Fungistatic Activity of Water-Soluble Fluorescent Pigments of Fluorescent Pseudomonads

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ABSTRACT

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Water-soluble fluorescent pigments of 156 fluorescent *Pseudomonas* isolates of plant pathogenic and saprophytic species inhibited the growth of *Geotrichum candidum*. Other fungal species that showed sensitivity to the pigments produced by four *Pseudomonas* isolates were *Rhizoctonia solani, Sclerotinia sclerotiorum, Phymatotrichum omnivorum, Phytophthora megasperma,* and *Pythium aphanidermatum*. A direct correlation was established between the quantity of a partially purified pigment obtained from an isolate of *Pseudomonas fluorescens* and its fungistatic activity against *P. aphanidermatum*. The inhibition could be counteracted by adding iron to the medium in excess of chelating capacity of the added pigment. Results show that fungistatic activity of the pigment is due to its ability to remove iron from the medium by forming an iron-pigment complex. The pigment exhibited properties similar to those of known microbial iron-chelating substances (siderophores).

Water-soluble, yellow-green fluorescent pigments produced by many isolates of fluorescent pseudomonads in certain synthetic media have been used for identification and classification of this group of bacteria (12). These pigments have been reported to be produced in media containing low levels of iron (2,4–6, 11,14). The chemical structures of these pigments are not known.

Meyer et al (8,9) have shown that a fluorescent pigment produced by an isolate of *Pseudomonas fluorescens* is an effective chelator of trivalent iron and exhibited properties similar to those of siderophores. We recently reported that a water-soluble fluorescent pigment produced by 156 isolates of fluorescent pseudomonads exhibited fungistatic properties against a number of important plant pathogenic fungi in vitro by chelating iron from the media (10). Kloepper et al (3) also reported that siderophores produced by fluorescent pseudomonads inhibit the growth of an isolate of *Erwinia carotovora* and an isolate of *Escherichia coli* by chelating iron from the medium. Some siderophores also are reported to be capable of stimulating growth of some aboveground fungi such as *Ustilago sphaerogena* (1), *Aspergillus* sp. (15), and *Colletotrichum muscae* (7).

The objectives of this study were to determine quantitatively the fungistatic and iron-chelating activities of a water-soluble fluorescent pigment of an isolate of *P. fluorescens* and to compare its physicochemical properties with those of a fluorescent pigment characterized by Meyer et al (8).

MATERIALS AND METHODS

Fungistatic activity of the crude pigment. One hundred eighty-six representative isolates of the following fluorescent *Pseudomonas* species were tested for production of fluorescent pigments and for fungistatic activity against *Geotrichum candidum*: *Pseudomonas phaseolicola, P. glycinea, P. lachrymans, P. syringae, P. cichorii, P. marginalis, P. marginata, P. fluorescens,* and *P. putida*. Bacterial isolates were streaked across the center of culture plates containing 15 ml of King's medium (proteose peptone, 2.0%; glycerol, 1.0%; K_2HPO_4, 0.15%; $MgSO_4$, 0.15%; agar, 2.0%). Plates were incubated at room temperature for 72 hr and were spray inoculated with a suspension containing approximately 10^4 spores per milliliter of *G. candidum* produced on potato-dextrose agar (PDA). Plates were incubated at room temperature for about 24 hr and were examined for the presence of the fluorescent pigment and the size of inhibition zones. Sensitivity of *Rhizoctonia solani, Sclerotinia sclerotiorum, Phymatotrichum omnivorum, Pythium aphanidermatum,* and *Phytophthora megasperma* to the pigments produced by four *Pseudomonas* isolates were also tested according to the following procedure: King's medium B culture plates were streaked about 3 cm from the center of the plates in a rectangular pattern (6 × 6 cm), with each of the four *Pseudomonas* isolates. These plates were inoculated 24 hr later with each of the above fungi by placing a small disk, taken from a 4-day-old PDA culture, in the center of the plates. Culture plates inoculated with the fungi alone served as control. The diameter of fungal colonies grown in the presence and the absence of the bacteria were measured after 4 days of incubation at 26–28 C in the dark.

To determine the effect of iron on the production and fungistatic activity of the fluorescent pigment, petri dishes containing 15 ml of medium B, supplemented with 1.0, 1.5, 2.0, and 3.0 μg of ferrous sulfate or ferric chloride per milliliter of the medium, were inoculated with four *Pseudomonas* isolates and with each of the above fungi, according to the procedure described above, and zones of inhibition or the diameter of fungal colonies were determined. Ferrous sulfate used throughout this study contained less than 0.1% Fe^{+3} based on analysis using ammonium thiocyanate reaction (13).

