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IMMUNOLOGICAL PATTERNS OF DISTRIBUTION OF BACTERIAL DENITRIFYING ENZYMES*

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Key Word Index—*Rhodobacter sphaeroides* f.sp. *denitrificans*; immunological techniques; denitrifying enzymes.

Abstract—Highly specific polyclonal antibodies against respectively nitrate, nitrite and nitrous oxide reductases from a photosynthetic denitrifying bacterium *Rhodobacter sphaeroides* f.sp. *denitrificans* were raised in rabbits. IgG fractions purified from antisera were routinely used in ELISA and Western immuno-blotting procedures to determine the distribution of these denitrifying enzymes in various bacteria. Molybdenum-containing nitrate reductase and multi-copper nitrous oxide reductase appear to be common proteins among the denitrifying species tested. The pattern of immunological crossreactivity for nitrite reductase confirmed that there are two immunologically different forms of the enzyme, namely cytochrome cd_1 and the copper-containing nitrite reductase. Although two forms of copper-containing nitrite reductases have been described, nevertheless they are immunologically identical.

INTRODUCTION

Denitrification involves at least a three-step reduction of nitrate via nitrite and nitrous oxide to dinitrogen. Three key denitrifying enzymes, namely nitrate (NO_3^-R), nitrite (NO_2^-R) and nitrous oxide (N_2OR) reductases have been characterized from a range of gram-negative bacteria (for review see [1] and refs within) and have recently been purified from a phototrophic denitrifying bacterium *Rhodobacter sphaeroides* f.sp. *denitrificans* [2–4].

Membrane bound dissimilatory nitrate reductases purified from a variety of bacteria [5] are composed of three subunits designated α, β and γ. The α-subunit (M_r 120000–150000) has a catalytic function, while the β-subunit (M_r 60000) has a structural role in membrane attachment. The γ-subunit (M_r 20000), which is not found in heat-released preparations, is a *b*-type cytochrome. Heat-released NO_3^-R from *R. sphaeroides* f.sp. *denitrificans* is composed of a major α-subunit (M_r 120000) and β-subunit of 60000 [2].

Three types of the nitrite reductases are known [6]: (i) cytochrome cd_1 enzyme in *Pseudomonas* and *Paracoccus denitrificans*; (ii) copper (type 1)-containing enzyme in *Alcaligenes* and (iii) copper (type 1 and 2) protein from '*Achromobacter cycloclastes*'. NO_2^-R from *R. sphaeroides* f. sp. *denitrificans* is composed of two non-identical subunits (M_r 37500 and 39500): one subunit contains Cu^{2+} type 1, the other Cu^{2+} type 2 [3].

Nitrous oxide reductase is considered to be an exclusive enzyme of denitrifying bacteria, N_2OR from *R. sphaeroides* f.sp. *denitrificans* is a soluble monomeric enzyme (M_r 73000 in denatured form) containing four copper atoms per mol with an electron paramagnetic resonance (EPR) spectrum of a type 2 Cu^{2+} site(s) [4]. Similar proteins were characterized from *Rhodobacter capsulatus* [7], *Achromobacter xylosoxidans* [8], *Pseudomonas perfectomarina* [9] and *Paracoccus denitrificans* [10].

The nucleotide sequences of 5S rRNA from seven denitrifying bacteria have recently been determined and the grouping of these bacteria, based on these sequences, appears to be related to the type of the nitrite reductase present [6].

In this study polyclonal antibodies raised against denitrifying enzymes from *R. sphaeroides* f.sp. *denitrificans* were used in immunological assays to determine the distribution of these enzymes in various bacteria. Immunological patterns of distribution of denitrifying enzymes are an extension to the genetic characteristics of denitrifying bacteria and are of particular value in evaluating the relatedness among them.

