Ammonia Fermentation, a Novel Anoxic Metabolism of Nitrate by Fungi*

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The induction of fungal denitrification by Fusarium oxysporum requires a minimal amount of O2, although excess O2 completely represses this process (Zhou, Z., Takaya, N., Sakairi, M. A. C., and Shoun, H. (2001) Arch. Microbiol. 175, 19–25). Here we describe another metabolic mechanism of nitrate in fungal cells, termed ammonia fermentation, that supports growth under conditions more anoxic than those of denitrification. The novel nitrate metabolism of eukaryotes consists of the reduction of nitrate to ammonium, coupled with the catabolic oxidation of electron donors to acetate and substrate-level phosphorylation. F. oxysporum thus has two pathways of dissimilatory nitrate reduction that are alternatively expressed in response to environmental O2 tension. F. oxysporum prefers O2 respiration when the O2 supply is sufficient. We discovered that this fungus is the first eukaryotic, facultative anaerobe known to express one of three distinct metabolic mechanisms closely depending on environmental O2 tension. We also showed that ammonia fermentation occurs in many other fungi that are common in soil, suggesting that facultative anaerobes are widely distributed among fungi that have been considered aerobic organisms.

Rapid changes in O2 supply are an ongoing challenge for many organisms living in environments such as soil. Facultative anaerobes are widely distributed among prokaryotes and can adapt immediately to rapid changes in aeration by altering their energy metabolism. On the contrary, much less is known about such adaptive techniques in eukaryotes, although many lower eukaryotes can survive under anoxic conditions (1–3). Most of these anaerobic eukaryotes have adapted permanently to extreme environments such as swamps and intestines where the O2 supply is always poor. Thus, they are obligate, not facultative, anaerobes.

Nitrate is generally metabolized by organisms in assimilatory and dissimilatory reductive pathways. Bacteria, fungi, and plants reduce nitrate to ammonium to assimilate nitrogen into their biomass (Scheme 1). Dissimilatory reduction (nitrate respiration) is performed by many bacteria in which nitrate is used as an alternative electron acceptor for respiration when O2 is not available. One form of nitrate respiration results in denitrification (Scheme 2), a strategy that is extensive in facultative anaerobic bacteria (4–6). Another form (see Scheme 1) has been identified in enterobacteria and other proteobacteria (7).

\[
\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+ \\
\text{SCHEME 1}
\]

\[
\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2 \rightarrow \text{N}_2O \\
\text{SCHEME 2}
\]

Many fungi can perform denitrification (8–10). Although the anaerobic process was initially thought to be only a prokaryotic feature (11), the fungal denitrifying system is localized to mitochondria where it acts as a mechanism for anaerobic respiration similar to that of bacteria (12). The finding of denitrification in fungi suggests that such organisms are eukaryotic facultative anaerobes. Here, we present evidence for ammonia fermentation, a second form of dissimilatory nitrate metabolism in denitrifying fungi, and for the alternative expression of ammonia fermentation and denitrification under anaerobic conditions in response to the O2 supply. The results show that many fungi, which are common in soil and which have been considered aerobic organisms, should really be classified as facultative anaerobes.

**EXPERIMENTAL PROCEDURES**

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transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium supplemented with 600 mM ethanol and 10 mM ammonium and/or nitrate. The flask was sealed with a rubber stopper after purging air from the medium and head space by flushing with argon gas (anoxic conditions) or sealed with a cotton plug without purging the air (aerobic condition). Dry cell weight was determined after drying washed cells at 94 °C for 24 h.

Preparation of Cells for Enzyme Analyses—Enzyme activities related to nitrate metabolism were determined with each subcellular fraction prepared from the fungal cells grown under various aeration conditions. F. oxysporum was rotary-shaken in a 5-liter Erlenmeyer flask containing 1 liter of medium containing 10 mM nitrate under each aeration condition. Anoxic (ammonia-fermenting) and aerobic (nitrate-assimilatory) conditions were obtained, respectively, as described above. Sealing the flask with a rubber stopper without replacing head space air generated hypoxic (denitrifying) conditions (8). Non-induced cells were prepared by incubating under anoxic conditions in the absence of nitrate (nitrate was replaced with ammonium). Fungal cells were harvested at the early stage of exponential growth and then disrupted and fractionated into subcellular fractions as reported (12).

