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Membrane-Bound Nitrate Reductase Is Required for Anaerobic Growth in Cystic Fibrosis Sputum▽†

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The autosomal recessive disorder cystic fibrosis (CF) affects approximately 70,000 people worldwide and is characterized by chronic bacterial lung infections with the opportunistic pathogen *Pseudomonas aeruginosa*. To form a chronic CF lung infection, *P. aeruginosa* must grow and proliferate within the CF lung, and the highly viscous sputum within the CF lung provides a likely growth substrate. Recent evidence indicates that anaerobic microenvironments may be present in the CF lung sputum layer. Since anaerobic growth significantly enhances *P. aeruginosa* biofilm formation and antibiotic resistance, it is important to examine *P. aeruginosa* physiology and metabolism in anaerobic environments. Measurement of nitrate levels revealed that CF sputum contains sufficient nitrate to support significant *P. aeruginosa* growth anaerobically, and mutational analysis revealed that the membrane-bound nitrate reductase is essential for *P. aeruginosa* anaerobic growth in an in vitro CF sputum medium. In addition, expression of genes coding for the membrane-bound nitrate reductase complex is responsive to CF sputum nitrate levels. These findings suggest that the membrane-bound nitrate reductase is critical for *P. aeruginosa* anaerobic growth with nitrate in the CF lung.

Cystic fibrosis (CF) is a heritable disease characterized by accumulation of large volumes of mucus (sputum) within the lungs and persistent colonization with opportunistic pathogens. While numerous bacterial species transiently colonize CF sputum or persist at low cell densities, the opportunistic pathogen *Pseudomonas aeruginosa* establishes a chronic infection and can attain densities of $>10^9$ cells/ml of sputum (19). Chronic *P. aeruginosa* infection is the leading cause of morbidity and mortality in CF patients (20). Once established, *P. aeruginosa* CF lung infections are highly refractory to antibiotic treatments, and even aggressive therapies fail to fully eradicate the bacterium from the lung (20). Several aspects of *P. aeruginosa* physiology are believed to contribute to its high antibiotic resistance in vivo, including biofilm formation and overproduction of the viscous exopolysaccharide alginate (23).

P. aeruginosa CF lung infections are frequently modeled under aerobic laboratory conditions; however, direct oxygen measurements within CF lung sputum in situ indicate that sputum contains hypoxic, and potentially anaerobic, regions (38). In addition, strict anaerobic bacteria have been detected in sputum samples from multiple CF patients (21, 29), suggesting that environments capable of sustaining these species are present in vivo. There are several factors that could potentially reduce oxygen levels within CF sputum: cultured CF lung epithelial cells consume oxygen at a higher rate than non-CF lung epithelial cells, oxygen diffusion through sputum is restricted, and oxygen is consumed by resident sputum microorganisms, including *P. aeruginosa* (38). Collectively these factors may effectively reduce oxygen to levels insufficient for aerobic respiration. Interestingly, antibodies to components of two putative anaerobic nitrate reductases of *P. aeruginosa*, NapA and NarG, have been detected in sera from CF patients (2), suggesting that *P. aeruginosa* produces these respiratory enzymes in vivo.

It is clear from several published reports that *P. aeruginosa* physiology is remarkably different during anaerobic growth and aerobic growth. For example, it has been proposed that *P. aeruginosa* "prefers" to reside in antibiotic-resistant biofilms under anaerobic conditions (40) and coincidently enhances production of the alginate capsule surrounding the biofilm under these conditions (5, 38). The structure and function of the outer membrane are also altered during anaerobic growth. Specifically, *P. aeruginosa* modifies the structure of its lipopolysaccharide from a highly electronegative surface to a neutral surface during anaerobic growth (30). This change provides enhanced resistance to cationic antimicrobials, such as aminoglycoside antibiotics (28), and potentially to naturally produced cationic peptides due to inhibition of charge-mediated uptake (17). Although the mechanism is unclear, the bactericidal effects of the noncationic antibiotics meropenem, ceftazidime, and colistin are also diminished under anaerobic growth conditions for *P. aeruginosa* CF lung isolates (18). These antibiotics are used in combination therapies for treatment of chronic *P. aeruginosa* lung infection (15). Taken together, these observations suggest that, if anaerobic microenvironments are present in the CF lung sputum layer, growth in these microenvironments may significantly impact *P. aeruginosa* CF lung infection.

To generate energy for processes such as biofilm formation and alginate production in vivo, *P. aeruginosa* must obtain carbon and energy from its CF sputum growth environment. *P. aeruginosa* is capable of utilizing a wide range of carbon sources in CF sputum (26) and possesses numerous putative and confirmed oxidases for respiratory growth (7). *P. aeruginosa* is a denitrifier and can utilize the nitrogenous oxides nitrate, nitrite, and nitrous oxide as electron acceptors for

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respiratory growth in the absence of oxygen (39). *P. aeruginosa* can also anaerobically metabolize arginine in a substrate level phosphorylation pathway known as arginine deimination, as well as ferment pyruvate; however, these pathways are likely not important for growth in vivo, since they support little or no bacterial growth (>40-h doubling time for arginine deimination and no net growth for pyruvate fermentation). Instead, these metabolic pathways are likely used for anaerobic cellular maintenance in the absence of nitrogenous electron acceptors (9, 34). Given the evidence that *P. aeruginosa* potentially encounters anaerobic microenvironments during the course of chronic CF lung infection and the impact of anaerobic growth on *P. aeruginosa* persistence and antibiotic resistance, we sought to determine the *P. aeruginosa* metabolic components critical for anaerobic growth in CF sputum. In this study, we identified and characterized a component of *P. aeruginosa* metabolism, the membrane-bound nitrate reductase, as a required determinant for *P. aeruginosa* anaerobic growth and nitrate reduction in an in vitro CF sputum medium.