RESULTS AND DISCUSSION

Three denitrifying enzymes, namely NO_3^-R, NO_2^-R and N_2OR purified from *R. sphaeroides* f.sp. *denitrificans* were used as antigens for production of antibodies in rabbits. Immunoglobulin (IgG) fractions (anti-NO_3^-R, anti-NO_2^-R and anti-N_2OR) were purified from antisera and tested for specificity by a cross-immunoelectrophoresis technique. They appeared to be monospecific since the immunoelectrophoresis resulted in single precipitin peaks with their antigens, i.e. NO_3^-R, NO_2^-R and N_2OR respectively (Fig. 1). The specificity of the IgG fractions was also tested by immuno-blotting technique. Samples of cell extracts of *R. sphaeroides* f.sp. *denitrificans* were separated in polyacrylamide gels by two-dimensional electrophoresis (Fig. 2A). Separated proteins were then

* This paper is dedicated to the memory of Dr Tony Swain, founder editor of *Phytochemistry*.

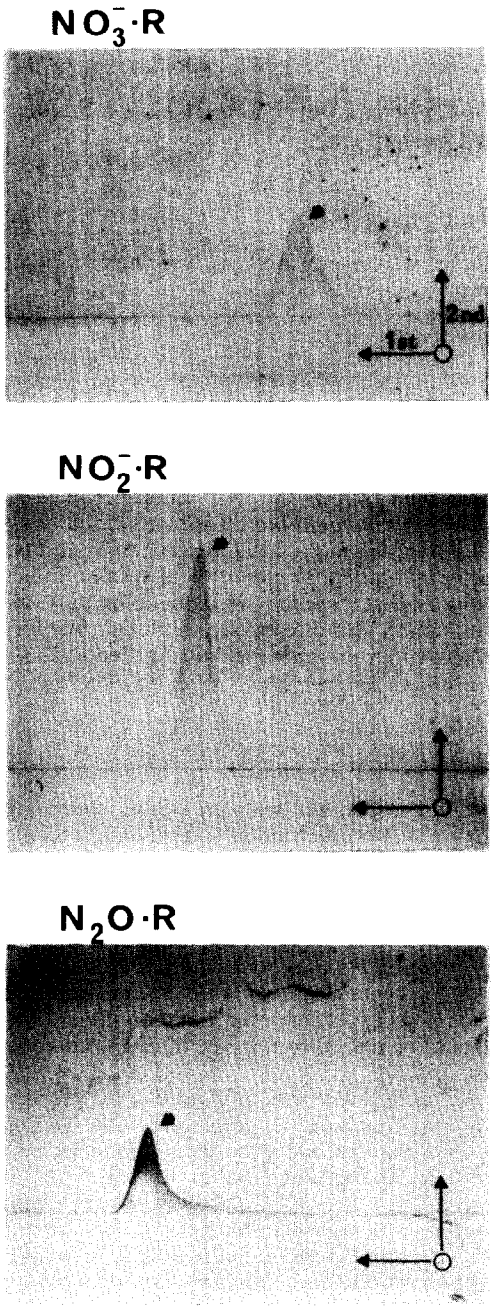

Fig. 1. Cross-immunoelectrophoresis of purified nitrate, nitrite and nitrous oxide reductases from *R. sphaeroides* f.sp. *denitrificans*. The enzymes (5 μg each) were subjected to electrophoresis in agarose gels (1st dimension, 10 V/cm for 90 min, at 15°, anode to the left). They were then electrophoresed into another agarose gel containing IgGs raised against each purified enzyme (2nd dimension, 15 μl IgG per cm^2, at 2 V/cm for 20 hr, at 15°, anode at top). After drying, gels were stained with Coomassie Brilliant Blue.

transferred onto nitrocellulose membranes and immunostained by an enzyme-conjugated system. Each of the antibodies detected only polypeptides with electrophoretic characteristics identical with those of subunits or apoproteins of denitrifying enzymes originally used as the antigens (Fig. 2B–D). None of the IgG fractions cross-reacted with a protein other than its antigen. Thus, antibodies raised against denitrifying enzymes proved to be monospecific and suitable for studying the distribution of these enzymes among denitrifying bacteria.

