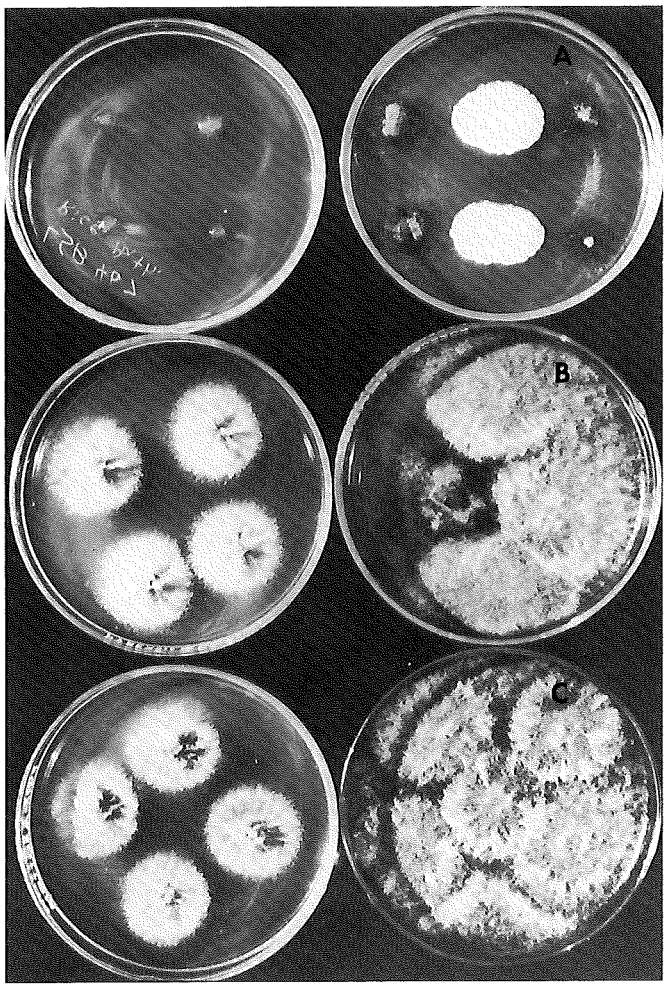

Fig. 2. Colonies that developed from single conidia of *Helminthosporium turcicum* on PDA after 7 days (right side) with **A,** colonies of the pseudomonad; **B,** a colony of diseased *H. turcicum*; and **C,** control *H. turcicum*. Subcultures (left side) of *H. turcicum* in the row on the right side. The subcultures of *H. turcicum* with the bacterium were diseased, whereas those of *H. turcicum* with diseased fungus and the controls were healthy.

TABLE 2. Reaction of colonies derived from single conidia of *Helminthosporium turcicum* placed on PDYA (0.5% and 1.2% peptone) and on NDA 10–12 mm from 7-day-old colonies of the pseudomonad and subcultured after 7, 14, and 16 days

Time (days)	Reaction of treated *H. turcicum* on: PDYA (0.5% peptone) (no. diseased/ no. subcultured)	NDA (no. diseased/ no. subcultured)	PDYA (1.2% peptone) (no. dead/ no. subcultured)
7	40/150	10/150	0/200
14	122/150	150/150	60/200[a]
16	...[b]	...[b]	200/200

[a] About 25% of the transfers tht grew were diseased.
[b] Disease development was extensive at 14 days; there was no need to record the 16-day observations.

