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Nitrite Reductase of *Nitrosomonas europaea* Is Not Essential for Production of Gaseous Nitrogen Oxides and Confers Tolerance to Nitrite

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A gene that encodes a periplasmic copper-type nitrite reductase (NirK) was identified in *Nitrosomonas europaea*. Disruption of this gene resulted in the disappearance of Nir activity in cell extracts. The nitrite tolerance of NirK-deficient cells was lower than that of wild-type cells. Unexpectedly, NirK-deficient cells still produced nitric oxide (NO) and nitrous oxide (N_2O), the latter in greater amounts than that of wild-type cells. This demonstrates that NirK is not essential for the production of NO and N_2O by *N. europaea*. Inactivation of the putative *fnr* gene showed that Fnr is not essential for the expression of *nirK*.

Oxidation of ammonia (NH_3) to nitrite (NO_2^-) is the first step in the nitrification process, which plays an essential role in the global nitrogen cycle. Nitrification involves the oxidation of NH_3, via NO_2^-, to nitrate (NO_3^-) by the combined actions of NH_3- and NO_2^--oxidizing bacteria. The NH_3-oxidizing bacteria are lithoautotrophs that harvest their vital free energy in the oxidation of NH_3 to NO_2^-. This process is carried out by the sequential actions of membrane-bound ammonia monooxygenase, which oxidizes NH_3 to hydroxylamine (NH_2OH), and periplasmic hydroxylamine oxidoreductase (HAO), which oxidizes NH_2OH to NO_2^- (24). NH_3-oxidizing bacteria utilize molecular oxygen both for the formation of NH_2OH from NH_3 and as the terminal acceptor of electrons from the respiratory chain (24). Alternatively, NO_2^- and nitric oxide (NO) may also be used as terminal electron acceptors via a putative denitrification pathway. Homologues of genes encoding the denitrification enzymes nitrite reductase (Nir) and nitric oxide reductase (Nor) are present in the genome of the NH_3-oxidizing bacterium *N. europaea* (22).

NH_3-oxidizing bacteria produce NO, nitrous oxide, and, in some cases, N_2 during growth on NH_3. The mechanisms that underlie the production of these nitrogenous gases include the dissimilatory reduction of NO_2^- (15–17). An enzyme with Nir activity has been isolated from *N. europaea* (3, 6, 12, 13, 18). The ability of *N. europaea* to use NO_2^- as an alternative electron acceptor suggests that the pathway may act as an alternative mode of respiration, as in the "true" denitrifying bacteria (1, 19). Alternatively, the denitrifying enzymes may serve to protect NH_3-oxidizing bacteria from the negative effects of NO_2^- produced during growth (16, 20). On the other hand, the nitrification pathway may also be involved in the production of NO and N_2O by NH_3-oxidizing bacteria. HAO of *N. europaea* has been shown to produce NO and N_2O during the oxidation of NH_2OH in vitro (6, 8). Whether HAO also contributes to the production of these gases in vivo is unclear

We disrupted the putative *nirK* gene of *N. europaea* to learn whether it encodes a functional Nir and to study the effects of mutagenesis of this gene on (i) the production of NO and N_2O and (ii) the tolerance of the cells toward NO_2^-.

Analysis of the *nirK* gene and adjacent loci. Preliminary sequence data were obtained from The DOE Joint Genome Institute (JGI) (http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html). An open reading frame (ORF) with homology to genes encoding copper-type Nir enzymes is present in *N. europaea*. This ORF, which we have designated *nirK*, is 930 bp in length and translates into a polypeptide of 309 amino acid residues. An alignment revealed that NirK of *N. europaea* is significantly shorter than other characterized Cu-type Nir proteins (about 50 N-terminal residues). The closest relative of the NirK of *N. europaea* characterized thus far is the outer membrane copper-type Nir of *Neisseria gonorrhoeae*, the precursor of which contains a prokaryotic membrane lipoprotein attachment site (11). However, analysis of the N terminus of NirK of *N. europaea* with the SignalP algorithm (http://www.cbs.dtu.dk/services/SignalP/) predicted the presence of a periplasmic target sequence, suggesting that this protein resides in the periplasm.

