Metabolic and Developmental Effects Resulting from Deletion of the citA Gene Encoding Citrate Synthase in Aspergillus nidulans

Sandra L. Murray and Michael J. Hynes*

Department of Genetics, University of Melbourne, Parkville, Victoria 3010, Australia

Received 15 December 2009/Accepted 9 February 2010

Citrate synthase is a central activity in carbon metabolism. It is required for the tricarboxylic acid (TCA) cycle, respiration, and the glyoxylate cycle. In Saccharomyces cerevisiae and Arabidopsis thaliana, there are mitochondrial and peroxisomal isoforms encoded by separate genes, while in Aspergillus nidulans, a single gene, citA, encodes a protein with predicted mitochondrial and peroxisomal targeting sequences (PTS). Deletion of citA results in poor growth on glucose but not on derepressing carbon sources, including those requiring the glyoxylate cycle. Growth on glucose is restored by a mutation in the creA carbon catabolite repressor gene. Methylercitrate synthase, required for propionyl-coenzyme A (CoA) metabolism, has previously been shown to have citrate synthase activity. We have been unable to construct the mcsAΔ citAΔ double mutant, and the expression of mcsA is subject to CreA-mediated carbon repression. Therefore, McsA can substitute for the loss of CitA activity. Deletion of citA does not affect conidiation or sexual development but results in delayed conidial germination as well as a complete loss of ascospores in fruiting bodies, which can be attributed to loss of meiosis. These defects are suppressed by the creAΔ204 mutation, indicating that McsA activity can substitute for the loss of CitA. A mutation of the putative PTS1-encoding sequence in citA had no effect on carbon source utilization or development but did result in slower colony extension arising from single conidia or ascospores. CitA-green fluorescent protein (GFP) studies showed mitochondrial localization in conidia, ascospores, and hyphae. Peroxisomal localization was not detected. However, a very low and variable detection of punctate GFP fluorescence was sometimes observed in conidia germinated for 5 h when the mitochondrial targeting sequence was deleted.

There has been increased interest in primary carbon metabolism in fungi in recent years. There are two main reasons for this. As fungal pathogens establish infection they must adapt their utilization of carbon sources to the substrates present in the new environment of the host cells (reviewed in reference 6). With many of the fungal genomes available, the number of genes encoding enzymes and transporters potentially involved in central metabolism has become apparent and is greater than might have been anticipated (for example, see reference 16). Deciphering this complexity requires not only genome-wide studies but also detailed studies of individual genes encoding these proteins in order to determine their regulation and the cellular localization of the proteins, as well as their roles in metabolism and development. Here we report molecular genetic analysis of the citA gene encoding citrate synthase (EC 4.1.3.7), a central enzyme of carbon metabolism, in the filamentous ascomycete Aspergillus nidulans.

Citrate synthase is required for the formation of citrate from acetyl-coenzyme A (CoA) and oxaloacetate in the tricarboxylic acid (TCA) cycle and is therefore necessary for respiratory growth as well as for the generation of intermediates for biosynthetic reactions. Together with aconitate, malate dehydrogenase, isocitrate lyase, and malate synthase, it is also an essential enzyme in the glyoxylate cycle, which is necessary for growth on carbon sources such as acetate, ethanol, and fatty acids which are catabolized via acetyl-CoA (reviewed in reference 26).

In Saccharomyces cerevisiae the mitochondrial Cit1 is the major citrate synthase of the TCA cycle. An additional enzyme, Cit2, is peroxisomally localized via a C-terminal peroxisomal targeting sequence (PTS1) (29). In response to mitochondrial dysfunction CIT2 is upregulated via the retrograde response mediated by RTGI, -2, and -3, while mitochondrial respiratory deficiency results in RTG-dependent expression of CIT1 as well as that of aconitase (ACO1) and isocitrate dehydrogenase (IDH1 and IDH2), all enzymes necessary for 2-oxoglutarate formation and hence the synthesis of glutamate required for amino acid biosynthesis (9, 15, 30). In addition a third gene, CIT3, encodes a mitochondrial enzyme with citrate synthase activity. This enzyme has greater activity with propionyl-CoA, forming methylercitrate, and is necessary for the mitochondrial methylercitrate cycle involved in the metabolism of propionate (24). Cit2 has also been proposed to have methylercitrate synthase activity (17).

In S. cerevisiae Cit2 also plays a role in the transfer of acetyl-CoA generated in peroxisomes by β-oxidation of fatty acids or by ethanol and acetate metabolism in the cytoplasm to the mitochondria for metabolism via the TCA cycle. There are two alternative pathways: transfer as acetyl-carnitine formed by the peroxisomal/mitochondrial carnitine acyltransferase Cat2, together with the cytoplasmic Yal1 and Yat2 carnitine acyltransferases, or transfer via citrate formed by Cit2 (45, 51, 52). Only disruption of both pathways (e.g., by deletion of CAT2 and CIT2) results in a growth defect on fatty acids. The fact that deletion of CIT2 is not essential for utilization of

* Corresponding author. Mailing address: Department of Genetics, University of Melbourne, Parkville, Victoria 3010, Australia. Phone: 61 3 83446239. Fax: 61 3 83445139. E-mail: mjhynes@unimelb.edu.au.

Published ahead of print on 19 February 2010.
carbon sources metabolized via acetyl-CoA indicates that mitochondrial citrate synthase activity can replace the peroxisomal activity in the glyoxylate cycle. In contrast, in the pathogenic yeast *Candida albicans*, there is a single gene for citrate synthase and it is mitochondrial, and acetyl-CoA transport to mitochondria is solely dependent on the carnitine pathway (43, 57). In the plant *Arabidopsis thaliana*, there are five genes encoding citrate synthase enzymes. Two are peroxisomal (CSY2 and CSY3) and required for fatty acid respiration and seed germination, indicating that citrate acetyltransferases are not required for shunting acetyl units to the mitochondria (37).

