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Fungal Ammonia Fermentation, a Novel Metabolic Mechanism That Couples the Dissimilatory and Assimilatory Pathways of Both Nitrate and Ethanol

ROLE OF ACETYL CoA SYNTHETASE IN ANAEROBIC ATP SYNTHESIS*

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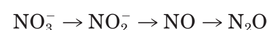
Fungal ammonia fermentation is a novel dissimilatory metabolic mechanism that supplies energy under anoxic conditions. The fungus *Fusarium oxysporum* reduces nitrate to ammonium and simultaneously oxidizes ethanol to acetate to generate ATP (Zhou, Z., Takaya, N., Nakamura, A., Yamaguchi, M., Takeo, K., and Shoun, H. (2002) *J. Biol. Chem.* 277, 1892–1896). We identified the *Aspergillus nidulans* genes involved in ammonia fermentation by analyzing fungal mutants. The results showed that assimilatory nitrate and nitrite reductases (the gene products of *niaD* and *niiA*) were essential for reducing nitrate and for anaerobic cell growth during ammonia fermentation. We also found that ethanol oxidation is coupled with nitrate reduction and catalyzed by alcohol dehydrogenase, coenzyme A (CoA)-acylating aldehyde dehydrogenase, and acetyl-CoA synthetase (Acs). This is similar to the mechanism suggested in *F. oxysporum* except *A. nidulans* uses Acs to produce ATP instead of the ADP-dependent acetate kinase of *F. oxysporum*. The production of Acs requires a functional *facA* gene that encodes Acs and that is involved in ethanol assimilation and other metabolic processes. We purified the gene product of *facA* (FacA) from the fungus to show that the fungus acetylates FacA on its lysine residue(s) specifically under conditions of ammonia fermentation to regulate its substrate affinity. Acetylated FacA had higher affinity for acetyl-CoA than for acetate, whereas non-acetylated FacA had more affinity for acetate. Thus, the acetylated variant of the FacA protein is responsible for ATP synthesis during fungal ammonia fermentation. These results showed that the fungus ferments ammonium via coupled dissimilatory and assimilatory mechanisms.

Most eukaryotic organisms obligatorily use oxygen (O_2) as a substrate for respiration and produce energy required for their biological activities and cell proliferation. Therefore, O_2 depletion is critical for their survival. Such organisms induce the expression of a set of genes to allow adaptation to anaerobic circumstances. Some lower eukaryotes such as yeasts and filamentous fungi induce these genes under anaerobic conditions to produce O_2-independent energy-producing metabolic mechanisms that support facultative anaerobic growth (1–4). This is in sharp contrast to higher eukaryotes that essentially require O_2.

Nitrate respiration and ammonia fermentation are examples of such mechanisms that are distributed among filamentous fungi (2–5). This is unusual, because these metabolic mechanisms had been, respectively, identified in facultative and obligate anaerobic bacteria and not in eukaryotic cells. Subsequent studies revealed that these fungi adapt to changes of environmental O_2 tension via these mechanisms along with O_2 respiration (6, 7). The fungus *Fusarium oxysporum* respires with nitrate under O_2-limited conditions through the sequential reactions of dissimilatory nitrate reductase (2, 7), dissimilatory nitrite reductase (2), and nitric-oxide reductase (8, 9), which reduce nitrate to nitrous oxide (N_2O) (nitrate respiration or denitrification) (Reaction 1).



$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$$

REACTION 1

$$NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$$

REACTION 2

These enzymes are located in mitochondria and are considered physiologically significant for producing ATP through the respiratory electron transfer chain (2, 7). *F. oxysporum* ferments ammonia under more anoxic conditions than nitrate respiration (5). Ammonia fermentation consists of the dissimilatory reduction of nitrate to ammonium (Reaction 2) coupled with the catabolic oxidation of electron donors (ethanol) to acetate and substrate-level phosphorylation that supports growth under anaerobic conditions. Furthermore, 15 of 17 tested fungal strains fermented ammonium under anaerobic conditions (5), suggesting that this activity is widely distributed among fungi.

Microorganisms metabolize nitrate via assimilatory and dissimilatory reductive pathways. Bacteria usually regulate these pathways independently and produce respective enzymes that catalyze these reactions (10, 11). Fungi also reduce nitrate to ammonium to assimilate nitrogen into their biomass. The nitrate-assimilating pathway in the fungus *Aspergillus nidulans* has been extensively studied (12), and assimilatory nitrate and nitrite reductases (encoded by *niaD* and *niiA* genes, respec-

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tively) located in the cytosol use NADH or NADPH (NAD(P)H) as an electron donor (13, 14). These properties are quite different from those involved in nitrate respiration (2, 7, 10) but are similar to those in ammonia fermentation (5). Genes encoding the nitrate and nitrite reductases involved in ammonia fermentation remain unidentified. Therefore, the functional relevance between nitrate and nitrite reductases in the assimilatory mechanism and in ammonia fermentation should be identified.

The original study of fungal ammonia fermentation used ethanol as a source of carbon and energy (5). Fungi use ethanol as a carbon source through the following processes (Reaction 3).

