

ATP-Citrate Lyase Is Required for Production of Cytosolic Acetyl Coenzyme A and Development in *Aspergillus nidulans*[∇]

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Acetyl coenzyme A (CoA) is a central metabolite in carbon and energy metabolism and in the biosynthesis of cellular molecules. A source of cytoplasmic acetyl-CoA is essential for the production of fatty acids and sterols and for protein acetylation, including histone acetylation in the nucleus. In *Saccharomyces cerevisiae* and *Candida albicans* acetyl-CoA is produced from acetate by cytoplasmic acetyl-CoA synthetase, while in plants and animals acetyl-CoA is derived from citrate via ATP-citrate lyase. In the filamentous ascomycete *Aspergillus nidulans*, tandem divergently transcribed genes (*aclA* and *aclB*) encode the subunits of ATP-citrate lyase, and we have deleted these genes. Growth is greatly diminished on carbon sources that do not result in cytoplasmic acetyl-CoA, such as glucose and proline, while growth is not affected on carbon sources that result in the production of cytoplasmic acetyl-CoA, such as acetate and ethanol. Addition of acetate restores growth on glucose or proline, and this is dependent on *facA*, which encodes cytoplasmic acetyl-CoA synthetase, but not on the regulatory gene *facB*. Transcription of *aclA* and *aclB* is repressed by growth on acetate or ethanol. Loss of ATP-citrate lyase results in severe developmental effects, with the production of asexual spores (conidia) being greatly reduced and a complete absence of sexual development. This is in contrast to *Sordaria macrospora*, in which fruiting body formation is initiated but maturation is defective in an ATP-citrate lyase mutant. Addition of acetate does not repair these defects, indicating a specific requirement for high levels of cytoplasmic acetyl-CoA during differentiation. Complementation in heterokaryons between *aclA* and *aclB* deletions for all phenotypes indicates that the tandem gene arrangement is not essential.

Acetyl coenzyme A (CoA) is a crucial metabolite involved in both intermediary carbon and energy metabolism as well as in biosynthetic pathways. Appropriate levels of this metabolite must be present in the correct cellular compartment for normal growth and development, and the pathways producing acetyl-CoA vary with the carbon source(s) available. When glycolytic carbon sources are present, pyruvate is the major source of acetyl-CoA in the mitochondrion as a result of pyruvate dehydrogenase activity (Fig. 1). Oxaloacetate, also derived from pyruvate via pyruvate carboxylase activity, is combined with acetyl-CoA by citrate synthase to form citrate, allowing the tricarboxylic acid (TCA) cycle to occur in mitochondria (Fig. 1). This results in the formation of biosynthetic intermediates and ATP generation via oxidative phosphorylation under aerobic conditions.

Cytoplasmic acetyl-CoA is essential as a building block for the biosynthesis of fatty acids and various secondary metabolites by virtue of its conversion to malonyl-CoA by cytoplasmic acetyl-CoA carboxylase and for sterol biosynthesis via the synthesis of hydroxymethyl glutaryl-CoA in the mevalonate pathway. It is also the substrate for acetylation of lysine residues in many proteins, including histones. The widespread occurrence of protein acetylation has recently been documented for both liver cells and *Salmonella enterica* to the extent that this post-translational modification may rival that of phosphorylation or ubiquitination in affecting protein function and stability (55, 61). There are two potential sources of cytoplasmic acetyl-

CoA: from citrate via ATP-citrate lyase (ACL), which depends on citrate entering the cytoplasm from the mitochondrion, or from acetate via acetyl-CoA synthetase (ACS) (Fig. 1). Acetate may be obtained from externally supplied acetate in the growth medium, or it may be produced from acetaldehyde derived from either pyruvate via pyruvate decarboxylase or from ethanol via alcohol dehydrogenase. A further potential source of acetyl-CoA is by β -oxidation of fatty acids in peroxisomes (not shown in Fig. 1) and, in this case, acetyl-CoA must exit the peroxisome into the cytoplasm.

There is evidence for the pyruvate-acetaldehyde-acetate pathway being the major source of cytoplasmic acetyl-CoA in *Saccharomyces cerevisiae*. Deletion of all three structural genes for pyruvate decarboxylase (*PDCl*, *PDC5*, and *PDC6*) results in loss of growth in defined glucose medium, and this can be restored by acetate or ethanol (15, 39). There are multiple genes for aldehyde dehydrogenase, and the situation is more complex. During anaerobic growth on glucose the cytoplasmic (Ald6) and the mitochondrial aldehyde dehydrogenases (Ald4 and Ald5) are the major sources of acetate (40). However, deletion of all three genes does not result in loss of viability or acetate formation, suggesting an unknown compensating pathway. Two ACSs, *Acs1* and *Acs2*, are present in *S. cerevisiae*, and loss of both enzymes is lethal on all carbon sources, indicating that this activity is essential (47, 52). *Acs2* is essential for growth on glucose, and the 5' region of *ACS2* contains binding sites for the global transcriptional activator Abf1, but it is not essential for growth on nonfermentable carbon sources, where *Acs1* is active as a result of transcriptional activation by Cat8 acting at a carbon source response element (CSRE) and by Adr1 (11, 24, 43, 45, 47). Mitochondrial localization of *Acs1* has been proposed, but some is present in the cytoplasm and

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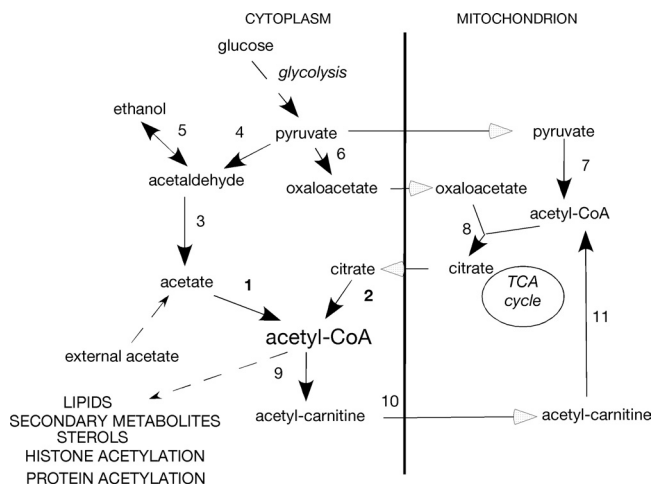


FIG. 1. Relevant metabolic pathways for acetyl-CoA generation. Enzymes are numbered as follows: 1, acetyl-CoA synthetase (EC 6.2.1.13); 2, ATP-citrate lyase (EC 2.3.3.8); 3, maldehyde dehydrogenase (EC 1.2.1.5); 4, pyruvate decarboxylase (EC 4.1.1.1); 5, alcohol dehydrogenase (EC 1.1.1.1); 6, pyruvate carboxylase (EC 6.4.1.1); 7, pyruvate dehydrogenase complex (EC 2.3.1.12); 8, citrate synthase (EC 2.3.3.1); 9, cytoplasmic carnitine acetyltransferase (EC 2.3.1.7); 10, acetyl-carnitine mitochondrial carrier; 11, mitochondrial/peroxisomal carnitine acetyltransferase (EC 2.3.1.7).

the nucleus and Acs2 is nuclear/cytosolic (47). Acs2 has been shown to be a major source of acetyl-CoA for histone acetylation on glucose, while on glycerol and ethanol this function can be replaced by Acs1. Neither peroxisomal β -oxidation nor mitochondrial pyruvate dehydrogenase activity was found to contribute to histone acetylation. The ability of a *Salmonella enterica* ACS targeted to different cell compartments to complement loss of Acs2 activity showed that cytoplasmic- or nuclear-localized activity was sufficient for growth on glucose but not mitochondrial localized activity. These data are consistent with cytoplasmic pools of acetyl-CoA being interchangeable with the nuclear but not the mitochondrial pool (47). The yeast pathogen *Candida albicans* also has two ACSs (6). However, the situation is different in that Acs2 is required not only for growth on glucose but also on ethanol, acetate, and lactate, and *acs2* is expressed on all of these carbon sources, while Acs1 is not essential. Acs2 is not required for growth on the fatty acid, oleate, or on glycerol, and *acs1* is only highly expressed on oleate. No effects on global histone acetylation were detected in that study (6). Two *acs* genes are predicted in the genome sequences of *Saccharomycotina* with the exception of *Yarrowia lipolytica* (see orthogroup 4318 at <http://www.broadinstitute.org/regev/orthogroups/>). A mutant of *Y. lipolytica* lacking acetate-inducible ACS activity has been found to be able to grow on glucose and glycerol but not on acetate (28).

