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Properties of a *Pseudomonas stutzeri* Outer Membrane Channel-Forming Protein (NosA) Required for Production of Copper-Containing N_2O Reductase

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A protein (NosA) in the outer membrane of *Pseudomonas stutzeri* that is required for copper to be inserted into N_2O reductase has been extracted and purified to homogeneity. The purified protein could form channels in black lipid bilayers. Like N_2O reductase, NosA contained copper and was only made anaerobically. In contrast to N_2O reductase, its synthesis was repressed by exogenous copper (but not by Mn, Co, Ni, Zn, or Fe). Also in contrast to N_2O reductase, NosA homologs were not immunologically detectable in *Pseudomonas aeruginosa*, *Pseudomonas mendocina*, *Pseudomonas alcaligenes*, or other strains of *P. stutzeri*.

Denitrification is a biological process that forms gaseous products, either nitrous oxide (N_2O) or dinitrogen (N_2), from terrestrial and aquatic nitrate (NO_3^-) through a series of anaerobic respirations for which nitrate, nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) are terminal electron acceptors (16). The process is unique to procaryotes. The final step is catalyzed by N_2O reductase, a copper-containing enzyme (7). An outer membrane protein (NosA) is required for insertion of copper into N_2O reductase; mutants defective in the encoding gene, *nosA*, produce N_2O reductase that is inactive because it lacks copper (14). In this paper we show that purified NosA can form channels in black lipid bilayers. We describe some of its properties and conditions of its synthesis.

MATERIALS AND METHODS

Bacterial strains and media. The *Pseudomonas* strains used in this study are listed in Table 1. LT medium was modified Luria-Bertani broth (6) supplemented with trace minerals solution (1 ml/liter) lacking added copper (14). LT-NO_3 medium contained 50 mM $NaNO_3$. LT-N_2O medium was N_2O-saturated LT. All concentrations were determined on a weight per volume basis.

Preparation of cell extracts and solubilization of NosA protein for immunological analysis. All incubations were at 37°C. For the preparation of cell extracts of anaerobically grown cells, an overnight culture of JM604 was diluted 1:100 in LT-NO_3 containing the appropriate concentration of $CuSO_4$. Cells were harvested by centrifugation at 4,000 × *g* for 20 min and suspended in 10 mM Tris buffer (pH 7.5 at 4°C) containing 5 mM $MgCl_2$. For the cell extracts of aerobically grown cells, an overnight culture was diluted in LT containing 100 μM $CuSO_4$ to give an A_{650} of 0.05. The culture was incubated with shaking (350 rpm) until the A_{650} reached 0.6; chloramphenicol was added (to 1 mg/ml), and the cells were harvested and suspended. The cell pastes were passed through a French pressure cell at 16,000 lb/in^2. Membrane fractions were collected by centrifugation at 40,000 × *g* for 1 h and suspended in Tris buffer. Triton X-100 was added to give a final concentration of 2%. The suspension was held at 4°C for 3 h, centrifuged, and suspended in Tris buffer. Triton X-100 and EDTA were added to give final concentrations of 2% and 10 mM, respectively. After overnight incubation at 4°C, the solubilized proteins in the supernatant were recovered following centrifugation.

Detection of NosA protein. Because of its lack of in vitro biochemical activity, the presence of NosA was determined as follows. During the early stages of purification, the protein preparations were mixed with a crude cell extract from JM753 (a strain that lacks NosA) and run on a two-dimensional O'Farrell gel (15). The NosA spot was located by comparing the pattern of spots with that of a NosA-producing strain. The amount of NosA was estimated from the size and intensity of the spot. Once purification had proceeded to the extent that NosA became a major protein, it was identified by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10).

Purification of NosA protein. Strain JM604 was grown anaerobically in 20 liters of LT-NO_3 for 24 h. Cells were harvested in a Sharples centrifuge. The cell paste was suspended in Tris buffer, and DNase and RNase (0.06 mg/g of cells) were added. The cells were broken by two passages through a French pressure cell at 16,000 lb/in^2. The membrane fraction was collected by centrifugation at 90,000 × *g* for 90 min and washed twice with Tris buffer. NosA was solubilized from the membrane fraction as described above, except that the centrifugations were at 90,000 × *g* for 90 min. All subsequent steps were performed at 4°C in Tris buffer containing 2% Triton X-100 and 10 mM EDTA.