Ethanol → acetaldehyde → acetate → acetyl-CoA

REACTION 3

Ethanol → acetaldehyde → acetyl-CoA → acetate

REACTION 4

The respective reactions of this ethanol utilization pathway of *A. nidulans* are catalyzed by alcohol dehydrogenase (Adh),[1] aldehyde dehydrogenase (Ald), and acetyl-coenzyme A (CoA) synthetase (Acs), which are encoded by the *alc*-family genes (15), *aldA* (16), and *facA* (17), respectively. The generated acetyl-CoA is subsequently used to synthesize various cellular components. Acetyl-CoA produced by this pathway under aerobic conditions also enters the tricarboxylic acid cycle that supplies redox equivalents for O_2 respiration and ultimately generates ATP. In contrast, ammonia fermentation by *F. oxysporum* uses another pathway to dissimilate ethanol (5) (Reaction 4). The key enzymes of this pathway are the CoA-acylating aldehyde dehydrogenase (Add) that catalyzes acetaldehyde + $NADP^+$ + CoA → acetyl-CoA + NADPH and acetate kinase (Ack) that catalyzes acetyl-CoA + ADP + P_i → acetate + ATP (5). However, the molecular properties of the fungal enzymes remain obscure.

Here, we identified ammonia fermentation activity in *A. nidulans* and investigated synthetic and naturally occurring mutations in genes of this fungus that might be involved in ammonia fermentation. We showed that the dissimilatory ammonia fermentation is obligatorily mediated by assimilatory nitrate and nitrite reductases. We also demonstrated that lysine residues of fungal Acs, which is required for ethanol and acetate utilization, are acetylated to catalyze the reverse reaction (acetyl-CoA-dependent ATP formation) during ammonia fermentation. These results showed that the fungus adapts to anaerobiosis by using assimilating enzymes for dissimilatory purposes. This study implies that the fungal assimilating and dissimilating reactions are intimately associated.

EXPERIMENTAL PROCEDURES

Strains, Cultures, and Media—A. nidulans strains FGSC26 (*biA1*), FGSC89 (*biA1*; *argB2*), FGSC691 (*biA1*; *niaD15*), FGSC713 (*yA2*; *pyroA4*; *niiA4*), FGSC952 (*biA2*; *alcR125*), and FGSC283 (*suA1adE20*, *yA2*, *adeE20*; *acrA1*; *galA1*; *pyroA4*; *facA303*; *sB3*; *nicB8*; *riboB2*) were obtained from the Fungal Genetic Stock Center (FGSC, University of Kansas Medical Center). *A. nidulans* TZ13 (*biA1*; *facA303*) and TZ34 (*biA1*; *argB2*; *facA303*) were progenies of a genetic cross between strains FGSC89 and FGSC283. Conidia (10^8) were transferred to 500-ml Erlenmeyer flasks containing 100 ml of MMDN (1% glucose, 0.6% $NaNO_3$, 10 mM KH_2PO_4, 7 mM KCl, 2 mM $MgSO_4$, 0.2% Hutner's trace metals (18)) and aerobically incubated at 30 °C for 20 h (preculture). Resultant mycelia were collected by centrifugation, washed twice with 0.7% NaCl, and then inoculated into 500-ml Erlenmeyer flasks containing 300 ml of MMEN (100 mM ethanol, 10 mM $NaNO_3$, 10 mM KH_2PO_4, 7 mM KCl, 2 mM $MgSO_4$, 0.2% Hutner's trace metals). The head space in the flasks was replaced with argon gas by purging the air for 15 min, then the flasks were sealed with butyl rubber stoppers and incubated at 30 °C at 120 rpm to induce ammonia fermentation. Aerobic conditions were maintained by agitating 100 ml of NMEN medium in flasks sealed with cotton plugs but without replacing the head-space air. Either 50 mM fructose or 10 mM NH_4Cl was added in some experiments. Growth of the auxotroph mutants was supported by adding the appropriate supplements according to the instructions provided by the FGSC.

Preparation of Cell-free Extracts—After the strains were cultured, mycelia were collected by filtration, suspended in buffer (10 mM Tris-HCl (pH 7.2), 0.6 M sucrose, 0.3 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 0.3 mM phenylmethylsulfonyl fluoride, 0.3 mM leupeptin) and homogenized with aluminum oxide powder as described previously (8). The homogenate was centrifuged at 10,000 × *g* for 60 min, and the supernatant (cell-free extract) was centrifuged at 105,000 × *g* for 90 min to obtain the soluble (supernatant) and mixed membrane (pellet) fractions.

Enzyme Assay—All reactions proceeded at 30 °C. We determined NADPH-dependent nitrate and nitrite reductase activity by measuring the amount of nitrite produced and changes in the absorption at 340 nm caused by NADPH, respectively (19, 20). Alcohol dehydrogenase (Adh), aldehyde dehydrogenase (Ald), and CoA-acylating aldehyde dehydrogenase (Add) were measured as described (5). Acetyl-CoA synthetase (Acs) activity was determined by a modification of the published method (5) as follows. Soluble fraction or purified FacA was added to a buffer (100 mM Tris-HCl (pH 9.0), 1.5 M KCl, 10 mM $MgCl_2$, 400 mM potassium acetate, 10 mM ATP, 1.5 mM CoA, 700 mM hydroxylamine HCl), and then acetyl-CoA production was colorimetrically monitored by measuring absorption at 540 nm as described (21). Soluble fraction or purified FacA was added to buffer (100 mM potassium phosphate (pH 7.2), 5 mM AMP, 5 mM potassium pyrophosphate, 5 mM acetyl-CoA) to start the reaction that generates acetate. Thereafter, ATP production was measured using a Rucifel-250 kit (Kikkoman, Tokyo), and chemiluminometry (Lumitester K-210, Kikkoman, Tokyo) and high performance liquid chromatography confirmed acetate formation.