ACL is present in only a few prokaryotes or *Archaea* but is widely present in algae, plants, and animals (reference 14 and references therein). Cytosolic ACL activity is the major source of cytoplasmic acetyl-CoA in plants, where tissue-specific expression is correlated with acetyl-CoA carboxylase expression (14). In mammalian cells, ACL is localized in both the cytoplasm and the nucleus, and small interfering RNA-mediated silencing of ACL expression affects the patterns of histone acetylation and lipid accumulation in adipocytes (56). Silencing

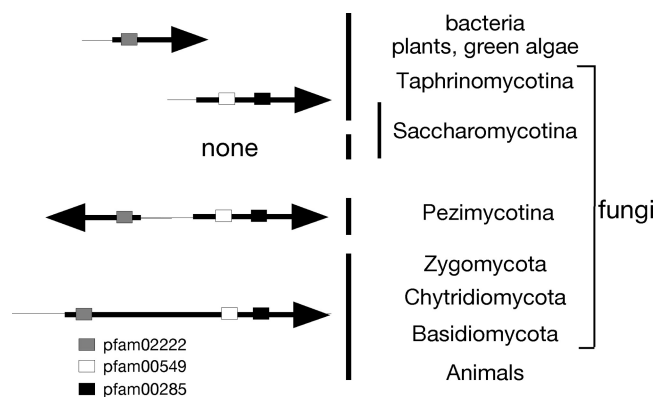


FIG. 2. ACL gene arrangements in different phyla. See reference 14 for information on bacteria, algae, plants, and animals. BlastP searches of fungal genomes (<http://www.broadinstitute.org/annotation/fungi/index.html>) using the predicted *A. nidulans* ACL-encoding genes AN2435.3 and AN2436.3 showed predicted divergently transcribed genes in the genomes of all species of the *Pezizomycotina*, e.g., *Magnaporthe grisea* (MGG_06720.5 and MGG_06719.5), *Scelerothia sclerotiorum* (SS1G_02378.1 and SS1G_02379.1), and *Botrytis cinerea* (BC1G_05991.1 and BC1G_05989.1). In the case of *Neurospora crassa* (NCU06783 and NCU06785), a very short dubious predicted gene (NCU06784) occurs between the genes. Single genes are found in *Zygomycota* (*Rhizopus oryzae* [RO3G_14197.1]), *Chytridiomycota* (*Batrachomyces dendrobatidis* [BDEG_06723]), and *Basidiomycota* (e.g., *Ustilago maydis* [UM01005.1], *Cryptococcus neoformans* [CNAG_04640.1], and *Coprinus cinerea* [CC1G_09654.1]). Analysis of fungal orthogroups (<http://www.broadinstitute.org/regev/orthogroups/>) showed no ACL homologues in the *Saccharomycotina*, with the exception of *Y. lipolytica* (YALI0D24431g and YALI0E34793g). *Schizosaccharomyces pombe* (*Taphrinomycotina*) contains two unlinked ACL genes (SPBC1703.07 and SPAC22A12.16). Conserved protein domains (<http://www.ncbi.nlm.nih.gov/sites/entrez>) are shown: pfam02222 (ATP grasp domain), pfam 00549 (CoA ligase), and pfam00285 (citrate synthase).

of ACS expression has little effect in the absence of added supraphysiological levels of acetate (56). Analysis of sequenced fungal genomes indicates that predicted ACL-encoding genes are present widely in fungi but are notably absent from sequenced members of the *Saccharomycotina* with the exception of *Y. lipolytica* (Fig. 2). A survey of a wide variety of (unsequenced) yeast species has found a correlation between those that accumulate high levels of lipids (oleaginous yeasts) and the presence of ACL activity, and these included some members of the *Saccharomycotina* as well as the *Basidiomycota* (4). A further correlation has been found between ACL activity and secondary metabolite and lipid production in filamentous fungi (57). ACL activity in *Aspergillus niger* has been found to be much higher than ACS activity, and the enzyme is inhibited by palmitoyl-CoA, indicating a role in fatty acid synthesis (38).

The primary sequences of ACL contain conserved domains for ATP and CoA binding as well as sequences related to the oxaloacetate binding and active site motifs of citrate synthase (14). However, ACL has been found to be either heteromeric or homomeric, and analysis of sequenced genomes reveals divergence in the distribution of these different structures in different phyla (Fig. 2) (see reference 14 for a phylogenetic tree). The *Arabidopsis thaliana* enzyme is a hetero-octamer containing four A-subunits (45 kDa) encoded by three genes and four B-subunits (65 kDa) encoded by two genes, while, in contrast, animal enzymes are homotetramers (14). Both ho-

omeric and heteromeric ACL structures are predicted in fungi. The filamentous ascomycete *Sordaria macrospora* has been shown to have two adjacent genes for different subunits of ACL separated by 3.4 kb, and these are divergently transcribed (34). ACL purified from *Aspergillus nidulans* has two dissimilar subunits of 70 and 55 kDa and is a hexamer containing three of each kind of subunit (1). This is supported by the presence of two predicted divergently transcribed genes in the *A. nidulans* genome sequence (see Fig. 3, below, and Results). This gene arrangement is also predicted for other *Peziizomycotina* species. Two ACL genes encoding dissimilar subunits are also predicted for *Y. lipolytica* as well as for *Schizosaccharomyces pombe*, but in these species the genes are unlinked. In contrast, only one gene encoding ACL is predicted in the other fungal phyla (Fig. 2), and ACL from the basidiomycete *Rhodotorula gracilis* has been shown to be a homotetramer (44). The most parsimonious evolutionary model is that the heteromeric structure is ancestral and a gene fusion in the common ancestor of fungi and animals occurred, resulting in the homomeric enzyme structure. The heteromeric structure was restored by a subsequent genome rearrangement in the ancestor of the ascomycetes that produced two genes, one for each kind of subunit. Subsequent gene loss occurred at least once in the *Saccharomycotina*.