(i) Ion-exchange chromatography. The solubilized proteins were applied to a column (2.6 by 47 cm) of DEAE-cellulose (DE-52; Whatman, Inc.). The column was eluted with 650 ml of a linear NaCl gradient (0 to 0.5 M), and fractions (5 ml) were collected. NosA eluted at 0.3 M NaCl. The fractions were analyzed for copper by atomic absorption spectroscopy. Fractions containing NosA and copper were pooled.

(ii) Ammonium sulfate fractionation. The pooled fractions were fractionated with solid $(NH_4)_2SO_4$. The precipitate formed between 60 and 80% saturation was dissolved in Tris buffer.

(iii) Gel filtration chromatography. The sample was applied to a Sephacryl S-200 Superfine column (1.6 by 100 cm), and fractions (4 ml) were collected. The NosA-containing fractions were pooled.

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TABLE 1. Bacterial strains

Species	Strain	Genotype or phenotype	Source or reference
Pseudomonas aeruginosa	PAO1	Wild type	T. C. Hollocher
Pseudomonas alcaligenes	14909	Wild type	ATCC[a]
Pseudomonas mendocina	25411	Wild type	ATCC
Pseudomonas stutzeri	JM299	Rough	B. A. Bryan and C. C. Delwiche
	JM300	Smooth variant	6
	JM604	*nal-7*	14
	JM753	*nal-7 nosA14*	14
	ZoBell	Wild type	8

[a] ATCC, American Type Culture Collection.

(iv) Second ion-exchange chromatography. The pooled fractions were loaded onto a DE-52 column (1.6 by 20 cm). The column was eluted with a 0 to 0.35 M linear gradient of NaCl. Those fractions containing NosA were pooled and loaded onto another gel filtration column. To decrease the concentration of detergent, the sample was applied to a DE-52 column (1.6 by 20 cm), washed with Tris buffer containing 0.1% Triton X-100, and eluted as before. The purified protein was concentrated by pressure ultrafiltration with a Diaflo PM10 membrane (Amicon Corp.).

Preparation of antibodies. Antibodies against purified NosA were raised in New Zealand White rabbits. NosA (1 mg) and 1 ml of Freund complete adjuvant (Difco Laboratories) were emulsified and injected subcutaneously into the back of the animal. After 3 and 4.5 weeks, 0.5 mg of protein in Freund incomplete adjuvant (0.5 ml) was administered. The rabbit was bled by cardiac puncture 1 week later. The immunoglobulin fraction was purified from the crude serum by ammonium sulfate fractionation and DEAE-cellulose chromatography (12). To remove nonspecific antibodies, the antibody solution was fractionated by affinity chromatography with Affi-Gel 10 (Bio-Rad Laboratories) as follows. Strain JM604 was grown anaerobically in LT-NO_3 containing 100 μM $CuSO_4$, and the cell extract was prepared as described above in 0.1 M phosphate buffer (pH 7.0). Membrane proteins were extracted with 2% Triton X-100 and 10 mM EDTA. After being dialyzed against phosphate buffer, the sample was mixed with the affinity gel and shaken (60 rpm) for 1 h at room temperature. The cytoplasmic soluble proteins were also mixed with affinity gel and shaken. The two gel mixtures were packed into one column (0.9 by 14 cm). After the column was washed, the concentrated immunoglobulin solution was applied, and fractions (2 ml) were collected. The fractions containing immunoglobulins were pooled and pressure concentrated.

N_2O reductase (NosZ) was purified by the method of Coyle et al. (7), and the corresponding antibody was obtained as described by Zumft et al. (19).

Immunological methods. Double-diffusion plates were prepared as described by Garvey et al. (9), except 50 mM Tris-acetate buffer (pH 8.2) containing 0.2% Triton X-100 and 2% polyethylene glycol 8000 was used.

Rocket immunoelectrophoresis was performed as described by Laurell (11). The buffer was the same as that used in the double-diffusion plates. Dried gels were stained with Coomassie brilliant blue R-250 for 20 min and destained to the desired contrast. The protein concentration was determined from a standard curve prepared with purified protein.

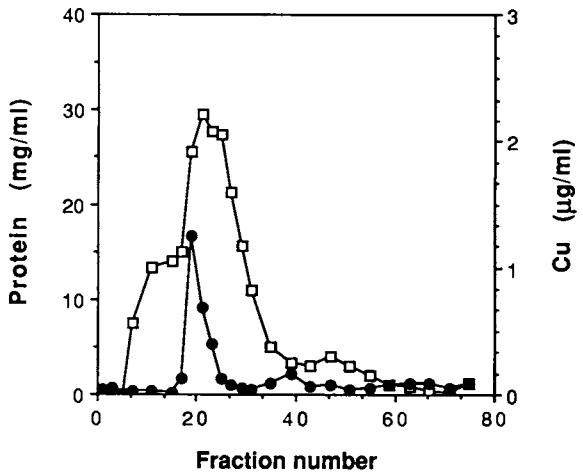

FIG. 1. Elution profile of ion-exchange chromatography of double-extracted membrane proteins. Symbols: □, protein concentration; ●, copper content. Fractions taken during sample application and column washing contained less than 50 ng of copper per ml of wash fluid.

