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Targeted gene replacement demonstrates that myristoyl-CoA:protein N-myristoyltransferase is essential for viability of *Cryptococcus neoformans*

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ABSTRACT **_Cryptococcus neoformans_ is a major cause of systemic fungal infection in immunocompromised patients. Myristoyl-CoA:protein N-myristoyltransferase (Nmt) catalyzes the transfer of myristate ($C_{14:0}$) from myristoyl-CoA to the N-terminal glycine of a subset of cellular proteins produced during vegetative growth of _C. neoformans_. A $Gly^{487} \rightarrow Asp$ mutation was introduced into _C. neoformans NMT_ by targeted gene replacement. The resulting strains are temperature-sensitive myristic acid auxotrophs. They are killed at 37°C when placed in medium lacking myristate and, in an immunosuppressed animal model of cryptococcal meningitis, are completely eliminated from the subarachnoid space within 12 days of initial infection. _C. neoformans_ and human Nmts exhibit differences in their peptide substrate specificities. These differences can be exploited to develop a new class of fungicidal drugs.**

Cryptococcus neoformans var. *neoformans* is an opportunistic pathogen which has emerged as a serious cause of systemic fungal infection in immunocompromised humans (1–3). Persistent *C. neoformans* infections are common in AIDS patients after completion of primary therapy with amphotericin B or fluconazole for meningitis. This leads to relapse rates of 50–60% and a shortened lifespan unless patients receive lifelong suppressive therapy, generally with fluconazole (4, 5). Both amphotericin B and fluconazole target ergosterol, the principal sterol in the organism's plasma membrane. Amphotericin B, a fungicidal macrolide, also binds to cholesterol in animal cell membranes, resulting in renal and other toxicities. Chronic suppressive therapy with the fungistatic drug fluconazole in severely immunocompromised hosts will most likely result in emergence of resistant strains (6). The search for alternative therapeutic targets in this organism would be helped by the ability to use targeted gene disruption to establish whether a given gene product is essential for viability. Protocols for high-efficiency transformation of *C. neoformans* have been reported (7–9). Gene replacement has remained elusive and may be strain- or locus-specific (9, 10).

Myristoyl-CoA:protein N-myristoyltransferase (Nmt) catalyzes the cotranslational transfer of myristate ($C_{14:0}$) from myristoyl-CoA to the amino-terminal glycine of a subset of eukaryotic cellular and viral proteins (11). *Saccharomyces cerevisiae* and human Nmts have an ordered bi-bi reaction mechanism: the apoenzymes bind to myristoyl-CoA, forming a high-affinity binary complex. Formation of this complex allows generation of a functional peptide binding site and subsequent generation of a myristoyl-CoA/Nmt/peptide ternary complex. After catalysis, CoA and then the myristoylpeptide product are released (12–14).

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NMT genes have been isolated from *S. cerevisiae*, *Candida albicans*, *Histoplasma capsulatum*, *C. neoformans*, and humans (15–18). All are present in a single copy per haploid genome. Several *S. cerevisiae* *N*-myristoylproteins are essential for viability and depend upon a covalently bound myristoyl group for expression of their biological functions. These include two ADP-ribosylation factors, Arf1p and Arf2p, which modulate several steps in vesicular/protein transport (19), and Gpa1p, the α subunit (G_α) of the heterotrimeric guanine nucleotide-binding protein involved in mating-factor signal transduction (20). Disruption of *NMT1* in *S. cerevisiae* causes recessive lethality (15). Metabolic labeling studies indicate that *C. neoformans* produces several cellular *N*-myristoylproteins during exponential growth (21). Two have been identified: an Arf (17) and a G_α homolog (22). *C. neoformans NMT* can complement the lethal phenotype of a *S. cerevisiae nmt* null allele (17).

We have used targeted gene replacement to establish that Nmt is an essential enzyme in *C. neoformans*. Moreover, sufficient differences exist in the peptide substrate specificities of the orthologous fungal and human enzymes to make Nmt an attractive target for development of fungicidal agents.

