

Comparative Genomics of *Pneumocystis* Species Suggests the Absence of Genes for *myo*-Inositol Synthesis and Reliance on Inositol Transport and Metabolism

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ABSTRACT In the context of deciphering the metabolic strategies of the obligate pathogenic fungi in the genus *Pneumocystis*, the genomes of three species (*P. carinii*, *P. murina*, and *P. jirovecii*) were compared among themselves and with the free-living, phylogenetically related fission yeast (*Schizosaccharomyces pombe*). The underrepresentation of amino acid metabolism pathways compared to those in *S. pombe*, as well as the incomplete steroid biosynthesis pathway, were confirmed for *P. carinii* and *P. jirovecii* and extended to *P. murina*. All three *Pneumocystis* species showed overrepresentation of the inositol phosphate metabolism pathway compared to that in the fission yeast. In addition to those known in *S. pombe*, four genes, encoding inositol-polyphosphate multikinase (EC 2.7.1.151), inositol-pentakisphosphate 2-kinase (EC 2.7.1.158), phosphoinositide 5-phosphatase (EC 3.1.3.36), and inositol-1,4-bisphosphate 1-phosphatase (EC 3.1.3.57), were identified in the two rodent *Pneumocystis* genomes, *P. carinii* and *P. murina*. The *P. jirovecii* genome appeared to contain three of these genes but lacked phosphoinositide 5-phosphatase. Notably, two genes encoding enzymes essential for *myo*-inositol synthesis, inositol-1-phosphate synthase (INO1) and inositol monophosphatase (INM1), were absent from all three genomes, suggesting that *Pneumocystis* species are inositol auxotrophs. In keeping with the need to acquire exogenous inositol, two genes with products homologous to fungal inositol transporters, ITR1 and ITR2, were identified in *P. carinii* and *P. murina*, while *P. jirovecii* contained only the ITR1 homolog. The ITR and inositol metabolism genes in *P. murina* and *P. carinii* were expressed during fulminant infection as determined by reverse transcriptase real-time PCR of cDNA from infected lung tissue. Supplementation of *in vitro* culture with inositol yielded significant improvement of the viability of *P. carinii* for days 7 through 14.

IMPORTANCE Microbes in the genus *Pneumocystis* are obligate pathogenic fungi that reside in mammalian lungs and cause *Pneumocystis* pneumonia in hosts with weakened immune systems. These fungal infections are not responsive to standard antifungal therapy. A long-term *in vitro* culture system is not available for these fungi, impeding the study of their biology and genetics and new drug development. Given that all genomes of the *Pneumocystis* species analyzed lack the genes for inositol synthesis and contain inositol transporters, *Pneumocystis* fungi, like *S. pombe*, appear to be inositol auxotrophs. Inositol is important for the pathogenesis, virulence, and mating processes in *Candida albicans* and *Cryptococcus neoformans*, suggesting similar importance within the *Pneumocystis* species as well. This is the first report to (i) characterize genes in the inositol phosphate metabolism and transport pathways in *Pneumocystis* species and (ii) identify inositol as a supplement that improved the viability of *P. carinii* in *in vitro* culture.

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The fungal pathogen *Pneumocystis jirovecii* causes a potentially lethal pneumonia, *Pneumocystis* pneumonia (PCP), in humans. PCP is an opportunistic infection and primarily occurs in patients with a compromised immune system, including those infected with the human immunodeficiency virus (HIV/AIDS), with chronic obstructive pulmonary disease (COPD) (1), and in other immunosuppressed populations, e.g., those receiving anti-tumor necrosis factor alpha (TNF) therapy for autoimmune diseases or immunosuppressive therapies after organ transplantation (2). Currently, the therapeutic options available for PCP are lim-

ited (3). These fungi are not susceptible to the commonly used antifungal agents, such as the azoles or amphotericin B, and despite concerted efforts to identify additional chemotherapeutic agents, the combination of trimethoprim and sulfamethoxazole (TMP-SMX) continues to be the standard prophylactic and therapeutic modality in use today, as it has been for the last few decades (4). However, TMP-SMX treatment can cause serious side effects, while other, second-line therapies are not as efficacious or are more toxic, like pentamidine isethionate (5).

The development of new drug therapies for treatment of PCP is

lagging because an *in vitro* cultivation system for any species of *Pneumocystis* that permits continuous passage does not exist. *In vitro* evaluation of new drugs for activity against *P. jirovecii* must be performed using surrogate *Pneumocystis* species obtained from laboratory immunosuppressed rodents, such as *Pneumocystis carinii*, which infects rats, and *Pneumocystis murina*, which infects mice, as there are no animal models for *P. jirovecii* and thus no culture stocks. The current *in vitro* systems are limited to short-term maintenance of the fungi in primary cultures, which does not allow determination of cidal or static effects. In addition, the use of *Pneumocystis* species from rodents leaves questions about how these drugs will perform in patients infected by *P. jirovecii*.

The *Pneumocystis* genus is comprised of obligate fungal pathogens that live only in the lungs of their mammalian hosts. The lack of a long-term *in vitro* culture indicates that some crucial nutrients are missing or undersupplemented in the culture medium. Previous metabolic studies indicated that the pathogen does not synthesize ergosterol and has to scavenge cholesterol from its host to thrive (6). More recent genome-sequencing studies pointed out that the fungus has dramatic underrepresentation of amino acid synthesis pathways (7). Despite the years of trials to supplement *Pneumocystis* culture with various sterols, amino acids, and other nutrients, the goal of obtaining a long-term *in vitro* culture of the fungus has remained beyond reach (8).

In this work, we conducted a comparative genome analysis of the three currently sequenced species of the *Pneumocystis* genus, *P. jirovecii*, *P. carinii*, and *P. murina*, and a free-living but phylogenetically related fungus, the fission yeast *Schizosaccharomyces pombe* (9). The analysis was focused on enzymes and metabolic pathways to better understand the metabolic strategies adopted by the obligate pathogen in the mammalian host milieu and, therefore, to identify nutrients that it may require from exogenous sources. The analysis identified an overrepresentation of the inositol phosphate metabolism pathway in the *Pneumocystis* genomes compared to that in the fission yeast. Genomic sequences and gene expression of the identified enzymes, as well as inositol transporters, were experimentally confirmed in *P. carinii* and *P. murina*, as *P. jirovecii* is not readily available. Supplementation with inositol in a primary *in vitro* culture system significantly improved the viability of *P. carinii* (as indicated by ATP levels) throughout a 14-day period, a time frame when ATP levels declined dramatically without this additive. These new data suggest that *Pneumocystis* species are inositol auxotrophs and that inositol is one of the critical nutrients needed for *in vitro* culture but in and of itself is not sufficient for continuous cultivation. Importantly, the lack of synthesis and reliance on exogenous sources of inositol provide a new attractive drug target for the pneumonia caused by these fungi.

