I, Laura Johnston, hereby submit this original work as part of the requirements for the degree of Master of Science in Biological Sciences.

It is entitled:
Optimization and Expression of the <i>Pneumocystis carinii erg6</i> Gene in a <i>Saccharomyces cerevisiae erg6</i> Deletion Mutant

Student’s name: Laura Johnston

This work and its defense approved by:

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Committee member: Jodi Shann, PhD
Committee member: James Stringer, PhD
Optimization and Expression of the *Pneumocystis carinii erg6* Gene in a *Saccharomyces cerevisiae erg6* Deletion Mutant

A thesis submitted to the Division of Research and Advanced Studies at the University of Cincinnati in partial fulfillment of the requirements for the degree of

Master of Science

In the Department of Biological Sciences
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ABSTRACT

*Pneumocystis* is a genus that causes a type of pneumonia, known as PCP, in immune deficient mammals. In contrast to most fungi, whose major membrane sterol is ergosterol, cholesterol scavenged from the mammalian host is the predominant sterol in *Pneumocystis*. Since *Pneumocystis* lacks ergosterol, and most traditional anti-fungal therapies target ergosterol biosynthesis, an alternate drug target is needed.

Although cholesterol is the major sterol in organisms grown in animals or culture media supplemented with mammalian sera, *Pneumocystis* produces *de novo* unique 28 and 29 carbon sterols with alkylations at the C-24 position of the sterol side chain. These 24-alkyl sterols have been shown to be important for *Pneumocystis* viability and proliferation. The S-adenosyl-L-methionine: sterol C-24 methyltransferase enzyme (SMT), coded by the *erg6* gene, catalyzes the transfer of methyl groups from S-adenosyl-L-methionine to the C-24 position of the sterol side chain. This enzyme, critical to *Pneumocystis*, is not present in the mammalian host, and therefore represents an intriguing chemotherapeutic target.

The *Pneumocystis carinii erg6* gene has been sequenced, cloned, and the recombinant SMT expressed in *E. coli*, and *T. thermophila*. These expression systems were of limited utility, so *Saccharomyces cerevisiae* was examined as a potential expression host. An *erg6* null strain of *S. cerevisiae* was commercially available and chosen as an expression host to eliminate the potential for endogenous SMT activity. Initial attempts to express the *P. carinii erg6* in *Saccharomyces* showed that the gene was transcribed, but the recombinant *P. carinii* SMT was not clearly present.

Codon usage was one of the potential factors affecting the production of the recombinant *P. carinii* SMT in *S. cerevisiae*. It was found that although *Pneumocystis* and *Saccharomyces*
both have A-T rich genomes, codon usage was actually quite different. Therefore a synthetic \textit{P. carinii} erg6 gene, optimized for expression in \textit{S. cerevisiae} was constructed, cloned into a \textit{S. cerevisiae} expression vector, and transformed into an erg6 null strain of \textit{Saccharomyces cerevisiae}. The native \textit{P. carinii} erg6 gene, and the \textit{S. cerevisiae} erg6 gene were also cloned and transformed in the same manner as controls. It was demonstrated that the \textit{P. carinii} SMT was expressed in \textit{S. cerevisiae}. This system will be useful for the production of sterols and recombinant SMT for further studies.
Acknowledgments

First and foremost, I would like to thank Dr. Edna Kaneshiro for believing that I could figure it out. I would like to thank my committee for their advice and support and reminding me that research is about working past problems. I would also like to thank my lab mates: Jenny Custer, Ted Wright and Dr. Steves Nkinin for the moral support and comradeship. I would like to thank Julie Stacey and Cathy Hayward for lending me equipment and supplies and always having candy. Thank you to Dr. Charlotte Paquin for all of the yeast advice. Thank you to my parents for being patient about my schedule and being supportive of my goals, whatever they have been and as often as they have changed. This research was supported in part by an NIH grant RO106084 to Dr. Kaneshiro.
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>SAM:SMT</td>
<td>S-adenosyl-L-methionine: sterol methyltransferase</td>
</tr>
<tr>
<td>SMT</td>
<td>Sterol methyltransferase</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>PnP</td>
<td>Pneumocystis carinii Pneumonia</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
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<tr>
<td>AID</td>
<td>Aids Defining Illness</td>
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<tr>
<td>OI</td>
<td>Opportunistic Infection</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TMP-SMX</td>
<td>Trimethoprim-Sulfamethoxazole</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>1N</td>
<td>Haploid</td>
</tr>
<tr>
<td>2N</td>
<td>Diploid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas-Liquid Chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>CAI</td>
<td>Codon Adaptation Index</td>
</tr>
<tr>
<td>JCAT</td>
<td>Java Codon Adaptation Tool</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>HIS</td>
<td>Histidine</td>
</tr>
<tr>
<td>S.c.-URA</td>
<td><em>S. cerevisiae</em> minimal media without uracil</td>
</tr>
<tr>
<td>YEPD</td>
<td>Yeast Extract Peptone Dextrose</td>
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<tr>
<td>TE</td>
<td>Tris-EDTA</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>MWM</td>
<td>Molecular weight markers</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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</table>
1. INTRODUCTION

1.1 Rationale

The *Pneumocystis* genus of opportunistic fungal pathogens causes a type of potentially lethal pneumonia known as PCP or pneumocystosis in immunodeficient hosts. Although progress in treatment and prophylaxis has been made, *Pneumocystis* continues to be a major cause of morbidity and mortality in AIDS patients (Grabar et al., 2008). Lack of a long term *in vitro* culture system has hindered biochemical and drug development. Drugs that have been found effective in clearing infections by ergosterol-synthesizing fungi have been shown to be ineffective against *Pneumocystis* (Kaneshiro et al., 2000; Bartlett et al. 1994) suggesting the need for alternative chemotherapeutic agents.

The objective of this study was to produce a system in which activity of the S-adenosyl-L-methionine: sterol C-24 methyltransferase (SAM:SMT) enzyme, an alternative target for development of anti-*Pneumocystis* therapies, could be further explored. The system was designed to overcome limitations of some previous systems by producing the recombinant SMT enzyme in an expression host that does not form insoluble inclusion bodies as in bacteria, have high proteolytic activity as in ciliates, or overcome codon bias as a barrier to expression of the recombinant gene.

1.2 History of *Pneumocystis*

Discovery

*Pneumocystis* was originally discovered in 1909, by the Brazilian discoverer of *Trypanosoma cruzi*, Carlos Chagas. He erroneously identified the organism as a pulmonary stage of *Trypanosoma cruzi* (Chagas, C., 1909). Later, Antonio Carini, observed that the organisms were not always associated with heavy *Trypanosome* loads, and sent samples to the Pasteur Institute
for further analysis (Carini, 1910). Delanöe and Delanöe (1912) determined that the organisms in question were not Trypanosomes and named the reclassified organism Pneumocystis carinii. After its description by the Delanöes in 1912, Pneumocystis was found in mice, rats, guinea pigs, monkeys and rabbits (Hughes, 2005).

**Association with Immunodeficiency and AIDS**

Pneumocystis was linked to an outbreak of untreatable pneumonia in premature, malnourished infants in nurseries throughout Europe (Hughes, 2005). The connection between Pneumocystis and compromised immune systems was not made until 1974 when a study took advantage of the fact that the only FDA-approved pentamidine treatment for PcP had to be obtained from the Center for Disease Control (CDC). Certain clinical data, such as underlying diseases were required in order to procure the drug. A correlation between PcP and compromised immune systems was established (Walzer *et al.*, 1974).

In 1981 Pneumocystis had the dubious honor of being the organism that led to the definition of AIDS. Masur *et al.* and Gottlieb *et al.* reported a total of 15 cases of PcP in otherwise healthy young men. It was known by then, that fulminate Pneumocystis infections were usually found only in the immunocompromised. The PcP had prompted them to look for an underlying immunodeficiency, which they found. This discovery of an acquired immune deficiency in these men led to the description of AIDS and the designation of PcP as an AIDS-defining illness (ADI). “In the 1980's as the HIV infection spread and AIDS became a serious public health problem in the USA and Europe; Pneumocystis pneumonia (PcP) concurrently emerged as the most prevalent opportunistic infection (OI) and the most common immediate cause of death among these patients” (Kaneshiro and Smulian, 2007). According to Gabrar *et al.* (2008) before the invention of highly active antiretroviral therapy (HAART), PcP was the most
frequent OI found in patients (15.6%), and after HAART became available, PcP was the second most frequent OI (19.1%). The vast majority (80%) of AIDS patients in the United States experience PcP during their convalescence (Kaneshiro and Smulian, 2007).

1.3 Pneumocystosis

Pathology

*Pneumocystis* trophic forms adhere tightly to the cell membrane of type I alveolar pneumocytes. This attachment and the proliferation of *Pneumocystis* in the alveoli alter permeability of the alveolar epithelium (Yoneda and Walzer, 1983). According to Beck et al. (1998) *Pneumocystis* burden can be fairly high without significant impairment of gas exchange. PcP was characterized by “edema, exudate, fibrosis, type II pneumocyte proliferation, and cellular infiltration of the alveolar wall” (Benfield et al., 1997). Eventually type I pneumocytes are damaged and the alveoli fill with foamy, eosinophilic material (Kaneshiro and Smulian, 2007). An inflammatory reaction accompanies heavy organism burden and seems to be the trigger for alveolar flooding and severe pneumonia (Beck et al., 1998).

Fulminate *Pneumocystis* infections usually occur in immunocompromised hosts. Malnourished children, chemotherapy patients, solid organ transplant recipients and agammaglobulinemics, in addition to HIV patients, are susceptible to *Pneumocystis* infections. Cell-mediated immunity, particularly T-cell deficiency is the most common immunologic deficiency associated with *Pneumocystis* (Kaneshiro and Smulian, 2007).

Epidemiology and Transmission

The majority (75%) of the human population in Europe and the United States has been exposed to *Pneumocystis* by the age of 4 (Kaneshiro and Smulian, 2007). A study in Chile by Vargas et al. (2001) found *Pneumocystis* DNA 32% of infants with mild respiratory infections, and 85% of
the infants in that same study had *Pneumocystis* antibodies by 20 months. Another study by Cushion *et al.*, (1999) showed that *Pneumocystis*-infected rats transmitted the infection to their pups within hours of birth. These studies show that *Pneumocystis* occurs worldwide, and mammalian host exposure happens early in life.

