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Paracoccidioides brasiliensis AND *Paracoccidioides lutzii*, A SECRET LOVE AFFAIR

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SUMMARY

To commemorate Prof. Carlos da Silva Lacaz's centennial anniversary, the authors have written a brief account of a few, out of hundreds, biological, ecological, molecular and phylogenetic studies that led to the arrival of *Paracoccidioides lutzii*, hidden for more than a century within *Paracoccidioides brasiliensis*. Lacaz's permanent interest in this fungus, and particularly his conviction on the benefits that research on paracoccidioidomycosis would bring to patients, were pivotal in the development of the field.

KEYWORDS: *Paracoccidioides brasiliensis; Paracoccidioides lutzii;* Biology; Dimorphism; Ecology; Molecular phylogeny.

INTRODUCTION

In 2006, a seminal paper on the genomic variability of *Paracoccidioides brasiliensis* was published[31] which brought a totally different perspective on the study of this fungal species. By then, Carlos da Silva Lacaz, that iconic figure in Latin American mycological circles, had been dead for four years, so that he could not greet the arrival of *Paracoccidioides lutzii* as a cryptic species within the genus *Paracoccidioides*[13,15,50,51], hidden as it was for a century under the guise of *P. brasiliensis*, ever since Adolpho Lutz described the fungus and its dimorphic nature for the first time in 1908[28].

As usual in science, *P. lutzii* arrived as the outcome of decades of experiments on biochemistry, ultrastructure and molecular biology in *P. brasiliensis*, a very brief account of which we intend to summarize herein.

… AND THE WALLS CAME TUMBLING DOWN

The changing morphology of *P. brasiliensis* and its direct relationship to pathogenicity (i.e., yeast-like phase as the morphological phase in lesions; mycelial phase as a saprophyte) inspired researchers since the early 1970s to analyze fungal structures directly responsible for the final shape of the cell. In so doing, the San-Blas's group at the Venezuelan Institute for Scientific Research (IVIC) devoted those years to break down yeast and mycelial walls as the most obvious shape-defining structures, in order to analyze their chemical composition, studies already initiated by KANETSUNA *et al*.[23] as a follow-up of CARBONELL's[12] ultrastructural analyses.

In fact, the possible role of α-1,3-glucan as a dimorphic determinant[24] and virulence factor[46] was first reported in this fungus. This polysaccharide is present in the *P. brasiliensis* yeastlike cell wall but disappears when the fungus changes to its mycelial phase, as it is substituted by a β-1,3-glucan which is almost exclusively present in this morphotype[45]. As in all pathogenic fungi, chitin was also a major structural component of the cell wall and therefore, subjected to analysis on its role on morphology and pathogenicity[42,43].

Chitin, the β-1,4-linked homopolymer of N-acetylglucosamine, is one of the major components of the fungal cell wall, with important functions in wall integrity as structural component, and involved in morphogenesis and conidiophore development[2]. In earlier times, its synthesis was proposed as a potential antifungal target by researchers in the field. However, the complexities of its synthesis, where multiple chitin synthases participate at different stages of growth, have cooled down the initial enthusiasm for the search of chitin-inhibitory antibiotics. In *Paracoccidioides* spp., seven chitin synthases, one for each of the fungal chitin synthase classes[15,33,34,57], have been reported.

β-1,3-glucan is also a structural component of the fungal cell walls, but unlike chitin, β-1,3-glucan synthesis appears less complex. In *P. brasiliensis* and *P. lutzii*, a single β-1,3-glucan synthase gene has been reported, which is overexpressed in the yeast phase of both species[48,58], a result that contradicts the fact that β-1,3-glucan content is higher in the cell wall of the mycelial phase. However, they agree with earlier *in vitro* biochemical data, that indicated a higher activity of *P. brasiliensis* β-1,3-glucan synthase in particulate preparations of yeast-like cells than those of mycelial cultures[49]. It might indicate a post-transcriptional regulation of the β-1,3-glucan synthesis in *Paracoccidioides* spp., perhaps requiring the participation of a regulatory subunit of the β-1,3-glucan synthase complex, as already reported in other fungi[48]. The partial inhibition of yeast growth in *P. brasiliensis* by echinocandin inhibitors (which target

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β-1,3-glucan synthases) and their higher growth inhibition in its mycelial phase, are in agreement with the relative content of this polysaccharide in each morphological phase[40,43]; it also indicates that despite the role of β-1,3-glucan as a structural component in fungal cell walls, its importance for the maintenance of the *Paracoccidioides* yeast cell wall is not enough to consider its synthesis as a promising antifungal target.

