A Morphological Mutant of *Paracoccidioides brasiliensis* Strain IVIC Pb9. Isolation and Wall Characterization

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SUMMARY

A morphological mutant of *Paracoccidioides brasiliensis* strain IVIC Pb9 was isolated after treatment of the yeast-like (Y) form with nitrosoguanidine. Colonies of the mutant grown at room temperature did not show the whitish cotton-like morphology typical of the mycelial form of the parental strain. Y-cells were much smaller than those produced by the parent and grew forming chains of different sizes. The main chemical difference in the wall of the Y-form was the replacement of the α -1,3-glucan, typical of the parental strain, by an amorphous 1,3-mannan in the mutant.

INTRODUCTION

Paracoccidioides brasiliensis, an imperfect fungus pathogenic to humans, undergoes thermal dimorphism (Nickerson, 1948). It develops a yeast-like (Y) form when grown at 37 °C and a mycelial (M) form at room temperature. Although both forms have been extensively studied by chemical and electron microscopic studies (Drouchet & Tapater, 1954; Carbonell & Rodriguez, 1965; Carbonell, 1969), the mechanism by which transformation takes place is not understood.

Quantitative analysis of the walls of both Y and M forms have shown that their chemical compositions are similar (Kanetsuna *et al.*, 1969) but that their glucans differ. α -1,3-Glucan has been reported as the main glucose polymer in the Y-form, while in the M-form this polysaccharide is replaced by a β -1,3-glucan (Kanetsuna *et al.*, 1972). These authors assumed that the spherical shape of the Y-like form of *P. brasiliensis* is due to the short rod-like fibres characteristic of the α -1,3-glucan and that the long narrow fibres of the β -1,3-glucan contribute to the mycelial form.

This paper describes a morphological mutant of the Y-form of *P. brasiliensis* strain IVIC Pb9 in which the fibrillar α -1,3-glucan is substituted by an amorphous 1,3 mannan. The cell wall of the yeast-like form of the parental strain *P. brasiliensis* IVIC Pb9 had a different chemical composition from that reported by Kanetsuna *et al.* (1969).

METHODS

Organism and growth conditions. *Paracoccidioides brasiliensis* strain IVIC Pb9, a human isolate (formerly strain 7193 of the Instituto Nacional de Tuberculosis, Caracas, Venezuela), has been maintained in our laboratory on Sabouraud Liquid Broth Modified (BBL) agar for several years. Exponentially-growing populations of the Y-form of this strain were

obtained by inoculating two loopfuls of a 3-day-old culture, from a Sabouraud agar slant kept at 37 °C, into 500 ml Erlenmeyer flasks containing 100 ml of brain-heart infusion broth (BHI), from BBL. Slants were subcultured at least 10 times every 3 days (San-Blas & Cova, 1975).

Mutagenesis and isolation of mutants. A late-exponential culture of the Y-form of *P. brasiliensis* IVIC Pb9, containing 10^6 colony forming units (c.f.u.)/ml in 100 ml BHI, was centrifuged, washed three times with 100 ml of 0.1 M-citrate buffer pH 5 and resuspended in 100 ml of this buffer with 50 µg of *N*'-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) (Sigma). The mixture was incubated in a 500 ml Erlenmeyer flask at 37 °C on a gyratory shaker for 30 min, centrifuged and washed three times in the same buffer to remove NTG. The pellet was next resuspended in 100 ml of fresh BHI broth and further incubated at 37 °C to allow expression of any mutant phenotype. After 72 h incubation, 0.1 ml portions of 10^{-1} and 10^{-2} dilutions of the culture were plated on GGY agar plates (Nickerson & Edwards, 1949) by the soft-agar-layer technique. Plates were incubated at 37 °C for 10 days to allow good growth of the colonies. Plates containing between 50 and 200 colonies were then further incubated at room temperature to allow transformation to the M-form. Plates were inspected several times over a period of 2 months to check for any possible variation from the wild-type morphology of the M-form colonies. Any M-form colony differing in morphology from the parental strain was allowed to transform back to the Y-form at 37 °C on Sabouraud Liquid Broth Modified agar slants.