Our interests are in the intracellular distribution of acetyl-CoA during growth on different carbon sources in *A. nidulans* (19, 20, 46). The extent to which mitochondrial and peroxisomal acetyl-CoA pools are separate from the cytoplasmic pool and the role of ACL is therefore of considerable importance. The pyruvate-acetaldehyde-acetate pathway is unlikely to be a major source of acetyl-CoA. Pyruvate decarboxylase was undetectable in glucose media (37). However, a single gene encoding pyruvate decarboxylase, together with *alcC*, encoding an alcohol dehydrogenase, has been found to be involved in a fermentation response under anaerobic conditions (22, 26). The utilization of ethanol as a sole carbon source is mediated by *AlcA* (alcohol dehydrogenase) and *AldA* (aldehyde dehydrogenase). The transcription of the genes for these enzymes is activated by the *AlcR* transcription factor, which responds to acetaldehyde as the inducer, and expression is repressed by carbon catabolite repression mediated by *CreA*, which represses *alcR* and *alcA* (16, 27). There is a single cytoplasmic ACS encoded by *facA*, and loss-of-function mutations in this gene result in loss of growth on acetate or ethanol as sole carbon sources but have no effect on growth on glucose or amino acids, such as proline or glutamate (3, 7, 42). Expression of *facA* is inducible by acetate mediated by the transcription factor *FacB*, which has considerable similarity to *Cat8* in *S. cerevisiae*, and it is also repressed by glucose (18, 21, 48, 49). Therefore, *FacA* is unlikely to be a source of cytoplasmic acetyl-CoA during growth on glucose or noninducing carbon sources. Acetyl-CoA is also produced in peroxisomes by fatty acid β -oxidation, and expression of the genes required is induced by fatty acids mediated by the transcription factors *FarA*, *FarB*, and *ScfA* (19, 20). Acetyl-CoA, produced in peroxisomes from fatty acids or in the cytoplasm from acetate, is transferred to the mitochondria via the acetyl-carnitine shuttle (Fig. 1) (9, 10, 46).

Overall, it is therefore predicted that, in *A. nidulans*, ACL is important for acetyl-CoA synthesis on some carbon sources

TABLE 1. Strains used in this study

Strain no.	Name	Genotype ^a
54	Wild type	<i>biA1 niiA4</i>
11036	TNO2A21	<i>pyroA4 nkuA::argB⁺ riboB2</i>
12393	<i>acl(AB)</i> Δ (original)	<i>pyroA4 nkuA::argB⁺ acl(AB)::bar riboB2</i>
12410	<i>acl(AB)</i> Δ	<i>acl(AB)::bar niiA4</i>
12408	<i>acl(AB)</i> Δ (empty vector)	<i>wA A. fumigatus riboB⁺/pyroA4 nkuA::argB⁺ acl(AB)::bar riboB2</i>
12404	<i>aclA⁺/acl(AB)</i> Δ	<i>wA A. fumigatus riboB⁺ aclA⁺/pyroA4 nkuA::argB⁺ acl(AB)::bar riboB2</i>
12406	<i>aclB⁺/acl(AB)</i> Δ	<i>wA A. fumigatus riboB⁺ aclB⁺/pyroA4 nkuA::argB⁺ acl(AB)::bar riboB2</i>
12400	<i>acl(AB)⁺/acl(AB)</i> Δ	<i>wA A. fumigatus riboB⁺ acl(AB)⁺/pyroA4 nkuA::argB⁺ acl(AB)::bar riboB2</i>
12438	<i>acl(AB)</i> Δ <i>veA⁺</i>	<i>biA1 acl(AB)::bar veA⁺ niiA4</i>
10593	<i>facA303</i>	<i>biA1 pyroA4 facA303 niiA4</i>
12445	<i>acuJ</i> Δ <i>acl(AB)</i> Δ	<i>biA1 acuJ::pyrG⁺ pyroA4 acl(AB)::bar riboB2</i>
12436	<i>acuH253 acl(AB)</i> Δ	<i>pabaA2 acuH253 acl(AB)::bar</i>
12431	<i>facC</i> Δ <i>acl(AB)</i> Δ	<i>acl(AB)::bar facC::argB⁺ riboB2</i>
12421	<i>facB101 acl(AB)</i> Δ	<i>wA3 acl(AB)::bar facB101 riboB2</i>

^a The presence of *nkuA::argB⁺* is not known for all strains. *wA* represents a disruption of the *wA⁺* locus as described in Fig. 3. All strains with the exception of 12438 contained the *veA1* mutation.

but not others. Impaired synthesis of lipid-based signaling molecules as well as secondary metabolites as a result of lowered cytoplasmic acetyl-CoA levels are likely to result in developmental defects. A mutation in one of the ACL structural genes was found to affect maturation of fruiting bodies in *Sordaria macrospora* (36). We have therefore investigated the effects of deleting the genes encoding ACL on growth and development. Growth is severely compromised in the absence of sources of cytoplasmic acetyl-CoA; asexual sporulation (conidiation) is greatly reduced and sexual development is absent.

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 (20). Minimal media contained the required supplements to allow auxotrophic strains to grow. All strains were derived from the original Glasgow strain and contained the *veA1* mutation unless otherwise indicated. The *facA303*, *acuH253*, *facC* Δ , and *facB101* mutations have been described elsewhere (3, 46, 49). The *acuJ* Δ deletant was generated by gene replacement using *pyrG* as the selectable marker (unpublished data). The genotypes of strains described here are presented in Table 1. Standard methods of *A. nidulans* genetic manipulations were as previously described (50) with the modification that all manipulations of *acl* Δ strains required the addition of acetate to the medium to allow growth. Preparation of protoplasts and transformation were as described previously (31) with the modification that the *acl(AB)* Δ strain was grown in 0.1% glucose minimal medium containing 50 mM acetate for the preparation of protoplasts. Recipient strains contained *nkuA4* to promote homologous integration events, and selectable markers were the *bar* gene (glufosinate resistance) and *riboB⁺* from *A. fumigatus* (31). 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 (19, 41).

DNA sequences and sequence analysis. *Aspergillus* spp. sequences were obtained from the genome sequences at 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>), NCBI (<http://www.ncbi.nlm.nih.gov/>), or the Broad Institute orthogroups database (<http://www.broadinstitute.org/regev/orthogroups/>). Analysis of potential mi-

