Saccharomyces cerevisiae Contains Four Fatty Acid Activation (FAA) Genes: An Assessment of Their Role in Regulating Protein N-Myristoylation and Cellular Lipid Metabolism

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Abstract. *Saccharomyces cerevisiae* has been used as a model for studying the regulation of protein N-myristoylation. MyristoylCoA:protein N-myristoyltransferase (Nmtlp), is essential for vegetative growth and uses myristoylCoA as its substrate. MyristoylCoA is produced by the fatty acid synthetase (Fas) complex and by cellular acylCoA synthetases. We have recently isolated three unlinked Fatty Acid Activation (*FAA*) genes encoding long chain acylCoA synthetases and have now recovered a fourth by genetic complementation. When Fas is active and *NMT1* cells are grown on media containing a fermentable carbon source, none of the *FAA* genes is required for vegetative growth. When Fas is inactived by a specific inhibitor (cerulenin), *NMT1* cells are not viable unless the media is supplemented with long chain fatty acids. Supplementation of cellular myristoylCoA pools through activation of imported myristate (C14:0) is predominantly a function of Faalp, although Faa4p contributes to this process. Cells with nmt181p need larger pools of myristoylCoA because of the mutant enzyme's reduced affinity for this substrate. Faalp and Faa4p are required for maintaining the viability of *nmt1-181* strains even when Fas is active. Overexpression of Faa2p can rescue *nmt1-181* cells due to activation of an endogenous pool of C14:0. This pool appears to be derived in part from membrane phospholipids since overexpression of Plblp, a nonessential lysophospholipase/phospholipase B, suppresses the temperature-sensitive growth arrest and C14:0 auxotrophy produced by *nmt1-181*.

None of the four known *FAA*s is exclusively responsible for targeting imported fatty acids to peroxisomal β-oxidation pathways. Introduction of a peroxisomal assembly mutation, *pas1*Δ, into isogenic *NMT1* and *nmt1-181* strains with wild type *FAA* alleles revealed that when Fas is inhibited, peroxisomes contribute to myristoylCoA pools used by Nmtlp. When Fas is active, a fraction of cellular myristoylCoA is targeted to peroxisomes. A *NMT1* strain with deletions of all four *FAA*s is still viable at 30°C on media containing myristate, palmitate, or oleate as the sole carbon source—indicating that *S. cerevisiae* contains at least one other *FAA* which directs fatty acids to β-oxidation pathways.

SACCHAROMYCES cerevisiae myristoylCoA:protein N-myristoyltransferase (Nmtlp)[1] is a 455 residue, monomeric enzyme located in the cytoplasm (16, 37). Nmtlp catalyzes the transfer of myristate (C14:0) from myristoylCoA to the amino-terminal Gly residue of ~12 cellular proteins (29). Several of Nmtlp's protein substrates are essential for viability and require a covalently bound myristoyl moiety for expression of their biological function. These include Gpalp, the α subunit of a heterotrimeric G protein involved in mating signal transduction (15, 58), and two functionally interchangeable ADP ribosylation factors, Arflp and Arf2p, which play important modulatory roles in protein and membrane trafficking (35, 51, 57). Disruption or deletion of the *NMT1* locus causes recessive lethality (16, 19).

Nmtlp has a highly ordered reaction mechanism (7, 53, 54). MyristoylCoA binds to the apo-enzyme forming a high affinity (K_b = 15 nM) myristoylCoA:Nmtlp binary complex. Formation of this complex induces formation of a functional binding site for nascent protein substrates. Once the ternary myristoylCoA:Nmtlp:peptide ternary complex is assembled, myristate is transferred from CoA and linked, via an

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1. *Abbreviations used in this paper*: CER cerulenin; FAA, Fatty Acid Activation; Fas, fatty acid synthetase; 5-FOA, 5-fluoro-orotic acid; GPD, glyceraldehyde 3-phosphate dehydrogenase; HPTLC, high performance thin layer chromatography; MYR, myristate; Nmtlp, myristoylCoA:protein N-myristoyltransferase; OLE, oleate; ORF, open reading frame; PAL, palmitate; X-ALD, X-linked adrenoleukodystrophy.

amide bond, to the amino-terminal Gly of a substrate. Finally, CoA and then the myristoylpeptide product are released.

Mutations in Nmt1p which reduce its affinity for myristoylCoA, such as $Gly^{451 \rightarrow Asp}$ in nmt181p, are associated with global defects in protein N-myristoylation, growth arrest at various stages of the cell cycle within 1 h after cells are shifted to the nonpermissive temperature (≥30°C) and lethality within 12 h (17, 31). The *nmt1-181* phenotype can be fully suppressed at 37°C by overexpressing nmt181p or Nmt1p, or by supplementing media with myristate (C14:0), but not shorter or longer chain saturated fatty acids (17, 28, 30, 43). The phenotype can be partially rescued at 30°C by overexpressing gene products that affect de novo production of myristoylCoA (17, 28, 31). This latter category of genes includes *FAS1* and *FAS2*, which encode the β and α subunits, respectively, of the $\alpha_6\beta_6$ fatty acid synthetase (Fas) complex (reviewed in reference 49), plus genes that either directly or indirectly regulate *FAS* transcription; e.g., *FAS1* and *FAS2* themselves (12, 31), as well as the phosphate-repressible *PHO5* gene which specifies the organism's principal acid phosphatase (31).

S. cerevisiae possesses at least two metabolic pathways that yield myristoylCoA. MyrisotylCoA accounts for ~5% of the acylCoAs produced by the cytoplasmic Fas complex during exponential growth at 15–37°C (26, 47). AcylCoA synthetases encoded by Fatty Acid Activation (*FAA*) genes can also produce myristoylCoA. Three *FAA* genes have been identified in *S. cerevisiae* (18, 30). In vitro assays of purified Faa1p, Faa2p, and Faa3p have shown that the myristoylCoA synthetase activities of Faa1p and Faa2p are equivalent and two orders of magnitude greater than that of Faa3p (38). The ability of these cellular acylCoA synthetases to generate myristoylCoA has been assessed using isogenic strains containing *NMT1* or *nmt1-181* plus all possible combinations of *faa1*, *faa2*, and *faa3* null alleles (30). The growth characteristics of these strains in the presence or absence of an active Fas complex, with or without supplementation of the media with fatty acids, suggests that Faa1p is the principal acylCoA synthetase responsible for activating imported myristate (30). However, *NMT1*, *faa1*Δ, *faa2*Δ, *faa3*Δ strains are viable on standard rich media supplemented with myristate, even when Fas is specifically inhibited with cerulenin (24), indicating that the *S. cerevisiae* genome contains at least one additional *FAA*. We have now isolated *FAA4* and, in a survey of *NMT1* or *nmt1-181* strains with various combinations of *faa* null alleles, assessed the role of the *FAA* genes in regulating protein N-myristoylation. We have also used these strains to determine if other pathways exist in *S. cerevisiae* for regulating myristoylCoA metabolism.

Materials and Methods

Strains and Media

All yeast strains were constructed by standard methods (55). The relevant genotypes of these strains are described in Table I.

YPD media consists of 1% yeast extract, 2% peptone, 2% dextrose. YP/glycerol is composed of 1% yeast extract, 2% peptone, 3% glycerol. YPD/agar plates, and YPD/agar plates supplemented with (*a*) fatty acids (NuCheck Prep) plus Brij58 (Sigma Chemical Co., St. Louis, MO; 1% wt/vol), and/or (*b*) 25 μM cerulenin (CER; Sigma Chemical Co.), were prepared as described in reference 18. The media used to induce β-oxidation in *S. cerevisiae* contains Tween 40 (0.015% wt/vol), yeast extract (0.3%), peptone (0.3%), KH_2PO_4 (0.7%), and myristate (MYR), palmitate (PAL), or oleate (OLE, all at a final concentration of 0.15% [wt/vol]). The growth characteristics of the various strains on YPD, YPD/fatty acid, YPD/CER/fatty acid, and "β-oxidation" plates were determined at 24°, 30°, and 37°C for 3–4 d. All experiments were repeated on at least two separate occasions.

