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Nitrate Reductase–Formate Dehydrogenase Couple Involved in the Fungal Denitrification by *Fusarium oxysporum*[1]

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Dissimilatory nitrate reductase (Nar) was solubilized and partially purified from the large particle (mitochondrial) fraction of the denitrifying fungus *Fusarium oxysporum* and characterized. Many lines of evidence showed that the membrane-bound Nar is distinct from the soluble, assimilatory nitrate reductase. Further, the spectral and other properties of the fungal Nar were similar to those of dissimilatory Nars of *Escherichia coli* and denitrifying bacteria, which are comprised of a molybdoprotein, a cytochrome *b*, and an iron-sulfur protein. Formate-nitrate oxidoreductase activity was also detected in the mitochondrial fraction, which was shown to arise from the coupling of formate dehydrogenase (Fdh), Nar, and a ubiquinone/ubiquinol pool. This is the first report of the occurrence in a eukaryote of Fdh that is associated with the respiratory chain. The coupling with Fdh showed that the fungal Nar system is more similar to that involved in the nitrate respiration by *Escherichia coli* than that in the bacterial denitrifying system. Analyses of the mutant species of *F. oxysporum* that were defective in Nar and/or assimilatory nitrate reductase conclusively showed that Nar is essential for the fungal denitrification.

Key words: denitrification, *Fusarium oxysporum*, hybrid respiration, mitochondria, nitrate reductase.

Eukaryotic cells have recently been found to participate in biological denitrification. Many fungi and yeasts were shown to be able to reduce nitrate and/or nitrite to nitrous oxide (N_2O) dissimilatorily (*1–3*). More recently, we showed that *Fusarium oxysporum* and many other soil fungi are also capable of anaerobic metabolism of nitrate to form ammonium (*4*). This so-called ammonia fermentation can support anoxic growth of fungi even under complete loss of oxygen supply. Under such anoxic conditions, the fungal cells were shown to contain many apparently immature mitochondria (*4*). By contrast, fungal denitrification requires a minimal amount of oxygen supply (*5*), and the denitrifying cells grown under the micro-aerobic (hypoxic) conditions contain apparently intact mitochondria (*6*), consistent with our previous observation that the fungal denitrification system is localized in mitochondria (*7*). Therefore, these soil fungi can adapt immediately to rapid changes in environmental oxygen supply by changing their energy metabolism: ammonia fermentation under anoxic conditions, denitrification under hypoxic conditions, and oxygen respiration under aerobic conditions (*4, 5*). Soil fungi have been thought to be generally obligatorily aerobic organisms, but these findings revealed that they would be predominantly facultative aerobes (or facultative anaerobes).

The denitrifying systems of two fungal species, *F. oxysporum* and *Cylindrocarpon tonkinense* are now being characterized (*7–12*). Nitric oxide reductase (Nor) of the fungal system is of cytochrome P450 (P450) type (*11*) and quite distinct from Nor of cytochrome *cb* type known among bacterial systems (*13*). Dissimilatory nitrate reductase (Nar) and nitrite reductase (Nir) activities were detected in the mitochondrial fractions of *F. oxysporum* and *C. tonkinense*, respectively, and shown to couple to the synthesis of ATP (*7*). Physiological respiration substrates such as malate plus pyruvate, succinate, and formate, and artificial electron donors such as reduced methylviologen (MVH), could support the Nar activity of the intact mitochondria of *F. oxysporum* (*7*). Treatment of the mitochondrial fraction with a detergent caused loss of the Nar activity that was supported by physiological respiration substrates (malate plus pyruvate), whereas it did not affect the MVH-dependent Nar activity. These results are highly indicative that the mitochondrial Nar activity is associated with the respiratory chain It is noteworthy that formate can support the mitochondrial Nar activity (*7*). The formate-nitrate oxidoreductase system is well characterized in the enteric bacterium *Escherichia coli* but not known among denitrifying soil bacteria.

Soluble and cytosoli NADH (or NADPH)-dependent nitrate reductase and nitrite reductase are involved in the ammonia fermentation by *F. oxysporum* (*4*). The properties

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of these reductases are very similar to those of assimilatory nitrate and nitrite reductases that are generally found in fungi, but distinct from the mitochondrial Nar and Nir involved in denitrification. Here we present conclusive evidence that *F. oxysporum* contains dissimilatory Nar that is distinct from the assimilatory nitrate reductase that is known to occur generally among fungi. We further show the first example in a eukaryote of formate dehydrogenase that is associated with the respiratory chain, providing electrons to Nar

MATERIALS AND METHODS

Fungal Strain–F. oxysporum MT-811, that was firstly shown as a fungal denitrifier (*1*), was used throughout this work.

