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Growth curves, morphology and ultrastructure of ten *Paracoccidioides brasiliensis* isolates

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Abstract

The yeast phase of ten *P. brasiliensis* isolates were studied to characterize their growth pattern, morphology and ultrastructure. Growth curves were determined after counts of total and viable fungi units (FU) during 20 days. Three growth patterns were observed: slow, reaching approximately $10-30 \times 10^6$ FU/tube (Pb 18, Pb 265 and PB 2); intermediate, reaching $60-150 \times 10^6$ FU/tube (IVIC Pb 9, IVIC Pb 267, Pb SN, Pb Vitor and Pb Campo Grande) and fast, reaching $180-370 \times 10^6$ FU/tube (Pb 2052 and Pb 192). The highest percentage of viable cells occurred on the 6th day of culture for Pb 192, Pb Campo Grande, Pb 2052 and IVIC Pb 9; on the 8th day for Pb Vitor, Pb SN, Pb 18 and IVIC Pb 267; on the 10th day for Pb 265 and on the 12th day of culture for Pb 2. Mean generation times varied from approximately 21.2 (Pb 2052) to 102.6 hours (Pb 265). The isolates showed similar morphology, except IVIC Pb 267 which did not present a typical yeast-phase at 35 °C and the two fast-growing isolates (Pb 2052 and Pb 192) that presented smaller cell sizes and less tendency to clump. The ultrastructure of the isolates was similar: the cell walls presented a width of 0.1 to 0.2 μ; the mitochondria presented few cristae and had equivalent patterns of distribution and morphology; the endoplasmic reticulum was scanty, presenting narrow cisternae; the vacuoles, empty or filled with electron-dense material, were numerous and two to five nuclei with pores were constantly observed.

Introduction

Paracoccidioides brasiliensis is a dimorphic pathogenic fungus which is the etiologic agent of paracoccidioidomycosis, a chronic granulomatous disease, endemic in Latin America [8]. The contact of humans with *P. brasiliensis* may cause clinically active disease or infection (without disease). This mycosis presents multiple clinical aspects, ranging from mild or localized to severe and disseminated forms.

The occurrence of paracoccidioidomycosis-infection, as well as the different clinical forms of the disease can be related to host intrinsic factors (e.g. age, sex, genetic background) or characteristics of the infecting agent [8].

Paracoccidioides brasiliensis has been the subject of numerous investigations concerning its ecology [19], thermal dimorfism [11, 15, 16, 17, 22], ultrastructure [5, 6, 7], growth characteristics [1, 13, 18, 23] and biochemistry [4, 12, 25, 26, 27]. These studies were undertaken employing various *P. brasiliensis* isolates, originally obtained from different patients living in distinct endemic areas.

The different morphological and physiological characteristics observed in those studies may be ascribed not only to the heterogeneity of the *P. brasiliensis* isolates, but also to the various experimental conditions employed.

Comparative studies among *P. brasiliensis* isolates are still scarce; consequently it is not known, at the moment, which biological properties are shared by the different *P. brasiliensis* isolates and which are characteristic of one isolate.

In the present report we proposed to study ten *P. brasiliensis* isolates, collected recently or many years ago from patients living in different endemic areas. These isolates were maintained in our laboratory and submitted to strictly the same procedures to allow comparison of their growth characteristics, morphology and ultrastructure.

Our results show that although the morphology and the ultrastructure of all studied isolates were roughly the same, the growth curves patterns of *P. brasiliensis* yeasts vary within the species.

Material and methods

Fungi

Ten isolates of *Paracoccidioides brasiliensis* were studied: Pb SN, Pb 265, Pb 18, Pb 192 and Pb 2, obtained from the culture collection of Departamento de Microbiologia, Universidade de São Paulo, Brasil; Pb Vitor and Pb Campo Grande a gift from Dr. Celeste Fava Netto (São Paulo, Brasil); Pb 2052, isolated by Dr. Arminda de Jesus Machado (Goiânia, Brasil); IVIC Pb 9 and IVIC Pb 267 kindly supplied by Drs. Gioconda and Felipe San-Blas (Venezuela). All these fungi were originally isolated from human patients, except IVIC Pb 267 which is a chemical mutant obtained after treatment of IVIC Pb 9 with nitrosoguanidine (G. and F. San-Blas, personal communication). The isolates were studied in their yeast phase.

