Role of Carnitine Acetyltransferases in Acetyl Coenzyme A Metabolism in Aspergillus nidulans

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The flow of carbon metabolites between cellular compartments is an essential feature of fungal metabolism. During growth on ethanol, acetate, or fatty acids, acetyl units must enter the mitochondrion for metabolism via the tricarboxylic acid cycle, and acetyl coenzyme A (acyl-CoA) in the cytoplasm is essential for the biosynthetic reactions and for protein acetylation. Acetyl-CoA is produced in the cytoplasm by acetyl-CoA synthetase during growth on acetate and ethanol while β-oxidation of fatty acids generates acetyl-CoA in peroxisomes. The acetyl-carnitine shuttle in which acetyl-CoA is reversibly converted to acetyl-carnitine by carnitine acetyltransferase (CAT) enzymes is important for intracellular transport of acetyl units. In the filamentous ascomycete Aspergillus nidulans, a cytoplasmic CAT, encoded by facC, is essential for growth on sources of cytoplasmic acetyl-CoA while a second CAT, encoded by the acuJ gene, is essential for growth on fatty acids as well as acetate. We have shown that AcuJ contains an N-terminal mitochondrial targeting sequence and a C-terminal peroxisomal targeting sequence (PTS) and is localized to both peroxisomes and mitochondria, independent of the carbon source. Mislocalization of AcuJ to the cytoplasm does not result in loss of growth on acetate but prevents growth on fatty acids. Therefore, while mitochondrial AcuJ is essential for the transfer of acetyl units to mitochondria, peroxisomal localization is required only for transfer from peroxisomes to mitochondria. Peroxisomal AcuJ was not required for the import of acetyl-CoA into peroxisomes for conversion to malate by malate synthase (MLS), and export of acetyl-CoA from peroxisomes to the cytoplasm was found to be independent of FacC when MLS was mislocalized to the cytoplasm.

The importance of understanding fungal carbon metabolism and its regulation has become increasingly apparent. This stems from studies of changes in metabolism accompanying fungal infections, development, and stress responses as well as the requirement for substrates for secondary metabolism (7, 9, 34, 35, 53). Furthermore, genome-wide studies of gene expression under different circumstances reveal the level of our ignorance of metabolic complexity. While carbon metabolism in the budding yeast, Saccharomyces cerevisiae, is best understood, it is widely recognized that this fungus is highly specialized in its preference for the fermentation of sugars to ethanol and the use of paralogous genes derived from a whole-genome duplication in its evolutionary history, to encode different forms of enzymes for particular enzymatic reactions (20). The metabolism of another hemiascomycete, Candida albicans, has received attention recently because of its importance as a human pathogen, and there are many shared features with S. cerevisiae. Of special interest, however, is the extent to which the regulation of the expression of genes has been rewired such that the S. cerevisiae transcription factors used for controlling fundamental metabolic pathways may be different in C. albicans, and some are more similar to those in euascomycetes (33, 49). Filamentous ascomycetes include the important pathogens Aspergillus fumigatus and Magnaporthe oryzae (grisea) in addition to the well-studied A. nidulans and Neurospora crassa, which serve as important models because of the availability of classical mutants and meiotic genetics as well as highly developed molecular genetics.

A fundamental feature of carbon metabolism is the existence of three cellular compartments—the cytoplasm, the mitochondria, where the tricarboxylic (TCA) cycle and respiration occur, and the peroxisomes, where a diversity of metabolic reactions can occur, including oxidative reactions such as β-oxidation of fatty acids and ones using acyl coenzyme A (acyl-CoA) derivatives as substrates. Entry of proteins and intermediary metabolites into mitochondria requires the presence of (usually) N-terminal mitochondrial targeting sequences (MTS) and specific mitochondrial carrier proteins, respectively (44). Specialized proteins called peroxins (Pex proteins) are required for protein import into the peroxisomal membrane, and two kinds of peroxisomal targeting sequences (PTS) occur: PTS1 sequences consisting of C-terminal tripeptides, which are recognized by Pex5, and PTS2 sequences, usually near the N terminus, which require Pex7 (5, 45). For some peroxisomal proteins, cryptic nonconsensus PTS are found. The many reactions carried out in peroxisomes require the flow of a diverse range of chemicals through the peroxisomal membrane, and this has been a controversial area (32, 52). The mechanisms for metabolite flux into and out of peroxisomes is of fundamental importance for understanding how particular carbon sources are metabolized and, in filamentous fungi, for understanding secondary metabolic biosynthetic pathways that involve peroxisomal enzymes (53).