Culture of the Fungal Strain—The basal medium contained in 1,000 ml of tap water, 10 g of sucrose, 1.36 g of KH_2PO_4, and 1 ml of a trace element solution. Its pH was adjusted to 7.5 with NaOH. The trace element solution consisted of 200 g of $MgSO_4 \cdot 7H_2O$, 10 g of $FeSO_4 \cdot 7H_2O$, 10 g of $FeCl_3 \cdot 6H_2O$, 2 g of $ZnSO_4 \cdot 7H_2O$, 0 5 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of $Na_2MoO_4 \cdot 2H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of H_3BO_3, and 10 g of citric acid, in 1,000 ml of distilled water. The seed culture was grown in 10 ml of the basal medium in a test tube supplemented with 0.2% peptone at 30°C for 3 days with shaking at 200 strokes·min^{-1}. The seed culture was then transferred to 300 ml of the same medium in a 500-ml Erlenmeyer flask and incubated on a rotary shaker at 120 rpm at 30°C for 3 days (preculture). For a small-scale experiment, 30 ml of the preculture was inoculated into 300 ml of the medium in a 500-ml Erlenmeyer flask and incubated at 30°C on a rotary shaker at 120 rpm. The flask was sealed with a cotton plug. For purification of Nar, the preculture (300 ml) was inoculated into 3 liter of the basal medium supplemented with 0.2% sodium nitrate and 0.2% soybean flour (*1*) in a 5-liter Erlenmeyer flask and incubated on a rotary shaker at 120 rpm at 30°C for 2 days. Then the flask was allowed to stand for 1 day without shaking. Mycelia were harvested by filtration (*1*) and stocked at –80°C until use.

To investigate the effects of mutations, mutant and wild-type strains were precultured as above except that 0.2% $NaNO_3$ was added to the medium. Mycelia were harvested by centrifugation and transferred to the MM medium (30 g/liter glycerol, 10 mM KH_2PO_4, 20 mM $NaNO_3$, and 1 ml of a trace element solution, pH 7.5) and incubated at 120 rpm at 30°C for 3 days.

Preparation and Fractionation of Cell-Free Extracts—The fungal cells were disrupted as reported (*1, 7*) by grinding with alumina in the presence of protease inhibitors (leupeptin, phenylmethylsulfonyl fluoride, and *N*-tosyl-L-phenylalanylchloromethylketone, 0.3 mM each). For the experiments to examine intracellular localization, the cells were disrupted under isotonic conditions (*7*). The extracts were then centrifuged at 700 ×*g* for 10 min, and the resulting supernatant was further centrifuged at 10,000 ×*g* for 60 min. The 700 ×*g* supernatant, the 10,000 ×*g* precipitate, and the 10,000 ×*g* supernatant were respectively used as the crude cell extract, large particle fraction, and soluble fraction, although the supernatant should also contain the microsomal fraction. For Nir assay, mycelia were disrupted as above in 100 mM sodium phosphate buffer (pH 7.2) containing 10% glycerol, 5 mM EDTA, and 10 μM FAD, and the supernatant arising from the centrifugation at 120,000 ×*g* for 60 min was used.

Enzyme Assays—Reduced methylviologen (MVH)-dependent nitrate reductase activities were assayed by determining the reaction product (nitrite) colorimetrically (*14*), as follows. The reaction mixture (final, 2.0 ml in 7-ml test tube) contained 10 mM sodium nitrate, 0.1 mM methylviologen, and 50–200 μl of enzyme solution in 0.1 M potassium phosphate buffer (pH 6.8; containing 0.1% Triton X-100). The test tube was sealed with a double butyl rubber stopper after degassing and replacing the head space gas with helium. The reaction was initiated by adding 200 μl of 0.1 M sodium dithionite solution in 0.1 M sodium bicarbonate at 30°C. The reaction was stopped by oxidizing the remaining dithionite, by unstopping the tube and mixing the solution vigorously with air. For examining electron donors, MVH was replaced with each electron donor at a concentration of 10 mM Formate dehydrogenase (Fdh) was assayed as follows. The large particle fraction prepared from denitrifying cells was suspended in 1.8 ml of the potassium phosphate buffer above containing an electron acceptor in a Thunburg cuvette. After degassing and replacing the head space gas with helium, the reaction was initiated by adding 0.2 ml of 0 1 M sodium formate at 30°C. The reaction was monitored by the change in absorbance due to the reduction of each electron acceptor. The ε value (mM^{-1}·cm^{-1}) for each electron acceptor between reduced and oxidized states (*15*) was: 21 at 600 nm for 2,6-dichlorophenol indophenol (DCIP), 13 at 600 nm for MV, 7.4 at 600 nm for benzyl viologen (BV), 6.22 at 340 nm for NADH and NADPH, and 12.5 at 275 nm for ubiquinone 2 (*16*). Assimilatory Nar and Nir was assayed under the aerobic conditions as described (*17, 18*). The activity of P450nor was assayed as described (*11*).

