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NOTES

Loss of Nitrous Oxide Reductase in *Pseudomonas aeruginosa* Cultured under N_2O as Determined by Rocket Immunoelectrophoresis

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The mass ratio of nitrous oxide reductase to total protein in the soluble protein fraction of *Pseudomonas aeruginosa* P2 was highest in cells grown on nitrate, decreased in cells grown on N_2O following the exhaustion of the initial charge of nitrate, and was nearly zero in cells exposed solely to N_2O.

Pseudomonas aeruginosa is unusual among denitrifiers in that it grows poorly on N_2O alone but can grow efficiently on the N_2O produced as a metabolic intermediate during the reduction of nitrate or nitrite to N_2 (1–3). Previous studies in our laboratory have determined that the immediate cause for poor growth on N_2O is a progressive loss of nitrous oxide uptake and reductase activities in cells grown under N_2O (7). In this study, we determined by using rocket immunoelectrophoresis (4) that the progressive loss of enzyme activity corresponds to a progressive decrease in the intracellular concentration of nitrous oxide reductase.

To raise antiserum, 1 ml of 98% pure nitrous oxide reductase (0.25 mg/ml) from *P. aeruginosa* P2 (SooHoo and Hollocher, unpublished data) in 0.15 M NaCl–50 mM potassium phosphate buffer (pH 7.0) was emulsified with 1 ml of Freund complete adjuvant and injected subcutaneously into the back of a New Zealand albino female rabbit. Booster injections of enzyme supplemented with Freund incomplete adjuvant were administered subcutaneously once a week for the next 3 weeks. The rabbit was then bled during weeks 5 through 8, and double immunodiffusion (4) was used to follow the antibody titer, which remained essentially the same during this period. Antiserum was clarified by centrifugation and stored frozen at −20°C until it was needed.

Immunoelectrophoresis in which 1% agarose gels were used was carried out as described in the Bio-Phoresis horizontal electrophoresis cell instruction manual (Bio-Rad). The volume of nitrous oxide reductase standard or soluble protein fraction was 5 μl per lane in all cases. Samples were diluted to give appropriate rocket heights, and plots of enzyme amount versus rocket height provided standard curves for purposes of quantification. After electrophoresis each gel was stained for 30 s in 0.05% Coomassie blue R250 in 50% methanol–10% glacial acetic acid and destained in 10% ethanol–15% glacial acetic acid overnight. The gels were dried in BioGelWrap sheets (Bio Design, Inc.) before rocket heights were measured. The bicinchoninic acid protein assay (Pierce) was used throughout, and nitrite was assayed by using a colorimetric method (6). Exhaustion of nitrate was judged to be complete when nitrite was no longer detectable.

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Bacteria were grown in 2-liter bottles that contained 1,500 ml of defined medium (1, 2) supplemented with nitrate. The bottles were sparged with N_2 inside an anaerobic glove bag (Coy Laboratories) before they were sealed with ring-clamped septa. The headspace gas in some bottles was replaced with N_2O (0.1 or 1 atm [ca. 10.13 or 101.3 kPa]) before autoclaving. *P. aeruginosa* grown anaerobically on 10 mM nitrate (1) was used as the inoculum (2%, vol/vol) for the 2-liter bottles, and cultures were incubated at 30°C in all cases. After exhaustion of nitrate and nitrite, the headspace gas was replaced in some cases with N_2O (1 atm), and the cultures were incubated for an additional 24 h. For aerobically grown cells, 1 liter of culture (without nitrate) was placed in a 4-liter flask and shaken at 140 rpm.

Cells were harvested by centrifugation, washed with 5 mM Tris hydrochloride buffer (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride, and finally suspended in the same buffer at volumes that were convenient for use with a French press or with a sonicator to break the cells. Because of low cell yields, cultures incubated under 0.1 atm of N_2O were sonicated with a microtip sonicator (Vibra cell model VC50; Sonics & Materials, Inc.) for 30 s (total time). Broken cell preparations were centrifuged at 100,000 × *g* for 1 h, and the supernatant (soluble protein fraction) was loaded into the gel.