Isolation of FAA4

YB517 was isolated as a strain containing *faa1*, *faa2*, and *faa3* null alleles. YB517 and YB518 were generated by sporulation of a diploid strain produced by mating YB498 and YB501 (cf. Table I). YB517, unlike the YB518, is not viable on YPD/CER media supplemented with 500 μM myristate. YB517 was transformed with pools A and C of a YCp50 based genomic library (50) and pools I, II, and III of a YEp24 based genomic library (11). Transformants were plated directly on selective media containing 25 μM CER plus 500 μM MYR and incubated at 30°C. Individual transformants were streaked onto synthetic complete media containing 25 μM CER, 500 μM MYR, and 0.1% (wt/vol) 5-fluoro-orotic acid (5-FOA; PCR Research Chemicals, Inc., Gainesville, FL) to determine the plasmid dependence of the phenotype. Plasmid DNA was isolated from transformants that demonstrated plasmid-dependent growth on CER/MYR at 30°C. The PCR and two degenerate oligonucleotides, 5'-TSYTTYTTGCCWYTRGCHCAT-3' and 5'-YTCHRRWGCRATRTATTCACC-3' (where H = A/C/T, R = A/G, S = G/C, W = A/T, and Y = C/T), were used to identify plasmids containing *FAA* sequences. These oligonucleotide primers were designed from conserved regions of *FAA1*, *FAA2*, and *FAA3* (30). Positive clones were rescreened using PCR, a different set of oligonucleotides derived from the open reading frame of *FAA1* (5'-CTGTTTTTTGCCACTAGCTCAT-3' and 5'-CTCGAGTGCGATATATTCACC-3'), and cycling conditions which result in amplification of *FAA1* but not *FAA2* or *FAA3*. Two plasmids with identical restriction patterns, derived from pool A of the YCp50 based library, tested positive with the degenerate oligonucleotides, but not the *FAA1*-specific oligonucleotides. One plasmid, designated pBB348, was examined further. The 729-bp PCR fragment derived from pBB348 was labeled with ^{32}P and used to probe a set of three nylon filters containing >90% of the *S. cerevisiae* genome in prime lambda clones (provided by L. Riles, Department of Genetics, Washington University, St. Louis, MO); and a filter containing electrophoretically separated *S. cerevisiae* chromosomes (Clonetech, Palo Alto, CA). The hybridization and washing stringencies used were identical to those described in reference 18. The relevant open reading frame (ORF) in pBB348 was sequenced on both strands using an Applied Biosystems Model 373A automatic sequencer and a Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Database Searching and Alignments

Homology searches were performed at the National Center for Biotechnology Information using the BLAST network service (3) and the following nonredundant databases: Brookhaven Protein Database (October, 1993 release), Swiss-Prot (Release 28.0), PIR (Release 40.0), and Genpept (Release 82.0). An alignment of the primary structures of Faa1p, Faa2p, Faa3p, Faa4p was generated with the algorithm included in GeneWorks (Release 2.2), a cost of five to open a gap, and a cost of 25 to lengthen a gap. Minimal diagonal length was set at four and the maximum diagonal offset at 10. GeneWorks was also used to search Faa1p, Faa2p, Faa3p, and Faa4p for potential sites of co- and posttranslational processing and for the presence of signal and mitrochondrial transit peptide sequences.

FAA4 and PAS1 Locus Alterations

The NcoI site at nucleotide +502 of *FAA4*'s ORF was changed to a NsiI site by treating NcoI-digested pBB348 with the Klenow fragment of DNA polymerase I and ligating the resulting blunt ends. The EcoRV site at nucleotide +1601 of *FAA4* was obliterated by inserting a SmaI/Pst linker (5'-GGGCTGCA-3'). A NsiI-PstI *FAA4* fragment was then ligated to NsiI/PstI-digested pGEM-5zf (Promega Biotec, Madison, WI). A HindIII-EcoRI fragment from the resulting recombinant plasmid, containing 321 bp of *FAA4*'s coding sequence (Gly^{215} to Phe^{322}) was replaced with a 4.8-kb HindIII-EcoRI fragment encompassing the *LYS2* gene (6, 20), yielding pBB355. *faa4ΔO.3::LYS2* was released from pBB355 with NsiI and PstI and used for a single step disruption (52) of *FAA4* in the yeast strains shown in Table I. A single step disruption of *PAS1* in strains YB322 and YB336 (Table I) was accomplished with pGR30 (21, 34). All locus alterations were verified by Southern blot analysis of genomic DNA.

Table I. Yeast Strains

Strain	Genotype	Source
YB332	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1 FAA2 FAA3 FAA4*	32
YB336	*MATa nmt1-181 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1 FAA2 FAA3 FAA4*	32
YB485	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1 faa2Δ0.5::LEU2 FAA3 FAA4*	32
YB492	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1 FAA2 faa3Δ0.8::LEU2 FAA4*	32
YB497	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 FAA2 FAA3 FAA4*	32
YB498	*MATa nmt1-181 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 FAA2 FAA3 FAA4*	32
YB499	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 faa2Δ0.5::LEU2 FAA3 FAA4*	32
YB501	*MATα NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1faa2Δ1.9::LEU2 faa3Δ0.8::LEU2 FAA4*	This work
YB503	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 FAA2 faa3Δ0.8::LEU2 FAA4*	32
YB505	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1 faa2Δ0.5::LEU2 faa3Δ0.8::LEU2 FAA4*	32
YB517	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 faa2Δ0.5::LEU2 faa3Δ0.8::LEU2* (No growth on MYR/CER)	This work
YB518	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 faa2Δ0.5::LEU2 faa3Δ0.8::LEU2 FAA4*	32
YB524	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1 FAA2 FAA3 faa4Δ0.3::LYS2*	This work
YB525	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 FAA2 FAA3 faa4Δ0.3::LYS2*	This work
YB526	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 faa2Δ0.5::LEU2 faa3Δ0.8::LEU2 faa4Δ::LYS2*	This work
YB527	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1 FAA2 FAA3 FAA4 pas1Δ::LEU2*	This work
YB528	*MATa nmt1-181 ura3 his3Δ200 ade2 lys2-801 leu2 FAA1 FAA2 FAA3 FAA4 pas1Δ::LEU2*	This work
YB529	*MATa NMT1 ura3 his3Δ200 ade2 lys2-801 leu2 faa1Δ1.9::HIS3 faa3Δ0.8::LEU2 faa4Δ::LYS2*	This work

Construction of Plasmids for Overexpressing of FAA4 and PLB1

A mutagenic oligonucleotide (5'-GAAGTACGCATCCATATGACCGAACAATATTCCG-3') and PCR were used to introduce a NdeI site at the initiator ATG codon of *FAA4* in pBB348. A 2.3-kb NdeI-SmaI fragment from the resulting plasmid, containing the entire *FAA4* ORF, was subcloned into pMON2670 (48), yielding pBB364. pBB365 was constructed by ligating a 2.3-kb XbaI fragment of pBB364 into XbaI-digested pBB358. (pBB358 is a low copy YCp-based plasmid containing the glyceraldehyde 3-phosphate dehydrogenase [*GPD*] promoter [8]. It was constructed from pBB307 [30] by eliminating its ClaI, NotI, and XbaI sites through cleavage with these restriction enzymes, treatment of the resulting DNA with Klenow, and religation. A new polylinker was introduced into pBB358 between its KpnI-SalI sites by annealing 5'-CTCTAGAATCGATGAATTCGGATCCGCGGCCGCCTGCAGG-3' and 5'-TCGACCTGCAGGCGGCCGCGGATCCGAATTCATCGATTCTAGAGGTAC-3' and religation.)

A *GPD-PLB1* expression vector was made by (*a*) releasing a SauI-DraI *PLB1* fragment from YEp24[*PLB1*], (*b*) treating the fragment with Klenow, and (*c*) ligating the blunt-ended restriction fragment to SmaI-digested pBB307.

Measurement of Faa2p Levels

Faa2p, containing a carboxy-terminal tag of six histidine residues (Faa2-6XHis), was expressed in a *fadR⁻fadD⁻* strain of *Escherichia coli* (LS6928; reference 46) and subsequently purified to apparent homogeneity by nickel-chelate affinity chromatography (38). Antibodies were raised against Faa2p-6xHis in two rabbits. The specificities of the resulting antibody preparations were surveyed by incubating preimmune and immune sera, at a final dilution of 1:1000, with protein blots containing purified Faa2p-6XHis, Faa1p-6xHis, and Faa3p-6xHis (38).

The ability of pBB325 (*GPD-FAA2*; reference 30) to produce increased levels of Faa2p in *S. cerevisiae* was investigated by transforming strain YB332 with this DNA or with the parental plasmid without insert (pBB307). Cells were grown at 24°C to mid-log phase in selective media and lysed using a protocol described in reference 42. Total cellular proteins (50 μg) were reduced, denatured, fractionated by SDS-polyacrylamide gel electrophoresis (40), and then transferred to nitrocellulose membranes (62). Blots were probed with anti-Faa2p-6XHis diluted 1:1,000 in Blotto. Antigen-antibody complexes were detected using ^{125}I-Protein A.