Growth conditions

All except two *P. brasiliensis* isolates were cultivated in semisolid Fava Netto's medium [9]; isolates IVIC Pb 9 and IVIC Pb 267 were maintained in semisolid peptone-yeast extract glucose medium (PYG) [27]. All the isolates were cultivated in their yeast phase at 35 °C, with the exception of IVIC Pb 267 which grows well at 23 °C but not at 35 °C and which has not a typical yeast-phase (G. and F. San-Blas, personal communication).

Growth curves determinations

The following procedures were performed for each growth curve determination: 30 tubes containing 6 ml of medium were seeded with fungi obtained from eight days old cultures. The initial inoculum was adjusted to 2.0×10^6 fungal units (FU) after counting in an hemocytometer; one fungal unit (FU) was defined as an isolate cell or a mother cell plus the attached buds; cellular viability was determined using the Janus Green B vital dye [3]. At two days intervals, during 20 days from the seeding, the total growth of three randomly selected tubes was collected flooding the agar surface with sterile PBS (pH 7.2–7.3) and gently scrapping its surface. F.U. total numbers were counted in a hemocytometer and cellular viability was determined.

The mean counts of triplicates were used to graph the growth curves and the mean generation times were calculated on the basis of the increment in the number of cells for all the studied isolates.

Preparation for light microscopy

Morphological studies under light microscopy were performed with yeast cells of each isolate collected at the point of maximum viability in the growth curve: Pb 2052, IVIC Pb 9, Pb 192 and Pb Campo Grande isolates were studied on the 6th day of culture; Pb Vitor, Pb 18, Pb SN and IVIC Pb 267 on the 8th day; Pb 265 and Pb 2 respectively on the 10th and on the 12th days.

Specimen preparation for electron microscopy

The method used by Baharaeen & Vishniac [2] was adapted to *P. brasiliensis* preparations as follows:

each *P. brasiliensis* isolate was cultivated as described for the growth curves determinations and used on the day of maximum viability. Each *P. brasiliensis* isolate was cultivated in triplicate, washed three times in PBS and fixed in a solution of 3% glutaraldehyde and 1.5% paraformaldehyde in 0.05 M cacodylate buffer at pH 7.2–7.4. The cells were maintained in this solution for 24 hours at 4 °C. After the initial fixation the cells of each tube were rinsed twice with PBS and once with 0.005 M cacodylate buffer and divided into three samples. One sample was resuspended in 6% potassium permanganate and left for one hour at room temperature and then post-fixed with 1% osmium tetroxide during 30 minutes at room temperature. The other samples were treated with either osmium tetroxide or potassium permanganate. After rinsing, the cells of each sample were mixed with a few drops of molten 4% agar. Small fragments of agar were dehydrated and embedded in Araldite. Ultrathin sections stained with led citrate were observed in Zeiss EM 9 and JEOL CX II electron microscopes.

