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-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 (PDC1, 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 these 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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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 was shown to be a major source of acetyl-CoA for histone acetylation and lipid accumulation in adipocytes (56). Silencing 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 sequences of all sequenced yeasts (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 contains 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-

![FIG. 1. Relevant metabolic pathways for acetyl-CoA generation.](image-url)

**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).
memonic 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 Pezizomycotina 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 *Saccharomyces*.

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-acetyldehyde-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 ScIa (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 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 axotrophic strains to grow. All strains were derived from the original Glasgow strain and contained the veA1 mutation unless otherwise indicated. The *facA303*, *acaH253*, *facC*, and *facB101* mutations have been described elsewhere (3, 46, 49). The *acaB* deletion 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 *acaB* 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 *acaBΔ* strain was grown in 0.1% glucose minimal medium containing 50 mM acetate for the preparation of protoplasts. Recipient strains contained *nkucA* 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/regew/orthogroups/). Analysis of potential mi-

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**Table 1. Strains used in this study**

| Strain no. | Name | Genotype* |
|------------|------|-----------|
| 54         | Wild type | biA1 nkuA pyroA4 nkuA::argB+ riboB2 |
| 11036      | TNO2A21 | pyroA4 nkuA::argB+ acl(AB):bar nkuA riboB2 |
| 12593      | acl(AB)Δ (original) | acl(AB):bar nkuA |
| 12410      | acl(AB)Δ | acl(AB):bar nkuA |
| 12408      | acl(AB)Δ (empty vector) | acl(AB):bar nkuA |
| 12404      | acuAΔ/acl(AB)Δ | acl(AB):bar nkuA |
| 12406      | acuB+/(acl(AB)Δ | acl(AB):bar nkuA |
| 12400      | acl(AB)+/(acl(AB)Δ | acl(AB):bar nkuA |
| 12438      | acl(AB)Δ veA4+ | biA1 acl(AB):bar veA+ nkuA |
| 10593      | facA303 | biA1 pyroA4 facA303 nkuA |
| 12445      | acuΔ acl(AB)Δ | biA1 acu::pyrG+ pyroA4 acl(AB):bar riboB2 |
| 12436      | acaH253 acl(AB)Δ | pabaK2 acaH253 acl(AB):bar |
| 1231      | facC acl(AB)Δ | acl(AB):bar facC::argB+ riboB2 |
| 12421      | facB101 acl(AB)Δ | acl(AB):bar facB101 riboB2 |

*The presence of nkuA::argB+ is not known for all strains. veA4 represents a disruption of the veA locus as described in Fig. 3. All strains with the exception of 12438 contained the veA1 mutation.*
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 glucosamine resistance, to generate the acl(A)Δ strain (Fig. 3B). Initially, transformants selected on protoplast medium (1% glucose–1 M sucrose) containing glucosamine 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(A)Δ strain, selecting for RiboB+ transformants, and screened for white conidia. In this way acl(A)+/acl(A)Δ, aclA+/acl(A)Δ, and aclB+/acl(A)Δ strains were isolated. Complementation of acl(A)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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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.
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-ketoadipyl-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-ketoadipyl-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)$\Delta$ were fertile in heterozygous crosses, yielding acl(AB)$\Delta$ progeny readily identified by a requirement for acetate and poor conidiation. We were unable to isolate acl(AB)$\Delta$ facA double mutants. In crosses to a strain containing the facA303 loss-of-function mutation (3), all acl(AB)$\Delta$ 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)$\Delta$, because acl(AB)$\Delta$ 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)$\Delta$ with loss-of-function mutations were isolated from crosses and tested for effects on acetate growth and proline media (Fig. 4B). In this way it was found that loss of FacC, the mitochondrial 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)$\Delta$. 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)$\Delta$-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 all strains of acl(AB)$\Delta$ 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)$\Delta$ 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 Pezizymycotina (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 fluorocetyl-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, conidal 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 fluorocetyl-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 conidal 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 fluorocetyl-CoA acting in a similar way to propionyl-CoA. The lack of growth inhibition by low
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 fluoroacetate 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.
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...
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 triacylcarnitine 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 Hule cell development (13), and the synthesis of polypeptide 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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