Nature of a Factor Causing Interstrain Lethality in Ustilago maydis

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ABSTRACT

A substance released by a strain of *Ustilago maydis* killed cells of a sensitive strain of this same organism. This phenomenon is called interstrain lethality. Conditions for maximal production of the lethal factor were different from those for maximal growth. The factor was a protein inactivated by pronase and chymotrypsin, but not by trypsin. Sensitive cells were killed immediately upon exposure to the lethal factor. Phytopathology 61: 50-53.

Ustilago maydis, a heterobasidiomycete, is the cause of the boil smut disease of corn. Characteristics of the organism and its development in corn have been summarized by Christensen (2).

Puhalla (7) isolated strains of this fungus which inhibited the growth of related strains (interstrain inhibition). Production of the inhibitory substance was shown to be controlled by cytoplasmic genetic elements. Strains which produced the inhibitory substance were designated "P1"; strains which did not, "P2." The P2 strains could be subdivided into two groups: P2s, strains sensitive to P1, and P2r, strains resistant to P1. All P1 strains were resistant to the inhibitory substance.

Somewhat similar inhibitors occur in other organisms; e.g., the killer factor in yeast (8), the killer factor in Paramecium (1), and the bacterial colicins (6). Similarities between the genetics of these systems and the production of inhibitor in *U. maydis* have been discussed (7).

Herein we report the isolation of the inhibitor substance, some of its properties and mode of action, and the nutrition of the P1 strain relative to production of the inhibitor.

Materials and Methods.—The P1 and the P2s strains were isolated from natural infections of corn (7).

The complete and minimal media were described by Holliday (4). Fresh cells were taken from streaks grown at 30 C for 24 hr on complete agar medium. Streaks were stored at 3 C as stock cultures and transferred monthly. Growth and nutrition studies employed unbuffered liquid minimal medium adjusted to pH 7.0 or minimal medium buffered with sodium phosphate-citric acid (3). Minimal medium, with 2% monosodium glutamate (w/v) and 2% agar, was used in the pad plate assay described below.

All studies employed 250-ml Erlenmeyer flasks containing 30 ml of liquid medium inoculated with fresh P1 cells. The flasks were shaken at 220 oscillations/min. After various periods, samples of the culture were centrifuged at 20,000 *g* for 20 min to remove cells, and the supernatant was analyzed for inhibitor activity. A pad plate technique was used to assay inhibitor activity. A suspension of ca. 10^6 fresh cells/ml of P2s were added to an equal volume of liquefied minimal agar medium with glutamate which had been cooled to 48 C. Two ml of this mixture was poured over a base layer of solid minimal medium containing 2% glutamate and allowed to harden. Sterile absorbent discs (12.7 mm diam; No. 740-E, Schleicher and Schuell, Keene, N.H.) were placed on the surface of the seeded overlay. To each disc was applied 0.05 ml of the P1 culture supernatant. Arbitrarily, 0.05 ml of culture supernatant was designated one unit of inhibitor activity. The plates were incubated at 30 C for 36 hr, and the diam of the zones of inhibition was measured.

Cell density was used as an index of growth in liquid medium. Suspensions were diluted and counted in a hemacytometer.

Pronase (B grade) and trypsin (salt free, crystallized, A grade) obtained from Calbiochem, Los Angeles, Calif.; and α-Chymotrypsin (Type II, 3 × crystallized) obtained from Sigma Chemical Co., St. Louis, Mo., were prepared in pH 7.0 phosphate buffer at a concn of 10 mg/ml.

Results.—The simplest medium in which the P1 strain grew and produced inhibitor was unbuffered minimal medium with 1% glucose as the sole source of carbon. A 48-hr shake culture at 30 C produced sufficient inhibitor to be assayable by the pad plate technique. Presence of inhibitor was indicated by a sharply delineated clear zone around the filter paper pad in an otherwise opaque lawn of P2s colonies. A straight line function was obtained when the diam of the zones of inhibition were plotted against dilutions of the culture supernatant (0 to 1:50) representing a range of 1 to 0.02 units. The inhibitor produced under these conditions was used as a temporary standard. Repeated plating of the standard on complete agar medium showed that no residual P1 cells were present. Sterilization of the inhibitory material was difficult by standard methods, as it was heat-labile and difficult to filter through a Millipore filter (0.40 μ pore size). Seitz filtration also resulted in excessive loss of activity, but was used in some experiments. Subsequent experiments on the nutrition of P1 cells relative to inhibitor production were evaluated in terms of the temporary described above.

