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Characteristics of 17 *Paracoccidioides brasiliensis* isolates

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We have studied the physiological and morphological features of 17 isolates of *Paracoccidioides brasiliensis* in order to define their phenotypes. The isolates were cultured at room temperature on potato dextrose agar (PDA , Difco) slants for mycelial growth and in 1% dextrose brain heart infusion agar (BHIA, Difco) at 37°C for the study of yeast forms. Most mycelial and yeast forms grew well between pH 5.6–9.4. In their response to osmotic pressure the isolates were separated in three groups: intolerant, intermediate and tolerant. They also varied in carbohydrate assimilation tests, which indicated important metabolic variation. No clear differences were observed in phenol oxidase tests, KNO_3, starch, casein and arbutin assimilation tests. Only 1 of the isolates, Bt-19, had gelatinase activity. No correlation was observed between the above differences and virulence. Two patterns of growth were observed in the mycelial cultures, glabrous and cottonous, the latter being correlated with increased virulence for ddY mice. Most yeast forms grew as cerebriform colonies, but Pb-HC and Bt-19 colonies had a cobblestone-like surface.

Key Words——morphology; *Paracoccidioides brasiliensis*; physiology.

Paracoccidioides brasiliensis (Splendore) Almeida is the causative agent of paracoccidioidomycosis, a deep mycosis limited to Latin America. This fungus grows as a yeast form at 35–37°C in vitro or in host tissue and as mycelial form at room temperature (Restrepo, 1985; San-Blas, 1993).

Studies on the morphological and physiological peculiarities of different isolates of *P. brasiliensis* are scarce. Requirement of amino acids and sulfur and their possible relation to dimorphism (Paris et al., 1985; Cano and Aguiar, 1991) as well as some of its protease activities are known (Albornoz and Campo-Aasen, 1971; Mendez-Giannini et al., 1990; Bedoya-Escobar et al., 1993; Vaz et al., 1994); and the nutritional requirements of *P. brasiliensis* have been compared with those of *Blastomyces dermatitidis* Gilchrist et Stokes (Gilardi and Laffer, 1962). DNA patterns of different isolates of *P. brasiliensis* by RAPD have been analyzed and it was suggested that the studied isolates could be separated in two different groups (Soares et al., 1995). In this paper we describe the morphological and physiological features of 17 different isolates of *P. brasiliensis* with the objective of defining phenotype variations that might be related to differences in genotype.

Materials and Methods

Isolates Thirteen Brazilian patients' *P. brasiliensis* isolates (Pb-9, IFM41620; Pb-18, IFM41621; Pb-265, IFM46466; Pb-339, IFM41630; Pb-HC, IFM46467; Bt-2, IFM41622; Bt-3, IFM41623; Bt-4, IFM41624; Bt-7, IFM41625; Bt-9, IFM41626; Bt-19, IFM41627; B-1183, IFM41628 and Recife, IFM41631), three Japanese patients' isolates (Aoki, IFM41632; Hachisuga, IFM 41633; and Wagura, IFM46215) and one Amazonian armadillo's isolate (Tatu, IFM46463) were maintained at room temperature on potato dextrose agar (PDA, Difco) slants and subcultured twice a year. They were transferred to tubes with 1% dextrose plus brain heart infusion agar (BHIA, Difco) at 37°C for 1 wk, then subcultured every 3 or 7 d for 2 wk. For the mycelial form studies they were subcultured on PDA slants at room temperature for 30 to 60 d.

Effects of pH Small amounts of mycelial forms were cultured for 30 d at 25°C on PDA plates plus glycine buffer and 0.2 M NaOH or 0.2 M HCl at a pH of 2.4, 4.0, 4.6, 5.6, 6.8, 7.6, 8.4, 9.2, 9.4, 9.6 or 10.4. Pinhole amounts of yeast forms were cultured for 7 d at 37°C on BHIA plates at pH 3.6, 4.6, 5.6, 6.8, 7.6, 7.8, 8.0, 8.4, 8.6, 8.8, 9.0 or 9.4. Growth was scored as present (+) or absent (−).