Metabolic Labeling of Cellular Lipids

Strains YB332, YB497, YB524, and YB525 (Table I) were grown in YPD at 24°C to OD_{600} ≈1. Cultures were transferred to tubes containing either [9,10(n)-^{3}H]myristate, or [9,10(n)-^{3}H]palmitate (33.5 Ci/mmol; 100 μCi/ml culture), and shaken for 1 h at 24°C. Cells were pelleted by centrifugation and cellular lipids extracted according to Bligh and Dyer (10), except that acid-washed glass beads (425–600-μm diam; Sigma Chemical Co.) were added in the initial step. Lipids were resuspended in chloroform/methanol (1:1), and aliquots of 100,000 dpm from each strain were spotted onto Silca Gel 60 high performance thin layer chromatography (HPTLC) plates (Merck, Sharpe, and Dohme, Rahway, NJ). Lipids were separated in a single dimension using methylacetate, isopropyl alcohol, chloroform, methanol, and 0.25% KCl (25:25:28:10:7). Lipid standards (Sigma Chemical Co.), included in each HPTLC plate, were visualized using iodine vapors. Radiolabeled lipids were detected by spraying the plates with EN^{3}HANCE and performing fluorography at −80°C.

Results

Isolation of FAA4

As noted in the Introduction, we had found that a *NMT1, faa1Δ,faa2Δ,faa3Δ* strain (YB518) is viable at 30°C when Fas is inhibited by the antibiotic cerulenin (5) and the YPD media is supplemented with 500 μM myristate. We were

A

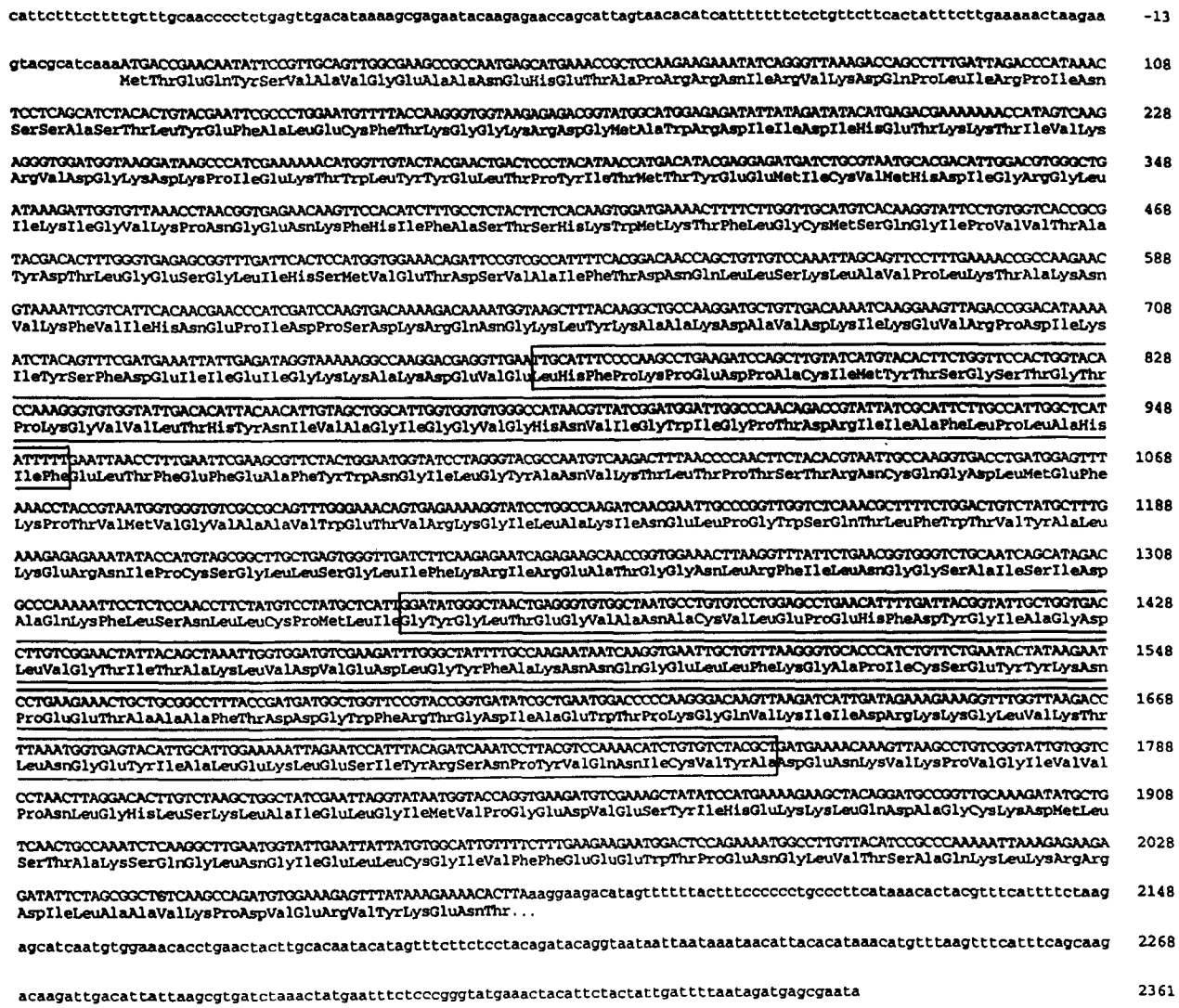
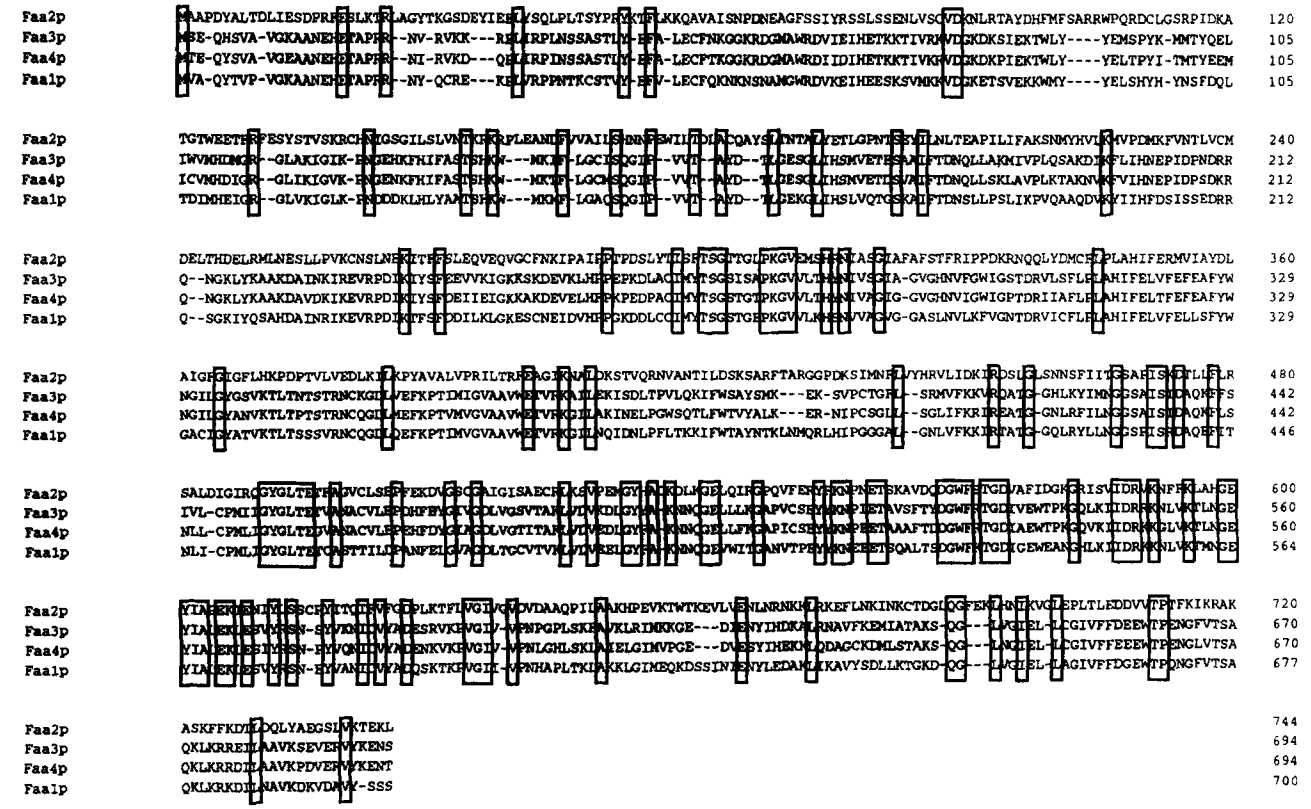