MATERIALS AND METHODS

Bacterial strains and growth media. *P. aeruginosa* strain PA14 and the isogenic *narG* and *napA* transposon insertion mutants were obtained from the MGH-Parabiosys:NHLBI Program for Genomic Applications (http://pga.mgh.harvard.edu/cgi-bin/pa14/mutants/retrieve.cgi). *P. aeruginosa* mPAO1 and the isogenic *narG* transposon insertion mutant were obtained from the University of Washington Genome Center (http://genome.washington.edu). Transposon insertion sites in PA14 and PAO1 mutants were confirmed using PCR. *P. aeruginosa* was routinely cultured on tryptic soy agar. *Escherichia coli* DH5α was used for routine cloning and was commonly grown in LB Miller broth (EMD Chemicals).

For *P. aeruginosa* anaerobic growth, a MOPS (morpholinepropanesulfonic acid)-buffered medium (50 mM MOPS, pH 7.2, 93 mM NH_4Cl, 43 mM NaCl, 3.7 mM KH_2PO_4, 1 mM $MgSO_4$, 3.5 μM $FeSO_4 \cdot 7H_2O$, 10 mM glycerol, 10 mM sodium succinate, and 0.001% yeast extract) or brain heart infusion (BHI) broth supplemented with $NaNO_2$ or KNO_3 was used. Anaerobic media were aliquoted into Balch tubes, capped, sealed, and heated for 30 min at 100°C. The heated tubes were boiled under vacuum to release residual oxygen from the media, and the headspace was replaced with nitrogen gas three times before the tubes were autoclaved. All incubations were performed at 37°C with shaking at 250 rpm. Where applicable, antibiotics were added at the following concentrations: kanamycin, 200 μg/ml; ampicillin, 50 μg/ml; and carbenicillin, 300 μg/ml.

Complementation of the *P. aeruginosa narG* mutant. The *narGHJI* genes were amplified from *P. aeruginosa* PA14 chromosomal DNA using the primers narGHJI-for (5′-GCTCTAGACAACCTCTGATTAGCGTTGTAAC-3′) and narGHJI-rev (5′-CCCAAGCTTGATCGATCACCTCGGTTCGGC-3′) (underlining indicates XbaI and HindIFF sites, respectively), using the Expand Long Template kit (Roche). The resulting 6,878-bp fragment containing *narGHJI* was digested with XbaI/HindIII and cloned into pUCP18 (29) to create pKP201. In this plasmid, *narGHJI* expression is under control of the constitutive *lac* promoter.

Anaerobic-growth experiments. Aerobic stationary-phase *P. aeruginosa* cells were inoculated to a final optical density at 600 nm (OD_{600}) of 0.001 in anaerobic BHI broth supplemented with various amounts of KNO_3. Growth was measured by OD_{600}, and samples were removed for nitrate analysis at 24 h postinoculation. For some experiments, anaerobic MOPS medium with or without 1 mM $NaNO_2$ was inoculated to 10^3 cells/ml with aerobic exponential-phase *P. aeruginosa* PA14. Samples were removed at 24 h postinoculation for viable-cell counts on tryptic soy agar and nitrite analysis.

To evaluate anaerobic growth in CF sputum, sputum was resuspended in deionized water as previously described (26). Sputum samples were obtained by expectoration from nonexacerbating adult CF patients with total *P. aeruginosa* titers of $<10^8$ cells/ml. Briefly, lyophilized CF sputum was resuspended in deionized water to a concentration of 20% (vol/vol) sputum, homogenized with a tip sonicator three to five times for 30 s each time, and centrifuged for 5 min at 16,000 × *g* before sterilization by filtration through a 0.45-μm-pore-size filter. Since CF sputum medium contains only 20% of the native nitrate levels of CF sputum, the CF sputum medium was amended with nitrate to levels commonly found in CF sputum harvested from the lung (400 μM). CF sputum medium with nitrate was incubated overnight in an anaerobic chamber (Coy Laboratories) prior to inoculation. *P. aeruginosa* strains were grown aerobically overnight on MOPS-buffered medium agar plates and subsequently transferred to an anaerobic chamber (Coy Laboratories). After 1 h of incubation at 37°C, *P. aeruginosa* cells were resuspended to an OD_{600} of 0.2 in MOPS-buffered medium with no added electron acceptor. After 4 to 8 h of incubation at 37°C in the anaerobic chamber, the cells were diluted to approximately 10^3 in anaerobic CF sputum medium with nitrate. Samples were removed for viable-cell counts immediately after inoculation and after 24 h of incubation at 37°C in the anaerobic chamber.

