INFECTION AND IMMUNITY, June 1983, p. 1134–1139
0019-9567/83/061134-06$02.00/0

Vol. 40, No. 3

Hydroxamate Siderophore Production by Opportunistic and Systemic Fungal Pathogens

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Received 18 January 1983/Accepted 25 March 1983

It has been suggested that siderophores may function as virulence factors. There have been few studies on production of siderophores by opportunistic and pathogenic fungi. We examined siderophore production by *Absidia corymbifera, Aspergillus niger, Rhizopus arrhizus, Rhizopus oryzae, Blastomyces dermatitidis, Histoplasma capsulatum, Sporothrix schenickii, Candida albicans*, and *Trichophyton mentagrophytes*. Fungi were cultured at 37 and 27°C in a chemically defined low-iron media (0.2 μM Fe). Culture supernatants were assayed for siderophores by two nonspecific methods [$FeCl_3$ and $Fe(ClO_4)_3$] and three chemically specific assays (catechol, 2,3-dihydroxybenzoate, and hydroxamate). All fungi secreted siderophores. Only siderophores of the hydroxamate type were found. More siderophore was produced at 27°C than at 37°C. The present study adds eight fungi to the list of known siderophore producers and confirms siderophore production by *H. capsulatum*.

Iron is necessary for microbial growth and is associated with the production of various microbial virulence factors (19). Iron in nature exists predominantly as the insoluble ferric form which is not readily available for assimilation. One mechanism by which microorganisms may acquire iron is through the secretion and reabsorption of specific iron chelators (15). These chelators are termed siderophores or siderochromes and have been shown to be produced by bacteria and nonpathogenic yeasts (2, 5, 7, 14).

Siderophores can be classified chemically into two major categories: the secondary hydroxamic acids and the phenolates (5, 20). Under controlled in vitro conditions, growth in an iron-deficient medium leads to an increased production of hydroxamates by hydroxamate-producing microorganisms (6) and phenolates by phenolate-producing microorganisms (13, 16, 17). It has been suggested that these siderophores may function as virulence factors by facilitating growth under the conditions of limited iron availability that are thought to exist in vivo (20). Siderophore chelates may also function as toxins (19).

Little, however, is known about siderophore production by fungi which cause disease in humans. To date, only one study has indicated that a systemic pathogenic fungus may produce siderophore. That report described a growth-promoting factor produced by *Histoplasma capsulatum* (3, 4). The purpose of this investigation was to determine whether opportunistic and pathogenic fungi which can infect humans have the capability to produce siderophore. This report describes the production of siderophore by eight different opportunistic and systemic fungal pathogens and confirms hydroxamate siderophore production by the pathogenic fungus *H. capsulatum*.

MATERIALS AND METHODS

Organisms. *Absidia corymbifera, Aspergillus niger, Rhizopus arrhizus, Rhizopus oryzae, Blastomyces dermatitidis, H. capsulatum*, two isolates of *Sporothrix schenckii, Candida albicans*, and *Trichophyton mentagrophytes* were maintained on Sabouraud dextrose agar. Spore suspensions used for inoculation were prepared for all fungi except *B. dermatitidis* and *H. capsulatum* by the technique developed by Reinhardt et al. (18), with the exception that sterile deionized water (18 M ohm cm^{-1}) was substituted for the antibiotic-containing collection fluid. The resulting spore preparation was concentrated to 0.5-ml packed volume by centrifugation and washed six times with 45 ml of sterile deionized water to remove and diminish the possibility of metal contamination. All apparatus used in the preparation of spores was cleaned and acid hardened with 4 N nitric acid before use to diminish metal contamination. Spore concentration was determined by direct count, and spore viability was determined by the dilution plating method.

