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Mutants of *Pseudomonas fluorescens* Deficient in Dissimilatory Nitrite Reduction Are Also Altered in Nitric Oxide Reduction

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Five Tn*5* mutants of *Pseudomonas fluorescens* AK-15 deficient in dissimilatory reduction of nitrite were isolated and characterized. Two insertions occurred inside the nitrite reductase structural gene (*nirS*) and resulted in no detectable nitrite reductase protein on a Western immunoblot. One mutant had Tn*5* inserted inside *nirC*, the third gene in the same operon, and produced a defective nitrite reductase protein. Two other mutants had insertions outside of this *nir* operon and also produced defective proteins. All of the Nir^- mutants characterized showed not only loss of nitrite reductase activity but also a significant decrease in nitric oxide reductase activity. When cells were incubated with ^{15}NO in $H_2{}^{18}O$, about 25% of the oxygen found in nitrous oxide exchanged with H_2O. The extent of exchange remained constant throughout the reaction, indicating the incorporation of ^{18}O from $H_2{}^{18}O$ reached equilibrium rapidly. In all nitrite reduction-deficient mutants, less than 4% of the ^{18}O exchange was found, suggesting that the hydration and dehydration step was altered. These results indicate that the factors involved in dissimilatory reduction of nitrite influenced the subsequent NO reduction in this organism.

Dissimilatory reduction of nitrite is the key step in the denitrification pathway; it is the point of divergence from assimilatory nitrogen metabolism (24). There are two types of nitrite reductases: one contains the cytochromes cd_1, and the other contains copper (6, 14, 20). Organisms containing the cytochrome cd_1 nitrite reductases are more frequently isolated from nature, whereas Cu-type nitrite reductases are found in organisms that exhibit more phylogenic diversity and occupy a wider range of ecological niches (6, 9).

Nitric oxide is the major product of nitrite reduction by purified nitrite reductases, and nitrous oxide is a minor product (4, 14, 17, 26). NO is generally accepted as one of the free and obligatory intermediates in the denitrification pathway (2, 5, 8, 10, 14, 20, 28), and NO reductases have been purified from *Pseudomonas stutzeri* Zobell (13) and *Paracoccus denitrificans* (4, 7). We recently showed that many denitrifiers containing Cu or cd_1 nitrite reductases are capable of undergoing O-atom exchange with $H_2{}^{18}O$ during the reduction of NO to N_2O (27) and that a labeled intermediate can be trapped with azide. These results suggest that a nitrosyl complex is formed during the reaction. It was also shown that the extent of the O-atom exchange reaction depended on the availability of electrons. The dependence of nitrosyl transfer upon the presence of NO during reduction of NO_2^- has been demonstrated (11).

Tn*5* was used by Zumft et al. (29) to generate mutants deficient in dissimilatory nitrite reduction (Nir^-) in *P. stutzeri* Zobell. All of the mutants isolated possessed normal NO reduction activity, indicating that NO reduction and nitrite reduction are distinct. The nitrite reductase gene (*nirS*) from *Pseudomonas aeruginosa* (21) and from two strains of *P. stutzeri* (Zobell [15] and JM 300 [23]) has been cloned. *nirM* encoding cytochrome c_{551} and *nirC* encoding a heme protein (also named ORF5 in *P. stutzeri*) are located immediately downstream of the nitrite reductase gene in *P. aeruginosa* (1, 15, 19). This is not the case in *P. stutzeri* (15), where *nirT* encoding a tetraheme protein and *nirB* encoding cytochrome c_{552} are located between the *nirS* and *nirM* genes.

We used Tn*5* to generate Nir^- mutants in *Pseudomonas fluorescens* and found that, in contrast to *P. stutzeri* Zobell, all five Nir^- mutants characterized not only lacked the ability to reduce nitrite but also showed a decreased ability to reduce NO. Furthermore, the extent of ^{18}O exchange with $H_2{}^{18}O$ during reduction of ^{15}NO to $^{15,15}N_2O$ was reduced to background levels in the mutants. These results suggest that the dissimilatory reductions of NO_2^- and NO are linked in this organism.

MATERIALS AND METHODS

Bacterial strains. *P. fluorescens* AK-15 is a gram-negative rod isolated from Capac loam soil from the Kellogg Biological Station, Kalamazoo County, Mich. This strain was identified based on its denitrifying ability, production of fluorescent pigments on King medium, growth at 4°C, lack of growth at 37°C, and fatty acid profiles. This strain was chosen for these studies because *P. fluorescens* strains are the most prevalent denitrifiers in nature and this strain has a high frequency of Tn*5* mutagenesis. Bacteria were grown at 25°C in tryptic soy broth (TSB; Difco Laboratories) supplemented with 0.15% KNO_3. A rifampin-resistant clone, YT101, was isolated from a spontaneous mutation.