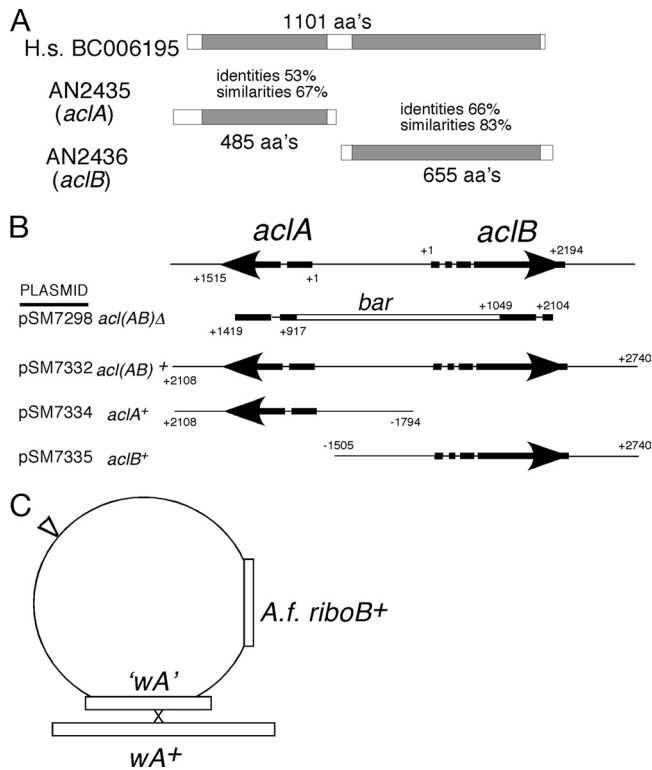


FIG. 3. Gene structure and deletion of ACL-encoding genes. (A) Comparison of the predicted products of the *aclA* and *aclB* genes with the human ACL (*H. sapiens* accession number BC006195). (B) Schematic of the deletion constructs. The structures of the divergently transcribed *aclA* and *aclB* genes are shown with the positions of introns indicated by gaps. The positions of start codons are indicated by +1 for each gene. The *bar* gene encoding glufosinate resistance replaced the indicated sequences in plasmid pSM7298, and a linear fragment generated by PCR was used to generate the initial *acl(AB)* deletion by transformation. pSM7332, pSM7334, and pSM7335 containing both *aclA*⁺ and *aclB*⁺ genes, *aclA*⁺ and *aclB*⁺, respectively, were constructed with the sequences inserted into the *wA* targeting vector shown. (C) The *wA* targeting vector contains the *A. fumigatus riboB* gene for selecting *RiboB*⁺ transformants and contains an internal fragment of the *wA* coding region (nt +812 to +2966), which allows screening for insertional inactivation of the *wA*⁺ gene based on the presence of white conidia. These plasmids were used to generate the *acl(AB)*⁺/*acl(AB)*Δ, *aclA*⁺/*acl(AB)*Δ, and *aclB*⁺/*acl(AB)*Δ transformants by transforming the original *acl(AB)*Δ strain. In addition, an empty *wA* targeting vector was used to generate a control *acl(AB)*Δ strain.

tochondrial targeting used TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) and MitoProt (<http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html>).

Deletion of *acl* genes. A 5.6-kb DNA fragment containing part of the AN2435 (*aclA*) and AN2436 (*aclB*) genes and the intergenic region (nucleotide [nt] +1419 of *aclA* to +2104 of *aclB*) was cloned by PCR using the primers 5'-AC CAGTGCAGTCTCTTCT-3' and 5'-GTTGAGGCCGAGTTCCTCAC-3' and was then inserted into EcoRV-cut pBluescript SK(+) (Stratagene) to create pSM7296. The *bar* gene encoding glufosinate resistance was isolated as an NcoI/SpeI fragment from pMT1612 (31) and inserted into NcoI/NheI-cut pSM7296 to generate pSM7298, in which the entire intergenic region as well as the region to nt +917 of *aclA* and to nt +1049 of *aclB* were removed and replaced by the *bar* gene. A linear PCR fragment using the above primers was used to transform the strain TNO2A21, selecting for glufosinate resistance to give the *acl(AB)*Δ strain (Fig. 3). A 6.7-kb DNA fragment containing both the *aclA* and *aclB* genes was cloned by the PCR using the primers 5'-CTCAATGGCCCTGGAATAAA-3' and 5'-CCATCTGGACGGCTTATGTT-3', and this was inserted into EcoRV-cut pBluescript SK(+) to create pSM7333 and also into the *wA* targeting vector

pRG6501 (Fig. 3C) cut with EcoICRI to create pSM7332 containing *aclA*⁺ and *aclB*⁺ genes. To isolate individual ACL genes, pSM7333 was cut with Sall and NotI, and the fragment was cloned into pRG6501 cut with XhoI and NotI to generate pSM7334 containing *aclA*⁺ (corresponding to nt -1794 to +2108 of *aclA*); alternatively, it was cut with XbaI and XhoI and the fragment was cloned into pRG6501 cut with XbaI and XhoI to generate pSM7335 containing *aclB*⁺ (corresponding to nt -1505 to +2740 of *aclB*). pSM7332, pSM7334, pSM7335, and pRG6501 were transformed into the *acl(AB)*Δ strain, selecting for *RiboB*⁺ transformants on medium containing 50 mM acetate, and screened for white conidiating colonies. In this way the *acl(AB)*⁺/*acl(AB)*Δ, *aclA*⁺/*acl(AB)*Δ, *aclB*⁺/*acl(AB)*Δ, and *acl(AB)*Δ (empty vector) strains were isolated (Table 1; Fig. 3B).

Conidial counts. Conidial suspensions in dilute Tween 80 solution (0.0005%) were added to 0.6% C-free minimal medium with 0.6% agar and added as a top layer to plates containing 1% glucose minimal medium containing 10 mM acetate and 10 mM ammonium tartrate as the nitrogen source or with 1 M sucrose added, and plates were incubated for 3 days to generate a lawn of conidiating mycelia. Three to four plugs for each plate were made with a 0.28-cm² glass tube, and conidia were resuspended by vortexing in 5 ml of dilute Tween 80. Suspensions were diluted and spread on complete medium containing 10 mM acetate, and the number of colonies was counted after 2 days of incubation. The number of viable colonies per cm² was calculated.

Microscopy. Images of conidia and cleistothecial development were captured on an Olympus SZX12 microscope.

RESULTS

Deletion of *aclA* and *aclB* genes. The AN2435 and AN2436 genes are predicted to encode ACL subunits with high similarity to the predicted sequences of the ACL subunits of *S. macrospora* (34) and to the single polypeptide of *Homo sapiens* (Fig. 3A). Neither polypeptide had predicted mitochondrial or peroxisomal targeting sequences consistent with cytoplasmic localization. The genes were designated *aclA* and *aclB*, respectively. Both genes were simultaneously inactivated by gene replacement with the *bar* gene, which encodes glufosinate resistance, to generate the *acl(AB)*Δ strain (Fig. 3B). Initially, transformants selected on protoplast medium (1% glucose-1 M sucrose) containing glufosinate required extended incubation because of extremely slow growth. Subsequently, it was found that growth was greatly enhanced by adding 50 mM acetate to the selection plates, and isolated transformants grew strongly on acetate as a sole carbon source (see below). The *aclA* and *aclB* genes were cloned together on a single fragment, and each gene was cloned individually (Fig. 3B). These fragments were inserted into a vector designed for targeting to the *wA* locus (Fig. 3C), transformed into the *acl(AB)*Δ strain, selecting for *RiboB*⁺ transformants, and screened for white conidia. In this way *acl(AB)*⁺/*acl(AB)*Δ, *aclA*⁺/*acl(AB)*Δ, and *aclB*⁺/*acl(AB)*Δ strains were isolated. Complementation of *acl(AB)*Δ for all phenotypes required the presence of both *aclA*⁺ and *aclB*⁺.

Effects of deletion of ACL genes on growth on different carbon sources. Deletion of the *acl* genes had major effects on growth in the absence of sources of cytoplasmic acetyl-CoA (Fig. 4A). Growth was greatly affected on glucose medium but restored by acetate. Growth of the *acl* deletions was only slightly better on rich complete medium, which contains 1% glucose, peptone, yeast extract, and casein hydrolysate, indicating that these supplements do not provide sufficient acetyl-CoA. Growth of the *acl* deletion strains was poor on carbon sources metabolized via 2-oxoglutarate, such as proline (or alanine, glutamate, or 2-aminobutyric acid [data not shown]), and acetate restored growth. In contrast, growth was normal on acetate or ethanol as sole carbon sources where cytoplasmic