RESULTS

Choice of the genome annotation tool. With the goal of unveiling the metabolic biological pathways encoded in the fungal genomes, two genome annotation tools, Blast2GO (10) and SHARKhunt (11), were validated for their ability to identify enzyme-coding genes within a well annotated fungal organism, *S. pombe*, which is phylogenetically close to *Pneumocystis* (9). Definitions of enzymes and metabolic pathways in *S. pombe* were retrieved from the KEGG Pathway database. The results in Fig. 1 illustrate the performance of both methods using various sequence homology cutoffs. Details of the distribution of the identified enzymes per path-

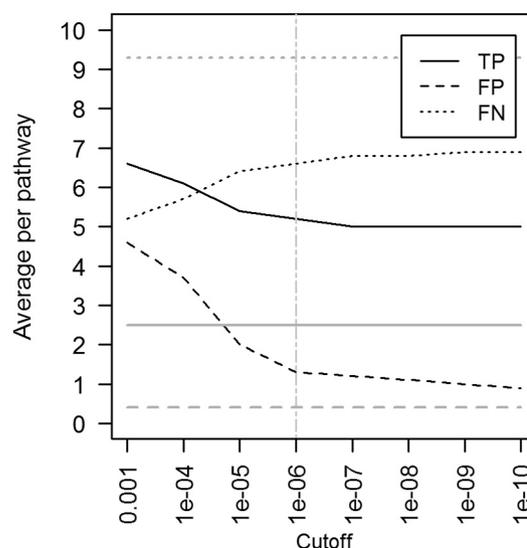


FIG 1 Identification of enzymes, as defined in the KEGG Pathway database, by Blast2GO (gray horizontal lines) and SHARKhunt (black curves) in *S. pombe* used as a gold standard organism. TP, true positives (enzymes that are known in *S. pombe* and which were identified by the tool); FP, false positives (not known in the fungus but were mistakenly found); FN, false negatives (known but were not identified). The numbers of found or missed enzymes are averages per metabolic pathway present in *S. pombe*. The vertical line indicates the sequence homology cutoff used in this work in the SHARKhunt search for enzymes within *Pneumocystis* genomes.

way can be found in Table S1 in the supplemental material or at the *Pneumocystis* Genome Project website (<http://pgp.cchmc.org/>).

The Blast2GO results (Fig. 1, horizontal gray lines) appeared to be independent of the homology cutoff used. Blast2GO showed very low sensitivity, finding only 2.5 enzymes on average per pathway, and yielded a high level of missing enzymes (9.3 enzymes on average). The low level of performance of Blast2GO and its independence from the homology cutoff used indicate either an outdated table of enzymes or a highly conservative heuristic used in the identification of enzymes based on the matched gene ontology (GO) categories. For example, it did not find any enzymes in steroid biosynthesis (KEGG Pathway identifier [ID] 00100) and found very few enzymes in glycerolipid metabolism (2 out of 15 known in *S. pombe*) or glycerophospholipid metabolism (4 out of 29) pathways (KEGG Pathway IDs 00561 and 00564, respectively; see Table S1 in the supplemental material). Of note, BlastX used in Blast2GO did show strong hits to genomic sequences of the enzymes of the steroid biosynthesis pathway of *S. pombe*. However, its subsequent enzyme annotation module failed to assign EC codes to them.

SHARKhunt showed a better performance than Blast2GO in recognizing known enzymes from the genomic sequences. At a homology cutoff with an *E* value of $\leq 10^{-6}$ (Fig. 1, vertical line), it reached an optimal balance between true- and false-positive instances, yielding 5.2, 1.3, and 6.6 enzymes on average for true positive, false positive, and false negative, respectively. Since SHARKhunt was more sensitive in finding enzymes, while sustaining a low level of false positives similar to that obtained with Blast2GO, we have chosen SHARKhunt as a search tool for subsequent annotation of *Pneumocystis* genomic sequences with respect to enzymes and metabolic pathways.

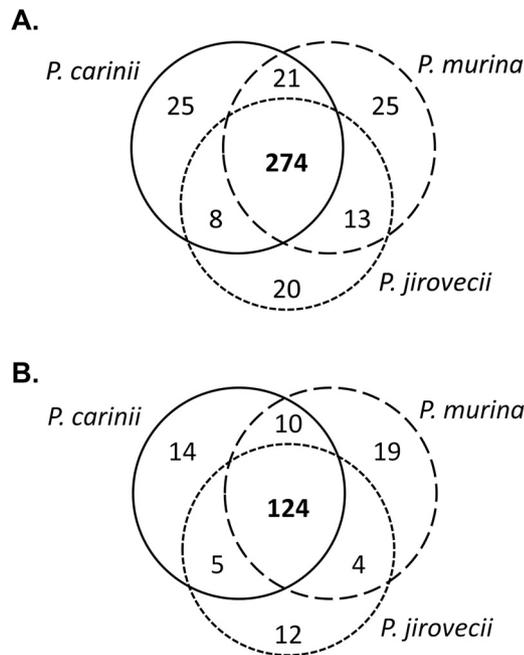


FIG 2 Overlap of identified enzymes between three *Pneumocystis* species. (A) Enzymes mapped into metabolic pathways defined in the KEGG Pathway database. (B) Enzymes not mapped into metabolic pathways.

Comparative genome analysis of the *Pneumocystis* genus.

There were 481, 490, and 460 total enzymes identified by SHARK-hunt as being encoded in the *P. carinii*, *P. murina*, and *P. jirovecii* genomes, respectively. Of these, 328, 333, and 315 enzymes were mapped to metabolic pathways. The majority of enzymes identified (274 mapped and 124 not mapped) were shared across all three species of *Pneumocystis* (Fig. 2). The entire list of identified enzymes and the corresponding pathways can be found in Table S2 in the supplemental material.

