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Growth-promoting factors for yeast cells of *Paracoccidioides brasiliensis*

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Low-density seedings of yeast cells of *Paracoccidioides brasiliensis* give poor growth (as assessed by plating efficiency test) on conventional mycological agar media, and therefore growth-promoting factors for this fungus were sought. Water-extracts of yeast cells of six *P. brasiliensis* isolates were all considerably effective in promoting the growth of low-density seedings of *P. brasiliensis* isolates Pb-18 and Hachisuga, but had little effect on isolate Bt-4. Horse serum, at a concentration range of 2-4%, moderately or considerably promoted the growth of these *P. brasiliensis* isolates. Combinations of the fungus cell extracts with horse serum were highly effective in promoting the growth of all of the fungal isolates. The fungus cell extracts showed siderophore (microbial iron carrier) activity. An iron-chelator, ethylenediaminetetraacetic acid, at a concentration of 100 μM also highly promoted the growth of the fungal isolates in the presence of horse serum, and ferric ion added to culture medium was considerably effective in the growth promotion. These results suggest that deficient utilization of external iron by the fungus cell is one of the growth-limiting processes for low-density seedings of yeast cells of *P. brasiliensis* on conventional mycological agar media.

Key Words——iron; iron chelator; *Paracoccidioides brasiliensis*; plating efficiency; siderophore.

Paracoccidioides brasiliensis (Splendore) Almeida, a thermally dimorphic fungus, is the causative agent of paracoccidioidomycosis, one of the major systemic mycoses in Latin America. It grows as yeast form in the host or when cultured at 37°C or somewhat lower temperatures. It is well known that plating efficiency (expressed as the ratio of colony forming units (CFU) to hemocytometer counts of viable fungal units) of low-density seedings of *P. brasiliensis* yeast cells is poor on standard mycological agar media. The reason for the poor plating efficiency is not yet been fully understood. Since improvement of the culture method to obtain high plating efficiency for *P. brasiliensis* yeast cells is of critical importance for studies of defense mechanisms of the host for this fungus, several efforts have been made to improve culture media for plating this fungus. Goihman-Yahr et al. (1980) found that horse serum, when added to brain heart infusion (BHI) agar at a concentration of 8%, moderately improved the plating efficiency of *P. brasiliensis* yeast cells. Castaneda et al. (1988) reported that culture filtrates of several *P. brasiliensis* isolates, when added individually to BHI agar or modified McVeigh-Morton medium in combination with horse serum, considerably increased the plating efficiency of some but not all *P. brasiliensis* isolates tested. Similar results were also reported by Singer-Vermes et al. (1992).

Recently Kurita et al. (1993) found that water-extract of yeast cells of a *P. brasiliensis* isolate, when added to BHI agar in combination with horse serum, was highly effective in improving the plating efficiency of yeast cells of *P. brasiliensis* isolates.

We investigated mechanisms of the growth-promotion of *P. brasiliensis* yeast cells. In the present paper, ferric ion (Fe^{3+}) and an iron-chelator (ethylenediaminetetraacetic acid, EDTA) as well as extracts of yeast cells of several *P. brasiliensis* isolates will be shown to exhibit, in combination with horse serum, considerable or excellent growth-promoting effects on *P. brasiliensis* yeast cells. Our results suggest that deficient utilization of external iron by the fungus cell is one of growth-limiting processes for low-density seedings of yeast cells of this fungus on conventional mycological agar media.

Materials and Methods

Fungi *Paracoccidioides brasiliensis* isolates Pb-18, B-1183, Bt-4 and Bt-9 were obtained from Brazilian patients with paracoccidioidomycosis, and isolate Tatu was obtained from the spleen of an armadillo (*Dasypus novemcinctus*) and shown to be pathogenic to hamsters (Naiff et al., 1986). *Paracoccidioides brasiliensis* Hachisuga was isolated from a Japanese patient with paracoccidioidomycosis. These *P. brasiliensis* isolates had been maintained in the yeast phase at 35°C on 1% glucose-

added BHI agar slants by subculturing at 4-d intervals. In some experiments yeast cells of these isolates were newly derived from the mycelial form and subcultured two times or more at 35°C before use, because their growing activity diminished after a long period of repeated subculture.