From the rocket heights and protein concentrations determined for the soluble protein fraction and the pure antigen, we calculated the mass fraction of antigen in the soluble fraction (Table 1). When *P. aeruginosa* was grown on 10 mM nitrate (final A_{660}, 0.25 to 0.32), the mass fractions of the antigen in the soluble fraction were 0.030 to 0.036. These values are in agreement with other estimates of the nitrous oxide reductase contents of denitrifying bacteria (5, 8). Growth on nitrate and N_2O together allowed maximum cell yields (A_{660}, ~0.8) to be reached and so was not oxidant limited. The final mass fraction of antigen in this case was also in the range expected for nitrate-supported growth (0.027) (Table 1). However, if exposure to N_2O followed the exhaustion of nitrate or if N_2O but not nitrate was added at the outset, then substantial decreases in the antigen level occurred. In addition, the cell yield at most only doubled in 24 h following the introduction of N_2O after exhaustion of nitrate. When inocula were exposed solely to N_2O for 4

TABLE 1. Mass fraction of nitrous oxide reductase in the soluble protein fraction of *P. aeruginosa* P2 as determined by rocket immunoelectrophoresis[a]

Culture conditions	A_{660}			Ratio of N_2O reductase to soluble protein	
	Initial	When the culture was switched to N_2O	Final	Observed	If caused solely by dilution[b]
10 mM nitrate	0.006		0.32 (24)[c]	0.036	NA
10 mM nitrate plus N_2O (1 atm)	0.006		0.76 (24)	0.027	NA
10 mM nitrate followed by N_2O (1 atm)	0.006	0.30 (24)	0.60 (48)	0.013	0.018
5 mM nitrate followed by N_2O (1 atm)	0.006	0.15 (24)	0.25 (48)	0.012	0.022
2 mM nitrate followed by N_2O (1 atm)	0.006	0.07 (24)	0.10 (48)	0.0056	0.025
N_2O (0.1 atm)	0.006		0.08 (96)	0.0027	0.0028

[a] Cultures were run in duplicate, and average values are reported. The levels of reproducibility for A_{660} and the ratio of nitrous oxide reductase to soluble protein were 10 and 20%, respectively.

[b] Assuming that biosynthesis of nitrous oxide reductase ceased under N_2O and that the enzyme was diluted as a result of subsequent cell growth. NA, Not applicable.

[c] The numbers in parentheses are culture ages or incubation times (in hours).

days, the cell mass increased only 13-fold (less than four doublings net), and the ratio of enzyme to soluble protein was so low (0.002 to 0.003) that quantification was difficult. The antigen was undectable in *P. aeruginosa* P2 grown as an aerobe on the same medium lacking nitrate.

In Table 1 the observed nitrous oxide reductase levels are compared with the values expected if these levels had resulted simply from the cessation of nitrous oxide reductase biosynthesis under N_2O and the subsequent dilution of this enzyme as a result of cell growth. Generally, the observed levels were lower than the expected levels. This could have occurred because some enzyme was lost by intracellular destruction or because of release following cell lysis. We believe that the former possibility is more plausible, inasmuch as the A_{660} of cell cultures decreased only very slowly after cultures had stopped growing.

In this study we found that the level of native (antigenic) nitrous oxide reductase decreased under N_2O and eventually became nearly undetectable. The results shown in Table 1 suggest that the growth of *P. aeruginosa* when N_2O is used as the sole energy source is nonlinearly related to the cellular content of antigenically competent nitrous oxide reductase. When the nitrous oxide reductase cellular content decreased in the presence of N_2O to 25 to 50% of the value observed during growth on nitrate, growth on N_2O became slow, with doubling times of 1 day or more. This kind of response is reminiscent of the nonlinear relationship observed previously between the growth of *P. aeruginosa* strains on N_2O and N_2O uptake activity (7). Why growth should virtually cease when N_2O respiration can and does continue at significant rates is unknown, but this phenomenon may be related in part to basal energy requirements for maintenance of cellular integrity without growth.

The pool of nitrous oxide reductase required for N_2O respiration evidently develops in *P. aeruginosa* only if upstream respiration occurs. In this sense the biosynthesis of the enzyme is regulated by nitrate or nitrite, but the mechanism remains to be determined.

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