Hydroxamic Acid from *Histoplasma capsulatum* That Displays Growth Factor Activity

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Growth factor(s) present in a spent liquid medium after culture of the yeast form of *Histoplasma capsulatum* enhanced both yeast and mycelial growth of nine isolates tested. Hydroxamic acid extracted from the culture fluid displayed growth factor activity.

Small inocula (few numbers of cells) of the dimorphic fungus *Histoplasma capsulatum* are unable to initiate growth on certain culture media even though growth of larger inocula on identical media may become confluent. Pine (17, 18) and Scherr (20) determined that at least 10^5 cells were required to initiate growth on the media used in their studies. This suggested to us that growth of *H. capsulatum* may be dependent upon activity of some growth-promoting factor(s) produced by the inoculated organism. It is only when cells exceed a certain number on a given medium that sufficient concentration of the factor accumulates to stimulate growth.

This report describes production of a growth factor(s) by *H. capsulatum* that is capable of enhancing both yeast and mycelial growth of small inocula. The factor is tentatively identified as a hydroxamic acid-type siderophore, a type of compound known to chelate and provide iron essential for cell metabolism (2, 6).

Evidence of growth factor production of *H. capsulatum* isolates is shown in Fig. 1. A yeast cell suspension of *H. capsulatum* 505 was prepared in sterile saline (0.15 N NaCl) from a slant culture grown at 37°C for 2 days on brain heart infusion agar (2% agar), supplemented with 1.0% dextrose, 0.01% cysteine-HCl, 5% out-dated human blood, and the following antibiotics, penicillin G (20 U/ml) and streptomycin sulfate (40 µg/ml) (BHIA/B). The cells were washed once in saline and diluted to the desired concentration after counting with a hemacytometer. Plates of BHIA/B were inoculated by spreading 200 cells of *H. capsulatum* 505 in 0.1-ml volumes. After the agar surface had dried, a heavy inoculum of each of three *H. capsulatum* cultures was deposited onto the center of the plates. The plates were incubated at 37°C for 7 days in a covered plastic wash basin to maintain adequate humidity. The appearance of satellite colonies of *H. capsulatum* 505 that surrounded the confluent

growth at the center of the plates was the indication of growth factor production by the three isolates tested. The growth-promoting activity appeared to be somewhat specific since growth of *H. capsulatum* was not seen about inocula of isolates of *Candida albicans* or *Cryptococcus neoformans* (data not shown).

Earlier we had observed growth factor activity in a spent brain heart infusion broth filtrate obtained after culture of yeast cells of *H. capsulatum*, and reported that incorporation of the broth filtrate into a solid medium enhanced colony formation of small inocula of several *H. capsulatum* isolates (W. R. Burt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, F62, p. 128; W. R. Burt and A. L. Underwood, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, F2, p. 313). Iron chelators (siderophores) function as growth factors for many microorganisms by their specific acquisition of ferric ion (2, 4, 6, 15). The possibility that the growth factor from *H. capsulatum* could be a hydroxamic acid-type siderophore therefore was considered.

Hydroxamic acids have been shown to be produced in large amounts by many saprophytic fungi in media deficient in iron (1, 4, 16). Growth factor, therefore, was produced by *H. capsulatum* 505 in a defined liquid medium described by McVeigh and Morton (13) and modified to contain 3×10^{-7} M iron (1). The culture was inoculated into 1-liter quantities of medium in Fernbach flasks and was incubated at 37°C with constant rotary shaking (120 rpm) for 14 days. After growth, the culture fluid was clarified by filtration through glass fiber filters (Type A/E, Gelman, Ann Arbor, Mich.) and sterilized by two passages through 0.45-µm membrane filters. Aliquots of the spent culture filtrate (growth factor) were stored frozen (−20°C).

