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Physiological characterization of ATP-citrate lyase in *Aspergillus niger*

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Abstract Acetyl-CoA, an important molecule in cellular metabolism, is generated in multiple subcellular compartments and mainly used for energy production, biosynthesis of a diverse set of molecules, and protein acetylation. In eukaryotes, cytosolic acetyl-CoA is derived mainly from the conversion of citrate and CoA by ATP-citrate lyase. Here, we describe the targeted deletions of *acl1* and *acl2*, two tandem divergently transcribed genes encoding subunits of ATP-citrate lyase in *Aspergillus niger*. We show that loss of *acl1* or/and *acl2* results in a significant decrease of acetyl-CoA and citric acid levels in these mutants, concomitant with diminished vegetative growth, decreased pigmentation, reduced asexual conidiogenesis, and delayed conidial germination. Exogenous addition of acetate repaired the defects of *acl*-deficient strains in growth and conidial germination but not pigmentation and conidiogenesis. We demonstrate that both Acl1 and Acl2 subunits are required to form a functional ATP-citrate lyase in *A. niger*. First, deletion of *acl1* or/and *acl2* resulted in similar defects in growth and development. Second, enzyme activity assays revealed that loss of either *acl1* or *acl2* gene resulted in loss of ATP-citrate lyase activity. Third, in vitro enzyme assays using bacterially expressed 6His-tagged Acl protein revealed that only the complex of Acl1 and Acl2 showed ATP-citrate lyase activity, no enzyme activities were detected with the individual protein. Fourth, EGFP-Acl1 and mCherry-Acl2 proteins were co-localized in the cytosol. Thus, *acl1* and *acl2* coordinately modulate the cytoplasmic acetyl-CoA levels to regulate growth, development, and citric acid synthesis in *A. niger*.

Keywords ATP-citrate lyase · Acetyl-CoA · Vegetative growth · Asexual conidiogenesis

Introduction

Acetyl-CoA, or activated acetate, is an important molecule in cellular metabolism, mainly used for energy production, biosynthesis of a diverse set of molecules, and protein acetylation [1, 5]. Appropriate levels of this intermediate metabolite must be present in the correct cellular compartment for normal growth and development. It has been well documented that acetyl-CoA is generated via three different mechanisms in nonphotosynthetic eukaryotic cell [8, 27]. In mitochondria, pyruvate dehydrogenase complex catalyzes the conversion of pyruvate into acetyl-CoA, which is subsequently incorporated into the TCA cycle and oxidized for energy production. Peroxisomal acetyl-CoA is generated during fatty acid beta-oxidation and then transported to the mitochondria for complete oxidation. In cytoplasm, acetyl-CoA synthetase (ACS) and ATP-citrate lyase (ACL) convert acetate and citric acid to acetyl-CoA, respectively, which is used as a precursor in fatty acids and mevalonate synthesis pathways. It has been reported recently that both ACS and ACL are also involved in the synthesis of acetyl-CoA in the nuclei, thus providing acyl groups for histone acetylation [27–29]. Such compartmentalized and distinct

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acetyl-CoA-generating systems would enable the organism to precisely regulate the supply of acetyl-CoA to different metabolic pathways.

ATP-citrate lyase is mainly a cytosolic enzyme that catalyzes the conversion of citrate and CoA into acetyl-CoA and oxaloacetate, along with the hydrolysis of ATP [5]. ACL is ubiquitous and presents in animals, plants, fungi, algae, and a few prokaryotes, but is notably absent from yeasts, which do not accumulate a significant amount of lipid. Animal ACLY is a homotetramer of 110–120-kDa subunits encoded by a single gene [7]. The expression of mammalian ACLY is mainly regulated by the transcription factor SREBP-1 [13, 16, 23]. The phosphorylation on The446, Ser450, and Ser454 residues contributes to ACLY stability [13, 16, 23]. It was reported that animal ACLY plays important roles in cell growth and transformation [3]. Alterations in its expressions or activity have been associated with several pathological conditions. In contrast, *Arabidopsis* ACL is structurally different from animal ACLY, composed of two distinct subunits, ACLA (45 kD) and ACLB (65 kD), probably in an A_4B_4 stoichiometry [8, 9]. In the *Arabidopsis* genome, three genes encode the ACLA subunit and two genes encode the ACLB subunit [9]. In situ hybridizations revealed that the mRNAs of ACLA and ACLB were coordinately accumulated in spatial and temporal patterns, consistent with the predicted physiological needs for cytosolic acetyl-CoA during plant development [8]. In fungi, ACL activity was identified decades ago [24], but the connections between the enzyme activity and gene function were not established until the rescue of *Sordaria macrospora* mutant per5 with a cosmid containing an ACL-like open reading frame [20]. Further investigation with *S. macrospora* revealed that its genome contains two adjacent ACL-like genes (*acl1* and *acl2*) encoding two polypeptides with homology to the N- and C-terminal parts of the animal ACLY polypeptide [19]. Functional characterization of fungal *acl1* and *acl2* indicates that these genes are involved in regulation of cell growth and development [12, 19]. Interestingly, mammalian ACLY was found in the nuclei, where it regulated histone acetylation by modulating the production of acetyl-CoA during adipocyte differentiation [29]. Recently, Acl1 and Acl2 proteins were observed in both the cytosol and nuclei in the pathogenic fungus *Gibberella zeae* [27]. Deletion of *acl1* and *acl2* resulted in reduced histone acetylation, demonstrating an important role of ACL in fungal epigenetics [27].

Aspergillus niger is an economically important filamentous fungus used for the production of chemicals and enzymes, serving as a model fungal fermentation process [2]. Currently, the complete genome sequences of three different strains of *A. niger* have been released, which enabled the prediction of gene functions and enriched our understanding of the biology of this species [2, 22]. However, a detailed genetic and biochemical analysis of individual genes is still required to assess their roles in overall cellular functions. Accumulating evidence suggests that genes involved in central carbon metabolism play important roles in the high productivity of *A. niger*. It was reported that the *A. niger acl* gene regulates succinic acid production [18]. However, whether *A. niger acl* plays an important role in the regulation of growth and development has yet to be addressed.

