Purification and characterization of the membrane-bound nitrate reductase isoenzymes of *Bradyrhizobium japonicum*

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Abstract Two respiratory membrane-bound nitrate reductase (NR) isoenzymes, NR_I and NR_{II}, have been purified for the first time from one single microorganism. Triton X-100-solubilized NRs were purified by a three-step procedure of differential centrifugation, Q-Sepharose chromatography, and gel filtration on Sephacryl S-300. Both isoenzymes were purified to homogeneity by the criteria of NR activity staining in polyacrylamide gels run under non-denaturating conditions and coincident staining of the protein band by silver nitrate. NR_I is composed of three subunits of 116 kDa, 68 kDa, and 56 kDa, whereas NR_{II} is composed of four subunits of 116 kDa, 68 kDa, 59 kDa, and 56 kDa. The 116-kDa subunit of NR_I and the 59-kDa subunit of NR_{II} exhibited immunological cross-reactivity with the respiratory NR of *Pseudomonas stutzeri* strain ZoBell.

Key words: Membrane isoenzyme; Nitrate reductase; Nitrate respiration; Purification; *Bradyrhizobium japonicum*

1. Introduction

Nitrate reductases (NRs) are molybdenum-containing enzymes that constitute a heterogeneous group according to their molecular mass and subunit composition, localization, stability, active site, structure, and specific activity. Monomeric enzymes have been purified from different origins such as *Plectonema boryanum* (molecular mass: 85 kDa) [1], *Azotobacter vinelandi* (molecular mass: 105 kDa) [2], or *Desulfovibrio desulfuricans* (molecular mass: 70 kDa) [3].

Periplasmic NRs [4–7] are composed of two subunits: one approximately 90 kDa that is the catalytic subunit and contains molybdenum, and one of 13 kDa which is a *c*-type cytochrome. Periplasmic NRs are constitutively expressed under both oxic and anoxic conditions [8]. These enzymes are structurally and functionally different from the membrane-bound respiratory enzymes and from the cytoplasmic assimilatory enzymes [7].

The best-studied NRs are the respiratory membrane-bound enzymes, characterized from the enteric bacterium *Escherichia coli* [9] and from *Paracoccus denitrificans* [10,11]. They are usually large membrane-bound molybdo/iron/sulfur proteins composed of three subunits, designated α, β, and γ, that in the native form can constitute a multimeric enzyme type $\alpha_2\beta_2\gamma_4$ [9]. The α subunit contains the catalytic site, while the β subunit has a structural function in membrane attachment. The third (γ), heme-*b*-containing subunit, transfers electrons to the iron–molybdenum centers of the enzyme, and thereby to nitrate [9,12]. Other respiratory membrane-bound NRs have been described which do not contain a cytochrome subunit in *Rhodobacter sphaeroides* forma sp. *denitrificans*, *Pseudomonas stutzeri*, and *Haloferax denitrificans* [13–15] which are composed of two subunits: a small one of 60 kDa and a larger one of approximately 116 kDa. Another aspect in the study of NR enzymes is the fact that some bacteria possess more than one enzyme [16,17] but, in these cases, the enzymes have a different cellular location and a different physiological role [17].

Previously, it has been shown that cells of *B. japonicum* strain PJ17 grown microaerobically with nitrate contain two NR enzymes bound to the membrane, NR_I and NR_{II} [18]. Both enzymes are required for nitrate-dependent, microaerobic growth of *B. japonicum* as two Tn*5*-induced mutants of PJ17, strains GRF110 and GRF3, which lack either NR_I or NR_{II}, in their membranes, are unable to grow microaerobically with nitrate [18]. In the present study, we have purified, for the first time from a single microorganism, the two membrane-bound NRs of *B. japonicum* PJ17 strains GRF110 and GRF3 that are expressed in anaerobic conditions and we have characterized both enzymes.