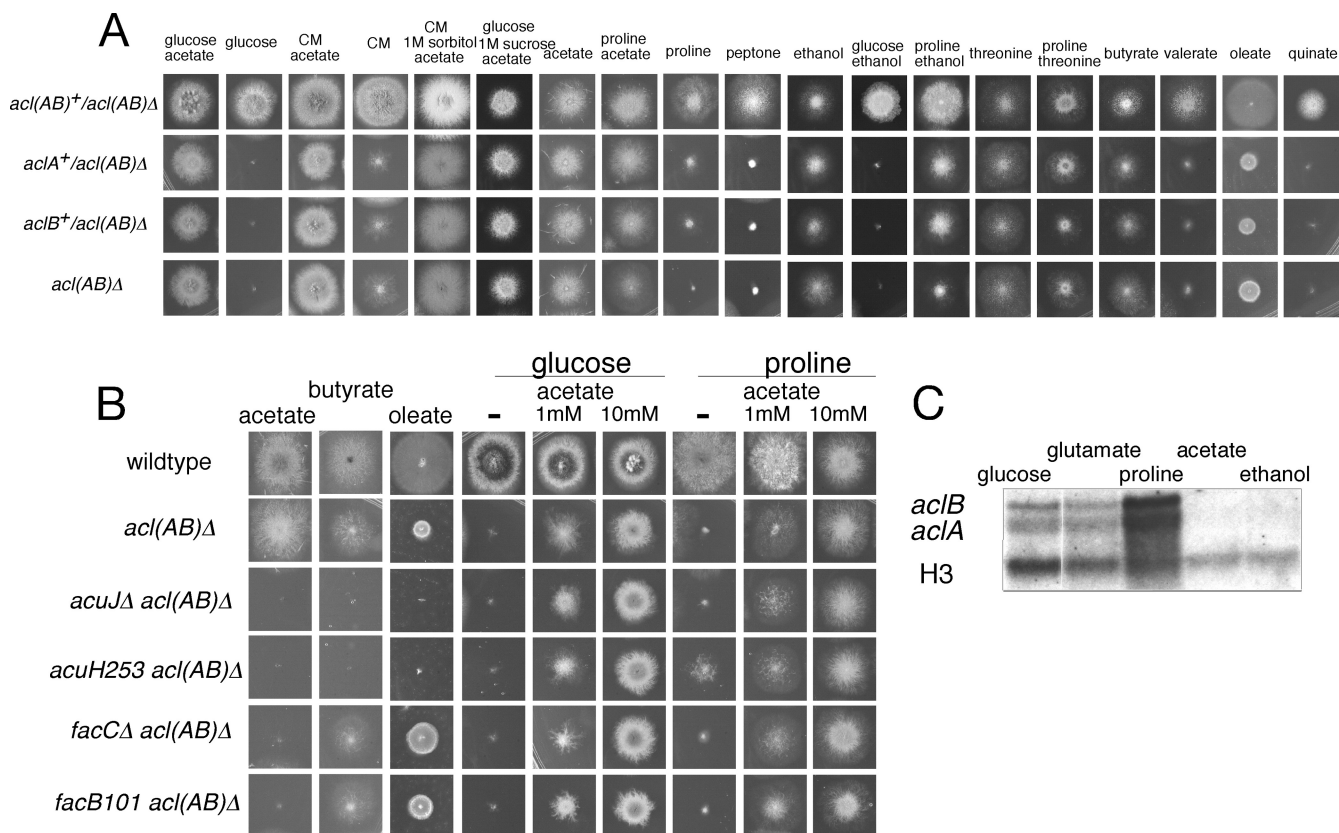


FIG. 4. Deletion of the *aclA* or *aclB* gene results in a requirement for cytoplasmic acetyl-CoA. (A) Growth on various carbon sources for 2 to 3 days at 37°C. The following carbon sources (concentrations shown in parentheses) were added to minimal medium with 10 mM ammonium chloride as the nitrogen source: glucose (1%); peptone (0.24%); acetate and proline (50 mM); ethanol (0.5%); quinate (0.25%); butyrate, valerate, and oleate (5 mM); threonine (20 mM). CM, complete medium containing 1% glucose. (B) Repair of *acl(AB)Δ* in mutant backgrounds affected in acetyl-CoA metabolism. Media were as for panel A, with acetate added at the indicated concentrations (with a dash indicating no added acetate). (C) Northern blot analysis of *aclA* and *aclB* expression. RNA was extracted from wild-type mycelia grown for 16 h in 1% glucose medium and then transferred to minimal medium containing 1% glucose, 50 mM glutamate, 50 mM proline, 50 mM acetate, or 0.5% ethanol as the sole carbon source, with 10 mM ammonium chloride as the nitrogen source for 4 h before harvesting and extraction. Ten micrograms of total RNA was run on a 1.2% agarose-formaldehyde gel, blotted, and probed sequentially with ³²P-labeled PCR products of *aclA* (primers 5'-CCAACACTCACA ACCCTCT-3' and 5'-GTTGAGGCCGAGTTCTTCAC-3') and *aclB* (primers 5'-GTTGAGGCCGAGTTCTTCAC-3' and 5'-ATGCCCTCTT CCACAACAAG-3'), with the EcoRI fragment of the histone H3 gene as a loading control.

acetyl-CoA is produced by ACS. While the presence of glucose did not prevent growth repair by acetate (see below), it completely prevented repair by ethanol, consistent with strong glucose repression of ethanol catabolism (27). Growth on threonine was unaffected, and threonine repaired growth on proline, consistent with catabolism of threonine via acetaldehyde and acetate (16). The deletion strains were able to grow on butyrate and to a much lesser extent on valerate and oleate. These fatty acids are metabolized via β -oxidation in peroxisomes (20), and therefore this result suggests that some acetyl-CoA can enter the cytoplasm from peroxisomes.

The observation that growth of the deletion strains on the aromatic quinate was completely abolished is of particular interest. This result was surprising because quinate is metabolized to acetyl-CoA and succinyl-CoA via the protocatechuate pathway (as characterized in bacteria [32]), which has been analyzed by the isolation of *pca* mutants unable to grow on either quinate or benzoate (25). The genes for the enzymes of this pathway have not been cloned. The last step in the pathway is catalyzed by β -ketoacyl-CoA thiolase, producing

acetyl-CoA and succinyl-CoA, and mutations in the proposed structural gene, *pcaF*, have been isolated (25). The likely gene has been identified as AN5698 by a BlastP search of the *A. nidulans* genome sequence using the *Escherichia coli* PcaF sequence (38% identity and 53% similarity), and this gene is on linkage group V, in accordance with the mapped location of the *pcaF* mutation (25). An N-terminal mitochondrial targeting sequence is predicted in the AN5698 sequence by Mitoprot and TargetP. Therefore, it is highly likely that the acetyl-CoA resulting from quinate catabolism is produced in the mitochondrion by β -ketoacyl-CoA thiolase. The inability of the *acl* deletants to grow on quinate is consistent with the cytoplasmic and mitochondrial pools of acetyl-CoA being distinct. Identification of all the genes encoding the enzymes of the protocatechuate pathway and the determination of their cellular localization is clearly worthwhile.

Effects of acetate utilization mutations on growth of *acl(AB)Δ*. The addition of acetate to glucose or proline medium allowed growth of the *acl* deletion mutants. It was likely that this resulted from the activity of cytoplasmic ACS encoded

by the *facA* gene. Strains containing *acl(AB)*Δ were fertile in heterozygous crosses, yielding *acl(AB)*Δ progeny readily identified by a requirement for acetate and poor conidiation. We were unable to isolate *acl(AB)*Δ *facA* double mutants. In crosses to a strain containing the *facA303* loss-of-function mutation (3), all *acl(AB)*Δ progeny were able to grow on acetate as the sole carbon source, i.e., *facA*⁺. This clearly indicated that ACS is required for growth repair by acetate. It was surprising that sufficient ACS activity was detected in the presence of glucose, which represses the expression of *facA* (3, 42). The FacB activator is responsible for acetate induction of *facA* (18, 42, 48). However, FacB was not required for acetate repair of growth of strains containing *acl(AB)*Δ, because *acl(AB)*Δ *facB101* double mutants were readily isolated from a cross and acetate was able to repair growth on both glucose and proline media (Fig. 4B). It has been recently shown that there is some FacB-independent expression of ACS activity in *A. niger* (29). It is not known whether this represents an unregulated constitutive level of expression or whether there is an unknown activation mechanism in the absence of acetate as inducer.

