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Anaerobic Activation of the Entire Denitrification Pathway in *Pseudomonas aeruginosa* Requires Anr, an Analog of Fnr

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The *Pseudomonas aeruginosa* gene *anr*, which encodes a structural and functional analog of the anaerobic regulator Fnr in *Escherichia coli*, was mapped to the *Spe*I fragment R, which is at about 59 min on the genomic map of *P. aeruginosa* PAO1. Wild-type *P. aeruginosa* PAO1 grew under anaerobic conditions with nitrate, nitrite, and nitrous oxide as alternative electron acceptors. An *anr* deletion mutant, PAO6261, was constructed. It was unable to grow with these alternative electron acceptors; however, its ability to denitrify was restored upon the introduction of the wild-type *anr* gene. In addition, the activities of two enzymes in the denitrification pathway, nitrite reductase and nitric oxide reductase, were not detectable under oxygen-limiting conditions in strain PAO6261 but were restored when complemented with the *anr*$^+$ gene. These results indicate that the *anr* gene product plays a key role in anaerobically activating the entire denitrification pathway.

Many species of gram-negative as well as gram-positive bacteria can use inorganic N oxides as alternative electron acceptors for respiratory growth under oxygen-limiting conditions by a process referred to as denitrification. The denitrification pathway is established as $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (17, 40, 44). A key step of denitrification is the conversion of nitrite to nitric oxide gas since this and the subsequent products are no longer usable by most organisms. Denitrification leads to significant losses of nitrogen fertilizer from the soil, and the process emits nitric and nitrous oxides that contribute to the destruction of the earth's ozone layer. The denitrifying process, however, can be used to aid bioremediation of environmental contaminants in aquifers where oxygen is limiting.

Denitrification is carried out by bacteria that preferentially grow in aerobic environments but that have an alternative capacity to use N oxides as respiratory electron acceptors when oxygen becomes scarce. Oxygen-limiting conditions (<10 μM O_2) are usually necessary for denitrification (33). However, some bacteria such as *Thiosphaera pantotropha* are able to denitrify in the presence of higher oxygen concentrations (4, 24). Although *Escherichia coli* and other enteric bacteria do not denitrify, they can respire nitrate and nitrite under anaerobic conditions and thus offer insight into how denitrification might be regulated by oxygen limitation. The *fnr* gene is essential for expression of genes involved in anaerobic metabolism under anaerobic conditions in *E. coli* (31, 35). A conserved symmetrical sequence, the Fnr box (consensus $TTGATN_4A$ TCAA), is located upstream of the Fnr-dependent genes and operons. Under low oxygen tension, the Fnr protein acts as a transcriptional activator by binding to the Fnr boxes (11). In *Pseudomonas aeruginosa*, a mutation in the *anr* gene (*anr*, for anaerobic regulation of arginine deiminase and nitrate reduction) results in the loss of anaerobic arginine degradation and nitrate reduction (10, 12). The *anr* gene encodes a protein with a structural and functional similarity to the Fnr protein from *E. coli* (26, 43). The Anr protein has a 51% identity with the Fnr protein in terms of their amino acid sequences, and the helix-turn-helix motifs at the C termini of Anr and Fnr are even more similar (12, 26, 43). Four essential cysteine residues proposed to be involved in Fe binding for Fnr (20, 27, 32) are also found in Anr. Anr acts on a sequence ($TTGACN_4ATCAG$) related to the consensus Fnr box to regulate expression of the arginine deiminase pathway under oxygen-limiting conditions in *P. aeruginosa* (10). The *arcDABC* operon, which is responsible for enzymes of this pathway, is rendered virtually noninducible by a deletion or an insertion in the Anr recognition site or by a mutation in the *anr* gene. Furthermore, the Anr protein activates transcription of anaerobically inducible, Fnr-dependent genes in *E. coli* (26).

To address whether Anr regulates the whole denitrification pathway under anaerobic conditions, we constructed a stable Anr deletion mutant of *P. aeruginosa* and characterized its ability to grow with inorganic N oxide compounds as alternative electron acceptors. We also physically mapped the location of *anr* on the chromosome of *P. aeruginosa* PAO.