Plates of Emmon modified Sabouraud dextrose agar (SDA) and BHIA/B were prepared with and without growth factor. The filter-ster-

ilized growth factor was added to a concentration of 2% after autoclaving and cooling of the media to 45°C. Saline suspensions containing 2×10^6 yeast cells per ml were made of each of the *H. capsulatum* isolates to be tested. Dilutions were made, and 200 and 2,000 cells in 0.1-ml volumes were spread onto the plated media. The plates incubated at 25°C were sealed with masking tape, and those incubated at 37°C were placed

in covered wash basins. Results are shown in Table 1. For most of the isolates tested, the growth factor-containing media (except the BHIA/B incubated at 25°C) more effectively supported growth of the smaller inocula than did control media lacking the growth factor. The colonies produced on the media with growth factor were uniform in size, whereas those growing on media without the factor were smaller
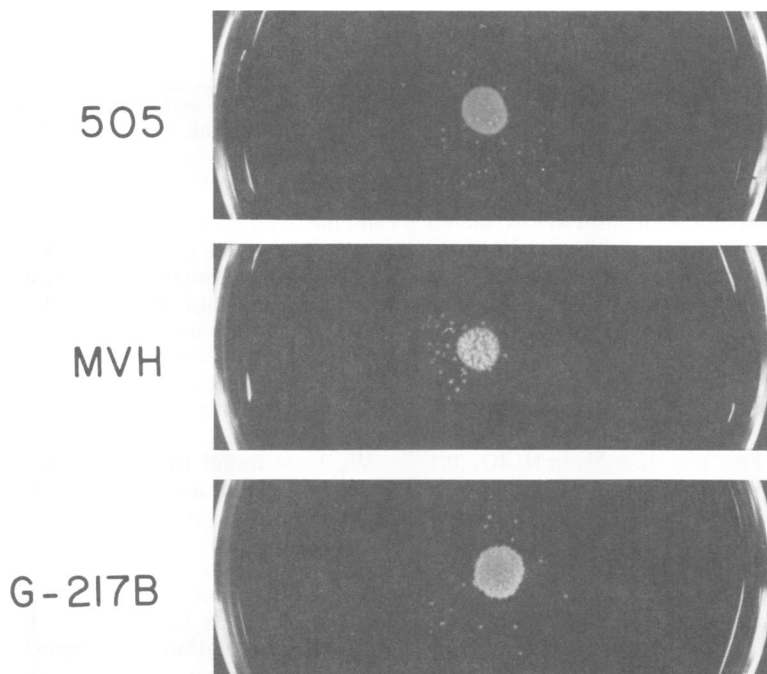

FIG. 1. *Development of satellite colonies of H. capsulatum 505 about confluent yeast growth of several isolates.*

TABLE 1. *Quantitation of standardized suspensions of H. capsulatum isolates on BHIA/B and SDA with and without growth factor*

Strain	CFU/ml determined on:					
	BHIA/B (37°C)[a]		BHIA/B (25°C)		SDA (25°C)	
	Growth factor	None	Growth factor	None	Growth factor	None
505	9.0×10^{5b}	2.0×10^3	1.1×10^6	8.9×10^5	9.1×10^5	2.3×10^5
MVH	1.4×10^6	1.2×10^5	1.7×10^6	1.5×10^6	1.5×10^6	8.8×10^5
G-217B	1.0×10^6	$<1 \times 10^{3c}$	7.3×10^5	4.5×10^5	4.7×10^5	$<1 \times 10^3$
G-217A	1.8×10^6	$<1 \times 10^3$	1.6×10^6	1.7×10^6	1.3×10^6	1.8×10^5
G-76	2.4×10^6	2.0×10^4	2.1×10^6	2.5×10^6	2.2×10^6	1.5×10^6
G-46	2.1×10^6	2.6×10^5	1.9×10^6	1.9×10^6	1.6×10^6	1.3×10^6
GS-2	1.3×10^6	1.2×10^4	9.9×10^5	7.2×10^5	4.0×10^4	2.0×10^3
GS-91	1.9×10^6	5.0×10^3	1.8×10^6	1.7×10^6	1.9×10^6	1.8×10^6
G-17	1.2×10^6	2.0×10^4	1.1×10^6	1.3×10^6	1.1×10^6	$<1 \times 10^3$

[a] Temperature of incubation. Plates were incubated at 37 and 25°C for 7 and 14 days, respectively.

[b] Number of colony-forming units (CFU) per milliliter in standardized suspensions as determined from plate counts of colonies forming on media. Each value was determined from the average of two plates.

[c] Indicates no growth on plates inoculated with 2,000 cells.

and more varied in size. The larger colonies on those media lacking the growth factor appeared to provide the factor to the surrounding smaller colonies in a manner similar to that shown in Fig. 1. The growth factor did not enhance colony formation of isolate GS-2 on SDA to the same extent that occurred with other isolates, which might indicate a higher concentration requirement of the factor for this isolate. Added growth factor was not necessary for maximal growth of the smaller inocula on BHIA/B incubated at 25°C since the colony-forming units per milliliter determined on this medium, which lacked the growth factor, equaled the values obtained on supplemented media.

