

Functional Characterization and Localization of *Pneumocystis carinii* Lanosterol Synthase^{∇†}

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Organisms in the genus *Pneumocystis* are ubiquitous, opportunistic pathogenic fungi capable of causing a lethal pneumonia in immunocompromised mammalian hosts. *Pneumocystis* spp. are unique members of the fungal kingdom due to the absence of ergosterol in their cellular membranes. Although these organisms were thought to obtain cholesterol by scavenging, transcriptional analyses indicate that *Pneumocystis carinii* encodes gene homologs involved in sterol biosynthesis. To better understand the sterol pathway in these uncultivable fungi, yeast deletion strains were used to interrogate the function and localization of *P. carinii* lanosterol synthase (*ERG7*). The expression of PcErg7p in an *ERG7*-null mutant of the yeast *Saccharomyces cerevisiae* did not alter its growth rate and produced a functional lanosterol synthase, as evidenced by the presence of lanosterol detected by gas chromatographic analysis in levels comparable to that produced by the yeast enzyme. Western blotting and fluorescence microscopy revealed that, like the *S. cerevisiae* Erg7p, the PcErg7p localized to lipid particles in yeast. Using fluorescence microscopy, we show for the first time the presence of apparent lipid particles in *P. carinii* and the localization of PcErg7p to lipid particles in *P. carinii*. The detection of lipid particles in *P. carinii* and their association with PcErg7p therein provide strong evidence that the enzyme serves a similar function in *P. carinii*. Moreover, the yeast heterologous system should be a useful tool for further analysis of the *P. carinii* sterol pathway.

Members of the fungal genus *Pneumocystis* can transiently colonize immunocompetent hosts, whereas those with immune deficiencies are particularly susceptible to developing a life-threatening pneumonia as a result of *Pneumocystis* infection (34, 50). Despite their fungal nature, *Pneumocystis* spp. are resistant to standard antifungal drugs that target the major fungal sterol, ergosterol, as well as enzymes involved in its biosynthesis. This lack of efficacy is attributed the lack of detectable ergosterol within its cellular membranes (15). The most abundant sterol found in *Pneumocystis* is cholesterol, which accounts for 81% of its total sterols (15). It is currently thought that most if not all of the cholesterol in *Pneumocystis* is scavenged from its mammalian host (52), but one report raises the possibility of cholesterol biosynthesis within *Pneumocystis* (53). Currently, there is no long-term in vitro culture method with which to grow and propagate these fungi, and attempts to functionally characterize genes or to establish effective drug targets have been impeded. Investigators in the field have had to rely on heterologous yeast systems, such as deletion strains of *Saccharomyces cerevisiae* (38), or the knockout of genes in the more complicated *Schizosaccharomyces pombe* system (30) to assess the function of *P. carinii* proteins.

Despite the lack of ergosterol in the membranes of *Pneumocystis* spp., several putative genes involved in sterol biosyn-

thesis were identified through the *Pneumocystis* Genome Project (11). These genes are likely to be functional based on transcriptional analysis (12), short-term in vitro inhibition studies (19), and the incorporation of radiolabeled squalene and mevalonate into *P. carinii* sterols (14, 20). The *P. carinii* sterol biosynthetic genes encoding the lanosterol 14 α demethylase enzyme (Erg11p) (38), the lanosterol synthase enzyme (Erg7p) (36), and the *S*-adenosyl methionine: C:24 sterol methyltransferase enzyme (Erg6p) (21) have been isolated, cloned, and expressed in heterologous yeast systems. Each of these enzymes was able to complement yeast strains containing a deletion of the respective gene, indicating that these *P. carinii* enzymes likely perform a similar function in *P. carinii*.

Erg7p is an essential enzyme of both the cholesterol and the ergosterol biosynthetic pathways. This enzyme is responsible for the conversion of 2,3-oxidosqualene, the last acyclic sterol precursor, into lanosterol, the first sterol intermediate of the mammalian and fungal sterol biosynthetic pathways. During this conversion, Erg7p performs a series of complex cyclization and rearrangement steps, resulting in the alteration of 20 bonds and the formation of four rings and seven stereocenters (42). *S. cerevisiae* Erg7p (ScErg7p) localizes to lipid particles and, when expressed in *S. cerevisiae*, Erg7p from the plant pathogen *Arabidopsis thaliana*, and the parasite *Trypanosoma cruzi* localized to lipid particles in an *S. cerevisiae* *ERG7* mutant (35, 36). Lipid particles are intracellular organelles consisting of a hydrophobic core of steryl esters and triglycerides surrounded by a phospholipid monolayer. The monolayer surrounding this cellular compartment contains 16 proteins all of which function in lipid metabolism (3). Several roles have been ascribed to lipid particles, including lipid metabolism and storage (3). Thus, it is not surprising that ergosterol biosynthesis is

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intrinsically linked to lipid particles and that yeast strains that lack lipid particles have a defect in ergosterol synthesis (46).

In silico sequence analysis of PcErg7p revealed that it contained residues that are essential for the catalytic activity of the ScErg7p (36) and, based on the incorporation of radiolabeled acetate into ergosterol, the same researchers showed that PcErg7p was able to functionally complement an *S. cerevisiae* ERG7-null mutant expressing PcErg7p. They further concluded that the *P. carinii* enzyme did not localize to lipid particles, after no enzymatic activity was detected in the isolated particles. It was our intent to provide a more complete picture of the function of this important enzyme by quantification of lanosterol production and analysis of the growth rate of yeast expressing PcErg7p and to resolve the cellular location of the protein. We show for the first time the presence of lipid storage compartments in *P. carinii* which are likely lipid particles and the localization of PcErg7p to this compartment in both yeast and in *P. carinii*.

MATERIALS AND METHODS

Cloning of ERG genes. PCR primers (sense, 5'-ATG ATT TAT GGG TAT ACC GAA AA-3'; antisense, 5'-AAT ATT ACC ATA TCT TTT CGA ATA CAT-3') were designed to amplify the open reading frame (ORF) of PcERG7 using the PcERG7 cDNA clone S18F10. ScERG7 was amplified from *S. cerevisiae* DNA using the primers (sense, 5'-ATG ACA GAA TTT TAT TCT GAC ACA-3'; antisense, 5'-AAG CGT ATG TGT TTC ATA TGC CCT GC-3'). The PCR products were each ligated into the galactose-inducible vector pYES2.1 (Invitrogen, Carlsbad, CA), followed by cloning into bacterial Top10F' cells (Invitrogen). Plasmid DNA from pYES2.1/PcERG7 and pYES2.1/ScERG7 was sequenced to verify the accuracy of the insert and proper orientation of the insert within the vector (CCHMC Genetic Variation and Gene Discovery Core Facility, Cincinnati, OH). The sequence for genomic PcERG7 sequence contained within contig 495 on the *Pneumocystis* Genome Project website (<http://pgp.cchmc.org/>) was aligned with the cDNA sequence using DNAMAN (Lynnon BioSoft, version 5.2.9) and MGAlign (28) to determine the number and location of introns within the coding sequence of PcERG7.