The effects of mutations affecting acetyl-CoA metabolism and cellular distribution via the acetyl-carnitine shuttle were investigated. Double mutant strains of *acl(AB)*Δ with loss-of-function mutations were isolated from crosses and tested for effects on acetate growth repair on glucose and proline media (Fig. 4B). In this way it was found that loss of FacC, the cytoplasmic carnitine acetyl-CoA transferase required for growth on acetate (Fig. 1, step 9) (46), AcuJ, the peroxisomal/mitochondrial carnitine acetyl-CoA transferase required for growth on both acetate and fatty acids (Fig. 1, step 11) (3, 19, 46), and AcuH, the mitochondrial acetyl-carnitine carrier protein required for growth on both acetate and fatty acids (Fig. 1, step 10) (3, 9, 10), did not affect the ability of acetate to repair growth of the *acl(AB)*Δ. In fact the effects of acetate were slightly enhanced, suggesting that, in wild-type strains, a low basal level of these activities on glucose or proline media containing acetate allows some transfer of acetyl-CoA into the mitochondrion, thereby reducing cytoplasmic acetyl-CoA levels.

Regulation of the expression of *aclA* and *aclB*. Northern blot analysis showed that both *aclA* and *aclB* were expressed when glucose, proline, or glutamate was present as the sole carbon source (Fig. 4C). Expression was not detected when either acetate or ethanol was the carbon source. This suggested that sources of cytoplasmic acetyl-CoA result in repression. These results were consistent with the finding that ACL enzyme activity was 9-fold higher in glucose-grown cells than acetate-grown cells (1) and also microarray data indicating that expression levels of both genes were about 20-fold higher in glucose than ethanol (8).

Developmental effects of loss of ACL. Deletion of the *acl* genes resulted in obvious reduced production of asexual spores (conidia) in colonies growing on all media, including acetate (Fig. 4A and B). This was partially relieved by high-osmotic medium (1 M sorbitol, 1 M sucrose, and 0.6 M KCl), which is known to enhance conidiation (17). Reduced conidiation was confirmed at the microscopic level (Fig. 5A) and quantitatively by counting viable conidia (Fig. 5B). *A. nidulans* is homothallic and sexual development can occur by selfing under appropriate conditions, and the presence of the *veA*⁺ allele increases the

level of sexual development (17). Outcrosses of *acl(AB)*Δ-containing strains to *acl(AB)*⁺ strains set up on media containing glucose and acetate were fully fertile. A complete loss of sexual development was found in selfings of *acl(AB)*Δ strains in both *veA1* and *veA*⁺ backgrounds, with no nurse cells (Hulle cells) or fruiting bodies (cleistothecia) produced (Fig. 5C). The fatty acid oleate has been found to greatly stimulate the production of cleistothecia, presumably by altering the level of fatty acid-derived oxylipins, which determine the balance between asexual and sexual reproduction (20, 51). *acl(AB)*Δ was completely insensitive to oleate-induced cleistothecia formation (Fig. 5D).

Complementation between *aclA* and *aclB* genes in trans. It has been suggested that divergently transcribed gene pairs are more commonly conserved in fungi than convergently orientated genes and that this may reflect functional relatedness and coexpression via bidirectional promoters (23). This situation is apparent for *aclA* and *aclB* in *A. nidulans* and for ACL genes in other members of the *Pezizomycotina* (Fig. 2). This might indicate a requirement for highly coordinated metabolic and developmental controls. We found that *aclA*⁺ and *aclB*⁺ genes could complement each other in *trans* in heterokaryons for conidiation (Fig. 6A), for growth on proline as a carbon source (Fig. 6B), and for sexual development (Fig. 6C). Therefore, *aclA*⁺ and *aclB*⁺ genes do not have to be adjacent for functional expression. However, it should be noted that each gene contains most of the intergenic upstream sequence, which presumably contains *cis*-acting sequences that determine appropriate transcriptional regulation.

Fluoroacetate inhibition of development. High levels of fluoroacetate in the absence of a repressing carbon source are toxic to *A. nidulans*. Loss-of-function mutations in the *facA* (ACS), *facC* (cytoplasmic carnitine acetyltransferase), and *facB* (acetate regulator) genes result in resistance (2, 3). The major mechanism of inhibition is probably due to conversion of fluoroacetate to fluoroacetyl-CoA by FacA and then to fluorocitrate by citrate synthase in the mitochondria, resulting in inhibition of aconitase and hence blocking the TCA cycle. We found that low concentrations of fluoroacetate inhibited development, but not growth, even in the presence of glucose (Fig. 7). Conidiation was inhibited on both glucose minimal medium and complete medium and, furthermore, conidial pigmentation was inhibited. The *facA303* mutation resulted in resistance to these effects (Fig. 7A). The effects of fluoroacetate on conidiation were reversed on high-osmotic medium, but conidial pigmentation was still inhibited (Fig. 7B). Loss-of-function mutations in *facC* or *facB* did not result in resistance (data not shown), indicating that FacB-independent expression of *facA* is responsible for the synthesis of cytoplasmic fluoroacetyl-CoA, which results in inhibition of conidiation. Fluoroacetate also resulted in complete inhibition of sexual development, and again the *facA303* mutation resulted in resistance (Fig. 7C). Propionyl-CoA, and to a lesser extent acetyl-CoA and butyryl-CoA, has been shown to inhibit ACL activity in *A. nidulans* (5). Propionate also inhibits the biosynthesis of polyketides, including the conidial pigment, via the formation of propionyl-CoA (59, 60). Therefore, it is likely that the effects of fluoroacetate on asexual and sexual development are mainly due to inhibition of ACL activity by fluoroacetyl-CoA acting in a similar way to propionyl-CoA. The lack of growth inhibition by low