Enzyme Analyses—All enzyme activities were measured anaerobically at 30 °C in subcellular fractions from fungal cells cultured under various conditions. Alcohol dehydrogenase (Ald) was assayed by following the production of NADH from 50 mM ethanol and 5 mM NAD⁺. Acetaldehyde dehydrogenase (acylating) (AddA) was assayed by determining the amount of acetyl-CoA formed during an incubation with 50 mM acetaldehyde, 5 mM NAD⁺, and 5 mM coenzyme A. Acetate kinase (Ack) was assayed by determining the amount of ATP formed from 5 mM ADP and 5 mM acetyl-CoA. Acetyl-CoA hydrolyase was assayed by determining the amount of acetyl-CoA from 5 mM acetyl-CoA. Acetalddehyde dehydrogenase activity was measured by incubating with 50 mM acetaldehyde and 5 mM NAD⁺, and the amount of formed acetate was measured. Acetyl-CoA synthetase was assayed by measuring the amount of acetyl-CoA formed during an incubation with 50 mM acetaldehyde, 5 mM coenzyme A, and 5 mM ATP. Nitrate reductase and nitrite reductase were, respectively, assayed by incubating with 10 mM sodium nitrate or sodium nitrite and 2 mM NADH, and the amount of generated nitrite or ammonium was determined. Buffers were as follows: 70 mM Tris-HCl (pH 7.2) for Ald, AddA, acetaldehyde dehydrogenase, and acetyl-CoA synthetase assays and 70 mM potassium phosphate (pH 7.2) for Ack, acetyl-CoA hydrolyase, nitrate reductase, and nitrite reductase assays.

Other Methods—Nitrate, nitrite, ammonium, and acetate were measured by ion chromatography using a 761 Compact IC (Metrohm). N₂O and CO₂ were determined by gas chromatography (13). ATp was assayed using a luminometer (Lumitester K-21; Kikkoman) (12). Acetyl-CoA was determined as reported (14). Transmission electron microcopy photographs were obtained as described (15) using a JEM-1200 EX transmission electron microscope (JEOL Ltd., Tokyo, Japan).

RESULTS

Alternative Anaerobic Metabolism of Nitrate by F. oxysporum—We showed that the induction of denitrifying activity in the fungus F. oxysporum MT-811 requires a minimal amount of aeration (hypoxic conditions) and that the recovery of nitrate-nitrogen into N₂O (denitrification yield) varies considerably depending on the extent of the O₂ supply (13). The coexistence of ammonium in the medium should improve the denitrification yield, because ammonium generally represses the assimilatory use of nitrate. Here we incubated hypoxic cultures (13) in the presence of both 10 mM nitrate and 10 mM ammonium to examine effects of ammonium on the denitrification yield (Fig. 1A). In agreement with prior observations (13), denitrification (N₂O formation) was induced over a long time course (stage 3). This time, however, we observed an unusual phenomenon during the lag period before denitrification was induced. Ammonium initially depressed nitrate use in assimilation, a characteristic of stage 1 cultures. At stage 2, nitrate levels began to decrease, and nitrate and ammonium concomitantly accumulated in the medium. At stage 3, nitrate decreased rapidly with concomitant evolution of N₂O. Gas chromatography-mass spectrometry with the stable isotope species of nitrate, ¹⁴NO₃⁻ and ¹⁵NO₃⁻, showed that ammonium ions, as well as N₂O (8), were derived from nitrate during anoxic culture (data not shown) (16). By contrast, the decrease or increase of each compound was linear over the entire course of the culture when ammonium was the sole nitrogen source (Fig. 1B).

Nitrate did not seem to be converted to ammonium for assimilatory purposes, because a sufficient amount of ammonium remained in the medium when the conversion began. On the other hand, cell growth was significantly increased when nitrate was added (cf. Fig. 1, A and B), indicating that nitrate metabolism is an energy-yielding process. These findings show that nitrate is metabolized in a dissimilatory pathway to form ammonium at stage 2 and N₂O at stage 3 (Fig. 1A). The evolution of N₂O was accompanied by the accelerated evolution of CO₂, indicating that the carbon source (ethanol) was decomposed as a respiration substrate for denitrification. In contrast, the rate of CO₂ evolution during stage 2 was much lower, suggesting that the reducing equivalent for the formation of ammonium was supplied by oxidizing ethanol to another C-2 compound.

Effects of Oxygen Supply on the Nitrate Metabolism—The effect of O₂ supply on nitrate metabolism by F. oxysporum MT-811 was examined using fed-batch cultures under various aeration conditions (0, 10, 20, or 30 ml O₂/h) in which nitrate was the only nitrogen source (Fig. 2). Ammonium (N₂O) was determined for each culture when nitrate and formed nitrite disappeared. The rate of nitrate conversion to ammonium was highest in the complete absence of an O₂ supply. With increasing aeration, the recovery of ammonium declined, and conversely, the evolution of N₂O increased. Less than 20% of nitrate was used for assimilation under these O₂-limited conditions. With an excess O₂ supply, neither ammonium nor N₂O was formed, although nitrate was consumed, indicating that
nitrate is utilized only for assimilation, and that accelerated cell-growth (cf. Fig. 3, right) depends on O₂ respiration.