A key metabolite of intermediary metabolism is acetyl coenzyme A (acyt-COA). During growth on glycolytic carbon sources, the pyruvate produced is used to generate acetyl-CoA in the mitochondrion by the action of pyruvate dehydrogenase as well as oxaloacetate in the cytoplasm via pyruvate carboxy-
lase. This allows citrate to be formed by mitochondrial citrate synthase, enabling the operation of the TCA cycle. Cytoplasmic acetyl-CoA is required for the biosynthesis of essential compounds such as fatty acids and sterols as well as for the acetylation of proteins, including histones. A fundamental difference between hemiascomycetes (with the known exception of Yarrowia lipolytica) and other fungi is how this pool of acetyl-CoA is generated. S. cerevisiae and C. albicans depend on cytoplasmic acetyl-CoA synthases (ACS) while other fungi use ATP-citrate lyase in the absence of external sources of acetyl-CoA such as ethanol, acetate, or fatty acids (10, 26, 56, 63).

The anaplerotic glyoxylate bypass is absolutely required for growth on carbon sources that produce acetyl-CoA, and this has been a major focus for studies of the metabolic requirements for fungal pathogenicity (14). Five enzyme activities are required for this pathway. Malate dehydrogenase, citrate synthase, and aconitase are also activities of the mitochondrial TCA cycle while isocitrate lyase (ICL) and malate synthase (MLS) are specific and are absolutely required for growth on sources of acetyl-CoA. Acetyl-CoA is produced in the cytoplasm by ACS when acetate or ethanol is the carbon source while fatty acid utilization depends on the production of acetyl-CoA in peroxisomes by β-oxidation. A feature of the glyoxylate pathway is that the localization of the enzymes in different cellular compartments necessitates the shuttling of intermediates between mitochondria, cytoplasm, and peroxisomes, and this varies between different fungi (reviewed in references 32 and 52). Furthermore, the location of particular enzyme activities can be altered by mutation without necessarily affecting growth. In S. cerevisiae ICL is cytoplasmic while MLS can be peroxisomal, but only during growth on oleate, and this localization is not essential for growth on oleate (31). Therefore, glyoxylate, the product of ICL (and the substrate for MLS), can passage the peroxisomal membrane, and acetyl-CoA, the other substrate for MLS, must be able to exit the peroxisome (either directly or indirectly) during growth on oleate. In C. albicans both ICL and MLS contain PTS1 sequences and are peroxisomal (46, 47, 48). However, when both of these enzymes are cytoplasmic in a pex5Δ mutant, growth on acetate and ethanol is not affected (46). In A. nidulans ICL, encoded by acuD, is peroxisomal and dependent on a cryptic PTS2 sequence because mislocalization results when pexG (7) is mutated (29). MLS, encoded by acuE, contains a PTS1 and is dependent on pexE (5) for localization (29). When both ICL and MLS are mislocalized in pex mutants, growth on acetate or ethanol still occurs, and mutation of the PTS1 of MLS does not prevent growth on fatty acids, acetate, or ethanol. Therefore, acetyl units generated by peroxisomal β-oxidation can exit the peroxisome for malate formation.

The shuttling of acetyl units within the cell is clearly a crucial factor in the growth of fungi on sources of acetyl-CoA. The key enzyme activity for this is carnitine acetyltransferase (CAT) in which acetyl-CoA and acetyl-carnitine are interconverted (30).

In S. cerevisiae there are two redundant pathways for the transfer of acetyl units into mitochondria, either by production of citrate or via the acetyl-carnitine shuttle. Two citrate synthases, mitochondrial Cit1 and peroxisomal Cit2, occur, and citrate produced in peroxisomes from acetyl-CoA and oxaloacetate during growth on sources of acetyl-CoA can enter the mitochondrial Cit1 and peroxisomal Cat2, cytosolic Yat2, and Yat1, which is associated with mitochondria (19, 60, 68, 70). Cat2 contains an MTS at the N terminus and a C-terminal PTS1 as well as a cryptic internal PTS1. Expression of the full-length protein results in mitochondrial targeting while the use of a second transcription start only under oleate-induced conditions results in a shorter protein lacking the MTS and targeted to peroxisomes (15). C. albicans lacks peroxisomal citrate synthase and is absolutely dependent on the carnitine shuttle for growth on fatty acids, acetate, and ethanol (58, 59, 72). Ctn2 is required on all sources of acetyl-CoA and is a peroxisomal/mitochondrial CAT with dual targeting mediated by an N-terminal MTS and a C-terminal PTS1. Ctn1 and Ctn3 are neither peroxisomal nor mitochondrial and can complement deletion mutants of yat1 and yat2, respectively, in S. cerevisiae. Deletion of ctn1 results in loss of growth on acetate and ethanol but not fatty acids. Puzzlingly, deletion of ctn3 does not result in a carbon utilization phenotype (58, 72).