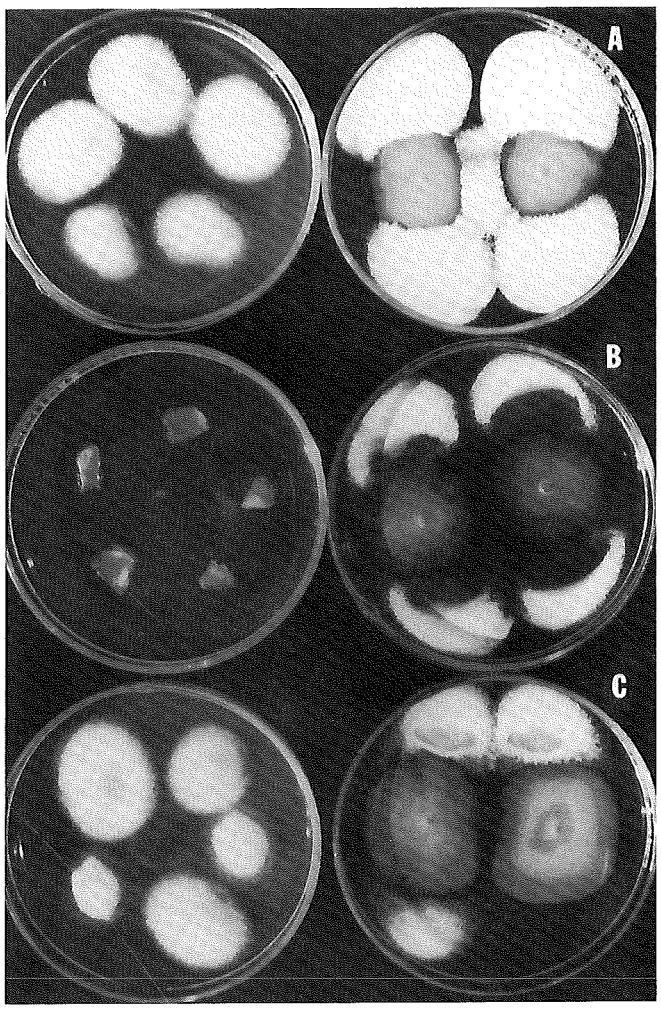

Fig. 3. Colonies of *Helminthosporium maydis* race T that developed from single conidia placed PDYA (1.2% peptone) (right side) with 7-day-old colonies of the pseudomonad after **A,** 1 wk and **B,** 2 wk, and **C,** with 10-day-old colonies of the pseudomonad. Colonies of *H. maydis* were symptomless and viable after 1 wk of growth with 7-day-old colonies of the pseudomonad, but lysed and died after 2 wk. Colonies of *H. maydis* with 10-day-old colonies of the pseudomonad showed some lysis, but were viable. Plates on the left side contain subcultures from corresponding plates of the treated fungus to the immediate right.

colonies on PDYA and NDA. Mycelial growth that developed from single conidia of *H. turcicum* on PDYA (0.5% peptone) and on NDA 10–12 mm from 4-day-old colonies of the pseudomonad was usually limited and stunted, but otherwise symptomless, after 7 days. Subcultures of the treated colonies of *H. turcicum* from both media were made 1 wk after placement of the conidia; about 25% of the transfers from PDYA were diseased and about 5% of those from NDA were diseased. Subcultures of the treated *H. turcicum* were made after 2 wk and all grew, but about 75% of those of *H. turcicum* from PDYA were diseased, while all subcultures of treated *H. turcicum* from NDA were diseased.

Single conidia of *H. turcicum* placed on PDYA (1.2% peptone) plated 10–12 mm from 4-day-old colonies of the pseudomonad grew more slowly than the controls without the bacterium, but otherwise the colonies were symptomless for about 1 wk. Subcultures made 1 wk after placement of the conidia grew readily and were apparently healthy. During the next 9 days, these colonies of *H. turcicum* gradually changed color from olivaceous gray to reddish brown and the mycelium collapsed and was covered with scattered droplets of brown exudate. Of 200 subcultures of these colonies of *H. turcicum* treated for 16 days, none grew. Subcultures made 14 days after placement of the conidia showed no growth after 24 hr, but most grew after an additional 24–48 hr (Table 2).

