Gas Chromatographic Assay for In Vitro Complementation of Pseudomonas aeruginosa Mutants Deficient in Nitrate Reduction

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An electron capture gas-chromatographic technique was developed to continuously measure nitrate (NO₃⁻) reduction during in vitro complementation tests with extracts from Pseudomonas aeruginosa mutants deficient in both assimilatory and dissimilatory nitrate reduction as a result of a single genetic mutation. The procedure involves coupling nitrate reduction to nitrous oxide (N₂O) evolution via a series of reactions specific to the denitrification pathway. The assay was dependent on nitrate concentration, and optimal activity was obtained with a final concentration of 0.2% potassium nitrate. The reduction exhibited a stoichiometry of 2:1 (NO₃⁻/N₂O), and succinate was the best electron source for the reaction, followed by glucose, pyruvate, and malate. The technique can also be used for continuously monitoring nitrate reduction. The optimal nitrite concentration in the nitrite reductase assay was 0.025%. The initial complementation studies of mutant extracts demonstrated that at least two genes are shared between the two nitrate reduction pathways in P. aeruginosa.

The assimilatory and denitrification pathways of nitrate reduction involve the initial reduction of nitrate to nitrite. Until recently, it had not been clearly demonstrated that gene components were shared between the assimilatory and denitrification pathways in Pseudomonas aeruginosa (4, 5). The selection of mutants by nitrosoguanidine mutagenesis and other treatments yielded isolates which carried multiple genetic lesions and, when genetically analyzed, yielded conflicting results (9, 10, 13, 14). We recently isolated a number of spontaneous mutants blocked in the ability to assimilate and denitrify at the expense of nitrate. Reversion and transductional analyses have revealed these mutants to be the result of a single mutation and not multiple genetic lesions (4). These mutants have been categorized into class I mutants, which are presumptive Mo cofactor mutants, and class II mutants, which carry a single genetic lesion that codes for an unknown function. We have developed a gas-chromatographic technique for performing in vitro complementation tests with extracts prepared from P. aeruginosa mutants. In this assay, the reduction of nitrate is coupled to N₂O evolution; the assay is ca. 50-fold more sensitive than the classic diazotization method (3, 11) by virtue of the high sensitivity of N₂O detection by electron capture gas chromatography (6). We report the optimal operating parameters of the in vitro assay; we used it to confirm our previous results, which indicated that at least two gene products are shared between the assimilatory and denitrification nitrate reduction systems in P. aeruginosa (4).

MATERIALS AND METHODS

Organisms and culture conditions. Mutants derived from parental strain P. aeruginosa PA01 were isolated as previously described and represent organisms unable to assimilate or dissimilinate nitrate because of a single genetic mutation (4). Starter cultures for all assays were grown as follows. Fernbach flasks (3 liters) containing 300 ml of tryptic soy broth supplemented with 0.5% glucose were inoculated with 12-h slants of P. aeruginosa; the mixtures were incubated with rotary shaking (200 rpm) at 37°C for 8 to 10 h in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Starter cultures were then used to inoculate a 10-liter vessel containing 3 liters of tryptic soy broth supplemented with 0.5% glucose; the mixture was incubated aerobically at 200 rpm and 37°C in a model MF 214 Microferm Fermentor (New Brunswick) for 2.5 to 3 h. Cells were harvested with a Sorvall RC2B centrifuge equipped with a GSA rotor at 8,000 × g for 10 min at 25°C and washed twice in Vogel-Bonner medium (15) supplemented with 1 μM sodium molybdate and 0.2% glucose. Cells were then derepressed and induced for both pathways as follows. Cells were resuspended in Vogel-Bonner medium supplemented with 1 μM sodium molybdate and 0.2% glucose at a density of 1 g (wet weight) of cells per 10 ml of medium, which was also supplemented with 0.2% KNO₃ (strain PA01) or 0.025% KNO₂ (mutants). The cell suspensions were transferred in equal volumes (40 to 50 ml) to 60-ml serum vials, which were sealed and flushed with argon to create anaerobic conditions; the suspension was then incubated at 37°C for 8 h. The derepressed and induced cells were harvested by centrifugation (8,000 × g at 25°C), washed twice in 0.05 M potassium phosphate buffer (pH 7.4), and divided into 3-g pellets, which were stored at −70°C until needed.