Purification of mutants. Once transformation to the Y-form was obtained, a loopful of the suspected mutant was inoculated into 5 ml BHI broth and incubated at 37 °C. After 72 h incubation, portions from the cultures were diluted in 0.85 % (w/v) NaCl and plated out on GGY agar and incubated at 37 °C. When good colony growth was observed, plates were transferred to room temperature to allow transformation of the colonies. Some of the colonies which showed the altered phenotype were again transformed to the Y-form and treated as above, until all colonies derived from a single one showed the mutant phenotype.

Preparation and analysis of walls. Both the wild type and the morphological mutant were analysed as described previously (San-Blas & Carbonell, 1974). The walls were separated into three fractions: an alkali-insoluble residue (fraction 1); an alkali-soluble fraction, precipitated by neutralization with acetic acid (fraction 2); and an alkali-soluble fraction, not precipitated by neutralization with acetic acid (fraction 3). Thin-layer chromatography using the developing system described by Lato *et al.* (1968) replaced paper chromatography. Release of formic acid in periodate-oxidized samples (25 mg samples suspended in 25 ml of 30 mM- NaIO_4) was followed by titration against 1 mM-NaOH. The uptake of periodic acid was followed by the spectrophotometric method of Aspinall & Ferrier (1957). The preparation of wall fractions for electron microscopy has been described elsewhere (San-Blas & Carbonell, 1974). Yeast mannan was prepared according to San-Blas & Cunningham (1974) and used for comparisons.

Sources of enzymes. α -1,3-Glucanase (EC. 3.2.1 group) and β -1,3-glucanase (EC. 3.2.1.6) were prepared from *Cladosporium resinae* and *Basidiomycetes* QM806, respectively, according to Reese & Mandels (1959). These exoenzymes were tested against β -1,3-glucan from *Aspergillus niger* and β -1,3-glucan from *P. brasiliensis* Pb9 (San-Blas & Carbonell, 1974).

Enzymic procedures. All glucose-containing samples were incubated with either α - or β -1,3-glucanase. The sample containing the equivalent of 1 mg of glucose was mixed with 1 mg of α - or β -1,3-glucanase in 1 ml of 0.05 M-acetate buffer pH 5.0. The mixture was

Table 1. Chemical composition of walls from yeast-like (Y) forms of *P. brasiliensis*, strains IVIC Pb9 and IVIC Pb140

The amount of each class of compound is expressed as a percentage of either the cell wall or the three fractions derived from it.

	IVIC Pb9*	IVIC Pb9†	IVIC Pb140
Wall			
Hexoses	38.4	41.5	36.5
Amino sugars	43.4	30.0	44.1
Amino acids	10.1	17.0	14.3
Lipids	11.0	10.0	5.0
Fraction 1			
Yield‡	40.0	41.5	84.7
Hexoses	19.0	16.9	34.3
Amino sugars	44.0	60.5	52.7
Amino acids	20.6	16.2	11.9
Fraction 2			
Yield‡	45.0	3.2	3.4
Hexoses	100.0	100.0	93.0
Amino sugars	0.0	0.0	0.0
Amino acids	0.0	0.0	6.7
Fraction 3			
Yield‡	5.0	55.3	11.3
Hexoses	25.0	20.4	22.3
Amino sugars	0.0	0.0	0.0
Amino acids	0.0	19.2	16.8
Lipids	—	60.0	60.7

* As reported by Kanetsuna & Carbonell (1970) and Kanetsuna *et al.* (1972).

† As found during the course of this work, after several years of subculturing *in vitro*.

‡ Fraction as a percentage of the weight of the wall.

incubated at 37 °C for 0, 0.5, 1, 2, 4, 6 or 24 h. At the indicated times, 0.1 ml of the reaction mixture was heated at 100 °C for 5 min to inactivate the enzyme. After adjusting the volume to 1 ml and filtering, the liberated glucose was estimated by the Somogyi method for reducing sugar (Somogyi, 1952).