Seven bacterial species were chosen for comparison with *R. sphaeroides* f.sp. *denitrificans* on the basis of their known denitrifying activity (Table 1). *Escherichia coli*, although incapable of denitrification, was also selected because of its capacity to reduce nitrate to nitrite via nitrate reductase [5].

The anaerobic culture conditions in the presence of nitrate led to expression of the denitrifying pathway as shown by *in vitro* activities of denitrifying enzymes. The activities of NO_3^-R, NO_2^-R and N_2OR, measured with reduced benzyl viologen dye as an electron donor, were detected in all bacterial strains tested, with the exception of *E. coli* which expressed only activity of nitrate reductase. The activities of denitrifying enzymes varied between strains but were well correlated with the amounts of these enzymes synthesized in cells, as quantified by the ELISA technique (Table 1). Although N_2OR was immunodetected in all strains (with the exception of *E. coli*), *in vitro* activity of the enzyme was always low, reflecting its known instability [4, 9, 10].

Electrophoretic separation of membrane proteins followed by the immunoblotting revealed that all bacterial strains analysed in this study contained proteins cross-reacting with anti-NO_3^-R/IgG. Molecular mass of immunoreactive proteins varied slightly between strains but in all bacteria two subunits, similar to α- and β-subunits of *R. sphaeroides* NO_3^-R, were detected (Fig. 3A). These results indicate that these bacteria have the same molybdenum enzyme. This is in accordance with a view that the structure of membrane-bound dissimilatory nitrate reductases is similar, if not identical, in most denitrifying bacteria [1, 2, 5].

The activities related to the enzymic reduction of nitrite were detected in all bacterial strains analysed (Table 1), with the exception of *E. coli* (this bacterium has assimilatory nitrite reductase since its synthesis was repressed when ammonium was present in the culture medium). Soluble protein fractions obtained from these bacteria were tested for the presence of nitrite reducing enzymes. Proteins crossreacting with anti-NO_2^-R/IgG were detected in *Alcaligenes denitrificans*, '*Achromobacter cycloclastes*', *Ps. denitrificans* and *Ps. alcaligenes* (Fig. 3B lines 1–5). Although two classes of copper-NO_2^-R enzymes, varying in their EPR spectra, have been described [6] results presented in this paper indicate that they all are immunologically identical (Fig. 3B).

As expected, none of the other strains included in this study (*P. denitrificans*, *Ps. perfectomarina* and *Ps. aeruginosa*) showed a positive reaction with anti-NO_2^-R IgG. It has been shown in several independent studies that these bacteria contain different type of respiratory nitrite reductase namely cytochrome cd_1 [6].

Until recently cytochrome cd_1 was considered to be an exclusive type of NO_2^-R found in *Pseudomonas*. However, Zumft *et al.* [11] have purified a copper-containing enzyme from *Ps. aerofaciens*. In this paper we report that copper-nitrite reductase was also detected in *Ps. alcaligenes*.

The tight taxonomic clustering of species of *Pseudomonas* has been demonstrated by several independent techniques, most recently by DNA–rRNA hybridization