2. Materials and methods

2.1. Growth of cells and preparation of membranes

B. japonicum strains GRF3 and GRF110 were grown in 20 l of aerobic batch cultures at 28°C in LMB medium [18] with 5 mM sodium glutamate as the nitrogen source. Cells were harvested at early stationary phase by tangential flow microfiltration followed by centrifugation at $8000\times g$ for 10 min at 4°C. For induction of membrane-bound NR activity, cells were incubated microaerobically (O_2/Ar, 2 : 98, v/v) at 28°C for 36 h in the incubation mixture described previously [18]. After incubation, the cells were collected by centrifugation as above, washed with 50 mM Tris-HCl (pH 7.5) until nitrite could not be detected in the supernatant, and then resuspended in the same buffer supplemented with 1 mmol/l phenylmethylsulfonyl fluoride (PMSF) to inhibit proteolysis. Cell suspensions were passed twice through a French press at about 120 MPa. Unbroken cells were removed by centrifugation at $10\,000\times g$ for 10 min at 4°C. Membranes were prepared by further centrifugation of the supernatant at $250\,000\times g$ for 1 h at 4°C. The membrane pellet was washed once with 50 mM Tris-HCl (pH 7.5), and dispersed by suspension to a final protein concentration of about 10 mg/ml in an identical buffer containing 4% (w/v) Triton X-100 and 1 mmol/l PMSF. After incubation on ice for 15 min, the solubilized membranes were obtained by removing Triton X-100-insoluble material by centrifugation at $270\,000\times g$ for 30 min at 4°C.

2.2. Purification of nitrate reductases

Aliquots (5 ml) of Triton X-100-solubilized membranes from GRF110 and GRF3 were supplemented with 25% (w/v) glycerol

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Abbreviations: NR, nitrate reductase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; U, activity unit(s)

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and, separately, applied to an anion-exchange column (Q-Sepharose Fast Flow, 2.6×10 cm; Pharmacia-LKB) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing NaCl 50 mM and 1% (w/v) Triton X-100. The column was washed with two column volumes of the equilibration buffer and developed with a gradient of 50–650 mM NaCl in five column volumes (300 ml) of equilibration buffer. Fractions that contained NR activity were pooled and concentrated to about 3 ml in an Amicon Diaflo Ultrafiltration Cell equipped with a YM-100 membrane. The concentrated pool was further applied to a Sephacryl S-300 column (2.6×100 cm; Pharmacia-LKB) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl and 1% Triton X-100, and eluted with 500 ml of the same buffer. The molecular weight of the NR enzymes was determined using a calibration kit from 29 to 700 kDa (Sigma). NR-containing fractions were concentrated and stored at −25°C. All chromatographic steps were carried out at 4°C.

2.3. Polyacrylamide gels

After gel filtration, purity of the enzymes was determined in samples subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE). Gradient gels of 4–10% acrylamide supplemented with 0.1% Triton X-100 were used. NR activity in gels was detected as a clear band of oxidized methyl viologen as described previously [18]. A high-molecular-weight calibration kit from Pharmacia-LKB was used for standard proteins. The subunit composition of each enzyme was determined by sodium dodecyl sulfate (SDS)–PAGE [19] using gradient gels of 7–15% and standard low-molecular-weight proteins obtained from Pharmacia-LKB. Native and SDS-PAGE gels were stained for protein with the silver staining method of Blum et al. [20]. Densitometry of stained gels was carried out by measuring absorbance at 560 nm.

2.4. Immunochemical methods

For Western blot analysis, proteins were transferred electrophoretically from acrylamide gels onto nitrocellulose membranes [21]. NR was detected by immunoblotting with a rabbit antiserum raised against purified respiratory NR from *Pseudomonas stutzeri* strain Zo-Bell [14]. Goat anti-rabbit IgG peroxidase conjugate was used as the secondary antibody and pre-stained proteins from Sigma were used as standard.

2.5. Analytical methods

Iron and molybdenum were determined by inductively coupled plasma emission spectrometry. Separate calibration curves were run for iron and molybdenum in Triton X-100. Acid-labile sulfide was assayed according to Brumby et al. [22]. The complementation assay of *Neurospora crassa nit-1* mutant was made according to [23]. Protein was determined by the method of Wang and Smith [24] using bovine serum albumin as a reference protein. Optical absorption spectra were recorded at room temperature with a Shimadzu UV-260 spectrophotometer. For chemical reduction, a few crystals of sodium dithionite were added. The isoelectric point of the NR enzymes was determined as described previously [25]. NR activity was determined at 30°C by measuring the reduction of nitrate to nitrite with dithionite-methyl viologen as the electron donor [18]. The reaction was started by addition of the dithionite and terminated after 5 min by vigorous shaking until samples had lost their blue color. Nitrite was determined by a diazotization procedure [26]. Activity units (U) correspond to nmol NO_2^- produced per min.