For *B. dermatitidis* and *H. capsulatum*, a fragmented mycelial suspension was prepared (11). After growth in Sabouraud dextrose broth, the mycelial matt was collected and washed six times with deionized water, resuspended in low-iron medium (LIM) (described below), and fragmented with a 16- by 124-mm, 7-ml-capacity Broeck ground-glass tissue grinder (Fisher Scientific Co., Pittsburgh, Pa.) that had been cleaned and hardened with 4 N nitric acid. The fragmentation process was discontinued when no clumps or aggregates could be visually detected within the

grinder. The inocula were standardized spectrophotometrically as previously described (11). Briefly, the absorbance density of the fragmented mycelium suspension was determined with a Beckman model 35 spectrophotometer set at a wavelength of 450 nm. The fungal suspension was then diluted with low-iron medium to an absorbance density of 0.600 and used immediately. The efficiency of the 0.600 absorbance density inoculum of both *B. dermatitidis* and *H. capsulatum* to initiate growth in Sabouraud broth at the 1:200 ratio of inoculum to medium used in this investigation was 100%.

LIM. LIM was prepared in deionized water (18 M ohm cm^{-1}). The constituents were dextrose (1.0 g/liter), $MgSO_4$ (0.030 g/liter), $CaCl_2$ (0.010 g/liter), K_2HPO_4 (0.400 g/liter), biotin (2×10^{-8} g/liter; Sigma Chemical Co., St. Louis, Mo.), thiamine (4×10^{-8} g/liter; Sigma), and 10 ml of nonessential amino acids solution per liter (100×) (no. 114, GIBCO, Grand Island, N.Y.). A 10× stock solution of K_2HPO_4 was prepared and passed over a Chelex 100 column (described below) to remove excess iron before dilution in the final medium. The medium was sterilized by filtration through a deionized water-washed 0.22 μM filter (Millipore Corp., Bedford, Mass.). Portions of the LIM and the K_2HPO_4 were retained for immediate iron analysis. The remainder was stored at 4°C in 50-ml plastic tubes (Falcon 2070; Falcon Plastics, Oxnard, Calif.).

Iron depletion by Chelex 100. A 2.5- by 22-cm column of Chelex 100 (Bio-Rad Laboratories; Richmond, Calif.) analytical-grade chelating resin, 200 to 400 mesh, sodium form was prepared. The resin was adjusted to pH 6.9 by rinsing the column with 0.5 M sodium acetate buffer and then washing with deionized water (five times bed volume). A 150-ml amount of the 10× stock solution of K_2HPO_4 was pumped through the column at a rate of 1 ml/min. The initial 40 ml collected was discarded, and the remainder was collected, pooled, and stored at 4°C in 50-ml plastic tubes (Falcon 2070).

Iron determination. Complete LIM and its various individual constituents were prepared in deionized water and lyophilized to dryness with a shelf-type lyophilizer (model 10-145 MR-BA; Virtis, Inc., Gardiner, N.Y.). The resulting residues were redissolved in a volume of deionized water that resulted in a 50-fold concentration of the original samples. Samples (0.125 ml) of the concentrated medium or constituents were added to disposable semi-micro cuvettes (VWR Scientific Apparatus, Atlanta, Ga.) followed by 0.5 ml of 0.5 M acetate buffer (pH 4.5). Iron was reduced to the ferrous form by the addition of 0.025 ml of 1.3 M ascorbic acid. The contents of the cuvette were mixed, and the absorbance at 562 nm was determined. After 5 min, 0.025 ml of ferrozine (Sigma) [3-(2-pyridyl)-5, 6-bis-4-phenyl-sulfonic acid)-1,2,4-triazine] was added. The final absorbance was determined 20 min later. The change in absorbance (final less initial) was compared to values obtained with standard iron solutions to determine the iron present. Analysis of the LIM by this method revealed that it contained 0.2 μM or 11 ng or iron per ml. Most of the iron (> 90%) was contained in the amino acid and K_2HPO_4 constituents.

Fungal cultures and collection of supernatants. All fungi were cultured in 10 ml of LIM contained within 50-ml loosely capped tubes (Falcon 2070). Growth was initiated with 5×10^5 spores or 50 μl of the fragmented mycelium (absorbance density, 0.600). The cultures were incubated at 37 or 27°C for periods of up to 2 months. Growth was estimated turbidimetrically at 450 nm by using a Beckman model 35 spectrophotometer. Culture fluid was separated from the fungal mass by centrifugation at 9,000 rpm for 20 min with a Beckman J-21 centrifuge equipped with a JA-14 head. The resulting supernatant was removed, filtered through a 0.22-μm filter, and either assayed immediately for siderophore or stored at 4°C.

