Review

Fungal denitrification and nitric oxide reductase cytochrome P450nor

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We have shown that many fungi (eukaryotes) exhibit distinct denitrifying activities, although occurrence of denitrification was previously thought to be restricted to bacteria (prokaryotes), and have characterized the fungal denitrification system. It comprises NirK (copper-containing nitrite reductase) and P450nor (a cytochrome P450 nitric oxide (NO) reductase (Nor)) to reduce nitrite to nitrous oxide (N2O). The system is localized in mitochondria functioning during anaerobic respiration. Some fungal systems further contain and use dissimilatory and assimilatory nitrate reductases to denitrify nitrate. Phylogenetic analysis of nirK genes showed that the fungal-denitrifying system has the same ancestor as the bacterial counterpart and suggested a possibility of its proto-mitochondrial origin. By contrast, fungi that have acquired a P450 from bacteria by horizontal transfer of the gene, modulated its function to give a Nor activity replacing the original Nor with P450nor. P450nor receives electrons directly from nicotinamide adenine dinucleotide to reduce NO to N2O. The mechanism of this unprecedented electron transfer has been extensively studied and thoroughly elucidated. Fungal denitrification is often accompanied by a unique phenomenon, co-denitrification, in which a hybrid N2 or N2O species is formed upon the combination of nitrogen atoms of nitrite with a nitrogen donor (amines and imines). Possible involvement of NirK and P450nor is suggested.

Keywords: denitrification by fungi; co-denitrification; P450nor; mitochondrial anaerobic respiration; NirK

1. INTRODUCTION

The nitrogen cycle performed by micro-organisms comprises three processes; nitrogen fixation, nitrification and denitrification. The cycle is very important for life and global environment, providing nitrogen to life as nutrition and maintaining homeostasis of the Earth. Denitrification is the reverse reaction of nitrogen fixation in the sense that it carries fixed nitrogen back to the atmosphere. The major source of global nitrous oxide (N2O) emissions are the microbial activities of nitrification and denitrification. Therefore, the control and understanding of microbial denitrification is most important for reducing N2O emissions. Features of bacterial-denitrifying systems are well characterized at a molecular level [1–3]. The bacterial-denitrifying system comprises four reducing steps; NO3⁻ → NO2⁻ → NO → N2O → N2, each of which is catalysed by a dissimilatory nitrate reductase (dNar), dissimilatory nitrite reductase (dNir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos), respectively. The reducing equivalents for these reactions are provided from the respiratory chain coupling to the synthesis of adenosine triphosphate (ATP), and thus bacterial denitrification functions as anaerobic respiration. Previously, organisms involved in the nitrogen cycle were thought to be restricted to bacteria (prokaryotes). About two decades ago, we showed that many fungi and yeasts (eukaryotes) also exhibit distinct denitrifying activities [4–6]. Before our finding, there were many papers reporting that fungi can evolve a small amount (at most 15%) of N2O from, in most cases, nitrite, and thus they may exhibit denitrifying activity [7]. However, these papers only reported the simple observation without providing any evidence that the small amount of N2O evolution by fungi is a biological reaction. In contrast, we have characterized the denitrifying system of fungi at the molecular level (identifying both proteins and genes), mainly employing two fungal species, Fusarium oxysporum strain MT811 (JCM11502) and Cylindrocarpon tonkinense IFO (NITE Biological Resource Center; NBRC) 30561. The most characteristic feature of the fungal-denitrifying system is the involvement of cytochrome P450 (P450) as nitric oxide reductase (P450nor) [8,9]. Since then, many papers from other groups have also shown that fungal denitrification functions in nature as a major process in the nitrogen cycle [10–13].

2. FUNGAL-DENITRIFYING SYSTEM

The denitrifying systems of F. oxysporum MT811 (JCM 11502) and C. tonkinense IFO (at present, NBRC)
30561 are depicted in Figure 1. They comprise the minimal couple, NirK (copper-containing dNir) and P450nor. The fungal system seems to lack Nos and thus the final product is N₂O. Both systems were shown to function as the mitochondrial anaerobic respiration [14].