MATERIALS AND METHODS

Replacement of _C. neoformans NMT_ with _nmt487D_. M049 is a serotype A *ade2*$^-$ strain of *C. neoformans* (9). Southern blot analysis indicated that M049's single-copy *NMT* gene is contained in a 6.2-kb *Hin*dIII fragment. A size-selected *Hin*dIII genomic library was generated from strain M049 and screened with a full-length Nmt cDNA (17) to recover *NMT*. A Gly → Asp mutation was subsequently engineered at codon 487 by using PCR and 5′-AACAAGGATCCAAGATCGACGTCGTCATG-3′ as the coding-strand mutagenic primer. An *nmt487D–ADE2* plasmid (pCN28-14; Fig. 1*A*) was constructed by ligating (*i*) the ≈4.5-kb *Hin*dIII–*Nco* I fragment containing *nmt487D*, (*ii*) a 3.0-kb *ADE2* fragment from pCnade2ΔApa (9), and (*iii*) an ≈2.4-kb *Bam*HI–*Hin*dIII fragment containing 3′ *NMT* flanking sequence into pCRII (Invitrogen). pCN28-14 was partially digested with *Hin*dIII, resulting in a mixture of DNAs which was used for projectile-mediated transformation of M049.

M049 was prepared for transformation by inoculating 50 ml of yeast extract/peptone/dextrose (YPD) medium with two or three colonies and vigorously shaking the culture for 48 hr. The late-logarithmic-phase cells were pelleted at 1000 × *g* and suspended in 17 ml of regeneration medium (1 M sorbitol/1 M mannitol/0.9% yeast nitrogen base without amino acids/2.6% glucose/2.67% YEP broth/0.13% gelatin; final cell density, 5×10^8 per ml). A 200-μl aliquot was spread on selective medium (0.67% yeast nitrogen base without amino acids/0.08% adenine-dropout mix (Bio 101)/1 M sorbitol/2%

Abbreviation: Nmt, myristoyl-CoA:protein N-myristoyltransferase.