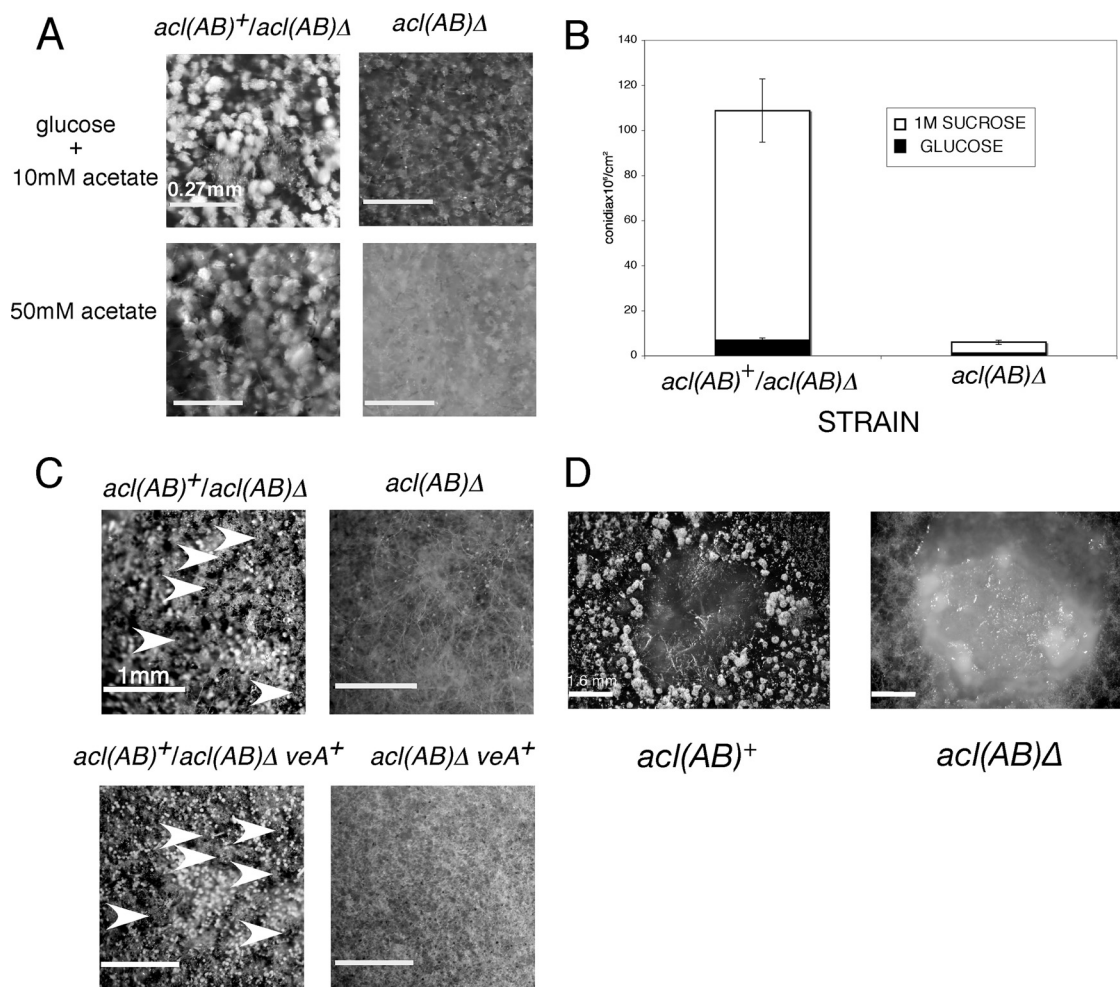


FIG. 5. Effects of deletion of *aclA* and *aclB* on conidiation and sexual development. (A) Reduced conidiation in the the *acl(AB)Δ* strain compared to the complemented strain. (B) Viable conidial counts of the *acl(AB)Δ* strain compared to the complemented strain. One molar sucrose increased the number of conidia for both strains: 7-fold for *acl(AB)Δ* and 20-fold for the complemented strain. Acetate at 10 mM was present in all media. (C) Loss of sexual development in the *acl(AB)Δ* strain. White arrowheads indicate mature cleistothecia. Sexual development was not restored in the *veA⁺* background. Selfed crosses were set up on 1% glucose medium containing 10 mM acetate and allowed to develop for 14 days. (D) Sexual development was not restored by oleate-Tween 80. Conidia of each strain were streaked on thick crossing plates containing 1% glucose and 10 mM acetate and incubated for 1 day, and then a drop of 1 M oleate mixed with a drop of Tween 80 was added to the plates. After a further incubation for 1 day, plates were sealed and incubated for 10 days. A dense ring of mature cleistothecia formed around the oleate in the wild-type strain but not in the *acl(AB)Δ* strain.

levels of fluoroacetate provides support for the hypothesis that higher levels of ACL activity are required for development than for hyphal growth. In addition it is possible that fluoroacetyl-CoA inhibits other enzyme activities that use acetyl-CoA as a substrate and that are involved in development.

DISCUSSION

Deletion of the *acl* genes results in loss of growth of *A. nidulans* in the absence of external sources of cytoplasmic acetyl-CoA, strongly suggesting that ACL activity is required to generate cytoplasmic acetyl-CoA. In contrast, *S. cerevisiae* and *C. albicans* lack ACL genes, and cytoplasmic ACS activity is essential (6, 47).

A source of cytoplasmic acetyl-CoA is required for fatty acid and sterol biosynthesis. In *A. nidulans* the gene *accA*, encoding

acetyl-CoA carboxylase, which is necessary for the conversion of acetyl-CoA to malonyl-CoA (and therefore for fatty acid biosynthesis and chain elongation), has been isolated, but attempts to delete the gene were unsuccessful (30). Furthermore, the ACC-specific inhibitor soraphen A inhibited growth even in the presence of added fatty acids, indicating that this enzyme is essential (30). Acetyl-CoA is a substrate for acetoacetyl-CoA thiolase (EC 2.3.1.9) and also for the subsequent synthesis of 3-hydroxy-3-methyl-glutaryl-CoA in the mevalonate-ergosterol biosynthetic pathway. Loss of or reduced acetylation of proteins, including histones, is likely to have significant effects on normal growth (see the introduction). It will be of interest to determine whether ACL is present in the nucleus for histone acetylation, as in mammalian cells (56), and to investigate changes in the patterns of global protein acetylation during growth on different carbon sources.

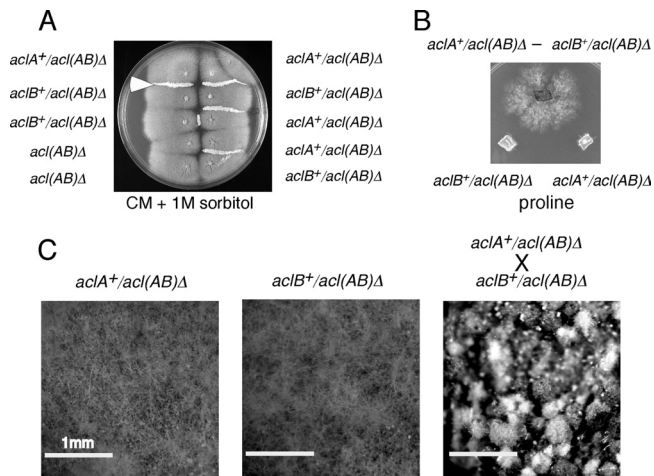


FIG. 6. Complementation between *aclA* Δ and *aclB* Δ . (A) Complementation for conidiation in heterokaryons. Strains were inoculated adjacent to each other on complete medium containing 1 M sorbitol and 10 mM acetate and incubated for 4 days. Strongly conidiating heterokaryotic mycelia were formed only when *aclA*⁺ and *aclB*⁺ strains were adjacent (an example is by the white arrow). (B) Complementation for growth on proline in a heterokaryon. An *aclB*⁺/*acl(AB)* Δ *nii44* strain was isolated by crossing and a heterokaryon with *aclA*⁺/*acl(AB)* Δ established by repeated transfers of mycelium to glucose minimal medium containing 10 mM acetate. An agar plug of the heterokaryon together with control agar plugs of *aclA*⁺/*acl(AB)* Δ and *aclB*⁺/*acl(AB)* Δ were transferred to medium containing 50 mM proline as the carbon source. Complementation was observed as strong growth on proline after 4 days of incubation. (C) Complementation for sexual development. A cross between the *aclB*⁺/*acl(AB)* Δ *nii44* strain and *aclA*⁺/*acl(AB)* Δ was set up on 1% glucose minimal medium with sodium nitrate as the sole nitrogen source and 10 mM acetate. Conidia and mature cleistothecia developed after 10 days of incubation, while few conidia and no cleistothecia were produced in the selfed deletion strains.