Effect of pH, temp, and length of incubation on inhibitor production.—After 2 days' growth of P1 cells in unbuffered minimal medium, the pH dropped from 7.0 to about 2.5. In order to test whether the pH of the medium affected the amt of inhibitor produced, a series of buffered minimal media were tested. The effect of different pH values on inhibitor production and growth

of the organism at 25 C and 30 C is shown in Fig. 1. Maximal growth occurred at pH 4.0, whereas maximal inhibitor production was at pH 7.0.

Temperature and length of incubation also had a profound effect on inhibitor production (Fig. 2). Maximum inhibitor production occurred in 72 hr at 25 C, whereas maximum growth was at 30 C. After prolonged incubation (120 hr), the inhibitor concn showed a decline.

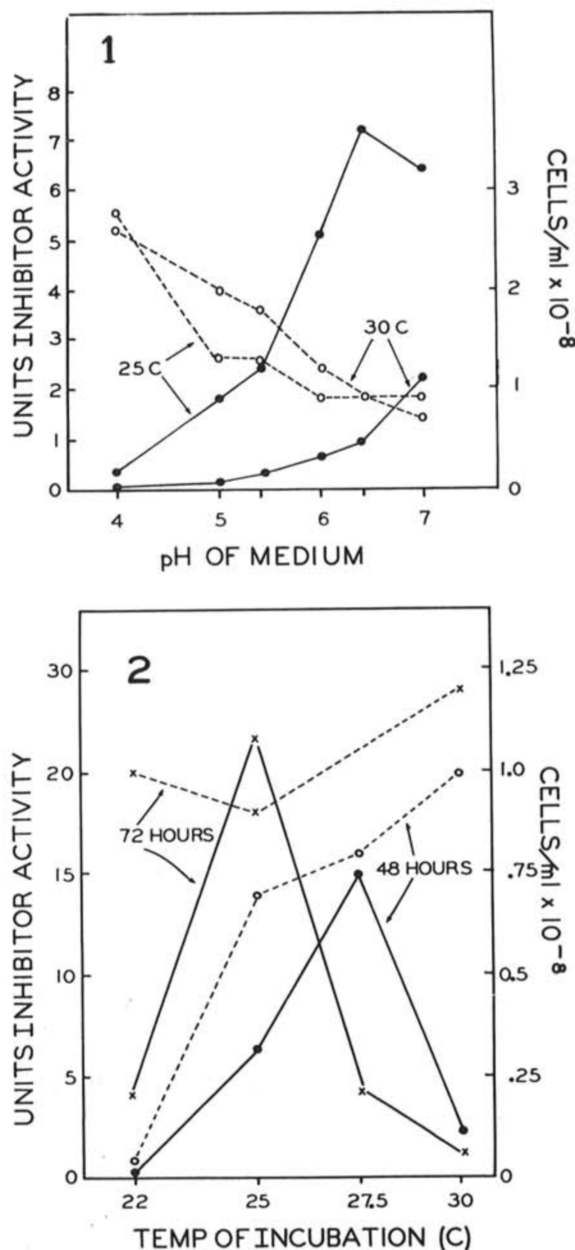

Fig. 1-2. Inhibitor production and growth of P1 (inhibitor producing) cells of *Ustilago maydis*. **1)** Effect of pH in shake culture incubated at 25 and 30 C for 48 hr; dotted lines indicate growth and solid lines indicate inhibitor production. There was little or no growth below pH 4.0. **2)** Effect of temp in shake culture at pH 7.0 and incubated for 48 and 72 hr; solid lines = inhibitor activity; dotted lines = growth.