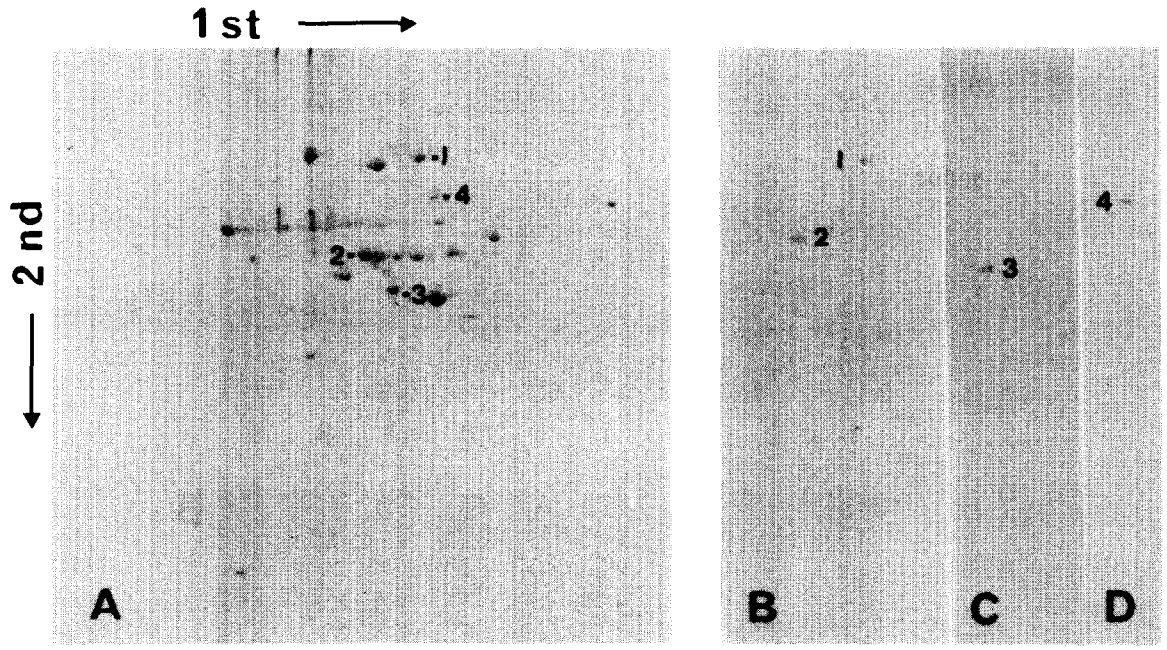

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis (A) and Western immuno-blot (B, C, D) of crude extract of *R. sphaeroides* f.sp. *denitrificans*. Denitrifying enzymes were detected with specific antibodies (see Fig. 1). 1 and 2, α- and β- subunits of nitrate reductase (M_r 120000 and 60000 respectively); 3, subunit of nitrite reductase (M_r 40000); 4, nitrous oxide reductase (M_r 75000). *Ca* 40 μg protein samples were separated in the gels. 1st dimension—isoelectrofocusing and 2nd dimension—SDS polyacrylamide gel electrophoresis.

Table 1. Immunological crossreactivity and catalytic activities of nitrate, nitrite and nitrous oxide reductases in denitrifying bacteria

		NO_3^-R		NO_2^-R		N_2OR	
Bacterial strain	ATCC number	Spec. activity‖ (nkat/mg)	ELISA†	Spec. activity‖ (nkat/mg)	ELISA†	Spec. activity‖ (nkat/mg)	ELISA†
1. *Rhodobacter sphaeroides* f.sp. *denitrificans*	(T. Satoh)	122	A	160	A	18	A
2. *Alcaligenes denitrificans* ss. *xylosoxidans*‡	27061	68	B	67	C	5	C
3. *Achromobacter cycloclastes*‡	15466	72	B	109	B	4	C
4. *Pseudomonas denitrificans*‡	13876	30	C	95	B	6	C
5. *Pseudomonas alcaligenes*	14909	42	C	7	C	4	C
6. *Esherichia coli B*§	23226	110	A	nd	nd	nd	nd
7. *Paracoccus denitrificans*	13543	145	A	16*	nd	20	A
8. *Pseudomonas perfectomarina*¶	14405	73	B	22*	nd	11	B
9. *Pseudomonas aeruginosa*	10145	57	B	10*	nd	5	C

*Although copper-NO_2^-R was not present in these strains the activity of cytochrome cd_1 nitrite reductase enzyme was detected with reduced benzyl viologen as an electron donor.