In this article, we report the deletion of *acl1* and *acl2* genes encoding two subunits of ATP-citrate lyase in *A. niger* using vectors harboring a selectable marker and a flanking marker for transformant screen. Characterization of *acl1* or/and *acl2*-deficient mutants reveals that these two genes are required for vegetative growth, pigmentation, asexual conidiogenesis, conidial germination, acetyl-CoA, and citric acid production. Our phenotypic characterization, enzyme activity assay, and protein subcellular location analysis suggest that both Acl1 and Acl2 subunits are required to form a functional ATP-citrate lyase complex in *A. niger*.

Materials and methods

Strains and culture conditions

The *A. niger* wild-type strain ATCC1015 obtained from Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences (Tianjin, China) was used to generate the gene deletion and overexpression strains. All the wild-type and mutant strains generated in this study are listed in supplementary material Table S1 and their relevant characteristics are described. For culture maintenance and conidiation, wild-type and mutant strains were grown on potato dextrose agar (PDA), potato dextrose broth (PDB), solid or liquid complete medium (CM) at 30 °C supplemented with 5 mM acetate when necessary as described [6]. *A. niger* transformants were cultured on CM containing 5 mM acetate, 200 μM cefotaxime, 100 μg/ml ampicillin, 100 μg/ml streptomycin, 250 μg/ml hygromycin B, or/and 10 μg/ml bleomycin for *acl* mutant screen based on the methods described previously [6, 25]. Conidia were harvested by scraping the biomass grown on PDA plates, re-suspended in sterile water, and purified by passing through Microcloth (Calbiochem, San Diego, CA, USA). Mycelia used for genomic DNA, total RNA, and total protein extraction were harvested from cultures grown in liquid CM at 30 °C for 2 days.

Escherichia coli DH5α cells and BL21 (DE3) cells used for molecular cloning and fusion protein expression, respectively, were both grown at 37 °C in Luria–Bertani medium (LB) supplemented with or without 100 μg/ml

kanamycin or 100 μg/ml ampicillin as described [15]. *Agrobacterium tumefaciens* AGL1 was grown on LB supplemented with 100 μg/ml kanamycin when required [15].

Cloning of the cDNA of *acl1* and *acl2*, and generation of *acl1*^{H305A} point mutation

Total RNA was prepared from wild-type *A. niger* using the TRIzol method as described [4, 6]. The RNA samples were treated with DNase at 37 °C for 30 min to clear the genomic DNA. First-strand cDNA synthesis was carried out with a PrimeScript RT Reagent Kit (TaKaRa, Mountain View, CA, USA). Two microliters of the cDNA mixture was used for PCR amplification of *acl1* and *acl2* genes with primer pairs *acl1*-cDNA-F/*acl1*-cDNA-R and *acl2*-cDNA-F/*acl2*-cDNA-R, respectively. The PCR products were separated by 1 % agarose gel electrophoresis, and subsequently excised from the gel and purified with Gel Extraction Kit (Qiagen, Hilden, Germany). The resultant *acl1* and *acl2* products were then digested and ligated into vector pIJ2925 to obtain pIJ2925-*acl1* and pIJ2925-*acl2*, respectively. The fidelity of cloned *acl1* and *acl2* genes were confirmed by sequencing.

For creating *acl1*^{H305A} point mutation, we introduced the desired mutations on the primers, which were used for PCR amplification as described [15]. Briefly, using pIJ2925-*acl1* as template, two fragments were PCR amplified with primer pairs *acl1*-cDNA-F/*acl1*-HA-R and *acl1*-HA-F/*acl1*-cDNA-R, respectively. The resultant PCR products were joined by splice overlap extension (SOE) PCR with primer pair *acl1*-cDNA-F/*acl1*-cDNA-R, which was purified by agarose gel electrophoresis and ligated into pIJ2925 to obtain pIJ2925-*acl1*^{H305A}. The introduction of the desired point mutation on *acl1* was confirmed by sequencing. The primers used in each instance in this study are listed in supplementary material Table S2.

Generation of *acl* overexpression strains

pLH41 and pLH43, which express EGFP and mCherry protein, respectively, were used to construct EGFP-Acl1 and mCherry-Acl2 fusion protein expression vectors. In brief, using pIJ2925-*acl1* as template, the *acl1* gene was PCR amplified with primer pair *acl1*-F/*acl1*-R, and the resultant PCR product was digested and ligated into pLH41 to obtain pLH41-*acl1*. Using pIJ2925-*acl2* as template, the *acl2* gene was PCR amplified with primer pair *acl2*-F/*acl2*-R, and the resultant PCR product was digested and ligated into pLH43 to obtain pLH43-*acl2*. The fidelity of cloned *acl1* and *acl2* was confirmed by sequencing. Using the *Agrobacterium*-mediated transformation method as described [6, 25], pLH41-*acl1* and pLH43-*acl2* were introduced into wild-type *A. niger* to obtain transformants that express an extra copy of *acl1* and *acl2*. The expression of EGFP-Acl1 or/and mCherry-Acl2 fusion proteins was confirmed by confocal microscopic observation of red and green fluorescence. Plasmids pLH41 and pLH43 are derived from pFGL59 [15], and their physical maps are shown in supplementary material Fig. S1. The primers used are listed in supplementary material Table S2, and the restriction enzyme sites are underlined.

Acl deletion and complementation

The *acl2*-deletion mutant was generated using the standard one-step gene replacement strategy as described [6, 15, 25]. Briefly, about 1 kb of upstream and downstream regions of *acl2* gene were PCR amplified with primer pairs *acl2*L-F/*acl2*L-R and *acl2*R-F/*acl2*R-R, respectively, and digested and ligated sequentially to flank the hygromycin resistance cassette in pFGL59Ble to obtain gene replacement construct pFGL59Ble-*acl2*. The pFGL59Ble-*acl2* was introduced into *A. niger* via *Agrobacterium*-mediated transformation [6, 25]. CM containing 5 mM acetate and 250 mg/ml hygromycin or/and 10 μg/ml bleomycin was used for selection. Correct gene replacement event was confirmed by PCR analysis and sequencing. The same strategy as used for the *acl2* deletion was followed to construct vectors pFGL59Hyg-*acl1* and pFGL59Ble-*acl1acl2*, and generate *Δacl1* single mutants and *Δacl1Δacl2* double mutants. The plasmids pLH41-*acl1*, pLH41-*acl1*^{H305A}, and pLH43-*acl2* expression cassettes were introduced into the corresponding mutants to perform rescue assays. The plasmids pFGL59Ble and pFGL59Hyg are derived from pFGL59, and their physical maps are shown in supplementary material Fig. S1.