Construction of the Plasmids—A DNA fragment encoding 6× histidine-tagged FacA (His-FacA) was amplified by PCR using the primers 5′-ccgaattcatgcatcaccatcaccatcacagtggacggaccaattgccc-3′ and 5′-cctctagatgtatttgcctcgggaagcgtgcactgtggcgataattctc-3′ (restriction sites are underlined). The 3-kbp fragment was specifically amplified, blunt-ended, and inserted to the SmaI site of pUC118 to generate pFAC1. Plasmid pFAC2 was constructed by ligating the 3-kb EcoRI-XbaI fragment of pFAC1 into the same restriction sites of vector pSSH18. Plasmid pSSH18 was constructed by inserting the 2.2-kbp SalI fragment of the *argB* gene into the SalI site of pUC18. The plasmid pALD1 for generating the *aldA* knock-out mutant TK11 was constructed as follows. The 2.8-kbp DNA fragment corresponding to part of *aldA* was amplified by PCR using the primers, 5′-ccgaattcgcgagctcttcaacgtgttttc-3′ and 5′-ggatctgcagcatacgtgctttctg-3′ and digested with EcoRI and EcoRV. The 700-bp DNA fragment was purified and inserted into the EcoRI-SmaI site of pSSH18. The resultant plasmid was digested with SphI and PstI and ligated with the 750-bp DNA fragment corresponding to the 3′-part of *aldA* that had been prepared by PCR using the primers, 5′-cctgcatgctacggcactggttgg-3′ and 5′-ggatctgcagcatacgtgctttctg-3′ and digested with SphI and PstI. The PCR conditions were 30 cycles of 94 °C for 30 s (denaturation), 56 °C for 40 s (annealing), and 72 °C for 90 s (elongation) in reactions containing 1 μg of *A. nidulans* FGSC26 total DNA. We confirmed the nucleotides by sequencing the inserted fragments.

Fungal Transformation—We transformed *A. nidulans* strains using standard procedures (22). *A. nidulans* TK21 and TK31 were obtained by introducing pFAC1 and pFAC2 into strains TZ13 and TZ34, respectively. Transformants were respectively selected according to restored absence of acetate utilization and arginine auxotrophic phenotypes. Prior to transformation, pFAC2 was linearized at its unique SacI site within the *argB* gene, and integration of the plasmid into the *argB* locus by a single crossover was confirmed by Southern blotting total DNA from TK31. We selected *A. nidulans* TK11 from the transformants obtained with FGSC89 and pALD1 and confirmed gene replacement.

Preparation of Recombinant FacA—A. nidulans TK31 was cultured under conditions of aerobic and ammonia fermentation, and the soluble fraction was prepared as described above. A nickel-nitrilotriacetic acid-agarose column (bed volume, 3 ml, Qiagen) was equilibrated with buffer (50 mM potassium phosphate-NaOH (pH 8.0), 300 mM NaCl) containing 25 mM imidazole and washed with 50 ml of the same buffer. Protein was

[1] The abbreviations used are: Adh, alcohol dehydrogenase; CoA, coenzyme A; Ack, acetate kinase; Acs, acetyl-CoA synthetase; Add, CoA-acylating acetaldehyde dehydrogenase; FacA, gene product of *facA*; $FacA^{aero}$, FacA prepared from aerobically grown cells; $FacA^{anaero}$, FacA prepared from anaerobically grown cells; FGSC, Fungal Genetic Stock Center.

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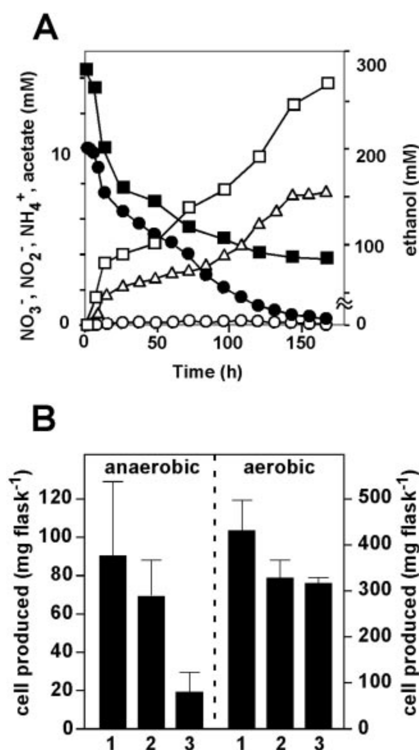

FIG. 1. **Nitrogen and carbon metabolism by *A. nidulans* during anoxic culture.** *A*, *A. nidulans* FGSC26 was cultured in medium containing 10 mM $NaNO_3$ (MMEN medium) as a nitrogen source. *Closed circles*, NO_3^-; *open circles*, NO_2^-; *triangles*, NH_4^+; *closed squares*, ethanol; *open squares*, acetate. *B*, cell growth during anaerobic (*left*) and aerobic (*right*) culture. *A. nidulans* FGSC26 was cultured in MMEN medium (*bar 1*), MMEN medium containing 10 mM NH_4Cl (*bar 2*), and MMEN medium that replaces $NaNO_3$ with NH_4Cl (*bar 3*).