Carbohydrate assimilation Yeasts form cells in suspension were inoculated on API20C auxanogram (bioMerieux, France) and incubated at 37°C for 4 d for evaluation of 19 carbohydrates: glucose (GLU), glycerol (GLY), 2-keto-D-gluconate (2KG), arabinose (ARA), xy-

lose (XYL), adonitol (ADO), xylitol (XLT), galactose (GAL), inositol (INO), sorbitol (SOR), α-methyl-D-glucoside (MDG), *N*-acetyl-glucosamine (NAG), cellobiose (CEL), lactose (LAC), maltose (MAL), saccharose (SAC), trehalose (TRE), melezitose (MLZ) and raffinose (RAF). Isolates Pb-265, Bt-19 and Pb-HC were not studied because of their poor growth on the system or the difficulty in obtaining yeast forms.

Halophilia PDA slants grown at 25°C for 30 d were used for the mycelial form and BHIA slants grown at 37°C for 30 d for the yeast form. Three subcultures were done for each medium, one without NaCl and one each with 1 or 3% NaCl.

Osmophilia Pinhole amounts of mycelial forms were cultured at 25°C for 30 d on PDA plates with 10, 20, 30 or 40% of glycerol.

Urease activity Yeast forms were spread on Christensen urea agar (Eiken, Tokyo) at 37°C for 4 d. A patient's isolate of *Cryptococcus neoformans* (Sanfelice) Vuillemin var. *neoformans* was cultured as a positive control.

Phenol oxydase activity Pinhole amounts of yeast forms were inoculated at 37°C for 4 d on PDA plates containing caffeic acid (Wako, Tokyo, Japan, 0.3 g/L). A patient's isolate of *C. neoformans* var. *neoformans* was used as a positive control.

KNO_3 assimilation Pinhole amounts of mycelial forms were cultured at 25°C for 30 d on plates of yeast carbon base (Difco) with 2% agar plus 0.78% KNO_3. Plates with the same medium plus 0.78% $(NH_4)_2SO_4$ as a nitrogen source were used as controls. Yeast forms were also cultured in the same media at 37°C for 30 d.

Starch assimilation Pinhole amounts of mycelial forms were cultured at 25°C for 30 d on plates of yeast morphology agar (Difco) plus 0.2% agar. Yeast forms were also cultured in the same media at 37°C for 30 d.

Casein hydrolysis test (skim milk assimilation) Pinhole amounts of mycelial forms were cultured at 25°C for 30 d on plates of skim milk (50 g/L, Difco) plus 2% agar. Yeast forms were also cultured in the same media at 37°C for 30 d. A patient's isolate of *Aspergillus fumigatus* Fesenius was used as control.

Arbutin assimilation Pinhole amounts of mycelial forms were cultured at 25°C for 30 d on plates of 0.5% arbutin (Wako) plus 1% yeast extract (Difco) and 2% agar, to which we added one or two drops of 10% $FeCl_3$. Yeast forms were also cultured on the same medium at 37°C for 30 d. The test indicates β-glucosidase activity.

Gelatinase activity Pinhole amounts of mycelial forms were cultured at 25°C for 60 d in tubes of Czapek-Dox solution (Difco) with 12% gelatin (Difco).

Mating test Mating was carried out on PDA plates at room temperature for 8 wk. Punct inoculation of each pair of isolates was placed on the center of the plate at 1 cm distance. The growth was observed gross and microscopically.

Morphology Mycelial cultures at 25°C for 2 mo on PDA slant and yeast form cultures at 37°C for 7 d on BHIA slants were grossly observed. Yeast cells were suspended in lactophenol cotton blue and spread on slides for microscopical observation.

Pathogenicity Three Japanese patients' isolates (Aoki, Hachisuga and Wagura) were evaluated for pathogenicity in ddY mice as described previously using a scoring method obtained from organ culture 4 wk after intravenous inoculation with 10^6 yeast cell colony forming units from groups of 5 mice (Sano et al., 1991).

