Nitrite Reductase NirS Is Required for Type III Secretion System Expression and Virulence in the Human Monocyte Cell Line THP-1 by *Pseudomonas aeruginosa*[▽]

Nadine E. Van Alst,[1] Melanie Wellington,[2] Virginia L. Clark,[1] Constantine G. Haidaris,[1] and Barbara H. Iglewski[1]*

Department of Microbiology and Immunology[1] and Department of Pediatrics,[2] University of Rochester Medical Center, Rochester, New York 14642

The nitrate dissimilation pathway is important for anaerobic growth in *Pseudomonas aeruginosa*. In addition, this pathway contributes to *P. aeruginosa* virulence by using the nematode *Caenorhabditis elegans* as a model host, as well as biofilm formation and motility. We used a set of nitrate dissimilation pathway mutants to evaluate the virulence of *P. aeruginosa* PA14 in a model of *P. aeruginosa*-phagocyte interaction by using the human monocytic cell line THP-1. Both membrane nitrate reductase and nitrite reductase enzyme complexes were important for cytotoxicity during the interaction of *P. aeruginosa* PA14 with THP-1 cells. Furthermore, deletion mutations in genes encoding membrane nitrate reductase (Δ*narGH*) and nitrite reductase (Δ*nirS*) produced defects in the expression of type III secretion system (T3SS) components, extracellular protease, and elastase. Interestingly, exotoxin A expression was unaffected in these mutants. Addition of exogenous nitric oxide (NO)-generating compounds to Δ*nirS* mutant cultures restored the production of T3SS phospholipase ExoU, whereas nitrite addition had no effect. These data suggest that NO generated via nitrite reductase NirS contributes to the regulation of expression of selected virulence factors in *P. aeruginosa* PA14.

The ubiquitous gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen responsible for both acute and chronic infections. *P. aeruginosa* is commonly an etiologic agent in ear infections (8), infections of burn wounds (27), corneal keratitis (21, 30), and pulmonary infections in patients with cystic fibrosis (16) and ventilator-associated pneumonia (5). *P. aeruginosa* is frequently resistant to conventional antibiotic therapy and the antimicrobial effector mechanisms of phagocytes (9), particularly in the biofilm mode of growth (15).

The establishment of *P. aeruginosa* infection is accompanied by the synthesis of a diverse array of virulence factors composed of various exoproteins, such as elastase and exotoxin A, as well as the mucoid exopolysaccharide alginate (20). The type III secretion system (T3SS), a mechanism whereby cytotoxic effector proteins are directly secreted into the host cell cytoplasm following contact of the bacterium with a target cell (11), has also been identified as a virulence determinant of *P. aeruginosa* that contributes significantly to the pathogenesis of acute infection (45, 46). The *P. aeruginosa* T3SS plays a major role in triggering cell death in phagocytes and epithelial cells (17) and is composed of at least 20 proteins, including (i) a secretory apparatus, (ii) machinery devoted to the direct translocation of effectors into the host cell cytoplasm, and (iii) four effector proteins, ExoS, ExoT, ExoU, and ExoY, that contribute to host cell toxicity (19, 46). Regulation of virulence factor expression in *P. aeruginosa* is hierarchical and highly complex, involving quorum sensing (37, 44) and responses to environmental signals, including stresses applied via host defense mechanisms (46).

P. aeruginosa is capable of growing anaerobically by using nitrate as a terminal electron acceptor through the denitrification pathway. The pathway and the operons (47) encoding the enzymatic steps are shown in Fig. 1. The pathway intermediate nitric oxide (NO) is highly reactive with iron-containing prosthetic groups of proteins such as heme and Fe-S clusters (18). NO detoxification by NO reductase during anaerobic growth is therefore important for maintenance of cell function. In addition, NO is a potent antimicrobial effector produced by phagocytes (4), and *P. aeruginosa* flavohemoglobin has an important role in NO detoxification under aerobic conditions (1). Hence, *P. aeruginosa* must control NO toxicity produced both internally and by the host in order to survive under different environmental conditions.

NO is also widely appreciated as an important signaling molecule in many biological systems (7). We recently demonstrated roles for membrane nitrate reductase and nitrite reductase, the first two enzymes in the denitrification pathway, in swarming motility, biofilm formation, and virulence in the surrogate nematode host *Caenorhabditis elegans* (43). These processes were assayed under aerobic conditions, indicating that the roles played by nitrate reductase and nitrite reductase were distinct from their contribution to the bioenergetics of *P. aeruginosa* anaerobic growth (43). The finding that similar phenotypes were displayed by a nitrate reductase (Δ*narGH*) mutant and a nitrite reductase (Δ*nirS*) mutant suggested that nitrite or NO served as a signaling molecule in these processes, since downstream NO reductase and nitrous oxide reductase mutants behaved comparably to wild-type *P. aeruginosa*.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Box 672, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642. Phone: (585) 275-3402. Fax: (585) 473-9573. E-mail: bigl@mail.rochester.edu.
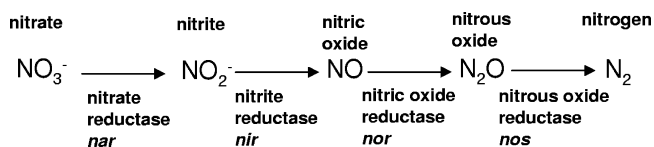
FIG. 1. Schematic of the denitrification pathway in *P. aeruginosa*. The representative operons are in italics.

In this study, we used a set of denitrification pathway mutants to evaluate the virulence of *P. aeruginosa* PA14 in a model of phagocyte interaction by using the human monocytic cell line THP-1 (41). We show that both membrane nitrate reductase and nitrite reductase enzyme complexes are important for virulence during the interaction of *P. aeruginosa* PA14 with THP-1 cells. Furthermore, the Δ*narGH* and Δ*nirS* deletion mutants were defective in the expression of T3SS components, extracellular protease, and elastase. Interestingly, exotoxin A expression was unaffected in these mutants. Addition of exogenous NO-generating compounds to Δ*nirS* mutant cultures restored production of T3SS phospholipase ExoU production, whereas nitrite addition had no effect. These data provide genetic and biochemical evidence indicating that NO generated via nitrite reductase contributes to the regulation of expression of selected virulence factors in *P. aeruginosa* PA14.

MATERIALS AND METHODS

Strains. *P. aeruginosa* strain PA14 (31) served as the parental strain for all studies. Mutant strains derived from PA14 are described in Table 1. For experiments analyzing exotoxin A production, *P. aeruginosa* PA103 was used as a positive control and *toxA* mutant PA103-29 (28) was used as a negative control.