RESULTS

During the procedure for the mutant isolation, 1250 Y-form colonies on GGY plates surviving the mutagenesis treatment were examined periodically for up to 8 weeks' incubation at room temperature. About 2 % of the surviving Y-form colonies assayed for transformation did not show the whitish cotton-like morphology typical of the M-form of the wild-type colonies of *P. brasiliensis* (Restrepo, 1970), although they adhered to the agar surface and were composed of compact interwoven mycelia. The mycelium appeared typical upon examination by phase contrast in smears. Those colonies which apparently differed from the parental strain IVIC Pb9 only in the morphology of the M-colonies, were chosen as presumptive mutants for further tests. Samples from each of these colonies were allowed to transform back to the Y-form at 37 °C. After one cycle of purification, half of these presumptive mutants gave M-form colonies showing the cotton-like appearance of the parental strain. M-form colonies from the remaining half retained their altered morphology through several purification cycles. One of these mutants, identified as strain IVIC Pb140, was selected for further studies. M-form colonies of this mutant showed a brownish brain-like morphology, lacking typical aerial mycelia and submerged growth (Fig. 1), as opposed to the

Fig. 1. Four-week-old M-like colony of *P. brasiliensis* IVIC Pb140 grown on Sabouraud agar at 22 °C. Bar marker represents 2 mm.

Fig. 2. Four-week-old M-like colony of *P. brasiliensis* IVIC Pb9 grown on Sabouraud agar at 22 °C. Bar marker represents 2 mm.

M-form colonies of the parental strain (Fig. 2). Reversion to the wild-type morphology was not observed. Y-form colonies on Sabouraud agar were brain-like and easily detachable from the agar surface, resembling those of the parental strain IVIC Pb9 (Carbonell & Rodriguez, 1965). Strain IVIC Pb140 was a slow-growing form in BHI broth and formed clumps, a typical feature of *P. brasiliensis* growing under the same conditions. However, the presence of an appreciable number of true colonies in the form of lifeboats was observed. They were similar to those usually observed in some wild-type colonies grown on agar plates at the same temperature. The formation of these colonies in broth was probably due to the fact that Y-like cells of this mutant tended to remain in chains growing in several directions (Fig. 3). These Y-like cells were much smaller than those of IVIC Pb9 (Fig. 4), and were round or oval with diameters of 1 to 3 μm . Most cells remained attached to one another, forming chains of different sizes. Cells showing multiple peripheral buds with the appearance of a ship's wheel were also observed.

Chemical analysis of walls and their alkali-extracted fractions produced the following results:

Whole walls. Table 1 summarizes the differences in chemical composition of *P. brasiliensis* IVIC Pb9 and IVIC Pb140, and includes figures reported by Kanetsuna *et al.* (1972) for *P. brasiliensis* IVIC Pb9. Paper chromatography of total acid hydrolysates of wall preparations

Fig. 3. Y-like cells of *P. brasiliensis* 1VIC Pb140 grown in BHI broth at 37 °C. Bar marker represents 10 μ m.

Fig. 4. Y-like cells of *P. brasiliensis* 1VIC Pb9 grown in BHI broth at 37 °C. Bar marker represents 10 μ m.

Fig. 5. Negatively-stained fraction 1 from *P. brasiliensis* 1VIC Pb9. A similar arrangement was

Fig. 6. Infrared absorption spectra of fungal polysaccharides. (1) Chitin; (2) fraction 1 from *P. brasiliensis* 1VIC Pb9 and 1VIC Pb140; (3) laminarin; (4) α -1,3-glucan from *P. brasiliensis* 1VIC Pb9; (5) mannan from *Hansenula holstii*; (6) 1,3-mannan from *P. brasiliensis* 1VIC Pb140.

showed the presence of glucose, galactose and mannose in both strains. Lipids appeared to be present in higher amounts in the parental strain than in the mutant, whereas both strains had similar amounts of amino sugars and amino acids. Shadowed electron micrographs of strain 1VIC Pb140 cell walls showed a fibrillar arrangement similar to that already described for 1VIC Pb9 (Carbonell, 1972).

Fraction 1. Although the mutant strain contained twice as much fraction 1 as the parental strain, the composition was similar (Table 1). Hydrolysates contained 50 to 60 % of the total fraction as amino sugars, which when analysed by paper chromatography showed only glucosamine. Infrared absorption of this fraction produced a pattern which corresponded to that of chitin (Fig. 6), with an absorption band at 890 cm^{-1} ; characteristic of β -linkages in glucans.