The *nirK* gene is clustered with three other ORFs in the genome of *N. europaea* (Fig. 1). ORF 1 translates into a protein of which the predicted N terminus of the mature form matched that of a previously isolated soluble blue copper oxidase that we sequenced in this study (EKREFDLSIEDTRIVLVGKRDFHTFAFNGQVPAPLIHVM) (3). ORFs 2 and 3 encode periplasmic c-heme-containing polypeptides that have been characterized by Whittaker and coworkers (22).

Biochemical characterization and complementation of a NirK-deficient mutant. *N. europaea* strain ATCC 19178 and NirK-deficient mutant XLnt (ATCC 19178 derivative; *nirK*::pNIRsu [this study]) were grown in liquid medium as

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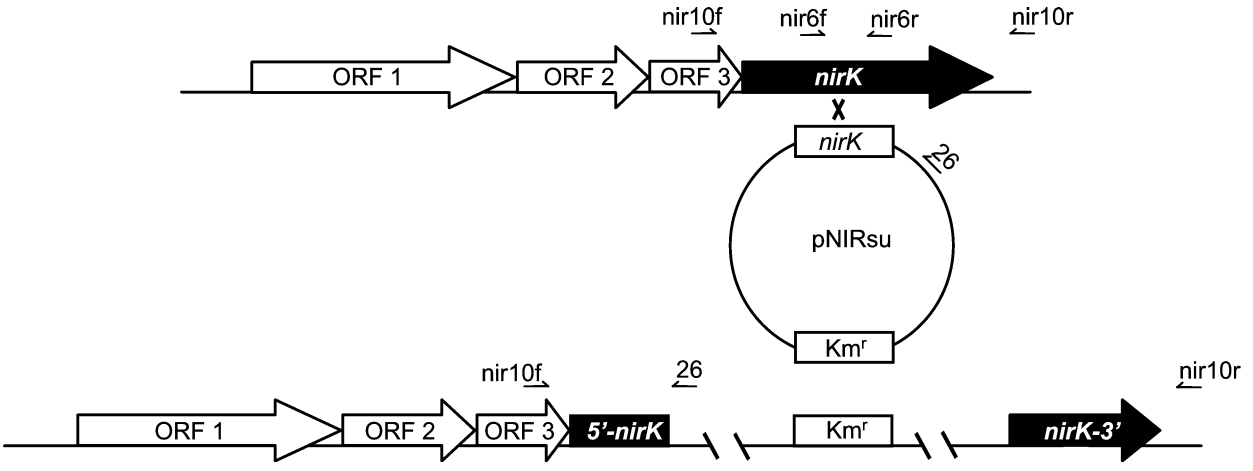

FIG. 1. Schematic maps of the *nirK* gene cluster in wild-type *N. europaea* (top) and in the NirK-deficient strain (bottom), the *nirK* gene of which was disrupted by integration of suicide vector pNIRsu via homologous recombination. The small arrows indicate the primers used for amplification of the internal *nirK* gene fragment (nir6f and nir6r) and the complete *nirK* gene (nir10f and nir10r) and for verification of the correct integration of suicide vector pNIRsu in the *nirK* gene (nir10f and 26). The PCR with primers nir10f and nir10r was specific for the wild-type organization.

described by Hyman and Arp (9) and on solid medium as described by Hommes et al. (5). Plasmids were transferred from *Escherichia coli* to *N. europaea* by means of conjugation by the method of T. Iizumi (personal communication). An internal fragment of the *nirK* gene was obtained by PCR and cloned into suicide vector pRVS3 (21). The resulting vector, pNIRsu, was transferred to wild-type cells of *N. europaea* (Fig. 1). Integration of this construct into the chromosomal copy of the *nirK* gene by homologous recombination resulted in the disruption of this gene. The correctness of the integration was confirmed by PCR (Fig. 1). Nir activity in cell extracts and periplasmic protein extracts was assayed with NH_2OH as an electron donor as described by Hooper (6). Nir activity was present in the extracts from wild-type cells but not in those from NirK-deficient cells (Table 1). The NirK-deficient mutant was complemented by the insertion of a broad-host-range vector (pEG400 [4]) that contained the *N. europaea nirK* gene under the control of the kanamycin acetyltransferase gene promoter. Presumably because the kanamycin acetyltransferase gene promoter was less active than the wild-type *nirK* promoter, the Nir activity in the periplasmic protein extract of this strain was only partially restored (to 0.29 mmol min^{-1} g of $protein^{-1}$). This assumption is corroborated by the relative amounts of NirK in extracts from wild-type and complemented NirK-deficient cells as visualized by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis discussed below (Fig. 2).