***P. aeruginosa*–THP-1 cell coculture system.** *P. aeruginosa*-phagocyte interactions were performed with the human monocytic cell line THP-1 (41). THP-1 cells (American Type Culture Collection, Manassas, VA) were routinely cultured in RPMI 1640 tissue culture medium supplemented with 1% heat-inactivated fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 2.5 g/liter glucose, 0.05 mM β-mercaptoethanol, penicillin-streptomycin, and GlutaMAX (Invitrogen Corp.). *P. aeruginosa* strains were grown overnight in LB medium and washed twice with phosphate-buffered saline. In a total volume of 100 μl of RPMI 1640 tissue culture medium supplemented with 1% heat-inactivated fetal bovine serum, 4×10^5 unstimulated THP-1 cells were mixed with 4×10^4 bacteria (multiplicity of infection [MOI] of 0.1) in 1-ml round-bottom culture tubes at 37°C. In some experiments, the MOI was increased to 1.0 or 10, as indicated in the figures. Organisms and THP-1 cells were brought together by spinning the tubes at $500 \times g$ for 7 min, after which they were cocultured at 37°C for various intervals of time. Immediately after the mixing of bacteria with THP-1

cells and at 2, 4, and 6 h of incubation, 650 μl sterile distilled H_2O was added to the tube, the tube contents were vortexed vigorously to lyse host cells, and aliquots were plated on LB agar to quantitate CFU. CFU were determined after 24 h of incubation at 37°C. Data are expressed as CFU per milliliter. In the absence of host cells, all bacterial strains grew to approximately 1×10^7 to 3×10^7 CFU/ml after 6 h of incubation (data not shown).

Cytotoxicity assay. The cytotoxicity of *P. aeruginosa* for THP-1 cells was determined by measuring lactate dehydrogenase (LDH) release following coculture. At an MOI of either 0.1 or 1.0, as indicated in the figures, strains were combined with THP-1 cells in the wells of an opaque 96-well microtiter dish with 4×10^5 THP-1 cells per well and a final volume of 100 μl per well. Organisms and THP-1 cells were brought together by spinning the plates at $500 \times g$ for 7 min, after which they were cocultured for 6 h at 37°C. After coculture, LDH release was measured by adding the CytoTox-One Homogeneous Membrane Integrity Assay reagent (Promega) in accordance with the manufacturer's instructions. As a negative control, THP-1 cells were incubated in the absence of *P. aeruginosa* (described as "untreated" [see Fig. 3]). In addition, wells containing only *P. aeruginosa* were included in each assay. LDH release in experimental samples was calculated as a percentage of the LDH released by uninfected THP-1 cells that had been lysed with the Triton X-100 provided in the kit.

Induction of the T3SS in axenic culture and immunodetection of secreted proteins. In accordance with the protocol of Lee et al. (23), all strains were grown overnight in LB broth, subcultured at a dilution of 1:1,000 in LB broth supplemented with 5 mM EGTA to chelate calcium and promote secretion of T3SS exoproducts, and grown at 37°C for 6 h with aeration. Cell densities were determined by measuring optical density at 660 nm (OD_{660}), and culture supernatants were collected. The final OD_{660} was approximately 0.7 to 0.8. Proteins from the supernatant were precipitated with 10% trichloroacetic acid and 0.02% sodium deoxycholate. Pellets were washed with acetone to remove detergent and lipids. Protein precipitates were solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer in a volume normalized to the cell density based on the OD_{660} of each culture and fractionated on a 12% SDS-polyacrylamide gel. Proteins were either stained with Coomassie blue or transferred to nitrocellulose for detection by Western blotting with monoclonal antibodies (MAbs) against either phospholipase ExoU or translocator protein PcrV or with rabbit polyclonal antiserum against bifunctional type III cytotoxin ExoT (generously provided by Dara W. Frank, Milwaukee, WI). In some experiments, the EGTA-treated LB medium was supplemented with the exogenous NO donor *S*-nitrosoglutathione (GSNO) (1, 3), sodium nitroprusside dehydrate (SNP) (1), or 2,2′-(hydroxynitrosohydrazono)bis-ethanimine (DETA-NO) (6) or with sodium nitrite. GSNO, SNP, DETA-NO, or sodium nitrite was added to cultures in EGTA-treated LB broth 1 h prior to the harvesting of proteins from the culture supernatant (10). All chemicals were from Sigma-Aldrich.

Measurement of NO concentrations. NO concentrations were measured with an Apollo 4000 Free Radical Analyzer with ISO NOP sensors (World Precision Instruments, Sarasota, FL). A standard curve for the conversion of picoamperes of electrical current to NO concentrations was generated by the addition of a range of concentrations of potassium nitrite (World Precision Instruments) to 0.1 M KI in 0.1 M H_2SO_4 (acidified KI), in accordance with the manufacturer's instructions. All standard curves and enzyme reactions were performed in temperature-controlled reaction vessels (World Precision Instruments) at a constant temperature of 37°C (6). Wild-type and Δ*nirS* mutant strain PA14 bacteria were

TABLE 1. Strains used in this study

Strain	Description	Reference(s)
PA14	Parental wild type	31
PA14 Δ*narGH*	Membrane nitrate reductase polar deletion mutant	43
PA14 Δ*nirS*	Nitrite reductase polar deletion mutant	43
Complemented PA14 Δ*narGH*	Δ*narGH*::mini-Tn7-*narX′K1K2GHJI*; insertion of wild-type copy of *narX′K1K2GHJI* downstream of *glmS* site in membrane nitrate reductase mutant	43
Complemented PA14 Δ*nirS*	Δ*nirS* complemented with cosmid pMO011424 containing entire *nir* operon; Tet^r Kan^r	32, 43
PA14 MAR2xT7::norB	NO reductase mutant; Gm^r	24
PA14 MAR2xT7::*nosZ*	Nitrous oxide reductase mutant; Gm^r	24
PA14 MAR2xT7::pcrV	T3SS mutant deficient in proper translocation of effector molecules; Gm^r	24
PA14 Δ*exoU*	T3SS effector mutant deficient in phospholipase activity	26
PA14 Δ*exoT*	T3SS effector mutant deficient in ADP ribosylation and small G-protein activating protein activity	26
PA103	Overproduces ToxA protein	28
PA103-29	ToxA-deficient mutant of PA103	28

grown in LB broth supplemented with 5 mM EGTA to an OD_{660} of 0.7 to 0.8. Cells were diluted to an OD_{660} of 0.04 in a total volume of 2 ml LB broth containing 3 mM SNP. Picoamperes of electrical current were measured at 1-s intervals for 5 min, allowing the cells to reach a steady state, and the picoamperes of electrical current were converted to nanomolar NO by using the standard curve described above. The NO concentration generated in the absence of *P. aeruginosa* was also determined.

