Identification and Physiological Studies of Soil Bacteria Causing Fungistasis[1]

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Introduction

Most fungi in soil are represented only by inactive propagules (12). Germination is not limited by lack of nutrients or other essential growth factors but by inhibitors. Dobbs and Hinson (5) described the phenomenon of fungistasis in soil and demonstrated that spores of most fungi fail to germinate because of this principle. Park (9), Jackson (6), and Lingappa and Lockwood (7) have confirmed this phenomenon. Jackson (6) suggested that fungi could be grouped into 3 categories, depending upon sensitivity, to the fungistatic principle. These include a) significant reduction in spore germination, b) spores unaffected and c) spore germination stimulated.

The fungistatic principle is assumed to be of biotic origin, since heat treatment, prolonged drying, treatment with organic solvents and elution with citrate buffer reduced or destroyed activity (5). Lingappa and Lockwood (7) showed that pH, redox potential, osmotic conditions and volatile substances from soil were not responsible for fungstasis.

The fungistatic principle has not been separated from soil. The failure of spores to germinate may be due either to the presence of some inhibitory factor or the absence of essential growth substance (2). Lockwood (8) found that certain *Streptomyces* spp. restored activity to sterile soil and suggested antibiotics may be involved but Brian (2) considered antibiotics to be significant only if soil was heavily enriched with organic matter. Dobbs and Hinson (5) found that the fungistatic effect could be overcome by the addition of glucose to soil but Chinn and Ledingham (3) found pure glucose ineffective. Enrichment with crude organic matter such as soybean meal reduced fungistasis, however.

Although it is assumed that the fungistatic principle is of biotic origin, little is known concerning which components of the soil microflora or fauna are responsible or the ecologic or physiologic conditions necessary for production of the principle.

The objectives of this research were to:

1) Isolate representative genera of bacteria and antinomycetes from soil and develop methods to assay for fungistatic activity in a cell-free medium and,

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2) To consider the influence of culture medium, cell age, population and metabolic inhibitors on production of the fungistatic principle.

Materials and Methods

The bacteria and actinomycetes used were isolated from soil and characterized by the conventional procedures. Some representative genera were from stock cultures.

Three different methods were used to assay for fungistatic activity *in vitro*. The test fungi were *Trichoderma viride* Pers. ex. Fries and *Pestalotia macrotricha* de Not. Conidial suspensions from 6-9 day old cultures were used in all tests with the criterion for fungistatic activity being the reduction in spore germination when compared to controls. The bacteria and actinomycetes were grown in shake culture in modified nutrient broth-yeast extract medium. Where they were incorporated in water agar, the agar was washed to remove any traces of nutrients.

Method 1. The bacteria were separated from the growth medium by filtration through a sterilized vacuum-type millipore apparatus. The pad (pore size 4.45u) and bacteria were washed with 200 ml of sterile deionized water to remove nutrients. The pad was then inverted in a sterile petri dish, and 2 discs (12 mm diam.) of sterile water agar were placed on the opposite side of the pad. Since the bacteria were separated from the test fungus, the active principle had to diffuse through the millipore pad. A drop of conidial suspension of *T. viride* was placed on each disc and incubated 24 hours as 26°C in a humidity chamber.

Method 2. The bacteria were separated from the medium at 7000 G for 5 min in a Servall centrifuge. The pellets were washed 3 times, using centrifugation and resuspension of cells, to remove nutrients. To assure uniform germination of the condiospores, the pH of the bacterial pellet was adjusted to 5.7-6.5 with phosphate buffer. An 8.5 ml aliquot of this cell system was added to 4 ml of cooled, 2X water agar. After the agar solidified, conidia of *T. viride* were spotted in 4 places, and the plates were incubated at 26° for 24 hrs. Agar blocks were cut from the plates after 24 hr and spore germination determined as in Method 1.

Method 3. The bacteria were not separated from the growth medium prior to testing. Ten 60 x 25 mm pads of Whatman #1 filter paper were stacked in a section of a deep (23 mm) petri dish along with a flat bottom crucible. After sterilization, 1 ml of water was added to the crucible to maintain humidity for spore germination. The bacterial suspension was harvested, pH adjusted to 5.7-6.5 and 5 ml used to saturate the filter paper pads. Two sterile water agar blocks (10 mm x 10 mm) were placed on the saturated filter paper stack and 1 drop of a conidial suspension of *T. viride* was pipetted onto each block. The plates were incubated for 24 hr and percent germination determined as in Method 1.