Figure 1.

able to identify a related *NMT1,faal*Δ,*faa2*Δ,*faa3*Δ strain (YB517) which is not viable under these growth conditions (see Materials and Methods). We used this latter strain to screen for suppressors of its lethal phenotype on YPD/MYR/CER at 30°C. Two identical plasmids were obtained from the YCp50-based genomic library described by Rose et al. (50). PCR using primers, derived from the conserved putative ATP binding domains of Faalp, Faa2p and Faa3p, yielded a 729-bp product whose nucleotide sequence was related to, but distinct from, that of the other *FAA* genes. The PCR fragment was used to probe blots of *S. cerevisiae* chromosomes as well as prime lambda clones incorporating >90% of the yeast genome. The amplified DNA reacted with chromosome XIII and lambda clone 1986. (Prime lambda clones are numbered according to the scheme of L. Riles and M. Olsen, manuscript in preparation. The clones are available from the American Type Tissue Culture Collection, Rockville, MD.) The putative *FAA4* gene was not linked to *FAA1* (chromosome XV, lambda clones 3748/2748), *FAA2* (chromosome V, lambda clone 6977), or *FAA3* (chromosome IX, lambda clone 4554).

Sequence analysis of the insert contained in the YCp50 plasmid revealed a 2,088-bp open reading frame, encoding a 694-amino acid protein (Fig. 1 *A*). Sequence alignments indicated that this protein has 78% identity with Faa3p, 61% identity with Faalp, and 23% identity with Faa2p (Fig. 1 *B*). (The ORFs of *FAA1* and *COT1*, a gene involved in cobalt accumulation [13], are separated by <2 kb. Cotlp is 60% identical to Zrclp, a protein which confers resistance to zinc and cadmium ions [36]. The ORF of *FAA4* is located on the same prime lambda clone as *ZRC1*. The close linkage of *FAA1* and *COT1*, and *FAA4* and *ZRC1* raises the possibility that they arose from a gene duplication event.)

Like the other *S. cerevisiae* Faas, Faa4p does not have amino-terminal sequences which are predicted to function as a mitochondrial transit peptide or elements which are known to function in directing proteins to peroxisomes (reviewed in reference 59).



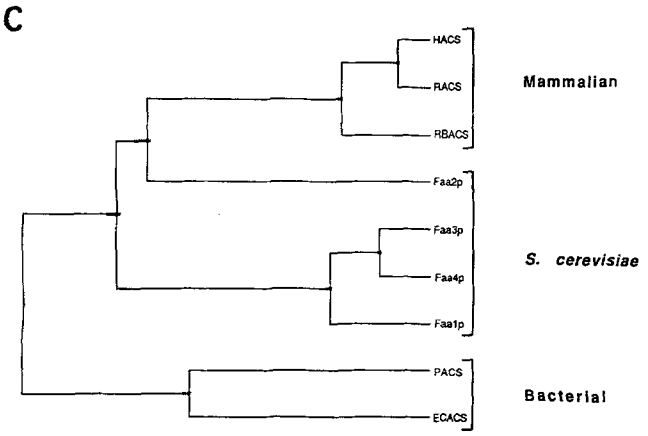


Figure 1. Structure of *FAA4* and a comparison of its protein product with other long chain acylCoA synthetases. (*A*) Sequence of *FAA4* and its predicted protein product. The amino acids shown in boxes represent two highly conserved regions in the known prokaryotic, yeast, and mammalian acylCoA synthetases (32, 60). These two domains may be involved in binding of ATP and/or hydrolysis of a pyrophosphate from ATP in the reaction catalyzed by acylCoA synthetases:

$$\text{Fatty acid} + \text{ATP} \xrightarrow{Mg^{2+}} [\text{Fatty acyl-AMP}] + PP_i \xrightarrow{\text{CoA}} \text{Fatty acylCoA} + \text{AMP}$$

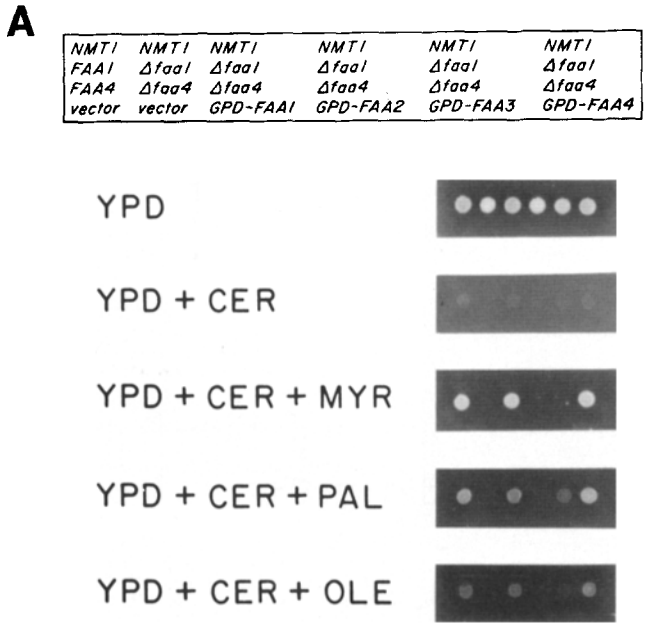
Note that the *FAA4* gene in pBB348 has 113 nucleotides 5′ of it predicted translation start site. Expression of *FAA4* in this YCp50-based plasmid is likely to be influenced by *cis*-acting elements located in the adjacent tetracycline resistance gene (50). (*B*) Multiple sequence alignment of the four known yeast Faas. (*C*) Dendogram representation of the sequence relationships between Faa1p, Faa2p, Faa3p, Faa4p, human acylCoA synthetase (HACS; reference 1): rat liver acylCoA synthetase (RACS; (reference 60); rat brain acylCoA synthetase (RBACS; reference 23); *Pseudomonas oleovarans* acylCoA synthetase encoded by the *alkK* gene (PACS; reference 63); and *E. coli* acylCoA synthetase (FadD; reference 9). This dendogram was generated using an algorithm incorporated into Geneworks. The length of horizontal lines is proportional to the magnitude of the difference in identity between two aligned sequences. Vertical lines have no significance.

A search of several protein databases with Faa4p revealed significant similarities to the three known mammalian long chain acylCoA synthetases plus the two reported prokaryotic acylCoA synthetases. The dendogram presented in Fig. 1 *C* shows that among the four *S. cerevisiae* Faas, Faa2p most closely resembles the mammalian long chain acylCoA synthetases.

FAA4 Is Not Essential

Deletion of *FAA4* has no detectable effect on the growth of a *NMT1,FAA1,FAA2,FAA3* strain at 24–37°C on YPD media (data not shown). Wild type strains of yeast are not viable at 24–37°C on YPD when their Fas complex is inhibited by cerulenin (Fig. 2 *A*). The ability of exogenous myristate, palmitate, or oleate to rescue growth of *NMT1,FAA1,FAA2, FAA3* cells on YPD/CER at 24–37°C is not impaired by deleting *FAA4* (data not shown).

To determine the role of each Faap in activating imported myristate (C14:0), palmitate (C16:0), and oleate (C18:1$^{\Delta 9}$), we examined the phenotypes of isogenic *NMT1* strains, with various combinations of *faa* null alleles, on YPD/CER/



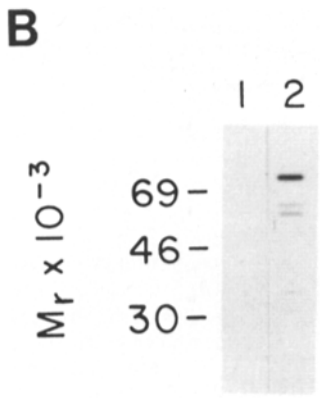


Figure 2. The growth characteristics of a *NMT1*, *faa1*Δ, *faa4*Δ strain with and without *GPD-FAA* episomes. (*A*) An equal number of YB525 (*NMT1*, *faa1*Δ, *faa4*Δ) cells, transformed with either *GPD-FAA1* (pBB330), *GPD-FAA2* (pBB325), *GPD-FAA3* (pBB 343), *GPD-FAA4* (pBB365), or the parental vector (pBB-307), were plated on YPD media, with or without 500 μM fatty acids (MYR, myristate; PAL, palmitate; OLE, oleate), and 25 μM cerulenin (CER). Plates were incubated for 4 d at 30°C. (*B*) Lysates were prepared from exponentially growing cultures of YB332 (*NMT1*, *FAA1*,*FAA2*,*FAA3*,*FAA4*), transformed with either *GPD-FAA2* (lane 2) or the parental vector without insert (lane *1*). Total cellular proteins (50 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The protein blot was then probed with a rabbit anti-Faa2p sera. Antigen-antibody complexes were detected with ^{125}I-labeled protein A.

