RESEARCH ARTICLE

The anti-sigma factor MucA of Pseudomonas aeruginosa: Dramatic differences of a mucA22 vs. a ΔmucA mutant in anaerobic acidified nitrite sensitivity of planktonic and biofilm bacteria in vitro and during chronic murine lung infection

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Abstract

Mucoid mucA22 Pseudomonas aeruginosa (PA) is an opportunistic lung pathogen of cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) patients that is highly sensitive to acidified nitrite (A-NO₂⁻). In this study, we first screened PA mutant strains for sensitivity or resistance to 20 mM A-NO₂⁻ under anaerobic conditions that represent the chronic stages of the aforementioned diseases. Mutants found to be sensitive to A-NO₂⁻ included PA0964 (pmpR, PQS biosynthesis), PA4455 (probable ABC transporter permease), katA (major catalase, KatA) and rhlR (quorum sensing regulator). In contrast, mutants lacking PA0450 (a putative phosphate transporter) and PA1505 (moaA2) were A-NO₂⁻ resistant. However, we were puzzled when we discovered that mucA22 mutant bacteria, a frequently isolated mucA allele in CF and to a lesser extent COPD, were more sensitive to A-NO₂⁻ than a truncated ΔmucA deletion (Δ157–194) mutant in planktonic and biofilm culture, as well as during a chronic murine lung infection. Subsequent transcriptional profiling of anaerobic, A-NO₂⁻-treated bacteria revealed restoration of near wild-type transcript levels of protective NO₂⁻ and nitric oxide (NO) reductase (nirS and norCB, respectively) in the ΔmucA mutant in...
contrast to extremely low levels in the A-NO$_2^-$-sensitive $mucA22$ mutant. Proteins that were S-nitrosylated by NO derived from A-NO$_2^-$ reduction in the sensitive $mucA22$ strain were those involved in anaerobic respiration (NirQ, NirS), pyruvate fermentation (UspK), global gene regulation (Vfr), the TCA cycle (succinate dehydrogenase, SdhB) and several double mutants were even more sensitive to A-NO$_2^-$. Bioinformatic-based data point to future studies designed to elucidate potential cellular binding partners for MucA and MucA22. Given that A-NO$_2^-$ is a potentially viable treatment strategy to combat PA and other infections, this study offers novel developments as to how clinicians might better treat problematic PA infections in COPD and CF airway diseases.

Introduction

Chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) are two chronic airway diseases complicated by life-threatening infections caused by the opportunistic Gram-negative bacterium, Pseudomonas aeruginosa (PA). In the United States alone, there are an estimated 15 million COPD patients (251 million worldwide (WHO estimates, [1]), while there are only 30,000 U.S. CF patients (70,000 world-wide, [2]). COPD is a highly deteriorative alveolar disease coupled with airway derangements causing an accumulation of thick mucus that is typically a consequence of long-term smoking. Cultures of respiratory secretions yield PA in 8.5–16.8% of patients who also experience more frequent COPD exacerbations [3, 4] and up to 18% of patients requiring mechanical ventilation [5] who have increased mortality. In contrast, CF airway disease is biphasic, involving an early oxidative, neutrophil-rich environment [6] (especially during acute exacerbations) [7] followed by a chronic hypoxic or anaerobic phase [8–10]. In contrast, chronically infected patients suffer from poor airway oxygenation resulting, in part, from intractable, antibiotic-resistant [11] bacterial communities known as biofilms that are formed by PA and other organisms embedded within thick microaerobic [12] or anaerobic airway mucus [8, 13–16].

A further reduction in lung function occurs when mucoid, $mucA22$ mutant alginate-overproducing mutant PA forms biofilms that are of the mode II variety [13, 17, 18]. The $mucA22$ allele results from a C residue deletion at position 430, leaving a truncated 15.8 kDa MucA protein [19]. Unlike mode I biofilms, where organisms attach directly to animate (e.g., cells) or inanimate (e.g., plastic, glass, etc.) surfaces, mode II biofilm bacteria are not attached to surfaces, but rather to themselves, developing as highly structured communities embedded within the thick mucus that mature into “soccer ball-shaped” micro- and/or macrocolonies in the infected airways [20]. Many research groups around the world are attempting to unravel the precise metabolic features of PA within mode II biofilms embedded within the CF and COPD airway mucus [17, 21, 22]. Metabolism of certain amino acids appears to be prevalent among PA isolates from chronically infected patients including aromatic amino acids, specifically phenylalanine and tyrosine [23]. Two seminal papers were published in 2002 by our group (Yoon et al., [8]) and Worlitzsch et al., [13] that indicate that oxygen tension within the thick mucus lining of the CF airways is significantly reduced and there are some niches that are likely completely anaerobic. For many CF and COPD pathogens, including PA, an alternative electron acceptor such as nitrate (NO$_3^-$) or nitrite (NO$_2^-$) is required. Both NO$_3^-$ and NO$_2^-$ can be detected in sufficient quantities for PA to undergo anaerobic respiration in both CF [24] and COPD [25]. Since our anaerobic biofilm theory of chronic CF lung disease was reported in 2002 [8], obligate anaerobes have been isolated from CF sputum (for recent review, see [18])
as well as in COPD sputum [26]. In fact, there have been many published manuscripts spanning over 40 years describing the isolation of obligate anaerobes from the CF airways, thereby supporting our anaerobic theory [10, 27, 28] (for mini-reviews, see [17, 18]). In conjunction with anaerobic nitrogen oxide metabolism, one very significant development in the potential treatment of mucoid PA was discovered in 2006 where we (Yoon et al., [9]) also showed that mucoid mucA22 mutant CF isolates were sensitive to acidified sodium nitrite (herein termed A-NO$_2^-$). A-NO$_2^-$ was used in the aforementioned study because we found that the pH of the CF airway surface liquid from explanted CF lungs was ~6.4–6.5 [9]. However, in that study, of nearly 100 mucoid PA isolates from 12 different North American CF clinics, expectedly none possessed true deletions of the mucA gene [9]. A second study, using the other two major COPD and CF pathogens methicillin-resistant Staphylococcus aureus and Burkholderia cepacia, as well as nonmucoid PA showed that A-NO$_2^-$ also kills these organisms, particularly under anaerobic conditions [29]. Relatedly, it should be noted that one feature of CF is reduced airway iNOS (inducible nitric oxide (NO) synthase) expression, especially in chronic CF [30]. iNOS is an enzyme that generates potentially antimicrobial levels of nitric oxide (NO) and is a major contributor to the host's innate immune system. In contrast, iNOS also appears to play a role in COPD, as iNOS mRNA levels were recently shown to be elevated in COPD patient's relative to nonsmokers and smokers without COPD [31].

There is a severe dearth of nearly any formal understanding of the genetics of adaptive mutations in PA that are acquired in COPD. Puzzlingly, given the estimated 3,600-fold reduced patient numbers worldwide for CF relative to COPD, vastly more is understood regarding mutations that emerge during the course of CF [32, 33]. For example, PA acquires 3 adaptive mutations during CF, lasR (early $x = 12$ years, rhlR ($x = 17$ years [34]) and mucA (early [in some cases 3 yrs old] and late (chronic) CF [9, 35] and likely also in COPD [36]. In 2002, we discovered yet another weakness in mutants frequently isolated from CF patient sputum. We discovered that anaerobic PA rhlR QS (controlled by the las system) mutants in PA anaerobic biofilms mysteriously committed an anaerobic metabolic suicide in biofilms by overproduction of toxic endogenous levels of respiratory NO [8]. Chronic, long-term infections are characterized by bacteria that have undergone a process known as mucoid conversion within the progressively thickened airway mucus [37]. This process involves mutations in a variety of genes including mucB (algN) (periplasmic protein that binds the anti-sigma factor, MucA, [38, 39]), algW (encoding a membrane protease that cleaves MucA [40]), and mucD (a periplasmic protease that degrades MucA via activation of MucP [39, 41]. However, the most abundant mutations that trigger mucoid conversion in both COPD [31, 36] and CF [9, 35] are within the mucA gene, encoding a cytoplasmic membrane-spanning anti-sigma factor [35]. The primary appreciated function of MucA is to sequester the extracytoplasmic sigma factor AlgT(U) near the cytoplasmic side of the inner membrane [42]. The most common mucA mutant allele is called mucA22 [9, 35], caused by a C deletion at base 430, resulting in a 15.8 kDa truncated protein that allows mucoid conversion by enabling AlgT(U) to activate transcription of genes involved in production of the viscous exopolysaccharide alginate, a linear $\beta$-1,4-linked co-polymer consisting of $\beta$-D-mannuronate and $\alpha$-L-guluronate [43, 44]. The production of alginate severely complicates the clinical course for CF patients [45], resulting in progressively worsened forced expiratory volume per second (FEV$_1$) measurements and poor pulmonary function tests (PFTs). Thus, mucoid conversion is often considered one, if not the most negative, clinical hallmarks precipitating a dramatic antibiotic regimen adjustment for patients infected by such organisms.

Given this important and comprehensive background information, in this study, we elected to first identify a series of transposon mutants that were either more susceptible or resistant to
defined concentrations of anaerobic A-NO₂⁻. During this process, we discovered a different and unexpected role of MucA in sensitivity to A-NO₂⁻ by generating not only mucA22 mutants, but also a truncated ΔmucA mutant (Δ157–194), the latter of which were surprisingly resistant to A-NO₂⁻ relative to its mucA22 counterpart. We used a combined transcriptional profiling and protein S-nitrosylation approach to identify potential mechanisms of A-NO₂⁻ sensitivity in wild-type, mucA22 vs. ΔmucA strains. First, the transcription levels of genes encoding nitrate (NO₃⁻), nitrite (NO₂⁻) and nitric oxide (NO) reductase (collectively NAR, NIR and NOR) were at or near wild-type levels in ΔmucA and wild-type bacteria when compared to the mucA22 mutant, which we show to be far lower in this study upon exposure to A-NO₂⁻. Consistent with these observations, we show that mucA22 bacteria are susceptible to A-NO₂⁻ during a chronic lung infection in mice, while ΔmucA bacteria were resistant. Our data also involved extensive bioinformatics analysis suggesting that MucA22, a truncated ~15.8 kDa protein, has an as yet unknown anaerobic function, but also confers a significant defect, a translationally-significant and marked sensitivity to A-NO₂⁻.

Results

Screening for PA strains that demonstrate enhanced sensitivity or resistance to A-NO₂⁻

A mariner Tn library representing a three genome coverage (~15,000 mutants) and previously constructed insertion or deletion mutants were used to screen for PA strains for sensitivity or resistance to 20 mM A-NO₂⁻ under anaerobic conditions (Table 1). Using wild-type PAO1 bacteria and strain FRD1 as respective A-NO₂⁻ resistant and sensitive controls, several Tn mutants that were identified to be more resistant to A-NO₂⁻. These included PA1504 (transcriptional regulator), PA0450 (probable PO₄³⁻ transporter), PA1370 (hypothetical protein), and PA0780 (proline utilization regulator). In contrast, A-NO₂⁻ sensitive strains included mutants lacking PA0964 (pmpR, regulator of PqsR-mediated quorum sensing, [46]), PA4455 (a putative ABC transporter permease [47]), ribonucleotide diphosphate reductase subunits

Table 1. Transposon (Tn) and gene replacement mutants found to be sensitive (S) or resistant (R) to 20 mM A-NO₂⁻ under anaerobic conditions after 24 hr incubation.

| Strain Gene | (Sensitive (S)/Resistant (R)) | Reference |
|-------------|------------------------------|-----------|
| FRD1 mucA22 | S                            | [51]      |
| PA01 mucA22 | S                            | [9], This study |
| PA01 pmpR::Tn-Gm | S                               | This study |
| PA01 norCB::Gm | S                               | [50]      |
| PA01 rhlR::Gm  | S                            | [52]      |
| PA01 katA::Gm  | S                            | [53]      |
| PA01 las::Tn-Gm | S                               | This study |
| PA01 ndtJa::Tn-Gm | S                               | This study |
| PA01 nuoK::Tn-Gm | S                               | This study |
| PA01 PA4455::Gm | S                               | [47], This study |
| PA01 ΔmucA (Δ157–194) (Hassett lab) | S | This study |
| PA01 ΔmucA (Δ157–194) (Schurr lab) | R | This study |
| PA01 PA1504::Tn-Gm | R                               | This study |
| PA01 PA0450::Tn-Gm | R                               | This study |
| PA01 PA1370::Tn-Gm | R                               | This study |
| PA01 PA0780::Tn-Gm | R                               | This study |
| PA01 PA0780::Tn-Gm | R                               | This study |

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(PA5496-nrdJa, 5497-nrdJb), mucA22, PA0779 (ATP-dependent protease), katA (major catalase KatA, [48]), rhlR (quorum sensing regulator, [49]), lon (Lon protease), nuoK (NADH dehydrogenase subunit), norCB (NO reductase, [50], clinical strain FRD1 and a proven A-NO₂⁻ sensitive mucA22 mutant [51], respectively. As a control, we constructed a deletion mutant of mucA (ΔmucA) and to our surprise, this strain was resistant to A-NO₂⁻.

