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Nitrate Sensing and Metabolism Modulate Motility, Biofilm Formation, and Virulence in *Pseudomonas aeruginosa*▽

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Infection by the bacterial opportunist *Pseudomonas aeruginosa* frequently assumes the form of a biofilm, requiring motility for biofilm formation and dispersal and an ability to grow in nutrient- and oxygen-limited environments. Anaerobic growth by *P. aeruginosa* is accomplished through the denitrification enzyme pathway that catalyzes the sequential reduction of nitrate to nitrogen gas. Mutants mutated in the two-component nitrate sensor-response regulator and in membrane nitrate reductase displayed altered motility and biofilm formation compared to wild-type *P. aeruginosa* PAO1. Analysis of additional nitrate dissimilation mutants demonstrated a second level of regulation in *P. aeruginosa* motility that is independent of nitrate sensor-response regulator function and is associated with nitric oxide production. Because motility and biofilm formation are important for *P. aeruginosa* pathogenicity, we examined the virulence of selected regulatory and structural gene mutants in the surrogate model host *Caenorhabditis elegans*. Interestingly, the membrane nitrate reductase mutant was avirulent in *C. elegans*, while nitrate sensor-response regulator mutants were fully virulent. The data demonstrate that nitrate sensing, response regulation, and metabolism are linked directly to factors important in *P. aeruginosa* pathogenesis.

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium capable of causing infection in the immunocompromised host. The types of infection caused by *P. aeruginosa* include otitis media (19), infection of burn wounds (37), and lung infection in cystic fibrosis (CF) patients (27). In many instances, infection by *P. aeruginosa* assumes the form of a biofilm, which is highly resistant to antibiotics and to attack by immune effector cells (20).

P. aeruginosa growth in biofilms is characterized by its ability to grow in nutrient- and oxygen-limited environments. Anaerobic growth by *P. aeruginosa* is accomplished through a denitrification enzyme pathway that catalyzes a four-step sequential reduction of nitrate to nitrogen gas, with nitrite, nitric oxide, and nitrous oxide, respectively, as intermediates. Two different nitrate reductase complexes mediate nitrate reduction to nitrite in *P. aeruginosa* (4, 68), a plasma membrane-bound nitrate reductase complex encoded by the *narK1K2GHJI* operon and a periplasmic nitrate reductase encoded by *napEFDABC*. Reduction of either nitrate or nitrite substrates provides energy for *P. aeruginosa* anaerobic growth, with nitrate reduction to nitrite via nitrate reductase contributing more significantly to proton motive force and hence energy production (4, 68). A well-described environment for *P. aeruginosa* growth under anoxic conditions is as a biofilm within the mucus of the CF lung (66). Nitrate and nitrite levels in CF mucus, generated in part by the host inflammatory response to infection, are sufficient to support anaerobic metabolism of *P. aeruginosa* (27).

Biofilm formation and organism dispersal leading to spread of infection by *P. aeruginosa* are dependent on motility. In vitro assays of motility have permitted genetic dissection of the process. Swimming by *P. aeruginosa* through semisolid agar is dependent upon flagella (26). The most complex motility function in *P. aeruginosa* is swarming on moist surfaces. Swarming is regulated by quorum sensing and is mediated by the combined action of flagella and type IV pili (32, 34, 42). Production of the biosurfactant rhamnolipid (33), or its precursor (hydroxyalkanoyloxy)alkanoic acid (18), serves as an essential aid to swarming motility (8) by acting as a wetting agent to overcome the surface tension of water and facilitate movement across the moist surface (26). Culture conditions can modulate rhamnolipid production and, consequently, swarming. Several reports demonstrate that nitrate is the best nitrogen source for rhamnolipid production in *P. aeruginosa* (reviewed in reference 55). Accordingly, nitrate promotes swarming by *P. aeruginosa* while ammonium does not (18). The basis for the preference for nitrate in rhamnolipid production and swarming is unknown (55). Furthermore, rhamnolipid production is initiated when nitrate is depleted from the culture medium (55).

Several studies using transcriptional profiling have demonstrated the importance of quorum sensing as a global regulator of nitrate metabolism, biofilm formation, and motility in *P. aeruginosa* (63, 65). In addition to quorum sensing, anaerobic growth via denitrification in *P. aeruginosa* is regulated by Anr (54, 67), an ortholog of Fnr (for "fumarate and nitrate reductase") in *Escherichia coli*. A consensus Anr sequence, TTGA CN_4ATCAG (56), is found in the intergenic region between the *narK1K2GHJI* membrane-bound nitrate reductase operon and the operon encoding the nitrate sensor-regulator *narXL*. The membrane-bound nitrate reductase *narK1K2GHJI* and its cognate two-component nitrate sensor-regulator *narXL* are organized as separate operons that are transcribed divergently (Fig. 1). NarX/NarL is a classic bacterial two-component regulatory system in which a histidyl-aspartyl phosphorelay controls gene expression. The periplasmic domain of the sensor protein, NarX, contains a highly conserved nitrate recognition

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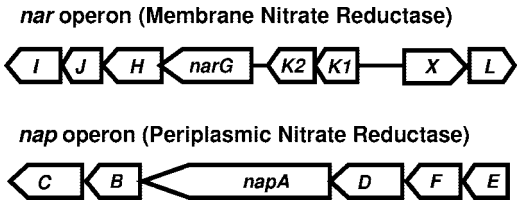

FIG. 1. Nitrate regulation and dissimilation operons. Genomic organization of the *P. aeruginosa* membrane nitrate reductase operon *narK1K2GHJI*, the two-component nitrate sensor-regulator operon *narXL*, and the periplasmic nitrate reductase operon *napEFDABC*. Arrows indicate the direction of transcription.

region termed the P box. NarX responds to P-box recognition of nitrate by autophosphorylating a conserved histidyl residue in its cytoplasmic transmitter domain. Transfer of phosphate to the conserved aspartyl residue in the receiver domain of the response regulator NarL results in activation or repression of target operon transcription (57). NarL is a typical helix-turn-helix transcriptional regulator that acts as a dimer, binding preferentially to a 7-2-7 consensus sequence of a heptamer (TACc/tNa/cT) organized as an inverted repeat separated by 2 bp (15, 57, 58). This motif is located upstream of *narK1K2GHJI* in *P. aeruginosa*, allowing NarL to contribute to the activation of the membrane nitrate reductase operon.

In this study, we utilized a set of *P. aeruginosa* deletion mutants to examine the roles of the nitrate sensor-response regulator NarX/NarL, the periplasmic nitrate reductase NapAB, and the membrane-bound nitrate reductase NarGHI in motility, biofilm formation, and virulence in the surrogate model host *Caenorhabditis elegans*. The data demonstrate that nitrate sensing and metabolism are linked directly to factors important in *P. aeruginosa* pathogenesis.