Nonspecific assay for siderophore in culture supernatants. Two methods of siderophore assay were employed. Method A: 50 to 150 μl of culture supernatant was mixed with 0.12 M $FeCl_3$ in 0.005 N HCl to give a final volume of 900 μl (12), and the absorbance at 425 nm was determined. Method B: 50 to 150 μl of culture supernatant was mixed with 5 mM $Fe(ClO_4)_3$ in 0.1 N HCl to give a final volume of 900 μl (7), and the absorbance at 425 nm was determined. For both assays, if siderophore was present, a yellow-orange color indicative of the iron-siderophore complex was produced.

Detection of catechol. The method used to detect catechol was based on that described by Arnow (1) in which catechol gives a yellow color when reacted with nitrous acid and changes to an intense orange-red when made strongly basic (~pH 10). To 1.0 ml of supernatant were added in order with mixing 1.0 ml of 0.5 N HCl, 1.0 ml of nitrite-molybdate reagent (10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of deionized water), 1.0 ml of 1.0 N NaOH, and 1.0 ml of deionized water. In this assay, 174 μM catechol (Aldrich Chemical Co., Milwaukee, Wis.) was used as a positive control. Dihydroxybenzoic acid (Aldrich), acetohydroxamic acid (Aldrich), benzohydroxamic acid (Aldrich), and deferoxamine (Ciba, Summit, N.J.) were used as negative controls.

Detection of 2,3-dihydroxybenzoate. The assay for 2,3-dihydroxybenzoic acid is based on its absorption at 318 nm (21). The UV absorption spectrum of each culture supernatant was determined and compared to the spectrum obtained from 200 μM 2,3-dihydroxybenzoic acid prepared in LIM and deionized water. Catechol, acetohydroxamic acid, benzohydroxamic acid, and deferoxamine were used as negative controls.

Detection of hydroxamic acids. The method used for detection of hydroxamic acids was based on that described by Emery and Neilands (7, 8), who demonstrated that the oxidation of certain hydroxamic acids yields a material with a very strong absorption at 264 nm. To 0.4 ml of culture supernatant were added in order 1.0 ml of 2.19 mM periodic acid, 2.6 ml of distilled water, and 2 drops of glycerol. The final volume was mixed, and the absorbance at 264 nm was determined. In this assay, 1.56 mM solutions of acetohydroxamic acid, benzohydroxamic acid, and deferoxamine were used as positive controls. Catechol and dihydroxybenzoic acid were used as negative controls.

RESULTS

Nonspecific determination of siderophore in culture supernatants. All of the fungi had grown to a visible mass after 11 days of incubation at 37 and 27°C. The quantity of fungal material was

