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Sterol biosynthesis and sterol uptake in the fungal pathogen *Pneumocystis carinii*

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by

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Abstract

Fungi in the genus *Pneumocystis* are the cause of a potentially life threatening pneumonia, *Pneumocystis* pneumonia (PCP). The understanding of the lifecycle, metabolism, and drug development has been hindered due to a lack of a long term *in vitro* culture system. Unlike most other fungi, members of the genus *Pneumocystis* do not appear to synthesize the major fungal sterol, ergosterol. However, genome scans and *in vitro* assays suggest the presence of functional genes involved in a sterol pathway. One of the goals of this work was to characterize the *P. carinii* sterol enzyme, lanosterol synthase (Erg7p), an essential enzyme of the sterol pathway. The activity of *P. carinii* Erg7p was assessed by heterologous expression of *P. carinii* Erg7p in a *Saccharomyces cerevisiae* Erg7p null mutant. Growth rates and lanosterol production were similar between *S. cerevisiae* expressing the *P. carinii* enzyme and *S. cerevisiae* expressing its own Erg7p under the same conditions, indicating that not only does *P. carinii* produce a functional Erg7p, but also that the enzyme functionally complements the *S. cerevisiae* enzyme. Western blotting and fluorescent localization studies revealed that *P. carinii* Erg7p localizes to lipid particles in *S. cerevisiae* as does *S. cerevisiae* Erg7p. A novel finding of these studies, was that *P. carinii* contains lipid particles, and that *P. carinii* Erg7p localizes to lipid particles in *P. carinii*. These studies indicate that *P. carinii* Erg7p functions similar to the *S. cerevisiae* enzyme, and may perform a similar function in *P. carinii*.

Biochemical analyses of sterols within the membranes of *P. carinii* have shown that it utilizes cholesterol rather than ergosterol as its bulk sterol. However, *P. carinii* does not appear to synthesize cholesterol from a *de novo* pathway, but rather scavenges

exogenous sterols from its mammalian host. *S. cerevisiae* is induced to undergo sterol scavenging under anaerobic conditions. Consequently, another goal of this work was to provide information on the effect of O_2 on sterol biosynthesis and sterol scavenging by *P. carinii*. ATP measurements revealed that the viability of *P. carinii* is severely decreased when maintained under hypoxic conditions, and this decrease correlated with an increase in drug susceptibility. We show that uptake of exogenous cholesterol by *P. carinii* occurred under normal O_2 tensions, indicating that sterol scavenging is not limited to anaerobic conditions. Microarray analysis indicated that hypoxic maintenance of *P. carinii* resulted in decreased transcription of several genes involved in sterol and lipid biosynthesis suggesting that while hypoxic conditions down-regulated genes involved in sterol biosynthesis, down-regulation of sterol biosynthesis is not a requirement for sterol scavenging in *P. carinii*. The ability of *P. carinii* to scavenge exogenous sterols under normal O_2 tensions at which the sterol pathway is unaffected provides evidence that sterol scavenging may be the primary means that *P. carinii* utilizes to obtain its sterols.

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Table of Abbreviations:

PCP	*Pneumocystis* pneumonia
HAART	Highly Active Antiretroviral Therapy
TMP-SMX	Trimethoprim Sulfamethoxazole
DHPS	Dihydropteroate Synthase
AIDS	Acquired Immune Deficiency Syndrome
HIV	Human Immunodeficiency Virus
CDC	Centers for Disease Control
rRNA	ribosomal Ribonucleic Acid
ICBN	International Code of Nomenclature
MSG	Major Surface Glycoprotein
PFGE	Pulse Field Gel Electrophoresis
SREBP	Sterol Regulatory Element Binding Protein
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
UPC2	Uptake Control Protein
ECM22	Extracellular Mutant
SRE	Sterol Response Element
ER	Endoplasmic Reticulum
ERG11	Lanosterol 14α demethylase
ERG6	Sterol C-24 methyltransferase
ERG7	Lanosterol synthase/oxidosqualene cyclase
ERG3	Sterol C-5 Desaturase

ERG1	Squalene epoxidase
ACAT	Acyl-Coenzyme Cholesterol Acylransferase
HAP1	Heme Activator Protein
ROX1	Repressor of Hypoxic Genes
SUT1	Sterol Uptake Gene
ARS1	Anaerobic Response Element
DAN1	Delayed Anaerobic Gene
CYC8	Cytochrome C
TUP1	Deoxythymidine Monophosphate Uptake Gene
AUS1	ABC protein involved in Uptake of Sterols
NMR	Nuclear Magnetic Resonance
DAVID	Database for Annotation, Visualization and Integrated Discovery
ER	Endoplasmic reticulum
ERAD	ER Associated Degradation

Chapter 1: Introduction

Members of the genus *Pneumocystis* are opportunistic fungi capable of causing a lethal pneumonia in mammalian hosts. *Pneumocystis* colonization of immunocompetent hosts appears to have minimal clinical consequences, but colonization in hosts with debilitated or compromised immune systems may result in the development of *Pneumocystis* pneumonia (PCP) (139,210). Prior to the AIDS epidemic in the early 1980s, PCP was a rare occurrence seen only in malnourished children, transplant recipients, cancer patients and those with immune deficiencies (65). Today, despite highly active anti-retroviral therapy (HAART), PCP is still one of the most common AIDS defining illness (73), making it a focus of research efforts designed to both control and eradicate the pathogen.

Numerous insights have been gained about the basic biology of *Pneumocystis* organisms, but the inability to grow the pathogen outside the mammalian lung has hindered advancements in the field of *Pneumocystis* research. The standard anti-fungal therapies are the azole class of anti-fungals and amphotericin B, which target the fungal sterol pathway or ergosterol, the end product of sterol biosynthesis in fungal cells. *Pneumocystis* spp. are resistant to these drugs, and the drugs used for the treatment of PCP include pentamidine, atovaquone, and a combination of trimethoprim and sulphamethoxazole (TMP-SMX) (70,97,98) which are typically used to treat bacterial and protozoal infections. Growing evidence suggests that resistance to sulfamethoxazole in *Pneumocystis jirovecii* may be occurring in human populations worldwide, due to mutations in the dihydropteroate synthase (DHPS) gene (29,55,86). Prophylactic treatment with sulfa has been causally linked to these mutations (175). The reasons

behind the inability of the organism to grow in culture has not been determined, but its resistance to standard antifungal drugs has been attributed in part to the lack of detectable ergosterol within the membranes of *Pneumocystis* (69). It is thought that *Pneumocystis* scavenges cholesterol from its mammalian host and incorporates it into its own membranes, making cholesterol rather than ergosterol the bulk sterol of *Pneumocystis* (215). Despite the lack of the ergosterol and the putative scavenging ability of *Pneumocystis*, non-host derived sterols have been isolated from *Pneumocystis carinii* (69,105,113,114) indicating that the organism may have a functional sterol pathway. The mechanisms of sterol uptake in *Pneumocystis* have not been determined, but in yeast, sterol scavenging occurs under anaerobic conditions (6). Conversely, in some filamentous fungi, sterol scavenging can occur under normal oxygen tensions (216). The presence of a putative functional sterol pathway in *Pneumocystis* suggests that novel anti-*Pneumocystis* drug targets may exist, but a better understanding of the *Pneumocystis* sterol pathway and its sterol scavenging abilities is necessary for adequate drug design. As a result, the goal of this project is to extend our knowledge of the basic biology of *P. carinii* by characterizing *P. carinii* lanosterol synthase, the first sterol producing enzyme of the *P. carinii* sterol pathway, and probing the effect of oxygen on sterol biosynthesis and scavenging mechanisms.

Historical Background

The first description of *P. carinii* occurred in 1909 when the Brazilian scientist Carlos Chagas observed cyst-like structures in the lungs of guinea pigs infected with *Trypanosoma cruzi*, and subsequently identified them as part of the life cycle of the

trypanosome (32). The following year, Antonio Carini observed similar structures on slides containing lung sections from *T. cruzi* infected rats (31). After observing a lack of these structures in rats with extensive trypanosomiasis, Carini was uncertain whether theses cystic forms were part of the trypanosome lifecycle, and sent his data along with samples to the Pasteur Institute for further study (87). At the institute, Pierre and Mme Delanoe identified the organism in the lungs of rats without trypansomes and established that the organisms observed by Chagas and Carini were a species altogether distinct from *T. cruzi* (53). The Delanoes named this new species *Pneumocystis carinii.* The genus name "*Pneumocystis*" was given due to the propensity of the organism for the lung and its cyst-like morphology, and the species name "*carinii"* was given in honor of Antonio Carini who provided the samples for study (53). After publication of the Delanoes paper describing *P. carinii*, both Chagas and Carini recanted their earlier observations, and accepted *P. carinii* as a unique species (87).

The organism was observed in the lungs of several mammalian species (8,35,150), but remained in relative obscurity until epidemics of an atypical interstitial plasma cell pneumonia began to occur in the 1940s in overcrowded European orphanages (87). The disease appeared limited to premature infants and to children who were born after full term fetal development, but subsequently became malnourished (65). In 1942 van der Meer and Brug identified *P. carinii* as the causative agent of the atypical pneumonia in infants (197). This finding went largely unnoticed until 1951 when Czechoslovakian researcher Vanek observed *Pneumocystis* organisms in alveolar exudate of infants dying from pneumonia (199). Vanek acknowledged the previous findings of van der Meer and Brug, and subsequent studies by Vanek, Jirovec, Lukes (200)

unambiguously showed that the organisms originally thought to be to innocuous were a major cause of disease and mortality in infants. The first description of *Pneumocystis* in adults was made by Hamperl in 1956 (75), and subsequent observations made in the following years (81,158) displaced the idea that the organism was a purely pediatric pathogen. The first documented case of PCP in the United Stated occurred in 1956 (48) and a publication by Gajdusek (65) the following year chronicling the epidemics in European orphanages formally introduced both the pathogen and the disease to American doctors and researchers.

In 1958 Ivady and Paldy used pentamidine, a drug commonly used to treat African trypanosomiasis, to treat PCP, and they observed that patients recovered from the disease (101). Consequently, pentamidine became the standard therapy for treatment of PCP. The epidemics of PCP in Europe declined after World War II (87), but the disease was identified in a new subset of patients soon after. Until the 1960s, the disease was largely confined to children, but the disease began to occur in the United States in both children and adults with cancer and underlying immune deficiencies (87). The link between immunosuppressive anti-cancer therapies and PCP was made upon evaluation of outbreaks of PCP in cancer treatment facilities (93,103,149). As organ transplantation became more common, PCP cases were observed in recipients who were immunosuppressed to prevent organ rejection (174,176). In 1974 Walzer took advantage of the link between pentamidine and PCP to provide further insight into disease susceptibility (210). Since pentamidine was the only drug available for treatment of the pneumonia, and the only source for obtaining the drug was the Centers for Disease Control (CDC), Walzer accessed a database containing information on requests for the

drug. He observed that most requests for pentamidine were made on behalf of patients with various forms of cancer, primary immune deficiencies, and organ recipients establishing that PCP was a disease predominately found in immunosuppressed populations (210). This same year, using Frenkel's rat model of PCP (64), Hughes observed that a combination of trimethoprim and sulfamethoxazole was highly effective against the pneumonia (98). Later in clinical trials, the drug combination was shown be equally effective against PCP in humans but with fewer adverse effects than pentamidine (94,95). In 1978, trimethoprim-sulfamethoxazole (TMP-SMX) was approved by the FDA for treatment of PCP.

In the late 1970s and in 1980 several men were identified as having PCP with no known risk factors for the pneumonia, and because the predisposing factor for PCP infection was immune suppression, physicians were prompted to search for an underlying immune deficiency. In 1982 the Acquired Immune Deficiency Syndrome (AIDS) was defined (1,122), and it was observed that PCP occurred in nearly half of those diagnosed with the disease (102). The following year, the Human Immunodeficiency Virus (HIV) was discovered and identified as the cause of AIDS (15). Consequently, research into the once rare pathogen intensified, and information about *Pneumocystis* increased significantly. The rise in PCP susceptible individuals, the adverse effects associated with pentamidine use, and sulfonamide-sensitive patients warranted development of new drug therapy for PCP. As a result, since the identification of HIV and AIDS, several drugs have been found to be effective against PCP including aerosolized pentamidine (129), dapsone (89,130,144), atovaquone (90,97) and clindamycin and primaquine (22,23,152). However, pentamidine can be toxic to some patients (13), dapsone is a sulfa containing

drug that has been associated with allergic response in some patients (92), strains of *P. jiroveci* are resistant to atovaquone (121), and though better tolerated, both clindamycin and primaquine have a limited range of utility as it is only prescribed for mild to moderate PCP (21).Thus despite newer drugs available for anti-PCP therapy, these drugs are associated with many adverse affects and indicates that there is still a great need for new anti-PCP drug therapy.

The application of molecular approaches for the study of *Pneumocystis* has greatly increased the knowledge of the organism. In 1988 Wakefield cloned and amplified *P. carinii* DNA using the polymerase chain reaction (PCR) (207,208). Because these organisms are intractable to *in vitro* culture, this made a great impact in the field of *Pneumocystis*, as it facilitated the detection of *Pneumocystis* organisms in both clinical and research settings (87). Another important advance in the *Pneumocystis* knowledge base came through sequence analysis and subsequent phylogenetic re-classification of members of the genus *Pneumocystis*. Placement of *Pneumocystis* in the protist kingdom had been questioned throughout the decades (203), but the initial classification of the organism as a protozoan by Chagas remained unchallenged. In 1988, sequencing of the *P. carinii* gene encoding the small subunit ribosomal RNA (16S) gene, and direct sequencing of *P. carinii* rRNA in 1989 revealed that the organism was more similar to fungal sequences than to any of the available protozoan species (57,180). Comparison of the *P. carinii* small ribosomal subunit rRNA with 38 other fungal rRNA sequences placed *P. carinii* in the fungal kingdom on the basal branch between the ascomycetes and the basidiomycetes (83,198).

The Organism

While genetic analysis clearly supports a fungal nature for *P. carinii,* it is considered an atypical fungus possessing several distinctive characteristics not found in other fungi to date. These include the lack of detectable ergosterol, the major fungal sterol (69), the lack of efficacy of clinically available anti-fungal drugs such as amphotericin B (41) and azoles (106,146), and the presence of no more than two copies of the gene encoding the 16S-like rRNA, whereas most fungi contain hundreds of copies (71). While knowledge of the organism has increased drastically since the HIV/AIDS era of the 1980s and 1990s, the acquisition of information on the basic biology of organism has been severely impeded due to the lack of a long term *in vitro* culture method. As a result, researchers have had to rely on the animal models of *Pneumocystis* infection (46) to study and characterize the organism. This section highlights information obtained as a result of bioinformatics, molecular, and biochemical analysis using short term *in vitro* maintenance of the organism (33), animal models, and heterologous expression systems.

Pneumocystis species

The definitive classification of *Pneumocystis* as a fungus came after sequencing the rDNA from organisms obtained from the lungs of rats (57,179), and since then, *Pneumocystis* have been found in a wide variety of mammalian species (162-166). Molecular evidence has shown that most mammalian species harbor at least one species of *Pneumocystis* (40). Comparison of nuclear small subunit RNA (srRNA), mitochondrial large ribosomal subunit RNA, and thymidylate synthase sequences among *Pneumocystis* derived from rat, human, mouse, ferret, and pig revealed differences that were similar to

those seen between different yeast species (115). Pulse Field Gel Electrophoresis (PFGE) of *Pneumocystis* chromosomes isolated from infected rat, mouse, human, and ferret lungs revealed a diversity of karyotypes among populations of *Pneumocystis* isolated from different hosts (178,213). These analyses revealed variations in the size and numbers of *Pneumocystis* chromosomes and in the sizes of the genomes (40,178,213). Using the same technique, it was found that two *Pneumocystis* species could co-infect rats (42,47). Both species were identified within the same rat colony, and both have been found to co-exist in the lungs of the same host (42). To date, co-infections with different species have not been observed in other mammals.

Frenkel made the first distinction between the rat species of *Pneumocystis* and the species infecting humans by naming the human species, *Pneumocytis jiroveci* in 1976 (62). At the 3rd International Workshop on *Pneumocystis* in 1994, it was proposed that the organisms be given a tripartite name based on the host of origin and in accordance with the International Botanical Code of Nomenclature (IBCN) for 'physiological variants' (187). Some of the names given were: *Pneumocystis carinii* f. sp. *carinii* for the *Pneumocystis* species found in most laboratory rats, *Pneumocystis carinii* f. sp. *ratti* for the second most common rat derived *Pneumocystis* species, *Pneumocystis carinii* f. sp. *muris* for the species infecting mice, *Pneumocystis carinii* f. sp *oryctolagi* for the species infecting rabbits, and *Pneumocystis carinii* f. sp. *hominis*, for the species infecting humans. Later a binomial naming system was adopted for naming *Pneumocystis* species, and in 1999 the first bionomial names were published by Frenkel (63). In his publication he designated the two former species *Pneumocystis carinii* f. sp. *hominis* and *Pneumocystis carinii* f. s*p. carinii* as *Pneumocystis jiroveci*, and *Pneumocystis carinii*

respectively. In 2006, Redhead validated Frenkel's earlier work, which did not use the ICBN guidelines for naming and typifications, and also corrected the spelling of *Pneumocystis jiroveci* to *Pneumocystis jirovecii* (153). Three other species of *Pneumocystis* have been formally named using the binomial system and these include *Pneumocystis wakefieldiae* (43) formerly named *Pneumocystis carinii* f. sp. *ratti*, *Pneumocystis murina* (117) formerly named *Pneumocystis carinii* f. sp. *muris*, and *Pneumocystis oryctolagi* formerly named *Pneumocystis carinii* f. sp. *oryctolagi* (51).

Morphology and Life Cycle of *Pneumocystis*

The complete life cycle of *Pneumocystis* is currently unknown, but the organism appears to replicate both sexually (140) and asexually by binary fission (Figure 1) (30,155). Sexual reproduction is thought to be mediated by mating types resulting in the formation of an ascus containing eight ascospores (172,189). Three morphological forms of *Pneumocystis* have been described: The trophic form, sporocyte, and the ascus. In the *Pneumocystis carinii*-infected lung, trophic forms outnumber ascus forms in a ratio of about ten to one (205). An environmental form of *Pneumocystis* has not been found, and with the lack of an *in vitro* culture method, there is no method to observe the morphological changes that take place during the life cycle of *Pneumocystis*. As a result, microscopic evaluation of organisms obtained from *Pneumocystis* infected lungs have provided the only information about these forms of the organism (38).

Trophic forms range in size from 1.5-5.0μm and can be divided into two categories: small and large (40). The smaller form is thought to be the product of spore release from an ascus and is the youngest of all morphological forms of *Pneumocystis*

(39,50). The larger form is thought to be the vegetative form of *Pneumocystis*, and is thought to reproduce by binary fission (Figure 1A) (30). Trophic forms are bound by two membranes which are separated by a thin electron-lucent space (50). The outermost membrane is embedded within a dense matrix that contains polysaccharides and the major surface antigen of *Pneumocystis*, [the major surface glycoproteins (MSG)] (49), and the inner membrane is the plasma membrane.

Conjugation of haploid trophic forms of opposite mating types is thought to result in the formation of the sporocyte form of *Pneumocystis* (37). Mating between two trophic forms results in a diploid zygote which proceeds through meiosis to produce four haploid nuclei that mitotically divide to yield eight nuclei (Figure 1B) (37,39). Maturation of the sporocyte into the ascus form is concomitant with a thickening of the cell wall via the formation of an electron-lucent middle layer (50). The cell wall of the sporocyte is composed of three layers, with the outermost electron dense layer embedded within a glycocalyx that is composed of polysaccharides and MSG, similar to that of the outer layer of the trophic form. The middle electron-lucent layer is composed of glucan while the inner membrane is composed of sterols (38,50).

The ascus (often referred to as a "cyst") is thought to be the final stage in the life cycle of *Pneumocystis*. The ascus form is spherical, 5-8μm in diameter, and contains eight ascospores (39). The spores contain a single nucleus with a double membrane, separated by a thin layer of electron-lucent space, similar to that seen in the trophic forms (50). Unlike the trophic form, the ascus form has an abundance of glucan within its cell wall (39). The cell wall of the ascus form is similar to the sporocyte cell wall, however the mature ascus contains a thickened portion that protrudes inward that is thought to be

part of the process by which spores are released (50). This is supported by the identification of a foramen-like structure in sections of the protrusion (220), and single pores within the cell walls of empty *Pneumocystis* asci, both of which may serve as exit points for the spores (100). The release of spores from the ascus results in eight small trophic forms which will ultimately perpetuate the life cycle of *Pneumocystis* (40).

Pneumocystis Major Surface Glycoprotein and Transmission

The surface of all *Pneumocystis* organisms is covered with an electron dense glycocalyx composed of heavily glycosylated, highly immunogenic proteins that have been termed the Major Surface Glycoproteins (MSG) of *Pneumocystis* (67). The genome of *P. carinii* contains approximately 73MSG genes (119) all of which are localized to the ends of chromosomes (184,194). MSGs range in size between 95 and 120 kilodaltons, and sequence analysis indicates that isoforms of MSG differ by an average of 19% (118,119). Despite the large number of MSG isoforms within the genome of *P. carinii*, several lines of evidence indicate that expression of MSG is limited to a single MSG per organism at any given time (177). Transcription of MSG occurs as result of proximity to a unique telomeric site within the genome called the expression site (56,184,186), and only the MSG located at the expression site is expressed at a given time (7). The expression site contains the Upstream Conserved Sequence (UCS), which is a sequence that is found at the beginning of all MSG mRNAs (56,206). The start codon for MSG mRNA is located in the sequence transcribed from the UCS (56,206). Thus, translation of MSG begins with the UCS and results in the production of a precursor MSG that undergoes further processing in the ER where it is glycosylated and proteolytically

cleaved (183). The UCS contains a signal sequence targeting the precursor protein to the ER, but is likely removed in the ER, as the UCS is not present on surface MSG (183).

A large variety of MSGs have been localized to the expression site, suggesting the possibility that recombination may play a role in the installation of MSG at the UCS (116,184,185). A site-specific recombination event involving the Conserved Recombination Junction Element (CRJE) has been implicated in the process of MSG translocation to the UCS (206). The CRJE is located at the expression site between the UCS and the expressed MSG, and at the beginning of MSG genes that are not expressed (donor MSGs) (184,206). Localization and conservation of CRJE suggests that it may function as a target of a site-specific event, such as a double-strand break that would increase recombination between the expression site and donor MSG genes (119). Repair of the double strand break would result in an alteration of the MSG located at the UCS and alter MSG surface expression.

