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# Hybrid Respiration in the Denitrifying Mitochondria of *Fusarium oxysporum*

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**Induction of the mitochondrial nitrate-respiration (denitrification) system of the fungus *Fusarium oxysporum* requires the supply of low levels of oxygen ($O_2$). Here we show that $O_2$ and nitrate ($NO_3^-$) respiration function simultaneously in the mitochondria of fungal cells incubated under hypoxic, denitrifying conditions in which both $O_2$ and $NO_3^-$ act as the terminal electron acceptors. The $NO_3^-$ and nitrite ($NO_2^-$) reductases involved in fungal denitrification share the mitochondrial respiratory chain with cytochrome oxidase. *F. oxysporum* cytochrome $c_{549}$ can serve as an electron donor for both $NO_2^-$ reductase and cytochrome oxidase. We are the first to demonstrate hybrid respiration in respiring eukaryotic mitochondria.**

**Key words: aerobic respiration, cytochrome *c*, *Fusarium oxysporum*, mitochondrion, nitrate respiration.**

Denitrification is a biological process that reduces nitrate ($NO_3^-$) or nitrite ($NO_2^-$) to nitrous oxide ($N_2O$) or dinitrogen ($N_2$) gas, and plays an important role in maintaining the global environment. It functions physiologically as anaerobic respiration that utilizes $NO_3^-$ and related nitrogen oxides as terminal electron acceptors (*1*). In bacterial denitrifying systems, $NO_3^-$ is successively reduced to $N_2$ *via* $NO_2^-$, nitric oxide (NO), and $N_2O$. Each reducing step is catalyzed by a distinct enzyme: $NO_3^-$ reductase (Nar), $NO_2^-$ reductase (Nir), NO reductase (Nor), and $N_2O$ reductase (Nos), respectively. All of these reductases are associated with the respiratory chain to function as terminal dehydrogenases. Denitrification was long considered a unique feature of bacteria until we discovered this process in the fungus *Fusarium oxysporum* (*2*). The fungal denitrifying system is localized to mitochondria and consists of three successive reducing steps catalyzed, respectively, by Nar, Nir, and Nor (*2–6*). The fungal system thus seems to lack Nos and produces $N_2O$ as the final denitrification product. A unique feature of the fungal system is that the reduction process is not wholly associated with the respiratory chain. The final step required to reduce NO to $N_2O$, is catalyzed by a unique cytochrome P450 (P450nor) (*3*, *5*, *7*, *8*). This enzyme receives electrons directly from NADH (or NADPH) (*3*), and thus is not associated with the respiratory chain.

Another unique feature of fungal denitrification is focused on its requirement for a small supply of oxygen ($O_2$) (*2*, *9*, *10*). This means that *F. oxysporum* generates more denitrifying activity under hypoxic than under totally anoxic conditions. Under most anoxic conditions, denitrification is replaced by another type of $NO_3^-$ metabolism, namely ammonia fermentation (*11*). We could not determine, however, the hypoxic conditions that are best for denitrification in terms of dissolved oxygen (DO) (*9–11*). *F. oxysporum* does not denitrify at DO levels above zero. Thus the best aeration is so low that it is indicated in terms of $O_2$ supply (volume) per cell mass per h (*10*, *11*). Under such aerating conditions the rate of incorporation of $O_2$ by the cells would be faster than the rate of $O_2$ diffusion into the medium. Fungal cells grown under such denitrifying conditions (hypoxic, in the presence of $NO_3^-$) seem to contain intact mitochondria, which is in contrast to the apparently immature mitochondria in cells that ferment ammonia under more anoxic conditions (*11*). Denitrifying mitochondria have cytochrome oxidase activities as well as Nar and Nir activities. These results indicate that fungal cells respire $O_2$ and $NO_3^-$ simultaneously under hypoxic conditions (*6*, *10*). The present study compares the mitochondrial respiratory chains of *F. oxysporum* incubated under aerobic and denitrifying conditions. The results indicate a hybrid type of respiration in fungal, hypoxic mitochondria.