Unless otherwise stated, the studies were done

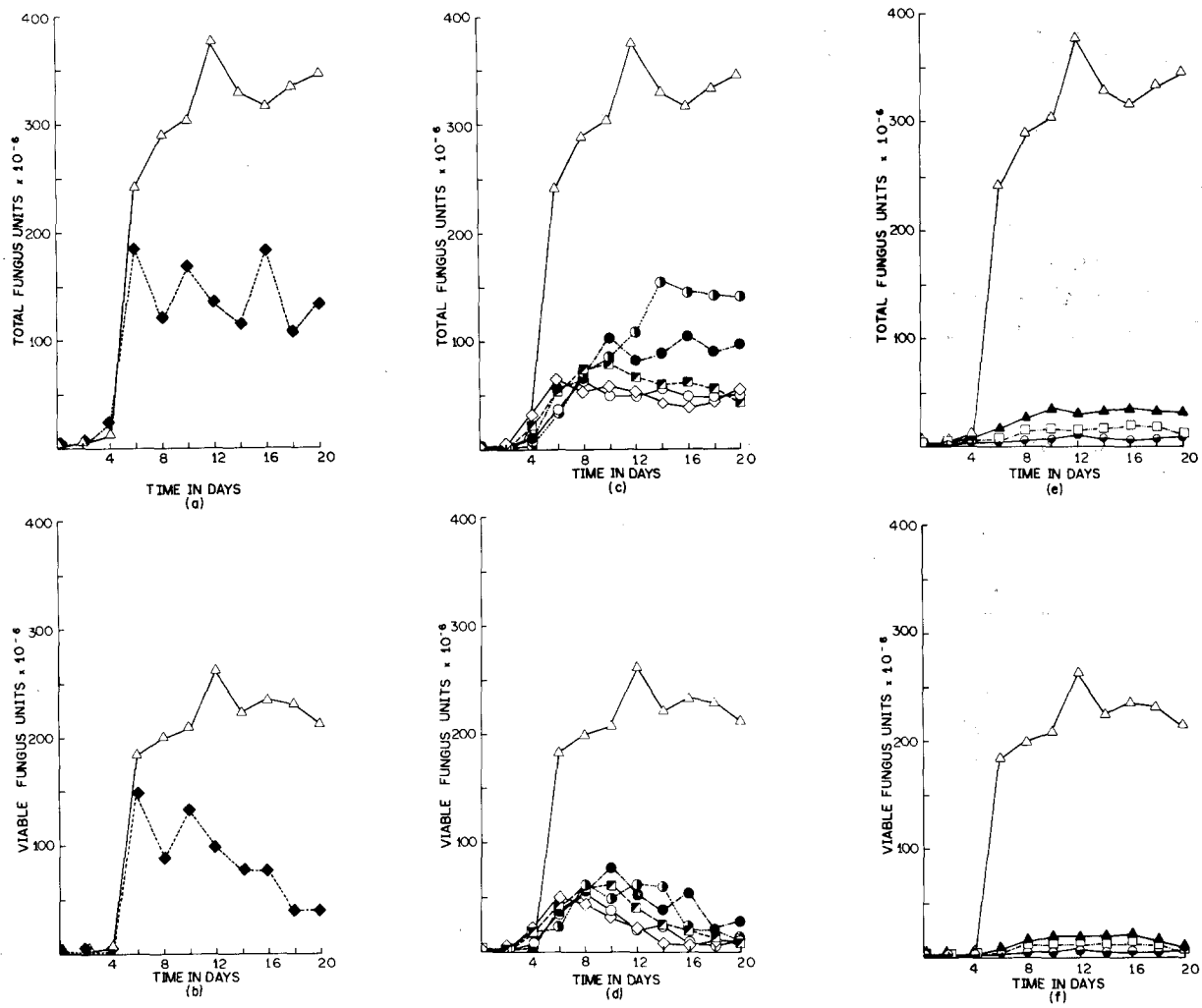

Fig. 1. Total (above) and viable (below) yeast cell counts of ten *P. brasiliensis* isolates showing curves with fast (a, b), intermediate (c, d) and slow (e, f) growth patterns. The intermediate and slow growth curve profiles are compared to Pb 192, the fastest growing isolate; (△——△) Pb 192; (◆– – –◆) Pb 2052; (●–·–·–●) Pb SN; (○——○) IVIC Pb 9; (◑– – –◑) IVIC Pb 267; (◙– – –◙) Pb Campo Grande; (◇——◇) Pb Vitor; (□–·–·–□) Pb 18; (◒——◒) Pb 265; (▲——▲) Pb 2.

using the samples fixed in potassium permanganate or in potassium permanganate and osmium tetroxide, since these two treatments provided the best preparations.

