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Compensatory periplasmic nitrate reductase activity supports anaerobic growth of *Pseudomonas aeruginosa* PAO1 in the absence of membrane nitrate reductase

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Abstract

Nitrate serves as a terminal electron acceptor under anaerobic conditions in *Pseudomonas aeruginosa*. Reduction of nitrate to nitrite generates a transmembrane proton motive force allowing ATP synthesis and anaerobic growth. Inner membrane-bound nitrate reductase NarGHI is encoded within the *narK1K2GHJI* operon and the periplasmic nitrate reductase NapAB is encoded within the *napEFDABC* operon. The role of the two dissimilatory nitrate reductases in anaerobic growth, and the regulation of their expression were examined by using a set of deletion mutants in *P. aeruginosa* PAO1. NarGHI mutants were unable to grow anaerobically, but plate cultures remained viable up to 120 hr. In contrast, nitrate sensor-response regulator mutant $\Delta narXL$ displayed growth arrest initially, but resumed growth after 72 hr and reached early stationary phase in liquid culture after 120 hr.

Genetic, transcriptional, and biochemical studies demonstrated that anaerobic growth recovery by the NarXL mutant was the result of NapAB periplasmic nitrate reductase expression. A novel transcriptional start site for *napEFDABC* expression was identified in the NarXL mutant grown anaerobically. Furthermore, mutagenesis of a consensus NarL-binding site monomer upstream of the novel transcriptional start site restored anaerobic growth recovery in the NarXL mutant. The data suggest that during anaerobic growth of wild type *P. aeruginosa* PAO1, nitrate response regulator NarL directly represses expression of periplasmic nitrate reductase, while inducing maximal expression of membrane nitrate reductase.

INTRODUCTION

Pseudomonas aeruginosa growth in biofilms is characterized by its ability to carry out respiration in oxygen-limited environments. Important for anaerobic respiration is the dissimilatory enzyme pathway that catalyzes the reduction of nitrate (NO_3^-) to nitrogen gas (N_2). Reduction of either nitrate or nitrite provides energy for *P. aeruginosa* growth in the absence of oxygen, with nitrate reduction to nitrite via nitrate reductase contributing more significantly to proton motive force and, hence, energy production, than nitrite reduction or arginine fermentation (Berks *et al.*, 1995; Zumft, 1997).

Respiratory nitrate reduction to nitrite can be mediated by two different nitrate reductase complexes in *P. aeruginosa* (Berks *et al.*, 1995; Zumft, 1997): an inner membrane-bound nitrate reductase complex encoded by the *narK1K2GHJI* operon and a periplasmic nitrate reductase encoded by the *napEFDABC* operon. NarGHI forms the core membrane-bound

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nitrate reductase. The γ -subunit NarI, anchored in the inner membrane, accepts electrons from the quinol pool and transfers them via β -hemes to the β -subunit NarG. The α - and β -subunits NarGH extend into the cytoplasm and associate with a molybdopterin cofactor (Philippot & Hojberg, 1999) and the Snr-1 protein (Kerschen *et al.*, 2001). NarJ is a chaperone involved in NarGHI assembly (Liu & DeMoss, 1997), and NarK1K2 are thought to be involved in nitrate and nitrite transport (Goddard *et al.*, 2008; Sharma *et al.*, 2006; Wood *et al.*, 2002). NapAB comprises the periplasmic nitrate reductase, with NapC, a cytochrome *c*-type protein, likely involved in electron transfer to NapAB. NapD is essential for NapAB activity and is postulated to be a chaperone involved in NapA maturation prior to export into the periplasm (Potter, 2001). NapF appears to be a non-heme, iron-sulfur protein (Nilavongse *et al.*, 2006; Olmo-Mira *et al.*, 2004) implicated in an energy-conserving role coupled to the oxidation of ubiquinol (Brondijk *et al.*, 2002), and may also play a role in the post-translational maturation of NapA (Nilavongse *et al.*, 2006; Olmo-Mira *et al.*, 2004). The physiological role of NapE has not yet been defined.

Adjacent to the 5' - region of the *narK1K2GHJI* operon, and divergently transcribed in *P. aeruginosa*, is the *narXL* operon encoding the two-component nitrate sensor-response regulator proteins, NarX and NarL (Hartig *et al.*, 1999; Stewart *et al.*, 1989). NarXL is a classic two-component bacterial regulatory system in which a histidyl-aspartyl phosphorelay controls gene expression in response to specific environmental stimuli (Varughese, 2002). The periplasmic domain of the sensor protein, NarX, contains a highly conserved nitrate recognition region termed the P-box. Nitrate recognition by the P-box results in autophosphorylation of a conserved histidyl residue in the NarX cytoplasmic transmitter domain. Transfer of phosphate to the conserved aspartyl residue in the receiver domain of the response regulator NarL results in transcriptional regulation of target operons (Stewart, 2003). NarL, in conjunction with oxygen regulator Anr and N-oxide regulator Dnr, induce *narK1K2GHJI* operon expression in *P. aeruginosa* (Schreiber *et al.*, 2007). The regulation of *napEFDABC* expression in *P. aeruginosa* is less well understood.

Using a set of *P. aeruginosa* deletion mutants, we corroborate previous studies (Schreiber *et al.*, 2007) that NarXL activates the *narK1K2GHJI* operon, and we further show that a deletion in *narGH* resulted in anaerobic growth arrest. In contrast, a deletion in *narXL* resulted in subsequent growth recovery, which was correlated with the expression of the *napEFDABC* operon. A novel transcriptional start site for *napEFDABC* expression was identified in the NarXL mutant grown anaerobically. Furthermore, mutagenesis of a consensus NarL-binding site monomer upstream of the novel transcriptional start site also restored anaerobic growth recovery in the NarXL mutant. The data suggest that during anaerobic growth of wild type *P. aeruginosa* PAO1, nitrate response regulator NarL directly represses expression of periplasmic nitrate reductase, while inducing maximal expression of membrane nitrate reductase.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

P. aeruginosa PAO1 was used throughout this study. Deletion mutants were generated in *P. aeruginosa* PAO1 as described in (Van Alst *et al.*, 2007), using techniques described by Schweizer and colleagues (Hoang *et al.*, 1998). All strains, corresponding mutants and plasmids used in this study are listed in Supplemental Table 1.