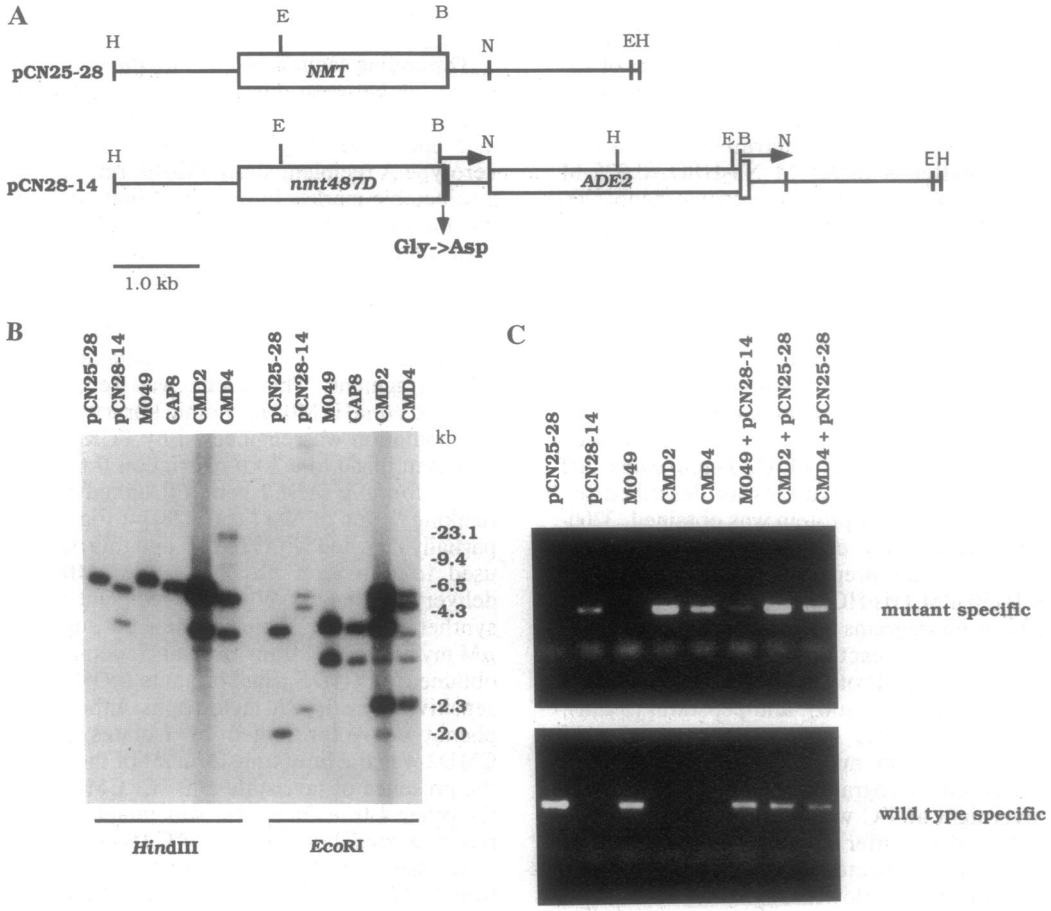

FIG. 1. Replacement of *C. neoformans NMT* with *nmt487D*. (*A*) Restriction maps of inserts in pCN25-28, containing the 6.2-kb *NMT* locus from *C. neoformans* strain M049, and pCN28-14, the *nmt487D–ADE2* plasmid used for Biolistic transformation. H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; N, *Nco* I. Horizontal arrows shown over a portion of pCN28-14 indicate the region of the *nmt487D* sequence which was duplicated for construction of this targeting vector. (*B*) Southern blot analysis of genomic DNA (5 μg) prepared from CMD2 and CMD4, the parental M049 strain, and CAP8, a stable ADE2 transformant (obtained by using pCnade2ΔApa; ref. 9) plus pCN25-28 and pCN28-14 DNA (100 pg). The blot was probed with a ^{32}P-labeled, 6.2-kb *Hin*dIII fragment from pCN25-28. In CMD4, the 6.2-kb endogenous *NMT Hin*dIII fragment is absent. The 6.0-, 5.5-, and 2.4-kb *Eco*RI fragments in CMD2 and CMD4 DNA comigrate with *Eco*RI fragments from pCN28-14. The 3.0-kb *Eco*RI fragment contains sequences from the extreme 5′ end of the *NMT* allele. The presence of extra *Hin*dIII and *Eco*RI bands in CMD2 and CMD4 suggested that portions of pCN28-14 had been integrated into more than one site. Digestion with *Nru* I, which does not cleave the 6.2-kb *NMT* fragment or the 3.0-kb *ADE2* fragment, established that there were two integration sites in CMD4. One site was at the *NMT* locus and contained a single copy of the mutant allele without associated vector sequences. There was one other extragenic site of integration that did contain vector sequences. PhosphorImager (Molecular Dynamics) scans of Southern blots disclosed that CMD2 contained 10 copies of the mutant allele at the *NMT1* locus (data not shown). (*C*) Allele-specific PCR. (*Upper*) One hundred picograms of plasmid DNAs, 1 μg of genomic DNA, or 1 μg genomic DNA plus 100 pg of plasmid DNA were used as the templates in PCRs with an *nmt487D*-specific coding-strand primer plus a noncoding-strand primer. (*Lower*) The same DNAs were used as templates in PCRs with an *NMT*-specific noncoding-strand primer and a coding-strand primer.

glucose/500 μM myristate/0.5% Brij/2% agar). DNA was precipitated onto gold beads by adding (in order with continuous vortexing) 3 μl of a 1-μg/ml solution of DNA, 30 μl of 2.5 M $CaCl_2$, and 6 μl of 1 M spermidine (free base) to 30 μl of gold beads (1 μm; Bio-Rad; 60 mg/ml of water). The beads were washed in ethanol and spread on macrocarriers (Bio-Rad; ≈10 μl of DNA-coated beads/macrocarrier). The macrocarriers were subsequently dried in a dessicator box and placed in a Bio-Rad Biolistic PDS-1000 particle delivery system, where they were subjected to 1300 psi (1 psi = 6.89 kPa) to deliver the DNA-coated beads to the plates of *C. neoformans* M049. After incubation at 24°C for 5–6 days, transformants were screened by (*i*) incubation at 37°C on plates of synthetic medium which lacked adenine and (*ii*) incubation at 24°C on synthetic medium which lacked adenine but contained 500 μM myristate.