Results

Growth curves

Growth curves showing total and viable cell counts of the ten studied *P. brasiliensis* isolates are depicted in Fig. 1. The isolates exhibited three patterns of growth: a) Pb 192 and Pb 2052 showed a rapid increase in total cell number from the 2nd day on; at the end of a sharp exponential phase, approximately 184.0 to 370 $\times$ 10^6 FU/tube were counted. Highest percentage of viable cells occurred on the 6th day; b) IVIC Pb 9, IVIC Pb 267, Pb SN, Pb Vitor and Pb Campo Grande showed an intermediate increase in total cell number; on the late logarithmic phase approximately 60.0 to 150.0 $\times$ 10^6 FU/tube were counted. Highest cellular viability was found at the 6th of 8th days of culture; c) Pb 2, Pb 18 and Pb 265 presented a low increase in the number of cells; the maximum total counts ranged from approximately 10.0 to 30.0 $\times$ 10^6 FU/tube. The highest cells viability occurred on the 10th of 12th days.

On the 20th day of culture, most of the isolates presented a significant decrease in the percentage of viable cells, except for Pb 192 (fast growth), Pb Vitor (intermediate growth) and Pb 265 (slow growth).

Differences in the mean generation times were also observed; these values ranged from 21.3 (Pb 2052) to 102.7 hours (Pb 265) (Table 1).

Light microscopy

Examination under light microscopy of the ten isolates revealed that they shared the following characteristics: most of the cells were oval, pear shaped or spherical, with dimensions ranging from 25 to 50 μ in the short axis and showing one or multiple buds. The mutant IVIC Pb 267 presented the above described morphology as well as filamentous cells (Table 1). Clumped cells were more frequently ob-

Table 1. Comparison of some *P. brasiliensis* isolates characteristics.

Isolate	Mean generation time (hours)	Day of highest viability	Highest percentage of viable cells	Highest total number of FU $\times$ 10^{-6}/tube	Percentage of viable cells on day 20	General morphology	Estimated size (short axis) μ
Pb 2052	21.3	6th	82.3%	184.7	30.9%	spherical, isolated cells	25
Pb Vitor	27.0	8th	79.0%	66.7	13%	spherical and oval cells	25 – 50
IVIC Pb 9	35.9	6th	90.4%	64.5	15.9%	spherical and oval cells	25 – 50
Pb 192	36.7	6th	75.3%	375.6	61.3%	spherical, isolated cells	25
Pb SN	40.0	8th	82.0%	98.9	28.7%	spherical and oval cells numerous clumped cells	25 – 50
Pb Campo Grande	42.5	6th	80.3%	78.7	14.3%	spherical and oval cells	25 – 50
IVIC Pb 267	48.5	8th	81.3%	155.9	6.1%	filamentous, spherical and oval cells; numerous clumped cells	25 – 50
Pb 2	55.3	12th	62.6%	34.7	22.6%	spherical and oval cells	25 – 50
Pb 18	58.4	8th	83.3%	16.5	48.9%	spherical, oval and pear shaped cells; numerous clumped cells	25 – 50
Pb 265	102.4	10th	85.6%	10.2	68.4%	spherical and oval cells	25 – 50

served in preparations of Pb SN, Pb 18 and IVIC Pb 267 isolates.

Ultrastructural studies

The comparative ultrastructural investigations showed no significant difference between the studied *P. brasiliensis* isolates. A typical ultrastructural aspect is shown in Fig. 2.

The thickness of the cell wall measured from 0.1 to 0.2 μ. The outermost wall layer was thin, electron-dense, with electron-opaque deposits on the external surface. A less electron-dense, broad inner layer was observed. In most preparations, this region was homogeneous throughout its entire thickness, but in some sections another thin, electron-dense sub-layer could be seen (Fig. 3).