Construction of ERG7 mutant strains. The diploid *S. cerevisiae* ERG7 mutant strain (*MAT α /MAT α his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 lys2 Δ 0/+ met15 Δ 0/+ ura3 Δ 0/ura3 Δ 0 Δ ERG7) was obtained from the American Type Culture Collection and used to express pYES2.1, pYES2.1/PcERG7, or pYES2.1/ScERG7. The yeast were grown overnight in yeast extract-peptone-dextrose medium containing 200 μ g of G418/ml, and transformation and sporulation were performed according to the method of Morales et al. (38). Spores from each strain were released by sonication, and spores obtained from strains expressing either pYES2.1/PcERG7 or pYES2.1/ScERG7 were plated on uracil-deficient minimal medium containing 2% galactose and 200 μ g of G418/ml to select for spores with the wild-type ERG7 deletion. Spores containing the empty vector were plated on similar medium lacking G418 to select for spores containing ERG7 at the wild-type locus. Haploid yeast strains were identified by using multiplex PCR as previously reported by Huxley et al. (17). Upon verification of the haploid yeast colonies from strains expressing pYES2.1/PcERG7 and pYES2.1/ScERG7, PCR was performed to verify the expression of either PcERG7 or ScERG7 in the absence of chromosomal ScERG7 using primers designed to amplify the ORF of the gene. PCR verification of the absence of *S. cerevisiae* ERG7 in the haploid yeast colony was achieved by using a primer from the 5' untranslated region (5'UTR) of ScERG7 (5'-GCTTAGTTTTGTCCATCTCATTG 3') and an antisense primer to the *KanMX* gene (5' CTG CAG CGA GGA GCC GTA AT 3').*

Growth rate analysis. Yeast colonies containing wild-type ScERG7, pYES2.1, pYES2.1/PcERG7, and pYES2.1/ScERG7 were inoculated into either glucose-containing minimal medium or galactose-containing minimal medium lacking uracil to induce protein expression and to maintain pYES2.1 in vector containing haploid strains. The cultures were maintained at 30°C in a shaking incubator, and aliquots of each culture were taken at 4, 8, 12, 24, 48, and 72 h of growth in liquid medium. The optical density at 600 nm of each aliquot was measured to assess growth of the respective cultures using the POLARstar Optima (BMG Labtech, Durham, NC). The results are expressed as the mean of three separate experiments, each performed in triplicate.

In silico TM analysis. Determination of hypothetical transmembrane (TM)-spanning domains was performed by using HMMTOP2 online software (<http://www.enzim.hu/hmmtop/html/submit.html>) (47, 48), MINNOU online software (<http://polyview.cchmc.org>) (8), and SOSUI online software (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) (16). Hydrophobicity analysis was performed according to the method of Kyte and Doolittle (26) with a window size of 19 amino acids and using TopPred online software (<http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>) (49).

PcErg7p purification and polyclonal antibody production. PcERG7 cDNA was cloned and expressed in the pET30 vector (Novagen, Madison, WI). PcErg7p expression was induced by using IPTG (isopropyl- β -D-thiogalactopyranoside), and the protein was purified from inclusion bodies within *Escherichia coli* according to the manufacturer's instructions. Briefly, the cells were harvested by centrifugation and resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). The cell suspension was sonicated, and the soluble protein fraction was separated from the insoluble fraction by centrifugation. The insoluble protein fraction was solubilized overnight at 4°C in binding buffer containing 6 M urea, and PcErg7p was extracted from the insoluble protein extract by rapid affinity chromatography using His-Bind resin (Novagen). Urea was removed with sequential washes, and PcErg7p was eluted from the column by using 1 M imidazole. Western analysis with an S protein-horseradish peroxidase-conjugated antibody (Novagen) confirmed the presence of purified PcErg7p in the eluted fraction, and purified PcErg7p was sent to Cocalico Biologicals (Reamstown, PA) for polyclonal antibody production. The specificity of the polyclonal antiserum was determined via Western blotting using *P. carinii* cell lysates and recombinant PcErg7p as a positive control. The antibody fraction of the antiserum was precipitated by using ammonium sulfate and reconstituted in phosphate-buffered saline (PBS). A fluorescent PcErg7p antibody was developed by labeling the antibody fraction with Alexa Fluor 488 dye (Invitrogen) according to manufacturer's instructions.

Lanosterol quantification. Wild-type yeast, pYES2.1/PcERG7, and pYES2.1/ScERG7 containing yeast were inoculated and cultured for up to 3 days, and aliquots were taken after 24, 48, and 72 h of growth. The cells were collected, homogenized, and placed into glass vials. A Lowry assay (31) was performed on aliquots of the homogenates to determine the protein concentration. Mass cellular lanosterol was quantified by using gas chromatography with cholesterol as an internal standard, and sterol extraction was performed as previously reported (39, 44). Alcoholic KOH (940 μ l of ethanol and 60 μ l of 50% KOH) was added to each vial; the vials were capped, placed in a 65°C water bath for 2 h, and cooled; and 5 μ g of cholesterol was added to each sample. The lipid content of each sample was extracted by using 3 ml of petroleum ether and recovered by evaporation of petroleum ether under a stream of air. The lipids were resuspended in 15 μ l of hexane, and 2 μ l of each extract was injected into a GC-17A gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD), and the amount of lanosterol present in each sample was calculated based on cholesterol and lanosterol peaks. These experiments were performed twice, and the data are expressed as micrograms of lanosterol per milligram of protein.