***NMT* Allele-Specific PCR.** PCR mixtures contained genomic DNA (1 μg), a mutant-specific coding-strand primer (5′-CCAAACAAGGATCCAAGATCGA-3′, where the 3′ A of the primer is the mutated nucleotide in codon 487), a noncoding-strand primer (5′-CACGGCTCGAGCGGCCG-CAAAATATGCATGTCATAG-3′), 2.5 mM $MgCl_2$, and 1 unit of AmpliTaq DNA polymerase (Cetus). The wild-type *NMT* allele was identified with a noncoding-strand primer (5′-GGATTTTAGAGCATGACGACGC-3′), a coding-strand primer (5′-AAAGTTCGGACCAGGCGATG-3′), and 1 mM $MgCl_2$. Thermocycling conditions were 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for 25 cycles.

Purification of Human and *C. neoformans* Nmts from *Escherichia coli*. Human Nmt was expressed in *E. coli* and purified to apparent homogeneity as described (23). *C. neoformans* Nmt was also expressed in and purified from *E. coli*. An amino-terminal tag of six histidine residues followed by a factor Xa cleavage site (IEGR) was engineered by PCR using 5′-GCCCATGGCACACCATCACCATCACCATATC-GAAGGTCGCGATTCCTCTGATAACAAGGCC-3′ as the mutagenic primer and pCN1-18 (17) as the template DNA. The resulting *MHHHHHHIEGR*-Nmt *C. neoformans* DNA

was placed in pMON22310 (Str^R, *ori322*) under the control of the *tac* promoter, yielding pCN30-2. *E. coli* JM101 transformed with pCN30-2 was incubated at 24°C in 4 liters of LB containing streptomycin (30 μg/ml) and isopropyl β-D-thiogalactopyranoside (100 μM) until an OD_{600} of 0.8 was achieved. Cells were harvested by centrifugation and suspended in 80 ml of buffer A (0.025 M Na_2HPO_4/0.025 M NaH_2PO_4, pH 7/0.3 M NaCl/10 mM 2-mercaptoethanol/200 μM Pefabloc (Boehringer Mannheim)/2 μM leupeptin/2 μM pepstatin) with 40 mM imidazole. Cells were lysed in a French pressure cell, cellular debris was removed by centrifugation, and the resulting supernatant (870 mg of protein) was added to 0.5 ml of Ni^{2+} NTA-agarose (Qiagen), preequilibrated with buffer A/40 mM imidazole. The mixture was shaken for 90 min at 4°C. Bound protein was eluted with a step gradient of 60 mM to 150 mM imidazole in buffer A. Fractions were assayed for Nmt activity using an octapeptide derived from the amino terminus of *C. neoformans* Arf (GLSVSKLL-amide) and the enzyme assay described below. Six hundred micrograms of protein was obtained, 3200-fold enriched in Nmt activity compared with the crude bacterial lysate. The enzyme preparation (400 μg) was dialyzed into buffer B (50 mM Tris·HCl, pH 8.0/100 mM NaCl/1 mM $CaCl_2$). Sixteen micrograms of factor Xa (Boehringer Mannheim) was added in a reaction volume of 330 μl and incubated at 4°C overnight. Proteolysis was stopped with Pefabloc (200 μM), leupeptin (2 μM), and pepstatin (2 μM), and the solution was dialyzed against buffer A/40 mM imidazole. One hundred fifty micrograms of protein was recovered. One hundred micrograms of the dialyzed enzyme was incubated overnight at 4°C with 0.25 ml of Ni^{2+} NTA-agarose preequilibrated in buffer A/40 mM imidazole. The flowthrough fraction was collected and bound protein was eluted as before. Nmt in the flowthrough was 11,000-fold purified (yield, 50%).

***In Vitro* Nmt Assays.** The peptide substrate specificities of human and *C. neoformans* Nmts were compared in an *in vitro* assay system (12) that contained 7–70 ng of the 11,000-fold purified *C. neoformans* Nmt or 6–60 ng of 1000-fold purified human Nmt, 0.23 μM [^{3}H]myristoyl-CoA, and 182 μM peptide. Assays were performed in triplicate and repeated on at least two occasions, each time with GLSVSKLL-amide as a reference control.