The mucA gene, mucoidy and alginate production in mucA22 vs. ΔmucA strains

The immediate goal of our experimental plan moving forward was to assess the evolutionary “rationale” underlying why the mucA22 mutation occurs so frequently in PA derived from sputum from patients suffering from both CF and COPD airway disease [9, 36] and evaluate whether a complete deletion of the mucA gene differed with respect to alginate production and sensitivity to the potential translational CF and COPD therapeutical agent, A-NO₂⁻. As a reminder, the mucA gene encodes a cytoplasmic membrane spanning anti-sigma factor that binds the extra-cytoplasmic sigma factor AlgT(U) in the cytoplasm and is one of the most common mechanisms for the conversion to mucoidy in both of the aforementioned diseases [35]. The mucA gene is the second gene of an 11.524 kb alginate gene regulation operon located between 831,301 and 842,825 bp on the PA chromosome (Fig 1, www.pseudomonas.com). Other genes indicated by a minus sign (-) allow for mucoid conversion or alginate gene regulation when mutated (e.g., negative regulators mucB,C,D, etc. [38, 39, 54].

Isogenic mucA1 and ΔmucA mutants in the PAO1 background were visibly mucoid on L-agar plates (Fig 2A). The ΔmucA mutant (Δ157–194) was created in both the Hassett and Schurr labs and confirmed by sequence analysis, S1A–S1C Fig). However, we must emphasize that a complete in-frame deletion could not be constructed despite numerous attempts by both laboratories and equipped with the appropriate mutagenesis constructs. Additionally, the PA mucA22 and ΔmucA mutants were complemented in trans to the nonmucoid phenotype using the wild-type mucA gene inserted into the arabinose-inducible pHERD20T plasmid (Fig 2A). Surprisingly, the ΔmucA mutant was found to generate significantly less alginate than mucA22 (p = 0.012) but greater than the nonmucoid strain, PAO1 (Fig 2B). We also tested the alginate stability phenotype in mucA22 vs. ΔmucA bacteria for it is well known that the process of mucoid reversion (mucoid-to-nonmucoid phenotype) occurs when bacteria are grown under aerobic static conditions [56]. Fig 2C indicates that mucoid stability is greater in mucA22 versus ΔmucA bacteria after incubated for 48 hr.

Mucoidy and A-NO₂⁻ planktonic sensitivity of mucA22, ΔmucA, and strain FRD1 (mucA22) relative to PA

The sensitivity of each strain used in Fig 2A–2C to A-NO₂⁻ in anaerobic planktonic cultures was assessed, as well as the well-known CF mucA22 isolate known as FRD1 [9]. Surprisingly, the ΔmucA mutant was found to be more resistant to A-NO₂⁻ relative to a sensitive mucA22 PA mutant. Strain FRD1 was even more sensitive to 15 mM A-NO₂⁻ than its mucA22 PA PAO1-based mutant counterpart (Fig 3).

A-NO₂⁻ sensitivity of mucA22 vs. ΔmucA in anaerobic biofilms

We have previously shown that anaerobic conditions favor more robust biofilm formation by PA than aerobic conditions and that anaerobic conditions can exist in the thick CF airway surface liquid [8], thereby limiting the overall efficacy of the powerful aminoglycoside, tobramycin and other front-line antibiotics [57]. Thus, we next assessed the overall efficacy of A-NO₂⁻
against mature, pre-formed anaerobic biofilms in strains PA, mucA22, ΔmucA and their complemented strains. Fig 4A shows confocal laser scanning microscopy (CLSM) analysis results of 1 day old biofilms of bacteria that were used for the planktonic A-NO$_2^-$ susceptibility assay. These bacterial biofilms were continuously grown in LBN-pH 6.5 (control condition, NO$_3^-$) or LBN-pH 6.5 (treated condition, NO$_3^-$ 15 mM NO$_2^-$) for 2 additional days. As shown in the planktonic susceptibility assay, mucA22 strains were also more sensitive to A-NO$_2^-$ than the ΔmucA strain in bacterial biofilms that showed 37% and 25.5% cell death compared to control, respectively ($p = 0.029$, Fig 4A and 4B). A-NO$_2^-$ resistance complementation was achieved using pHERDmucA. In some cases, the biofilm structure often was slightly altered, especially in mucA-complemented bacteria which may be a function of multiple copies of cellular MucA. Taken together, our results showed that mucA22 is more sensitive to A-NO$_2^-$ than ΔmucA in both planktonic and biofilm cultures.

**Sensitivity of mucA22 but not ΔmucA PA to A-NO$_2^-$ during chronic murine lung infection**

To test the role of MucA in A-NO$_2^-$ sensitivity in an animal model of infection, a chronic murine lung infection model was employed as previously described with the exception that strain PAO1 was not used for it is nonmucoid with a wild-type mucA allele [9]. Infected mice
were treated twice daily with 15 mM A-NO$_2^-$ for 5 days and the mice were then sacrificed. Fig 5 demonstrates that mucA22 were very sensitive to A-NO$_2^-$ treatment when compared to ΔmucA mutant bacteria. This is consistent with the planktonic and biofilm A-NO$_2^-$ susceptibility assay results.

Microarray analysis of wild-type, mucA22 and ΔmucA PA upon exposure to A-NO$_2^-$

Given that mucA22 mutant bacteria were more sensitive to A-NO$_2^-$ than ΔmucA bacteria in planktonic and biofilm culture as well as during murine airway infection, we next performed transcriptional profiling experiments using wild-type PAO1, mucA22 and ΔmucA bacteria to help elucidate the mechanism(s) underlying this apparent paradox. First, considerable efforts were made to isolate quality RNA from A-NO$_2^-$ treated organisms. After many attempts and various protocol adjustments, all three strains were grown for 24 hr under anaerobic conditions in LBN, pH 6.5, followed by treatment of each organism with 15 mM A-NO$_2^-$ for 20 min.
The number of genes in each functional class comparing (i) PAO1 vs. mucA22 (Fig 6A, S1 Table), (ii) PAO1 vs. ΔmucA (Fig 6B, S2 Table) and (iii) mucA22 vs. ΔmucA (Fig 6C, S3 Table) are depicted, respectively. Interestingly, genes involved in NIR and NOR activity showed significantly reduced expression in mucA22 bacteria anaerobic induction conditions, while the expression of these genes was elevated in the ΔmucA mutant. This is consistent with the A-NO₂⁻ sensitivity phenotype of the mucA22 strain as this could lead to accumulation of toxic NO₂⁻/NO in the bacteria as a result of inefficient use of the anaerobic respiratory pathway involved in A-NO₂⁻ metabolism: A-NO₂⁻ > NO > N₂O. Fig 6D is a Venn diagram synopsis of gene overlap, the colors of which and the genes are also shown in Table 2.
Role of MucA in acidified nitrite sensitivity in P. aeruginosa

Fig 4. (A) Assessment of A-NO$_2$ on PA (wild-type), mucA22 and ΔmucA mutant strains viability when grown anaerobically as biofilms. Bacterial suspensions were diluted 100-fold in LBN, pH 6.5 and grown in Costar glass-bottomed, chambers amenable to confocal laser scanning microscopic analysis. The planktonic cells were washed from one-day old biofilms. The bacterial biofilms were separated into 2 conditions; control (LBN (NO$_3^-$), pH 6.5) and treated (LBN (NO$_3^-$), pH 6.5 plus 15 mM A-NO$_2$ or NO$_3^-$/NO$_2^-$) conditions and continuously grown for 2 more days under anaerobic condition. Both top and sagittal views of each live/dead stained biofilm are depicted after CLSM. Live cells are green while red cells are dead (B) The ratio of percent cells dead was calculated by comparison between control and treated conditions. The experiments were performed 3 times independently (n = 3).

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Identification of S-nitrosylated proteins in A-NO$_2^-$-treated bacteria

To further evaluate the response of PA to A-NO$_2^-$, a unique proteomic approach was used to measure cysteine S-nitrosylation of cellular proteins mediated by NO generated from A-NO$_2^-$ reduction, known as SNOSID (S-nitrosylation, SNO Site Identification, [58]). A-NO$_2^-$ treatment of bacteria allows for significant generation of what are termed S-nitrosylated proteins (or SNO proteins, [59]). This technique was used to monitor PA proteins that had increased or decreased levels of SNO-proteins in wild-type, mucA22 and ΔmucA strains. The data collected are based upon SNO proteins in the 2-D gels shown in S2A–S2C Fig. Twelve SNO proteins are presented in tabular form after mass spectrometric identification and their relative levels expressed in each of the aforementioned strains listed in Table 3. Interestingly, several of the proteins that showed differing levels of S-nitrosylation include those involved in anaerobic metabolism (NirQ, NirS, NrdJB) or anaerobic survival (UspK, FdnG), alginate...
production (GDP-mannose dehydrogenase), the TCA cycle (succinate dehydrogenase, SdhB) and virulence (Vfr).

Given that PA usp (universal stress protein) genes are necessary for long-term anaerobic survival [60], we postulated that a double mucA22 uspK would be hyper-sensitive to anaerobic A-NO$_2^-$ treatment. Fig 7 clearly confirms our hypothesis that this mutant is indeed more susceptible to A-NO$_2^-$ treatment than mucA22 mutant bacteria. We also identified that the FdnG (formate dehydrogenase-O, major subunit, [61]) that is physically associated with NAR in vivo [62], was identified to be S-nitrosylated. In comparison to the mucA22 uspK mutant, the mucA22 fdnG double mutant was even more sensitive to A-NO$_2^-$ (Fig 7).

Why is CF clinical strain FRD1 hypersensitive to A-NO$_2^-$ relative to PAO1 mucA22?

To determine whether mucoid CF strain FRD1 (i.e., a well-studied, chronically adapted CF strain, [51]) had mutations other than mucA22 that might reveal clues as to why it is so sensitive to A-NO$_2^-$ relative to PA mucA22, we assessed differences in the PA vs. FRD1 genome, the latter of which was recently sequenced [63]. Specifically, we assessed potential mutations in genes encoding anaerobic respiratory/regulatory genes in strain FRD1 that are not in strain PA [63]. The PAO1 genome is comprised of 6,264,404 bp while that of the FRD1 genome has 6,712,339 bp total nucleotides, with a percent G+C content of ~66%, based upon data compiled from 133 contigs [63, 64]. A search and analysis of genes predicted to be involved in A-NO$_2^-$, resistance showed numerous single base pair substitutions. These included missense mutations in genes such as several of the nar genes, as well as in nirS, and norCB among others that would hypothetically affect A-NO$_2^-$ sensitivity. However, during anaerobic conditions, mutations within the norB or norC genes would likely influence sensitivity to A-NO$_2^-$.
Differences in the coding sequence of the PAO1 and FRD1 norB gene revealed a deletion of Arg300 in the latter [63]. In strain PAO1, Arg300 is positioned in a highly basic loop that consists of 4 consecutive arginine residues linking Helix IX and Helix X on the cytoplasmic side (Fig 8). Arg300 forms a hydrogen bond with the carbonyl of Lys228 that caps the C-terminus of Helix VI. Therefore, it is possible that loss of this interaction might result in conformational changes that distort the binuclear centers of NorB, specifically His207, which is positioned on Helix VI and is a ligand for FeB. When cloned into a PAO1 norCB mutant, the FRD1 norCB genes could not rescue a normal anaerobic growth density (S4 Table).