Fusarium oxysporum MT811 also contains dNar that resembles the bacterial counterpart, NarGHI. The fungal system of F. oxysporum MT811 is also unique in that dNar is supported by a ubiquinone-dependent formate dehydrogenase (UQFdh) [15,16]. The couple of dNar and UQFdh from Escherichia coli is well characterized but not known among denitrifying bacteria. We suggested that the same electron transport system comprising PfL, UQFdh and dNar is also functioning in F. oxysporum MT811 [17]. The importance of formate as the electron donor to the fungal-denitrifying system in natural environments was recently demonstrated [11]. Fungal denitrification requires a minimal amount of oxygen supply [18]. Under such conditions oxygen respiration and denitrification occur simultaneously (hybrid respiration) [16] in intact mitochondria [18] (Figure 1). Fusarium oxysporum MT811 contains aNar that resembles the bacterial counterpart, aNar. The assimilatory nitrate-reducing system is ubiquitously distributed among plants and micro-organisms to provide the nitrogen atoms of nitrate as nutrition for life. Assimilatory and dissimilatory nitrate-reducing systems were previously thought to function independently of one another. Therefore, involvement of aNar or assimilatory nitrate-reducing system (aNar and aNir) in denitrification [20] and ammonia fermentation [18] are the first instance of use of the assimilatory system for dissimilatory purpose (for producing ATP). In addition to the difference between the denitrifying system of F. oxysporum and C. tonkinense, their carbon sources are also different. In F. oxysporum, denitrification is repressed and heterolactic acid fermentation dominates when glucose is available under anoxic conditions [19]. In C. tonkinense, denitrification is not repressed by glucose, but works in parallel with glycolysis via the pentose phosphate shunt [21]. Nicotinamide adenine dinucleotide phosphate (NADPH)-specific P450nor isozyme (P450nor2), which is localized in the cytosol, functions as an electron sink for the pentose phosphate shunt.

The eukaryotic NirK protein and its gene were firstly isolated from F. oxysporum MT811 [22,23]. Membrane-bound dNir protein was partially purified from C. tonkinense [24]. The mitochondrial dNir (C. tonkinense) along with dNar (F. oxysporum) activities were shown to be associated with the respiratory chain coupling to the synthesis of ATP [14] (Figure 1). This is the first proof of the occurrence of anaerobic respiration in mitochondria [25]. Recent genome analyses have revealed the presence of nirK gene homologues in many genomes of eukaryotes,
including fungi, protozoa and green algae. We showed that all of these eukaryotic homologue genes along with the nirK genes of *F. oxysporum* and *C. tonkinense* [26] form a closely related group (clan) sharing the same ancestor, in sharp contrast to the random distribution of nirK and nirS (encoding cytochrome cd_{1} type dNir) genes among denitrifying proteobacteria [23]. Further, no gene homologous to nirS is found among fungal genomes. From these results, we proposed the possibility that eukaryotic nirK genes along with fungal-denitrifying systems originate from the protomitochondrion (the endosymbiont that gave rise to the mitochondrion) [23].

The genome analyses have also revealed that not only nirK homologue genes but also the genes homologous to CYP55 (P450nor) and nap (periplasmic nitrate reductase) are found in many fungal genomes. Our **BLAST** results indicated that out of 72 fungal genomes 19 contained nirK homologues, 16 contained CYP55 and 15 contained *nap* homologues (26.4–20.8%). And many of these genomes contained both nirK and CYP55. The high ratio of the appearance of nirK and CYP55 homologues suggests that the denitrifying system comprising NirK and P450nor is widely distributed among fungi. By contrast, no genome contained a *narGHI* homologue, suggesting that the fungal-denitrifying system that contains NarGHI like that of *F. oxysporum* MT811 is minor. The significance of the presence of *nap* homologues in many fungal genomes remains to be elucidated.

