Alteration of Fungal Morphology Induced by a Substance from *Bacillus cereus*

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SUMMARY

A strain of *Bacillus cereus* inhibited growth of some fungi and induced the formation of spherical cells. The substance responsible was identified as a thermostable protein active between pH values of 4.0 to 7.0. The substance affected fungi from various classes, but was not equally effective with different fungi. Leakage from cells exposed to the substance was demonstrated. The relation of this substance to the lysins of *B. cereus* is discussed.

INTRODUCTION

Difficulties in incorporation of large molecules during studies on sexual morphogenesis in *Schizophyllum commune* (Raper, 1966; Raper & Raper, 1968) have stimulated a search for agents capable of inducing sphaeroplast formation. In the course of this search an inhibitory effect caused by a bacillus was noticed. The bacillus, identified as *Bacillus cereus*, also induced the formation of spherical cells. Studies were therefore initiated to characterize the inducing substance and to determine its effect on fungi.

Bacillus cereus is known to produce various bacteriolytic substances (lysins) (Greenberg & Halvorson, 1955; Norris, 1957; Bernheimer & Grushoff, 1967*a, b*; Bernheimer, Grushoff & Avigad, 1968; Koga & Kusaka, 1968; Kusaka, 1969). However, it will be shown that the substance inducing the formation of spherical cells in fungi differs from the bacteriolytic substances produced by the same organism.

The induction of spherical cells in fungi by substances of low molecular weight and by unknown agents in the presence of other micro-organisms has been described earlier (e.g. Porter, 1924; Bent & Morton, 1963). The present study provides a partial characterization of the specific agent formed by *Bacillus cereus* that induces the formation of spherical cells in fungi, and demonstrates its proteinaceous nature.

METHODS

Organisms. The bacillus inducing the alteration of fungal morphology was isolated as a contaminant from an agar plate of *Schizophyllum commune* and identified as *Bacillus cereus* according to the diagnostic criteria described in *Bergey's Manual for Determinative Bacteriology* (1957). Other bacilli used were obtained from the collection of the Department of Food and Biotechnology of the Israel Institute of Technology.

The strains of *Schizophyllum commune* were from the collection of Dr John R. Raper of Harvard University. All other fungi used, with the exception of *Collybia*, were obtained from Dr M. Chortin, Department of Plant Pathology, Volcani Institute for Agricultural

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Research, Beit-Dagon, Israel. *Collybia* was obtained from the collection of Dr G. Simchen, Genetics Laboratory, Hebrew University, Jerusalem, Israel.

Culture media. The bacteria were grown in a medium (designated henceforth as YE) containing 2% (w/v) glucose, 0.5% yeast extract and 0.5% peptone. *Schizophyllum* was grown in complete medium (CM) and minimal medium (MM) described by Raper & Miles (1958). All other fungi used in the study were grown on potato dextrose agar (PDA).

Production and characterization of the inducing substance. Cultures of *Bacillus cereus* were grown in 250 ml. flasks containing YE broth on a rotary shaker at 30°. The flasks were inoculated with 1 ml. of a suspension of a 16 h. culture. Twenty h. after inoculation the broth was centrifuged (25,000 g for 15 min.) and the supernatant was assayed for activity after filtration through a Millipore filter (0.45 µm.).

Activity was assayed as follows: One ml. samples of the supernatant were distributed in sterile test tubes into which agar blocks containing fungal mycelium were introduced. After incubation at 25° for a minimum of 10 h. the mycelium was examined microscopically for the presence of spherical cells.

Characterization of the active substance followed its separation from the medium by ammonium sulphate fractionation and dialysis of the precipitate against 10⁻³ M-citrate-phosphate buffer (pH 5.6) and in some instances following chromatography on Sephadex G-200 with the same buffer.

Tests for induction of leakage from the cells were performed with prelabelled germlings of *Schizophyllum*. The spores were obtained from a fruiting dikaryon and the germlings were labelled by incorporation of [¹⁴C]glucose, [³H]phenylalanine, or [³H]deoxyuridine during growth in MM in the presence of each of the labelled compounds. To increase the efficiency of incorporation 'cold' glucose and asparagine were omitted from the medium when labelled glucose and phenylalanine respectively were used. After 54 h. growth in the presence of the labelled compound the germlings were harvested on a Millipore filter, washed with the corresponding 'cold' compound and placed in a scintillation vial into which 2 ml. of YE, or YE and the substance inducing the alteration of fungal morphology (MAS), was added. The vials with the germlings were incubated at 25° and the leakage of labelled compounds from the cells was determined at various times by measuring the radioactivity, in filtered 0.1 ml. samples of YE and YE+MAS, in a liquid scintillation counter (Packard Model 3310).

