Dissimilatory Nitrate Reduction Metabolisms and Their Control in Fungi

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Most fungi grow under aerobic conditions by generating ATP through oxygen respiration. However, they alternatively express two pathways of dissimilatory nitrate reduction in response to environmental oxygen tension when the oxygen supply is insufficient. The fungus Fusarium oxysporum expressed the pathway of respiratory nitrate denitrification that is catalyzed by the sequential reactions of nitrate reductase and nitrite reductase. These enzymes are coupled with ATP generation through the respiratory chain and produce nitric oxide. Fungal nitric oxide reductase uses NADH as the direct electron donor in contrast to bacterial systems and thus might function in regeneration of NAD+ and detoxification of the toxic radical, nitric oxide. Another pathway of nitrate dissimilation by fungi reduces nitrate to ammonium and couples acetogenic reaction with substrate-level phosphorylation. This metabolic mechanism is also in feature of a variety of fungi and it is called ammonia fermentation. Thus, fungi adapt to various aerated conditions using these pathways of nitrate dissimilation in addition to conventional oxygen respiration.

FIG. 1. Comparison of the denitrifying systems of bacteria and fungi. Nar, Nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; N2Or, nitrous oxide reductase.
of some enterobacteria are membrane-bound complexes that generate a proton electrochemical gradient, and functions in anaerobic (NO₃⁻) respiration. In contrast, ammonification by obligate anaerobes such as clostridia is not linked to the respiratory chain and occurs in the cytoplasm (4, 5). The reaction is slower than that by the enterobacteria and since it is coupled to substrate level phosphorylation, it might be a fermentative metabolic system (ammonia fermentation) (4, 5).

For over a century, denitrification and ammonification had been considered processes of prokaryotes until Shou et al. identified denitrification in Fusarium oxysporum (6) and in other fungi (7) which were long thought to be strictly aerobic. Fungal denitrification is apparently a hypoxic metabolism as it proceeds in the presence of NO₃⁻ under a lower O₂ concentration (8). Thereafter, we also showed that ammonia fermentation occurs in F. oxysporum and in many other fungi under anaerobic conditions (9). These results initially indicated that eukaryotes can use a complex ATP-producing system under hypoxic or anoxic conditions (1–3) except for some obligatory anaerobic fungi that inhabit anoxic environments such as swamps and intestines (10). This review describes recent studies on the dissimilatory fungal mechanisms of NO₃⁻ and discusses their physiological significance. Mechanisms by which the fungus can regulate the metabolisms for survival under conditions of depleted O₂ are also discussed.

I. DENITRIFICATION BY FUNGI

Since fungal denitrification was reported in 1991 (6), the denitrifying system of F. oxysporum has been intensely studied. This fungus denitrifies NO₃⁻ to N₂O whereas others evolve N₂ by co-denitrification (11). F. oxysporum consistently denitrifies in line with the criteria for respiratory denitrifiers proposed by Mahne and Tiedje (12). These are recovery of nitrogen atoms of NO₃⁻ to gaseous nitrogen species (N₂O) of over 80% and a production rate above 10 μmol N₂O min⁻¹ g protein⁻¹ (6, 8). Diauxic growth is observed in cultured F. oxysporum during the transition from O₂ respiring to denitrifying conditions (13). Mitochondrial Nir activity is coupled to the synthesis of ATP (described below). These findings indicated that fungal denitrification physiologically acts as anaerobic (NO₃⁻) respiration. The cell-free activities of Nar, Nir, and Nor could be reconstituted using cell-free extracts of F. oxysporum (6, 14, 15), that are responsible for N₂O production. The overall denitrifying system of the fungus is compared with that of bacteria in Fig. 1.

Respiratory reduction of nitrate and nitrite Cell-free Nir activities were reconstituted using the NADH-phenadinedemethosulfate system as the electron donor with F. oxysporum (15), Cylindrocopron tonkinense (14), and Fellomycetes fuzhouensis (16). The Nir of C. tonkinense catalyzes the reduction of NO₃⁻ to NO coupled to ATP synthesis in the mitochondria (14), suggesting that Nir is involved in respiratory denitrification by this fungus. In contrast to Nir, Nar is rare in fungi. The Nar of F. oxysporum might be localized to the mitochondria and supported by common respiration substrates such as malate, pyruvate and sucrose.