Besides its role on cell wall maintenance, β-1,3-glucan has been shown to be an immunostimulatory molecule, inducing TNFα production by macrophages and recognized by the pattern-recognition receptor (PRR) dectin-1, a C-type lectin expressed on the surface of many mammal cell types, including macrophages, monocytes, neutrophils and T-cells[8,37,62]. The drastic reduction of β-1,3-glucan in the cell wall of the pathogenic yeast phase of *Paracoccidioides*, and its replacement by α-1,3-glucan as an outermost layer, could work as an evolved mechanism to avoid host recognition by the fungus.

Alpha-1,3-glucan, also present as the outermost layer in *Histoplasma capsulatum*, has been shown to block innate immune recognition by host cells in this fungus[37], reinforcing SAN-BLAS *et al.*[46] original proposal of this polysaccharide as a fungal virulence factor, and its emergence as a potential antifungal target. In *P. brasiliensis*, α-1,3-glucan is essentially a linear polysaccharide, with less than 3% of α-1,4-linked glucose branches, occasionally attached as single units to the α-1,3-backbone. A sole α-1,3-glucan synthase, Ags1, has been identified as responsible for the polysaccharide synthesis in *Paracoccidioides*[48]. Also one α-1,3-glucanase, Agn1, has been identified in the *Paracoccidioides* spp. genomes as the only hydrolase gene present in them[60].

A second element with a probable role in the synthesis of α-1,3-glucan has been identified. We refer to *P. brasiliensis* Amy1, a cytoplasmic α-1,4-amylase homologous to the *H. capsulatum* Amy1 (involved in the synthesis of α-1,3-glucan in the latter), that complements an *H. capsulatum* Amy1 mutant[11]. The enzyme showed a relatively higher hydrolyzing activity on amylopeptin than in starch, producing oligosaccharides 4 to 5 glucose residues long. The role of *P. brasiliensis* Amy1 in the synthesis of α-1,3-glucan is still unclear, although the authors suggest that it could be related to the generation of oligosaccharides that might act as primers for the biosynthesis of this polysaccharide by Ags1p[11].

The absence of α-1,3-glucan from the natural fungal host, its role in virulence and the apparent simplicity of the mechanisms of synthesis and hydrolysis of this polysaccharide (one synthase, one hydrolase), make both processes attractive targets for the development of specific drugs. Blocking the mechanism of synthesis (by inhibiting either Ags1 or Amy1 activities), or increasing its degradation (by stimulating Ang1 activity), might result in the depression of fungal virulence, and trigger the natural immune response of the infected organism against the fungus[11].

Despite its role in shaping the cell, the fungal wall is a dynamic structure. Rather than a rigid structure shaped only by polysaccharides such as chitin and β-1-3-glucan, the cell wall is a plastic and permeable structure, where those structural polysaccharides serve as scaffolds for proteins involved in signaling, cell-cell interactions, host attachment, as well as lipids related to the fungal secretion system, playing roles in the maintenance and modulation of the wall[30], all of which gives the fungal cell the ability to adapt to environmental changes.

Contrary to the abundant information on cell wall polysaccharides in *Paracoccidioides* spp., data on the composition of cell wall proteins and lipids are scarce, mostly going back to the earlier and general data of KANETSUNA and SAN-BLAS. Recently, the PUCCIA's group in São Paulo, have started a thorough work on lipid composition of *P. brasiliensis* cell wall, comparing isolates Pb3 and Pb18, which belong to the PS2 and S1 species, respectively, isolates whose infection profiles in murine models are different[27,36]. They found 49 phospholipid species in Pb3 and 38 in Pb18, phosphatidylcholine and phosphatidylethanolamine being the most abundant phospholipidic molecules in both isolates. Brassicasterol was the most abundant sterol in the cell walls of both isolates, a compound already reported as the main sterol in the cytoplasmic membrane of *P. brasiliensis* yeast phase[61].

Strategies for studies on gene function have proven cumbersome in *Paracoccidioides* spp., and although antisense RNA technology has recently emerged to overcome problems with gene knockout, the multinucleated nature of *Paracoccidioides* cells makes it difficult to apply it as a universal technique, since it only appears to work in a few strains. Progress towards development of molecular tools for gene function studies that work in all *Paracoccidioides* cryptic species are still needed, in order to provide information for definitely determine the roles of genes associated with regulation, synthesis and hydrolysis of the cell wall on growth, morphogenesis and pathogenesis of these important human pathogens. Not all has been said about their cell walls and therefore, we must expect further developments to arrive in due time.