Conjugation and isolation of mutants. The rifampin-resistant strain of wild-type *P. fluorescens*, YT101, was used as the recipient, and *Escherichia coli* S-17 carrying the pSUb2021 plasmid containing Tn*5* was used as the donor (22). Mating was carried out at 25°C overnight. Exconjugants were selected on TSB plates supplemented with rifampin (50 μg/ml) and kanamycin (50 μg/ml). Colonies were replica plated onto TSB plates containing 0.15% KNO_3 and grown

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overnight in an anaerobic glovebox. Plates were then taken out of the anaerobic glovebox, and a piece of Whatman no. 42 filter paper was laid on top of each plate and covered with a thin layer of cooled agar. N_2 gas bubbles appeared over denitrifying colonies in 30 to 60 min. If a thick top agar layer was used without the Whatman paper, it took more than 12 h before N_2 gas bubbles appeared and they were more difficult to distinguish. Colonies that did not produce gas bubbles were picked and further tested for their ability to produce bubbles by growth in culture tubes containing inverted Durham tubes.

Growth conditions and activity assays. Strain YT101 was grown in the presence of rifampin (50 μg/ml). Mutant strains were grown in the presence of both kanamycin (50 μg/ml) and rifampin (50 μg/ml). Anaerobic cells were grown overnight at 25°C in serum bottles containing 100 ml of TSB and 0.15% KNO_3. They were harvested by centrifuging at 8,000 × *g* for 15 min and were washed twice with 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.3). Crude extracts were prepared by sonication and subsequent centrifugation at 10,000 × *g* for 20 min at 4°C. The supernatant was assayed for nitrite and NO reductase activities by measuring the rate of NO and N_2O appearance. The reaction mixture for NO reduction by the crude extract included 10 mM EDTA, 40 μM phenazine methosulfate (PMS), 4 mM NADH, and 50 mM HEPES (pH 7.3). To assay the NO reduction activities in whole cells, anaerobically grown cells were suspended in 1 ml of TSB in an 8-ml serum bottle with an anaerobic atmosphere created by repeated evaculation and filling with argon. NO was injected to start the reaction. All reaction vials were shaken on their sides at 100 rpm to facilitate the rate of gas-liguid transfer. NO and N_2O were detected by gas chromatography with ^{63}Ni electron capture detectors (16). The protein concentration of the crude extract was determined by the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co.). The amount of protein in whole cells was estimated with the Folin and Ciocalteu phenol reagent (Sigma) after alkaline lysis with 1 N NaOH (12).

DNA sequencing. To determine the Tn*5* insertion site in the Nir$^-$ mutants, subclones in pUC18 containing the left inverted repeat region of Tn*5* were sequenced with a primer made to a region near the end of the inverted repeat. DNA was sequenced by the dideoxy method with a sequencing kit (U.S. Biochemical Corp.).

Western and Southern blots. Western immunoblots were developed with antibodies against nitrite reductase from *P. aeruginosa* as described previously (6). Genomic DNA isolation, restriction enzyme digestion, electrophoresis, and Southern blotting were done as described by Maniatis et al. (18). Plasmid pSUP2021 containing Tn*5* was isolated by alkaline lysis (18) and labeled by nick translation (Boehringer Mannheim). The nitrite reductase gene from *P. aeruginosa* in a 3.5-kb *Eco*RI fragment in pEMBL18 was provided by M. C. Silverstrini (21).

Isotope analysis. ^{15}NO preparation, gas chromatography-mass spectrometry measurements, and calculation of ^{18}O exchange were performed as described previously (25, 27).

RESULTS

Isolation of Nir$^-$ and Nos$^-$ mutants. Deficiency in any step of denitrification leads to a lack of N_2 formation. The top agar method used previously to isolate denitrifying deficient mutants in *P. stutzeri* (29) did not work well with *P. fluorescens* AK-15, perhaps because of the slower rate of N_2 production in strain AK-15. Whatman filter paper applied on top of colonies before a thin layer of top agar was added proved to be more efficient in trapping the N_2 gas. Mutants that could not produce bubbles were categorized as Nir$^-$ (deficient in NO_2^- reduction) or Nos$^-$ (deficient in N_2O reduction) based on the characteristics summarized in Table 1. The frequencies of Nir$^-$ and Nos$^-$ phenotypes were about 0.032 and 0.024%, respectively. No nitric oxide reduction-deficient (Nor$^-$) mutants were found. All Nir$^-$ and Nos$^-$ mutants could grow with NO_3^- as the electron acceptor, but they could not produce N_2 bubbles because of a block in either nitrite reduction or N_2O reduction. Mutants deficient in nitrite reduction produced bubbles in the presence of both NO_3^- and N_2O, suggesting that these mutants could convert N_2O to N_2. Nitrate was included in this test because *P. fluorescens* AK-15 could not grow well on N_2O alone. After overnight growth on nitrate, Nir$^-$ mutants accumulated NO_2^-, whereas Nos$^-$ mutants accumulated N_2O. Two Nos$^-$ mutants (YT15 and YT25) were used for control studies.