### 3. CO-DENITRIFICATION

Fungal denitrification is often accompanied by a unique phenomenon, co-denitrification, in which a hybrid N_{2} or N_{2}O species is formed upon combination of nitrogen atoms from nitrite and other nitrogen compounds (nitrogen donor) [5,27]. A similar phenomenon was later found in the anammox reaction [28]. The ratio of denitrification and co-denitrification varies depending on the conditions (fungal strains and nitrogen donors). The co-denitrification product (N_{2} or N_{2}O) varies depending on the redox state of the nitrogen donor. Amines provide N_{2} [5] whereas imines or azide form N_{2}O [27] as the co-denitrification product, as shown below.

\[
^{15}\text{NO}_{2} + \text{R}_{14}\text{NH}_{2} \rightarrow ^{15}\text{N}^{14}\text{N}
\]

\[
^{15}\text{NO}_{2} + ^{15}\text{NHOOH} \rightarrow ^{15}\text{N}^{14}\text{NO}
\]

P450nor was shown to catalyse the co-denitrification reaction forming N_{2}O and N_{2} from NO and azide [29]. An external electron donor such as nicotinamide adenine dinucleotide (NADH) is not necessary, indicating that the nitrogen donor also functions as an internal electron donor to reduce NO. It would therefore appear that the direct reactant is NO rather than nitrite in the co-denitrification reaction. In co-denitrification, nitrite would be reduced to NO by dNir (NirK), followed by the co-denitrification reaction by P450nor.

**Fusarium solani** IFO (NBRC) 9425 exhibits potent co-denitrification activity [5]. Among the three fungal strains tested (*F. oxysporum* MT811, *C. tonkinense* and *F. solani* IFO 9425), *F. solani* exhibited the highest co-denitrification activity against the nitrogen donor (aniline; figure 2). The recovery of nitrogen atoms of nitrite and aniline into N_{2} is high (usually more than 50%), as shown in figure 2. Inhibitors of NirK (diethylidithiocarbamate and cyanide) strongly inhibited co-denitrification, suggesting involvement of NirK in the reaction. The same product (N_{2}) was formed when nitrite was replaced with NO (L. Jiang & H. Shoun 2009, unpublished results), again suggesting that the direct reactant is NO rather than nitrite. Therefore, the fungal-denitrifying system can produce N_{2} as the co-denitrification product, although it cannot form N_{2} by denitrification. Laughlin & Stevens [10] reported fungal dominance of denitrification and co-denitrification in a grassland soil. It therefore appears that both

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*Phil. Trans. R. Soc. B* (2012)
co-denitrification as well as fungal denitrification occur generally in nature.

4. CYTOCHROME P450nor (FUNGAL NITRIC OXIDE REDUCTASE)
P450nor was first isolated from *F. oxysporum* MT811 as a haem protein possessing lipoxygenase activity and the properties of P450 [30]. Of course, the real function of the haem protein as Nor was not then known. The serendipitous finding that the P450 is specifically induced by nitrate (or nitrite) [31] led us to discover fungal denitrification [4]. Isolation of the gene showed that the haem protein belongs to the P450 superfamily, with the family number 55 (CYP55) being identified [32]. Interestingly, in spite of its eukaryotic origin CYP55 exhibits a closer relationship to bacterial P450s than to eukaryotic P450s (figure 3). The amino acid sequence of CYP55 shows sequence identities to the bacterial (actinomycetes) CYP105 members as high as about 40 per cent. So we suggested that the fungus had acquired the P450 gene from actinomycetes by horizontal transfer [32]. Once the fungal denitrification was found, it was rather easy to find the physiological function of CYP55 as Nor (P450nor) [8] because it was involved in denitrification. However, it took 10 years after its isolation to elucidate its physiological function. Surprisingly, P450nor could receive electrons directly from NADH. This phenomenon seemed to oppose the central dogma of physiological electron transfer, because two electrons of NADH are transferred simultaneously as a hydride ion (H−) and thus a one-electron redox centre such as haem can never receive the two electrons directly. P450 usually receives electrons from NAD(P)H via an electron transport system (redox partner) containing a flavoprotein. P450 can be classified depending on the type of redox partner (figure 4). Bacterial and mitochondrial P450s are supplied with electrons by the couple ferredoxin reductase and ferredoxin, whereas eukaryotic (microsomal) P450s are supplied by a P450 reductase containing FAD and FMN. P450nor is an exceptional P450 [33] that does not require a redox partner (direct electron transfer from NAD(P)H).