Table 2. Overlapping differential gene expression corresponding to Fig 6D*.

| PA0025_aroE_at | PA0523_norC_at | PA0525_at | PA1059_at | PA1083_flgH_at | PA1837_at |
|----------------|----------------|-----------|-----------|----------------|-----------|
| A.(18)         | PA1838_cysL_at | PA2667_pfeS_at | PA3413_at | PA3530_at | PA3630_at | PA3632_at |
| PA3880_at      | PA4155_at      | PA4156_at | PA4158_fepG_at | PA4161_fepG_at | PA4896_at |
| PA0126_at      | PA0291_oprE_at | PA0293_at | PA0320_at | PA0425_mexA_at | PA0431_at |
| PA0548_tktA_at | PA0549_at      | PA0792_prpD_at | PA0793_at | PA0794_at | PA0795_prpC_at |
| PA0796_prpB_at | PA0797_at      | PA1135_at | PA1136_at | PA1137_at | PA1239_at |
| PA1805_ppuD_at | PA2015_at      | PA2287_at | PA2288_r_ at | PA2657_at | PA2659_at |
| PA2840_at      | PA2934_at      | PA3008_at | PA3118_leuB_at | PA3119_at | PA3120_leuD_at |
| B.(57)         | PA3195_gapA_at | PA3748_at | PA3787_at | PA3932_at | PA4030_at | PA4059_at |
| PA4129_at      | PA4130_at      | PA4131_at | PA4132_at | PA4234_usvA_at | PA4636_at |
| PA4768_smpB_at | PA4769_at      | PA4839_speA_at | PA4853_fis_at | PA4915_at | PA4932_rplL_at |
| PA4933_at      | PA4935_rpsF_at | PA5125_nrcF_at | PA5147_mut_at | PA5162_rmlD_at | PA5136_rmlA_at |
| PA5304_dadA_at | PA5383_at      | PA32667_3372284_at |
| PA0069_at      | PA0082_at      | PA0083_at | PA0124_at | PA0161_at | PA0224_at |
| PA0286_at      | PA0353_lyvD_at | PA0410_pill_at | PA0412_pillK_at | PA0413_at | PA0526_at |
| PA0558_at      | PA0669_at      | PA0670_at | PA0731_at | PA0765_mucC_at | PA0906_at |
| PA0910_at      | PA0911_at      | PA0933_ygcA_at | PA0938_at | PA1048_at | PA1431_rsal_at |
| PA1541_at      | PA1588_sucC_at | PA1589_sucD_at | PA1746_at | PA2277_arlsB_at | PA2290_gcd_at |
| PA238_1_at     | PA2445_gcvP2_at | PA2639_nuoD_at | PA2640_nuoE_at | PA2641_nuoF_at | PA2642_nuoG_at |
| PA2643_nuoH_at | PA2646_nuoK_at | PA2648_nuoM_at | PA2664_fhp_at | PA2667_at | PA2705_at |
| C.(83)         | PA2706_at      | PA2718_at | PA2788_at | PA2796_tal_at | PA2846_at | PA2869_at |
| PA2946_at      | PA2948_cobM_at | PA3011_atopA_at | PA3012_at | PA3013_foaB_at | PA3066_at |
| PA4493_at      | PA4499_at      | PA4625_at | PA4803_at | PA4812_fdnG_at | PA4919_pncB1_at |
| PA3067_at      | PA3181_at      | PA3392_nosZ_at | PA3471_at | PA3567_at | PA3575_at |
| PA3620_muteS_at | PA3859_at      | PA3972_at | PA4006_at | PA4006_at | PA4400_at |
| PA4045_at      | PA4046_at      | PA4061_at | PA4068_at | PA4180_at | PA4440_at |
| PA4493_at      | PA4499_at      | PA4625_at | PA4803_at | PA4812_fdnG_at | PA4919_pncB1_at |
| PA5479_gIPlP_at | PA5496_at      | PA5557_atopH_at | PA5564_gidB_at | PA5565_gidA_at |
| PA0510_at      | PA0515_at      | PA0516_nirF_at | PA0518_nirM_at | PA0520_nirQ_at | PA0764_mucB_at |
| D.(12)         | PA0766_mucA_at | PA1423_lasI_at | PA2830_htxX_at | PA3971_at | PA4810_fdnI_at | PA5429_aspA_at |
| PA0201_at      | PA0280_cysA_at | PA0281_cysW_at | PA0283_sbp_at | PA0284_at | PA0396_piuL_at |
| E.(12)         | PA0524_norB_at | PA2599_at | PA3446_at | PA3450_at | PA3931_at | PA4443_cysD_at |
| PA0045_at      | PA0179_at      | PA0432_sahH_at | PA0546_metK_at | PA0547_at | PA0671_at |
| PA1132_at      | PA1423_at      | PA1587_lpdD_at | PA1865_at | PA2644_nuoI_at | PA2658_at |
| F.(25)         | PA2662_at      | PA3472_at | PA3551_algA_at | PA3747_at | PA4033_at |
| PA4630_at      | PA4971_at      | PA5203_gshA_at | PA5250_at | PA5251_at | PA5252_at |

*Overlapping gene groups (probe set ID) are colored as the Venn diagram showing in Fig 6D.

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Anaerobic A-NO$_2$-sensitivity: intuitive and nonintuitive gene products involved in its defense

The mucA22 allele in PA is the most common mutation (deletion of a C residue at position 430) found during the course of chronic CF and COPD, resulting in a truncated MucA protein of ~15.8 kDa and resulting mucoid conversion (alginate-overproduction). A little over 13 years ago, we discovered that mucoid, mucA22 mutant PA were uniquely susceptible to A-NO$_2$- at the slightly acidic pH that is consistent with that of the CF (pH 6.4–6.5 [9, 66] and COPD airway surface liquid [67], effectively revealing a previously unrecognized “Achilles heel” of these highly refractory organisms. This weakness was a significant translational development given that mucA22 organisms are formidably antibiotic- and phagocyte-resistant. Prior to that work, two seminal papers describing the anaerobic biofilm mode of growth in CF were published in 2002 [8, 13]. Since then, dedicated research efforts have ensued to unravel the biology of the ever-evolving organisms cultured from anaerobic CF and COPD airway biofilms. In addition, the slightly acidic nature of CF and COPD airways allows for A-NO$_2$- to be reduced to NO, that we have shown is known to kill not only mucA22 mutant PA, but also S. aureus (MRSA) and B. cepacia [29].

Thus, given these clinically relevant and translational developments (U.S. Patent # 8,557,300 B2 by corresponding author), the primary goal of this study was to identify additional genes involved in A-NO$_2$- sensitivity or resistance. Using both transposon and strategic insertion or deletion mutagenesis approaches, a series of mutants displaying the aforementioned traits were discovered. First, we showed that the major catalase, KatA, is required for anaerobic A-NO$_2$- resistance because of its surprisingly inherent ability to buffer the NO derived from A-NO$_2$- reduction [48]. Second, a mutant defective in the rhl quorum sensing circuit regulator (rhlR), that we have also found previously to commit an anaerobic suicide by overproduction of endogenous respiratory NO in biofilms [8], was also found to be sensitive to exogenous anaerobic A-NO$_2$-.

**Table 3. Identification of SNO proteins from the different bacteria used in this study.** For example, the fold change in spot 2A (FdnG) in the mucA22 mutant is 2.2-fold more S-nitrosylated than wild-type bacteria.

| Spot ID | Gene/protein name | Fold change$^*$ | mucA22 vs PAO1 | ΔmucA vs PAO1 | ΔmucA vs mucA22 |
|---------|------------------|-----------------|----------------|---------------|-----------------|
| 2A      | fdnG             | 2.2             | Up 2.4         | Down          | -               |
| 2B      | fdnG             | 13              | -              | 6.3           | Down 2.1        |
| 5       | ggbC            | -               | -              | -             | 1.5             |
| 6A      | nrdJB           | -               | 1.4           | -             | 1.3             |
| 6B      | nrdJB           | -               | 1.9           | -             | 1.6             |
| 7       | priC or opdA     | 1.7             | -              | 1.7           | -               |
| 8       | algD             | -               | 1.7           | 1.5           | 1.4             |
| 11      | Vfr              | -               | 6.7           | -             | 7.1             |
| 13      | nirS             | -               | -             | 2.3           | -               |
| 15      | oprH             | -               | 3.2           | -             | 2.9             |
| 17      | prpB or bcpA     | -               | 1.2           | 1.4           | 1.7             |
| 18      | nirQ             | 1.7             | -             | -             | 1.7             |
| 19      | galU             | -               | 1.4           | -             | 1.3             |

$^*$ = Relative SNO protein expression levels.

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**Discussion**

**Anaerobic A-NO$_2^-$ sensitivity: intuitive and nonintuitive gene products involved in its defense**

The mucA22 allele in PA is the most common mutation (deletion of a C residue at position 430) found during the course of chronic CF and COPD, resulting in a truncated MucA protein of ~15.8 kDa and resulting mucoid conversion (alginate-overproduction). A little over 13 years ago, we discovered that mucoid, mucA22 mutant PA were uniquely susceptible to A-NO$_2^-$ at the slightly acidic pH that is consistent with that of the CF (pH 6.4–6.5 [9, 66] and COPD airway surface liquid [67], effectively revealing a previously unrecognized “Achilles heel” of these highly refractory organisms. This weakness was a significant translational development given that mucA22 organisms are formidably antibiotic- and phagocyte-resistant. Prior to that work, two seminal papers describing the anaerobic biofilm mode of growth in CF were published in 2002 [8, 13]. Since then, dedicated research efforts have ensued to unravel the biology of the ever-evolving organisms cultured from anaerobic CF and COPD airway biofilms. In addition, the slightly acidic nature of CF and COPD airways allows for A-NO$_2^-$ to be reduced to NO, that we have shown is known to kill not only mucA22 mutant PA, but also S. aureus (MRSA) and B. cepacia [29].

Thus, given these clinically relevant and translational developments (U.S. Patent # 8,557,300 B2 by corresponding author), the primary goal of this study was to identify additional genes involved in A-NO$_2^-$ sensitivity or resistance. Using both transposon and strategic insertion or deletion mutagenesis approaches, a series of mutants displaying the aforementioned traits were discovered. First, we showed that the major catalase, KatA, is required for anaerobic A-NO$_2^-$ resistance because of its surprisingly inherent ability to buffer the NO derived from A-NO$_2^-$ reduction [48]. Second, a mutant defective in the rhl quorum sensing circuit regulator (rhlR), that we have also found previously to commit an anaerobic suicide by overproduction of endogenous respiratory NO in biofilms [8], was also found to be sensitive to exogenous anaerobic A-NO$_2^-$. Other genes that were necessary for optimal A-NO$_2^-$

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resistance included another quorum sensing regulator gene, pmpR (regulator of the Pseudomonas quinolone signal (PQS), [68]), lon (encoding the Lon protease), PA4455 (a putative ABC transporter, [47]), and nrdJa, b (encoding anaerobic ribonucleotide reductase). The Lon protease has previously been shown to be required for optimal activity of Fhp (flavohemoglobin), an aerobically expressed flavohemoglobin with aerobic but not anaerobic NO detoxifying properties [69, 70]. Thus, it makes perfect sense that the PA0779 (asrA, a Lon peptidase) mutant is also sensitive to A-NO₂⁻. However, the most intriguing results of this study and given the predominance of mucA mutations from predominantly CF and to a lesser extent COPD were from A-NO₂⁻ sensitivity and insensitivity results comparing mucA22 vs. ΔmucA bacteria, the latter of which was found to be paradoxically resistant to it in planktonic, biofilm and during a chronic murine lung infection. Thus, the remainder of this study focused on understanding the mechanism underlying this puzzling development.

Microarray and SNO protein analyses and interpretations upon exposure of bacteria to A-NO₂⁻

To better understand why wild-type and ΔmucA strains were less sensitive to A-NO₂⁻ than mucA22 bacteria, we strategically performed transcriptional profiling of wild-type, mucA22

![Graph showing the sensitivity of anaerobic planktonic PAO1 (diamonds) mucA22 mutant (squares) with secondary mutations in uspK (strikethrough squares) and fdnG (triangles). We must note that there were no observable differences in the growth patterns of mucA22 and the double mutants.](https://doi.org/10.1371/journal.pone.0216401.g007)
and ΔmucA strains exposed to anaerobic A-NO₂⁻. We found that wild-type bacteria and the ΔmucA mutant had far higher transcriptional levels of protective nir (encoding NIR), nor (encoding NOR) and fhp (encoding flavohemoglobin) genes when compared to the very low transcription of such genes in the mucA22 mutant. This is consistent with the very low transcription of nir and nor genes in anaerobic clinical isolate FRD1 (a mucA22 mutant) exposed to A-NO₂⁻ as we have described previously [9]. In addition, many nuo genes (encoding NADH dehydrogenase) were also upregulated in the ΔmucA mutant. It is not surprising that the nuoABDFHIJKMN genes are also required for anaerobic growth with both NO₃⁻ and NO₂⁻ as terminal electron acceptors [71, 72]. Thus, the wild-type and ΔmucA mutant have a greater ability to detoxify both NO₂⁻ and NO. Another interesting observation from the transcriptional profiling was that wild-type PAO1 and the muc22 mutant have normal expression of mucD, the last gene of the algT/U operon, but the ΔmucA mutant has 5-fold less expression. MucD is a periplasmic protease that serves as a weak negative regulator of AlgW protease, which senses periplasmic stress, leading to cleavage/activation of MucA [73]. This alteration in the AlgT(U) (σ²²) pathway might affect expression of nir, nor, and fhp genes, which is currently under investigation. However, the ΔmucA strain has 27 extra amino acids than the mucA22 mutant, begging the question as to whether these amino acids are involved in the anaerobic respiratory pathway vis a vis protection against NO.

To complement our transcriptomic data, our S-nitrosylation analysis also revealed mechanistic clues from the protein level. As a reminder, S-nitrosylation is a mechanism of signal
transduction in eukaryotes based upon a covalent bond between NO and cysteine residues, thereby serving as a post-translational modification [74]. In bacteria, however, S-nitrosylation can actually be used to trigger anaerobic gene transcription mediated, as seen by SNO-mediated activation of OxyR in *E. coli* [75]. The most S-nitrosylated proteins (an event that can compromise protein function) have significant links to the overall anaerobic respiratory cascade of *PA*. These include NrdG (a regulator of *ndrJa* genes), NirS (NIR), NirQ [76], NrdJa (anaerobic ribonucleotide reductase (RNR)) and NuoL (NADH dehydrogenase chain L).