TABLE 1. Characteristics of Nir$^-$ and Nos$^-$ mutants from *P. fluorescens* AK-15

Strain	Accumulation of NO_2^- from NO_3^-[a]	Bubble formation from:		Accumulation of N_2O from NO_3^-[a]
		NO_3^-	N_2O plus NO_3^-	
YT101	−	++	++	−
YT2511	+	−	++	−
YT2471	+	−	++	−
YT3221	+	−	++	−
YT31	+	−	++	−
YT4221	+	−	++	−
YT25[b]	−	−	−	++
YT15	−	−	−	++

[a] Accumulation of NO_2^- and N_2O was tested after overnight growth.
[b] Four additional mutants with the same phenotype were also isolated.

Physical characterization of Nir$^-$ mutants. To detect Tn*5* in these mutants, Tn*5* was labeled and used to probe the genomic DNA from the wild-type and mutant strains. When genomic DNA was cut with *Eco*RI and *Bam*HI, two bands, which varied in sizes in different mutants, hybridized to the Tn*5* probe, suggesting that single copies of Tn*5* had been inserted into different positions in the genome (data not shown). One of the *Eco*RI and *Bam*HI fragments of each mutant strain with the intact *neo* gene of Tn*5* was subcloned in pUC19 by selection for resistance to kanamycin. Subclones from YT2511, YT2471, and YT3221 hybridized to a common 12-kb *Eco*RI DNA fragment, which also hybridized to structural genes of nitrite reductases from *P. aeruginosa* (21) and *P. stutzeri* JM300 (23), suggesting that all three Tn*5* insertion sites were clustered in this *nir* operon. By obtaining DNA sequences from regions flanking the Tn*5* insert in pUC19 subclones and comparing them with published *nir* operon sequences (15, 21, 23), we found that Tn*5* was inserted near the middle of the nitrite reductase gene in YT2511 and near the carboxyl terminus in YT2471. In YT3221, Tn*5* was located near the heme binding site in *nirC*, the third gene in the operon. Subclone fragments from mutants YT4221 and YT31 did not hybridize to the 12-kb *Eco*RI genomic DNA fragment containing the nitrite reductase gene and had no homology with each other.

Biochemical characterization of Nir$^-$ mutants. Crude cell extracts of all Nir$^-$ mutants showed no nitrite reductase activity (Table 2). When a Western blot was developed with antibodies against the nitrite reductase, mutants YT4221,

TABLE 2. Rates of NO_2^- and NO reduction and extents of ^{18}O exchange by wild-type and mutant strains of *P. fluorescens* AK-15[a]

Strain	nmol of N mg^{-1} min^{-1}			^{18}O Exchange[b] (%)
	Crude extract		Cells (+NO)	
	$+NO_2^-$	+NO		
YT101	110	17.4	38.2	25.5 ± 1.3
YT2511	0[c]	6.4	10.1	1.3 ± 0.4
YT2471	0	8.2	8.0	1.5 ± 0.5
YT3221	0	7.1	12.8	1.3 ± 1.3
YT31	0	7.7	10.4	1.2
YT4221	0	8.4	17.6	3.9 ± 2.5
YT25	ND[d]	ND	37.6	23.3 ± 6.4
YT15	ND	ND	39.4	23.7 ± 5.0

[a] Data are means of duplicate samples for crude extracts and triplicate samples for whole cells. The nitrite reductase activity assay mixture had about 0.04 mg of protein and 1 μmol of nitrite. The NO reductase assay contained 0.08 mg of protein and 2 μmol of NO. The same amount of NO was used in the whole-cell assay.

[b] Reaction mixtures contained 4 mg of whole-cell protein, 5.14% $H_2{}^{18}O$, and 1.5 μmol of ^{15}NO in an 8-ml serum bottle with 1 ml of TSB solution. The reaction was stopped with 0.1 ml of NaOH (10 N) after 40 min. The data are obtained from triplicate samples.

[c] The detection limit for specific activity was about 0.13 nmol mg^{-1} min^{-1}.