Representative genera of the bacteria and actinomycetes were grown on 3 media to determine the effect of medium on fungistatic activity *in vitro*. The 3 media used were glucose-yeast extract (GYE), glucose-peptone (GP) and Medium A, a modification of GP.

Glucose Yeast Extract Broth (GYE)

Glucose	2.00 gm	NH_2NO_3	0.20 gm
Yeast extract	0.20 gm	$MgSO_4$	0.20 gm
Peptone	0.07 gm	$FeCl_3$	0.001 gm
Beef extract	0.04 gm	$CaCl_2$	0.010 gm
K_2HPO_4	0.40 gm	A to Z solution	1.0 ml
KH_2PO_4	0.40 gm	H_2O	1.01

Glucose Peptone Broth (GP)

Glucose	5.0 gm
Peptone	5.0 gm
DL Methionine	0.1 gm
H_2O	1.01

Medium A

Glucose	10.0 gm
Peptone	1.25 gm
Beef extract	0.75 gm
NH_4NO_3	0.50 gm
K_2HPO_4	0.25 bm
KH_2PO_4	0.75 gm
$MgSO_4$	0.02 gm
$FeCl_3$	0.001 gm
H_2O	1.01

The bacteria were grown 4 days in the 3 media and a comparison of fungistatic activity determined, using method 2.

The genera used in the study of the effect of age upon production of the fungistatic principle were grown 1 and 8 days in shake culture to obtain cells in the logarithmic stage of growth and as resting cells. The cells were harvested after the predetermined incubation period and tested for fungistatic activity, using Method 2.

In the population density experiment, the lowest population of bacteria/ml necessary to cause total inhibition of spore germination was determined. The bacteria were washed 3 times, using centrifugation and resuspension. An aliquot of the washed bacteria was added to a Petroff-Hausser counting chamber with a Pasteur pipette to estimate the number of bacteria per ml. Serial dilutions of 10^9 to 10 cells/ml were made and an 8.5 aliquot of each population was drawn and tested for activity, as described in Method 2.

The effect of temperature on production of the fungistatic principle by selected bacteria was limited to the range at which 80% spore germination of the test fungi occurred as well as the range of growth of the bacteria. Temperature range for germination of the conidiospores of the 2 test fungi was determined by incubation of spores at 4° to 28°C at 4°C intervals. The bacteria were prepared as outlined in Method 2. The predetermined minimal temperature for 80% germination of *T. viride* and *P. macrotricha* was 16°C and 12°C, respectively. The test plates were incubated at 4°C below the minimum for each test fungus, at the minimum temperature and at 26°C.

The metabolic inhibitors NaN_3 and KCN were used to determine if the biosynthesis of the fungistatic principle was dependent on aerobic respiration. By this means some indication of the nature of the principle might be learned. Washed, 8-day-old cells of species of selected genera of bacteria were suspended in concentrations of 1×10^{-4} to 1×10^{-6} M of the metabolic inhibitors and incorporated into water agar. The dosages were below the levels to which the fungi were sensitive. After incorporation of the bacteria and inhibitors in

water agar, the test conidia were placed on the agar and the assay for fungistatic activity carried out by Method 2.

Results

The genera of bacteria and actinomycetes from soil and selected from stock culture that produce the fungistasis are given in Table 1. In most cases, the same genera occurred in all 3 soils. If a genus taken from each soil produced nearly the same degree of fungistasis, only 1 was included in the later experiments. The genus *Pseudomonas* was in every sample and consistently produced fungistasis. Therefore, it is referred to as *Pseudomonas* spp. If isolates of a genus from different soils or the same soil produced varying degrees of fungistasis, the identity of the isolate was maintained, as *Acromobachter* C14, *Achromobachter* F99, etc.

TABLE 1

The Genera of Bacteria and Actinomycetes from Soil that Produced Fungistasis

Chalmers Silty Clay Loam	Soil of Unknown Origin	Warsaw Loam
Achromobacter C14	*Achromobacter* F99	*Arthrobacter*
Alcaligenes	*Achromobacter* ET	*Achrobacter*
Arthrobacter CA	*Arthrobacter* E2	*Brevibacterium* W10
Pseudomonas	*Bacillus* FZ	*Bacillus*
Micrococcus CQ	*Brevibacterium* ESI	*Rhizobium*
Sarcina C17	*Micrococcus* N2	*Nocardia*
Bacillus	*Nocardia* UO	*Protaminobacter*
Brevibacterium	*Streptomyces*	*Pseudomonas*
Streptomyces	*Arthrobacter* W4	*Micrococcus*
Nocardia CO	*Pseudomonas*	
	Sarcina FS	

The 3 methods used to determine fungistatic activity, **in vitro**, gave comparable results. Method 2 was used throughout these experiments because of its simplicity and ease of preparation.