## MATERIALS AND METHODS

*Strains, Culture, and Media*—We preincubated *F. oxysporum* MT-811 (JCM11502, Japan Collection of Microorganisms) (*2*) at 120 rpm on a reciprocal shaker at 30°C for 72 h in 100 ml of glycerol peptone medium [3% glycerol, 0.2% polypeptone and inorganic salts (*2*)]. Aerobic culture was carried out by growing the fungal cells at 30°C for 6 h on a rotary shaker (120 rpm) after inoculating the preculture (100 ml) into 1000 ml of glycerol-peptone medium in a 5 liter Erlenmeyer flask sealed with a cotton plug. The hypoxic cultures were achieved in a similar manner to the aerobic culture above, but the flask contained 3000 ml (before inoculation) of medium and the

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Table 1. **Specific activities of enzymes involved in the respiratory chain of *F. oxysporum* mitochondria.**

| Mitochondrial source (cellular environment) | NADH dehydrogenase (Complex I) | Succinate dehydrogenase (Complex II) | UQH2-cytochrome *c* oxidoreductase (Complex III) | Cytochrome *c* oxidase (Complex IV) |
|---|---|---|---|---|
| | (units $mg^{-1}$) | (units $mg^{-1}$) | (units $mg^{-1}$) | (units $mg^{-1}$) |
| Aerobic (+ 20mM $NO_3^-$) | 8.4 ± 0.4 | 1.9 ± 0.2 | 0.46 ± 0.08 | 900 ± 100 |
| Aerobic | 9.0 ± 0.1 | 1.1 ± 0.3 | 0.38 ± 0.08 | 1000 ± 100 |
| Micro-aerobic (+ 20mM $NO_3^-$) | 1.8 ± 0.1 | 0.3 ± 0.1 | 0.40 ± 0.12 | 1600 ± 200 |
| Micro-aerobic | 2.7 ± 0.3 | 0.5 ± 0.2 | 0.41 ± 0.08 | 1400 ± 200 |

Data are presented as mean values of three experiments. One unit is defined as 1 μmol product $min^{-1}$.

incubation time was prolonged (72 h). Denitrifying cultures were achieved under the conditions used for hypoxic culture in medium supplemented with 0.2% $NaNO_3$.

*Preparation of Mitochondria*—All manipulations proceeded at 4°C or on ice. *F. oxysporum* cells harvested after each culture described above were homogenized with quartz sand in buffer A [0.8 M sucrose, 0.1% bovine serum albumin, 10 mM Tris-HCl (pH 7.2)] in the presence of protease inhibitors as reported (*4*). The homogenate was centrifuged at 1,500 ×*g* for 10 min, and then the supernatant was further separated by centrifugation at 10,000 ×*g*. The resulting pellet was resuspended in buffer A and fractionated by sucrose density gradient centrifugation as described (*4*). The interface between the 34 and 43% (weight/weight) sucrose layers was collected and used as the mitochondrial fraction. This fraction was resuspended in buffer B [10 mM Tris-HCl (pH 7.4), 4 mM ATP, and 4 mM $MgCl_2$], ultrasonicated and centrifuged at 250,000 ×*g* for 60 min to obtain the membrane (pellet) and soluble (supernatant) fractions.

*Enzyme Assay*—Levels of NADH-ubiquinone (UQ) reductase (complex I) were determined using coenzyme $Q_2$ as the electron acceptor (*12*). Succinate dehydrogenase (complex II) was assayed by measuring the reduction of 2,6-dichloroindophenol (*13*). Ubiquinol ($UQH_2$)–cytochrome *c* reductase (complex III) was determined by following the change in absorbance at 550 nm due to the reduction of cytochrome *c* (*14*). Cytochrome oxidase activity (complex IV) was measured as described (*15*) by monitoring the change in absorbance at 549 nm due to the oxidation of ferrocytochrome $c_{549}$ (*16*). *F. oxysporum* cytochrome $c_{549}$ was prepared as described (*16*). The activities of $UQH_2$-$NO_3^-$ reductase (*6*) and NADH-dependent NO reductase (P450nor) (*3*) were determined as described. Cytochrome *c*–$NO_2^-$ reductase was determined by measuring the amount of substrate ($NO_2^-$) remaining after the reaction (*17*). The reaction mixture (1.9 ml) in test tubes contained 6.3 μM cytochrome $c_{549}$, 10.5 μM $NaNO_2$, 0.2 mg mitochondrial fraction and 100 mM potassium phosphate (pH 7.5). The mixture was degassed, the headspace was replaced with helium, and the tube was sealed with a double butyl rubber stopper. The reaction was started by adding 0.1 ml of 10 mM sodium hydrosulfite. The amount of $NO_2^-$ reduced without cytochrome $c_{549}$ served as the control reaction. An artificial electron donor, NADH/phenazine methosulfate (PMS)–dependent Nir activity was assayed as reported (*16*).