A membrane-bound Nar from the mitochondrial fraction of F. oxysporum was recently solubilized, partially purified and characterized (16). The enzyme activity of Nar was supported by a physiological electron donor in the respiratory chain (ubiquinol), showing that it is distinct from the soluble, NADH-dependent NO₃⁻ reductase that is used for assimilating NO₃⁻. Furthermore, the spectral properties, molecular weight and cofactors for the fungal Nar were similar to those of the dissimilatory Nar of Escherichia coli or denitrifying bacteria that is comprised of molybdopterin, a cytochrome b, and an iron-sulfur protein (3, 16). Formate-NO₃⁻ oxidoreductase activity was detected in the mitochondria, which arose from the coupling of formate dehydrogenase (Fdh), Nar, and the ubiquinone/ubiquinol pool. This is the first report of a eukaryotic Fdh that is associated with the respiratory chain. Coupling with Fdh indicated that the fungal Nar system is more similar to that involved in the NO₃⁻ respiration (ammonification) by E. coli than that in the bacterial denitrifying system (16).

NO reduction — NO detoxification or electron sink? In denitrifying F. oxysporum cells, cytochrome P450nor (P450nor) that catalyzes reduction of NO to N₂O has been thought to function as Nor (6, 17). The involvement of cytochrome P450 is the most characteristic feature of the fungal system, since it is in marked contrast to the bacterial Nor of cytochrome bc (1–3). P450nor have been purified from several fungi such as F. oxysporum (17), C. tonkinense (18) and Trichosporon cutaneum (19) and their corresponding genes have been cloned. The crystal structure of F. oxysporum P450nor is essentially similar to that of proteins in the cytochrome P450 superfamily (20). Unlike Nar and Nir, the fungal Nor (P450nor) receives electrons directly from NADH, suggesting that it is not directly associated with the mitochondrial respiratory chain (6, 14, 17).

Two isoforms of P450nor (P450norA and P450norB) are encoded by the CYP55 gene in F. oxysporum (21). They differ in terms of their initiation sites for translation: P450norA is translated from the initiation codon followed by a putative mitochondrial targeting signal, whereas P450norB is translated from an initiation codon without the signal sequence located downstream from that of P450norA (22). I examined their intracellular localization by enzymatic, spectroscopic, and immunological means to show that both mitochondria and the cytosol contain P450nor. Translational fusions between the putative mitochondrial targeting signal of P450norA and E. coli β-galactosidase resulted in significant β-galactosidase activity in the mitochondrial fraction, suggesting that P450norA locates in the anaerobic mitochondria of F. oxysporum and reduces NO (22). The other isoform (P450norB) is localized in the cytosol. Since NO can be dispersed in biological membranes, NO produced by mitochondrial Nir leaks into the cytosol where it is used as a substrate by the cytosolic isoforms P450norB.

To establish the physiological significance of P450nor, F. oxysporum mutants lacking the P450nor gene were constructed (23). The mutants apparently lost P450nor protein, Nor activity, denitrifying activity and failed to evolve N₂O under denitrifying conditions (23). The level of Nir activity in the mycelia was normal and NO was evolved under deni-
trifying conditions. These results presented unequivocal genetic evidence supporting the notion that P450nor is essential for the reduction of NO to N\textsubscript{2}O (23). Since P450nor might be an electron sink as well as a system that can detoxify the reactive nitrogen radical NO under denitrifying conditions (6, 14), that the loss of P450nor did not affect cell growth under denitrifying conditions is perplexing. One explanation might be the involvement of flavohemoglobin (fhh), which has recently been identified in this fungus.