Results

Mycelial growth was completely prevented at pH 2.4 and the majority of the isolates grew well from pH 5.6 to 9.4 (Table 1). Most of the isolates had inhibited growth at pH 10.4. Isolate Wagura showed excellent growth at pH 8.4–9.4, but it did not grow at pH 5.6 or below. Isolates Pb-265, Aoki and Hachisuga were not tolerant to higher pH.

Yeast forms did not grow below pH 3.6. All isolates in the yeast form showed good growth from pH 5.6 to 9.4 (Table 2).

Glucose was well assimilated by all isolates tested. Maltose was also assimilated by 12 out of 14 isolates tested. Some of the isolates also assimilated glycerol, 2-keto-D-gluconate, arabinose, xylose, adonitol, xylitol, galactose, inositol, sorbitol, α-methyl-D-glucoside, *N*-acetyl-glucosamine, cellobiose, lactose, maltose, trehalose, melezitose and raffinose. Isolates Bt-3, Bt-4, Bt-7, Tatu and Wagura assimilated more than eight carbohydrates (Table 3).

Both the mycelial and yeast forms were tolerant of 1% NaCl added to the medium but not to 3%.

Isolates Pb-9, Pb-265, Pb-339, Bt-3, Bt-19, Recife and Wagura were intolerant to osmotic pressure: isolates Pb-18, Bt-9, B-1183, Aoki and Hachisuga were intermediate and isolates Pb-HC, Bt-4, Bt-7 and Tatu were tolerant strains to osmotic pressure (Table 4).

All tested isolates in yeast form were positive for urease test. Phenol oxidase test was positive in all tested isolates in yeast form, but the growth was inhibited. Mycelial and yeast forms were positive in the KNO_3 assimilation test, also with growth inhibition. Both mycelial and yeast forms did not assimilate starch. Four mycelial-form of isolates, Pb-339, Pb-HC, Bt-9 and Bt-19, were negative in the casein assimilation test. In the yeast form, all but two isolates (Pb-HC and Wagura) were negative in this test (Table 4).

All mycelial-form isolates were positive in the arbutin assimilation test with growth inhibition; in yeast form they were negative with the exception of isolate Bt-7 (Table 4). Only, Bt-19 in mycelial form had gelatinase activity. All matched cultures fused well. No characteristic architecture, such as ascospores or clamps were observed under the microscope. On gross examination, the majority of the isolates in mycelial form grew as white, whitish pink, whitish gray or beige cottonous colonies with central fissures. Exceptional isolates were Pb-265, Pb-339, B-1183 and Bt-19 which had gray glabrous surface without central fissures. Isolate Recife had glabrous and cottonous parts. On the reverse side, all of the cultures were brown or brownish pink in color. In

Table 1. Effect of pH on *Paracoccidioides brasiliensis* mycelial growth.

Isolate	2.4	4.0	4.6	5.6	6.8–9.4[a)]	9.6	10.4
Pb-9	−	−	+	+	+	+	+
Pb-18	−	−	+	+	+	+	+
Pb-265	−	−	+	+	+	−	−
Pb-339	−	−	+	+	+	+	+
Pb-HC	−	−	+	+	+	+	+
Bt-2	−	−	−	+	+	+	+
Bt-3	−	+	+	+	+	+	+
Bt-4	−	−	+	+	+	+	+
Bt-7	−	−	+	+	+	+	+
Bt-9	−	−	+	+	+	+	+
Bt-19	−	−	+	+	+	+	+
B-1183	−	+	+	+	+	+	+
Recife	−	−	+	+	+	+	+
Aoki	−	+	+	+	+	+	−
Hachisuga	−	+	+	+	+	+	−
Tatu	−	−	−	+	+	+	+
Wagura	−	−	−	−	+	+	+

a) Growth at pH 6.8, 7.6, 8.4, 9.2 and 9.4 was examined.

Table 2. Effect of pH on *Paracoccidioides brasiliensis* yeast form growth.