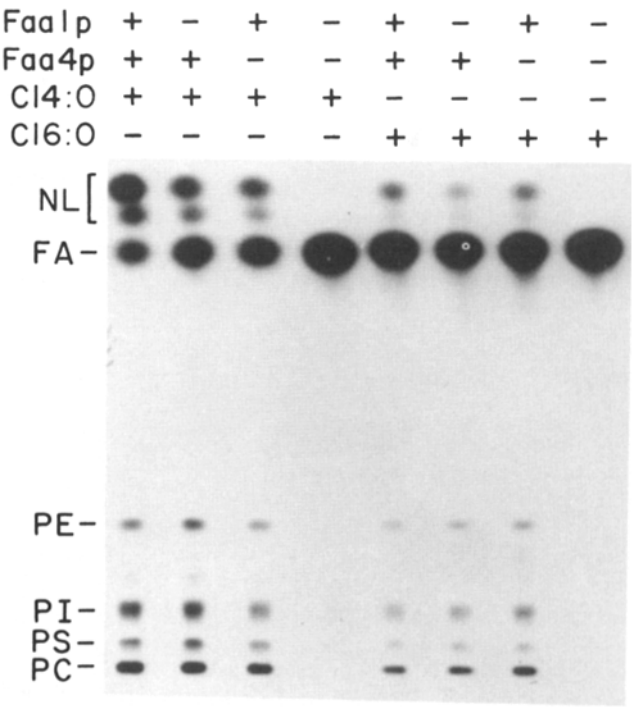


Figure 3. Metabolic labeling of cellular lipids in isogenic *NMT1* strains containing various combinations of wild type or null *FAA1* and *FAA4* alleles. Total cellular lipids were prepared from wild type (YB332), *faa1*Δ (YB497), *faa4*Δ (YB498), and *faa1*Δ,*faa4*Δ (YB525) strains harvested during exponential growth at 24°C in selective media containing [^{3}H]myristate (C14:0) or [^{3}H]palmitate (C16:0). Lipids were separated in a single dimension by HPTLC and the plates subjected to autoradiography for 20 h. The position of migration of lipid standards are shown: *NL*, neutral lipids; *FA*, unesterified fatty acids; *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *PC*, phosphatidylcholine.

FATTY ACID plates at 24–37°C. The only *FAA* which produces a phenotype when deleted alone is *FAA1*. *NMT1*, *faa1*Δ cells show varying degrees of growth retardation at 30–37°C on YPD/CER depending upon the chain length and concentration of the fatty acid included in the media: growth is greater in the presence of myristate compared to palmitate which, in turn, is more efficiently used than oleate (data not shown; cf. reference 30). Strains with deletions of both *FAA1* and *FAA4* are not viable at any of these temperatures, even when YPD/CER is supplemented with up to 500 μM of each fatty acid (Fig. 2 *A*). No other combination of two *faa* null alleles produces inviable cells under these conditions. In fact, *faa2*Δ,*faa4*Δ, and *faa3*Δ,*faa4*Δ cells show no growth retardation compared to isogenic wild type strains.

Activation of Imported Fatty Acids Is Primarily Accomplished by Faa1p and Faa4p

The results presented in the preceding paragraph suggest that Faa1p and Faa4p are both able to activate imported long chain fatty acids with Faa1p being the principal acylCoA synthetase responsible for this function. To test this hypothesis, we compared the incorporation of exogenous tritiated C14:0 and C16:0 into cellular phospholipids produced by a wild type (*NMT1*,*FAA1*,*FAA2*,*FAA3*,*FAA4*) strain and three isogenic derivatives: one with a *faa1* null allele, one with a *faa4* null allele, and another with *faa1*,*faa4* null alleles. There is no impairment in the incorporation of either fatty acid in strains with *faa1*Δ or *faa4*Δ during exponential growth at 24°C in YPD (Fig. 3). However, when both *FAA1* and *FAA4* are deleted, there is a dramatic reduction in the incorporation of both ^{3}H-labeled fatty acids into cellular phospholipids and a marked increase in free fatty acid (Fig. 3).

FAA4 and FAA1 Are Functionally Interchangeable

We examined the ability of *FAA1*, *FAA2*, *FAA3*, and *FAA4* to rescue growth of *NMT1*,*faa1*Δ cells on YPD/CER/FATTY ACID plates at 30–37°C. The *FAA* ORFs was placed under the control of the strong, constitutive *GPD* promoter contained in a centromeric plasmid. Each of the four *GPD-FAA* plasmids, or the parental vector without insert, was intro-

duced into YB525 (*NMT1,faal*Δ,*faa4*Δ). Equal numbers of cells were then plated at 30° and 37°C on YPD/CER supplemented with 500 μM myristate, palmitate, or oleate. *GPD-FAA1* or *GPD-FAA4* are able restore growth at 30°C to levels equivalent to that of an isogenic wild type strain. Partial rescue can be achieved with *GPD-FAA3* but only when palmitate or oleate is used (Fig. 2 *A*). Similar results were obtained at 30° and 37°C (data not shown).

The inability of *GPD-FAA2* to rescue *NMT1,faal*Δ,*faa4*Δ cells (Fig. 2 *A*) was not due to an inability to overexpress to the protein. When protein blots of cell lysates, prepared from a wild type strain transformed with *GPD-FAA2*, were probed with a rabbit polyclonal antibody raised against purified Faa2p, a marked increase in the steady state level of this acyl-CoA synthetase was observed (Fig. 2 *B*).

Under Certain Growth Conditions, FAA1 Plus FAA4 Are Necessary for Growth Even When Fas Is Active

Wild type strains of *S. cerevisiae* can grow on glycerol as the sole carbon source. This requires conversion of glycerol to glyceraldhyde-3-phosphate (for entry into the glycolytic pathway) and adequate mitochondria respiration so that the NADH generated by glycolysis can be removed (33). We incubated isogenic *NMT1* strains, with all combinations of *faa* null alleles, at 24–37°C on YP media containing 3% glycerol. When Fas is active, cells with a single *faa* null allele are able to grow at rates comparable to that of wild type cells (data not shown). *FAA1* and *FAA4* have to be deleted before there is any impairment of growth. There is slightly diminished growth at 30°C while virtually no growth is evident at 37°C (Fig. 4; and data not shown). The biochemical basis for this growth retardation is not known although it is possible that deletion of *FAA1* and *FAA4* may affect mitochondrial function.

Assessment of the Ability of FAAs to Rescue the Temperature-sensitive Growth Arrest and Myristic Acid Auxotrophy Produced by nmt1-181

A *nmt1-181* strain with *faa1,faa2*, and *faa3* null alleles cannot grow at 24–37°C on YPD, even when the de novo pathway for acylCoA biosynthesis is intact and the media is supplemented with 500 μM myristate (reference 30; and data not shown). Deletion of *FAA1* alone produces moderate growth retardation at 24°C on YPD and YPD/MYR when compared to an isogenic *nmt1-181*, *FAA1* strain. In contrast, deletion of *FAA4* alone (or *FAA2* or *FAA3*) has no detectable effect under these growth conditions (reference 30; and data not shown). We were unable to delete *FAA4* in a *nmt1-181,faal*Δ strain using a single step gene disruption protocol and obtain cells which were viable on selective media at 24°C. Therefore, diploid *nmt1-181* cells, homozygous for *faal*Δ and heterozygous for *faa4*Δ, were sporulated and the phenotypes of haploid daughter cells determined by standard tetrad analysis. The results confirmed that *nmt1-181,faal*Δ,*faa4*Δ haploid daughters are not viable.

Introduction of *GPD-FAA1*, or *GPD-FAA3*, or *GPD-FAA4* into a *nmt1-181* strain with wild type *FAA* alleles (YB336) does not rescue or enhance growth at 24–37°C on YPD or on YPD/CER supplemented with 125–500 μM myristate and palmitate (Fig. 5; and data not shown).

GPD-FAA2 is unique among the *GPD-FAAs* in two respects. First, it is the only *GPD-FAA* which can augment growth of a *nmt1-181,FAA1,FAA2,FAA3,FAA4* strain at 30°C on YPD without fatty acid supplementation (Fig. 5). It also enhances growth of this strain at 30°C on YPD/CER containing 125 μM MYR. Second, *GPD-FAA2* is the only *GPD-FAA* which can enhance growth of an isogenic *NMT1,FAA1, FAA2,FAA3,FAA4* strain on YPD/CER supplemented with myristate, palmitate, or oleate at concentrations which normally are not sufficient to support growth when Fas is blocked (e.g., see YPD/CER/palmitate [125 μM] in Fig. 5).