WHEN ONE BECAME TWO… OR MORE

The observation that distinct isolates of *P. brasiliensis* were variable in mycological, antigenic and virulence aspects did not pass unnoticed through the years[17,20,25,29,47]. The first molecular typing studies, such as RAPD analysis, had already indicated that the genetic variability among *P. brasiliensis* isolates might be beyond the species level[10,19,21]. After applying the Phylogenetic Species Concept (PSC) in studies of Multi-Locus Sequence Type (MLST)[31], it became clear that instead of a unique species, *P. brasiliensis* might contain several different cryptic species, a fact that was confirmed by additional studies[13,32,50,54]. Initially, three phylogenetic species were recognized: S1, a paraphyletic and recombining species; PS2, a monophyletic and recombining species; and PS3, a monophyletic and clonal species[31,32]. Once additional isolates, mainly from the Brazilian Central-Western Amazonia, were subjected to the same[13,50] or different molecular analyses[54,55], a fourth more diverging phylogenetic species (Pb01-like) emerged, later designated as *P. lutzii*[51,52].

Genomic analysis of *P. brasiliensis* and *P. lutzii* pointed to significant differences. While the genome sizes of the two cryptic species (S1/Pb18 and PS2/Pb03) of *P. brasiliensis* are similar (30.0 Mb and 29.1 Mb respectively), the *P. lutzii* genome is nearly 3 Mb larger (32.9 Mb). The total number of initial predicted genes varies between 7,875 in *P. brasiliensis* to 9,132 in *P. lutzii*. The three genomes are highly syntenic, though S1/Pb18 and PS2/Pb03 of *P. brasiliensis*, share a significantly higher percentage of sequence similarity (96%) to each other in comparison to *P. lutzii* (90%). Transposons may be one of the reasons for the enlargement of *P. lutzii* genome, as they constitute 16% of it, twice as much as that of *P. brasiliensis* S1/Pb18 and PS2/Pb03 genomes (8-9%)[15].

Differences between *P. lutzii* and *P. brasiliensis* (S1, PS2 and PS3 species) were also detected in their proteomic yeast profiles by means of 2D electrophoresis and mass spectrometry. Out of 343 protein spots, 267 were differentially expressed, and 193 proteins were identified. Glycolysis/gluconeogenesis and alcohol fermentation-related proteins were more abundant in *P. lutzii*, indicating a higher use of anaerobic pathways for energy production. Antigenic proteins such as GP43 and 27-kDa, were less abundant in *P. lutzii* and PS2 genotype of *P. brasiliensis*[35]. The *P. lutzii* GP43 orthologue is poorly expressed and contains few epitopes in common with the *P. brasiliensis* immunodominant antigen GP43[26], contributing to serological diagnostic difficulties in patients infected with *P. lutzii*[6].

Before *P. lutzii* was actually recognized as a new species, HAHN *et al*.[19] observed differences in pathology and response to treatment in paracoccidioidomycosis patients from the Central-Western region of Brazil where most *P. lutzii* isolates have been reported so far, as compared with other regions in which *P. brasiliensis* predominates (see below). This is a field that deserves urgent attention, in order to preserve the life of patients afflicted by this severe and frequently lethal disease.

FAMILIES ARE MESSY

Morphological and molecular findings indicate that both *Paracoccidioides* species might be classified as Ascomycota, order Onygenales, family Ajellomycetaceae[59], in a natural group that also includes other vertebrate-associated fungi of the genera *Histoplasma*, *Blastomyces*, *Emmonsia* and *Lacazia*[22,44]. This fungal family presents some common mycological and ecological features, such as dimorphism, arthroconidia production and occurrence in restricted geographic areas, and affinities for animal product derivatives or remnants like feces and uric acid[9].

TWO HOMES, TWO WORLDS IN TENSION

The ecological niche of *Paracoccidioides* spp. is not completely understood[18,38]. *P. brasiliensis* has been isolated sporadically from soil and related materials (animal feces and dog food mixture with soil), and frequently from armadillos[5]. Several other domestic and wild mammals appear to be infected by the fungus, as determined by intradermic test, serology and molecular procedures[7,16,39]. The fungus has been demonstrated by molecular tools in soil samples from burrows of the nine-banded-armadillo, *Dasypus novemcinctus*[4,14,53,56], and also in aerosol samples, confirming the spread of the fungus by inhalation[1].