Membrane structures were well preserved when potassium permanganate (with or without osmium tetroxide) was used as fixative agent. The plasma membrane was observed as a unit membrane, showing a trilayered structure. In some isolates, cells showed multiple invaginations of their plasma membrane. These invaginations constituted vesicles and tubular structures and sometimes seemed associated with septum formation between mother and daughter cells during the budding process.

Large numbers of mitochondriae were scattered throughout the citoplasm of the cells of all isolates. These organelles had few cristae and no inclusions were observed in their matrix. In some cells, the

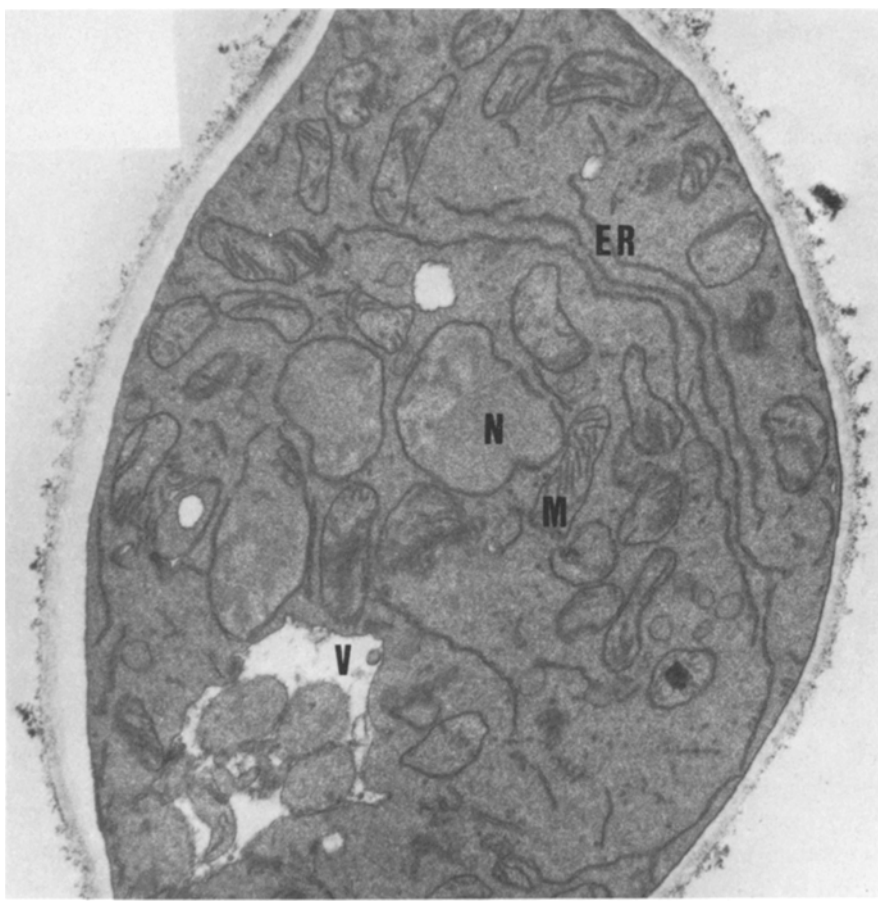

Fig. 2. Typical ultrastructural aspect of *P. brasiliensis* yeast cells. Isolate IVIC Pb 267. M = mitochondria; ER = endoplasmic reticulum; V = vacuole; N = nucleus. (×9500).