Expression and purification of 6His-Acl protein

A standard protocol was followed to express and purify 6His-Acl proteins [11]. Briefly, using pIJ2925-*acl1* as template, the *acl1* gene was PCR amplified with primer pair *acl1*-F-*Nde*I/*acl1*-R-*Eco*RI, and the resultant PCR products were digested and ligated into pET28a(+) to obtain pET28a-*acl1*. *E. coli* BL21 (DE3) was transformed with the vector pET28a-*acl1*, and the protein expression was induced by 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG) at 20 °C for 18 h. The cultures were chilled on ice and the cell pellet was harvested by centrifugation at 12,000 rpm at 4 °C for 10 min, and then re-suspended in 1 × Ni–NTA buffer (50 mM NaH_2PO_4, 500 mM NaCl, 5 mM imidazole, 10 % glycerin, pH 8.9). The cell suspension was sonicated (4 s each with 6 s cooling between successive bursts in 20 min). The resulting lysate was centrifuged at 12,000 rpm at 4 °C for 20 min. One milliliter of the supernatant was applied onto a 10-ml column filled

with 1.5 ml of His-select Ni-Chelating affinity gel (Qiagen, Hilden, Germany) pre-conditioned with 1 × Ni–NTA buffer. The elution was performed with 5 ml of 1 × bind buffer containing 50, 100, 300, and 500 mM imidazole sequentially. The same strategy as used for the 6His-Acl1 expression and purification was followed to produce 6His-Acl1^{H305A} and -Acl2 proteins. The purity of recombinant proteins was analyzed by SDS-PAGE, and the concentration was determined by the Bradford protein assays (Bio-Rad Laboratories, Hercules, CA, USA).

ACL enzyme activity assay

ACL enzyme activities were determined using a malate dehydrogenase (MDH)-coupled assay system as described [14]. In brief, a certain amount of protein (6His-Acl1, 6His-Acl1^{H305A}, His-Acl2, the lysate of wild-type, *Δacl1*, *Δacl2* or *Δacl1Δ acl2* stains) was added into the mixture consisting of 200 mM Tris–HCl (pH 8.4), 20 mM sodium citrate, 5 U/ml malate dehydrogenase, 10 mM $MgCl_2$, 10 mM DTT, 0.25 μM NADH, and 10 mM ATP. The reaction was started by adding 0.2 mM CoA and incubated at 37 °C. The decrease in absorption at 340 nm was measured.

Acetyl-CoA assay

The acetyl-CoA levels in respective strains were determined using Acetyl-Coenzyme A Assay kit (Sigma-Aldrich, St Louis, MO, USA). Wild-type, *acl* deletion, and overexpression strains were grown in PDA medium supplemented with 5 mM acetate for 3 days to obtain sufficient cell mass, and then shifted to PDA medium and cultured for another 2 days. One hundred milligrams of mycelia of each strain was ground in liquid nitrogen and re-suspended in 100 μl 1 M $HClO_4$ buffer. After centrifugation at 13,000 rpm at 4 °C for 10 min, the supernatant was collected and pH was adjusted with 3 M $KHCO_3$ to pH 7. Fifty microliters of each sample was used for acetyl-CoA assay as directed by the manual.

Citric acid assay

Ten million conidia of wild-type, *acl*-deletion, and -overexpression strains were grown in PDA medium supplemented with 5 mM acetate for 3 days, then shifted to shake flasks containing fermentation medium (10 % glucose, 0.25 % NH_4NO_3, 0.1 % $MgSO_4{\cdot}7H_2O$, 0.1 % KH_2PO_4, and 0.05 % yeast extract), and cultured at 30 °C at 200 rpm for another 5 days. Citric acid in the fermentation broth was examined with high-performance liquid chromatography (HPLC, column: Aminex HPX-87H, 300 mm × 7.8 mm) at wavelength 210 nm [21].

Chemicals and antibiotics

Ethanol, glycerol, succinic acid, malic acid, fumaric acid, 2-ketoglutaric acid, citric acid pyruvic acid, fructose, xylose, rhamnose, lactose, sucrose, maltose, raffinose, and sorbitol were from Sangon Biotech (Shanghai, China). Hygromycin B, bleomycin, kanamycin, ampicillin, 4′,6-diamidino-2-phenylindole (DAPI) and IPTG were from Sigma-Aldrich (St. Louis, MO, USA).

Confocal microscopy

The mycelium of *A. niger* strains over-expressing EFGP-Acl1 and mCherry-Acl2 was used for confocal microscopic observation. Images were captured using a confocal laser scanning microscope (Leica, Heidelberg, Germany). DAPI was used to stain the nuclei.

Results

Efficient deletions of *acl1* and *acl2* in *A. niger* with a flanking marker

ANI_1_76094 and *ANI_1_78094*, designed as *acl1* and *acl2*, are two tandem divergently transcribed genes interrupted by a 2,686-bp of non-coding sequence in *A. niger* chromosome (Fig. 1a). cDNAs cloned from *acl1* and *acl2* loci contain an ORF encoding polypeptides with 656 aa and 485 aa in length, respectively. Acl1 is similar to C-terminus of human ACLY polypeptides, while Acl2 is similar to the N-terminus (Fig. 1b). The His760 residue required for ACLY activity is conserved in Acl1(His305), while the Thr446, Ser450, and Ser454 residues involved in phosphorylation and regulation of ACLY stability are not retained in Acl1 or Acl2 (Fig. 1b). To address the physiological functions of *acl1* and *acl2* in *A. niger*, we deleted these two genes using a newly developed strategy for highly efficient screening of gene replacement events. Briefly, we introduced bleomycin resistance as a flanking marker next to the disrupted plasmid-borne copy of *acl1* and *acl2* genes (Fig. 1c). In this case, double-crossover homologous recombinants will express HPH used to disrupt the target gene but not the bleomycin resistance flanking marker. To this end, we screened the individual strains obtained by *Agrobacterium*-mediated transformation and identified six isolates with proper gene replacement out of 78 primary transformants for *acl1*, four isolates out of 52 for *acl2*, and five isolates out of 81 for *acl1/acl2* double mutants. The PCR-based selection of T7, an *acl2* gene-replacement event, is shown in Fig. 1d.