Expression of *P. carinii* MSG results in the induction of both humoral and cell mediated immune responses to the pathogen (173). Thus switching of surface MSG by recombination may be a method used by the pathogen to evade the host immune system. "Antigenic variation" has been implicated as a means to circumvent the immune response in other pathogenic microbes (16), and the structure and expression of the MSG gene family in *Pneumocystis* indicates that it can function to generate surface antigen variants at a frequency that exceeds the occurrence of random mutation events. In addition to its putative role in immune evasion, MSGs have been shown to interact with host proteins and mediate attachment to host cells and also participate in binding to alveolar cells and macrophages (59,136,137,151).

Pneumocystis proliferate inside the lungs of mammalian hosts where they attach to type I alveolar epithelial cells (218). Attachment to type I cells occurs predominately with trophic forms of *Pneumocystis* (4,17,52), and is mediated in part by fibronectin-binding integrins on lung epithelial cells (4,151) and fibronectin-containing domains within the *Pneumocystis* MSG (151). Attachment of *Pneumocystis* appears to be required for initiation of infection (19,20), and transmission of *Pneumocystis* occurs solely between individuals within the same mammalian species (5,66). Neither the infectious form of the organism nor an environmental reservoir for *Pneumocystis* has been identified (40), but studies by Walzer and Hughes have indicated that *Pneumocystis* is transmitted through an airborne route (88,211). In these studies, rats and mice demonstrated to be free of *Pneumocystis* were found to acquire PCP after being housed either in separate cages within the same room, or after being housed in the same cage with rats or mice infected with *Pneumocystis* (88,211). Several other studies (142,201,202) have indicated that *Pneumocystis* organisms are transmitted from host to host, and it has been established that as few as ten organisms are needed to transmit infection between hosts (44).

***Pneumocystis* and PCP**

Fungi in the genus *Pneumocystis* are the causative agents of PCP, and while *Pneumocystis* organisms have been isolated from a large number of various mammalian species, PCP is most commonly found in those with depressed immune systems (210). Consequently, PCP is limited mainly to specific patient groups including those with AIDS and those having received organ transplants, and chemotherapy (210). Symptoms

of PCP include: progressive dyspnea, nonproductive cough, and low-grade fever (188), however, these symptoms differ between AIDS and non-AIDS patients. Patients with AIDS and PCP have a much higher burden of *Pneumocystis* and fewer neutrophils within their lungs than do patients with PCP without AIDS (134). The large yield of *Pneumocystis* organisms from patients with AIDS allow for an easier diagnosis of PCP from induced sputum and bronchoalveolar-lavage samples (188), and the decreased numbers of neutrophils in these patients result in better oxygenation as a result of decreased inflammation (135). However, those that have PCP in the absence of AIDS are at an increased risk of lung damage due to a massive influx of inflammatory cells (135). Thus, the severity of PCP is often correlated with the extent of neutrophilic invasion where higher levels of neutrophils result in inflammation leading to extensive lung damage, impaired gas exchange and respiratory failure (188). Death as result of PCP is largely due to the detrimental inflammation caused by the presence of *Pneumocystis* organisms in the lung rather than organism burden itself (135).

The host immune response to *Pneumocystis* is mediated by several immune effector cells with CD4+ T cells being critical to this response. CD4+ cell depletion has been shown to predispose animal models to PCP (157,168), and loss of CD4+ cells during HIV infection results in the onset of AIDS in which PCP is the most common AIDS defining illness, and a common opportunistic infection associated with AIDS (161). CD4+ cells recruit and activate other immune effector cells including macrophages to the lung during PCP infection. Alveolar macrophages are the main effector cells responsible for killing *Pneumocystis* organisms. Macrophage activation in response to *Pneumocystis* can be mediated by the interaction between β-glucan on the surface of

Pneumocystis cysts and the dectin 1 receptor on marophages (159). Activated macrophages take up the organisms and incorporate them into phagolysosomes for subsequent degradation (133). Loss of macrophage function results in reduced clearance of *Pneumocystis* in both human subjects and animal models of PCP (124,133). Activated macrophages produce inflammatory cytokines, chemokines, reactive oxygen species and other metabolites in response to phagocytosis of *Pneumocystis* (133), and though these immune cells aid in eliminating the pathogen, they also facilitate a potentially life-threatening inflammatory cascade in the alveolar environment.

A chest radiograph is often used to evaluate patients suspected as having PCP (85), but a definitive diagnosis of PCP is made after microscopic identification of the organisms obtained from induced sputum, BAL samples, or lung samples (85,188). In these samples *Pneumocystis* organisms can be identified with a variety of stains. The cysts or asci can be visualized with toluidine blue, methenamine silver and cresyl echt violet while trophic forms can be identified by staining with a modified Papanicolaou, a rapid variant of the Wright–Giemsa stain, or Gram–Weigert stain (39,188). The trophic forms cannot be visualized with stains that rely on cyst wall carbohydrates, but cysts are visible when stained with preparations to view trophic forms, such as Diff-quick (123). The treatment of choice for PCP is TMP-SMX (95), but in patients with allergies to sulfa containing drugs or who fail to resolve PCP after taking TMP-SMX, pentamidine isethionate may be used for treatment of the pneumonia (87,94). Alternate treatments for PCP infection include dapsone (89,143) and atovaquone (91,96). Corticosteriods have been used for patients with severe PCP due to their ability to decrease inflammation resulting in subsequent improvement in lung function (145,148). Patients at an increased

risk for developing PCP are often put on prophylaxis during their period of risk, and these treatments include the same drugs used to treat the disease (188).

Autophagy in Fungal Organisms

The detection and removal of damaged or nonessential organelles and cytoplasmic content is a vital process for maintenance of cellular homeostasis. In eukaryotic cells, two major degradation pathways exist to rid the cell of unnecessary contents: the ubiquitin proteasome system and autophagy (125). The ubiquitin proteasome system is responsible for the degradation of specific short-lived proteins, while autophagy is involved in the bulk degradation of long-lived cytosolic proteins and organelles (219). Autophagy aids in maintaining normal cellular homeostasis by helping to maintain the balance between synthesis, degradation and recycling of cellular material. Additionally, autophagy is induced as an adaptive response to conditions of oxygen deprivation, cellular stress, and nutrient deprivation (79). The process of autophagy involves the use of double-membrane autophagosomes to sequester organelles and cytosolic material and deliver them to vacuoles for degradation. In the event of starvation, the resulting macromolecules can be recycled for re-use, making autophagy a major mechanism used by starving cells to regain homeostasis (219).

In contrast to the non-specific bulk degradation of organelles that occurs during autophagy, specific cytoplasmic bulk degradation occurs through the cytoplasm to vacuole pathway (Cvt). In addition, different terms have been used to describe autophagy according to the cargo contained within the autophagosome. Autophagic degradation of the mitochondria has been termed mitophagy, while autophagy occurring in ribosomes,

peroxisomes, and the ER, have been termed ribophagy, pexophagy, and reticulophagy, respectively (79). The Cvt pathway is mechanistically similar to autophagy, and many of the genes involved in this pathway overlap with autophagic genes (12,99,127). More than 30 autophagy related genes have been identified in the *S. cerevisiae* genome (170), and of these, 17 of them form the core of the autophagy machinery and participate in all types of autophagy (125). Currently, there has been no research into autophagy or autophagic processes in *Pneumocystis*, however, the studies described in this work identify several genes with homology to autophagy related genes in the genome of *P. carinii* and suggest that autophagy may be induced in *P. carinii* as a result of either hypoxic maintenance or ER stress.

Sterol Biosynthesis and Regulation: Fungal vs. Mammalian Cells

Sterols are vital components of all eukaryotic cell membranes, and are essential for cell growth and viability. Ergosterol, the major sterol found in fungal cell membranes, functions in the same capacity as cholesterol, the major sterol found in mammalian cell membranes (80). Sterols have many roles in eukaryotic membranes including: establishing appropriate membrane fluidity (128), regulating membrane bound enzymes (34), and maintaining membrane permeability (14). The sterol biosynthetic pathway in fungi and mammals is strikingly similar, but differences in the later steps of both pathways result in two structurally different molecules. Both ergosterol and cholesterol (Figure 2) have a –OH group on C-3 of the sterol ring and a double bond at C-5 of the ring. However, the synthesis of ergosterol has three additional steps resulting in two additional double bonds at C-7 and C-22 and a methyl group at C-24 of the ergosterol

side chain. These structural differences make cholesterol and ergosterol remarkably suited for fulfilling both the cellular and membrane requirements of the organism in which they are most abundant (82).

All enzymes of the post-squalene or committed sterol pathway are conserved between mammals and fungal organisms until after the formation of zymosterol. After the formation of zymosterol, the ergosterol pathway proceeds in a linear fashion to the production of ergosterol (Figure 3), but the cholesterol pathway proceeds to cholesterol via two distinct routes (1) through demosterol or (2) through lathosterol (Figure 4). These divergent routes to sterol production result in sterols that are uniquely suited for mammalian and fungal cells. In mammalian cell membranes, cholesterol is arranged in a bilayer conformation allowing external forces to be distributed more efficiently (84), while in fungal cell membranes, ergosterol is arranged in a monolayer conformation, causing the membrane to be more rigid, and less flexible than mammalian cell membranes (84). These differences can be attributed to the lack of a cell wall in mammalian cells and the presence of one in fungal cells. The cell wall is located outside the cell membrane and provides structural integrity and protection from external forces. The lack of a cell wall surrounding mammalian cells allows mammalian cell membranes to be more flexible than fungal cells, and the divergence of the sterol pathways contribute to the nature of these two membranes. In ergosterol, two additional double bonds formed by the actions of the C-5 desaturase and C-22 desaturase enzymes (9,171) contribute to the rigidity of fungal cell membranes whereas the cholesterol molecule lacks these additional modifications providing the mammalian cell membrane with more flexibility (84).

In mammalian cells, cholesterol can be obtained either from exogenous sources or synthesized *de novo*, and the rate of *de novo* cholesterol synthesis is directly correlated with exogenous cholesterol intake (61,191). The mechanisms by which a mammalian cell detects changes in cholesterol levels and how these changes directly influence the transcription of genes involved in cholesterol biosynthesis have been elucidated. HMG CoA reductase catalyzes the first committed step of the mevalonate pathway from which cholesterol and other isoprenoids are produced, and is the rate-limiting enzyme of sterol biosynthesis (72). During periods of high intracellular sterol concentration, the activity of HMG Co-A reductase is low, and the activity of the enzyme is high during periods of low intracellular sterol concentration (147). The proteins responsible for regulation of HMG CoA reductase in mammals are Sterol Regulatory Element Binding Proteins (SREBPs). SREBPs are transcription factors that regulate genes involved in the synthesis and uptake of cholesterol and fatty acids (27). Genes regulated by SREBPs include, HMG CoA synthase, low density lipoprotein receptor (LDLR) and squalene synthase (27,72,74). When cholesterol is abundant in mammalian cells, SREBPs remain in the endoplasmic reticulum (ER) where they are associated with SREBP Activating Protein (SCAP) and the ER retention protein Insig (217). High levels of cholesterol facilitate SREBP ER retention and the degradation of HMG CoA reductase in a ubiquitin-mediated process (167).

The regulation of ergosterol biosynthesis in fungi has not been as extensively studied as cholesterol biosynthesis regulation. Like the genes involved in cholesterol biosynthesis, the genes encoding enzymes for ergosterol biosynthesis are transcriptionally regulated in response to the need for ergosterol (54). Within recent years, two SREBPs

have been identified in *S. cerevisiae* that are involved in the regulation of sterol synthesis in yeast. *Upc2p* and *Ecm22p* are members of the $Zn[II]_2$-Cys6 binuclear cluster of family of fungal transcription factors (190) that bind to the promoters of ergosterol biosynthetic (*ERG*) genes and activate these genes upon sterol depletion (204). *Upc2* and *Ecm22p* are known to bind to a 7-bp Sterol Response Element (SRE) in the promoters of sterol C-8 isomerase (ERG2) and C-5 sterol desaturase (ERG3), but the presence of the SRE in several other promoters of genes involved in sterol biosynthesis indicates that they may be able to regulate transcription of other genes along the ergosterol pathway as well (204). Thus it has been speculated that Upc2p and Ecm22p may be the major transcriptional regulators of sterol-responsive genes in the sterol biosynthetic pathway of yeast (204).

Evidence for Sterol Pathway in *P. carinii*

Although ergosterol is the major sterol found in fungal cell membranes, biochemical analysis has shown that cholesterol, rather than ergosterol, is the main sterol found in *Pneumocystis* (60,69,107). Cholesterol accounts for up to 81% of the total sterols isolated from the lungs of *Pneumocystis* infected rats, and it has been postulated that most if not all the cholesterol is scavenged from the host (69). Conversely, one report speculates that *P. carinii* may synthesize cholesterol through a *de novo* pathway (222), but to date there is no definitive evidence to suggest that the organism contains all of the genes necessary to synthesize either cholesterol or ergosterol. Despite the lack of detectable ergosterol, *Pneumocystis* organisms contain putative gene homologs that are present in the sterol biosynthetic pathway of other fungi (45).

Several studies support the existence of a *de novo* sterol pathway in *P. carinii* (60,68,69,109). Sterols from *P. carinii* became radioactive after incubation with radioactive sterol precursors such as acetate, mevalonate, squalene, HMG-CoA, and isopentenyl diphosphate (58,60,109,182). As a result, the acetate mevalonate pathway was identified as the pathway by which *de novo* sterol synthesis occurs (58,60,181,182) This pathway leads to the formation of C_{28} and C_{29} Δ^7 sterols such as fungisterol and stigmast-7-en-3β-ol (60). To date these sterols have only been found in *T. cruzi* (132) and the plant pathogenic rust fungi of the class Uredinales (212). In addition to these rare sterols, the organism appears to synthesize its own unique sterols, including [(24*Z*)-ethylidenelanost-8-en-3β-ol] (pneumocysterol) (60,105,108,195) that may be necessary for survival. Because these sterols are synthesized *de novo* by the organism despite its apparent ability to scavenge available sterols, these sterols have been called "metabolic sterols" (78,108). Since these sterols appear to be unique to *Pneumocystis*, they may not only provide excellent drug targets (78), but may have potential as possible markers for PCP infection (105).

The lack of detectable ergosterol in *P. carinii* may explain why several classes of drugs specifically aimed at ergosterol and enzymes along the ergosterol biosynthetic pathway are not effective against the pathogen. Drugs such as Amphotericin B, a commonly used antifungal agent, bind directly to ergosterol and disrupt cellular permeability, but are not clinically effective against *Pneumocystis*. However, at sufficient concentrations of the drug, Amphotericin B will bind cholesterol and other membrane sterols, suggesting that Amphotericin B is effective against *Pneumocystis*, but only at levels that are toxic to host cell membranes (106). It was also observed both *in vitro* and

in vivo that six commonly used imidazoles, a class of drug that specifically targets the enzyme lanosterol demethylase (*Erg11* gene) of the ergosterol pathway, are also ineffective against *Pneumocystis* (18). Conversely, in an *in vitro* study of *P. carinii* sterol biosynthesis inhibitors, two proprietary imidazoles produced by GlaxoSmithKline (GR 40317A and GR 42539X) were shown to be effective against *P. carinii,* whereas the commonly prescribed imidazoles, such as fluconazole were ineffective against the pathogen (106). These data suggest that *P. carinii Erg11* may still be a viable drug target, and that newer drugs targeting the gene may reduce the viability of *Pneumocystis* organisms. Despite the lack of detectable ergosterol in *Pneumocystis*, several genes involved in sterol synthesis have been found within its genome (45), but to date, only three of these genes, *Erg6* (sterol C-24 methyl transferase), *Erg11* (lanosterol 14α demethylase), and *Erg7* (lanosterol synthase) have been the focus of investigation.

Pneumocystis carinii sterol enzyme characterization

Lanosterol synthase (Erg7p) Lanosterol synthase or *Erg7p* is the enzyme responsible for converting the last acyclic sterol precursor into lanosterol, the first cyclic sterol precursor. Loss of lanosterol synthase function results in nonviable yeast mutants, and Erg7p inhibitors reduced *P. carinii* viability (106). *P. carinii ERG7* was cloned and expressed in an *S. cerevisiae ERG7* mutant strain (141). *In silico* analysis of *P. carinii Erg7p* demonstrated that the enzyme retained amino acid residues that are known to be catalytically essential for the *S. cerevisiae* enzyme (141). Using ^{14}C labeled acetate, Milla *et al*. demonstrated that the sterol biosynthetic pathway of the *P. carinii ERG7* expressing

S. cerevisiae strain remained functional despite the lack of the wild type gene. The group found that after three hours, the bulk of [^{14}C] acetate had been incorporated into ergosterol, establishing that *P. carinii* lanosterol synthase was able to complement the *S. cerevisiae* enzyme.

In *Saccharomyces cerevisiae*, lanosterol synthase is localized to lipid particles which are intracellular neutral lipid stores and that are thought to participate in lipid biosynthesis and trafficking mechanisms (11). However, this study concluded that the *P. carinii* enzyme did not localize to lipid particles based on the presence of six putative transmembrane spanning domains which would make the enzyme ill suited for insertion into the lipid particle monolayer; the lack of *PcErg7p* activity in lipid particles of the yeast mutant strain expressing *PcErg7p;* and the lack of an 83kDa band in a Coomasie stained gel containing lipid particle proteins isolated from *PcErg7p* expressing yeast (141). This group was chiefly interested in localization of lanosterol synthase within lipid particles, thus no other functional studies were done to further characterize the enzyme or determine its cellular location.

Lanosterol 14α demethylase (*Erg11p)*

P. carinii lanosterol 14α-demethlyase (*Erg11p*), the enzyme responsible for removing a methyl group from C-14 of lanosterol and also the target of azole antifungal drugs, was cloned and expressed in an *S. cerevisiae ERG11* null mutant (146). Sequence analysis comparing the translated open reading frame of *P. carinii Erg11p* to other fungal *Erg11p* proteins revealed the presence of amino acid substitutions at positions 113 and 125 of the highly conserved substrate recognition site (146). These substitutions are also found in a

fluconazole resistant *Candida albicans* strain (10). Consequently, functional analysis of the *P. carinii ERG11* gene expressed in the *S. cerevisae ERG11* mutant revealed that *P. carinii Erg11p* required a 2.2-fold higher dose of voriconazole and a 3.5-fold higher dose of fluconazole than *S. cerevisiae Erg1p1* expressed under similar conditions to achieve a 50% reduction in growth (146). Based on these data, the group concluded that *P. carinii Erg11p* is inherently resistant to azole anti-fungals (146). However, this study utilized a high copy plasmid in their analyses and they did not assess the copy number of either the control plasmid containing *ScERG11* or the plasmid containing *PcERG11*. Therefore, it is difficult to determine whether the associated differences in drug susceptibility were attributed to differences in *ERG11* copy number between the two strains or the amino acid substitutions found in *PcErg11p.*

Sterol methyltransferase (*Erg6p)*

The *P. carinii* sterol C-24 methyl transferase gene (*ERG6*), which catalyzes the alkylation of C-24 of the sterol nucleus, was cloned and expressed in *E. coli* (111). During this study it was shown that *P. carinii Erg6p,* unlike other fungal *Erg6* enzymes that use the sterol metabolite zymosterol as a substrate, has a preference for lanosterol and 24-methylenelanosterol (110). As a result, this group speculated that lanosterol to 24-methylenelanosterol is the major post lanosterol pathway in *P. carinii.* This would indicate that lanosterol demethylation by *Erg11* occurs after C-24 alkylation by *Erg6* in *P. carinii,* and that substrates for *P. carinii Erg11* are 24-alkylsterols and not lanosterol (112). This is not an unlikely occurrence, as this alternate pathway was observed in the previously mentioned fluconazole resistant *C. albicans* strain (10).

Sterol uptake: Mammals vs Fungi

Though mammals are able to up-regulate *de novo* cholesterol synthesis via increased gene transcription, they also have mechanisms in place to selectively take up cholesterol to maintain cellular homeostasis, and decrease the amount of circulating plasma cholesterol (28,77). SREBPs play a major role in mediating sterol uptake by up-regulating the transcription of the low density lipoprotein receptor (LDLR) (27). The role of the LDLR is to transport cholesterol-carrying lipoprotein particles into the cell (26). The ligand of the LDLR is low density lipoprotein (LDL), the major carrier of cholesterol in the blood. LDL is responsible for cholesterol transport to peripheral tissues and thus the regulation of intracellular *de novo* cholesterol synthesis. The effects of LDL are mediated through its binding to the LDLR and uptake into cells. The apoB-100 component of LDL binds to the LDLR on the cell membrane (221), and the receptor-ligand complex is taken up into the cell via endocytosis (26). Endocytosis and invagination of the cell membrane containing LDLR and its ligand result in the formation of an endosome which then fuses with lysosomes (26). Enzymes within the lysosome free the receptor and allow it to circulate back to the cell surface to continue transporting LDL particles into the cells (26). Lysosomal lipases can hydrolyze cholesterol esters in LDL to release free cholesterol which can then be used for the creation of membranes in cells where cholesterol is lacking (26). Cholesterol can also be re-esterified by acyl-coenzyme cholesterol acylransferase (ACAT) to create an intracellular storage form of cholesterol to accommodate excess cholesterol for later use by the cell (25). At sufficient quantities

of intracellular cholesterol, transcription of the LDL receptor is inhibited by retention of SREBPs within ER membranes (160).

Many aspects of the regulatory pathway of ergosterol remain unknown, but what is known is that ergosterol biosynthetic genes are regulated in response to oxygen tension, making ergosterol biosynthesis an oxygen dependent process (6). When grown under aerobic conditions, yeast are able to synthesize sterols, and are unable to acquire exogenous sterols (156), a phenomenon known as aerobic sterol exclusion (131). While yeast are only able to take up exogenous sterols during anaerobic growth, some filamentous fungi like *Aspergillus fumigatus* are able to take up sterols under aerobic conditions (216). The molecular mechanisms behind aerobic sterol exclusion have not been elucidated, but heme has been implicated in the process. *S. cerevisiae* contains many genes that are repressed under aerobic conditions and are induced when the supply of oxygen is limited (131,223). Cells are able to sense oxygen availability through the levels of heme, which is produced in an oxygen dependent mechanism (223). Heme stimulates transcription of HMG CoA reductase via the *Hap1p* transcriptional activator, and both heme and *Hap1p* are involved in aerobic ergosterol biosynthesis via the regulation of *Erg11p* (193) and HMG CoA reductase (76,192). *Hap1p* is also responsible for aerobic induction and anaerobic repression of ROX1 (196), a well known repressor of hypoxic genes (138) that is activated upon expression of Hap1 in a heme-dependent mechanism (120).