SDS-PAGE analyses of periplasmic protein fractions (prepared as described by Witholt et al. [23]) revealed a protein band of the size that was predicted for the NirK protein (31 kDa) in the wild-type pattern that was absent in that of the mutant (Fig. 2). Quadrupole time of flight mass spectrometer (Q-TOF-MS) analyses of the mass spectrum and amino acid sequence of this protein band, as obtained from the soluble protein fraction of wild-type cells, revealed that it contained the *nirK* gene product. All of the sequenced fragments were 100% identical to the predicted NirK sequence (four fragments, 56 residues sequenced). One of the analyzed NirK fragments had the sequence KTVQVTLHAVETDVAYDNK. Apparently, the N-terminal lysine was retained during the digestion with trypsin due to inefficient cleavage. The lack of additional amino acids upstream of this lysine residue, together with the absence of a trypsin site adjacent to the lysine, demonstrates that the lysine residue is the N-terminal amino acid of the mature NirK protein and that the signal peptide (MYLIYTKRTVFMKNSISLFSSYRFTHIILMLIVLALIPLT SQA) is cleaved as we predicted. This was in accordance with the finding that Nir activity resides in the periplasmic space.

Production of NO and N_2O. The concentrations of NO and N_2O were measured in the headspace of batch cultures that were in the early stationary phase of growth. These cultures were incubated in 150 ml of medium in 500-ml bottles that were sealed with rubber stoppers and incubated on an angled (70°) rotary shaker (175 rpm) at 30°C in the dark. The NO concentrations measured in the headspace gas of cultures of wild-type and NirK-deficient cells with an NO_x analyzer were

TABLE 1. Biochemical and physiological properties of wild-type and NirK-deficient cells of *N. europaea*

N. europaea strain	Nitrate reductase activity (mmol min^{-1} g of $protein^{-1}$)[a]	N_2O concn (mM)[b]	N_2O production rate [μmol h^{-1} (OD · ml)$^{-1}$][c]	O_2 uptake rate [mmol min^{-1} (OD · ml)$^{-1}$][d]
Wild type	1.33	0.11	0.27	0.50
NirK deficient	0.00	0.30	1.12	0.40

[a] Measured in periplasmic protein fractions.
[b] Measured in the headspace of cultures in the early stationary phase of growth.
[c] Measured in the headspace of exponentially growing cultures. OD, optical density.
[d] Measured with a Clark-type electrode in the cultures used for measurement of the N_2O production rate.

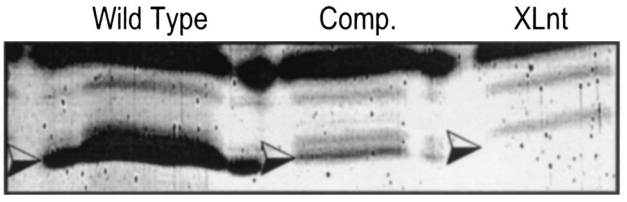

FIG. 2. SDS-PAGE of periplasmic protein fractions. WT, wild type; Comp., complemented NirK deficient; XLnt, NirK deficient. The arrowheads indicate the position of the NirK protein band, which is not present in the extract from NirK-deficient cells.

on the same order of magnitude, around 5 μM. NO production rates were also monitored by using oxygenated hemoglobin, which reacts rapidly with NO, resulting in a shift in the UV-visible light absorption spectrum of hemoglobin (14). When headspace gas from either wild-type or NirK-deficient cell cultures was led through an oxygenated hemoglobin solution, this shift occurred at similar rates, illustrating that the NO production rates of the two strain were similar.