Solubilization and Partial Purification of Dissimilatory Nitrate Reductase (Nar)—All procedures were done below 4°C. The large particle fraction was suspended in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M KCl and 10% glycerol with a protein concentration of 10 mg/ml. The suspension was mixed with 2% Triton X-100 and stirred for 3 h, then centrifuged at 100,000 ×*g* for 60 min. The resulting supernatant (solubilized enzyme) was subjected to further purification. All buffers used for purification contained 10% glycerol and 0.1% Triton X-100. The solubilized enzyme solution prepared from 1.0 kg of wet cells was dialyzed against 20 mM Tris-HCl buffer (pH 7.2), then applied to a Q-Sepharose (Pharmacia, Sweden) column (30 × 100 mm) equilibrated with the same buffer. The column was washed with the buffer containing 0.2 M KCl, and the enzyme was eluted with a gradient of 0.2–0 4 M KCl. The active fraction was dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and applied to a hydroxyapatite column (30 × 100 mm; Wako Pure Chemicals, Osaka) equilibrated with the same buffer. The column was eluted with a linear gradient of 10–200 mM potassium phosphate (pH 7.0). The active fraction was concentrated with Diaflo 8400 or 8050 (Amicon, USA) using an Ultra filter (50000-cut; Advantec Toyo, Tokyo), and dialyzed against 20 mM Tris-HCl buffer (pH 7.2). The fraction was applied to FPLC (Fast Protein Liquid Chromatograph; Pharmacia, Sweden) equipped with a Mono Q column (5 × 50 mm) equilibrated with the same buffer, and eluted with a linear gradient of 0.15–0.35 M

KCl. By this procedure the activity was divided into two fractions (Nar1 and Nar2). They were utilized for further characterizations as the solubilized and partially purified preparations.

Polyacrylamide Gel Electrophoresis (PAGE) and Activity Staining—Native PAGE was performed in a similar manner to SDS-PAGE (*19*) except that the buffer system contained 0.1% Triton X-100 instead of SDS. The large particle fraction was solubilized with 1% Triton X-100 prior to PAGE. Each sample was applied to electrophoresis without boiling and visualized by nitrate reductase–dependent activity staining (*20*) with modification. After electrophoresis the gel was soaked in 100 ml of 50 mM potassium phosphate buffer (pH 6 8) containing 50 mM sodium nitrate and 5 mM MV, then 5 ml of dithionite solution (0.2 g/ml in 0.1 M sodium bicarbonate) was added. The solution was shaken gently to reduce all MV to MVH, left to stand until the blue color in the active bands in the gel was bleached, and then fixed by adding 50 ml of 1% 2,4,5-triphenyltetrazorium chloride solution

Isolation and Characterization of Chlorate-Resistant Mutants—Chlorate-resistant mutants were isolated by the method of Correll *et al* (*21*) with the following modification. Conidia of *F. oxysporum* MT-811 (10 to 50 μl of suspension) were spotted on plates containing PDC medium (potato dextrose medium (Difco) with 2% sucrose and 100 mM sodium chlorate) and incubated for 5 to 7 days at 30°C. Mycelial sectors appearing after the incubation (chlorate-resistant mutants) were replicated to PDC plates and single colonies were isolated from replicas by serial dilution. This single colony isolation procedure was repeated three times and resultant strains were used as mutants. For testing auxotrophy, small portions of mycelia were transferred to the basal medium containing 10 mM indicated nitrogen compounds as a sole source of nitrogen and growth was monitored after incubating at 30°C for 3 days.

Other Determinations—Protein was determined by a modification of the method of Lowry *et al.* (*22*) according to Wang *et al.* (*23*), which enables the determination in the presence of Triton, or by Bio-Rad Protein Assay (Bio-Rad, USA). Spectrophotometric measurements were done with a Beckman DU 7500 spectrophotometer.