The carnitine shuttle has not been fully characterized in filamentous ascomycetes. In the rice pathogen M. oryzae, a mutant with impaired virulence was found to result from a mutation in a gene, phh-2, encoding a CAT, and this mutation greatly affected growth on acetate and fatty acids (4, 50). A green fluorescent protein (GFP)-tagged Pth2 was found to be peroxisomal; however, reannotation of the gene indicated an additional 5’ exon encoding an MTS, suggesting an additional mitochondrial form of the enzyme (see Fig. 1). In A. nidulans CAT activity is crucial for growth on sources of acetyl-CoA. Citrate synthase, encoded by citA, is mitochondrial, and no major function for a minor peroxisomal form has been found (39). Mutations in facC result in loss of growth on acetate but not on fatty acids (3, 57), and facC encodes a CAT with no predicted MTS or PTS, strongly indicating that cytoplasmic activity is not essential for fatty acid utilization (57). Mutations in acud cause loss of growth on fatty acids as well as acetate, and an acud facC double mutant completely lacks CAT activity (3, 38, 57). Acu1 is therefore predicted to be a mitochondrial/peroxisomal CAT (57). Here, we describe the cloning and deletion of the acud gene and show that the product is localized to peroxisomes and mitochondria and that this is independent of the carbon source supplied.

In S. cerevisiae the shuttling of acetyl units into mitochondria via citrate is accounted for by the occurrence of mitochondrial transporters such as Ctp (reviewed in reference 44). Acetyl-carnitine transport into mitochondria is accomplished by Crc1, which is essential for growth on sources of acetyl-CoA in the absence of Cit2 (42, 68). Similarly, in A. nidulans the acudH gene, originally identified by acetate/fatty acid nonutilizing mutants, encodes a mitochondrial acetyl-carnitine transporter (3, 12, 13). No peroxisomal transporters for acetyl-carnitine are known. The extent to which the peroxisomal membrane forms a barrier to the transport of acetyl units, not only to the mitochondria but also to the cytoplasm and from the cytoplasm to the peroxisome, is an important question. We have addressed this question by examining the effects of mislocalizing Acu1 from the peroxisomes to the cytoplasm.
**MATERIALS AND METHODS**

*Aspergillus nidulans* strains, media, transformation, and molecular and genetic techniques. Media and conditions for growth of *A. nidulans* were as described previously (29). All strains were derived from the original Glasgow strain and contained the veA1 mutation, and standard *A. nidulans* genetic manipulations were as previously described (67). Isolation of *fac*Δ, *pecC::bar* disruption, *acuA211*, and *acuE210* mutant strains has been described previously (3, 29, 57). The isolation of a *gfp::acuE* (stop) strain, in which a functional GFP-AcuE fusion protein is mislocalized, and *gfp::acuE* (SKL), its control strain, has been described previously (29). Where appropriate, double mutant strains were confirmed by out-crosses. Preparation of protoplasts and transformation selection markers were as described previously (40). DNA from transformants was analyzed by Southern blotting to confirm predicted integration events. Standard methods for DNA manipulations, DNA isolation, nucleic acid blotting, and hybridization have been described previously (28, 54).