Yeast lipid particle isolation. Lipid particles were obtained according to a previously published method (35). Briefly, yeast cells were grown to early stationary phase and treated with zymolyase 20T to create yeast spheroplasts. Spheroplasts were washed twice with 20 mM potassium phosphate (pH 7.4) and 1.2 M sorbitol and then homogenized in breaking buffer (10 mM MES-Tris [pH 6.9], 12% Ficoll 400, 0.2 mM EDTA) at a final concentration of 0.5 ml per g of wet cell weight. The homogenate was centrifuged at 5,000 \times g, and the supernatant was overlaid with breaking buffer and centrifuged at 100,000 \times g in an SW28 swing-out rotor. The floating layer (top layer) was collected, overlaid with 10 mM MES-Tris (pH 6.9)–8% Ficoll 400–0.2 mM EDTA, and centrifuged for 30 min at 100,000 \times g. The top layer was collected, overlaid with 10 mM MES-Tris (pH 6.9)–0.25 M sorbitol–0.2 mM EDTA, and centrifuged for 30 min at 100,000 \times g. The top layer of the gradient containing a highly purified yeast lipid particle fraction was collected for analysis.

Immunoblotting. Yeast colonies were grown to late log phase, and *P. carinii* organisms and late-log-phase yeast cells were lysed by using Y-PER reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. Protein concentrations were determined by using a BCA protein assay (Pierce), and equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted as described previously (29). Erg7p was detected by using a 1:5,000 dilution of polyclonal PcErg7p antiserum IgG, followed by a 1:10,000 dilution of goat anti-rabbit-horseradish peroxidase conjugate. Reactive protein bands were visualized by using TMP1 component HRP membrane substrate (BioFX Laboratories, Owings Mills, MD). For lipid particle immunoblotting, lipid particle fractions were collected in two 1-ml aliquots from the top of the gradient (lipid

particle isolation described above), and PcErg7p and ScErg7p were blotted and detected as stated above.

Fluorescent localization of PcErg7p in yeast. Colonies containing pYES2.1/*PcERG7* were inoculated into minimal medium containing 2% galactose and lacking uracil, and yeast colonies containing *ScERG7*-GFP (Invitrogen) were inoculated into yeast extract-peptone-dextrose. The cultures were allowed to grow for 2 days in a 30°C shaking incubator. Cells containing pYES2.1/*PcERG7* were pelleted, washed with PBS, and permeabilized with 1% dimethyl sulfoxide in PBS. The cells were collected via centrifugation and washed to remove the residual dimethyl sulfoxide. Nonspecific binding sites were blocked in pYES2.1/*PcERG7*-expressing cells using 10% (wt/vol) bovine serum albumin (BSA) in PBS, and the cells were collected and resuspended for 1 h in an anti-V5-fluorescein isothiocyanate (FITC)-conjugated antibody (Invitrogen). The cells were washed with PBS containing 0.1% Tween 20, and pYES2.1/*PcERG7*-containing yeast and *ScERG7*-GFP-containing yeast were incubated for 1 h in 1 μ M Nile Red in PBS. After incubation with Nile Red, the cells were washed, dropped onto microscope slides coverslipped, and visualized with a Nikon Eclipse E600 fluorescence microscope. FITC images were viewed by using excitation filters at 465 to 495 nm and emission filters at 515 to 555 nm, and Nile Red images were viewed by using excitation filters at 540 to 580 nm and emission filters at 600 to 660 nm.

Fluorescent localization of PcErg7p in *P. carinii*. Cryopreserved *P. carinii* were thawed, centrifuged, and resuspended in PBS, and the cells were permeabilized and blocked similar to the yeast cells (described above). *P. carinii* organisms were collected by centrifugation, resuspended in 1% BSA in PBS solution, and incubated for 1 h with polyclonal PcErg7p antiserum conjugated with Alexa Fluor 488. The cells were washed twice with PBS, resuspended in 6% BSA in PBS, and incubated for 1 h with Qdot 525 goat F(ab')₂ anti-rabbit IgG conjugate. The cells were centrifuged at 10,000 \times g, washed twice with PBS, and incubated for 1 h with 1 μ M Nile Red in PBS. After centrifugation, the organisms were washed once with 0.1% Tween 20 in PBS, incubated twice in PBS, and visualized with a Nikon Eclipse E600 fluorescence microscope. Qdot 525 and Alexa Fluor 488 images were viewed by using excitation filters at 465 to 495 nm and emission filters at 515 to 555 nm.

Statistical analysis. Statistical analyses were performed using GraphPad v.4 (GraphPad Software, Inc., La Jolla, CA), and significance was assessed by using analysis of variance and the Tukey-Kramer multiple-comparison post test.

RESULTS

PCR was used to amplify the entire ORF of *PcERG7* from a *PcERG7* cDNA clone, and a 2,160-bp product corresponding to the size of the *PcERG7* ORF (36) was detected. The genomic sequence for *PcERG7* was found to be 2,564 nucleotides in length, and alignment of the *PcERG7* genomic sequence with the *PcERG7* cDNA sequence revealed that the gene contains 10 exons and 9 introns ranging in length between 9 and 622 nucleotides and between 41 and 49 nucleotides, respectively. We confirmed that the PCR product was of *P. carinii* origin by hybridization of a ³²P-labeled *PcERG7* cDNA probe to a contoured clamped homogeneous electrical field blot containing the chromosomes of seven karyotype forms of *P. carinii* and the single *P. wakefieldiae* karyotype (43). The radiolabeled probe bound to a single 620-kb chromosome in all karyotype forms of *P. carinii* (see Fig. S1, black arrow, lanes 2 to 9, in the supplemental material) and to a chromosome of 550 kb in *P. wakefieldiae* (see Fig. S1, open arrow, lane 10, in the supplemental material). These chromosomes correspond to chromosome 3 in both genomes, indicating that the *ERG7* gene is located on the same chromosome in both *P. carinii* and *P. wakefieldiae*, a novel finding since genes are rarely located on the same chromosome in both genomes (10).

Multiple sequence comparisons of the in silico-translated ORF of PcErg7p to the same protein from other fungal species indicate a high degree of conservation in the amino acid sequence of lanosterol synthases across the fungal kingdom

(Fig. 1). Our bioinformatics analysis confirms that PcErg7p contains the squalene cyclase domain that is responsible for catalyzing the cyclization reaction that results in the conversion of lanosterol from the linear molecule 2,3-oxidosqualene (1, 32, 33, 51) (Fig. 1). As previously reported, within this domain are amino acid residues that are essential for the catalytic activity of ScErg7p: aspartate 456, histidines 146 and 234, tyrosine 410, and valine 454 (36). The amino acid sequence of PcErg7p and ScErg7p are 49% identical and 65% similar, indicating a significant degree of conservation between these two proteins.