Isolation and purification of the pigment. An isolate of *P. fluorescens* (ATCC No. 13525) was used throughout this study. Succinate medium (K_2HPO_4, 0.6%; KH_2PO_4, 0.3%; $(NH_4)_2SO_4$, 0.1%; $MgSO_4$, $7H_2O$, 0.02%; succinic acid, 0.4%) was adjusted to pH 7.2 by addition of 2N NaOH prior to sterilization. Flasks (250-ml) containing 50 ml of the medium were inoculated with the bacterium and incubated at 24–26 C for 38 hr without shaking. Bacterial cells were removed by centrifugation at 10,000 *g* for 20 min. Three hundred milliliters of cellfree extract was centrifuged at 10,000 *g* for 20 min after addition of two volumes of acetone. The fluorescent pigment was precipitated from the extract by adding two additional volumes of acetone followed by centrifugation at 10,000 *g* for 20 min. The pigment was washed three times with reagent-grade acetone and dissolved in 12 ml of 0.03 M acetone buffer, pH 5.2. Two-tenths milliliter of pigment solution was placed at the top of a column (1.5 × 25 cm) of Sephadex G25. The column was eluted with water at a rate of 1.0 ml/min at 24–25 C. Absorption spectra of each 1-ml fraction was determined by a

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recording spectrophotometer.

Determination of chelating potential of the free pigment. Determination of iron-chelating potential of the free pigment solution (minimum amount of Fe^{+2} or Fe^{+3} required to convert all of the free pigment to Fe-pigment complex) was done according to a procedure described by Meyer et al (8). The method utilizes an increase in absorption at 450 nm with increases in the amount of Fe-pigment complex. Twenty-four microliters of the standard pigment solution (defined later) and 1.17 ml of 0.03 M acetate buffer, pH 5.2, were added to a cuvette (1-cm light path). Ten-microliter aliquots of 2.5×10^{-3} M solutions of ferric chloride or ferrous sulfate were added successively to the cuvette, and after mixing, absorption at 450 nm was measured. The pigment was considered to be completely chelated when addition of iron solutions did not cause an increase in absorbance at 450 nm. **Chelating potential of the crude pigment in 38- and 96-hr-old cultures of the test bacterium on medium B also was determined according to the above procedure.**

The term "standard pigment solution" used herein is defined as a solution of free partially purified pigment from a 38-hr-old medium B culture of an isolate of *P. fluorescens* (ATCC No. 13525) having an absorbance of 0.59 at 403 nm at 50-fold dilution. The iron-**chelating potentials of 24 μl of standard pigment solution in 1.17 ml** of 0.03 M acetate buffer, pH 5.2, were 246 and 426 nmoles of Fe^{+2} and Fe^{+3}, respectively. The data from four replications were analyzed by using linear regression analysis and the mean maximum absorbance in the presence of excess iron.

Fungistatic activity of the pigment. The pigment was isolated from a 38-hr-old stationary culture of the test bacterium and was partially purified according to the procedure described above. Different volumes (ranging from 50 to 100 μl) of filter-sterilized standard pigment solution were added to 5 ml of sterile liquid King's medium B in 50-ml flasks. In another experiment, 50-ml flasks containing 5 ml of sterile liquid medium B were supplemented with 100 μl of sterile standard pigment solution and 0, 25, 50, 75, 100, 125, and 150 nmoles of either ferric chloride or ferrous sulfate. Flasks containing 5 ml of the medium without the pigment and iron served as controls. The pH of the above media was 7.20 ± 0.2. Cultures were inoculated with a small disk taken from a 2- to 3-day-old culture of *P. aphanidermatum* and were **incubated in a shaker bath at 32 C for 48 hr. Fungal mycelia were collected on oven-dried, preweighed weighing papers, dried at 60 C,** and dry weights were determined. King's medium B, which is a bacteriological medium, was chosen for this experiment because, unlike other mycological media tested, it contains a very small amount of iron. The medium supported the growth of *P. aphanidermatum* quite well.