RESULTS

Effect of MAS on various micro-organisms. *Bacillus cereus* inhibited growth of *Schizophyllum commune* and induced the formation of spherical cells; the effect was not limited to the terminal cells. Growth inhibition and spherical cell formation on exposure to MAS were also shown by *Alternaria tenuis*, *Aspergillus niger*, *Claviceps paspali*, *Fusarium moniliforme*, *Pullularia* sp. and *Trichoderma viride*. However, *Aspergillus tereus*, *Collybia velutipes*, *Nematoctonus* sp., *Penicillium notatum* and *Phytophthora parasitica* were insensitive to MAS. Thus not all fungi were equally affected, and the induction of spherical cells occurred among representatives of different classes. Among the organisms tested *F. moniliforme* was extremely sensitive (Pl. 1).

To determine if inhibition of growth and the induction of spherical cell formation in *Schizophyllum commune* was exclusive to *Bacillus cereus* the effect of various bacilli was examined. *Bacillus subtilis*, *B. mycoides* and *B. thuringiensis* neither inhibited the growth of the fungus nor induced the formation of spherical cells. *Bacillus megaterium* and *B. polymyxa* inhibited the growth of the fungus but did not induce the formation of spherical cells. Only

the *B. cereus* isolated originally from the plates of *Schizophyllum*, and other isolates of *B. cereus* from the bacterial stock collection inhibited the growth of *S. commune* and induced the formation of spherical cells.

EXPLANATION OF PLATE

The effect of the substance (MAS) produced by *Bacillus cereus* on fungal mycelium.

Fig. 1. *Schizophyllum commune* exposed to MAS.

Fig. 2. Normal mycelium of *Schizophyllum commune*.

Fig. 3. *Fusarium moniliforme* exposed to MAS.

Fig. 4. Normal mycelium of *Fusarium moniliforme*.

Fig. 5. Reformation of the mycelium of *Schizophyllum commune* from detached cells after long exposure to MAS.

Nature and properties of MAS

A cell-free filtrate of the medium in which the *Bacillus* was grown contained the active substance. The substance was precipitated by ammonium sulphate. Activity was distributed between the precipitate and the supernatant at 50 % extinction, but only in the precipitate of the 70 % fraction.

The molecular size was not small, as the substance was non-dialysable. Furthermore, in column chromatography on Sephadex G-200, with Blue Dextran and cytochrome *c* as markers, it was eluted nearer to Blue Dextran (Fig. 1). The substance was excluded (i.e. was in the first fraction) from a Sephadex G-100 column. Thus the molecular weight of the active substance is probably within the range of 1 to 2×10^5 .

Activity was recovered in fractions with negligible extinction at 260 nm. and maximal extinction (E) at 280 nm. The ammonium sulphate fractionation and the extinction characteristics of the active fractions thus suggest that MAS was a protein.

Fig. 1. Molecular exclusion chromatography of the fraction precipitated by $(\text{NH}_4)_2\text{SO}_4$ (70 % saturation) on a 30 cm. Sephadex G-200 column (10^{-3} M-citrate-phosphate buffer, pH 5.6). Cytochrome *c* was characterized by its extinction spectrum.

In tests at temperatures between 4° and 55° the substance was active between 12° and 37° . However, whereas at the lowest temperature tested the mycelium of the indicator fungus (*Schizophyllum commune*) was viable, this was not the case at the highest temperature. Only following clarification of the biochemical effect of the substance will it be possible to determine the upper limit of activity.

In tests at a range of pH values from 3.0 to 8.5 at intervals of 0.5 units the substance was active between pH 4.0 and pH 7.0 with a decline in activity at the two extremes. Maximal activity was obtained at pH 5.0 and 5.5.

