Pearls

Genomic Insights into the Fungal Pathogens of the Genus *Pneumocystis*: Obligate Biotrophs of Humans and Other Mammals

Philippe M. Hauser*

Institute of Microbiology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland

Pneumocystis organisms were first believed to be a single protozoan species able to colonize the lungs of all mammals. Subsequently, genetic analyses revealed their affiliation to the fungal Taphrinomycotina subphylum of the Ascomycota, a clade which encompasses plant pathogens and free-living yeasts. It also appeared that, despite their similar morphological appearance, these fungi constitute a family of relatively divergent species, each with a strict specificity for a unique mammalian species [1]. The species colonizing human lungs, Pneumocystis jirovecii, can turn into an opportunistic pathogen that causes Pneumocystis pneumonia (PCP) in immunocompromised individuals, a disease which may be fatal. Although the incidence of PCP diminished in the 1990s thanks to prophylaxis and antiretroviral therapy, PCP is nowadays the second-most-frequent, life-threatening, invasive fungal infection worldwide, with an estimated number of cases per year above 400,000 [2]. Despite this clinical importance, studies of P. *jirovecii* progressed slowly, at least in part because of the lack of a continuous culture system. Nevertheless, recent genomic findings provided insights into the lifestyle of these fungi.

Genome Sequences

Despite the absence of culture, the nuclear genome sequences of three *Pneumocystis* spp. have been recently released to the public. That of P. carinii, the species infecting rats, was obtained from resected lungs of an infected rat and purification of P. carinii cells (pgp.cchmc.org). This provided sufficient amounts of relatively pure DNA for conventional cloning and sequencing of chromosomes separated on gel, as well as for high throughput DNA sequencings (HTS). The nuclear genome sequence of P. murina, the species infecting mice, was obtained from resected lungs followed by HTS (Pneumocystis murina Sequencing Project, Broad Institute of Harvard and the Massachusetts Institute of Technology, http://www.broadinstitute.org/). Thanks to cell immunoprecipitation and whole genome amplification, the P. jirovecii nuclear genome sequence was obtained using HTS from a single bronchoalveolar lavage fluid sample of a single patient with PCP [3]. Because of the low DNA purity, the assembly necessitated an innovative approach using iterative identification of the fungal homologies among the reads from the host and lung microbiota. The genome sequences of the mitochondria were also obtained from the same sequencings, as well as by PCR [4,5].

Genomic Assemblies

The nuclear genome assembly of *P. carinii* is still highly fragmented, whereas that of *P. murina* is made of 17 contigs likely to correspond to the 17 chromosomes composing this genome (Table 1). The *P. jirovecii* nuclear assembly presents an intermediary fragmentation, which results from the difficulty of assembling it out of a mixture of reads. Because of their repetitive nature,

telomeres were not assembled. The nuclear genomes of the three *Pneumocystis* spp. are approximately 8 Mb.

The mitochondrial genomes of *P. murina* and *P. carinii* are approximately 25 kb, whereas that of *P. jirovecii* is around 35 kb (Table 1). This size difference is due to a supplementary region that is non-coding and highly variable in size and sequence among isolates [5]. The circular structure of the *P. jirovecii* mitochondrial genome differs from the linear one present in the two other *Pneumocystis* spp. The biological significance of this difference remains unknown.

Genome Content

Using gene models specifically developed, the three Pneumocystis spp. nuclear genomes were predicted to encode approximately 3,600 protein coding genes (Table 1). Mapping of the genes onto the chromosomes is achieved for P. murina because contigs correspond to chromosomes, as well as for P. carinii because isolated chromosomes were sequenced, but not for P. jirovecii. Functional annotation was optimized by the use of transcription data as well as of carefully chosen fungal proteomes as intermediary data for mapping onto the Kyoto Encyclopedia of Genes and Genomes (KEGG) atlas of biochemical pathways [3,6]. About 30%-40% of the genes were reported to encode hypothetical proteins without significant homology with the databases, but this proportion is decreasing as new fungal genome sequences are released. A maximum likelihood phylogeny from the alignment of 458 concatenated orthologs revealed Taphrina deformans and the members of the Schizosaccharomyces genus as the closest relatives of Pneumocystis spp. [3,7]. The genome content of the three Pneumocystis spp. covered most of the biochemical pathways corresponding to the basal metabolism and standard cellular processes [6,8]. Specific features included the presence of a single operon encoding the ribosomal RNA (Table 1), such as T. deformans [7], which contrasts with the tens or hundreds in other fungi, and the lack of common fungal

Editor: Joseph Heitman, Duke University Medical Center, United States of America

Published November 6, 2014

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Funding: The author's research is funded by the Swiss National Science Foundation (grant 310030-146135). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The author has declared that no competing interests exist.

