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Evidence for Gene Sharing in the Nitrate Reduction Systems of *Pseudomonas aeruginosa*

MARTHA GOLDFLAM AND JOHN J. ROWE*
Department of Biology, University of Dayton, Dayton, Ohio 45469

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Pseudomonas aeruginosa mutants unable to assimilate or dissimilate nitrate were isolated. Transduction and reversion analyses of these mutants revealed that single genetic lesions are responsible for the double phenotypes. The mutants were divided into two classes based on the ability to utilize hypoxanthine. It can be concluded from this study that at least two genes are shared between the two nitrate reduction systems.

Pseudomonas aeruginosa possesses two different nitrate reduction systems. When nitrate is the sole nitrogen source, the assimilatory pathway serves to reduce nitrate to ammonia, which is utilized for amino acid synthesis and eventually for the biosynthesis of all nitrogen-containing compounds necessary for cell function.

P. aeruginosa can also utilize nitrate as a terminal electron acceptor to support anaerobic respiration in a dissimilatory process termed denitrification. In contrast to the assimilatory pathway, the dissimilatory pathway is derepressed only under anaerobic conditions even when nitrate is absent and derepression is not affected by the presence of ammonia or amino acids. The dissimilatory reduction of nitrate is sensitive to chlorate, which is believed to be reduced by nitrate reductase to the toxic compound chlorite.

Although the two pathways for nitrate reduction are not directly related functionally, they do have a common reaction, i.e., the reduction of nitrate to nitrite. It has been speculated that in bacteria possessing both pathways some gene products may be shared. Among the few reports concerning nitrate reduction in *P. aeruginosa*, there are arguments for (28, 29) and against (25, 26) such a hypothesis. The evidence presented in this report supports the concept that gene products are shared between the nitrate reduction systems of *P. aeruginosa*.

P. aeruginosa PAO41, from which all mutants were derived, was used in this study. The wild type is sensitive to chlorate when grown anaerobically. The growth media included the basal salts medium of Vogel and Bonner (31) with 0.5% glucose as the carbon source, which was solidified with 2% Bacto-Agar (Difco Laboratories, Detroit, Mich.) when required. This solid medium will henceforth be abbreviated VBA. The complex medium was nutrient broth or nutrient agar (NA) supplemented with 0.5% glucose.

The following nitrogen sources were added to the basal salts medium or complex medium when appropriate: 0.1% NH_4Cl, 1.0% KNO_3, 0.025% KNO_2, and 0.1% hypoxanthine. Potassium chlorate ($KClO_3$) was added at a concentration of 0.1%. All plates were incubated for 48 h at 37°C unless otherwise stated. Anaerobic conditions were maintained using a carbon dioxide-hydrogen atmosphere (Gaspak; BBL Microbiology Systems, Cockeysville, Md.).

Isolation of mutants. Twenty-one mutants were employed in this study. Each had three pertinent characteristics: (i) resistance to chlorate anaerobically, (ii) inability to reduce nitrate, but ability to reduce nitrite anaerobically, (iii) inability to use nitrate, but ability to use nitrite as the sole nitrogen source aerobically (Table 1).

Mutant EJ1 was previously isolated in this laboratory (by Eric Beck, unpublished data) by selection for chlorate resistance after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. All the other strains reported here were spontaneous mutants isolated in the following way. Nutrient broth (20 ml) was inoculated with 5 ml of an overnight nutrient broth culture of PAO41 and incubated at 37°C and 200 rpm in a reciprocal shaking dry-air incubator (New Brunswick Scientific Co., New Brunswick, N.J.) for 2 to 3 h. The cells were harvested by centrifugation (SS-34 rotor; Ivan Sorvall Inc., Norwalk, Conn.) at 5,000 × *g* for 10 min at 25°C and suspended in 2.5 ml of 0.85% saline. Samples of the cell suspension (0.1 ml) were spread on NA plates supplemented with KNO_2 and $KClO_3$ and incubated anaerobically. Presumptive chlorate-resistant mutants were streaked to quadrants of NA containing KNO_2 and $KClO_3$ and incubated

TABLE 1. Anaerobic and aerobic utilization of inorganic nitrogen sources by wild-type PAO41 and mutants