Detection of *P. aeruginosa* exotoxin A secretion. Trypticase soy broth (TSB) treated with Chelex was prepared as described previously (28) and used for exotoxin A production. Overnight cultures grown in TSB treated with Chelex were diluted 1:100 in fresh medium and allowed to grow for 24 h at 37°C with aeration. Cells were harvested by centrifugation, and the supernatant was collected for analysis of exotoxin A secretion by Western blotting. Proteins were separated on a 12% Tris-glycine SDS-polyacrylamide gel, transferred to membrane filters, and probed with polyclonal rabbit anti-exotoxin A antiserum (a generous gift from Abdul N. Hamood, Lubbock, TX) at a 1:4,000 dilution. Purified exotoxin A (1 μg) was used as a positive control in the Western blot assays.

Quantitation of total protease and LasB elastase secretion. Total protease secretion was determined by the method of Rinderknecht et al. (34). A 50-μl volume of supernatant from an overnight culture of *P. aeruginosa* grown in peptone-TSB was added to 2 ml of 10 mM HEPES buffer (pH 7.5) containing 20 mg of hide powder blue (Sigma-Aldrich) as a substrate. Release of bound dye was indicative of protease activity. The suspension was mixed at 37°C for 1 h, insoluble substrate was removed by centrifugation, and the A_{595} of the supernatant was determined. Absorbance readings were normalized to the protein concentration of the culture supernatant determined by the bicinchoninic acid method (Pierce).

A similar experiment was performed to evaluate LasB elastase secretion (40). A 100-μl volume of supernatant from an overnight culture grown in peptone-TSB was added to 2 ml of 0.1 M Tris-maleate with 0.1 mM $CaCl_2$ (pH 7.0) containing 10 mg elastin-Congo red (Sigma-Aldrich). The suspension was mixed at 37°C for 18 h, insoluble substrate was removed by centrifugation, and the A_{495} of the supernatant was determined. Absorbance readings were normalized to the protein concentration of the culture supernatant determined by the bicinchoninic acid method (Pierce).

Reverse transcription (RT)-PCR. For detection of transcripts encoding *P. aeruginosa* exoproducts from axenic cultures, organisms were grown in calcium-chelated LB medium as described above. After 6 h, RNA was extracted from 2 ml of culture with RNAprotect Bacteria Reagent and RNeasy Mini Spin columns in accordance with the manufacturer's instructions (Qiagen). For detection of transcripts during interaction with phagocytes, *P. aeruginosa*–THP-1 cell cocultures were incubated at an MOI of either 0.1 or 1.0 at 37°C; after 2 h of incubation, RNA was extracted in accordance with the manufacturer's instructions. RNA was treated with RNase-free DNase I (Invitrogen). PCR with oligonucleotide primers GroEL F1 and GroEL R1 (Table 2) was used to confirm the absence of contaminating DNA in the purified RNA. In addition, reverse transcriptase was omitted from negative control RT-PCRs to confirm the absence of contaminating DNA in the RNA samples. The oligonucleotides used in the RT-PCRs and the predicted sizes of the respective amplicons are shown Table 2.

Statistical analysis. CFU, cytotoxicity, elastin-Congo red (elastase), and hide powder blue (protease) assays were performed in triplicate in three independent experiments. Data presented are the mean ± the standard deviation (SD) of all nine samples. For cytotoxicity assays, mean blank values were subtracted from mean results, and the results were normalized to percent lysis relative to maximum-lysis samples. For these calculations, the final SD was calculated by standard error propagation methods. For all assays, statistical comparisons were performed with the Student *t* test, and *P* values of ≤0.05 were considered significant.

RESULTS

Membrane nitrate reductase (Δ*narGH*) and nitrite reductase (Δ*nirS*) mutants are sensitive to killing by the human monocyte cell line THP-1. We showed previously that both the *P. aeruginosa* membrane nitrate reductase (Δ*narGH*) mutant and the nitrite reductase (Δ*nirS*) mutant were avirulent in the nematode *C. elegans* slow-kill and fast-kill assays compared to parental strain PA14 (43). In a model of the *P. aeruginosa*-phagocyte interaction, we evaluated the survival of the wild-

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′→3′)	Predicted amplicon size (bp)
narH RT S narH RT AS	5′GAGATCGACGACTACTACGAACC 3′ 5′CACCTGCTCCACGCTCAACC 3′	677
GroEL F1 GroEL R1	5′CGCTCGCAAGAAAATGCTGGTC 3′ 5′CGACGGACAGTTCGTTTTCCAG 3′	550
nirS RT S nirS RT AS	5′AGGTCGTTGAGCTGTTTCTTCG 3′ 5′GCCAAGGACGACATGAAAGC 3′	437
ExoU RT S Exo U RT AS	5′GGGAATACTTTCGGGAAGTT 3′ 5′CGATCTCGCTGCTAATGTGTT 3′	428
PcrV RT S PcrV RT AS	5′ATGGAAGTCAGAAACCTTAATGCC 3′ 5′AAATCCTTGATCGACAGCTTGC 3′	629
ExoT RT S ExoT RT AS	5′CATATTCAATCATCTCAGCAGAACC 3′ 5′AGTCTCTCCTCTGTCAAAGTCG 3′	398
RhlA RT S RhlA RT AS	5′GAAAGTCTGTTGGTATCGGTTTGC 3′ 5′TATTGCCGACGGTCTCGTTG 3′	494
LasB RT S LasB RT AS	5′TTTCTACGCTTGACCTGTTGTTCG 3′ 5′TACTTGCCGATCTTCTGGTTGC 3′	635
ToxA RT S ToxA RT AS	5′TCAGTCATCGCCTGCATTTC 3′ 5′CTCGCGTTGATAGGGTGACC 3′	460

type PA14, Δ*narGH* and Δ*nirS* mutant, and several other mutant strains (Fig. 2) after cocultivation with the human monocytic cell line THP-1 (41). We initiated our studies at a low MOI of 0.1 to provide a strong challenge to the bacterium by the phagocyte line. In coculture with THP-1 cells, wild-type PA14 organism numbers increased by several orders of magnitude during the incubation period. In contrast, the Δ*narGH* and Δ*nirS* mutants were almost completely eliminated by the THP-1 human monocyte line after 6 h of incubation (Fig. 2A), whereas the genetically complemented Δ*narGH* and Δ*nirS* mutants grew similarly to wild-type PA14 (data not shown). A T3SS phospholipase (Δ*exoU*) mutant was killed comparably to the Δ*narGH* and Δ*nirS* mutants (Fig. 2B). However, the *MAR2xT7::norB* and *MAR2xT7::nosZ* transposon mutants, defective in the nitrate dissimilation pathway downstream of nitrite reductase, grew similarly to wild-type PA14 during cocultivation with THP-1 cells (Fig. 2B). In the absence of host cells, all bacterial strains grew to approximately 1×10^7 to 3×10^7 CFU/ml after 6 h of incubation (data not shown). At increased MOIs of 1.0 and 10, both the Δ*narGH* and Δ*nirS* mutants were killed to a significantly greater degree than wild-type PA14 in the THP-1 cell–*P. aeruginosa* coculture (*P* ≤ 0.01) (Fig. 2C). Furthermore, the Δ*nirS* mutant was killed more effectively than the Δ*narGH* mutant by THP-1 cells at MOIs of 1.0 (*P* ≤ 0.05) and 10.0 (*P* ≤ 0.01).