3. Results

3.1. Purification of nitrate reductases

The respiratory NRs, NR_I and NR_{II}, were purified from membrane extracts of *B. japonicum* mutant strains GRF110 and GRF3, respectively, after solubilization with Triton X-100, followed by differential centrifugation, anion-exchange chromatography through Q-Sepharose, and gel filtration on Sephacryl S-300. A summary of the results is presented in Table 1 and Fig. 1. Ion-exchange chromatography was the most effective purification step. NR_I eluted from the Q-Sepharose column at 270–290 mM NaCl, whereas NR_{II} eluted at 310–320 mM NaCl. The average specific activity of NR_I after gel filtration was 4638 U/mg protein, representing a 21.6-fold purification and a yield of about 27.6%. NR_{II} was purified 15.9-fold with a yield of 26.7%, and had a specific activity of 4316 U/mg protein (Table 1).

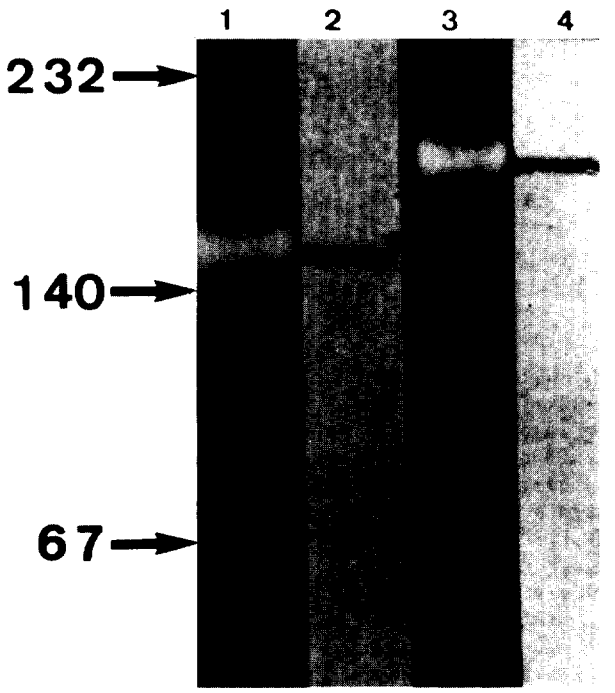

Fig. 1. Native PAGE of purified NR_I and NR_{II} from *B. japonicum* GRF110 and GRF3, respectively, run on a 4–10% polyacrylamide gel. Lanes 1 and 3, NR_I and NR_{II}, respectively, stained for NR activity. Lanes 2 and 4, NR_I and NR_{II}, respectively, stained for protein. Numbers indicate the molecular mass of standard proteins in kDa.

3.2. Subunit composition and immunological study

Both enzymes were purified to homogeneity according to native PAGE, which revealed a single band when the gel was stained with silver nitrate, that corresponded to a single band of NR activity (Fig. 1). The molecular mass of the homogeneously purified protein and NR activity band was 160 kDa for NR_I and 200 kDa for NR_{II} in a non-denaturating gel. These molecular masses are essentially the same as those previously determined in membrane extracts of PJ17 after solubilization with Triton X-100 [18].

Analysis of NR_I by SDS-PAGE revealed the presence of three protein-staining bands of molecular masses 116, 68, and 56 kDa (Fig. 2). Distribution of the stained protein by densitometer scans showed that 42% is associated with the larger band, while the relative concentration of the medium and the smaller subunits were 25% and 33%, respectively (data not shown). When purified preparations of NR_{II} were analyzed on SDS-PAGE, four protein-staining bands of molecular masses 116, 68, 59, and 56 kDa were present (Fig. 2). The relative concentration of each band was 32%, 21%, 19%, and 28% (data not shown). Assuming that all bands stained equally with silver stain, these data are consistent with a probable 1 : 1 : 1 ratio of the NR_I subunits and a 1 : 1 : 1 : 1 ratio for the subunits of NR_{II}. The presumed molecular masses of 240 kDa for NR_I and of 299 kDa for NR_{II} are not in agreement with those observed after gel electrophoresis, perhaps

because of the non-denaturating conditions used in that assay, where the proteins migrated in function of their charge, size, and form. However, the theoretical molecular masses correlated very well with the observed 236-kDa and 285-kDa after gel filtration through Sephacryl S-300, which is a more precise method than gel electrophoresis.

