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# Identification of Coprogen B and Its Breakdown Products from *Histoplasma capsulatum*

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Iron added to a chemically defined liquid medium suppressed hydroxamic acid production at 37°C by yeast cells of *Histoplasma capsulatum*. Four hydroxamic acids, HA-I, HA-II, HA-III, and HA-IV, present in the low-iron fluid after the culture of *H. capsulatum* were isolated by extraction and cation-exchange chromatography through cellulose phosphate (0.35% formic acid). Visible spectra of prepared ferrihydroxamates indicated that HA-II and HA-III were monohydroxamates, whereas HA-I and HA-IV were identified as di- and trihydroxamates, respectively. Reductive hydrolysis of HA-I (the major hydroxamic acid isolated) yielded ornithine. Hydrolysis of HA-IV in water or in 0.1 N NaOH resulted in the formation of HA-I (dihydroxamic acid) and HA-II (monohydroxamic acid). Based on their charge at pH 5.2 and 2 determined by paper electrophoresis, $R_f$ values on thin-layer chromatography, infrared spectra, and reactivity to ninhydrin, three of the isolated hydroxamic acids were identified as deferricoprogen B (HA-IV) and its breakdown products, dimerumic acid (HA-I) and *trans* fusarinine (HA-II). HA-I and HA-IV exhibited growth factor activity for both yeast and mycelial forms of growth of *H. capsulatum*.

Iron serves essential functions in a number of metabolic processes of microbial cells (4, 13). In neutral or alkaline environments or in body fluids, however, this metal is in a bound or precipitated form and as such remains inaccessible for cell utilization. Many microorganisms have been shown to acquire iron by secreting a class of chelating compounds. These relatively small molecular weight ligands, collectively called siderophores, characteristically have high affinity for the specific binding of ferric ion and subsequently are able to solubilize the metal for use by cells. Those siderophores studied to date have been shown to be of two chemical types, namely, the phenolates (catechols) and the hydroxamic acids. Phenolate-type siderophores are produced only by bacteria; the latter are produced by several bacterial species, including actinomycete species, and by fungi (6, 16). Some hydroxamic acids (formerly called sideromycins), such as the albomycins, exhibit antibiotic activity toward microbial cells. Others (formerly called the sideramines) exhibit growth factor activity for cells by their scavenging of iron (6, 16).

Hydroxamic acids have been isolated and characterized from many saprophytic species of fungi (1, 2, 5–8, 10, 12, 16, 20). Recently, we reported on the production of hydroxamic acid by the pathogenic fungus *Histoplasma capsulatum*, which exhibited growth factor activity for both yeast and mycelial forms of growth (3). In this communication, data are presented that three of the hydroxamic acids produced by *H. capsulatum* are deferricoprogen B and its breakdown products, dimerumic (dimerum) acid and *trans* fusarinine, compounds previously isolated and characterized from other saprophytic fungi (5, 6).

## MATERIALS AND METHODS

**Organisms.** *H. capsulatum* isolates used in this study were kindly furnished by R. P. Tewari, Southern Illinois University School of Medicine, Springfield, Ill. and by J. Funkhouser, Miami Valley Hospital, Dayton, Ohio. The organisms were maintained in their yeast form by frequent transfer onto slants of brain heart infusion agar (2% agar, wt/vol) containing 1% (wt/vol) dextrose, 0.01% (wt/vol) cysteine-hydrochloride, 5% (vol/vol) outdated human blood, and antibacterial antibiotics (pencillin G, 20 U/ml and streptomycin sulfate, 40 μg/ml) (BHIA/B). The blood and antibiotics were added after autoclaving and cooling the medium to 45°C in a water bath.