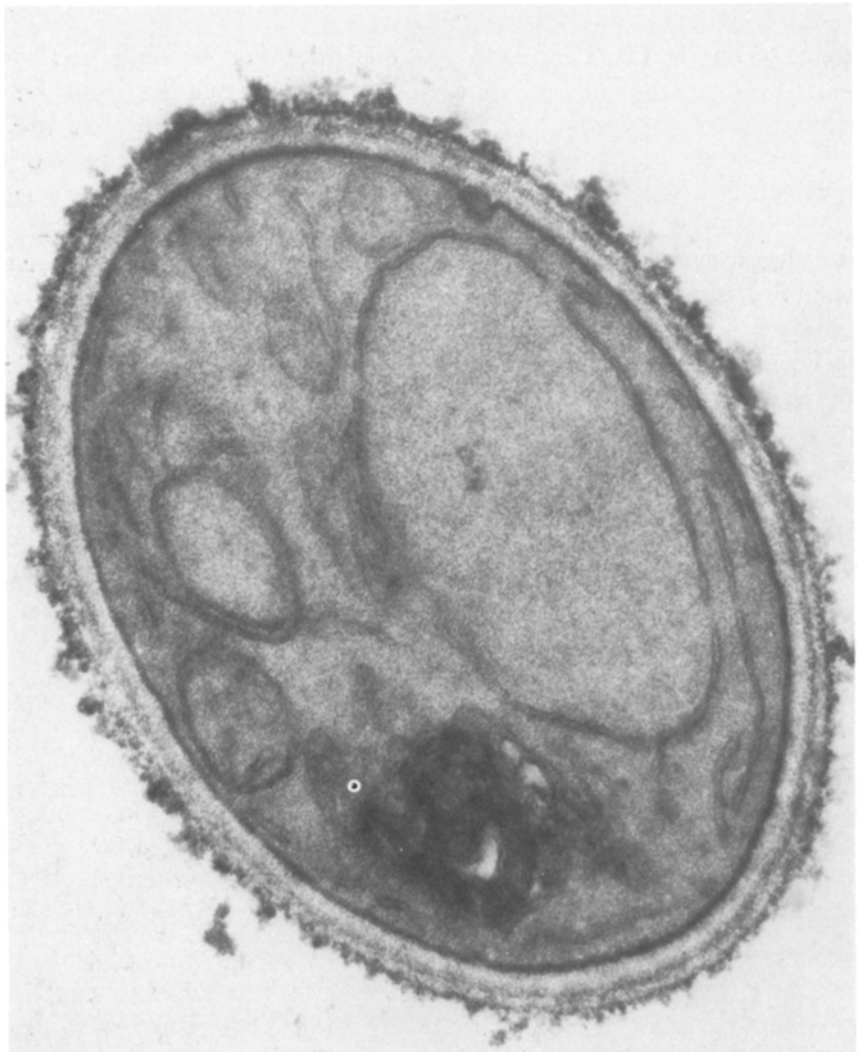

Fig. 3. Ultrastructural aspect of the trilayered wall of *P. brasiliensis* yeast cell. Isolate Pb 18. (×20000).

mitochondria predominated near the cell surface (Fig. 2).

The endoplasmic reticulum appeared as long, undilated cisternae. No membrane-associated ribosomes were observed, independently from the fixation method applied. Free ribosomes were observed in the citoplasm of cells post-fixed with osmium tetroxide only (Fig. 4).

Structures resembling Golgi apparatus could not be identified in any of the preparations obtained from the different *P. brasiliensis* isolates.

Vacuoles of different sizes and forms, some of them containing abundant electron-dense material were usually observed.

P. brasiliensis yeast cells are multinucleated. Two to five nuclei percell were usually observed and pores were evident along the nuclear membrane. The nuclei contained mostly euchromatin and no nucleolus was observed (Fig. 2).

Discussion

In the present report, the growth curves of ten *P. brasiliensis* isolates were determined under strictly the same conditions and three distinct patterns of behaviour were observed. This fact indicates that the growth efficiency of *P. brasiliensis* sub-