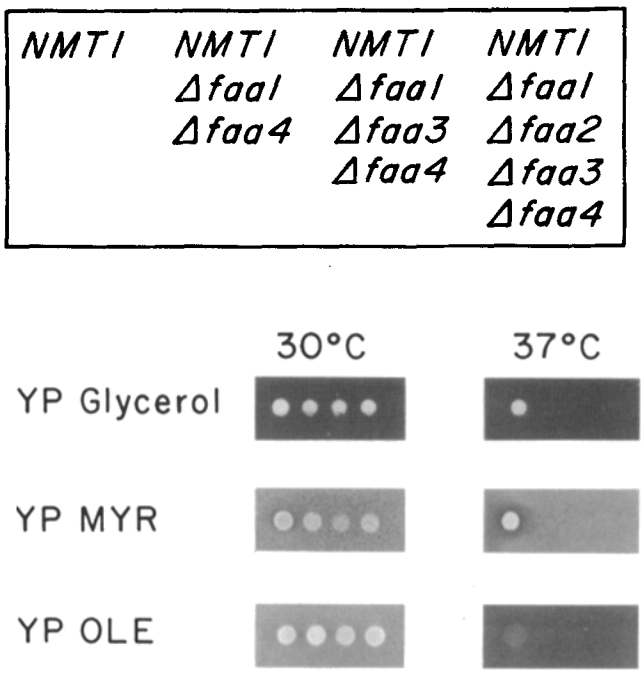


Figure 4. Assessment of growth of *faa*Δ strains on a nonfermentable carbon source. Isogenic wild-type (YB332), *NMT1,faal*Δ,*faa4*Δ (YB525), *NMT1, faal*Δ,*faa3*Δ,*faa4*Δ (YB529), and *NMT1 faal*Δ, *faa2*Δ*faa3*Δ,*faa4*Δ(YB526) strains were streaked onto media containing either glycerol, myristate, or oleate as the sole carbon source and incubated at 30°C or 37°C for 4 d.

It is important to note that *GPD-FAA2* is unable to rescue a *NMT1,faal*Δ,*faa4*Δ strain on YPD/CER supplemented with up to 500 μM myristate, palmitate, or oleate (Fig. 2 *A*) even in the face of a >100-fold increase in steady state levels of Faa2p (Fig. 2 *B*).

These findings led us to the following conclusions. First, Faalp and Faa4p appear to be essential for maintaining the viability of *nmt1-181* cells even when Fas is actively synthesizing myristoylCoA. Second, the ability of *GPD-FAA2* to rescue a *nmt1-181* strain on YPD appears to be due to activation of endogenous pools of myristate. The rescue cannot be accounted for by activation of imported fatty acids based on our observation that over-expressing Faa2p in a *NMT1, faal*Δ,*faa4*Δ strain fails to rescue growth on YPD/CER/FATTY ACID plates.

Overexpression of the Phospholipase Encoded by PLB1 Can Rescue nmt1-181 Cells

What is the source of endogenous free fatty acids which are activated by Faas, resulting in augmentation of cellular myristoylCoA pools? One possibility is that phospholipids

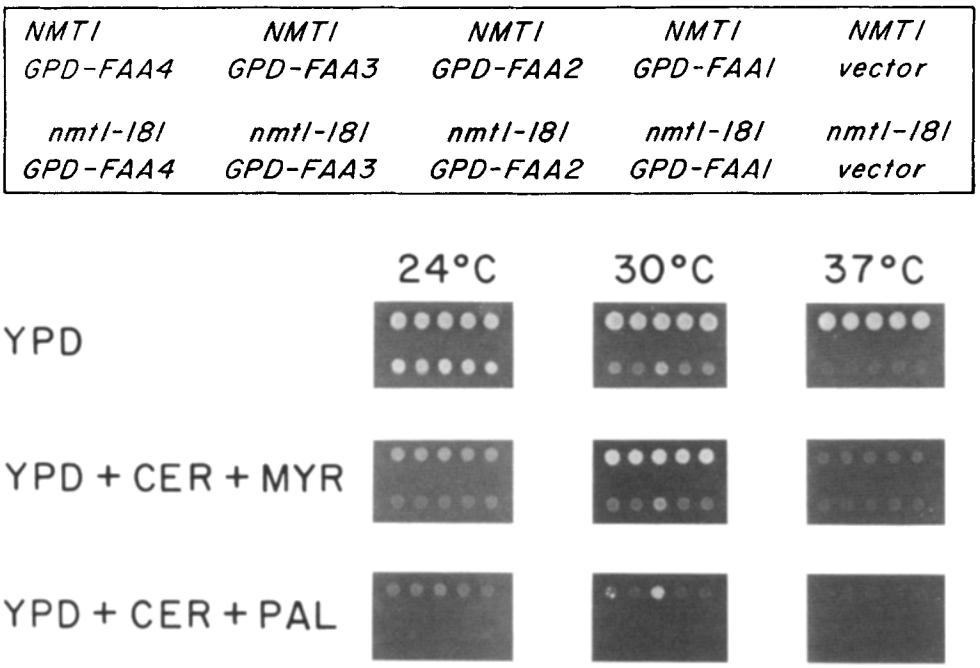


Figure 5. Comparison of the phenotypes of isogenic *NMT1* and *nmt1-181* strains containing *GPD-FAA* episomes. Isogenic strains YB332 (*NMT1*, *FAA1,FAA2,FAA3,FAA4*) and YB336 (*nmt1-181,FAA1,FAA2, FAA3,FAA4*), containing either *GPD-FAA1*, *GPD-FAA2*, *GPD-FAA3*, *GPD-FAA4*, or the parental vector without a *GPD-FAA* insert, were plated in equal numbers on YPD media with or without 125 μM fatty acids (MYR, myristate; PAL, palmitate) and 25 μM cerulenin (CER). Plates were incubated for 4 d at 24°, 30°, or 37°C.

and/or triacylglycerols serve as repositories for acyl chains which can be metabolically processed to myristoylCoA (cf. reference 61). This hypothesis was tested by transforming isogenic *NMT1* and *nmt1-181* strains containing wild type *FAA* alleles (YB332 and YB336) with *GPD-PLB1* or *GPD-TGL1* episomes. *PLB1* encodes a 664–amino acid lysophospholipase/phospholipase B which is apparently responsible for deacylation of phosphatidylcholine and phosphatidylethanolamine but not phosphatidylinositol (41). Plb1p exists in three isoforms: two are associated with the plasma membrane while the other is secreted through the periplasmic space into the media (66, 67). *plb1*Δ cells have no residual cellular lysophospholipase/phospholipase B activity yet exhibit no detectable growth defects (41). *TGL1* encodes a 548-amino acid protein with homology to mammalian triglyceride lipase (2).

GPD-TGL1 has no effect on the phenotypes of the *NMT1* or *nmt1-181* strains when they are incubated at 24°, 30°, or 37°C on YPD (data not shown). In contrast, *GPD-PLB1* rescues growth of the *nmt1-181* strain on YPD at 30°C (Fig. 6). This rescue is also achieved at 30°C on YPD/CER containing 125 μM myristate (a concentration which does not normally sustain growth at this temperature). This latter finding on cerulenin-containing media indicates that the mechanism of Plb1p's rescue does not involve changes in Fas activity. *nmt1-181* strains containing deletions of any one of the four *FAAs* exhibit the same degree of rescue by *GPD-PLB1* on YPD at 30°C as the isogenic strain with wild type *FAA* alleles (data not shown).

These results establish that acyl chains derived from membrane-associated phosphatidylcholine and/or phosphatidylethanolamine can be liberated by Plb1p and processed to myristoylCoA by one or more of the cell's Faas. The myristoylCoA produced in this fashion is accessible to cellular Nmt.