RESULTS

Distinct Species of Nitrate Reductase—MVH-dependent nitrate reductase activity could be detected in both the soluble and large particle fractions prepared from the cells grown under denitrifying conditions, as shown in Table I. However, the activities showed marked contrast in the specificity for other electron donors. NADH and NADPH were effective only for the activity recovered in the soluble fraction, whereas succinate and formate could support only the activity in the large particles Both fractions contained no activity when the cells were grown without nitrate (preculture; zero time in Fig. 1), indicating that these activities are inducible by nitrate The results clearly demonstrated the presence of distinct species of nitrate reductase, the properties of which are consistent with assimilatory (soluble) and dissimilatory (membrane-bound) nitrate reductases, respectively. The activity-staining native PAGE (Fig. 2A) further confirmed the heterogeneity of nitrate reductase activity. The activity in the soluble fraction exhibited little mobility under the conditions, whereas the solubilized large particle fraction contained two mobile, active bands.

Formate Dehydrogenase (Fdh) Activity—Formate-dependent Nar activity detected in the large particle fraction (Table I) suggested presence of Fdh coupling to the Nar reaction. As shown in Table II, Fdh activity could be detected

TABLE I. **Electron donors of nitrate reductase activities.** *Cell-free extracts were prepared from the cells grown under denitrifying conditions and assayed for nitrate reductase activity as described in "MATERIALS AND METHODS"*

Electron donor (10 mM)	Relative activity (%)	
	Soluble	Large particles
MVH	100[a]	100[b]
NADH	33	0
NADPH	36	0
Succinate	0	25
Formate	0	25

[a]0 397 nmol·min^{-1}·mg protein^{-1} [b]0.684 nmol min^{-1}·mg protein^{-1}

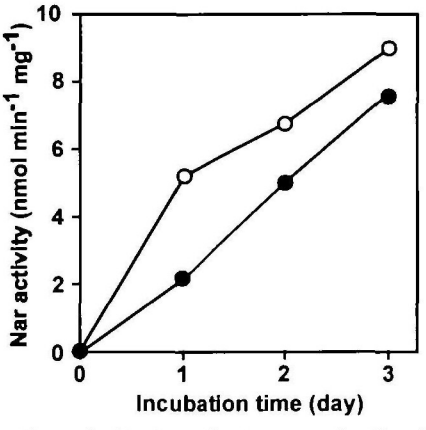

Fig 1 **Induction of nitrate reductase production by nitrate.** MVH-dependent nitrate reductase activity in the subcellular fractions was measured for each fraction after incubation of the cells in the nitrate-containing medium for indicated periods. (○), large particle fraction, (●), soluble fraction

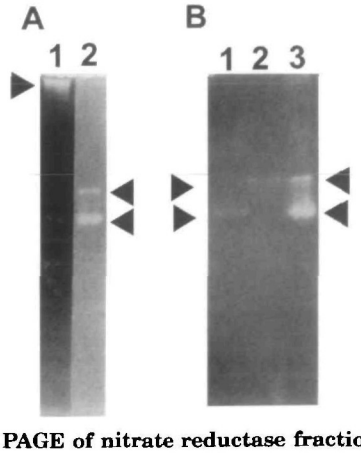

Fig. 2 **Native PAGE of nitrate reductase fractions.** After electrophoresis, enzymes were visualized by activity-staining. (A) Soluble (lane 1) and large particle (lane 2) fractions. (B) Nar2 (lane 1), Nar1 (lane 2), and the Nar pool after Q-Sepharose (lane 3) fractions in Table III

in the large particle fraction by employing the phenazine methosulfate (PMS)–DCIP system as the electron acceptor. In addition to the artificial system, a physiological electron acceptor, ubiquinone 2, was also effective in reconstituting the activity. Very slight activity could be reconstituted when BV was utilized, whereas MV, NAD^+, and $NADP^+$ were quite inert.