Preparation of a uniform standard.—The preceding experiments on the relationship of time, temp, and length of incubation to inhibitor production permitted a determination of optimal conditions for maximum inhibitor production. These were a pH of 7.0, an incubation temp of 25 C, and an incubation time of 72 hr. This regime resulted in a 21-fold increase in inhibitor concn over the temporary standard. A large amt of culture fluid prepared under these conditions was frozen, and samples were used as a new uniform standard in the following experiments.

Characteristics of inhibitor.—The effect of heat on the inhibitor was determined, and a rapid decrease in activity was found with increase in temp. No activity was detected after treatment for 30 sec at 80 C. At 60 C, 64% of the activity remained after 1 min and then decreased to 2% after 10 min. A 50% loss in activity was detected after treatment for 10 min at 50 C. At 40 C, however, the inhibitor was very stable, even after 2-hr incubation.

The inhibitor solution was adjusted to either pH 2 or pH 11 and allowed to remain at 25 C. After various time intervals, aliquots were taken, the pH was adjusted to 7.0, and their inhibitory activities were tested. After 2 hr at pH 11, there was a 77% loss of activity; after 5 hr, no activity remained. On the other hand, even after 24 hr at pH 2.0 there was only a 40% loss of activity. Inhibitor kept at pH 7.0 showed no loss of activity after 24 hr.

Four standard units of inhibitor (0.4 ml volume) were treated with 1 mg of pronase, trypsin, or chymotrypsin in pH 7.0 buffer at 30 C for 4 hr. There was complete loss of activity with the pronase and chymotrypsin treatment, but none with trypsin. Doubling the trypsin concn had no effect. Boiled controls insured that inactivation was dependent on the catalytic activity of the enzymes.

The inhibitor substance was not dialyzable through cellophane. No loss of activity resulted when the inhibitor was concd by lyophilization. Protein determination by the Lowry et al. method (5) of several inhibitor samples indicated less than 0.1 mg of protein/ml in the uniform standard inhibitor. Since the amount of protein in the inhibitor sample was so low, the technique of precipitation of proteins with ammonium sulfate was not feasible as a method for isolation of the inhibitor substance. Preliminary tests, however, showed that almost all of the activity could be precipitated by bringing the culture fluid to 40% saturation with ammonium sulfate. The inhibitor activity was eluted at the solvent front when chromatographed on either G-50 or G-25 Sephadex with 0.05 M phosphate buffer as eluting agent.

Mode of action of the inhibitor.—P2s cells (sensitive) were exposed to various concn of inhibitor in shake culture for time periods up to 2 hr. After exposure, the cells were washed with water and tested for viability by plating on complete medium. The amount of residual inhibitor in the system was tested immediately after

TABLE 1. Percentage kill of P2s *Ustilago maydis* cells exposed to various concn of inhibitor produced by P1 cells[a]

Exposure time (min)	Concn of inhibitor/ml					
	5.24	1.05	0.52	0.11	0.05	0.02
0.5	100	50	39	0	0	0
15	100	>99	98	40	17	10
60	100	>99	>99	60	38	33
120	100	>99	99	73	41	27

[a] Concentration of P2s cells was 1.2×10^6/ml in a total test volume of 10.5 ml in pH 5.0 buffered minimal medium. Incubation temp was 30 C.

the P2s cells were added to the inhibitor and again after an incubation of 2 hr at 30 C. Results of this experiment are given in Table 1. This experiment showed that the sensitive cells were killed and not merely inhibited. Furthermore, there was a direct relationship between concn of inhibitor and killing of P2s cells. The level of residual inhibitor in the system dropped dramatically as killing occurred. For example, at an initial inhibitor concn of 5.24 units/ml, the P2s cells (1.2×10^6 ml) removed 32% of the activity as soon as they contacted the inhibitor. After an incubation of 2 hr at 30 C, the cells removed 55% of the inhibitory activity. At lower concn of inhibitor (1.05 and 0.52 units/ml), all of the activity was removed by the same concn of P2s cells after 2 hr. Sensitive cells which were killed by boiling apparently removed inhibitor activity from solution as effectively as live cells.