The cytotoxicity of the Δ*narGH* and Δ*nirS* mutants for THP-1 cells is diminished at low MOIs. To determine whether the decreased survival of the Δ*narGH* and Δ*nirS* mutants correlated with reduced cytotoxicity against THP-1 cells, we compared the abilities of the mutants and their respective complemented strains with that of wild-type PA14 to induce release of LDH from THP-1 cells. Both the Δ*narGH* and Δ*nirS* mutants displayed significantly less cytotoxicity against THP-1 cells than did wild-type PA14 and the complemented strains (*P* ≤ 0.01)
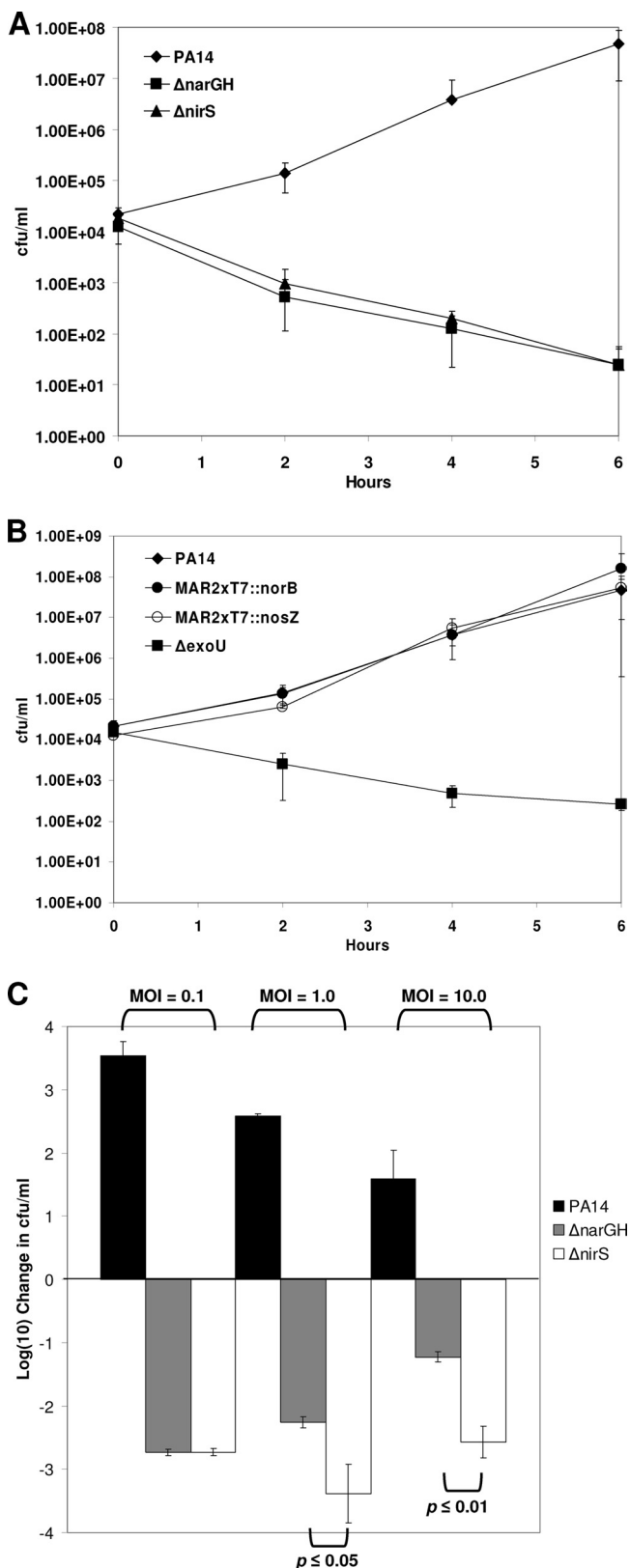
at an MOI of 0.1 (Fig. 3). As expected, the Δ*exoU* and *MAR2xT7::pcrV* T3SS mutants also displayed significantly reduced cytotoxicity compared to that of wild-type PA14 ($P \leq$ 0.01). In contrast, the Δ*exoT*, *MAR2xT7::norB*, and *MAR2xT7:: nosZ* mutants displayed levels of cytotoxicity similar to that of wild-type PA14 (Fig. 3A).

To determine whether increasing the MOI would affect cytotoxicity, we compared LDH release from THP-1 cells treated with *P. aeruginosa* at MOIs of 0.1 and 1.0 in a separate experiment (Fig. 3B). Even though the Δ*narGH* and Δ*nirS* mutants were killed to a significant degree by THP-1 cells at an MOI of 1.0 compared to wild-type PA14 (Fig. 2C), the increased MOI resulted in a level of LDH release comparable to that of wild-type PA14 for both mutants, as well as for the Δ*exoU* mutant. These data suggested induction of other cytotoxic effectors at the higher MOI and led us to examine the mutants for expression of *P. aeruginosa* virulence factors with cytotoxic potential.

The Δ*narGH* and Δ*nirS* mutants display decreased secretion of T3SS exoproducts ExoU, ExoT, and PcrV. To explore the possibility that reduced T3SS exoproduct secretion contributed to the increased sensitivity of the Δ*narGH* and Δ*nirS* mutants to phagocyte killing and their decreased cytotoxicity, we compared the production of selected T3SS exoproducts by the mutants and their respective genetically complemented strains to that by wild-type PA14. Supernatants of strains grown in calcium-depleted LB medium were analyzed by Western blotting with antibodies against ExoU, ExoT, or PcrV. The Δ*narGH* and Δ*nirS* mutant strains displayed no detectable ExoU, ExoT, or PcrV secretion (Fig. 4A to C), whereas the complemented mutants and wild-type PA14 showed comparable degrees of secretion of each T3SS exoproduct. The *MAR2xT7::norB* and *MAR2xT7::nosZ* mutants also secreted levels of T3SS exoproducts comparable to those of wild-type PA14. Western blotting of whole-cell lysates from the Δ*narGH* and Δ*nirS* mutants failed to detect the T3SS exoproducts, indicating that the products were not retained in the cells due to a secretion defect (data not shown).

These data indicate that a factor contributing to the decreased survival of the Δ*narGH* and Δ*nirS* mutants following coculture with the THP-1 human monocyte line is their inability to generate and secrete T3SS exoproducts. Normal secretion of T3SS exoproducts by the *MAR2xT7::norB* and *MAR2xT7::nosZ* mutants suggests that the lack of a NO signal in the Δ*narGH* and Δ*nirS* mutants contributes to this phenomenon and implicates endogenous NO production by nitrite reductase as an important regulator of the T3SS in *P. aeruginosa* PA14.

