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Analysis of the Novel Nitrate Dissimilatory Mechanisms by Filamentous Fungi

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Filamentous fungi are important in Japanese fermentation industries and their roles in the fermentation processes have been extensively studied. Most of them grow well under the aerobic conditions and thus they have been long thought to be aerobic organisms, however, a variety of filamentous fungi were shown to employ the anaerobic metabolic mechanisms, nitrate (NO_3^-) respiration and ammonia (NH_3) fermentation. These metabolic mechanisms had been exclusively described in facultative and restricted anaerobic bacteria, respectively, thus these findings are surprising and implicate that the filamentous fungi might adapt into changes of environmental oxygen (O_2) tension by using these complicated mechanisms for growth. Until now, studies of Dr. Shoun and his colleagues have revealed the components involved in the mechanisms. The fungus *Fusarium oxysporum* respires with NO_3^- under the O_2 limited conditions by the sequential reaction of NO_3^- reductase, nitrite (NO_2^-) reductase, and nitric oxide (NO) reductase, by which NO_3^- is reduced to nitrous oxide (N_2O) (NO_3^- respiration). These enzymes are located in mitochondrion and are considered to be physiologically significant for producing ATP for growth through respiratory electron transferring chain. The fungal NH_3 fermentation is occurred under more anoxic conditions than NO_3^- respiration. It was originally found in *F. oxysporum*. This process consists of NO_3^- reduction to NH_4^+ coupled with the catabolic oxidation of electron donors (ethanol) to acetate and substrate-level phosphorylation that supports growth under anaerobic conditions. This process resembles ammonification by the obligatory anaerobe *Clostridium*, and is termed NH_3 fermentation. It was also shown that 15 of 17 tested fungal strains ferment NH_3 under anaerobic conditions, suggesting that this activity is widely distributed among fungi. In the mechanism, cytosolic NAD(P)H-dependent NO_3^- and NO_2^- reductases, which are similar to assimilating NO_3^- and NO_2^- reductases, are responsible for substrate level phosphorylation.

This study describes properties of the genetically manipulated and the naturally occurred mutants of the fungi that have lesions in the possible genes involved in the fungal anaerobic metabolisms. The data presented here give a conclusive evidence of the components and physiological significance of these fungal metabolic mechanisms.

1. Physiological function of NO reduction and oxidation by *F. oxysporum*

Although NO_3^- respiration (denitrification) is important for microorganisms to grow under anoxic conditions, it could often produce NO, which is hazardous for cellular components and their activity since NO is a free radical concerning to various biological functions. Most microorganisms have an NO degrading enzyme flavohemoglobin (fhb), to survive against the damaging effects of exogenous NO. This study demonstrated that the fungus *F. oxysporum* produced NO under the NO_3^- respiratory condition and induced production of two hemoproteins, fhb and cytochrome P450nor (P450nor). Both proteins have been known to metabolize NO to less hazardous forms, N_2O and NO_3^-, respectively. Detailed investigation of the mutants defective in either protein showed that they had a redundant function for supporting cell growth against highly toxic NO stress. In contrast, only P450nor rescued mitochondrial damages such as inhibition of respiration and breakage of cellular iron-sulfur proteins caused by NO, indicating that fhb and P450nor could have different targets dependent with the targets for eliminating NO stress. They must constitute a dual heme NO degrading system that is specific for filamentous fungi since both the hemoproteins are unique for microorganisms and P450nor is only seen in filamentous fungi. This observation is also significant on P450 biology since P450nor is the first example that might make evolution for detoxifying.

2. Fungal NH_3 fermentation and genes involved in the process

Studies on NH_3 fermentation mechanisms in *F. oxysporum* showed that the key reactions of NH_3 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 *Clostridium* and they do not appear to be a feature of eukaryotic cells. Here I investigated the mechanisms by using *Aspergillus nidulans* as a model fungus. Mutant strains in the loci required for NO_3^- assimilation were tested for ability of NH_3 fermentation. The results showed that *niaD* and *niiA* that code for NO_3^- and NO_2^- reductases, respectively were critical for NH_3 fermentation as well as for NO_3^- assimilation. These correspond well to the previous observation of *F. oxysporum* NH_3 fermentation system and suggested both enzymes function in both assimilation and dissimilation of NO_3^-.

The activities of AddA and Ack were able to be reconstituted using cytosolic fractions of *A. nidulans* as well as those of *F. oxysporum*. Interestingly, the loss-of function mutant in the *facA* gene, which codes for acetyl CoA synthetase (Acs), completely lost Ack activity and fermentative production of NH_3. Since Ack catalyzes reverse reaction of Acs, it is suggested that *facA* is responsible for Ack activity under the NH_3 fermentation conditions in *A. nidulans*.

3. Post-transcriptional modification of FacA regulates enzymic reactions

Acs is widely distributed from prokaryotic to eukaryotic cells and the reaction is shown to be reversible. Recombinant FacA protein purified from normal aerobically-growing cells of *A. nidulans* exhibited higher Acs activity and little Ack activity under the physiological concentration of the substrates. By contrast, FacA protein purified from the NH_3-fermenting *A. nidulans* showed less Acs activity than that from aeroically growing cells. It is surprising that the latter FacA protein exhibited considerably high Ack activity. Steady state kinetics showed that the difference of these forms of Acs is dependent with affinity (K_m values) for the substrates. The FacA protein from the NH_3-fermenting cells showed slightly higher molecular mass on SDS-PAGE and was reacted more strongly with anti-acetyl lysine antibody. These results indicates that *A. nidulans* acetylates some lysine residue(s) of FacA protein in response to the environmental O_2 concentration to regulate its affinity for the substrates, which reversed the reaction from Acs to Ack. Detailed regulating mechanisms of the FacA protein is now under investigation.