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Potential new drug targets and mating process of the human pathogenic fungus Pneumocystis jirovecii

Luraschi Amanda

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Faculté de biologie et de médecine

Institut de Microbiologie, CHUV (Centre hospitalier universitaire vaudois)

Potential new drug targets and mating process of the human pathogenic fungus *Pneumocystis jirovecii*

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

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Biologiste diplômé ou Master de l'Université de Lausanne

Jury

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Dr. Frederic Lamoth, Expert
Prof. Gilles Nevez, Expert

Lausanne
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PhD Thesis Report

presented to the

Faculty of Biology and Medicine
Lausanne University

by

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Potential new drug targets and mating process of the human pathogenic fungus *Pneumocystis jirovecii*

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Prof. Pierre-Yves Bochud

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Summary

Pneumocystis organisms are extracellular fungal parasites that colonize the lungs of mammals. They constitute a group of species, each displaying strict host specificity for a given mammalian species. *Pneumocystis jirovecii* infects specifically humans. It is an opportunistic pathogen causing severe pneumonia in immunocompromised individuals that can be fatal if not treated. 1,3 and 1,6 -β glucans are essential constituents of cell walls of most fungi. Enzymes involved in their synthesis represent ideal drug targets, especially as inhibitors of these enzymes are known. The first part of my thesis project consisted in the identification and functional characterization of these two potential drug targets in the human pathogen *P. jirovecii* by rescue of the null allele of the orthologous gene in *Saccharomyces cerevisiae.*

Echinocandins are a class of antifungal drugs composed by caspofungin, micafungin and anidulafungin, that inhibits the catalytic subunit Gsc1 of the enzymatic complex ensuring the synthesis of 1,3-β glucan. Even if caspofungin efficacy was demonstrated against *P. carinii* and *P. murina* in the rodent model, its efficacy against *P. jirovecii* is controversial in the clinical studies presently available. Moreover, the response to caspofungin of *P. jirovecii* could be different than those of *P. carinii* and *P. murina* because these species have a mean genetic divergence of ca. 20% at nucleotide level. In the fungal pathogen *Candida albicans,* resistance to caspofungin is conferred by point mutations leading to two amino acid substitutions within the hotspot no. 1 of mutations of Gsc1. We used site-directed mutagenesis to introduce the corresponding mutations within the *gsc1* gene of the three *Pneumocystis* species. Upon expression of *Pneumocystis gsc1* genes on plasmid in the *S. cerevisiae* null mutant, a partial restoration of the wild type growth was observed on medium containing caspofungin in presence of one substitution, and increased more in presence of the two substitutions. Our results suggest that the Gsc1 enzyme of *P. jirovecii* is sensitive to caspofungin, similarly to those of *P. carinii* and *P. murina*.

In the third and last part, we investigated the sexual reproduction of *Pneumocystis* organisms, that would be essential for the formation of asci, the particles necessary for the spread of the infection. Recent comparative genomic analysis performed by our group suggested the existence of a single mating type locus that includes both plus (P) and minus (M) genes, suggesting that *Pneumocystis* species are primary homothallic organisms. However, the mating type of single cells remained unknown (M or P, or M and P at the same time). The aim of the third part was to analyze the expression of the M-factor receptor (*mam2*) and the P-factor receptor (*map3*) genes, and to verify if both are present at the same time at the surface of single *Pneumocystis* cells. Several bronco-alveolar lavage (BAL) fluid samples from patient with *P. jirovecii* pneumonia were analyzed for *mam2* and *map3* expression. The majority of them resulted positive for both pheromone receptors, suggesting that they are concomitantly expressed during *Pneumocystis* infection. *P. jirovecii* Mam2 and Map3 pheromone receptors were afterwards observed at the cellular surface using a specific immunofluorescent staining. The majority of the presumed trophic cells we identified were positive for both pheromone receptors, suggesting that each cell is of M and P mating types at the same time, and consequently that any cell can mate with any other cell present in the population.

Resumé

Les organismes du genre *Pneumocystis* sont des champignons parasites extracellulaires qui peuvent coloniser les poumons des mammifères. Ils font partie d'un groupe d'espèces ayant chacune une spécificité stricte pour un mammifère donné, *Pneumocystis jirovecii* étant celle qui infecte spécifiquement l'homme. Cette dernière est un pathogène opportuniste, qui peut causer une pneumonie très sérieuse chez les individus dont le système immunitaire est déficient, à savoir la pneumonie à *Pneumocystis* ou pneumocystose (PCP). Les glucans sont des constituantes essentielles de la paroi de la cellule fongique dans une grande majorité des champignons. Les enzymes impliquées dans leur synthèse représentent des cibles thérapeutiques idéelles, spécialement parce que les inhibiteurs de ces enzymes sont connus. La première partie de mon projet de thèse a consisté en l'identification et la caractérisation fonctionnelle de deux cibles thérapeutiques potentielles du pathogène humain *P. jirovecii* grâce la complémentation fonctionnelle d'une souche de *Saccharomyces cerevisiae* avec les allèles orthologues deletés.

Les échinocandines sont une classe de médicaments antifongiques qui comprend la caspofungine, la micafungine et l'anidulafungine. Ces antifongiques inhibent spécifiquement la sous unité catalytique Gsc1 du complexe enzymatique nécessaire à la synthèse du 1,3-β glucane. Même si l'efficacité de la caspofungin a été démontrée contre *P. carinii et P. murina* dans le modèles animaux, l'utilisation de ce médicament contre *P. jirovecii* est controversé dans les études clinique disponibles. En plus, la réponse de *P. jirovecii* à ce traitement pourrait être différente de *P. carinii* et *P. murina,* parce que ces espèces ont une divergence génétique moyenne de 20% aux niveau nucléotidique. Chez le champignon pathogène *Candida albicans,* des mutations spécifiques dans Gsc1 amenant à la substitution de deux acides aminées, confèrent une résistance à la caspofungine. En utilisant un système de mutagenèse dirigé, j'ai introduit les mutations correspondantes dans les gènes *gsc1* de *P. jirovecii, P. carinii* et *P. murina.* J'ai ensuite exprimé ces gènes dans la souche *S. cerevisiae* deletée pour *GSC1*. Comme déjà observé précédemment, l'expression des gènes *gsc1* des différents *Pneumocystis* permet une restauration partielle de la croissance par rapport à la souche sauvage. Cette restauration de croissance augmente en présence d'une mutation, et augmente encore plus en présence des deux mutations. Nos résultats suggèrent que l'enzyme Gsc1 de *P. jirovecii* serait sensible à la caspofungin à un niveau similaire que ces de *P. carinii* et *P. murina*.

Dans la troisième et dernière partie de ma thèse, j'ai étudié la reproduction sexuée des organismes du genre *Pneumocystis*, lequel semble être indispensable pour la formation de l'asque, qui peut infecter un nouvel hôte par transmission aérienne. Les analyses de génomiques comparatives qui ont été réalisées par notre groupe, ont démontré l'existence d'un seul locus de mating type, lequel inclus les gènes plus (P) et minus (M). Cela impliquerait que *Pneumocystis* est un organisme homothallique primaire, et par conséquent que chaque souche serait auto-fertile. Toutefois, le mating type de chaque cellule reste inconnu (M ou P, ou M et P à la fois). Le but de cette troisième partie était d'analyser l'expression des récepteurs pour le facteur de mating M (mam2) et pour le factor P (map3), et de vérifier si un seul ou les deux récepteurs étaient présents ensemble à la surface de chaque cellule de *P. jirovecii*. J'ai testé l'expression de mam2 et map3 sur plusieurs lavages broncho-alvéolaire (LBA) venant de patients souffrants de pneumonie causée par *P. jirovecii*. La majorité des LBA était positive pour

les deux récepteurs. Ces résultats suggèrent que les deux récepteurs sont exprimés de façon concomitante pendant la pneumonie. Mam2 et Map3 de *P. jirovecii* ont été observés à la surface des cellules trophiques en utilisant une coloration immuno-fluorescente spécifique pour chacun des récepteurs. La majorité des cellules trophiques présumées étaient positives pour les deux récepteurs. Ces résultats suggèrent que chaque cellule est des deux mating types (M et P) à la fois, et de fait, chaque cellule pourrait s'accoupler dans les poumons avec n'importe quelle autre cellule de *Pneumocystis* présente.

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General introduction

History

Pneumocystis organisms were first described in 1909 by Carlos Chagas (Chagas, 1909). *Pneumocystis* cysts were found in the lungs of trypanosome-infected animals and were misinterpreted as a new form of schizogonic stages of *Trypanosoma cruzi*, an animal parasite. *Pneumocystis* started to be considered pathogenic for humans in 1942, when the organism was found during autopsy studies. In particular, *Pneumocystis* was attributed as the cause of fatal pneumonia cases associated with the presence of cysts inside pulmonary alveoli (van der Meer & Brug, 1942; Vanek 1951). In the 1950s, the number of pneumonia caused by *Pneumocystis* dramatically increased in Europe (Morris *et al.,* 2004). The victims of this disease were mainly immune-deficient children and patients under immunosuppressive therapy. At the end of the 1980s, *Pneumocystis* pneumonia became an important public health problem being the most frequent opportunistic infection in individuals with AIDS (Acquired Immune Deficiency Syndrome) (Millis, 1986). *Pneumocystis* pneumonia is nowadays one of the most frequent cause of death and pathological state in immunocompromised patients.

Phylogeny and Taxonomy

Pneumocystis organisms are extracellular parasites that colonize the lungs of mammals (Thomas *et al.,* 2004; Cushion & Stringer, 2010; Gigliotti *et al.,* 2014). They constitute a group of species, each displaying strict host specificity for a given mammalian species. By now, there are five species which have been described: *Pneumocystis jirovecii* in *Homo sapiens* (human being) (Frenkel, 1999)*, Pneumocystis carinii* in *Rattus norvegicus* (rat) (Frenkel, 1999), *Pneumocystis wakefieldiae* in *Rattus norvegicus* (Cushion *et al.,* 1993; Cushion *et al.,* 2004) and *Mus musculus* (mouse) (Keely *et al.,* 2004) and *Pneumocystis oryctologi* in *Oryctolagus cuniculus* (Old World rabbit) (Dei-cas *et al.,* 2006). The existence of other specific species in other mammals as macaques, ferrets, bats, shrews, horses, pigs and dogs is suggested by genetic studies (Banerji *et al.,* 1994; Peters *et al.,* 1994; Christensen *et al.,* 1996; English *et al.,* 2001; Guillot *et al.,* 2004). Initially, *Pneumocystis* species were wrongly classified as protozoans, but DNA sequence homology analysis revealed that they are fungi (Edman *et al.,* 1988; Stringer *et al.,* 1989). *Pneumocystis* organisms belong to the subphylum Taphrinomycotina of the Ascomycota, to which yeasts of the genus *Schizosaccharomyces* also belong (Hancock, 2002). Interestingly, *Pneumocystis* species are the only animal pathogens among the Taphrinomycotina members, which are all phyto-pathogens.

Biology

An *in vitro* long-term culture method is still not available for this pathogen. Schildgen *et al.* (2014) described a novel system of co-culture on airway epithelial cells, but it remains to be established. This lack complicated the study of *P. jirovecii*, mostly on research of new drug targets. However, several tasks are now possible because of the release of the genome sequence of *P. jirovecii* by two groups (Cissé *et al.,* 2012; Ma *et al.,* 2016).

Pneumocystis organisms miss several synthesis and assimilation pathways as revealed by genome sequence analysis, suggesting that their life-style is obligate parasitism, without free-living forms (Hauser *et al.,* 2010; Cissé *et al.,* 2012; Cissé *et al.,* 2014; Porollo *et al.,* 2014; Hauser 2014). The loss of metabolic genes also correlates with an important dependency of the parasite on its host, and explains in part the strict host specificity of *Pneumocystis*, because the scavenging of essential compound requires a remarkable host adaptation. Accordingly, the entire life cycle of *Pneumocystis* species occurs inside the host's lungs. Moreover, there is no evidence of an environmental source, strongly supporting the hypothesis of obligate parasitism of *Pneumocystis*, with mammals as unique reservoir.

Pneumocystis pneumonia

The agent of *Pneumocystis jirovecii* pneumonia (PCP) PCP in humans is *P. jirovecii* (Figure 1). Patients at risk of PCP are individuals immunocompromised, as HIV (Human Immunodeficiency Virus) infected people, or transplant recipients. Pathogens that take advantages of the impaired immune state of an individual are called opportunistic. Normally, these pathogens do not cause disease in healthy hosts. The symptoms of PCP include fever, cough, difficulty breathing, loss of weight and sweating during the night. In children, PCP has been suggested to aggravate the sudden infant death syndrome (Vargas *et al.,* 2007), infant bronchiolitis (Tristram *et al.,* 1988), and chronic pulmonary infections (Morris *et al.,* 2004). PCP is nowadays the second most frequent life-threatening invasive fungal infection worldwide with above 400'000 annual cases world-wide, representing a severe public health problem, as the mortality rate rises as high as 80% if untreated (Brown *et al.,* 2012).

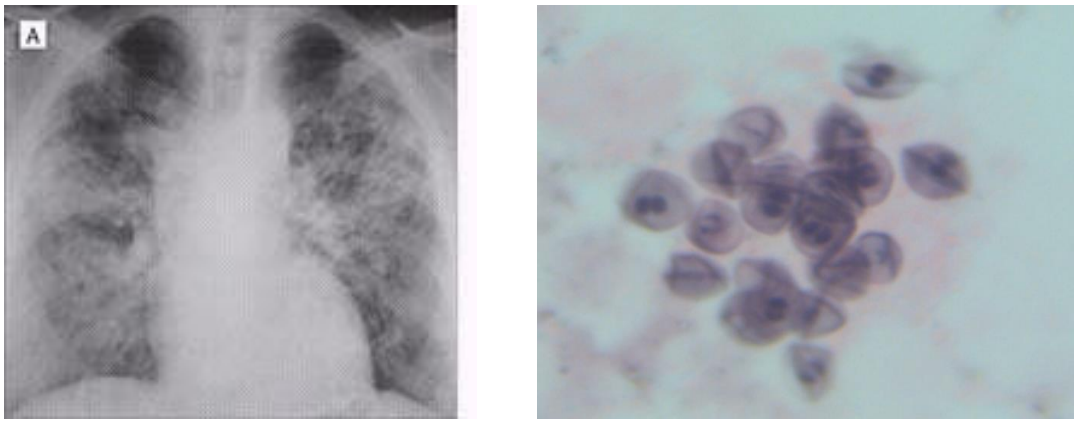

Figure 1. Left panel: chest radiography showing bilateral infiltration of *P. jirovecii* inside the lungs of an infected patient (Kovacs *et al.,* 2011). Right panel: cluster of *P. jirovecii* asci from a bronco alveolar lavage sample from an infected patient, stained with methenamine silver staining (CHUV).

Source and transmission

Two hypotheses about the origin of a PCP episode occurring in a given patient have been proposed: (i) the reactivation of a latent infection, supposedly acquired during early childhood, when the immune system is not entirely efficient, and (ii) *de novo* exposure to new sources of *Pneumocystis* organisms. Despite the fact that more than 90% of infants develop a *Pneumocystis* primo-infection, it is more likely that the hypothesis of *de novo* infection is the real cause of PCP (Morris *et al.,* 2002). Cases of transmission of *P. jirovecii* due to the occurrence of PCP case clusters in hospital strongly support this second hypothesis (Nevez *et al.,* 2008). Production and transmission of infecting forms of *Pneumocystis* is guaranteed by its host, which is a dynamic reservoir, more or less susceptible to the pathogen depending on its immune state. Besides infected patients that develop a PCP, a contribution to dissemination of the parasite is attributed to healthy colonized individuals, which in most cases are not aware of their status. The rodent model proved experimentally that transmission from an infected individual to a new host occurs through inhalation of infecting particles by the airborne route (Hughes, 1982; Cushion *et al*., 2010), representing a unique case among pathogenic fungi (Chabé *et al.,* 2009; Aliouat-Denis *et al.,* 2008). Transmission might also occur from the mother to the foetus via the trans-placental route (Ceré *et al.,* 1997; Sanchez *et al.,* 2007; Montes-Cano *et al.,* 2009). Because of the strict host species specificity, the transmission of this parasite from animals to humans is very unlikely (Chabé *et al*., 2011).

Diagnostics

Because of the absence of a culture method for *P. jirovecii in vitro,* diagnosis for PCP is based on the detection of the pathogen in patient's samples, such as sputum, broncho-alveolar lavage fluid (BAL) or lung tissues. The staining methods used are methenamine silver (Figure 1, right panel), toluidine blue-O, Giemsa stain (Morris, 2012), or immunofluorescence staining (Ng, 1990). Nowadays, PCR techniques are more and more used (Huggett *et al.,* 2008).

Treatments

Standard antifungals targeting the fungal cell membrane integrity or synthesis are not effective against *P. jirovecii.* This is probably due to presence of cholesterol instead of ergosterol in its membrane. Nowadays, the most efficient treatment for *P. jirovecii* infection is cotrimoxazole. This drug is a combination of trimethoprim and sulfamethoxazole, both targeting enzymes involved in the folic acid biosynthesis pathway. Folic acid is a metabolite required for the synthesis of essential cellular compounds. The use of this drug for prophylaxis and treatment of PCP since the late 1980s correlated with the identification of specific mutations within the active site of the target of sulfamethoxazole (Kovacs *et al.,* 2001; Lane *et al.,* 1997; Ma *et al.,* 1999; Nahimana *et al.,* 2003), as well as of the target of trimethoprim (Nahimana *et al.,* 2004). These mutations are believed to confer resistance to cotrimoxazole to a level sufficient to provoke failure of prophylaxis (Lane *et al.,* 1997; Ma *et al.,* 1999; Queener *et al.,* 2013). Moreover, cotrimoxazole can cause important side effects in some patients. For these reasons, the research of new treatments, and consequently new drug targets, is of the utmost importance.

New drug targets

The lack of a culture method complicates the identification of new drug targets in *P. jirovecii*. The strategy used so far has been to identify potential drug targets in the genome of *P. carinii*, the species infecting rats, which was used as a model (Fox & Smulian, 1999; Kottom & Limper, 2004; Lo Presti *et al.,* 2007; Cockel *et al.,* 2009; Hauser & Macreadie, 2006). The function of the potential targets was characterized by complementation of the deletion mutant of the orthologous gene in the model yeasts *S. cerevisiae* or *Schizosaccharomyces pombe*. This strategy proved useful but presents the drawback that *P. carinii* is relatively distant from *P. jirovecii*, with a mean divergence of gene sequences at the nucleotide level of ca. 20% (Stringer, 1996), so that the sensibility to drugs of the targets may vary between the two species. The recent releases of *P. jirovecii* genome sequences (Cissé *et al.*, 2012; Ma *et al.,* 2016) opened a new era for the search of new drug targets. Indeed, it offers the opportunity to identify the genes encoding the actual targets to inhibit within the *P. jirovecii* genome*,* and no more those of the model *P. carinii.*

The fungal cell wall represents an ideal drug target because (i) is essential for the microorganisms, (ii) it is absent in human cells, and (iii) because it is easy reachable by the drugs since it is localized on the surface of the cell. Thus, the search of new drugs targets among the genes responsible for the synthesis and metabolism of the fungal cell wall constitutes a valid strategy. A schematic representation of this wall is shown in Figure 2. The most important structural components of the fungal cell wall are the β-1,3 glucans which are synthetized by the enzyme β-1,3 glucan synthase including the catalytic subunit Gsc1. β-1,6 glucans are less abundant than β-1,3 glucans, synthetized by the enzyme Kre6, and act as a flexible glue between the other components of the wall. A layer of chitins is present between the β-glucans and the membrane. Yeast cell walls contain also mannoproteins, glycosylated proteins with chains rich in mannose, integrated into the wall. Most wall proteins have a function of structure and cell shape maintaining (Bowman and Free, 2006).

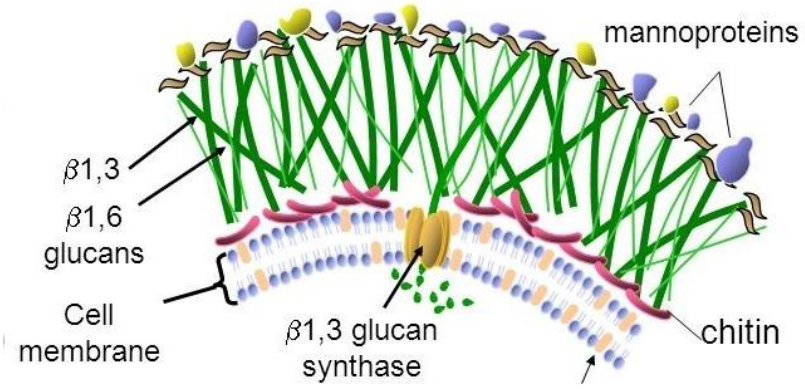

Figure 2. Schematic representation of the fungal cell wall (Mandell and Diamond, 2000. Atlas of Infectious Disease: Fungal infections, DOI: 10.1007/978-1-4757-9313-0).

Life cycle

Although *P. jirovecii* is an important cause of mortality of immuno-compromised patients, there is still no *in vitro* long-term culture method available for this pathogen. A system of co-culture on human pseudostratified airway epithelial cells has been recently described (Schildgen *et al.,* 2014), but it remains to be widely established. Because of this lack, the life cycle of *Pneumocystis* species remains hypothetical and mostly based on microscopic and molecular observations. Figure 3 shows the most recently proposed hypothetical cycle (Hauser and Cushion, 2018). As obligate parasites, the entire cycle of *Pneumocystis* species seems to occur within the host's lungs. The infection begins with the inhalation of an infectious particle (the ascus or the ascospores), coming from the respiratory tract of another infected individual. The ascus releases eight ascospores which will become eight trophic cells, that represent the predominant forms inside the lungs during the infection (90 to 98% of the cells; Aliouat-Denis *et al.,* 2009). Trophic cells are mononuclear and mostly haploid (Stringer and Cushion 1998; Wyder *et al.,* 1998; Martinez *et al.,* 2011), they have a variable diameter of 2 to 8 µm (Dei-cas *et al.,* 2004) and are apparently devoid of cell wall. Trophic cells can multiply by binary fission or start a sexual cycle. Trophic cells might also multiply by a process called endogeny, in which a larger trophic form contains an undefined number of smaller trophic cells, but this type of reproduction have been observed only in two studies and remains poorly understood (Vossen *et al.,* 1978, Yoshida 1989).

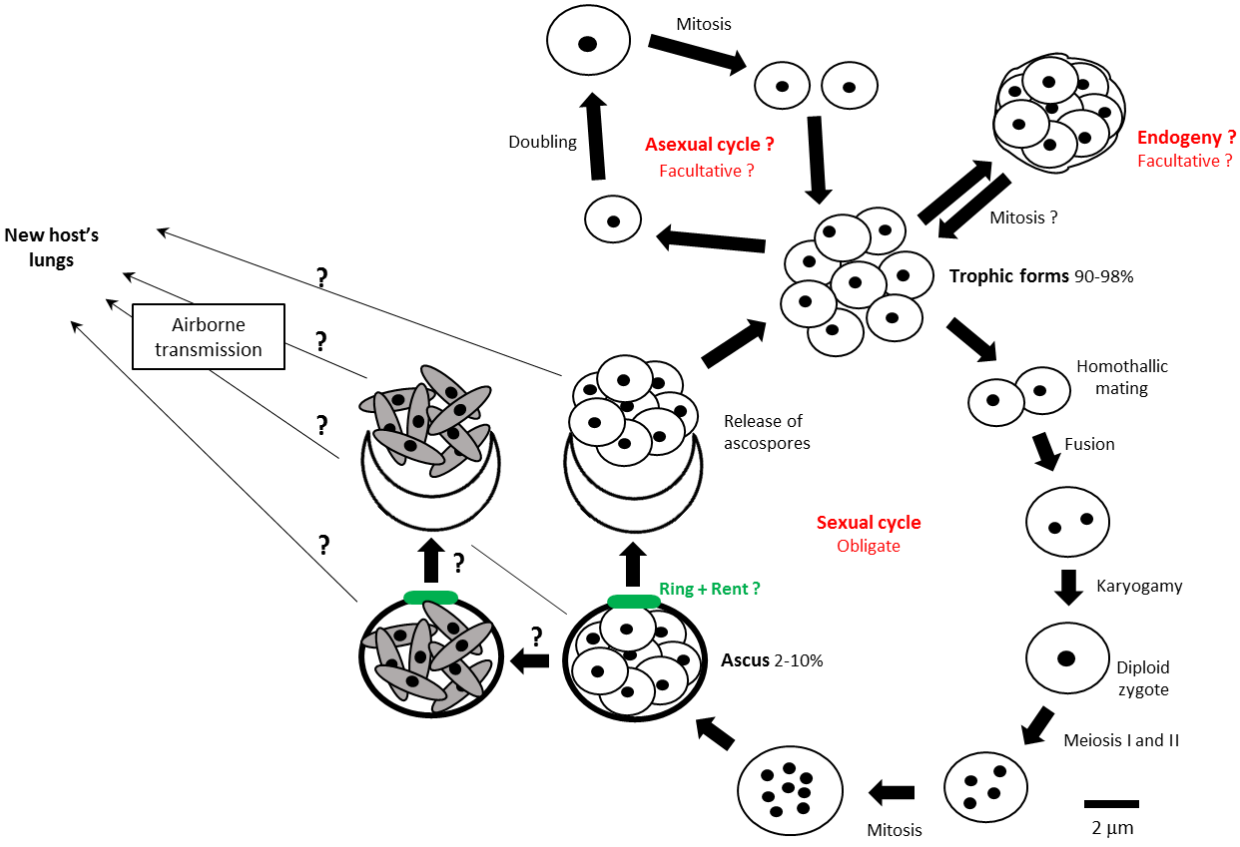

Figure 3. *Pneumocystis* hypothetical life cycle. Black dots represent nuclei of cells. The "?" indicate processes hypothesized but not yet confirmed or only poorly supported. Circular structure called rent or ring is shown in green. Figure and legend from Hauser & Cushion, 2018.

The effective occurrence of the asexual cycle remains an open question: observations on trophic cells shows fusion at the level of cell membrane as well as at the level of the nuclear membrane, suggesting the occurrence of mating rather than mitosis. Moreover, a study showed that sexual reproduction was sufficient to account for the number of cells issued from reproduction, suggesting that the asexual cycle may not happen at all (Aliouat *et al.,* 1999). Occurrence of the asexual cycle through binary fission or endogeny might be facultative, or take place only during some specific phases of the infection or under specific conditions. Trophic cells are also supposed to be able to enter in a latency phase, which might be crucial for the survival of the organism (Hauser and Cushion, 2018).

Sexual cycle

The sexual cycle would involve mating of two compatible trophic cells, which enter into sexual cycle and then undergo meiosis followed by a mitosis that gives rise to a new cell: the ascus. Asci represent only 2 to 10 % of the *Pneumocystis* cells population. Asci measure 4 to 6 µm and have a rounded shape, they are surrounded by a thick wall and contain eighth daughter cells, the ascospores. The ascospores are typically rounded in shape, similar to trophic cells, but they have been also observed as elongated and more condensed cells (Figure 3, round ascospores are white, while elongated ascospores appear in grey). Ascospores are presumably released through a circular structure, called rent or ring, that opens a hole in the ascus cell wall (Figure 3).The supposed function of this ring is to operate as a pressure valve, that allows the expansion of the ascus promoting the ascospores release. In morphological studies, ascospores have been observed close to collapsed and empty asci, which take the form of a crescent moon. They presumably remain in the proximity of the ascus once released. The release of ascospores inside the lungs does not exclude that some asci leave the host through the airborne route to reach a now host.

The occurrence of sexual cycle is supported by several facts: (i) the identification of synaptonemal complex, the protein structure that mediate the chromosome pairing during meiosis (Matsumoto *et al.,* 1984; Peter *et al.,* 2001); (ii) the demonstration of expression of sex-related genes (Cushion *et al.,* 2007; Vohra *et al.,* 2004; Richard *et al.,* 2018); (iii) the fact that asci are present in the vast, if not all, majority of human infections, since the staining of their wall is used as diagnostic tool; and (iv) the supposed essentiality of asci to be necessary to the transmission of the pathogen to new hosts as aerially transported particles (Cushion *et al.,* 2010; Martinez *et al*., 2013). Thus, trophic cells of compatible mating type have to be present during the infection to allow sexual conjugation.

Two modes of sexual reproduction are observed among fungi: heterothallism involving two cells of opposite and compatible mating type, and homothallism involving a single mating type that is self-compatible. In heterothallic fungi, divergent genes of opposite mating type are located in separate cells. Each cell contains only one of the *MAT* locus, and expresses *MAT* genes that define one mating type (Figure 4A). To mate, two cells have to be of the opposite mating type (Ni *et al.,* 2011). On the other hand, homothallic reproduction is more complex and composed of two modalities. The primary homothallic fungi present both mating type loci in the same genome (Figure 4B). Secondary homothallic fungi, such as the yeast models *S. cerevisiae* and *S. pombe,*

present three *MAT* loci in their genome, only one being active at a time while the other two are silenced (Figure 4C and Figure 5). Regions of homology allow the exchange of the expressed cassette. This results in a cell that can switch from one mating type to the other.

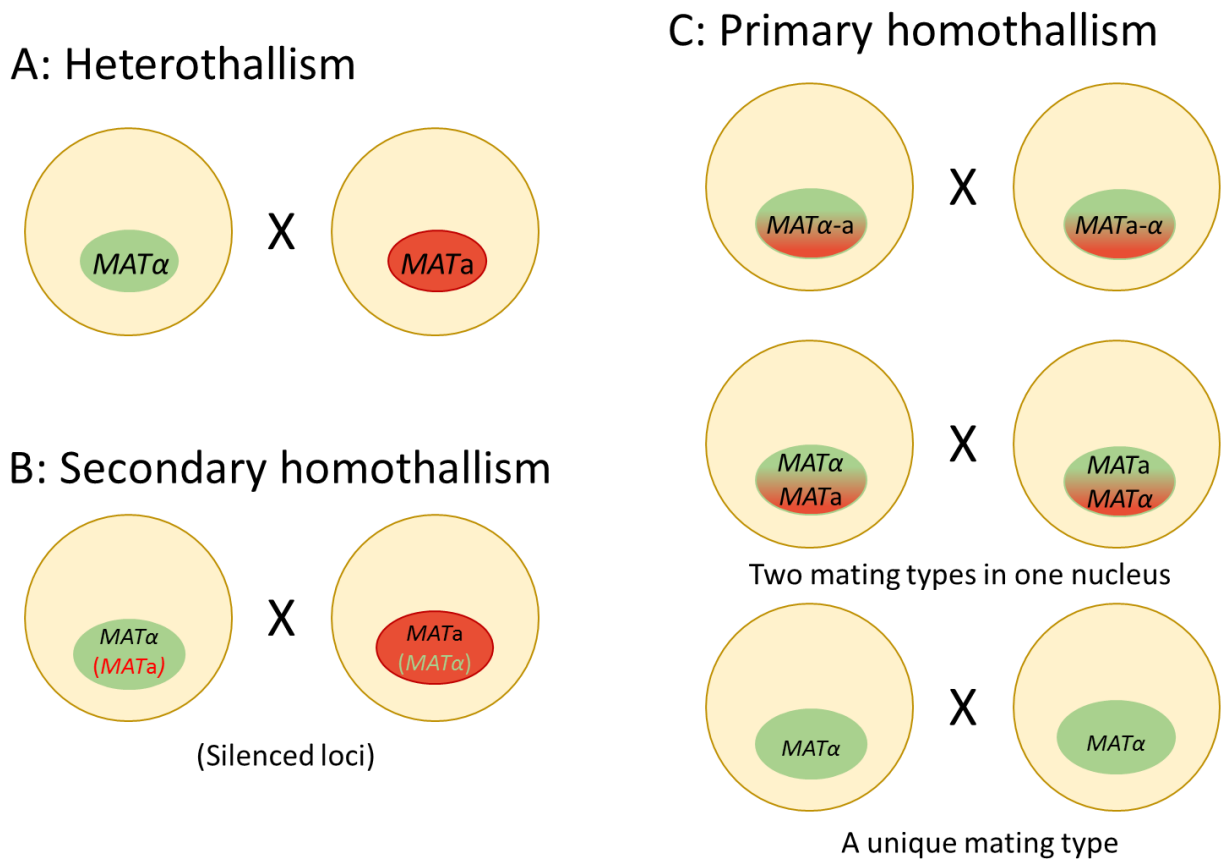

Figure 4. Representation of heterothallic (**A**), secondary homothallic (**B**), and primary homothallic (**C**). *MAT* a, mating type a; *MAT* α, mating type α. Each cross represent a mating event. Adapted from Ni *et al.*, 2011.

Our group recently investigated the mode of sexual reproduction of *Pneumocystis* species by comparative genomics. Sex-related genes of *S. pombe,* a closely related species among the Taphrinomycotina members, were used as query sequences to identify homologous genes in the genomes of *P. jirovecii* and *P. carinii.* Only three candidates of the four *MAT* genes types present in *S. pombe* were detected. These genes are implicated into the sexual differentiation into plus (P) or minus (M) mating type. The three candidate homologues that were found are *matMc,* a transcription factor with high-mobility-group domain, *matPi,* a transcription factor with homeobox domain, and *MatMi,* a mating type M-specific polypeptide. These three putative *MAT* genes were located on a single DNA molecule. Their proximity suggests a fusion of two MAT loci, one of the type M, composed by the genes *matMc* and *matMi*, and the other of type P, incomplete because composed by only *matPi,* but missing the *matPc* gene. Interestingly, the inspection failed to detect any putative *cis*-acting sequence motifs, homologous to the ones that flank the *S. pombe MAT* loci and implicated in the switching from one mating type to the other, neither motifs implicated in the silencing of *MAT* loci in *S. pombe*. Figure 5 shows a schematic representation of the MAT loci in the genome of *S. pombe, P. jirovecii* and *P. carinii.* The *P. murina MAT* genes are presumably distributed as the ones of the other *Pneumocystis* species (results not showed in Figure 5, Almeida *et al.,* 2015). All these considerations suggested that *Pneumocystis* species are primary homothallic organisms, i.e. each strain is self-fertile and able to produce asci on its own. This is markedly different from the reproduction process of *S. pombe* (Inderbitzin *et al.,* 2015). Recently, our group ascertained the function of the *P. jirovecii* and *P. carinii matMc* genes by restoration of sporulation in the corresponding mutant of *S. pombe* (Richard *et al.,* 2018). Consistently with primary homothallism, the three *MAT* genes were often expressed at the same time during human infections, and the same single *MAT* locus was found in all *P. jirovecii* isolates. Nevertheless, many aspects of the process of this homothallic sexual reproduction remains to be ascertained and further characterized. In particular, it should be determined if each cell is only M, only P, or both at the same time.

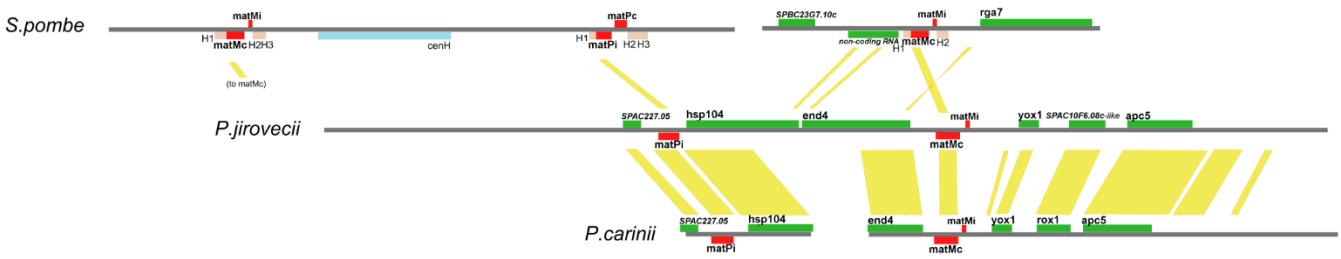

Figure 5. Schematic representation of the distribution of the *MAT* genes in *S. pombe, P. jirovecii* and *P. carinii*. From Almeida *et al.,* 2015.

Projects of the PhD thesis

My PhD thesis includes three parts:

Part 1: Identification and functional ascertainment of the *Pneumocystis jirovecii* potential drug targets Gsc1 and Kre6 involved in glucan synthase

Part 2: Site-directed mutagenesis of the 1,3-β glucan synthase catalytic subunit of *Pneumocystis jirovecii* and susceptibility assay suggest its sensitivity to caspofungin

Part 3: *Pneumocystis* primary homothallism involves trophic cells carrying both Plus and Minus pheromone receptors

Parts one and two are presented in this report as two original published articles. The third part is presented as a scientific article format that is in preparation. The three articles are summarized here below (chapter "summaries of the articles") and presented *in extenso* as annexes. I performed all the experiments reported in this thesis (except some PCRs in part 3 that were performed by our laboratory technician S. Richard) and accordingly I am first author of all three publications.

Summaries of the articles

Identification and functional ascertainment of the *Pneumocystis jirovecii* potential drug targets Gsc1 and Kre6 Involved in glucan synthesis

Abstract

Glucans are essential constituents of the cell wall in most fungi. Echinocandins are a class of antifungal drugs inhibiting the catalytic subunit Gsc1 of the enzymatic complex ensuring the synthesis of 1,3-β glucan. Besides, inhibitors of the enzyme Kre6 involved in the synthesis of 1,6-β glucan were recently described. We identified and functionally characterized these two potential drug targets in the human pathogen *P. jirovecii* by rescue of the null allele of the orthologous gene in *Saccharomyces cerevisiae*. The *P. jirovecii* proteins Gsc1 and Kre6 were identified using those of the relative *Pneumocystis carinii*. The expression of their encoding genes on plasmid rescued the increased sensitivity to respectively caspofungin or calcofluor white of the corresponding *S. cerevisiae* null allele. The uniqueness and likely essentiality of these proteins suggest that they are potential good drug targets.

Full text article is in Annexe 1.