To test this possibility, the Δ*nirS* mutant was grown in EGTA-treated LB medium for 5 h and then supplemented

of wild-type PA14, as measured by CFU per milliliter. (B) Wild-type PA14 and NO reductase (*MAR2xT7::norB*), nitrous oxide reductase (*MAR2xT7::nosZ*), and T3SS (Δ*exoU*) mutants were cocultured with THP-1 cells as for panel A. Only Δ*exoU* displayed reduced viability over time. (C) Comparison of wild-type PA14 and Δ*narGH* and Δ*nirS* mutant strain interactions with THP-1 cells at different MOIs. At all of the MOIs used, the two mutants displayed a reduction in viability compared to that of wild-type PA14. At MOIs of 1.0 and 10, the viability of the Δ*nirS* mutant was significantly less than that of the Δ*narGH* mutant.

FIG. 2. *P. aeruginosa* interaction with human monocytic cell line THP-1. (A) Wild-type PA14 and membrane nitrate reductase (Δ*narGH*) and nitrite reductase (Δ*nirS*) mutant strains were cocultured with THP-1 cells at an MOI of 0.1 for up to 6 h. The Δ*narGH* and Δ*nirS* mutants displayed reduced viability over time compared to that
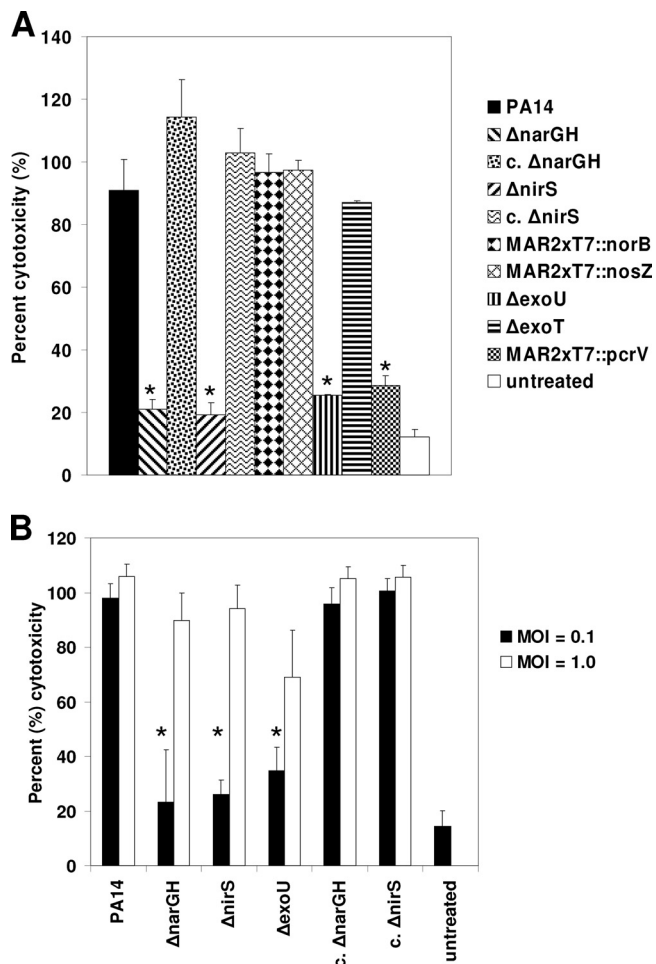
FIG. 3. Cytotoxicity of *P. aeruginosa* for THP-1 cells. (A) Cytotoxicity for THP-1 cells was determined by measuring LDH release following interaction with *P. aeruginosa*. Wild-type PA14 and denitrification pathway mutants were cocultured with THP-1 cells at an MOI of 0.1 for 6 h. T3SS (Δ*exoU*, Δ*exoT*, and *MAR2xT7::pcrV*) mutants were also examined. Compared to wild-type PA14, the membrane nitrate reductase (Δ*narGH*) and nitrite reductase (Δ*nirS*) mutants induced significantly less cytotoxicity against THP-1 cells, similar to the Δ*exoU* and *MAR2xT7::pcrV* T3SS mutants. In contrast, the genetically complemented Δ*narGH* and Δ*nirS* mutant strains (c. Δ*narGH* and c. Δ*nirS*, respectively), as well as the Δ*exoT*, *MAR2xT7::norB*, and *MAR2xT7::nosZ* T3SS mutants, induced cytotoxicity comparably to that of wild-type PA14. (B) Comparison of cytotoxicity against THP-1 cells at MOIs of 0.1 and 1.0. At the higher MOI of 1.0, all of the strains induced comparable levels of cytotoxicity. There was no significant release of LDH from strain PA14 in the absence of THP-1 cells (data not shown).
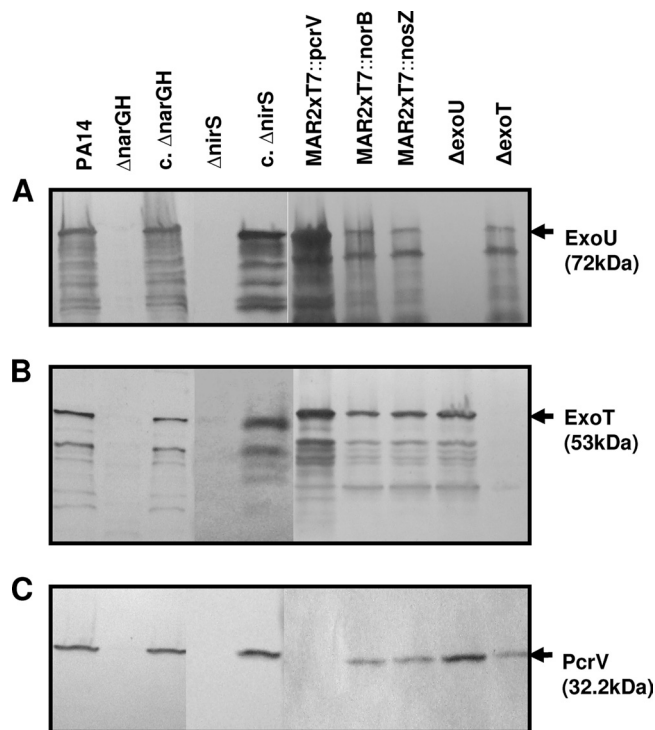

FIG. 4. Detection of T3SS exoproduct secretion by Western blotting. All strains were grown for 6 h in LB broth supplemented with 0.5 M EGTA to chelate calcium and induce expression of T3SS exoproducts (23). Culture supernatant proteins were precipitated and analyzed by Western blotting with a mouse MAb against either ExoU (A) or PcrV (C) or with rabbit polyclonal antiserum to ExoT (B). Representative Western blot assays are shown. T3SS exoproducts were not detected in the supernatant from either the Δ*narGH* or the Δ*nirS* mutant. Supernatants from the Δ*exoU* (A), Δ*exoT* (B), and *MAR2xT7::pcrV* (C) mutant control strains were also negative for secretion of the corresponding exoproducts. c. Δ*narGH* and c. Δ*nirS*, genetically complemented Δ*narGH* and Δ*nirS* mutant strains, respectively.

with either GSNO, SNP, or DETA-NO at a final concentration of 3 mM (1). The culture was allowed to grow for an additional hour before the precipitation of proteins from the supernatant. The T3SS exoproduct ExoU was detected in culture supernatants following exposure to all three NO donors (Fig. 5A). In contrast, addition of increasing concentrations of sodium nitrite to Δ*nirS* mutant cultures did not stimulate ExoU expression (Fig. 5A). Thus, exogenous addition of a NO donor can rescue the T3SS expression deficiency of the Δ*nirS* mutant.