Nitrate and nitrite measurements. Nitrate concentrations in CF sputum were determined by anion-exchange high-performance liquid chromatography (HPLC) using a Dionex ion chromatography system. Sputum samples from seven adult CF patients were lyophilized, reconstituted to 25% (vol/vol) in deionized water, and homogenized and sterilized as described above prior to HPLC analysis. For analyses of nitrate concentrations in *P. aeruginosa* culture supernatants, bacterial cells were removed from cultures by centrifugation for 5 min at 7,500 × *g*, and the supernatant was filtered through a 0.45-μm-pore-size syringe filter prior to HPLC analysis. Nitrite concentrations in *P. aeruginosa* culture supernatants were determined using the Nitric Oxide Assay Kit (Calbiochem) according to the manufacturer's instructions.

RNA transcript analyses by reverse transcription (RT)-PCR. Total RNA was isolated from late-exponential-phase cultures (OD_{600}, 0.6) of *P. aeruginosa* PA14 grown in anaerobic BHI broth with 20 mM KNO_3 using an RNeasy miniprep kit (QIAGEN). DNA contamination was removed by digestion with RQ1 DNase (Promega). DNA contamination was monitored by PCR amplification of the *P. aeruginosa rplU* gene with the primers rplU-for (5′-CGCAGTGATTGTTACC GGTG-3′) and rplU-rev (5′-AGGCCTGAATGCCGGTGATC-3′). RNA integrity was monitored by agarose gel electrophoresis. Synthesis of cDNA with the random primer $(NS)_5$ was performed with SuperScript II (Invitrogen) according to the manufacturer's instructions with 100 ng RNA template. A standard PCR was performed with the Expand Long Template kit (Roche) with 25 ng chromosomal DNA, 100 ng RNA, or 25 ng cDNA templates with primer sets overlapping putative cotranscribed coding regions (see Fig. 3A). For PCRs using primer set 8 (see Fig. 3A), 50 ng cDNA template was used.

Microarray analyses. *P. aeruginosa* was inoculated to an OD_{600} of 0.001 in CF sputum medium with 10% (vol/vol) CF sputum as previously described (26). Briefly, lyophilized CF sputum was sterilized by UV treatment for 20 min, resuspended to a final concentration of 10% (vol/vol) in a MOPS-buffered base (50 mM MOPS [pH 7.2], 93 mM NH_4Cl, 43 mM NaCl, 3.7 mM KH_2PO_4, 1 mM $MgSO_4$, and 3.5 μM $FeSO_4 \cdot 7H_2O$) and homogenized by sonication as described above. CF sputum medium was supplemented with 100 mM KNO_3 for aerobic and anaerobic microarray experiments. The microarray experiments were performed in duplicate for each condition. Cells were harvested for RNA isolation at an OD_{600} of 0.1. RNA was isolated and monitored for integrity and DNA contamination as described above. Microarray analysis with Affymetrix *P. aeruginosa* GeneChips was performed as previously described (26).

Transcriptional regulation of the *nar* operon. A 688-bp region upstream of *narK1* was amplified from *P. aeruginosa* PA14 chromosomal DNA using the Expand Long Template kit (Roche) with the primers nar prom-for (5′-GGGG TACCGGCTAAACTCTCTGCACGGAC-3′) and nar prom-rev (5′-GAAGAT CTGCACCGTGCTGAGCAGTTGCG-3′) (underlining indicates KpnI and BglII sites, respectively). The resulting fragment was digested with KpnI/BglII and cloned upstream of a promoterless *lacZ* gene on the reporter plasmid pQF50 (10) to create pKP301. Clones were verified by restriction enzyme digestion and DNA sequencing at the Laboratory for Genomics and Bioinformatics at the University of Oklahoma Health Sciences Center.

To monitor aerobic expression of *narG*, *P. aeruginosa* PA14 carrying pQF50 or pKP301 was inoculated to an OD_{600} of 0.01 in 10 ml BHI broth with or without added nitrate (400 μM or 20 mM KNO_3) in a 125-ml Erlenmeyer flask. β-Galactosidase activity was monitored during exponential (OD_{600}, 0.2 and 0.6) and early stationary (OD_{600}, 1.6) phases. To monitor anaerobic expression of *narG*, *P. aeruginosa* PA14 carrying pQF50 or pKP301 was inoculated to an OD_{600} of 0.01 in 75 ml BHI broth in a 1-liter flask and incubated with shaking at 37°C. At an OD_{600} of 0.2, 3 ml cells was injected into prewarmed anaerobic Balch tubes containing KNO_3 or water so that the final added KNO_3 concentration in cultures was 0 μM, 400 μM, or 20 mM. After 4 h of incubation, cells were harvested for analysis of β-galactosidase activity as previously described (24).

Microarray data accession number. The microarray data have been deposited in the EMBL-EBI data bank (www.ebi.ac.uk/miamexpress) under experiment accession number E-MEXP-1051.

