Disruption of *narH*, *narJ*, and *moaE* Inhibits Heterotrophic Nitrification in *Pseudomonas* Strain M19

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Interruptions in three nitrate reductase-related genes, *narH*, *narJ*, and *moaE*, inhibited heterotrophic nitrification in *Pseudomonas* strain M19. No nitrate was detected in the medium, and nitrification proceeded in the presence of a nitrate reductase inhibitor. Heterotrophic nitrification was greatly stimulated by the addition of nitrate.

Nitrification is a key step in the global nitrogen cycle; it alters both the mobility and utilization potential of nitrogen in soil (21). The chemoautotrophic bacteria *Nitrosospira* and *Nitrobacter* are typically identified as the main agents of nitrification, but many heterotrophic bacteria and fungi can also nitrify (8, 13). The physiological role of heterotrophic nitrification is unknown (8). In addition, because many microorganisms are not amenable to traditional culturing techniques (2), the phylogenetic diversity and ecological significance of heterotrophic nitrifiers are not known. Knowledge of the molecular mechanisms of heterotrophic nitrification would facilitate cultivation-independent studies of these organisms and provide information about the physiological role of this process.

Reports of the molecular mechanisms of heterotrophic nitrification have presented either functional or sequence data, but not both. Moir et al. (14) used heterologous expression to show that the *Paracoccus denitrificans* ammonia monooxygenase consists of two subunits and is a quinol oxidase. Ono et al. (16) purified a 115-kDa pyruvic oxime dioxygenase containing three 40-kDa subunits from *Alcaligenes faecalis*. Other studies (11, 15) have reported the purification of hydroxylamine oxidase proteins from *Paracoccus denitrificans* and *Pseudomonas* strain PB16. None of these reports have provided corollary sequence data. Daum et al. (7) used Southern analysis and reported a weak hybridization of *Pseudomonas putida* genomic DNA with *Nitrosomonas europaea amoA*. They did not provide any functional data to demonstrate that this gene product was involved in heterotrophic nitrification. This is the first study that provides both functional and sequence analyses of genes involved in heterotrophic nitrification.

Bacterial strains and plasmids. *Pseudomonas* strain M19 was isolated with a glutamate enrichment from a tundra dry meadow soil at the Niwot Ridge long-term ecological research site in the front range of the Colorado Rocky Mountains. It was determined that M19 was a heterotrophic nitrifier by testing for the production of nitrite (see below) after growth in nitrification broth (22). A partial sequence of the 16S rRNA gene of *Pseudomonas* strain M19 was generated (accession number AF539808), and BLAST (1) searches revealed that this sequence is 99% identical to the 16S rRNA gene from *Pseudomonas fluorescens*.

Escherichia coli strain S17-1 carrying pJFF350 was kindly provided by Sven Valla. S17-1 has been described elsewhere (20) and features chromosomally encoded *tra* genes for conjugation. pJFF350 (9) carries the Omegon-Km transposon containing the *aphA* kanamycin resistance gene and an *E. coli* origin of replication.

Conjugation. Transposon mutagenesis was used to interrupt the genome of *Pseudomonas* strain M19 to identify genes required for heterotrophic nitrification. M19 was conjugated with S171-1/pJFF350 to mobilize the Omegon-Km transposon into M19. Approximately 4×10^8 of both the *E. coli* S171-1/pJFF350 donor cells and the M19 recipient cells were mixed on a 0.2-μm-pore-size filter. The cells were incubated on Luria-Bertani (LB) agar medium for 16 h at room temperature. The filter was resuspended in 10 ml of 0.9% NaCl, and 100-μl aliquots were plated on *Pseudomonas* agar B (2% peptone, 1% glycerol, 0.15% $MgSO_4 \cdot 7H_2O$, 0.15% K_2HPO_4, 1.5% agar) containing ampicillin (100 μg/ml) and chloramphenicol (5 μg/ml) (to select against S17-1/pJFF350) and kanamycin (100 μg/ml) (to select against M19). Approximately 9,500 recombinant colonies were screened for the lack of nitrification activity. Cells were grown in selective LB medium in deep 96-well plates at room temperature for 20 h, and cultures were tested for nitrite. Nitrite analysis was performed by a modified version of the method of Bundy and Meisinger (5). Briefly, 100 μl of diazotizing reagent (0.5 g of sulfanilamide in 100 ml of 600 mM HCl) and 100 μl of coupling reagent [0.3 g of *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml of 120 mM HCl] were added to 100-μl samples of the cultures. A pink color was considered a positive indication of the presence of nitrite. Three mutants that were incapable of nitrification, mt54, mt97, and mt105, were obtained.