The influence of the cultural medium on the production of the fungistatic principle by bacteria is given in Table 2.

In some cases, the medium apparently had no effect on production of the fungistatic principle. For example, *Pseudomonas*, *Brevibacterium* ESI, *Arthrobacter* ES2 and *B. subtilis* completely inhibited spore germination regardless of the medium. *Arthrobacter* CA inhibited spore germination when grown on glucose peptone broth and glucose yeast extract but was less active on Medium A. *Rhizobium* spp. and *Sarcina* C17 produced similar results. *Nocardia* spp. and *Proteus vulgaris* do not produce a strong fungistatic effect, but the activity was similar on the 3 media.

In the age studies it was desirable to determine if the production of the fungistatic principle occurred in the logarithmic phase of bacterial growth and if the principle was maintained in resting (8-day-old) cells. Table 3 presents the results.

Most of the isolates, for example, *Pseudomonas* spp., *Nocardia* spp., *Sarcina* FS, *Achromobacter* F99, *Alcaligenes* spp., *Erwinia* spp., *Protam-*

TABLE 2
Influence of Culture Medium on the Production of the Fungistatic Principle

Source	Percent Germination					
	Medium A Broth		Glucose Peptone Broth		Glucose Extract Broth	Yeast
	1[a]	2[b]	1	2	1	2
Arthrobacter CA	48[c]	57	0	0	0	0
Bacillus FL-Z	0	0	5	0	0	0
Nocardia UO	20	41	31	48	29	55
Arthrobacter W4	0	0	0	21	0	1
Proteus vulgaris	23	48	28	59	27	43
Micrococcus N2	0	0	0	9	0	0
Rhizobium spp.	55	77	0	0	0	0
Sarcina C17	42	15	5	3	0	0
Brevibacterium ESI	0	0	0	0	0	0
Pseudomonas spp.	0	0	0	0	0	0
Arthrobacter ES-2	0	0	0	0	0	0
Bacillus subtilis	2	4	0	0	0	0

[a]Conidia of Pestalotia macrotricha used in assay
[b]Conidia of Trichoderma viride used in assay
[c]Average of 2 replications

TABLE 3
The Influence of Age of Bacterial Cells Upon Production of the Fungiastic Principle

Microorganism	Percent Germination			
	P. macrotricha	T. viride	P. macrotricha	T. viride
	1-day-old cells		8-day-old cells	
Brevibacterium W2	68	86	19	93
Arthrobacter ES2	0	20	0	0
Pseudomonas spp.	0	0	0	0
Nocardia CQ	0	0	0	0
Sarcina FS	0	0	0	0
Achromobacter F99	0	0	0	0
Alcaligenes spp.	1	2	0	0
Erwinia spp.	2	5	0	0
Achromobacter 14C	57	0	43	0
Arthrobacter WL4Y	15	48	0	68
Micrococcus CQ	10	74	18	86
Proteminobacter	0	0	0	0
Streptomyces	0	0	0	0

inobacter spp. and *Streptomyces* spp. inhibited germination of conidia of the test fungus after growth for 1 day and the 8 day-old cells were also fungistatic. *Brevibacterium* W2 was slightly inhibitory to *P. macrotricha* after 1 day but increased in fungistatic activity after 8 days. There was no change in the effect with *T. viride*. *Arthrobacter* W4 increased in its fungistatic activity in the 8-day-old cells toward *T. viride*, but decreased in the degree of fungistasis toward *P. macrotricha* in the resting cells.

The cell population necessary to completely inhibit spore germination

of the test fungi was 10^9 cells/ml. The population density of the bacteria did not affect spore germination until concentrations of 10^4 to 10^8 cells/ml were achieved. At a population of 10^7 cells/ml, *Arthrobacter* CA and *Brevibacterium* W10 were fungistatic to *T. viride; Sarcina* FS was fungistatic to *P. macrotricha;* and *Pseudomonas* spp. was totally fungistatic to spores of both test fungi. *Arthrobacter* CA, *protaminobacter*, *Brevibacterium* W10, and *Achromobacter* F99 were totally fungistatic to the test fungi at a poplation of 10^8 cells/ml. When the population had reached 10^9 cells/ml, the bacteria except *Rhizobium* spp. were fungistatic to the test fungi. *Rhizobium* was completely fungistatic to *T. viride* at 10^9 cells/ml, but it was not fungistatic toward *P. macrotricha* at any concentration.