The filamentous ascomycete *Aspergillus nidulans* has both citrate synthase-encoding and methylcitrate synthase-encoding genes, *citA* and *mcsA*, respectively (8, 36). In both *A. nidulans* and *Aspergillus fumigatus* it has been shown that *McsA* is mitochondrial and has both methylcitrate and citrate synthase activities and is required for propionyl-CoA metabolism (8, 22, 31). Cell fractionation studies have shown that citrate synthase activity colocalizes with the mitochondrial fraction (35), and an N-terminal mitochondrial targeting sequence is predicted by the gene sequence (36). However, *CitA* has a putative C-terminal peroxisomal targeting sequence (PTS1 AKL), and genes in some filamentous ascomycetes also have potential PTS1 sequences (see below). The role of peroxisomal citrate synthase activity is not at all clear. The acuI-encoded peroxisomal/mitochondrial carnitine acetyltransferase is required for growth on both fatty acids and acetate, while the facC-encoded cytoplasmic enzyme is required for growth on acetate (1, 20, 42). Therefore, like *C. albicans*, the carnitine shuttle is absolutely required for acetyl-CoA intracellular transport.

Because of our interest in the role of peroxisomes in fatty acid and acetate metabolism in *A. nidulans* (21), we have investigated phenotypes resulting from deletion of the *citA* gene. Our results indicate that loss of CreA-mediated carbon repression allows expression of *mcsA*, resulting in the restoration of sufficient citrate synthase activity to suppress growth and developmental defects resulting from *citAΔ*. We have also investigated the role of peroxisomal localization of CitA and found this is at most extremely low and does not play a major role.

**MATERIALS AND METHODS**

*A. nidulans* strains, media, transformation, and molecular and genetic techniques. Media and conditions for growth of *A. nidulans* were as described previously (21). All strains were derived from the original Glasgow strain and contained the velaI mutation, and standard *A. nidulans* genetic manipulations were as previously described (48, 49). Preparation of protoplasts and transformation were as described previously (32). Recipient strains contained *nhkAΔ* to promote homologous integration events, and selectable markers were the *bar* gene (glucosamine resistance) and *rifoB* from *A. fumigatus* (32). A strain containing *mcsAΔ* (RY011-56) was obtained from Matthias Brock. DNA from transformants was analyzed by Southern blotting to confirm predicted integration events. Standard methods for DNA manipulations, RNA isolation, nucleic acid blotting, and hybridization have been described previously (20, 39).

DNA sequences. *Aspergillus* sequences were obtained from the genome sequences available from the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). Other fungal sequences were derived from either specific genome sequences available at the Broad Institute (http://www.broadinstitute.org/annotation/fungi/index.html) or at NCBI (http://www.ncbi.nlm.nih.gov/).

Deletion of *citA*. The *citA* gene (coordinates –910 to +2570) was amplified using the primers 5′-GGCTTGCGTAACTTTTGAA and 5′-AGTTGGGAC GAAACAGTCC and cloned into pBluescript SK(+) (Stratagene, La Jolla, CA), forming pSM7337. A deletion construct was made by replacing a partial Xhol-partial PstI fragment, corresponding to amino acids +56 to 420 of the predicted protein, with a partial Xhol-partial PstI fragment of the *bar* selectable marker, creating pSM7341. A linear PCR product generated using the above primers was transformed into strain TNO2A21 (32), and transformants were selected for glucosamine resistance.

Gene targeting to the *wA* locus. The plasmid pRG6501 contains from +812 to +2666 of the *wA* coding region (amplified using the primers 5′-TATGTTGCC AATCCACCGG and 5′-TGATGGAAGATCCTGGCC) and was cloned into a plasmid containing the *A. fumigatus* rifoB gene inserted into the Spel site of pBluescript SK(+). Upon transformation into a recipient *wA*. *A. nidulans* strain, insertion of the circular plasmid by homologous recombination causes a disruption of the *wA* gene, which can be detected by the presence of white conidia.

Complementation and *wA* targeting with citA*. A fragment containing the *citA* gene (coordinates –910 to +2570) was cloned into pRG6501 for targeting to the *wA* locus. This was transformed into the *citAΔ* strain grown overnight in 0.05% glucose plus 50 mM acetate liquid medium, and a transformant with integration of a single plasmid copy at *wA* was obtained (citA+/citAΔ). A transformant with pRG6501 integrated at *wA* was obtained as a control.

Construction of *citA* and *citAAK*-encoding strains. For *citAAK*-encoding strains, the primers 5′-TTCTGTAACGCCTACGTCG and 5′-CCTGTTGGGCCTAAGCTATA were used to change A1829 to T, which resulted in the amino acid change from K468 to a stop codon. For *citAΔEK*, the primers 5′-CTTAGCGCGCACAAGCGTGG and 5′-TAAATTTCTTGAACGACTGAGG were designed to delete the codon +1847 to +1849 (L474). Inverse PCR using Phusion (Finzymes) and these primers was performed on the plasmid pSM7340 containing +1173 to +2236 of the *citA* gene. The resulting product was ligated to form the plasmids pSM7344 and pSM7319 for *citAΔEK* and *citAAK*-encoding strains, respectively. Full-length versions of these constructs were reconstituted by replacing the 624-bp PstI-EcoRI fragment from the *citA* gene with that from pSM7344 and pSM7319, and these full-length versions were cloned into the *wA*-targeting plasmid pRG6501 and transformed into the *citAΔ* strain to generate *citAΔEK/citAΔ* and into TNO2A21 to generate *citAAK+/citAΔ*.

Construction of *citA-gfp* and *citAAK-gfp* strains. For localization studies, a fragment encoding AcGFP (Clontech Laboratories, Inc.) was used to replace bp +823 to +972, corresponding to amino acids +174 to +226 of *citA* and *citAΔEK*, forming *citA-gfp* and *citAAK-gfp*, respectively. These were inserted into the *wA* targeting vector and transformed into TNO2A21, generating *citA-gfp/citAΔ* and *citAAK-gfp/citAΔ*.

Construction of *cit5-sΔA* and *cit5-sΔA-citAAK*-encoding strains. For *cit5-sΔA* the primers 5′-GCCATATGCATCTAAACAC and 5′-CTCTACGGCAAGACAGAAG were designed to delete bp +6 to +101, corresponding to amino acids 3 to 34 of *CitA*. Inverse PCR was performed with pSM7337 as template using Phusion (Finzymes), and the product was ligated to form pSM7480. The 1.1-kb XbaI fragment from pSM7480 containing the deletion was used to replace the corresponding region in both the *citA* and *citA-gfp* sequences in the *wA*-targeting plasmids for transformation into TNO2A21 to generate *cit5-sΔA/citAΔ* and *cit5-sΔA-citAAK-gfp/citAΔ*.