†Amount of immunoreactive protein as detected by ELISA technique: A, >8 μg/mg protein; B, 2-4 μg/mg; C, <2 μg/mg; nd, not detectable.

‡The genera *Achromobacter/Alcaligenes* are ill-defined and genus *Achromobacter* was not mentioned in the Approved Lists of Bacterial Names [21]. *Alcaligenes denitrificans* ss. *xylosoxydans* was formerly classified as *Achromobacter xylosoxydans* [18]. *Achromobacter cycloclastes* seems to be different from the genera *Achromobacter/Alcaligenes* and should be transferred in future to another genus [6]. *Pseudomonas denitrificans* has been classified as *Alcaligenes sp.* [22] and then re-classified as *Achromobacter xylosoxydans* [1].

§*E. coli* is not a denitrifying bacterium.

¶On the basis of DNA/DNA homology *Pseudomonas perfectomarina* it is proposed that it be transferred to the species *Pseudomonas stutzeri* [23].

‖Enzyme activity is expressed in units of Katals (kat), the conversion of one mol of substrate per sec, 1 nkat = 10^{-9} kat.

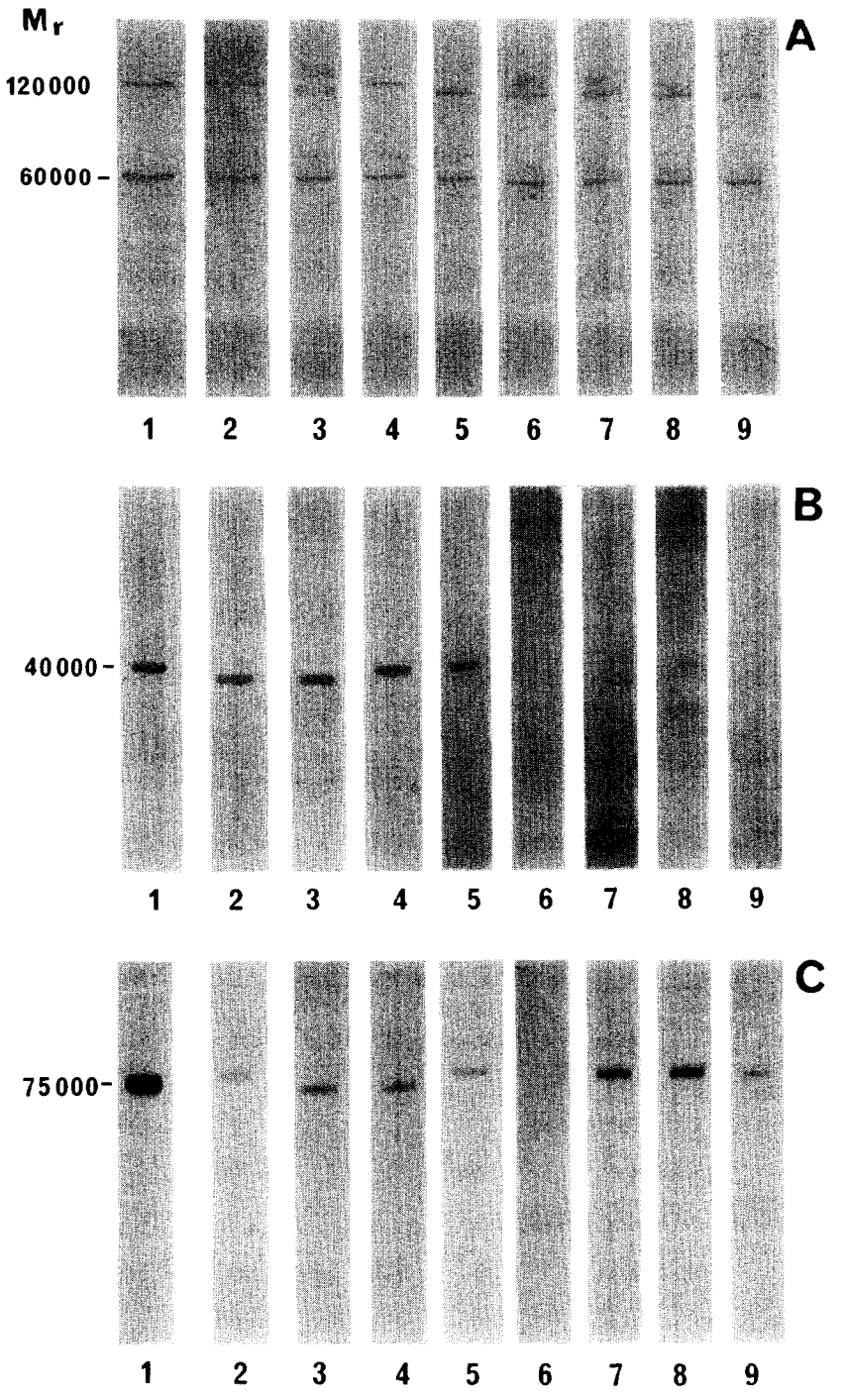

Fig. 3. Western immuno-blot for (A) nitrate reductase, (B) nitrite reductase and (C) nitrous oxide reductase. 1-*R. sphaeroides* f.sp. *denitrificans*, 2-*Alcaligenes denitrificans*, 3-*Achromobacter cycloclastes*, 4-*Pseudomonas denitrificans*, 5-*Pseudomonas alcaligenes*, 6.-*Esherichia coli*, 7-*Paracoccus denitrificans*, 8-*Pseudomonas perfectomarina*, 9-*Pseudomonas aeruginosa*. Samples of membrane fractions (A, 10 μg protein each) as well as soluble fractions B and C, 5 μg protein each) from bacterial strains listed above were prepared as described in Experimental.

[12]. Since all denitrifying *Pseudomonas* examined in this study belong to Pelleroni's rRNA group I [13], it is of interest that not all of them have the same type of NO_2^-R, indicating variability of this enzyme even among closely related species.