Based on the assumption that hydroxamic acid produced by *H. capsulatum* is the growth factor for the organism, hydroxamic acid in the iron-limited spent culture medium was extracted by procedures described by Atkin and co-workers (1). Culture filtrate was concentrated 20-fold by pervaporation and adjusted to pH 4.5 by the addition of 1 HCl. The hydroxamic acid was extracted into the organic phase with phenol-chloroform (1:1), and then into the aqueous phase by addition of excess ether and several volumes of water. Hydroxamic acid was assayed by reaction with $Fe(ClO_4)_3$ (5 mM) in $HClO_4$ (0.1 M) (1). Bound hydroxylamine, indicative of a hydroxamic acid functional group, was detected in the spent culture filtrate and the culture extract using the Csaky test (3), and this confirmed the presence of hydroxamic acid.

Results (Table 2) demonstrate a direct relationship between growth factor activity and hydroxamic acid concentration since both remained in approximately equal proportions in the culture filtrate and extract. Spent culture fluid and extracted hydroxamic acid were diluted to produce an absorbance (480 nm) of 0.1 as determined in the assay for hydroxamic acid. The solutions were filter sterilized and diluted serially through a series of twofold dilutions. Volumes of 0.1 ml of each solution were spread onto separate BHIA/B plates (60 by 15 mm), which were allowed to dry at 4°C overnight. Each plate was then inoculated with 50 cells of *H. capsulatum* 505 and incubated at 37°C for 7 days. The number of colonies counted on the media receiving the diluted samples was an approximation of relative growth factor activity of the preparation.

In our studies, an extracellular product(s) from *H. capsulatum* 505 was found to enhance both yeast and mycelial growth of inocula containing relatively few cells of *H. capsulatum* isolates on BHIA/B (37°C) and SDA (25°C) (Table 1). It is postulated that, for growth of *H. capsulatum* on certain media, the inoculated

TABLE 2. *Comparison of growth factor activity of culture filtrate and extracted hydroxamic acid added to BHIA/B*

Reciprocal of dilution[a]	No. of colonies formed	
	Culture filtrate	Hydroxamic acid extract
2	25	17
4	26	38
8	17	23
16	0	12
32	1	0
64	0	0
128	0	0

[a] Culture filtrate and extract were adjusted to 0.1 absorbance (480 nm), filter sterilized, and diluted serially (twofold). Volumes of 0.1 ml of each dilution were added to separate BHIA/B plates.

cells must produce a diffusible factor that ultimately stimulates their own growth. The factor is able to affect growth only when it accumulates to or above some minimum threshold concentration. Accumulation of the growth factor to that concentration in a medium would be expected to occur most efficiently from densely inoculated cells, whereas this would not occur from fewer inoculated cells.

We have found that addition of the growth factor present in a spent, low-iron culture medium supported growth of the smaller number of inoculated cells. In studies by other workers, inocula containing 4×10^4 to 2×10^6 cells were required to initiate growth on certain media (17, 18, 20). Yen and Howard (21) observed that optimum germination of *H. capsulatum* at 23°C occurred only when yeast cells were present in concentrations in excess of 5×10^6 per ml. The exogenously supplied growth factor was not required for growth on BHIA/B at 25°C since mycelial growth occurred on the medium without growth factor and equaled growth on the supplemented media (Table 1). These results confirm those of Loosli (10, 11) and others (5, 12) who reported that recovery of *H. capsulatum* from clinical materials was accomplished most efficiently on blood-enriched media when incubated at room temperature. SDA has been found to be unsatisfactory for the primary isolation of *H. capsulatum* from clinical specimens (7–9, 12, 19).

The culture of *H. capsulatum* 505 produced hydroxamic acid in the iron-limited medium as determined by reaction with $Fe(ClO_4)_3$ and by detection of bound hydroxylamine. Production of hydroxamic acid-type siderophores by a number of fungal species and bacteria was reported previously (1, 2, 4, 14–16). This is the first report of siderophore production by a pathogenic fungus. Our study suggests that an association exists

between growth factor activity and the content of hydroxamic acid since both remained in approximately equal proportions in the spent culture filtrate and the extracted hydroxamic acid (Table 2). The hydroxamic acid from *H. capsulatum* serves as a growth factor presumably by chelating and supplying iron essential to cell growth.

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