Among the enzymes not shared between all three species, two observations are worth mentioning. The *Pneumocystis* species that inhabit rats and mice (*P. carinii* and *P. murina*) have five additional enzymes in the tryptophan metabolism pathway (KEGG Pathway ID 00380) compared to *P. jirovecii* residing in humans. These are indoleamine 2,3-dioxygenase (EC 1.13.11.52), 3-hydroxyanthranilate 3,4-dioxygenase (EC 1.13.11.6), kynurenine 3-monooxygenase (EC 1.14.13.9), arylformamidase (EC 3.5.1.9), and kynureninase (EC 3.7.1.3) that constitute the functional module tryptophan → kynurenine → 2-aminomuconate (KEGG Module ID M00038). Moreover, *P. carinii* has an additional enzyme in this pathway, alpha-ketoglutarate dehydrogenase (EC 1.2.4.2). On the other hand, *P. jirovecii* has three enzymes in the steroid biosynthesis pathway (KEGG Pathway ID 00100) that are specific to this species, namely, lathosterol 5-desaturase (EC 1.14.21.6; Erg3) and 7-dehydrocholesterol reductase (EC 1.3.1.21), which are the two final steps in cholesterol biosynthesis (KEGG Module ID M00101), and cycloeucaenol cycloisomerase (EC 5.5.1.9), which is an enzyme in the phytosterol branch of the steroid biosynthesis pathway and may represent a false-positive artifact of SHARK-hunt predictions. The actual presence of these enzymes, as well as their expression, has yet to be confirmed. Their functional significance and biological implications, such as rele-

vance to host specificity, will be the subject of future studies and are beyond the scope of this work.

***Pneumocystis* metabolic pathways and their comparison with those of *S. pombe*.** To reveal the metabolic strategies employed by the obligate *Pneumocystis* species in contrast to those of a free-living fungus, all identified enzymes have been mapped to metabolic pathways in KEGG Pathways and compared to the known pathways in fission yeast. Table 1 represents metabolic pathways defined for *S. pombe* in KEGG; for the complete list refer to Table S3 in the supplemental material.

Besides the remarkable representation of the tryptophan metabolism pathway in the two rodent *Pneumocystis* species compared to that in the human *Pneumocystis* species, *S. pombe* is also missing this functional module. Otherwise, metabolic pathways constituting amino acid metabolism lack many enzymes in the *Pneumocystis* genomes compared to these pathways in the fission yeast. This has recently been noted for *P. carinii* (7) and *P. jirovecii* (12), and the present work extends these observations to *P. murina*. While amino acids appear to be an important ingredient of the culture medium, they do not suffice to support the long-term *in vitro* viability of *Pneumocystis*. Most growth media previously tested have been serum based and contained amino acids as essential components in their formulations.

Metabolic pathways of carbohydrate metabolism are well represented in all *Pneumocystis* genomes, and the expression of the genes from this category has been previously confirmed for *P. carinii* using an expressed sequence tag (EST) library (13). In contrast to this previous study, more specific metabolic pathways in this category are now identified, along with the larger number of enzymes comprising them. Three pathways are worth mentioning here: starch and sucrose metabolism (KEGG Pathway ID 00500), amino sugar and nucleotide sugar metabolism (KEGG Pathway ID 00520), and inositol phosphate metabolism (KEGG Pathway ID 00562). These pathways, in addition to enzymes shared with the fission yeast, have 6, 4, and 4 additional enzymes in *P. carinii*, 7, 1, and 4 in *P. murina*, 5, 3, and 3 in *P. jirovecii*, respectively. Since inositol is an essential nutrient in eukaryotes and a critical proliferative factor for many pathogens, the increased number of *Pneumocystis*-specific genes in the inositol phosphate metabolism pathway in comparison to those in fission yeast was of great interest to us (Fig. 3). The enzymes highlighted in yellow in Fig. 3 are those shared between all fungi and those in red are *Pneumocystis* specific, whereas inositol polyphosphate 1-phosphatase (EC 3.1.3.57) is specific to *P. carinii* and *P. murina*. In addition to a large set of genes for inositol-metabolizing enzymes, *Pneumocystis* genomes contain sequences homologous to inositol transporter genes, including ITR1_SCHPO, ITR2_SCHPO, and putative inositol transporters in other fungi of the *Aspergillus* genus. These findings are highly significant in light of the fact that genes encoding inositol-1-phosphate synthase (INO1, EC 5.5.1.4), an enzyme essential for the synthesis of inositol from glucose-6-phosphate to *myo*-inositol 1-phosphate, were absent from each of the 3 genomes, suggesting that *Pneumocystis* fungi, like *S. pombe*, require an external source of inositol and are therefore inositol auxotrophs. The enzyme that catalyzes the next step in the synthesis, inositol monophosphatase (INM1, EC 3.1.3.25), which dephosphorylates *myo*-inositol 1-phosphate to *myo*-inositol, was also absent from the *Pneumocystis* and *S. pombe* genomes.

In the effort to reduce the possibility that the apparent lack of genes for *myo*-inositol-synthesizing enzymes in the *Pneumocystis*

TABLE 1 Representation of metabolic pathways of the three *Pneumocystis* species compared to those in *S. pombe* by the numbers of enzymes identified