Preparation of extracts. Cell pellets (3 g [wet weight]) were thawed and suspended in 9 ml of 0.05 M potassium phosphate buffer (pH 7.4). The suspension was placed in a glass sonicator vessel, which was then immersed in an ice water bath, and the suspension was disrupted by the application of five 30-s bursts at 1-min intervals. The crude extract was centrifuged twice at 12,000 × g for 10 min (each time) at 4°C, and the supernatant fraction was collected and maintained on ice.

Assay. The reaction mixture was typically composed of 0.3 ml of extract, 0.1 ml of reducing source, and 0.5 ml of 0.05 M potassium phosphate buffer (pH 7.4) in a 13.4-ml serum vial, which was sealed and flushed with acetylene (1, 17). Either potassium nitrate (0.1 ml) or potassium nitrite (0.1 ml) at various concentrations was injected into the vial to initiate the reaction. This mixture was incubated for 20 min at room temperature, and N₂O evolution was analyzed by electron capture gas chromatography. Headspace samples were analyzed for N₂O with a model 3700 Varian electron capture gas chromatograph equipped with a 0.1-ml
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FIG. 1. Evolution of nitrous oxide as a function of time in P. aeruginosa PA01 extracts with potassium nitrate (0.2%) and glucose (0.2%) as the electron acceptor and electron donor, respectively.

sample loop. The column support system was Porapak Q (80 to 100 mesh) in a stainless steel column (2.46 m by 3 mm [outer diameter]). Operating conditions were as follows: column temperature, 55°C; injection port temperature, 60°C; and detector temperature, 350°C. The carrier gas was 95% Ar–5% CH₄ at a flow rate of 20 ml/min.

A 1-ml syringe was used to periodically sample the headspace of each assay vessel. Peak heights recorded with a strip recorder were measured manually, and N₂O concentrations were determined from a standard curve. N₂O values were corrected for gas solubility in the sample liquid phase, assuming the solubility values (Ostwald coefficients) established for pure water (α = 0.594 at 25°C). The values reported represent the averages of at least three replicates of all experiments.

**Protein determination.** Protein determinations for extracts were made by the method of Spector (12).

FIG. 2. Evolution of nitrous oxide as a function of time in P. aeruginosa PA01 extracts with potassium nitrate (0.025%) and glucose (0.2%) as the electron acceptor and electron donor, respectively.

FIG. 3. Evolution of nitrous oxide as a function of protein concentration in P. aeruginosa PA01 extracts with potassium nitrate (0.2%) and glucose (0.2%) as the electron acceptor and electron donor, respectively. Samples were incubated for 30 min.

**RESULTS AND DISCUSSION**

The reduction of nitrate or nitrite as measured by N₂O evolution in extracts of P. aeruginosa was linear as a function of time (Fig. 1 and 2). No nitric oxide (NO) was detected in any of the assays performed, even though operating conditions were specifically set to detect the presence of N₂O and NO. The reaction was dependent on the presence of an electron donor (data not shown).

The reaction was also dependent on protein concentration (Fig. 3). The stoichiometry of the reaction was determined to be approximately 2NO₃⁻/N₂O, as expected (Table 1).

The effect of various concentrations of nitrate and nitrite on the in vitro generation of N₂O was studied to determine the optimal concentration of each required for the assay. As can be seen in Fig. 4A and B, the optimal concentrations were determined to be 0.2% nitrate and 0.025% nitrite. High concentrations of both nitrate and nitrite inhibited the production of N₂O. Nitrite inhibition has been suggested to be a result of nitrite binding to heme compounds such as cytochromes, thus preventing the flow of electrons through the respiratory chain (8, 16).