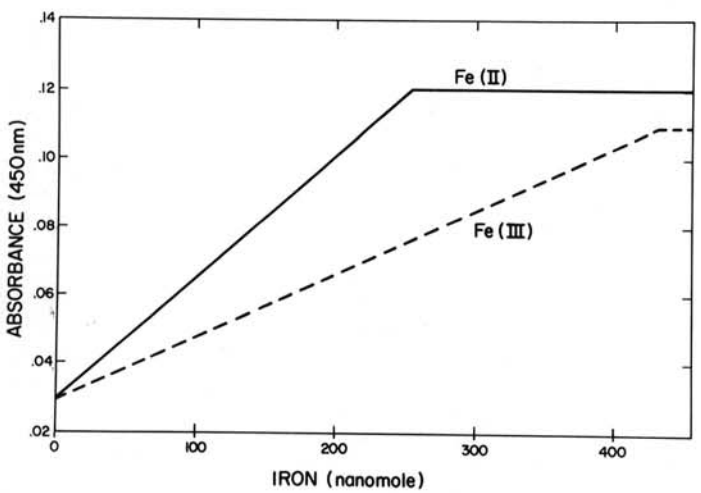

Fig. 1. Increase in absorbance of the purified pigment as a function of the amounts of added iron. Ten-microliter aliquots of 2.5×10^{-3} M solutions of ferrous sulfate (Fe^{+2}) or ferric chloride (Fe^{+3}) were added successively to a cuvette containing 24 μl of standard pigment solution and 1.17 ml of 0.03 M acetate buffer, pH 5.2, and changes of absorbance at 450 nm were recorded. Each point used in determining the line represented an average of four measurements in four replications. Regression equations for added iron were $A_{450\,nm} = 0.00037$ nM $Fe^{+2} + 0.029$, $r^2 = 0.98$, and $A_{450\,nm} = 0.00019$ nM $Fe^{+3} + 0.028$, $r^2 = 0.95$. Absorption maxima at 450 nm for Fe^{+2} and Fe^{+3} pigment complex were 0.121 ± 0.005 and 0.109 ± 0.005, respectively.

RESULTS

Fungistatic activity of the crude pigment. Of the 186 isolates of fluorescent pseudomonads, representative members of plant pathogenic and saprophytic species, 156 isolates produced water-soluble fluorescent pigments in medium B that inhibited the growth of *G. candidum*. The remaining 30 isolates did not produce pigment or inhibit growth of the fungus. There were gradual decreases in the intensity of fluorescent pigments produced by four *Pseudomonas* isolates in medium B and in the level of inhibition of all the following fungi as the concentration of added ferric chloride or ferrous sulfate in the medium increased: *G. candidum, R. solani, S. sclerotiorum, P. omnivorum, P. megasperma,* and *P. aphanidermatum*. Neither fluorescent pigments nor fungistatic activity were detected in 3-day-old medium B cultures containing 2.0 or 3.0 μg of either ferrous sulfate or ferric chloride per milliliter of solution.

Isolation and purification of the pigment. Chromatographic separation of the acetone-precipitated pigment on Sephadex G-25 yielded one peak. The purity of the pigment was not enhanced by chromatographic separation of the pigment as judged by its absorption characteristics. The absorption spectrum of the purified free pigment in water had two main peaks, one at 210 nm and the other at 403 nm. Absorption characteristics of the pigment were very similar to those of a fluorescent pigment isolated from *P. fluorescens*. (8).

Determination of the chelating potential of the pigment. Addition of ferrous sulfate and ferric chloride to partially purified pigment resulted in an increase in absorbance at 450 nm, indicating that both Fe^{+2} and Fe^{+3} were chelated by the pigment (Fig. 1); however, the pigment was capable of binding more Fe^{+3} than Fe^{+2}. The ratio of iron-chelating potential of the free pigment for Fe^{+3} to that for Fe^{+2} was 1.71. The respective figures for crude pigments from 38- and 96-hr-old cultures of the test bacterium were 1.47 and 1.53. Results of linear regression analysis of data related to Fe^{+2}- and Fe^{+2}-induced changes in absorbance of the pigment showed that there were slight changes in Fe^{+2}- and Fe^{+3}-chelating potential of the crude and purified pigments isolated from 38- and 96-hr-old cultures of the test bacterium. Unlike the free pigment, Fe^{+2}- and Fe^{+3}-pigment complexes did not fluoresce under UV light, were not fungistatic, and were slightly brown. The characteristic changes observed with iron were not observed with the following divalent cations: Al, Cr, Co, Ni, Pb, Mn, Zn, Cu, Mg, and Ca.