Strains were stored at -80°C in 10% skim milk. Aliquots were plated onto either PTSB (50 g/L peptone, 2.5 g/L tryptic soy broth) agar plates or LB (5 g/L yeast extract, 10 g/L NaCl, and 10 g/L tryptone). Growth studies were performed using either reduced LB or reduced NY (25 g/L nutrient broth and 5 g/L yeast extract) (Difco, Detroit, MI) broth supplemented with 100 mM KNO_3 , 10 mM KNO_2 (VWR International, West Chester, PA) or 40 mM arginine (Sigma-

Aldrich, St. Louis, MO) as indicated. Reduced liquid medium was prepared by boiling the water for 10 minutes, bubbling N₂ gas through the prepared broth for 10 minutes, and sterilization by autoclaving. All antibiotics were obtained from Sigma-Aldrich. Carbenicillin was used at 400 µg/ml.

Aerobic overnight cultures were used to inoculate 200 ml of medium in a 500 ml baffled Erlenmeyer flask to an initial optical density at 660 nm (OD₆₆₀) of 0.05. Cultures were incubated in a shaking water bath at 250 rpm at 37°C. Anaerobic growth studies were performed in a sealed tent with a gas mix of 85% (v/v) nitrogen, 10% (v/v) hydrogen and 5% (v/v) carbon dioxide. Aerobic overnight cultures were used to inoculate 200 ml of reduced medium in 250 ml bottles at OD₆₆₀ of 0.05. Anaerobic cultures were grown at 37°C with mixing using a magnetic stir plate. For all growth studies, no more than 10% (v/v) of the starting culture volume was removed over the course of the experiment to monitor growth as measured by OD₆₆₀ (Wagner *et al.*, 2003).

Preliminary studies demonstrated that wild type *P. aeruginosa* PAO1 required at least 50 mM nitrate for optimal anaerobic growth, and 100 mM nitrate did not appear to exert a toxic effect during aerobic growth. Identical results were observed using NY medium instead of LB (data not shown), suggesting medium components in LB and NY other than nitrate did not influence anaerobic growth in these strains of *P. aeruginosa*. For all subsequent experiments, media were supplemented with 100 mM nitrate.

Recombinant DNA manipulations—Plasmid and cosmid DNA was isolated using the QIAprep spin kit (QIAGEN, Valencia, CA). Restriction endonucleases, T4 DNA polymerase, calf intestinal phosphatase, and T4 DNA ligase were used as specified by the supplier (New England Biolabs, Beverly, MA). DNA fragments were isolated from agarose gels using Freeze and Squeeze Kit (Bio-Rad, Hercules, CA). PCR was performed using the iProof polymerase kit (Bio-Rad) in an ABI model 2700 thermal cycler (Foster City, CA). Oligonucleotide primers (Sigma Genosys) used in this study are listed in Supplemental Table 2.

Complementation of PAO1 *nar* and *nap* mutants was performed in single copy by integration into the chromosome. Integration was performed as described previously (Choi *et al.*, 2005) by mini-Tn7 insertion at the *att* Tn7 site located downstream of *glmS* using mini-Tn7 elements that carry the respective chromosomal DNA fragments. Complementing genes were expressed from their native promoters.

Construction of *lacZ* transcriptional fusions

Both the intact *narXL* and Δ *narXL* constructs contain *narK1* and *narK2*, as well as the first 49 nucleotides of *narG*, with the putative *narL* box upstream of *narK1*. Transcriptional fusions Φ (*narXL-narG-lacZ*):pLP170 and Φ (Δ *narXL-narG-lacZ*):pLP170 were created by subcloning into the *Eco*RI site of plasmid pLP170 upstream of a promoterless *lacZ* (Preston *et al.*, 1997).

A *napD* transcriptional fusion was created using primers napoperon 1 (sense) and napoperon 2 (antisense) which amplified region *upnapE* containing *oprH5'*, *napE*, *napF*, and *napD5'* (Supplemental Figure 4A). *upnapE* was subcloned into pLP170 to generate Φ (*napD-lacZ*):pLP170. The construct was electroporated into either the wild type PAO1, PAO1: Δ *narXL*, and PAO1: Δ *narGH* backgrounds to evaluate *nap* operon expression via β -galactosidase activity. As a negative control, pLP170 alone was also electroporated into all three strains.

β-galactosidase assays

β-galactosidase assays (Preston *et al.*, 1997) were performed on organisms grown under either anaerobic or aerobic conditions over a 120 hr period. All solutions were freshly prepared on the day of the assay. Activity is expressed in arbitrary (Miller) units based on the formula $100 \times [A_{420}/(T \times V \times OD_{660})]$ where T = time in minutes, V = volume of reaction in ml, and OD₆₆₀ corresponds to the optical density of the culture used (Miller, 1977). Assays were performed in triplicate in two separate experiments.

RNA isolation, RT-PCR, and mapping of *nap* operon transcriptional start sites by 5' RACE primer extension

200 ml cultures were grown anaerobically in NY supplemented with 100 mM KNO₃. Both PAO1 and *ΔnarXL* were harvested at midlog phase (OD₆₆₀=0.6) at 8 and 96 hr, respectively; *ΔnarGH* was harvested at 96 hr (OD₆₆₀=0.12). Total RNA was isolated using RNA Whiz (Ambion; Austin, TX) per manufacturer's instructions. Contaminating genomic DNA was removed using Turbo DNA-free kit (Ambion) per the manufacturer's protocol.

RT-PCR was performed using Ambion RETROscript along with Ambion SuperTaq Plus. Removal of contaminating DNA was confirmed by PCR using *GroEL* oligonucleotides, *GroEL* F1 and *GroEL* R1, and omitting reverse transcriptase from the RT-PCR reaction. RNA integrity was confirmed by RT-PCR using *GroEL* primers, *GroEL* F1 and *GroEL* R1, which yield a 550 bp amplicon. Oligonucleotides used to detect *narH* transcripts were *narH* S nested 2 and *narH* AS nested 2, which yield a 390 bp amplicon. Oligonucleotides used to detect *napA* transcripts were *napA* RT-PCR S and *napA* RT-PCR AS, which yield a 677 bp amplicon.