Water-extracts of fungus cells Yeast cells of *P. brasiliensis* isolates for water-extraction were grown at 35°C for 4 d on 1% glucose-added BHI agar plates. The yeast cells of individual fungi were harvested and suspended in distilled water to a density of 10% (w/v). The suspensions were autoclaved at 120°C for 15 min and allowed to stand at room temperature for 3 d. After centrifugation at 1000 x g for 15 min, the supernatants were removed and kept at room temperature under sterile conditions until use.

Reagents Ethylenediamine-di-*o*-hydroxyphenyl acetic acid (EDDHA), desferrioxamine mesylate (DFOM) and EDTA disodium salt were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). $FeCl_3 \cdot 6H_2O$, $AlCl_3 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, $MnCl_2 \cdot 4H_2O$, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$ and $SnCl_2 \cdot 2H_2O$ were obtained from Wako Pure Chemicals Ltd. (Tokyo, Japan). All these inorganic salts and DFOM were individually dissolved in distilled water and sterilized by membrane filtration before adding to culture media. EDDHA and EDTA disodium salt were added to culture media before autoclaving the media.

Horse serum was purchased from GIBCO Laboratories, Life Technologies Inc. (Grand Island, NY, USA). Horse serum was added to the autoclaved basal medium which had been cooled to 56°C.

Plating efficiency test To assess growth-enhancing activity of test substances, the plating efficiency test was employed. The procedure has been described elsewhere (Kurita et al., 1993). Briefly, yeast cells of *P. brasiliensis* isolates Pb-18, Bt-4 and Hachisuga to be used as inocula in the plating efficiency test were grown at 35°C for 5 d on 1% glucose-added BHI agar slants and suspended in phosphate-buffered saline (PBS, pH 7.2). The fungus cell suspensions were passed through six layers of gauze to remove clumps of fungus cells, and fungal units were counted in a hemocytometer. The fungal units consisted of single cells, single cells with daughter cells, and two- to five-cell units. More than 97% of cell units of the *P. brasiliensis* isolates thus prepared were found viable (Kurita et al., 1993). Fungus cell suspensions were adjusted to a density of 300 or 500 fungal units/ml for use. A volume of 1 ml of the fungus cell suspension was plated on each agar plate to be tested. The agar plates were placed in a humidified incubator at 35°C for 6 d, and colonies formed were counted. Plating efficiency expressed in percent was calculated as follows: (colonies formed/fungal units counted in a hemocytometer) x 100.

Statistical analyses Analyses were performed by the Student's *t*-test, where $p<0.05$ is statistically significant.

Results and Discussion

Effect of water-extracts of *P. brasiliensis* isolates As is well known, the plating efficiency of *P. brasiliensis* was poor on BHI agar without supplement. Horse serum, when added to the medium to a concentration of 4%, moderately improved the plating efficiency of *P. brasiliensis* isolates Pb-18 and Hachisuga, and fairly improved that of isolate Bt-4 (Table 1). As reported by Castaneda et al. (1988), higher concentrations of horse serum did not further improve the plating efficiency, and essentially the same results were obtained with 2% concentration of horse serum (data are not shown). In addition to water-extract of *P. brasiliensis* Pb-18, extracts of *P. brasiliensis* isolates Tatu, Bt-4, Bt-9, Hachisuga and B-1183 considerably increased the plating efficiencies of *P. brasiliensis* isolates Pb-18 and Hachisuga. By contrast, these fungus cell extracts had little effect on the growth of isolate Bt-4. When combined with horse serum, the fungus cell extracts were highly effective in improving the plating efficiency of all the *P. brasiliensis* isolates employed (Table 1). Figure 1 shows the growth-promoting effect of the fungus cell extract and horse serum on isolate Pb-18. In another line of investigation, water-extracts of yeast cells of *Histoplasma capsulatum* Darling and *Blastomyces dermatitidis* Gilchrist & Stokes, and water-extracts of hyphae of *Aspergillus fumigatus* Fresenius and *Penicillium decumbens* Thom were also highly effective in promoting the growth of *P. brasiliensis* yeast cells especially in the presence of 2-4% (v/v) horse serum (unpublished data). These results appear to indicate that the putative growth factor(s) for *P. brasiliensis* yeast cells

Table 1. Effect of water-extracts of *P. brasiliensis* yeast cells on plating efficiency of *P. brasiliensis*.