Solubilization and Partial Purification of the Membrane-Bound Nitrate Reductase (Nar) Activities—The Nar activity in the large particles was solubilized and partially purified for further characterization. The activity was separated into two fractions after the final step (Mono Q), which were tentatively termed Nar1 and Nar2 (Table III, Fig. 3). Nar1 and Nar2 exhibited different motilities on the native PAGE (Fig. 2B). Each band agreed well in its mobility with the respective active band derived from the particle fraction (Fig. 2A, lane 2) or in the Q-Sepharose pool before Nar1 and Nar2 were separated (Fig 2B, lane 3). So it can be concluded that this purification process separated the heterologous Nar activities in the large particle fraction. The amount of protein recovered after the final step was, however, too low to determine their concentrations or the subunit compositions by SDS-PAGE

Absorption Spectrum—The absorption spectrum of the Nar fraction after hydroxyapatite chromatography is indicated in Fig. 4 The spectrum was characteristic of a cytochrome with absorption peaks at 556, 525, and 425 nm in its dithionite-reduced form, and closely resembles that of

TABLE II **Electron acceptors of formate dehydrogenase.** The large particle fraction was assayed for each Fdh activity

Electron acceptor[a]	Relative activity (%)
PMS-DCIP	100[b]
MV	0
Benzylviologen	1
NAD^+	0
$NADP^+$	0
Ubiquinone 2	35

[a]1 mM except for PMS (0 4 mM)–DCIP (0 05 mM) system [b]0.366 nmol·min^{-1}·mg protein^{-1}

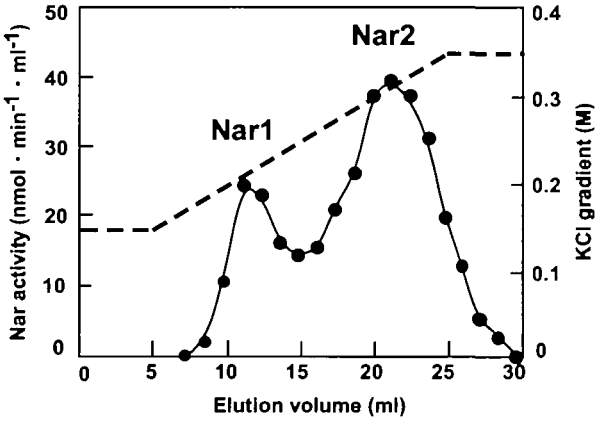

Fig. 3. **Separation of Nar fractions by anion exchange chromatography on Mono Q.** (●) Nar activity (nmol min^{-1} ml^{-1}); (-----), KCl gragient (M)

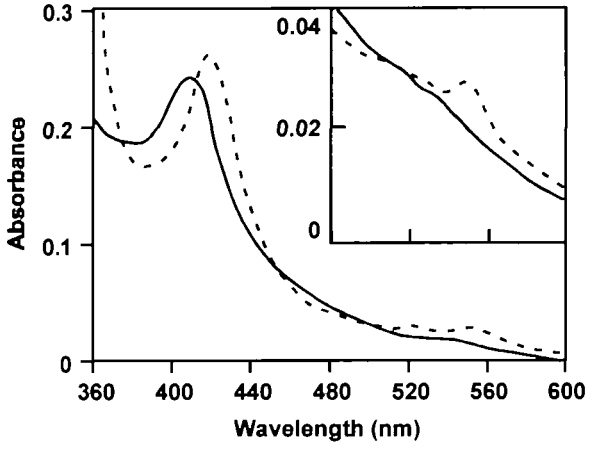

Fig. 4 **Absorption spectra of nitrate reductase fraction after hydroxyapatite column chromatography.** (——), oxidized form as prepared (Table III), (----), dithionite-reduced form Inset, expanded

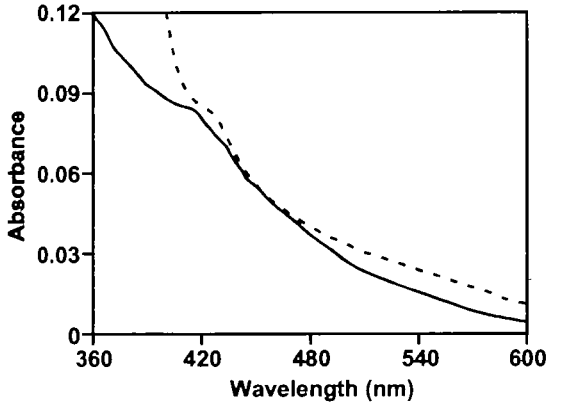

Fig 5 **Absorption spectra of Nar2 fraction after Mono Q column chromatography.** (——), oxidized form as prepared (Table III), (-----), dithionite-reduced form

TABLE III **Purification of nitrate reductase from the large particle fraction of *F. oxysporum*.**

Step	Total protein (mg)	Total activity (nmol·min^{-1})	Specific activity (nmol min^{-1}·mg^{-1})	Recovery (%)
Large particle	1,900	7,700	4.05	100
Solubilized Fraction	870	5,470	6 27	71
Q-Sepharose	69	5,470	79 2	71
Hydroxyapatite	12	4,130	344	53 5
Mono Q				
Nar1	–[a]	461	–	6 0
Nar2	–	769	–	10 0

[a]Not determinable.

cytochrome b_1 of bacterial Nar (*24–27*). By further purification, the activity was divided into Nar1 and Nar2 fractions. Their absorption spectra showed that Nar1 retained cytochrome *b*, whereas Nar2 lost the cytochrome and displayed a spectrum characteristic of an iron-sulfur protein (Fig. 5) (*24, 25, 28*).