MATERIALS AND METHODS

Media, strains, and culture conditions. For routine growth, organisms were plated onto LB (5 g/liter yeast extract, 10 g/liter NaCl, and 10 g/liter tryptone) agar plates. All antibiotics were obtained from Sigma-Aldrich. Carbenicillin was used at 400 μg/ml, gentamicin at 40 μg/ml, and tetracycline at 100 μg/ml. Deletion mutants were generated in *P. aeruginosa* PAO1 for the motility and biofilm studies and in PA14 for the assays of virulence in *C. elegans*. All *P. aeruginosa* strains and corresponding mutants used in this study are listed in Table 1. The *E. coli* strains OP50 (6) and SM10 (17) have been described previously.

Recombinant DNA manipulations and genetic techniques. For construction of mutant strains, internal gene deletions were generated within the *nar* and *nap* operons by a two-step splice overlap extension PCR strategy (30). In-frame deletions were generated for *narX* and *narL* (Table 1); otherwise, all mutations generated were polar. Deletion mutants were created by allelic exchange via conjugation between *E. coli* SM10 (17) and *P. aeruginosa*, as described by Schweizer and colleagues (28).

The Tn::*norC* mutant was identified in a genomic screen of transposon mu-

TABLE 1. *P. aeruginosa* strains

Strain	Description[a]	Source or reference
P. aeruginosa PAO1		
PAO1	WT strain	62
Δ*narXL*	Nitrate sensor-response regulator mutant	This study
Δ*narGH*	Membrane nitrate reductase mutant	This study
Δ*narL*	Nitrate response regulator mutant	This study
Δ*napA*	Periplasmic nitrate reductase mutant	This study
Δ*napA*:Δ*narGH*	Double dissimilatory nitrate reductase mutant	This study
Δ*nirS*	Nitrite reductase mutant	This study
Δ*narX*	Nitrate sensor mutant	This study
Tn::*norC*	Nitric oxide reductase transposon mutant	22
Δ*rhlI*	Rhamnolipid autoinducer synthesis mutant	7
fliC	Flagellin type B mutant	21
Δ*narXL*-C	Δ*narXL*::mini-Tn*7*-*narXLK1′*; insertion of WT copy of *narXLK1* downstream of the *glmS* site in the nitrate sensor-response regulator mutant	This study
Δ*narX*-C	Δ*narX*::mini-Tn*7*-*narXLK1′*; insertion of WT copy of *narXLK1* downstream of the *glmS* site in the nitrate sensor mutant	This study
Δ*narL*-C	Δ*narL*::mini-Tn*7*-*narXLK1′*; insertion of WT copy of *narXLK1* downstream of the *glmS* site in the nitrate response regulator mutant	This study
Δ*narGH*-C	Δ*narGH*::mini-Tn*7*-*narX′K1K2GHJI*; insertion of WT copy of *narX′K1K2GHJI* downstream of the *glmS* site in the membrane nitrate reductase mutant	This study
Δ*napA*-C	Δ*napA*::mini-Tn*7*-*napEFDABC*; insertion of WT copy of *napEFDABC* downstream of the *glmS* site in the periplasmic nitrate reductase mutant	This study
Δ*napA*:Δ*narGH*-C	Δ*napA*:Δ*narGH*::mini-CTX-*napEFDABC*::mini-Tn*7*-*narX′K1K2GHJI*; insertion of WT copy of *napEFDABC* at the *attB* site and insertion of WT copy of *narX′K1K2GHJI* downstream of the *glmS* site in the double periplasmic and membrane nitrate reductase mutant	This study
P. aeruginosa PA14		
PA14	WT strain	47
Δ*narXL*	Nitrate sensor-response regulator mutant	This study
Δ*narGH*	Membrane nitrate reductase mutant	This study
Δ*narL*	Nitrate response regulator mutant	This study
Δ*napA*	Periplasmic nitrate reductase mutant	This study
Δ*narGH*-C	Δ*narGH*::mini-Tn*7*-*narX′K1K2GHJI*; insertion of WT copy of *narX′K1K2GHJI* downstream of the *glmS* site in the membrane nitrate reductase mutant	This study

[a] WT, wild type.

tants in PAO1 based on the inability to grow under anaerobic conditions with nitrate as a terminal electron acceptor (22).

Complementation of PAO1 *nar*, *nap*, and *nir* mutants was performed in single copy by integration into the chromosome. Integration at the *attB* site was performed as described previously (29) or by mini-Tn*7* insertion at the *att* Tn*7* site located downstream of *glmS* using mini-Tn*7* elements that carry the respective chromosomal DNA fragments (10). Complementing genes were expressed from their native promoters.

Swarming motility. Swarming medium consisted of 0.5% (wt/vol) Bacto agar, 8 g/liter nutrient broth, and 5 g/liter glucose (49). Swarm plates were allowed to set overnight at room temperature. Cultures were grown overnight aerobically in LB and adjusted to an optical density at 660 nm (OD_{660}) of 1.1. Swarm plates were inoculated with 1 μl of cell suspension on the center of the agar surface and incubated at 37°C for 18 h. The zone of swarming was measured and photographed. Each strain was assayed in triplicate platings, and three experiments were performed.

Swimming motility. Swimming medium consisted of 10 g/liter tryptone, 5 g/liter NaCl, and 0.3% (wt/vol) Bacto agar (49). Swim plates were made the day of use at a thickness of 3 mm and allowed to dry at room temperature for 3 h. Cultures were grown overnight aerobically in LB and adjusted to an OD_{660} of 1.1. Swim plates were inoculated with a sterile needle into the middle of the agar and incubated at 30°C for 24 h, covered in plastic wrap. The zone of swimming was measured and photographed. As with the swarming assays, each strain was assayed in triplicate platings, and three experiments were performed.

Rhamnolipid production. To quantify the amount of rhamnolipid produced by each strain, the orcinol rhamnolipid assay was used (40, 45). Cultures were grown overnight in peptone-tryptic soy broth. Cells (1 ml) were harvested, washed, and resuspended in an equal volume of modified Guerra-Santos (GS) (25) medium. GS medium was inoculated with overnight cultures to an OD_{660} of 0.15, and cultures were incubated for 82 h. Cultures were harvested by centrifugation at 16,000 × *g* for 5 min, and the supernatant was filtered with a Millipore 0.22-μm filter. Supernatant (100 μl) was extracted with 200 μl of double-distilled water (ddH_2O) and 600 μl of diethyl ether and mixed by shaking for 30 s, and the ether phase was removed and saved. The aqueous phase was extracted twice more with diethyl ether, and the ether layer was removed each time and saved. The ether layers were pooled (approximately 1,800 μl) and extracted with 300 μl of 20 mM HCl. The ether layer containing the extracted rhamnose was transferred to a fresh tube and allowed to evaporate to dryness in an N_2 atmosphere overnight. Dried rhamnose was resuspended in 1 ml of ddH_2O for quantitation in the orcinol assay. The orcinol assay was carried out by mixing 100 μl of extracted sample with 100 μl of 1.6% orcinol and 800 μl of 60% H_2SO_4. The solution was heated at 80°C for 30 min and allowed to cool to room temperature. After 10 min, the OD_{420} was recorded and the rhamnose concentration was determined by comparison to a standard curve generated with a series of rhamnose concentrations.