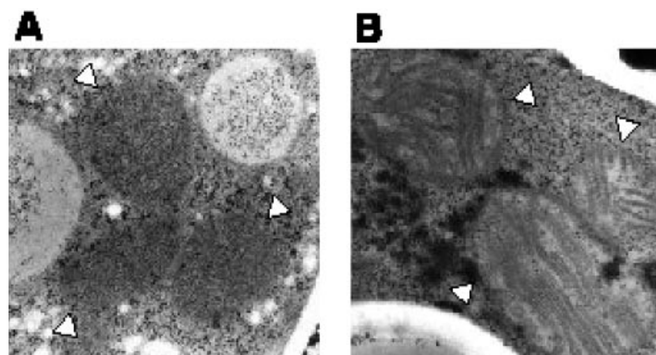

FIG. 2. **Transmission electron micrographs of *A. nidulans* cells.** Aerobic cells (*A*) were cultured in MMEN medium at 30 °C for 12 h. Anoxic cells (*B*) were incubated the under ammonia-fermenting conditions. Magnification, ×25,000. *Arrowheads* indicate mitochondria.

eluted through the column from the soluble fraction using the same buffer containing 250 mM imidazole. The fraction containing His-FacA was eluted through a Resource Q column (Amersham Bioscience) that had been equilibrated with 20 mM Tris HCl (pH 8.0) using a linear gradient of 0 to 0.5 M NaCl in the same buffer. The resultant fractions were gel-filtrated (Superdex™ 200HR, Amersham Biosciences) using 50 mM sodium phosphate (pH 7.0) containing 150 mM NaCl.

Other Methods—Nitrate, nitrite, and ammonium were determined by ion exchange chromatography as described previously (5). Acetate and acetaldehyde were measured by high performance liquid chromatography as described (5). Ethanol was determined enzymatically using alcohol dehydrogenase and NAD^+ as described (23). Dry cell weight was determined after drying the cells at 80 °C for 3 h. Purified proteins (1 μg) were Western-blotted using the ECL Western blot detection system (Amersham Bioscience) according to the instructions provided with the kit and a 1:1000-diluted anti-acetyl lysine antibody (Upstate Biotechnology). Transmission electron micrographs were obtained as described (24) using a JEOL-1200 EX transmission electron microscope (JEOL Ltd., Tokyo, Japan).

RESULTS

Anaerobic Ammonia Fermentation by A. nidulans—We showed that various species of fungi ferment ammonia under anaerobic conditions (5), although the ammonia-fermenting activity of *A. nidulans* has not been described. We incubated anaerobic cultures of *A. nidulans* FGSC26 in the presence of 10 mM nitrate and 300 mM ethanol to examine its ammonia-fermenting activity (Fig. 1*A*). The culture consumed nitrate and produced ammonium over the incubation period, but little nitrite was detected. Of the consumed nitrate, 80% was metabolized to ammonium. The remaining 20% might have been converted to biomass, because nitrate was the sole nitrogen source for the culture. Ammonia production was accompanied by acetate formation at a 1:2 ratio throughout the incubation. This is consistent with the stoichiometry of the reaction products of ammonia fermentation by *F. oxysporum* that is described as: NO_3^- + 2 ethanol → NH_4^+ + 2 acetate (5).

The nitrate-dependent growth of the culture in the absence of O_2 (Fig. 1*B*) indicates that nitrate is critical for the anoxic cell growth of the *A. nidulans*. Because little N_2O and nitrite was detectable during the culture, *A. nidulans* does not seem to respire using nitrate and thus cannot produce ATP through nitrate respiration unlike *F. oxysporum* (5). This indicated that *A. nidulans* produces ATP through ammonia fermentation.

Morphology of Mitochondria—Fig. 2 shows transmission electron micrographs of *A. nidulans* mitochondria. The number, size, shape, and membranous structure of the mitochondria did not significantly differ between cells producing ammonium anaerobically and those growing aerobically. However, the electron density of most mitochondria in the former was lower than that of the latter cells. These properties are typical of immature mitochondria in *F. oxysporum* that ferments ammonia (5) and are consistent with the notion that this anoxic ammonium formation of *A. nidulans* is a non-respiratory type of fermentation that generates cytosolic ATP (ammonia fermentation).