Fhb have been isolated from various microorganisms as well as from denitrifying \emph{F. oxysporum} (24, 25). It catalyzes the reduction of NO to NO\textsubscript{2} and its physiological function might be to scavenge hazardous NO (25). P450nor and fhb share the same substrate, namely, NO\textsubscript{3}, which is produced by Nar in this denitrifying fungus. It was recently found that the mutant strain does not produce either fhb or P450nor, that its growth was reduced and its respiratory systems were damaged. The introduction of either of the genes encoding fhh or P450nor restored these functions, suggesting that these proteins comprise an NO degrading system that is essential for growth and which counteracts the toxic effects of NO (Takaya, unpublished result). The filamentous fungi would adapt to the conditions of NO\textsubscript{3} respiration that produce harmful nitrogen oxide species as reaction intermediates and/or byproducts by diversifying the functions of cytochrome P450.

**Distribution of fungal denitrifiers** Subsequent to the initial screening by Kim et al. (7), I systematically searched and identified fungal denitrifiers (26). Among 67 fungal strains tested, sixteen of them, including \emph{F. fuzhouensis} JCM7367, \emph{T. cutaneum} JCM2390, and \emph{T. cutaneum} JCM2391 evolved measurable amounts of N\textsubscript{2}O from NO\textsubscript{2} (14). P450nor and its gene have recently been isolated from \emph{T. cutaneum} (19) although no cell-free Nor activity has yet been detected in \emph{F. fuzhouensis}, suggesting a different mechanism of denitrification. The genera \emph{Fellomyces} and \emph{Trichosporon} are anamorphs of basidiomycetous yeast (26) while the strains found by Kim et al. are ascomycetes and zygomycetes (7). Thus, fungal denitrifiers are randomly, rather than systematically distributed among fungi.

**II. AMMONIA FERMENTATION BY FUNGI**

Another mechanism of NO\textsubscript{3} metabolism in \emph{F. oxysporum} cells was described. This process consists of NO\textsubscript{3} reduction to NH\textsubscript{4}\textsuperscript{+} coupled with the catabolic oxidation of electron donors (ethanol) to acetate and substrate-level phosphorylation that supports growth under anaerobic conditions (Fig. 2). This process resembles ammonification by the obligatory anaerobe \emph{Clostridium}, and is termed ammonia fermentation (9). It was also shown that 15 of 17 tested fungal strains ferment ammonia under anaerobic conditions, suggesting that this activity is widely distributed among fungi (9). The key reactions of ammonia fermentation are the conversion of acetaldehyde to acetyl-CoA by acetaldehyde dehydrogenase (AddA) and the hydrolysis of acetyl-CoA by the ATP-forming acetate kinase (Ack). These reactions have been identified only in the obligatory anaerobe \emph{Clostridium} (5) and they do not appear to be a feature of eukaryotic cells. The activities of these and the other enzymes involved in ammonia fermentation were reconstituted using cytosolic fractions of \emph{F. oxysporum}, which supported the notion that ammonia fermentation is coupled to substrate-level phosphorylation (9). The NADH-dependent properties of NO\textsubscript{3} and NO\textsubscript{2} reductase activities (Fig. 2) are quite distinct from those of the dissimilatory mitochondrial Nar and Nir of denitrifying cells (14), but are similar to the assimilatory reductases generally found among fungi.

**III. REGULATION OF FUNGAL HYPOXIC METABOLISM**

Since fungal denitrification appeared to proceed under hypoxic conditions, I examined the effects of O\textsubscript{2} on the denitrification and ammonia fermentation activities of \emph{F. oxysporum} (8). The results revealed that the fungal denitrifying activity requires a minimal amount of O\textsubscript{2} for induction, and is repressed by excess O\textsubscript{2}. The optimal O\textsubscript{2} supply differed between the denitrification substrates: 690 and 250 μmol O\textsubscript{2} h\textsuperscript{−1} g\textsuperscript{−1} dry cells for NO\textsubscript{3} and NO\textsubscript{2}, respectively. With an optimal O\textsubscript{2} supply, 80% and 52% of nitrogen atoms in NO\textsubscript{3} and NO\textsubscript{2} respectively were recovered as the denitrification product N\textsubscript{2}O. A minimal O\textsubscript{2} requirement is a unique feature of \emph{F. oxysporum} denitrification that differs from that of bacterial denitrifiers that function exclusively under anoxic conditions. Unlike denitrification, ammonia fermentation by the fungus is induced under conditions that are more anoxic than those of denitrification (0 μmol O\textsubscript{2} h\textsuperscript{−1} g\textsuperscript{−1} dry cell) (9). \emph{F. oxysporum} thus has two pathways of dissimilatory NO\textsubscript{3} reduction, which are alternatively expressed in response to environmental O\textsubscript{2} tension when the O\textsubscript{2} supply is sufficient. These hypoxic metabolisms are adaptive mechanisms that function when the fungus could not respire O\textsubscript{2}.