BHI agar supplemented with	Horse serum (4%)	Plating efficiency (%)[c]		
		P. brasiliensis isolates		
		Pb-18	Bt-4	Hachisuga
Nil	−	21± 3	1±1	12±2
Nil	+	43± 7	74±4	46±3
Pb[a]-18 Ext[b]	−	73± 3	1±1	70±6
Pb-18 Ext	+	97±10	99±2	97±8
Pb Tatu Ext	−	56± 2	0.5±0.5	68±2
Pb Tatu Ext	+	90± 6	99±5	87±4
Pb Bt-4 Ext	−	42± 7	1±1	77±2
Pb Bt-4 Ext	+	85± 5	98±3	91±2
Pb Bt-9 Ext	−	52± 4	1±0.4	60±8
Pb Bt-9 Ext	+	90± 4	101±7	98±4
Pb Hachisuga Ext	−	47± 6	1±0.5	66±4
Pb Hachisuga Ext	+	89± 4	102±8	96±7
Pb B-1183 Ext	−	74± 8	Not done	Not done
Pb B-1183 Ext	+	95± 7	Not done	Not done

a) Pb=*P. brasiliensis*.
b) Ext = Water-extract. Water-extract was added to a concentration of 5%.
c) Mean plating efficiency ±SD of quadruplicate cultures in single experiments.

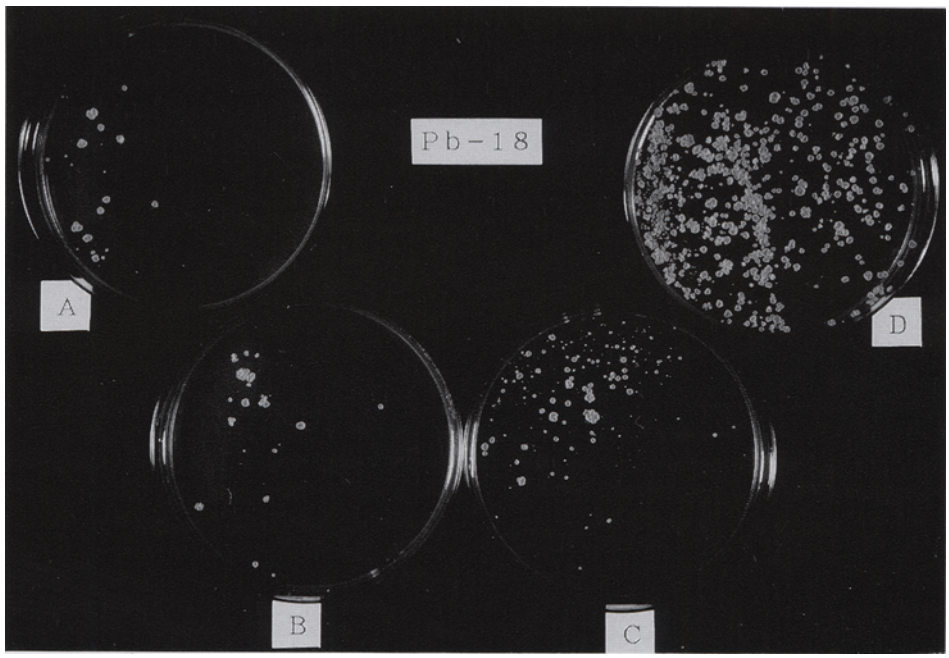

Fig. 1. Plating efficiency for *P. brasiliensis* Pb-18.
Culture media were BHI agar with no supplement (A), supplemented with 4% horse serum (B), with 5% water-extract of *P. brasiliensis* Bt-4 (C), and with 4% horse serum plus 5% water-extract of *P. brasiliensis* Bt-4 (D). Five hundred fungal units in 1 ml of PBS were plated on each agar plate. The photograph was taken at day 7 of incubation at 35°C.

is neither species- nor strain-specific.