Semicontinuous RT-PCR analysis. RNA extractions and reverse transcription PCRs (RT-PCRs) were carried out as described previously (55). The annealing temperature for all primer sets was 58°C. *citA* extraction was amplified with the primers 5′-ACCAGAATTTTGCGGAC and 5′-GCCATAGGTTAGCAC GAGAG to yield a cDNA (364 bp) using 24 cycles. *mcsA* was amplified with the primers 5′-ATCTTAGCTAGTGAAC and 5′-CCAGCGGGCAGACAGCAG to yield a cDNA (380 bp) using 28 cycles. benA (loading control) was amplified with the primers 5′-ATGTTGTACCCACGGGCAG and 5′-GCTC CGGTGTTACAAAGG to yield a cDNA (209 bp) using 26 or 25 cycles.

Germination tests. Conidia were grown in 24-well plates containing 1% glucose minimal liquid medium or minimal medium containing 50 mM acetate at 37°C. Photos were taken at the indicated time points by using an inverted microscope, and germinated conidia were counted (detectable germ tube emergence). At least three replicates were performed, and a minimum of 50 conidia were counted for each replicate, but most often there were more than 100.

Western blotting. For hyphal samples, strains were 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 50 mM acetate and 10 mM NH4Cl. For germinated conidia, strains were grown for 5 h at 37°C in 1% glucose minimal liquid medium. Total protein extraction and Western blotting were performed as previously described (50). Aliquots of 100 μg of total protein for both hyphal and conidial germination samples were separated by SDS-PAGE. CitA-GFP was detected using a 1:5,000 dilution of anti-GFP rabbit polyclonal antibody (Millipore) and a 1:4,000 dilution of anti-rabbit IgG–horseradish peroxidase antibodies (Promega) as primary and secondary antibodies, respectively. Signals were...
FIG. 1. Methylcitrate synthase compensates for the loss of citrate synthase under conditions of carbon derepression. (A) Deletion of *citA* results in reduced growth on glucose. Colonies of the strains shown were inoculated onto glucose minimal medium and grown for 4 days at 37°C. The complemented strain contained a single copy of *citA* inserted at the *wA* locus, and the empty vector strain contained the *wA*-targeting vector alone inserted at the *wA* locus. The enlargement shows that *citAΔ* does not prevent conidiation. (B) Growth of *citAΔ* is equivalent to wild type on derepressing carbon sources except for ethanol. Carbon sources were added to minimal medium with 10 mM ammonium chloride as the nitrogen source at the following concentrations: glucose and peptone (1%); acetate, proline, γ-aminobutyric acid (GABA), and glutamate (50 mM); ethanol and quinate (0.5%); butyrate and oleate (10 mM); –, no added carbon source; CM, complete medium containing 1% glucose. (C) Suppression of the *citAΔ* phenotype by *creA204*. Medium was as for panel B, with xylose, fructose, lactose, and arabinose added at 1% and glycerol at 0.5%. Note that the *creA204* mutation results in a compact colony morphology. (D) *citAΔ* is resistant to the toxic effects of propionate. Medium was as for panels B and C, with propionate added at the indicated concentrations. Note that *mcsAΔ* results in extreme sensitivity to propionate. In all cases (B to D) growth was for 2 to 3 days at 37°C. (E) Derepression of *mcs4* RNA levels, based on RT-PCR analysis of total RNA from wild-type (WT) and *creA204* strains. RNA was extracted from mycelia grown in glucose minimal medium with ammonium tartrate as the nitrogen source and transferred to the indicated carbon sources for 4 h. Primers and cycle numbers are described in Materials and Methods. The tubulin gene (*benA*) was used as a loading control.

Deletion of the *citA* gene. The structural gene for citrate synthase (*citA*) of *A. nidulans* has previously been cloned and sequenced (36). This corresponds to AN8275 in the genome sequence (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). A 3.4-kb fragment containing this gene was cloned, and a deletion construct was made by replacing sequences with coordinates (relative to the ATG) +235 to +1684 (corresponding to amino acids 56 to 420) with the *bar* gene encoding glufosinate resistance. A 4-kb linear fragment generated by PCR was transformed into an *nkuAΔ* strain, and transformants were selected for glufosinate resistance on protoplast medium, which contains 1 M sucrose and 1% glucose. The few large resistant colonies appearing on the plates were subsequently found by Southern blot analysis to be due to integration events without generating a gene deletion. A longer incubation period yielded small resistant colonies which were found to grow more strongly on glufosinate medium when acetate was the sole carbon source. Southern blot analysis of three of these transformants showed that the predicted gene replacement event had occurred, and one was used in subsequent analyses. The *citAΔ* mutant phenotype on glucose medium was very slow growth. Extended incubation showed that conidiation was not greatly affected (Fig. 1A). All *citAΔ* phenotypes were complemented by targeting the 3.4-kb *citA*+ fragment (containing 910 bp of the 5’-untranslated region [UTR] and 718 bp of the 3’-UTR) to the *wA* locus (Fig. 1A and B). Integration of the empty *wA*-targeting vector did not restore normal growth on glucose (Fig. 1A).

Growth of *citAΔ* on carbon sources. The small colony phenotype of *citAΔ* was observed on both minimal medium and on rich complete medium, which contains 1% glucose. In contrast, growth on gluconeogenic carbon sources, including amino acids, acetate, and fatty acids (butyrate and oleate), was only slightly less than the wild type, with the exception of ethanol, on which growth was extremely poor (Fig. 1B). Growth of...
**citΔ** was also restored when glycerol, fructose, lactose, or arabinose, but not xylose, was provided as sole carbon source (Fig. 1C). This pattern of growth correlated with glucose and xylose as strong sources of carbon catabolite repression, as previously described (2).