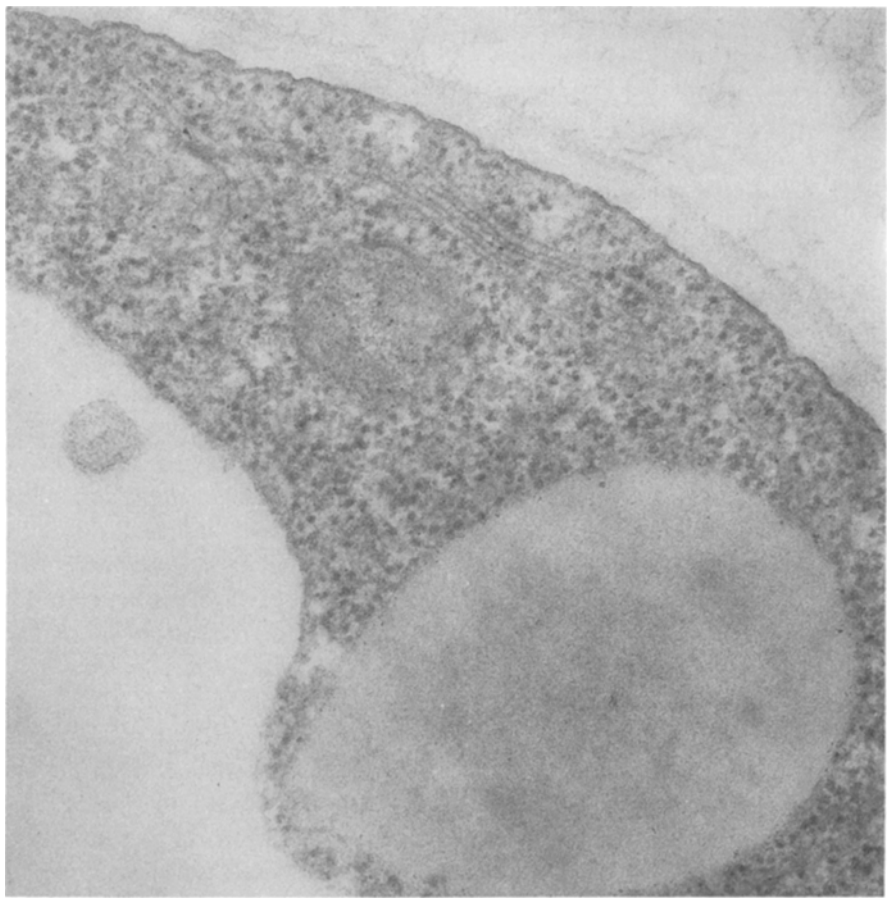

Fig. 4. Ultrastructural aspect of *P. brasiliensis* yeast cell free ribosomes. Isolate Pb 2052. (×40000).

populations, under controlled conditions, is dependent on intrinsic biological properties of these fungi. Other authors [1] also reported that *P. brasiliensis* isolates obtained from different sources behaved differently even when subjected to the same conditions.

Such differences of growth are usually attributed to adaptation to "*in vitro*" conditions which is dependent on the time elapsed since the first isolation of the fungus. However our data show that this is not a general phenomenon: Pb 18 and Pb 192 isolates, both maintained "*in vitro*" for more than 26 years had different growth profiles; the maximum number of yeasts attained by Pb 192 was 23 times higher than that attained by Pb 18. On the other hand, Pb 2052, which was isolated more recently, grew well, showing high numbers of total yeasts (9.5 times more than Pb 18).

Although none of the *P. brasiliensis* isolates of the present study showed an abrupt decrease in the number of viable yeasts, comparable to that obtained by others [1, 23], a significant decrease in the percentage of viable fungi was always detected on the 20th day of culture. This loss in viability could not be ascribed only to the exhaustion of the culture media: Pb 192 and Pb 2052, the fastest growing isolates showed respectively high and low viability. It has been suggested [1, 23] that a lethal metabolite excreted by *P. brasiliensis* yeasts may be involved in such phenomena. The *P. brasiliensis* isolates of the present study may differ in the degree of production of such a metabolite.

Total counts of *P. brasiliensis* yeasts obtained under light microscopy did not allow us to distinguish

dead from living cells in most part of the curves, as these fungi showed no remarkable morphological changes. A true expression of the kinetics of growth was obtained using vital dye staining. Previous data showed that growth curves of *P. brasiliensis* constructed using viable cells are either as reliable as [23] or better than [20] those constructed using the colony-forming unit (CFU) technique. For this reason, as well as for being technically simple and rapid, total and viable yeast counts were done under light microscopy.