[d] ND, not determined.

YT31, and YT3221 showed a positive band corresponding in size to the wild-type nitrite reductase protein, indicating that defective proteins were made (Fig. 1). However, mutants YT2511 and YT2471 showed no such band. This was consistent with the physical characterization in that these two mutants had Tn*5* inserted inside *nirS*. The fact that a defective protein was made by YT3221 suggested that *nirC* or a gene(s) further downstream is essential for the production of active nitrite reductase.

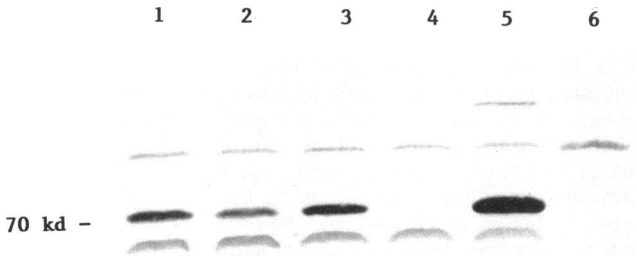

FIG. 1. Western blot of proteins from wild-type and mutant strains. The blot was developed with polyclonal antibodies raised against the nitrite reductase purified from *P. aeruginosa*. Samples of 5 μg of protein were used per blot for all strains. Lanes 1 through 6 contained strains YT4221, YT31, YT3221, YT2511, YT101, and YT2471, respectively.

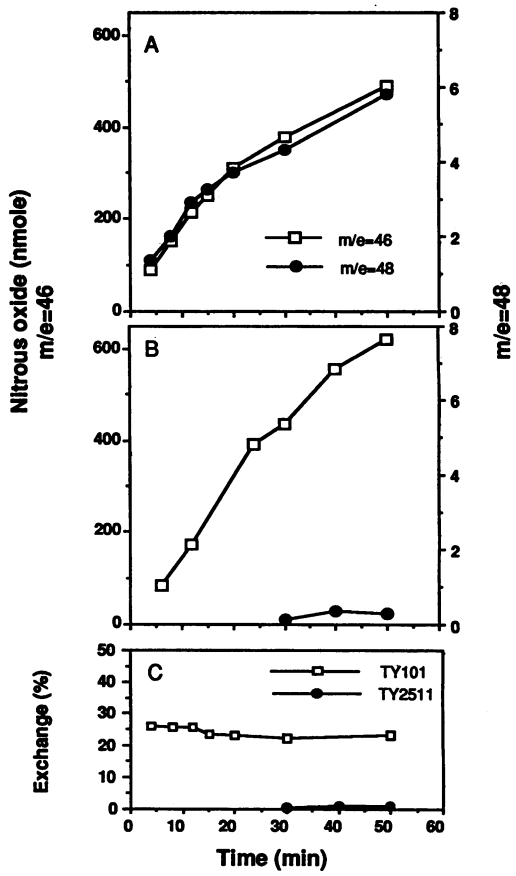

FIG. 2. The time course of ^{18}O exchange in resting cells of the YT101 (A) and YT2511 (B) mutant strains. The extent of exchange (C) was calculated based on the data in panels A and B. Data are means of duplicate samples. The amounts of cells were equivalent to 1 mg of protein for the wild type and 4 mg of protein for YT2511. In YT2511, the $^{15,15}N_2{}^{18}O$ species could not be detected until after 20 min.

The NO reduction activity in resting cells in Nir^- mutants of *P. fluorescens* AK-15 was reduced by about three- to fourfold relative to that of the Rif^r wild-type strain YT101. When crude cell extracts were assayed in the presence of artificial electron donors (PMS and NADH), the NO reduction activity was reduced by about two- to threefold (Table 2). The NO reduction activity in Nos^- mutants was not altered. Thus, there is a consistent reduction in NO reduction activity only in Nir^- strains.

^{18}O-exchange studies to probe the mechanism of NO reduction. To study whether mutations in *nir* genes also affected the mechanism of nitric oxide reduction, the extent of oxygen atom exchange during the reduction of ^{15}NO in resting cells was measured. The ratio of $^{15,15}N_2{}^{18}O$ (*m/e*, 48) to the total amount of nitrous oxide produced reflects the extent of exchange (27); this was about 25% in the YT101 and Nos^- mutants (YT25 and YT15) (Table 2). However, in Nir^- mutants the extent of exchange was reduced to background levels except in YT4221, which was slightly above the background. The percentage of exchange remained constant at 25% throughout the course of reduction of NO to N_2O, indicating that the hydration-dehydration reaction rap-

idly reached equilibrium (Fig. 2). Since Nir$^-$ mutants had a reduced rate of NO reduction, four times more cells from mutant YT2511 were used in the time course study. Only a very small amount of $^{15,15}N_2{}^{18}O$ was observed, suggesting that the decreased ^{18}O exchange for YT2511 (Table 2) reflects an intrinsic difference in mechanism rather than simply a difference in the rate at which isotopic equilibrium was reached.