Site-directed mutagenesis of the 1,3-β glucan synthase catalytic subunit of *Pneumocystis jirovecii* suggests its sensitivity to caspofungin

Abstract

The echinocandin caspofungin inhibits the catalytic subunit Gsc1 of the enzymatic complex synthetizing 1,3-β glucan, an essential compound of the fungal wall. Studies in rodents showed that caspofungin is effective against *Pneumocystis* asci. However, its efficacy against asci of *Pneumocystis jirovecii,* the species infecting exclusively humans, remains controversial. The aim of this study was to assess the sensitivity to caspofungin of the *P. jirovecii* Gsc1 subunit*,* as well as of those of *Pneumocystis carinii* and *Pneumocystis murina* infecting respectively rats and mice. In absence of an established *in vitro* culture method for *Pneumocystis* species*,* we used functional complementation of the *Saccharomyces cerevisiae gsc1* deletant. In the fungal pathogen *Candida albicans,* mutations leading to amino acid substitutions in Gsc1 confer resistance to caspofungin. We introduced the corresponding mutations into the *Pneumocystis gsc1* genes using site-directed mutagenesis. In spot dilution tests, the sensitivity to caspofungin of the complemented strains decreased with the number of mutations introduced, suggesting that the wild-type enzymes are sensitive. The minimum inhibitory concentrations of caspofungin determined by E-test® and Yeastone® for strains complemented with *Pneumocystis* enzymes were identical to those upon complementation with the enzyme of *C. albicans* for which caspofungin presents low MICs. However, they resulted lower than the MICs upon complementation with the enzyme of the resistant species *Candida parapsilosis*. Sensitivity levels of Gsc1 enzymes of the three *Pneumocystis* species were similar, suggesting that *P. jirovecii* is sensitive to caspofungin during infections, as *P. carinii* and *P. murina*.

Full text article is in Annexe 2.

Pneumocystis primary homothallism involves trophic cells carrying both Plus and Minus pheromone receptors

Abstract

Both asexual and sexual cycle are believed to be part of the life cycle of fungi of the genus *Pneumocystis.* Trophic cells, the predominant cellular forms inside the lungs during the infection, can multiply by binary fission or start a sexual cycle, which involves the mating of two trophic cells. Recent comparative genomic analysis revealed the existence of a single mating type locus that includes both plus (P) and minus (M) genes, suggesting that *Pneumocystis* species are primary homothallic organisms. This would imply that each strain is self-fertile. The genes encoding the receptors for P and M pheromones have been identified in the *Pneumocystis* genome. However, the mating type of single cells remains unknown: M, P, or M and P at the same time. The aim of the present work was to analyze the expression of the M-factor receptor (*mam2*) and the P-factor receptor (*map3*) genes, and to investigate if both are present at the same time at the cellular surface of single *Pneumocystis* cells. Ten broncho-alveolar lavage (BAL) fluid samples from patients with *Pneumocystis jirovecii* pneumonia were analyzed by reverse transcriptase-PCR for expression of the *mam2* and *map3* genes. The majority of them were positive for both pheromone receptors. The same was observed in a sample of lungs of mice infected with *Pneumocystis murina.* These results suggest that both pheromone receptors are most often concomitantly expressed during *Pneumocystis* infection. Both *P. jirovecii* Mam2 and Map3 pheromone receptors were observed at the cellular surface of the vast majority of presumed trophic cells observed using specific immunofluorescent stainings. These observations strongly suggest that each cell is of both M and P mating types at the same time, and consequently that any cell can mate with any cell present in the population.

Full text article is in Annexe 3.

Conclusions

The first part of this work focused on the identification of new drug targets to treat *Pneumocystis jirovecii* infections. We identified and isolated the genes encoding two new potential targets involved in cell wall metabolism and integrity, Gsc1 and Kre6. The *gsc1* gene is apparently unique in the genome of *P. jirovecii*, thus is likely to be essential, so that the protein it encodes could be a good drug target. This enzyme is the target of the antifungal echinocandins. Even if this gene is apparently mostly active in the ascus form, it represents a good drug target because asci are an obligate stage of the life cycle of *Pneumocystis* organisms. Similarly, *kre6* represents a good drug target because of its uniqueness in the *Pneumocystis* genome and apparently essentiality for the life of the pathogen, even if it is expressed exclusively in asci.

In the second part of this work, we used genetic engineering to demonstrate that the Gsc1 enzyme of *P. jirovecii* is sensitive to caspofungin in a way similar to those of the rodent pathogens. Moreover, the level of sensitivity of the *Pneumocystis* Gsc1 enzymes were similar to that of the human pathogen *Candida albicans,* which is currently treated with caspofungin in patients. This suggests that the sensitivity level of *Pneumocystis* enzymes is interesting from a clinical point of view. Echinocandins are not intended for *Pneumocystis* pneumonia treatment for the moment, even if these antifungals are sometimes used as alternative or additional therapy. Our results suggest the need of implementation of clinical trials in humans, as we bring new arguments in favor of the use of these antifungals as potential treatment of PCP. Echinocandins could be useful to limit the spread of the infection in hospitals, as they inhibit the formation of asci, the infectious particle responsible of the propagation of the infection. However, the use of these antifungals as prophylaxis is complicated by their parenteral administration and high cost. Furthermore, our results support the high relevance of the use of animal models to understand the effect of drugs on the human pathogen *P. jirovecii.*

In the third part, we studied the sexual reproduction of *Pneumocystis* organisms. We showed that the two pheromone receptors involved in mating are often concomitantly expressed during infection, and that they are both present on the surface of the majority of supposed single trophic cells. This suggest that each cell is of both mating types at the same time, and consequently that any cell can mate with any cell present in the population. Moreover, this might allow mating between different *P. jirovecii* strains inside the lungs, as most human infections are polyclonal, further increasing the genetic diversity compared to the self-fertilization of a single strain. New knowledge about the *Pneumocystis* sexual reproduction can also be helpful for identification of new pathways and enzymes that could be used as drug target.

Taken all together, our efforts to better understand this pathogen should be useful to fight *Pneumocystis* infection in the future.

Perspectives

S. cerevisiae strains expressing the *Pneumocystis* glucan synthase proved useful to test the sensitivity to caspofungin, and could be used to test sensitivity to others echinocandins, i.e. micafungin, anidulafungin and the recently discovered rezafungin. However, *S. cerevisiae* contains a second glucan synthase, that replaces the function of the deleted one and that is less sensitive to other echinocandins, preventing the observation of *Pneumocystis* gene sensitivity. The second synthase gene could be deleted by replacing its natural promoter with a cassette containing the repressible tetO promoter. This will allow the inhibition of the expression of this second synthase and consequently to test the sensitivity of *Pneumocystis* Gsc1 to other echinocandins.

S. cerevisiae expressing *Pneumocystis* pheromone receptors could result useful for a receptor signaling assay to detect *Pneumocystis* pheromones. The assay would consist in measuring the activity of the β-galactosidase reporter gene fused to the genes activated directly by the mating cascade. This cascade occurs only if all the components of the pheromone-induced pathway are present and functional, and only if exposed to mating factors. BALs from infected patient with a high fungal load could be used as source of *Pneumocystis* P and M mating factors.

Comparative genomic analysis recently performed by our group (Almeida *et al.,* 2015) revealed that several sex related genes present in the closely related species *S. pombe* are present in *Pneumocystis* genomes. Some of them, as genes involved in cell fusion, signal transduction, meiosis, deserve further investigation. Their expression during PCP could be verified in a similar way we did here for the pheromone receptors.

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Annexe 1

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ORIGINAL ARTICLE

Identification and Functional Ascertainment of the *Pneumocystis jirovecii* Potential Drug Targets Gsc1 and Kre6 Involved in Glucan Synthesis

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ABSTRACT

The most efficient drug against the human pathogenic fungus *Pneumocystis jirovecii* is cotrimoxazole targeting the folate biosynthesis. However, resistance toward it is emerging and adverse effects occur in some patients. Studies in rodent models suggested that echinocandins could be useful to treat *Pneumocystis* pneumonia. Echinocandins inhibit the catalytic subunit Gsc1 of the enzymatic complex ensuring the synthesis of 1,3-β glucan, an essential constituent of cell walls of most fungi. Besides, inhibitors of the enzyme Kre6 involved in the synthesis of 1,6-β glucan, another essential component of fungal walls, were recently described. We identified and functionally characterized these two potential drug targets in the human pathogen *P. jirovecii* by rescue of the null allele of the orthologous gene in *Saccharomyces cerevisiae.* The *P. jirovecii* proteins Gsc1 and Kre6 identified using those of the relative *Pneumocystis carinii* as the query sequence showed high sequence identity to the putative fungal orthologs (53–97% in conserved functional domains). The expression of their encoding genes on plasmid rescued the increased sensitivity to, respectively, caspofungin or calcofluor white of the corresponding *S. cerevisiae* null allele. The uniqueness and likely essentiality of these proteins suggest that they are potential good drug targets.

PNEUMOCYSTIS organisms are fungal parasites that colonize the lungs of mammals (Skalski et al. 2015; Thomas and Limper 2004). Each *Pneumocystis* species displays strict host specificity for a single mammalian species. The species infecting humans is *Pneumocystis jirovecii*. In immuno-compromised patients, *P. jirovecii* can turn into an opportunistic pathogen causing *P. jirovecii* pneumonia (PCP), a severe disease which can be fatal. Standard antifungals targeting the fungal cell membrane integrity or synthesis are not effective against *P. jirovecii*, possibly because of the presence of cholesterol instead of ergosterol in its membrane (Kaneshiro et al. 1994; Russian and Kovacs 1998). The most efficient drug currently used is cotrimoxazole, a combination of trimethoprim and sulfamethoxazole. These two molecules are inhibitors of enzymes involved in the biosynthesis of folic acid, a metabolite required for the biosynthesis of essential cellular compounds. Cotrimoxazole is active against both cellular forms present during *Pneumocystis* infection: the trophic form which is the predominant one and apparently devoid of wall, and the ascus form which would be issued from a sexual cycle and has a thick wall (Aliouat-Denis et al. 2009; Itatani and Marshall 1988; Thomas and Limper 2007). The use of cotrimoxazole for prophylaxis and treatment of PCP since the late 1980s (Kovacs et al. 2001) correlated with the detection of specific mutations within the active site of the dihydropteroate synthase, the target of sulfamethoxazole (Lane et al. 1997; Ma et al. 1999; Nahimana et al. 2003). Because of the emerging resistance to cotrimoxazole and because this drug can cause side effects such as intolerance and toxicity, it is of the utmost importance to find new treatments and to identify new drug targets to fight *P. jirovecii*.

One promising class of antifungals is echinocandins. Caspofungin (CAS), anidulafungin, and micafungin are cyclic hexapeptides with fatty acyl side chains which are

noncompetitive inhibitors of the catalytic subunit of the 1,3-β glucan synthase enzymatic complex (Schmatz et al. 1990). β-glucan molecules are homopolymers of β-1,3 linked D-glucose with β-1,6 linked D-glucose side chains in minority. β-glucans are important components of the fungal cell wall, together with mannoproteins and chitins (Bowman and Free 2006). All these components are usually involved in the host immune recognition in typical fungal pathogens (Amarsaikhan and Templeton 2015; Cambi et al. 2008). In contrast, *Pneumocystis* organisms would be unable to synthesize chitin, and harbor only β-glucans and mannoproteins, which, however, lack hyper-mannose glycosylation (Ma et al. 2016). The 1,3-β glucan synthase enzymatic complex is an ideal drug target to fight pathogenic fungi because it (i) is essential for these microorganisms, (ii) has no human counterparts, and (iii) is easily reachable by drugs because of their localization at the fungal surface, anchored in the cellular membrane. Four studies reported initially the efficacy of echinocandins and related compounds against *Pneumocystis carinii,* the species infecting rats (Powles et al. 1998; Schmatz et al. 1990, 1995; Sun and Zhaohui 2014). Consistent with their lack of or poor glucan content, trophic forms appeared to be much less sensitive to CAS than asci (Cushion et al. 2010). Combining caspofungin with cotrimoxazole provided an addictive effect, improving the clearance of infection by *Pneumocystis murina,* the species infecting mice (Lobo et al. 2013). However, the response to echinocandins of the human pathogen *P. jirovecii* could be different than those of the models *P. carinii* and *P. murina*. Indeed, the genomic coding sequences of the two latter species present a mean divergence of ca. 20% at nucleotide level from those of *P. jirovecii* (Stringer 1996). The available clinical reports concerning the issue are contradictory so that the efficacy of caspofungin against *P. jirovecii* remains unclear. Indeed, the combination of caspofungin with cotrimoxazole was reported to have cleared several PCP episodes, two in HIV-positive patients (Ceballos et al. 2011; Lee et al. 2016), seven in transplant recipients (Tu et al. 2013; Utili et al. 2007), and one in a pediatric case (Beltz et al. 2006). Caspofungin alone was reported to have been successful in a patient undergoing bone marrow transplantation (Annaloro et al. 2006), in a Wegener's granulomatosis patient (Hof and Schnülle 2008), as well as in eight out of ten HIV-infected patients (Armstrong-James et al. 2011). However, failure of treatment using caspofungin alone was described in four HIV-negative (Kim et al. 2013) and two cancer patients (Kamboj et al. 2006).

In *Saccharomyces cerevisiae,* the *GSC1*, *GSC2*, and *GSC3* genes (also called *FKS1* to 3) encode different catalytic subunits of the 1,3-β glucan synthase enzymatic complex. The *GSC1* gene is active during the vegetative growth, whereas *GSC2* and *GSC3* are expressed during sporulation (Bowman and Free 2006; Mazur et al. 1995). The presence of a unique *gsc1* gene encoding the 1,3-β glucan synthase catalytic subunit in the genome of *P. carinii* and the inhibition of the Gsc1 protein by the pneumocandin L-733,560, a compound structurally very close to echinocandins, were demonstrated (Kottom and Limper 2000). These observations also suggest that echinocandins might become useful for prevention and treatment of PCP in the future.

1,6-β glucans are less abundant than 1,3-β glucans in fungal cell walls, and act as a flexible glue between the other components of the wall (Kollár et al. 1997). At least 10 genes are involved in the biosynthesis of 1,6-glucan in *S. cerevisiae* (Shahinian and Bussey 2000). These genes have also no homologs in human genome. Inhibitors of the 1,6-β glucan synthesis were recently discovered (Kitamura 2010; Kitamura et al. 2009). These inhibitors target the membrane protein *kre6* and show an important in vitro activity against *Candida* species and *S. cerevisiae* (Kitamura 2010). *Saccharomyces cerevisiae* harbors the Kre6 enzyme as well as a homolog called Skn1 sharing 66% sequence identity with Kre6. Unlike the *KRE6* gene, the deletion of the *SKN1* gene does not show any effect on growth or 1,6-β glucan levels within the wall, suggesting that the Skn1 enzyme is less important than the Kre6 enzyme, at least in the laboratory conditions (Roemer et al. 1993). The presence of a unique *kre6* gene and the sensitivity of the Kre6 enzyme to the 1,6-β glucan inhibitors were recently demonstrated in *P. carinii* (Kottom et al. 2015). These observations suggest that 1,6-β glucan inhibitors might become useful new drugs against *P. jirovecii*.

The identification and characterization of the *P. jirovecii gsc1* and *kre6* genes has not been carried out yet. Despite that the in vitro culture method for this pathogen published by Schildgen et al. (2014) is not widely established yet, this task is now possible because of the recent release of the genome sequence of *P. jirovecii* by two groups (Cissé et al. 2012; Ma et al. 2016). In this study, we report the identification of these two genes within the *P. jirovecii* genome sequence, as well as the assessment of their function by the complementation of the respective deleted orthologous gene of *S. cerevisiae*.

MATERIALS AND METHODS

Strains and growth conditions

Y05251 is an *S. cerevisiae* haploid strain with a deletion of the 1,3-β glucan synthase catalytic subunit gene (*GSC1*, also called *FKS1)* that encodes the Gsc1 protein (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLR342w::kanMX4*). This strain is herein named the *gsc1* deletant. It was obtained from Euroscarf (European *S. cerevisiae* Archive for Functional Analysis [http://www.euroscarf.de/]). Strains of *S. cerevisiae* with deletion of *GSC1* exhibit a slow growth rate and impaired growth in the presence of low doses of caspofungin (CAS) (Markovich et al. 2004). The parental strain of the *gsc1* deletant, strain BY4741 from Euroscarf (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), was used as the control in the complementation tests. This strain is herein named the wild type strain.

Y05574 is an *S. cerevisiae* haploid strain with a deletion of the *KRE6* gene that encodes the Kre6 protein which is

one of the genes involved in the 1,6-β glucan biosynthesis (*Mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPR159w::kanMX4*). This strain is herein named the *kre6* deletant. It was also obtained from Euroscarf. Strains of *S. cerevisiae* with deletion of *KRE6* exhibit defects in cell wall integrity, which induces an impaired growth in the presence of Calcofluor White M2R (CFW) (Roemer and Bussey 1991). Strain BY4741 is also the parental strain of the *kre6* deletant and was used as the control in the complementation tests.

Strains were grown on complete yeast extract-peptone-dextrose (YEPD) medium (1% [wt/vol] Difco yeast extract, 2% Difco peptone, 2% glucose).

Source of *Pneumocystis jirovecii* gene sequence

The *P. carinii* Gsc1 protein (primary accession number UniProt ID Q9HEZ4) was used as the query sequence in BLASTp searches against the two available *P. jirovecii* proteomes at http://blast.ncbi.nlm.nih.gov/Blast.cgi. A single putative ortholog was detected in each proteome, that is, no homolog with a lower identity was present. The two *P. jirovecii* gene sequences encoding the Gsc1 protein identified were then retrieved from the European Nucleotide Archive (http://www.ebi.ac.uk/ena) (Brooksbank et al. 2014). The *gsc1* gene corresponds to the PNEJI1_001061 locus in the *P. jirovecii* genome assembly version ASM33397v2 (Cissé et al. 2012), and to the T551_02309 locus in the *P. jirovecii* genome assembly version Pneu_jiro_RU7_V2 (Ma et al. 2016).

The *P. jirovecii* gene sequences encoding the Kre6 protein were similarly retrieved using the *P. carinii* Kre6 protein (UniProt ID Q6UEI2) as the query sequence. A single putative ortholog was detected in each proteome. The *kre6* gene corresponds to the PNEJI1_003487 locus in the ASM33397v2 assembly, and to the T551_02808 locus in Pneu_jiro_RU7_V2 assembly.

Multiple sequence alignments of *P. jirovecii*, *P. carinii*, *S. cerevisiae*, and *Schizosaccharomyces pombe* proteins were generated using T-Coffee (Notredame et al. 2000). Conserved domains were found using the NCBI's search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Sequences identity % of whole proteins and conserved domains has been calculated with Align Sequence Protein BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Cloning of the *Pneumocystis jirovecii gsc1* and *kre6* genes

Since the *P. jirovecii gsc1* and *kre6* genes include several introns (three and seven, respectively), the corresponding cDNAs were synthesized by GeneCust Europe (Ellange, Luxembourg). The sequences of the alleles present in the ASM33397v2 assembly were used (primary accession number UniProt ID L0PD34 and L0P8X6, respectively). Their sizes without introns are, respectively 5,760 and 2,040 bps. Both cDNAs were synthesized with flanking XbaI and SalI restriction sites for subsequent cloning. Both genes were digested with XbaI and SalI restriction enzymes and cloned using T4 ligase (New England Biolabs, Basel, Switzerland) into the p416GPD yeast expression vector (Mumberg et al. 1995) previously digested with the same restriction enzymes. The recombinant plasmids were introduced into *Escherichia coli* DH5α-competent cells and minipreparations of plasmid DNA was performed according to Birnboim and Doly (Birnboim and Doly 1979).

Cloning of the *Saccharomyces cerevisiae GSC1* and *KRE6* genes

To perform complementation controls, the *S. cerevisiae GSC1* and *KRE6* genes were amplified by PCR from DNA from the wild type strain BY4741 extracted as described previously (Lo Presti et al. 2007). The genes are respectively 5,631 and 2,163 pbs. PCR primers and conditions are given in Tables S1 and S2, respectively. Primers were synthesized by Microsynth (Balgach, Switzerland). For oriented cloning, the primers were designed to create unique restriction sites at ends of the PCR products. The latter were extracted using the QIAquick gel extraction kit (Qiagen, Basel, Switzerland), digested by BamHI and PstI restriction enzymes and cloned into the p415GPD expression vector (*GSC1*), or digested by BamHI and SalI and cloned into p416GPD (*KRE6*).

Transformation of *Saccharomyces cerevisiae* deletants

The recombinant plasmids p416GPD.*Pjgsc1*, p416GPD.*Pjkre6*, p415GPD.*ScGSC1*, and p416GPD.*ScKRE6* were introduced into their corresponding *S. cerevisiae* deletant by transformation for uracil (p416GPD) or leucine (p415GPD) prototrophy using the one-step method (Chen et al. 1992). To be used as controls in the complementation tests involving selection of plasmids, the deletants and the wild type strain BY4741 were transformed with the empty p416GPD or p415GPD. Transformants were selected on solid yeast nitrogen base (YNB) medium (0.67% [wt/vol] yeast nitrogen base, 2% glucose, 2% Gibco agar) supplemented with complete supplement mixture (CSM) lacking uracil or leucine (MP Biomedicals Luzern, Switzerland). Transformants clones were randomly chosen and purified by growth on the same selective medium.

Complementation tests

Functional complementation of the *S. cerevisiae gsc1* deletant was assessed by growth on YNB selective medium lacking uracil or leucine and supplemented with or without 75 ng/ml CAS (Fluka, Chemie AG, Buchs Switzerland). Transformant clones were grown overnight in the selective medium to avoid the loss of plasmid. Cells were then diluted at an absorbance at 540 nm of 0.5 in NaCl 0.9% (wt/vol)(ca. 3.5×10^6 cells), four serial 10-fold dilutions in NaCl 0.9% were prepared, and 3 µl of each dilution were spotted onto the medium. Spots were observed and photographed after 5 d at 30 °C.

Functional complementation of the *S. cerevisiae kre6* deletant on plasmid was assessed using exactly the same

Table 1. Sequence identity (%) of the *Pneumocystis jirovecii* Gsc1 and Kre6 proteins to the putative orthologs of *Pneumocystis carinii*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*

	Gsc1			Kre6	
	Whole protein	1,3-β glucan synthase domain 1	1,3-β glucan synthase domain 2	Whole protein	GH16 fungal kre6 glucanase domain
P. carinii	90	94	97	70	83
S. cerevisiae	61	70	71	48	53
S. pombe	60	65	73	51	59

procedure as for *gsc1*, except that the selective medium was supplemented with 35 μg/ml CFW (Sigma Life Science, Buchs, Switzerland). CFW inhibits the growth of the deletant by binding to the residues of chitin remaining exposed due to the improper 1,6-β glucan cell wall deposition (Roemer and Bussey 1991). Since CFW is photosensitive, Petri dishes were wrapped in aluminum paper just after their preparation and kept away from light as much as possible.

PCR primers and conditions used to assess the presence or absence of complementing genes are given in Tables S1 and S2, respectively. Positive controls consisted in the amplification of the genomic genes from DNA extracted using QIAamp DNA blood kit (Qiagen, Basel, Switzerland) from a BAL fluid of a patient with PCP.

RESULTS

Identification and cloning of the *Pneumocystis jirovecii gsc1* and *kre6* genes

A single Gsc1 protein was identified within each of the two *P. jirovecii* proteomes available by a homology search using the Gsc1 protein of *P. carinii* as the query sequence. The two *gsc1* gene sequences were identical, except (i) three synonymous single-nucleotide polymorphisms (SNP), and (ii) a second exon longer in the Ma et al. assembly than in the Cissé et al. assembly (Fig. S1A). The supplementary sequence of the exon presented five bp deleted relatively to Cissé et al. which eliminate a STOP codon in the frame. This resulted in a segment of 27 amino acids, which is absent in the *P. jirovecii* Gsc1 protein predicted by Cissé et al., but present and exactly identical in that of *P. carinii* (Fig. S2A). This segment is not part of the conserved glucan synthase domains. The allele of the Cissé et al. assembly was investigated in this study (only this one was described at the beginning of this study). The translation product of the ORF bears the highest degree of identity with the Gsc1 protein of *P. carinii* (90%), and a weaker degree of identity with that of *S. cerevisiae* (61%) and with the *S. pombe* Bgs4 protein which is orthologous to Gsc1 (60%). The identity was higher in the two conserved functional domains, supporting that the four proteins were orthologs (Table 1). Since *S. cerevisiae* does not process *Pneumocystis* introns, a synthetic *P. jirovecii gsc1* gene without introns was cloned into the expression vector p416GPD.

A single *P. jirovecii* Kre6 protein was identified in each proteome using the Kre6 protein of *P. carinii* as the query sequence. The two *kre6* gene sequences were identical, except (i) four synonymous SNPs, and (ii) an ORF shorter in Ma et al. than in Cissé et al. which ended within the last intron predicted in the latter assembly (Fig. S1B). This resulted in a predicted protein lacking the last segment of 23 amino acids present in that of Cissé et al. (Fig. S2B). The allele of the Cissé et al. assembly was investigated in this study. The gene encompasses seven introns. The translation product of the ORF bears the highest degree of identity with the Kre6 protein of *P. carinii* (70%), while it shares a weaker degree of identity with those of *S. cerevisiae* (48%) and with the *S. pombe* Yepb protein which is orthologous to Kre6 (51%) (a second homolog Yeob presenting a high identity with *S. cerevisiae* Skn1 is also present in *S. pombe*). The identity was higher in the functional domain (Table 1; Fig. S2B), supporting that the four proteins were orthologs. A synthetic *P. jirovecii kre6* gene without introns was cloned into p416GPD.

Functional complementation of the *Saccharomyces cerevisiae gsc1* deletant with the *Pneumocystis jirovecii gsc1* gene

The recombinant plasmids p416GPD.*Pjgsc1* and p415GPD.*ScGSC1* were introduced into the *S. cerevisiae gsc1* deletant, whereas the empty p416GPD and p415GPD vectors were introduced into both the deletant and the wild type strain (*S. cerevisiae GSC1* could not be cloned into p416GPD because of restriction sites issues). The deletant shows a paradoxical phenotype: an increased susceptibility to CAS in absence of the target of this antifungal. This results from the involvement of this gene in the cell wall integrity pathway (Markovich et al. 2004; Reinoso-Martín et al. 2003). On medium containing CAS, a complete restoration occurred in the presence of p415GPD.*ScGSC1*, but not in the presence of the empty vector (Fig. 1A). On the other hand, a partial restoration of the wild type growth was observed with p416GPD.*Pjgsc1*, that is, the number of colonies growing at each concentration was reduced. Because of the instability of the plasmids requiring constant selection of their auxotrophy marker as well as of the partial complementation obtained upon expression of the *P. jirovecii gsc1* gene, it was crucial to check by PCR the presence or absence of the complementing gene in the different strains (Fig. 2A). The *P. jirovecii gsc1* gene or the *S. cerevisiae GSC1* gene was present in the functionally complemented strains but not in the deletant harboring the empty vector. Amplifying the polylinker without insert further identified the empty vector. As a positive control, *P. jirovecii gsc1* gene with introns was amplified from a BAL fluid of a patient with PCP. These observations demonstrated that expression of the *P. jirovecii* gene rescued the function of the deleted *S. cerevisiae GSC1* gene encoding the Gsc1 protein.

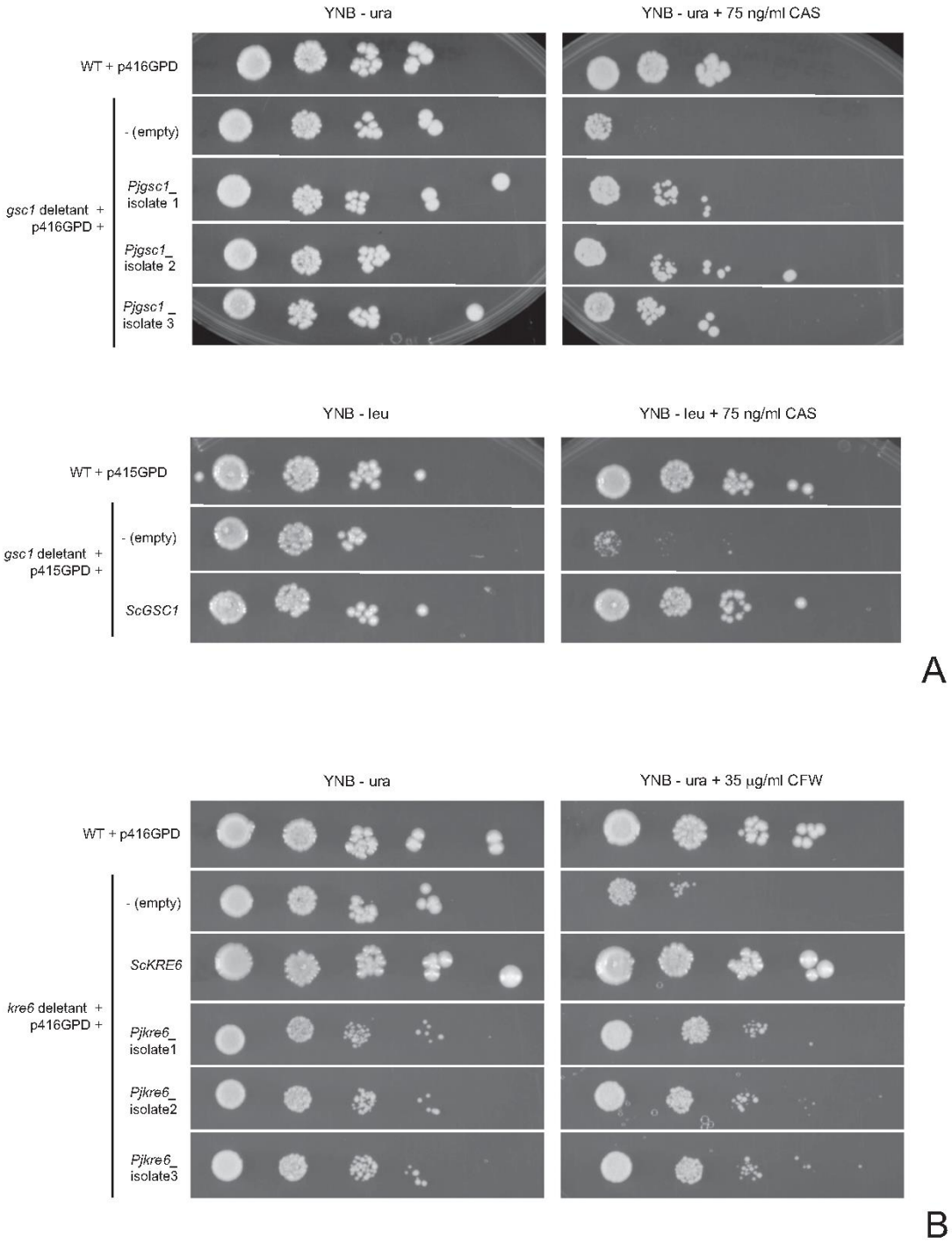
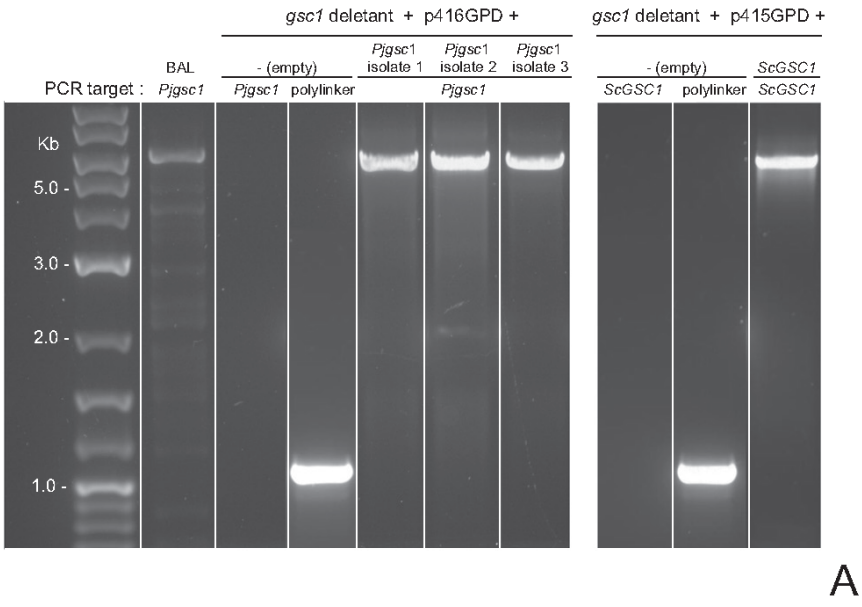


Figure 1 Complementation of the *Saccharomyces cerevisiae gsc1* (**A**) and *kre6* (**B**) deletants by expression of the *Pneumocystis jirovecii gsc1* or *kre6* gene on plasmid. Control complementation by expression of the *S. cerevisiae GSC1* or *KRE6* gene is also shown. Log dilutions of a suspension of cells at 3.5×10^6/ml were spotted on minimal selective medium without (left) or with (right) CAS or CFW, and incubated for 5 d at 30 °C. The most concentrated suspension is on the left. YNB + CSM ura was used for p416GPD selection, YNB + CSM leu for p415GPD selection.



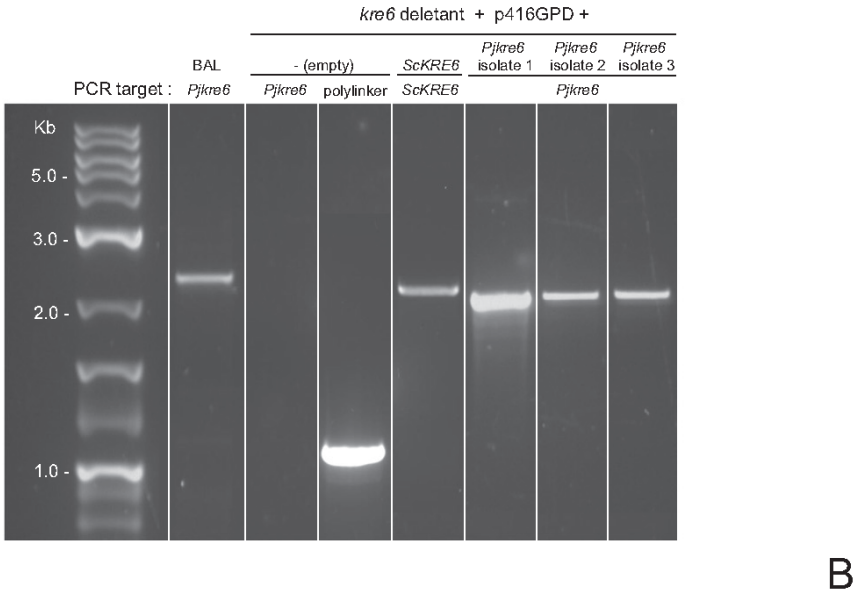


Figure 2 PCR assessment of the presence or absence of the *Pneumocystis jirovecii gsc1* and *kre6* and *Saccharomyces cerevisiae GSC1* and *KRE6* genes in the constructed strains. (**A**) The presence of the *P. jirovecii gsc1* gene (PCR product with introns of 6,017 bp) was confirmed in the DNA from the BAL fluid of a patient with PCP and in the three transformant clones of *S. cerevisiae gsc1* deletant bearing p416GPD.*Pjgsc1* (PCR product without introns of 5,782 pb), whereas the gene was absent in the *gsc1* deletant bearing the empty vector. The presence of the *S. cerevisiae GSC1* gene was confirmed in the DNA of the clone bearing p415GPD.*ScGSC1*, whereas the gene was absent in the clonebearing the empty vector. To confirm the presence of the empty vector in the clones bearing p416GPD or p415GPD, the polylinker without insert (1,106 pb) was amplified. (**B**) The presence of the *P. jirovecii kre6* gene (PCR product with introns of 2,341 bp) was confirmed in the DNA from the BAL fluid of a patient with PCP and in the three transformant clones of *S. cerevisiae kre6* deletant bearing p416GPD.*Pjkre6* (PCR product without introns of 2,040 pb), whereas the gene was absent in the kre6 deletant bearing the empty vector. The presence of the *S. cerevisiae KRE6* gene was confirmed in the DNA of the clone bearing p416GPD.*ScKRE6*. The presence of the empty vector was confirmed by amplification of the polylinker without insert. All negative controls did not generate products.

Functional complementation of the *Saccharomyces cerevisiae kre6* deletant with the *Pneumocystis jirovecii kre6* gene

The assay and PCR controls for complementation of the *kre6* deletant were performed exactly as described above for the *gsc1* deletant, except that the recombinant plasmids p416GPD.*Pjkre6* and p416GPD.*ScGSC1* and CFW in the medium were used. A partial and a complete restoration of the wild type growth occurred in the presence of respectively p416GPD.*Pjkre6* and p416GPD.*ScKRE6*, but not in the presence of the empty vector (Fig. 1B). The reduced size of the colonies revealed that the growth rate of the deletant carrying the recombinant plasmid p416GPD.*Pjkre6* was lower than that of the wild type strain, in presence or not of CFW. PCRs showed that the *P. jirovecii kre6* gene or the *S. cerevisiae KRE6* gene was present in the functionally complemented strains but not in the deletant harboring the empty vector (Fig. 2B). These observations demonstrated that expression of the *P. jirovecii* gene rescued the function of the deleted *KRE6* gene encoding the Kre6 protein.

DISCUSSION

In this study, we identified the *P. jirovecii gsc1* gene and proved its function by rescue of the null allele of the orthologous gene in *S. cerevisiae*. We also identified another potential drug target among the enzymes involved in cell wall biosynthesis, the Kre6 protein implicated in the 1,6-β glucan biosynthesis, and also characterized its function by complementation. Both these heterologous complementations were partial, whereas those with the homologous *S. cerevisiae* gene were complete. This could be explained by the fact that we used a vector with a strong promoter for the complementation, and that the high expression of the gene was toxic (Romanos et al. 1992). However, we repeated the experiments using a weaker promoter and the results were similar (data not shown). Such partial complementation is not surprising since it often occurred in our previous complementation studies. Indeed, the *S. cerevisiae dhfs* and *brl1* deletants complemented with the *P. jirovecii* and *P. carinii* genes presented a reduced growth rate (Lo Presti et al. 2007; Luraschi et al. 2015). Partial complementation may result from a poor fitness of the enzyme in the heterologous cellular environment. For example, the reduced growth rate of the complemented *kre6* deletant could be due to a persistent stress in the cell wall, leading to a perturbation of the cell wall integrity. Impaired synthesis due to a different codon usage could also play a role. We reported partial complementation also in homologous complementation of *brl1* (Lo Presti et al. 2009). This suggests the possible importance of a different gene dosage due to the plasmidic rather than chromosomic location, or of the use of a non natural promoter which provides a constitutive rather than temporally regulated expression, and/or an inadequate level of expression.