TABLE 1. Anaerobic metabolism genes differentially regulated during anaerobic growth in CF sputum

ORF[a]	Gene[a]	Product[a]	Pathway[b]	Regulation[c]
PA3875	*narG*	Respiratory nitrate reductase alpha chain	Denitrification	16
PA1174	*napA*	Periplasmic nitrate reductase protein NapA	Denitrification	12
PA0519	*nirS*	Nitrite reductase precursor	Denitrification	122
PA0523	*norC*	Nitric oxide reductase subunit C	Denitrification	135
PA3392	*nosZ*	Nitrous oxide reductase precursor	Denitrification	35
PA5171	*arcA*	Arginine deiminase	Arginine deimination	40
PA0927	*ldhA*	D-Lactate dehydrogenase (fermentative)	Pyruvate fermentation	1
PA0836	*ackA*	Acetate kinase	Pyruvate fermentation	6
PA0835	*pta*	Phosphate acetyltransferase	Pyruvate fermentation	3

[a] From the *P. aeruginosa* genome website, http://www.pseudomonas.com. ORF, open reading frame.
[b] Sources: denitrification, reference 42; arginine deimination, 34; pyruvate fermentation, 9.
[c] Regulation (*n*-fold) of genes during *P. aeruginosa* aerobic and anaerobic growth in CF sputum medium with 100 mM nitrate; a positive number indicates up-regulation of the gene during anaerobic growth.

RESULTS

***P. aeruginosa* induces genes encoding two putative anaerobic nitrate reductases during anaerobic growth in an in vitro CF sputum medium.** CF sputum serves as an in vivo growth substrate for *P. aeruginosa* in the CF lung (19, 25), and we recently developed a method for utilizing CF lung sputum as an in vitro growth substrate for *P. aeruginosa* (26). Transcriptome analyses of CF sputum-grown *P. aeruginosa* indicated that amino acids are the likely in vivo aerobic growth substrates (26). Since anaerobiosis commonly influences how bacteria acquire necessary carbon and energy, it is important to understand how anaerobic growth in CF sputum might impact *P. aeruginosa* metabolism. To determine the impact of anaerobic conditions on *P. aeruginosa* gene expression during growth in CF sputum, we conducted GeneChip microarray analyses of *P. aeruginosa* grown aerobically and anaerobically in CF sputum medium amended with 100 mM nitrate. This nitrate concentration was chosen to provide *P. aeruginosa* with ample electron acceptor for growth. Approximately 3% of the genes within the *P. aeruginosa* genome (167 genes) were differentially regulated over 10-fold when aerobic and anaerobic CF sputum medium-grown bacteria were compared (see the supplemental material). Results from these analyses suggest that, similar to aerobic growth, amino acids likely serve as the carbon substrates for anaerobic growth in CF sputum (see the supplemental material), since expression of previously reported amino acid catabolism genes (26) was unchanged.

Among the genes differentially regulated during anaerobic growth in CF sputum medium were those encoding factors required for anaerobic respiration with nitrate, anaerobic arginine deimination, and pyruvate fermentation. These genes were highly up-regulated during anaerobic growth in CF sputum medium (Table 1). Interestingly, genes encoding two predicted nitrate reductases, one associated with the cytoplasmic membrane (*narGHJI*) and one located in the periplasm (*napAB*), were induced during anaerobic growth in CF sputum. These results contrast with a previous transcriptome study of *P. aeruginosa* PAO1 grown in common laboratory medium, in which the periplasmic nitrate reductase and genes important for nitrate transport (*narK2*) (31) were down-regulated during anaerobic growth with nitrate, while no differential regulation of the membrane-bound nitrate reductase was observed (12).

Genes encoding respiratory enzymes that utilize oxygen as the terminal electron acceptor were also differentially regulated during anaerobic growth in CF sputum medium. Transcript levels for genes encoding the oxygen-scavenging cbb_3-2 cytochrome oxidase (PA1555 to -1557) (data not shown), which are positively regulated by the anaerobic transcriptional regulator Anr, were increased four- to eightfold (7). Conversely, expression of genes encoding homologues of cytochrome *o* ubiquinol oxidase (PA1317 to -1321), which is important for aerobic respiratory energy generation in *E. coli* under highly aerated conditions (6), were decreased approximately threefold (data not shown).

The *narG* gene is required for anaerobic growth with multiple nitrate concentrations. Similar to *P. aeruginosa*, *E. coli* possesses both cytoplasmic membrane-bound and periplasmic nitrate reductases. In *E. coli*, nitrate levels dictate the transcription of genes encoding these two nitrate reductases. Genes encoding the periplasmic nitrate reductase are maximally transcribed anaerobically in the presence of micromolar nitrate concentrations, while genes encoding the membrane-bound nitrate reductase are maximally transcribed anaerobically with millimolar nitrate levels (36). Based on these findings, we reasoned that the levels of nitrate in CF sputum are critical to elucidating the roles of *P. aeruginosa* nitrate reductases in the proposed anaerobic niches of the CF lung. To examine this possibility, we used anion-exchange HPLC to measure nitrate levels within sputum samples collected from multiple CF patients (Table 2). These studies indicate that CF sputum nitrate concentrations fall within an approximate 10-fold range (73 to 792 μM), with an average concentration of 348 μM. Based on these data, we chose 400 μM nitrate as a physiologically relevant nitrate concentration for further ex-