RESULTS AND DISCUSSION

Introduction of an *nmt487D* Allele into *C. neoformans* by Homologous Recombination. *C. neoformans* is a haploid basidomycete. Although it can be mated and sporulated, tetrad analysis is not a practical way of demonstrating recessive lethality. Therefore, a temperature-sensitive mutation was introduced into *C. neoformans NMT* so that we could assess whether Nmt is essential. The five reported Nmt sequences all contain conserved glycine 5 residues from their carboxyl terminus (17). Gly^{451} → Asp substitution in *S. cerevisiae* Nmt1p produces a temperature-dependent reduction in the enzyme's affinity for myristoyl-CoA (24). Within 1 hr after shifting to nonpermissive temperatures (≥30°C), *S. cerevisiae* strains with this mutant allele (*nmt1-181*) exhibit global defects in protein N-myristoylation and undergo growth arrest at various stages of the cell cycle. *nmt1-181* cells can be rescued at 37°C by overexpression of nmt181p or Nmt1p and/or by supplementing the medium with myristate, but not shorter- or longer-chain saturated fatty acids (24, 25). Gly^{487} in *C. neoformans* Nmt corresponds to Gly^{451} in *S. cerevisiae* Nmt1p. *C. neoformans* Nmt containing a genetically engineered Gly^{487} → Asp mutation exhibits temperature-dependent reductions in its activity when coexpressed with *C. neoformans* Arf in *E. coli*, a bacterium with no endogenous Nmt (17). *C. neoformans nmt487D* also produces temperature-sensitive growth arrest and myristate auxotrophy when used to complement a *S. cerevisiae nmt1*Δ allele (17).

The coding sequences of *C. neoformans* genes can vary by up to 5% between different strains, particularly those with different serotypes (26). The *NMT* allele used for mutagenesis and targeted gene disruption was recovered from a serotype A recipient strain (M049; ref. 9). *ADE2* (9), used as a selectable marker in the disruption vector, was recovered from a serotype D strain (B-3501; ref. 9). We reasoned that these choices should reduce the likelihood of recombination at M049's *ade2* allele and increase the likelihood of recombination at its *NMT* locus.

Southern blots of M049 genomic DNA indicated that the entire ≈2.5-kb *NMT* locus was contained in a 6.2-kb *Hin*dIII DNA fragment. This locus was recovered from a size-selected *Hin*dIII M049 genomic library (Fig. 1*A*). A Gly^{487} → Asp mutation was engineered by PCR. The *nmt487D* allele was then fused to a 3-kb restriction fragment containing the *C. neoformans ADE2* gene (9) linked to 2 kb from the 3′ flanking region of *NMT* (pCN28-14; Fig. 1*A*). pCN28-14 was partially digested with *Hin*dIII and this mixture of DNAs was used to transform *C. neoformans* M049 with a particle delivery system (9). When the recipient cells were plated on synthetic medium lacking adenine but supplemented with 500 μM myristate, 187 transformants (adenine prototrophs) were obtained. Six *ADE* transformants (≈3%) were temperature-sensitive myristic acid auxotrophs, although with two distinct phenotypes, represented by isolates CMD2 and CMD4. CMD2 was the only transformant of the 6 that could grow in the presence of myristate at 37°C. CMD4, representative of the other 4 transformants, was unable to grow at 37°C and required myristate even at 24°C (Fig. 2).

Southern blot analysis of uncut genomic DNA prepared from CMD2 and CMD4 indicated that all of the transforming DNA had been integrated into the hosts' chromosomes (data not shown). The blots revealed (Fig. 1*B*) 5.9- and 4.1-kb *Hin*dIII fragments, corresponding to the *nmt487D* allele, in both CMD2 and CMD4. PCR confirmed that only the mutant *nmt487D* allele was present in CMD2 and CMD4 (Fig. 1*C*).

A second set of transformations was performed to determine which type of DNA fragment among the mixture of DNAs used in the original Biolistic transformation was best