* Email: Philippe.Hauser@chuv.ch



Citation: Hauser PM (2014) Genomic Insights into the Fungal Pathogens of the Genus *Pneumocystis*: Obligate Biotrophs of Humans and Other Mammals. PLoS Pathog 10(11): e1004425. doi:10.1371/journal.ppat.1004425

Table 1. Statistics of *Pneumocystis* spp. nuclear and mitochondrial genomic assemblies^a.

	P. jirovecii	P. murina	P. carinii	
Nuclear				
Assembly size [Mb] ^b	8.1	7.45	6.3	
Contigs	358	17	4′278	
N50 [kb]	41.6	491	2.2	
Mean GC content [%]	28.4	26.9	32.5	
Protein coding genes ^c	3'898	3′557	4′591	
Coding regions [%]	68.9	66.3	50.0	
rRNA operon	1	1	1	
tRNA genes	77	47	36 ^d	
Mitochondrial				
Assembly size [kb]	35.1	24.6	26	
Structure	circular	linear	linear	
Contigs	1	1	1	
GC content [%]	25.7	29.8	29.8	
Protein coding genes	14	14	14	
Orfs (unknown function)	1	2	4	
rRNA genes	2	2	2	
tRNA genes	25	28	25	

^aThe data of the nuclear genomes are from Cissé et al. [3], except those of *P. murina* which are from broadinstitute.org. The data of the mitochondrial genomes are from Sesterhenn et al. [4] and Ma et al. [5].

^bThe nuclear assemblies of the three species are considered to be incomplete and do not include repetitive elements.

^cThe numbers for *P. jirovecii* and *P. carinii* overestimate proteome size because some peptides are from incomplete coding DNA sequences at ends of contigs.

^dOnly complete copies in *P. carinii* assembly were considered.

doi:10.1371/journal.ppat.1004425.t001

virulence factors such as the glyoxylate cycle and polyketide synthase clusters [3,6,8]. The mitochondrial genomes of the three *Pneumocystis* spp. encode 15 to 17 proteins (Table 1).

Genomic Insights by Comparative Genomics

Relevant features of the P. carinii genome were compared to those of the free-living yeast S. cerevisiae and of the extreme fungal obligate parasite Encephalitozoon cuniculi [9]. Intermediate values of gene number, genome size, and mean intergenic space suggested that P. carinii might be in the process of becoming dependent on its host. The use of Schizosaccharomyces pombe genome sequence as a control for genomic annotation revealed the absence of most of the enzymes specifically dedicated to the synthesis of amino acids in *Pneumocystis* spp. [3,6]. Whole genome comparison to other representatives of the Taphrinomycotina subphylum revealed several supplementary gene losses in P. carinii and P. jirovecii (P. murina could not be analyzed because its genome sequence was released prior to publication under specific terms). The hypothetical gene repertoires of ancestors were reconstructed using maximum parsimony and the irreversible Dollo model of evolution [10]. The approach identified approximately 2,000 genes presumably lost by the common ancestor of Pneumocystis and Taphrina genera during its evolution towards the Pneumocystis genus. Analysis of these genes revealed losses of genes that impair the biosynthesis of thiamine, the assimilation of inorganic nitrogen and sulfur, and the catabolism of purines. In addition, lytic proteases, which are believed to be crucial to fungal virulence, were underrepresented. The absence of the genes was ascertained by extensive gene searches in partially overlapping data sets expected to cover 100% of the genomes, and by the fact

that it was observed in both *P. carinii* and *P. jirovecii*. Nevertheless, it is important to keep in mind that we cannot firmly exclude the presence of genes of a previously unknown origin that have not been observed in other organisms so far, and thus would be undetectable because they are absent from the databases. These gene losses constitute an important signature of the lifestyle of *Pneumocystis* spp.

Obligate Parasitism

The loss of biosynthetic pathways of essential molecules is a hallmark of obligate parasitism in both eukaryotic and prokaryotic organisms [11,12]. These losses are believed to be allowed by the availability of the end products of the pathways within the host environment. Similarly, the absence of substrate may explain the loss of assimilation pathways. The loss of amino acids and thiamine biosyntheses in *Pneumocystis* spp. strongly suggests that they are obligate parasites. Thus, their entire cycle probably takes place within the host lungs, and no free-living form of these fungi would exist. In addition to amino acids and thiamine, Pneumocystis spp. are believed to scavenge cholesterol from their host to build their own membranes [13]. In bacteria, gene losses associated with obligate parasitism imply a reduction of the genome size linked to a reduction of the guanine-cytosine (GC) content [14]. Likewise, Pneumocystis spp. genomes have smaller genome size and GC content than their free-living and facultative parasite relatives, respectively Schizosaccharomyces spp. and T. deformans (Table 2).