TABLE 2. Nitrate levels in CF sputum

CF sputum sample no.	NO_3^- (μM)
1	73
2	340
3	682
4	792
5	120
6	89
7	342
Avg	348

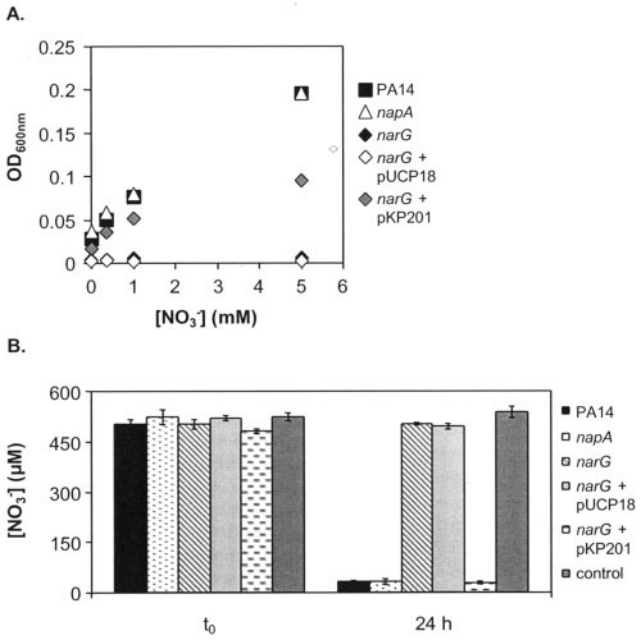

FIG. 1. *P. aeruginosa* requires the *narG* gene for anaerobic growth with nitrate. *P. aeruginosa* PA14, the PA14 isogenic *napA* and *narG* mutants, and the PA14 *narG* mutant harboring pUCP18 or pUCP18 expressing *narGHJI* in *trans* (pKP201) were grown anaerobically in BHI broth in the presence of various nitrate concentrations. (A) Growth at 24 h postinoculation as measured by OD_{600}. Error bars representing standard deviations from the mean are too small to be seen. (B) Nitrate levels immediately after inoculation (t_0) and 24 h postinoculation as measured by anion-exchange chromatography in anaerobic *P. aeruginosa* cultures supplemented with 400 μM nitrate. Uninoculated controls were included to evaluate nonbiological reduction of nitrate. The error bars represent standard deviations from the mean.

periments. The concentrations of nitrate observed in this study were within a fivefold range of previous studies examining nitrate levels in CF sputum; however, it should be noted that our results were obtained by direct measurement of nitrate in CF sputum, while previous investigations measured nitrate levels by first enzymatically converting nitrate to nitrite (16, 22).

Based on *E. coli* physiology and the observation that micromolar levels of nitrate are present in CF sputum, we hypothesized that the *P. aeruginosa* periplasmic nitrate reductase (NapAB) would be required for anaerobic growth in CF sputum. Conversely, our hypothesis predicted that the membrane-bound nitrate reductase complex (NarGJHI) would be required for anaerobic growth of *P. aeruginosa* with nitrate concentrations not relevant to CF disease. To test this hypothesis, we compared anaerobic growth of isogenic *P. aeruginosa napA* and *narG* mutants to wild-type *P. aeruginosa* in the presence of various nitrate concentrations (Fig. 1A). A linear relationship was observed between the wild-type *P. aeruginosa* growth yield and the concentration of nitrate provided. Contrary to our hypothesis, the *napA* mutant demonstrated no anaerobic growth defect while the *narG* mutant demonstrated a severe anaerobic growth defect at all nitrate concentrations, including concentrations normally found in CF sputum (Fig. 1A). We confirmed that the transposon insertion in *narG* was the basis for this growth defect by complementation analysis. Expression of the *narGHJI* genes, encoding the entire membrane-bound nitrate reductase, in *trans* restored growth of the *narG* mutant to near wild-type levels, while the presence of the complementation vector alone had no impact on growth (Fig. 1A). Complementation analysis was performed with the entire membrane-bound nitrate reductase coding region, since the transposon insertion in the *narG* mutant exerts polar effects on *narHJI*. Similar anaerobic growth results were obtained when wild-type *P. aeruginosa* strain PAO1 was compared to the PAO1 *narG* mutant, indicating that this growth defect is not specific to strain PA14 (data not shown).

Since nitrate serves as the terminal electron acceptor in these experiments, nitrate loss can be used as a marker for anaerobic nitrate reduction. Based on the previous growth yield experiments, we predicted that the *narG* mutant would not reduce nitrate while the wild-type and the *napA* mutant would exhibit similar nitrate reduction profiles. Measurement of nitrate concentrations in cell supernatants of wild-type *P. aeruginosa*, the *napA* mutant, and the *narG* mutant grown anaerobically with 400 μM nitrate indicated that after 24 h of growth, wild-type *P. aeruginosa* and the *napA* mutant reduced virtually all the nitrate present (Fig. 1B). As expected, the *narG* mutant was deficient for anaerobic nitrate reduction, and expression of *narGHJI* in *trans* restored this ability. Collectively, these data indicate that *P. aeruginosa* requires the membrane-bound nitrate reductase for anaerobic growth and nitrate reduction at nitrate concentrations relevant to the CF lung.