**Production and extraction of hydroxamic acids.** Yeast cells of *H. capsulatum* strain 505 were inoculated into 1-liter quantities of a defined liquid medium described by McVeigh and Morton (MM medium) (14). Ferrous ammonium sulfate was omitted from their formulation; $3 \times 10^{-7}$ M iron was added to the medium from a solution described by Atkin and coworkers (2). Cultures in Fernbach flasks were incubated at 37°C with constant rotary shaking (120 rpm) for 14 days, after which time the cells were removed from

the culture medium by filtration through glass fiber filters (type A/E; Gelman Sciences, Inc., Ann Arbor, Mich.). The culture fluid then was passaged twice through 0.45-μm membrane filters (Gelman) to ensure the complete removal of yeast cells. The hydroxamic acid-containing filtrate then was concentrated approximately 20-fold by flash evaporation at 40°C. Hydroxamic acid in the concentrated culture filtrate was extracted into the organic-layer phase with phenol and chloroform (1:1, wt/vol) and then was recovered in the aqueous phase by the addition of excess ether and several small volumes of water (1). The aqueous phase then was extracted three times with ether and dried by flash evaporation. The resulting residue was triturated several times with small volumes of methanol, which then were passaged through a glass fiber filter (type A/E) to remove a nonsoluble, nonhydroxamic acid material. The methanol was removed by flash evaporation, leaving a light brown oily residue (hydroxamic acid).

**Cation-exchange chromatography.** Hydroxamic acids were purified further by cation-exchange chromatography through cellulose phosphate (1.00 meq/g; Sigma Chemical Co., St. Louis, Mo.). The hydroxamic acid preparation was dissolved in approximately 3 ml of 0.35% (vol/vol) formic acid, and this was applied to a column (2 by 50 cm) of the adsorbent previously equilibrated with the same buffer. Hydroxamic acids were eluted from the column by passage of the formic acid solution with a flow rate of 25 ml per h, and fractions (6.5 ml) were collected with a Gilson fractionator (Gilson Medical Electronics, Middleton, Wis.). Hydroxamic acid-containing fractions were pooled and dried with the flash evaporator.

**Analytical methods.** Chromatography on commercially prepared thin-layer plates (E. M. Laboratories, Cincinnati, Ohio) was used to assess the purity of the isolated compounds and to identify hydrolysis products. The solvents used were: (i) water-saturated *n*-butanol; (ii) *n*-butanol–ethanol–water (4:1:5, vol/vol/vol, upper phase); (iii) *n*-butanol–acetic acid–water (100:12:25, vol/vol/vol); (iv) pyridine–water (65:35, vol/vol); and methanol–water–diethylamine (80:20:4, vol/vol/vol). Development was in the ascending direction. Compounds also were analyzed by paper electrophoresis in pyridine–acetic acid–water (14:10:930, vol/vol/vol; pH 5.2) and in 3.5% formic acid (pH 2.0) according to the procedures of Emery (7). Electrophoresis was carried out at 32 V/cm for 30 min. Hydroxamic acids were detected by spraying with 10% $FeCl_3$ (7). Ninhydrin (0.5% in acetone) was used to detect ninhydrin reaction compounds. For comparison, an authentic sample of dimerumic acid was a gift from J. B. Neilands, University of California, Berkeley. Fusarinine was isolated from *Fusarium roseum*, which was supplied by T. Emery, Utah State University, Logan.

**Hydrolysis.** Several milligrams of isolated hydroxamic acid were hydrolyzed in freshly regenerated hydriodic acid (9) in evacuated, sealed glass tubes according to the procedures of Emery and Neilands (8). Samples of hydrolysate were analyzed by thin-layer chromatography and by paper electrophoresis, as outlined previously. Compounds used as standards were purchased from Sigma Chemical Co.

**Chemical assay.** Hydroxamic acid was estimated by combining 0.5 ml of sample with 2.5 ml of 5 mM $Fe(ClO_4)_3$ in 0.1 M $HClO_4$ and measuring the absorbance at 480 nm of the ferrihydroxamate reaction product (1).

**Spectroscopy.** Ferrihydroxamates were prepared as outlined by Moore and Emery (15). Solutions were adjusted to pH 1.5 and 7.0 by the addition of HCl or KOH. Visible spectra of the chelated compounds were determined with a Perkin-Elmer 550 spectrophotometer connected to a recorder. Infrared (IR) spectra of purified compounds, pressed into KBr pellets, were performed by J. Goldfinger, Inland Corp., Dayton, Ohio, using a Perkin-Elmer model 580 spectrometer.