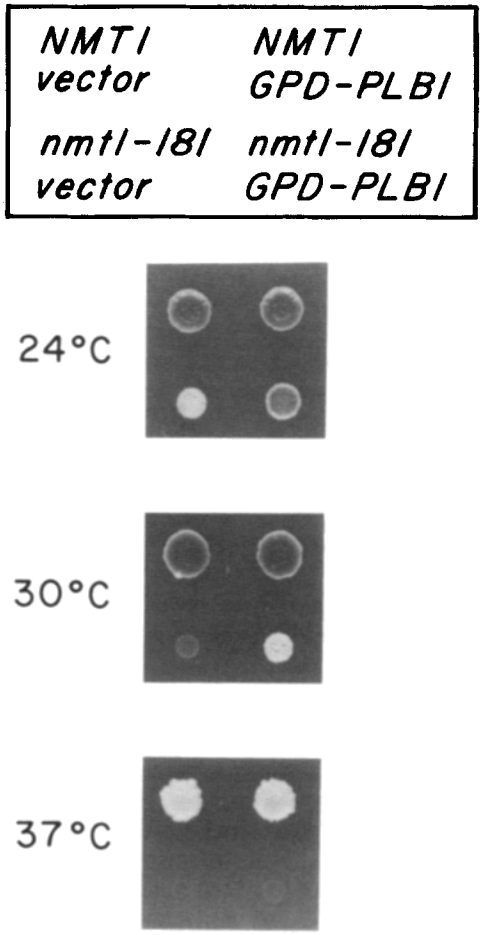


Figure 6. Rescue of a *nmt1-181* strain by *GPD-PLB1*. Isogenic wild-type (YB332; *NMT1,FAA1,FAA2,FAA3,FAA4*) and *nmt1-181* (YB336; *nmt1-181,FAA1,FAA2,FAA3,FAA4*) strains, transformed with either *GPD-PLB1* or the parental vector without insert, were plated on YPD media and incubated for 4 d at 24°, 30°, or 37°C.

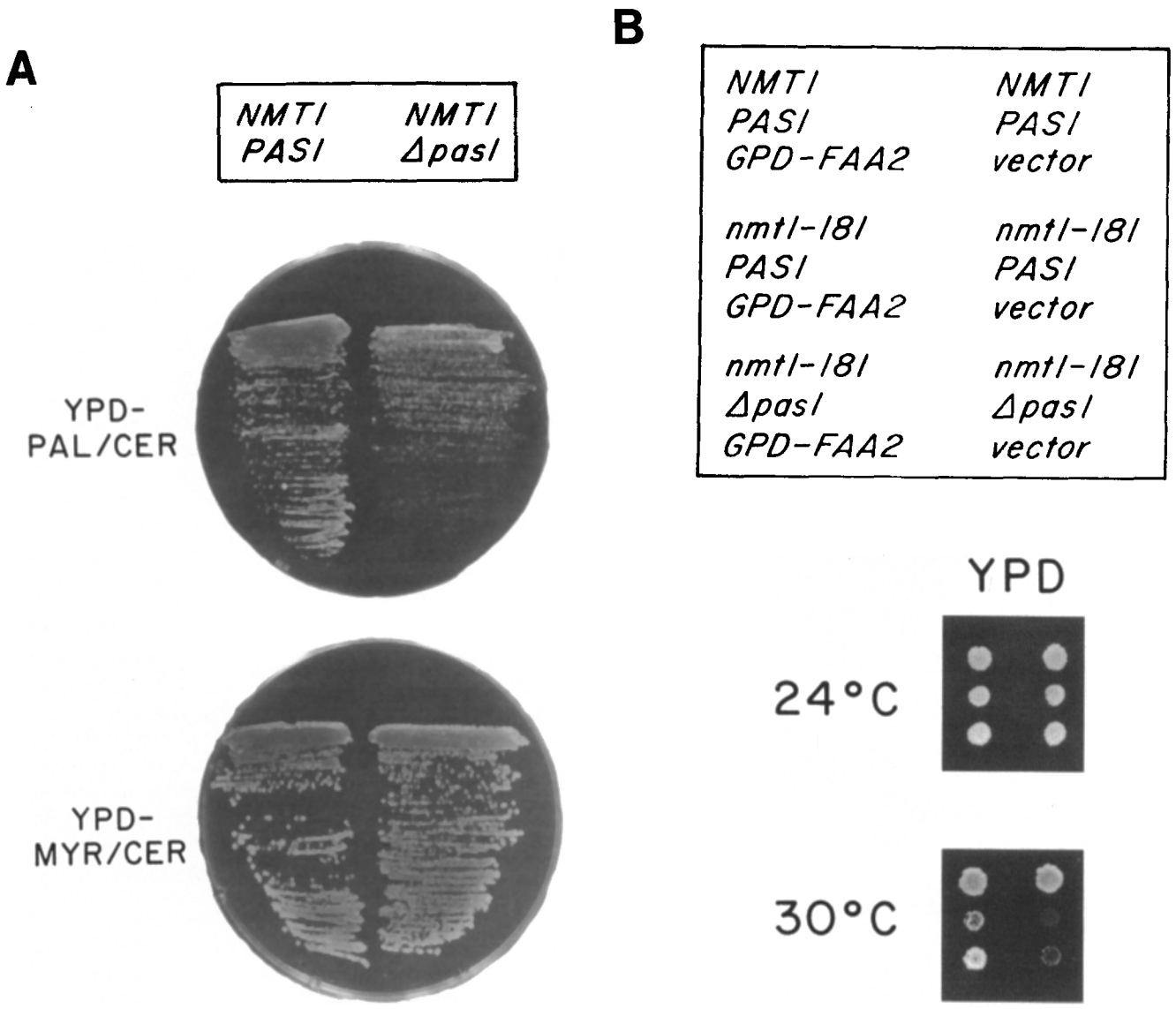


Figure 7. Effects of *pas1*Δ on growth of *NMT1* and *nmt1-181* strains. (*A*) Isogenic *NMT1,PAS1* (YB332) and *NMT1,pas1*Δ (YB527) strains were streaked to YPD media supplemented with 25 μM cerulenin (CER) and either 125 μM palmitate (PAL) or 125 μM myristate (MYR). Plates were incubated at 30°C for 3 d. (*B*) Isogenic *NMT1,PAS1* (YB332), *nmt1-181,PAS1* (YB336), and *nmt1-181,pas1*Δ (YB528) cells, containing either *GPD-FAA2* or the parental vector without insert, were plated on YPD media and incubated for 4 d at 24° or 30°C.

The Role of Faas in β-Oxidation of Fatty Acids: Evidence for a FAA5

NMT1 strains with wild type *FAA* alleles can grow on media containing myristate, palmitate, or oleate as the sole carbon source (30, 64). Growth requires that the exogenous fatty acids be imported, activated to their CoA derivatives by cellular Faa(s), and metabolically processed via β-oxidation in peroxisomes (21, 49). *NMT1* strains containing single *faa* null alleles are able to grow at 30–37°C on media containing any of these fatty acids. Deletion of all four *FAA* alleles in a *NMT1* strain still allows growth on YP/myristate, YP/palmitate, or YP/oleate at 24–30°C (Fig. 4; data not shown). These results suggest that *S. cerevisiae* contains at least one other *FAA* (*FAA5*) which is able to activate imported fatty acids and direct them to β-oxidation pathways.

Analysis of Peroxisomal Assembly Mutants

nmt1-181 strains with wild type *FAA* alleles are viable at 24–37°C on media containing myristate as the sole carbon source (i.e., YP/myristate; data not shown). They are not viable on YP/palmitate at these temperatures, indicating that imported palmitate cannot be metabolically processed via β-oxidation to myristoylCoA in amounts sufficient to overcome the catalytic defects of nmt181p. (The fact that an isogenic *NMT1* strain containing wild type *FAA* alleles is not viable on YPD/CER but is viable on YPD/CER/PAL [Fig. 2 *A*] indicates that the exogenous palmitate can yield sufficient amounts of myristoylCoA to satisfy the needs of Nmt1p.)

We used a peroxisomal assembly mutant (*pas1*) to examine the contribution of peroxisome-based β-oxidation activities

to cellular myristoylCoA pools. Strains with deletions of *PAS1* do not contain morphologically identifiable peroxisomes and are unable to grow on YP/myristate, YP/palmitate, or YP/oleate at 24–37°C (references 21, 22; and data not shown). Introduction of a *pas1* null allele into a *NMT1* strain with wild type *FAA* alleles results in a marked reductions in growth on YP-DEXTROSE/CER/palmitate at 30°C. No such growth retardation is seen on YP-DEXTROSE/CER/myristate (Fig. 7 *A*). (*pas1*Δ does not effect growth when cells are plated on YP-DEXTROSE/palmitate or YP-DEXTROSE/myristate, i.e., when Fas is active.) These results indicate that when Fas is inhibited, peroxisomes contribute to myristoylCoA pools used by Nmt1p, even when a fermentable carbon source (dextrose) is available.