It is unknown by what type of infectious propagule *Pneumocystis* is transmitted. Studies by Walzer *et al.* (1977) and Hughes (1982) using immunocompromised rodents showed that *Pneumocystis* transmission is not through water or food, but is likely airborne. One can infer that the infectious form could be expelled from the respiratory tract of infected individuals by coughing or wheezing, and dispersed to other hosts (Cushion, 2005). *Pneumocystis* appears to be capable of survival and remaining infective outside the host (Kaneshiro and Maiorano 1996, Chin *et al.*, 1999). PCR has been used to detect *Pneumocystis* in air, both indoor and outdoor (Bartlett *et al.*, 1997; Wakefield, 1996). Most likely the organisms are taken in during inhalation and infection is initiated in the alveolus (Kaneshiro, 2002).

*Pneumocystis* can initiate an infection in either of two ways: (1) latent organisms in the host can be reactivated, and (2) a host can be reinfected with organisms from a different source (Kaneshiro, 2002). Most exposure to *Pneumocystis* does not result in morbidity. In young children, still developing their immune systems, *Pneumocystis* seems to cause mild respiratory systems (Vargas *et al.*, 2001). Pregnant women have been shown to asymptptomatically carry *Pneumocystis* and transmit it to their infants (Vargas *et al.*, 2003). Pneumocystosis can be triggered if the host becomes immunocompromised by disease or immunosuppressive therapy (Kaneshiro, 2002). Immunocompetent individuals that are colonized by *Pneumocystis* are asymptomatic, and clear the infection fairly quickly (Vargas *et al.*, 1995; Maskell *et al.*, 2003).
Chemotherapeutic Agents

Although *Pneumocystis* is a fungus, the drugs that are used to treat PcP are usually used for the treatment of protozoan and bacterial infections (Joffrin and Cushion, 2010). TMP-SMX is usually the first line therapy for pneumocystosis, while pentamidine, atovaquone, trimetrexate, and clindamycin are considered second line therapies (Larsen *et al.*, 2005) (Table 1). Corticosteroids are usually administered to combat the inflammation associated with fulminate pneumocystosis (Larsen *et al.*, 2005).

The gold standard of treatment for *Pneumocystis* pneumonitis is trimethoprim-sulfamethoxazole (TMP-SMX; Bactrim®). TMP-SMX is a combination of the two drugs (Table 1), both of which inhibit enzymatic reactions in the folate biosynthesis pathway. TMP-SMX is effective because *Pneumocystis* synthesizes folate *de novo*, and it is not capable of scavenging folate from the mammalian host (Larsen *et al.*, 2005). The affinity of TMP for the *Pneumocystis* dihydrofolate reductase enzyme is much higher than the human form (Larsen *et al.*, 2005). This allows trimethoprim to be used as an antibiotic. The enzymatic target for sulfamethoxazole is not present in the mammalian host, an ideal characteristic for an antimicrobial agent. TMP-SMX is highly effective, with a therapeutic success rate of at least 75%; unfortunately upwards of 50% of AIDS patients have adverse reactions to the drug (Hughes, 1992).

In 1958, Ivaldy and Paldy found the first drug to be effective in clearing *Pneumocystis* infections, pentamidine isethionate. It is now used as a parenteral alternative to TMP-SMX for
<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Drug Name</th>
<th>Biochemical Target</th>
<th>Mechanism of Action</th>
</tr>
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<tbody>
<tr>
<td>Bactrim® (TMP-SMX)</td>
<td>Trimethoprim-sulfamethoxazole</td>
<td>Dihydrofolate Reductase (DHFR)</td>
<td>inhibits folate biosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dihydropteroate Synthase (DHPS)</td>
<td>inhibits folate biosynthesis</td>
</tr>
<tr>
<td>Nebupent®</td>
<td>Pentamidine isethionate</td>
<td>unknown</td>
<td>unknown</td>
</tr>
<tr>
<td>Dapsone®</td>
<td>Dapsone (w/ TMP)</td>
<td>DHPS</td>
<td>inhibits folate biosynthesis</td>
</tr>
<tr>
<td>Mepron®</td>
<td>Atovaquone</td>
<td>Cytochrome B Complex</td>
<td>ATP synthesis</td>
</tr>
<tr>
<td>Neutrexin®</td>
<td>Trimetrexate (w/ leukovorin)</td>
<td>DHFR</td>
<td>inhibits folate biosynthesis</td>
</tr>
<tr>
<td>Dalcin®</td>
<td>Clindamycin (w/ primaquine)</td>
<td>ribosomal translocation</td>
<td>inhibits protein elongation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(50s rRNA in bacteria)</td>
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</table>
treating *Pneumocystis* pneumonia (Fishman, 1998). It is as effective as TMP-SMX. The mechanism of action is not well described. Pentamidine is a fairly toxic drug and 80% of patients have some sort of adverse reaction, however it can be used in sulfa-allergic patients (Larsen *et al.*, 2005).

A sulfone drug, dapsone, can be used in combination with trimethoprim in patients who do not tolerate sulfa drugs such as sulfamethoxazole. Dapsone, like sulfamethoxazole, blocks folate synthesis by inhibition of dihydropteroate synthase (Hughes, 1998). It is noteworthy that in a comparative study with TMP-SMX, 13% of the patients treated with dapsone-TMP experienced major toxicity, as opposed to 51% of patients treated with TMP-SMX.

Trimetrexate originally developed as an antineoplastic agent, blocks dihydrofolate reductase in *Pneumocystis*, but must be administered with folinic acid in order to prevent severe neutropenia (Fishman, 1998). Trimetrexate is another inhibitor of the DHFR enzyme and therefore folate metabolism (Fishman, 1998). It has been shown to be better tolerated, but not as effective as TMP-SMX (Larsen, 2005).

Two therapeutic options that do not target folate biosynthesis are atovaquone and clindamycin administered with primaquine. Atovaquone, an analog of ubiquinone (CoQ) interferes with the cytochrome B complex and prevents mitochondrial electron transport (Larsen, 2005). Most often used to treat malaria, it is fairly well tolerated. Clindamycin is a lincosamide antibiotic and in bacteria, prevents protein biosynthesis by binding to the 50S subunit of the ribosomal RNA, (rRNA) (Gold, 1999). When combined with primaquine, it is effective in clearing *Pneumocystis*, but has the unfortunate side effect of increasing susceptibility to *Clostridium difficile* cholitis (Fishman, 1999).
Although effective therapies for *Pneumocystis* infections are available, patients with long-term immunodeficiency experience recurrent *Pneumocystis* infections and there is growing concern over the development of drug resistant *Pneumocystis* (Giner et al., 2002). The current therapies are not universally well tolerated and are associated with unpleasant side effects (Giner et al., 2002). New drugs need to be developed to combat these problems.

1.4 The *Pneumocystis* Organisms

Systematics and Phylogeny

*Pneumocystis* is an enigmatic organism with characteristics of both fungi and protozoa. Recently, molecular phylogenetic analyses have lead to the consensus that the *Pneumocystis* are atypical fungi. Where *Pneumocystis* falls in the phylogenetic tree of fungi still seems to be a topic of debate (Redhead et al., 2006) (Table 2). A monumental effort by Hibbert et al. (2007) concludes that *Pneumocystis* is a member of the subkingdom Dikarya, phylum Ascomycota, and seems to be the most comprehensive effort at phylogenetic classification of the fungi. There are currently five named species of *Pneumocystis*, *P. carinii* (Delanöe & Delanöe, 1912), *P. jirovecii* (Frenkel, 1999), *P. murina* (Keely et al., 2004), *P. wakefieldiae* (Cushion et al. 2004) and *P. oryctolagi* (Dei-Cas et al., 2006) (Table 3). Other putative species known to infect different mammals are designated with tripartite names (Table 3) until enough data is available to warrant formal description (Redhead et al., 2006). Phylogenetic data suggest that the radiation of pathogenic forms of *Pneumocystis* occurred about 100 million years ago, around the same time that primates and rodents diverged from each other. This is highly suggestive of co-evolution between *Pneumocystis* and its mammalian hosts (Keely et al., 2003). *Pneumocystis* species have evolved closely with their host species and show a high level of host-species specificity. Even in
Table 2. Synopsis of *Pneumocystis* Systematics (Redhead et al. 2006).

<table>
<thead>
<tr>
<th>Taxonomic Group</th>
<th>Designation</th>
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<tbody>
<tr>
<td>Kingdom</td>
<td>Fungi</td>
</tr>
<tr>
<td>Phylum</td>
<td>Ascomycota</td>
</tr>
<tr>
<td>Class</td>
<td>Pneumocystomycetes</td>
</tr>
<tr>
<td>Order</td>
<td>Pneumocystidales</td>
</tr>
<tr>
<td>Family</td>
<td>Pneumocystidaceae</td>
</tr>
</tbody>
</table>
Table 3. Nomenclature of *Pneumocystis* organisms with their host. Those organisms with a binomial name have formally been described as a distinct species. The tripartite names are provisional pending sufficient formal Latin characterization and descriptions to warrant a novel species name (Keeley and Stringer, 2004; Dei-Cas *et al.*, 2006).

<table>
<thead>
<tr>
<th>Host Species</th>
<th><em>Pneumocystis</em> Species Name</th>
</tr>
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<tbody>
<tr>
<td>Ferret</td>
<td><em>P. carinii f. sp. mustelae</em></td>
</tr>
<tr>
<td>Horse</td>
<td><em>P. carinii f. sp. equi</em></td>
</tr>
<tr>
<td>Human</td>
<td><em>P. jirovecii</em> Frenkel 1999</td>
</tr>
<tr>
<td>Monkey (Goeldi’s monkey)</td>
<td><em>P. carinii f. sp. callimico</em></td>
</tr>
<tr>
<td>Monkey (Geoffroy’s marmoset)</td>
<td><em>P. carinii f. sp. callithrix</em></td>
</tr>
<tr>
<td>Monkey (red-handed tamarin)</td>
<td><em>P. carinii f. sp. midas</em></td>
</tr>
<tr>
<td>Monkey (Weddell’s tamarin)</td>
<td><em>P. carinii f. sp. fusciolis</em></td>
</tr>
<tr>
<td>Monkey (squirrel monkey)</td>
<td><em>P. carinii f. sp. sciureus</em></td>
</tr>
<tr>
<td>Mouse</td>
<td><em>P. murina</em> Keeley <em>et al.</em> 2004</td>
</tr>
<tr>
<td>Pig</td>
<td><em>P. carinii f. sp. suis</em></td>
</tr>
<tr>
<td>Rabbit</td>
<td><em>P. oryctolagi</em> Dei-Cas <em>et al.</em> 2006</td>
</tr>
<tr>
<td>Rat</td>
<td><em>P. carinii</em> Delanöe &amp; Delanöe 1912</td>
</tr>
<tr>
<td>Rat</td>
<td><em>P. wakefieldiae</em> Cushion <em>et al.</em> 2004</td>
</tr>
<tr>
<td>Shrew</td>
<td><em>P. carinii f. sp. sorex</em></td>
</tr>
</tbody>
</table>
immunocompromised hosts, *Pneumocystis* is not transmissible between species (Dei-Cas et al., 1998).