We compared NO consumption between PA14 and the

Δ*nirS* mutant in the presence of 3 mM SNP with a NO detector. A steady-state NO level is achieved in LB medium supplemented with SNP when the generation of NO by SNP is in equilibrium with the production of NO_2^-, as NO reacts with O_2. The steady-state level of NO production from SNP alone plateaued within 150 s at 1.6 μM (Fig. 5B). The NO production of PA14 peaked at 1.3 μM and reached a steady-state level of 200 nM NO. In contrast, that of the Δ*nirS* mutant peaked at 300 nM and reached a steady-state NO level of 100 nM (Fig. 5B). These data suggest that the increased NO level in wild-type PA14 compared to that in the Δ*nirS* mutant is due to conversion of NO_2^- to NO by the functional NirS protein in PA14. In contrast, the Δ*nirS* mutant has only one source of available NO, that generated by SNP. In the absence of a NO donor, endogenous NO must be produced from NO_2^- by NirS. We determined the nitrite concentration in LB medium alone to be 5.5 μM (data not shown), indicating that wild-type strain PA14, but not the Δ*nirS* mutant, can produce endogenous NO under these culture conditions.

***P. aeruginosa* Δ*narGH* and Δ*nirS* mutants display decreased total protease and elastase secretion but normal exotoxin A secretion.** To determine whether the secretion of other *P.*
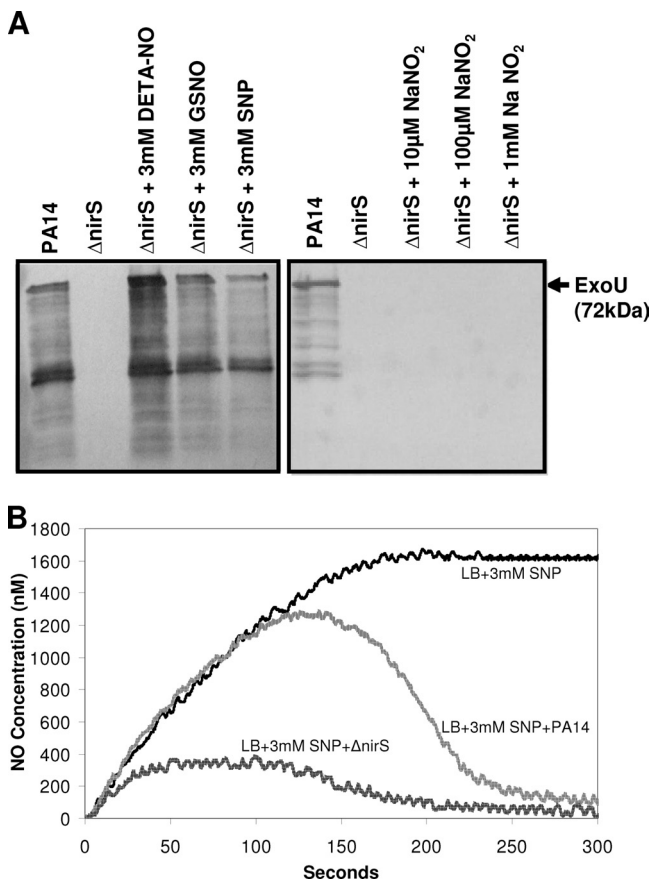
FIG. 5. Restoration of ExoU expression in the Δ*nirS* mutant by supplementation with NO donors. (A) All strains were grown for 6 h in LB broth supplemented with 0.5 M EGTA to chelate calcium and induce the expression of T3SS exoproducts (23). Cultures were supplemented with either a NaNO₂ or a NO donor (DETA-NO, GSNO, or SNP) 1 h prior to supernatant harvesting, as indicated. Culture supernatant proteins were precipitated and analyzed by Western blotting with a mouse MAb against ExoU. T3SS exoproducts were not detected in supernatants from the Δ*nirS* mutant supplemented with NaNO₂. In contrast, exoproducts were detected in the supernatant of the Δ*nirS* mutant supplemented with all of the NO donors. (B) NO consumption by PA14 and the Δ*nirS* mutant in the presence of 3 mM SNP. The steady-state level of NO release by SNP occurred within 150 s, with a plateau at 1.6 μM (black line). The wild-type PA14 NO level peaked at 1.3 μM and reached a steady state at 200 nM (dark gray line). The Δ*nirS* mutant NO level peaked at 300 nM and reached a steady state at 100 nM.

aeruginosa exoproducts known to contribute to virulence was altered in the Δ*narGH* and Δ*nirS* mutant strains, we evaluated the total protease, elastase (LasB), and exotoxin A secretion by these mutants. Both the Δ*narGH* and Δ*nirS* mutants displayed a significant decrease ($P \leq 0.01$) in total protease (Fig. 6A) and LasB elastase secretion (Fig. 6B) compared to that of wild-type PA14 and the complemented mutant strains. Wild-type PA14; the complemented Δ*narGH* and Δ*nirS* mutants; the *MAR2xT7::norB*, *MAR2xT7::nosZ*, and T3SS mutants; and the Δ*exoU*, Δ*exoT*, and *MAR2xT7::pcrV* mutants all secreted comparable levels of both enzymes. In contrast, wild-type PA14, as well as all of the mutant strains derived from PA14, secreted comparable levels of exotoxin A (Fig. 6C). Purified exotoxin A and culture supernatant
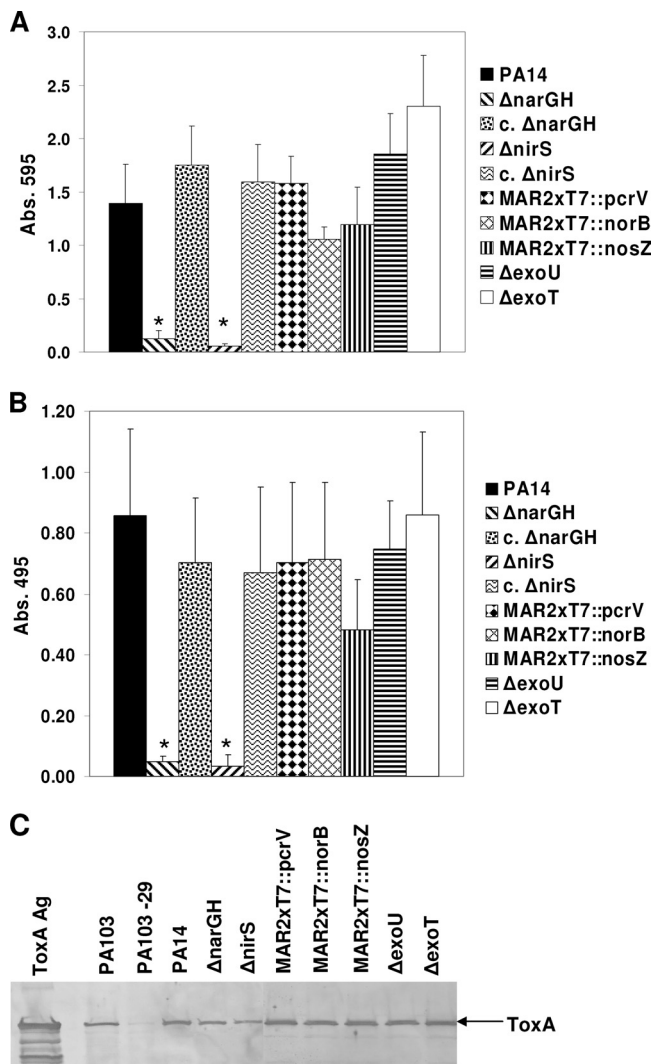