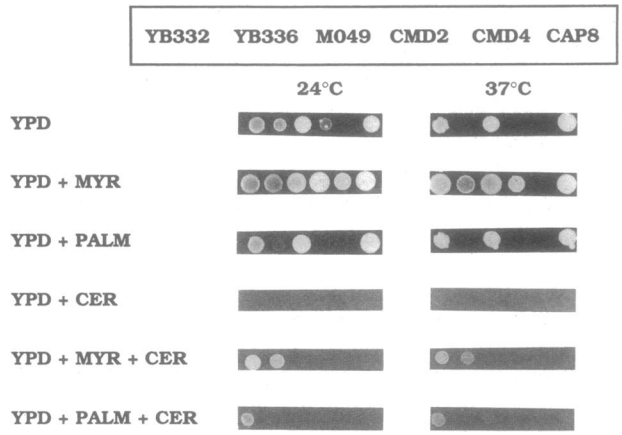

FIG. 2. Comparison of the phenotypes of isogenic *C. neoformans NMT* and *nmt487D* strains and isogenic *S. cerevisiae NMT1* and *nmt1-181* strains. Single colonies of *C. neoformans* or *S. cerevisiae* were diluted in 1× yeast nitrogen base/1 M sorbitol. A sterile replicator was used to transfer equivalent numbers of cells to agar plates which were subsequently incubated at 24°C or 37°C for 2 days. The order of the strains is shown in the box at the top. YB332 and YB336 are isogenic *NMT1* or *nmt1-181* strains of *S. cerevisiae*. MYR + PALM, 500 μM myristate and palmitate; CER, 25 μM cerulenin. Shown is a representative result obtained from two independent experiments, each performed in duplicate.

suited for homologous recombination with *NMT*. There were only modest differences in the fraction of transformants (adenine prototrophs) that were temperature-sensitive myristic acid auxotrophs: (*i*) 4/63 (6.3% yield) with the purified 10-kb (*Hin*dIII) *nmt487D–ADE2–NMT* 3′ fragment, (*ii*) 4/118 (3.4%) with the (*Hin*dIII) linearized recombinant plasmid, and (*iii*) 3/120 (2.5%) with supercoiled DNA. These results indicate that homologous recombination is reasonably efficient at this locus in the recipient serotype A strain.

Phenotypic Analysis of Isogenic *NMT* and *nmt487D* Strains. *C. neoformans* isolate CMD2 (*nmt487D*) is viable at 24°C on YPD medium. Growth at 37°C requires supplementation with myristate. Palmitate ($C_{16:0}$) at 500 μM not only fails to rescue viability at the nonpermissive temperature (37°C) but also suppresses growth at 24°C. The parental strain, M049, is able to grow at both temperatures in the absence or presence of myristate or palmitate. These growth characteristics mimic those seen with the corresponding *S. cerevisiae NMT1* and *nmt1-181* strains (Fig. 2).

The CMD2 genome contains 10 copies of *nmt487D* (Fig. 1*B*). CMD4 contains 2 copies of *nmt487D* and is unable to grow on YPD at 24°C without myristate (Fig. 2). The phenotypic differences between CMD2 and CMD4 are most likely explained by a gene dosage effect, since overexpression of nmt181p rescues *S. cerevisiae nmt1-181* strains.

When grown at nonpermissive temperatures, *S. cerevisiae nmt1-181* strains require that their intracellular myristoyl-CoA pools be maintained by contributions from at least two metabolic pathways: (*i*) *de novo* production by the fatty acid synthetase (Fas) complex and (*ii*) activation of imported myristate by two of the organism's four known long-chain acyl-CoA synthetases, Faa1p and Faa4p (27). Cerulenin is a specific inhibitor of Fas. It blocks growth of *nmt1-181* (and *NMT1*) strains. Growth can be restored by adding myristate to YPD/cerulenin medium (Fig. 2). Isogenic *NMT* and *nmt487D* strains of *C. neoformans* are also unable to grow on YPD/cerulenin at 24°C or 37°C. Unlike *S. cerevisiae NMT* and *nmt1-181* cells, the corresponding *C. neoformans* strains cannot be rescued on YPD/cerulenin by 500 μM myristate (Fig. 2). These results suggest potential differences in the regulation of protein N-myristoylation and/or fatty acid utilization between *S. cerevisiae* and *C. neoformans*.

The myristic acid auxotrophy of *C. neoformans nmt487D* cells is a stable phenotype. When CMD4 cells are grown at 24°C in YPD/myristate to stationary phase and then plated on YPD, the rate of reversion to myristate prototrophy is $<10^{-8}$.

When myristate is withdrawn and the cells are incubated in YPD alone at 37°C, the parental strain, M049 (*NMT*) continues to double at a normal rate, but CMD2 and CMD4 (*nmt487D*) begin to die within 4 hr and are completely dead within 24 hr (Fig. 3*A*). These results establish that Nmt is essential for the viability of *C. neoformans*.