**Growth studies.** The effect of iron on hydroxamic acid production was determined in MM medium. All glassware was cleaned with concentrated nitric acid and thoroughly rinsed with double-distilled water to eliminate contaminating traces of iron. Flasks (250 ml) each containing 50 ml of the liquid MM medium were supplemented with filter-sterilized iron solution (2) to contain $10^{-7}$ to $10^{-4}$ M iron. Media were inoculated with yeast cells of *H. capsulatum* strain 505 and incubated at 37°C for 14 days with constant rotary shaking (120 rpm). Cells in 20 ml of the culture fluid were then removed by vacuum filtration through tared, sintered glass filters (fine porosity, Pyrex filtering crucibles). The weight of cells was determined after drying at 80°C to constant weight. Hydroxamic acid was estimated as outlined previously.

To determine the growth factor activity of the purified hydroxamic acids, filter-sterilized solutions were made that produced absorbances of 0.1 to 0.2 in the assay for hydroxamic acid. Solutions were added to 2% (vol/vol) to brain heart infusion agar containing 0.1 (wt/vol) bovine serum albumin, fraction V (Sigma) (BHIA/BSA) in place of the blood addition and to Sabouraud dextrose agar (Emmon modified; Difco Laboratories, Detroit, Mich.) (SDA). The media were dispensed into plastic petri plates (100 by 15 mm). A suspension of yeast cells of isolates of *H. capsulatum* was prepared from cultures grown on BHIA/B slants for 3 days. Cells were washed once with 0.15 N saline and counted in a hemacytometer. Approximately 200 cells in 0.1-ml volumes were spread onto the plated media. To prevent their drying out, SDA plates were sealed with masking tape and incubated at 25°C for 14 days; those containing BHIA/BSA were incubated at 37°C for 7 days in a covered wash basin. The number of colonies forming on the media was the indication of the growth factor activity of the respective hydroxamic acid preparations.

## RESULTS

**Effect of iron on hydroxamic acid production.** Iron added to the liquid medium suppressed the production of hydroxamic acid by yeast cells of *H. capsulatum* strain 505 (Fig. 1) in a manner similar to that which occurs in other fungi (2, 7, 16). The suppression was evident in the medium containing added iron at concentrations in excess of $3 \times 10^{-7}$ M. Elevating the amount of iron to $10^{-4}$ M, however, resulted in increased cell yields, as indicated by the determination of the dry weight of cells.

**Production and purification of hydroxamic acids.** Hydroxamic acid reached maximal concentration (0.088 absorbance at 480 nm) in the

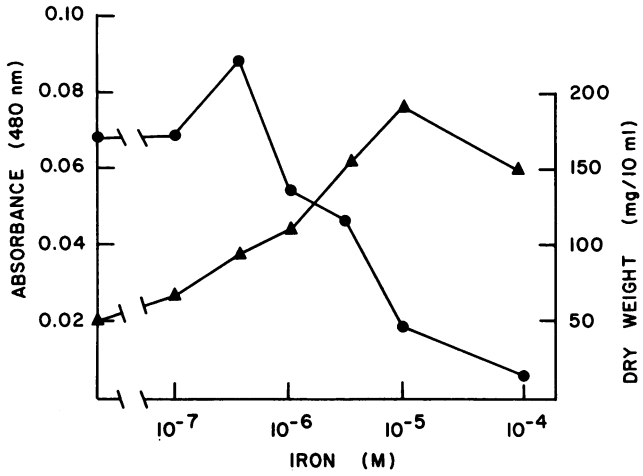


FIG. 1. Effect of iron concentration on production of hydroxamic acid by *H. capsulatum* strain 505. Symbols: ●, hydroxamic acid (absorbance at 480 nm); ▲, cell dry weight.