Soluble protein fractions from all denitrifying bacteria tested (with the exception of *E. coli*) contained a protein which crossreacted with anti-N_2OR/IgG (Fig. 3C). Molecular masses of the immunoreactive proteins varied between species but were very similar with that of N_2OR

from *R. sphaeroides* f.sp. *denitrificans* [4]. Korner *et al.* [14] have reported that antibodies against *Ps. perfectomarina* N_2O R did not react with the enzymes from *P. denitrificans* and *R. sphaeroides* f. sp. *denitrificans*. In our hands however the antibodies against *R. sphaeroides* f. sp *denitrificans* N_2O R immunoreacted with the enzyme from *Ps. perfectomarina* (Fig. 3C, line 8). Although there are many similarities between denitrifying enzymes from these bacteria, it has been pointed out that immunological responses to N_2O reducing enzymes seems to be related to whether or not their purification was carried out under anaerobic conditions [10].

In this study we have compared by immunological techniques representative species of the denitrifying bacteria for the presence of denitrifying enzymes, crossreacting with the specific antisera to these proteins purified from *R. sphaeroides* f. sp. *denitrificans*. Nitrate and nitrous oxide reductases seem to be common proteins among the denitrifying species tested. The pattern of immunological crossreactivity for NO_2^-R confirmed that there are two immunologically different forms of enzyme, namely the cytochrome cd_1 and the copper-containing nitrite reductase. Although the EPR spectra of copper are different in the copper-containing nitrate reductases [6] nevertheless they appeared to be immunologically identical.

EXPERIMENTAL

Bacterial strains. Strains used in this studies were obtained from the American Type Culture Collection (ATCC) and are listed in Table 1. *Rhodobacter sphaeroides* f. sp. *denitrificans* strain IL106 was kindly supplied by Dr T. Satoh (Department of Biology, Tokyo Metropolitan University, Tokyo 158, Japan).