**RESULTS**

**Localization of AcuJ.** Cloning and sequencing of the *acuJ* gene (see Materials and Methods) allowed prediction of the gene structure, which was subsequently confirmed by annotation of AN6279 in the genome sequence (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html; http://www.aspergillusgenome.org/). Intron positions were confirmed by sequencing of the insert of a lambda gt10 DNA clone and by sequencing the protoplast base PCR using intron-flanking primers. An *acuJΔ* mutation was generated by gene replacement. A BamHI-EcoRV fragment containing the pycG* gene was inserted into pB3B1 cut with BglII and NruI. Transformation of strain A770 (pyrG89 pabaB22 riboB2) using the *AcuJ* gene was annotated and found to correspond to AN6279 in the genome sequence (http://www.aspergillusgenome.org/). Intron positions were confirmed by sequencing of the insert of a lambda gt10 DNA clone and by sequencing the protoplast base PCR using intron-flanking primers. An *acuJΔ* mutation was generated by gene replacement. A BamHI-EcoRV fragment containing the pycG* gene was inserted into pB3B1 cut with BglII and NruI. Transformation of strain A770 (pyrG89 pabaB22 riboB2) selecting for uracil/uridine prototroph resulted in a transformant with the phenotype of loss of function *acuJ* mutants and was confirmed by Southern blotting to contain the predicted event. This resulted in replacement of the sequence from +83 to +939 (relative to the predicted first ATG) with the *pycG* gene. The *acuJ*Δ gene segregated as a single gene in meiotic crosses. Complementation by the *acuJ*Δ gene was observed by cotransforming an *acuJΔ riboB* strain with pB3B1 together with pPL3 (containing the riboB* gene). Approximately half of the RibO* transformants had an *AcuJ*+ phenotype on acetate as a sole carbon source.

*GFP tagging of AcuJ*. GFP-encoding sequences were amplified from pALX191 with the primers 5′-CCATGTAAGCAAACAGGCCG-3′ and 5′-ACCTTGTAACGTCGTCACT-3′ and cloned into pB3B1 cut with NruI (coordinate +939 relative to the predicted first ATG) using the *AcuJ* gene. The *AcuJ* gene was inserted in frame at the sequence corresponding to aromatic amino acid (aa) 267 of *acuJ*, pNB8E was made by digesting pNB4E with ClaI and self-ligated, thereby deleting the sequence encoding 58 aa at the C terminus, including the predicted PTS1 (AKL). These plasmids were cotransformed into a riboB2 strain with pPL3, selecting for RibO* to generate the strains TNBE4-4 and TNBE5-3.

**Mislocalization of AcuJ.** pB3B1 was cut with BglII and self-ligated to create pSM6008. This was used as a template for inverse PCR with the primers acuJ1 and pSM6008. This was used as a template for inverse PCR with the primers acuJ1 and acuJ2. Using mouse anti-GFP (at 1/4000) and anti-mouse IgG-horseradish peroxidase (HRP) at 1/4000 antibodies (Promega Corp.) as primary and secondary antibodies, respectively. Anti-tubulin was used to detect β-tubulin as a loading control as previously described (61). Signals were detected using Fujifilm image reader LAS-3000 (Berthold Australia Pty Ltd).

**Microscopy.** For confocal microscopy, strains were grown on coverslips for 16 h at 37°C in 1% glucose minimal liquid medium and then transferred to minimal medium containing 1% glucose, 50 mM proline, 50 mM acetate, 5 mM butyrate, or 2.5 mM oleate. Total protein extraction and Western blotting were as previously described (66). Fifty micrograms of total protein from the samples was separated by SDS-PAGE. AcuJ-GFP was detected using mouse anti-GFP (1/4,000) and anti-mouse IgG-horseradish peroxidase (HRP) at 1/4,000 antibodies (Promega Corp.) as primary and secondary antibody, respectively. Anti-tubulin was used to detect β-tubulin as a loading control as previously described (61). Confoocal microscopy was performed as described previously (39).

For determination of mislocalized GFP-AcuJ, strains were grown on 1% glucose minimal solid medium (1% agar) on microscope slides at 37°C for 16 h. Microscopy and capture of images were as described previously (61).

**Localization of AcuJ.** Cloning and sequencing of the *acuJ* gene (see Materials and Methods) allowed prediction of the gene structure, which was subsequently confirmed by annotation of AN6279 in the genome sequence (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html; http://www.aspergillusgenome.org/). Intron sequences were confirmed by RT-PCR and cDNA sequencing. Deletion of *acuJ* (Materials and Methods) resulted in a loss of growth on acetate and short- and long-chain fatty acids but not on glucose or gluconeogenic carbon sources such as proline or glycerol, which are not metabolized via acetyl-CoA. The phenotype was equivalent to that observed for the *acuJ221* mutant (3, 57). It is important to note that, unlike some other mutants affected in acetyl-CoA and peroxisome metabolism (26, 29, 39), *acuJΔ* strains have no obvious developmental phenotypes.