Although the precipitation and extinction characteristics suggest that MAS is a protein, it remained active after exposure to 100° for 70 min. The stability at this temperature is

reminiscent of spore proteins (for review see Kornberg, Spudich, Nelson & Deutscher, 1968). However, the degree of inactivation of MAS by high temperature cannot be accurately quantified before defining a biochemical reaction in which it participates. It is conceivable that the substance is a complexed protein in which the protein portion of the molecule is stabilized. Additional indications to this effect were found in tests to determine the effect of proteolytic enzymes on MAS. After treatment with trypsin (400 $\mu\text{g./ml.}$) and chymotrypsin (100 $\mu\text{g./ml.}$) for 5 h. at pH 8.5 no discernible decline of activity was observed. MAS was totally inactivated by pronase (100 $\mu\text{g./ml.}$) only after exposure to the proteolytic enzyme for 5 h. at pH 8.5.

Fig. 2

Fig. 3

Fig. 2. Leakage from cells of *Schizophyllum commune* induced by MAS. □, Prelabelled with [¹⁴C]glucose and exposed to MAS; ■, control. ○, Prelabelled with [³H]phenylalanine and exposed to MAS; ●, control. Δ, Prelabelled with [³H]deoxyuridine and exposed to MAS; ▲, control.

Fig. 3. Time of induction of leakage from cells of *Schizophyllum commune* labelled with [³H]-phenylalanine. ○, Cells exposed to MAS; ●, control.

Cholesterol and ergosterol (10 to 100 $\mu\text{g./ml.}$) had no effect on the activity of MAS. However, the surface-active agents Tween 40 and Tween 80 (0.1 to 1.0%, v/v) inhibited the activity of MAS.

The effect of MAS on fungal and bacterial cells. Long exposure of mycelium of *Schizophyllum* to MAS (5 to 10 days) resulted in the dissociation of the spherical cells from the mycelium. Groups of these were found in the supernatant after slow centrifugation (2000 g for 5 min.). Dissociation was extensive if the mycelium was kept in the presence of MAS on a rotary shaker. The individual cells could germinate and reform the mycelium (Pl. 1). If germination occurred in the presence of MAS new spherical cells were formed; if germination occurred in the absence of MAS normal growth was resumed. Only some spherical cells exhibited osmotic sensitivity to distilled water. Addition of up to 10^{-2} M-EDTA did not increase the proportion of cells sensitive to such osmotic conditions.

By the use of various metabolic precursors it was shown that MAS induced leakage from the cells of *Schizophyllum* (Fig. 2, 3) within 2 h. of exposure (Fig. 3). The results of such a test for leakage often reflect the state of the membrane. However, the instability of the membrane may not have resulted from a direct effect of MAS, but rather from an alteration of wall structure induced by MAS.

Because various lysins isolated from *Bacillus cereus* were reported to induce lysis of *B. cereus* and *B. subtilis* (Bernheimer *et al.* 1968; Kusaka, 1969) the effect of MAS on bacteria of these two species was examined to evaluate the relations of MAS to the various lysins. Exposure of the bacteria to MAS for 24 h. did not affect the viability and therefore did not induce lysis.

DISCUSSION

The substance isolated from *Bacillus cereus*, which induced formation of spherical cells of fungi, differed from the bacteriolytic substances associated with spore-forming aerobic bacteria (Greenberg & Halvorson, 1955; Norris, 1957; Koga & Kusaka, 1968; Kusaka, 1969) in that: (a) it was thermostable, (b) its effect was shown in the presence of sterols and (c) it did not induce lysis in bacteria. The only common property found thus far is the proteinaceous nature of both the lysins and MAS.

The effects of MAS on fungi were the induction of formation of spherical cells, the dissociation of the mycelium and leakage from the cells. However, osmotic sensitivity was not uniform among the spherical cells, which suggested that these cells retained at least part of the cell wall and thus retained their rigidity. The effect of MAS on fungal cell wall is being currently examined by both chemical and cytological analysis. Although it has recently been shown that cells of temperature-sensitive mutants of *Aspergillus nidulans* with defective synthesis of amino sugars assume a spherical shape (Cohen, Katz & Rosenberger, 1969) the effect of MAS may be of a degradative nature rather than inhibition of synthetic processes since the effect was not restricted to the terminal cells.

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