The Gsc1 subunit catalyzes the polymerization of uridine diphosphate-glucose into the 1,3-β glucan core, an insoluble molecule required for the cell wall assembly (Shematek et al. 1980). The *GSC1* and *GSC2* genes are a redundant essential pair, that is, the double deletion mutant is not viable, while the single mutants is viable (Inoue et al. 1995; Mazur et al. 1995). Thus, the growth of the *gsc1* deletant in absence of CAS in our experiments was probably supported by the expression of *GSC2*. In *P. carinii,* only one *gsc1* gene was found using Southern blot analyses (Kottom and Limper 2000). The *gsc1* gene is also unique in *P. jirovecii* according to our BLAST searches in the two proteomes presently available. Consequently, this gene is likely to be essential in *P. jirovecii* and the protein it encodes a good potential drug target. This is true even if this gene is active mostly in the asci, because it is thought that production of asci constitutes an obligate stage of the life cycle (Cushion et al. 2010; Hauser 2014), and that these forms might be the particles responsible for the transmission of the fungus between hosts via the airborne route (Cushion et al. 2010; Martinez et al. 2013).

The Kre6 enzyme is anchored to plasma, Golgi, and reticulum endoplasmic membranes. It is involved in the synthesis of 1,6-β glucan which is also an essential component of the fungal cell wall. The function of this enzyme remains unclear. It is thought to be a transglucosidase contributing to the construction of a protein-bound glucan-structure which acts as an acceptor site for the 1,6- β- glucan addition at cell surface (Montijn et al. 1999). In *S. cerevisiae,* this Golgi membrane glycoprotein has a homolog, Skn1, which is structurally related and has a similar function but which plays a less important role. The double deletant of *KRE6* and *SKN1* genes shows a noticeable alteration of the cell wall structure not observed in the single mutants, suggesting the essentiality of the function (Roemer et al. 1994). Only one *kre6* gene was found in *P. carinii* using Southern blot analyses (Kottom et al. 2015) and our BLAST searches suggest that this gene is also unique in *P. jirovecii.* Thus, this gene is possibly essential and the protein it encodes a good candidate drug target against *P. jirovecii,* even if it is expressed mostly or exclusively in asci. The pyridobenzimidazole derivatives inhibiting Kre6 enzymes recently described (Kitamura et al. 2009), may become a useful new treatment against *P. jirovecii* as well as against other pathogenic fungi, possibly in combination with cotrimoxazole.

The *gsc1* and *kre6* genes and their encoded proteins predicted in the two available *P. jirovecii* genomes were not identical. In both cases, the few synonymous SNPs observed can be attributed to natural polymorphisms between these isolates originating from Switzerland and USA. As far as the Gsc1 proteins are concerned, the 27 amino acids segment missing within one of them may also be a natural polymorphism because (i) the shortened enzyme is functional as demonstrated in the present work, (ii) the segment is not part of the conserved glucan synthase domains, and (iii) the absence is associated to five bp inserted in a region spanning only 24 bp, a configuration which is unlikely for sequencing errors. As far as

the Kre6 proteins are concerned, the 23 amino acids shorter end of one of them may result from a prediction error because (i) the sequence of the gene is identical in both assemblies, and (ii) the missing segment is almost fully conserved in the *P. carinii* enzyme. These observations suggest that further assessment of the two available *P. jirovecii* genomes and proteomes is warranted.

The sensitivities of Gsc1 and Kre6 enzymes toward respectively echinocandins and the specific inhibitors of Kre6 were demonstrated in *P. carinii* (Kottom et al. 2015; Schmatz et al. 1990). Although active sites of enzymes are generally conserved, one cannot exclude that these sensitivities vary between *P. carinii* and *P. jirovecii* because these species are relatively distant. Clinical reports are contradictory but insights can be obtained by studying the *P. jirovecii* enzymes. We plan to use the complemented strains constructed here to determine these sensitivities.

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TRANSPARENCY DECLARATION

None to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Oligonucleotide primers used for PCR amplification.
Table S2. PCR conditions.
Figure S1. Multiple sequence alignment of *gsc1* (A) and *kre6* (B) gene sequences of the two *P. jirovecii* genome assemblies (Cissé et al. 2012; indicated as Cisse; Ma et al. 2016, indicated as Ma).
Figure S2. Multiple-sequence alignment of Gsc1 (A) and Kre6 (B) proteins.

Supplementary data of annexe 1

Target [a]	Primer	5' - 3' nucleotide sequence	Fragment amplified size with / without introns (bp)	Description
Pjgsc1 ORF	Pj*gsc1*start	CCGCGGTCGACATGTCACAACGACAGC[b]	6017 / 5782	First 16 nt
	Pj*gsc1*end	CCCCCCCCCGGGCTATGTAAACATCTTATATG[b]		Last 20 nt
Pjkre6 ORF	Pjkre6start	ATGAAAAAAAGTCATTGGTC	2341 / 2040	First 20 nt
	Pjkre6end	TTAACATTCGTTCATAAGTC		Last 20 nt
ScGSC1 ORF	Sc*GSC1*start	GCGGGATCCATGACCACTGATCAACAACC[d]	5652 / NA [c]	First 20 nt
	Sc*GSC1*end	CCCCCCCTGCAGTTATTTTATAGTTGACCAGG[d]		Last 20 nt
ScKRE6 ORF	ScKRE6start	GCGGGATCCATGCCTTTGAGAAAATCTAAC[d]	2184 / NA	First 21 nt
	ScKRE6end	CCCCCCGTCGACAGAGGATAACTTGAACTTCG[d]		Last 20 nt
Plasmids'	M13 forward	GTAAAACGACGGCCAGT	1106 / NA	NA
polylinker	M13 reverse	CAGGAAACAGCTATGAC		

Table S1. Oligonucleotide primers used for PCR amplification

[a] Pj, P. *jirovecii*; Sc, *S. cerevisiae*.

[b] These primers include the underlined restriction sites and five or six upstream bases allowing restriction [b]ecause there were intended for an oriented cloning which we did not use in the present work.

[c] NA, not applicable.

[d] These primers include the underlined restriction sites and three or six upstream bases allowing restriction for oriented cloning.

				35 cycles							
		Initial denaturation		Denaturation		Annealing		Elongation		Final extension	
Target[a]	$MgCl_2$ (mM)	sec	°C	sec	°C	sec	°C	sec	°C	sec	°C
Pjgsc1[b]	1.75	180	94	25	94	15	44	360	68	360	72
Pjkre6	7.5	180	94	30	94	30	52	150	72	600	72
ScGSC1	3.0	180	94	30	94	30	53	360	72	600	72
ScKRE6	3.0	180	94	30	94	30	52	135	72	600	72
Plasmids' polylinker	3.0	180	94	30	94	30	55	70	72	600	72

Table S2. PCR conditions for *gsc1* and *kre6* genes amplification

[a] Pj, P. *jirovecii*; Sc, *S. cerevisiae*.

[b] The Kapa Long Rage HotStart polymerase (Kapa Biosystems, Baden, Switzerland) was used for amplification of *Pjgsc1*, whereas all the other PCRs were performed using the High Fidelity Expand polymerase (Roche, Basel, Switzerland).

A

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Pjgsc1_Cisse_ORF          1 ATGTCACAACGACAGCATTATTACGATGATTCTTATCCAAGTCAAACAGATCCATATTATGCTGAC
Pjgsc1_Cisse_genomic      1 ATGTCACAACGACAGCATTATTACGATGATTCTTATCCAAGTCAAACAGATCCATATTATGCTGAC
Pjgsc1_Ma_ORF             1 ATGTCACAACGACAGCATTATTACGATGATTCTTATCCAAGTCAAACAGATCCATATTATGCTGAC
Pjgsc1_Ma_genomic         1 ATGTCACAACGACAGCATTATTACGATGATTCTTATCCAAGTCAAACAGATCCATATTATGCTGAC

Pjgsc1_Cisse_ORF         67 AATGGCTATAATAATGCAGATTTTCACGGGTCTTCATATGCACCCGAAGGGTATGATCATCAGGGA
Pjgsc1_Cisse_genomic     67 AATGGCTATAATAATGCAGATTTTCACGGGTCTTCATATGCACCCGAAGGGTATGATCATCAGGGA
Pjgsc1_Ma_ORF            67 AATGGCTATAATAATGCAGATTTTCACGGGTCTTCATATGCACCCGAAGGGTATGATCATCAGGGA
Pjgsc1_Ma_genomic        67 AATGGCTATAATAATGCAGATTTTCACGGGTCTTCATATGCACCCGAAGGGTATGATCATCAGGGA

Pjgsc1_Cisse_ORF        133 GCTTATCATCCTATGGAATACGGACAAGAGTATTATGATGAAGGATATGATAATGGGCAGGTACCA
Pjgsc1_Cisse_genomic    133 GCTTATCATCCTATGGAATACGGACAAGAGTATTATGATGAAGGATATGATAATGGGCAGGTACCA
Pjgsc1_Ma_ORF           133 GCTTATCATCCTATGGAATACGGACAAGAGTATTATGATGAAGGATATGATAATGGGCAGGTACCA
Pjgsc1_Ma_genomic       133 GCTTATCATCCTATGGAATACGGACAAGAGTATTATGATGAAGGATATGATAATGGGCAGGTACCA

Pjgsc1_Cisse_ORF        199 TATGATGCACGTGCTTTTGATATGTATTCACCATCGGATGATGCATATTATCGTCAAGAAAATGCC
Pjgsc1_Cisse_genomic    199 TATGATGCACGTGCTTTTGATATGTATTCACCATCGGATGATGCATATTATCGTCAAGAAAATGCC
Pjgsc1_Ma_ORF           199 TATGATGCACGTGCTTTTGATATGTATTCACCATCGGATGATGCATATTATCGTCAAGAAAATGCC
Pjgsc1_Ma_genomic       199 TATGATGCACGTGCTTTTGATATGTATTCACCATCGGATGATGCATATTATCGTCAAGAAAATGCC

Pjgsc1_Cisse_ORF        265 TATTATGATTATCCTGCAGATGCATATGCAACGGATGTGTATG-----------------------
Pjgsc1_Cisse_genomic    265 TATTATGATTATCCTGCAGATGCATATGCAACGGATGTGTATGGTACGTTTTTTTATAAAATTCAG
Pjgsc1_Ma_ORF           265 TATTATGATTATCCTGCAGATGCATATGCAACGGATGTGTATG-----------------------
Pjgsc1_Ma_genomic       265 TATTATGATTATCCTGCAGATGCATATGCAACGGATGTGTATGGTACGTTTTTTTATAAAATTCAG

Pjgsc1_Cisse_ORF        307 -------------------------------------ATCCATATGGAATGCCAATTGCTGATCAAC
Pjgsc1_Cisse_genomic    331 AAGTAATTTGTTATTTACTATTTTTTGAATTATTAGATCCATATGGAATGCCAATTGCTGATCAAC
Pjgsc1_Ma_ORF           307 -------------------------------------ATCCATATGGAATGCCAATTGCTGATCAAC
Pjgsc1_Ma_genomic       331 AAGTAATTTGTTATTTACTATTTTTTGAATTATTAGATCCATATGGAATGCCAATTGCTGATCAAC

Pjgsc1_Cisse_ORF        338 ACCCTCTTCAATATTTTCAAGATCATGGGAATTATATGTATAATCGTAAAGGGAAGCATAGAGGAT
Pjgsc1_Cisse_genomic    397 ACCCTCTTCAATATTTTCAAGATCATGGGAATTATATGTATAATCGTAAAGGGAAGCATAGAGGAT
Pjgsc1_Ma_ORF           338 ACCCTCTTCAATATTTTCAAGATCATGGGAATTATATGTATAATCGTAAAGGGAAGCATAGAGGAT
Pjgsc1_Ma_genomic       397 ACCCTCTTCAATATTTTCAAGATCATGGGAATTATATGTATAATCGTAAAGGGAAGCATAGAGGAT

Pjgsc1_Cisse_ORF        404 CATCTGAAGGTTCAGAAGCATTTTCCGACTTTACAATGCGTTCTGATATGGCTCGTGCGGCGGAAT
Pjgsc1_Cisse_genomic    463 CATCTGAAGGTTCAGAAGCATTTTCCGACTTTACAATGCGTTCTGATATGGCTCGTGCGGCGGAAT
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Pjkre6_Cisse_genomic      265 GAAGATGAACCAGCATATCAAACAAAACAAGAAAATGTGGAGTATTATGATAATTCTTTTAGCTCT
Pjkre6_Ma_ORF             265 GAAGATGAACCAGCATATCAAACAAAACAAGAAAATGTGGAGTATTATGATAATTCTTTTAGCTCT
Pjkre6_Ma_genomic         265 GAAGATGAACCAGCATATCAAACAAAACAAGAAAATGTGGAGTATTATGATAATTCTTTTAGCTCT

Pjkre6_Cisse_ORF          331 CAGTCTCCTCGAAATGTATATACAGATGGGTATAAAGCTTATAACGATATACAGGATTATGCATCT
Pjkre6_Cisse_genomic      331 CAGTCTCCTCGAAATGTATATACAGATGGGTATAAAGCTTATAACGATATACAGGATTATGCATCT
Pjkre6_Ma_ORF             331 CAGTCTCCTCGAAATGTATATACAGATGGGTATAAAGCTTATAACGATATACAGGATTATGCATCT
Pjkre6_Ma_genomic         331 CAGTCTCCTCGAAATGTATATACAGATGGGTATAAAGCTTATAACGATATACAGGATTATGCATCT

Pjkre6_Cisse_ORF          397 CTTGATTACAAAAGGAAATCTTATATGAATTATCCTGAAGAACCAGATAATAATTATTGGCATGAA
Pjkre6_Cisse_genomic      397 CTTGATTACAAAAGGAAATCTTATATGAATTATCCTGAAGAACCAGATAATAATTATTGGCATGAA
Pjkre6_Ma_ORF             397 CTTGATTACAAAAGGAAATCTTATATGAATTATCCTGAAGAACCAGATAATAATTATTGGCATGAA
Pjkre6_Ma_genomic         397 CTTGATTACAAAAGGAAATCTTATATGAATTATCCTGAAGAACCAGATAATAATTATTGGCATGAA

Pjkre6_Cisse_ORF          463 CCGCAAGAAAGTGTGTATACAGAAGAATATATAGAACCCGAATCAAGAAAAACAAATAAAGGATCT
Pjkre6_Cisse_genomic      463 CCGCAAGAAAGTGTGTATACAGAAGAATATATAGAACCCGAATCAAGAAAAACAAATAAAGGATCT
Pjkre6_Ma_ORF             463 CCGCAAGAAAGTGTATATACAGAAGAATATATAGAACCCGAATCAAGAAAAACAAATAAAGGATCT
Pjkre6_Ma_genomic         463 CCGCAAGAAAGTGTATATACAGAAGAATATATAGAACCCGAATCAAGAAAAACAAATAAAGGATCT

Pjkre6_Cisse_ORF          529 TTTAATACATACAAAAATACCGCTAAATCTGATGTTGGAAATAACCTTGATACATTATGGGATCCT
Pjkre6_Cisse_genomic      529 TTTAATACATACAAAAATACCGCTAAATCTGATGTTGGAAATAACCTTGATACATTATGGGATCCT
Pjkre6_Ma_ORF             529 TTTAATACATACAAAAATACCGCTAAATCTGATGTTGGAAATAACCTTGATACATTATGGGATCCT
Pjkre6_Ma_genomic         529 TTTAATACATACAAAAATACCGCTAAATCTGATGTTGGAAATAACCTTGATACATTATGGGATCCT

Pjkre6_Cisse_ORF          595 ACTGTAACAGAACCCGATGATTATCTTCACAACCCAACCTTTAAGGATAGAAAAAAAGATTATTAT
Pjkre6_Cisse_genomic      595 ACTGTAACAGAACCCGATGATTATCTTCACAACCCAACCTTTAAGGATAGAAAAAAAGATTATTAT
Pjkre6_Ma_ORF             595 ACTGTAACAGAACCCGATGATTATCTTCACAACCCAACCTTTAAGGATAGAAAAAAAGATTATTAT
Pjkre6_Ma_genomic         595 ACTGTAACAGAACCCGATGATTATCTTCACAACCCAACCTTTAAGGATAGAAAAAAAGATTATTAT

Pjkre6_Cisse_ORF          661 TTTTTTACAAAACGTGGTATATTTAATATAGGATCTCTTGTGTTTTTAATACTTGGTGTAATGTTT
Pjkre6_Cisse_genomic      661 TTTTTTACAAAACGTGGTATATTTAATATAGGATCTCTTGTGTTTTTAATACTTGGTGTAATGTTT
Pjkre6_Ma_ORF             661 TTTTTTACAAAACGTGGTATATTTAATATAGGATCTCTTGTGTTTTTAATACTTGGTGTAATGTTT
Pjkre6_Ma_genomic         661 TTTTTTACAAAACGTGGTATATTTAATATAGGATCTCTTGTGTTTTTAATACTTGGTGTAATGTTT

Pjkre6_Cisse_ORF          727 GTTTTTATTGGTTACCCTATTATGTTATATATTCGCAGGGCATATGATGATGCTCATAGTTGTCCT
Pjkre6_Cisse_genomic      727 GTTTTTATTGGTTACCCTATTATGTTATATATTCGCAGGGCATATGATGATGCTCATAGTTGTCCT
Pjkre6_Ma_ORF             727 GTTTTTATTGGTTACCCTATTATGTTATATATTCGCAGGGCATATGATGATGCTCATAGTTGTCCT
Pjkre6_Ma_genomic         727 GTTTTTATTGGTTACCCTATTATGTTATATATTCGCAGGGCATATGATGATGCTCATAGTTGTCCT

Pjkre6_Cisse_ORF          793 AATTGTATACGAACACTGCCTATAGATTTATTGGATGCAACAAGAAGCCTTATTGATCCGGATACA
Pjkre6_Cisse_genomic      793 AATTGTATACGAACACTGCCTATAGATTTATTGGATGCAACAAGAAGCCTTATTGATCCGGATACA
Pjkre6_Ma_ORF             793 AATTGTATACGAACACTGCCTATAGATTTATTGGATGCAACAAGAAGCCTTATTGATCCGGATACA
Pjkre6_Ma_genomic         793 AATTGTATACGAACACTGCCTATAGATTTATTGGATGCAACAAGAAGCCTTATTGATCCGGATACA
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Pjkre6_Cisse_ORF          859 CCGTTAGAATTTTATGAACGGAAGAGTAAAGATGGAAAAATTTATAAAATTGTATTTTCAGATGAA
Pjkre6_Cisse_genomic      859 CCGTTAGAATTTTATGAACGGAAGAGTAAAGATGGAAAAATTTATAAAATTGTATTTTCAGATGAA
Pjkre6_Ma_ORF             859 CCGTTAGAATTTTATGAACGGAAGAGTAAAGATGGAAAAATTTATAAAATTGTATTTTCAGATGAA
Pjkre6_Ma_genomic         859 CCGTTAGAATTTTATGAACGGAAGAGTAAAGATGGAAAAATTTATAAAATTGTATTTTCAGATGAA

Pjkre6_Cisse_ORF          925 TTTAATAAAAATGGAAGAACATTCTATCCAGGTGATGATCAGTTTTG------------------
Pjkre6_Cisse_genomic      925 TTTAATAAAAATGGAAGAACATTCTATCCAGGTGATGATCAGTTTTGGTATATATTAAATATGTCT
Pjkre6_Ma_ORF             925 TTTAATAAAAATGGAAGAACATTCTATCCAGGTGATGATCAGTTTTG------------------
Pjkre6_Ma_genomic         925 TTTAATAAAAATGGAAGAACATTCTATCCAGGTGATGATCAGTTTTGGTATATATTAAATATGTCT

Pjkre6_Cisse_ORF          972 ------------------------GGAAGCAGTCGATTTACATTACTGGTCAACTATGAGTATAGA
Pjkre6_Cisse_genomic      991 TTGAATTTTAATAAATTTGAATAGGGAAGCAGTCGATTTACATTACTGGTCAACTATGAGTATAGA
Pjkre6_Ma_ORF             972 ------------------------GGAAGCAGTCGATTTACATTACTGGTCAACTATGAGTATAGA
Pjkre6_Ma_genomic         991 TTGAATTTTAATAAATTTGAATAGGGAAGCAGTCGATTTACATTACTGGTCAACTATGAGTATAGA

Pjkre6_Cisse_ORF         1014 ATGGTATGATCCTGATGCTATAACTACAAATGGAGGTTTTTTGGAGATACGACTTGATGCTTTTCG
Pjkre6_Cisse_genomic     1057 ATGGTATGATCCTGATGCTATAACTACAAATGGAGGTTTTTTGGAGATACGACTTGATGCTTTTCG
Pjkre6_Ma_ORF            1014 ATGGTATGATCCTGATGCTATAACTACAAATGGAGGTTTTTTGGAGATACGACTTGATGCTTTTCG
Pjkre6_Ma_genomic        1057 ATGGTATGATCCTGATGCTATAACTACAAATGGAGGTTTTTTGGAGATACGACTTGATGCTTTTCG

Pjkre6_Cisse_ORF         1080 AAATCATGATCTTAATTATAGATCAG----------------------------------------G
Pjkre6_Cisse_genomic     1123 AAATCATGATCTTAATTATAGATCAGGTATTTTTTGCAATTAAATTTTAATTTTGACATGTATAGG
Pjkre6_Ma_ORF            1080 AAATCATGATCTTAATTATAGATCAG----------------------------------------G
Pjkre6_Ma_genomic        1123 AAATCATGATCTTAATTATAGATCAGGTATTTTTTGCAATTAAATTTTAATTTTGACATGTATAGG

Pjkre6_Cisse_ORF         1107 TATGCTTCAAAGTTGGAATAAACTATGTTTTAAAGGAGGTATTATTGAAGCATCTATTTCACTTCC
Pjkre6_Cisse_genomic     1189 TATGCTTCAAAGTTGGAATAAACTATGTTTTAAAGGAGGTATTATTGAAGCATCTATTTCACTTCC
Pjkre6_Ma_ORF            1107 TATGCTTCAAAGTTGGAATAAACTATGTTTTAAAGGAGGTATTATTGAAGCATCTATTTCACTTCC
Pjkre6_Ma_genomic        1189 TATGCTTCAAAGTTGGAATAAACTATGTTTTAAAGGAGGTATTATTGAAGCATCTATTTCACTTCC

Pjkre6_Cisse_ORF         1173 AGGTAGAGGAGACATTTCTGGATT-----------------------------------------
Pjkre6_Cisse_genomic     1255 AGGTAGAGGAGACATTTCTGGATTGTAGGATAATATATGTATATATATATTATATTATTTATTACT
Pjkre6_Ma_ORF            1173 AGGTAGAGGAGACATTTCTGGATT-----------------------------------------
Pjkre6_Ma_genomic        1255 AGGTAGAGGAGACATTTCTGGATTGTAGGATAATATATGTATATATATATTATATTATTTATTACT

Pjkre6_Cisse_ORF         1197 -----TTGGCCAGCATTTTGGGCAATGGGAAATCTTGGTCGTCCTGGCTTTGGTGCTTCTACTGAT
Pjkre6_Cisse_genomic     1321 ATTAGTTGGCCAGCATTTTGGGCAATGGGAAATCTTGGTCGTCCTGGCTTTGGTGCTTCTACTGAT
Pjkre6_Ma_ORF            1197 -----TTGGCCAGCATTTTGGGCAATGGGAAATCTTGGTCGTCCTGGCTTTGGTGCTTCTACTGAT
Pjkre6_Ma_genomic        1321 ATTAGTTGGCCAGCATTTTGGGCAATGGGAAATCTTGGTCGTCCTGGCTTTGGTGCTTCTACTGAT

Pjkre6_Cisse_ORF         1258 GGAGTATGGCCATATAGTTATGATACATGTGAT-----------------------------------
Pjkre6_Cisse_genomic     1387 GGAGTATGGCCATATAGTTATGATACATGTGATGTAAGATTATATATTTGTTTTTTAAAATCTTAT
Pjkre6_Ma_ORF            1258 GGAGTATGGCCATATAGTTATGATACATGTGAT-----------------------------------
Pjkre6_Ma_genomic        1387 GGAGTATGGCCATATAGTTATGATACATGTGATGTAAGATTATATATTTGTTTTTTAAAATCTTAT

Pjkre6_Cisse_ORF         1292 -------GTTGGAATTACACCTAATCAATCAGATTCTAACGGCATATCATCTCTTCCTGGAATGAG
Pjkre6_Cisse_genomic     1453 AATTTAGGTTGGAATTACACCTAATCAATCAGATTCTAACGGCATATCATCTCTTCCTGGAATGAG
Pjkre6_Ma_ORF            1292 -------GTTGGAATTACACCTAATCAATCAGATTCTAACGGCATATCATCTCTTCCTGGAATGAG
Pjkre6_Ma_genomic        1453 AATTTAGGTTGGAATTACACCTAATCAATCAGATTCTAACGGCATATCATCTCTTCCTGGAATGAG

Pjkre6_Cisse_ORF         1350 ATTTCCTAACTGTGTATGTCCTAATTCAGATCATCCAAGCCCAGGAAAAGGACGAGGTGCACCAGA
Pjkre6_Cisse_genomic     1519 ATTTCCTAACTGTGTATGTCCTAATTCAGATCATCCAAGCCCAGGAAAAGGACGAGGTGCACCAGA
Pjkre6_Ma_ORF            1350 ATTTCCTAACTGTGTATGTCCTAATTCAGATCATCCAAGCCCAGGAAAAGGACGAGGTGCACCAGA
Pjkre6_Ma_genomic        1519 ATTTCCTAACTGTGTATGTCCTAATTCAGATCATCCAAGCCCAGGAAAAGGACGAGGTGCACCAGA

Pjkre6_Cisse_ORF         1416 AATAGATATAATTGAAGCATCTGTTGATCTTAGTTTTCGTCTTGGAGAAGCATCACAATCAGTACA
Pjkre6_Cisse_genomic     1585 AATAGATATAATTGAAGCATCTGTTGATCTTAGTTTTCGTCTTGGAGAAGCATCACAATCAGTACA
Pjkre6_Ma_ORF            1416 AATAGATATAATTGAAGCATCTGTTGATCTTAGTTTTCGTCTTGGAGAAGCATCACAATCAGTACA
Pjkre6_Ma_genomic        1585 AATAGATATAATTGAAGCATCTGTTGATCTTAGTTTTCGTCTTGGAGAAGCATCACAATCAGTACA

Pjkre6_Cisse_ORF         1482 GTTTGCACCTTTTGACGACCTTTATACACCAAATTATGAACATATGAAAATATATAATAAAGAAAA
Pjkre6_Cisse_genomic     1651 GTTTGCACCTTTTGACGACCTTTATACACCAAATTATGAACATATGAAAATATATAATAAAGAAAA
Pjkre6_Ma_ORF            1482 GTTTGCACCTTTTGACGACCTTTATACACCAAATTATGAACATATGAAAATATATAATAAAGAAAA
Pjkre6_Ma_genomic        1651 GTTTGCACCTTTTGACGACCTTTATACACCAAATTATGAACATATGAAAATATATAATAAAGAAAA

Pjkre6_Cisse_ORF         1548 AACTCATATAAATAATTATCGTGGCAATTCTTTTCAACAAA-------------------------
Pjkre6_Cisse_genomic     1717 AACTCATATAAATAATTATCGTGGCAATTCTTTTCAACAAAGTAAAAATATAATATTTCTATTTTT
Pjkre6_Ma_ORF            1548 AACTCATATAAATAATTATCGTGGCAATTCTTTTCAACAAA-------------------------
Pjkre6_Ma_genomic        1717 AACTCATATAAATAATTATCGTGGCAATTCTTTTCAACAAAGTAAAAATATAATATTTCTATTTTT
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Pjkre6_Cisse_ORF        1589 ------------------CATTTTCATGTATCACGTACTTAAATAATGAATGGTATGATGGACGTA
Pjkre6_Cisse_genomic    1783 AATTTACTTATTATTTAGCATTTTCATGTATCACGTACTTAAATAATGAATGGTATGATGGACGTA
Pjkre6_Ma_ORF           1589 ------------------CATTTTCATGTATCACGTACTTAAATAATGAATGGTATGATGGACGTA
Pjkre6_Ma_genomic       1783 AATTTACTTATTATTTAGCATTTTCATGTATCACGTACTTAAATAATGAATGGTATGATGGACGTA

Pjkre6_Cisse_ORF        1637 AATTTCAAACATATTCTCTTGAGTATGAACCTGGTAAAAATGGTTTTATTCAATGGTATATTGGTG
Pjkre6_Cisse_genomic    1849 AATTTCAAACATATTCTCTTGAGTATGAACCTGGTAAAAATGGTTTTATTCAATGGTATATTGGTG
Pjkre6_Ma_ORF           1637 AATTTCAAACATATTCTCTTGAGTATGAACCTGGTAAAAATGGTTTTATTCAATGGTATATTGGTG
Pjkre6_Ma_genomic       1849 AATTTCAAACATATTCTCTTGAGTATGAACCTGGTAAAAATGGTTTTATTCAATGGTATATTGGTG

Pjkre6_Cisse_ORF        1703 ATAATCCAACGTGGATGATGAAAGCTGAATCAGTAGGACCAAATGGGAAAATTGGACAAAGGCTCA
Pjkre6_Cisse_genomic    1915 ATAATCCAACGTGGATGATGAAAGCTGAATCAGTAGGACCAAATGGGAAAATTGGACAAAGGCTCA
Pjkre6_Ma_ORF           1703 ATAATCCAACGTGGATGATGAAAGCTGAATCAGTAGGACCAAATGGGAAAATTGGACAAAGGCTCA
Pjkre6_Ma_genomic       1915 ATAATCCAACGTGGATGATGAAAGCTGAATCAGTAGGACCAAATGGGAAAATTGGACAAAGGCTCA

Pjkre6_Cisse_ORF        1769 TATCTGAAGAGCCCATG---------------------------------------------GCATT
Pjkre6_Cisse_genomic    1981 TATCTGAAGAGCCCATGGTGAGATTAACTTATATCTTTGTTTTTTTTTTGACCTGATACAGGCATT
Pjkre6_Ma_ORF           1769 TATCTGAAGAACCCATG---------------------------------------------GCATT
Pjkre6_Ma_genomic       1981 TATCTGAAGAACCCATGGTGAGATTAACTTATATCTTTGTTTTTTTTTTGACCTGATACAGGCATT

Pjkre6_Cisse_ORF        1791 CGTAATTAACCTTGCAATGTCAGAGTCTTTTGCTAAAATAGAATGGGGAAGATTACAATTTCCTGC
Pjkre6_Cisse_genomic    2047 CGTAATTAACCTTGCAATGTCAGAGTCTTTTGCTAAAATAGAATGGGGAAGATTACAATTTCCTGC
Pjkre6_Ma_ORF           1791 CGTAATTAACCTTGCGATGTCAGAGTCTTTTGCTAAAATAGAATGGGGAAGATTACAATTTCCTGC
Pjkre6_Ma_genomic       2047 CGTAATTAACCTTGCGATGTCAGAGTCTTTTGCTAAAATAGAATGGGGAAGATTACAATTTCCTGC

Pjkre6_Cisse_ORF        1857 TATTATGCGAGTAGATTGGGTCCGCATTTACCAAGAAACGCCTATGATTACATGTGATCCTCCTGG
Pjkre6_Cisse_genomic    2113 TATTATGCGAGTAGATTGGGTCCGCATTTACCAAGAAACGCCTATGATTACATGTGATCCTCCTGG
Pjkre6_Ma_ORF           1857 TATTATGCGAGTAGATTGGGTCCGCATTTACCAAGAAACGCCTATGATTACATGTGATCCTCCTGG
Pjkre6_Ma_genomic       2113 TATTATGCGAGTAGATTGGGTCCGCATTTACCAAGAAACGCCTATGATTACATGTGATCCTCCTGG

Pjkre6_Cisse_ORF        1923 ATATCCAACTACAAAGTATATTAAAGA----------------------------------------
Pjkre6_Cisse_genomic    2179 ATATCCAACTACAAAGTATATTAAAGAGTATGTTGTTTTTATTATATAATTTGCAAATTTAACAGA
Pjkre6_Ma_ORF           1923 ATATCCAACTACAAAGTATATTAAAGAGTATGTTGTTTTTATTATATAA-----------------
Pjkre6_Ma_genomic       2179 ATATCCAACTACAAAGTATATTAAAGAGTATGTTGTTTTTATTATATAA-----------------

Pjkre6_Cisse_ORF        1954 ------ACATCCTATAGCTTATTATAACAATAATATTACTACTTGGGAGAATACCGGCTATCAATG
Pjkre6_Cisse_genomic    2245 TATCAGACATCCTATAGCTTATTATAACAATAATATTACTACTTGGGAGAATACCGGCTATCAATG
Pjkre6_Ma_ORF           1965 ------------------------------------------------------------------
Pjkre6_Ma_genomic       2221 ------------------------------------------------------------------

Pjkre6_Cisse_ORF         2010 GCCTAAAAATCGACTTATGAACGAATGTTAA
Pjkre6_Cisse_genomic     2311 GCCTAAAAATCGACTTATGAACGAATGTTAA
Pjkre6_Ma_ORF            1965 -------------------------------
Pjkre6_Ma_genomic        2221 -------------------------------
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Figure S1. Multiple sequence alignment of *gsc1* (**A**) and *kre6* (**B**) gene sequences of the two *P. jirovecii* genome assemblies (Cissé *et al.,*2012, indicated as Cisse; Ma *et al.,* 2016, indicated as Ma). T-Coffee was used (Notredame *et al.,* 2000). Residues shown in bold are synonymous SNPs. Dashes indicate gaps. **A.** Alignment of *gsc1* ORF and genomic sequences. The second exon of the allele of Ma *et al.,*is 81 bp longer than that of Cissé et al., and presents five bp deleted relatively to the genomic sequence of Cissé *et al.,*from position 2581 to 2604. **B.** Alignment of *kre6* ORF and genomic sequences. The last exon of the allele of Ma *et al.,*ends within the last intron of the allele of Cissé *et al.,*, resulting in a ORF 69 bp shorter.

A

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P.jirovecii        1 MSQRQHYYDDSYPSQTDPYYADNGYNNADFHGSSYAPEGYDHQGAYHPMEYGQEYYDEGYDNGQVPYDARAF
P.carinii          1 MSQQQHYYDDSYGGQNGGYYGEHSYDNTGFNNGSYGSGVYE-QGGYYGPEYGQEYYDE-YDGGGAMYNGQGH
S.cerevisiae       1 MNTDQQ-----------PYQGQTDYTQ----------------------GPGNGQSQE-----------QDY
S.pombe            1 MSGNN-----------ENVSGITGHDAVSDDQYAYDSEVYD-DQNAYQRQPADAYSNE-EFLDQADYD----
                       *.  :              .  .:                          .:   :*

P.jirovecii       73 DMYSPSDDAYYRQENAYYDYPAD--AYATDVYDPYGMPIADQHPLQYFQDHGNY-MYNRKGK-HRGSSEGSE
P.carinii         71 EMYNSGEEGYYRQEEGYYDYPQD--GYVG---DTYGIKKDILRGNGYFQGQDEYYTYDRKGK-RRGSSEASE
S.cerevisiae      29 DQY--GQPLYPSQADGYYD-PNVAAGTEA---DMYGQQPPN---ESYDQDYTNG-EYYGQPP-NMA-AQDGE
S.pombe           56 -------SMYGEGYNG-YDYPTG----VT---ESYGDEYTP--VDTASSGINQY-S-TEKGKFTRPSDEYES
                          *    :. ** *          : **           ..  :     :       :  .

P.jirovecii      141 AFSDFTMRSDMARAAEFDAYGRFDEQYRSYAPSTESLNQMA-SR-RGYYP-DSSQISYTGNRSSGASTPVYG
P.carinii        137 TFSDFTMRSDMVRAAEYDSYGRFDERYRSYEPSTESLNQMA-SRQRGYRP-D-SQISYTGNRSSGASTPIYG
S.cerevisiae      89 NFSDFSSYGPP-GTPGYDSYG-------------------------GQYT-A-SQMSYGEPNSSGTSTPIYG
S.pombe          109 EYSDYNAQPSD--ANNF-YNLRGDGRYNAYDPSSDSLANVYNSVPYGSSPYDFSNSSFVGN--SGSGTPLDG
                     :**:.       :  :                              *  .   *: *:     **:.**: *

P.jirovecii      210 MEYNQAAMMTSARSREPYPAWTAENQIPISKEEIEDIFIDLTNKFGFQRDSMRNMYDHMMVLLDSRASRMTP
P.carinii        206 MYYNQAAMMTSARSREPYPTWTAENQIPISKEEIEDIFIDLTNKFGFQRDSMRNMYDHMMVLLDSRASRMTP
S.cerevisiae     133 NYDPNAIAM--ALPNEPYPAWTADSQSPVSIEQIEDIFIDLTNRLGFQRDSMRNMFDHFMVLLDSRSSRMSP
S.pombe          176 DSGSFYADSANLTNREPYPAWTPENELPLTKEEIEDIFIDLTNKLGFQRDSMRNMYDFFMCLLDSRASRMTP
                             .****:**.:.: *:: *:**********::**********:*.:* *****:***:*

P.jirovecii      282 NQALLSLHADYIGGDNANYRNWYFAAQLDLDDAVGFSNMDFEKN-KKTNHSQKF------------------
P.carinii        278 NQALLSLHADYIGGDNANYRNWYFAAQFDLDDAVGFSNMDLDKN-RKSNYSQKS------------------
S.cerevisiae     203 DQALLSLHADYIGGDTANYKKWYFAAQLDMDDEIGFRNMSLGKLSRKARKAKKK------------------
S.pombe          248 DQALLTLHADYIGSDIANYKKWYFASQMDREDAVGLANVGIYGG-KVTSIKEKGKFFSRNKKAPKVVKPPRK
                     :****:*******.* ***::****:*:* :* :*: *:.:    : :   :*

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P.jirovecii      335 ------SKS---Q-KNTTAKDILQALESDNPLESAIYRWKTKCSQMSQYDRARELALYLLCWGEANQVRFTP
P.carinii        331 ------SKKF--Q-KNSASKSILQALDGDNSLESAIYRWKTRCTQMSQYDRARELALYLLCWGEANQVRFTP
S.cerevisiae     257 ------NKKAMEEANPEDTEETLNKIEGDNSLEAADFRWKAKMNQLSPLERVRHIALYLLCWGEANQVRFTA
S.pombe          319 SRFKRKKKK---E-QPEEAEDEYIDVNTDDSLESAEYRWRSHMRSMTQFERAQQIALWLLLWGEANNVRFMP
                           .*.   : :   ::.    :: *:.**:* :**:::  .::  :*.:.:**:** *****:*** .