FIG. 6. Total protease, elastase, and exotoxin A secretion by denitrification pathway mutants. Culture supernatants from wild-type PA14 and denitrification pathway and T3SS mutants were compared for total protease (A) and elastase (B) activities. Only the Δ*narGH* and Δ*nirS* mutants displayed significantly lower levels of protease and elastase secretion than wild-type PA14. Protease secretion was restored to normal in the genetically complemented Δ*narGH* and Δ*nirS* mutant strains (c. Δ*narGH* and c. Δ*nirS*, respectively). Supernatants from each strain were tested in triplicate in three separate experiments, and the data represent the mean ± SD. In contrast, wild-type PA14 and all of the mutant strains derived from PA14 secreted ToxA into their culture supernatants, as detected by Western blotting (C). *P. aeruginosa* PA103 and PA103-29 were used as positive and negative controls, respectively, for the production of exotoxin A; ToxA Ag is the purified *P. aeruginosa* exotoxin A (1 μg) used as a positive control for Western blotting. Abs. 495, absorbance at 495 nm.

from *P. aeruginosa* PA103 were used as positive controls in the Western blot assay; culture supernatant from exotoxin A-deficient mutant *P. aeruginosa* PA103-29 (28) was used as a negative control (Fig. 6C).

Virulence factor expression is regulated at the level of transcription in the Δ*narGH* and Δ*nirS* mutants. To determine whether lack of exoproduct secretion in the Δ*narGH* and Δ*nirS* mutants was regulated at the level of transcription, we com-

pared their steady-state mRNA levels to that of wild-type PA14 by RT-PCR both under T3SS-inducing culture conditions and during the *P. aeruginosa*–THP-1 cell interaction. *groEL*, *narH*, and *nirS* primers were used as positive controls in the RT-PCR; omission of reverse transcriptase served as a control for DNA contamination.

Under T3SS-inducing, axenic culture conditions (Fig. 7A), transcripts encoding the T3SS exoproducts ExoU, ExoT, PcrV, and PopD were not detected by RT-PCR in either the Δ*narGH* or the Δ*nirS* mutant. The *lasB* elastase transcript was also not detected in the Δ*narGH* and Δ*nirS* mutants. Earlier studies (43) demonstrated that the Δ*narGH* mutant was defective in rhamnolipid production; accordingly, *rhlA* transcript expression was not detected in either the Δ*narGH* or the Δ*nirS* mutant. As predicted from the Western blot assay data (Fig. 6C), *toxA* transcripts were detected in all three strains (Fig. 7A). Analysis of transcripts from the *P. aeruginosa*–THP-1 cell coculture at an MOI of either 0.1 (Fig. 7B) or 1.0 (Fig. 7C) also demonstrated lack of expression of T3SS exoproducts, *rhlA*, and *lasB* by the Δ*narGH* and Δ*nirS* mutants. The *toxA* transcript was not detected at an MOI of 0.1 in wild-type PA14 or the Δ*narGH* or Δ*nirS* mutant (Fig. 6B) but was present in all three strains at an MOI of 1.0 (Fig. 6C). To control for RNA integrity, *groEL* transcript was detected in all of the RT-PCR assays performed.

Taken together, the results suggest that the increased susceptibility of the Δ*narGH* and Δ*nirS* mutants to killing by THP-1 cells and the decreased cytotoxic capacity of these mutants are due to the lack of expression of T3SS exoproducts, and possibly lack of proteolytic enzyme expression, at an MOI of 0.1. However, the cytotoxicity against THP-1 cells at the higher MOI of 1.0 by the Δ*narGH*, Δ*nirS*, and Δ*exoU* mutants (Fig. 3B) was likely attributable to induction of normal levels of exotoxin A expression (25, 29). The data are consistent with the hypothesis that endogenous NO generated by nitrite reductase contributed to regulation of expression at the level of transcription of T3SS exoproducts, rhamnolipid, protease, and elastase, but not exotoxin A, in *P. aeruginosa* PA14.

DISCUSSION

Dissimilation of nitrogen oxides, particularly nitrate and nitrite, drives energy metabolism under anaerobic conditions in a variety of bacteria, including *P. aeruginosa* (35). In addition to its role in anaerobic growth of *P. aeruginosa*, our previous work has shown that the nitrate dissimilation pathway modulates factors important in pathogenesis under conditions where oxygen is apparently not limiting, including motility, initiation of biofilm formation, and virulence in a surrogate model host, the nematode *C. elegans* (43). In the present study, we extended these observations to the *P. aeruginosa*-phagocyte interaction by using the pathogenic strain PA14 (31). Mutants deficient in membrane nitrate reductase and nitrite reductase, the first two steps of the nitrate dissimilation pathway (Fig. 1), were readily killed by the human monocytic cell line THP-1 (41), while mutants deficient in NO reductase and nitrous oxide reductase, the last two steps in the pathway, grew unchecked by THP-1 cells, similarly to wild-type PA14 (Fig. 2A and B).