Electron Donors—The Nar fraction retained the ubiquinol 2-dependent Nar activity until the step of hydroxyapatite in the purification process (Table IV). Another physiological electron donor, menadiol, supported reconstitution of a low but distinct activity. In addition to MVH, ascorbate-PMS was effective as an artificial electron donor. This ubiquinol-dependent Nar activity was lost in the Nar2 fraction but retained in the Nar1 fraction (data not shown), showing that the cytochrome *b* component is essential for the activity. So the heterogeneity of Nar activity in the large particle fraction can be ascribed to the presence of two assembly forms containing or not containing the cytochrome *b* component (corresponding to the γ-subunit in the Nar of *E. coli*).

Inhibitors—Effects of inhibitors on the Nar activities were examined (Table V). The formate-dependent Nar activity in the large particle fraction was moderately inhibited by toluene 3,4-dithiol or 2-heptyl-4-hydroquinoline *N*-oxide (HQNO). Toluene 3,4-dithiol is a chelator of molybdenum cofactor, and HQNO is an analogue of quinones and inhibits ubiquinone/ubiquinol-dependent enzyme reactions. Some inhibitors (cyanide, azide, and toluene 3,4-dithiol) inhibited these Nar fractions to different extents Cyanide and azide considerably inhibited the activity in the hydroxyapatite fraction, whereas they did not inhibit at all the formate-dependent activity in a membrane-bound form. It is possible that access of these inhibitors is blocked when the enzymes are membrane-bound. The profile of inhibition of these Nar activities by these inhibitors agrees well with the properties of the molybdenum-containing dissimilatory nitrate reductases of bacteria (*29,30*).

Effects of Defective Mutation of the Nitrate-Assimilation on the Dissimilatory Reduction of Nitrate—Next we investigated the fungal metabolism of nitrate from a different aspect to show the occurrence of distinct nitrate reductase species in *F. oxysporum*. It has been reported that fungal mutants defective in assimilatory nitrate reductase can be isolated by their chlorate-resistance (*21*). We selected spontaneous mutants that are able to grow on the chlorate-containing plates and characterized them (Table VI). They were classified into three groups by their ability to utilize different nitrogen sources for growth. The group of mutants represented by strain M10 showed little growth on the medium containing nitrate as a sole nitrogen source but could grow on nitrite. These strains exhibited significant decrease in NADPH-dependent nitrate reductase activity (Table VII), indicating that they had a lesion in the structural gene for assimilatory nitrate reductase (NR), *niaD* (*31*). On the other hand, strain M10 showed less but significant MVH-dependent Nar activity along with the N_2O-forming denitrifying activity, which indicated that dissimilatory reduction of nitrate occurs independently from assimilatory nitrate reductase and that dissimilatory and assimilatory nitrate reductases are regulated by independent genes. Strain M1 utilized the same nitrogen sources as strain M10 with the exception of hypoxanthine This phenotype is typical of a deficiency in biosynthesis of molybdenum cofactor (*21*). The loss of MVH-dependent Nar activity along with the N_2O-forming denitrifying activity in this strain (Table VII) is consistent with our proposal that dissimilatory Nar is a molybdenum protein and the Nar species is critical for the dissimilatory reduction of nitrate in this fungus.