Table 4 presents the results of the influence of temperature upon the production of the fungistatic principle.

TABLE 4
Influence of Temperature on the Production of the Fungistatic Principle

Microorganism	Percent Germination[a]					
	P. macrotricha			T. viride		
	8°C	1212°C	26°C	8°C	1212°C	26°C
Pseudomonas spp.	0[a]	0	0	0	0	0
P. vulgaris	0	66	27	2	39	43
Arthrobacter W4	0	0	0	0	0	0
Achromobacter EST	0	6	68	4	40	82
Nocardia CO	0	27	0	0	17	0
Bacillus spp.	0	18	0	0	30	0
Micrococcus NL2	0	0	0	0	34	0
Brevibacterium ESI	0	20	0	0	70	0
Rhizobium spp.	0	18	90	13	57	0
Sarcina FS	0	0	0	0	91	0
Achromobacter F99	0	0	0	0	5	0
E. coli K12B	0	8	0	10	37	0

[a]Average of 3 replications

Preliminary experiments showed that the lowest temperature which allowed 80% germination of the spores of *T. viride* and *P. macrotricha* was 16°C and 12°C, respectively. The plates of the test bacteria spotted with conidia of *P. macrotricha* were incubated at 8°C, 12°C and 26° C and the plates spotted with conidia of *T. viride* at 12°C, 16°C, and 26°C.

The 16°C temperature altered the fungistasis of *Sarcina* FW, *Brevibacterium* ESI, *Micrococcus* N2, *Rhizobium* spp., *Nocardia* spp., *Bacillus* spp., and *E. coli* toward *T. viride.* Except for *Brevibacterium* EST, and *Nocardia* spp., the fungistatic activity of these bacteria toward *P. macrotricha* was not affected by the low temperature (16°C). *Rhizobium* spp., which was not fungistatic to *P. macrotricha* at room temperature (22-24°C), was inhibitory at 12°C. *Achromobacter* EST was not fungistatic at 26°C but was inhibitory to the spores at the low temperatures.

The results of studies using selected metabolic inhibitors to alter production of the fungistatic principle are given in Table 5. The figures

represent the percent germination of conidia of *T. viride* using KCN and NaN_3 as the inhibitors.

TABLE 5
Effect of Metabolic Inhibitors on the Production of the Fungistatic Principle by Selected Genera of Bacteria

	Percent Germination					
	NAN_3			KCN		
	10^{-4}M	10^{-5}M	10^{6}M	10^{-4}M	10^{-5}M	10^{6}M
Achromobacter F99	81[b]	10	3	11	2	4
Rhizobium spp.						
Pseudomenas spp.	87	42	9	7	1	3
Micrococcus N2	0	0	0	0	0	0
Achromobacter ET	64	30	1	20	2	0
Pseudomonas spp.	0	0	0	0	0	0
Bacillus FZ	2	2	0	0	0	0
Control	80	80	95	95	90	87

[a]Conidia of Trichoderma viride used as test fungus
[b]Results of 2 replications

Concentrations of 1 x 10^{-4} M NaN_3 and 1 x 10^{-6} M KCN, 80% spore germination or greater occurred.

The fungistatic activity of *Achromobacter* F99, *Achromobacter* ET, and *Rhizobium* spp. was markedly reduced after the addition of 1 x 10^{-4} M NaN_3 to the washed cells. As the concentration of the inhibitor decreased from 1 x 10^{-4} to 1 x 10^{-5} M NaN_3, a sharp reduction in spore germination occurred. The addition of the KCN had little or no effect upon the test bacteria. *Pseudomonas* Spp., *Bacillus* FZ and *Micrococcus* N2 were not affected by added metabolic inhibitors.

Discussion

There are 3 orders of bacteria that are prominent in the soil, namely, Pseudomonadales, Eubacteriales and Actinomycetales (1). This study reveals that soil isolates of 12 genera of these 3 orders produce a fungistatic principle. This suggests that the fungistasis phenomenon is widespread among soil borne bacteria.

In some cases, there were differences in the fungistatic activity of the different isolates of a given genus. Also, the 2 test fungi were not equally sensitive to all isolates. For example, the isolates of the genus *Achromobacter* varied in fungistatic activity and the isolate of *Rhizobium* spp. was not fungistatic to the spores of *Pestalotia macrotricha* but inhibited germination of spores of *Trichoderma viride*.

The difference in the fungistatic activity among the genera and isolates of the same genus suggests that the various bacteria either produce different fungistatic substances or the same substance in varying quantities.