Loss of *ERG7* results in an inviable phenotype in yeast, and previous studies (36) have shown that the expression of *PcERG7* in *ERG7*-null yeast restores viability. To better study the enzyme, pYES2.1/*PcERG7* was expressed in an *ERG7*-null mutant, and Western analysis was used to verify protein expression in the mutant. PcErg7p was predicted to be 83 kDa (36), and a polyclonal antibody raised against PcErg7p detected the protein in *P. carinii* and yeast containing pYES2.1/*PcERG7* (Fig. 2, lanes 1 and 2, respectively). Due to the conservation between the two proteins, ScErg7p (Fig. 2, lanes 3 to 5) was detected using the same antibody. The larger-molecular-weight bands detected in lanes 2 and 4 were no longer present when a higher dilution of the polyclonal antibody was used (see Fig. S2, lane 3, in the supplemental material), indicating the band was likely due to a nonspecific protein-antibody interaction. However, at this concentration ScErg7p was not detected in wild-type cells where ScErg7p is expressed at basal levels (see Fig. S2, lane 1, in the supplemental material), indicating that either higher protein concentrations or a more concentrated antibody is necessary to detect basal levels of ScErg7p. To determine whether the expression of exogenous PcErg7p in the null yeast mutant resulted in any growth differences, the growth rates of haploid wild-type yeast, and yeast containing pYES2.1, pYES2.1/*PcERG7*, and pYES2.1/*ScERG7* were assessed. All strains reached stationary phase 48 h after inoculation, with the exception of the strain containing only the pYES2.1 vector, which reached the stationary phase 24 h after inoculation (Fig. 3). There were no significant differences in the growth rates of the strains expressing pYES2.1/*PcERG7* and pYES2.1/*ScERG7* at any of the time points analyzed (Fig. 3), indicating that the timing for entry into both the log and stationary phases of growth was identical. Thus, the PcErg7p appeared to supply sufficient levels of lanosterol necessary for normal growth of *S. cerevisiae*.

The conversion of 2,3-oxidosqualene into lanosterol by Erg7p is the first sterol-producing step in ergosterol biosynthesis, and PcErg7p was able to sustain ergosterol biosynthesis in the absence of the wild-type enzyme in yeast (36). The similar growth rates of yeast containing PcErg7p and ScErg7p indicate that lanosterol production by the enzymes may be similar. To quantify the amounts of lanosterol directly, gas chromatography was used to measure lanosterol produced by the *P. carinii* and *S. cerevisiae* enzymes in each of the strains. Lanosterol quantities between the wild-type strain and that containing pYES2.1/*PcERG7* were not significantly different at any of the time points (Fig. 4). The amount of lanosterol produced by the pYES2.1/*ScERG7*-containing strain was not significantly different from that produced by the pYES2.1/*PcERG7* strain after 24 and 48 h of growth but did produce statistically higher

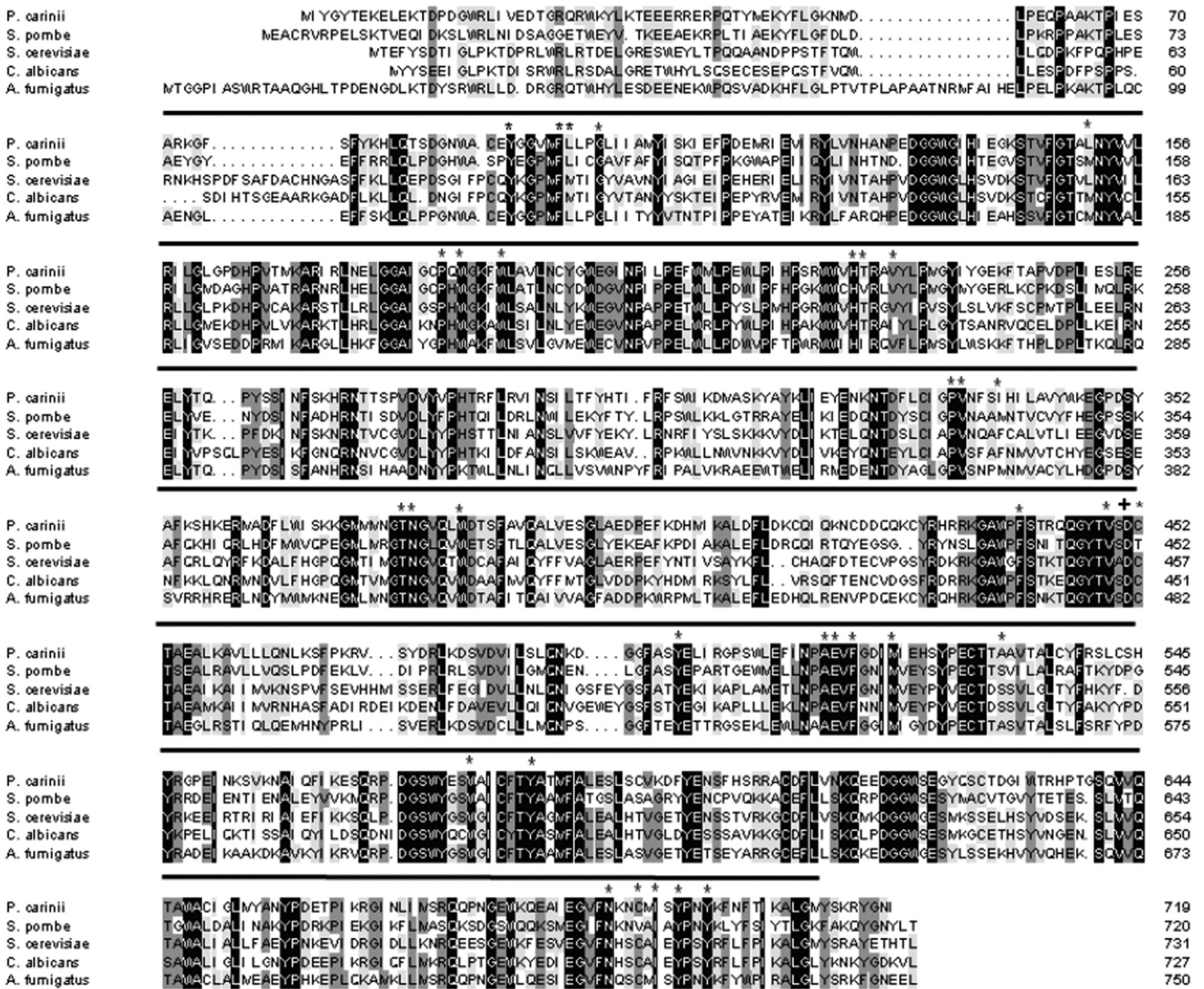


FIG. 1. Multiple sequence alignment comparing predicted amino acid sequence of PcErg7p to the Erg7p amino acid sequence from *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus fumigatus*. Dark-shaded regions indicate areas of homology within the amino acid sequences of all species represented, while light-shaded regions indicate regions of sequence similarity between two or more sequences. The black bar above the amino acid alignment corresponds to the squalene cyclase domain of PcErg7p, which lies between amino acids 71 and 711. Asterisks correspond to conserved amino acid residues within the squalene cyclase domain of PcErg7p according to the Conserved Domain Database (32, 33). The plus sign at residue 451 corresponds to the catalytic aspartic acid that is responsible for the initiation of the ring cyclization reaction of lanosterol synthesis.

levels after 72 h (Fig. 4). These data were consistent with real-time PCR data, indicating that *ScERG7* demonstrated higher gene expression than *PcERG7* at this time point (data not shown). This increase may be indicative of a difference in the copy number of pYES2.1 between the two strains rather than a decreased efficiency of the *P. carinii* enzyme.