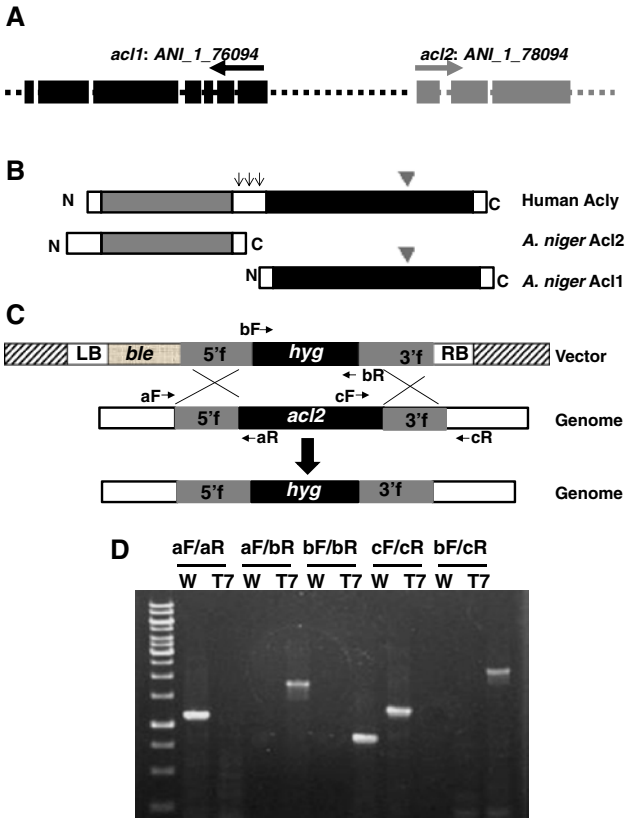


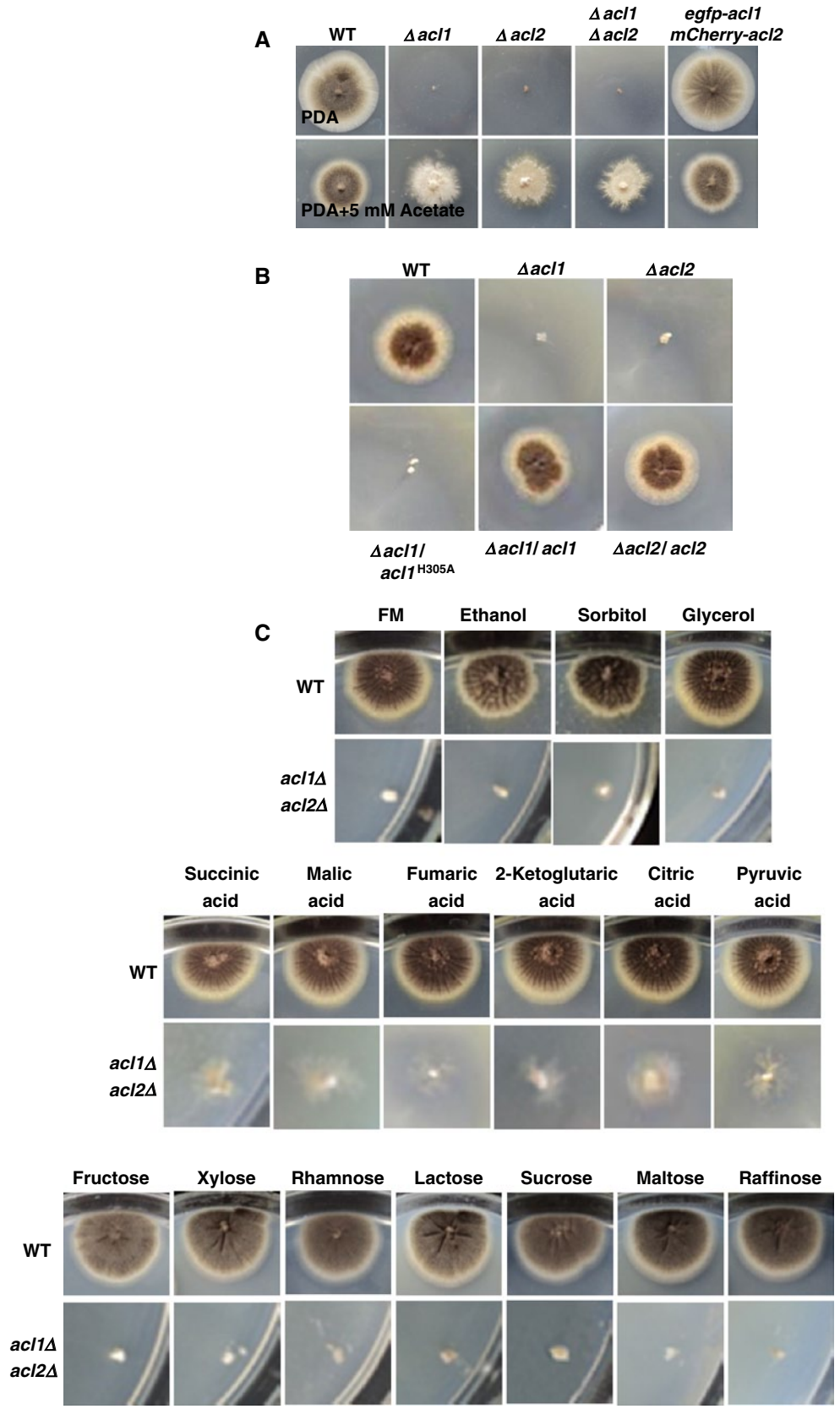
Fig. 1 Generation of *acl*-deletion strains in *A. niger*. **a** Schematic representation of *acl1* and *acl2* loci in the wild-type strain. *Closed bars* indicate the coding regions. *Arrows* represent the transcription direction of the *acl1* and *acl2* genes. **b** Homologies between human and *A. niger* ACL polypeptides. Homologous regions of human ACLY polypeptides, and *A. niger* Ac1 and Acl2 polypeptides are shaded in *black and grey*, respectively. The conserved histidine in catalytic centre is labeled with an *arrowhead*. Three phosphorylation sites in human ACLY are labeled with *arrows*. **c** Outline of *acl2* gene deletion in *A. niger*. 5′f and 3′f represent the upstream and downstream flanking sequences of *acl2* gene. *RB* and *LB* represent the right and left border sequences, respectively, of T-DNA. *ble* and *hyg* represent the bleomycin and hygromycin-resistant genes, respectively. *Arrows* represent the loci of primers (aF, aR, bF, bR, cF, and cR) on the vector and *A. niger* genome. **d** PCR confirmation of the deletion of *acl2* gene. Genomic DNA of wild type and transformant 7 (T7) were used for PCR assay