The *narG* mutant does not have a general anaerobic growth defect. Denitrification is the progressive reduction of nitrate to dinitrogen, and nitrate reduction to nitrite is the first step (42). Our data indicate that the *P. aeruginosa narG* gene is required to carry out this initial reaction. *P. aeruginosa* can also use the denitrification intermediates nitrite and nitrous oxide as anaerobic electron acceptors (39). Previous studies of NarG in *Pseudomonas fluorescens* revealed that genetic inactivation of *narG* also resulted in an inability to reduce nitrate, but it also negatively impacted nitrite reduction (14). Based on these studies, it was possible that inactivation of *narG* in *P. aeruginosa* resulted in a generalized anaerobic growth defect not specific to nitrate respiration. To determine if the growth defect of the *narG* mutant was specific to nitrate, we tested the ability of this mutant to grow anaerobically using 1 mM nitrite as the terminal electron acceptor. The results from these experiments indicate that the *narG* mutant reaches similar cell densities (averages, 6.6×10^7 CFU/ml for wild-type and 5.0×10^7 CFU/ml for the *narG* mutant) and reduces nitrite to levels (<10 μM) similar to those for wild-type *P. aeruginosa*. These data indicate that the *narG* mutant does not possess a general anaerobic growth defect but instead has a nitrate-specific defect.

Nitrate levels in CF sputum support significant anaerobic *P. aeruginosa* growth, and the *narG* gene is required for this growth. Previous work in our laboratory indicated that aerobic growth in CF sputum significantly impacts the physiology of *P. aeruginosa* (26). Having established that *narG* was required for anaerobic growth of *P. aeruginosa* in a common laboratory medium using physiologically relevant concentrations of nitrate, we sought to determine whether growth in CF sputum would influence the anaerobic requirement for *narG*. For these experiments, wild-type *P. aeruginosa* and the *narG* mutant were

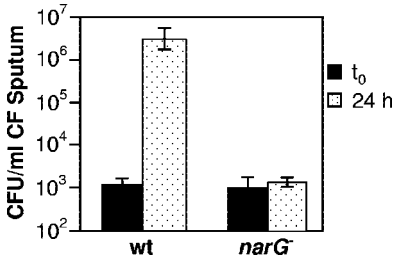

FIG. 2. The *narG* gene is required for anaerobic growth in CF sputum. *P. aeruginosa* PA14 and the isogenic *narG* mutant were grown anaerobically in 25% CF sputum medium containing 400 μM nitrate. Viable-bacterial-cell counts immediately after inoculation (t_0) and after 24 h of anaerobic growth are shown. The error bars represent standard deviations from the mean. wt, wild type.

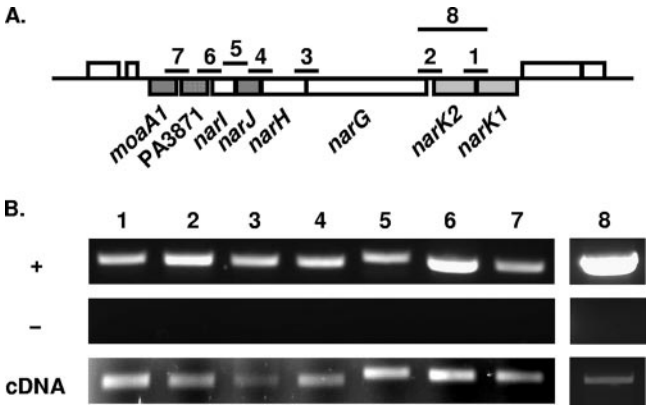

FIG. 3. The *narG* gene is cotranscribed with other genes implicated in anaerobic nitrate reduction. (A) Chromosomal organization of genes surrounding *narG* (http://www.pseudomonas.com). The numbered horizontal bars represent regions amplified by primer sets overlapping coding regions of putative cotranscribed genes (see Table S1 in the supplemental material). (B) RT-PCR analysis of *narG* transcript structure. *P. aeruginosa* PA14 was grown anaerobically and harvested for RNA isolation. RNA was used as a template for amplification of cDNA by random priming, and cDNA was subsequently used as a template for standard PCR with primer sets overlapping coding regions as shown in panel A. Appropriate positive (+; *P. aeruginosa* chromosomal DNA) and negative (−; RNA) controls were included. Product 8, spanning *narK1* to *narG*, is presented separately due to the larger size of the amplicon.

grown in an in vitro CF sputum medium containing nitrate at levels (400 μM) commonly found in CF sputum. Viable-cell counts were then performed to examine the importance of *narG* for anaerobic growth in CF sputum. The results from these experiments indicated that wild-type *P. aeruginosa* grows well anaerobically in CF sputum medium, exhibiting an ~3.5-log-unit increase in cell numbers (from 1×10^3 to 5×10^6 bacteria/ml); however, inactivation of *narG* eliminated the ability of *P. aeruginosa* to grow anaerobically in CF sputum (Fig. 2). Collectively, these data indicate that the concentration of nitrate commonly found in CF sputum is sufficient to support significant growth of *P. aeruginosa* and that the membrane-bound nitrate reductase is required for *P. aeruginosa* anaerobic growth in CF sputum medium.