Static petri dish biofilms. Cultures were grown overnight in LB medium and subcultured the following morning. Organisms were allowed to grow to early stationary phase (OD_{660} = 1.2) and diluted to an OD_{660} of 0.5 in modified FAB-citrate medium (63). Two milliliters of diluted culture was used to inoculate 35-mm culture dishes with glass coverslips incorporated into the bottom (MatTek Corp.), and the dishes were incubated at 37°C for 24 h aerobically. Biofilms were gently washed by replacement modified FAB-citrate medium, 1 ml at a time for three washes, followed by staining for 5 min with 1 ml of SYTO 9 and propidium iodide, as described by the manufacturer (Molecular Probes, Inc., Eugene, OR). For each biofilm, five independent 250- by 250-μm fields at ×40 magnification were imaged by confocal microscopy, with approximately 24 1- to 2-μm optical sections analyzed per field. Data were analyzed with Matlab software. Experiments were performed in triplicate.

***P. aeruginosa*-*C. elegans* interactions.** *C. elegans* Bristol N2 wild-type strain was cultured as described previously (6, 59). A 2-ml overnight culture of the PA14 strains, as well as *E. coli* OP50, was grown in LB, and 10 μl of culture was applied to the appropriate agar type in a 5.5-cm petri plate. After 16 h at 37°C and an additional 24 h at 25°C, each plate was seeded with 20 to 30 L4-stage *C. elegans* organisms. The slow killing assay was performed with NG medium (60). Once the *C. elegans* organisms were seeded, they were allowed to incubate at 25°C. Plates were scored after 3 days, and three replicates per experiment were performed. A worm was considered to be dead when it no longer responded to touch with a metal probe. The fast killing assay was performed as described for the slow killing assay, except that high-osmolarity PGS medium was used (60). Plates were scored after 8 h, and three replicates per experiment were used (60). Results are presented as percentages of live organisms compared to survival on the *E. coli* OP50 control strain.

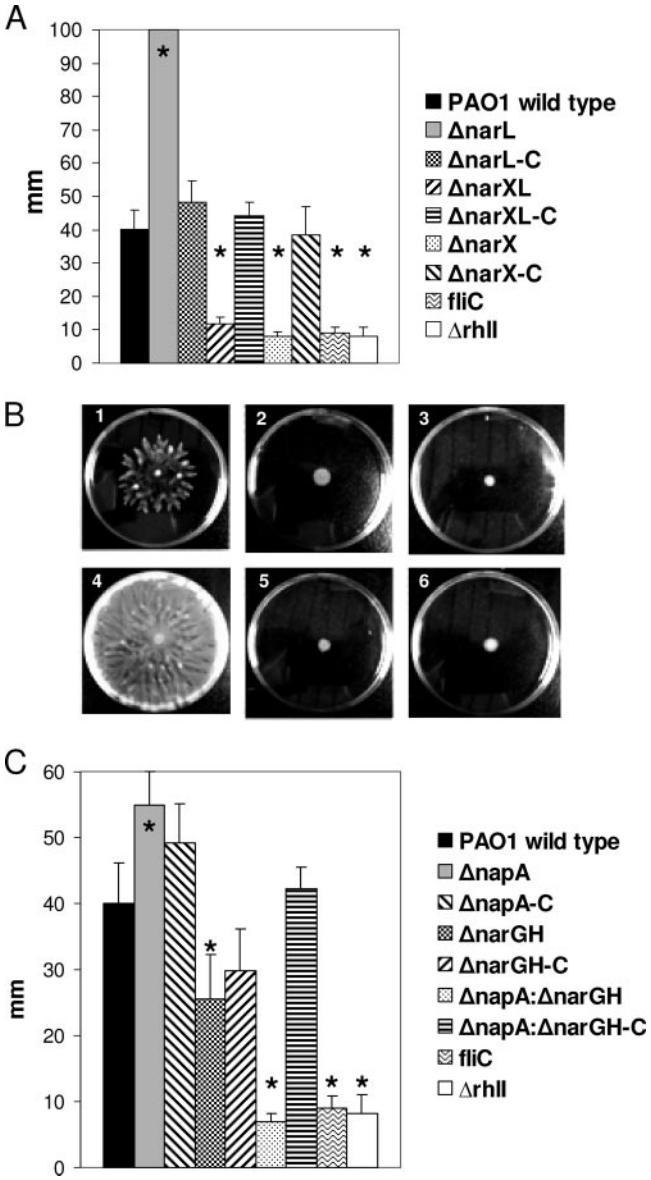

FIG. 2. Swarming in the regulatory and nitrate reductase mutants. (A) Swarm assay of PAO1 *nar* regulation mutants. *fliC* and Δ*rhlI* mutants were used as negative control strains. Each mutant was tested in quadruplicate, and the data represent the mean ± SD from three independent experiments. (B) Representative examples of the swarm assay of *nar* regulation mutants after 18 h. 1, PAO1 wild type; 2, PAO1:Δ*narXL*; 3, PAO1:Δ*narX*; 4, PAO1:Δ*narL*; 5, PAO1:*fliC*; 6, PAO1:Δ*rhlI*. (C) Swarm assay of PAO1 *nar* and *nap* nitrate reductase mutants and control strains. Each mutant was tested in quadruplicate, and the data represent the mean ± SD from three independent experiments.

Statistical analysis. Student's *t* test was performed to determine statistical significance between pairs of experimental groups. A P value of <0.05 was considered statistically significant. In each figure, an asterisk indicates a statistically significant difference from the wild-type value.

RESULTS

***nar* mutants display altered swarming.** Swarming in *P. aeruginosa* is mediated primarily via flagellar motion across a solid surface, facilitated by the production of the biosurfactant