The third group, typified by strain M7, was defective in utilizing both nitrate and nitrite as well as in NADPH-dependent nitrate reductase and Nir activities (Table VII). The mutations causing this phenotype are called Nit and thought to be derived from mutations in the regulatory loci of nitrate-inducible genes in *F. oxysporum* (*21*) and other

TABLE IV **Electron donors of partially purified Nar.** The Nar fraction after the hydroxyapatite stop in Table III was assayed for each activity

Electron donor	Concentration (mM)	Relative activity (%)
Dithionite-MV	10 + 0 1	100[a]
Dithionite	10	1 0
Ascorbate-PMS	10 + 0.1	10 8
Ascorbate	10	1 1
Ubiquinol-2	1 0	12 4
Menadiol	1 0	1 4
Duroquinol	1 0	0
None	0	0

[a]0.344 μmol min^{-1} mg $protein^{-1}$

TABLE V **Inhibitors of formate- and MVH-dependent Nar activities.** The Nar fraction after the hydroxyapatite step in Table III was assayed for each activity

Inhibitor (1 0 mM)	Relative activity (%) (Electron donor/Nar fraction)	
	Formate/large particle	MVH/hydroxyapatite[a]
None	100[b]	100[c]
KCN	98	15
NaN_3	100	17
EDTA	91	80
α,α′-Dipyridyl	97	97
8-Hydroxyquinoline	–[d]	100
o-Phenanthroline	–	100
Toluene 3,4-dithiol	39	14
HQNO	46	100

[a]Same Nar fraction as in Table IV [b]0 173 nmol·min^{-1}·mg $protein^{-1}$ [c]0 344 μmol·min^{-1}·mg $protein^{-1}$ [d]Not tested

TABLE VI **Nitrogen assimilation by the chrolate-resistant mutants.** The strains were replicated on the basal medium containing indicated compounds as a nitrogen source. +, growth was observed –, no or little growth

Strains	Genotype	Growth on					
		Nitrate	Nitrite	Hypoxantine	Glutamine	Uric acid	Ammonia
MT-811	WT	+	+	+	+	+	+
M1	Moco	–	+	–	+	+	+
M10	NR	–	+	+	+	+	+
M7	Nit	–	–	+	+	+	+

TABLE VII **Enzyme activity and denitrification by the *F. oxysporum* strains.** To measure assimilatory NADPH-dependent nitrate reductase (NR) and Nir, the strains were cultured in the basal medium with nitrate as a nitrogen source. To analyze denitrification (N_2O production) and MVH-dependent Nar, the strains were cultured in the basal medium with sodium nitrate and peptone. Other conditions were described in "MATERIALS AND METHODS." Data are mean values of three experiments.

Strain	Genotype	Enzyme activity				N_2O production (mmol flask^{-1})
		MVH-Nar/NR (nmol NO_2^- min^{-1} mg^{-1})	NADPH-NR (nmol NO_2^- min^{-1} mg^{-1})	NADPH-Nir (nmol NADPH min^{-1} mg^{-1})	P450nor (mmol N_2O min^{-1} mg^{-1})	
MT-811	WT	10 4	3 1	16 5	0 60	153
M10	NR	4 6	0 3	5 3	1 2	23
M1	Moco	0 4	N D*	13 9	0 60	N D
M7	Nit	0 5	N D	N D	N D	N D

*Not detectable

fungi (*32*). It is of interest that the strain simultaneously lost the activities related to the dissimilatory metabolism, that is, MVH-Nar, P450nor-dependent Nor, and the N_2O-forming denitrification. Immunoblot analysis also showed lack of production of P450nor in the strain M7 (data not shown), which is consistent with nitrate-specific induction of the Nor-encoding gene (*33*). These results suggest that the fungal nitrate respiration system involving Nar and Nor shares with the genes for assimilation of nitrate/NO_2^- the transcription machinery that controls their expression.

DISCUSSION

The results of enzymatic and genetic analyses presented here are all consistent with the presence of at least two distinct species of nitrate reductase in *F. oxysporum*, dissimilatory nitrate reductase (Nar) and assimilatory nitrate reductase. It was also shown that Nar is essential for the fungal denitrification (Tables VI and VII). The assimilatory reduction of nitrate by fungi has long been well characterized (*31, 32*), whereas little data were available characterizing the eukaryotic Nar of fungi. A few papers have reported the occurrence of Nar in fungi by detecting the MVH-dependent Nar activity (*1, 7, 17, 34*). As is evident from the results in Table I, however, the MVH-dependent activity cannot discriminate Nar activity from assimilatory nitrate reductase activity. So the detection of the MVH-dependent activity does not provide enzymatic evidence for the occurrence of Nar. Here we showed that ubiquinol is the specific electron donor for the mitochondrial Nar, which should be the physiological electron donor of Nar associated with the mitochondrial respiratory chain (*7*). It would appear from the absorption spectrum (Figs. 4 and 5) along with other results that the fungal Nar has a similar subunit composition to the bacterial Nars of *E. coli* and *Paracoccus denitrificans*, which are also involved in nitrate respiration (denitrification). They are comprised of α, β, and γ subunits, which respectively contain molybdocofactor, iron-sulfur redox center, and cytochrome *b* (*24–28, 35*) Nar1 fraction seems to be complete with respect to the quaternary structure, because it retained the ubiquinol-dependent activity, and loss of the cytochrome *b* component in Nar2 accompanied loss of the activity.