### Suppression of citΔ by creA204
The CreA gene mediates carbon catabolite repression, and the creA204 mutation, resulting from a mutation affecting the DNA binding domain of the CreA repressor, results in relief of repression (40). Therefore, a citΔ creA204 strain was constructed by crossing and tested for growth on various carbon sources (Fig. 1C). Although the creA204 mutation results in a more compact colony, it was clear that relief of carbon catabolite repression substantially restored growth on the repressing carbon sources glucose and xylose. No effect was seen for growth on ethanol.

### Expression of the mcsA gene provides an alternative citrate synthase activity.
In both *A. nidulans* and *A. fumigatus* it has been shown that the product of the *mcsA* gene has both methylcitrate synthase and citrate synthase activities, and the expression of this gene is increased on nonrepressing carbon sources (8, 22, 31). This strongly suggested that derepression of *mcsA* expression by growth on nonrepressing carbon sources or by the creA204 mutation results in suppression of the effects of loss of citrate synthase activity. *mcsAΔ* does not affect growth on carbon sources other than leading to propionate sensitivity due to the requirement for methylcitrate synthase in propionyl-CoA metabolism (Fig. 1D) (7, 8). Interestingly, citΔ resulted in increased utilization and greater resistance to propionate than the wild type (Fig. 1D), indicating that loss of CitA, which has no methylcitrate synthase activity (8), may allow more efficient metabolism of propionyl-CoA via McsA. McsA is methylcitrate synthase from *A. nidulans*. The asterisks above CreA indicate the residues that were replaced by stop codons. Lack of effect of the citA46* mutation on growth. Growth tests were conducted as described for Fig. 1, with propionate present at 10 mM. The citA46*/citAΔ strain contains the citA46* mutant gene targeted to the *wA* locus in a *citAΔ* background, while the *citAΔ/citAΔ* strain contains *citAΔ* targeted to the *wA* locus in a *citAΔ* background.

![Figure 2](http://ec.asm.org/)

**FIG. 2.** Effect of deletion of the potential peroxisome-targeting sequence of CitA. (A) C-terminal sequences of citrate synthase from various ascomycetes: *S. cerevisiae* Cit1 (YNR001C), Cit2 (YCR005C), and Cit3 (YPR001W); *N. crassa* Neurospora crassa (NCU01692); *P. anserina* (CAC12961); *M. grisea* Magnaporthe grisea (MGG_0720); *Y. lipolytica* (YAL1E02684g); *C. albicans* (orf19.4393). McsA is methylcitrate synthase from *A. nidulans*. The asterisks above CreA indicate the residues that were replaced by stop codons. (B) Lack of effect of the citA46* mutation on growth. Growth tests were conducted as described for Fig. 1, with propionate present at 10 mM. The citA46*/citAΔ strain contains the citA46* mutant gene targeted to the *wA* locus in a *citAΔ* background, while the *citAΔ/citAΔ* strain contains *citAΔ* targeted to the *wA* locus in a *citAΔ* background.

Sequence. Therefore, the function of this protein remains unknown.

### Effects of mutation of the potential citΔ PTS1.
Strains containing citΔ are able to grow on carbon sources metabolized via acetyl-CoA and which therefore require the glyoxylate cycle. Since McsA can substitute for CitA, as described above, and this activity is mitochondrial, a functional glyoxylate cycle does not depend on cytoplasmic or peroxisomal citrate synthase activity. Nevertheless CitA has a potential C-terminal peroxisomal targeting sequence (AKL) and is conserved in other *Aspergillus* spp. and in *Penicillium chrysogenum*. The C-terminal sequences of some ascomycetes are shown in Fig. 2A. PTS1 sequences are not found in the citrate synthases of other ascomycetes, apart from the defined peroxisomal Cit2 of *S. cerevisiae*, although the situation for *Podospora anserina* and *Neurospora crassa* is ambiguous, because KKL has the potential to act as a PTS1 in the appropriate context (4). The methylcitrate synthases McsA and Cit3 lack PTS1 sequences, and *Candida albicans* lacks an ortholog of Cit3. A mutation (citA46*) changing the C-terminal L to a stop codon and therefore altering the putative PTS1 was able to fully complement citΔ for all growth phenotypes (Fig. 2B). Furthermore, no effect was seen on the utilization of fatty acids or acetate, which require enzyme activities located in peroxisomes (21).
Developmental effects of *citAΔ*. As noted above, no effects of *citAΔ* on conidiation on any medium were observed. However, sexual spore development was affected. *A. nidulans* is homothallic, and therefore strains can be selfed. In addition, each fruiting body (cleistothecium) produces ascospores arising from a single initial diploid zygoete undergoing sequential meiotic divisions (48). Crosses are normally set up by initially growing strains on glucose-containing complete medium and transferring to glucose minimal medium plates which are tapped to exclude air (48). Because *citAΔ* results in poor growth on glucose, we set up crosses on media with either acetate or proline as the carbon source before transferring to glucose minimal medium plates. It was found that sexual development was initiated in *citAΔ* selfed crosses. However, cleistothecia were small and obscured by the nurse cells surrounding the cleistothecia (Hulle cells), and this was also shown by comparing sizes of cleaned isolated cleistothecia (Fig. 3A and B). Measurement of sizes showed that cleistothecia resulting from sexual spore development was affected. *citAΔ* conidia was delayed. Germination of the *citAΔ* strain in acetate medium was extremely poor for unknown reasons. Germination of the *citAAK*/*citAΔ* strain was similar to that of the *citAΔ* strain, showing that peroxisomal localization is not required. As shown below, GFP-tagged CitA was detected in ungerminated conidia.