Site of action of inhibitor.—An attempt was made to recover viable inhibitor-treated P2s cells by a subsequent treatment with the enzyme pronase. Sensitive cells were treated with inhibitor; and after specified time intervals, the cells were treated with pronase solution for 3 hr at 30 C. There was no reversal of inhibition with this treatment.

DISCUSSION.—Such cultural factors as temp, pH, and incubation period markedly affected the growth of P1 *U. maydis* cells and production of inhibitor. In no instance did conditions for maximal inhibitor production coincide with maximum growth. The lower amt of inhibitor produced at pH 4 seems to be associated with decreased synthesis rather than with greater instability of the substance. Even prolonged incubation of inhibitor at pH 2 caused relatively little loss in activity. The optimal temp for inhibitor production was 25 C, whereas optimal temp for growth was 30 C. It appears, therefore, that there is a qualitative difference in the metabolic pathways used by the organism at these two temp.

Puhalla (7) found that P1 cells growing on unbuffered solid minimal medium did not produce inhibitor unless 2% of glutamate was added to the medium. We have shown that inhibitor was produced in unbuffered liquid minimal medium without supplements. These data may be reconciled on the basis of our experiments relating pH of the medium to inhibitor production. In unbuffered liquid medium the pH dropped rapidly from 7 to 2.5. In contrast, if this medium was supplemented with 2% glutamate, the pH rose from 7 to 8 during growth of the P1 cells. Such changes in pH undoubtedly occurred on solid media, but the change was localized around the developing P1 colony. The sharp drop in pH around the colony on the unbuffered medium could prevent synthesis of inhibitor. If this supposition was correct, buffering the solid minimal medium at pH 7.0 should have the same effect as adding glutamate. We found this to be true.

Previous studies (7) also showed that a P1 strain of *U. maydis* will mate with a P2s strain in the corn plant, whereas they do not mate on agar plates. This may be explained in part on the basis that the plant sap is not at the optimal pH for inhibitor production. If inhibitor is produced by the P1 strain in the plant, the concn may be too dilute to prevent mating. There is also the possibility that as low amt of inhibitor are produced they are either translocated or inactivated at the site of elaboration by the plant.

Our results suggest that the inhibitor is protein in nature. It is heat-labile, nondialyzable, alkali-labile, but relatively stable to acid. It can be precipitated by ammonium sulfate and by cold acetone. On the other hand, we have not been able to detect substantial amt of protein in culture fluids of *U. maydis* cells. Thus, the potency of the inhibitor per unit of protein appears to be extremely high.

Activity of the inhibitor is destroyed by treatment with pronase, an enzyme which degrades protein to the level of amino acids. The more selective proteolytic enzyme, chymotrypsin, also destroyed the inhibitor, but trypsin did not, which suggests a method for examining the amino acid linkages of the inhibitor molecule.

Since sensitive cells did not recover from even a short exposure to inhibitor, and were stained dark blue with a dilute solution of methylene blue, we concluded that the cells are not merely inhibited, but are killed. Living cells, on the other hand, reduced the stain to a colorless form. The interaction between a P1 and P2s strain of *U. maydis* has been called interstrain inhibition (7), but since the P2s cells are killed, we propose that the phenomenon be called interstrain lethality.

The rapidity with which cells were killed and the rapidity with which the lethal substance was removed by both living and dead sensitive cells strongly indicated that the substance acted at least initially at the surface of the cell. Substances similar to the lethal factor of *U. maydis* are the killer factor in yeast (8) and the colicins of enteric bacteria (6). All are proteins and are secreted into the culture medium. In contrast to the lethal factor of *U. maydis*, the killer factor in yeast is quite labile even under normal laboratory conditions and must be stabilized with gelatin. The colicins ex-

hibit a surface interaction with the bacterial cells. When bacterial cells exposed to colicin were subsequently treated with trypsin, however, they revived. We have not found such a reversal in the *U. maydis* system.

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