Nitrate and Nitrite Reduction in Ammonia Fermentation—We determined the enzyme activity involved in nitrate metabolism through ammonia fermentation. The soluble fraction of cell-free extracts prepared from ammonia-fermenting *A. nidulans* FGSC26 exhibited significant NAD(P)H-dependent nitrate and nitrite reductase activity (Table I). Little activity was detected in the mixed membrane fraction (data not shown), which is consistent with the notion that ammonia is fermented in the cytosol but is not a respiratory product produced in mitochondria. Both NAD(P)H-dependent nitrate and nitrite reductases are specific under anaerobic (ammonia-fermenting) conditions (Table I), indicating that they contribute to ammonia fermentation. These properties are essentially similar to our previous observations of *F. oxysporum* that ferments ammonia (5). We examined the effect of mutations in the *niaD* and *niiA* genes (13) that encode assimilatory NAD(P)H-dependent nitrate and nitrite reductases, respectively, on ammonia fermentation by *A. nidulans*. Both enzyme activities disappeared in the respective mutants that produced neither ammonia (Fig. 3*A*) nor acetate (data not shown) under ammonia fermentation conditions, indicating that these metabolic reactions are coupled. Fig. 3*A* also shows that these mutations considerably repressed cell growth. Because we supplemented the culture medium with ammonium in addition to nitrate as an additional nitrogen source, the growth defect was caused by a loss of ammonia fermentation activity rather than a defect in assimilating nitrogen. These results unequivocally demonstrated that the fungus ferments ammonia by obligatory me-

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TABLE I
Effect of mutations on nitrate and nitrite reductase activity by A. nidulans

Cells collected from the preculture were transferred to the MMEN medium, which contained 10 mM $NaNO_3$ and 10 mMNH_4Cl as nitrogen sources, and incubated under the anaerobic conditions for 12 h. Enzyme activity in the soluble fraction was measured by using NADPH as an electron donor. Data are mean values of three measurements.

Strain	Relevant genotype	Culture condition	Specific activity	
			Nitrate reductase	Nitrite reductase
			nmol min^{-1} mg^{-1}	
FGSC26	Wild type	Anaerobic	19 ± 4	10 ± 3
		Aerobic	<0.1	<0.1
FGSC691	*niaD15*	Anaerobic	<0.1	11 ± 5
		Aerobic	<0.1	<0.1
FGSC713	*niiA4*	Anaerobic	15 ± 3	<0.1
		Aerobic	<0.1	<0.1

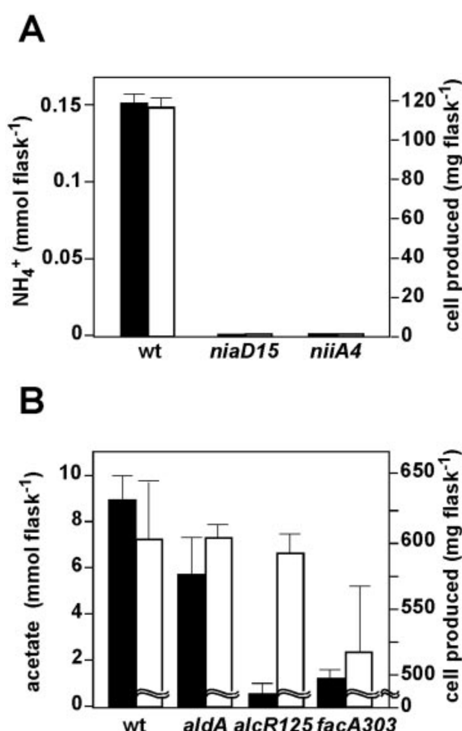

FIG. 3. **Effect of mutation on ammonia fermentation activity by *A. nidulans*.** Strains were cultured for 12 h under ammonia-fermenting conditions. MMEN medium containing 10 mM NH_4Cl (*A*) and 100 mM fructose (*B*) to support mutant growth. *Closed bars*, production of ammonium (*A*), and acetate (*B*); *open bars*, cell growth. Relevant genotypes are shown below the *bars*.

diation via the gene products of *niaD* and *niiA*, which are assimilatory nitrate and nitrite reductases.

Specific production of the *niaD* and *niiA* gene products under conditions of ammonia fermentation was of interest, because whether *niaD* and *niiA* expression is regulated under anoxic conditions is unknown. We cultured fungal strains in the presence of ammonium and nitrate (Table I). The fungus normally represses expression of the genes for nitrate assimilation in the presence of ammonium (14, 25), and the undetectable nitrate and nitrite reductase activities in aerobic cells are consistent with this fact (Table I). Meanwhile nitrate and nitrite reductase activities were evident under conditions of ammonia fermentation, and they were apparently dependent upon *niaD* and *niiA*. This indicates that ammonium does not repress *niaD* and *niiA* under anaerobic conditions.

Ethanol Oxidation Pathways in Ammonia Fermentation—We measured enzyme activities involved in the acetate-forming reaction by *A. nidulans* FGSC26 (Table II). Over 90% activity of all tested enzymes was recovered in the soluble fraction (data not shown), indicating their location in the cytosol. Activity of Add was induced under the conditions of ammonia fermentation like that in *F. oxysporum* (5). The acetyl-CoA-dependent ATP producing activity of *A. nidulans* requires AMP as a phosphorylation substrate. ADP did not support the activity. This activity is distinguishable from that of *F. oxysporum*, which produces ATP through ADP (5). *A. nidulans* constitutively produced enzymes involved in ethanol utilization (Adh, Ald, and acetyl-CoA-producing Acs) (Table II). Fig. 3*B* shows that the knockout mutant of the *aldA* gene, which is essential for ethanol utilization and which encodes assimilatory aldehyde dehydrogenase (16), produced 30% less acetate than the wild type. This indicated that most (70%) of the acetate was produced through Add and that 30% was produced through the *aldA*-dependent reduction of acetaldehyde that should not be linked to ATP formation. The mutation in the *alcR* locus affected not only Ald but also Adh activity (Table II). This is consistent with the fact that *alcR* encodes a transcription factor essential for expression of the Adh and Ald genes and for the fungus to utilize ethanol (26). Acetate formation by this mutant was more decreased than that by the *aldA* mutant (Fig. 3*B*), indicating that Adh is important for ammonia fermentation. These results demonstrated that the main pathway for ethanol oxidation is catalyzed by a sequential reaction of Adh, Add, and Acs. The involvement of Acs is unique in the ammonia fermentation mechanism of *A. nidulans*.

