Pseudomonas aeruginosa PA1006, Which Plays a Role in Molybdenum Homeostasis, Is Required for Nitrate Utilization, Biofilm Formation, and Virulence

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Abstract

Pseudomonas aeruginosa (Pae) is a clinically important opportunistic pathogen. Herein, we demonstrate that the PA1006 protein is critical for all nitrate reductase activities, growth as a biofilm in a continuous flow system, as well as virulence in mouse burn and rat lung model systems. Microarray analysis revealed that ΔPA1006 cells displayed extensive alterations in gene expression including nitrate-responsive, quorum sensing (including PQS production), and iron-regulated genes, as well as molybdenum cofactor and Fe-S cluster biosynthesis factors, members of the TCA cycle, and Type VI Secretion System components. Phenotype Microarray™ profiles of ΔPA1006 aerobic cultures using Biolog plates also revealed a reduced ability to utilize a number of TCA cycle intermediates as well as a failure to utilize xanthine as a sole source of nitrogen. As a whole, these data indicate that the loss of PA1006 confers extensive changes in Pae metabolism. Based upon homology of PA1006 to the E. coli YhhP protein and data from the accompanying study, loss of PA1006 persulfuration and/or molybdenum homeostasis are likely the cause of extensive metabolic alterations that impact biofilm development and virulence in the ΔPA1006 mutant.

Introduction

P. aeruginosa (Pae) is a ubiquitous opportunistic nosocomial pathogen that infects individuals with pre-disposing conditions such as cancer, AIDS, burns, and importantly, Cystic Fibrosis (CF). Pae is highly clinically relevant since it causes ~10% of the 2 million life-threatening nosocomial infections that occur annually in the United States [1]. Pae possesses numerous virulence factors that contribute to pathogenesis including proteases, exotoxin A, hydrogen cyanide, and phenazines [2,3]. In addition, despite intensive treatment, eradication of Pae is extremely difficult due to its intrinsic ability to resist a variety of antimicrobial agents [4,5].

Mounting evidence indicates that Pae experiences microaerobic as well as anaerobic environments during biofilm development and during infection in vivo [6,7,8,9,10,11,12,13,14]. In biofilms, cell density and secreted factors may contribute to oxygen depletion/availability. During infection, a mucoidy phenotype, resulting from the expression of the polysaccharide alginate, often develops. It has been speculated that this dense covering provides a barrier to oxygen diffusion. In the CF lung, a failure in the Cl channel CFTR (Cystic Fibrosis transmembrane conductance regulator) produces salt imbalances that allow thick mucus to develop. Excess mucus hinders the beating of epithelial cilia likely allowing bacteria to settle. Pae appears well-suited for this niche. Experiments measuring the effect of prepared mucus on Pae biofilms in vitro suggest that the dense mucus prevalent in a CF lung may exert a positive effect by slowing swimming, increasing the local concentration of autoinducers, and restricting access of host factors such as lactoferrin [13]. Reduced oxygen tension does not appear to be a problem in infection, since biofilms can form under anaerobic conditions [8]. Compounding matters, biofilms and anaerobic growth also appear to contribute to increased antibiotic resistance [6]. Proteins that enable biofilm development and anaerobic metabolism in vivo (in the host) are not well defined and likely also contribute to virulence potential.

In the absence of oxygen, Pae may respire by utilizing nitrate or nitrite as alternative terminal electron acceptors via denitrification [16]. Denitrification enzymes reduce nitrate (NO3−) to nitrite (NO2−), and subsequently to nitric oxide (NO), nitrous oxide (N2O), and finally, dinitrogen gas (N2). In addition to denitrifica-
tion enzymes \textit{per se}, nitrate alters the expression of a number of genes involved in virulence factor production [17]. Consistent with a correlation between virulence, biofilms, and anaerobic growth by denitrification, NarGHI (the membrane associated nitrate reductase) is required for growth in the CF lung [18]. NarGHI is also required for biofilm formation in the flow system as well as for virulence in the \textit{C. elegans} model of infection [19]. It is also notable that NO elicits biofilm dispersal suggesting that denitrification pathways appear to contribute to virulence; [8,19], we set out to thoroughly characterize the role of PA1006 in \textit{Pae}. To confirm specificity of the original Tn mutant and further investigate the function of PA1006, an in-frame deletion mutant (\textbf{ΔPA1006}) was constructed [25] in \textit{Pae} strain PAO1. For anaerobic growth studies, aerobically grown overnight cultures of PAO1, the \textbf{ΔPA1006} mutant and a complemented mutant (\textbf{ΔPA1006(attBPA1006)} containing a single copy of the wild-type (WT) \textit{PA1006} gene reintroduced onto the chromosome [26], were used to inoculate pre-reduced NY media supplemented with 100 mM KNO$_3$ as the terminal electron acceptor. Aerobic growth was unaffected (Fig. 1A), in contrast to the WT strain PAO1, the \textbf{ΔPA1006} mutant was unable to grow anaerobically (Fig. 1B). Even after 24 hrs, no significant change in optical density was observed (data not shown). Single copy chromosomal complementation of the \textbf{ΔPA1006} mutant with the WT gene restored anaerobic growth (Fig. 1B) indicating that loss of \textit{PA1006} was solely responsible for the anaerobic growth defect with nitrate.

Since \textit{Pae} can use electron acceptor sources other than nitrate for anaerobic growth, the ability of the mutant to use alternative sources for anaerobic growth was analyzed. Notably, \textbf{ΔPA1006} was fully capable of growing anaerobically with nitrite and arginine (File S1). These data suggest that \textit{PA1006} is specifically involved in the reduction of nitrate.

\textit{Pae} can reduce nitrate either through assimilation or dissimilation. In \textit{Pae}, dissimilatory reduction of nitrate (where nitrate acts as a terminal electron acceptor) normally occurs under anaerobic conditions but may also occur during aerobic growth [16,27,28]. Previously, microarray data indicated the nitrate reductase genes (\textit{nar} and \textit{nap}) are induced aerobically when 100 mM KNO$_3$ is added to the media [17]. Therefore, nitrate reductase activity was assayed using whole cell suspensions from cultures grown aerobically in the presence of nitrate [29,30]. Methyl viologen was used as the electron donor avoiding oxygen inhibition. Membrane (\textit{Nar}) and periplasmic (\textit{Nap}) activities were differentiated by their respective sensitivity or insensitivity to sodium azide (Na$_3$NO$_2$) which only inhibits the membrane bound nitrate reductase NarGHI [30]. Apparent Nar and Nap-dependent nitrate reductase activities were measurable in WT suspensions whereas \textbf{ΔPA1006} failed to show significant levels of either activity (Fig. 1C). Once again, the complemented \textbf{ΔPA1006} strain showed activity equivalent to WT (Fig. 1C).