The N_2O concentration in the headspace of batch cultures of wild-type and NirK-deficient cells was measured with a gas chromatograph (Table 1). Remarkably, the concentration of N_2O in the headspace of the NirK-deficient cultures was approximately threefold higher than that in the headspace of the wild-type cell cultures. Next, equal amounts of exponentially growing cells in liquid cultures were transferred to new flasks (50 ml) that were sealed with rubber stoppers and incubated at 30°C for 50 min with continuous shaking (175 rpm). Subsequently, samples were taken from the headspace at 10-min intervals for measurement of the N_2O concentration. The NirK-deficient cells produced N_2O at a rate that was approximately four times greater than that of the wild-type cells (Table 1). The NH_3-dependent oxygen uptake rates of the cultures used in this experiment were found to be similar, indicating that the respiratory potentials of the wild-type and NirK-deficient cells were comparable (Table 1).

Growth characteristics of the NirK-deficient mutant. The growth characteristics of wild-type and NirK-deficient cells of *N. europaea* were determined by measurement of the time-dependent increase in turbidity of aerobic batch cultures (Fig. 3a and b). The cultures (150 ml) were incubated in 500-ml flasks (with semiloose caps to facilitate gas exchange) on an angled (70°) rotary shaker (175 rpm) at 30°C in the dark. The specific growth rates of wild-type and NirK-deficient cells were similar, approximately 0.1 h^{-1}. The NirK-deficient cells reached a maximal biomass concentration that was approximately 90% of that of the wild-type cells.

Effect of NO_2^- on growth. Involvement of NirK in the tolerance of cells of *N. europaea* toward NO_2^-, which can be toxic to the cells (20), was assessed by examination of the growth characteristics of wild-type and NirK-deficient cells in a series of batch cultures to which increasing amounts of NO_2^- were added at the start of culturing (Fig. 3a and b). Culturing conditions were as described above. The addition of increasing amounts of NO_2^- had increasing negative effects on the specific growth rate and maximal biomass concentration of cultures of wild-type and NirK-deficient cells. However, these effects were more profound for the NirK-deficient cells than for the wild-type cells at each given NO_2^- concentration. At the highest concentration tested (100 mM), wild-type cells were still capable of growth while NirK-deficient cells were not. This demonstrates that NirK confers tolerance to NO_2^-. The concentration of NO_2^- in the growth medium was measured during growth of the cultures to which 0 and 10 mM NO_2^- had been added (Fig. 3c). Under both conditions, the concentration of NO_2^- in the cultures of wild-type cells exceeded those in the medium of the NirK-deficient cells at all measured points. It is likely that this was due to the increased negative effects of NO_2^- on the growth of NirK-deficient cells. This observation indicates that the mechanism by which NirK reduces the negative effects of NO_2^- on growth does not involve lowering of the amount of extracellular NO_2^- to which the cells were exposed.

Role of Fnr in regulation of the *nirK* gene. The genome of *N. europaea* contains a gene that encodes a protein with a high degree of homology to transcription activators that belong to the Fnr/Crp family. Fnr of *N. europaea* contains the cysteine