For transcriptional mapping, 5' RACE was performed as per manufacturer's protocol (Invitrogen; Carlsbad, CA). Oligonucleotides used to identify the start of transcription upstream of *napEFDABC* were *nap* GSP-3, *nap* GSP-4, and *nap* GSP-5 which yielded two amplicons in PAO1:Δ*narXL* (400 bp and 150 bp) and one in wild type PAO1 (150 bp) grown under anaerobic conditions.

Nitrite Quantitation

Wild type PAO1 and *ΔnarXL* were grown in nitrate supplemented NY medium for 150 hr. Samples were taken and cells were removed by filtration. Nitrite concentration was determined in the filtrate for each strain at each time point against a standard curve of known nitrite concentrations using the Measure – iT High Sensitivity Nitrite Assay Kit (Molecular Probes).

Nitrate reductase activity

Nitrate reductase activity was determined in whole cell suspensions (MacGregor *et al.*, 1974; Stewart *et al.*, 2002). Cultures were grown anaerobically in NY supplemented with 100 mM KNO₃. To inhibit protein synthesis, 1.5 ml of 50 μg/ml chloramphenicol was added to 1.5 ml of culture. To inhibit further nitrate reduction by the membrane nitrate reductase NarGHI, 1.5 μl of 50 mM NaN₃ was added as indicated. Cells were centrifuged, washed twice, and re-suspended in an equal volume of 50 mM phosphate buffer, pH 7.2, and OD₆₆₀ was determined. An 800 μl aliquot of cells was mixed with 100 μl freshly prepared, 0.5 mg/ml methyl viologen solution. Nitrate reduction was initiated by adding 100 μl of a solution containing 4 mg/ml sodium dithionite, 4 mg/ml sodium bicarbonate, and 100 mM KNO₃. Control reactions replaced sodium dithionite with water. Reactions were incubated at room temperature for 5 min, and stopped by vortexing until the solution became clear, indicating the electron donor was oxidized. 1 ml of 1% w/v sulfanilic acid in 20% HCl was added immediately to the stopped reaction, and vortexed for 15 sec. 1 ml of 1.3 mg/ml N-(1-naphthyl) ethylenediamine-HCL was added to allow formation of red azo dye, and the suspension was centrifuged to pellet

debris. A₅₄₀ of the supernatant was measured spectrophotometrically to quantitate dye formation, and A₄₂₀ was measured to account for absorbance due to light scattering by residual cells or cell fragments. Activity is expressed in arbitrary units based on the formula $100 \times [A_{540} - (0.72 \times A_{420})] / (T \times V \times OD_{660})$ (Stewart & Parales, 1988). T = time in minutes, V = volume of reaction used in ml, OD₆₆₀ corresponds to the optical density of the culture used. Assays were performed in triplicate in three separate experiments.

Site directed mutagenesis of putative NarL binding sites upstream of the *napEFDABC* operon

The plasmid *napEFDABC*:pUCP18 was used to construct two independent, site-directed mutant NarL binding sites upstream of *napEFDABC*, *narLBS1napEFDABC* and *narLBS2napEFDABC* (Supplemental Table 1). Site directed mutagenesis of the two potential NarL binding sites were accomplished by SOE-PCR. *narLBS1napEFDABC* was generated by mutating the wild type sequence CAGGGTACTGAA, denoted as BS1 by the solid underline in Figure 7A, to CAGGGTGCATAA using the oligonucleotides napoperon#1, pnarL1 Sense, pnarL1 AS, and napoperon #11. *narLBS2napEFDABC* was generated by mutating the wild type sequence TACTACAGTA, denoted as BS2 by the dashed underline in Figure 7A, to CATCATAGTA using the oligonucleotides napoperon#1, pnarL2 Sense, pnarL2 AS, and napoperon#11.

Statistical analysis

Statistical analysis was performed using the paired Student's *t* test to determine statistical significance between strains in the β-galactosidase assay and whole cell nitrate reduction assay. In all cases, *P* values of ≤ 0.05 were considered significant.

RESULTS

P. aeruginosa nitrate sensor-response regulator mutant recovers from anaerobic growth arrest

Deletion mutants in the nitrate reductase operon *narK1K2GHJI* and nitrate sensor-response regulator operon *narXL* in *P. aeruginosa* strain PAO1 were generated by allelic exchange (Hoang *et al.*, 1998) to examine their growth under anaerobic conditions. These mutants included a deletion spanning the junctions of *narG* and *narH* ($\Delta narGH$), *narJ* and *narI* ($\Delta narJI$), a deletion encompassing the bulk of *narXL* ($\Delta narXL$) and a deletion in *narL* alone ($\Delta narL$). The positions of the respective deletions in the PAO1 chromosome of the mutants are listed in Supplemental Table 1, and deletions were confirmed by Southern analysis (data not shown).

Over 24 hr of aerobic culture, $\Delta narXL$ and $\Delta narGH$ grew comparably to the wild type PAO1 strain. In contrast, after 24 hr growth under anaerobic conditions, $\Delta narXL$ and $\Delta narGH$ displayed growth arrest compared to parental strain PAO1 (Figure 1). Genetic complementation of the two mutants with the appropriate genomic DNA fragments showed slightly delayed anaerobic growth compared to wild type PAO1, but all three strains reached similar densities after 24 hr. $\Delta narGH$ was not an anaerobically lethal mutant. Replica plates of $\Delta narGH$ cultures were incubated anaerobically up to 120 hr, with a plate removed daily and shifted to aerobic conditions. Following overnight aerobic incubation, 98% (61 out of 62 colonies) of the $\Delta narGH$ colonies displayed growth recovery by increasing colony size after 120 hr incubation (data not shown). The $\Delta narJI$ mutant displayed a comparable phenotype to $\Delta narGH$ in all cases (data not shown), and was not studied further. A possible explanation for the persistence of the $\Delta narGH$ mutant may be due to pyruvate fermentation as suggested in (Eschbach *et al.*, 2004).