Isolate	3.6	4.6	5.6–9.0[a)]	9.4
Pb-9	−	+	+	+
Pb-18	−	+	+	+
Pb-265	−	+	+	+
Pb-339	−	+	+	+
Pb-HC	−	+	+	+
Bt-2	−	−	+	+
Bt-3	−	−	+	+
Bt-4	−	+	+	+
Bt-7	−	+	+	+
Bt-9	−	+	+	+
Bt-19	−	+	+	+
B-1183	−	+	+	+
Recife	−	+	+	+
Aoki	−	+	+	+
Hachisuga	−	+	+	+
Tatu	−	+	+	+
Wagura	−	+	+	+

a) Growth at pH 5.6, 6.8, 7.6, 7.8, 8.0, 8.4, 8.6, 8.8 and 9.0 was examined.

isolates Pb-18 and Wagura, a dark brown pigment diffused into the medium.

In the yeast form at 37°C, the colonies on BHIA slants had cerebriform surface, with the exception of isolates Pb-HC and Bt-19, which grew slowly and as cobblestone-like hard colonies (Fig. 1). Microscopically, the majority of the isolates grew as yeasts with multiple budding; isolates Pb-339 and Aoki, however, grew as a mixture of yeasts and hyphae, and isolates Pb-HC and Bt-19 grew only as hyphae without multiple budding yeast forms (Table 5).

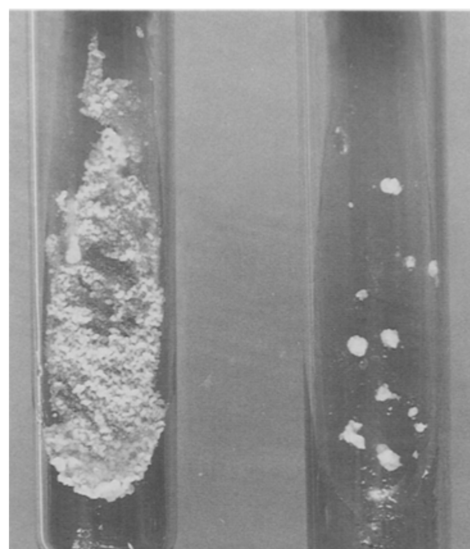

Fig. 1. Yeast form colonies of isolates Pb-HC and Tatu grown at 37°C for 7 d.
Note cobblestone-like colonies of isolate Pb-HC (right).

The pathogenicities of isolates Aoki, Hachisuga and Wagura obtained by organ culture scoring were 2.0, 5.4 and 2.6, respectively.

Discussion

The mycelial forms of different isolates of *P. brasiliensis* differed in their adaptability to pH. However, no mycelial form grew below pH 2.4, and the majority of the iso-

Table 3. Assimilation of carbohydrates[a)] in *Paracoccidioides brasiliensis* yeast form by API20C auxanogram.

Isolate	0	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF
Pb-9	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Pb-18	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Pb-339	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Bt-2	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−
Bt-3	−	+	+	+	+	−	+	+	+	−	+	−	+	−	+	+	−	−	−	−
Bt-4	−	+	−	+	+	−	+	−	−	+	+	−	−	+	−	+	−	+	+	+
Bt-7	−	+	−	−	+	−	+	−	−	−	+	−	+	+	−	+	−	+	+	+
Bt-9	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
B-1183	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Recife	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aoki	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Hachisuga	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Tatu	−	+	+	+	+	−	+	−	−	−	+	+	+	+	+	+	−	+	+	+
Wagura	−	+	−	+	+	+	+	−	+	−	−	−	+	−	−	+	−	−	−	−

a) GLU, glucose; GLY, glycerol; 2KG, 2-keto-D-gluconate; ARA, arabinose; XYL, xylose; ADO, adonitol; XLT, xylitol; GAL, galactose; INO, inositol; SOR, sorbitol; MDG, α-methyl-D-glucoside; NAG, *N*-acetyl-glucosamine; CEL, cellobiose; LAC, lactose; MAL, maltose; SAC, saccharose; TRE, trehalose; MLZ, melezitose; RAF, raffinose.

Table 4. Physiological characteristics of *Paracoccidioides brasiliensis*.