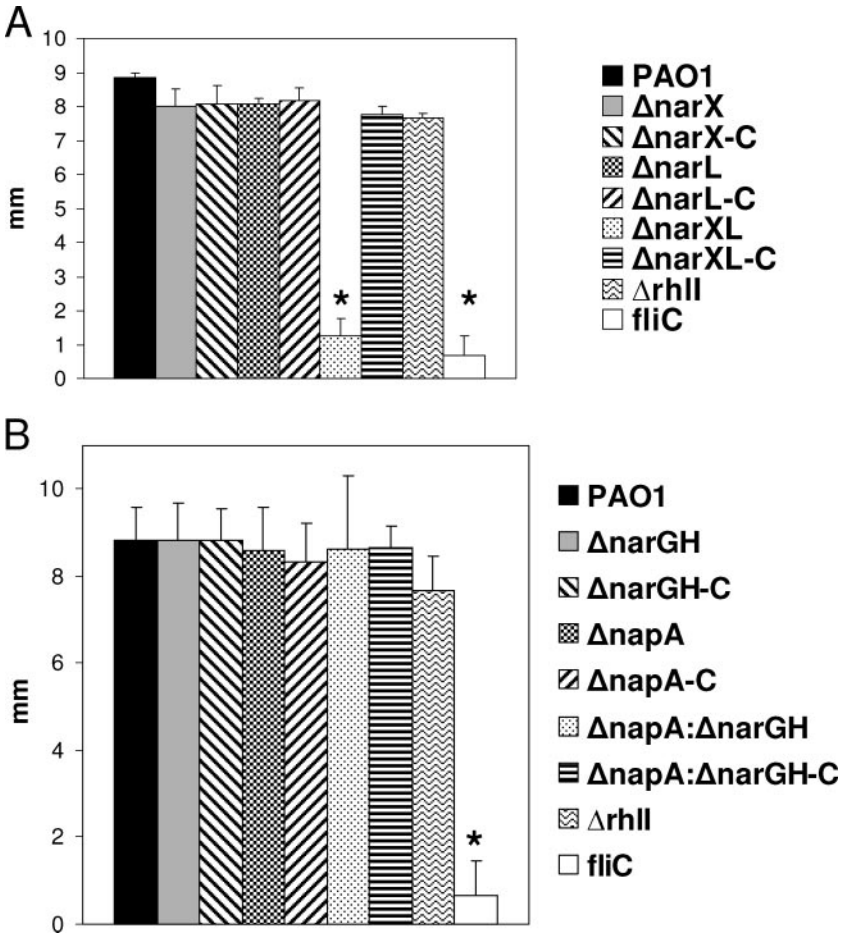

FIG. 3. Swimming in the regulatory and nitrate reductase mutants. (A) Swim assay of nitrate sensor-regulator mutants. Swimming distance was measured after 24 h. (B) Swim assay of *nar* and *nap* nitrate reductase mutants. In all cases, each strain was tested in quadruplicate and the data represent the mean ± SD from three independent experiments.

rhamnolipid. Since rhamnolipid production is modulated by nitrogen metabolism (18, 38, 40, 41), we examined swarming in *nar* mutants. Zones of swarming were measured for each strain after 18 h of aerobic incubation at 37°C. For clarity of presentation, data on nitrate sensor-response regulator mutants and nitrate reductase mutants are shown separately. Compared to wild-type PAO1, the regulatory mutants Δ*narX* and Δ*narXL* demonstrated significantly decreased swarming ($P < 0.001$), similar to a rhamnolipid synthase mutant, Δ*rhlI* (7), and a *fliC* flagellar mutant (21), which were used as negative control strains. In contrast, the Δ*narL* mutant produced an increased zone of swarming compared to wild-type PAO1 (Fig. 2A and B), so that by 18 h it had reached the edge of the 100-mm petri plate. Representative images of hyperswarming by the Δ*narL* mutant and decreased swarming by the Δ*narX* and Δ*narXL* mutants are shown in Fig. 2B. Swarming by the periplasmic nitrate reductase mutant, Δ*napA*, was slightly elevated compared to that of wild-type PAO1 ($P < 0.001$), while the membrane nitrate reductase mutant, Δ*narGH*, was deficient in swarming ($P < 0.01$). However, swarming was completely ablated ($P < 0.001$) in the double nitrate reductase mutant, Δ*napA*:Δ*narGH*, comparable to the negative control strains, Δ*rhlI* and *fliC* (Fig. 2C). In each case, genetic complementation restored the swarming phenotype of the mutants to that of the wild type (Fig. 2A and C). These data demonstrate that the nitrate sensor NarX is required for swarming. Furthermore, swarming is dependent on nitrate reduction.

PAO1:Δ*narXL* is deficient in swimming motility. Unlike swarming, swimming motility requires flagella but not rhamnolipid production. Swim motility plate assays were performed to investigate whether the different swarm phenotypes seen were a result of altered flagellum-induced motility. Regulatory mutants, Δ*narL* and Δ*narX*, had swim zones comparable to that of PAO1. Interestingly, Δ*narXL* showed a marked decrease ($P < 0.001$) in swimming that was comparable to the *fliC* negative control strain (Fig. 3A). These results suggest that decreased swarming in Δ*narXL* may be due, in part, to deficient flagellar motion, as indicated by decreased swimming. Unlike swarming, none of the nitrate reductase mutants displayed diminished swimming (Fig. 3B). As predicted, nitrate supplementation of swim medium did not enhance swimming of wild-type PAO1 or any of the mutants (data not shown), since swimming does not involve the production of rhamnolipid. The reduced swimming motility by the double nitrate sensor-regulator mutant Δ*narXL* suggests that both a functional nitrate sensor and a regulator are required for flagellar

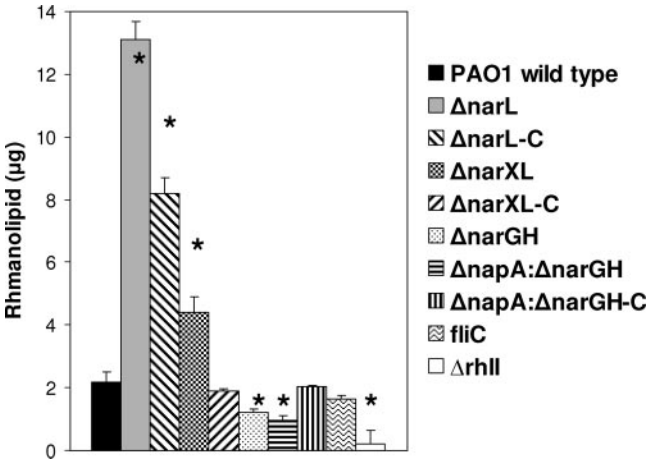

FIG. 4. Quantitation of rhamnolipid production with the orcinol assay. Supernatants from wild-type PAO1 and each mutant were tested in duplicate, and the data represent the mean ± SD from three independent experiments.

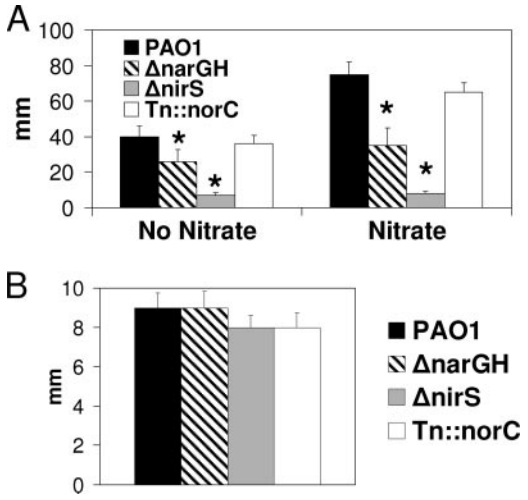

FIG. 5. Nitrate supplementation does not enhance swarming of the membrane nitrate reductase mutant (Δ*narGH*) and the nitrite reductase mutant (Δ*nirS*). Swarming (A) and swimming (B) of *nar* and *nir* mutants are shown.

movement. Cross talk with other sensors or response regulators, respectively, may explain why the individual Δ*narL* and Δ*narX* mutants swam normally.