Thin-layer chromatography showed glucose as the main neutral sugar in both preparations, with traces of galactose. Digestion of fraction 1 from either strain with β -1,3-glucanase released all the glucose residues as glucose, suggesting that this occurred as β -1,3-glucan.

Table 2. *Sugars in the walls of P. brasiliensis* IVIC Pb9 and IVIC Pb140 (yeast-like form)

	IVIC Pb9	IVIC Pb140
Fraction 1	Glucose as β -1,3-glucan <i>N</i> -acetylglucosamine as chitin Traces of galactose	Glucose as β -1,3-glucan <i>N</i> -acetylglucosamine as chitin Traces of galactose
Fraction 2	Glucose as α -1,3-glucan	Mannose as 1,3-mannan
Fraction 3	Galactose and mannose as galactomannan Traces of glucose	Glucose and galactose (polymeric association not studied)

Amino acids were also present in similar amounts in both preparations although it was not determined whether they were discrete proteins or glycoproteins.

Electron micrographs (Fig. 5) showed fibrils shorter than those described by Carbonell, Kanetsuna & Gil (1970) for the chitin and β -1,3-glucan of the parental strain IVIC Pb9.

Fraction 2. Both strains formed about the same amount of this fraction. However, their compositions differed (Table 2). Thin-layer chromatography of an acid hydrolysate from fraction 2 of the parental strain gave a single spot of glucose, shown by enzymic analysis to be exclusively α -1,3-linked. Electron micrographs of this fraction showed fibrils which resembled those reported before for α -1,3-glucan (San-Blas & Carbonell, 1974). The mutant strain contained mannose as the only sugar component. Periodate oxidation of this material produced no reaction, suggesting a 1,3-mannan. Its anomeric configuration was not determined. The infrared spectrum of this material (Fig. 6) showed a band at 800 cm^{-1} which has been reported as characteristic of mannans (Michell & Scurfield, 1970).

Electron micrographs of the mannan found in fraction 2 of the mutant strain showed an amorphous material in which no fibrils could be recognized, unlike the α -1,3-glucans.

Fraction 3. The alkali-soluble, non-precipitable fraction of both forms contained proteins and polysaccharides. In fraction 3 of the parental strain, thin-layer chromatography showed the presence of galactose, mannose and traces of glucose, whereas in the mutant strain, glucose and galactose were the only sugars present. The possible linkage between these sugars was not studied. It is also possible that they form complexes with the relatively high amounts of proteins present in this fraction. This material was not organized as a fibrillar structure, being amorphous under the electron microscope.

DISCUSSION

Variations in the morphology of both Y- and M-form colonies of *P. brasiliensis* grown *in vitro* are routinely observed and are probably influenced by environmental conditions, although no systematic description of spontaneous variation in colony morphology in *P. brasiliensis* has so far appeared. The same phenomenon often occurs in other dimorphic pathogenic fungi. Berliner & Burdo (1973) and Berliner (1973) used the effect of continuous light and total darkness to separate *Histoplasma capsulatum* into two strains (brown and albino) which differ in both morphological and physiological characteristics. An extensive study of the natural variability of *Coccidioides immitis* has revealed such extreme variations in the morphology of the colonies as to indicate that many of the strains should not have been typed as *C. immitis* (Huppert, Sun & Bailey, 1967).

The induction of mutants of dimorphic pathogenic fungi has also been demonstrated. In *C. immitis*, two distinct auxotrophic avirulent mutants of this fungus were obtained after induction of the wild-type prototrophic virulent strain with X-ray irradiation (Foley,

Berman & Smith, 1960). The isolation of biochemical and morphological mutants of this fungus was facilitated by the synergistic effects of ultraviolet light and alkylating chemical agents (Walch & Walch, 1967).

The mutant described in this paper, strain IVIC Pb140, was one of several morphological and biochemical mutants which have been isolated in our laboratory and are being characterized.

Y-like cells of *P. brasiliensis* growing in chains are commonly observed when grown at 28 °C, suggesting a transitional morphological state between the Y- and M-form. The fact that Y-like cells of strain IVIC Pb140 tend to remain in chains at 37 °C may be due to an altered division process at this temperature. Both these matters will be further investigated.