The purified enzymes were tested in an immunoblot assay against antibodies raised against the respiratory NR of *P. stutzeri* strain ZoBell [14]. The 116-kDa subunit of NR_I and the 59-kDa subunit of NR_{II} cross-reacted with the polyclonal antiserum (Fig. 2). The fact that the 116-kDa subunit of NR_{II} gave no hybridization signal allow us to conclude that this subunit is different in both NR enzymes of *B. japonicum* PJ17.

3.3. Enzymes properties

The purified NR_I and NR_{II} were brownish in color and had a broad absorption spectrum in the visible range as shown in Fig. 3. In addition to the absorbance maximum due to protein at 278 nm, there was a shoulder at 325 nm and a broad band centered around 412 nm, a typical feature of proteins containing iron–sulfur groups. Reduction of the enzymes by dithionite decreased the absorbance in the visible range. A small shoulder persisted at 420 nm. No sign of the presence of a cytochrome moiety in the area of 500–600 nm was observed in the spectra of the purified enzymes either as isolated or in the dithionite-reduced state. Because *b*-type cytochromes are easily lost during enzyme purification and also because they may escape detection by SDS electrophoresis after extraction with detergents [10,27], electronic spectra were recorded from samples obtained after solubilization of the membranes with Triton X-100 and after passage through Q-Sepharose and Sephacryl S-300 columns. None of the NR preparations gave indications of the presence of cytochromes (data not shown).

Four separate determinations on two preparations of each NR_I and NR_{II} were made for iron, molybdenum and acid-labile sulfide (Table 2). Both the molybdenum and iron analyses took into account standards tested with quantities of Triton X-100 equivalent to those present in the samples. These data indicated that the molybdenum/iron/acid-labile sulfide ratio was 1 : 8 : 8 per molecular mass 240 kDa of NR_I, and 1 : 12 : 12 per molecular mass 299 kDa of NR_{II}, suggesting that NR_I had one molybdenum atom and two iron–sulfur clusters, and that for each molybdenum there was at least three iron–sulfur clusters in NR_{II} (Table 2). The proposed molybdenum/iron/sulfur ratios for NR_I and NR_{II} have been reported for the metal contents of NRs from *Bacillus licheniformis* [28], *B. halodenitrificans* [29], *P. stutzeri* [14], and *E. coli* [27].

The purified enzymes were tested for the activity in the apo-(*nit-1*) NR assay [23]. In our conditions, the activities were

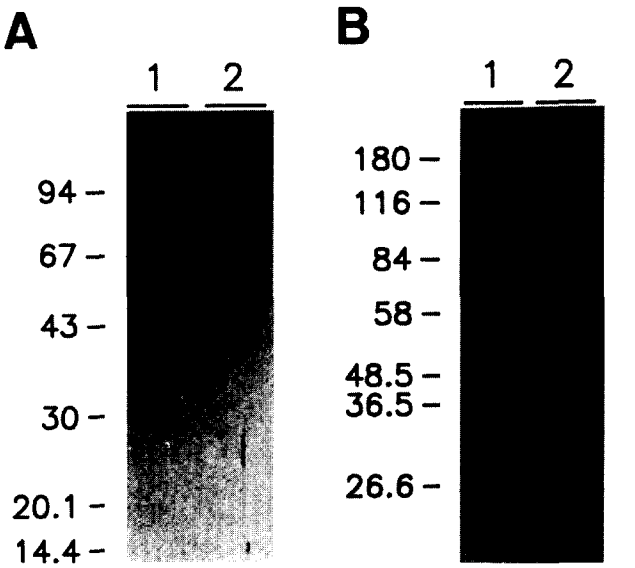

Fig. 2. Subunit composition and inmunoblot analysis. (A) 7–15% SDS-PAGE of the purified enzymes stained with silver nitrate. (B) Western blot assay. Both panels: lane 1, NR_I; lane 2, NR_{II}. Numbers in the left indicate the molecular mass of standard proteins in kDa.