NAD-dependent Fdh is known to occur widely among eukaryotes, but Fdh associated directly with the respiratory chain has not hitherto been reported. The present results are the first to show the occurrence of formate-ubiquinone oxidoreductase in a eukaryote. We previously showed that formate can support the mitochondrial Nar activity (*7*), and here we show that the fungal Fdh can supply electrons via quinol/quinone pool to Nar. A similar electron-supplying pathway to nitrate-respiration is well known in *E. coli*. A nitrate-inducible, membrane-bound formate-menaquinone oxidoreductase (formate dehydrogenase-N) supplies electrons *via* a quinol/quinone pool to a menaquinol-nitrate oxidoreductase (membrane-bound nitrate reductase-A) (*35*). Since menaquinone might not be applicable to eukaryotic cells as a mitochondrial electron-transferring component, the fungal Fdh-Nar system might have adapted to utilize ubiquinone instead of menaquinone. Such an Fdh-Nar coupling system has not hitherto been reported among denitrifying soil bacteria. So it is of extreme interest that the fungal denitrifying system contained an Fdh-Nar coupling system that occurs among bacteria only in the nitrate respiration system (in *E. coli*) of the non-denitrifying type.

Since complete purification of Nar from *F. oxysporum* seems very difficult, simultaneous studies by other approaches is also required to clarify the novel respiration system in the fungal mitochondrion. Recently, Kurakov and coworkers showed that a Nar species distinct from assimilatory nitrate reductase should be induced concomitantly with denitrifying activity in the same fungal species, mainly from the results using metabolic inhibitors (*17*). Isolation of the Nar gene, as well as purification of the Nar protein, should be the most important tools for characterizing the fungal Nar. Our present results on the defective mutants (Tables VI and VII) demonstrated that such genetic approaches would be possible, for example, by screening a cDNA library for a cDNA that compensates for the Nar deficiency.

We previously showed that a small amount of oxygen (O_2) supply is essential for inducing denitrifying activity in the fungal cells as well as forming intact mitochondria in which the denitrifying system is localized (*5, 6*). The mitochondria formed under such hypoxic conditions also contained cytochrome oxidase (cytochrome aa_3) (*7*). Therefore, it would appear that nitrate respiration (denitrification) and O_2 respiration are acting simultaneously in such hypoxic mitochondria. The fungal denitrifying system seems to lack Nor (cytochrome *cb*) and N_2O reductase (Nos) of bacterial type, and its Nor (P450nor) receives electrons directly from NADH. So the fungal system forms NO as the final product of nitrate respiration from nitrate, in which Nar-Fdh couple and Nir should be involved. Among the enzymes involved in the full denitrifying process of bacteria, only Nar is oriented to the cytoplasm side and its reaction contributes to the proton-motive force (*36*). Other enzymes (Nir, Nor, and Nos) are oriented or localized to the periplasm side and thus their reactions cancel the proton-

motive force (*35*). Therefore, if O_2 is available, it should be energetically more favorable for the respiratory chain to avoid donating electrons to Nir, Nor, and Nos downstream of the complex III. Employment of Fdh as the electron-donating system to Nar is also energetically favorable, since the formate-ubiquinone reductase reaction due to Fdh can contribute to a proton-motive force It would therefore appear that the eukaryotic microorganisms (fungi) have constructed the own respiration system during evolution to adapt to hypoxic conditions, a hybrid respiration system, in which the energetically favorable portion of nitrate respiration is combined with O_2 respiration.

We have isolated and/or characterized several components that are involved in the fungal denitrification by *F oxysporum*: Nor (P450nor) (*11*), Nir (*9*), cytochrome c_{549} (*9*), flavohemoglobin (*37*), Nar (*7*, and present study), and Fdh (*7*, and present study). P450nor has not been found in bacteria, whereas flavohemoglobin is more universal among prokaryotic and eukaryotic microorganisms (*38*). As noted above, the Nar-Fdh couple seems to resemble the bacterial counterpart involved in the nitrate respiration of non-denitrifying type in *E. coli*. These components in the fungal system seem to be a medley from various origins. Isolation of their genes should clarify more about the origin and evolution of the fungal denitrifying system.

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