Phenotype characterization of *acl1* and *acl2* gene-deletion strains

ATP-citrate lyase performs quite important functions in variant species [5, 10, 12]. Here, we found that deletion of *acl1* or/and *acl2* caused severe growth defects in *A. niger* grown on PDA medium, in which glucose is the main source of carbon. The addition of 5 mM acetate into PDA medium repaired the growth defects of these gene-deficient strains (Fig. 2a). Interestingly, in this condition, *acl1* or/and *acl2* mutants grew well, but they lost the pigment-producing ability. The mycelium of wild type was dark while *acl1* or/and *acl2* mutants produced white color mycelium in the presence of acetate (Fig. 2a). Introduction of *acl1* and *acl2* expression cassette into *Δacl1* and *Δacl2* mutants, respectively, rescued their defects, suggesting that loss of *acl1* or *acl2* contributed to the defects observed in the mutants (Fig. 2b). Significantly, expression of $Acl1^{H305A}$, in which the histidine required for enzyme activity was replaced by alanine failed to rescue the defects of *Δacl1* mutants, further confirming the role of *acl1* in vegetative growth in *A. niger* (Fig. 2b). The growth profiles of *Δacl1Δacl2* stains were further investigated by comparing the wild type and the mutants grown on variant fermentation conditions in which glucose was replaced by different alcohols, fatty acids, and sugars (Fig. 2c). The addition of sorbitol slightly repaired the growth defect of *Δacl1Δacl2* mutants. Surprisingly, the addition of ethanol failed to repair the growth defect of *acl*-deficient *A. niger* stains (Fig. 2c), which is different from the observations in *acl*-deletion *A. nidulans* stains [12, 19]. Interestingly, growth of *Δacl1Δacl2* mutants was significantly improved in the presence of TCA cycle intermediates. In contrast, growth of *Δacl1Δacl2* mutants was poor on media containing different sugars as carbon sources.

Few conidia were observed on the surface of aerial hyphae of *Δacl1* and *Δacl2* mutants grown on PDA medium (Fig. 3a). Even in the presence of 5 mM or higher concentration of acetate, these mutants produced approximately fivefold less numbers of conidia than the wild type (Fig. 3a, b). Promoters of *glaA* from *A. niger* and *gdpA* from *A. nidulans* have been used for protein expression in *A. niger* and other filamentous fungi [26]. Here, we constructed *acl* overexpression strains by introduction of *gdpA-egfp-acl1* and *glaA-mCherry-acl2* expression cassettes into wild-type *A. niger* (Table S1, Fig. S1). Compared to the wild-type strain, overexpression of *acl1* or/and *acl2* showed no significant effects on conidiogenesis in *A. niger* either cultured on PDA or PDA supplemented with 5 nm acetate (Fig. 3a, b). Examination of conidiophore development at the structural level showed that *Δacl1Δacl2* mutants formed vesicles and fewer metulae that failed to differentiate to produce phialides (Fig. 3c), the flask-shaped projections that produce conidia. The addition of 5 mM acetate induced *Δacl1Δ acl2* mutants to produce phialides, which are different from those of wild type in that they produce fewer conidiospore (Fig. 3c).

Delayed conidial germination was observed in *Δacl1Δacl2* stains. Germ tubes were formed by the conidia of wild type after 12 h incubation, while short germ tubes were not observed in *Δacl1* and *acl2Δ* mutants until 18-h incubation. Interestingly, we observed that the conidia of *Δacl1Δacl2* stains swelled and became much larger than the conidia of the wild type during conidial germination (Fig. 3d). These defects of *Δacl1Δacl2* strains on conidial


A
WT
Δacl1
Δacl2
Δacl1 Δacl2
egfp-acl1 mCherry-acl2
PDA
PDA+5 mM Acetate
B
WT
Δacl1
Δacl2
Δacl1/ acl1H305A
Δacl1/ acl1
Δacl2/ acl2
C
FM
Ethanol
Sorbitol
Glycerol
WT
acl1Δ acl2Δ
Succinic acid
Malic acid
Fumaric acid
2-Ketoglutaric acid
Citric acid
Pyruvic acid
WT
acl1Δ acl2Δ
Fructose
Xylose
Rhamnose
Lactose
Sucrose
Maltose
Raffinose
WT
acl1Δ acl2Δ

◀ **Fig. 2** Growth profiles of *acl-deletion* strains. **a** Growth defects of *acl*-deletion strains. The indicated strains were grown on PDA and PDA supplemented with 5 mM acetate, respectively. **b** Rescue of *Δacl1* and*Δacl*2 with the expression cassette of *acl1*, *acl1*^{H305A}, and *acl2*, respectively. All strains were grown on PDA medium. **c** Wild-type and *Δacl1Δacl2* mutants were grown on fermentation media (FM) in which glucose was replaced by indicated chemicals (the concentration of ethanol, glycerol, succinic acid, malic acid, fumaric acid, 2-ketoglutaric acid, citric acid and pyruvic acid was 10 mM, and the concentration of fructose, xylose, rhamnose, lactose, sucrose, maltose, raffinose, and sorbitol was 1 %)

germination and swelling were rescued by the addition of 5 mM acetate into their conidial suspension (Fig. 3d). *Δacl1* and *Δacl2* showed the same defect in conidial germination as observed with *Δacl1Δacl2* (data not shown). Thus, phenotype characterizations of *acl1* and *acl2* gene-deficient strains suggest that these two genes regulate growth, pigmentation, phialide development, asexual spore formation, and conidial germination in *A. niger*.