We are uncertain whether the basal expression of *PcERG7* would produce sufficient lanosterol to sustain growth of *S. cerevisiae* because attempts to place *PcERG7* under the control of the *ScERG7* promoter via homologous recombination were unsuccessful. Deletion of *ERG7* from yeast is lethal, suggesting that there were no other lanosterol-producing enzymes within the cells. Therefore, the detection of lanosterol in the strain containing PcErg7p indicates that *PcERG7* produces a lanos-

terol-synthesizing enzyme in yeast and likely performs a similar role in *P. carinii*.

ScErg7p localizes to lipid particles in yeast, and a previous analysis revealed that most proteins associated with lipid particles lack TM domains or contain only one of these domains (3). Thus, it has been proposed that proteins containing multiple TM domains are unable to associate with the monolayer membrane surrounding lipid particles (3). Based on the in silico predictions of HMMTOP 2.0 and Kyte and Doolittle, PcErg7p was predicted to have six hypothetical TM domains (36), making the enzyme ill suited for insertion into the phospholipid monolayer of lipid particles (3, 36). In contrast, we found highly variable results using the same protein prediction models in addition to others (Table 1). Our in silico analysis of

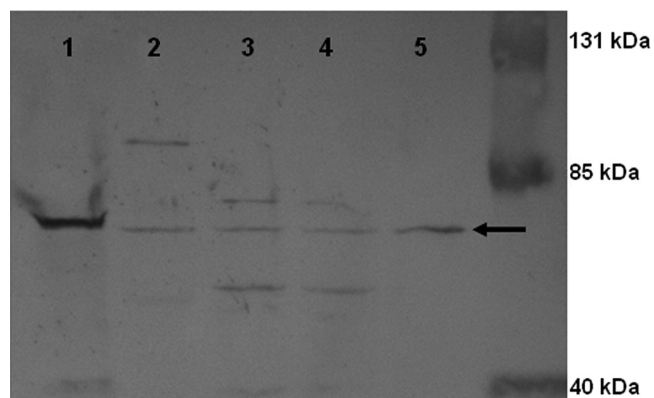


FIG. 2. Detection of wild-type ScErg7p, recombinant PcErg7p and ScErg7p. Protein extracts from *P. carinii*, *S. cerevisiae*, and *S. cerevisiae* containing either pYES2.1/PcERG7 or pYES2.1/ScERG7, were blotted and probed with PcErg7p antiserum. Lanes 1 to 5 correspond to protein lysates from *P. carinii*, yeast containing pYES2.1/PcERG7, yeast containing pYES2.1/ScERG7, yeast containing pYES2.1, and wild-type yeast, respectively. PcErg7p and ScErg7p were detected as 83-kDa proteins, and the arrow indicates 83-kDa band corresponding to Erg7p detected in the lysates.

the protein sequences of both PcErg7p and ScErg7p show that the number of TM domains range from zero to six depending on the program (Table 1). These data indicate that PcErg7p could fit the profile of a protein that is capable of insertion into the phospholipid monolayer of a lipid particle and highlight the fact that characterization of protein structure based on *in silico* data may not always be accurate or consistent.

The significant degree of similarity between the protein sequences of PcErg7p and ScErg7p, the similar growth rates, and the similar lanosterol production of yeast containing the enzymes led us to examine whether PcErg7p localizes to lipid particles in yeast as does the native protein. Lipid particles from haploid wild-type yeast and yeast containing pYES2.1, pYES2.1/ScERG7, and pYES2.1/PcERG7 were isolated and analyzed by Western blotting. The results from the pYES2.1/ScERG7 strain and the pYES2.1/PcERG7 strain are shown in

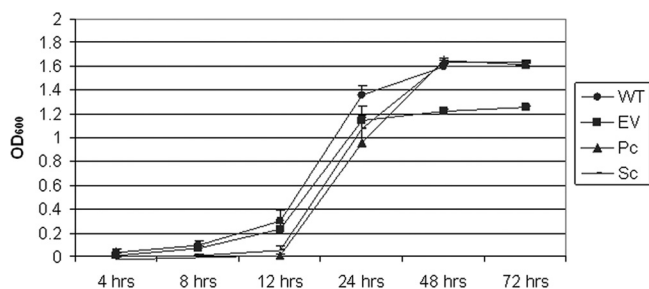


FIG. 3. Growth curve comparing growth of wild-type yeast (WT) and yeast containing, pYES2.1 (EV), pYES2.1/PcERG7 (Pc), or pYES2.1/ScERG7 (Sc) cultured in liquid medium at 30°C. Each data point represents the mean of three independent studies. Error bars represent the standard deviation of each group. Statistical significance ($P \leq 0.05$) was noted for all strains compared to the WT strain at all time points analyzed with two exceptions: WT compared to EV at 12 h and WT compared to Sc at 72 h. Statistical significance was not detected when comparing pYES2.1/PcERG7 and pYES2.1/ScERG7 at any of the time points in the study.

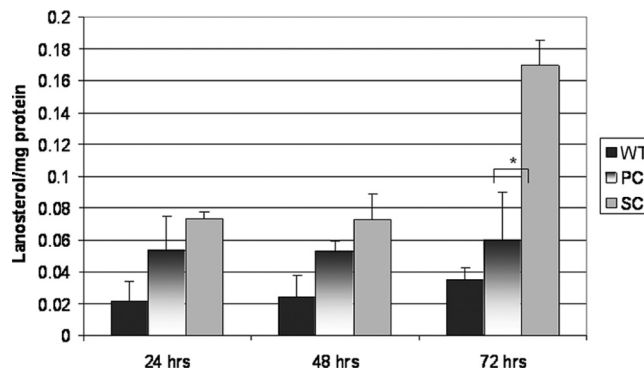


FIG. 4. Lanosterol production by wild-type yeast (WT) or yeast containing either pYES2.1/ScERG7 (SC) or pYES2.1/PcERG7 (PC). Lanosterol levels were assessed by gas liquid chromatography, and asterisks indicate statistical significance. Values represent the mean of each group, and error bars represent the standard deviation of each group.