*Spectroscopy*—Low temperature absorption spectra of the mitochondrial fractions were measured under liquid nitrogen using a HITACHI 557 Double Wavelength Double Beam Spectrophotometer. The concentration of each sample was adjusted to 5 mg protein/ml using buffer A. The specific content of each cytochrome was determined by measuring its respective -band in the redox difference spectrum (dithionite-reduced minus oxidized) (*18*). The carbon monoxide (CO) difference spectrum was used for spectral detection and the determination of cytochrome P450 (P450) (*19*).

## RESULTS AND DISCUSSION

*Mitochondrial Respiratory Chain of Fusarium oxysporum Incubated under Aerobic and Hypoxic Conditions*—We examined the effect of incubating *F. oxysporum* under aerobic or hypoxic conditions in the presence or absence of $NO_3^-$, on the mitochondrial respiratory chain. Table 1 shows that the activities of enzyme complexes I to IV were detected in all mitochondrial fractions prepared from cells grown under all tested conditions. However, the activities of complexes I (NADH dehydrogenase) and II (succinate dehydrogenase) were lower under hypoxic conditions whereas those of complexes III and IV were not significantly affected by changes in the culture conditions. The activities of complexes I and II were the lowest under denitrifying conditions (hypoxic with $NO_3^-$), in sharp contrast to the activities of complexes III and IV.

*The Mitochondrial Denitrifying System*—We compared the activities of enzymes involved in fungal denitrification between the mitochondrial fractions of aerobic and denitrifying cells. The activities of all enzymes were greater in the denitrifying mitochondria, whereas higher activities (except NADH/PMS-Nir) than predicted were expressed in aerobic mitochondria (Table 2). The results

Table 2. **Specific activities of denitrifying enzymes in mitochondria of *F. oxysporum*.**

| Mitochondrial source (cells) | $UQH_2$-nitrate reductase | NADH-PMS–nitrite reductase | Cytochrome *c*–nitrite reductase | Nitric oxide reductase |
|---|---|---|---|---|
| | (units $mg^{-1}$) | (units $mg^{-1}$) | (units $mg^{-1}$) | (units $mg^{-1}$) |
| Aerobic | 35 ± 8.3 | <0.1 | 5.0 ± 0.8 | 80 ± 10 |
| Denitrifying | 64 ± 12 | 3.3 ± 0.4 | 7.8 ± 0.2 | 350 ± 180 |

Cytochrome *c*–nitrite reductase activities are represented as the amount of nitrite consumed at the end (2 hours) of the reaction. One unit is defined as 1 nmol product $min^{-1}$. Values are the means of three experiments.

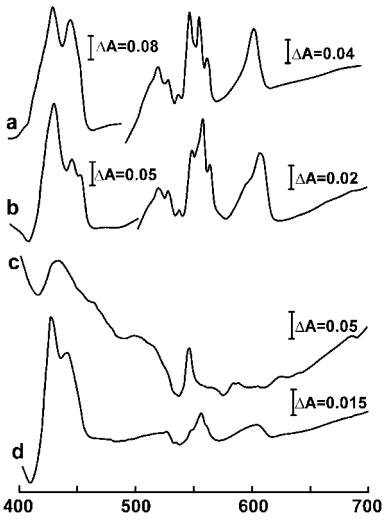


Fig. 1. **Reduced minus oxidized difference spectra of *F. oxysporum* mitochondria and submitochondrial fractions at low temperature.** Mitochondria were prepared from aerobic (a) and denitrifying (b) cells of *F. oxysporum* MT-811. The denitrifying mitochondria were further fractionated into soluble (c) and membrane (d) fractions. Each sample contained 5 mg/ml protein.

suggest that these enzymes are constitutively expressed at low levels even under aerobic conditions. We previously showed that P450nor is expressed at a very low level under non-denitrifying conditions (*5*), although the expression was too low to be detected spectrally (CO-difference spectrum) (*20*) or immunologically (Western blot) (*5*). We previously showed that the catalytic turnover of P450nor is very high at low enzyme (P450nor) concentrations, but saturates as the enzyme concentration is increased (*3*). This means that the Nor activity is not necessarily proportional to the P450nor concentration employed for the enzyme assay. The low Nor activity in the aerobic mitochondria (Table 2) suggests the presence of a very low level of P450nor that could not be detected either spectrally or immunologically.