Many genes involved in the later steps of ergosterol biosynthesis require molecular oxygen for catalysis, and as a result these enzymes are down-regulated as the supply of oxygen declines. Likewise, since heme production is dependent on the supply

of oxygen, heme-mediated *Rox1p* repression of hypoxic genes declines as oxygen levels decrease resulting in increased expression of nearly all *Rox1p* repressed genes (126). The up-regulation of hypoxic genes and down-regulation of ergosterol biosynthetic genes results in exogenous sterol up-take. Two genes have been implicated in sterol uptake during anaerobic conditions: UPC2 and SUT1. Though expressed aerobically, transcription of UPC2 is induced under anaerobic conditions (2), and upon induction *Upc2p* binds to the anaerobic response element (ARS1) in anaerobically expressed genes to induce transcription during hypoxia (2). *Sut1p*, in contrast to *Upc2p* does not bind directly to DNA, but rather binds to *Cyc8p* of the *Cyc8p-Tup1p* complex, which like *Rox1p* is an aerobic repressor of hypoxic genes, and relieves repression of hypoxic genes (154). Transcription of SUT1 is increased 9.6 fold under anaerobic conditions (169), and over-expression of SUT1 results in a 2.6 fold increase in sterol uptake under aerobic conditions (24). *Sut1p*, however, is unable to mediate sterol uptake unless both *Dan1p* and *Aus1p* are functionally expressed (3). AUS1 encodes a member of the ATP-binding-cassette family of transporters that is necessary for sterol uptake and that requires ATP to facilitate the uptake (214), and *Dan1p* is a cell wall mannoprotein that is up-regulated in response to SUT1 over-expression, and thus has been identified as a hypoxia regulated gene (3).

Evidence for Sterol Uptake in *P. carinii*

It has already been noted that it is thought *Pneumocystis* may scavenge host derived sterols such as cholesterol, in addition several plant sterols have been biochemically detected in *P. carinii* including: campesterol, β-sitosterol, brassicasterol

and stigmasterol (69). It is proposed that these plant sterols are incorporated into the lung by the host, and may be subsequently taken up by *P. carinii* and incorporated into its membranes (104). Cholesterol and plant sterols are taken up by *P. carinii* and incorporated unchanged in *P. carinii* membranes, however there is evidence that suggests that the pathogen can remodel host-derived sterols using the *P. carinii Erg6* sterol enzyme (69). NMR analysis of HPLC isolated *P. carinii* sterols revealed that *P. carinii* contains a total of 43 sterols, and of these, 32 contained a methyl group on C-24 of the sterol side chain (69). The thought that *Pneumocystis carinii* remodels host-derived sterols is due to the presence of a large number of Δ^5 alkylated C-24 sterols in these isolated fractions (69). C-24 sterol methyltransferase (*Erg6*) is the enzyme responsible for C-24 alkylation of the sterol structure, and C-5 desaturase (*Erg3*) is the enzyme responsible for destauration of C-5 and the formation of triene sterols. Mammals are unable to alkylate the C-24 position of the sterol nucleus, and the lack of triene sterols in *Pneumocystis* (69) suggests that the organism is not able to destaurate C-5. The gene encoding the C-5 desaturase enzyme has not been identified in the *P. carinii* genome. This has lead to the thought that Δ^5 sterols found in the isolated fractions were first scavenged from the host by *Pneumocystis*, and then remodeled by the *P. carinii* Erg6p.

Purpose and Specific Aims

Drug therapy has successfully reduced the number of deaths attributed to PCP infection, but there are still groups that are unable to tolerate these drugs due to allergies or harsh side effects. The potential for resistance to sulfamethoxazole and dapsone (36) is prompting the need for a new arsenal of drugs for anti-PCP therapy. The abundance of

cholesterol found in isolated fractions of *P. carinii* sterols, the presence of sterol biosynthetic genes within the *P. carinii* genome in addition to the unique sterols found in *P. carinii* together indicate that while the sterol pathway of *P. carinii* may have similarities to other fungi, it also involves deviations from the typical sterol pathway found in other fungal species. The steps involved in ergosterol and cholesterol synthesis have been determined for both fungi and mammals, respectively. However, to date the complete *P. carinii* sterol pathway has not been determined. The unique sterols isolated from the pathogen indicate that the sterol biosynthetic pathway of *Pneumocystis* may hold novel drug targets that have not been identified. Limited efforts have been put forth to characterize this pathway in *P. carinii*, and while some genes have been characterized, many have not. As a result, one of the goals of this project was to characterize the lanosterol synthase gene from *P. carinii* using a yeast heterologous systems and biochemical analyses. This enzyme was chosen because it is responsible for the formation of the first sterol of both the cholesterol and ergosterol biosynthetic pathway, and the essential nature of sterols in eukaryotic cells makes this enzyme critical to survival of the organism.

Although it is commonly accepted that *P. carinii* scavenges cholesterol from its mammalian host, there is very little data about how or when the organism obtains this cholesterol. To date, it has not been determined whether the sterol pathway in *P. carinii* is dependent on oxygen or if the scavenging ability of the organism occurs as a result of impaired oxygenation. It has been proposed that when grown under anaerobic conditions, *P. carinii* mimics the sterol scavenging ability *S. cerevisiae* resulting in the absorption of significant amounts of cholesterol from the host (222). *P. carinii* resides in the host lung

where it is attached to type 1 cells that are responsible for facilitating gas exchange across the mammalian lung. In human subjects, the attachment of *Pneumocystis* to type 1 cells impedes gas exchange in the lung, and results in impaired oxygenation and diffusing capacity, alterations in lung compliance, as well as changes in both total lung capacity and vital capacity (209). Thus another goal of this project was to determine the effects of oxygen on viability, drug susceptibility, sterol biosynthesis and sterol uptake in *P. carinii*.

Aim 1: Express and characterize *Pneumocystis carinii* lanosterol synthase in a yeast heterologous system

As the ability of the *P. carinii* lanosterol synthase enzyme to complement the *S. cerevisiae* enzyme has already been shown, the *working hypothesis* is that *P. carinii* lanosterol synthase functions the same way as *S. cerevisiae* lanosterol synthase. These studies were designed to functionally characterize and to determine the cellular localization of the *P. carinii* enzyme. The open reading frame of the *P. carinii* lanosterol synthase gene was amplified, and the translated cDNA sequence was aligned against a fungal protein database using BlastX on the NCBI website to identify regions of homology and conserved functional domains within the amino acid sequence of *P. carinii* lanosterol synthase. *P. carinii* lanosterol synthase cDNA was cloned into the yeast shuttle vector pYES2.1 and transformed into a null lanosterol synthase yeast mutant. Using this system both the enzymatic function, and localization of *P. carinii* lanosterol synthase was assessed and compared to the *S. cerevisiae* enzyme to determine similarities between the two enzymes.

Commercial strain used:

YHR072W Genotype: MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0 ΔERG7 (Obtained from ATCC)

Aim 2: Determine the effects of oxygen on *P. carinii* sterol biosynthesis and sterol scavenging ability.

The *working hypothesis* is that sterol scavenging is a survival mechanism of *P. carinii*, and will occur under normal oxygen tensions, but oxygen availability will limit viability and sterol gene expression of *P. carinii*. To test this hypothesis, the effect of oxygen on the viability of *P. carinii* was assessed using the ATP bioluminescent assay to determine the ATP content of organisms (33) under standard tissue culture conditions (constituting aerobic conditions), and microaerophillic and anaerobic conditions. The ability of *P. carinii* to scavenge exogenous sterols was assessed under normal tissue culture conditions, and gene expression profiles of *P. carinii* under standard tissue culture and anaerobic conditions were assessed using microarray analysis to determine the effect of oxygen tension on sterol gene expression profiles.

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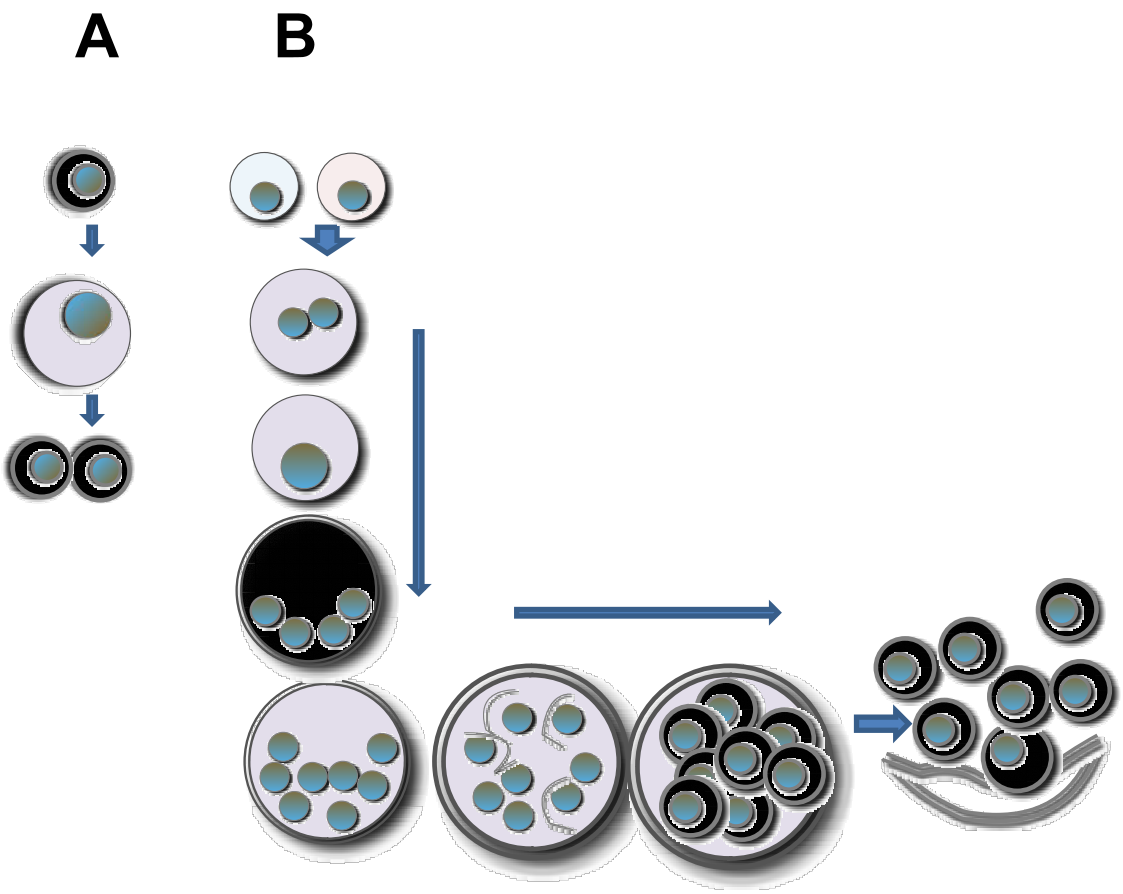

Figure 1. Proposed Lifecycle of *Pneumocystis*

A. Haploid trophic forms duplicate their nuclear material and are thought to replicate asexually by binary fission which results in two genetically identical trophozoites.

B. Two presumptive mating types conjugate, undergo karyogamy and produce a diploid zygote which proceeds through meiosis and an additional mitosis to produce 8 nuclei. Nuclei are packaged into spores presumably by invagination of the ascus cell membrane. Spores are released from the ascus and become vegetative forms that ultimately perpetuate the *Pneumocystis* lifecycle. (The life cycle was composed using SmartDraw, San Diego, CA)

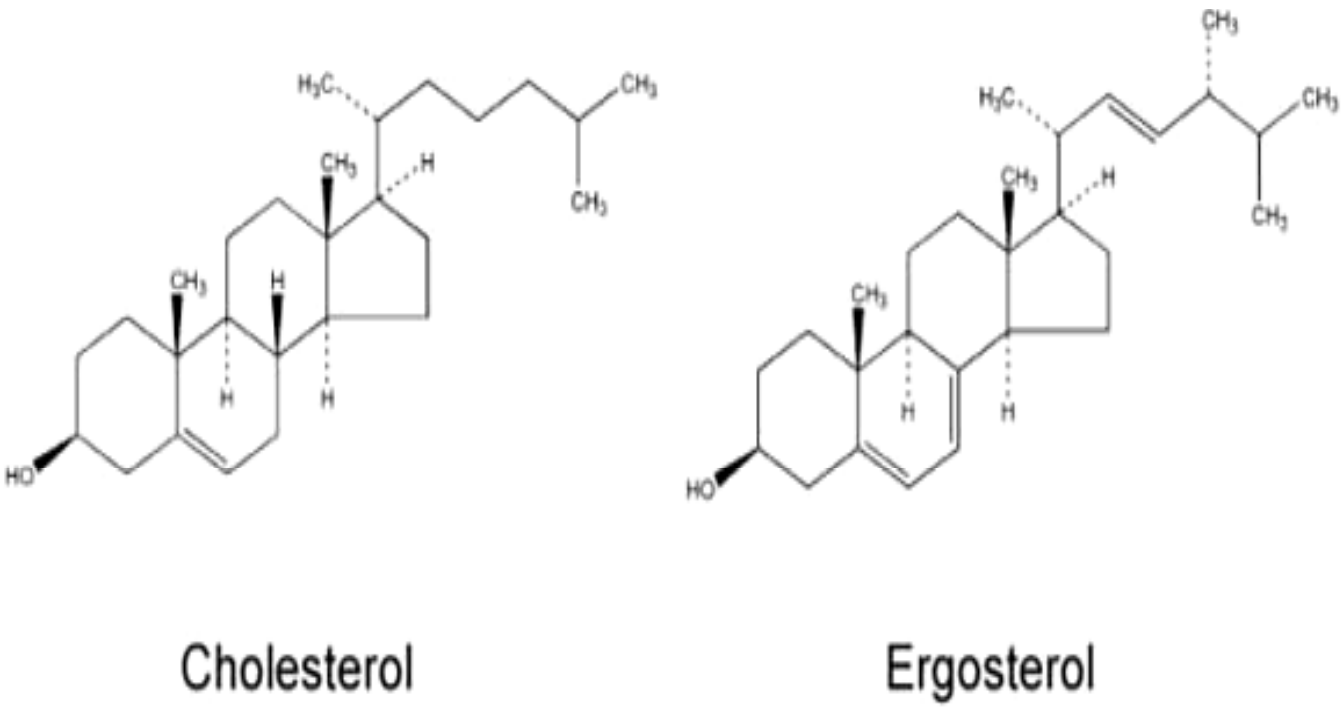

Figure 2. The molecular structure of cholesterol and ergosterol

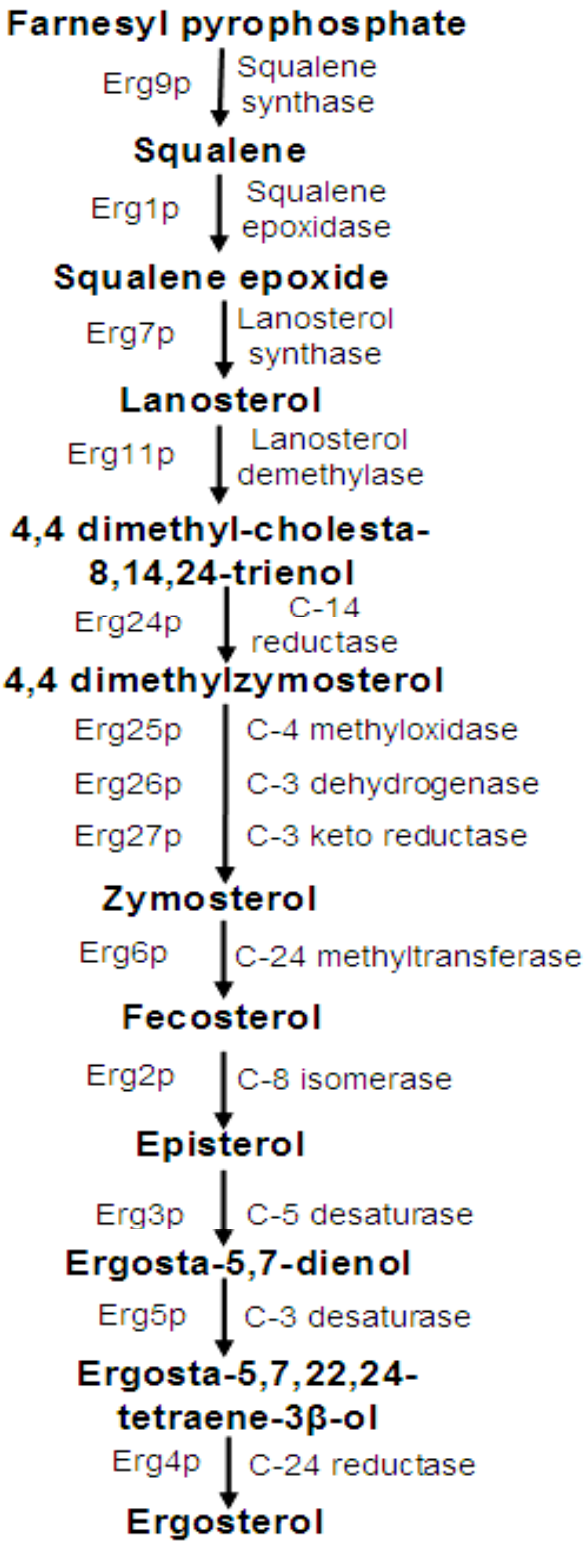

Figure 3. Committed ergosterol biosynthetic pathway

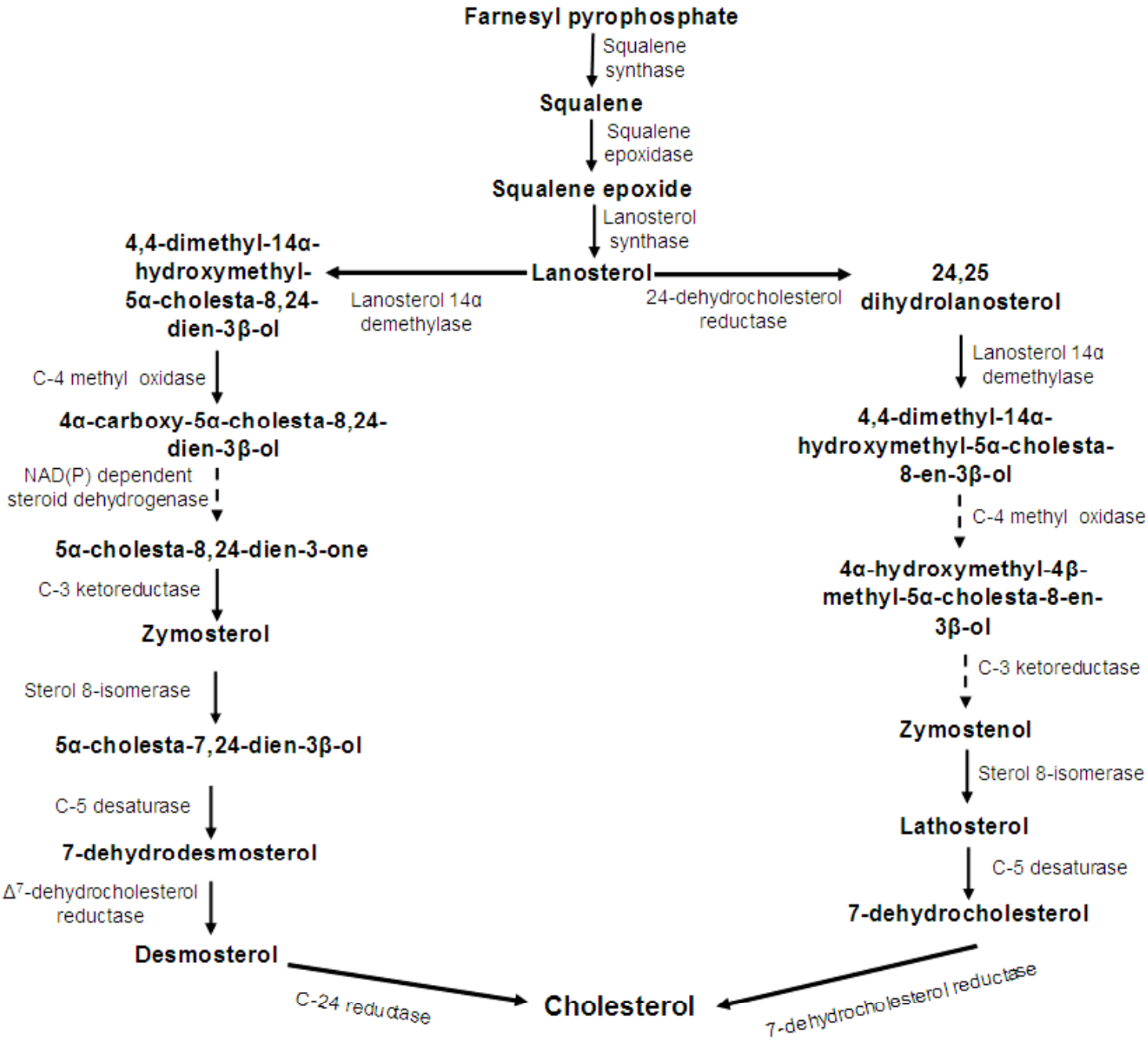

Figure 4. Committed cholesterol biosynthetic pathway

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Chapter 2. Functional characterization and localization of *Pneumocystis carinii* lanosterol synthase

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Abstract

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 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*). 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.

Introduction

Members of the fungal genus *Pneumocystis* can transiently colonize immunocompetent hosts, while those with immune deficiencies are particularly susceptible to developing a life threatening pneumonia as a result of *Pneumocystis* infection (35,51). Despite their fungal nature, *Pneumocystis* are resistant to standard anti-fungal 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 most if not all of the cholesterol in *Pneumocystis* is scavenged from its mammalian host (53), but one report raises the possibility of cholesterol biosynthesis within *Pneumocystis* (54). 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* (39) or knockout of genes in the more complicated *Schizosaccharomyces pombe* system (31) to assess the function of *P. carinii* proteins.

Despite the lack of ergosterol in the membranes of *Pneumocystis*, several putative genes involved in sterol biosynthesis 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 14α demethylase enzyme (Erg11p) (39), the lanosterol synthase enzyme (Erg7p) (37), 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 ergosterol biosynthetic pathways. This enzyme is responsible for the conversion of 2,3-oxidosqualene, the last acyclic sterol precursor, into lanosterol, the first cyclic 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 (43). *Saccharomyces cerevisiae* Erg7p (ScErg7p) localizes to lipid particles, and when expressed in *S. cerevisiae*, Erg7p from the plant pathogen *Arabidopisis thaliana*, and the parasite *Trypansoma cruzi* localized to lipid particles in an *S. cerevisiae ERG7* mutant (36,37). 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 intrinsically linked to lipid particles, and that yeast strains that lack lipid particles have a defect in ergosterol synthesis (47).