                     ----------------------1,3-β glucan synthase domain 1---------------------
P.jirovecii      397 ECLCFIFKCANDYLNSPQCQAMVEPVPEGSYLNDIITPLYIYMRDQGYEIINGKYVRRERDHNKIIGYDDIN
P.carinii        394 ECLCFIFKCANDYLNSPQCQAMVEPAPEGSYLNDVITPLYAYMRDQGYEIINGRYVRRERDHNKIIGYDDIN
S.cerevisiae     323 ECLCFIYKCALDYLDSPLCQQRQEPMPEGDFLNRVITPIYHFIRNQVYEIVDGRFVKRERDHNKIVGYDDLN
S.pombe          387 EVIAFLFKCAYDYIISPEAQNVTEPVPEGYYLDNIVSPLYQYMHDQQFEIINGKYVRRERPHDQLIGYDDIN
                     * :.*::*** **: ** .*   ** *** :*: :::*:* ::::* :**::*::*:*** *::::****:*

                     ---------------
P.jirovecii      469 QLFWYSEGIERIVLSDKTRIIDLPPEQRYLRLKDVVWKKVFFKTYRETRSWFHLFTNFNRIWIIHITVYWFY
P.carinii        466 QLFWYPEGIQRIVLSDKTRMVDLPLDQRYPRFKDVVWKKAFFKTYRETRSWFHLFTNFNRIWIIHITVYWFY
S.cerevisiae     395 QLFWYPEGIAKIVLEDGTKLIELPLEERYLRLGDVVWDDVFFKTYKETRTWLHLVTNFNRIWVMHISIFWMY
S.pombe          459 QLFWHAEGIARLIFEDGTRLIDIPASERFHRLPEVQWNRAFYKTYYESRSWFHLITNFNRIWVIHFGMFWYF
                     ****:.*** ::::.* *::::* .:*: *: :* *. .*:*** *:*:*:**.*******::*: ::* :
                                                                    --------transmembrane-

P.jirovecii      541 TAANSPTVYTHNYQQSLDNQPPFAYRMSAVSFGGGVASLLMIIATLAEWAYVPRKWAGAQHLTRRLLFLILF
P.carinii        538 TAANSPTVYTHNYQQSLDNQPPFAYRMSAVGFGGGVASLLMIVATLAEWAYVPRKWPGAQHLTRRLLFLILF
S.cerevisiae     467 FAYNSPTFYTHNYQQLVDNQPLAAYKWASCALGGTVASLIQIVATLCEWSFVPRKWAGAQHLSRRFWFLCII
S.pombe          531 TAFNSPTLYTKPFHQRDGPKPTGASQWAAVACTSVVSCIIMAAASLCEYLFVPRRFPGSKPIWKRLCIIVLI
                      * ****.**: ::*  . :*  * : :: .  . *:.::   *:*.*: :***::.*:: : :*: :: ::
                     ----------           ------transmembrane------                 -trans-

P.jirovecii      613 LIINVAPGVYVIKFAPWKPKVSVVTTLISIIHFLIAMFTFLFFAIMPLGGLFGNYLYKKTRRYVASQTFTAN
P.carinii        610 FIINVAPGVYVIKFAPWKPNVSIVTTLISIMHFLIAIFTFLFFAIMPLGGLFGNYLYKKTRRYVASQTFTAN
S.cerevisiae     539 FGINLGPIIFVFAYDKDT-VYSTAAHVVAAVMFFVAVATIIFFSIMPLGGLFTSYMKKSTRRYVASQTFTAA
S.pombe          603 AIINLIPIVYIFGFSSKHQQRSGRRIAVGVVAFLMSIATYVYFSLVPLQSTFGKLSVKDSRKYLANKYFTSN
                       **: * :::: :       *     :. : *:::: * ::*:::** . * .   *.:*:*:*.: **:
                     -membrane------       -----------transmembrane-----------
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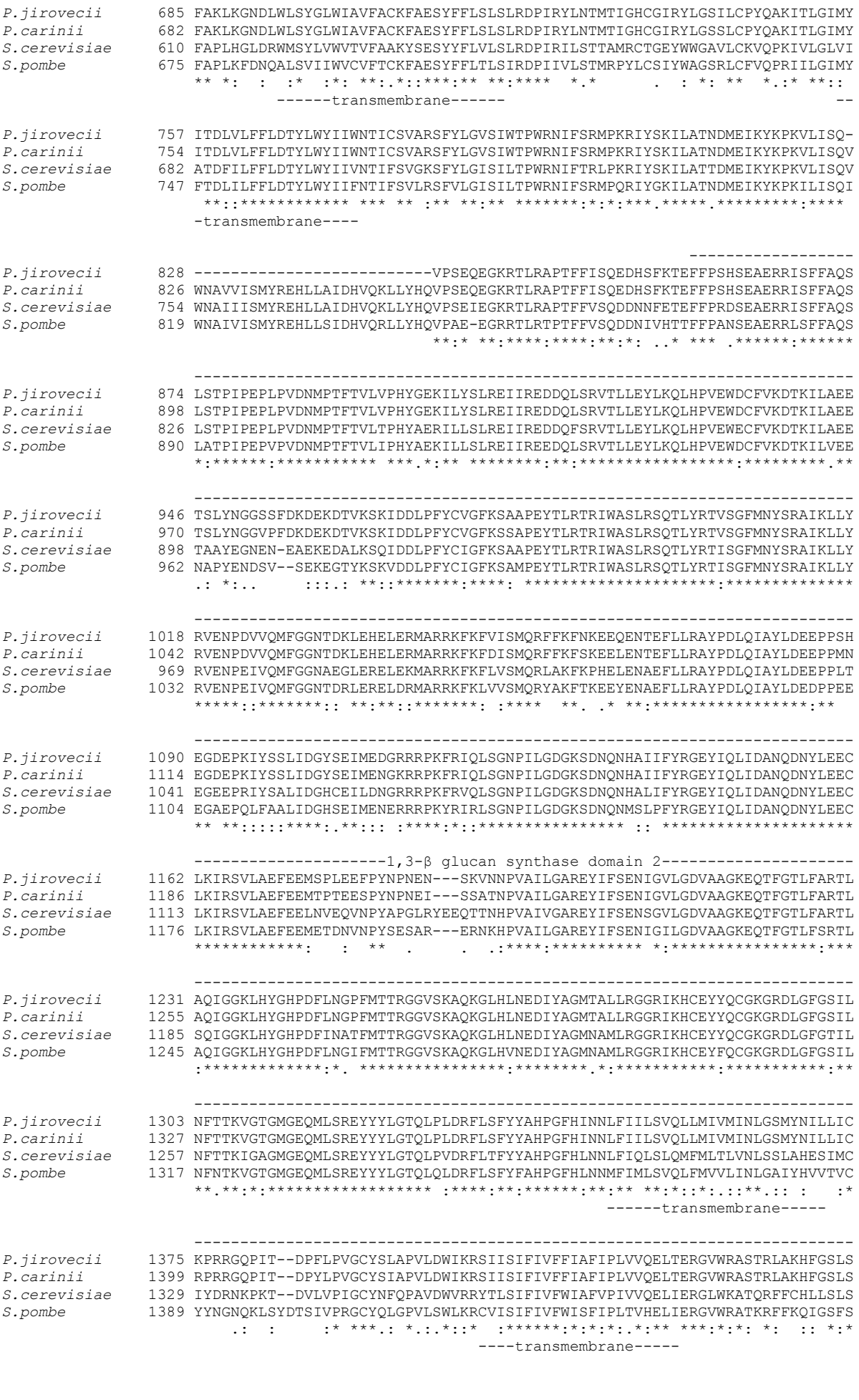
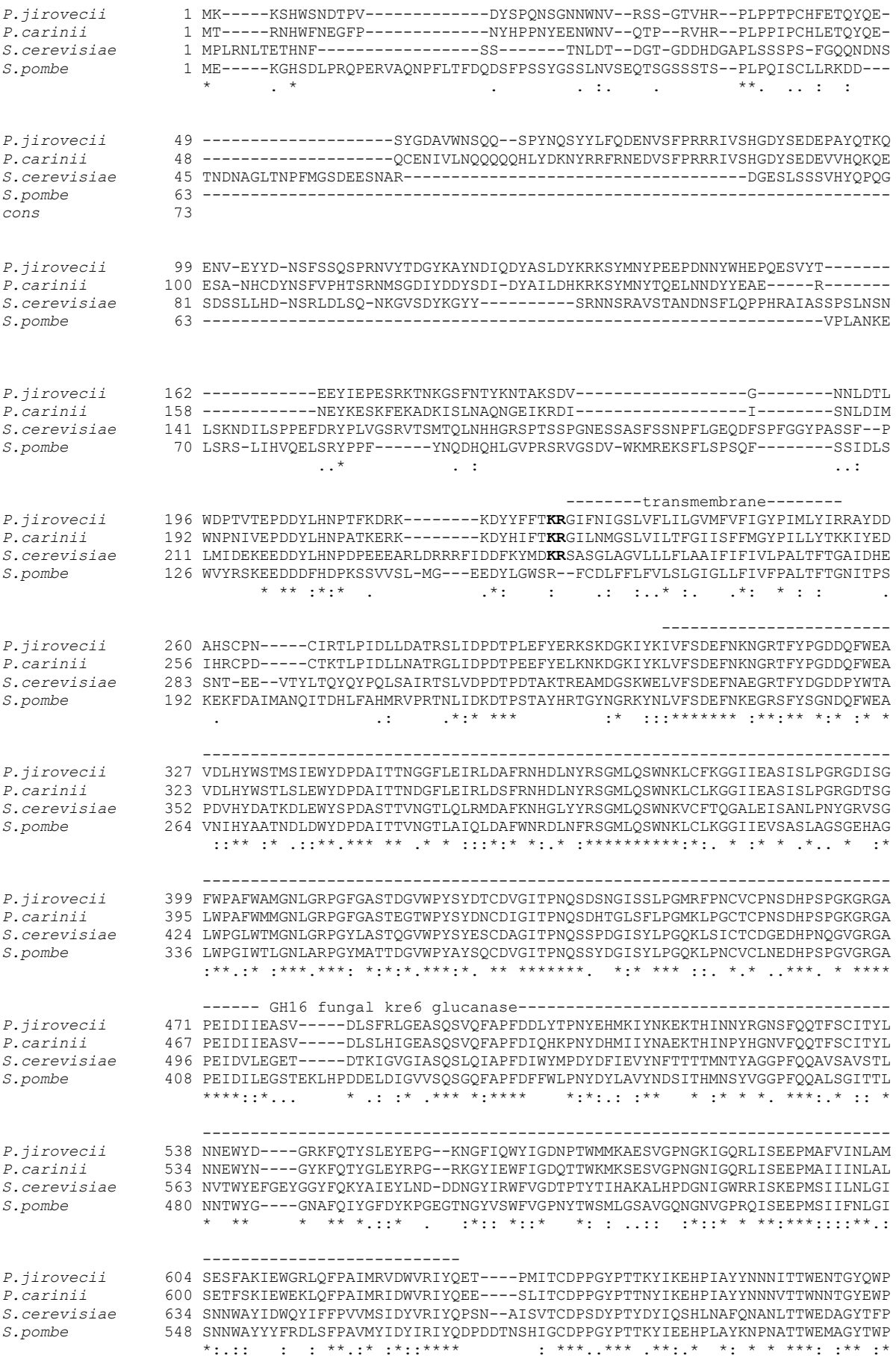

P.jirovecii 685 FAKLKGNDLWLSYGLWIAVFACKFAESYFFLSLSLRDPIRYLNTMTIGHCGIRYLGSILCPYQAKITLGIMY
P.carinii 682 FAKLKGNDLWLSYGLWIAVFACKFAESYFFLSLSLRDPIRYLNTMTIGHCGIRYLGSSLCPYQAKITLGIMY
S.cerevisiae 610 FAPLHGLDRWMSYLVWVTVFAAKYSESYYFLVLSLRDPIRILSTTAMRCTGEYWWGAVLCKVQPKIVLGLVI
S.pombe 675 FAPLKFDNQALSVIIWVCVFTCKFAESYFFLTLSIRDPIIVLSTMRPYLCSIYWAGSRLCFVQPRIILGIMY
------transmembrane------
P.jirovecii 757 ITDLVLFFLDTYLWYIIWNTICSVARSFYLGVSIWTPWRNIFSRMPKRIYSKILATNDMEIKYKPKVLISQ-
P.carinii 754 ITDLVLFFLDTYLWYIIWNTICSVARSFYLGVSIWTPWRNIFSRMPKRIYSKILATNDMEIKYKPKVLISQV
S.cerevisiae 682 ATDFILFFLDTYLWYIIVNTIFSVGKSFYLGISILTPWRNIFTRLPKRIYSKILATTDMEIKYKPKVLISQV
S.pombe 747 FTDLILFFLDTYLWYIIFNTIFSVLRSFVLGISILTPWRNIFSRMPQRIYGKILATNDMEIKYKPKILISQI
-transmembrane----
P.jirovecii 828 -------------------------VPSEQEGKRTLRAPTFFISQEDHSFKTEFFPSHSEAERRISFFAQS
P.carinii 826 WNAVVISMYREHLLAIDHVQKLLYHQVPSEQEGKRTLRAPTFFISQEDHSFKTEFFPSHSEAERRISFFAQS
S.cerevisiae 754 WNAIIISMYREHLLAIDHVQKLLYHQVPSEIEGKRTLRAPTFFVSQDDNNFETEFFPRDSEAERRISFFAQS
S.pombe 819 WNAIVISMYREHLLSIDHVQRLLYHQVPAE-EGRRTLRTPTFFVSQDDNIVHTTFFPANSEAERRLSFFAQS
P.jirovecii 874 LSTPIPEPLPVDNMPTFTVLVPHYGEKILYSLREIIREDDQLSRVTLLEYLKQLHPVEWDCFVKDTKILAEE
P.carinii 898 LSTPIPEPLPVDNMPTFTVLVPHYGEKILYSLREIIREDDQLSRVTLLEYLKQLHPVEWDCFVKDTKILAEE
S.cerevisiae 826 LSTPIPEPLPVDNMPTFTVLTPHYAERILLSLREIIREDDQFSRVTLLEYLKQLHPVEWECFVKDTKILAEE
S.pombe 890 LATPIPEPVPVDNMPTFTVLIPHYAEKILLSLREIIREEDQLSRVTLLEYLKQLHPVEWDCFVKDTKILVEE
P.jirovecii 946 TSLYNGGSSFDKDEKDTVKSKIDDLPFYCVGFKSAAPEYTLRTRIWASLRSQTLYRTVSGFMNYSRAIKLLY
P.carinii 970 TSLYNGGVPFDKDEKDTVKSKIDDLPFYCVGFKSSAPEYTLRTRIWASLRSQTLYRTVSGFMNYSRAIKLLY
S.cerevisiae 898 TAAYEGNEN-EAEKEDALKSQIDDLPFYCIGFKSAAPEYTLRTRIWASLRSQTLYRTISGFMNYSRAIKLLY
S.pombe 962 NAPYENDSV--SEKEGTYKSKVDDLPFYCIGFKSAMPEYTLRTRIWASLRSQTLYRTISGFMNYSRAIKLLY
P.jirovecii 1018 RVENPDVVQMFGGNTDKLEHELERMARRKFKFVISMQRFFKFNKEEQENTEFLLRAYPDLQIAYLDEEPPSH
P.carinii 1042 RVENPDVVQMFGGNTDKLEHELERMARRKFKFDISMQRFFKFSKEELENTEFLLRAYPDLQIAYLDEEPPMN
S.cerevisiae 969 RVENPEIVQMFGGNAEGLERELEKMARRKFKFLVSMQRLAKFKPHELENAEFLLRAYPDLQIAYLDEEPPLT
S.pombe 1032 RVENPEIVQMFGGNTDRLERELDRMARRKFKLVVSMQRYAKFTKEEYENAEFLLRAYPDLQIAYLDEDPPEE
P.jirovecii 1090 EGDEPKIYSSLIDGYSEIMEDGRRRPKFRIQLSGNPILGDGKSDNQNHAIIFYRGEYIQLIDANQDNYLEEC
P.carinii 1114 EGDEPKIYSSLIDGYSEIMENGKRRPKFRIQLSGNPILGDGKSDNQNHAIIFYRGEYIQLIDANQDNYLEEC
S.cerevisiae 1041 EGEEPRIYSALIDGHCEILDNGRRRPKFRVQLSGNPILGDGKSDNQNHALIFYRGEYIQLIDANQDNYLEEC
S.pombe 1104 EGAEPQLFAALIDGHSEIMENERRRPKYRIRLSGNPILGDGKSDNQNMSLPFYRGEYIQLIDANQDNYLEEC
----------1,3-β glucan synthase domain 2----------
P.jirovecii 1162 LKIRSVLAEFEEMSPLEEFPYNPNEN---SKVNNPVAILGAREYIFSENIGVLGDVAAGKEQTFGTLFARTL
P.carinii 1186 LKIRSVLAEFEEMTPTEESPYNPNEI---SSATNPVAILGAREYIFSENIGVLGDVAAGKEQTFGTLFARTL
S.cerevisiae 1113 LKIRSVLAEFEELNVEQVNPYAPGLRYEEQTTNHPVAIVGAREYIFSENSGVLGDVAAGKEQTFGTLFARTL
S.pombe 1176 LKIRSVLAEFEEMETDNVNPYSESAR---ERNKHPVAILGAREYIFSENIGILGDVAAGKEQTFGTLFSRTL
P.jirovecii 1231 AQIGGKLHYGHPDFLNGPFMTTRGGVSKAQKGLHLNEDIYAGMTALLRGGRIKHCEYYQCGKGRDLGFGSIL
P.carinii 1255 AQIGGKLHYGHPDFLNGPFMTTRGGVSKAQKGLHLNEDIYAGMTALLRGGRIKHCEYYQCGKGRDLGFGSIL
S.cerevisiae 1185 SQIGGKLHYGHPDFINATFMTTRGGVSKAQKGLHLNEDIYAGMNAMLRGGRIKHCEYYQCGKGRDLGFGTIL
S.pombe 1245 AQIGGKLHYGHPDFLNGIFMTTRGGVSKAQKGLHVNEDIYAGMNAMLRGGRIKHCEYFQCGKGRDLGFGSIL
P.jirovecii 1303 NFTTKVGTGMGEQMLSREYYYLGTQLPLDRFLSFYYAHPGFHINNLFIILSVQLLMIVMINLGSMYNILLIC
P.carinii 1327 NFTTKVGTGMGEQMLSREYYYLGTQLPLDRFLSFYYAHPGFHINNLFIILSVQLLMIVMINLGSMYNILLIC
S.cerevisiae 1257 NFTTKIGAGMGEQMLSREYYYLGTQLPVDRFLTFYYAHPGFHLNNLFIQLSLQMFMLTLVNLSSLAHESIMC
S.pombe 1317 NFNTKVGTGMGEQMLSREYYYLGTQLQLDRFLSFYFAHPGFHLNNMFIMLSVQLFMVVLINLGAIYHVVTVC
------transmembrane-----
P.jirovecii 1375 KPRRGQPIT--DPFLPVGCYSLAPVLDWIKRSIISIFIVFFIAFIPLVVQELTERGVWRASTRLAKHFGSLS
P.carinii 1399 RPRRGQPIT--DPYLPVGCYSIAPVLDWIKRSIISIFIVFFIAFIPLVVQELTERGVWRASTRLAKHFGSLS
S.cerevisiae 1329 IYDRNKPKT--DVLVPIGCYNFQPAVDWVRRYTLSIFIVFWIAFVPIVVQELIERGLWKATQRFFCHLLSLS
S.pombe 1389 YYNGNQKLSYDTSIVPRGCYQLGPVLSWLKRCVISIFIVFWISFIPLTVHELIERGVWRATKRFFKQIGSFS
----transmembrane-----

```
                 ------------------------------------------------------------------------
P.jirovecii      1445 PLFEVFVSQIYANSLLQNLAFGGARYIGTGRGFATTRIPFSILFSRFAGASIYLGSRTLIMLLFATVTMWIP
P.carinii        1469 PLFEVFVSQIYANSLLQNLAFGGARYIGTGRGFATTRIPFSILFSRFAGASIYLGSRTLIMLLFATVTMWIP
S.cerevisiae     1399 PMFEVFAGQIYSSALLSDLAIGGARYISTGRGFATSRIPFSILYSRFAGSAIYMGARSMLMLLFGTVAHWQA
S.pombe          1461 PLFEVFTCQVYSQAITSDLAYGGARYIGTGRGFATARLPFSILYSRFAVPSIYIGARFLMMLLFGTMTVWVA
                      *:****. *:*:.:: .:** ******.*******:*:*****:**** .:**:*:* ::****.*:: * .
                                                          ----transmembrane----   --

                 ------------------------------------------------------------------------
P.jirovecii      1517 HLVYFWVSVLALCICPFIFNPHQFSWTDFFVDYREFIRWLSRGNSRSHANSWIGYCRLSRTRITGFKRKALG
P.carinii        1541 HLVYFWVSVLALCISPFIFNPHQFSWTDFFVDYREFIRWLSRGNSRSHANSWIGYCRLSRTRITGFKRKALG
S.cerevisiae     1471 PLLWFWASLSSLIFAPFVFNPHQFAWEDFFLDYRDYIRWLSRGNNQYHRNSWIGYVRMSRARITGFKRKLVG
S.pombe          1533 HLIYWWVSIMALCVAPFLFNPHQFDWNDFFVDYREFIRWLSRGNSRSHANSWIGYCRLTRTRITGYKRRVLG
                       *:::*.*: :* ..**:****** * ***:***::********.: * ****** *::*:****:**: :*
                      --transmembrane-----

                 ------------------------------------------------------------------------
P.jirovecii      1589 QPSEKLSGDIPRAGFNNVFFSEVIGPLILVILSLVPFCFMNSRPGFEPFGK--------SNPARNGSNPLIR
P.carinii        1613 QPSEKLSGDIPRAGFSNVFFSEVIGPMILVLLSLVPYCFINSRPGFEPFGK--------SNPAKNGSNPLIR
S.cerevisiae     1543 DESEKAAGDASRAHRTNLIMAEIIPCAIYAAGCFIAFTFINAQTGVKTTD-------------DDRVNSVLR
S.pombe          1605 QPSDKISMDTPRAKFTNVFFSDVLIPALLAAGAIIPYFFINSQPGNPMFITDPNNPSPYVHDTKTGTNPILR
                      : *:* : * .**  .*:::::::   : .  .::.: *:*::.*                     *.::*
                                      -----transmembrane------                        ---

                 --------------------------------
P.jirovecii      1653 IAIVSFAPICVNALVAFVFFGMACCMGPILTICCKKFGAVLATISHAIAVIVLVAFFEVLWFLEGWSFSKTI
P.carinii        1677 IAIVSFAPICVNAMVAFVFFGMACCMGPILTICCKKFAAVLATISHAIAVIILVTFFEVLWFLEGWSFSKTI
S.cerevisiae     1602 IIICTLAPIAVNLGVLFFCMGMSCCSGPLFGMCCKKTGSVMAGIAHGVAVIVHIAFFIVMWVLESFNFVRML
S.pombe          1677 LVIISLIPIAAGFGMSGFFGGMACCLGPAFGLCCKKFPSIFAAIAHTIQIFIFIAIFEVCWFLDGWSLPKTV
                      : * :: **...  :  .  **:** ** : :****  :::* *:* : ::: :::* * *.*:.:.: : :
                      --transmembrane----------          -------transmembrane-------       -

P.jirovecii      1725 LGLVTMISLQRAFLKMLTIMILTREFKHDGSNLAWWTGRWYSNNLGVHAMSQPAREFVCKVIELSLFAADFC
P.carinii        1749 LGLVTMISLQRAFLKILTIMILTREFKHDGSNLAWWTGRWYSNNLGVYAMSQPAREFVCKVIELSLFAADFC
S.cerevisiae     1674 IGVVTCIQCQRLIFHCMTALMLTREFKNDHANTAFWTGKWYGKGMGYMAWTQPSRELTAKVIELSEFAADFV
S.pombe          1749 LAFCAVTAIHRFIFKILTLLCLSREVKQDSANISWWSGKWYGKGYGYHAFTLPAREFVCKAIELNLFATDFF
                      :.. :    :* ::: :* : *:**.*:* :* ::*:*:**.:. *  * : *:**:..*.***. **:**
                      -transmembrane--------                                          ---trans-

P.jirovecii      1797 LGHLLLFILTPILAIPYIDRWHSMLLFWLRPSRQIRPPIFSLKQNKLRKRIVRRYATLFFGLFLLFLMIILV
P.carinii        1821 LGHLLLFILTPILAIPYIDRWHSMLLFWLRPSRQIRPPIFSLKQNKLRKRIVRRYATLFFGLFLLFLMIILV
S.cerevisiae     1746 LGHVILICQLPLIIIPKIDKFHSIMLFWLKPSRQIRPPIYSLKQTRLRKRMVKKYCSLYFLVLAIFAGCIIG
S.pombe          1821 LGHLLLFFMLPVICIPYIDRWHSVLLFWLRPSRQIRPPIFSTKQNRLRKRIVRRYSALYFSILVIFLILIIV
                      ***::*:   *:: ** **::**::****:*********:* **.:****:*::*.:*:* :: :*   *:
                      -membrane----------                                    ----transmembrane-

P.jirovecii      1869 PAVGHSKFPKSLNNIPALKNLGLIQPSNDPRGATG---RTTRPANSNGTYKM--F-----------T
P.carinii        1893 PALGHSKFPKSLNNIAFLKNLGLIQPSNDPRGATG---RTTRPGNSNGTYKL--FI----------Y
S.cerevisiae     1818 PAVASAKIHKHIGDSLDGVVHNLFQPINTTNNDTGSQMSTYQSHYYTHTPSLKTWSTIK--------
S.pombe          1893 PLAAGAEIRQGLTASEAVAKG-AV-GWNQTNSSIGSGIIQPRDTNYTANYSF--WYDRYHFEFNTTY
                      *  . ::: : :           .   * ...  *      :    . . .:  :
                      ------
```

B


P.jirovecii
P.carinii
S.cerevisiae
S.pombe
cons
transmembrane
GH16 fungal kre6 glucanase

```
                    coiled coil
P.jirovecii      672 KNRLMNEC---------
P.carinii        668 KNRLMNKC---------
S.cerevisiae     704 KNILTGKCTSSKFKLSS
S.pombe          620 KNSLMHKCNT-------
                     ** *  :*
```

Figure S2. Multiple-sequence alignment of Gsc1 (**A**) and Kre6 (**B**) proteins. T-Coffee was used (Notredame *et al.,*2000). Identical, strongly and weakly conserved residues are indicated by asterisks, double points, and single points, respectively. Dashes indicate gaps. **A.** Alignment of the Gsc1 proteins of *P. jirovecii* of Cissé *et al.* (primary accession number L0PD34), *P. carinii* (Q9HEZ4), *S. cerevisiae* (P38631), and the *S. pombe* (Bgs4; O74475). The 1,3-β glucan synthase domains 1 and 2 as well as transmembrane helix domains 1 to 16 are shown respectively above and under the alignment. **B.** Alignment of the Kre6 proteins of *P. jirovecii* (L0P8X6), *P. carinii* (Q6UEI2), *S. cerevisiae* (P32486), and *S. pombe* (O13941). The transmembrane signal-anchor for type II membrane protein domain is shown. The two basic residues shown in bold are implicated in the cytoplasmic localization (Roemer and Bussey 1991). The Kre6 glucanase domain related to the glycoside hydrolase family 16 (GH16) and a coiled coil are also shown.

Annexe 2

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SUSCEPTIBILITY

Site-Directed Mutagenesis of the 1,3-β-Glucan Synthase Catalytic Subunit of *Pneumocystis jirovecii* and Susceptibility Assays Suggest Its Sensitivity to Caspofungin

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ABSTRACT The echinocandin caspofungin inhibits the catalytic subunit Gsc1 of the enzymatic complex synthesizing 1,3-β-glucan, an essential compound of the fungal wall. Studies with rodents showed that caspofungin is effective against *Pneumocystis* asci. However, its efficacy against asci of *Pneumocystis jirovecii*, the species infecting exclusively humans, remains controversial. The aim of this study was to assess the sensitivity to caspofungin of the *P. jirovecii* Gsc1 subunit, as well as of those of *Pneumocystis carinii* and *Pneumocystis murina* infecting, respectively, rats and mice. In the absence of an established *in vitro* culture method for *Pneumocystis* species, we used functional complementation of the *Saccharomyces cerevisiae* gsc1 deletant. In the fungal pathogen *Candida albicans*, mutations leading to amino acid substitutions in Gsc1 confer resistance to caspofungin. We introduced the corresponding mutations into the *Pneumocystis gsc1* genes using site-directed mutagenesis. In spot dilution tests, the sensitivity to caspofungin of the complemented strains decreased with the number of mutations introduced, suggesting that the wild-type enzymes are sensitive. The MICs of caspofungin determined by Etest and YeastOne for strains complemented with *Pneumocystis* enzymes (respectively, 0.125 and 0.12 μg/ml) were identical to those upon complementation with the enzyme of *C. albicans*, for which caspofungin presents low MICs. However, they were lower than the MICs upon complementation with the enzyme of the resistant species *Candida parapsilosis* (0.19 and 0.25 μg/ml). Sensitivity levels of Gsc1 enzymes of the three *Pneumocystis* species were similar. Our results suggest that *P. jirovecii* is sensitive to caspofungin during infections, as are *P. carinii* and *P. murina*.

KEYWORDS echinocandins, drug sensitivity, drug resistance, heterologous functional complementation, *Pneumocystis*

The *Pneumocystis* genus comprises fungal species that colonize the lungs of mammals (1–4). Each of them displays strict host specificity for a single mammalian species. The species infecting humans is *Pneumocystis jirovecii*, an opportunistic pathogen that can cause fatal pneumonia (*Pneumocystis* pneumonia [PCP]) if not treated. The most effective drug against *P. jirovecii* is currently co-trimoxazole, a combination of sulfamethoxazole and trimethoprim, two inhibitors of enzymes that are involved in the folic acid biosynthesis pathway. However, potential resistance is emerging due to the selection of strains carrying specific mutations in the active site of the targets of both molecules (5–9). Moreover, co-trimoxazole can cause important side effects in some patients, such as intolerance and toxicity. For these reasons, it is crucial to find new drugs to treat PCP.

Echinocandins constitute an alternative class of antifungal drugs to consider for the treatment of PCP. This class includes caspofungin (CAS), anidulafungin, and micafungin. They are cyclic hexapeptides with fatty acyl side chains and act as noncompetitive inhibitors of the catalytic subunit Gsc1 of the 1,3-β-glucan synthase enzymatic complex

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TABLE 1 Sequence identity of Gsc proteins to their orthologs and paralogs

Protein	Ortholog or paralog	% identity		
		Whole protein	1,3-β-Glucan synthase domain 1	1,3-β-Glucan synthase domain 2
P. jirovecii Gsc1[a]	*P. carinii* Gsc1	90	94	97
	P. murina Gsc1	91	95	96
	S. cerevisiae Gsc1	59	70	73
S. cerevisiae Gsc1[b]	*S. cerevisiae* Gsc2	87	81	94
	S. cerevisiae Gsc3	51	57	60

[a]Alignment of *P. jirovecii* Gsc1 to its ortholog or paralog is shown in Fig. S1.
[b]Alignment of *S. cerevisiae* Gsc1 to its ortholog or paralog is shown in Fig. S2.

(10). The decrease of the β-glucan synthesis results in the loss of cell integrity and rigidity, which can lead to cell lysis. β-Glucan molecules are components of the cell wall that are homopolymers of β-1,3-linked D-glucose with β-1,6-linked D-glucose side chains present in minority. The Gsc1 protein of *Pneumocystis carinii*, the species infecting rats, was first reported to be inhibited by the compound L-733,560, a molecule structurally close to echinocandins (11) but never used clinically. More recently, Cushion et al. (12) reported the efficacy of the echinocandins (caspofungin, micafungin, and anidulafungin) in reducing cysts number within the lungs of the host. Recently, we identified and functionally ascertained the function of the Gsc1 subunit of *P. jirovecii* using complementation of the orthologous gene of *Saccharomyces cerevisiae* (13). The presence of a unique *gsc1* gene in the genome of *P. jirovecii*, as in that of *P. carinii*, further suggests that the Gsc1 subunit is a potential interesting drug target to fight PCP.

In *S. cerevisiae*, the 1,3-β-glucan synthase catalytic subunit is encoded by two different genes, *GSC1* and *GSC2*. A third paralog, *GSC3*, is also present, but it is involved only during sporulation. The two subunits *GSC1* and *GSC2* are functionally redundant, but their expression is differentially regulated. The expression of *GSC1* is constitutive and responsible for cell wall synthesis during the vegetative growth, while that of *GSC2* is induced by glucose deprivation or pheromones and is also involved in cell wall synthesis during sporulation. The *GSC1* and *GSC2* genes have an essential overlapping function; i.e., only disruption of both genes is lethal. Importantly, the *GSC2* gene can replace the function of the *GSC1* gene during vegetative growth in the case of loss by mutation or deletion (14). The *S. cerevisiae* strain with a deletion of the *GSC1* gene shows a reduced and impaired growth in the presence of CAS (15) or anidulafungin (16) but not of micafungin (16). On the other hand, the *S. cerevisiae* wild type (WT) shows normal growth in the presence of low doses of CAS and anidulafungin, but its growth is severely impaired in the presence of micafungin. These observations showed that the *S. cerevisiae* Gsc1 and Gsc2 subunits have different sensitivities to each echinocandin despite the fact that their identity at the amino acid sequence level is as high as 87% over the whole protein, with 81% and 94% identity at the level of 1,3-β-glucan synthase domains 1 and 2, respectively (Table 1). To our knowledge, the polymorphisms responsible for these different sensitivities have not been determined so far.

Spontaneous mutants resistant to echinocandins were initially isolated in *S. cerevisiae* and *Candida albicans* (17–19). Rare clinical isolates of *C. albicans* were also found to be resistant (20, 21). A specific change of a serine in position 645 to a proline (S645P) was identified in all spontaneous and most clinical resistant *C. albicans* isolates (21). It is localized within a highly conserved region of the Gsc1 protein in which other mutations conferring resistance to CAS were also identified in *C. albicans* (21). This "hot spot no. 1" of mutations starts at residue 641 and ends at residue 649 of *C. albicans* Gsc1. A second but less relevant hot spot of mutations conferring resistance has been identified in another region of the enzyme, from residue 1357 to residue 1364. The S645P substitution has been most frequently observed, a phenylalanine-to-serine substitution in position 641 (F641S) being the second most frequent substitution (22).

The mutation corresponding to the *C. albicans* S645P substitution introduced by site-directed mutagenesis was found to confer reduced susceptibility to CAS *in vitro* to the mold *Aspergillus fumigatus* (23, 24).

Although demonstrated to reduce efficiently the asci during *P. carinii* and *P. murina* infections (10, 12, 25–28), the efficacy of CAS against *P. jirovecii* remains controversial. Indeed, clinical reports documented the clearance of PCP treated with CAS alone (29–31) or used in combination with co-trimoxazole (32–36) or clindamycin (37). However, failures of CAS treatment were also described (38, 39). Despite the generally high conservation of active sites among orthologous enzymes, one cannot exclude that the sensitivity to CAS may vary among *P. jirovecii* and the two *Pneumocystis* species infecting rodents because these species are relatively distant from each other (20% mean divergence at nucleotide level in genomic coding sequences [40]). The *P. jirovecii* Gsc1 subunit bears 90% and 91% identity with those of *P. carinii* and *P. murina*, respectively (Table 1). At the level of 1,3-β-glucan synthase domains 1 and 2, i.e., the active sites, the identities are from 94% to 97%. These values are comparable to those between the Gsc1 and Gsc2 subunits of *S. cerevisiae* (see above), which present drastically different sensitivities to the different echinocandins.

The aim of the present study was to determine if the Gsc1 subunit of *P. jirovecii* is sensitive to the echinocandin CAS, as are those of *P. carinii* and *P. murina*. To investigate the issue, we analyzed the level of sensitivity of *S. cerevisiae* strains functionally complemented by the expression of the wild-type or mutated enzymes of the three *Pneumocystis* species.

(The present work was submitted by A. Luraschi as a partial fulfillment of a Ph.D. degree at the Faculty of Biology and Medicine of the University of Lausanne.)