Other than nirS, it should be noted that all of the aforementioned genes are essential for anaerobic growth [77], especially the Class II RNRs [78]. Thus, we believe that S-nitrosylation is a consequence of exposure to A-NO$_2^-$. However, we also believe that the NO generated by A-NO$_2^-$ reduction overwhelms the machinery involved in the transcription of the protective *norCB* genes (encoding NO reductase). In 2007, we showed that high (~16 μM) endogenous levels of NO inactivate the ANR/DNR regulatory cascade in a *norCB* mutant, leading to abysmally poor growth under anaerobic conditions [50].

**What could be the mechanism of A-NO$_2^-$ toxicity in anaerobic mucA22 mutant PA relative to that of a ΔmucA mutant?**

There are at least five possible mechanisms of cell injury and death from A-NO$_2^-$ in mucA22 mutant bacteria, (a) inhibition of heme-enzyme(s), (b) destruction of iron-sulfur (Fe-S) centers (e.g., the master anaerobic transcriptional regulator ANR, an NO-sensitive 4Fe-4S cluster protein, [50]), (c) disruption of cellular iron homeostasis (with a,b,c being primarily via the formation of dinitrosyliron complexes [48]), (d) oxidative injury (upon introduction to an aerobic environment), and (e) protein adduction (nitration, nitrosation). NO reacts with only two groups of species under biological conditions, other radicals (such as O$_2$ and O$_2^-$) and transition metals. At first glance (however *vide infra*), oxygen radical-based death can be ruled out because of the strict anaerobiosis enforced in these studies given the fact that both CF and COPD airways have anaerobic airway pockets, leaving likely transition metal interactions as the major mechanism(s) of NO toxicity. Most cellular metal ions are “shielded” from NO and thus are not likely targets. The “classic” metal targets are iron and heme-containing (those with an open ligation position), Fe-S centers, and the “chelatable iron pool” (CIP) [79–84].

**Final questions that urgently need answers**

What might be the biological functions of MucA vs. MucA22: are there potentially other binding partners during anaerobic growth other than the extracytoplasmic sigma factor, AlgT(U) and the periplasmic negative regulator MucB? The most intriguing finding of this work was the discovery that mucA22 and ΔmucA bacteria behave very differently *vis a vis* A-NO$_2^-$ susceptibility patterns. Our results suggest that the 15.8 kDa truncated MucA22 protein has an unknown function that is possibly to dysregulate the anaerobic respiratory regulatory and enzymatic genetic circuitry. In support of this notion, and based upon this pioneering work, we have previously shown that NIR and NOR genes are dramatically down-regulated in mucA22 bacteria relative to wild-type organisms [9]. As a final attempt to build the hypothesis-driven platform for a future study, we elected to use bioinformatic techniques to elucidate further clues as to why mucoid mucA mutants are more sensitive to A-NO$_2^-$ than wild-type, nonmucoid bacteria. To accomplish this goal, our microarray data were analyzed to identify differentially expressed genes (DEGs) between three conditions (anaerobic *PA*, mucA22 and ΔmucA exposed to 15 mM A-NO$_2^-$, pH 6.5 in pairs. The significant DEGs were selected by the criteria that fold change values are larger than two and the *p*-value is lower than 0.05. We also compared the significant DEGs under each comparison design based upon the microarray
data and looked for overlapping genes to investigate if the transcriptional profile changes were contributing to the hypersensitivity of A-NO$_2^-$ in mucA22 mutant bacteria when compared to wild-type and ΔmucA). The functional annotation clustering of the gene set was conducted by DAVID to see if any functional pathway was related with alternative gene expression. If the overlapping genes were also found in the mucA22 vs. ΔmucA analyses, it indicates that the genes expressed differently should have a tight interaction with MucA. To elucidate the mechanism underlying A-NO$_2^-$ sensitivity as well as identifying potential interacting partners of MucA, we searched an on-line PA PAO1 protein-protein interaction (PPI) database (http://research.cchmc.org/PPIdatabase) using the query word MucA. This database contains prediction results by a random forest classifier that was trained on nine genomic features (co-essentiality, co-expression, co-functionality, co-localization, domain-domain interaction, co-pathway involvement, transmembrane helices, co-operon and co-gene cluster involvement. [85]). This assessment resulted is a large-scale PPI network in PA with significant coverage and high accuracy, i.e., 57,746 potential protein interactions covering 4,256 PA PAO1 proteins [86]. Among the DEGs, we selected the potential PPI partners of MucA from the interactome database. By querying MucA in the database, we found 17 proteins with which it is predicted to interact (S5 Table, Fig 9). We also searched the STRING database (https://string-db.org) for MucA interactors. The top 20 interactors of high confidence interaction with MucA were used for the following analysis. Five proteins are in common between the interactors predicted by two sources. In total, 32 MucA interactors were predicted (S6 and S7 Tables). We then investigated if these interactors changed expression in the ΔmucA or mucA22 mutants compared to wild type PAO1. Five interactors were found significantly altered expression in PA vs ΔmucA, including three muc genes (mucBCD), algA and nirC, respectively. In contrast, when PAO1 gene expression was compared to the mucA22 mutant, only levels of algA and nirC transcription were found significantly changed. Interestingly, only the nirC gene was found differentially expressed in all three strains comparisons. In fact, nirC expression was highest in the mucA22 mutant and down-regulated in the ΔmucA mutant when compared to PAO1 expression. This pattern indicates that the expression of the nirC gene is more likely to be influenced by any changes in the mucA gene, and the expression level may be correlated to the A-NO$_2^-$ sensitivity phenotype. We identified possible anaerobic binding partners for MucA, NirC and NirM. Both NirC and NirM are periplasmic c-type cytochromes that are known to donate electrons to NIR, thereby promoting efficient anaerobic respiration [87]. These data indicate that there is a distinct possibility that anaerobic MucA22 has a function other than binding to AlgT (U) and MucB. Relatedly, an anaerobic protein “interactome” has been shown linking the primary motility protein, flagellin (FliC) with NirS and DnaK [88]. Very complex yet interesting biology was revealed in the aforementioned work. First, a nirS mutant could not form a flagellum and as such was impaired in swimming motility. Conversely, if the flagellum and anaerobic respiration are coordinately regulated in an as yet unappreciated pathway, then a fliC mutant should be impaired in anaerobic respiration—which was not the case. The other interesting feature between flagellar expression and mucoidy is that fliC is repressed by the AlgT (U)-dependent regulator AmzR that directly represses the fleQ gene, the product of which is required for fliC transcription [89]. We have previously shown that mucoid mucA22 mutant bacteria grow poorly anaerobically because they harbor dramatically lower respiratory NAR and NIR activity [9].

Closing remarks

Finally, this multi-disciplinary study revealed a fascinating paradox in that ΔmucA mutant bacteria possess an anaerobic A-NO$_2^-$ resistant phenotype relative to that of mucA22 mutant
bacteria. This is consistent with the myriad of strains (>300) that have been sequenced in several studies indicating that no mucoid strains would be classified as true deletion mutants and we could not engineer a complete deletion despite numerous attempts [9, 35]. Given the numerous discoveries and scientific disciplines used in this study, we elected to provide a synopsis flow chart that is detailed in Fig 10 as a refresher and eliminate any potential confusion. This figure is divided into (i) initial screen, (ii) unexpected discovery, (iii) mechanistic evaluation and (iv) future studies. Lastly, future studies are designed to identify putative anaerobic MucA and potentially MucA22 binding partners in the context of better understanding the important translational implications of A-NO\textsubscript{2}\textsuperscript{-} treatment for killing of highly refractory airway infectious bacteria in CF and COPD.

Fig 9. Integrated protein interaction networks. A. Inset. Simplified version of the integrated protein interaction networks. B. Fifteen proteins encoded by the genes involved in A-NO\textsubscript{2}\textsuperscript{-} sensitivity (green nodes; overlapping DEGs of PA vs mucA22 and ΔmucA vs mucA22) and 32 predicted MucA interactors (red nodes) were used to build an integrated protein interaction network. The genes labeled in red were also found differentially expressed between PA vs ΔmucA.

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Materials and methods

Bacterial strains and growth conditions

All bacteria, including newly constructed mutants and plasmids used in this study are listed in Table 4. Organisms were routinely grown in either Luria-Bertani broth (L-broth) or L-broth plus 100 mM KNO\textsubscript{3} (LBN) or LBN pH 6.5 which is LBN containing 50 mM potassium phosphate. Aerobic cultures were grown at 37°C with shaking at 250 rpm at a 1/10 volume to total Erlenmeyer flask ratio. Media were solidified with 1.5% Bacto-agar. Bacteria were grown
under anaerobic conditions at 37˚C in a dual-port Coy Laboratories anaerobic chamber. Frozen bacterial stocks were stored at -80˚C in a 1:1 mixture of 30% glycerol and stationary-phase bacterial suspension.

### Table 4. Bacterial strains, plasmids and oligonucleotides used in this study.

| Strain, plasmid or oligonucleotide | Description (relevant genotype or phenotype) or sequence (5' to 3') | Company source or reference |
|------------------------------------|--------------------------------------------------|-----------------------------|
| **E. coli**                        |                                                  |                             |
| DH5α                              | Φ80dlacZΔM15 endA1 recA1 hsdR17(rK− mK+) supE44 thi-1 gyrA96 Δ(lacZYA-argF)U169 | Invitrogen                   |
| S17-1 λ pir                       | Pro− Res− Mod+ recA; integrated RP4-Tet::Mu-Kan::Tn7, Mob+ | [91]                        |
| **P. aeruginosa**                 |                                                  |                             |
| PAO1                              | Wild-type laboratory strain                       | [92]                        |
| FRD1                              | Mucoid CF clinical isolate with mucA22 allele     | [93]                        |
| mucA22                            | PAO1 mucA22 mutant, mucoid                        | [94]                        |
| ΔmucA                             | PAO1ΔmucA mutant (Δ157–194) (Hassett and Schurr labs) | This study                  |
| mucA22 uspK                       | mucA22, uspK::TnGm                                 | This study                  |
| mucA22 fhnG                       | mucA22, fhnG::TnGm                                 | This study                  |
| moaA2                             | moaA2::TnGm                                      | This study                  |
| PA4455                            | PA4455::Gm                                       | [47]                        |
| PA0964                            | PA0964::TnGm                                     | This study                  |
| PA0450                            | PA0450::TnGm                                     | This study                  |
| rhIR                              | rhIR::Gm                                         | [52]                        |
| norCB                             | norCB::Gm                                        | [50]                        |
| lon                               | lon::TnGm                                        | This study                  |
| muoK                              | muoK::TnGm                                       | This study                  |
| **Plasmids**                      |                                                  |                             |
| pBT20                             | Mini-Tn delivery vector, ApR, GmR                 | [95]                        |
| pUCGM                             | Source for Gm cassette, ApR, GmR                  | [96]                        |
| pEX100T-KS                        | Pseudomonas gene replacement suicide vector with modified multiple cloning site, sacB, oriT, CbR | [97]                        |
| pEXΩ100T                          | GmR cassette from pUCGM was inserted into unique Scal site of pEX100T-KS, sacB, oriT, GmR | This study                  |
| pEXΩ100TΔmucA                     | 1kb upstream and downstream fragments flanking mucA gene were cloned into pEXΩ100T, sacB, oriT, GmR | This study                  |
| pHERD20T                          | Escherichia-Pseudomonas shuttle vector, ApR       | [98]                        |
| pHERDmucA                         | mucA cloned into pHERD20T                        | This study                  |
| **Nucleotides**                   |                                                  |                             |
| AD2                               | 5’-cangctwsgtntscaa                               |                             |
| Gm447                             | 5’-tgcaagcagacgatggtgacgat                      |                             |
| Gm464                             | 5’-tgagccgccccgggtggtgctc                       |                             |

| ApR, ampicillin resistant; CbR, carbenicillin resistant; GmR, gentamicin resistant |

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### Manipulation of recombinant DNA and genetic techniques

All plasmid and chromosomal nucleic acid manipulations were performed by standard techniques [99]. Plasmid DNA was transformed into *E. coli* strain DH5α (Protein Express, Cincinnati, OH). To detect the presence of insert DNA, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml) was added to agar media. Restriction endonucleases, the Klenow fragment of DNA polymerase I, T4 DNA polymerase, and T4 DNA ligase were used as specified by the vendor (Invitrogen/ Gibco-BRL Corp., Gaithersburg, MD). Plasmid DNA was
isolated by using plasmid miniprep isolation kits (Qiagen), and restriction fragments were recovered from agarose gels by using SeaPlaque low-melting-point agarose (FMC BioProducts, Rockland, ME). PCRs were performed by using Pfu DNA polymerase (BRL) and appropriate primers in an MJ Research thermal cycler, with 30 cycles of denaturation (2 min, 94˚C), annealing (1 min, 54˚C), and extension (1 min 30 s, 72˚C). Amplified DNA fragments were gel purified, cloned into pCR2.1 (Invitrogen), and sequenced.