In silico sequence analysis of PcErg7p revealed that it contained residues that are essential for the catalytic activity of the ScErg7p (37), and based on the incorporation of radiolabeled acetate into ergosterol, the same group 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 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 reaction 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 (29) 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 MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0 ΔERG7 was obtained from ATCC and used to express pYES2.1, pYES2.1/*PcERG7* or pYES2.1/*ScERG7*. The yeast were grown overnight in yeast extract peptone dextrose (YEPD) media containing 200μg/mL G418, and transformation and sporulation were performed according to the method published by Morales (39). 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 200μg/ml G418 to select for spores containing the wild type *ERG7* deletion. Spores containing the empty vector were plated on similar media lacking G418 to select for spores containing *ERG7* at the wild type locus. Haploid yeast strains were identified using multiplex PCR as previously reported by Huxley et al. (17). Upon verification of haploid yeast colonies from strains expressing pYES2.1/*PcERG7* and pYES2.1/*ScERG7*, PCR was performed to verify 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 using a primer from the 5' UTR of *ScERG7* (5'

GCTTAGTTTT TGTCCATCT CATTG 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 media lacking uracil to induce protein expression and to maintain pYES2.1 in vector containing haploid strains. The cultures were maintained at 25^{o}, 30^{o}, and 37^{o} in a shaking incubator, and aliquots of each culture were taken at 4, 8, 12, 24, 48, and 72 hours of growth in liquid media. The OD_{600} of each aliquot was measured to assess growth of the respective cultures using the POLARstar Optima (BMG Labtech, Durham, NC). Results are expressed as the mean of 3 separate experiments each performed in triplicate.

In silico transmembrane analysis. Determination of hypothetical transmembrane spanning domains were performed using HMMTOP2 online software (http://www.enzim.hu/hmmtop/html/submit.html) (48,49), 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 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) (50).

PcErg7p Purification and Polyclonal Antibody Production. *PcERG7* cDNA was cloned and expressed in the pET30 vector (Novagen, Madison, WI). PcErg7p expression

was induced using IPTG, and the protein was purified from inclusion bodies within *E. coli* following manufacturers instructions. Briefly, the cells were harvested by centrifugation and re-suspended in binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 m*M* 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 4^0 in binding buffer containing 6M 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 using 1M imidazole. Western analysis using an S protein-HRP 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 Blot using *P. carinii* cell lysates and recombinant PcErg7p as a positive control. The antibody fraction of the antiserum was precipitated using ammonium sulfate and reconstituted in 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 three days, and aliquots were taken after 24, 48, and 72 hours of growth. The cells were collected, homogenized and placed into glass vials. A Lowry Assay (32) was performed on aliquots of the homogenates to determine protein concentration. Mass cellular lanosterol was quantified

using gas chromatography with cholesterol as an internal standard, and sterol extraction was performed as previously reported (40,45). Alcoholic KOH (940µl ethanol and 60µl 50% KOH) was added to each vial, the vials were capped, placed in a 65^0 water bath for two hours, cooled, and 5µg of cholesterol was added to each sample. The lipid content of each sample was extracted using 3ml of petroleum ether, and recovered by evaporation of petroleum ether under a stream of air. The lipids were re-suspended in 15µl of hexane and 2µl of each extract was injected into a GC-17A gas chromatograph (Shimadzu Scientific Instruments, Columbia Maryland), 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 following the method previously published (36). Briefly, yeast cells were grown to early stationary phase and treated with zymolyase 20T to create yeast spheroplasts. Spheroplasts were washed twice with 20mM potassium phosphate (pH 7.4) and 1.2M sorbitol and homogenized in breaking buffer (10mM MES/Tris (pH 6.9), 12% Ficoll 400, 0.2mM EDTA) at a final concentration of 0.5ml per gram of wet cell weight. The homogenate was centrifuged at 5,000xg, and the supernatant was overlaid with breaking buffer and centrifuged at 100,000xg in an SW-28 swing-out rotor. The floating layer (top layer) was collected, overlaid with 10mM MES/Tris (pH 6.9), 8% Ficoll 400, and 0.2mM EDTA, and centrifuged for 30 minutes at 100,000xg. The top layer was collected, overlaid with 10mM MES/Tris (pH 6.9), 0.25M sorbitol, and 0.2mM EDTA, and centrifuged for 30

minutes at 100,000xg. 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 using Y-PER reagent (Pierce, Rockford, IL) according to manufacturers instructions. Protein concentrations were determined using a BCA protein assay (Pierce) and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted as described previously (30). Erg7p was detected 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 using TMP1 Component HRP Membrane Substrate (BioFX Laboratories, Owings Mills, MD). For lipid particle immunoblotting, lipid particle fractions were collected in two, one milliliter 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 pYES.2.1/*PcERG7* were inoculated into minimal media containing 2% galactose and lacking uracil, and yeast colonies containing *ScERG7*-GFP (Invitrogen) were inoculated into YPD. The cultures were allowed to grow for two days in a 30^0 shaking incubator. Cells containing pYES2.1/*PcERG7* were pelleted, washed with PBS, and permeabilized with 1% DMSO in PBS. The cells were collected via centrifugation and washed to remove residual DMSO. Non-specific binding sites were blocked in pYES2.1/*PcERG7* expressing cells

using 10% w/v BSA in PBS, and the cells were collected and re-suspended for one hour in an anti-V5-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 one hour in 1µM 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 fluorescent microscope. FITC images were viewed using excitation filters at 465-495nm and emission filters at 515-555nm and Nile Red images were viewed using excitation filters at 540-580nm and emission filters at 600-660nm.

Fluorescent Localization of PcErg7p in *P. carinii*. Cryopreserved *P. carinii* were thawed, centrifuged and re-suspended in PBS, and the cells were permeabilized and blocked similar to the yeast cells (described above). *P. carinii* organisms were collected by centrifugation and re-suspended in 1% BSA in PBS solution and incubated for one hour with polyclonal PcErg7p antiserum conjugated with Alexa-Fluor® 488. The cells were washed twice with PBS, and re-suspended in 6% BSA in PBS and incubated for one hour with Qdot® 525 goat $F(ab')_2$ anti-rabbit IgG conjugate. The cells were centrifuged at 10,000xg, washed twice with PBS, and incubated for one hour with 1µM Nile Red in PBS. After centrifugation, the organisms were washed once with 0.1% Tween-20 in PBS, twice in PBS, and visualized with a Nikon Eclipse E600 fluorescent microscope. Qdot® 525 and Alexa-Fluor® 488 images were views using excitation filters at 465-495 and emission filters at 515-555.

Statistical analysis. Statistical analyses were performed using GraphPad v.4 (GraphPad Software, Inc., La Jolla, CA) and significance was assessed using ANOVA and the Tukey-Kramer Multiple Comparisons Test post test.

RESULTS

PCR was used to amplify the entire open reading frame (ORF) of *PcERG7* from a *PcERG7* cDNA clone, and a 2160-bp product corresponding to the size of the *PcERG7* ORF (37) was detected. The genomic sequence for *PcERG7* was found to be 2564 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 9-622 nucleotides and 41-49 nucleotides, respectively. We confirmed that the PCR product was of *P. carinii* origin by hybridization of a ^{32}P labeled *PcERG7* cDNA probe to a CHEF blot containing the chromosomes of 7 karyotype forms of *P. carinii* and the single *P. wakefieldiae* karyotype (44). The radiolabeled probe bound to a single 620kb chromosome in all karyotype forms of *P. carinii* (Fig. 1 black arrow lanes 2-9) and to a chromosome of 550kb in *P. wakefieldiae* (Fig. 1 open arrow lane 10). These chromosomes correspond to chromosome number three 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. 2). 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 (1,33,34,52) (Fig. 2). As previously reported, within this domain are amino acid residues that are essential for the catalytic activity of ScErg7p, namely: aspartate 456, histidine 146 and 234, tyrosine 410, and valine 454 (37). 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 (37) have shown that 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 83kDa (37), and a polyclonal antibody raised against PcErg7p detected the protein in *P. carinii* and yeast containing pYES2.1/*PcERG7* (Fig. 3, lanes 1 and 2, respectively**).** Due to the conservation between the two proteins, ScErg7p (Fig. 3, Lanes 3-5) was detected using the same antibody. The larger molecular weight bands detected in lanes 2 and 4 were no longer present when using a higher dilution of the polyclonal antibody (Fig. 4, Lane 3) indicating the band was likely due to a non-specific protein-antibody interaction. However, at this concentration ScErg7p was not detected in wild type cells where ScErg7p is expressed at basal levels (Fig. 4, Lane 1), indicating that either higher protein concentrations or a more concentrated antibody is necessary to detect basal levels of ScErg7p. To determine whether 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 hours after inoculation with the exception of the strain containing only the pYES2.1 vector which reached stationary phase 24 hours after inoculation (Fig. 5). There were no significant differences in the growth rates of the strains expressing pYES2.1/*PcERG7* and pYES2.1/Sc*ERG7* at any of the time points analyzed (Fig. 5) 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 (37). 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 employed 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. 6). The amount of lanosterol produced by the pYES2.1/*ScERG7*-containing strain was not significantly different than the pYES2.1/*PcERG7* strain after 24 and 48 hours of growth, but did produce statistically higher levels after 72 hours (Fig. 6). These data were consistent with real time PCR data indicating that *ScERG7* had a 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 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 lanosterol 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 transmembrane (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 (37) making the enzyme ill suited for insertion into the phospholipid monolayer of lipid particles (3,37). In contrast, we found highly variable results using the same protein prediction models in addition to others (Table 1). Our *in silico* analysis of the protein sequences of both PcErg7p and ScErg7p show that the number of TM domains range from 0-6 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 highlights 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 ask 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 analysis. Results from the pYES2.1/*ScERG7* strain and the pYES2.1/*PcERG7* strain are shown in Figure 7C and Figure 7D, respectively. The presence of an 83kDA band in Figure 7D, 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. 7 Panel D, 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. 7 Panel C, lanes 1 and 2). The lower bands may be degradation products of PcErg7p as these were detected upon purification of the native protein which 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. 7A and B, respectively) using our polyclonal antibody.

The presence of PcErg7p in lipid particles in yeast is in contrast to a previous report (37). 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 which could be detected by a FITC- conjugated V5 antibody. Lipid particles consist of a hydrophobic core of neutral lipids which can be readily stained with the fluorescent dye Nile Red (4,13,25,47). FITC staining of pYES2.1/*PcERG7* containing yeast revealed a punctate staining pattern (Fig. 8B, Panel 1) similar to that of a GFP-Erg7p containing yeast strain (Fig 8A, Panel 1), used for visual comparisons. When FITC stained PcErg7p in the *ERG7* yeast mutant were overlayed with the Nile Red stained lipid particles (Fig. 8B, Panel 2), the two fluorophores merged within the cell confirming that PcErg7p is localized to lipid particles in yeast (Fig. 8B, Panel 3) similar to that seen in the GFP-Erg7p control yeast strain (Fig. 8A, Panel 3).

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. 8C, Panel 2) in a punctate pattern similar to that seen in yeast stained with Nile Red (Fig. 8A and B, Panel 2) indicating that *P. carinii* does appear to house stores of neutral lipids. To visualize PcErg7p within *P. carinii*, we used the fluorescent dye AlexaFluor® 488 conjugated to an anti-PcErg7p antibody, and Qdot 525® was used as a secondary antibody to enhance detection of PcErg7p. PcErg7p was localized to discrete regions within *P. carinii* (Fig. 8C, Panel 1) in a pattern similar 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. 8C, Panel 3), indicating localization of the PcErg7p to lipid particles in *P. carinii*.

DISCUSSION

We, like previous investigators showed that PcErg7p was able to complement a null yeast Erg7p mutant (37). In contrast, we found that PcErg7p was localized to lipid particles in yeast and in *P. carinii* using western blotting and fluorescent localization studies. The previous group concluded that PcErg7p does not localize to lipid particles based on three observations: the presence of six putative transmembrane 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 83kDa band, the predicted size of PcErg7p, in a Coomasie stained gel containing lipid particle proteins isolated from PcErg7p expressing yeast. The differences between our two studies were likely due to sensitivities of the techniques employed. We used polyclonal antisera to detect the presence of PcErg7p, while 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. Erg1p was shown to be active in the ER, but inactive in lipid

particles (28). 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 (7,46,52), 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 (36,37). 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 transmembrane domains or as many as six transmembrane domains. Another study (38) characterizing TM domains in ergosterol biosynthetic enzymes from *S. cerevisiae* using programs not used in this study indicates that ScErg7p contains between 0 and 4 transmembrane 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 though 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 14α demethylase enzyme, the target of azole anti-fungal drugs, was biochemically characterized, and sequence analysis comparing the translated open reading frame of PcErg11p to other fungal Erg11 proteins revealed the presence of two amino acids that are thought to confer resistance to azole anti-fungal drugs (39). 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 substrates for *P. carinii* Erg11 are 24-alkylsterols and not lanosterol (Fig. 9). 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 endoplasmic reticulum (42), and enzymes involved in the committed sterol pathway were found to localize to the ER (42). Interestingly, these investigators also found that several enzymes: Erg1p, Erg7p, Erg6p, and Erg27p were localized both to the ER and lipid particles. 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 (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 (18,23,24,41) 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 (47). 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, as the isolation procedure required a minimum of one liter of late log phase *S. cerevisiae* to isolate sufficient quantities of lipid particles. Despite this, the observation that *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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Table 1. *In silico* transmembrane helix predictions for PcErg7p and ScErg7p. The number of transmembrane spanning domains within the protein sequences of PcErg7p and ScErg7p were predicted using various protein structure prediction models.

Transmembrane Predictions

Server	PcErg7p	ScErg7p
HMMTOP2	6	6
SOSUI	1	0
TopPred2	3	3
MINNOU	0	1

Figure Legends

Figure 1. Chromosomal localization of *PcERG7*. (Left) CHEF gel containing separated chromosomes from 9 karyotype forms of *Pneumocystis.* Numbers on the left indicate molecular weight derived from the MW ladder in Lane 1. (Right) CHEF blot hybridized with radiolabeled *PcERG7* cDNA showing localization of *ERG7* to chromosome 3 in both the *P. carinii* and *P. wakefieldiae* genomes. Lanes 2-9 represent different karyotype forms of *P. carinii*, lane 10 corresponds to chromosomes isolated from *P. wakefieldiae*, and lane 11 corresponds to a co-infection of *P. carinii* and *P. wakefieldiae*. Black arrow indicates hybridization of *ERG7* to a chromosome in *P. carinii*, while the open arrow indicates hybridization of *ERG7* to a chromosome in *P. wakefieldiae*. Chromosome sizes were calculated by linear regression based on the migration of 48.5 kb lambda ladder.

FIG. 2. Multiple sequence alignment comparing predicted amino acid sequence of PcErg7p to the Erg7p amino acid sequence from *Schizosaccromyces pombe*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus fumigatus*. Darker shaded regions indicate areas of homology within the amino acid sequences of all species represented while lighter shaded regions indicate regions of sequence similarity between 2 or more sequences. The black bar above the amino acid alignment corresponds to the squalene cyclase domain of PcErg7p which lies between amino acid 71 and 711. Asterisks correspond to conserved amino acid residues within the squalene cyclase domain of PcErg7p according to the Conserved Domain Database (33,34). The plus sign

at resiude 451 corresponds to the catalytic aspartic acid that is responsible for the initiation of the ring cyclization reaction of lanosterol synthase.

FIG. 3. 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 1-5 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 83kDa proteins, and the arrow indicates 83kDa band corresponding to Erg7p detected in the lysates.

FIG. 4. Detection of wild type ScErg7p, recombinant PcErg7p and ScErg7p. Lanes 1-4 correspond to protein lysates from WT yeast, yeast expressing pYES2.1/*ScErg7*, yeast expressing pYES2.1/*PcErg7*, and *P. carinii* respectively. PcErg7p and ScErg7p were detected as 83kDa proteins, and the arrow indicates 83kDa band corresponding to Erg7p detected in the lysates.

FIG. 5. Growth curves 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^{o}. Each data point represents the mean of 3 independent studies. Error bars represent the standard deviation of each group. Statistical significance (p value ≤ 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 hours and WT compared to Sc at 72 hours. Note:

Statistical significance was not detected when comparing pYES2.1/*PcERG7* and pYES2.1/*ScERG7* at any of the time points in the study.

FIG. 6. 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. 7. PcErg7p localizes to lipid particles in yeast. Lipid particles were isolated from wild type yeast, and yeast containing either pYES2.1/*PcERG7* or pYES2.1/*ScERG7*. The floating layer was removed in 1 milliter aliquots, and 5µg of protein from the top two fractions (indicated as 1 and 2) were subjected to western analysis. (A) WT, (B) pYES2.1, (C) pYES2.1/*ScERG7*, (D) pYES2.1/*PcERG7*.

FIG. 8. Fluorescent localization of PcErg7p in yeast and *P. carinii*. GFP-ScErg7p was localized to lipid particles in yeast using an *S. cerevisiae* Erg7p-GFP yeast strain and Nile Red (Panel A). Left represents GFP-ScErg7p in *S. cerevisiae* (Panel A1), middle panel represents Nile Red stained GFP-ScErg7p yeast (Panel A2), right panel represents merged GFP and Nile Red images of GFP-ScErg7p (Panel A3). Panel B shows *S. cerevisiae* containing pYES2.1/*PcERG7*. Left represents PcErg7p stained with V5-FITC conjugated antibody (Panel B1), middle panel represents Nile Red stained PcErg7p in yeast, right panel represents merged FITC and Nile Red images. Fluorescent 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*. Left image represents PcErg7p stained with AlexFuor® 488 and Q-dot 525 (Panel C1). Middle image represents Nile Red stained *P. carinii* (Panel C2), and the image on the right represents the merged image (Panel C3). Arrows indicate areas of co-localization. Magnification bars = 10μm.

FIG. 9. Putative *P. carinii* sterol pathway. A putative sterol biosynthetic pathway indicating genes that have been cloned and functionally characterized (gray, bold-face), 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). Note: Two post lanosterol pathways are proposed for *P. carinii* , one leading the formation of pneumocyterol (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.

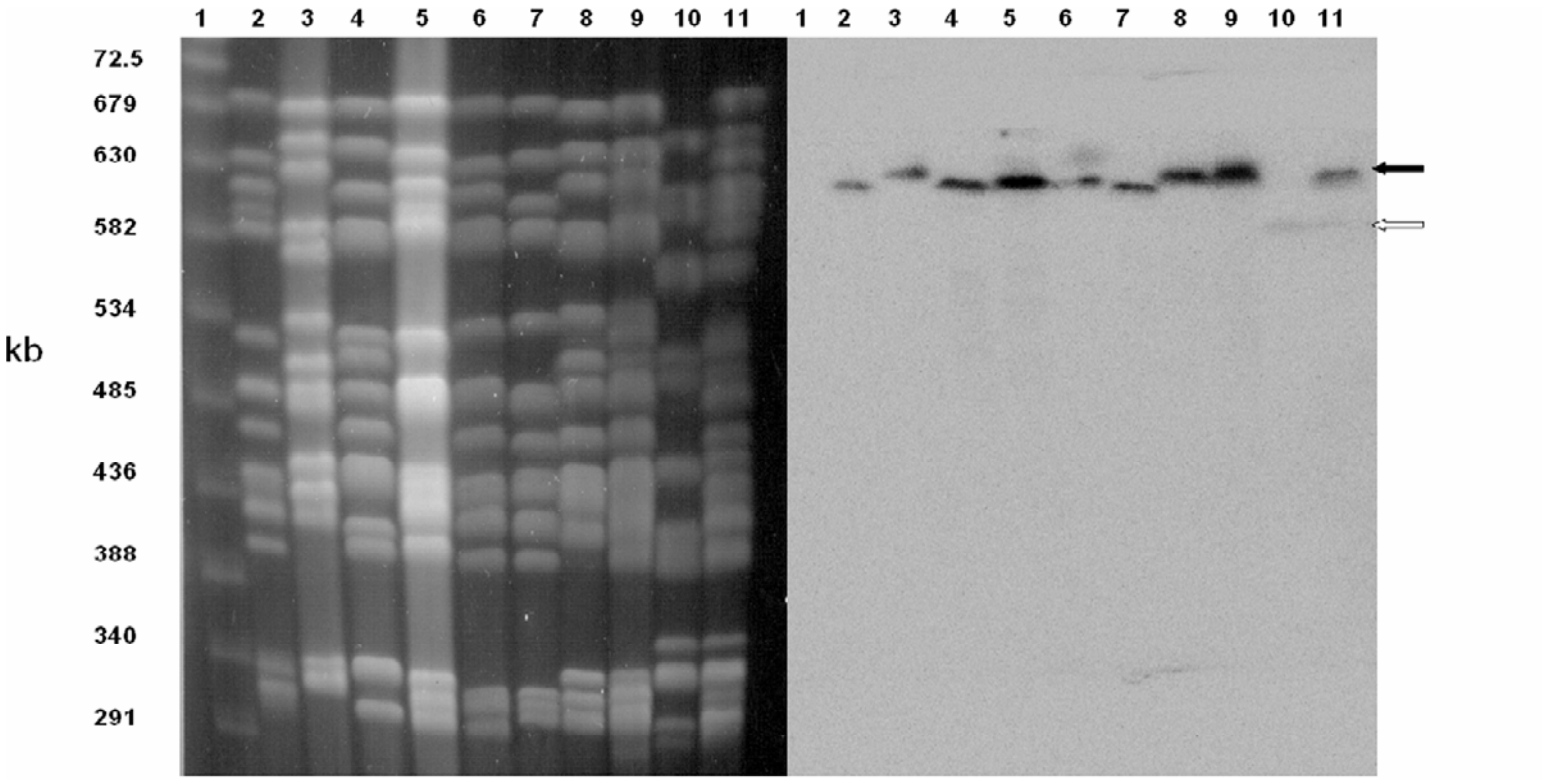

Figure 1. CHEF Blot of *P. carinii* chromosomes and PcERG7 localization to chromosome three