The *facA* gene was originally identified as encoding fungal Acs that is essential for assimilating acetate and ethanol under aerobic conditions (17). However, the non-functional mutation in the *facA* gene (*facA303*) eliminated the enzyme activities for both ATP-dependent acetyl-CoA formation and acetyl-CoA-dependent ATP production (Table II). Both activities were restored by introducing plasmids containing either the *facA* (pFAC1, data not shown) or the histidine-tagged *facA* (His-FacA) gene (pFAC2, Table II). The mutation almost completely repressed acetate formation and significantly decreased the cell growth coupled with ammonia fermentation (Fig. 3*B*), indicating that the *facA* product is responsible for the acetate-forming Acs activity that catalyzes a key reaction (ATP formation) in the ammonia fermentation mechanism. More acetate was formed by Acs activity in a *facA*-dependent manner when the cells were cultured under anaerobic conditions, whereas Acs activity formed more acetyl-CoA when cultured under aerobic conditions (Table II). These results showed that the aerating conditions of the culture regulated the production of these enzymatic activities of the *facA* gene product (FacA).

Regulation of the Reversible Reaction of the facA Gene Product—These results and the observation that fungal Acs uses AMP for ATP production indicate that FacA would catalyze the acetate-forming Acs reaction via the reversed acetyl-CoA-forming Acs reaction. We cultured the *A. nidulans* strain TK31 that produced histidine-tagged FacA under aerobic and anaerobic conditions and purified recombinant FacA. Each chromatography step gave a single peak of Acs activity, and the homogeneity of the purified preparations was confirmed by SDS-PAGE. The molecular mass of FacA prepared from cells grown under anaerobic conditions (FacA^{anero}) was slightly higher (75.5 kDa) than that prepared from the cells grown under aerobic conditions (FacA^{aero}) (74.8 kDa) (Fig. 4*A*). This could be due to post-translational modification of the FacA^{anero} (see the next section). Both proteins eluted from the gel filtration column at a position of 75 kDa, indicating that they were monomer. Under the standard assay conditions, the enzyme activity of FacA^{aero} was 7.1 and 3.4×10^{-3} μmol min^{-1} mg^{-1} for acetyl-

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TABLE II
Specific activities of the enzymes involvement in ammonia fermentation

Cells were cultured in the medium with 300 mM ethanol and 50 mM fructose as carbon sources for 12 h. Reaction products of the enzyme reactions are shown in parenthesis. Data are mean values of three measurements.

Strain	Relevant genotype	Culture condition	Specific activity				
			Add (acetyl CoA)	Acs (acetate)	Acs (acetyl CoA)	Adh (acetaldehyde)	Ald (acetate)
			nmol min^{-1} mg^{-1}				
FGSC26	Wild type	Anaerobic	850 ± 180	150 ± 23	22 ± 7	37 ± 5	28 ± 6
		Aerobic	<0.1	42 ± 7	120 ± 32	74 ± 13	35 ± 10
TZ13	*facA303*	Anaerobic	520 ± 50	<0.1	<0.1	25 ± 7	20 ± 9
		Aerobic	<0.1	<0.1	<0.1	42 ± 2	38 ± 3
TK31	*facA303::his-facA*	Anaerobic	660 ± 90	95 ± 35	20 ± 14	40 ± 5	19 ± 7
		Aerobic	<0.1	25 ± 3	120 ± 27	66 ± 9	46 ± 12
TK11	Δ*aldA::argB*	Anaerobic	310 ± 94	74 ± 10	10 ± 6	37 ± 2	<0.1
		Aerobic	<0.1	15 ± 5	63 ± 8	47 ± 12	<0.1
FGSC952	*alcR125*	Anaerobic	95 ± 14	68 ± 10	20 ± 4	5.5 ± 1.5	1.5 ± 0.5
		Aerobic	<0.1	13 ± 1	57 ± 5	4.5 ± 3.0	1.0 ± 0.2

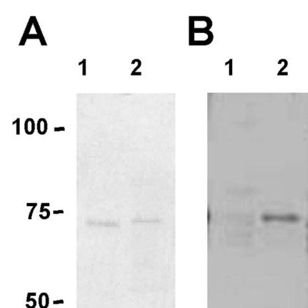

FIG. 4. **Purification of His-FacA from the aerobic and the anaerobic cells.** His-FacA was purified from *A. nidulans* TK31 cells cultured under aerobic (*lane 1*) and anaerobic (*lane 2*) conditions. Protein (1 μg) was resolved by SDS-PAGE, stained with CBB (*A*) and Western blotted (*B*) against anti-acetyl-lysine antibodies. The positions of molecular mass markers (kDa) are shown *left*.