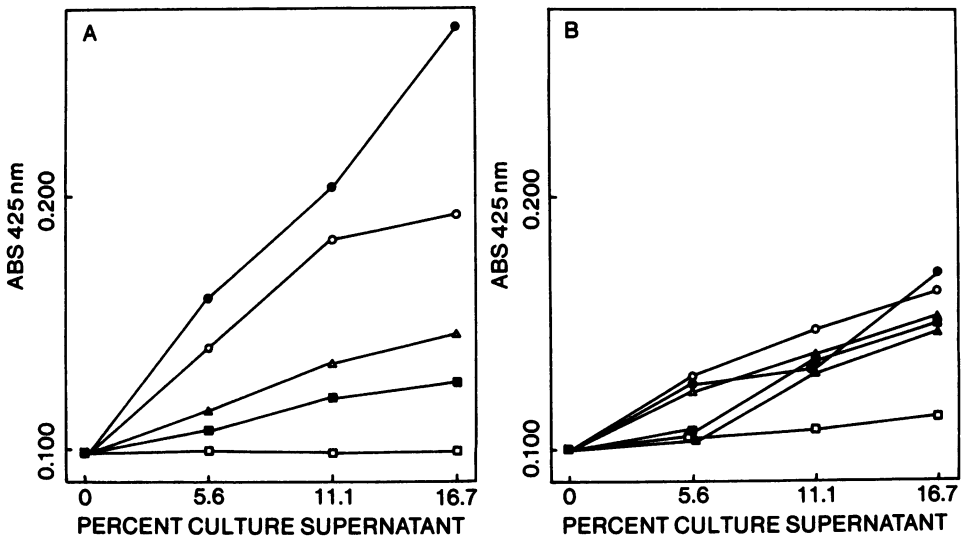

FIG. 1. Iron-binding activity of fungal culture supernatants indicating the presence of secreted siderophore after 8 weeks of growth in LIM at 27°C. Iron perchlorate (0.5 mM) was used to form the colored iron siderophore complex. (A) Opportunistic fungi: *A. niger* (●), *R. oryzae* (○), *A. corymbifera* (△), *R. arrhizus* (■), and medium control (□). (B) Pathogenic fungi: *H. capsulatum* (○), *B. dermatitidis* (△), *S. schenckii* 1 (●), *S. schenckii* 2 (▲), *T. mentagrophytes* (■), and *C. albicans* (□). ABS, Absorbance.

noticeably greater in the cultures inoculated with *A. corymbifera, A. niger, R. arrhizus, R. oryzae*, and *T. mentagrophytes*. Growth of *B. dermatitidis* and *H. capsulatum*, which was mycelial, was barely detectable. The 11-day culture supernatants from *A. niger* grown at both 27 and 37°C produced an intense, stable, yellow color after the addition of iron. There was some hint of a reaction with *R. oryzae* and *A. corymbifera*. There was no detectable reaction with the other culture supernatants.

After 8 weeks of incubation at 27 and 37°C, *B. dermatitidis* and *H. capsulatum* had grown sufficiently so that the mycelium was readily visible. The mycelial mass of the other fungi had increased only slightly. Supernatant culture fluid from all 10 fungal cultures produced a stable yellow color measurable at 425 nm when mixed with $FeCl_3$ or $Fe(ClO_4)_3$, indicating the presence of siderophore. Uninoculated LIM control cultures produced no color change, indicating that they did not contain siderophore. It was concluded that all 10 fungi had the capacity to produce and secrete siderophore when grown in LIM at either 27 or 37°C.

When the amount of culture supernatant added to excess iron reagent was plotted against absorbance of the colored siderophore iron complex, a linear relationship was found (Fig. 1). From this relationship, the iron-binding activity of the various supernatants could be semi-quantitatively compared. Iron-binding activity was expressed as the change in absorbance at 425 nm per percent supernatant, which is the slope of the plot. Slope values for both nonspecific siderophore assays are tabulated in Table 1 for the supernatants collected after 8 weeks of growth at 27°C. The coefficient of determination for the $FeCl_3$ assays ranged from 0.755 to 0.886. For $Fe(ClO_4)_3$, it ranged from 0.920 to 1.00, indicating that the $Fe(ClO_4)_3$ assay more accurately indicated iron-binding activity. By using the $Fe(ClO_4)_3$ slope values, it can be seen that the culture supernatants from the opportunistic fungi *R. orzyae* and *A. niger* contained the greatest iron-binding activity, and *C. albicans* contained the least. The remaining culture supernatants had intermediate values. The iron-binding slope values ranged from 18 to 47% lower at 37°C, indicating that the quantity of siderophore produced by a given fungal agent was less at 37 than at 27°C. The rank order of the iron-binding activity of the supernatants, however, was the same at 27 and 37°C.

Detection of phenolate-type siderophore in culture supernatants. Two methods were used to determine whether siderophore detected by the nonspecific iron-binding method was of the phenolate type. In the first method, the criterion for the presence of dihydroxybenzoate derivatives is a specific absorption band at 318 nm (21). Pure dihydroxybenzoate absorbed at 318 nm. Catechol, dihydroxybenzoic acid, acetohydroxamic acid, benzohydroxamic acid, and deferoxamine did not. None of the 10 culture supernatants produced an absorbance band at 318 nm, sug-

TABLE 1. Nonspecific and specific assays for siderophore[a]