Molecular techniques. Southern hybridization analysis was used to determine the number of transposon insertions in the mutant strains. DNA was extracted from the three mutants using Genomic-tips (Qiagen, Valencia, Calif.). Genomic DNA (750 ng) was digested with *Eco*RI (Promega, Madison, Wis.) and used for Southern hybridization by standard protocols

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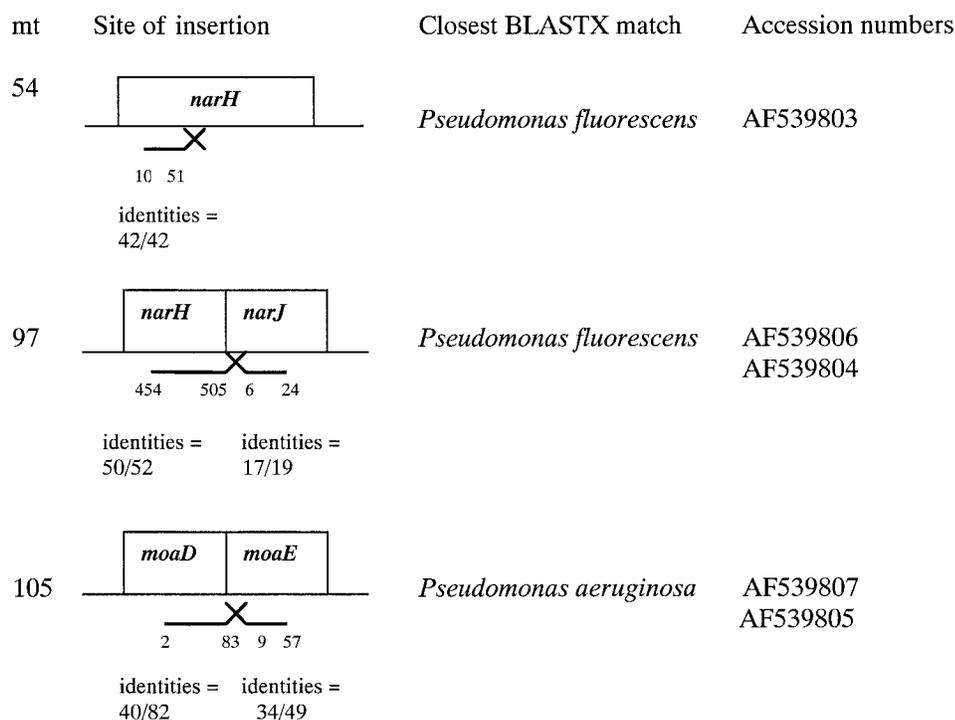
FIG. 1. Sites of transposon insertions in mt54, mt97, and mt105 mutants. The transposon insertion site (X) and sequenced areas (lines) are shown. The numbers below the lines are amino acid positions. Values for identities refer to amino acid sequences. The closest BLASTX match to the mutants and GenBank accession numbers are also shown.

(19). An *aphA* probe was generated using the PCR digoxigenin probe synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany) and the primers aphAF (5′-CAAGATGGATTGCACGCAGGT-3′) and aphAR (5′-GAACTCGTCAAGAAGGCGAT-3′) following the manufacturer's directions. For mutants mt54 and mt97, a single band of approximately 10 kb hybridized to the probe, signifying a single transposon insertion. For mutant mt105, two bands (approximately 12 and 9 kb) hybridized to the probe, suggesting that two transposons had integrated into the genome.