Acl1 and Acl2 are required for ATP citrate lyase activity

The presence of ATP-citrate lyase activity in *A. niger* was reported previously [24]. To address whether loss of *acl1* or/and *acl2* gene(s) affects the ATP-citrate lyase activity in *A. niger*, ATP-citrate lyase activities of the parental strain and the mutants were determined using a malate dehydrogenase-coupled assay system. The cell lysate was prepared from strains grown in PDA medium supplemented with 5 mM acetate for 3 days, and the individual ATP-citrate lyase activity was examined. As shown in Fig. 4a, *Δacl1*, *Δacl2*, and *Δacl1Δacl2* strains had negligible ACL activity compared with the wild type, while *acl*-overexpression strain showed higher ACL activity than the wild type, suggesting that *acl1* and *acl2* are responsible for ACL enzyme activity in *A. niger*. We further examined the in vitro enzyme activity using bacterially expressed 6His-Acl1, -Acl1^{H305A}, and -Acl2 proteins (Fig. 4b). Activity assay indicated that only the complex of Acl1 and Acl2 had ACL activity (Fig. 4c). The individual protein or the complex of Acl1^{H305A} and Acl2 exhibited negligible activities (Fig. 4c). We conclude that both Acl1 and Acl2 subunits are required to form a functional ATP-citrate lyase in *A. niger*. Moreover, the conserved His305, which is supposed to form a phosphohistidine species during catalysis, is essential for Acl1 enzyme activity.

Compartmentation of Acl1 and Acl2

ATP-citrate lyase is generally regarded as a cytoplasmic enzyme [5]. Interestingly, this enzyme was recently found in the nuclei in both mammalian cells and filamentous fungus [27, 29]. Here, we constructed a strain expressing EGFP-Acl1 and mCherry-Acl2 to investigate the cellular localization of these fusion proteins. Microscopic observation revealed that these two proteins were colocalized in the cytoplasm with a reticular pattern (Fig. 5). We also observed the colocalization of these fusion proteins in the hyphal tip (data not shown). These suggest that Acl1 and Acl2 form a complex and principally function in the cytoplasm in *A. niger*.

Deletions of *acl1* and *acl2* affect acetyl-CoA accumulation and citric acid production

The effects of deletion or overexpression of *acl1* and *acl2* genes on acetyl-CoA synthesis were addressed. Wild-type, *acl*-deletion, and overexpression strains were grown in PDA medium supplemented with 5 mM acetate for 3 days to obtain sufficient cell mass, and then shifted to PDA medium and cultured for another 2 days. The samples for acetyl-CoA assay were prepared from cultures of 0 days, 1 day, and 2 days grown in PDA medium without addition of acetate. The acetyl-CoA levels were comparable between the wild-type and *acl* mutants at 0 h, then started to decrease both in wild-type and *acl*-deficient strains in the absence of external acetate. However, *acl*-deletion strains showed a significantly faster decline of acetyl-CoA than the wild type (Fig. 6a). Two-day culture in the absence of extracellular acetate resulted in the final concentration of acetyl-CoA 2.6, 1.3, 1.0, and 0.75 ng/mg in wild-type, *Δacl1*, *Δacl2* and *Δacl1Δacl2* strains, respectively. Moreover, *acl*-overexpression strains showed significantly higher levels of acetyl-CoA than the wild type in all culture conditions (Fig. 6a). ATP-citrate lyase catalyzes the conversion of citrate and CoA into acetyl-CoA. Interestingly, we found that deletion of *acl1* or *acl2* genes in *A. niger* nearly aborted citric acid synthesis, while overexpression of these two genes resulted in a 32 % increase of citric acid production (Fig. 6b). Our data suggest that *acl1* and *acl2* genes regulate acetyl-coA synthesis and citric acid production in *A. niger*.

Discussion

ATP-citrate lyase activity has been identified in cell-free extracts from several filamentous fungi. It is reported that activity of ACL in *A. niger* is independent of a carbon source, unlike ACL in *A. nidulans* [1, 24], where ACL activity is affected by the choice of carbon source [1, 24]. Consistent with the finding in *S. macrospora*, *G. pulicaris*, and *A. nidulans* [12, 19, 27], the adjacent genes, *acl1* and *acl2*, encode two polypeptides with homology to the N- and C-terminal parts of the animal ATP-citrate lyase polypeptide in *A. niger*. Using a selection marker and a flanking