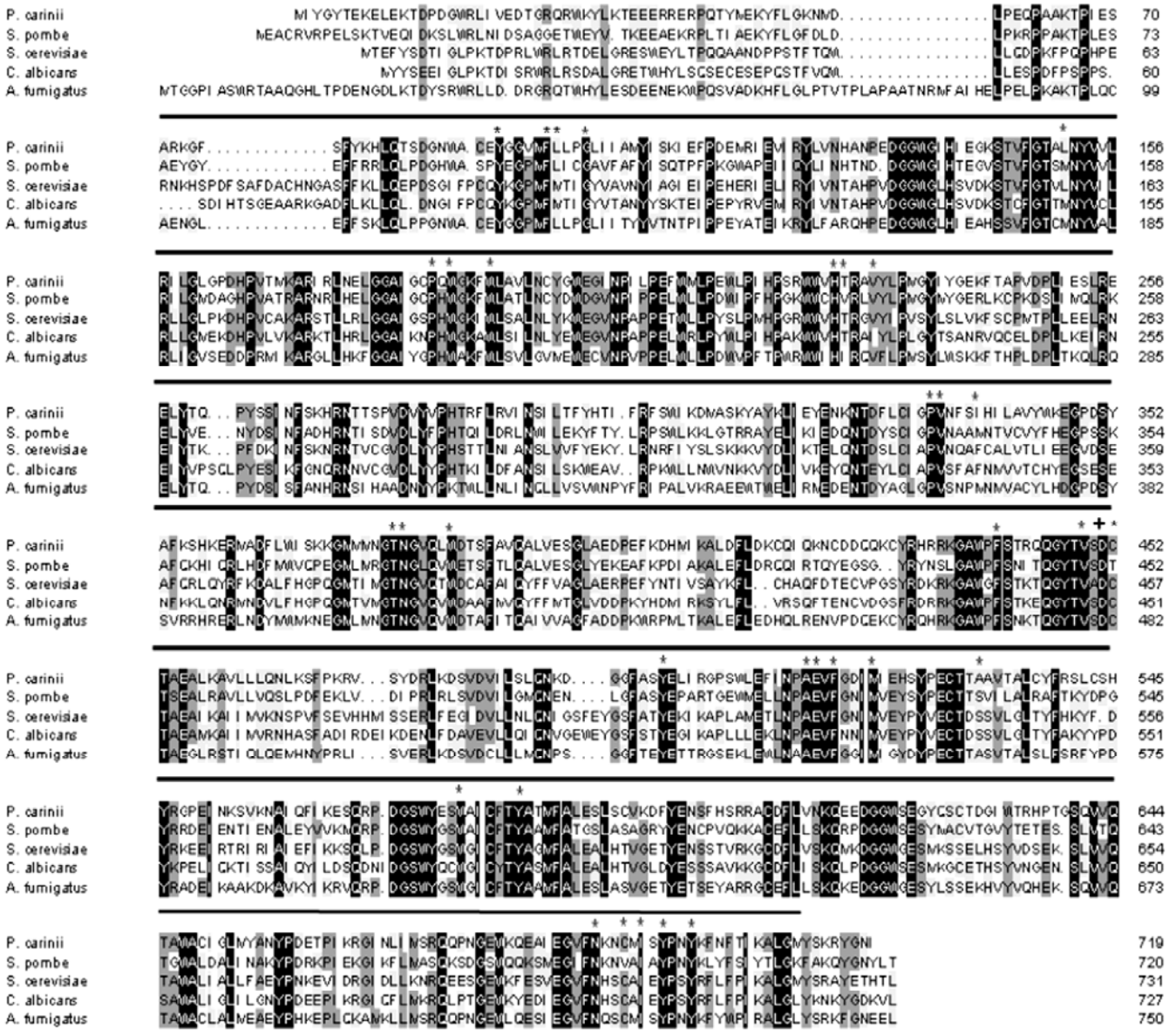

Figure 2. Multiple sequence alignment of fungal lanosterol synthases

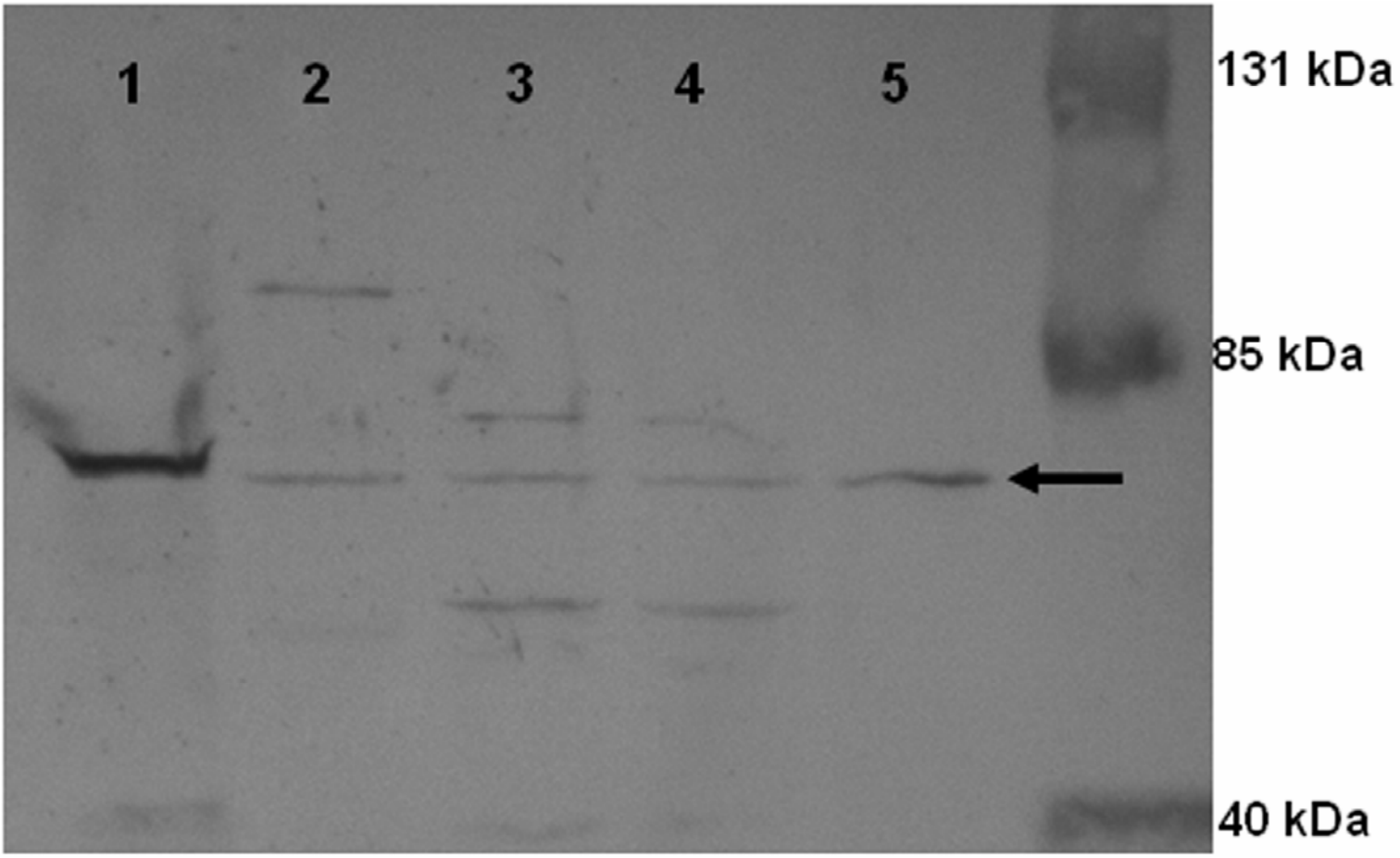

Figure 3. Detection of wild type ScErg7p, recombinant PcErg7p and ScErg7p in yeast and *P. carinii* lysates.

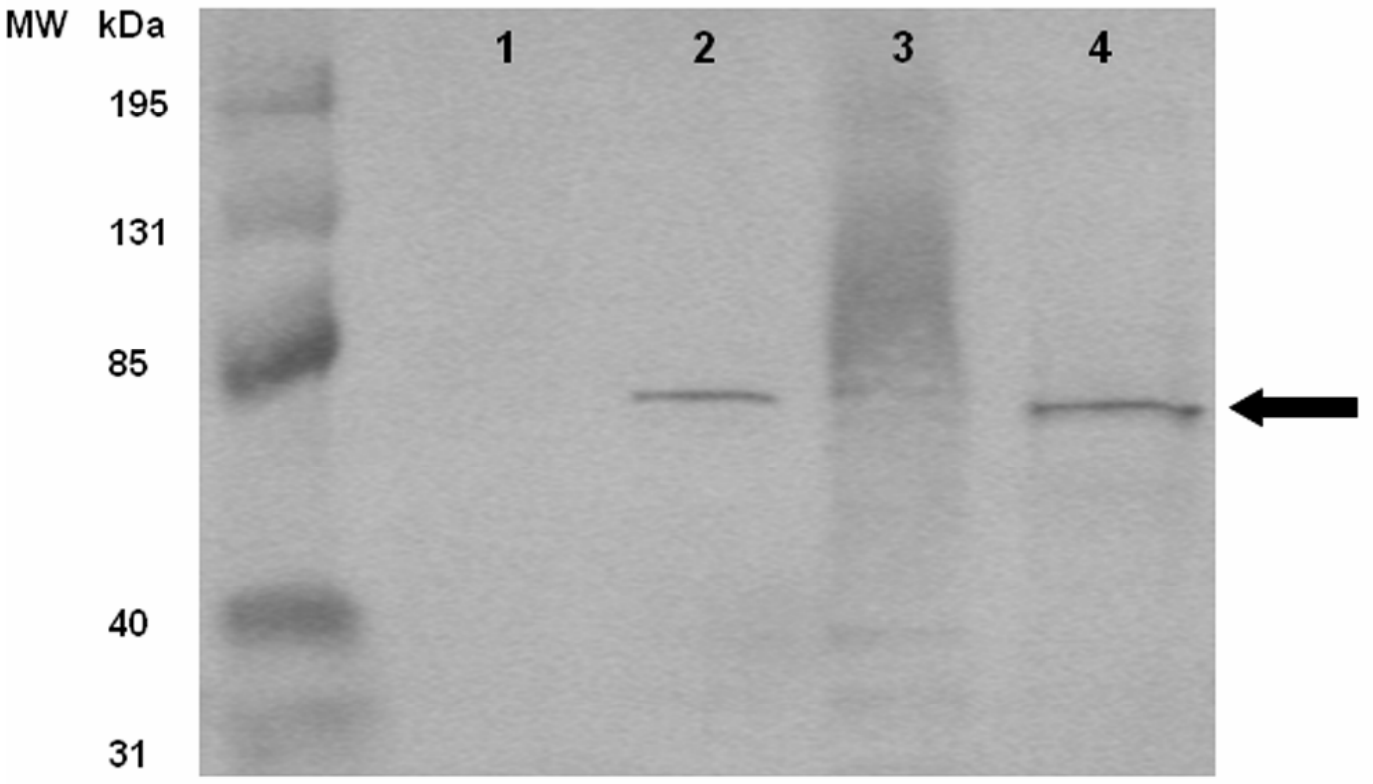

Figure 4. Detection of wild type ScErg7p, pYES2.1/ScErg7p, pYES2.1/PcErg7p, and *PcErg7* in *P. carinii*

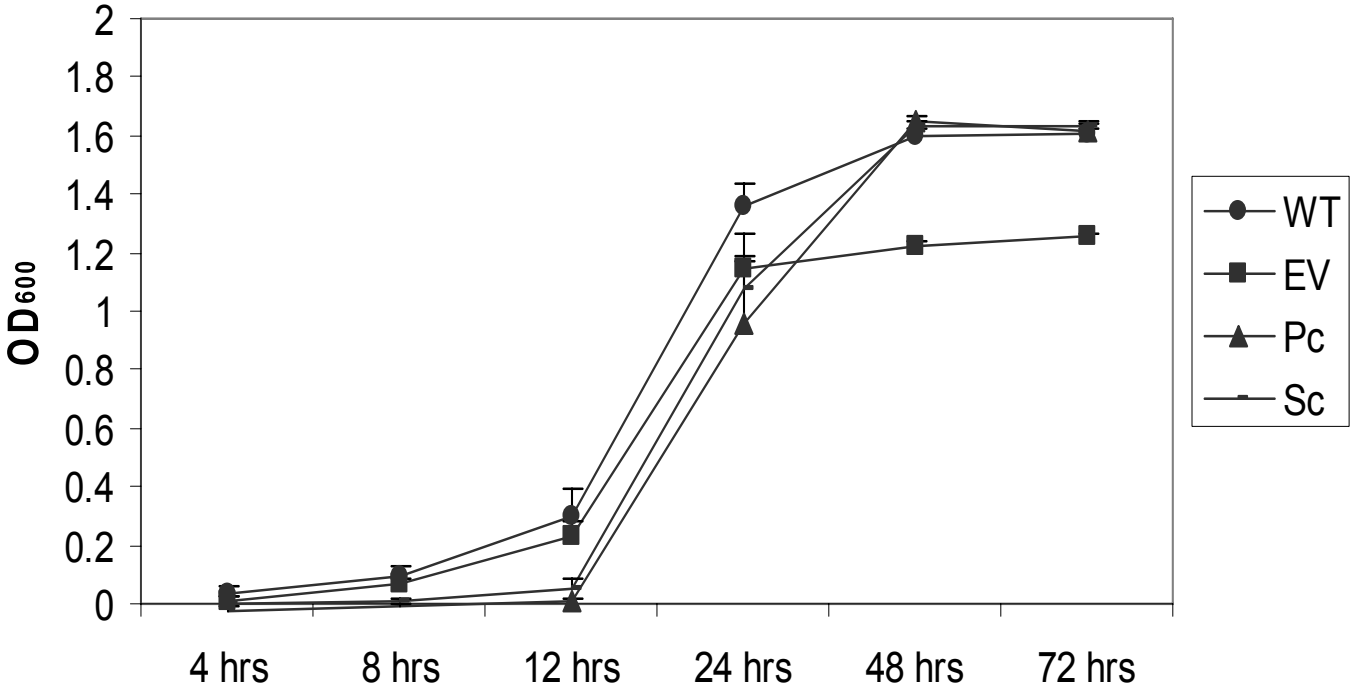

Figure 5. Growth curve comparing growth of wild type yeast (WT) and yeast containing, pYES2.1 (EV), pYES2.1/*PcERG7* (Pc), or pYES2.1/*ScERG7* (Sc)

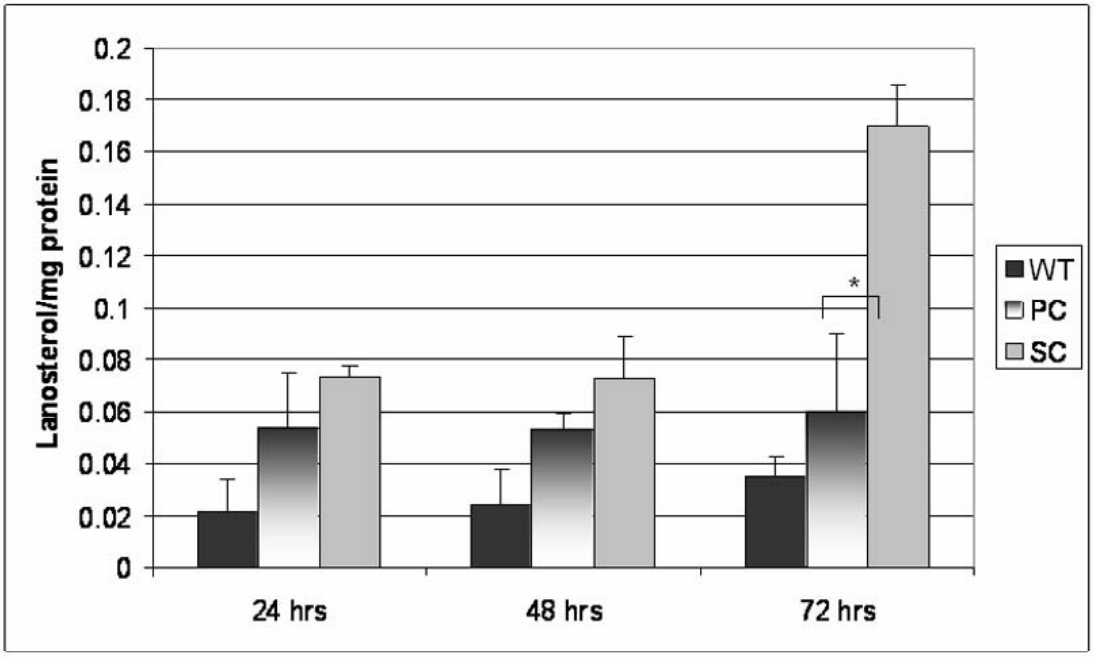

Figure 6. Lanosterol production by wild type yeast (WT) or yeast containing either pYES2.1/*ScERG7* (SC) or pYES2.1/*PcERG7* (PC).

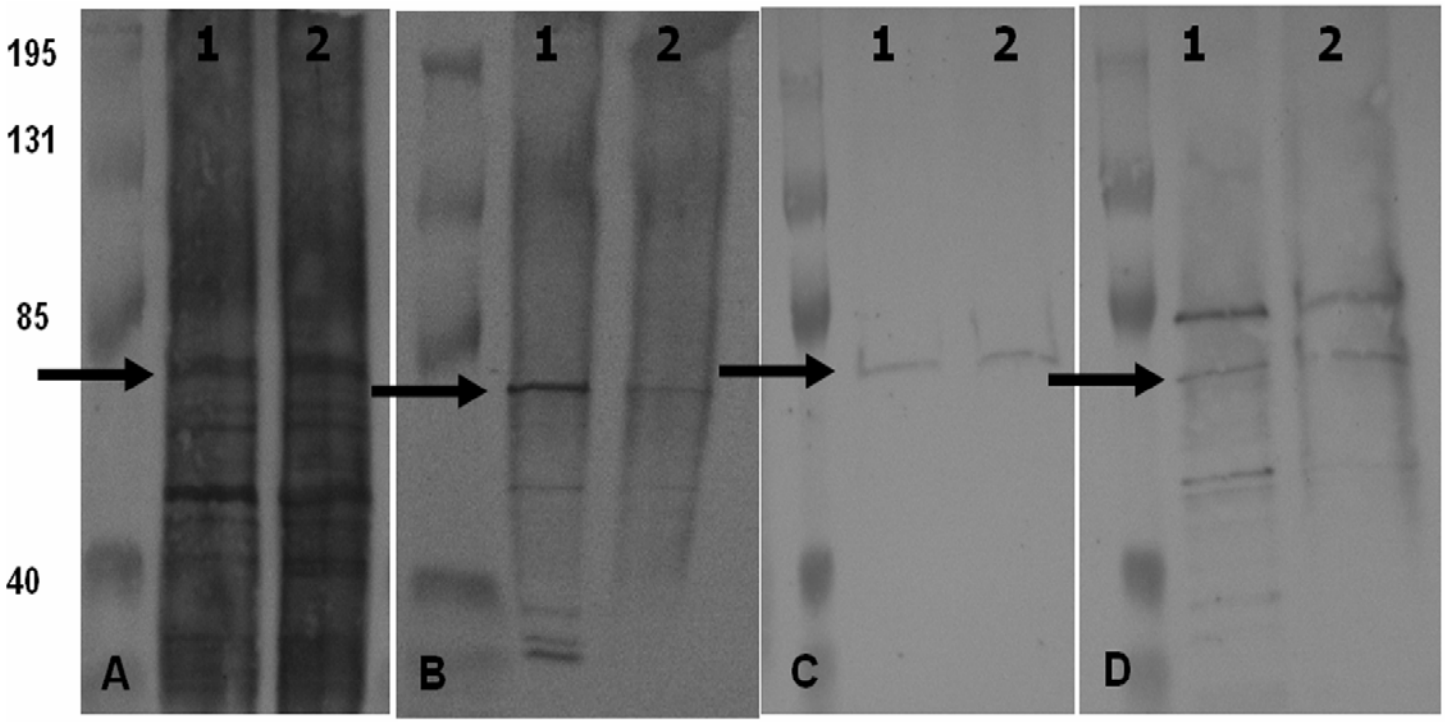

Figure 7. PcErg7p localization to lipid particles in yeast.

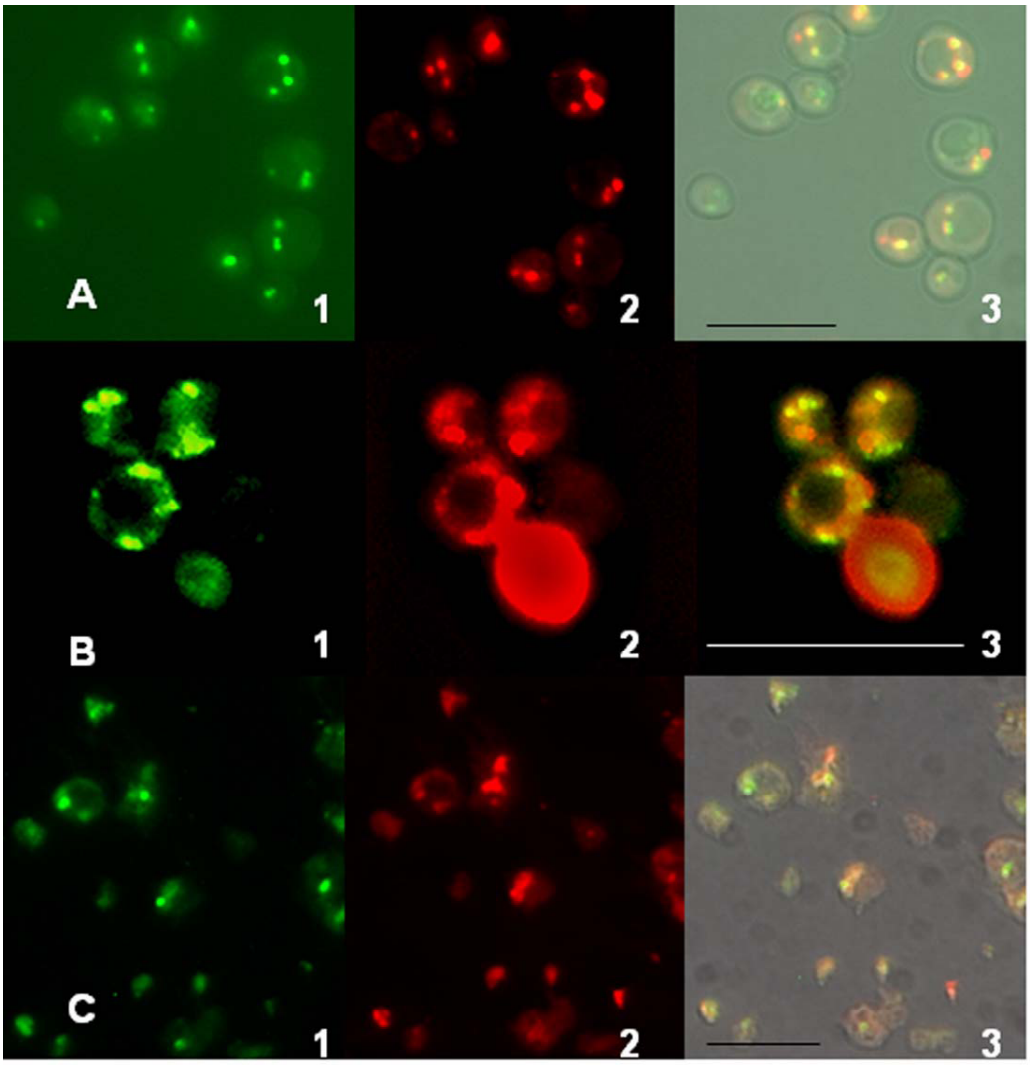

Figure 8. Fluorescent *P. carinii* localization to lipid particles in yeast and *P. carinii*