Strain	Utilization of inorganic nitrogen					
	Anaerobic			Aerobic		
	NA with KNO_3	NA with KNO_2	NA with KNO_2 with $KClO_3$	VBA with KNO_3	VBA with KNO_2	VBA with NH_4Cl[a]
PAO41	+	+	−	+	+	+
EJ1	−	+	+	−	+	+
MJ1–MJ20	−	+	+	−	+	+

[a] Growth was tested in the presence of 0.1% NH_4Cl (excess) and 0.001% NH_4Cl (limiting).

anaerobically. To confirm that the chlorate-resistant isolates were mutants unable to utilize nitrate anaerobically and to screen for mutants also unable to utilize nitrate as a sole nitrogen source aerobically (mutants with double phenotypes), single colonies of each isolate were "picked" to the following plates: VBA with KNO_3, VBA with KNO_2, VBA with NH_4Cl, and VBA with 0.001% NH_4Cl, which were incubated aerobically, and to NA with KNO_3, NA with KNO_2, and NA with both KNO_2 and $KClO_3$, which were incubated anaerobically. All chlorate-resistant mutants (100%) were able to utilize nitrite, but not nitrate, for anaerobic respiration. Of 199 such spontaneous mutants, 10% (20:199) were also unable to use nitrate as the sole nitrogen source aerobically (Table 1). The 20 isolates were designated MJ1 to MJ20. The aerobic NH_4Cl plates were included in the system to be certain that there were no defects in the ability of the mutants to assimilate NH_3 that might falsely be expressed as assimilatory nitrate reduction negative. All mutants were able to utilize high (0.1%) and limiting (0.001%) concentrations of ammonia as the sole nitrogen source (Table 1).

Genetic analysis. To determine whether a single gene was responsible for the double phenotypes observed in the 20 spontaneous mutants and EJ1, transduction and reversion studies were performed. Wild-type PAO41 lysates of bacteriophages B3 and G101 (11, 12) with titers of 10^{11} PFU ml^{-1} were used for transduction as previously described (1, 3). Fifty transductants of each cross were picked to NA with KNO_3 and to NA with KNO_2, which were incubated anaerobically, and to VBA containing KNO_3, which was incubated aerobically. All plate cultures were incubated for 3 to 5 days at 37°C. Appropriate controls were included.

The results of the transduction studies demonstrate that 100% of the transductants for six mutants, initially selected for their ability to assimilate nitrate, simultaneously regained the capacity to dissimilate nitrate. Transductants were scored for on VBA medium with KNO_3 incubated under aerobic conditions. Transduction frequency ranged from 1.6×10^{-4} to 5.4×10^{-6}. Of the transductants (150) from the PAO(B3) × EJ1 cross, 100% utilized nitrate on NA with KNO_3 and also grew on NA with KNO_2 anaerobically, and 100% utilized nitrate on VBA with KNO_3 aerobically. Of the transductants (50 each) from crosses PAO(G101) × EJ1, PAO(B3) × MJ1, PAO(G101) × MJ1, PAO(B3) × MJ2, PAO (G101) × MJ2, PAO(B3) × MJ3, PAO(G101) × MJ3, PAO(B3) × MJ4, PAO(G101) × MJ4, PAO(B3) × MJ6, PAO(G101) × MJ6, 100% utilized nitrate on NA with KNO_3 and also grew on NA with KNO_2 anaerobically, and 100% utilized nitrate on VBA with KNO_3 aerobically. When revertants of 11 of the mutants were selected for the assimilatory nitrate reduction function, the dissimilatory function was also recovered. Of the revertants (100 each) from mutants MJ1, MJ2, MJ3, MJ6, MJ9, MJ12, and MJ20, 100% utilized nitrate on NA with KNO_3 and on NA with KNO_2 anaerobically, and 100% utilized nitrate on VBA with KNO_3 aerobically. Of the revertants from mutants MJ10, MJ7, MJ8, and MJ4 (62, 61, 30, and 29 tested, respectively), 100% utilized nitrate on NA with KNO_3 and also grew on NA with KNO_2 anaerobically, and 100% utilized nitrate on VBA with KNO_3 aerobically. The total number of revertants reported was obtained from two different experiments. Reversion frequency ranged from 1.3×10^{-9} to 5.0×10^{-11}. These combined results indicate that a single gene was responsible for the double phenotypes in the following mutants: EJ1, MJ1, MJ2, MJ3, MJ4, MJ6, MJ7, MJ8, MJ9, MJ10, MJ12, and MJ20. No revertants were obtained for MJ11 and MJ14 after several attempts, perhaps owing to a deletion at the mutation site. The reversion frequencies for MJ5, MJ13, MJ16, MJ18, and MJ19 were too low to obtain a significant number of revertants, and the results are therefore not included. When cultures of MJ15 and MJ17 were scored for revertants, background growth prevented distinct picking of the revertants possibly because of leaky mutations.