culture fluid by day 17 after the inoculation of strain 505. Thereafter, by day 21, the concentration of hydroxamic acid decreased only slightly to produce an absorbance of 0.082 (data not shown). After the removal of cells, the spent culture fluid was concentrated about 20-fold, and the hydroxamic acid was extracted as outlined above. The extracted material was separated into four iron-binding compounds by cation-exchange chromatography through cellulose phosphate (Fig. 2). After flash evaporation of the iron-binding fractions, the compound from peak I, which was the major hydroxamic acid isolated from the culture filtrate, dried to a white residue in the flask. This was dissolved in a small volume of hot methanol. On holding the methanolic solution at −20°C for several days, a white crystalline material formed and was collected (HA-I yield, 28 mg/liter). This compound was soluble in 1 N NaOH, 1 N HCl, water, pyridine, dimethylforamide, and dimethylsulfoxide, but not in less polar solvents tested, such as methanol, ethanol, *n*-propanol, *t*-butanol, *n*-butanol, acetone, or chloroform, and had a

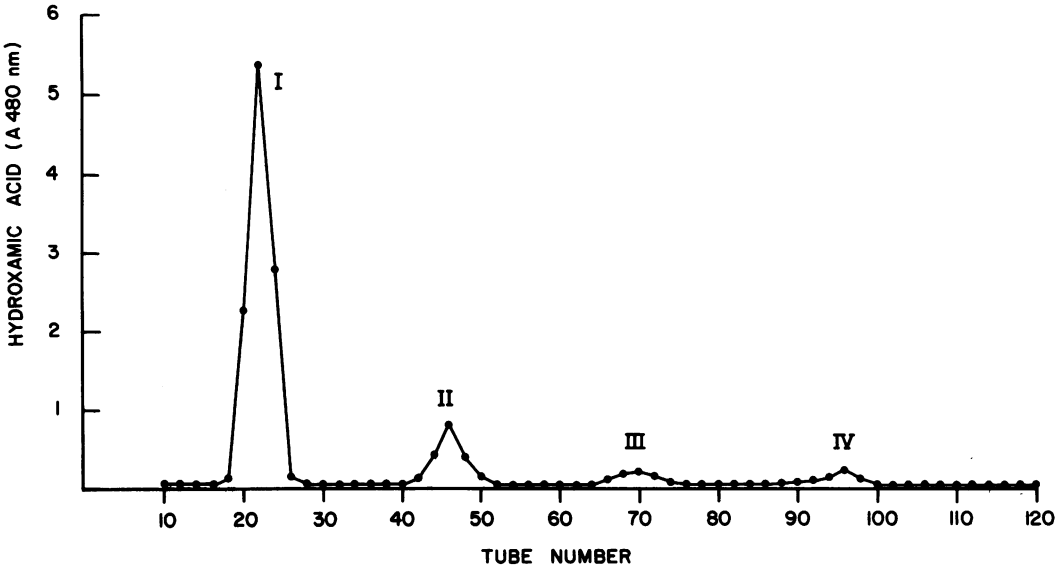


FIG. 2. Separation of hydroxamic acids by cation-exchange column (2 by 50 cm) chromatography through cellulose phosphate in 0.35% formic acid.

melting point of 120 to 121°C. The other isolated compounds, HA-II, HA-III, and HA-IV, were clear to light-tan oils, which eventually dried, forming thin films on glass.

**Identification of hydroxamic acids.** The purity of the isolated hydroxamic acids was confirmed by thin-layer chromatography, using solvent systems i, ii, and iii (Table 1). On paper electrophoresis at pH 5.2, HA-I, HA-II, and HA-III were neutral, whereas compound HA-IV appeared to be positively charged, migrating toward the cathode. At the lower pH of 2, all compounds appeared to be positively charged, with the exception of HA-I, which remained neutral. Fusarinine served as a control, being neutral at pH 5.2 and positively charged at pH 2.0 (7). Based on their migration on electrophoresis, HA-II and HA-III most likely have both free amino and carboxyl groups, whereas HA-IV appeared to have a preponderance, at least, of free amino groups. The neutral compound, HA-I, apparently had neither amino nor carboxyl groups, since it remained neutral at either pH. Of the four isolated compounds, HA-II, HA-III, and HA-IV were reactive with ninhydrin, a unique property also shared by the fusarinines, compounds isolated from *F. roseum* (6).