The Omegon Km-based *aphA* gene and *E. coli* origin of replication were used to generate plasmids containing the interrupted genes. Serial dilutions of *Eco*RI-digested genomic DNA were ligated using T4 DNA ligase (Promega) overnight at 16°C. For each mutant, we pooled the ligation dilution series and transformed the plasmids into TOP10 (Invitrogen, San Diego, Calif.) chemically competent cells following the manufacturer's directions. We plated the bacteria on LB containing kanamycin (100 μg/ml) to select for intramolecular ligations that contained the transposon-encoded origin of replication and *aphA* gene. For each mutant, 10 transformants were selected for analysis. Plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen) and were screened with *Eco*RI/*Bam*HI, *Eco*RI/*Apa*I, and *Eco*RI/*Sma*I (Promega) restriction digestion. For both mt54 and mt97, all 10 plasmids generated identical restriction patterns. For mutant mt105, two restriction patterns corresponded to the 12-kb *Eco*RI restriction fragment and the 9-kb *Eco*RI restriction fragment (data not shown).

Plasmids were sequenced using primers OmegonOri (5′-G

TCAGAGGTTTTCACCGTCATC-3′) and aphA2 (5′-GCTCACAGCCAAACTATC-3′) with the BigDye Terminator Cycle Sequencing kit version 2.0 (PE Biosystems, Foster City, Calif.) following the manufacturer's directions. Sequencing products were analyzed at the Iowa State University DNA Sequencing Facility. Sequences were edited with Sequencher 4.1 (Gene Codes Co., Ann Arbor, Mich.) and used to search GenBank with BLASTX (1). The transposons interrupted *narH* in mt54 and *narJ* in mt97. In mt105, one transposon interrupted a genomic region with no significant BLASTX matches and the other transposon interrupted *moaE* (Fig. 1). *Pseudomonas* strain M19 is closely related to *P. fluorescens*, and the interrupted *nar* genes were highly identical to *P. fluorescens* sequences (Fig. 1). No *moaE P. fluorescens* sequences are available in GenBank; therefore, the sequence identity for the closest BLAST match is not as high (Fig. 1).

All interrupted genes are required for the function of the molybdo-bis(molybdopterin guanine dinucleotide) (MobisMGD) containing membrane-bound nitrate reductase NAR (for a recent review, see reference 18). NarH is an Fe-S-containing electron transfer subunit (18). NarJ is a system-specific chaperone involved in the acquisition of the MobisMGD cofactor by the NarGH complex (4). MoaE is required for molybdopterin biosynthesis (17).

To rule out nitrate reduction as the source of nitrite, the medium was tested for the presence of nitrate. Nitrate analysis was performed by a reduction to nitrite; 200 μl of the medium was mixed with 1,000 μl of 0.1 M NH$_4$Cl, and the solution was passed over a copperized cadmium column. Nitrite was measured as described above. This assay is sensitive to 20 μM

TABLE 1. Nitrite accumulation in *Pseudomonas* strain M19 and mutant cells after growth for 68 h at room temperature

Strain	Amt (mM) of nitrite accumulated[a]			
	Peptone[b]	Ammonium[c]	Nitrate[d]	Nitrate + peptone[e]
M19	0.03 ± 0.00 (HETN)	0.02 ± 0.00 (HETN)	4.40 ± 0.43 (NAP/NAR)	17.16 ± 2.32 (HETN/NAP/NAR)
mt54	0	0	0.05 ± 0.00 (NAP)	0
mt97	0	0	0.04 ± 0.00 (NAP)	0
mt105	0	0	NG[f]	0

[a] Nitrite accumulated in cultures grown with nitrogen sources. Values are the mean $\pm$ standard deviation for three cultures. Proposed pathways for nitrite production are indicated in parentheses. Pathway abbreviations: HETN, heterotrophic nitrification; NAP, periplasmic nitrate reductase; NAR, membrane-bound nitrate reductase.
[b] Nitrification broth with peptone (22).
[c] Minimal medium with succinate and ammonium (10).
[d] Minimal medium with succinate (10) supplemented with 10 mM sodium nitrate.
[e] Nitrification broth with peptone (22) supplemented with 10 mM sodium nitrate.
[f] NG, no growth.