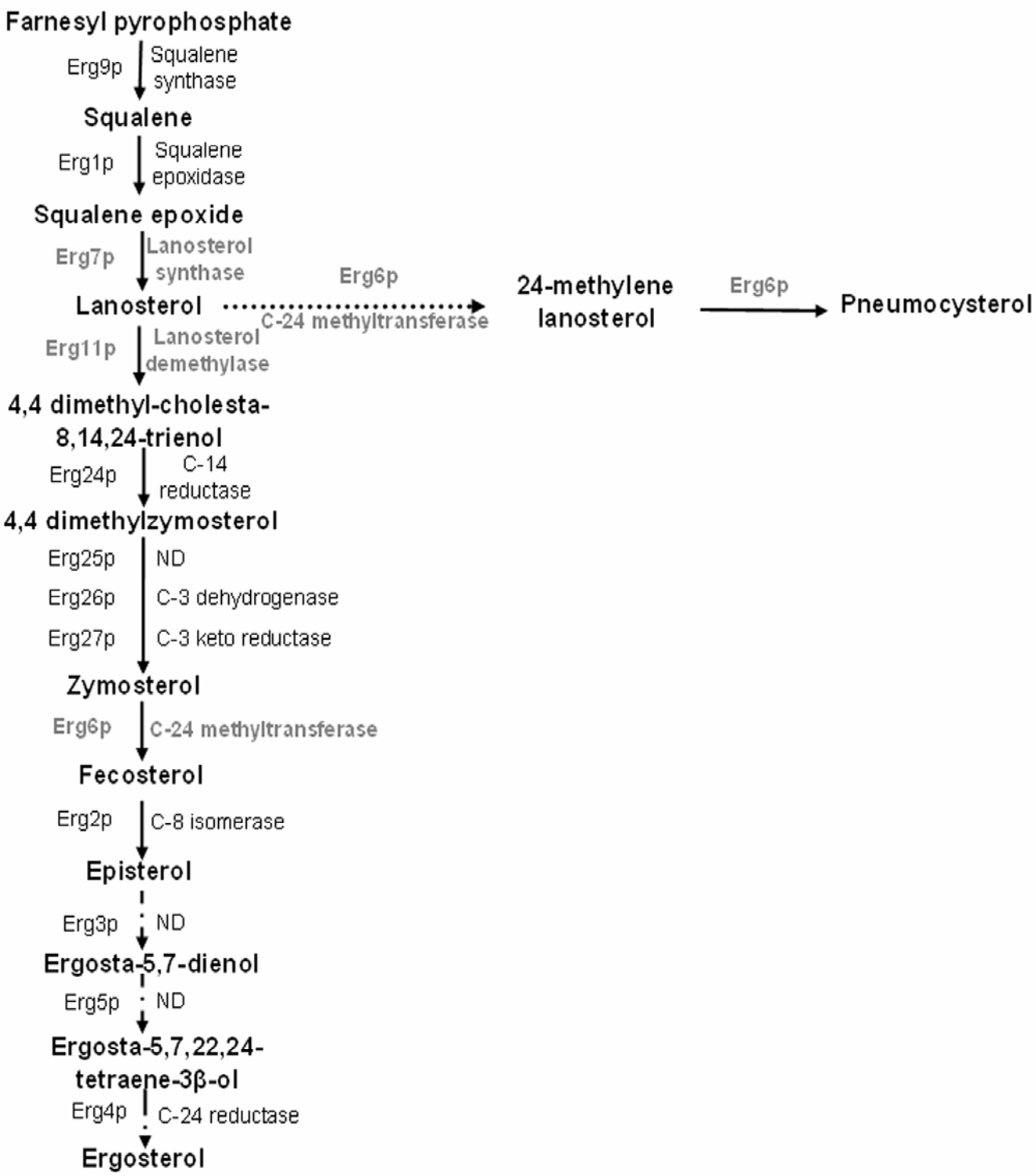

Figure 9. Putative sterol biosynthetic pathway of *P. carinii*

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Chapter 3. The effect of oxygen on viability, sterol uptake, and transcriptional responses in *Pneumocystis carinii*

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Abstract

Unlike other fungi, *Pneumocystis* spp. lack ergosterol and instead use cholesterol as their bulk sterol. It is thought that *Pneumocystis carinii* scavenges sterols from its host, but despite the lack of ergosterol, *P. carinii* also retains functional sterol biosynthetic genes within its genome. Due to oxygen requiring sterol enzymes, the ergosterol pathway is nonfunctional under anaerobic conditions in *Saccharomyces cerevisiae*, and as a consequence, sterol scavenging becomes the major mechanism for obtaining sterols. In the present study, we explored the effects of oxygen on *P. carinii* viability, transcriptional profile, and sterol scavenging ability. The ATP content of *P. carinii* maintained under microaerophillic conditions (10-15% O_2 and 7-15% CO_2) was significantly higher than that of *P. carinii* maintained under standard conditions (21% O_2 and 5% CO_2), and hypoxic maintenance (<1 % O_2 and 10%CO_2) of *P. carinii* resulted in marked decline of the ATP of *P. carinii* suggesting that *P. carinii* can survive under conditions of limited, but not absent environmental oxygen. Using the fluorescent cholesterol analog NBD-cholesterol we determined that *P. carinii* is able to rapidly scavenge cholesterol under standard oxygen tensions, and that scavenged cholesterol is trafficked to cellular membranes within *P. carinii*. Comparison of the transcriptional profiles of *P. carinii* under standard and hypoxic conditions indicated that hypoxic maintenance results in down-regulation of genes involved in sterol and lipid metabolism, protein folding, and stress response, and up-regulation of genes involved in autophagy. These studies indicate that the sterol pathway of *P. carinii* is down-regulated as a result of decreased oxygen tension, but, decreased oxygen is not necessary to initiate sterol scavenging in *P. carinii*. In addition, these studies identify putative genes with homology to genes involved in

autophagy, and suggest potential mechanisms that may result in induction of this pathway in *P. carinii*.

Introduction

Pneumocystis spp. are atypical fungi due to the presence of large amounts of cholesterol, the major mammalian sterol, and a lack of detectable ergosterol, the major fungal sterol (24). The lack of ergosterol makes the organism resistant to anti-fungal drugs that target either ergosterol or ergosterol biosynthesis. Despite the lack of detectable ergosterol in *Pneumocystis carinii*, these organisms contain putative gene homologs that encode enzymes involved in sterol biosynthesis (12). Targeted inhibition of these enzymes in *in vitro* assays resulted in decreased viability of *P. carinii* indicating that these enzymes are functional (37). Several unique sterols have been isolated from *P. carinii* including C_{28} and C_{29} Δ^7 24-alkysterols such as fungisterol and stigmast-7-en-3β-ol (20,38), but to date the complete sterol pathway has not been determined. In addition to cholesterol and these unique sterols, *P. carinii* also contains a large number of Δ^5 alkylated C-24 sterols (24). C-24 sterol methyltransferase (*Erg6*) is the enzyme responsible for C-24 alkylation of the sterol structure, and C-5 desaturase (*Erg3*) is the enzyme responsible for destauration of C-5 and the formation of triene sterols. Mammals are unable to alkylate the C-24 position of the sterol nucleus, and the lack of triene sterols in *P. carinii* (24) suggests that the organism is not able to destaurate C-5. Consistent with this idea is the absence of a gene encoding the C-5 desaturase enzyme in the *P. carinii* genome. This has lead to the belief that these Δ^5 sterols, which include cholesterol, were first scavenged from the host by *P. carinii*.

The presence of oxygen requiring enzymes in the sterol biosynthetic pathway makes sterol biosynthesis an aerobic process in *Saccharomyces cerevisiae* (3). Under hypoxic conditions, sterol biosynthesis is inhibited, and the viability of yeast under these conditions is completely dependent on exogenous sterol availability (3). As a result, *S. cerevisiae* scavenges exogenous sterols and incorporates these sterols into cellular membranes. The regulation of genes induced under hypoxic conditions has been studied extensively in the budding yeast *S. cerevisiae* and the fission yeast, *Schizosaccharomyces pombe*, and while similar genes are induced in response to hypoxic conditions, the mechanism of induction of these genes differs between the two organisms. In *S. cerevisiae*, heme biosynthesis, which is intrinsically linked to oxygen availability, directly correlates with induction of the transcriptional regulator, HAP1 (27). Hap1 induces genes required for cellular respiration, the oxidative stress response and HMG CoA reductase 1 (83), but Hap1 is also responsible for aerobic induction of the transcriptional repressors ROX1 and MOT3 (78). Under aerobic conditions Rox1 and Mot3 repress the transcription of hypoxic genes through the action of the Tup1-Ssn6 complex (43,53). Hypoxic conditions prevent heme biosynthesis causing Hap1 to bind to and repress transcription of its own gene. Repression of Hap1 results in decreased expression of Rox1 and Mot2 concomitant with increased transcription of anaerobic genes in *S. cerevisiae*. Hypoxic conditions not only results in a decrease in heme, but also decreases sterol availability. Consequently, both *S. cerevisiae* and *S. pombe* sense oxygen levels through sterol availability as sterol biosynthesis is decreased under hypoxic conditions. Oxygen sensing through sterol biosynthesis occurs through Upc2 in *S. cerevisiae* and Sre1 in *S. pombe*. Upc2 is induced under hypoxic conditions as a result of

decreased sterol biosynthesis, and induces expression of genes involved in sterol biosynthesis (13) and sterol uptake (80). Under hypoxic conditions, Sre1, activates genes required for both anaerobic adaptation and sterol biosynthesis, the latter activation being similar to mammalian Sterol Regulatory Element Binding Proteins (SREBPs) (29). Sre1 is regarded as the principle regulator of anaerobic gene induction in *S. pombe* and is responsible for the induction of itself as well as genes required for non-respiratory oxygen-consumptive pathways under hypoxic conditions (84).

P. carinii resides in the host lung where it is attached to type 1 cells (1,5). Type I cells facilitate the exchange of oxygen and carbon dioxide across the alveolar surface, and during *Pneumocystis* infection, attachment of the organisms to these cells and subsequent proliferation within the lung results in impaired oxygenation, respiratory alkalosis and subsequent type I cell damage (87). During *Pneumocystis* infection, large clusters of organisms fill the alveolar lumen, and under these conditions, pockets or microenvironments of differing O_2 and CO_2 levels may be created which could potentially alter the lung environment. It has been proposed that when grown under hypoxic conditions, *P. carinii* mimics the sterol scavenging ability of *S. cerevisiae* resulting in the accumulation of significant amounts of cholesterol from the host (92). To date there have been no studies to determine whether the *P. carinii* sterol pathway is dependent on oxygen or if the scavenging ability of the organism is a result of impaired oxygenation. We sought to determine the effect of hypoxic maintenance on both the viability and the transcriptional profile and sterol scavenging ability of *P. carinii*. The transcriptional profile of *P. carinii* maintained under hypoxic conditions suggested an up-regulation of genes involved in autophagic processes, and a down-regulation of genes

involved in the stress response and protein folding. Additionally, our analyses indicated that hypoxic maintenance of *P. carinii* resulted in a down-regulation of sterol biosynthesis suggesting that *P. carinii* may not employ mechanisms to up-regulate sterol biosynthesis under these conditions as do other fungi. Using NBD cholesterol we showed that *P. carinii* was able to scavenge exogenous cholesterol from the media under normal oxygen tensions, indicating that sterol scavenging occurs under conditions that allow sterol biosynthesis in the organism. This work provides the first look at *P. carinii* under hypoxic conditions, and suggests that these conditions down-regulate sterol genes, induce ER stress and autophagy in *P. carinii*. Additionally, this work provides evidence that exogenous sterol scavenging is not a consequence of decreased oxygenation in *P. carinii*, and suggests that sterol scavenging may be a major mechanism used by *P. carinii* to obtain sterols.

Materials and Methods

Environmental culture conditions

Biobag disposable environmental chambers (Fisher Scientific, Cincinnati, OH) were used to simulate microaerophilic (10-15% O_2 and 7-15% CO_2) and anaerobic (<1 % O_2 and 10% CO_2) conditions. Standard tissue culture conditions were maintained at 21% O_2 and 5% CO_2 in a humidified incubator. Cryopreserved *P. carinii* were thawed and immediately resuspended in RPMI-1640-based medium supplemented with 20% calf serum, IX MEM vitamins, non-essential amino acids, L-glutamine, 100 IU penicillin and 100 μg/mL streptomycin (10). The concentration of organisms was adjusted to 5 x 10^7 *P. carinii*/mL in multi-well plates maintained under the three atmospheric conditions at 36

ºC. After 1, 3, 5, and 7 days of culture, plates under microaerophillic and hypoxic conditions were removed from the Biobags and 50 µl aliquots were taken from triplicate wells from each of the plates and assayed for ATP content using the luciferin-luciferase ATP bioluminescent assay as previously described (9). Plates were not placed back into the Biobags. Rather, other plates were removed at subsequent time points. Plates under standard conditions were used at each time point as described for hypoxic and microaerophillic conditions.

Microarray based transcriptional profiling of *P. carinii*

P. carinii organisms were collected after 24 hours of incubation under standard and hypoxic conditions, and RNA was isolated using the *MasterPure™Yeast RNA Purification kit* (Epicentre, Madison, Wisconsin). RNA purity and quantity was determined using a NanoDrop ND1000 spectrophotometer and submitted to the University of Cincinnati Microarray Core (http://microarray.uc.edu) for analysis with the Agilent Bioanalyzer. The RNA was subjected to a 2 fold amplification process, converted to cDNA, labeled with Cy3 and Cy5, and hybridized to *P. carinii* custom-printed microarray slides which were created from a 70-mer oligonucleotide signature library (Illumina Inc. San Diego, CA) representing 3067 unique putative open reading frames (approximately 45% of the genome). The slides were scanned with the Axon GenePixPro 5.0 software, and the PMTs were set to 600 in both 635nm (Cy5) and 532nm (Cy3) channels. Each analysis was performed in triplicate and for one of the triplicate slides, the Cy-3 and Cy-5 labeling was switched for the control and experimental target RNAs. After a 3-step normalization process, data were analyzed using the open source

environment "R" for clustering and analysis. Probability scores were used to identify significant changes, and genes with P-values greater than 0.05 were not used in subsequent analyses.

Putative *P. carinii* genes that were significantly regulated in response to changes in O_2 tension were identified using Spot IDs which were assigned based on the original 70-mers used to design the custom microarray slides. Since the genome of *P. carinii* is not fully annotated, many of the genes were identified based on sequence homology to other fungal homologs using BLASTX (2) analysis. UniProt IDs were assigned to each gene, and the IDs were submitted into the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Database Gene ID Conversion Tool (14,28) and converted to unique DAVID gene IDs. David gene IDs were then used to assay for pathways over-represented in the submitted gene set.

***P. carinii* NBD-cholesterol uptake**

P. carinii were incubated for two hours in either serum free or serum containing medium followed by the addition of NBD-cholesterol (Avanti Polar Lipids) at 5μg/ml. The organisms were incubated with NBD-cholesterol for up to 4 hours and aliquots were taken from each sample at multiple time points and washed to remove exogenous NBD-cholesterol. The amount of NBD-cholesterol taken up over time was quantified using the POLARstar Optima (BMG Labtech). Microscopic analysis of NBD-cholesterol in *P. carinii* was assessed using a Nikon Eclipse E600 fluorescent microscope and a sample from organisms incubated for one hour in serum free medium containing 5μg/ml NBD-cholesterol.

Fluorescent NBD localization

P. carinii were incubated for one hour in serum free medium containing 5μg/ml NBD-cholesterol. The organisms were washed with PBS to remove exogenous NBD-cholesterol and then counterstained with Mitotracker® Red (Invitrogen) to visualize mitochondria, and with Nile Red to visualize lipid particles.

Results

Although no long term *in vitro* culture method exists for any species of *Pneumocystis* (spp), the ATP content of *Pneumocystis* can be retained or slightly increased using a short term maintenance medium (9). However, during this time the number of organisms do not markedly increase (9). Thus, the ATP content of organisms maintained under standard, hypoxic, and microaerophillic conditions was used to assess the effect of oxygen on the viability of *P. carinii*. The ATP levels of the organisms maintained under standard conditions were used as the basis of comparison among the different atmospheric conditions as this has been the standard *in vitro* conditions used in ours and other laboratories. The ATP levels of both the standard and microaerophillic organisms peaked on day-three and began to decline on day five (Fig. 1). However, at all time points, the levels of ATP in the microaerophillic organisms remained significantly higher than those of the organisms maintained under standard conditions (Fig. 1). Notably, maintenance of *P. carinii* under hypoxic conditions resulted in dramatic declines in ATP after a single day of exposure, and the ATP content of *P. carinii* under

hypoxic conditions was significantly lower than the two other conditions at all time points.

In yeast, the plasma membrane is impermeable to exogenous sterols when oxygen is readily available, but the pathogenic fungus *Aspergillus fumigatus* is able to take up cholesterol under these same conditions (89). The rapid loss of ATP in *P. carinii* maintained under hypoxic conditions suggests that the viability of *P. carinii* is decreased under these conditions, and given the fact that *P. carinii* contains cholesterol, but does not appear to be able to synthesize cholesterol, we sought to determine whether the presence of oxygen prevents cholesterol uptake in *P. carinii*. The ability of *P. carinii* to take up exogenous cholesterol under standard conditions was assessed by incubating *P. carinii* with NBD-cholesterol for up to 4 hours in either serum free or serum containing medium. *P. carinii* readily imported NBD-cholesterol in as early as five minutes, and while the uptake of the fluorescent cholesterol occurred more rapidly when NBD-cholesterol was added to organisms maintained in serum free medium, the labeled cholesterol was still taken up by *P. carinii* maintained in medium containing serum indicating that competition between labeled and unlabeled cholesterol in the serum likely accounted for this difference (Fig. 2). Fluorescent microscopy was used to visualize NBD-cholesterol transport and localization in *P. carinii*. Microscopic evaluation indicated that *P. carinii* took up the labeled cholesterol and incorporated it into discrete regions of the cell (Fig. 3). Dual staining was used to visualize some of these regions. Counterstaining *P. carinii* that have taken up NBD-cholesterol with the mitochondrial stain Mitotracker® Red, indicated that the labeled cholesterol was trafficked to the mitochondria, while counterstaining with Nile Red showed that the scavenged cholesterol

may be processed into cholesterol esters and stored in intracellular lipid particles. Taken together, these data indicate that the ability of *P. carinii* to take up exogenous cholesterol when both oxygen and unlabeled sterol are in abundance emphasizes the necessity of cholesterol for *P. carinii* viability and suggests that cholesterol scavenging is a major mechanism employed by *P. carinii* to obtain sterols.

Hypoxic maintenance of *P. carinii* resulted in drastic, but not complete declines in ATP after 24 hours, however it is unclear what cellular processes are induced as a result of limited oxygen. Therefore, we compared the transcriptional profiles of *P. carinii* under hypoxic conditions and *P. carinii* under standard conditions to identify cellular processes that are affected by hypoxia. We identified 1056 genes with statistically significant changes in expression as a result of hypoxic maintenance, and of these 551 were up-regulated while 505 were down-regulated. BLASTX comparison of the translated nucleotide sequences of genes that were regulated in response to hypoxic conditions was used to identify putative *P. carinii* genes based on homology to known proteins in the fungal protein database. As a result, we were able to identify 385 genes that were up-regulated and 387 genes that were down-regulated in response to hypoxia.

The oxygen dependent post-squalene pathway of sterol biosynthesis utilizes 25% of non-respiratory oxygen in *S. cerevisiae* (74), and decreased activity of several enzymes in the post-squalene pathway under hypoxic conditions leads to the transcriptional up-regulation of genes involved in sterol biosynthesis in *S. cerevisiae* and *C. albicans*. Previous studies have shown that hypoxic maintenance results in up-regulation of genes involved in sterol biosynthesis in fungal organisms (75,79,84). Consistent with previous studies in *C. parapsilosis* (75) and *S. pombe* (84), the *P. carinii* sterol gene squalene

epoxidase (ERG1) was up-regulated under hypoxic conditions in our microarray analysis. However, unlike previous analyses, genes involved in sterol biosynthesis were significantly down-regulated under hypoxic conditions in *P. carinii*. Sterol genes encoding HMG CoA reductase, the rate limiting enzyme of sterol biosynthesis in mammals and fungi, and sterol C-24 methyltransferase (Erg6) were significantly down-regulated. HMG CoA reducatase is the rate limiting step of sterol biosynthesis in mammals and fungi, while Erg6 is a highly active enzyme in the sterol pathway of *P. carinii* (24,39,39). The sterols of *P. carinii* consist mostly of 24-alkylsterols, the products of the enzymatic activity of Erg6, and down-regulation of Erg6 suggests that these sterols may have decreased in abundance as a result of hypoxic maintenance of *P. carinii*. In total, eight genes involved in sterol biosynthesis and nine genes involved in fatty acid and lipid biosynthesis were down-regulated in our transcriptional analysis, indicating that both lipid and sterol biosynthesis are down-regulated in *P. carinii* under hypoxic conditions.

Hypoxic conditions resulted in up-regulation of genes involved in intracellular transport, particularly those required for autophagy and the cytoplasm to vacuole transport pathway (Table 1). While autophagy plays a role in normal cellular growth and homeostasis, this process is also a major mechanism used by starving cells to facilitate vacuolar degradative recycling of organelles and macromolecular material in response to changes in overall cellular metabolic function (21). SEC24, SEC18, and SNX4 encode enzymes required for autophagy in *S. cerevisiae* (31,60) and putative homologs of these genes were up-regulated in response to hypoxic conditions in *P. carinii* (Table 1). The cytoplasm to vacuole pathway is another pathway utilized by *S. cerevisiae* to sequester

and deliver cytoplasmic material to the vacuole for degradation and subsequent recycling under conditions of nutrient deprivation (41). SNX4 and VPS34 encode enzymes that have been implicated in both autophagy and the cytoplasm to vacuole pathway in *S. cerevisiae*, and putative homologs of both genes were up-regulated in *P. carinii* under hypoxic conditions. VPS34 encodes phosphatidylinositol 3-kinase (PtdIns 3-kinase), and is required for both autophagy (40,60,67) and cytoplasm to vacuole transport (60,68). In yeast, VPS34 encodes the sole PtdIns 3-kinase, and is essential for the formation of autophagosomal structures in *S. cerevisiae* (40). Mutation of VPS34 results in a lack of accumulation of autophagic bodies, and a lack of mature ApeI, a hallmark of the cytoplasm to vacuole transport pathway (66). Up-regulation genes involved in autophagy and the cytoplasm to vacuole and autophagy pathways suggests that these pathways are active in *P. carinii* under hypoxic conditions.