Effects of *citAAK* on colony growth. Although the growth of colonies arising from the inoculation of mass conidia of the *citAAK*/*citAΔ* strain was not detectably affected at 2 to 3 days of incubation (Fig. 2B), it was observed that colonies arising from single ascospores or conidia developed more slowly than *citAΔ* strains, with colonies barely visible at approximately 24 h (Fig. 4A, B, and C). This suggested a possible role for the peroxisomal localization of CitA during the early stages of growth. Colony development from single conidia and ascospores was also investigated in pex mutants, in which peroxisomal localization of matrix proteins is affected (21). Deletion of the *pexE* gene, encoding the receptor required for the localization of PTS1-containing proteins but not PTS2 proteins (21), resulted in slow colony growth from both conidia and ascospores (Fig. 4E). In contrast, the complete loss of peroxisomes resulting from deletion of *pexC* or the loss of targeting of all matrix proteins resulting from mutation of *pexF* did not result in greatly slowed colony development (Fig. 4E). It should be noted that *pex* mutants show delayed conidial germination, but at 5 to 6 h the germination percentages are similar to the wild type (G. S. Khew, unpublished data). Colony growth arising from single conidia or ascospores of loss-of-function mutants affected in the glyoxylate cycle (*acuE* and *acuF*, for phosphoenol pyruvate carboxykinase) was not significantly different from wild type (results not shown). Therefore, an essential role for peroxisomal CitA in the utilization of gluconeogenic carbon sources during the early stages of growth following conidial germination is unlikely.

Cellular localization of CitA. CitA was tagged by inserting a sequence encoding GFP (between coordinates +823 and +972, corresponding to amino acids +174 to +226), and the resulting plasmid was targeted to the *wA* locus. In hyphae, GFP fluorescence colocalized with mitochondria stained with Mito Tracker Red (Fig. 5A). Fluorescence was not detected in separate organelles that might correspond to peroxisomes. This was particularly clear for oleate-grown hyphae, where we have previously shown that peroxisomes proliferate, forming large clusters (21). Furthermore, we have been able to detect dual localization to both mitochondria and peroxisomes with other GFP-tagged proteins (reference 46 and unpublished data). Therefore, under these conditions, either there is no peroxisomal localization or it is not detectable due to peroxisomes being obscured by mitochondria combined with a low level of expression of the peroxisome-localized form of the enzyme. Western blotting showed expression of CitA-GFP, and this was 2- to 3-fold higher in acetate-grown than in glucose-grown hyphae (Fig. 5E). Expression was not affected by the *citAAK* mutation. Both resting conidia and ascospores showed appar-
FIG. 3. Effects of citA mutations on sexual development and conidial germination. (A) Sexual development in selfings of the indicated genotypes. White arrows indicate cleistothecia. Bar, 273 μm. (B) Isolated cleistothecia. Bar, 273 μm. (C) Size distribution of cleistothecia from selfings of \textit{citA}^+ and \textit{citAΔ} strains, indicating a bimodal distribution of sizes. (D) Squashed cleistothecia from selfings, showing that \textit{citAΔ} results in loss of ascospore production without affecting cleistothecin production. This phenotype is suppressed by the \textit{creA204} mutation. (E) Germination of conidia on glucose and acetate minimal media. Conidial dilutions were germinated, and images were captured with an inverted microscope. Conidia visibly germinated were counted and are expressed as a percentage of the total. At least 50 spores were counted, and values are the averages of at least three replicates with standard error bars shown.
ent mitochondrial localization of CitA-GFP (Fig. 5B and C). MitoTracker Red staining was not possible, presumably due to mitochondria being inactive (47). The pattern of localization was not affected by mutation of the putative PTS1.

To increase the possibility of detecting peroxisomal targeting we deleted sequences for the predicted mitochondrial targeting sequence (amino acids 3 to 34) in both citA and citA-gfp and targeted the mutant genes to the wA locus in a citA background. Not surprisingly, isolates of genotype citA3-34/citAΔ, obtained by crossing to a citAΔ strain, were unable to grow on glucose medium, indicating that mitochondrial localization of CitA is essential. CitA3-34-GFP fluorescence was not detectable in hyphae or resting conidia and ascospores (Fig. 5A to C), and no expression was detected by Western blotting (Fig. 5E). Therefore, loss of mitochondrial targeting may result in protein instability.

As noted above we observed slower colony development resulting from the citAAK* mutation. Therefore, we investigated conidia germinated for 5 h, when polarized growth is evident. Western blotting detected high levels of CitA-GFP in both citA-gfp and citAAK*-gfp strains, and also a low level of expression was clearly detected in the citA3-34Δ-gfp strain, unlike in hyphae (Fig. 5E). GFP fluorescence apparently localized to mitochondria was observed in citA-gfp germinated conidia. In the absence of the mitochondrial targeting sequence in the citA3-34Δ-gfp strain, punctate fluorescence was observed in a minority of germinated conidia and not in every experiment. Some examples are shown in Fig. 5D. This suggested some peroxisomal localization of CitA at this stage of growth, which was observable in the absence of highly fluorescent mitochondria in citA-gfp germinated conidia. Support for a requirement for peroxisomal localization of CitA for colony extension was provided by the finding that the citA3-34Δ mutation could complement the citAAK* mutation for colony extension in a diploid that was also homozygous for citAΔ (Fig. 4D). Presumably, peroxisomal CitA resulting from citA3-34Δ can compensate for the loss of peroxisomal targeting due to the citAAK* mutation.

**DISCUSSION**

Deletion of citA results in greatly impaired growth on glucose-containing media but not on a variety of derepressing carbon sources. Expression of the mcsA gene encoding methylecitrlate synthase is elevated under carbon-derepressing conditions. The creA204 mutation, which is derepressed for glucose repression (40), suppresses the glucose phenotype and results in derepression of mcsA. Therefore, McsA activity can replace the requirement for CitA, and this is supported by our inability to obtain a citA/mcsA double mutant.

Consistent with these results, purified McsA from A. nidu-

---

**FIG. 4.** Colony development is slower in the citAAK* mutant. Spores were diluted, plated on complete medium, incubated at 37°C, and photographed at the indicated times. (A) Conidia; (B) ascospores from selfed strains. (C) Colony diameters (in mm) of the indicated strains resulting from plating conidia on complete medium were measured at the indicated times during incubation at 37°C. The same 10 colonies were counted at each time point. Standard error bars are shown. (D) Complementation in a diploid of the colony development phenotype of citAAK* by citA3-34Δ, encoding CitA lacking the mitochondria-targeting sequence. Conidia were plated on complete medium. (E) Colony development is slower in the pexE mutant. Ascospores from selfed pex mutants and conidia from pex mutants were plated and photographed as for panels A and B.
fungus has a similar substrate specificity (31), and enzyme activity with propionyl-CoA as substrate and mcsA transcription is greatly increased by growth on peptone, a limiting carbon source (22). Analysis of the genomes of other filamentous fungi showed the presence of predicted mitochondria-targeted methylcitrate synthases (31). In P. anserina, deletion or loss-of-function mutants of the citrate synthase gene cit1 can grow on standard medium that contains dextrin, presumably a limiting carbon source, and 25% of wild-type citrate synthase activity is present in the mutants. Furthermore, Western blot assays showed a second weaker cross-reacting band in extracts from all strains when a polyclonal antibody to S. cerevisiae Cit1 was used (38). This additional citrate synthase is likely to be methylcitrate synthase, and a predicted gene is found in the P. anserina genome (PODANSg3467; accession no. CAP65504.1).