8 U/mg protein for NR_{II} and 5.7 U/mg protein for NR_I. As control we used solubilized membranes of the wild-type strain that showed an activity of 23.3 U/mg protein. These data showed that both dissimilatory NRs of *B. japonicum* contain a molybdenum cofactor capable of activating apo-(*nit-1*) NR and agree well with the data of other authors who proved that the dissimilatory NR from *E. coli*, *Pseudomonas carboxydoflava*, and *Rhodobacter sphaeroides* have a modified molybdenum cofactor which contain a molybdopterin guanosine dinucleotide that is active in the *nit-1* cofactor assay [30].

NR_I and NR_{II} had K_m values for nitrate of 300 μM and 430 μM, respectively, with reduced methyl viologen as the electron donor. The enzymes were also active with benzyl viologen. Neither of them used NADPH or NADH for nitrate reduction in the presence or the absence of flavin adenine dinucleotide. Azide was a strong inhibitor of both enzymes; K_i values were 0.9 mM for NR_I and 0.7 mM for NR_{II}. Cyanide also inhibited NR_I and NR_{II}, although at higher concentration than azide. K_i value was 0.3 mM for NR_I and 0.2 for NR_{II}. The isoelectric point of NR_I was 6.2 and that of NR_{II} was 6.8.

4. Discussion

Our previous results have shown that *B. japonicum* PJ17 grown microaerobically with nitrate expresses two NRs,

Table 1
Purification of membrane-bound NR_I and NR_{II} from *B. japonicum* mutant strains GRF110 and GRF3, respectively

Fraction	Total protein		Total activity		Specific activity		Yield	
	NR_I (mg)	NR_{II} (mg)	NR_I (U)	NR_{II} (U)	NR_I (U/mg)	NR_{II} (U/mg)	NR_I (%)	NR_{II} (%)
Membranes	132.6	136.7	28509	37182	215	272	100	100
Triton-100 solubilized membranes	31.3	28.6	24790	30831	792	1078	86.9	82.9
Q-sepharose	5.3	4.0	11411	13412	2153	3353	40.0	36.1
Sephacryl S-300	1.7	2.3	7885	9927	4638	4316	27.6	26.7

NR_I and NR_{II}, in the cytoplasmic membrane [18]. Despite our attempts to purify the enzymes from membranes of *B. japonicum* PJ17, no discrete separation of the two enzymes was achieved either through gel filtration or ion-exchange chromatography. To avoid this problem we have used two Tn*5* mutant strains that lack either NR_I or NR_{II} as sources to purify and characterize both enzymes. Although different NR enzymes have been purified from different microorganisms, this is the first time that two dissimilatory NR with the same cellular location have been purified from a single bacterium.

NR_I and NR_{II} contain three subunits of 116, 68, and 56 kDa, and in addition NR_{II} had a fourth subunit of 59 kDa. We could assume that the 116-kDa subunit contains the catalytic site such as the described enzymes of *P. stutzeri*, *R. sphaeroides* and *H. denitrificans* [13–15]. If this is so, the fact that the 116-kDa subunits of NR_I and NR_{II} are antigenically dissimilar (Fig. 2) suggests differences in their active sites. Furthermore, NR_I and NR_{II} differed in affinity for nitrate, pI, sensitivity to azide and cyanide, and the lower content of iron and molybdenum of NR_I (Table 2).

The *b*-type cytochromes of other NRs are easily lost during the purification. In our case, we did not detect any cytochrome by SDS-PAGE, where subunits around 20 kDa or smaller were not detected in a 7–15% gradient gel. In the absorption spectra, there were no peaks in the area of 500–600 nm, typical for cytochrome b_{556} of the NR of *E. coli*. The NRs we have described here are clearly different from those in which cytochrome is an intrinsic component of the complex. Cytochrome is, however, essential for microaerobic growth of *B. japonicum*, because mutants with lesions in the gene *cycA*, that encodes for the soluble cytochrome c_{550}, are unable to grow microaerobically with nitrate as the terminal electron acceptor [31]. NR_I and NR_{II} resemble the enzymes of *P. stutzeri*, *R.sphaeroides*, and *H. denitrificans* in the absence of a cytochrome subunit as well as in the presence of a large subunit of approximately 116 kDa.