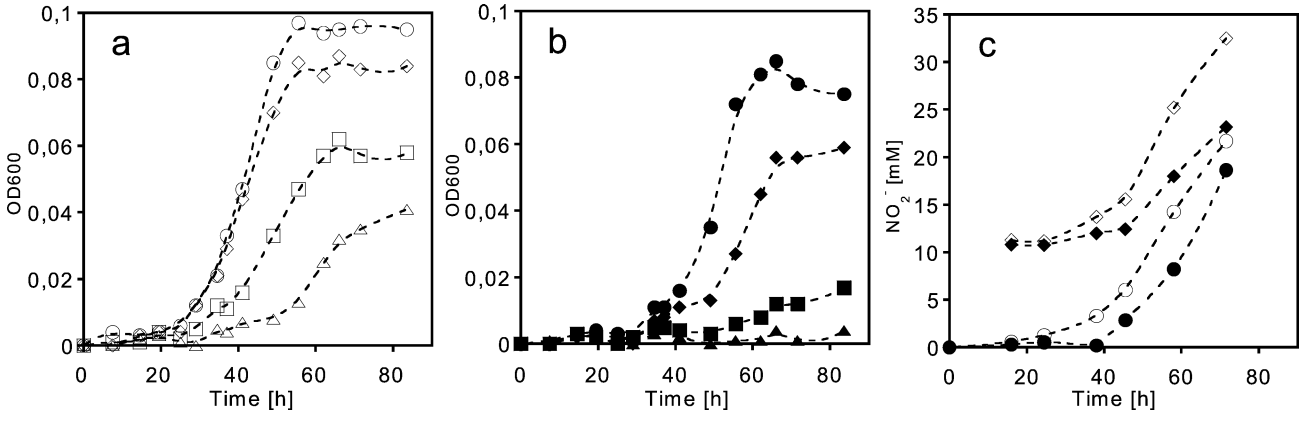

FIG. 3. Growth curves of wild-type (a) and NirK-deficient (b) cells of *N. europaea* incubated in aerobic batch cultures with 0 mM (circles), 10 mM (diamonds), 50 mM (squares), and 100 mM (triangles) $NaNO_2$ present in the growth medium at the start of the incubation. OD600, optical density at 600 nm. (c) Concentrations of NO_2^-, measured in a comparable experiment, in the growth medium of batch cultures of wild-type (open symbols) and NirK-deficient (filled symbols) cells of *N. europaea* with 0 mM (circles) and 10 mM (diamonds) $NaNO_2$ present in the medium at the start of the incubation. All experiments were done in triplicate, and representative curves are shown.

residues that align with those in *E. coli* Fnr and which are involved in the ligation of the oxygen-sensing [4Fe-4S] cluster. Fnr is involved in control of the expression of the denitrification enzymes in various bacteria (10, 25). For this reason, we constructed and analyzed an Fnr-deficient strain. NirK was still expressed in this mutant, which demonstrates that the putative Fnr of *N. europaea* is not essential for transcription of the *nirK* gene.

Conclusions. *N. europaea* possesses a gene that encodes a functional copper-type NirK that resides in the periplasmic space. Based on the findings presented here, we conclude that NirK is essential for the production of neither NO nor N_2O in *N. europaea*. The absence of NirK resulted in a decreased tolerance to NO_2^-, indicating that it may serve to protect the cell from the negative effects of this product of NH_3 oxidation. A model of the respiratory network of *N. europaea* that encompasses a linear denitrification pathway comprising NirK and Nor predicts that absence of NirK would render the system incapable of the production of NO and N_2O. Clearly, *N. europaea* has an alternative pathway for the production of these gases. Since HAO has been demonstrated to produce NO and N_2O in vitro, this key enzyme of the nitrifying pathway is also likely to be involved in this alternative pathway in vivo (6, 7).

The finding of *nirK* gene homologues in oceanic NH_3-oxidizing bacteria illustrates that NirK is not unique to *N. europaea* but appears to be widespread among the group of NH_3-oxidizing bacteria (2).