Overproduction of rhamnolipid correlates with hyperswarming by the Δ*narL* mutant. Unlike the reduced swimming seen with Δ*narXL*, Δ*narL* demonstrated swimming at a level comparable to that of the wild type (Fig. 3A). This suggested that flagellar motility was likely not playing a role in hyperswarming by Δ*narL*. We therefore examined the other major contributor to swarming in *P. aeruginosa*, rhamnolipid production. To assess whether increased swarming by Δ*narL* was correlated with overproduction of rhamnolipid, we quantitated rhamnose levels as a correlate of rhamnolipid production in the wild type and the *nar* mutants using the orcinol assay, as described by Ochsner (39) (Fig. 4). The rhamnolipid-deficient Δ*rhlI* mutant (7) was used as a negative control strain. The Δ*narL* strain produced significantly (approximately sixfold) more rhamnolipid than did the wild type ($P < 0.001$). Complementation of the mutant with *narXL* significantly reduced rhamnolipid production compared to Δ*narL* ($P < 0.02$), although levels in the complemented strain still exceeded wild-type levels ($P < 0.001$). The weakest swarmer, Δ*napA*:Δ*narGH*, produced significantly lower levels of rhamnolipid than did the wild type ($P < 0.02$) and was comparable to the Δ*rhlI* negative control strain ($P = 0.06$ [not significant {NS}]). Interestingly, Δ*narXL* produced approximately twice as much rhamnolipid as did the wild type ($P < 0.001$); rhamnolipid production by the complemented mutant was equivalent to that of the wild type ($P = 0.13$ [NS]). These data suggest that increased rhamnolipid production may play a role in hyperswarming by Δ*narL* and that reduced rhamnolipid production may contribute to diminished swarming by Δ*narGH* and Δ*napA*:Δ*narGH*. The reduced swarming by Δ*narXL* cannot be attributed simply to a reduction in rhamnolipid production, since its production is elevated compared to that of the wild type.

Nitrate augmentation of swarming requires membrane nitrate reductase. Nitrate stimulates rhamnosyl transferase A (*rhlA*) expression (18) in *P. aeruginosa*. To gauge the effect of nitrate supplementation on swarming, 100 mM KNO_3 was added to swarming agar. Preliminary studies demonstrated that this level of nitrate supported robust anaerobic growth of strain PAO1 and had no effect on aerobic growth. A markedly increased zone of swarming was seen not only in wild-type PAO1 ($P < 0.05$) but also in Δ*narXL*, Δ*narX*, and Δ*napA* mutants. In contrast, Δ*narGH* and Δ*napA*: Δ*narGH* mutants did not have an increased zone of swarming when grown on swarm medium supplemented with 100 mM KNO_3 compared to nonsupplemented swarm medium. The zone of swarming for Δ*narGH* was 26 ± 6 mm (mean ± standard deviation [SD]) in standard swarming medium, compared to 35 ± 8 mm in nitrate supplemented medium (Fig. 5A).

Nitric oxide production is essential for swarming but not swimming in *P. aeruginosa*. The swarming and swimming assays were performed aerobically, indicating that the motility defects observed were not likely due to diminished anaerobic energy metabolism. This suggested that nitrate reduction regulated swarming, possibly via a signaling mechanism generated by nitrate dissimilation. One possible signaling molecule is nitric oxide (NO), the product of nitrite reductase.

To determine whether swarming was affected by NO production, a deletion mutation was created within the *nirS* gene in the nitrite reductase operon. Swarming by Δ*nirS* was compared to that of wild-type PAO1 and a transposon mutant mutated in nitric oxide reductase (22) (Tn::*norC*), which is immediately downstream of nitrite reductase in the nitrate dissimilatory pathway. On conventional swarming medium and swarming medium supplemented with nitrate, the nitrite reductase mutant showed significantly decreased swarming compared to that of wild-type PAO1 (Fig. 5A, left panel), similar to that of Δ*napA*:Δ*narGH* in Fig. 2C. In contrast, Tn::*norC* swarmed comparably to PAO1. Nitrate supplementation increased swarming of both wild-type PAO1 and Tn::*norC* but not of either Δ*narGH* or Δ*nirS*. Swimming motility was comparable to that of wild-type PAO1 for all of the mutants (Fig. 5B). This suggests that NO may be an important signaling

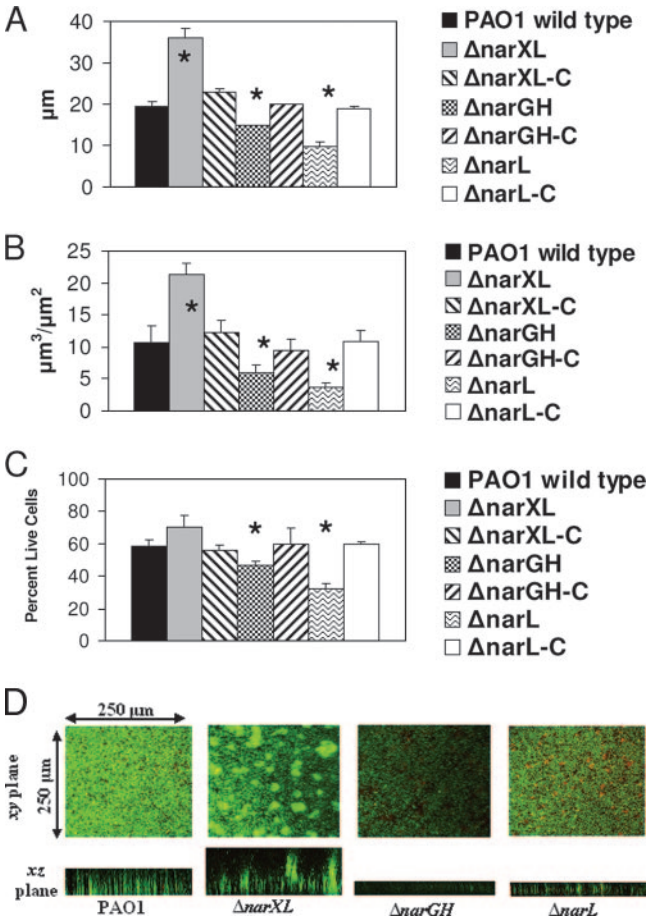

FIG. 6. Analysis of static biofilms. (A) Average maximum biofilm thickness. (B) Average total biofilm biomass. (C) Average percent live cells. Biofilms were analyzed with Matlab software. The data represent the mean ± SD from three independent experiments. (D) Images of representative biofilms stained with SYTO 9 (green, live cells) and propidium iodide (red, dead cells) (Molecular Probes, Inc.). Biofilms were incubated at 37°C in FAB-citrate medium after 24 h. Each field size was 250 μm by 250 μm at ×40 magnification. Images shown are overlays of optical sections taken in the *xy* plane (top panel), to reveal microcolony formation, and the *xz* plane (bottom panel), to measure biofilm depth. 1, PAO1 wild type; 2, PAO1:Δ*narXL*; 3, PAO1:Δ*narGH*; 4, PAO1:Δ*narL*.

molecule for swarming, by activation of rhamnolipid production through induction of *rhlAB* expression, but does not modulate swimming.