Immunoblot analysis was performed with the Immuno-Blot (goat anti-rabbit–horseradish peroxidase) kit from Bio-Rad Laboratories.

Lipid bilayer experiments. The method of Armstrong et al. (1) was used for the black lipid bilayer experiments. The zero-current potential measurements were made as described by Benz et al. (4).

Chemical methods. Protein content was determined by the method of Smith et al. (17) with bovine serum albumin as the standard. Copper content was determined with an atomic absorption spectrophotometer (Perkin Elmer 5000) at 324.7 nm.

Gel separations. One-dimensional SDS-PAGE (10) and O'Farrell gels (15) were used as described before.

RESULTS

Purification of NosA protein. NosA was extracted from a membrane fraction of cells that were grown anaerobically in a low-copper medium (LT-NO_3), conditions that maximize its expression. The protein was extracted from the membrane fraction by 2% Triton X-100 and 10 mM EDTA. Subsequent purification was performed by DEAE-cellulose column chromatography, ammonium sulfate fractionation and gel filtration column chromatography. NosA eluted from the DEAE-cellulose column at 0.3 M NaCl, accompanied by a copper peak (Fig. 1). A copper peak also accompanied the elution profile of NosA from the gel filtration column.

The purified protein was electrophoretically homogeneous (Fig. 2) and migrated to the known location of NosA on an O'Farrell gel. The identity of the purified protein with NosA was further confirmed by Western blots (immunoblots; with anti-NosA) of O'Farrell gels of the total cell extracts from the wild type and a *nosA* mutant (JM753). A single spot developed at the known location of NosA on the gel containing the wild-type extract; no protein was detected on a gel containing an extract of JM753 (data not shown). The M_r of NosA was 65,000; its copper concentration was 0.75 ± 0.25 (mean ± standard deviation) mol of copper per mol of protein. The amount of copper per milligram of protein was monitored at all steps of the purification; it increased progressively, suggesting a firm binding of copper to NosA.

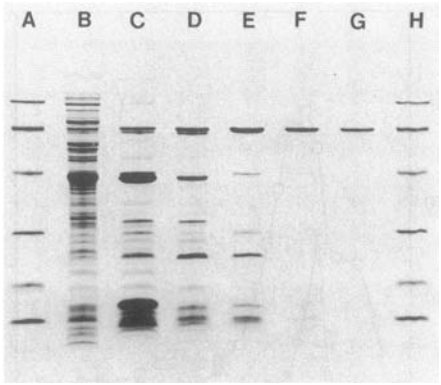

FIG. 2. Purification of NosA. Lanes: B, total lysate (90 μg); C, double-extracted membrane proteins (90 μg); D, pooled fractions from DEAE-cellulose column (50 μg); E, 60 to 80% ammonium sulfate fraction (18 μg); F, pooled fractions from gel filtration column (8 μg); G, purified NosA (4.5 μg). Lanes A and H, M_r markers (top to bottom): rabbit muscle phosphorylase *b*, 97,400; bovine serum albumin, 66,200; hen egg white ovalbumin, 42,699; bovine carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; hen egg white lysozyme, 14,400.

Expression of *nosA*. The effect of various environmental factors on the expression of *nosA* was investigated by monitoring NosA levels with O'Farrell gels and rocket immunoelectrophoresis. Exogenous copper repressed *nosA* expression (Fig. 3). The highest expression was seen in LT-NO_3 medium, which was found by atomic absorption spectroscopy to contain 1.0 μM copper (14). The level of expression was sharply decreased with 3 μM $CuSO_4$; with 11 μM $CuSO_4$, no detectable protein was made. Repression of *nosA* expression by copper appears to be specific. Of five other cations tested (Mn, Co, Ni, Zn, Fe^{2+}, and Fe^{3+}) at 100 μM, none repressed expression of *nosA* (data not shown).

Another set of experiments determined the effect of exogenous copper on the expression of *nosA* in cells grown in LT medium containing N_2O as the electron acceptor. Under these conditions, *nosA* expression was more sensitive to exogenous copper. Complete repression was observed even at 1.7 μM $CuSO_4$ (data not shown).