In contrast to $\Delta narGH$ and $\Delta narJI$, $\Delta narXL$ began to recover from growth arrest after approximately 72 hr, and reached the maximum growth levels observed for wild type after 120 hr (Figure 1). Little anaerobic growth was observed by either wild type PAO1 or $\Delta narXL$ in NY medium alone or NY medium supplemented with 40 mM arginine (Supplemental Figure 1), illustrating the need for nitrate supplementation under these conditions. Arginine fermentation in NY medium alone did not support *P. aeruginosa* PAO1 growth under anaerobic conditions over 5 days. *P. aeruginosa* is an obligate respirer and, hence, does not have the ability to ferment pyruvate unless provided with a terminal electron donor such as nitrate. Pyruvate does not sustain *P. aeruginosa* anaerobic growth but permits anaerobic survival up to 18 days (Eschbach *et al.*, 2004).

Genetic evidence that delayed growth recovery of $\Delta narXL$ is due to the expression of NapAB

It has been suggested that the periplasmic nitrate reductase encoded by *napAB* is expressed only under aerobic and microaerobic conditions, and is thought to function in the transition from aerobic to anaerobic metabolism in denitrifying bacteria (Potter, 2001). However, recent work by Stewart, *et al.* demonstrated that *napAB* is expressed under anaerobic conditions in *E. coli* K-12 (Stewart *et al.*, 2002). We hypothesized that if anaerobic growth recovery of $\Delta narXL$ was due to expression of *napAB*, a deletion in *napA* in the $\Delta narXL$ background would fail to grow anaerobically. Deletions in *napA* in both parental PAO1 ($\Delta napA$) and $\Delta narXL$ ($\Delta narXL:\Delta napA$) were constructed, and the deletions confirmed by Southern analysis.

Strain $\Delta napA$ grew similarly to wild type PAO1. In contrast, strain $\Delta napA:\Delta narXL$ failed to demonstrate anaerobic growth recovery in NY supplemented with nitrate, with the double mutant displaying growth arrest similar to $\Delta narGH$ (Figure 1). Strains $\Delta narXL$ and $\Delta narGH:\Delta narXL$ displayed full growth recovery after 120 hr (Figure 1). $\Delta napA:\Delta narXL$ complemented *in trans* with *narXLK1K2G5':pUCP18* also grew similarly to wild type (Supplemental Figure 2). When complemented *in trans* with *napEFDABC:pUCP18*, $\Delta napA:\Delta narXL$ reached a growth plateau after 48 hr under anaerobic conditions after a slight initial delay (Supplemental Figure 2). All strains grew comparably aerobically in NY supplemented with nitrate (data not shown). These data provide genetic evidence for the hypothesis that *napAB* expression is required for anaerobic growth recovery by $\Delta narXL$. Several approaches were taken to understand the basis of the recovery from growth arrest by $\Delta narXL$ under anaerobic conditions.

Delayed growth recovery by $\Delta narXL$ is not due to a defect in the remainder of the denitrification pathway

To test whether the delayed growth recovery by $\Delta narXL$ was due to a defect in nitrate reduction to nitrite, or within subsequent steps in the reduction of nitrogen oxide species by the denitrification pathway ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), we compared anaerobic growth of wild type PAO1 and $\Delta narXL$ in NY liquid medium supplemented with either nitrate or nitrite. Wild type PAO1 and $\Delta narXL$ were grown in NY supplemented with nitrate under anaerobic conditions for either 4 hr or 24 hr. Cultures were maintained anaerobically, and diluted to O.D.₆₆₀ of ~0.05 into fresh anaerobic NY supplemented with either 100 mM KNO₃ or 10 mM KNO₂. After 4 hr of anaerobic adaptation, PAO1 reached early stationary phase (O.D.₆₆₀ = 1.4) within 24 hr when subcultured back into NY supplemented with 100 mM KNO₃ (Figure 2A). PAO1 subcultured in NY supplemented with 10 mM KNO₂, as well as $\Delta narXL$ subcultured into either medium, reached an O.D.₆₆₀ of ~0.25 after 48 hr. In contrast, after 24 hr of adaptation to anaerobic conditions (when subcultured into either medium) $\Delta narXL$ reached a growth plateau comparable to wild type PAO1 after 48 hr, compared to 96 hr for cells not pre-adapted to anaerobic growth on nitrate. The growth of $\Delta narXL$ was slightly delayed compared to wild type PAO1 grown under nitrate supplementation, but comparable to wild type grown under nitrite supplementation (Figure 2B). These data suggest that wild

type PAO1 becomes induced to grow on nitrite between 4 and 24 hr. $\Delta narXL$ displays a growth profile comparable to wild type PAO1 sub-cultured in medium supplemented with 10 mM KNO_2 after 24 hr of anaerobic adaptation, suggesting that the remainder of the denitrification pathway downstream of nitrate reduction was still functional in $\Delta narXL$. Hence, the growth delay seen initially in $\Delta narXL$ grown in nitrate (Figure 1) was likely due to differences in the conversion of nitrate to nitrite.

Delayed growth recovery by $\Delta narXL$ is due in part to differences in nitrite metabolism

Next, we examined whether the conversion of nitrate to nitrite was different in wild type PAO1 compared to $\Delta narXL$ by quantitation of the nitrite concentration in culture supernatant when the strains were grown under anaerobic conditions. Wild type PAO1 and $\Delta narXL$ were grown in nitrate supplemented NY medium for 150 hr. Samples were taken and cells were removed by filtration. The nitrite concentration in the supernatant was determined for each strain at each time point. Wild type PAO1 nitrite concentration peaked after 6 hours of incubation under anaerobic conditions (335 μM) (Supplemental Figure 3A) coinciding with wild type PAO1 mid-log growth. In contrast to wild type PAO1, nitrite started accumulating in $\Delta narXL$ around 72 hours (64 μM) and peaked at 111 hr (87 μM) coinciding with the beginning of the delayed growth recovery phenomenon exhibited by $\Delta narXL$. The area under the wild type growth curve was similar to the area under the $\Delta narXL$ growth curve. The corresponding anaerobic growth curve for the nitrite concentration assay is shown in Supplemental Figure 3B. These data suggest that the delayed growth recovery by $\Delta narXL$ is due to a slow conversion of nitrate to nitrite, and that a minimum amount of nitrite accumulation is required to activate the nitrite reductase and, hence, the rest of the denitrification pathway.