KEGG Pathway and ID	Metabolic pathway	No. of enzymes present in:										
		<i>S. pombe</i>		<i>P. carinii</i> ^c			<i>P. murina</i>			<i>P. jirovecii</i>		
		Per KPD ^a	Per SH ^b	Per SH	Also in <i>S. pombe</i>	Not in <i>S. pombe</i>	Per SH	Also in <i>S. pombe</i>	Not in <i>S. pombe</i>	Per SH	Also in <i>S. pombe</i>	Not in <i>S. pombe</i>
Amino acid metabolism												
00250	Alanine, aspartate, and glutamate metabolism	21	11	11	10	1	12	11	1	12	11	1
00260	Glycine, serine, and threonine metabolism	22	16	10	7	3	9	7	2	9	7	2
00270	Cysteine and methionine metabolism	27	13	10	6	4	9	5	4	8	4	4
00280	Valine, leucine and isoleucine degradation	8	5	5	4	1	8	4	4	7	5	2
00290	Valine, leucine and isoleucine biosynthesis	7	2	0	0	0	0	0	0	1	1	0
00300	Lysine biosynthesis	13	6	1	0	1	0	0	0	0	0	0
00310	Lysine degradation	8	5	4	4	0	4	3	1	3	3	0
00330	Arginine and proline metabolism	29	21	8	4	4	8	5	3	8	4	4
00340	Histidine metabolism	12	3	1	0	1	1	0	1	1	0	1
00350	Tyrosine metabolism	13	6	1	1	0	1	1	0	1	1	0
00360	Phenylalanine metabolism	8	5	2	2	0	3	2	1	2	2	0
00380	Tryptophan metabolism	9	7	8	3	5	7	2	5	2	2	0
00400	Phenylalanine, tyrosine and tryptophan biosynthesis	20	12	10	10	0	8	8	0	10	10	0
Carbohydrate metabolism												
00010	Glycolysis/gluconeogenesis	24	10	16	14	2	15	14	1	14	13	1
00020	Citrate cycle (TCA cycle)	16	9	16	15	1	15	13	2	14	12	2
00030	Pentose phosphate pathway	15	7	11	10	1	10	10	0	11	11	0
00040	Pentose and glucuronate interconversions	4	3	3	2	1	3	2	1	3	2	1
00051	Fructose and mannose metabolism	13	6	10	8	2	10	8	2	11	8	3
00052	Galactose metabolism	11	5	8	5	3	5	4	1	6	4	2
00500	Starch and sucrose metabolism	14	12	16	10	6	16	9	7	14	9	5
00520	Amino sugar and nucleotide sugar metabolism	17	10	15	11	4	12	11	1	14	11	3
00562	Inositol phosphate metabolism	10	8	12	8	4	12	8	4	11	8	3
00620	Pyruvate metabolism	18	11	11	9	2	14	10	4	11	8	3
00630	Glyoxylate and dicarboxylate metabolism	10	9	9	6	3	9	6	3	8	6	2
00640	Propanoate metabolism	12	6	6	5	1	5	4	1	5	4	1
00650	Butanoate metabolism	8	3	2	2	0	3	2	1	3	2	1
Energy metabolism												
00190	Oxidative phosphorylation	9	7	9	8	1	10	8	2	10	8	2
00680	Methane metabolism	13	4	8	6	2	9	7	2	7	5	2
00910	Nitrogen metabolism	11	6	4	2	2	5	3	2	4	2	2
00920	Sulfur metabolism	14	8	5	2	3	3	1	2	4	2	2
Glycan biosynthesis and metabolism												
00510	<i>N</i> -Glycan biosynthesis	21	7	11	11	0	10	10	0	10	10	0
00511	Other glycan degradation	1	1	1	0	1	0	0	0	1	0	1
00513	Various types of <i>N</i> -glycan biosynthesis	13	5	5	5	0	4	4	0	4	3	1
00514	Other types of <i>O</i> -glycan biosynthesis	2	1	1	1	0	1	1	0	1	1	0
00563	Glycosylphosphatidylinositol anchor biosynthesis	5	2	2	2	0	2	2	0	2	2	0
Lipid metabolism												
00061	Fatty acid biosynthesis	5	4	4	2	2	4	2	2	3	1	2
00062	Fatty acid elongation	6	1	2	1	1	3	1	2	2	1	1
00071	Fatty acid degradation	5	4	2	2	0	2	2	0	2	2	0
00072	Synthesis and degradation of ketone bodies	2	1	2	2	0	2	2	0	2	2	0
00100	Steroid biosynthesis	16	6	9	7	2	9	7	2	11	7	4

(Continued on following page)

TABLE 1 (Continued)

KEGG Pathway and ID	Metabolic pathway	No. of enzymes present in:										
		<i>S. pombe</i>		<i>P. carinii</i> ^c			<i>P. murina</i>			<i>P. jirovecii</i>		
		Per KPD ^a	Per SH ^b	Per SH	Also in <i>S. pombe</i>	Not in <i>S. pombe</i>	Per SH	Also in <i>S. pombe</i>	Not in <i>S. pombe</i>	Per SH	Also in <i>S. pombe</i>	Not in <i>S. pombe</i>
00561	Glycerolipid metabolism	15	9	6	3	3	7	3	4	6	3	3
00564	Glycerophospholipid metabolism	29	13	11	11	0	13	12	1	11	11	0
00565	Ether lipid metabolism	10	4	4	3	1	5	3	2	4	2	2
00590	Arachidonic acid metabolism	7	6	2	1	1	1	1	0	0	0	0
00591	Linoleic acid metabolism	5	2	1	1	0	2	1	1	1	0	1
00600	Sphingolipid metabolism	8	5	6	2	4	5	2	3	7	2	5
01040	Biosynthesis of unsaturated fatty acids	6	2	3	1	2	4	1	3	3	1	2
Metabolism of cofactors and vitamins												
00130	Ubiquinone and other terpenoid-quinone biosynthesis	6	2	3	0	3	4	0	4	1	0	1
00670	One carbon pool by folate	13	5	9	8	1	8	7	1	9	8	1
00730	Thiamine metabolism	6	4	4	3	1	2	2	0	3	2	1
00740	Riboflavin metabolism	10	2	8	6	2	8	6	2	7	6	1
00750	Vitamin B ₆ metabolism	7	5	2	2	0	2	2	0	2	2	0
00760	Nicotinate and nicotinamide metabolism	10	6	5	3	2	6	4	2	5	3	2
00770	Pantothenate and coenzyme A biosynthesis	12	7	7	5	2	7	5	2	8	6	2
00780	Biotin metabolism	6	2	3	1	2	3	1	2	3	1	2
00785	Lipoic acid metabolism	3	1	3	3	0	3	3	0	1	1	0
00790	Folate biosynthesis	8	2	7	6	1	8	6	2	7	6	1
00860	Porphyrin and chlorophyll metabolism	15	12	14	10	4	16	10	6	14	10	4
Metabolism of other amino acids												
00410	Beta-alanine metabolism	8	4	2	1	1	3	2	1	2	1	1
00430	Taurine and hypotaurine metabolism	2	2	2	1	1	1	1	0	1	1	0
00450	Selenocompound metabolism	6	6	4	3	1	4	3	1	3	2	1
00460	Cyanoamino acid metabolism	4	3	2	2	0	2	2	0	2	2	0
00480	Glutathione metabolism	16	11	8	6	2	9	7	2	7	6	1
Metabolism of terpenoids and polyketides												
00900	Terpenoid backbone biosynthesis	17	10	13	12	1	13	11	2	11	10	1
00909	Sesquiterpenoid and triterpenoid biosynthesis	2	0	1	1	0	1	1	0	2	1	1
Nucleotide metabolism												
00230	Purine metabolism	42	29	34	27	7	32	28	4	34	28	6
00240	Pyrimidine metabolism	26	18	20	16	4	19	16	3	18	15	3
Translation												
00970	Aminoacyl-tRNA biosynthesis	23	13	21	21	0	21	21	0	21	21	0
Xenobiotic biodegradation and metabolism												
00627	Aminobenzoate degradation	0		3	0	3	4	0	4	4	0	4
00983	Drug metabolism: other enzymes	0		5	0	5	5	0	5	5	0	5

^a KPD, defined in the KEGG Pathway database.

^b SH, identified by SHARKhunt.