RESULTS

Functional ascertainment of the *P. carinii* and *P. murina gsc1* genes by complementation of the *S. cerevisiae gsc1* deletant. We identified a single Gsc1 protein within the *P. murina* proteome by a homology search using the Gsc1 protein of *P. carinii* as the query sequence. To ascertain the function of the *P. carinii* and *P. murina gsc1* genes, recombinant plasmids expressing them were introduced into the *S. cerevisiae gsc1* deletant. The identities of the Gsc1 proteins studied relatively to that of *S. cerevisiae* are given in Table S3 in the supplemental material. Serial dilutions of the transformed strains were spotted onto medium containing or not containing CAS (spot dilution test [Fig. 1]). The deletion of the *GSC1* gene in *S. cerevisiae* causes a paradoxical effect, i.e., an increased susceptibility to CAS, though the target of CAS is absent (15). This is due to the replacement of Gsc1 by Gsc2, an enzyme that is more sensitive to CAS (14). On the other hand, this replacement allows the growth of the deletant strain on medium without CAS. On medium supplemented with CAS, a complete restoration of the wild-type growth was observed in the presence of the control *S. cerevisiae GSC1* gene, but not in the presence of the empty vector (Fig. 1, *S. cerevisiae gsc1* deletant + empty plasmid). A partial restoration was observed in the presence of the *P. carinii* or *P. murina* gene, as we previously reported for *P. jirovecii* and reproduced here (Fig. 1). These observations demonstrated that the expression of *P. carinii* and *P. murina gsc1* genes rescued the function of the deleted *S. cerevisiae GSC1* gene, demonstrating their function. In order to investigate the sensitivity to CAS of the three *Pneumocystis* enzymes, we used site-directed mutagenesis to introduce mutations that correspond to those conferring resistance in other fungi.

Sensitivity to CAS of the *S. cerevisiae* strains complemented with the *Pneumocystis* Gsc1 mutated proteins. Mutants resistant to echinocandins carrying mutations F641S and S645P within hot spot no. 1 of Gsc1 have been described for the pathogenic fungus *C. albicans* (17–21). The sequences of this hot spot of mutations of the *P. jirovecii*, *P. carinii*, and *P. murina* Gsc1 protein were aligned with those of *C. albicans*, *S. cerevisiae*, and *C. parapsilosis* (Fig. 2; alignment of the complete proteins is shown in Fig. S1). This alignment identified the positions in the three *Pneumocystis gsc1* genes corresponding to the *C. albicans* F641S and S645P substitutions. Site-directed mutagen-

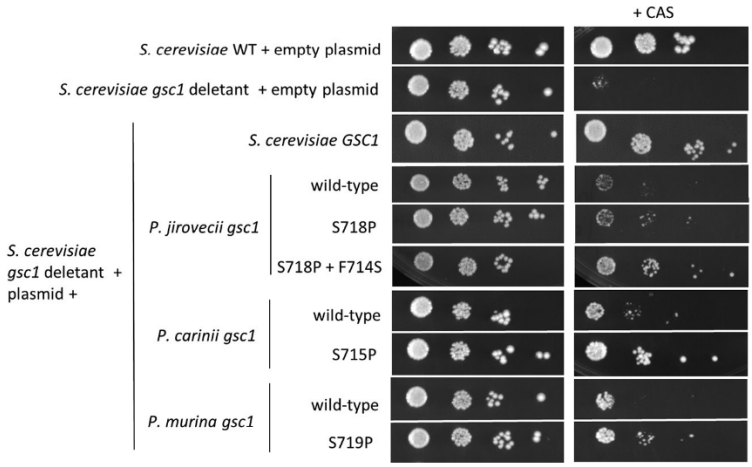

FIG 1 Sensitivity to caspofungin (CAS) of *S. cerevisiae* WT and functionally complemented *gsc1* deletant strains using the spot dilution test. The complementing genes expressed on plasmids, wild type or encoding the indicated amino acid substitution, are listed on the left. The growth of the *S. cerevisiae* deletant strain on medium without CAS was due to the activity of the orthologous gene *GSC2*, which replaces the function of the deleted *GSC1* gene. Log dilutions of a suspension of cells at ca. 7.5×10^5/ml were spotted on minimal selective medium without (left) or with (right) 150 ng/ml of CAS and incubated for 3 days at 30°C. The most concentrated suspension is on the left. The complementing gene was expressed on plasmid p416GPD, except that of *S. cerevisiae*, which was expressed on p415GPD because of restriction site issues. YNB minimal selective medium supplemented with CSM without uracil was used to select for p416GPD, while YNB supplemented with CSM without leucine was used to select p415GPD. Three independent isolates of each strain were analyzed; results for one representative isolate are shown.

esis was used to introduce one or two mutations for the corresponding substitutions within the *gsc1* gene of *P. jirovecii*, *P. carinii*, or *P. murina* (the polymorphisms introduced at the nucleotide sequence level are described in Table S1).

The partial restoration of the wild-type growth on CAS observed with the *P. jirovecii* Gsc1 enzyme increased in the presence of one mutation (Fig. 1, compare the S718P mutant to the wild type) and increased more upon introduction of the two mutations simultaneously (compare the S718P and F714S double mutant to the S718P mutant and the wild type). Similarly, the partial restoration with the *P. carinii* or *P. murina* enzyme increased in the presence of a single mutation (compare the S715P and S719P mutants to their respective wild types). This increase of complementation efficiency corresponds to a decrease of sensitivity to CAS. This demonstrated that the three wild-type *Pneumocystis* enzymes present a certain level of sensitivity to CAS.

MIC assessment using Etest and Sensititre YeastOne. We determined the MICs of CAS for the *S. cerevisiae* WT and complemented *gsc1* deletant strains. To assess the sensitivity of the two methods, we also analyzed *C. albicans* and *C. parapsilosis*, as well

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                                                    ---------
C.albicans          611 NYIKLKGLDMWMSYLLWFLVFLAKLVESYFFLTLSLRDPIRNLSTMTMR-CVGEVWYKDIVCRNQAKIVLGL  681
S.cerevisiae        609 AFAPLHGLDRWMSYLVWVTVFAAKYSESYYFLVLSLRDPIRILSTTAMR-CTGEYWWGAVLCKVQPKIVLGL  679
A.fumigatus         644 SFPRLHGNDMWMSYGLWVCVFGAKLAESYFFLTLSFKDPIRILSPMQIHQCAGVKYIGNVLCHKQPQILLGL  715
P.jirovecii         684 NFAKLKGNDLWLSYGLWIAVFACKFAESYFFLSLSLRDPIRYLNTMTIG-HCGIRYLGSILCPYQAKITLGI  754
P.carinii           681 NFAKLKGNDLWLSYGLWIAVFACKFAESYFFLSLSLRDPIRYLNTMTIG-HCGIRYLGSSLCPYQAKITLGI  751
P.murina            685 NFAKLKGNDLWLSYGLWIAVFACKFAESYFFLSLSLRDPIRYLNTMTIG-HCGIRYLGSALCPYQAKITLGI  755
C.parapsilosis      622 NFVKLRGLDMWMSYLLWVLVFLAKLVESYFFLTLSLRDAIRNLSKTTMR-CTGEVWYGDIVCRQQAKIVLGL  692
                          :  *:* * *:** :*. ** .*  ***:** **::*.** *.   :    *  :    :*  *.:* **:
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FIG 2 Multiple-sequence alignment of the region encompassing hot spot no. 1 of mutations of Gsc1 proteins. T-Coffee was used (48). The identical, strongly, and weakly conserved residues are indicated by asterisks, colons, and periods, respectively. Dashes indicate gaps. The hot spot no. 1 of mutations (21) is shown above the alignment by the dashed line. Residues F641 and S645 conferring CAS resistance in *C. albicans* and the corresponding residues in the other proteins are in bold. The natural resistance of *C. parapsilosis* is due to the polymorphism P660A at the end of the same hot spot of mutations. The alignment of the complete proteins is shown in Fig. S1.

TABLE 2 MICs of caspofungin (CAS) for the *S. cerevisiae* WT and functionally complemented *gsc1* deletant strains, as well as for *Candida* species[a]

Strain	MIC (μg/ml)	
	E-test	YeastOne
S. cerevisiae WT + empty plasmid	0.250	0.25
S. cerevisiae gsc1 deletant + empty plasmid	0.125	0.12
S. cerevisiae gsc1 deletant + plasmid		
+ *S. cerevisiae GSC1*	0.250	0.25
+ *P. jirovecii gsc1* wild type	0.125	0.12
+ S718P	0.125	0.12
+ S718P + F714S	0.125	0.12
+ *P. carinii gsc1* wild type	0.125	0.12
+ S715P	0.125	0.12
+ *P. murina gsc1* wild type	0.125	0.12
+ S719P	0.125	0.12
+ *C. albicans GSC1*	0.125	0.12
+ *C. parapsilosis GSC1*	0.190	0.25
C. albicans	0.380	0.12
C. parapsilosis	0.500	0.50

[a]One isolate among three of each complemented strain was chosen randomly for analysis. One out of two experiments that gave similar results is reported here. Although obtained using various methods that have various sensitivities, the MICs previously published correspond roughly to ours for the *S. cerevisiae* WT (0.25 versus 0.03 to 0.4 μg/ml [15, 16, 21]) and *gsc1* deletant (0.12 to 0.125 versus 0.0015 to 0.1 μg/ml [15, 16, 21]), as well as for *C. albicans* (0.12 to 0.380 versus 0.12 to 0.25 μg/ml [22, 41]) and *C. parapsilosis* (0.50 versus 0.25 to 8 μg/ml [41 -43]).

as the *S. cerevisiae gsc1* deletant complemented with the *GSC1* gene of these two *Candida* species. CAS presents low MICs for the former *Candida* species, whereas it presents high MICs for the latter (41, 42). The natural high MICs for *C. parapsilosis* are due to a polymorphism at the end of the hot spot no. 1 that has not been observed in *C. albicans* so far (43) (proline to serine in position 660 [Fig. 2]). According to Espinel-Ingroff et al. (41) and Canton et al. (42), the MICs of CAS for *C. parapsilosis* that we obtained were below the epidemiological cutoff values for both Etest and YeastOne methods (0.5 versus 4 and 0.5 versus 2 μg/ml, respectively). The MICs for *C. albicans* we obtained were also below the epidemiological cutoff values of wild-type isolates (0.38 versus 0.5 and 0.12 versus 0.25 μg/ml, respectively). In agreement with the spot dilution results described here, we observed using both Etest and YeastOne a decreased MIC for the *S. cerevisiae gsc1* deletant compared to that for the WT (respectively, 0.125 and 0.12 versus 0.250 and 0.25 μg/ml [Table 2]; the Etest results are shown in Fig. S3). All *S. cerevisiae* strains complemented with the *Pneumocystis* wild-type or mutated genes had MICs identical to those of the *gsc1* deletant (0.125 μg/ml for Etest and 0.12 μg/ml for YeastOne). The increase of MIC of CAS conferred by the mutations introduced was not detected using Etest or YeastOne. Thus, these methods are less sensitive than the spot dilution test, since the latter always allowed detection of this decrease in several experiments. The MICs for *C. albicans* whole cells using Etest and YeastOne were similar to those for the *S. cerevisiae* WT strain (0.380 and 0.12 μg/ml versus 0.250 and 0.25 μg/ml), whereas, consistent with its reported high MICs, *C. parapsilosis* had higher MICs also in our hands using both methods (0.500 and 0.50 μg/ml). The increased MICs for *C. parapsilosis* were also detected using both methods upon heterologous expression of its Gsc1 subunit in *S. cerevisiae* (respectively, 0.190 and 0.25 μg/ml, versus 0.125 and 0.12 μg/ml for *C. albicans* Gsc1), despite the fact that MICs were systematically lower using heterologous expression than whole cells. Using the heterologous expression system, the wild-type *Pneumocystis* Gsc1 subunits had MICs identical to those of *C. albicans* Gsc1 (0.125 and 0.12 μg/ml), whereas *C. parapsilosis* Gsc1 presented higher MICs (0.190 and 0.25 μg/ml). These observations suggested that the sensitivity to CAS of the three *Pneumocystis* enzymes is similar to that of *C. albicans*, which presents low MICs for CAS.

DISCUSSION

Because of the absence of an *in vitro* culture method, testing for sensitivity to CAS cannot be performed directly on whole *Pneumocystis* cells. A study reported the effects of echinocandins against *P. murina* and *P. carinii* using suspension and biofilm culture methods (44). Unfortunately, these methods are not established for *P. jirovecii*. Consequently, we studied the Gsc1 enzymes of three *Pneumocystis* species in the heterologous system of expression of *S. cerevisiae*. We used site-directed mutagenesis to introduce into the *Pneumocystis* enzymes the substitutions corresponding to those conferring resistance to CAS in *C. albicans*. This revealed that despite the divergence among their active sites, the three *Pneumocystis* Gsc1 enzymes present low MICs for CAS, and this to similar levels. Because CAS has been demonstrated to be effective in reducing *P. carinii* and *P. murina* asci during infections (12, 27), this observation suggested that CAS could also be effective against *P. jirovecii*. Moreover, MIC determination showed that the level of sensitivity of *Pneumocystis* Gsc1 was similar to that of the *C. albicans* enzyme, suggesting that the sensitivity of the *Pneumocystis* enzymes is at a level that is usable clinically. It is of course difficult to translate our results obtained at the enzyme level to the whole-cell level. Nevertheless, Gsc1 is a cell surface enzyme that is easily reachable by drugs and thus more likely to behave similarly among the three *Pneumocystis* species. A structural difference of the cell wall could induce various sensitivities to CAS of the Gsc1 subunit among the three *Pneumocystis* species. However, there is presently no obvious reason to think that the wall of *P. jirovecii* is different from those of *P. carinii* and *P. murina*. The efficacy of echinocandins, and specifically of CAS, to treat *P. jirovecii* infections remains controversial, and accordingly, the American drug and European medical agencies do not advise their use for that purpose. Our results bring new arguments in favor of the use of this class of antifungals for the treatment of PCP, suggesting the need to implement clinical trials in humans. Finally, our results support the high relevance of the animal models as tools to understand the effect of CAS on the human pathogen *P. jirovecii*.

Studies with animal models showed that echinocandins provoke the disappearance of *P. carinii* and *P. murina* asci but not of the trophic forms, probably because the latter cells have no or little cell wall made of 1,3-β-glucan (12). Thus, the treatment did not eradicate the infection, and its cessation resulted in the repopulation in asci from the remaining trophic cells. Consequently, it is likely that CAS is useful only in combination with another therapy targeting trophic forms, or both cellular forms, such as cotrimoxazole. CAS inhibited efficiently the dissemination of the pathogen in animal models (12), which is consistent with the fact that asci are believed to be the transmission particles (12, 44).

In conclusion, our results demonstrate that the Gsc1 enzyme of the human pathogen *P. jirovecii* is sensitive to caspofungin, similar to the enzymes of the animal pathogens *P. carinii* and *P. murina*. This suggests that echinocandins might be a good alternative to treat PCP in humans when used in combination with an established treatment. The use of echinocandins to fight *Pneumocystis* infections deserves further investigation.

MATERIALS AND METHODS

Strains and growth conditions. Y05251 is an *S. cerevisiae* haploid strain in which the 1,3-β-glucan synthase catalytic subunit gene *GSC1* (also called *FKS1*) was deleted (*MAT***a** *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 YLR342w::kanMX4*). It was obtained from Euroscarf (European *S. cerevisiae* Archive for Functional Analysis [http://www.euroscarf.de]). The strain, called the *gsc1* deletant here, exhibits an impaired growth in the presence of low doses of CAS (14). The parental strain of the *gsc1* deletant is BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 uraΔ0*) and was also obtained from Euroscarf (here referred to as the wild type [WT]). The latter was used as a control in the sensitivity tests and in MIC assays. Strains were grown on complete yeast extract-peptone-dextrose (YEPD) medium (1% [wt/vol] Difco yeast extract, 2% Difco peptone, 2% glucose).

Single colonies of *Candida albicans* (ATCC 10231) and *Candida parapsilosis* (*sensu stricto*, i.e., group I of the *C. parapsilosis* complex; ATCC 22019) were streaked on Sabouraud medium (0.5% [wt/vol] casein peptone, 0.5% meat extract peptone, 2% glucose) and then grown on minimal solid yeast nitrogen base (YNB) medium (0.67% [wt/vol] yeast nitrogen base, 2% glucose, 2% Gibco agar) supplemented with a

complete supplement mixture (CSM; MP Biomedicals). *C. albicans* and *C. parapsilosis* were chosen because CAS presents, respectively, low and high MICs for them.

Cloning of the fungal *gsc1* genes. To identify the *P. murina gsc1* gene, the *P. carinii* Gsc1 protein (Q9HEZ4) was used as query sequence in BLASTp search against *P. murina* proteome at http://blast.ncbi.nlm.nih.gov/Blast.cgi. A single putative ortholog was detected (locus tag PNEG_03180). The *P. murina* gene sequence encoding the Gsc1 protein was then retrieved from the European Nucleotide Archive (http://www.ebi.ac.uk/ena). The cloning of the *P. jirovecii* and *S. cerevisiae gsc1* genes was previously described (13). Since the *P. carinii* and *P. murina gsc1* genes include each three introns, their cDNAs were synthesized and cloned into the p416GPD vector (45) by GeneCust Europe (Ellange Luxemburg). Their sizes without introns are, respectively, 5,835 bp and 5,847 bp.

To perform a control of sensitivity of our heterologous expression model, the *GSC1* genes of *C. albicans* (GenBank accession number D88815) and *C. parapsilosis* (European Nucleotide Archive accession number EU221325) were amplified by PCR from yeast genomic DNA extracted as described previously (46). The detailed procedures for PCR amplification using the proofreading high-fidelity Expand polymerase (Roche Diagnostics) and cloning were described previously (47). Their sizes are, respectively, 5,694 and 5,730 bp. PCR primers and conditions are listed in Tables S1 and S2. Because these primers were intended for oriented cloning, they were designed to create unique restriction sites at ends of the PCR products. After the PCRs, the products were extracted using a QIAquick gel extraction kit (Qiagen, Basel, Switzerland). For cloning each *Candida GSC1* gene into the p416GPD expression vector, the double restriction described in Table S1 were used.

Site-directed mutagenesis. The Gsc1 protein sequences of *C. albicans* (UniProt identifier O13428), *S. cerevisiae* (P38631), *P. jirovecii* (L0PD34, locus tag PNEJI1_001061), *P. carinii* (Q9HEZ4), *P. murina* (M7P3D9, locus tag PNEG_03180), and *C. parapsilosis* (A9YLC3) were aligned using T-Coffee (48). This alignment allowed determination of the positions within the *Pneumocystis* genes corresponding to the mutations F641S and S645P conferring resistance to CAS on *C. albicans* (Fig. 2; alignment of the complete proteins is shown in Fig. S1). To perform site-directed mutagenesis, two different kits were used. The QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) was used to create the mutation in the *P. jirovecii gsc1* gene leading to the change of the serine at position 718 of the Gsc1 protein to a proline (S718P). The Q5 site-directed mutagenesis kit (BioLabs) was used to introduce the F714S/S718P double substitution in *P. jirovecii*, the S715P substitution in *P. carinii*, and the S719P substitution in *P. murina*. Mutagenesis was performed according to the manufacturers' instructions. Minipreparations of plasmid DNA were subsequently carried out (49). In order to verify the presence of the desired mutations, an internal segment of the *gsc1* genes was amplified and subsequently sequenced. Primers for mutagenic reactions and PCR amplifications are listed in Table S1. Mutagenesis amplification reactions and PCR conditions are described in Table S2. Sequencing of both strands was performed using the two primers used for amplification, as well as the BigDye Terminator DNA sequencing kit and ABI PRISM 3100 automated sequencer (both from PerkinElmer Biosystems).

Transformation of the *S. cerevisiae gsc1* deletant. Transformation with plasmids containing the *P. jirovecii gsc1* or the *S. cerevisiae GSC1* gene were previously described (13). The *S. cerevisiae GSC1* gene could not be cloned in the p416GPD plasmid because of restriction sites issues, but it was cloned into p415GPD (*leu* marker instead of *ura*). The recombinant p416GPD plasmids containing the *Pneumocystis* mutated *gsc1* alleles, as well as the *C. albicans* or *C. parapsilosis GSC1* gene, were introduced into the *gsc1* deletant by transformation for uracil prototrophy using the one-step method (50). Transformants were selected on solid YNB medium supplemented with CSM (MP Biomedicals) lacking uracil. In order to be used as controls in the sensitivity tests and in the MIC assays, the *gsc1* deletant and the WT were transformed with the empty p416GPD plasmid. Three transformants of each constructed strain were randomly chosen and purified by growth on the same selective medium.

Test of complementation and susceptibility to caspofungin. Before studying the sensitivity to echinocandins, we had to assess the function of the *P. carinii* and the *P. murina gsc1* genes, as we previously did for the *P. jirovecii gsc1* gene (13). Functional complementation of the *gsc1* deletant was proven by the spot dilution test on YNB selective medium lacking uracil and supplemented or not with 150 ng/ml of CAS (Fluka Chemie AG). CAS appeared sensitive to temperature variation in our study. Consequently, the medium was cooled down to 54°C before addition of CAS. In addition, petri dishes containing CAS were stored at room temperature but not at 4°C. The concentration of CAS used in our experiments was selected after several trials. To that aim, transformant isolates carrying the *P. carinii gsc1* or *P. murina gsc1* gene were grown overnight in YNB selective medium supplemented with CSM lacking uracil to avoid the loss of the plasmid. Cells were then diluted at an optical density at 540 nm (OD_{540}) of 0.1 in 0.9% (wt/vol) NaCl (ca. 7.5×10^5 cells/ml). Four serial 10-fold dilutions in 0.9% NaCl were prepared, and 3 μl of each dilution was spotted onto the medium. Spots were observed after 3 to 4 days of incubation at 30°C. The same procedure was used to assess the functionality and sensitivity to CAS of the strains complemented with the mutated *gsc1* alleles. The sensitivity to micafungin could not be studied because the *gsc1* deletant had no phenotype to complement on this drug, i.e., the Gsc2 subunit is resistant. Anidulafungin could also not be studied because, for underdetermined reasons, the results were not reproducible in our hands.

MIC assessment using Etest. Assays were performed according to the manufacturer's instructions. Each strain was grown overnight in YNB selective medium plus CSM lacking uracil, or leucine for the *S. cerevisiae GSC1* gene, and then adjusted in 0.9% NaCl to an OD_{540} of 0.2 ($\sim 1.5 \times 10^6$ cells/ml). One hundred microliters of this dilution was spread on fresh YNB solid medium plus CSM lacking uracil or leucine. A single strip of Etest caspofungin (bioMérieux) was then applied on each petri dish. MICs were

read after 2 days of incubation at 30°C, or at 35°C for the *Candida* species. The MIC was defined as the concentration at which no growth was observed on both sides of the Etest strip.

MIC assessment using Sensititre YeastOne. Assays were performed according to the manufacturer's instructions, except that the incubation time was increased from 24 to 48 h for all *S. cerevisiae* strains because of their low growth rate. Each strain was grown overnight in YNB selective medium and then adjusted in 0.9% NaCl to an OD_{540} of 0.2 (ca. 1.5×10^6 cells/ml). Twenty microliters of this dilution was then diluted into 11 ml of YeastOne inoculum broth in order to obtain ca. 3×10^3 cells/ml. One hundred microliters was then transferred into each well of a YeastOne plate (Thermo Fisher Scientific). Plates were observed and MICs determined after 24 or 48 h of incubation at 30°C, or at 35°C for the *Candida* species. The MIC was defined as the first well in which no pellet of cells was observable.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01159-18.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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Supplementary data of annexe 2

Experiment	Target [a]	Primer	5'-3' nucleotide sequence	Fragment amplified size (bp)	Description
Single substituion introduction	Pj*gsc1*	Pj*GSC1*t2152cStart	TAGGATCTCGCAGAG**G**AAGAGATAGAAAAAAGTATGATTCTGCA[b, c]	11564	Pj*gsc1* position 2124 to 2167 containing the t2152c substitution (S718P in the protein)
		Pj*GSC1*t2152cEnd	TGCAGAATCATACTTTTTTCTATCTCTT**C**CTCTGCGAGATCCTA[b,c]		
Control mutagenesis	Pj*gsc1* internal fragment	Pj*gsc1*control mutation A	CTCCATGGAAACCGAACCTC	511	Pj*gsc1* position 1880 to 1894
		Pj*gsc1*control mutation B	TATGTTTCTCCAAGGCGTCC		Pj*gsc1* position 2372 to 2391
double substituion introduction	Pj*gsc1*	Pj*gsc1*t2141c/t2152cStart	CTCTT**C**CTCTGCGAGATCCTATTAG[d]	11564	Pj*gsc1* position 2147 to 2171 containing the t2152c substitution (S718P in the protein)
		Pj*gsc1*t2141c/t2152cEnd	ATAGA**G**AAAAGTATGATTCTGCAAAC[b, d]		Pj*gsc1* position 2121 to 2146 containing the t1241c substitution (F714S in the protein)
Single substituion introduction	Pc*gsc1*	Pc*gsc1*t2143cStart	CTTGTCTCTT**C**CTTTGAGAGATC[b,d]	11413	Pc*gsc1* position 2133 to 2155 containing the t2143c substitution (S715P in the protein)
		Pc*gsc1*t2143cEnd	AAGAAATAGGATTCTGCAAATTTG[d]		Pc*gsc1* position 2109 to 2132
Control mutagenesis	Pc*gsc1* internal fragment	Pc*gsc1* control mutation A	TGCTCCATGGAAACCGAATGTTAG	471	Pc*gsc1* position 1869 to 1892
		Pc*gsc1* control mutation B	TGACCTAGCGACTGAACAAATAG		Pc*gsc1* position 2318 to 2340
Single substituion introduction	Pm*gsc1*	Pm*gsc1*t2155cStart	CTTGTCTCTT**C**CTTTGAGAGATC[b, d]	11625	Pm*gsc1* position 2145 to 2167 containing the t2155c substitution (S719P in the protein)
		Pm*gsc1*t2155cEnd	AAGAAATAGGATTCAGCAAAC[d]		Pm*gsc1* position 2124 to 2144
Control mutagenesis	Pm*gsc1* internal fragment	Pm*gsc1* control mutation A	GGAAACCGAATGTCAGCGGTC	462	Pm*gsc1* position 1889 to 1905
		Pm*gsc1* control mutation B	GATCTGGCGACAGAACAAATAG		Pm*gsc1* position 2330 to 2351

Table S1. Primers for mutagenesis and controls.
[a] Pj, *P. jirovecii* ; Pc*, P. carinii*, Pm, *P. murina*.
[b] Bold residues represent the nucleotide to be substituted.
[c] Designed with QuikChange Primer Design Program (http://www.agilent.com/genomics/qcpd).
[d] Designed with NEBaseChanger program (http://nebasechanger.neb.com/).
[e] These primers include the underlined restriction sites for oriented cloning and three or six upstream bases allowing restriction.

	Initial denaturation		Denaturation		Annealing		Elongation		Final extension	
Target / Substitution[a]	sec	°C	sec	°C	sec	°C	sec	°C	sec	°C
Pj*gsc1* / S718P[b]	60	95	50	95	50	60	700	68	420	68
Pj*gsc1* / F714S/S718P[c]	30	98	10	98	30	56	360	72	120	72
Pc*gsc1* / S715P[c]	30	98	10	98	30	57	360	72	120	72
Pm*gsc1* / S715P[c]	30	98	10	98	30	57	360	72	120	72
Pj*gsc1* 1880-2391[d]	180	94	30	94	30	57	40	72	600	72
Pc*gsc1* 1869-2340[d]	180	94	30	94	30	63	40	72	600	72
Pm*gsc1* 1889-2351[d]	180	94	30	94	30	60	60	72	600	72

Table S2. Conditions of mutagenesis and PCR reactions.
[a] Pj, *P. jirovecii* ; Pc, *P. carinii*; Pm, *P. murina*.
[b] QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies).
[c] Q5 Site-Directed Mutagenesis Kit (BioLabs).
[d] Amplification using the Expand high fidelity (Roche, Basel Switzerland), 35 cycles.
[e] Amplification using the Kapa Long Range HotStart polymerase (kappa Biosystem, Baden, Switzerland), 35 cycles.

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C.albicans         1 MSYNDNNNH---YYDPNQQGGMPPHQG----------GEGYYQQQYDDMGQQ--P-HQQDYYDP-NA-----   50
S.cerevisiae       1 MNTD---QQ---PYQGQTDYTQGP--G----------NGQSQEQDYDQYGQPLYPSQADGYYDP-NVAAGTE   53
A.fumigatus        1 MSGY---QQGGGHYNDGYGHQEHG------------------------DSFYQDEHGQAYYDH--DYGDGY   42
P.jirovecii        1 MSQR---QH---YYDDSYPSQTDPYYADNGYNNADFHGSSYAPEGYDHQGAYHPMEYGQEYYDEGYDNGQVP   66
P.carinii          1 MSQQ---QH---YYDDSYGGQNGGYYGEHSYDNTGFNNGSYGSGVY-EQGGYYGPEYGQEYYDE-YDGGGAM   64
P.murina           1 MSQQ---QH---YYDESYPGQNGPYYGEHGYGNSGFNNGSYASGVYDEQGEYYAPEYGQEYYDE-YDSGGVM   65
                   1 *.     ::    *:                                 .       : ***           72

C.albicans        51 ---------------QYQQQPY---------------------------DMDGYQDQA---NYGGQPMNAQG   77
S.cerevisiae      54 A-------------DMYGQQPP---------------------------NESYDQDYTN-GEYYGQ-PPNM-   82
A.fumigatus       43 YDRSG---YYGPDGNHNQQEGGYYDAGQPHDDYYGDHY--------------YDQGNGQ-QGY-DNRGRRRG   95
P.jirovecii       67 YDARAFDMYSPSDDAYYRQENAYYDY--PADAYATDVYDPYGMPIADQHPLQYFQDHGN-YMY-NRKGKHRG  134
P.carinii         65 YNGQGHEMYNSGEEGYYRQEEGYYDY--PQDGYVGDTY---GIKKDILRGNGYFQGQDEYYTY-DRKGKRRG  130
P.murina          66 YDGQGREMYNGGEDGYYRQEDRYYDY--PQDAYIADTYDPYGVPMADQRPLQYFEGQDEYHMY-DRKGKRRG  134
                  73                  *:                                   :.      * ..       144

C.albicans        78 YN-ADPEAFSDFSYGGQTPGTPGYDQYGT------------------------QYT-PSQMSYGGDPRSSGA  123
S.cerevisiae      83 AA-QDGENFSDFSSYGP-PGTPGYDSYGG------------------------QYT-ASQMSYGEP-NSSGT  126
A.fumigatus       96 DSEEDSETFSDFTMRSETARAADMDYYGRGDERYNSYADS-----QYGGRGYGYRPPSSQISYGAN-RSSGA  161
P.jirovecii      135 SS-EGSEAFSDFTMRSDMARAAEFDAYGRFDEQYRSYAPSTESLNQMASR-RGYYPDSSQISYTGN-RSSGA  203
P.carinii        131 SS-EASETFSDFTMRSDMVRAAEYDSYGRFDERYRSYEPSTESLNQMASRQRGYRP-DSQISYTGN-RSSGA  199
P.murina         135 SS-EGSETFSDFTMRSDMARAAEFDSYGRFDERYRSYGPSTESLNQMASRQRGYRP-DSQISYTGN-RSSGA  203
                 145       * ****:  .    :.  * **                              .  **:**    .***:  216

C.albicans       124 STPIYGGQGQGYDPTQFNMSSNLPYPAWSADPQAPIKIEHIEDIFIDLTNKFGFQRDSMRNMFDYFMTLLDS  195
S.cerevisiae     127 STPIYGNYDPN-AI--AMALPNEPYPAWTADSQSPVSIEQIEDIFIDLTNRLGFQRDSMRNMFDHFMVLLDS  195
A.fumigatus      162 STPVYGMDYGN-A-LPAGQRSREPYPAWASDGQVPVSKEEIEDIFLDLVNKFGFQRDSMRNMYDHLMTMLDS  231
P.jirovecii      204 STPVYGMEYNQ-AAMMTSARSREPYPAWTAENQIPISKEEIEDIFIDLTNKFGFQRDSMRNMYDHMMVLLDS  274
P.carinii        200 STPIYGMYYNQ-AAMMTSARSREPYPTWTAENQIPISKEEIEDIFIDLTNKFGFQRDSMRNMYDHMMVLLDS  270
P.murina         204 STPIYGMYYNQ-AAMMTSARSREPYPAWTAENQIPVSKEEIEDIFIDLTNKFGFQRDSMRNMYDHMMVLLDS  274
                 217 ***:**             .. ***:*::: * *:. *.*****:**.*::**********:*::*.:***  288

C.albicans       196 RSSRMSPAQALLSLHADYIGGDNANYRKWYFSSQQDLDDSLGFANMTLGKIGRKARKASKKSKKARKAAEEH  267
S.cerevisiae     196 RSSRMSPDQALLSLHADYIGGDTANYKKWYFAAQLDMDDEIGFRNMSLGKLSRKARKAKKKNKKAME--EAN  265
A.fumigatus      232 RASRMTPNQALLSLHADYIGGDNANYRRWYFAAHLDLDDAVGFANMKLGKADRKTRKARKAAKKAAQQ---N  300
P.jirovecii      275 RASRMTPNQALLSLHADYIGGDNANYRNWYFAAQLDLDDAVGFSNMDFEK-NKKTNHSQKFSK-SQKN----  340
P.carinii        271 RASRMTPNQALLSLHADYIGGDNANYRNWYFAAQFDLDDAVGFSNMDLDK-NRKSNYSQKSSKKFQKN----  337
P.murina         275 RASRMTPNQALLSLHADYIGGDNANYRNWYFAAQFDLDDAVGFSNMDLGK-NRKSSYSQKSSKKFQKN----  341
                 289 *:***:* ***************.***:.***::: *:** :** ** : * .:*:  : *  *   :      360

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C.albicans       268 GQDVDALANELEGDYSLEAAEIRWKAKMNSLTPEERVRDLALYLLIWGEANQVRFTPECLCYIYKSATDYLN  339
S.cerevisiae     266 PEDTEETLNKIEGDNSLEAADFRWKAKMNQLSPLERVRHIALYLLCWGEANQVRFTAECLCFIYKCALDYLD  337
A.fumigatus      301 PENVEETLEALEGDNSLEAAEYRWKTRMNKMSQHDRVRQLALFLLCWGEANQVRFLPECLCFIFKCADDYYN  372
P.jirovecii      341 -TTAKDILQALESDNPLESAIYRWKTKCSQMSQYDRARELALYLLCWGEANQVRFTPECLCFIFKCANDYLN  411
P.carinii        338 -SASKSILQALDGDNSLESAIYRWKTRCTQMSQYDRARELALYLLCWGEANQVRFTPECLCFIFKCANDYLN  408
P.murina         342 -SASKNILQALDGDNSLESAIYRWKTRCTQMSQYDRARELALYLLCWGEANQVRFTPECLCFIFKCANDYLN  412
                 361     .   : ::.* .**:*  ***:: ..::  :*.*.:**:** ********* .****:*:*.* ** :  432

                         1,3-β glucan synthase domain 1
                 _____________________________________________________________________________
C.albicans       340 SPLCQQRQEPVPEGDYLNRVITPLYRFIRSQVYEIYDGRFVKREKDHNKVIGYDDVNQLFWYPEGISRIIFE  411
S.cerevisiae     338 SPLCQQRQEPMPEGDFLNRVITPIYHFIRNQVYEIVDGRFVKRERDHNKIVGYDDLNQLFWYPEGIAKIVLE  409
A.fumigatus      373 SPECQNRVEPVEEFTYLNEIITPLYQYCRDQGYEIVDGKYVRRERDHNQIIV-SDMNQLFWYPEGIERIALE  443
P.jirovecii      412 SPQCQAMVEPVPEGSYLNDIITPLYIYMRDQGYEIINGKYVRRERDHNKIIGYDDINQLFWYSEGIERIVLS  483
P.carinii        409 SPQCQAMVEPAPEGSYLNDVITPLYAYMRDQGYEIINGRYVRRERDHNKIIGYDDINQLFWYPEGIQRIVLS  480
P.murina         413 SPQCQAMVEPAPEGSYLNDVITPLYTYMRDQGYEIINGRYVRRERDHNKIIGYDDINQLFWYPEGIERIVLS  484
                 433 ** **   **  *  :** :***:* : *.* *** :*::*:**:***:::  .*:******.*** :* :.  504

C.albicans       412 DGTRLVDIPQEERFLKLGEVEWKNVFFKTYKEIRTWLHFVTNFNRIWIIHGTIYWMYTAYNSPTLYTKHYVQ  483
S.cerevisiae     410 DGTKLIELPLEERYLRLGDVVWDDVFFKTYKETRTWLHLVTNFNRIWVMHISIFWMYFAYNSPTFYTHNYQQ  481
A.fumigatus      444 DKTRLVDIPPAERWTKLKDVVWKKAFFKTYKETRSWFHMITNFNRIWVIHLGAFWFFTAFNAQSLYTDNYQQ  515
P.jirovecii      484 DKTRIIDLPPEQRYLRLKDVVWKKVFFKTYRETRSWFHLFTNFNRIWIIHITVYWFYTAANSPTVYTHNYQQ  555
P.carinii        481 DKTRMVDLPLDQRYPRFKDVVWKKAFFKTYRETRSWFHLFTNFNRIWIIHITVYWFYTAANSPTVYTHNYQQ  552
P.murina         485 DKTRMVDLPLDQRYPRFKDVVWKKAFFKTYRETRSWFHLFTNFNRIWIIHITVYWFYTAANSPTVYTHNYQQ  556
                 505 * *:::::*  :*: :: :* *...*****:* *:*:*:.*******::*   :*:: * *: :.**.:* *  576

C.albicans       484 TINQQPLASSRWAACAIGGVLASFIQILATLFEWIFVPREWAGAQHLSRRMLFLVLIFLLNLVPPVYTFQIT  555
S.cerevisiae     482 LVDNQPLAAYKWASCALGGTVASLIQIVATLCEWSFVPRKWAGAQHLSRRFWFLCIIFGINLGPIIFVFAYD  553
A.fumigatus      516 QVNNKPPGYRIWSAVGFGGALSSFIQIAATICEWMYVPRRWAGAQHLTKRLMFLILVFVINLAPGVFVFAYS  587
P.jirovecii      556 SLDNQPPFAYRMSAVSFGGGVASLLMIIATLAEWAYVPRKWAGAQHLTRRLLFLILFLIINVAPGVYVIKFA  627
P.carinii        553 SLDNQPPFAYRMSAVGFGGGVASLLMIVATLAEWAYVPRKWPGAQHLTRRLLFLILFFIINVAPGVYVIKFA  624
P.murina         557 SLDNQPPFAYRMSAVGFGGGVASLLMIIATLAEWAYVPRRWPGAQHLTRRLLFLILFLIINVAPGVYVIKFA  628
                 577  ::::*      :: .:** ::*:: * **: ** :***.*.*****::*: ** :.: :*: * ::.:     648