Owing to the fact that studies of others were made using different isolates of *P. brasiliensis*, their results cannot be easily compared to ours. Even when the same isolate is employed, comparisons become difficult due to the different culture media used, as well as to the distinct methods of growth measurement employed. This is the case of the work by San-Blas *et al.* [23] who cultivated IVIC Pb 9 in liquid media, assessing total numbers of yeasts by turbidimetric measurements and viable yeasts by CFU and vital staining. In the present work we determined total and viable yeasts of IVIC Pb 9 on semisolid culture media using microscopic methods. The sharp decrease in viable yeasts from the 10th day on obtained by those authors, that did not occur in our model may be explained by the earlier exhaustion of nutrients or by facilitated diffusion of eventual toxic metabolite in the liquid medium. The better growth efficiency attained in the present work may be related not only to physico-chemical characteristics of the media used but also to the initial inocula seeded. Instead of inoculating loopfuls of yeasts, a low and known number of viable cells was used, possibly allowing better conditions to cellular reproduction. The strict control of the number of viable cells of the inoculum may render the method more reproducible.

The growth of IVIC Pb 9 was also compared to that of IVIC Pb 267, a chemical mutant originated from this isolate. Their kinetics of growth were similar in spite of very marked differences in the macroscopic aspects of the colonies and microscopic cell morphology: IVIC Pb 267 preparations presented yeasts, as well as yeast-micelium cells, whereas the parental IVIC Pb 9 isolate showed the typical morphology of *P. brasiliensis* yeast cells. The morphological aspects described for the isolates of this study agree with those of the literature, confirming that the characteristic multiple budded double wall structures are constant in the yeast-phase of this fungus, irrespective of variations in the cultural conditions.

The several *P. brasiliensis* isolates showed similar ultrastructural features; significant differences were not observed even when IVIC Pb 267 was compared to other isolates. The present observations agree with those of others [5, 6, 7] regarding the general aspect of the cell organelles as well as the absence of a Golgi apparatus. In some isolates, cells showed multiple invaginations of their plasma membranes. Such membrane configurations resembled structures designed either myelin figures, mesosomes or intracytoplasmic membrane systems which may be involved in septal formation, DNA replication or respiratory processes of pathogenic fungi [10]. Our observations seems to agree with those by Szaniszlo *et al.* [28] who suggested that such membrane structures are possibly involved in the budding process. Differently from other authors, nucleoli could not be found. Lamellar bodies were observed in some preparations. Since the fixation of *P. brasiliensis* proved to be difficult and several fixation techniques had to be employed, the lack of observation of nucleoli may indicate the need of other types of fixations; similarly, the presence of lamellar bodies could be a fixation artifact, as suggested by Garrison [10].

The relationship between the "*in vitro*" and "*in vivo*" characteristics of some *P. brasiliensis* isolates was compared previously [13] and a dissociation between the "*in vitro*" growth patterns and the pathogenicity to susceptible inbred mice was observed: isolates Pb 18 and Pb 265 which behaved similarly "*in vitro*", showing low counts of fungi and long mean generation times, were respectively the most and the least pathogenic isolates; Pb 2052 and IVIC Pb 267, which grew abundantly "*in vitro*" were, respectively pathogenic and apathogenic; isolates Pb SN and IVIC Pb 9 behaved similarly both "*in vitro*" and "*in vivo*", displaying an intermediate pattern of pathogenicity and growth. The present results also indicate that there is no direct correlation between the "*in vitro*" growth

pattern of *P. brasiliensis* isolates and the "*in vivo*" behaviour of the fungi: Pb Vitor and Pb Campo Grande that were isolated about 5 years ago from patients with different clinical forms of the disease (respectively pulmonar and disseminated), both showed similar intermediate growth pattern "*in vitro*".

Finally, the results of our study can provide some data for a more adequate choice of *P. brasiliensis* isolates to be used in research or in diagnostic procedures.

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