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REFERENCES

1. **Abeliovich, A., and A. Vonshak.** 1992. Anaerobic metabolism of *Nitrosomonas europaea*. Arch. Microbiol. **158:**267–270.
2. **Casciotti, K. L., and B. B. Ward.** 2001. Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **67:**2213–2221.
3. **DiSpirito, A. A., L. R. Taaffe, J. D. Lipscomb, and A. B. Hooper.** 1985. A 'blue' copper oxidase from *Nitrosomonas europaea*. Biochim. Biophys. Acta **827:**320–326.
4. **Gerhus, E., P. Steinrucke, and B. Ludwig.** 1990. *Paracoccus denitrificans* cytochrome c_1 gene replacement mutants. J. Bacteriol. **172:**2392–2400.
5. **Hommes, N. G., L. A. Sayavedra-Soto, and D. J. Arp.** 1996. Mutagenesis of hydroxylamine oxidoreductase in *Nitrosomonas europaea* by transformation and recombination. J. Bacteriol. **178:**3710–3714.
6. **Hooper, A. B.** 1968. A nitrite-reducing enzyme from *Nitrosomonas europaea*. Preliminary characterization with hydroxylamine as electron donor. Biochim. Biophys. Acta **162:**49–65.
7. **Hooper, A. B., K. R. Terry, and P. C. Maxwell.** 1977. Hydroxylamine oxidoreductase of *Nitrosomonas*. Oxidation of diethyldithiocarbamate concomitant with stimulation of nitrite synthesis. Biochim. Biophys. Acta **462:**141–152.
8. **Hooper, A. B., T. Vannelli, D. J. Bergmann, and D. M. Arciero.** 1997. Enzymology of the oxidation of ammonia to nitrite by bacteria. Antonie Van Leeuwenhoek **71:**59–67.
9. **Hyman, M. R., and D. J. Arp.** 1992. $^{14}C_2H_2$- and $^{14}CO_2$-labeling studies of the de novo synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. J. Biol. Chem. **267:**1534–1545.
10. **Lissenden, S., S. Mohan, T. Overton, T. Regan, H. Crooke, J. A. Cardinale, T. C. Householder, P. Adams, C. D. O'Conner, V. L. Clark, H. Smith, and J. A. Cole.** 2000. Identification of transcription activators that regulate gonococcal adaptation from aerobic to anaerobic or oxygen-limited growth. Mol. Microbiol. **37:**839–855.
11. **Mellies, J., J. Jose, and T. F. Meyer.** 1997. The *Neisseria gonorrhoeae* gene *aniA* encodes an inducible nitrite reductase. Mol. Gen. Genet. **256:**525–532.
12. **Miller, D. J., and D. J. D. Nicholas.** 1985. Characterization of a soluble cytochrome oxidase/nitrite reductase from *Nitrosomonas europaea*. J. Gen. Microbiol. **131:**2851–2854.
13. **Miller, D. J., and P. M. Wood.** 1983. The soluble cytochrome oxidase of *Nitrosomonas europaea*. J. Gen. Microbiol. **129:**1645–1650.
14. **Murphy, M. E., and E. Noack.** 1994. Nitric oxide assay using hemoglobin method. Methods Enzymol. **233:**240–250.
15. **Poth, M.** 1986. Dinitrogen production from nitrite by a *Nitrosomonas* isolate. Appl. Environ. Microbiol. **52:**957–959.
16. **Poth, M., and D. D. Focht.** 1985. ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. Appl. Environ. Microbiol. **49:**1134–1141.
17. **Remde, A., and R. Conrad.** 1990. Production of nitric oxide in *Nitrosomonas europaea* by reduction of nitrite. Arch. Microbiol. **154:**187–191.
18. **Ritchie, G. A., and D. J. Nicholas.** 1974. The partial characterization of purified nitrite reductase and hydroxylamine oxidase from *Nitrosomonas europaea*. Biochem. J. **138:**471–480.
19. **Schmidt, I., and E. Bock.** 1997. Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. Arch. Microbiol. **167:**106–111.
20. **Stein, L. Y., and D. J. Arp.** 1998. Loss of ammonia monooxygenase activity in *Nitrosomonas europaea* upon exposure to nitrite. Appl. Environ. Microbiol. **64:**4098–4102.
21. **van Spanning, R. J., A. P. De Boer, D. J. Slotboom, W. N. M. Reijnders, and A. H. Stouthamer.** 1995. Isolation and characterization of a novel insertion sequence element, IS1248, in *Paracoccus denitrificans*. Plasmid **34:**11–21.
22. **Whittaker, M., D. Bergmann, D. Arciero, and A. B. Hooper.** 2000. Electron transfer during the oxidation of ammonia by the chemolithotrophic bacterium *Nitrosomonas europaea*. Biochim. Biophys. Acta **1459:**346–355.
23. **Witholt, B., H. van Heerikhuizen, and L. de Lije.** 1976. How does lysozyme penetrate trough the bacterial outer membrane? Biochim. Biophys. Acta **443:**534–544.
24. **Wood, P. M.** 1986. Nitrification as bacterial energy source, p. 39–62. *In* J. I. Prosser (ed.), Nitrification. Society for General Microbiology, IRL Press, Oxford, United Kingdom.
25. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.