Delayed anaerobic growth recovery of $\Delta narXL$ and $\Delta narL$ is due to an alternative anaerobic energy metabolism pathway

Two approaches were taken to determine whether anaerobic growth recovery of $\Delta narXL$ and $\Delta narL$ was due to the induction of the *narK1K2GHJI* operon by an alternative transcriptional regulator or whether it was due to an alternative energy metabolism pathway exclusive of *narK1K2GHJI*.

First, transcriptional fusions were generated in which the first fifty base pairs of *narG* coding sequence were fused to *lacZ*. Two constructs were made, one containing an intact *narXL* locus, $\Phi(narXL-narG-lacZ)$, and the other containing a *narXL* deletion, $\Phi(\Delta narXL-narG-lacZ)$ (Figure 3A). Each construct was cloned into pLP170 (Preston *et al.*, 1997) and used to transform wild type PAO1 and $\Delta narXL$, respectively. Wild type PAO1 and $\Delta narXL$ were also transformed with vector pLP170 alone to serve as negative controls. Because the *narXL* locus is intact in $\Phi(narXL-narG-lacZ)$, we were able to use this construct as both a plasmid-based reporter fusion for *narK1K2GHJI* expression, and to complement $\Delta narXL$. We hypothesized that if *narK1K2GHJI* expression was induced by an alternative transcriptional regulator, β -galactosidase levels would gradually increase over time in $\Delta narXL:\Phi(\Delta narXL-narG-lacZ)$. However, β -galactosidase levels in $\Delta narXL:\Phi(\Delta narXL-narG-lacZ)$ were only 19% of wild type ($p \leq 0.01$) after 24 hr (Figure 3B), and we did not see an increase in β -galactosidase levels in this strain through 120 hr. A possible explanation why β -galactosidase expression in $\Delta narXL:\Phi(\Delta narXL-narG-lacZ)$ was 19% of wild type may be due to either gratuitous transcription or that Anr is responding to anaerobiosis. Strains provided with intact *narXL* *in trans* demonstrated high levels of β -galactosidase activity after 24 hr, confirming the function of the reporter fusion (Figure 3B).

Second, if *narK1K2GHJI* expression in $\Delta narXL$ was regulated by a transcriptional activator other than NarL, we predicted that a $\Delta narXL:\Delta narGH$ double mutant would abate anaerobic growth recovery. However, this double mutant retained anaerobic growth recovery after 72 hr

similar to $\Delta narXL$ (Figure 1). The studies using $\Phi(\Delta narXL-narG-lacZ)$ in either a wild type or $\Delta narXL$ background demonstrated that NarXL activated *narK1K2GHJ* expression (Figure 3B). Diminished β -galactosidase expression in $\Delta narXL:\Phi(\Delta narXL-narG-lacZ)$, and the growth recovery by $\Delta narXL:\Delta narGH$, suggests growth recovery by $\Delta narXL$ and $\Delta narL$ seen in Figure 1 is due to an alternative anaerobic respiratory pathway exclusive of the membrane nitrate reductase NarGHI. Therefore, we hypothesized that the expression of the NapAB periplasmic nitrate reductase could be responsible for growth recovery in $\Delta narXL$ and $\Delta narL$.

Transcriptional and biochemical evidence that delayed growth recovery in $\Delta narXL$ is due to the expression of periplasmic nitrate reductase NapAB

To support the genetic evidence that *napAB* expression was required for delayed growth recovery seen in $\Delta narXL$ under anaerobic conditions, three additional experiments were performed. First, using RT-PCR, we examined *napA* and *narH* transcript levels in wild type PAO1, $\Delta narXL$, and $\Delta narGH$ under anaerobic conditions in NY supplemented with nitrate. The expression of the *napA* transcript was detected only in $\Delta narXL$, while the *narH* transcript was detected only in wild type PAO1 (Figure 4). As a control for RNA integrity, the *groEL* transcript was detected in all three strains under anaerobic conditions. PCR products from genomic DNA using each primer pair are shown in lanes marked (+) and, reverse transcriptase was omitted from reactions shown in lanes marked (−).

Second, to confirm that *nap* operon expression was induced in $\Delta narXL$ under anaerobic conditions in the presence of nitrate, $\Phi(napD-lacZ)$ transcriptional fusions were constructed (Supplemental Figure 4A) and used to transform wild type PAO1, $\Delta narXL$, and $\Delta narGH$. Each strain was also transformed with vector pLP170 alone to serve as a negative control. In wild type PAO1 and $\Delta narGH$, β -galactosidase levels remained low at 4000 Miller units and 5000 Miller units, respectively, under anaerobic conditions. In contrast, β -galactosidase levels were significantly elevated in $\Delta narXL$ compared to wild type PAO1 and $\Delta narGH$ ($p < 0.001$) after 24 hr of anaerobic growth (Supplemental Figure 4B), and remained consistently elevated at 30,000 Miller units for the entire 120 hr growth period. Combined with the RT-PCR data, these results indicated that expression of the *napEFDABC* operon is induced in $\Delta narXL$, and suggested that *napEFDABC* operon expression was responsible for growth recovery seen in $\Delta narXL$ under anaerobic conditions.