concentrations of nitrate, and no nitrate was detected in the medium. *Pseudomonas* strain M19 produced 100 μM concentrations of nitrite after 20 h in this medium at room temperature (data not shown). Thus, trace amounts of nitrate in the medium could not account for the production of nitrite. Furthermore, concentrations of sodium azide (3) that are inhibitory to membrane-bound nitrate reductase did not inhibit nitrite production in M19 (data not shown). It is possible that nitrogen was first oxidized to nitrate and then reduced to nitrite by the membrane-bound nitrate reductase. If this were the case, then nitrate would accumulate in the mutant strains in which the NAR activity was blocked. However, no nitrate accumulated in the mutant strains (data not shown). The periplasmic nitrate reductase NAP is also found in strains of *Pseudomonas* and is insensitive to sodium azide (18). However, this is also a Mo-bisMGD-containing enzyme that would be inactive in the *moaE* mutant, yet we detected no nitrate accumulation by this strain (data not shown). Taken together, these results imply that the nitrite production by M19 was not the result of nitrate reduction.

To further investigate the role of nitrate reductase genes in heterotrophic nitrification, nitrite accumulation was assayed after growth on different nitrogen sources with and without nitrate (Table 1). Heterotrophic nitrification was not specific to organic nitrogen; *Pseudomonas* strain M19 also produced nitrite when grown on ammonium, while mutants mt54, mt97, and mt105 were incapable of this activity (Table 1). M19 was also able to reduce nitrate; millimolar quantities of nitrite accumulated in these cultures. No nitrite accumulated in cultures of the mutants grown on nitrate supplemented with peptone. However, the *nar* mutants (mt54 and mt97) produced micromolar quantities of nitrite when grown on nitrate alone, suggesting that the NAP pathway was active. The *moaE* mutant was incapable of growth and nitrite production on nitrate alone (Table 1), implying that the NAR and NAP pathways were inactive. Nitrite production due to heterotrophic nitrification in cultures grown without nitrate was low; micromolar quantities accumulated in cultures supplemented with peptone or ammonium (Table 1). Interestingly, nitrate addition greatly enhanced heterotrophic nitrification, increasing nitrite accumulation from the micromolar to millimolar range in cultures grown with peptone. The nitrite accumulation in these cultures was much greater than the sum of nitrite yield from separate cultures supplemented with nitrate and peptone (Table 1).

Because the yield of nitrite was greater than the amount of nitrate in the medium, the difference must be due to an increase in heterotrophic nitrification rather than an increase in nitrate reduction.

Conclusions. Interruptions in the *narH*, *narJ*, and *moaE* genes all inhibit heterotrophic nitrification in *Pseudomonas* strain M19. It is possible that these gene products are involved in both nitrate reduction and nitrogen oxidation. Indeed, the processes of aerobic denitrification and heterotrophic nitrification are linked in many microbes (13). In addition, subunits of the *Nitrobacter hamburgensis* nitrite oxidoreductase, which catalyzes both nitrate reduction and nitrite oxidation, share sequence similarity with NarH and NarG (12). Furthermore, Crossman et al. (6) found that heterologous expression of nitrate reductase genes resulted in the production of nitrite even though no nitrate was added to the medium. These researchers provided no data to demonstrate that the quantities of nitrite produced could be explained by trace amounts of nitrate in the medium. These results suggest a role for nitrate reductase genes in heterotrophic nitrification. In addition, heterotrophic nitrification was stimulated in *Pseudomonas* strain M19 with the presence of nitrate. Nitrate reductase genes are induced by nitrate, probably increasing both their reductive and oxidative roles in M19. However, although *narH*, *narJ*, and *moaE* are required for nitrification, they are not sufficient to catalyze this reaction. Indeed, several bacteria, including *E. coli*, contain all of these genes and are not capable of heterotrophic nitrification. More work is needed to elucidate the mechanism and physiological role of heterotrophic nitrification.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
3. **Berks, B. C., S. J. Ferguson, J. W. B. Moir, and D. J. Richardson.** 1995. Enzyme and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. Biochim. Biophys. Acta **1232:**97–193.
4. **Blasco, F., J.-P. D. Santos, A. Magalon, C. Frixon, B. Guigliarelli, C.-L. Santini, and G. Giordano.** 1998. NarJ is a specific chaperone required for molybdenum cofactor assembly in nitrate reductase A of *Escherichia coli*. Mol. Microbiol. **28:**435–447.