CoA- and the acetate-forming reactions, respectively, whereas those of FacA^{anaero} were 2.6×10^{-3} and 3.9 μmol min^{-1} mg^{-1} (data not shown), indicating that the direction of the enzymatic reaction was regulated at the enzyme level. Because the specific activity of acetate formation catalyzed by FacA^{aero} and that of acetyl-CoA formation catalyzed by FacA^{anaero} linearly increased with increasing concentrations of the substrate (acetyl-CoA and acetate, respectively) up to 25 mM, we could not determine kinetic constants for these reactions. However, our results demonstrated that the K_m value of FacA^{aero} for acetate was more than 33-fold lower than that for acetyl-CoA and that the K_m value of FacA^{anaero} for acetyl-CoA was over 23-fold lower than that for acetate (Table III). Thus, affinity for the substrates mainly determines the direction of the reaction catalyzed by FacA.

The bacterium *Salmonella enterica* acetylates Acs on the conserved lysine residue to repress its activity (27). The same lysine residue was also conserved in *A. nidulans* FacA protein (Lys^{629}, data not shown). We examined whether a similar modification occurs in the fungal FacA (Fig. 4*B*). The results of immunoblotting showed that anti-acetyl-lysine antibody reacted with purified FacA^{anaero}. In contrast, no signal was generated with FacA^{aero}. These results demonstrated that the fungus acetylates FacA in its lysine residue(s) under anaerobic conditions to regulate affinity for acetate and acetyl-CoA and that this modification regulates the direction of the reversible reaction catalyzed by FacA.

DISCUSSION

This study established the first genetic basis of the fungal ammonia fermentation mechanism that is summarized in Fig. 5. The mechanism essentially consists of nitrate-reducing and ethanol-oxidizing pathways. Although they are essentially similar to those of *F. oxysporum* (5) and the former is suggested in *Clostridium* (28), the components involved in each pathway are distinguishable. *A. nidulans* reduces nitrate to ammonium through the nitrate and nitrite reductases (*niaD* and *niiA* gene products) that are required for the assimilatory reduction of nitrate (13, 14). In contrast, the *Clostridium* system uses specific enzymes for dissimilatory nitrate reduction (28). Although whether *F. oxysporum* requires the *niaD* and *niiA* genes for ammonia fermentation remains unknown, the NAD(P)H-dependent activity and cytosolic location of the nitrate and nitrite reductases (5) suggest that assimilatory nitrate and nitrite reductases are involved in the mechanism of ammonia fermentation. We recently proposed that *Cylindrocarpon tonkinense*, a close relative of *F. oxysporum*, reduces nitrate to nitrous oxide in a dissimilatory manner (denitrification) that involves NAD(P)H-dependent assimilatory nitrate reductase (29). These findings imply that nitrate assimilatory enzymes function in fungal dissimilation mechanisms under anaerobic conditions. That the fungi use assimilatory nitrate and nitrite reductases for fermenting ammonium is physiologically reasonable, because the assimilatory and ammonia-fermenting enzymes are located in the cytosol and catalyze the same reaction through which cytosolic NADH is oxidized. This is significant for the fungus to remove excess electron equivalents (NADH) under anoxic conditions; that is, the reaction should function as an electron sink during energy metabolism.

Nitrate reduction accompanies ethanol oxidation to acetate that is coupled with substrate level phosphorylation (Fig. 5). One key reaction in the ethanol-oxidizing pathways is catalyzed by Add that is specifically produced under anaerobic conditions to generate acetyl-CoA (Table II). This is a common component between fungal mechanisms of ammonia fermentation (5). Meanwhile the acetogenic system of *A. nidulans* is unique in that it involves Acs in ATP synthesis. Microorganisms catalyze acetogenic reactions under anaerobiosis by two pathways. One is catalyzed by the acetate kinase-phosphotransacetylase system that uses acetyl phosphate as an intermediate to phosphorylate ADP. The other is catalyzed by Acs. The acetogenic reaction catalyzed by Acs is AMP-dependent in bacteria but ADP-dependent in archaea (30) and in the obligatory anaerobic protist *Giardia lamblia* (31). Acs produced by *A. nidulans* under anaerobic conditions (FacA^{anaero}) is the first known eukaryotic acetogenic Acs that is dependent upon AMP. Furthermore, *A. nidulans* is the first known organism that uses Acs for ammonia fermentation. These results showed that fungal ammonia fermentation is a novel collection of acetogenic mechanisms in microorganisms.

To grow on acetate as a sole source of carbon, *A. nidulans*

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TABLE III
Kinetic properties of Acs activity of the FacA proteins

Mean values of duplicate results are shown.

Enzyme	Acetyl CoA production			Acetate production		
	K_m for acetate	V_{max}	V_{max}/K_m	K_m for acetyl-CoA	V_{max}	V_{max}/K_m
	mM	*mmol min⁻¹ mg⁻¹*		*mM*	*mmol min⁻¹ mg⁻¹*	
$FacA^{aero}$	0.76	7.1	9.4	≥25	ND[a]	NA[b]
$FacA^{anaero}$	≥25	ND	NA	1.1	3.9	3.5

[a] ND, not determined.
[b] NA, not applicable.