**Morphology and Life Cycle**

The morphology of *Pneumocystis* varies considerably depending on life cycle stage. Since *Pneumocystis* was still classified as a protozoan until the 1980s, protozoan terminology lingers, particularly in reference to life cycle. There are three main life cycle stages of *Pneumocystis*: the 7-8 µm pleomorphic trophic form, the 0.5-6 µm sporocyte (spore;); and the 4-7 µm thick-walled cyst (spore case;)(Dei-Cas et al., 2005).

The thin-walled trophic forms are the presumptive vegetative life cycle stage of *Pneumocystis* (Figure 1). The larger trophs have an irregular shape, while the smaller trophs appear more rounded and are presumed to be recently excysted. The trophic cell wall consists of one electron-dense layer (Dei-Cas et al., 2005). Trophic forms also exhibit typical eukaryotic organelles such as a nucleus surrounded by a nuclear envelope and nuclear pores, endoplasmic reticulum, ribosomes, and filopodia called tubular extensions (Yoshida, 1989; Kaneshiro and Baughman, 2001a; Cushion, 2004). These trophic forms are haploid (Wyder et al. 1998) and thought to be capable of asexually reproducing by binary fission (Yoshida, 1989). Additionally they may be capable of fusion to produce diploid forms, which undergo meiosis and develop into cysts containing haploid intracystic bodies (Yoshida, 1989).

Sporocytes (precysts) range in size and have thicker cell walls than the trophic forms (Figure 2). These forms tend to be spherical. Presumptive later sporocyte stages have inner electron lucent layers in their cell wall in addition to the electron dense layer found in
Figure 1. Transmission electron microscopy of *Pneumocystis carinii* trophic forms adhering to a type I alveolar pneumocyte. Trophic forms are not adhered to the type II alveolar pneumocyte (II). Trophic forms (T), type I alveolar pneumocyte (I), type II alveolar pneumocyte (II) (S. G. Langreth and E. S. Kaneshiro, unpublished).
trophs (Dei-Cas et al. 2005). Synaptonemal complexes, a hallmark of meiosis have been observed in these forms (Yoshida, 1989; Kaneshiro and Baughman, 2001a).

Thick-walled spore cases (cysts) containing 8 spores (intracystic bodies) are probably the mature spore cases (Figure 3) (Kaneshiro and Smulian, 2007; Ruffolo, 1994). These cysts have the two-layer cyst walls with inner electron-lucent layers and outer electron-dense layers and are spherical (Dei-Cas et al., 2005). A thickened area in the cyst wall is presumed to be the area in which a pore develops through which spores can be released during excystation (Figure 4). Thick-walled cysts with banana-shaped spores and thin-walled cysts with pleomorphic spores have also been observed (Figure 5). These forms are not well understood (Kaneshiro and Smulian, 2007).

In the absence of a reliable in vitro culture method for Pneumocystis there is considerable ambiguity concerning Pneumocystis’ life cycle. The distinct forms found in the mammalian lung, examined by static micrographs, have been the basis for proposed Pneumocystis life cycles (Kaneshiro and Smulian, 2007). Figure 6 shows one proposed scheme relating the different morphological forms.
Figure 2. Transmission electron microscopy of *Pneumocystis carinii* early sporocyte.

Sporocyte (S), and trophic forms (T), (S. G. Langreth and E. S. Kaneshiro, unpublished).
Figure 3. Transmission electron micrograph of *Pneumocystis carinii* spore cases. Thick walled, mature, spore cases (cysts) containing eight spores (intracystic bodies). Outer electron dense layer and inner electron lucent layer are also apparent. Spore cases (S), trophic forms (T), electron-dense layer (D), electron lucent layer (L), (S. G. Langreth and E. S. Kaneshiro, unpublished).
Figure 4. Transmission electron micrographs of *Pneumocystis carinii* mature spore case with thickening of the cell wall and putative pore for excystation, and empty spore case after excystation. Mature spore case (S), trophic form (T), thickened cell wall (W), pore (P), empty spore case (E) (S. G. Langreth and E. S. Kaneshiro, unpublished).
Figure 5. Transmission electron micrograph of *P. carinii* thin-walled spore case containing pleomorphic forms (P), (S. G. Langreth and E. S. Kaneshiro, unpublished)
Figure 6. Proposed intra-pulmonary life cycle of *Pneumocystis* (Hunt, 2006; modeled after Yoshida, 1989; Cushion, 2004); 1N and 2N indicate ploidy for the life cycle stage.
1.5 Sterols

General Overview

Sterols are large non-polar lipids with three principal components: a 3β-hydroxyl group, tetracyclic nucleus and an aliphatic side chain with at least 8 carbons. Double bonds can vary in number and location, usually at the 5, 7 or 8 position of the sterol nucleus, and the 22 or 24 position of the side chain. Side groups can also be found on the backbone and vary in number and location. Carbons are numbered starting with the sterol nucleus, followed by the side chain and last carbons attached to the nucleus. Figure 7 is an example of this numbering system. Sterols are named according to total number of carbons, locations of double bonds and location of side groups.

Sterols contribute significantly to eukaryotic cell membranes and are essential for cell growth and viability (Kaneshiro, 1998). They play important roles such as maintaining fluidity and permeability in the membrane as well as regulation of membrane-bound enzymes (Bard et al., 1978). For example, cholesterol, the primary mammalian sterol is found in a bilayer arrangement, although it has been also suggested that the planar molecule can reside between the lipid monolayers. This arrangement allows flexibility in mammalian membranes, which is critical to allow for proper membrane function (Joffrion and Cushion, 2010). In contrast it has been suggested that ergosterol, the major sterol found in fungi, occurs as a monolayer conformation making fungal cell membranes more rigid (Joffrion and Cushion, 2010).

Pneumocystis Sterols

In 2002 Giner et al. comprehensively identified 43 individual sterols in Pneumocystis carinii by using thin-layer and liquid chromatography to isolate individual sterols and nuclear
Figure 7. Numbering system following standard sterol nomenclature (Nkinin, 2006).
magnetic resonance (NMR) spectroscopy for structural analysis. NMR can provide “definitive structural characterizations, including elucidation of double bond positions and the detection of stereoisomers” (Giner et al., 2002). The structures of these sterols are shown in Figure 8.

Although Pneumocystis is a fungus, ergosterol, the major sterol in most fungal cell membranes, was not detected, nor were the \( \Delta^{5,7} \) sterol nuclei, trienes and tetraenes that are direct ergosterol precursors (Giner et al., 2002). This provided an explanation for the ineffectiveness of antifungal drugs that target either ergosterol or sterol biosynthesis.

Cholesterol, the major mammalian sterol, comprised 82% of the total sterols detected in P. carinii (Giner et al., 2002). Evidence to support the suggestion that cholesterol is scavenged from the mammalian host is abundant (Worsham et al., 2003; Zhao et al., 2003; Kaneshiro, 2004; Giner et al., 2004) and scavenging of host compounds is commonly encountered among endoparasites. Phytosterols, presumably from the host’s diet have also been detected in P. carinii, which supports the idea that Pneumocystis scavenges a significant portion of its sterols from the host (Amit and Kaneshiro, 2001). The majority of the sterol species detected (32 of 43) had alkyl groups at the C-24 position of the sterol side chain. Mammals are not capable of forming these sterols as they lack the SMT enzyme. The high percentage of 24-alkyl sterols suggests that the SAM:SMT enzyme that performs this alkylation is highly active in Pneumocystis (Giner et al., 2002). A study by Urbina et al. (1997) demonstrated that C-24 alkyl sterols were essential to Pneumocystis viability. Thus, SMT presents an attractive target for the development of new antifungal/anti-Pneumocystis therapies (Giner et al., 2002).
Figure 8. Pneumocystis carinii sterols: 4,4-dimethyl and 4α-methyl sterols (A-K). Δ sterols (S-EE). Other sterols (FF-QQ). (Giner et al. 2002).
Saccharomyces cerevisiae Sterols

In 2011, Nkinin et al. identified 25 sterols, including ergosterol, in Saccharomyces cerevisiae and erg11 deletion mutants expressing the Pneumocystis carinii or the S. cerevisiae erg11 cDNA. The structures of these sterols are shown in Figure 9. S. cerevisiae is a more typical fungus and ergosterol was found by gas liquid chromatography (GLC) analysis to comprise 71.6% of the total sterols (Nkinin et al., 2011). Twenty of the 25 sterols described were 24-alkyl sterols, suggestive that SMT is also a highly active enzyme in Saccharomyces. S. cerevisiae erg6 deletion mutants were shown to accumulate zymosterol and cholesta-5,7,22,24-tetraen-3β-ol (Bard et al., 1978).  