The *narG* gene is cotranscribed with other genes implicated in anaerobic nitrate reduction. A recent study reported that antibodies to *P. aeruginosa* NarG are present in sera from CF patients (2), suggesting that *narG* is expressed by *P. aeruginosa* in vivo. These data, along with our data demonstrating that *narG* is required for anaerobic growth in CF sputum medium, underscore the need to identify conditions under which *narG* is transcribed. Such studies will provide important information regarding the microenvironments of the CF lung where the membrane-bound nitrate reductase may be produced. To examine *narG* transcription, it was important to first identify the genomic context of the gene. The *narG* gene is the third gene in a putative operon implicated in anaerobic nitrate reduction (31). Aside from *narG*, this potential operon includes two homologues of the nitrate/nitrite antiporter gene *narK*, the molybdopterin cofactor synthesis genes PA3871 (*nifM*) and *moaA1*, and *narHJI*, which encode other components of the membrane-bound nitrate reductase (Fig. 3A) (http://www.pseudomonas.com). In silico analysis supports cotranscription of these eight genes, as the start codon and the stop codon of adjacent genes are localized within 75 bp of each other. To experimentally examine the *nar* operon structure, RT-PCR was used to examine the *narG* transcript structure. The results from these experiments demonstrate that a transcript containing *narK1*, *narK2*, *narG*, and likely the five other downstream genes exists (Fig. 3B and C). These results also suggest that the promoter controlling *nar* transcription lies upstream of *narK1*.

Expression of *narG* is induced during anaerobic growth with physiologically relevant nitrate levels. Is *nar* operon expression induced by anaerobiosis and/or by physiologically relevant nitrate concentrations? To address this question, a 688-bp region upstream of *narK1* was amplified and cloned into the β-galactosidase reporter plasmid pQF50 to generate a *nar*::*lacZ* transcriptional fusion. *P. aeruginosa* carrying *nar*::*lacZ* was then used to monitor induction of the *nar* genes under aerobic and anaerobic conditions with or without added nitrate. Our results indicate that anaerobic growth induces transcription of the *nar* operon approximately 10-fold, and this induction is enhanced further anaerobically with the addition of nitrate (Fig. 4A). More importantly, this enhanced induction with nitrate was observed using 400 μM nitrate, indicating that *nar* expression is responsive to nitrate levels commonly found in the CF lung.

In other bacteria, transcriptional regulation in response to oxygen levels and nitrate is attributed to FNR-type proteins and NarL, respectively (33, 35). Previous studies have shown that two FNR-type regulatory proteins, Anr and Dnr, are required for *P. aeruginosa* denitrification (1, 41). As expected from the *nar* promoter results described above, in silico examination of the region upstream of *narK1* revealed the presence of consensus binding sites for FNR-type proteins (1) and NarL (33) (Fig. 4B). The *narXL* genes are divergently transcribed from *narK1*, suggesting that the putative FNR and NarL consensus binding sites in their intergenic region may serve to regulate either or both sets of genes. As predicted by the operon structure, no consensus binding sites for FNR-type proteins or NarL were found in the intergenic regions within the *nar* operon (Fig. 4A and data not shown), suggesting that the promoter upstream of *narK1* likely regulates *nar* operon expression.

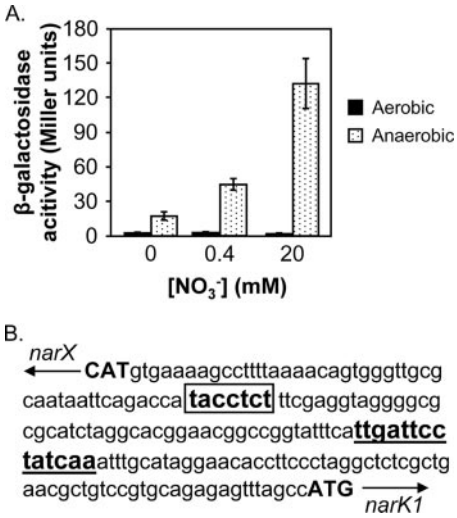

FIG. 4. (A) Expression of *narG* is induced during anaerobic growth with 400 μM nitrate. The DNA region upstream of *narK1* was cloned upstream of a promoterless *lacZ* gene on pQF50 to generate *nar*::*lacZ*. *P. aeruginosa* PA14 harboring pQF50 or the *nar*::*lacZ* fusion was grown aerobically and anaerobically in BHI broth with 0 μM, 400 μM, or 20 mM added nitrate. Assays for β-galactosidase (24) were performed for anaerobic and aerobic bacteria during exponential growth. The error bars represent standard deviations of the mean. Average background β-galactosidase activity (33 activity units) mediated by promoterless pQF50 was not significantly altered by the presence or absence of nitrate under aerobic or anaerobic conditions, with the exception of an ~2-fold increase in activity in anaerobic cultures in the presence of 20 mM nitrate. (B) In silico analysis of the *nar* operon promoter reveals nitrate- and oxygen-responsive operators. The DNA sequence upstream of *narK1* possesses consensus binding sites for FNR-type transcriptional regulators (TTGAT . . . . ATCAA, underlined) and the nitrate-responsive transcriptional regulator NarL (TACYNKT, boxed). The predicted translational start codons of *narK1* (ATG) and the divergently transcribed *narX* (CAT) are shown.