Isolate	Osmophilia Glycerol (%)				Casein assimilation[a)]		Arbutin assimilation[a)]	
	10	20	30	40	M	Y	M	Y
Pb-9	+	−	−	−	+	−	+	−
Pb-18	+	+	−	−	+	−	+	−
Pb-265	+	−	−	−	+	−	+	−
Pb-339	+	−	−	−	−	−	+	−
Pb-HC	+	+	+	−	−	+	+	−
Bt-2	+	+	−	−	+	−	+	−
Bt-3	+	−	−	−	+	−	+	−
Bt-4	+	+	+	−	+	−	+	−
Bt-7	+	+	+	−	+	−	+	+
Bt-9	+	+	−	−	−	−	+	−
Bt-19	+	−	−	−	−	−	+	−
B-1183	+	+	−	−	+	−	+	−
Recife	+	−	−	−	+	−	+	−
Aoki	+	+	−	−	+	−	+	−
Hachisuga	+	+	−	−	+	−	+	−
Tatu	+	+	+	−	+	−	+	−
Wagura	+	−	−	−	+	+	+	−

a) M, mycelial form; Y, yeast form.

lates grew well between pH 5.6 and 9.4. The mycelial form of isolate Wagura did not grow at pH 5.6 or below; this isolate was difficult to subculture on blood agar at pH 7.3 (Nakajima, 1993). Both mycelial and yeast forms grew from pH 5 to 9 (Gilardi and Laffer, 1962; Restrepo, 1985).

Carbohydrate assimilation by yeast forms varied among the isolates studied. Some assimilated few carbohydrates, for example, Pb-9, Pb-18, Pb-339, Bt-2, Bt-9, B-1183, Recife, Aoki and Hachisuga, whereas others (Bt-3, Bt-4, Bt-7, Tatu and Wagura) were capable of assimilating the majority of carbohydrates tested. This is an indication of important metabolic variation among different isolates of Pb. It has been reported that D-glucose, D-fructose, D-galactose and D-mannose were assimilated by *P. brasiliensis* both in solid and liquid medium, but D-ribose, L-arabinose, D-xylose, L-rhamnose, D-maltose, sucrose, lactose, D-trehalose, D-cellobiose, melibiose, D-raffinose, melzitose, D-sorbitol and D-mannitol were not (Gilardi and Laffer, 1962). Contrary to this report, the majority of our isolates assimilated maltose and some of them also assimilated arabinose, xylose,

Table 5. *Paracoccidioides brasiliensis* colonies in mycelial forms.

Isolate	Mycelial form colony (PDA slant)			Pathogenicity
	Color	Texture	Reverse color	
Pb-9	White	Cottonous	Brown	2.6[b]
Pb-18	White	Cottonous	Dark browm	2.4[b]
Pb-265	Gray	Glabrous	Brown	Low[c]
Pb-339	Gray	Glabrous	Brown	ND[d]
Pb-HC	White	Cottonous	Brown	ND
Bt-2	White-pink	Cottonous	Brown-pink	ND
Bt-3	White	Cottonous	Brown	ND
Bt-4	White-pink	Cottonous	Brown-pink	4.6[b]
Bt-7	White	Cottonous	Brown	0.8[b]
Bt-9	White	Cottonous	Brown	5.4[b]
Bt-19	Gray	Glabrous	Brown	ND
B-1183	Gray	Glabrous	Brown	0.4[b]
Recife	White-gray	Cottonous[a]	Brown	ND
Aoki	White	Cottonous	Brown	2.0
Hachisuga	White	Cottonous	Brown	5.4
Tatu	White	Cottonous	Brown	ND
Wagura	Beige	Cottonous	Dark brown	2.6

a) The isolate was cottonese with a glabrous part.
b) The data were cited form Sano et al., 1991.
c) The datum was cited from Vaz et al., 1994.
d) ND: not done.

xylitol, sorbitol, cellobiose, lactose, trehalose, melezitose and raffinose. The API20C kit appears to be very important to carbohydrate assimilation evaluation.

The fact that mycelial and yeast forms of *P. brasiliensis* grew in 1% NaCl does not indicate a halophilic tendency, since this concentration is very close to physiological saline. Osmophilia permitted separation of our isolates into three groups: low, moderate and high tolerance.