1.6 The S-adenosyl-L-methionine: Sterol C24-methyltransferase (SAM:SMT) Enzyme

Sterol biosynthesis is one of few areas where differences in metabolism can be found between fungi and animals (Nes, 2000). The SAM:SMT enzyme catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the C-24 position of the sterol side chain (Figure 10). Since SAM donates the methyl group for approximately 95% of the transmethylation reactions occurring in cells, it is considered the universal methyl group donor (Merali, 2000). SMTs are common in plants and fungi, however do not occur in mammals, which suggests that they could be exploited therapeutically (Nes, 2000). There are two classes of SMTs, based on the ability of the enzyme to methylate 24-methylene sterol substrates to produce 24-ethylene sterols. The type 1 SMT is not able to perform this function, however the type 2 enzyme can perform this methylation. Some plants have separate enzymes for the two reactions (Nes, 2003). In S. cerevisiae the SMT protein is coded by the erg6 gene. The sequences for the P.
Figure 9. *Saccharomyces cerevisiae* sterols. Lanosterol (A), 14-Desmethyllanosterol (B), 4α,14α-Dimethylcholesta-8,24-dienol (C), 4α,14α-Dimethylergosta-8,24(28)-dienol (D), 4α-Methylcholesta-8,24-dienol (E), 4α-Methylergosta-8,24(28)-dienol (F), (24R)-Ergosta-5,7,22-trienol (Ergosterol) (G), Ergosta-7,24(28)-dienol (Episterol) (H), (24S)-Ergosta-5,7-dienol (Dihydroergosterol) (I), Ergosta-8,24(28)-dienol (Fecosterol) (J), Cholesta-8,24-dienol (Zymosterol) (K), Ergosta-5,7,24(28)-trienol (L), (24S)-ergost-7-enol (Fungisterol) (M), (24R)-Ergosta-5,7,9(11),22-tetraenol (N), (24R)-Ergosta-7,22-dienol (O), (24S)-Ergost-8-enol (P), Ergosta-7,22,24(28)-trienol (Q), (24S)-Ergosta-5,8-dienol (R), Ergosta-5,7,22,24(28)-tetraenol (S), (24S)-Ergosta-5,7,9(11)-trienol (T), 1-(1>10)-abeo-(24R)-Ergosta-5,7,9(11),22-tetraenol (U), (24R)-Ergosta-5,8,22-trienol (V), Ergosta-5,7,9(11),24(28)-tetraenol (W), Ergosta-5,8,24(28)-trienol (X), Ergosta-6,8,24(28)-trienol (Y). (Nkinin et al., 2011)
Figure 10. The sterol methyltransferase reaction. The *Pneumocystis carinii* SMT is capable of transferring a methyl group to the C-24 position of the lanosterol side chain, and also catalyzing a second methyl group transfer to 24-methylene lanosterol to produce pneumocysterol.

The SMT from *Saccharomyces cerevisiae* has been characterized. In 1998 Nes *et al.* over-expressed this SMT in *E. coli* and found that the protein had a pI of 5.95, native molecular mass of 172,000 Da and was arranged as a tetramer with 4 identical, 43.4 kDa subunits. The same study showed that zymosterol was the preferred substrate for the *S. cerevisiae* SMT. The *S. cerevisiae* SMT, like most fungal SMTs is considered a type 1 SMT (Nes, 2003).

The sequence of the *P. carinii* erg6 cDNA, provided by Rosenfeld *et al.* (2001) is available at NCBI, locus number AY032981. The 1,134 base-pair gene codes for a 377 amino acid protein with a predicted MW of 43.2 kDa and isoelectric point of 6.2. Putative sterol-binding sites were shown to be unique (Kaneshiro *et al.*, 2002). Initial enzyme kinetic characterization showed the preferred substrates were lanosterol and 24-methylene lanosterol, a preference that is distinct from other fungi (Kaneshiro *et al.*, 2002). The *Pneumocystis* SMT seems to function best around human physiological temperature and pH (Kaneshiro *et al.*, 2002).

1.7 *Saccharomyces cerevisiae* Expression System

*Saccharomyces cerevisiae*, often referred to as brewer’s yeast has been widely studied because of its significance to the brewing and baking industries. It is a model organism for scientific research and has the advantage of growing quickly. A typical generation time for yeast is 90 min. It can be grown on defined media, which allows manipulation of the organism’s chemical environment. It is a eukaryote with a fully sequenced genome [http://www.yeastgenome.org](http://www.yeastgenome.org) and genetic manipulation is relatively straightforward because yeast can be grown in both haploid and diploid states.
The yeast synthesizes a variety of lipids and lipid content can be manipulated genetically, which makes them suitable for production of specific lipids (Blagovic et al., 2001). Industrially, yeasts have been used to produce a variety of flavors, essences, proteins and enzymes (Blagovic et al., 2001). The ability of yeast to undergo genetic manipulation in order to produce recombinant enzymes and withstand manipulation of its lipid content makes it an ideal system for expression of an enzyme involved in the formation of sterols. Saccharomyces is also advantageous as an expression system because it does not form insoluble inclusion bodies or have high proteolytic activity. Unlike bacterial expression systems, eukaryotes have the ability to modify proteins post transcriptionally.

Winzeler et al. created a series of yeast deletion mutants in 1999. These systematically deleted genes allowed characterization of the yeast genome and also provided a deletion mutant of interest to for this study. It was desirable to study this enzyme in a yeast deletion background so that endogenous Saccharomyces SMT activity would not mask activity of the recombinant Pneumocystis SMT. The haploid form of the deletion mutant was viable, demonstrating that SMT is not an essential enzyme for viability of this yeast (Winzler et al., 1999). This allows any SMT activity to be directly attributed to the recombinant enzyme, since the haploid form would lack the endogenous gene and therefore lack the SMT enzyme. These yeast deletions mutants are commercially available from ATCC.
2. MATERIALS AND METHODS

2.1 Genetic Analyses

Comparision of SMTs

Physiochemical properties of *P. carinii* and *S. cerevisiae* SMT were evaluated and compared using the ProtoParam tool made available on the Expert Protein Analysis System Server of the Swiss Institute of Bioinformatics (http://ca.expasy.org/tools/protoparam.html). Predictions of physiochemical properties were made from cDNA sequences taken from NCBI.

Analysis of *Pneumocystis* and *Saccharomyces* codon usage

Previous work had shown that although a recombinant *Pneumocystis carinii* erg6 gene expressed in *Saccharomyces cerevisiae* was transcribed, the protein product was not detected (Kaneshiro *et al.*, 2001). Codon usage was examined as a possible explanation for this observation. The *S. cerevisiae* codon usage chart, created using the GCG program Codon Frequency by J. Michael Cherry, was obtained and used as the basis of comparison for codon usage analysis. Each codon in the *P. carinii* erg6gene was examined for frequency of use by *Saccharomyces*. An excel spreadsheet was created to compare the frequency of each *Pneumocystis* codon with the frequency of the most preferred *Saccharomyces* codon. Frequency of use of the codon in the native *P. carinii* erg6 sequence was divided by the frequency of the preferred codon in the reference set. This Codon Adaptation Index (CAI) for each codon was used to evaluate candidate codons for optimization.

Codon Adaptation Index Value and GC content of the entire *P. carinii* erg6 gene were analyzed using Java Codon Adaptation Tool (J CAT) (Grote *et al.*, 2005). The same program was used to provide a version of the *P. carinii* erg6 gene fully optimized for expression in *S. cerevisiae*, and a partially optimized version where only the most influential codons were
modified. The fully optimized version was created for custom gene synthesis while the partially optimized version was created to evaluate the possibility of site-directed mutagenesis. The optimization algorithm employed primarily used codon bias of the expression organism for creating the optimized gene sequence; GC content and repetitive sequences were also taken into account. The gene is here designated as *Pneumocystis carinii erg6* optimized gene (Pcerg6\textsuperscript{OPT}).

**Synthesis of the Pcerg6\textsuperscript{OPT} gene**

The *P. carinii erg6* optimized (Pcerg6\textsuperscript{OPT}) gene was commercially synthesized by Genewiz, Inc (South Plainfield, NJ). “The synthesis strategy itself couples oligonucleotide synthesis, oligo-annealing, PCR amplification, and cloning to yield the final construct” (Schwarz, 2011).

**Obtaining the Scerg6\textsuperscript{NAT} gene**

The *Saccharomyces cerevisiae erg6* (Scerg6\textsuperscript{NAT}) gene sequence is known (NCBI). Since the gene was not available to our lab in plasmid form, strain BY4742 (ATCC, Manassas, VA) wild type for the erg6 gene, was used to provide template RNA to obtain the necessary erg6 cDNA. RNA was extracted using the RNeasy\textsuperscript{®} Mini Kit and was used to produce cDNA from which to amplify the target gene. Reverse transcriptase (RT) reactions were performed using 1 µg template RNA, 1 µM Oligo-dT Primers (Qiagen Sciences, Valencia, CA), and 4 units of Reverse Transcriptase Inhibitor. Otherwise reactions were set up as specified by the Omniscript\textsuperscript{®} manual. cDNA produced by the Reverse Transcriptase reaction was used as the template for the amplification of the *Saccharomyces cerevisiae erg6* gene by the polymerase chain reaction (PCR). PCR primers to the *S. cerevisiae erg6* gene were designed using the Web-Primer program from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/cgi-bin/web-primer). PCR products were isolated on 1% agarose gels to verify the size of the target
amplicon. PCR products of the correct size were TOPO® cloned into the pYES2.1 plasmid and transformed into chemically competent E. coli (Invitrogen, Carlsbad, CA) for propagation. A description of cloning protocol was as follows. Ten E. coli colonies were selected and grown overnight in LB broth containing 100 µg/ml ampicillin. Cultures were screened by PCR (as follows) for the presence of the insert and eight clones were sent to Genewiz, Inc (South Plainfield, NJ) for sequence analysis. One clone was found to have the correct sequence and orientation and was propagated for further DNA extractions.