Anoxic Cell Growth Coupled to the Ammonia Formation—The nitrogen source-dependent growth of flask cultures in the absence of O₂ (Fig. 3, left) indicates that nitrate supports more growth than ammonium. Because only slight denitrification occurs without O₂ (Fig. 2), nitrate-mediated anoxic growth must depend on nitrate metabolism to form ammonium. Aerobic cultures in the same medium increased enormously the yield of the cells (Fig. 3, right), supporting the conclusion derived from Fig. 2 that the anaerobic nitrate metabolism (ammonia formation and denitrification) is replaced by O₂ respiration when the O₂ supply is sufficient.

Morphology of Mitochondria—Fig. 4 shows transmission electron microscopy observations of mitochondria. Most mitochondria in the anoxic cells that formed ammonium (Fig. 4B) were immature and exhibited low electron density, in sharp contrast to the apparently intact mitochondria of cells grown under aerobic conditions (Fig. 4A) or in denitrifying cells (15). These results demonstrate that anoxic nitrate metabolism forms ammonium in a non-respiratory system that produces ATP. We term this method of eukaryotic nitrate metabolism “ammonia fermentation.”

Effect of Carbon Source on Ammonia Fermentation—We examined ammonia fermentation by F. oxysporum cultured in flasks under anoxic conditions with ethanol, glycerol, or glucose as the carbon source (Table I). Under these conditions, most nitrate-nitrogen was recovered into ammonium, consistent with the results shown in Fig. 2, and recovery of ammonium was high in the presence of ethanol. Ammonia fermentation was accompanied by acetate accumulation, as predicted above from the results in Fig. 1A. The stoichiometry of the formed ammonium and acetate was exactly 1:2 except for the culture with glucose. This

**FIG. 2.** Effects of aeration on nitrate metabolism. *F. oxysporum* was examined in fed-batch cultures in medium supplemented with 10 mM nitrate (without ammonium) under various aeration conditions (0, 10, 20, or 30 ml O₂/h). Ammonium (●) or N₂O (○) was determined after one cycle of each culture and expressed as % recovery of nitrogen atoms against consumed (i.e. added) nitrate. Nitrate utilized for assimilation (■) was estimated by subtracting total amounts of formed ammonium and N₂O from amount of initially added nitrate. Dry cell matter was estimated as average before and after a one-cycle culture.

**FIG. 3.** Cell growth during anoxic (left) and aerobic (right) cultures. *F. oxysporum* was incubated in the flask for 3 days in the presence of 10 mM ammonium and/or nitrate under anoxic or aerobic conditions. Dry cell matter was represented as the net growth that was obtained by subtracting the mass of the inoculated cells (0.4 g) from that after the culture.

**FIG. 4.** Photographs by transmission electron microscopy of *F. oxysporum* cells. The aerobic cells (A) were cultured in a 500-ml Sakaguchi flask containing 100 ml of medium (with 10 mM nitrate) at 30 °C for 24 h on a reciprocal shaker. The anoxic cells (B) were grown under ammonia-fermenting conditions as in Fig. 3 (left) in medium supplemented with only nitrate and harvested when ammonium began to form in the medium. Magnification, ×36,000. Arrowheads indicate mitochondria.

**TABLE I**

Stoichiometry between the products because of ammonia fermentation by *F. oxysporum*

The cells were incubated by the anoxic flask culture as in Fig. 3 (left) in the medium containing only nitrate (10 mM, 1 mmol) for 5 days, and each product was determined. Nitrate was completely consumed by the end of each culture with the exception of that with glucose in which a half of nitrate still remained.

| Carbon source | NH₄⁺ | NO₃⁻ | NH₄⁺ | N₂O | CO₂ | Acetate |
|---------------|-----|-----|-----|-----|-----|--------|
| Ethanol       | 0.8 ± 0.05 | 80 | 0.05 ± 0.02 | 10 | 0.45 ± 0.1 | 1.6 ± 0.2 |
| Glycerol      | 0.7 ± 0.05 | 70 | 0.06 ± 0.02 | 12 | 0.61 ± 0.1 | 1.4 ± 0.2 |
| Glucose       | 0.2 ± 0.05 | 40 | 0.03 ± 0.01 | 12 | 6.8 ± 0.8 | 0.6 ± 0.1 |

*% yield against consumed nitrate-N.
indicates that the reducing equivalent derived from 4-electron oxidation to form acetate is utilized in the 8-electron reduction of nitrate to ammonium, consistent with the oxidation of ethanol to acetate. The recovery of ammonium was low in cells cultured with glucose, and instead, much more CO₂ evolved, suggesting that alcohol fermentation was predominant.