The reaction mechanism of P450nor has been extensively studied [34,35]. The turnover of the overall reaction, 2NO + NADH + H+ ! N2O + H2O + NAD+, is very rapid: 1000 s−1 at 10°C, and thus should be of the order of 105 min−1 or more at 25°C [34]. The overall reaction can be divided into three steps (figure 5). The first substrate (NO) binds to ferric (Fe3+) P450nor to form a ferric-NO complex (Fe3+–NO). Fe3+–NO is then reduced with NADH to form a specific intermediate (I) with a Soret absorption peak at 444 nm (Fe3+–NO + NADH + H+ ! I + NAD+). Finally, I interacts with the second NO to form N2O (I + NO ! N2O + H2O). The chemical entity of I was proposed to be ferric-hydroxylamine radical complex, as shown in figure 5 [35]. The reaction mechanism (figure 5) is also supported by a quantum-chemical calculation [36].

P450nor is localized to both mitochondria and cytoplasm in the fungal cells. *Fusarium oxysporum* and
C. tonkinense localize P450nor in different manners. Two P450nor isoforms of F. oxysporum are derived from a single gene (CYP55A1). P450norA of F. oxysporum is translated from the first initiation codon of the gene including the mitochondrial targeting signal, whereas P450norB is translated from the second initiation codon below the targeting signal and is thus localized to cytosol [37,38]. Cylindrocarpon tonkinense contains two P450nor genes: for P450nor1 (CYP55A2) and P450nor2 (CYP55A3) [39]. CYP55A2 contains a sequence for a mitochondria-targeting signal, whereas CYP55A3 does not. P450nor1 specifically employs NADH as the electron donor, while P450nor2 prefers NADPH to NADH, although NADH can afford sufficient activity [40]. The electron donor specificity depends on the amino acid residues at two positions in the B'-helix (73rd and 75th positions in the case of CYP55A1) [41,42]. Steric hindrance due to side chains of Ser73 and Ser75 in CYP55A1 excludes the 2'-phosphate moiety of NADPH from the site. In P450nor2 of C. tonkinense, Ser75 is replaced with Gly, permitting accommodation of NADPH. Double mutation at these sites in P450nor of F. oxysporum (S73G/S75G; GG mutant) markedly improved the specificity against NADPH [43].

Direct electron transfer from NADH to the haem of P450nor was conclusively demonstrated by kinetic analysis [44] and by the determination of a crystal structure of a P450nor (GG mutant) complexed with an NADH analogue (nicotinic acid adenine...
The structure of the P450nor–NAAD complex is compared with that of the ferric–NO complex of P450nor [45] in figure 6. Little difference was observed between the structures of P450nor in the ferric resting state [46] and in the ferric–NO complex [45]. By contrast, a remarkable conformational change in the protein was induced upon binding of NAAD (figure 6). The entrance gate of the haem distal pocket is closed. Two Arg residues, Arg64 and Arg174, play a key role in the binding [41] by putting the pyrophosphate moiety of NAAD between them. Glu71, Arg64 and Asp88 form a salt bridge network to stabilize the protein structure [44,47]. The interaction between Arg64 and Asp88 is broken upon binding of NAAD to destabilize the protein. This makes a driving force to exclude rapidly an NAD$^+$ molecule from the active site after electron transfer is finished. One of the propionate side chains of the haem moves up accompanying the movement of Arg292, which fixes the nicotinamide ring stereochemically. The conserved Thr residue (Thr243) in the I-helix interacts with the carboxyl of nicotinic acid ring to fix it stereochemically. This interaction of Thr243 together with the propionate of haem moving upward restricts the conformation of the nicotinic acid ring so that the pro-R side of C4-hydrogens faces the haem, which is consistent with the pro-R hydrogen-specific hydride transfer [35]. A hydrogen bond network is formed to deliver a proton from solvent to Ser286 that is located in the close vicinity of haem [45]. However, the network is rearranged to form a proton channel upon binding of NAAD, and the bound NADH (NAAD) which is itself involved in the network [43] (figure 7), suggesting that a proton is supplied to the enzymatic reaction via the proton channel before formation of the intermediate (444 nm species; in the second step in figure 5). This is because the hydrogen bond network containing NAD$^+$ would be degraded after the release of NAD$^+$ (the last step in figure 5). The proton supply to form the intermediate is consistent with the structure of the intermediate (ferric-hydroxyl amine radical complex).