**PCR of the Pcerg6NAT gene**

Previous work by Nicole Worsham and Akhelish Pandy in the Kaneshiro laboratory produced clones of E. coli transformed with the pTWIN1 plasmid containing the native Pneumocystis carinii erg6 gene (Pcerg6NAT). These clones were the source for Pcerg6NAT template DNA. Five-ml broth cultures were grown overnight in LB media containing 100 µg/ml ampicillin. DNA was extracted using the QIAprep® Spin Miniprep Kit (Qiagen Sciences, Valencia, CA). Sequence integrity was confirmed using sequencing primers complimentary to the regions up and downstream of the target gene on the pTWIN1 plasmid, designed by New England Biolabs® (Ipswich, MA). Genewiz, Inc (South Plainfield, NJ) performed the sequence analysis. Sequence data was compared to the published Pneumocystis carinii erg6 sequence. Once the integrity of the P. carinii erg6 sequence was confirmed, PCR primers were designed using Mac Vector (Mac Vector, Inc., Carey, NC) to amplify the target gene (Table 4), while
Table 4. PCR primers for erg6 genes used to produce PCR products for cloning into pYES2.1/V5-His-TOPO®.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Gene Amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scerg6F</td>
<td>ATGAGTGAAAACAGAATTGAGA</td>
<td>Scerg6\textsuperscript{NAT}</td>
</tr>
<tr>
<td>Scerg6R</td>
<td>TTATTGAGTTGCTTTCTTGGG</td>
<td></td>
</tr>
<tr>
<td>Pcerg6F</td>
<td>ATGTCTTTTGAACTGATATAGAGAAG</td>
<td>Pcerg6\textsuperscript{NAT}</td>
</tr>
<tr>
<td>NSerg6R</td>
<td>AACCAAAAGGTGTTTCTACAAACCATAATTG</td>
<td></td>
</tr>
<tr>
<td>GAL1F</td>
<td>AATATACCTCTATACCTTAACGTC</td>
<td>Pcerg6\textsuperscript{OPT}</td>
</tr>
<tr>
<td>V5R</td>
<td>ACCGAGGAGGGGTAGGGAT</td>
<td></td>
</tr>
</tbody>
</table>
omitting the stop codon at the end of the sequence. This was done to allow the recombinant
Pcerg6Nat gene to be expressed in frame with a C-terminal fusion tag.

2.2 Saccharomyces cerevisiae Expression System

The pYES2.1 TOPO® TA Expression Kit (Invitrogen™, Carlsbad, CA) was used for the
expression of the target erg6 genes in S. cerevisiae (Figure 11). This system makes use of a C-
terminal fusion tag containing a proximal V-5 epitope followed by a distal 6x HIS tag. The V-5
epitope is “derived from the P and V proteins of the paramyxovirus” and allows for detection of
the recombinant protein via Western blot analysis. The 6x HIS tag can be used for purification
of the recombinant protein by Immobilized Metal Affinity Chromatography (IMAC).
Expression of the recombinant gene is under control of a galactose inducible promoter and has a
uracil selectable marker.

2.3 Cloning and Transformation

Production of PCR Products for Cloning

PCR primers for the Pneumocystis genes were designed using Mac Vector (Mac Vector,
Inc., Carey, NC ) software. Primers for the S. cerevisiae erg6 were designed using the Web-
Primer program from the Saccharomyces Genome Database (http://www.yeastgenome.org/cgi-
bin/web-primer). In order to express the C-terminal fusion tags, anti-sense primers were
designed to exclude the native stop codon in the Pcerg6 Optimized and Pcerg6 Native genes,
however the stop codon was retained in the Scerg6 Native gene in order to produce an untagged
recombinant Saccharomyces SMT. Integrated DNA Technologies® (Coralville, IA) synthesized
primers. Primers were diluted to a stock concentration of 50 pmoles/µl for a final concentration
Figure 11. The pYES2.1/V5-His-TOPO® plasmid (Invitrogen, Carlsbad, CA). The erg6 genes from *P. carinii* and *S. cerevisiae* were cloned into this vector for transformation and expression in *S. cerevisiae* strain YML008C (ATCC).
of 0.4 µM in a 25 µl reaction. The reaction consisted of 12.5 µl GoTaq® Hot Start Colorless Master Mix (Promega, Madison, WI), 2X, 1 µl each of sense and anti-sense primer, 1 µl template DNA and 14.5 µl nuclease-free water (Promega, Madison, WI). A negative control with 1 µl of water substituted for the template DNA and a positive control was plasmid or genomic DNA previously shown to contain an amplifiable erg6 gene by the respective primers. GoTaq® Hot Start Master Mix (Promega, Madison, WI) was chosen for ease of reaction preparation and to reduce amplification artifacts during the initial heating cycle. PCR amplifications were performed using the erg6 PCR program (Table 5). Before cloning, an aliquot of the products from the erg6 as well as from the control PCRs were analyzed on a 1.0% agarose gel to verify amplicon size.

**TOPO® TA Cloning and Transformation**

Cloning and transformation were performed according to the pYES2.1 Expression Kit User Manual. One µl of fresh PCR product was incubated for 5 min with 1 µl salt solution (1.2M NaCl, 0.06M MgCl₂), 1 µl TOPO® vector and 2 µl sterile water. The reaction was carried out on ice and immediately followed by transformation. Two µl of the cloning reaction were added to one vial of TOP 10 One Shot® Chemically Competent E. coli (Invitrogen, Carlsbad, CA) and mixed by stirring gently with the pipette tip. This tube was incubated on ice for 5 min, followed by heat shock for 30 s at 42 °C without shaking. The tube was immediately placed on ice then 250 µl of room temperature super optimal broth with catabolite repression medium (SOC; Invitrogen, Carlsbad, CA) was added, the tubes were capped and shaken horizontally at 200 rpm, 37 °C, for 60 min. Ten µl of the cloning reaction was plated at room-temperature on Petri plates containing LB + ampicillin (100 µg/ml)-selective media, and incubated overnight at 37 °C.
Table 5. PCR program for erg6 genes and transformant analysis. Annealing temperature depends on primers used. PCR was performed for a total of 30 cycles and held at 4 °C until the reaction was removed from the thermocycler.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
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<td>94</td>
<td>5</td>
<td>hot start (denaturation)</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>initial annealing</td>
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<tr>
<td>72</td>
<td>2</td>
<td>initial extension</td>
</tr>
<tr>
<td>94</td>
<td>.75</td>
<td>denaturation</td>
</tr>
<tr>
<td>50</td>
<td>.75</td>
<td>annealing</td>
</tr>
<tr>
<td>72</td>
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<td>extension</td>
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<tr>
<td>72</td>
<td>10</td>
<td>final extension</td>
</tr>
<tr>
<td>4</td>
<td>indefinite</td>
<td>holding</td>
</tr>
</tbody>
</table>
Selection and Screening of E. coli Transformants

After overnight incubation, 10 well-spaced colonies were sampled and screened by PCR for the presence of the erg6 insert in the pYES2.1 plasmid by using the GAL1 Forward and V5 C-terminal Reverse primers supplied with the kit (Invitrogen, Carlsbad, CA) (Table 6). The PCR product was isolated on a 1% agarose gel. Colonies that appeared to have inserts of the correct size were transferred to 5 ml of Luria broth containing 100 µg/ml ampicillin and shaken at 200 rpm overnight at 37 °C. Plasmid DNA was extracted from the broth cultures using the QIAprep® Spin Miniprep Kit (Qiagen Sciences, Valencia, CA).

Plasmid DNA was sent to Genewiz, Inc. for sequencing using the above primers. Sequence data was analyzed using DNAMAN Version 7.110 (Lynnon Corporation, Pointe-Claire, Quebec) and one colony shown to have the correct sequence and orientation was grown up in 1 L of LB-ampicillin broth for large-scale plasmid DNA preparation. Microgram quantities of plasmid DNA containing the recombinant erg6 genes were extracted from 1L cultures using the Wizard® Maxiprep DNA purification kit (Promega) for use in subsequent Saccharomyces transformations.

Transformation of Saccharomyces cerevisiaeerg6 Knockout Mutant

Deletion mutant Saccharomyces cerevisiae strain YML008C BY4742 (ATCC), genotype MATalpha his3delta1 leu2delta0 lys2delta0 ura3delta0 deltaERG6 was chosen as the expression host. This haploid strain was chosen primarily for its knockout of the endogenous erg6 gene, which allowed any SMT activity to be ascribed to the recombinant enzyme. Resistance to geneticin conferred by the knockout construct allowed the strain to be grown in selective media containing 200 µg/ml geneticin. The ura3 deletion was complemented by the plasmid allowing
selection for transformants. Strain BY4742, the parental strain of YML008C and an ura3-positive strain BR1669 were used as controls.

Initial transformations were performed by the lithium acetate method, according to Geitz and Woods (2002). A single colony of strain YML008C was selected from a YEPD/geneticin plate and grown at 30 °C for 4 h in 5 ml of YEPD broth, while being shaken at 150 rpm. Cells were counted using an improved Neubauer hemocytometer and 50 ml of YEPD broth was inoculated with enough cells to produce a culture with cell density of 2 x 10⁷ cells/ml the next morning. Cells were harvested at 4,000 x g for 5 min at 4 °C. The culture medium was decanted and cells were washed in 25 ml of sterile water, which was followed by a second spin at 3,000 x g for 5 min at 4 °C. Cells were resuspended in 1 ml sterile water and transferred to a sterile 1.5 ml microfuge tube in which they were spun at 10,000 x g for 30 s. The supernatant was removed and the cells were resuspended in sterile water to a total volume of 1 ml. One hundred µl aliquots of this preparation, each containing about 10⁸ cells were transferred by pipette into 1.5 ml microfuge tubes. These tubes were centrifuged at 10,000 x g for 30 s and the supernatant removed, leaving the cells for use in the transformation protocol.

Plasmid DNA was diluted in nuclease-free water (Promega) to a concentration of 29.4 ng/µl. A total of 1 µg of plasmid DNA was used in each reaction. Reagents were added to the microfuge tubes containing cells in the following order: 240 µl polyethylene glycol 3500 (PEG 3500) 50% w/v, 36 µl 1.0 M lithium acetate, 50 µl boiled single-stranded carrier DNA, and finally 34 µl plasmid DNA. Cells were resuspended in the transformation mixture by vigorous vortex agitation. The tubes were incubated at 42 °C in a water bath for 40 min then centrifuged at 13,000 x g for 30 s and the supernatant was removed. One ml of sterile water was used to gently resuspend the pellet. Ten µl of transformed cells were transferred using a wide-bore tip
pipette into a 90 µl pool of sterile water on a pre-warmed selective plate and gently spread. Selective plates were made with S.c.-URA medium containing 200 µg/ml geneticin. Transformants were incubated for 5 days at 30 °C.