The predicted structure of *acuJ* and the N-terminal amino acid sequence are shown in Fig. 1A. A predicted MTS is present in the N-terminal sequence of AcuJ, and cleavage of the signal peptide predicted by Mitoprot (11) yields a polypeptide of 68.5 kDa. Initiation of translation at the second methionine gives a polypeptide of 69.2 kDa. These are in complete agreement with the size (69 kDa) of a carnitine acyltransferase enzyme purified from *A. nidulans* grown in propionate or acetate medium (M. Brock, personal communication). The predicted sequence of AcuJ is highly conserved in other *Aspergillus* spp. although the first exon has not been annotated correctly in all species (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). N-terminal sequences of orthologs from other fungi also contain predicted MTS and the potential for two translation start points. Alignments are shown for *N. crassa*, *M. oryzae*, *C. albicans*, and *S. cerevisiae* in Fig. 1B. A C-terminal peroxisome targeting sequence (PTS) is present in AcuJ (Fig. 1A) as for orthologs from other fungi. Therefore, like the genes of *S. cerevisiae* and *C. albicans*, *acuJ* is proposed to encode two polypeptides, one containing an MTS and a PTS and targeted to mitochondria and one containing only the PTS and targeted to peroxisomes.

Localization of AcuJ was studied by the use of a fusion protein in which GFP was inserted in frame at residue 267 of Western blotting. The strain TNBE4-4 was grown for 16 h at 37°C in 1% glucose minimal liquid medium and transferred for 4 h to either the same medium or minimal medium including 10 mM NH4Cl and either 50 mM proline, 50 mM acetate, 5 mM butyrate, or 2.5 mM oleate. Total protein extraction and Western blotting were as previously described (66). Fifty micrograms of total protein from the samples was separated by SDS-PAGE. AcuJ-GFP was detected using mouse anti-GFP (1/4,000) and anti-mouse IgG-horseradish peroxidase (HRP) at 1/4,000 antibodies (Promega Corp.) as primary and secondary antibody, respectively. Anti-tubulin was used to detect β-tubulin as a loading control as previously described (61). Signals were detected using Fujifilm image reader LAS-3000 (Berthold Australia Pty Ltd).
AcuJ, and expression was driven by the native promoter (Fig. 2). MitoTracker Red containing clearly indicated localization of GFP fluorescence to mitochondria (Fig. 2A). Peroxisomal localization was observed as fluorescence in punctate dots, which, in the presence of fatty acids (butyrate and oleate), proliferated to form clusters, as observed previously for peroxisomes (29). This peroxisomal localization was not observed in the absence of 67 C-terminal amino acids including the PTS1 (Fig. 2B). MitoTracker Red costaining clearly indicated localization of GFP fluorescence to mitochondria (Fig. 2A). Peroxisomal localization was observed as fluorescence in punctate dots, which, in the presence of fatty acids (butyrate and oleate), proliferated to form clusters, as observed previously for peroxisomes (29).

**Effects of mislocalization of peroxisomal AcuJ.** The cytoplasmic CAT encoded by facC is essential for acetate but not for fatty acid utilization, while AcuJ is required for growth on acetate as well as fatty acids (3, 57). Furthermore, the acetyl-carnitine mitochondrial carrier, the product of acuH, is essential for growth on both acetate and fatty acids (3, 12, 13). It is clear that the mitochondrial form of AcuJ is required for the conversion of imported acetyl-carnitine to acetyl-CoA, allowing entry into the TCA cycle via coupling with oxaloacetate via citrate synthase. The requirement for peroxisomal AcuJ during growth on acetate and on both short- and long-chain fatty acids has not been established.

We mutated the acuJ-encoded PTS1 (AKL to AK*). This mutation was shown to result in loss of the peroxisomal localization of AcuJ with GFP fused at the N terminus (Fig. 3A). Cotransformation of an acuJΔ strain yielded 12 cotransformants able to grow on acetate but not on fatty acids from a total of 19 transformants. One of these cotransformants was found to contain a single ectopic copy of acuJ containing the AK* mutation by Southern blot analysis, and the acetate and fatty acid phenotypes segregated as a single gene in crosses. This acuJ AK* mutation was crossed into various backgrounds for growth testing on acetate and fatty acids (Fig. 3B).