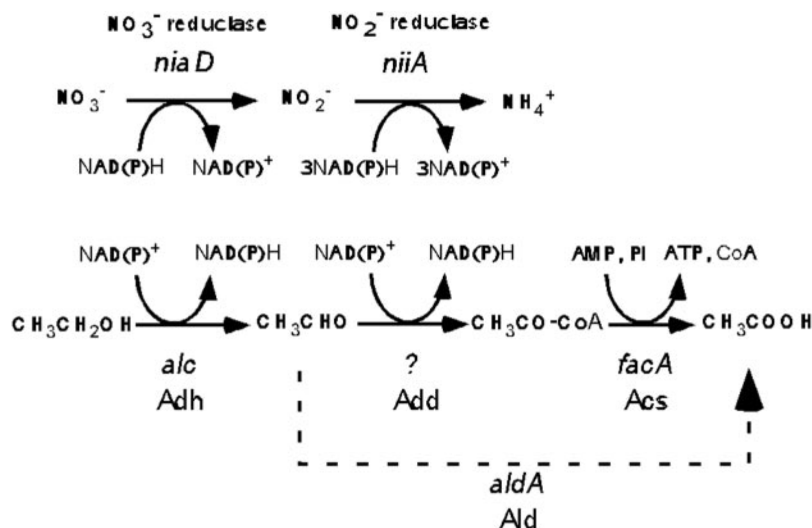

FIG. 5. **Metabolic system and genes involved in fungal ammonia fermentation.** Genes and enzymes involved in each reaction are *Adh*, alcohol dehydrogenase; *Add*, CoA-acylating aldehyde dehydrogenase; *Ald*, aldehyde dehydrogenase; *Acs*, acetyl-CoA synthetase. The *broken line* indicates minor non-productive pathway that does not generate ATP.

requires *facA* (19). Our findings showed that *facA* is also required for the fungus to ferment ammonia (Fig. 3). These observations indicate that *A. nidulans* shares FacA to activate acetate in the acetate assimilation mechanism and for the acetogenic reaction in ammonia fermentation. Both reactions are the reverse of the other, and the fungus adapts to environmental changes in O_2 tension by modulating the direction of the reaction. To our knowledge, we are the first to describe a reversible reaction of which the direction is regulated *in vivo*. This regulation seems a reasonable way for the fungus to switch cellular metabolism in response to changes in O_2 availability. Under anaerobic conditions, fungal growth is usually limited by external electron acceptors and cellular metabolism should be down-regulated to support slow growth. Acetyl-CoA is considered a key compound that mediates numerous biosynthetic and energy-yielding metabolic pathways as well as regulates several key metabolic reactions. Thus, modulation of FacA so that it catalyzes reverse reaction of acetyl-CoA synthetase ($FacA^{anaero}$) should efficiently decrease the intracellular acetyl-CoA concentration through which fungal cells could adapt to anaerobic slow growing conditions. Furthermore, this reaction is a benefit to the fungus, because the generated ATP can support anaerobic cell growth.

We provided evidence for novel roles of *niaD*, *niiA*, and *facA*, which raises several interesting questions such as how acetylation of the lysine residue modulates the Acs activity of FacA. Acs catalyzes a two-step reaction in which an acetyl-AMP intermediate is formed in the initial half of the reaction. The acetyl-AMP intermediate is the substrate for the second half of the reaction in which CoA displaces AMP to yield acetyl-CoA. A recent report describing *S. enterica* Acs showed that acetylation inactivated only the initial half of the reaction (27). Our findings that FacA acetylation decreases affinity for acetate are consistent with this report (Table III). We also found that acetylation increased the affinity for acetyl-CoA, which has not observed in other Acs and implicates that acetylation affects the second half of the reaction. Further biochemical and structural studies of *A. nidulans* FacA are underway. Apparently, ammonium does not repress *niaD* and *niiA* expression under ammonium fermenting conditions, but how the fungus releases ammonium repression of the gene expression under the anaerobic conditions remains unknown. Perhaps O_2 affects ammonium repression. This important issue should be studied to understand fungal nitrogen metabolism. How fungi evolved ammonium fermentation and nitrate respiration mechanisms also remains obscure. The fermentative nitrate reduction of *Clostridium* is regarded as a primitive form of anaerobic respiration (32). The potential of the assimilatory nitrate and nitrite reductases for acting as dissimilatory enzymes in fungal ammonia fermentation suggested that this process has evolved to not only nitrate respiration but also nitrate assimilation systems. Until now, nitrate respiration by *A. nidulans* has not been proven in contrast to *F. oxysporum*, which performs both nitrate respiration and ammonia fermentation. The nitrate metabolic mechanisms of *A. nidulans* might be more primitive than those of *F. oxysporum*. The components of mechanisms among fungal species can now be compared.

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Fungal Ammonia Fermentation, a Novel Metabolic Mechanism That Couples the Dissimilatory and Assimilatory Pathways of Both Nitrate and Ethanol: ROLE OF ACETYL CoA SYNTHETASE IN ANAEROBIC ATP SYNTHESIS

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