Subsequent transformations were done via electroporation using a modification of the Gottschilling Lab electroporation protocol (http://labs.fhcrc.org/gottschling/Yeast%20Protocols/ytrans.html). A single colony was selected from a YEPD-geneticin plate and used to inoculate 50 ml of YEPD broth. This was grown for 18 h (until early stationary phase) at 30 °C, 150 rpm shaking to a cell density between 0.6 and 2 x 10⁸ cells/ml. Cell density was verified using a hemocytometer and light microscope. Cells were harvested by centrifugation at 3,000 x g for 5 min at 4 °C and kept on ice throughout the procedure. Cells were washed with 40 ml of ice-cold sterile water, packed into pellets at 2,500 rpm for 5 min at 4°C and the supernatant was decanted and the cells were resuspended in 20 ml of sterile, ice-cold water. Cells were again concentrated into pellets and then resuspended in 5 ml of ice-cold sterile 1 M sorbitol. Cells were concentrated into pellets as described and then resuspended to a total volume of 1.0 ml in the sorbitol solution and maintained on ice.

Cells suspended in 160 µl sorbitol and then 20 µl of DNA at a concentration of 29.4 ng/µl (in water, not TE buffer) were added to a sterile 0.2 cm electroporation cuvette. One pulse of 10 µF x 10 capacitance, 480 ohms, 1.5 kV of resistance was delivered to each sample. One ml of ice-cold 1 M sorbitol was immediately added after electroporation and the sample was aseptically transferred to a sterile microfuge tube and placed on ice while the other electroporation procedures were being performed. Ten-µl aliquots of each reaction mixture were spread onto plates containing selective media and incubated as above.
Screening and Characterization of *Saccharomyces* Transformants

Five colonies from each transformation procedure were picked from the selective plates and transferred to *S.c.* URA broth with 200 µg/ml geneticin and shaken overnight at 4 °C, 150 rpm. One µl of each culture was added directly to a hot start PCR, program shown in Table 5, using the GAL1 Forward and V5 Reverse primers (Table 6) to verify the presence of the pYES2.1 plasmid in the transformants. Aliquots of the PCR product were isolated on 1.0% agarose gels and size of the insert of the estimated using LowRanger 100-bp DNA Ladder (NorgenBiotek Corporation, Thorold, ON). Another PCR was performed using primers that bind to regions upstream of the *erg6* locus and in the middle of the *erg6* knockout construct. PCR performed using these primers “YML008C-A and Kan-B” was performed to verify the presence and location of the knockout cassette, which demonstrates that the strain was truly the *erg6* knockout. Plasmid DNA was prepared as above from cultures verified to be the knockout strain and contained the pYES2.1 plasmid with appropriate size inserts. This plasmid DNA was sent for sequencing by Genewiz, Inc. and analyzed as above.

2.4 Expression and Detection of the Recombinant SMT

Expression of the Recombinant SMT

Expression of the recombinant SMT was under the control of a galactose-inducible promoter. A single colony was used to inoculate 5 ml of *S.c.* URA broth with 2% glucose as the carbon source. After 48 h this culture was transferred to 50 ml of *S.c.*-URA with 2% raffinose as the carbon source to remove the glucose repression of the recombinant *erg6* gene. The cells
Table 6. Primers used for molecular analyses of YML008C/pYES2.1*erg6* transformants.

GAL1F and V5R were used to verify the presence of the insert in the plasmid and for sequencing plasmid DNA. YML008C-A and YML008C-B were used to verify the integrity of the *S. cerevisiae erg6* gene in strain BY4742. YML008C-A and KAN-B were used to verify the presence of the knockout cassette at the YML008C locus.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Binding</th>
<th>Expected Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(5’-3’)</td>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>GAL1-F</td>
<td>AATATACCTCTATTTAACGTC</td>
<td>Plasmid</td>
<td>1283 b.p.</td>
</tr>
<tr>
<td>V5-R</td>
<td>ACCGAGGAGGGTTAGGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YML008C-A</td>
<td>CTGTTGCCGATAACTTCTTCATTCC</td>
<td>Chrom13.</td>
<td>373 b.p.</td>
</tr>
<tr>
<td>YML008C-B</td>
<td>TATCGGTCTACCATCCCAATTTCTCA</td>
<td><em>erg6</em></td>
<td></td>
</tr>
<tr>
<td>YML008C-A</td>
<td>CTGTTGCCGATAACTTCTTCATTCC</td>
<td>Chrom. 13</td>
<td>508 b.p.</td>
</tr>
<tr>
<td>KAN-B</td>
<td>CTGCAGCGAGGAGCCGTAAT</td>
<td></td>
<td><em>kan</em></td>
</tr>
</tbody>
</table>

40
were grown on raffinose for 48 h and then transferred to 1 L of S.c. URA medium with 2% galactose as the carbon source to induce expression of the recombinant gene. Cells were grown for 48 h and an aliquot was removed for verification of induction of recombinant SMT expression.

Detection of the Recombinant *P. carinii* SMT

A 5-ml aliquot was taken from 1 L of culture grown for 48 h on S.c. URA medium with 2% galactose. Untransformed strain YML008C was used as a control. Cells were harvested at 3,000 x g for 10 min at 4 °C in a tabletop centrifuge (Sorvall, Asheville, NC). Cells were transferred into 1.5-ml microfuge tubes and washed once with sterile water. The cells were resuspended in 1.0 ml of yeast lysis buffer (Ness, 2004) and transferred to microfuge tubes containing 0.5 ml of 0.5 mm glass beads (Mo Bio Laboratories, Inc., Carlsbad, CA). Cells were homogenized in a Mini-Bead Beater™ (BioSpec Products, Bartlesville, OK) for a total of 5 x 1-min cycles with 1 min of cooling on ice between cycles. The cell lysate was clarified by centrifugation at 13,000 rpm in a tabletop microfuge (Fisher Scientific, Waltham, MA) and the supernatant was retained for further analysis. Protein content of the clarified lysate was determined spectrophotometrically on a Nano Drop® ND-1000 spectrophotometer (Thermo Scientific, Asheville, NC) using the *RC DC™* Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Cell lysates were solubilized by the addition of an equal volume of 2X Laemmli’s sample buffer (20% glycerol, 10% β-mercaptoethanol, 6% sodium dodecyl sulfate (SDS), 125 mM Tris-HCl pH 6.8 and 7.5% bromophenol blue) and boiling for three min. Samples were loaded onto a discontinuous 4%-10% gel and separated by SDS- polyacrylamide gel electrophoresis (PAGE) for 90 min at 100 V in a Mini-Protein III apparatus (Bio-Rad Laboratories, Hercules, CA).
ColorBurst™ pre-dyed molecular weight marker (Sigma Aldrich, St. Louis, MO) was used to estimate sample molecular weights.

After separation, the proteins were electrophoretically transferred to nitrocellulose membrane (Bio-Rad Laboratories) at 100 V for 60 min in the Mini-Protein III apparatus (Bio-Rad Laboratories). Visualization of the pre-dyed molecular weight markers (MWM) used to monitor progress of the sample through the gel was also used to verify successful transfer of the protein to the nitrocellulose membrane.

Membranes were blocked overnight for non-specific protein binding in blocking buffer consisting of 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 ml/L Tween-20 (TBST) with 5% non-fat dry milk (NFDM). The nitrocellulose was then transferred to a primary antibody solution containing at 1:5,000 dilution of anti-V5-HRP-conjugated antibodies in TBST with 0.5% non-fat dry milk (NFDM) and shaken at room temperature for 60 min on an orbital shaker (Bellco, Vineland, NJ). After incubation with the antibody the membrane was rinsed 3 times for 15 min each in 0.5% NFDM-TBST and the recombinant SMT was visualized with stabilized TMB (3,3,5,5-tetramethylbenzidine) substrate for horseradish peroxidase (Promega, Madison, WI).

2.5 Growth and Harvesting of Cultures

Growth of Yeast Strains

Ten-ml cultures of S. cerevisiae strains were started from frozen glycerol stocks in S.c.-URA broth with 2% glucose as the carbon source (S.c.-URA/glucose). The medium for strain BY4742, the parental strain, was supplemented with 20 mg/L uracil. The medium for strain YML008C, the erg6 knockout strain, was supplemented with 20 mg/L uracil and 200 µg/ml geneticin (Gibco, Grand Island, NY). The medium for the YML008C/pYES2.1erg6
transformants was supplemented with 200 µg/ml geneticin and the uracil was omitted. Cultures were grown 48 h at 30 °C, 200 rpm in an Excella E25 Shaking Incubator (New Brunswick Scientific, Edison, NJ). The cells were transferred into 250 ml Erlenmeyer flasks containing 50 ml of their respective media with 2% raffinose as the carbon source and grown under the same conditions for an additional 48 h. They were then transferred to 2.8-L Fernbach flasks containing 1 L of their respective induction media (S.c.-URA/galactose). Cultures were grown for 48 h under the same conditions.

**Harvesting of Yeast Cells**

The cultures were harvested by centrifugation at 3,000 x g for 5 min at 4 °C using a Fiberlite® F14-6X250Y rotor in a Sorvall® RC 6 Plus centrifuge (Thermo Scientific, Asheville, NC). Cells from 3-5 L of culture were regularly pooled to obtain enough lipids for extraction. The pellets were washed in sterile ddH₂O (double-distilled water) and transferred to 50-ml polypropylene tubes. Cells were spun under the same conditions in a BiofugeStratos tabletop centrifuge (Thermo Scientific, Asheville, NC), the supernatant decanted and the weight of the packed cells was determined. Pellets were either used immediately for lipid extraction or frozen at -20°C for later use.

**2.6 Lipid Methods**

**Extraction and Purification of Total Lipids**

A monophasic extraction by the method of Bligh & Dyer (1959) was used to extract total lipids. In short, cells were resuspended in ddH₂O and the volume of the cell suspension was used as the water fraction, to produce a solvent mixture with the ratio 1.2:0.8, chloroform (CHCl₃): methanol (CH₃OH): water (ddH₂O). The monophasic solvent suspension was stirred overnight at room temperature. The monophasic extraction mixture was centrifuged at 1,500...
rpm in a BiofugeStratos tabletop centrifuge and the pellet discarded. A Folch biphasic partition was prepared from the monophasic Bligh & Dyer extraction supernatant to purify the lipids. Lipid purification was achieved by adding chloroform and ddH$_2$O to form a mixture with a final ratio of CHCl$_3$:CH$_3$OH:ddH$_2$O, 2:1:0.75 (v/v/v) (Folch et al., 1957). The Folch partition mixture was allowed to sit overnight at room temperature in a separatory funnel until the organic and aqueous phases clearly separated. The organic (lower) phase was collected in a round-bottom flask and concentrated in a Rotovap reduced pressure concentrator (Brinkmann Instruments, Westbury, NY).