In a previous study we tested the virulence of 6 of the 17 isolates in ddY mice (Sano et al., 1991). Isolates Bt-9 and Bt-4 were the most virulent (5.4 and 4.6), whereas Pb-9 and Pb-18 had moderate (2.6 and 2.4), and Bt-7 and B-1183 low virulence(0.8 and 0.4). Isolate Hachisuga had high virulence (5.4) and isolates Aoki and Wagura had intermediate ones (2.0 and 2.6). Virulence of Pb-265 is known to be low (Vaz et al., 1994). No correlation was observed between virulence and osmophilia.

Our urease activity test results are similar to the report of Gilardi and Laffer (1962), who studied 9 *P. brasiliensis* isolates. Contrary to their results, our 17 isolates were capable of using KNO_3 as source of nitrogen.

Our results of the starch assimilation test are similar to those reported (Gilardi and Laffer, 1962); our isolates also did not assimilate starch in yeast or mycelial forms.

The majority of our isolates in the mycelial form assimilated casein, whereas only two isolates in the yeast form did. Isolate Pb-HC was the exception: it assimilated casein in the yeast form, but not in the mycelial form.

Gelatinase activity by electrophoresis of culture supernatants from four Pb isolates resulted in different band patterns that correlated with virulence (Vaz et al., 1994). We used a different method but did not find the reported gelatinase activity in Pb-18, Pb-265 and Pb-339 isolates. Furthermore, Pb-18 without gelatinase activity by our method was only moderately virulent to ddY mice (Sano et al., 1991). Virulence of *P. brasiliensis* may vary with culture methods, storage and cold stress (Sano et al., 1994). These facts must be kept in mind when comparing contradictory results.

Definite data on the taxonomy of *P. brasiliensis* have yet to be reported. Some of its morphological features on freeze-etching preparations, such as cytoplasmic invaginations, do not permit classification of *P. brasiliensis* among basidiomycetes or ascomycetes (Takeo et al., 1990). Other features such as melanin biosynthesis, reduction of tetrazolium and canavaline-glycine-bromothymol blue reaction are similar to those of *Cryptococcus neoformans*, a basidiomycete (Assis et al., 1995a, b, c). However, RNA sequence comparisons suggest that *P. brasiliensis* is phylogenically close to *Histoplasma capusulatum* Darling and *Blastomyces dermatitidis*, both ascomycetes (Leclerc et al., 1994). *Paracoccidioides brasiliensis* and *B. dermatitidis* belong to the same genus according to Gilardi and Laffer (1962). Therefore, there is not enough available information to classify *P. brasiliensis* as basidiomycetes or ascomycetes.

The physiological features of 17 isolates of *P. brasiliensis* studied indicated that an ideal culture medium for *P. brasiliensis* has yet to be found, unlike the case

of *Cryptococcus neoformans* for which niger seed media are used (Kwon-Chung and Bennett, 1992). Physiological features studied do not appear to be correlated with virulence.

No perfect stage of *P. brasiliensis* is known. Fusing may be a way to stimulate the appearance of the perfect stage in fungi. All of our 17 isolates fused well but no perfect stage was detected. Recently, several natural isolates of *P. brasiliensis* were obtained from nature (Naiff et al., 1986; Ferreira et al., 1990; Camargo and Taborda, 1993; Vergara et al., 1996). Mating tests with these new isolates and isolates from patients may offer a further possibility of finding the perfect stage.

Gross morphology of mycelial forms permits separation of our 17 isolates into two groups: colonies of Pb-265, Pb-339, Bt-19 and B-1183 were glabrous, and all the others cottonous. Virulence appears to be correlated with the cottonous pattern of growth, since all the virulent isolates we studied in the past (Sano et al., 1991) and the present study had this feature. To confirm this correlation, more glabrous isolates should be studied. All our yeast form cultures were cerebriform with multiple budding, except for Pb-HC and Bt-19, that had cobblestone-like surface without multiple budding.

In conclusion, further genetical studies are required to detect the correlation between the various phenotypes and the genotype of *P. brasiliensis*.

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