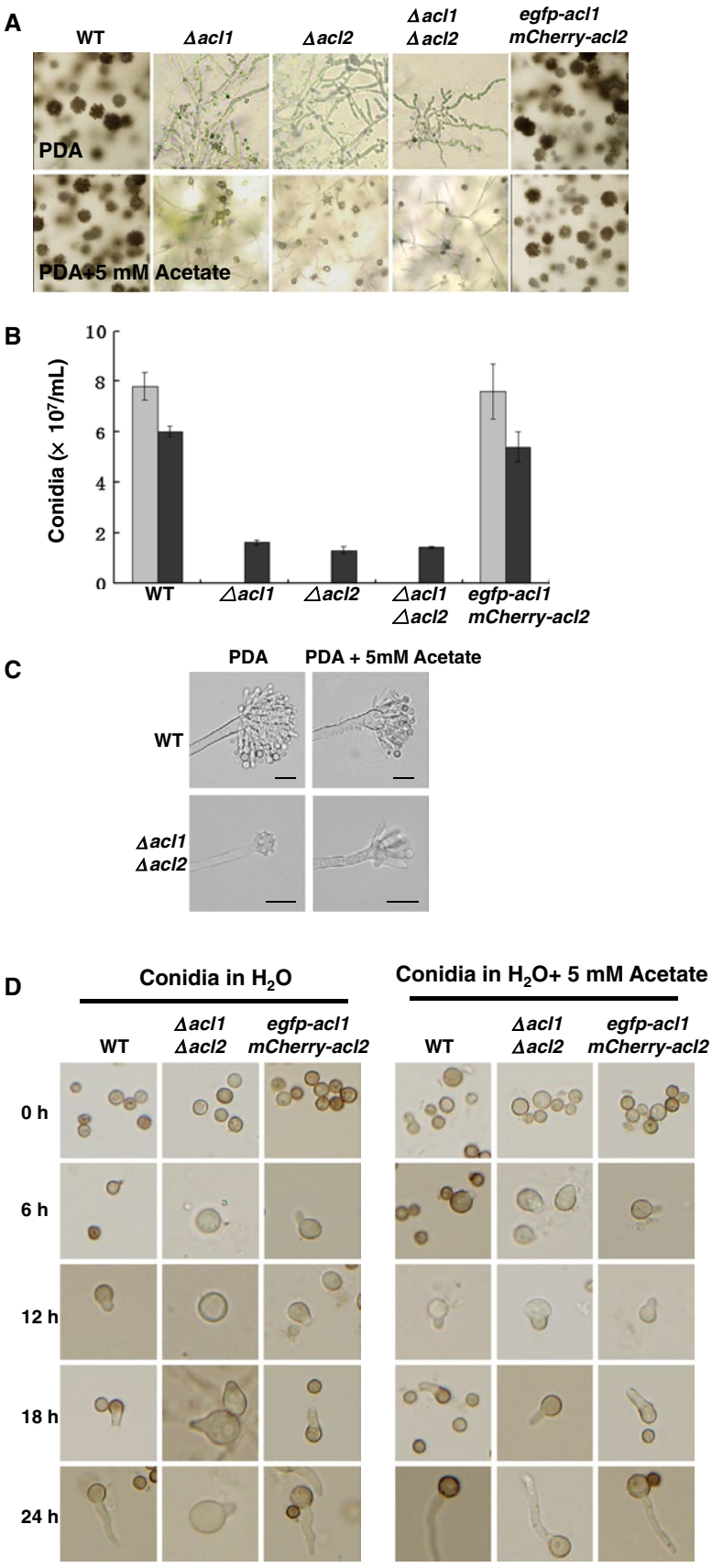


Fig. 3 Conidiogenesis and conidial germination of *acl*-deletion strains. **a** Evaluation of conidiogenesis. Strains of the indicated genotypes were cultured on PDA and PDA supplemented with 5 mM acetate for 6 days at 30 °C, respectively. Aerial hyphae on the surface of the colonies were imaged. **b** Conidiation defects in *acl*-deletion strains. Conidia produced by the indicated strains were harvested and quantified with a haemocytometer as described [22]. *Light grey bars* and *dark grey bars* represent the strains grown on PDA and PDA supplemented with 5 mM acetate, respectively. *Data* represent the mean values from three independent experiments. **c** Structural morphology of conidiophore of wild-type and *ΔacI1ΔacI2* mutants. **d** *acl* is required for conidial germination. Conidia of the indicated strains were suspended in distilled water with or without 5 mM acetate. A total of 20 μl of the individual conidial samples was inoculated on glass slides and incubated on room temperature. Conidial germinations were imaged at the indicated time point

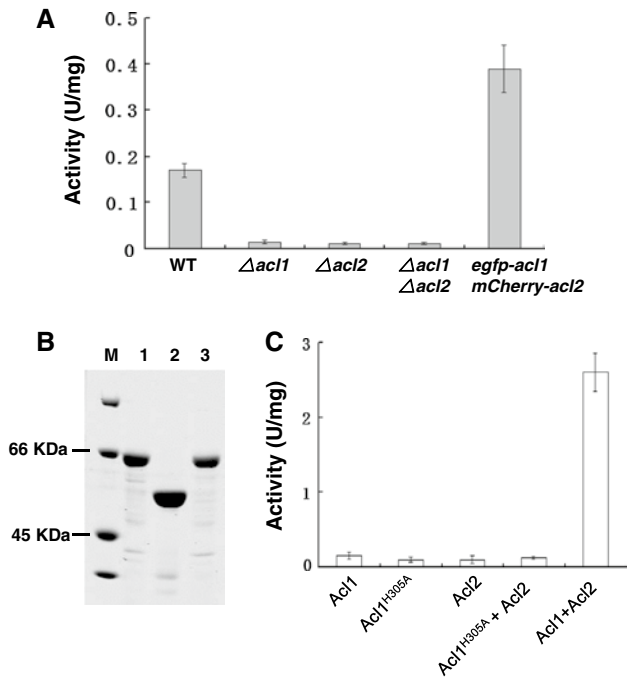


Fig. 4 Both *Acl1* and *Acl2* subunits are required for ACL enzyme activity. **a** Acl1 and Acl2 are required for ACL enzyme activity. Protein lysate was prepared from the indicated strains grown on PDA supplemented with 5 mM acetate. The ACL enzyme activity of each sample was examined. **b** Expression and purification of Acl proteins. 6His-tagged Acl proteins were expressed in *E. coli* BL21 (DE3) and purified. The quality of the proteins was determined by SDS-PAGE. 1, 6His-Acl1; 2, 6His-Acl2; and 3, 6His-Acl1^{H305A}. **c** Acl1 and Acl2 are required for ACL enzyme activity in vitro. ACL enzyme activity assays were performed with Acl1, Acl1^{H305A}, Acl2, the complex of Acl1 and Acl2, and the complex of Acl1^{H305A} and Acl2. Data (**a** and **c**) represent the mean values from three independent experiments

marker, *acl1* and *acl2* were deleted with efficiencies around 6–8 % in *A. niger*. Deletion of *acl1* or *acl2* genes results in the loss of ACL enzyme activity, which is consistent with the genomic prediction that *acl1* and *acl2* are the only two genes encoding ATP-citrate lyase subunits in *A. niger* [22]. This also suggests that both subunits are required for ACL activity in *A. niger*. This hypothesis was supported by our in vitro enzyme activity assay. ACL activity was only detected with the complex of Acl1 and Acl2 but not Acl1^{H305A} and Acl2 complex or individual protein Acl1 or Acl2. Autophosphorylation of the conserved histidine residue is essential for animal ACLY activity. Thus, despite the formation by two subunits, *A. niger* ACL may have a similar catalytic mechanism as animal ACLY, which is a single peptide.

The single and double mutants of *acl1* and *acl2* genes showed pleiotropic defects in vegetative growth, pigmentation, conidiogenesis, and conidial germination. Our results are consistent with the phenotypes of *acl* mutants of *A. nidulans*, *G. zeae*, and *Cryptococcus neoformans* but in contrast with the properties of an *S. macrospora* mutant [10, 12, 20, 27], in which loss of ACL activity showed no evident effects on vegetative growth. Significantly, characterization of the structural morphology of conidiophore indicated that *acl* genes are essential for phialide formation in *A. niger* (Fig. 3c). In *A. niger*, these defects are correlated with decreased acetyl-CoA levels in the mutant strains. Colocalization of Acl1 and Acl2 in the cytoplasm suggests that ACL is most likely involved in regulation of cytosolic acetyl-CoA synthesis in *A. niger*. In *A. niger*, the gene *acs* encoding ACS, which is supposed to convert acetate to acetyl-CoA in cytosol, is constitutively expressed during vegetative growth in liquid PDA and CM media (data not shown). We found that the addition of 5 mM acetate into PDA medium repaired the defects of *acl*-deficient strains in vegetative growth and conidial germination but not pigmentation and conidiogenesis. These results suggest that the deficiency of cytosolic acetyl-CoA in *acl*-deletion mutants could be partially complemented by ACS pathway. This is consistent with presence of acetyl-CoA synthetase activity generating cytoplasmic acetyl-CoA in *A. niger* [17]. *A. niger* has two homologs of carnitine acetyltransferase-encoding genes (CATs, CAK45472, and EHA18856) [22]. Phenotype analysis of the single-gene deletion mutants grown in PDA and CM medium revealed that both of the