Lipids were rinsed from the flask 3 times with a solution of chloroform:methanol (2:1; v/v). The rinses were pooled in a tared shell vial and dried under a stream of nitrogen in an Organomation, N-Evap (Berlin, MA). After weighing the sample was flushed with nitrogen, closed with a teflon-lined screw cap and stored at -20 °C. **Fractionation of Total Lipids**

Adsorption column chromatography, using silicic acid (Unisil, Clarkson Chemical Co, Williamsport, PA) as the stationary phase, was performed to separate the neutral lipids from the total lipid fraction (Kates, 1986). A glass column was prepared with a glass wool plug at the narrow end. Unisil, acid washed, activated, silicic acid was suspended in an excess of chloroform to form a slurry (Clarkson). This slurry was applied to the glass column until about 10 cm of Unisil was suspended by the glass wool. Great care was taken to ensure that the sufficient chloroform was always covering the Unisil so as not to let it run dry. The column was topped with a minimal amount of sand and the column was washed with about 10 ml of chloroform.
The total lipid sample was reconstituted in about 1 ml of chloroform and loaded onto the column and 100 ml of chloroform was used to elute the sterol-containing neutral lipid fraction. This fraction was collected in a round bottom flask and concentrated on a Rotovap, (Brinkmann Instruments, Westbury, NY). This sample was then transferred to a tared shell vial, dried under a stream of nitrogen, and weighed as above. Fifty ml of methanol was then used to elute the polar lipid fraction, which was collected, concentrated, and frozen in case it was needed later.

**Saponification of Neutral Lipids**

The neutral lipid fraction was subjected to mild alkaline hydrolysis in order to hydrolyze steryl esters and produce total free sterols (Kaneshiro et al., 1994). The sample was reconstituted in 0.6 ml of a solution of 2 chloroform:1 methanol (v/v) then 0.6 ml of a 4% solution of methanolic potassium hydroxide was added to the resuspended sample and the reaction was swirled and capped under a stream of nitrogen. The saponification reaction was allowed to proceed at room temperature for 2 h. The reaction was acidified with 0.5 ml 6N HCl then 0.6 ml of chloroform, 0.2 ml of methanol and 0.4 ml of ddH2O was added to the vial to create a Folch mixture for biphasic partitioning. This was allowed to separate and the lower, organic phase was removed and transferred into a tared shell vial. One ml of the Folch lower solution (86 chloroform:14 methanol:1 ddH2O, v/v/v) was added to the reaction vial, allowed to separated and the lower phase removed. The sample was extracted three times and the organic phases containing the sterols, pooled into the tared shell vial, dried in an N-Evap (Berlin, MA) and the sterol weight taken.

**Gas-Liquid Chromatography**

Sterols were analyzed in a Hewlett Packard 6890 (Agilent Technologies, Palo Alto, CA) gas chromatograph (GC). The GC was equipped with a J&W Scientific HP-Ultra 2 column, 25
m in length with an internal diameter of 0.2 mm coated with 0.33 µm film (Agilent Technologies, Palo Alto, CA). Sterols were resuspended in hexane. Column conditions were as follows: oven temperature was 250 °C, injection port and flame ionization detector 290 °C. Helium was used as the carrier gas. Relative elution retention times of sterol standards were determined and calibrated relative to the retention time of cholestane (Steraloids Inc., New Port, RI). Authentic standards of ergosterol, zymosterol and lanosterol were also used to identify the GLC peaks in the different yeast sterol analyses.

3. RESULTS

3.1 Comparision of SMTs

After comparison using the ProtoParam tool from the Swiss Institute of Bioinformation, the SMT from *P. carinii* was found to be compatible for expression in *S. cerevisiae*. Physiochemical properties of the respective SMTs were also predicted (Table 7). The *P. carinii* SMT was predicted to have a molecular weight of 43.1 kDa, pI of 6.2 and an instability index of 36.84 if expressed in *Saccharomyces*. The *S. cerevisiae* SMT was predicted to have a molecular weight of 48.1, pI of 5.5 and an instability index of 30.10. Instability indexes less than 40 are considered stable.
Table 7. Predicted physiochemical properties of SMTs prepared using the ProtoParam tool at the Swiss Institute of Bioinformatics (http://ca.expasy.org/tools/protoparam.html). SAM:SMT enzymes vary greatly but have highly conserved sterol and SAM binding motifs. Instability indices are calculated for expression in Saccharomyces cerevisiae, an index less than 40 is considered stable.

<table>
<thead>
<tr>
<th>SAM:SMT</th>
<th># Amino Acids</th>
<th>pI</th>
<th>MW (kDa)</th>
<th>t_{1/2} (h)</th>
<th>Instability Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumocystis carinii</td>
<td>377</td>
<td>6.2</td>
<td>43.2</td>
<td>20+</td>
<td>36.84</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>383</td>
<td>5.5</td>
<td>48.1</td>
<td>20+</td>
<td>30.10</td>
</tr>
<tr>
<td>Leishmania Amazonensis</td>
<td>353</td>
<td>6.1</td>
<td>39.9</td>
<td>20+</td>
<td>46.96</td>
</tr>
</tbody>
</table>
3.2 Codon Usage Analysis

Codon usage was analyzed using codon usage charts generated by the GCG program Codon Frequency and Java Codon Adaptation Tool (JCAT) (Grote et al., 2005). The P. carinii genome was found to have a relatively high AT content (35.4%) and the S. cerevisiae genome was found to have an even higher AT content (39.8%) in comparison to other common model systems such as E. coli (Table 8). Analysis of the P. carinii erg6 gene on a codon-by-codon basis revealed that 139 out of 377 (37%) amino acids were coded by codons that are not preferred by S. cerevisiae. Four of the 13 codons with the poorest codon adaptation index scores were located within the first 50 codons of the gene.

3.3 Optimization and Synthesis of the Pcerg6OPT gene

Optimization of the P. carinii erg6 gene was performed using JCAT. Analysis of the P. carinii erg6 sequence showed that more codons would need alteration than would be practical by site-directed mutagenesis. After optimization the GC content of the Pcerg6OPT gene was 41%. The sequence of the Pcerg6OPT gene is shown in Figure 12. Figure 13 shows a graphical representation of the Pcerg6OPT gene and the Pcerg6NAT gene showing the change in GC content and distribution. Genewiz Inc commercially prepared the Pcerg6OPT gene.

The Pcerg6NAT gene was obtained by PCR from existing stock plasmids and the Scerg6NAT gene was obtained by RT-PCR using oligo-dT primers for the reverse transcriptase and primers specific to the S. cerevisiae erg6 gene for PCR. It was found that RNase inhibitor was necessary for the RT reaction and subsequent PCR to be completed successfully (Figure 14). PCR products from the amplification of the Scerg6NAT gene were TOPO® cloned into the pYES2.1 plasmid and transformed into chemically competent E. coli (Invitrogen, Carlsbad, CA).
Table 8. Codon usage in *P. carinii, S. cerevisiae, E. coli* and *T. thermophila*. Genomic GC content was calculated using coding regions of the genome. Untranslated regions were not used. Codon usage charts, generated by the GCG Codon Frequency program, were used to determine preferred major codon for each given amino acid. Codon preferences are given in percents identical relative to *Pneumocystis carinii* preferred codons.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pneumocystis carinii</em></td>
<td>35.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>39.8</td>
<td>48</td>
<td>63</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>49.7</td>
<td>38</td>
<td>n/a</td>
</tr>
<tr>
<td><em>Tetrahymena Thermophila</em></td>
<td>33.1</td>
<td>52</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Figure 12. The sequence of the Pcerg6\textsuperscript{OPT} gene synthesized by Genewiz Inc.
These transformed *E. coli* were screened by PCR and 4 colonies were found to have inserts of the correct size (Figure 14). Sequencing of plasmid DNA extracted from these clones (Genewiz, Inc., South Plainfield, NJ) confirmed that one of the clones had the correct sequence and orientation. This plasmid was used for subsequent transformations.

### 3.4 Transformation by the Lithium Acetate Method and Electroporation

Transformation of strain YML008C by the lithium acetate method was found to be very inefficient and unreliable. The best attempt yielded a transformation efficiency of 0.8 transformants/µg pYES2.1 plasmid. Strain BY4742 transformed more efficiently yielding 176 transformants/µg pYES2.1 plasmid (Table 9).

Transformation by electroporation was found to be significantly more efficient than the lithium acetate method. Strain YML008C transformed with an efficiency of 866 colonies/µg pYES2.1 plasmid (Table 9).

In both transformation procedures, the ability to grow on uracil-deficient medium was conferred to the transformations by the complementation of the *ura3* gene carried on the pYES2.1 plasmid. The ability to grow on medium containing geneticin was conferred by the knockout cassette, which contains a gene for geneticin resistance. This allowed selection of transformants carrying both the knockout structure and the exogenous plasmid (Figure 15).

#### 3.5 Characterization of YML008C/pYES2.1*erg6* Transformants

After microscopic examination (Figure 16) colonies of YML008C transformed with the pYES2.1 plasmids containing the respective *erg6* genes were screened by PCR and found to contain the target plasmid, with an insert of the correct size. They were also screened for the presence of the knockout structure in the YLM008C (*erg6*) locus (Figure 17). Initial
Figure 14. Obtaining the Scerg6NAT gene. A. Lanes 1 & 2: RT-PCR reaction using oligo DT primers for the RT step, erg6 specific primers for the PCR with (+) RNase Inhibitor. Lanes 4 & 5: RT-PCR reaction using erg6 specific primers for the RT step, erg6 specific primers for the PCR without (-) RNase Inhibitor B. PCR screening of E. coli transformant clones for erg6 (~1,200 bp) insert. Lanes 9, 10, 11 and 13 contain the full-length erg6 gene (MWM are indicated in bp).
Table 9. Transformation efficiencies of lithium acetate and electroporation methods. The pYES2.1 plasmid contained the target *erg6* genes and the YEP24 plasmid was used as a control.