C.albicans       556 KLV-IYSKSAYAVSIVGFFIAVATLVFFAVMPLGGLFTSYMNKRSRRYIASQTFTANYIKLKGLDMWMSYLL  626
S.cerevisiae     554 KDT-VYSTAAHVVAAVMFFVAVATIIFFSIMPLGGLFTSYMKKSTRRYVASQTFTAAFAPLHGLDRWMSYLV  624
A.fumigatus      588 KSMGISKTIPLIVGIVHFFVALATFVFFSVMPLGGLFGSYLKKHGRQYVASQTFTASFPRLHGNDMWMSYGL  659
P.jirovecii      628 PWKPKVSVVTTLISIIHFLIAMFTFLFFAIMPLGGLFGNYLYKKTRRYVASQTFTANFAKLKGNDLWLSYGL  699
P.carinii        625 PWKPNVSIVTTLISIMHFLIAIFTFLFFAIMPLGGLFGNYLYKKTRRYVASQTFTANFAKLKGNDLWLSYGL  696
P.murina         629 QWKPNVSVVTTLISIMHFLIAIFTFLFFAIMPLGGLFGNYLYKKTRRYVASQTFTANFAKLKGNDLWLSYGL  700
                 649       .  .  :. : *::*: *::**::******* .*: *  *:*:******* :  *:* * *:** :  720
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                          Hot spot 1
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C.albicans       627 WFLVFLAKLVESYFFLTLSLRDPIRNLSTMTMRCV-GEVWYKDIVCRNQAKIVLGLMYLVDLLLFFLDTYMW  697
S.cerevisiae     625 WVTVFAAKYSESYYFLVLSLRDPIRILSTTAMRCT-GEYWWGAVLCKVQPKIVLGLVIATDFILFFLDTYLW  695
A.fumigatus      660 WVCVFGAKLAESYFFLTLSFKDPIRILSPMQIHQCAGVKYIGNVLCHKQPQILLGLMFFMDLTLFFLDSYLW  731
P.jirovecii      700 WIAVFACKFAESYFFLSLSLRDPIRYLNTMTIGHC-GIRYLGSILCPYQAKITLGIMYITDLVLFFLDTYLW  770
P.carinii        697 WIAVFACKFAESYFFLSLSLRDPIRYLNTMTIGHC-GIRYLGSSLCPYQAKITLGIMYITDLVLFFLDTYLW  767
P.murina         701 WIAVFACKFAESYFFLSLSLRDPIRYLNTMTIGHC-GIRYLGSALCPYQAKITLGIMYITDLVLFFLDTYLW  771
                 721 *. ** .*  ***:** **::**** *..  :    *  :    :*  *.:* **::   *: *****:*:*  792

C.albicans       698 YIICNCIFSIGRSFYLGISILTPWRNIFTRLPKRIYSKILATTEMEIKYKPKVLISQIWNAIVISMYREHLL  769
S.cerevisiae     696 YIIVNTIFSVGKSFYLGISILTPWRNIFTRLPKRIYSKILATTDMEIKYKPKVLISQVWNAIIISMYREHLL  767
A.fumigatus      732 YIICNTVFSVARSFYLGVSIWSPWRNIFSRLPKRIYSKVLATTDMEIKYKPKVLISQVWNAIIISMYREHLL  803
P.jirovecii      771 YIIWNTICSVARSFYLGVSIWTPWRNIFSRMPKRIYSKILATNDMEIKYKPKVLISQ---------------  827
P.carinii        768 YIIWNTICSVARSFYLGVSIWTPWRNIFSRMPKRIYSKILATNDMEIKYKPKVLISQVWNAVVISMYREHLL  839
P.murina         772 YIIWNTICSVARSFYLGVSIWTPWRNIFSRMPKRIYSKILATNDMEIKYKPKVLISQVWNAVVISMYREHLL  843
                 793 *** * : *:.:*****:** :******:*:*******:***.:**************              864

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C.albicans       770 AIDHVQKLLYHQVPSEIEGKRTLRAPTFFVSQDDNNFETEFFPRNSEAERRISFFAQSLATPMPEPLPVDNM  841
S.cerevisiae     768 AIDHVQKLLYHQVPSEIEGKRTLRAPTFFVSQDDNNFETEFFPRDSEAERRISFFAQSLSTPIPEPLPVDNM  839
A.fumigatus      804 AIDHVQKLLYHQVPSEQEGKRTLRAPTFFVSQEDQSFKTEFFPPGSEAERRISFFAQSLSTPMPEPLPVDNM  875
P.jirovecii      828 ------------VPSEQEGKRTLRAPTFFISQEDHSFKTEFFPSHSEAERRISFFAQSLSTPIPEPLPVDNM  887
P.carinii        840 AIDHVQKLLYHQVPSEQEGKRTLRAPTFFISQEDHSFKTEFFPSHSEAERRISFFAQSLSTPIPEPLPVDNM  911
P.murina         844 AIDHVQKLLYHQVPSEQEGKRTLRAPTFFISQEDHSFKTEFFPSHSEAERRISFFAQSLSTPIPEPLPVDNM  915
                 865             **** ************:**:*:.*:*****  **************:**:*********  936

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C.albicans       842 PTFTVFTPHYSEKILLSLREIIREDDQFSRVTLLEYLKQLHPVEWDCFVKDTKILAEETAAYENGDDSEKLS  913
S.cerevisiae     840 PTFTVLTPHYAERILLSLREIIREDDQFSRVTLLEYLKQLHPVEWECFVKDTKILAEETAAYEGN-ENEAEK  910
A.fumigatus      876 PTFTVLIPHYSEKILLSLREIIREDEPYSRVTLLEYLKQLHPHEWDCFVKDTKILADETSQFNGE--PEKSE  945
P.jirovecii      888 PTFTVLVPHYGEKILYSLREIIREDDQLSRVTLLEYLKQLHPVEWDCFVKDTKILAEETSLYNGGSSFDKDE  959
P.carinii        912 PTFTVLVPHYGEKILYSLREIIREDDQLSRVTLLEYLKQLHPVEWDCFVKDTKILAEETSLYNGGVPFDKDE  983
P.murina         916 PTFTVLVPHYGEKILYSLREIIREDDQLSRVTLLEYLKQLHPVEWDCFVKDTKILAEETSLYNGGVPFDKDE  987
                 937 *****: ***.*:** *********:  ************** **:**********:**: ::.    :  . 1008

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C.albicans       914 EDGLKSKIDDLPFYCIGFKSAAPEYTLRTRIWASLRSQTLYRTVSGFMNYARAIKLLYRVENPELVQYFGGD  985
S.cerevisiae     911 EDALKSQIDDLPFYCIGFKSAAPEYTLRTRIWASLRSQTLYRTISGFMNYSRAIKLLYRVENPEIVQMFGGN  982
A.fumigatus      946 KDVAKSKIDDLPFYCIGFKSAAPEYTLRTRIWSSLRSQTLYRTVSGFMNYSRAIKLLYRVENPEVVQMFGGN 1017
P.jirovecii      960 KDTVKSKIDDLPFYCVGFKSAAPEYTLRTRIWASLRSQTLYRTVSGFMNYSRAIKLLYRVENPDVVQMFGGN 1031
P.carinii        984 KDTVKSKIDDLPFYCVGFKSSAPEYTLRTRIWASLRSQTLYRTVSGFMNYSRAIKLLYRVENPDVVQMFGGN 1055
P.murina         988 KDTVKSKIDDLPFYCVGFKSSAPEYTLRTRIWASLRSQTLYRTVSGFMNYSRAIKLLYRVENPDVVQMFGGN 1059
                1009 :*  **:********:****:***********:**********:******:************:.:** ***: 1080

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C.albicans       986 PEGLELALERMARRKFRFLVSMQRLSKFKDDEMENAEFLLRAYPDLQIAYLDEEPALNEDEEPRVYSALIDG 1057
S.cerevisiae     983 AEGLERELEKMARRKFKFLVSMQRLAKFKPHELENAEFLLRAYPDLQIAYLDEEPPLTEGEEPRIYSALIDG 1054
A.fumigatus     1018 SEKLERELERMARRKFKIVVSMQRYAKFNKEERENTEFLLRAYPDLQIAYLDEEPPVNEGEEPRLYSALIDG 1089
P.jirovecii     1032 TDKLEHELERMARRKFKFVISMQRFFKFNKEEQENTEFLLRAYPDLQIAYLDEEPPSHEGDEPKIYSSLIDG 1103
P.carinii       1056 TDKLEHELERMARRKFKFDISMQRFFKFSKEELENTEFLLRAYPDLQIAYLDEEPPMNEGDEPKIYSSLIDG 1127
P.murina        1060 TDKLEHELERMARRKFKFVISMQRFFKFSKEELENTEFLLRAYPDLQIAYLDEEPPMNEGDEPKIYSSLIDG 1131
                1081 .: **  **:******:: :****  **. .* **:********************.  *.:**::**:**** 1152

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C.albicans      1058 HCEMLENGRRRPKFRVQLSGNPILGDGKSDNQNHAVIFHRGEYIQLIDANQDNYLEECLKIRSVLAEFEEMN 1129
S.cerevisiae    1055 HCEILDNGRRRPKFRVQLSGNPILGDGKSDNQNHALIFYRGEYIQLIDANQDNYLEECLKIRSVLAEFEELN 1126
A.fumigatus     1090 HCELLENGMRKPKFRIQLSGNPILGDGKSDNQNHSIIFYRGEYIQVIDANQDNYLEECLKIRSVLAEFEELT 1161
P.jirovecii     1104 YSEIMEDGRRRPKFRIQLSGNPILGDGKSDNQNHAIIFYRGEYIQLIDANQDNYLEECLKIRSVLAEFEEMS 1175
P.carinii       1128 YSEIMENGKRRPKFRIQLSGNPILGDGKSDNQNHAIIFYRGEYIQLIDANQDNYLEECLKIRSVLAEFEEMT 1199
P.murina        1132 YSEIMENGKRRPKFRIQLSGNPILGDGKSDNQNHAIIFYRGEYIQLIDANQDNYLEECLKIRSVLAEFEEMT 1203
                1153 :.*::::* *:****:********************::**:******:************************:. 1224

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C.albicans      1130 VEHVNPYAPNLKSEDNNTKKDPVAFLGAREYIFSENSGVLGDVAAGKEQTFGTLFARTLAQIGGKLHYGHPD 1201
S.cerevisiae    1127 VEQVNPYAPGLRYEE-QTTNHPVAIVGAREYIFSENSGVLGDVAAGKEQTFGTLFARTLSQIGGKLHYGHPD 1197
A.fumigatus     1162 TDNVSPYTPGIPS----TNTNPVAILGAREYIFSENIGVLGDVAAGKEQTFGTLFARTLAQIGGKLHYGHPD 1229
P.jirovecii     1176 PLEEFPYNPNENS----KVNNPVAILGAREYIFSENIGVLGDVAAGKEQTFGTLFARTLAQIGGKLHYGHPD 1243
P.carinii       1200 PTEESPYNPNEIS----SATNPVAILGAREYIFSENIGVLGDVAAGKEQTFGTLFARTLAQIGGKLHYGHPD 1267
P.murina        1204 PIEESPYNPNEVS----SAANPVAILGAREYIFSENIGVLGDVAAGKEQTFGTLFARTLAQIGGKLHYGHPD 1271
                1225   .  ** *.       .  .***::********** **********************:************ 1296

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C.albicans      1202 FLNATFMLTRGGVSKAQKGLHLNEDIYAGMNAMMRGGKIKHCEYYQCGKGRDLGFGSILNFTTKIGAGMGEQ 1273
S.cerevisiae    1198 FINATFMTTRGGVSKAQKGLHLNEDIYAGMNAMLRGGRIKHCEYYQCGKGRDLGFGTILNFTTKIGAGMGEQ 1269
A.fumigatus     1230 FLNGIFMTTRGGISKAQKGLHLNEDIYAGMNAMIRGGRIKHCEYYQCGKGRDLGFGSILNFTTKIGTGMGEQ 1301
P.jirovecii     1244 FLNGPFMTTRGGVSKAQKGLHLNEDIYAGMTALLRGGRIKHCEYYQCGKGRDLGFGSILNFTTKVGTGMGEQ 1315
P.carinii       1268 FLNGPFMTTRGGVSKAQKGLHLNEDIYAGMTALLRGGRIKHCEYYQCGKGRDLGFGSILNFTTKVGTGMGEQ 1339
P.murina        1272 FLNGPFMTTRGGVSKAQKGLHLNEDIYAGMTALLRGGRIKHCEYYQCGKGRDLGFGSILNFTTKVGTGMGEQ 1343
                1297 *:*. ** ****:*****************.*::***:******************:*******:*:***** 1368

                                    1,3-β glucan synthase domain 2
                 ____________________________________________________________________________
C.albicans      1274 MLSREYFYLGTQLPLDRFLSFYYGHPGFHINNLFIQLSLQVFILVLANLNSLAHEAIMCSYNKDVPVTDVLY 1345
S.cerevisiae    1270 MLSREYYYLGTQLPVDRFLTFYYAHPGFHLNNLFIQLSLQMFMLTLVNLSSLAHESIMCIYDRNKPKTDVLV 1341
A.fumigatus     1302 MLSREYYYLGTQLPLDRFLSFYYAHPGFHINNMFIMLSVQMFMIVLINLGALKHETITCRYNPDLPITDPLR 1373
P.jirovecii     1316 MLSREYYYLGTQLPLDRFLSFYYAHPGFHINNLFIILSVQLLMIVMINLGSMYNILLICKPRRGQPITDPFL 1387
P.carinii       1340 MLSREYYYLGTQLPLDRFLSFYYAHPGFHINNLFIILSVQLLMIVMINLGSMYNILLICRPRRGQPITDPYL 1411
P.murina        1344 MLSREYYYLGTQLPLDRFLSFYYAHPGFHINNLFIILSVQLLMIVMINLGSMYNILLICRPRRGQPITDPYL 1415
                1369 ******:*******:****:***.*****:**:** **:*:::::.: **.:: :  : *    . * **    1440
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Figure S1. Multiple sequence alignment of Gsc1 proteins. T-Coffee was used (Notredame *et al.,*2000). The identical, strongly, and weakly conserved residues are indicated by asterisks, double points, and single points, respectively. Dashes indicate gaps. Residues F641 and S645 of *C. albicans* Gsc1 and the corresponding residues in the other proteins are shown in bold. The hot spots 1 and 2 (Park *et al.,*2005) of mutations as well as the 1,3-β glucan synthase domains 1 and 2 are indicated above the alignment.

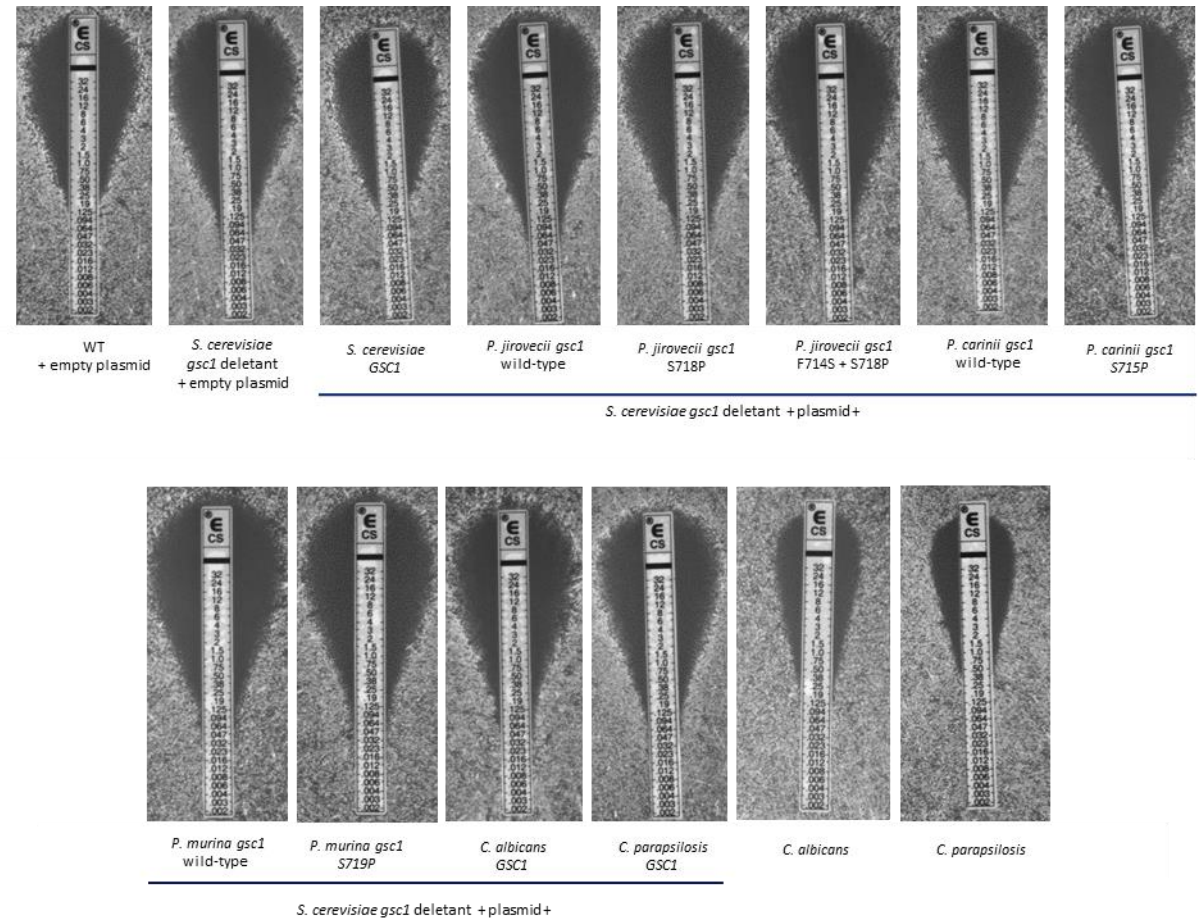

Figure S2. E-test determination of the minimum inhibitory concentration (MIC) of caspofungin (CAS) for the S. cerevisiae WT and functionally complemented gsc1 deletant strains, as well as for Candida species. One hundred microliters of a suspension of cells at ca. 1.5 x 106 cells/ml were spread on minimal selective medium lacking uracil, or lacking leucin for the S. cerevisiae GSC1 gene. The CAS E-test strip was deposited, and the plate was incubated at 30°C, or 35°C for the Candida species. The concentration at which no growth was observed on both sides of the E-test strip was defined as the MIC.

Annexe 3

Pneumocystis primary homothallism involves trophic cells carrying both Plus and Minus pheromone receptors

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Introduction

Pneumocystis organisms are extracellular parasites colonizing the lungs of mammalian species (Thomas *et al.,* 2004*,* Cushion and Stringer *et al.,* 2010; Gigliotti *et al.,* 2014). An important feature of these fungi is their host specificity for a single mammalian species. The species infecting humans is *P. jirovecii*, while *P. murina* and *P. carinii* infect respectively mice and rats. If the immune system of the host is impaired, *P. jirovecii* can turn into an opportunistic pathogen that causes a severe pneumonia (*Pneumocystis* pneumonia, PCP), which can be fatal if not treated. This disease is nowadays the second most frequent life-threatening invasive fungal infection worldwide, with more than 400'000 annual cases (Brown *et al.,* 2012).

Despite the gravity of PCP, an *in vitro* long-term culture method for *Pneumocystis* organisms is still not available. Schildgen *et al*. (2014) described a system of co-culture with human airway epithelial cells, but, to our knowledge, nobody could reproduce it so far. Consequently, the life cycle of these pathogens remains hypothetical and mostly deduced from microscopic and molecular studies on the model *P*. *carinii* (Hauser and Cushion, 2018). Several synthesis and assimilation pathways are missing in the *Pneumocystis* genomes, as revealed by sequence analysis, suggesting that these organisms are obligate parasites without free-living forms (Hauser *et al.,* 2010*;* Cissé *et al.,* 2012; Cissé *et al.,* 2014; Hauser 2014; Porollo *et al.,* 2014). Accordingly, their entire life cycle would occur inside the host's lungs. It is believed to include both asexual and sexual cycles. The asexual cycle involves trophic cells, which are the predominant cellular form during the infection (up to 98% of the cell population). They are mononuclear and mostly haploid (Dei-Cas *et al.,* 2004). This type of cell is apparently devoid of a cell wall, and would divide by binary fission resulting in two daughter cells. The sexual cycle is more complex and would involve the mating of two compatible trophic cells that start the mating process by fusing, then undergo meiosis and mitosis (Skalski *et al.,* 2015). This process would end with the formation of a new cellular structure, the ascus (formerly called cyst), which is surrounded by a thick wall and contains eight daughter cells, the ascospores. Asci are found in the majority of human infections, and staining of their wall is used as a diagnostic tool. Asci and/or ascopspores are necessary for the transmission of the pathogen to new hosts as aerially transported particles (Cushion *et al.,* 2010; Martinez *et al.,* 2013).

There are two modes of sexual reproduction in fungi: heterothallism and homothallism. Heterothallic fungi present two different kinds of strains, each expressing genes of opposite mating type from their *MAT* locus. Two cells of opposite compatible mating type are needed to mate (Ni *et al.,* 2011). Homothallic reproduction involves self-fertile strains and includes two modalities. Primary homothallic fungi present the genes of both mating types in their genome, which can be closely located or not. Secondary homothallic fungi, such as *Schizosaccharomyces pombe,* harbor three *MAT* loci, of which only one is active while the other two are silenced. The expressed locus can be exchanged with a silenced one by a mechanism of switching, so that a cell can switch from one mating type to the other.

Our group recently investigated the mode of sexual reproduction of *Pneumocystis* species by comparative genomics (Almeida *et al.,* 2015). Despite the lack of an established culture method for *Pneumocystis* organisms, this task could be accomplished because of the release of the genome sequence of *P. jirovecii* by two groups (Cissé *et al.,* 2012; Ma *et al.,* 2016). Sex-related genes of the closely related species *S. pombe* were used as query sequences to identify homologous genes in the genomes of *P. jirovecii* and *P. carinii.* In *S. pombe,* four genes involved in mating type differentiation are present: *matMc*, a transcription factor with high-mobility-group domain; *matMi,* a mating type M-specific polypeptide; *matPc,* a transcription factor with high-motility group; and *matPi,* a transcription factor with homeobox domain. In the *Pneumocystis* genomes, only three candidates of the four *MAT* genes present in *S. pombe* were detected: *matMc, matPi,* and *matMi*. These three putative *MAT* genes were located on a single DNA molecule. Their proximity suggested a fusion of two *MAT* loci, one of the type minus (M), composed of the genes *matMc* and m*atMi*, and the other of type plus (P), composed of m*atPi* only, and incomplete because of the absence of *matPc.* Importantly, we did not detect any putative *cis*-acting sequence motifs, homologous to the ones that flank the *S. pombe MAT* loci and implicated in the switching from one mating type to the other. These observations suggested that *Pneumocystis* organisms are not secondary homothallic organisms. Heterothallism could also be excluded, because it is incompatible with the presence of both mating loci on the same DNA molecule. Consequently, *Pneumocystis* species are probably primary homothallic organisms, meaning that each strain is self-fertile and able to produce asci on its own. *P. jirovecii MAT* genes have been further investigated by our group (Richard *et al.,* 2018). We ascertained the function of *P. jirovecii* and *P. carinii matMc* genes by restoration of sporulation in a *S. pombe* strain in which the corresponding gene was deleted. Using PCR analysis, we evidenced the same *MAT* locus in different *P. jirovecii* isolates, and showed a frequent concomitant expression of the three *MAT* genes during infection. Our results strongly suggested that sexuality through primary homothallism is obligate to accomplish the life cycle.

Sexual reproduction of fungi always involves mating factors (pheromones) signaling and specific receptors to recognize them. The pheromones receptors are located at the cell surface, anchored in the cellular membrane, and bind pheromones secreted by cells of the opposite mating type. In heterothallic reproduction, a cell of mating type P release P pheromone which is detected by P receptors on the surface of a M cell, and vice-versa. Receptors for both mating factors have been identified in the *Pneumocystis* genomes (Almeida *et al.,* 2015), strongly suggesting that cells of both mating types are present during the infection. However, the pheromone receptors may be expressed separately by some uncharacterized mechanisms, only P or only M receptors being on the surface on single cells (Figure 1A). Alternatively, they could be expressed both in each

cell (Figure 1B). In the present study, we analyzed both receptors of *P. jirovecii* and *P. murina*, encoded respectively by *mam2* and *map3* genes. Our aims were (i) to investigate their expression during infection, in human and mouse, and (ii) to determine if only P, only M, or both mating receptors are present at the same time at the surface of single trophic cells.

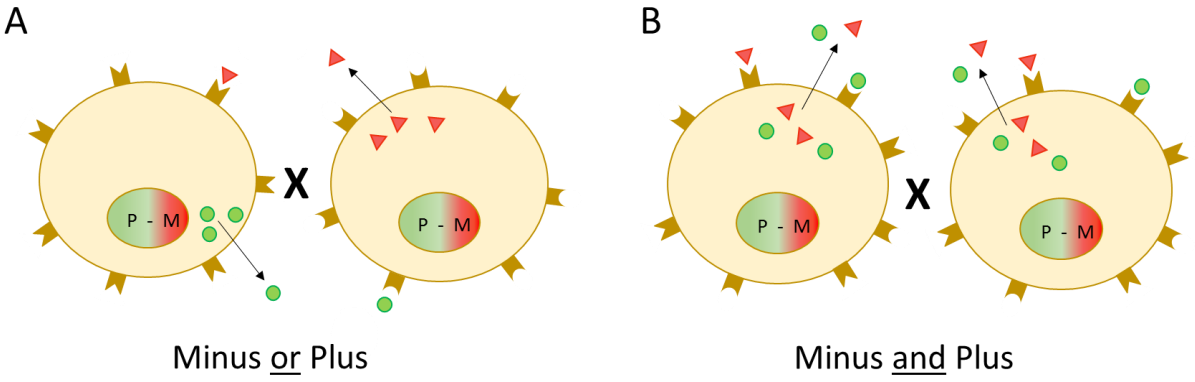

Figure 1. Schematic representation of hypotheses concerning the expression of the pheromone receptors on cells of primary homothallic fungi. *Pneumocystis* cells are represented: both mating loci are present and fused in a single locus in the nucleus. (**A**) Only P or only M receptors are present on the cell surface. (**B**) Both pheromone receptors are present at the same time on the cell surface. Each cross represents the involvement of two cells in the mating process. Figure adapted from Reece *et al.*(2014).

Material and methods

Strain and growth conditions

SY2011 is an *S. cerevisiae* haploid strain in which both mating receptors *STE2* and *STE3* are deleted (*MATa ste3Δ ste2Δ mfa1Δ mfa2Δ::FUS1-LacZ*) (Boone *et al.,* 1993). It was grown at 30°C on complete yeast extract-peptone-dextrose (YEPD) medium (1% [wt/vol] Difco yeast extract, 2% Difco peptone, 2% glucose). SY2011 grows as pale pink colonies. The coloration is due mutation in *ade1* or *ade2* genes of the adenine biosynthesis pathway. Mutations cause accumulation of an intermediate that is converted in the red pigment.

Source of the *Pneumocystis mam2* and *map3* genes

To identify the *P. murina* and *P. jirovecii mam2* genes encoding the P mating receptor, the *P. carinii* Mam2 protein (Uniprot ID A2TJ26, also called *Ste2*) was used as the query sequence in BLASTp searches at http://blast.ncbi.nlm.nih.gov/Blast.cgi against the two available proteomes of *P. jirovecii* and that of *P. murina*. A single putative ortholog was detected in each proteome: PNEJI1_000211 locus in the *P. jirovecii* genome assembly version ASM33397v2 (Cissé *et al.,* 2012; hereafter called the Cissé assembly); T551_00015 locus in the assembly version Pneu-jiro_RU7_V2 (Ma *et al.,* 2016; hereafter called the Ma assembly). A single putative ortholog was found for *P. murina* (PNEG_03148). The *P. murina* and *P. jirovecii* gene sequences encoding the Mam2 proteins were then retrieved from the European Nucleotide Archive (http://www.ebi.ac.uk/ena). The same procedure was applied to retrieve the *P. murina* and *P. jirovecii map3* genes encoding the M mating factor using the *P. carinii* Map3 protein (Q9HDG3). A single putative ortholog was detected in each *P. jirovecii* proteome: PNEJI1_002694 locus in the Cissé assembly, and T551_02750 locus in the Ma assembly. A single putative ortholog was found for *P. murina* (PNEG_03013). The same *P. jirovecii mam2* and *map3* genes were already identified by Almeida *et al*. (2015), but only in the Cissé assembly. The alleles of the Cissé assembly were investigated in the present study. Alignments of the two *P. jirovecii mam2* and *map3* genes are shown in Figure S1. Because of the presence of two introns in each *mam2* or *map3* gene, their cDNAs were synthesized by GeneCust Europe (Ellange Luxemburg). This step was necessary because *S. cerevisiae* does not process *Pneumocystis* introns. The *mam2* genes were cloned into p415GPD, while the *map3* genes were cloned into p416GPD (both plasmids from Mumberg *et al.,* 1995). Two plasmids with different markers were used in order to be introduced at the same time in the SY2011 strain by selection for leucine (p415GPD) and uracil (p416GPD) prototrophy.

Source of the *P. murina mat genes*

The *P. murina matMc* and *matPi* genes were identified using the *P. jirovecii* MatMc (locus T551_0262) and MatPi (T551_02159) proteins as query sequences in a BLASTp search against the proteome of *P. murina.* A single putative ortholog of each Mat protein was detected: PNEG_02275 (MatMc), PNEG_02273 (MatPi). Because it is highly divergent, the *P. murina matMi* was identified as described in Almeida *et al*. (2015)(coordinates AFWA02000013: 80789-80905, provided by J. Almeida). Primers and PCR conditions for their amplification are listed in Table S1 and S2.

PCR amplification

In order to avoid contaminations, PCRs were set up and analyzed in separate rooms, and negative controls were systematically performed at each experiment. PCRs were performed on genomic DNA and cDNA obtained from bronchoalveolar lavage (BAL) fluid samples of patients with *Pneumocystis* pneumonia (these samples have been already investigated by our group for the expression of *MAT* genes, Richard *et al.,* 2018). Genomic DNAs were extracted using the QIAamp DNA blood kit (Qiagen). Total RNAs were extracted from the BALs using the RiboPure yeast kit (A*MBio*n). These clinical samples were previously stock at - 80°C in RNAlater (A*MBio*n) as quickly as possible upon reception. cDNAs were synthesized from each RNA preparation using the REPLI-g WTA Single Cell kit involving random amplification (Qiagen). The resulting cDNA were purified using LiCl-ethanol precipitation in presence of glycogen (Qiagen supplementary protocol). The random amplification of cDNA included in the kit were necessary for the detection of *P. jirovecii* transcripts among those of the patient because of their small amount. For the amplification of *P. murina* pheromone receptors genes, genomic DNA was extracted from lung homogenate of infected immunosuppressed mice using the Blood and Tissue Kit (Qiagen). Total RNA was extracted using TRIzol™ Reagent (Thermofisher), and cDNAs were synthesized using the SuperScript IV ViloMaster Mix Kit (Invitrogen). PCRs were designed to overlap one intron in order to analyze its splicing. Primers used and conditions are listed in Table S1 and Table S2.

Transformation of the *S. cerevisiae ste2/ste3* double deletant strain

The recombinant plasmids p415GPD containing *P. jirovecii mam2* gene and p416GPD containing *P. jirovecii map3* gene were co-introduced into the SY2011 *S. cerevisiae* strain by transformation for uracil and leucine prototrophy using the one-step method (Chen *et al.,* 1992). The same was done using recombinant plasmids containing *mam2* and *map3* genes of *P. murina*. As control, SY2011 was co-transformed with empty p415GPD and p416GPD plasmids. Transformants were selected on solid yeast nitrogen base (YNB) medium (0.67% [wt/vol] yeast nitrogen base, 2% glucose, 2% Gibco agar) supplemented with a complete supplement mixture lacking uracil and leucine (CSM, MP Biomedicals).

Antibodies choice and preparation

The protein sequences of *P. jirovecii, P. carinii* and *P. murina* Mam2 or Map3 were aligned to that of *S. cerevisiae* (Figure 2). The transmembrane domains in the *Pneumocystis* receptors were predicted by Uniprot (highlighted in grey in Figure 2). Their localizations corresponded to those of the *S. cerevisiae* proteins. As reported by Vohra *et al.* (2004) for *P. carinii map3*, the extracellular and intracellular domains of *Pneumocystis* Mam2 and Map3 proteins were deduced according to those of *S. cerevisiae* proteins (Uddin *et al*., 2017). Antibodies against the extracellular domains of *P. jirovecii* Mam2were prepared by the company Eurogentec by immunizing rabbits with the two peptides derived from the following sequences: C+TVNQTVILKNSHGEK; C+LSDFDMFSLSRAQ (peptides 1

and 2 in Figure 2A). Antibodies against the extracellular domains of *P. jirovecii* Map3 were prepared by immunizing rats with peptides derived from the following sequences: WFNGVEAKSPGYIYC; QSKINYSWDHVHQWG+C (peptides 1 and 2 in Figure 2B). Exactly the same procedure was used for the preparation of the antibodies against *P. murina* Mam2 using WFNGSKSTYRGDLYC and KSNIKYSWSDVHNWN+C peptides, and against Map3 using C+PTNQTIFLKNSKGET and LSDFDEFSLSRAQ+C peptides. Each pair of peptides was injected into two animals (Figure 3). The sera of the two animals were mixed for the staining experiments. Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor Plus 488 (green, Invitrogen), and Goat Anti-Rat IgG H&L Alexa Fluor® 594 (red, Eurogentec) were used as secondary reagents to detect the primary antibodies.

A

B

Figure 2. Multiple sequence alignment of Mam2 and Map3 proteins. (**A**) Alignement of Mam2 proteins of *S. cerevisiae* (Uniprot ID:D6VTK4), *P. jirovecii* (L0PDU6; translation product of the Cissé assembly), *P. carinii* (A2TJ26), and *P. murina* (M7P3B3). (**B**) Alignement of Map3 proteins of *S. cerevisiae* (Uniprot ID: P06783*), P. jirovecii* (L0PBZ8; translation product of Cissé *et al.,*allele), *P. carinii* (Q9HDG3), and *P. murina* (M7NMS4). T-coffee was used (Notredame *et al.,* 2000). The identical, strongly, and weakly conserved residues identified are indicated by asterisks, double points, and single points, respectively. Dashes indicate gaps. The transmembrane domains are highlighted in grey. Extracellular domains are indicated in green above the alignment, intracellular domains are indicated in blue. Peptides 1 and 2 are shown in green for Mam2 and red for Map3, and the corresponding residues are in bold in *P. jirovecii* and *P. murina* sequences.

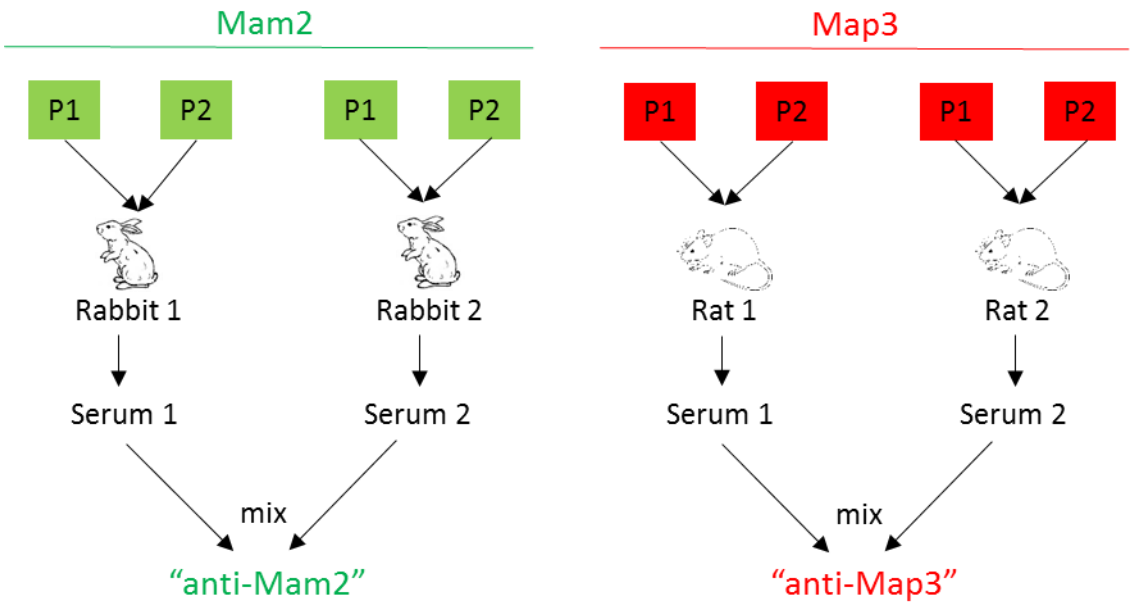

Figure 3. Antibodies anti-Mam2 and anti-Map3 preparation. This procedure was used by Eurogentec for the preparation of the antibodies used in the present study.