Sample	Nonspecific[b]		Specific				
				Catechol[d]		Hydroxamate[e]	
	$FeCl_3$	$Fe(ClO_4)_3$	DHB[c] (318 nm)	Color with acid	Color with base	Color	264 nm
LIM	0.476	−0.180	None	None	None	None	0.009
A. corymbifera	71.7	3.09	None	Yellow	None	None	0.216
A. niger	65.1	10.04	None	Yellow	None	None	1.22
B. dermatitidis	71.0	3.56	None	Yellow	None	None	0.417
C. albicans	65.0	1.17	None	Yellow	None	None	0.230
H. capsulatum	69.7	4.10	None	Yellow	None	None	0.440
R. arrhizus	58.5	2.07	None	Yellow	None	None	2.80
R. oryzae	76.0	6.15	None	Yellow	None	None	0.563
S. schenckii 1	65.2	4.39	None	Yellow	None	None	0.365
S. schenckii 2	56.5	3.60	None	Yellow	None	None	0.354
T. mentagrophytes (ATC 18748)	60.0	3.55	None	Yellow	None	None	0.466
Catechol			None	Yellow	Red	Yellow	0.085
Dihydroxybenzoic acid			Peak	Yellow	Red	Yellow	0.091
Acetohydroxamic acid			None	Yellow	None	None	−0.003
Benzohydroxamic acid			None	Yellow	None	None	0.498
Deferoxamine			None	Yellow	Blue	None	2.11

[a] Supernatants from LIM cultures inoculated for 8 weeks at 27°C.
[b] Slope of iron-binding activity equals the absorbance at 425 nm × 10^3 divided by percent culture supernatant.
[c] Dihydroxybenzate (DHB) derivatives are indicated by an absorbance peak at 318 nm.
[d] Catechol derivatives are detected by formation of a yellow color at acid conditions and a red color at basic conditions.
[e] Hydroxamate derivatives are detected by the formation of nitrosodimers which absorb specifically at 264 nm.

gesting that the siderophores secreted into the LIM did not belong to the phenolate class (Table 1).

The second method was based on the chemical detection of catechol. The criterion consists of the production of a yellow color upon acidification with nitrous acid followed by a change to orange-red when the mixture is made basic (1). By this criterion, none of the culture supernatants was positive for catechol, whereas both pure catechol and dihydroxybenzoate standards produced a positive reaction (Table 1). The negative controls (acetohydroxamic acid, benzohydroxamic acid, and deferoxamine) did not produce the orange-red change. Interestingly, the hydroxamate siderophore deferoxamine produced a notable blue color reaction.

Detection of hydroxamate derivatives in culture supernatants. The indication for the presence of secondary hydroxamates is based on the detection of a strong absorption band at 264 nm after the formation of a nitrosodimer (7, 8). All of the culture supernatants and the benzohydroxamic acid and deferoxamine controls produced a positive reaction. Dihydroxybenzoate and catechol produced a yellow color which absorbed slightly at 264 nm. Interestingly, the acetohydroxamic acid control showed no evidence of reaction. LIM showed no reaction. These results (Table 1) indicate that the siderophores produced and detected for all 10 fungi by the nonspecific iron-binding assay method were of the hydroxamate type.

DISCUSSION

Siderophore secretion by several species of nonpathogenic fungi has been reported (2, 6). There has been only one report of siderophore production by a pathogenic fungus (3, 4). In this report, we demonstrate that the opportunistic fungi *A. corymbifera, A. niger, R. arrhizus*, and *R. oryzae* and the pathogenic fungi *B. dermatitidis, C. albicans, H. capsulatum, S. schenckii*, and *T. mentagrophytes* produce siderophores of the hydroxamate type (Table 1). We found no indication that these fungi produced phenolate-class siderophores.

The presence of siderophore was first detected by observing the development of the characteristic yellow-orange color reaction which results when the siderophore chelates iron. By this criterion, all 10 fungi secreted siderophore after 8 weeks of growth in medium containing 0.2 μM iron. In contrast, only *A. niger* had clearly secreted siderophore after 11 days of growth. The sensitivity of this assay is not known, but based on an extinction determination with deferoxamine, benzohydroxamic acid, and acetohy-

droxamic acid, we speculate that it is in the range of 0.2 to 0.4 mM of siderophore for the various fungi. It should be noted that we did not concentrate any culture supernatants. Concentrating the early culture supernatants before assay might have clearly revealed the presence of siderophores produced by the fungi at an earlier time. Because there was a hint of a reaction with the 11-day culture supernatants of *R. oryzae* and *A. corymbifera*, we feel that siderophore production and secretion may not necessarily be a delayed-in-time event.