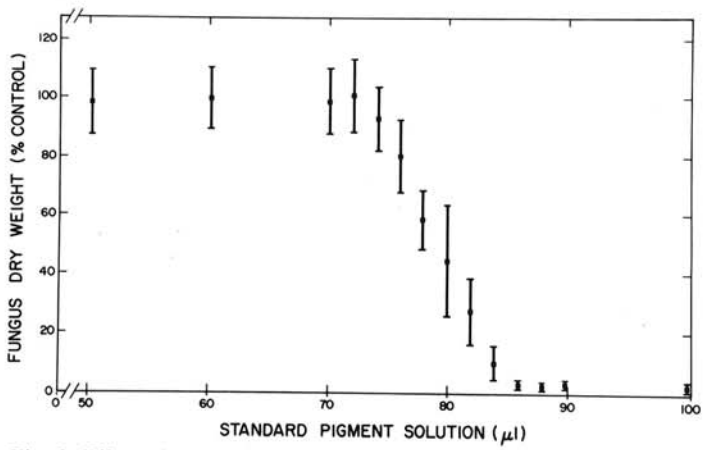

Fig. 2. Effect of partially purified pigment on the growth of *Pythium aphanidermatum*. Different levels of the pigment were added to 5 ml of liquid medium B. Media were inoculated with the fungus and dry weights of the cultures were determined after 48 hr of incubation at 32 C. Each point represents an average of six measurements in three replications.

Fungistatic activity of the purified pigment. A direct correlation was established between the levels of the partially purified fluorescent pigment in the medium and growth inhibition of *P. aphanidermatum* (Fig. 2). In media containing inhibitory levels of the partially purified pigment, growth inhibition was indirectly correlated with the level of Fe^{+2} or Fe^{+3} in the medium (Fig. 3). Moreover, the growth of *P. aphanidermatum*, inhibited in the presence of the pigment, was resumed soon after addition of ferrous sulfate or ferric chloride to the medium in excess of chelating capacity of the added pigment. Reversal of pigment-induced fungistasis could not be achieved with the following divalent cations: Al, Cr, Co, Ni, Pb, Mn, Zn, Cu, Mg, and Ca. These results show that the growth inhibiting activity of the pigment is due to its ability to remove iron from the medium by forming an iron-pigment complex. Moreover, the pigment possesses fungistatic rather than fungicidal properties.

Purified pigments from two additional isolates of *P. fluorescens* and one isolate of *P. marginalis* exhibited chelating and fungistatic properties similar to those of the pigment isolated from the *P. fluorescens* isolate (ATCC No. 13525).

DISCUSSION

A fluorescent pigment produced by an isolate of *P. fluorescens* has been found to be involved in chelation and transport of iron into bacterial cells (8,9). Results of this study confirm and extend this finding and show that iron chelation is a consistent property of fluorescent pigments produced by 156 isolates of fluorescent pseudomonads tested, which were representative members of plant pathogenic and saprophytic species. We are also reporting for the first time that the fluorescent pigments produced by fluorescent pseudomonads exhibit fungistatic properties by virtue of being siderophores. Evidence presented in this paper supports our view that fungistatic property of the fluorescent pigment is due to its ability to chelate iron from the environment, creating an iron deficiency.

Kloepper et al (3) have shown that addition of a purified siderophore from a plant growth-promoting strain of fluorescent *Pseudomonas* to the soil resulted in an increase in the growth of potato plants while the ferric complex of the siderophore was not effective. They also reported that a siderophore produced by an isolate of plant growth-promoting fluorescent *Pseudomonas* exhibited in vitro antibiosis against *Erwinia carotovora* and *Escherichia coli* only in the absence of added ferric chloride. However, the antifungal activity of the siderophore was not tested. On the basis of these results they postulated that the growth-promoting activity of some fluorescent pseudomonads might be due to their ability to produce siderophores that complex environmental iron, making it less available to certain rhizoplane microorganisms including some quasiphytopathogens. The hypothesis advanced by Kloepper et al (3) is supported by our observation that a partially purified fluorescent siderophore produced by *P. fluorescens* inhibited the growth of a number of important soilborne pathogenic fungi in vitro whereas its ferrous and ferric complex did not. However, the hypothesis is weakened by the lack of direct evidence for the production of the siderophores by these bacteria in the rhizosphere and/or rhizoplane. These siderophores are produced only in the presence of low levels of iron (8,9). Therefore, we are currently attempting to establish whether the level of iron around roots is low enough to allow pigment production.