Methods used to construct PA mutant strains

Screening of more sensitive or resistance A-NO₂⁻ strains using Transposon (Tn) mutagenesis. PA strain PAO1 was subjected to transposon (Tn) mutagenesis using the mariner transposon vector, pBT20 [95]. The transposon within pBT20 was conjugally transferred by biparental mating using E. coli S17-1 λ pir into strain PAO1 as previously described [95]. An overnight broth culture of the donor strain (2 ml of stationary phase culture) and 0.5 ml of the recipient strain were clarified by centrifugation and resuspended in 0.5 ml of L-broth. This concentrated suspension was then spotted on the center of a L-broth plate, allowed to dry, and incubated for ~18 hr at 37˚C. Mating mixtures were scraped and resuspended in 1 ml of L-broth. Suspensions (300 µl) were spread evenly onto Pseudomonas isolation agar (PIA) containing gentamicin (Gm) at 150 µg/ml and incubated at 37˚C for 48 hr. The resulting growth was scraped from the plate and resuspended in 2 ml of 0.9% saline and serial dilutions plated onto freshly prepared LBN plates, pH 6.5 containing 15 mM acidified NaNO₂ (A-NO₂⁻). Transposon insertion sites were determined through sequencing the flanking region of the transposon by a semi-random PCR method, as described previously [100] using random primer AD2 and transposon-specific primer Gm447, followed by the nested primers Gm464 and AD2 (Table 1). Individual colonies were patched to L-broth plates and L-broth A-NO₂⁻, pH 6.5 plates containing 15 mM KNO₃ with either 15 mM or 25 mM NaNO₂ for selection of sensitive and resistance strains, respectively. Cells were grown aerobically for 24 hr and anaerobically for up to 48 hr. Those organisms that grew on the 25 mM NaNO₂ plates were considered A-NO₂⁻ resistant while cells that did not grow on 15 mM NaNO₂ plates were considered A-NO₂⁻ sensitive. Confirmation of the A-NO₂⁻ sensitive or resistance phenotype was followed by an A-NO₂⁻ killing assay by enumeration of remaining CFU after treatment. Briefly, the overnight culture was diluted 1000-fold into L-broth pH 6.5, 50 mM phosphate buffer containing either 15 mM KNO₃ or 15 mM NaNO₂, respectively. Cell viability was monitored daily for 3 days. All experiments were performed at least 3 times and reproducible mutants were then assessed for the identity of the specific gene interrupted by the transposon. The genomic DNA was isolated and the identification of the transposon integration site was initiated by semi-random PCR. The resulting PCR amplification products were subjected to DNA sequence analysis at the Cincinnati Children’s Hospital DNA core (Cincinnati, OH).

Allelic exchange and sucrose counter-selection for construct of mutants. The strategy for insertional inactivation of PA genes (Table 1) was facilitated by gene disruption with an 850-bp GmR cassette from pUCGM (52), and the gene replacement vector pEX100T-KS (29), the latter of which allowed for selection of double-crossover events within putative recombinants cultured on agar containing 5–6% sucrose. To facilitate construction of an unmarked nonpolar mucA deletion mutant, the GmR cassette from pUCGM was inserted into unique Scal site of pEX100T-KS, creating plasmid pEXΩ100T. Approximately 1 kb of upstream and downstream fragments of the mucA gene was PCR amplified, and cloned into the HindIII and SpeI sites of pEXΩ100T. The resultant plasmid, pEXΩ100TΔmucA, was used to construct a mucA deletion mutant (herein termed ΔmucA) that contained a downstream constitutive promoter to ensure transcription of the mucB gene. All mutants were confirmed by DNA
sequencing of amplified PCR products. Two independent mucA deletion mutants from the D. J.H. and M.J.S. laboratories were also confirmed by Illumina sequencing (S1 Fig) and the contigs were assembled using the PATRIC alignment program. Other mutants that we suspected to be sensitive to A-NO$_2^-$ from previous studies and our own literature-based hypotheses are also listed in Table 4. Some other mutants listed in Table 4 were also constructed using this method.

**Planktonic culture measurements of A-NO$_2^-$ sensitivity.** Overnight cultures of PA and various Tn and/or allelic exchange mutants were 1:100 diluted into either LB broth (pH 6.5) or LBN broth (pH 6.5, the pH of CF airway surface liquid, [9]) supplemented with varying concentrations of NaNO$_2$ (0, 5, 10, 15, 20, 25 and 30 mM, hence the term A-NO$_2^-$) and grown anaerobically for 48 hr. Five µl of cells from each culture was serially diluted and spotted onto LB agar plates and incubated aerobically for 24 hr at 37˚C. The plates were then scanned for enumeration of CFU. (ii) Some strains were also cultured anaerobically for 72 hr in LBN broth (pH 6.5) supplemented with either 0, 20, or 25 mM NaNO$_2$ for strain PAO1, ΔmucA and mucA22 mutants, as well as mucA22 uspK and mucA22 fdnG, respectively. Cultures were processed daily, and serial cell dilutions were spotted onto L-agar plates. Surviving bacteria were enumerated after a 24 hr incubation at 37˚C.

**Anaerobic biofilm A-NO$_2^-$ sensitivity measurements.** Bacteria were grown aerobically in LB broth to stationary phase followed by a 1:100 dilution into 3 ml of LBN in confocal friendly glass bottomed chambers (Costar). Static bacterial biofilms were allowed to develop under anaerobic condition as previously described [8]. After 24 hr, biofilms were washed with sterile PBS to remove planktonic cells, and fresh LBN broth (pH 6.5) containing 15 mM KNO$_3$ (control), or 15 mM KNO$_3$ plus 15 mM NaNO$_2$ was added to the bacteria biofilm cultures. The biofilms were then incubated under anaerobic conditions for an additional of 48 hr, washed 2 times with PBS, and stained with Live/Dead BacLight bacterial viability kit (Invitrogen, Eugene, OR). Biofilm images were viewed by confocal laser scanning microscopy using a Zeiss LSM 710 confocal microscope and visualized the live cells in green and the dead cells in red. The excitation and emission wavelengths for green fluorescence (live cells) were 488 nm and 500 nm, while those for red fluorescence (dead cells) were 490 nm and 635 nm, respectively. All biofilm experiments were repeated at least 3 times independently. The live/dead ratio of the biofilms were calculated using imageJ 1.46r software following the guidelines by the University of Chicago Integrated Light Microscopy Core. The results are presented as the differences in the dead/live ratio comparing A-NO$_2^-$ treated versus control conditions.

**Transcriptional profiling using Affymetrix GeneChips of PA, mucA22 and ΔmucA strains exposed to A-NO$_2^-$**. PA, mucA22 and ΔmucA bacteria were grown anaerobically for 24 hr in LBN, pH 6.5, followed by the addition of 15 mM NaNO$_2$ (A-NO$_2^-$) for an additional 20 min. Organisms were then pelleted by centrifugation at 13,000 x g for 5 min and the pellets resuspended in RNAlater (Ambion) to prevent bacterial RNA degradation and to stabilize the bacterial mRNA. To assess quantitative gene expression analysis of PA, Affymetrix GeneChips were used. RNA from PA was isolated by using Qiagen RNeasy columns according to the manufacturer’s protocol for isolation of total RNA. RNA from three independent samples was isolated for hybridization on three independent Pseudomonas GeneChips. Once the RNA was eluted from the Qiagen RNeasy column, then RNA was treated with 2 U of DNase I (Ambion) for 15 min at 37˚C. The reaction was stopped by the addition of 25 µl of DNase stop solution (50 mM EDTA, 1.5 M sodium acetate and 1% SDS). The DNase I was removed by phenol/chloroform extraction followed by ethanol precipitation. The approximate amount of RNA isolated was quantified using spectrophotometer. To determine the quality of the RNA, samples were analyzed on an Agilent bioanalyzer 2100. The quality of RNA was determined by examining the 16S and 23S rRNA bands on the electrophoretogram that should be at a 1:1
ratio. Ten μg of total RNA was used for cDNA synthesis, fragmentation and labeling according to the Affymetrix GeneChip PA Genome Array Expression Analysis Protocol. Briefly, random hexamers (Invitrogen) were added (final conc. 25 ng/μl) to the 10 μg of total RNA along with in vitro transcribed B. subtilis control spikes (as described in the Affymetrix GeneChip PA Genome Array Expression Analysis Protocol). cDNA was synthesized using Superscript II (Invitrogen) according to the manufacturer’s instructions under the following conditions: 25˚C for 10 min, 37˚C for 60 min, 42˚C for 60 min, 70˚C for 10 min. RNA was removed by alkaline treatment and subsequent neutralization. The cDNA was purified by a QIAquick PCR purification kit (Qiagen) and eluted in 40 μl of elution buffer (10 mM Tris-HCl, pH 8.5). The cDNA was then fragmented by DNase I (0.6 U per μg cDNA, Amersham) at 37˚C for 10 min and end-labeled with Biotin-ddUTP using the Enzo BioArray Terminal Labeling kit (Affymetrix) at 37˚C for 60 min. Proper cDNA fragmentation and biotin labeling were determined by gel mobility shift assay using NeutrAvidin (Pierce) and on a 5% polyacrylamide gel stained with SYBR Green I (Roche). The labeled cDNA was hybridized to the Affymetrix Pseudomonas GeneChip according to the manufacturer’s protocol. Microarray data was generated using Affymetrix (www.affymetrix.com) protocols as we have done previously [72, 101]. We used Affymetrix data previously obtained from PA grown from three independent samples as the control for gene expression comparisons.

**Microarray data analysis.** Microarray data were generated and analyzed using Affymetrix protocols as previously described [102–104]. Probe set summarization (.CHP) files were generated using the Affymetrix MicroArray Suite 5.0 (MAS 5.0) algorithm. The absolute expression transcript levels were normalized for each chip by globally scaling all probe sets to a target signal intensity of 500. Three statistical algorithms (detection, change call, and signal log ratio) were used to identify differential gene expression in experimental and control samples. The decision of a present, absent, or marginal identification for each gene was determined by using MicroArray Suite software (version 5.0; Affymetrix). Those transcripts that received an “absent” designation were removed from further analysis. A t test was used to isolate those genes whose transcriptional profile was statistically significant (P < 0.05) between the control and experimental conditions. Pair-wise comparisons between the individual experimental and control chips were done by batch analyses using MicroArray Suite to generate a change call and signal log ratio for each transcript. A positive change was defined as a call whereby more than 50% of the transcripts increased or marginally increased for up-regulated genes or decreased or marginally decreased for down-regulated genes. Lastly, the median value of the signal log ratios for each comparison was calculated and only transcripts that had a value greater than or equal to 1 for up-regulated and less than or equal to 1 for down-regulated genes were placed on the final list of transcripts whose profile had changed. The signal-log ratio was converted and expressed as the change (n-fold). The microarray data are available on the GEO (Gene Expression Omnibus) website at http://www.ncbi.nlm.nih.gov/projects/geo (GEO accession no. GSE128220).

**Bioinformatic analysis of differential gene expression**

The microarray data were analyzed to identify differentially expressed genes (DEGs) between three conditions (anaerobic PA, mucA22 and ΔmucA exposed to 15 mM A-NO₂⁻, pH 6.5 for 20 min) in pairs. The significant DEGs were selected by the criteria that fold change values are larger than two and the p-value is lower than 0.05. To elucidate the mechanism underlying A-NO₂⁻ sensitivity as well as identifying potential interacting partners of MucA, we searched an online PA PAO1 protein-protein interaction (PPI) database (http://research.cchmc.org/PPIdatabase) using the query word MucA. This database contains prediction results by a
random forest classifier that was trained on eight genomic features (co-essentiality, co-expression, co-functionality, co-localization, domain-domain interaction, co-pathway involvement, transmembrane helices, co-operon and co-gene cluster involvement). The result is a large-scale PPI network in PA with significant coverage and high accuracy, i.e., 57,746 potential protein interactions covering 4,256 PA PAO1 proteins [86]. Among the DEGs, we selected the potential PPI partners of MucA from the interactome database. We also compared the significant DEGs under each comparison design and looked for overlapping genes, to investigate if the transcriptional profile changes were contributing to the hypersensitivity of A-NO$_2$ in mucA22 mutant bacteria when compared to wild type and ΔmucA). The functional annotation clustering of the gene set was conducted by DAVID to see if any functional pathway was related with alternative gene expression. If the overlapping genes were also found in the mucA22 vs. ΔmucA analyses, it indicates that the genes expressed differently should have a tight interaction with MucA. Among the DEGs, we selected the PPI partners for MucA from the interactome database. We also searched the STRING database (https://string-db.org) for predicted MucA interacting partners. The top 20 interactors with high confidence were used for in our analysis. To investigate if the transcriptional profile changes were contributing to the hypersensitivity of A-NO$_2$ in mucA22 comparing to wild type and ΔmucA, we also compared the significant DEGs under each comparison design and looked for overlapping genes. The GO function enrichment of the gene set was conducted on the overlapping DEGs and the predicted MucA interactors, in order to see if any functional pathway was related with altered gene expression.