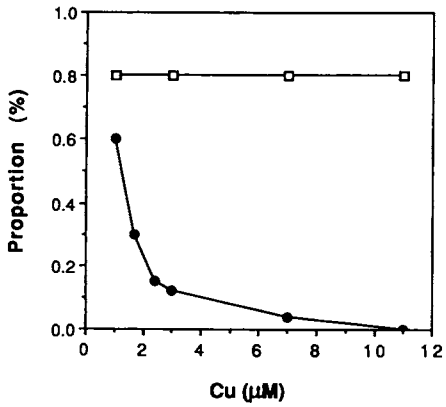

FIG. 3. Effect of exogenous copper on synthesis of NosA and N_2O reductase. Vertical axis represents the fraction of total cell protein that is NosA (●) or N_2O reductase (□) in *P. stutzeri* JM604 as determined by rocket immunoelectrophoresis. Horizontal axis represents the concentration of copper present in growth medium as judged by atomic absorption spectroscopy.

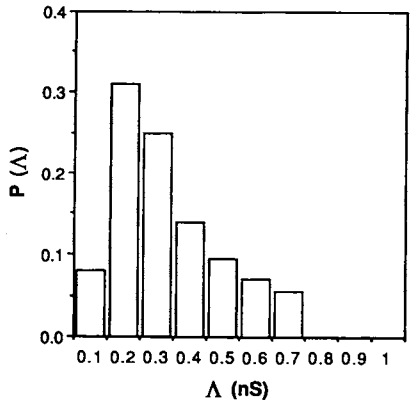

FIG. 4. Distribution of conductivity increments in a single-channel experiment. Membrane conductance (Λ) is given in nanosiemens. P (Λ) is the number of observed steps of the given conductance divided by the total number of steps (n = 243 events). The applied voltage was 20 mV. The aqueous phase (1 M KCl) contained 1 to 20 μg of purified NosA protein.

We also tested the effect of exogenous copper on the expression of the *nosZ*-encoded protein, nitrous oxide reductase itself. Although the activity of this protein is dependent on its copper component, exogenous copper had no effect on its expression (Fig. 3).

Expression of both NosA and NosZ was repressed by aerobiosis. In fully aerobic cultures, neither gene product was detectable by rocket immunoelectrophoresis (data not shown).

Channel-forming activity of NosA. Since NosA is located in the outer membrane and mutant strains that lack it produce copper-free N_2O reductase (14), the possible channel-forming activity of NosA was tested with an artificial black lipid bilayer. Adding homogeneous NosA (1 to 20 μg) to an aqueous solution (1 M KCl) divided by a black lipid bilayer caused the electrical conductance through the membrane to increase. An analysis of the distribution of the individual conductance increments is shown in Fig. 4. The average single-channel conductance (0.32 nS) was similar to that caused by the small-pore form of *P. aeruginosa* protein F (0.36 nS) (18). At higher concentrations of added NosA, a typical macroscopic conductance curve was observed (a smooth conductance increase with time rather than single steps of conductance increase). There was a linear relationship between the voltage applied and the current at applied voltages up to 100 mV. This result suggests that channels were not voltage gated. The ion selectivity (P_c/P_a [5]) calculated from zero-current membrane potential measurements was 3.8 ± 1.9. For comparison, the ion selectivity of nonspecific porins, such as protein F from *P. aeruginosa* and OmpF from *Escherichia coli*, is 2.5 (3) and 3.9 (5), respectively. Thus, under the conditions of these measurements, NosA shows only a slight selectivity for passing cations compared with anions. However, these data show that NosA has the ability to form ion-conducting channels in a membrane.

Presence of NosA protein in denitrifying bacteria. The presence of NosA-like proteins in various denitrifying pseudomonads was tested with double-diffusion plates (Fig. 5). Membrane extracts from the *P. stutzeri* strains JM300 and JM299 gave a strong cross-reaction with antibody against NosA protein from JM604, but similar extracts from *P. mendocina*, *P. alcaligenes*, *P. aeruginosa*, and *P. stutzeri*