Siderophores have been classified as hydroxamic acids and phenolates. Three methods were used to determine the chemical class of siderophore. The first method involved examining the UV spectrum of the siderophore-containing supernatants for absorption at 318 nm. A peak at this wavelength would suggest the presence of a phenolate siderophore of the dihydroxybenzoic acid variety (21). We found no evidence for the presence of phenolates by this method. It should be noted that catechol, which is also phenolate derived, did not absorb at 318 nm, suggesting that the method was specific for dihydroxybenzoic acid derivatives.

The second method employed a reaction described by Arnow (1). With this reaction, if the siderophore is a phenolate, it will first form a yellow product when reacted with nitrous acid that will then turn orange-red when it is made basic by the addition of NaOH. Both catechol and dihydroxybenzoic acid, which are phenolate derivatives, produced this color change, whereas the hydroxamic acid standards, acetohydroxamic acid, and benzohydroxamic acid did not. Interestingly, the siderophore deferoxamine formed a blue product in this reaction after the addition of 0.5 N NaOH. Arnow alluded to the possibility that this reaction could yield other colors that were specific for certain nonphenolate structures. The blue reaction for the hydroxamic acid derivative deferoxamine was not described. Thus, the possibility exists that, in addition to phenolates, this assay might be useful for identifying hydroxamate-type siderophores that are closely related to deferoxamine. None of the culture supernatants shown to be siderophore positive by the nonspecific iron-binding method changed color in this test, suggesting that the siderophores were not phenolates.

The third method was based on the detection of nitrosodimers which are formed when hydroxamates are reacted with period acid (7, 21). A recent comparison of four methods used to detect the presence of hydroxamic acid derivatives indicates that this method is sensitive for determining whether unknown siderophores are of the hydroxamate type (9). Because the intensity of the absorbance at 264 nm varies with the types of nitrosodimer produced, the method is only qualitative. Siderophore-containing supernatants from all 10 fungi, as well as the benzohydroxamic acid and deferoxamine standards, produced the typical absorbance at 264 nm, indicating that a nitrosodimer had been formed and that the siderophores belonged to the hydroxamate class (Table 1). Interestingly, the acetohydroxamic acid standard did not react, suggesting that this structure does not form a nitrosodimer. This would seem to be consistent with the observation that primary hydroxamic acids and hydroxylamine do not form nitrosodimers (9, 10). We also noted that both dihydroxybenzoic acid and catechol absorbed slightly at 264 nm. There was, however, no change in their absorbance after reaction with periodic acid, confirming the specificity of the reaction for secondary hydroxamates.

In this study, it was not possible to determine the quantity of siderophore produced, since we did not have the various siderophores produced by each fungus available as standards. We also have not determined whether more than one type of hydroxamate siderophore was produced. However, the data plotted in Fig. 1 can be used within certain limitations to compare the iron-binding activity of the various siderophore-containing supernatants. Large slope values indicate either more siderophore or more iron-binding sites. Assuming a reasonably constant number of iron-binding sites, *A. niger* and *R. oryzae* were the most active siderophore producers. Interestingly, *C. albicans*, the only yeast tested, showed the least reactivity.

Although this report clearly indicates that a variety of opportunistic and pathogenic fungi can produce siderophores when grown in a chemically defined LIM, it has not been determined whether these fungi produce siderophores under physiological conditions or in vivo. It is also not known whether these siderophores function as virulence factors or whether they are incidental by-products of fungal metabolism. Preliminary data in our laboratory, however, indicate that several of these fungi, when grown in serum at physiological conditions of temperature and pH, produce a factor that is antagonistic to the fungal growth inhibitory activity mediated by human transferrin.

ACKNOWLEDGMENTS

We thank Tim Granade for maintaining the fungal cultures and Lisa McDonough for her assistance in preparing the manuscript.

This work was supported by Public Health Service grant 1 R01 A1 15779-01A1 BM from the National Institute of Allergy and Infectious Diseases.

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