^c For *Pneumocystis* species, first, the total number of enzymes identified by SHARKhunt; second, the number of these enzymes overlapping with *S. pombe* genes defined in the KEGG Pathway database; and third, the number of enzymes not known in *S. pombe*.

genomes is not a false-negative finding, a genome of another closely related fungus was retrieved that might have these two enzymes. *Taphrina deformans* belongs to the same subphylum (Taphrinomycotina) as *S. pombe* and *Pneumocystis* spp. and, according to the genome annotation project maintained by the Joint Genome Institute, is predicted to have both these enzymes (see EC 5.5.1.4 and 3.1.3.25; [http://genome.jgi.doe.gov/cgi-bin/metapathways?db=Tapde1_1&map=MAP00562&models=0&](http://genome.jgi.doe.gov/cgi-bin/metapathways?db=Tapde1_1&map=MAP00562&models=0&col=1&batchid=Tapde1_1%3A1)

[col=1&batchid=Tapde1_1%3A1](http://genome.jgi.doe.gov/cgi-bin/metapathways?db=Tapde1_1&map=MAP00562&models=0&col=1&batchid=Tapde1_1%3A1)). Sequence homology searches, both on the DNA and protein levels, for *T. deformans* EC 5.5.1.4 and 3.1.3.25 did not yield homologous hits to any of the three *Pneumocystis* genomes.

Given the significance of inositol and its metabolites in the proliferation and virulence of other pathogenic fungi, as well as the lack of synthetic enzymes in *Pneumocystis*, we focused on further investigation of the inositol phosphate metabolism pathway.

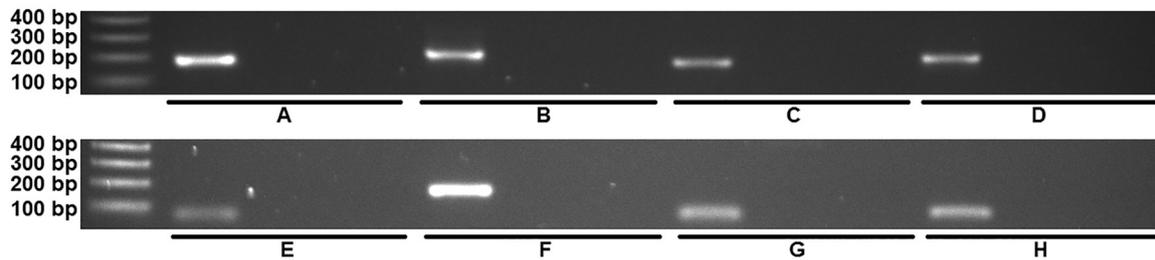


FIG 4 Representative products from reverse transcriptase real-time PCR of genes in the inositol phosphate metabolism pathway that were found to be conserved between *P. murina* and *P. carinii* but were not present in *S. pombe*. All products were found to be of the expected size, and all negative controls (mouse/rat cDNA or no cDNA) were negative. (A) *P. murina* inositol-polyphosphate multikinase, mouse cDNA negative control, and no-cDNA negative control. (B) *P. murina* inositol-pentakisphosphate 2-kinase, mouse cDNA negative control, and no-cDNA negative control. (C) *P. murina* phosphoinositide 5-phosphatase, mouse cDNA negative control, and no-cDNA negative control. (D) *P. murina* inositol-1,4-bisphosphate 1-phosphatase, mouse cDNA negative control, and no-cDNA negative control. (E) *P. carinii* inositol-polyphosphate multikinase, rat cDNA negative control, and no-cDNA negative control. (F) *P. carinii* inositol-pentakisphosphate 2-kinase, rat cDNA negative control, and no-cDNA negative control. (G) *P. carinii* phosphoinositide 5-phosphatase, rat cDNA negative control, and no-cDNA negative control. (H) *P. carinii* inositol-1,4-bisphosphate 1-phosphatase, rat cDNA negative control, and no-cDNA negative control.

ITR2 was identified in *P. murina* (PmITR2) and *P. carinii* (PcITR2) but not in *P. jirovecii* (Table 3). The PmITR2 gene had 1,960 bp of genomic sequence, and the PcITR2 gene had 1,968 bp. Both had six introns, ranging from 44 to 50 bp in length, which were located in almost exactly the same places in each genome. The putative amino acid sequences and the genomic DNA (gDNA) were about 90% homologous.

Reverse transcriptase real-time PCR of *Pneumocystis* inositol transporter genes. Reverse transcriptase real-time PCR was utilized to confirm the expression of the two transporter genes involved in IPM that were found to be conserved between *S. pombe*, *P. murina*, and *P. carinii*. The results of the real-time PCR on the inositol transporter genes were analyzed in the same fashion as the results for the inositol metabolism pathway, and the ΔC_T value between the inositol transporter gene and TS was calculated (Table 4). All of the ITR genes were expressed at higher levels than the TS gene in both *P. murina* and *P. carinii*. When representative products were run on an agarose gel, bands of the expected size were observed, indicating that all genes were expressed (Fig. 5).

Culture supplementation with inositol. The ATP bioluminescence assay is used to assess the viability (total ATP content) of *P. carinii* in inositol-supplemented medium versus unsupplemented medium. This method has been routinely used to assess

the effects of anti-*Pneumocystis* agents with the anticipation that effective drugs will decrease the viability of treated versus untreated organisms (4). In the case of supplements, the desired outcome is an increase in the ATP levels versus the results for untreated organisms. In this set of experiments, a wide range of *myo*-inositol concentrations, from 10,000 to 0.01 $\mu\text{g/ml}$, were assessed for their effects on the ATP content. The most consistent results were achieved with concentrations ranging between 1,000 and 10 $\mu\text{g/ml}$ (Fig. 6). All readings of the assays were performed in triplicate wells and analyzed using analysis of variance (ANOVA) with Tukey's posttest and significance set to a *P* value of <0.05 .

As observed by the results in Fig. 6, the ATP content as measured by the output of relative light units (RLU) was significantly increased at all concentrations of the *myo*-inositol supplementation (note the log scale). Greater effects were observed after 7 days in culture, when the viability of the unsupplemented control *P. carinii* dramatically declined in ATP content. A clear dose response was not obvious with the different concentrations of *myo*-inositol. This was likely due to the presence of *myo*-inositol in both the medium and serum, which was not controlled for in these studies and may have interfered with the ability to evaluate the optimal concentration in that context. Although the inositol clearly improved the viability of the *P. carinii* extracted from the

TABLE 3 Structural analysis of inositol transporter genes in *Pneumocystis* species

Gene product and fungal species	gDNA size (bp)	No. of introns (position[s])	% gDNA identity			No. of amino acids	% Amino acid identity		
			<i>P. carinii</i>	<i>P. murina</i>	<i>P. jirovecii</i>		<i>P. carinii</i>	<i>P. murina</i>	<i>P. jirovecii</i>
ITR1									
<i>P. carinii</i>	1,568	1 (94–140)		90.26	79.87	507		89.35	75.83
<i>P. murina</i>	1,571	1 (96–142)	90.26		78.81	507	89.35		75.24
<i>P. jirovecii</i>	1,582	1 (97–136)	79.87	78.81		513	75.83	75.24	
ITR2									
<i>P. carinii</i>	1,968	6 (95–143, 225–272, 742–786, 1166–1217, 1504–1549, 1593–1637)		89.28		558		89.29	
<i>P. murina</i>	1,960	6 (98–145, 224–267, 741–785, 1161–1210, 1497–1540, 1586–1630)	89.28			560	89.29		

DNAMAN version 5.2.9 was used to analyze the putative *Pneumocystis* ITR genes.