**Identification of anaerobic S-nitrosylated proteins in PA, mucA22 and ΔmucA strains using the SNOSID technique (SNO Site Identification)**

PA PAO1, mucA22 and ΔmucA were grown in L-broth under aerobic conditions at 37˚C for 18 hr. Bacteria were then further diluted 1000-fold in L-broth, pH 6.5 (50 mM potassium phosphate) containing 15 mM KNO$_3$ for 24 hr under anaerobic conditions. Bacteria were then exposed to 15 mM NaNO$_2$ for 1 hr. The cell pellet was lysed with B-PER plus 0.1 mM EDTA and 0.5 mM PMSF at room temperature for 10 min. Next, identical protein levels were used to evaluate S-nitrosylation using the “biotin switch” method as described by Jaffrey and Snyder [105]. Briefly, protein lysates were placed in blocking solution (2.5% SDS and 0.1% methanethiosulfonate, MMTS) prepared in dimethylformamide (DMF) with 9 volumes of HEN buffer (250 mM HEPES-NaOH pH 7.7, 1 mM EDTA and 0.1 mM neocuproine) in the dark at 50˚C for 20 min with frequent vortex. The excess MMTS was removed by precipitation with 3 volumes of cold acetone. After centrifugation, the protein pellet was washed with 70% cold acetone 4 times and then resuspended into HEN buffer containing 1% SDS, 2.5 mg/ml biotin-HPDP and 200 mM sodium ascorbate. The mixtures were incubated in the dark at 25˚C for 1 hr with intermittent vortex. The biotinylated nitrosothiol proteins were then precipitated with acetone. Again, after washing 4 times with 70% cold acetone, the protein pellet was dissolved in 0.1X HEN buffer containing 1% SDS and 3 volumes of neutralization buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) was added followed by 50 μl of pre-washed avidin affinity resin. The mixture was then incubated at 4˚C for 18 hr. The resin was extensively washed 4 times with 1 ml of washing buffer (20 mM HEPES, pH 7.7, 600 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) and the resin was fully dried via gentle aspiration with a 28-gauge needle. Bound protein was then eluted with 50 μl of elution buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, 100 mM 2-mercaptoethanol). The samples were concentrated and desalted using Amicon® Ultra centrifuge filter devices (3 K) and the buffer exchanged 2 times with 50 μl of GE Healthcare Life Sciences DeStreak buffer. The
protein concentration was determined using a Pierce 660 nm protein assay and prepared for 2-dimensional gel electrophoresis. Then, 27 μg of purified protein was loaded onto 7 cm IPG strips, pH 3–10 NL (non-linear) and subjected to electrophoresis according to a standardized procedure for the Invitrogen zoom apparatus with no streaking in the first dimension. After running denaturing polyacrylamide gel electrophoresis (SDS-PAGE) for the second dimension, proteins were visualized after silver staining. Gel images were compared using Progenesis SameSpots V3.2 software ((Nonlinear Dynamic, Inc., Durham, NC). The protein spots that revealed significant differences in pairs between PA and mucA22, PA and ΔmucA, or mucA22 and ΔmucA were then analyzed using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. The proteins were then identified by searching the Swiss-Prot PA mass spectrometric database.

Alginate assays
Selected bacteria were grown in L-broth under aerobic conditions for 24 hr at 37˚C with shaking at 250 rpm. The alginate isolation and assay was performed base on the protocol of Ma et. al., [106] with minor modifications. Briefly, the overnight bacterial cultures were mixed 1:1 ratio with PBS and then centrifugation at 16,000 x g for 10 min. One volume of 2% cetylpyridinium chloride was added to the supernatant to precipitate the alginate. After centrifugation at the same speed for 5 min, he pelleted was resuspended in 2 volumes of 1 M NaCl and alginate was precipitated using 2 volumes of cold isopropanol. After centrifugation and air drying, the alginate pellet was resuspended in 200 μl of saline. Alginate concentration as mg/ml was calculated using the carbozole assay [107].

Infection of mouse airways and effects of A-NO₂⁻ on bacterial viability
Six-week old male Balb/C mice (8 per cohort) were purchased from Harlan Laboratories, Inc. Approximately ~5 x 10^6 of isogenic mucA22 or ΔmucA strains were resuspended in 0.9% saline containing purified PA alginate at final concentration of 1.1 mg/ml and used 50 μl to inject into mouse tracheas nonsurgically using a 21-gauge ball-end needle to the back of the tongue above the tracheal opening as previously described [108]. The successful delivery of bacteria into the lungs was manifested by a slight gag reflex by the mice exhibited immediately after instillation followed by a pattern of rapid breathing. After 24 hr of incubation, mouse lungs were instilled with 25 μl of 15 mM A-NO₂⁻ at pH 6.5 (in 0.1 M phosphate buffer) intranasally twice daily. On the fifth day, the mice were sacrificed, and the viable bacteria from serially diluted lung homogenates were enumerated.

Ethics statement
All animal studies were performed in accordance with the protocols approved by the Animal Care Committee at the University of Illinois at Champagne-Urbana. The animal study was 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 (IACUC) at the University of Illinois at Urbana-Champaign (Protocol Number: 15171).

Supporting information
S1 Fig. Two independently generated mucA deletions mapped to an identical position on the PAO1 genome. PAO1 ORFs, PA PAO1 open reading frames; Red boxes indicate the mucA deletion regions. A. PAO1 ΔmucA (M.J.S. lab), Illumina reads from PAO1 ΔmucA
aligned to the PAO1 chromosome from the Schurr laboratory; B. PAO1 ΔmucA (D.J.H.), Illumina reads aligned to PAO1 from PAO1 ΔmucA from corresponding author Hassett’s laboratory. C. Alignment of wild-type, mucA22 and ΔmucA alleles.

(S2 Fig. 2-D gel Western blots for SNO proteins in anaerobically grown strains treated with 15 mM A-NO \textsubscript{2}- \textsuperscript{2}. A. PAO1; B. mucA22; C. ΔmucA. SNO-proteins were separated using Immobiline DryStrip pH 3–10 NL (non-linear) gels and then silver stained. SNO-proteins revealing differences in signal intensity from each set of protein spots were extracted from the gels and identified by mass spectrometry. The identification of each circled protein is listed in Table 3 with the fold up or down values given.

(S1 Table. Fold change in gene expression of anaerobic PAO1 vs. mucA22 upon exposure to 15 mM A-NO \textsubscript{2}. The change up/down are values in the mucA22 mutant relative to that of strain PAO1. IG, intragenic region.

(S2 Table. Fold change in gene expression of anaerobic PAO1 vs. ΔmucA upon exposure to 20 mM A-NO \textsubscript{2}. The change up/down are values in the ΔmucA mutant relative to that of strain PAO1.

(S3 Table. Fold change in gene expression of anaerobic mucA22 vs. ΔmucA bacteria upon exposure to 15 mM A-NO \textsubscript{2}. The change up/down are values in the ΔmucA mutant relative to that of strain mucA22.

(S4 Table. Inability of the FRD1 norCB genes to complement an anaerobic growth defect of a PAO1 norCB mutant.

(S5 Table. Overlapping differentially expressed genes (DEGs) in 3 comparisons.

(S6 Table. Predicted interactors of MucA. Differentially expressed genes (DEGs) are in red.

(S7 Table. GO function enrichment of overlapping DEGs in mucA22 vs ΔmucA and PAO1 vs mucA22, and predicted MucA interactors.

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**References**

1. Pawankar R. Allergic diseases and asthma; a global public health concern and a call to action. World Allergy Organization Journal. 2014; 20147:12, https://doi.org/10.1186/1939-4551-7-12.

2. Cutting GR. Cystic fibrosis genetics: from molecular understanding to clinical application. Nat Rev Genet. 2015; 16(1):45–56. https://doi.org/10.1038/nrg3849 PMID: 25404111; PubMed Central PMCID: PMCPMC4364438.

3. Rosell A, Monso E, Soler N, Torres F, Angrill J, Riise G, et al. Microbiologic determinants of exacerbation in chronic obstructive pulmonary disease. Arch Intern Med. 2005; 165(8):891–7. Epub 2005/04/27. 165/8/891 [pii] https://doi.org/10.1001/archinte.165.8.891 PMID: 15851640.

4. Almagro P, Salvado M, Garcia-Vidal C, Rodriguez-Carballeira M, Cuchi E, Torres J, et al. Pseudomonas aeruginosa and mortality after hospital admission for chronic obstructive pulmonary disease. Respiration. 2012; 84(1):36–43. Epub 2011/10/15. 000331224 [pii] https://doi.org/10.1159/000331224 PMID: 21996555.

5. Roche N. Characteristics of obstructive respiratory diseases (asthma and COPD) in the elderly. Rev Mal Respir. 2007; 24(6):803–5. Epub 2007/07/17. MDPI-RMR-06-2007-24-6-0761-8425-101019-200629017 [pii]. PMID: 17632444.

6. Wollitzsch D, Herberth G, Ulrich M, Doring G. Catalase, myeloperoxidase and hydrogen peroxide in cystic fibrosis. Eur Respir J. 1998; 11(2):377–83. Epub 1998/04/29. PMID: 9551742.

7. McGrath LT, Mallon P, Dowey L, Silke B, McClean E, McDonnell M, et al. Oxidative stress during acute respiratory exacerbations in cystic fibrosis. Thorax. 1999; 54(6):518–23. Epub 1999/05/21. https://doi.org/10.1136/thx.54.6.518 PMID: 10335006.

8. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, et al. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: Relationships to cystic fibrosis pathogenesis. Dev Cell. 2002; 3(4):593–603. PMID: 12408810.
9. Yoon SS, Coakley R, Lau GW, Lymar SV, Gaston B, Karabulut AC, et al. Anaerobic killing of mucoid *Pseudomonas aeruginosa* by acidified nitrite derivatives under cystic fibrosis airway conditions. J Clin Invest. 2006; 116:436–46. https://doi.org/10.1172/JCI24684 PMID: 1640061

10. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med. 2008; 177(9):995–1001. https://doi.org/10.1164/rccm.200708-1151OC PMID: 18263800.

11. Field TR, White A, Elborn JS, Tunney MM. Effect of oxygen limitation on the in vitro antimicrobial susceptibility of clinical isolates of *Pseudomonas aeruginosa* grown planktonically and as biofilms. Eur J Clin Microbiol Infect Dis. 2005; 24(10):677–87. https://doi.org/10.1007/s10096-005-0031-9 PMID: 16249934.

12. Alvarez-Ortega C, Harwood CS. Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. Mol Microbiol. 2007; 65(1):153–65. https://doi.org/10.1111/j.1365-2958.2007.05772.x PMID: 17581126.

13. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Reduced oxygen concentrations in airway mucus contribute to the early and late pathogenesis of *Pseudomonas aeruginosa* cystic fibrosis airway infection. J Clin Invest. 2002; 109:27–35. https://doi.org/10.1172/JCI13870 PMID: 11827991.

14. Hassett DJ, Cuppoletti J, Trapnell B, Lymar SV, Rowe JJ, Sun Yoon S, et al. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Adv Drug Deliv Rev. 2002; 54 (11):1425–43. PMID: 12458153.

15. Hassett DJ, Lymar SV, Rowe JJ, Schurr MJ, Passador L, Herr AB, et al. Anaerobic metabolism by *Pseudomonas aeruginosa* in cystic fibrosis airway biofilms: role of nitric oxide, quorum sensing and alginate production. In, Strict and Facultative Anaerobes: Medical and Environmental Aspects. 2004:87–108.

16. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol. 2002; 184(4):1140–54. https://doi.org/10.1128/JB.184.4.1140-1154.2002 PMID: 11807075.

17. Hassett DJ, Korthagen TR, Irvin RT, Schurr MJ, Sauer K, Lau GW, et al. *Pseudomonas aeruginosa* biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. Expert Opin Ther Targets. 2010; 14(2):117–30. Epub 2010/01/09. https://doi.org/10.1517/14728222.2012.708025 PMID: 22793158.

18. Su S, Hassett DJ. Anaerobic *Pseudomonas aeruginosa* and other obligately anaerobic bacterial biofilms growing in the thick airway mucus of chronically infected cystic fibrosis patients: an emerging paradigm or “Old Hat”? Expert Opin Ther Targets. 2012; 16(9):859–73. Epub 2012/07/17. https://doi.org/10.1517/14728222.2012.708025 PMID: 22793158.

19. Bragonzi A, Wiehlmann L, Klockgether J, Cramer N, Worlitzsch D, Doring G, et al. Sequence diversity of the mucA/B/D locus in *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Microbiology. 2006; 152(Pt 11):3261–9. https://doi.org/10.1099/mic.0.29175-0 PMID: 17074897.

20. O’Toole GA. To build a biofilm. J Bacteriol. 2003; 185(9):2687–9. https://doi.org/10.1128/JB.185.9.2687-2689.2003 PMID: 12700246.