A total of 14 genes required for endoplasmic reticulum (ER) to golgi transport, endocytosis and vacuolar homeostasis were up-regulated in this analysis (Table 1). Up-regulation of *P. carinii* genes encoding putative homologs to Sec17, Sec18, Sec24, Sec31 and Uso1 indicate an increase in vesicular transport from the ER to the golgi. Sec24 and Sec31 are components of the coat protein complex II (COPII) which has been implicated in the secretory pathway in *S. cerevisiae* (45,76). While Sec17 and Sec18 have a role in ER to golgi transport (59,65), both have been implicated as necessary for vacuolar fusion events (31,56,57). Additionally, *P. carinii* genes encoding the putative HSE1 and VPS36 genes, which are part of the Endosomal Sorting Complex Required for Transport (ESCRT), ESCRT-0 and ESCRT-II complexes respectively, were up-regulated (Table 1). ESCRT complexes sort ubiquitinated membrane proteins into multivesicular bodies, a key

step in the lysosomal degradation pathway (81). Other transport genes up-regulated in *P. carinii* under hypoxic conditions include FAB1 which is involved in the endocytic vacuolar pathway and is a regulator of vacuolar homeostasis (11), GGA2, a regulator of trafficking of proteins between the golgi and the vacuole (25), and MON2 which has been implicated in trafficking between the late golgi and early endosome (15) and is required for endocytosis and maintenance of vacuolar structure (8,35) (Table 1). In addition to genes involved in intracellular transport, a gene with homology to the pro-apoptotic serine protease NMA11(18) was up-regulated in *P. carinii* under hypoxic conditions (Table 1). A putative FIS1 homolog was also up-regulated in *P. carinii* after hypoxic maintenance (Table 1). In *S. cerevisiae* FIS1 encodes a protein involved in apoptosis (42), mitochondrial fission (42) and peroxisome abundance (46). Previous studies have indicated that deletion of Fis1 is associated with a decrease in peroxisome abundance (46). Thus it can inferred that up-regulation of Fis1 may be associated with peroxisome abundance. In *S. cerevisiae* Snx4 has been implicated in peroxisome degradation or pexophagy (52), and it has been demonstrated that peroxisomes are delivered to the vacuole by mechanisms of both the cytoplasm to vacuole pathway and autophagy(30). Thus up-regulation of genes involved in these three pathways suggests a common molecular signal leading to the induction of these pathways in *P. carinii*.

Up-regulation of putative gene homologs involved in transport pathways in *P. carinii* as a result of hypoxic maintenance indicates that the organism may be under stress- inducing conditions, and that processes involved in the stress response may be down-regulated under hypoxic conditions. Consistent with this is the fact that we observed down-regulation of a total of 26 genes involved in the stress response and

unfolded protein response (Table 2). Putative genes encoding proteins involved in protein folding were down-regulated in *P. carinii* under hypoxic conditions. Protein folding genes down-regulated in our analysis include: JEM1 (63), SSB1 (58), CCT7 (82), CCT8 (82), MGE1 (88), and PHB1 (61). Homologs of *S. cerevisiae* genes required for targeting and degradation of misfolded proteins down-regulated in our analysis include: MNS1 (85,86), HRD3 (69), JEM1 (63), CDC48 (34), ATE1 (4), PSA2 (16), DDI1 (33), KAR2 (73), DER1 (26), GRR1 (47), RPT2 (49), and PUP5 (23) (Table 2). MNS1, HRD3, DER1, JEM1, CDC48, and KAR2 are part of quality control mechanisms in the ER that selectively retain misfolded proteins in an effort to allow for proper folding (62). These mechanisms facilitate the eventual translocation of proteins that cannot be properly folded to the cytosol for ubiquitination and subsequent degradation by the proteasome (62). Interestingly, our transcriptional analysis indicates that subunits of the proteasome including RPT2 (49), PUP2 (23), and PRE8 (16), and regulatory subunits of the proteasome including: RPN1 and BLM10 are also down-regulated in *P. carinii* under hypoxic conditions (Table 2). Other putative *P. carinii* genes down-regulated under hypoxic conditions include those involved in the stress response in mitochondria such as SSC1 (77), HSP60 (70), SOD2 (50), and ERV1 (54), and the stress induced genes MSN2 (51), and PSR2 (36) (Table 2). Taken together these results suggest that hypoxic conditions prevent not only proper folding of proteins in the ER, but also prevent their subsequent degradation resulting in a potential abundance of misfolded proteins both in the ER and mitochondria.

Discussion

Over 500 genes have been found to be affected by environmental levels of oxygen in the yeast *Saccharomyces cerevisiae*, and these play a role in numerous cellular functions including fermentation, cellular respiration, heme biosynthesis, and fatty acid and sterol biosynthesis (32). While the genome of *P. carinii* is not completely annotated, and the information presented in these analyses include only a portion of the genome, these studies provide the first assessment of *P. carinii* under hypoxic conditions, and indicate that *P. carinii* is an obligate aerobe. Fungal pathogens like *C. albicans* and *C. neoformans* can dwell in multiple tissues within the mammalian host, and as a result can be exposed to varying oxygen concentrations (17). Unlike these organisms, extrapulmonary dissemination of *Pneumocystis* spp. is rare, and is found most often in patients in the late stages of Acquired Immune Deficiency Syndrome who have not received prophylactic treatment (87). Thus exposure of *Pneumocystis* to hypoxic conditions is rare, and *P. carinii* may have limited mechanisms to adapt to these conditions. These studies indicate that the viability of *P. carinii* is severely attenuated under hypoxic conditions.

The increase in ATP levels of the *P. carinii* organisms maintained under microaerophillic conditions *in vitro* suggests that these organisms may be able to survive in similar levels of CO_2 *in vivo*. These data suggest that the metabolism of *P. carinii* may be altered under conditions of raised CO_2 levels providing alternative methods for acquiring necessary sterols. Our assessment of sterol uptake revealed that the ability of *P. carinii* to take up exogenous cholesterol under normal oxygen tensions resembles that of the filamentous fungal pathogen *Aspergillus fumigatus* (89). *A. fumigatus* was

demonstrated to have the ability to take up exogenous sterols under aerobic conditions, and the addition of serum to media enhanced growth and cholesterol uptake (89). In our analyses, much of the imported cholesterol appeared to localize to putative lipid particles in *P. carinii* suggesting it was stored as cholesterol ester, similar to findings of cholesterol import in *A. fumigatus* (89). Import of exogenous cholesterol by *A. fumigatus* after the addition of itraconazole resulted in decreased efficacy of the anti-fungal, indicating that cholesterol import attenuated the effect of the drugs that target the sterol pathway (89).

P. carinii is resistant to clinically used azole anti-fungals, however, studies have indicated that the organism is susceptible to proprietary azoles, and other drugs targeting sterol biosynthesis (37). It is unknown whether targeting sterol enzymes of *P. carinii* will increase cholesterol import, however, these studies suggest that inhibition of sterol biosynthesis in *P. carinii* is not a prerequisite for cholesterol import. The ability of *P. carinii* to import exogenous cholesterol under normal oxygen tensions suggests that the sterol pathway of *P. carinii* does not produce a sterol that is comparable to fulfill the membrane requirements of *P. carinii*. Thus, the ability of *P. carinii* to import exogenous cholesterol is necessary for viability these fungi. Consequently, unpublished results from our lab indicate that maintenance of *P. carinii* in medium lacking serum, which is a significant source of cholesterol, results in rapid loss of viability. Our studies indicate that *P. carinii* employs a robust sterol scavenging mechanism that is independent of environmental oxygen concentration. These studies suggest that while *P. carinii* encodes functional homologs involved in sterol biosynthesis, both import of exogenous sterols and *de novo* sterol biosynthesis are necessary to provide the sterol requirements for *P.*

carinii.

The transcriptional assessment of *P. carinii* presented here indicated that maintenance of *P. carinii* under hypoxic conditions resulted in a down-regulation of genes required for sterol biosynthesis in *P. carinii*. With the exception of ERG1, we did not observe up-regulation in sterol genes whose products require molecular oxygen for activity. Transcription of lanosterol C-14 demethylase (Erg11), sterol C-4 methyloxidase (Erg25), sterol C-5 desaturase (Erg3) , and sterol C-22 desaturase (Erg5) were up-regulated in previous analyses of the transcriptional response of *C. albicans* (79) and *S. pombe* under hypoxic conditions (84). The activity of these enzymes is dependent on molecular oxygen, and as a result, hypoxic conditions result in decreased activity of these enzymes. The decreased activity of these enzymes leads to transcriptional up-regulation of the genes encoding these enzymes in *S. pombe* and *S. cerevisiae* and is mediated by the Sre1 and Upc2, respectively. Sre1 is the principal activator of anaerobically expressed genes and is required for expression of every anaerobically up-regulated sterol enzyme downstream of lanosterol in *S. pombe* (84). Homologs for neither UPC2 nor SRE1 have been identified within the *P. carinii* genome. In *S. pombe* Erg1 was regulated independently of Sre1 (84), suggesting an unknown mechanism of induction in both *S. pombe* and *P. carinii*. Although the *P. carinii* sterol gene encoding Erg11 has been identified and characterized, the genes encoding Erg25, Erg3, and Erg5 have not been identified within the its genome. Consequently, we speculate that down-regulation of sterol genes in *P. carinii* is likely due to the apparent lack of their hypoxic inducers concomitant with an inability of *P. carinii* to adapt hypoxic conditions.

The transcriptional profile of *P. carinii* under hypoxic conditions suggests a loss of ER quality control mechanisms that may contribute to the decreased viability of *P. carinii* under low oxygen conditions. Proper cellular homeostasis requires functional quality control systems that assist cells in eliminating terminally misfolded proteins via the help of molecular chaperones (44). Accumulation of misfolded proteins is potentially toxic to cells due to exposure of hydrophobic and free cysteine residues which can lead to protein aggregation and induce ER stress (44,62). Therefore, if molecular chaperones are unable to facilitate proper folding of proteins, terminally misfolded proteins are removed from the ER via ER-associated degradation (ERAD) (62). Our transcriptional analysis indicates that hypoxic maintenance of *P. carinii* resulted in the down-regulation genes encoding multiple molecular chaperones, and transcription of the molecular chaperone KAR2 was decreased the most drastically in our analysis. Kar2 has been implicated as a mediator of ERAD substrate selection, and all known substrates for ERAD require Kar2 for degradation (19,62). Loss of Kar2 function results in aggregation and significant impairment of degradation (64). In addition to down-regulation of Kar2, several other genes required for ERAD function were down-regulated suggesting that hypoxic maintenance of *P. carinii* may result in an accumulation of misfolded proteins in the ER. Accumulation of such proteins combined with the down-regulation of genes required for their removal may result in loss of cellular homeostasis in *P. carinii*.

The ERAD pathway is the primary mechanism cells use to handle misfolded proteins that accumulate in the ER (55), and it has been speculated that autophagy may function as an alternate in the event that this accumulation overwhelms the ERAD capacity (91). Addition of dithiothreitol, an inhibitor of protein folding and inducer of

ER stress, resulted in autophagy in yeast cells (90). ER stress has been linked to autophagy in several studies (6,7,48,91) implicating autophagy as another ER quality control mechanism. Yeast have other autophagy-like pathways in addition to bulk autophagy including: the cytoplasm to vacuole pathway and pexophagy (71,72), and genes involved in all three of these pathways were up-regulated in *P. carinii* under hypoxic conditions. While we did not determine whether hypoxic conditions result in accumulation of misfolded proteins, we speculate that down-regulation of genes responsible for ensuring proper protein folding concomitant with down-regulation of genes required for ERAD would permit misfolded proteins to accumulate in the ER and result in ER stress in *P. carinii*. Under stress conditions, cells initiate multiple pathways that lead to either cell death or survival, and autophagy has been implicated as having either a pro-death function or pro-survival function. In the event that cells are unable to regain proper homeostasis, cell death will occur despite the initiation of pro-survival mechanisms (22). Thus, we propose that hypoxic conditions induce ER stress in *P. carinii* resulting in induction of autophagy, however, despite induction of autophagy, *P. carinii* is unable regain proper homeostasis, resulting in loss of viability.

These studies provide insight into mechanisms that are affected by hypoxic maintenance of *P. carinii*, and suggest for the first time the presence of genes involved in the bulk degradative processes of autophagy, cytoplasm to vacuole transport and pexophagy, in addition to the ER quality control mechanism of ERAD. Additionally, these studies confirm that *P. carinii* encodes genes involved in sterol biosynthesis, and establish that the cholesterol present in the membranes of *P. carinii* is likely scavenged from its host. These studies show *P. carinii* is a dynamic pathogen that is able to respond

to changes in environmental conditions. Future molecular and microscopic analyses are necessary to determine the presence of marker proteins indicating the activity of these pathways and to visualize the presence of autophagosomal complexes in *P. carinii*.

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Table 1. Putative *P. carinii* genes up-regulated in response to hypoxic conditions

Genes invovled in transport

Gene Name	Gene Description	Fold Δ Avs5	p Avs5
FAB1	1-phosphatidylinositol-3-phosphate 5-kinase FAB1	2.001	0.018
SEC31	Protein transport protein Sec 31	4.735	0.000
USO1	Intracellular protein transport protein USO1	1.549	0.008
GGA2	ADP-ribosylation factor-binding protein GGA2	2.932	0.000
MON2	Protein MON2	2.191	0.000
COG4	Conserved oligomeric Golgi complex subunit 4	1.621	0.000
SEC2	Rab guanine nucleotide exchange factor SEC2	1.821	0.005
SEC17	Alpha-soluble NSF attachment protein	1.762	0.043
SEC24	Protein transport protein sec24	3.486	0.000
VPS34	Phosphatidylinositol 3-kinase VPS34	2.112	0.008
HSE1	Class E vacuolar protein-sorting machinery protein HSE1	1.865	0.005
VPS36	Vacuolar protein-sorting-associated protein 36	2.289	0.000
SNX4	Sorting nexin-4	2.406	0.048

Genes involved in apoptosis and autophagy

Gene Name	Gene Description	Fold Δ Avs5	p Avs5
SEC18	Vesicular-fusion protein sec18	1.683	0.026
NM111	Pro-apoptotic serine protease nma111	1.812	0.001
FIS1	Mitochondria fission 1 protein	1.907	0.003
ATG15	Putative lipase atg15	3.952	0.000

Table 2. Putative *P. carinii* genes down-regulated under hypoxic conditions

Gene involved in Lipid Biosynthesis

Gene Name	Gene Description	Fold Δ Avs5	p Avs5
FAA1	Long-chain-fatty-acid--CoA ligase 1	-3.705	0.000
ALE1	Lysophospholipid acyltransferase	-2.273	0.001
IFA38	3-ketoacyl-CoA reductase	-1.870	0.010
PSD2_SCHPO	Phosphatidylserine decarboxylase proenzyme 2	-1.715	0.025
ACL1_SCHPO	Probable ATP-citrate synthase subunit 1	-1.532	0.002
ETR1_SCHPO	Probable trans-2-enoyl-CoA reductase	-1.478	0.002
LCB2	Serine palmitoyltransferase 2	-1.425	0.049
APT1	Acyl-protein thioesterase 1	-1.370	0.017
ACL2_SCHPO	Probable ATP-citrate synthase subunit 2	-1.360	0.033

Gene involved in Sterol Biosynthesis

Gene Name	Gene Description	fold Δ Avs5	p Avs5
RAR1	Putative mevalonate kinase	-2.689	0.001
ERG13	Hydroxymethylglutaryl-CoA synthase	-2.543	0.009
ERG6	Sterol 24-C-methyltransferase (Erg6)	-2.166	0.000
IDI1	Isopentenyl-diphosphate Delta-isomerase	-2.096	0.000
HMG1	hydroxy-3-methylglutaryl-coenzyme A reductase 1	-2.013	0.005
ERG10	Acetyl-CoA acetyltransferase (Erg10)	-1.915	0.002
FPP1	Farnesyl pyrophosphate synthetase	-1.800	0.007
ERG9	Squalene synthetase (Erg9)	-1.323	0.025

Genes involved in the stress response

Gene Name	Gene Description	Fold Δ Avs5	p Avs5
MNS1	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase	-2.688	0.000
KAR2	KAR2/BIP	-6.146	0.000
HRD3	ERAD-associated E3 ubiquitin-protein ligase component HRD3	-2.299	0.007
JEM1	DnaJ-like chaperone JEM1	-3.187	0.003
CDC48	Cell division control protein 48	-1.876	0.049
ATE1	Arginyl-tRNA--protein transferase 1	-2.893	0.000
DER1	ER-associated proteolytic system protein Der1, putative	-1.452	0.005
RPT2	protease regulatory subunit 4 homolog	-1.536	0.014
MGE1	GrpE protein homolog, mitochondrial	-1.717	0.031
GRR1	E3 ubiquitin ligase complex F-box protein GRR1	-1.626	0.005
DDI1	DNA damage-inducible protein 1	-2.419	0.001
BSD2	Metal homeostatis protein	-1.932	0.016
VPS24	Vacuolar protein sorting-associated protein 24	-1.555	0.011
CCT7	T-complex protein 1 subunit eta	-2.261	0.000
CCT8	T-complex protein 1 subunit theta	-1.485	0.045
TPS2	Trehalose-phosphatase	-2.699	0.006
MRS11	Mitochondrial import inner membrane translocase subunit TIM10	-1.638	0.037
SOD2	Superoxide dismutase [Mn], mitochondrial	-1.775	0.007
PHB1	Prohibitin-1	-1.885	0.000
HSP60	Heat shock protein 60	-1.817	0.001
SSB1	Heat shock protein SSB1	-4.053	0.000
ERV1	Mitochondrial FAD-linked sulfhydryl oxidase ERV1	-1.863	0.013
MSN2	Zinc finger protein MSN2	-1.475	0.014
PSR2	Probable phosphatase PSR2	-1.474	0.004
ABC1	Protein ABC1, mitochondrial; Flags	-2.004	0.044
SSC1	Heat shock protein SSC1	-2.042	0.000

Genes encoding proteasomal subunits

Gene Name	Gene Description	Fold Δ Avs5	p Avs5
RPN1	26S proteasome regulatory subunit rpn1	-3.215	0.041
BLM10	Proteasome activator BLM10	-2.217	0.001
PRE8	Proteasome component Y7	-4.382	0.004
PUP2	Proteasome component PUP2	-2.043	0.000

Figure Legends

Fig. 1. Effect of oxygen tension on *P. carinii* viability. ATP levels of *P. carinii* organisms under standard (S), microaerophilic (MA), and anaerobic (AN) conditions were assessed by the ATP bioluminescent assay over the course of seven days. Data represent the means ± SD, n = 3. Asterisks indicate statistically significant differences ($P < 0.05$) between the ATP levels of the MA and AN to the S at the same number of incubation days.

Fig. 2. *P. carinii* NBD-cholesterol uptake. *P. carinii* was maintained under standard conditions in either serum free or serum containing medium for two hours followed by incubation with NBD-cholesterol. The amount of intracellular NBD-cholesterol acquired over time was quantified using the POLARstar Optima, and the red bars indicate intracellular NBD-cholesterol from *P. carinii* maintained in serum free medium, while the blue bars indicate the amount of NBD-cholesterol in *P. carinii* maintained in serum containing medium.

Fig. 3. *P. carinii* NBD-cholesterol localization. *P. carinii* were incubated in serum free medium for one hour prior to the addition of NBD-cholesterol. Intracellular NBD-cholesterol was visualized using a Nikon Eclipse E600 fluorescent microscope. Panel 1. (A) DIC image of *P. carinii* (B) Intracellular NBD-cholesterol (green) within *P. carinii* (C) Merged DIC and NBD-cholesterol images. Panel 2 (A) DIC image of *P. carinii* (B) Intracellular NBD-cholesterol (green) (C) Mitochondria of *P. carinii* (red) (D) Merged DIC, NBD-cholesterol, and mitochondrial images. Yellow images indicate regions of co-

localization between the two fluorophores, and the arrow in Panel 2D indicates an area of co-localization between NBD-cholesterol and Mitotracker Red®. Panel 3 (A) DIC image of *P. carinii* (B) Intracellular NBD-cholesterol (green) (C) Nile Red stained lipid particles of *P. carinii* (red) (D) Merged DIC, NBD-cholesterol, and Nile Red images. The arrow in Panel 3D indicates an area of co-localization between NBD-cholesterol and Nile Red.