The lack of nitrate reductase activity in the \textbf{ΔPA1006} strain may be due to the lack of expression of the \textit{nar} and \textit{nap} transcripts or proteins. To determine if the \textbf{ΔPA1006} strain expressed NarGHI peptides, Western blot analysis was performed using rabbit polyclonal \textit{\textgamma;}-NarGHI antisera. The \textbf{ΔPA1006} mutant displayed peptides that reacted with \textit{\textgamma;}-NarGHI at levels comparable to WT (Fig. 1D). These data suggest that the \textbf{ΔPA1006} strain is capable of expressing the transcripts and protein subunits for the membrane tethered nitrate reductase NarGHI. In this case, the lack of nitrate reductase activity may be due to an inability to post-translationally process and/or assemble functional nitrate reductase enzymes.
**Figure 1. PA1006 is critical for nitrate reductase activity.** A/B) PA1006 does not appear to affect aerobic growth in rich media but is required for anaerobic growth with nitrate. (●) WT; (○) ΔPA1006; (▲) ΔPA1006:attb:PA1006. Growth curves were performed in duplicate as indicated in the Methods average values are plotted. Data showed excellent agreement. C) ΔPA1006 whole cell suspensions lack periplasmic and membrane nitrate reductase activity. D) Western blot with α-NarGH antisera of whole cell extract of wild-type (wt) and ΔPA1006 (Δ) cells indicates that the membrane nitrate reductase is present but inactive. E) Summary of nitrate and nitrite reductases in *Pae*, their cofactors, and what is known about functionality in the ΔPA1006 mutant.

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Because nitrate reduction can also occur aerobically via the assimilatory Nas complex [31], and Nas also contains iron-sulfur clusters and molybdobium cofactor, we investigated if PA1006 also played a role in this process. ΔPA1006 was unable to grow aerobically when nitrate was provided as the sole nitrogen source (Files S1 and S8). These data indicate that the assimilatory nitrate reductase is also nonfunctional. Figure 1E is a compilation of Paε nitrate reductases and the cofactors required by each enzyme. A summary of the results of the growth and enzyme assays for the ΔPA1006 strain are also shown. Loss of both assimilatory and dissimilatory nitrate reductase activities suggests that essential co-factors common to the nitrate reductases such as iron-sulfur clusters or molybdobium cofactor (we will use the generic term “MoCo” throughout this manuscript) may be lacking.

Given that the PA1006 gene exists in an operon with PA1007 which appears to be a transmembrane protein, it is possible that the PA1007 protein may cooperate with PA1006. To address this point, we obtained and confirmed the ΔPA1007 transposon mutant from the PAO1 transposon insertion strain collection from the University of Washington. Since the ΔPA1007 mutant was able to grow anaerobically with nitrate (data not shown), we did not perform additional analyses of the ΔPA1007 mutant since it displayed non-equivalence with the ΔPA1006 deletion strain.

PA1006 is Required for Virulence

Paε virulence has been examined by using different animal as well as plant and invertebrate models [32,33,34,35,36,37,38]. Since the PA1006 To mutant previously displayed diminished virulence in the lettuce leaf model [21], we hypothesized that PA1006 may play a role in Paε pathogenesis in other model systems.

The burned mouse model has been extensively used to examine the pathogenesis of Paε infection of burn wounds as well as to demonstrate the critical roles of virulence factors [38]. We employed the burned-mouse model to test the requirement of PA1006 for Paε pathogenesis. Three groups of mice (n = 15 total) were burned and inoculated with approximately \(3 \times 10^6\) CFU of each of the strains as described in the materials and methods. Three separate experiments were performed with each strain. Survival of the mice was followed for a total of 5 days. [38]. As shown in Figure 2A, the parental WT PAO1 strain caused a significantly higher percent mortality than the ΔPA1006 mutant (48 hours, p = 0.077, 72 hours, p = 0.0092, 96 hours, p = 0.0092, 120 hours, p = 0.0025). Importantly, complementation of the ΔPA1006 mutant, with a single copy of the PA1006 gene integrated into the chromosome, fully restored virulence to wild-type levels (93%) confirming a role for PA1006 in Paε virulence and infection in this acute infection animal model. Reduced mortality observed by the PA1006 mutant may have been due to the result of reduced dissemination (the systemic spread) of Paε within the mice. The systemic spread of PAO1, ΔPA1006, and the complemented mutant were examined by determining the numbers of CFU of these strains within the livers of the burned and infected mice. As shown in Figure 2B, at 24 h post burn infection, the numbers of bacteria (CFU per gram of tissue) that were recovered from the livers of the ΔPA1006 mutant-infected mice were significantly lower than that of WT PAO1- or complemented mutant-infected, with almost a 3-log difference (p<0.01). Colonies were obtained from the livers of the mice were examined for the ability to grow anaerobically (data not shown). One hundred percent of the colonies examined retained the mutant phenotype, which confirmed that the ΔPA1006 mutant did not revert to WT with concomitant ability to grow anaerobically. These results suggest that a mutation in PA1006 interferes with the systemic spread of Paε within the burned and infected mice. Next, we chose to evaluate the role of PA1006 in the agar bead rat lung model which is representative of the chronic infection observed in the CF lung [35]. Quantitative bacteriology (colony forming units recovered from tissue) showed that the ΔPA1006 mutant survived as well as the WT bacteria when the mutant was embedded in agar beads and placed in rat lungs (Figure 2C). However, the ΔPA1006 mutant appeared to cause significantly (p<0.001) less damage to the lung tissue compared to the WT strain (Figure 2C). The WT strain showed ~38% lung inflammation whereas the mutant displayed about half as much inflammation (~16%). The decrease in inflammation was completely restored in the complemented strain (ΔPA1006catB:PA1006), indicating expression of PA1006 is required for full virulence of Paε in the lung model. These data suggest that PA1006 may also be a critical determinant of chronic pathogenesis in the CF patient’s lung.

PA1006 is Critical for Biofilm Maturation

Bacterial biofilms have been implicated in chronic lung infections caused by Paε [39,40]. Since PA1006 is required for anaerobic growth with nitrate and virulence, we hypothesized that PA1006 could play a role in biofilm formation. To test this hypothesis, PAO1, ΔPA1006, and the complemented strains (ΔPA1006catB:PA1006) were tagged with the green fluorescent protein (GFP) [41]. We first tested biofilm formation in the “static-dish” assay which does not entail a flow system and relatively immature biofilms may form 24 h after initial inoculation. Confocal scanning laser microscopy (CSLM) and COMSTAT analysis were used to analyze the biofilms that covered the glass window at the bottom of the petri plate. In this system, WT, ΔPA1006 mutant and complemented strains all appeared identical with mean thicknesses of approximately 10 μm each (File S2).