There is a third gene encoding a putative citrate synthase in A. nidulans (AN1079). This is more closely related to bacterial proteins than to CitA or McsA and lacks an obvious mitochondrial targeting sequence. There are related genes in other Aspergillus spp. with additional copies in the three sequenced A. niger strains (16, 44). These additional putative citrate synthases are not present in either N. crassa or Magnaporthe grisea (44). Our results indicate that this enzyme cannot substitute for CitA, and its function remains unknown.

Deletion of citA does not prevent growth on acetate, butyrate, or oleate, which are carbon sources generating acetyl-CoA and therefore requiring the glyoxylate cycle for growth. Citrate synthase activity is essential for this pathway, and therefore the mitochondrial McsA can substitute for CitA. Furthermore the citA<sup>AK<sup>*</sup></sup> mutation, lacking the potential peroxisomal targeting sequence, does not prevent growth on these carbon sources. Therefore, peroxisomal activity is not required, despite the peroxisomal localization of the unique enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase in A. nidulans (21). This is consistent with the conclusion that glyoxylate cycle intermediates must be able to shuttle between cellular compartments in S. cerevisiae (26) and in C. albicans, which lacks peroxisomal citrate synthase. Unlike S. cerevisiae, in which peroxisomal Cit2 is redundant with the acetyl-carnitine shuttle (51, 52, 45), there is clearly no major role for CitA in the transfer of acetyl-CoA from peroxisomes into mitochondria. This is consistent with the absolute requirement for carnitine-acetyltransferases during growth on acetate or fatty acids for A. nidulans (42, 20).

Deletion of citA resulted in greatly reduced utilization of ethanol, which is metabolized via acetate to produce acetyl-CoA in the cytoplasm and therefore also requires the glyoxylate cycle. Microarray experiments show mcsA expression during growth on ethanol (10). A partial explanation for this result may be that the levels of mitochondrial acetyl-CoA resulting from ethanol catabolism are much lower than in acetate-grown cells and the lower citrate synthase activity of McsA is insufficient for adequate flux through the TCA cycle. McsA, as an essential enzyme of the methylcitrate cycle, is required for growth on propionate, and deletion of mcsA leads to sensitivity to the toxic effects of propionyl-CoA (5, 7, 8, 56). citA<sup>Δ</sup> resulted in increased growth on propionate and resistance to its toxicity. It is likely that, in propionate-grown wild-type strains, when both methylcitrate synthase and high citrate synthase activity

---

**FIG. 5.** Localization and expression of CitA-GFP. (A) CitA-GFP is localized in mitochondria in hyphae. Mycelium was grown in 1% glucose minimal liquid medium for 16 h and then transferred for a further 4 h to the same medium or to minimal medium containing 30 mM acetate or 0.5% Tween 80 as a source of oleate. Mitochondria were visualized with MitoTracker Red CMXRos (Invitrogen). CitA-GFP and CitA<sup>AK<sup>*</sup></sup>-GFP are present in resting conidia (B) and resting ascospores (C). CitA<sup>3-34A</sup>-GFP is not detectable. Bar, 10 μM (A and B) or 5 μM (C). (D) CitA<sup>3-34A</sup>-GFP is visible as punctate dots in germinated conidia. Conidia were grown on coverslips in 1% glucose minimal liquid medium at 37°C for 5 h. Window, 10 μM. (E) Western blot of CitA-GFP-tagged strains on glucose and acetate. CitA<sup>3-34A</sup>-GFP is not detectable in hyphae but is detectable at a low level in germinated conidia. Strains were grown as described for microscopy. Tubulin was used as a loading control.

---

*C. lans* has been shown to have activity with both propionyl-CoA ($K_m$, 1.7 μM) and acetyl-CoA ($K_m$, 2.5 μM). The presence of potential CreA-binding sites in the 5′ end of mcsA suggested catabolite repression of expression (8). Purified McsA from *A.
activities are present, there is competition between the enzymes for the substrate oxaloacetate, leading to less conversion of propionyl-CoA to methylcitrate. Loss of CitA prevents this competition, allowing a balanced use of substrates and resulting in increased flux through both TCA and methylcitrate cycles. TCA cycle enzymes interact with each other and with the mitochondrial membrane (53, 41). This suggests that competition between CitA and McsA for binding to other TCA cycle enzymes (particularly aconitase) might reduce propionate utilization in the wild type. In the absence of CitA such competition would be eliminated.

Resting conidia of *A. nidulans* and *A. fumigatus* have been shown to contain stored mRNAs which are translated upon germination, and this also triggers transcription of specific genes (3, 27, 33, 34). It has also been suggested that germination results in a shift from a maintenance fermentative metabolism to respiration (27). Inhibitors of the respiratory chain severely delay germination of *A. fumigatus* conidia, and active mitochondria are found very early in germination (47). In microarray analysis of *A. nidulans* conidial germination, *citA* mRNA has been detected in ungerminated and germinated spores (3 h and 5 h) while *mcsA* expression was not detected (deposited data of reference 3). Hybridization to *A. fumigatus* macroarrays of PCR fragments from approximately one-third of the genes showed that citrate synthase mRNA was present in resting spores and also increased during the first 30 min following germination, while methylcitrate synthase RNA, although detected before germination, was not present in the mRNA population at 30 min (27). We have detected mitochondrial localized CitA-GFP in both ungerminated conidia as well as 5 h after germination and found that germination of *citAD* conidia was significantly delayed. Loss of the putative CitA PTS1 did not affect germination. Of particular interest was the finding that the *creA204* mutation suppressed the *citAD* germination defect, suggesting that derepressed *mcsA* expression could substitute for the loss of CitA. This implies that CreA-mediated carbon repression of *mcsA* expression operates during the synthesis of stored conidial mRNAs during conidial formation and/or during the early stages of germination. Upon germination, stored trehalose is rapidly mobilized (11) and may result in carbon repression. Studies with GFP-labeled McsA would be of interest.