<table>
<thead>
<tr>
<th>Method</th>
<th>Strain</th>
<th>pYES2.1</th>
<th>YEP24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium Acetate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BY4742</td>
<td>1.4</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td>YML008C</td>
<td>.08</td>
<td>17.6</td>
</tr>
<tr>
<td>Electroporation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BY4742</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>YML008C</td>
<td>866</td>
<td>716</td>
</tr>
</tbody>
</table>
Figure 15. Selection of transformants. Geneticin resistance conferred by the \textit{erg6} knockout cassette, uracil selective marker on the pYES2.1 plasmid carrying the recombinant \textit{P. carinii erg6} gene.
characterization of the strain YML008C by gas-liquid chromatography showed that the knockout strain had fewer sterol species than BY4742, the parental strain. Expression of the recombinant SMT was verified via western blot using α-V5 antibodies conjugated to horseradish peroxidase to detect the recombinant protein (Figure 18). The recombinant protein was present. The molecular weight of the recombinant protein was approximately 43 kDa.

4. DISCUSSION

4.1 Codon Analysis and Gene Optimization

Both *Pneumocystis* and *Saccharomyces* have relatively AT-rich genomes. Although GC content is not drastically different between *P. carinii* and *S. cerevisiae* (35.4% and 39.8%, respectively), codon usage was fairly different. When analyzed on a codon-by-codon basis it seems that *Pneumocystis* and *Saccharomyces* both prefer AT rich codons. Between the AT-rich codons, codon preference is not synonymous. It is plausible that codon bias led to low levels of expression in previous attempts to express the *P. carinii* erg6 in *Saccharomyces*. By adjusting codon usage in the Pcerg6\textsuperscript{NAT} gene to create the Pcerg6\textsuperscript{OPT} gene, codon bias can be eliminated as a confounding factor, therefore increasing the likelihood that the recombinant Pcerg6\textsuperscript{OPT} will be translated in detectible quantity. The *P. carinii* SAM:SMT enzyme is predicted to be stable when expressed in *S. cerevisiae* (Table 7), which suggests that degradation of the recombinant enzyme is not likely to be a significant problem. Gene synthesis proved to be a fast, reliable and relatively inexpensive means of obtaining plasmid DNA containing the desired gene. Optimization of the target gene
Figure 16. Micrograph of *S. cerevisiae* cells transformed with the pYES2.1Pcer66OPT plasmid taken under light field optics (x400 magnification). Note budding of cells and absence of other organisms.
Figure 17. Molecular characterization of yeast transformants for presence of knockout structure; (A) expected amplicon size 373 bp. Lane 1 Pcerg6\textsuperscript{OPT}, Lane 2 Pcerg6\textsuperscript{NAT}, Lane 3 Scerg6\textsuperscript{NAT}. Lane 4 positive control, Lane 5 negative control. (B) pYES2.1erg6 plasmid, expected amplicon size ~1200 bp. Lane 1 Pcerg6\textsuperscript{OPT}, Lane 2 Pcerg6\textsuperscript{NAT}, Lane 3 Scerg6\textsuperscript{NAT}. Lane 4 positive control, Lane 5 negative control.
Figure 18. Western blot analysis using α-V5-HRP antibodies. α-V5 antibodies bind to the V5-epitope tag on the recombinant protein demonstrating that the recombinant *P. carinii* SAM:SMT is expressed and is of the correct size.
by site-directed mutagenesis would not have been economical nor would it be practical due to the large number of codons (37%) needing to be optimized.

4.2 Transformation Methods

Transformation of the recombinant \textit{erg6} genes into strain YML008C proved to be the greatest challenge in this study. Although the lithium acetate method is reputed to be the best method for transforming yeast, and control strains transformed readily, strain YML008C did not frequently survive the transformation procedure. Transformation efficiency for the target strain by the lithium acetate method was extremely low, 0.8 transformants/µg plasmid DNA compared to 176 transformants/µg plasmid DNA in the control strain. Strain YML008C is an \textit{erg6} knockout that was shown to have fewer sterols (presumably less 24-alkyl sterols) than the parental strain. Lack of these sterols could have significant effects on the health of the cell and the integrity of the cell membrane. Since the plasmid has to pass through holes in the cell membrane in order to produce a transformant, it is possible that this strain does not have a sturdy enough membrane to withstand this treatment and transform reliably.

Transformation by electroporation produced many more transformants in the strain YML008C than by the lithium acetate method. A transformation efficiency of 866 colonies/µg plasmid DNA was obtained with much less effort. Transformations with all three of the target genes were successful. This method was found to be simple, fast and repeatable, a significant improvement over the lithium acetate method.

4.3 Characterization of Transformants

Characterization of transformants was performed on three levels: media, microscopic and molecular. First, transformants were grown on media under two selective pressures. Media contained geneticin that should only allow strain YML008C (or other strains containing the gene
for geneticin resistance) to grow. Aside from having the erg6 gene knocked out, strain YML008C has the ura3 gene knocked out. Untransformed YML008C cannot grow on media without uracil. The ura3 gene on the plasmid complements the knockout allowing the transformant to synthesize uracil and therefore grow in uracil-deficient media.

Transformant cultures were examined by microscopy and verified to be pure cultures of Saccharomyces cerevisiae. No contamination was found.

Molecular characterization of the transformants examined the presence and location of the knockout cassette. Amplicons of the expected size were found for each of two PCRs. The first was to verify the presence of the plasmid with the correct size insert in the transformants. Primers that annealed to the GAL1 promoter and V5 epitope on the plasmid confirmed that the plasmid was in the transformants and the size of the amplicon, expected to be around 1,200 base pairs, demonstrated that an insert of the correct size was present in the plasmid. Presence and location of the knockout cassette was determined by PCR using one primer that annealed to the chromosome upstream of the Saccharomyces erg6 gene and a second primer that annealed within the knockout cassette. The positive result by this method determined that the knockout cassette was inserted at the erg6 locus, therefore the erg6 gene was disrupted.

The presence of the recombinant SAM:SMT was verified by Western blot analysis, demonstrating that the target enzyme is being produced. The recombinant SMT was shown to have a MW of approximately 43 kDa, which is in agreement with the molecular weight predicted by ProtoParam. Thus, the P. carinii SAM:SMT produced in S. cerevisiae remained intact. This project represents the first demonstration of the recombinant P. carinii SAM:SMT expression in S. cerevisiae.
4.4 Future Directions

The aim of this study was to create a system in which the \textit{P. carinii} SAM:SMT enzyme could be studied. Direct biochemical analyses of \textit{Pneumocystis} enzymes are difficult since no direct culture method exists for \textit{Pneumocystis} and significant quantities of \textit{Pneumocystis} organisms are difficult to obtain. By producing the \textit{Pneumocystis} enzymes in an expression host that is easily cultured, these enzymes can be produced in large quantities and evaluated biochemically. Rational drug development to target these enzymes may eventually be possible.

Biochemical analysis of the sterols produced by these transformants is the logical first step. Characterization of the sterols by GLC, mass spectrometry and nuclear magnetic resonance spectroscopy can definitively confirm whether 24-alkyl sterols are absent in the untransformed YML008C and definitively confirm the structures of the sterols produced by the \textit{erg6} transformants.

Preliminary GLC analyses of the sterols in \textit{S. cerevisiae} \textit{erg6} deletion mutant expressing the \textit{P. carinii} SAM:SMT show major differences from those in wild-type yeast (Figure 19). Thus, unlike the high level of complementation seen with the \textit{P. carinii} sterol 14\textalpha{}-demethylase (coded by the \textit{erg11} gene) (Nkinin et al. 2011), the \textit{P. carinii} SAM:SMT enzyme in \textit{S. cerevisiae} appears to differ considerably from the SAM:SMT of yeast. This is not unexpected as the \textit{P. carinii} is capable of adding the first and the second methyl group to the sterol side chain thus synthesizing 24-methylene and methyl as well as ethylidene and ethyl products (Kaneshiro et al, 2002). In contrast, the \textit{S. cerevisiae} SAM:SMT synthesizes only 24-methylene and methyl products since it can transfer only one methyl group to that site (SMT 1). Furthermore, the \textit{P. carinii} SAM:SMT has a sterol substrate preference for lanosterol and 24-methylene lanosterol.
STEROL ANALYSIS – GLC TRACINGS

Figure 19. Sterol Analysis-GLC tracings of *S. cerevisiae* strains. A. Parental strain. B. *S. cerevisiae erg6* knockout mutant. C. *S. cerevisiae erg6* knockout mutant expressing the *P. carinii* SAM:SMT.
whereas the enzyme in *S. cerevisiae* prefers zymosterol (Kaneshiro et al., 2001). These observations are consistent with the suggestion that the *P. carinii* SAM:SMT has a unique properties and further elucidation of its structure and function could lead to specific inhibitors of this enzyme (Kaneshiro et al. 2002).

Enzyme kinetics of the recombinant *P. carinii* SAM:SMT can build on previous studies and inhibitors of the 24-SMT can be tested in yeast. Also, kinetic studies can be performed on purified SAM:SMT and the activity can be evaluated without interference from other enzymes. The purified enzyme could also be used for crystallographic analyses to obtain the 3-D molecular structure, which might identify sites specific for the protein that can be used for the development of potential anti-*Pneumocystis* drugs.

Now that a reliable method of transforming *S. cerevisiae* ML008C has been developed, any number of *erg6* genes could be transformed into this *erg6* knockout mutant. Transformation of the *P. jirovecii* *erg6* gene into this system might allow analysis of this SAM:SMT of *Pneumocystis* that is pathogenic to humans. Transformation of *erg6* genes from *trypanosomes* or other organisms that produce 24-alkylated sterols could allow for evaluation of their SAM:SMTs in this system.
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Appendix 1.

Evidence for DNA Synthesis in Pneumocystis carinii Trophozoites Treated with the β-1,3-Glucan Synthesis Inhibitor Pneumocandin L-693-989

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ABSTRACT

Pneumocandins inhibit β-1,3-glucan synthesis preventing the development of Pneumocystis cysts that are absent from the lungs of treated rats. To determine whether treated trophozoites are capable of DNA replication, cytochemical analyses were performed on 4’,6-diamidino-2-phenylindole (DAPI) and DB181-stained Pneumocystis carinii isolated from pneumocandin L-693-989-treated rats. Fluorescence intensities of trophozoite nuclei from drug-treated rats were greater than those of untreated controls, suggesting that DNA replication was not inhibited but that cytokinesis and perhaps karyokinesis were blocked.