Absorption characteristics of the pigment reported here are very similar to the pigment recovered from an isolate of *P. fluorescens* by Meyer et al (8). Both pigments exhibited large increases in absorbance at 450 nm upon forming Fe^{+3}-complex. However, the pigment isolated by Meyer et al (8) was specific to Fe^{+3} and did not bind Fe^{+2}, whereas the pigment reported here did not exhibit such specificity. These results show that the two pigments are similar, but not identical. However, the possibility that the observed differences are due to the presence of impurity in our preparations cannot be ruled out. Moreover, accurate information on the specific properties of the pigment, including its chelating potential, can be obtained only after further purification. The partially-purified pigment described here exhibited a slight change in chelating potential for divalent and trivalent iron with culture age. The pigment isolated by Meyer et al (8) also was reported to break down under mild alkaline conditions into several pigmented decomposition products.

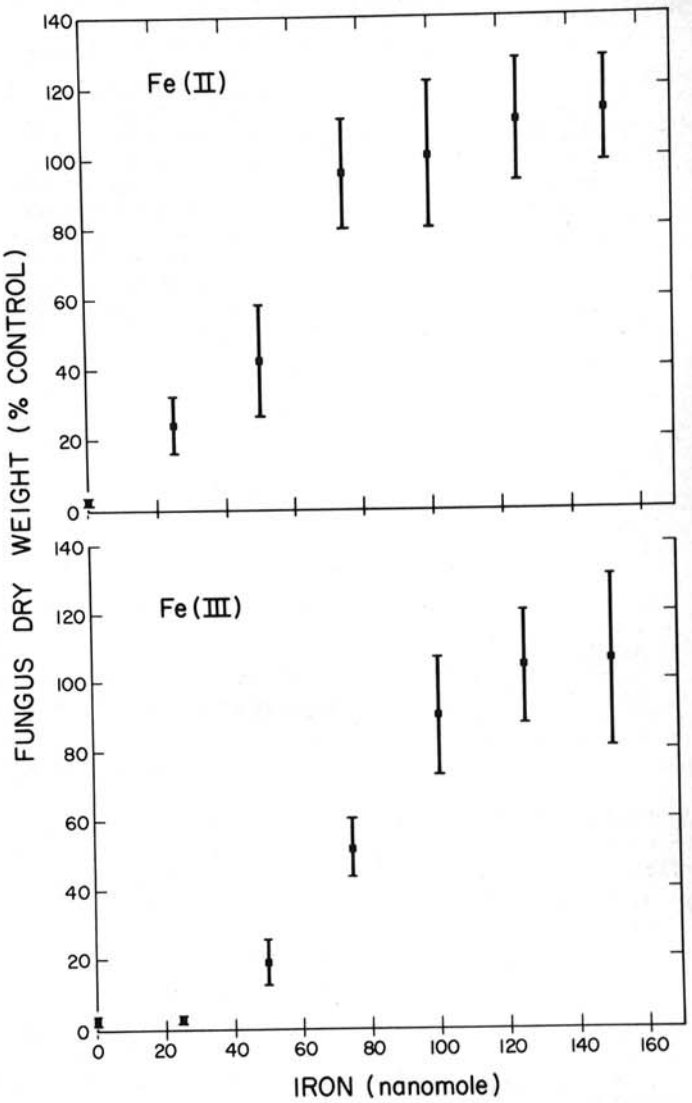

Fig. 3. Counteraction of pigment-induced inhibition by iron. Different levels of ferrous sulfate (Fe^{+2}) or ferric chloride (Fe^{+3}) were added to 5 ml of liquid medium B containing 0.1 ml of standard pigment solution. Media were inoculated with *Pythium aphanidermatum* and dry weights of the cultures were determined after 48 hr of incubation at 32 C. Each point represents an average of six measurements in three replications.

Results of this study are significant because fluorescent pseudomonads, which are present ubiquitously in agricultural soils and on plant surfaces, might be able to compete effectively with some plant pathogenic organisms for a limited supply of iron. It might, therefore, be possible to reduce the incidence of certain soilborne diseases by increasing population of certain fluorescent pseudomonads, particularly in alkaline soils where available iron is very limited.

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