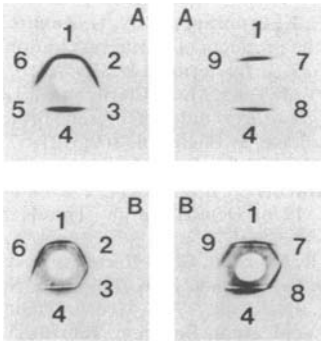

FIG. 5. Immunodiffusion tests with NosA and N_2O reductase antibodies. (A) Each outer well contained 20 μg of double-extracted outer membrane proteins, and the center well contained NosA antibody. (B) Each outer well contained 130 μg of total cell protein, and the center well contained N_2O reductase antibody. Wells: 1 and 4, *P. stutzeri* JM604; 2, *P. stutzeri* JM299; 3, *P. mendocina*; 5, blank; 6, *P. stutzeri* JM300; 7, *P. aeruginosa*; 8, *P. alcaligenes*; 9, *P. stutzeri* strain ZoBell.

strain ZoBell showed no cross-reaction. In contrast, antibody made against N_2O reductase from JM604 gave a strong reaction with extracts from all of these strains.

DISCUSSION

The copper content of N_2O reductase is essential for its activity. Wild-type cultures grown in copper-free medium (13) and certain strains carrying mutations in the structural gene *nosZ* (19) produce copper-free N_2O reductase and are unable to reduce N_2O to N_2. Similarly, mutant strains that are unable to make NosA produce an inactive copper-free N_2O reductase (14).

We have speculated (14) that NosA might take copper from the external medium and insert it into N_2O reductase, which our experiments have indicated is located in the periplasm of *P. stutzeri* JM604. In the present paper we show that NosA is a channel-forming protein. Its essentiality for the synthesis of copper-containing N_2O reductase, its copper content, and its specific repressibility by exogenous copper suggested that NosA brings copper into the cell. And since *nosA* mutants are like the wild type except for their inability to reduce N_2O, the only obvious function of NosA is to bring copper to N_2O reductase. Moreover, the repressibility of NosA synthesis by exogenous copper and the copper content of the purified protein further suggest that NosA functions to bring copper into the cell when external copper concentrations are low. Yet the black lipid bilayer studies presented here do not indicate that NosA channels are specific for copper. Their weak fourfold selectivity for cations is quite similar to the cation selectivity (3.9) of OmpF (5), the nonspecific porin from *E. coli*.

Possibly, the specificity of NosA for copper uptake may be conferred by another protein. For example, a high-affinity periplasmic copper-binding protein might bind to NosA in a manner analogous to the binding of periplasmic maltose-binding protein to the outer membrane LamB maltoporin (2). Such binding would decrease the concentration of free copper ions in the periplasm and thus maintain the concentration gradient for passage of copper ions through the NosA pore. The existence of such a protein is currently being investigated.

Alternatively, NosA may be a bifunctional protein, containing both a copper-binding and a pore-forming domain. This seems plausible because NosA has a higher molecular weight than any porin investigated to date, i.e., 65,000 M_r compared with the previous maximum of 48,000 M_r for maltoporin (2) and the phosphate-specific protein of *P. aeruginosa* (4). Our results suggest, however, that the NosA channel is not copper specific: the channel was not cation selective, and in preliminary experiments, Cu^{2+} did not influence conductance of K^+ or Cl^- through the channels. Nevertheless, purified NosA has copper bound tightly to it. The presence of a copper-binding domain on NosA could assist copper uptake through the NosA channel. For example, if the copper-binding site were at the external surface of the outer membrane, it could serve to attract copper ions to the vicinity of the channel. Alternatively, if it were on the periplasmic side of the outer membrane, it could serve a function equivalent to periplasmic binding proteins by maintaining a concentration gradient across the outer membrane at low external concentrations of copper.

In spite of the intimate metabolic connection between NosA and N_2O reductase, control of their expression is quite different. Synthesis of NosA is completely repressed by 11 μM exogenous copper; expression of N_2O reductase is unaffected. The reason that *nosA* mutants make copper-free N_2O reductase even with NosA-repressing levels of exogenous copper is unexplained. The *nosA* mutants that have been studied (14) were generated by a frameshift mutation and selected for an inability to grow with N_2O and to produce NosA. Possibly, the mutants that have been studied are polar mutants that fail to produce one or more proteins other than NosA. One of these other putative proteins may be needed to incorporate copper into N_2O reductase when the exogenous copper supply is elevated. Studies to identify this protein are in progress.

We have asked whether the NosA system is common to that of denitrifiers that are closely related to our strain of *P. stutzeri*. This does not seem to be the case. If the related organisms *P. mendocina*, *P. alcaligenes*, *P. aeruginosa*, and *P. stutzeri* strain ZoBell do contain a NosA homolog, they are not related closely enough to cross-react.

ACKNOWLEDGMENTS

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