DISCUSSION

Among the five characterized Nir$^-$ mutants, three had Tn*5* inserted in the *nir* operon containing the nitrite reductase structural gene (*nirS*). Insertion of Tn*5* into the *nirS* genes in YT2511 and YT2471 resulted in no nitrite reductase activity and the absence of identifiable nitrite reductase protein in a Western blot (Fig. 1). Insertions in the *nirC* genes and two other regions, however, produced defective nitrite reductase proteins, suggesting that three or more genes are involved in the production of an intact nitrite reductase. These may include genes involved in the synthesis of the unique heme d_1 chromophore or for assembling or processing of the polypeptide.

All five Nir$^-$ mutants isolated from this strain of *P. fluorescens* showed a decrease in the rate of NO reduction and a lack of ^{18}O exchange with $H_2{}^{18}O$ during reduction of NO (Table 2). The decrease in NO reduction rate was about 3- to 4-fold in whole cells, but all mutants except YT4221 showed at least a 10-fold reduction in ^{18}O exchange, suggesting that there is a genetic and/or mechanistic relationship between the dissimilatory reduction of NO_2^- and that of NO. This relationship cannot, however, be due simply to mutations in the regulatory region of the genome: YT2511 and YT2471 had Tn*5* inserted inside the nitrite reductase structural gene, yet they had the same decrease in NO reduction and ^{18}O exchange as the others. Further, Tn*5* mutants deficient in N_2O reduction (Nos$^-$) exhibited normal rates of NO reduction and ^{18}O exchange, indicating that the Tn*5* insertion event per se does not result in the observed effects on NO reduction.

Possible explanations for these results include the following. (i) In normal cells in vivo, a substantial portion of the NO to N_2O flux is catalyzed by the nitrite reductase, such that Nir$^-$ mutants exhibit reduced NO reduction levels with different ^{18}O exchange characteristics due to the functional NO reductase. Although definitive data with *P. fluorescens* have not yet been obtained, studies on *P. stutzeri* have shown that purified nitrite reductase is incapable of reducing NO either by itself or in the presence of NO_2^- (17). Furthermore, quantitative studies of NO concentrations during reduction of NO_2^- are consistent with the bulk of the nitrogen flux of denitrification occurring via NO (28). Thus, this explanation seems highly improbable. (ii) Production of N_2O from NO_2^- is a two-step process involving NO production by the nitrite reductase and subsequent NO reduction to N_2O by NO reductase. These two enzymes may associate with each other in vivo to channel products from one to another. Loss of functional nitrite reductase may lead to disruption of this association, resulting in some loss of NO reductase activity and the absence of ^{18}O exchange. (iii) A third possibility is that a functional nitrite reductase or functional nitrite reduction system is necessary for full expression of NO reductase activity at the functional or genetic level. (iv) Similarly, a functional nitrite reductase might be necessary for the synthesis of one or more electron transfer proteins, such as cytochromes, that are specific electron donors for the NO reductase.

The results of Zumft et al. (29) with Tn*5*-induced mutants of *P. stutzeri* Zobell may be consistent with the last interpretation. Although they found no significant changes in NO reduction activity, they did find two Nir$^-$ mutants that had significant changes in the amount of cytochrome c_{552} and/or alpha-peak *c*-type cytochrome, and they suggested that these cytochromes exhibited a functional and/or regulatory interdependence. However, *P. fluorescens* AK-15 and *P. stutzeri* Zobell differ both physiologically and genetically. For example, the former does not grow efficiently on N_2O alone. This phenomenon is often observed in *P. aeruginosa* (14). The structure of the *nir* operon in *P. fluorescens* AK-15 consists of *nirS* followed immediately by *nirM* and *nirC*, similiar to the *nir* operon in *P. aeruginosa* (unpublished results). The data, however, do not allow us to distinguish explanations ii, iii, and iv.

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ADDENDUM

During the reviewing process for this paper, a Nor$^-$ mutant of another species was isolated by gene replacement (3). This mutant blocks the denitrification pathway at nitric oxide, indicating that N_2O cannot be formed from nitrite directly via the nitrite reductase and thus providing more convincing evidence to reject explanation i. The Nor$^-$ mutant isolated is conditionally lethal, which explains why no Nor$^-$ mutants were found in this study.

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