Immunofluorescence staining and microscopy

In order to avoid the loss of the two plasmids, strain SY2011 expressing *Pneumocystis* pheromone receptors were grown overnight in selective minimal medium YNB lacking uracil and leucine. Cells were diluted at an optical density (OD) at 540 nm of 1 in the same medium (ca. 7.5 x 10^6 cells). Cells were then centrifuged for 5 min at 5000 rpm and washed once with PBS (phosphate buffered saline, NaCl 0.68%, KH_2PO_4 0.04%, Na_2HPO_4 0.15%). Cells were resuspended in nuclease-free water, and 15 µl were deposed on a microscope slide, previously washed with EtOH 100% and dried. Once completely air dried at room temperature, cells were heat-fixed by repeated rapid passages of the back side of the slide over a flame. Slides were treated with 100 µl blocking buffer (PBS/NGS 5% [normal goat serum, Gibco life technologies]/BSA 3% [bovine serum albumin]) for 30 to 45 min at room temperature. Slides were then washed and covered-with a mix of the antibodies anti-Mam2 and anti-Map3 primary antibodies with a final dilution of 1/25 in washing buffer (PBS/NGS 5%/BSA 0.01%). were then incubated for 1h at room temperature in a humid dark chamber. The humid dark chamber consisted in a plastic box containing wet absorbent paper with plastic supports on which slides were leaned. Antibodies were removed by washing the slide several times with washing buffer. Subsequently, slides were treated with a mix of two secondary antibodies (anti-rabbit and anti-rat) at a final dilution of 1/200 in washing buffer, and incubated for 1h at room temperature in the humid dark chamber. Secondary antibodies were removed and slides were washed several times with PBS. Slides were then observed with a fluorescent microscope (Zeiss, Axioplan 2). To visualize the *P. jirovecii* cells present in a BAL of a patient, the Merilfluor® coloration (Meridian, Bioscience Europe) was used according to manufacturer's instructions. This staining uses fluorescent antibodies directed against cell wall and matrix antigens and is currently used as diagnostic tool. FITC filter was used to visualize Mam2 receptors and Merifluor staining (green fluorescence), whereas FRITC filter was used to visualize Map3 receptor (red fluorescence). All the pictures were taken at 1000x magnification, with a Spot RT3 camera (Visitron System). ImageJ was used to handle the images.

Results

Identification of the *P. jirovecii* and *P. murina mam2* and *map3* genes

A unique Mam2 protein was identified within each of the two *P. jirovecii* proteomes available using the *P. carinii* Mam2 protein as the query sequence. The *mam2* genomic DNA sequences of the two genome assemblies were identical except that the first of the two introns is predicted only in the Cissé assembly (Figure S1A). Similarly, a single Map3 protein was identified within each of the two *P. jirovecii* proteomes. The *P. jirovecii map3* genes from the two assemblies presented several differences (Figure S1B). First, the ORF predicted in the Ma assembly was 156 bps longer than that in Cissé assembly, 70 bps upstream and 84 bps downstream. These regions are identical in the Cissé assembly (Figure S1B, in grey), except on one non-synonymous polymorphism (SNP) at position 32 of the Ma sequence (Figure S1B, in bold). Second, the first of the two introns is predicted only in the Cissé assembly (Figure S1B). Third, two synonymous SNPs are present at positions 201 and 324 of the Cissé sequence, and one non-synonymous at position 391 (Figure S1B, in bold). Similarly, a single putative Mam2 and Map3 proteins were identified within the proteome of *P. murina.* The *mam2* and *map3* encoding genes present respectively one and two introns (Figure S2). Consistent with their expression, one potential TATA box and one potential cap-signal were identified upstream the start codons of the *P. jirovecii* and *P. murina mam2* and *map3* ORFs (Figure S3). These elements were also present upstream of the of *P. jirovecii map3* ORF of the Ma assembly (Figure S3, in light blue). The *mam2* and *map3* gene alleles from the Cissé assembly were investigated in the present study. Alignments of the Mam2 and Map3 proteins of closely related organisms are shown in Figures 2A and 2B, respectively. The identities of the Mam2 and Map3 proteins of *P. carinii, P. murina, S. cerevisiae,* and *S. pombe* relatively to those of *P. jirovecii* are given in Table 1**.** Despite the low identity to *S. cerevisiae* and *S. pombe* receptors (22 to 28%), the orthology of all these proteins is suggested by the presence of the same number of transmembrane domains, with the exception that the seventh in *P. carinii* and *P. murina* Mam2 is not predicted in Uniprot (Figure 2A). The presence of these transmembrane domains strongly suggests that these proteins are localized within the cell membrane. Since *S. cerevisiae* does not process *Pneumocystis* introns, synthetic *Pneumocystis mam2* and *map3* genes without introns were cloned into plasmids for heterologous expression.

	Protein sequence identity (%)	
	Mam2	Map3
P. carinii	63	57
P. murina	64	61
S. cerevisiae	22	22
S. pombe	28	23

Table 1. Sequence identity (%) of the *P. jirovecii* Mam2 and Map3 proteins to the putative orthologs of *P. carinii, P. murina, S. cerevisiae,* and *S. pombe*.

The *Pneumocystis mam2* and *map3* genes are often expressed concomitantly during infection

The expression of the two pheromone receptors was expected to occur during *Pneumocystis* infection because mating is required to produce the asci which are most often, if not always, present. This would be consistent with the presence of transcription motifs upstream of the ORFs (Figure S3). To investigate this issue in humans, we used reverse transcriptase-PCR analysis of total RNAs extracted from ten BALs of ten patients with *Pneumocystis* pneumonia. Our group previously investigated these samples for the expression of the *MAT* genes, and ensured that the RNAs did not contain genomic DNA by (i) the lack of amplification in absence of reverse transcription, and (ii) the lack of intron in the PCR product from the unrelated gene encoding β-tubulin (*β-tub*)(Richard *et al.,* 2018). As a control for the experiments of the present study, we repeated the latter amplification and consistently obtained the same results (Figure 4A). Of the ten patients, four were positive for the expression of both *P. jirovecii mam2* and *map3* genes, three for only one receptor gene, and three negative for both receptor genes (Table2; Figure 4A). The latter three patients were also negative for the *MAT* and *β-tub* transcripts, suggesting that RNA degradation may have occurred during the uncontrolled period between collection of the samples from the patients and their arrival in our laboratory. The three patients positive for only one receptor may reflect low expression resulting from collection of the BAL after the peak of expression, possibly at a late stage of infection. The size of the PCR product from *P. jirovecii mam2* transcripts revealed that the predicted intron no 1 was not removed, whereas two PCR products were systematically obtained from the *map3* transcripts, one containing the intron no 1, the other not (Figure 4A). These results could be explained with occurrence of alternative-splicing events. We also investigated the expression of the two *P. murina* receptors in one sample from infected lungs of a single mouse. The sample resulted positive for expression of both pheromone receptors, with removal of the predicted intron (Table 2; Figure 4B). These observations suggested that the two pheromone receptors are most often concomitantly expressed during each *Pneumocystis* infection. This is consistent with the existence of two mating types during sexual reproduction.

		MAT transcription factors			Pheromone receptors	
	β-tub	*matMc*	*matMi*	*matPi*	*mam2*	*map3*
P. jirovecii cDNA patient no.						
1	+	+	+	+	+	+
2	-	-	-	-	-	-
3	+	-	+	-	+	-
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	-	-	+	+	-
7	+	+	+	+	+	+
8	+	-	-	+	-	-
9	-	-	-	-	-	-
11	+	+	+	+	-	+
P. murina cDNA	+	+	+	+	+	+

Table 2. . PCR amplification of *Pneumocystis mam2* and *map3* transcripts from 10 BALs fluid samples of 10 patients with PCP, and from infected mouse lungs. Amplification of *β-tub* was used as control. The amplification results for the *P. jirovecii MAT* genes are from Richard *et al.* (2018) (BAL of patient no. 10 could not be analyzed because it was no more available). +, positive PCR result; -, negative PCR result.

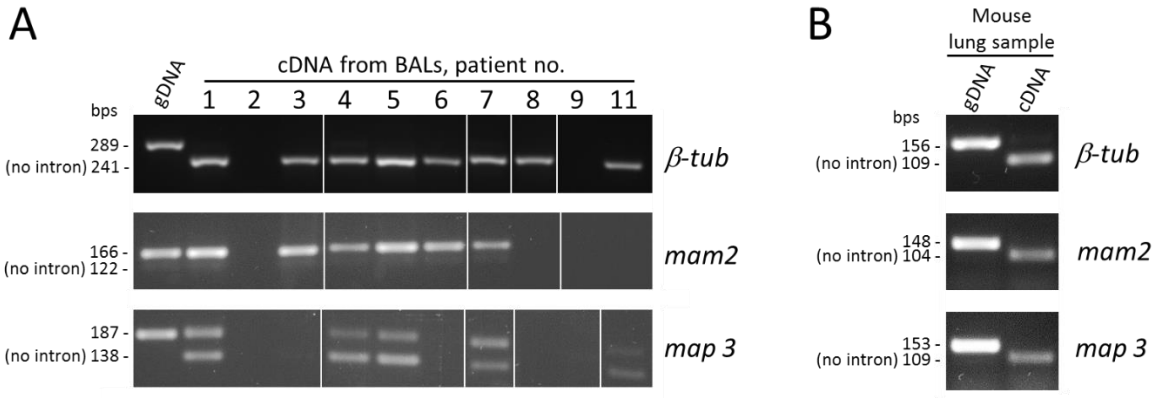

Figure 4. Amplification of the *mam2*, *map3* and *β*-tub transcripts of *Pneumocystis* by reverse transcriptase-PCR. (**A**) Analysis of cDNAs obtained from 10 BALs fluid samples of 10 patients with *Pneumocystis* pneumonia. Genomic DNA from patient no. 3 was used as positive control. (**B**) Analysis of cDNA from a sample of infected mouse lungs. The PCR products were of the expected sizes shown next to the bands. bps, bases pairs. gDNA, genomic DNA.

The *Pneumocystis* pheromone receptors Mam2 and Map3 localize at the cell surface when expressed in *S. cerevisiae*

We intended to visualize the pheromone receptors at the surface of *P. jirovecii* trophic cells using specific antibodies. In order to validate the staining tools needed for that purpose, we performed experiments on *S. cerevisiae* strain SY2011 expressing the *Pneumocystis* pheromone receptors. Strain SY2011 has both endogenous mating pheromone receptors deleted. Each recombinant strain expressing both Mam2 and Map3 of *P. jirovecii* or of *P. murina* was stained using two specific antibodies and two secondary antibodies of different immunofluorescent colors, and their cell surface was examined under the microscope. A strain carrying both empty vectors was used as a negative control. Strong green and red signals were observed at the surface of the vast majority of the cells expressing *P. murina* or *P. jirovecii* pheromone receptors, but not on the cells of the control containing the empty vectors (Figure 5). Consistent with the expected localization of the receptors within the cellular membrane, the coloration appeared as little dots all around the cells at their surface, as shown by the cells enlarged at the bottom-left side of each image of Figure 5. These observations were consistent with a localization of *Pneumocystis* Mam2 and Map3 pheromone receptors within the cellular membrane of *S. cerevisiae*, and validated the immunofluorescent staining tools to be used on *Pneumocystis* cells.

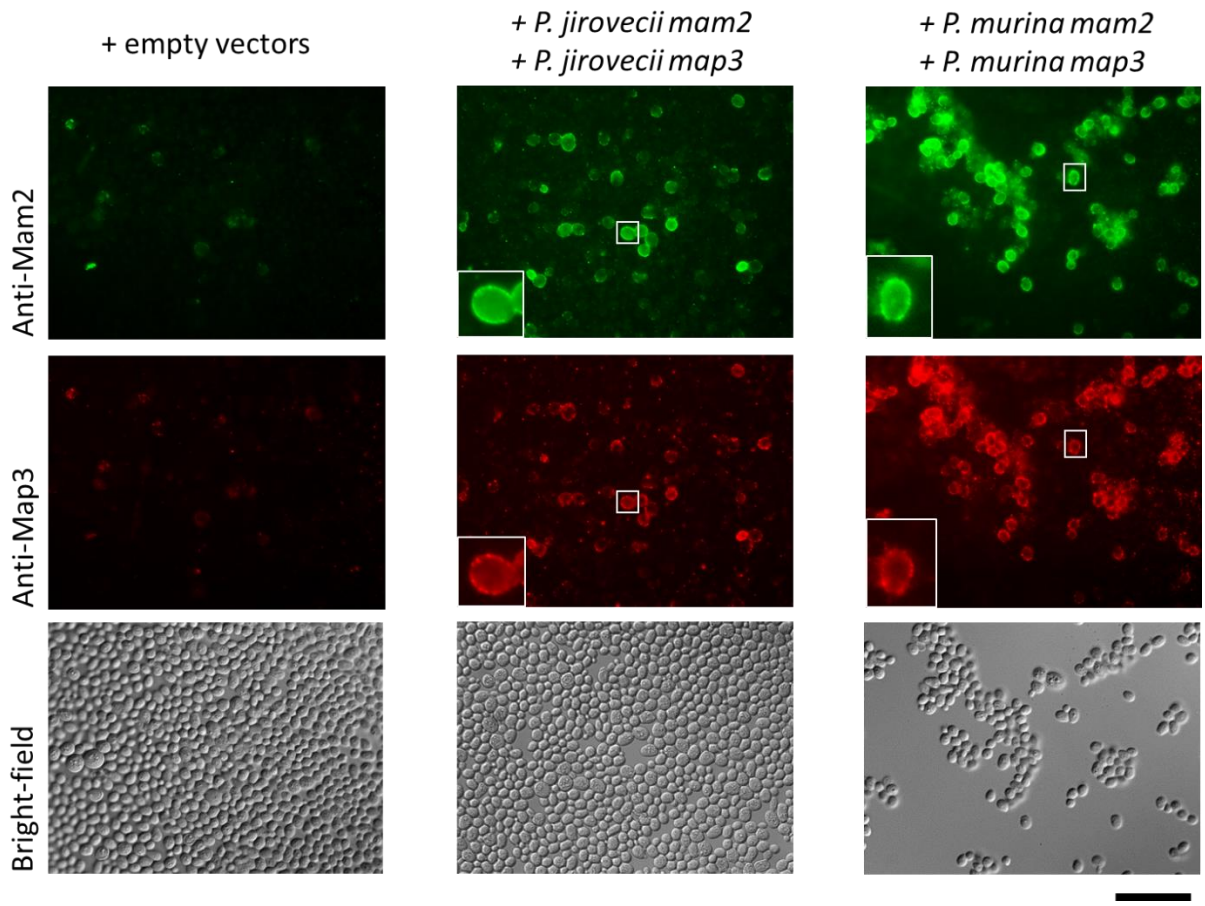

Figure 5. Immunofluorescent microscopic analysis of *Pneumocystis* pheromone receptors Mam2 and Map3 expressed in *S. cerevisiae* SY2011 strain. Recombinant strains harbored plasmids expressing the indicated heterologous gene, or empty vectors. FITC filter (green) was used to visualize Mam2, FRITC filter (red) to visualize Map3. The squared cells are enlarged at the bottom of the image. The scale bar underneath the images on the right is 50 µm.

The Mam2 and Map3 receptors are frequently both present on the surface of single *P. jirovecii* trophic cells

Our reverse transcriptase-PCR analyses showed that both *mam2* and *map3* genes are often expressed concomitantly during *Pneumocystis* infection. Nevertheless, this finding is compatible with two possibilities: each cell expresses only one of the two receptors, or each cell expresses both receptors (Figure 1). To investigate this issue, we performed immunofluorescent stainings of *P. jirovecii* cells from the BAL of patient no. 1 (which had the highest fungal load among our samples, Richard *et al.,* 2018). We also tried to stain *P. murina* cells in smears of infected mouse lungs, but our attempts remained unsuccessful, only *P. jirovecii* cells could be stained. In order to differentiate trophic cells from asci, we used the Merifluor® kit relying on an antibody directed against the cell wall of all *P. jirovecii* cell types (trophic forms, asci, and ascospores). This kit also stains the extracellular matrix surrounding the cells within clusters. Using this kit, the isolated cells that are smaller than those forming the clusters are mostly trophic cells (2 to 8 μm), whereas the large rounded cells within the clusters are mostly asci (4 to 6 μm; Figure 6A and 6B, green stain). Co-staining with Merifluor® and anti-Map3 could be performed because of distinct fluorescent colors. It revealed that the identified trophic cells were most often also positive with the Map3 staining (Figure 6B). Co-staining with anti-Mam2 and anti-Map3 antibodies revealed that the majority of the identified trophic cells were positive with both staining (Figure 6C). In both experiments, we observed about 20 cells positive with both stainings, only two or three were positive with only one. Although we cannot exclude that the latter cells expressed only one receptor, they could have resulted from the relatively fast fading of the immunofluorescence. The trophic cells were often the most strongly stained, consistent with the expected expression of the receptors only by these cells (compare trophic cells and asci of Figure 6B and 6C). As expected, the receptors were sometimes clearly located at the surface in some trophic cells, as shown by the enlarged cells in Figure 6. These observations suggested that Mam2 and Map3 receptors are often both present on the surface of each trophic cell.

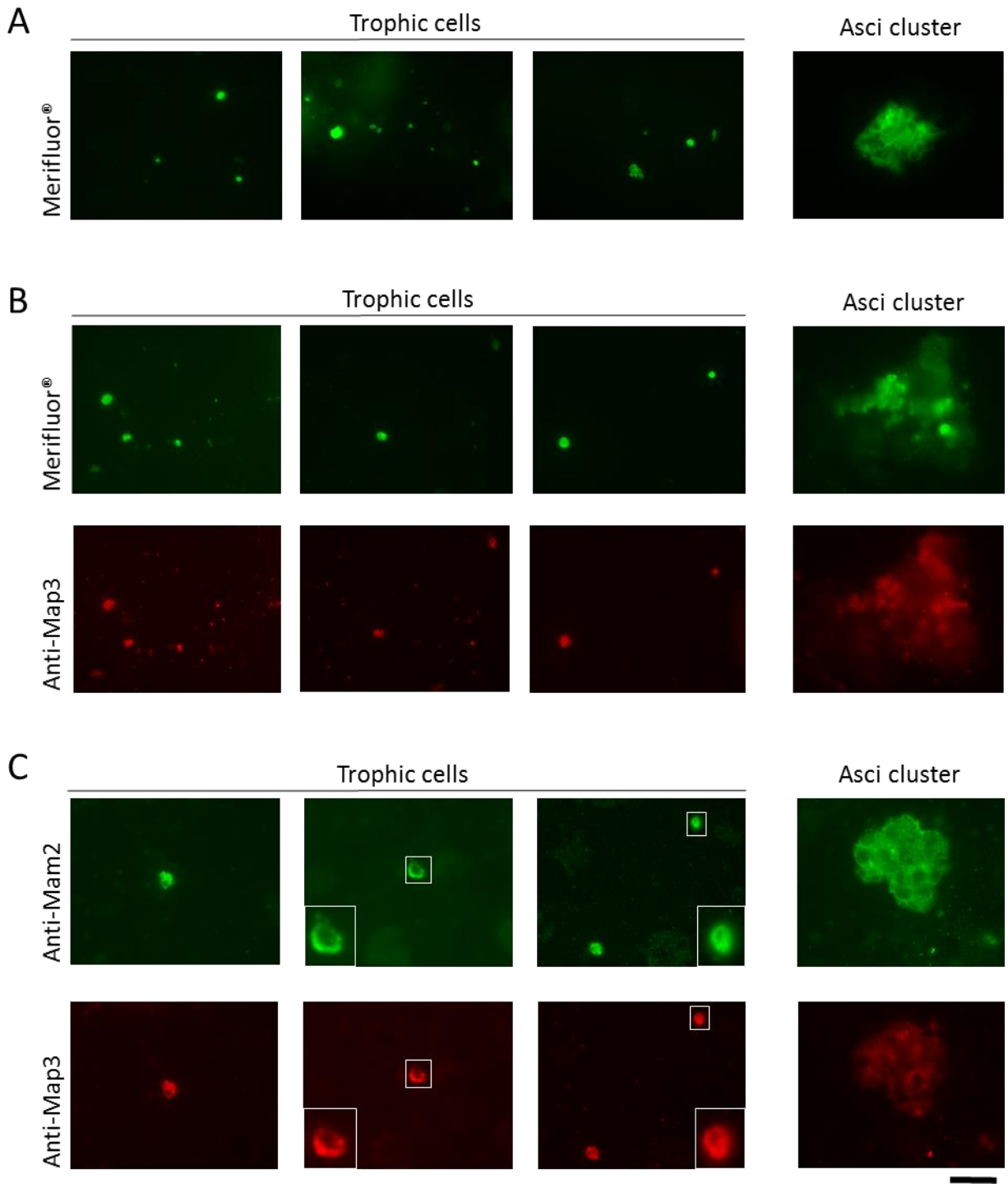

Figure 6. Immunofluorescent microscopic analysis of Mam2 and Map3 pheromone receptors on *P. jirovecii* cells from a BAL fluid sample of patient with pneumonia. FITC filter (green) was used for Merifluor® staining and to visualize Mam2, FRITC filter (red) to visualize Map3. The squared cells are enlarged at the bottom of the image. (**A**) Merifluor® staining. (**B**) Co-staining Merifluor® and anti-Map3. (**C**) Co-staining anti-Mam2 and anti-Map3. The scale bar underneath the images on the right is 25 µm.

Discussion

The mode of sexual reproduction of *Pneumocystis* organisms is most probably primary homothallism. In the present study, we investigated the expression of the *Pneumocystis* genes *mam2* and *map3* that encode the receptors of the P and M mating factors. We found that both genes are most often expressed concomitantly during infection in humans as well as in mice. In addition, immuno-stainings revealed that both pheromone receptors are most often present at the same time at the surface of trophic cells, as represented schematically in Figure 1B. Thus, *Pneumocystis* mating involves cells which are of both mating types P and M at the same time. This suggests that each trophic cell might excrete both pheromones P and M. We could not study this because the genes encoding these pheromones could not be identified, consistently with their notoriously important divergence among fungi (Almeida *et al.,* 2015).

P. jirovecii mam2 transcripts included the first predicted intron, while two *map3* transcripts were observed, one including the predicted intron, the other not. The ORF was conserved upon retention of both these introns and no STOP codons were present. For both genes, the translated product of the intron is located in the fifth transmembrane domain (respectively at position 200 and 177 in the alignments of Figure 2), but it is unknown whether the longer proteins are functional. These observations can be attributed to the occurrence of alternative splicing events, a mechanism which was previously described for the *map3* transcripts in *P. carinii* (Smulian *et al.,* 2001). A high-level of alternative splicing events has been reported in *Pneumocystis* species, with intron retention as the most common mechanism (42 to 49% of introns concerned; Ma *et al.,* 2016). The different splicing variants could be associated with the different *Pneumocystis* cellular forms (Ye *et al.,* 2001). Surprisingly, despite the presence of typical acceptor and donor motifs (Figure S1), the two introns were predicted only in the Cissé assembly, not in the Ma assembly. This difference remains unclear, and might be linked to the intron retention we observed. Alternative splicing is believed to be useful to increase the diversity of transcripts, possibly to respond to different environments, and regulate transcription of genes, intron retention being involved in the regulation of protein isoform production (Jacob *et al.,* 2017). As far as *P. murina* is concerned, the predicted introns were removed from the *mam2* and *map3* transcripts. These two introns contains a STOP codon (positions 781-783 and 544-546 respectively in *mam2* and *map3* genomic DNA sequence, Figure S2, highlighted in red). The transcripts with retention of these introns are possibly eliminated by the nonsense mediated mRNA decay machinery, a pathway existing in all eukaryotes, including *Pneumocystis* (Ma *et al.,* 2016).

Primary homothallism is believed to be advantageous for pathogenic fungi because it avoids the need to find a compatible partner to mate, while still providing evolutionary advantages (Heitman, 2010). Indeed, despite that it involves a single strain, primary homothallism can avoid accumulating deleterious mutations and increase genetic diversity as well as virulence (Roach and Heitman, 2014). This strategy seems to concern also *Pneumocystis* organisms. In the case of *P. jirovecii*, the presence of both pheromone receptors P and M on each trophic cell could enable to mate with the other strains which are most often present during human infections (Alanio et al., 2016). This might allow increasing further the genetic diversity relatively to self-fertilization of a single strain. The situation might be different in *P. murina* infections because these are apparently monoclonal (M.T. Cushion, unpublished data; Cissé *et al.,* 2018). Monoclonality might be reflect an adaptation to the

laboratory conditions of this animal model of infection. It would be interesting to study *P. murina* infections in wild populations of mice.

In the closely related organism *S. pombe,* the expression of the Map3 pheromone receptor is regulated by the MatPc transcription factor. This could not be the case in *Pneumocystis* organisms because no MatPc homolog was found (Almeida *et al.,* 2015). The absence of the latter suggested that *Pneumocystis* populations might be composed by only M cells, but our results demonstrate that cells are also of the P mating type. Thus, the expression of Map3 and genes specific for the P mating type are probably regulated by another transcription factor than MatPc in *Pneumocystis* organisms. Besides, in *S. pombe*, the fusion between two cells of opposite mating type allows the formation of a complex composed of Pi and Mi transcription factors, Pi originating from the P cell, Mi from the M cell (Vještica *et al*., 2018). The Pi-Mi complex bind to the promoter of *mei3*, a gene which is involved in meiosis induction and inhibition of re-fertilization. The latter mechanisms remain unclear in *Pneumocystis* organisms because (i) the *mei3* gene is not present in *Pneumocystis* genomes (Almeida *et al.,* 2015), and (ii) the two MatMi and MatPi transcription factors are present and expressed in the same cell so that the Pi-Mi complex might be always present. Thus, the mechanisms of repression and de-repression of meiosis are most probably different from those of *S. pombe.* These observations are not surprising because rewiring of *MAT* pathways is a phenomenon frequently observed among fungi (Reedy *et al.,* 2009; Sherwood *et al.,* 2014).

In conclusion, we showed that both Mam2 and Map3 pheromone receptors are most often both present together on the majority of trophic *P. jirovecii* cells, strongly suggesting that each cell is of both M and P mating types at the same time. This might facilitate mating events because they could happen between any cells of the population. Further experiments are necessary to decipher the mechanisms involved in *Pneumocystis* sexuality.

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Supplementary data of annexe 3

Table S1. PCR primers

Target[a]	Primer name	Primer 5' - 3' nucleotide sequence	PCR product size (bp) with intron/without intron	Description
Pj β-tub	Pj-Btub-for	TTTTCAGTGGTTCCCTCACC	150/103	Amplification of internal fragment from position 677 to 827 on genomic DNA, this region encompasses the intron no 3 of 47 bps
	Pj-Btub-rev	AGAATGTTTCATCGGAATTTTCA		
Pj mam2	Pj-mam2-for	GCTGCTATTCAAAATTCAATGG	169/140	Amplification of internal fragment from position 508 to 677 on genomic DNA, this region encompasses the first intron of 29 bps
	Pj-mam2-rev	TGTCTACGGTAAATTGCG		
Pj map3	Pj-map3-for	TCTGGCCCCCTATTTTTGG	186/139	Amplification of internal fragment from position 488 to 674 on genomic DNA, this region encompasses the first intron of 47 bps
	Pj-map3-rev	AGACGTACAAATCTAGAAAGTGTC		
Pm β -tub	Pm-Btub-for	CATACAATGCGACGCTTTCT	156/109	Amplification of internal fragment from position 878 to 1034 on genomic DNA, this region encompasses the intron no 6 of 47 bps
	Pm-Btub-rev	GGAAGCTTTAATGTTCGCATAC		
Pm mam2[d]	Pm-mam2-for	ATTATGAGCTGTCAAACATTG	148/104	Amplification of internal fragment from position 721 to 869 on genomic DNA, this region encompasses the unique intron of 44 bps
	Pm-mam2-rev	ATTACGACGAGCGCTTGAG		
Pm map3	Pm-map3-for	CGATCATGGCTAGCTGTG	153/109	Amplification of internal fragment from position 457 to 610 on genomic DNA, this region encompasses the first intron of 44 bps
	Pm-map3-rev	CATTTCGCTTTTTGAAGTATG		
Pm matMc	Pm-matMc-for	GAATCCTCCACGACCACCTA	151 (no intron)	Amplification of internal fragment from position 234 to 384
	Pm-matMc-rev	TCGCTGTTTTACAGCTGGTG		
Pm matMi	Pm-matMi-for	TGTTTACCATTTCACCTTCACC	100 (no intron)	Amplification of internal fragment from position 2 to 101
	Pm-matMi-rev	TTTTCACTGGCTAATGCATGG		
Pm matPi	Pm-matPi-for	CAACAAGGAATTGTCGGAGAC	164 (no intron)	Amplification of internal fragment from position 171 to 334
	Pm-matPi-rev	TCCGACATAAATCCGACAGA		

[a] *Pj, P. jirovecii; Pm, P. murina*

Table S2. PCR conditions. All the PCRs were performed using the High Fidelity Expand polymerase (Roche)

	Final concentration Mg2+	Initial denaturation		Denaturation		Annealing		Elongation		Final extension	
Target[a]	mM	sec	°C	sec	°C	sec	°C	sec	°C	sec	°C
Pj β-tub	4.5	180	94	30	94	30	58	30	72	600	72
Pj mam2	4.5	180	94	30	94	30	58	30	72	600	72
Pj map3	4.5	180	94	30	94	30	58	30	72	600	72
Pm β-tub	3	180	94	30	94	30	60	30	72	600	72
Pm mam2	3	180	94	30	94	30	56	30	72	600	72
Pm map3	3	180	94	30	94	30	56	30	72	600	72
Pm matMc	3	180	94	30	94	30	60	30	72	600	72
Pm matMi	3	180	94	30	94	30	60	30	72	600	72
Pm matPi	3	180	94	30	94	30	60	30	72	600	72

[a] Pj*, P. jirovecii; Pm, P. murina*

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Pjmam2_Cissé_ORF       1 ATGTCTCTTTCTACAGTAAACCAGACTGTCATTCTTAAGAATTCTCATGGAGAGAAGGTCAAATTT   66
Pjmam2_Cissé_gen       1 ATGTCTCTTTCTACAGTAAACCAGACTGTCATTCTTAAGAATTCTCATGGAGAGAAGGTCAAATTT   66
Pjmam2_Ma_ORF          1 ATGTCTCTTTCTACAGTAAACCAGACTGTCATTCTTAAGAATTCTCATGGAGAGAAGGTCAAATTT   66
Pjmam2_Ma_gen          1 ATGTCTCTTTCTACAGTAAACCAGACTGTCATTCTTAAGAATTCTCATGGAGAGAAGGTCAAATTT   66
                       1 ************************************************************   66

Pjmam2_Cissé_ORF      67 TTATTGTCAGATTTTGACATGTTTTCTCTTTCTAGAGCACAAACATCCATGATATTTTCTGCACAA  132
Pjmam2_Cissé_gen      67 TTATTGTCAGATTTTGACATGTTTTCTCTTTCTAGAGCACAAACATCCATGATATTTTCTGCACAA  132
Pjmam2_Ma_ORF         67 TTATTGTCAGATTTTGACATGTTTTCTCTTTCTAGAGCACAAACATCCATGATATTTTCTGCACAA  132
Pjmam2_Ma_gen         67 TTATTGTCAGATTTTGACATGTTTTCTCTTTCTAGAGCACAAACATCCATGATATTTTCTGCACAA  132
                      67 ************************************************************  132

Pjmam2_Cissé_ORF     133 TGTGCAATGAGTGCTTTGTTAGCAATTATTCTTCTATTAACATCAAAACGTGAAAAAGCAAAAACA  198
Pjmam2_Cissé_gen     133 TGTGCAATGAGTGCTTTGTTAGCAATTATTCTTCTATTAACATCAAAACGTGAAAAAGCAAAAACA  198
Pjmam2_Ma_ORF        133 TGTGCAATGAGTGCTTTGTTAGCAATTATTCTTCTATTAACATCAAAACGTGAAAAAGCAAAAACA  198
Pjmam2_Ma_gen        133 TGTGCAATGAGTGCTTTGTTAGCAATTATTCTTCTATTAACATCAAAACGTGAAAAAGCAAAAACA  198
                     133 ************************************************************  198

Pjmam2_Cissé_ORF     199 TTTCTTTTCTTTTTAAACATGGCTGGACTAATATCTGTATTTATACGAGGATGCCTTCAATGTGCT  264
Pjmam2_Cissé_gen     199 TTTCTTTTCTTTTTAAACATGGCTGGACTAATATCTGTATTTATACGAGGATGCCTTCAATGTGCT  264
Pjmam2_Ma_ORF        199 TTTCTTTTCTTTTTAAACATGGCTGGACTAATATCTGTATTTATACGAGGATGCCTTCAATGTGCT  264
Pjmam2_Ma_gen        199 TTTCTTTTCTTTTTAAACATGGCTGGACTAATATCTGTATTTATACGAGGATGCCTTCAATGTGCT  264
                     199 ************************************************************  264

Pjmam2_Cissé_ORF     265 TATTTAACTGGTACATGGACAAGCTATAGTGTTCAATTTCTCGGAGAATTCGAGTTGTTATCATAT  330
Pjmam2_Cissé_gen     265 TATTTAACTGGTACATGGACAAGCTATAGTGTTCAATTTCTCGGAGAATTCGAGTTGTTATCATAT  330
Pjmam2_Ma_ORF        265 TATTTAACTGGTACATGGACAAGCTATAGTGTTCAATTTCTCGGAGAATTCGAGTTGTTATCATAT  330
Pjmam2_Ma_gen        265 TATTTAACTGGTACATGGACAAGCTATAGTGTTCAATTTCTCGGAGAATTCGAGTTGTTATCATAT  330
                     265 ************************************************************  330

Pjmam2_Cissé_ORF     331 AATGATTTCTATGTCTCAATTATTGCATCATGCATGCCTATTTTTATCATCTTATTTATTGAGCTT  396
Pjmam2_Cissé_gen     331 AATGATTTCTATGTCTCAATTATTGCATCATGCATGCCTATTTTTATCATCTTATTTATTGAGCTT  396
Pjmam2_Ma_ORF        331 AATGATTTCTATGTCTCAATTATTGCATCATGCATGCCTATTTTTATCATCTTATTTATTGAGCTT  396
Pjmam2_Ma_gen        331 AATGATTTCTATGTCTCAATTATTGCATCATGCATGCCTATTTTTATCATCTTATTTATTGAGCTT  396
                     331 ************************************************************  396

Pjmam2_Cissé_ORF     397 TCTCTTCTTATTCAAATTAGAGTAATCTACGCATCACACAGAAAGTTACGAATGCCGCTCACAATA  462
Pjmam2_Cissé_gen     397 TCTCTTCTTATTCAAATTAGAGTAATCTACGCATCACACAGAAAGTTACGAATGCCGCTCACAATA  462
Pjmam2_Ma_ORF        397 TCTCTTCTTATTCAAATTAGAGTAATCTACGCATCACACAGAAAGTTACGAATGCCGCTCACAATA  462
Pjmam2_Ma_gen        397 TCTCTTCTTATTCAAATTAGAGTAATCTACGCATCACACAGAAAGTTACGAATGCCGCTCACAATA  462
                    397 ************************************************************  462

Pjmam2_Cissé_ORF     463 ATTTCTTGTGTCATAATATCAGTAGTTATACTATTTTGGGTAATTGCTGCTATTCAAAATTCAATG  528
Pjmam2_Cissé_gen     463 ATTTCTTGTGTCATAATATCAGTAGTTATACTATTTTGGGTAATTGCTGCTATTCAAAATTCAATG  528
Pjmam2_Ma_ORF        463 ATTTCTTGTGTCATAATATCAGTAGTTATACTATTTTGGGTAATTGCTGCTATTCAAAATTCAATG  528
Pjmam2_Ma_gen        463 ATTTCTTGTGTCATAATATCAGTAGTTATACTATTTTGGGTAATTGCTGCTATTCAAAATTCAATG  528
                     463 ************************************************************  528

Pjmam2_Cissé_ORF     529 GCGATTTTGTCTCAAACACATTTTGGAAGCAGTGGTATTTGGGGTGCACCTTGGCCATATACCGCC  594
Pjmam2_Cissé_gen     529 GCGATTTTGTCTCAAACACATTTTGGAAGCAGTGGTATTTGGGGTGCACCTTGGCCATATACCGCC  594
Pjmam2_Ma_ORF        529 GCGATTTTGTCTCAAACACATTTTGGAAGCAGTGGTATTTGGGGTGCACCTTGGCCATATACCGCC  594
Pjmam2_Ma_gen        529 GCGATTTTGTCTCAAACACATTTTGGAAGCAGTGGTATTTGGGGTGCACCTTGGCCATATACCGCC  594
                     529 ************************************************************  594

                                  intron 1
                         _____________________________
Pjmam2_Cissé_ORF     595 GCCC-----------------------------GATGTTTAGTTTTTGTTTCAAAATTATTTTTC  630
Pjmam2_Cissé_gen     595 GCCCGTATTTCATTTGTTTTTAGTGTATGTTTAGGATGTTTAGTTTTTGTTTCAAAATTATTTTTC  660
Pjmam2_Ma_ORF        595 GCCCGTATTTCATTTGTTTTTAGTGTATGTTTAGGATGTTTAGTTTTTGTTTCAAAATTATTTTTC  660
Pjmam2_Ma_gen        595 GCCCGTATTTCATTTGTTTTTAGTGTATGTTTAGGATGTTTAGTTTTTGTTTCAAAATTATTTTTC  660
                     595 ****                         ******************************  660

Pjmam2_Cissé_ORF     631 GCAATTTACCGTAGACATAAAATGGGTATCAAAGATTTTGGACCAATGCAAATCATATTTATTACA  696
Pjmam2_Cissé_gen     661 GCAATTTACCGTAGACATAAAATGGGTATCAAAGATTTTGGACCAATGCAAATCATATTTATTACA  726
Pjmam2_Ma_ORF        661 GCAATTTACCGTAGACATAAAATGGGTATCAAAGATTTTGGACCAATGCAAATCATATTTATTACA  726
Pjmam2_Ma_gen        661 GCAATTTACCGTAGACATAAAATGGGTATCAAAGATTTTGGACCAATGCAAATCATATTTATTACA  726
                     661 ************************************************************  726
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                                                                  intron 2
Pjmam2_Cissé_ORF     697 AGCTGTCAAACATTAATTATTCCTG----------------------------------------  720
Pjmam2_Cissé_gen     727 AGCTGTCAAACATTAATTATTCCTGGTATCTTTTTTTTTCTTTTTTTCCCTTTACTCTTAACACTT  792
Pjmam2_Ma_ORF        727 AGCTGTCAAACATTAATTATTCCTG----------------------------------------  750
Pjmam2_Ma_gen        727 AGCTGTCAAACATTAATTATTCCTGGTATCTTTTTTTTTCTTTTTTTCCCTTTACTCTTAACACTT  792
                     727 ************************                                        792