Next, biofilm maturation was studied using a flow cell system which allows a relatively more mature biofilm to develop. Mature biofilms consist of a progression from microcolonies to tower-like structures that resemble mushrooms and are able to withstand the shear forces associated with the flow system. In addition to more complex architectures, water channels are also apparent. In the flow system assay, biofilm architecture was followed over a period of 3 days by CSLM and data analyzed using COMSTAT. At 24 h and thereafter up to 72 h, the WT and complemented strains formed microcolonies and channels characteristic of mature Paε biofilms. In contrast to the static system, the ΔPA1006 mutant displayed a severe defect in the continuous flow biofilm system (Fig. 3). Even after 72 h, very few bacteria were observed in the ΔPA1006 chamber and the few cells present appeared as a thin layer of undifferentiated cells that failed to form microcolonies. Quantitative analysis with COMSTAT confirmed that the ΔPA1006 mutant displayed severely reduced biomass, lower average thickness, and lower surface coverage compared to WT and complemented strains (Fig. 3). The calculated roughness coefficient, reflecting heterogeneity in thickness of the biofilm, was significantly greater for the ΔPA1006 mutant (data not shown). Since complementation of ΔPA1006 fully restored normal biofilm architecture, this dramatic phenotype is clearly due to the loss of PA1006. It should also be noted that the media used to grow biofilms in both static and flow-systems were identical and contained ammonia as the nitrogen source. Thus, failure of the ΔPA1006 mutant to form a biofilm in the flow-system is not simply due to a failure to grow or utilize nitrate as a nitrogen source. More likely, the ΔPA1006 mutant is less able to withstand the shear forces imposed by the flow system.

Since flagellar-based swimming and swarming motilities as well as type IV pilus-mediated twitching motility are important for Paε...
biofilm formation and dispersal [42], we investigated whether the ΔPA1006 mutation influenced these forms of motility. The ΔPA1006 mutant exhibited twitching (17 mm ± 1 mm) and swimming (32 mm ± 1 mm) motilities undistinguishable from the WT strain PAO1 (twitching; 18 mm ± 1 mm) and (swimming; 32 mm ± 1 mm), indicating that the defects in biofilm maturation are not due to deficiencies in motility. Next, we measured swarming. Initially, the ΔPA1006 mutant appeared to fail to exhibit swarming motility compared to WT PAO1. However, the original swarm agar plates contained nitrate as the sole source of nitrogen and growth was minimal. Given the failure of the ΔPA1006 mutant to utilize nitrate, we substituted glutamate as the nitrogen source. In this case, swarming of the ΔPA1006 mutant (10 mm ± 1 mm) was comparable to WT (9 mm ± 1 mm). Since rhamnolipid production also affects virulence, biofilms, as well as swarming motility, we also examined rhamnolipid production. Cells were grown for 90 h in M 8 minimal salts-based media containing glucose and glutamate as the sole sources of carbon and nitrogen respectively [43] and rhamnolipid production was determined by extraction of culture supernatants with diethyl ether followed by the orcinol assay as previously described [44]. Similar to swarming, rhamnolipid production by the ΔPA1006 mutant appeared equivalent to WT when glutamate was substituted for nitrate as the source of nitrogen (all cell culture supernatants contained ~100±10 mg/mL rhamnolipids). These data indicate that the ΔPA1006 mutant does not resemble other previously characterized mutants defective in biofilm formation, such as those deficient in flagella, Type IV pilus, GacA, or Crc which display poor surface attachment or alterations in swimming, swarming, or twitching [42,45].

Pae virulence and biofilms have been affected by changes in or a loss of lipopolysachharide (LPS) [36,44]. When LPS profiles were analyzed and compared, the parent, mutant, and complemented strains expressed identical LPS profiles, confirming that the reduced virulence and dissemination as well as failure to form a mature biofilm in the flow systems observed with the ΔPA1006 mutant were not due to altered LPS expression (data not shown).

Loss of PA1006 Affects Global Gene Expression

The combination of reduced virulence and biofilm phenotypes displayed by the ΔPA1006 mutant suggested that expression of additional genes or activities of other proteins may be influenced by PA1006. In addition, previous transcriptome analysis from our laboratory revealed that expression of greater than 500 genes were
altered by the addition of nitrate at early stationary phase under aerobic conditions [17]. Since the ΔPA1006 mutant failed to dissimilate or assimilate nitrate, we were interested to test the effect of nitrate on gene expression in the ΔPA1006 mutant. Microarray analysis was performed on both WT and the ΔPA1006 mutant grown aerobically in the presence and absence of nitrate (Tables 1, 2; Files S4, S5, S6). It should be noted that the list of genes presented in Table 1 is not exhaustive, but rather lists genes that showed robust changes or those whose products are involved in denitrification, or represent known or putative virulence factors. The complete set of genes that showed altered expression can be found in Files S4 and S5.

In the absence of nitrate supplementation (NY media), 73 and 16 genes displayed decreased or increased expression respectively in the ΔPA1006 mutant (using a 2-fold cutoff). Especially notable were significantly higher levels of RNAs for the Nar gene cluster.
which codes for the membrane nitrate reductase, accessory factors, and nitrate transport proteins (PA3071-PA3077), as well as genes involved in molybdopterin biosynthesis (PA3914-18; and PA3870; Table 1). This was unexpected because nitrate reductase activity appeared to be lacking in the ΔPA1006 mutant. Also notable was the fact that the ΔPA1006 mutant also displayed reduced expression of several Type-VI Secretion System components (PA0074-75, PA0077-78, PA0085, PA0090, PA0262, and PA3294). Since Type-VI Secretion System components have already been implicated in P. aeruginosa virulence [46] these data may be correlated with the loss of virulence potential displayed by the ΔPA1006 mutant in the mouse burn and rat lung.

Next, we examined the effect of NO3− on gene expression. 230 genes displayed increased expression and 328 genes displayed decreased or increased expression respectively (with a 2-fold cutoff) in the ΔPA1006 mutant compared to the WT when 100 mM KNO3 was added to the growth media. In this case, other denitrification pathway genes such as nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) displayed dramatically reduced expression in the ΔPA1006 mutant (PA0509-26 and PA3391-96 clusters; Table 2). Generally, compared to WT, it appears that denitrification pathway enzymes downstream of Nar are not induced by nitrate in the ΔPA1006 mutant. Loss of PA1006 also altered the expression of several other notable genes (Table 2). For example, ΔPA1006 cells also showed increased expression of the

Table 1. Notable nitrate metabolism and virulence genes whose expression levels are altered in the ΔPA1006 mutant in the absence of nitrate.