Physiological studies carried out with mutant strains GRF3 and GRF110 revealed that NR_{II} showed features typical of the membrane-bound respiratory NRs; the enzyme was induced by microaerobiosis and nitrate, was inhibited by oxygen, and was insensitive to repression by ammonia. In contrast, NR_I was induced by microaerobiosis alone and was insensitive to repression by ammonia and to induction by nitrate [18]. Therefore, NR_{II} could be the biochemical equivalent of NRs from nitrate-respiring and nitrate-denitrifing microorganisms. NR_I might be a second type of NR with a yet to be defined biochemical role. The fact that strains GRF110 and GRF3 are unable to grow microaerobically with nitrate [18] indicates that the two enzymes cannot substitute for the physiological role of each other. Our previous results [18] and the results presented in this study allow us to confirm that *B. japonicum* PJ17 has two NR isoenzymes that are differentially regulated.

Table 2
Metal analysis of purified NR_I and NR_{II}

Metal	Mol of metal/mol of enzyme			
	NR_I		NR_{II}	
	Range	Average	Range	Average
Mo	0.61–0.81	0.73	0.76– 1.02	0.89
Fe	6.61–9.43	8.02	11.74–13.28	12.51
S^{2-}	6.09–8.23	7.16	11.22–12.62	11.92

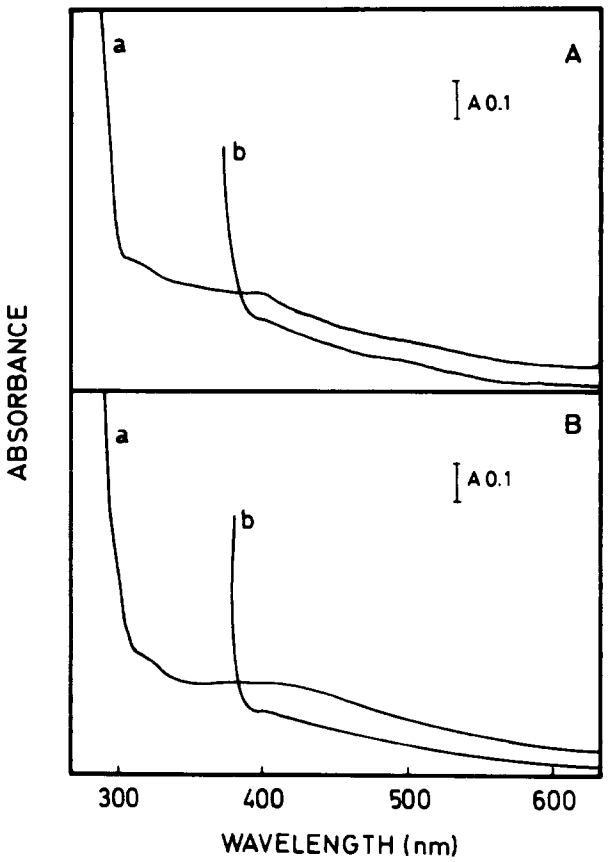

Fig. 3. Spectra of purified NR_I (A) and NR_{II} (B) (0.576 mg protein/ml) in 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, (a) and the same sample reduced with dithionite (b).

Nitrate assimilation and respiration have been established as distinct processes in bacteria such as *K. pneumoniae* [32,33] and *P. aeruginosa* [34,35]. It has not been established, however, whether the same or separate enzymes catalyze assimilatory and respiratory nitrate reduction in *B. japonicum*, as deduced from the fact that strain GRF110 is unable to grow with nitrate under aerobic conditions [18]. To determine the exact physiological role of both isoenzymes in the nitrate respiration process and their possible involvement in the aerobic or anaerobic nitrate assimilation further studies are needed. The Tn*5* mutant strains, GRF3 and GRF110, are being used to isolate the Tn*5*-tagged genes. The resulting genetic evidence combined with the biochemical characterization of the NR isoenzymes, we present here, will allow us to demonstrate the relative contributions of each enzyme to N assimilation and aerobic growth.

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