*A. nidulans* is homothallic, and sexual development is promoted by glucose and low levels of aerobic respiration (18). We have found that sexual development is not affected by *citAD*, with normal production of Hulle cells, cleistothecia, and the red pigment, cleistothecin. This indicates that either there is no requirement for citrate synthase activity or that McsA is expressed in developing tissue and can provide sufficient activity. However, the cleistothecia produced in *citAD* selfed crosses were small and devoid of ascospores, and this is an autonomous property of cleistothecia produced in *citAΔ X citA−* crosses. This shows that loss of citrate synthase activity results in a block in meiosis. Ascospore production was restored in the *creA204 citAΔ* double mutant, consistent with derepression of *mcsA* expression relieving the meiotic block. However, selfed crosses of *citAΔ* strains on the carbon sources lactose or arabinose did not produce ascospores, showing that derepression of *mcsA* expression in developing cleistothecia was not affected by the exogenous carbon source. These observations provide strong evidence for an intrinsic CreA-mediated carbon repression operating in a specific cell type, the ascogenous hyphae, within the developing cleistothecia. It has been proposed that, during sexual development, monosaccharides, released from cell walls by lytic enzymes produced by the Hulle cells, are used as the carbon source by the developing cleistothecia, and specific expression of a hexaside transporter gene in ascogenous hyphae has been observed (54).

It has been found that amino acid starvation and defects in cross-pathway signaling of amino acid starvation leads to a block in sexual development in *A. nidulans* and other fungi (19). Amino acid biosynthesis could be blocked in *citAΔ* as a result of loss of intermediates generated by the TCA cycle. This explanation for our results seems unlikely, since the amino acid starvation block is at an earlier stage, with only poorly pigmented microcleistothecia, approximately 20 μm in diameter, formed, in contrast to the fully mature cleistothecia devoid of ascospores formed in *citAΔ* selfed crosses.

A requirement for citrate synthase activity during meiosis is compatible with observations in other fungi. In both *Schizosaccharomyces pombe* and *S. cerevisiae*, respiration is required throughout meiosis, while in *S. cerevisiae* it has been shown that a nonfermentable carbon source is required for the early stages of meiosis (23). In *N. crassa* inactivation of complex I of the respiratory chain by mutation results in an early block in meiosis (12). In contrast to *A. nidulans*, the heterothallic fungus *P. anserina*, like other Sordariomycetes, forms open fruiting bodies (perithecia) and is amenable to detailed studies of meiosis. Respiration mutants are female sterile (13). The effects of mutations in the citrate synthase gene, *cit1*, on sexual development in the heterothallic fungus *P. anserina* have been subject to detailed study (38). Immunofluorescence studies demonstrated the presence of mitochondrial localized protein in asci and ascospores. In homozygous crosses between most complete loss-of-function mutants of *cit1*, including a deletion, there is a block at a particular stage of meiotic prophase I as well as slowed pachytene and occasional defects in crozier formation. A *cit1* mutation, resulting in the deletion of a single amino acid, produced only a partial loss of enzyme activity and did not affect meiosis. Both a mutation producing a truncated protein and an induced point mutation leading to inactive full-length protein had severe meiotic defects, but the specific block in meiotic prophase was not complete. A single amino acid substitution in a conserved domain almost completely lacks ascus development and shows no specific meiotic arrest, and this has been attributed to the mutant protein severely interfering with mitochondrial metabolism. A role for the Cit1 protein as opposed to the activity has been proposed. As noted above it is likely that in *P. anserina* citrate synthase activity is also provided by a methylcitrate synthase, and it is not known whether this is expressed during meiosis and the observed results are due to variable and incomplete interference with this activity by the different Cit1 variants. Our results do not support a role for CitA protein, as opposed to citrate synthase activity, in meiosis in *A. nidulans*.

The role of peroxisomal CitA is not at all clear. Deletion of the putative C-terminal PTS1 sequence does not affect carbon source utilization or developmental phenotypes, including germination. Furthermore, there does not seem to be a conserved role for peroxisomal citrate synthase in fungi, since *M. grisea*...
and *C. albicans* lack obvious PTS1 sequences (Fig. 3). Of course it cannot be excluded that there is an additional targeting sequence. It has been reported that Cit2 in *S. cerevisiae* can be targeted to both mitochondria and peroxisomes by a cryptic N-terminal targeting sequence that is unmasked when the PTS1 sequence is deleted (28). The sequences involved are not conserved in CitA. We have not been able to detect CitA-GFP-labeled peroxisomes in hyphae, conidia, or ascospores. This may represent a very low level of peroxisomal localization.

In proteins with dual mitochondrial and peroxisomal targeting, this may represent a very low level of peroxisomal localization. For example, with one downstream of the mitochondrial targeting sequence, and these often arise from the use of two transcription start points. Examples include *S. cerevisiae* Cat2 (14, 52) and *A. nidulans* IdpA (46). There are no downstream ATG codons in *citA* that maintain the open reading frame, and mapped expression sequence tags do not indicate alternative transcription start points. Therefore, any peroxisomal localization must result from import of either full-length protein or protein with the mitochondrial signal sequence removed. This is highly likely to result in very inefficient import and might account for our difficulty in detecting peroxisomal CitA-GFP. However, *Penicillium chrysogenum* CitA (which contains the PTS1 AKL) has been detected in purified peroxisomal proteins by liquid chromatography-tandem mass spectrometry analysis (25).