Third, nitrate reductase activity was measured in permeabilized cells using methyl viologen (MV) as an artificial electron donor. To determine which nitrate reductase was functional in a given strain, 50 μ M sodium azide was added to selected cultures. NarGHI activity is azide-sensitive, whereas NapAB activity is azide-resistant (Jones & Garland, 1977; MacGregor *et al.*, 1974; Stewart *et al.*, 2002). Using either benzyl viologen (BV) or methyl viologen (MV) as an electron donor, periplasmic nitrate reductase NapAB would be predicted to function in either the presence or absence of azide, whereas membrane nitrate reductase NarGHI would function only in the absence of azide. Organisms grown under anaerobic conditions were collected during mid-log growth at an OD_{660} of ~ 0.6 with exception of $\Delta narGH$ and $\Delta napA:\Delta narXL$, which were collected at an OD_{660} of ~ 0.12 . In either the absence or presence of sodium azide, $\Delta narXL$ utilized MV comparably as an electron donor to reduce nitrate to nitrite (Figure 5), indicating increased periplasmic nitrate activity in this strain. As predicted, $\Delta narGH$ and $\Delta napA:\Delta narXL$ demonstrated no nitrate reductase activity using MV under any conditions tested. In contrast, wild type PAO1 and $\Delta napA$ demonstrated a significantly reduced ($p < 0.002$) ability to utilize MV as an electron donor in the presence of sodium azide compared to $\Delta narXL$, suggesting that the only functional nitrate reductase in wild type PAO1 under anaerobic conditions is the membrane nitrate reductase NarGHI. Similar results were obtained when BV was utilized as an electron donor. Increased NapAB periplasmic nitrate reductase

activity in $\Delta narXL$ corresponded to the increased levels of *napA* transcription observed in this mutant.

NarL represses the expression of periplasmic nitrate reductase NapAB under anaerobiosis in *P. aeruginosa* PAO1

Our genetic, transcriptional, and biochemical studies demonstrated that anaerobic growth recovery by the nitrate sensor-response regulator mutant $\Delta narXL$ was the result of NapAB periplasmic nitrate reductase expression. These results would predict a regulatory role for NarL in NapAB expression. Under high nitrate concentrations, *E. coli* NarL competes with orthologous nitrate response regulator NarP for binding consensus regulatory motifs upstream of the periplasmic nitrate reductase operon, causing the repression of periplasmic nitrate reductase expression (Darwin & Stewart, 1995; Overton *et al.*, 2006; Potter *et al.*, 1999; Stewart & Bledsoe, 2003; Stewart *et al.*, 2003). Two different putative NarL binding sites were identified upstream of the *P. aeruginosa* PAO1 periplasmic nitrate reductase *napEFDABC* operon. Rather than being organized as the consensus inverted heptamer (TACYNMT) repeats separated by two random base pairs (8, 35, 36), the heptamer pairs overlap slightly (Supplemental Figure 5A). The first heptamer pair BS1 (solid underlines), more distal from the start site of *napE* translation, contains 6/7 consensus bases in the forward strand and 5/7 consensus bases in the reverse strand. The second, proximal heptamer pair BS2 (dashed underlines) contains 6/7 consensus bases in each strand.

To determine whether either BS1 or BS2 were involved in regulation of *napEFDABC* expression, site directed mutations in each of the two sites were generated in cloned fragments containing the upstream *oprH-napE* intergenic region and *napEFDABC*. The two mutant constructs, along with the wild type fragment, were cloned into pUCP18 and used to transform $\Delta napA:\Delta narXL$ and $\Delta napA:\Delta narGH$. The six strains were grown under anaerobic conditions in NY supplemented with 100 mM KNO₃ (Supplemental Figure 5B). We predicted that a mutation in either BS1 or BS2 important in NarL regulation of *napEFDABC* would undergo anaerobic growth recovery in a $\Delta napA:\Delta narGH$ background. In the presence of functional NarL and an intact *oprH-napE* intergenic regulatory region, $\Delta narGH$ is unable to grow anaerobically (Supplemental Figure 5B) and the $\Delta napA:\Delta narGH$ double mutant displays the same phenotype (data not shown). As expected, introduction of the wild type construct into $\Delta napA:\Delta narGH$ did not restore anaerobic growth. Interestingly, mutation of the distal putative NarL binding site BS1, but not the proximal BS2, restored anaerobic growth in $\Delta napA:\Delta narGH$ (Supplemental Figure 5B). When the same plasmid constructs were introduced into a $\Delta napA:\Delta narXL$ background, all strains grew comparably to the $\Delta narXL$ strain under anaerobic conditions (data not shown), demonstrating that the plasmids themselves did not interfere with anaerobic growth, and that growth recovery occurs when *napEFDABC* is provided *in trans* in the absence of NarL (data not shown).

Based on these results, the distance of BS1 from the start of *napEFDABC* translation suggested the possibility of an alternative transcriptional start site during anaerobic growth of $\Delta narXL$. Wild type PAO1 and $\Delta narXL$ were grown under anaerobic conditions in NY medium supplemented with 100 mM KNO₃, and RNA was isolated from cells grown to mid-log phase (OD₆₆₀ = 0.6). Using a set of *napE*-specific antisense primers in Rapid Amplification of cDNA Ends-based PCR followed by nucleotide sequencing, we identified a transcriptional start site common to both wild type PAO1 and $\Delta narXL$ 25 bases upstream of the start site of translation. A second transcriptional start site unique to $\Delta narXL$ was identified 118 bases upstream of the start site of translation (Supplemental Figure 5A).

These genetic and transcriptional mapping data support the model (Figure 6) that, in wild type PAO1, phospho-NarL binds to BS1 and represses transcription of *napEFDABC* and stimulates transcription of *narK1K2GHJI*. Repression of *napEFDABC* is lifted either by the absence of

NarL or the alteration of BS1, permitting increased transcription of *napEFDABC* and thereby supporting anaerobic growth over time. We suggest that the transcription of *napEFDABC* initiated downstream of BS2, and common to both wild type PAO1 and $\Delta narXL$, represents a constitutive basal level of transcription corresponding to that reported in (Schreiber *et al.*, 2007). However, this level of *napEFDABC* transcription is insufficient to support robust anaerobic growth of a $\Delta narGH$ mutant.