**Enzymes Involved in Ammonia Fermentation**—The possible metabolic pathway described in Fig. 5 is based upon the above finding that ethanol is the best electron donor for ammonia fermentation and that the stoichiometry between the fermentation products, ammonium and acetate, is exactly 1:2. We determined the enzyme activities involved in each step using subcellular fractions prepared from cells cultured under aerobic (assimilatory, with respect to nitrate), hypoxic (denitrifying), or anoxic (ammonia-fermenting) conditions (Table II). AddA and the ATP-forming Ack activities were specifically induced only in anoxic cells that fermented ammonia, whereas other acetogenic activities (acetyl-CoA hydrolyase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase) were particularly low (or absent) in the cells. These results demonstrated that ethanol is oxidized successively by Ald, AddA, and Ack to acetate, coupled with ATP production and the reduction of nitrate to ammonia (Fig. 5), and that ammonia fermentation is an adaptive metabolism of the fungus *F. oxysporum*. These activities (Ald, AddA, and Ack) were recovered in the soluble and microsome fractions but not in the mitochondrial fraction, which would be consistent with the increasing population of damaged mitochondria in the anoxic cells (Fig. 4). NADH-dependent properties of nitrate reductase and nitrite reductase activities (Scheme 1) are quite distinct from those of the mitochondrial dissimilatory nitrate and nitrite reductases of the denitrifying cells (12) but are similar to the assimilatory reductases generally found among fungi.

**Screening of Other Ammonia-fermenting Fungi**—Ammonia-fermenting activity was screened in 17 fungal strains closely or distantly related to *F. oxysporum* MT-811 cultured under anoxic flask conditions as shown in Fig. 3 (left). Only two among the 17 strains tested did not exhibit this activity, and the following 15 strains distinctly converted nitrate to ammonium under anoxic conditions: *Talaromyces rotundus* (IFO; 9142), *Trichophyton rubrum* (IFO; 5807), *Hyalodendron* sp. (IFO; 31243), *Penicillium abeum* (IFO; 6239), *Petrosiella guttulata* (IFO; 8613), *Calonectria hyotensis* (IFO; 8962), *Hypocreanigricans* (IFO; 31290), *Hypomycetes trichothecoides* (IFO; 6892), *Neurospora crassa* (IFO; 6067), *F. oxysporum* (IFO; 30710), *F. oxysporum* (IFO; 9968), *Cylindrocarpon tonkinense* (IFO; 30561), *Gibberella fujikuroi* (IFO; 6349), *F. oxysporum* (Institute of Applied Microbiology, University of Tokyo; 5009), *Fusarium lini* (Institute of Applied Microbiology, University of Tokyo; 5008), and the following two strains did not: *Podospora carbonaria* (IFO; 31850), *Orbiinymes spectabilis* (IFO; 32157). Most of these strains are found in subdivisions of ascomycetina and ascomyceteous imperfect fungi with the exceptions of *Hyalodendron* sp. (Mastigomycotina) and *P. carbonaria* (Zygomyxotina).

**DISCUSSION**

The present study presents evidence that the denitrifying fungus *F. oxysporum* contains another anaerobic type of nitrate metabolism, which we refer to as ammonia fermentation. We also showed that ammonia fermentation and denitrification are alternatively expressed depending on the extent of the O₂ supply. Ammonia fermentation is expressed under the most anoxic conditions even in the complete absence of O₂ supply. Small but distinct cell growth during ammonia fermentation (see Fig. 1 and Fig. 3) should depend on substrate-level phosphorylation by Ack (acetate kinase), which is specifically induced in cells that ferment ammonia (Table II). Anoxic nitrate metabolism (ammonia fermentation) is replaced by denitrification (Fig. 2) with concomitant formation of intact mitochondria when the O₂ supply is limited, and under a sufficient supply of O₂, denitrification is further replaced by aerobic (O₂) respiration. Thus the fungus *F. oxysporum* that expresses diversified pathways of