It is of interest that the growth responsiveness of isolate Bt-4 to the fungus cell extracts was different from that of isolates Pb-18 and Hachisuga.

Detection of siderophore The putative growth-promoting factor in the *P. brasiliensis* cell extracts was found to be heat-stable because autoclaving (120°C for 15 min) did not abolish or diminish the growth-promoting activity of the fungus cell extracts. In another line of investigation, the growth-promoting factor was found to be dialysable, indicating that it is a low molecular weight substance.

Castaneda et al. (1988) reported that siderophores (iron-carriers) produced by *B. dermatitidis* and *Ustilago sphaerogena* Burrill moderately improved the plating efficiency of *P. brasiliensis* yeast cells in combination with horse serum. Although their results appear to suggest that siderophores could be the growth-promoting factor in the fungus cell extracts, they did not show that *P. brasiliensis* yeast cells produced a siderophore. Thus we first examined the *P. brasiliensis* cell extract for siderophore activity. EDDHA and DFOM possess a highly specific capacity to bind iron, the complex stability constant (log K) for Fe^{3+} being 33.91 for EDDHA (L'Eplattenier et al., 1967) and 30.6 for DFOM (Prelog, 1963). These iron-chelators are potent growth-inhibitors for *P. brasiliensis* yeast cells (Table 2), probably because this fungus can not utilize iron chelated by these compounds. If the fungus cell extract contains a siderophore utilizable by *P. brasiliensis* yeast cells and the siderophore possesses a high affinity for Fe^{3+} which is comparable to those of EDDHA and DFOM, it should reverse the growth-inhibitory effect of these iron-chelators (Miles et al., 1975). As shown in Table 2, the water-extract of *P. brasiliensis* Pb-

Table 2. Antagonism of water-extract of *P. brasiliensis* against iron chelators in effect on plating efficiency of *P. brasiliensis*.

BHI agar supplemented with	Plating efficiency (%)[c]		
	P. brasiliensis isolates		
	Pb-18	Bt-4	Hachisuga
Nil	24± 3	1± 1	14± 3
EDDHA	0± 0	0± 0	2± 1
EDDHA+Pb-18 Ext[a]	65± 5	1± 1	69± 4
EDDHA+HS[b]	0± 0	4± 2	2± 1
EDDHA+Pb-18 Ext+HS	91± 6	87± 3	93± 5
EDDHA+$FeCl_3$ (10 μM)	0± 0	0± 0	2± 1
EDDHA+$FeCl_3$ (100 μM)	47± 4	0± 0	53± 3
DFOM	0± 0	0± 0	0± 0
DFOM+Pb-18 Ext	41± 3	0± 0	51± 1
DFOM+HS	0± 0	3± 1	0± 0
DFOM+Pb-18 Ext+HS	97±11	85± 5	93± 4
DFOM+$FeCl_3$ (10 μM)	0± 0	0± 0	0± 0
DFOM+$FeCl_3$ (100 μM)	38± 3	0± 0	54± 3

EDDHA and DFOM were added to a concentration of 100 μM.
a) Pb-18 Ext = water-extract of Pb-18 yeast cells added to a concentration of 5%.
b) HS = horse serum added to a concentration of 2%.
c) Mean plating efficiency ± SD of quadruplicate cultures in single experiments.

18 yeast cells fairly reversed the growth inhibition of isolates Pb-18 and Hachisuga by the iron-chelators. Fe^{3+}, added at equimolar concentration with the iron-chelators, also reversed the inhibitory effect of the chelators on these *P. brasiliensis* isolates as expected (Table 2). However, iron concentration of the *P. brasiliensis* cell extract was as low as 2.86 μM as determined by a conventional atomic absorption technique. Iron contained in the fungus cell extract should have little effect on the growth inhibition with 100 μM of EDDHA or DFOM because even 10 μM concentration of Fe^{3+} added along with 100 μM of EDDHA or DFOM had no effect in this regard (Table 2). Therefore it was apparent that the cell extract of the *P. brasiliensis* isolate contained a siderophore. Similar results were also obtained with the cell extracts of the other *P. brasiliensis* isolates, the iron contents of which were lower than 3.1 μM (data are not shown). By contrast, horse serum failed to reverse the inhibitory effect of EDDHA and DFOM on isolates Pb-18 and Hachisuga (Table 2), indicating that horse serum did not contain a siderophore utilizable by these *P. brasiliensis* yeast cells or that, if it did, the siderophore had low affinity for iron as compared with EDDHA and DFOM.