Loss of peroxisomal AcuJ in acuJ AK* acuJΔ strains had no effect on acetate utilization. This showed that, during growth on acetate, acetyl-CoA entry into peroxisomes for metabolism via MLS is not dependent on acetyl-carnitine formation because the absence of peroxisomal AcuJ for conversion of acetyl-carnitine to acetyl-CoA had no effect. The ability of the facC Δ acuJ AK* double mutants to utilize acetate showed that AcuJ mislocalized to the cytoplasm was able to replace the requirement for the cytoplasmic FacC enzyme for growth on acetate. This result was supported by the finding that loss of peroxisomes due to disruption of the pexC gene (corresponding to pex3 of S. cerevisiae [29]) and therefore resulting in the presence of cytoplasmic AcuJ suppressed the requirement for FacC for acetate utilization (Fig. 3C).

Mislocalization of AcuJ in strains expressing only the AcuJ AK* form of the protein (acuJ AK* acuJΔ) resulted in loss of growth on both short- and long-chain fatty acids (Fig. 3B). This indicated that conversion of acetyl-CoA formed by β-oxidation in peroxisomes to acetyl-carnitine is essential for fatty acid utilization and that cytoplasmic FacC or AcuJ cannot replace this requirement. It has been previously shown that mislocalization of MLS (AcuE) to the cytoplasm does not abolish growth on acetate or fatty acids (29). Therefore, the substrate acetyl-CoA, formed in peroxisomes by β-oxidation, must be able to enter the cytoplasm. If conversion to acetyl-carnitine is the only way acetyl units can exit peroxisomes, then reconversion to acetyl-CoA by FacC might be required. However, we found that the facC Δ
did not prevent growth of a strain with mislocalized AcuE on fatty acids (Fig. 3D).

DISCUSSION

AcuJ is targeted to mitochondria and to peroxisomes, and this dual targeting is independent of the carbon source. Mitochondrial activity is essential for production of acetyl-CoA from imported acetyl-carnitine during growth on both acetate and fatty acids. For both acetate and fatty acids, the acetyl-carnitine mitochondrial carrier (AcuH) is required (3, 12, 13). Formation of acetyl-carnitine by cytoplasmic CAT activity, normally provided by facC, is necessary for mitochondrial import during growth on acetate. This requirement can also be provided by mislocalized AcuJ. Peroxisomal CAT activity (AcuJ) is essential for growth on fatty acids, indicating that acetyl-carnitine formation in peroxisomes from acetyl-CoA produced by β-oxidation is required for transport of acetyl units into mitochondria. This requirement cannot be replaced by cytoplasmic activity resulting from mislocalized AcuJ. The possibility that acetyl-carnitine transport from peroxisomes to mitochondria is mediated by a direct interaction between the organelles warrants investigation.

In addition to a peroxisomal pathway, A. nidulans also has a pathway for β-oxidation of short-chain fatty acids in mitochondria, and both pathways are essential for growth on butyrate.
Butyryl-CoA was found to be a substrate for acuJ but not facC-dependent CAT activity in assays of crude extracts, giving 40 to 60% of the activity when acetyl-CoA was the substrate (results not shown). Butyrate utilization is independent of both FacA and FacC, indicating that butyrate can enter both mitochondria and peroxisomes directly without formation of cytoplasmic butyryl-carnitine, while the transfer of butyryl-CoA, produced in the penultimate step in peroxisomal β-oxidation of even-chain fatty acids, to mitochondria is likely to occur via AcuJ. Propionyl-CoA produced by peroxisomal β-oxidation of valerate or long-chain fatty acids with odd numbers of carbons is also likely to be dependent on AcuJ for transfer to mitochondria for metabolism via the methyl-citrate cycle because purified enzyme has very high activity with propionyl-CoA as a substrate (M. Brock, personal communication).

Malate formation from glyoxylate and acetyl-CoA occurs in peroxisomes via MLS (AcuE). Therefore, acetyl units must enter the peroxisome during growth on acetate. While this might normally occur via acetyl-carnitine, it is clear that this is not essential because peroxisomal AcuJ is not required for growth on acetate. Furthermore, acetyl-carnitine export from peroxisomes and conversion to acetyl-CoA in the cytoplasm by FacC are not necessary for growth on fatty acids when AcuE is mislocalized to the cytoplasm. The proposed situations for wild-type, facC, and acuJ strains are diagrammed in Fig. 4.