Construction of mutant PAO6261. We previously engineered a chromosomal *anr* insertion mutation in *P. aeruginosa* PAO6215 (43). However, this insertion was unstable in the absence of antibiotic selection. To obtain a stable *anr* mutant, we introduced a defined *anr* deletion from plasmid pME3584 (43) into the chromosomal *anr* locus of *P. aeruginosa* by gene replacement. The mobilizable, ColE1-based suicide plasmid pME3087 (19, 36) was used to clone a 2.9-kb *Sal*I-*Bam*HI fragment which carries the *P. aeruginosa anr* gene with an internal 0.33-kb deletion (Fig. 1). In the resulting construct, pME3585, the sequences upstream and downstream of the mutated *anr* gene provided sufficient homology for marker exchange (Fig. 1). Plasmid pME3585 was mobilized from an exponential culture of transfer-proficient *E. coli* S17-1 (28) into *P. aeruginosa* PAO1 grown to stationary phase at 43°C; the mating conditions were done according to the method of Rella

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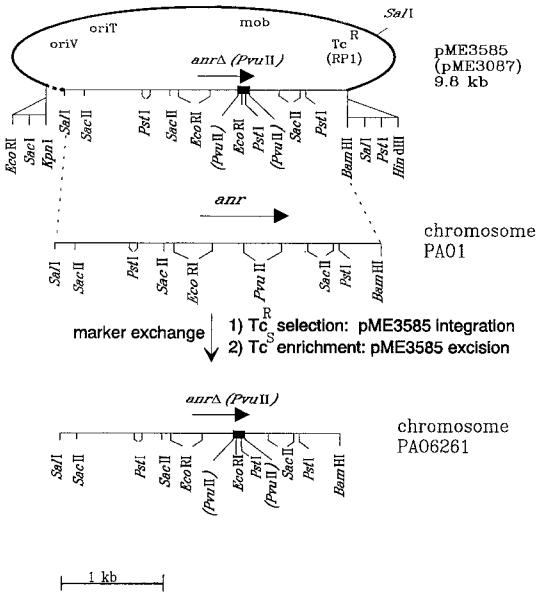

FIG. 1. Introduction of the *anr* (Δ*Pvu*II) mutation into the chromosome of *P. aeruginosa* PAO1. The 2.9-kb *Sal*I-*Bam*HI fragment (—) from pME3584 (43) was extended, at the left end, by a short *Kpn*I-*Sal*I fragment (- - -) from pBluescript and inserted into pME3087 (19, 36) cleaved with *Kpn*I and *Bam*HI (▬). The internal *Pvu*II deletion and accompanying linker insertion (▬) in *anr* have been previously described (43). Restriction sites shown in parentheses were lost during cloning. oriV, origin of ColE1 replication; oriT, origin of transfer; mob, genes required for conjugative mobilization of ColE1 (5); Tc^R, tetracycline resistance determinant from the IncP plasmid RP1 (37); Tc^S, tetracycline sensitive.

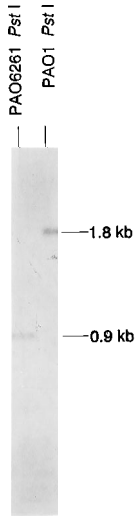

FIG. 2. Southern hybridization demonstrating the deletion in the chromosomal *anr* gene of strain PAO6261. Genomic DNA from wild-type PAO1 and engineered mutant PAO6261 (see text) was digested with *Pst*I, electrophoresed in a 0.6% agarose gel, and hybridized to an *anr* probe, as previously described (43). The 1.8-kb *Pst*I fragment spanning the *anr* wild-type gene in PAO1 was shortened to 0.9 kb as a consequence of the 0.3-kb *Pvu*II deletion and the newly introduced *Pst*I site in the linker replacing the deleted fragment (Fig. 1).

et al. (22). Selection for tetracycline (TC) resistance (on minimal medium E containing 50 μg of TC per ml) at 37°C produced PAO1 transconjugants carrying a chromosomally integrated copy of pME3585 at about 10^{-7} per donor. TC-sensitive derivatives were then obtained by enrichment with D-cycloserine (13). Excision of pME3585 gave the *anr* mutant PAO6261 (Fig. 1). The 0.33-kb deletion in the *anr* gene was confirmed by Southern hybridization (Fig. 2).

Mapping of *anr*. Previously, the *anr* gene was mapped by classical genetic methods to the 60-min region of the PAO chromosome (10). We have now determined the physical location of *anr* on the PAO chromosome; to this end, the 1.3-kb *Sac*II fragment containing *anr* (Fig. 1) was isolated from plasmid pME3580 (10). The fragment was labelled with ^{32}P and used as a probe in Southern hybridization of *Spe*I-digested chromosomal DNA separated by pulsed-field gel electrophoresis (21). The *anr* probe hybridized to the *Spe*I fragment which is made up of fragments Q and R of the same size (150 kb) (25, 34). To distinguish between these, we repeated the hybridization with *Dpn*I-digested DNA, and hybridization was to *Dpn*I fragment C (696 kb), within which the *Spe*I fragment R is located (25). Thus, *anr* was shown to be located in *Spe*I fragment R, which is at about 59 min (21). This confirms the *anr* location determined by conjugation (10).