21. Storey DG, Ujacak EE, Mitchell I, Rabin HR. Positive correlation of algD transcription to lasB and lasA transcription by populations of *Pseudomonas aeruginosa* in the lungs of patients with cystic fibrosis. Infect Immun. 1997; 65(10):4061–7. PMID: 9317008.

22. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Walsh MJ, Greenberg EP. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature. 2000; 407:762–4. https://doi.org/10.1038/35037627 PMID: 11827991.

23. Palmer KL, Aye LM, Whiteley M. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. J Bacteriol. 2007; 189(22):8079–87. https://doi.org/10.1128/JB.01138-07 PMID: 17873029.

24. Grasmann H, Ioannidis I, Tomkiewicz RP, de Groot H, Rubin BK, Ratjen F. Nitric oxide metabolites in cystic fibrosis lung disease. Arch Dis Child. 1998; 78(1):49–53. https://doi.org/10.1136/adc.78.1.49 PMID: 9534676.

25. Liu J, Sandrini A, Thurston MC, Yates DH, Thomas PS. Nitric oxide and exhaled breath nitrite/nitrates in chronic obstructive pulmonary disease patients. Respiration. 2007; 74(6):617–23. https://doi.org/10.1159/000106379 PMID: 17643055.

26. Jassem E, Kedzia A, Rek M, Wolska-Goszka L, Szelezynski K. Occurrence of non-spore forming anaerobic bacteria in the upper airways of patients with chronic obstructive pulmonary disease. Med Dosw Mikrobiol. 1996; 48:49–54. Epub 1996/01/01. PMID: 8926767.
27. Bittr F, Richet H, Dubus JC, Reynaud-Gaubert M, Stremler N, Sarles J, et al. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. PLoS One. 2008; 3(8):e2908. Epub 2008/08/07. https://doi.org/10.1371/journal.pone.0002908 PMID: 18682840.

28. Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2003; 41(8):3546–58. Epub 2003/08/09. https://doi.org/10.1128/JCM.41.8.3548-3558.2003 PMID: 12904354.

29. Major TA, Panmanee W, Mortensen JE, Gray LD, Hoglen N, Hassett DJ. Sodium nitrite-mediated killing of the major cystic fibrosis pathogens Pseudomonas aeruginosa, Staphylococcus aureus, and Burkholderia cepacia under anaerobic planktonic and biofilm conditions. Antimicrob Agents Chemother. 2010; 54(11):4671–7. Epub 2010/08/11. AAC.00379-10 [pii] https://doi.org/10.1128/AAC.00379-10 PMID: 20896868.

30. Dotsch J, Puls J, Klimmek T, Rascher W. Reduction of neuronal and inducible nitric oxide synthase gene expression in patients with cystic fibrosis. Eur Arch Otorhinolaryngol. 2002; 259(4):222–6. PMID: 12064512.

31. Corradi M, Mutti A. Nitric oxide synthase isoforms in lung parenchyma of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2010; 181(1):3–4. https://doi.org/10.1164/rccm.200908-1279ED PMID: 20026749.

32. Hogardt M, Heesemann J. Adaptation of Pseudomonas aeruginosa during persistence in the cystic fibrosis lung. Int J Med Microbiol. 2010; 300(8):557–62. Epub 2010/10/15. S1438-4221(10)00086-3 [pii] https://doi.org/10.1016/j.ijmm.2010.08.006 PMID: 20943439.

33. Hogardt M, Hoboth C, Schmidolt S, Henke C, Bader L, Heesemann J. Stage-specific adaptation of hypermutable Pseudomonas aeruginosa isolates during chronic pulmonary infection in patients with cystic fibrosis. J Infect Dis. 2007; 195(1):70–80. Epub 2006/12/08. JID36448 [pii] https://doi.org/10.1086/509821 PMID: 17152010.

34. Bjarnsholt T, Jensen PO, Jakobsen TH, Phipps R, Nielsen AK, Rybtke MT, et al. Quorum sensing and virulence of Pseudomonas aeruginosa during lung infection of cystic fibrosis patients. PLoS One. 2010; 5(4):e10115. Epub 2010/04/21. https://doi.org/10.1371/journal.pone.0010115 PMID: 20404933.

35. Martin DW, Schurr MJ, Mudd MH, Govan JR, Holloway BW, Deretic V. Mechanism of conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients. Proc Natl Acad Sci U S A. 1993; 90(18):8377–81. https://doi.org/10.1073/pnas.90.18.8377 PMID: 8378309.

36. Valderrey AD, Pozuelo MJ, Jimenez PA, Macia MD, Oliver A, Rotger R. Chronic colonization by Pseudomonas aeruginosa of patients with obstructive lung diseases: cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease. Diagn Microbiol Infect Dis. 2010; 68(1):20–7. Epub 2009/08/24. S0732-8893(10)00131-8 [pii] https://doi.org/10.1016/j.diagmicrobio.2010.04.008 PMID: 20727465.

37. Govan JRW, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol Rev. 1996; 60:539–74. PMID: 8840786.

38. Goldberg JB, Gorman WL, Flynn JL, Ohman DE. A mutation in algN permits trans activation of alginate production by algT in Pseudomonas aeruginosa. J Bacteriol. 1993; 175:1303–8. https://doi.org/10.1128/jb.175.5.1303-1308.1993 PMID: 844793.

39. Boucher JC, Martinez-Salazar J, Schurr MJ, Mudd MH, Yu H, Deretic V. Two distinct loci affecting conversion to mucoidy in Pseudomonas aeruginosa in cystic fibrosis encode homologs of the serine protease HtrA. J Bacteriol. 1996; 178(2):511–26. https://doi.org/10.1128/jb.178.2.4990-4996.1996 PMID: 8755047.

40. Wood LF, Leech AJ, Ohman DE. Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in Pseudomonas aeruginosa: Roles of sigma (AlgT) and the AlgW and Prc proteases. Mol Microbiol. 2006; 62(2):412–26. Epub 2006/10/06. MM05390 [pii] https://doi.org/10.1111/j.1365-2958.2006.05390.x PMID: 17025880.

41. Qiu D, Esinger VM, Rowe DW, Yu HD. Regulated proteolysis controls mucoid conversion in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2007; 104(19):8107–12. Epub 2007/05/02. 0702660104 [pii] https://doi.org/10.1073/pnas.0702660104 PMID: 17470813.

42. Xie Z-D, Hershberger CD, Shankar S, Ye RW, Chakrabarty AM. Sigma factor-anti sigma factor interaction in alginate synthesis: inhibition of AlgT by Muca. J Bacteriol. 1996; 178:4990–6. https://doi.org/10.1128/jb.178.16.4990-4996.1996 PMID: 8759865.

43. Skjak-Braek G, Grasdalen H, Larsen B. Monomer sequence and acetylation pattern in some bacterial alginites. Carbohydr Res. 1986; 154:239–50. PMID: 3098421.

44. Gacesa P. Alginates. Carbohydr Polym. 1988; 8:161–82.
45. Pedersen SS, Hoiby N, Espersen F, Koch C. Role of alginate in infection with mucoid Pseudomonas aeruginosa in cystic fibrosis. Thorax. 1992; 47(1):6–13. https://doi.org/10.1136/thx.47.1.6 PMID: 1539148.

46. Liang H, Li L, Dong Z, Surette MG, Duan K. The YeBC family protein PA0964 negatively regulates the Pseudomonas aeruginosa quinolone signal system and pyocyanin production. J Bacteriol. 2008; 190(18):6217–27. Epub 2008/07/22. JB.00428-08 [pii] https://doi.org/10.1128/JB.00428-08 PMID: 18641136.

47. McDaniel C, Su S, Panmanee W, Lau GW, Browne T, Cox K, et al. A Putative ABC Transporter Permease Is Necessary for Resistance to Acidified Nitrite and EDTA in Pseudomonas aeruginosa under Aerobic and Anaerobic Planktonic and Biofilm Conditions. Front Microbiol. 2016; 7:291. https://doi.org/10.3389/fmicb.2016.00291 PMID: 27064218; PubMed Central PMCID: PMCPMC4817314.

48. Su S, Panmanee W, Wilson JJ, Mahtani HK, Li Q, Vanderwielen BD, et al. Catalase (KatA) Plays a Role in Protection against Anaerobic Nitric Oxide in Pseudomonas aeruginosa. J Bacteriol. 2014; 196:2044–54. https://doi.org/10.1128/jb.196.7.2044-2054.1994 PMID: 8144472.

49. Ochsner UA, A.K. K, Fiechter A, Reiser J. Isolation and characterization of a regulatory gene affecting Ochsner UA, A.K. K, Fiechter A, Reiser J. Isolation and characterization of a regulatory gene affecting

50. Yoon SS, Karabulut AC, Lipscombe JD, Hennigan RF, Lymar SV, Groce SL, et al. Two-pronged survival strategy for the major cystic fibrosis pathogen, Pseudomonas aeruginosa, lacking the capacity to degrade nitric oxide during anaerobic respiration. Embo J. 2007; 26(15):3662–72. https://doi.org/10.1038/sj.embj.7601787 PMID: 17627281.

51. Ohman DE, Chakrabarty AM. Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a Pseudomonas aeruginosa cystic fibrosis isolate. Infect Immun. 1981; 33:142–8. PMID: 6790439.

52. Hassett DJ, Ma J-F, Elkins JG, McDermott TR, Ochsner UA, West SEH, et al. Quorum sensing in Pseudomonas aeruginosa controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Mol Microbiol. 1999; 34:1082–93. PMID: 10594832.

53. Ma J-F, Ochsner UA, Klotz MG, Nanayakkara VK, Howell ML, Johnson Z, et al. Bacterioferritin A modulates catalase A (KatA) activity and resistance to hydrogen peroxide in Pseudomonas aeruginosa. J Bacteriol. 1999; 181:3730–42. PMID: 10368148.

54. Boucher JC, Schurr MJ, Yu H, Rowen DW, Deretic V. Pseudomonas aeruginosa in cystic fibrosis: role of mucC in the regulation of alginate production and stress sensitivity. Microbiology. 1997; 143(Pt 11):3473–80. https://doi.org/10.1099/00221287-143-11-3473 PMID: 9387225.

55. Yorgey P, Rahme LG, Tan MW, Ausubel FM. The roles of mucD and alginate in the virulence of Pseudomonas aeruginosa in plants, nematodes and mice. Mol Microbiol. 2001; 41(5):1063–76. PMID: 11555287.

56. Hassett DJ. Anaerobic production of alginate by Pseudomonas aeruginosa: alginate restricts diffusion of oxygen. J Bacteriol. 1996; 178:7322–5. https://doi.org/10.1128/jb.178.23.7322-7325.1996 PMID: 8954520.

57. Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS. Oxygen limitation contributes to antibiotic tolerance of Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother. 2004; 48(7):2659–64. https://doi.org/10.1128/AAC.48.7.2659-2664.2004 PMID: 15215123.

58. Hao G, Derakhshan B, Shi L, Campagne F, Gross SS. SNOSID, a proteomic method for identification of cysteine S-nitrosylation sites in complex protein mixtures. Proc Natl Acad Sci U S A. 2006; 103(4):1012–7. https://doi.org/10.1073/pnas.0508412103 PMID: 16418269.

59. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraiki O, Michel T, et al. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci U S A. 1992; 89(1):444–8. https://doi.org/10.1073/pnas.89.1.444 PMID: 1346070.

60. Schreiber K, Boes N, Eschbach M, Jaensch L, Wehland J, Bjarnsholt T, et al. Anaerobic survival of Pseudomonas aeruginosa by pyruvate fermentation requires an Usp-type stress protein. J Bacteriol. 2006; 188(2):659–68. https://doi.org/10.1128/JB.188.2.659-668.2006 PMID: 16385055.

61. Godfrey C, Gadfly PM, Thomson AJ, Greenwood C, Coddingston A. Electron-paramagnetic-resonance and magnetic-circular-dichroism studies on the formate dehydrogenase-nitrate reductase particle from Pseudomonas aeruginosa. Biochim J. 1987; 243(1):241–8. https://doi.org/10.1042/bj2430241 PMID: 3038083; PubMed Central PMCID: PMCPMC1147838.

62. Godfrey C, Greenwood C, Thomson AJ, Bray RC, George GN. Electron-paramagnetic-resonance spectroscopy studies on the dissimilatory nitrate reductase from Pseudomonas aeruginosa. Biochim J. 1984; 224(2):601–8. https://doi.org/10.1042/bj2240601 PMID: 6097225.
Wood LF, Ohman DE. Independent regulation of MucD, an HtrA-like protease in Pseudomonas aeruginosa Mucoid Strain FRD1. Isolated from a Cystic Fibrosis Patient. Genome Announc. 2015; 3(2). https://doi.org/10.1128/genomeA.00153-15 PMID: 25792066; PubMed Central PMCID: PMC4395061.