Most previous work concerned with fungistasis used soil as the source of the principle. Because of the chemical and physical complexities of the soil, fungistatic assays are difficult to duplicate. Consequently, one of our objectives was to develop methods for assay of the fungistatic activity of soil isolates in a soil-free medium.

When fungistasis is studied *in vitro*, it is important that this phenomenon is not confused with the effects caused by the accumulation of metabolic end products and autolysis of the bacterial cells. By collecting the bacteria on a sterile millipore pad, washing the pad with sterile water and inverting the pad in a petri dish (Method 1) this was avoided. In this position the pad prevented intimate contact between the bacteria and spores but provided moisture for the diffusion of the fungistatic principle. In the other assay methods used, the effects of other metabolites and autolysis were minimized by repeated washing of the cell suspensions by centrifugation and resuspension before testing *in vitro*. The test period was short (24 hr.) so that there was little time for accumulation of metabolic by products which might interfere with the measurement of fungistatic activity.

The pH range of the medium on which fungistatic assays were conducted was critcal for conidia of *T. viride*. If the bacterial suspension to be used in an assay was above pH 6.9 spore germination was curtailed. For accurate measurement of the fungistatic activity, it was necessary to adjust the pH to 5.7-6.5 Treatments were discarded where there was a change in the pH during the 24 hr. incubation period for spore germination.

The number of bacteria per unit volume of medium required to produce total fungistasis of the test fungi was 10^7 to 10^9 cells/ml. The total number of bacteria per gram of soil as determined by direct microscopy provide values on the order of 10^8 to 10^9 cells/gm (1). The total population of any single species in soil is far less than the population used in the fungistatic assays because of the lack of nutrients, space, and/or due to antagonism. This suggests that the diverse population of soil bacteria produces a common substance inhibitory to spore germination. Another explanation is that there are populations of soil bacteria localized in the ecological niches where food and other factors are not limiting, thus giving sufficiently large populations to provide the concentrations necessary for spore inhibition.

Bacteria were subjected to metabolic inhibitors to determine the effect on production of the fungistatic principle. The concentrations of metabolic inhibitors used were not high enough to be fungistatic to conidia of the test fungi. It was assumed that the fungistatic principle was produced during aerobic respiration. Cyanide acts as an inhibitor when the enzyme occurs as ferricytochrome oxidase, whereas, azide inhibits enzyme action when it occurs as ferricytochrome oxidase, (11). Cytochrome oxidase of bacteria may differ from that found in other cells and may differ between species as well (4).

With *Achromobacher* F99, the highest concentration of azide suppressed the production of the fungistatic principle as evidenced by the sharp decrease in spore germination when a 10-fold dilution of the metabolic inhibitor was assayed. *Bacllius* FZ was not affected by the metabolic inhibitors at any concentrations used. These results agree with Clifton (4) in that there might be a difference in the cytochromes involved in the various bacteria. The results also suggest that the cytochrome enzymes are not involved in the aerobic respiration of the bacteria and the subsequent synthesis of the fungistatic principle. This

can be speculated from the standpoint that the cyanide failed to suppress the bacterial production of the fungistatic principle. Smith (10) pointed out that, in a number of instances, observations on the effects of inhibitors on the respiration of bacteria indicated that the cytochrome C oxidase system could not be present. Our results in this study showed that some of the most active producers of fungistatic principle, namely *Pseudomonas* spp., *Micrococcus* N2 and *Bacillus* FZ, were not affected in the presence of the metabolic inhibtors.

Summary

Isolates of 12 different genera of bacteria and actinomycetes from soil were fungistatic to the test fungi *Trichoderma viride* and *Pestalotia macrotricha*. Differences in fungistatic activity occurred among the genera and, in some cases, among isolates of the same genus. The 2 test fungi were not equally sensitive to all isolates. This suggests that either different fungistatic substances are involved or the same substance is produced in varying quantities.

In most cases, the growth medium had no effect upon production of the fungistatic principle, *in vitro*. The principle was produced by the bacteria in the logarithmic phase of growth and as resting cells. The cell population necessary to completely inhibit spore germination of the test fungi was from 10^7 to 10^9 cells/ml. Fungistatic activity was highest when the bacteria were grown at optimum temperatures.

The metabolic inhibitors sodium azide and potassium cyanide interfered with production of the fungistatic principle with certain organisms. It is suggested that either the bacteria have different cytochromes or that cytochrome oxidase is not involved in aerobic respiration and the subsequent synthesis of the fungistatic principle.

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