Pjmam2_Cissé_ORF     721 ----CAATCTTTATTATTATTGATTTCTGGGTAGATATAACCGGATTTAGTTCATTGACTCAAGCG  783
Pjmam2_Cissé_gen     793 TTAGCAATCTTTATTATTATTGATTTCTGGGTAGATATAACCGGATTTAGTTCATTGACTCAAGCG  858
Pjmam2_Ma_ORF        751 ----CAATCTTTATTATTATTGATTTCTGGGTAGATATAACCGGATTTAGTTCATTGACTCAAGCG  813
Pjmam2_Ma_gen        793 TTAGCAATCTTTATTATTATTGATTTCTGGGTAGATATAACCGGATTTAGTTCATTGACTCAAGCG  858
                     793     ************************************************************  858


Pjmam2_Cissé_ORF     784 TTTGTTGTAATGTCTTTACCATTATCTTCTCTTTGGGCATCATCTAAAATAGAAAAAAATAAAAAT  849
Pjmam2_Cissé_gen     859 TTTGTTGTAATGTCTTTACCATTATCTTCTCTTTGGGCATCATCTAAAATAGAAAAAAATAAAAAT  924
Pjmam2_Ma_ORF        814 TTTGTTGTAATGTCTTTACCATTATCTTCTCTTTGGGCATCATCTAAAATAGAAAAAAATAAAAAT  879
Pjmam2_Ma_gen        859 TTTGTTGTAATGTCTTTACCATTATCTTCTCTTTGGGCATCATCTAAAATAGAAAAAAATAAAAAT  924
                     859 ************************************************************  924


Pjmam2_Cissé_ORF     850 AGCATGGCACAGCCATACAGTGAGCGTATAAATAGCAAGGATTATAGTGTTAAAAGCTCTCCAACT  915
Pjmam2_Cissé_gen     925 AGCATGGCACAGCCATACAGTGAGCGTATAAATAGCAAGGATTATAGTGTTAAAAGCTCTCCAACT  990
Pjmam2_Ma_ORF        880 AGCATGGCACAGCCATACAGTGAGCGTATAAATAGCAAGGATTATAGTGTTAAAAGCTCTCCAACT  945
Pjmam2_Ma_gen        925 AGCATGGCACAGCCATACAGTGAGCGTATAAATAGCAAGGATTATAGTGTTAAAAGCTCTCCAACT  990
                     925 ************************************************************  990


Pjmam2_Cissé_ORF     916 TCGCTAAGTAAATCATCTTATATCGACTTTAAACAGCCCCCTTGTTATTTAGATTCTGGGAAATCA  981
Pjmam2_Cissé_gen     991 TCGCTAAGTAAATCATCTTATATCGACTTTAAACAGCCCCCTTGTTATTTAGATTCTGGGAAATCA 1056
Pjmam2_Ma_ORF        946 TCGCTAAGTAAATCATCTTATATCGACTTTAAACAGCCCCCTTGTTATTTAGATTCTGGGAAATCA 1011
Pjmam2_Ma_gen        991 TCGCTAAGTAAATCATCTTATATCGACTTTAAACAGCCCCCTTGTTATTTAGATTCTGGGAAATCA 1056
                     991 ************************************************************ 1056


Pjmam2_Cissé_ORF     982 CCATGTATTCCTTCTTTTGAATATAATGGAAATCCTTTTGATCAATTTTATGAAAATGACAGAAAT 1047
Pjmam2_Cissé_gen    1057 CCATGTATTCCTTCTTTTGAATATAATGGAAATCCTTTTGATCAATTTTATGAAAATGACAGAAAT 1122
Pjmam2_Ma_ORF       1012 CCATGTATTCCTTCTTTTGAATATAATGGAAATCCTTTTGATCAATTTTATGAAAATGACAGAAAT 1077
Pjmam2_Ma_gen       1057 CCATGTATTCCTTCTTTTGAATATAATGGAAATCCTTTTGATCAATTTTATGAAAATGACAGAAAT 1122
                    1057 ************************************************************ 1122


Pjmam2_Cissé_ORF    1048 AGACTCAATATATTTATAGAAGAATCAGTAGATATATCTTCAGAAAAAGCATAA 1101
Pjmam2_Cissé_gen    1123 AGACTCAATATATTTATAGAAGAATCAGTAGATATATCTTCAGAAAAAGCATAA 1176
Pjmam2_Ma_ORF       1078 AGACTCAATATATTTATAGAAGAATCAGTAGATATATCTTCAGAAAAAGCATAA 1131
Pjmam2_Ma_gen       1123 AGACTCAATATATTTATAGAAGAATCAGTAGATATATCTTCAGAAAAAGCATAA 1176
                    1123 ********************************************************* 1176
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B

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Pjmap3_Cissé_ORF       1 ------------------------------------------------------------------    2
Pjmap3_Cissé_gen       1  ATGTATTTGGGTTAACGGAGCATATAAGTTATTTTCAAGTATTACGTGTCCAATTTTTTGTTGAT    2
Pjmap3_Ma_ORF          1  ATGTATTTGGGTTAACGGAGCATATAAGTTGTTTTCAAGTATTACGTGTCCAATTTTTTGTTGAT   66
Pjmap3_Ma_gen          1  ATGTATTTGGGTTAACGGAGCATATAAGTTGTTTTCAAGTATTACGTGTCCAATTTTTTGTTGAT   66
                       1  ****************************** ***********************************   66

Pjmap3_Cissé_ORF       3 ----ATGGGCGATGTATTTTACGTTATTTATTCTTTTATTGGATTTATATGCTCAGTTATACCTTC   62
Pjmap3_Cissé_gen       3 CAAGATGGGCGATGTATTTTACGTTATTTATTCTTTTATTGGATTTATATGCTCAGTTATACCTTC   62
Pjmap3_Ma_ORF         67 CAAGATGGGCGATGTATTTTACGTTATTTATTCTTTTATTGGATTTATATGCTCAGTTATACCTTC  132
Pjmap3_Ma_gen         67 CAAGATGGGCGATGTATTTTACGTTATTTATTCTTTTATTGGATTTATATGCTCAGTTATACCTTC  132
                      67 ************************************************************** 132

Pjmap3_Cissé_ORF      63 TATTTGGCACTGGAAATATCGTAATGTAGCACCACTATGTCTTATTTTTTGGATCTCTGCTTGCAG  128
Pjmap3_Cissé_gen      63 TATTTGGCACTGGAAATATCGTAATGTAGCACCACTATGTCTTATTTTTTGGATCTCTGCTTGCAG  128
Pjmap3_Ma_ORF        133 TATTTGGCACTGGAAATATCGTAATGTAGCACCACTATGTCTTATTTTTTGGATCTCTGCTTGCAG  198
Pjmap3_Ma_gen        133 TATTTGGCACTGGAAATATCGTAATGTAGCACCACTATGTCTTATTTTTTGGATCTCTGCTTGCAG  198
                     133 ************************************************************** 198

Pjmap3_Cissé_ORF     129 CTTAATATGTTTTATAAACTCTATCGTCTGGTTTAATGGAGTTGAAGCAAAATCTCCGGGTTATAT  194
Pjmap3_Cissé_gen     129 CTTAATATGTTTTATAAACTCTATCGTCTGGTTTAATGGAGTTGAAGCAAAATCTCCGGGTTATAT  194
Pjmap3_Ma_ORF        199 CTTAATATGTTTTATAAACTCTATCGTCTGGTTTAATGGAGTTGAAGCAAAATCTCCGGGTTATAT  264
Pjmap3_Ma_gen        199 CTTAATATGTTTTATAAACTCTATCGTCTGGTTTAATGGAGTTGAAGCAAAATCTCCGGGTTATAT  264
                     199 ************************************************************** 264

Pjmap3_Cissé_ORF     195 ATACTGTGATATTGCTACAAAAATTATACTTGGGTCTACCTCAGGAGAATTAGGTGCTATTGCAGC  260
Pjmap3_Cissé_gen     195 ATACTGTGATATTGCTACAAAAATTATACTTGGGTCTACCTCAGGAGAATTAGGTGCTATTGCAGC  260
Pjmap3_Ma_ORF        265 ATACTGCGATATTGCTACAAAAATTATACTTGGGTCTACCTCAGGAGAATTAGGTGCTATTGCAGC  330
Pjmap3_Ma_gen        265 ATACTGCGATATTGCTACAAAAATTATACTTGGGTCTACCTCAGGAGAATTAGGTGCTATTGCAGC  330
                     265 ****** ************************************************************ 330

Pjmap3_Cissé_ORF     261 TATTTCACATTATCTTTCAAAAATCATGAGCCCTGTACATTCTTCTGTACAAACTAAAACAATACG  326
Pjmap3_Cissé_gen     261 TATTTCACATTATCTTTCAAAAATCATGAGCCCTGTACATTCTTCTGTACAAACTAAAACAATACG  326
Pjmap3_Ma_ORF        331 TATTTCACATTATCTTTCAAAAATCATGAGCCCTGTACATTCTTCTGTACAAACTAAAACAATCCG  396
Pjmap3_Ma_gen        331 TATTTCACATTATCTTTCAAAAATCATGAGCCCTGTACATTCTTCTGTACAAACTAAAACAATCCG  396
                     331 ************************************************************** ** 396

Pjmap3_Cissé_ORF     327 CAGAAGACAGGCTATAGAAGATCTTCTTATGAGTTTTACATGCCCAATCATCATGATATGTTTACA  392
Pjmap3_Cissé_gen     327 CAGAAGACAGGCTATAGAAGATCTTCTTATGAGTTTTACATGCCCAATCATCATGATATGTTTACA  392
Pjmap3_Ma_ORF        397 CAGAAGACAGGCTATAGAAGATCTTCTTATGAGTTTTACATGCCCAATCATCATGATATGTTTATA  462
Pjmap3_Ma_gen        397 CAGAAGACAGGCTATAGAAGATCTTCTTATGAGTTTTACATGCCCAATCATCATGATATGTTTATA  462
                     397 ************************************************************** * 462

Pjmap3_Cissé_ORF     393 TTATGTTATTCAATCTGCAAGGTATGTAATAAATGGTGTCAATGGGTGTGTACCATGGTCCGATCA  458
Pjmap3_Cissé_gen     393 TTATGTTATTCAATCTGCAAGGTATGTAATAAATGGTGTCAATGGGTGTGTACCATGGTCCGATCA  458
Pjmap3_Ma_ORF        463 TTATGTTATTCAATCTGCAAGGTATGTAATAAATGGTGTCAATGGGTGTGTACCATGGTCCGATCA  528
Pjmap3_Ma_gen        463 TTATGTTATTCAATCTGCAAGGTATGTAATAAATGGTGTCAATGGGTGTGTACCATGGTCCGATCA  528
                     463 ************************************************************** 528

Pjmap3_Cissé_ORF     459 ATCATGGCCAACAGTAATCATCGTTTTAATCTGGCCCCCTATTTTTGGTTCAATCAGTGCTTATTA  524
Pjmap3_Cissé_gen     459 ATCATGGCCAACAGTAATCATCGTTTTAATCTGGCCCCCTATTTTTGGTTCAATCAGTGCTTATTA  524
Pjmap3_Ma_ORF        529 ATCATGGCCAACAGTAATCATCGTTTTAATCTGGCCCCCTATTTTTGGTTCAATCAGTGCTTATTA  594
Pjmap3_Ma_gen        529 ATCATGGCCAACAGTAATCATCGTTTTAATCTGGCCCCCTATTTTTGGTTCAATCAGTGCTTATTA  594
                     529 ************************************************************** 594

                                                intron 1
Pjmap3_Cissé_ORF     525 TTCAG----------------------------------------------CTAAAGTAATATA  542
Pjmap3_Cissé_gen     525 TTCAGGTACACCATTTGCACGGTTTATAATAAATGTATTTATCATTTTATTAGCTAAAGTAATATA  590
Pjmap3_Ma_ORF        595 TTCAGGTACACCATTTGCACAGTTTATAATAAATGTATTTATCATTTTATTAGCTAAAGTAATATA  660
Pjmap3_Ma_gen        595 TTCAGGTACACCATTTGCACAGTTTATAATAAATGTATTTATCATTTTATTAGCTAAAGTAATATA  660
                     595 *****                                             ************* 660
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Pjmap3_Cissé_ORF     543 CCTATATTTCAAAAAACAAAAAGAATTTCAAAATGTTTTAAGAGATTCTAAAACATCTATGACACT  608
Pjmap3_Cissé_gen     591 CCTATATTTCAAAAAACAAAAAGAATTTCAAAATGTTTTAAGAGATTCTAAAACATCTATGACACT  656
Pjmap3_Ma_ORF        661 CCTATATTTCAAAAAACAAAAAGAATTTCAAAATGTTTTAAGAGATTCTAAAACATCTATGACACT  726
Pjmap3_Ma_gen        661 CCTATATTTCAAAAAACAAAAAGAATTTCAAAATGTTTTAAGAGATTCTAAAACATCTATGACACT  726
                     661 ************************************************************  726

Pjmap3_Cissé_ORF     609 TTCTAGATTTGTACGTCTTATAGGCATATGCTCCCTTTTAGTCACTGTTTATTTACCATTAAATAT  674
Pjmap3_Cissé_gen     657 TTCTAGATTTGTACGTCTTATAGGCATATGCTCCCTTTTAGTCACTGTTTATTTACCATTAAATAT  722
Pjmap3_Ma_ORF        727 TTCTAGATTTGTACGTCTTATAGGCATATGCTCCCTTTTAGTCACTGTTTATTTACCATTAAATAT  792
Pjmap3_Ma_gen        727 TTCTAGATTTGTACGTCTTATAGGCATATGCTCCCTTTTAGTCACTGTTTATTTACCATTAAATAT  792
                     727 ************************************************************  792

Pjmap3_Cissé_ORF     675 TTACATGTTATATACGAATATATTTCTAATTATTCAAAGCAAAATAAACTATTCATGGGACCATGT  740
Pjmap3_Cissé_gen     723 TTACATGTTATATACGAATATATTTCTAATTATTCAAAGCAAAATAAACTATTCATGGGACCATGT  788
Pjmap3_Ma_ORF        793 TTACATGTTATATACGAATATATTTCTAATTATTCAAAGCAAAATAAACTATTCATGGGACCATGT  858
Pjmap3_Ma_gen        793 TTACATGTTATATACGAATATATTTCTAATTATTCAAAGCAAAATAAACTATTCATGGGACCATGT  858
                     793 ************************************************************  858

Pjmap3_Cissé_ORF     741 TCATCAATGGGGTCATGGTATTGCTTATTTGAAAAATGATAAAATATCTTTTAATCTATGGCTTAT  806
Pjmap3_Cissé_gen     789 TCATCAATGGGGTCATGGTATTGCTTATTTGAAAAATGATAAAATATCTTTTAATCTATGGCTTAT  854
Pjmap3_Ma_ORF        859 TCATCAATGGGGTCATGGTATTGCTTATTTGAAAAATGATAAAATATCTTTTAATCTATGGCTTAT  924
Pjmap3_Ma_gen        859 TCATCAATGGGGTCATGGTATTGCTTATTTGAAAAATGATAAAATATCTTTTAATCTATGGCTTAT  924
                     859 ************************************************************  924

Pjmap3_Cissé_ORF     807 ACCGTCAAATAGTATTGTTGTTTTCATTTTTTTTGGTATGGGAAGTGATGCCATTGTCATGTATAA  872
Pjmap3_Cissé_gen     855 ACCGTCAAATAGTATTGTTGTTTTCATTTTTTTTGGTATGGGAAGTGATGCCATTGTCATGTATAA  920
Pjmap3_Ma_ORF        925 ACCGTCAAATAGTATTGTTGTTTTCATTTTTTTTGGTATGGGAAGTGATGCCATTGTCATGTATAA  990
Pjmap3_Ma_gen        925 ACCGTCAAATAGTATTGTTGTTTTCATTTTTTTTGGTATGGGAAGTGATGCCATTGTCATGTATAA  990
                     925 ************************************************************  990

Pjmap3_Cissé_ORF     873 AGAAGTGGCAAGAAAACTGTATATAATTAAATTTTTTGATTTCTTTAAAAGAATGTTCAAAAGAAA  938
Pjmap3_Cissé_gen     921 AGAAGTGGCAAGAAAACTGTATATAATTAAATTTTTTGATTTCTTTAAAAGAATGTTCAAAAGAAA  986
Pjmap3_Ma_ORF        991 AGAAGTGGCAAGAAAACTGTATATAATTAAATTTTTTGATTTCTTTAAAAGAATGTTCAAAAGAAA 1056
Pjmap3_Ma_gen        991 AGAAGTGGCAAGAAAACTGTATATAATTAAATTTTTTGATTTCTTTAAAAGAATGTTCAAAAGAAA 1056
                     991 ************************************************************ 1056

Pjmap3_Cissé_ORF     939 GACTCAGGATGTCAGTAACAAAGACTATTATAACAGCTATAATTTCGAAAAATCATTAGACAG---  999
Pjmap3_Cissé_gen     987 GACTCAGGATGTCAGTAACAAAGACTATTATAACAGCTATAATTTCGAAAAATCATTAGACAGGTA 1052
Pjmap3_Ma_ORF       1057 GACTCAGGATGTCAGTAACAAAGACTATTATAACAGCTATAATTTCGAAAAATCATTAGACAG--- 1117
Pjmap3_Ma_gen       1057 GACTCAGGATGTCAGTAACAAAGACTATTATAACAGCTATAATTTCGAAAAATCATTAGACAGGTA 1122
                    1057 ************************************************************    1122

                          ___________intron 2_____________________
Pjmap3_Cissé_ORF    1000 ----------------------------------------ATGTCCACCATTGTTTTATAACCAAG 1027
Pjmap3_Cissé_gen    1053 AGACACATCAGTTCTAAGGTATCTTTTTACAGGCTTCCAGATGTCCACCATTGTTTTATAACCAAG 1118
Pjmap3_Ma_ORF       1118 ----------------------------------------ATGTCCACCATTGTTTTATAACCAAG 1145
Pjmap3_Ma_gen       1123 AGACACATCAGTTCTAAGGTATCTTTTTACAGGCTTCCAGATGTCCACCATTGTTTTATAACCAAG 1188
                    1123                                         ************************* 1188

Pjmap3_Cissé_ORF    1028 TACGCGATGCACAGATTATAGAAAACAACTCTTTTAGCGACCATCCTGCAATCCCTCCAATATACA 1093
Pjmap3_Cissé_gen    1119 TACGCGATGCACAGATTATAGAAAACAACTCTTTTAGCGACCATCCTGCAATCCCTCCAATATACA 1184
Pjmap3_Ma_ORF       1146 TACGCGATGCACAGATTATAGAAAACAACTCTTTTAGCGACCATCCTGCAATCCCTCCAATATACA 1211
Pjmap3_Ma_gen       1189 TACGCGATGCACAGATTATAGAAAACAACTCTTTTAGCGACCATCCTGCAATCCCTCCAATATACA 1254
                    1189 ************************************************************ 1254

Pjmap3_Cissé_ORF    1094 TGGAACACAGCAAACCATACACCTTTACAGAT---------------------------------- 1122
Pjmap3_Cissé_gen    1185 TGGAACACAGCAAACCATACACCTTTACAGATGTACCTATTTATTCCCATAGCAAAAACACCTATT 1213
Pjmap3_Ma_ORF       1212 TGGAACACAGCAAACCATACACCTTTACAGATGTACCTATTTATTCCCATAGCAAAAACACCTATT 1277
Pjmap3_Ma_gen       1255 TGGAACACAGCAAACCATACACCTTTACAGATGTACCTATTTATTCCCATAGCAAAAACACCTATT 1320
                    1255 ********************************                               1320

Pjmap3_Cissé_ORF    1123 ------------------------------------------------- 1125
Pjmap3_Cissé_gen    1214 CCATGCCCTTTGAAAAATACCAATATGAATTTAGAAATGATAAAATATAA 1216
Pjmap3_Ma_ORF       1278 CCATGCCCTTTGAAAAATACCAATATGAATTTAGAAATGATAAAATATAA 1327
Pjmap3_Ma_gen       1321 CCATGCCCTTTGAAAAATACCAATATGAATTTAGAAATGATAAAATATAA 1370
                    1321                                                  1370
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Figure S1. Multiple sequence alignment of *mam2* (**A**) and *map3* (**B**) ORF and genomic (gen) gene sequences of the two *P. jirovecii* genome assemblies (Cissé *et al.*, 2012, indicated as Cissé; Ma *et al.,* 2016, indicated as Ma). T-Coffee was used (Notredame *et al.,* 2000). The identical residues are indicated by asterisks. Dashes indicate gaps. Introns are highlighted in grey and indicated as "intron 1" or "2". Acceptor and donor canonical sequences of *Pneumocystis* introns are in bold. **A**. Alignment of *mam2* ORF and genomic sequences. The first intron predicted in Cissé assembly is not predicted in Ma assembly and considered as part of the ORF (residues 600 to 629 in the genomic sequences). **B**. Alignment of *map3* ORF and genomic sequences. ORF and genomic sequences of Ma assembly are longer than Cissé sequences, 70 bps upstream and 83 bps downstream. The first intron predicted in Cissé assembly is not predicted in Ma assembly and considered as part of the ORF (residues 600 to 647 in the Ma genomic sequence) Residues shown in bold are synonymous SNPs. The ORF sequences of Cissé were investigated in the present study.

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Pmmam2_ORF       1 ATGGCATTTTCTCCAACAAACCAGACAATATTTCTTAAGAACTCTAAAGGAGAAACAATTCCATTTTTATTATCGGAT   78
Pmmam2_gen       1 ATGGCATTTTCTCCAACAAACCAGACAATATTTCTTAAGAACTCTAAAGGAGAAACAATTCCATTTTTATTATCGGAT   78
                 1 ************************************************************************   78

Pmmam2_ORF      79 TTCGATGAATTTTCACTCTCTAGAGCTCAAACGTCTATGATATTTTCAGCACAATGTGCAATGAGCTTATTATTGGCA  156
Pmmam2_gen      79 TTCGATGAATTTTCACTCTCTAGAGCTCAAACGTCTATGATATTTTCAGCACAATGTGCAATGAGCTTATTATTGGCA  156
                79 ************************************************************************  156

Pmmam2_ORF     157 CTTGTCCTTATGTTGACATCAAAAAGAGAAAAAAGAAAAACATTGCTTTTCTTTTTAAACATAGGTGGATTAGTAACA  234
Pmmam2_gen     157 CTTGTCCTTATGTTGACATCAAAAAGAGAAAAAAGAAAAACATTGCTTTTCTTTTTAAACATAGGTGGATTAGTAACA  234
               157 ************************************************************************  234

Pmmam2_ORF     235 GTATTTATAAGAGCATGTCTTCAATGTGCTTATTTGTCAGGCACCTGGGTAAGCTATAGTGTCCAATTTCTTGGAGAA  312
Pmmam2_gen     235 GTATTTATAAGAGCATGTCTTCAATGTGCTTATTTGTCAGGCACCTGGGTAAGCTATAGTGTCCAATTTCTTGGAGAA  312
               235 ************************************************************************  312

Pmmam2_ORF     313 TTTGAATTATTGTCGCAAAAAGACTTTTATATATCAATTATCGCATCATGTATTCCAATTTTTATTATTTTGTTCATC  390
Pmmam2_gen     313 TTTGAATTATTGTCGCAAAAAGACTTTTATATATCAATTATCGCATCATGTATTCCAATTTTTATTATTTTGTTCATC  390
               313 ************************************************************************  390

Pmmam2_ORF     391 GAACTTTCTCTTCTCATTCAAATTAGAGTAGTATATGCAACTGATAAAAGATTACAGATACCATTGACAATATTTTTT  468
Pmmam2_gen     391 GAACTTTCTCTTCTCATTCAAATTAGAGTAGTATATGCAACTGATAAAAGATTACAGATACCATTGACAATATTTTTT  468
               391 ************************************************************************  468

Pmmam2_ORF     469 TCTATAATAATAATTATCGTTATAACCTTTTGGATTTTAGCTGCTGTTCAAAACTCAATGGCAGTTTTATCTCAAACA  546
Pmmam2_gen     469 TCTATAATAATAATTATCGTTATAACCTTTTGGATTTTAGCTGCTGTTCAAAACTCAATGGCAGTTTTATCTCAAACA  546
               469 ************************************************************************  546

Pmmam2_ORF     547 CATTTTGGACATAGCGGTGTATGGGGCGCACCCTGGCCTTATACAGTAGCACGCATATCTTTTGCTTTTAGTATATTT  624
Pmmam2_gen     547 CATTTTGGACATAGCGGTGTATGGGGCGCACCCTGGCCTTATACAGTAGCACGCATATCTTTTGCTTTTAGTATATTT  624
               547 ************************************************************************  624

Pmmam2_ORF     625 ATAGGATGTATTGTTTTTATTTATAAATTGCTTATCACCATTTATCGAAGACATAAAATGGGAGTCAAAGAATTTGGA  702
Pmmam2_gen     625 ATAGGATGTATTGTTTTTATTTATAAATTGCTTATCACCATTTATCGAAGACATAAAATGGGAGTCAAAGAATTTGGA  702
               625 ************************************************************************  702

                                                                              intron 1
Pmmam2_ORF     703 CCAATACAAATTATATTTATTATGAGCTGTCAAACATTGATCATCCCTG------------------------------  750
Pmmam2_gen     703 CCAATACAAATTATATTTATTATGAGCTGTCAAACATTGATCATCCCTGGTATTTTTTGTCACATTCTGTTTTATTTC  780
               703 *************************************************                              780

Pmmam2_ORF     751 ----------------CCATTCTCATTCTTATCGATTTTGGAGTAAAAATAACAGGTTTTAGCTCATTAACTCAAGCG  813
Pmmam2_gen     781 TAATATTATTTTATAGCCATTCTCATTCTTATCGATTTTGGAGTAAAAATAACAGGTTTTAGCTCATTAACTCAAGCG  858
               781                 ************************************************************  858

Pmmam2_ORF     814 CTCGTCGTAATGTCTTTACCTTTATCTTCTCTTTGGGCATCATCTAAAGTCGAAAAAAATAAAAACAACGTAACACCA  891
Pmmam2_gen     859 CTCGTCGTAATGTCTTTACCTTTATCTTCTCTTTGGGCATCATCTAAAGTCGAAAAAAATAAAAACAACGTAACACCA  936
               859 ************************************************************************  936

Pmmam2_ORF     892 ACATATTATAAAGATATGAAGAGCATTGGAGATTATAGTATTGAAAGCACACCGAGTTCATTCACTAAACCATCTTAT  969
Pmmam2_gen     937 ACATATTATAAAGATATGAAGAGCATTGGAGATTATAGTATTGAAAGCACACCGAGTTCATTCACTAAACCATCTTAT 1014
               937 ************************************************************************ 1014

Pmmam2_ORF     970 ATAGGGTTTAGAAAACCATCGTATTTCTCGGAATATTCTAAAAGTCCTTTCTATGATGAGTATTTTGATGATAATGGG 1047
Pmmam2_gen    1015 ATAGGGTTTAGAAAACCATCGTATTTCTCGGAATATTCTAAAAGTCCTTTCTATGATGAGTATTTTGATGATAATGGG 1092
              1015 ************************************************************************ 1092

Pmmam2_ORF    1048 TCTAAACTTGATATACTAGTGGAAAAATCTCTAAATGTGTTTCCAGAAAAGAAATAG---------------------- 1102
Pmmam2_gen    1093 TCTAAACTTGATATACTAGTGGAAAAATCTCTAAATGTGTTTCCAGAAAAGAAATAGTTAGAAAGTTTTTAGCACGTT 1170
              1093 ********************************************************                     1170

Pmmam2_ORF    1103    1104
Pmmam2_gen    1171 CA 1172
              1171    1172
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B

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Pmmap3_ORF       1 ATGGGAGAAGTGTTTTATATCTTTTTTTGCTTGATTGGATTTTTATGTTCAATTATACCTTCTATCTGGCACTGGAAA   78
Pmmap3_gen       1 ATGGGAGAAGTGTTTTATATCTTTTTTTGCTTGATTGGATTTTTATGTTCAATTATACCTTCTATCTGGCACTGGAAA   78
                 1 ********************************************************************************   78

Pmmap3_ORF      79 TATCGAAATGTCGCGCCTTTATGTCTTATTTTTTGGATATCTTCAACTAATTTAATATATTTCATTAATTCTATCATC   156
Pmmap3_gen      79 TATCGAAATGTCGCGCCTTTATGTCTTATTTTTTGGATATCTTCAACTAATTTAATATATTTCATTAATTCTATCATC   156
                79 ********************************************************************************  156

Pmmap3_ORF     157 TGGTTTAACGGATCTAAATCAACATATCGTGGTGATCTATATTGTGACATTGTGACTAAGCTTATACTCGGATCGGTT   234
Pmmap3_gen     157 TGGTTTAACGGATCTAAATCAACATATCGTGGTGATCTATATTGTGACATTGTGACTAAGCTTATACTCGGATCGGTT   234
               157 ********************************************************************************  234

Pmmap3_ORF     235 ACTGGAGAATTAGGTGCTACTGTTGCTATTACACATTACCTTTCGAAAATTATGAAGTCTTCATATTCATCTATCCAG   312
Pmmap3_gen     235 ACTGGAGAATTAGGTGCTACTGTTGCTATTACACATTACCTTTCGAAAATTATGAAGTCTTCATATTCATCTATCCAG   312
               235 ********************************************************************************  312

Pmmap3_ORF     313 TCTAAAATAACTCGTAGAAATCAAGCAATAGAAGATATTCTTTTTAGTTTTACATGTCCTATTATAATAATGTCTTTA   390
Pmmap3_gen     313 TCTAAAATAACTCGTAGAAATCAAGCAATAGAAGATATTCTTTTTAGTTTTACATGTCCTATTATAATAATGTCTTTA   390
               313 ********************************************************************************  390

Pmmap3_ORF     391 CATTATATCGTTCAGCCAGCAAGGTATGTAATCGATGGAATTAGTGGATGCATGCCATGGACGGATCGATCATGGCTA   468
Pmmap3_gen     391 CATTATATCGTTCAGCCAGCAAGGTATGTAATCGATGGAATTAGTGGATGCATGCCATGGACGGATCGATCATGGCTA   468
               391 ********************************************************************************  468

Pmmap3_ORF     469 GCTGTGATTATTGTTTTATTATGGCCTCCTGTATTTGGTAGTATCAGTGCTTACTATTCAG----------------   528
Pmmap3_gen     469 GCTGTGATTATTGTTTTATTATGGCCTCCTGTATTTGGTAGTATCAGTGCTTACTATTCAGGTATATTCTTCGTATGA   546
               469 ************************************************************                   546

                     intron 1
Pmmap3_ORF     529 ----------------------------TTAAAGTAATTATTTCATACTTCAAAAAGCGAAATGAATTTCAAACTATT   579
Pmmap3_gen     547 TTTATAATAAATGTATTCATTTTATTAGTTAAAGTAATTATTTCATACTTCAAAAAGCGAAATGAATTTCAAACTATT   624
               547                             ************************************************  624

Pmmap3_ORF     580 TTAAAAGATTCAAAATCGTCTATGACTTTATCAAGATTCATACGTCTTATAGGCTTATCATCTCTAATAATAGCCATT   657
Pmmap3_gen     625 TTAAAAGATTCAAAATCGTCTATGACTTTATCAAGATTCATACGTCTTATAGGCTTATCATCTCTAATAATAGCCATT   702
               625 ********************************************************************************  702

Pmmap3_ORF     658 TACTTGCCACTTAATATTTACTTGTTAGCTATAAACATAGCCCAGATTATTAAAAGCAACATCAAATATTCATGGTCA   735
Pmmap3_gen     703 TACTTGCCACTTAATATTTACTTGTTAGCTATAAACATAGCCCAGATTATTAAAAGCAACATCAAATATTCATGGTCA   780
               703 ********************************************************************************  780

Pmmap3_ORF     736 GATGTTCATAATTGGAATTCTAGTATTTTTTATCTTCCAAAAAGCAACATGCCTTTCAATCGCTGGCTTTCGCCATCT   813
Pmmap3_gen     781 GATGTTCATAATTGGAATTCTAGTATTTTTTATCTTCCAAAAAGCAACATGCCTTTCAATCGCTGGCTTTCGCCATCT   858
               781 ********************************************************************************  858

Pmmap3_ORF     814 AATGGCATTATTGTTTTTATCTTTTTTGGTATGGGTAATGATGCAATACTCATGTATAAAGAAATAGCAAGAAAATTG   891
Pmmap3_gen     859 AATGGCATTATTGTTTTTATCTTTTTTGGTATGGGTAATGATGCAATACTCATGTATAAAGAAATAGCAAGAAAATTG   936
               859 ********************************************************************************  936

Pmmap3_ORF     892 TACATTACTCAATTTTTCCATTTTGTCCAAAAGAAAATTTTCAAAAAAAAAAACAGAAGATAACAAAAATTCACAGGAT   969
Pmmap3_gen     937 TACATTACTCAATTTTTCCATTTTGTCCAAAAGAAAATTTTCAAAAAAAAAAACAGAAGATAACAAAAATTCACAGGAT  1014
               937 ********************************************************************************  1014

                                                                        intron 2
Pmmap3_ORF     970 TATTATAATAGCTATAGTTTTGAAAAATCATTAAACAG----------------------------------------  1007
Pmmap3_gen    1015 TATTATAATAGCTATAGTTTTGAAAAATCATTAAACAGGTAAGATAAATCAATCTTTTAATATCCTTTTCCTTACAAA  1092
              1015 ************************************                                          1092

Pmmap3_ORF    1008 ---------TTGTCCTCCATTATTTTATAATCAAACACGAGACGTAAGAATTCTTGAAAATGGTTCTTTAAATGATTA  1076
Pmmap3_gen    1093 TATATTTAGTTGTCCTCCATTATTTTATAATCAAACACGAGACGTAAGAATTCTTGAAAATGGTTCTTTAAATGATTA  1170
              1093          ************************************************************************  1170

Pmmap3_ORF    1077 TTCTTCTCCTCCTATTTATACAGATCATGACAAGTACAACTTAGATTTATCAATCTATAATCAGTATTACAGAGACAA  1154
Pmmap3_gen    1171 TTCTTCTCCTCCTATTTATACAGATCATGACAAGTACAACTTAGATTTATCAATCTATAATCAGTATTACAGAGACAA  1248
              1171 ********************************************************************************  1248
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Pmmap3_ORF     1155 TTCTAATATAAATAATAAATACGGGCCTCGCAAATAA 1191
Pmmap3_gen     1249 TTCTAATATAAATAATAAATACGGGCCTCGCAAATAA 1285
                    ************************************* 1285
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Figure S2. Sequence alignment of *P. murina mam2* (**A**) and *map3* (**B**) ORF and genomic (gen) gene sequences. T-coffee was used (Notredame *et al.,* 2000). The identical residues are indicated by asterisks. Dashes indicate gaps. Introns are highlighted in grey and indicated as "intron 1" or "2". Acceptor and donor canonical sequences of *Pneumocystis* introns are in bold. Stop codons within introns are in red.

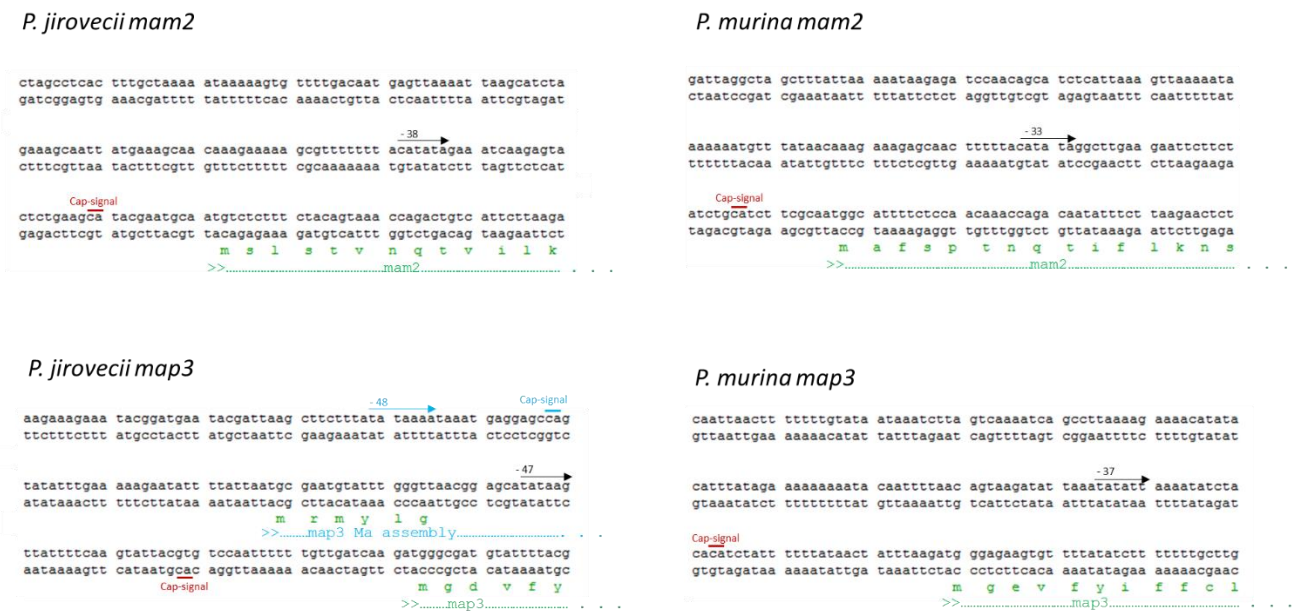

Figure S3. Potential TATA boxes and Cap-signal upstream the start codon of *mam2* and *map3* genes of *P. jirovecii* and *P. murina.* Potential TATA boxes were identified by visual inspection by matching with the descriptions in Bucher *et al.,* 1990*.* TATA-boxes are shown with an arrow oriented toward the ORF, and their distance from the start codon of the ORF are shown in bps. Potential cap-signals are shown in red. In *P. jirovecii map3,* the begin of the ORF and promoter elements in the Ma assembly version are in blue.