Other fungi. Several *Helminthosporium*, spp. *H. maydis* races O and T, *H. victoriae, H. carbonum*, and *H. cynodontis*, were co-cultured with mature colonies of the pseudomonad on PDA. The growth of all of these species was altered markedly compared to unexposed control colonies. Growth of *H. maydis* race T, for example, was limited and mostly subsurface, with only short terminals of aerial hyphae. Normal *H. maydis* race T has abundant, heavy, aerial mycelium. Subcultures of all of these treated helminthosporia were, however, apparently normal. Since the reaction of *H. turcicum* to the pseudomonad activity on PDA was different from that on PDYA, single conidia of *H. maydis* race T were placed on PDYA, 1.0 and 1.2% peptone, 10–12 mm from 7-day-old colonies of the pseudomonad. The rate of growth of the fungus was slightly less than that of normal *H. maydis* on control plates, but nevertheless vigorous and apparently healthy. The mycelium advanced to, and made contact with the bacterium; very little mycelium penetrated the bacterial colonies. The *H. maydis* colonies remained symptomless for 1 wk and all subcultures of these treated colonies grew and were apparently healthy. On day 7 or 8, growth stopped abruptly and the fungus mycelium collapsed and lysed. During the next 3–4 days, lytic activity continued in the young cells around the entire edge of the colony and especially in the more mature mycelium adjacent to the bacteria; lysed portions of the *H. maydis* colonies became almost translucent.

Two weeks after placement of single conidia the treated colonies of *H. maydis* were subcultured by cutting out large pieces of the lysed fungal mycelium (up to 1 cm^2), which were transferred to fresh PDA. Of a total of 200 subcultures of *H. maydis* from PDYA with 1.2% peptone, in four experiments 190 failed to grow after 24 hr and 166 failed to grow after 72 hr. Of 200 transfers from *H. maydis* on PDYA with 1.0% peptone, 94 failed to grow after 24 hr and 74 failed to grow after 72 hr. All of the subcultures that grew were apparently healthy (Fig. 3). Average diameter of the bacterial colonies was larger (37 mm) on 1.0% peptone than those (31 mm) on 1.2% peptone.

Single conidia of *H. maydis* race T also were placed on PDYA (1.2% peptone) 10–12 mm from 10-day-old colonies of the pseudomonad. The rate of growth of the colonies of *H. maydis* was slower than normal controls, but symptom development was less severe than *H. maydis* on PDYA when single conidia were placed 10–12 mm from 7-day-old colonies of the pseudomonad. In four experiments, among 200 subcultures of the *H. maydis* grown with 10-day-old cultures of the pseudomonad, 28 failed to grow after 24 hr and 17 failed to grow after 72 hr. All of the subcultures that failed to grow were from one experiment; all subcultures showed mycelial growth after 24 hr in the other three experiments (Table 3 and Fig. 3).

Activity in PDYA was traced beyond 10 days by transplanting 24-hr-old colonies of *H. maydis* to plates containing 14- and 21-day-old pseudomonad. The transplants on 14-day plates grew about as well as colonies from single conidia on plates with 10-day-old colonies of the pseudomonad, but transplants on 21-day plates grew very slowly (~3 mm in diameter) after 2 wk of growth. The treated *H. maydis* was subcultured 14 days after transplanting. Twenty-five subcultures were made of the *H. maydis* from each treatment in each of three experiments and all grew. The very small, but viable, colonies of *H. maydis* seemed to show that activity of the agent produced by the pseudomonad on PDYA had disappeared after 21 days (Table 3).

The pseudomonad bacterium. The pseudomonad is a Gram-negative, aerobic rod with a single polar flagellum. It produces a yellow, water-insoluble pigment; produces acid from glucose, maltose, sucrose, and xylose; and is oxidase-negative. The bacterium was obtained from corn leaves infected with *H. turcicum*, but pathogenicity trials on corn were negative. These characteristics warrant its placement in the genus *Pseudomonas*. Additional characteristics of the organism were determined with Minitek tests (BBL Division of Becton, Dickinson and Co., Cockeysville, MD 21030) (Table 4).