Compounds having either one to three hydroxamic acid functional groups per molecule exhibit differing abilities to chelate iron at low pH (1, 7), and this may be determined by their absorbance spectra on lowering the pH from 7 to 1.5. The change in absorbance maxima exhibited by HA-II and HA-III was suggestive of their having a single hydroxamate group per molecule (Table 1). The spectra of HA-I were typical of those produced by a dihydroxamic acid, such as dimerumic acid or rhodotorulic acid. HA-I was identified as a trihydroxamate; the absorption maxima for such compounds characteristically remain close to 430 nm at the two pH values.

In 0.2 M EDTA, the solution of ferrihydroxamates, HA-II and HA-III, quickly became colorless. The bleaching occurred more slowly with compound HA-I, whereas HA-IV was resistant to the bleaching action of EDTA. Typically, compounds having multiple hydroxamate functional groups per molecule are correspondingly more resistant to the removal of ferric ion by EDTA (7, 15).

TABLE 1. $R_f$ values of hydroxamic acids

| Hydroxamic acid | $R_f$ value in solvent: | | |
|---|---|---|---|
| | i[a] | ii[b] | iii[c] |
| HA-I | 0.51 | 0.58 | 0.59 |
| HA-II | 0.26 | 0.45 | 0.42 |
| HA-III | 0.17 | 0.33 | 0.27 |
| HA-IV | 0.27 | 0.47 | 0.45 |
| Dimerumic acid | 0.49 | 0.58 | 0.59 |
| Fusarinine | 0.28 | 0.45 | 0.43 |

[a] Water-saturated *n*-butanol.
[b] *n*-Butanol–ethanol–water (4:1:5, vol/vol/vol).
[c] *n*-Butanol–acetic acid–water (100:12:25, vol/vol/vol).

TABLE 2. Absorbance maxima of isolated ferrihydroxamates

| Hydroxamate | λ maximum (nm) at pH: | |
|---|---|---|
| | 1.5 | 7.0 |
| HA-I | 490 | 434 |
| HA-II | 525 | 422 |
| HA-III | 517 | 430 |
| HA-IV | 434 | 430 |

Most hydroxamic acids produced by fungi are derivatives of δ-*N*-hydroxyornithine. Reductive hydrolysis of HA-I in HI yielded a single ninhydrin-positive compound on analysis by thin-layer chromatography. Its identification as ornithine was confirmed by chromatography in the five solvent systems described and by paper electrophoresis (see above).

It appeared likely that HA-I and HA-II or HA-III or both were breakdown products of the trihydroxamic acid, HA-IV. Subsequently, it was found that heating HA-IV dissolved in water or in 0.1 N NaOH at 100°C for 1 h resulted in the formation of compounds having $R_f$ values identical to those of the dihydroxamic acid HA-I and the monohydroxamic acid HA-II, similar to what would be expected to occur with coprogen B (see Fig. 4). The IR spectrum of HA-I was similar to that obtained with authentic dimerumic acid (Fig. 3), and that obtained for HA-IV was similar to the reported spectrum of coprogen B (5).

The acyl function of fungal hydroxamic acids has been found to be either an acetyl group or a derivative of mevalonic acid. Because of its αβ unsaturation, -C=C-, the presence of the latter group may be detected by its characteristically strong absorption at 225 nm. An αβ unsaturation of HA-II was indicated by an observed absorption maximum at 225 nm, a characteristic also shared by the monohydroxamic acid, fusarinine (7). The charge and $R_f$ values of HA-II were similar to those of fusarinine.

The fusarinines are characteristically unstable when heated to 60°C in 0.5 N HCl. Emery (7) has attributed this to the ease of lactone formation of the acyl group because of its *cis* configuration. Differing from fusarinine, HA-II was stable in 0.5 N HCl (data not shown), as were HA-I, HA-III, and HA-IV, which indicated that the acyl function for these compounds most likely is in the *trans* configuration, similar to *trans* fusarinine, a component of coprogen B (5).