Wang D, Hildebrand F, Ye L, Wei Q, Ma LZ. Genome Sequence of Mucoid Pseudomonas aeruginosa Strain FRD1. Genome Announc. 2015; 3(2). https://doi.org/10.1128/genomeA.00376-15 PMID: 25908149; PubMed Central PMCID: PMCPMC4408350.

Hino T, Matsumoto Y, Nagano S, Sugimoto H, Fukumori Y, Murata T, et al. Structural basis of biological N2O generation by bacterial nitric oxide reductase. Science. 2010; 330(6011):1666–70. https://doi.org/10.1126/science.1195591 PMID: 21106633.

Coakley RD, Grubb BR, Paradiso AM, Gatzky JT, Johnson LG, Kreda SM, et al. Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. Proc Natl Acad Sci U S A. 2003; 100(26):16083–8. https://doi.org/10.1073/pnas.2634339100 PMID: 14668433.

Borrill ZL, Roy K, Vessey RS, Woodcock AA, Singh D. Non-invasive biomarkers and pulmonary function in smokers. Int J Chron Obstruct Pulmon Dis. 2008; 3(1):171–83. Epub 2008/05/21. PMID: 18488441.

Cao H, Krishnan G, Goumnerov B, Tsongalis J, Tompkins R, Ramsey LG. A quorum sensing-associated virulence gene of Pseudomonas aeruginosa encodes a LysR-like transcription regulator with a unique self-regulatory mechanism. Proc Natl Acad Sci U S A. 2001; 98(25):14613–8. https://doi.org/10.1073/pnas.251465298 PMID: 11724939.

Arai H, Hayashi M, Kuroi A, Ishii M, Igarashi Y. Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of Pseudomonas aeruginosa. J Bacteriol. 2005; 187(12):3960–8. Epub 2005/06/07. 187/12/3960 [pii] https://doi.org/10.1128/JB.187.12.3960-3968.2005 PMID: 15937158.

Koskenkorva T, Aro-Kakkainen N, Bachmann D, Arai H, Frey AD, Kallio PT. Transcriptional activity of Pseudomonas aeruginosa fhp promoter is dependent on two regulators in addition to FhpR. Arch Microbiol. 2008; 190(4):385–96. Epub 2007/11/29. https://doi.org/10.1007/s00203-007-0329-3 PMID: 18043907.

Filiatrault MJ, Picard KO, Ngai H, Passador L, Iglewski BH. Identification of Pseudomonas aeruginosa genes involved in virulence and anaerobic growth. Infect Immun. 2006; 74(7):4237–45. https://doi.org/10.1128/IAI.02014-05 PMID: 16790798.

Platt MD, Schurr MJ, Sauer K, Vazquez G, Kukavica-Ibrulj I, Potvin E, et al. Proteomic, microarray, and signature-tagged mutagenesis analyses of anaerobic Pseudomonas aeruginosa at pH 6.5, likely representing chronic, late-stage cystic fibrosis airway conditions. J Bacteriol. 2008; 190(8):2739–58. https://doi.org/10.1128/JB.01683-07 PMID: 18488441.

Wood LF, Ohman DE. Independent regulation of MucD, an HtrA-like protease in Pseudomonas aeruginosa, and the role of its proteolytic motif in alginate gene regulation. J Bacteriol. 2006; 188(8):3134–7. https://doi.org/10.1128/JB.188.8.3134-3137.2006 PMID: 16585775; PubMed Central PMCID: PMCPMC1447020.

Anand P, Stamler JS. Enzymatic mechanisms regulating protein S-nitrosylation: implications in health and disease. J Mol Med (Berl). 2012; 90(3):233–44. https://doi.org/10.1007/s00109-012-0878-z PMID: 22361849; PubMed Central PMCID: PMCPMC3379879.

Seth D, Hausladen A, Wang YJ, Stamler JS. Endogenous protein S-Nitrosylation in E. coli: regulation by OxyR. Science. 2012; 336(6080):470–3. Epub 2012/04/28. 336/6080/470 [pii] https://doi.org/10.1126/science.1215643 PMID: 22539721.

Hayashi NR, Arai H, Kodama T, Igarashi Y. The nirQ gene, which is required for denitrification of Pseudomonas aeruginosa, can activate the RubisCO from Pseudomonas hydrogenosphera. Biochim Biophys Acta. 1998; 1381(3):347–50. PMID: 9729445.

Crespo A, Gavalda J, Julian E, Torrents E. A single point mutation in class III ribonucleotide reductase promoter renders Pseudomonas aeruginosa PAO1 inefficient for anaerobic growth and infection. Sci Rep. 2017; 7(1):13350. https://doi.org/10.1038/s41598-017-14051-2 PubMed Central PMCID: PMCPMC5645315. PMID: 29042684.

Crespo A, Pedraz L, Astola J, Torrents E. Pseudomonas aeruginosa Exhibits Deficient Biofilm Formation in the Absence of Class II and III Ribonucleotide Reductases Due to Hindered Anaerobic Growth. Front Microbiol. 2016; 7:688. https://doi.org/10.3389/fmicb.2016.00688 PMID: 27242714; PubMed Central PMCID: PMCPMC4860495.

Adgent MA, Squadrito GL, Bailinger CA, Krzywanski DM, Lancaster JR, Postlethwait EM. Desferrioxamine inhibits protein tyrosine nitration: Mechanisms and implications. Free Radic Biol Med. 2012; 53(4):951–61. Epub 2012/06/19. S0891-5849(12)00338-3 [pii] https://doi.org/10.1016/j.freeradbiomed.2012.06.003 PMID: 22705369.
80. Bosworth CA, Toledo JC Jr., Zmijewski JW, Li Q, Lancaster JR Jr. Dinitrosyliron complexes and the mechanism(s) of cellular protein nitrosothiol formation from nitric oxide. Proc Natl Acad Sci U S A. 2009; 106(12):4671–6. Epub 2009/03/06. 0710416106 [pii]. https://doi.org/10.1073/pnas.0710416106 PMID: 19261856.

81. Staple R, Owusu BY, Brandon A, Cusick M, Rodrigue C, Marques MB, et al. Erythrocyte storage increases rates of NO and nitrate scavenging: implications for transfusion-related toxicity. Biochem J. 2012; 446(3):499–508. Epub 2012/06/23. BJ20120675 [pii]. https://doi.org/10.1042/Bj20120675 PMID: 22720637.

82. Lancaster JR Jr., Langrehr JM, Bergonia HA, Murane N, Simmons RL, Hoffman RA. EPR detection of heme and nonheme iron-containing protein nitrosylation by nitric oxide during rejection of rat heart allograft. J Biol Chem. 1992; 267(16):10994–8. Epub 1992/06/15. PMID: 1375994.

83. Reddy D, Lancaster JR Jr., Cornforth DP. Nitrite inhibition of Clostridium botulinum: electron spin resonance detection of iron-nitric oxide complexes. Science. 1983; 221(4612):769–70. Epub 1983/08/19. PMID: 6308761.

84. Toledo JC Jr., Bosworth CA, Hennon SW, Mahtani HA, Bergonia HA, Lancaster JR Jr. Nitric oxide-induced conversion of cellular chelatable iron into macromolecule-bound paramagnetic dinitrosyliron complexes. J Biol Chem. 2008; 283(43):28926–33. Epub 2008/05/16. M707862200 [pii]. https://doi.org/10.1074/jbc.M707862200 PMID: 18480062.

85. Zhang M, Su S, Bhatnagar RK, Hassett DJ, Lu LJ. Prediction and Analysis of the Protein Interactome Complex of the Nitrite Reductase NirS, the Chaperone DnaK, and the Flagellum Protein FliC Is Essential for Flagellum Assembly and Motility in Pseudomonas aeruginosa. J Bacteriol. 2015; 197(19):3066–75. https://doi.org/10.1128/JB.00415-15 PMID: 26170416; PubMed Central PMCID: PMC4560289.

86. Tart AH, Blanks MJ, Wozniak DJ. The AlgT-dependent transcriptional regulator AmrZ (AlgZ) inhibits flagellum biosynthesis in mucoid, nonmotile Pseudomonas aeruginosa cystic fibrosis isolates. J Bacteriol. 2006; 188(18):6483–9. https://doi.org/10.1128/JB.00636-06 PMID: 16952938; PubMed Central PMCID: PMC1595476.

87. Horner-de Acuna JM, Molinarí G, Rohde M, Dammeyer T, Wissing J, Jansch L, et al. A Periplasmic Fumarate Re-ductase NAD(P)H Oxidoreductase (QFr) Is Essential for the Production of the Biofilm Matrix in Pseudomonas aeruginos a. J Bacteriol. 2001; 183:5555–64. https://doi.org/10.1128/JB.183.18.5555-5564.2001 PMID: 11398180.

88. Simon R, Priefer U, Puehler A. A broad host range mobilization system for gene transfer in gram negative bacteria. Bio-Technology. 1983; 1:784–91.

89. Holloway B. Genetic recombination in Pseudomonas aeruginos a. J Gen Microbiol. 1955; 13:572–81. https://doi.org/10.1099/00221287-13-3-572 PMID: 13278508.

90. Goldberg JB, Ohman DE. Cloning and expression in Pseudomonas aeruginos a of a gene involved in the production of alginate. J Bacteriol. 1984; 158:1115–21. PMID: 6427188.

91. Mathee K, Ciofu O, Sternberg C, Lindum PW, Campbell JI, Jensen P, et al. Mucoid conversion of Pseudomonas aeruginos a by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiology. 1999; 145:1349–57. https://doi.org/10.1099/13500872-145-6-1349 PMID: 10411261.

92. Kulašekara HD, Ventre I, Kulašekara BR, Lazdunski A, Filloux A, Lory S. A novel two-component system controls the expression of Pseudomonas aeruginos a fimbrial cup genes. Mol Microbiol. 2005; 55 (2):368–80. Epub 2005/01/22. MM4402 [pii]. https://doi.org/10.1111/j.1365-2958.2004.04402.x PMID: 15659157.

93. Schweizer HP. Small broad-host-range gentamicin resistance gene cassettes for site-specific insertion and deletion mutagenesis. Biotechniques. 1993; 15:831–3. PMID: 8267974.

94. Schweizer HP, Hoang TT. An improved system for gene replacement and xy/E fusion analysis in Pseudomonas aeruginos a. Gene. 1995; 158:15–22. PMID: 7789804.
98. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD. pBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. Appl Environ Microbiol. 2008; 74(23):7422–6. Epub 2008/10/14. AEM.01369-08 [pii] https://doi.org/10.1128/AEM.01369-08 PMID: 18849445.

99. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; 1982.

100. Caetano-Anollés G. Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl. 1993; 3(2):85–94. Epub 1993/10/01. PMID: 8268791.

101. Fung C, Naughton S, Turnbull L, Tingpej P, Rose B, Arthur J, et al. Gene expression of *Pseudomonas aeruginosa* in a mucin-containing synthetic growth medium mimicking cystic fibrosis lung sputum. J Med Microbiol. 2010; 59(Pt 9):1089–100. Epub 2010/06/05. jmm.0.019984–0 [pii] https://doi.org/10.1099/jmm.0.019984-0 PMID: 20522626.

102. Frisk A, Schurr JR, Wang G, Bertucci DC, Marrero L, Hwang SH, et al. Transcriptome analysis of *Pseudomonas aeruginosa* after interaction with human airway epithelial cells. Infect Immun. 2004; 72(9):5433–8. https://doi.org/10.1128/IAI.72.9.5433-5438.2004 PMID: 15322041; PubMed Central PMCID: PMCPMC17424.

103. Lizewski SE, Schurr JR, Jackson DW, Frisk A, Carterson AJ, Schurr MJ. Identification of AlgR-regulated genes in *Pseudomonas aeruginosa* by use of microarray analysis. J Bacteriol. 2004; 186(17):5672–84. https://doi.org/10.1128/JB.186.17.5672-5684.2004 PMID: 15317771.

104. Morici LA, Carterson AJ, Wagner VE, Frisk A, Schurr JR, Zu Bentrup KH, et al. *Pseudomonas aeruginosa* AlgR Represses the Rhl Quorum-Sensing System in a Biofilm-Specific Manner. J Bacteriol. 2007; 189(21):7752–64. https://doi.org/10.1128/JB.01797-06 PMID: 17766417.

105. Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. Sci STKE. 2001; 2001(86):PL1. https://doi.org/10.1126/stke.2001.86.pl1 PMID: 11752655.

106. Ma S, Selvaraj U, Ohman DE, quarless R, Hassett DJ, Wozniak DJ. Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. J Bacteriol. 1998; 180(4):956–68. PMID: 9473053.

107. Knutson CA, Jeanes A. A new modification of the carbazole analysis: application to heteropolysaccharides. Anal Biochem. 1968; 24:470–81. PMID: 5723302.

108. Hoffmann N, Rasmussen TB, Jensen PO, Stub C, Hentzer M, Molin S, et al. Novel mouse model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis. Infect Immun. 2005; 73(4):2504–14. Epub 2005/03/24. 73/4/2504 [pii] https://doi.org/10.1128/IAI.73.4.2504-2514.2005 PMID: 15784597.