DISCUSSION

A disease was produced in *H. turcicum* grown in plates of PDA with 1-, 2-, or 3-wk-old colonies of a pseudomonad. The disease developed whether or not mycelium of *H. turcicum* touched the bacterial colonies and was cell-free production of disease. The disease or change in *H. turcicum* was permanent, subcultures were always diseased, and the pathogenic agent appeared to be transmitted to healthy *H. turcicum* by hyphal contact inoculation with diseased mycelium. There was strong evidence that the lethal

TABLE 3. *Helminthosporium maydis* race T colonies, started from single conidia, on PDYA (1.2% peptone) 10–12 mm from 7- and 10-day-old colonies of the pseudomonad and transplants of 24-hr-old colonies of *H. maydis* on PDYA with 14- and 21-day-old colonies of the pseudomonad. The treated colonies of *H. maydis* were subcultured 2 wk after placement of conidia or transplants

Time after subculture (hr)	Subcultures (no. apparently dead/no. subcultured) of treated *H. maydis* on fresh PDA after 14 days with pseudomonad cultures that were:			
	7 days old	10 days old	14 days old	21 days old[a]
24	190/200	28/200	0/75	0/75
48	178/200	20/200	...	...
72	166/200[b]	17/200	...	...

[a] Colonies of *H. maydis* averaged 3 mm in diameter.
[b] Few apparently dead transfers grew after 72 hr.

TABLE 4. Diagnostic characteristics of the *pseudomonas* sp. bacterium

Characteristics	Reaction
Lactose utilization	–
Glucose (anaerobic)	–
Arginine dihydrolase activity	+
Lysine decarboxylase activity	–
Ornithine decarboxylase activity	–
ONPG (β-galactosidase) activity	–
Phenylalanine deaminase activity	–
Indole production	–
NO_3 reduction	+
N_2 production	–
Starch hydrolysis	–
Citrate utilization	+
Triple-sugar/iron-agar reaction	alk/alk
Growth on eosin-methylene-blue agar	–
Acid production from	
glucose	+
maltose	+
sucrose	+
xylose	+
Oxidase	–

agent also multiplied either on or in the mycelium *H. maydis* race T.

Two opposing mechanisms were considered to explain the production of disease in *H. turcicum* and the killing of *H. turcicum* and *H. maydis*: induction and transmission. Induction implies the activation of something in an organism that begins to reproduce and cause a permanent change. The level of disease produced in *H. turcicum* was much lower when the fungus was exposed to 4-wk-old bacterial colonies on PDA. Induction of a lethal agent would have required that both *H. maydis* and *H. turcicum* possess such an agent: either an integrated viral agent or a gene that caused self destruction and that could be activated by the pseudomonad. For these reasons, induction as a cause of disease and death in these fungi seemed implausible.

Disease in *H. turcicum* on PDA and the death of colonies of *H. maydis* and *H. turcicum* on PDYA (1.2% peptone) all appeared to be the result of infection by an agent produced by the pseudomonad. If this is true, it raises two important biological questions. First, whether other bacteria can be reservoirs and vectors of agents infectious to other organisms, and second, whether an agent can cause infection, produce severe symptoms and even death of cells, yet not reproduce a transmissible entity. Death of *H. maydis* cells after exposure to the activity of the pseudomonad could be classified as a hypersensitive reaction. The local lesion reaction of plants to inoculation with plant viruses is regarded as a hypersensitive reaction, but the infecting virus reproduces, is present in the necrotic tissue of the lesions, and is capable of causing reinfection. The production by bacteria of bacteriocins, which bind and are lethal to, but fail to reproduce in, other closely related strains, resembles the activity of the pseudomonad against *H. maydis*.

The production of disease in *H. turcicum* treated with the pseudomonad occurred in fewer days on PDA than on NDA, suggesting that medium influences the interaction.

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