DISCUSSION

Several studies using transcriptional profiling have demonstrated the importance of quorum sensing as a global regulator of nitrate metabolism and biofilm formation in *P. aeruginosa* (Schuster *et al.*, 2003; Wagner *et al.*, 2003; Wagner *et al.*, 2004). In addition to quorum sensing, anaerobic growth via denitrification in *P. aeruginosa* is regulated by Anr (Ye *et al.*, 1995), an ortholog of Fnr (fumarate and nitrate reductase) in *E. coli* (Sawers, 1991). A consensus Anr sequence TTGACN₄ATCAG (Spiro & Guest, 1990) and the consensus NarL binding sequence TACc/tNa/cT (Tyson *et al.*, 1993) are found in the intergenic region between the *narK1K2GHJI* membrane-bound nitrate reductase operon and the operon encoding the nitrate sensor-response regulator *narXL*. NarXL activation of the membrane nitrate reductase *narK1K2GHJI* operon has been reported in both the denitrifying pseudomonad *P. stutzeri* (Hartig *et al.*, 1999) and in *P. aeruginosa* (Schreiber *et al.*, 2007). A third study demonstrated that Anr and NarL act in concert with Integration Host Factor to activate transcription of the *hema* gene, which encodes the first enzyme in heme biosynthesis during anaerobic growth in *P. aeruginosa* (Krieger *et al.*, 2002).

The consensus heptamer binding site for gene activation by NarL (Tyson *et al.*, 1993) is organized as an inverted repeat separated by two nucleotides, the 7-2-7 motif. However, some variants of this motif are functional in *P. aeruginosa* (Krieger *et al.*, 2002). *In silico* analysis of the *P. aeruginosa* PAO1 genome using the web-based Regulatory Sequence Analysis-Tools program (RSAT) 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 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 the NADH dehydrogenase complex I, which participates in electron transfer via ubiquinone to the membrane-bound nitrate reductase NarGHI (Berks *et al.*, 1995; Zumft, 1997). NADH dehydrogenase complex I is essential for anaerobic growth in *P. aeruginosa* (Filiatrault *et al.*, 2006). NADH dehydrogenase complex I and ubiquinone perform a similar function aerobically by coupling to the electron transport chain of the bacterial cytochrome complex (Richardson *et al.*, 2001).

In *P. fluorescens*, *nuo* mutants are deficient in plant root colonization (Camacho Carvajal *et al.*, 2002), 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 mutant in *E. coli* demonstrated that the *nuo* operon is part of the NarL regulon (Constantinidou *et al.*, 2006). In contrast, over 10% of intergenic regions in *P. aeruginosa* PAO1 have at least one consensus NarL half-site (www.pseudomonas.com). The overall importance of full 7-2-7 to half-site recognition in transcription regulation by NarL in *P. aeruginosa* is unknown. However, in the report cited above (Krieger *et al.*, 2002), mutation of a single NarL heptamer half site in the *P. aeruginosa hemA* intergenic region was sufficient to abolish *hemA* expression. Recently, Benkert *et al.* (Benkert *et al.*, 2008) showed that NarL represses the expression of the arginine fermentation pathway *arcDABC* in *P. aeruginosa*. Additionally, they identified a heptamer half site for binding by NarL centered at position -56 upstream of *arcDABC* rather than the traditional 7-2-7 binding site.

Studies in *E. coli* support our conclusion that NarL plays a role in regulation of *P. aeruginosa* *napEFDABC* operon expression. Darwin and Stewart (Darwin & Stewart, 1995) identified a consensus NarP/NarL binding site upstream of the periplasmic nitrate reductase operon and determined that NarL directly repressed *E. coli* *napFDAGHBC* operon expression (Potter, 2001; Stewart *et al.*, 2003). Subsequently, Potter and Cole (Potter & Cole, 1999) demonstrated that a strain containing a functional periplasmic nitrate reductase, but lacking a functional membrane nitrate reductase response regulator NarL (*napA*⁺*narL*⁻), grew better anaerobically than a strain that contained both a functional periplasmic nitrate reductase and NarL (*napA*⁺*narL*⁺). The failure of the *narL*⁺*nap*⁺ strain to grow well in batch culture was partly attributed to NarL-dependent repression of *nap* operon expression by nitrate. Furthermore, the *narGHI*⁻ *napA*⁺ strain grew better anaerobically under nitrate limiting conditions. In contrast, the *narGHI*⁺ *napA*⁻ strain grew better in high nitrate concentrations (Stewart *et al.*, 2002; Wang *et al.*, 1999). In addition, *E. coli* NarL can bind to sites other than the preferential 7-2-7 site (Darwin & Stewart, 1995; Darwin *et al.*, 1997) to either promote or repress transcription. The sequences TTACAATTG and TAACCACAC, found upstream of the *nrfA* operon in *E. coli* at positions -50 and -22, respectively, are single heptamers where NarL can bind and act as a repressor in presence of nitrate (Darwin *et al.*, 1997; Darwin, 1996). Dong *et al* (Dong *et al.*, 1992) demonstrated in *E. coli* that single base substitutions in these sequences significantly reduced NarL binding.

Regulation of periplasmic nitrate reductase operon *napEFDABC* expression by response regulator NarL in *P. aeruginosa* has not been widely considered because, to date, NarL has only been shown to be important in the activation of expression of the membrane nitrate reductase *narK1K2GHJI* operon (Schreiber *et al.*, 2007). Unexpectedly, we observed anaerobic growth recovery over time in Δ *narXL*. Genetic, transcriptional, and biochemical studies presented here demonstrated that the delayed anaerobic growth recovery in Δ *narXL* was attributed to the activity of periplasmic nitrate reductase NapAB. A novel transcriptional start site for *napEFDABC* expression was identified in Δ *narXL* grown anaerobically. Furthermore, mutagenesis of a consensus NarL-binding half-site upstream of the novel transcriptional start site also restored anaerobic growth recovery in Δ *narXL*. Taken together, these data support a model (Figure 6) in which the nitrate response regulator NarL directly represses expression of periplasmic nitrate reductase NapAB, while inducing maximal expression of membrane nitrate reductase NarGHI during anaerobic growth of wild type *P. aeruginosa* PAO1.

Studies of *Paracoccus pantotrophus* and *E. coli* suggest why *P. aeruginosa* Δ *narXL* displayed a delayed growth phenotype even though NapAB expression was induced very early under anaerobic conditions. *P. pantotrophus* expressing only a functional periplasmic nitrate reductase supported anaerobic respiration and growth on nitrate at levels approximately one-third that of the wild type (Bell *et al.*, 1993). Also, *E. coli* (Stewart *et al.*, 2002) used periplasmic nitrate reductase and fumarate reductase equally well in the respiratory chain involving glycerol 3-phosphate oxidation. In this case, the fumarate reductase is not a coupling site for generating a proton motive force, suggesting that the periplasmic nitrate reductase is also not a coupling site for generating a proton motive force via quinol oxidation.