The regulatory system of \emph{CYP55} gene expression, which encodes P450nor, was investigated (27). A promoter-reporter analysis of \emph{F. oxysporum} \emph{CYP55} using the fusion gene between the gene promoter for \emph{CYP55} and the \emph{E. coli} β-galactosidase gene showed that the region between nu-
presses hypoxic genes under aerobic conditions) is responsible for the binding consensus sequence of the yeast Rox1p that represses transcription factors.

Results indicate that the fungus adapts to denitrifying conditions through a combination of NirA- and Rox1-like transcriptional factor NirA, which induces expression of the NO$_3^-$assimilatory genes of Aspergillus nidulans in the presence of NO$_3^-$ (Fig. 3). This indicates that CYP55 expression is concomitantly regulated with the NO$_3^-$assimilatory genes. This mechanism makes sense as both NO$_3^-$ assimilation and denitrification use NO$_3^-$ as a substrate and as an inducer in this fungus. Deletion studies also indicate that the nucleotide sequence between –118 and –107 (which is similar to the binding consensus sequence of the yeast Rox1p that represses hypoxic genes under aerobic conditions) is responsible for CYP55 repression under hypoxic conditions. These results indicate that the fungus adapts to denitrifying conditions through a combination of NirA- and Rox1-like transcription factors.

IV. APPLICATION TO WASTE WATER TREATMENT

The atmospheric concentration of the greenhouse gas, N$_2$O, is continuously increasing. This gas is evolved from artificial processes such as waste water treatment plants where nitrogen contaminants are removed by denitrifying bacterial activity in activated sludge. As described above, N$_2$O is the final product of fungal denitrification, whereas most bacterial denitrifiers produce N$_2$O due to insufficient anaerobiosis, since the reducing step of N$_2$O is the most sensitive among all the reduction steps involved in denitrification (1–3). To overcome this, I genetically manipulated a denitrifying bacterium to suppress N$_2$O production during denitrification (28). The typical denitrifier Pseudomonas stutzeri strain producing F. oxysporum fhb emitted less N$_2$O than the wild-type strain under both aerobic and anaerobic conditions. The rate of N$_2$ production was higher than that in the wild-type strain after the depletion of O$_2$ in culture, suggesting that fhb enhances the reduction of N$_2$O to N$_2$ (28). At present, the mechanism by which fhb suppress N$_2$O production has not been clarified, but fhb might affect directly or indirectly denitrification through NO-metabolism. The novel strains should be useful tools with which to design processes that do not produce N$_2$O during denitrification.

CONCLUDING REMARKS

This review briefly described recent advances in studies of fungal dissimilatory NO$_3^-$ reduction systems. After the discovery of fungal denitrification, hypoxic dissimilatory mechanisms were discovered in microorganisms previously thought to be strictly aerobes. Denitrification by actinomycetes (29) and the dissimilatory NO$_3^-$ reduction to NO$_2^-$ by Bacillus subtilis (30) are examples. These findings imply that other aerobic microorganisms have the potential for anaerobic respiration. The fungal NO$_3^-$ dissimilation described here also evokes several questions from the perspectives of evolution and ecology. Current dogma states that eukaryotic mitochondria developed an O$_2$ respiration system from a denitrifying system after protomitochondria were generated by symbiosis with a bacterial denitrifier. This begs the questions as to whether the mitochondrial denitrification system of the fungus is a recent development or a remnant of the protomitochondria and whether fungal NO$_3^-$ dissimilation is so widespread in nature as to affect global nitrogen. Future investigations into fungal hypoxic dissimilation should help answer these questions.

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