Biotrophy

Two categories of parasites are recognized: biotrophs, which secrete low amounts of lytic enzymes and obtain food from living Table 2. Some features of selected microorganisms with various lifestyles.

Kingdom	Lifestyle	Organism	Genome size (Mb)	% GC	Minimum missing pathways	Reference
Fungi	Obligate animal biotroph	Pneumocystis spp.	8	30	amino acids synthesis	3, 10
					thiamine synthesis	
					nitrogen+sulfur assimilation	
					purine degradation	
	Obligate animal parasite	Encephalitozoon cuniculi	3	47	amino acids synthesis	6
					vitamins synthesis	
					nucleotides synthesis	
					lipids synthesis	
	Free-living	Saccharomyces cerevisiae	13	38	-	6
		Schizosaccharomyces spp.	14	36	nitrogen+sulfur assimilation	10
	Facultative plant biotrophs	Taphrina deformans	13	50		7
		Ustilago maydis	20.5	54	-	16
	Obligate plant biotrophs	Blumeria graminis	120	44	thiamine synthesis	16
					nitrogen+sulfur assimilation	
		Golovinomyces orontii	160	50	thiamine synthesis	16
					nitrogen+sulfur assimilation	
		Puccinia graminis	89	46	thiamine synthesis	17
					nitrogen+sulfur assimilation	
Protista	Obligate animal parasite	Plasmodium falciparum	23	21	amino acids synthesis	18
		Leishmania major	33	55	amino acids synthesis	18
		Cryptosporidium hominis	10	32	amino acids synthesis	18
-						

doi:10.1371/journal.ppat.1004425.t002

host cells, and necrotrophs, which secrete many degrading enzymes and toxins and obtain food from dead host cells [15]. The missing pathways of *Pneumocystis* spp. are shown in Table 2 together with those of selected microorganisms with various lifestyles. The requirement in thiamine and the absence of inorganic nitrogen and sulfur assimilation are hallmarks of obligate plant biotrophs [16]. Several other biological characteristics of Pneumocystis spp. are also hallmarks of obligate biotrophs: (i) the absence of destruction of host cells during colonization as well as during pathogenic infection, (ii) the lack of known virulence factors, (iii) a sex life cycle occurring within the host, and (iv) the difficulty to be cultured in vitro so far [8,16,17]. On the other hand, the loss of the catabolism of purines and of the amino acids syntheses revealed in *Pneumocystis* spp. has not been observed in fungal biotrophs so far. The first loss might be specific to Pneumocystis spp., but the second is a hallmark of organisms feeding on other animals, such as obligate parasites of humans [18] (Table 2). This latter loss might be related to the adaptation to animal hosts and suggests that there are more proteins available in animal than plant hosts. Thus, the genomic and biological characteristics of Pneumocystis spp. suggest the working hypothesis that they are obligate biotrophic parasites of mammals. This hypothesis has been already proposed previously on the basis of the analysis of P. carinii transcriptome [8], as well as of the biological characteristics of Pneumocystis spp. [1]. Experiments are required in order to test this hypothesis. In particular, the computational predictions supporting obligate biotrophy need to be verified. *Pneumocystis* spp. would be the first fungal biotrophs of animals recognized so far. Of note, the lifestyle of *P. jirovecii* differs from the other fungal obligate parasites of humans, *Malassezia* and *Candida* spp., which are obligate commensals and opportunistic necrotrophs.

The reduction of genome size in *Pneumocystis* spp. contrasts with the increase of this parameter in some fungal obligate plant biotrophs (Table 2). This latter increase corresponds to a proliferation of retrotransposons, which may create genetic variability and diversity including panels of effectors involved in virulence [16]. As in *Ustilago maydis* (Table 2), the genome size reduction in *Pneumocystis* spp. might be compensated by the genetic diversity generated by sexuality, suggesting that they might be obligate sexual organisms. This hypothesis would fit the fact that the asci issued from the sexual cycle might be the unique airborne particles responsible for the transmission of the fungus [19].

Epidemiological Consequence

Obligate parasitism of *P. jirovecii* has important implications in the management of immunocompromised patients susceptible to PCP. Indeed, the fungus is most probably restricted to the lungs of humans so that the only sources of the infection are patients with PCP and colonized humans, such as infants experiencing primo-infection and transitory carriers, as well as, possibly, pregnant women and elderly people [20].

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Acknowledgments

I thank Michel Monod, Joseph Kovacs, Liang Ma, Ousmane Cissé, and Melanie Cushion for critical reading of the manuscript and fruitful comments.

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