The recent genome survey of the *Paracoccidioides* species proved that this pathogen has the metabolic apparatus to degrade cellulosic plant material in the soil[35], making soil a probable saprobe habitat for *Paracoccidioides*. But more important is to elucidate its environmental requirements, tolerance and interaction with other species, in a multidimensional space. *Paracoccidioides* spp. and other Ajellomycetacean fungi, except *Lacazia loboi*, typically present at least two ecological niches, because of their dual saprobic and parasitic life style. During the saprobic mycelial phase these pathogens interact with different conditions and resources, such as periodic changes in temperature and humidity, competition with other microorganisms, and probable predation by amoebae and nematodes, commonly found in soil; during the parasitic yeast phase they are exposed to the mammal tissues, with temperature increase, hormone influences and response to the immune system.

As noticed in previous paragraphs, there are much more studies about the parasitic niche of *Paracoccidioides* than in its saprobic phase, reason for which BUSTAMANTE *et al.*[9] or TERÇARIOLI *et al.*[53], among others, evaluated the growth and conidia production of *P. brasiliensis* on soil in order to understand how the pathogen interacts with some abiotic factors, such as soil texture and availability of water. *P. brasiliensis* grows well both in clay and sand soil, provided they are saturated with water, a limiting factor for the fungal growth. It was also observed that soil containing high amount of exchangeable aluminium (H+Al) and low base saturation inhibit growth of the fungus; at the same time, it turned out that armadillos infected with *P. brasiliensis* were more easily found in soils low in H+Al, sandy and with medium to low concentrations of organic matter[3]. Most isolates were able to produce arthroconidia though a lower conidia production in PS2 genotype was observed in comparison with S1 isolates, a fact that could explain the unbalanced proportion of 1:9 (PS2:S1) isolates found in the same endemic area of Botucatu, SP, Brazil[55].

Additional ecological studies are pending in order to detect and differentiate the cryptic species in soil, a survey that is already under way in several regions of the Brazilian geography, i.e., ARANTES *et al.*'s[1] detection of *P. lutzii* in a hyperendemic area for *P. brasiliensis* by means of nested PCR amplicons from the ITS region, that clearly differentiates both species[50]. Such studies will help strengthen phylogeographic information on *Paracoccidioides*, operational to design a possible evolutionary scenario for the speciation process of this genus[4].

So far, phylogeographic inferences revealed simultaneous geographic expansions of S1 isolates[55], one of which is now considered a S1 subspecies, represented by Venezuelan isolates[41]. Another expansion gave rise to the Colombian PS3 species, geographically isolated in the Andes, once they emerged around eight million years ago. As there are no clear geographic barriers in the Brazilian Shield, there are no obvious geological allopatric events to explain the speciation processes that gave rise to S1, PS2, and *P. lutzii*. Probably this is a kind of microallopatry, where the ecological differences in the same geographic area may play an important role in the isolation and divergence of the species.

CLOSING REMARKS

When Carlos da Silva Lacaz was born in 1915, seven years had already passed since *P. brasiliensis* made its début in the annals of the Latin American medical mycology[28]. The fungus turned out to be the agent of one of the most relevant fungal diseases of the region, in terms of frequency and devastating consequences both for patient and society. The efforts and funds invested in state-of-art research on the subject have paid in the huge amount of information now available on biology, immunology, ecology, phylogeny and much more, leading to the design of better treatment procedures for the benefit of paracoccidioidomycosis patients, either from *P. brasiliensis* or *P. lutzii*. However, the puzzle is far from been solved, and improved protocols still await further fundamental and applied research to comply with one apothegm of the Hyppocratic Oath: *ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν* (abstain from doing harm), popularized in a Latin version as "*Primum non nocere*", (first of all, do not harm), a maxim that Lacaz always had in mind to act accordingly.

RESUMO

Paracoccidioides brasiliensis e *Paracoccidioides lutzii*, um caso secreto de amor

Para comemorar o centenário de aniversário do Prof. Dr. Carlos da Silva Lacaz, os autores fazem um breve relato dos estudos sobre a biologia, ecologia e filogenia molecular que culminaram na revelação da espécie *Paracoccidioides lutzii*, que havia permanecido escondida por mais de um século ao lado de *Paracoccidioides brasiliensis*. O professor Lacaz exerceu papel central no desenvolvimento desta área do conhecimento, pois manteve interesse permanente nas pesquisas deste fungo e da paracoccidioidomicose, visando principalmente proporcionar benefícios aos pacientes acometidos por esta micose.

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