When grown under anaerobic conditions, *P. aeruginosa* favors expression of the membrane nitrate reductase NarGHI (Schreiber *et al.*, 2007) since its activity is coupled to energy production (Berks *et al.*, 1995; Zumft, 1997). The two component sensor-regulator system NarXL is required for the expression of the membrane nitrate reductase as demonstrated in Figure 3B and shown in (Schreiber *et al.*, 2007). Hence, Δ *narXL* and Δ *narGH* show no growth over the first 72 hours of incubation under anaerobic conditions (Figure 1). However, delayed growth recovery was observed in Δ *narXL* but not in Δ *narGH*. This phenomenon was due to the expression of the periplasmic nitrate reductase (Figure 5). The delayed onset of growth by

$\Delta narXL$ is likely to be a multifaceted process requiring first the de-repression and expression of the periplasmic nitrate reductase. Even though periplasmic nitrate reductase is expressed as early as 3 hr (Supplemental Figure 4B), the onset of the delayed growth recovery by $\Delta narXL$ does not occur until approximately 72 hours under anaerobic conditions suggesting that the expression, quantity, and/or functionality of the periplasmic nitrate reductase may be sub-optimal. This may be due in part to a dosage effect which can be overcome by providing the *napEFDABC* operon *in trans* on a multi-copy plasmid as illustrated in Supplemental Figure 5 and Supplemental Figure 2.

Alternatively, the delayed growth recovery observed in $\Delta narXL$ may be due to a threshold of nitrite concentration accumulating that must be met. Because there may be a slow generation of nitrite by $\Delta narXL$ due to the slow conversion of nitrate to nitrite by NapAB, the subsequent induction of the rest of denitrification pathway may be delayed as seen in Supplemental Figure 3. Maximal nitrite accumulation in wild type PAO1 occurred after 6 hr of anaerobic growth, coinciding with mid-log phase (Supplemental Figure 3A). Nitrite accumulation in $\Delta narXL$ began at 72 hr and reached a maximum concentration at 111 hr of anaerobic growth, also coinciding with mid-log phase in this mutant. However, following 24 hr pre-adaptation of $\Delta narXL$ in medium supplemented with nitrate and subculture into medium supplemented with nitrite, both wild type PAO1 and $\Delta narXL$ (Figure 2B) grew comparably, suggesting that the activity of periplasmic nitrate reductase is the rate limiting step for growth, since the rest of the denitrification pathway is not induced unless an adequate concentration of nitrite is generated. Hence, under normal conditions in wild type *P. aeruginosa* PAO1, the shift from aerobic to anaerobic growth prefers membrane nitrate reductase utilization to drive energy metabolism. However in $\Delta narXL$, a shift from aerobic to anaerobic growth requires the electron transport system to be remodeled because the periplasmic nitrate reductase is not a direct coupling site. Hence, *P. aeruginosa* must rely on the remainder of the denitrification pathway and the arginine fermentation pathway for this purpose. (Eschbach *et al.*, 2004; Schreiber *et al.*, 2006; Vander Wauven *et al.*, 1984).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Anaerobic growth curves of PAO1 *nar* and *nap* deletion mutants in NY + 100 mM KNO₃.

Figure 2.

Anaerobic growth curves of wild type PAO1 (diamonds) and $\Delta narXL$ (squares) pre-adapted to anaerobic growth in NY + 100 mM KNO₃ after 4 hours (**A**) or 24 hours (**B**). Solid shapes represent anaerobic subculture into NY + 100 mM KNO₃. Open shapes represent anaerobic subculture into NY + 10 mM KNO₂.

Figure 3.
A. Diagram of transcriptional fusion $\Phi(narXL-narG-lacZ)$ and $\Phi(\Delta narXL-narG-lacZ)$, containing the *nar* operon regulatory region upstream of *narK1*. **B.** β -galactosidase expression of *P. aeruginosa* PAO1 and $\Delta narXL$ transformed with fusion $\Phi(narXL-narG-lacZ)$ or $\Phi(\Delta narXL-narG-lacZ)$ and grown under anaerobic conditions in NY + 100mM KNO_3 for 120 hours. As a negative control for background expression levels of β -galactosidase, each strain was transformed with pLP170 alone.

Figure 4.

Detection by RT-PCR of *groEL*, *narH*, and *napA* transcripts in *P. aeruginosa* PAO1, $\Delta narXL$, and $\Delta narGH$ grown under anaerobic conditions in NY + 100 mM KNO₃. Wild type PAO1 and $\Delta narXL$ were grown to mid-log phase of OD₆₆₀ = 0.6 (taken at 6 hours and 96 hours, respectively). $\Delta narGH$ was grown to OD₆₆₀ = 0.1 (taken at 96 hours).

Figure 5.

Nitrate reductase activity in *P. aeruginosa* PAO1 *nar* and *nap* deletion mutants grown under anaerobic conditions in NY + 100 mM KNO₃. Wild type PAO1 and *narXL* were grown to mid-log phase of OD₆₆₀ = 0.6 (taken at 6 hours and 96 hours, respectively). $\Delta narGH$ was grown to OD₆₆₀ = 0.1 (taken at 96 hours). The electron donors benzyl viologen and methyl viologen are denoted as BV and MV, respectively, in legends. Data are expressed as nmol nitrite min⁻¹ mg⁻¹ protein. As a negative control, sodium dithionite was omitted from the nitrate reductase reaction.

Figure 6.

Proposed model of *P. aeruginosa* NarL regulation under anaerobic conditions in the presence of nitrate. Square in *narK1-narX* intergenic region indicates consensus 7-2-7 NarL binding site; double diamonds in *napE-oprH* intergenic region indicates pair of overlapping NarL half-sites. Phospho-NarL acts as a transcriptional activator of *narK1K2GHJI* and a repressor of *napEFDABC*.