Characterization of PAO6261. To examine whether the deletion in the *anr* gene will result in a loss of denitrification capability, mutant PAO6261 was grown under anaerobic conditions with nitrate, nitrite, or nitrous oxide. Mutant PAO6261 could not use nitrate and nitrite as electron acceptors for growth under anaerobic conditions, while wild-type PAO1 could (Table 1). Nitrous oxide supported poor but significant growth for wild-type *P. aeruginosa* under anaerobic conditions. *P. aeruginosa* PAO has long been known to grow poorly on N_2O as the electron acceptor, apparently because of the inactivation of nitrous oxide reductase (3, 30). No growth, however, was observed for the Anr-negative strain PAO6261 with nitrous oxide as the only electron acceptor. Growth with nitrate, nitrite, or nitrous oxide was restored in strain PAO6261 after complementation with the wild-type *anr* gene carried by plasmid pME3580 (Table 1). These results indicate that the deletion in *anr* renders *P. aeruginosa* incapable of using any of these substrates as alternative electron acceptors under anaerobic conditions.

To further examine the effects of the *anr* deletion on the denitrification pathway under oxygen-limiting conditions, the ability of the wild-type organism and the *anr* deletion mutant to convert nitrate, nitrite, and nitrous oxide to N_2 gas was tested by measuring the end product of the denitrification pathway, N_2. Wild-type PAO1 was capable of reducing all three inorganic N compounds to N_2 gas, while the mutant strain PAO6261 could not (Table 2). Introduction of the wild-type *anr* gene into the mutant PAO6261 restored N_2 production

TABLE 1. Growth of PAO1 strains with different electron acceptors under anaerobic conditions[a]

Strain	Optical density at 600 nm (mean ± SD)		
	NO_3^-	NO_2^-	N_2O
PAO1	1.24 ± 0.04	0.82 ± 0.01	0.083 ± 0.002
PAO6261	0.0025 ± 0.0001	0.0015 ± 0.0005	0.003 ± 0.0012
PAO6261(pME3580)	1.02 ± 0.015	0.57 ± 0.03	0.055 ± 0.004

[a] Strains of *P. aeruginosa* were grown in 150-ml serum bottles containing 100 ml of tryptic soy broth supplemented with 20 mM KNO_3 or 15 mM $NaNO_2$ as indicated. Anaerobic conditions were achieved by repeatedly evacuating and flushing the sample bottles with argon gas. For the testing of N_2O as the electron acceptor, filter-sterilized N_2O was added to the headspace to achieve 1 atm (1 atm = 101.29 kPa). The cultures were grown at 37°C with shaking. Optical density data are means and standard deviations of results from four replicate bottles.

TABLE 2. Formation of N_2 gas by PAO1 strains with different electron acceptors under anaerobic conditions[a]

Strain	Mean N_2 gas formation (μl) ± SD		
	NO_3^-	NO_2^-	N_2O
PAO1	95 ± 26	144 ± 40	53 ± 12
PAO6261	0	0	0
PAO6261(pME3580)	85 ± 33	118 ± 13	30 ± 4

[a] Strains of *P. aeruginosa* were grown in 10 ml of tryptic soy broth in 15-ml culture tubes with an inverted Durham tube inside. The growth medium was supplemented with different electron acceptors as described in footnote *a* to Table 1. The cultures were incubated at 37°C without shaking. The amount of N_2 gas produced was measured by the volume of the bubble inside the Durham tube. Previous analysis of such bubbles has shown them to be composed almost entirely of N_2; any N_2O produced is dissolved in the liquid. Gas formation data are means and standard deviations of results from three replicate tubes.

from all three alternative electron donors. Because of *P. aeruginosa*'s poor growth on N_2O, this experiment provides more extensive evidence that nitrous oxide reductase is regulated by Anr and that the whole pathway is functional only in the presence of Anr.