An immunosuppressed rabbit model of cryptococcal meningitis has many features of the human disease (28). Therefore, 10^8 colony-forming units of CMD2, CMD4, or an isogenic *NMT ADE2* transformant of M049 (CAP8 in Figs. 1*B* and 2) were inoculated intracisternally into cortisone-treated New Zealand White rabbits. Between days 2 and 7 after inoculation, there was rapid and equal clearance of the two *nmt487D* strains. The cerebrospinal fluid was sterile by day 12. In contrast, the *NMT* transformant CAP8 produced continuous infection, comparable to the wild-type parent strain H99. Although a direct experimental comparison was not made, previous work indicates that the rate of elimination of CMD2 and CMD4 is more rapid than that seen with amphotericin B or fluconazole treatment of wild-type H99 infections in the rabbit model (29, 30). This *in vivo* study suggests that inhibition of *C. neoformans* Nmt would produce a rapid fungicidal response in cerebrospinal fluid, even if the host were severely immunosuppressed.

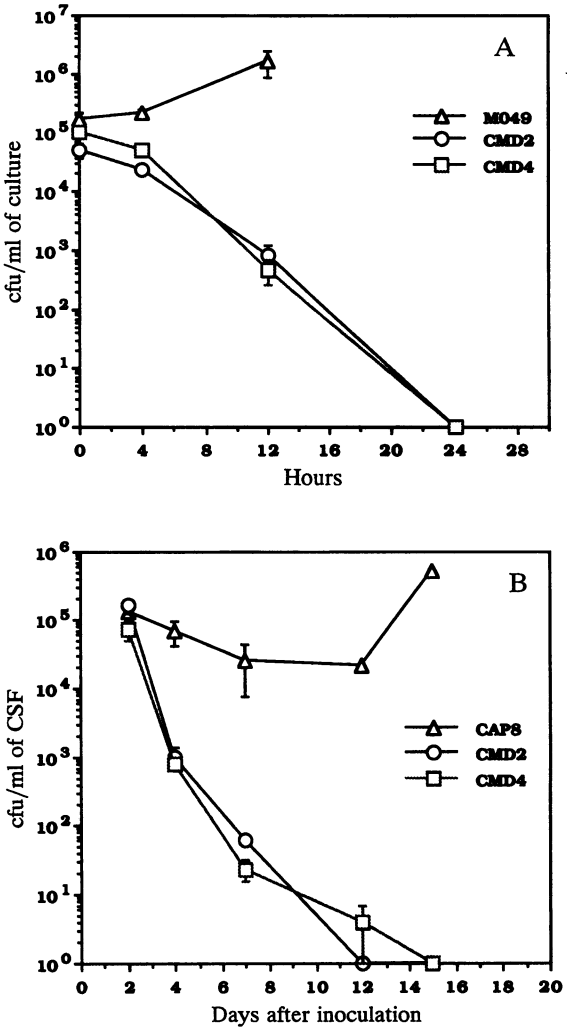

FIG. 3. Evidence that *C. neoformans* Nmt is essential for viability. (*A*) Cultures were grown to early logarithmic phase at 24°C in YPD/500 μM myristate. Cells were washed four times in water suspended in YPD, and shaken at 37°C. The number of viable colony-forming units (cfu) per ml was measured by plating aliquots of the culture, recovered at various time points after withdrawal of myristate, on YPD/myristate/agar and incubating the plates for 3 days at 24°C. Mean values (±SD) obtained from triplicate assays are plotted at each time point. A representative experiment is shown (n = 2 independent determinations). (*B*) Clearance of *C. neoformans nmt487D* during experimental meningitis. New Zealand White rabbits were inoculated intracisternally with 10^8 cfu of CMD2, CMD4, or CAP8, an *ADE2 NMT* transformant of M049 (n = 3 animals per isolate). Rabbits were treated 1 day before inoculation (and daily thereafter) with an intramuscular injection of cortisone acetate (2.5 mg/kg). Cerebrospinal fluid (CSF) was removed at 2, 4, 7, 12, and 15 days of infection and plated on YPD/myristate/agar at 24°C. Mean values ± (SE) are plotted. Note that two rabbits in the CAP8 group died after 7 days.