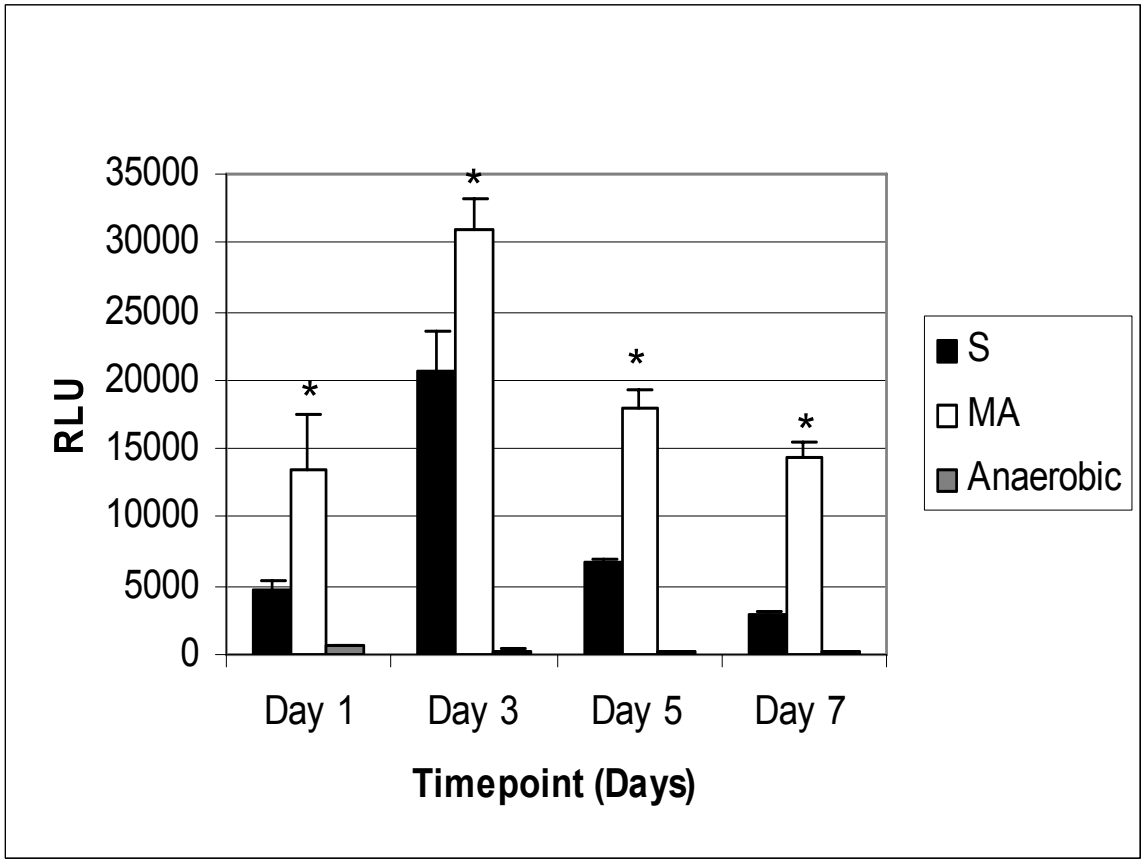


Figure 1. Effect of oxygen tension on *P. carinii* viability

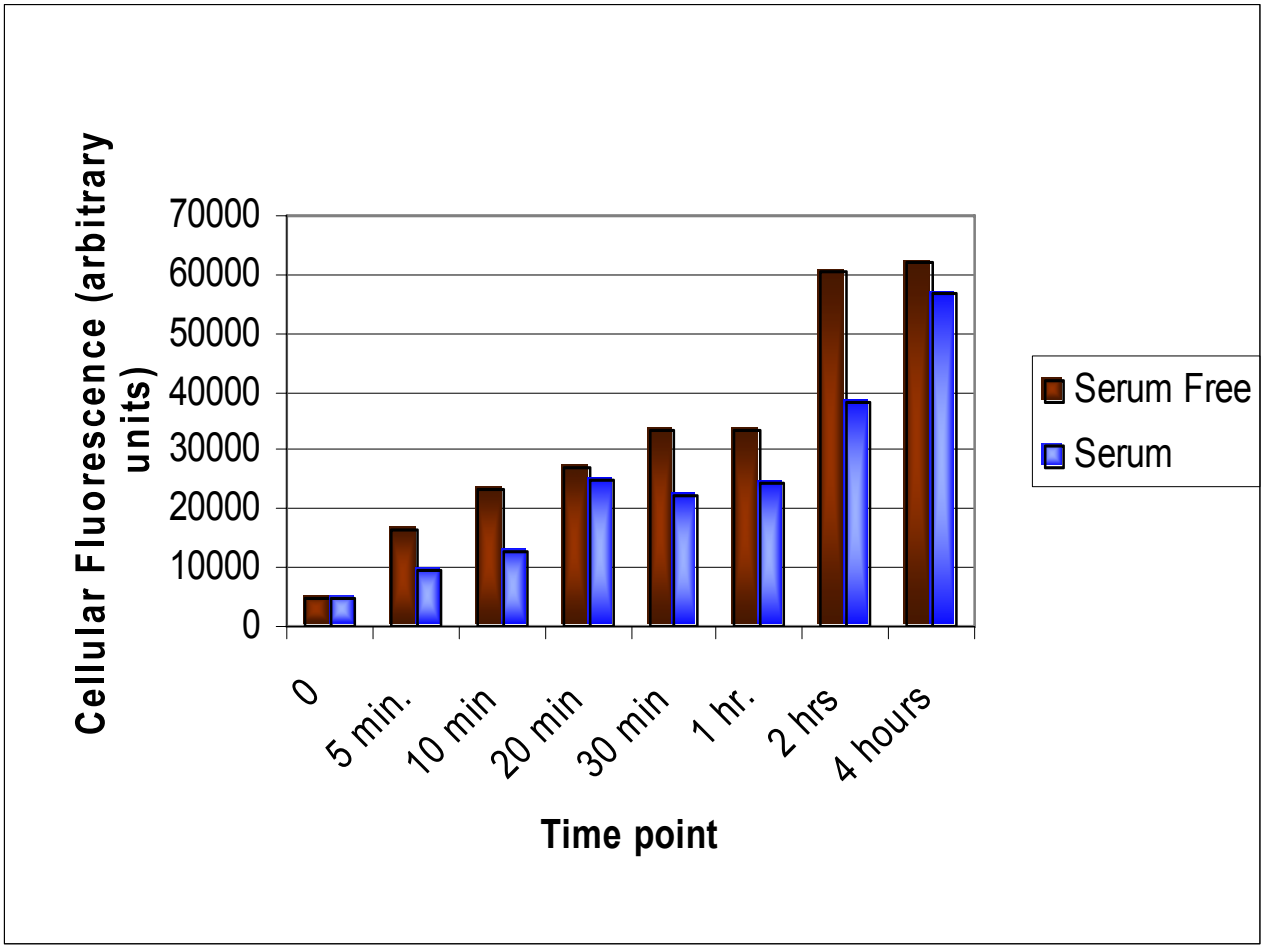
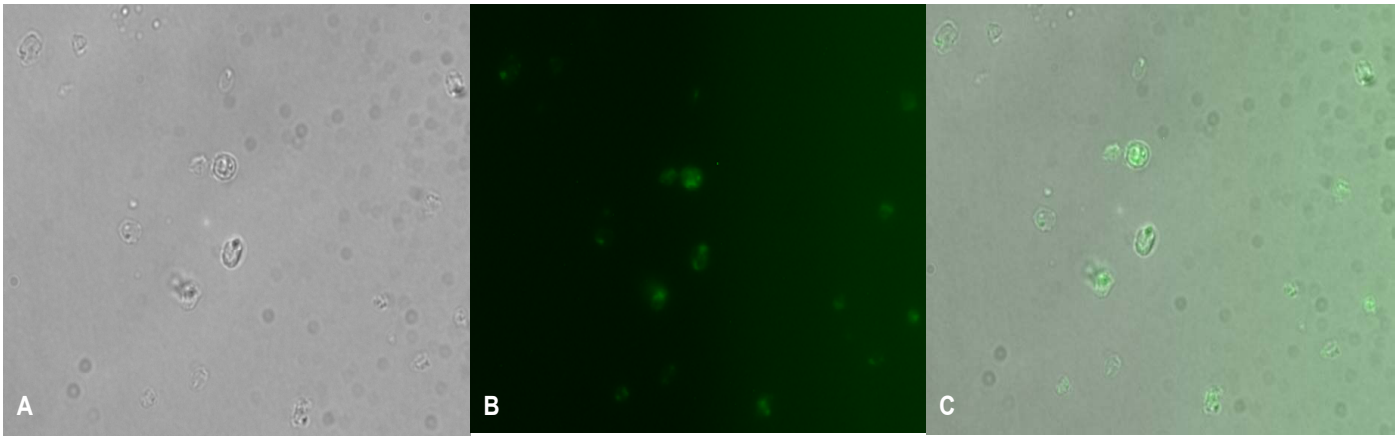
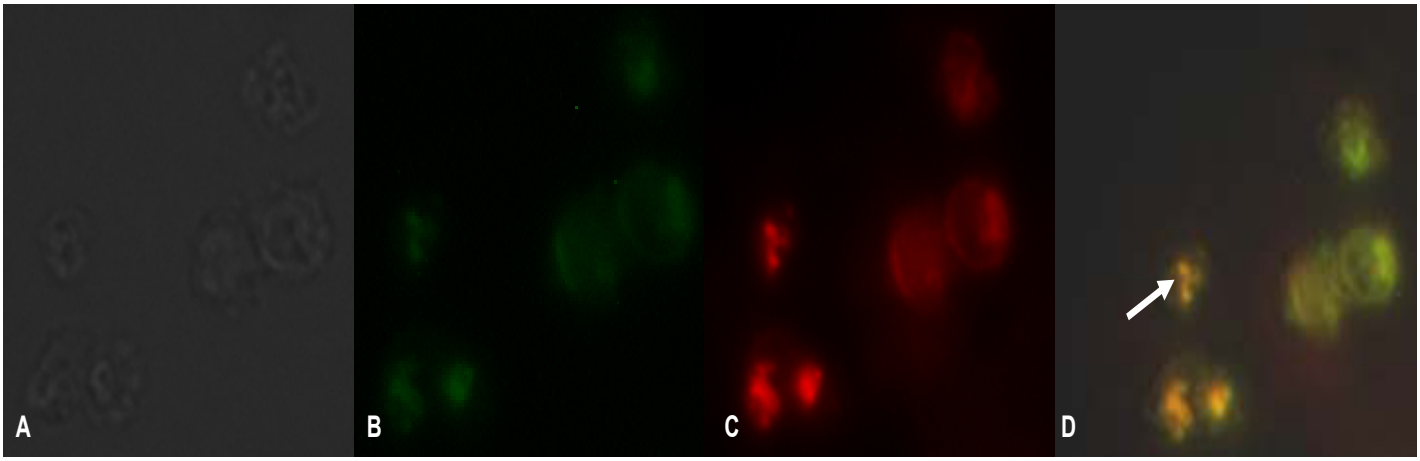


Figure 2. *P. carinii* NBD-cholesterol uptake

Panel 1



Panel 2



Panel 3

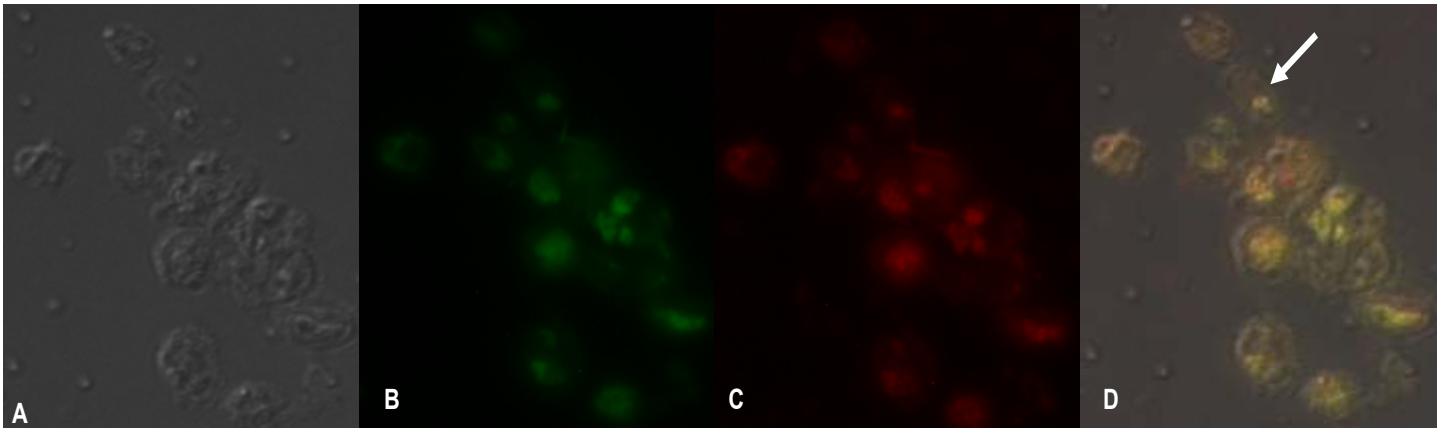


Figure 3. *P. carinii* NBD-cholesterol localization

Conclusions and Future Directions

The placement of *Pneumocystis* spp. in the fungal kingdom was a major step towards understanding the biology of these organisms, but much is still unknown about these atypical fungi. The inability of *P. carinii* to synthesize ergosterol, the substitution of cholesterol as the bulk sterol, combined with the lack of efficacy of standard anti-fungal drugs that target the sterol pathway of *P. carinii* would seem to indicate that *de novo* sterol synthesis does not occur in *P. carinii*. The studies detailed in Chapter two confirm that *P. carinii* encodes a functional lanosterol synthase that is similar in both function and localization to that of the model fungal organism *S. cerevisiae*. The high degree of amino acid conservation is consistent with it's placement within the fungal kingdom. The reaction catalyzed by mammalian lanosterol synthase has been called the most complex reaction in human biology (10). Lanosterol synthase 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, lanosterol synthase 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 (22). While the crystal structure of a fungal lanosterol synthase has not been resolved, the intricacy of the reaction catalyzed by the mammalian enzyme and the importance of the enzyme to sterol biosynthesis requires not only the retention of the enzyme, but also a high degree of conservation within the primary amino sequence to facilitate the reaction. Thus the ability of *P. carinii* to produce a functional lanosterol synthase that is similar to that of other fungal homologs is consistent with the thought that *P. carinii* is able to synthesize sterols via *de novo* sterol pathway.

Lanosterol synthases are widely regarded as integral membrane proteins (3,24,27), 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 (18,19). In contrast, a previous study of *P. carinii* lanosterol synthase failed to show localization to these organelle-like structures, but rather found the enzyme in yeast microsomal fractions (19). In the present work, we showed that *P. carinii* lanosterol synthase does localize to lipid particles, both in the heterologous yeast system and in the native fungus, *P. carinii.* In *S. cerevisiae* a functional sterol 3-ketoreductase (Erg27p) is necessary for lanosterol synthase localization and activity in lipid particles (21). Erg27 is an essential enzyme in *S. cerevisiae*, and previous studies have shown that yeast strains lacking a functional Erg27p, accumulate squalene, and produce a truncated lanosterol synthase that mislocalizes to microsomal compartments in the yeast rather than lipid particles (21). Additionally, yeast cells that lack lipid particles are defective in sterol biosynthesis due to the loss of activity of the sterol enzyme squalene epoxidase (26). As a result there was a marked decrease in the incorporation of ergosterol into the plasma membrane, and the mutant was hypersensitive to the anti-fungal drug terbinafine (26). Consequently, both Erg27p and lipid particles are necessary for sterol biosynthesis in *S. cerevisiae*.

The identification of lipid particles in *P. carinii* implies the existence of a cellular trafficking system that includes a chaperoning step via Erg27p to cytoplasmic lipid particles. We have identified a homolog of PcErg27p in the *Pneumocystis* genome, and the identification of lipid particles and a functional lanosterol synthase in *P. carinii* suggests that a similar mechanism involved in trafficking lanosterol synthase to lipid

particles exists in *P. carinii*. Sequestration of PcErg7p in lipid particles also suggests the presence of an activation mechanism and the proximity of Erg7 to other sterol pathway proteins in lipid particles suggests an operational sterol pathway. A determination of the enzymes contained within lipid particles in *P. carinii* may help to provide further insight on *de novo* sterol biosynthesis in the organism. In addition, it is intriguing to note that localization of *P. carinii* lanosterol synthase to lipid particles in the yeast heterologous system implies that *S. cerevisiae* Erg27p may have been involved in the transport of *P. carinii* lanosterol synthase to lipid particles. Further studies are warranted to determine whether there is a specific interaction between *P. carinii* lanosterol synthase and *S. cerevisiae* sterol Erg27p that facilitates localization of *P. carinii* lanosterol synthase to lipid particles in yeast.

The overall environment and the microenvironments within the mammalian lung alveoli are complex and it is apparent that studies of *Pneumocystis* spp. should be put in the context of these conditions. During infection, *Pneumocystis* attaches to type I pneumocytes in the lung. Type I cells facilitate O_2 and CO_2 exchange across the alveolar surface, and *Pneumocystis* infection is thought to impede this exchange. Enzymes in the sterol pathway require oxygen. Within the *Pneumocystis* infected lung, it has been suggested that there are microenvironments comprised of differing O_2 and CO_2 content (in biofilms, e.g.) (Figure 1). To begin to dissect the biology of these fungi under conditions more relevant to their *in vivo* setting, we set out to determine conditions under which *P. carinii* scavenges sterols, and to determine the effect of oxygen on *P. carinii* viability, drug response, gene expression and scavenging mechanisms. These studies

investigated the role such conditions had on *P. carinii* gene expression profiles, viability and drug response.

The studies in Chapter three detail the affects of both O_2 and CO_2 on the viability and drug response of *P. carinii*. The viability of organisms maintained under anaerobic conditions was significantly reduced, and anaerobic conditions also resulted in increased susceptibility to anti-*Pneumocystis* drugs. In contrast, maintenance under microaerophillic conditions did not result in a drastic decline in ATP, and while anti-*Pneumocystis* drugs resulted in a loss of viability, the effect was delayed. The ability of fungal pathogens to respond to changes in CO_2 is critical for virulence. For instance, at high levels of CO_2 such as those found in human tissues (5% vs. 0.036% in ambient air), the fungal pathogen *Candida albicans* produces hyphae (25) while *Cryptococcus neoformans* produces a polysaccharide capsule(28). Both hyphal and capsular production are absolutely necessary for the virulence of their respective organism (9,23). The effects of CO_2 are mediated through adenylyl cyclase (AC), but prior to signaling through AC, CO_2 must be converted to bicarbonate, the biologically active form of CO_2. The conversion of CO_2 to bicarbonate occurs spontaneously albeit at a slow rate when the concentration of CO_2 is low such as atmospheric concentrations of CO_2. Organisms that dwell in ambient conditions overcome this by expression of the enzyme carbonic anhydrase (CA) which accelerates the conversion 10 million-fold(20), but CA is dispensable for growth at high concentrations of CO_2 such as those found in human tissues (1,2,15). A gene with homology to CA has been not found within the genome of *P. carinii* suggesting that *P. carinii* is unaccustomed to low levels of CO_2. Consistent with this, is the fact that an environmental reservoir for *Pneumocystis* spp has not been

located, and the only known niche for *Pneumocystis* spp is the mammalian lung. Additionally, unpublished data from our lab indicates that ATP levels of *P. carinii* decrease drastically under ambient conditions, and while increases in pH may be a factor in this decline, it cannot be ruled out that the low levels of CO_2 affect the viability of *P. carinii* under these conditions. The studies in Chapter three suggest that *P. carinii* may be exposed to high levels of CO_2 levels in the lung, and therefore the organism is amenable to high levels of CO_2.

The decrease in viability due to anaerobic conditions is likely, at least in part, due to a decreased ability to respond to ER stress. Protein folding and ERAD are normal cellular processes that aid the cell in maintaining proper homeostasis, however decreased protein folding capabilities overwhelm ERAD leading to ER stress. Down-regulation of genes involved in these processes up-regulate autophagy. The studies outlined in Chapter 4 suggest that autophagy, and the autophagy like pathways of pexophagy and the cytoplasm to vacuole transport are active pathways in *P. carinii*, and are induced in response to anaerobic conditions. Autophagy is an evolutionarily conserved pathway in virtually all eukaryotic organisms, and is the primary mechanism for degrading and recycling ageing proteins and organelles (17). In yeast, autophagy occurs in response to both extracellular stress conditions such as: nutrient starvation, hypoxia, and high temperature, as well as intracellular stress conditions such as: accumulation of damaged or superfluous organelles and cytoplasmic components (17). Thus, the data presented in Chapter three suggest that the mechanism of autophagy induction is similar to induction in *P. carinii*. Autophagy has been implicated as both a pro-survival and pro-death mechanism in response to nutrient starvation, hypoxia, and stress (6,7). Autophagy has

been classified as a form of cell death that is induced as a response to these conditions (16), but its induction under these conditions also implicate autophagy as a pro-survival mechanism that functions to facilitate the reestablishment of cellular homeostasis (6). Nevertheless, in the event that proper homeostasis cannot be restored, cell death is eminent, and induction of the putative apoptotic serine protease NM11 (5) in *P. carinii* along with FIS1 which plays a role in early apoptotic events (14) suggest that *P. carinii* is unable to retain homeostasis under anaerobic conditions and induces an apoptotic cascade.

The levels of environmental O_2 affect many cellular processes, and in *S. cerevisiae* sterol scavenging occurs under conditions where oxygen is limited due to the down-regulation of enzymes involved in ergosterol biosynthesis. The presence of cholesterol within the membranes of *Pneumocystis* indicates that the organism may not only employ mechanisms to synthesize sterols *de novo*, but the organism has the ability to scavenge cholesterol from its mammalian host. In Chapter three the ability of *P. carinii* to scavenge cholesterol was assessed using the fluorescent cholesterol analog, NBD-cholesterol. In contrast to what has been observed in *S. cerevisiae*, *P. carinii* was able to take up NBD-cholesterol and traffic the exogenous sterol to cellular membranes under normal O_2 tensions. The ability of *P. carinii* to scavenge cholesterol under these conditions is intriguing in light of the fact that the sterol pathway is functional under these conditions and suggests that sterol scavenging is a survival mechanism of *P. carinii*. Thus, despite the lack of ergosterol in *P. carinii*, and extensive sterol scavenging mechanisms employed by the organism, *P. carinii* retains sterol genes and the ability to scavenge sterols because both are necessary for the viability of *P. carinii* under normal

O_2 tensions. This emphasizes the fact that drugs targeting the sterol pathway of *P. carinii* may be of limited use.

The goals of this work were to characterize the *P. carinii* lanosterol synthase, determine conditions under which *P. carinii* scavenges sterols, and to determine the effect of oxygen on *P. carinii* viability, drug response, and gene expression. The essential nature of sterols in eukaryotic organisms has made the ergosterol pathway an attractive drug target for anti-fungal therapy. The work described here highlights the similarities between *P. carinii* lanosterol synthase and the same enzyme in *S. cerevisiae* indicating a functional sterol pathway in *P. carinii*. This work also highlights the robust sterol scavenging mechanism of *P. carinii* which is employed under conditions that prevent sterol scavenging in *S. cerevisiae*. However it is unknown how inhibition of *P. carinii* sterol enzyme might affect this process or sterol biosynthesis. Although the lack of ergosterol has made *Pneumocystis* (spp) resistant to polyene antifungal drugs that target ergosterol, other studies have shown the organisms are susceptible to drugs targeting sterol enzymes (4,12,13). The *P. carinii* C-24 methyltransferase sterol enzyme has been proposed to be a novel anti-*Pneumocystis* drug target due to the lack of the enzyme in the mammalian sterol pathway (13) and the fact that the organisms contains a large variety of 24-alklyated sterols (8). Additionally, despite the presence of lanosterol synthase in mammalian cells, it *P. carinii* and mammalian enzymes have varying sensitivities to drugs that target the enzyme (11). Many of the studies identifying putative anti-*Pneumocystis* drug targets within the sterol pathway were performed *in vitro*, but the utility of these inhibitors against *Pneumocystis in vivo* remains in question due to the ability of the organism to scavenge cholesterol and other sterols from the host

environment. Therefore future studies analyzing the sterol pathway as a potential anti-*Pneumocystis* drug target should account for both sterol biosynthesis and sterol scavenging mechanisms in *Pneumocystis* spp.

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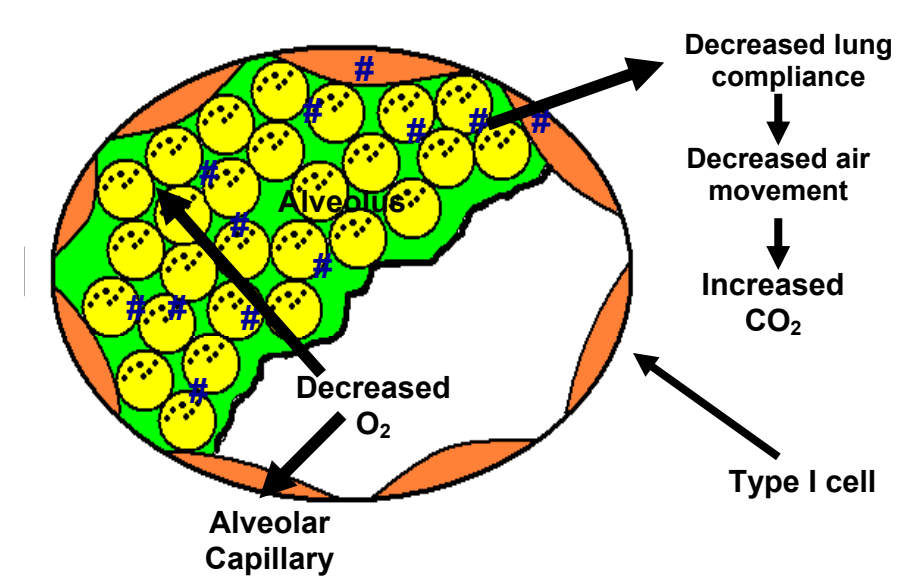

Figure 1. Working model of *Pneumocystis* exposure to low oxygen and high carbon dioxide concentrations in the lung.