***Nar* mutants display altered biofilm architecture.** Since swarming and swimming are critical for both initiation of biofilm formation and dispersal of organisms from biofilms (43, 48), we investigated whether the *nar* mutants displayed an altered biofilm architecture compared to wild-type PAO1. The Δ*narXL* mutant displayed increased average thickness ($P = 0.006$) and increased average total biomass ($P = 0.002$) (Fig. 6A and B, respectively) compared to wild-type PAO1 and other nitrate reductase mutants, while the percentage of live cells in the Δ*narXL* biofilm was comparable to that of the wild type (Fig. 6C). Formation of microcolony aggregates by Δ*narXL* with increased thickness distinguished this mutant from the other strains (Fig. 6D). Genetic complementation with *narXL* reduced biofilm thickness and biomass to near-wild-type levels and diminished microcolony formation. In contrast to Δ*narXL*, average thickness, average total biomass, and percentage of live cells were each significantly reduced in Δ*narGH* and Δ*narL* compared to the wild type (Fig. 6). Genetic complementation of Δ*narGH* and Δ*narL* with *narGH* and *narL*, respectively, restored biofilm thickness, biofilm biomass, and the percentage of live cells to wild-type levels. The data suggest that the two-component sensor-regulator mutant Δ*narXL* does not readily disperse due to its inefficient flagellar movement, resulting in an increased biofilm mass. In contrast, the regulator mutant Δ*narL* cannot form a robust biofilm, as a consequence of the overproduction of rhamnolipid. The data also suggest that biofilm formation by the membrane nitrate reductase mutant Δ*narGH* is inhibited due to its inability to dissimilate nitrate.

Membrane nitrate reductase is required for *P. aeruginosa* virulence in *C. elegans*. Altered motility and biofilm formation in the nitrate reductase mutants led us to examine whether the mutants demonstrated diminished virulence. The nematode *C. elegans* has been used as a surrogate host to examine the virulence of *P. aeruginosa* (1, 47, 60, 61). Since wild-type PAO1 is does not readily kill *C. elegans*, we constructed a set of deletion mutants in the virulent *P. aeruginosa* PA14 strain. Δ*narXL*, Δ*narL*, Δ*narGH*, Δ*napA*, and Δ*napA*:Δ*narXL* mutations were constructed in *P. aeruginosa* PA14 by allelic exchange (28). All mutations were confirmed by Southern analysis, and the mutants displayed the growth profiles seen for the PAO1 mutants (data not shown).

PA14:Δ*narGH* is avirulent in *C. elegans*. To determine whether any of these nitrate reductase mutants were deficient in infecting and killing *C. elegans*, the slow kill assay was performed on NG minimal medium, in which the mutants were compared to negative control *E. coli* OP50 and virulent wild-type *P. aeruginosa* PA14. After 3 days, *C. elegans* seeded on PA14:Δ*narGH* showed 94% viability, a significantly greater survival level compared to that on wild-type PA14, which was 15% ($P = 0.03$) (Fig. 7A). The remaining mutants, Δ*narXL*, Δ*narL*, Δ*napA*, and Δ*napA*:Δ*narXL*, killed *C. elegans* in the slow kill assay comparably to wild-type PA14.

To determine whether any of the nitrate reductase mutants were deficient in inducing toxicity-mediated death in *C. elegans*, the fast kill assay was performed on high-osmolarity PGS medium. Plates were scored after 8 h to determine *C. elegans* viability. *C. elegans* on *E. coli* OP50 remained 100% viable, compared to the 18% viability of *C. elegans* on PA14 ($P = 0.006$). In contrast, *C. elegans* seeded on PA14:Δ*narGH* was 94% viable. This level of survival was similar to that seen on *E. coli* OP50 ($P = 0.4$ [NS]) and was significantly higher than that on PA14 ($P = 0.03$). Again, the Δ*narXL*, Δ*narL*, Δ*napA*, and Δ*napA*:Δ*narXL* mutants caused toxicity-mediated death in *C. elegans* at levels comparable to that of PA14 (Fig. 7B). Genetic complementation of PA14:Δ*narGH* restored killing in each assay to wild-type PA14 levels (Fig. 7C and D). Because PA14:Δ*narGH* was avirulent in the *C. elegans* model, we compared its swarming and swimming motilities with wild-type PA14. The PA14:Δ*narGH* mutant displayed reduced swarming motility and normal swimming motility compared to wild-type PA14, a behavior similar to that of the PAO1:Δ*narGH* mutant (Fig. 2C). The virulence studies with *C. el-*

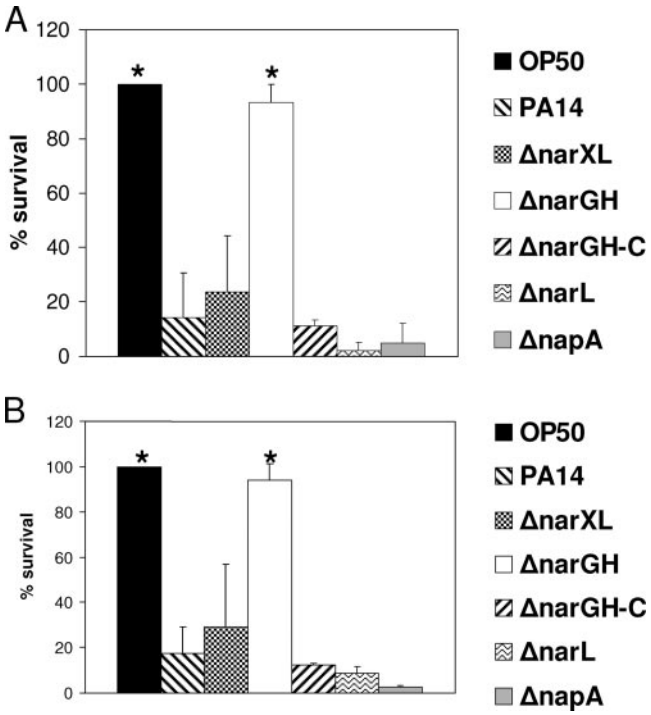

FIG. 7. Virulence of mutants in *C. elegans*. (A) Slow kill assay with *C. elegans*. (B) Fast kill assay with *C. elegans*. The data represent the mean ± SD from three independent experiments.

egans demonstrate that the membrane nitrate reductase, but not the nitrate sensor-response regulator, is important for virulence in *C. elegans*. Thus, even in the absence of the response regulator NarL, a basal level of membrane nitrate reductase activity may generate sufficient NO to function as a signaling molecule for expression of virulence. This suggestion is supported by the observation that the nitrite reductase mutant PA14:Δ*nirS* is comparably avirulent in both the fast and slow kill assays in *C. elegans* (data not shown). A model for the roles of the nitrate sensor-response regulator and the components of the nitrate dissimilation pathway in motility, biofilm formation, and virulence in *P. aeruginosa* is presented in Fig. 8.

DISCUSSION

Nitrate sensor-response regulator mutants. Transcriptional profiling in *E. coli* (11, 44) indicates a regulatory role for NarL that goes beyond anaerobic energy metabolism, with NarL activating, directly or indirectly, the transcription of 51 operons and the repression of 41 operons. A global analysis of the NarX/NarL regulon has not yet been reported for *P. aeruginosa*. As with *E. coli*, the phenotypes of the regulatory mutants strongly suggest that the *P. aeruginosa* PAO1 NarX/NarL regulon is broader than genes involved in anaerobic energy metabolism.