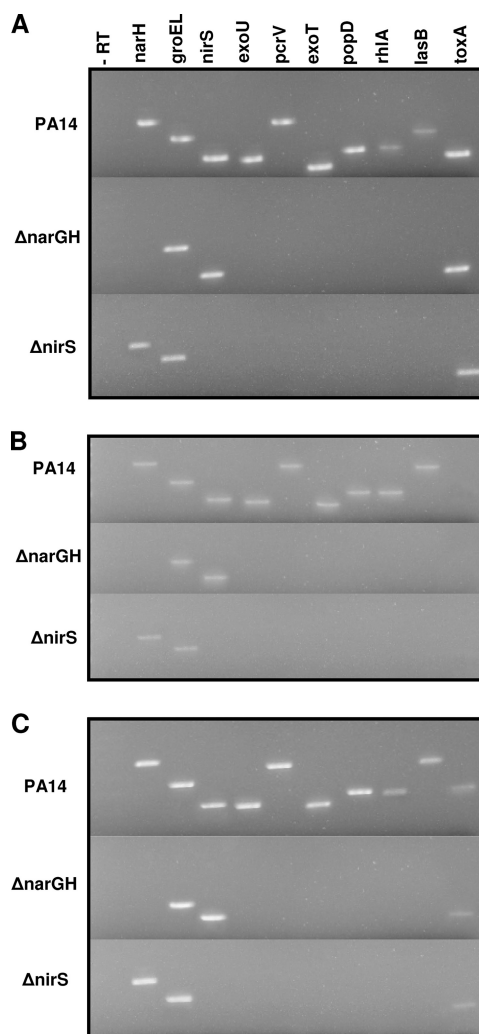We demonstrated that NirS expression in *P. aeruginosa*

FIG. 7. Detection of *P. aeruginosa* exoproduct gene expression by RT-PCR. Table 2 describes the oligonucleotides used for RT-PCR and the predicted size of each amplicon. (A) RT-PCR of mRNA extracted from axenic culture under T3SS-inducing conditions after 6 h of growth. (B) RT-PCR of mRNAs extracted from *P. aeruginosa* strains cocultured with the human monocytic cell line THP-1 at an MOI of 0.1. (C) RT-PCR of mRNAs extracted from *P. aeruginosa* strains cocultured with the human monocytic cell line THP-1 at an MOI of 1.0. In accordance with their secreted protein profiles, transcripts for proteases and T3SS exoproducts were not detected by RT-PCR in the Δ*narGH* and Δ*nirS* mutants under any of the conditions tested. *toxA* transcripts were detected in wild-type PA14 and the Δ*narGH* and Δ*nirS* mutants under T3SS-inducing culture conditions (A) and during the *P. aeruginosa* interaction with THP-1 cells at an MOI of 1.0 (C) but not at an MOI of 0.1 (B). The *toxA* expression profiles of the Δ*narGH* and Δ*nirS* mutants correlate with the cytotoxicity data shown in Fig. 3B. −RT, control without reverse transcriptase.

PA14 is required to induce the T3SS that translocates effector proteins ExoT and ExoU into host cells. A *nirS* mutant unable to express the T3SS could be rescued by the exogenous addition of a NO donor, suggesting that the product of nitrite reductase activity may act as a signaling molecule, allowing *P. aeruginosa* PA14 to induce the T3SS. Thus, the defects in virulence can be attributed to an inability to induce the T3SS in the *nir* mutant.

Transcriptional regulation of the *P. aeruginosa* T3SS is complex (46), but all T3SS genes are under the direct transcriptional control of ExsA, an AraC family transcriptional activator. ExsA activation is antagonized by two antiactivators, ExsD and PtrA. T3SS transcription is coupled to T3SS activity, controlled through modulation of cyclic AMP biosynthesis, repressed by a variety of stresses, involved with multiple two-component regulatory systems, and inversely related to biofilm formation (46). We propose that an additional environmental signal involved in T3SS regulation is NO.

Only a limited number of transcriptional regulators have been demonstrated to interact with NO (see reference 35 for a review of transcription factors regulating the denitrification pathway). *P. aeruginosa* has four members of the Crp/FNR family of transcriptional activators (22); Vfr is an ortholog of Crp, ANR is an ortholog of FNR and responds to anaerobiosis, and DNR responds to NO (12, 33). ANR regulates *nar* and *dnr* expression, while DNR regulates *nirS*, *nirQ*, *norBC*, and *nos* expression (2, 35). These four denitrification genes are the only ones predicted to be regulated by DNR (35). Direct binding of gaseous NO to a ferrous heme cofactor regulates the activity of DNR in *P. aeruginosa* (12). *Brucella melitensis* NnrA is a member of this family, and it is thought that NO may serve as the signal sensed by NnrA (14).

Under aerobic conditions, the *P. aeruginosa* flavohemoglobin (*fhp*) detoxifies NO and is regulated by the NO-responsive regulator FhpR (1). FhpR is an ortholog of *Escherichia coli* NorR, a σ^{54}-dependent member of the NtrC family of transcriptional activators. Binding of NO to a nonheme iron center in the GAF domain results in mononitrosyl-iron complex formation and converts NorR to its active form (42). NorR regulons are small, and it has been predicted that *P. aeruginosa* NorR (FhpR) regulates only itself and the *fhp-nnrS* operon (35).

A third type of NO-responsive regulator is NsrR, a member of the Rrf2 family of transcriptional repressors. Although *P. aeruginosa* contains several members of the Rrf2 family (Pfam02082), it does not have a protein closely related to NsrR (35). Thus, it is unlikely that an Rrf2 family repressor is the NO-responsive regulator that affects T3SS expression. Other possibilities for NO regulation of virulence factor expression in *P. aeruginosa* PA14 would include a two-component sensor-response regulator that senses NO similarly to the sensing of nitrate by the sensor-response regulator NarX-NarL (39). Our previous work (43) showed that a *C. elegans* Δ*narXL* mutant was fully virulent, likely precluding a role for this two-component system in virulence factor expression. However, the numerous "hypothetical" two-component sensor-response regulators in the PA14 genome are potential candidates.

Recent work by Schreiber et al. (36) indicates that, under aerobic conditions, a *nirS-lacZ* fusion in *P. aeruginosa* PAO1 expressed low but measurable levels of β-galactosidase activity, even without the addition of exogenous nitrate. Furthermore, denitrifying bacteria, such as *P. stutzeri*, avoid NO toxicity by using the enzymes in the nitrate dissimilation pathway to maintain a low, nanomolar, steady-state concentration of free NO in the cell (13). We propose that *P. aeruginosa* maintains a similar physiological level of NO via basal nitrate and nitrite reductase activities to regulate the expression of selected virulence factors. We believe that the 5 μM concentration of nitrite in LB broth is sufficient to provide substrate to NirS, which has very low activity in aerobically grown cells, resulting in a low steady-state level of NO.

The comparisons of the transcriptional profiles of the Δ*narGH* and Δ*nirS* mutants with that of wild-type PA14 currently in progress, combined with nitrosylation profile comparisons (38) of these strains, should provide insight into the mechanisms of NO regulation of virulence factor expression in *P. aeruginosa* PA14. Understanding these mechanisms may suggest new therapeutic options for inhibiting virulence factor expression in *P. aeruginosa*.

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