Cytochrome $c_{549}$ was previously isolated from denitrifying cells of *F. oxysporum* (*16*). We cloned its gene and cDNA (unpublished data) and found that cytochrome $c_{549}$ comprises 106 amino acid residues with a deduced amino acid sequence very similar to mitochondrial cytochromes *c* of other fungi. Here we show that cytochrome $c_{549}$ of *F. oxysporum* donates electrons to mitochondrial cytochrome oxidase (Table 1) as well as to Nir (Table 2). These results show that cytochrome $c_{549}$ is a component of the mitochondrial respiratory chain of *F. oxysporum*. The electron transfer from cytochrome *c* to Nir is consistent with our previous observation that denitrification by intact cells (*2*) and the mitochondrial Nir activity supported by physiological electron donors (*4*) are specifically inhibited by antimycin A (an inhibitor of complex III).

*Spectroscopic Analysis of Denitrifying Mitochondria*—We analyzed the components of fungal mitochondria by measuring differences in the redox spectra (dithionite-reduced minus oxidized as prepared) at low temperature (Fig. 1, a and b). Mitochondrial fractions prepared from cells grown under both aerobic and denitrifying conditions contained similar cytochrome components, although at different ratios. The absorption peaks at 444 nm and around 605–610 nm are characteristic of cytochrome $aa_3$ (cytochrome oxidase). We previously showed that fungal mitochondria contain at least three species of cytochrome $b_5$–like hemeproteins (*21*), the presence of which was spectrally confirmed by the peak at 556 nm. The peak at 562 nm should be derived from other cytochromes *b*. The peak at 546 nm must be derived from cytochrome $c_{549}$. The minor difference in the peak position would be due to a blue shift caused by the low temperature. The specific contents of these cytochromes were calculated from the extent of each α-band in the difference spectra (Fig. 1) and the results are listed in Table 3. Spectral evidence for the presence of P450 in the mitochondrial fraction of denitrifying *F. oxysporum* was provided previously by the CO-difference spectrum (*20*). This absorption peak in the difference spectrum is never seen in non-denitrifying cells (*2*, *9*, *20*, *22*).

Table 3. **Cytochrome components in the aerobic and denitrifying mitochondria of *F. oxysporum*. Cytochrome contents were calculated from the data in Fig. 1.**

| Mitochondrial source (cells) | Cytochromes (nmol/mg protein) | | | | |
|---|---|---|---|---|---|
| | *a* | *b* | $b_5$ | *c* | P450[a] |
| Aerobic | 1.34 | 0.74 | 0.32 | 0.87 | – |
| Denitrifying | 0.70 | 0.45 | 0.16 | 0.21 | 0.44 |

[a]From Ref. *20*.

Denitrifying mitochondria were further fractionated into soluble (Fig. 1c) and membrane (Fig. 1d) fractions. All cytochromes except cytochrome *c* were recovered in the membrane fraction. These results are consistent with the general localization of cytochromes in the respiratory chain. Therefore, the denitrifying mitochondria of *F. oxysporum* contain the same cytochrome components as aerobic mitochondria except that denitrifying mitochondria also contain P450nor.

*Hybrid Respiration in Fungal Mitochondria*—These results showed that both $O_2$- and $NO_3^-$-respiration systems function simultaneously in fungal cells under hypo-