TABLE 4 ΔC_T values for inositol transporter genes versus the thymidylate synthase gene^a

Species	Gene product	ΔC_T
<i>P. murina</i>	ITR1	-0.30
<i>P. carinii</i>	ITR1	-2.76
<i>P. murina</i>	ITR2	-4.56
<i>P. carinii</i>	ITR2	-1.13

^a Reactions were performed in triplicate, and the average threshold cycle (C_T) value of the three replicates was determined. ΔC_T values were calculated by subtracting the average C_T value of the thymidylate synthase gene from the average C_T value of the gene from the inositol phosphate metabolism pathway.

rat lung, it was insufficient as the sole supplement to sustain a high level of viability throughout the 14-day assay period.

DISCUSSION

myo-inositol is an essential nutrient in eukaryotes, where it is involved in at least 3 vital functions (14). As a constituent of phosphatidylinositol, it is a component of biological membranes and, when phosphorylated, participates in exocytosis, cytoskeletal restructuring, and intracellular membrane trafficking; phosphatidylinositol-4,5-bisphosphate is critical in signal transduction pathways that control many cellular function; and finally, it is a precursor of glycosylphosphatidylinositol membrane anchors which affix glycolipids to cell surfaces (15).

myo-inositol is also a critical proliferative factor for many pathogens, including mycobacteria, fungi, and protozoa, which adopt different strategies of acquiring inositol either from the host or by synthesizing it (16). In infections with *Candida albicans* and *Cryptococcus neoformans*, inositol (17) and inositol transporters (18) have been associated with pathogenesis and virulence. Inositol has also been implicated in the mating processes of *S. pombe* and *C. neoformans* (19). In the case of *C. neoformans*, inositol secreted by plants stimulates mating, as does exogenous inositol imported by transporters in *S. pombe* (20). The connection of inositol with mating in *S. pombe* and *C. neoformans* is additionally intriguing, as *Pneumocystis* species reproduce sexually within the lung (9). *myo*-inositol may then play more than a nutritional role in supporting the growth of *Pneumocystis* and could potentially function as a signal or stimulant for initiation of the sexual cycle.

The lack of inositol-1-phosphate synthase and inositol monophosphatase, two enzymes critical to the synthesis of *myo*-inositol, and the presence of inositol transporters and enzymes involved in the metabolism of inositol strongly suggest that *Pneumocystis* species cannot synthesize their own inositol and must rely on the host for its exogenous sources.

Saccharomyces cerevisiae can both synthesize inositol and import it via two distinct transporter genes, ITR1 and ITR2 (21). *C. albicans* also has the ability to synthesize inositol but only has a

single high-affinity inositol transporter (17). Remarkably, *C. neoformans* possesses 10 or 11 inositol transporters, depending on the variety (19), which is thought to result from the requirement for inositol from plants in its native environment. *C. albicans* has a number of opportunities to salvage inositol from its human host. Dietary intake of *myo*-inositol is about 1 g/day, making for a reliable source for *C. albicans* as an intestinal commensal (17). In the disseminated state, *C. albicans* can be exposed to 15 to 70 μ M in human serum and up to 200 to 270 μ M in liver and lymphatic tissue as ready sources. Within the lung, *Pneumocystis* would be exposed to *myo*-inositol from serum due to alveolar damage induced by the pneumonia, with subsequent leakage of serum into the alveolar compartments, and also from catabolism of phosphatidylinositol, which comprises approximately 1.5% of pulmonary surfactant (22).

Conclusions. A comparative genome analysis has provided critical new insights into the *Pneumocystis* genus with respect to enzymes and metabolic pathways. The genomes of three species of *Pneumocystis* (*P. carinii*, *P. murina*, and *P. jirovecii*) were compared among themselves and with the free-living, phylogenetically related fungus *S. pombe*. *Pneumocystis* species and *S. pombe* are both members of the monophyletic Taphrinomycotina (Archiascomycota) (23, 24).

The majority of the computationally identified enzymes were shared across *Pneumocystis* species. We chose to focus on the inositol phosphate metabolism pathway as it appeared that *Pneumocystis* species could not synthesize *myo*-inositol due to a lack of INO1 and INM1 genes in their genomes. Four enzymes and two inositol transporters were found in the rodent *Pneumocystis* genomes but not in *S. pombe*. At the same time, *P. jirovecii* lacked both the second inositol transporter (ITR2) and phosphoinositide 5-phosphatase (EC 3.1.3.36) that are found in *P. murina* and *P. carinii*. These differences support the genetic literature that contends the rodent species are more closely related to each other on a phylogenetic level than they are to the species inhabiting humans.

Supplementation of *in vitro* culture with inositol statistically improved the viability of *P. carinii* during the later days of culture, 7 through 14, compared to the viability of the fungus in unsupplemented medium. However, it also appears that inositol, together with amino acids and the additional cofactors and ingredients in the RPMI 1640 medium and serum, are still not sufficient to maintain a long-term culture. Nonetheless, the strategy of comparative genomics employed here holds the greatest promise to date to identify all of the essential nutrients that *Pneumocystis* fungi require to live and thrive both in the mammalian lung and in an artificial environment. Further biochemical characterization of the nutrient acquisition mechanisms identified should lay the

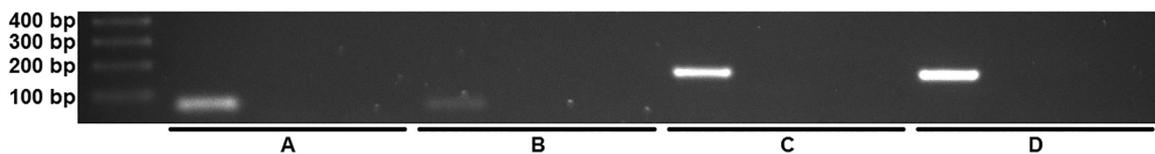


FIG 5 Representative products from reverse transcriptase real-time PCR of *Pneumocystis* ITR genes. All products were found to be of the expected size, and all negative controls (mouse/rat cDNA or no cDNA) were negative. (A) *P. murina* ITR1, mouse cDNA negative control, and no-cDNA negative control. (B) *P. carinii* ITR1, rat cDNA negative control, and no-cDNA negative control. (C) *P. murina* ITR2, mouse cDNA negative control, and no-cDNA negative control. (D) *P. carinii* ITR2, rat cDNA negative control, and no-cDNA negative control.