Media and growth conditions. Rhodobacter sphaeroides f. sp. *denitrificans* was grown photoheterotrophically in a mineral medium containing 10 mM potassium nitrate as described previously [15]. The *Pseudomonas* strains were grown anaerobically in a culture medium described by Korner *et al.* [14]. All other strains were grown anaerobically in Luria Broth medium supplemented with 1 g sodium nitrate per liter. Cultures in completely filled 10 ml culture tubes were inoculated with 10% (v/v) inoculum and incubated at 30° for 24 hr.

Purification of enzymes and production of antibodies. Nitrate reductase (EC 1.7.99.4.), nitrite reductase (EC 1.7.2.1.) and nitrous oxide reductase (no EC entry) were purified to homogeneity according to the procedures described previously [2–4]. These enzymes were used as antigens for the production of antibodies in rabbits. The antigens were administered in 3 to 5 consecutive injections as described in [2]. The IgG fractions were purified from anti-sera by affinity chromatography on Protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals) run according to the recipe supplied by the manufacturer. Specificity of purified IgG fractions was tested by a cross-immunoelectrophoresis technique [16].

Preparation of cell fractions and electrophoresis. Crude extracts as well as soluble and membrane fractions were prepared from washed cells as described previously [15]. Details of one- and two-dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS, 0.1%, w/v) as well as sample preparation procedures are described in [15].

Immunological techniques. (a) *ELISA.* The denitrifying enzymes were quantified in cell fractions by enzyme-linked immunosorbent assay (ELISA) techniques. Indirect non-competitive enzyme immunoassay procedures with antigens or antibodies immobilized on the solid phase were employed [17]. The goat anti-rabbit IgG peroxidase conjugate (Sigma) was used in all immunoassays.

(b) *Western immuno-blotting* used to detect the denitrifying enzymes in cell fractions of various bacteria was performed according to a modified procedure of ref. [18]. Protein samples separated in 1 mm thick polyacrylamide gels (10 and 12.5%, w/v) were electrophoretically transferred to nitrocellulose membranes at 4° in Mini-Protein II transfer apparatus (BioRad) operating at 175 mA for 90 min. Transfer buffer contained 20% (v/v) ethanol, 192 mM glycine and 25 mM Tris–base (pH 7.4). Molecular weight protein markers (labelled with fluoresceine isothiocyanate, FITC, Sigma; [19]) were used to calibrate the gels as well as to evaluate successful protein transfer. The remaining protein binding sites of nitrocellulose membranes were saturated during 3- to 5-hr incubation in 5% (w/v) solution of powdered non-fat milk resuspended in 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl and 1 mM Na_2EDTA (TBS buffer). The nitrocellulose sheets were then incubated overnight at 4° with antibodies (rabbit IgG fractions) diluted 100-fold in TBS buffer. They were washed three times in TBS buffer containing 0.1% (v/v) Triton X-100 and incubated for 2 hr at room temperature with a anti-rabbit goat antibodies [$F(ab')_2$ fragment of IgG] conjugated with alkaline phosphatase (Sigma). The sheets were washed again (at least 4 times) and incubated with an alkaline phosphatase substrate solution containing 100 mM Tris–HCl buffer (pH 8.3), 100 μM naphthol AS-Bl phosphate and 1 mg/ml Fast Red TR salt (both from Sigma).

Enzyme assays. Nitrate reductase, nitrite reductase and nitrous oxide reductase activities were assayed spectrophotometrically in cell fractions as described previously [15]. All assays were carried out under anaerobic conditions with reduced benzyl viologen as an electron donor. Specific activities calculated from the initial rates of reduced benzyl viologen oxidation were related to protein content of bacterial cells. Protein content was determined by the method of Bradford [20], using bovine serum albumin as a standard protein.

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