With regard to isolate Bt-4, water-extract of *P. brasiliensis* cells added alone did not reverse the inhibitory effect of EDDHA or DFOM, as expected from results shown in Table 1 (the fungus cell extract failed to promote the growth of isolate Bt-4). Horse serum added alone was only slightly effective in this regard. By contrast, a combination of horse serum and the fungus cell extract effectively antagonized EDDHA and DFOM (Table 2). It is not yet clear why the combination was effective while each alone was not effective. The effects of the fungus cell extract and horse serum appeared to be cooperative, and not simply additive.

Table 3. Growth-promoting activity of ethanol-soluble and insoluble fractions of *P. brasiliensis* extract.

BHI agar supplemented with	Plating efficiency(%)[d] (Pb—18)
Nil	28±3
HS[a](2%)	44±3[e]
Pb-18 Ext(2%)	71±3[e]
EtOH-S[b](2%)	56±4[e]
EtOH-I[c](2%)	38±2[e]
Pb-18 Ext(2%)+HS(2%)	96±4[f]
EtOH-S(2%)+HS(2%)	98±3[f]
EtOH-I(2%)+HS(2%)	48±5
EDDHA	0±0
EDDHA+Pb-18(4%)	46±4
EDDHA+EtOH-S(4%)	31±3
EDDHA+EtOH-I(4%)	1±1

EDDHA was added to a concentration of 100 μM.
a) HS=horse serum.
b) EtOH-S=ethanol-soluble fraction reconstituted to the original volume of Pb-18 extract solution.
c) EtOH-I=ethanol-insoluble fraction reconstituted to the original volume of Pb-18 extract solution.
d) Mean plating efficiency±SD of quadruplicate cultures in single experiments.
e) $p<0.01$ vs 'Nil'.
f) $p<0.01$ vs 'HS(2%)'.

The Pb-18 cell extract was lyophilized then fractionated by ethanol-extraction. The ethanol-soluble fraction accounted for 28% of the original fungus cell extract by weight. The ethanol-soluble and insoluble fractions were examined for the growth-promoting activity and antagonizing activity to EDDHA. As shown in Table 3, both the growth-promoting and antagonizing activities resided in the ethanol-soluble fraction. By contrast, the ethanol-insoluble fraction showed only a modest growth-promoting activity and little antagonizing activity. These results are consistent with the possibility that a siderophore produced by the fungus cell is a growth-promoting factor for *P. brasiliensis* yeast cells, as in the case of *H. capsulatum* yeast cells (Burt et al., 1981). Further purification and characterization of the growth-promoting factor is in progress in our laboratory.

Effect of inorganic cations and EDTA To determine whether iron utilization by the fungus cell is involved in the mechanism of growth promotion of *P. brasiliensis* yeast cells, we examined Fe^{3+} and several other inorganic cations for growth-promoting activity for *P. brasiliensis*. The inorganic cations were freshly added to the basal medium as their chlorides. Among the cations tested, only Fe^{3+} was highly effective, at a concentration of 100 μM, in promoting the growth of *P. brasiliensis* isolates Pb-18 and Hachisuga in the presence of horse serum. In the absence of horse serum, Fe^{3+} was moderately effective, at a concentration of 100 μM but not at 10 μM, on these isolates (Table 4). These results appear to indicate that efficient utilization of external iron by the fungus cell is involved in the growth promotion of these fungus isolates.

It should be noted that unsupplemented BHI agar, i.e., the basal medium employed here, is not deficient in

Table 4. Effect of iron and EDTA on plating efficiency of *P. brasiliensis*.