Interestingly, and rather unexpectedly, the phenotypes of the nitrate sensor-response regulator Δ*narXL* double mutant and the response regulator Δ*narL* mutant differed. The increased biofilm phenotype displayed by the Δ*narXL* double mutant suggests an inability of the mutant to disperse from the biofilm once incorporated into the biofilm structure, due to inefficient motility. In contrast, the Δ*narL* response regulator mutant displayed a hyperswarming phenotype as a likely consequence of the overproduction of rhamnolipid, which in turn modulated biofilm formation (5, 16, 31).

There are at least two potential mechanisms for elevated rhamnolipid production by Δ*narL*. In the absence of its cognate response regulator, NarL, NarX may activate an alternative response regulator that, either directly or indirectly, activates the rhamnosyltransferase operon *rhlAB* to drive rhamnolipid production. Analysis of the domain architectures of bacterial response regulators performed by Galperin (24) identified NarL-like response regulators to be among the more abundant classes in bacteria. Including NarL, the PAO1 genome contains 16 probable NarL-like response regulators. Other than NarL, the only one characterized to date is the global regulator GacA (50, 53, 61); the remaining 14 are "hypothetical." One or more of this pool of response regulators may possess a receiver domain that could lead to recognition and activation by NarX. This type of sensor-regulator cross talk may also contribute to regulation of flagellum-mediated swimming, since the double Δ*narXL* mutant was defective in swimming, but the respective single deletion mutants mutated in either *narX* or *narL* swam comparably to wild-type PAO1.

Alternatively, NarL may repress *rhlAB* expression. Induction of rhamnolipid production as a consequence of nitrate depletion may be directly related to diminished production and/or diminished phosphorylation of NarL (Fig. 8A). Observations in both *E. coli* and *P. aeruginosa* provide support for this potential mechanism. The consensus NarL 7-2-7 binding motif has been identified upstream of the membrane nitrate reductase operon encoded by *narK1K2GHJI* in *P. aeruginosa* (15, 57, 58). However, studies with *E. coli* have also shown that NarL can bind to sites other than the preferential 7-2-7 site (13, 14), to either promote or repress transcription.

In silico analysis of the *P. aeruginosa* PAO1 genome using the web-based Regulatory Sequence Analysis Tools program (http://rsat.ulb.ac.be/rsat/) demonstrates that, in addition to the regulatory region of the *narK1K2GHJI* operon, only six sites were identified upstream of a predicted coding region with the full 7-2-7 NarL consensus binding motif (maximum of one mismatch). One 7-2-7 site identified is the region upstream of *nuoA-N* encoding NADH dehydrogenase complex I, which participates in electron transfer via ubiquinone to the membrane-bound nitrate reductase NarGHI (4, 68) and is essential for anaerobic growth in *P. aeruginosa* (22). NADH dehydrogenase complex I and ubiquinone perform similar functions aerobically by coupling to the electron transport chain of the bacterial cytochrome complex (51). In *P. fluorescens*, *nuo* mutants are deficient in plant root colonization (9), suggesting that NADH dehydrogenase complex I may be involved in aerobic motility and biofilm formation in addition to involvement in anaerobic growth. Transcriptome analysis of a NarX/NarL mutation in *E. coli* demonstrated that the *nuo* operon is part of the NarL regulon (11). A second 7-2-7 site is upstream of PA3349, a probable chemotaxis protein that could also influence motility.

In contrast to the small number of 7-2-7 consensus sites, over 10% of the intergenic regions in *P. aeruginosa* PAO1 have at least one consensus NarL half-site. The overall importance of the full 7-2-7 to half-site recognition in transcription regulation