Various concentrations of pyruvate, succinate, glucose, and L-malate were tested as potential physiological electron donors for nitrate reduction (Fig. 5). Their effectiveness as electron donors for the assay were, in decreasing order, as follows: 0.1% succinate, 0.2% glucose, 0.1% pyruvate, and

**TABLE 1. Stoichiometry of the reduction of nitrate to nitrous oxide**

<table>
<thead>
<tr>
<th>NO₃⁻ (nmol)</th>
<th>N₂O (nmol)</th>
<th>NO₃⁻/N₂O</th>
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</thead>
<tbody>
<tr>
<td>1,000</td>
<td>489.5 ± 12.5</td>
<td>2.04</td>
</tr>
<tr>
<td>1,000</td>
<td>512.4 ± 17.2</td>
<td>1.95</td>
</tr>
<tr>
<td>1,000</td>
<td>501.0 ± 14.8</td>
<td>1.99</td>
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</table>

*The reducing source was 0.1 ml of 0.2% glucose. The reaction was initiated by the addition of 0.1 ml of potassium nitrate (1,000 nmol). At various times, samples were analyzed as described in the text until it was established that the reaction had been completed. The average values of all experimental data were as follows: NO₃⁻, 1,000 nmol; N₂O, 500.9 nmol; NO₃⁻/N₂O, 1.99. Each value represents the average of three replicates of a single experiment.*
able to complement each other and reduce nitrate, with consequent \( \text{N}_2\text{O} \) evolution (Table 2), thus confirming the previous finding that at least two gene products are shared between the two nitrate reduction systems in \( P. \text{aeruginosa} \) (4).

In summary, the gas-chromatographic assay reported here represents a simple, sensitive method for the analysis of nitrate or nitrite reduction. Using this system, we have been able to detect as little as \( 10^{-11} \text{ mol} \) of \( \text{N}_2\text{O} \), whereas with the traditional diazotization method, at best \( 10^{-9} \text{ mol} \) of nitrite can be detected. Furthermore, this assay allows for the continuous monitoring of nitrate and nitrite reduction without having to disturb or destroy the sample. There is also no

0.1% malate. All four electron donors caused an inhibition of \( \text{N}_2\text{O} \) evolution at higher than optimal concentrations.

It is interesting to note that succinate reduction takes place through a flavoprotein which is commonly part of the membrane-bound electron transport chain. Previous in vitro studies of the nitrogen oxide reductases indicated that the addition of flavin adenine dinucleotide and flavin mononucleotide to extracts enhances reduction (2, 7). Thus, succinate may be the primary physiological reducing source for nitrogen oxide reduction.

The assay was used to perform in vitro complementation tests with extracts from mutants unable to assimilate or dissimilate nitrate because of a single genetic mutation to corroborate that at least two gene products are shared between the two nitrate reduction systems in \( P. \text{aeruginosa} \). The mutants used have been previously categorized into two classes on the basis of growth studies (4), class I mutants (Mo cofactor mutants) and class II mutants (unknown function). When mixed in equal volumes, these two classes were
need for colorimetric reagents and, if proper controls are included, there is no possibility of false-positive readings. It has been used successfully in this study for in vitro complementation testing of extracts from *P. aeruginosa* mutants unable to assimilate or dissociate nitrate because of a single genetic lesion, thus corroborating our earlier conclusion that at least two genes are shared between the two systems.

### ACKNOWLEDGMENTS

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### LITERATURE CITED


<table>
<thead>
<tr>
<th>Mutant class</th>
<th>Mutant</th>
<th>N₂O evolution (nmol/mg of protein) from:</th>
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</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>Nitrite</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>MJ2</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>MJ4</td>
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<tr>
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<td></td>
<td>MJ13 + MJ3</td>
<td>45.6</td>
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</table>

* Each 13.4-ml serum vial (sealed and flushed with acetylene) contained equal volumes (0.3 ml) of each mutant extract, 0.2 ml of 0.5 M potassium phosphate buffer (pH 7.4), and 0.1 ml of 0.2% glucose. The reaction was initiated by the injection of 0.1 ml of 0.2% potassium nitrate into each vial. * ND, None detected.