BHI agar supplemented with	Plating efficiency(%)[b]		
	P. brasiliensis isolates		
	Pb-18	Bt-4	Hachisuga
Nil	27±2	1±1	14±3
HS[a](2%)	43±7[c]	79±5[c]	48±5[c]
$FeCl_3$(10 μM)	26±3	1±0.5	15±3
$FeCl_3$(100 μM)	63±4[c]	1±1	57±3[c]
$FeCl_3$(100 μM)+HS(2%)	92±2[c]	58±3[c]	91±8[c]
EDTA(100 μM)	79±8[c]	3±1[d]	33±4[c]
EDTA(100 μM)+HS(2%)	97±3[c]	105±2[c]	89±9[c]

a) HS=horse serum.
b) Mean plating efficiency±SD of quadruplicate cultures in single experiments.
c) $p<0.01$ vs 'Nil'.
d) $p<0.05$ vs 'Nil'.

any nutrients, including iron, that is indispensable to the growth of *P. brasiliensis* yeast cells, because all the *P. brasiliensis* isolates employed showed normal abundant growth within several days at 35°C on this medium when high-density inocula of *P. brasiliensis* yeast cells were used. In this case, it can reasonably be assumed that *P. brasiliensis* yeast cells efficiently utilize iron and other nutrients in the medium for their growth. Therefore it appears that a defect in utilization of external iron by *P. brasiliensis* cells, but not insufficiency of iron in the medium, is one of growth-limiting processes for low-density seedings of these fungal isolates on unsupplemented BHI agar. Presumably, insufficient siderophores are available for *P. brasiliensis* yeast cells to grow when low-density seedings are used on conventional mycological agar media, whereas sufficient siderophores are provided by the large number of fungus cells in the vicinity when high-density inocula are used. With regard to isolate Bt-4, by contrast, Fe^{3+} failed to exhibit the growth-promoting effect in the presence or absence of horse serum (Table 4).

Of the other inorganic cations, manganous ion (Mn^{2+}) was fairly growth-inhibitory on these fungal isolates both in the presence and absence of horse serum, while the other cations (Al^{3+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+} and Sn^{2+}) showed no significant effect on any of these *P. brasiliensis* isolates (data not shown).

EDTA is an iron-chelator and the stability constant (log K) of EDTA-Fe^{3+} complex was reported to be 25.1, indicating that EDTA possesses a relatively high affinity for Fe^{3+} (Prelog, 1963). Higher concentrations of EDTA are highly toxic for *P. brasiliensis*, as is well known with other microorganisms (Kida et al., 1992). EDTA, at a concentration of 3 mM or higher, completely suppressed the growth of yeast cells of this fungus in the presence or absence of horse serum (data not shown). By contrast, EDTA at a concentration of 100 μM, unlike the same concentration of EDDHA and DFOM, highly promoted the growth of all the *P. brasiliensis* isolates employed in the presence of horse serum, and in the absence of horse serum EDTA moderately promoted the growth of isolates Pb-18 and Hachisuga, but had only a slight effect on isolate Bt-4 (Table 4). The effectiveness pattern of EDTA on the growth of these *P. brasiliensis* isolates resembled those of the fungus cell extracts (Table 1), suggesting that the growth-promoting factor in the fungus cell extracts is an iron-chelator, i.e., a siderophore.

To our knowledge, BHI agar supplemented with EDTA (50-200 μM) plus horse serum (2-4%) would be the simplest and most reliable culture medium with high plating efficiency for yeast cells of *P. brasiliensis*.

The present study showed that the fungus cell extracts possessed siderophore activity and that the fungus cell extracts, EDTA and freshly added Fe^{3+} were effective in promoting the growth of *P. brasiliensis* yeast cells. These results, taken together, strongly suggest that utilization of external iron by the fungus cell is one of the growth-limiting processes for low-density seedings of this fungus.

In preliminary experiments, the growth-promoting factor(s) in horse serum was found to be nondialysable, i.e., a high molecular weight substance. Characterization of growth factors and studies of mechanisms of the growth-promoting effect of horse serum is in progress in our laboratory.

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