Although *NMT1,FAA,pas1*Δ cells show no growth defects on YP-DEXTROSE, YP-DEXTROSE/myristate, or YP-DEXTROSE/palmitate when the de novo pathway for acylCoA synthesis is active, this is not the case with isogenic *nmt1-181* cells. Introduction of *pas1*Δ into *nmt1-181* cells with wild type *FAA* alleles results in a slight, but reproducible, rescue of growth at 30°C on YPD alone (Fig. 7 *B*). This finding provides genetic evidence that when Fas is active, some fraction of cellular myristoylCoA is targeted to peroxisomes and is therefore not available to nmt181p.

We also used the *pas1*Δ allele to examine the mechanism by which overproduction of *FAA2* results in a partial rescue of *nmt1-181* cells on YPD at 30°C. *GPD-FAA2* or the *GPD* episome without *FAA2* insert was introduced into isogenic *nmt1-181* strains containing wild type *FAAs*, with or without a *pas1*Δ allele. Cells were plated on YPD at 24° and 30°C. When peroxisome assembly is blocked, the rescue of *nmt1-181* cells produced by *FAA2* overexpression is enhanced (Fig. 7 *B*). This finding is consistent with the notion, presented in the preceeding paragraph, that when Fas is active, a fraction of myristoylCoA is targeted to peroxisomes: i.e., augmentation of cellular myristoylCoA pools through Faa2p overexpression is maximized because peroxisomal sequestration/utilization of myristoylCoA is reduced by *pas1*Δ.

Discussion

Faas and Regulation of the Efficiency and Specificity of Protein N-Myristoylation

By generating strains with multiple *faa* null alleles and by controlling the activity of the de novo pathway for acylCoA production with cerulenin, we have been able to assess the role of cellular acylCoA synthetases in regulating the efficiency of protein N-myristoylation. Even with deletion of the four known *FAA* genes, Nmt1p is able to support adequate levels of protein N-myristoylation for vegetative growth using myristoylCoA produced by Fas. When the myristoylCoA requirements for Nmt are increased by mutations that reduce the enzyme's affinity for this substrate, then contributions from the Faas become necessary to maintain viability. Based on an analysis of *nmt1-181* strains containing various combinations of *faa* null alleles, it appears that *FAA1* is the principal acylCoA synthetase involved in this supplementation of cellular myristoylCoA pools. Faa1p's contribution cannot be simply accounted for by activation of imported fatty acids since *nmt1-181* cells can grow on completely synthetic media.

The acyl chain specificity of protein N-myristoylation in vivo does not appear to be fully determined by the in vitro acylCoA specificities of Nmt. Heterogeneous acylation of certain mammalian N-myristoylproteins with alternative Nmt substrates—C12:0, C14:1$^{\Delta 5}$, and C14:2$^{\Delta 5,8}$—has been shown to be cell lineage specific (14, 32, 39, 45). This has led to the notion that the availability of various acylCoAs influences the nature of acyl chains transferred by Nmt to its protein substrates. Faa1p, Faa2p, and Faa3p purified from *fadD*$^-$ strains of *E. coli* have distinct fatty acid substrate specificities and pH optima in vitro (38). Differences in the substrate specificities and intracellular locations of Faas could account for the nature of the acylCoA species available to Nmt1p in vivo.

The fact that the membrane-associated phospholipase, Plb1p, is able to partially rescue a *nmt1-181* strain also points to the importance of considering where C14:0 is deposited in cells. Although *NMT1,plb1*Δ cells have no detectable phenotypic abnormalities, the efficiency (and specificity) of protein N-myristoylation may be influenced by the ability to induce a phospholipase which generates fatty acid substrates for one or more of the Faas.

Faas and the Regulation of Cellular Lipid Metabolism

It appears that when cells are grown on YPD/CER/FATTY ACID plates, Faa1p or Faa4p are required to activate imported long chain fatty acids. When Faa4p is overexpressed, it can replace the functions provided by Faa1p under these growth conditions. The inability of Faa2p to rescue when overexpressed provides additional support for the notion that it does not have access to imported fatty acids and/or that it is not involved in delivering activated, exogenously derived fatty acids to intracellular metabolic pathways.

The data obtained from overexpressing *FAA1* or *FAA4* in *faa1*Δ,*faa4*Δ cells also suggest that Faa1p does not have to form a heterodimer with Faa4p to be functional. A similar conclusion about not having to form Faa1p:Faa2p or Faa1p:Faa3p heterodimers for activity can be made from earlier experiments that involved overexpression of *FAA1* in isogenic *NMT1,faa2*Δ and *NMT1,faa3*Δ strains (30).

One intriguing possibility is that Faa1p and/or Faa4p function in the translocation of fatty acids across cellular membranes. There is precedent for invoking this hypothesis. Transport of palmitate across rat liver peroxisomal membranes requires prior activation to palmitoylCoA by a palmitoylCoA synthetase associated with the cytoplasmic face of the membrane (56). The availability of yeast strains with and without *faa1*Δ and/or *faa4*Δ alleles may allow the relationship between fatty acid activation and passage through membranes to be examined directly when cells are grown on a fermentable carbon source.

When *NMT1,Faa1*Δ,*Faa2*Δ,*Faa3*Δ,*faa4* cells are grown on media containing fatty acid as the sole carbon source, Faa5p and/or other Faas are apparently able to activate imported fatty acids. The lack of viability of *NMT1,faa1*Δ, *FAA2,FAA3,faa4*Δ and *NMT1,faa1*Δ,*faa2*Δ,*faa3*Δ,*faa4*Δ cells on YP-DEXTROSE/CER/FATTY ACID media indicates that *FAA5* itself, or in combination with *FAA2* and *FAA3*, is not able to supply sufficient acylCoAs to cellular metabolic pathways to sustain viability in the absence of an active Fas complex. Our metabolic labeling studies also suggest that *FAA5* is unable to direct imported myristate or pal-

mitate to phospholipid biosynthetic pathways when cells are grown on a fermentable carbon source. Alternatively, *FAA5* may be specifically induced when fatty acids are present as the sole carbon source or it may be repressed by glucose.

These observations emphasize the importance of noting the effects of growth conditions as well as growth phase when considering the role of Faas in yeast lipid metabolism. Once *S. cerevisiae* cells reach stationary phase or are deprived of nutrients, they are able to survive for several months without significant loss of viability (65). Under these conditions, profound changes occur in phospholipid and triacylglycerol content (e.g., references 25, 27, 61). The failure of *GPD-TGL1* to rescue *nmt1-181* cells during exponential growth may reflect the inability of acyl chains associated with triglycerides to be processed to myristoylCoA and delivered to nmt181p, or it may reflect the failure of *GPD-TGL1* to produce increases in cellular triglyceride lipase activity. A more likely explanation is that since triglyceride pools only accumulate as cells approach stationary phase (61), Tg11p cannot contribute to myristoylCoA pools in exponentially growing *nmt1-181* cells due to a lack of substrate.

The relative contributions of Fas and the Faas to regulating lipid metabolism (and protein N-myristoylation) during stationary phase have not been defined. The availability of isogenic strains with various *faa* deletions now allow such an analysis to be performed. Preliminary experiments indicate that deletion of *FAA1* in a *NMT1* strain (YB497) produces a 13-fold decrease in the number of viable cells after a 50-d incubation at room temperature in water, when compared to an isogenic wild type strain (YB332). Deletion of both *FAA1* and *FAA2* (YB499) results in a 21-fold decrease in viability over this time period (data not shown).

Use of S. cerevisiae Strains with faa Null Alleles to Identify and Characterize Mammalian AcylCoA Synthetases

The phenotypes described above for *S. cerevisiae* strains with various combinations of *faa* null alleles provide an opportunity to isolate new mammalian acylCoA synthetases by complementation and/or to assign functions to the growing list of known mammalian acylCoA synthetases. This exercise may also shed light on the pathogenesis of certain human metabolic diseases. For example, X-linked adrenoleukodystrophy (X-ALD) is associated with the accumulation of very long chain fatty acids in cells (e.g., C24:0) and impairments in peroxisomal β-oxidation (56). X-ALD was thought to be due to a selective deficiency in peroxisomal lignoceroylCoA synthetase (56). Recently, a subset of patients with X-ALD have been found to have a mutation which affects a member of the ABC family of transporters. This protein may be involved in the import or anchoring of a peroxisomal very long chain acylCoA synthetase (4, 44). Since it appears that *S. cerevisiae* has at least one Faa devoted exclusively to activating fatty acids destined for peroxisomal β-oxidation (i.e., Faa5p), it may be possible to identify human peroxisomal long chain acylCoA synthetases by complementation of *S. cerevisiae* strains with *faa* null alleles.

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