**5. CONCLUDING REMARKS**

Fungal denitrification is involved in the nitrogen cycle in nature as a major pathway. This is supported by the distribution of nirK (dNir) and CYP55 (P450nor) gene homologues in many fungal genomes (more than 20%), together with several recent papers showing the predominance of fungal denitrification in various environments. Since the final product of fungal denitrification is N$_2$O, it appears that fungal denitrification is one of the major sources of N$_2$O emissions. Acidification of environments, for example, by acid rain and excess use of ammonia fertilizer, promotes fungal activity resulting in an increase in N$_2$O emissions.

Most of fungal-denitrifying systems seem to contain NirK and P450nor as essential components. These two genes are the minimum pair to ensure denitrification from nitrite to N$_2$O. Some fungi further use dNar (Nar GHI type in the case of *F. oxysporum* MT811) and/or aNar, which also enable denitrification of nitrate. NirK and dNar are associated with the mitochondrional respiratory chain, coupled to the synthesis of ATP. This is the first example of the occurrence of anaerobic respiration in mitochondria. By contrast, P450nor and aNar receive electrons directly from NAD(P)H and thus are not associated with the respiratory chain. Thus, P450nor and aNar function as an electron sink under anoxic conditions. Thus ATP-producing metabolism, being inefficient in ATP production, reflects the strategy of fungi for survival under anoxic conditions, in which preference is for speed of metabolism over energy efficiency. The significance of the presence of Nap-homologue genes in many fungal genomes remains to be elucidated.

Eukaryotic nirK and its homologue genes obviously originate from the same ancestor, possibly the proto-mitochondrion which harboured NirK-type (but not NirS-type) dNir [23]. Thus, the fungal and bacterial-denitrifying systems share the same origin. However, P450nor is unique to the fungal system. It appears that the mitochondrial-denitrifying system replaced the original Nor protein with P450nor, whose gene was initially obtained from bacteria (actinomycetes) by horizontal gene transfer. The prototype P450 gene would have encoded the usual monooxygenase, whereas fungi would have modulated the gene to give Nor activity, because P450nor is now not found among bacteria.

P450 proteins belonging to the P450 superfamily are among the most diversified enzyme proteins. However, even among such diversified P450 proteins, the function of P450nor is peculiar [33]. The function of P450nor is thus atypical of most diversified P450 proteins. The mechanism of the stereospecific transfer of H$^-$ from NADH to the haem of P450nor has been elucidated. In addition to Nor activity, P450nor will catalyse the co-denitrification reaction. We have also found that P450nor exhibits NADH-peroxidase activity (H$_2$O$_2$ + NADH + H$^+$ → 2H$_2$O + NAD$^+$; S. Nakaya & H. Shoun 2008, unpublished data). P450nor is therefore a multi-functional detoxifying enzyme. P450nor is also related to the pathogenicity of a fungus [48].

Co-denitrification is the first process to show the formation of a hybrid N$_2$ or N$_2$O species [5,27]. It will
Figure 7. NADH and proton channels. (a) Perpendicular to and (b) parallel with the haem plane. Water molecules forming the proton channel are shown as spheres.

depend on the reaction of NO with a nitrogen donor, which is possibly catalysed by P450nor and thus characteristic of fungal denitrification. Co-denitrification products (N2 or N2O) vary depending on the redox state of the nitrogen donor: N2 is formed from amines and N2O from imines or azide. This suggests that the nitrogen donors also act as an internal electron donor. Thus, the co-denitrification process can be considered to be a kind of Nor reaction employing amines or imines as an internal electron donor (and nitrogen donor). The molecular mechanism of co-denitrification needs further elucidation. The mechanism of co-denitrification by the bacterium Streptomyces antibioticus [49], in which a very small amount of a hybrid N2 species is formed, also remains to be elucidated, because P450nor is not found in bacteria.

This work is the result of collaborations of the author (H.S.) with many researchers and students of University of Tsukuba, the University of Tokyo, Riken, University of Konstanz, Osaka Prefecture University and Chiba University. This work was supported by Grants-in-aid for scientific research from the Japan Society for the Promotion of Science and the Research and Development Programme for New Bio-Industry Initiatives. We thank the staff of Photon Factory and SPring-8 for X-ray data collection.

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