DISCUSSION

This study provides evidence that the membrane-bound nitrate reductase is required for *P. aeruginosa* anaerobic growth in an in vitro CF sputum medium. Genes encoding this enzyme complex are induced under anaerobic conditions in the presence of nitrate concentrations commonly found in CF sputum (16, 22). Based on these results, we anticipate that *P. aeruginosa* will express *narG* in the CF lung when exposed to anaerobic microenvironments. These data also suggest that *P. aeruginosa* requires the membrane-bound nitrate reductase to generate energy in vivo under anaerobic conditions when nitrate is available. A recent study aimed at identifying transposon mutants in *P. aeruginosa* PAO1 unable to grow anaerobically with nitrate did not identify the membrane-bound anaerobic nitrate reductase as essential (11). The reason for this apparent inconsistency is not strain differences, as *P. aeruginosa* PAO1 also requires *narG* for anaerobic growth with nitrate (data not shown); however, since the previous study did not perform a saturating mutagenesis, it is possible that mutants in the *nar* operon were not examined.

Given that previous studies have found considerably lower levels of nitrite than of nitrate in CF sputum (16, 22), nitrate would likely be the electron acceptor used for anaerobic respiratory growth of *P. aeruginosa* in vivo. This nitrate is presumably derived from the spontaneous oxidation of reactive nitrogen species produced by components of the innate immune system (8). Since nitrate is present in CF sputum, it could be argued that *P. aeruginosa* does not utilize endogenous nitrate as a terminal electron acceptor in the CF lung. It should be noted that the samples used in this study contained low levels of *P. aeruginosa* ($<10^8$ cells/ml sputum) and sufficient nutrients to allow substantial bacterial growth. It should also be noted that the CF lung is not a batch growth system (like that used in these studies) but more analogous to a chemostat or fed-batch system. Under these growth conditions, sputum nutritional components may be continually replenished by endogenous nitrate production. In any case, our results indicate that a *P. aeruginosa* protein produced in individuals with CF is required for anaerobic growth using the preferred anaerobic electron acceptor (nitrate) present in the CF lung. This study also provides the first experimental evidence that CF sputum nitrate levels are sufficient to support significant anaerobic growth of *P. aeruginosa*.

Antibodies to NapA were also detected in sera from CF patients (2), indicating that the periplasmic nitrate reductase is also produced by *P. aeruginosa* in vivo. The results of our study indicate that the periplasmic nitrate reductase is not required for anaerobic energy generation, since no growth defect or nitrate reduction defect is observed in a *napA* mutant. In other microorganisms, the periplasmic nitrate reductase fills a variety of metabolic roles (27). Some bacteria possess and use only the periplasmic nitrate reductase for anaerobic growth with nitrate (3, 4). As mentioned above, *E. coli* possesses both membrane-bound and periplasmic enzymes but utilizes the periplasmic enzymes only at low substrate concentrations (32, 36). Alternatively, some microorganisms use the periplasmic nitrate reductase to balance cellular oxidation-reduction processes when the concentration of cellular reducing equivalents is high and oxygen concentrations are low (13, 27). It is possible that *P. aeruginosa* utilizes the periplasmic nitrate reductase for this purpose.

While *P. aeruginosa* prefers respiratory energy production in anaerobic environments where nitrate is available, this study does not preclude the potential role of arginine deimination and pyruvate fermentation in the CF lung. It is possible that *P. aeruginosa* could encounter anaerobic low-nitrate environments in vivo. However, the inability of pyruvate fermentation to support *P. aeruginosa* anaerobic growth (9) and the observation that an arginine deimination mutant shows no anaerobic growth deficiency in CF sputum (data not shown) suggest that these pathways would be primarily used for acquiring cell maintenance energy or balancing oxidation-reduction processes and not bacterial growth in vivo.

P. aeruginosa possesses five putative or confirmed terminal oxidases that could potentially utilize oxygen for respiratory growth (7). Given the apparent respiratory flexibility of *P. aeruginosa* during aerobic growth, it is surprising that one physiological component is required for anaerobic growth with nitrate in an in vitro CF sputum medium. These results are encouraging and suggest that the membrane-bound nitrate reductase is a potential candidate for the targeted development of therapies specific for *P. aeruginosa* growing anaerobically. Anaerobic respiration as a drug target has been proposed for other bacteria, including *Mycobacterium bovis* (37). *M. bovis*

requires the membrane-bound nitrate reductase for chronic lung infection, thus emphasizing the potential importance of this enzyme complex in persistent infections. Since clinically relevant phenotypes, such as mucoidy and biofilm formation, are influenced by the respiratory and metabolic states of *P. aeruginosa*, investigations such as this examining *P. aeruginosa* physiology during growth with in vivo substrates and various oxygen concentrations are critical to the development of effective treatment strategies.

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