| ORF     | Gene              | Fold change | Protein description                                      |
|---------|-------------------|-------------|---------------------------------------------------------|
| PA3870  | mopaA1            | +17.6       | molybdopterin biosynthetic protein A1                   |
| PA3871  |                   | +21.5       | probable peptidyl-prolyl cis-trans isomerase            |
| PA3872  | narI              | +22.2       | respiratory nitrate reductase gamma chain              |
| PA3873  | narJ              | +26.9       | respiratory nitrate reductase delta chain              |
| PA3874  | narH              | +34.2       | respiratory nitrate reductase beta chain               |
| PA3875  | narG              | +38.0       | respiratory nitrate reductase alpha chain             |
| PA3876  | narK2             | +51.8       | nitrite extrusion protein 2                            |
| PA3877  | narK1             | +63.9       | nitrite extrusion protein 1                            |
| PA3911  | yhbT              | +7.0        | conserved hypothetical protein                          |
| PA3912  | yhbV              | +11.2       | conserved hypothetical protein                          |
| PA3913  | yhbU              | +15.1       | probable protease                                      |
| PA3914  | mopaE1            | +209.4      | molybdenum cofactor biosynthesis                       |
| PA3915  | mopaB1            | +52.8       | molybdopterin biosynthetic protein B1                   |
| PA3916  | mopaE2            | +11.6       | molybdopterin converting factor                        |
| PA3917  | mopaD             | +10.1       | molybdopterin converting factor                        |
| PA3918  | mopaC             | +10.5       | molybdopterin biosynthetic protein C                   |
| PA0074  | ppkA              | -2.4        | Ser/Thr protein kinase- TypeVI SS                      |
| PA0075  | ppmA              | -2.0        | Ser/Thr protein phosphatase- TypeVI SS                 |
| PA0077  | icmM              | -2.0        | TypeVI SS component                                   |
| PA0078  |                   | -2.4        | TypeVI SS component                                   |
| PA0085  | Hcp1              | -2.0        | TypeVI SS component                                   |
| PA0090  | cpaV              | -2.0        | TypeVI SS component                                   |
| PA0262  |                   | -2.0        | TypeVI SS component                                   |
| PA3294  |                  | -2.0        | TypeVI SS component                                   |

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Table 2. Notable nitrate metabolism and virulence genes whose expression levels are altered in the ΔPA1006 mutant in the presence of nitrate.

| ORF     | Gene              | Fold change | Protein description                                      |
|---------|-------------------|-------------|---------------------------------------------------------|
| PA0996  | pglA              | +11.8       | probable coenzyme A ligase                              |
| PA0997  | pglB              | +6.4        | 3-oxoacyl-acyl-carrier-protein                          |
| PA0998  | pglC              | +6.0        | 3-oxoacyl-acyl-carrier-protein                          |
| PA0999  | pglD              | +4.6        | 3-oxoacyl-acyl-carrier-protein synthase III             |
| PA1000  | pglE              | +9.2        | quinolone signal response protein                       |
| PA1001  | phnA              | +8.0        | anthranilate synthase component I                       |
| PA1002  | phnB              | +5.1        | anthranilate synthase component II                      |
| PA1172  | napC              | +2.4        | cytochrome c-type protein                               |
| PA1173  | napB              | +3.4        | Nitrate reductase cytochrome c-type subunit             |
| PA1174  | napA              | +3.7        | periplasmic nitrate reductase protein                   |
| PA1175  | napD              | +4.8        | NapD protein of periplasmic nitrate reductase           |
| PA1176  | napF              | +4.3        | ferredoxin protein NapF                                 |
| PA1177  | napE              | +4.6        | periplasmic nitrate reductase protein                   |
| PA1871  | lasA              | +13.1       | LasA protease precursor                                 |
| PA2193  | hcnA              | +8.1        | hydrogen cyanide synthase HcnA                         |
| PA2194  | hcnB              | +5.3        | hydrogen cyanide synthase HcnB                         |
| PA2195  | hcnC              | +5.1        | hydrogen cyanide synthase HcnC                         |
| PA3478  | rhsB              | +30.0       | rhassymofltransferase chain B                           |
| PA3479  | rhsA              | +10.5       | rhassymofltransferase chain A                           |
| PA3724  | lasB              | +3.8        | elastase LasB                                          |
| PA3878  | narX              | +2.0        | two-component sensor NarX                              |
| PA5170  | arcD              | +3.3        | arginine/ornithine antiporter                           |
| PA5171  | arcA              | +3.0        | arginine deiminase                                      |
| PA5172  | arcB              | +2.3        | ornithine carbamoyltransferase, catabolic               |
| PA0509  | nirN              | −9.4        | probable c-type cytochrome                             |
| PA0510  | nirE              | −9.2        | probable methyltransferase                             |
| PA0511  | nirI              | −9.1        | heme d1 biosynthesis protein                            |
| PA0512  | nirH              | −10.1       | conserved hypothetical protein                          |
| PA0514  | nirL              | −11.1       | heme d1 biosynthesis protein                            |
| PA0515  | nirD              | −8.3        | probable transcriptional regulator                     |
| PA0517  | nirC              | −6.2        | probable c-type cytochrome precursor                   |
| PA0518  | nirM              | −7.7        | cytochrome c-551 precursor                              |
| PA0519  | nirS              | −7.0        | nitrite reductase precursor                             |
| PA0520  | nirQ              | −10.6       | regulatory protein                                     |
| PA0521  | norE              | −56.9       | probable cytochrome c oxidase subunit                   |
| PA0522  |                  | −15.3       | hypothetical protein                                   |
| PA0523  | norC              | −294.4      | nitric-oxide reductase subunit C                        |
| PA0524  | norB              | −348.9      | nitric-oxide reductase subunit B                        |
| PA0525  | norD              | −306.6      | probable denitrification protein                       |
| PA0526  |                  | −7.4        | hypothetical protein                                   |
| PA3391  | nosR              | −140.3      | regulatory protein NosR                                 |
| PA3392  | nosZ              | −165.9      | nitrous-oxide reductase precursor                      |
| PA3393  | nosD              | −42.7       | nitrous oxide accessory protein                         |
| PA3394  | nosF              | −71.8       | ABC-type transport system                               |
| PA3395  | nosY              | −117.9      | copper enzyme maturation, permease                     |
| PA3396  | nosL              | −124.7      | predicted lipoprotein, nitrous oxide reduction         |

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Pseudomonas quinolone signal (PQS) biosynthesis genes (PA0996-PA1001) as well as anthranilate components (PA1001-1002) when grown in the presence of nitrate. Rhamnolipid biosynthesis (rhlAB; PA3479-70) and elastase (lasI/PA1071 and lasI/PA3724) also showed significantly increased expression in the \( \Delta PA1006 \) mutant compared to WT when nitrate was added. It is notable that \( rhlAB \) levels appeared to be increased since rhamnolipid production appeared unaltered (see above). This apparent discrepancy may be due to differences in growth and media conditions required to measure rhamnolipid production versus microarray analyses. Several other QS-regulated genes [47,48,49] including hydrogen cyanide biosynthesis components (hcnA-C; PA2193-95), the cbb-3 type cytochrome c oxidase (PA4133), a putative sulfite reductase (PA4130), cyt P450 (PA3331), and fabH2 (PA3333) also displayed aberrantly high expression levels when the \( \Delta PA1006 \) mutant was grown with nitrate (Table 1). Normally, in the WT strain, QS and denitrification (nitrate-responsive) genes display reciprocal expression patterns [17,47,50]. That is, in the presence of nitrate, QS regulated genes are normally repressed. However, in the \( \Delta PA1006 \) mutant, many QS genes that are normally repressed by nitrate supplementation remain expressed at relatively high levels.