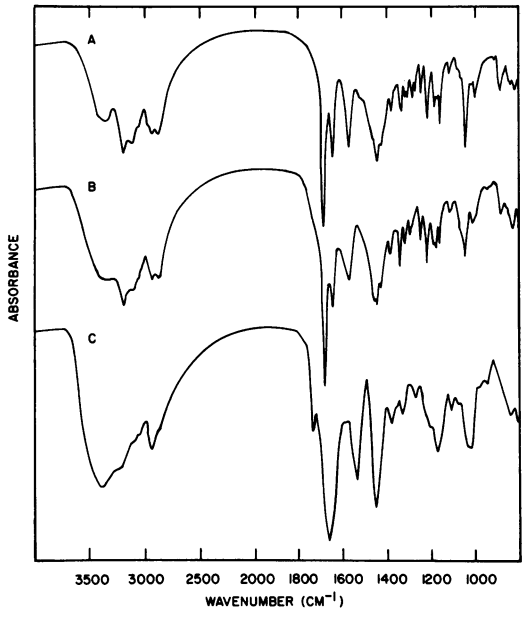


FIG. 3. IR spectra of HA-I (A), dimerumic acid (B), and HA-IV (C).

Based on their charge characteristics at pH 5.2 and 2.0, $R_f$ values in three solvent systems, reaction with ninhydrin, and visible and IR spectra, HA-I, HA-II, and HA-IV were identified as dimerumic acid, *trans* fusarinine, and deferricoprogen B, respectively (Fig. 4). The monohydroxamic acid, HA-III, which was isolated in very small amounts, remains unidentified.

**Growth factor activity.** In an earlier report, an extract containing hydroxamic acid from a low-iron culture medium produced by *H. capsulatum* was found to enhance the colony formation of several *Histoplasma* isolates on solid media (3). To determine which of the four hydroxamic acids isolated from *H. capsulatum* might have growth factor activity, solutions of the compounds were added to a solid medium as described (see above). The compounds identified as di- and trihydroxamates, HA-I and HA-IV, respectively, and authentic dimerumic acid were found to facilitate both yeast and mycelial colony formation of the three *H. capsulatum* strains tested (Table 3). Slight mycelial growth factor activity was attributed to HA-III for the three strains tested.

## DISCUSSION

Four distinct hydroxamic acids were isolated from a low-iron 37°C culture of the yeast form of *H. capsulatum* strain 505. Based on their electrophoretic charge, the number of hydroxamate functional groups per molecule, thin-layer chromatographic data, and IR spectra, three were identified as deferricoprogen B and its breakdown products, dimerumic acid and *trans* fusarinine (Fig. 4). Of the four isolated compounds, HA-IV and HA-I exhibited growth factor activity for *H. capsulatum*.

Diekmann (5) previously isolated and characterized coprogen B and its components from culture fluids of *Fusarium dimerum*. Dimerumic acid has since been synthesized chemically (24). Growth factor activity of the structurally related trihydroxamates; coprogen (12) and triornicin (10, 11), and the dihydroxamic acid, rhodotorulic acid (1), remains unknown at present. Although speculative at this point, the possibility remains that the diketopiperazine ring moiety of