Categorization of the mutants based on the molybdenum cofactor. To better characterize the mutants, they were analyzed for their ability to assimilate hypoxanthine aerobically as the sole

nitrogen source. Isolated colonies were picked from 48-h plate cultures of mutants EJ1 and MJ1 to MJ20 and wild-type PAO41 to the following: VBA with KNO_3, VBA with KNO_2, VBA with NH_4Cl, and VBA with 0.1% hypoxanthine. All cultures were incubated aerobically at 37°C and observed for positive or negative growth at 24-h intervals for 96 h. Results remained constant after the first observation.

Hypoxanthine was chosen because the enzyme xanthine dehydrogenase is involved in the oxidation of hypoxanthine and this enzyme, like nitrate reductase, contains a molybdenum (Mo) cofactor. From previous studies of *Neurospora crassa, Aspergillus nidulans, Rhizobium meliloti*, and *Escherichia coli* (5, 10, 15, 20), mutations which result in a nonfunctional Mo cofactor for nitrate reductase also affect other molybdoproteins such as xanthine dehydrogenase and formate dehydrogenase. It is thought that all molybdoproteins (with the exception of nitrogenase) may, in fact, contain a genetically conserved Mo cofactor (2, 4, 6, 13, 14, 17, 21–24). The utilization of hypoxanthine allowed us to place the mutants into one of two general classes (Table 2). Of the 21 mutants, 11 were unable to assimilate hypoxanthine as a sole nitrogen source and were placed in class I, whereas those able to assimilate hypoxanthine were placed in class II.

From these results, it can be surmised that at least two genes are shared between the assimilatory and dissimilatory nitrate reduction systems. Although class I mutants have at least one gene in common (presumably responsible for synthesizing the Mo cofactor), it is unknown what gene product(s) is shared between the two nitrate reduction systems of the class II mutants. It is unlikely that a regulatory protein is shared because the pathways are expressed under completely different conditions and have different physiological functions. Because nitrate reductase is membrane bound during denitrification, whereas the assimilatory nitrate reductase is soluble, the membrane attachment component for dissimilatory nitrate reduction can also be eliminated as a potential shared component.

TABLE 2. Utilization of hypoxanthine as a sole nitrogen source aerobically by wild-type PAO41 and class I and class II mutants

Utilization of hypoxanthine			
Class I		Class II	
Strain	VBA and 0.1% hypoxanthine	Strain	VBA and 0.1% hypoxanthine
EJ1[a]	−	MJ2[a]	+
MJ1[a]	−	MJ4[a]	+
MJ3[a]	−	MJ7[a]	+
MJ5[a]	−	MJ8[a]	+
MJ6[a]	−	MJ11	+
MJ9[a]	−	MJ13	+
MJ10[a]	−	MJ15	+
MJ12[a]	−	MJ16	+
MJ14	−	MJ17	+
MJ18	−	MJ19	+
MJ20[a]	−		
PAO41	+		

[a] Mutants for which it has been shown that single-site mutations are responsible for the pleiotropic effects.

Besides the Mo cofactor, a few other components can be considered candidates for shared gene products. One such candidate is cytochrome *b*. Biochemical analyses of both assimilatory and dissimilatory nitrate reductase enzymes have indicated that cytochrome *b* is an integral part of the nitrate reductase enzyme structure and is necessary for physiological functioning (7, 8, 9, 16, 18, 19, 27). Cytochromes are genetically conserved proteins, and it is likely that in an organism possessing both the assimilatory and dissimilatory pathways, the same genes could code for the cytochrome involved in both systems.

Another possible candidate is one or more of the peptides that comprise nitrate reductase (30). Also, it can be speculated that the protein or proteins responsible for the transport of nitrate through the membrane could be common to both pathways.

In conclusion, the evidence presented here suggests that at least two genes are shared between the assimilatory and dissimilatory nitrate reduction systems of *P. aeruginosa*. Class I mutants represent mutations in the Mo cofactor processing system, whereas the shared gene product(s) represented by the class II mutants is unknown.

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