In addition to nitrate-responsive and QS-regulated genes, we also observed altered gene expression of several Fur-regulated genes [51] in the \( \Delta PA1006 \) mutant only when grown in the presence of nitrate (File S6). In WT, Fur-regulated genes are derepressed when nitrate is added, whereas these genes remain repressed in the \( \Delta PA1006 \) mutant (suggesting that when nitrate is added, Fe is replete in the \( \Delta PA1006 \) mutant yet depleted in WT). The IscR operon consisting of genes involved in sulfur trafficking (PA3815-PA3808) is another notable example of a set of genes whose expression remains at the basal level in \( \Delta PA1006 \) but increased levels in WT when grown with nitrate. Taken together, the data suggests that the \( \Delta PA1006 \) mutant fails to respond to nitrate.

While we cannot presently explain deregulation of the QS genes, inactivation by reactive nitrogen species such as NO may account for the effects seen with Fur-regulated genes. Consistent with \( E. coli \) studies [52], in WT \( Pae \) grown with nitrate, denitrification will generate reactive nitrogen species such as NO which can inactivate Fur allowing the expression of Fur-regulated genes. In the \( \Delta PA1006 \) mutant, reactive nitrogen species such as NO would not be generated from \( NO_3 \), since the mutant cannot utilize \( NO_3 \), and Fur-regulated genes remain repressed. These data suggest that \( Pae \) may employ strategies to balance QS signaling with denitrification needs and products, and vice versa. Disruption of this balance may also prevent growth as a biofilm or in a host organism.

Transcriptional changes observed by the microarray analyses were validated using transcriptional lacZ fusions. While the WT strain displays reduced expression of \( \Delta rhlA, \Delta rhlI, \text{and} \Delta rhlR \), when grown aerobically in the presence of nitrate, the \( \Delta PA1006 \) mutant continues to express these transcripts at similar levels regardless of whether nitrate is added to the growth medium. (File S3). These data confirm that the \( \Delta PA1006 \) mutant over-expresses genes required for rhamnolipid production when grown in the presence of nitrate.

**PA1006 Affects Production of PQS**

Since \( \Delta PA1006 \) cells also showed an increased expression of the PQS biosynthetic genes in the presence of nitrate, we examined PQS production by \( \Delta PA1006 \) compared to WT. TLC analysis revealed that \( \Delta PA1006 \) cells continue to produce PQS when grown in the presence of nitrate, whereas nitrate suppresses PQS production in WT and complemented strains (Fig. 4). These data further validate the microarray analysis results and also show that PQS production may be regulated by nitrate availability (or downstream products of denitrification). Since PQS levels were altered in the presence of nitrate, we also evaluated the production of other QS signaling molecules 3-oxo-C12-homoserine lactone (C12-HSL), and 3-oxo-C4-homoserine lactone (C4-HSL). Both C12-HSL and C4-HSL levels appeared similar to WT (data not shown) unlike the PAO-JP-2 mutant (\( \Delta lasI/\Delta lasH \)) which fails to produce autoinducers [53]. Therefore, a failure to produce autoinducers can be ruled out as the root cause of biofilm instability in the \( \Delta PA1006 \) mutant.

While the reason for aberrant regulation of PQS in the presence of nitrate remains unknown, PQS deregulation may also have a significant impact on the \( \Delta PA1006 \) mutant’s metabolism. Aside from its role as a QS signal [54], recent studies revealed other functions for PQS. It has been shown that excess PQS appears to block denitrification by inhibiting nitrate reductase activity [55]. PQS can also bind to iron (III) and may sequester it near the surface of the cell [56]. Since addition of excess iron relieved the inhibition of denitrification activity by PQS, it was suggested that PQS production may be regulated by nitrate availability (or downstream products of denitrification). Since PQS levels were altered in the presence of nitrate, we also evaluated the production of other QS signaling molecules 3-oxo-C12-homoserine lactone (C12-HSL), and 3-oxo-C4-homoserine lactone (C4-HSL). Both C12-HSL and C4-HSL levels appeared similar to WT (data not shown) unlike the PAO-JP-2 mutant (\( \Delta lasI/\Delta lasH \)) which fails to produce autoinducers [53]. Therefore, a failure to produce autoinducers can be ruled out as the root cause of biofilm instability in the \( \Delta PA1006 \) mutant.

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free PQS levels must be tightly regulated to control iron (III) surface levels or availability during denitrification [55]. In order to rule out the possibility that iron sequestration by PQS was causing defects in nitrate reductase, we supplemented NY+NO3 media with additional iron (50 μM each of FeSO4 and FeCl3) and tested anaerobic growth. Additional iron failed to restore anaerobic growth/nitrate utilization by the ΔPA1006 mutant (data not shown) indicating that chelation of iron by PQS is not likely the root cause of a failure to grow anaerobically. The failure to rescue nitrate utilization with additional iron also agrees with the microarray data which clearly show that, in the presence of nitrate, Fur-regulated genes remain repressed in the ΔPA1006 mutant (even though extracellular PQS remains in excess) indicating that intracellular iron is not being significantly depleted by the PQS.

**PA1006 Displays an Altered Metabolic Profile**

The microarray gene expression profile indicated that the ΔPA1006 mutant displayed changes in enzymes associated with metabolism. Therefore, to study the ΔPA1006 mutant’s metabolic capacity in more detail, Biolog Phenotype Microarrays TM, which have been used successfully to probe *Pae* metabolism [57,58], were used to comparatively assess the metabolic requirements of the ΔPA1006 PAO1 mutant versus WT strain. Biolog results are provided in File S7. In addition, functional annotation of genes that showed expression changes in microarray experiments and substrates that showed altered utilization in Biolog experiments were superimposed upon KEGG pathway maps [59] and this extended analysis is provided in Files S7, S8, S9. Several global observations could be made which indicated several trends. For example, many TCA cycle (or nearby) intermediates such as succinate, citrate, fumarate, malate, and α-ketoglutarate could not be utilized as effectively as sole sources of carbon by the ΔPA1006 mutant. In addition, the ΔPA1006 mutant appeared less capable of utilizing acetate as a sole source of carbon suggesting that the key metabolic enzyme isocitrate lyase (encoded by *PA2634*) or other glyoxylate cycle enzymes may also be compromised [60,61]. Similarly, several purines such as adenosine, guanine, guanosine, xanthine, and xanthosine were not utilized as effectively as sole sources of nitrogen by the ΔPA1006 mutant. In fact, the most severe deficiencies in ΔPA1006 were found with nitrate and xanthine as nitrogen sources. When nitrate or xanthine was supplied as the nitrogen source, the tetrazolium dye color did not exceed background in ΔPA1006 whereas WT gave a relatively robust signal (we also confirmed in separate experiments that the ΔPA1006 mutant is unable to grow on hypoxanthine as a sole nitrogen source (data not shown)). These data may be revealing with regard to PA1006 function since an inability to utilize xanthine combined with a loss of all nitrate reductase activities is consistent with ‘shared nitrate reductase mutants” where MoCo is lacking [62].