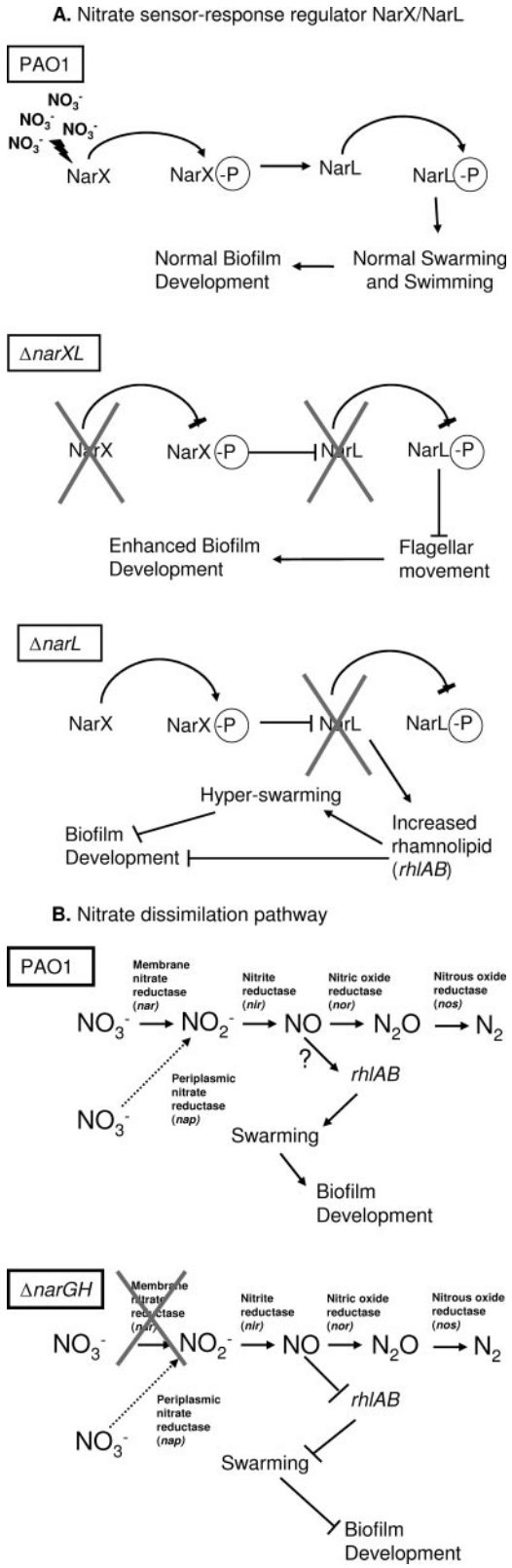

FIG. 8. Proposed models for nitrate regulation of motility, biofilm formation, and virulence in *P. aeruginosa* PAO1. (A) Nitrate sensor-response regulator function in motility and biofilm formation. (Top) In wild-type *P. aeruginosa* PAO1, nitrate activates the autophosphorylation of the nitrate sensor NarX. Transfer of the phosphate to the receiver domain of the response regulator NarL results in either activation or repression of target operon transcription. Pathways leading to motility and biofilm development are operative. (Middle) In the double mutant Δ*narXL*, loss of the nitrate sensor NarX results in an inability to activate NarL. Swimming and swarming are both diminished in Δ*narXL*, suggesting impairment of flagellar synthesis and/or function. As a consequence, biofilm thickness and biomass are enhanced compared to the wild type, possibly due to a defect in organism dispersal from the biofilm. The membrane nitrate reductase operon *narK1K2GHJI* is not activated in the nitrate sensor-response regulator double mutant. However, derepression of the periplasmic nitrate reductase operon *napEFDABC* in Δ*narXL* permits growth under microaerobic and anaerobic conditions (unpublished observations), allowing biofilm formation. (Bottom) In the response regulator mutant Δ*narL*, the cognate sensor target is absent. Inability of NarX to activate NarL results in a significant increase in rhamnolipid production. As a consequence, Δ*narL* displays a hyperswarming phenotype that likely diminishes its ability to form a biofilm. It is unknown whether an alternative sensor activates NarL to stimulate rhamnolipid production or whether phospho-NarL represses rhamnolipid production. Cross talk with other sensors or response regulators that modulate flagellar function may also explain why the individual Δ*narL* and Δ*narX* mutants swam normally while swimming of the double mutant Δ*narXL* was impaired. Despite alterations in biofilm formation and swimming in the regulatory mutants, all were as virulent as wild-type PAO1 in *C. elegans*. (B) Nitrate dissimilation in motility, biofilm formation, and virulence. (Top) In wild-type PAO1, both membrane nitrate reductase and periplasmic nitrate reductase contribute to nitrite formation. We propose that further reduction of nitrite to nitric oxide (NO) provides a signal to stimulate rhamnolipid production, resulting in swarming and normal biofilm formation. It is unknown whether the regulation of rhamnolipid synthesis by NO is direct or indirect. (Bottom) This model is supported by the phenotypes of the nitrate dissimilation pathway mutants. The membrane nitrate reductase mutant Δ*narGH* is defective in rhamnolipid production, swarming, and biofilm formation but not in swimming. Hence, it appears that NO is not involved in regulation of flagellar synthesis and/or function. While the periplasmic nitrate reductase mutant Δ*napA* shows no phenotypic differences from wild-type PAO1, swarming is completely ablated in the double nitrate reductase mutant Δ*narGH*:Δ*napA*. Swarming was also ablated in the nitrite reductase mutant Δ*nirS* but not in the nitric oxide reductase mutant Tn::*norC*. For clarity, the latter two mutants are not indicated in the diagram. The absence of an adequate level of NO signaling results in avirulence of both Δ*narGH* and Δ*nirS* in *C. elegans*.

by NarL in *P. aeruginosa* is unknown. However, mutation of a single NarL heptamer half-site in the *P. aeruginosa hemA* intergenic region was sufficient to abolish *hemA* expression (35). Relevant to patterns of rhamnolipid production observed in the present study, NarL half-sites are found upstream of genes for both the response regulator *rhlR* and the rhamnosyltransferase component *rhlA*. Future studies will determine the role of the NarL half-sites in regulation of global gene expression in *P. aeruginosa*.

Complex phenomena in *P. aeruginosa* such as swarming and biofilm formation are certain to be controlled by a hierarchy of regulatory mechanisms operating at multiple levels. Transcriptional profiling, accompanied by biochemical analysis of response regulator function, will begin to address the respective role of NarL in regulation of swarming and biofilm formation and help determine whether its regulatory role is direct or indirect.

Nitrate reductase mutants. Our initial expectation was that the phenotypes of the nitrate reductase mutants would be similar, if not identical, to that of the double nitrate sensor-

regulator mutant. However, unlike the nitrate sensor-regulator mutant, the membrane nitrate reductase mutant and the membrane/periplasmic nitrate reductase double mutant swam normally. The major motility defect in the nitrate reductase mutants was in swarming. Since the swarming assay was performed aerobically, bioenergetic constraints were likely not contributing significantly to the motility phenotypes. While portions of the swarming colony may be microaerobic or anaerobic, we favored the interpretation that nitrate reduction provided a signal that enhanced swarming. An obvious choice of signal was NO, the product of nitrite reductase activity (Fig. 8B).

The role of NO as an intracellular signal in bacteria is well established in the regulation of the nitrate dissimilation pathway (2, 46). The regulatory role of nitrate metabolites, particularly NO, in response to oxidative and nitrosative stress in *P. aeruginosa* has also been investigated (52). Arai et al. (2) have demonstrated NO-responsive regulation of flavohemoglobin and suggest that the physiological role of flavohemoglobin in *P. aeruginosa* is detoxification of NO under aerobic conditions. A role for NO in regulation of pathways related to motility and biofilm formation is just beginning to be explored. A recent report by Barraud et al. (3) demonstrated that NO is important in the dispersal of *P. aeruginosa* biofilms and that exogenous NO can stimulate both swarming and swimming. In support of an intracellular signaling role for NO in swarming, the nitrite reductase mutant Δ*nirS* also displayed a severe swarming defect. However, the swarming of the nitric oxide reductase transposon mutant Tn::*norC* was comparable to that of the wild type. Furthermore, nitrate supplementation enhanced swarming of the wild type and Tn::*norC* but not of Δ*narGH* and Δ*nirS* (Fig. 5). Together with the observation that biofilm formation is diminished in Δ*narGH* (Fig. 6), genetic evidence provided herein supports a role for NO signaling in the regulation of *P. aeruginosa* motility and biofilm architecture.

Because *P. aeruginosa* PAO1 is not as virulent as strain PA14 in *C. elegans* (36), we utilized strain PA14 as the parent strain in the killing assays and for construction of selected deletion mutants. The most surprising observation was that the membrane nitrate reductase and nitrite reductase mutants were avirulent in the *C. elegans* model while the regulatory mutants and periplasmic nitrate reductase mutant were as virulent as the wild type. A possible explanation for this difference is that membrane nitrate reductase is expressed constitutively at low levels in the regulatory and periplasmic nitrate reductase mutants to provide the nitrite substrate for NO generation by nitrite reductase and downstream signaling of virulence factor expression. This explanation is supported by the avirulence of the nitrite reductase mutant in *C. elegans*. Nitrate reductase function contributes to virulence in other pathogenic bacteria as well. Membrane nitrate reductase mutants of *Salmonella enterica* serovar Typhi and *Mycobacterium bovis* BCG have a decreased survival rate in macrophages and diminished tissue persistence (12, 23, 64), suggesting that the membrane nitrate reductase is important in intracellular survival and/or replication.

In summary, we describe a role for nitrate metabolism in motility, biofilm architecture, and virulence *P. aeruginosa* PAO1 by using a set of deletion mutants. The data suggest that the regulon of the two-component nitrate sensor-response regulator pair NarX/NarL extends beyond regulation of nitrate metabolism and influences the related phenomena of motility and biofilm formation. Furthermore, the data also suggest that nitrate dissimilation modulates motility, biofilm formation, and virulence of *P. aeruginosa* PAO1, seemingly independently of the regulatory function of NarX/NarL. Continued analysis of the mutants described in this study will increase our understanding of the connection between the basic metabolic pathways of nitrate dissimilation and aspects of *P. aeruginosa* biology related to its pathogenesis.

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