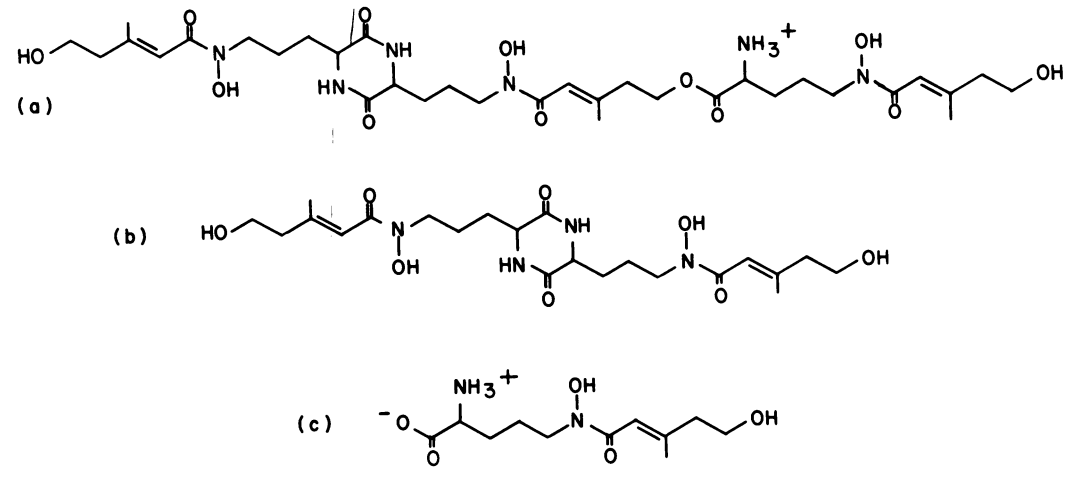


FIG. 4. Coprogen B (a), dimerumic acid (b), and *trans* fusarinine (c).

TABLE 3. Growth factor activity of purified hydroxamic acids

| Fungal isolate | No. of colonies formed on: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SDA (25°C)[a] with: | | | | | | BHIA/BSA (37°C)[b] with: | | | | | |
| | None | HA-I | HA-II | HA-III | HA-IV | Dimerumic acid | None | HA-I | HA-II | HA-III | HA-IV | Dimerumic acid |
| 505 | 34 | 147 | 36 | 123 | 120 | 156 | 0 | 173 | 0 | 0 | 130 | 142 |
| G-217B | 0 | 134 | 0 | 35 | 115 | 78 | 0 | 138 | 0 | 3 | 142 | 130 |
| MVH | 29 | 129 | 45 | 74 | 148 | 156 | 0 | 180 | 0 | 18 | 138 | 80 |

[a] Incubation for 14 days.
[b] Incubation for 7 days.

these compounds and of coprogen B and dimerumic acid affects the ferric siderophore complex and fungal cell membrane interaction for iron utilization.

Previous reports by Pine (17, 18) and Scherr (21) and from this laboratory (3) found that small inocula of *H. capsulatum* were unable to grow on certain media, even though when larger numbers of cells were plated on identical media, the resulting fungal growth was confluent. Based on these findings, we proposed that the growth of *H. capsulatum* requires the production of a factor which subsequently is able to facilitate cell growth. Fewer inoculated cells spread onto a medium would be unable to produce the growth factor sufficiently to stimulate colony formation. However, from larger inocula adequate production would be attained. Evidence obtained in this study suggests that the hydroxamic acids, HA-IV (deferricoprogen B) and HA-I (dimerumic acid), produced by *H. capsulatum* serve this function, presumably by chelating and supplying iron essential for cell metabolism.

In recent studies, the iron-binding proteins, transferrin and lactoferrin, were shown to inhibit 10- to 20-fold the yeast growth of *H. capsulatum* (21; M. C. Sutcliff and R. H. Alford, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D25, p. 42). Hydroxamic acid-type siderophores (growth factors) produced by *H. capsulatum*, therefore, might enhance fungal virulence in infected tissues by facilitiating iron acquisition from iron-binding proteins, analogous to mechanisms described for bacterial siderophores (23, 25).

The mycelial growth stimulation of *H. capsulatum* by HA-IV and HA-I and also HA III has important ecological implications. Powell et al. (19) found various hydroxate levels in extracts of numerous different soil types tested. In soils harboring *H. capsulatum*, there may be a special prevalence of certain hydroxates, such as coprogen B or dimerumic acid or both, produced by various companion soil saprophytic fungi. Indeed, these compounds have been shown to be produced by a variety of fungi, including *F. dimerum*, *Nectria cinnabarina*, *Myrothecium* sp., and *Neurospora crassa* (5, 6). Possibly other hydroxamates produced by fungi or soil actinomycetes might also have growth factor activity for *H. capsulatum*.

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