**Discussion**

The goal of these studies was to shed light on the biological role of the PA1006 protein in *Pae* metabolism and virulence. Our data suggest the ΔPA1006 mutant phenotype is due to metabolic differences. Since the most robust changes in gene expression in the ΔPA1006 mutant reflect a loss of nitrate responsiveness (and lack of coordination of denitrification with QS regulation) it is likely that a loss of signaling via denitrification pathway intermediates contributes significantly to the ΔPA1006 mutant phenotype. A concrete example of this idea is inactivation of Fur by reactive nitrogen species such as NO and how it affects Fur-regulated gene expression. E. coli studies [52] predict that *Pae* denitrification would generate reactive nitrogen species such as NO which can inactivate Fur allowing the expression of Fur-regulated genes. In the ΔPA1006 mutant we find that Fur-regulated genes remain repressed (in contrast to WT which are induced by nitrate) most likely because reactive nitrogen species such as NO are not generated from NO3 since the mutant cannot reduce NO3. In further support of the idea that a loss of denitrification signaling pathways critically alters *Pae* metabolism, *AnacGH* and ΔmutS mutants were also shown to be defective in biofilm formation and virulence [19,63]. Reciprocally, addition of *excess* denitrification intermediate NO was also shown to disperse biofilms that had already formed [20]. Therefore, the differences in Fur-regulated gene expression that we observe in the ΔPA1006 mutant may only be indicative of other significant changes affected by loss of denitrification that we cannot appreciate presently.

Given the additional alterations in metabolic gene expression and substrate utilization profile revealed by the Biolog studies, metabolic changes beyond the loss of denitrification may further contribute to the ΔPA1006 mutant phenotype. For example, recent publications have revealed that excess carbon sources (such as the TCA cycle intermediate succinate), as well as D-amino acids (generated by racemases) are all effective in dispersing biofilms [64,65]. Similarly, the loss of virulence displayed by the ΔPA1006 mutant may also result from global metabolic changes. For example, two genes (PA0867 and PA3234) that showed decreased RNA levels in the ΔPA1006 mutant in the presence of nitrate were reciprocally up-regulated in CF clinical isolates of *Pae* in three separate reports [12,13,66,67]. While the function of PA3234 is unknown, PA0867 or AcsA (acetyl-coA synthase) is particularly interesting because acetyl-coA is an important metabolite that connects many aspects of central metabolism [68]. It may also be noteworthy that the metabolite/signaling molecule c-di-GMP has been shown to mediate NO-induced dispersal of biofilms [69] and is also linked to Type VI secretion systems [70], both of which are altered in the ΔPA1006 mutant.

When we initiated the studies presented in this manuscript, the function of PA1006 could not be predicted using a bioinformatics approach and the robust phenotypes we observed in virulence and biofilm assays were entirely unexpected. In the companion manuscript, we provide evidence that PA1006 may function in a similar but not identical manner compared to YhhP/TusA [24]. First, we clearly demonstrate that PA1006 is modified by a persulfide group in *Pae* on the highly conserved Cys that in YhhP/TusA is also modified as a persulfide. Although sulfur trafficking via persulfide sulfur carriers is a relatively new concept, many metabolites such as Fe-S clusters, MoCo, thiamine, and 2- or 4-thiouridine derivatives are concrete examples of metabolites that require sulfur trafficking for their biosynthesis and additional metabolites may be forthcoming [71]. In this light, one possible explanation of our data is that the loss of PA1006 may affect sulfur trafficking for MoCo biosynthesis or Fe-S cluster assembly. Both MoCo and Fe-S clusters are essential cofactors for electron transfer in the catalytic subunits of assimilatory and dissimilatory nitrate reductase enzyme complexes, the periplasmic nitrate reductase of *Pae* (see Fig. 1E and ref. [72]), as well as xanthine dehydrogenase [73], and all of these activities are lacking in the ΔPA1006 mutant. In the accompanying paper, we also demonstrate that PA1006 interacts with several MoCo biosynthesis factors, and that PA1006 is required for molybdenum homeostasis [24]. These data are consistent with the loss of nitrate reductase activity and a failure to utilize xanthine/hypoxanthine displayed by the ΔPA1006 mutant detailed herein. Together, these studies provide a novel and
unexpected connection between metabolism, biofilm formation, and virulence.

Methods

Bacterial Strains and Growth Conditions

_P. aeruginosa_ strain PAO1 was maintained at 37°C on NY (2.5% nutrient broth and 0.5% yeast extract) agar plates supplemented with 100 mM KNO3 or peptone tryptic soy broth (PTSB) agar plates [74]. The transposon mutant containing a disruption in NapA was obtained from PA14 Mutant Library (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi? [75]). Anaerobic growth of _P. aeruginosa_ was performed as previously described [47]. Anaerobic growth of _P. aeruginosa_ was performed in a Coy anaerobic chamber (85% N2, 10% H2, and 5% CO2; Coy Laboratories Inc.). For anaerobic growth, Luria-Bertani broth was used to prepare aerobic media and cell cultivation was performed as previously described [74]. Anaerobic growth of _P. aeruginosa_ was determined by plating on VBMM plates containing either 100 mM KNO3, 10 mM KNO2, or NH4Cl, as the sole nitrogen source, and incubating under aerobic conditions for 48 h [62,77]. _E. coli_ cultures were grown using LB agar plates and broth. When necessary, media was supplemented with ampicillin (100 μg per ml) for _E. coli_ and carbenicillin (200 μg per ml) for _Pae_. Anaerobic growth was determined by plating on VBMM plates containing either 100 mM KNO3, 10 mM KNO2, or NH4Cl, as the sole nitrogen source, and incubating under aerobic conditions for 48 h [62,77].

Recombinant DNA Techniques

Cloning was performed using standard methods [78]. _Pseudomonas_ chromosomal DNA was isolated using a previously described method [79]. DNA sequences of all constructs were obtained using automated DNA sequencing with Big Dye terminator mix (U. of Rochester Core Facility).