Fig. 5C and D, respectively. The presence of an 83-kDa band in Fig. 5D, lanes 1 and 2, indicates that PcErg7p localizes to lipid particles in yeast. The presence of the larger-molecular-weight band detected in lipid particles from the pYES2.1/PcERG7 strain (Fig. 5D, lanes 1 and 2) is likely due to the presence of the V5 epitope on the protein. The larger-molecular-weight band was seen as a faint band on the blot containing lipid particles from the pYES2.1/ScERG7 strain, but the band was not readily apparent after imaging (Fig. 5C, lanes 1 and 2). The lower bands may be degradation products of PcErg7p since these were detected upon purification of the native protein that was used for generation of the PcErg7p polyclonal antibody. We were also able to detect ScErg7p from wild-type strains and strains containing pYES2.1 in lipid particles isolated from these strains (Fig. 5A and B, respectively) using our polyclonal antibody.

The presence of PcErg7p in lipid particles in yeast is in contrast to a previous report (36). To verify our findings, we sought to visualize the enzyme within the yeast mutant using fluorescent markers to the protein and lipid particles. Expression of PcErg7p from the pYES2.1 vector allowed us to create a PcErg7p-V5 epitope fusion protein that could be detected by an FITC-conjugated V5 antibody. Lipid particles consist of a hydrophobic core of neutral lipids that can be readily stained with the fluorescent dye Nile Red (4, 13, 25, 46). FITC staining of pYES2.1/PcERG7-containing yeast revealed a punctate staining pattern (Fig. 6B1) similar to that of a GFP-Erg7p-containing yeast strain (Fig. 6A1) used for visual comparison.

TABLE 1. *In silico* TM helix predictions for PcErg7p and ScErg7p^a

Server	No. of TM predictions	
	PcErg7p	ScErg7p
HMMTOP2	6	6
SOSUI	1	0
TopPred2	3	3
MINNOU	0	1

^a The number of TM-spanning domains within the protein sequences of PcErg7p and ScErg7p were predicted using various protein structure prediction models.

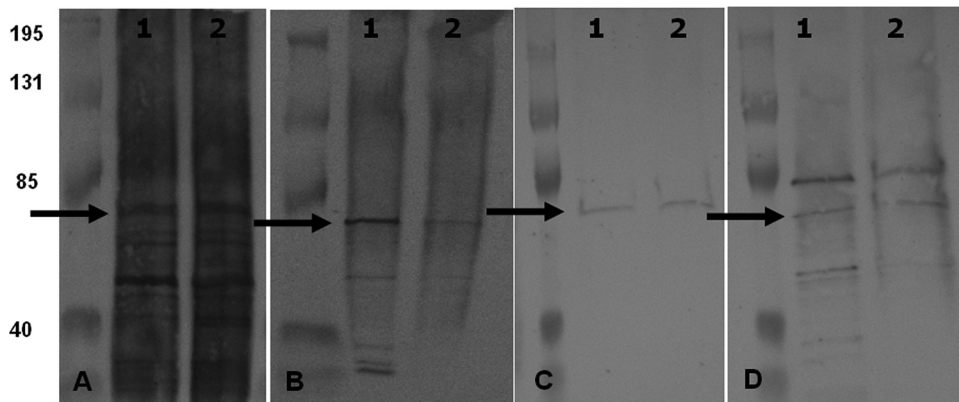


FIG. 5. PcErg7p localizes to lipid particles in yeast. Lipid particles were isolated from wild-type yeast and yeast containing pYES2.1, pYES2.1/PcERG7, or pYES2.1/ScERG7. The floating layer was removed in 1-ml aliquots, and 5- μ g portions of protein from the top two fractions (indicated as lanes 1 and 2) were subjected to Western analysis. (A) Wild type; (B) pYES2.1; (C) pYES2.1/ScERG7; (D) pYES2.1/PcERG7.

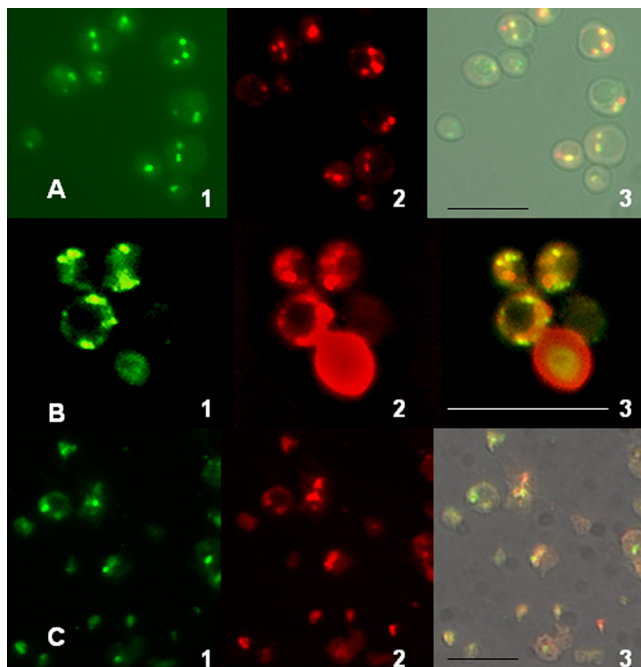


FIG. 6. Fluorescence localization of PcErg7p in yeast and *P. carinii*. (A) GFP-ScErg7p was localized to lipid particles in yeast using an *S. cerevisiae* Erg7p-GFP yeast strain and Nile Red. The left panel shows GFP-ScErg7p in *S. cerevisiae* (A1), middle panel shows Nile Red-stained GFP-ScErg7p yeast (A2), and the right panel shows merged GFP and Nile Red images of GFP-ScErg7p (A3). (B) *S. cerevisiae* containing pYES2.1/PcERG7. The left panel shows PcErg7p stained with V5-FITC conjugated antibody (B1), the middle panel shows Nile Red-stained PcErg7p in yeast (B2), and the right panel shows merged FITC and Nile Red images (B3). (C) Fluorescence localization of PcErg7p in *P. carinii* was performed using PcErg7p antisera conjugated with Alexa Fluor 488 and Qdot 525 to identify PcErg7p and Nile Red to identify lipid particles in *P. carinii*. The left image shows PcErg7p stained with Alexa Fluor 488 and Qdot 525 (C1), the middle image shows Nile Red-stained *P. carinii* (C2), and the image on the right shows the merged image (C3). Arrows indicate areas of colocalization. Scale bars, 10 μ m.