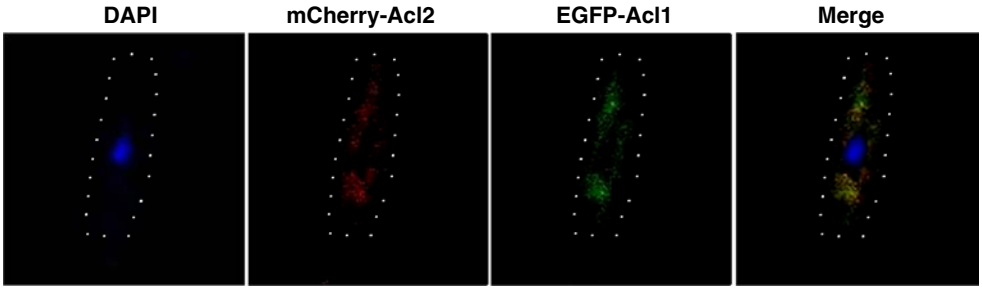


Fig. 5 Colocalization of *Acl1* and *Acl2* in the cytoplasm. The wild-type strain containing *mCherry-acl1* and *egfp-acl2* expression cassettes were grown in PDA for 3 days. The fluorescence was observed with a confocal microcopy. *Red and green colors* represent mCherry-Acl2 and EGFP-Acl1, respectively. DAPI was used to stain DNA (*blue*)

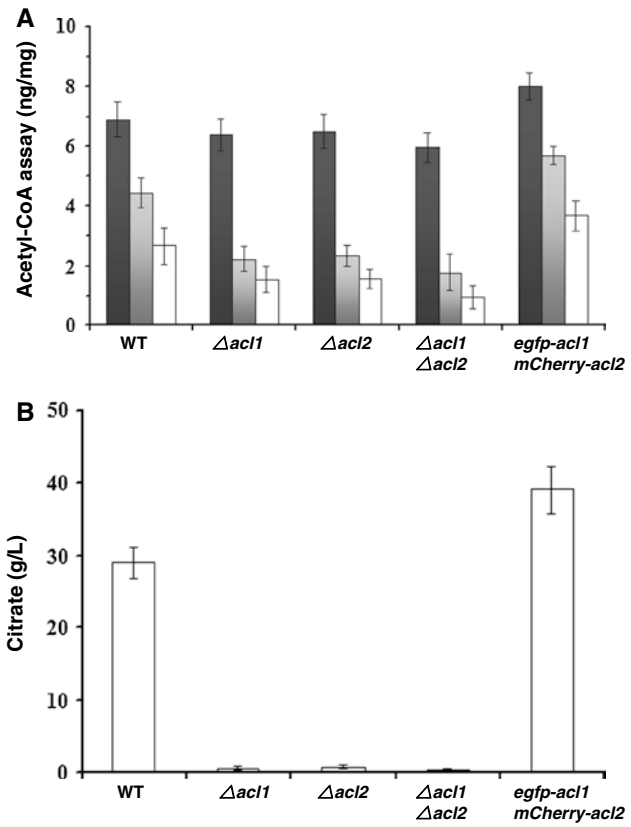


Fig. 6 *acl* genes are required for acetyl-CoA and citric acid production in *A. niger*. **a** *acl* genes regulate acetyl-CoA synthesis. The indicated strains were grown in PDA medium supplemented with 5 mM acetate for 3 days and then shifted to PDA medium and cultured for another 2 days. Acetyl-CoA assay were performed using Acetyl-Coenzyme A Assay kit ("Materials and methods") with samples prepared from cultures of 0 (*dark grey bar*), 1 day (*light grey bar*), and 2 days (*white bar*) grown in PDA medium without addition of acetate. **b** The indicated strains were grown in PDA medium supplemented with 5 mM acetate for 3 days, and then shifted to fermentation medium and cultured for another 5 days. The concentration of citric acid in each fermentation broth was determined using HPLC. Data (**a** and **b**) represent the mean values from three independent experiments

CATs are involved in regulation of vegetative growth and development (data not published). This suggests that translocation of acetyl-CoA between different organelles also plays important roles in growth, development, and metabolism in *A. niger*.

ACL cleaves citric acid to produce acetyl-CoA and oxaloacetate. Interestingly, our batch cultivations showed that deletion of *acl* genes resulted in loss of citric acid production, while overexpression of *acl* increased citric acid synthesis in *A. niger*. It seems reasonable to interpret that *acl*-deletion strains have defects to produce citric acid in that these mutants grow poorly on medium containing glucose as the primary source of carbon. Analysis of the fermentation broth revealed that around 96 % glucose remained in the fermentation broth of *acl*-deficient strains (data not shown), indicating that the ability of these mutants to convert glucose to citrate was compromised. The increased citric acid production in *acl*-overexpression strains is likely related to the glycolysis process. This hypothesis is supported by the previous report that ACLY positively modulates the expression of three key regulators of glycolysis, hexokinase 2, phosphofructokinase-1, and lactate dehydrogenase A, in mammalian cells [29]. Here, the cytoplasmic localization of EGFP-Acl1 and mCherry-Acl2 was observed, however, it remains to be confirmed whether a very low level of these proteins, which is out of the sensitivity of our microscopy, are localized in the nuclei to modulate the expression of genes involved in glycolysis pathway and TCA cycle in *A. niger*.

In conclusion, we have demonstrated that both Acl1 and Acl2 subunits are required for the ACL enzyme activity. Loss of *acl* genes results in significant decrease in acetyl-CoA synthesis and severe defects in vegetative growth, pigmentation, asexual development, conidial germination, and citric acid production. External acetate partially repairing the defects of *acl*-deletion strains suggests that *acl* genes have some unique functions that can not be complemented by other pathways in *A. niger*.

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Conflict of interest The authors declare that they have no competing interests.

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