Construction of the ΔPA1006 Mutant

An in-frame deletion of PA1006 (Gene ID: 879448) was created by overlap extension PCR as described previously [80,81] with the following modifications. Two DNA fragments consisting of a region of PAO1 genomic DNA containing approximately 1.5 kb upstream and 0.75 kb downstream respectively, of PA1006 were obtained from PCR-amplification of p1E1, a plasmid from the PA1006 insertion at the attB:PA1006 (Gene ID: 879448) was created. All efforts were made to minimize suffering in all animal experiments. Mouse experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center (Protocol Number: 07044). Rat experiments were conducted according to the guidelines of the Canadian Council of Animal Care for the care and use of experimental animals containing 5% sucrose. The deletion was verified using PCR and Southern blotting.

Complementation of ΔPA1006 Unmarked Deletion Mutant

For single copy chromosomal insertion complementation, the plasmid p1E1 (see above) was used to subclone the complete PA1006 ORF, its putative promoter region, and flanking DNA with flanking _Pae_ sites. The _Pae_ fragment was then moved into pUCP18. pUCP18 containing the PA1006 fragment was digested with _SphI_ and a resulting 650 bp fragment was made blunt with T4 polymerase and ligated into _EcoRV_-digested miniCTX1 [26]. The resulting plasmid was verified by restriction analysis and PCR and introduced into _E. coli_ strain SM10. This strain was used to mobilize the complementing fragment into the ΔPA1006 mutant by conjugation, as described previously [26]. Chromosomal insertion at the _attB_ site was verified by PCR and Southern blot analyses.

Virulence Assays

_Lethality in burned mouse model_. The virulence of the PAO1 strains was examined by using the burned-mouse model as described [82]. Briefly, _Pae_ strains were subcultured and grown at 37°C to an optical density at 540 nm of approximately 0.9. Cells were then pelleted, washed, and serially diluted in PBS. Thermal injury was induced by scalding 15% of the body surface in 90°C water for 10 s and 1×10^5 CFU of each _Pae_ strain was injected subcutaneously within the burn eschar. Mortality was monitored for 5 days. The Fisher’s exact test (Statview; Abacus Concepts, Inc.) was used to determine the significant differences between groups of mice for the mortality experiments.

_Dissemination in burned mouse model_. At 24 h post burn infection, the mice were euthanized by intracardial injection of 0.2 ml of Sleepaway (sodium pentobarbital-7.8% isopropyl alcohol euthanasia solution; Fort Dodge Laboratories, Inc. Fort Dodge, Iowa). The livers of each animal were obtained from both control and challenged mice. Livers were weighed, suspended in PBS, and homogenized (Wheaton overhead stirrer; Wheaton instruments, Millville, NJ). A 100 μl aliquot of each homogenate was plated on LB agar plates to determine the number of post burn infection CFU. The number of CFU from each liver was calculated per gram of tissue. The student t-test was used to determine the significant differences between the strains.

_Rat lung model_. The rat chronic-lung infection assay was performed as previously described [32,35]. Briefly, rats (Sprague-Dawley male 180–200 g) were inoculated with approximately 1×10^3 cfu of either the wild-type bacteria, the ΔPA1006 mutant, or the complemented mutant (ΔPA1006 attB:PA1006) encased in agar beads and placed in the left lungs of the rats. At 10 days post infection, lungs were removed for either quantitative bacteriology or quantitative pathological analysis [32,35]. Sections were coded, analyzed and scored as previously described [32,35].

Ethics Statement

All efforts were made to minimize suffering in all animal experiments. Mouse experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center (Protocol Number: 07044). Rat experiments were conducted according to the guidelines of the Canadian Council of Animal Care for the care and use of experimental animals.
approved by the University of Calgary Animal Care Committee (Protocol Number: M000099).

Biofilm Analysis

**Static dish biofilms.** Static dish biofilms assays were carried out as described previously [19]. Plasmid pTDKgfp [41], which constitutively expresses green fluorescent protein (GFP), was introduced into each strain by electroporation. Overnight cultures containing the plasmid pTDK-gfp were grown in LB and then diluted into modified FAB-citrate media (final OD_{600} = 0.5) prior to inoculation. Next, 2.0 ml of cells (OD_{600} = 1.2) were inoculated into 35 mm petri plates with glass coverslips incorporated into the bottom chamber (Matek Corp.) to allow visualization. After 24 h incubation at 37°C under aerobic conditions, media was carefully removed and replaced with three consecutive 1.0 mL portions of modified FAB-citrate. Next, biofilms were visualized by a Leica inverted laser scanning confocal microscopy (CSLM). Analysis of biofilm images was done with COMSTAT [85]. Five separate fields measuring 250 × 250 μm were analyzed for each condition.

**Continuous flow biofilms.** Biofilms were cultivated in flow chambers at 37°C as described [41], with slight modifications. Plasmid pTDKgfp [41], which constitutively expresses GFP, was introduced into each strain by electroporation. Early stationary phase cultures (OD_{600} = 1.2) were used as inoculum. After bacteria adhered to the glass slides for 2 h, medium was pumped at a constant flow rate of 0.2 ml/min. At the designated time, flow cells were visualized with a Leica laser scanning confocal microscope (URMC Pathology Imaging Core, Rochester, NY). Analysis was performed as described above for static biofilms. It is worth noting that in the FAB-citrate media, the nitrogen source is ammonia and not nitrate. Moreover, we found that the ΔPA1006 mutant was able to utilize ammonia as a sole source of nitrogen in minimal media (data not shown). Therefore, in these biofilm studies, a lack of nitrate utilization displayed by the ΔPA1006 mutant would not prevent the ΔPA1006 mutant from obtaining nitrogen.

**Twitching and Motility Assays**

Twitching motility was assessed on LB (1%) agar plates at 37°C as described by Glesner [83]. Swimming assay was performed via the method described by Rashid by inoculating plates containing 1% tryptone, 0.5% NaCl, and 0.3% agar and incubating at 30°C for 16 h [84]. Swarming motility was performed as described by Kohler et al. [43].