When FITC-stained PcErg7p in the *ERG7* yeast mutant was overlaid with the Nile Red-stained lipid particles (Fig. 6B2), the two fluorophores merged within the cell, confirming that PcErg7p is localized to lipid particles in yeast (Fig. 6B3) similar to what was seen in the GFP-Erg7p control yeast strain (Fig. 6A3).

The presence of lipid particles within *P. carinii* has never been evaluated; therefore, we stained *P. carinii* organisms with Nile Red to establish whether *P. carinii* organisms contain these neutral lipid stores. Nile Red staining was detected in *P. carinii* (Fig. 6C2) in a punctate pattern similar to that seen in yeast stained with Nile Red (Fig. 6A2 and B2), indicating that *P. carinii* does appear to house stores of neutral lipids. To visualize PcErg7p within *P. carinii*, we used the fluorescent dye Alexa Fluor 488 conjugated to an anti-PcErg7p antibody, and Qdot 525 was used as a secondary antibody to enhance the detection of PcErg7p. PcErg7p was localized to discrete regions within *P. carinii* (Fig. 6C1) in a similar pattern to that seen in yeast. To resolve whether these regions represent lipid particles, *P. carinii* images of Nile Red-stained lipid particles and Qdot 525-stained PcErg7p were merged. We observed a dual localization, as indicated by the resulting yellow image (Fig. 6C3), indicating localization of the PcErg7p to lipid particles in *P. carinii*.

DISCUSSION

We, like previous investigators, showed here that PcErg7p was able to complement a null yeast Erg7p mutant (36). In contrast, however, we found that PcErg7p was localized to lipid particles in yeast and in *P. carinii* by using Western blotting and fluorescence localization studies. The previous group of researchers concluded that PcErg7p does not localize to lipid particles based on three observations: the presence of six putative TM spanning domains, which would make the enzyme ill suited for insertion into the lipid particle monolayer; the lack of PcErg7p enzymatic activity in lipid particles of the yeast mutant strain expressing PcErg7p; and the lack of an 83-kDa band, the predicted size of PcErg7p, in a Coomassie blue-

stained gel containing lipid particle proteins isolated from PcErg7p expressing yeast. The differences between these two studies were likely due to the sensitivities of the techniques used. We used polyclonal antisera to detect the presence of PcErg7p, whereas the previous study relied on detection of the protein in a stained polyacrylamide gel, which likely did not have the sensitivity necessary to detect the protein. In addition, the lack of PcErg7p activity may have been due to the inactive state of the *P. carinii* enzyme. Inactivation of enzymes in lipid particles has been shown for *S. cerevisiae* squalene epoxidase (Erg1p), which localizes to both lipid particles and the endoplasmic reticulum (ER). Erg1p was shown to be active in the ER but inactive in lipid particles (27). These same investigators found that addition of lipid particles from a wild-type strain to microsomes from an Erg1p-disrupted strain resulted in partial restoration of Erg1p activity in the lipid particles, indicating a working relationship between these two cellular compartments that may be destroyed upon mechanical separation of the two compartments. Our study did not assess the activity of PcErg7p in lipid particles, and therefore we cannot rule out the possibility that the enzyme may not be active in these organelles.

Lanosterol synthases are widely regarded as integral membrane proteins (7, 45, 51), and lanosterol synthases from yeast and *Trypanosoma cruzi* and cycloartenol synthase from *Arabidopsis thaliana* have all been cloned and expressed in yeast and found to localize to lipid particles in lanosterol synthase yeast mutants (35, 36). Characterization of lipid particle proteins from yeast revealed that most lipid particle proteins lack TM domains or contain only one of these domains (3). Our in silico analyses revealed that PcErg7p or ScErg7p may contain as few as zero TM domains or as many as six TM domains. Another study (37) characterizing TM domains in ergosterol biosynthetic enzymes from *S. cerevisiae* using programs not used in the present study indicates that ScErg7p contains between zero and four TM domains. In light of these highly variable results, the use of TM domains to predict localization to lipid particles seems to be of little use.

Despite the ability of *P. carinii* to scavenge cholesterol from the host, evidence is mounting that suggests the organism has a functional sterol pathway, and although a complete sterol biosynthetic pathway for *P. carinii* has not been elucidated, numerous insights about the pathway have been gained as a result of biochemical analysis and heterologous expression of three of the genes involved in sterol biosynthesis. The *P. carinii* lanosterol 14 α demethylase enzyme, the target of azole antifungal drugs, was biochemically characterized, and sequence analysis comparing the translated ORF of PcErg11p to other fungal Erg11 proteins revealed the presence of two amino acids that are thought to confer resistance to azole antifungal drugs (38). Functional analysis of PcErg11p expressed in an *S. cerevisiae* Erg11p mutant revealed that PcErg11p required a 2.2-fold-higher dose of voriconazole and a 3.5-fold-higher dose of fluconazole than *S. cerevisiae* Erg11p for a 50% reduction in growth. The *P. carinii* S-adenosyl-L-methionine:C-24 sterol methyltransferase (*ERG6*) gene has also been cloned and heterologously expressed in yeast and *E. coli* (21, 22). These studies revealed that PcErg6p has a preference for lanosterol as its substrate, unlike other fungal *Erg6* enzymes that use the sterol metabolite zymosterol as a substrate. As a result, it was proposed that the flux of sterols in *P. carinii* may be lanosterol

to 24-methylenelanosterol to pneumocysterol, the latter being a result of a second methylation by PcErg6p upon 24-methylenelanosterol (22). This would indicate that lanosterol demethylation by Erg11p occurs after C-24 alkylation by Erg6p in *P. carinii* and that the substrates for *P. carinii* Erg11 are 24-alkylsterols and not lanosterol (Fig. 7). This is not unlikely given the fact that the product of the yeast Erg11 enzyme, 4,4-dimethyl-cholesta-8,14,24-trienol, was not detected in a comprehensive analysis of *P. carinii* sterols (15) and that this alternative pathway has been observed in a fluconazole-resistant strain of *C. albicans* (2).