5. **Bundy, L. G., and J. J. Meisinger.** 1994. Nitrogen availability indices, p. 951–984. *In* R. W. Weaver, S. Angle, P. Bottomley, D. Bezdicek, S. Smith, A. Tabatabai, and A. Wollum (ed.), Methods of soil analysis. Part 2. Microbiological and biochemical properties. Soil Science Society of America book series no. 5. Soil Science Society of America, Madison, Wis.

6. **Crossman, L. C., J. W. B. Moir, J. J. Enticknap, D. J. Richardson, and S. Spiro.** 1997. Heterologous expression of heterotrophic nitrification genes. Microbiology **143:**3775–3783.

7. **Daum, M., W. Zimmer, H. Papen, K. Kloos, K. Nawrath, and H. Bothe.** 1998. Physiological and molecular biological characterization of ammonia oxidation of the heterotrophic nitrifier *Pseudomonas putida*. Curr. Microbiol. **37:**281–288.

8. **De Boer, W., and G. A. Kowalchuk.** 2001. Nitrification in acid soils: microorganisms and mechanisms. Soil Biol. Biochem. **33:**853–866.

9. **Fellay, R., H. M. Krisch, P. Prentki, and J. Frey.** 1989. Omegon-Km: a transposable element designed for in vivo insertional mutagenesis and cloning of genes in Gram-negative bacteria. Gene **76:**215–226.

10. **Harms, N. E., G. E. de Vries, K. Maurer, E. Veltkamp, and A. H. Stouthamer.** 1985. Isolation and characterization of *Paracoccus denitrificans* mutants with defects in the metabolism of one-carbon compounds. J. Bacteriol. **164:**1064–1070.

11. **Jetten, M. S. M., P. de Bruijn, and J. G. Kuenen.** 1997. Hydroxylamine metabolism in *Pseudomonas* PB16: involvement of a novel hydroxylamine oxidoreductase. Antonie Leeuwenhoek **71:**69–74.

12. **Kirstein, K., and E. Bock.** 1993. Close genetic relationship between *Nitrobacter hamburgensis* nitrite oxidoreductase and *Escherichia coli* nitrate reductases. Arch. Microbiol. **160:**447–453.

13. **Kuenen, J. G., and L. A. Robertson.** 1988. Ecology of nitrification and denitrification, p. 161–218. *In* J. A. Cole and S. J. Ferguson (ed.), The nitrogen and sulphur cycles. Cambridge University Press, Cambridge, United Kingdom.

14. **Moir, J. W. B., L. C. Crossman, S. Spiro, and D. J. Richardson.** 1996. The purification of ammonia monooxygenase from *Paracoccus denitrificans*. FEBS Lett. **387:**71–74.

15. **Moir, J. W. B., J.-M. Wehrfritz, S. Spiro, and D. J. Richardson.** 1996. The biochemical characterization of a novel non-haem-iron hydroxylamine oxidase from *Paracoccus denitrificans* GB17. Biochem. J. **319:**823–827.

16. **Ono, Y., A. Enokiya, D. Masuko, K. Shoji, and T. Yamanaka.** 1999. Pyruvic oxime dioxygenase from the heterotrophic nitrifier *Alcaligenes faecalis*: purification and molecular and enzymatic properties. Plant Cell Physiol. **40:**47–52.

17. **Pitterle, D. M., and K. V. Rajagopalan.** 1989. Two proteins encoded at the *chlA* locus constitute the converting factor of *Escherichia coli chlA1*. J. Bacteriol. **171:**3373–3378.

18. **Richardson, D. J., B. C. Berks, D. A. Russell, S. Spiro, and C. J. Taylor.** 2001. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell. Mol. Life Sci. **58:**165–178.

19. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

20. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.

21. **Vitousek, P. M., J. R. Gosz, C. C. Grier, J. M. Melillo, and W. A. Reiners.** 1982. A comparative analysis of potential nitrification and nitrate mobility in forest ecosystems. Ecol. Monogr. **52:**155–177.

22. **White, J. P., and G. T. Johnson.** 1982. Aflatoxin production correlated with nitrification in *Aspergillus-flavus* group species. Mycologia **74:**718–723.