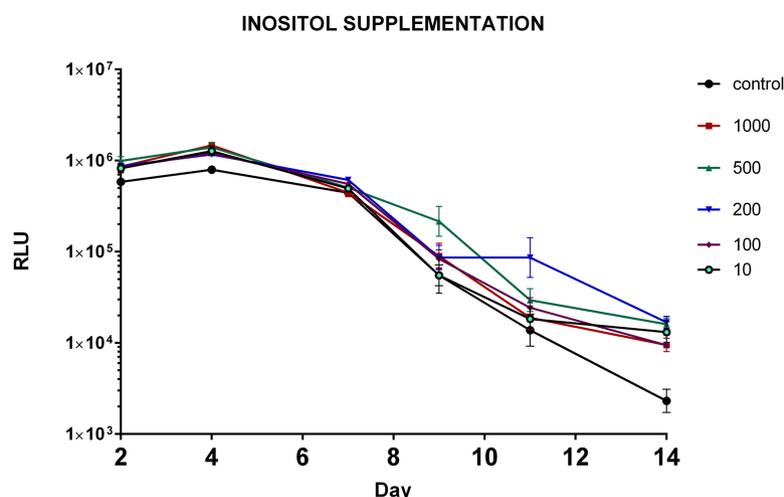


FIG 6 ATP content of *myo*-inositol-supplemented versus unsupplemented *P. carinii* *in vitro*. The ATP content of *P. carinii* in an RPMI 1640-based axenic culture that was supplemented with *myo*-inositol at concentrations ranging from 1,000 to 10 µg/ml (or not supplemented, control) over a 14-day time period were measured using a bioluminescence-based assay which emits light (relative light units [RLU]) in a linear relationship with the amount of ATP present. Cultures were run in triplicate, and significance ($P < 0.05$) of the results for supplemented versus unsupplemented medium was found at all time points (note the log scale).

groundwork for rational drug design of a completely new class of anti-*Pneumocystis* agents.

MATERIALS AND METHODS

Genome annotation. Genomic sequences were retrieved from the following sources: *P. carinii* sequences from the *Pneumocystis* Genome Project (<http://pgp.cchmc.org/>), *P. murina* sequences from the *Pneumocystis murina* Database (http://www.broadinstitute.org/annotation/genome/Pneumocystis_group/MultiHome.html), and *P. jirovecii* and *S. pombe* sequences from the NCBI Genome database (http://www.ncbi.nlm.nih.gov/genome?LinkName=pubmed_genome&from_uid=23269827 and <http://www.ncbi.nlm.nih.gov/genome/14>, respectively).

P. murina genomic sequences used in this study are revealed and reported here with permission of the genome holders. Genome-wide anal-

ysis and identification of putative enzymes is in concordance with the National Institutes of Health (NIH) policy of genomic data sharing.

Annotation of genomic sequences was conducted using sequence homology-based approaches implemented in BlastX (25), Blast2GO (10), and SHARKhunt (11). Sequence homology search was performed across the NCBI nr database and using the PRIAM enzyme-specific sequence profiles (26). Identified enzymes were mapped to metabolic pathways defined in the KEGG Pathway database (27) and compared with a reference genome, that of *S. pombe*, using the EC2KEGG tool (28).

Reverse transcriptase real-time PCR of the inositol phosphate metabolism pathway. RNA was isolated from cryogenically preserved *P. murina* and *P. carinii* organisms with TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. cDNA was immediately synthesized from the extracted RNA using SuperScript II

TABLE 5 Primers used for real-time PCR analysis of genes in the inositol phosphate metabolism pathway that were found in the *P. murina*, *P. carinii*, and *P. jirovecii* genomes but not in the *S. pombe* genome

Species and target	Primer		Annealing temp (°C)
	Left	Right	
<i>P. murina</i>			
Inositol-polyphosphate multikinase (EC 2.7.1.151)	AGAGCCTGATTTTGACATCGA	AGCCTCTTCCCATATCTTTTGCA	55
Inositol-pentakisphosphate 2-kinase (EC 2.7.1.158)	GGCATATTTCTCAGAAGGAAACCG	ATGAAAGAGGGGAGTCACAACC	55
Phosphoinositide 5-phosphatase (EC 3.1.3.36)	TTGATAGGGCCAATGAATTTTC	TCTGACTTATAATTTTCGCCCTGA	55
Inositol-1,4-bisphosphate 1-phosphatase (EC 3.1.3.57)	TTTGCAAAGCCATGCACCG	ACCTGCAGCATGATCCCAAG	56
<i>P. carinii</i>			
Inositol-polyphosphate multikinase (EC 2.7.1.151)	TGGCTATATAAAGGGTTTGC GA	AGCCTCTTCCCATATCTTTTGCA	60
Inositol-pentakisphosphate 2-kinase (EC 2.7.1.158)	AGTCGTAACCTCCCTCTTTCAT	CGCATGTGTTTCGTTAATGTCT	55
Phosphoinositide 5-phosphatase (EC 3.1.3.36)	AAAGGCTCTAATTACGTCAACT	TGATCAGAAAATGTCAATGGTGA	50
Inositol-1,4-bisphosphate 1-phosphatase (EC 3.1.3.57)	TGTTAGCACGTGGGGAATGT	ACCTGCTGCATGATCCCAA	60

TABLE 6 Primers used for real-time PCR analysis of the putative inositol transporter genes in *P. murina* and *P. carinii*

Target	Primer		Annealing temp (°C)
	Left	Right	
PmITR1	CAGCATTGTACTGACCACA	AATCCAAACCAAGCACCA	50
PcITR1	TGCATTGTACTGACCACA	ACAATCCAAATCCAAGCACCT	60
PmITR2	GACCTGTCCGTGGGCATTA	GGCTACTCCCTTTGCTCGAA	55
PcITR2	GGAACAAGGTTTATCGATTCTAC	ACCGGGTACAATTTGAATCAA	50

reverse transcriptase (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. The remaining RNA and the freshly synthesized cDNA were stored at -70°C .

Oligonucleotide primers, based on sequences from the *Pneumocystis murina* Database (PmD, http://www.broadinstitute.org/annotation/genome/Pneumocystis_group.2/MultiHome.html) and Pneumocystis Genome Project (PGP, <http://pgp.cchmc.org/>), were designed to amplify genes in the inositol phosphate metabolism pathway that were found to be conserved between *P. murina* and *P. carinii* but not present in *S. pombe*. When possible, primers were designed to span an intron junction to prevent amplification of genomic DNA. In addition to the primers designed to amplify these genes, reactions were also performed with sets of primers designed to amplify the *Pneumocystis* thymidylate synthase (TS) gene, which is present as a single copy in the genome, as a comparison for expression of the inositol phosphate metabolism pathway genes, and the *Pneumocystis* mitochondrial large ribosomal subunit (mtLSU), as a positive control. Each set of primers also had a negative control that contained no cDNA template.