The effects of mislocalization of S. cerevisiae Cat2 have not been studied. However, the original observation in S. cerevisiae that Ctn2 is sufficient for growth on oleate in a cat2Δ strain indicates that the requirement for acetyl-CoA in the peroxisome can be satisfied by acetyl-CoA produced in the peroxisomes in the absence of the carnitine shuttle (60, 70). In C. albicans it has been recently shown that loss of mitochondrial Ctn2 results in loss of growth on acetate, ethanol, and oleate, consistent with the acetyl-carnitine shuttle into mitochondria being essential on these carbon sources (58). Ctn2 was also altered by mutating the PTS1 and the second initiating codon, thereby eliminating peroxisomal localization such that the enzyme was present only in mitochondria. This situation differs from our experiments where AcuJ was mis-localized to the cytoplasm (Fig. 3D).
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FIG. 4. Proposed pathways for acetyl-CoA distribution between cellular compartments during growth on acetate and fatty acids in different mutant backgrounds. (A) In wild-type, acetyl-CoA formed from acetate by ACS (FacA) is converted to acetyl-carnitine by FacC for transport into mitochondria via the mitochondrial transporter (AcuH), where it is converted back to acetyl-CoA for metabolism via the TCA cycle and into peroxisomes for malate formation by MLS (AcuE). Acetyl-CoA formed from fatty acids by β-oxidation in peroxisomes is converted to malate by AcuE and to acetyl-carnitine by AcuJ and transported to mitochondria via AcuH, possibly by direct interaction between peroxisomes and mitochondria (dashed line). Some exchange of acetyl-CoA between the cytoplasm and peroxisomes can occur. (B) Loss of FacC prevents conversion of cytoplasmic acetyl-CoA to acetyl-carnitine and results in lack of growth on acetate but not fatty acids. For growth on acetate, indirect transfer of acetyl units to mitochondria via the formation of peroxisomal acetyl-carnitine is insufficient. (C) Mislocalization of AcuJ to the cytoplasm prevents conversion of peroxisomal acetyl-CoA, produced by fatty acid β-oxidation, to acetyl-carnitine and results in loss of growth on fatty acids but not on acetate. Acetyl-CoA entry into peroxisomes and conversion to malate by peroxisomal AcuE is not eliminated. Replacement of cytoplasmic FacC activity by mislocalized AcuJ allows growth on acetate. For growth on fatty acids, transfer of acetyl-CoA from the peroxisomes to the cytoplasm is insufficient for efficient formation of acetyl-carnitine for mitochondrial import. For simplicity, formation of succinate and glyoxylate by ICL (AcuD) in peroxisomes is not shown.

Overall, our results, together with those for C. albicans, show that conversion of acetyl-CoA to acetyl-carnitine is not absolutely essential for the transport of acetyl units through the peroxisomal membrane, contrary to the original proposal for S. cerevisiae (70). There is no direct evidence for acetyl-CoA being unable to traverse the peroxisomal membrane. It has been suggested that acetate, generated from peroxisomal acetyl-CoA by thioesterases, might be able to exit the peroxisome (24, 58). A number of predicted thioesterases occur in C. albicans and in A. nidulans while in S. cerevisiae a single peroxisomal enzyme (Tes1) is regulated by fatty acid induction, and deletion of TES1 results in slightly reduced growth on oleate (41). Alternatively, acetyl-CoA hydrolase could generate acetate (58). However, in both C. albicans and A. nidulans, this protein is mitochondrial, and its activity is as a CoA transferase that results in detoxification of acetate that enters the mitochondrion under acid pH (17, 18). If acetate exits the peroxisome, then formation of acetyl-CoA by cytoplasmic ACS and then of acetyl-carnitine by cytoplasmic CAT activity would be required. The ACS enzymes of C. albicans (Acs1 and Acs2) are not normally required for growth on oleate (although ACS1 is induced by oleate), and neither Ctn1 nor Ctn3 is required for growth on oleate (10, 58, 72). Importantly, these results also indicate that the essential provision of cytoplasmic acetyl-CoA from peroxisomal acetyl-CoA generated during growth on oleate is independent of CAT or ACS activity. If acetyl units enter the peroxisomes as acetate, then activation to acetyl-CoA by ACS would be required. The ACS proteins of S. cerevisiae, C. albicans, and A. nidulans (FacA) lack obvious peroxisomal targeting sequences. Furthermore, loss of peroxisomal ATP, which is essential for ACS activity, in ant1 mutants lacking the ATP/AMP carrier (see below) does not affect growth on acetate in A. nidulans (29).