Comparison of the Substrate Specificities of *C. neoformans* and Human Nmts. Human and *C. neoformans* Nmts were expressed in and purified from *E. coli*. The enzymes were assayed *in vitro* together with [^{3}H]myristoyl-CoA and a panel of octapeptides (Fig. 4). Several of these peptides were excellent substrates for human Nmt but very poor substrates for *C. neoformans* Nmt: e.g., GNIFGNLL-amide (residues 1–8 of human ARF3) and GNAAAARR-amide (a derivative of the catalytic subunit of bovine protein kinase A). The two Nmts had no significant differences in their specific activities when incubated with GCTVSTQT-amide (from *S. cerevisiae* Gpa1p) and GNIFANLF-amide (from human ARF1). Six

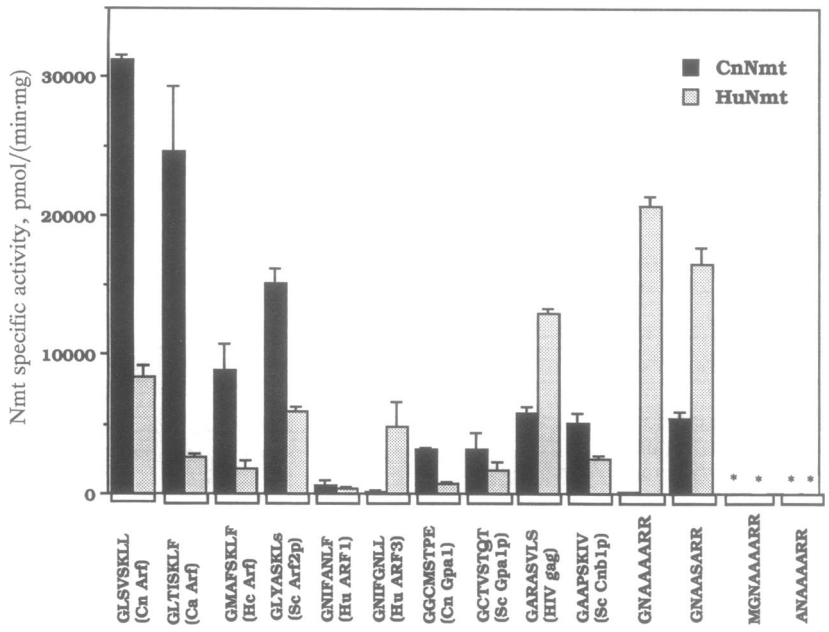

FIG. 4. Comparison of the peptide substrate specificities of purified human and *C. neoformans* Nmts. Octapeptides were surveyed in a discontinuous *in vitro* Nmt assay system. Mean values (± 1 SD) are plotted. An asterisk indicates that the peptide is not a substrate. Cn, *C. neoformans*; Ca, *Candida albicans*; Hc, *Histoplasma capsulatum*; Sc, *S. cerevisiae*; Hu, human; HIV, human immunodeficiency virus.

octapeptides were superior substrates for the *C. neoformans* enzyme. They represent the amino-terminal sequences of *C. neoformans* Arf, the *C. neoformans* G_α homolog, and orthologous fungal Arfs.

Analysis of the CoA derivatives of $C_{8:0}$ to $C_{16:0}$ fatty acids, plus a series of myristic acid analogs with single triple bonds (2- to 13-tetradecynoyl-CoAs) and single cis double bonds (*Z2*- to *Z12*-tetradecenoyl-CoAs), using radiolabeled GARASVLS-amide as the peptide acceptor, indicated that human and *C. neoformans* Nmts have similar acyl-CoA binding sites: i.e., (*i*) they prefer myristoyl-CoA, although they can readily accommodate a one-methylene variation in acyl chain length; (*ii*) palmitoyl-CoA is not a substrate for either enzyme; and (*iii*) there is a principal bend at C5–C6 of the bound hydrocarbon which prevents 5-tetradecynoyl-CoA from forming a binary complex with these Nmts (31). Together, these results suggest that it may be possible to design specific competitive inhibitors of *C. neoformans* Nmt which react with its peptide binding site and are fungicidal.

Note Added in Proof. Transformations of M049 with linear pCN28-14 DNA have yielded strains with a single copy of *nmt487D* integrated at the *NMT* locus without additional integration sites elsewhere in the genome. The phenotype of these strains is similar to that of CMD4.

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