Real-time PCR was performed in an Applied Biosystems 7500 fast real-time PCR system (Life Technologies, Grand Island, NY). The reactions were performed in triplicate in a final volume of $22\ \mu\text{l}$ containing $1\times$ SYBR green PCR master mix (Life Technologies, Grand Island, NY) and $0.8\ \text{mM}$ of the appropriate primer pair (Table 5). Undiluted cDNA from either *P. murina* or *P. carinii* was used as the template. The reactions were initially incubated at 50°C for 2 min and then at 95°C for 10 min, followed by 40 to 45 cycles of 95°C for 15 s and 50 to 60°C (Table 5) for 1 min. Fluorescence data were captured during the 50 to 60°C step. Representative reaction products were run on a 1.0% agarose gel prestained with ethidium bromide and visualized with UV light to confirm the presence of products of the predicted size.

Assembly and completion of the putative *P. carinii* ITR1/2 sequence. Two putative sequences for the *P. murina* ITR1 and ITR2 (PmITR1 and PmITR2), as determined by the Broad Institute and their collaborators, were retrieved from the PmD (Pm B123: PNEG_01850.1 and PNEG_00943.1). These sequences were used in homology searches against the sequence database of the PGP using the Basic Local Alignment Search Tool (BLAST) to identify sequences with homology to PmITR1 and PmITR2. The retrieved sequences were assembled and trimmed in DNAMAN version 5.2.9 (Lynnon Corporation, Quebec, Canada) to produce two putative *P. carinii* ITR contigs, designated PcITR1 and PcITR2. The contigs were analyzed by BLAST against the GenBank Fungal database to ensure that they were homologous to other fungal ITRs. This analysis also identified the sequence for a putative *P. jirovecii* ITR from GenBank (accession number CCJ30095).

After assembly from the PGP sequence database, the putative PcITR1 sequence was found to have a sequencing gap. To complete the sequence, oligonucleotide primers were designed that flanked the sequence gap starting at base pair 49 (PcITR1-L, AAG GAA GCA GTA GAA AGT GA, and PcITR1-R, CAT AAT GAC CTC CAA TAG CCA). PCR was performed in a $20\text{-}\mu\text{l}$ reaction mixture containing $1\times$ Jump-Start REDTaq ReadyMix (Sigma, St. Louis, MO), $0.8\ \text{mM}$ of the PcITR1-L and PcITR1-R oligonucleotide primers, and genomic *P. carinii* DNA as the template. The PCR conditions were as follows: 95°C hot start for 3 min, a 95°C denaturing step for 30 s, 50°C annealing step for 30 s, and 72°C extension step for 1 min for 45 cycles, and a final extension of 5 min at

72°C . Reaction products were run on a 0.7% agarose gel prestained with ethidium bromide and visualized with UV light for the presence of a product of the predicted size. PCR products were ligated into the pCR2.1-TOPO vector (Life Technologies, Grand Island, NY) and transformed into TOP10F' cells (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Plasmids were purified, screened for inserts of the proper size, and then sequenced on an ABI Prism 3730xl DNA analyzer (Life Technologies, Grand Island, NY) at a local facility.

Reverse transcriptase real-time PCR of inositol transporter genes. Primers for real-time PCR were designed as described above based on sequences of the PmITR1, PmITR2, PcITR1, and PcITR2 genes (Table 6). Real-time PCR was performed on the inositol transporter genes as described above. Representative reaction products were run on a 1.0% agarose gel prestained with ethidium bromide and visualized with UV light to confirm the presence of products of the predicted size.

Culture media and supplementation. (i) Compound preparation. Serial dilutions of *myo*-inositol (Sigma-Aldrich, St. Louis, MO) ranging from $10,000$ to $0.01\ \mu\text{g}/\text{ml}$ were made in RPMI 1640 containing 20% calf or horse serum, 1% MEM (minimal essential medium) vitamin solution, 1% minimal essential medium with nonessential amino acids (MEM-NEAA), $2,000$ units/ml penicillin-streptomycin, and $50\ \mu\text{g}/\text{ml}$ vancomycin. Negative controls included medium without added inositol and with $10\ \mu\text{g}/\text{ml}$ ampicillin. There was no vehicle control as the *myo*-inositol was miscible in the medium.

(ii) *P. carinii* ATP assay. Cryopreserved and characterized (ATP content, no microbial contaminants) *P. carinii* cells isolated from rat lung tissue were distributed into triplicate wells of 48-well plates with a final volume of $1,000\ \mu\text{l}$ of RPMI 1640 supplemented with 20% calf serum and other additives and a *P. carinii* cell total of $5\times 10^7/\text{ml}$ as previously described (29). Plates were incubated at 37°C with $5\% \text{CO}_2$ in a water-jacketed incubator.

At the assigned time points, 5% of the well volume ($50\ \mu\text{l}$) was removed and washed, and the ATP content was measured using the ATP-liteM luciferin-luciferase assay (PerkinElmer) according to the vendor's instructions. The luminescence generated by the ATP content of the samples was measured by a BMG Polarstar optima spectrophotometer. Samples of each group were examined microscopically to rule out the presence of bacteria or other microbial contaminants that may have been introduced during the culture process. Cultures were refed every other day by removing 50% of the medium and replacing it with fresh medium.

Statistical analysis. Background luminescence was subtracted, and triplicate well readings of the assays were analyzed using ANOVA with a Tukey posttest. Significance was accepted at a *P* value of <0.05 .

Quench control test. Inositol levels were checked for any direct effect on the ATP bioluminescence assay. The ATP standard was diluted and added to the medium with the inositol dilutions used in the assays. No quenching of the enzyme reaction was observed.

Genomic and protein sequences. Sequences of genes, transcripts, and proteins of the *Pneumocystis*-specific enzymes from the inositol phosphate metabolism pathway and inositol transporters are available in Text S1 and S2, respectively, in the supplemental material. New sequences of *P. carinii* inositol transporters are published in the NCBI GenBank with the following accession numbers: PcITR1 (KM263199) and PcITR2 (KM263200).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01834-14/-DCSupplemental>.

- Text S1, TXT file, 0.02 MB.
- Text S2, TXT file, 0.02 MB.
- Table S1, XLSX file, 0.01 MB.
- Table S2, XLSX file, 0.02 MB.
- Table S3, XLSX file, 0.02 MB.

ACKNOWLEDGMENTS

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