With regard to the chemical composition of the walls, previous reports (Kanetsuna & Carbonell, 1970; Kanetsuna *et al.*, 1972) indicated that most glucans in the Y-like form of *brasiliensis* were structured in the α -1,3-configuration (95 %), with only 5 % as β -1,3-glucans (Table 1). However, it was observed in our laboratory that during continuous subculturing *in vitro* of this strain, its pathogenicity decreased and at the same time its cell wall composition varied remarkably. A complete analysis of this strain was then required and results are summarized here for strain IVIC Pb9. This study confirmed that the α -1,3-glucan (i.e. the alkali-soluble fraction 2) was reduced. In fact, it declined from 40 % (Kanetsuna *et al.*, 1972) to 3 % of the total cell wall. At the same time, a galactomannan present only in the M-form of *P. brasiliensis* (Kanetsuna *et al.*, 1969) appeared in the Y-like form of strain IVIC Pb9 after subculturing *in vitro* in such a way that the whole quantity of sugars in the cell wall was about the same for both forms: 39 % (Kanetsuna *et al.*, 1969) and 41.5 % (Table 1). Amino sugar and amino acid contents remained almost unchanged in both cases.

According to these results, it seems that the peripheral α -1,3-glucan is only required by the fungus when the pathogenic Y-like form is infecting the host, being reduced when continuously subcultured *in vitro*. Preliminary experiments also suggest that this α -1,3-glucan reappears when strain IVIC Pb9 is successfully injected in animals or when it is cultured in medium to which foetal calf serum is added (G. San-Blas, D. Vernet and F. San-Blas, unpublished results). There may be a relationship between this α -1,3-glucan and pathogenicity.

On the other hand, the substitution of α -1,3-glucan by galactomannan in these two strains of IVIC Pb9 may be related to the decrease of pathogenicity observed in the strain extensively cultured *in vitro*. Azuma *et al.* (1974) demonstrated the serological activity of galactomannans from various pathogenic fungi, including *P. brasiliensis*; they pointed out that the Y-form of *P. brasiliensis* was devoid of galactomannan and that the galactomannan obtained from the mycelial cells of *Histoplasma*, *Paracoccidioides* and *Blastomyces* species were common antigens of these fungi. Whether the galactomannan found in *P. brasiliensis* (Y-form) after successive subculturing *in vitro* belongs to the same group of antigens, has still to be investigated.

The composition of the wall of the mutant strain differed from that of the parent strain in that the fibrillar α -1,3-glucan was replaced by an amorphous 1,3-mannan. This mannan was recognized not only by chemical analysis such as chromatography and periodate oxidation, but also by infrared spectroscopy of the polymer. Of the features common to all the yeast spectra, the band in the region 812 to 800 cm^{-1} is the most interesting in relation to mannans. Barker, Bourne & Whiffen (1956) claimed that it was characteristic of both α - and β -mannans, and Marchessault (1962) regarded its presence in the spectra of polysaccharide mixtures as indicative of mannans. Michell & Scurfield (1970) confirmed Marchessault's conclusion and showed this band in the spectra of mixtures of mannans

with various polysaccharides and proteins. The presence of an absorption band at 1530 cm^{-1} in IVIC Pb140, fraction 2 is also indicative of a protein component (Michell & Scurfield, 1970), which was revealed in the chemical analysis of its hydrolysates (Table 1). The possible linkage between the mannan and this protein was not studied although it is possible that the mannan found in IVIC Pb140 may have such a linkage, like the yeast mannan-protein complex described elsewhere (Sentandreu & Northcote, 1968; León de Pinto & San-Blas, 1975).

The β -1,3-glucan present in very low amounts (5 %) in the Y-like form of the original strain (Kanetsuna *et al.*, 1969) was present in larger amounts in both the parental strain reported here and in the mutant. The β -1,3-glucan has been described as being responsible for the elongated shape of the mycelium (Kanetsuna *et al.*, 1972). However, the presence of large amounts (about 30 %) of this polysaccharide in the Y-like forms of the strains studied here cast some doubts on the validity of this assumption.

From the results discussed in this paper, it can be said that mechanisms which regulate the formation of either the yeast-like or the mycelial form in *P. brasiliensis* are not yet well understood and that further investigation is required in order to elucidate the role of each polysaccharide on these fungal walls.

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