Previously, it has been shown that expression of dissimilatory nitrate reductase depends on *anr* (10). To examine whether dissimilatory nitrite reductase and nitric oxide reductase are also regulated by Anr, we assayed these enzymatic activities in crude extracts prepared from cells induced for denitrification by oxygen depletion during growth in sealed serum bottles (10, 39). Mutant PAO6261 showed little nitrite reductase activity compared with that of the wild type (Table 3). No gram-negative denitrifying bacteria, including *P. aeruginosa*, can grow with NO alone because of its toxicity. Thus, growth experiments cannot be used to test for the role of Anr in the NO reduction step. However, the data of Remde and Conrad (23) and Kalkowski and Conrad (17) suggest that NO reductase of *P. aeruginosa* is inducible under anaerobic conditions. We found a lack of NO reductase activity in the crude extract of strain PAO6261, indicating that NO reduction is also regulated by the Anr protein (Table 3). These results demonstrate that Anr is required for the expression of these two enzymes.

Loss of all four steps in the denitrification pathway in mutant PAO6261 indicates that the *anr* gene product is a common transcriptional activator for denitrification. The Anr protein may directly act on the transcription of the reductases as well as on the expression of genes responsible for the synthesis of electron donors, cofactors, and other regulatory proteins involved in the pathway. This is supported by the independent finding (2) that Anr is required for the expression of both *nirS* (nitrite reductase gene) and *nirQ* (regulator for nitrite and nitric oxide reduction). This evidence was obtained by measuring the activity of catechol-2,3-dioxygenase from *nirS-xylE* and *nirQ-xylE* fusions in wild-type and Anr^- strains. Similar studies with promoter probes for genes involved in other steps of the pathway have not been done.

TABLE 3. Activities of nitrite and nitric oxide reductases in different strains of *P. aeruginosa*[a]

Strain	Mean sp act (nmol of N/min · mg) ± SD[b]	
	Nir	Nor
PAO1	354 ± 26	12 ± 1.0
PAO6261	<1.0	<0.16
PAO6261(pME3580)	296 ± 81	3 ± 0.3

[a] Strains of *P. aeruginosa* were grown in 150-ml serum bottles containing 80 ml of tryptic soy broth. The serum bottles were capped with stoppers after inoculation and were incubated at 37°C with shaking. Induction of denitrifying enzymes in the growing cultures occurred after the oxygen was exhausted. Enzymatic assays were performed as described previously (39), except that nitrite disappearance was measured for the nitrite reductase activity. The specific activity data are means and standard deviations of results from three replicate vials.

[b] Nir, nitrite reductase; Nor, nitric oxide reductase.

The Anr protein or its analogs may play similar roles in denitrifying bacteria other than *P. aeruginosa*. This hypothesis is supported by the presence of Fnr boxes in the promoter regions of nitrite reductase genes (2, 15, 29, 41), of nitric oxide and nitrous oxide reductase genes in *Pseudomonas stutzeri* (7, 45), of *azu* and pseudo-*azu* encoding possible electron donors in *Alcaligenes faecalis* (14), and of *nosR* and *nirQ* encoding two potential regulatory proteins in *P. stutzeri* (7, 16). This bacterium appears to have two *fnr*-like genes (8). By contrast, in *P. aeruginosa*, there is only one *fnr*-like gene, *anr*. This gene has now been shown to be required for the arginine deiminase pathway, hydrogen cyanide biosynthesis, and all steps in denitrification, suggesting its global role in regulating anaerobic metabolism.

Although the effects of N oxides on the activation of genes involved in denitrification have not been investigated in this study, other authors have found that the nitrite reductase genes of *P. aeruginosa* can be activated by nitrate under both anaerobic (1, 42) and aerobic (42) conditions. The latter results help explain some physiological studies which show denitrification activity under aerobic conditions in *P. aeruginosa* (9, 23). More extensive studies in O_2-controlled chemostats with other species of denitrifiers, i.e., *P. stutzeri* (18) and *Achromobacter cycloclastes* (6), have shown that all denitrifying enzymes assayed are detectable when both N oxides and low to intermediate levels of O_2 (depending on the enzyme) are present. These studies indicate that while anaerobiosis is not an absolute requirement for the synthesis of denitrifier enzymes, synthesis and especially pathway activity greatly increase as O_2 decreases. It can be concluded that in *P. aeruginosa*, full activation of the denitrification pathway requires both N oxide substrates and low O_2 levels, with Anr being the major and obligatory regulator. Anr has low but measurable activity under aerobic conditions in *P. aeruginosa* (38). The low level of denitrification activity observed in the presence of oxygen may thus be attributable to the residual activity of Anr. It is unknown, however, whether aerobic denitrification in *T. pantotropha* also depends on an Fnr-like regulator (4, 24).

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