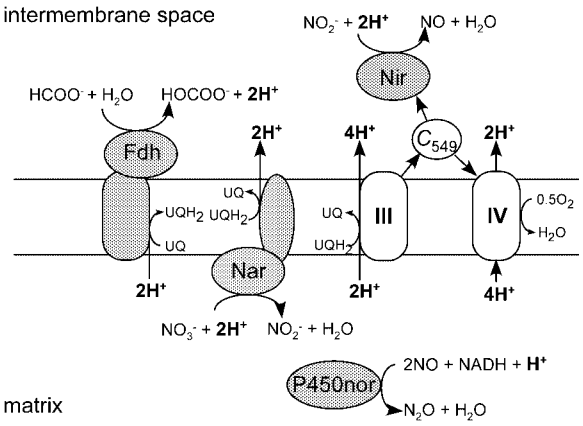


Fig. 2. **A hypothetical model of the fungal hybrid respiration system.** The localization of each component is according to our previous reports (*4*, *6*, *20*). The same component topology as their bacterial counterparts (*23*) is assumed. NO, the reaction product of Nir and the substrate of P450nor, is assumed to penetrate spontaneously into the cytoplasmic and matrix space according to its gradient.

xic, denitrifying conditions. Both respiration systems share the mitochondrial respiratory chain, which means that a kind of hybrid respiration works in which both cytochrome oxidase and denitrifying enzymes (Nar, Nir) function as the terminal oxidase and dehydrogenases (Fig. 2). We showed that the formate dehydrogenase (Fdh)-Nar couple localizes to mitochondria and is involved in the fungal $NO_3^-$-respiration system (*6*). This couple is involved in the $NO_3^-$-respiration system of non-denitrifying *Escherichia coli*, but has never been identified in bacterial denitrification systems. Fdh transfers electrons from formate to Nar *via* the UQ/$UQH_2$ pool, and yields by itself a proton-motive force (*23*). Among four terminal dehydrogenases (reductases) (Nar, Nir, Nor, and Nos) in the typical bacterial denitrifying system, only Nar functions on the cytoplasmic side to contribute to the generation of a proton motive force. The other three reductases localize to the periplasmic side and receive electrons from cytochrome *c*, the reactions of which thus consumes protons in the periplasm and decreases the proton motive force. However, these three reductases receive electrons downstream of complex III (cytochrome $bc_1$ complex), which yields more proton motive force than each reductase (Nir, Nor, or Nos) consumes, in contrast to Nar, which receives electrons from $UQH_2$. Thus the electron flow from $UQH_2$ to four nitrogen oxides ($NO_3^-$, $NO_2^-$, NO, and $N_2O$) *via* four terminal reductases (Nar, Nir, Nor, and Nos) results in the same stoichiometry of net proton translocation (2 protons per 2 electrons) (*23*) (*cf.* Fig. 2).

Assuming that the components of the mitochondrial $NO_3^-$-respiration system have a similar topology to that of the components of bacterial systems (*23*) (Fig. 2), only Nir consumes protons in the intermembrane space of mitochondria. In the fungal hybrid system, electrons downstream of complex III are divided into only two pathways: one to $O_2$ *via* cytochrome oxidase and the other, to $NO_2^-$ *via* Nir. It seems reasonable to consider that the system has been constructed in order to avoid the energetically unfavorable reactions downstream of complex III by bacterial types of Nor and Nos and to make electrons flow to $O_2$ as much as possible. Fungal Nar can receive electrons from at least three pathways, that is, from Fdh as well as complexes I and II. The low activities of complexes I and II in the hybrid system (Table 1) support our previous assumption that the Fdh-Nar couple functions predominantly in the fungal $NO_3^-$-respiration system (*6*). The result also seems reasonable assuming that NADH is reserved for P450nor, the reactions of which have physiological relevance to NO detoxification and an electron sink under hypoxic conditions (*3*).

We, therefore, conclude that in the fungal hybrid respiration system, an energetically favorable portion of $NO_3^-$-respiration (Fdh-Nar couple) is combined with $O_2$-respiration (cytochrome oxidase). The reactions of both Fdh and Nar can yield a proton motive force (Fig. 2). Formate would be formed from pyruvate by the reaction of pyruvate-formate lyase (*1*, *23*) (Kuwazaki *et al*., submitted). The electron flow downstream of complex III employs $O_2$ in addition to $NO_2^-$ as the electron acceptor, which yields a greater proton motive force than the electron flow to only denitrifying enzymes (Nir, Nor, and Nos). Excess electrons generated under hypoxic conditions are mainly dumped onto NO, the final product of fungal $NO_3^-$-respiration, by the reaction of P450nor to produce the denitrification product $N_2O$. Thus, *F. oxysporum* has generated a unique system of energy production for survival under hypoxic conditions that is distinct from its bacterial counterparts.

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