Our results are in contrast with the properties of an *S. macrospora* mutant isolated based on a defect in sexual development and found to have a mutation in one of the ACL-encoding genes (34, 36). This mutation did not affect vegetative growth. This could result from media differences or residual ACL activity (about 4% of wild type). A more interesting possibility is that sufficient acetyl-CoA is formed by the pyruvate-acetaldehyde-acetate pathway during growth on glucose. In *Neurospora crassa* significant levels of ethanol are formed even during aerobic growth on glucose, and expression of the genes for pyruvate decarboxylase and one of the alcohol dehydrogenases is higher in glucose medium compared to carbon-starved conditions (58). It is likely therefore that some of the acetaldehyde formed from pyruvate may be converted to acetate by basal levels of aldehyde dehydrogenase and then to acetyl-CoA by ACS. This pathway may operate in the closely related *S. macrospora* (35). This could be tested by determining whether the *S. macrospora* ACL-deficient mutant is affected in growth on carbon sources such as glutamate and proline, where the production of pyruvate by glycolysis cannot occur.

The FacA-dependent acetate repair of the growth of the *acl* deletants even in the presence of glucose and independent of FacB is consistent with recent observations in *A. niger* (29). Apparently there is sufficient constitutive ACS present to allow acetyl-CoA formation when acetate is present. This may be a scavenging mechanism when small amounts of acetate are present, although no energy is saved, since one molecule of ATP is consumed by both ACL and ACS reactions. The inability of the *acl* deletants to grow on quinate, which we propose results in the formation of acetyl-CoA in the mitochondrion, is in accord with a separation of mitochondrial and cytoplasmic acetyl-CoA pools. However, citrate synthesized in the mitochondrion must be able to enter the cytoplasm for ACL activity to occur, and investigations of the mechanisms involved in mitochondrial-cytoplasmic shuttling are needed. The ability of the *acl* deletion strains to grow to some extent on

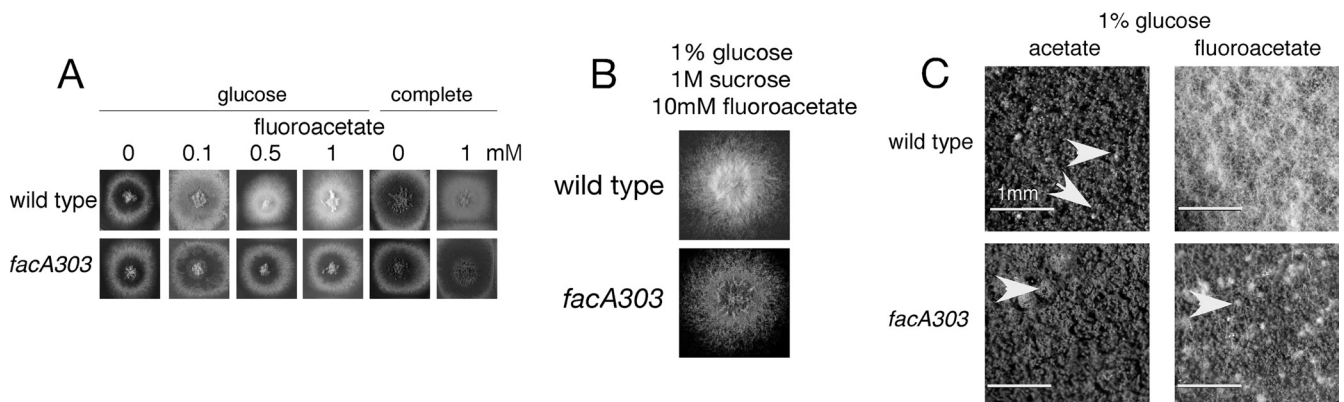


FIG. 7. Inhibition of conidiation and sexual development in the presence of fluoroacetate. (A) Inhibition of conidiation. The indicated amounts of fluoroacetate were added to 1% glucose minimal medium with 10 mM ammonium tartrate as the nitrogen source or to complete medium, and strains were inoculated and grown for 2 days. Fluoroacetate inhibited conidiation and conidial pigment formation in the wild-type strain but not in *facA303*. (B) Osmotic remediation of the effects of fluoroacetate on conidiation. Colonies were inoculated on glucose medium containing 1 M sucrose and 10 mM fluoroacetate and incubated for 2 days. Conidiation was not inhibited, although conidia lacked pigment in the wild-type strain, but not in the *facA303* strain. (C) Inhibition of sexual development by fluoroacetate. Strains were streaked onto 1% glucose minimal medium with 10 mM ammonium tartrate as the nitrogen source, plates were taped to exclude air after 2 days of incubation, and cultures were incubated for an additional 10 days. Either acetate or fluoroacetate was present at 50 mM. Development of cleistothecia (white arrows) and conidia was inhibited by fluoroacetate in the wild type but not the *facA303* strain.

the fatty acids butyrate and oleate indicates that peroxisomal and cytoplasmic pools of acetyl-CoA are not completely separate. This is in accord with our previous observation that mislocalization of the glyoxylate cycle enzyme malate synthase to the cytoplasm does not affect growth on fatty acids (20). It is of interest that loss of FacC, the cytoplasmic carnitine acetyltransferase, does not prevent growth of the *acl(AB)*Δ on butyrate or oleate. This shows that some acetyl-CoA can pass through the peroxisomal membrane and does not require conversion to acetyl-carnitine, as previously suggested for *S. cerevisiae* (53).

Loss of ACL results in greatly reduced production of conidia, even with the provision of acetate as a source of acetyl-CoA. At least one reason for this could be insufficient levels of the siderophores necessary for iron import and intracellular transport required for conidiation (12, 13, 54). The production of both triacetylfusarinine C and ferricrocin requires mevalonate synthesis, and there are also acetyl-CoA-dependent steps in their respective pathways (12). A complete loss of sexual development was found in *acl* deletion strains, with none of the specialized cell types that occur under inducing conditions being observed. Fatty acid-derived oxylipins are key signaling molecules for development (51). However, it is unlikely that this is the only reason for a requirement for ACL, because oleate did not induce cleistothecial formation in the *acl(AB)*Δ mutant. Siderophores are important for cleistothecial but not Hulle cell development (13), and the synthesis of polyketide secondary metabolites is important for fruiting body formation in *N. crassa* and *S. macrospora* (33). Clearly a number of acetyl-CoA-dependent pathways are essential in *A. nidulans*. The isolated *acl* mutant of *S. macrospora* is not completely deficient in sexual differentiation but only affects later stages (36). As discussed above, this may arise from residual activity in the mutant combined with some acetyl-CoA production from acetate allowing limited development, or it may reflect a real difference in developmental controlling molecules. The phenotype of *acl* gene deletions would be interesting to determine.

In *S. macrospora* ACL activity has been found to be present constitutively in medium used for promoting fruiting. However, activity increases at 48 h, corresponding to the initiation of perithecial development, and manipulation of mycelial density to delay the timing of initiation also delayed the increase in activity. This correlated with protein levels and transcription and strongly indicates developmental control of *acl* gene expression (36). We have demonstrated metabolic regulation of *acl* gene expression in *A. nidulans* with strong repression resulting from growth on sources of acetyl-CoA. The molecular basis of this novel control mechanism is entirely unknown and warrants further detailed study. Evidence for one or more developmental controls is provided by the observation that low levels of fluoroacetate inhibit conidiation and sexual development without greatly affecting growth on glucose and that conidiation is reduced in the *acl(AB)*Δ mutant even during growth on acetate. Divergent transcription of adjacent *acl* genes is highly conserved in the *Pezizomycotina*. This may reflect a need for strict coordinate regulation by *cis*-acting bidirectional regulatory sequences to prevent undesirable metabolic effects of unbalanced synthesis of the individual subunits. We have shown that the *acl* genes do not have to be adjacent to

function. Deletion analyses of the intergenic region aimed at determining the extent to which metabolic and developmental regulation during both asexual and sexual reproduction are separable will be of great interest. Our results emphasize the need for fungi to alter their central metabolic pathways during the differentiation of reproductive cell types.

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