Progress in solving the long-standing problem of movement of small molecules through the peroxisomal membrane has been enhanced by the discovery of pore-forming channels which would permit free passage of metabolites of less than 400 kDa, such as glyoxylate cycle intermediates including acetyl-carnitine and acetyl-CoA (1, 2, 21). However, larger molecules, such as the cofactors FAD, NAD(P), and CoA, would require specific transport proteins in the peroxisomal
membrane. Binding of FAD and NAD(P) to their cognate enzymes and subsequent transport as folded proteins might offer a mechanism for FAD and NAD(P) entry (52). CoA and acetyl-CoA are likely to require specific carrier proteins. Ant1, the ATP/AMP exchange protein, has been well characterized (43, 69). The other identified transporter is the Pxa1/2 ABC transporter, which transports long-chain fatty acids into peroxisomes (23). Mutations in genes for other peroxisomal transporter proteins have not been identified, and this could be explained by functional redundancy, disruption of peroxisomal integrity, or both peroxisome and mitochondrial function. Full analysis of all peroxisomal membrane proteins is likely to yield answers to these questions.

The functions of the facC, acuJ, and acuH genes are reflected in their regulation. The facC gene is required only for acetate utilization and is subject to acetate induction mediated by FacB (28, 57, 65). The acuJ and acuH genes are required for growth on both acetate and fatty acids and, in common with the glyoxylate cycle genes acuD and acuE, are induced by acetate (FacB) and by fatty acids mediated by the FarA and FarB transcription factors (28). Induction via FacB has been suggested to be mediated (at least in part) by the levels of cytoplasmic acetyl-carnitine rather than of acetate itself (25, 57). This is mediated by transcriptional induction of facB because the expression of a facB-lacZ fusion gene is lower when acetyl-carnitine cannot be formed (in facA and facC mutants) and is high when acetyl-carnitine accumulates in an acuJ loss-of-function mutant; this is supported by Western blot analysis of the production of an HA epitope-tagged FacB (M. J. Hynes, unpublished data). The nature of this regulatory circuit affecting FacB expression remains to be determined.

In S. cerevisiae YAT1 and YAT2 contain carbon source response elements (CSREs) and are regulated by Cat8/Sip4 in response to carbon limitation (22, 55, 62, 71). Expression of CAT2, encoding the peroxisomal/mitochondrial enzyme, and CRC1, encoding the acetyl-carnitine mitochondrial carrier, is regulated by Cat8/Sip4 and also by Oaf1 and Pip2, the transcription factors mediating fatty acid induction (51). For C. albicans it has been previously suggested that the peroxisomal/mitochondrial CAT (Ctn2) as well as ICL and MLS are regulated by an ortholog of the A. nidulans FarA (28). C. albicans lacks orthologs of Oaf1 and Pip2, and the predicted conserved ortholog of FarA, named Ctf1, is required for fatty acid but not acetate or ethanol utilization and for oleate induction but not acetate induction of ICL1 (49). Deleting possible orthologs of CAT8 and ADR1 had no effect on growth on acetate, ethanol, or oleate or on expression of ICL1 (49). Northern blot analysis showed acetate and oleate induction of CTN1 and CTN3 while CTN2 was strongly induced only by oleate (49).

In A. nidulans loss-of-function mutations in the specific glyoxylate cycle genes, acuD and acuE, or in facC, acuJ, or acuH do not prevent growth on glycerol (3). In S. cerevisiae neither ICL nor MLS is required for glycerol utilization (37). However, any one of Yat1, Yat2, or Cat2 is essential for glycerol utilization but only in a cit2Δ background (60). This suggests that entry of acetyl units from cytoplasmic acetyl-CoA produced by pyruvate via pyruvate decarboxylase, aldehyde dehydrogenase, and ACS (63) into mitochondria either as citrate or acetyl-carnitine is required, possibly because production of mitochondrial acetyl-CoA from imported pyruvate via pyruvate dehydrogenase is limiting. The situation for growth of C. albicans on nonfermentable carbon sources is even less clear, and growth tests indicate significant temperature sensitivity (48). Although icΔΔ mutants are affected in growth on glycerol (48), the effects of mutations in CTN1, CTN2, or CTN3 have not been reported. Growth on citrate requires both isocitrate lyase and the acetyl-carnitine shuttle, but the pathway for citrate breakdown is obscure (47, 48, 58). It should be noted that A. nidulans grows extremely slowly on citrate as a sole carbon source. An interesting question is whether, for any carbon sources, acetyl groups can be transferred from mitochondria to the cytoplasm by a reversal of the carnitine shuttle. Overall, these observations indicate the considerable gaps in our knowledge of the details of carbon utilization in fungi. Fundamental differences between hemiascomycetes and other fungi in acetyl-CoA metabolism are of considerable interest.

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