**Table II**

| Fraction       | Activity (nmol each product indicated/min/mg protein⁻¹) | *NH₄⁺* | *NO₂⁻* | *NO₃⁻* | *NH₄⁺* |
|----------------|-------------------------------------------------------|-------|--------|--------|-------|
|                 | Aerobic (assimilation)                                |       |        |        |       |
| Soluble         | Ald (CH₃CHO) 175 ± 20 AddA (CH₃CO-CoA) 0 Ack (ATP) 0 | 400 ± 20 | 1250 ± 100 | 3125 ± 120 | 60 ± 9  |
|                 | Microsome 562 ± 40                                   | 310 ± 10 | 300 ± 20 | 760 ± 26 | 10 ± 2  |
|                 | Mitochondrion 0                                      | 0      | 0      | 40      | 20 ± 5  |
|                 | Hypoxic (denitrification)                            |       |        |        |       |
| Soluble         | Ald (CH₃CHO) 24 ± 4 AddA (CH₃CO-CoA) 3 ± 2 Ack (ATP) 5 ± 3 | 260 ± 15 | 1000 ± 30 | 1040 ± 88 | 24 ± 5  |
|                 | Microsome 76 ± 10                                    | 240 ± 20 | 260 ± 12 | 241 ± 14 | 8 ± 2   |
|                 | Mitochondrion 0                                      | 0      | 0      | 30 ± 4  | 0      |
|                 | Anoxic (Ammonia fermentation)                        |       |        |        |       |
| Soluble         | Ald (CH₃CHO) 4 ± 1 AddA (CH₃CO-CoA) 30 ± 12 Ack (ATP) 25 ± 4 | 130 ± 10 | 20 ± 5  | 40 ± 10 | 12 ± 6  |
|                 | Microsome 11 ± 3                                    | 205 ± 14 | 0      | 12 ± 6  | 0      |
|                 | Mitochondrion 0                                      | 0      | 0      | 18 ± 3  | 0      |
|                 | Anoxic (non-induced cells)                           |       |        |        |       |
| Crude extract   | 2 ± 2                                                | 34 ± 8 | 21 ± 5 | 10 ± 6  | 9 ± 4   |
|                 | Mitochondrion 0                                      | 2 ± 1  | 0      | 0      | 0      |

**Fig. 5.** Possible metabolic pathway for the fungal ammonia fermentation coupled to acetogenic oxidation of ethanol and substrate-level phosphorylation. Nir, nitrite reductase; Nar, Nitrate reductase; Add, Acetaldehyde dehydrogenase; Acs, Acetyl-CoA synthetase; Ath, Acetyl-CoA hydrolyase; Pi, inorganic phosphate.
Ammonia fermentation is limited when glucose was the carbon source (Table I). The ammonium recovery was low, but far more CO₂ evolved, suggesting that alcohol fermentation predominates over ammonia fermentation when glucose is available. *F. oxysporum* ferments alcohol, which has been understood for some time (17). The recovery of ammonium was highest when ethanol was the electron donor (Table I), indicating that ammonia fermentation acts physiologically as a secondary fermentation method that replaces primary alcohol fermentation when nitrate is available.

This fungal process is similar to acetogenic fermentation in prokaryotes, a reaction that is coupled to nitrate reduction and substrate-level phosphorylation reactions (18, 19). However, bacterial nitrate metabolism is highly restricted, arising only in a single genus of obligate anaerobes, *Clostridium*, and it is regarded as a primitive form of anaerobic respiration in these bacteria (20). It is therefore surprising to find anoxic nitrate metabolism (ammonia fermentation) in eukaryotes (fungi) that have been considered to date as aerobic organisms.

Recent advances in genome projects, along with our screening results (21), have revealed that cytochrome P450nor, a characteristic enzyme in fungal denitrifying systems that functions to produce energy. This study is the first to show that an organism (or eukaryote) can use a multimodal type of respiration (or ATP-producing) system to rapidly adapt to changes in the oxygen supply.

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Recent advances in genome projects, along with our screening results (21), have revealed that cytochrome P450nor, a characteristic enzyme in fungal denitrifying systems that functions as nitric-oxide reductase, is expressed widely among fungi. This indicates that denitrification is a key mechanism of nitrate metabolism in fungi. We further demonstrate here that ammonia is also fermented in the denitrifying fungus *F. oxysporum* MT-811 and in many other fungi that are common to soil. These results demonstrated that many such fungi should really be classified as facultative, rather than as obligate aerobes, although most fungi are in fact so far recognized as obligate aerobes. Advances in soil microbiology have revealed that the microbial biomass of temperate soils is usually dominated by fungi (22). Our present conclusion that many soil fungi are facultative anaerobes is consistent with this fact. The fungal prosperity in soil should be supported by a multirespiratory system, because the natural environment in soil with respect to oxygen supply is highly variable, and the ability to immediately adapt to rapid changes should be a potent tool for survival. The present results should thus evoke interest in how eukaryotes have evolved such a variety of metabolic mechanisms to produce energy.

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