Cellular localization is an important factor in determining the function, regulation, and interactions with other proteins within cellular compartments. A large-scale study using green fluorescent protein to target enzymes involved in yeast lipid synthesis has revealed that enzymes involved in the early steps of ergosterol biosynthesis are cytosolic with the exception of Hmg1p and Hmg2p, which are found in the ER (41), and enzymes involved in the committed sterol pathway were found to localize to the ER (41). Interestingly, these investigators also found that several enzymes—Erg1p, Erg7p, Erg6p, and Erg27p—were localized both to the ER and to lipid particles. A total of 80% of yeast Erg6p was localized almost exclusively in lipid particles, with only 20% being localized to the ER (27). If the proposed sterol pathway of *P. carinii* follows the order proposed by Kaneshiro et al. (22), and PcErg6p is also localized to lipid particles in *P. carinii*, then PcErg7p would be in close proximity to this next enzyme of the pathway, which would help to facilitate sterol biosynthesis in *P. carinii*.

Our study is the first to localize a *P. carinii* sterol enzyme and the first to suggest that *P. carinii* contains intracellular lipid particles. Because little is known about the sterol pathway in *P. carinii*, the discovery that *P. carinii* contains lipid particles has important implications for sterol biosynthesis in this organism. Upon their initial isolation from yeast, lipid particles were considered a storage compartment for triglycerides (TAG) that provide energy and steryl esters (STE) that could be hydrolyzed for membrane synthesis (9). This view has been challenged with the discovery of TAG lipases (5, 6, 18, 25) and STE-hydrolyzing enzymes (18, 23, 24, 40), indicating that lipid particles may function not only in sterol biosynthesis but may also help to regulate the flux of sterols between lipid particles and the plasma membrane (46). The presence of this cellular compartment indicates that *P. carinii* may be able to replenish sterols to sterol-depleted membranes via hydrolysis of lipid particle steryl esters and also to store and provide energy through the formation of and degradation of TAGs. Upon inhibition of the sterol pathway of *P. carinii* or under conditions of nutrient deprivation, the organism may be able to control the sterol composition of its membranes, as well as to provide energy to maintain cellular processes such as membrane biogenesis and sterol biosynthesis. Consequently, a determination of the contents of *P. carinii* lipid particles may help to elucidate more about the *P. carinii* sterol biosynthetic pathway, but this may be a formidable task due to the lack of an in vitro culture system. Our attempts to isolate sufficient quantities of lipid particles from *P. carinii* were severely hindered for this reason, since the isolation procedure required a minimum of 1 liter of late-log-phase *S. cerevisiae* to isolate sufficient quantities of lipid particles. Despite this, the observation that

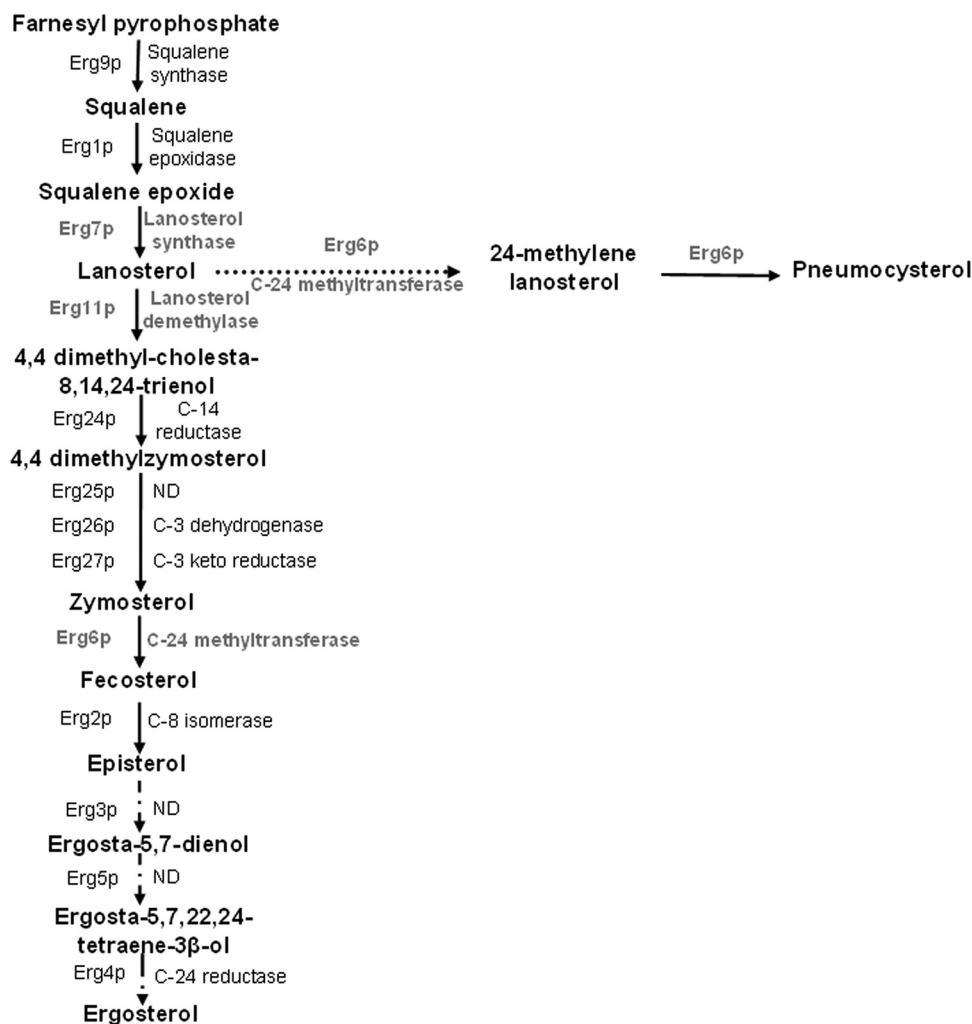


FIG. 7. Putative *P. carinii* sterol pathway. A putative sterol biosynthetic pathway indicating genes that have been cloned and functionally characterized (gray, boldface) and genes that have not been detected in analyses (ND) is represented. Other genes listed include those where either genomic or cDNA sequences have been identified by the *Pneumocystis* genome project (<http://pgp.cchmc.org/>), but the genes have not been characterized. However, the sterol products of these reactions have been identified in previous analyses (15). Two post-lanosterol pathways are proposed for *P. carinii*, one leading the formation of pneumocystol (22), as indicated by the dotted arrow, and another leading to the formation of episterol. Hatched arrows indicate steps that have not been determined due to the lack of detection of the genes involved.

P. carinii contains lipid particles is novel, and the localization of PcErg7p to lipid particles may indicate that other sterol biosynthetic enzymes such as Erg6p and Erg1p may be localized there as well.

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