We found that the *citA*Δ*K*+ mutation results in a reduced rate of colony extension arising from single ascospores and conidia. This phenotype was complemented by the *citA*Δ*34Δ* mutation, which results in a protein retaining the PTS1 but with loss of mitochondrial targeting. In the *citA*Δ*34Δ*-*gfp* strain, we observed rare punctate GFP localization in 5-h germinated conidia but not in hyphae, and this correlated with detection of some protein in this strain at this stage. It can therefore be suggested that a low level of peroxisomal CitA in germinated hyphae enhances colony growth. It is not obvious what role this activity plays in metabolism. While complete loss of peroxisomes in a *pexC* mutant does not affect colony extension, loss of PTS1 protein targeting in a *pexE*Δ*5Δ* does. Mutants with loss of the glyoxylate cycle or gluconeogenesis do not show this phenotype. Therefore, utilization of gluconeogenic substrates provided by mobilization of carbon sources stored in spores is unlikely. At present we do not have a satisfactory explanation for these data.

Overall we have shown that the putative CitA PTS1 sequence, which is conserved in other *Aspergillus* spp., does not play a major role in carbon source utilization or in the developmental functions of CitA. It is clear that peroxisomal citrate synthase activity is dispensable for the glyoxylate cycle. Furthermore, CitA function can be replaced by the citrate synthase activity of the mitochondria-localized methylcitrate synthase McsA when carbon catabolite repression is relieved, either by growth on derepressing carbon sources or by the loss of CreA repressor function. Of particular interest is the finding that derepression of *mcsA* due to the *creA*204 mutation, but not by derepressing carbon sources, suppresses the *CitA* requirement for the production of ascospores within cleistothecia. CreA has previously been regarded as responding to the externally supplied carbon source to regulate the transcription of genes required for the utilization of alternative carbon sources. Our results show that CreA-mediated repression can be an intrinsic property of the metabolism of particular cell types.

**ACKNOWLEDGMENTS**

This work was supported by the Australian Research Council. Assistance by Khanh Nguyen and Quentin Lang and provision of the mcsAΔ strain and helpful comments by Matthias Brock are gratefully acknowledged.

**REFERENCES**

tome to the genome: the microbe of penicillin-producing Penicillium 

central role for the peroxisomal membrane in gyoxylate cycle function. 

of the exit from dormancy of Aspergillus fumigatus conidia. BMC Genomics 
9:417.

functional analysis of the cryptic N-terminal targeting signal for both mito-
chondria and peroxisomes in yeast peroxisomal citrate synthase Cit2p. J. Bio-
chem. 140:121–133.

the CIT2 gene of Saccharomyces cerevisiae is peroxisomal. Mol. Cell. Biol. 
10:1399–1405.

30. Liu, Z., and R. A. Butow. 1999. A transcriptional switch in the expression of 
yeast tricarboxylic acid cycle genes in response to a reduction or loss of 

synthase from Aspergillus fumigatus propionyl-CoA affects polyketide synthe-


conidial-enriched transcripts in Aspergillus nidulans using suppression sub-

pyruvate carboxylase and of some other enzymes in Aspergillus oryzae. Eur. 

and characterization of the creA gene encoding the mitochondrial citrate 

peroxisomal citrate synthase is required for fatty acid respiration and seed 


laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold 
Spring Harbor, NY.

in the creA gene involved in carbon catabolite repression in Aspergillus 


42. Stemple, C. J., M. A. Davis, and M. J. Hynes. 1998. The facC gene of 
Aspergillus nidulans encodes an acetate-inducible carnitine acetyltransferase. 

43. Strijbis, K., C. W. van Roermund, W. F. Visser, E. C. Mol, J. van den Burg, 
D. M. MacCallum, F. C. Odds, E. Paramonova, B. P. Krom, and B. Distel. 
2008. Carnitine-dependent transport of acetyl coenzyme A in Candida albii-
cans is essential for growth on nonfermentable carbon sources and contrib-

44. Sun, J., X. Lu, U. Rinas, and A. P. Zeng. 2007. Metabolic peculiarities of 
Aspergillus nidulans disclosed by comparative metabolic genomics. Genome 
Biol. 8:R182.

nitine-dependent metabolic activities in Saccharomyces cerevisiae; three car-
nitine acetyltransferases are essential in a carnitine-dependent strain. Yeast 
18:585–595.

46. Szewczyk, E., A. Andrianopoulos, M. A. Davis, and M. J. Hynes. 2001. A 
single gene produces mitochondrial, cytoplasmic, and peroxisomal NADP-
dependent isocitrate dehydrogenase in Aspergillus nidulans. J. Biol. Chem. 
276:37722–37729.

47. Taubitz, A., B. Bauer, J. Heesemann, and F. Ebel. 2007. Role of respiration 
in the germination process of the pathogenic mold Aspergillus fumigatus. 

Aspergillus nidulans: meiotic progeny for genetic analysis and strain construc-

49. Todd, R. B., M. A. Davis, and M. J. Hynes. 2007. Genetic manipulation of 
Aspergillus nidulans: heterokaryons and diploids for dominance, complemen-
tation and haploidization analyses. Nat. Protoc. 4:822–830.

Nuclear accumulation of the GATA factor AreA in response to complete 
1655.

51. van Roermund, C. W. T., Y. Elgersma, N. Singh, R. J. A. Wanders, and H. 
Tabak. 1995. The membrane of peroxisomes in Saccharomyces cerevisiae is 
impermeable to NAD(H) and acetyl-CoA under in vivo conditions. EMBO 
J. 14:3480–3486.

52. van Roermund, C. W., E. H. Hettema, M. van den Berg, H. F. Tabak, and R. 
J. Wanders. 1999. Molecular characterization of carnitine-dependent 
transport of acetyl-CoA from peroxisomes to mitochondria in Saccharomyces 
cerevisiae and identification of a plasma membrane carnitine transporter, 

structure between Krebs TCA cycle enzymes: a model for the metabolon. 

2004. A putative high affinity hexose transporter, Inx1, of Aspergillus nidi-
ulans is induced in vegetative hyphae upon starvation and in ascogenous 

control of mnr4 by the bZIP transcription factor MeaB reveals a new level 


57. Zhou, H., and M. C. Lorenz. 2008. Carnitine acetyltransferases are required 
for growth on non-fermentable carbon sources but not for pathogenesis in 