**Nitrate Reductase Activity**

Nitrate reductase activity was determined in whole cell suspensions [29,30]. Cultures were grown aerobically in NY with 100 mM KNO_3. To inhibit protein synthesis, 1.5 ml of 50 μg/ml chloramphenicol was added to 1.5 ml of culture. If indicated, 1.5 μl of 50 mM NaN_3 was added to inhibit the membrane nitrate reductase NarGHI. Cells were centrifuged, washed twice, and reinitiated by adding 100 μl of a solution containing 4 mg/ml sodium dithionite, 4 mg/ml sodium bicarbonate, and 100 mM KNO_3. 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 s. 1 ml of 1.3 mg/ml N-(1-naphthyl) ethylenedi-amine-HCl was added to allow formation of red azo dye, and the suspension was centrifuged to pellet debris. Optical density at 540 nm (OD_{540}) of the supernatant was measured spectrophotometrically to quantitate dye formation, and optical density at 420 nm (OD_{420}) 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 × (OD_{540} - (0.72 × OD_{420}))/74 × T × V × OD_{660} [85]. T= time in minutes, V= volume of reaction used in ml, OD_{660} corresponds to the optical density of the culture used. Assays were performed in triplicate in three separate experiments. To detect NarGHI protein, Western Blot analysis was performed. Overnight cultures grown in LB were diluted to OD_{660} = 0.05 in NY+100 mM KNO_3 broth and were grown to OD_{660} ~ 1.0. Cells were pelleted and resuspended in SDS gel loading buffer containing 1% SDS (3× volume of cell pellet) and boiled. Approximately 20 mg of total cell lysate were resolved by SDS PAGE. Rabbit polyclonal α-NarGHI primary was kindly provided by Axel Magalon (CNRS, Marseille FR). The secondary antibody was conjugated to HRP and the blot was developed using chemiluminescence.

**Microarray Analysis**

Parental PAO1 WT and mutant ΔPA1006 strains were grown aerobically at 37°C in nutrient broth supplemented with yeast extract containing 100 mM KNO_3 or lacking KNO_3. RNA was extracted at early stationary phase (OD_{600} = 1.2) using TRI-reagent (Ambion, Austin, TX). Residual DNA was removed with amplification grade DNase I (Invitrogen) and RNA was concentrated using a MinElute kit (Qiagen). RNA integrity was assessed using a Bioanalyzer (Agilent Technologies, Foster, City, CA). cDNA synthesis, labeling, fragmentation, hybridization, and chip scanning was performed as described previously [47]. Microarray Suite 5.0 (Affymetrix, Santa Clara, CA), GeneSpring 6.2 (Silicon Genetics, Redwood City, CA) and Significance Analysis of Microarrays (SAM) version 1.15 [86] were used to analyze the data. Microarrays were globally scaled to a target intensity of 500 and filtered for transcripts present in two of three arrays. Statistical significance was determined using a t-test with p value of <0.05. Differentially expressed genes showing ≥2-fold change were filtered using the above criteria and are listed in Table 2. For SAM analyses, transcripts which demonstrated >2-fold change or greater and a false discovery rate of 5% were considered statistically significant. SAM analyses results are available in Files S4 and S5. Microarray data were deposited into Gene Expression Omnibus repository (GEO) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and may be found with the following accession numbers: GSM711446- PA01 delta PA1006 strain grown in NY media, replicate 1; GSM711447- PA01 delta PA1006 strain grown in NY media, replicate 2; GSM711448- PA01 wild-type strain grown in NY media, replicate 1; GSM711449- PA01 wild-type strain grown in NY media, replicate 2; GSM711450- PA01 delta PA1006 strain grown in NY+NO3 media, replicate 1; GSM711451- PA01 delta PA1006 strain grown in NY+NO3 media, replicate 2; GSM711452- PA01 delta PA1006 strain grown in NY+NO3 media, replicate 3; GSM711453- PA01 wild-type strain grown in NY+NO3 media, replicate 1; GSM711454- PA01 wild-type strain grown in NY+NO3 media, replicate 2; GSM711455- PA01 wild-type strain grown in NY+NO3 media, replicate 3.

**Metabolic Profile Analysis**

Phenotype Microarray (Biolog; Hayward, CA) PM1 and PM2 (carbon), PM3B (nitrogen), and PM4A (sulfur and phosphorus) plates were employed to assess the ability of the strains to utilize
various sources of carbon, nitrogen, sulfur, and phosphorus. Experiments were performed according to the manufacturer’s instructions. Provided minimal media was used to grow overnight cultures of WT and PA1006 PAO1 and these were used to inoculate the 96-well plates provided by Biolog. These were incubated without shaking at 37°C and growth was monitored by optical density of the tetrazolium dye at 590 nm at 24 and 48 h in a SpectraMax M5 plate reader (Molecular Devices; MDS Analytical; Toronto, ON).

Pseudomonas Quinolone Signal (PQS) Analysis

PQS was assayed as previously described [54]. Briefly, bacteria grown aerobically overnight in NY or NY supplemented with 100 mM KNO₃ were extracted twice with 0.001% glacial acetic acid-acidified ethyl acetate. Extracts were evaporated and re-suspended in ethyl acetate:acetonitrile 50:50. Samples were separated by Thin Layer Chromatography on silica gel plates and visualized using a hand-held UV lamp [54].

Supporting Information

File S1 Table showing that PA1006 is not required for anaerobic growth with nitrite or arginine. (PDF)

File S2 PA1006 is not required for biofilm formation in a static dish system. Biofilms were grown and analyzed as indicated in methods. A) Representative confocal images of Pae strains expressing GFP in flow-cell biofilms. Images were taken at random locations of each flow cell using confocal laser scanning microscope. B) COMSTAT analysis of biofilms. (PDF)

File S3 Confirmation of altered rhlA, rhlI, and rhlR gene expression in the ΔPA1006 mutant to WT when grown in the presence of nitrate. β-galactosidase -promoter fusion reporter constructs were used to determine expression levels. (PDF)

File S4 Microarray analysis of gene expression comparing ΔPA1006 mutant to WT PAO1 in the absence of nitrate (worksheet 1). This shows SAM analysis of microarray data of gene expression comparing ΔPA1006 mutant to WT PAO1 in the absence of nitrate (worksheet 2). (XLSX)

File S5 Microarray analysis of gene expression comparing ΔPA1006 mutant to WT PAO1 in the presence of nitrate (worksheet 1). This shows SAM analysis of microarray data of gene expression comparing ΔPA1006 mutant to WT PAO1 in the presence of nitrate (worksheet 2). (XLS)

File S6 Iron-regulated genes (with putative fur-boxes) show altered expression in the ΔPA1006 mutant compared to WT PAO1. (XLSX)

File S7 Phenotype Microarray™ analysis (Biolog) comparing metabolic profiles of ΔPA1006 mutant to WT PAO1 in the absence of nitrate (excel spreadsheet). (XLSX)

File S8 KEGG Pathway analysis results in Excel Spreadsheet format. (XLSX)

File S9 KEGG Pathway analysis results in PowerPoint file format. (PPT)

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Author Contributions

Conceived and designed the experiments: MJF GT VEW NVA KR PS BHI. Performed the experiments: MJF GT VEW NVA KR PS. Analyzed the data: MJF GT VEW NVA KR PS BHI. Contributed reagents/materials/analysis tools: MJF GT VEW NVA KR PS JMS BHI. Performed the experiments: MJF GT VEW NVA KR PS. Analyzed the data: MJF GT VEW NVA KR PS BHI. Wrote the paper: MJF GT BHI.

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